FC-MEDIATED ANTIBODY FUNCTIONS AND FC-RECEPTOR POLYMORPHISM

EDITED BY: Guido Ferrari, Georgia Tomaras, R. Keith Reeves and Gabriella Scarlatti

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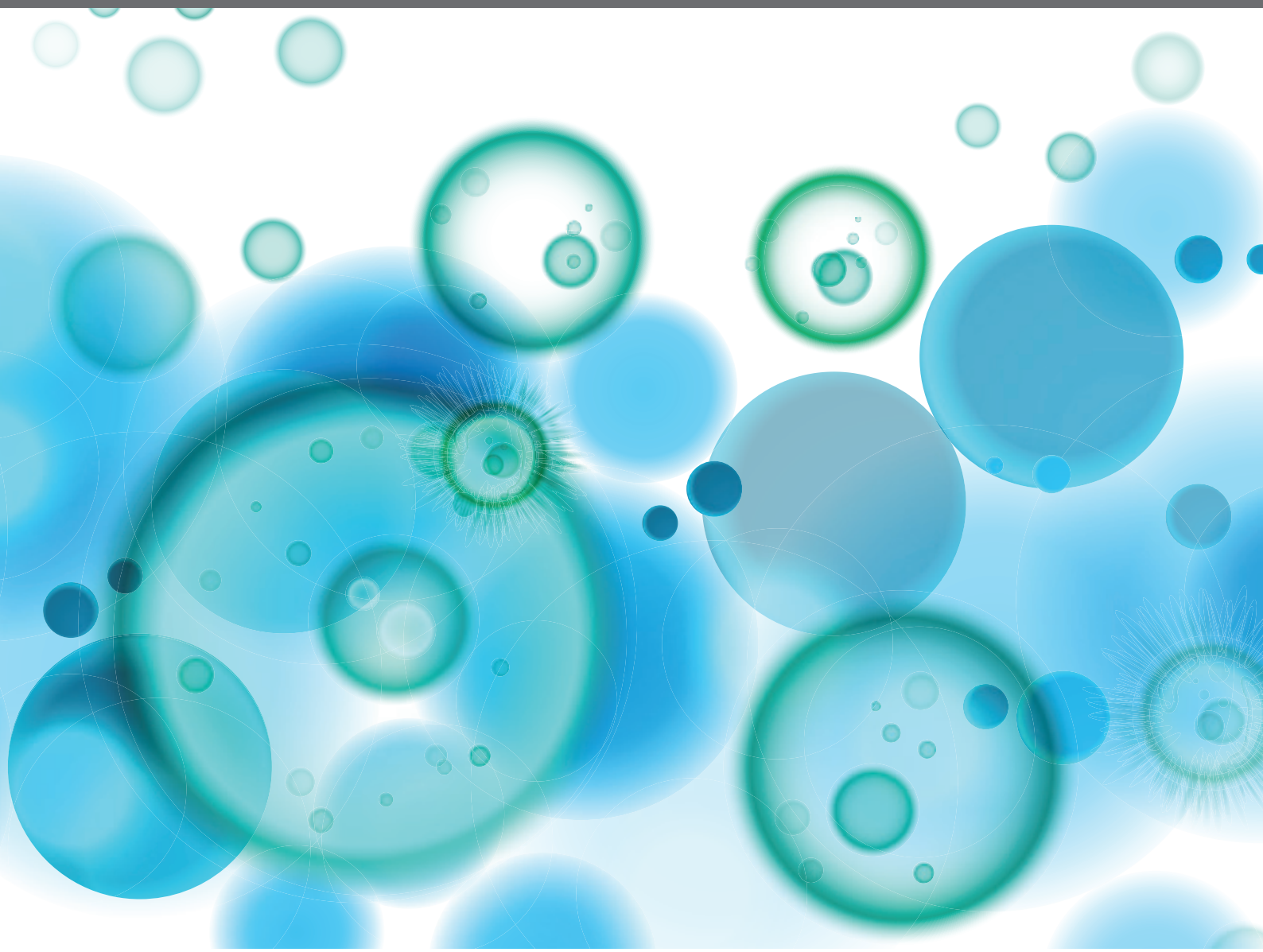

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FC-MEDIATED ANTIBODY FUNCTIONS AND FC-RECEPTOR POLYMORPHISM

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Antibody Epitope Specificity for dsDNA Phosphate Backbone Is an Intrinsic Property of the Heavy Chain Variable Germline Gene Segment Used

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Analysis of protein sequences by the informational spectrum method (ISM) enables characterization of their specificity according to encoded information represented with defined frequency (F). Our previous data showed that F(0.367) is characteristic for variable heavy chain (VH) domains (a combination of variable (V), diversity (D) and joining (J) gene segments) of the anti-phosphocholine (PC) T15 antibodies and mostly dependent on the CDR2 region, a site for PC phosphate group binding. Because the T15 dsDNA-reactive U4 mutant also encodes F(0.367), we hypothesized that the same frequency may also be characteristic for anti-DNA antibodies. Data obtained from an analysis of 60 spontaneously produced anti-DNA antibody VH domain sequences supported our hypothesis only for antibodies, which use V gene segment in germline configuration, such as S57(VH31), MRL-DNA22, and VH11, members of the VH1 (J558) and VH7 (S107) gene families. The important finding is that out of seven V gene segments used by spontaneous anti-DNA antibodies, F(0.367) is only expressed by the germline configuration of these three V gene segments. The data suggest that antibody specificity for the phosphate group moiety delineated as F(0.367) is the intrinsic property of the V germline gene segments used, whereas paratope/epitope interaction with antigens bearing this epitope, such as PC or dsDNA, requires corresponding antibody VH conformation that is susceptible to somatic mutation(s).

Keywords: anti-DNA antibodies, anti-PC antibodies, VH germline genes, Characterization of antibody specificity by ISM, dsDNA reactive antibodies

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INTRODUCTION

Natural autoantibodies, mainly IgM whose heavy chains are encoded by unmutated VDJ genes, play a role in immune system homeostasis, provide the first line of defense against infections, and may play a role in autoimmune disease as somatically mutated IgG autoantibodies (1, 2). The highly diverse CDR3 loops are assumed as the key determinant of specificity in antigen recognition, but in nonsomatically mutated antibodies, binding sites may consist of germline-encoded CDR1 and CDR2 sequences dominating in a number of contacts, whereas light chains play a subsidiary role to heavy chains (3, 4). It was also suggested that in contrast to antigen specificity determined by CDR3 (5),

germline-encoded CDR1 and CDR2 sequences accommodate binding to a number of different unrelated antigens (6). The analyses also showed that despite the potential to generate almost unlimited variability, the CDR regions exhibit a small number of core main chain conformations termed "canonical structures" (7). In particular, a limited repertoire of the main chain adopted conformations dependent on the loop length and a few key conserved residues at defined positions (8) has been assigned to CDR1 and CDR2 regions (9).

One of the best studied primary antibody responses to phosphocholine (PC) is T15 antibody expressing heavy and light chain products of the T15(V1) and Vk22 germline genes in mice (10–13). It is of interest that in ontogeny, T15 predominant clonotypes appear about 1 week after birth (14), whereas PC-specific responses or precursors were detected as early as 1 day after birth (15). An important finding is that the heavy chains of T15 and other PC binding proteins bearing M603 and M167 idiotypic determinants are derived from a single germline T15(V1) gene segment and three light chains, i.e., T15 (VK22), M603 (VK8), and M167 (VK24) (13, 16, 17).

Crystallography studies of the anti-PC binding antibody provide evidence for the PC contact residues, revealing that favorable interaction of the choline moiety is with CDR1 Glu-35, whereas specific interactions occur between the phosphate group and charged groups such as CDR2 Arg-52 that produce a large favorable electrostatic interaction and Lys-54 that helps neutralize the PC negative charge (18, 19). The data obtained from mutagenesis experiments conferred importance of CDR2 Arg-52 as a site for interaction with the PC phosphate group (20), whereas interaction with the carrier involves different sites (21). The role of CDR2 H52-H56 motif in nucleic acid binding was also demonstrated by analyses of monoclonal autoantibodies derived from lupus-prone mice (22).

On the other hand, T15 CDR2 sequence VH50-60 region, a part of the self-binding domain (homophilicity), enhances antibody potency (23). The CDR2 of T15 antibody, according to our view, may also have an immunoregulatory role in the ontogeny of natural Tregs and consequently in the control of T15 and some anti-DNA antibody diversification (24).

Anti-DNA antibodies recognize a considerable number of different epitopes, and their exact nature is only partially known (25). Anti-dsDNA antibodies may react with linear and conformational determinants exposed on the double helix of DNA and cross-react with different antigens (26). For example, a similar arrangement of phosphate groups in the DNA sugar-phosphate backbone and phospholipids may explain cross-reactivity (27).

Sequence analysis of anti-dsDNA antibodies from autoimmune mice revealed a high frequency of mutations and the presence of basic amino acids in the CDRs, such as Arg and Lys and polar Asn with the potential to interact with structures within dsDNA (28–31) or, when gained during affinity maturation, be critical for CDR3 region interaction with histone-DNA complex (32–34). This complex according to a hapten-carrier-like model, may initiate production of both anti-dsDNA and other anti-nucleosome antibodies [reviewed in (35)].

In prior studies, we have shown that antibody VH domains of anti-PC T15 and T15 dsDNA binding somatic mutant, U4 (13), encode characteristic sequence information represented with F(0.367) (36). In this report, we extended this finding by showing that F(0.367) is also expressed by several anti-DNA antibody VH domains that use V germline or somatically mutated S57(VH31), MRL-DNA22, and VH11 gene segments of the VH1 (J558) and VH7 (S107) gene families, as well as that protein sequences of these germline genes in addition to T15(V1) encode an intrinsic epitope specificity represented by F(0.367). Obtained data suggests that as long as the frequency is expressed by an antibody VH domain (a) the corresponding conformation for paratope/epitope interaction might be preserved despite somatic mutations and (b) because of somatic mutation(s), interaction with another antigen bearing the same epitope might be achieved and vice versa, loss of the characteristic frequency may cause achievement of a new epitope specificity.

METHOD

The sequence analysis was performed by applying the informational spectrum method (ISM). The physicomathematical basis of ISM was described in detail elsewhere (37), and here, we will only point the basic steps involved by the method. According to the ISM approach, also denoted as resonant recognition model (RRM) (38), protein sequences are transformed into signals by assignment of numerical values of each amino acid. These values correspond to electron–ion interaction potential (39) determining electronic properties of amino acids that are responsible for their intermolecular interactions (40–43). The signal obtained is decomposed in periodical function by Fourier transformation. The result is a series of frequencies and their amplitudes (the informational spectrum, IS). Detailed steps (43) that precede obtaining the IS by the ISM are explained in the **Supplementary Information**. The obtained frequencies correspond to the distribution of structural motifs with defined physicochemical characteristics determining the biological function of the sequence. When comparing proteins that share the same biological function, the technique allows detection of code/frequency pairs in IS, which are specific for their common biological properties. This common information is represented by characteristic peaks in the cross-spectrum (CIS) of proteins. The method is insensitive to the location of the motifs and, thus, does not require the previous alignment of the sequence. A measure of similarity for each peak is a signal-to-noise ratio (S/N), which represents a ratio between signal intensity at one particular IS frequency and the mean value of the whole spectrum which depends on the number of the sequences used in the analysis.

Abbreviations: CDR, Hypervariable region; CIS, Cross-spectral analysis; EIIP, Electron-ion interaction potential; ISM, Informational spectrum method; IS, Informational spectrum; RRM, Resonant recognition model; S/N, Signal-to-noise ratio; VH, Variable heavy chain.

RESULTS

Our previous data showed that VH domain of the anti-PC T15 idiotype antibody that uses an unmutated copy of the V germline gene T15(V1) (16, 17), as well as anti-PC binding antibodies of different idiotypes, encode information represented with F(0.367) in short F(0.37) (36). We also showed that F(0.37), is independent of a single substitution-glutamic acid to alanine, at position 35 in the T15 antibody CDR1 region, causing reactivity acquisition for dsDNA (13) but depends on mutations in CDR2 region (36). In this report, seven V germline gene amino acid sequences used by spontaneous anti-DNA antibodies (31) were analyzed; of which, only three showed F(0.367) in individual spectra such as S57(VH31) (30), MRL-DNA22 (44) germline gene segments members of the VH1(J558) gene family, and VH11 (45) member of the VH7(S107) gene family. The CIS of the T15(V1), S57(VH31), MRL-DNA22, and VH11 V germline gene segment amino acid sequences is presented in **Figure 1A**, revealing a peak at F(0.367). The T15(V1) V germline gene segment from the VH7(S107) gene family is introduced because VH domains of antibodies that express F(0.367), as we have shown previously (36), use this V gene segment in germline configuration (13, 16, 17). The CIS of the four V germline gene segments used by anti-DNA antibodies (31), such as BWDNA16, 2F2, BWDNA7, and VH283, which do not express F(0.367) is presented in **Supplementary Table 1**, revealing that characteristic peak is not at F(0.367).

In this report, the analysis was performed on 60 spontaneous anti-DNA VH domain sequences (31); of which, 20 are encoded by the V gene segments that express F(0.367). However, F(0.367) expression is limited, because only six antibody VH domains retained this characteristic (30%). Thus, we found that F(0.367) is expressed by IgG 74.c2 out of three individually analyzed VH domains of anti-DNA antibodies that use VH11 V gene segment or by IgG 17s-c2 out of nine analyzed anti-DNA antibodies that use S57(VH31) as well as IgG 17s.83, IgG 17s-c3, IgM 111.185, and IgM 165.27 out of eight analyzed VH domains encoded by MRL-DNA22 V gene segment. The CIS of the VH domains of these antibodies is shown in **Figure 1B** revealing a dominant peak at F(0.367). It might be concluded that some anti-DNA antibodies encoded by these V gene segments have lost F(0.367) as the result of somatic mutations.

In **Figure 1C**, CIS of VH domains for 54 anti-DNA VH domains is shown which, in an individual spectrum, does not express F(0.367) and thus do not encode epitope specificity for phosphate groups of dsDNA backbone. It should be emphasized that a peak at F(0.023) with dominant S/N value is detected for the V gene segments (**Figure 1A**), whereas it is a unique peak in the CIS (**Figure 1C**) obtained for anti-DNA VH domains, whose individual sequences do not express F(0.367) a feature relevant for the specificity here analyzed. An analysis of antibodies reactive with ssDNA, Z-DNA, and chromatin further confirms the connection between F(0.367) expression and antibody specificity for the phosphate group of B DNA backbone as shown in **Supplementary Figures 1B–D**.

It is of interest to note that comparison of the V gene segments and VH domain contribution to S/N value for the peak at F(0.367) revealed an insignificant CDR3 region contribution (**Table 1**).

We further made an attempt to determine peptide position in the V gene segment sequences mostly contributing to the F(0.367) expression. The data obtained showed that for T15(V1), VH11(VH7), S57[VH31(VH1)], and MRL-DNA22(VH1) V germline gene segments, these peptides involve residues at positions 35–66, 36–67, 46–65, and 46–77, respectively. The most important finding is that selected peptides include CDR2 regions that are abundant in basic residues (**Table 2**), indicating an CDR2 role in both F(0.367) expression and interaction with an antigenic determinant shared by the PC hapten and dsDNA.

The data obtained from the VH domains analysis of two preimmune natural polyreactive autoantibodies, E7 and D23 (46), which react with antigens such as DNA, myosin, actin, tubulin, spectrin, and trinitrophenol, revealed that F(0.367) was not expressed (**Figure 1D**), meaning that epitope specificity of these antibodies differs from dsDNA-reactive anti-DNA antibodies here analyzed. The CDR2 regions of these autoantibodies are in germline configuration and with a reduced number of basic residues.

DISCUSSION

Previously, using ISM for protein sequence analysis (37), we showed that antibody VH domains of T15 PC binding antibody and U4 dsDNA binding antibody encode information determining sequence specificity represented with characteristic frequency F(0.367), in short F(0.37) (36). We also showed that this frequency is dependent on the type of residues in the CDR2 region and insensitive to a residue substitution in CDR1 (36) of the T15 U4 mutant (13). In this report, we extend these findings by showing that F(0.367) is not only expressed by VH domains of T15 and some spontaneous anti-DNA antibodies from autoimmune mice but is found to be also intrinsic for the V germline gene segments used by these antibodies.

It has been shown that anti-PC binding antibody VH encoded by T15(V1) V gene segment of the VH7(S107) germline gene family (13, 16, 17) form strong interactions between the PC phosphate group and charged residues in the CDR2 region, such as Arg-52 and Lys-54, whereas CDR1 region Glu-35 is involved in choline binding (17, 18). Therefore, F(0.367) expressed by antibodies such as T15, T15 somatic mutant U4, and some anti-DNA antibodies may characterize epitope specificity, that is, specificity for phosphate groups present on different antigens such as PC hapten and dsDNA. Furthermore, the data presented showed that expression of the $S/N_{F(0.367)}$ mostly depends on antibody V gene segments, and thus, a contribution of the CDR3 regions is insignificant (**Table 1**). It should be emphasized that IgG V gene segments of anti-DNA antibodies expressing F(0.367) can be close to germline configuration such as antibodies 74.c2 and 17s.83 encoded by the V gene segment VH11 of the VH7 (S107) gene family and MRL-DNA22 of the gene family VH1 (J558), respectively (31), suggesting that some mutations are tolerable as they do not affect the specificity delineated by the F(0.367). However, they differed in CDR3 regions (31), and their

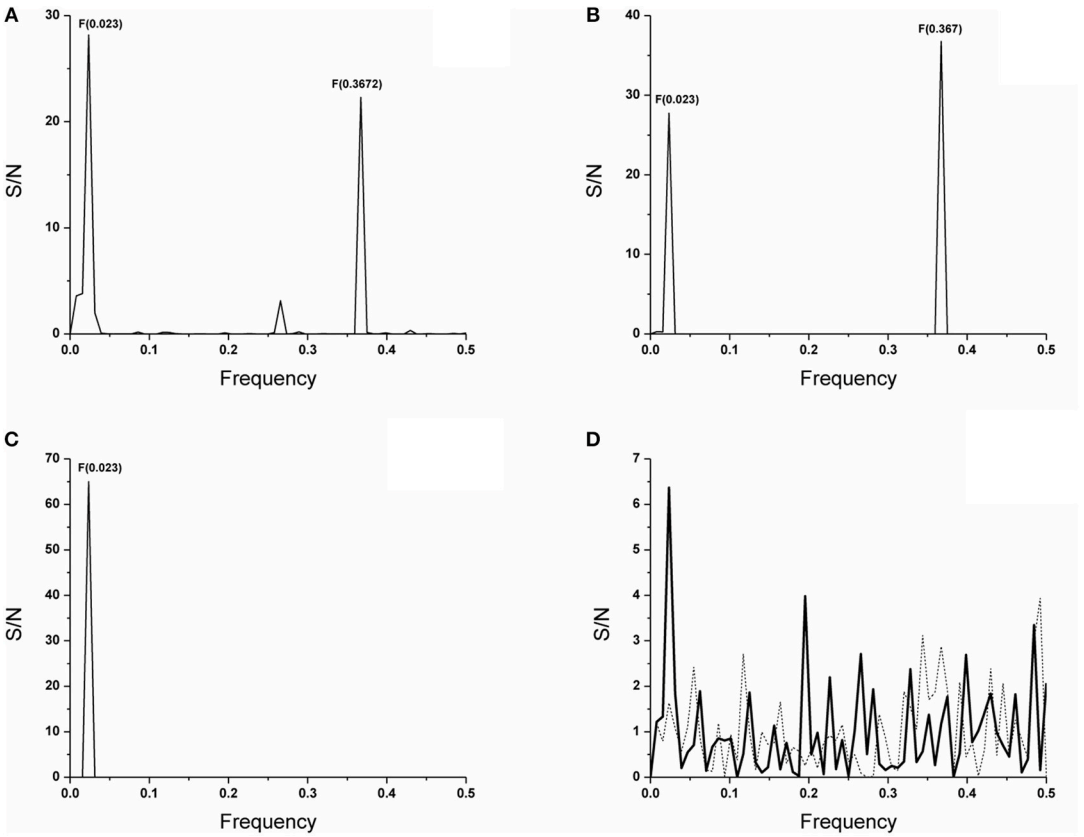

FIGURE 1 | ISM analysis of the V germline genes and antibody VH domain protein sequences. The CIS of the V germline genes segments of VH1, VH11, S57(VH31) and MRL-DNA22 which shows characteristic F(0.367) relevant for the biological activity here followed and activity irrelevant F(0.023) **(A)**. The CIS of the VH domains of antibodies 74.c2 encoded by the V gene segment VH11, a member of the VH7 (S107) gene family, 17s-c2 encoded by the V gene segment S57(VH31) and 17s.83, 17s-c3, 111.185 and 165.27 encoded by V gene segment MRL-DNA22 of the VH1 (J558) gene family **(B)**. CIS of all anti-DNA antibodies which in individual spectrum does not express F(0.367) **(C)**. The IS of the preimmune natural polyreactive autoantibodies which use V gene segments from VH2 (Q52) gene family such as D23 VH domain (___) and superimposed IS of the E7 VH domain (……) **(D)**. The abscissa represents the frequencies from the Fourier transform of the sequence of electron-ion interaction potential (EIIP). The lowest frequency is 0.0 and the highest is 0.5. The ordinate represents the signal to noise ratio (S/N) corresponding to each frequency component in the informational spectrum (IS).

TABLE 1 | Contribution of antibody VH domains and corresponding V gene segments to $S/N_{F(0.367)}$.

Antibodies	Antibody isotype	V germline gene segment used	VH gene family	$S/N_{F(0.367)}$	
				Domains	V gene segments
T15	IgG	T15(V1)	VH7 (S107)*	3.727	3.544
U4	IgG	T15(V1)	VH7 (S107)*	3.950	3.821
74.c2	IgG	VH11	VH7 (S107)*	5.622	5.304
17s-c2	IgG	S57(VH31)	VH1 (J558)*	4.148	4.008
17s.83	IgG	DNA22	VH1 (J558)*	3.789	3.383
17s-c3	IgG	DNA22	VH1 (J558)*	4.434	3.944
111.185	IgM	DNA22	VH1 (J558)*	3.963	3.623
165.27	IgM	DNA22	VH1 (J558)*	3.731	3.259

**Old nomenclature for VH gene families is given in parenthesis.*

contribution to F(0.367) expression is insignificant (**Table 1**), whereas IgM 111.185 (MRL-DNA22) anti-DNA antibody (31) retains V gene segment in germline configuration. The data presented may be in accord with the idea that V germline gene segments prone to bind a dsDNA epitope should be less dependent on CDR3 regions (48).

Anti-dsDNA antibodies derived from autoimmune mouse models revealed that they have undergone somatic mutations suggesting their role in achievement of the corresponding conformation. Thus, an important finding obtained from sequence analysis showed the presence of basic amino acids Arg, Lys, and His and, perhaps, the uncharged Asn in CDRs (28–31),

TABLE 2 | Sequence alignment for CDR2 regions of the V gene segments.

V gene segments	Germline configuration	Antibody	CDR2 region amino acid sequence																		
						abc															
MRL-DNA22	+		N	I	Y	P			G	S	S	S	T	**N**	Y	**N**	E	**K**	F	**K**	S
MRL-DNA22		111.185	–	–	–	–			–	–	–	–	–	–	–	–	–	–	–	–	–
MRL-DNA22		165.27	–	–	–	–			–	–	–	–	–	–	–	–	–	–	–	–	–
MRL-DNA22		17s.83	N	–	–	–			–	–	I	I	–	**H**	F	**N**	–	**K**	–	**K**	**N**
MRL-DNA22		17s-c3	E	–	–	–			**R**	–	G	**N**	I	Y	Y	**N**	–	**K**	–	**K**	G
						abc															
S57(VH31)	+		W	I	Y	S			G	S	G	**N**	T	**K**	Y	**N**	E	**K**	F	**K**	D
S57(VH31)		17s-c2	–	–	–	P			–	–	–	**N**	–	**K**	–	**N**	–	**K**	–	**K**	–
VH11	+		L	I	**R**	**N**	**K**	A	**N**	G	Y	T	T	E	Y	S	A	S	V	**K**	G
VH11		74-c2	–	–	**R**	**N**	**K**	–	**N**	**D**	–	–	–	–	–	–	–	–	–	**K**	–
						a	b	c													
T15(V1)	+		**A**	**S**	**R**	**N**	**K**	**A**	**N**	**D**	**Y**	**T**	**T**	**E**	**Y**	**S**	**A**	**S**	**V**	**K**	**G**
T15(V1)		T15	–	–	**R**	**N**	**K**	–	**N**	–	–	–	–	–	–	–	–	–	–	**K**	–
T15(V1)		U4	–	–	**R**	**N**	**K**	–	**N**	–	–	–	–	–	–	–	–	–	–	**K**	–
VH1210.7	+		Y	I	S	Y	S	G	S	T	Y	Y	**N**	P	S	L	**K**	S			
VH1210.7		E7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–			
VH101	+		V	I	W	S	G	G	S	T	D	Y	**N**	A	A	F	I	S			
VH101		D23	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–			

The CDR2 region sequences (aa 50-65) for V germline genes and epitope specific anti-DNA antibodies shown in the single letter code were taken from Tillman et al. (31). Origin of these V germline gene sequences are: S57(VH31) (30), MRL-DNA22 (44), VH11 (45). Sequences for T15(V1), as well as E7 and D23 antibodies are taken from Diamond and Scharff (13), Crews et al. (16), Rudikoff et al. (17), and Baccala et al. (46) respectively. Within the compared antibody V gene segments dashes indicate identity with the reference germline gene sequences, while basic residues are shown in bold. Numbering is according to Kabat et al. (47).

whereas Arg in the CDR3 has an important contribution in DNA specificity for DNA-histone complexes (32–34). However, cationic amino acids were not necessary for immune deposit formation (49). Thus, another goal of this study was to examine the role of the CDR2 regions in F(0.367) expression and in particular the content of basic residues in the CDR2 regions of V germline genes used by anti-DNA antibodies. The data obtained showed that peptides within sequences mostly contributing to F(0.367) expression cover residues at position 35–66 for T15(V1), 36–67 for VH11, 46–65 for S57(VH31), and 46–77 for MRL-DNA22. It should be emphasized that these peptide sequences include the CDR2 region enriched in basic residues of T15(V1), VH11, S57(VH31), and MRL-DNA22 germline genes and are also present in CDR2 regions of antibody VH domains (**Table 2**). It can be seen that type and positions of the basic residues for CDR2 of the T15(V1) and VH11 V germline gene segments and anti-DNA antibody V gene segments are the same and S57(VH31) differs slightly. The MRL-DNA22 anti-DNA IgM isotypes are close to germline configuration, whereas IgG differs in the type and position of basic residues (**Table 2**). The CDR2 regions of natural polyreactive autoantibodies are in germline configuration and have one or two basic residues, confirming that both the number and position in CDR2 regions are important for antibody epitope specificity.

The findings presented indicate that antibody specificity for an antigenic determinant (epitope) in the context of different antigens might be identified by the ISM approach (37).

The method applied made a possible correlation between primary antibody structure and specificity delineated by a characteristic frequency.

The main conclusion is that antibody VH domain sequences can encode ability expressed as characteristic frequency, to interact with non-protein structures of various molecules after achievement of the corresponding conformation by somatic mutations.

AUTHOR CONTRIBUTIONS

TS-R and RM developed the study design, analyzed the data and wrote the paper. HK and VJ revised the paper.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Expression of a Recombinant High Affinity IgG Fc Receptor by Engineered NK Cells as a Docking Platform for Therapeutic mAbs to Target Cancer Cells

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Anti-tumor mAbs are the most widely used and characterized cancer immunotherapy. Despite having a significant impact on some malignancies, most cancer patients respond poorly or develop resistance to this therapy. A known mechanism of action of these therapeutic mAbs is antibody-dependent cell-mediated cytotoxicity (ADCC), a key effector function of human NK cells. CD16A on human NK cells has an exclusive role in binding to tumor-bound IgG antibodies. Though CD16A is a potent activating receptor, it is also a low affinity IgG Fc receptor (FcγR) that undergoes a rapid downregulation in expression by a proteolytic process involving ADAM17 upon NK cell activation. These regulatory processes are likely to limit the efficacy of tumor-targeting therapeutic mAbs in the tumor environment. We sought to enhance NK cell binding to anti-tumor mAbs by engineering these cells with a recombinant FcγR consisting of the extracellular region of CD64, the highest affinity FcγR expressed by leukocytes, and the transmembrane and cytoplasmic regions of CD16A. This novel recombinant FcγR (CD64/16A) was expressed in the human NK cell line NK92 and in induced pluripotent stem cells from which primary NK cells were derived. CD64/16A lacked the ADAM17 cleavage region in CD16A and it was not rapidly downregulated in expression following NK cell activation during ADCC. CD64/16A on NK cells facilitated conjugation to antibody-treated tumor cells, ADCC, and cytokine production, demonstrating functional activity by its two components. Unlike NK cells expressing CD16A, CD64/16A captured soluble therapeutic mAbs and the modified NK cells mediated tumor cell killing. Hence, CD64/16A could potentially be used as a docking platform on engineered NK cells for therapeutic mAbs and IgG Fc chimeric proteins, allowing for switchable targeting elements and a novel cancer cellular therapy.

Keywords: FcR, ADCC, NK cell, immunotherapy, antibody

INTRODUCTION

Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system that target stressed, infected, and neoplastic cells (1). In contrast to the diverse array of receptors involved in natural cytotoxicity, human NK cells mediate ADCC exclusively through the IgG Fc receptor CD16A (FcγRIIIA) (2–4). This is a potent activating receptor and its signal transduction involves the association of the transmembrane and cytoplasmic regions of CD16A with FcRγ and/or CD3ζ (4–9). Unlike other activating receptors expressed by NK cells, the cell surface levels of CD16A undergo a rapid downregulation upon NK cell activation during ADCC and by other stimuli (10–14). CD16A downregulation also occurs in the tumor environment of patients and contributes to NK cell dysfunction (15–19). A disintegrin and metalloproteinase-17 (ADAM17) expressed by NK cells plays a key role in its downregulation by cleaving CD16A in a *cis* manner at a specific location proximal to the cell membrane upon NK cell activation (13, 14, 20).

There are two allelic variants of CD16A that have either a phenylalanine or valine residue at position 176 (position 158 if amino acid enumeration does not include the signal sequence). The CD16A-176V variant has a higher affinity for IgG (21, 22), but CD16A-176F is the dominant allele in humans (23). Clinical analyses have revealed a positive correlation between the therapeutic efficacy of tumor-targeting therapeutic mAbs and CD16A binding affinity. Patients homozygous for the CD16A valine variant (CD16A-V/V) had an improved clinical outcome after treatment with anti-tumor mAbs compared to those who were either heterozygous (CD16A-V/F) or homozygous (CD16A-F/F) for the lower affinity FcγRIIIA isoform [as reviewed in Wang et al. (4)]. These findings establish that increasing the binding affinity of CD16A for anti-tumor mAbs may lead to improved cancer cell killing.

CD64 (FcγR1) binds to monomeric IgG with 2–3 orders of magnitude higher affinity than CD16A (24–26). CD64 recognizes the same IgG isotypes as CD16A and is expressed by myeloid cells, including monocytes, macrophages, and activated neutrophils, but not NK cells (24, 26). We generated the novel recombinant receptor CD64/16A that consists of the extracellular region of human CD64 for high affinity antibody binding, and the transmembrane and intracellular regions of human CD16A for mediating NK cell signal transduction. CD64/16A also lacked the membrane proximal ADAM17 cleavage site found in CD16A. In this study, we stably expressed CD64/16A in NK92 cells, a cytotoxic human NK cell line that lacks endogenous FcγRs (27), and in induced pluripotent stem cells (iPSCs) that were then differentiated into primary NK cells. We show that in these two NK cell platforms, this novel recombinant FcγR is functional and can capture soluble monomeric IgG therapeutic mAbs that provide targeting elements for tumor cell ADCC.

MATERIALS AND METHODS

Antibodies

All mAbs to human hematopoietic and leukocyte phenotypic markers are described in **Table 1**. All isotype-matched negative control mAbs were purchased from BioLegend (San Diego, CA). APC-conjugated $F(ab')_2$ donkey anti-human or goat anti-mouse IgG (H+L) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The human IgG1 mAbs trastuzumab/Herceptin and rituximab/Rituxan, manufactured by Genentech (South San Francisco, CA), and cetuximab/Erbitux, manufactured by Bristol-Myers Squibb (Lawrence, NJ), were purchased through the University of Minnesota Boynton Pharmacy. Recombinant human L-selectin/IgG1 Fc chimera was purchased from R&D Systems (Minneapolis, MN).

TABLE 1 | Antibodies.

Antigen	Clone	Fluorophore	Company
CD56	HCD56	PE-CY7	BioLegend, San Diego, CA
CD3	HIT3a	PE	BioLegend
CD16	3G8	APC	BioLegend
CD16	3G8	none	Ancell, Bayport, MN
CD7	CD7-6B7	PE/CY5	BioLegend
CD336/NKp44	P44-8	APC	BioLegend
CD335/NKp46	9E2	APC	BioLegend
CD159a/NKG2A	Z199	APC	Beckman Coulter, Brea, CA
CD314/NKG2D	1D11	PerCP/Cy5.5	BioLegend
CD158a/KIR2DL1	HP-MA4	PE	BioLegend
CD158b1/KIR2DL2/L3	DX27	PE	BioLegend
CD158e1/KIR3DL1	DX9	PE	BioLegend
CD94	DX22	PE	BioLegend
CD64	10.1	APC	BioLegend
CD64	10.1	none	Ancell
CD34	561zz	PE	BioLegend
CD45	2D1	APC	BioLegend
CD43	CD43-10G7	APC	BioLegend
CD62L/L-selectin	LAM1-116	APC	Ancell

Generation of Human CD64/16A

Total RNA was isolated from human peripheral blood leukocytes using TRIzol total RNA isolation reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized with the SuperScript preamplification system (Invitrogen). The recombinant CD64/16A is comprised of human CD64 extracellular domain and CD16A transmembrane and cytoplasmic domains. PCR fragments for CD64 (885 bps) and CD16A (195 bps) were amplified from the generated cDNA. The PCR fragments were purified and mixed together with the forward primer 5′- CGG *GAA TTC* GGA GAC AAC ATG TGG TTC TTG ACA A-3′, the reverse primer 5′- CCG *GAA TTC* TCA TTT GTC TTG AGG GTC CTT TCT-3′ (underlined nucleotides are EcoR I sites), and Pfx50 DNA polymerase (Invitrogen) to generate the recombinant CD64/16A receptor. CD64/CD16A and CD16A cDNA (CD16A-176V variant) was inserted into the retroviral expression vector pBMN-IRES-EGFP and virus was generated for NK92 cell transduction, as previously described (14). For this study, the transduced NK92 cells were sorted

by flow cytometry to derive populations with homogenous expression of CD16A or CD64/16A, but consisted of a mixed clonal population to avoid the effects of random genomic integration of vector DNA in a single NK92 transductant, as we have done in previous studies (14, 20). Additionally, CD64/CD16A cDNA was inserted into a pKT2 sleeping beauty transposon vector and used along with SB100X transposase for iPSC transduction, as previously described (14). The nucleotide sequences of all constructs were confirmed by direct sequencing from both directions on an ABI 377 sequencer with ABI BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

Cells

Fresh human peripheral blood leukocytes from plateletpheresis were purchased from Innovative Blood Resources (St. Paul, MN). Peripheral blood mononuclear cells were further enriched using Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and NK cells were purified by negative depletion using an EasySep human NK cell kit (StemCell Technologies, Cambridge, MA), as per the manufacturer's instructions, with >95% viability and 90–95% enrichment of $CD56^+CD3^-$ lymphocytes. Viable cell counting was performed using a Countess II automated cell counter (Life Technologies Corporation, Bothell, WA). The human NK cell line NK92 and the ovarian cancer cell line SKOV-3 were obtained from ATCC (Manassas, VA) and cultured per the manufacturer's directions. The NK92 cells required IL-2 for growth (500 IU/ml), which was obtained from R&D Systems and the National Cancer Institute, Biological Resources Branch, Preclinical Biologics Repository (Frederick, MD). Heat inactivated Gibco FBS was purchased from Thermo Fisher Scientific (Waltham, MA), and heat inactivated horse serum was purchased from Sigma-Aldrich (St. Louis, MO). For all assays described below, cells were used when in log growth phase.

The iPSCs UCBiPS7, derived from umbilical cord blood CD34 cells, have been previously characterized and were cultured and differentiated into hematopoietic progenitor cells as described with some modifications (14, 28–31). iPSC culture and hematopoietic differentiation was performed using TeSR-E8 medium and a STEMdiff Hematopoietic Kit (StemCell Technologies), which did not require the use of mouse embryonic fibroblast feeder cells, TrypLE adaption, spin embryoid body formation, or $CD34^+$ cell enrichment. To passage iPSCs, cells were dissociated with Gentle Cell Dissociation Reagent (StemCell Technologies) and aggregates $\geq$50 μm in diameter were counted with a hemocytometer and diluted to 20–40 aggregates/ml with TeSR-E8 medium. Each well of a 12-well plate was pre-coated with Matrigel Matrix (Corning Inc., Tewksbury, MA) and seeded with 40–80 aggregates in 2 ml of TeSR-E8 medium. Cell aggregates were cultured for 24 h before differentiation with the STEMdiff Hematopoietic Kit, as per the manufacturer's instructions. At day 12 of hematopoietic progenitor cell differentiation, the percentage of hematopoietic progenitor cells was determined using flow cytometric analysis with anti-CD34, anti-CD45, and anti-CD43 mAbs. NK cell differentiation was performed as previously described (32). The iPSC-derived NK cells, referred to here as iNK cells, were expanded for examination using γ-irradiated K562-mbIL21-41BBL feeder cells at a 1:2 ratio in cell expansion medium [60% DMEM, 30% Ham's F12, 10% human AB serum (Valley Biomedical, Winchester, VA), 20 μM 2-mercaptoethanol, 50 μM ethanolamine, 20 μg/ml ascorbic Acid, 5 ng/ml sodium selenite, 10 mM HEPES, and 100–250 IU/ml IL-2 (R&D Systems)], as described previously (14, 29–31).

Cell Staining, IFNγ Quantification, and Flow Cytometric Analysis

For cell staining, 0.5×10^6-1×10^6 cells were stained with the indicated mAbs (**Table 1**) using flow buffer (dPBS containing 2.5% FBS and 0.02% sodium azide) and 1×10^4-5×10^4 cells per sample were acquired, as previously described (11, 14, 20). For controls, fluorescence minus one was used as well as appropriate isotype-matched antibodies since the cells of interest expressed FcRs. A FSC-A/SSC-A plot was used to set an electronic gate on leukocyte populations and an FSC-A/FSC-H plot was used to set an electronic gate on single cells. A Zombie viability kit was used to assess live vs. dead cells, as per the manufacturer's instructions (BioLegend).

To assess the capture of soluble trastuzumab, rituximab, cetuximab, or L-selectin/Fc chimera, transduced NK cells were incubated with 5 μg/ml of antibody for 2 h at 37°C in MEM-α basal media (Thermo Fisher Scientific) supplemented with IL-2 (200 IU/ml), HEPES (10 mM), and 2-mercaptoethanol (0.1 mM), washed with MEM-α basal media, and then stained on ice for 30 min with a 1:200 dilution of APC-conjugated $F(ab')_2$ donkey anti-human IgG (H + L). To detect recombinant human L-selectin/Fc binding, cells were stained with the anti-L-selectin mAb APC-conjugated Lam1-116. To compare CD16A and CD64/16A staining levels on NK92 cells, the respective transductants were stained with a saturating concentration of unconjugated anti-CD16 (3G8) or anti-CD64 (10.1) mAbs (5 μg/ml), washed extensively with dPBS containing 2% goat serum and 2 mM sodium azide, and then stained with APC-conjugated $F(ab')_2$ goat anti-mouse IgG (H+L). This approach was used since directly conjugated anti-CD16 and anti-CD64 mAbs can vary in their levels of fluorophore labeling. IFNγ quantification was performed by a cytometric bead-based Flex Set assay (BD Biosciences, San Jose, CA), per the manufacturer's instructions. The minimum and maximum limit of detection for the assay was 1.8 and 2,500 pg/ml, respectively. All flow cytometric analyses were performed on a FACSCelesta instrument (BD Biosciences). Data was analyzed using FACSDIVA v8 (BD Biosciences) and FlowJo v10 (Ashland, OR).

Cell-Cell Conjugation Assay and ADCC

NK92 cells used in these assays were transduced with pBMN-IRES-EGFP empty vector or vector containing CD64/16A or CD16A cDNA. For all transductants, nearly 100% of the cells expressed GFP. The NK92 transductants were initially serum-starved for 2 h at 37°C in MEM-α basal media (Thermo Fisher Scientific, Waltham, MA) supplemented with IL-2 (200 IU/ml),

HEPES (10 mM), and 2-mercaptoethanol (0.1 mM). SKOV-3 cells were labeled with CellTrace Violet (Molecular Probes, Eugene, OR) per the manufacturer's instructions, incubated with 5 μg/ml trastuzumab for 30 min and washed with MEM-α basal media. NK92 cells and SKOV-3 cells were then resuspended in the supplemented MEM-α basal media at 1×10^6 and 2×10^6/ml, respectively. For a 1:2 Effector:Target (E:T) ratio, 100 μl of each cell type was mixed together, centrifuged for 1 min at 20 × g and incubated at 37°C for the indicated time points. After each time point, the cells were gently vortexed for 3 s and immediately fixed with 1% paraformaldehyde in dPBS at 4°C. Cells were immediately analyzed by flow cytometry for which 2×10^4 cells per sample were acquired for analyses. The percentage of conjugated NK cells was calculated by gating on GFP and CellTrace Violet double-positive events.

To evaluate ADCC, a DELFIA EuTDA-based cytotoxicity assay was used according to the manufacturer's instructions (PerkinElmer, Waltham, MA). Briefly, target cells were labeled with Bis(acetoxymethyl)-2-2:6,2 terpyridine 6,6 dicarboxylate (BATDA) for 30 min in their culture medium, washed in culture medium, and pipetted into a 96-well non-tissue culture-treated U-bottom plates at a density of 8×10^3 cells/well. A tumor targeting mAb was added at the indicated concentrations and NK cells were added at the indicated E:T ratios. The plates were centrifuged at 400 × g for 1 min and then incubated for 2 h in a humidified 5% CO_2 atmosphere at 37°C. At the end of the incubation, the plates were centrifuged at 500 × g for 5 min and supernatants were transferred to a 96 well DELFIA Yellow Plate (PerkinElmer) and combined with europium. Fluorescence was measured by time-resolved fluorometry using a BMG Labtech CLARIOstar plate reader (Cary, NC). BATDA-labeled target cells alone with or without therapeutic antibodies were cultured in parallel to assess spontaneous lysis and in the presence of 2% Triton-X to measure maximum lysis. ADCC for each sample is represented as Percent Specific Release and was calculated using the following formula:

$$Percent\ Specific\ Release = \frac{(Experimental\ release - Spontaneous\ release)}{(Maximal\ release - Spontaneous\ release)} * 100$$

For each experiment, measurements were conducted in triplicate using two-three replicate wells.

Statistical Analyses

Statistical analyses were performed by use of GraphPad Prism (GraphPad Software, La Jolla, CA, USA). After assessing for approximate normal distribution, all variables were summarized as mean ± *SD*. Comparison between 2 groups was done with Student's *t*-test, with $p < 0.05$ taken as statistically significant.

RESULTS

Expression and Function of CD64/16A in NK92 Cells

We engineered a recombinant FcγR that consists of the extracellular region of human CD64 and the transmembrane and cytoplasmic regions of human CD16A, referred to as CD64/16A (**Figure 1A**). The human NK cell line NK92 stably expressing this recombinant receptor were initially used to examine its function. These cells lack endogenous FcγRs but can mediate ADCC when expressing recombinant CD16A (14, 20, 27). As shown is **Figure 1B**, NK92 cells expressing CD64/16A were positively stained by an anti-CD64 mAb, whereas parental NK92 cells or NK92 cells expressing CD16A were not. CD16A is known to undergo ectodomain shedding upon NK cell activation resulting in its rapid downregulation in expression (10–13, 20). CD16A as well as its isoform CD16B on neutrophils is cleaved by ADAM17 (10), and this occurs at an extracellular region proximal to the cell membrane (13, 14). The ADAM17 cleavage region of CD16A is not present in CD64 or CD64/16A (**Figure 1A**). We found that CD16A underwent a >50% decrease in expression upon NK92 stimulation by ADCC, whereas CD64/16A demonstrated little to no downregulation (**Figure 1C**).

To establish the functional activity of CD64/16A, we determined its ability to promote E:T conjugation, induce ADCC, and stimulate IFN-γ production. SKOV-3 cells, an ovarian cancer cell line that expresses HER2, were used as the target. A two-color flow cytometric approach was used to quantify the conjugation of NK92-CD64/16A cells and SKOV-3 cells in the absence and presence of the anti-HER2 therapeutic mAb trastuzumab. A bicistronic vector was used for CD64/16A as well as GFP expression, and its fluorescence was used to identify the NK92 cells. SKOV-3 cells were labeled with the fluorescent dye CellTrace Violet. The incubation of NK92-CD64/16A cells with SKOV-3 cells alone resulted in a low level of conjugation after 60 min of exposure (**Figure 2A**). E:T conjugation was markedly increased in the presence of trastuzumab, and this was effectively disrupted by the presence of the anti-CD64 mAb 10.1 (**Figure 2A**), which blocks IgG binding (33). The addition of trastuzumab, however, did not enhance E:T conjugation by NK92 cells transduced with an empty vector expressing only GFP (NK92 control cells) (**Figure 2A**).

An increase in SKOV-3 cell conjugation by NK92-CD64/16A cells corresponded with increased cytolytic activity. We determined direct target cell killing by NK92-CD64/16A cells using an ADCC assay in which various concentrations of trastuzumab and E:T ratios were examined. We show in **Figure 2B** effective SKOV-3 cytotoxicity by NK92-CD64/16A cells in the presence of trastuzumab that decreased with lower mAb concentrations and lower E:T ratios. To confirm the role of CD64/16A in the induction of target cell killing, we also performed the assay in the presence the anti-CD64 mAb 10.1, which effectively blocked ADCC (**Figure 2C**).

Cytokine production is also induced during ADCC and NK cells are major producers of IFNγ (4, 34). NK92-CD64/16A cells exposed to SKOV-3 cells and trastuzumab produced considerably higher levels of IFNγ than when exposed to SKOV-3 cells alone (**Figure 2D**). Taken together, the above findings demonstrate that the CD64 component of the recombinant receptor engages

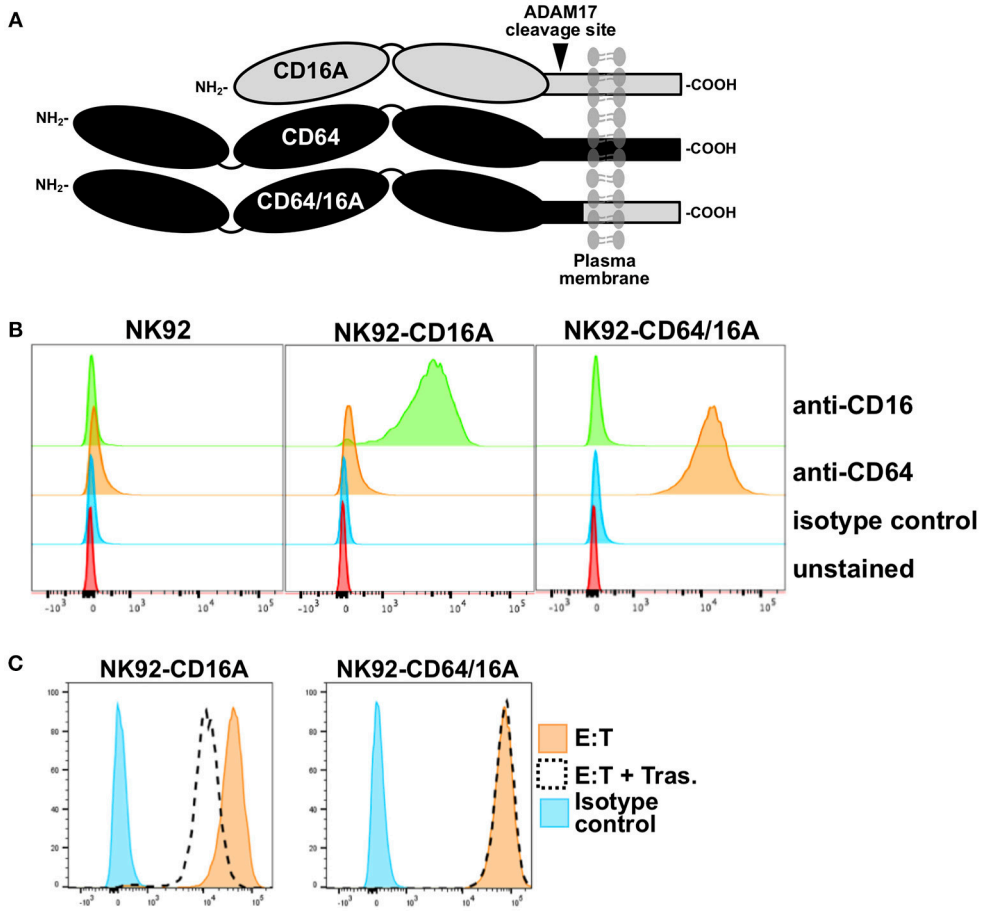

FIGURE 1 | Expression of CD64/16A by NK92 cells**. (A)** Schematic representation of the cell membrane forms of CD16A, CD64, and CD64/16A. CD16A undergoes ectodomain shedding by ADAM17 at a membrane proximal location, as indicated, which is not present in CD64 and CD64/16A. **(B)** NK92 parental cells, NK92-CD16A cells, and NK92-CD64/16A cells were stained with an anti-CD16, anti-CD64, or an isotype-matched negative control mAb and examined by flow cytometry. **(C)** NK92-CD16A and NK92-CD64/16A cells were incubated with SKOV-3 cells with or without trastuzumab (5 μg/ml) at 37° C (E:T = 1:1; 5×10^5 cells to 5×10^5 cells) for 2 h. The NK92-CD16A and NK92-CD64/16A cells were then stained with an anti-CD16 mAb or an anti-CD64 mAb, respectively, and examined by flow cytometry. Non-specific antibody labeling was determined using the appropriate isotype-negative control mAb. **(B,C)** Data is representative of at least three independent experiments.

tumor-bound antibody, and that the CD16A component promotes intracellular signaling leading to degranulation and cytokine production.

CD64/16A as a Docking Platform For Antibodies

CD64 is distinguished from the other FcγR members by its unique third extracellular domain, which contributes to its high affinity and stable binding to soluble monomeric IgG (26). We compared the ability of NK92 cells expressing CD64/16A or the high affinity variant of CD16A (CD16A-176V) to capture soluble therapeutic mAbs. NK92 cell transductants expressing similar levels of CD64/16A and CD16A (**Figure 3A**) were incubated with trastuzumab for 2 h. After which, excess antibody was washed away and the cells were stained with a fluorophore-conjugated anti-human IgG antibody and then evaluated by flow cytometry. As shown in **Figure 3B**, NK92-CD64/16A cells captured considerably higher levels of trastuzumab than did the NK92-CD16A cells (8.1-fold increase ± 1.3, mean ± *SD* of 3 independent experiments). In addition, the NK92-CD64/16A cells efficiently captured the tumor-targeting mAbs Erbitux/cetuximab and Rituxan/rituximab, as well as the fusion protein L-selectin/Fc (**Figure 3C**). We then tested whether NK92-CD64/16A cells with a captured tumor-targeting mAb mediated ADCC. For this assay, we compared equal numbers of NK92-CD64/16A and NK92-CD16A cells that were incubated with the same concentration of soluble trastuzumab, washed, and exposed to SKOV-3 cells. We observed that target cell killing by NK92-CD64/16A cells with captured trastuzumab was significantly higher than by these same cells in the absence trastuzumab and was far superior to NK92-CD16A cells at all E:T ratios examined (**Figure 3D**). In contrast, SKOV-3 cytotoxicity by NK92-CD16A and NK92-CD64/16A cells was not significantly different if trastuzumab was present

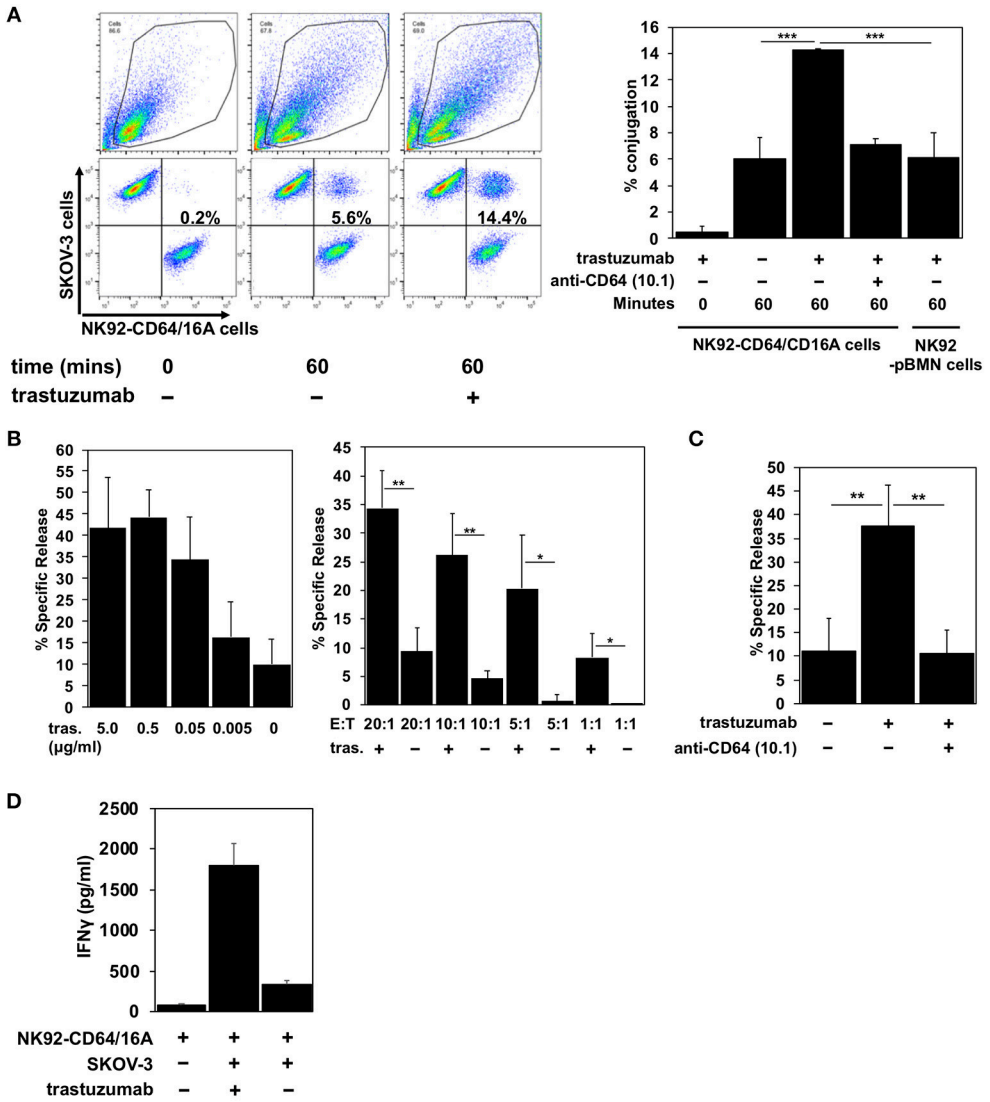

FIGURE 2 | CD64/16A promotes target cell conjugation, ADCC, and IFNγ production**. (A)** NK92 control cells or NK92-CD64/16A cells, both expressing GFP, and SKOV-3 cells, labeled CellTrace Violet, were mixed at an E:T ratio of 1:2 (1×10^5 cells to 2×10^5 cells) in the presence or absence of trastuzumab (5 μg/ml) and the anti-CD64 mAb 10.1 (10 μg/ml), incubated at 37°C up to 60 min, fixed, and then analyzed by flow cytometry, as described in the Materials and Methods. For the flow cytometric data, representative data are shown. For the bar graphs, mean ± *SD* of three independent experiments is shown. Statistical significance is indicated as ***$p < 0.001$. **(B)** NK92-CD64/16A cells were incubated with SKOV-3 cells (E:T = 20:1; 1.6×10^5 cells to 8×10^3 cells) and trastuzumab (tras.) at the indicated concentrations (left panel), or with SKOV-3 cells at the indicated E:T ratios in the presence or absence of trastuzumab (5 μg/ml) (right panel) for 2 h at 37°C. Data are represented as % specific release and the mean ± SD of 3 independent experiments is shown. Statistical significance is indicated as *$p < 0.05$, **$p < 0.01$. **(C)** NK92-CD64/16A cells were incubated with SKOV-3 cells (E:T = 20:1; 1.6×10^5 cells to 8×10^3 cells) in the presence or absence of trastuzumab (5 μg/ml) and the anti-CD64 mAb 10.1 (10 μg/ml), as indicated, for 2 h at 37°C. Data are represented as % specific release and the mean ± *SD* of 3 independent experiments is shown. Statistical significance is indicated as **$p < 0.01$. **(D)** NK92-CD64/16A cells were incubated with SKOV-3 cells (E:T = 1:1; 1×10^5 cells to 1×10^5 cells) with or without trastuzumab (5 μg/ml) for 2 h at 37°C. Secreted IFNγ levels were quantified by ELISA. Data is shown as mean of 2 independent experiments.

during the assay and not initially docked to the transductants (**Figure 3E**). This demonstrates that the different transductants had an equivalent cytolytic capacity. Taken together, these findings show that NK92 cells expressing CD64/16A can stably bind soluble anti-tumor mAbs and IgG fusion proteins, and that these can serve as targeting elements to kill cancer cells.

Expression and Function of CD64/16A in iPSC-Derived NK Cells

We also examined the function of CD64/16A in engineered primary NK cells. Genetically modifying peripheral blood NK cells by retroviral or lentiviral transduction at this point has been challenging (35). Embryonic stem cells and iPSCs can be differentiated into cytolytic NK cells *in vitro* (28–31, 36),

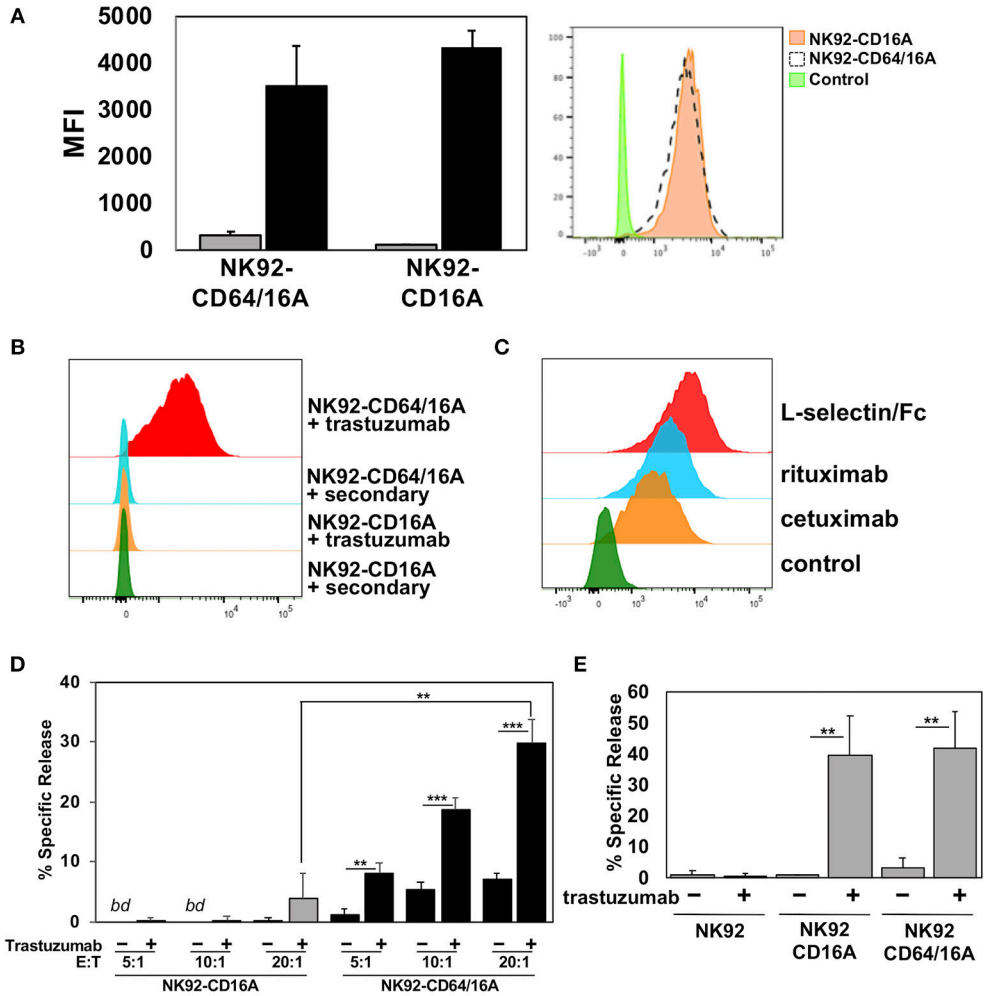

FIGURE 3 | CD64/16A attaches to soluble tumor-targeting mAbs and IgG fusion proteins**. (A)** Relative expression levels of CD16A and CD64/16A on NK92 cells were determined by cell staining with anti-CD16 and anti-CD64 mAbs (black bars), respectively, or an isotype-matched negative control antibody (gray bars). The bar graph shows mean fluorescence intensity (MFI) ± *SD* of three independent experiments. Representative flow cytometric data are shown in the histogram overlay. The dashed line histogram shows CD64 staining of NK92-CD64/16A cells, the orange-filled histogram shows CD16A staining of NK92-CD16A cells, and the green-filled histogram shows isotype control antibody staining of the NK92-CD16A cells. **(B)** NK92-CD16A and NK92-CD64/16A cells were incubated with or without trastuzumab (5 μg/ml) for 2 h at 37°C, washed, stained with a fluorophore-conjugated anti-human secondary antibody, and analyzed by flow cytometry. Data is representative of at least 3 independent experiments. **(C)** NK92-CD64/16A cells were incubated with cetuximab or rituximab (5 μg/ml for each), washed, and then stained with a fluorophore-conjugated anti-human secondary antibody. Control represents cells stained with the anti-human secondary antibody only. NK92-CD64/16A cells were also incubated with L-selectin/Fc (5 μg/ml), washed, and then stained with a fluorophore-conjugated anti-L-selectin mAb. NK92 cells lack expression of endogenous L-selectin (data not shown). All staining was analyzed by flow cytometry. Data shown are representative of 3 independent experiments. **(D)** NK92-CD16A and NK92-CD64/16A cells were incubated in the presence or absence of trastuzumab (5μg/ml), washed, and exposed to SKOV-3 cells (8×10^3) at the indicated E:T cell ratios for 2 h at 37°C. Data is shown as mean ± SD of 3 independent experiments. Statistical significance is indicated as $^{**}p < 0.01$, $^{***}p < 0.001$. *bd* = below detection, (i.e., < spontaneous release by negative control cells). **(E)** NK92-CD16A and NK92-CD64/16A cells were incubated with SKOV-3 cells (E:T = 10:1; 8×10^4 cells to 8×10^3 cells) in the presence or absence of trastuzumab (5 μg/ml), as indicated, for 2 h at 37°C. Data is shown as mean ± SD of 3 independent experiments. Statistical significance is indicated as $^{**}p < 0.01$.

and these cells are highly amendable to genetic engineering (14, 30, 37, 38). Undifferentiated iPSCs were transduced to express CD64/16A using a sleeping beauty transposon plasmid for non-random gene insertion and stable expression. iPSCs were differentiated into hematopoietic cells and then iNK cells by a two-step process that we have previously described (14, 28, 29). For this study, we modified the hematopoietic differentiation method to streamline the procedure by using a commercially available media and hematopoietic differentiation kit, as described in the Materials and Methods. $CD34^+CD43^+CD45^+$ cells were generated, further differentiated into iNK cells, and these cells were expanded for analysis using recombinant IL-2 and K562-mbIL21-41BBL feeder cells. $CD56^+CD3^-$ is a hallmark phenotype of human NK cells, and these cells

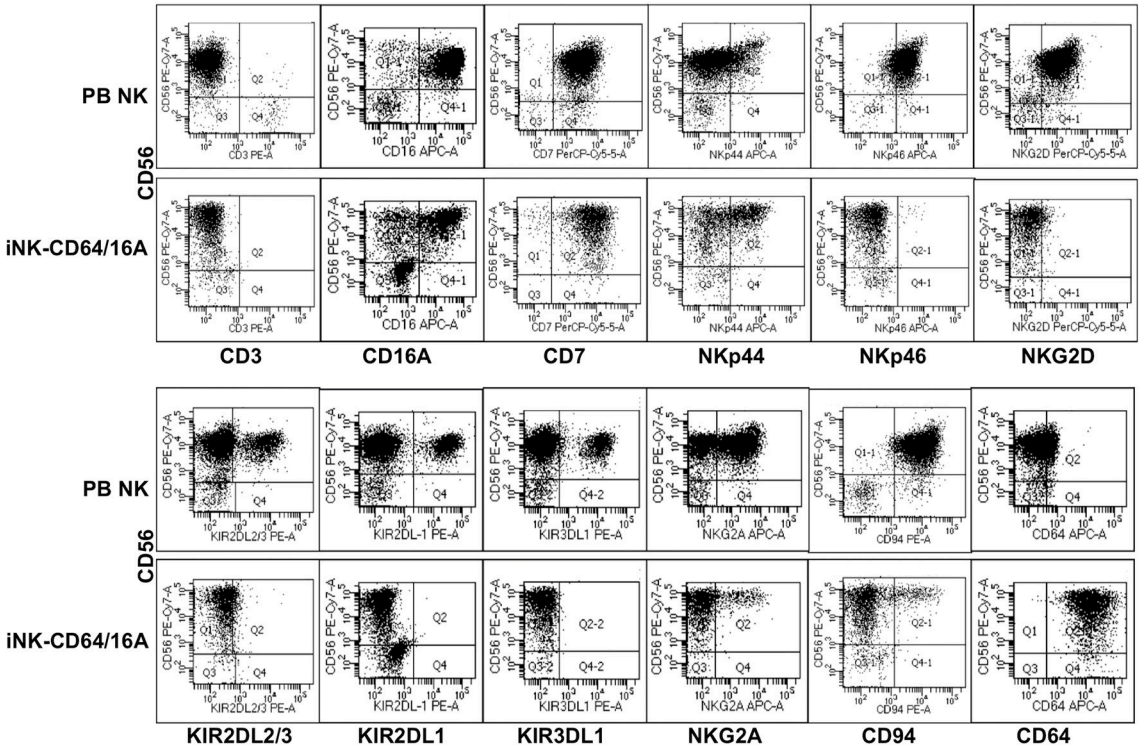

FIGURE 4 | Generation of iNK cells expressing CD64/CD16A. iPSCs were transduced to stably express CD64/16A, differentiated into NK cells, and then expanded using K562-mbIL21-41BBL feeder cells, as described in the Materials and Methods. iNK-CD64/16A cells and freshly isolated peripheral blood (PB) NK cells were stained for CD56, CD3 and various inhibitory and activating receptors, as indicated. CD64/16A expression was determined by staining the cells with an anti-CD64 mAb. For the iNK-CD64/16A cells, representative data from at least three independent experiments are shown. For the PB NK cells, three separate blood donors were examined and data from one donor is shown.

composed the majority of our differentiated cell population (**Figure 4**). We also assessed the expression of a number of activating and inhibitory receptors on the iNK cells and compared this to freshly enriched, peripheral blood NK cells. The latter cells have heterogenous phenotypes among individuals and in particular for their inhibitory receptors (39–41). In contrast to the subsets of peripheral blood NK cells expressing the inhibitory KIR receptors KIR2DL2/3, KIR2DL1, and KIR3DL1, the expanded iNK cells lacked expression of these receptors and the activating receptors NKp46 and NKG2D (**Figure 4**). Of importance is that unlike peripheral blood NK cells, essentially all of the iNK cells were stained with an anti-CD64 mAb (**Figure 4**), demonstrating the expression of CD64/16A.

To assess the function of CD64/16A, we compared iNK cells derived from iPSCs transduced with either a pKT2 empty vector or pKT2-CD64/16A. The NK cell markers mentioned above were expressed at similar levels and proportions by the two iNK cell populations (data not shown), including CD16A (**Figure 5A**), but only the iNK-CD64/16A cells were stained by an anti-CD64 mAb (**Figure 5A**). Both iNK transductants demonstrated increased SKOV-3 cell killing when in the presence of trastuzumab, yet iNK-CD64/16A cells mediated significantly higher levels of ADCC than did the iNK-pKT2 control cells (**Figure 5B**). The anti-CD16 function blocking mAb 3G8, but not the anti-CD64 mAb 10.1, effectively inhibited ADCC by the iNK-pKT2 cells (**Figure 5B**). Conversely, 10.1, but not 3G8, blocked ADCC by the iNK-CD64/16A cells (**Figure 5B**). These findings show that the generated iNK cells were cytolytic effectors responsive to CD16A and CD64/16A engagement of antibody-bound tumor cells. We also treated iNK-CD64/16A and iNK-pKT2 cells with soluble trastuzumab, washed away excess antibody, and exposed them to SKOV-3 cells. Under these conditions, ADCC by the iNK-CD64/16A cells was markedly higher than the iNK-pKT2 cells (**Figure 5C**), further establishing that CD64/16A can capture soluble anti-tumor mAbs that serve as a targeting element for tumor cell killing.

DISCUSSION

CD16A has an exclusive role in inducing ADCC by human NK cells (2–4). The affinity of antibody binding and the expression levels of this IgG Fc receptor modulate NK cell effector functions and affect the efficacy of tumor-targeting therapeutic mAbs (4, 11, 19, 20). To enhance anti-tumor antibody binding by NK cells, we expressed a novel recombinant FcγR consisting of the extracellular region of the high affinity

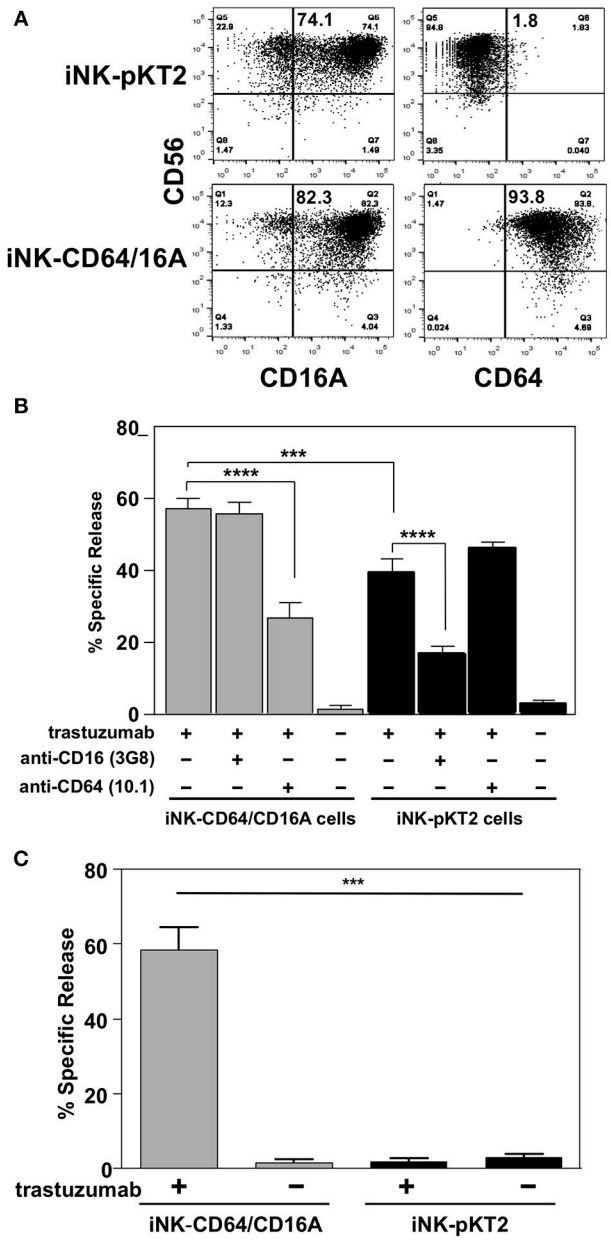

FIGURE 5 | iNK-CD64/16A cells show enhanced ADCC compared to iNK-pKT2 control cells**. (A)** iNK cells derived from empty vector (iNK-pKT2) or CD64/16A (iNK-CD64/16A) transduced iPSCs were stained for CD56, CD64, and CD16A, as indicated. The percentage of $CD56^+$ cells positively staining for CD16A or CD64 are indicated in the top right quadrant. **(B)** iNK-pKT2 or iNK-CD64/16A cells were incubated with SKOV-3 cells (E:T = 10:1; 8×10^4 cells to 8×10^3 cells) in the presence or absence of trastuzumab (5μg/ml), the function blocking anti-CD16 mAb 3G8 (5 μg/ml), and the function blocking anti-CD64 mAb 10.1 (5 μg/ml), as indicated, for 2 h at 37°C. Data is shown as mean ± SD of three independent experiments. Statistical significance is indicated as $^{***}p < 0.001$; $^{****}p < 0.0001$. **(C)** iNK-pKT2 and iNK-CD64/16A cells were incubated in the presence or absence of trastuzumab (5 μg/ml), washed, and exposed to SKOV-3 cells (E:T = 10:1; 8×10^4 cells to 8×10^3 cells) for 2 h at 37°C. Data is shown as mean ± *SD* of three independent experiments. Statistical significance is indicated as $^{***}p < 0.001$.

FcγR CD64 and the transmembrane and intracellular regions of CD16A. NK cells expressing CD64/16A facilitated cell conjugation with antibody-bound tumor cells, cytotoxicity, and IFNγ production, demonstrating function by both components of the recombinant FcγR. CD64/16A lacks the ADAM17 cleavage region found in CD16A and it did not undergo the same level of downregulation in expression during ADCC. Moreover, consistent with the ability of CD64 to stably bind soluble monomeric IgG, NK cells expressing CD64/16A could capture soluble anti-tumor therapeutic mAbs and kill target cells.

We demonstrate that CD64/16A was functional in two human NK cell platforms, the NK92 cell line and primary NK cells derived from iPSCs. NK92 cells lack inhibitory KIR receptors and show high levels of natural cytotoxicity compared to other NK cell lines derived from patients (42). NK92 cells have been widely used to express modified genes to direct their cytolytic effector function, have been evaluated in preclinical studies, and are undergoing clinical testing in cancer patients (42, 43). iPSCs are also very amendable to genetic engineering and can be differentiated into NK cells expressing various receptors to direct their effector functions (14, 30, 37, 38). For this study, we streamlined and standardized the hematopoietic differentiation step using a commercial kit. Though the generated iNK cells lacked several inhibitory and activating receptors compared to peripheral blood NK cells and iNK cells in previous studies (29–31), the majority of the cells were $CD16A^+$, which is expressed by mature NK cells (44), and mediated ADCC, demonstrating they were cytotoxic effector cells. The particular phenotype of the iNK cells will be important for the desired effector functions. However, to better direct and enhance their anti-tumor activity through the expression of engineered receptors and reduce their off-target effects, it may be advantageous for the iNK cells not to express endogenous inhibitory and activating receptors. Tumor cell ADCC by iNK cells expressing CD64/16A cells was significantly blocked by an anti-CD64 mAb. Interestingly, in contrast to pKT2 vector control iNK cells, ADCC by the iNK-CD64/16A cells was not blocked by an anti-CD16 mAb. Why endogenous CD16A in the iNK-CD64/16A cells did not have a role in the *in vitro* ADCC assay is unclear at this time. This may be due to a competitive advantage by CD64/16A over endogenous CD16A in binding antibody and/or in utilizing the same pool of downstream signaling factors.

An individual NK cell can kill multiple tumor cells in different manners. This includes by a process of sequential contacts and degranulations, referred to as serial killing (45, 46), and by the localized dispersion of its granule contents that kills surrounding tumor cells, referred to as bystander killing (47). Further studies are required to determine the effects of CD64/16A expression on these killing processes during ADCC. Inhibiting CD16A shedding has been reported to slow NK cell detachment from target cells and reduce serial killing by NK cells *in vitro* (48). Due to the CD64 component and its lack of ectodomain shedding, NK cells expressing CD64/16A could be less efficient at serial killing

and more efficient at bystander killing. An important next step will be to assess the anti-tumor activity of NK cells expressing CD64/16A *in vivo*, which will include the use of NK92-CD64/16A cells and iNK-CD64/16A cells in tumor xenograft models.

Therapeutic mAbs have become one of the fastest growing classes of drugs, and tumor-targeting mAbs are the most widely used and characterized immunotherapy for hematologic and solid tumors (49). NK cells expressing CD64/16A have several potential advantages as a combination therapy, as their capture of anti-tumor mAbs, either individually or when mixed, prior to adoptive transfer provides diverse options for switchable targeting elements. Modifying NK cells expressing CD64/16A with an antibody would also reduce the dosage of therapeutic antibodies administered to patients. We showed that fusion proteins containing a human IgG Fc region, such as L-selectin/Fc, can also be captured by CD64/16A, which may provide further options for directing the tissue and tumor antigen targeting of engineered NK cells. Advantages of the NK92 and iNK cell platforms for adoptive cell therapies is that they can be readily gene modified on a clonal level and expanded into clinical-scalable cell numbers to produce engineered NK cells with improved effector activities as an off-the-shelf therapeutic for cancer immunotherapy (36, 37, 42, 43, 50).

AUTHOR CONTRIBUTIONS

BW and JW collected, assembled, analyzed, and interpreted the data, and wrote the manuscript. KS collected, analyzed, and interpreted the data, and revised the manuscript. RH, HM, DM, YL, and AR collected, analyzed, and interpreted the data. DK analyzed the data and revised the manuscript. All authors contributed to manuscript preparation, read, and approved the submitted version.

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VH1-69 Utilizing Antibodies Are Capable of Mediating Non-neutralizing Fc-Mediated Effector Functions Against the Transmitted/Founder gp120

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Multiple antibody effector functions arise in HIV-1 infection that could be harnessed to protect against infection or clear the persistent reservoir. Here, we have investigated the genetic and functional memory B cell and antibody landscape present during early infection in six individuals infected with either subtype A, C, or an A/C recombinant HIV-1. These individuals demonstrated varying levels of plasma autologous neutralization (nAb) against the transmitted/founder envelope (T/F Env) pseudovirus and non-neutralizing Fc-mediated effector function (nnFc) antibody-dependent cell-mediated cytotoxicity (ADCC) against the T/F Env gp120 protein at ~7 months after infection. Genetic analysis of the immunoglobulin heavy (VH) and light (VL) chain variable domain gene segments from 352 autologous T/F Env gp120-specific single B cells recovered at this same 7-month time-point revealed an over-representation of the VH1-69 germline in five of six individuals. A defining feature of the VH1-69 utilizing gp120-specific antibodies was their significantly more hydrophobic complementarity-determining region-2 (CDRH2) regions compared to other VH CDRH2 sequences from each individual. While none of the VH1-69 antibodies possessed strong neutralizing activity against virions pseudotyped with the autologous T/F Env, almost a third were capable of mediating high ADCC activity, as assayed by intracellular granzyme B activity in CEM.NKr.CCR5 target cells coated with autologous T/F Env gp120. High ADCC mediating VH1-69 antibodies exhibited shorter complementarity-determining region-3 (CDRH3) lengths and a more neutral isoelectric point than antibodies lacking this function. In the individual that developed the highest autologous ADCC responses, the high granzyme B producing antibodies bound to surface expressed envelope in the absence of CD4 and were not enhanced by the addition of soluble CD4. Overall, VH1-69 utilizing antibodies are commonly induced against gp120 in diverse HIV-1 infections and a subset of these antibodies can mediate ADCC functions, serving as a bridge between the innate and adaptive immune response to HIV-1.

Keywords: HIV-1, gp120, VH1-69, non-neutralizing Fc-mediated effector function, neutralization

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INTRODUCTION

The potential antibody repertoire in humans is a stunning example of the creative capacity of the evolutionary process. Differential combinations of heavy-chain germline V, D, and J gene segments, kappa or lambda light-chain V and J combinations, all combined with the additional junctional diversity found within both the heavy and light-chain rearrangements, results an estimated 1×10^8 to 1×10^{15} potential B cell receptor combinations (1, 2). This diversity is the foundation for B cell receptor repertoires in individuals that can interact, to some degree, with essentially any pathogen encountered over the course of a lifetime. From this starting point, reactive B cells evolve via somatic hypermutation and affinity maturation, generating antibodies with increasing affinity for specific epitopes associated with specific pathogens, and ultimately the ability to exert neutralizing and/or non-neutralizing effector functions, to not only clear current infections, but prevent or reduce the pathogenicity of future infections by the same (or similar) pathogen (3, 4). While this process has been capitalized on by numerous vaccination strategies to generate protective antibody responses in the absence of true primary infection, we have yet to generate a highly protective prophylactic vaccine to HIV-1. An HIV-1 vaccine will need to be given to a genetically diverse human population, and induce a protective immune response against HIV-1 variants of unprecedented genetic diversity (5, 6). However, the antibody response to HIV-1 infection is not necessarily consistent or predictable between individuals. Neutralizing (nAb) and non-neutralizing Fc-mediated (nnFc) activities against the autologous virus develop at different rates between individuals (7–13). Autologous nAb are initially strain-specific and target a single epitope that differs across individuals. In contrast, broadly neutralizing antibodies (bnAbs) are capable of neutralizing a large percentage of genetically diverse HIV-1 variants; however, they develop only after several years of infection in a small percentage of infected individuals, utilize a number of different heavy and light chain germline genes and combinations, and often exhibit exceptionally rare features (14–18). Nevertheless, bnAbs remain a major goal for some HIV vaccine development efforts. A hopeful strategy for the induction of bnAbs is to target germlines of interest and guide the evolution of these specific lineages along a pre-defined trajectory. This remains an uphill battle considering the randomness and diversity inherent in the normal human antibody selection and maturation process (19). Thus, anything resembling immunological convergence, especially within the context of HIV-1 infection, could be a source of cautious optimism.

In HIV-1 infection, nnFc activities such as antibody-dependent cell-mediated cytotoxicity (ADCC), have been associated with slower disease progression and elite control of viral replication, as well as reduced transmission in a number of studies in humans and non-human primates (20–27). Unfortunately, relatively little is known about the genetic and biochemical characteristics of antibodies mediating these responses during natural infection, or the defining features of antigens that are capable of inducing them. The immunoglobulin VH1 subgroup has been implicated in two analyses of vaccine-induced monoclonal antibodies (mAbs) capable of anti-HIV ADCC activity (28, 29). More specifically, VH1-69 germline derived antibodies are of interest, due to their propensity to combine a hydrophobic CDRH2 with an acidic CDRH3 that facilitates binding to CD4-induced (CD4i) epitopes on gp120, with the potential to mediate ADCC (30, 31). However, this has yet to be explored in depth within the context of mAb interactions with autologous T/F gp120 in early natural infection.

Here, we have capitalized on a unique opportunity to shed light on the B cell and antibody landscape in six individuals early in Subtype A ($n = 2$), Subtype C ($n = 2$), or Subtype A/C ($n = 2$) HIV-1 infection, where the development of neutralization breadth in plasma at 3-years post-infection was previously characterized (32). While autologous nAb and ADCC activity generally developed by 3 years post-infection in most individuals, marked diversity in both nAb and ADCC responses was observed between these individuals at ∼7 months post-infection. An analysis of 352 memory B cells specific for the autologous T/F Env gp120 surface subunit isolated at this same time-point revealed the VH1-lineage was dominant, with VH1-69 heavy-chain germline genes over-represented in five of six individuals, regardless of subtype of the infecting variant or of individual host VH1-69 allelic variation. A subset (28%) of these dominant VH1-69 utilizing antibodies were capable of mediating high ADCC activity against CEM.NKr.CCR5 target cells coated with autologous T/F gp120. This subset displayed genetic features and epitope specificity distinct from the low ADCC VH1-69 mAbs. These results suggest in HIV-1 infection, VH1-69 utilizing mAbs with distinctive genetic features could be immunological "first-responders," mediating ADCC effector function against autologous gp120 while nAb develops.

METHODS

Ethics Statement

The participants, R774F, R53F, Z1800M, Z1047M, R1142F, and R66M, were among a group of individuals enrolled in heterosexual discordant couple cohorts that were selected for study based on rapid screening of adults with recent history of HIV exposure in Rwanda and Zambia. After obtaining written informed consent, blood samples were collected from participants longitudinally, every 1–3 months, depending on enrollment date. All couples in the cohort were provided monthly counseling and testing prior to the HIV-negative partner becoming positive. The procedures for written informed consent and research activities were approved by institutional review boards at all collaborating clinical research centers, with further compliance to human experimentation guidelines set forth by the United States Department of Health and Human Services. The study was reviewed and approved by the Republic of Rwanda National Ethics Committee, Emory University Institutional Review Board, and the University of Zambia Research Ethics Committee. To protect confidentiality, all subject identification numbers were anonymized by assigning a coded ID that removes any identifying information.

Blood was also obtained from normal, HIV-1 seronegative human volunteers at Emory University through an Institutional Review Board approved phlebotomy protocol. This protocol also anonymized volunteers by assigning coded IDs to remove any identifying information.

Study Population

The subjects studied here were enrolled in Protocol C, a uniform vaccine-preparedness study developed and implemented by the International AIDS Vaccine Initiative (IAVI; http://www.iavi.org) that was carried out at multiple sites in Africa, including the Zambia-Emory HIV Research Project (ZEHRP, coded IDs Z1800M, Z1047M) and Projet San Francisco (coded IDs R774F, R53F, R1142F, R66M) (33, 34). Projet San Francisco subjects were associated with epidemiologically linked donors, while the two subjects from ZEHRP were not. These subjects were also included in our previous study (32).

Cell Line Authentication

Genetica Cell Line Testing was used to authenticate 293 and TZM-bl cell lines. 293T cells: 88.9% identity, 80.00% match to HEK 293T/17. Expi293F: 90.32% identity, 93.33% match to HEK-293 [293] (ATCC CRL-1573). TZM-bl: 100.00% identity, 100% match to HeLa (ATCC CCL-2). All lines were mycoplasma negative. CEM.NKr.CCR5 cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: CEM.NKr.CCR5 Cells from Dr. Alexandra Trkola (35–37).

Neutralization Assays

Generation of Env pseudoviruses and the experimental protocol for the TZM-bl neutralization assay have been described previously (9, 11, 38–49). As R66M began antiretroviral therapy at ∼1.25 years post-infection, which can give a false impression of "neutralization" in the TZM-bl assay, IgG antibodies were purified from the 36.5-month plasma sample (Time Point 3) using a GE Healthcare Life Sciences Ab SpinTrap, according to the manufacturer's instructions (GE 28-4083-47). The concentration of the purified IgG was determined by ELISA and adjusted, and was then used in place of plasma in neutralization assays for this time point. In all other assays, plasma was used to evaluate polyclonal autologous neutralization. 2000 IU of each titered Env pseudovirus (in DMEM with ∼3.5% (vol/vol) FBS (Hyclone, # SH30088.03) and 40 μg/mL DEAE-dextran) was mixed with five-fold serial dilutions (beginning at 1:100) of heat-inactivated plasma samples or purified polyclonal IgG and assayed for its inhibitory potential against the appropriate autologous Env pseudovirus using the TZM-bl indicator cell line, with luciferase as the readout. The average background luminescence from a series of uninfected wells was subtracted from each experimental well. All assays contained duplicate wells and were repeated at least once independently. For mAb neutralization, 2000 IU pseudovirus with 10 μg/ml of mAb and added to plated TZM-bl cells. Samples were run in triplicate, and when antibody yield was sufficient, this was duplicated. "Antibody only" wells were also included as a negative control to monitor for any potential cytotoxic activity of the mAb and/or elution buffer. HIV bnAb VRC01 was used as a positive control and anti-influenza HA mAb EM4C04 was used as a negative control, both at 10 μg/ml.

Granzyme B Activity Assay

A panel of potential HIV-negative effector cell donors at Emory University were screened for CD16 158V/F polymorphisms via flow cytometry, similar to a method previously described (50). Donor PBMC were stained with APC conjugated anti-human CD56 (BioLegend, #318309), PacBlue anti-human CD3 (BioLegend, #300330), and either FITC anti-human CD16 (Thermo, #MHCD1601) for total CD16, or a FITC anti-human CD16 158V-specific clone MEM-154 (Thermo, #MAI-19563) and analyzed on an LSRII (BD Bioscience) instrument and with FlowJo 10.4 software (Treestar). The resulting ratio of the mean fluorescence of FITC-positive, CD56, CD3, cells (MEM-154:Total) was used to eliminate 158F homozygous donors, and identify 158V/F or 158V/V donors for downstream use in the GzB assay. Two donors were selected (Donor 190, Donor 457), and PBMC were isolated via Ficoll centrifugation and cryopreserved in liquid nitrogen (90% FBS, 10% DMSO) until needed.

Assessment of GzB activity was performed as previously described (51). T/F Env gp120 proteins with a C-terminal 6XHis-tag were synthesized by GENEART (ThermoFisher). Genes were synthesized and inserted into pcDNA3.3 and transfected into FreeStyle™ 293 cells. Proteins were purified via 5 ml HisTrap™ FF column, linear gradient from 20 to 500 mM Imidazole in PBS, 500 mM NaCl at 6 days post-transfection, with a HiLoad 26/600 Superdex200 polishing step. Cryopreserved PBMC from uninfected donors were recovered in RPMI (10% FBS (Hyclone) Pen/strep/l-glut) the night before the assay. To ensure consistency across assays, effector cell viability was required to be >80% on the day of the assay to proceed. CEM.NKr.CCR5 cells were coated with 8 μg gp120/million cells at RT for 45 min. Dyes TFL4 and NFL1 were added according to the manufacturer's instructions for 15 min in a 37 degree C water bath (OncoImmunin, Inc. GranToxiLux PLUS! and NFL1). CEM.NKr.CCR5 target cells were washed 2x in complete RPMI media, then mixed with primary donor PBMC cells at a 30:1 Effector:Target ratio (25 μl 300,000 Effector cells; 25 μl 10,000 Target cells), along with 75 μl of GzB substrate, and incubated for 5 min at RT. Twenty five microliters of five-fold serially diluted plasma (beginning at a dilution of 1:200), 50 μg/ml HIV-Ig [NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: from NABI and NHLBI (cat# 3957) (52)] positive control, or 50 μg/ml commercial IgG (SouthernBiotech, #0150-01) negative control were added, and incubated at RT for 15 min. Tubes were spun at 300 × *g* for 1 min, and incubated at 37°C, 5% CO2, for 1 h. Fixed cells (final concentration 1.85% formaldehyde, 10 min) were washed, then resuspended in buffer for flow cytometric analysis. Because fixation could result in loss of GzB signal, samples were immediately analyzed on an LSRII (BD Bioscience) instrument and with FlowJo 10.4 software (Treestar). Target cell only controls were run to ensure proper gating of targets vs. effector cells. Target with added effector cells in the absence of antibody (media only) were included

to quantify background GzB signaling. Values that became negative after subtraction of background were normalized to 0%. Negative (commercial IgG) and positive (HIV-Ig) controls were included in every assay run. Assays were not valid unless the percentage of GzB positive cells in the positive control tube were >10%, after subtraction of background activity. Only results from valid assays are included. Example Granzyme B assay flow-plots are included in **Supplementary Figure 1**. To test for inherent differences across the gp120 proteins that could influence the assay, negative and positive control GzB activity was examined. There were no statistical differences between the T/F gp120 proteins in background (Target + Effector cell only samples), negative control (commercial IgG), or positive control (HIV-Ig) GzB activity (**Supplementary Figures 2A–C**). There was also no statistical difference in all experimental values between the two effector cell donors, as values obtained from each donor were not statistically different (**Supplementary Figure 2D**, Mann–whitney, $p > 0.05$) and were highly correlated (**Supplementary Figure 2E**, Spearman, $p < 0.0001$, $r = 0.83$). To measure GzB activity induced by mAbs, the protocol was repeated in an identical manner, with all corresponding positive and negative controls, but with experimental mAbs tested at five-fold serial dilutions beginning at 5 μg/ml.

B Cell Sorting

The same preparations of T/F Env gp120 proteins as used in the GzB assay, described in detail above, were used for single B cell sorting. Cryopreserved PBMC obtained from the sites in Rwanda and Zambia (∼5–10 million cells/vial) were washed and resuspended in PBS + 2% FBS. After counting, cells were incubated with 8 μg of the autologous T/F gp120 per million cells and stained with: live/dead Aqua (Invitrogen, # L34957), anti-CD3 Pacific Blue (BD Pharmingen, # 558124), anti-CD19 BV650 (BioLegend, # 302237), anti-IgG FITC (BD Pharmingen, #555786), anti-His PE (Miltenyi Biotec, # 130-098-810), and single-cell sorted into 96-well plates containing 20 μl cell lysis buffer (Superscript III RT buffer, Tween, DTT, Invitrogen, #18080-044) (RNaseOUT, Invitrogen, #100000840). Sorts were carried out using a FACS Aria Cell Sorter and the following gating strategy: size, singlets, live, CD3-, CD19+, IgG+, gp120/His+. CD3+ gp120/His+ cells were monitored as positive controls for gp120 binding and detection.

Immunoglobulin heavy and light chain variable domain regions were PCR-amplified as previously described, using oligo-dT (ThermoScientific, #AM5730G) and SuperScriptIII (Invitrogen, #18080-044) (53). IgG-specific primer was also included in the RT reaction for individuals R53F, R774F, and Z1047M to increase recovery of heavy chain regions. Five microliters of the RT reaction was used to amplify heavy (IgG only), kappa, and lambda chain variable regions using high performance liquid chromatography (HPLC) purified primers as described in Liao et al. (53) and Phusion Hotstart II High Fidelity DNA Polymerase (Thermo Scientific, #F537S) for first round nested PCR. For the second round PCR reaction, 2.5 μl of first-round was used as a template. PCR amplified variable regions were gel purified (Qiagen, #28706) and combined with a CMV promoter containing DNA fragment, and the appropriate corresponding constant region DNA fragment (including a polyA tail) via overlapping PCR (53). Plasmids HV0024, HV0023, HV0025, and HV0026 were kindly provided by Dr. Larry (Huaxin) Liao at the Duke Human Vaccine Institute. The assembled full-length heavy- and light-chain segments were then cloned into pCR2.1TOPO-TA (ThermoFisher, # K4500-40) for long-term storage and mAb expression. VH and VL plasmid pairs were co-transfected at a 1:2 ratio into 6-well plates containing Expi293F cells (Thermo Fisher Scientific, Expi293 Expression System Kit, #A14635). Five to seven days post-transfection, mAbs were purified from the cell culture supernatant using Ab SpinTrap with Protein G Sepharose High Performance (GE Healthcare, # 28408347). The concentrations of the purified mAbs were quantified on an Octet RED96 using Anti-Human IgG Fc AHQ biosensors (ForteBio, #18-5001). mAb expression plasmids that could be stably amplified and resulted in transfections with yields >100 μg/ml were carried forward with antibody effector function characterizations.

PCR and Sequence Analysis of Immunoglobulin Variable Domains

The nucleotide sequence of each PCR amplicon was determined by Beckman Coulter Genomics or Sequetech using the following primers: H-R474 (5′- GCTGTGCCCCCAGAGGTG-3′) or K-R405 (5′-GACAGATGGTGCAGCCACAGTTCG-3′) or L-R400 (5′-CAGAGTGACCGAGGGGGCAGC-3′). These partial sequences were analyzed with NCBI IGBLAST for putative germline gene identification (https://www.ncbi.nlm.nih.gov/igblast/igblast.cgi) (**Supplementary Table 1**) (54). After assembly of the PCR fragments and cloning, plasmids were sequenced with the appropriate reverse primer, as well as CL-F681 (5′- TCTGGGTTCCAGGTTCCACTGGTGAC-3′). The resulting sequences were re-analyzed using IMGT vquest (http://imgt.org/IMGT_vquest/vquest) for germline gene verification, framework and CDR mapping, quantification of percent identity to germline, CDR H3 and L3 amino acid length, molecular mass, and pI (**Supplementary Table 1**) (55, 56). Circos diagrams were generated via the Circos online tool (http://mkweb.bcgsc.ca/tableviewer/) (57). CDRH2 grand average of hydropathy (GRAVY) scores were calculated using an online tool (http://www.gravy-calculator.de/). CDRH3 tyrosine sulfation sites were enumerated with the Sulfinator online tool (http://web.expasy.org/sulfinator/) (58). Sequences were uploaded to Genbank under the following accession numbers: MK269362–MK270058.

Assessment of Affinity and Epitope Binning Using Biolayer Interferometry (BLI)

Affinity assays were performed on an Octet RED96 (ForteBio, Inc, Menlo Park, CA) at 30°C, with 1,000 rpm agitation. Each mAb was immobilized on an Anti-hIgG Fc Capture (AHC) biosensor (Fortebio, # 18-5060) at a concentration of 25 μg/ml for 300 s, and a baseline reading was recorded for 60 s in kinetics buffer (PBS with 0.01% BSA, 0.02% Tween20, and 0.005% sodium azide). Sensors were then immersed in varying molarities of the corresponding autologous T/F gp120 protein for 300 s,

then allowed to dissociate in kinetics buffer for 600 s. Sensors were regenerated before each kinetics assay. Human IgG at 25 μg/ml dipped into kinetics buffer was included on every assay plate for reference during analysis (SouthernBiotech, #0150-01). Molarity (range 5–0.06 μM), association time (range 300–600 s), and dissociation time (range 600–1,200 s) were varied as necessary to obtain appropriate X^2 and R^2 values. Anti-influenza HA antibody EM4C04 (kindly provided by Dr. Jens Wrammert, Emory University) and/or commercial IgG were included to survey for non-specific mAb binding against each gp120.

K_D values were calculated using ForteBio Data Analysis 9.0. After reference subtraction, Y-axis data was aligned to the 50–59.9 s of the baseline reading. For inter-step correction, data was aligned to the dissociation step. Data was then processed with Savitzky-Gola filtering, and curve fitting was performed using a 1:1 binding model. K_D values were averaged from ≥2 reads (average number of reads ≥3) with appropriate X^2 and R^2 (median $X^2 = 0.11$, range = 0.00–1.94; median $R^2 = 0.99$, range = 0.93–1.0) (**Supplementary Table 3**).

Binning (competition) assays were performed at 30 degrees C, with 1,000 rpm agitation. Synthesized gp120 (R66M T/F Env O20) at a concentration of 25 μg/ml was immobilized on an Anti-Penta-HIS (HIS1K, ForteBio, # 18-5120) biosensor for 300 s, and a baseline reading was recorded for 30 s in kinetics buffer. Sensors were then immersed in 0.66 μM primary (R66M-derived) mAb for 10 min. R66M-derived mAbs that resulted in a primary signal of <0.1 at 0.66 uM (lower affinity mAbs) were increased in molarity, as production allowed, to reach that threshold. mAbs that could not be produced at this concentration were not analyzed. After a second baseline reading for 30 s in kinetics buffer, sensors were immersed in 0.66 μM secondary antibody ("reference mAb"—VRC01, 3074, PGT121, R66M 7C12) (VRC01 mAb Heavy Chain Expression Vector and VRC01 mAb Light Chain Expression Vector was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: from Dr. John Mascola (cat# 12035 and 12036) (59, 60); 3074, kindly provided by Xiangpeng Kong, NYU School of Medicine; PGT121, PGT 121 was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Anti-HIV-1 gp120 Monoclonal (PGT121) catalog #12343 (61) to assess reference mAb binding capacity in the presence of primary R66M mAb binding. Reference mAb as primary and secondary mAb was included as a positive control for competition (reduced reference binding). EM4C04 (anti-influenza mAb) primary and reference mAb secondary was used as an additional control for non-specific competition (peak reference binding). Binning values were calculated using ForteBio Data Analysis 9.0. R66M mAbs capable of reducing reference mAb binding to <50% of reference binding in the presence of EM4C04 primary (set at 100% binding), were considered competitors for that reference mAb.

Flow Cytometric Analysis of mAb Binding to Surface Env With and Without sCD4

293T cells were transfected with a plasmid encoding the T/F *env* gene isolated from R66M (the same plasmid utilized for generation of R66M Env pseudovirus) using Fugene HD (Promega). At 48-h after transfection, cells were washed with PBS, and briefly trypsinized. After washing again with PBS, cells were aliquoted and resuspended in flow buffer (PBS + 5% FBS), with or without 25 μg/ml recombinant human sCD4 (R&D Systems, 514-CD), and allowed to incubate for 30 min at RT. Cells were then incubated with 25 μg/ml polyclonal IgG (SouthernBiotech, #0150-01), 5 μg/ml PGT121, 10 μg/ml of CD4i mAb17b, or an R66M mAb at RT for 30 min. After washing with flow buffer, samples were then incubated with Goat Anti-Human IgG Fc biotin (SouthernBiotech, # 2014-08) at 1:200 for 20 min at RT. After washing with flow buffer, samples were incubated with 1:1,000 PE-conjugated streptavidin (BD Pharmingen, #554061) for 20 min at RT, while protected from light. Cells were then washed and fixed for 10 min (3.7% formaldehyde). The percentage of PE/Env positive cells were determined via quantification on an LSRII (BD Bioscience) instrument and with FlowJo 10.4 software (Treestar). All samples were analyzed on at least three independent occasions. PGT 121 was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Anti-HIV-1 gp120 Monoclonal (PGT121) (61). 17b was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Anti-HIV-1 gp120 Monoclonal (17b) from Dr. James E. Robinson (62–67).

Statistical Analyses

All graphing, calculations, and analyses were performed in Prism 6.0. Non-parametric statistical tests were used. A Mann–Whitney test was used to compare two groups; a Kruskal–Wallis test was used to compare more than two groups. Ordinary one-way ANOVA, Tukey's multiple comparison test was used for multiple comparisons. Chi-square test was used for contingency analyses. Spearman rank test was used to assess correlations.

RESULTS

Marked Variation in the Development of Plasma Autologous nAb and Non-neutralizing Fc Activity During Early HIV-1 Infection

In a previous study, 21 HIV-1 infected individuals from serodiscordant couple cohorts in Zambia (Z) and Rwanda (R) were evaluated for viral and host factors that contributed to the development of plasma nAb breadth at 3-years after infection (32). Here, six of those subjects were included in a more focused investigation of autologous nAb and ADCC activity against the respective T/F Env. These six individuals were selected because they were infected by the types of HIV-1 variants that predominate in these cohorts, subtype A (R774F and R53F), subtype C (Z1800M and Z1047M) and A/C recombinant HIV-1 (R1142F and R66M), and because sample quality, quantity, and availability was suitable for the study, which included sorting of antigen-specific B cells from cryopreserved PBMC. Plasma samples used in the analyses were collected at Time Point 1 (average of 0.89 months post-antigen-positive test date, range 0–2.9 months), Time Point 2 (average

of 7.5 months post-infection, range 5.1–8.7 months), and Time Point 3 (average of 37.5 months post-infection, range 36.4–40.2 months). The same plasma sample was used to measure both nAb and ADCC activity in each case. The T/F Env for each individual had been derived previously using reverse transcription and single genome PCR amplification of plasma collected at an average of 34 days after the estimated date of infection (range 22–65 days), as described in Smith et al. (32). Sequence analysis was used to identify an amplicon representing the T/F Env consensus sequence, which was cloned into an expression plasmid for generation of pseudovirus for use in the neutralization assay (32). The T/F Env gp120 coding sequence of each individual was used to manufacture the protein for use in the ADCC activity assay and subsequent single B cell sorting.

Figure 1 shows the combined nAb and ADCC activity for the three longitudinal plasma samples against the autologous T/F Env for the six subjects. At Time Point 1 (**Figure 1A**, 0.89 months), three of the six individuals exhibited clear nAb activity that reached ∼20% at the lowest dilutions (green, solid lines, left Y-axis). The highest levels of nAb were associated with individuals whose samples were obtained further from transmission (R774F, 1.4 months and R1142F, 2.9 months). ADCC activity was negligible in these early plasma samples, except for R1142F, which reached 4.6% GzB positive cells (green, dashed lines, right Y-axis). Thus, both nAb and ADCC antibody functions can be detected using the autologous T/F Env as early as 2.9 months after infection.

At Time Point 2 (**Figure 1B**, 7.5 months), nAb activity against the T/F Env exceeded 50% in five of six individuals at a 1:100 dilution of plasma, and was more than 80% in two individuals (red, solid lines, left Y-Axis). The exception was R774F, whose plasma nAb activity had not increased from that present at Time Point 1. ADCC activity was also readily detectable in five of six individuals at Time Point 2, with the average peak percentage of GzB positive target cells ranging from 8.1 to 20.2% at various plasma dilutions. In contrast, ADCC activity in R53F plasma remained indistinguishable from background, even though 50% neutralization of the T/F Env was achieved at this time point, suggesting that these two antibody populations were distinct.

At Time Point 3 (**Figure 1C**, 37.5 months), very potent autologous nAb was present in five of six individuals, exceeding 80% neutralization at the lower plasma dilutions, with two individuals exhibiting complete neutralization of the T/F Env (blue, solid lines, left Y-axis). nAb in R66M plasma was notably low at this later time point. High ADCC responses, peaking at 14.7–30.2% positive for GzB at various plasma dilutions, were present in all six individuals. Of note, when this data is compared with previously reported neutralization breadth at 3-years post-infection for this small set of individuals, no association between autologous ADCC activity and the development of neutralization breadth is apparent (18, 32). Overall, autologous nAb and ADCC activity developed in all six subjects to varying degrees, but the individual trajectory and magnitude varied.

Memory B Cells With VH1-69 Dominate the Early Anti-gp120 Response in Five of Six Individuals

To understand the early antibody responses to the autologous HIV-1 T/F Env gp120 at higher resolution, variable heavy (VH) and variable light (VL) gene segments were recovered from single IgG+ memory B cells sorted for reactivity with the autologous T/F Env gp120 protein using cryopreserved PBMC collected at 7.5 months after infection (Time Point 2). This approach was designed to capture a wide variety of Env-specific B cells that were activated and selected for survival during the first 7 months of HIV-1 infection, and had the capacity to contribute to the plasma nAb and ADCC functions observed at this time point (**Figure 1B**). A total of 352 Env-specific memory B cells were recovered, and the VH and VL germline gene usage and pairings present in each subject are illustrated in **Figure 2**. In the Circos plots, each colored ribbon indicates a unique VH/VL combination, and the width is proportional to frequency (57). Broad diversity of VH and VL usage and pairing was observed in each individual, supporting that a highly polyclonal antibody response against epitopes in gp120 had occurred during the first months of infection.

Notably, VH1-derived B cells were commonly elicited in these individuals (**Figure 2**, shades of blue), similar to what has been described in chronic HIV-1 infection, VH1-derived B cells (68–72). Specifically, VH1-69 was the dominant VH germline gene utilized by T/F gp120-specific memory B-cells in five of the six individuals studied (**Figure 2**, royal blue). The frequency of VH1-69 B cells in those five individuals ranged from 21 to 51% (**Figure 3A**). Indeed, the VH1-69-lineage was not only dominant, but also over-utilized in all five individuals when compared to frequencies obtained from circulating B cells with successfully rearranged IGHV genes [reported range 3.1–9.7 (73)], or a previous analysis of eight HIV-1 vaccine volunteers pre-vaccination [reported range 0.7–8.2% IgG peripheral blood (74)]. Therefore, we investigated in more detail the dominance of VH1-69 to understand the impact of this immunological phenomenon on the early antibody responses.

Pathogenically cross-reactive VH1-69 lineages likewise dominated a DNA prime/trivalent gp140 protein boost HIV vaccination regimen (74). And, hydrophobic interactions between VH1-69 CDRH2 and hydrophobic cavities within HIV-1 gp120 have also previously been observed (30). In the data collected here, grand average hydropathy (GRAVY) scores of isolated T/F gp120-specific VH1-69 CDHR2 amino acid sequences ranged from 0.34 to 2.7, with median GRAVY scores ranging from 1.28 to 1.89, indicating high hydrophobicity (**Figure 3B**, **Supplementary Table 1**). In contrast, the median CDRH2 GRAVY score for the corresponding non-VH1-69 T/F gp120-specific B cells for each individual was significantly lower, ranging from −1.16 to −0.46 (Ordinary one-way ANOVA, Tukey's multiple comparisons test: R53F $p = 0.018$, all others $p < 0.0001$). These observations reinforce the concept that high CDRH2 hydrophobicity is a unique and universal feature of the VH1-69 antibodies.

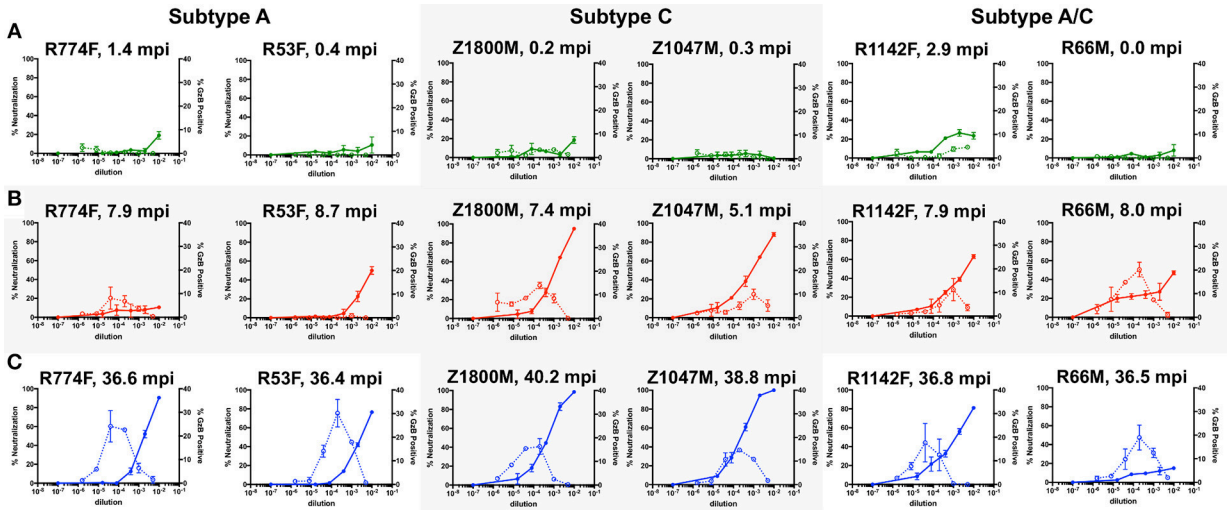

FIGURE 1 | Evaluation of longitudinal nAb and ADCC capacity of plasma samples against the autologous T/F Env. nAb and ADCC activity in plasma collected at chronologic time points 1 (**A**, 0.87 months post infection), 2 (**B**, 7.5 months), and 3 (**C**, 37.5 months); All graphs show the percent neutralization of autologous T/F Env pseudovirus (closed symbols with solid line, left Y-axis) and percentage of fluorogenic GzB substrate positive CEM.NKr.CCR5 cells coated with autologous T/F Env gp120 (open symbols with dashed line, right Y-axis). The subjects are grouped by HIV-1 subtype, which is labeled on the top. The time points shown are 0 to 2.9 months after the first antigen-positive test (green), 5.1–8.7 months after the first antigen positive test (red lines), and 36.4–40.2 months after the first antigen positive test (blue lines). The X-axis indicates the reciprocal dilution of the individual's plasma plotted on a $\log_{10}$ scale. Symbols and error bars represent the mean and standard error of mean, respectively, between at least two replicate experiments for neutralization, and the mean and standard error of mean of independent experiments utilizing cells from two different donors for the GzB assay. Control data for (i) plasma nAb against a negative control VSV-g Env pseudovirus and (ii) GzB mediated by normal human serum against the T/F Env gp120 can be found in **Supplementary Figure 2F**.

During influenza infection, the presence of a phenylalanine (F) or leucine (L) polymorphism at position 54 can strongly influence the frequency of VH1-69 utilization in anti-influenza antibodies, and their resulting functions (75). Though individuals' genomes were not sequenced to determine specific alleles within this study, this information was inferred from the isolated VH1-69 sequences. Within this group of individuals, VH1-69 containing 54F (associated with neutralization of influenza) comprised the majority of anti-gp120 VH1-69 sequences in five of the six individuals (**Figure 4**, green). These findings reflect previous analyses that noted the 54L-homozygous genotype is rarely found in African populations, with 54F-homozygous and 54F/L-heterozygous alleles composing the vast majority of VH1-69 genotypes (2, 76). Interestingly, the 54L polymorphism was observed in the majority of VH1-69 sequences from one individual, R66M (73%, **Figure 4**, **Supplementary Tables 1**, **2**), while it was at a much lower frequency of between 0 and 14% in the other individuals. VH1-69 sequences that contained an amino acid other than F or L at position 54 were isolated from three individuals: R774F, Z1800M, and R66M, further demonstrating the polymorphic nature of VH1-69 alleles.

VH1-69 Utilizing mAbs Mediate ADCC Effector Functions but Are Poorly Neutralizing

In the context of influenza, "innate-like" VH1-69 lineage mAbs can be not only broadly reactive, but broadly neutralizing as well (77). To explore the potential antiviral functions of the dominant anti-gp120 VH1-69 antibody response in early HIV-1 infection, mAbs were produced by cloning VH and VL regions into expression vectors that provided a common IgG1 constant region (53). Thus, all mAbs had identical Fc regions, allowing us to attribute differences solely to the variable domains. Neutralizing activity was present against the autologous T/F Env in five of the six plasma samples contemporaneous to the antibody isolation (**Figure 1B**). We therefore quantified the neutralization capacity of 105 VH1-69 mAbs against virions pseudotyped with the corresponding autologous T/F Env in the same manner. It should be noted that only one VH1-69 mAb was regenerated from subject R53F, due to the genetics of the B cell response (**Figure 2**). At 10 μg/ml, none of the mAbs demonstrated potent autologous neutralizing activity, when defined as the ability to reduce infectivity of the T/F Env to below 50% (**Figure 5A**, **Supplementary Table 4**). These VH1-69 antibodies are therefore probably not responsible for the observed plasma nAb activity.

Additionally, ADCC activity was also present in the 7.5-month plasma samples in all individuals except R53F (**Figure 1B**). We therefore evaluated 94 VH1-69 mAbs (overlapping almost completely with the 105 analyzed for nAb activity) for their capacity to mediate ADCC functions in the same manner, using concentrations of 5, 1, and 0.2 μg/ml of each mAb against CEM.NKr.CCR5 cells coated with the autologous T/F Env gp120 (**Supplementary Table 4**). In contrast to the universally low neutralization capacity exhibited by the VH1-69 mAbs, 27 VH1-69 mAbs mediated high GzB activity, defined as having a peak of GzB positive cells >10% over the course

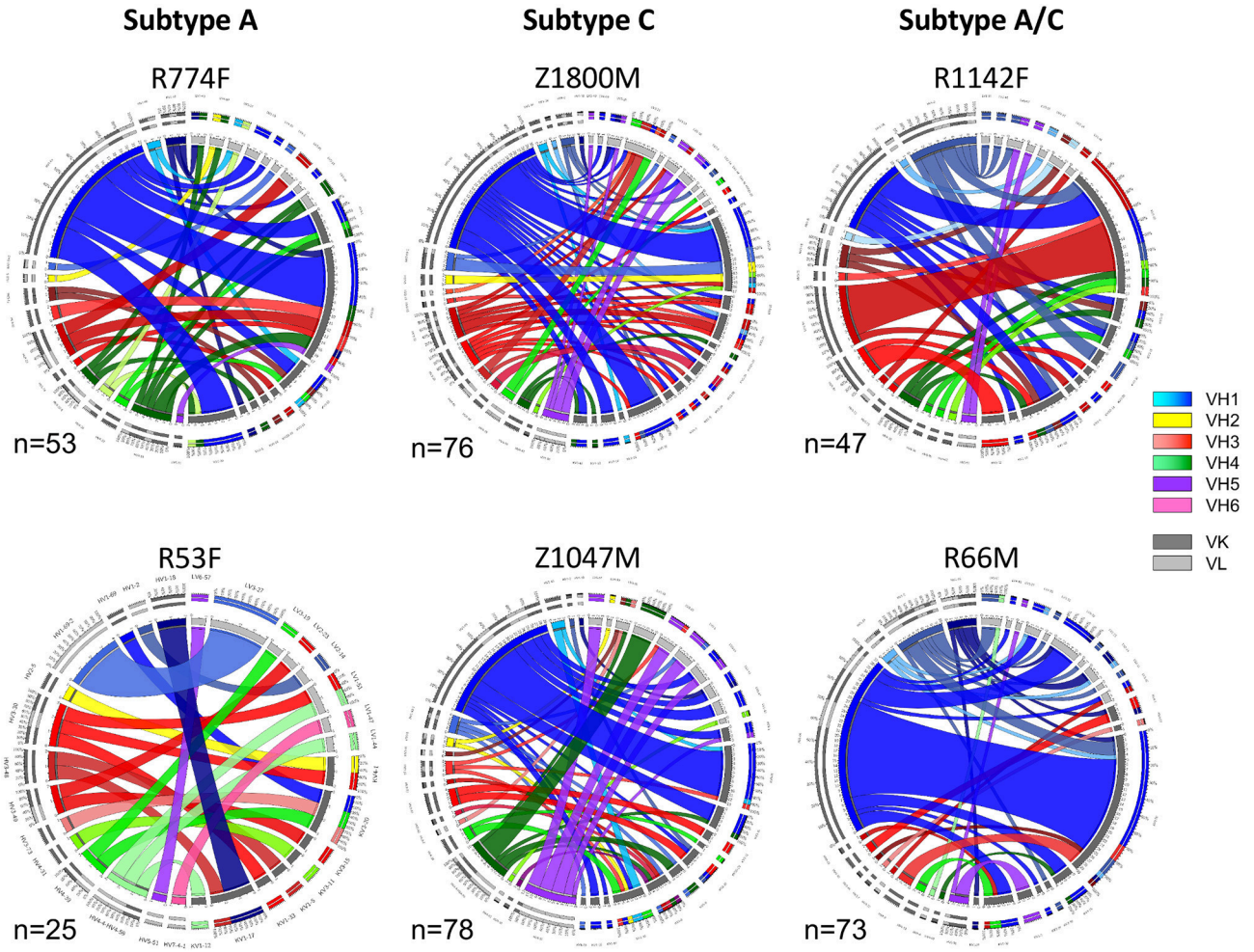

FIGURE 2 | Circos diagrams of VH and VL pairing networks at 7.5-months post-infection (time point 2). Germline genes were assigned to each VH and VL sequence from each gp120-reactive single B cell using NCBI IGBLAST and vquest analysis. Circos diagrams were utilized to illustrate the network of VH germline (shades of blue VH-1, yellow VH-2, shades of red VH-3, shades of green VH-4, purple VH-5, pink VH-6) with the paired VL κ (dark gray) or λ (light gray) germline from individuals infected by HIV-1 Subtype A (left column), Subtype C (center column), and Subtype A/C unique recombinants (right column). Band width is proportional to the number of paired sequences that contain those VH and VL pairs. The total number of paired sequences analyzed for each individual is shown.

of the dilution series (**Figure 5B**, **Supplementary Table 4**), a conservative threshold based on natural variation in "target + effector only" control values between patient-derived gp120s (**Supplementary Figure 2**). Furthermore, "high GzB" VH1-69 mAbs were present in the same five individuals that exhibited plasma ADCC activity (**Figure 1B**). The largest proportion of high ADCC-mediating VH1-69 mAbs was observed in Z1800M (56% of tested mAbs), while the highest magnitude was observed in a subset of R66M mAbs (more than 30% peak GzB signaling). As noted above, only one VH1-69 antibody was recovered and tested from subject R53F. Interestingly, R53F was also the only subject that did not have detectable plasma GzB activity at the 7.5-month time point (**Figure 1B**). Thus, some anti-gp120 VH1-69 mAbs exhibit high ADCC-mediated effector capabilities but poor neutralizing activity in early HIV-1 infection, based on the analysis of memory B cells in these six individuals. These VH1-69 antibodies could contribute to the GzB activity mediated by the contemporaneous plasma.

High ADCC Activity Is Associated With Shorter CDRH3 Regions and Higher Isoelectric Point in VH1-69 mAbs

Because all of the VH1-69 mAbs studied here have the same Fc region, any differences in the ability to mediate ADCC effector function such as GzB signaling must be attributable to the variable regions. Therefore, all of the VH1-69 mAbs shown in **Figure 5B** were divided into two groups: those with peak GzB positive cells >10% (after subtraction of background activity), and those below 10%, to search for defining features. When stratified in this manner, there was no statistical difference between the two groups in a number of genetic and functional features, including VH identity to germline,

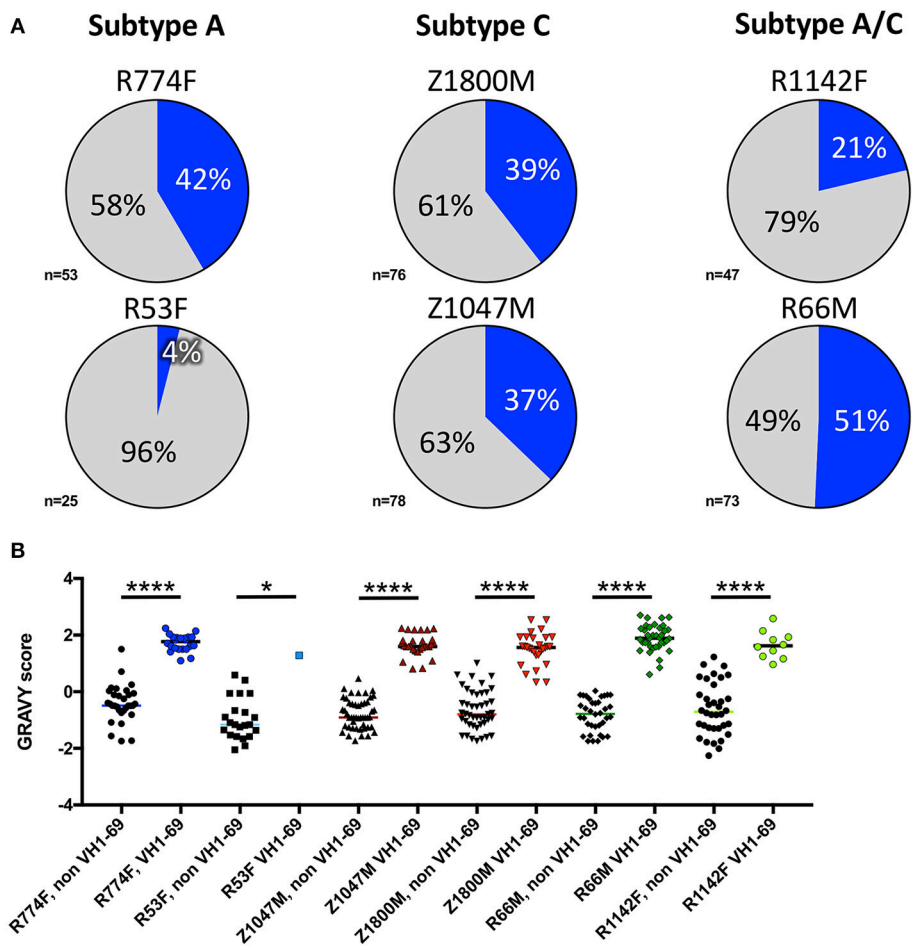

FIGURE 3 | VH1-69 is over-represented among anti-gp120 memory B cells at 7.5-months post-infection. **(A)** Percentages of VH1-69 germline usage (blue graph sections) vs. all other variable heavy-chain germline genes (gray graph sections) isolated from memory B cells reactive to autologous T/F gp120 in six individuals infected with Subtype A (left column), Subtype C (center column), and Subtype A/C unique recombinants (right column). The total number of heavy chain sequences analyzed for each individual is shown. **(B)** To determine whether these VH1-69 sequences maintained a hydrophobic complementarity-determining region-2 (CDRH2) associated with VH1-69, grand average hydropathy (GRAVY) scores (Y-axis) were calculated from non-VH1-69 (black symbols) and VH1-69 sequences (colored symbols) obtained from each individual (http://www.gravy-calculator.de), bars represent median values. Individual codes are listed below the X-axis. VH1-69 CDRH2 were significantly more hydrophobic (positive values) than the non-VH1-69 CDRH2 in every individual (Ordinary one-way ANOVA, Tukey's multiple comparisons test: R53F $p = 0.018$, all others $p < 0.0001$). Raw values are reported in **Supplementary Table 1**.

number of tyrosine sulfation sites within CDRH3 (30, 58), CDRH2 GRAVY score, kappa or lambda VL pairing, or binding affinity for the autologous T/F gp120 (**Supplementary Figure 3**, **Supplementary Tables 1**, **4**). However, there was a significant difference between high and low ADCC VH1-69 antibodies in autologous neutralization capacity, with some of the low GzB antibodies capable of weak autologous neutralization ($p = 0.0015$, **Supplementary Figure 3**, **Supplementary Table 4**). It is important to note that, despite this difference, neither group of antibodies was capable of potent autologous neutralization at 10 μg/ml, which is a relatively high concentration (GzB <10% median = 97% infectivity; GzB >10% median = 106% infectivity).

In contrast, CDRH3 length did emerge as a defining feature of high Gzb activity, with antibodies capable of mediating high ADCC activity having statistically shorter CDRH3 amino acid length ($p = 0.0007$, **Figure 6A**, **Supplementary Tables 1**, **2**). However, the median CDRH3 length for these high GzB antibodies was not unusually short compared to the median length determined for rearranged human B cells (median CDRH3 length for GzB >10% = 14 AA vs. reported median value = 15 AA) (70). This observation suggests that long CDRH3 regions may be less conducive to antibody-Fc interactions as measured in this setting.

To further investigate the association of CDRH3 length and high ADCC function, we focused on the impact of the J region, which is selected during VDJ rearrangement and contributes to the length of CDRH3. While there are six J_H regions in the human repertoire, it is striking that bnAbs commonly use the longest J region, J6, including b12, 3BNC117, 8ANC131, PGT145, PG9,

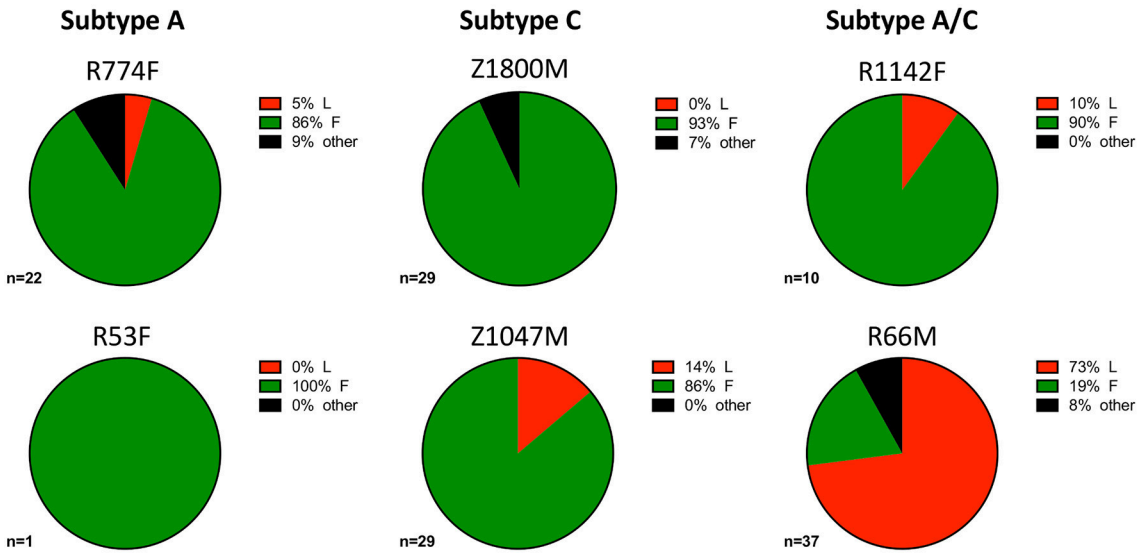

FIGURE 4 | VH1-69 polymorphisms at amino acid 54 within complementarity-determining region-2 (CDRH2)**.** The frequency of 51p1-like 54F (green), hv1263-like 54L (red), and other amino acids in six individuals infected with Subtype A (left column), Subtype C (center column), and Subtype A/C unique recombinants (right column) is illustrated. The 54F polymorphism was dominant except for R66M (bottom right), where 54L was present in the majority of the anti-gp120 VH1-69 sequences and R53F where only one VH1-69 sequence was present. A minority of VH1-69 sequences contained "other" amino acids (black) at this position in three individuals: R774F, Z1800M, and R66M. The total number of VH1-69 sequences analyzed for each individual is shown. Raw values are reported in **Supplementary Table 2**.

PGT121, PGT151, and 2F5 (16). We therefore examined J region usage in the VH1-69 mAbs to gain a better understanding of how CDRH3 length was generated. Using the VH1-69 mAbs grouped by low or high GzB activity as shown in **Figure 6A**, similar J1, J3, and J4 usage was found between the two groups (**Figure 6B** low and **Figure 6C** high, **Supplementary Tables 2, 4**). Conversely, J6 was more frequently utilized by low GzB antibodies (22.0%) compared to the high GzB group (3.9%), the latter of which had higher J5 usage. None of the VH1-69 mAbs contained J2. The overall distribution of J chains was significantly different between the two groups of VH1-69 antibodies (Chi-square, $p = 0.0004$, J2 was excluded due to the zero frequency). Thus, the infrequent presence of J6 could contribute to shorter CDRH3 regions in this antibody population. There was also a significant difference in the calculated isoelectric point (pI) for the heavy chain of the antibodies able to mediate high GzB activity vs. those that did not ($p = 0.035$, **Figure 6D**, **Supplementary Table 2**). The pI is the pH at which the antibody has no net charge, and can be estimated based on the amino acid sequence. pI can influence such parameters as tissue distribution and clearance *in vivo*. Interestingly, mAbs that mediated higher GzB activity exhibited a higher, closer to neutral, median isoelectric point (4.58 vs. 6.15). Thus, at a physiological pH, the high GzB mAbs would be less negatively charged than the low GzB mAbs, perhaps influencing their recognition of certain epitopes. Overall, this analysis revealed that VH1-69 antibodies that mediate higher GzB activity against target cells coated with the autologous T/F Env gp120 protein have unique features that include relatively shorter CDRH3 regions, associated with J chain preference, and a closer to neutral isoelectric point than those with low GzB activity.

mAb Epitope Does Not Define High Autologous ADCC Activity

To investigate the epitopes that were targeted on gp120 by high ADCC-mediating mAbs, a series of binding competition experiments were carried out with VH1-69 mAbs from the individual with the highest ADCC signaling, R66M. We utilized bnAbs with known specificities against the CD4bs, V3, and the mannose patch/base of V3 to gain information about the R66M mAb epitopes. CD4bs mAbs are thought to be unable to mediate ADCC in the gp120 coating assay because the protein is bound to cell-surface CD4. Therefore, to test this hypothesis, 28 R66M VH1-69 mAbs were assessed for their ability to compete with the CD4bs bnAb VRC01 for binding to the R66M T/F gp120 protein using biolayer interferometry. Overall, the majority of VH1-69 mAbs from R66M did not compete with VRC01. Only 2 of 28 mAbs reduced VRC01 binding to <50% (**Figure 7A**, **Supplementary Table 4**). As expected, these two antibodies belonged to the low GzB signaling group; however, there was no statistical difference between the two groups in their ability to compete with VRC01 binding (**Figure 7A**).

We next tested competition against a V3-directed bnAb, 3,074, that recognizes a linear epitope conserved among HIV-1 clades A, B, and C (78). In general, the VH1-69 mAbs recovered from R66M competed only weakly 3,074 (**Figure 7B**, **Supplementary Table 4**). However, the level of V3-directed competition was greater in the low GzB group (**Figure 7B**, Mann–Whitney, $p = 0.005$). Thus, these findings contradict the idea that CD4 binding in the gp120-coating assay preferentially measures V3-mediated GzB activity (**Figure 7B**). Furthermore, none of the six high GzB mAbs from R66M appeared to target V3.

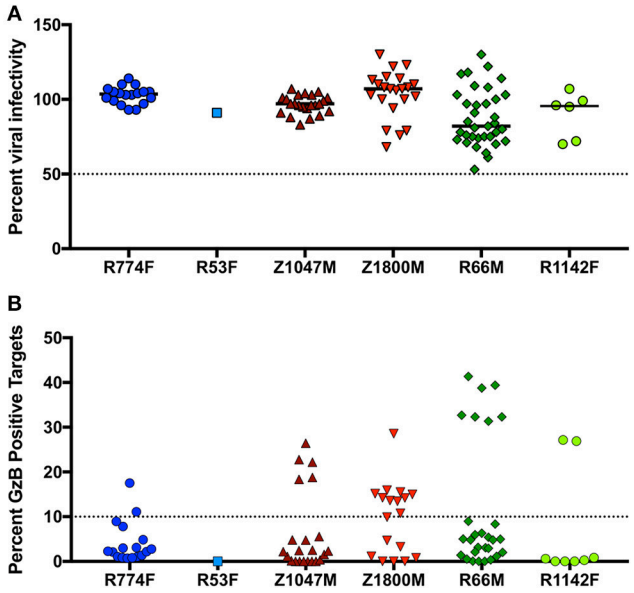

FIGURE 5 | Effector function capabilities of VH1-69 antibodies**. (A)** A concentration of 10 μg/ml of each mAb was evaluated in the TZM-bl neutralization assay against the autologous T/F Env pseudovirus for each individual (labeled below the X-axis). Percent residual infectivity of the T/F Env pseudotyped virus for individual mAbs, relative to virus-only controls, is shown (Y-axis). Symbols represent the mean of triplicate wells, bars represent the median of all mAbs from each individual. **(B)** Each mAb was used in a five-fold dilution series of 5, 1, and 0.2 μg/ml against CEM.NKr.CCR5 cells coated with autologous T/F Env gp120. The peak percentage of cells positive for Granzyme B (GzB) activity over this dilution series, after subtraction of background activity (percentage of GzB positive cells in target + effector reactions), is shown on the Y-axis. Each symbol represents the mean of two separate assays, utilizing effector cells from two independent HIV-negative effector cell donors. The activity of each mAb against target cells coated with the autologous gp120 are labeled for each individual subject below the X-axis. "High" activity was defined as achieving GzB activity >10% (dashed line, Y-axis). Bars indicate the median value for each subject. Raw values are reported in **Supplementary Table 4**.

The R66M mAbs were next competed against bnAb PGT121, which recognizes an epitope formed by V3-proximal glycans and the V3 base. A relatively large proportion of R66M VH1-69 mAbs competed to varying degrees with PGT121 for gp120 binding. In terms of GzB activity, 13 mAbs were capable of reducing PGT121 binding to <50%, including two from the high GzB group and 11 from the low GzB group. A significant difference in PGT121 competition was observed between the two groups (**Figure 7C**, $p = 0.027$). Thus, antibodies with epitopes overlapping that of PGT121 did not commonly mediate high GzB activity. However, two R66M VH1-69 antibodies that competed with PGT121 did mediate high Fc-mediated GzB activity. This finding is significant in that it demonstrates that mAbs arising in the same individual can recognize a similar epitope on gp120 yet some exhibit high ADCC activity while others do not.

To explore this concept further, VH1-69 mAbs from R66M were competed against an autologous mAb 7C12, which was chosen because its CDRH3 length was the median for R66M (20 AA), it exhibited high affinity binding to the R66M T/F gp120, and this mAb lacked ADCC activity. Many of the VH1-69 R66M mAbs competed with 7C12 (75% of all those tested) indicating that they likely recognize overlapping epitopes. When stratified by GzB activity, the VH1-69 mAbs in the low GzB group competed with 7C12 to a greater degree than the high GzB group (**Figure 7D**, $p = 0.006$). However, three mAbs in the high GzB group also strongly reduced 7C12 binding (**Figure 7D**, **Supplementary Table 4**: mAbs 1D10, 6C12, and 7B7). These findings provide additional evidence that the angle of binding and other factors extrinsic to the epitope are important for mediating Fc functions. They further suggest that a novel, immunodominant epitope exists within the R66M gp120 protein that is highly susceptible to autologous ADCC activity.

Additionally, an important observation from these studies is that the gp120 coating assay detected high Fc-mediated GzB activity by antibodies with epitopes that are not expected to require induction by CD4, such as those antibodies competing with PGT121 (**Figure 7C**). This was somewhat unexpected due to the nature of the assay (gp120 pre-bound to surface CD4), and the preponderance of previously described CD4i mAbs that utilize VH1-69, including 17b, c12/SB1/X5, 23e, 47e/412d/E51, and M16 (30). To investigate more directly whether the six R66M VH1-69 mAbs capable of mediating high GzB signaling within this assay are enriched for CD4i epitopes, we first assessed the ability of 16 R66M mAbs to recognize endogenously produced, processed, membrane-bound autologous T/F Env presented on the surface of transfected 293T cells. This panel of mAbs included six that mediated high GzB activity and nine that did not. All of the R66M mAbs tested were capable of recognizing membrane bound, autologous R66M T/F Env except for mAb 6C12, which did mediate ADCC and clearly bound to soluble gp120 in OctetRED and ELISA assays (ELISA data not shown) (**Supplementary Figure 4**).

We next determined whether binding of the remaining 15 mAbs to surface expressed Env was influenced by addition of soluble human CD4 (sCD4). Flow cytometry was performed with R66M mAbs from the high ($n = 6$) and low ($n = 9$) GzB groups to determine the percent of Env-positive 293T cells in the absence and presence of sCD4. Differences in binding, whether an increase (suggestive of a CD4i epitope) or decrease (suggestive of a CD4bs epitope) were compared to PGT121 binding, which is not sensitive to the addition of sCD4. Binding of mAb 17b, which recognizes a CD4i epitope, was used for comparison (**Figure 7E**). Interestingly, the CD4i antibody 17b bound to surface expressed R66M Env in the absence of sCD4, perhaps suggesting this Env trimer is natively in a more "open" conformation. However, 17b binding was significantly increased more than 10% upon pre-incubation of the Env-expressing cells with sCD4 (**Figure 7E**). Remarkably, binding of the six R66M mAbs capable of mediating high ADCC activity (red) to surface-expressed Env was not enhanced by sCD4, while several low ADCC mAbs (blue) did display altered binding profiles in the presence of sCD4 (**Figure 7E**). This latter group included mAb 1F7, which had reduced binding in the presence of sCD4 and also competed with VRC01, although it did not mediate potent neutralization. There were also several mAbs that displayed a

significant increase in binding in the presence of sCD4, similar to 17b, yet none of these mAbs mediated high ADCC activity in the gp120 coating assay.

DISCUSSION

VH1-69 utilizing antibodies have gained notoriety as first-responders in a number of viral infections. An over representation of VH1-69 antibodies has been described in influenza infection, hepatitis C associated cancers, respiratory syncytial virus infection, and HIV-1 infection (70, 77, 79–82). A widely accepted explanation for the ability of VH1-69 antibodies to readily interact with diverse viral proteins is the inherent hydrophobic CDRH2 region that is unique to VH1-69 alleles (30, 75). Here, we also observed an over representation of VH1-69 germline-utilizing antibodies that recognize autologous T/F gp120 proteins, in five of six individuals infected with diverse HIV-1 variants prevalent in Sub-Saharan Africa (Subtype A, C, and A/C recombinants). A unique feature of our study is that it was carried out at ∼7-months post-infection and demonstrates that the VH1-69 response occurs early. The broad predominance of anti-gp120 VH1-69 antibodies in the subjects studied here, infected with diverse HIV-1 variants, prompted us to delve deeper into the features and antiviral functions of individual VH1-69 mAbs. Within ∼100 VH1-69 mAbs from the six HIV-1 infected individuals that were tested for *in vitro* Fc-mediated GzB activity, nearly one-third were able to mediate high ADCC activity against cells coated with autologous T/F gp120. In contrast, none of the VH1-69 antibodies were capable of potent mAb neutralization capacity. Importantly, despite the hypothesis that VH1-69-utilizing mAbs are "innate like," the absence of substantial ADCC activity in the plasma near the time of infection illustrates affinity maturation is required to acquire effector functions.

The VH1-69 mAbs in our study also exhibited CDRH2 regions that were significantly more hydrophobic than the non-VH1-69 anti-gp120 sequences from the same individuals. This observation is consistent with the concept that VH1-69 alleles have evolved in humans to provide a "first-line response" to viral infections (83). The VH1-69 locus itself is genetically diverse, with at least 14 alleles described thus far, and copy number variation from 2 to 4 copies per individual (2, 76, 84–86), perhaps hinting that it might be under selective pressure to continue to diversify within the human population. Further supporting this is the observation that genetic variation within this locus can influence the anti-viral response. In the context of human influenza infection, antibodies that contain phenylalanine at amino acid position 54 are associated with neutralization, while those that contain leucine at this position are not only infrequently induced, but also lack neutralization capacity in comparison to the 54F variants (2, 76). If this division in function remained prominent for other viral infections, such as HIV-1, it could have substantial implications for vaccine design, as these alleles are differentially globally distributed (2). While VH1-69 allelic variation has not been exhaustively defined, especially in African populations relevant to the individuals studied here, we were able to quantify the number of anti-gp120 VH1-69 sequences that contained F, L, or another amino acid at position 54. As previously reported, the 54F-containing sequences were the most frequently utilized, and generally composed the majority of VH1-69 antibodies even when the 54L-containing sequences were also present in the same individual. The one exception to this observation was R66M, where 54L-containing alleles were the dominant VH1-69 genotype. In contrast to influenza infection, where the 54L genotype results in fewer VH1-69 anti-viral antibodies, in R66M, anti-gp120 VH1-69 utilizing antibodies containing both 54F and 54L made up 51% of the isolated sequences (76). This suggests that the dearth of 54L VH1-69 antibodies observed in individuals during influenza infection does not necessarily translate to a similar bias in HIV-1 infection. Also in contrast to influenza infection, where the 54F genotype is associated with neutralization, the ability to mediate neutralization or ADCC effector functions against HIV-1 was not stratified by VH1-69 genotype (76). Both 54F and 54L were found in the high and low ADCC-mediating groups. This could be a reason for cautious optimism, in the context of vaccines specifically designed to elicit polyfunctional antibody responses, that include ADCC effector functions in humans (87). However, VH1-69 allelic diversity is only one component of host genetics that influences ADCC. Other factors likely influence ADCC *in vivo*, such as FcγRIIIa allelic variation (88, 89). For example, polymorphisms present at position 158 in FcγRIIIa, such as valine (V) or phenylalanine (F), can differ in affinity for antibody Fc. This could alter the potency and efficacy of ADCC *in vivo*, even if appropriate ADCC-mediating antibodies are present. The assay utilized here to measure ADCC activity included effector cells from HIV-negative donors, who were screened to eliminate low affinity (F/F homozygous) donors. Though these and other differences in host genetics could alter the performance of these antibodies *in vivo*, we have identified and described a number of antibodies, with distinct genetic features, present in five of six individuals, that have the potential to mediate autologous ADCC effector function against HIV-1 T/F gp120. These antibodies also bind to endogenously expressed Env on the cell surface, and are not dependent on the presence of CD4 to bind to their epitope or mediate Fc-mediated GzB signaling. It will be of interest to further define the novel epitopes that are susceptible to ADCC, whether they are conserved across HIV-1 variants, and what features of Env are important to elicit them.

Interestingly, a number of genetic and biochemical features did not distinguish VH1-69 antibodies capable of mediating high ADCC activity from those that did not. Importantly, and supporting the idea that VH1-69 interactions with viral glycoproteins are innate-like, there was no difference in somatic hypermutation (i.e., percent identity to germline) and likewise, no difference in binding affinity for the autologous T/F gp120 protein between VH1-69 ADCC-mediating and non-mediating antibodies. Thus, it was not necessarily routine selection and affinity maturation that lead to mAbs capable of ADCC, nor generic "ELISA-like" binding of antibody to gp120 that resulted in ADCC effector functions observed here. All of the VH1-69 antibodies bound to soluble gp120, but only a subset could mediate ADCC using the exact same protein coated onto the

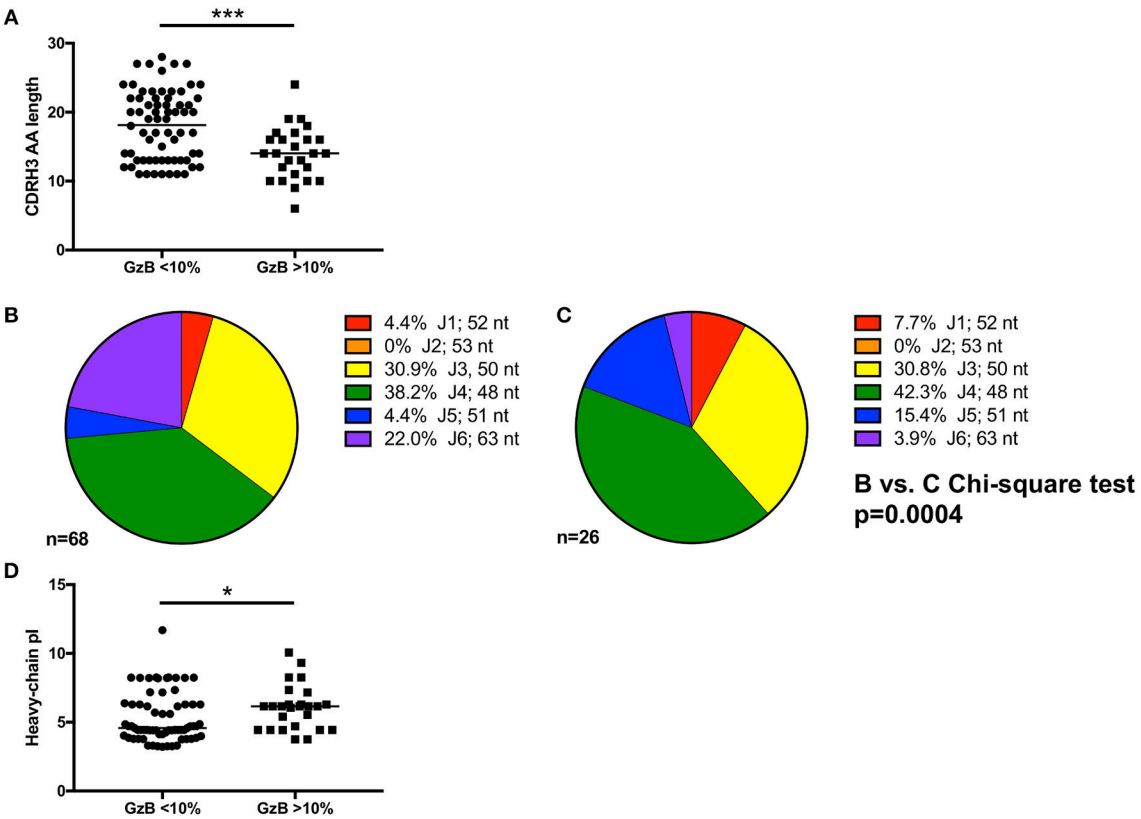

FIGURE 6 | Genetic signatures of VH1-69 mAbs capable of mediating high ADCC activity. **(A)** Amino acid length of mAb complementarity-determining region-3 (CDRH3) (Y-axis) of mAbs with GzB <10% are longer than those from mAbs that mediate high ADCC activity (Mann–Whitney, $p = 0.0007$). **(B)** Distribution of J1 (red), J2 (orange), J3 (yellow), J4 (green), J5 (blue), and J6 (purple) utilization of VH1-69 mAbs that mediated low ADCC activity compared to **(C)** J-region utilization in mAbs that mediated high ADCC activity revealed differential J-region usage between the two groups of mAbs (Chi-square test, $p = 0.0004$). Number of sequences analyzed and percentage of each J-region utilized is indicated. **(D)** A modest difference in isoelectric point (Y-axis) of the heavy chain of the antibodies able to mediate high ADCC vs. low ADCC also emerged (Mann–Whitney, $p = 0.035$). $^{*}p \leq 0.05$, $^{***}p \leq 0.001$.

target cell surface. A possible explanation is that some mAbs bind to gp120 at an angle that facilitates a sufficient number of antibody-Fc interactions with effector cells needed to trigger granzyme B release, i.e., an "angle of attack" conducive to mediating ADCC functions, while other antibodies bind in a manner that was not favorable for Fc receptor engagement. Though structural studies will be necessary to fully appreciate the importance of specific epitopes and angle-of-binding to those epitopes, we describe clear instances where antibodies that share similar epitopes display divergent ADCC activity. Similar findings were reported in previous analyses of anti-HIV ADCC activity, where the importance of not only the recognized epitope, but also the angle of antibody binding, was emphasized (90). Certainly, this is an important observation in the context of HIV-1, but it is also an important concept to consider when generating any therapeutic mAb with a desired ADCC mechanism of action.

A genetic feature that was strongly associated with high ADCC activity in this study was shorter CDRH3 length. In stark contrast with bnAbs, which sometimes include exceptionally long CDRH3 regions, VH1-69 mAbs capable of mediating ADCC had, on average, shorter CDRH3 regions than non-mediating mAbs (16). However, these "short" CDRH3s averaged 14 AA in length, near the expected average lengths of CDRH3 in "control" human antibodies [reported value 15 AA (70)]. This "average" CDRH3 length appeared to be related to a lower frequency (but not absence) of J6 usage in the high ADCC mAb group, which can contribute to longer CDRH3 length [63 nt addition, (91)] and is frequently found in bnAbs with long CDRH3 (16). The infrequent use of J6 was compensated for mainly by a three-fold increase in J5 usage, which is of average size (51 nt addition). An additional genetic metric that was modestly distinctive of high ADCC mAbs was a higher isoelectric point of the heavy chain that was closer to neutral. The isoelectric point of mAbs has been strongly associated with their *in vivo* clearance and half-life (92). Antibodies mutated within the VH to have lower isoelectric points exhibited improved half-lives over the original unmutated mAbs, without modifying the constant region or light chain (92). Considering the lack of protection observed in non-human primate studies that utilized non-neutralizing antibodies (93, 94), it would be tempting to hypothesize that targeted reduction of isoelectric point of established non-neutralizing, ADCC-mediating antibodies could potentially improve the outcome of

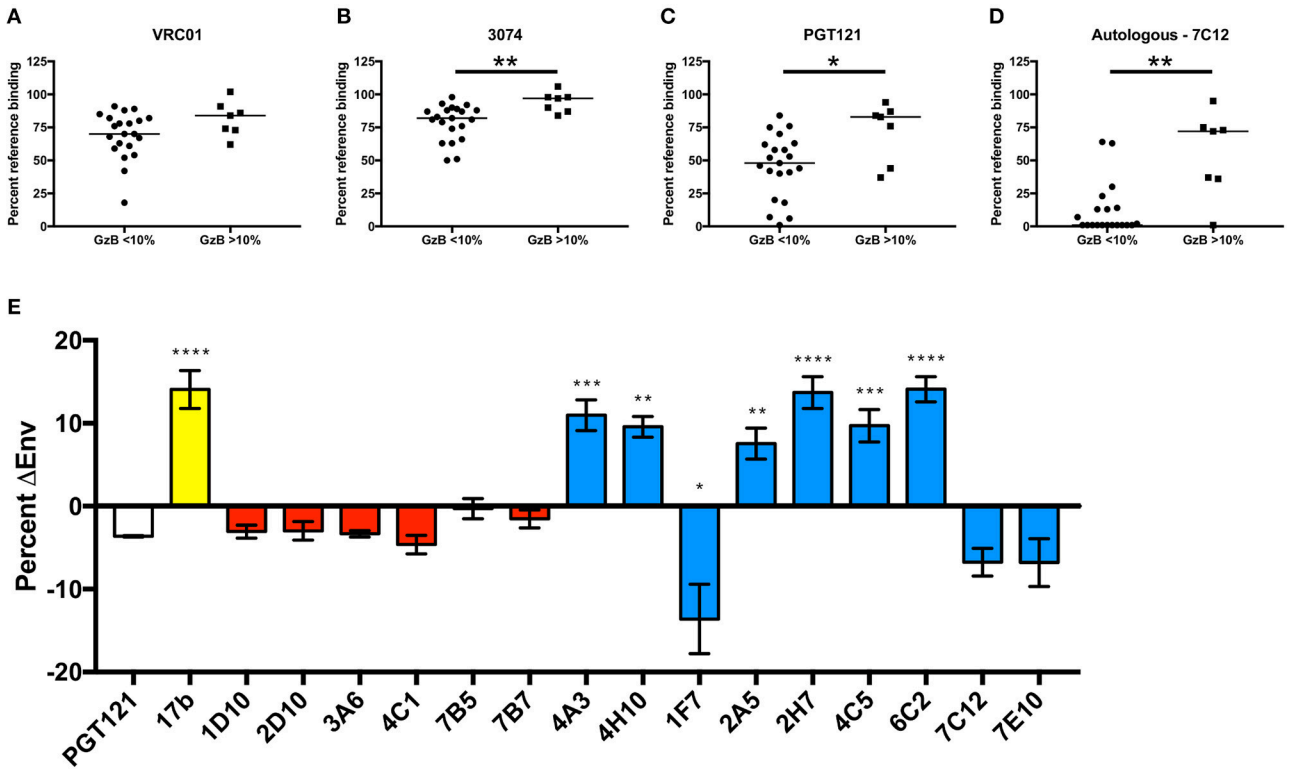

FIGURE 7 | Definition of putative epitopes of R66M VH1-69 mAbs through competition**.** In order to gain insight into the putative T/F Env gp120 epitopes targeted by R66M VH1-69 mAbs, the ability of low ADCC (GzB <10%) and high ADCC (GzB>10%) mAbs to reduce binding of reference mAbs **(A)** VRC01 (CD4 binding site), **(B)** 3074 (V3), **(C)** PGT121 (N332-glycan supersite, V3 base), and **(D)** a "median" autologous R66M mAb 7C12 to the R66M T/F gp120 protein was assessed via epitope binning on the Octet RED96. Percent residual binding of the reference antibody (top label) in the presence of each tested R66M mAb is reported, bar indicates median value. Statistically significant differences between low and high ADCC mAbs was observed for competition with 3074 (Mann–Whitney, $p = 0.005$), PGT121 ($p = 0.02$) and with the autologous mAb, 7C12 (Mann–Whitney, $p = 0.002$). **(E)** CD4bs and CD4i epitopes were identified via quantification of R66M Env-positive transfected 293T cells in the absence and presence of sCD4. Y-axis represents the increase (positive values) or decrease (negative values) of Env-positive cells in the presence of sCD4 relative to no sCD4. Binding of bnAb PGT121 (white bar) to R66M Env is not substantially affected by sCD4, thus was used as a reference. Control CD4i mAb 17b binding (yellow bar) significantly increased when transfected cells were pre-incubated with sCD4 (Ordinary one-way ANOVA, $p = 0.0001$). R66M high ADCC mAbs (red bars) were not significantly affected by sCD4, however several low ADCC mAbs (blue bars) demonstrated likely CD4i epitopes (4A3, $p = 0.0004$; 4H10, $p = 0.0014$; 2A5, $p = 0.0047$; 2H7, $p = 0.0001$; 4C5, $p = 0.0005$; 6C2, $p = 0.0001$) or CD4bs epitope (1F7, $p = 0.027$). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

these trials. Thus, the data presented here begin to generate a picture of antibodies that can mediate ADCC-mediated functions against the autologous Env, providing tangible features that distinguish them from non-ADCC antibodies. Furthermore, these findings highlight three important points. First, the R66M mAbs that mediated high ADCC activity were generally able to recognize membrane-bound Env in the absence of CD4, in addition to their capacity to bind to soluble gp120. Secondly, the high ADCC-mediating mAbs were not directed toward CD4i epitopes, thus this experimental approach was not biased to detect Fc-mediated activity against epitopes exposed by gp120 binding to CD4. Finally, the recognition of CD4i epitopes was not the primary determinant of Fc-mediated GzB activity for R66M mAbs in the gp120 coating assay. Together, these findings suggest that there are many more facets of Fc-mediated antibody activity than are currently appreciated, and that the use of varied *in vitro* assays and multiple approaches can enrich this body of knowledge. Furthermore, the use of novel antibodies and autologous antibody/protein combinations may be key to furthering these discoveries of ADCC epitopes and antibody binding modes.

The data presented here can be considered in the context of unanswered questions. For example, it remains to be seen what impact these ADCC antibodies have on the viral population and disease progression. Plasma neutralization capacity can clearly influence the evolution of an individual's viral quasispecies, while HIV-1 Env's capacity to tolerate mutations and maintain viral fitness makes the influence that nAb and has on disease progression and pathogenesis is less clear (95–97). There is also the question as to whether these mAbs with ADCC function, present pre-exposure, could reduce transmission rates or improve disease progression post-infection using an autologous challenge virus. Alternatively, there is a possibility, especially within the context of the ADCC assay utilized here, that ADCC mAbs present as pre-existing immunity could have a detrimental effect on disease progression and CD4+ T-cell counts, as soluble

gp120 could adhere to the surface of uninfected CD4+ T-cells and target them for cell death (though it should be noted that in the referenced study, uninfected "bystander cells" could not be differentiated from cells bound to virions, in the process of becoming infected) (98). It will also be interesting to investigate whether any of these early autologous ADCC-mediating mAbs, with conservative identities to germline and average affinities for autologous T/F gp120, display ADCC breadth, or whether they display strain specificity, which is frequently observed with early autologous neutralizing antibodies. The ability to recognize and exert multiple antiviral functions against a broad range of genetically and geographically distinct HIV-1 variants is almost certainly required for protection against infection. Although only autologous nAb and ADCC activity were assessed in the current study, we have identified common genetic features of early VH1-69 anti-gp120 antibodies induced in distinct host genetic backgrounds against diverse HIV-1 variants that are associated with higher GzB activity. Future studies will be required to determine whether these antibodies are cross-reactive and if they can also mediate ADCC functions in other assays. The dominance of VH1-69 in these HIV-1 infected individuals suggests an immunologic convergence that could be harnessed to elicit antibodies capable of mediating ADCC activity against gp120, suggesting that a better understanding of this process could inform the design of vaccine immunogens. Understanding the complexities of how ADCC mediating antibodies arise in infected and vaccinated individuals, and carry out effector functions, will likely require the use of varied *in vitro* assays and multiple approaches. The discovery of new antibodies with novel epitopes and binding modes will facilitate this process, expanding our knowledge beyond commonly used prototypical antibodies and viral strains.

AUTHOR CONTRIBUTIONS

CD and SS: conceptualization, methodology, and writing—original draft; CD, SS, and SB: formal analysis; CD: funding acquisition, project administration, and supervision; SS and SB: investigation; WK, SL, EK, MP, and SA: resources; CD, SS, WK, SL, EK, MP, and SA: writing—review & editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.03163/full#supplementary-material

Supplementary Table 1 | Genetic characterization of paired heavy- and light-chain sequences isolated from autologous T/F gp120 reactive single memory B cells sorted from PBMC collected at 7.5 months after infection (Time Point 2). Sequences from paired heavy- and light-chains from autologous T/F Env gp120-reactive single B cells were analyzed via IMGT vquest (http://imgt.org/IMGT_vquest/vquest) to determine (i) Variable heavy (VH) germline and allele, (ii) VH identity to germline, (iii) CDRH2 sequence determination, (iv) CDRH3 length, (v) CDRH3 sequence determination, (vi) Variable light (VL) germline and allele. CDRH2 GRAVY scores were calculated via (http://www.gravy-calculator.de).

Supplementary Table 2 | Genetic features of VH1-69 utilizing antibody sequences. VH1-69 sequences were analyzed in further detail, due to their overrepresentation in isolated anti-gp120 memory B cells. Variable Joining region (VJ) alleles and heavy-chain isoelectric points were determined via IMGT vquest (http://imgt.org/IMGT_vquest/vquest). Amino acid 54 within CDRH2 is highlighted in bold. CDRH2 GRAVY scores were calculated via (http://www.gravy-calculator.de). The number of tyrosine sulfination sites contained within CDRH3 was quantified via the Sulfinator online tool (http://web.expasy.org/sulfinator/) (58).

Supplementary Table 3 | Quantification of VH1-69 mAb binding affinity for autologous T/F gp120. The affinity of VH1-69 utilizing mAbs for binding to the autologous T/F Env gp120 protein was determined on an Octet RED96. The molarity of gp120 utilized for measurement, calculated KD and KD error, kon and kon error, kdis and kdis error, X^2, and R^2 values are reported. Average KD, kon, and kdis values are reported for each set of measurements. Median X^2 (and range), R^2 (and range), KD, kon, and kdis are reported for each individual.

Supplementary Table 4 | Neutralizing and non-neutralizing Fc-mediated effector function of VH1-69 utilizing antibodies. The ability of virions pseudotyped with autologous T/F Env to infect TZM bl indicator cells in the presence of 10 μg/ml of each mAb was assayed in triplicate, and repeated independently as quantity allowed. Non-neutralizing Fc-mediated effector function was quantified via GranToxiLux assay, in a mAb dilution series of 5, 1, and 0.2 μg/ml against CEM.NKr.CCR5 cells coated with autologous T/F Env gp120. Values reported are the mean peak percentage of cells positive for Granzyme B (GzB) activity over this dilution series, after subtraction of background activity, utilizing effector cells from two independent HIV-negative effector cell donors.

Supplementary Figure 1 | Representative Granzyme B control and experimental flow-plots. CEM.NKr.CCR5 cells were pulsed with gp120 proteins (R66M gp120 illustrated here). After gating for size (targets) and NFL4 negative (live) cells, determination of Granzyme B activity was calculated by drawing gates based on target cells incubated alone (Targets Alone). The percentage calculated within "Targets + Effectors" was inferred to be background GzB activity, and subtracted from all other positive percentages to calculate final positive percentage. Negative

and Positive controls were included in all assays. In groups of tested patient derived mAbs, ADCC high (R66M 1D10) and low (R66M 1F7) were observed.

Supplementary Figure 2 | Impact of T/F Env gp120 proteins and effector cell donor variation on ADCC and autologous plasma control assays. The percentage of GzB positive cells between individuals was compared in reactions containing **(A)** only T/F Env gp120 coated CEM.NKr.CCR5 target cells and donor effector cells (background GzB activity), **(B)** negative control 50 μg/ml commercial polyclonal IgG (minus background), and **(C)** positive control 50 μg/ml HIV-Ig (minus background). There were no significant differences in these parameters between the six T/F Env gp120 proteins (Kruskal–Wallis, ns), although there was ∼2- to 3-fold variation in HIV-Ig responses as would be expected. Bars illustrate standard error of the mean. **(D)** There was no significant difference between GzB percentages between results obtained using Donor 190 and 457 effector cells (Mann–Whitney, ns), and **(E)** the GzB results were highly correlated between the two donors (Spearman, $p < 0.0001$, $r = 0.82$). **(F)** Negative control data for **Figure 1**. nAb and ADCC activity in plasma collected at chronologic Time Points 1, 2, and 3; (i) nAb against a negative control VSV-g Env pseudovirus (closed symbols, solid lines), and (ii) GzB mediated by normal human serum against the T/F Env gp120 (open symbols, dashed lines). Symbols and error bars represent the mean and standard error of mean, respectively, between at least two replicate experiments for neutralization, and the mean and standard error of mean of independent experiments utilizing cells from two different donors for the GzB assay.

Supplementary Figure 3 | Genetic and functional comparison of VH1-69 low ADCC mAbs vs. high ADCC mAbs. VH1-69 mAbs were stratified into two groups, based on low (peak GzB percentage <10%) or high (peak GzB percentage >10%) ADCC activity. No significant differences emerged between these two groups in a number of metrics: **(A)** heavy-chain percent nucleotide identity to germline (Mann–Whitney, $p = 0.74$); **(B)** the number of tyrosine sulfation sites found within the heavy-chain complementarity-determining region-3 (CDRH3) (Mann–Whitney, $p = 0.18$) (30); **(C)** kappa or lambda light-chain pairing (Fisher's exact test, $p = 0.38$); **(D)** heavy-chain complementarity-determining region-2 (CDRH2) grand average hydropathy (GRAVY) scores (Mann–Whitney, $p = 0.89$); **(E)** binding affinity for autologous T/F gp120 (Mann–Whitney, $p = 0.76$). **(F)** There was a significant difference between the two groups and their ability to neutralize virions pseudotyped with the autologous T/F Env (Mann–Whitney, $p = 0.0015$). $^{**}p \leq 0.01$.

Supplementary Figure 4 | Antibody recognition of R66M T/F Env transfected cells in the absence and presence of sCD4. 293T cells were transfected with R66M T/F Envelope expression plasmid. 48-h post-transfection, cells were stained with control and R66M VH1-69 mAbs (indicated above plots), either **(A)** without or **(B)** with pre-incubation with sCD4. X-axis indicates PE intensity, Y-axis indicates side scatter area (SSC-A). "Env-positive" cells are found in the lower right quadrant (Q3), percentage of total events per quadrant are indicated. **(C)** Percent Env-positive cells, minus IgG background, for each mAb in the absence (white) and presence of sCD4 (gray). Columns indicate mean of a minimum of three independent experiments, bars represent SEM.

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REVIEW
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Antibody-Dependent Cellular Phagocytosis in Antiviral Immune Responses

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Antiviral activities of antibodies may either be dependent only on interactions between the antibody and cognate antigen, as in binding and neutralization of an infectious virion, or instead may require interactions between antibody–antigen immune complexes and immunoproteins or Fc receptor expressing immune effector cells. These Fc receptor-dependent antibody functions provide a direct link between the innate and adaptive immune systems by combining the potent antiviral activity of innate effector cells with the diversity and specificity of the adaptive humoral response. The Fc receptor-dependent function of antibody-dependent cellular phagocytosis (ADCP) provides mechanisms for clearance of virus and virus-infected cells, as well as for stimulation of downstream adaptive immune responses by facilitating antigen presentation, or by stimulating the secretion of inflammatory mediators. In this review, we discuss the properties of Fc receptors, antibodies, and effector cells that influence ADCP. We also provide and interpret evidence from studies that support a potential role for ADCP in either inhibiting or enhancing viral infection. Finally, we describe current approaches used to measure antiviral ADCP and discuss considerations for the translation of studies performed in animal models. We propose that additional investigation into the role of ADCP in protective viral responses, the specific virus epitopes targeted by ADCP antibodies, and the types of phagocytes and Fc receptors involved in ADCP at sites of virus infection will provide insight into strategies to successfully leverage this important immune response for improved antiviral immunity through rational vaccine design.

Keywords: antibody effector functions, antibody-dependent cellular phagocytosis (ADCP), Fc receptors, phagocytes, antiviral antibodies

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INTRODUCTION

Antibodies are a key component of the human adaptive immune system, and the elicitation of antibodies has been correlated with vaccine efficacy in many diseases (1). Passively infused antibodies have been used in anti-toxin, anti-viral, and anti-inflammatory treatments; and monoclonal recombinant antibodies are also currently being pursued for prevention of HIV-1 infection in large Phase IIb clinical trials (NTC02716675 and NCT02568215). Antibodies can exert

their protective functions via a multitude of mechanisms. Some functions, such as neutralization, mainly depend on interaction of the Fv domain (**Figure 1A**) with antigen and are therefore predominantly Fc domain independent. Other functions, including antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP), require interactions between the antibody Fc domain with other proteins or immune effector cells via recognition by Fc receptors (9–11) (**Figures 1A–C**). These Fc receptor-dependent antibody functions provide a direct link between the innate and adaptive immune systems, harnessing the potent anti-pathogen functions of the innate immune system, and overcoming its inherent limited pattern recognition capacity by utilizing the diversity and specificity of the adaptive immune response. Fc receptor-dependent antibody functions are important components of the immune response that provide mechanisms for clearance of infected host cells, immune complexes, or opsonized pathogens. Fc receptor-dependent antibody functions are also involved in activation of downstream adaptive immune responses by facilitating antigen presentation or by stimulating the secretion of inflammatory mediators (12, 13). This review is focused on the antibody Fc receptor-dependent effector function ADCP in immune responses against viruses and targets three areas of interest: (1) discussion of the biophysical factors that influence ADCP including the properties of the receptors, antibodies, and effector cells; (2) survey and interpretation of evidence supporting a potential role for ADCP in either inhibiting or enhancing viral infection; and (3) description of current approaches used to measure ADCP with consideration for the translation of studies performed in animal models.

ANTIBODY AND Fc RECEPTOR INTERACTIONS INVOLVED IN ADCP

Immune complexes formed between antigen and antibody are capable of engaging a diversity of Fc receptors on innate immune cells. The type I IgG Fc receptors are activatory FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa, FcγRIIIb, and inhibitory FcγRIIb (**Figure 1C**). Other IgG Fc receptors include the non-classical (type II) IgG Fc receptors CD209 and CD23, neonatal FcR (FcRn) which is involved in IgG transport and recycling, and the cytosolic Fc receptor TRIM21. IgA antibodies are specifically engaged by FcαRI (**Figure 1C**), and the specific receptor for IgE is FcεRI—which is involved in rapid allergic responses. Immune complexes may also interact with other receptors that have been described to bind to immunoglobulins but have been relatively uncharacterized, including FCA/MR (14), FCMR (15), IgD-R (16), CD71 (17), secretory component receptors (18), asialoglycoprotein receptors (19), and M cell receptors (20).

When engaged, most Fc receptors are capable of cytoplasmic signaling. For instance, as shown in **Figure 1C**, FcγRIIa and FcγRIIc signal via their immunoreceptor tyrosine-based activation motif (ITAM) domains, whereas FcγRI and FcγRIIIa lack ITAM domains but associate with an FcRγ signaling chain (γ_2) and signal via its ITAM domain. The inhibitory FcγRIIb signals via an immunoreceptor tyrosine-based inhibition motif (ITIM) domain. The IgA receptor, FcαRI, also associates with the FcRγ signaling chain (**Figure 1C**), but this seems to be dispensable for signaling, and signaling is dependent on dephosphorylation of the intracellular domain of FcαRI (21). Downstream signaling pathways are complex and dependent on the Fc receptor, cell type, and stimulation mechanism, but generally act via increasing intracellular calcium cation concentration, activation of PKC, or activation of ras (22).

As a result of the ubiquitous presence of antibody in both the systemic and mucosal microenvironments, regulatory systems are required to prevent constitutive Fc receptor signaling. This is achieved via several mechanisms that are either intrinsic to Fc receptor signal pathways, impacted by external soluble signals detected by the phagocyte, or established at the genetic level (**Figure 2**). A key intrinsic regulator is the inability of free antibody to activate Fc receptor signaling. The low-affinity Fc receptors, including FcγRIIa, FcγRIIb, FcγRIIc, and FcγRIIIa require multiple coordinated interactions for sufficient binding avidity, and thus can only be triggered by multivalent antibody-antigen immune complexes (22). Even for the high-affinity FcγRI, which is able to bind monomeric IgG, binding does not trigger signaling through its associated γ-chain; instead, signaling requires receptor clustering and cross-linking (23). Moreover, activatory Fc receptors can also produce inhibitory signals when engaged at a low level–the mechanisms for this phenomenon are not well-understood but may involve ITAM monophosphorylation which activates the inhibitory SHIP-1 (24) rather than the diphosphorylation required for activatory Syk engagement (25). Similarly, immunostimulatory and immunoinhibitory Fc receptors are often co-expressed on the same cell, thus the outcome of antibody-mediated signaling is often dependent on the balance of activating or inhibiting signals. The abundance of these receptors on the cell-surface and their ability to interact with immune complexes is influenced by soluble signaling molecules, which allows the local inflammatory milieu to also contribute to the regulation of Fc receptor-dependent responses of phagocytes (26–28). Finally, Fc receptor signaling is regulated at the genetic level. Single nucleotide polymorphisms in human Fc receptors affect interactions with antibody Fc, resulting in Fc receptor variants with lower or higher relative affinities for immune complexes (2, 6, 11, 12, 29). Several Fc receptor polymorphisms have been associated with the occurrence or progress of disease resulting from infection with viruses including dengue virus (30, 31), influenza virus (32), human coronavirus (33), Epstein-Barr virus (EBV) (34), Kaposi's Sarcoma virus (KSV) (35), and HIV-1 (36–38).

The specific Fc receptor engagements involved in ADCP of different viruses or virus-infected cells at sites of infection and throughout antiviral immune responses have not been completely defined, and are not expected to follow a generalizable rule. As will be described in the following sections, the receptors involved will differ depending on the characteristics of the antibody forming the complex —such as isotype, subclass, and glycosylation— as well as on the particular type of phagocyte encountering the immune complex.

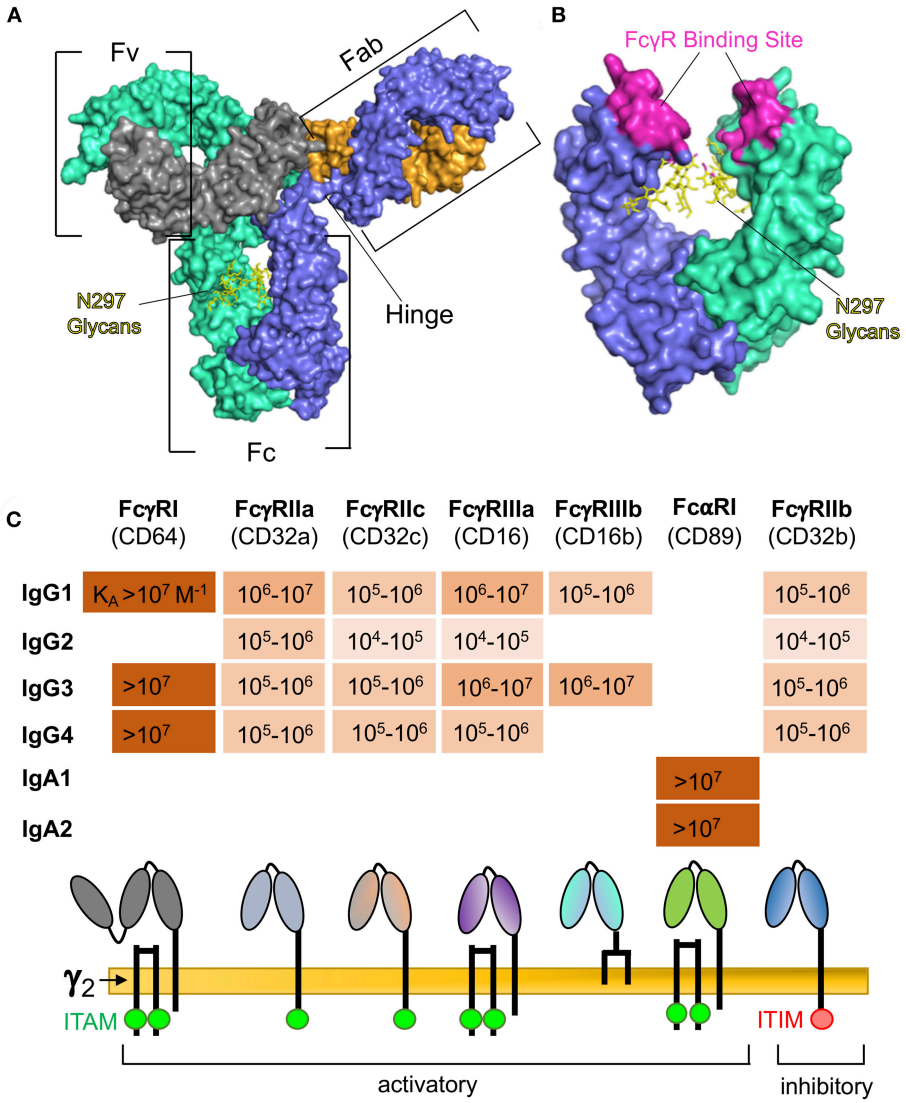

FIGURE 1 | (A) Surface representation of human IgG1 indicating: the Fv regions, which are the portions of the Fab arms required for antigen binding; the Fc region, which interacts with immunoproteins and specific receptors for effector functions; and the hinge region, which provides flexibility for the Fab and Fc regions. The IgG heavy chain 1 is depicted in blue, heavy chain 2 in teal, light chain 1 in orange, and light chain 2 in gray. Glycans are represented by yellow sticks. Rendering was made in PyMOL software using protein data bank ID 1HZH. **(B)** Expanded view of human IgG1 Fc region after 180° rotation, indicating residues involved in binding to Fcγ receptors (2–5), and the glycosylation site at asparagine 297 (N297). **(C)** Binding affinities (6–8) of Fc receptors commonly implicated in ADCP and comparison of receptor composition and signaling domains. Absence of values indicates no detectable binding.

ANTIBODY CHARACTERISTICS THAT IMPACT ADCP

In addition to the Fc receptor regulatory mechanisms described above, the ability of interactions between immune complexes and Fc receptors to result in Fc receptor signal transduction is further regulated by specific characteristics of the antibody Fc region. Antibody isotype serves as the principal level of regulation, as most Fc receptors are specific for only one isotype of antibody. Within isotype, there is additional regulation at the level of antibody subclass (**Figure 1C**). For the human IgG isotype, IgG3 has the highest affinity for most of the type I FcγRs, followed by IgG1, then IgG4, then IgG2 (6). In contrast, subclass is not a predominant source of regulation for ADCP by IgA, as human FcαR has been demonstrated to have similar affinity for IgA1, and IgA2 (7).

Additional regulation of Fc–Fc receptor interactions required for ADCP occurs via diversity in glycosylation of the antibody Fc (39). The IgG Fc region contains an N-linked glycan at asparagine 297 (**Figure 1B**) that impacts the conformation of the antibody and affinity for Fc receptors (39). As described in a comprehensive recent review by Jennewein and Alter, for IgG antibodies there are 36 possible glycoforms and 4 different subclasses, yielding a total of 144 possible unique Fc regions

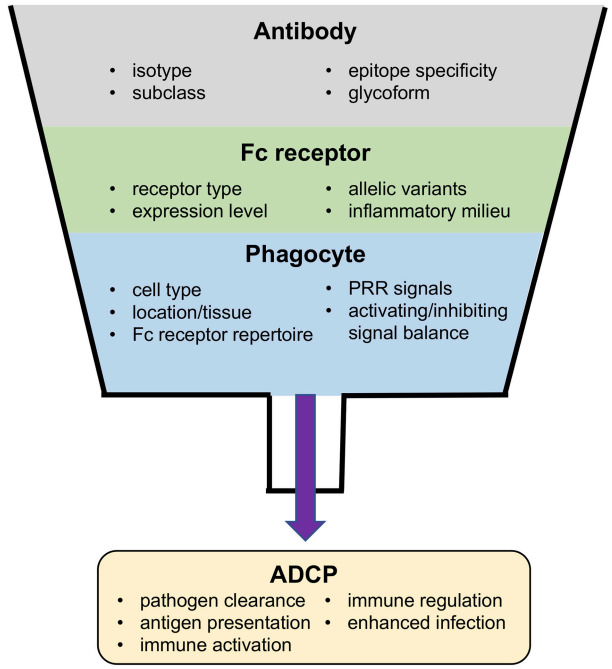

FIGURE 2 | Factors impacting ADCP and potential outcomes of the ADCP response to virus infection.

(40). Importantly, antibody glycovariation can be modulated by inflammatory responses, allowing the immune system to adapt and adjust antibody Fc glycoforms to modulate biologic activities in response to infection (40). Changes in antibody glycosylation have been described during the course of HIV-1 infection (41, 42), and in response to influenza vaccination (43, 44), but for many viral diseases the role of glycovariation in antiviral antibody responses has yet to be defined.

Fc RECEPTOR SIGNALING FOR ADCP

Leukocytes involved in ADCP must express at least one type of Fc receptor. This includes monocytes, macrophages, neutrophils, and eosinophils; canonically referred to as professional phagocytes. In ADCP the phagocyte is engaged by antibody, either directly via Fc receptor or indirectly via antibody-fixed complement, to engulf one or more opsonized particles or molecules, which typically including pathogens, infected cells, and their derivatives (45, 46). The internalization in most cases leads to the destruction of the internalized target by phagolysosomal degradation, though it is important to note that several human pathogens have evolved to co-opt this process and survive within phagocytes (47–50). Phagocytosis leads to different immune outcomes depending on the cell type—for instance, antibody-mediated phagocytosis by macrophages leads to enhanced pathogen destruction and antigen presentation, whereas antibody-mediated phagocytosis by plasmacytoid dendritic cells leads to enhanced secretion of interferon alpha (51–53).

In Fc receptor-mediated phagocytosis, ligated and aggregated Fc receptor become phosphorylated via Src family tyrosine kinases (54) on their ITAM domains (either their own or from an associated γ-subunit), forming a docking site for Syk (55) and triggering a signaling cascade involving PKC (56, 57), PI3K (58–60), and synthesis of PI(4,5)P2, (3,4,5)-PIP3, and DAG (61). These lead to actin cytoskeleton remodeling (62), allowing the advance of the phagocytic cell over the target. The strength of early signaling events is proportional to the number of engaged Fc receptors, whereas late signaling events required to complete phagocytosis require a concentration threshold of 3′PI to be satisfied (63).

PHAGOCYTES INVOLVED IN VIRUS ADCP

The type of phagocyte involved in an ADCP responses to virus infection depends not only on the profile of Fc receptors expressed by the cell and characteristics of the antiviral antibodies as described above, but also on the phagocyte being present at, or recruited to, sites of infection. Professional phagocytes are differentially distributed in the circulation and tissues (64–67), and inflammatory signaling can promote both ingress and egress of immune effector cells (68). Therefore, cells present at the site of infection and involved in the antiviral response likely change over time. Transgenic mouse models and cell-type specific depletions can help to identify essential cell populations, but a clear view of the specific interactions involved in the tissue at the site of virus infection is often limited, especially in humans. Despite the difficulties inherent to accessing and studying immune cells *in situ* within human tissues, a remarkable study by Sips et al. defined the distribution and frequency of Fc receptor expressing immune cells in mucosal and lymphoid tissues (69). They identified differential distribution of professional phagocytes —with macrophages being the dominant phagocyte population in lymph nodes, and intestinal tissues and neutrophils representing the dominant phagocyte population in tissues from the lower female reproductive tract. Using a novel tissue phagocytosis assay, they compared the HIV-1-specific ADCP activity of neutrophils and macrophages from the colon and cervix. They found that although abundant in the colon, colon-resident macrophages were deficient in ADCP compared to colon- and cervix-resident neutrophils as well as cervix-resident macrophages. This seminal study likely only partially defines the diversity of professional phagocytes, both for phenotype and functionality, within tissues that can be encountered by antibody-virus immune complexes during virus infection, and that inevitably impacts outcome of these encounters.

BALANCE OF ACTIVATORY AND INHIBITORY SIGNALS DETERMINE HOW PHAGOCYTES RESPOND TO IMMUNE COMPLEXES

Importantly, most phagocytes are capable of other Fc-dependent effector functions in addition to ADCP. Thus, the outcome of each interaction between phagocytes and immune complexes

is determined by a combination of signals (**Figure 2**). Many phagocytes express more than one type of Fc receptor, often expressing both activatory and inhibitory Fc receptors. The balance of these divergent signal pathways is critical to simulating and regulating each potential effector response. Signals mediated by other types of receptors also contribute to determining the type of effector response a phagocyte will mount. Among them, information from pattern recognition receptors (PRRs) that can detect molecular patters associated with different types of pathogens are integrated in the response to Fc receptor stimuli. For example, Toll-like receptor 3, 7, 8, and 9 have the ability to detect viral nucleic acids and activate immune cells (70), and are therefore able to potentiate effective antiviral responses including ADCP. In contrast, alternative inhibitory signals, such as CD47 SIPα can negatively regulate phagocytosis. Intriguingly, some viruses express homologues of CD47, which may act to prevent activation of professional phagocytes as a strategy for immune evasion (71, 72).

Apart from direct antiviral activity through uptake and elimination of virus or infected cells, antibody-dependent phagocytosis is also important in the development and regulation of immune responses themselves (**Figure 2**) (12). Pathogen-associated molecular patterns (PAMPs) derived from virus antigens can be released upon phagocytosis and digestion of antibody–virus immune complexes. The released virus PAMPs can prime an inflammatory response upon sensing by PRRs, which may then stimulate additional immune cells and activate subsequent immune responses (12, 13). For instance, in adenovirus infection of the respiratory tract, alveolar macrophages are responsible for internalizing adenovirus and initiating early pro-inflammatory signaling (73). A similar response, termed antibody-induced inflammation, has been demonstrated to have an important role in protection against influenza infection (74). There are also direct roles for ADCP in modulation of adaptive immunity. ADCP of immune complexes by dendritic cells via FcγRIIa promotes MHC class I and II antigen presentation and induces cellular and humoral immune responses, while uptake through FcγRIIb prevents dendritic cell maturation and does not promote immune activation (75). Antibody production and affinity is also regulated by FcγRIIb-mediated induction of B-cell apoptosis, which helps to eliminate B cells with low affinity B cell receptors (75). Based on the diverse roles of ADCP in immune responses it is unsurprising that ADCP has been shown to be an important component of immune responses to infection by many different viruses.

EVIDENCE FOR THE IMPORTANCE OF ADCP IN IMMUNE RESPONSES AGAINST VIRUSES

Phagocytosis has traditionally been known for its role in clearance of bacteria and fungi, as evidenced by the fact that persons with defects in phagocytosis are susceptible to common bacterial and fungal infections (76). It is important to note that these observations relate to total phagocytosis (antibody dependent and antibody independent) and thus do not allow for dissection of the specific contribution of ADCP to protection from bacterial and fungal infection. The role of ADCP in immune responses against viruses is similarly complex and difficult to dissect given its association with other antibody functions in the settings of infection, vaccination, and passive immunization.

HIV-1

In the context of natural infection with HIV-1, the first line of evidence for a role of ADCP in the antiviral immune response is associations between Fc receptor genetics and disease progression or risk of infection. Forthal and collaborators (37) performed FcγRIIa genotyping of a large cohort HIV-1 infected men ($n = 559$), over 90% of which were entered into the study prior to the availability of antiretroviral therapy, and all of whom were enrolled into the cohort with $CD4^+$ T cell counts above $500/mm^3$. They found that homozygosity for the low affinity allele of FcγRIIa (R/R131), the Fc receptor implicated in IgG-mediated ADCP activity of antibody responses against HIV-1 (77, 78), significantly predicted an accelerated rate of disease progression —defined as $CD4^+$ T cell counts under $200/mm^3$– when compared to subjects that were heterozygous for this allele (H/R131), or homozygous for the high affinity allele (H/H131) of FcγRIIa (37). No such correlation was observed for FcγRIIIa allelic variants, which is conventionally regarded as the primary Fc receptor involved in natural killer (NK) cell ADCC. Associations between Fc receptor genetics and risk of HIV-1 infection have also been studied in the setting of vertical transmission. Using samples collected from antiretroviral-naïve HIV-seropositive mothers and paired infants in western Kenya, Brouwer et al. (36) identified infant homozygosity for the high affinity allele of FcγRIIa as a risk factor for perinatal HIV-1 infection. They observed no impact of maternal FcγRIIa alleles on transmission. Recently, a similar study was conducted using samples collected from 79 HIV-1 transmitting mothers, 234 non-transmitting mothers, and their offspring, in a South African cohort with contrasting results (79). In this latter study, the infant FcγRIIa acquisition risk factor identified by Brouwer et al. was not recapitulated, but instead the authors reported that mothers with the high affinity allele for FcγRIIIa (homozygous or heterozygous) were associated with a significantly lower risk of HIV-1 vertical transmission (79). Taken collectively, these three observations highlight the complexity in interpreting correlative studies. How can the seemingly contradictory findings be explained? Many factors are likely contributing to the divergent outcomes including differential requirements for immune control of disease progression vs. infection when comparing the Forthal study to the two studies investigating vertical transmission. Within the transmission studies, the authors of the South African study put forth the hypothesis that differences in timepoints used for determination of HIV infection may have resulted in inclusion of more postnatal breastfeeding transmission events in the Brouwer study, which likely have different requirements for protection compared to infections occurring in utero or perinatally. Although this may be the case, it is also important to reiterate that Fc receptor genetics is only one level of regulation for ADCP and other Fc receptor-dependent immune responses. As previously described,

further regulation occurs at the level of the cell (type of phagocyte and combination of receptors expressed), within tissue immune environment (presence or absence of inflammatory signals), and via the antibody comprising the immune complex (specificity, isotype, subclass, and glycoforms). Thus, although studies based on genetic factors alone may provide insight into the potential for ADCP and other Fc receptor-dependent antibody functions to contribute to immune responses to HIV-1 they are limited by an inability to account for the myriad of factors that impact these antibody effector functions *in vivo*. Other studies have helped to address some of these limitations and provide additional evidence that ADCP plays an important antiviral role against HIV-1. By comparing ADCP activity of polyclonal IgG collected from HIV-1 infected individuals and healthy controls, Ackerman and collaborators determined that phagocytosis activity was higher for IgG from viremic patients and HIV-1 controllers compared to IgG collected from patients on highly active antiretroviral therapy. Importantly, they found that the antibodies from controllers were able to outcompete phagocytic activity of antibodies from viremic individuals and were biased toward interactions with activatory FcγRIIa over interactions with inhibitory FcγRIIb (78). In follow-up studies, they demonstrated that ADCP was a component of a polyfunctional response in HIV-1 controllers that included NK cell activation, ADCC, and complement deposition (80), and that was likely impacted by skewing of antibody glycoforms (42). Unlike that observed for HIV controllers, impaired phagocytosis is one of the hallmarks of chronic viremic HIV-1 infection (81–83), and may be related to a loss of FcγRII expression on monocytes and dendritic cells (77).

Preclinical and clinical trials of candidate HIV-1 vaccines have provided more opportunities to evaluate the importance of ADCP in protection against SIV/SHIV/HIV-1 infection. Immune correlates analyses suggest that protection against infection as well as inhibition of virus replication after establishment of infection is mediated not only by direct neutralization, but also by Fc-mediated antibody effector functions (80, 84–92).

In rhesus macaque SIV and SHIV preclinical animal models, non-broadly neutralizing antibody functions, including phagocytosis, correlated with reduced risk of infection as measured by increased number of low-dose challenges to infection (86, 90, 93). Of particular interest is the elegant study by Ackerman and colleagues, which identified distinct immune signatures of vaccine-mediated protection dependent on the route of immunization (94). In this study, rhesus macaques were immunized with a DNA prime-Ad5 SIVmac239 Env-based vaccine regime, either via the intramuscular (IM) route, or intranasally in an aerosol (AE) formulation. Equivalent levels of vaccine efficacy (∼70%) against repeated low dose smE660 intra-rectal challenge were observed for both the IM and AE immunization groups, although unique humoral immune profiles and correlates of risk were identified. ADCP however, was identified as a correlate of reduced infection risk in both the IM and AE vaccine groups. Remarkably, although ADCP was a common immune function linked to protection independently of the route of immunization, the phagocytes and antibody isotypes associated with ADCP differed. For animals vaccinated by the IM route, monocyte ADCP and IgG were associated with reduced risk of infection, while ADCP by neutrophils (termed antibody-dependent neutrophil phagocytosis, ADNP) and IgA were associated with reduced risk of infection in animals vaccinated via the AE route. Importantly, a cross study validation of the ADCP correlate was preformed, and ADCP was also identified as associated with reduced risk of infection by low dose SHIV challenge in rhesus macaques vaccinated with an ALVAC prime gp120-boost vaccine regimen (93, 94), providing evidence for ADCP in vaccine-elicited protection afforded by different vaccine regimens and routes of inoculation, and against different challenge viruses.

Consistent with the observations from efficacious preclinical studies performed in rhesus macaque models, an immune correlates analysis of the partially efficacious RV144 vaccine human clinical trial provided evidence that non-neutralizing antibodies contributed to reduced risk of infection. Vaccine-elicited variable region 1 and 2 (V1/V2) IgG antibodies correlated with decreased risk of HIV-1 infection (85, 89, 91, 95) and these V1/V2 antibodies were not broadly neutralizing but were capable of multiple antiviral functions, such as ADCC, virion capture, ADCP, and tier-1 neutralization (91, 96–98). Notably, the RV144 vaccine regimen elicited antibodies that exhibited coordinated Fc-mediated effector responses (87, 91). Fc receptor polymorphisms also influenced RV144 vaccine efficacy (99), although these polymorphisms were for FcγRIIc and have not been associated with ADCP. Other HIV-1 vaccine efficacy trials that showed no efficacy either lacked a coordinated Fc receptor-dependent effector response (87) or lacked evidence of strong Fc-mediated antibody functions (100, 101).

When considered collectively, the results from non-human primate and HIV-1 candidate vaccine clinical trials provide strong evidence that ADCP is an achievable and potentially protective antiviral immune response to induce by preventative HIV-1 vaccines. As efforts continue toward development of vaccines that can induce broad-neutralizing antibodies it will be important to ensure that ADCP and other Fc receptor-dependent antibody responses are elicited. Fortunately, although HIV-1 broadly neutralizing antibodies (bnAbs) are defined based on their ability to neutralize a broad range of viruses, many bnAbs are also capable of mediating Fc receptor-dependent antiviral functions including ADCC and ADCP (69, 102–104).

Passive immunization trials have also show that antibody-mediated protective activity is not solely due to neutralization, but also in part due to Fc receptor-dependent functions. In non-human primate (NHP) passive immunization studies, with both high and low dose vaginal challenge of rhesus macaques with SHIV162p3, protection decreased by about 50% when the administered passive antibody was incapable of binding Fc receptors (105, 106). Similarly, in passive immunization studies performed with humanized mice, antibodies with enhanced ability to bind activating Fc receptors gave greater protection than their epitope-matched counterparts (107, 108). Although the specific role of ADCP in these observations has not been determined, when combined with the evidence provided from studies of HIV-1 virus control and disease progression, and from candidate vaccine trials, there is strong evidence to support

ADCP as contributing to antibody-mediated protection from HIV-1. However, a recent study by Parsons and collaborators demonstrated that for the highly potent neutralizing antibody PGT121, Fc-receptor dependent functions were dispensable for maximal protection (109). These results suggest that the extent to which Fc receptor-dependent antibody functions contribute to protection is variable, and likely dependent on multiple factors including neutralization potency and/or characteristics of the virus challenge (110).

Influenza Virus

Huber and colleagues used a murine vaccination and challenge model to provide evidence supporting a role of ADCP in protective immune responses to influenza virus infection (111). Normal BALB/c mice and BALB/c mice engineered to lack expression of the Fc receptor γ-chain—and thus deficient in Fc receptor signaling—were given identical influenza immunizations and challenges. Mice lacking the Fc receptor γ-chain were incapable of antibody-mediated phagocytosis, and were highly susceptible to influenza infection, despite the presence of normal levels of cytokines (IFN-γ and IL-10) and antibodies. The condition was not reversed even in the presence of passively-infused anti-influenza antibodies from immune wild type mice, indicating that a defect in interactions of antibody with immune effector cells caused the susceptibility. The effector mechanism was narrowed down by demonstrating protection after passive transfer of immune sera into CD3ε-transgenic mice that lack T cells and NK cells, thereby showing lack of dependence on NK cells and suggesting against ADCC as the mechanism of inhibition. In contrast, macrophages were observed to be ingesting opsonized virus particles, implicating ADCP in inhibition of influenza infection (111). Subsequent work demonstrated that neutrophils may be an essential phagocyte that interacts with influenza specific antibody in protection from disease (112).

Another line of evidence showed that depletion of lung phagocytes, i.e., neutrophils and alveolar macrophages, caused uncontrolled growth of influenza in mice, and caused mortality at doses that were sub-lethal to normal mice (112, 113). Passive immunization studies performed in normal mice or neutrophil-depleted mice demonstrated that the effector mechanism responsible for control of influenza infection by phagocytes was likely antibody-dependent, since control of infection was enhanced by passively infused antiserum (112). Consistent with these results, DeLillo and collaborators used humanized Fcγ receptor mice in a passive immunization model to demonstrate that influenza hemagglutinin protein stalk-specific neutralizing human monoclonal antibodies (see Epitope section below) were dependent on interactions with activatory Fcγ receptors for protection from influenza challenge (114). The antiviral mechanisms of this protection were found to likely be dependent on both ADCC (114) and ADCP (115). Passive immunization experiments performed by He and collaborators, and in a second study by DiLillo and collaborators, also demonstrated a requirement for Fc receptor interactions in order to achieve maximal protection with both neutralizing, and non-neutralizing influenza-specific antibodies (74, 116). He and collaborators found that protection was dependent on alveolar macrophages, and that these cells were capable of ADCP of immune complexes formed with influenza virus and non-neutralizing or neutralizing antibodies, suggesting a role for ADCP in the observed protective efficacy of passive immunization.

The contribution of ADCP to protection from influenza infection observed in passive immunization studies supports antibodies capable of ADCP as a desirable outcome of active immunization to protect against influenza infection. A paramount goal for the armamentarium against influenza pandemics is development of a universal vaccine that is efficacious against the high diversity of influenza subtypes and strain variants that result from antigenic drift. Many approaches toward this goal attempt to focus the immune response on highly conserved influenza antigens. Results of testing such candidate vaccine regimens have suggested that ADCP may be an important immune response elicited by antibodies that target conserved influenza epitopes. Using influenza virus like particles (VLPs) as an immunogen to elicit responses to the highly conserved matrix protein 2 (M2), Song and colleagues demonstrated, in mice, that M2 VLP immunes sera induced cross protection across heterologous influenza A viruses including the 2009 H1N1 pandemic virus, as well as the heterosubtypic H3N2 and H5N1 influenza viruses. They also found that the immune sera responsible for this cross-protection required the presence of dendritic cells and macrophages (117). Another group independently identified similar observations and reached similar conclusions. Using a vaccine approach based on the conserved M2e influenza epitope, they showed that passive immunization by M2e-specific antibodies depends on the presence of alveolar macrophages with intact FcγRI and FcγRIII for immune protection (118). The authors of this study point out that the M2 protein is highly expressed on the surface of infected cells, but limited on influenza virions. They therefore hypothesized that the anti-M2e antibodies preferentially target influenza infected cells and eliminate them via ADCC and ADCP prior to virus propagation and release by budding. ADCP of infected cells is an understudied aspect of immune responses to all viral infections, and it should be further investigated in this context. Collectively, these preclinical candidate vaccine studies provide evidence that protection by Fc receptor-dependent processes including ADCP may be broad and stretch across influenza subtypes. However, ADCP activity has also been shown to be a component of the non-cross-reactive immune response induced by the seasonal trivalent influenza vaccine (119), and anti-influenza sera ADCP activity is common in healthy adults (120). It remains unknown how ADCP and other Fc receptor-dependent functions contribute to persistent natural and vaccine-induced responses to seasonal influenza.

While most evidence from studies performed in animal models suggests that phagocytes and ADCP contribute to protective immune responses against influenza virus there may also be aspects of their function that augment influenza infection. For example, inhibition or removal of the ability of phagocytes to produce reactive oxygen species improved the resolution of lung influenza infection in mice (121), and the high affinity allele

of FcγRIIa was found to be a risk factor for severe pneumonia during the 2009 A/H1N1 pandemic (32).

Herpesviruses

ADCP of herpes simplex virus-infected fibroblast by both neutrophils and monocytes has been observed *in vitro* (122), and several studies have implicated ADCP as having a role in immune responses to herpesvirus infections. ADCP was recently identified as a component of the antibody response elicited by vaccination with a cytomegalovirus (CMV) subunit vaccine. Nelson and collaborators investigated the humoral components of reduced risk of CMV acquisition observed in the moderately (∼50%) efficacious clinical trial of the CMV glycoprotein gB/MF59 vaccine conducted in postpartum women (123, 124). Consistent with previous observations (125), they found that this vaccine induced only modest neutralizing antibody responses. However, the vaccine-elicited antibodies were demonstrated to bind to the surface of infected cells and mediate robust ADCP of both gB-protein coated beads and fluorescently-labeled whole CMV virions (124). Interestingly, the vaccine elicited high magnitude gB-specific IgG3 responses, which likely contributes to the high levels of ADCP observed as the IgG3 isotype has previously been shown to be superior to IgG1 for virion ADCP in studies performed with HIV-1 (104). The study by Nelson et al. is novel in that it implicates non-neutralizing antibody responses as having an important role in antibody responses against CMV, and therefore may help to open new pathways toward development of highly effective next-generation CMV vaccines.

A genetic link providing evidence of a role ADCP in immune responses to herpesviruses was described for EBV. The low affinity allele of FcγRIIa was found to be enriched in EBV infection and correlated with the expression of the latency protein LMP1, suggesting that this allele may be a risk factor for latent EBV infection (34).

Collectively these studies provide evidence that ADCP contributes to vaccine-elicited and natural immune responses to herpesvirus infection. Due to the prevalence of herpesviruses worldwide, and the need for additional strategies for treatment and prevention, additional research in this area is warranted.

Other Virus Infections

Although the ADCP has not been comprehensively studied in the context of most virus infections, it may be a common component of antiviral humoral immune responses to diverse types of viruses as supported by the study of respiratory syncytial virus (RSV), ebola virus, human papilloma virus (HPV), foot-and-mouth disease virus (FMDV), severe acute respiratory syndrome coronavirus (SARS-CoV), and West Nile virus (WNV).

For RSV and ebola virus, evidence of ADCP in immune responses is limited to *in vitro* assessment of virus-specific monoclonal antibodies. ADCC and ADCP activity was observed for RSV G protein-specific monoclonal antibodies produced by B cells from healthy (presumably RSV exposed and immune) adults (126). For ebola virus, glycoprotein-specific monoclonal antibodies produced by B cells in response to ebola virus glycoprotein DNA prime and virus-vectored boost vaccination were shown to have ADCP activity (127).

The contribution of ADCP activity to protection from HPV infection was reported for anti-HPV neutralizing monoclonal antibodies. Using passive transfer experiments in mouse models, Wang and collaborators provided evidence that HPV-specific IgG monoclonal antibodies can cross the vaginal epithelial at sites of local disruption, and that this IgG had ADCP activity (128). They propose that ADCP augmented protection against vaginal HPV infection resulting from virus neutralization because protection was less efficacious when passive transfers were performed with $F(ab')_2$ instead of whole IgG, when performed in Fcγ-deficient mice, or with mice depleted of neutrophils and $Gr1^+$ macrophages.

FcγRII genotyping of 180 people previously infected with SARS-CoV and 200 region-matched normal donors was used to identify homozygosity for the low affinity allele of FcγRIIa as significantly associated with severe SARS-CoV infection (33). Further evidence supporting a role for ADCP in the immune response that attenuates SARS-CoV infection comes from depletion studies that demonstrated a requirement for infiltrating and tissue resident macrophages, as well as SARS immune sera, for clearance of SARS-CoV from pulmonary cells in a mouse model (129). NK cells were not required, suggesting against ADCC as the immune mechanism involved in the reduction of infection (129). The SARS immune sera use in these experiments had the ability to neutralize SARS-CoV. Thus, this study provides another example of ADCP contributing to the protection mediated by antibodies with the ability to neutralize virus, as described above for HPV and HIV-1. Similar observations have been made for FMDV (129–131). Taken together, these observations suggest the compelling possibility that for many viruses where neutralization has been shown to have the ability prevent or control infection, ADCP or other Fc receptor-dependent effector functions may also contribute and enhance their protective function to an extent that has yet to be fully defined.

For WNV, passive antibody transfer of WNV non-structural protein-1 (NS1)-specific monoclonal antibodies into normal mice and Fcγ receptor knock-out mice demonstrated Fc receptor-dependent protection (132). Protection was maintained in mice lacking only FcγRIII, suggesting that NK cell ADCC was not essential. The authors demonstrated that murine macrophages were able to internalize NS1-expressing target cells in the presence of anti-NS1 antibodies, generating the hypothesis that ADCP contributed to protection against WNV infection in this model system. Interestingly, this study demonstrates a role for ADCP of virus-infected cells in protection, which is understudied when compared to ADCP of whole virus or virus proteins. It is possible that infected-cell and virion ADCP may in some contexts have divergent contributions to antiviral responses as it has been shown that virion ADCP promotes higher replication of WNV and other flaviviruses using *in vitro* cell culture models (133, 134).

POTENTIAL FOR ADCP TO ENHANCE VIRAL INFECTION

ADCP is not always associated with beneficial or protective immune responses. In some cases, ADCP has been demonstrated to promote infection. Termed antibody-dependent enhancement (ADE), this has predominantly been described for viruses from the genus *Flavivirus* including dengue virus, yellow fever virus, Japanese encephalitis virus, and zika virus (135–140). Fc receptor-dependent ADE is a particular concern for infections by dengue virus (141–143). There are four serotypes of dengue virus, and antibodies generated in response to infection with one serotype have variable degrees of cross reactivity to the other three serotypes. Upon re-challenge with the original serotype, antibodies are protective. However, the pre-existing antibody response will augment infection and promote more severe disease upon secondary infection with any of the other serotypes (143). Moreover, ADE resulting from cross reactivity may not be limited to secondary infection with another strain of the same virus; ADE might also occur when the secondary infection is a different but closely related virus. For example, the results of recent studies have suggested that dengue virus-specific antibodies may be capable of enhancing zika virus infection (144), and reciprocally, that zika virus-specific antibodies may be capable of enhancing dengue virus infection (140). These observations have been met with skepticism (145), and additional work will be required to demonstrate that this type of ADE has clinical relevance to dengue and zika incidence and disease. ADE is thought to occur when non-neutralizing antibodies, or suboptimal concentrations of neutralizing antibodies, bind and opsonize virus, and are subsequently internalized by Fc receptor expressing cells via ADCP. Within the cells these immune complexes may perturb normal antiviral immune functions resulting in the virus not being destroyed. Instead, the virus exploits ADCP as a tool to expand access to host cells, resulting in higher infection burden and often stimulating an overt inflammatory response that contributes to disease pathogenesis (143). How do viruses such as dengue subvert the process of ADCP to promote increased infection? One potential mechanism that has been proposed to allow for dengue virus to escape Fc receptor-mediated antiviral signaling is through engaging the inhibitory receptor leukocyte immunoglobulin-like receptor-B1 (LILRB1) (146). Thus, for dengue and other viruses, the outcome of each interaction between antibody-virus immune complexes and phagocytes depends on the balance of activating signals, which are expected to promote ADCP and virus elimination, and inhibitory signals, which have the potential to enhance infection.

In the studies of HIV-1 infection, early papers reported that complement and antibodies at low titers could enhance HIV-1 infection *in vitro* (147–150). Since monocytes and macrophages can be infected with HIV-1, one theory is that antibody-mediated phagocytosis may have caused increased viral infection of these phagocytes. More specifically, enhancement may have been due solely to weakly or non-neutralizing antibodies that remain capable of engaging but not neutralizing HIV-1 virus (151). However, it is notable that no enhancement of infection in the vaccine arm was observed in the VAX003 and VAX004 clinical trials, both of which elicited high levels of non-neutralizing antibodies (152, 153).

As described in a comprehensive review of the topic by Taylor and collaborators (143), there is evidence of ADE for many other viruses including influenza, SARS-CoV, RSV, and ebola virus—for all of which there also exists evidence of ADCP as being part of beneficial antiviral antibody responses. These dichotomous observations emphasize the balancing act faced by the immune system as it attempts to respond to virus infection. Immune responses of sufficient specificity and potency are required to protect against infection and disease, but suboptimal responses can be exploited by pathogens resulting in higher levels of infection and more severe disease. Because of this, ADE remains a concern for the development of vaccines against viruses for which potent neutralizing antibody response cannot be elicited.

VIRAL EPITOPES RECOGNIZED IN ADCP

One of the best examples of how epitope specificity can impact ADCP was described for influenza-specific antibodies. Two distinct epitope regions of the influenza hemagglutinin protein are recognized by influenza-specific antibodies: the immunodominant and antigenically variable head domain; and the more antigenically conserved stalk region (**Figure 3**). DiLillo and colleagues demonstrated a requirement for antibody–Fcγ receptor interactions for protection by the stalk-specific antibodies, but not for antibodies recognizing the head domain (114). They further demonstrated that the stalk-specific antibodies were capable of activating NK cells for ADCC, and that the protective efficacy of stalk-specific antibodies could be improved with mutations that selectively enhance engagement with FcγRIIa and FcγRIIIa (114). Mullarkey and colleagues followed-up on these insights and identified a similar epitope-dependent dichotomy of anti-hemagglutinin antibody responses in antiviral assays performed with neutrophils as the source of innate effector cells. Using recombinantly-produced chimeric antibodies with mouse Fv and human Fc regions, they demonstrated that stalk-specific, but not head-specific, antibodies induced ADCP, and reactive oxygen production by neutrophils. Similar observations were made for antibodies made both as IgG1, and as IgA, indicating that the epitope dichotomy for interaction with Fc receptors is conserved for Fcγ and Fcα receptors (115). Their use of recombinantly produced antibodies with identical Fc regions confirms that at least for influenza virus, antibody epitope specificity can play an essential role in establishing downstream antiviral effector responses. A subsequent study by DiLillo and collaborators expanded upon their initial observations, and demonstrated that the requirement for Fc receptor involvement for protection *in vivo* was not restricted only to stalk-specific antibodies, but instead was a common feature of antibodies capable or recognizing a breadth of influenza strains. Using mouse models, Fc receptor dependent protection was shown for both broadly neutralizing stalk and head specific antibodies, as well as broadly-binding non-neutralizing head-specific antibodies (116). Thus,

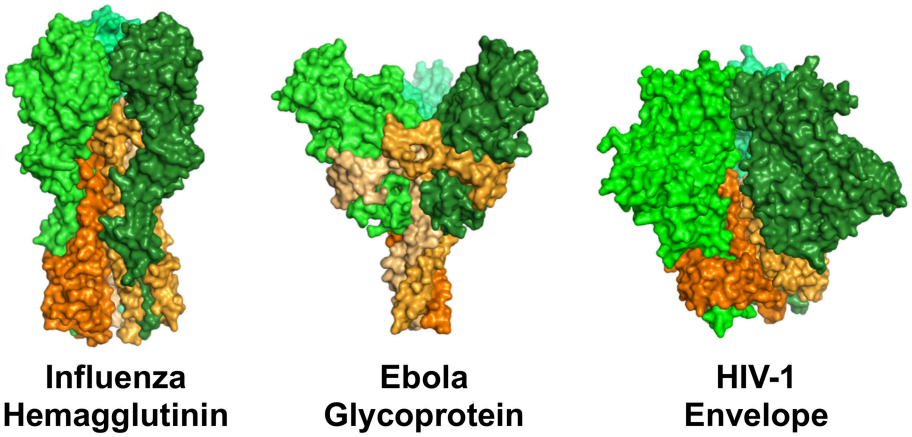

FIGURE 3 | Surface representation of structures of the viral fusion proteins of influenza virus, ebola virus, and HIV-1 that are targets of antiviral antibodies. Perspective is from the side, such that the proteins are standing atop the virus envelope. These envelope proteins share a general structural architecture as trimers of dimeric proteins comprised of a receptor binding domain (head, represented by shades of green) and a viral membrane-bound domain (stem/stalk, represented by shades of orange). Renderings were made in PyMOL using protein data bank ID 3GBN (influenza), 5JQ3 (ebola), and 5FYL (HIV-1).

there exists a yet to be explained relationship between breadth and Fc receptor-dependence in protective antibody response to influenza. Sera from human donors has the ability to mediate anti-influenza ADCP (119, 120), it will therefore be important for future research to further map the specificities of natural human influenza ADCP antibodies, and to explain the mechanisms underlying the differential functions of the strain-specific head antibodies from multi-strain specific head and stalk antibodies.

A similar epitope dichotomy has been described for ebola virus. The ebola virus envelope glycoprotein is similar to the envelope fusion glycoproteins of influenza and HIV-1 in that they share a common architecture of trimers of dimeric proteins. The dimers are comprised of a distal receptor-binding head region, and a membrane-bound stem/stalk region (**Figure 3**). The stem/stalk regions are less variable than the head regions, however the head regions are generally regarded as immunodominant. Ebola stalk-specific specific antibodies were demonstrated to be capable of mediating multiple Fc receptor-dependent effector functions including NK cell activation and ADCP by monocytes or neutrophils, while glycan cap-specific mAbs lacked this functionality (154). Thus, it will be interesting to study parallels and differences between broad-binding influenza and ebolavirus stalk-specific ADCP antibodies as the biomolecular interactions underlying the relationships between epitope and function are elucidated.

Diverse HIV-1 epitopes can be targeted for virion phagocytosis, including broadly neutralizing epitopes such as the gp41 MPER (stem region), and gp120 head-region targets such as the CD4 binding site, V2 glycan, and trimer apex. Non-broadly neutralizing epitopes such as the gp41 principal immunodominant domain (stem region), V1/V2 loop and V3 loop (gp120 head region) (104, 155–157) are also targets of ADCP antibodies. The CD4-induced epitopes found on HIV-infected cells are not engaged alone for phagocytosis, but can be engaged synergistically, at least with V2 epitopes, for virus engagement (98). The ability of these epitopes to be engaged for phagocytosis varies by strain, indicating the substantial heterogeneity between viruses. Furthermore, antibodies targeting the same epitope can vary in their phagocytosis potency, emphasizing the fact that the rules of antibody-epitope engagement that allow for ADCP are not completely understood. These may include antibody Fc availability which can be affected by antigen binding valency and angle of approach (158). It is also likely that there are non-neutralizing antigenic targets on the HIV-1 virion surface that are involved in ADCP or other Fc-dependent functions but have not been discovered. Our understanding of the HIV-1 envelope protein (Env) surface is strongly biased toward neutralizing epitopes, since the search for anti-HIV-1 antibodies typically involves the selection and characterization antibodies positive for neutralization. However, these typically make up only a portion of the antibodies that are elicited in response to HIV-1 infection or vaccination, but the non-neutralizing specificities capable of binding and engaging Env are typically not further studied. In addition, monomeric gp140 or gp120 proteins, and even the current generation of stabilized trimeric proteins that are typically used as hooks for antibody selection likely do not fully recapitulate the diversity of natural Env forms on the virion envelope, which may include both functional trimers and non-functional forms of envelope (159). If so, large-scale unbiased antibody screening may identify additional crucial antibody specificities for Fc-mediated effector functions against virions that may be targeted by vaccines or passive immunotherapy. It is important to note that most virion ADCP methods do not differentiate between infectious and non-infectious virion particles–thus, an open question remains as to whether non-neutralizing antibodies that appear to mediate virus ADCP do indeed cause uptake of infectious viruses, or whether they merely engage non-infectious epitopes on defective particles.

Virus-infected cells and virion particles are distinct biological targets of antibody-mediated internalization. The epitope specificities involved for each process may differ, given different epitope exposure on viruses and infected cells. For instance, in the

context of HIV-1, the gp41 principal immunodominant domain targeted by antibodies such as 7B2 and F240 is frequently found on virions, but not on infected cells, whereas the converse is true for the conserved region 1 conformational domain targeted by antibodies such as A32 and C11 (104, 160, 161). The majority of studies describing the ADCP response to HIV-1 have been focused on gp120/gp140 proteins or whole virions, thus there is much less known about ADCP of HIV-1 infected cells and the epitopes that may be involved in this process. Additional research in this area is needed for HIV-1 and other viruses.

Comparatively less has been described regarding the fine epitope specificity of ADCP antibodies in the immune responses to other virus infections or vaccination. As a general rule, for ADCP of virions the epitope must be present on the surface of the virions, while for ADCP of infected cells any epitope expressed on the surface of a virus-infected cell may be a potential target. For most viruses limited antibody specificities have been described to mediate ADCP: CMV gB (124) protein, RSV G protein (126), ebola virus glycoproteins (127), HPV L2 protein (128), and WNV NS1 protein (132). In most of these studies, the ADCP activity was identified by testing available antibodies, or antibodies selected for alternative functionality such as neutralization. Therefore, many potential specificities remain unexplored and it is likely that antibodies that target other epitope regions are capable of ADCP activity but have not yet been identified. Overall, there is a dearth of knowledge regarding specific epitopes that can be targeted by ADCP antibodies against most viruses, and more insight into this area may inform new strategies for rational design of vaccines intended to elicit ADCP responses.

APPROACHES TO MEASURE VIRUS ADCP

Virus ADCP is typically measured via a cell-based assay, where the amount of viral target internalized by a phagocyte is quantified. Sample monoclonal or polyclonal antibodies are first pre-mixed with the viral target to form immune complexes. Phagocytes are then introduced for antibody-dependent phagocytosis to occur. Such phagocytes include primary monocytes, macrophages, neutrophils, or corresponding cell lines (104, 162, 163). Phagocytes can be spinoculated (164) with the immune complexes to increase the signal-to-noise ratio, though this is not necessary to achieve detectable signal in the case of antibody-dependent phagocytosis of HIV-1 virions (165). Non-internalized virus particles are then washed off, and the amount of internalized viral target is quantified.

In theory, any method capable of quantifying target particles (e.g., nucleic-acid based RT-PCR, protein-based ELISA, or fluorescence) can be used as the assay readout, as long as internalized virus particles can be separated from extracellular or cell-attached particles. In practice, fluorescence-based approaches are the most popular due to the capability for high-throughput readout via flow cytometry (162). In fluorescence-based approaches, the target particle could be a live fluorescently-labeled virion (104, 120, 166) or a virus-derived protein conjugated to a fluorescent bead (120, 162). Where virions are labeled, this can be done with direct labeling [including viral membrane labeling which would exclude detection of membrane-fused viruses (167)], or internal labeling via co-transfection of genes encoding fluorescent non-structural proteins, resulting in their random incorporation into packaged virus particles (168). Fluorescence labels can also be engineered to report internalization, either by the addition of a non-cell-permeable fluorescence quencher during the wash step (169), or by using a pH-sensitive dye.

It is as yet unclear how results compare between different types of viral targets. Notably, phagocytosis signaling pathways and particle uptake dynamics are impacted by the physical properties of the target, including ligand spacing (170, 171), size (170, 172), shape (173), stiffness (174), and antigen height (175). Since virus particles differ from protein-coated beads in a number of these attributes, it remains unclear how these differences affect Fc-Fc receptor signaling, or how they influence the range of virus epitope-Fab conformations that are capable of mediating phagocytosis. In the case of HIV-1, one group has argued that HIV-1 virus particles cannot undergo ADCP due to insufficient surface ligand density (176). This was based on their observation of undetectable internalization of HIV-1 viruses by HIV-specific antibodies as compared with an anti-phosphatidylserine antibody. However, this result is at odds with our work demonstrating epitope-specific internalization of HIV-1 virus particles using a variety of HIV-specific monoclonal antibodies of different Env specificities (100), as well as polyclonal antibodies from sera and breast milk (165). Since the experimental procedures used by both groups were similar, the reasons for this discrepancy are unclear, and may involve the method of virus preparation.

Moving forward, it will be important to determine how the nature of the immune complex affects phagocytosis. Current assays do not routinely measure the size of the immune complex engulfed, but only the number of intracellular or intravesicular virions, which may be either a single immune complex or an amalgamation of multiple co-phagocytosed viruses. In fact, for many viruses, it is not even clear whether antibody-dependent virion internalization is strictly a process of phagocytosis, or whether other processes including endocytosis and macropinocytosis are involved. It will also be important to determine the signaling outcomes of such phagocytosis in multiple cell types and inflammatory conditions, in order to determine the role of phagocytosis in the recruitment of other effector functions as well as the subsequent development of the local inflammatory response and longer term adaptive immune priming. For highly polymorphic viruses including HIV-1 and influenza, it will also be useful to develop a panel of representative viral strains (fluorescent if necessary) for use as a reproducible measure of the breadth of ADCP activity which can be compared across different settings and clinical trials. Indeed, just as HIV-1 antibody neutralization potency against tier 1 viruses did not necessarily predict neutralization breadth, ADCP breadth may provide surprising clues for the development of vaccines that elicit strong Fc effector function.

In contrast to ADCP of virus particles, methodology for ADCP of virus-infected cells remains underexplored, as

most current ADCP assays have been developed to measure internalization of immune complexes formed with virions, or protein-coated beads. Thus, it remains difficult to quantify how antibody epitope specificity and Fc profile contribute toward their potency for ADCP of virus-infected cells. Methodologies to investigate ADCP of virus-infected cells both *in vitro* and *in vivo*, in particular differentiating such events from non-antibody-mediated phagocytosis of virus-infected cells, will also be important to determine whether infected-cell ADCP results in virus inhibition or spread.

In addition to ADCP, trogocytosis is another potential outcome of Fc-dependent interactions between antibodies and virus-infected cells. Two assays developed to measure HIV/SIV-specific ADCC responses have been shown to also detect antibody-mediated interactions between monocytes and virus-infected or protein-coated cells (177, 178). Both assays use fluorescent dyes to label infected or protein-coated target cells. Flow cytometric analysis of cell populations after incubation of these target cells with antiviral antibodies and innate effector cells identified $CD14^+$ cells present in the effector cell population as having acquired the fluorescent dye used to label the target cells —indicating transfer of target cell membrane (trogocytosis) and/or ADCP of cells or cell fragments. More recently, an *in vitro* HIV-1 trogocytosis assay has been developed, using flow cytometry to specifically measure the transfer of membrane fragments from a gp120-coated $CD4^+$ T cell line to the THP-1 monocytic cell line (179). The relationship between trogocytosis and phagocytosis remains unclear. It may be that these two processes are not mutually exclusive, and that trogocytosis occurs as a result of "aborted phagocytosis" (180). Alternatively, antibody-dependent trogocytosis may represent an alternative mechanism for elimination of target cells (181–183). Presently, the roles of trogocytosis in immune responses to virus infection are not known, and additional research will be needed to determine how this process impacts virus pathogenesis and to further explore potential integrations with ADCP.

CONSIDERATIONS FOR TRANSLATION OF STUDIES PERFORMED IN ANIMAL MODELS

There are significant barriers to the translation of Fc effector functions between animals and humans. The most notable is the difference between the Fc/Fc receptor systems of rhesus, mice, and humans. The differences between the mouse and human Fc/Fc receptor system have been reviewed previously (184). While rhesus and human Fc/Fc receptor systems might be expected to be more similar, there remain substantial differences. Fc differences are apparent when comparing IgA and IgG subclasses between rhesus macaques and humans (**Figure 4**). Rhesus macaques have only one IgA gene, whereas humans have two IgA genes, IgA1 and IgA2. While both rhesus macaques and humans have four IgG subclasses, rhesus IgGs are not structurally or functionally analogous to human IgG1-4. In fact, rhesus IgG subclasses are genetically more similar to each other than they are to their human homologues (**Figure 4A**), and the diversity in the germline immunoglobulin genes is higher in rhesus macaques than in humans (188, 189). Structural differences between rhesus and human antibodies are best exemplified by the IgG3 subclass. The human IgG3 C_H domain encodes repeats of a hinge region exon, resulting in a hinge that is approximately four times longer (in number of amino acids) than that of human IgG1 (**Figure 4B**) (190). There is no such exon duplication and hinge elongation in rhesus macaque IgG3 (188). The structure of the hinge dictates the flexibility of the antibody (185), and therefore impacts biological functions and has implications for translation of studies performed in humans and rhesus macaques. For example, vaccine-elicited HIV-specific IgG3 was identified as a correlate of reduced risk of infection in the RV144 clinical trial (87, 89), and *in vitro* studies have demonstrated higher ADCP activity for IgG3 HIV monoclonal antibodies compared to other IgG subclasses (104). Given the differences between human and rhesus IgG3 it is unlikely that preclinical studies performed in the rhesus model would have predicted that vaccine-elicited IgG3 could play a crucial role in reducing the risk of HIV infection as observed in RV144. Caveats such as this must be considered when using animal models.

There are also differences in how human and rhesus macaque antibodies interact with Fc receptors. For instance, rhesus IgG2 and IgG4 retain strong binding to Fc receptors (191, 192), whereas human IgG2 and IgG4 have severely attenuated binding (6). Thus, there is more similarity in Fc receptor interactions and effector functions (191) across rhesus IgG compared to that observed for humans. This suggests that rhesus macaques do not use IgG subclasses to tune antibody responses to the same extent as humans, and highlights the need for caution when comparing subclass profiles in humoral responses across these species. In addition, rhesus macaque Fc receptors are more highly polymorphic than human Fc receptors (189, 192) and the functional implications of many of these polymorphisms have yet to be defined. Importantly, while rhesus and human Fc and Fc receptors can cross-react in binding, antibody binding to FcγRIIa is different between humans and rhesus macaques when comparing antibody Fc mutants, suggesting that antibodies may not behave similarly against human and rhesus macaque FcγRIIa (193), which is commonly engaged for ADCP (162). Despite these differences, our preliminary data indicate that there remains cross-reactivity and some level of functional homology for phagocytosis between humans and rhesus macaques, with similar rank order of phagocytosis activity across species when human IgG and IgA isotypes/subclasses are tested against human or rhesus monocytes and neutrophils (Pollara et al., unpublished observations).

Further studies are required on the interactions between Fc and Fc receptors in different species, their effects on phagocytosis, and the subsequent effects of phagocytosis to determine what findings from preclinical studies can be translated. Passive immunotherapy may be simpler to model since there appears to be functional homology for phagocytosis between human and rhesus systems when human Ig is used. Humanized mice will also be valuable for these types of studies. Specifically, the mouse model developed by Smith and collaborators in the laboratory of Dr. Jeffrey Ravetch is expected to provide the most utility (194).

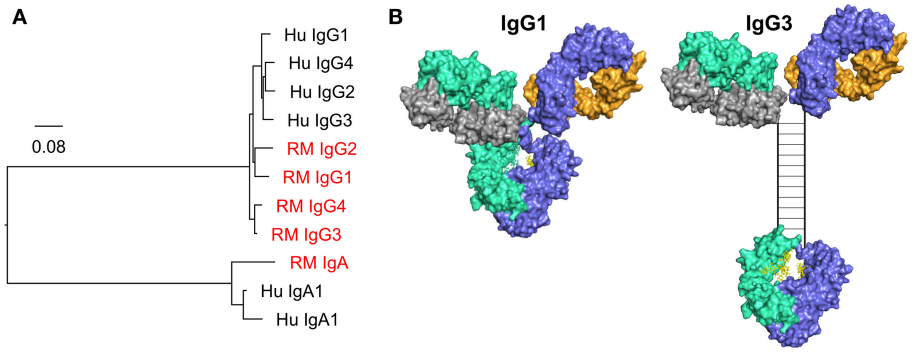

FIGURE 4 | (A) Phylogenic tree of human (Hu) and rhesus macaque (RM, red) IgG and IgA C_H genes. Scale bar represents the percentage sequence dissimilarity as a measure of phylogenetic distance. **(B)** The human IgG3 hinge region is elongated when compared to IgG1. The hinge region in the hybrid surface/schematic rendering of human IgG3 is drawn to scale, with an estimated hinge length of 80 angstroms, as estimated by Lu and colleagues using small angle x-ray scattering data, and consistent with prior estimates from electron microscopy data (185, 186). Disulfide bonds ($n = 11$) are represented by rungs in the ladder structure (187).

In their model, the mouse Fcγ receptor genes have been deleted and human Fcγ receptor transgenes have been inserted. This results in mice that express functional human Fcγ receptors on the same types of cells and at the same levels as found in humans. The primary limitations of this model are that only human Fcγ receptors are expressed. Thus, Fcα-receptors, FcεRI, Fc receptor neonatal, Type II Fc receptors, and other Fc-binding proteins remain as native mouse forms. Moreover, repeated infusion of human IgG can result the development of anti-human IgG antibodies in these mice (195).

CONCLUSIONS AND FUTURE PERSPECTIVES

ADCP is an Fc receptor-dependent function of antibodies that is likely common to immune responses elicited by virus infection and in response to vaccination. Importantly, there is substantial evidence that supports ADCP as contributing either to protection from infection, or reduction in disease severity for diverse types of viruses. In most cases, it is likely that ADCP works in tandem with additional Fc-independent and Fc-dependent antiviral activities as part of an effective polyfunctional humoral response. In fact, for many viruses that have been demonstrated to have antibody-dependent correlates of protection it is highly likely that ADCP is involved, but perhaps was not thoroughly explored. Additional investigation into the role of ADCP in protective viral responses, and the specific virus epitopes targeted by ADCP antibodies, may provide insight into strategies for rational vaccine design to elicit these types of antibody responses while avoiding deleterious ADE activity. Additionally, identifying the types of phagocytes and Fc receptors involved in ADCP at sites of virus infection within tissues, throughout the course of infection and virus clearance, remains an understudied aspect of host and virus interactions. Finally, as most ADCP assays used to measure this immune response *in vitro* quantify uptake of virus subunits, virions, and in some cases infected cells, there remains a gap in knowledge regarding the outcome of phagocytosis. Further research into this area could determine if protection by ADCP is dependent on clearance and elimination of virus and virus infected cells, or by potentiating subsequent immune responses via antigen presentation or immune signaling. As our understanding of ADCP grows, it is likely that approaches to successfully leverage this important immune response for improved antiviral immunity will be discovered.

AUTHOR CONTRIBUTIONS

MT and JP conceived and wrote the manuscript. KW performed protein modeling for the figures and wrote the manuscript.

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Impact of Human FcγR Gene Polymorphisms on IgG-Triggered Cytokine Release: Critical Importance of Cell Assay Format

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Monoclonal antibody (mAb) immunotherapy has transformed the treatment of allergy, autoimmunity, and cancer. The interaction of mAb with Fc gamma receptors (FcγR) is often critical for efficacy. The genes encoding the low-affinity FcγR have single nucleotide polymorphisms (SNPs) and copy number variation that can impact IgG Fc:FcγR interactions. Leukocyte-based *in vitro* assays remain one of the industry standards for determining mAb efficacy and predicting adverse responses in patients. Here we addressed the impact of FcγR genetics on immune cell responses in these assays and investigated the importance of assay format. FcγR genotyping of 271 healthy donors was performed using a Multiplex Ligation-Dependent Probe Amplification assay. Freeze-thawed/pre-cultured peripheral blood mononuclear cells (PBMCs) and whole blood samples from donors were stimulated with reagents spanning different mAb functional classes to evaluate the association of FcγR genotypes with T-cell proliferation and cytokine release. Using freeze-thawed/pre-cultured PBMCs, agonistic T-cell-targeting mAb induced T-cell proliferation and the highest levels of cytokine release, with lower but measurable responses from mAb which directly require FcγR-mediated cellular effects for function. Effects were consistent for individual donors over time, however, no significant associations with FcγR genotypes were observed using this assay format. In contrast, significantly elevated IFN-γ release was associated with the *FCGR2A*-131H/H genotype compared to *FCGR2A*-131R/R in whole blood stimulated with Campath ($p \leq 0.01$) and IgG1 Fc hexamer ($p \leq 0.05$). Donors homozygous for both the high affinity *FCGR2A*-131H and *FCGR3A*-158V alleles mounted stronger IFN-γ responses to Campath ($p \leq 0.05$) and IgG1 Fc Hexamer ($p \leq 0.05$) compared to donors homozygous for the low affinity alleles. Analysis revealed significant reductions in the proportion of $CD14^{hi}$ monocytes, $CD56^{dim}$ NK cells ($p \leq 0.05$) and FcγRIIIa expression ($p \leq 0.05$), in donor-matched freeze-thawed PBMC compared to whole blood samples, likely explaining the difference in association between FcγR genotype and mAb-mediated cytokine release

in the different assay formats. These findings highlight the significant impact of *FCGR2A* and *FCGR3A* SNPs on mAb function and the importance of using fresh whole blood assays when evaluating their association with mAb-mediated cytokine release *in vitro*. This knowledge can better inform on the utility of *in vitro* assays for the prediction of mAb therapy outcome in patients.

Keywords: Fc gamma receptors, antibody immunotherapy, Fc gamma receptor polymorphism, cytokine release syndrome, cytokine release assays

INTRODUCTION

The advent of monoclonal antibodies (mAb) has revolutionized the treatment of malignant and autoimmune disease (1, 2). However, there is considerable variability in response to mAb therapy, as some patients may not respond to treatment whilst others experience toxic side effects, of which cytokine release syndrome (CRS) is the most detrimental to patient safety (3). CRS is characterized by rapid immune cell activation and systemic elevations of proinflammatory cytokines, in particular IFN-γ, TNF-α, and IL-6. CRS has been observed with the clinical use of several antibodies, including muromonab (anti-CD3), TGN1412 (anti-CD28), Rituximab (anti-CD20) and alemtuzumab (Campath-1H, anti-CD52) (3–6).

In the first-in-man trial of TGN1412, rapid, life-threatening CRS was observed in healthy volunteers (6). Preclinical *in vitro* testing using soluble TGN1412 to stimulate human whole blood or purified peripheral blood mononuclear cells (PBMCs) failed to predict this toxicity (7). Following these failures, there has been a concerted effort to develop predictive *in vitro* assays that enable a better understanding of mAb *in vivo* action and potential toxicity (8–10). mAb target density, immunoglobulin G (IgG) isotype, tissue microenvironment and Fc gamma receptor (FcγR) expression levels are all key to the outcome of therapy (11). Importantly, several studies have reported that *in vivo* expression levels and distribution of FcγR profoundly influence mAb effector function (12–15). Recapitulating the *in vivo* interaction of the mAb with FcγR *in vitro* is therefore of significant value.

Six FcγR are present in humans, consisting of high and low affinity receptors. The high-affinity FcγRI (CD64) is encoded by *FCGR1A* on chromosome 1q21. The low-affinity receptors, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa, and FcγRIIIb are encoded by genes *FCGR2A*, *FCGR2B*, *FCGR2C*, *FCGR3A*, and *FCGR3B*, respectively, in a 200 kb region on chromosome 1q23-24. These genes are subject to numerous single nucleotide polymorphisms (SNPs) and copy number variation (CNV) (16). There are four reported copy number regions (CNRs) in the low-affinity locus, each encompassing a differing combination of genes (17). Genetic variation can impact upon receptor function and associations have been made between FcγR genetic variants and disease. SNPs in *FCGR2A* (rs1801274; 131H) and *FCGR3A* (rs396991; 158V) increase receptor affinity for IgG (18) while CNV can alter the level of FcγR expressed at the cell surface available for IgG binding. SNPs altering receptor affinity have been associated with superior responses in some cohorts of cancer patients treated with mAb immunotherapy (19–24). *FCGR2A*-131R (25) and *FCGR2B*-232T (26) have been implicated in increased risk of systemic lupus erythematous, while *FCGR3B* HNA 1B and decreased copy number of *FCGR3B* have been associated with reduced immune complex clearance and increased risk of autoimmunity (25, 27).

Given the impact of FcγR SNPs and CNV on receptor function via IgG Fc:FcγR interactions on immune cells (28, 29), studies investigating treatment efficacy and side effect profile in the context of FcγR genotypes and expression levels are warranted. We hypothesized that since the *FCGR2A*-131H and *FCGR3A*-158V alleles in particular, markedly enhance receptor affinity for IgG (18), mAbs and an IgG1 Fc Hexamer construct, have the potential to elicit enhanced cytokine release amongst individuals possessing these gene variants. Furthermore, we aimed to determine the association of several FcγR SNPs with the magnitude of IgG Fc triggered cytokine release in two widely used assay formats: freeze-thawed precultured PBMCs and whole blood. The whole blood assay (but not freeze-thawed PBMC) format recapitulates immune cell subset frequencies, FcγR cellular distribution and expression levels at physiological levels more accurately, revealing associations between FcγR genotype and magnitude of cytokine release in response to mAb treatment. These findings highlight FcγR genotype characterization paired with *vitro* assessment of mAb therapeutics may indeed better predict the magnitude, and variability of responses observed in clinical settings and inform on enhanced therapy design.

MATERIALS AND METHODS

Healthy Donor Cohorts and Ethical Approval

This study comprises two independent cohorts of anonymous healthy donors (total $n = 271$). The Southampton cohort (30), consisted of 178 anonymous healthy donors entering local transfusion services (National Blood Service, Southampton, UK). This study was approved by the University of Southampton Faculty of Medicine Ethics Committee and the National Research Ethics Service Committee South Central, Hampshire, UK. The UCB cohort consisted of 93 anonymous healthy donors based at UCB Celltech, Slough, UK. Blood samples obtained from these donors were taken with informed consent under UCB Celltech UK HTA license number 12504. All donors gave written informed consent in accordance with the Declaration of Helsinki.

PBMC Preparation and Blood Collection

PBMCs were sourced from leukocyte cones (National Blood Service, Southampton, UK) and whole blood was collected

from the UCB donor cohort in lithium heparin vacutainers (BD). PBMCs were isolated from these samples immediately by density gradient centrifugation (Lymphoprep, Axis-Shield). Samples were subsequently frozen in 10% DMSO and 90% fetal bovine serum (FBS, Sigma-Aldrich) and stored in liquid nitrogen for 3–24 months.

Genomic DNA Extraction and Multiplex Ligation-Dependent Probe Amplification (MLPA) Assay

Frozen PBMC samples were rapidly thawed and genomic DNA (gDNA) was extracted (DNeasy Blood and Tissue Kit, Qiagen, GmbH, Hilden, Germany). DNA quality was assessed by UV spectrophotometry.

CNV and SNPs in the low-affinity FcγR locus were measured as previously described (30). 100 ng DNA was analyzed in triplicate using the SALSA MLPA P110 and P111 probe mixes (MRC-Holland, Amsterdam, The Netherlands). PCR products were analyzed using the Genetic Analysis System CEQ 8800 capillary electrophoresis machine and GenomeLab software (Beckman Coulter, High Wycombe, UK). CNV across the locus and SNPs in *FCGR2A* 131R/H (rs1801274), *FCGR3A* 158F/V (rs396991), *FCGR2B* 232I/T (rs1050501), *FCGR2C* 57X/Q (rs759550223), *FCGR3B* HNA 1A/B/C isoforms were assessed.

Intra-sample data normalization was performed using the Coffalyser.NET software (MRC-Holland) by comparing the peak heights of PCR products generated by probes detecting regions of interest against the peak heights of PCR products targeting control genes of known normal copy number. Inter-sample normalization was performed by comparing test cases against a reference sample of 96 pooled European Collection of Cell Cultures (ECACC) Human Random Control panel 1 (Porton Down, Public Health England, UK) gDNA samples. Normalized MLPA data was analyzed using Microsoft Excel 2010.

Antibodies and IgG1 Fc Hexamer

Avastin (Bevacizumab) was sourced from Genentech. Hybridoma cells expressing OKT3 (mouse IgG2a) were obtained from the American Type Culture Committee (ATCC) and mAb isolated from tissue culture media by standard procedures in-house. TGN1412 was produced in-house using published sequences (US patent number US7585960). Variable regions were sub-cloned into expression vectors (pEE6.4 heavy chain and pEE12.4 light chain; Lonza) containing constant regions of human IgG4. Heavy- and light-chain vectors were sub-cloned together before transfection into 293F cells for transient production or CHO-K1 cells for stable production. mAb was purified on Protein A-Sepharose, and aggregates were removed by gel filtration. Campath-1H (Campath) human IgG1 was sourced from Professor Geoffrey Hale (University of Cambridge, UK).

To generate a recombinant hexameric Fc construct (IgG1 Fc hexamer), human IgG1 Fc with mature N-termini starting with an IgG1 core hinge (CPPC) were directly fused at their C-terminal lysine residues to the 18 amino-acid C-terminal extension or "tail-piece" (PTLYNVSLVMSDTAGTCY) of human IgM, which promotes covalent multimerization. IgG1 Fc hexamer was expressed transiently in CHO cells and purified using Protein A and S200 size exclusion chromatography as described previously (28, 29). IgG1 Fc hexamer fraction purity was >98% on analytical HPLC after size exclusion chromatography (SEC). IgG1 Fc hexamer was stored at 4°C in PBS or frozen in aliquots at −80°C.

Endotoxin levels for all antibodies and IgG1 Fc Hexamer used in this study were measured and found to be <1 ng/mg protein (Endosafe-PTS, Charles River Laboratories).

PBMC Assay

Frozen PBMCs were rapidly thawed at 37°C and cultured for 24 h in a flat-bottomed 24-plate at high density (HD), defined as 1.5×10^7 cells/well (total volume 1.5 mL/well), in serum-free medium (CTL-Test Medium, CTL Europe GmbH, Bonn, Germany) supplemented with glutamine (2 mM), pyruvate (1 mM), penicillin, and streptomycin (100 IU/mL), at 37°C in 5% CO_2. PBMCs were washed and cultured in CTL-Test medium at 1×10^5 cells per well, in a round-bottomed 96-well plate. These cultures were then stimulated with soluble Avastin (5 μg/mL), OKT3 (5 μg/mL), TGN1412 (5 μg/mL), Campath (5 μg/mL), or IgG1 Fc Hexamer (100 μg/mL) and incubated at 37°C in 5% CO_2. T-cell proliferation was quantified at 72 h and cytokine release was quantified 24 h post-stimulation.

Whole Blood Assay

Blood from healthy human volunteers was collected into lithium heparin vacutainers (BD) and used within 2 h of the blood draw. Minimally-diluted blood was stimulated with either 100 μg/mL of IgG1 Fc hexamer or 10 μg/mL Campath (Genzyme). Briefly, 12.5 μL of 20x final concentration Avastin (5 μg/mL), OKT3 (5 μg/mL), TGN1412 (5 μg/mL), Campath (5 μg/mL), or IgG1 Fc Hexamer (100 μg/mL) was transferred to a 96-well round bottom tissue culture plate (Costar). 237.5 μL of whole blood was added and mixed gently by pipetting. Plates were incubated at 37°C, 5 % CO_2, 100 % humidity for 24 h, centrifuged at 300 g for 5 min and plasma collected for cytokine analysis. Plasma not analyzed immediately was stored at −80°C until analysis.

Flow Cytometry

1×10^6 PBMCs or 100 μL of whole blood (diluted 1:2 with PBS) were stained with the appropriate fluorochrome-conjugated mAb for 30 min at 4°C and washed once. Samples were stained with anti-CD3 PerCP (clone: SK7), anti-CD56–PE (clone: HCD56), anti-CD19 APC-Cy7 (clone: HIB19), anti-CD14–Pacific Blue (clone: M5E2) and IgG1κ-FITC (clone: MOPC-21) isotype control (all from BioLegend). FcγR staining was carried out using anti-FcγRI FITC (clone: 10.1, $F(ab')_2$), anti-FcγRIIa FITC (clone: E08, $F(ab')_2$), anti-FcγRIIb FITC (clone: 6G11, $F(ab')_2$), anti-hFcγRIIIa FITC (clone: 3G8, $F(ab')_2$), and isotype control human IgG1 FITC (clone: FITC8 $F(ab')_2$), (generated from published sequences in-house or sourced from BioInvent International AB). Results are shown as geometric mean fluorescent intensity (MFI) for FcγR expression on B cells (FSC-A^{lo}SSC-$A^{lo}$$CD19^+CD3^-$), NK cells (FSC-$A^{lo}$SSC-$A^{lo}$$CD56^{dim}CD3^-$, $CD56^{bright}CD3^+$ or $CD56^{hi}CD3^+$), classical

monocytes (FSC-A^{int}SSC-A^{int}CD14^{hi}), non-classical monocytes (FSC-A^{int}SSC-A^{int}CD14^{lo}) and granulocytes (CD14^{-}SSC^{hi}), (see **Supplementary Figure 1** for FACS gating strategy). FcγR expression levels were corrected by subtracting the geometric MFI of the corresponding isotype control staining.

Intracellular IFN-γ staining of PBMCs was carried out by culturing mAb-treated PBMCs with Golgi plug (BD Biosciences) for 24 h. Cells were stained with anti-CD4 Pacific blue (clone: SK3, BioLegend), anti-CD8 V500 (clone: RPA-T8, BD Biosciences) and anti-CD56 PE (clone: HCD56, BioLegend). PBMCs were fixed with FOXP3 Fix/Perm buffer (BioLegend) and permeabilized with FOXP3 Perm buffer before staining with anti-IFN-γ PE-Cy7 (clone: 4S.B3, eBiosciences). Samples were analyzed on a BD FACSCanto II (BD Biosciences) and data was analyzed using FlowJo Version 9.4.11 (Tree Star).

T-Cell Proliferation Assay

PBMCs were labeled with 2 μM carboxyfluorescein succinimidyl ester (CFSE). Cells were cultured in a 24-well plate at 1×10^7/mL for 24 h prior to the stimulation assays. Cells were transferred into round-bottomed 96-well plates at 1×10^5 per well. On day 3, cells were labeled with anti-CD8-APC (clone: SK1, BioLegend) and anti-CD4-PE (clone C4/120: in-house), and proliferation was assessed by CFSE dilution on a FACSCalibur or FACSCanto flow cytometer (BD Biosciences). CD4$^+$ and CD8$^+$ T cell division is defined as a percentage of total cells excluding the parent population (first peak).

Cytokine Determination

Supernatants from PBMC and plasma from whole blood assays were taken 24 h post-stimulation. IFN-γ, TNFα, IL-1β, and IL-6 levels were determined using the V-plex Proinflammatory Panel 1 (human) 4-plex Kit (Cat No: K15052D-2, Meso Scale Discovery) as per the manufacturer's protocol.

Statistical Analysis

Chi-squared tests were used to compare cohorts in terms of genotype frequency and to test for Hardy-Weinberg equilibrium. Where appropriate to conform to the assumption of "Normality" and constant variance, continuous data was log-transformed prior to analysis, results back-transformed to give geometric means and cytokine release data plotted with a logarithmic axis. One-way analysis of variance (ANOVA) with *post-hoc* pairwise comparisons was used to compare donor groups with different *FCGR* alleles. Two-way ANOVA with *post-hoc* pairwise comparisons or a paired student's *t*-test were used to compare groups where the same donors were used in each group (e.g., comparing immune cells subset frequencies in donor matched whole blood and freeze-thawed PBMCs). As a large number of statistical tests have been carried out in a range of contexts, there may be an issue with multiplicity of *p*-values. Formal multiplicity adjustments have not been used, so *p*-values should be interpreted with care and within the overall scientific context. Data analysis was carried out using the Graphpad Prism version 8.0.1 software. Statistical significance defined as $^*p < 0.05$, $^{**}p < 0.01$ $^{***}p < 0.001$ and $^{****}p < 0.0001$ and ns = non-significant.

RESULTS

Low-Affinity FcγR Gene Locus Characterization in Two Independent Cohorts

We assessed the frequency of common SNPs in the low-affinity FcγR locus in two independent cohorts using an FcγR-specific MLPA assay (**Supplementary Table 1**). Genotype frequencies for the combined cohorts are displayed in **Table 1**. Genotyping of the genes not reported to be affected by CNV, *FCGR2A* and *FCGR2B*, showed allele frequencies of 23.99% RR, 52.77% RH and 21.4% HH, and of 77.86% II, 20.66% IT and 0.37% TT, respectively. Genotypes reported for *FCGR3A, FCGR2C,* and *FCGR3B* include those with CNV. Frequencies for *FCGR3A* were 33.58% FF, 49.45% FV, and 8.86% VV; *FCGR2C* were 53.14% XX, 21.03% XQ and 2.21% QQ; and *FCGR3B* were 4.43% AA, 43.54% AB, and 31.37% BB. Reported genotypes were within Hardy-Weinberg equilibrium.

Copy gain (25.8%) across the locus was more prevalent than copy loss (20.2%). We found alterations in CNRs 1 and 2, with CNR2 the most prevalent event (**Table 2**). Frequencies of CNV and CNR events for each cohort are described individually in **Supplementary Tables 2, 3** and for samples with available functional data in **Supplementary Tables 4, 5**.

Immune Cell Subset Frequencies and FcγR Expression on Healthy Donor PBMCs

Using flow cytometry (see **Supplementary Figure 1** for FACS gating strategy), we assessed the cellular constituents and FcγR expression in freeze-thawed PBMC samples from 107 healthy individuals from the Southampton cohort. These donors were selected to generate a cohort with the full range of FcγR SNPs and CNV status with the potential to confer low- and high-affinity IgG binding. As expected, T cells were the most abundant cell type in these samples (median 59.8%, range 38.8–81.8%), followed by monocytes (median 12.3%, range 5.1–30.2%), CD56^{dim} NK cells (median 7.6%, range 1.8–14.3%), CD3$^+$ NK cells (median 5.9%, range 1.7–15.1%) and B cells (median 3.9%, range 1.1–10.1%). CD4$^+$ T cells (median 47.6%, range 25.2–71.2%) were more abundant than CD8$^+$ T cells (median 10.5%, range 3.6–37%) in all donor samples. The frequencies of CD56^{bright} NK cells and dendritic cells (DCs) in these samples were <0.01% of total cells (**Figure 1A**).

Monocytes can be categorized into classical and non-classical subsets on the basis of CD14 high (CD14^{hi}) or low (CD14^{lo}) expression, respectively. FcγR expression levels on each monocyte population were assessed separately. As previously reported (31), CD14^{hi} monocytes abundantly expressed FcγRI and FcγRIIa, but were low or negative for FcγRIIb and FcγRIIIa (**Figure 1B**). In contrast, the less frequent CD14^{lo} non-classical monocytes expressed lower levels of FcγRI and FcγRIIa and higher levels FcγRIIb and FcγRIIIa (**Figure 1C**). B cells expressed high levels of FcγRIIb in comparison to non–classical monocytes but did not express any other FcγR (**Figure 1D**). CD56^{dim} NK cells expressed very variable levels of FcγRIIIa (**Figure 1E**, median MFI 2242, and range 97-8987). Finally, CD3$^+$ T and

TABLE 1 | Combined genotype frequencies of common low-affinity FcγR genes in the combined cohorts.

Gene	SNP(s)	Genotype	*N*	%	*p*-value	Chi^2 test
FCGR2A	rs1208724	RR	65	23.99	0.42	0.51
		RH	143	52.77		
		HH	58	21.40		
		Failed	5	1.85		
FCGR3A	rs396991	FF	91	33.58	0.42	0.66
		FV	134	49.45		
		VV	24	8.86		
		F	1	0.37		
		V	1	0.37		
		FFF	6	2.21		
		FFV	1	0.37		
		VVV	1	0.37		
		Failed	12	4.43		
FCGR2C	rs759550223	XX	144	53.14	0.48	0.5
		XQ	57	21.03		
		QQ	6	2.21		
		X	17	6.27		
		Q	9	3.32		
		XXX	25	9.23		
		XXQ	4	1.48		
		XQQ	1	0.37		
		QQQ	4	1.48		
		XXXX	1	0.37		
		Failed	3	1.11		
FCGR3B	HNA isoforms (rs200688856 and rs5030738)	AA	12	4.43	0.09	2.86
		AB	118	43.54		
		BB	85	31.37		
		A	8	2.95		
		B	15	5.54		
		AAA	4	1.48		
		AAB	11	4.06		
		ABB	10	3.69		
		BBBB	1	0.00		
		Failed	7	0.37		
FCGR2B	rs1050501	II	211	77.86	0.9	0.02
		IT	56	20.66		
		TT	1	0.37		
		Failed	3	1.11		

HWE was calculated based on diploid genotypes within the population. A p-value >0.05 (Chi^2 >3.84) was considered to be within HWE.

TABLE 2 | Copy number variation event frequencies across low-affinity FcγR genes in the combined cohorts.

Gene	Gene copy	Total (n)	Total (%)
FCGR3A	1	2	0.7
	2	243	90.7
	3	7	2.6
FCGR2C	1	26	9.7
	2	207	77.2
	3	31	11.6
	4	1	0.4
FCGR3B	1	23	8.6
	2	215	80.2
	3	25	9.3
	4	1	0.4
Event	**Gain (%)**	**Loss (%)**	**Total (%)**
CNR1	7 (12.1)	2 (3.4)	9 (15.5)
CNR2	25 (43.1)	23 (39.7)	48 (82.8)
CNR1/2	1 (1.7)	0 (0)	1 (1.8)

$CD3^+$ NK cells were negative for any cell surface FcγR expression (data not shown).

mAb Mediated T-Cell Proliferation and Cytokine Release Using a PBMC-Based Assay Format

Pre-stored frozen PBMC samples are widely used in academia and industry, to facilitate genotyping and subsequent analysis. In the current study, PBMC samples from healthy donors, were frozen and then re-thawed for use in a PBMC-based cytokine release assay. In these assays, we opted to test the T cell-targeting antibodies, OKT3 (anti-CD3, mouse IgG2a) and TGN1412 (anti-CD28, human IgG4), renowned for inducing CRS in human subjects (5, 6). In order to establish a methodology to predict such CRS *in vitro*, Hunnig et al. developed a modified PBMC-based assay. They showed that PBMCs precultured at high density (HD), but not fresh PBMCs or whole blood, respond to TGN1412 with cytokine release *in vitro*, mimicking the proinflammatory effects observed in the clinic (8). We subsequently reported that the response to TGN1412 was a consequence of a pronounced upregulation of FcγRIIb on monocytes in the HD PBMC culture (10). Given these results and the accepted importance of FcγRs in mediating mAb effector functions, we utilized this PBMC-based HD preculture assay to assess the impact of donor FcγR genotype on T-cell proliferation and cytokine release in response to mAbs of differing functional classes which bind functionally disparate targets.

Consequently, OKT3, TGN1412 and the clinically relevant anti-CD52 (Campath, human IgG1) were used to assess the magnitude and variability of the cytokine release across our donor cohort using this assay platform. We observed a predominant but highly variable, $CD8^+$ T-cell division in response to the anti-CD3 mAb (**Figure 2A**, range 10-92% division of total $CD8^+$ cells). In contrast, TGN1412 predominantly induced $CD4^+$ T-cell division (**Figure 2A**, range 12–74% of total $CD4^+$ cells). T-cell proliferation in response to the control anti-VEGF mAb Avastin was negligible in both T cell subsets (**Figures 2A,B**).

We next assessed cytokine release in response to OKT3, TGN142 and Campath. All 3 mAb induced strong IFN-γ, TNF-α, IL-1β, and IL-6 responses in comparison to Avastin. Cytokine responses to the T-cell specific mAb OKT3 and TGN1412 were

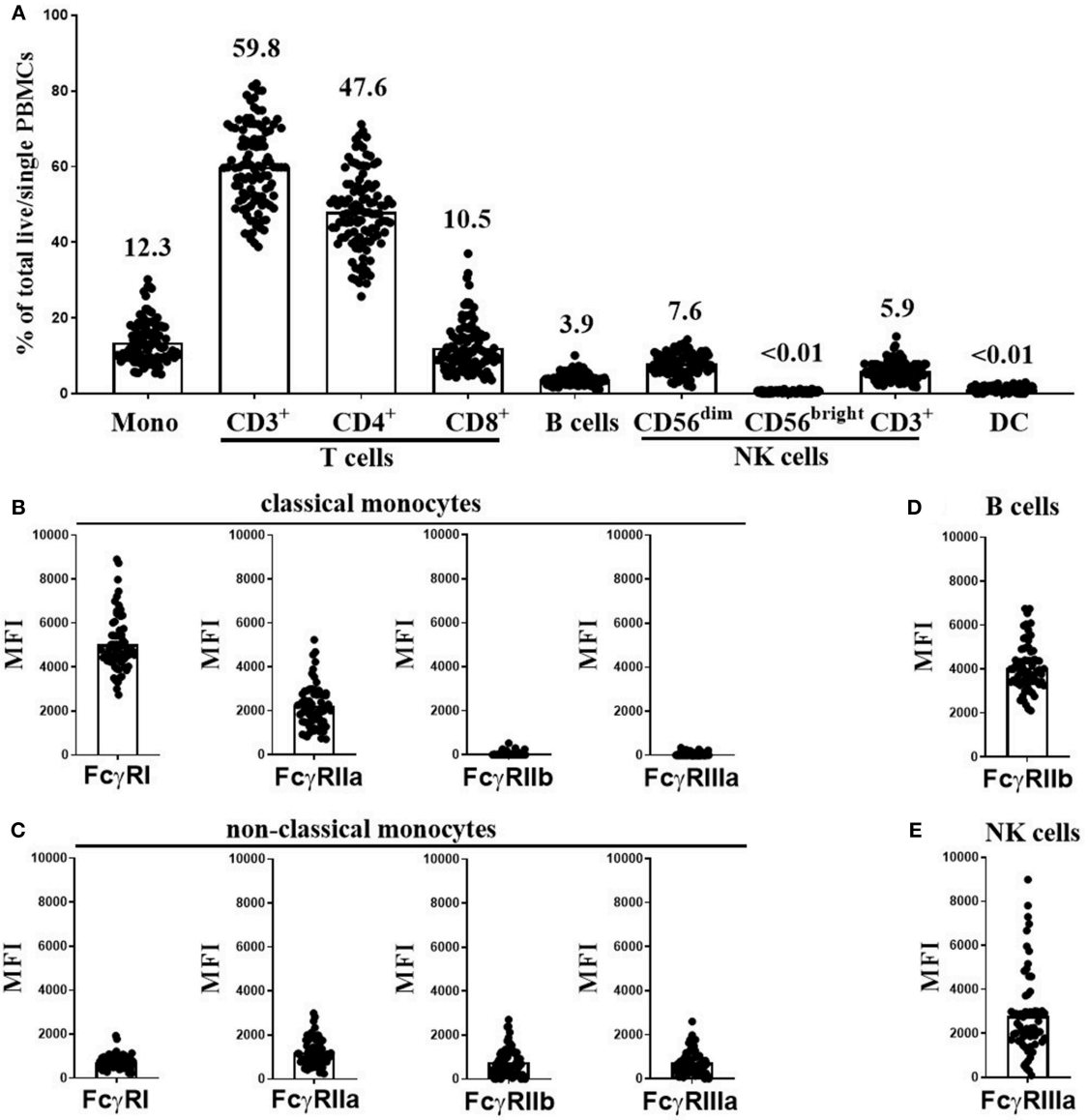

FIGURE 1 | Immune cell subset frequencies and FcγR expression on PBMCs from healthy donors. Immune cell subset frequencies and FcγR expression on freeze-thawed PBMCs using flow cytometry. **(A)** Quantification of monocytes (Mono), $CD3^+$, $CD4^+$, and $CD8^+$ T cells, B cells, $CD56^{dim}$, $CD56^{bright}$, and $CD3^+$ NK cells and dendritic cell (DC) frequencies in freeze-thawed PBMCs from healthy human subjects (mean frequency of each cell subset stated above bar). FcγR expression on **(B)** classical, and **(C)** non-classical monocytes, **(D)** FcγRIIb expression on B cells and **(E)** FcγRIIIa expression on $CD56^{dim}$ NK cells ($n = 107$). Each point represents a donor, bars represent group means.

stronger than those observed in Campath-stimulated cultures. However, there was marked variability in the magnitude of donor responses to all three mAb treatments (**Figures 2C–F**). IFN-γ responses to OKT3, TGN1412 and Campath were stable over time, since stimulating PBMCs from the same donor on five separate occasions (over a 6-month period) revealed a similar response on each occasion (**Supplementary Figure 2**). The IFN-γ response to OKT3, TGN1412, and Campath, was significantly correlated with TNF-α, IL-1β, and IL-6 release ($R^2 = 0.27$–0.83, $p \leq 0.002$–0.0001 for IFN-γ vs. the other three cytokines (except IFN-γ vs. TNF-α for Campath), **Supplementary Figure 3**), and therefore we chose to present IFN-γ release as an exemplar read-out to assess all further cytokine responses in these assays.

To assess the cell populations responsible for this IFN-γ release, we performed intracellular cytokine staining on permeabilized cells stimulated with OKT3, TGN1412, or Campath. Flow cytometry was used to identify and determine the percentage of each cell population secreting IFN-γ. The source of IFN-γ was $CD8^+$ T cells in response to OKT3, both $CD4^+$ and $CD8^+$ T cells in response to TGN1412 and NK cells

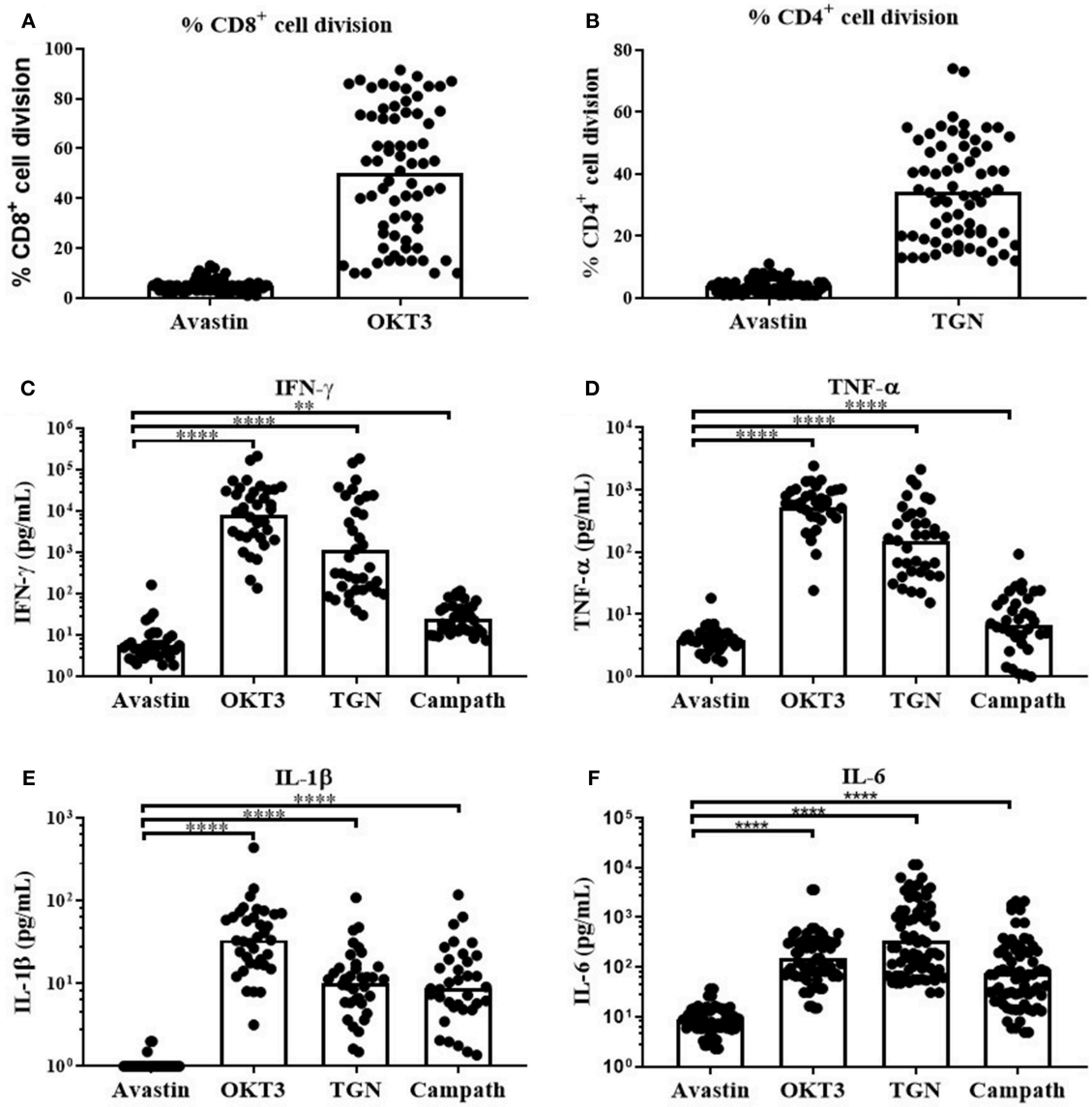

FIGURE 2 | *In vitro* T-cell proliferation and cytokine release in response to mAb stimulation of freeze-thawed PBMCs. T-cell proliferation and cytokine release in response to mAb stimulation. **(A)** % $CD8^+$ cell division in PBMC cultures stimulated with Avastin or OKT3 and **(B)** % $CD4^+$ cell division in PBMC cultures stimulated with Avastin or TGN1412 (TGN), ($n = 69$, bars represent group means). **(C)** IFN-γ, **(D)** TNF-α, **(E)** IL-1β, and **(F)** IL-6 release by PBMCs stimulated with Avastin, OKT3, TGN1412 or Campath ($n = 36$, bars represent group geometric means). $^{**}p < 0.01$ and $^{****}p < 0.0001$.

in response to Campath (**Figures 3A,B**). We next assessed the impact of donor FcγR genotype on the magnitude and variability of these responses observed using this HD freeze-thawed PBMC assay format.

FcγR Genotype Does Not Significantly Impact on mAb-Mediated IFN-γ Secretion in a HD PBMC Assay Format

Three key FcγR polymorphisms have been previously associated with mAb effector capacity, defining high or low affinity receptors for FcγRIIa, FcγRIIIa and a stop codon in FcγRIIc (20–24). We therefore determined the effects of the FcγRIIa 131H/R, FcγRIIIa 158V/F, and FcγRIIc Q/X polymorphisms on IFN-γ release in response to Campath and TGN1412 using the HD PBMC-based cytokine release assay in 36 donors. When stimulating PBMCs with Campath or TGN1412, no significant association of increased IFN-γ release was observed with any of the FcγRIIa, FcγRIIIa, or FcγRIIc alleles (**Figures 4A–F**). Furthermore, no significant associations were observed between FcγR SNPs and magnitude of OKT3 or TGN1412 mediated T-cell proliferation (data not shown). We hypothesized that key properties of the relevant FcγR-expressing immune cell subsets may have been altered during the isolation and storing/thawing/culture

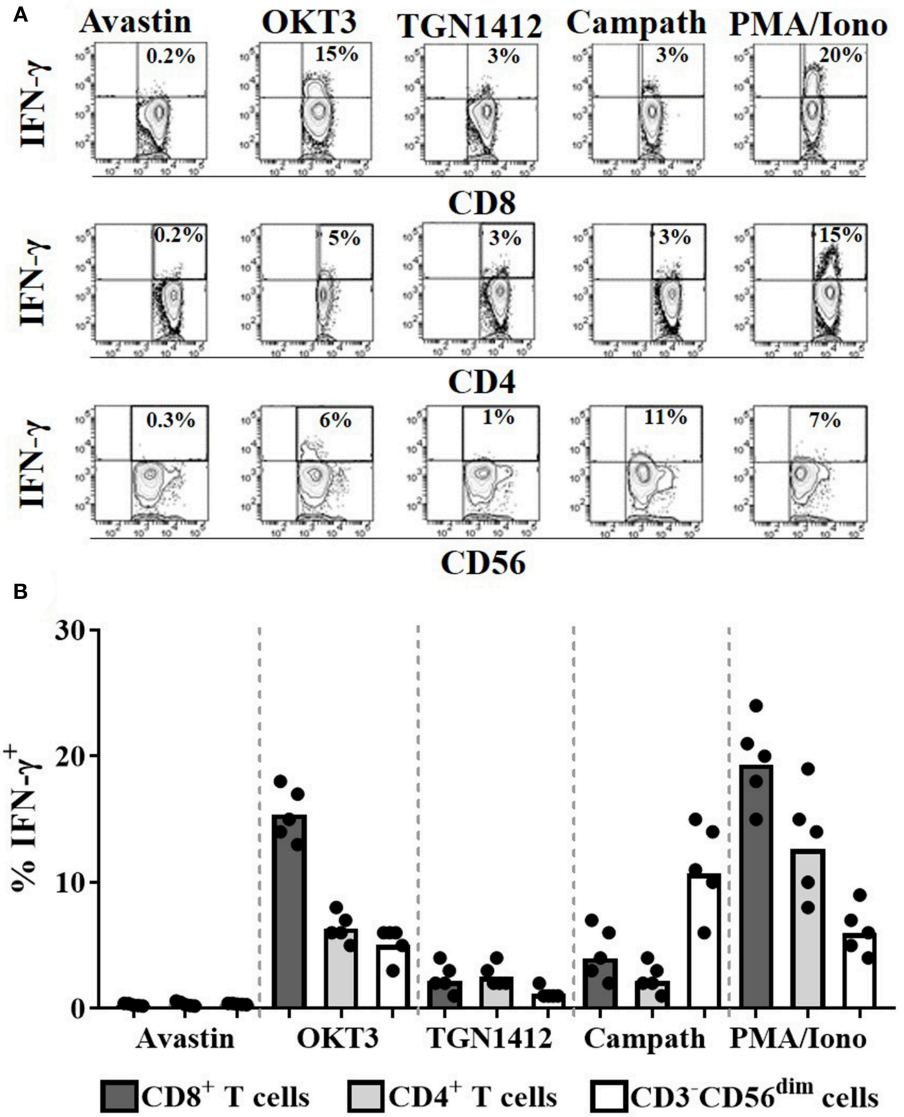

FIGURE 3 | Cellular source of the IFN-γ in response to mAb stimulation. Intracellular IFN-γ staining of PBMCs stimulated for 24 h with Avastin, OKT3, TGN1412, Campath or PMA/Ionomycin (PMA/Iono). **(A)** Representative FACS contour plots of IFN-γ vs. CD8, CD4, or CD56 staining of PBMC cultures stimulated with the aforementioned treatments. **(B)** % $CD8^+$ T cells, $CD4^+$ T cells, and $CD56^{dim}$ NK cells that are IFN-γ^+ post-stimulation ($n = 5$, bars represent group means).

of PBMCs, potentially compromising any associations between FcγR genotype and magnitude of mAb mediated IFN-γ response. To address this, we next compared the frequencies of immune cell subsets between donor matched whole blood and freeze-thawed PBMC samples.

Freeze-Thawed PBMCs Display Altered Immune Subset Frequencies and FcγR Expression Profiles Compared to Matched Whole Blood Samples

As expected, whole blood had a significantly higher frequency of granulocytes in comparison to donor matched freeze-thawed (frozen) PBMC samples as a percentage of total live cells (**Figure 5A**); (Granulocyte median = 54.38% in whole blood compared to 1% for frozen PBMCs, $p < 0.0001$). The proportions of T cells (median = 44.41% for whole blood, 50.04% for frozen PBMCs, $p < 0.05$) were significantly enriched in frozen PBMC samples (**Figure 5B**). In contrast significant reductions in $CD14^{hi}$ classical monocytes (median = 7.6% for whole blood and 5.5% for frozen PBMCs, **Figure 5C**), B cells (median = 11.7% for whole blood, 8.9% for frozen PBMCs, **Figure 5E**) and $CD56^{dim}$ NK cells (median = 5.9% for whole blood, 5.3% for frozen PBMCs, **Figure 5F**) were observed when comparing whole blood to frozen PBMC samples ($p < 0.05$, $p < 0.001$ and $p < 0.05$, respectively). $CD14^{lo}$ non-classical monocyte frequencies were not significantly altered in frozen PBMC when compared to whole blood samples (**Figure 5D**). The proportions of $CD56^{bright}$

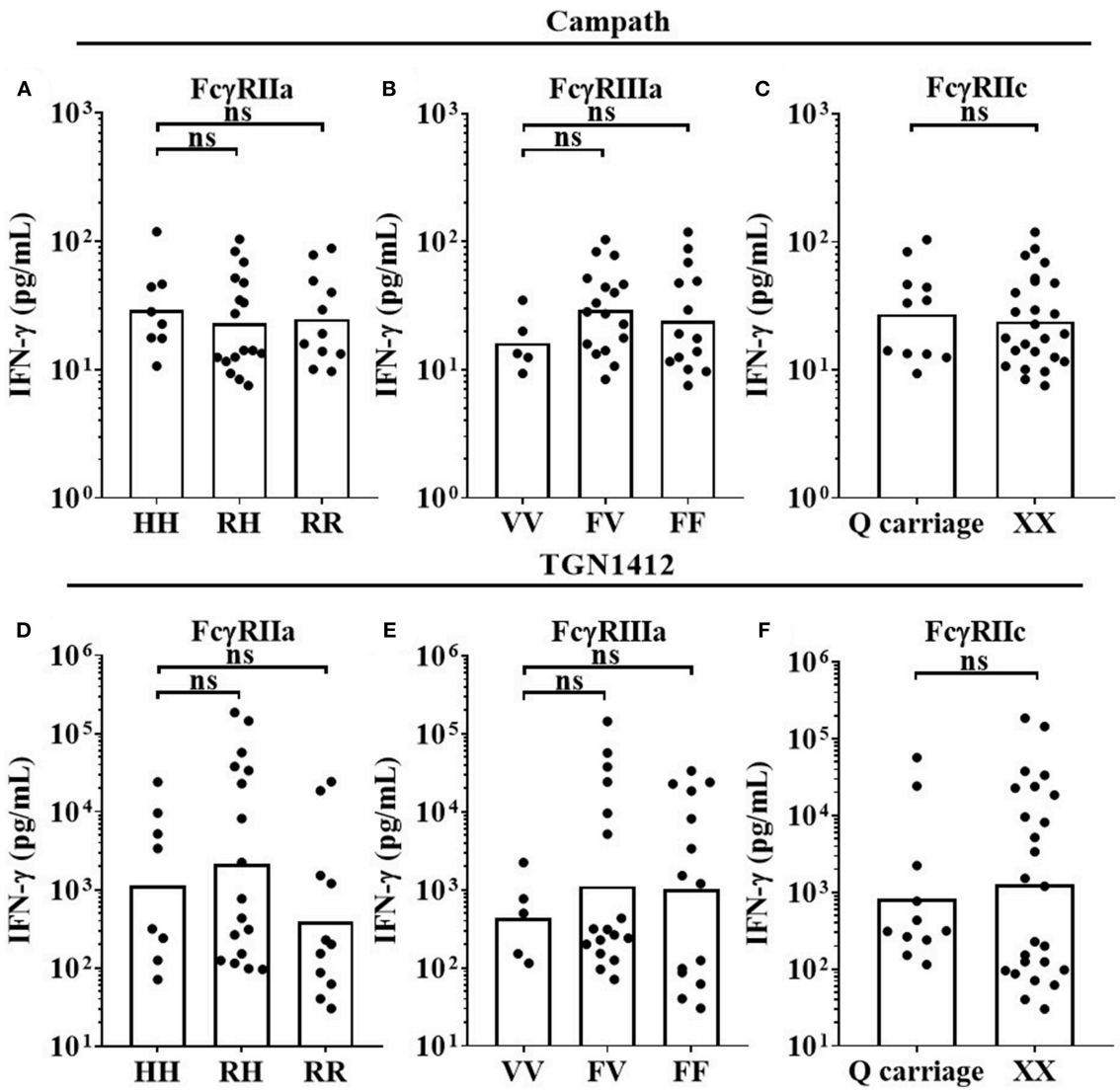

FIGURE 4 | Impact of FcγR polymorphisms on mAb mediated IFN-γ release in a PBMC based assay format. Healthy donors were grouped by FcγRIIa polymorphisms; HH ($n = 8$), RH ($n = 17$) and RR ($n = 11$), FcγRIIIa polymorphisms; VV ($n = 5$), FV ($n = 17$) and FF ($n = 14$), and FcγRIIc polymorphisms; QQ/XQ (n = 11) and XX ($n = 25$). PBMCs were stimulated with **(A–C)** Campath or **(D–F)** TGN1412 and IFN-γ release was quantified 24 h post-stimulation. Each point represents a donor and bars represent group geometric means. ns = non-significant.

NK cells (median = 0.15% for whole blood, 0.4% for frozen PBMCs, $p < 0.01$) and $CD3^+$ NK cells (median = 2.4% for whole blood, 4.3% for frozen PBMCs, $p < 0.001$) were significantly enriched in frozen PBMC samples (**Figures 5G,H**).

We also observed significant change in FcγR expression levels when comparing whole blood to frozen PBMCs. Significant reductions in FcγRIIb on B cells (median = 2,904 for whole blood, 2115 for frozen PBMCs, $p < 0.01$ **Figure 6A**) and FcγRIIIa (median = 4,618 for whole blood, 3,144 for frozen PBMCs, $p < 0.05$, **Figure 6B**) on NK cells were observed in frozen PBMCs compared to whole blood. FcγRI expression remained constant whereas FcγRIIa was significantly upregulated on classical monocytes in frozen PBMCs (median = 327 for whole blood, 1,124 for frozen PBMCs, $p < 0.0001$, **Figure 6C**). FcγRIIa (median = 701 for whole blood, 2,167 for frozen PBMCs, $p < 0.0001$) and FcγRIIb (median = 264 for whole blood, 733 for frozen PBMCs, $p < 0.01$) expression levels were both significantly upregulated on non-classical monocytes (**Figures 6D,E**) in frozen PBMCs, whereas FcγRIIIa expression levels remained unaltered (**Figure 6G**). Granulocytes (present only in whole blood), did not express FcγRI and FcγRIIb, but did express low levels of FcγRIIa and high, but variable levels of FcγRIIIB (**Figure 6H**).

FcγR expression on freeze-thawed PBMCs was also assessed pre- and post-HD culture. As previously reported (10), FcγRI expression on monocytes was not significantly altered (**Supplementary Figure 4A**). In contrast FcγRIIb was markedly

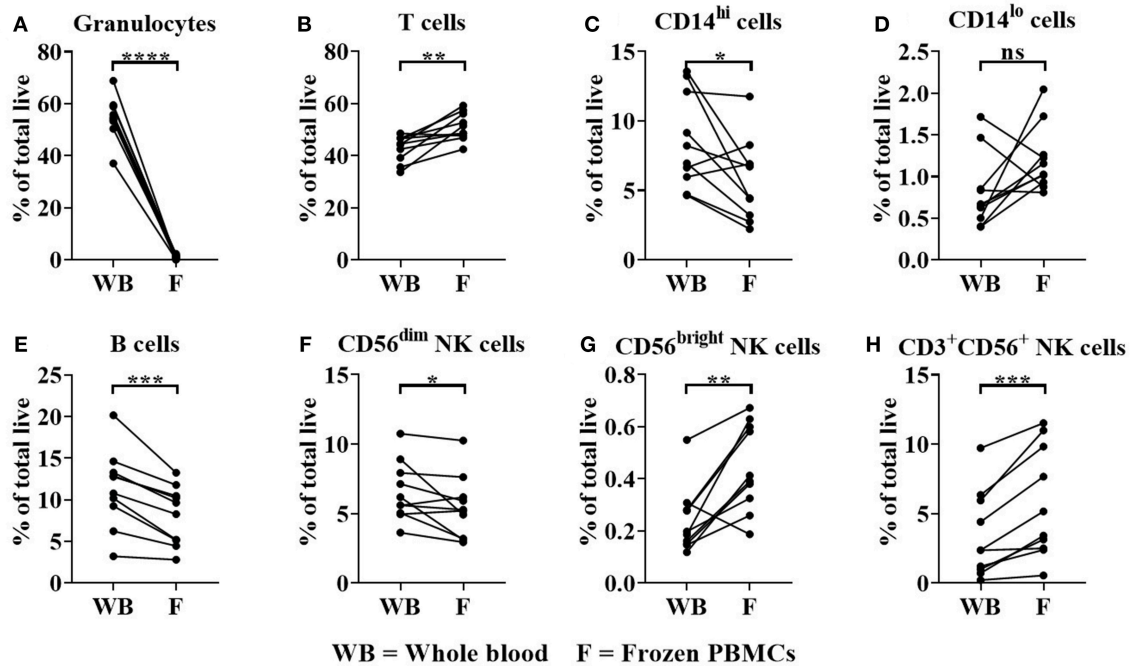

FIGURE 5 | Immune cell subset frequencies in donor matched whole blood (WB) and frozen (F) PBMC samples. Immune cell subset frequencies were quantified in donor matched whole blood, fresh and frozen PBMCs using flow cytometry. Immune cell frequencies were quantified as % of total live/single cells **(A)** % Granulocytes, **(B)** T cells, **(C)** $CD14^{hi}$ monocytes, **(D)** $CD14^{lo}$ monocytes, **(E)** B cells, **(F)** $CD3^{-}CD56^{dim}$, **(G)** $CD3^{-}CD56^{bright}$, and **(H)** $CD3^{+}CD56^{+}$ NK cells of total live/single cells. Each point represents a donor, bars represent group means, ($n = 10$). *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$ and ****$p < 0.0001$ and ns = non-significant.

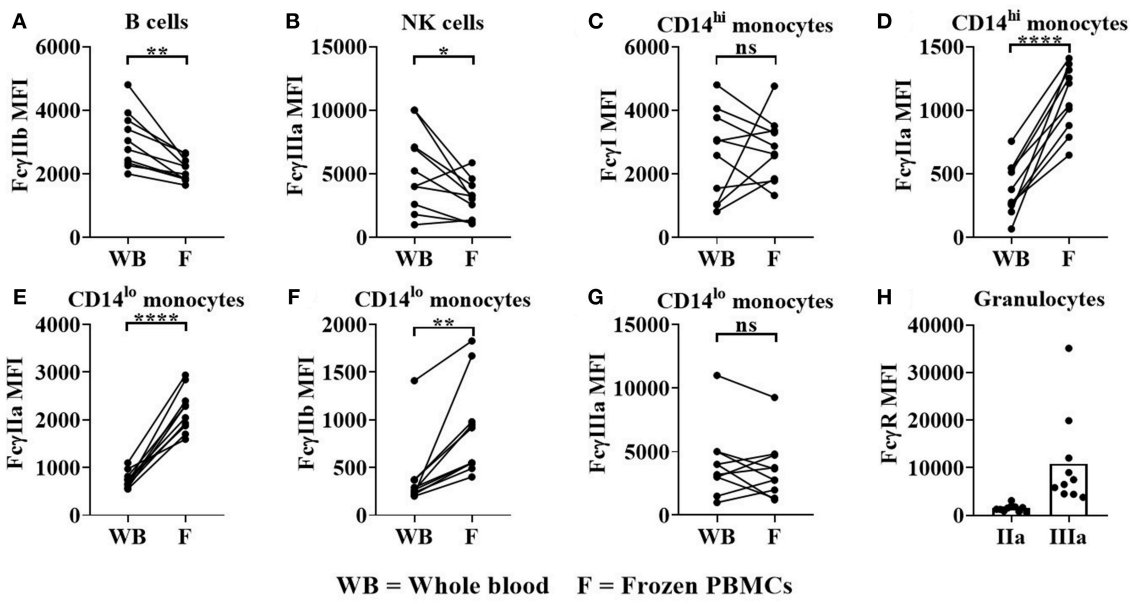

FIGURE 6 | FcγR expression in donor matched whole blood (WB) and frozen (F) PBMCs. Using flow cytometry, FcγR expression was quantified on **(A)** B cells **(B)** NK cells, **(C,D)** classical monocytes, **(E–G)** non-classical monocytes and **(H)** granulocytes in donor matched WB and frozen PBMC samples. FcγRIIa (IIa) and FcγRIIIa (IIIa) expression on granulocytes was quantified in WB only. Each point represents a donor, bars represent group means, ($n = 10$). *$p < 0.05$, **$p < 0.01$, ****$p <$ 0.0001 and ns = non-significant.

increased (100-fold increase post-HD culture, $p < 0.01$), whereas increases in FcγRIIa (0.75-fold increase, $p < 0.05$) and FcγRIIIa (3-fold increase, $p < 0.05$) were less pronounced (**Supplementary Figures 4B–D**). FcγRIIb expression on B cells (**Supplementary Figure 4E**) and FcγRIIIa expression on NK cells were also not significantly modified in freeze-thawed PBMCs after HD culture (**Supplementary Figure 4F**).

These effects of freeze-thawing on FcγR expression and NK cell/monocyte frequencies in PBMC samples prompted us to re-assess the IFN-γ response of those therapeutics which induce cytokine release via Fc:FcγR interactions, using a whole blood assay format.

FcγR Polymorphisms Determine the Magnitude of mAb Dependent IFN-γ Secretion in a Whole Blood Assay Format

To determine whether the impact of FcγR polymorphisms on IFN-γ release in response to Campath stimulation (an effect largely mediated by Fc:FcγR interactions) can be assessed *in vitro*, we utilized the whole blood assay format and samples from 88 UCB cohort donors. Furthermore, to restrict ourselves solely to Fc:FcγR effects, without a bias from the target receptor (e.g., CD52) we also stimulated these whole blood samples with an IgG1 Fc hexamer, which is a recombinant human IgG1 Fc construct generated by fusing the human IgG1 Fc domain to the tail-piece domain of human IgM (28). The IgG1 Fc hexamer was designed as a high-avidity FcγR blocking agent, but was demonstrated to induce high levels of pro-inflammatory cytokine release in whole blood *in vitro* assays, but not PBMC assays via a mechanism dependent on the presence of neutrophils and interactions with FcγRIIa and FcγRIIIb (29). We used this IgG1 Fc Hexamer here as a target receptor-independent mimic for ordered immune complexes which may form after mAb infusion and pose a CRS risk.

IFN-γ responses to Campath (median = 4,051 pg/ml) and the IgG1 Fc hexamer (5,588 pg/mL, $p < 0.001$) for both reagents were significantly stronger than PBS-treated controls, (11.72 pg/ml, **Supplementary Figure 5A**). Furthermore, there was a significant correlation ($R^2 = 0.53$, $p < 0.0001$) of IFN-γ release between Campath and IgG1 Fc Hexamer treatment (**Supplementary Figure 5B**). Longitudinal assessment of IFN-γ release over a period of four months using repeat whole blood samples from nine heathy donors, in five separate assays, revealed stable responses from all donors to Campath and IgG1 Fc hexamer stimulation (**Supplementary Figures 6A,B**)—again indicating a stable donor-specific response profile, with potential value as a prognostic test.

This assay format revealed that donors homozygous for the high-affinity *FCGR2A*-131H allele mounted significantly stronger IFN-γ responses to Campath (median = 5,273 pg/mL), than individuals homozygous for the low IgG affinity *FCGR2A*-131R allele (median = 2,788 pg/mL, $p < 0.01$ when comparing HH individuals with RR individuals, **Figure 7A**). Heterozygous individuals (RH) elicited intermediate responses (median = 3,331 pg/mL, $p < 0.05$ when comparing HH individuals with RH individuals, **Figure 7A**). There were no statistically significant differences in IFN-γ release, between the high affinity homozygous *FCGR3A*-158V allele, heterozygous VF and homozygous *FCGR3A*-158F low IgG affinity donors, in response to Campath stimulation. However, this may have been due to the low numbers of donors homozygous for the high affinity *FCGR3A*-158V allele in our cohort (median; VV = 6,005 pg/mL, VF = 3,847 pg/mL and FF = 2,869 pg/mL, $p = 0.26$ when comparing VV vs. FF donor responses, **Figure 7B**). Significantly greater IFN-γ release was observed in response to Campath stimulation amongst donors homozygous for both high affinity *FCGR2A*-131H and *FCGR3A*-158V SNPs (median = 9204 pg/mL) in comparison to donors homozygous for the low IgG affinity *FCGR2A*-131R and *FCGR3A*-158F alleles (median = 2,684 pg/mL, $p < 0.05$, **Figure 7C**). When comparing *FCGR2C*-57Q SNP-carrying donors, who are predicted to express this additional activatory FcγR on NK cells, with *FCGR2C*-57X homozygous donors who are FcγRIIc negative, no significant associations with IFN-γ release were observed ($p = 0.34$, when comparing QQ/QX with XX donors, **Figure 7D**). Furthermore, no significant associations between the *FCGR3B* and *FCGR2B* SNPs and Campath induced IFN-γ release were revealed using this assay format (**Figures 7E,F**).

For the IgG1 Fc hexamer, *FCGR2A*-131H homozygous high affinity donors mounted significantly stronger IFN-γ responses (median = 7,867 pg/mL) when compared to *FCGR2A*-131R low affinity homozygous donors (median = 3,470 pg/mL, $p < 0.05$). The *FCGR2A*-131RH heterozygous donors mounted an intermediate response (median = 5,066 pg/mL, **Figure 8A**). There were no statistically significant differences in IFN-γ release between the homozygous high affinity *FCGR3A*-158V allele, heterozygous VF and homozygous *FCGR3A*-158F low IgG affinity donors, in response to IgG1 Fc Hexamer stimulation. (median IFN-γ responses (pg/mL); VV = 7441, VF = 5,066 and FF = 5,443.14, **Figure 8B**). IFN-γ release in response to IgG1 Fc hexamer amongst donors homozygous for both the high affinity *FCGR2A*-131H and *FCGR3A*-158V alleles were significantly elevated compared to donors homozygous for the low IgG affinity alleles (median for HH/VV = 9,472 and RR/FF = 4,324 pg/mL, $p < 0.05$, **Figure 8C**). No significant associations between the *FCGR2C*, *FCGR3B* and *FCGR2B* SNPs and IgG1 Fc hexamer induced IFN-γ release were observed using this assay format (**Figures 8D–F**). Assessing the effects of *FCGR3A*, *FCGR2C,* and *FCGR3B* gene CNV with FcγR expression on immune cells or mAb mediated cytokine release was not possible in the current study due to the limited size of donor cohorts and relative rarity of gene CNV >2.

Finally, we stimulated matched whole blood and precultured freeze-thawed PBMC cultures with OKT3, TGN1412, Campath and the IgG1 Fc Hexamer, to directly compare both assay formats and modified IFN-γ release amongst five high IgG affinity *FCGR2A*-131H homozygous donors compared to five low IgG affinity *FCGR2A*-131R homozygous donors. A trend toward increased IFN-γ release was observed in the *FCGR2A*-131H homozygous donors. However, with such small donor numbers a clear statistically significant relationship could not be confirmed using either assay format (data not shown). Altogether, these results demonstrate the importance of donor assay format and

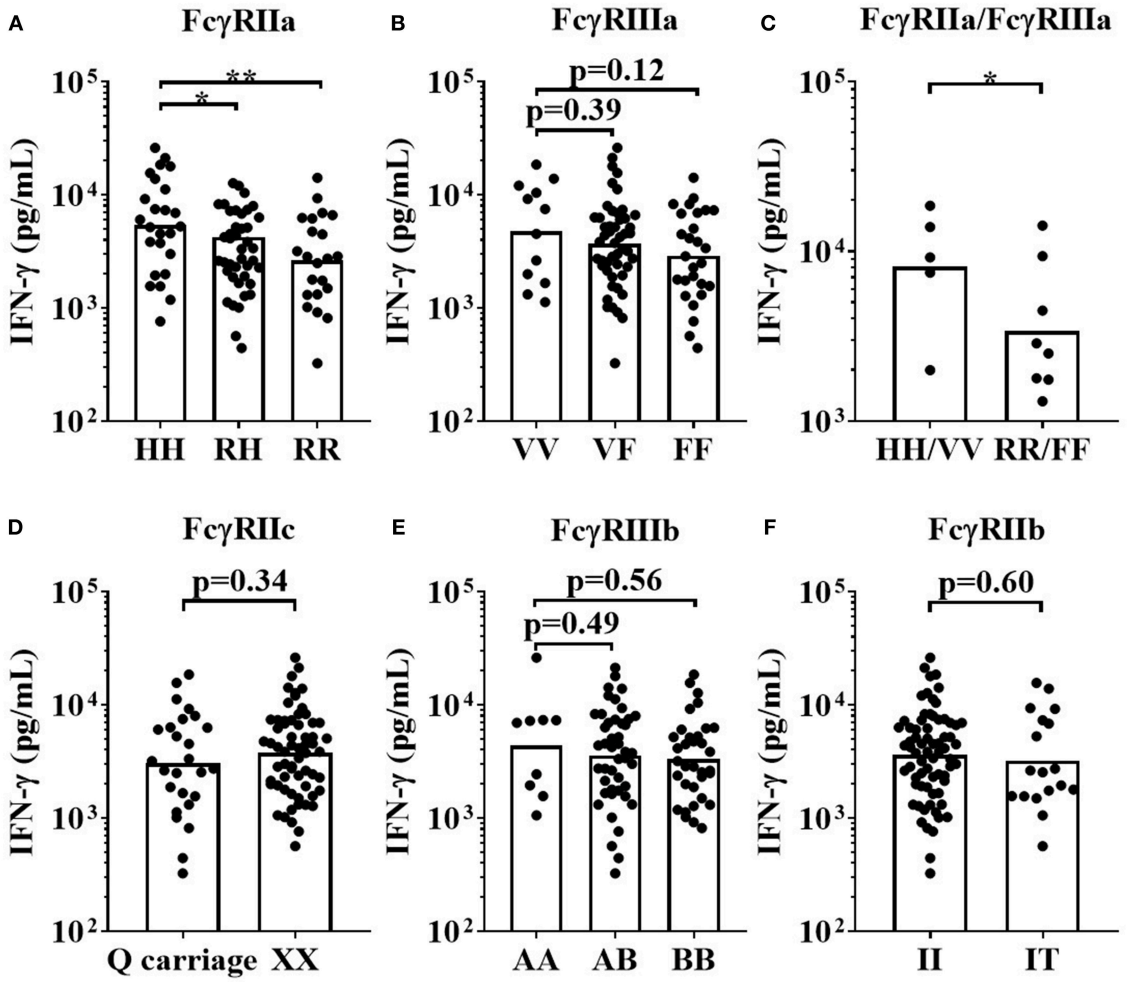

FIGURE 7 | Impact of FcγR polymorphisms on Campath induced IFN-γ release in a whole blood assay format. Healthy donors were grouped by **(A)** FcγRIIa polymorphisms; HH ($n = 25$), RH ($n = 41$) and RR ($n = 22$), **(B)** FcγRIIIa polymorphisms; VV ($n = 12$), FV ($n = 49$) and FF ($n = 27$), **(C)** high affinity FcγR polymorphisms HH/VV ($n = 5$) and low affinity FcγR polymorphisms RR/FF ($n = 8$), **(D)** FcγRIIc polymorphisms; Q carriage ($n = 26$) and XX ($n = 62$), **(E)** FcγRIIIb polymorphisms; AA ($n = 9$), AB ($n = 45$) and BB ($n = 34$) and **(F)** FcγRIIb polymorphisms; II ($n = 70$) and IT ($n = 18$). Whole blood from each donor was stimulated with Campath and IFN-γ release was quantified 24 h post-stimulation. Each point represents a donor, bars represent group geometric means. $^*p < 0.05$ and $^{**}p < 0.01$.

sufficient sample numbers when determining the impact of *FCGR2A* and *FCGR3A* polymorphisms on the magnitude of the IFN-γ response elicited by antibodies and IgG constructs with CRS-inducing potential.

DISCUSSION

Beyond their effects resulting from specific binding of cell surface antigens, mAb possess additional biological activity mediated through their Fc:FcγR interactions which are typically critical for efficacious antibody immunotherapy in patients. mAb interactions with FcγRIIa and FcγRIIIa mediate antibody dependent cellular cytotoxicity (ADCC) and antibody dependent cellular phagocytosis (ADCP) by tumor targeting mAb such as Rituximab, Herceptin and Campath. SNPs in *FCGR2A* and *FCGR3A* genes influencing mAb affinity for FcγR have previously been shown to modify antibody immunotherapy in cancer patients (32). Here we report that whole blood assays are potentially more sensitive for hazard identification of mAb mediated cytokine release, as well as the assessment of the impact of *FCGR2A* and *FCGR3A* SNPs on the magnitude of this cytokine release *in vitro*.

MLPA remains the current gold standard assay for comprehensive FcγR genotyping. Using PBMC samples from healthy donors, we observed considerable variability across the FcγR locus in the form of SNPs and CNV, with homology between FcγR genes further complicating analysis as a result of the ancestral segmental duplication (33, 34). SNP frequencies described in this study are in line with others (35, 36) and while the reported SNP frequencies are within Hardy-Weinberg equilibrium, it is not optimized for loci with

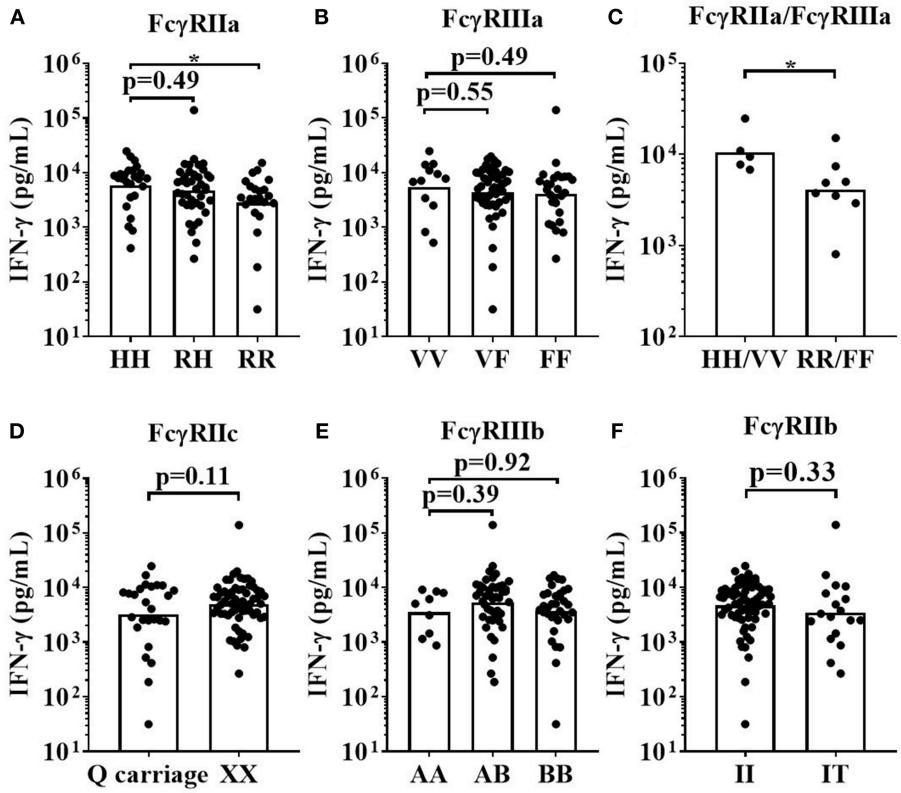

FIGURE 8 | Impact of FcγR polymorphisms on IFN-γ release induced by IgG1 Fc Hexamer stimulation in a whole blood assay format. Healthy donors were grouped by; **(A)** FcγRIIa polymorphisms; HH ($n = 25$), R/H ($n = 41$), and RR ($n = 22$), **(B)** FcγRIIIa polymorphisms; VV ($n = 12$), FV ($n = 49$) and FF ($n = 27$), **(C)** high affinity FcγR polymorphisms HH/VV ($n = 5$) and low affinity FcγR polymorphisms RR/FF ($n = 8$), **(D)** FcγRIIc polymorphisms; Q carriage ($n = 26$) and XX ($n = 62$), **(E)** FcγRIIIb polymorphisms; AA ($n = 9$), AB ($n = 45$) and BB ($n = 34$) and **(F)** FcγRIIb polymorphisms; II ($n = 70$) and IT ($n = 18$). Whole blood from each donor was stimulated with IgG1 Fc Hexamer and IFN-γ release was quantified 24 h post-stimulation. Each point represents a donor, bars represent group geometric means. *$p < 0.05$.

CNV. CNV represents a significant source of genetic diversity and can affect the function of FcγR gene products. Alterations in copy number of FcγR genes have reported gene dosage effects on protein expression (27, 37). CNV has been described in *FCGR3A*, *FCGR2C,* and *FCGR3B* (30, 35, 37), with rare events reported to affect *FCGR2B* (34). Regions of copy number alteration, CNR1-4, encompassing multiple genes in the locus have been described (17). As previously described (17, 35), CNV of *FCGR3A* is rare (3.6% of individuals), with alterations affecting *FCGR2C* and *FCGR3A* the most common CNV events. To date, these CNV have not been associated with mAb-mediated effects in the clinic, perhaps due to their relative rarity, leading to insufficient statistical power. Similar deficiencies were observed here in our study, with several 100 donors being required to study the impacts of CNV comprehensively. While many studies have reported associations between the high affinity *FCGR2A* and *FCGR3A* alleles and greater mAb efficacy in numerous cancers (20, 23), others have not observed such associations (38, 39), perhaps due to differing and complex biological backgrounds. We postulated that FcγR genotypes may correlate with *in vitro* IFN-γ responses to mAb stimulation and further enable prediction of CRS risk in the clinic.

In recent years, the limited value of rodent models for predicting treatment responses in humans set in motion intensive research to establish *in vitro* assays using human PBMCs; for example to predict the magnitude of cytokine release induced by therapeutic mAb (40). Assessment of mAb function and toxicity *in vitro* often utilizes banked frozen PBMC samples in both commercial pharmaceutical and academic settings. We previously used a PBMC-based assay format in which freeze-thawed PBMCs are first cultured at high density prior to stimulation with mAb. The high density preculture step promotes PBMC sensitivity to TGN1412 which otherwise elicits no immune cell activation in fresh untouched PBMC cultures (8, 10). Thus using this assay format which allows for the assessment of a CRS-inducing mAb (TGN1412), we sought to determine the impact of FcγR SNPs on mAb-induced cytokine release. Although assays were reproducible and stable per donor over time (indicating an inherent factor underpinning the level of response), no significant impact of *FCGR2A*, *FCGR2C,* or *FCGR3A* polymorphisms was observed with any mAb with respect to the magnitude of cytokine release (**Figure 4**). Larger cohorts would be required to accurately study the impact of CNV at the low-affinity locus given the low-frequency of events

in *FCGR3A*, for example, whose gene product, FcγRIIIa, is an important mediator of NK cell-mediated ADCC.

This prompted a detailed comparison of immune cell subset frequencies and FcγR expression in whole blood and previously frozen PBMC samples. Significant reductions in monocytes, NK cells ($CD3^{-}CD56^{dim}$ cells) and FcγRIIIa expression on the latter were observed in freeze-thawed PBMCs relative to whole blood (**Figures 5C,F**, **6B**). These observations were in concordance with previous studies reporting reduction in FcγRIIIa positive NK cells in freeze-thawed PBMC samples (41). In addition, FcγRIIa expression was significantly increased on monocytes in PBMC samples (**Figures 6D,E**), however, monocyte frequencies were reduced (**Figure 5C**), further impacting the likelihood of observing any *FCGR2A* SNP association with mAb-mediated cytokine release. The absence of FcγR-bearing neutrophils, platelets, donor IgG and complement proteins from serum in PBMC samples further justifies the utility of whole blood assays when determining the effects of FcγR SNPs on mAb-mediated cytokine release.

In the current study minimally diluted whole blood (95% blood / 5% mAb diluent) combined with aqueous mAb presentation was shown to be a useful and promising format, with only minimal sample and mAb manipulation aiming to preserve the natural peripheral blood molecular and cellular composition. We used this system to test Campath which binds CD52, a cell surface membrane antigen abundantly expressed on the surface of B cells, T cells, and monocytes (42). Campath triggering of FcγRIIIa on NK cells directly leads to IFN-γ release (12). We also tested an IgG1 Fc Hexamer construct which interacts with FcγRs, not target antigen. We have previously reported that cytokine release associated with this construct is primarily via interaction with FcγRIIa and FcγRIIIb and dependent on the presence of neutrophils (28, 29). Thus, both Campath and the IgG1 Fc Hexamer were suitable candidates for the assessment of the impact of FcγR SNPs on cytokine release in this assay format.

FcγRIIa is a monomeric receptor possessing an ITAM in its intracellular domain. It is the most broadly distributed FcγR, being expressed on monocytes, macrophages, platelets, and neutrophils and also in a soluble form (FcγRIIa2), (43). Our quantification of FcγRIIa expression in whole blood confirmed expression is restricted to monocytes and neutrophils (**Figure 6**). IgG triggering of FcγRIIa-mediated ITAM signaling results in cellular activation, phagocytosis, oxidative burst and the production of pro-inflammatory cytokines by monocytes and neutrophils (44). In whole blood, we observed a significantly elevated IFN-γ release in response to Campath and IgG1 Fc Hexamer stimulation amongst *FCGR2A*-131H/H donors compared to R/R donors. As Campath primarily stimulates cytokine release by triggering FcγRIIIa on NK cells (12), it was unexpected to observe a significant association with the *FCGR2A*-131H allele (**Figure 7A**). This enhanced IFN-γ release amongst the *FCGR2A*-131H homozygous donors may therefore be an indirect consequence of Campath triggered FcγRIIa activation on monocytes and neutrophils leading to pro-inflammatory cytokine release in these cell types, that then activates NK cells to secrete IFN-γ (45). We have previously demonstrated that the IgG1 Fc hexamer stimulates IFN-γ production in whole blood in a neutrophil-dependent manner, in contrast to the response to Campath which was not affected by depletion of neutrophils from whole blood (29). Isolated neutrophils have also been shown to be capable of producing pro-inflammatory cytokines (29, 46) and TLR-independent neutrophil-derived IFN-γ is important for host resistance to intracellular pathogens (28), emphasizing the importance of maintaining the presence of these FcγR bearing cells in *in vitro* cytokine release assays, especially when testing reagents with the potential to form immune complexes. Neutrophils express both FcγRIIa and FcγRIIIb and this study, along with our previous data, suggests both receptors are important in this immune-complex induced cytokine response and that polymorphisms in FcγRIIa in particular may modulate this. This is in agreement with earlier studies indicating a complex interplay between FcγRIIa and FcγRIIIb haplotype and sensitivity of neutrophils to IgG-induced respiratory burst (47).

FcγRIIIa is a type I transmembrane receptor and signals via its association with the ITAM-expressing FcRγ chain, encoded by the *FCER1G* gene (48). In whole peripheral blood its expression is largely restricted to $CD3^{-}CD56^{dim}$ NK cells, and non-classical monocytes (**Figures 6B,C**). Additionally, FcγRIIIa is also abundantly expressed on macrophages (not present in whole blood) as well as on tumor-associated macrophages (49). FcγRIIIa has been reported to be the most potent activating receptor on freshly isolated peripheral blood NK cells, able to elicit potent ADCC and cytokine production in response to Campath treatment (50). Although not significant, we observed enhanced IFN-γ responses to Campath and IgG1 Fc hexamer stimulated whole blood cultures sourced FCGR3A-158V/V donors relative to *FCGR3A*-158V/F and *FCGR3A*-158F/F donors. Given the lower frequency of the *FCGR3A*-158V/V genotype in Western European populations (<10%), large sample size is essential for these studies to achieve statistically significant associations with mAb mediated cytokine release. In the current study only 12/88 donors (UCB cohort) possessed the *FCGR3A*-158V/V genotype, likely explaining the lack of statistical significance.

In the whole blood assay format, mAb-mediated effector functions are profoundly influenced by simultaneous mAb interactions with FcγRIIa, FcγRIIIa, FcγRIIb, and FcγRIIIb. To partially address the impact of IgG Fc interaction with more than one FcγR species, we analyzed a subset of donors homozygous for high or low affinity *FCGR2A*-131 and *FCGR3A*-158 alleles. The HH/VV donors had a 4-fold higher IFN-γ response to Campath and a 2-fold higher response to IgG1 Fc Hexamer, in comparison to the RR/FF donors. Encouragingly, these significant differences were observed with a relatively low number of donors and were again stable over time (indicating a stable donor-specific response profile), with potential value for development of prognostic tests. However, greater donor numbers are required for sufficient statistical powering for other associations, especially when also taking into account the low frequency of certain SNPs and large variability of cytokine responses to mAb stimulation of whole blood cultures. Based upon power calculations on our data to date, we recommend ≥20 donors for each FcγR SNP for assessing associations of FcγR genotype with mAb-mediated

cytokine release hazard identification. This gives more than 80% power to detect a 3-fold difference between groups, using the whole blood IFN-γ release assay (**Supplementary Figure 7**).

Using the whole blood assay format, we did not observe statistically significant associations between *FCGR2C*, *FCGR2B,* or *FCGR3B* SNPs with the magnitude of Campath or IgG1 Fc hexamer-mediated cytokine release. FcγRIIc expression has been reported on NK cells, however, using flow cytometry we observed negligible or no FcγRIIc expression on NK cells, in >100 PBMC or whole blood samples (manuscript in preparation). This may explain the lack of significant association of *FCGR2C* SNPs with the magnitude of cytokine release. In whole blood samples, FcγRIIb expression is almost entirely restricted to B cells, which are unlikely to contribute to Campath or IgG1 Fc Hexamer induced IFN-γ release. Furthermore, it is worth recollecting that FcγRIIb is an ITIM-signaling inhibitory receptor, more likely to restrict cytokine release mediated by ITAM signaling on cell types co-expressing activatory and inhibitory FcγR. In addition, the I232T *FCGR2B* SNP leading to lack of inhibitory signaling, is extremely rare in Caucasian populations and so would require an extremely large cohort to study (26). Although FcγRIIIb has been reported to play a role in IgG1 Fc Hexamer induced cytokine release (29), we observed large variability in the expression levels of this receptor on neutrophils (MFI range 3771-35100) between donor samples. This may have compromised observing significant associations of *FCGR3B* SNPs with the extent of cytokine release in the IgG1 Fc Hexamer treated samples.

In summary, while there is considerable variability in the magnitude of cytokine responses elicited by cytokine storm-inducing IgG1 antibodies and Fc constructs in the whole blood assay format, key cell populations such as NK cells, monocytes and neutrophils remain intact and express FcγR at physiological levels. Our findings suggest that high-throughput genotyping combined with whole blood assays may be a powerful pharmacogenetic approach to predict both mAb therapy outcome and hazard identification but requires sufficient donors of each FcγR genotype if these associations are sought.

AUTHOR CONTRIBUTIONS

KH, CH, TR, KL, and JMS performed experiments. KH, CH, and JS performed statistical analyses. KH, CH, TR, MG, JCS, and MC designed experiments. KH and CH wrote the manuscript with contributions from TR, DH, and MC. All authors contributed to manuscript revision and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00390/full#supplementary-material

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REVIEW
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Fc-Mediated Antibody Effector Functions During Respiratory Syncytial Virus Infection and Disease

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Respiratory syncytial virus (RSV) is a major cause of severe lower respiratory tract infections and hospitalization in infants under 1 year of age and there is currently no market-approved vaccine available. For protection against infection, young children mainly depend on their innate immune system and maternal antibodies. Traditionally, antibody-mediated protection against viral infections is thought to be mediated by direct binding of antibodies to viral particles, resulting in virus neutralization. However, in the case of RSV, virus neutralization titers do not provide an adequate correlate of protection. The current lack of understanding of the mechanisms by which antibodies can protect against RSV infection and disease or, alternatively, contribute to disease severity, hampers the design of safe and effective vaccines against this virus. Importantly, neutralization is only one of many mechanisms by which antibodies can interfere with viral infection. Antibodies consist of two structural regions: a variable fragment (Fab) that mediates antigen binding and a constant fragment (Fc) that mediates downstream effector functions via its interaction with Fc-receptors on (innate) immune cells or with C1q, the recognition molecule of the complement system. The interaction with Fc-receptors can lead to killing of virus-infected cells through a variety of immune effector mechanisms, including antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). Antibody-mediated complement activation may lead to complement-dependent cytotoxicity (CDC). In addition, both Fc-receptor interactions and complement activation can exert a broad range of immunomodulatory functions. Recent studies have emphasized the importance of Fc-mediated antibody effector functions in both protection and pathogenesis for various infectious agents. In this review article, we aim to provide a comprehensive overview of the current knowledge on Fc-mediated antibody effector functions in the context of RSV infection, discuss their potential role in establishing the balance between protection and pathogenesis, and point out important gaps in our understanding of these processes. Furthermore, we elaborate on the regulation of these effector functions on both the cellular and humoral side. Finally, we discuss the implications of Fc-mediated antibody effector functions for the rational design of safe and effective vaccines and monoclonal antibody therapies against RSV.

Keywords: RSV, antibody, Fc gamma receptor, Fc-mediated effector functions, antibody functionality, ADCC, ADCP, vaccine

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INTRODUCTION

Respiratory syncytial virus (RSV) infection is a major cause of severe respiratory illness requiring hospitalization in young infants (1). Hospitalization for severe RSV-mediated disease peaks between 6 weeks and 6 months of life (2, 3), when infants mainly depend on their innate immune system and maternal antibodies for protection against infectious diseases. However, the exact role of RSV-specific maternal antibodies is unclear. Some studies show that high titers of maternal antibodies are associated with protection against RSV infection (4–6); whereas others indicate that high maternal antibody titers do not have a beneficial effect or even associate with an increased risk of recurrent wheezing (7–11). It is important to note that the antibody titers in these studies are determined by *in vitro* binding or neutralization assays, while additional antibody effector functions are not taken into account.

For nearly all licensed vaccines, antibodies are the presumed correlate of protection, but the underlying mechanisms of protection often remain unknown (12). Recent research suggests that, in addition to binding and neutralization, antibody effector functions are important contributors to protective immunity against several viruses, including influenza virus (13–15), HIV (16, 17), and Ebola virus (18, 19).

In contrast to their beneficial role in providing protection against infection and disease, antibodies have also been implicated in disease enhancement. For example, non-neutralizing dengue-specific antibodies have been shown to mediate antibody-dependent enhancement (ADE) of disease (20, 21). Interestingly, the 1960's formalin-inactivated (FI) RSV vaccine induced poorly-neutralizing antibodies which have been suggested to be involved in vaccine-enhanced disease upon natural infection (22–24). These examples illustrate the possibility that virus-specific antibodies contribute to pathogenesis when failing to protect.

Currently, the RSV field lacks a comprehensive overview of antibody effector functions in the context of RSV infection and disease. Here, we review what is known about various antibody effector functions during RSV infection, discuss their potential role in establishing the balance between protection and pathogenesis, and point out important gaps in our understanding of these processes. Moreover, we elaborate on the regulation of these effector functions on both the cellular and humoral side. Finally, we discuss the implications of antibody-mediated effector functions for the rational design of safe and effective vaccines and monoclonal antibody therapies against RSV. A thorough understanding of the role of antibodies in protection or disease during RSV infection is crucial for the development of new and improved vaccination strategies and may provide much-needed new insights into the precise mechanisms of antibody-mediated protective immunity.

FC-MEDIATED ANTIBODY EFFECTOR FUNCTIONS

Antibody effector functions are an important part of the humoral immune response and form an essential link between innate and adaptive immunity. Most of these effector functions are induced via the constant (Fc) region of the antibody, which can interact with complement proteins and specialized Fc-receptors. The latter can induce activating or inhibitory pathways, depending on the type of receptor, and are found on B cells and most innate immune cells in various combinations. The most well-known Fc-mediated antibody effector functions are antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC). In addition, antibodies have been found to mediate inflammation and immunomodulation through the induction of cellular differentiation and activation. Each of these functions is described in detail below and a schematic overview is depicted in **Figure 1**.

ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY (ADCC)

ADCC is induced when Fc gamma receptors (FcγRs) on innate effector cells are engaged by the Fc domain of antibodies that are bound to viral proteins on the surface of virus-infected cells. This interaction induces the release of cytotoxic granules (containing perforins and granzymes) resulting in killing of infected cells (25). Multiple innate effector cells, including natural killer (NK) cells, neutrophils, monocytes, and macrophages, are capable of ADCC *in vitro*. However, the most important contributors to ADCC *in vivo* are thought to be NK cells, which express only FcγRIIIA. **Figure 2** shows a schematic representation of ADCC.

In the field of tumor immunology, ADCC has been recognized as an important mechanism of action for therapeutic monoclonal antibodies (mAbs) that target tumor cells [as reviewed by (26)]. For infectious diseases, ADCC only recently started to gain attention. ADCC has been shown to form a critical component of effective immunity against HIV and influenza virus. ADCC-inducing HIV-specific antibodies were identified as a key correlate of protection in the RV144 HIV vaccine trial (27–29). Moreover, HIV-infected individuals who control the virus without antiretroviral therapy demonstrated a broader polyfunctional humoral immune response including ADCC activity compared to viremic individuals (30–33). There has been much debate about the role of ADCC during influenza-induced disease. Some studies point to the protective capacity of ADCC-inducing antibodies (34, 35), whereas others do not show any role for NK cells in antibody-mediated protection (36), or even suggest involvement of ADCC in exaggeration of the immune response (37–39). For multiple other clinically important viral infections, including dengue virus and Ebola virus, research into the effect of ADCC is ongoing (40–42). Taken together, ADCC seems to be involved in the immune response against multiple viruses and is therefore potentially of interest in the context of RSV infection.

ADCC in RSV Infection

NK cells are the most important contributors to ADCC *in vivo* and important effector cells during RSV infection. In mice, increased numbers of NK cells are present in the lungs early after RSV infection (43–45). In RSV-infected infants, the

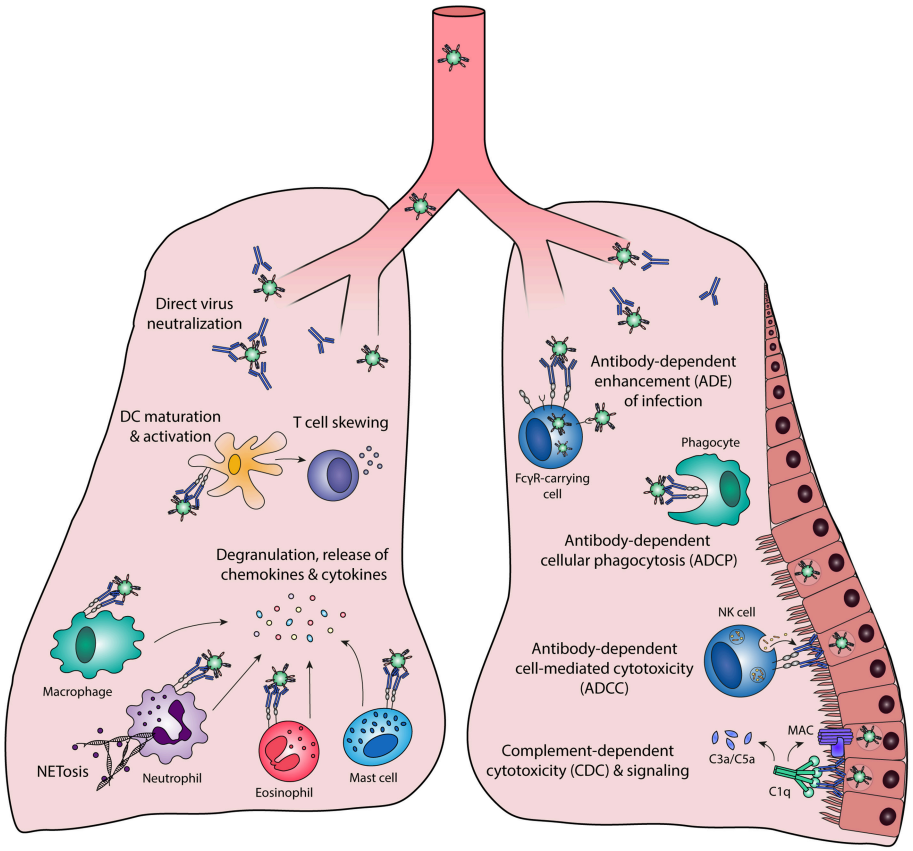

FIGURE 1 | Fc-mediated antibody effector functions. Antibodies elicit a wide range of effector functions during viral infections. These include but are not necessarily limited to the functions depicted in this figure. DC, dendritic cell; FcγR, Fc gamma receptor; MAC, membrane attack complex; NK cell, natural killer cell.

proportion of NK cells has been reported both to be decreased (46–49) or increased (50, 51) in comparison with healthy controls or infants with mild symptoms. Since maternally-derived antibodies are virtually always present during primary RSV infection and antibody-coated virus-infected cells are a trigger for ADCC, it can be assumed that ADCC occurs during primary RSV infection.

Although NK cells are thought to be the most important mediators of ADCC against virus-infected cells, this has never been shown for RSV. All studies mentioned below are performed with peripheral blood mononuclear cells (PBMCs), without distinction between different cell types. RSV-specific immunoglobulin G (IgG) has been shown to induce ADCC toward RSV-infected epithelial cells *in vitro* (52, 53). The major surface antigens of RSV are the fusion (F) and the attachment (G) protein which are both required for infectivity *in vivo*. The RSV F protein has two conformational states: post-fusion (post-F) and pre-fusion (pre-F), of which the latter is a potent target for neutralization (54). Multiple studies show that anti-RSV G antibodies are efficient inducers of ADCC *in vitro* (55, 56), and the involvement of this process in virus clearance *in vivo* has been proposed (57, 58). In contrast, anti-RSV F antibodies do not efficiently induce ADCC *in vitro* (55), although it must be noted that no distinction between pre- and post-F antibodies was made and the ADCC potential could differ between the two functional states of the F protein.

Antibodies from breast milk, cord blood, and nasopharyngeal secretions and serum from RSV-infected infants show ADCC activity *in vitro* (52, 53, 59). This shows that the antibodies that are present *in vivo* are capable of eliciting ADCC activity *in vitro*. Two studies showed that the level of ADCC activity measured *in vitro* was independent of clinical symptoms and age, suggesting that ADCC is not a determining factor in the varying clinical manifestations of primary RSV infection (53, 59). Interestingly, the ADCC capacity of serum antibodies from RSV-infected infants rapidly declines over time, whereas the neutralization capacity remains more stable. If ADCC is important in protection against infection, this decline could partly explain the susceptibility to repeated infections throughout life.

Limited evidence is present on the occurrence of ADCC during RSV infection *in vivo*. The most convincing data is provided by mouse studies performed with anti-RSV G protein-specific Fab- or $F(ab')_2$ fragments lacking the complete Fc domain, or aglycosylated antibodies lacking the glycosylation site

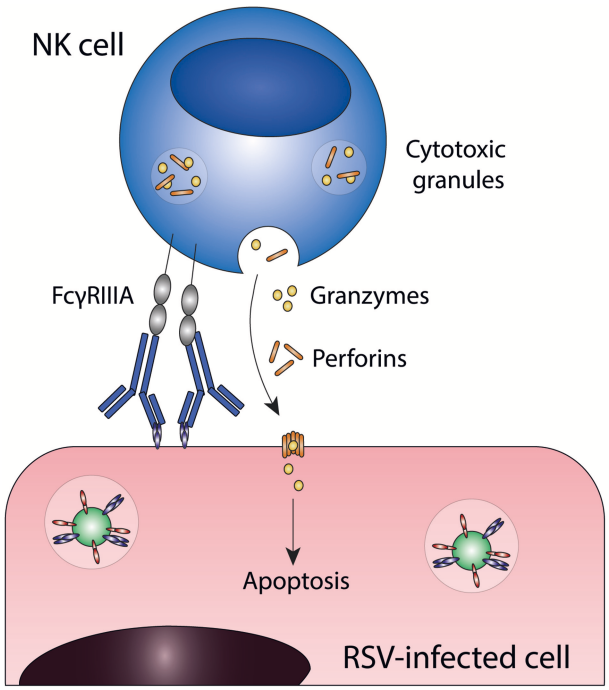

FIGURE 2 | Antibody-dependent cell-mediated cytotoxicity (ADCC). Fc gamma receptors present on for example natural killer (NK) cells engage antibody-bound infected cells and induce target cell death through the release of cytotoxic granules. FcγRIIIA, Fc gamma receptor IIIA; NK cell, natural killer cell; RSV, respiratory syncytial virus.

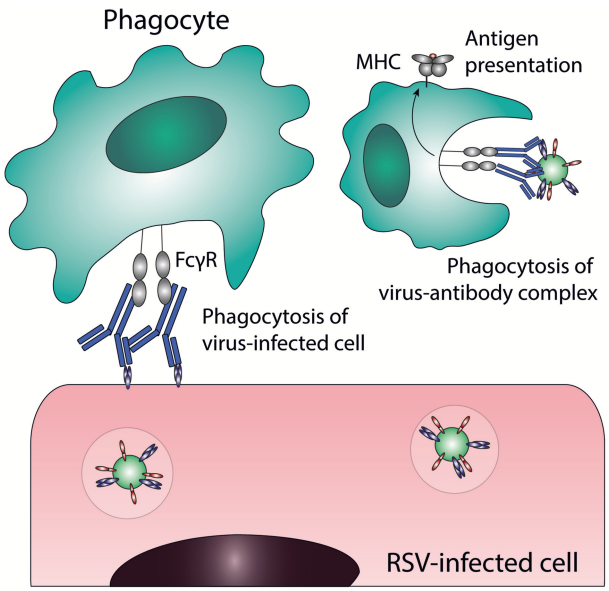

FIGURE 3 | Antibody-dependent cellular phagocytosis (ADCP). Phagocytes can clear virus-infected cells and immune complexes that are engaged by Fc gamma receptors through phagocytosis. Uptake of viral particles or proteins leads to antigen presentation, which induces the adaptive immune system. FcγR, Fc gamma receptor; MHC, major histocompatibility complex; RSV, respiratory syncytial virus.

that is required for efficient FcγR and complement interactions (58, 60, 61). It was shown that Fab fragments of the 1812A2B anti-RSV G antibody and $F(ab')_2$ fragments of the 131-2G anti-RSV G antibody do not reduce viral load, whereas the corresponding intact antibodies do confer protection (58, 60). The authors propose that virus clearance by the 131-2G antibody is mediated through ADCC, however, the involvement of other Fc-mediated effector functions in this study cannot be ruled out. In an attempt to ascertain the role of ADCC by NK cells in the protective mechanisms of the anti-RSV G antibody 18A2B2, SCID beige mice (which are deficient in NK cell activity) were passively immunized with the full antibody (60). In this study, the absence of NK cells had no effect on the protective capacity of 18A2B2, pointing to the involvement of other Fc effector functions. Further research is needed to study the exact role of ADCC for other mAbs and RSV-immune serum. Passive immunization with aglycosylated 1C2 anti-RSV G antibodies reduced virus titers significantly but were not as effective as wildtype antibodies, indicating that protection by the 1C2 antibody is mediated by both Fc-dependent and Fc-independent mechanisms (61). Although these studies highlight the importance of Fc-mediated antibody effector functions in protection against RSV infection in the case of these specific anti-RSV G mAbs, the role of ADCC in protection or pathogenesis during natural RSV infection remains to be determined.

ANTIBODY-DEPENDENT CELLULAR PHAGOCYTOSIS (ADCP)

ADCP or opsonophagocytosis is the uptake of virus-antibody complexes or antibody-coated virus-infected cells by phagocytic cells (for a schematic representation of this process see **Figure 3**). Phagocytic cells, including monocytes, macrophages, neutrophils, eosinophils and dendritic cells (DCs), express FcγRI, FcγRII, and FcαRI, which can all mediate immune complex uptake. The exact phagocytic capacity of effector leukocytes is dependent upon the cell type, differentiation stage, and level of FcγR expression. ADCP results in the clearance of immune complexes from the infected host, by trafficking of the complexes to lysosomes for degradation and antigen processing for presentation on Major Histocompatibility Complex (MHC)-molecules on the cell surface. Interestingly, some viruses have exploited this mechanism to infect phagocytes by escaping from lysosomal degradation (described below in "Antibody-dependent enhancement of infection").

ADCP has been extensively described for its role in protection against bacteria, but its importance during viral infections is unclear. Some studies have been performed for influenza virus, showing that phagocytosis by (alveolar) macrophages may contribute to protection from infection in mice (36, 62) and potentially plays a role in the recovery from severe infections in humans (15, 63). Also for cytomegalovirus (CMV), it was shown that vaccine-induced antibodies play an important role in vaccine efficacy, independent of neutralization or ADCC capacity (64). In accordance with these results, a study by Nelson et al. showed no

role for neutralization or ADCC, while robust ADCP induction was observed (65). Antibody-mediated clearance by phagocytes *in vivo* has also been suggested for HIV (66, 67), adenovirus (68), West Nile Virus (WNV) (69), and foot-and-mouth disease virus (70, 71).

ADCP in RSV Infection

Phagocytosis of RSV-antibody complexes or RSV-infected cells has to our knowledge never been directly explored as a protective immune mechanism for RSV. *In vitro* studies show phagocytosis of RSV immune complexes by neutrophils (56, 72, 73) and eosinophils (74). Varying levels of phagocytic activity have been observed for different RSV-specific monoclonal antibodies, suggesting that ADCP activity depends on epitope and/or affinity (56, 73). An *in vivo* mouse study has shown that macrophages are essential in conferring antibody-mediated restriction of RSV replication, whereas neutrophil depletion did not significantly affect pulmonary viral replication (75). This suggests that Fc-mediated effector functions executed by macrophages rather than neutrophils are important in protection against RSV infection in a mouse model.

Besides the uptake of viral particles, phagocytosis initiates the activation of cells. This can result in the release of a broad range of effector molecules (72–74), which will be described in detail in "Antibody-dependent immunomodulation during RSV infection." Although there is limited evidence on the role of ADCP during RSV infection, the importance of macrophages in antibody-mediated protection in mice provides a basis for further investigation.

ANTIBODY-MEDIATED COMPLEMENT ACTIVATION

Besides ADCC and ADCP, antibodies can also induce complement activation. The complement cascade contributes to pathogen elimination either directly, by means of complement-dependent cytotoxicity (CDC), or indirectly, through phagocytic clearance of complement-coated targets and the induction of an inflammatory response. Activation of the classical complement pathway results from binding of the recognition molecule C1q to the Fc domain of antibodies bound to virus-infected cells (76, 77), as depicted in **Figure 4**. Upon binding of C1q, the proteases of the classical pathway are activated, leading to cleavage of C2 and C4. Together, the resulting cleavage products form the C3 convertase (C4bC2a) that cleaves C3 into C3a and C3b. One of the mechanisms by which the complement cascade is regulated, is cleavage of active C4b, which serves as a marker for complement activation. The release of anaphylatoxins C3a and C5a stimulates a pro-inflammatory environment by inducing the recruitment of immune effector cells and the activation of leukocytes, endothelial cells, epithelial cells, and platelets (78, 79). The highly reactive C3b binds to pathogens and infected cells, leading to immune complex clearance and phagocytosis through complement receptors found on immune cells. The terminal complement components will assemble into the membrane attack complex (MAC), resulting in lysis of the infected cell. Besides direct antiviral activity, the complement system can also regulate B cell responses. The binding of complement-coated immune complexes to complement receptor 2 on B cells is reported to lower the B cell activation threshold, thereby promoting long-lived adaptive immunity and higher antibody levels (80, 81).

Complement can have both a protective and pathogenic role during viral infections. The protective capacity of poorly neutralizing antibodies during WNV infection is mediated by the complement system, as was shown using knockout mice (69). The presence of complement even enhances the neutralization capacity of WNV-specific antibodies (82). In addition, an important role for complement has been shown in the protective capacity of (monoclonal) antibodies against influenza virus (38, 83), vaccinia virus (84), CMV (85), and HIV (66, 67). In contrast, complement activation has also been suggested to contribute to disease severity in dengue virus (86, 87) and HIV infection (88, 89).

Antibody-Mediated Complement Activation in RSV Infection

The complement system consists of multiple components and elicits its effector functions through different pathways. Early studies have shown antibody and complement deposition on nasopharyngeal cells of RSV-infected infants (90). Whether this contributed to viral clearance or disease was not determined. Studies in complement-deficient mice have shown that complement is important in antibody-mediated protection against RSV infection (60, 75). A number of different mechanisms have been suggested for this complement-enhanced protection. Firstly, direct enhancement of the neutralization capacity of antibodies by fixation of complement components to virus-antibody complexes may increase the steric hindrance of bound antibodies (91). Another mechanism that could be at play is complement-dependent opsonization of virus-infected cells, which leads to subsequent uptake by phagocytes. Finally, complement has also been shown to increase the CD4(+) T cell response in the presence of RSV immune serum in an *in vivo* mouse model (92).

Besides its potential role in the clearance and/or pathogenesis of natural RSV infection, complement activation has been suggested to contribute to disease enhancement induced by natural infection following FI-RSV vaccination. C3a receptor (C3aR)-deficient mice had decreased airway hyperresponsiveness (AHR) and less mucus production in an FI-RSV vaccination-challenge model (93). In this study, C3aR expression was enhanced in C5-knockout mice, showing that the balance in activation of different complement factors (C3a vs. C5a) is important in determining disease outcome. Moreover, Polack et al. demonstrated the co-localization of IgG and C3 in the lungs of mice with enhanced RSV disease, but not in control mice (22). In addition, both C3- and B cell-knockout mice showed a decrease in bronchoconstriction compared to WT mice vaccinated with FI-RSV. Therefore, in a mouse model of vaccine-enhanced disease, the presence of C5 seems protective, whereas C3a promotes enhanced disease. This is also supported by the

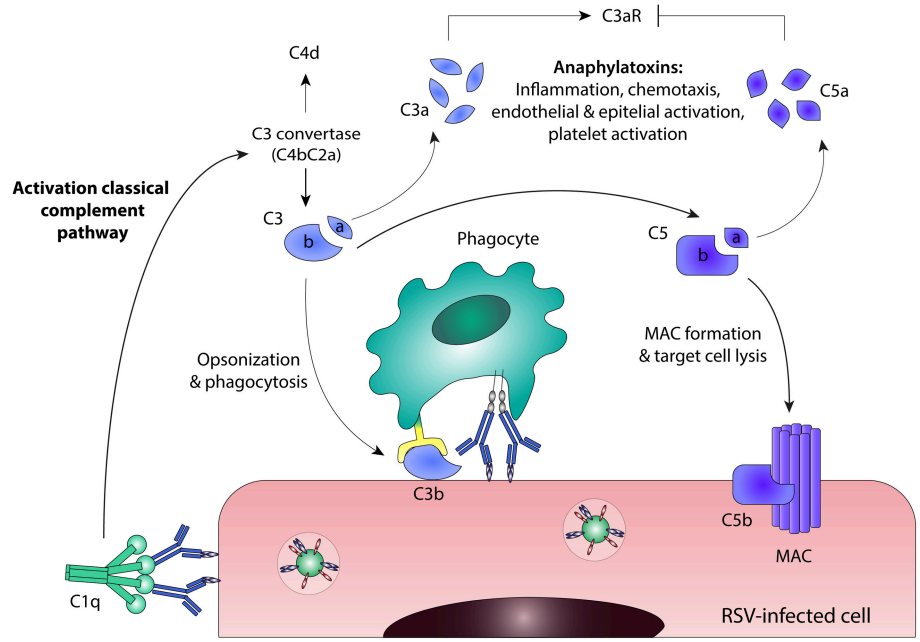

FIGURE 4 | Antibody-mediated complement activation. Binding of C1q to antibody-bound virus-infected cells leads to activation of the classical complement pathway. C3 convertase (C4bC2a) is formed and cleaves C3 into C3a and C3b. Active C4b can be cleaved into the enzymatically inactive form C4d, which serves as a marker for complement activation. Further downstream in the classical complement pathway, C5 is cleaved into C5a and C5b. C3a and C5a are anaphylatoxins that stimulate a pro-inflammatory environment, although they act in different ways: C3a induces C3aR signaling, whereas C5a inhibits C3aR expression. C3b binds to pathogens and infected cells, leading to phagocytosis through complement receptors found on immune cells. The terminal complement components will assemble into the membrane attack complex (MAC), resulting in direct lysis of the infected cell. C3aR, C3a Receptor; MAC, membrane attack complex; RSV, respiratory syncytial virus.

limited data available on complement activation during vaccine-enhanced disease in infants. Lung sections of the two children who died of vaccine-enhanced disease had extensive deposition of complement cleavage product C4d, which serves as a stable marker for complement activation (22). The presence of C4d provides evidence for complement activation during vaccine-enhanced disease in infants, but it remains to be determined whether there is a causal relation between complement activation and vaccine-enhanced disease. Finally, mouse studies point to the involvement of complement components in the development of AHR and asthma upon RSV infection (94, 95). Taken together, the complement system seems to be important in antibody-dependent protection *in vivo*, but it also potentially contributes to (vaccine-enhanced) disease and asthma, suggesting a dual role in RSV infection that requires further investigation.

ANTIBODY-MEDIATED IMMUNOMODULATION

Besides the well-defined classical Fc-mediated effector functions (ADCC, ADCP, CDC), immune complexes can also promote immune cell maturation and activation, leading to a wide range of effector activities and production of pro-inflammatory and immunomodulatory mediators (a limited overview is depicted in **Figure 5**). Some of these pro-inflammatory cytokine responses correlate with protection as has been shown for influenza (62) and HIV (96). The importance of FcγRs in this process has been shown by the use of FcγR-deficient mice [as extensively reviewed in (97)]. In contrast to the pro-inflammatory responses caused by immune complexes, injection with intravenous immunoglobulin (IVIg) can induce an anti-inflammatory state. It is proposed that this anti-inflammatory effect is partly due to the presence of sialylated antibodies in IVIg, which induce expression of FcγRIIB (the only inhibitory FcγR) and thereby dampen the inflammatory response (98).

Immune complexes can also regulate cellular maturation and activation. The balance between inhibitory and activating FcγR interactions is crucial in regulating B cell IgG responses (99–101), and skewing APC maturation and antigen presentation (102–105), which can modulate T cell activation. Immune complexes have also been shown to bias the macrophage immune response toward a Th2-like phenotype (106).

Antibody-Mediated Immunomodulation in RSV Infection

RSV-antibody complexes can lead to activation of phagocytes either directly or after phagocytosis, resulting in the production of reactive oxygen species (ROS), thromboxane, (pro-inflammatory) cytokines, and chemokines (72, 73, 107), which may contribute to viral clearance. However, these mediators can also have immunopathological effects, including tissue damage, platelet aggregation, and bronchoconstriction. Given that neutrophils are the predominant airway leukocytes

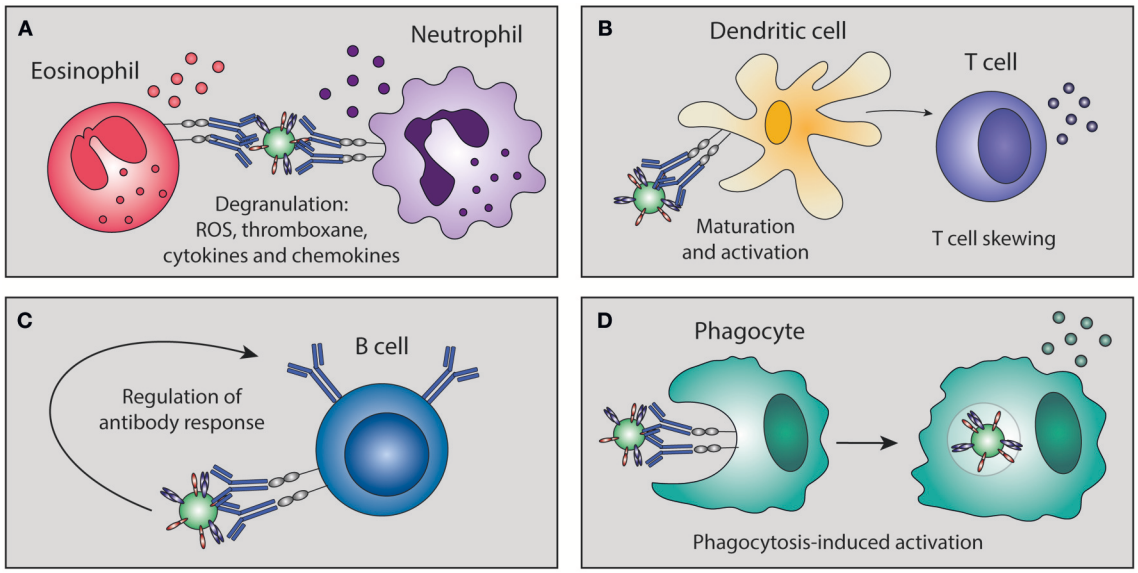

FIGURE 5 | Antibody-mediated immunomodulation. Immune complexes can skew immune cell maturation and activation of granulocytes, dendritic cells, T cells, B cells, and phagocytes. This immunomodulation can lead to **(A)** degranulation, **(B)** skewing of T cell responses, **(C)** regulation of B cell antibody responses, and **(D)** phagocytosis-induced secretion of immunomodulatory mediators. ROS, reactive oxygen species.

present in RSV-infected infants, their activation is suggested to be involved in the induction of severe RSV disease (108). Interestingly, in contrast to RSV immune complexes, it has been reported that RSV alone does not lead to ROS production by granulocytes (107) and can even inhibit this process (73, 109). It has been suggested that anti-RSV G mAbs are less potent inducers of ROS and cytokine production than anti-RSV F mAbs (73), but this was based on experiments with only two RSV-specific antibodies. Notably, differences in the capacity to induce a response may not be due to antigen-specificity *per se* but rather due to epitope localization, as described in the paragraph "Important epitopes in RSV infection."

Excessive eosinophilic activation has been suggested to play a role in the immunopathology of FI-RSV-induced disease in mice (22). Whether the non-neutralizing antibodies induced by the FI-RSV vaccine play a role in this activation remains unknown. *In vitro* studies have shown that eosinophils can phagocytose RSV-antibody complexes, leading to degranulation (74). The use of heat-inactivated serum abolished this effect, indicating complement involvement.

Besides an immunomodulatory effect on granulocytes, RSV-antibody complexes can also affect T cell responses. Kruijsen et al. show in an *in vivo* mouse model that IFN-γ secretion by CD4(+) T cells is increased in the presence of RSV immune serum (92). This increase is dependent on both FcγRs and the complement system. Additional *in vitro* experiments indicate that both anti-RSV G, as well as anti-RSV F antibodies can induce this enhanced CD4(+) T cell response, whereas CD8(+) T cells are only activated by the presence of anti-RSV G antibodies. Another *in vitro* study found that DCs primed with complexes composed of RSV and F-specific antibodies displayed an impaired capacity to activate CD8(+) and CD4(+) T cells (110).

RSV-antibody complexes also contribute to antibody-mediated immunomodulation through the induction or inhibition of cytokine and chemokine production in PBMCs. In an *in vitro* study, RSV-antibody complexes inhibited IFN-α production in PBMCs, whereas these complexes enhanced IFN-α production of PBMCs in the absence of CD14(+) cells (111). Another *in vitro* study showed that, compared to RSV alone, RSV immune complexes induce increased IFN-α, IFN-γ, CXCL10, and CXCL11 production in monocytes (112). In infant PBMCs, only CXCL10 production was significantly enhanced. CXCL10 can mediate a neutrophil-dependent excessive pulmonary inflammation (113), which could contribute to RSV pathogenesis. This indicates that immune complexes can potentially also activate neutrophils indirectly, through the induction of chemokines and cytokines in PBMCs. Altogether, these studies show that immune complexes are able to skew the RSV-specific immune response in multiple ways, but more research is needed to clarify the exact contribution of antibody-mediated immunomodulation to protection and disease during RSV infection.

ANTIBODY-DEPENDENT ENHANCEMENT (ADE) OF INFECTION

ADE refers to a phenomenon in which virus-specific antibodies promote, rather than inhibit, infection and/or disease. In ADE of infection, also known as extrinsic ADE (114), the number of virus-infected cells is increased in the

presence of (natural or monoclonal) antibodies that are non-neutralizing or present in sub-neutralizing concentrations. ADE of infection requires the presence of FcγRs on target cells and is an efficient *in vitro* tool to assess Fc-FcγR interactions. However, while ADE of infection has been observed for many viruses *in vitro* [as extensively reviewed in (115)], its significance *in vivo* remains uncertain. A schematic representation of ADE of infection is depicted in **Figure 6**.

ADE of RSV Infection

ADE of RSV infection has been demonstrated *in vitro* for both mAbs and RSV-immune serum in monocytic cell lines, PBMCs, neonatal, and adult NK cells, and primary mouse and cotton rat immune cells (110, 116–120). However, whether the ADE of infection observed *in vitro* is related to *in vivo* disease outcome is doubtful. No correlation has been found between disease severity in infants and the capacity of serum antibodies to induce ADE of RSV infection *in vitro* (119). Furthermore, ADE of infection has never been demonstrated *in vivo*. However, it must be noted that this has never been assessed during FI-RSV vaccine-enhanced disease.

ANTIBODY-DEPENDENT ENHANCEMENT (ADE) OF DISEASE

ADE of disease, or instrinsic ADE (114), refers to a process in which the presence of pathogen-specific antibodies contributes to disease severity. For example, immune complexes might bind to FcγR-expressing immune cells, modulating the immune response, and subsequently leading to enhanced inflammation. ADE of disease has been a presumed cause of severe disease following various viral infections and vaccinations (37, 114, 121–123). However, the underlying mechanisms are largely unknown and *in vivo* data supporting these claims are often lacking. However, for dengue virus infection some first clues to unravel the mechanism underlying ADE of disease have recently been published. Wang et al. have been able to show a correlation between FcγRIIIA binding capacity of dengue virus antibodies and disease severity *in vivo* (21). The dengue-specific antibodies are thought to cross-react with platelet antigens and induce thrombocytopenia. Suggested underlying mechanisms are FcγR-mediated platelet activation, phagocytosis, or ADCC, but further investigation is needed to confirm these hypotheses. In addition, Katzelnick et al. have shown that high dengue-specific antibody titers correlate with protection, whereas intermediate antibody titers correlate with severe dengue disease (124). Although low or no antibody titers are not protective, they do not enhance disease. It is possible that RSV-specific antibodies show a similar pattern, as illustrated schematically in **Figure 7**.

ADE of RSV Disease

Although *in vitro* ADE of infection does not seem to be a determinant for severe RSV disease (119), other antibody-mediated mechanisms could be involved, as has recently been shown for dengue virus infection (21). Many animal studies on RSV infection highlight the role of an excessive immune response in FI-RSV vaccine-enhanced disease. It is likely that poorly-neutralizing vaccine-induced antibodies play a role in the development of FI-RSV vaccine-enhanced disease (22–24), although it remains uncertain which Fc-mediated effector functions are involved.

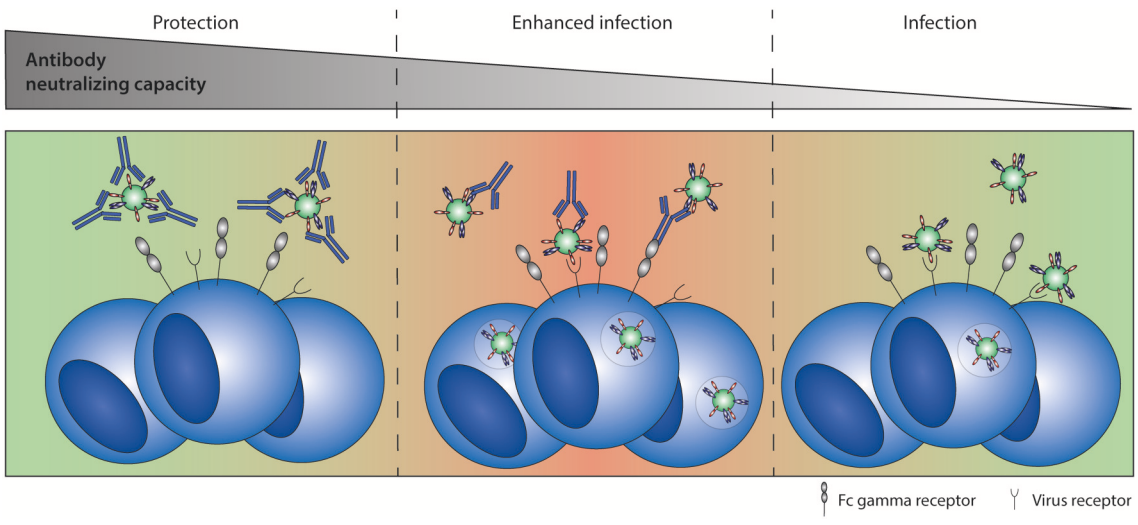

FIGURE 6 | Antibody-dependent enhancement (ADE) of infection. ADE of infection has been shown *in vitro* for multiple viruses, including RSV. High antibody titers neutralize the virus completely. Sub-neutralizing antibody titers form immune complexes that can interact with both the virus receptor and Fc gamma receptors, leading to enhanced infection levels compared to infection in the absence of antibodies.

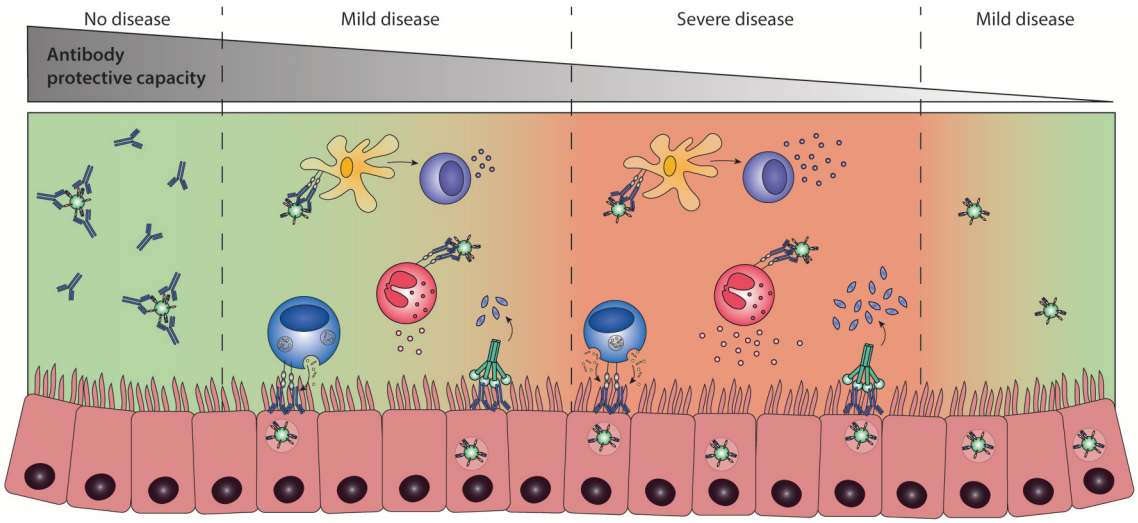

FIGURE 7 | Antibody-dependent enhancement (ADE) of disease. ADE of disease refers to a process in which the presence of pathogen-specific antibodies contributes to disease severity. Highly neutralizing antibodies result in sterile immunity, preventing infection and disease (left panel). The presence of low levels of protective antibodies allows for viral replication and leads to an Fc-mediated immune response that can either contribute to protection (second panel) or potentially lead to more severe disease (third panel) compared to infection in the absence of antibodies (right panel). It is currently unknown whether Fc-mediated effector functions can lead to severe disease and which immunological mechanisms determine the difference between protective or enhancing Fc-mediated responses.

Little is known on the involvement of (maternal) antibodies in the development of severe disease after natural RSV infection. Severe RSV infections are most frequently seen in the first 6 months of life when infants have circulating maternal RSV-specific antibodies (2). This suggests that RSV-specific antibodies may contribute to the induction of severe RSV disease. Results from animal studies with Fab fragments and FcγR-knockout mice indeed show the involvement of antibody-mediated effector functions both in protection against viral replication (58, 60, 61) and in promoting inflammation (92).

Some studies have reported enhanced RSV disease to occur in the presence of waning immunity. Murphy et al. reported enhanced pulmonary pathology 3 months after immunization with a RSV F glycoprotein vaccine (125), which was not seen 1 week after immunization (126). In a follow-up study, enhanced lung pathology was observed upon immunization with low doses of recombinant F protein, mimicking waning immunity (127). Interestingly, the enhanced disease was independent of the presence of a Th1- or Th2-biasing adjuvant.

Taken together, there are clear indications suggesting that Fc-mediated antibody effector functions may contribute to severe RSV disease. Complement activation has been linked to vaccine-enhanced disease and asthma, and may therefore also be involved in severe RSV disease upon natural infection. In addition, the immunomodulatory effects of immune complexes can lead to a pro-inflammatory environment, which is thought to be the underlying cause of RSV-mediated pathology. However, more research on the involvement of individual Fc-mediated effector functions in disease outcome following RSV infection is needed.

REGULATION OF FC-MEDIATED EFFECTOR FUNCTIONS

Fc-mediated antibody effector functions play an important role in shaping the immune response and their active regulation is crucial to prevent excessive immune activation. A number of determinants have been found to influence Fc-mediated effector functions on both the cellular and antibody side of the Fc-Fc receptor (FcR) interaction. Important antibody characteristics are the isotype, subclass, glycosylation pattern, and antigen specificity, while important cellular determinants are the epitope position relative to the target cell membrane and FcR expression and polymorphisms on the effector cell, which together determine the capacity of the antibody to interact with specific FcRs. Most antibodies are not specifically eliciting a single effector function, and therefore the combination of all these characteristics determines the outcome of the various Fc-FcR interactions and the interaction with the complement system.

ANTIBODY ISOTYPE AND SUBCLASS

Antibodies consist of two functional domains: the variable antigen-binding fragment (Fab) and the constant fragment (Fc) that interacts with FcRs and C1q. The isotype of the Fc domain (IgA, IgD, IgE, IgG, and IgM) represents the major determinant of Fc-mediated effector functions. Of these isotypes, IgG is the most important when it comes to Fc-mediated effector functions, as this is the only isotype known to interact with the widely expressed FcγRs. Whereas, the majority of antibodies in serum are of the IgG subtype, IgA is the major isotype present in mucosal secretions. This isotype interacts with its specific

receptor FcαRI, which is present on neutrophils, eosinophils, monocytes, and macrophages [extensively reviewed in (128)]. Activation of FcαRI by IgA-opsonized pathogens can induce ADCC, phagocytosis, degranulation, and cytokine release. Other important isotypes to briefly mention are IgM, which is a potent complement activator (76), and IgE, which has been linked to various allergic diseases.

In humans, four different IgG subclasses (IgG1-IgG4) are known. These subclasses differ in amino acid sequence, which influences their capacity to interact with certain classes of FcγRs and complement components as depicted in **Table 1**. Production of different isotypes and subclasses is tightly regulated and dependent on differentiation of the B cell, which can be influenced by cytokines and interactions with pattern-recognition receptors. The response to protein antigens usually involves T cell help and induces class switching to IgG1 or IgG3, whereas polysaccharide antigens induce class switching to IgG2 in the absence of T cell help (132). Viral infections, including RSV infections, mostly induce IgG1 and IgG3 antibody responses (133–135).

IgG1 and IgG3 have the highest affinity for FcγRs and are potent activators of complement, ADCC and phagocytosis (129, 136, 137). IgG3 is the subclass with the highest potential to activate both FcγRs and complement, but due to its short half-life the preferred subclass for therapeutic cytotoxic activity is IgG1 (138). In contrast, receptor-blocking antibodies are often of the IgG2 or IgG4 subclass to avoid Fc-mediated cytotoxic side effects (139). Induction of specific subclasses can have major effects on the outcome of vaccine trials as has been shown for the HIV RV144 and VAX003 vaccines. RV144 recipients produced highly functional IgG3 antibodies that provided 31.2% efficacy, whereas VAX003 recipients elicited a monofunctional IgG4 antibody response that was not protective at all (140).

Antibody Isotype and Subclass in RSV Infection

Severe RSV-mediated disease is most prevalent in infants below 6 months of age. These children mainly rely on maternally-derived IgG for protection against infectious diseases, but the correlation between serum IgG levels and protection against RSV disease is poor (7, 9–11). A recent study by Habibi et al. found that mucosal IgA titers are a better predictor of susceptibility to RSV infection than serum IgG levels in an adult challenge model (141). In addition, they showed a hampered IgA memory B cell response to RSV, which may explain the life-long susceptibility to repeated RSV infections. In accordance with these results, lower levels of nasal IgA were found in naturally RSV-infected adults compared to healthy controls (142). These findings highlight the importance of mucosal IgA in protection against RSV infection. However, it is questionable whether IgA plays a role in protection or disease during primary infection. IgA antibodies to RSV are only found in secretions after 4 months of age, confirming they are synthesized as a result of (primary) infection (143). RSV-specific IgA has been shown to induce antibody-mediated effector functions. Although palivizumab-IgA demonstrated slightly higher lysis of RSV-infected HEp2 cells by neutrophils (but not monocytes) *in vitro,* there was a somewhat decreased efficacy *in vivo* compared to palivizumab-IgG (144). Additional experiments with FcαRI transgenic mice suggest that IgA-mediated protection is Fc receptor-independent. No further research with RSV-IgA immune complexes has been published to date and therefore their role in protection or disease remains to be investigated.

Another interesting isotype is IgE, as the results from multiple studies suggest the involvement of this isotype in the development of RSV-mediated bronchiolitis and wheezing (145–148). In a mouse model, RSV-specific IgE has been shown to enhance airway hyperresponsiveness (149). Since all infants produce IgE in response to RSV infection (150), it is thought that the height and duration of the IgE response are important for the induction of subsequent immunopathology (148, 151, 152). Mast cells abundantly express the IgE-specific Fc receptor (FcεRI) and were shown to play an important role in IgE-induced airway hyperresponsiveness in an RSV reinfection mouse model (149).

In addition to studies on isotypes, extensive studies have been performed on the presence of IgG subclasses during RSV infection. Wagner et al. have performed some early studies into the antibody subclass response to the RSV F and G glycoproteins in both infants and adults (133, 153, 154). Primary RSV infections predominantly gave rise to IgG1 and IgG3 antibodies, whereas subsequent infection only led to an increase in IgG1 and IgG2 titers (133). RSV infection led to a poor IgG4 antibody response in all subjects. RSV F protein was the most immunogenic, leading to higher antibody titers compared to the RSV G protein (154). The IgG1/IgG2 ratio of antibody titers to the RSV F protein was fourfold higher than to the RSV G protein after the first three RSV infections in infants. This difference was thought to be due to the extensive glycosylation of the G

TABLE 1 | Binding capacity and functionality of IgG subclasses.

Subclass	Serum abundance (%)	FcγRI	FcγRIIa	FcγRIIb	FcγRIIIa	FcγRIIIb	C1q	Effector functions
IgG1	60	+++	+++	+	++	+++	++	ADCC, ADCP, CDC
IgG2	32	–	++	–	–	–	+	
IgG3	4	++++	++++	++	++++	++++	+++	ADCC, ADCP, CDC
IgG4	4	++	++	+	–	–	–	

(129–131). ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; CDC, complement-dependent cytotoxicity; FcγR, Fc gamma receptor; IgG, immunoglobulin G.

protein, resulting in IgG2 antibodies. IgG1 and IgG3 are the most potent FcγR-binding subclasses. This suggests that the majority of anti-RSV F antibodies are effective inducers of Fc-mediated effector functions, in contrast to the IgG2 subset of anti-RSV G antibodies. Experimental RSV infection in adults showed similar subclass responses to RSV F and G protein (153). A recent study confirms the findings of Wagner et al. showing a strong IgG3 response in infants younger than 4 months, despite the presence of high levels of maternal antibodies (155). A rise in RSV-specific IgG1 and IgG2 was only observed in infants older than 7 months.

Besides human studies, several mouse studies have been performed to investigate the subclass antibody response. Although some homology between mouse and human IgG subclasses has been found, it is unclear whether they induce the same downstream immune responses. In mice, neonatal IgG responses to RSV infection are significantly skewed toward mIgG1 (homologous to human IgG4), indicating a Th2 bias (156), whereas primary infection in adult mice leads to a balanced mIgG2a/mIgG1 response (homologous to human IgG1/IgG4) (157). Compared to wild-type RSV infection, immunization with inactivated or non-replicative RSV led to a low mIgG2a/mIgG1 ratio (24, 158). The largest proportion of antibodies directed at the RSV-F protein was mIgG2 (homologous to human IgG1), whereas the G protein response had a significantly lower proportion of mIgG2 (158). These results indicate that both the age of the host and the antigens determine the subclass response. However, it is remarkable that RSV infection leads to a poor IgG4 antibody response in humans, but to a high mIgG1 (homolog of human IgG4) response in (neonatal) mice. Thus, caution is warranted in the translation between human and mouse antibody studies.

Although extensive studies have been performed on the presence of specific subclasses, evidence on the role of these different subclasses during RSV infection is limited. One study describes a direct comparison between the functionality of palivizumab-IgG1 and -IgG2 (159). The neutralizing potential of both subclasses was comparable. However, the IgG2 antibody showed negligible binding to murine FcγRs and human C1q, resulting in less efficacy *in vivo* as measured by increased viral lung titers in challenged cotton rats (159). This finding underscores the protective potential of IgG1-mediated effector functions during RSV infection.

ANTIBODY GLYCOSYLATION

Glycosylation of the antibody Fc domain is another important regulator of Fc-mediated effector functions. Each IgG molecule contains a highly conserved asparagine at position 297 (N297) that functions as a glycosylation site that can harbor a variety of glycans, consisting of varying combinations of mannose, (bisecting) N-acetylglycosamine (GlcNAc), fucose, galactose, and sialic acid (**Figure 8**). The complete absence of this glycan leads to a conformational state that is non-permissive for FcγR or complement binding, thereby impairing Fc-mediated antibody effector functions.

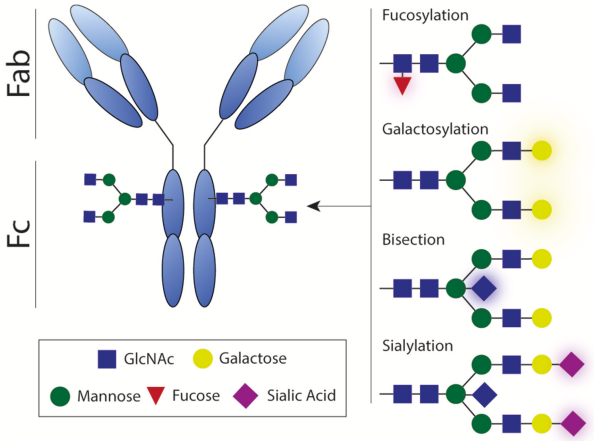

FIGURE 8 | Antibody glycosylation. Each IgG molecule contains a glycosylation site that can harbor a variety of glycans, consisting of varying combinations of mannose, (bisecting) N-acetylglycosamine, fucose, galactose, and sialic acid. Antibody effector activity is substantially impaired in absence of this glycan. Fab, antigen-binding fragment; Fc, crystallizable fragment; GlcNAc, N-acetylglycosamine.

Afucosylation has the most straightforward influence on antibody effector functions. The absence of the core fucose on the Fc-glycan directly boosts ADCC activity by enhancing the interaction with FcγRIIIA (**Figure 2**) (160–162). Interestingly, afucosylated mAbs have shown to be more protective against various infectious agents (163, 164) and more efficacious in cancer therapy (165, 166). However, increased levels of afucosylation are also associated with severe disease during secondary dengue infection (21).

Another biologically important modification to the Fc glycan is sialylation. The presence of sialic acid inhibits FcγR binding and is reported to be partly responsible for the anti-inflammatory activity of IVIg (98, 167). Besides having anti-inflammatory properties, sialylated Fc glycans have also been shown to induce the production of high-affinity broadly neutralizing antibodies against influenza virus (101).

Besides its effect on Fc receptor interactions, Fc glycosylation also affects complement C1q binding to immune complexes. A recent study shows that elevated galactosylation and sialylation increase C1q-binding, downstream complement deposition, and complement dependent cytotoxicity (168). In contrast, agalactosylated IgG has also been suggested to elicit enhanced complement activation, considering its role in several autoimmune diseases (169). These findings suggest that activation of complement potentially contributes to pathogen clearance, but can also contribute to inflammation in autoimmune disease, highlighting the dual role of complement.

Fc glycosylation is subject to active regulatory mechanisms that control the composition of the glycan structure. Major changes in glycosylation occur during pregnancy (170, 171), upon vaccination (101, 172), and during certain viral infections (101). Therefore, insight in the glycosylation pattern during RSV infection and disease may provide valuable clues on the cause of severe RSV disease.

Antibody Glycosylation in RSV Infection

To our knowledge, only one group has investigated the effect of glycosylation in the response toward RSV infection. Hiatt et al. compared the original Palivizumab mAb with an afucosylated and agalactosylated plant-produced glycovariant (G0) in different *in vitro* and *in vivo* assays (159). The G0 glycovariant showed enhanced binding to murine FcγRs but less binding to human C1q compared to the parental Palivizumab, whereas neutralization capacity was comparable. The *in vivo* protective capacity of the G0 glycovariant was improved compared to the original, as evidenced by decreased pulmonary viral titers. In conclusion, this study suggests that the influence of Fc-glycosylation may be important in the protective capacity of RSV-specific antibodies but this needs to be studied in more detail for other mAbs and virus- and vaccine-induced antibodies.

EPITOPE POSITION

Next to antibody structure and glycosylation, the location of the antibody-bound epitope with respect to the membrane of the infected cell has been shown to be pivotal in determining Fc-mediated effector functions. Since the use of mAbs, it has been noticed that different mAbs binding the same target protein can elicit different effector mechanisms (173). Antibodies binding to epitopes closer to the membrane (membrane proximal epitopes) mediate ADCC and CDC activity more efficiently, whereas antibodies that target membrane distal epitopes are often highly neutralizing and efficient ADCP-inducers (13, 174–176). More specifically, recent research suggests that ADCP is most efficiently triggered when antibodies bind within 10 nm from the cell surface (177), indicating that the optimal ADCP-inducing epitope is located neither too close, nor too far away from the cell membrane. These studies suggest that besides the common need for particular Fc-FcγR interactions, there are fundamental differences in the activation requirements of specific Fc-mediated effector functions. For CDC activity, stabilization of complement components on the cell surface is essential. This would require a short distance from epitope to cell membrane. During ADCC, the formation of an immune synapse is essential. This small synapse can only be formed when the NK cells engage antibodies bound in close proximity to the cell membrane, explaining the need for membrane proximal epitopes (175).

Important Epitopes in RSV Infection

Neutralization of RSV is mainly established by antibodies against the RSV F and RSV G protein (178). Antibodies against the SH and N protein have also been described (179, 180) and although these antibodies are not involved in neutralization, they may have other important (Fc-mediated) functions (181). Capella et al. recently showed that antibodies against the pre-F protein were the most prevalent RSV-specific serum antibodies in infants below 2 years of age (182). Both serum IgG levels against anti-RSV pre-F and G correlated with disease severity in this study.

Various antigenic sites (named Ø and I-VIII) have been described for the two conformational states of the RSV F protein (183, 184). Pre-F-specific antibodies are better neutralizers than post-F-specific antibodies (185). However, not all pre-fusion F antibodies have similar neutralizing activity (183). The most potent neutralizing antibodies bind to distal epitopes, suggesting that the neutralizing potential of anti-RSV F antibodies not only relies on the conformation of F on which the epitope is present (e.g., pre- vs. post-F), but may also depend on the location of the epitope relative to the viral or cellular membrane. As described above, the proximity to the membrane determines the efficiency of Fc-mediated effector functions (13, 174, 175). This suggests that potently-neutralizing antibodies, binding to distal epitopes, may also be efficient inducers of ADCP. Antibodies binding to proximal epitopes are generally less neutralizing, but may be more potent in inducing ADCC and CDC.

The most important antigenic site for the RSV G protein is the central conserved domain (CCD). Despite the high variability of RSV G, antibodies against the CCD are broadly neutralizing against both RSV A and B strains (186). The G protein CCD binds to the CX3CR1 receptor, leading to attachment of RSV to its target cells (187). Antibodies against this receptor-binding domain efficiently neutralize RSV infection and decrease pathogenesis by binding soluble G protein, an immune evasion protein secreted by RSV-infected cells (56, 57, 188). Soluble G protein has been found to inhibit Fc-mediated antiviral effects of macrophages and complement (75), and to modulate trafficking of CX3CR1(+) cells (189). Next to the important roles mentioned above, antibodies against the CCD domain are also able to induce Fc-mediated effector functions like ADCP and ADCC (56).

Taken together, not only the antigen but also the epitope determines the efficacy of antibodies. Interestingly, evidence suggests that targeted epitopes may differ between infants and adults (190), but the effect of these changes on the efficacy of the antibody response is unknown. Further research may uncover the relation between antigenic site and effector functions against RSV infection, and thereby reveal preferred antibody-binding sites for protection against RSV disease.

FCγR EXPRESSION AND POLYMORPHISMS

Another regulator of Fc-mediated effector functions is the expression pattern and polymorphisms of FcγRs. The majority of leukocytes express more than one FcγR type with varying downstream signaling activities. The level and variety of FcγR expression is tightly regulated during leukocyte development and can be modulated by certain mediators present during infection, inflammation, or even vaccination (103, 191). As stated before, the balance between inhibitory and activating FcγR interactions is crucial in regulating B cell IgG responses (99–101) and skewing APC maturation and antigen presentation (102–105). Additionally, co-engagement and signaling through other receptors such as TLRs may influence the activation threshold (192). Altogether, this points out the importance of receptor expression patterns on effector cells.

Besides variation in FcγR expression patterns, single nucleotide polymorphisms (SNPs) in FcγRs occur. Although

many SNPs have been identified, only few have been shown to impact receptor function (193). One of the functional SNPs has been identified in FcγRIIa. Only the R131H allelic variant of this receptor is capable of interacting with IgG2, enabling efficient phagocytosis (194, 195). Another SNP affecting binding affinity has been characterized for FcγRIII, which has two co-dominantly expressed allotypes: V158 and F158. The presence of a valine residue at position 158 increases the affinity for IgG1 and IgG3, augmenting for example NK cell activity (196, 197).

FcγR Expression and Polymorphisms in RSV Infection

Different FcγRs can have opposing effects on the immune response, as has also been shown for RSV. In FcγR$^{-/-}$ mouse models, Gomez et al. demonstrate that murine FcγRIII (homolog of human FcγRIIA) contributes to viral replication and airway inflammation, whereas murine FcγRIIb (homolog of human FcγRIIb) has a protective effect as was shown by a decrease in viral titers (110). *In vitro,* RSV infection has been found to increase mFcγRII and mFcγRIII expression in murine macrophage cultures which subsequently showed enhanced phagocytosis (198).

Although the clinical relevance of FcγR SNPs has been studied intensively for auto-immune diseases (199), cancer treatment (200) and various viral infections (201–204), there is no data on the role of these polymorphisms in RSV infection or disease. In a genetic association study, performed to identify genes that are involved in RSV susceptibility, a SNP in FCER1A was found (205). This polymorphism had previously been found to be associated with altered FcεRI expression levels and allergic disease, supporting the involvement of IgE in RSV-mediated disease.

IMPLICATIONS FOR VACCINE AND mAb DEVELOPMENT

Currently there are no market-approved vaccines or antivirals available against RSV. The only available treatment is the administration of a prophylactic F protein-specific mAb (Palivizumab) to reduce hospitalization in high-risk infants (206). However, the use of Palivizumab is restricted and its cost-effectiveness is often discussed (207). Improved mAbs with higher efficacy rates are thus highly needed and many research efforts are ongoing to develop these mAbs. A recent clinical trial with a pre-F-specific mAb (Suptavamab) failed to demonstrate efficacy in pre-term infants although the mAb was superior to Palivizumab in neutralization tests *in vitro* and in reducing viral load in the cotton rat model (208) (press release Regeneron, August 14, 2017). The failure of this highly neutralizing mAb indicates that protection against RSV-mediated disease, which is known to be immunopathological in nature, depends on more than just neutralization of the virus.

In addition to efforts made to develop improved therapeutic mAbs, there is an extensive pipeline of vaccines that are currently being tested in different phases of clinical development (https://www.path.org/resources/rsv-vaccine-and-mab-snapshot/). The development of vaccines is of great importance, especially for developing countries where RSV-related mortality is high and mAb therapy is inaccessible due to high costs. The majority of vaccine candidates currently in clinical trials are designed to induce systemic IgG, mostly against the RSV F protein. The results of the pre-F-specific Suptuvamab and the recent failures of two F-specific vaccine candidates tested in elderly, imply that a broader and more polyfunctional immune response may be needed to confer protection against RSV-mediated disease (209, 210) (press release Novavax, September 15, 2016).

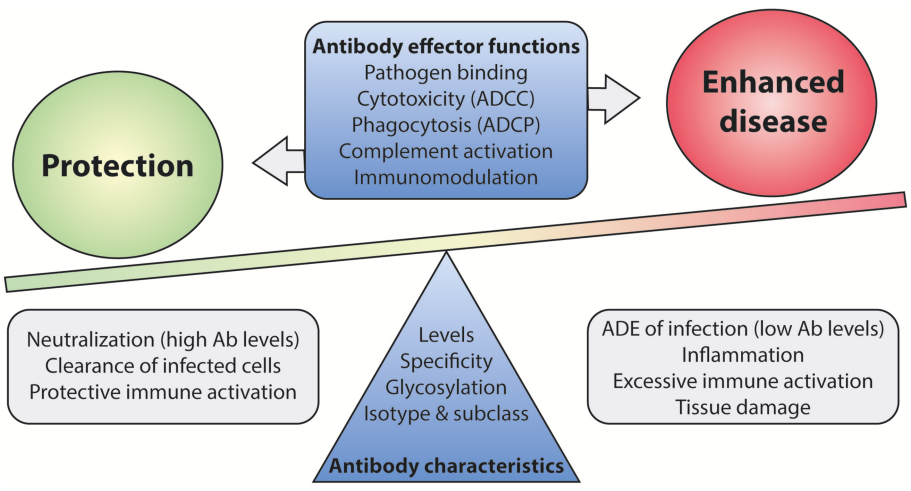

FIGURE 9 | The balance between Fc-mediated protection and enhanced disease. Antibody effector functions, regulated by differences in antibody characteristics, are suspected to play a role in disease outcome upon RSV infection. Immune activation by Fc-mediated effector functions is likely needed for efficient viral clearance. However, excessive activation may lead to inflammation and tissue damage. A balanced and contained immune response is most likely the key to protection upon infection. Ab, antibody; ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; ADE, antibody-dependent enhancement.

To this date, no accurate correlate of protection has been defined for RSV infection as virus-specific antibody levels or neutralization titers do not seem of use in this respect. The lack of a well-defined correlate of protection complicates the development of new vaccines, as efficacy now has to be demonstrated in expensive large-scale clinical trials. Mounting evidence suggests that antibody effector functions beyond neutralization can contribute to both protection and disease (110, 159, 181, 211). A balanced activation of different Fc-mediated effector functions is key to prevent excessive inflammation and tissue damage (**Figure 9**). It will be of importance to implement assays that identify Fc-mediated effector functions of mAbs and vaccine-induced antibodies. Studies in FcγR-knockout mice have indicated the importance of Fc-FcγR interactions for protection against RSV infection (110, 181), but the testing of mAbs and vaccines demands high-throughput approaches. Systems serology captures a wide array of antibody characteristics and effector functions. It has proven effective in identifying antibody features that contribute to protection for various (viral) pathogens (19, 212, 213). Such an approach will provide detailed information on the characteristics that are required for a protective RSV antibody response.

The ability to generate an antibody profile that selectively binds particular epitopes and FcγRs is important to enable the induction of only the desired antibody effector functions. Recent developments now allow targeted modifications to mAbs that can lead to enhancement or inhibition of specific Fc-mediated antibody effector functions through glyco-engineering or the induction of specific antibody subclasses or isotypes (159). In the future, this might also be possible for vaccines.

One can conclude from the studies presented above that Fc-FcR interactions are an integral component of the immune response against RSV and should be considered in the rational design of next generation RSV-specific mAbs and vaccines. Only limited data is available on the effect of specific Fc-mediated antibody effector functions during RSV infection, but it is clear that these can be both beneficial and detrimental for protection against RSV infection and disease outcome. In the future, Fc-mediated effector functions might be harnessed to optimize the efficacy of RSV-specific mAbs and vaccine-induced antibodies. However, our current knowledge on the precise role of individual effector functions in RSV disease is too limited to rationally design such antibodies and vaccines. Therefore, until the individual contributions of Fc-mediated effector functions to protection and disease are unraveled, aiming to induce highly neutralizing antibodies seems the safest approach. These antibodies will need to halt the infection at the site of entry and thereby prevent excessive (antibody-mediated) immune activation. It remains to be seen whether complete neutralization can be achieved via the induction of serum IgG alone, or whether the induction of mucosal IgA is necessary for reliable neutralization activity. The many clinical trials that are currently ongoing with maternal and neonatal vaccine candidates will show whether these approaches indeed result in protection during the first, most vulnerable, months of life.

CONCLUDING REMARKS

Neutralizing antibody titers do not adequately correlate with protection against RSV disease. Interestingly, antibodies have additional Fc-mediated effector functions besides neutralization, but this area of research is currently underappreciated in the RSV field. With this review, we aim to encourage a paradigm shift from neutralization-based studies toward functional studies examining the precise role of Fc-mediated antibody effector functions in vaccine efficacy and RSV disease. We have evaluated the current literature on the effect of RSV-specific antibodies on NK cells, phagocytes, the complement system, cytokine production, and B- and T-cell skewing. Multiple *in vivo* studies using FcγR-knockout mice or modified RSV-specific antibodies indicate the importance of Fc-mediated effector functions in protection from RSV infection and disease (110, 159, 181, 211). In addition, Fc-mediated effector functions might have a role in ADE of RSV disease (22, 23). However, most studies into vaccine and mAb efficacy still only report antibody (neutralization) titers and disregard any Fc-mediated effector functions. The importance of these antibody effector functions has already been shown for multiple clinically important viral pathogens and is only starting to be explored for RSV. In our view, a better understanding of the broad range of effector mechanisms that are induced by RSV-specific antibodies will greatly contribute to the much-needed development and testing of next generation mAbs and vaccines against this virus.

AUTHOR CONTRIBUTIONS

EvE, WL, and PvK conceived the topic and scope of this review. EvE researched the literature and designed the figures. EvE and PvK drafted the first version of the manuscript. WL and GF critically reviewed the manuscript. All authors approved the final manuscript.

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Induction of Fc-Mediated Effector Functions Against a Stabilized Inner Domain of HIV-1 gp120 Designed to Selectively Harbor the A32 Epitope Region

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Recent clinical trials and studies using nonhuman primates (NHPs) suggest that antibody-mediated protection against HIV-1 will require α-HIV envelope humoral immunity beyond direct neutralization to include Fc-receptor (FcR) mediated effector functions such as antibody-dependent cellular cytotoxicity (ADCC). There is also strong evidence indicating that the most potent ADCC response in humans is directed toward transitional non-neutralizing epitopes associated with the gp41-interactive face of gp120, particularly those within the first and second constant (C1–C2) region (A32-like epitopes). These epitopes were shown to be major targets of ADCC responses during natural infection and have been implicated in vaccine-induced protective immunity. Here we describe the immunogenicity of ID2, an immunogen consisting of the inner domain of the clade A/E 93TH057 HIV-1 gp120 expressed independently of the outer domain (OD) and stabilized in the CD4-bound conformation to harbor conformational A32 region epitopes within a minimal structural unit of HIV-1 Env. ID2 induced A32-specific antibody responses in BALB/c mice when injected alone or in the presence of the adjuvants Alum or GLA-SE. Low α-ID2 titers were detected in mice immunized with ID2 alone whereas robust responses were observed with ID2 plus adjuvant, with the greatest ID2 and A32-specific titers observed in the GLA-SE group. Only sera from groups immunized in the presence of GLA-SE were capable of mediating significant ADCC using NKr cells sensitized with recombinant BaL gp120 as targets and human PBMCs as effectors. A neutralization response to a tier 2 virus was not observed. Altogether, our studies demonstrate that ID2 is highly immunogenic and elicits A32-specific ADCC responses in an animal host. The ID2 immunogen has significant translational value as it can be used in challenge studies to evaluate the role of non-neutralizing antibodies directed at the A32 subregion in HIV-1 protection.

Keywords: HIV envelope, ID (Inner domain) immunogen, ADCC (Antibody dependent cellular cytotoxicity), A32 epitope, Fc-mediated effector function

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INTRODUCTION

The design of immunogens which induce broadly protective antibody responses against human immunodeficiency virus type 1 (HIV-1) is a major goal of HIV-1 vaccine development. This goal is formidable as HIV-1 evades immune surveillance via a number of escape mechanisms (1–3). Over the last few years neutralizing humoral responses have been observed to overcome some of these obstacles and provide protection in a subpopulation of chronically infected individuals (1, 4–9). Despite the significant progress in identification and characterization of broadly neutralizing antibodies (bnAbs) there are still multiple, challenging obstacles in the design of a successful candidate immunogen which, when coupled with appropriate immunization strategies, can induce effective neutralizing responses *in vivo* (10). These challenges are primarily linked to the unusual structural features associated bnAbs; such as the long complementary determining region 3 (CDR H3) and the high level of somatic mutation of the variable (V) domain. The frequency of B cells for these unusual antibodies is very low and the time required for their full development from progenitors is remarkably long (11), making them very complex candidates for vaccine design.

By contrast, less is known about mechanisms of vaccine induced humoral responses that act solely through Fc-mediated effector functions, including antibody-dependent cell-mediated cytotoxicity (ADCC). Epitopes involved solely in Fc-mediated processes are usually exposed late during viral entry and are thus targeted by antibodies that lack direct neutralizing activity. One group of these potent ADCC targets constitute the CD4-inducible (CD4i) epitopes within the gp120 molecule, referred to as Cluster A epitopes (12–16). These epitopes become exposed on the target cell surface during viral entry after envelope trimers engage the host CD4 receptor and they persist on newly infected cell surfaces for extended periods of time (17–20), reviewed in (21–23). They are also expressed at the surface of infected cells, but only in cell populations that retain some levels of the CD4 receptor which is required for triggering envelope trimers on budding virions (15, 16, 23). We recently isolated and characterized, at the molecular level, the complexes of CD4-triggered gp120 with a number of monoclonal antibodies (mAbs) known to be capable of potent Fc-receptor mediated function from memory B cells of HIV-1 infected individuals that recognize the A32-like epitope within the Cluster A epitope region (14, 24, 25). Based on these studies, we mapped the A32 epitope into the highly conserved constant regions 1 and 2 (C1–C2) of the gp120 inner domain in the CD4-bound conformation. We also found that A32-like antibodies differ significantly from those involved in neutralization as they mostly possess moderate length CDR H3 loops and low degrees of V affinity maturation and therefore bypass the frequently observed somatic hypermutation hurdle in eliciting a protective antibody response (12, 26, 27). The high sequence conservation of the A32 epitope among different HIV isolates indicates the possibility that ADCC responses specific for this epitope region may be cross-reactive and multiple strains would therefore undergo limited immune escape. Indeed, the recent vaccination strategy tested in the RV144 vaccine trial partially confirmed these predictions. A32-like responses were induced with the RV144 vaccine and ADCC responses directed to the A32 epitope region were implicated in its protective effect (28). In the absence of IgA responses, ADCC correlated with a reduced infection risk (29, 30) with a very narrow array of antibody specificities involved in the protective effect. RV144 ADCC specificities included the linear epitopes in the V2 loop region (31) and the CD4-inducible conformational epitopes within the A32 region (32, 33), confirmed by blocking the plasma ADCC activity with the A32 Fab (32). Furthermore, most ADCC mAbs (19 of 23) isolated from vaccine recipients targeted multiple related but distinct conformational epitopes in the A32 region (31, 32). These antibodies displayed low levels of V_H chain somatic mutation (0.5–1.5%) and mediated cross-clade ADCC activity; clade B and CRF01 AE, as well as clade C, which was not represented in the vaccine (32); a canarypox ALVAC prime with the E.92TH023 gp120 membrane anchored insert and an AIDSVAX B/E gp120 boost.

Here we describe the immunogenicity of a gp120 sub domain immunogen, referred to as ID2, designed by our group to stimulate humoral responses involving solely FcR-effector mechanisms designed to elicit an ADCC response in the absence of a neutralizing response (34). ID2 consists of the inner domain (ID) of the clade A/E HIV-1 gp120 93TH057 isolate and was made to confer the minimal structural unit of gp120 stably presenting the non-neutralizing epitopes in the A32 region without any other known epitopes present (34). When injected into BALB/c mice, ID2 was able to elicit cross-clade A32-like antibody responses with ADCC activities against $gp120_{BaL}$ coated cells.

MATERIALS AND METHODS

ID2 Immunogen Expression and Purification

A HEK 293 cell line stably expressing the ID2 immunogen was generated using the plasmid previously used for transient protein production, as in (34). Freestyle 293 medium (Gibco) from cells grown for 6–7 days (8% CO2 at 37°C in shaker flasks rotating at 145 rpm) after inoculation (1×10^6 cells/ml) was collected and passed through a 0.45 μm filter. ID2 was purified from the media using an N5-i5 IgG affinity column, which was made by coupling N5-i5 IgG to protein A resin using the Pierce protein A IgG plus orientation kit (Thermo Fisher Sci.). Media was passed over the column after equilibration in phosphate buffered saline (PBS) pH 7.2. The column was washed with 5–10 column volumes of PBS pH 7.2 and ID2 protein eluted with 0.1 M glycine pH 3.0. Elution fractions were concentrated and dialyzed against PBS pH 7.2 prior to use in animal studies.

Immunization and Blood Collections

BALB/c mice were purchased from The Jackson Laboratory and housed in the animal facility managed by BIOQUAL's, Inc., Rockville, MD. The mice were cared for in accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) standards and all procedures involving animals were approved by the University

Committee on Use and Care of Animals (UCUCA) of BIOQUAL, Inc. 6–8 weeks old BALB/c mice (male and female, 6 animals per group) were immunized at week 0, 2, 4, and 8 via IP injections of 20 μg of ID2 protein in different adjuvants. The control group received ID2 immunogen in PBS and two adjuvants were also trialed; ID2 immunogen in Alum (2% aluminum hydroxide wet gel suspension, InvivoGen, Catalog # vac-alu-250), and ID2 immunogen in GLA-SE adjuvant (stable oil-in water emulsion containing TLR-4 agonist developed by Infectious Disease Research Institute, Catalog # IDRI-GLA-SE, known also under the name EM082). Serum samples were collected prior to immunization and 2 weeks after each immunization according to the scheme shown in **Figure 1**.

Detection of Serum Immunoglobulin Specific for ID2

The presence and titers of total IgG, IgG1, IgG2a, IgG2b or IgG2c, IgG3, IgA, and IgM antibodies, specific for ID2 recombinant protein in sera of immunized mice were determined by an Enzyme Linked Immunosorbent Assay (ELISA) using a 100-μL-per-well volume format. Blocking Buffer (Tris-buffered saline (TBS; 10 mM Tris and 100 mM NaCl; pH 8.0) with 5% no fat dry milk and 0.1% Nonidet P-40) was used as blocking solution and as diluting solution for sera and detecting Abs. TBS-T buffer (TBS with 0.1% Tween-20) was used as washing solution. ELISAs were performed as follows: ID2 recombinant protein (0.5 μg/ml) was adsorbed onto ELISA plates (Immunoblot 2HB Thermo, Milford, MA) overnight at 4°C. The plates were washed three times and incubated with 100 μl blocking buffer per well for 2 h at room temperature. Serially diluted sera, beginning at 1:100, were then added and allowed to react with the coated antigen for 2 h at 37°C. Sera was removed, the plates washed, and alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma cat#A3562), IgG1, IgG2a, IgG2b, IgG2c, and IgG3 (SouthernBiotech cat# 1071-04, 1081-04, 1091-04, 1078-04, 1103-04, respectively), IgA and IgM antibodies (SouthernBiotech cat #1040-04 and 1021-04, respectively) diluted 1:1000 in blocking buffer, were added followed by incubation for 1 h at 37°C. After removal of unbound antibody and washing, the Blue Phos Microwell Phosphatase Substrate System (KPL 50–88-00) was used as a substrate to quantitate bound antibody. After 15 min incubation at room temperature, the reaction was stopped using APstop Solution (KPL 50-89-00) and the optical density was read on a microplate reader (SpectraMax Paradigm Multi-Mode Detection Platform Molecular Devices) at 620 nm. The anti-ID2 antibody half-max binding serum titer was calculated using a Microsoft Excel iteration formula.

Competition ELISAs

To determine if the immunization with ID2 immunogen had induced Cluster A like serum Abs, sera from the terminal bleed were tested for their ability to compete with Cluster A mAbs (A32, N5-i5) for the binding to ID2 in an ELISA setting. Plates were coated o/n at 4°C with 0.5 μg/ml of ID2. Ten-fold serial dilutions of immune sera and pre-immunization sera were mixed 1:1 with biotinylated human CD4i anti-envelope mAb at a concentration correspondent to the half max binding concentrations for each tested mAb; 0.66 μg/mL of A32 and 0.2 μg/ml of N5-i5. The mixtures were then added to previously washed and blocked plates. As a control, a 10-fold dilution of unbiotinylated CD4i mAbs A32 and N5-i5, starting at 10 μg/mL, were tested in the same assay. Sera and Abs were prepared as 2x solutions. After 2 h incubation at r.t. assay wells were washed and incubated with avidin-AP (Invitrogen 1:1,000 dilution) and then with the Blue Phos Microwell Phosphatase Substrate System. Biotinylated-mAb binding was determined by measuring absorbance at 620 nm. Competition percentage was calculated using GraphPad Prism as follows: 0% inhibition was defined as the mean OD value of the lowest serial dilution of the pre-immunization sera ($1{:}10^6$) while 100% inhibition was defined as the mean OD value of the highest concentration tested for unbiotinylated mAbs A32 and N5-i5 (10 μg/ml).

Sera Reactivity With Denatured ID2 Protein

To assess if immunization with ID2 recombinant protein elicited serum antibodies recognizing conformational epitopes on ID2, pooled sera collected 2 weeks after the last immunization were incubated in solution o/n at 4°C with 1 μg/mL of denatured ID2. ID2 recombinant protein denaturation was performed as previously described in Moore et al. (35), with little modification. Briefly ID2 protein (final concentration 200 μg/ml) was mixed with 10 mM DTT, 0.1% SDS (Sigma-Aldrich), 0.1% FBS (GIBCO-Termo Fisher Scientific), and incubated at 70°C for 10 min. After incubation, the denatured protein was diluted 1:10 in TBS and stored at −20°C until used. The mixtures of denatured protein and pooled sera were then added to a plate coated with ID2. Pooled sera from each group were used as a control. Goat anti-mouse IgG Alkaline Phosphatase conjugated was used as secondary Ab. Plates were read at 620 nM after addition of Blue Phos Microwell Phosphatase Substrate System as previously described.

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

The ADCC activity of immunoglobulins present in mouse sera collected at week 10 were tested with the optimized rapid fluorometric antibody-dependent cellular cytotoxicity (RFADCC) assay (36). Briefly, EGFP-CEM-NKr-CCR5SNAP cells sensitized with recombinant BaL gp120 were used as targets and human PBMCs were utilized as effectors. Sera were serially diluted three-fold starting at 1:100 through 1:1,968,300 together with control mAbs (N5-i5-positive and Synagis-negative controls). After 2 h of incubation the samples were fixed and collected (at approximately 20,000 events per sample) on a Fortessa Special Order instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). ADCC activity (shown as % cytotoxicity) was defined as the percentage of EGFP-CEM-NKr-CCR5-SNAP target cells that lost GFP staining but retained the CCR5-SNAP tag staining. The results represent the average of the samples tested in triplicate and normalized to the N5-i5 positive control. Max lysis was defined as the maximum percent lysis at any sera concentration. Ec_{50} was determined using a GraphPad prism formula of Log(agonist) vs. Normalized response for a variable slope.

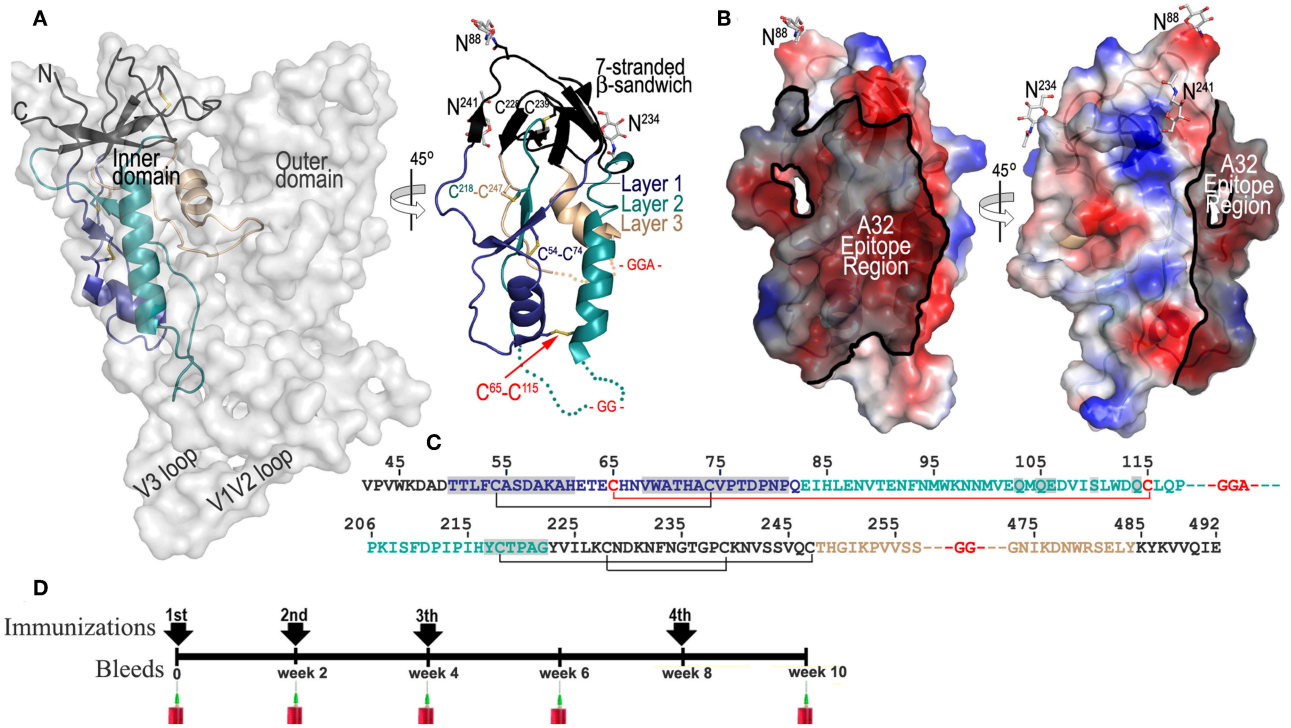

FIGURE 1 | ID2 immunogen design and immunization scheme. **(A)** Putative and crystallographic structure of ID2 immunogen. Putative structure of ID2 is shown overlaid over the structure of full length gp120 from the CD4-triggered BG505 SOSIP trimer (PDB code:1U1F) and a 45° rotation shows the crystal structure of ID2 immunogen from the ID2-Fab A32 complex structure (PDB code:4YC2). "Layered" architecture of gp120 inner domain is shown with the 7-stranded β-sandwich colored black, layer 1 in blue, layer 2 in cyan, layer 3 in light orange. The C^{65}-C^{115} disulfide bond introduced to stabilize ID2 in the CD4-bound conformation and GGA(GG)-linkers are shown in red. Sugars at positions 88, 234, and 241 are shown as sticks. The region of ID2 disordered in the ID2-Fab A32 complex are show as broken lines. **(B)** The A32 epitope region in the context of ID2 immunogen. The gp120 residues involved in binding of A32 and A32-like antibodies N5-i5, 2.2c, N60-i3, JR4 are highlighted in black over the ID2 molecule. The molecular surface is displayed over the ID2 molecule and the electrostatic potential is shown as red for negative, blue for positive, and white for apolar. **(C)** Primary sequence of the ID2 construct (from gp120 sequence of clade A/E 93TH057 isolate) with disulfide bonds and –GG—linkers shown. Residues forming the A32 epitope region are highlighted in gray. **(D)** Schematic of immunization protocol.

Neutralization Assay

Mouse sera collected 2 weeks after the 4th immunization and pre-immune sera, were tested in a TZM-bl assay for the presence of neutralizing antibodies. Briefly, 3-fold serial dilution (starting from 1/100 dilution) of sera were mixed with JR-FL pseudo-virus (TCID of 45000 Relative Luminescence Unit) and incubated at 37°C in 5% CO_2 atmosphere for 60 min at room temperature. TZM-bl cells (10,000/well in complete RPMI with 11 μg/mL DEAE-Dextran) were then added to the sera-pseudo virus mix and plates were incubated for 48 h at 37°C in 5% CO_2 atmosphere. One hundred and Fifty Microliter of supernatant were removed and 100 μL/well of BrightGlo was then added to each well and after a 2 min incubation to allow complete cell lysis, 100μL from each well was transferred to 96 well black plates. Plates were read with a luminometer using the Promega BrightGlo program. Percent neutralization was determined by calculating the difference in average relative luminescence units (RLU) between virus control (no serum/antibody) and test wells (cells + serum or antibody sample + virus), dividing this result by the difference in average RLU between virus control (cell + virus) and cell only wells, and multiplying by 100.

Statistical Analysis

Differences in responses between ID2 alone, ID2+Alum, and ID2+GLA-SE were analyzed using a Two-way ANOVA with Bonferroni post-test comparing every sample to every other sample. All statistical analysis was carried out using GraphPad Prism (Version 5 for Windows, San Diego, CA, USA). ****represents statistical significance of $P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$. Blue stars represent the difference between ID2 alone and ID2 + Alum, red stars represent differences between ID2 alone and ID2 + GLA-SE and purple stars represent differences between ID2 + Alum and ID2 + GLA-SE.

RESULTS

ID2 as an Immunogen Candidate Selectively and Stably Presents the A32 Region

ID2 was designed to stably present the conformational CD4-inducable epitopes of the A32 region within a minimal structural

unit of gp120 without any other known (neutralizing or non-neutralizing) epitopes present (34). The design of ID2 was guided by detailed analysis of the epitope structures of A32 and several A32-like antibodies (14, 24, 25) that involve the Env antigen binding residues exclusively within the gp120 inner domain of the constant regions 1 and 2 (C1–C2). Through several steps of structure-guided design we obtained a construct consisting of only 154 residues of the gp120 inner domain which is stabilized in the CD4-bound conformation by the addition of a C_{65}-C_{115} disulfide bond (**Figures 1A–C**). In the ID2 construct the outer domain, variable loops and receptor binding sites were removed to form a minimal structural unit which engrafts only the A32 epitope region. **Figures 1A,B** show the putative and crystallographic structure of ID2, determined previously in a complex with the Fab of the A32 antibody. ID2 constitutes only one third of the full length gp120 molecule with the A32 epitope region mapping to almost half of the ID2 surface. In addition, a significant area of the ID2 face which does not harbor the A32 epitope is masked by N-glycosylation (asparagines at positions 88, 234, and 241) most likely rendering this part of molecule immunologically silent (**Figures 1B,C**). We showed previously that ID2 is folded to fully preserve the conformation of the inner domain as seen in the context of CD4-triggered gp120 and stably presents the functional A32 epitopes within the C1–C2 region and thus constitutes a novel immunogen candidate for selective induction of A32-like responses (34).

To evaluate if ID2 indeed is capable of selective induction of humoral response to the desired A32 region we performed immunogenicity studies using the recombinant preparation of ID2 obtained by mammalian cell culture (to preserve wild-type glycosylation) and BALB/c mice as an animal host. Purified ID2 protein, 20 μg per injection, was used to immunize groups of mice (6 animals per group) in the absence of adjuvant (in PBS) and with two adjuvant choices; Alum (2% aluminum hydroxide wet gel suspension) and GLA-SE (a stable oil-in water emulsion containing TLR-4 agonist adjuvant developed by Infectious Disease Research Institute). Immunizations were done according to the immunization scheme shown in **Figure 1D** with three immunizations in 2-week intervals and a fourth at week 8. Sera was collected 2 weeks after each immunization and analyzed individually for each mouse.

Immunization With Recombinant ID2 Protein Induced Specific Anti-ID2 Responses in BALB/c Mice

Sera collected 2 weeks after each immunization were tested in a sandwich ELISA to assess the presence of specific anti-ID2 serum antibodies. As shown in **Figures 2A–D**, immunization with ID2 with and without adjuvant elicited an anti-ID2 humoral immune response in all immunized mice, although with significant differences among the 3 immunization groups. Some mice immunized with ID2 without adjuvant developed a weak anti-ID2 specific humoral immune response with a half max binding titers above 1:500, but only after the 4th immunization (**Figure 2E**). In contrast, titers and kinetics of the humoral immune response was quite different in mice immunized with ID2 delivered alongside Alum or GLA-SE adjuvants. In both groups the immunization induced a detectable specific humoral immune response after the 1st immunization, however, only mice injected with ID2+Alum had higher levels of specific IgG compared to ID2 injected alone (**Figure 2A**). After 2 and 3 injections, both adjuvants induced significant levels of ID2-specific IgG above mice injected with ID2 alone (**Figures 2B,C**) and following the final injection, specific IgG levels were significantly higher in the GLA-SE adjuvant group compared to the Alum group (**Figure 2D**). For mice immunized in GLA-SE, all sera showed an enhancement in the anti-ID2 specific antibody immune response from 1 to 2 logs after each immunization with half-max binding titers ranging from 1:3,400 to 1:36,000 after the 4th immunization (**Figures 2D,E**). Altogether, these results clearly indicate that recombinant protein ID2 is immunogenic and that such immunogenicity can be improved by administrating the recombinant protein in combination with an adjuvant. As for the adjuvant, our data clearly suggested that mice immunized with ID2 in GLA-SE produced higher titers of anti-ID2 serum antibodies.

Immunization With ID2 Immunogen Induces an Antibody Response Specific for the Conformational Epitopes Within the A32 Region

By design ID2 consists of two faces; a face that harbors the conformational epitopes of the A32 region and a face that is exposed by the removal of the OD which does not have any known epitope targets. Although the epitopes within the newly exposed face are not known, this face might harbor epitopes that are rendered immunodominant by their exposure. To assess if the antibody response induced by ID2 is indeed specific for the desired A32 epitope region we tested sera collected after the final immunization in a competition ELISA with mAb A32 and the A32-like antibody N5-i5 (14). Our assay format was designed to detect if serum antibodies inhibited the binding of mAb A32 or N5-i5 to the recombinant ID2 protein immobilized on microplates. As shown in **Figures 3A,B**, the immune sera of mice immunized with ID2 alone were not able to inhibit the binding of the tested mAbs to the coated ID2 protein. In contrast, mice immunized either with ID2 in Alum or in GLA-SE elicited antibodies capable of blocking A32 (**Figure 3A**) and N5-i5 (**Figure 3B**) at a significantly higher level than sera from mice injected with ID2 alone. Sera from mice immunized with the GLA-SE adjuvant inhibited the binding of over 80% of both N5-i5 and A32, significantly higher than mice injected in the presence of Alum. These data indicate that immunization with ID2 leads to elicitation of a serum antibody response specific for Env targets that overlap with epitopes recognized by the A32 region antibodies A32 and N5-i5 with the adjuvant GLA-SE eliciting the most robust humoral responses.

Next, we asked if the elicited antibody responses were directed toward conformational or linear epitopes within the ID2 immunogen. Pooled sera collected after the final immunization were incubated in solution with denatured ID2 (dID2) protein

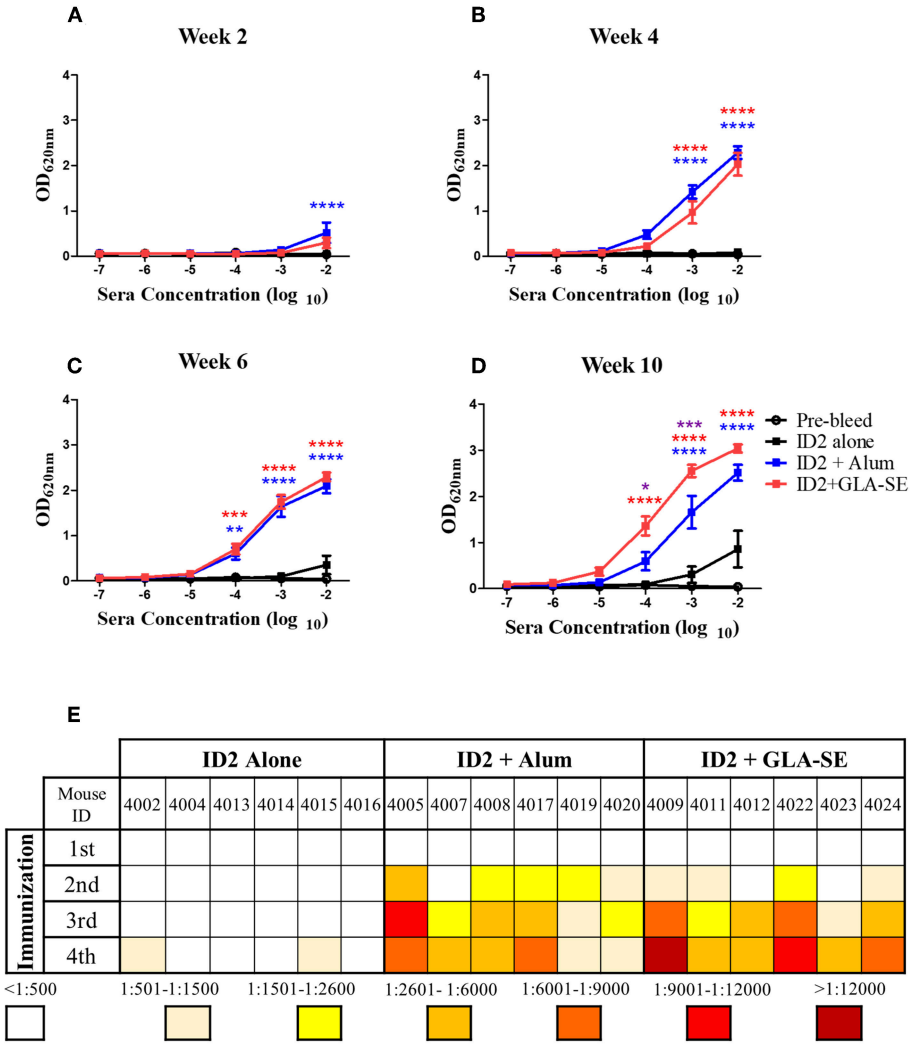

FIGURE 2 | Kinetics and titers of anti-ID2 immune response in immunized mice. Sera collected 2 weeks after each immunization were tested in ELISA for the presence of anti-ID2 specific immune responses (total αID2 IgG). Each sample was assayed in duplicate, displayed is the Mean ± SEM of the 6 mice in each group for **(A)** Week 2, **(B)** Week 4, **(C)** Week 6, and **(D)** Week 10. **(E)** The half-max binding for each individual mouse, identified with a unique number, at each time point following an immunization (1–4) was calculated. $n = 6$ mice for each group. **** represents statistical significance of $P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$. Blue stars represent the difference between ID2 alone and ID2 + Alum, red stars represent differences between ID2 alone and ID2 + GLA-SE and purple stars represent differences between ID2 + Alum and ID2 + GLA-SE.

and then probed in ELISAs with non-denatured ID2 immunogen coated on microplates. As shown in **Figure 3C**, after being adsorbed with denatured ID2 in solution, sera of all 3 immunization groups were still able to bind the non-denatured ID2. The residual sera of mice immunized with ID2 + GLA-SE adjuvant showed higher levels of binding to conformationally intact ID2 than mice injected with ID2 alone and GLA-SE led to significantly higher levels than alum. This indicates that immunization with ID2 protein alone or in combination with adjuvants leads to elicitation of sera antibodies that recognize and bind conformational ID2 epitopes with a higher titer of conformational antibodies present in sera of mice immunized with the GLA-SE adjuvant.

Sera of Mice Immunized With ID2 in GLA-SE Mediates ADCC

The ID2 immunogen was designed to harbor A32 or A32-like epitopes involved in potent ADCC responses against target cells during the earliest stage of viral entry i.e., at the interaction of gp120 of the Env trimer with the host cell receptor CD4 (14, 21–23, 37) and HIV infected/budding cells which retain CD4 at the target cell surface. Antibodies recognizing the A32 region epitopes were shown to lack conventional neutralizing activities [(12–16, 38), reviewed in (21–23)]. To test if ID2 elicited antibodies capable of ADCC against CD4 inducible (CD4i) targets of a cross clade gp120 we characterized the terminal sera of immunized mice with the optimized RFADCC assay

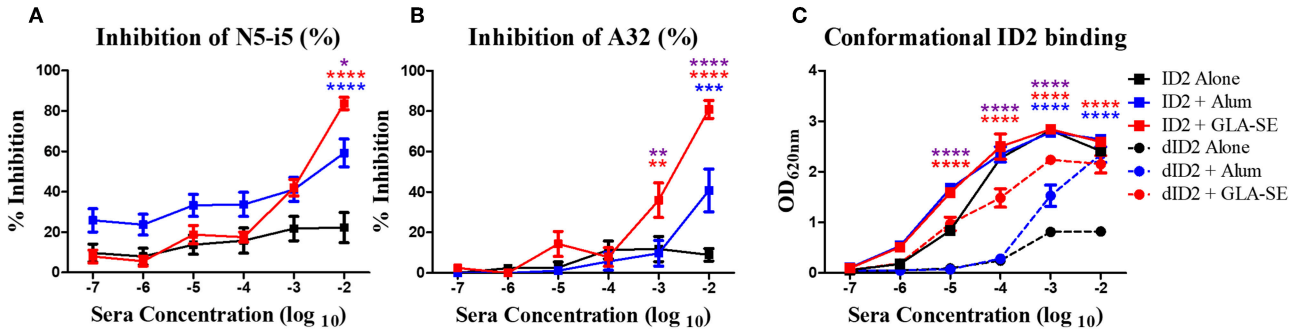

FIGURE 3 | Epitope and conformational specificity of serum antibodies elicited in immunized mice. Sera collected 2 weeks after the final immunization (week 10) were tested for their ability to compete with Cluster A biotinylated mAbs for the binding to ID2 recombinant protein in ELISA. Plates were coated with 0.5 μg/mL of ID2 and serum was incubated with **(A)** A32 and **(B)** N5-i5 (right panel) at half max binding concentrations. Each sample was assayed in duplicate before calculation of % inhibition from the average. **(C)** To assess if immunization with ID2 alone or in combination with adjuvants induced serum antibodies recognizing conformational or linear epitopes different dilutions of pooled sera collected 2 weeks after the last immunization (week 10) were mixed o/n at 4°C with a fixed concentration of denatured ID2. Sera were then reacted in ELISA on ID2 coated plates. Anti-mouse IgG alkaline phosphatase conjugated was used to detect the binding of serum antibodies. **** represents statistical significance of $P < 0.0001$, ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$. Blue stars represent the difference between ID2 alone and ID2 + Alum, red stars represent differences between ID2 alone and ID2 + GLA-SE and purple stars represent differences between ID2 + Alum and ID2 + GLA-SE.

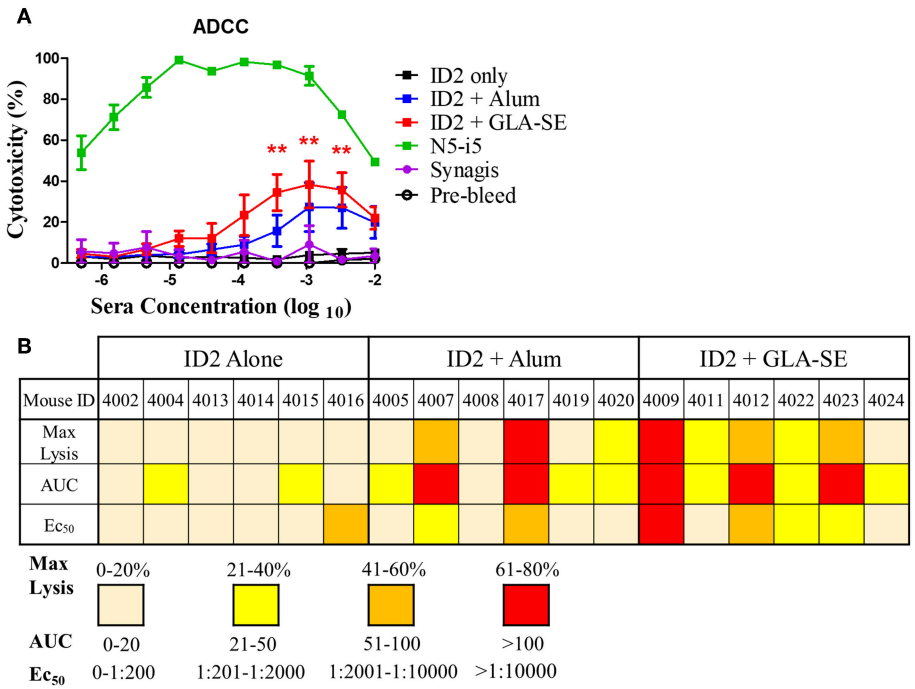

FIGURE 4 | ADCC in sera from mice immunized with ID2 with and without adjuvant. **(A)** Pre-immune sera and sera collected after the last immunization were tested in Rapid Fluorescence ADCC assay against EGFP-CEM-NKr-CCR5SNAP cells sensitized with recombinant BaL gp120 to assess their ability to mediate cytotoxicity. Results are reported as % Cytotoxicity, in relation to maximal cytotoxicity by N5-i5. All samples were analyzed in triplicate before calculating the % cytotoxicity from the mean. No statistical difference existed between ID2+Alum and ID2+GLA-SE at any sera concentration, determined using a Two-way ANOVA test with Bonferroni post-test. **(B)** The maximum lysis (%), Area under the curve (AUC) and Ec_{50} for each individual mouse, identified with a unique number, at each time point following the final immunization was calculated. Max lysis was defined as the maximum percent lysis at any sera concentration. Area under the curve (AUC) was calculated using an Excel formula. Ec_{50} was determined using a GraphPad prism formula of Log(agonist) vs. Normalized response for a variable slope. $N = 6$ for each group. ** represents statistical significance of $P < 0.01$. Red stars represent differences between ID2 alone and ID2 + GLA-SE.

(36) using NKr cells sensitized with recombinant BaL gp120. As shown in **Figure 4A**, sera from mice immunized with ID2 in the absence of adjuvant showed no ADCC activity, with cytotoxicity readings comparable to the negative control Synagis and the pre-bleed samples. Sera analyzed from mice immunized with ID2 + Alum were capable of modest but not significant ADCC

above ID2 alone samples with peaks of cytotoxicity for sera dilutions of 10^2-10^4. In contrast, sera from mice injected with ID2 + GLA-SE elicited significant ADCC when compared to ID2 alone (**Figures 4A,B**).

ID2 was designed specifically to contain no known neutralizing epitope targets (34) in order to closely mimic the response observed in the RV144 vaccine trial in which ADCC in the absence of neutralization was associated with protection (28). To test if the ID2 immunized sera contained any neutralizing antibodies we performed a standard neutralization TZM-bl assay against the Tier 2 clade B virus JRFL. This virus was selected to represent a biologically relevant strain for demonstrating neutralization. The recent publication by Montefiori et al. (39) concludes that tier 2 viruses represent the Env conformation of most circulating viruses and are therefore the most appropriate for determining neutralization potential of antibodies. Using sera collected after the last immunization and mAbs A32 and P7 as respective negative and positive controls, we were able to determine that none of the tested sera was able to robustly neutralize the JRFL virus (**Figure 5**).

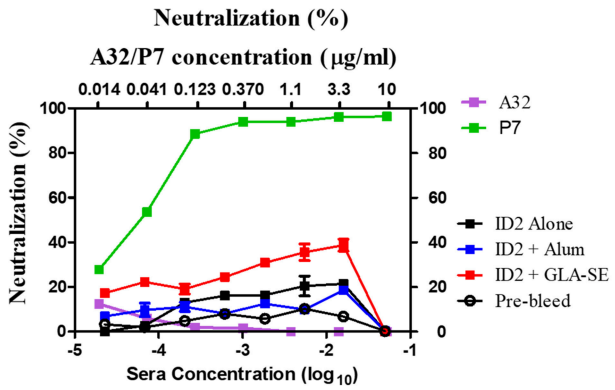

FIGURE 5 | Neutralization in sera from mice immunized with ID2 with and without adjuvant. Sera were tested in a TZM-bl assay for the presence of neutralizing activities against clade B Tier 2 JRFL pseudo-virus. Sera from Pre-bleed (Hollow circle), ID2 Alone (Black square), ID2 + Alum (Blue square), and ID2 + GLA-SE (Red square) were diluted and assayed for their ability to neutralize a clade B Tier 2 JR pseudo-virus (Bottom X axis). These samples were run alongside diluted A32 antibody (orange square) for a negative control and P7 antibody (green square) as a positive control, beginning at 10 μg/ml (Top X axis).

Mice Immunized With ID2 in GLA-SE Show a Larger Diversity of Anti-ID2 Ig Subclasses

In addition to showing that ADCC in the absence of robust neutralization was adequate for protection against HIV, the RV144 trial also highlighted the importance of the type of antibody repertoire raised. A strong IgG response in the absence of IgA conferred the best protection (28). We therefore aimed to determine the antibody repertoire triggered in response to ID2 in the presence and absence of adjuvants. We tested sera collected after the last immunization (week 10) for the presence of anti-ID2 specific IgG1, IgG2a, IgG2b, IgG2c, IgG3, IgA, and IgM (**Figures 6A–G**). As expected, the IgG1 subclass was detected in all immunization groups albeit with different titers. Mice immunized with ID2 alone had very low half-max binding titers, ranging between 1:341 and 1:2,344 (**Figure 6H**). The addition of Alum increased the level of ID2-specific IgG1 with half-max binding titers ranging from 1:7,597 to 1:66,337 in these sera. The half-max binding titers were similar for mice immunized in the presence of GLA-SE, ranging between 1:16,678 and 1:72,864. In contrast, significant differences existed in titers of IgG2a and 2b subclasses between the Alum and GLA-SE adjuvant groups. Mice immunized in the presence of Alum generated a very low titer for both IgG2a and 2b, with half max binding titers for both below 1:500 in all but two mice, while the addition of GLA-SE resulted in half max binding titers for IgG2a ranging between 1:1,833 and 1:20,585 and for IgG2b between 1:572 and 1:3,139 (**Figures 5B,C,H**). Low levels of anti-ID2 specific IgG2c, IgG3, IgA, and IgM responses were detected only in sera of mice immunized in presence of GLA-SE adjuvant (**Figures 6D–G**). Combined, these data indicate that immunizations with GLA-SE adjuvant induce a larger diversity of anti-ID2 IgG subclasses as compared to Alum.

DISCUSSION

Antibodies capable of effective Fc-mediated effector functions, including ADCC, have recently received increasing interest as important components of a vaccine induced humoral response. Although the enthusiasm for this type of antibody function was mostly evoked by the RV144 trial, the evidence also exists from vaccination strategies with Env immunogens in non-human primates (NHP) that link FcR effector functions of antibodies with post-infection control of viremia and/or blocking HIV-1 acquisition, often in the absence of neutralization (40–45), reviewed in (27).

Correlate analyses of the infection risk in the RV144 trial have indicated two gp120 epitope regions; the conformational C1-C2 and the linear V2 loop epitopes, as the major players involved in the Fc-mediated protective response (21, 22, 46). Although only the ADCC response of antibodies directed at the crown of the V2 loop region directly correlated with a lower risk of infection (29–31) the non-neutralizing C1-C2-specific A32-like antibodies synergized with the weakly-neutralizing V2 antibodies (33) to deliver ADCC against neutralization resistant tier 2 isolates. The synergistic crosstalk between antibodies directed at these two epitopes was recognized to be an important component of the protective effect of the RV144 vaccine trial suggesting that C1-C2- and V2-specifc antibodies may act in tandem in a polyfunctional antibody profile to deliver a broad and potent Fc-effector response (33).

Similarly, antibodies specific for Cluster A epitope region (including the A32 subregion) and the co-receptor binding site correlated with sterilizing heterologous protection against SHIV162p3 in NHPs immunized with the conformationally constrained gp120 immunogen, full-length single chain (FLSC) (41, 47). As with the RV144 trial, no correlation

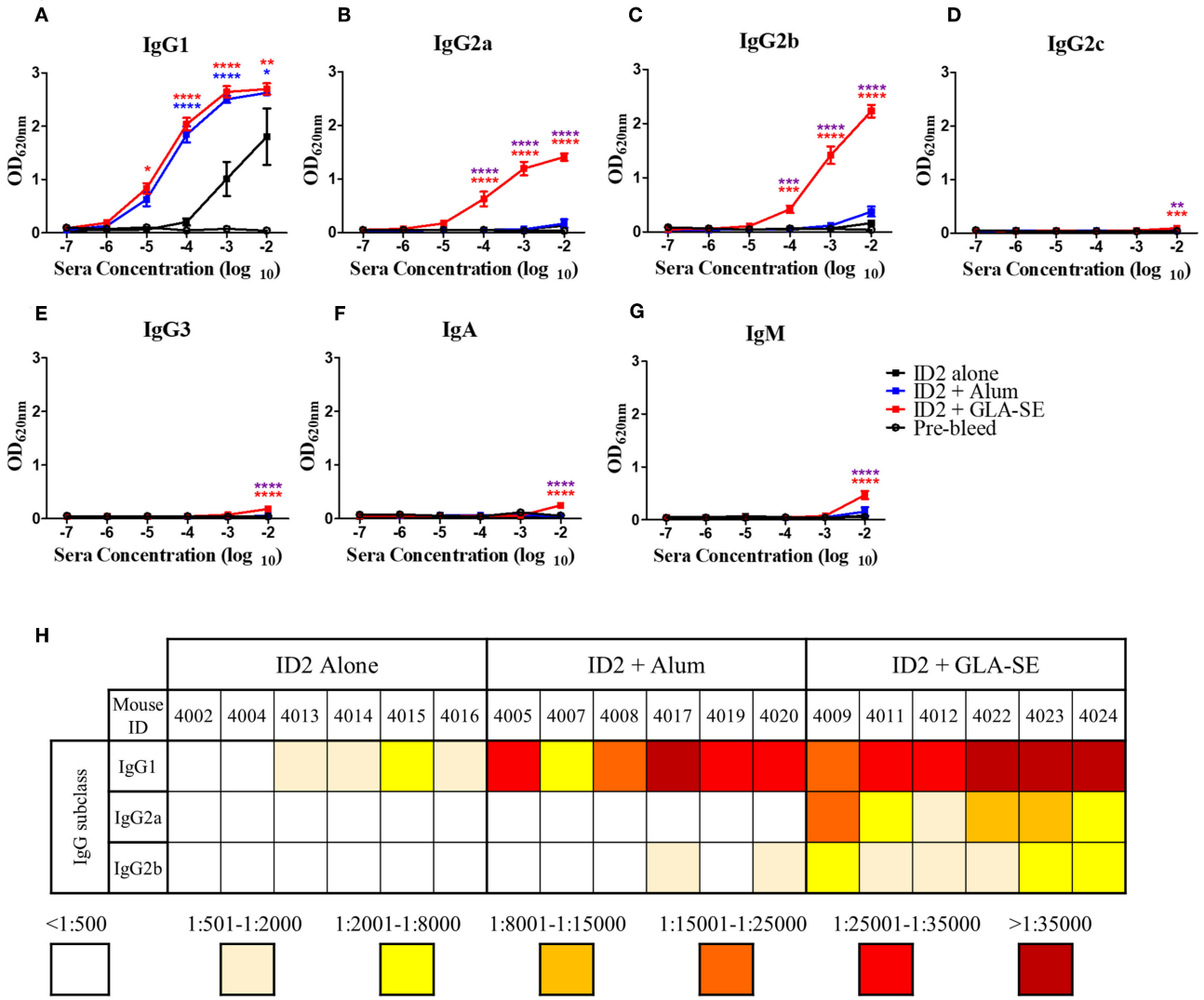

FIGURE 6 | Evaluation of IgG subclasses, IgA and IgM elicited in immunized mice. 10-fold dilutions of pre-immunized (pre-bleed, Black circle) and immunized mice sera collected after last immunization (week 10), were evaluated in ELISA to assess the IgG subtypes; **(A)** IgG1, **(B)** IgG2a, **(C)** IgG2b, **(D)** IgG2c, **(E)** IgG3, as well as **(F)** IgA, and **(G)** IgM induced with no adjuvant (Black square), with Alum (Blue square), or with GLA-SE (Red square). **(H)** The half-max binding for each individual mouse sera sample, identified by an individual number, for the terminal bleed was calculated and displayed for IgG1, IgG2a, and IgG2b. $n = 6$ mice for each group. **** represents statistical significance of $P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$. Blue stars represent the difference between ID2 alone and ID2 + Alum, red stars represent differences between ID2 alone and ID2 + GLA-SE and purple stars represent differences between ID2 + Alum and ID2 + GLA-SE.

between neutralizing activity and protection was observed in these studies pointing again at a role for Fc-mediated effector function in protection against SHIV-1 transmission. In both the RV144 and FLSC trials described above the C1–C2 specific antibodies acted in tandem with antibodies directed at other Env epitopes to contribute to the vaccine efficacy through Fc effector mechanisms. The question remains open if antibodies directed at these non-neutralizing epitope targets could alone afford protection or if they act only as a component of a polyclonal response with antibodies targeting other epitopes. This question has not been addressed, mostly due to the lack of an appropriate immunogen which could selectively bear only this conformational epitope target.

In this study, we investigated the immunogenicity and ability to induce an effective cross-clade ADCC response of a new immunogen candidate ID2, developed previously in our laboratory, as a minimal structural unit of HIV Env stably presenting the non-neutralizing epitopes within the A32 region (C1–C2 epitopes). ID2 consists of the inner domain construct stabilized in CD4-bound conformation by C_{65}-C_{115} disulfide bond preserving the A32 region epitopes in the context of CD4-triggered full length gp120, derived from a clade A/E strain, without the complication of any other known neutralizing

epitopes. We immunized BALB/c mice intraperitoneally (IP) with 4 doses of ID2 (20 μg /dose) over a period of 8 weeks with three immunizations with 2 weeks intervals and one final immunization delivered 4 weeks later to assess the induction of a memory antibody response. ID2 was administered either alone or with an adjuvant: Alum or GLA-SE. The resulting immune sera were evaluated for the presence of anti-ID2 antibodies (total IgG, IgG subclasses, IgA, and IgM) as well as for their capacity to bind to conformational A32 region epitopes and compete for the binding with mAbs specific for those epitopes. Immune sera were also tested in our RFADCC assay for their ability to mediate ADCC with CEM-NKr-CCR5 target cells sensitized with recombinant gp120 from the Clade B HIV-1_{BaL} isolate and to neutralize a Tier 2 strain of HIV-1. Our results showed that immunization with ID2 alone elicited no or very low anti-ID2 serum antibodies and those elicited were only after the 4th immunization, whereas mice in the groups immunized with ID2 in adjuvants showed robust anti-ID2 responses. Of the two adjuvants, mice immunized with ID2 in GLA-SE exhibited higher anti-ID2 titers at the end of study (week 10) as compared with the Alum group. Interestingly, both groups showed comparable titers after the 3rd immunization, but while in the GLA-SE group the 4th immunization boosted the immune response significantly above the alum group, mice in the Alum group presented a comparable titer to week 6. These data indicate that administration of ID2 in combination with GLA-SE induces enhanced and easily boosted responses to the target antigen. This agrees with the data regarding GLA-SE as an adjuvant, which was designed to promote strong and long lasting T_H1 responses to protein vaccine antigens (48, 49). Quality analyses of the immune responses elicited by ID2 with adjuvants indicated that antibodies elicited by ID2 with GLA-SE were directed more toward conformational epitopes within the A32 region as shown by the ability of these sera to inhibit the binding of mAb A32 and the A32-like mAb N5-i5 to ID2 and by the residual binding of sera previously incubated with a denatured/linear ID2. Differences were also detected in terms of the IgG isotypes of the elicited antibodies. Mice immunized with ID2 in Alum produced mostly IgG1 antibodies, whereas the immunization with ID2 in GLA-SE induced a broader range and a higher titer of IgG isotypes IgG1, IgG2a, and IgG2b, together with low but detectable IgG3 in addition to IgA and IgM. This difference in antibody isotypes between the two adjuvant groups likely explains the significant higher titers of total IgG in the GLA-SE adjuvant group over the alum group following the final immunization.

ADCC was only significantly increased over the negative control in sera isolated from the GLA-SE adjuvant group, indicating that high titers of ID2-specific mouse IgG1, IgG2a, and IgG2b in addition to small amounts of other isotypes are required for effective RFADCC. In addition to generating more diverse isotypes of ID2 specific antibodies, immunizing with GLA-SE also led to the generation of more specific antibodies – as indicated by significantly higher percent competition for both A32 and N5-i5 antibodies. Interestingly, some sera displayed high ADCC yet low levels of ID2-specific IgG and an absence of class switching from IgG1. One example of this is sample 4,007; when looking at the individual data for the competition ELISA, 4,007 sera inhibits the binding of A32 by 39.7% at the highest sera concentration and N5-i5 by 45%, an average amount for A32 and below average for N5-i5 when compared to other mice in the Alum group. One possibility for a more potent ADCC response is that antibodies to different epitopes were raised in this particular mouse. While the design of ID2 prevents the elicitation of classical C11-like antibodies, some antibodies may be specific for C11-like epitopes which would not have been identified by the competition ELISAs carried out. One known examples of an antibody which could be raised to this area of ID2 is JR4 (24). As previously reported, the RFADCC assay (36) uses antigen sensitized NKr cells and human PBMCs, in which mostly monocyte activity (through their FcγRIIA receptors) is measured. Although interactions between mouse immunoglobulins and human FcγRs are largely understudied there are reports indicating that human FcγRIIA binds mouse IgG1, 2a, and 2b, but not mouse IgG3 (50). All together this suggests that the presence of IgG1 and IgG2a in immunized mouse sera is sufficient to stimulate human PBMCs to mediate the killing of cross-clade antigen sensitized NKr cells.

In conclusion, these results indicate that ID2 can be highly immunogenic, especially when administered together with GLA-SE as an adjuvant, and that the elicited immune response can mediate ADCC in a RFADCC assay using human PBMCs as effector cells. None of the immune sera showed robust neutralization activity against the tier 2 JRFL virus indicating that ID2 as an immunogen induces mostly a humoral immune response against the desired non-neutralizing epitopes within the A32 region. ID2 in combination with GLA-SE was successfully utilized to demonstrate that Fc-effector mechanisms in the absence of strong neutralizing antibodies could be directed toward the A32 subregion which has the potential for HIV-1 protection and ID2 could therefore be utilized for future studies in a NHP challenge study.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

MV, NG, WT, and MP designed, performed research, and analyzed the data. AD, CO, RF, and GL carried out assays and analyzed the ADCC data. RS analyzed the data. MV, RS, and MP wrote the paper. All authors read the manuscript and provided comments or revisions.

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REVIEW
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Mind the Gap: How Interspecies Variability in IgG and Its Receptors May Complicate Comparisons of Human and Non-human Primate Effector Function

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The field of HIV research relies heavily on non-human primates, particularly the members of the macaque genus, as models for the evaluation of candidate vaccines and monoclonal antibodies. A growing body of research suggests that successful protection of humans will not solely rely on the neutralization activity of an antibody's antigen binding fragment. Rather, immunological effector functions prompted by the interaction of the immunoglobulin G constant region and its cognate Fc receptors help contribute to favorable outcomes. Inherent differences in the sequences, expression, and activities of human and non-human primate antibody receptors and immunoglobulins have the potential to produce disparate results in the observations made in studies conducted in differing species. Having a more complete understanding of these differences, however, should permit the more fluent translation of observations between model organisms and the clinic. Here we present a guide to such translations that encompasses not only what is presently known regarding the affinity of the receptor-ligand interactions but also the influence of expression patterns and allelic variation, with a focus on insights gained from use of this model in HIV vaccines and passive antibody therapy and treatment.

Keywords: non-human primate, rhesus, cynomolgus, HIV, SIV, IgG, Fcγ receptor, neonatal Fc receptor

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INTRODUCTION

Advances in the treatment and prevention of HIV have relied heavily on the macaque animal model. Infection of animals with simian immunodeficiency virus (SIV) or chimeric simianized human immunodeficiency virus (SHIV) allows researchers to both model transmission and induce in a variety of macaque species a disease state characterized by sequelae closely resembling those found over the course of human HIV infection (1–5), against which therapeutic interventions can be compared. The similarities of the macaque model to humans has permitted the screening of drug candidates prior to elaborate and expensive human trials, which has contributed to the development of lifesaving drugs such as tenofovir (6, 7). In addition to its successes in the development of small molecule inhibitors, work in macaques has demonstrated that vaccines and antibodies are capable of mediating protection against SIV/SHIV infection, offering important

insights into the characteristics of effective immunological responses that serve as a model for vaccine research and development.

However, the high-profile failures of all but one of the vaccine candidates tested in humans to protect against HIV-1 infection also suggest the limitations of the macaque model. For all of the beneficial contributions the model has made to the understanding and treatment of HIV, discrepancies between outcomes in macaque studies and in the clinic suggest that a better understanding of how human and macaque immunobiology differ may be necessary in order to more reliably draw translatable conclusions from animal studies. Fundamental differences between humans and macaques, here focusing particularly in the area of humoral immune responses, mean that in some cases, the result of a vaccination study or a passive transfer experiment conducted in macaques may not be recapitulated if attempted in humans. Promisingly, with a more complete understanding of this interspecies diversity, it should be possible to better translate observations likely to be meaningful to human protection and therapy. While numerous Fc receptors for IgG exist, many of these, including FcRL5, mucins, and TRIM21 (8–10), have yet to be evaluated thoroughly in the macaque model. However, recent work has greatly enhanced our knowledge of IgG, FcγR, and FcRn biology in rhesus macaques. This review summarizes the current understanding of what differences exist in the setting of receptor and antibody interactions between humans and non-human primates, with a particular emphasis on those facets that have the potential to affect the evaluation of candidate vaccines and antibody-based strategies for the prevention or treatment of HIV-1 infection in humans.

ANTIBODIES IN HIV PREVENTION AND THERAPY

A wealth of data from non-human primate studies has demonstrated that monoclonal antibodies can provide sterilizing protection from viral challenge (11–13); additionally, because humoral immune responses are often correlates of vaccine-mediated protection, it has become clear that closer inspection of immunoglobulin biology in macaques has the potential to significantly advance research and development of vaccines and therapeutic antibodies. This potential was made all the more apparent following the conclusion of the single successful human vaccine trial to date, the RV144 trial conducted in Thailand, in which a moderate degree of protection was observed (14). A subsequent study of vaccine-induced immune responses identified binding, rather than broadly neutralizing, antibodies directed against the envelope glycoprotein as a correlate of reduced risk of infection (15). Furthermore, additional correlates analysis suggested that binding antibody responses with certain characteristics, such as a bias toward IgG3, away from IgA, and with antibody-dependent cell-mediated cytotoxicity (ADCC), and complement cascade-initiating activity were associated with reduced risk (15–18). These findings reinforce those of numerous other studies in both humans and macaques (summarized in **Table 1**) that have concluded that innate immune effector functions, such as ADCC, correlate with and may directly contribute to improved outcomes at all stages of HIV infection, from potentially blocking acquisition of the virus altogether to maintaining a lower viral load and delaying the onset of AIDS.

One of the most illustrative examples is the work of Hessell et al. which compared the protective efficacy of the broadly neutralizing antibody b12 with variants engineered to lack affinity for Fcγ receptors (FcγR) and/or the complement cascade-initiating protein C1q. Passive transfer of the native b12 was able to successfully block infection of rhesus macaques (27). Comparative analysis of the engineered variant lacking affinity for cellular antibody receptors to the crystallizable fragment (Fc) of IgG demonstrated that effector function driven by FcγRs, but not complement, contributed to protection. Similarly, humanized mouse models of HIV prevention and therapy have led to similar observations regarding the contribution of effector functions to the *in vitro* effect of other broadly neutralizing antibodies (69–71). However, given the lack of impact of effector function on the protection afforded by the neutralizing antibody PGT121 (59), and the inability of Fc glycosylation changes that result in enhanced ADCC activity specifically to enhance b12's protective efficacy (64), the generalizability of this observation to other antibodies, and the specific FcγR-dependent effector activities that may play a role in protection, remain to be determined. Nonetheless, a wealth of diverse data from studies of vaccines, monoclonal antibodies, and natural infection converge to point toward the importance of Fc receptor-mediated antiviral activities *in vivo*. Thus, fully leveraging the macaque model of HIV to identify vaccine and antibody candidates that bear desirable effector function profiles requires accounting for the ways in which immunoglobulins and their receptors differ between non-human primates and humans.

ANTIBODY RECEPTORS

The considerable interspecies diversity existing between the members of the FcγR family (itself a member of the immunoglobulin superfamily) (**Figure 1**) dramatically complicates comparisons of immune responses in humans and macaques. When cross-linked through their binding of antigen-antibody immune complexes, FcγRs transmit activating or inhibitory signals to the immune cell, depending on the identity of the signaling domain associated with the receptor in question. By enabling innate immune effector cells to potently respond to antigenic targets that the humoral immune system has adapted to recognize, FcγRs serve as a critical bridge between the arms of the immune system. Among other outcomes, FcγR engagement can lead to the release of cytokines, the direct killing of virus-infected, antibody-opsonized cells via ADCC, and phagocytosis and subsequent antigen processing and display (72).

This review will also briefly consider a receptor unrelated to the FcγR family—the MHC class I-like neonatal Fc receptor (FcRn). Much like the Fcγ receptors, FcRn acts as an important link between arms of the immune system, connecting humoral,

TABLE 1 | A partial summary of human and NHP studies that investigated the role of antiviral antibody effector functions in the context of (S)HIV infection.

Year	Species	Setting	Relevant findings, quoted	References
Fc RECEPTOR-MEDIATED EFFECTOR FUNCTION IS ASSOCIATED WITH REDUCED ACQUISITION OF HIV-1/SIV/SHIV				
2018	*M. mulatta*	Vaccination	• Reduced risk of infection was associated with IgG-driven antibody-dependent monocyte-mediated phagocytosis in the [intramuscular] vaccinees, but with vaccine-elicited IgA-driven neutrophil-mediated phagocytosis in [aerosol]-immunized animals	(19)
2017	*M. mulatta*	Vaccination	• Systems serology of the antibody responses identifies plasma antibody binding to HIV-infected cells, peak ADCC antibody titres, NK cell-mediated ADCC and antibody-mediated activation of MIP-1b in NK cells as the four immunological parameters that best predict decreased infection risk	(20)
2015	*M. mulatta*	Vaccination	• Protective efficacy correlated with the functionality of Env-specific antibody responses • These data demonstrate robust protection by Ad/Env vaccines against acquisition of neutralization-resistant virus challenge in rhesus monkeys	(21)
2015	*M. mulatta*	Vaccination	• Protection correlated with antibody-dependent cellular cytotoxicity specific for CD4-induced epitopes, provided that the concurrent antivaccine T-cell responses were minimal. Protection was lost in instances when T-cell responses were high or when the requisite antibody titers had declined	(22)
2014	*H. sapiens*	Vaccination	• These data suggest that subclass selection differences associated with coordinated humoral functional responses targeting strain-specific protective V2 loop epitopes may underlie differences in vaccine efficacy observed	(16)
2013	*M. mulatta*	Vaccination	• Protection against acquisition of infection correlated with vaccine-elicited binding, neutralizing, and functional non-neutralizing antibodies	(23)
2012	*M. mulatta*	Vaccination	• Measures of ADCC activity were higher among the SIVΔnef-inoculated macaques that remained uninfected than among those that became infected	(24)
2012	*H. sapiens*	Vaccination	• The binding of IgG antibodies to variable regions 1 and 2 (V1V2) of HIV envelope proteins (Env) correlated inversely with the rate of HIV-1 infection	(15)
2011	*M. mulatta*	Vaccination	• All protected animals showed gp41-specific vaginal IgAs with HIV-1 transcytosis-blocking properties and vaginal IgGs with neutralizing and/or [ADCC] activities • Plasma IgGs totally lacked virus-neutralizing activity	(25)
2007	*H. sapiens*	Vaccination	• The level of vaccine-induced ADCVI activity correlated inversely with the rate of acquiring HIV infection • ADCVI correlated poorly with neutralizing or CD4-gp120-blocking Ab activity • degree to which the ADCVI Ab response predicted the rate of infection was influenced by polymorphisms at the FcgR2a and FcgR3a gene loci	(26)
2007	*M. mulatta*	Passive transfer	• There is a dramatic decrease in the ability of a broadly neutralizing antibody to protect macaques against SHIV challenge when Fc receptor and complement-binding activities are engineered out of the antibody • No loss of antibody protective activity is associated with the elimination of complement binding alone	(27)
1998	*M. mulatta*	Passive transfer	• SIV hyperimmune sera given subcutaneously prior to oral SIV inoculation protected 6 newborns against infection • SIV hyperimmune sera was given… 3 weeks after oral SIV inoculation, viremia was not reduced	(28)
Fc RECEPTOR-MEDIATED EFFECTOR FUNCTION IS ASSOCIATED WITH REDUCED VIREMIA AND/OR HIGHER CD4$^+$ T CELL COUNTS				
2018	*M. mulatta*	Challenge model	• There was a significant reduction in the seeding of virus to the lymph nodes and a decrease in plasma viremia in the HIV antibody-infused macaques compared with the control antibody-infused animals	(29)
2016	*H. sapiens*	Infected patients	• [elite controllers] demonstrated polyfunctional humoral immune responses able to coordinately recruit ADCC, other NK functions, monocyte and neutrophil phagocytosis, and complement	(30)
2016	*M. mulatta*	Vaccination	• gp140-specific IgG3 Abs of females but not males were correlated with [ADCC] against gp120 targets ($p = 0.026$) and with [ADCP] ($p = 0.010$) • IgG3 Ab of females but not males also correlated with decreased peak viremia ($p = 0.028$)	(31)
2015	*M. mulatta*	Passive transfer	• Passive infusion of each of the three antibodies significantly reduced the number of [transmitted/founder] genomes	(32)
2011	*M. mulatta*	Vaccination	• Pre- and post-challenge memory B cells were correlated with functional antibody responses including [ADCC], [ADCVI], and transcytosis inhibition • Post-challenge, Env-specific IgG and IgA memory B cells were correlated with reduced chronic viremia	(33)
2011	*H. sapiens*	Infected patients	• ADCC responses to whole gp140 Env protein were strongly associated with a slower decline in CD4 T cell loss	(34)
2013	*H. sapiens*	Infected patients	• Found significantly higher levels of ADCC antibodies in controllers verses viremic subjects ($p = 0.017$)	(35)

(Continued)

TABLE 1 | Continued

Year	Species	Setting	Relevant findings, quoted	References
2010	*M. mulatta*	Vaccination	• Both ADCVI and percent ADCC killing prechallenge were significantly correlated with reduced acute viremia • [percent ADCC killing prechallenge], as well as post-challenge ADCVI and ADCC, was also significantly correlated with reduced chronic viremia	(36)
2009	*M. mulatta*	Vaccination	• The higher ADCC and ADCVI activities seen in the Tat/Env group provide a plausible mechanism responsible for the greater chronic-phase protection	(37)
2009	*M. mulatta*	Vaccination	• Reduced acute viremia was significantly correlated with higher serum binding titer, stronger [ADCC] activity, and peak prechallenge and 2-week-postchallenge [ADCVI]	(38)
2009	*H. sapiens*	Infected patients	• ADCC was detectable in all controllers tested and was significantly higher than in viremic individuals ($P < 0.0002$)	(39)
2005	*M. mulatta*	Vaccination	• *In vitro* ADCC activity correlated with *in vivo* reduced acute viremia after a mucosal challenge with pathogenic SIV	(40)
2004	*H. sapiens*	Infected patients	• Women with [cervical-lavage] ADCC activity had lower genital viral loads than did women with serum ADCC activity only	(41)
2001	*H. sapiens*	Infected patients	• Serum titers of anti-HIV-1 ADCC antibodies bear a significant ($P < 0.05$) positive correlation with the peripheral blood CD+ T cell counts and a negative one with the number of copies of HIV-1 RNA in the plasma of HIV-infected individuals	(42)
2001	*H. sapiens*	Infected patients	• ADCC effector cell function…correlated inversely with viral load ($R = -0.42$, $p = 0.007$) and directly with $CD4^+$ cell counts ($R = 0.52$, $p = 0.001$) • ADCC reduced virus yields from CD4+ lymphocytes infected with a primary HIV isolate	(43)
2001	*H. sapiens*	Infected patients	• The magnitude of this effector cell-mediated antiviral antibody response was inversely associated with plasma viremia level	(44)
1994	*H. sapiens*	Infected patients	• Individuals with CD4 counts <200/mm^3 were found to have the lowest titres of [ADCC-mediating] antibodies in their sera • ADCC-effector function of the [PBMCs] of HIV-infected individuals was significantly ($p < 0.05$) reduced as compared to PBMC from healthy, HIV-seronegative individuals	(45)
1990	*H. sapiens*	Infected patients	• Early ADCC responses were associated with high mean %$CD4^+$ T cell numbers and absence of lymphadenopathy throughout the 2-year observation period	(46)
Fc RECEPTOR-MEDIATED EFFECTOR FUNCTION IS ASSOCIATED WITH IMPROVED CLINICAL STATUS AND/OR DELAYED PROGRESSION TO (S)AIDS				
2017	*H. sapiens*	Infected patients	• [elite controllers] had higher levels of HIV Env-specific antibodies capable of binding Fc RIIIa, activating NK cells, and mediating granzyme B activity (all $P < 0.01$) than viremic subjects	(47)
2013	*H. sapiens*	Infected patients	• Although the magnitude of ADCC responses in the LTSP cohort were not higher and did not correlate with CD4 T-cell depletion rates, the LTSP cohort had significantly broader ADCC responses compared with the non-LTSP cohort	(48)
2002	*M. mulatta*	Challenge model	• Our study shows a correlation between humoral response, ADCC activity, and disease progression (as measured by $CD4^+$ T cell counts) • In these animals, ADCC activity is associated with delayed progression to AIDS	(49)
1999	*H. sapiens*	Vertical transmission	• Titres of [ADCC] were similar in transmitting vs. non-transmitting mothers… however, high [ADCC] titres were correlated with a good clinical status of children	(50)
1996	*H. sapiens*	Infected patients	• Rapid progessors had significantly lower titres of Abs that mediate ADCC against HIV-1 gp120 than those of non-rapid progressors…or…non-progressors • High titres of Abs that mediate ADCC correlate with successful host defense against AIDS	(51)
1993	*H. sapiens*	Vertical transmission	• Presence and titres of ADCC mediating and/or neutralizing antibodies in maternal sera did not predict HIV-1 infection in their respective children • Significantly higher frequency of ADCC was seen in the seropositive non-AIDS children compared with the AIDS children	(52)
1990	*H. sapiens*	Vertical transmission	• ADCC antibody frequencies were much higher (70%) in the non-AIDS group than in the AIDS group (30%) • HIV-specific ADCC and neutralizing antibodies do not seem to protect against transmission of HIV from mother to child but are significantly correlated with a better clinical stage of childhood HIV infection	(53)
1988	*H. sapiens*	Infected patients	• All sera of asymptomatic individuals… had a higher mean ADCC titer as compared to sera from patients progressing to AIDS or ARC	(54)
1987	*H. sapiens*	Infected patients	• ADCC titers were lower in patients with [AIDS] than in asymptomatic carriers	(55)
1987	*H. sapiens*	Infected patients	• Sera from healthy HTLV-III/LAV seropositive individuals in the presence of mononuclear cells from healthy HTLV-III/LAV seronegative donors exhibited significantly higher levels of ADCC activity compared to sera from patients with AIDS	(56)

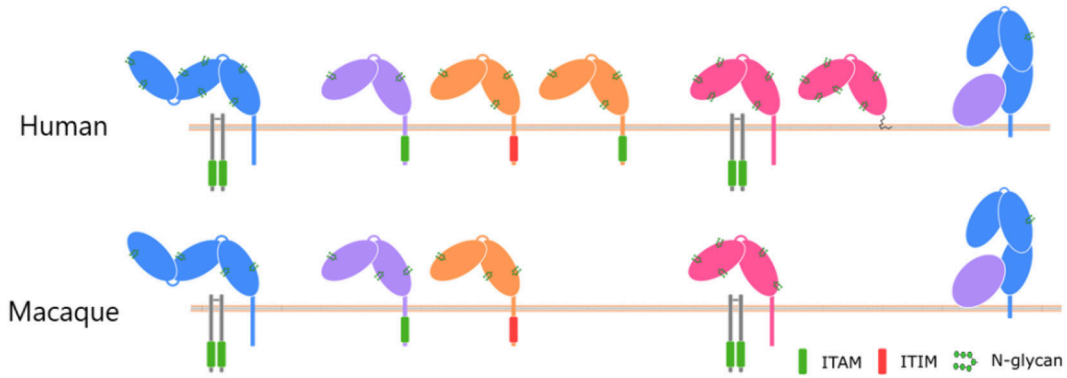
(Continued)

TABLE 1 | Continued

Year	Species	Setting	Relevant findings, quoted	References
Fc RECEPTOR-MEDIATED EFFECTOR FUNCTION IS ASSOCIATED WITH A REDUCED RISK OF MORTALITY				
2015	*H. sapiens*	Vertical transmission	• ADCC levels were higher in uninfected than infected infants, although not significantly • Increase in ADCC antibody activity in infected infants was associated with reduced mortality risk	(57)
1999	*H. sapiens*	Infected patients	• High baseline ADCC (>median) was associated with improved survival ($P = 0.05$)	(58)
Fc RECEPTOR-MEDIATED EFFECTOR FUNCTION IS DISPENSIBLE TO PROTECTION AGAINST HIV-1/SHIV				
2018	*M. mulatta*	Challenge model	• The single V2 antibody at the dose given did not significantly reduce the number of infections	(29)
2018	*M. nemestrina*	Challenge model	• The potent neutralizing capacity of PGT121 renders the Fc-dependent functions of the Ab at least partially redundant	(59)
2016	*M. mulatta*	Passive transfer	• CH31 IgG1 and IgA2 isoforms infused before high-dose SHIV challenge were completely to partially protective, respectively, while [non-neutralizing]Abs (CH54 IgG1 and CH38 mIgA2) were non-protective	(60)
2016	*H. sapiens*	*Ex vivo* model	• CD4 binding site bnAbs b12 IgG1 and CH31 IgG1 and IgA2 isoforms potently blocked HIV-1JR-CSF and HIV-1Bal26 infection. However, IgG1 and IgA nnAbs, either alone, or together, did not inhibit infection despite the presence of FcR-expressing effector cells in the tissue	(60)
2015	*M. mulatta*	Passive transfer	• 7B2 IgG1 or A32 IgG1, each containing mutations to enhance Fc function, was administered passively to rhesus macaques but afforded no protection against productive clinical infection	(32)
2014	*M. mulatta*	Passive transfer	• Passive transfer of a low-dose of ADCC inducing antibodies did not protect from infection following SHIV-SF162P3 challenge	(61)
2013	*M. mulatta*	Challenge model	• Despite virus-specific suppressive activity of the non-NAbs having been observed *in vitro*, their passive immunization post-infection did not result in SIV control *in vivo* • Virion binding and ADCVI activity with lack of virus neutralizing activity were indicated to be insufficient for antibody-triggered non-sterile SIV control	(62)
2014	*M. mulatta*	Vaccination	• We identify blocking CD4+ T cell recruitment to thereby inhibit local expansion of infected founder populations as a second correlate of protection • Virus-specific immune complex interactions with the inhibitory FcgRIIb receptor in the epithelium lining the cervix initiate expression of genes that block recruitment of target cells	(63)
2012	*M. mulatta*	Passive transfer	• NFb12 had higher affinity for human and rhesus macaque FcgR3a and was more efficient in inhibiting viral replication and more effective in killing HIV-infected cells in an ADCC assay • Despite these more potent *in vitro* antiviral activities, NFb12 did not enhance protection *in vivo* against repeated low-dose vaginal challenge in the [SHIV/macaque] model compared to wild-type b12	(64)
2011	*M. mulatta*	Challenge model	• Compared with control animals, the protection by [neutralizing] b12 achieved statistical significance, whereas that caused by [non-neutralizing] F240 did not	(65)
2005	*M. mulatta*	Passive transfer	• Six neonatal macaques were infused [subcutaneously] with immune IgG...positive for ADCC and Ab-dependent cell-mediated viral inhibition • No protection, assessed by viral burdens, CD4 counts, and time to euthanasia was observed	(40)
1994	*H. sapiens*	Vertical transmission	• High levels of anti-HIV-1 ADCC antibody at birth are not protective against vertical transmission of HIV-1	(66)
1990	*H. sapiens*	Infected patients	• There is no significant difference in ADCC values between those who remained asymptomatic and those who progressed to disease	(67)
1989	*H. sapiens*	Infected patients	• Levels of serum and effector-cell ADCC activity do not predict whether an individual will develop AIDS	(68)

and cellular immunity through its diverse activities. The receptor's capacity to distinguish monomeric IgG from that found in immune complex both imparts IgG with a lengthy serum half-life (73), and ensures that captured antigens are efficiently processes and presented to T cells (74, 75).

The differences in the sequences, ligand binding affinities, and expression patterns of human and macaque IgG and IgG receptors raise the possibility that a potent effector function-driven immune response may have a different phenotypic character in each species. This situation is further complicated by the frequent use of species-mismatched reagents and cell lines for *in vitro* activity assays. For example, the high baseline activity of macaque natural killer cells has driven most analyses of the ADCC potential found in the sera of vaccinated animals to be performed using human cell lines (40, 76). Similarly, the macaque/SHIV model is routinely used to evaluate the ability of passively transferred human antibodies to protect against infection (11–13). Absent a convincing case that the subclass of the transfused antibody or the receptor profile of the effector cell lines do not differ meaningfully between species, caution is required when attempting to extrapolate the findings of such studies from one species to another. Such risks may be mitigated, however, if given the means to more confidently translate observations between primate species and humans.



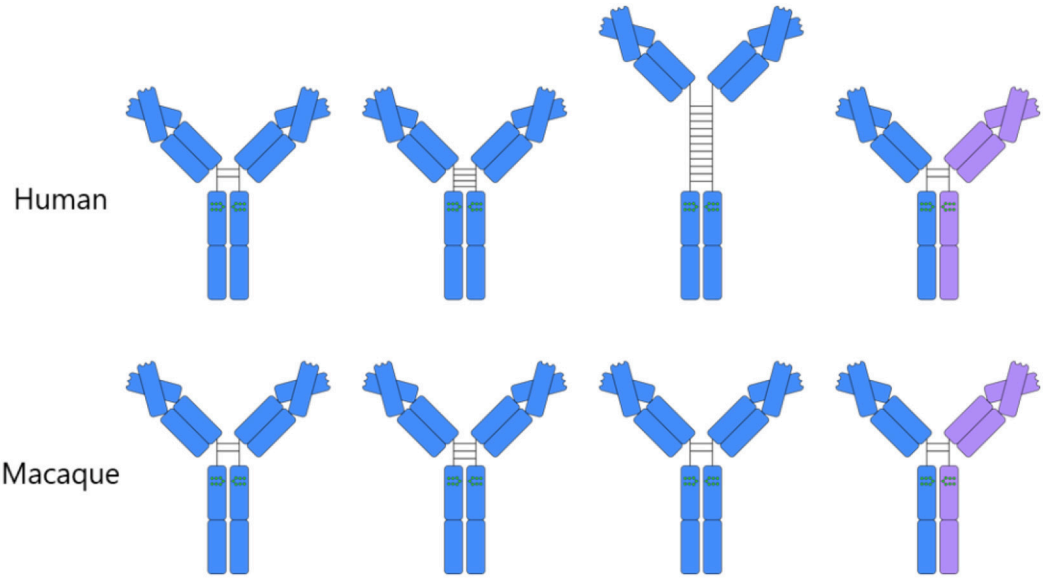
		FcγR1	FcγR2a	FcγR2b	FcγR2c	FcγR3a	FcγR3b	FcRn [β_2M]
Total Expressed Variants	Human	1	2	2	1	2	3	1 [1]
	Rhesus	3	4	2	–	3	–	1 [1]
Total N-glycan motifs	Human	7	2	3	3	5	6	1 [0]
	Macaque[1]	4	3	3	–	5	–	1 [0]
Amino Acid Substitutions[2] – Human vs. Rhesus	Extracellular Domain	15 – 17	20 – 24	18 – 20	–	15 – 18	–	7 [16]
	Transmembrane and Cytoplasmic Domains	6 + Δ17[3]	15	6	–	6	–	3 [–]

FIGURE 1 | A comparison of selected Fc receptors between humans and rhesus macaque species. Differences in protein coding sequences reflects insertions, deletions, and sites of polymorphism.
(1) The number of N glycan motifs is conserved across rhesus and cynomolgus macaques.
(2) The ranges of values represent the minimum and maximum changes involved in shifting from human to rhesus; exact numbers depend on the allelic variants being compared.
(3) Rhesus macaque FcγRI is truncated by 17 amino acids at its C terminus relative to the human variant.

DISTINCTIONS BETWEEN HUMAN AND NHP IgG TYPES

The other participant in the FcγR-antibody interaction, immunoglobulin G, introduces an additional dimension that must be accounted for when translating observations between macaques and humans. Primate species nominally share the same IgG subclasses, having IgG1 through IgG4, numbered according to serum prevalence, but in the case of humans and macaques, the similarities largely end with this convention. Macaque IgG subclasses are in fact a much less structurally and functionally diverse group of proteins than those found in humans (**Figure 2**). For example, human IgG3 features an elongated hinge, up to 62 amino acids in length (81, 82), for which no analog has been found in any species of macaque. Human IgG2, on the other hand, has a shortened hinge, and exists in several disulfide bond-based structural isoforms whose activity profile can differ (83). The similarities between macaque subclasses do not end with their sequences, however; the functional activity of macaque IgG, assessed by the ability to promote monocyte phagocytosis and natural killer cell degranulation, and binding to FcγR is relatively consistent across each of the four subclasses in rhesus (80) and cynomolgus (77), as compared to the more distinct activity and binding profiles observed for human IgG subclasses.

The human IgG response, conversely, can progress through better delineated phases during the course of infection, phases which may have arisen because they can collectively lead to an immune response activity profile that rises and falls when appropriate – fighting infection early on, and having the capacity to wane before the damage caused by inflammation is too severe. Within this framework, the role of IgG3, the first subclass within the immunoglobulin locus and the form that tends to be observed early on in infection (84), is to rapidly activate the immune response, particularly the effector cells of the innate immune system. In keeping with this role, human IgG3 strongly interacts with Fcγ receptors (85) and is a potent promoter of phagocytosis and degranulation. Switching downstream in the locus to IgG1 gives rise to this abundant, long-lived molecule. In fact, it has been theorized that the relative affinities of the human subclasses differ as beneficial mutations accumulate and switching to downstream subclasses occurs (86). By virtue of their generally reduced affinity for FcγR (85), subsequent or direct class-switching to IgG2 or IgG4 reduces activating potential, leading to the formation of less potent immune complexes (78). IgG4 may be particularly well-suited for this role because of its ability to undergo the process of Fab arm exchange, through which naturally-occurring bispecific antibodies are produced (87, 88). Because bivalent targeting normally aids in immune complex formation, the inability to avidly bind may further reduce the inflammatory potential of IgG4. Indeed, in humans, switching to IgG4 is often associated with amelioration of antibody-mediated allergy (89–92). Cynomolgus macaque



		IgG1	IgG2	IgG3	IgG4
Mass (kDa)	Human	146	145	150 – 156	145
	Rhesus	146	146	146	146
Hinge length [disulfide bonds]	Human	15 [2]	12 [4]	32 – 63 [5 – 11]	12 [2]
	Rhesus	18 [2]	12 [3]	17 [3]	10 [2]
Half-life (days)	Human	21	21	7 | 21[1]	21
Closest Human Homolog (% AA match)	Rhesus	IgG1 (90.7)	IgG1 (88.5)	IgG1 + 4 (89.2)	IgG4 (90.2)
In vivo Depletion[2]	Rhesus	++	++	+	+

FIGURE 2 | Comparison of human and rhesus macaque subclass properties. Human IgG3 is depicted with the most commonly expressed number of hinge unit repeats. IgG4 coloration reflects the potential for Fab arm exchange, which is a feature of human and rhesus IgG4, but not of cynomolgus macaques. References: (77–79).

(1) The allotype-dependent inclusion or absence of His435 determines half-life of human IgG3, with histidine-containing having a longer half-life.

(2) Plus signs denote the relative degree and durability of the decline in the $CD8^+$ T cell population based on treatment with subclass-switched anti-CD8 monoclonal antibody (80).

antibodies cannot perform this Fab arm exchange (77, 93), and the quantity of IgG4 in macaques is thought to be quite minor (79, 80, 94).

Further, while the IgG subclasses are generally ordered from most to least activating in the immunoglobulin locus of humans (IgG3, IgG1, IgG2, IgG4), they are ordered from least to most activating in rhesus (IgG4, IgG3, IgG2, IgG1) (80, 95), though again, the differences in activity across rhesus and cynomolgus macaque subclasses are considerably less pronounced than in humans, and IgG sequences suggest this may hold in other macaque species as well (96). Left unanswered is the question of what significance observed polymorphisms in macaque IgG subclasses may hold—evidence of polymorphisms despite limited sequencing efforts may suggest the presence of significant allotypic diversity in rhesus and cynomolgus macaques (95, 97). It is likely that, to date, immunoglobulin sequence variation existing within macaque species has been undersampled.

While little is known about the progression of subclass switching over time in macaques, in humans, an early but waning IgG3 response has been observed in the setting of both natural infection and vaccination (16, 98, 99). Repetitive protein boosts in the setting of vaccination have also been observed to increase the magnitude of the IgG4 response (16, 17, 99). Most importantly, the range of effector activity differences and structural distinctions among subclasses are considerably more broad in humans than in macaques (80, 100). When making immunological comparisons between species, it is important to consider the ways in which human antibodies have been functionally honed that are not shared by the non-human primates relied on as research models.

DISTINCTIONS BETWEEN HUMAN AND NHP FcγR

Members of the FcγR family share several common traits. Typically, two extracellular C-like domains are responsible for binding residues of IgG located in the lower hinge region (101–105). A molecule of IgG is bound by a single Fcγ receptor (106–108) through contacts made with both heavy chain constituents of the IgG homodimer. The character of those contacts differs between the protomers, with each side tending to favor a different class of intermolecular interactions (109). The extracellular domains of the receptors are followed by a transmembrane domain and a cytoplasmic domain, which may contain an activating or inhibitory signaling motif. In the absence of an intrinsic signaling motif, the task of communicating the antibody-mediated binding and receptor clustering is handled through association with the Fc receptor γ chain or other accessory receptors. An overview of the similarities and differences between receptors can be found in **Figure 1**.

Organization of this review will follow according to the type of signal a receptor transduces. Fcγ receptor crosslinking may lead to the generation of an activating signal though the involvement of an immunoreceptor tyrosine-based activating motif (ITAM) located in the cytoplasmic domain of the receptor or on the Fc receptor γ chain [reviewed in (72, 110–112)]. Briefly, activating receptors include the human and macaque FcγRI, FcγRIIa, and FcγRIIIa receptors. FcγRIIb, bearing an immunoreceptor tyrosine-based inhibitory motif (ITIM), is then the sole member of the inhibitory category of FcγR. The FcγRIIIb receptor found exclusively on human granulocytes, and apparently not present in the macaque genome, represents a special case as it lacks the normal transmembrane and cytoplasmic domains and is instead anchored to the cell membrane via a lipid chain. Although not as well-characterized as the other human Fcγ receptors, much of the available evidence suggests that FcγRIIIb contributes to the activation of granulocytes and that it is most appropriately included in the ITAM-bearing category of receptors.

FcγRI

Among members of the Fcγ receptor family, FcγRI stands apart by virtue of its three extracellular domains and high ligand-binding affinity. As in humans, the receptor's elevated affinity in rhesus and cynomolgus macaques, has been measured in the low nanomolar to high picomolar range (77, 100), allows it to readily bind circulating monomeric IgG (85). As **Figure 1** illustrates, the cytoplasmic domain of FcγRI lacks an intrinsic ITAM and this receptor instead utilizes the Fc receptor common γ chain to activate the cell (113, 114).

Characterization of FcγRI's role initially presented a challenge to researchers as the failure to express the receptor in humans apparently does not lead to any obvious immunodeficiency or associate with more frequent infections (115). Furthermore, the high concentration of IgG in circulation coupled with the receptor's high affinity suggested that FcγRI would be fully occupied by monomeric IgG at steady state (110). However, the conservation of this receptor across primate species and the rarity of individuals with expression defects suggests an important biological role. It is now thought that a primary role of FcγRI may be in antigen processing and display, as well as triggering the release of cytokines (116–118).

The single major allele of FcγRI expressed by humans (NP_000557) shares a high degree of amino acid sequence identity (∼90%) with the macaque variants characterized to date, although several noteworthy differences do exist. The three major rhesus variants and one cynomolgus variant differ from the human receptor at four predicted contact residues and lack three N-linked glycosylation motifs found in the human molecule. Furthermore, in rhesus macaques (see NP_001244233), but not cynomolgus (NP_001270969), there is a 17 amino acid truncation at the intracellular C terminus. The consequences of this deletion, if any, for the receptor's association with the common γ chain have not been determined.

Within the macaque genus, FcγRI's extracellular domain phenotype is extremely similar, with the most common rhesus allele (FcγRI-1) and the major cynomolgus allele sharing identical extracellular domains. Furthermore, the rhesus FcγRI-2 allele differs from these at only two extracellular residues and the FcγRI-3 allele at just one. None of these polymorphic positions are predicted to contact the IgG Fc based on homology models. This high degree of conservation is also thought to extend to FcγRI's pattern of expression by immune cells. In keeping with its role in antigen presentation, the receptor is expressed by dendritic cells (117, 119), monocytes, and macrophages (120) (**Table 2**). Granulocytes are also capable of expressing FcγRI following induction by interferon γ (130, 131).

In light of the high degree of homology shared by human and macaque FcγRI, it should come as no great surprise that the receptors have strikingly similar preferences for IgG subclasses. Within a fully human context (receptor and IgG), FcγRI binds IgG1 and IgG3 with similarly high affinities, and has an intermediate preference for IgG4. The receptor demonstrates significantly reduced affinity for IgG2 (77, 85). In rhesus macaques, however, FcγRI has nearly equivalent affinities for all four subclasses of rhesus IgG (100); this trend is also observed in cynomolgus macaques (77). The cause of this difference is not the sequence of the receptors, but the IgG. This becomes apparent when mixing receptors and antibodies of different species—human FcγRI has relatively uniform affinity for different subclasses of macaque IgG while macaque receptors exhibit preferences between human subclasses (77, 100). Human IgG2 contains a unique motif (VAGP) in its upper CH2 domain, a motif not present in any macaque subclass or other human subclasses [instead—LLGGP, for all but human IgG4; accession numbers: [human] P01857; P01859; P01860; P08161 [rhesus macaque] see (79)], that impairs binding to FcγRI and accounts for this observation (136). Interestingly, human IgG4 has a slightly altered motif (FLGGP) at this position, which may explain the intermediate recognition of this subclass.

The high degree of FcγRI conservation between humans and macaques imposes fewer restrictions on the comparison process than is the case for other receptors handled in this review. IgG1, which is perhaps the most likely candidate for comparison due to its prevalence *in vivo* and among licensed therapeutics (137–139), is a particularly straightforward case

TABLE 2 | Expression of members of Fcγ receptor family by immune cell subsets in humans and macaques.

Lineage	Role	Cell type	Species	FcγRI	FcγRIIa	FcγRIIb	FcγRIIc	FcγRIIIa	FcγRIIIb
Myeloid	Granulocytes	Neutrophils	*H. sapiens*	I	+	+ / −	+	−	+
			M. fascicularis	ND	○	○	−	−	−
		Eosinophils	*H. sapiens*	ND	+	−	ND	−	+
			M. fascicularis	ND	○	○	−	−	−
		Basophils	*H. sapiens*	−	+	+	ND	−	+
			M. fascicularis	ND	○	○	−	−	−
		Mast Cells	*H. sapiens*	I	+	−	+/−	−	+
			M. fascicularis	ND	○	○	−	−	−
	Phagocytes	Monocytes/Macrophages	*H. sapiens*	I	+	+/−	−	+	−
			M. mulatta	+	○	○	−	+	−
		Dendritic Cells	*H. sapiens*	+	+	+	−	−	−
			M. mulatta	+	○	○	−	−	−
		Platelets	*H. sapiens*	ND	+	−	−	ND	ND
			M. mulatta	ND	ND	ND	−	ND	−
Lymphoid		B Cells	*H. sapiens*	−	−	+	+	−	−
			M. mulatta	−	○	○	−	−	−
		T Cells	*H. sapiens*	−	−	+/−[1]	−	+/−[2]	−
			M. mulatta	−	○	○	−	−	−
		Natural Killer Cells	*H. sapiens*	−	−	−	+	+	−
			M. mulatta	−	○	○	−	+	−

○ Positive for FcγR2 but a/b not determined.
I, Inducible by interferon γ.
ND, Not determined.
+ / − Expressed by a low percentage of cells, or by a specific subset.
Subset-specific expression is:
(1) FcγRIIb has been observed on human memory CD8$^+$ T cells (121).
(2) FcγRIIIa has been observed on activated human CD4$^+$ T cells (122).
References: (72, 117, 119–135).

and FcγRI-bearing macaque cells are expected to recognize a passively transfused human antibody near natively. In the case of human IgG2 and IgG4 however, there is evidence to suggest that while macaque FcγRI mirrors the human receptor's hierarchy of subclass preference outlined above, the receptor's affinity may be up to 4-fold stronger than in humans (77, 100). Since human IgG2 and IgG4 Fc domains have been used for monoclonal antibodies in instances where a silent Fc profile is desired (139), as well as for extending the half-life of therapeutic proteins by expression as Fc fusions, the heightened inflammatory potential of these molecules when evaluated in a macaque model may need to be taken into account.

FcγRIIa

A sharp increase in complexity, both with and between species, occurs when surveying the most widely expressed activating receptor—FcγRIIa (140). This receptor plays a central role in the phagocytosis of antibody-opsonized antigens, which it triggers by means of an intrinsic ITAM located in its cytoplasmic domain (110).

Humans express two major allelic variants of FcγRIIa, which are distinguished by the identity of the amino acid found at position 131 of the extracellular domain (141). Expression of an arginine at this location results in weakened binding of IgG, particularly for IgG2 (142). Conversely, the histidine-containing variant demonstrates substantially higher affinity across multiple subclasses, including IgG2, and can effectively bind immune complexes composed of this subclass (85, 143). The consequences of this polymorphism can be dramatic. In the treatment of cancer, multiple monoclonal antibodies indicated for a range of malignancies have demonstrated differing responses based on the allelic makeup of the recipients (144). Protection against extracellular bacteria, often mediated by IgG2 targeting polysaccharide antigens, can also be impaired by the lower affinity allele; this may lead to an increase in the frequency and severity of certain infections among R131 individuals, particularly those homozygous for the allele (145–147). In the context of HIV, infected individuals that are homozygous for the lower affinity R131 variant experience a more rapid decline in CD4$^+$ T cells than subjects having one or two copies of the high affinity H131 allele (148). Interestingly, no impact of this polymorphism on risk of infection was observed in the Vax004 HIV vaccine trial (149).

In macaques, a considerably greater number of major allelic variants have been characterized. Based on records in GenBank, numerous variants of this receptor have been defined in rhesus, pigtailed and cynomolgus macaques. While H131 is the minor allele in several human populations (150, 151), most macaque FcγRIIa alleles feature a histidine at this position and therefore

resemble the high affinity human receptor in sequence and antibody recognition (100). The FcγRIIa-4 allele of rhesus macaques is an important exception to this trend, having a proline substitution at this residue that is likely detrimental to the receptor's affinity for IgG (100). Similarly, although the FcγRIIa-1 allele includes the critical histidine, it also contains an additional N-linked glycan motif at position 128, a residue that contacts the Fc region of IgG in the cocrystal structure of human proteins (152), which apparently interferes with the receptor's IgG affinity (100). The consequence of these differences is that alleles 1 (at least when produced recombinantly) and 4 have reduced affinity for macaque IgG compared to alleles 2 and 3 (100), creating high and low affinity variation analogous to the R131/H131 polymorphism in humans. Although the exact allelic frequencies have not been reported, the allotypic variants have been roughly numbered by prevalence (100). Accordingly, the FcγRIIa-1 allele is expected to occur most frequently, and its impact on passive transfer experiments and vaccine studies could prove to be substantial, provided that the native receptor is glycosylated similarly to the recombinantly-produced receptor utilized in *in vitro* studies. Relevant to the potential for differences between macaque and human FcγRII to drive differences in outcomes of antibody infusions between species, it is clear from evaluation of the binding profiles of engineered IgG sequence variants across human and rhesus receptors, that greater phenotypic differences are observed for FcγRII as compared to FcγRIII between species (153).

Receptor variation is not limited to the extracellular domain of FcγRIIa. While the four reported receptor alleles of rhesus macaques are identical intracellularly, they differ from the human and cynomolgus macaque variants in the sequence of their signaling domains. The activating ITAM motif takes the form of two semi-conserved sequences separated by six to eight amino acids. In the available sequences reviewed ([human] P12318; [rhesus] H9BMP0; [cynomolgus] Q8SPW4), the first ITAM unit takes the form Tyr-Met-Thr-Leu. Rhesus macaques repeat this sequence for the second iteration of the motif. Humans and cynomolgus macaques on the other hand use the slightly altered sequence of Tyr-Leu-Thr-Leu (154). This is in addition to a handful of residues flanking the second ITAM that are conserved among the available macaque sequences, but that differ when compared to the human receptors. The consequences of these substitutions, if any, are not known.

The comparison of sequence diversity alone is not sufficient to capture all of the differences between human and macaque FcγRIIa; among other factors, the patterns of expression must also be accounted for when translating observations between species. In humans, FcγRIIa is the most commonly expressed Fc receptor of monocytes and macrophages (155), and is also expressed by dendritic cells and granulocytes. Analysis of expression in macaques has been complicated by the fact that the extracellular domain of FcγRIIa and the inhibitory FcγRIIb receptor have even more highly conserved amino acid sequences (95%) than is the case in humans (92%) (100) ([human] P12318 vs. P31994; [rhesus] H9BMP0 vs. F7GVR0; [cynomolgus] Q8SPW4 vs. Q8SPW3). Difficulty in obtaining flow cytometry reagents capable of reliably discriminating between the receptors has meant that much of the literature published to date omits the specific form of the receptor expressed by a given cell type, reporting instead only the presence or absence of FcγRII (156). Even so, there is data that suggests that cynomolgus and pigtail macaque neutrophils express 3–5 fold more total FcγRII than their human counterparts (77, 156), which are known to express only the activating form of the receptor (132). Given this known difference in expression levels and additional differences potentially associated with activating vs. inhibitory and allotypic FcγRII composition, there is the potential for altered neutrophil biology in non-human primate model systems.

FcγRIII

In many of the studies summarized in **Table 1**, subjects producing antibodies capable of carrying out directed killing of virally-infected cells through the process of antibody-dependent cell-mediated cytotoxicity (ADCC) experienced significantly better clinical outcomes. In macaques and most, but not all, humans, the cell type most commonly associated with performing ADCC, natural killer (NK) cells, exclusively express FcγRIIIa, making the receptor a potent potential contributor to antiviral effector function. Humans express two major allelic variants of FcγRIIIa that are distinguished based on differing levels of activity attributed to a single polymorphic residue in the extracellular domain—position 158 in this case (157, 158). Expression of a valine at this position results in greater affinity for human IgG1 and IgG3, and to a lesser extent IgG4, as compared to the phenylalanine-containing variant (85). Although it is found less frequently in the population (159), the V158 allele can confer significant benefits. For example, V158 individuals respond more strongly to anti-tumor monoclonal antibody therapy, an observation attributed to enhanced ADCC directed against the tumor cells (160–162).

The extracellular domain of rhesus macaque FcγRIIIa also exhibits a polymorphism at position 158. However, the isoleucine expressed by the FcγRIIIa-1 and−2 alleles has similar side chain chemistry to the valine of FcγRIIIa-3 and as a result, the extracellular domains of known rhesus alleles resemble the high affinity human receptor in both sequence and affinity (153). Interestingly, the FcγRIIIa-2 allele, which is identical to FcγRIIIa-1 extracellularly but carries a pair of polymorphisms that introduce valine residues to the transmembrane domain and membrane-proximal region of the cytoplasmic domain (positions 229 and 233, respectively), is associated with more complete depletion of B cells following treatment with rituximab (163). More broadly, the amino acid sequences of the non-human primates surveyed maintained > 90% homology to humans in their extracellular domains. In each of the macaque sequences though, there is an altered residue that leads to the elimination of a N-glycan motif known to be modified in humans. This is potentially offset by the introduction of a new motif close by, but it is not known whether this new site is accessible for glycosylation.

Based on biophysical and functional data gathered thus far (77, 100, 153), one can reasonably conclude that the macaque forms of the receptor are a fair approximation of the high affinity

human allele, FcγRIIIa V158. Unfortunately, the prevalence of the lower affinity F158 allele in humans exceeds that of the high affinity allele (164–166). All else being equal, this difference in allelic frequencies suggests that most humans may be likely to generate a less robust ADCC response than a macaque would based on the lower affinity of their FcγRIIIa receptor. Significantly, human polymorphic variation at this position has been associated with rate of infection among low risk HIV vaccine recipients, and development of Kaposi's sarcoma, an AIDS defining illness, in HIV infected men (148, 167).

Human granulocytes uniquely express a variant of FcγRIII that is apparently not found in any species of macaque—FcγRIIIb (168). The receptor's homology to the extracellular domain of human FcγRIIIa and the weight of experimental evidence generated to date argue for the inclusion of FcγRIIIb alongside the activating Fcγ receptors. This classification has been far from straightforward however (169), in part because FcγRIIIb uniquely lacks both a transmembrane domain and cytoplasmic signaling domains, instead being tethered to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor (168). Even so, upregulation of FcγRIIIb in response to engagement has been observed as well as an association with Syk signaling in neutrophils (170). In order to transduce such signals, FcγRIIIb would require an accessory signaling element, the proposed candidates for which include the complement receptor (171) and FcγRIIa (172). The latter receptor has been hypothesized to act in synergy with FcγRIIIb to increase the flux of Ca^{2+} into granulocytes and enhance their effector functions (173), which include the release of cytotoxic granules (174) and phagocytosis (175, 176). Conversely, a competing view of FcγRIIIb holds that the receptor merely serves as a decoy that competes with activating receptors for immune complexes. Experimental support for this model lies in the observation that enhancing the affinity of IgG for FcγRIII broadly resulted in lower levels of neutrophil ADCC (177). This alternate hypothesis raises the possibility that human granulocytes may have a means to receive an inhibitory input that is not available to macaque effector cells, potentially further skewing signaling in neutrophils.

INHIBITORY RECEPTOR

FcγRIIb

All humans and macaques express a single form of inhibitory FcγR—FcγRIIb. The receptor contains an intrinsic ITIM in its cytoplasmic domain that allows it to rein in immune responses. FcγRIIb is abundantly expressed by phagocytic cells (120, 132) and is typically the sole Fcγ receptor found on human B cells (132, 133). It is additionally expressed by liver sinusoidal endothelial cells, where it is thought to play an role in recognition and uptake of immune complexes (178, 179). Evaluation of the role of this receptor on immune cells has been especially hampered by the limited availability of staining reagents capable of discriminating FcγRIIa from FcγRIIb. In humans, an FcγRIIb-specific reagent is available (132), however, its specificity toward macaque receptors has not been reported. All major human variants of FcγRIIb feature identical extracellular domains and contain an arginine at residue 131 (P31994), making them phenotypically more similar to the low affinity variant of the activating FcγRIIa (85, 153). Of the two human alleles which have been characterized, the rarer variant contains an isoleucine to threonine mutation in the transmembrane domain, at residue 232. This change renders the receptor incompatible with inclusion in lipid rafts and as a result, unable to signal (180, 181).

In contrast to humans, both reported alleles of rhesus macaque FcγRIIb feature a histidine at position 131 and therefore more closely resemble the high affinity variants of FcγRIIa. This is superceded, however, in the FcγRIIb-2 allele by a leucine to proline substitution at residue 88, a site immediately adjacent to a predicted Fc contact residue, which abrogates the receptor's affinity for all subclasses of both rhesus macaque and human IgG (100). While this variant is the less common of the two alleles, its precise frequency has yet to be reported. However, it is possible that the inclusion of FcγRIIb-2 animals in a study could have significant consequences. Intracellularly, FcγRIIb is well-conserved between humans and macaques; the cytoplasmic domains differ at just two residues ([human] P31994; [rhesus] F7GVR0; [cynomolgus] Q8SPW3). Of these differences, one is a conservative change from isoleucine in humans to valine in rhesus macaques. Furthermore, neither of the mutations occurs proximal to the signaling motif.

That FcγRIIb is the sole inhibitory Fc receptor in humans and macaques means the consequences of the interspecies FcγRIIb differences have the potential to be far-reaching. For example, according to one proposed model of SIV and HIV infection, FcγRIIb engagement at the mucosa somewhat paradoxically has been suggested to contribute to protection by generating an inhibitory signal that slows the migration of $CD4^+$ T cells to the region and deprives the virus of host cells for replication (63). However, the high affinity phenotype of macaque FcγRIIb could mean that the receptor stands on an equal footing (or greater, in the case of FcγRIIa alleles 1 and 4) with the activating receptors in the competition for immune complexes—a scenario that is not recapitulated in humans where even FcγRIIa-R131 has higher affinity for IgG than FcγRIIb (85, 100).

ADDITIONAL RECEPTORS

FcγRIIc

In ∼20–45% of humans (182, 183), an unequal genetic crossover between the activating cytoplasmic domain of FcγRIIa and the extracellular domain of FcγRIIb results in the expression of the receptor FcγRIIc (184) on B cells and natural killer cells (134, 135). FcγRIIc provides an additional means of activating immune cells and is capable of triggering ADCC in natural killer cells (185). In the context of HIV infection, the receptor is noteworthy based on the observation that a single nucleotide polymorphism located in an intron of FcγRIIc was associated with greater vaccine efficacy in the RV144 trial (186). A similar receptor has not been reported in macaques.

Neonatal Fc Receptor (FcRn)

The similarities between macaques and humans that make these non-human primates a tractable model for the study of HIV also make them useful for determining the pharmacokinetic

properties of biologic drugs. Cynomolgus macaques in particular are a widely utilized species for the evaluation of antibodies and Fc fusion proteins (187), biologics that depend heavily on FcRn to achieve half-lives that can extend beyond 3 weeks (73, 188–191).

FcRn is a heterodimeric protein composed of a β_2-microglobulin (β_2m) subunit and an MHC class I-like heavy chain (192–194). Diverse tissues and cell types express FcRn (195–198), but it functions principally in intestinal epithelia, vascular endothelia, and syncytiotrophoblasts (199–203) where the receptor's ability to bind to the Fc portion of IgG in a pH-dependent manner, having high affinity under acidic conditions (pH $<$ 6.5) and relatively low affinity at a physiological pH of 7.4 (204–206), permits the uptake, transfer, and release of IgG across compartments of the body. This property of FcRn enables the maternofetal transfer of IgG across the placenta (207) and the absorption of IgG present in maternal milk by the intestinal epithelium of neonatal rodents (204, 208). FcRn's high level of expression in vascular endothelial cells leads to the sorting and recycling of endocytosed IgG from acidified endosomes, where the receptor's affinity for IgG is high, to circulation, where the physiological pH causes the release of IgG (73, 189–191). Additionally, FcRn can contribute to the accelerated processing of antigens derived from multivalent immune complexes and their subsequent presentation by both forms of major histocompatibility complex (74, 75).

The heavy chain of FcRn is well-conserved among the macaque species commonly used in biomedical research; the most frequent variants are identical to one another (accession numbers: NM_001257520, NM_001284551, and XM_011768102). Among the ten residues that differ between humans and macaques, none occur in a location known to contact the Fc, or in the receptor's N-linked glycan sequon (209). Similarly, of the four polymorphisms observed in cynomolgus macaques, only two occur in the extracellular domain and neither involves putative IgG Fc contact residues (210). Interestingly, a promoter polymorphism in FcRn has been identified to associate with reduced levels of serum IgG, though the impact of this polymorphism on the half-life or biodistribution of passively administered mAb or polyclonal samples remains to be defined (211).

The β_2-microglobulin subunit that makes up the remainder of the heterodimeric receptor is somewhat more diverse than the heavy chain, both between species of macaques and when macaques are compared to humans; the N-terminal isoleucine required for interaction with IgG (212) is present in all species under review, and the impact of sequence diversity in this protein has been less well-studied in all species.

CONCLUSIONS

The sheer number of factors to consider when translating observations between macaques and humans makes the process a challenging, multidimensional one. Differences in the structures and activities of IgG subclasses, and polymorphisms in protein sequence and post-translational modification of antibody receptors are a subset of the many relevant considerations. Copy number variation, splice variants, and alleles with sequence variation outside of coding regions have been associated with a diversity of phenotypes in humans (183, 213–217), and are presumed to exist in NHP. A number of differences in the patterns of cellular expression of FcγRs between species are well-established, and while genetically-associated differences in expression levels likely exist, they have not been fully characterized. Linkage disequilibrium between FcRs and a diversity of major and minor alleles make it difficult to define the potential effect of these polymorphisms with high confidence even in large cohorts. Thus, the process of precisely translating results between species is a complex and potentially intractable challenge. However, the highly significant contributions to drug development demonstrates that macaques can nonetheless serve as an excellent preclinical model.

When considering exceptional scenarios, several simplifying assumptions may help to smooth the process. The first—treating the members of the macaque genus largely interchangeably—has already achieved widespread adoption (156) as the shifting landscape of cost, conservation, and ethical considerations have driven changes in the decades since the model's introduction in the preferred macaque species, and among NHP more generally, for biomedical research (218). In light of the considerable similarities between macaque species (including shared allotypic diversity) outlined in this review, it is our view that under common research circumstances, this practice serves as a useful simplifying assumption; however, exceptions do exist. For example, evaluation of passive infusion of human IgG4 in NHP is one such example. Human IgG4 can undergo Fab arm exchange with endogenous human IgG4, but would not exchange with any serum antibody in some, though not all macaque species. Similarly, outcomes of vaccination studies in humans have been associated with the subclasses of antibodies induced. However, the extensive differences between the repertoire of subclasses in humans and the relatively more monolithic structural and activity profiles of NHP IgG types may pose challenges to easy translation of vaccine studies. These differences may preclude attempts to model means to induce specific subclasses by altered regimens, adjuvants, and immunogens, or to explore the relevance of class-switching patterns observed in humans to challenge outcomes.

Of the cell types and FcγR-mediated immune processes potentially impacted by the differences in human and macaque biology, two in particular stand out as deserving of additional comment. Unlike the low affinity phenotype of human FcγRIIb, the inclusion of a histidine at a critical contact residue in macaques grants equivalent affinity (or greater, in the case of alleles 1 and 4) to that of the activating FcγRIIa. While this has the potential to alter the ratio of activating and inhibitory signals relative to what is observed in humans for any cell type expressing a mix of receptors, the effect could be even more pronounced in B cells where FcγRIIb is the only member of the family expressed.

Neutrophil biology is another aspect of macaque immunity likely to differ significantly from that of humans based on differences in Fcγ receptors. The reasons for this are twofold. The first is the substantially higher level of expression of

FcγRII receptor that has been observed on macaque neutrophils. This expression profile potentially contributes to a stronger response by these cells in macaques, although this assumes that macaque neutrophils, like those of humans, express FcγRIIa, and do not express inhibitory the FcγRIIb, which could offset the larger number of activating receptors. The second major difference is the unique expression of FcγRIIIb, which is absent from macaques, by human neutrophils, although the effect of expression of this receptor is more difficult to determine as its role has not been definitively determined.

The recent history of biomedical research well-illustrates the utility of the members of the macaque genus for modeling human diseases and evaluating therapeutic interventions. Much of the progress left to be made in understanding the non-human primate model resides on the margins and need only be considered under particular circumstances. However, consideration of the interspecies variability that has been defined to date and the gaps in our present understanding, if filled, have the potential to make macaques an even more enabling model organism. These insights in turn may increase the rate at which pre-clinical observations are converted to clinical success stories.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Chronic HIV-1 Infection Alters the Cellular Distribution of FcγRIIIa and the Functional Consequence of the FcγRIIIa-F158V Variant

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Chronic HIV-infection modulates the expression of Fc gamma receptors (FcγRs) on immune cells and their antibody-dependent effector function capability. Given the increasingly recognized importance of antibody-dependent cellular cytotoxicity (ADCC) in HIV-specific immunity, we investigated the cellular distribution of FcγRIIIa on cytotoxic lymphocytes—natural killer cells and $CD8^+$ T cells—and the effect of the FcγRIIIa-F158V variant on ADCC capacity in HIV-infected individuals ($n = 23$) and healthy controls ($n = 23$). Study participants were matched for F158V genotypes, carried two copies of the *FCGR3A* gene and were negative for FcγRIIb expression on NK cells. The distribution of $CD56^{dim}FcγRIIIa^{bright}$ and $CD56^{neg}FcγRIIIa^{bright}$ NK cell subsets, but not FcγRIIIa surface expression, differed significantly between HIV-1 negative and HIV-1 positive donors. NK cell-mediated ADCC responses negatively correlated with the proportion of the immunoregulatory $CD56^{bright}FcγRIIIa^{dim/neg}$ cells and were lower in the HIV-1 positive group. Intriguingly, the FcγRIIIa-F158V variant differentially affected the NK-mediated ADCC responses for HIV-1 negative and HIV-1 positive donors. Healthy donors bearing at least one 158V allele had higher ADCC responses compared to those homozygous for the 158F allele (48.1 vs. 34.1%), whereas the opposite was observed for the HIV-infected group (26.4 vs. 34.6%), although not statistically significantly different. Furthermore, $FcγRIIIa^+CD8^{bright}$ and $FcγRIIIa^+CD8^{dim}$ T cell subsets were observed in both HIV-1 negative and HIV-1 positive donors, with median proportions that were significantly higher in HIV-1 positive donors compared to healthy controls (15.7 vs. 8.3%; $P = 0.016$ and 18.2 vs. 14.1%; $P = 0.038$, respectively). Using an HIV-1-specific GranToxiLux assay, we demonstrate that $CD8^+$ T cells mediate ADCC through the delivery of granzyme B, which was overall lower compared to that of autologous NK cells. In conclusion, our findings demonstrate that in the presence of an HIV-1 infection, the cellular distribution

of FcγRIIIa is altered and that the functional consequence of FcγRIIIa variant is affected. Importantly, it underscores the need to characterize FcγR expression, cellular distribution and functional consequences of FcγR genetic variants within a specific environment or disease state.

Keywords: NK cells, CD8 T cells, antibody-dependent cellular cytotoxicity, Fc gamma receptor, polymorphism, HIV, infection

INTRODUCTION

Receptors for the Fc domain of immunoglobulin G (IgG), so called Fc gamma receptors (FcγRs), link the specificity of IgG with potent effector functions of the innate immune system. FcγRs comprise a family of activating (FcγRI, FcγRIIa, and FcγRIIIa) and inhibiting (FcγRIIb) receptors that are differentially expressed on innate immune cells such as natural killer (NK) cells, monocytes, dendritic cells, neutrophils, and granulocytes (1–3). During an infection, these receptors play an important role in activating IgG-induced protective inflammatory processes and regulating immune responses (4–7).

Increasing evidence support an important role for FcγR-mediated effector functions, in particular antibody-dependent cellular cytotoxicity (ADCC), in HIV-1-specific immunity (6, 8). In adults, ADCC has been associated with a reduced risk of HIV-1 acquisition in the RV144 vaccine trial, whereas in infants born to HIV-1 infected mothers, passively acquired ADCC activity associated with reduced mortality (9). Similarly, ADCC responses associate with slower disease progression in HIV-1 infected adults (10–14).

In the periphery, $CD56^{dim}$FcγRIIIa^{bright} NK cells are the primary effectors of ADCC responses (15). During a chronic HIV-1 infection, however, shedding of FcγRIIIa from the surface of cytotoxic $CD56^{dim}$FcγRIIIa^{bright} NK cells together with an increase in the $CD56^{neg}$FcγRIIIa^{bright} NK cell subset leads to a significant reduction in NK cell function (16, 17). It is unknown whether this loss in NK cell-mediated ADCC capacity is compensated for by other cytotoxic cells, where FcγR expression is induced upon immune cell activation. For chronic hepatitis C virus or Epstein Barr virus infections, for instance, it has been shown that FcγRIIIa expression is induced on an effector memory $CD8^+$ T cell subset (18, 19). This FcγRIIIa^+CD8^+ T cell subset acquires NK cell-like functional properties, including ADCC activity accompanied by the release of pore forming perforin and serine protease granzyme B (18, 19). The presence and role of this $CD8^+$ T cell subset in HIV-1 infection is currently undefined.

In addition to the effect of an actively replicating virus on FcγRIIIa expression, host genetics also contribute to variability of FcγRIIIa expression and/or ADCC responses. *FCGR3A* copy number variation directly correlates with the surface density of FcγRIIIa, with individuals bearing a single *FCGR3A* copy and correspondingly lower FcγRIIIa surface densities, having reduced ADCC responses compared to individuals with two or more gene copies (20). In addition, a phenylalanine (F) to valine (V) substitution at amino acid 158 in the proximal Ig-like domain of FcγRIIIa confers increased binding for IgG1, IgG3, and IgG4, which has been associated with higher NK cell activation and ADCC responses (21–23). Unlike *FCGR3A* copy number and the FcγRIIIa-F158V variant, a deletion of a copy number variable region (CNR) encompassing *FCGR3B* and *FCGR2C*—known as CNR1—does not affect FcγRIIIa directly (24). However, it juxtaposes the 5′-regulatory sequences of *FCGR2C* with the open reading frame of *FCGR2B*, creating a chimeric *FCGR2B'* gene (25). This results in the expression of the inhibitory FcγRIIb on NK cells where it regulates FcγRIIIa-mediated ADCC responses (25, 26).

FCGR variants are rarely adjusted for in studies that compare NK cell-mediated ADCC capacity between HIV-positive and HIV-negative individuals. Moreover, it is unclear if the altered immune milieu accompanying an HIV-1 infection modulates the functional consequences of the aforementioned variants. In this study, we sought to characterize FcγRIIIa expression on cytotoxic lymphocytes—NK cells and $CD8^+$ T cells—and associated ADCC responses in healthy donors and viraemic HIV-1 individuals matched for *FCGR* genetic variants.

MATERIALS AND METHODS

Cohort

All study participants were black South Africans recruited from the city of Johannesburg, Gauteng province, South Africa (**Table 1**). Self-reported HIV-1 uninfected individuals who did not have an acute or chronic illness at the time of sample collection were prospectively recruited from the National Institute for Communicable Diseases as healthy controls. Viraemic, treatment naïve HIV-1 infected individuals were identified from an existing cohort recruited from hospitals in Johannesburg and Soweto. This study was carried out in accordance with the recommendations of the National Health Research Ethics Council (NHREC) of the South African Department of Health. The protocol was approved by the University of the Witwatersrand Ethics Committee (Ethics clearance certificate no. M1511102). All participants provided written informed consent in accordance with the Declaration of Helsinki.

Abbreviations: NK, natural killer cell; IgG, immunoglobulin G; HIV-1, human immunodeficiency virus 1; ADCC, antibody-dependent cellular cytotoxicity; *FCGR*, Fc gamma receptor gene; FcγR, Fc gamma receptor protein; CNR1, gene copy number variable region 1; EDTA, ethylenediaminetetraacetic acid; HNA, Human neutrophil antigen; MLPA, multiplex ligation-dependent probe amplification; FMO, flurescence minus one; PBMCs, peripheral blood mononuclear cells; KIR2DL2/L3, killer immunoglobulin-like receptor 2 DL2/L3; NKG2A/D, natural killer group 2A or−2D.

TABLE 1 | Clinical and demographic characteristics of study cohort.

	HIV-1 negative	HIV-1 positive	*P*-value
N	23	23	
Age [mean (SD)]	36.3 [8.5]	40.2 (8.8)	0.143*
Gender [% Females]	69.6	82.6	0.491*
HIV-1 VIRAL LOAD [MEDIAN (IQR)]			
158FF	–	6,931 (3,598–15,090)	0.563†
158FV/VV	–	4,732 (1,134–11,430)	
CD4 T CELL COUNT [MEAN (*SD*)]			
158FF	–	508 (267)	0.629†
158FV/VV	–	562 (261)	
OTHER DISEASES OR INFECTIONS			
Mycobacterium tuberculosis	0 (0%)	2 (8.7%)	

*Comparison between HIV-1 negative and HIV-1 positive groups.
†Comparison between genotypes.

FCGR Variant Genotyping

Genomic DNA was isolated from ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood. Study participants were genotyped for *FCGR* variants using the *FCGR*-specific multiplex ligation-dependent probe amplification (MLPA) assay (MRC Holland, Amsterdam, The Netherlands) as previously described (27, 28). This assay detects the genomic copy number of *FCGR2A*, *FCGR2B*, *FCGR2C*, *FCGR3A*, and *FCGR3B* and functional allelic variants that include FcγRIIa-H131R (alias H166R, c.497A>G, rs1801274); FcγRIIb-I232T (c.695T>C, rs1050501), FcγRIIIa-F158V (alias F176V, c.634T>G, rs396991), FcγRIIIb-HNA1a/b/c, *FCGR2C* gene expression variants c.169T>C (X57Q) and c.798+1A>G (rs76277413), and the *FCGR2B/C* promoter variants c.-386G>C (rs3219018) and c.-120T>A (rs34701572) in two multiplex reactions. Capillary electrophoresis was used to separate the MLPA assay amplicons on an ABI Genetic Analyzer 3500. Data were analyzed with Coffalyser.NET software created by the MLPA assay manufacturer, MRC Holland.

Monoclonal Antibodies and Flow Cytometric Analysis

The following monoclonal antibodies were used: CD3-PerCP (SK7), CD56-AF647 (B159), CD8-APC-H7 (SK1), CD16-PE (3G8), CD16-FITC (NKP15), CD32-FITC (2B6), and CD32-PE (FLI8.26). All antibodies were obtained from BD Biosciences (San Jose, CA). Dead cells were labeled with the BD Horizon™ Fixable Viability Stain 510. Samples were acquired on a BD Fortessa X20 (BD biosciences) and analyzed on FlowJo version 9.8.1 software (Tree Star, San Carlos, CA). Fluorescence-minus-one (FMO) controls were used to set the appropriate gates for analyses.

Isolation of Specific Cell Populations

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated whole blood using Ficoll-Paque™ PLUS density gradient centrifugation (GE Healthcare) and stored at −80°C. NK cells and $CD8^+$ T cells were positively selected from overnight rested PBMCs using MACS® magnetic cell separation technology (Miltenyl Biotec). To limit the presence of NK cells in the enriched $CD8^+$ T cell preparation, NK cells were first isolated from PBMCs using $CD56^+$ beads prior to isolation of $CD8^+$ T cells with $CD8^+$ beads. Viability and number of each participant's NK and $CD8^+$ T cells were determined by trypan blue exclusion through direct cell counting on a haemocytometer. The purity of enriched cell fractions was determined by flow cytometric phenotyping assays.

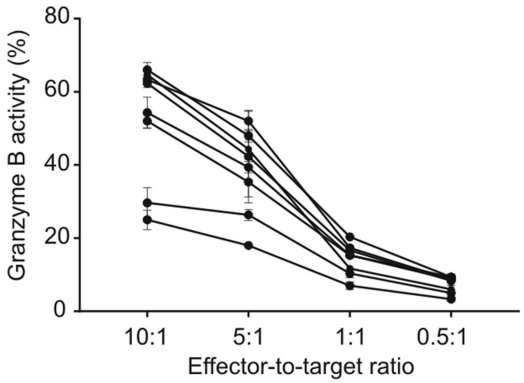
Antibodies

ADCC capacity was evaluated using pooled IgG isolated from the HIV-1 infected individuals included in the study. IgG was isolated from plasma using the Melon Gel IgG Purification kit according to the manufacturer's instructions (Thermo Scientific) and quantitated using the Bicinchoninic acid (BCA) assay. HIV immune globulin (HIVIG; NIH AIDS reagent program) was used as a positive control.

ADCC

NK cells and $CD8^+$ T cells both utilize the pore forming perforin and serine protease granzyme B during cytotoxic responses. It is therefore possible to evaluate ADCC responses of both NK cells and $CD8^+$ T cells with the HIV-specific GranToxiLux assay (15). In brief, CEM.NK^R.CCR5 cells were coated with recombinant HIV-1 ConC gp120, followed by opsonisation with HIV-1-specific antibodies and incubation for 45 min in the presence of NK cells or $CD8^+$ T cells at an effector-to-target (E:T) ratio of 10:1. The optimal concentration of isolated IgG (30 μg/ml) was determined empirically. Samples were acquired on BD Fortessa X20 flow cytometer and data analyzed on FlowJo version 9.8.1 software (Tree Star, San Carlos, CA). Granzyme B activity in the absence of any antibody (background killing) was determined for each subject—NK cells and $CD8^+$ T cells—and subtracted from granzyme B activity in the presence of antibody. The inter assay coefficient of variation, as calculated from a HIVIG-specific ADCC response measured for a single donor included in every run, was <10%.

To assess $CD8^+$ T cell mediated granzyme B responses, HIV-1 positive donors with <5% NK cell contamination in their enriched $CD8^+$ T cell fractions were identified and selected for further analysis. This proportion of NK cells equates to a <0.5:1 NK-to-target ratio in a 10:1 CD8-to-target preparation. In our experience, the NK cell-mediated granzyme B activity at 0.5:1 is on average 14.1% (standard deviation [SD]: 2.8%) of that observed for NK cells at a ratio of 10:1 (**Figure 1**). Using these data, a positivity threshold for $CD8^+$ T cell-mediated granzyme B responses was calculated relative to autologous NK cell-mediated granzyme B responses whereby the mean granzyme B activity for autologous NK cells (E:T = 10:1) was multiplied with 22.4% (14.1% + 3 × SD:2.8%).



	Granzyme B activity		
	1:10	0.5:1	0.5:1 / 1:10
Donor 1	66%	9%	0.136
Donor 2	62%	8%	0.129
Donor 3	30%	5%	0.167
Donor 4	65%	6%	0.092
Donor 5	52%	9%	0.173
Donor 6	54%	9%	0.167
Donor 7	63%	9%	0.143
Donor 8	25%	3%	0.120
		Mean	0.141
		Mean + 3*STD	**0.224**

FIGURE 1 | NK cell-mediated ADCC responses at different effector-to-target (E:T) cell ratios. NK cells isolated from eight HIV-1 negative donors were tested at different E:T ratios in an HIV-specific GranToxiLux assay using an ADCC-mediating monoclonal antibody (A32) at 2.5 μg/ml. The mean fraction of Granzyme B activity observed at an E:T of 0.5:1 relative to 10:1 was used to calculate a 99% confidence level cut-off value for NK cell-mediated ADCC responses at an E:T of 0.5:1. Data points represent the mean of triplicate measures and the standard deviation indicated by error bars.

Statistics

All statistical analyses were performed using GraphPad Prism 7 software version 7.04 (GraphPad Software). *P*-values from 2-tailed tests <0.05 were considered statistically significant. The significance of differences between unpaired data sets were analyzed with the Mann-Whitney U tests and paired data sets with the Wilcoxon matched-pairs signed rank test. The significance of differences between more than two data sets were analyzed using Kruskal-Wallis tests. Correlation analyses of data between two groups were assessed using the non-parametric Spearman rank correlation coefficient. To determine the role of FcγRIIIa-F158V alleles in ADCC responses, the V allele was studied under a dominant model due to the low prevalence of VV homozygotes (29).

RESULTS

Study Population

Thirty-seven chronic HIV-1 infected, viraemic, and antiretroviral naïve individuals with sufficient sample available were identified from an existing cohort of HIV-1 positive individuals (**Figure 2**). Following *FCGR* genotyping, seven individuals were excluded due to the possession of an *FCGR3A* gene duplication ($n = 1$), *FCGR3A* gene deletion ($n = 2$), or CNR1 deletion that results in the expression of the inhibitory FcγRIIb on NK cells ($n = 4$). To ensure that individuals carrying an undetectable CNR1 deletion—possess a duplication of this region on a single chromosome and a deletion on the other—were also excluded, expression of FcγRIIb on NK cells was monitored using flow cytometry. During the eligibility screening of individuals, such an individual was indeed identified and excluded from the study; thus, five HIV-1 positive individuals in total expressed FcγRIIb on their NK cells and were excluded.

Within the HIV-1 positive group, FcγRIIIa-158FF donors were paired with FcγRIIIa-158FV donors according to their HIV-1 RNA plasma viral load. Twenty-three HIV-1 positive individuals were eligible for further analysis, with the FcγRIIIa-F158V genotype distribution closely resembling that observed in the general black population from the same region in South Africa (29). As a comparative group, thirty-three HIV-1 negative individuals were genotyped for *FCGR* variants. Six individuals were excluded due to the possession of an *FCGR3A* duplication ($n = 1$) or CNR1 deletion ($n = 5$). Twenty-three eligible HIV-1 negative individuals were subsequently paired with HIV-1 positive individuals based on the FcγRIIIa-F158V genotype. None of the study participants expressed the activating FcγRIIc on their NK cells, as determined by the *FCGR2C* c.798+1A>G splice-site variant, or carried the *FCGR3A* intragenic haplotype previously associated with increased FcγRIIIa surface density (26, 30).

Age and gender did not differ significantly between HIV-1 negative and positive donors (**Table 1**). Two HIV-1 positive individuals had tuberculosis, of which one was an FcγRIIIa-158FF donor and the other an FcγRIIIa-158FV donor. No other infections were noted for these patients. Human cytomegalovirus (HCMV) infection status was not determined for study participants. However, the prevalence is likely 100% for the HIV-1 positive individuals and >85% for HIV-1 negative individuals as observed in other cohorts in rural and urban South Africa [(31) and Tiemessen, unpublished data]. Other sexually transmitted infections were not tested for in the study cohort.

FcγRIIIa Expression on NK Cell Subsets

NK cell subsets were defined based on the relative surface expression of CD56 and FcγRIIIa. Four subsets were identified that include $CD56^{bright}FcγRIIIa^{dim/neg}$, $CD56^{dim}FcγRIIIa^{bright}$, $CD56^{neg}FcγRIIIa^{bright}$, and $CD56^{dim}FcγRIIIa^{dim/neg}$ (**Figure 3A**). The distribution of these subsets within the NK cell population differed significantly between HIV-1 positive and HIV-1 negative donors. Compared to the HIV-1 negative group, the HIV-1 positive group had a smaller median proportion of $CD56^{dim}FcγRIIIa^{bright}$ cells (60.2 vs. 77.0%, $P = 0.0002$, **Figure 3B**) that was offset by a larger median

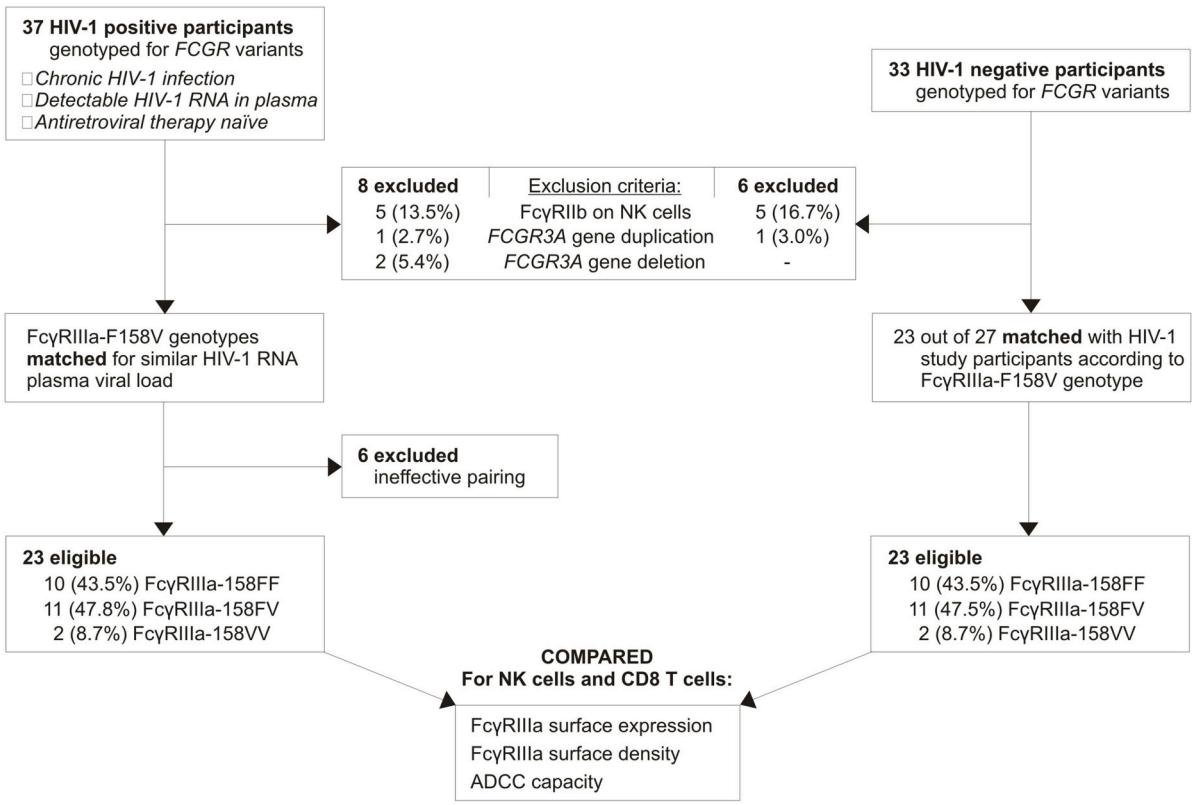


FIGURE 2 | Study participant selection.

proportion of $CD56^{neg}Fc\gamma RIIIa^{bright}$ cells (14.9 vs. 2.1%, $P < 0.0001$, **Figure 3B**). The inverse relationship between the NK cell subsets in HIV-1 positive individuals, but not HIV-1 negative individuals, was further observed in a correlation analysis ($R = -0.633$, $P = 0.001$, **Figure 3F**). The relative proportions of $CD56^{bright}Fc\gamma RIIIa^{dim/neg}$ and $CD56^{dim}Fc\gamma RIIIa^{dim/neg}$ did not differ significantly between HIV-1 positive and HIV-1 negative donors. In contrast to the observed differences in NK cell subsets, FcγRIIIa surface density on neither cytotoxic $CD56^{dim}Fc\gamma RIIIa^{bright}$ nor $CD56^{neg}Fc\gamma RIIIa^{bright}$ NK cells differed between the two groups ($P = 0.948$ and $P = 0.486$, respectively; **Figure 3C**).

NK Cell-Mediated ADCC Responses

The capacity of $CD56^{+}$ NK cells to mediate ADCC was tested in an HIV-1-specific granzyme B assay in the presence of pooled IgG isolated from HIV-1-infected South Africans. ADCC responses, measured as granzyme B activity in target cells, were reduced in the HIV-1 positive group compared to the HIV-1 negative group, although not statistically significantly different (31.0 vs. 43.3%, $P = 0.184$, **Figure 3D**). In both groups, the ADCC responses were affected by the FcγRIIIa-F158V variant (**Figure 3E**). Healthy donors bearing at least one V allele had a higher median NK cell-mediated granzyme B activity compared to those homozygous for the F allele (48.1 vs. 34.1%, $P = 0.284$). This trend was, however, not observed in the HIV-1 positive group. In contrast, HIV-1 positive donors bearing at least one V allele had reduced granzyme B activity compared to those homozygous for the F allele (26.4 vs. 34.6%, $P = 0.522$).

ADCC responses of both HIV-1 negative and HIV-1 positive donors negatively correlated with the proportion of immunoregulatory $CD56^{bright}Fc\gamma RIIIa^{dim/neg}$ cells ($R = -0.486$, $P = 0.019$; and $R = -0.454$, $P = 0.030$, respectively; **Figure 3F**). Furthermore, ADCC responses of HIV-1 negative donors, but not HIV-1 positive donors, positively correlated with the proportion of $CD56^{dim}Fc\gamma RIIIa^{bright}$ cells ($R = 0.665$, $P = 0.0005$; and $R = 0.233$, $P = 0.284$, respectively; **Figure 3F**). FcγRIIIa expression levels on the cytotoxic $CD56^{dim}Fc\gamma RIIIa^{bright}$ cell subset did not correlate with ADCC responses in either HIV-1 negative or HIV-1 positive group.

Expression of FcγRIIIa on $CD8^{+}$ T Cells

During the course of characterizing FcγRIIIa expression on peripheral leukocytes in whole blood obtained from a preliminary cohort of HIV-1 negative and HIV-1 positive donors, we observed a $CD8^{bright}$ T cell subset expressing FcγRIIIa in both groups (**Figure 4**). The proportion of $Fc\gamma RIIIa^{+}CD8^{bright}$ T cells within the $CD8^{bright}$ T cell population varied extensively, ranging from 4.4 to 45.9%, with the median proportion significantly higher in the HIV-1 infected group compared to the healthy control group (17.8 vs. 9.8%, $P = 0.002$; **Figure 4**).

In the present validation study, two $CD8^{+}$ T cell subsets are identified, designated $CD8^{bright}$ and $CD8^{dim}$ (also referred to as

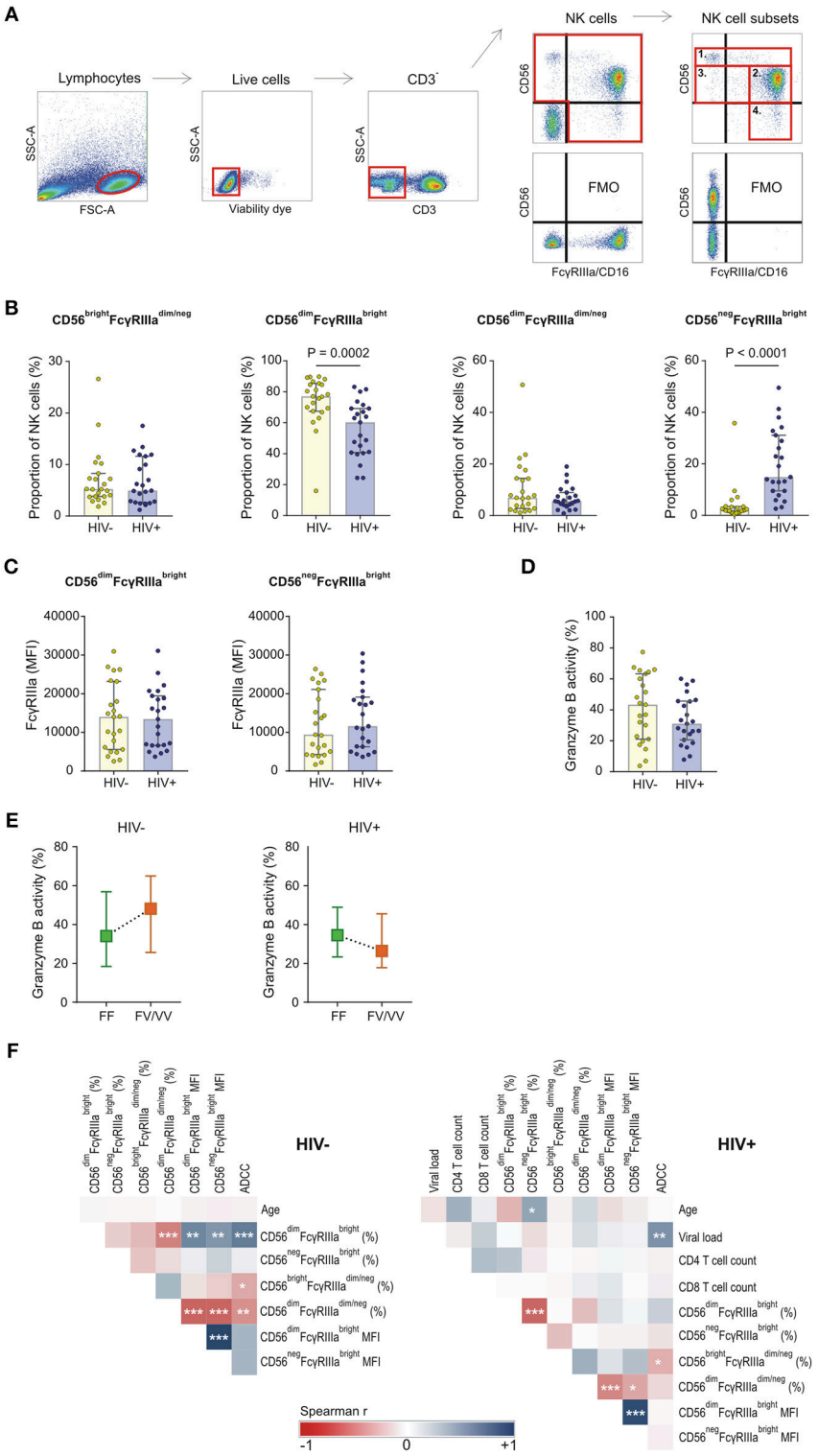


FIGURE 3 | FcγRIIIa expression on NK cell subsets in HIV-1 uninfected and HIV-1 infected individuals matched for *FCGR* genetic variants. **(A)** Gating strategy for defining four NK cell subsets: 1. CD56^{bright}FcγRIIIa$^{dim/neg}$, 2. CD56^{dim}FcγRIIIa^{bright}, 3. CD56^{dim}FcγRIIIa$^{dim/neg}$, and 4. CD56^{neg}FcγRIIIa^{bright}; **(B)** Comparison of NK cell subsets between HIV-1 uninfected and infected individuals; **(C)** FcγRIIIa surface density on FcγRIIIa^{bright} NK cell subsets; **(D)** ADCC activity of NK cells at a target-to-effector cell ratio of 10:1 with isolated IgG pooled from HIV-1 study participants; **(E)** Median ADCC responses for individuals homozygous for the FcγRIIIa-158F allele and individuals bearing at least one FcγRIIIa-158V allele; **(F)** Correlation analysis between demographic, clinical, phenotypic and functional variables in HIV-1 uninfected and HIV-1 infected individuals (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$).

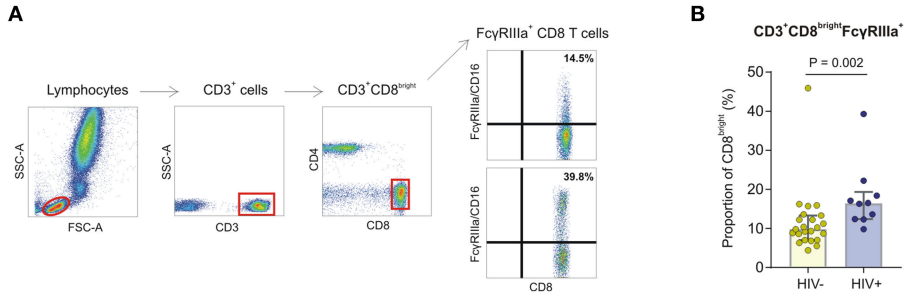


FIGURE 4 | FcγRIIIa expression on CD8 T cells in whole blood obtained from HIV-1 negative and HIV-1 positive donors. **(A)** Gating strategy showing two representative individuals with low and high proportions of FcγRIIIa^{+}CD8^{bright} T cell subsets. **(B)** Frequencies of FcγRIIIa^{+}CD8^{bright} T cells in whole blood isolated from HIV-negative healthy controls ($n = 23$) and HIV-infected individuals ($n = 10$).

CD8^{high} and CD8^{low}), according to the relative expression of CD8 on CD3^{+} T cells (**Figure 5**). The CD8^{dim} subset accounted for 13.9 and 13.4% of the total CD8^{+} T cell population in HIV-1 negative and HIV-1 positive donors, respectively ($P = 0.978$). In agreement with our observations in whole blood, 2–44.8% of CD8^{bright} cells and 2–68.4% of CD8^{dim} cells expressed FcγRIIIa. Compared to its FcγRIIIa^{+}CD8^{bright} counterpart, the FcγRIIIa^{+}CD8^{dim} subset expressed significantly higher levels of FcγRIIIa in both the HIV-1 positive and negative group (471 vs. 1610, $P < 0.001$; and 377 vs. 740, $P < 0.001$, respectively; data not shown).

Compared to the HIV-1 negative group, the HIV-1 positive group had a significantly higher median proportion of FcγRIIIa^{+}CD8^{bright} and FcγRIIIa^{+}CD8^{dim} T cell subsets (15.7 vs. 8.3%; $P = 0.016$ and 18.2 vs. 14.1%; $P = 0.038$, respectively) and correspondingly higher median FcγRIIIa surface densities on both FcγRIIIa^{+}CD8^{+} T cell subsets (471 vs. 377, $P = 0.031$; and 1,610 vs. 740, $P = 0.021$, respectively) (**Figure 5B**). The proportion of FcγRIIIa^{+}CD8^{bright} and FcγRIIIa^{+}CD8^{dim} cells positively correlated in the HIV-1 negative group ($R = 0.671$, $P = 0.001$; **Figure 5C**). However, this relationship was not observed for the HIV-1 positive group. In the latter group, neither the presence nor levels of FcγRIIIa correlated with CD4^{+} T cell count or HIV-1 plasma viral load (**Figure 5C**). However, the proportion of FcγRIIIa on CD8^{bright} T cells negatively correlated with age in both groups, although this correlation was only statistically significant for the HIV-1 positive group ($R = -0.510$, $P = 0.013$; **Figure 5C**).

CD8^{+} T Cells Mediate ADCC

To assess CD8^{+} T cell-mediated ADCC responses, CD8^{+} T cells were positively selected from PBMCs after depletion of NK cells. The proportion of potential contaminating NK cells was determined using flow cytometry and six HIV-1 positive donors were identified with <5% NK cells in their enriched CD8^{+} T cell fractions (mean: 1.3%; range: 0.3–2.9%). A threshold for positive CD8^{+} T cell-mediated granzyme B responses (5.6%) was calculated based on the corresponding NK cell granzyme B responses at an NK-to-target ratio of 10:1 (described in section Materials and Methods). Five out of six donors had positive CD8^{+} T cell-mediated granzyme B responses that ranged from 7.5 to 25.6% (mean: 14.5%; **Figure 5E**). Overall, the CD8^{+} T cell-mediated granzyme B activity was comparatively lower than that observed for their NK cell counterparts ($P = 0.031$).

DISCUSSION

This study set out to comprehensively characterize FcγRIIIa variability and its effect on ADCC responses in HIV-1 positive and HIV-1 negative South African individuals, the population that bears the largest HIV-1 epidemic. Accurate assessment of FcγRIIIa variability requires careful selection of study participants to exclude confounding genetic variables that modify FcγRIIIa expression and FcγRIIIa-mediated cell activation. Study participants within the comparative groups were further matched for the FcγRIIIa-F158V variant that not only alters ADCC responses, but also affects measurements of FcγRIIIa surface expression with the most commonly used anti-FcγRIIIa antibody, clone 3G8 (32).

In comparing a cohort of Black South African HIV-1 negative and HIV-1 positive donors matched for genotypic variants, we confirmed previously described significant differences in the distribution of NK cell subsets, while differences in FcγRIIIa surface density on cytotoxic NK cells were not observed. NK cell-mediated ADCC responses of HIV-1 positive donors were both reduced and differentially affected by the FcγRIIIa-F158V variant compared to HIV-1 negative donors. In addition, FcγRIIIa expression was identified on a subset of cytotoxic CD8 T cells where it potentially contributes to HIV-1-specific ADCC responses in a granzyme B-dependent manner.

In healthy individuals, NK cells typically comprise a dominant CD56^{dim}FcγRIIIa^{bright} population and minor populations that include CD56^{bright}FcγRIIIa$^{dim/neg}$, CD56^{dim}FcγRIII$^{dim/neg}$ and CD56^{neg}FcγRIIIa^{bright}. Perturbation of NK cell subsets in the presence of an HIV-1 infection has been extensively described (16, 17, 33). In HIV-1 infected individuals, the cytotoxic CD56^{dim}FcγRIIIa^{bright} subset contracts with an associated expansion of the CD56^{neg}FcγRIIIa^{bright} subset. The latter is characterized by higher levels of inhibitory NK cell receptors, lower levels of natural cytotoxicity receptors, and reduced secretion of cytokines compared to the CD56^{dim}FcγRIIIa^{bright}

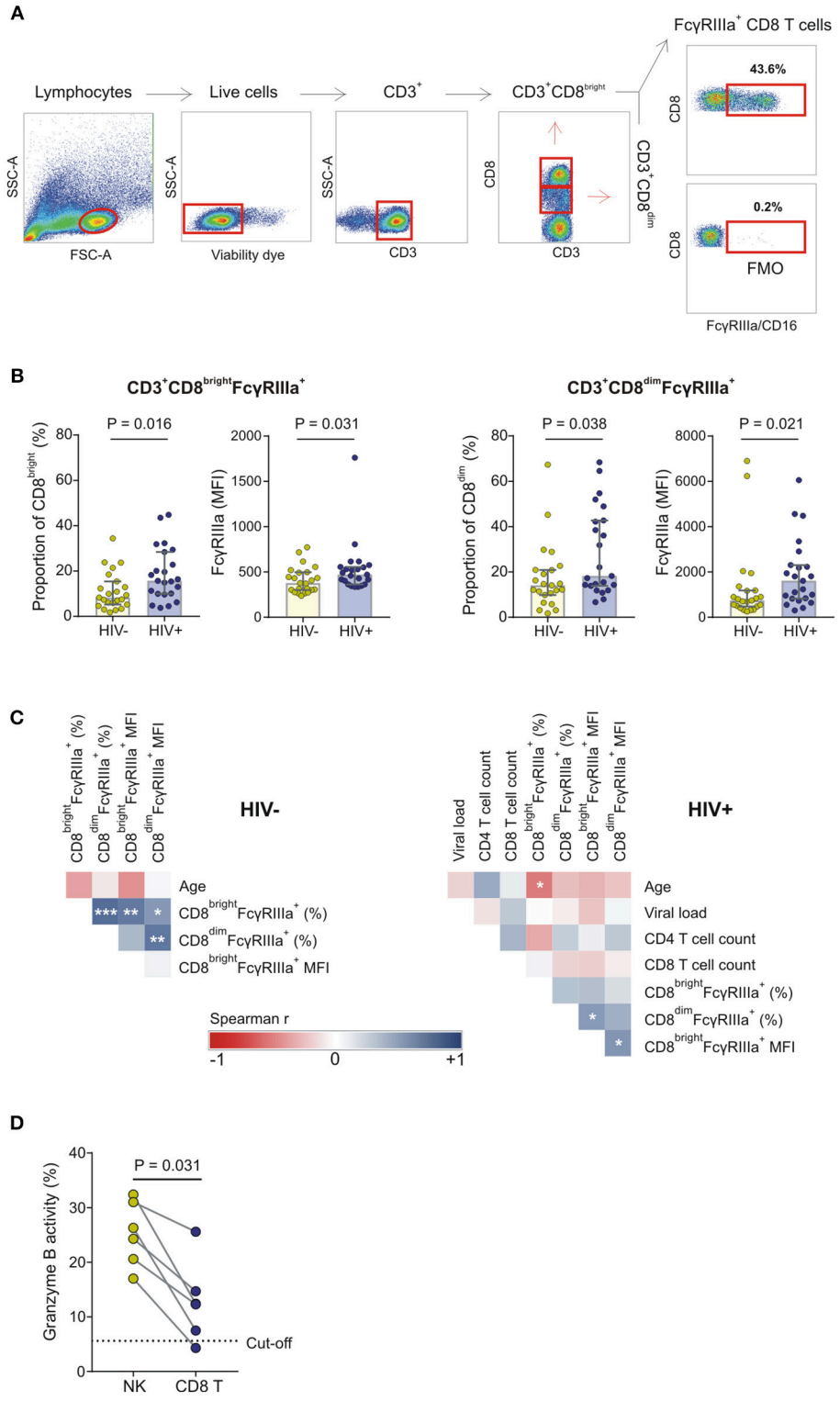


FIGURE 5 | FcγRIIIa expression on CD8 T cells in HIV-1 uninfected and HIV-1 infected individuals matched for *FCGR* genetic variants. **(A)** Gating strategy; **(B)** The proportion of FcγRIIIa^{+}CD8^{bright} and FcγRIIIa^{+}CD8^{dim} T cells and corresponding median fluorescence intensity (MFI) of FcγRIIIa on these FcγRIIIa^{+}CD8^{+} T cell subsets in a cohort of HIV-1 uninfected and infected individuals; **(C)** Correlation analysis between demographic, clinical, phenotypic, and functional variables in HIV-1 uninfected and HIV-1 infected individuals; **(D)** CD8^{+} T cell-mediated ADCC responses of HIV-1 positive donors relative to autologous NK cell-mediated ADCC responses at the same effector-to-target cell ratio (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$).

subset (16, 17). This hyporesponsive NK cell subset was similarly increased in the South African HIV-1 positive cohort; however, it did not associate with HIV-1 viral load as observed by Mavilio et al. (16, 34). The lack of an association with HIV-1 viral load may be explained by an ∼5- to 6-fold lower median HIV-1 plasma viral load of the South African cohort compared to the other cohorts. Moreover, in the present study, NK cell subsets were studied for overnight rested PBMCs as opposed to freshly isolated negatively-selected NK cells.

The dysregulation of NK cell subsets is typically associated with reduced ADCC responses in HIV-1 infected individuals (16, 17, 35, 36). In the present study, lower ADCC responses were similarly observed for HIV-1 infected individuals. Since ADCC capacity was studied for $CD56^+$ NK cells it precluded an analysis of the association between the $CD56^{neg}Fc\gamma RIIIa^{bright}$ subset and ADCC responses, but not for the $CD56^{dim}Fc\gamma RIIIa^{bright}$ and $CD56^{bright}Fc\gamma RIIIa^{dim/neg}$ subsets. A negative correlation observed between the $CD56^{bright}Fc\gamma RIIIa^{dim/neg}$ subset and ADCC responses for both HIV-1 negative and positive donors would suggest that ADCC capacity is similarly affected by the immunoregulatory $CD56^{bright}Fc\gamma RIIIa^{dim/neg}$ subset in both groups. However, as demonstrated by a positive correlation between the cytotoxic $CD56^{dim}Fc\gamma RIIIa^{bright}$ subset and ADCC responses for HIV-1 negative donors, but not HIV-1 positive donors, not all factors modulating ADCC capacity may be shared between healthy individuals and HIV-1 infected individuals.

Given the association of ADCC-mediating antibody responses with HIV-1 protective immunity, it could be hypothesized that genetic determinants of NK cell-mediated ADCC capacity, in particular the FcγRIIIa-F158V variant, may associate with HIV-1 acquisition risk or disease progression. The FcγRIIIa-158V isoform has greater avidity for complexes comprising IgG1, IgG3, and IgG4 than the FcγRIIIa-158F isoform and confers increased NK cell activation and ADCC responses in healthy individuals (21–23). Despite its potential contribution to ADCC responses, the FcγRIIIa-158V isoform is yet to be positively associated with HIV-1 acquisition and disease progression. On the contrary, the FcγRIIIa-158V isoform has been associated with an increased risk of HIV-1 infection (37), disease progression (37), and HIV-1-associated Kaposi's sarcoma (KS) and Cryptococcal disease (38, 39). Furthermore, homozygosity for the FcγRIIIa-158V allele associated with a higher rate of HIV-1 infection among vaccinated men in the VAX004 trial (40), while homozygosity for the FcγRIIIa-158F allele associated with greater protection from HIV-1 disease progression in male participants in the RV144 vaccine trial (41). Taken together, these findings are more indicative of FcγRIIIa-158V-mediated antibody-dependent enhancement of infection rather than improved ADCC responses to the benefit of the individual. Alternatively, it is possible that the functional consequence of the FcγRIIIa-F158V isoforms may be different in the presence of an HIV-1 infection and that a different mechanism(s) may underlie the aforementioned associations.

FcγRIIa/FcγRIIIb polymorphic variants, for example, show distinct differences in oxidative burst responses of resting neutrophils; however, once neutrophils were pre-activated with IFNγ and G-CSF these differences were no longer observed (42). Other non-FcγR genetic variants have also been shown to differentially affect gene expression or cytokine production of activated and resting immune cells (43). In the present study, we show a similar trend for the FcγRIIIa-F158V variant, higher ADCC responses for HIV-1 negative donors bearing the FcγRIIIa-158V allele compared to those homozygous for the FcγRIIIa-158F allele, whereas in HIV-1 positive donors this trend was lost or even slightly reversed. The effect of the variant on ADCC responses was, however, not significant in either group. It is possible that the independent effect size of the FcγRIIIa-F158V variant is too small to detect with the current sample size. Larger cohort studies that adjust for other NK cell activation and inhibitory receptors are required to further define the role of this variant in HIV-1 infection. Nonetheless, these findings may partially explain the inconclusive role of FcγRIIIa genetic variants in HIV-1-specific immunity [reviewed by Cocklin and Schmitz (44)].

In addition to modulating the function of an allelic variant, infection can lead to the induction of FcγRIIIa on other cytotoxic cells, including $CD8^+$ T cells. An $Fc\gamma RIIIa^+CD8^+$ T cell population was first described in the 1980's and has since been characterized in the context of hepatitis C virus and Epstein Barr virus infections (18, 19, 45). These terminally differentiated $CD8^+$ T cells belong to the T effector memory $CD45RA^+$ lymphocyte subset, are perforin positive, directly mediate ADCC *ex vivo*, and increase *in vivo* during hyperlymphocytosis (18, 19). In addition to FcγRIIIa, this cell subset also has increased expression of other NK-like receptors including NKG2A, NKG2D, KIR2DL2/L3 and KIR2DL1/S1 when compared to $Fc\gamma RIIIa^-CD8^+$ T cells (46). The present study validates the expression of FcγRIIIa on cytotoxic $CD8^+$ T lymphocytes and is in agreement with other studies that have consistently showed an increase in the proportion of $Fc\gamma RIIIa^+CD8^+$ T cells in the presence of a virus infection (18, 19, 46). Compared to $CD8^{bright}$ cells, the surface density of FcγRIIIa was significantly higher on $CD8^{dim}$ cells, a subset characterized by higher activation levels, increased cytotoxicity and increased cytokine production (47–49). The higher proportions of $Fc\gamma RIIIa^+CD8^+$ T cells in HIV-1-infected individuals suggests that HIV-1 in its own right is a driver of these cell expansions, whereas increasing age associated with reduced proportions of FcγRIIIa-expressing $CD8^+$ T cells. The increase in FcγRIIIa expression on $CD8^+$ T cells in HIV infection contrasts with decreased proportions of FcγRIIIa expressing NK cells. This suggests the development of an ADCC capacity by $CD8^+$ T cells that could compensate to some extent for the reduction in NK cell ADCC function.

Attributing the otherwise innate cell function of ADCC to $CD8^+$ T cells—mediated through expression of FcγRIIIa and engagement of HIV-1-specific antibodies—is reminiscent of other known examples of innate-like unconventional T cell populations. Among these are invariant NKT cells, mucosal-associated invariant T cells, and γδT cells, that recognize foreign/self-lipid presented by non-classical MHC molecules (50, 51). Another interesting unconventional $CD8^+$ T cell subset is one with a prominent innate/memory phenotype identified by co-expression of eomesodermin (Eomes) and KIR/NKG2A

(52). The current study highlights the addition of another unconventional $CD8^+$ T cell population, capable of ADCC function, that warrants further investigation.

In conclusion, our findings underscore the importance of expanding studies of HIV-specific antibodies to include the influence of different host cell types that share expression of FcγRs (constitutive or induced), the respective functional cellular capabilities, as well as host genotypes, in the context of the presiding immune milieu which is altered as a consequence of chronic HIV infection. Continuing investigations are warranted to further define effector functions, cytokine production and activation status of these different $FcγRIIIa^+$ NK and CD8 T cell subsets in similarly selected individuals.

AUTHOR CONTRIBUTIONS

NP recruited HIV-1 negative donors, performed the majority of the experiments, analyzed the data and wrote the manuscript. RL designed the study, contributed to the data analysis, and writing of the manuscript. BD contributed to the flow cytometry experiments. ZW and NM recruited HIV-1 positive donors, CT in her capacity as head of the laboratory, allocated funds toward the study, supervised the research, and provided the necessary infrastructure to perform the work.

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REVIEW
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Effect of Fc Receptor Genetic Diversity on HIV-1 Disease Pathogenesis

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Fc receptor (FcR) genes collectively have copy number and allelic polymorphisms that have been implicated in multiple inflammatory and autoimmune diseases. This variation might also be involved in etiology of infectious diseases. The protective role of Fc-mediated antibody-function in HIV-1 immunity has led to the investigation of specific polymorphisms in FcR genes on acquisition, disease progression, and vaccine efficacy in natural history cohorts. The purpose of this review is not only to explore these known HIV-1 host genetic associations, but also to re-evaluate them in the context of genome-wide data. In the current era of effective anti-retroviral therapy, the potential impact of such variation on post-treatment cohorts cannot go unheeded and is discussed here in the light of current findings. Specific polymorphisms associating with HIV-1 pathogenesis have previously been genotyped by assays that captured only the single-nucleotide polymorphism (SNP) of interest without relative information of neighboring variants. With recent technological advances, variation within these genes can now be characterized using next-generation sequencing, allowing precise annotation of the whole chromosomal region. We herein also discuss updates in the annotation of common FcR variants that have been previously associated with HIV-1 pathogenesis.

Keywords: next-generation sequencing, polymorphism, disease association, Fc receptors, HIV-1

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INTRODUCTION

Fc receptors comprise a class of cell surface receptors expressed on various hematopoietic cells that bind to the Fc portion of antibodies to form immune complexes and recruit the complement and/or effector system to defend the body against pathogens. The Fc receptors are classified based on their binding to the Fc domain of immunoglobulin (Ig). The most abundant Ig in serum is IgG which can bind to different classes of FcγR. Other types of FcR including FcεR, Fcα/μR, and FcαR1 are receptors for other Ig classes such as IgE, IgM, and IgA.

More recently vaccine studies in infectious diseases point to a critical role of non-neutralizing antibody functions, which is the ability of an antibody to interact with other immune components and effector cells via their Fc portions to mediate killing or control of the pathogen. These mechanisms include, but are not limited to, antibody dependent cellular cytotoxicity (ADCC), antibody-dependent cell-mediated virus inhibition (ADCVI), antibody dependent

cellular phagocytosis (ADCP), and antibody dependent complement deposition (ADCD) (1, 2). These functions are mediated by three distinct classes of FcγRs that are expressed on most human immune cells, with varying levels of expression dependent on cell type such as monocytes, macrophages, natural killer cells, eosinophils, neutrophils, B cells but not on T cells (3). These receptors include FcγRI (CD64), FcγRIIa/b/c (CD32), and FcγRIIIa/b (CD16), which bind the different IgG subclasses with varying proficiency, and can cause either activation or inhibition of the effector cell.

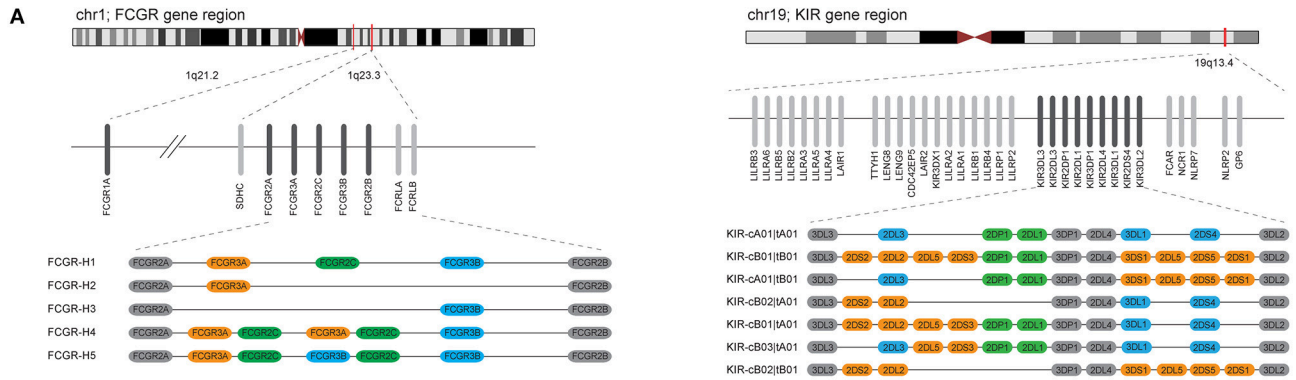
Polymorphisms in the FcγR have been shown to affect binding affinity to the Fc region of IgG and can trigger a range of effector and immunoregulatory functions. Such variation has been shown to play a crucial role in the pathogenesis of a range of chronic inflammatory and autoimmune diseases, as well as susceptibility to infectious pathogens (4, 5). An overview of FcγR biology has been recently summarized and so our focus for this review will be to evaluate the effect of genetic variation in the human Fcγ receptors and their role specifically in HIV-1 disease pathogenesis (6). Although there is evidence that the neonatal Fc receptor (FcRn), an MHC class I-related molecule expressed on many cells, functions in HIV-1 vaccination and infection (7, 8), no significant genetic variation has been identified for this locus, and we have not included it in this review.

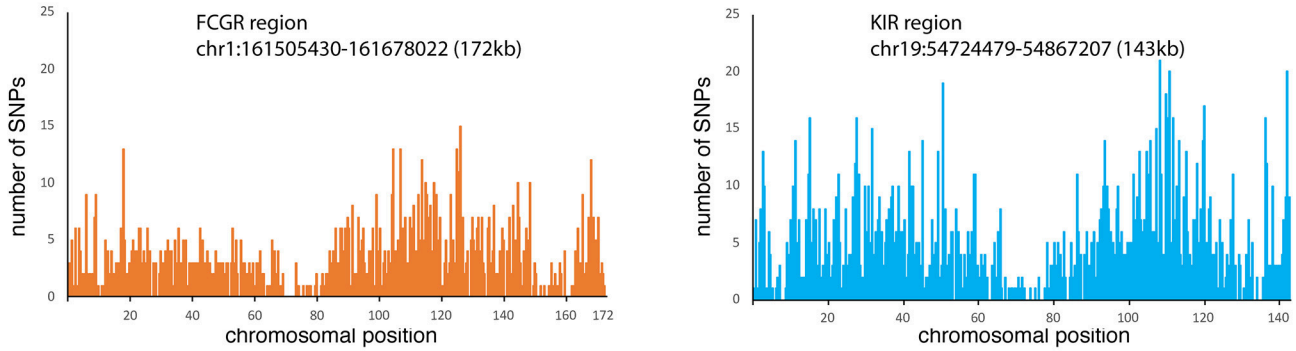
FCγ RECEPTOR GENETIC DIVERSITY

The Fcγ receptors are encoded by the FCGR genes located on chromosome 1 in humans, including five FCGRs in a tandem arrangement within ∼200 kb of genomic sequence (**Figure 1A**). A sixth gene, FCGR1A, is located ∼12 Mb distant from the five gene cluster. Genetic variation at the FCGR gene cluster bears similarity to the Killer Ig-like receptor (KIR) region which is shown in comparison to emphasize both the types and extent of copy number and allelic variation (**Figures 1A,B**) (9). Like KIR, the genes in the FCGR cluster are arranged in haplotypes containing both invariant framework and copy number variant genes. An examination of total nucleotide variation in FCGR from a recent genome build indicates extensive depths of SNP variation, similar in overall extent to KIR and the vast majority of which has not been functionally characterized (**Figures 1B,C**). Both gene families encode receptors for other central components of the immune response (KIR and MHC class I; FCGR and IgG constant domains) placing them in distinct roles but perhaps of equivalent importance in investigations of host genetics and its relationship to immune function. Given the parallels in significance and the similar physical characteristics of both copy number variation and allelic polymorphism, a major difference is that the allelic variations for FCGR genes have been less examined, curated and annotated. This review in part is attempting to address this deficit as an organizing framework of characterized variation possibly guided by established methods for structural and allelic annotation as currently employed for the KIR system (10).

In the FCGR family of six genes, several nonsynonymous single nucleotide polymorphisms (SNP), SNPs encoding altered splice sites, and copy number variants (CNVs) encoding addition, or deletion of one or more gene have been functionally characterized (**Figure 1**, **Table 1**). Variation in the FCGR1 gene is limited, with the most frequent minor allele characterized at <4%, but there is considerable diversity in the other FCGR genes. The most studied SNPs in the FCGR genes over the past two decades have centered on nonsynonymous substitutions that contribute to differential binding affinity for subclasses of IgG. FCGR2A has two allele variants encoding arginine or histidine at amino acid position 166 (rs1801274), with the latter resulting in a higher affinity for IgG1, and IgG2 (11–13). FCGR3A also has two common allele variants differing by a single SNP, altering codon 176 from phenylalanine to valine (rs396991) resulting in a higher affinity for IgG1, IgG2, IgG3, and IgG4 for the 176V variant (11, 12, 14). This stronger binding affinity is associated with functional capacity of the receptor in different experimental and clinical contexts (15–17). Two adjacent nonsynonymous SNPs in FCGR2A (together altering codon 63 from Q to W; rs201218628) have been studied, although their functional consequence is less clear, and their frequency is rare (18). FCGR2B has an isoleucine to threonine change at position 232 (rs1050501), which alters the transmembrane region, with the 232T allele inhibiting the association of FCGR2B with lipid rafts in a human B cell line measuring downstream function (19). The frequency of this alteration is low at ∼1% in Caucasians, and while more prevalent among African Americans and Asians (5–11%) has been less studied.

A major studied polymorphism in the FCGR2C gene is a SNP in exon 3 (rs759550223) that encodes a glutamine or a stop codon resulting in the presence or absence of protein expression (20–22). The frequency of the minor allele varies between populations, and studies have suggested it is expressed on NK cells and is capable of inducing ADCC after receptor cross-linking on purified NK cells as measured by their ability to lyse the target P815 cell line (20, 23). In addition, both alleles have been associated with both null and surface expression on NK cells as measured by anti-FCGR2B/C specific mAb 2B6 (22). However, there may be some confusion regarding this SNP as it is identical to rs10917661, which is assigned to FCGR2B, in a reference SNP identification (rs id) segment where the two genes have identical sequences except at the variant position. Sequence identity between FCGR2B and FCGR2C may lead to incorrect assignment of SNPs to these two loci. Also, rs759550223 has a very low minor allele frequency defined in the SNP database (dbSNP), and the minor allele assigned is identical to the FCGR2B-derived sequence, suggesting the possibility that the SNP has been falsely generated by a combination of variants between two distinct loci. FCGR2C has been previously reported to have arisen from an unequal recombination between the FCGR2A and FCGR2B genes, and encoded a functional molecule that exhibited differential expression in natural killer cells (21, 24, 25). However, FCGR2C is also classified as a gene/pseudogene in the NCBI gene database. These inconsistencies further emphasize the need of validation of the FCGR genes by a combination of methods such as next generation sequencing (NGS) technologies





C

region	bases in region (GRCh38.p12)	total dbSNPs (151)	total dbSNPs (151)	>0.005 (1000 genome)	>0.005 (1000 genome)
chr1:161505430-161678022 (FCGR; 1q23.3)	172593	14732	0.09	1202	0.0070
chr19:54724479-54867207 (KIR)	142729	16268	0.11	1558	0.011
All chr	3088269832	322806648	0.10	17856079	0.0058

FIGURE 1 | Structure and polymorphism of the FCGR genes. **(A)** Approximate chromosomal locations of the FCGR gene complex containing 5 FCGR genes and the FCGR1A gene located at ∼12 Mb centromeric to the FCGR cluster. In comparison, the KIR gene family is illustrated immediately to the right including adjacent genes (FCAR) and gene clusters. For both FCGR and KIR, alternative haplotypes identified in populations are illustrated with the colored bars depicting genes present in a subset of haplotypes and shaded bars depicting genes present in all haplotypes (framework genes). Figure depicting KIR has been reproduced with permission from the Oxford University Press (9). **(B)** The plots show the abundance of SNPs at each position of the indicated regions from chr1 and chr19, using data derived from the UCSC genome browser. **(C)** Approximate number of SNPs in both regions are listed including a summary from all chromosomes for comparison.

as discussed below, in addition to precise curating of allelic variation and flow cytometry phenotyping.

A triallelic nonsynonymous SNP at codon 66 (66R, 66L, 66H; rs10127939) of the FCGR3A gene has also been found to affect affinity for immune complexes (ICs), with the FCGR3A-66R and 66H alleles exhibiting higher affinity (26). Other nonsynonymous SNPs have been identified but none have been characterized functionally or in association analyses. FCRG3B polymorphisms were first described as the human neutrophil antigen (HNA)-1 system (27, 28). The three major HNA-1 variants have differential affinity for IgG1 and IgG3, with the higher affinity HNA-1a and lower affinity HNA-1b differing at 4 nonsynonymous codon positions (rs2290834, rs200688856, rs448740, rs147574249). Consistent with this differential affinity, phagocytosis was lower with HNA-1b through analysis of antibacterial IgG subclass antibodies and with IgG1 and IgG3 anti-Rhesus D (29, 30). A third isoform, termed HNA-1c, of unknown function is identical to the HNA-1b isoform except at the rs5030738 polymorphic site, where it encodes an asparagine rather than alanine residue (31). Other variants of the HNA-1 antigen system have also been described but to date no functional or association studies interrogating them have been reported (32, 33).

CNV is a hallmark of multicopy gene family genomic regions, including notably among them, those encoding immune response genes (34). In the FCGR region, CNVs include at least five haplotypes with varying combinations of deletions and duplications of the FCGR2C, FCGR3A, and FCGR3B genes, flanked by the invariant framework FCGR2A and FCGR2B genes (**Figure 1A**, *left*) (34–36). FCGR-H1 forms the most common among these haplotypes, containing the five loci, with FCGR-H2 being the most commonly observed CNV [equivalent to CNR1 in Nederer et al. (36)] and FCGR-H3 less prevalent (equivalent to CNR2 or CNR3). Although variants FCGR-H4 and -H5 have

not been explicitly described, they form predicted reciprocal structures of the H2 and H3 deletion variants (34). Individuals with FCGR gene copy numbers that may be consistent with those structures have been described (22, 34–38). As discussed above, older genotyping methods focusing on specific regions of the genes may have misassigned SNPs, and the lack of a standard nomenclature of common FGCR coding variants may lead to misinterpretation when comparing different studies. Newer NGS technologies have allowed for updated annotation of the FCGR genes as per the current Human genome database reference hg38. SNPs with a minor allele frequency >0.01 are shown in **Table 1**.

FCGR VARIATION AND HIV-1 DISEASE PATHOGENESIS

The first report of the effect of polymorphisms in the FCGR genes on HIV-1 disease progression was in two natural history HIV-1 cohorts consisting of anti-retroviral therapy (ART) naïve individuals (39). Since then, functional SNPs in the FCGR2A (rs1801274) and FCGR3A (rs396991) genes that affect binding affinity to the Fc domain of IgG have been evaluated in the context of HIV-1 acquisition, disease progression, and vaccine efficacy. Now that most HIV-1 infected individuals are on ART, there is an opportunity to evaluate disease outcomes after ART initiation. With increased high-throughput sequencing, targeted SNP genotyping is being replaced by whole gene and genome sequencing. This gives the opportunity to evaluate previous host genetic findings in the light of genome wide findings and also examine other SNPs in nearby genes. We will discuss the effects of genetic polymorphisms in the FCGR genes and their impact on HIV-1 disease progression, acquisition, post-ART and vaccine outcomes in the next sections.

FCGR Polymorphisms and HIV-1 Disease Progression

Candidate Gene Studies

Forthal et al. identified an association between the FCGR2A low binding RR (rs1801274) genotype and a faster rate of CD4+ T cell decline and progression to AIDS using samples and data from the Multicenter AIDS Cohort Study (MACS) consisting of more than 500 HIV-1 infected males of mostly Western European ancestry (40). Paradoxically, the same RR genotype was also found to associate with a decreased risk of Pneumocystis jiroveci (carinii) pneumonia, an AIDS defining illness, when compared to the HH genotype in the same cohort. At the functional level, cells from RR homozygous carriers demonstrated less efficient phagocytosis of HIV-1/IgG complexes. There was no association of the FCGR2A genotype with viral load setpoint (spVL), defined as the number of HIV-1 RNA copies/ml in a plasma sample collected 18 months after the first seropositive test. The absence of association of FCGR2A variation with spontaneous viral load control was also confirmed in an HIV-1 seroconverting cohort including 253 Kenyan women, in which the associations with disease progression and CD4+ T cell decline were not replicated (41).

No association was observed by Forthal et al. in the MACS cohort between a specific FCGR3A genotype (rs396991) and spontaneous viral control or disease progression (40). Similarly, Weis et al. did not identify any significant genetic associations of FCGR3A variation with disease progression or spVL in the Kenyan's women cohort (41). There is one report of the VV genotype of FCGR3A being overrepresented in 43 untreated controllers compared to 59 HIV positive progressors on ART (42). However, since the HIV positive progressors were on ART, analyses with measures of spVL or CD4+ T cell counts could not be performed and this finding remains inconclusive.

A more consistent finding has been reported by two independent groups showing the association between the FCGR3A FF genotype and decreased risk of Kaposi's sarcoma (KS) (39, 40). In the first study, FCGR3A genotyping was performed in two small cohorts consisting of 119 and 131 HIV-1 infected males of Western European ancestry. A significant association with protection was identified in each cohort independently and in the combined analysis. Forthal et al. replicated this finding in the MACS cohort. KS is the most frequent malignant condition associated with HIV-1 related immunosuppression, and alterations in the cytokine balance have been suggested to play a critical role in its pathogenesis. Differences in genotype have been shown to alter IgG binding that could influence cytokine levels, with the V allele having higher affinity than the F. The authors concluded that FF homozygous individuals might be at lower risk of KS because of a less vigorous proinflammatory response. The VV genotype has been associated with an increased risk of cryptococcal disease in 164 HIV-1 infected men, again in the MACS cohort (43). Of note, this observation extends beyond HIV-1 infection, because the VV genotype was previously associated with cryptococcal disease in non-HIV-infected individuals in a separate study (44).

Genome-Wide Testing

The associations with HIV-1 natural history described above were tested using a candidate gene study design in cohorts with relatively small sample size. The current availability of genome-wide genotyping and sequencing data provides an opportunity to reassess the potential involvement of FCGR variation in HIV-1 disease in larger cohorts, by applying more stringent standards for significance level and including robust population stratification (45). We therefore accessed previously published data generated from cohorts and studies that contributed to the International Collaboration for the Genomics of HIV (ICGH) (46–48) and assessed genetic associations with HIV-1 disease outcomes in the FCGR2A and FCGR3A regions.

The potential associations between FCGR2A or FCGR3A variants and spVL were evaluated using a fixed-effect inverse-variance weighted meta-analysis across cohorts, including a total of 7,266 HIV positive patients of Western European ancestry. We tested all common polymorphisms (minor allele frequency >5%) in a 50 kb window around the gene. In line with previous studies, no significant association with spVL was observed. An additional analysis was performed in a subset of ICGH, consisting of 467 long-term non-progressors (individuals with CD4+ T cell counts consistently above 500 cells/mm (3) for >10 years without treatment) and 517 rapid progressors

TABLE 1 | Characterization of variation in the Fc Receptor genes.

Chr. location (GRCh38.p7)	dbSNP rs#	MAF	Function	RefSeqGene (genomic DNA)	RefSeqGene (mRNA)	SNP variants[a]	RefSeqGene (Protein)	Amino acid change
FCGR2A				NG_012066	NM_021642.3		NP_067674.2	
161506414-161506415	rs201218628	0.006	Missense	6000_6001	184_185	CA>TG	62	Gln>Trp
161509955	rs1801274[d]	0.442	Missense	9541	497	A>G	166	His>Arg
161510070	rs150311303	0.008	Insertion	9657_9658	613_614	insTTC	205	Gln>GlnLeu
161510859	rs11810143	0.055	synonymous	10445	642	A>G	214	Pro>Pro
161510928	rs140474146	0.009	Synonymous	10514	711	G>A	237	Leu>Leu
161518073	rs12029217	0.121	Synonymous	17659	876	C>T	292	Pro>Pro
161518091	rs6694457	0.026	Synonymous	17677	894	T>C	298	Asp>Asp
FCGR2B				NG_023318	NM_004001.4		NP_003992.3	
161671501	rs148030870	0.010	synonymous	13387	243	C>T	81	Ser>Ser
161671594	rs6665610	0.138	Synonymous	13480	336	G>A	112	Thr>Thr
161671618	rs367584808	0.005	Synonymous	13504	360	C>A	120	Leu>Lue
161672984	rs200112434	0.109	Missense	14870	401	T>G	134	Val>Gly
161673192	rs2298022	0.029	Synonymous	15078	609	G>A	203	Thr>Thr
161673195	rs182968886	0.110	Synonymous	15081	612	G>A	204	Leu>Lue
161674008	rs1050501	0.186	Missense	15894	695	T>C	232	Ile>Thr
161675262	rs28651835	0.032	Missense	17148	766	C>T	256	Pro>Ser
FCGR2C				NG_011982	NM_201563.5[b]		NP_963857.3	
161589466	rs114945036	0.245	Intron	13128		C>T		
161589597	rs759550223	0.002	Nonsense	13259	169	C>T	57	Gln> *Ter
161589781	rs138747765	0.194	Missense	13443	353	C>T	118	Thr>Ile
161589930	rs78603008	0.195	Intron	13592		G>A		
161591153	rs370748254	0.134	Missense	14815	401	T>G	134	Val>Gly
161591361	rs74341264	0.199	Synonymous	15023	609	G>A	203	Thr>Thr
161591366	rs76016754	0.034	Missense	15028	614	A>T	205	Tyr>Phe
161599629	rs430178	0.259	Splice acceptor	23291		C>G		
161599779	rs138731942	0.075	Synonymous	23441	948	C>T	316	Asn>Asn
FCGR3A				NG_009066	NM_001127593.1		NP_001121065.1	
161543083	rs115866423	0.006	Missense	12541	694	A>T	232	Asn>Tyr
161544752	rs396991[d]	0.351	Missense	10872	526	T>G	176	Phe>Val
161548509	rs150808747	0.012	Synonymous	7115	231	C>T	77	Asp>Asp
161548524	rs114535887	0.019	Synonymous	7100	216	G>A	72	Ser>Ser
161548543	rs10127939	0.039	Missense	7081	197	T>G/T>A	66	Leu>Arg/Leu>His

(Continued)

TABLE 1 | Continued

Chr. location (GRCh38.p7)	dbSNP rs#	MAF	Function	RefSeqGene (genomic DNA)	RefSeqGene (mRNA)	SNP variants[a]	RefSeqGene (Protein)	Amino acid change
FCGR3B				NG_032926	NM_000570.4		NP_000561.3	
161626224	rs71632957	0.022	Synonymous	10740	498	T>C	166	Asp>Asp
161626242	rs114169903	0.025	Synonymous	10722	480	A>G	160	Pro>Pro
161629781	rs2290834	0.447	Missense	7183	316	A>G	106	Ile>Val
161629800	rs368410676	0.023	Synonymous	7164	297	G>T	99	Pro>Pro
161629853	rs147574249	0.284	Missense	7111	244	A>G	82	Asn>Asp
161629864	rs5030738	0.083	Missense	7100	233	C>A	78	Ala>Asp
161629903	rs448740	0.467	Missense	7061	194	A>G	65	Asn>Ser
161629983	rs527909462	0.135	Synonymous	6981	114	T>C	38	Leu>Leu
161629989	rs200688856	0.128	Missense	6975	108	C>G	36	Ser>Arg
FCGR1A				NG_007578	NM_000566.3		NP_000557.1	
149784005	rs138447715	0.034	Missense	6274	55	A>G	19	Thr>Ala
149784064	rs80039899	0.017	Synonymous	6333	114	C>T	38	Thr>Thr
149784065	rs7531523	0.005	Missense	6334	115	G>A	39	Val>Ile
149784139	rs149926813	0.005	Synonymous	6408	189	T>C	63	Thr>Thr
149784147	rs144081076	0.004	Missense	6416	197	C>T	66	Ser>Leu
149784224	rs74315310	0.004	Nonsense	6493	274	C>T	92	Arg> *Ter
149788436	rs138510822	0.008	Synonymous	10705	378	G>A	126	Ala>Ala
149790290	rs587727639	0.008	Missense	12559	796	G>A	266	Asp>Asn
FCAR				Na[c]	NM_002000.3		NP_001991.1	
54885265	rs61735068	0.006	Missense		101	A>C	34	Lys>Thr
54885488	rs1865096	0.261	Synonymous		324	G>A	108	Arg>Arg
54885501	rs11666735	0.055	Missense		337	G>A	113	Asp>Asn
54888276	rs61735069	0.042	synonymous		631	T>C	211	Leu>Leu
54889758	rs77103719	0.006	Synonymous		759	G>A	253	Thr>Thr
54889796	rs61735070	0.006	Missense		797	C>T	266	Pro>Leu
54889804	rs16986050	0.155	Missense		805	A>G	269	Ser>Gly

[a]*Only SNP variants with a MAF of >0.01 are shown.*

[b]*The status of FCGR2C has been recently changed from gene to pseudogene in genbank.*

[c]*No unique genome reference sequence has been defined for FCAR in genbank at this point.*

[d]*rs1801274 and rs396991 have been commonly referenced in the literature as FCGR2A-131R/H and FCGR3A-158F/V, respectively.*

(individuals with two or more CD4+ T cell counts below 300 cells/mm (3) within 3 years after the last seronegative test result). We were unable to replicate findings by Forthal et al. and did not observe associations with HIV-1 progression (40). These analyses included the FCGR2A rs1801274 polymorphism; however, the FCGR3A polymorphism rs396991 was not available from the ICGH meta-analysis as it is not directly genotyped on most genotyping arrays and could not be reliably imputed. Thus, in order to evaluate its association with HIV-1 spVL, we reassessed a standard genome-wide association study (GWAS) using exome sequencing data from 395 individuals of European descent in the Swiss HIV Cohort Study (SHCS), following the procedures described in McLaren et al. (48). This analysis did not show any association between rs396991 and HIV-1 spVL ($p = 0.21$). Additionally, the rs1801274 did not show any association with spVL ($p = 0.54$) in the same cohort (**Figure 2**). The inability to replicate previous findings could be attributed to differences in sample size, clinical definition and statistical rigor employed. Globally, these new analyses confirm the previous findings that common human genetic variants in FCGR2A or FCGR3A are not associated with spontaneous control of HIV-1 infection.

Influence of FCGR Diversity on HIV-1 Acquisition

There are no conclusive studies reporting associations of FCGR polymorphisms with HIV-1 acquisition. Two independent mother-to-child transmission cohorts reported contrasting findings of FCGR2A genotypes associating with increased infection risk in children with the high-affinity HH (rs1801274) genotype (49, 50).

Here again, we evaluated potential associations between FCGR2A and FCGR3A variants and susceptibility to HIV-1 infection by accessing the results of a previous GWAS of HIV-1 acquisition that compared 6,300 HIV-1 infected individuals and 7,200 controls of European ancestry (46). The rs1801274 polymorphism did not show any sign of association with HIV-1 acquisition ($p = 0.81$), and all other tested polymorphisms in a 50 kb window around both FCGR2A and FCGR3A were also non-significant after correction for multiple testing. The FCGR3A SNP (rs396991) was not included on the genotyping chip and could not be reliably imputed and so was not tested directly. This analysis in the largest acquisition cohort published to date adds substantial evidence to the lack of involvement of common FCGR2A and FCGR3A polymorphisms in HIV-1 acquisition.

Role of FCGR Polymorphisms on Outcomes After ART Initiation

Variation in genotype and expression of host genes is well established to impact HIV-1 susceptibility and disease progression in ART- naïve individuals (47). Initiation of ART in acute HIV-1 infection can limit establishment of viral reservoirs and induces post-treatment control in some individuals (51, 52).

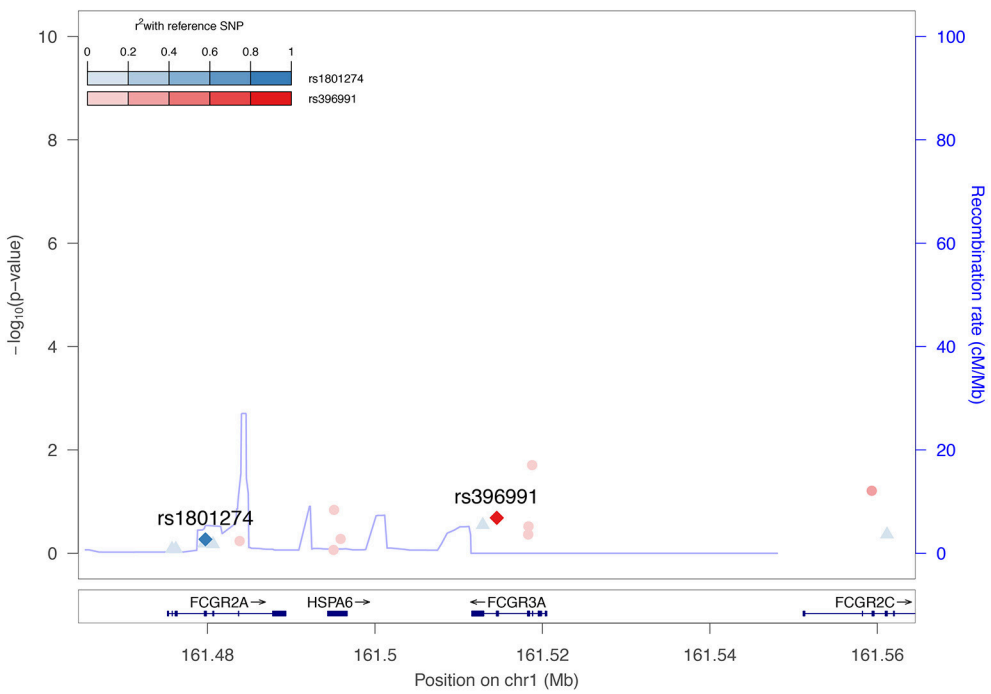


FIGURE 2 | No association between FCGR2A or FCGR3A polymorphisms on HIV-1 set point viral load. Regional association plot highlighting the association between the FCGR2A (rs1801274) and FCGR3A (rs396991) polymorphisms and HIV-1 spVL across 395 exome sequenced patients (48). Color intensities represent the linkage disequilibrium (r^2) of other SNPs in the region with rs1801287 and rs396991, respectively. The blue line indicates the estimated recombination rate in cM/Mb from The International HapMap Consortium (2007).

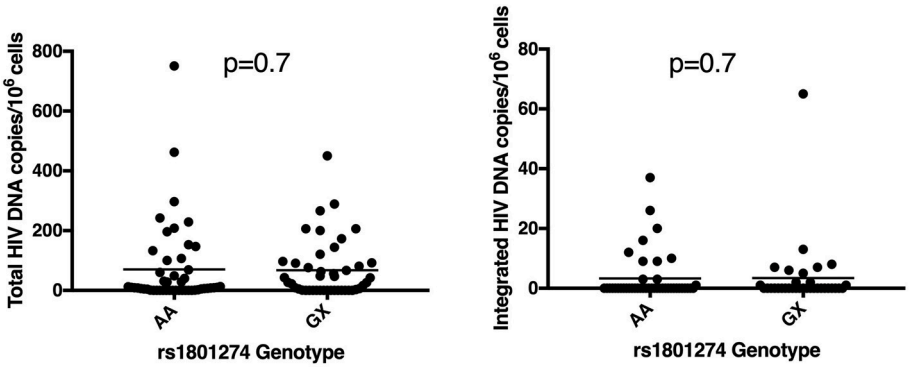


FIGURE 3 | Polymorphism in FCGR2A (CD32a) does not associate with reservoir size. The rs1801274 SNP variant did not associate with levels of total or integrated HIV DNA, determined in $N = 93$ and $N = 78$ of the patients, respectively (55, 56).

Host variation that influences viral reservoir size or reactivation during ART has not been definitively studied and has potential to significantly advance HIV cure research. There is at least one report indicating that broadly neutralizing antibodies (bNAbs) can interfere with establishment of a silent reservoir by Fc-FcR mediated mechanisms in humanized mice when administered early in the infection (53). Recently, Descours et al. identified CD32a (FCGR2A) as a marker of latently infected CD4 T cells (54). Given the previous associations of FCGR2A with HIV-1 disease pathogenesis, we hypothesized that polymorphisms in this gene might affect the size of the viral reservoir in patients that went on ART early in acute infection (55, 56). We examined genetic variation in the FCGR2A gene, characterizing polymorphisms in 436 ART-suppressed patients from the RV254 cohort. We screened for 18 variants in the extracellular domains of FCGR2A including rs1801274 and did not find associations with total or integrated HIV DNA ($p > 0.05$) (**Figure 3**). Surprisingly, recent reports from several independent groups confirm that they were unable to replicate the original findings from the Descours et al. study, showing that associations with post-ART control continue to be elusive (57–60).

Effect of FCGR Variation on HIV-1 Vaccine Efficacy

Variation in host genes can impact vaccine outcomes, and other than HLA, the only other gene to impact HIV-1 vaccine efficacy was in the FCGR locus (61–63). Two studies of HIV-1 vaccine efficacy revealed a remarkable coincidence of FCGR polymorphism associated with opposing directions for efficacy, suggesting that the effect of FCGR genetic variation may be specific to vaccine regimens. The FCGR2C association study of Li et al. (62), used a direct sequencing approach to identify FCGR2C SNPs that associated with vaccine efficacy (VE) against HIV-1 in the RV144 vaccine trial, that showed modest efficacy (64). Individuals with at least one minor allele of three FCGR2C SNPs (rs114945036, rs138747765, and rs78603008) had a vaccine efficacy of 64% against any HIV-1 subtype and 91% against the CRF01-AE subtype with the protective 169K HIV-1 variant identified previously by sieve analysis (65). Although the functional mechanisms underlying the association were not revealed in this study, a subsequent examination of the FCGR2C SNPs showed rs114945036 correlated with expression levels of FCGR2A/C (66). This effect was found across different populations and was specific to the rs114945036 SNP located in the intron. Further, rs114945036 also associated with the expression of the Fc receptor-like A (FCRLA) gene, an FCGR related gene located within a gene cluster adjacent to FCGR (see **Figure 1A**). These results suggest that the FCGR expression is either influenced by this SNP through an undefined mechanism, or is in linkage with other causal variants that directly affect expression levels.

In the second study, four FCGR2C SNPs significantly modified the hazard ratio in the HVTN505 trial that did not show protection against HIV-1 acquisition (62). Three of the SNPs were common with those previously identified in RV144. In contrast to the RV144 study, in HVTN505 among the recipients carrying the FCGR2C minor alleles, HIV-1 acquisition risk was higher in the vaccine group than in the placebo group, in precisely the opposite direction of that observed in RV144 (efficacy against HIV-1 acquisition hazard ratios (HR) of 9.79 ($p = 0.035$) and 0.36 ($p = 0.04$), respectively). It is not clear how polymorphisms in a pseudogene functions during HIV-1 vaccination and their associations with FCGR expression may provide a novel avenue for further investigation.

Two additional studies of outcomes in Vax004, a trial testing recombinant gp120 vaccination in preventing sexually acquired HIV infection, also implicated FCGR variation in HIV infection and vaccine efficacy. Both studies tested the classical FCGR2A or FCGR3A variants comprised of the FCGR2A-R/H (rs1801274) and FCGR3A-F/V (rs396991) alleles. The first study found that lower affinity receptors (FCG2A-RR or HR and FCGR3A-FF) were associated with higher serum ADCVI activity, which itself predicted the rate of infection (67). A second study by the same group, showed the FCGR3A-VV genotype distinguished the lowest behavioral risk group from the high-risk behavioral group (68). The low risk group had a higher infection rate than low risk

vaccinees with one or two F alleles (HR = 3.52; $p = 0.002$) while the high-risk group showed no association. Functional studies may be directed by these findings to interrogate quantitative and qualitative effects on FCGRs and associated antibody production. At a minimum, the intersection of these studies suggests that the impact of FCGR genetic variations on vaccine efficacy should be further investigated.

FCGR AND IGHG—FUTURE DIRECTIONS FOR GENETIC ANALYSIS

It is apparent that the genomic complexity of the FCGR region presents a major challenge for uncovering the underlying causal FCGR variants. Different FCGR have distinct functions and mechanisms of regulation but share highly similar sequences. While FCGR genetic variations are clearly linked to host defense against infectious diseases and other important immune functions as discussed, current approaches measure only a small portion of the existing FCGR variation. The HVTN505 and RV144 studies referenced above were by far the most comprehensive in that regard, measuring ∼10 kb of the FCGR region, including functional exons encoding external protein domains and flanking intron sequences from the five FCGR genes. However, the complete FCGR region extends over 200 kb leaving open the likelihood of additional causal variation (**Figure 1A**). Indeed, the lack of phasing of the over 20,000 SNPs documented in the FCGR region significantly limits its direct utility for association analysis and the ultimate goal of identifying causal variants (69). Complete haplotype-resolved FCGR genomic sequences across human populations by approaches such as those used for defining variability in the KIR region may be necessary in order to provide a complete analysis of these loci (70–72).

When considering FCGR host genetics and its relationship to HIV-1 susceptibility and vaccine efficacy—or any association with disease—a natural but not often considered extension to host genetic association studies interrogating FCGR variability lies within the human immunoglobulin constant heavy G chain (IGHG) gene region on chromosomal segment 14q32.3 (73). This region encoding the human IG heavy constant genes (IGHG3, IGHG1, IGHG2, IGHA2, and the IGH locus on chromosome 14) provides access to a system for understanding immunogenicity of the polymorphic IG chains (74, 75). The evident functional relationship between FCGR and IgG constant region variability, which itself is substantial (74), argues strongly for host genetic studies of FCGR to be paired with analysis of IGHG. Although little genomic characterization for the IGHG system is available at present, we anticipate that NGS technologies will rapidly fill that void. Of course, once provided with high quality and high resolution data, significant effort will need to be invested in new sophisticated analytical approaches examining multiple factors simultaneously to find the causal variation revealing operative biological mechanisms.

CONCLUDING REMARKS

Disease pathogenesis of HIV-1 has been shown to be modulated by allelic variants in the FCGR genes. However, such findings have not always been robust, as they were not replicated or in some cases were contradictory. There is however considerable interest in the role of Fc-mediated antiviral functions such as ADCC, ADCP, ADCD, and ADCVI in protective immunity against HIV-1 (76). Host genetics of the Fc receptors that bind to the Fc domain of the IgG antibody might modulate the functional antiviral antibody responses to HIV-1 vaccination. ADCC was previously identified as a correlate of protection in the RV144 human efficacy trial (64). More recently, ADCP has been shown to correlate with protection against acquisition of SIV/SHIV/HIV-1 in multiple preclinical and human efficacy trials (77–80). Given such associations, it would be critical to investigate association of host variation in FCGR genes and such Fc-mediated antiviral functions that are now being generated using technologies such as systems serology (1). Such findings might shed light on the role of Fc gene and receptor genotypes on HIV-1 disease pathogenesis.

AUTHOR CONTRIBUTIONS

RT conceptualized, organized the content of the review and wrote sections Introduction, FCGR polymorphisms and HIV-1 disease progression, Influence of FCGR diversity on HIV-1 acquisition, Role of FCGR polymorphisms on outcomes after ART initiation, and Concluding Remarks. DG contributed to sections Fcγ receptor genetic diversity, Effect of FCGR variation on HIV-1 vaccine efficacy, and FCGR and IGHG–future directions for genetic analysis. JF and CT drafted sections FCGR polymorphisms and HIV-1 disease progression, Influence of FCGR diversity on HIV-1 acquisition. All authors participated in editing and revising the manuscript.

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REVIEW
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A Case for Antibodies as Mechanistic Correlates of Immunity in Tuberculosis

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Tuberculosis infects one quarter of the world's population and is the leading cause of death by a single infectious agent, responsible for a reported 1.3 million deaths in 2017. While *Mycobacterium tuberculosis* is treatable with antibiotic therapy, the increased prevalence of drug resistance, coupled with the variable efficacy of the only widely approved vaccine, has highlighted the need for creative approaches to therapeutic and vaccine development. Historically, a productive immune response to *M. tuberculosis* has been thought to be nearly entirely cell-mediated, with humoral immunity being largely dismissed. However, in this review, we will discuss the historical skepticism surrounding the role of the humoral immune response to *M. tuberculosis*, and examine more recent evidence suggesting that antibodies may play a valuable role in host defense against the pathogen. Despite the amount of data portraying antibodies in a negative light, emerging data have begun to highlight the unexpected role of antibodies in *M. tuberculosis* control. Specifically, it has become clear that antibody features of both the variable and constant domain (Fc) ultimately determine the extent to which antibodies modulate disease. Thus, a more precise definition of the antigen-binding and innate immune recruiting functions of antibodies that contribute to *M. tuberculosis* restriction, are sure to help guide the development of next-generation therapeutics and vaccines to curb this global epidemic.

Keywords: tuberculosis, antibodies, cell-mediated immunity, Fc effector function, innate immune system, humoral immunity

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INTRODUCTION

Mycobacterium tuberculosis (*Mtb*), the causative agent of tuberculosis, is the leading cause of death from single infectious agent globally. *Mtb* infects one quarter of the global population, and caused ∼1.3 million deaths worldwide in 2017 (1). Importantly, tuberculosis infection does not always lead to tuberculosis disease, as tuberculosis presents as a spectrum of infection states. These range from an asymptomatic state, referred to as latent infection (LTBI), to the deadlier active disease (ATB).

While tuberculosis is treatable with antibiotics, the immense global burden, as well as the rise of drug resistance, has highlighted the need for improved methods for disease treatment and prevention. Bacillus Calmette-Guérin (BCG), developed nearly a century ago, remains the only licensed tuberculosis vaccine. Prepared using a live attenuated strain of *Mycobacterium bovis*, its protective efficacy is remarkably inconsistent (2, 3). BCG shows consistent protection against severe forms of tuberculosis disease, such as tuberculosis meningitis and miliary tuberculosis, in infants (4). However, the vaccine exhibits limited protection against pulmonary tuberculosis,

and importantly, does not protect teenagers and adults who are most likely to spread *Mtb* (5, 6). Given that BCG vaccination is widely given, yet tuberculosis remains the largest infectious disease killer globally, it is clear that a more effective vaccine is urgently needed to control the disease globally. Thus, creative approaches to therapeutic and vaccine development are critical to change the trajectory of the ongoing tuberculosis epidemic.

Cellular-mediated immunity (CMI), in particular $CD4^+$ T cells, are unequivocally important in restricting tuberculosis progression, and are seen as the primary immunologic axis mediating host immunity to *Mtb*. Both CD4 knock-out studies in animal models (7–9), as well as epidemiologic data documenting increased rates of active disease among HIV-infected patients with low $CD4^+$ T cell counts (10, 11), clearly demonstrate the lack of bacterial control in the absence of this pivotal immune effector. Thus, historically, the vast majority of vaccine design efforts have focused on the development of strategies that harness T cell immunity to drive protection or control of *Mtb*.

Conversely, while antibodies represent the correlate of immunity following most clinically approved vaccines (12), humoral immunity has been understudied in the context of *Mtb* vaccine design due to its perceived insignificance for anti-microbial control (13–15). Yet, the idea that the humoral immune response plays little role in *Mtb* infection is in part related to the perceived dichotomy between humoral and cellular immunity. Specifically, the paradigm dictates that Th1 responses counter intracellular pathogens by driving CMI, while humoral immunity is largely responsible for the control and clearance of extracellular pathogens (16). Consequently, in the absence of unambiguous evidence proving a protective role for antibodies, it has been assumed that due to their extracellular canonical mode of action, antibodies must not be relevant or critical for protection against *Mtb*. Moreover, despite our emerging appreciation for a role for antibodies in driving cellular cytotoxicity via the recruitment of the innate immune system as well as additional anti-microbial mechanisms, the perceived insignificance of the humoral immune response to *Mtb* remains pervasive in the field.

However, a growing body of literature has provided evidence indicating that *Mtb*-specific antibodies modulate tuberculosis disease. Specifically, evidence from passive transfer, monoclonal therapeutic, cohort, and vaccine studies each individually, and collectively argue that antibodies can positively shape the immune response to *Mtb*. Here we will discuss the uncertainties that have long surrounded the antibody response to *Mtb*, as well as examine the evidence suggesting that antibodies represent a wealth of untapped potential against this global killer.

A CASE FOR ANTIBODIES FROM PASSIVE TRANSFER STUDIES

The positive results of serum therapies against a range of infectious diseases in the late 1800s spawned a plethora of human and animal transfer experiments attempting to cure tuberculosis by the same methodology. In the 1890s, the Henry Phipps Institute immunized cows with a heat-killed concentrate of *Mtb* bacilli (17). However, the administration of the cow serum failed to show any benefit in tuberculosis patients (18, 19). Similar work performed by Viquerat and De Schweinitz aimed at exploring the impact of administration of immune sera from different animals (horse, cow, donkey) on disease, again, showed little benefit following passive transfer (18, 19). Moreover, in a more recent study, New Zealand rabbits were intravenously infected with *Mtb* in order to generate immune serum. When this serum was administered to mice challenged with BCG, disease was actually enhanced (20), arguing for a deleterious effect of *Mtb*-specific antibodies. Over time, studies, such as these have helped to construct the narrative that antibodies are not beneficial, and may even be detrimental.

Yet, in the wake of these disappointments, several studies had in fact shown a beneficial, bactericidal activity of antibodies both *in vitro* and *in vivo*. For example, immune guinea pig serum was reported to have complete bactericidal activity against *Mtb in vitro* (18). Moreover, early passive transfer of immune bovine serum in 412 subjects with tuberculosis, was reported to induce complete resolution of disease in 16% of treated subjects, to ameliorate clinical symptoms in 40% of subjects, and to mediate the clearance of sputum bacteria in 43% of patients treated with serum (21). Similarly, immune donkey serum was also reported to cure 83% of treated subjects in another study (22). Finally, the use of horse serum was shown to have significant disease benefit in more than 80% of treated individuals in one study, however the same serum had limited benefit in additional clinical studies (23, 24), calling into question comparability across studies (19). Thus, in reality, small sample sizes, differences in disease severity, differences in clinical endpoint analyses, and the lack of control groups in many of these passive transfer studies resulted in mixed findings, ranging from no benefit to complete resolution of disease (6, 19, 22–24). Furthermore, antibiotics began to gain traction at this time showing consistent anti-microbial effects (25, 26), casting further doubt, not on the anti-microbial activity of some sera, but in the utility of these therapeutics in light of simpler treatment regimens with drugs.

Yet, despite the intermittent signals of efficacy, little attention focused on the underlying biological differences across studies. Specifically, little attention was paid to potential species-specific differences in efficacy across passively transferred antibodies, the impact of sensitization approaches, the critical potential impact of differences in antibody-specificities across immune sera, or the overall anti-microbial potency of the transferred sera. Thus, rather than demonstrating a lack of clinical benefit, collectively, the body of passive transfer studies instead clearly highlighted that not all antibodies are protective, and that qualitative nuances exist across humoral responses that are critical determinants of humoral protection (19).

Despite the confusing historical data, a number of passive transfer studies performed in the past two decades support the protective nature of serum-transfer. In a passive transfer study by Guirado et al., a liposome packaged preparation of *Mtb* extract in combination with rifampicin and isoniazid was administered to *Mtb* infected mice (27). Hyperimmune serum was then extracted from these mice and applied to previously *Mtb* infected SCID mice (lacking functional T cell, B cells, and NK cells) that

had received antibiotics. Through 10 weeks post-infection, the hyperimmune serum treated mice maintained lower CFUs in the lungs than control groups (27). Thus, even in the setting of a compromised immune response in the SCID mice, passive transfer was able to reduce *Mtb* burden *in vivo* independent of CMI.

Another significant passive transfer phenotype resulted from the administration of human intravenous immunoglobulin (IVIg) to *Mtb*-infected mice. IVIg is derived from the serum of large numbers of individuals, and often harbors immunoglobulin from *Mtb* exposed individuals either due to BCG vaccination, undiagnosed latent infection, or environmental mycobacterial exposure. Using an IVIg preparation with *Mtb*-specific antibodies, investigators observed a significant decrease in *Mtb* burden in mice following the passive administration of IVIg (28). Interestingly, this anti-microbial function was observed when the pool of antibodies was administered both early and late in infection (28). Given the emerging appreciation for the critical protective role of antibody IVIg Fc-glycosylation in autoimmune disease (29), Olivares et al. profiled the impact of Fc-deglycosylated IVIg. Removal of the Fc-glycan using EndoS, resulted in significantly reduced anti-microbial control, highlighting for the first time that the Fc-activity of transferred antibodies was critical to their protective function (30). Prior work by Olivares et al. found that intranasal IVIg was protective in mice and that this effect was eliminated when the inoculant was depleted of *Mtb*-specific antibodies, indicating that protection was mediated by antibody binding and subsequent Fc-receptor interactions (31). Thus, these data pointed to functions beyond simple binding and blockade of infection, in control and elimination of *Mtb*.

Finally, an intriguing study by Li et al. demonstrated that some, but not all, sera from infected or even sensitized-but-uninfected individuals harbor protective antibodies in serum transfer studies (32). Specifically, sera from healthcare workers with latent infection or from healthcare workers that were highly exposed, but remained negative in clinical diagnostic tests, were transferred to mice prior to aerosol challenge. Upon challenge, antibodies from half a dozen healthcare workers, including both latently and uninfected donors, conferred protection, resulting in a 2- to 3-fold decrease in lung CFUs compared to mice receiving serum from actively infected donors. Surprisingly, $CD4^+$ T cells were also critical for serum-based protection, suggesting a novel axis by which antibodies and T cells may work synergistically to drive anti-microbial function. Moreover, beyond this observation, the data reported from this study were the first to highlight that not all sera are equally protective, and that even sera from highly-exposed, but uninfected individuals may harbor the key protective humoral immune responses that can drive anti-microbial function *in vivo*.

Taken together, despite early inconsistencies in preclinical and clinical results following polyclonal passive transfer studies, emerging data clearly highlight the protective nature of some—but not all—serum preparations. These data clearly motivate a re-examination of the specific features of polyclonal antibody responses able to drive protection following serum transfer.

A CASE FOR ANTIBODIES FROM MONOCLONAL THERAPEUTIC STUDIES

Due to their defined antigen specificity and modifiable Fc domains, monoclonal antibodies allow the precise determination of antibody features contributing to disease control. In the late 1990's, an array of murine-derived monoclonal antibodies were created and assessed for their ability to mediate protection in mice that largely exhibited progressive disease (33), representing a high bar for antibody-mediated protection. While not all tested monoclonal antibodies conferred protection *in vivo*, antibody clone 9d8, specific to the capsular antigen arabinomannan (AM), significantly improved survival over time (34). While this antibody did not reduce *Mtb* burden, it prolonged survival via improved granulomatous containment of *Mtb*. Interestingly, rather than a canonical blocking function of the antibody, the authors speculated that this effect was attributable to antibody-mediated enhancement of cellular immunity. These data were among the first to highlight the protective activity of monoclonal antibodies, as well as the possibility that these molecules may confer protection through unexpected mechanisms of action.

Since this study, additional monoclonal antibody passive transfer experiments with antibodies targeting different *Mtb* antigens have resulted in various forms of protective activity. Hamasur et al. observed that antibody clone SMITB14, specific to the cell well-glycolipid lipoarabinomannan (LAM), prolonged survival upon administration to mice (35). In contrast to the previous study with the 9d8 antibody to AM, the LAM-specific SMITB14 antibody resulted in a significant reduction in bacterial burden in the lungs and spleen of infected mice. Importantly, when the $F(ab')_2$ domain of SMITB14 was administered, following the removal of the antibody constant domain that is required for the recruitment of innate immune functions, survival was similar to full length antibodies, suggesting that the binding activity of the antibody was sufficient to provide protection against disease. In contrast to the work with IVIg, this data pointed to the importance of binding and potential blockade of infection by LAM-specific antibodies, analogous to the neutralization of viruses (36), that is independent of Fc-interactions within the immune system (35).

Two antibodies targeting *Mtb* protein antigens have additionally been shown to modulate disease *in vivo*. Passive transfer of a monoclonal antibody binding heparin-binding hemagglutinin adhesin (HBHA), a surface exposed mycobacterial adhesin (37, 38), was shown to prevent mycobacterial extrapulmonary dissemination in mice (38). Additionally, a second antibody targeting the heat shock protein X (HspX), a stress-induced intracellular and cell wall protein (39), was shown to reduce lung *Mtb* burden in mice (40). Interestingly, the HspX-specific antibody was of the human IgA1 isotype, and importantly, protection was only observed in mice transgenic for human CD89 (FcαR1), the primary receptor for human IgA. Monoclonal antibody administered to the CD89-negative littermates did not demonstrate any measurable level of protection, strongly suggesting that the effect was Fc-mediated.

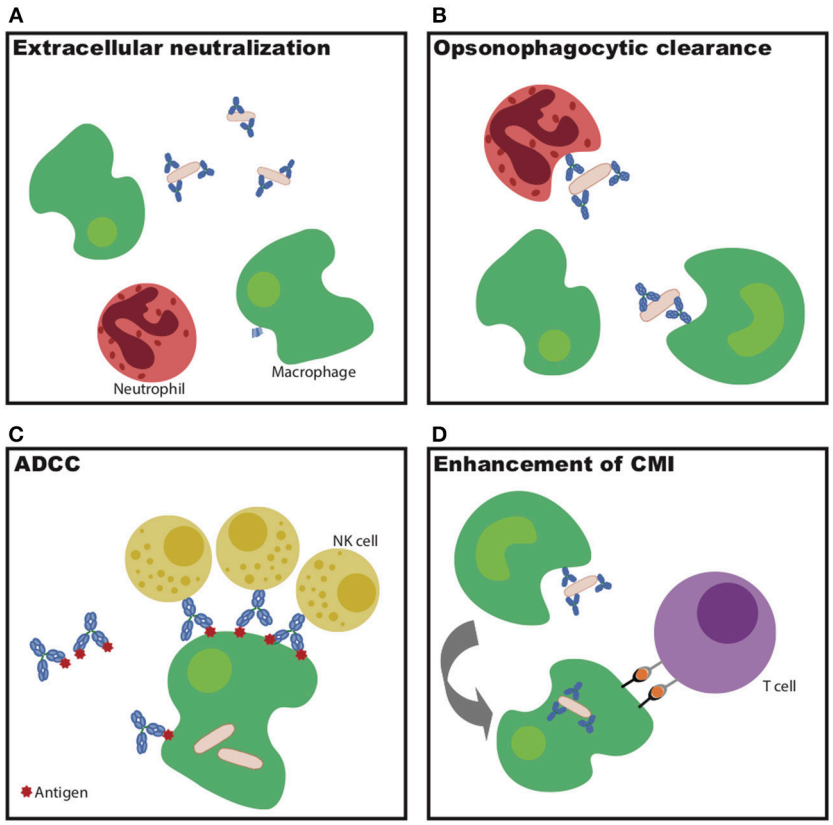


FIGURE 1 | Potential mechanisms of antibody-mediated Mtb restriction. **(A)** Antibody binding to extracellular bacteria prevents entry into cells. **(B)** Antibody-dependent cellular phagocytosis drives increased bacterial killing during uptake. **(C)** Antibody-dependent cellular cytotoxicity drives infected cell and/or bacterial killing. **(D)** Antibodies potentiate cell-mediated immunity via enhanced antigen presentation.

Overall, this monoclonal therapeutic work demonstrates that multiple antibody specificities are able to confer protection against *Mtb in vivo*. However, the protective mechanisms diverge by specificity, highlighting the multiple humoral mechanisms—some via strict blockade and others via innate immune engagement (**Figure 1**)—that may be harnessed to control and ultimately eliminate the bacteria *in vivo*.

A CASE FOR ANTIBODIES FROM COHORT STUDIES

Arguments for and against antibodies have additionally emerged based on observations from human cohort studies. A study in China showed that patients with X-linked agammaglobulinemia (XLA) do not have increased susceptibility to tuberculosis despite lacking mature B cells and normal antibody titers (41). Similarly, patients with defective Bruton's tyrosine kinase genes, manifesting in compromised humoral immunity, also show no indication that humoral immune deficiencies predispose individuals to increased risk of tuberculosis disease (12, 42). However, critically, many of the study subjects, lacking humoral immune components, were given IVIg therapy (12, 41, 42), transferring *Mtb*-specific antibodies; a clear confounding factor given the protective effect of IVIg described above (28, 30, 31). Other arguments against humoral immunity cite the finding that humans receiving B cell-depleting monoclonal antibody, rituximab, do not have a measurable increase in risk for tuberculosis disease (43, 44). While appealing, the argument does not take into consideration the fact that rituximab has a limited to negligible impact on depleting antibody-secreting cells, plasma cells, that reside in the bone marrow and do not express CD20. Moreover, rituximab treatment does not alter antibody levels that remain relatively stable over time (43, 44).

In contrast to the arguments against antibodies as correlates of *Mtb* control, both historical and more recent cohort studies have indicated a potential role for antibodies in combating tuberculosis disease. While increased *Mtb*-specific antibody levels have traditionally been associated with progressive disease (12), in the 1990s, a study in children from the UK and southeast Asia found that lipoarabinomannan (LAM)-specific IgG titers correlated with decreased risk of disseminated disease independent of age and geographic origin (45). Troughs in LAM IgG titer levels coincided with peak incidence of bacterial dissemination, and serum derived from the disseminated group also showed significantly ($p < 0.05$) decreased titers to purified LAM as measured by LAM binding ELISA. These data pointed

to specific antibody populations, rather than total *Mtb*-specific antibodies, as a correlate of disease control.

More recently, building on qualitative differences in *Mtb*-specific antibodies, it was observed that individuals with latent and active tuberculosis generate distinct functional antibody profiles (46). Given the ability of antibodies to deploy the innate immune system to drive pathogen clearance, the study broadly profiled the Fc-profile of *Mtb*-specific antibodies. The study noted nearly distinct antibody Fc profiles across the groups, with differences largely driven by distinct IgG Fc-glycosylation patterns. Specifically, purified IgG from LTBI patients exhibited enhanced binding levels to FcγRIIIa, the Fc-receptor found on NK cells, which resulted in increased antibody-dependent cellular cytotoxicity (ADCC) and increased NK cell activation. Intriguingly, these differences in biophysical and functional differences also corresponded with an increased ability for antibodies from LTBI patients to drive intracellular *Mtb* killing in macrophages. Consistent with these data, a recent multi-cohort analysis that aimed to identify immune factors associated with asymptomatic LTBI, observed that LTBI control was associated with higher signaling via FcγRIIIa, and enhanced NK cell activity (47), corroborating the potential role of NK cell recruiting antibodies in long-term *Mtb* control.

Taken together, while human cohort studies have been commonly pointed to as evidence against a protective role for antibodies in *Mtb* infection, increasing evidence argues against this. Human cohort studies have been consistent with monoclonal and passive transfer studies, identifying LAM as a productive antibody target, as well as identifying the Fc-domain and antibody effector functions as critical qualities that modulate disease outcome. Thus, specifically manipulating both the specificity and functionality of the *Mtb*-specific humoral immune response represent tractable approaches for the design of next-generation therapeutics and vaccines.

A CASE FOR ANTIBODIES FROM VACCINE STUDIES

Despite the fact that nearly all successful vaccines to date function by eliciting protective antibody responses, the majority of tuberculosis vaccines in use and in development, have been focused on the induction of CMI (48). Recent work by Hansen et al. demonstrates that a cytomegalovirus vector vaccine expressing *Mtb* antigens protects against *Mtb* infection without eliciting detectable antibody levels in the blood (49). However, a number of vaccine studies in murine and macaque preclinical models, as well as large-scale clinical trials in humans, have suggested a potentially protective role for antibodies in *Mtb* vaccine efficacy.

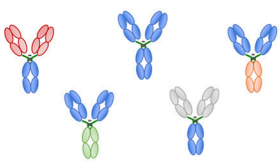
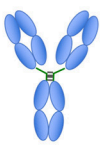
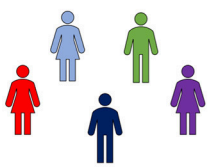
Study type	Evidence	
 Passive transfer studies	Evidence for	❖ Hyperimmune serum from vaccinated mice reduces *Mtb* burden in SCID mice upon passive transfer (27) ❖ IVIg passive transfer reduces *Mtb* burden in mice in an antigen-specific, and Fc-dependent manner (28-30) ❖ Immune serum from highly exposed healthcare workers decreases respiratory bacterial burden in mice upon passive transfer (32)
	Evidence against	❖ Administration of immune sera from various animals failed to ameliorate tuberculosis disease *in vivo* (17-20)
 Monoclonal therapeutic studies	Evidence for	❖ Monoclonal antibodies specific to AM, LAM, HBHA, and HspX ameliorate *Mtb* disease in mice upon passive transfer (34,35,38,40)
	Evidence against	❖ LAM and mAGP-specific IgM antibodies did not significantly protect mice upon passive transfer (34)
 Cohort studies	Evidence for	❖ Increased LAM-specific IgG titers correlated with decreased risk of disseminated tuberculosis disease (45) ❖ Purified IgG from LTBI patients exhibits increased binding to FcγRIIIa, increased ADCC activity, and increased ability to drive intracellular *Mtb* killing compared to purified IgG from ATB patients (46)
	Evidence against	❖ B cell and antibody depleted patients do not show increased risk of developing tuberculosis (12,41,42, 43,44)
 Vaccine studies	Evidence for	❖ Antibodies elicited by an AM-conjugate vaccine have protective activity in mice (50) ❖ *Mtb*-specific IgA levels in the lungs represent a primary correlate of vaccine-mediated protection in rhesus macaques (51) ❖ Vaccine-induced Ag85A-specific IgG titers correlate with reduced risk of developing ATB (52)
	Evidence against	❖ Significant antibody levels not detected to select *Mtb* antigens following protective vaccination of rhesus macaques with a cytomegalovirus-based vaccine (49)

FIGURE 2 | A table summarizing the key findings for and against a role for antibodies in *Mtb* disease control.

Beginning in the murine model, Prados-Rosales et al. performed one of the few studies that rationally designed a tuberculosis vaccine to selectively induce a mycobacteria-specific antibody response (50). Specifically, the capsular polysaccharide AM was conjugated to either *Mtb* Ag85b or the *B. anthracis* protective antigen. Both polysaccharide-conjugate vaccines significantly reduced lung bacterial burden in *Mtb* infected mice. Additionally, passive transfer of immune serum from AM-vaccinated mice provided protection in the form of reduced lung bacterial burden when administered prior to *Mtb* challenge of naïve mice. These data therefore provided concrete evidence for a protective role of vaccine induced AM-specific antibodies *in vivo*, pointing to the potential utility of a polysaccharide conjugate vaccine against *Mtb*.

In the rhesus macaque model, a localized, lung specific vaccine-induced antibody response was linked to protection (51). Specifically, rhesus macaques were vaccinated with BCG either intradermally, representing the standard route of immunization, or by the mucosal route via endobronchial instillation. Following repeated low-dose challenge with *Mtb*, the group receiving the mucosal vaccination demonstrated decreased lung CFUs and pathology. Intriguingly, the mucosal group also mounted a unique and robust PPD-specific antibody response, including enhanced PPD-specific IgA levels, locally in the bronchoalveolar lavage fluid. This compartment-specific induction of immunoglobulin in the lung represented a primary correlate of protective immunity, pointing to a humoral correlate of protection—focused at the site of infection—for the first time following BCG vaccination.

Finally, in humans, antibodies have emerged as a correlate of protection in a number of different tuberculosis vaccine clinical trials. In 2009, a phase 2b trial was conducted in BCG primed individuals, using a recombinant Vaccinia Ankara virus modified to express Ag85A (MVA85A) to boost cellular immune responses (52). While results confirmed that the treatment was safe and tolerable, vaccine efficacy was not significantly higher than BCG vaccination alone. Despite the lack of protective efficacy over BCG, a 2016 correlates analysis found that the presence of Ag85A-specific IgG titers correlated with a reduced risk of developing TB (53), pointing to the unexpected presence of a humoral immune correlate of protection in this human vaccine study.

More recently, protection was observed using an adjuvanted fusion-protein vaccine strategy in the M72/AS01$_E$ vaccine trial (54). The trial tested the safety and protective efficacy of a vaccine comprised of a fusion of *Mtb* antigens *Mtb*32A and *Mtb*39A, two mycobacterial virulence factors poised to induce robust T cell immunity, delivered with AS01 as the adjuvant. This phase 2b trial showed an exciting 54% protection against progression to active tuberculosis disease in *Mtb*-infected adults. Interestingly, while expected T cell immunity was observed, the vaccine induced a robust anti-M72 protein-specific IgG response that remained 26-fold higher than the pre-vaccination levels even 12 months following vaccination (55). Given that these proteins may contribute to mycobacterial virulence, it is plausible that both canonical virulence blocking antibodies as well as potentially non-canonical antibody functions could contribute to vaccine efficacy. Thus, additional work is required to identify more precisely the potential mechanistic role of antibodies in M72-mediated protection from infection.

Overall, data from vaccine studies in mice, primates, and humans, have hinted at a potentially protective role for antibodies in mediating protection. While it must be acknowledged that in each of these cases a robust T cell response was also observed, the antibody responses should not be overlooked and may function independently of, and/or in coordination with CMI to confer protection. Thus, vaccines designed to induce antigen-specific immunoglobulins merit further consideration.

FUTURE PERSPECTIVES FOR ANTIBODIES

Together, the studies examined above illustrate that antibodies can modulate *Mtb* disease *in vivo*, and that this protective effect can manifest in numerous ways (**Figure 2**). Across numerous categories of experimental evidence, antibodies binding specific antigens, including polysaccharide surface antigens (AM and LAM), and virulence factors (HBHA and Ag85) demonstrate particular promise. Nevertheless, relative to the large number and broad landscape of *Mtb* antigens, very little is known about additional antigens that may represent productive antibody targets. Moving forward, continued generation and testing of *Mtb*-specific monoclonal antibodies against a diverse array of *Mtb* antigens will allow this further antigenic characterization and the evolution of vaccine design, beyond the empirical approach, to a rational process founded on knowledge of protective targets in *Mtb*.

Beyond recognition of specific antigens by the antibody variable domain, evidence indicates that antibody Fc-mediated signaling also plays a critical part in *Mtb* control. Mice unable to signal through activating Fc-gamma receptors have increased bacterial burden in the lung and spleen following *Mtb* infection, as well as decreased survival compared to wild-type mice (56). Consistent with this finding, lines of evidence across passive transfer, cohorts, and vaccine studies all indicate a likely role for the antibody Fc in antibody-mediated protection (40, 45–47, 56). As alluded to above, this Fc-mediated action may function through enhanced opsonize bacterial uptake, through the recruitment of innate immune cell killing of infected macrophages, or through the potentiation of adaptive immunity through the delivery of antigens for immune priming via antigen presentation (**Figure 1**) (51). Expanding our understanding of the mechanisms by which antibodies may selectively leverage distinct immune effector mechanisms of action will be critical for the rational design of next generation monoclonals or vaccines to combat *Mtb* disease.

Future studies leveraging animal models will remain key to uncover antibody mechanisms of action, and will advance the development of vaccines and therapeutics able to fully harness the humoral immune system to fight the bacteria. Non-human primates represent the ideal animal model for antibody studies due to similar disease manifestations (spectrum of disease) as observed in humans (57), as well as their highly homologous

Fc-receptor repertoire and function (58). However, due to the limited number of BL3 facilities able to conduct *Mtb* infections in NHPs, as well as the high cost associated with running NHP studies, the model remains under-utilized. Nevertheless, mice represent an imperfect but potential first model to study the role of antibodies in *Mtb* disease. While mice do not display the same spectrum of tuberculosis infection states as humans, numerous consistencies between mouse and human work have advocated for their continued use. For example, LAM-specific antibodies have been demonstrated to ameliorate *Mtb* disease in mice (35), and in humans, high LAM antibody titers are associated with less severe tuberculosis disease (45). Moreover, Li et al. demonstrated that antibodies from latently infected, and highly exposed, but uninfected healthcare workers reduced *Mtb* burden in mice upon passive transfer as compared to IgG from ATB patients, mimicking results observed *in vitro* in an *Mtb* human whole-blood restriction assay (32). Furthermore, given the analogous response of human and innate immune cells to human IgG1 antibodies (59), the mouse model offers an opportunity to explore the mechanistic function of anti-*Mtb* IgG antibodies *in vivo*. Finally, human Fc-receptor knock-in mice, able to respond to both IgGs (60) and IgAs (40) offer additional opportunities to screen and explore human protective antibody responses *in vivo*.

CONCLUDING REMARKS

Though historically humoral immunity has often been overlooked, and in some instances wrongfully discredited, in the context of *Mtb* infection, the evidence amassed from passive transfer, monoclonal therapeutics, cohort and vaccine studies has coalesced into a compelling argument for the importance of antibodies and for their continued study (**Figure 2**). Ultimately, expanding on this work will provide a more complete picture of the immunological drivers of protection against *Mtb*, beyond simple CMI, and may precipitate the development of novel therapeutics and vaccines against this global killer.

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REVIEW
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Monkeying Around: Using Non-human Primate Models to Study NK Cell Biology in HIV Infections

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Natural killer (NK) cells are the major innate effectors primed to eliminate virus-infected and tumor or neoplastic cells. Recent studies also suggest nuances in phenotypic and functional characteristics among NK cell subsets may further permit execution of regulatory and adaptive roles. Animal models, particularly non-human primate (NHP) models, are critical for characterizing NK cell biology in disease and under homeostatic conditions. In HIV infection, NK cells mediate multiple antiviral functions via upregulation of activating receptors, inflammatory cytokine secretion, and antibody dependent cell cytotoxicity through antibody Fc-FcR interaction and others. However, HIV infection can also reciprocally modulate NK cells directly or indirectly, leading to impaired/ineffective NK cell responses. In this review, we will describe multiple aspects of NK cell biology in HIV/SIV infections and their association with viral control and disease progression, and how NHP models were critical in detailing each finding. Further, we will discuss the effect of NK cell depletion in SIV-infected NHP and the characteristics of newly described memory NK cells in NHP models and different mouse strains. Overall, we propose that the role of NK cells in controlling viral infections remains incompletely understood and that NHP models are indispensable in order to efficiently address these deficits.

Keywords: HIV, SIV, non-human primates, innate immunity, natural killer cells, animal models

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INTRODUCTION

Natural killer (NK) cells have previously been thought simplistically and aptly named, but recent characterizations suggest their roles in both innate and adaptive immunity are in fact quite diverse and complex. In humans, traditional phenotyping identifies NK cells as large non-B, non-T cells expressing CD56 and CD16, and in peripheral blood they are broadly classified into two subpopulations—$CD56^{bright}$ cytokine-secreting and $CD56^{dim}CD16^{+}$ cytotoxic cells. The major function of NK cells in viral infections and cancer is lysis of target cells by rapidly releasing cytolytic mediators such as perforin and granzyme B and/or secretion of inflammatory cytokines which include but are not limited to interferon (IFN)-γ, tumor growth factor (TGF), tumor necrosis factor (TNF), interleukin (IL)-6, IL-10, granulocyte macrophage-colony stimulation factor (GM-CSF), and G-CSF. NK cell functions are controlled by a balance of activating receptors such as

natural cytotoxicity receptors (1) (NKp30, NKp44, and NKp46), activating killer immunoglobulin receptors (KIRs) and C-type lectin receptors (NKG2D and NKG2C), and inhibitory receptors including inhibitory KIRs and NKG2A (2, 3). Recent studies in humans and mouse models have uncovered the existence of an array of NK cell subsets of diverse phenotypes and differential functions. Indeed, NK cell diversity in a single individual could range from 6,000 to 30,000 distinct phenotypes (4) and their functional repertoire now includes long lived memory-like responses, antigen specific memory responses and immunoregulatory roles in addition to their previously known innate functions (5–13). Given their unique nuances in phenotype, maturation, and function in blood and different tissue compartments, it is imperative to understand the role of NK cells in infections, specifically mucosal infections such as human immunodeficiency virus (HIV) and others. To this end, animal models, both mice and non-human primates (NHP), have proved useful in deepening our knowledge on NK cell biology, subsets, and tissue specific responses in health and disease. In the context of animal models to recapitulate the role of NK against HIV-1 infection, this review will primarily focus on these aspects of NK cells in human responses and its analogous modeling in non-human primates (NHP), which has been summarized in **Figure 1**.

CAVEATS OF MODELING HUMAN NK CELL BIOLOGY IN MICE

Mouse models have played significant and historic roles in understanding the interplay of the immune system and infections, with basic NK cell biology as a particularly notable example. However, critical differences between human and murine NK cells can sometimes complicate direct comparisons. Murine NK cells do not express CD56 but have approximate functional homologs—$CD11b^{low}CD27^{high}$ and $CD11b^{high}CD27^{low}$ NK cells have been correlated to the human $CD56^{bright}$ and $CD56^{dim}$ subsets respectively (14). However, the $CD27^{low}$ subset is not capable of antibody dependent cell cytotoxicity functions (ADCC). Murine NK cells lack NKp44 and NKp30 expression altogether, and indeed NKp46 is the only NCR that is expressed on both murine and human NK cells (15). Further, while both murine and human NK cells express NKG2D, the ligands differ between the species. Murine NKG2D binds to 3 members of the minor histocompatibility family, 5 members of the retinoic acid early inducible gene 1 (Rae-1) family of proteins and murine UL-16-binding protein-like transcript (MULT1). Human NKG2D ligands include MHC I-like (MIC) molecules, MHC-I chain-related A, MHC-I chain-related B and UL16 binding (ULBP) protein family (16). The MIC family proteins are highly polymorphic with more than 70 alleles, and the NKG2D ligands of both species diversified independently, and thus are not orthologous (17). The trafficking markers, and hence the tissue distribution, also vary between the two species. For example, human NK cells are generally present homeostatically in lymph nodes (LN), albeit at low levels, whereas NK cells are observed in murine LN only after stimulation (18). A major difference, as well as an evolutionary disparity, is the recognition of their cognate MHC class I molecules. Murine NK cells use the Ly49 family of proteins which have C-type lectin domains, for cognition of MHC I molecules (19). Human NK cells lack Ly49 proteins and rather express the highly divergent Ig superfamily receptors called KIR that recognize MHC-I (20). Both Ly49 and KIRs act as functional analogs, but they vary to a large extent in their genetic and structural properties and exhibit qualitative differences in their MHC-I interaction. While mouse studies have significantly expanded our current knowledge of NK cell subsets and their functionality, some of these significant differences complicate the modeling of NK cell biology for some

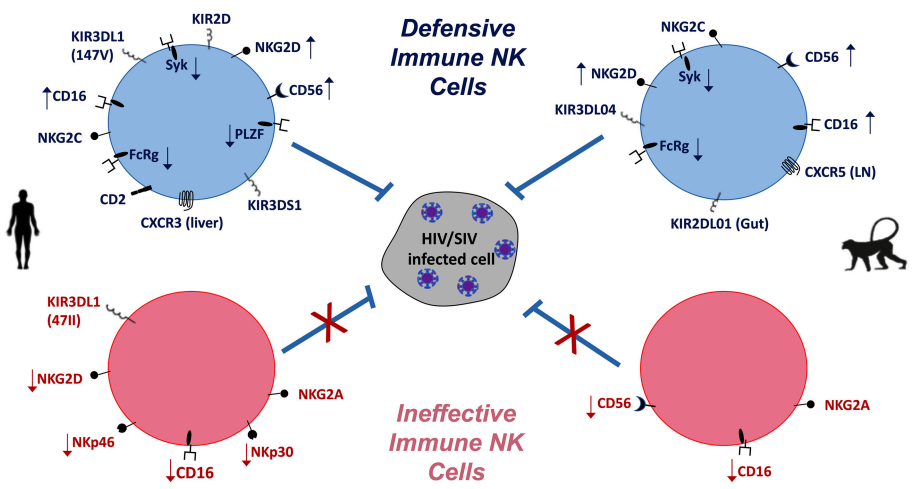

FIGURE 1 | NK cells in HIV/SIV infections of humans **(Left)** and macaques **(Right)**. Differential expression (↑ increase, ↓ decrease) of NK cell markers indicative of defensive and effective anti-HIV/SIV functions (blue) are compared to virus-induced impaired (pink) functions. Overall, the balance of activating and inhibitory receptors is indicative of NK cell functionality and contribution to pathogenic outcomes.

human diseases. Indeed, the limited lifespan, differences in antibody repertoires, and most critically for HIV-1 infection, the lack of lentiviral tropism in mice, has restricted their use as an animal model to understand the role of NK cells against HIV-1.

MODELING NK CELL BIOLOGY IN NHP

NHP NK cells are generally much more similar to human NK cells than murine NK cells and the possibility of *in-vivo* manipulations, such as depleting NK cell numbers, offer opportunities to specifically address NK cell biology. The peripheral NK cell frequency in Old World monkeys, which includes rhesus, cynomolgus and pig-tailed macaques, sooty mangabeys and African green monkeys (AGM), averages ∼10% of blood lymphocytes similar to humans. Whereas, in neotropical primates such as common marmosets and cotton-top tamarins, the NK frequency is typically < 5% (21–26). Phylogenetic studies comparing multiple mammalian species have identified KIR3DL as the first ancestral gene originating from simian primates (27). Similar to human NK cells, great apes and Old World monkeys have a rich diversity of KIR3DL1, whereas the New World monkey KIRs diverged from the Old World monkeys, apes and humans, and their KIR3DL1 is more specific to their species. NHP NK cells also have a few dissimilarities such as the low expression of CD56, universal expression of CD8α and NKG2A/C by all subsets of NK cells compared to human NK cells (21, 25, 26, 28, 29). Due to this, the major delineating markers commonly used to identify NK cells in Old World and New World monkeys are CD8α/NKG2A/C and NKp46 respectively.

NHP NK cells, particularly those in rhesus macaques (MAC), have been studied in detail over the last two decades. Gating for CD56 and CD16 expression on circulating NKG2A/C$^+$ MAC NK cells, defines three distinct populations: CD56$^+$CD16$^-$ cells which are functionally equivalent to human CD56^{bright} NK cells; CD56$^-$CD16$^+$ cells corresponding to the human CD56^{dim} NK cells and the CD56$^-$CD16$^-$ (DN) cells for which an analogous phenotype in humans is not yet clearly defined (30, 31). Although NK cell differentiation is dynamic, the CD56 expression pattern can denote the functional maturation of human NK cells, whereby downregulation of CD56 expression indicates a mature differentiated cytotoxic profile (32–34). Hong et al. (35) identified expression patterns in MAC NK cell subsets similar to human NK cells by transcriptional analysis. Expression pattern of transcripts in MAC CD56$^+$ cells were consistent with primitively differentiated cytokine producing cells evidenced as IL-7R, TNF receptor super family member 1B, GATA-3, TCF-7, CD53, amphiregulin, and Granzyme K among others. Conversely, transcripts of effector proteins, such as CCL3, CCL4, and CCL5, were highly expressed in CD16$^+$ cells. Interestingly, Hong et al. (35) found the DN subset to be an intermediary stage between the CD56$^+$ and CD16$^+$ subsets based on the transcriptional profile. While CD57 has also been proposed as a marker of mature, functionally distinct population of NK cells in humans (36), a simian analog has not been identified yet. Overall, the phenotypic, functional and transcriptional profiling has shown that NHP NK cells are well-suited to model their human counterparts as it will be discussed in the following sections.

NK CELL MODULATION OF HIV AND SIV INFECTIONS VIA KIR/HLA

Epidemiological studies of long-term non-progressors and elite controllers of HIV infection have indicated that the co-expression of KIR3DS1 and a specific HLA-B haplotype known as the HLA-Bw480I correlates with lower viral load, a slower decline of CD4$^+$ T-cell counts and delayed progression to AIDS (37–39). In fact, the NK cell subsets upregulate KIRs and KIR-like molecules in their effort to control virus replication as demonstrated by the protective role of HLA-Bw480I that can potentially bind KIR3DL1 on the membrane of NK cells, contribute to their expansion (40) and increase their cytolytic function (41). In addition to the polymorphism in the HLA-Bw4 variants associated with protection from disease progression, it has been recently reported that a single isoleucine-to-valine substitution in position 47 (I47V) of the KIR3DL1 was responsible for a less protective role in controlling HIV-1 infection compared to the 47VV (not reaching significance) and a significantly more protective role than the 47II genotype (42); the protective role was confined to its interaction with the HLA-B*57:01 and not with the HLA-B*57:03. These data suggest that the KIR-HLA interaction is specifically tuned to impact control of HIV-1 replication. These observations are also supported by the findings that both KIRDL2 and KIRDL3 expressing NK cells can mediate control of HIV-1 via interaction with HLA-C molecules (43, 44). In addition to the polymorphism of the KIR receptor, higher copy numbers of KIR3DS1 and KIR3DL1 in the presence of their ligands were associated with lower viral set point. NK cells from individuals with multiple copies of KIR3DL1 in the presence of KIR3DS1 and their ligands were able to inhibit *in vitro* replication more robustly (37).

Similar observations on the importance of the interaction between KIR and class I HLA molecules have been reported in MAC models and linked with their ability to control SIV infection. The polymorphisms of MAC KIR differs from human (45), but several activating KIR, defined as KIR3DL or KIR3DH, have been associated with lower virus load and longer survival alone or in association with the class I Mamu-A1*001 allele (46) [see also review by Walter and Ansari FI 2015 (47)]. Enhanced copies of KIR3DL04 (or KIR3DH04) were also reported to associate with decreased loss of CD4$^+$ T cells and increased CD56/16 DN NK production of IFN-γ (48). Lastly, a unique profile of circulation and tissue accumulation of the KIR3DL01$^+$ NK subset during acute SIV infection was reported to indicate that this subset (and not the KIR3DL05$^+$) accumulate in gut tissues. These cells also displayed higher proliferation, activation, and antiviral function during chronic infection (49). The study did not correlate the findings with the outcome on control of viremia or disease progression, but raises the important issue of the dynamic changes that take place within the NK subsets during infection, with regard to their frequencies and tissue distribution. An important outcome for the recognition of infected cells by NK

cells through the KIR/HLA interaction is related to the ability of NK cells to exert immune pressure on HIV-1 sequence (50, 51), similar to what was described for the $CD8^+$ T cell responses (52, 53).

As discussed above, KIRs play important roles in controlling both HIV and SIV infections. However, one must be judicious when using NHP models to study the impact of KIRs on retroviral infection, due to the disparities between the KIR repertoires of human and that of NHP (54). For instance, the KIR subtype with only one extracellular domain, KIR1D, has been identified in MAC, which seems to be unique to NHP and there is no corresponding counterpart in human (55). Even though the nucleic acid sequence contains two Ig-like domains, the expressed protein is truncated at the second domain due to a frame shift mutation (55). Consequently, the KIR1D subtype lacks the cytoplasmic domain as well, and although it's *in vivo* function is still currently unknown, it is speculated to be secreted extracellularly (56). Another major feature that differentiates the human and NHP KIR is their complexity of KIR2D and KIR3D subtypes. Based on the structure and MHC molecule specificity, the KIR genes can be divided into 4 main lineages, I, II, III, and V (54, 57). While only 3 distinct lineage II KIR3D genes have been characterized in human, MAC has been reported to have a highly diverse lineage II repertoire, consisting of 10 KIR3DL genes and 9 KIR3DS genes (56, 58, 59). This may have evolved to complement the expanded HLA-A and B genes that are observed in NHP, and indeed, some of the KIR3DL/S has been shown to bind to HLA-A/B molecules (27, 58, 60). Conversely, humans have more diverse lineage III KIR genes that are absent in Old-World monkeys, such as MAC (55, 61), which is consistent with the higher variability of HLA-C molecules in human (62). Thus, the KIR gene repertoire for NHP seems to be much more complex than that of humans. The latest studies identified a total of 22 different KIR genes for MAC (59, 63), as compared to 15 KIR genes and 2 pseudogenes in humans (64). In general, the complexity of KIR genotype and expression in relation to the class I HLA molecules should be carefully considered when expression of KIR on human and NHP NK subsets are evaluated for their correlation with the outcome of retroviral infections. Furthermore, it is clear that, in both humans and NHP, KIR, and HLA class I polymorphism can influence the outcome of infection, but the evolutionary advantage of these molecules has not been elucidated yet.

FC-RECEPTOR (FCR) MEDIATED NK CELL FUNCTIONS IN HIV/SIV INFECTIONS

Among the immunomodulatory and effector functions mediated by NK cells, their role as effector cells in Fc-dependent antibody functions represented by ADCC is very important in HIV-1 and SIV infections. This is best highlighted by the correlation between vaccine-induced ADCC responses and control of virus replication (65–68) and protection (69), and in pre-clinical studies conducted in MAC and recent observations in mother-to-child transmission (70, 71). Moreover, the only human vaccine clinical trial that provided limited success, the RV144 study conducted in Thailand, suggested a crucial role for non-neutralizing Ab responses capable of mediating ADCC as correlates with lower risk of infection (72, 73). The NK cells provide the effector cell component to these type of responses, upon engagement of their FcγR III (CD16) by the Fc region of an antibody that can recognize antigens expressed on the membrane of infected cells (74). In humans as well as in NHP, the canonical ADCC-mediating effector NK cell subset has been described as those that are lineage negative (lacking expression of markers defining major T and B cell subsets) and $CD16^+$, which are the peripheral $CD56^{dim}CD16^{bright}$ cells in humans and $CD3^-CD20^-CD8^+NKG2A/C^+CD16^+$in MAC. The effector function of these cellular subsets is in general regulated by the fine interaction between the Ab subclasses and the polymorphisms of the FcRs that present substantial differences between the human and NHP (75–77). In addition to the classical Fc-FcR engagement of ADCC effector cell subsets, it has also been reported that recognition of the infected target cells requires engagement of NKG2D-receptor, suggesting that the NKG2D may serve as a co-receptor for ADCC-mediated NK cell functions (78). In addition to the $CD8\alpha^+CD16^+$ NK subset, it has been reported that the $CD8\alpha^-$ NK cells can also be potent ADCC effector cells in MAC and co-express the CD56, CD16, NKG2D, and KIR2D receptors. These cells represent approximately 35% of the macaque $CD8\alpha^-$ cells and are responsive to stimulation by IL-15 to upregulate the CD69 receptor and produce IFN-γ and TNF- cytokines, providing additional functions to the cytotoxicity (79).

IMPACT OF HIV AND SIV INFECTION ON NK DISTRIBUTION AND FUNCTION

In healthy humans, tissue NK cells are more heterogeneous, complex and less studied than their peripheral blood counterparts due to limited access to human tissues. Tissue-resident NK cells differ by their pattern of chemokine and adhesion receptors, which are specialized based on their homing properties and/or *in-situ* maturation (80, 81). $CD56^{bright}$ NK cells in human blood express trafficking markers CD62L, CCR7, CXCR3, and CXCR4 that allow their migration into secondary lymphoid organs, inflamed tissues, and tumors, whereas tissue resident $CD56^{bright}$ NK cells do not express CD62L but other adhesion markers such as CD49a and CD103 (18, 82–84). The $CD56^{dim}$ subset expresses receptors that are necessary for migration into inflamed sites including CXCR4, CX3CR1, CXCR2, and CXCR3 and low levels of CD62L and no CCR7. On the other hand, $CD56^{bright}$ NK cells express high levels of CCR7 and CD62L and constitute a large proportion of NK cells in the lymph node because of their affinity to high endothelial venules (HEV) (18, 85). In fact, LNs have been proposed as a site of maturation for some NK cells (86). $CD56^{bright}$ NK cells are also the predominant population in the gut and participate in the gut homeostasis (87). However, multiple pathogens including

HIV-1, can disrupt the overall homeostatic NK cell distribution in tissues.

It has been previously reported that NK cells undergo redistribution amongst different tissue compartments during the acute phase of HIV infection, as indicated by the increased frequency of circulating $CD3^{neg}CD56^{neg}CD16^{pos}$ NK cells with perturbed functional profiles and reduced presence of $CD3^{neg}CD56^{pos}$ NK cells (88). Despite this body of evidence that suggests an important role for NK cells in the control of HIV-1 replication, they are not able to clear the infection. This may be attributed, at least in part, to the overall subversion of the immune system caused by HIV-1, where NK cells are not only altered functionally but may be impaired in trafficking and tissue infiltration. In fact, it was described very early that NK cells were dysfunctional in HIV-1 infected subjects (89) and this effect could be detected at the level of their ability to perform FcR-mediated functions (90), as well as expression of KIR, activation markers, and cytokine production (91, 92). The impairment of NK cells could be due to the direct effect of HIV-1 or, due to the effect of cytokine milieu on NKp30 and NKG2D expression (93) and/or CD4 dysfunction. The latter has been recently demonstrated to be the case because blockade of PD-1 and IL-10 pathways can restore the HIV-1 specific CD4 T cells *in vitro* and enhance cytokine expression and cytolytic function of the NK subsets (94). The presence of impaired NK subsets is not solely observed during HIV-1 infection as reported by Meier et al. (95). In fact, they described a similar alteration of the NK subsets in HIV-1 and Hepatitis C virus (HCV) infections with a clear decline in the frequency of the $CD56^{dim}$ NK that resulted in reduction of IFN-γ production and cytotoxic function (95). Similar observations on the impairment of NK cell function has been described in SIV-infected MAC related to differentiation, cytokine secretion, and expression of activation/homing markers (96, 97). The importance of these dysfunctions in the context of hampering the ability of NK subsets to fully control retroviral infection is indicated by the demonstration that in chronically SIV-infected MAC, the frequency of CD56/16 DN NK cells in the spleen and liver of infected animals with high virus load was significantly lower than in animals with lower virus load (98). Moreover, frequency of the liver-resident $CXCR3^+$ NK cells and circulating $NKG2D^+$ cells were inversely correlated with plasma viremia (98). These data suggest that differences in the location and function of the NK subsets have a relevant impact on the outcome of virus replication in the SIV model and the same may occur in HIV-1 infection. To support the complexity of this reality, data was collected from the animals that can naturally survive the SIV infection. In fact, the analysis of the distribution of NK cells within the LN in the pathogenic and non-pathogenic SIV-infection models represented by the MAC and AGM, respectively, revealed unique differential aspects (99). In the pathogenic model, the NK cells were found in a random distribution and did not accumulate in the follicles, whereas a significantly higher frequency of NK cells was observed in the AGM LN mostly around or within the follicles. The AGM NK cells also expressed CXCR5 and the frequency of $CXCR5^+$ NK cells in the AGM LN was significantly higher. This distribution persisted throughout the time of observation of the infected animals and was associated with a significantly higher frequency of cells with membrane-bound IL-15 in the AGM. Anti-IL-15 treatment of AGM depleted NK cells from LN, spleen, and gut, and it induced a significantly increased plasma viral load as well as the amount of cell-associated viral RNA and DNA in the LN, compared to the untreated animals. Collectively, these data indicated that the unique control of virus in non-pathogenic AGM is at least partially mediated by NK cells.

IMPACT OF *IN VIVO* NK DEPLETION IN SIV-INFECTED ANIMALS

A major argument for the importance of $CD8^+$ T cell responses in control of retroviral infection was initially provided by seminal studies conducted by Letvin and collaborators, who reported the immediate rebound in SIV replication in MAC upon depletion of $CD8^+$ T cells by infusion of targeted monoclonal antibodies (100). Similar experiments have now been conducted in MAC models to address the role of NK cells in virus control, but thus far have provided contrasting results. Initially, $CD16^+$ NK depletion performed using the 3G8 mAb 24 h before infection with SIV did not impact the level of viremia observed in the infused animals during the first 11 days of infection compared to those receiving a control mAb. These data suggested that $CD16^+$ NK cells did not contribute to initial control of SIV replication (101), although the assay had several caveats, such as the emergence of idiotypic antibodies (102) and the lack of $CD16^-$NK cells depletion from lymph nodes that are largely responsible for controlling virus replication (85, 103). More recently, depletion of NK cells in the periphery and in intestinal mucosal tissues following administration of JAK3 inhibitors induced a modest but significant increase of plasma viral load in all six animals tested and in tissue viral load in 5 out of 6 animals. The latter was not related to an increase in frequency of $CD4^+$ T cells, suggesting an increased production of the virus on a per cell basis. (104). A follow-up study investigated prolonged administration of the JAK3 inhibitor during the acute phase of infection and recapitulated the finding of significant higher virus replication during the chronic phase (>12 weeks) of infection in JAK3-treated MAC, but not during the acute phase of infection. One caveat to the latter study was related to the concomitantly observed partial depletion of $CD8^+$ T cell subsets, among other immune cells, and the unresolved contribution that this could have had on the outcome of the study, mainly implicating the depletion of NK subsets in the gastrointestinal tissue (105). The different outcomes of these studies could either be related to the stage of infection, acute vs. chronic infection, or to the depletion procedure, that could impact different NK cell subsets. Overall, the data do not provide a definitive determination of the impact that NK cells could have on the control of SIV infection in MAC. The anti-IL-15 neutralization approach to deplete NK cells has been shown to be effective in AGM and MAC (99, 106), but full evaluation has not been performed during acute and chronic infection of a pathogenic species.

MEMORY NK CELLS AND HIV/SIV

The possibility of NK cells with adaptive features perhaps emerged from an unexpected observation by Boehncke et al. (107) wherein wild-type (WT) and T-cell deficient mice responded similarly to 4-dinitro-1-fluorobenzene (DNFB)-induced contact hypersensitivity (CHS). NK cell memory, as an emerging field of study, was further solidified by O' Leary et al. (108) in a CHS mouse model with the observation of T- and B-cell independent adaptive immunity that was mediated by NK cells. These responses were elicited by haptens and persisted for at least 4 weeks following sensitization. The same group later demonstrated (109) that liver-resident NK cells in mice were not only capable of generating a memory pool against haptens but also against influenza, vesicular stomatitis virus (VSV) and HIV; and that the chemokine receptor CXCR6 plays a critical role in this process. Subsequently, the phenomenon has since been observed in other mouse models, non-human primates, as well as in humans (reviewed in (12, 110, 111)).

As our understanding of the memory NK cell response expands, multiple subpopulations that may mediate antigen recall through differing mechanisms have emerged. These different subtypes are, however, somewhat fluid. For practical purposes, we will comment on four categorizations of these cells:

1) True *antigen-specific memory NK* cells respond to an antigen presented analogously to classical adaptive cells. Antigens include haptens (112), cytomegalovirus (CMV) (13, 113–115), HIV (13, 109), and others (109, 116, 117).
2) *Cytokine-induced memory* NK cells seem to respond to specific cytokines (IL-12, IL-15, and IL-18) with a brief pre-activation period followed by enhanced activity in response to cytokine receptor stimulation (118). Cytokine-induced memory NK cells have been reported against influenza virus (119), leukemia (120) and melanoma (121). A recent article also reports the generation of "tumor-induced memory-like" (TIML-) NK cells (122).
3) *Memory-like* (adaptive) NK cells comprise a unique subset of NK cells that have reduced expression of the CD16 adaptor molecules, FcR γ-chain, and Syk, specifically induced in responses to CMV infection (123–127). Memory-like NK cell numbers have also been shown to expand in HIV (128, 129), HCV (130) and Epstein Bar Virus (EBV) (131–133) infections.
4) *Evolved memory* (adaptive) NK cells overlap with antigen-specific and memory-like NK cells with a response induced by CMV infection. A critical difference is the expression of a specific receptor, Ly49H, by NK cells that interact with MCMV glycoprotein m157 (134, 135) in mouse models, and more recently, Ly49I and Ly49C were also shown to interact with specific MCMV peptides (136). An analogous $NKG2C^{+}$ cell type has also been described in humans although the mechanisms are less well-defined (137). Collectively, an evolved memory NK cell can be considered as one that expresses a receptor (or perhaps a precise combination of known and unknown receptors) that is induced to control a specific pathogen.

Paust et al. (109) demonstrated that murine NK cells can develop memory against HIV antigens, a virus which cannot infect mice, and thus there is no evolutionary component. Primed hepatic NK cells (but not splenic NK cells) mounted a vigorous recall response in recipient mice, and the chemokine receptor CXCR6 was deemed critical for this function. Later, our group showed that NK cells in MAC were capable of mounting a recall response against SIV/SHIV and HIV vaccine antigens (13), and NKG2C was indicated to play a critical role in this process.

To our knowledge, memory-like FcR γ-chain deficient NK cells have not been reported in mice. One possibility is the exclusive association of CD16 to FcR γ-chain homodimers in mice, compared to the association with homodimers and heterodimers of FcR γ-chain and CD3ζ in humans (138). Thus, murine NK cells might not be able to respond to CMV infection in a manner similar to human NK cells. Nonetheless, the prevalence of other subtypes of NK cells (cytokine-induced and antigen-specific) suggests murine NK cells have likely devised alternate strategies to control viral infections. Other such subtle variations of these cells almost certainly exist, but the exact interplay between the pathogen and NK cells that induces each population still needs significant assessment.

The defining characteristics of "memory-like" NK cells are the lack of the FcR γ signaling chain and Syk adaptor proteins, likely resulting from epigenetic reprogramming of these subsets of NK cells by CMV (126, 139). The initial observation made by Leeansyah et al. suggested persistent lack of FcR γ-chain expression in NK cells from HIV-1 positive subjects receiving cART (140); however, CMV infection status of these patients was not reported in this study. A follow-up study by the same group suggested that these cells had significantly reduced NCR (NKp46 and NKp30) expression and showed greater ADCC against opsonized targets (128), but the role of HIV *per se* in the induction of these cells is not clear. CMV is likely the primary source of induction of these cells (124, 125), but how HIV can also modulate these phenotypes in NK cells is unclear. Our own assessment in MAC suggests SIV infection does not have a significant impact on total numbers of $\gamma\text{-chain}^{-}\text{Syk}^{-}$ NK cells, which is modulated by rhesus CMV (rhCMV), but the migration into tissues was heavily influenced by SIV infection (127). In blood, rhCMV titers were correlated to adaptive NK cell numbers in both rhCMV-infected, as well as rhCMV/SIV co-infected animals (127). This observation was similar to observations made by Zhou et al. (128), where adaptive NK cell prevalence generally correlated with CMV antibody titers, but was further modulated by HIV infection. This discrepancy could simply be explained by host species-specific differences, or perhaps by HIV specific responses exerted by NK cells in humans. Furthermore, SIV was able to subvert the enhanced responses of adaptive NK cells by suppressing the alternate signaling mechanism induced by rhCMV (127). The data explaining the effect of HIV/SIV infection on adaptive NK cells, in the absence of prior CMV infection, are sorely lacking. It is imperative to address the skewed observations in SIV infection, which could have arisen due to the confounding effects of co-infection with CMV. Most importantly, we need to address the question of protective

features of CMV induced NK cells against other viral infections such as HIV-1.

Similar questions have been posited for other memory NK cell subsets as well, particularly NKG2C$^+$ evolved NK cells induced by CMV infection (141). Similar to the expansion of γ-chain$^-$Syk$^-$ NK cell populations in SIV/HIV$^+$ subjects, the expansion of the NKG2C$^+$ population in HIV-1$^+$ subjects is attributed to concurrent HCMV infection (142). A recent study suggests that adaptive NK cells induced by CMV in HIV-1 infected individuals are further modulated and marked by reduced expression of the transcription factor promyelocytic leukemia zinc finger (PLZF) (129, 129) and that these cells are distinguished from other adaptive NK cells expressing NKG2C or CD57. Intriguingly, HIV might be directly affecting NKG2C$^+$ NK cell numbers, since p24 has been reported to stabilize HLA-E expression on lymphocytes of HIV$^+$ patients (143). Overall, the questions regarding the protective ability of memory/adaptive/cytokine-induced NK cell subsets against HIV remain unanswered. Information on the unadulterated effect of HIV on the NK cell receptor repertoire, functional abilities, epigenetic reprogramming and specific subset expansion *in vivo* needs significant in-depth investigation and may direct specific preventative and curative strategies against HIV infections.

CONCLUDING REMARKS

Many studies have highlighted the crucial role of NK cells in mediating control of HIV transmission, dissemination, disease, and reciprocally virus-mediated subversion of NK cells. Unlike many other pathogens, mouse models have contributed in a more limited way to this body of knowledge, due to the lack of tropism of lentiviruses and caveats of humanized mouse models. These circumstances have created one of the best examples of the significant utility of studying immunology in NHP, specifically SIV infection of various MAC species. Overall, these studies have revealed multiple layers of NK cell-virus interplay in lentivirus infection including: (1) KIR-HLA; (2) induction of CD2 and NKG2-related molecules; (3) interaction of Fc-receptor bearing NK cells and Ab-opsonized virus infected cells; and (4) development of HIV/SIV-specific NK cell memory-like responses. Although many unanswered questions remain regarding NK cell correlates of virus control, the significant contribution of NHP models cannot be overstated and rapidly evolving *in vivo* and *ex vivo* manipulations will undoubtedly continue to advance studies of HIV vaccine and other therapeutic modality development.

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CM, SS, JN, and GF contributed to writing of specific sections. RR and GF oversaw overall preparation of the manuscript, contributed to writing. RR edited the final version of the manuscript.

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REVIEW
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Conceptual Approaches to Modulating Antibody Effector Functions and Circulation Half-Life

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Antibodies and Fc-fusion antibody-like proteins have become successful biologics developed for cancer treatment, passive immunity against infection, addiction, and autoimmune diseases. In general these biopharmaceuticals can be used for blocking protein:protein interactions, crosslinking host receptors to induce signaling, recruiting effector cells to targets, and fixing complement. With the vast capability of antibodies to affect infectious and genetic diseases much effort has been placed on improving and tailoring antibodies for specific functions. While antibody:antigen engagement is critical for an efficacious antibody biologic, equally as important are the hinge and constant domains of the heavy chain. It is the hinge and constant domains of the antibody that engage host receptors or complement protein to mediate a myriad of effector functions and regulate antibody circulation. Molecular and structural studies have provided insight into how the hinge and constant domains from antibodies across different species, isotypes, subclasses, and alleles are recognized by host cell receptors and complement protein C1q. The molecular details of these interactions have led to manipulation of the sequences and glycosylation of hinge and constant domains to enhance or reduce antibody effector functions and circulating half-life. This review will describe the concepts being applied to optimize the hinge and crystallizable fragment of antibodies, and it will detail how these interactions can be tuned up or down to mediate a biological function that confers a desired disease outcome.

Keywords: antibody engineering, Fc optimization, therapeutic antibodies, biologics, passive immunity, immunotherapy

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INTRODUCTION

Since the approval of the first monoclonal antibody by the FDA in 1986 (1), there has been a rapid increase in the number of available monoclonal antibodies or antibody derivatives. In 2015 there were 44 antibodies approved for human use in the United States and Europe (2). Consistent with an expected annual approval rate of six to nine additional antibodies (3), the number of approved antibodies and antibody-like biologics in the United States has climbed to more than 70 (1, 4). It is estimated that global sales of antibody-based products approach $60–75 billion in any given year (2–8). Therefore, many pharmaceutical companies are including antibody-like molecules in their development portfolio due to their high capacity to generate revenue.

Basic science continues to discover the underlying mechanisms of genetic disorders, cancer, and infectious diseases (9, 10). Elucidation of these mechanisms fuels the development of

antibody-based biologics to counteract the abnormal biologic process that is causing disease. How the antibody counteracts the biologic process can be optimized for selectivity and potency by modifying the sequence of the antibody-based molecule to enhance or abrogate its interaction with the host immune system (11, 12). This concept is the foundation of antibody optimization efforts in industry laboratories as well as academic research laboratories. While most approved biologics are traditional antibodies, optimized antibodies like Orencia® (abatacept), Soliris® (eculizumab), Nplate® (romiplostim), and Removab® (catumaxomab) have paved the way for optimized antibodies as treatment options (11).

To optimize an antibody one must understand how the antibody is constructed and the role of each of its parts. An intact full-length antibody consists of two 50 kD heavy chains and two 25 kD light chains resulting in a 150 kD full-length, soluble immunoglobulin (13). Each heavy chain associates with a light chain through disulfide bonds and non-covalent interactions to form a heterodimer (14). The two heterodimers are paired together via disulfide bonds between the heavy chains (15, 16). Each heavy and light chain heterodimer includes the antigen binding fragment (Fab) composed of the light chain paired to the variable region of the heavy chain and the CH1 domain of the heavy chain constant region (17, 18). C-terminal to the Fab is the hinge, and the crystallizable fragment (Fc) (17, 18). The hinge region can be subdivided into upper, core, and lower hinge regions (19). The Fc includes the CH2 and CH3 domains of the heavy chain constant region (14).

The constant region of antibodies also contributes to the sequence variation of the heavy chain. The variable region of the heavy chain recombines with the heavy chain constant region to produce a full-length heavy chain (20, 21). The antibody can vary in isotype depending on whether the alpha, mu, gamma, epsilon, or delta constant region gene segment is recombined with the variable region (22). Among the human gamma gene segments there are 4 different subclasses designated as gamma 1, 2, 3, and 4, which are approximately 90% identical to each other (1). In a clinical context, each subclass is important since each subclass specializes in the elimination of different types of pathogens (23). For example, there is an association between deficiency in IgG2 antibodies and infection with encapsulated bacteria (24). The molecular basis of the association may be a diminished antibody response to polysaccharide antigens in individuals lacking IgG2 antibodies (25). For antibody engineering, the different isotypes and subclasses are important for antibody optimization since the sequence variation occurs at sites that determine affinities and specificities for FcRn, Fc alpha receptor, Fc gamma receptors, and complement protein C1q (26). There are 5 Fc gamma receptors (FcγR) that activate effector cells upon binding to IgG. Among the activating receptors there are FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa, and FcγRIIIb (27). There is one inhibitory Fc gamma receptor—FcγRIIb (28, 29). The FcγRs are polymorphic, where certain alleles exhibit higher affinity for Fc than others. For example Val158 allelic variants of FcγRIIIa bind with higher affinity to IgG1 Fc than the Phe158 allelic variant (30). Antibody binding to these receptors can facilitate the recruitment of effector cells to opsonized target cells or opsonized pathogens for clearance [**Figure 1A**; (2)]. Therefore, changes to the sequence and post-translational modification of the Fc and hinge regions of antibodies allows one to manipulate the effector functions and circulation of a given antibody or antibody-like protein (31). In addition to sequence variation, the Fc region also contains an N-linked glycosylation site at residue 297, which is important for Fc structure and function (32). Most clinically approved antibody-based products are of the gamma isotype, subclass 1 (IgG1) (2). There are currently 3 IgG2 antibodies that are approved for use in the United States (2). IgG3 has long hinge region prone to proteolytic cleavage (23), and exhibits a reduced half-life relative to other IgG subclasses (33). For these reasons it has it has not been the subclass of choice for biologics. Since most clinically-approved antibodies are of the gamma isotype (2), the optimization of antibody binding to FcγRs has been the major focus of the Fc engineering field. However, it is important to note that IgA, IgM, and IgE isotypes have Fc receptors as well, which can be exploited by Fc engineering as discussed below (34–36).

In this review, the approaches utilized to optimize or eliminate Fc interactions with host proteins will be discussed. This review will focus on changes to Fc sequence and glycosylation as a means to modulate Fc function. While Fc optimization is presented as two distinct categories of either enhancement or abrogation of Fc binding the review will describe how a single mutation can have both effects; thus, the two categories are not mutually exclusive. Ultimately, the reader will gain knowledge of how to alter the Fc region of an antibody to change its immunologic properties.

ANTIBODY FC MUTATIONS FOR THE IMPROVEMENT OF EFFECTOR FUNCTIONS

Enhanced FcγR Binding

Engagement of FcγRs is required for antibody effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) (37). Below, modifications that affect binding to FcγRs which result in enhanced ADCC and ADCP are described.

Point Mutations to Enhance FcγR Binding

The Fc of antibodies has been optimized using multiple approaches in attempts to increase binding affinity to selected FcγR (**Table 1** and **Figure 2**). Guided by the 3.2 Å structure of the Fc of IgG1 (61), Shields et al. performed alanine scanning mutagenesis of the solvent exposed amino acid residues on Fc (38). Antibodies encoding Fc regions with alanine mutations were screened for their binding to FcγRI, FcγRIIa, FcγRIIb, FcγRIIIa, and FcRn. Each individual mutation was subdivided into improved or reduced binding to each FcγR and FcRn. Twenty-seven individual mutations increased binding to at least one FcγR or FcRn. In an attempt to engineer an Fc that bound strongly to FcγRIII—a receptor that mediates ADCC (62, 63)—alanine mutations at different sites were combined into one modified Fc. The combination of Ser298Ala, Glu333Ala, and Lys334Ala mutations (sometimes referred to as the AAA mutations) had an additive improvement on the affinity of IgG1

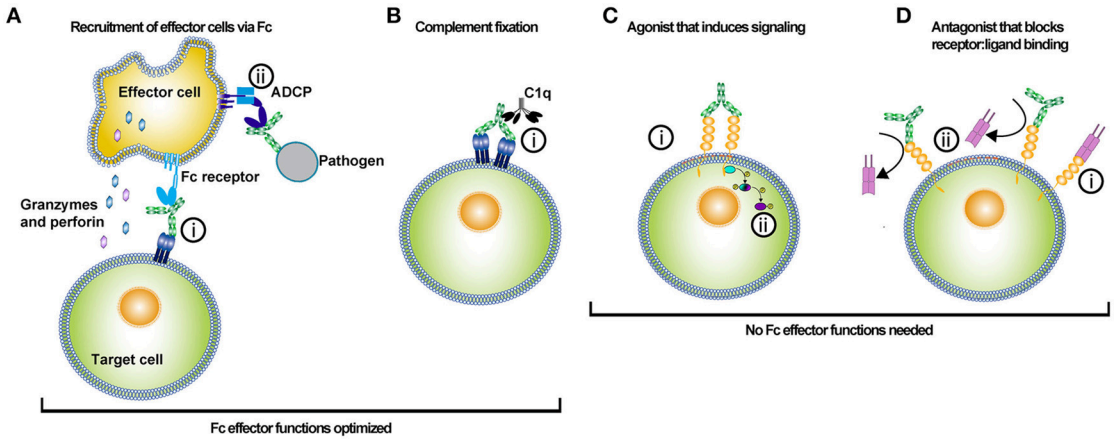

FIGURE 1 | Uses of antibody-based biologics. The type of Fc optimization undertaken for an antibody depends on the desired function of the antibody *in vivo*. Antibody-based biologics are principally used as adaptor molecules for effector cell recruitment to targets, inducers of the classical complement pathway, agonists, or antagonists. **(A)** Antibody biologics can be used to opsonize target cells or pathogens and recruit effector cells to kill or phagocytose the target cell or pathogen. (i) Antibodies bind to the antigen via their Fabs and recruit effector cells via their Fc interacting with Fc receptors. Recruitment of the effector cell to the target cell and release of cytotoxic molecules, such as granzyme and perforin results in target cell killing. Alternatively, (ii) effector cells can bind to the Fc of an antibody that has opsonized a pathogen, and subsequently phagocytose the pathogen via antibody-dependent cellular phagocytosis (ADCP). Much of the Fc engineering effort has been focused on improving Fc affinity for various activating FcγRs. The improvement of Fc affinity for FcγRs has led to augmented ADCC and ADCP activity for the optimized antibody-based biologic. **(B)** Antibodies can kill target cells through the initiation of the complement pathway. The initial step in initiating the complement pathway is the binding of complement protein 1q (C1q). (i) C1q binds to the Fc of antibodies in complex with antigen. Specific mutations have been introduced into the Fc of antibodies to enhance Fc binding affinity to C1q. **(C)** Agonistic antibody-based molecules are designed to bind to ligand and induce signaling by crosslinking a membrane-bound receptor. (i) Binding of both Fab arms crosslinks the cell receptor and (ii) subsequent phosphorylation of intracellular signal transducers potentiates the receptor signaling. These types of antibody-based biologics do not require Fc function; hence these molecules are prime candidates for Fc optimization that silences FcγR and complement binding. **(D)** Antagonistic antibody-based biologics bind to a target molecule and prevent the function of that protein either directly or by blocking its ligand from binding. (i) Typically the ligand binds to its receptor, (ii) but in the presence of the antagonistic antibody the receptor binding site is competitively or allosterically blocked. The lack of ligand binding prevents intracellular signaling by the membrane-bound receptor. In this instance, Fc effector functions are not needed and thus mutations that silence Fc effector function can be employed to optimize these types of biologics.

for FcγRIIIa (**Table 1**) (38). The improved binding to FcγRIIIa translated to 50–100-fold more potent killing *in vitro* of Her2+ cells by the antibody Herceptin when Ser298Ala, Glu333Ala, and Lys334Ala mutations were incorporated.

In a directed evolution approach, Lazar et al. used a computational algorithm to calculate amino acid substitutions that would be predicted to improve the interaction between Fc and FcγRIIIa (39). They also generated a set of quality improvement mutations that would be predicted to improve stability and solubility. Ser239Asp and Ile332Glu in the CH2 domain individually improved FcγRIIIa binding affinity by one log compared to the wildtype Fc (**Figure 2A**). To maximize binding these two mutations were combined into one Fc construct which resulted in an approximately 2-log enhancement in binding affinity for FcγRIIIa compared to the wildtype Fc. However, an unwanted increase in binding to the inhibitory FcγRIIb was also conferred by the Ser239Asp/Ile332Glu double mutant (39). This undesired effect was partially negated by adding an Ala330Leu mutation to the Ser239Asp/Ile332Glu variant (**Figure 2A**). The triple mutant Ser239Asp/Ile332Glu/Ala330Leu (commonly referred to as DLE) was introduced into the Fc of anti-cancer antibody Trastuzumab. Compared to wildtype Trastuzumab, Trastuzumab with the DLE mutations had 2-log more potent ADCC killing of Her2+ cancer cell lines expressing low or high levels of cancer antigen (39). The DLE mutations also increased ADCC activity of an anti-integrin antibody, MEDI-522, against a human melanoma cell line (64). Similarly, these mutations increased ADCP by the anti-CD20 cancer antibody Rituximab (39). At the cellular level the DLE mutations function to recruit higher numbers of natural killer cells per target cell coated with the DLE-optimized antibody compared to wildtype antibody (65). Approximately 90% of the recruited NK cells kill cell the target upon first contact, and then move to a second cell for killing (65). Thus, single NK cells kill more target cells per contact and more target cells over time (65). This increase in recruitment and more efficient killing conferred by the DLE-optimized antibody was shown to be due to enhanced FcγRIII-mediated signaling as measured by ZAP70 phosphorylation (65).

The crystal structure of the Fc containing the Ser239Asp/Ile332Glu/Ala330Leu mutations was solved to understand how these mutations affect FcγRIIIa binding. The structure showed an open conformation of the Fc where the two CH2 domains were separated from each other by an additional 30 Å compared to wildtype Fc (66). Thermostability measurements suggested that the opening of the CH2 domain could be because the CH2 domain was less stable or more flexible upon addition of the Ser239Asp/Ile332Glu/Ala330Leu mutations (66). The structure of the optimized Fc was modeled interacting with FcγRIIIa,

TABLE 1 | Fc modifications to enhance antibody effector function.

Modifications or mutations (reference)	Abbreviated name	Phenotype	Enhanced effector function
Ser298Ala/Glu333Ala/Lys334Ala (38)	AAA	• Enhanced FcγRIIIa affinity	ADCC
Ser239Asp/Ala330Leu/Ile332Glu (39, 40)	DLE	• Increased FcγRIIIa affinity • Low binding to inhibitory FcγRIIb	ADCC ADCP
Ser239Asp/Ile332Glu (39, 40)	DE	• Increased FcγRIIIa • Strong binding to inhibitory FcγRIIb	ADCC ADCP
Gly236Ala/Ser239Asp/Ala330Leu/Ile332Glu (41–43)	GASDALIE	• Increased binding affinity to FcγRIIa and FcγRIIIa • Only a small increase to FcγRIIb	ADCC
Gly236Ala (40)	GA	• Increases FcγRIIa affinity • No change in FcγRIIb affinity • Decreased FcγR1	ADCP
Ser239Asp/Ile332Glu/Gly236Ala (40)	DAE	• Recovers FcγRI binding lost by Gly236Ala • Increases FcγRIIIa and FcγRIIa • Enhanced FcγRIIb binding	ADCC ADCP
Leu234Tyr/Gly236Trp/Ser298Ala (44)	YWA	• Improved FcγRIIIa affinity when present in 1 heavy chain constant region • Used in asymmetric Fc design with DLE	ADCC
Phe243Leu, Arg292Pro, Tyr300Leu, Val305Ile, and Pro396Leu (45)	Variant 18	• Enhanced FcγRIIa and FcγRIIIa off-rates • Less than 2 fold enhancement of FcγRIIb	ADCC
Lys326Trp/Glu333Ser (46)		• Increased C1q binding • CDC activity was comparable to Lys326Trp, but improved versus wildtype Fc • Decreased ADCC activity	CDC
Lys326Ala/Glu333Ala (46)		• Increased C1q binding • Preserved ADCC activity	CDC
Lys326Met/Glu333Ser (46)		• Increased CDC activity • Preserved ADCC activity	CDC
Cys221Asp/Asp222Cys (47)		• Increased C1q binding • Preserves FcγRIII affinity and ADCC	CDC
Ser267Glu, His268Phe, and Ser324Thr (48)	EFT	• Increased C1q binding • Ser267Glu increased inhibitory FcγRIIb affinity • Decreased ADCC/ADCP	CDC
His268Phe and Ser324Thr (48)	FT	• Improved CDC • Functions with ADCC and ADCP enhancing mutations • Less potent CDC than EFT	CDC
Glu345Arg (49)	Arg345	• Increased C1q binding • IgG1 hexamer formation	CDC
IgG1/IgG3 cross-subclass (50)	1133 1131	• Increased C1q binding • Preserves ADCC activity	CDC
IgG2/IgG3 cross-subclass (51)	IgG 3-3-3/2-3 IgG 2-2-3-2	• Increases C1q and C4b binding	CDC
4-domain cross-isotype (52)	γγγα	• Decreased FcγRI binding • Decreased Polymeric Ig receptor binding • Decreased half-life	CDC
Tandem cross-isotype (53)	IgG1/IgA2	• Bound to FcγRs, FcαRI, and FcRn • Decreased C1q binding	ADCC
Chimeric cross-isotype (54)	IgGA	• Bound to FcγRI, FcγRIIa, FcαRI • Lost FcRn	ADCC ADCP CDC
Multimeric IgG (55)		• Increased C1q • Increased FcγRI and FcγRIII	CDC
Galactosylation (56, 57)		• Increased C1q	CDC
Biantennary glycan at N297 (58, 59)		• Improved binding to FcγRIIIa	ADCC
Afucosylated glycan at N297 (60)		• Increased binding to FcγRIIIa	ADCC

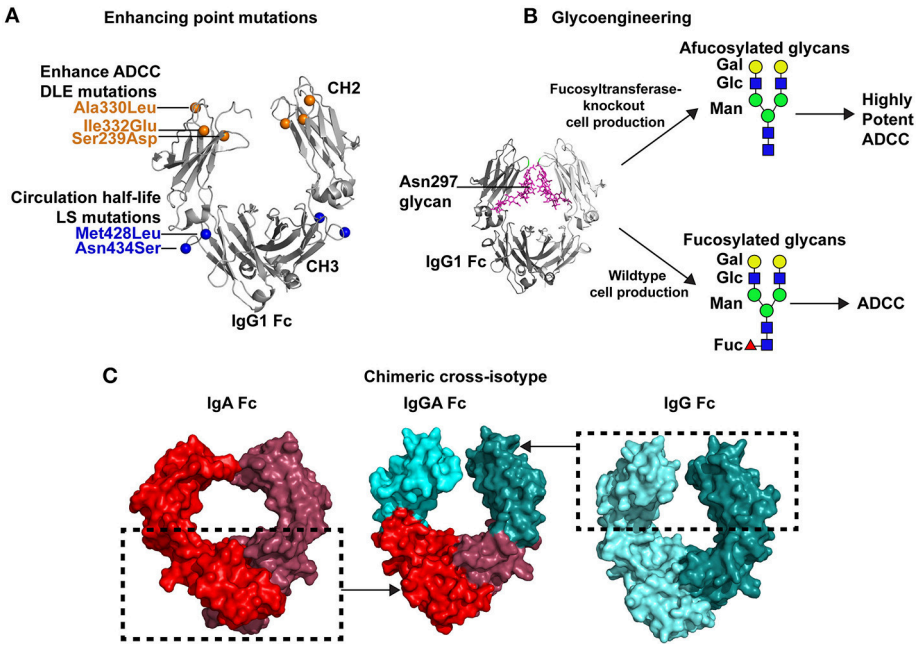

FIGURE 2 | Strategies for improving antibody Fc-mediated effector functions. **(A)** Multiple point mutations have been identified that improve binding affinity of Fc for specific FcγRs. In some instances a single FcγR, such as FcγRIIIa is the receptor of interest. Directed evolution, alanine scanning, or structure-guided design have been used to identify these mutations. An example of these mutations is the DLE (Ser239Asp/Ile332Glu/Ala330Leu) set of mutations that are shown in the crystal structure of the Fc by orange spheres (PDB:3DO3; 42). These mutations improve ADCC activity. Additionally, mutations can be inserted that improve antibody circulation *in vivo*. The LS mutations, depicted by blue spheres, (Met428Leu/Asn434Ser) are one example of antibody half-life extension mutations. **(B)** Antibody effector functions can be enhanced by glycoengineering the Fc domain. The Fc domain contains a N-linked glycan at position 297. A crystal structure of the IgG1 Fc (gray) and the N297 glycan (magenta) are shown (PDB:4BYH). Expression of antibody in wildtype cells results in a fucosylated complex glycans present at N297. However, specialized cells have been created with fucosyltransferase knocked out, which results in afucoylated glycans at Asn297. Antibodies with afucosylated glycans exhibit upto 50-fold more potent ADCC than the same antibody with a fucosylated glycan at Asn297 (60). Green circles, mannose; blue squares, GlcNAc2; yellow circles, galactose; and red triangles, fucose. **(C)** Antibody effector functions can be improved by expanding the breadth of Fc receptors capable of interacting with Fc. To improve antibody effector function the Fc of a single antibody can be engineered to bind to Fc receptors for multiple antibody isotypes. This concept has led to the design of cross-isotype IgGA antibodies (center) where the IgG1 CH2 a1 loop residues 245–258 and the IgG1 CH3 domain (cyan) were exchanged with the structurally analogous regions of IgA (54). The regions inside the dashed box were combined to create a chimeric cross-isotype Fc. The IgG1 segments are colored light and dark cyan (right, PDB: 3DO3), and the IgA segments are colored light and dark red (left, PDB: 1OW0). The cross-isotype Fc is capable of binding to FcγRI and FcαRI, hence either of these Fc receptors can be used to recruit diverse effector cells to target cells (54).

to determine whether the Ser239Asp/Ile332Glu/Ala330Leu mutations created additional interactions with the FcγRIIIa. Indeed, the structural model suggested additional hydrogen bonds between Ser239Asp/Ile332Glu in the Fc and Lys158 in the FcγRIIIa. Ala330Leu potentially created more hydrogen bonds with Ile85 in the FcγRIIIa as well (66). Additional electrostatic and hydrophobic interactions were also suggested by the structural model (66).

Mimoto et al. engineered an asymmetric Fc that combined the DLE mutations with their newly-identified Fc optimization mutations (44). In a large saturating mutagenesis screen, they examined the binding of 1,000 single Fc mutants to identify mutations that improved FcγRIIIa binding when present in only one of the heavy chains within an IgG molecule. They selected three mutations, Leu234Tyr, Gly236Trp, and Ser298Ala (termed YWA mutations), from their screen to incorporate into one heavy chain constant region. Since the DLE mutations had been shown to increase FcγRIIIa binding they incorporated these mutations into the other heavy chain constant region. Antibodies bearing YWA mutations in one heavy chain and DLE in the other mediated ADCC of tumor antigen-expressing cells *in vitro* more potently than symmetrical antibodies that contained only the YWA or DLE mutations (44). Thus, this asymmetric Fc design enables one to incorporate multiple optimization mutations to additively improve Fc function.

Macrophages utilize FcγRIIa to phagocytose antibody-opsonized antigens (67). To increase Fc receptor binding to FcγRIIa, Richards et al. screened 900 Fc variants for binding to FcγRIIa and identified Gly236Ala substitution alone increased the binding affinity approximately 6-fold for both His131 and Arg131 alleles of FcγRIIa (40). Unfortunately, the addition of Gly236Ala into IgG1 Fc reduced the IgG1 affinity for the activating receptor FcγRI (40). To recover the FcγRI binding, previously reported Ser239Asp/Ile332Glu mutations were introduced into the IgG1 Fc. This triple combination of mutations showed a 3-fold increase in FcγRI up to 70-fold increase in affinity for FcγRIIa, and up to a 31-fold increase in affinity for FcγRIIIa. The Ser239Asp/Ile332Glu/Gly236Ala

mutations enhanced *in vitro* FcγRIIa-dependent phagocytosis and FcγRIII-dependent ADCC activity of an IgG1 targeting adenocarcinoma cell lines (40).

The activating receptor FcγRIIa is 90% similar to the inhibitory receptor FcγRIIb (68), and thus the increase in FcγRI and FcγRIIIa affinity for Ser239Asp/Ile332Glu/Gly236Ala was accompanied by a 13-fold enhancement in binding to FcγRIIb. To compare the binding of both the activating and inhibitory FcγRIIs the ratio of binding was determined. The ratio of binding between the activating FcγRIIa and inhibitory FcγRIIb receptors was higher for Gly236Ala and the Ser239Asp/Ile332Glu/Gly236Ala than wildtype IgG1 (40). Thus, the ratio may be most important for determining the final functional activity of antibodies encoding the Ser239Asp/Ile332Glu/Gly236Ala mutations. Smith et al. attempted to improve the binding ratio of FcγRIIa to FcγRIIb by combining related sets of mutations to generate Gly236Ala/Ser239Asp/Ala330Leu/Ile332Glu (referred to as GASDALIE) (41). This collection of mutations increased binding affinity to FcγRIIIa encoding the low affinity allele Phe158 by 30-fold, most likely because of increased electrostatic interactions between the Fc and FcγRIIIa (41, 42). Similarly, binding affinity to FcγRIIa was improved 25-fold (41). FcγRIIb binding affinity affinities were only slightly increased, which resulted in a FcγRIIa to FcγRIIb affinity ratio of 11.6 compared to 1.6 for wildtype IgG1 (41). In a second experiment, investigators attempted to optimize Fc while avoiding any mutations that increased FcγRIIb binding. Using yeast display the investigators identified mutations that increased FcγRIIIa binding and reduced FcγRIIb binding. Upon making their mutant libraries and expressing them on the surface of yeast they used bead depletion to remove antibody Fc variants that bound to FcγRIIb. After FcγRIIb-bead depletion, the library of remaining Fc regions was screened for binding to recombinant FcγRIIIa. Using two different libraries, seven single mutations appeared to lack FcγRIIb binding while improving FcγRIIIa binding. These mutations were introduced into the Fc region of IgG1 individually as well as in various combinations. Combinations of Phe243Leu, Arg292Pro, Tyr300Leu, Val305Ile, and Pro396Leu mutations slowed the off-rates of Fc binding to FcγRIIa and FcγRIIIa relative to wildtype Fc without increasing binding to the inhibitory FcγRIIb receptor. The Fc that included all five mutations—termed variant 18—had a 10-fold improvement in affinity for FcγRIIa and FcγRIIIa, and <2-fold increase in FcγRIIb affinity. The variant 18 Fc exhibited potent ADCC activity against colon, ovarian, and breast cancer cell lines *in vitro* for several different antibodies (45). *In vivo*, an IgG1 encoding Phe243Leu, Arg292Pro, Tyr300Leu, Val305Ile, and Pro396Leu mutations conferred a significant increase in survival in a lethal ovarian tumor transplantation model (45). These mutations have shown promise in treatment of cancer in humans as well. Anti-HER2 monoclonal antibody Margetuximab, which contains Phe243Leu/Arg292Pro/Tyr300Leu/Val305Ile/Pro396Leu optimization mutations, exhibits improved ADCC activity compared to the standard of care antibody trastuzumab (69). In one clinical trial 78% of the response-evaluatable patients who received Margetuximab showed a reduction in tumor size (70), highlighting the potential promise for Fc optimization to improve disease treatment.

Glycoengineering to Enhance FcγR Binding

The Fc of IgG1 contains a single N-linked glycosylation site at position 297. The glycan present at N297 typically consists of two N-acetylglucosamine (GlcNAc), three mannose, and two more GlcNAc linked to the mannose to form a biantennary complex glycan (71). The two GlcNAc are linked to mannose through either a β1,2 linkage to α-3 or α-6 of the mannose. Thus, each arm of the glycan can be distinguished as the α1,3 or α1,6 arm depending upon how the mannose and $GlcNAc_2$ are linked (71). Additional fucose, galactose, sialic acid, and GlcNAc can be added to the core glycan structure (**Figure 2B**). IgG found circulating in human sera are generally fucosylated, however during recombinant IgG production the glycan composition can be altered by expressing the antibody in plant cells, knocking in or knocking out specific glycosidases, or *in vitro* enzymatic digestion of the glycosylated IgG (**Figure 2B**) (72). Since both heavy chains are glycosylated it is possible for a single IgG molecule to have significant glycan heterogeneity (71). The glycan has direct effects on FcγR binding. The Asn297 glycan on the Fc can clash with glycans on the FcγRIII protein, which results in poor engagement of effectors cells that mediate ADCC. Also, nuclear magnetic resonance studies have shown that Fc regions containing different glycans at Asn297 adopt different hinge region conformations (73). Since the hinge region is contacted by FcγRs, the glycosylation of N297 indirectly affects the ability of the Fc to interact with FcγRs. Thus, optimization of Fc glycosylation has been important for producing antibody biologics with a desired function.

To modulate antibody activity several studies have modified antibody Fc glycosylation by expressing or inhibiting enzymes in the producer cells. Expression of β(1,4)-N-acetylglucosaminyltransferase III when expressing IgG gives an antibody glycosylated at N297 that has a biantennary glycan and has better ADCC activity (58). Davies et al. produced anti-CD20 IgG1 under these conditions and found the antibody had 10–20-fold more potent FcγRIIIa-dependent killing of CD20+ cells (59). Despite these results the importance of bisecting GlcNAc is debated, and the removal of fucose has been asserted as an alternative hypothesis [**Figure 2B**; (74)]. Antibodies deficient in fucose have been shown to have 50-fold higher binding to FcγRIIIa and enhanced ADCC activity (60). The enhancement of afucosylated antibody binding to FcγRIIIa is higher for the high affinity Val158 allele compared to the Phe158 allele, but both alleles show an increase in binding to afucosylated IgG1 compared to fucosylated IgG1 (40, 75). The most dramatic increase in binding by afucosylated Fc is for glycosylated FcγRIIIa (76), with the removal of the Asn162 glycan in FcγRIIIa completely abrogating this enhanced binding (76). The mechanism for glycosylated FcγRIII recognition was later determined by structural studies, which showed that the afucosylated Asn297 glycan interacts with the Asn162 glycan on FcγRIII (77). The addition of fucose to the Fc glycan creates clashes with the $GlcNAc_2$ on FcγRIIIa, providing a structural explanation for why afucosylated antibodies bind

better to FcγRIIIa (77). The approval of mogamulizumab (POTELIGEO®) in Japan marked the first approval for human use of an afucosylated antibody with enhanced ADCC activity (78–80).

Enhancing FcγR Binding by Exchange of Fc Domains Across Isotypes (Cross-Isotype Antibodies)

In addition to increasing affinity for receptors by introducing point mutations or modifying glycans, the Fc can be optimized to engage a wider range of Fc receptors (**Figure 2C**). As stated above, Fc receptors for isotypes other than gamma exist on particular leukocytes. By creating a Fc region that can interact with multiple Fc receptors, such as FcγR and FcαRI, one creates an antibody with expanded, novel abilities to engage effector cells (2). Neutrophils are the most abundant leukocyte in the body, and they engage Fc of IgA antibodies via the FcαRI (81, 82). Single domains of IgA2 were appended to end of the gamma 1 constant region creating a four-domain constant region (CH1g-CH2g-CH3g-CH3a) (52) in an attempt to engage FcγRs and FcαRI. To make the constant region more similar to the alpha constant region the CH1 domain of gamma 1 was substituted for the alpha 1 constant region domain (CH1a-CH2g-CH3g-CH3a). These four-domain cross-isotype IgGA chimeric antibodies bound to J chain similar to natural IgA2 but had reduced transport by polymeric Ig receptor. The four-domain, cross-isotype antibodies also had a 3–5-fold decrease in FcγRI affinity, and possessed the short serum half-life of IgA2 instead of the protracted serum circulation of IgG1. Despite these shortcomings, the four-domain cross-isotype IgGA design was capable of mediating complement-dependent lysis of sheep red blood cells and appeared to be more pH-resistant than IgG1 (52). In a similar approach a second type of cross-isotype Fc has been created by fusing the gamma 1 and alpha constant regions together to create a tandem G1-A Fc region (53). In this design the hinge, CH2, and CH3 of IgA2 is fused to the C-terminus of IgG1. This tandem cross-isotype IgG/IgA design had similar expression levels, antigen binding, and thermostability as antibodies made in the IgG1 format. *In vitro*, the tandem cross-isotype IgG/IgA bound to FcαRI and FcγRI, FcγRII, FcγRIIIa, and FcRn with affinities similar to wildtype IgA and IgG, respectively. This binding to various FcRs translated to the antibody mediating ADCC activity with polymorphonuclear cells and NK cells. C1q binding to the tandem cross-isotype IgG/IgA was reduced 3-fold compared to the IgG1 format of the antibody. Lastly, in BALB/c mice, tandem IgG/IgA circulated with a half-life similar to that of IgG1. In a third design, Kelton et al. created a cross-isotype antibody by exchanging the CH3 domain and CH2 α1 loop residues 245–258 (PKPKDTLMISRTPE) of the gamma 1 constant region with that of the alpha constant region [**Figure 2C**; (54)]. The chimeric Fc possessed the ability to bind to FcγRI, FcγRIIa, and FcαRI (54). Antibodies made in this IgGA format were capable of mediating ADCC with polymorphonuclear cells, mediating ADCP with macrophages, and activating complement (54). However, this design lacked binding to neonatal Fc receptor which regulates antibody half-life (54). Thus, further optimization would be required for effective *in vivo* use of this design. In total, these designs show the promise of the concept of engaging a wide range of effector cells for antibody Fc optimization.

IgG Multimerization Augments FcγR Binding

Multimerizing IgG has shown promise in the treatment of autoimmune diseases (83, 84). The IgG multimers are constructed in various ways including the addition of heterologous multimerization domains such as isoleucine zippers (83), another hinge region at the N-terminus of the natural hinge, or another hinge region at the C-terminus of the CH3 domain (83). Similarly, hexamers of IgG have been created by appending the IgM tailpiece to the C-terminus of the IgG1 Fc and creating a cysteine bond at position 309 (85, 86). The multimeric IgG formed by the addition of the IgM tailpiece bound strongly to FcγRI, FcγRIIa, and FcγRIIIa and bound weakly to FcγRIIb and FcγRIIIb (85, 86). Across the various designs, multimeric IgG bound to a higher magnitude than monomeric IgG to FcγRI, FcγRIIb, and FcγRIII (83, 85). Such molecules have shown promise in preclinical models of arthritis, neuropathy, and autoimmune myasthenia gravis (83, 84, 87). Hence the multimeric IgG platform is being further optimized to fine-tune the immune receptors, such as FcRn that can interact with the multimer (55).

Enhancing Complement Fixation

Point Mutations to Increase C1q Binding and Complement-Dependent Cytotoxicity (CDC)

Antibodies can exert cytotoxic effects by engaging the complement pathway. The initial step of this process is the binding of C1q to the CH2 domain of an antibody-opsonized antigen [**Figure 1B**; (88)]. Alanine scanning mutagenesis of the human IgG1 Fc identified Asp270, Lys322, Pro329, and Pro331 as essential for C1q binding to the Fc (89), although Fc from different species utilize different residues for binding to C1q (90). To increase Fc binding to C1q Idusogie et al. identified Lys326 and Glu333 as proximal to the core binding site of C1q within the Fc. The importance of these residues was first tested by alanine mutagenesis. Introducing Lys326Ala and Glu333Ala increased C1q binding and CDC activity by 50%. To optimize the Fc for binding to C1q various amino acids were introduced at 326 and 333 individually and in combination. The combination of Lys326Trp and Glu333Ser increased C1q binding by 5-fold (46). However, CDC activity conferred by the Lys326Trp/Glu333Ser double mutant Fc was the same as the Lys326Trp single mutant, and the Fc lost the ability to mediate ADCC. In instances when ADCC activity is also important, mutating positions 326 and 333 to two alanines, or mutating positions 326 and 333 to methionine and serine, respectively, provided an increase in CDC without hindering ADCC (46). Similar mutagenesis experiments have been done for the hinge region to determine whether it affects C1q binding and CDC activity. The mutation of the hinge region was not intuitive since C1q binds to the CH2 domain below the hinge region. However, it was shown that in the upper hinge region substituting Trp in various combinations at positions 222, 223, 224, and 225 increased C1q binding and increased CDC activity relative to wildtype IgG1 (47). ADCC activity and FcγRIIIa binding were unchanged by these modifications (47).

Cys221Asp and Asp222Cys alone or in combination with Trp substitutions also increased C1q and CDC activity (47). Thus, the hinge of human IgG1 modulated C1q binding to the CH2 domain (47).

To identify other point mutations that improve CDC activity of IgG1, Moore et al. made 38 Fc variants of an anti-CD20 antibody and screened them *in vitro* for their ability to mediate CDC against Raji cells (48). Among the 38 variants, three variants encoding Ser267Glu, His268Phe, and Ser324Thr (termed the EFT mutations) changes were identified as having more potent CDC activity vs. wildtype IgG1 (48). The largest improvement in CDC activity was achieved when the three mutations were combined into one Fc variant (48). Correlation analyses suggested the improvement in CDC potency was due to increased C1q binding (48). The triple EFT mutations had increased binding to the inhibitory FcγRIIb, which presumably limited its ADCC and ADCP activity. The addition of ADCC and ADCP-enhancing mutations to the EFT mutations restored ADCC and ADCP function back to wildtype IgG1 levels, but did not confer an improvement (48). The increased binding to FcγRIIb could be reduced by eliminating the Ser267Glu from the EFT mutations, however this change came a cost of reduced CDC potency. The His268Phe and Ser324Thr mutations were then capable of being combined with ADCC and ADCP-enhancing mutations to create a single Fc with improved CDC, ADCC, and ADCP activity (48). This study highlights the complicated balance between optimizing one effector function without decreasing another effector function.

Insertions and Deletions to Increase to Increase C1q Binding and Complement-Dependent Cytotoxicity (CDC)

Hinge length is important for C1q recognition of antibodies or antibody-based proteins. IgG3 has a distinct extended hinge of 62 amino acids that arises from the duplication of 3 exons that encode for part of the core hinge region (91). For IgG3 antibodies complement activation is increased by shortening its hinge region (92). While complete removal of the hinge ablates CDC function, a hinge of 15 amino acids instead of 62 amino acids exhibited 10-fold more potent CDC activity (92). More specifically, removal of the three repeats regions within the core hinge does not eliminate CDC, but instead improved CDC potency for anti-bacterial antibodies (93). This result for IgG3 is in contrast to IgG1 where two amino acid deletions in the core hinge region reduced C1q binding, CDC activity, and ADCC (47).

Cross-Subtype Antibodies to Improve C1q Binding

IgG1 is the preferred subclass for antibody biopharmaceuticals over IgG3 since the long hinge of IgG3 complicates large scale production of the antibody (2). However, IgG3 possesses the best *in vitro* binding to C1q (94). As a means to enhance IgG1 C1q binding, domains of IgG3 where substituted for IgG1 domains to create IgG1/G3 cross-subtype antibodies (50). These chimeras eliminated the difficulty of purifying antibodies with long hinge regions but capitalized on IgG3 effector functions. In one of the best variants, termed 1133, the CH1 and hinge region from IgG1 was fused to the Fc from IgG3 (50). Its ADCC activity and antigen binding were unchanged, while, its CDC activity and C1q binding were enhanced relative to wildtype IgG1 or IgG3 (50). Furthermore, the 1133 design allowed for CDC activity when the antigen levels were low (95). However, the 1133 Fc variant lacked protein A binding, which is important for easy purification of the antibody. Thus, an antibody with the CH1, hinge, and CH3 of IgG1 and CH2 of IgG3 was constructed since C1q binds the CH2 domain and protein A binds the CH3 domain. This variant had improved CDC activity and the ability to bind protein A (50). The molecular basis for the improved binding of the chimeras is presumed to be the amino acid differences in the CH2 domain that are proximal to the C1q binding site in the tertiary structure of the Fc. In mutagenesis experiments aiming to define the amino acids required for C1q binding to IgG1 and IgG3 K322 was found to be important for both subclasses, but dependence on other amino acids varied between subclasses. For example, P331 was required for CDC activity of IgG1 (89), but had only a modest effect on IgG3 CDC activity (96). These results indicate that C1q binding differs between IgG3 and IgG1 thus chimeric antibodies may be able to enhance binding by combining Fc:C1q interactions from both gamma subclasses. While the goal of Fc designs has been to boost IgG1 activity, cross-subclass designs have also been used to confer activity to functionally silent subclasses. IgG2 and IgG4 have very little ability to mediate CDC compared to IgG1 or IgG3 (51). However, replacing the CH2 domain of IgG2 with that of IgG3 can instill CDC activity to the otherwise IgG2 Fc (51). Similarly, IgG4 differs from IgG1 at position 331, which has been shown to be proximal to the C1q binding site (90). Changing the IgG4 residue at position 331 to match IgG1 conferred a moderate level of CDC. Therefore, if one knows the key residues for mediating an effector function they can be introduced into functionally silent Fc domains to confer specific functions.

IgG1 Hexamer Formation Boosts C1q Binding and CDC Activity

The multimerization of IgG by binding to antigen is known to enhance C1q binding substantially (97). To engender multimerization of the IgG in the absence of antigen, analysis of IgG structures identified position 345 as an amino acid that could facilitate multimerization between the Fc regions of different antibodies (49). The IgG structure suggested that the introduction of a positively charged amino acid would confer Fc:Fc interactions. Thus, a Glu345Arg mutation was introduced into the IgG1 Fc. Electron microscopy and mass spectrometry confirmed that this mutation resulted in monomeric IgG1 as well as multimeric IgG1 linked via the Fc (49). The multimeric IgG possessed higher binding to C1q and more potent lysis of a Burkitt's lymphoma cell line (49). Interestingly, the Glu345Arg mutation not only increased CDC activity by IgG1, but also IgG2, IgG3, and IgG4. Multimers of IgG1 have also been described by the addition of IgM tailpiece and Cys309 in the IgG1 Fc as stated above (see IgG multimerization augments FcγR binding). IgG hexamers created using the IgM tailpiece strategy also showed improved binding to C1q and C5b relative to wildtype IgG1 (55). Thus, multimerization of IgG is another method in addition

to point mutations and IgG1/IgG3 cross-isotype antibodies to increase C1q binding affinity.

Glycoengineering to Improve Complement Binding

The Asn297 glycan within the CH2 domain of the Fc can be modified to improve CDC activity (56, 57, 98–101). In a large screen of 20 different glycoforms of anti-trinitrophenyl hapten IgG1 Fc an overabundance of galactosylation increased C1q binding and CDC activity compared to the unmodified glycoform of IgG1 (57). Galactosylation appeared to be the principal glycan residue that affected CDC activity as significant positive correlations were observed between abundance of galactosylation on Fc and CDC potency (57). In a separate study Peschke et al. confirmed the importance of galactosylation for CDC activity using a different antibody specificity and multiple IgG subclasses. Galactosylation of the IgG1 Fc improved CDC activity of the anti-CD20 antibody Rituxumab against Raji B cells *in vitro* (56). The improved CDC activity conferred by galactosylation of the Fc was applicable to IgG3, but was not applicable to IgG2 or IgG4 in this *in vitro* model (56). The improved CDC activity conferred by galactosylated Fc was not due to changes in antigen binding, but instead was associated with enhanced C1q binding (56, 57). Overabundance of galactosylation on IgG1 Fc also improved thermostability when measured by differential scanning calorimetry (99). Thus, galactosylating the Fc is one strategy for producing a stable biologic with highly potent CDC activity.

IMPROVED ANTIBODY HALF-LIFE CIRCULATION

In addition to improving antibody effector functions by increasing affinity for activating FcγR and C1q, Fc optimization efforts have also tried to improve antibody circulation *in vivo*. *In vivo* IgG catabolism is regulated by its interaction with the neonatal Fc receptor (FcRn) (102). The FcRn binds to IgG at the junction of the CH2 and CH3 domains in a pH dependent manner (102–104). IgG is endocytosed by cells where it can be shuttled to lysosomes or recycled back to the cell surface (105). Binding of IgG to FcRn at low pH (pH < 6.5) in the endosomes allows the antibody to be trafficked with the FcRn back to the cell surface (106, 107). Poor binding to FcRn at pH < 6.5 results in the antibody being trafficked to the lysosome and degraded (105). At the physiologic pH of the extracellular environment IgG has weak affinity for FcRn which results in its release from the FcRn back into circulation (105). The pH dependent binding is regulated by protonation of His310, 435, 436 in the Fc at low pH (108). The protonation creates positively charged residues that can bind to negatively charged Glu117, Glu132, and Asp137 in the FcRn (109).

Point Mutations to Increase FcRn Binding Affinity

In a global approach to increasing IgG1 half-life, alanine scanning mutagenesis of the Fc was performed. In this screen 17 amino acids that affect IgG Fc binding to FcRn were identified (38). Among the 17 amino acids Asn434 and Glu380 showed large increases in affinity when mutated to alanine (38). The Asn434Ala mutation has been useful for countering the poor FcRn affinity that can result from the introduction of FcγR affinity-optimizing mutations (38), thus Asn434Ala is typically added to Ser298Ala, Glu333Ala, and Lys334Ala to create a AAAA variant with enhanced FcγR binding and normal or slightly improved FcRn binding (**Table 2**) (38). Additional half-life mutations were identified by sequentially performing random and directed evolution screens of phage libraries. The phage binding was done at pH 6 to mimic endosomal pH, and elution was done at pH 7.4 to find variants that did not bind at physiologic pH. Six collections of mutations were identified that improved FcRn binding across three different assays including Glu294deletion/Thr307Pro/Asn434Tyr (termed C6A-66) and Thr256Asn/Ala378Val/Ser383Asn/Asn434Tyr (referred to as C6A-78) (116). Asn434Tyr was among the most common mutations found in each collection (114). One of the differences between these collections of mutations was the ability to bind to FcγRIIIa, thus one could extend half-life while also retaining ADCC activity or knocking out ADCC activity (114). In more recent work, the C6A-66 collection of mutations were analyzed further since it showed only a moderate increase in FcRn binding, but had the best serum half-life *in vivo*. The collection of mutations were studied as individual mutations to elucidate the function of the deletion of Glu294 (115). This deletion resulted in higher sialylation of the Asn297 glycan on the Fc (115). The abundance of sialic acid was necessary for the increase in antibody half-life *in vivo* (115). Thus, increased FcRn binding was not the only factor that contributed to increased half-life. Sialylation also has a role in regulating serum half-life (115).

Ghetie et al. also created large libraries of random mutations of Thr252, Thr253, and Thr254 in the mouse Fc and screened them for binding to mouse FcRn using a bacteriophage display platform (113). The three sites were chosen based on their proximity to the FcRn binding site on Fc. From the phage library the collection of Thr252Leu, Thr253Ser, and Thr254Phe was identified that had significantly longer half-life in wildtype mice. These three mutations, termed LSF, did not affect association rates of Fc with FcRn but did slow the dissociation rate of Fc from FcRn at pH6 (113). This result indicated for mouse antibodies that that position 252, 254, and 256 could be manipulated to increase antibody half-life. Hence in a later study, phage display libraries of human IgG1 were used to identify analogous mutations at positions 252, 254, and 256 (111). In the human IgG1 Met252Tyr, Ser254Thr, and Thr256Glu was observed in a high abundance among the clones isolated from the phage library. Inclusion of these 3 mutations, often called YTE, in the IgG1 Fc resulted in a 10-fold slower dissociation rate of Fc and FcRn. Overall, the YTE mutations enhanced the apparent equilibrium rate constant 3-fold for Fc binding to FcRn. To determine whether the increased binding to FcRn *in vitro* translated to improved pharmacokinetics in primates, wildtype IgG1 or a YTE variant were infused into cynomolgus monkeys and serum half-life was compared. The YTE antibody possessed a 4-fold increase in serum half-life as compared to the wildtype antibody (64). Additionally, there was a higher

TABLE 2 | Fc modifications to improve antibody circulation half-life.

Modifications or mutations (reference)	Abbreviated name	Phenotype	Enhanced function
Arg435His (110)	His435	• Increased binding to FcRn at low pH	Extended half-life
Asn434Ala (38)	A	• Increased binding to FcRn at pH6	Extended half-life
Met252Tyr/Ser254Thr/Thr256Glu (111)	YTE	• Slowed off-rate for Fc and FcRn • Increased FcRn affinity • Decreased ADCC	Extended half-life
Met428Leu/Asn434Ser (112)	LS	• Increased affinity to and slowed off-rate for FcRn at pH6 • No change in ADCC	Extended half-life
Thr252Leu/Thr253Ser/Thr254Phe (113)	LSF	• Increased binding to FcRn at pH < 6.5	Extended half-life
Glu294delta/Thr307Pro/Asn434Tyr (114)	C6A-66	• Increased binding to FcRn at pH < 6 • No binding to FcRn at pH7.4 • Decreased FcγRIIa binding and ADCC	Extended half-life
Thr256Asn/Ala378Va l/ Ser383Asn/Asn434Tyr (114)	C6A-78	• Increased binding to FcRn at pH < 6 • No binding to FcRn at pH7.4	Extended half-life
Glu294delta (114, 115)	Del	• Increased sialylation	Extended half-life

concentration of infused IgG measured in bronchioalveolar lavage fluid when the Fc included the YTE mutations (64). The pharmacokinetic profile of an IgG1 possessing the YTE mutations was determined in a phase 1, double-blind, dose escalation study. In this study the YTE-variant of IgG1 had a serum half-life of 80–112 days (117). Based on the typical serum half-life of IgG1 being 21 days, the YTE mutation seems to increase half-life 4–5-fold in humans (117). In a second study YTE was introduced into motavizumab and their half-life was directly compared in humans. Consistent with the first study, serum half-life was extended 2–4-fold compared to wildtype IgG. Remarkably, the individuals that received the YTE variant had functional therapeutic antibody present in their serum 240 days after antibody infusion (118). Thus, the YTE mutations raise the possibility of long-acting antibody-based biologics that could suppress or protect from disease in humans. While YTE improves half-life of the antibody, it also eliminates ADCC activity of the antibody (64). This pitfall can be counteracted by the addition of ADCC-enhancing mutations, such as DLE (64). Therefore, YTE mutations should be used alone only when the antibody does not need to mediate ADCC.

Another set of mutations that improve antibody half-life was discovered by Zalevsky et al. using rational protein design. Introduction of Met428Leu and Asn434Ser mutations (referred to as the LS mutations) in IgG1 Fc resulted in a decrease in the dissociation rate and an 11-fold improvement in binding affinity between Fc and human FcRn at pH6 [**Figure 2A**; (112)]. In contrast to YTE, LS mutations did not significantly reduce ADCC activity (119). In cynomolgus macaques, the LS mutations conferred a 3-fold increase in antibody half-life (112). A similar increase of 3–4-fold in serum antibody half-life was seen in human FcRn transgenic mice (112). The improvement in function conferred by the LS mutations was tested by engrafting tumors into the human FcRn transgenic mice and infusing wildtype or LS-mutant IgG1 (112). For two different cancer immunotherapeutic antibodies the LS mutant IgG1 inhibited tumor growth significantly better than the wildtype IgG1 (112). Since the initial description of the LS mutations, multiple groups have shown these mutations boost antibody half-life in cynomolgus macaques (120, 121) and rhesus macaques (119, 122). The LS mutations have been helpful in sustaining protection against HIV-1 infection in animal models (119, 122, 123). The incorporation of LS resulted in increased antibody concentrations at mucosal sites and prolonged serum half-life (119, 122). Together these attributes resulted in improved protection afforded by optimized IgG1 in macaque models of HIV-1 infection (119, 122, 123). Clinical trials are planned to administer anti-HIV-1 IgG1 antibodies encoding the LS mutation. The extent to which the improved pharmacokinetic profile translates from macaques to humans will be determined, and whether longer half-life improves therapeutic efficacy will be evaluated. Novel mutations to improve antibody half-life are still being pursued. Approaches to improve upon the LS mutations include finding mutations that completely eliminate Fc binding to FcRn at physiologic pH, while also enhancing binding at low pH.

Cross-Subclass Point Mutations to Enhance FcRn Binding

IgG1 makes these 3 productive Fc:FcRn contacts and has a half-life of 21 days (109). In contrast to IgG1, IgG3 alleles typically encode arginine at position 435 instead of histidine. IGHG3*17, IGHG3*18, and IGHG3*19 alleles are the exceptions, which encode histidine like IgG1. The presence of arginine vs. histidine confers a serum half-life of only 7 days (124). *In vitro* competition assays suggest that IgG1 with His435 outcompetes IgG3 with Arg435 for FcRn binding (110). Furthermore, Arg435 seems to increase binding of IgG3 to FcRn at physiologic pH (110), which could result in more IgG3 being absorbed to epithelial cells expressing FcRn hindering the ability of IgG3 to freely circulate in serum. To increase IgG3 half-life, position

435 was changed to histidine, which boosted FcRn binding at low pH (110). Consistent with the increased binding, serum concentrations of IgG3 are higher in individuals who express an allelic variant of IgG3 encoding His435 and infusion of IgG3 encoding His435 is more efficient (110, 125). Thus, modulation of the pH sensing ability of IgG3 Fc is one mechanism for boosting its serum half-life.

ANTIBODY FC ENGINEERING FOR THE ABLATION OF EFFECTOR FUNCTIONS

While Fc optimization has focused heavily on gain-of-function modifications, in certain situations it can be beneficial to eliminate antibody Fc function. These situations include antibodies that are used as **(1)** receptor agonists to crosslink receptors and induce signaling, **(2)** receptor antagonists to block receptor:ligand interactions to prevent signaling, or **(3)** drug delivery vehicles to deliver drug to antigen-expressing target cells (**Figures 1C,D**). In these instances Fc engagement of receptors on effector cells or engagement of C1q is not wanted, because it can lead to undesired killing of biologically-important cells expressing the receptor or recruitment of drug-conjugated antibodies to off-target cells (126, 127). Below, strategies to eliminate FcγR binding and complement protein C1q binding are described.

Ablation of FcγR Binding

Point Mutations to Ablate FcγR Binding

One of the earliest antibodies used in humans was OKT3 to prevent transplant rejection (1). Despite humanization of the antibody, this antibody induced proinflammatory cytokine secretion, which resulted in toxicity (128, 129). The cytokine secretion was due to binding of OKT3 to CD3 followed by crosslinking of FcγRs on T cells (129). To alleviate the cytokine induction from T cells the Fc region of the antibody was mutated to eliminate FcγR binding (129). A single mutation of Leu235Glu was sufficient for knocking out binding to Fc receptors on U937 cells (129). Furthermore, the 100-fold reduction in binding to FcγR also resulted in lower T cell activation and proliferation in the presence of the Leu235Glu Fc mutant IgG1. Building upon this initial mutation it was found that the combination of Leu234Ala and Leu235Ala (commonly called LALA mutations) eliminated FcγRIIa binding [**Table 3** and **Figure 3A**; (130, 131)]. These two mutations were later shown to eliminate detectable binding to FcγRI, IIa, and IIIa for both IgG1 and IgG4 (153). The use of LALA appears to be more effective than either Leu234Ala or Leu235Ala alone. Some groups have seen that the Leu235Glu mutation knocks out FcγRI binding (129). However, there are reports that this high affinity receptor still binds to IgG1 Fc when this mutation is present (132). Similarly, Leu234Ala single mutant Fc still possessed detectable binding to the high affinity Fc receptor FcγRI at least in some assays (131). Both the single and double mutations at position 234 and 235 reduced ADCC activity mediated by PBMCs and nearly ablated ADCC mediated by monocytes (132). Nonetheless, the LALA mutations have been tested in humans in a phase I clinical trial. Anti-CD4 antibody OKT3 encoding the LALA mutations was administered for the treatment of acute renal allograft rejection. The IgG1 with LALA mutations caused minimal adverse reactions and was able to reverse allograft rejection in 6 of 7 individuals (154). One common pairing, in addition to the LALA pairing, is Ser228Pro paired with Leu235Glu, which has been called the SPLE or PE mutations (133). The SPLE mutations have been introduced into IgG4, which has low binding to FcγR initially perhaps due to the Phe234 residue that differs from Leu234 found in IgG1 (155). This combination dramatically reduced FcγRI binding to IgG Fc to barely detectable levels by surface plasmon resonance (133), without reducing circulating half-life in rats (155). The effect of the Ser228Pro is thought to be mostly to improve stability of IgG4 (155).

The LALA mutations have provided a foundation for the addition of other mutations or new modifications to Leu235. Building upon the LALA mutant phenotype, Oganesyan et al. mutated Leu234 and Leu235, but also added Pro331Ser to the Fc design to completely abrogate binding between Fc and FcγRs (135). The triple mutant Pro331Ser, Leu234Glu, and Leu235Phe eliminated all FcγR binding (135) without disrupting the overall conformation of the Fc (135). Similarly, Pro329Gly mutation was added to the LALA mutations, which inhibited binding to murine FcγRI, II, and III by IgG2a Fc (134, 156). The amino acid at 329 was changed, because this residue makes contact with Trp108 and Trp131 of FcγRIIIa (61). The LALA-PG was an improvement over LALA mutations alone in that they nullified Fc function in mouse and human IgG (134), whereas LALA alone still retains murine FcγRIII binding to murine IgG2a (157). The significance of the LALA-PG mutations are that observed results in murine models are expected to more accurately translate to humans since the mutations confer a similar phenotype for both murine IgG2a and human IgG1 (134).

The LALA mutations are among the most common point mutations used to disrupt Fc receptor binding, however other sites have been reported to knockout Fc receptor binding. Using a panel of 32 site-directed alanine mutations in IgG Fc Lund and colleagues showed that Gly237 and Glu318 were required for FcγRII binding (131, 138, 158). This lack of binding resulted in poor phagocytosis *in vitro* (138). Additional alanine scanning mutagenesis experiments determined 9 different amino acid substitutions that resulted in loss of binding to FcγRI, IIa, IIb, and IIIa. Notably, Asp265Ala and Glu233Pro mutations reduced binding to all 4 receptors by >80% (38). Ala318, Ala237, Ala265, and Pro233 represent a collection of mutations that can be used in various combinations to eliminate Fc receptor binding to Fc (134, 137, 138).

Cross-Subclass Fc Designs Eliminate FcγR Binding

To silence the effector functions of Fc, large portions of Fc regions from different subclasses have been exchanged to generate cross-subclass Fc regions (141). These designs aim to silence the Fc effector functions by combining CH domains from different subclasses that lack opposing functions. For example, IgG2 has poor FcγR binding but binds C1q, and IgG4 lacks C1q binding but reacts with FcγRs (142). Hence, combinations of IgG2 and

TABLE 3 | Fc modifications to silence antibody effector function.

Modifications or mutations (reference)	Abbreviated name	Phenotype	Reduced effector function
Leu235Glu (129)	LE	• Decreased binding to cell surface FcγRs	ADCC
Leu234Ala/Leu235Ala (130–132)	LALA	• Decreased binding to FcγRI, II, III	ADCC ADCP CDC
Ser228Pro/Leu235Glu (133)	SPLE in IgG4	• Decreased FcγRI binding • Half-life was unchanged	
Leu234Ala/Leu235Ala/Pro329Gly (134)	LALA-PG	• Eliminated binding to FcγRI, II, III, C1q	ADCP
Pro331Ser/Leu234Glu/Leu235Phe (135, 136)	TM	• Decreased binding to FcγRI, II, III and C1q	CDC
Asp265Ala (134, 137)	DA	• Decreased binding to FcγRI, II, III	ADCC ADCP
Gly237Ala (138)		• Decreased binding to FcγRII	ADCP
Glu318Ala (138)		• Decreased binding to FcγRII	ADCP
Glu233Pro (38)		• Decreased binding to FcγRI, II, and III	
Gly236Arg/Leu328Arg (139, 140)	GRLR	• Decreased binding to all FcγR	ADCC
IgG2-IgG4 cross-subclass (141, 142)	IgG2/G4	• Decreased binding to FcγRs and C1q	
His268Gln/Val309Leu/Ala330Ser/Pro331Ser (143, 144)	IgG2m4	• Decreased binding to all FcγR • Decreased C1q binding	ADCC ADCP CDC
Val234Ala/Gly237Ala/Pro238Ser/ His268Ala/Val309Leu/Ala330Ser/Pro331Ser (144)	IgG2σ	• Near complete elimination of FcγRI, IIa, IIb, and IIIa binding • Decreased C1q binding • Binds FcRn	ADCC ADCP CDC
Leu234Ala/L235Ala/Gly237Ala/P238Ser/ His268Ala/Ala330Ser/Pro331Ser (144–146)	IgG1σ	• Near complete elimination of FcγRI, IIa, IIb, and IIIa binding • Binds FcRn	ADCC CDC
Ala330Leu (89)	AL	• Decreased C1q binding • Part of DLE mutations	CDC
Asp270Ala (89)		• Decreased C1q binding	CDC
Lys322Ala (89)		• Decreased C1q binding	CDC
Pro329Ala (89)		• Decreased C1q binding	CDC
Pro331Ala (89)		• Decreased C1q binding	CDC
IgG2-IgG3 cross-subclass (51)		• Decreased C1q binding	CDC
High mannose glycosylation (147, 148)		• Decreased C1q binding	CDC
Val264Ala (137)		• Decreased C1q binding	CDC
Phe241Ala (137)		• Decreased C1q binding	CDC
Asn297Ala or Gly or Gln (32, 149–152)		• Decreased binding to FcγRI and IIIa • Decreased C1q binding	ADCC ADCP CDC
S228P/Phe234Ala/Leu235Ala (144)	IgG4 PAA	• Decreased binding to FcγRI, IIa and IIIa	ADCC CDC

IgG4 CH domains have been constructed that are devoid of both C1q and FcγR binding (141, 142). Typically, in the IgG2/G4 chimeras the hinge and CH1 domain originates from IgG2 and the CH2 and CH3 domains are from IgG4 (141, 142).

Using a different approach to the same concept An and colleagues compared the amino acid sequences of different IgG subclasses and introduced mutations into IgG2 that would completely eliminate FcγR binding. The aim of this approach is to introduce natural amino acids into the Fc so that the Fc would not be immunogenic. The investigators made conservative changes in the IgG2 primary sequence resulting in His268Gln/Val309Leu/Ala330Ser/Pro331Ser mutations (143). This cross-subclass design was termed IgG2m4 and lacked binding to all FcγR (143). The circulating half-life of this antibody was comparable to wildtype IgG in macaques, which suggested the transplantation of IgG4 residues did not make the IgG2 more immunogenic (143).

More recently, Vafa et al. combined many of the mutations that have been discovered over the last 25 years to create an engineered construct called G2σ (**Figure 3B**). This construct included Val234Ala/Gly237Ala/Pro238Ser/His268Ala/Val309Leu/Ala330Ser/Pro331Ser mutations where many of the mutations were previously established as silencing mutations and the remaining mutations were selected as cross-subclass mutations that introduced IgG4 residues into IgG2 (144). In direct comparisons with IgG1, IgG2, IgG4, and IgG2m4, IgG2σ had

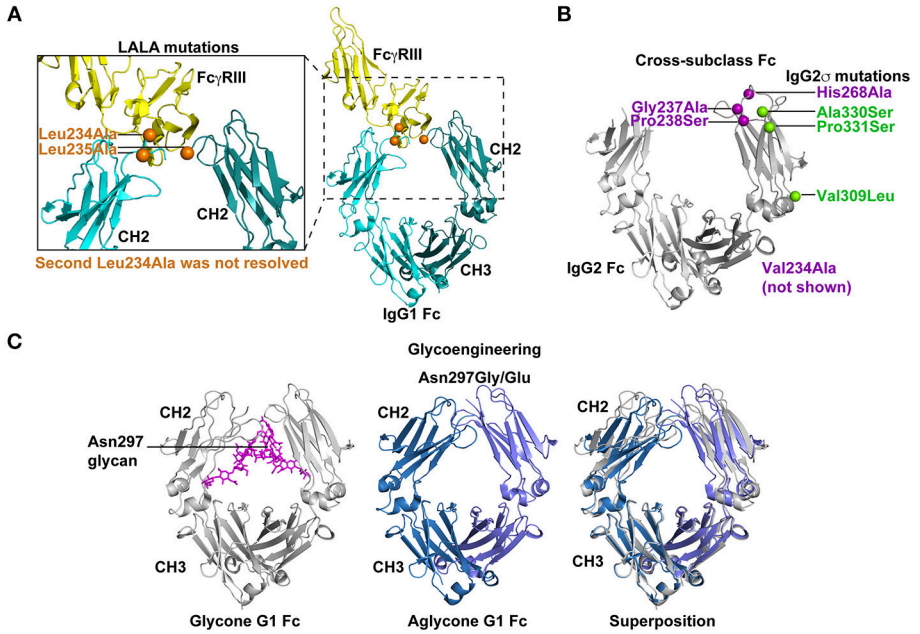

FIGURE 3 | Strategies for silencing antibody effector functions. **(A)** Point mutations in the Fc have been identified that disrupt antibody effector functions. The elucidation of key amino acids in the interaction of Fc with FcγRs has led to collections of point mutations that can eliminate or drastically reduce Fc binding to specific FcγRs. The Leu234Ala/Leu235Ala (LALA) mutations are perhaps the most commonly used mutations for disrupting antibody effector function (130, 131). As shown in the co-crystal structure (PDB: 1T83) with orange spheres the LALA mutations are proximal to FcγRIII (yellow) when it binds the IgG1 Fc (light and dark teal). These mutations can be combined with other effector function silencing strategies to engineer a Fc that is devoid of any FcγR binding or C1q binding. Leu 234 was only resolved in one of the chains of the Fc region. **(B)** Effector functions can be disrupted by exchanging amino acids between two Fc molecules from different IgG subclasses. These cross-subclass Fc designs rationally combine mutations that knockdown binding to a given FcγR or complement protein. IgG2m4 and IgG2σ are two examples of engineered Fc regions that were generated by this approach (143, 144). IgG2σ is perhaps the most effector function silent Fc and it combines cross-subclass mutations Val309Leu, Pro331Ser, and Ala330Ser (green spheres) with four additional mutations not naturally found in human Fc sequences (purple spheres) (144). The crystal structure of the IgG2 Fc (gray) encoding these mutations (green and blue spheres) showed the CH2 domains moved farther apart from each other. Also, Asp270 and Pro329, which are essential for binding to FcγR and C1q, were repositioned (PDB:4L4J; 145). Position 234 was not visible in the crystal structure and is not shown in figure. **(C)** Removal of the Asn297 glycan in the IgG Fc severely reduces Fc binding to FcγRs by inducing a Fc closed conformation. Several Fc designs have removed the N-linked glycosylation site at position 297 by introducing Asn297Gly or Asn297Glu changes (151, 152). The crystal structure of glycosylated IgG1 Fc (gray) is shown with the N297 glycan (magenta; PDB:4BYH). The introduction of a Gly or Glu residue at position 297 produces an aglycone IgG1 Fc (blue and lilac; PDB:3S7G). Superposition of the glycone and aglycone Fc crystal structures shows the lilac and blue CH2 domains in the aglycone are closer in proximity than the gray CH2 domains. The altered CH2 conformation has been hypothesized to be the structural explanation for reduced FcγR binding by the aglycone Fc.

the most profound elimination of binding to FcγRI, IIa, and IIIa (144). However, both IgG2m4 and IgG2σ lacked *in vitro* ADCC activity mediated by human PBMCs effector cells and possessed very little ADCP activity against breast cancer cell lines (144). Given the success of IgG2σ at ablating Fc effector functions, the design was translated to IgG1 and IgG4 (145, 146, 159). The IgG1σ (Leu234Ala/L235Ala/Gly237Ala/P238Ser/His268Ala/Ala330Ser/Pro331Ser), IgG2σ, and an IgG4 Fc encoding S228P/Phe234Ala/Leu235Ala mutations (termed IgG4 PAA) versions of Fc were compared for binding to FcγRs from multiple species (144, 145). IgG1σ and IgG2σ lacked binding to FcγRI and III (145). For FcγRIIa and IIb extremely weak binding could be seen to IgG1σ and IgG2σ at high concentrations of antibody (145). Both IgG1σ and IgG2σ exhibited lower binding to FcγRs than IgG4 PAA (145). IgG4 PAA also showed species-specific differences in binding to FcγRs, whereas IgG1σ and IgG2σ lacked binding for human, macaque, and mouse FcγRs. Therefore, IgG1σ and IgG2σ are among the most effective mutations for knocking out Fc effector function.

Mimoto et al. sought to use FcγRIIb as a way to capture immune complexes on the surface of FcγRIIb-expressing B cells (160). Thus, they engineered the Fc to selectively bind to FcγRIIb with a 200-fold increase in affinity, and a 10-fold lower affinity for the other FcγRs (160). The improved affinity for FcγRIIb conferred the desired boost in B cell presentation of peptides to T cells *in vitro* (160). Since FcγRIIb is an inhibitory receptor these mutations could be used to silence effector function by changing the ratio of Fc binding to activating vs. inhibitory receptors.

Ablation of C1q Binding to Reduce Complement Dependent Cytotoxicity (CDC)

Point Mutations to Ablate Complement Binding

Inducing the complement cascade has been associated with antibody injection site adverse reactions (161, 162). Therefore, eliminating C1q binding to Fc—the initial event in the activation of antibody-dependent complement cytotoxicity (163)—has been

a goal of Fc optimization. One of the benefits of the mutations engineered to eliminate FcγR binding is that many of them eliminate C1q binding too. In a structure-guided screen of Fc mutations, an Ala330Leu mutation was observed to decrease C1q binding (39). As stated above this mutation also eliminated FcγRIIb binding (39). In *in vitro* assays the Ala330Leu mutation reduced the ability of the antibody to mediate complement-dependent cytotoxicity (CDC) of target cells, presumably because Ala330Leu disrupted C1q binding to Fc. However, not all amino acids introduced at position 330 disrupted C1q binding, thus the effect was specific to the introduction of only certain amino acids at position 330 (39). It should be noted that Ala330Leu is one of the mutations in the set of mutations comprising the DLE mutations (Ser239Asp Ile332Glu Ala330Leu) that improve FcγR-mediated effector functions. The DLE optimization eliminates CDC activity mediated by the antibody, which can be rescued by removing the Ala330Leu mutation from the set. In instances where CDC causes adverse reactions to antibody administration the DLE mutations may be able to enhance FcγR-mediated effector functions and eliminate injection site reactions (39).

Additional amino acids that reduce C1q binding were identified by an alanine scan of the Fc. Asp270, Lys322, Pro329, and Pro331 were all implicated as sites in IgG that confer binding to C1q (89). Among these amino acid positions Asp270Ala and Pro329Ala, showed the most pronounced deficiency in complement activation and C1q binding across multiple concentrations of serum C1q (89). The Leu234Glu/Leu235Phe/Pro331Ser triple mutant Fc lacks binding to FcγRs, but also these mutations eliminate Fc binding to C1q (135). Similarly, the creation of IgG2m4 not only eliminates FcγR binding but also eliminates C1q binding (143). Vafa et al. examined the CDC activity of IgG2m4 (His268Gln/Val309Leu/Ala330Ser/Pro331Ser) and IgG2σ (Val2 34Ala/Gly237Ala/Pro238Ser/His268Ala/Val309Leu/Ala330Ser/ Pro331Ser) formats of Rituxan (144). Neither antibody format conferred CDC against a lymphoma cell line using human serum complement (144) showing both are potential designs for eliminating complement-mediated functions. The structure of IgG2σ Fc was solved to 1.9 angstroms and showed that it is in a more open conformation meaning the CH2 domains of the Fc are spaced relatively far apart (144). Moreover, the loop containing Leu328 is repositioned compared to wildtype IgG2 Fc. Thus, it is postulated that the change in conformation results in reorientation of Asp270 and Pro329, which eliminates FcγR and C1q binding to IgG2σ (144).

Cross-Subclass Fc Regions to Ablate Complement Activation

Fundamental knowledge of how each IgG subclass interacts with complement allows for fine tuning of CDC. For example, IgG2 can have moderate to low CDC activity (51). Thus to reduce IgG3 CDC activity the CH2 domain of IgG2 can be used to replace the CH2 domain of IgG3 (51). Similarly, to ablate IgG1 CDC activity a Pro331Ser mutation was introduced based on the fact that IgG4 has Ser331 and lacks CDC activity (136). These mutations are an example of how basic science can be applied to the design of antibody-based biologics. Also many of the point mutations made to knockout C1q binding are cross-subclass mutations (**Table 3**).

Glycoengineering to Ablate FcγR and C1q Binding

The Fc of IgG contains an N-linked glycosylation site at position 297 [**Figure 3C**; (72)]. Typically the glycan present at N297 is a complex biantennary glycan (164, 165). The modification of this glycan to high mannose glycan reduced the affinity of IgG1 Fc for C1q, which in turn reduced CDC activity (147, 148). Inhibiting the incorporation of galactose or sialic acid into carbohydrate synthesis did not dramatically silence immune effector functions of the antibody Fc (147). While glycans devoid of galactose or sialic acid appear to function normally, mutations in the Fc that knockout C1q and FcγRI binding can also lead to an increase in galactosylation and sialylation of the Asn297 glycan eliminate (137). Galactosylation and sialylation-increasing mutations include Phe241Ala, Val264Ala, and Asp265Ala mutations (137). Whether the change in glycosylation profile has a role in reducing C1q binding or is an unrelated bystander effect is not clear. In one study, sialylation of the Fc reduced C1q binding 4-fold suggesting hypersialylation could directly impair CDC responses (101). However, hypersialylation reduces terminal galactosylation complicating which factor contributes to the reduced C1q binding (101).

Another common method to eliminate Fc effector function has been to completely remove the glycosylation site by substituting alanine, glutamine, or glycine at position 297 (32, 149–152). The removal of the glycosylation site dramatically reduced IgG1 binding to FcγRI and C1q [**Figure 3C**; (137, 152, 166)]. In the context of IgG3, the Fc lacking glycosylation—the aglycone Fc—has reduced binding to FcγRI and C1q. *In vitro*, the aglycone IgG3 Fc loses the ability to mediate ADCC via FcγRIIIa (151, 152). However, removal of Asn297 glycan reduces, but does not eliminate, binding to mouse Fc receptors (157). Additionally, it is thought that avidity can overcome the low binding affinity to FcγRI conferred by mutating Asn297 (144). Therefore, on monocytes and macrophages where FcγRI is expressed at a high density the Asn297Gly mutation may not be sufficient to eliminate all binding. Lo et al. combined the Asn297Gly mutation with Asp265Ala mutation to further reduce FcγR binding to Fc. As stated above each of these mutations reduces FcγR and C1q binding individually and when combined showed a further reduction in Fc binding to FcγRs and C1q (134). Combinations such as the Asn297Gly/Asp265Ala are useful for near complete knockout of binding between Fc and FcγRs or C1q.

The mechanism behind the aglycone reducing Fc binding to C1q and FcγRs is not fully understood. The aglycone Fc is more susceptible to protease cleavage, which suggests the structure of the glycone Fc differs from that of the aglycone (152). Nuclear magnetic resonance studies have similarly suggested structural perturbations are present in the aglycone Fc (32, 73, 151). The clearest evidence for structural changes in the agylcosylated Fc was provided by the crystal structure of mouse Fc without the N297 glycan (167). The CH3 domains appeared identical, but the CH2 domains of aglycone Fc and glycosylated Fc differed in position by 10 to 14 angstroms (167). In the aglycone Fc the CH2 domains were closer together than in the

glycosylated Fc, thus the aglycone had a "closed" conformation. This closed conformation was not unique to mouse Fc. Crystal structures of both aglycosylated human IgG1 Fc (168) and IgG4 Fc (169) have shown the CH2 domains of a single Fc to undergo rigid-body movements of 10–20 angstroms to be closer in proximity to each other when aglycosylated (**Figure 3C**). The closed conformation of the aglycone Fc is mediated, at least in part, by the perturbed C'E loop (149, 150, 167, 168, 170). In total, eliminating Fc glycosylation induces a closed conformation that confers silencing of the Fc effector functions.

CONCLUSIONS

Many approaches including phage display, alanine scanning mutations, and structure-based design have all been successful in optimizing the Fc of antibody-based biologics (38, 39, 113). Underlying the optimization of the Fc is modulating its ability to bind to Fc receptors, C1q, and FcRn. These interactions can be modulated by the introduction of point mutations, inserting or deleting amino acids, modifying glycan composition, or appending protein domains (171). Overall, strengthening or disrupting Fc interactions with its binding partners as measured by *in vitro* affinity has translated to the desired outcome *in vivo*. The optimization of Fc for specific functions not only can improve *in vivo* functions, but it also provides a means to dissect the importance of specific Fc receptors (172) and downstream CDC, ADCC, phagocytosis, or circulation half-life in treating specific diseases (119, 173). While antibody-based biologics have been successful in the treatment of disease, new opportunities exist for antibody biologics as durable prevention strategies for infectious diseases (174–176). The antibody-mediated prevention (AMP) study will test the efficacy of monoclonal antibody passive infusion to prevent HIV-1 infection in 2,700 participants (NCT02716675). This phase 2b trial could provide the first proof-of-concept that neutralizing antibodies can provide protection from HIV-1 infection in humans. The VRC01 antibody that will be used contains a wildtype Fc; however, the next generation of HIV-1 antibody protection studies will likely include combinations of Fc-optimized HIV-1 neutralizing antibodies with prolonged circulation half-life (116), since longer half-life was important for protection in preclinical nonhuman primate studies (119, 122, 123). The ability to optimize the Fc region of antibodies continues to be a powerful approach for combating heritable diseases, infectious diseases, and cancer.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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FCGR3A and FCGR2A Genotypes Differentially Impact Allograft Rejection and Patients' Survival After Lung Transplant

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Fc gamma receptors (FcγRs) play a major role in the regulation of humoral immune responses. Single-nucleotide polymorphisms (SNPs) of *FCGR2A* and *FCGR3A* can impact the expression level, IgG affinity and function of the CD32 and CD16 FcγRs in response to their engagement by the Fc fragment of IgG. The CD16 isoform encoded by *FCGR3A* [158V/V] controls the intensity of antibody-dependent cytotoxic alloimmune responses of natural killer cells (NK) and has been identified as a susceptibility marker predisposing patients to cardiac allograft vasculopathy after heart transplant. This study aimed to investigate whether *FCGR2A* and *FCGR3A* polymorphisms can also be associated with the clinical outcome of lung transplant recipients (LTRs). The SNPs of *FCGR2A* ([131R/H], rs1801274) and *FCGR3A* ([158V/F], rs396991) were identified in 158 LTRs and 184 Controls (CTL). The corresponding distribution of genotypic and allelic combinations was analyzed for potential links with the development of circulating donor-specific anti-HLA alloantibodies (DSA) detected at months 1 and 3 after lung transplant (LTx), the occurrence of acute rejection (AR) and chronic lung allograft dysfunction (CLAD), and the overall survival of LTRs. The *FCGR3A* [158V/V] genotype was identified as an independent susceptibility factor associated with higher rates of AR during the first trimester after LTx (HR 4.8, $p < 0.0001$, 95% CI 2.37–9.61), but it could not be associated with the level of CD16- mediated NK cell activation in response to the LTR's DSA, whatever the MFI intensity and C1q binding profiles of the DSA evaluated. The *FCGR2A* [131R/R] genotype was associated with lower CLAD-free survival of LTRs, independently of the presence of DSA at 3 months (HR 1.8, $p = 0.024$, 95% CI 1.08–3.03). Our data indicate that FCGR SNPs differentially affect the clinical outcome of

LTRs and may be of use to stratify patients at higher risk of experiencing graft rejection. Furthermore, these data suggest that in the LTx setting, specific mechanisms of humoral alloreactivity, which cannot be solely explained by the complement and CD16-mediated pathogenic effects of DSA, may be involved in the development of acute and chronic lung allograft rejection.

Keywords: Fc-gamma receptors, natural killer cells, lung transplantation, chronic lung allograft dysfunction, HLA antibodies, allograft rejection

INTRODUCTION

Lung transplantationi (LTx) remains a challenging therapeutic option for patients with end-stage pulmonary disease. Significant improvement in immunosuppressive strategies has led to decreased lung allograft loss in the early post-transplant period. When compared to other solid organ transplantation settings, LTx remains associated with the lowest survival rates with a median survival of 6 years after transplant (1). Chronic lung allograft dysfunction (CLAD) is the main cause of chronic lung allograft rejection and is characterized by an irreversible loss of lung function associated with a high prevalence of complications such as bronchiolitis obliterans syndrome (BOS) and restrictive allograft syndrome (RAS) (2). Factors that relate to the HLA mismatch, to the graft procedure (ischemia, unilateral or bilateral surgery) and initial lung disease can lead to highly variable levels of recipient immune response to the lung allograft. Inflammatory biomarkers and antibodies, occurrence of acute cellular rejection (ACR), infections/colonization, auto-immunity, and air pollution have been analyzed for their potential predictive value in anticipating development of immunological responses associated with CLAD. Toll receptors, pro-inflammatory cytokines and non-classical HLA molecules, i.e., HLA-G and HLA-E polymorphisms or haplotypes, have been associated with CLAD (3–9), but the underlying mechanisms involved in this devastating outcome of LTx in a given recipient are still poorly understood. Antibody-mediated rejection (ABMR) has been associated with a higher incidence of chronic lung allograft dysfunction (CLAD) and mortality after LTx but the specific mechanisms and histological or immunological biomarkers that allow to define the clinical ABMR entity still await further comprehension (10). Detection of preexisting or development of *de novo* donor-specific human leukocyte antigen (HLA) alloantibodies (DSAs) have been extensively investigated for their potential value as biomarkers of humoral responses that may predict adverse outcome of LTx.

Various studies suggest that the pre-transplant detection of circulating DSA is not associated with an increased risk of developing CLAD or related death if prospective cross-match testing was negative (11, 12). The pretransplant detection of DSA and antibodies directed againd non-HLA antigens such as angiotensin type 1 receptor (AT1R) and endothelin type A receptor (ETAR) is reported to have a negative impact on lung transplant outcome (13).

Early detection of *de novo* DSA detected at 1 month after LTx has been significantly associated with a worse outcome (14). Multiplex solid phase single antigen bead assay (SAFB) detection of *de novo* DSAs, notably anti-HLA DQ DSA, have also been associated with acute cellular rejection (ACR) (15) CLAD (16–18). The presence of circulating DSA at the time of lung allograft biopsy has been identified as a risk factor for graft loss (19).

Although there is more and more direct and indirect evidence regarding the role of *de novo* DSA in CLAD occurrence, the mechanisms that sustain the variable toxicity of these anti-HLA antibodies to the lung allograft are still unclear. One of the well-known cytotoxic mechanisms of DSA occurs through the IgG-mediated activation of the complement cascade that results in C4d deposition within the graft. C4d staining in lung allograft biopsies is considered as a diagnosis criteria for ABMR (10, 20–28). However, although recent evidence suggests that detection of complement-binding DSA can be associated with lung allograft failure (29), the predictive value of complement-binding DSA on LTx clinical outcome remains to be firmly demonstrated.

IgG antibodies can also activate the immune system through ligation of functional Fc gamma receptors (FcγRs). Since these receptors are expressed by a variety of immune cells, including B cells, natural killer cells, platelets, dendritic cells and macrophages, FcR receptors constitute a major checkpoint that regulates the intensity of auto- and allo-immune responsiveness of the host in response to infectious and humoral threats (30, 31). As infections and the immune alloreactivity of the recipient toward the transplant remain a leading cause of graft rejection and death during the first year after LTx, there is a need for biomarkers that may improve the monitoring of early humoral responses in LTRs (32–34) and open therapeutic perspectives to dampen antibody-driven inflammation in immunized patients (35, 36).

Polymorphisms of FcγRIIA (*FCGR2A* [131R/H], rs1801274) FcγRIIIA (*FCGR3A* [158V/F], rs396991) genes, that respectively, encode for the CD32 and CD16 receptors for the Fc segment of IgG, have an impact on the level of expression and immune function of these activating FcγRs. Various reports have highlighted the clinical relevance of these SNPs in controlling the host immune response to monoclonal antibody therapy.

Abbreviations: HLA, Human Leukocyte Antigen; LTx, Lung Transplant; LTRx, Lung Transplant Recipient; Abs, Antibodies; Ag, Antigen; DSA, Donor-Specific Antibodies; BOS, Bronchiolitis Obliterans Syndrome; CLAD, Chronic Lung Allograft Dysfunction; ADCC, Antibody dependent cell cytotoxicity; CT, Computed Tomography; RAS, Restrictive Allograft Syndrome; ABMR, Antibody Mediated Rejection; ACR, Acute Cellular Rejection; SAFB, Single-Antigen Flow Beads; D, Days; M, Month; CMV, Cytomegalovirus; MFI, Mean Fluorescence Intensity; OS, Overall Survival; DFS, Disease Free Survival; CDC, Complement-Dependent Cytotoxicity; CF, Cystic Fibrosis.

The presence of a histidine (H) rather than an arginine (R) at position 131 results in higher affinity for IgG1 and IgG2. The *FCGR2A* [131H/H] genotype has been associated with higher efficacy of Rituximab-mediated B cell depletion strategies before transplant of ABO-incompatible organs. This *FCGR2A* [131H/H] SNP has also been identified as a susceptibility marker associated with the severity of community-acquired pneumonia (37).Genetic variation in the *FCGR2A* gene has also been shown to be associated with an increased prevalence of invasive pneumococcal diseases and respiratory infections after LTx (38, 39), regardless of the site of pneumococcal infection (40). Pro-inflammatory effects associated with the *FCGR2A* [131R/R] genotype have also been reported as a consequence of activating signals that result from the dynamic interactions between CD32 and its cognate C-reactive protein (CRP) and immunoglobulin ligands.

The clinical relevance of CD16 inflammatory pathways in antibody-mediated rejection of kidney and heart allografts has also been illuminated by studies that deciphered the molecular landscape of immune and endothelial cell transcripts that associate with ABMR lesions evaluated in allograft biopsies (41–43). Ligation of the Fc domain of IgG has also been identified as a mechanism that allows CD16-dependant clearance of viral pathogens by immune NK cells (44). Recent studies suggest that the Fc fragment of DSA can indeed exert its adverse cytotoxic and inflammatory effects though a complement-independent mechanism that relies on level of expression of the NK-cell surface CD16 receptor and its affinity for the Fc fragment of alloantibodies. The intensity of host antibody dependent cell cytotoxicity (ADCC) is known to be conditioned by the capacity of NK effector cells to form conjugates with antibody-coated allogeneic cells, which is in part influenced by the *FCGR3A* [158V/F] genotype (45–47). We and others have recently shown that polymorphic variation in the *FCGR3A* genes is also likely to affect the pathogenic effects of IgG alloantibodies by controlling the level of the CD16 dependent recipient's immunological cytotoxic responses to allogeneic donor cells exposed to chronic alloantibody threat (35, 48–51).

Genotypic variation in the *FCGR3A* receptor can thus impact the strength of FcR-dependent ADCC responses of NK cells and regulate their capacity to secrete inflammatory cytokines and release CD107a/Lamp1^{+} cytotoxic granules containing perforin and granzyme (50, 52, 53). The inter-individual variability in the functional CD16 receptor-dependent engagement by the Fc fragment of alloantibodies has been shown to condition the intensity of antibody-dependent cellular cytotoxicity (ADCC) of NK cells and the response to IgG immunotherapy.

The strength of FcR-mediated ADCC can also be affected by the isotype and glycosylation status of alloantibodies. The IgG1 and IgG3 isoforms have higher affinity for the CD16 *FCGR3A* [158V/V] variant when compared to *FCGR3A* [158F/F], thereby spurring increased effector cell activity in response to these IgG subclasses. We have shown that this high-affinity homozygous *FCGR3A* [158V/V] genotype is an independent predictor of cardiac allograft vasculopathy (48) and may be a clinically relevant underlying mechanism that sustains the level of DSA-mediated allograft injury (48–50). The recent evidence that the NK cell infiltration of the graft can predict kidney graft failure also sustains the clinical relevance of these NK cell mediated mechanisms of allograft injury (54).

Considering the central contribution of polymorphisms affecting FcγRIIA and FcγRIIIA genes and receptor function in the individual shaping of antibody-mediated inflammatory and cytotoxic alloimmune responses of the recipient, deciphering the mechanisms and FcγR susceptibility profiles that may be associated with LTx outcome is necessary to optimize risk stratification (8).

This study thus aimed to investigate whether FcγR polymorphisms may be linked to the pathogenic mechanisms of DSA toxicity and be associated with adverse clinical complications that impair patient and allograft outcome after LTx.

MATERIALS AND METHODS

Participants and Study Design

We conducted a retrospective single-center study enrolling 158 adult patients who underwent lung transplants (LTx) at the Marseille Lung Transplant Center between December 2006 and December 2013. All patients from the French cohort (COLT, *Cohort in Lung Transplantation*, l'Institut du Thorax, INSERM UMR1087/CNRS UMR 6291, CNIL 911142) were recruited in this study. All subjects gave written informed consent in accordance with the Declaration of Helsinki. A group of 184 healthy unrelated volunteer French bone marrow donors were also recruited to constitute a control cohort, allowing the analysis of the FcγR genotype. Blood donations were collected in the "Etablissement Francais du Sang," in accordance with BSL-2 practices. A medical interview was carried out prior to blood donation to exclude donors with medical contraindications. This study was carried out in accordance with the French Public Health Code (art L1221-1), approved by an institutional ethics committee and conducted in compliance with the Good Clinical Practice Guidelines declaration of Helsinki and Istanbul.

Post-transplant Clinical Management

All recipients received a similar standardized immunosuppressive regimen in accordance with our institutional protocols. Induction therapy consisted of intravenous administration (IV) of rabbit anti-thymocyte globulins (rATG, Pasteur Merieux, Lyon, France) given for the first 3 post-operative days [except when daily lymphocyte count was below 200/mm^3, and when there were cytomegalovirus (CMV) and/or EBV mismatches (i.e., seronegative recipient and seropositive donor)] and high dose methylprednisolone (6 mg/kg/d Day 1, 2 mg/kg/d Day 2 and Day 3, and 1 mg/kg/d thereafter). The standard triple maintenance immunosuppressive regimen consisted of tacrolimus (adjusted to maintain whole blood levels varying between 12 and 14 ng/ml), mycophenolate mofetil in 5 patients (adjusted to a white blood cell count above 4,000 mm^3), and steroids (prednisone) tapered to 0.25 mg/kg/d over the first 3 months and stopped around 12 months after surgery.

According to CMV recipient positive (R+) and donor positive/recipient negative (D+/R–) status, patients received CMV prophylaxis with IV ganciclovir switch to oral valganciclovir.

Postoperatively, recipients received prophylactic or preemptive anti-infection treatment (antibiotic, antiviral, and antifungal therapies) according to their preoperative and/or concomitant infectious status.

Recipients had regular visits to the transplant center for clinical radiological and functional evaluation. At our institution, surveillance transbronchial biopsies are routinely performed at the end of the first month, or earlier if clinically indicated. All transbronchial biopsies were graded for ACR (A grade) and lymphocytic bronchiolitis (B grade) (LB) by lung transplant pathologists. ACR and LB were defined, respectively, as perivascular or peribronchial mononuclear inflammation according to the International Society for Heart and Lung Transplantation (ISHLT) criteria. Histologic appraisal of ACR and LB was conducted in accordance with accepted ISHLT standards in terms of the minimum number of biopsy samples and exclusion of opportunistic infection (27, 28). Pulmonary function tests (PFTs) were routinely conducted at our center on a monthly basis for the first 12 post-operative months, at M2 intervals in the second year and at M3 intervals thereafter. The baseline FEV_1 value was calculated as the average of the 2 best FEV_1 values at least 2 measure gap. Baseline values of total lung capacity (TLC) and FEV_1/FVC were defined as the average of the 2 measurements obtained at the same time as the best 2 FEV_1 measurements. Chronic lung allograft dysfunction (CLAD) was defined according to the standardized international criteria (55). The phenotype BOS or RAS was specified according to ISHLT guidelines (56). The following histological patterns compatible with AMR were also analyzed: (i) neutrophil capillaritis and (ii) acute lung injury with or without diffuse alveolar damage and with or without organizing pneumonia.

HLA Antibody Screening and Identification Protocol

Recipient serum samples from 2006 to 2013 were collected routinely prior to transplant, at the minimum when the patient was placed on the waiting list and before the transplant procedure (D0: Day 0) and serially after LTx (at month 1 and 3). All sera samples obtained from the 158 recipients were further assessed using Luminex single-antigen flow beads (SAFB) to determine antibody specificity using Single Antigen—One Lambda reagents (*LABScreen*® Single Antigen class I or *LABScreen*® Single Antigen class II, One Lambda, Thermo Fisher Scientific, Canoga Park, California, USA) according to the recommendations of the manufacturers. The mean fluorescence intensity (MFI) used the baseline formula proposed by the Fusion™ v 3.2 software. All beads with a normalized MFI threshold >1,000 were considered positive. Since detected circulating alloantibodies were often directed against distinct HLA class I and/or class II antigens, the cumulative mean fluorescence intensity (cMFI) of DSA was calculated as the sum of the MFI for each of the individual DSAs detected. DSA were considered to be HLA antibodies directed against donor HLA antigen. Putative HLA-Cw and -DQA1 DSA were identified in accordance with the conventional linkage disequilibrium that is reported between HLA-B and HLA-C loci and between HLA-DQB1 and HLA-DQA1 loci, as respectively, described in the French population (57) and by supplementary extensive genotyping of HLA- genes in the recipient. Putative HLA-DR51, -DR52 and -DR53 DSA were determined in accordance with the linkage disequilibrium between DRB3, DRB4, DRB5 loci and DRB1 loci of recipient. The allelic specificities of HLA-Cw, DQA and -DR51, -DR52 and -DR53 DSA were assigned accordingly.

C1q Detection

All patients with HLA antibodies were tested for the presence of C1q. The binding level was determined by the C1qScreen™ assay per manufacturer instructions (One Lambda, Thermo Fisher Scientific, Canoga Park, California, USA). Fluorescence intensity was measured using Luminex-based LABScan™ 100 flow analyzer. C1q specific antibody specificity and binding levels were analyzed and determined through the Fusion™ v 3.2 software. C1q MFI > 1,000 was considered positive.

FCGR2A and *FCGR3A* Genotyping

The detection of single nucleotide polymorphisms (SNPs) allowed for the genotyping of *FCGR3A* ([158V/F], rs396991) and *FCGR2A* ([131R/H], rs1801274) as described (5, 48) using the SNAPSHOT technique. Genomic DNA was extracted from a 200-µl whole blood sample using the QIAmp Blood DNA Mini kit (Qiagen, Courtaboeuf, France) according to manufacturer instructions. A previously described homemade primer extension method (58) was used to simultaneously analyze the SNPs of the FcγR genes. The forward and reverse primers sequences were, respectively: *FCGR3A*-F 5′ TCCTAA TAGGTTTGGCAGTG 3′ and *FCGR3A*-R 5′ AAATGTTCA GAGATGCTGCT 3′, *FCGR2A*-F 5′ CCAGGAGGGAGAAAC CATC 3′ and *FCGR2A*-R 5′ CTCTTCTCCCCTCCCTACAT 3′. The extension primers are, respectively: *FCGR3A*_176-F: 30T-CCTACTTCTGCAGGGGGCTT and *FCGR2A*_166F 57T-CCAAAAGCCACACTCAAAGA. Data were analyzed using GeneMapper 4.0 with specific detection parameters. Using an in-house computer program, output files (.txt) exported from GeneMapper 4.0 were automatically formatted into files readable by the "Phenotype" application of the Gene[Rate] computer tool package (http://geneva.unige.ch/generate). For each different allele obtained, PCR products were sequenced on both strands using the BigDye Terminator v1.1 Sequencing Kit (Applied Biosystems) and analyzed on an automated fluorescence-based ABI PRISM 3130 XL genetic analyzer according to manufacturer protocol. FcR polymorphisms at these 4 loci were analyzed for allele frequencies, or homozygous or heterozygous allelic combination of FcγR alleles defining the corresponding genotype.

HLA Genotyping

Recipients and deceased donors were genotyped for low resolution HLA-A, -B, -DRB1, and -DQB1 loci by LABType® SSO (One Lambda, Thermo Fisher Scientific, Canoga Park,

California, USA) according to the manufacturer's specifications and the retrieved output was analyzed for allele identification using HLA Fusion^{TM} v 1.2.1. Software (One Lambda, Thermo Fisher Scientific, Canoga Park, California, USA).

Phenotypic Analysis of Antibody-Dependent NK Cell Activation

Evaluation of the CD16-dependent alloreactive potential of DSA was assessed using the previously described NK cell humoral activation test (NK-CHAT) (50). The level of serum-induced CD16 engagement and the degranulating potential of NK cells was assessed by flow cytometry analysis of the level of CD16 cell surface expression within the $CD3^{-}CD56^{+}$ NK cell compartment, gated within PBMC effector cells after exposure to target B cells that had been precoated in the presence of LTRs or control sera. Effector cells used in the standardized assay were prepared from a healthy donor displaying the *FCGR3A* [158V/V] encoding the high-affinity CD16 variant. The HLA typing of the two B cell lines used as targets were A2/A2, B44/B56, Cw1/Cw5, DR1/DR4, DR53, DQ5/DQ7 and A3/A3 B7/B35, Cw4/Cw5, DR10/DR15, DR51, DQ5/DQ6). Sera were selected for the presence of DSA recognizing a limited set of HLA antigens expressed by EBV target cell lines. Briefly, 500,000 target B cells (B-EBV cell lines expressing the cognate DSA target alloantigens detected in LTR) were incubated with control (CTL) unsensitized human male AB serum (Lonza) to block FcR, then rinsed and incubated for 15 min in the presence of 50% LTR serum [or CTL serum supplemented or not with 10 γg/ml of Rituximab IgG (positive control)] and then rinsed to remove unbound antibodies. Incubation of effector PBMC and pre-coated allogeneic B-EBV cell targets (1:1 ratio) was performed for 3 h at 37°C in presence of GolgiStop (Becton Dickinson 554724) and CD107-PC5 (Becton Dickinson 555802). Cells were then washed and labeled with CD3-ECD (Beckman Coulter A07748), CD16-PE (Beckman Coulter A07766), CD56-PC7 (Beckman Coulter A21692) for 15 min at room temperature protected from light. After 1 wash step, cells were resuspended in 500 µl PBS 2% SVF. Data acquisition and analysis was performed on a Beckman Coulter Navios cytometer. The mean fluorescence intensity of CD16 and percent of NK cells expressing Lamp1/CD107a expression was analyzed within the CD3-CD56+ NK cell subset. The NK-CHAT CD16 down regulation index (CD16DRI) was evaluated as a ratio between CD16 MFI measured on NK cells incubated with target cells in presence of effector cell autologous CTL serum/CD16 MFI of NK cells incubated with target cells and recipient serum to be tested. CD16DRI = (MFI CD16 control serum)/(MFI CD16 test serum). Alternatively, when serum at time of LTx was available for the assay, the baseline CD16 expression was evaluated in reference to the DSA negative serum collected at time of LTx. Sera containing HLA-DQ7 DSA obtained from kidney transplant recipients (KTR) with antibody-mediated rejection (ABMR) at time of diagnosis, previously described to induce high levels of CD16-dependent NK cell alloreactivity, were introduced as positive controls of the experiments, indexing the CD16 down regulation induced by LTx sera.

Statistical Analyses

Variables used to perform univariate and multivariable analyses included preoperative donor variables (donor age, sex, CMV status, HLA typing), preoperative donor–recipient matching parameters (age, sex, CMV status, and HLA mismatch), operative variables such as ischemia time, type of procedure (single vs. bilateral LTx) and preoperative and post-operative recipient variables (initial lung disease, HLA typing, pre- and post-transplant immunization status, occurrence of AR BOS, RAS, CLAD, or infectious bacterial episodes occurring during the first year post-transplant), and notably during the early post-Tx period at months 1 (M1) and 3 (M3) after LTx (**Table 1**).

Continuous variables were presented as median values and 25–75 interquartile ranges or mean ± sd according to their distribution evaluated using the Agostino & Pearson omnibus normality test performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, California, USA). Categorical variables were presented as percentages. Fisher's exact test and the chi-square test were used to compare categorical data and *t*-tests or Mann Whitney tests were used for the comparison of continuous variables.

The primary endpoints of this study were overall survival (OS) and disease/CLAD-free survival (DFS). OS was defined as the interval between the date of transplant and the last follow-up visit or death. DFS was defined as the time interval from transplant to the first event: either the graft failure or diagnosis of acute rejection or CLAD in living recipients, or the death of the patient. The Kaplan-Meier method was used to estimate overall survival and rejection-free survival. The log-rank test was used to assess the univariate effects on OS and DFS. For all analyses, a 2-sided $p < 0.05$ was considered statistically significant. Variables with a $p < 0.2$ were also considered to construct multivariate regression models. Multivariate analyses were performed using Fine and Gray's proportional hazards regression model. All analyses were performed using IBM SPSS 15.0 software (SPSS Inc., Chicago, IL) and the cmprsk package (developed by Gray, June 2001) on R2.3.0 software (http://www.R-project.org).

RESULTS

Characteristics of the LTR Cohort

The demographic characteristics and clinical features of the 158 adult LTRs (median age: 42 years, 75 females and 83 males) are summarized in **Table 1**. Patients received a first LTx for cystic fibrosis (37%), emphysema (29%), pulmonary fibrosis (22%), or another diagnosis (12%). The median follow-up time was 38 months after LTx. Median survival time of the 94 patients who were alive at time of follow-up was 4.2 years. Sixty-four patients (40.5%) died during the study follow-up (median time before death 12.6 months, 25–75 interquartile range: 1.8–35.5 years) and 5 patients experienced CLAD-associated graft failure with indication of a second lung transplant or death. Among deceased patients, 32 patients died during the first year post-LTx, among which 12 patients died during the first month post-LTx and 9 between the first and third months post-LTx. Acute rejection diagnosis was confirmed in the lung transplant biopsy of 53 patients (36%), the first rejection episode occurring before the

TABLE 1 | Demographic and Clinical characteristics of LTRs.

Recipient, *n*	**158**
Recipient age at LTx (years), median(25–75)	42 (30–54)
Recipient Age ≥ 50, *n* (%)	54 (34)
Donor Age, median (25–75)	43 (29–54)
Male Gender, *n* (%)	83 (53)
Native Lung Disease	
Cystic Fibrosis, *n* (%)	59 (37)
Fibrosis, *n* (%)	35 (22)
Emphysema, *n* (%)	46 (29)
Other, *n* (%)	18 (12)
Transplantation type	
Bilateral LTx, *n* (%)	118 (74)
Single LTx, *n* (%)	36 (24)
Other, *n* (%)	4 (2)
CMV Risk	
CMV R- (%)	50
CMV Mismatch D+R- (%)	21
Immunization Status	
HLA Mismatch, median (25–75)	7 (6–7)
DSA M1, *n*	49
MFI DSAM1, median (25–75)	13,000 (5,500–18,750)
DSA M3, *n*	27
MFI DSA M3, median (25–75)	7,100 (3,500–13,500)
M1 DSA persisting at M3, *n*	21
C1q DSAM1, *n*	20
C1q DSAM3, *n*	7
Bacterial Infections	
M1, *n*	20
M3, *n*	11
First year, *n*	35
Transplant Outcome	
Time follow up post-LTx (months), median (25–75)	38.5 (23–62)
Rejection events	
Biopsy proven Acute rejection Day 0-M3, *n* (%)	41 (28)
Biopsy proven Acute rejection first year, *n* (%)	53 (35)
Time before Acute Rejection event (Days), median (25–75)	42 (22–179)
CLAD, *n*	40 (25)
Time before CLAD (months), median (25–75)	22 (13–32)
BOS, *n* (%)	35 (22)
RAS, *n* (%)	5 (3)
Allograft and Patients' Survival	
CLAD- or Graft failure-free Survival, *n* (%)	71 (45)
Death, *n* (%)	64 (40)
Time before Death (months), median (25–75)	13 (1.8–35)
Death or Graft failure with 2nd LTx, *n* (%)	68 (43)

Data are presented as a number and %, or median (25–75 Interquartile ranges). BOS, Bronchiolitis obliterans syndrome; RAS, restrictive allograft syndrome; CLAD, chronic lung allograft dysfunction; M1 and M3, month 1 and 3 post-LTx.

third month in 41 LTRs (occurring before the fourth month in 24 LTRs and during the third month and the twelfth month post-LTx in 11 LTRs). The occurrence of acute rejection could be associated with the presence of DSA in 49% of these patients ($n = 20$). During the study period, 40 LTRs (25%) developed CLAD, of which 35 (87%) had BOS and 5 (12%) had RAS. Seventeen of the patients with a CLAD diagnosis did not survive and 5 were considered for a second transplant procedure. CLAD-free survival of the graft was observed in 71 patients (45%) with a median disease-free survival time of 4.2 years (25–75 percentile: 3.1–5.8).

Distribution of *FCGR3A* and *FCGR2A* Polymorphisms

Genotyping of the *FCGR2A* and *FCGR3A* polymorphisms was performed in 158 LTRs and analyzed in reference to a control cohort of 184 healthy donors (CTLs) (**Figure 1**). The *FCGR2A*-H allele was detected in 75.3% of LTRs and 83.1% of CTLs ($p = 0.0734$) while the -R allele was detected in 67.7% of LTRs and in 76% of CTLs ($p = 0.0851$). Although the distribution of *FCGR2A* [131R/R] and *FCGR2A* [131H/H] was not significantly altered in LTRs, the frequency of *FCGR2A* [131H/R] was found to be significantly lower in LTRs analyzed in reference to CTLs (chi-square $p = 0.0028$). The distribution of *FCGR3A* alleles did not significantly differ between patients and controls; presence of the -F allele was detected in 81% of LTRs and in 83.7% of CTLs while the -V allele distribution was in 64.6% of LTRs and in 68.5% in CTLs. The genotype frequencies of the various genotype combinations resulting from analysis of these SNPs in the LTR and CTL cohorts are illustrated in **Figure 1**. The frequency of the *FCGR3A* [158F/F] genotype was positively correlated with the *FCGR2A* [131R/R] genotype in CTLs and in LTRs ($p = 0.008$ and $p < 0.0001$, respectively). Presence of the *FCGR2A* [131R/R] was thus significantly associated with lower rates of patients with the *FCGR3A* [158V/V] good responder genotypes in LTRs ($p = 0.038$), but this inverse correlation did not reach significance in the control cohort (**Table 2**). Analysis of the groups stratified according for FCGR SNPs did not reveal any significant difference regarding the age of the LTR and type of underlying initial lung disease. The *FCGR2A* [131H/R] SNP with was found to be associated with recipient gender ($p = 0.029$). The *FCGR2A* [131H/H] genotype was observed to be significantly more common in women LTRs (32 out of 51, $p = 0.008$).

DSA Immunization During the First 3 Months Following Lung Transplant Is Associated With FCGR Polymorphism

During the first 3 months post-transplant, 55 patients developed *de novo* circulating DSA. During the first month post-LTx (M1), DSA developed in 49 LTRs (12 HLA class I, 20 HLA class II, and 17 HLA class I and II). The median MFI of DSA detected at M1 was 7,000 for HLA Class I DSA and 12,100 for HLA Class II DSA and 12 patients (8%) exhibited high cMFI values of DSA (over the 18,750 threshold corresponding to the 75 percentile value observed for MFI analyzed at M1) (**Table 1**). Circulating DSA could be detected in 27 LTRs analyzed at month 3 (M3) post-LTx (11 HLA class I, 13 HLA class II and 3 HLA class I and II) (**Figure 2**). The development of *de novo* DSA was observed at M3 in 6 LTRs with no detectable DSA at M1 (4 HLA class I, 1

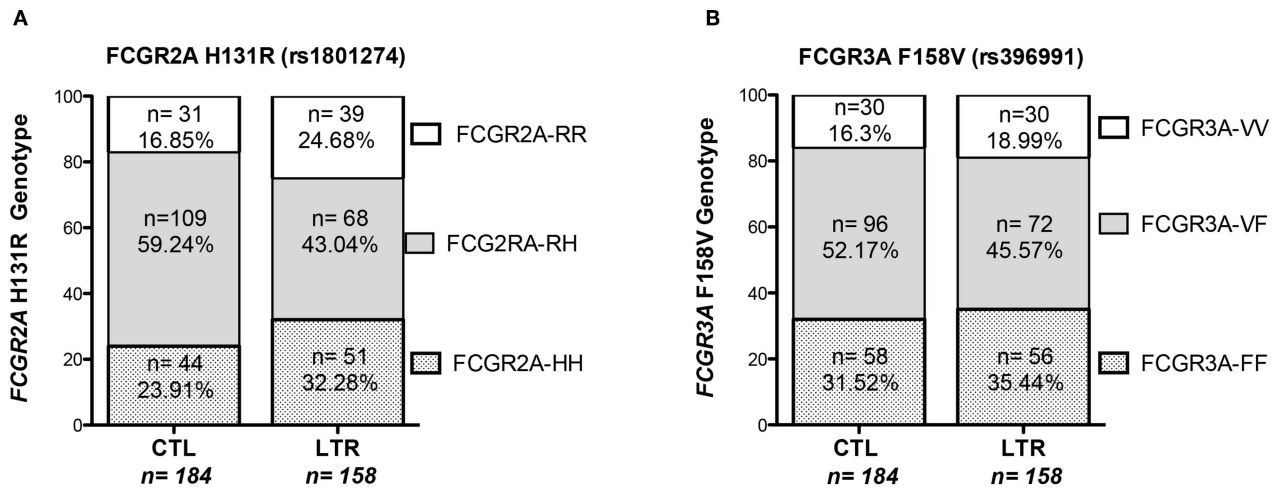

FIGURE 1 | Distribution of *FCGR3A* and *FCGR2A* genotypes in LTRs and CTLs. The proportion of the *FCGR2A* (left Panel, **A**) and *FCGR3A* (right panel, **B**) genotypes resulting from the SNP allelic combination were analyzed in a cohort of 158 LTRs and 184 CTLs recruited in southern France.

TABLE 2 | Association between the *FCGR2A* [131R/R] and FCGR3A [158V/F] genotypes observed in LTRs and CTLs.

		***FCGR2A* genotype LTR**			
		HH ***n* = 51**	**HR** ***n* = 68**	**RR** ***n* = 39**	***p*-value FCGR2A-RR vs. FCGR3A**
FCGR3A genotype	FF, $n = 56$	8	24	24	$p < 0.001$
	VF, $n = 72$	30	30	12	$p = 0.032$
	VV, $n = 30$	13	14	3	$p = 0.038$
		***FCGR2A* genotype CTL**			
		HH ***n* = 44**	**HR** ***n* = 109**	**RR** ***n*= 31**	***p*-value FCGR2A-RR vs. FCGR3A**
FCGR3A genotype	FF, $n = 58$	8	34	16	$P = 0.008$
	VF, $n = 96$	24	59	13	$p = 0.211$
	VV, $n = 30$	12	16	2	$p = 0.103$

HLA class II and 1 HLA Class I and Class II). The median MFI values of DSA detected at M1 and at M3 were, respectively, 13,000 and 7,100 (**Table 1**, **Figure 2**). DSA detected at M1 persisted at M3 in 21 LTRs (44% of DSA-positive MTR at M1) and 4 of these M3 DSA were also detectable 1 year post-transplant. HLA-DQ DSA were the only HLA class II persisting at M3. The median cMFI level of DSA detected at M1 and persisting at M3 (14,600, 25–75 percentile, 7,000–23,250) could not be linked to their persistence at M3 and did not significantly differ from the cMFI of DSA observed at M1 but undetectable at M3 (median MFI: 12,200, 25–75 percentile 4,000–16,000). Circulating DSA directed against DQ specificities were detected in 33 LTRs at M1 (median DQ-DSA MFI: 12,000 25–75 percentile 7,600–14,500) and in 14 LTRs at M3 (median 12,000, 5,000–15,850, 11 HLA-DQ DSA detected at M1 persist at M3 while 3 of the 14 HLA-DQ DSA detected at M3 were *de novo* DSA). C1q Binding DSA at M1 were detected in 20 LTRs (2 HLA class I, 15 HLA class II, and 3 HLA class I and II) with a median cMFI of C1q-binding DSA detected at M1 of 23,000 (25–75 percentile 6,300–30,000). Seven of the 27 DSA detected at M3 were found to bind C1q (1 HLA class I, 5 class II HLA DSA and 1 HLA class I and class II), with a median cMFI of 17,150 (25–75 percentile 7,000–31,000). All C1q binding DSA detected at M3 were also detected at M1.

The *FCGR2A* [131R/R] genotype was also to found to correlate with the detection of circulating DSA at M3 ($p = 0.011$) and notably to the development of *de novo* DSA between the first and second months post-transplant (4 out of the 6 *de novo* DSA detected at M3 were genotyped as *FCGR2A* [131R/R], $p = 0.017$). *FCGR3A* [158V/V] tends to be inversely associated with the persistence of DSA at M3 ($p = 0.07$, **Table 3**), as only 1 out of the 21 patients with persistent DSA at M3 was genotyped as *FCGR3A*

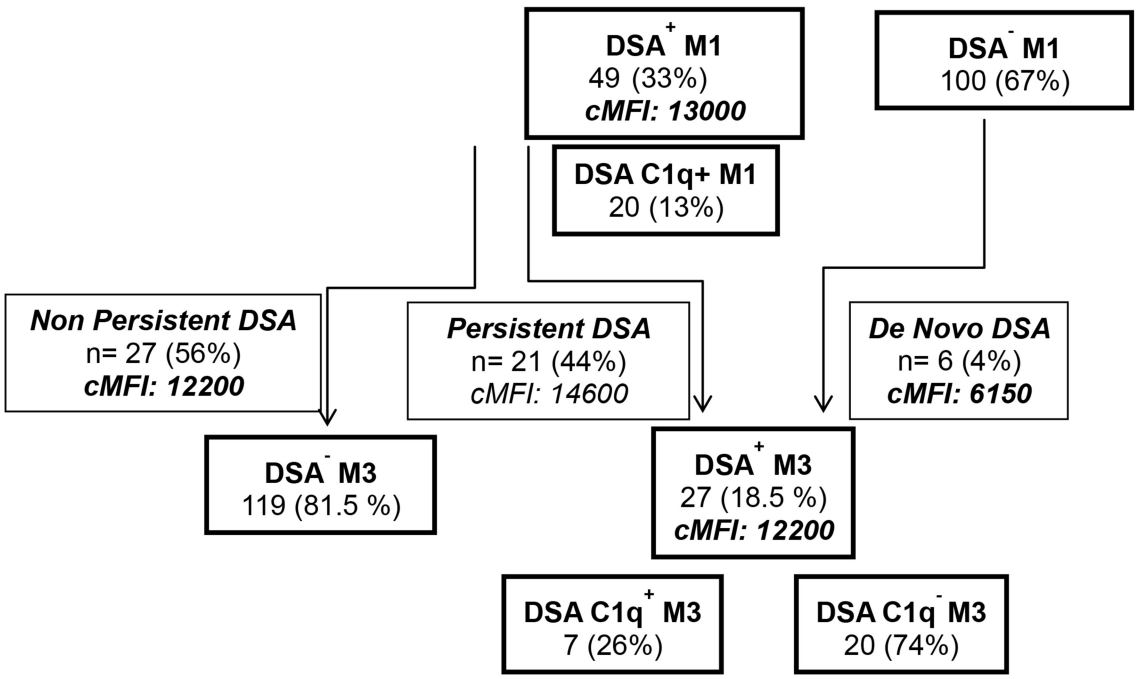

FIGURE 2 | Study flow chart analysis of the detection by single antigen flow bead Luminex Assay of *de novo* DSA and C1q binding activity of DSA at M1 and at M3 post-LTx.

TABLE 3 | Patients immunization characteristics according to the *FCGR2A* and *FCGR3A* genotypes.

	FCGR2A			FCGR3A		
	HH/RH	**RR**	***p*-value**	**FF/VF**	**VV**	***p*-value**
n = 158	119	39		128	30	
DSA M1, *n* = 49	33	16	0.161[t]	40	9	0.926, ns
Median MFI DSA M1	13,000	13,500	0.639, ns	12,500	13,800	0.477, ns
C1q DSA M1, *n* = 24	18	6	0.919, ns	19	5	0.869, ns
DSA M3, *n* = 27	15	12	0.011	26	1	0.024
DQ DSA M3, *n* = 14	7	7	0.022	13	1	0.225, ns
M1 DSA persisting at M3, *n* = 21	13	8	0.146[t]	20	1	0.07[t]
Median MFI DSA M3	6,300	9,500	0.494, ns	7,500	4,500	NA
C1q DSA M3, *n* = 7	5	2	0.840, ns	7	0	0.187[t]

Continuous variables are expressed as median (25–75 percentile ranges). p-values of the statistical tests comparing groups (Chi-2 or non-parametric Mann-Whitney test) were considered as significant when p-values were <0.05, tendency ([t]) when p > 0.05 and <0.2 and non-significant (ns) when p > 0.2 or NA when non-applicable. M1 and M3, month 1 and 3 post-LTx.

[158V/V]. The *FCGR3A*-F allele was significantly associated with the detection of circulating DSA at M3 (26 out of 27 LTRs with detectable circulating DSA at M3 were positive for the *FCGR3A*-F allele, $p = 0.024$) (**Table 3**).

FcGR3A and *FcGR2A* Polymorphisms Are Differentially Associated With Acute and Chronic Lung Allograft Rejection

The *FCGR3A* [158V/V] was also identified as a susceptibility genotype associated with a higher risk of acute rejection (log rank test for equality of survivor functions $p = 0.0009$, **Figure 3A**). Multivariate Cox regression analysis also revealed that the susceptibility conferred by the *FCGR3A* [158V/V] genotype (OR 4.8, $p < 0.0001$, 95% CI 2.375–9.607) was independent from the development of DSA at M3 and their C1q binding activity. *FCGR2A* [131R/H] was not observed to affect the rate of acute rejection. The *FCGR2A* [131R/R] genotype was instead associated with the detection of DSA occurring in the absence of acute rejection ($p = 0.029$).

The DSA immunization status of the patient evaluated during the first year post- LTx could not be associated with CLAD. Although the number of observations was small, 4 of the 5 LTRs

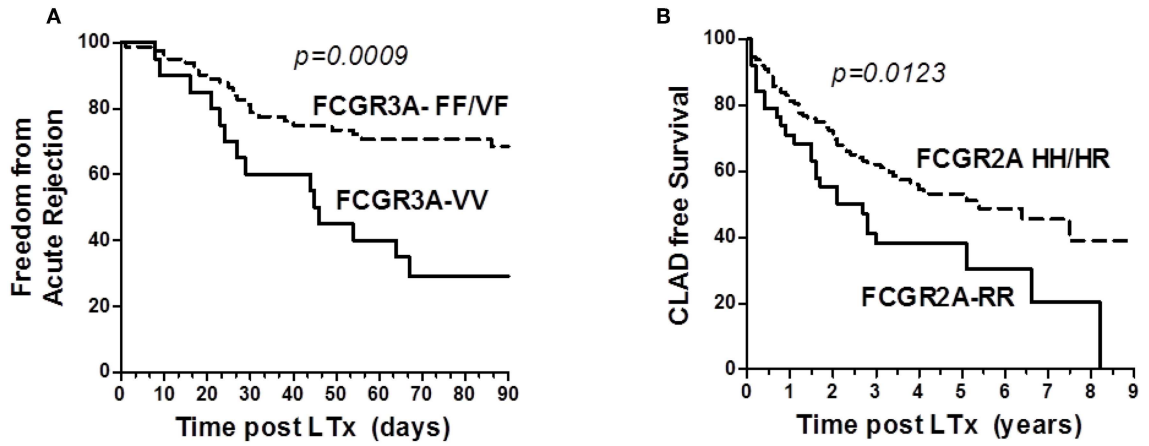

FIGURE 3 | Analysis of the influence of Fc-gamma Receptor polymorphisms on Rejection Free survival. **(A)** Impact of *FCGR3A* genotype on freedom from acute lung rejection. Kaplan–Meir Survival analysis links the *FCGR3A* [158V/V] (solid line, $n = 20$) to lower acute-rejection-free survival after 3 months post-LTx in LTRs, when compared with the *FCGR3A* [158V/F] and *FCGR3A* [158F/F] group (dashed line, $n = 81$). LTRs with the *FCGR3A* [158V/V] show an increased rate of acute rejection events occurring during the first 3 months post-LTx. **(B)** Impact of *FCGR2A* genotype on CLAD free survival. Kaplan–Meir Survival analysis links the *FCGR2A* [131R/R] (solid line, $n = 39$) to a lower CLAD-free survival rate in LTRs over time or study follow-up (years, x axis), when compared with the *FCGR2A* [131H/H] and *FCGR2A* [131R/H] group (dashed line, $n = 119$).

who developed RAS-associated CLAD were homozygous for the *FCGR3A*–FF allele ($p = 0.034$). While the *FCGR3A* [158V/F] could not be significantly associated with the risk of CLAD, *FCGR2A* [131R/R] was identified as a susceptibility marker associated with lower CLAD-free survival of LTRs ($n = 87$, logrank test for equality of survivor functions $p = 0.0123$, **Figure 3B**). Multivariate Cox regression models adjusting co-variables identified using univariate analysis further identified *FCGR2A* [131R/R] as an independent susceptibility marker associated with CLAD (OR 2.2; $p = 0.022$, 95CI 1.126–4.435) (**Table 4**). Multivariate Cox analysis further showed that the *FCGR2A* [131R/R] genotype is significantly associated with the enhanced risk of developing a composite LTx adverse outcomes (CLAD, graft failure or death), independently of other factors such as detection of DSA at M3 or native emphysema lung disease (**Table 4**).

LTx DSAs Have No Major Impact on CD16-Dependent NK Cell Cytotoxic Activation

Considering our previous finding that identifies the potential value of *FCGR3A* [158V/F] in predicting cardiac allograft vasculopathy (48), we further investigated whether the presence of association of acute rejection events with high affinity *FCGR3A* [158V/V] genotype could be associated with the DSA-mediated pathogenic effects that promote acute rejection mechanisms of lung allograft. Using the NK-cellular humoral activation test (NK-CHAT), previously designed to index the level of DSA-mediated engagement of CD16 and unravel potential ADCC-driven pathogenic effects of circulating DSA (41), we therefore aimed to investigate whether circulating DSA detected at M1 or M3 in LTRs have the potential to stimulate NK cell alloreactivity. Evaluation of the DSA-induced down regulation of NK cell CD16 expression (CD16 Down Regulation Index or CD16DRI) was performed in a standardized NK-CHAT assay analyzing alloreactivity of NK cells toward serum-coated $CD20^+$ B lymphocytes expressing the cognate HLA target alloantigen, using 23 serum samples obtained from 20 distinct LTRs (**Table 5**). Thirteen sera were collected at M1 and ten at M3. Four sera had detectable DSA that persisted at M3. Seven DSA at M1 and Four DSA at M3 were C1q positive.

Surprisingly, the CD16DRI values induced by the DSA^+ LTx sera were very low when compared to those obtained in response to Rituximab. These CD16 DRI values could not be associated with the intensity of DSA MFI and C1q binding activity of DSA. Furthermore, the CD16DRI of LTRs (LTR 17, 18, and 20) with HLA DQ7 DSA were lower than those obtained of ABMR kidney transplant recipients (KTR) with HLA DQ7 DSA and with similar MFI (**Figure 4**).

In contrast to the results previously observed in the kidney and heart transplant setting, the association of the *FCGR3A* [158V/V] genotype with acute rejection appears to be independent of the presence of circulating DSA and the DSA MFI levels. In contrast to the KTR serum, NK-CHAT evaluation of the serum of immunized LTRs did not reveal significant alloreactive DSA toxicity and did not allow for determination of their pathogenic potential in eliciting CD16 and NK-cell cytotoxic activation.

FCGR2A Polymorphism Impacts Patients and Allograft Survival

Occurrence of acute and chronic events or infectious episodes was not shown to have a major impact on overall survival in the analyzed cohort.

Early development of DSA that persist at M3 ($n = 21$) were associated with lower graft or patient survival rates ($p = 0.002$) and were associated with the presence of the low-affinity F allelic

TABLE 4 | Univariate analysis and multivariate Cox regression analysis of risk factors associated with acute or chronic rejection events.

Risk covariables	Univariate analysis *p-value*	Hazard ratio	Std. Err.	z	*P* > z	(95% Conf.interval)		
Acute Rejection in the first 3 months post-LTx, *n*= 41								
FCGR3A [158 V/V]	0.003	4.8	1.70	4.39	**<0.0001**	**2.375**	–	**9.607**
Native Lung Disease Emphysema	0.037	0.4	0.18	−2.05	**0.040**	**0.173**	–	**0.961**
M3 C1q Binding DSA	0.009	4.4	2.87	2.28	**0.023**	**1.231**	–	**15.789**
Single lung Tx	0.091	2.6	0.96	2.56	**0.010**	**1.251**	–	**5.352**
FCGR3A [158 V/F]	0.003	4.4	1.54	4.29	**<0.0001**	**2.247**	–	**8.761**
Native Lung Disease Emphysema	0.037	0.4	0.17	−2.17	**0.03**	**0.161**	–	**0.909**
M3 DSA^{+}	Ns ($p = 0.251$)	3.1	1.27	2.82	**0.005**	**1.417**	–	**6.917**
Single lung Tx	0.091	2.9	1.12	2.89	**0.004**	**1.419**	–	**6.243**
CLAD, *n= 40*								
FCGR2A [131R/R]	0.185	2.23	0.781	2.3	**0.022**	1.126	–	4.435
Native Lung Disease Emphysema	0.177	2.65	0.900	2.86	**0.004**	1.360	–	5.157
Recipient Gender: Female	0.142	1.62	0.531	1.47	0.141	0.852	–	3.080
Acute Rejection 1st Year	0.050	1.93	0.621	2.03	**0.042**	**1.024**	–	**3.625**
Composite adverse outcome: CLAD, graft loss or death *n* = 87								
FCGR2A [131R/R]	0.185^{t}	1.8	0.475	2.3	**0.024**	**1.080**	–	**3.028**
Native Lung Disease Emphysema	0.177	2.1	0.514	2.95	**0.003**	**1.279**	–	**3.439**
M3 DSA	0.075	1.9	0.557	2.44	**0.015**	**1.145**	–	**3.080**

Covariables (risk covariables) used to explain the Lung transplant outcome primary variable (Acute Rejection, CLAD or adverse composite outcome that includes CLAD or Graft loss or death) are listed on the left column. The bold text refers to covariables that retained independent significant p-values in multivariate cox regression models.

variant of CD16. Persistence of DSA at M3 was thus lower in *FCGR3A* [158V/V] LTRs ($p = 0.029$). Other parameters such as high MFI DSA detected at M1 (>18,750 MFI, 75 percentile value of DSA MFI at M1 post-LTx, $p = 0.109$) or FCRGR2A [131R/R] ($p = 0.052$) tended to be associated with the risk of death. While the FCGR3A genotype could not be associated to overall survival, the FCRGR2A [131R/R] genotype was identified as a risk factor associated with the composite adverse outcome combining graft failure or patient death ($n = 68$, log rank test for equality of survivor functions $p = 0.0417$, **Figure 5**). Multivariate analysis of variables associated with death from all causes after LTx ($n = 64$, 40.5% of the LTR cohort) retained initial diseases other than cystic fibrosis ($p = 0.048$) DSA with MFI values > to the 75 percentile 18,750 MFI value for DSA detected at M1 as significant risk factors (**Table 6**). The FCRGR2A [131R/R] genotype was shown to be an independent factor associated with lower overall survival of LTRs using multivariate logistic Cox regression models (HR: 1.8, $p = 0.047$ 95% CI: 1.008–3.121, **Table 6**).

DISCUSSION

FcγR constitute a major marker of immune activation in response to infections, antibodies and CRP inflammatory ligands. Recent studies suggest that the engagement of FcR by the Fc fragment of alloantibodies can influence the cytotoxic activation level of NK cells toward the heart or kidney allograft. This study aimed to investigate whether the combined evaluation of *FCGR3A* [158F/V] and *FCGR2A* [131H/R] SNPs and biomarkers that index the level of CD16-dependent ADCC of NK cells might also be relevant to stratify patients at higher risk of lung allograft failure.

Our results show evidence that the *FCGR3A* [158V/V] genotype can be identified as a genetic marker that predisposes LTRs to acute rejection, independently of their DSA immunization status. Fifteen out of thirty patients with the *FCGR3A* [158V/V] genotype indeed developed acute rejection during the first trimester. Since this profile has been associated with higher NK cell responsiveness, we further investigated how it may relate to the NK cell mediated cytotoxic effects of DSA toward the lung allograft. Various studies have shown that the detection of circulating DSA with an ability to ligate the C1q component and activate the complement cascade can be associated with a greater risk of acute rejection and allograft loss after heart or kidney transplant. These complement-dependent mechanisms of DSA-mediated cytotoxic effects have also been recently documented in the lung transplant setting (19, 20). As previously reported (29), all C1q DSA detected at M3 were directed toward donor HLA-DQ alloantigens and C1q positive were shown to be significantly associated with acute rejection in the study cohort. A limitation of our study is the lack of elements allowing the distinction of cellular acute rejection (ACR) from ABMR. Endothelial deposition of C4d and microvascular inflammation were shown to be reliable markers of ABMR in renal and cardiac allografts, but the clinical relevance of C4d staining within lung allograft biopsies still remains controversial for lungs. These diagnostic criteria for ABMR were not always available on a routine basis at the time of evaluation until the Banff nomenclature released more accurate histologic features that characterize lung allograft grafts biopsies of DSA-positive LTRs.

TABLE 5 | NK-CHAT: Evaluation of DSA reactivity toward B cell targets expressed in sera collected from LTR and KTR, used as positive controls for NK-CHAT.

LTR	Serum Time post-LTx	DSA HLA class I/II	MFI DSA	MFI C1q DSA	Serum induced CD107 up regulation (CD107URI)	Serum induced CD16 down regulation index (CD16DRI)	CLAD	AR	Death
LTR 1	M1	Class I	A3:8,500 B7:8,500	A3:14,000 B7: > 15,000	1	1	na	1	1
LTR 2	M1	Class I	A2:7,000 B44:3,000	A2: 3,000 B44: 10,000	1	1	1	1	0
LTR 3	M1	Class I	A2: 3,200	Negative	1	1	1	1	0
LTR 4	M1	Class I	A3: 2,300	Negative	1	1	0	0	0
LTR 5	M3	Class I	B7: 3,000	Negative	1.6	1	0	1	0
LTR 6	M1	Class I and II	A2: 3,500 DQ7: 13,000	DQ7: 10,000	0.4	1	0	0	0
	M3	Class II	DQ7: 9,000	Negative	0.5	1	0	0	0
LTR 7	M1	Class II	DQ2:10,000 DR53:3,000	Negative	3.3	1.2	na	0	1
LTR 8	M1	Class II	DQ5: 9,500	Negative	1.6	1	1	1	1
LTR 9	M1	Class II	DQ6: 10,000	Negative	1.4	1.2	0	0	0
LTR 10	M1	Class II	DQ7 > 15,000	DQ7 > 15,000	0.9	1.3	0	0	0
LTR 11	M1	Class II	DQ7:11,000 DQ9:4,000	DQ7:11,500 DQ9:11,500	0.9	1	1	0	1
LTR 12	M1	Class II	DQ7: 13,000	Negative	1.4	1.1	0	0	0
LTR 13	M3	Class II	DQ5: 14,000	DQ5: 9,000	0.9	1.4	0	0	0
LTR 14	M3	Class II	DQ5: 13,000	Negative	<1	<1	na	0	**1**
LTR 15	M3	Class II	DQ5 4,500	Negative	<1	<1	0	0	0
LTR 16	M3	Class II	DR53: 3,000	Negative	1	1	0	0	0
LTR 17	M3		DQ7: 13,400	DQ7 11,000	1	1			
LTR 18	M3	Class II	DQ7: 16,000	DQ7, 14,000	3	2.1	na	0	1
LTR 19	M1	Class II	DQ5:7,500 DQ6:6,000	DQ5: 7,000 DQ6: 6,500	1.2	<1	0	1	0
	M3	Class II	DQ5: 3,500	Negative	<1	<1			
LTR 20	M1	Class II	DQ7: 9,000	DQ7: 6,000	1.5	1.9	0	0	0
	M3	Class II	DQ7: 12,500	DQ7: 9,000	1.4	1.9			
KTR 1 ABMR	At time of ABMR	Class II	DQ7: 9,000	nt	6.5	29	na	na	na
KTR 2 ABMR	diagnoss	Class II	DQ7: 13,000	nt	7	42	na	na	na

AR, acute rejection; na, not applicable; nt, non-tested.

However, the association between the *FCGR3A* [158V/V] genotype and presence of circulating DSA at M1 or at M3 did not appear to confer a higher risk of acute rejection, whatever their MFI and their C1q binding activity status, thus suggesting that the graft damage associated with the *FCGR3A* [158V/V] genotype is independent from DSA immunization status. This finding was unexpected, as we have previously shown that the intensity of antibody-dependent CD16 activation of NK cells, indexed by the non-invasive NK-CHAT, is significantly enhanced in recipients that bear the *FCGR3A* [158V/V] genotype and constitute a relevant cytotoxic mechanism sustaining toxic effects of DSA and allograft vasculopathy (48, 50). In contrast, NK-CHAT evaluation of DSA from LTRs actually shows evidence that the engagement of CD16 by LTx DSA is low when analyzed in reference to anti-HLA class II DSA that are found in the serum of kidney transplant recipients at time of ABMR diagnosis and that exhibit comparable HLA-DQ7 specificities and MFI values. This low potential of LTx DSA to induce CD16-mediated NK cell activation appears to be independent of their C1q binding activity and could not be associated with adverse outcome of LTx in the present study. As CD16 exhibits higher affinity for IgG1 and IgG3 alloantibodies, this failure of DSA to engage CD16 mediated cytotoxic functions may be due to the IgG2 and/or IgG4 isotypes of DSA, which have been shown to exhibit lower affinity for CD16. This finding may also indicate that the glycosylation pattern of IgG1 DSA interferes with the FcγRs-mediated recognition of the Fc fragment of IgG by immune cells or complement factors. These hypotheses are supported by the observation that only 30% of circulating DSA detected at M1 and at M3 were found to bind C1q in this study cohort and by previous findings that report high IgG4 levels in patients with cystic fibrosis lung disease (59). These data suggest that, in contrast to previous findings, the *FCGR3A* [158V/V] susceptibility genotype does not appear as a major mechanism of

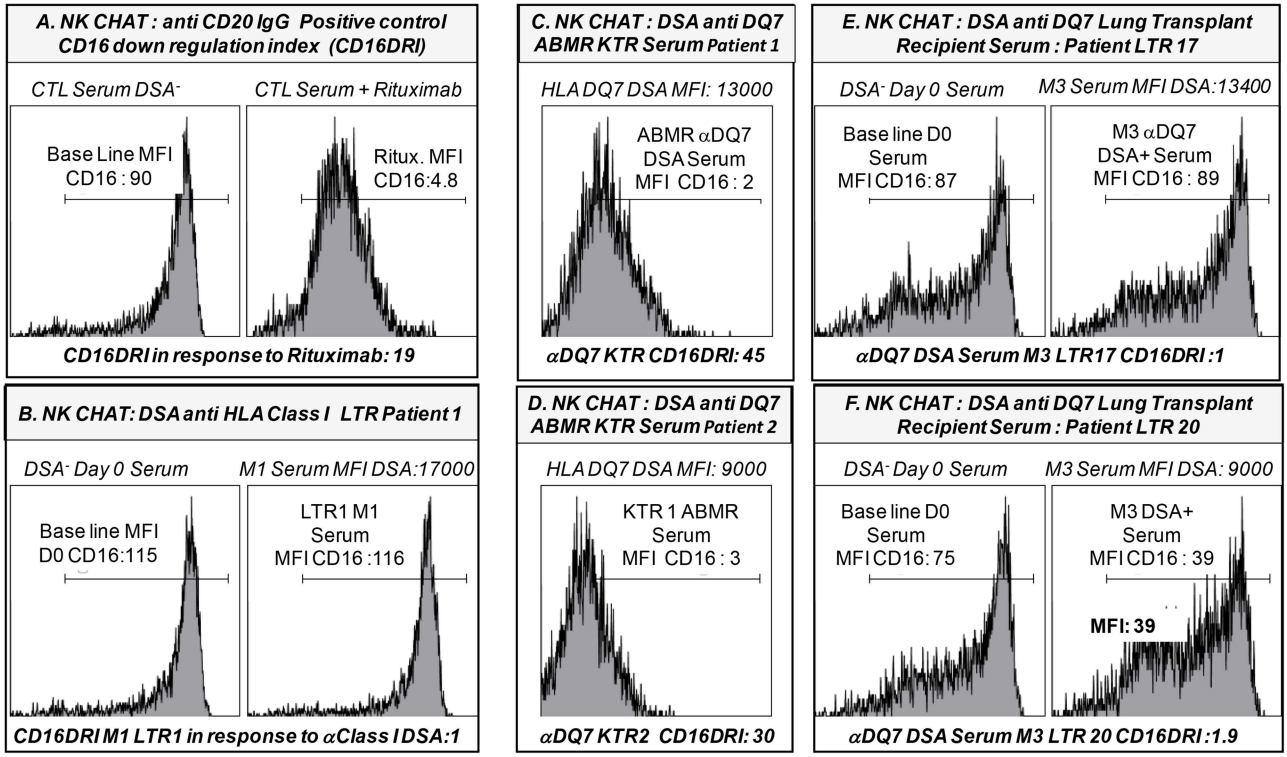

FIGURE 4 | Representative illustration of the NK-Cellular Humoral test (NK-CHAT) used to evaluate serum- and DSA- induced NK cell activation toward B cell lines expressing cognate HLA class I or HLA-DQ7 donor-specific allo-antigens. **(A)** PBMC effectors obtained from *FCGR3A* [158 V/V] CTL were exposed to B-EBV cell lines previously coated with DSA-negative serum. This allowed for evaluation of the baseline expression level of CD16 expression (CD16MFI) within the CD3-CD56+CD16+ NK cell compartment when PBMC were exposed to B cell targets. As a positive control indexing the level of IgG-induced CD16 down regulation (CD16DRI), the same B-EBV cell lines were coated in the presence of 10γ/ml Rituximab (anti CD20 IgG) prior to exposure to the effector PBMC. This allowed for calculation of the CD16 down regulation index (CD16DRI: baseline CD16MFI NK cells exposed to B cells coated with CTL DSA-negative serum/CD16 MFI of NK cells exposed to the same B targets pre-coated in presence of Rituximab). In this context, CD16DRI in response to Rituximab = 19, i.e., 90/4.8. **(B)** The CD16 DRI of NK cells was evaluated in response to the serum collected at M1 in one LTR (LTR 1, **Table 5**) with detectable levels of DSA recognizing the HLA-A3 and -B7 expressed on the B cell targets. Non-immunized serum (DSA-negative) collected from LTR 1 at time of LTx (D0) was used as the reference baseline CD16 MFI value to calculate the CD16DRI. Despite a cumulative MFI of DSA >17,000, DSA detected at M1 in LTR1 failed to induce CD16-dependent NK cell alloreactivity (CD16DRI = 1). **(C)** The CD16 DRI of NK cells was evaluated in response to the serum collected at time of ABMR diagnosis in one kidney transplant recipients (KTR 1, **Table 5**) with detectable levels of DSA recognizing the HLA-DQ7 (MFI: 13,000) expressed on the B cell targets. The CD16DRI of KTR1 (CD16DRI: 45) was greater than that observed in response to the Rituximab (CD16DRI:19, **A**). **(D)** The CD16 DRI of NK cells was evaluated in response to the serum collected at time of ABMR diagnosis in a second KTR (KTR 2, **Table 5**) with detectable levels of DSA recognizing the HLA-DQ7 (MFI: 9,000) expressed on the B cell targets. The CD16DRI of KTR1 (CD16DRI: 30) was greater than that observed in response to the Rituximab (CD16DRI:19, **A**). **(E)** The CD16 DRI of NK cells was evaluated in response to the serum collected at M3 (time of acute rejection) in a second LTR (LTR 17, **Table 5**) with detectable levels of DSA recognizing the HLA- DQ7 (MFI: 13,400) expressed on the B cell targets. Non-immunized serum (DSA-negative) collected from LTR 17 at D0 was used as the reference baseline CD16 MFI value to calculate the CD16DRI (CD16DRI = 1). The MFI DSA of LTR 17 (MFI: 13,400) was similar to the MFI DSA of ABMR KTR 1 serum as illustrated in **(C)**. **(F)** The CD16 DRI of NK cells was evaluated in response to the serum collected at M3 (time of acute rejection) in a third LTR (LTR 20, **Table 5**) with detectable levels of DSA recognizing the HLA- DQ7 (MFI DSA: 9,000) expressed on the B cell targets. The DSA-negative serum of LTR 20 collected at D0 was used as a baseline CD16 MFI value to calculate the CD16DRI (CD16DRI = 1.9). The MFI DSA of LTR 20 (MFI: 9,000) was similar to the MFI DSA of ABMR KTR 2 serum evaluated in **(D)**.

DSA-mediated lung allograft injury. CD16-dependent activation of NK cells in *FCGR3A* [158V/V] individuals that develop acute rejection may nevertheless be mediated by allo- or auto-antibodies that target non-HLA antigens and may not be revealed in the standardized NK-CHAT assay revealing CD16 cellular activation toward B lymphocyte cell targets. Several reports have indeed described ABMR lesions of the graft that occur in the absence of detectable levels of circulating DSA in the serum of kidney or heart transplant recipients. Antibodies directed against endothelial antigens or stress-induced antigens, such as vimentin, collagen V, Kα1 tubulin, AT1R, and MICA have also been reported in transplant recipients but the role of these non-HLA antibodies in the destruction and accelerated dysfunction of lung allograft remains poorly addressed (60). These reports have nevertheless raised interest in chronic injury resulting from humoral responses targeting non-HLA antigens, as these may be underestimated by the standard monitoring of patients' immunization status which is mainly restricted to the detection of anti-HLA alloantibodies (52). While the rate of acute rejection during the first year was previously identified as a risk factor

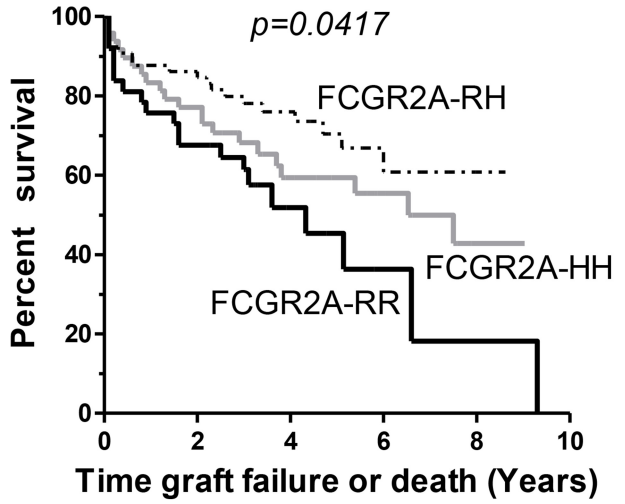

FIGURE 5 | Kaplan-Meir survival analysis of lung allograft survival stratified according to *FCGR2A* [131R/H] genotypes. The *FCGR2A* [131R/R] homozygous genotype (solid black line, $n = 39$) is associated with lower survival rates in LTRs when compared to *FCGR2A* [131H/R] (gray line, $n = 68$) or the *FCGR2A* [131H/H] (dashed line, $n = 51$).

for CLAD occurrence, occurrence of acute rejection in the first trimester could not be associated with an enhanced risk of developing chronic rejection nor with patient or graft survival in the present cohort. The F allelic variant of CD16 with low affinity for the Fc fragment of IgG was associated with the early development during the first month post-LTx of DSA that persist at M3. Persistence of DSA at M3 was thus less frequent in *FCGR3A* [158V/V] LTRs and was shown to be associated with lower survival times in LTR.

Our observations also identify a link between the presence of circulating DSA at M3 and the *FCGR2A* [131R/R] genotype, thus suggesting that FCGR2 polymorphisms may actually be associated with the persistence of harmful DSA at M3 rather than in the development of anti-HLA antibodies *per se*. The *FCGR2A* [131R/R] genotype was reported to be associated with shorter allograft survival in immunized kidney transplant recipients (KTR) (61, 62). We find that, independently of the risk associated with the *FCGR2A* [131R/R] genotype, detection and persistence of DSA at M3 constitute independent predictors of the adverse clinical composite outcome comprising CLAD or the patient's death. The persistence of DSA has been reported as a risk factor linked to BOS and to LTR death (16). A recent report showed that a majority of patients who were positive for *de novo* DSA during the first year after LTx developed BOS and were at higher risk of graft failure or death (63). In line with these reports, our study suggests that, independently of the presence of the susceptible *FCGR2A* [131R/R] genotype, the presence at M1 of DSA with high affinity for donor antigens (MFI DSA M1 > 18,750) can be identified as a risk factor associated with lower survival of LTRs.

The role of the complement-dependent pathogenicity of DSA is less well-documented in the lung transplant setting (19, 20). In a murine model of BOS, complement activation by antibodies to HLA class I was not required for the development of obliterative airway disease (OAD) that is similar to BOS in human LTx. Interestingly, in this study, at 90 days after LTx, only one out of 5 BOS patients had C1q DSA detected by SAFB whereas 3 out of 11 stable patients had C1q DSA. However, in our study, C1q DSA detected at M1 and M3 were mostly directed against HLA class II antigenic targets (90%) and do not appear as major contributors associated with the development of CLAD.

Although this is a limitation of the study, we cannot exclude the idea that auto-antibodies that target non-HLA antigens which were associated with the development of DSA, such as autoantibodies against K-α1 tubulin (K-α1T) and collagen V (ColV) (64), can participate in the chronic FcR-dependent reaction of recipient cells toward the lung allograft. This is supported by studies that report that development of col (V)-specific TH-17 cells may contribute to the pathogenesis of BOS (25, 59). Indeed, our standardized NK-CHAT evaluation of the DSA induced NK-cell activation was conducted toward B cell lines that express cognate HLA alloantigens. As B cells may not be relevant to evaluate the deleterious impact of non-HLA alloantibodies toward the lung allograft, this is a limit that prompts further studies that uses serum-coated lung epithelial or endothelial cells targets to evaluate NK cell ADCC.

FCGR2A [131R/H] is considered to be a heritable risk factor for a variety of infectious and inflammatory autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, malaria, multiple sclerosis, and anti-neutrophil cytoplasmic auto-antibody positive systemic vasculitis (65). The CD32 membrane receptor is expressed by a variety of immune cells that orchestrate the humoral immune response to pathogens, including B-lymphocytes, natural killer cells, macrophages, mast cells, and neutrophils. Its capacity to recognize IgGs bound to pathogens or infected cells has a protective effect against infections. While the *FCGR2A* allelic isoforms exhibit similar affinity for IgG1, the *FCGR2A* [131H/H] is the only FcγR variant that recognizes IgG2 subclasses, thus suggesting that the capacity to sense IgG2 antibodies may lead to impairment of pathogen surveillance in patients that lack the H allele. This SNP has been shown to play a role in the susceptibility to bacterial infections as *FCGR2A* [131H/H] individuals have greater potential to mediate IgG2-dependent bacterial phagocytosis than patients genotyped as *FCGR2A* [131R/R]. A distinct SNP (rs12746613) within the *FCGR2A* gene was previously associated with a higher risk of respiratory infections and mortality after LTx, but this variant was not associated with the risk of developing CLAD (34). Unlike other studies, our analysis of the present LTR cohort did not reveal any significant association between *FCGR2A* [131R/H] and occurrence of respiratory infections or the number of infection-related deaths. This discrepancy may relate to preventive and curative treatment of bacterial infections that differ between the transplantation centers. It may also reflect a complex interaction of these FCGR genotypes that encourages the overall survival of LTR for patients that have better capacities to thwart infections and overcome early acute rejection events. The observed finding of a inverse link between the *FCGR2A* [131R/R] susceptibility genotype and presence of the FCGR3A-V allele encoding the

TABLE 6 | Univariate analysis and multivariate Cox regression analysis of variables associated with graft loss and patient death post-LTx.

Covariables	Univariate analysis *p-value*	Hazard ratio	Std. Err.	z	P>z	(95% Conf.interval)		
Death, All causes, *n* = 64								
FCGR2A [131R/R]	0.114	**1.8**	**0.511**	**1.99**	**0.047**	**1.008**	**–**	**3.121**
Native Lung Disease: Cystic Fibrosis	**0.048**	**0.5**	**0.53**	**−2.25**	**0.024**	**0.278**	**–**	**0.915**
MFI DSA M1 > 18,750 (75 percentile)	0.109	**2.7**	**1.123**	**2.36**	**0.018**	**1.184**	**–**	**6.093**
Graft loss or LTR death, *n* = 68								
FCGR2A [131R/R]	**0.052**	**1.85**	**0.552**	**1.99**	**0.047**	**1.008**	**–**	**3.392**
MFI DSA M1 > 18,750 (75 percentile)	0.166	**2.8**	**1.259**	**2.29**	**0.022**	**1.159**	**–**	**6.759**
RAS	**0.009**	**2.8**	**1.385**	**2.08**	**0.037**	**1.062**	**–**	**7.384**
Native Lung Disease: Cystic Fibrosis	**0.034**	0.53	0.181	−1.86	0.063	0.272	–	1.034
DSA at M1 persisting at M3	0.153	1.37	0.550	0.78	0.436	0.622	–	3.009
Bilateral Lung Transplant	0.161	0.92	0.319	−0.24	0.814	0.467	–	1.818

Covariables (risk covariables) used to explain the Lung transplant outcome primary variable (Death all cause or Graft loss/death) are listed on the left column. The bold text refers to covariables that retained independent significant p-values in multivariate cox regression models.

CD16 receptor variant with higher affinity for the IgG Fc fragment in LTRs, may in part explain a lack of association of the FcCR3A-VV genotype with DSA- mediated chronic lung allograft dysfunction. In this study, the presence of the *FCGR2A* [131H/H] was observed to be strongly associated with the presence of the "high IgG1 responder" *FCGR3A* [158V/V] genotype, and such linkage disequilibrium may in part explain how this intricate distribution of susceptible and protective FCR SNPs may participate in the complex tuning of the host immune response to early infectious and humoral challenges and may be associated with enhanced survival and lower rates of CLAD in patients who have the protective *FCGR2A* [131H/R or H/H] genotype, notably in female LTRs.

In addition to this protective role against pathogens, the SNP dependent affinity of CD32 for the Fc fragment of IgG and/or CRP ligands was also identified as promoting inflammation. CD32-dependent triggering of immune cells is in part conditioned by the polymorphism and the expression profile of this functional receptor at the surface of immune cells. As is expressed by most leukocytes/macrophages that infiltrate the lung graft, *FCGR2A* [131R/H] could also influence the acquisition of an inflammatory-activated profile that favors tissue recruitment of activated lymphocytes to the lung (66). Interestingly, the *FCGR2A* [131R/R] susceptible genotype identified in this study has been associated with higher CRP binding avidity for the CD32 receptor expressed at the surface of monocytes and neutrophils.

Considering the growing evidence of the key role of CRP as an inflammatory mediator involved in the development of atherosclerosis and endothelial dysfunction, it is expected that CRP may be more powerful in triggering the pro-inflammatory function of CD32-expressing cell subsets such as platelets, endothelial cells, monocytes, and leukocytes in *FCGR2A* [131R/R] LTRs.

In conclusion, these data highlight that *FCGR2A* and *FCGR3A* polymorphisms constitute predisposing factors that are associated with the outcome of lung allografts. This study suggests that the combined assessment of the FCGR genotype and CRP or IgG ligands is thus an intriguing prospect to further decipher the complex mechanisms that shape the alloimmune and inflammatory responses in response to infectious and humoral threats. As shown in other organ transplant settings, our study indicates FCGR genotyping may favor early stratification of patients at risk and may create new perspectives to adapt personalized preventive and therapeutic approaches to prevent adverse outcomes of lung transplants.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All patients from the French cohort (COLT, Cohort in Lung Transplantation, l'Institut du Thorax, INSERM UMR1087/CNRS UMR 6291, CNIL 911142) were recruited in this study and gave their written informed consent to participate to the study in accordance with the Declaration of Helsinki. A group of 184 healthy unrelated of volunteer French bone marrow donors were also recruited to constitute a control cohort allowing analysis of FcγR genotype. Blood donations were collected in the Etablissement Francais du Sang, in accordance with BSL-2 practices. A medical interview was carried out prior to blood donation to exclude donors with medical contraindications. This study was carried out in accordance with the French Public Health Code (art L1221-1), approved by institutional ethics committee and conducted in compliance with the Good Clinical Practice Guidelines, declaration of Helsinki and Istanbul.

AUTHOR CONTRIBUTIONS

CP and PPa designed and coordinated the study, analyzed the data, and wrote the paper. PPe, LL, and JD performed experiments. AL contributed to the methodological and statistical analysis. MP, AB, FD-G, and JC contributed to the research design. PT and MR-G contributed to the collection of patient material and to the clinical aspects of the study.

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REVIEW
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Influenza and Antibody-Dependent Cellular Cytotoxicity

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Despite the availability of yearly vaccinations, influenza continues to cause seasonal, and pandemic rises in illness and death. An error prone replication mechanism results in antigenic drift and viral escape from immune pressure, and recombination results in antigenic shift that can rapidly move through populations that lack immunity to newly emergent strains. The development of a "universal" vaccine is a high priority and many strategies have been proposed, but our current understanding of influenza immunity is incomplete making the development of better influenza vaccines challenging. Influenza immunity has traditionally been measured by neutralization of virions and hemagglutination inhibition, but in recent years there has been a growing appreciation of other responses that can contribute to protection such as antibody-dependent cellular cytotoxicity (ADCC) that can kill influenza-infected cells. ADCC has been shown to provide cross-strain protection and to assist in viral clearance, making it an attractive target for "universal" vaccine designs. Here we provide a brief overview of the current state of influenza research that leverages "the other end of the antibody."

Keywords: ADCC—antibody dependent cellular cytotoxicity, influenza, antibodies, animal models, vaccine targets, mAb

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INTRODUCTION

Influenza causes 3–5 million cases of severe illness and 290,000–650,000 deaths annually (1). Influenza is caused by orthomyxoviruses that have a segmented, negative-strand RNA genome that encodes its own RNA-dependent RNA polymerase that results in ∼1 error per replicated genome (2–4). Accumulating errors cause small changes over time that allow viral escape (5, 6), a process called antigenic drift. Reassorting of the segmented influenza genome can produce novel influenza strains to which there is no preexisting immunity in the human population, a process called antigenic shift, and it is thought this mechanism produced the 1918, 1957, and 1968 pandemics (7).

The primary means of combatting both seasonal and pandemic influenza are quarantine and isolation (8), strict hygiene (9), and vaccination (10–12). Influenza vaccines were developed starting in the 1940s (13–16), and seasonal vaccines now include multiple antigens either as inactivated or live-attenuated products (17–20). Selection of representative influenza strains requires the ongoing worldwide analysis of circulating influenza (21), leading to potential mismatch with low vaccine efficacy (22). Public health leaders have called for the development of "universal" influenza vaccines (23), but at this time it is not clear what kinds of immunity such a vaccine should elicit.

Vaccines can prevent symptomatic disease, reduce disease duration, and reduce viral shedding, and infectivity to other persons. To accomplish these, a vaccine can elicit responses that inhibit

influenza virions and/or enhance the clearance of influenza-infected cells. The influenza virion has three virally-encoded surface antigens (**Figure 1A**): a trimeric glycoprotein hemagglutinin (HA) that binds to sialic acid on cell surface receptors promoting virion endocytosis followed by fusion of viral and host cell membranes, a tetrameric neuraminidase (NA) that cleaves sialic acid to release virions from infected cells, and enhance passage through respiratory mucins, and a proton channel (M2 matrix) that helps the release of the viral genome after acidification in endocytic vacuoles. These proteins are present in most split-virus vaccine products (24), but the exact contribution of each of these, or of other influenza proteins that are not surface expressed, to vaccine efficacy is unclear.

ADCC ACTIVITY AGAINST INFLUENZA

Antibody specificity is mediated by binding of Fab (fragment antigen binding) domains to their antigenic target, while the other end of the antibody is a constant region, the Fc (fragment crystallizable) domain, that provides a link between antibody recognition of infected cells and effector cells. NK cells, monocytes/macrophages, and neutrophils all have Fc-receptors (FcRs) on their surface, and the combination of both activating and inhibitory signals direct the immune response (25). In humans, NK cells are generally thought of as the primary effector for ADCC, but ADCC of influenza-infected cells has been demonstrated using neutrophils, monocytes, lymphocytes, and cord blood cells (26–28).

An increasing body of literature has demonstrated the possible protective role of non-neutralizing antibodies for pathogens as diverse as HIV-1 (29, 30), herpes simplex virus (31–33), Ebola (34, 35), and influenza (36–38). Influenza-infected cells can be recognized by antibodies that bind to proteins that play important roles within the viral life cycle. As noted above, HA initiates the viral life cycle by binding sialic acid and mediating viral entry into cells via fusion after receptor-mediated endocytosis (2). During this process, M2 matrix channels promote pH equilibration, a critical step in the release of the viral genome from the endosome (39). After influenza genome replication and protein synthesis, viral components are transferred to the plasma membrane where viral budding occurs. NA removes sialic acid from glycoproteins allowing the release of newly assembled virions from the surface of infected cells and preventing aggregation (2). HA has been the focus of most vaccine designs because HA evolves more rapidly than other antigens (40), but more conserved antigens like NA, M2, and nucleoprotein (NP) have also been considered attractive targets for vaccine designs. Antibodies against these other antigens do not appear to directly prevent infection but can target infected cells (19), and antibodies against all of these proteins have mediated killing of infected cells *in vitro* (**Figure 1B**).

Antibody-dependent cellular cytotoxicity (ADCC) against influenza was described by Greenberg et al. (26) and ADCC activity can be mediated by multiple cell types (27, 28, 41) and arises after infection or vaccination (41–43). Most early work suggested that ADCC was primarily directed against HA (44), although the contribution of other antigens to ADCC could not be ruled out (43). Study of influenza-specific monoclonal antibodies (mAbs) have shown that antibodies directed against the globular head often lack breadth while those directed against the more conserved HA stem can recognize multiple strains and subtypes of HA (4). Both specificities of HA antibodies have mediated ADCC (**Figure 1B**) but antibodies with neutralization and hemagglutination inhibition (HAI) activity tend to be directed against the HA head domain (**Figure 1A**).

ADCC-mediating antibodies also target other proteins on the virus surface (**Figure 1B**). Murine mAbs against influenza B NA mediated ADCC and provided protection in mouse challenge (45), but whether this activity contributes significantly to human protection is not known. NA antibody titers have correlated with protection against influenza infection in humans (46–50), but NA responses have not been studied at the same level of molecular detail as HA responses (51). NA antibodies are thought to act by decorating infected cells thus making them targets for complement fixation and ADCC, and by inhibiting enzymatic activity and preventing virions from escaping respiratory mucins (19). NA is present in most vaccines derived from influenza virions (24), but few vaccine candidates focus on NA antigens and their relative contribution to protection is not well-quantified. There is growing interest in NA as a component vaccine-mediated protection, and the recent formation of the NAction! working group (51) is focused on filling key knowledge gaps.

M2 is a highly conserved antigen necessary for the virus life cycle (39) and a number of studies showed antibodies to M2 mediated ADCC and protected in mouse studies (36, 52, 53). M2 is present at low copy number on virions and may be occluded by the larger HA and NA proteins (19) (**Figure 1A**), and while vaccine candidates based on M2 have advanced to human clinical trials (54, 55), it is not yet known whether these approaches will provide broad cross-protection when tested in human efficacy trials.

Curiously, a protein that might not be expected on the surface of either virions or virus-infected cells has been shown to mediate ADCC *in vitro*. Influenza nucleoprotein (NP) is involved in replication and packaging of virus RNA segments into virions (56), but studies have shown that NP is surface expressed on infected cells (57, 58). Humans given seasonal vaccine demonstrated H7N9 cross-reactive ADCC activity that correlated with binding to NP (59). Antibodies against both NP and the internal RNA-binding and structural matrix 1 (M1) protein did not mediate ADCC against target cells expressing those specific antigens, but immune complexes of those proteins primed natural killer (NK) cells to secrete cytokines (60) (**Figure 1C**); the authors suggested such activity could contribute to an anti-viral environment. Whether these kinds of activities play an important role in cross-protection against human infection is not yet known.

ADCC IN MOUSE MODELS

The receptors and cell types important for human immune response may not have the same effect or use the same pathways

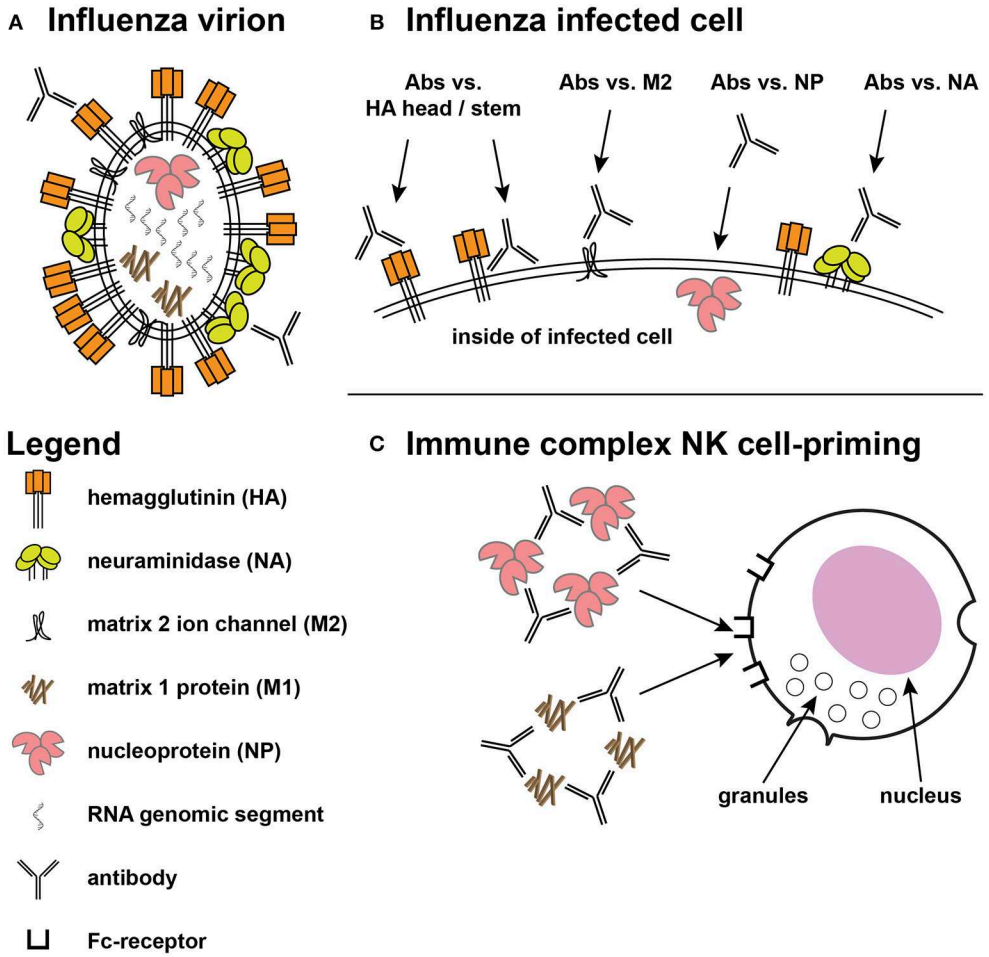

FIGURE 1 | Targets for protective influenza responses. **(A)** Influenza virion showing the tight packing of viral hemagglutinin (HA), neuraminidase (NA), and matrix 2 (M2) ion channels on the surface. Inside the virion are the 8 genomic segments, nucleoprotein (NP), and the structural matrix 1 (M1) protein. **(B)** The surface of an infected cell shows the same antigens spread out on the surface, providing access for ADCC-mediating antibodies directed at the HA head and stem, along with greater access to the M2 ion channel. Antibodies against NP have also shown ADCC activity. **(C)** Immune complexes of NP and M1 have been shown to prime NK cells to secrete cytokines.

in animal models. In fact, there are numerous differences among antibody isotypes and subclasses (61) in animal models typically used for immune studies, as well as in FcR sequences and cellular distributions (62), and such differences may result in species-specific mechanisms of ADCC activity against influenza.

With that caveat, mouse models have provided suggestive evidence for the kinds of immunity that might be protective in humans. DiLillo et al. administered human mAbs to transgenic mice that expressed human FcRs, and demonstrated that for both HA stem-directed antibodies (37) and HA head-directed antibodies (63) that Fc-FcR interactions were required for protection against lethal challenge. Interestingly, transgenic mice receiving neutralizing anti-HA and anti-NA antibodies were only protected against challenge if they were matched for Fc-Fc?R binding while strain specific anti-HA and anti-NA antibodies protected regardless of Fc-FcR matching (63). Similar studies of antibodies against M2 have also shown Fc-FcR dependence, with IgG1 M2-directed antibodies requiring matching FcRs to protect against lethal challenge (52, 53).

Wild-type mice have also demonstrated ADCC-mediating antibody protection. For example, murine antibodies generated against H7N9 influenza were protective, but ADCC-mediating antibodies required efficient Fc-FcR interaction to protect (64). Vaccination against H5N1 that elicited ADCC activity protected against lethal H5N8 challenge in mice (65), and protection against H7 influenza challenge also correlated with ADCC activity (66). Passive transfer of HA stem-specific human sera demonstrated protection against lethal challenge in a manner highly correlated with ADCC activity (67). Protection is not limited to HA-directed antibodies, as antibodies against influenza B NA also mediate ADCC and protect against lethal virus challenge (45).

However, not all mouse studies have suggested a protective effect of ADCC. Evaluation of candidate vaccines that targeted

specific epitopes on the HA head domain were capable of eliciting potent ADCC responses *in vitro*, but mice given these immunogens were more sensitive to lethal challenge (68, 69), and examination of lung tissue suggested damage caused by the immune response. Whether this kind of damage could occur in humans is not known.

OTHER NON-PRIMATE MODELS

Swine are susceptible to influenza and they are thought to be a key species in development of new strains by antigenic shift (70). Because of the major economic impact of swine influenza disease, vaccination is common and new vaccine candidates are tested in swine (71). Experimental infection models have been used to test swine for protection against challenge following vaccination (72) and as a model of enhanced influenza disease (73). Studies in this latter model have suggested that a lack of neutralizing activity against the challenge strain combined with ADCC-mediating antibodies can produce enhanced disease in swine (73, 74), suggesting that caution may be appropriate in the development of vaccines that do not elicit traditional correlates of influenza protection (75). Furthermore, differences between swine and human FcRs make direct measures of ADCC in swine challenging, and have confounded passive infusion studies of human antibodies (76). The swine model is important for improving our understanding of influenza pathogenesis, but it remains to be seen whether success or failure in the swine model will directly translate to human trials.

The ferret model may also provide insights into influenza immunity, although direct measures of ADCC activity in ferrets is also challenging. For example, immunization of ferrets to elicit HA stem-directed antibodies showed that ADCC in a reporter assay correlated with protection, though the assay used human FcRs due to the lack of ferret-specific reagents (77). Passive transfer of immune sera directed against the HA stem (78), infusion of ADCC-mediating mAbs (79), and immunization to induce ADCC-mediating antibodies (65) have all protected ferrets against heterologous influenza challenges. Unfortunately, the lack of ferret-specific reagents limits the depth of investigation possible at this time, and as with swine, it is not clear whether these studies will translate directly to human trials.

NON-HUMAN PRIMATE STUDIES

Non-human primate models have not traditionally been used for influenza research due to high cost and the low level of symptoms following experimental infection (80), but recently a number of studies have evaluated the protective capacity of ADCC in non-human primates. For example, protection was observed in rhesus macaques infected with a pre-pandemic H1N1 A/Kawasaki/173/2001 who were subsequently challenged with H1N1 A/California/04/2009; in this study ADCC activity correlated with control of the second H1N1 infection (81). Protection was also observed in a study of H5 immunization of rhesus macaques challenged with pandemic H1N1, and ADCC activity correlated with reduced viral shedding after infection (82). In cynomolgus macaques, vaccines that elicit ADCC-mediating antibodies have been shown to decrease shedding of influenza (83), and passive infusion of a human ADCC-mediating mAb protected against infection (84). However, given the differences between human and non-human primate Fc-FcR biology (85), it is not clear if non-human primate studies will be predictive of human study outcomes.

HUMAN STUDIES

Human studies have focused on examining people after natural infection, vaccination, and/or the isolation of human monoclonal antibodies. Natural infection studies have shown that influenza can imprint the immune system in a manner that provides protection against heterologous influenza exposure. For example, healthy adults and children in the US were found to have ADCC-mediating antibodies against H5N1 and H7N9 strains (86), despite the fact that those strains have not circulated in the US. Intravenous immune globulin, a product derived from plasma donation, contained ADCC-mediating antibodies that cross-reacted with multiple influenza strains; e.g., immune globulin collected from donors prior to the emergence of the 2009 pandemic strain was active against the newly emergent strain (87). During the last influenza pandemic a number of studies suggested that older adults were less likely to be infected by the newly emergent strain, and older persons were more likely to have ADCC-mediating antibodies against the 2009 H1N1 pandemic strain (88), suggesting that these responses may have contributed to the relative protection observed. However, such antibodies were not well correlated with protection in children (89, 90), indicating that *in vitro* activity alone may not provide an accurate correlate of efficacy.

ADCC-mediating antibodies that arise during severe infection may also correlate with outcomes. A study of seasonal and H7N9 human infections demonstrated that persons who did not survive infection were more likely to have low ADCC activity (91). It is not known whether ADCC-mediating antibodies being present prior to infection would have been protective.

Vaccine studies have examined the ability of different constructs to elicit cross-reactive ADCC-mediating antibodies. For example, seasonal vaccination of older adults indicated that they had strong boosting of ADCC activity, including activity against H5 and H7 strains (92). Similarly, a study of H7 vaccination found ADCC activity against many group 2 influenza strains (93), and H5 vaccination induced ADCC responses to multiple strains (94).

Testing of many of human-derived antibodies showed protection in mouse studies as described above and have helped define vaccine design targets. For example, antibodies against the vestigial esterase domain of H3 HA (95) or influenza B (96) mediate ADCC and block other viral activities, but whether such antibodies can be easily elicited by vaccination and protect is not known. Others have examined why HA head-directed antibodies are less efficient at mediating ADCC (97) or why antibodies that bind at or near the sialic acid receptor binding site of HA lack ADCC activity (98, 99). Data suggested that ADCC

activity against influenza-infected cells requires that sialic acid on NK cells must also bind HA. Several studies have identified possible vaccine targets on HA, including a pH-sensitive epitope on H7 HA (100), receptor binding site antibodies for influenza B (79), and the influenza B HA stem (101). Furthermore, recently isolated human antibodies against epitopes in the HA trimer interface provided protection in an Fc-dependent manner (38, 102), and such antibodies were elicited in animal models by vaccination (103). These studies suggest that probing the human immune system could identify additional targets for vaccine design.

Most human studies measure responses to influenza infection or vaccination, and some correlate those responses with epidemiologic data, but far fewer perform experimental infectious challenge to correlate a response with protection. In 2016, Jegaskanda et al. reported that high levels of pre-existing ADCC-mediating antibodies protected against experimental infection (104), suggesting that if ADCC activity is present prior to infection it can protect. However, it is not clear whether ADCC activity alone is protective, and as of this writing, there do not appear to be any reports of human challenge studies investigating the protective capacity of passively administered ADCC-mediating influenza antibodies. Essentially all humans over the age of 6 years have evidence of prior influenza infection (105), meaning that vaccination or passive infusion studies of ADCC-based protection in humans have to be interpreted in the light of prior immunity. Despite this, the studies described in this review and other work are leading to new vaccine designs, and it is hoped that one or more of these new designs will provide long-lasting protection to all people worldwide.

CONCLUSIONS

Influenza remains an important cause of human disease that often resists effective control by current vaccination strategies and there is a current push for the development of "universal vaccine" candidates. Antibody binding to Fc receptors with effector cell activation has been shown to protect in numerous animal models and harnessing this activity could be an important component of universal vaccine designs. ADCC and other activities based on the "other end of the antibody," in combination with traditional activities like neutralization, might be harnessed to contribute to protection from this ever-changing pathogen.

AUTHOR CONTRIBUTIONS

TV wrote the first draft and participated in editing. MM edited the draft and prepared the final version.

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Concurrent Exposure of Neutralizing and Non-neutralizing Epitopes on a Single HIV-1 Envelope Structure

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The trimeric envelope spikes on the HIV-1 virus surface initiate infection and comprise key targets for antiviral humoral responses. Circulating virions variably present intact envelope spikes, which react with neutralizing antibodies; and altered envelope structures, which bind non-neutralizing antibodies. Once bound, either type of antibody can enable humoral effector mechanisms with the potential to control HIV-1 infection *in vivo*. However, it is not clear how the presentation of neutralizing vs. non-neutralizing epitopes defines distinct virus populations and/or envelope structures on single particles. Here we used single-virion fluorescence correlation spectroscopy (FCS), fluorescence resonance energy transfer (FRET), and two-color coincidence FCS approaches to examine whether neutralizing and non-neutralizing antibodies are presented by the same envelope structure. Given the spatial requirements for donor-acceptor energy transfer ($\leq$10 nm), FRET signals generated by paired neutralizing and non-neutralizing fluorescent Fabs should occur via proximal binding to the same target antigen. Fluorescent-labeled Fabs of the neutralizing anti-gp120 antibodies 2G12 and b12 were combined with Fabs of the non-neutralizing anti-gp41 antibody F240, previously thought to mainly bind gp41 "stumps." We find that both 2G12-F240 and/or b12-F240 Fab combinations generate FRET signals on multiple types of virions in solution. FRET efficiencies position the neutralizing and non-neutralizing epitopes between 7.1 and 7.8 nm apart; potentially fitting within the spatial dimensions of a single trimer-derived structure. Further, the frequency of FRET detection suggests that at least one of such structures occurs on the majority of particles in a virus population. Thus, there is frequent, overlapping presentation of non-neutralizing and neutralizing epitope on freely circulating HIV-1 surfaces. Such information provides a broader perspective of how anti-HIV humoral immunity interfaces with circulating virions.

Keywords: single HIV-1 virion, epitope exposure, neutralizing and non-neutralizing epitopes, two-color coincidence fluorescence correlation spectroscopy (FCS), FRET-FCS

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INTRODUCTION

Intensive efforts are underway to develop preventive vaccines and therapeutic strategies based on humoral immunity against the HIV-1 envelope (Env). Such efforts logically consider directing antibody responses toward replication competent viral particles. Success in this regard demands an understanding of the epitope patterns expressed by virions and virus populations. On HIV-1 particles, the virus envelope spike is a heavily glycosylated trimer of three heterodimers containing gp120 surface subunits and gp41 transmembrane proteins. Gp120 binds the host cell receptor CD4 and a co-receptor, which triggers gp41 to mediate membrane fusion and viral entry. These antigens exhibit high variability in sequence and structure, driven by and allowing escape from immune pressure (1–13). At the same time, Env antigens can express highly conserved epitopes of various types, e.g., within glycan domains; within the CD4 or co-receptor binding sites; or on gp41. These epitopes are highly attractive targets for vaccine design as human antibodies (bNAbs) against them can be very broadly neutralizing (14–16) and provide potent sterilizing protection against SHIV challenge in macaque infection models (17–19).

The most highly conserved, functional and immutable Env epitopes are not neutralizing, often because they are structurally occluded on free trimers (20–22). Many of these epitopes are exposed as a consequence of natural virus-cell attachment mechanisms (21–23) but it is unclear how they are presented by free virions. One possibility is that non-neutralizing epitopes are expressed on "aberrant," non-functional envelope structures (24–26). Even if disconnected from productive attachment and entry processes, such structures could still mediate antiviral immunity if they appear on replication competent virions. Previous studies showed that non-neutralizing humoral responses directed against non-HIV antigens placed on functional virions mediated protection from SIV or HIV-1 infection in macaque and humanized mouse models, respectively (27–31). Thus, the presence and nature of any Env structure appearing in a virus population warrants careful evaluation in the context of antiviral immunity.

Numerous attempts have been made to characterize the prevalence of non-neutralizing vs. neutralizing epitope presentation in populations of HIV-1 virions and/or to partition HIV-1 virion populations into replication-competent vs. non-functional particles based on differential epitope presentations (9, 25, 26, 32–35). Such work has relied heavily on some manner of virion capture by anti-Env antibodies bound to a substrate. In general, findings from this approach have suggested that virus preparations can contain subpopulations of virions presenting variable mixtures of neutralizing and non-neutralizing epitopes. Subpopulations favoring non-neutralizing epitopes (presumably harboring a large amount of defective or degraded envelope) tend to be poorly- or non-infectious. Although useful, capture systems inform the nature of virions after some sort of adsorption procedure. Associated caveats include altered immunoreactivity patterns caused by the process of substrate attachment; altered virion characteristics caused by the capture manipulations. With some techniques, captured material may represent aggregates of particles as well as single virions. Moreover, capture methods cannot directly reveal whether individual virions present neutralizing and non-neutralizing epitopes on common surface structures, and/or show how frequently such scenarios occur within a virion population.

Previously we developed an analytical method based on fluorescence correlation spectroscopy (FCS) that allows the direct evaluation of mAb binding to HIV-1 virions continuously in solution (36). More recently we adapted this method to enable dual color detection of two different fluorescent-labeled mAbs bound to a single virion as well as detection of Förster resonance energy transfer (FRET) from fluorophores closely localized on single Env spikes. These tools can be used to investigate the relative presentation of neutralizing and non-neutralizing epitopes in virion populations and/or single spikes on an individual particle.

In the present study, the broadly cross-reactive, non-neutralizing F240 epitope (37) in the "Cluster I" domain of gp41 served as the focal point for our experiments. The F240 epitope is located within the immunodominant disulfide loop region of gp41 (38, 39), which is commonly immunoreactive on the surfaces of free virions (33, 34, 36, 40, 41). Although the F240 epitope is occluded on intact Env trimers, it seems to be exposed on undefined Env structures in which gp41 is oxidized (41). Passive transfer of mAb F240 exhibited a marginal degree of protective efficacy against SHIV challenge in macaques (42); a mAb against a related Cluster I epitope, 246D, mediated protection against HIV-1 infection in humanized mice (31); another related mAb, 7B2, (41) reduced the number of transmitted/founder SHIV variants in passively immunized macaques (43). Here we report that the antibodies directed against the F240 epitope and neutralizing gp120 epitopes bind concurrently to single virions via a shared Env-derived structure. Further, our data suggest that most particles in a virus population harbor such structures.

MATERIALS AND METHODS

HIV-1 Pseudovirus Production

HIV-1 BaL and HIV-1 JRFL pseudoviruses were generated by co-transfection of HEK293T cells with an Env-deficient HIV-1 backbone plasmid pNL4-3-ΔE-EGFP along with Env-expression plasmids (44, 45) pHIV-1-BaL 0.1 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID) and pCAGGS-JRFL (kindly provided by J. Binley, Torrey Pines Institute of Molecular Studies, San Diego, CA). Transfections were accomplished using FuGENE 6 (Roche, Indianapolis, IN) transfection reagent at a 3:1 reagent-DNA ratio. To produce the infectious molecular clone of transmitted/founder (T/F) HIV-1 AD17 virus (46), HEK293T cells were transfected with the AD17 plasmid (kindly provided by B. Hahn, University of Pennsylvania) at a FuGENE-to-DNA ratio of 3:1. Virions-containing supernatant was harvested after 3 days, and concentrated about 10-fold by incubating with PEG-*it*™ virus precipitation solution (System Biosciences, Mountain View, CA) for 18 h at 4°C as recommended by vendor.

The antigen content of all virion preparations was quantified using p24 and gp120 antigen capture ELISAs. Infectivity was established using standardized procedures (47) and quantified as function of $TCID_{50}$ in TZM-bl cells. HIV-1 BaL and HIV-1 JRFL pseudoviruses with gp120 to p24 ratio of 1:10–1:50, and 200,000–500,000 TCID50/mL; HIV-1 AD17 T/F with gp120 to p24 ratio of 1:200, and 600,000 to 1,000,000 TCID50/mL were used for FCS measurements. The Aldrithiol-2 (AT-2) inactivated (48, 49) HIV-1 BaL virus produced in SupT1-CCR5 CL.30 cells was generously provided by Dr. Jeff Lifson (AIDS Vaccine Program/NCI, Frederick, MD). pEGFP-Vpr (cat# 11386 from Dr. Warner C. Greene) plasmid (50) obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, was used to generate fluorescent HIV-1 BaL pseudoviruses.

Antibodies

mAbs b12, 2G12, PG9, and F240 were purchased from Polymun Scientific (Vienna, Austria). mAbs 17b and N49P7 (14) were expressed from plasmid clones in HEK293T cells using an IgG1 backbone for heavy-chain variable regions and either a κ- or λ-chain expression vector for light-chain variable regions. mAbs were purified from culture supernatants by protein-A chromatography. Fabs of b12, 2G12, PG9, N49P7 or F240 were prepared from purified IgG (10 mg/ml) by proteolytic digestion with immobilized papain (Pierce, Rockford, IL) and purified using protein A (GE Healthcare, Piscataway, NJ), followed by gel filtration chromatography on a Superdex 200 16/60 column (GE Healthcare, Piscataway, NJ). All mAbs or Fabs were fluorescently labeled and purified with Alexa 488, 568, or 647 monoclonal Antibody Labeling Kit (Invitrogen, Molecular Probes, Eugene, OR). Briefly, the Alexa dye has a succinimidyl ester moiety that reacts efficiently with primary amines of antibody to form stable dye-protein conjugates. Each labeling reaction was performed with 100 μg of a mAb or Fab. The labeled antibody was separated from unreacted dye by centrifugation through a spin column at 1,100x g for 5 min. Recovered antibodies were dialyzed against phosphate buffered saline as necessary. Labeled mAbs or Fabs were quantified by a UV-vis spectrometer (Nanodrop 2000, Thermo-Scientific, Wilmington, DE). Dye to protein ratios were determined by measuring absorbance at 280 nm (protein) vs. absorbance at corresponding wavelength for Alexa 488, 568, or 647. Conjugated mAbs or Fabs used in our experiments had an optimal dye to protein ratio in the range of 1–2 of dye per molecule of mAb or Fab.

Two-Color Fluorescence Correlation Spectroscopy

Fluorescence Correlation Spectroscopy (FCS) is a methodology that allows real-time detection of multiple protein-protein interactions in solution, by measuring diffusion and reaction kinetics of fluorescently-labeled biomolecules (51). Two-color FCS (52) has been used to monitor two different mAbs bound to HIV-1 virions. The binding of Alexa 488 or 647-labeled mAbs b12, 2G12, 17b, F240, and anti-RSV antibody, Synagis (negative control) to HIV-1 virions was monitored by tracking diffusion of their fluorescent label across the observation area, where unbound antibodies will diffuse much faster than those that bound viral particles as described in Ray et al. (36). Briefly, HIV-1 BaL pseudovirions were diluted to 10 μg/mL p24 equivalent in a 100-μL reaction volume (gp120:p24 ratio of 1:50), and were first incubated with 100 μg/mL non-specific IgG1 (1.5 μL of a 7 mg/mL stock) for 90 min at 37°C to block non-specific binding. Then 1 μL of the test Alexa conjugated mAbs (2 μg/mL of each mAb) was introduced and allowed to interact with pseudovirions for 90 min at 37°C. For spectroscopic measurements, 11 μL of the reaction mixture was loaded onto an FCS slide reservoir, sealed, then placed on a time-resolved confocal microscope (ISS Q2) with a high numerical aperture (NA = 1.2) water objective (60x magnification). The excitation source was a Fianium SC-400 super-continuum laser. NKT super-select acousto-optical tunable filter (AOTF) filter was used to select the excitation wavelengths. The beam after AOTF was passed through narrow bandpass clean-up filters. The samples were excited with two coincident excitation at 470 and 635 nm, and fluorescence signals from the Alexa 488 (A488) or Alexa 647 (A647) mAbs were collected in two separate detection channels in the 500–550 nm and 650–720 nm region over 60 s in a constant detection volume (∼1 fL) that is continuously replenished. ISS Vista vision software was used to generate the autocorrelation function of the fluorescent fluctuations of the Alexa labeled mAbs. The autocorrelation function of fluorescence intensities is given by the product of the mAb intensity at time t, $I(t)$ with the intensity after a delay time τ, $I(t+\tau)$, typically in the range from 10^{-2} to 10^2 ms, averaged over the 60 s of measurement.

For experiments in which only the conjugated mAbs and no virions were present, the autocorrelation function was fitted with a single species diffusion model equation. Diffusion coefficients of the fluorescent species under these conditions were routinely determined to be 60 $\mu m^2/s$. These values matched what was predicted for 150 kD IgG molecules in solution. In reactions with mAbs and virions, the autocorrelation was fit to a two-species diffusion model. In this operation, one species had the unbound mAb diffusion coefficient of 60 $\mu m^2/s$, and the second a diffusion coefficient of 6 $\mu m^2/s$, matching the predicted behavior of fluorescent mAbs bound to a 100 nm retroviral particle. The fitting equations were also used to determine the percentage of total mAb exhibiting the slower diffusion rate in reactions with virions. The mathematical derivation and application of the equations is described in (36). The cross-correlation measures (52, 53) between the two separate detection channels determined if two signal intensities were correlated; i.e., fluctuated in concert or independently. Only pairs of co-incident photon counts from two distinct channels (500–550 and 650–720 nm in the present case) will show positive correlation amplitude. Only dual-color cross-correlation data of two different antibodies in the presence of HIV-1 virion showing positive correlation amplitude were taken as evidence that two different antibodies bound to the same virion particle.

Characterization of Single Virion FCS Measures

As dual-label FRET measures cannot distinguish the number of targets based on signal intensity, they are most clearly interpreted when there are limiting amount of virions in the focal volume. This situation can be favored by appropriate dilutions of the

virus stock. In theory, the amount of p24 in a virus preparation may be used for this purpose, assuming 10^4 virus particles per picogram of p24 (54, 55). To better determine how p24 measures could be used in this manner with our virus preparations, we generated HIV-1 BaL pseudoviruses using the usual methods, but also containing an eGFP.Vpr fluorescent marker. The eGFP.Vpr HIV-1 BaL stock contained 3 μg/ml of p24, which based on 10^4 virus particles/picogram of p24 (54, 55), converts to 3×10^7 particles/μL or roughly 0.03 virus particle per femtoliter assuming that virions are evenly dispersed. Serially dilutions of virus were then analyzed by FCS (**Figure S1**), which detected the eGFP.Vpr signal and thereby quantified the number of particles in the focal volume at any one time. The number of fluorescent virions were determined by fitting the autocorrelation plot and extracting the correlation amplitude G(0) (51, 53). The number of molecules detected in the FCS focal volume is inverse of the correlation amplitude (51, 53). The diffusion coefficient of the eGFP.Vpr HIV-1 BaL pseudoviruses signals (5 $\mu m^2/s$) matched those previously determined for single pseudovirus particles (36). As shown in **Figure S1**, there was the expected linear relationship between p24 concentration and number of virions measured by FCS. Notably, the FCS analyses detected substantially more virions in the focal volume than what was predicted by p24 measures (for example, although 3 μg/ml was predicted to translate into 1 virion/33fL; FCS indicated there was 1 virion/1.6 fL). FRET-FCS can only be performed with unlabeled virions as the signal from the eGFP fluorescent virion will interfere with the signal from fluorescent tagged mAbs or Fabs. Thus, a conversion factor was determined and used for experiments with other unlabeled viruses (see below) to normalize p24 concentrations to the probable numbers of virions (∼1.5) being seen in the FCS focal volume of 1fL.

FRET-FCS Measurements

For FRET measurements, the Fabs (b12, 2G12, PG9, N49P7 or F240) were labeled with either donor (Alexa 488) or acceptor (Alexa 568) probes (Invitrogen mAb labeling kit). Dye-to-protein ratios were determined by measuring absorbance at 280 nm (protein) vs. 488 or 577 nm (dye). The dye-to-protein ratios were between 1 and 2. We specifically aimed to keep this low label of dye labeling as we are using a single molecule fluorescence method and minimally perturb the functionality of the protein. FRET measurements were performed in a confocal microscope (ISS Q2) equipped with a supercontinuum laser and AOTF in order to excite the molecules during its diffusion through the confocal volume. ISS Vistavision software was used to generate the FRET histogram and further analyses. FRET measurements were performed after forming complex with the HIV-1 BaL, HIV-1 JRFL, AT-2 inactivated HIV-1 BaL or HIV-1 AD17 T/F virions with donor-labeled Fab and acceptor labeled Fab. The number of virions at any given time was around 1.5 particle in the 1fL focal volume as described above.

Fluorescence responses from the donor and the acceptor molecules were separated by a dichroic beam splitter and detected by two avalanche photodiode detectors (APD) using the method of time-correlated single photon counting and the Time-Tagged Time-Resolved (TTTR) mode of the Becker and Hickl SPC-150 module. High quality bandpass (Chroma) filters were used for laser clean-up and recording donor and acceptor fluorescence in two separate detection channels. The collected single photon data was binned by 3.4 ms (corresponding to the diffusion time of the 100 nm diameter virion particle) in each channel (donor or acceptor), which resulted in intensity-time traces recorded for 120 s. Threshold values in each channel were used to identify the single molecule bursts from the corresponding background signal level. Fluorescence bursts were recorded simultaneously in donor and acceptor channels and FRET efficiencies were calculated using $E = I_A/(I_A + \gamma I_D)$ where I_D and I_A are the sums of donor counts and acceptor counts for each burst, taking into account the possible difference in the detection efficiency (γ) in two separate channels (56–60). The analyses revealed fractional quantity of FRET efficiency events for a specified bin and recorded time of the donor-acceptor intensity traces. For a measurement time of 120 s and sampling frequency of 300, total number of 36,000 events can be possibly obtained. It is important to note that an event is likely no more than two virions in the FCS observation volume of 1fL based on input concentration of p24 as shown in **Figure S1**. For each sample containing donor Fabs, acceptor Fabs and HIV-1 virions, fractions of FRET events relating to the total possible events for a given bin time or sampling frequency and measurement time were determined and subsequently the number of occurrences vs. FRET efficiency histogram plots were generated. The donor-to-acceptor distance (*r)* in terms of efficiency of energy transfer (*E*) and Förster Distance (R_0) is given by $r = R_0\ [1/E - 1]^{1/6}$. We have used the value of R_0 of 6.2 nm for the Alexa 488 (donor) and Alexa 568 (acceptor) pair for estimating the donor-to-acceptor distances. In addition to FRET measurements we have also performed FCS measurements to assess the *in vitro* binding of Fab fragments to HIV-1 virions. Consequently, we determined the translational diffusion coefficients of Alexa 488 or 568 labeled Fabs and the corresponding bound virion complexes from FCS measurements. The FCS measurements and analyses were performed as previously reported (21, 36, 57–60).

Assembly of Structural Models of b12 and 2G12 Bound to HIV Env

The model was assembled based on the available CryoEM structure of the virion associated HIV-1 trimer complexed with b12 Fab [PDB: 3DNL, (61)] and crystallographic structure of 2G12 Fab bound to $Man_9GlcNAc_2$ [PDB code: 6N2X, (62)]. 2G12 Fab was modeled into the b12 Fab-HIV-1 trimer by superimposition of the $Man_9GlcNAc_2$ moiety of the 2G12 Fab-$Man_9GlcNAc_2$ complex to the trimer at N-linked glycan at position 332 (62). The distances are measured from the center of each variable domain of Fab.

RESULTS

Previously we used FCS and fluorescent labeled proteins to examine the binding of individual anti-envelope mAbs or sCD4 to HIV-1 particles representing various strains with all reactants

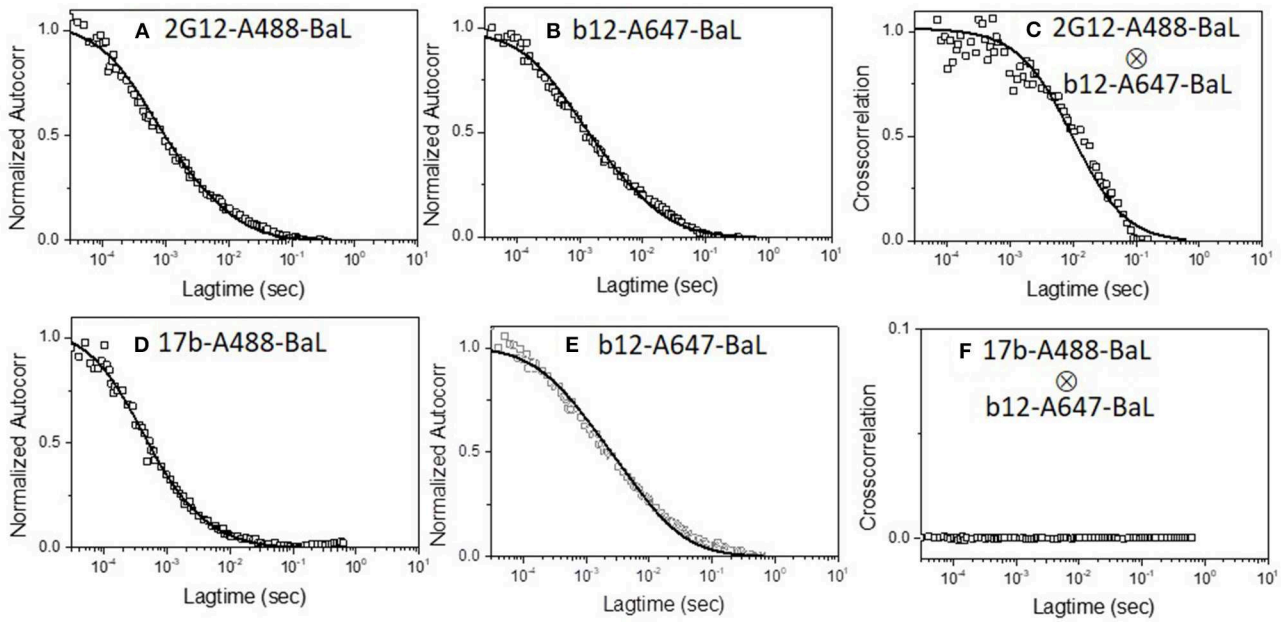

FIGURE 1 | Dual-color correlation curves of **(A–C)** 2G12-A488 and b12-A647 and **(D–F)** 17b-A488 and b12-A647 with HIV-1 BaL virions. **(A,D)** show autocorrelation plots of Alexa 488 labeled mAbs. **(B,E)** show autocorrelation plots of Alexa 647 labeled mAbs. **(C,F)** show cross-correlation curves of b12-A647 and 2G12-A488 or b12-A647 and 17b-A488 with HIV-1 BaL virions. Two color excitation wavelengths at 470 and 635 nm were used. All experiments were repeated three times with similar results.

in solution (21, 36, 41). These studies showed that the Alexa -labeled anti-gp120 bNAbs 2G12 (63) and b12 (64), and the non-neutralizing anti-gp41 mAb F240 (37, 41), bound efficiently and consistently to virions (21, 36). However, these studies did not address whether two antibodies, each of different specificity, bind to the same virion or to the same Env structure on a particle surface. We reasoned that dual color detection and FRET-FCS should afford a means to address this question.

Epitope Exposure on Single Virions by Dual Color FCS

We first applied the dual color detection method to explore the binding of two different mAbs to single HIV-1 BaL pseudovirus particles. We employed anti-envelope mAbs including b12 [a broadly neutralizing CD4 binding site antibody (64)], 2G12 [against a carbohydrate cluster on gp120 (63)], and F240 [against a cluster 1 epitope in gp41 (37, 41)] labeled with either Alexa 488 or Alexa 647. Monoclonal antibody 17b was tested as a negative control. This mAb recognizes a CD4-induced epitope on gp120 (65), binds weakly to HIV-1 BaL in the absence of sCD4, and partially competes with b12 for gp120 binding due to partial epitope overlap (20, 66). Thus, mAbs 17b and b12 are unlikely to bind the same virion except through non-specific processes. **Figure 1** shows the dual-color FCS measurements of Alexa-488 labeled 2G12 and Alexa-647 labeled b12 binding. Autocorrelation plots (**Figures 1A,B**) showed that in the reaction 42 and 45% of b12 or 2G12 mAbs, respectively, adopted the slower diffusion coefficient (6 $\mu m^2/s$) marking virion-bound mAb. Similar binding efficiencies for these mAbs were reported previously (36). Importantly, cross-correlation analyses (51, 53) (**Figure 1C**) of signals simultaneously detected in the two channels could also be fitted to the same single diffusion coefficient 6 $\mu m^2/s$. Such findings reflect that both 2G12 and b12 being bound to the same object, having the size of a retrovirus particle. In comparison, analyses of b12-A647 and 17b-A488 mixed with HIV-1 BaL virions showed no cross-correlation in binding signals (**Figures 1D–F**). Taken together, the data obtained with mAb pairs 2G12 and b12 indicated that the dual color coincidence FCS assay system could reflect the epitope exposure patterns on virus particles in solution.

A second set of experiments examined whether a single virion binds 2G12 neutralizing mAbs along with mAb F240. In these experiments, mAb F240 was labeled with Alexa-488 and mAb 2G12 with Alexa-647, the targets were again HIV-1 BaL pseudoviruses. **Figure 2** shows the dual-color FCS measurements of Alexa-488 labeled F240 and Alexa-647 labeled 2G12 binding to HIV-1 BaL virions. Autocorrelation plots (**Figures 2A,B**) showed that in the reaction 35 and 45% of F240 or 2G12 mAbs, respectively, adopted the slower diffusion coefficient (6 $\mu m^2/s$) marking virion-bound mAb. Similar binding profiles of fluorescently labeled F240 or 2G12 to HIV-1 BaL were reported previously (36). As in the experiments pairing 2G12 with b12 (**Figure 1**), cross-correlation analyses showed simultaneous signal detection in the two channels (**Figure 2C**) fitting a single diffusion coefficient of ~6 $\mu m^2/s$, indicating that 2G12 and F240 bound to the same virion particle. To verify these results further, the binding of both 2G12 and F240 mAbs was assessed with AT-2 inactivated HIV-1 BaL virions produced and

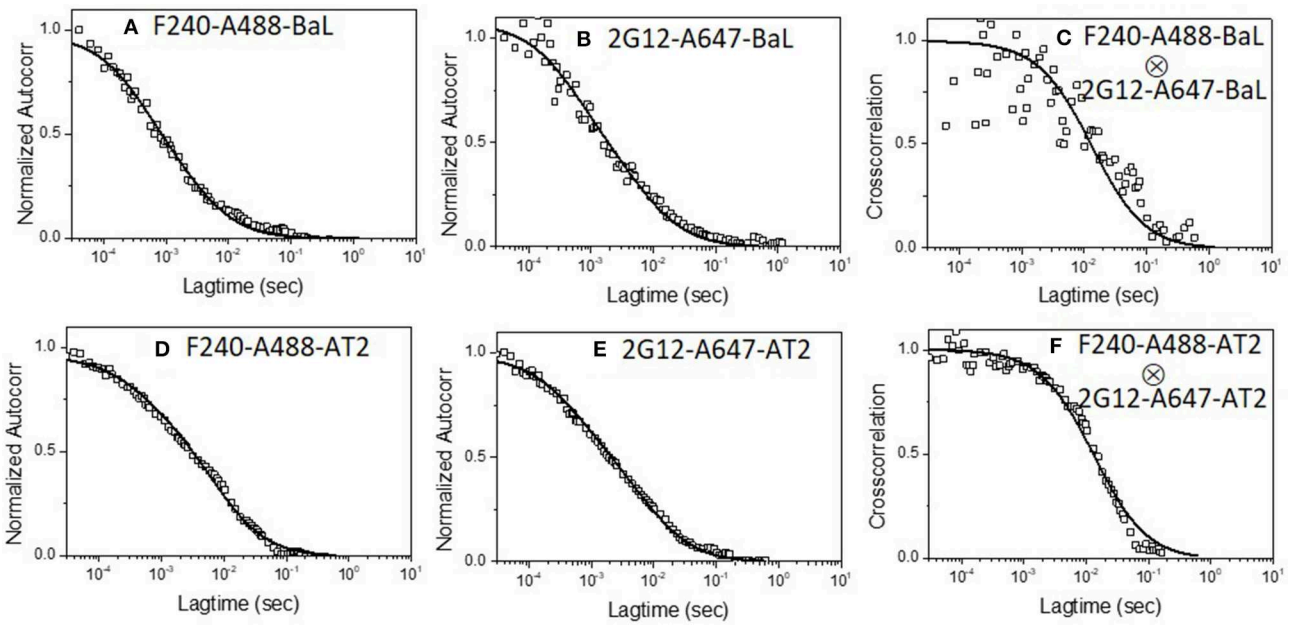

FIGURE 2 | Dual-color correlation curves of F240-A488 and 2G12-A647 with **(A–C)** HIV-1 BaL, and **(D–F)** AT-2 HIV-1 BaL virions. **(A,D)** show autocorrelation plots for F240-A488 with HIV-1 BaL and AT-2 HIV-1 BaL virions; **(B,E)** show autocorrelation plots of 2G12-A647 with HIV-1 BaL, and AT-2 HIV-1 BaL virions; **(C,F)** show cross-correlation curves of F240-A488 and 2G12-A647 with HIV-1 BaL and AT-2 HIV-1 BaL virions. The experiment was repeated three times with similar results.

purified in a different manner (41, 49). Similar auto-correlation (**Figures 2D,E**) and cross-correlation (**Figure 2F**) profiles were observed with Alexa-488 labeled F240 and Alexa-647 labeled 2G12 binding to AT-2 inactivated HIV-1 BaL virions.

Epitope Exposure on Individual Env Structures on HIV-1 Virions by FRET-FCS

Having determined that the above mAb pairs concurrently bind single virus particles, we next examined whether combinations of the above antibodies were attaching to the same Env structure on the virion. The FRET-FCS method relies on standard dipole-dipole interactions between paired donor and acceptor fluorophores, which occurs within the distance range of 2–10 nm. Thus, paired fluorescent mAbs will not create FRET signals unless they bind to tightly localized epitopes. To mitigate spatial differences caused by the probe length (i.e., whole IgG) and more precisely estimate the donor-acceptor distances, the FRET-FCS experiments employed labeled Fab fragments. The use of Fabs also enabled interpretations of FRET data vs. available structural data for Fab-Env complexes. The fluorophores pairs used in all experiments were Alexa-488 (A488 donor) and Alexa-568 (A568 acceptor). The test viruses (see below) were used at final concentrations of 10 μg/ml, which, based on relationship shown in **Figure S1**, was predicted to produce roughly 1.5 virions in the focal volume at any time.

The FRET-FCS system was first applied toward analyses of neutralizing epitopes. Reaction mixtures were constructed with fluorescent labeled 2G12 (A488 donor) and b12 (A568 acceptor) Fabs and HIV-1 BaL virions (see methods). As shown in **Figure 3**, FRET signals from the acceptor probe were indeed detected, following a diffusion coefficient of 6 $\mu m^2/s$ clearly distinguished from the ∼80 $\mu m^2/s$ value expected for an unbound Fab. The FRET signals fit a Gaussian profile with a mean FRET efficiency of ∼25% (**Figure 3A**). These data indicated that the two Fabs occupied a highly localized space on bound virions. FRET efficiency was not biased by the donor/acceptor labeling configuration as swapping the donor-acceptor labeling between b12 Fabs and 2G12 Fabs yielded highly similar measures (**Figure 3B**). According to Forster's equation (see methods) the calculated mean FRET efficiencies were consistent with a situation where the donor and acceptor Fabs bound to virions at an average distance of 7.4 nm apart. To validate these findings, we used existing structural information (61, 67) to model (see methods) the binding of 2G12 and b12 Fabs to a single HIV-1 trimer (**Figure 4**). This exercise predicts that the Fabs would be spaced ∼7.1 nm apart if bound to the same protomer and ∼7.4 nm apart if each is bound to a different protomer in the same trimer. In either case, the modeling predictions closely match those calculated from the FRET efficiencies. However, it must be noted that the Alexa conjugation chemistry does not place fluorophores at single, specified positions along the length of Fabs. Thus, FRET efficiencies, and derivative donor-acceptor distances, reflect aggregate measures of spatial ranges between paratopes and fluorophores on the two Fab probes. Given that Fabs are ∼3 nm long, up to 3 nm in extra distance between epitopes could be added when fluorophores are positioned at maximum distances from paratopes on each probe. However, even in this extreme situation the two epitopes can still be placed within the spatial constraints of a single trimer.

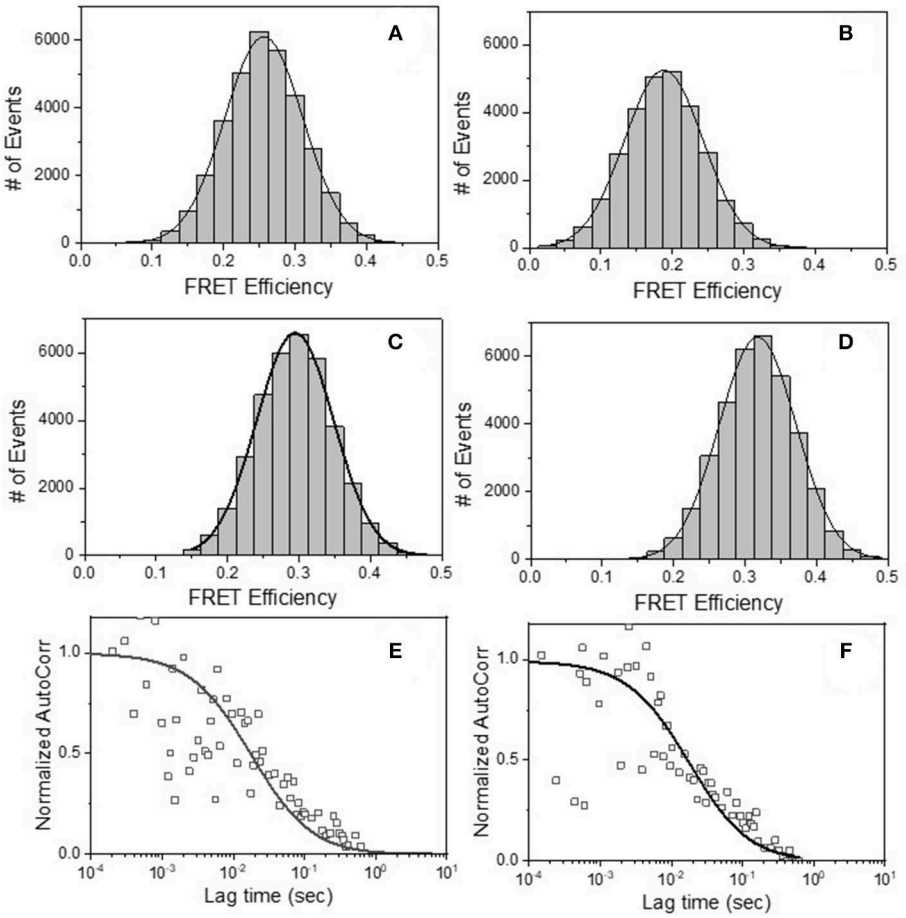

FIGURE 3 | FRET histograms of **(A)** b12 Fab-A488 and 2G12 Fab-A568 **(B)** 2G12 Fab-A488 and b12 Fab-A568, **(C)** b12 Fab-A488 and F240 Fab-A568 and **(D)** F240 Fab-A488 and b12 Fab-A568 with HIV-1 BaL virions. The solid lines in **(A–D)** are fit with a Gaussian distribution to the experimental FRET histogram data. Autocorrelation plots of the acceptor channel for **(E)** b12 Fab-A488 and 2G12 Fab-A568 and **(F)** b12 Fab-A488 and F240 Fab-A568 with HIV-1 BaL virions. The solid lines in **(E,F)** represent the fit to the experimental data. All experiments were repeated three times with similar results.

The prevalence of the above associations in the virus population was considered by comparing the events that exhibited any degree of FRET signal (**Figures 3A,B**) to the total number of events possibly observed under the reaction conditions used (see methods for calculation). These analyses indicated that in reactions with b12 Fab-A488 and 2G12 Fab-A568 or 2G12 Fab-A488 and b12 Fab-A568, donor-acceptor pairs, FRET signals covered 70–75% of the total possible observable events under the experimental system. Taken together, the data indicated that b12 and 2G12 Fabs bound to a single Env structure presented by the majority of particles observed in the FCS system.

We next applied FRET-FCS to reaction mixtures containing Fab fragments of b12 and F240 mixed with the HIV-1 BaL virions. The labeling of the two Fabs (A488 donor vs. A568 acceptor) were reciprocally interchanged to confirm that any FRET measures were not biased by the donor/acceptor labeling strategy. Moreover, FRET signals were not detected in reactions with donor (A488) and acceptor (A568) labeled Fabs in the absence of HIV-1 virions (data not shown). This was expected, as there was no reason for the Fabs to remain in association outside of random diffusion such that a FRET signal could be generated. As shown in **Figure 3**, FRET signals were again detected when combinations of b12 Fab-A488 and F240 Fab-A568 (Panel C) or F240 Fab-A488 and b12 Fab-A568 (Panel D) were mixed with HIV-1 BaL. Single diffusion coefficients calculated for the acceptor channel were 6 $\mu m^2/s$, consistent with signals emanating from objects with the size of HIV virions (**Figures 3E,F**). As above, the number of events that exhibited any degree of FRET signal (**Figures 3C,D**) were compared to the total number of events possibly observed in the system considering the recording time and sampling frequency (see methods). The analyses showed that in reactions with b12 Fab-A488 and F240 Fab-A568 or F240 Fab-A488 and b12 Fab-A568 donor-acceptor pairs, FRET signals covered 75 and 72% of the total possible observable events in the system. Gaussian fitting to the FRET histogram data (**Figures 3C,D**) reflected FRET efficiencies of 30–35% regardless of dye pairing. This in turn

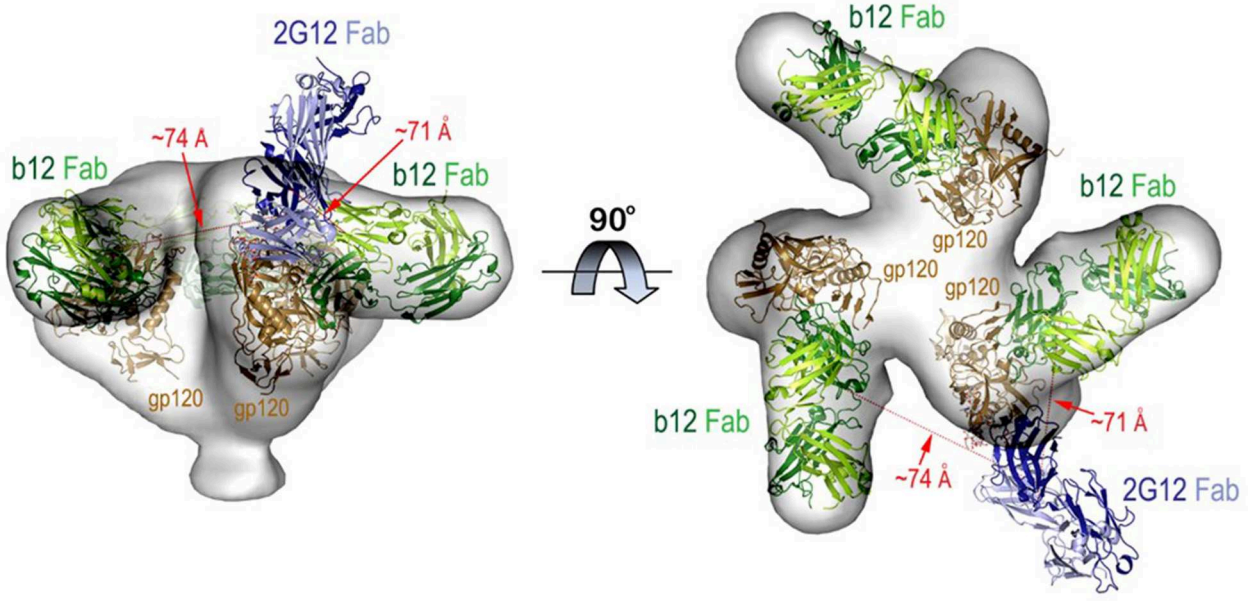

FIGURE 4 | Molecular model for b12 and 2G12 Fabs bound to the same HIV-1 trimer. The model was built using cryoEM structure of the virion associated HIV-1 trimer complexed with b12 Fab and 2G12 Fab-$Man_9GlcNAc_2$ complex as described in Methods. The relative distances (shown as red dotted lines) between the Fabs were measured from the centers of variable domains of each Fab. Molecular surface is displayed over the HIV-trimer and Fabs are shown in ribbon diagrams.

indicates that b12 Fab and F240 Fab bind in close proximity; ∼7.1 nm apart (in the range of 6.6–8 nm) on some sort of gp120-gp41 complex.

Similar experiments were carried out with 2G12 Fab-A488 and F240 Fab-A568 pairs. **Figure 5A** shows the FRET histogram of the Fabs with HIV-1 BaL virions. Again, the histogram plot reflected 30% FRET efficiency and Fab positions ∼7.1 nm apart (in the range of 6.4–8.1 nm). The FCS auto-correlation curve in the acceptor channel exhibited only a single diffusion corresponding to a HIV-1 BaL virion. To determine whether the FRET-FCS measures with HIV-1 BaL virions were generalizable, additional experiments were conducted in which the targets were either AT-2 inactivated HIV-1 BaL virus (produced in SupT1-CCR5 CL.30 cells) (41, 49) or a transmitted founder (T/F) infectious molecular clone (AD17) produced in HEK293T cells (36). As shown in **Figure 5**, both viruses exhibited FRET signals when reacted with 2G12 Fab-A488 and F240-Fab-A568 donor-acceptor pairs. The FRET pattern with the AT-2 inactivated HIV-1 BaL (**Figure 5C**), and predicted binding distance of the Fabs were comparable to what was detected with the HIV-1 BaL pseudovirus (**Figure 5A**). Between the two viruses, a slightly lower FRET efficiency was observed for HIV-1 AD17 (**Figure 5B**). One possible explanation is that HIV-1 BaL and AD17 present slightly different gp120-gp41 surface structures coincidently reactive with both Fab F240 and Fab 2G12. In any case, FRET-linked concurrent binding of the Fabs comprised 66, 58, and 58% of the total possible events in the system (see methods) for the HIV-1 BaL; HIV-1 AD17 or AT-2 inactivated HIV-1 BaL virions, respectively. These FRET-reactive fractions of the virus population were lower than what was observed with co-localized binding of b12 and F240 Fabs (see above).

HIV-1 BaL is a "tier 1b" virus, meaning it has a relatively "open" Env structure more sensitive to neutralization by a wider variety of anti-Env mAbs. Thus, we performed additional FRET-FCS experiments with a tier-2, CCR5 tropic JRFL pseudovirus; i.e., one expressing a "closed" Env structure resistant to neutralization by most mAbs. Reactions were run with fluorescent labeled neutralizing Fabs of 2G12, b12, N49P7 [a CD4 binding site potent broadly neutralizing mAb (14)] and PG9 [a potent broadly neutralizing mAb targeted to quaternary structure in the V1-V2 loops of gp120 (68–70)] along with F240 Fab. The PG9 gp120 epitope is predicted to be at a substantial distance (∼9 nm) from F240 in a single trimer, approaching the limit where FRET becomes undetectable. Nevertheless, PG9 Fab was tested as it interacts with two gp120 protomers in the trimer (68–70) and thus could inform the nature of the target antigen. Specifically, any FRET between PG9 and F240 Fab pairs could indicate the presence of two gp120s in the cognate Env structure. As shown in **Figure 6**, FRET signals were detected when combinations of 2G12 Fab-A488 and F240 Fab-A568 (Panel A); b12 Fab-A488 and F240 Fab-A568 (Panel B) were reacted with HIV-1 JRFL. The average FRET efficiencies for the 2G12-F240 and b12-F240 combinations were 25 and 20%, respectively similar to those observed with the tier-1 HIV-1 BaL virions. Accordingly, the calculated average distances for the 2G12-F240 and b12-F240 Fab pair combinations with HIV-1 BaL virion were 7.4 and 7.8 nm, respectively. FRET signals for the 2G12-F240 and b12-F240 Fab pairs with HIV-1 JRFL virions

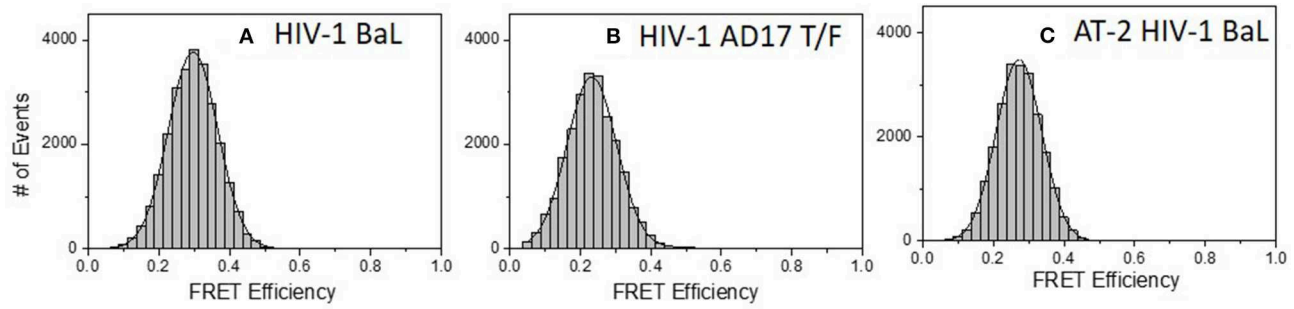

FIGURE 5 | FRET histograms of 2G12 Fab-A488 and F240 Fab-A568 reacted with different virus types: **(A)** HIV-1 BaL, **(B)** HIV-1 AD17 transmitted/founder, and **(C)** AT-2 HIV-1 BaL. The solid lines in **(A–C)** are fit with a Gaussian distribution to the experimental FRET histogram data. All measurements were repeated three times with similar results.

comprised 54 and 61% of the total possible observable events in the system.

FRET signals and efficiencies of 20% between N49P7 Fab-A488 and F240 Fab-A568 with HIV-1 JRFL virions (**Figure 6C**) reflected an average distance of 7.8 nm (in the range of 6.6–8.6 nm) between the two Fabs. This distance is similar to the average distance observed with b12-F240 Fab pairs, as expected, considering both b12 and N49P7 are CD4bs mAbs. FRET signals for the N49P7-F240 Fabs with HIV-1 JRFL virions comprised 56% of the total possible observable events in the system. The histogram for the combinations of PG9 Fab-A488 and F240 Fab-A568 mixed with HIV-1 JRFL (**Figure 6D**) reflected a FRET efficiency of about 10% for PG9-F240 pairs suggesting an average distance of 9 nm (in the range of 7.5–9.5 nm) between the two Fabs. As noted above, this result is in accordance with the available structural information of the distance between the PG9 epitope in the V1-V2 region of gp120 and F240 epitope in gp41 (41, 68–70). Notably, FRET signals from the PG9-F240 Fabs with HIV-1 JRFL virions comprised only 32% of the total possible observable events. The fit to autocorrelation plots (**Figures 6E–H**) for the acceptor channel for all the neutralizing and non-neutralizing Fab combinations showed single diffusion coefficients of 6 $\mu m^2/s$, consistent with signals emanating from objects with the size of HIV-1 virions.

DISCUSSION

Our previous FCS experiments showed that neutralizing mAbs 2G12, b12, and PG9; and the non-neutralizing anti-gp41mAb F240, bind often and efficiently to various pseudoviruses and full length infectious molecular clones produced in different ways (36). However, those analyses did not distinguish whether each mAb bound a specific subset of virions, nor did they reflect how individual virions react with multiple mAb specificities. Under the FCS conditions used here, we were able to make such determinations as each measured fluorescence event stemmed from roughly 1–2 particles in the focal volume being assayed (**Figure S1**). The diffusion coefficients of the collected signals further affirmed that they arose from objects the size of retroviral particles.

Dual color FCS established that the neutralizing mAbs 2G12 and b12 concurrently bound the majority of individual HIV-1 BaL pseudovirions in the population (**Figure 1**), and frequently enabled FRET signals (**Figure 3**) indicating occupancy of the same functional trimer, consistent with structural predictions obtained by *in silico* modeling (**Figure 4**). Observations of neutralizing antibodies bind concurrently to the same trimer are not particularly surprising from a virological standpoint; but the data as such support the utility of the approach. Moving to FRET analyses of other epitopes, a more unexpected yet consistent observation was the concurrent and highly localized binding of F240 Fab and 2G12 Fab to HIV-1 BaL and JRFL viruses; AT-2 inactivated HIV-1 BaL virions from another source; and the HIV-1 AD17 T/F molecular clone (**Figures 5**, **6**). We also detected tightly localized binding of F240 Fab and N49P7 or PG9 Fab on the Tier 2 HIV-1 JRFL pseudoviruses (**Figure 6**).

The F240 epitope is occluded on intact Env trimers (41) and is often assumed to reside only on gp41 "stumps" on the virion surface (26). We cannot eliminate the possibility that some virions present such gp41 structures. Nevertheless, our data suggest the existence of another, previously unexpected type of Env structure that expresses the F240 epitope within 10 nm of a variety of broadly neutralizing sites, spaced to avoid steric competition between cognate Fabs. It seems implausible that multiple Env-derived structures (e.g., a gp41 "stump" and non-specifically adsorbed gp120 monomers of some sort) could develop a surface structure comprising the epitope positioning we measured. A more likely possibility is that the F240 and gp120 epitopes are co-located on a misfolded or partially denatured trimer segment that remains membrane-anchored, retains one or more gp120 protomers, and exposes the gp41 Cluster I domain. Detection of FRET with F240 and PG9 Fabs suggests the presence of two closely spaced gp120 protomers in the target antigen. However, FRET signals for these Fabs were detected less often (roughly 30%) among the total possible observable events, compared to the other epitope pairs. One explanation for the difference is that F240 and PG9 are co-expressed on a relatively infrequent structure, compared to a more common one expressing F240 with 2G12, b12, or N49P7. Another explanation could be

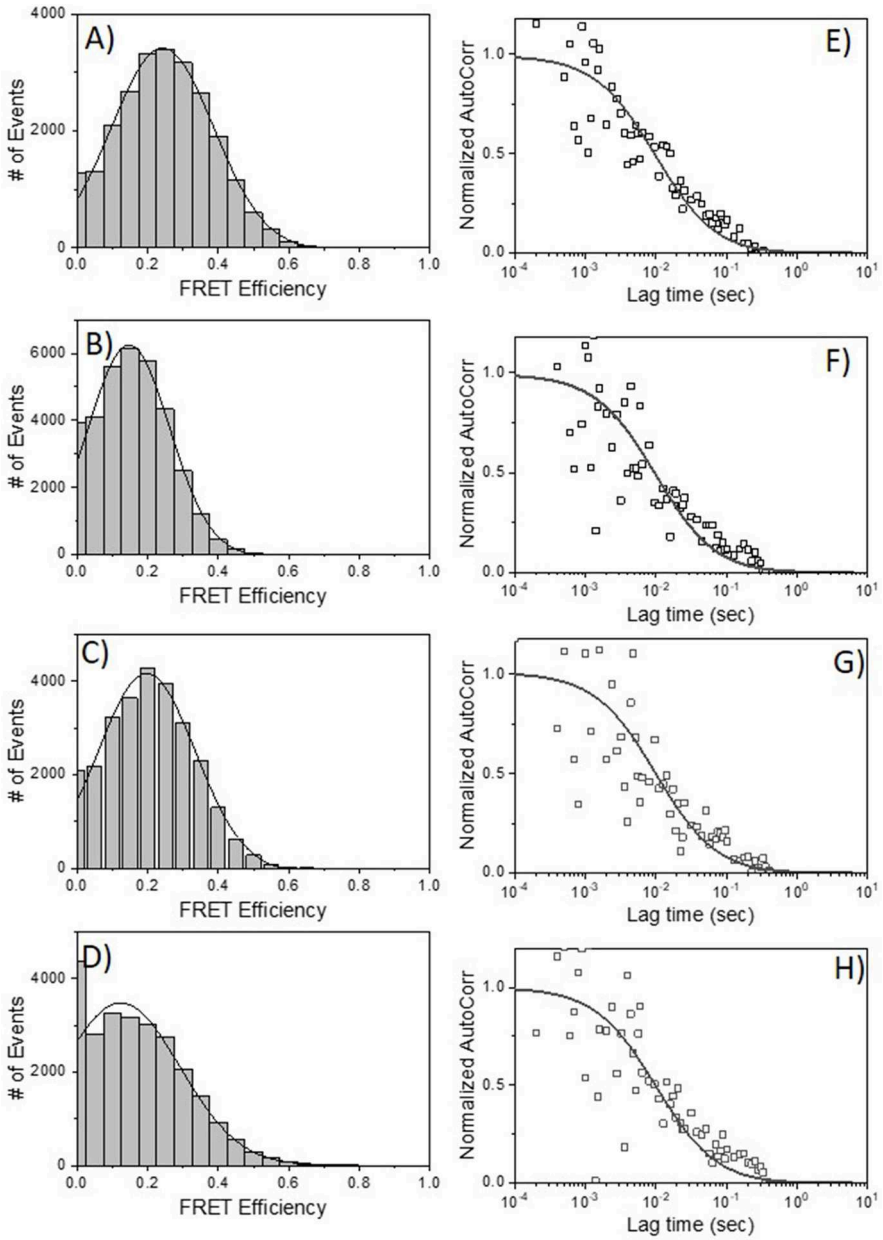

FIGURE 6 | FRET histograms of Fab pairs tested with Tier 2 HIV-1 JRFL virions. **(A)** 2G12 Fab-A488 and F240 Fab-A568; **(B)** b12 Fab-A488 and F240 Fab-A568; **(C)** N49P7 Fab-A488 and F240 Fab-A568 and **(D)** PG9 Fab-A488 and F240 Fab-A568. The solid lines in **(A–D)** are fit with a Gaussian distribution to the experimental FRET histogram data. Autocorrelation plots of the acceptor channel for **(E)** 2G12 Fab-A488 and F240 Fab-A568; **(F)** b12 Fab-A488 and F240 Fab-A568; **(G)** N49P7 Fab-A488 and F240 Fab-A568 and **(H)** PG9 Fab-A488 and F240 Fab-A568 with HIV-1 JRFL virions. The solid lines in **(E–H)** represent the fit to the experimental data. All measurements were repeated three times with similar results.

dynamic and heterogeneous positioning of PG9 Fab vs. F240 Fab, sometimes outside the FRET window, within the virion population. **Figure 7** summarizes potential scenarios for F240 and neutralizing anti-gp120 epitope presentation on virions that are consistent with the FCS data considered above. It must be noted that these sorts of Env antigens have not been apparent via virion capture approaches. One possible explanation is that the gp120-gp41 interactions in these structures are too fragile to withstand the capture process without experiencing further degradation.

Humoral immunity includes multiple Fab- and/or Fc-driven mechanisms capable of suppressing viral infections. Thus, any conserved viral epitopes on replication-competent viruses, even if non-neutralizing, are potential points of vulnerability. Humoral responses against influenza and Ebola viruses are important cases in point, where protection from infection by

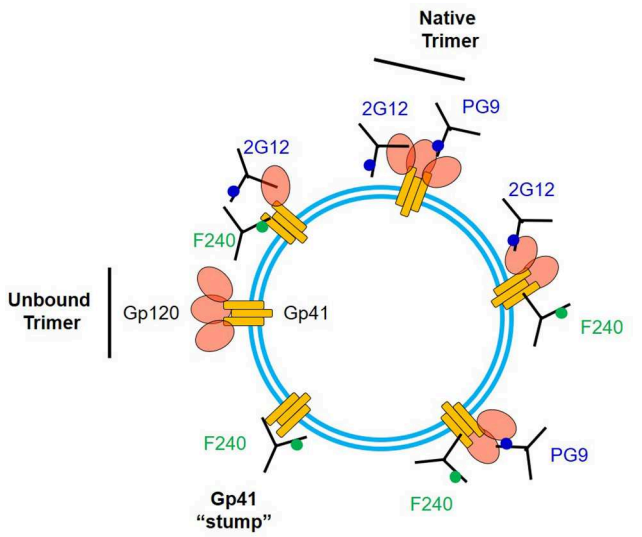

FIGURE 7 | Array of some possible structures on virion surfaces including ones that could co-express F240 and neutralizing epitopes. 2G12 and PG9 epitopes are shown as examples of the latter.

non-neutralizing antibodies has been repeatedly demonstrated (71–75). In the HIV-1 system, non-neutralizing epitopes are often considered in the context of aberrant Env structures (e.g., gp41 "stumps") on replication-defective particles while broadly neutralizing epitopes are often taken as a marker for replication-competent virions (9, 25, 26, 33, 34). However, our investigation of unadulterated, single virions in solution reveals that there is overlapping expression of both sorts of epitopes on the same virion and even on the same surface Env structures. Further, this overlap can be apparent on the majority of virions in a population. Such data point toward the potential value of developing vaccines that elicit polyclonal anti-Env responses comprising both neutralizing and non-neutralizing antibodies, which in concert may guide multiple effector mechanisms to viruses or virus infected cells.

Going a step further, our data provide a virological basis for considering whether and how certain non-neutralizing responses alone may effectively block infection, by vaccination or other preventive measure targeting gp41. In various animal models of HIV-1 infection, antibodies to F240 and related/overlapping Cluster I epitopes on gp41 have already been linked to varying degrees of resistance (31, 42, 43, 76, 77). Our data indicate that such *in vivo* effects may involve selective activity against virions, particularly given other evidence that Cluster I epitopes are poorly expressed on infected cells (41). Vaccines that generate such responses, particularly at points of mucosal exposure, merit exploration and may now be developed using new information concerning Env structures on free virions.

DATA AVAILABILITY

All data sets generated for this study are included in the manuscript and/or the **Supplementary File**.

AUTHOR CONTRIBUTIONS

KR designed the research, performed experiments, analyzed the data, and wrote the manuscript. MM and CO performed experiments. MP performed structural modeling. GL analyzed the data. AD designed the research, analyzed data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01512/full#supplementary-material

Figure S1 | Number of fluorescent eGFP.vpr HIV-1 BAL virions in the FCS focal volume (~1 fL) as a function of input p24 concentration. The measurements were performed in triplicates and average values are shown. Error bars indicate standard deviations.

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REVIEW
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Deciphering Fc-mediated Antiviral Antibody Functions in Animal Models

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Longstanding discordances and enigmas persist as to the specificities and other properties of antibodies (Abs) most effective in preventing or limiting many viral infections in mammals; in turn, failure to decipher key complexities has added to headwinds for both Ab-based therapeutic approaches and rational vaccine design. More recently, experimental approaches have emerged—and continue to emerge—for discerning the functional role of Ab structure, especially the Fc portion of antibody, in combating viral infections *in vivo*. A wide range of *in vitro* measures of antibody activity, from neutralization to antibody-dependent cell mediated cytotoxicity (ADCC)—each of these terms representing only an operational notion defined by the particulars of a given assay—are poised for assignment of both relevance and reliability in forecasting outcomes of infection. Of the several emergent technical opportunities for clarity, attention here is drawn to three realms: the increasing array of known modifications that can be engineered into Abs to affect their *in vivo* activities; the improvement of murine models involving knockouts and knock-ins of host genes including Fc receptors; and the development of additional virological design tools to differentiate Abs that act primarily by inhibiting viral entry from antibodies that mainly target viral antigens (Ags) on cell surfaces. To illustrate some of the opportunities with either zoonotic (emerging, spillover) or ancient human-adapted viruses, we draw examples from a wide range of viruses that affect humans.

Keywords: virus, antibody, Fc, FcR, neutralization, ADCC, animal models

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INTRODUCTION

The network of interactions between virus and host is not only complicated, it represents a complex adaptive system of which Ab-mediated immunity is only one important part. Despite the bewildering complexity, some useful generalizations have emerged: *in vivo veritas*; or in a colloquial tautology used in reference to viral vaccines and therapies, *the only correlate of protection is protection*. Direct testing of effectiveness in human trials is the ideal benchmark for licensure of vaccines and therapies for human use; however, in cases for which human testing is unfeasible or unethical (1, 2), indirect rationales for product licensure must be established on the basis of animal models. That is, where human health is the predominant ultimate concern of research and development, and is the standard benchmark of its relevance, the most meaningful *in vitro* assays along with non-human models of disease are sometimes necessary surrogates for human efficacy trials. And while *in vitro* assays can be highly useful as possible statistical correlates of

protection (3), they can also be poor reflections of complex realities: witness the abundant examples in which neutralization, binding titer, or hemagglutination-inhibition assays can be inadequate at best, misleading at worst (4–6). The fullness of what we wish to know about antiviral Abs is to be found in how Abs limit or sometimes exacerbate virus-precipitated disease in the body of an animal.

The scientific narrative on immunity to microbial pathogens has proceeded in waves, with peaks and troughs of emphasis on phagocytic cells, Abs, T cells, innate immunity, regulatory signaling, genomic analyses of immune repertoires, mechanisms of pathogen evasion of host responses, and so forth. Confounding the shifts in perceived importance of various aspects of immunity, there are differences in understanding of operational terms and their acronyms; a few of them used in this manuscript, and their intended meaning, are shown in **Box 1**. It is in this context, and with recognition that there already exist excellent recent reviews on discrete aspects of FcR-dependent antiviral immunity (8–16), that we aspire to offer a brief and possibly more holistic view of just one important aspect of virus-host interactions: the interactions between Abs, viral Ags, FcR, and FcR-bearing cells. We share in the anticipation and excitement of how emerging technologies may offer new experimental insights into complex processes that were previously suspected but unapproachable.

OVERARCHING QUESTIONS

Some viruses yielded long ago to empirical approaches to vaccines and Ab therapies, and those who led such progress (e.g., Jenner, Pasteur, Theiler, Salk, Sabin, Hilleman) are due tremendous credit for their insights, inventiveness, boldness, and dogged determination. Many other viruses have not surrendered so easily to either serendipity or brilliance, and in the more advanced examples, promising vaccines or Ab therapies have not yet completed their costly and uncertain journeys to licensure. It is the intractable and previously orphaned problems at which research is now directed. Restricting attention here to Ab-mediated immunity to viral infections, three major and interrelated questions arise on the path to vaccines and therapies (**Figure 1**). What Ab specificities are responsible for protection and are most desirable for their breadth and safety? What other characteristics of Abs are important for protection, especially in the Fc part of the molecule? And when these answers are known, how might vaccine be configured to elicit the most desirable specificities and types of Abs? The latter questions of immunogenicity and immunodominance have proven problematic for the diverse human population, and rational shaping of immune responses (e.g., fine specificities, types, durability) remains perhaps the greatest challenge of immunology. Here, we focus on experimental approaches to the precursor questions of what *kinds* of Ab response are desirable, and more specifically how hypotheses drawn from provisional *in vitro* correlates of protection might withstand the test of *in vivo* veracity.

THE VIRUS-AB ENCOUNTER

In previous reviews, we probed in some depth the matter of what *in vitro* virus neutralization is and is not, and how it does—and sometimes does not—align with an Ab's capacity to prevent or mitigate viral disease *in vivo* (4). We emphasized the redundancy of protective mechanisms typified by cell-targeting antibodies (CTAbs), i.e., those Abs (neutralizing or not) that mark virus-infected cells for interaction with various populations of Fc receptor (FcR)-bearing cells as well as complement (5). Many of the complexities previously noted, along with the kinds of protective functions that may or may not require FcR interactions, are summarized in **Box 2**). To simplify the narrative in this manuscript, the term "FcR" is used as shorthand for Fc gamma receptor (FcγR) unless otherwise specified. For those steeped in the large array of known FcR on immune effector cells as well as non-classical Fc-binding receptor homologs on a wider array of cells, we can only acknowledge the choice of brevity over an even greater narrative complexity, and point toward some of the many reviews available [e.g., reference (18). and citations in tables therein]. In like fashion, for the sake of brevity and "simplicity," emphasis is on Fc's of IgG molecules despite the many important uncertainties about the antiviral, interfering, or synergizing roles of IgM, IgA, and even IgE in host immunity to viruses (19).

To remind readers who are not steeped in virology, some fundamental features of virus-cell interactions in the context of adaptive immune responses are illustrated (**Figure 2**) to draw attention to the importance of viral replication cycle, the time-and-location distinctions between where Ab-dependent neutralization and Ab-dependent cell-targeting may occur in the cycle, and the rationales by which parts of the immune system mitigate rather than prevent infection. The special case of early, entry-associated targets for ADCC, best described with HIV, was covered in previous reviews (17, 20–23). Parenthetically, we affirm the complementary and interactive natures of Ab and T cell immunity, but that larger topic is not considered here. Moreover, **Figure 2** depicts only the events at a single-cell level, and reserves for elsewhere a discussion of the larger complexities of localized and distant viral spread in the infected host, viral persistence, latency, and biological systems that drive the ultimate outcomes of host survival and viral spread to new hosts.

PARALLELS WITH TUMOR MODELS

In the realm of CTAb, expectations with antiviral Abs are at least partially informed by the literature on Ab-mediated suppression or elimination of tumor cell growth *in vivo*. In fact, the human data with Ab therapy designed to eliminate cells—or otherwise interact with cell surfaces to achieve a biological effect—are more advanced in oncology and autoimmunity than they are in virology, resulting in many more licensed Ab therapies for human use (12, 14, 24, 25). In the case of tumor cell elimination, functions such as ADCC, complement-mediated cytolysis (CMC), and monocyte/macrophage-mediated killing—all categories of activity that are well-dissected yet poorly understood—are appreciated as major immune mechanisms to

BOX 1 | A brief guide to some fraught language.

- **Neutralization:** (virus neutralization) An operational term typically referring to an observed Ab-dependent decrease in viral infectivity, gene product (antigen or tag), genome, spread, or other phenomenon in a particular *in vitro* assay.
- **ADCC:** Ab-dependent cell mediated cytotoxicity, a collective and operational (assay-defined) term rooted in many possible, varied, and nonredundant *in vitro* assays that measure *FeR-dependent activities facilitated by FeR-bearing cells* with readouts such as: target cell lysis; phagocytosis; trogocytosis; NK cell activation; granzyme release; or *ex vivo* FeR binding.
- **CMC:** Ab-dependent, complement-mediated cytotoxicity, typically referring to direct or indirect measurement of lysis of antigen-bearing cells in the presence of specific Ab along with heat-labile proteins known or presumed to execute the full complement cascade. Related assays but requiring addition of FeR-bearing cells include CDCC (complement-dependent cell-mediated cytotoxicity) and CDCP (complement-dependent cell-mediated phagocytosis)
- **Protection:** Here, this sometimes-ambiguous term refers to any of several favorable outcomes: (1) prevention of viral infection ("sterile" immunity); (2) post-infection control of viral load, with mitigation of acute disease (with or without viral clearance); or (3) in the case of latent or persistent infection, sustained remission of symptoms along with reduced viral load and diminished transmission.

**A common language, with agreement upon the meaning of terms, is often missing from discussions of Ab-mediated immunity to viruses. A few important terms are shown here, as used in this manuscript. Background discussions of neutralization and ADCC have been provided previously (4, 5), and CDCP elsewhere (7).*

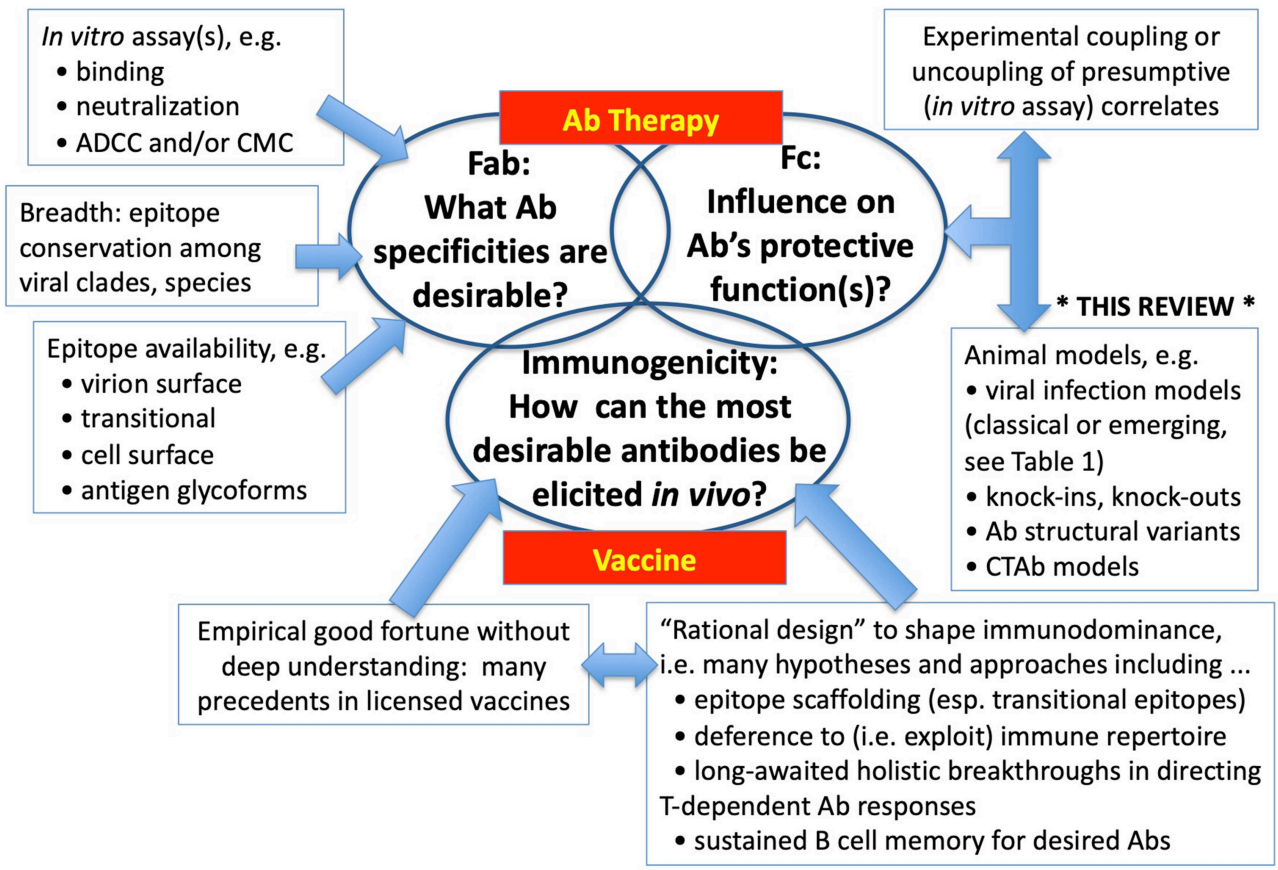

FIGURE 1 | Vaccines and Therapies: Central Questions in Ab-mediated Resistance to Viral Infections. Where empiricism and conventional strategies have not led to effective Ab-based therapies or vaccines, investigators turn to deeper understanding of: (1) the paratope (epitope-binding moiety) on the Fab; (2) the biological function-amplifying structures of Ig molecules located mostly in Fc; and (3) the unsolved complexities of how to construct vaccine Ags and microenvironments (e.g., adjuvants, cytokines) that induce Abs mirroring those most desirable.

be considered and manipulated. Routinely, developers seek to optimize Fc in order to maximize therapeutic effect and, when a proinflammatory response is undesirable, to minimize unhelpful inflammation in clinical trials. Similar clinical endeavors in human virology have been unfeasible or unethical. Nevertheless, where judged relevant, tumor models will be reflected in our considerations of how Abs act against virus-infected cells in Fc-dependent fashions.

BOX 2 | Structure-function considerations in antiviral antibodies that may confer protection*.

"Fc-independent" Ab-binding is sufficient. Fe serves only half-life and valency	"**Fc-dependent" Ab-binding is necessary but insufficient. Fe is required for one or more functions**
• "**Neutralization**" i.e., Abs that act solely as antagonists of viral binding, entry, or launch as in many common assays • **Aggregation** of virions to functionally suppress numbers of infectious units. • **Antagonism of viral assembly**, e.g., competitive inhibitors of trimmer formations or required cleavages. • **Antagonism of viral release** processes, e.g., anti-neuraminidase • **Antagonism of viral "virulence factors**" that otherwise exacerbate disease, promote intercellular spread, or aid transmission	**Opsonization:** Abs that exploit FeR to redirect infectious virions to insusceptible cells, e.g., neutrophils. • **Cell-targeting antibodies (CTAb)** that require Fe receptor (FeR) interaction for manifestation of antiviral effect. - **ADCC** as measured (17) for example by: direct lysis of infected cells; trogocytosis (RFADCC); phagocytosis; granzyme release/signaling by NK - **Complement activation:** lytic cascade; other pleotropic effects of partial complement activation • many other potential interactions

*See Box 1 for meaning of the term "Protection" in this manuscript. For discussions and additional references on these phenomena, refer to past reviews (4, 5).

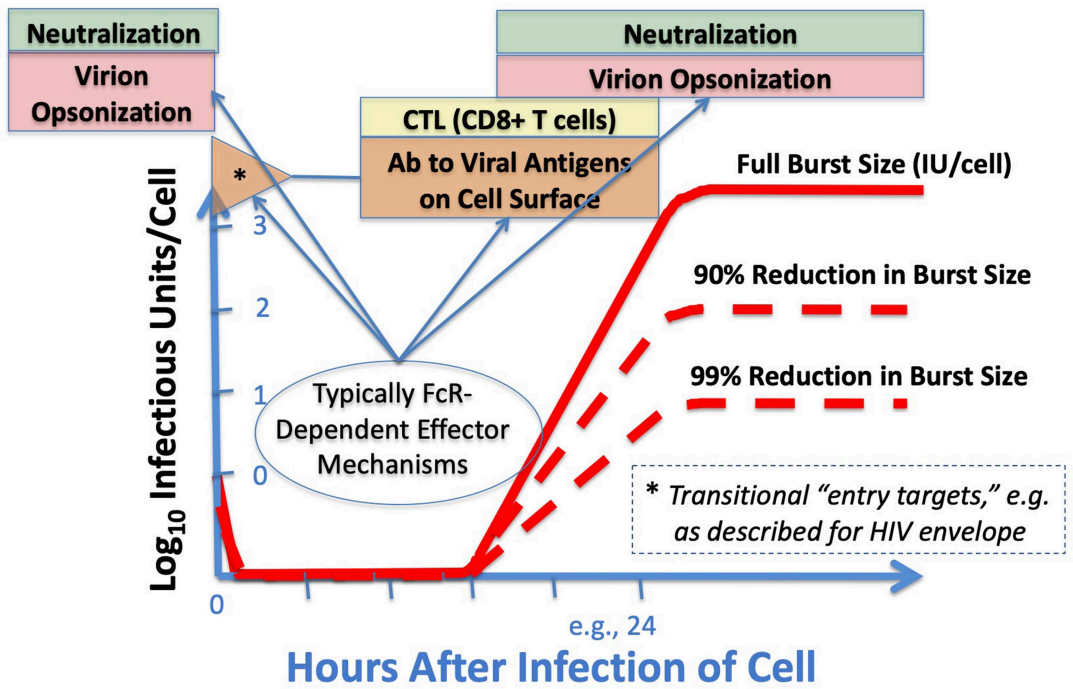

FIGURE 2 | Adaptive immunity at the cellular level, and windows of opportunity. As virus enters cells (here at a multiplicity of infection around 1 infectious unit [IU] per cell, thus non-infectious particles at even higher ratios), disassembles, and then makes new proteins and genomes on the way to making more virions, the targets available to the immune system change. Conventionally, "neutralizing Abs" inactivate or sequester virus extracellularly, either before infection or as virus emerges. FcR-bearing cells can facilitate such extracellular clearance when the FcR-bearing cells are virus-resistant, and additional proteins—such as those of the complement cascade—can augment this opsonization. Before and during viral replication, either T cells (recognizing MHC-associated peptides) or cell-targeting Abs (CTAbs, recognizing emergent and pre-assembly proteins) can disrupt cell integrity and thereby diminish the viral yield per cell (burst size) by many-fold; the sparing of uninfected cells accrues exponentially. In some cases (well-described with HIV), the entering virions display new and early CTAb targets (*) as the viral spike rearranges coincident with receptor engagement. Emphasis in this manuscript is upon recent and emerging experimental tools to decipher the *in vivo* effects of Fc-FcR interactions that result in protection, including those involving Abs shown to score positively with *in vitro* neutralization assays, ADCC assays, or both.

SOME PAST CHALLENGES IN DECIPHERING THE ROLE OF FC IN ANIMAL MODELS

Overwhelmingly, immunological data in non-human species have come from mouse models, where inbred mouse strains and myriad research reagents allow complex cell and Ab transfers. In virology, however, mice tend to be wholly or partially resistant to infection and disease caused by human pathogens of greatest interest; a compromise is sometimes found by serial passage of virus in mice to achieve some semblance of human disease and protection. For many years, even when there was

a palatable model of viral disease in mice, and early data suggested an important role for Fc in Ab-mediated resistance to certain of those viruses (4), data were generally unconvincing in assigning clear relevance to Fc because of technical limits, to wit: the necessary panels of virus-specific monoclonal antibodies (MAbs) having identical paratopes (Ag combining sites) but different Fc moieties were then unachievable. Consequently, a preponderance of evidence that murine MAbs of IgG2a subtype were generally most protective (especially for CTAbs) were less than definitive; Fab and $F(ab)_2$ fragments of Abs were almost exclusively non-protective (or poorly so) but were not directly comparable to intact Ab because the fragments (neutralizing *in vitro* or not) had short half-lives *in vivo*; attempts to deplete FcR-bearing cells *in vivo* were confounded by the overall toxicity and secondary effects of such depletions, so that truly appropriate controls were lacking; complement (C') depletion of mice typically left the protective capacity of whole Abs intact, but redundant mechanisms (ADCC as well as CMC) and incomplete C' depletion remained possibilities. Moreover, when cross-species transfers of Abs were made (e.g., human Abs into mice or non-human primates [NHP], mouse Abs into guinea pigs or NHP), positive protective results (e.g. Ab-mediated protection) were useful but negative results were fraught [not only do anti-Abs arise in a few days to eliminate xeno-Abs, but also the Fc-FcR interactions across species are problematic at best (26)].

ADVANCES IN VIROLOGY AND VIRAL PATHOGENESIS – BEYOND PASSAGE-ADAPTED VIRUSES TO ENGINEERED OR CHIMERIC CHALLENGE VIRUSES, OR VIRUS-SUSCEPTIBLE "HUMANIZED" MICE

In search of an animal model for viral disease, classical adaptation of virus by serial passage sometimes fails repeatedly, and the accumulating evidence on the nature of species barriers for any given virus may sometimes suggest that adaptation through mutation and selective pressure is highly improbable. At best, and through the lens of product licensure, Golding writes, "The establishment of animal models predictive of vaccine effectiveness in humans has been fraught with difficulties with low success rate to date."(1) Today, however, there are many ways to: (1) refashion genes of a human virus to become more likely to cause infection and disease in non-human species; (2) refashion genes of unrelated viral pathogens (e.g., ordinarily restricted to mouse or NHP) to express and incorporate presumptive "protective Ags" of human pathogens, in order to test mechanisms of immunity targeted against those antigens; (3) render a non-human species (especially mice) more human-like in susceptibility through engraftment of human cells, or through specific gene knock-ins (e.g., of human receptors for virus) and knock-outs (e.g., of host-range resistance factors such as interferon). A few examples and references are given in **Table 1**.

CTABS AND THE UTILITY OF CELLS THAT EXPRESS VIRAL AGS

As noted previously (5), the role of Fc-dependent Ab activities on viral clearance *in vivo* has too often been subordinated to an unfruitful "either-or" argument about relative importance of "neutralizing" vs. "non-neutralizing" Abs, when in fact many (but not all) neutralizing monoclonal MAbs (nMAbs) are also potent cell-targeting MAbs (CT-MAbs) see **Boxes 1**, **2**. To separate the effects of conventional nAbs from Abs that also (or exclusively) exert protective effects on viral Ag-expressing cells, the literature on tumor immunology provides useful guides in work that is already well-described and continuously evolving. For example, it is well-established that: (a) MAbs against tumor antigens (expressed on cell surfaces) can direct the elimination of antigen-expressing cells by mechanisms that require appropriate interactions between Fc and FcR (12, 36, 43); (b) anti-tumor activities (both *in vitro* and *in vivo*) can be either improved or diminished by making changes in the Fc portions of MAbs, including glycosylation (39, 44, 45); (c) complement as well as FcR can have a role in cell elimination, and experimental tools to untangle the two are improving (7, 15, 46–49); and (d) MAb interactions with inhibitory FcR can be important determinants of outcome, undermining protective effects and promoting Ag internalization (36, 50–53).

Obviously enough, many of the same experiments could be replicated—and rational improvements in antiviral Ab efficacies (and vaccines) possibly suggested—by testing the capacities of different Abs (in animal models with different FcR) to eliminate cells constitutively expressing viral membrane Ags of interest, as illustrated in **Figure 3B**. Indeed, such work has already begun (41, 42).

In addition to deeper understandings and new directions, practical rewards could arise from the establishment of model systems designed to find mechanistic correlates between *in vitro* assays and *in vivo* elimination of viral Ag-expressing cells: genuinely predictive models could facilitate bridging studies from animal models to human efficacy, otherwise a barrier for licensure of vaccines that require invocation of the "Animal Rule" (1).

CUMULATIVE, SYNERGISTIC, AND ANTAGONISTIC VARIABLES IN DISCERNMENT

As illustrated in **Figure 3**, animal models may be employed to examine effectiveness of Ab given at various times before (prophylactically) or after (therapeutically) the cognate Ag, in this case viral antigen either on virions or cells. Interpretations of outcomes are in some respects straightforward. However, behind the superficial simplicity of Abs including CTAb that may evoke Fc-dependent antiviral effector function, there exists a complex array of binding and signaling events, each with its own quantitative and qualitative dimensions. To simplify, these are illustrated in **Figure 4** as building blocks required to reach a

TABLE 1 | Classical and emerging approaches for deciphering Fc-mediated antiviral Ab functions in animal models.

General experimental approach, tool	Selected (Representative) examples
Where virus cannot cause meaningful infection or disease in a known animal model, *make a new virus*	simian/human HIV (SHIV) (27, 28); reconstructed (and controversial) influenza viruses (29, 30); surrogate live virus (e.g., VSV, vaccinia) expressing Ag from virus-of-interest
Where virus cannot cause meaningful infection or disease in a known animal model, *make a new animal*	Transduced (31) or transgenic (32) mice expressing MERS-CoV receptor; transgenic mice susceptible to hepatitis C (33, 34); multigenetic variants (35)
Identify and exploit naturally uncoupled (i.e., either/or) targets for anti-virion vs. anti-cell Abs	Alphaviruses, poxviruses, flaviviruses [reviewed in (4)]
For a given viral epitope of interest, construct *panel of MAbs having same paratope, different Fc isotypes.*	melanoma cells in mice (36); non-IgG isotypes underexplored; most viral systems reported to date lacked matching paratopes (4, 5, 37, 38)
For a given viral MAb of interest, construct *Ab variants ablated or augmented in FcR binding, complement activation, or other activity*.	tumor immunology (39, 40); influenza virus (6, 8); West Nile virus (38);
Limit examination to CTAbs by expressing one or more viral Ags on cells, then measuring *immune clearance of viral Ag-expressing cells*	HIV expressing influenza HA (41, 42)
Determine whether engineered (knockout) *mice lacking one or more FcR still retain protection by Ab*	West Nile virus (38), influenza (6), HIV (41)
Examine Ab-mediated *protection in mice lacking murine FcR but expressing one or more (knock-in) human FcR*	Mice so designed (26)
Examine Ab-mediated protection in (knockout) *mice lacking discrete FcR-bearing cell lineages*	Presaged in mice newly designed with knock-in human FcR to examine tumor immunity (26)

threshold of activity that is itself variable. Some of these are more widely familiar than others, as described next.

Proceeding from the bottommost variable illustrated in **Figure 4**, the importance of *Ab affinity for its cognate viral epitope* is well-known and foundational in immunology. As a general rule, higher affinity Abs are more active in most *in vitro assays*, and in protection. However, affinity is less an intrinsic property of Ab-epitope interaction than a measurement that can depend (over several orders of magnitude) upon epitope framework, conformation, epitope masking, and dynamic changes (rearrangements) that can occur for example when viral spikes encounter receptors, proteases, or pH changes (4, 5, 54).

Ab *concentration* is a familiar variable insofar as a concentration threshold is typically observed, below which a given Ab (monoclonal or polyclonal) is apparently ineffectual. Related, however, is the role of *Ab biodistribution* (its concentration at the necessary site of activity) which may undermine Ab effectiveness by providing insufficient concentration in critical sites including solid tumors, brain, retina, intestine, lung, and testes (55). Experimentally, as in animal models described here, there is a particular hazard at the opposite end of Ab concentration, i.e., the impulse to empirically screen for Ab effectiveness using high-dose treatments is contraindicated (as a singular approach) by the phenomenon of high-dose *prozone*, which presumably results from a diminution of the formation of complexes necessary for robust Fc-FcR interactions (56), akin to high-dose prozone in classical immunoprecipitation reactions. Another explanation offered [outside the binding-valency model (56)] is that the encounter between high Ab dose and high Ag burden results in an exhaustion of complement components (especially C2 and C4) with the consequence of diminished overall antibody effectiveness *in vivo* (15). In experimental animal models, a range of Ab doses is preferred.

Epitope accessibility refers to the longstanding observation, recapitulated in different ways with many viral Ags (4, 5, 54), that some viral epitopes are available to Ab (and thus to Fc-dependent immune effector mechanisms) only at selected times (or transiently) during the viral entry and growth cycle. We used the word "cryptic" in 1983 to describe epitopes that appear to be inaccessible on intact virions yet available on virus-infected cells; but "availability" may in other instances be an inadequate oversimplification of the dynamic nature of viral spike proteins.

Epitope topology and membrane proximity can determine spatial relationships and steric hindrances that favor one type of Fc-mediated effector function over another, illustrated for example in the development of therapeutic anti-CD20 Abs (40, 57) and strongly implied with a panel of influenza-specific MAbs (6). Some current data suggest that: "… complement-dependent cytotoxicity and Ab-dependent cellular cytotoxicity favored a membrane-proximal epitope, whereas Ab dependent cellular phagocytosis favored an epitope positioned further away." (57) More broadly, the favored (optimal) configuration for a given effector function may vary with different Ag-Ab pairs, exemplified by counter-examples in which HIV-specific MAbs against the "membrane proximal region" of envelope may have negligible effector function (22). Generalizations are difficult, as they are confounded by mechanistic differences between *in vitro* assays (17); *in vivo* efficacy will be decisive.

Ag concentration (or density) on target cells is a factor in shifting the apparent effectiveness of Ab, i.e., there is an inverse relationship between Ag expression on target cells and the amount of Ab required to meet a threshold of Fc-dependent activity. Part of anti-CD20 therapeutic efficacy is attributed to

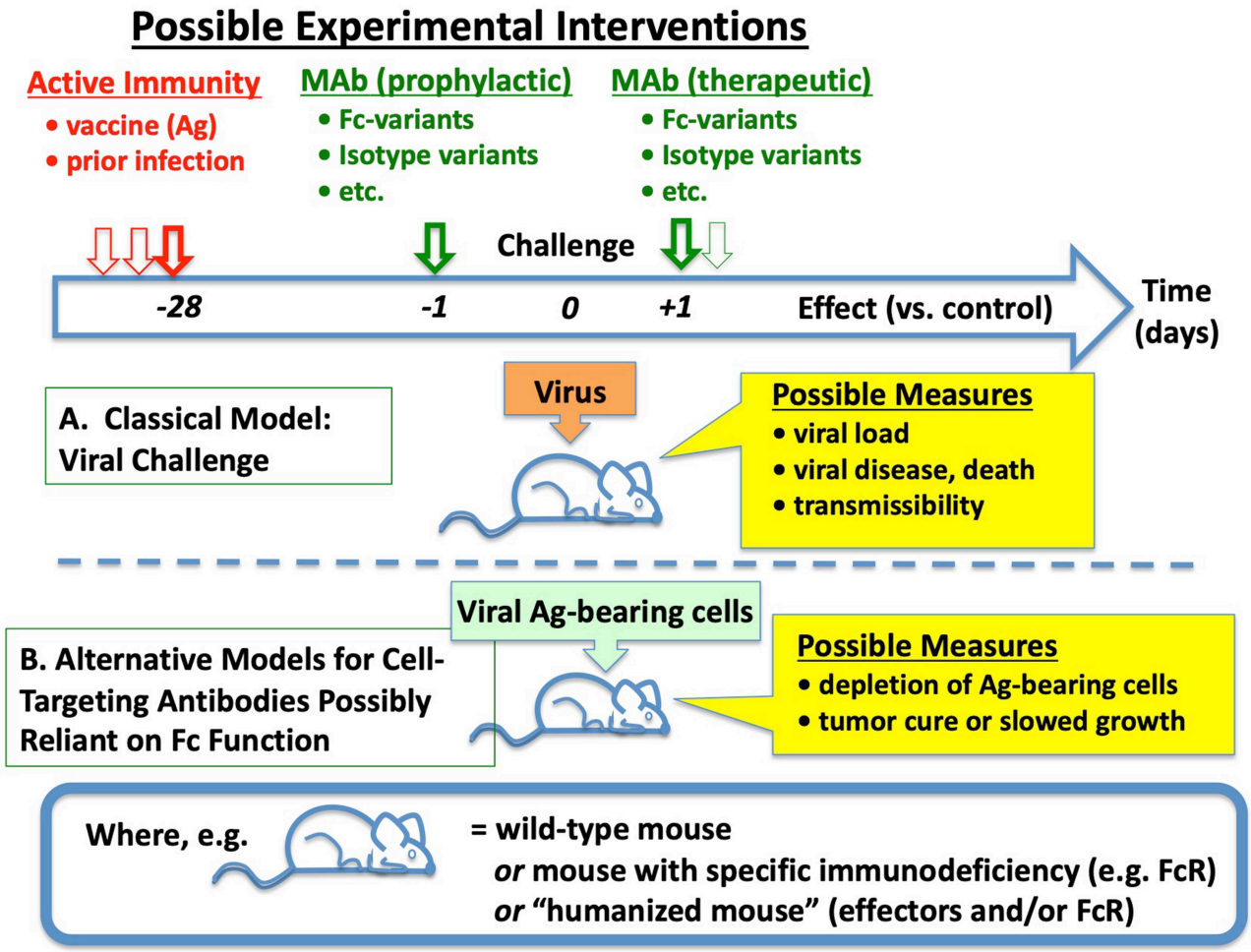

FIGURE 3 | Simplified Overview of Emerging Approaches for Discernment of Roles of Fc in Ab-mediated Immunity to Viruses. **(A)** In classical models, experimental animals (often mice) are challenged on day 0 with infectious virus, preceded (or sometimes followed) by intervention with active or passive immunization. **(B)** To disambiguate the collection of phenomena that only affect virions and their spread, and examine more closely the requirements for effective Fc-mediated effects against viral Ag-expressing cells, one can challenge mice instead with cells engineered to express viral Ags, somewhat mimicking prior models of tumor immunotherapy. See text and references for many of the potential complexities and opportunities in each step, including modified Abs, and highly modified mice.

high expression of CD20 on tumor cells (40), and it was recently shown that increased expression of target Ag on cells is one way to improve Fc-mediated cell clearance in mice otherwise compromised by persistent viral infection (58).

Fc-FcR affinity. We begin with a quote in a recent paper from the Ravetch group, leaders in the field of Fc-FcR interactions, and murine models with which to explore biological significance: "An antibody's Fc domain's relative affinity for the activating and inhibitory Fc**γ**R, called the A/I ratio, can determine its functional output, and is directly correlated to therapeutic efficacy *in vivo*. This has spawned recent efforts to engineer Abs with enhanced activating Fc**γ**R affinity." (26) Embedded in much of their work is the directly observed or implied importance not only of isotype (36) but also species matches in establishing Fc-FcR affinity: human, mouse, and non-human primate (NHP) FcR are non-equivalent in binding to any given Ab (typically, human IgG1 is the chosen type), and nomenclature of FcR in the various species is a poor guide to Ab affinity and function.

The current scientific literature is rich with the recognition of naturally occurring *Fc and FcR alleles or variants* (19, 59), followed by structural redesigns of Ab molecules to optimize *Ab glycosylation* (8, 12, 39) or *Ab half-life* (60). The details are outside the scope of this manuscript, but as with other variables cited above, we call attention to the caution with which the Fc-dependent possibilities or limitations of a single MAb may be viewed during the course of experiments.

Allosteric change in Fc is a phenomenon that remains incompletely resolved in terms of biological significance. However, improved tools in structural analysis restore the possibility that allostery is among the factors that may contribute additively or synergistically to Ab function. Thus, in a subset of MAbs and presumably in a subset of natural polyclonal Abs, the binding of Fab to its cognate epitope results in *allosteric*

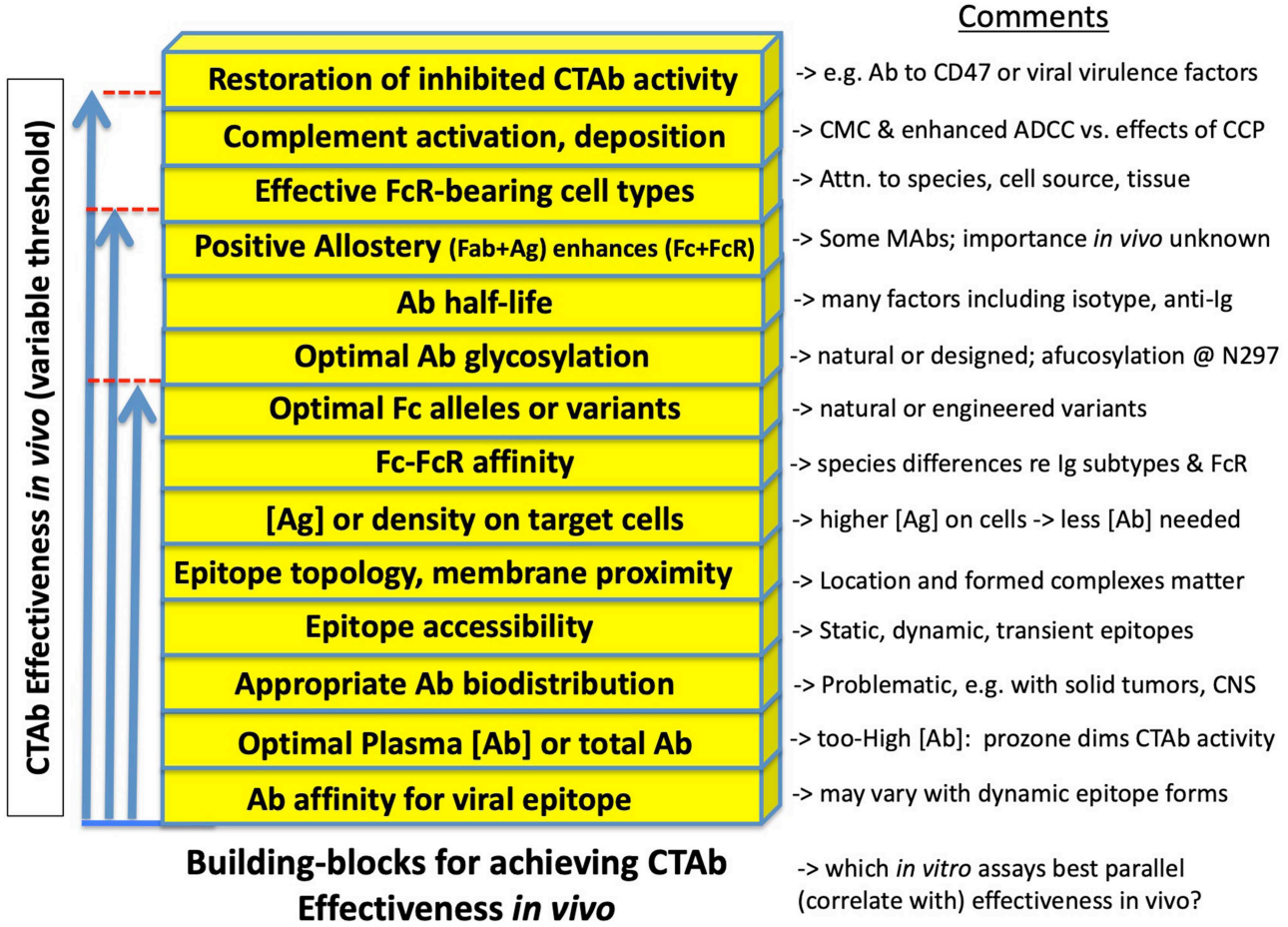

FIGURE 4 | Concepts represented as building blocks for effectiveness of cell-targeting Abs in immunity to viral infections. See text for details. Many factors—each with its own quantitative or qualitative dimension—contribute additively or synergistically to reaching a threshold of antiviral activity that meets a standard of protective efficacy (as defined in **Box 1**). The threshold is not a constant, but varies by individual, host species, virus biotype, dose and route of infection, etc. In short, the figure is intended to illustrate that Fc-mediated aspects of Ab-mediated immunity are interrelated parts of a complex adaptive system that arose during co-evolution of host and virus. To adequately exploit these concepts with vaccines or therapeutic Abs requires either empirical good fortune (the key to almost all licensed vaccines) or understandings that allow rational improvements in design. In the search for statistical or mechanistic correlates of protection, assays that combine several of these factors may be favored.

change in Fc, which in turn promotes higher affinity between Fc and FcR than is observed in the absence of Ag (61, 62). The implication is that, depending upon the assay, *in vitro* results may be an inadequate predictor of Fc-mediated functional activity of Ab. A different kind of allostery is seen when two MAbs synergize on the basis of how Fab binding to one part of an Ag molecule promotes binding of a second MAb recognizing a different epitope on the same Ag (63). Newer animal models may add clarity to the functional importance of such allosteric interactions in Ab-mediated protection against viruses.

The importance of *effective FcR-bearing cell types* should not be underestimated, especially in cross-species transfers, such as human Ig transferred into NHP or mice. The complexities are several, due not only to interspecies differences in FcR affinity for any given MAb, but interspecies differences in the cell types on which various FcR are found (26).

In virological circles, *complement activation and complement deposition* have received diminishing attention, presumably because ablation of complement (in murine models) tends to leave intact the protective antiviral capacity of an Ab (4, 5). However, the dismissal of complement is likely imprudent, as antibody therapies against human tumors show important additive and augmenting effects mediated by complement (39, 48, 49, 64). As signaled in **Figure 4**, attention must be paid to the additive and synergistic Fc-mediated effects of Abs, as these cumulatively determine whether a threshold is reached in which an Ab is effective *in vivo*.

The potential for *restoration of inhibited CTAb activity* is an emerging opportunity for understanding and improving the protective capacities of CTAb against viruses. Once again, tumor immunology has led the way, with anti-CD47 Abs already in clinical trials, and showing clinical promise by way

of dampening a "don't eat me" signal that otherwise spares tumor cells from destruction by monocytes and macrophages (65, 66). Many poxviruses explicitly express CD47 homologs, and other complex viruses such as herpesviruses express homologs of immunomodulatory proteins (67). The potential for anti-CD47 to shift the threshold for antiviral attention has not escaped notice (58), but this may be only the first of many opportunities to counteract known (67) and perhaps unknown viral proteins that undermine host immune responses including Fc-dependent activities. To venture a testable hypothesis, this could be part of the mechanism by which a herpesvirus subunit (HZ/su), consisting of one of the family of herpesvirus proteins (gE) that binds Fc (68), serves as an effective vaccine (68): by evoking Abs that restore otherwise-inhibited and FcR-dependent CTAb activity.

CONCLUDING REMARKS

While some elements of Ab-mediated antiviral immunity appear to be largely or completely independent of Fc function, others are highly reliant on Fc in order to exert biological effects that register as "protective" activity (**Boxes 1**, **2**). Such Abs (CTAbs) share with a number of therapeutic anti-cancer Abs the aim of arresting or destroying cells recognized by such Abs, and mechanistic relevance can be gleaned from the extensive research and clinical trials with anti-tumor CTAbs. However, the efficacies of antiviral CTAbs are differently complex due to both the typical incapacities to obtain human protection data and the longstanding problems inherent with classical animal models of viral disease. More recently, newer approaches (**Table 1**) have allowed increasing compatibility between a given virus (or its cell-expressed antigen), a susceptible animal model, Fc-FcR interactions, populations of FcR-bearing effector cells, etc. This brief review is intended to highlight and cite some of the recent literature that first points toward an almost bewildering complexity in the factors that intrude upon the subset of protective antiviral mechanisms that are Fc-dependent, and then to some feasible approaches to achieve clarity. Most likely, for a given virus with its unique structure and biology, and its unique pathogenetic profile in a chosen animal model, some Fc-dependent Ab-directed mechanisms will prove more reliably important than others. Moreover, each of the concepts illustrated in **Figure 4** is meant here to be understood as scalable in its importance, but rarely if ever sufficient by itself to achieve a threshold that achieves enough efficacy to adequately suppress viral replication and prevent viral disease. It is most encouraging that experimental tools, including those shown in **Table 1**, are emerging to affirm or refute the truth and biological importance of concepts previously mired in the complexities. To an increasing degree, rational selection and design of optimally effective MAbs (and the means to elicit them with vaccines) will inform antiviral research. Similarly, refinement and selection will continue to improve for *in vitro* assays that are not only statistical correlates but mechanistic correlates of protection. And still, the final proofs will continue to be an empirical matter of finding what is both safe and effective.

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AS, CO, and GL shared and discussed in lab meetings over a period of many months in the development of perspectives, emphases, and cited references for this manuscript. AS crafted the primary draft, which was improved, and approved by CO and GL.

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IgA and IgG1 Specific to Vi Polysaccharide of *Salmonella* Typhi Correlate With Protection Status in a Typhoid Fever Controlled Human Infection Model

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Vaccination against *Salmonella* Typhi using the Vi capsular polysaccharide, a T-cell independent antigen, can protect from the development of typhoid fever. This implies that antibodies to Vi alone can protect in the absence of a T cell-mediated immune response; however, protective Vi antibodies have not been well-characterized. We hypothesized that variability in the biophysical properties of vaccine-elicited antibodies, including subclass distribution and avidity, may impact protective outcomes. To interrogate the relationship between antibody properties and protection against typhoid fever, we analyzed humoral responses from participants in a vaccine efficacy (VE) trial using a controlled human infection model (CHIM) who received either a purified Vi polysaccharide (Vi-PS) or Vi tetanus toxoid conjugate (Vi-TT) vaccine followed by oral challenge with live *S.* Typhi. We determined the avidity, overall magnitude, and vaccine-induced fold-change in magnitude from before immunization to day of challenge of Vi IgA and IgG subclass antibodies. Amongst those who received the Vi-PS vaccine, Vi IgA magnitude (FDR $p = 0.01$) and fold-change (FDR $p = 0.02$) were significantly higher in protected individuals compared with those individuals who developed disease ("diagnosed"). In the Vi-TT vaccine group, the responses of protected individuals had higher fold-change in Vi IgA (FDR $p = 0.06$) and higher Vi IgG1 avidity (FDR $p = 0.058$) than the diagnosed Vi-TT vaccinees, though these findings were not significant at $p < 0.05$. Overall, protective antibody signatures differed between the Vi-PS and Vi-TT vaccines, thus, we conclude

that although the Vi-PS and Vi-TT vaccines were observed to have similar efficacies, these vaccines may protect through different mechanisms. These data will inform studies on mechanisms of protection against typhoid fever, including identification of antibody effector functions, as well as informing future vaccination strategies.

Keywords: typhoid fever, *Salmonella* Typhi (*S.* Typhi), Vi polysaccharide, correlates of protection (CoP), humoral immunity, vaccination, antibody, avidity

INTRODUCTION

The bacterial pathogen *Salmonella enterica* serovar Typhi (*S.* Typhi) is the leading cause of enteric fever world-wide and is responsible for nearly 20 million infections and ∼200,000 deaths annually. Infection predominantly affects travelers and citizens from low-income countries, where children under 5 years of age face the highest disease burden (1, 2). Historically, antibiotics have been the standard treatment for infection. However, antibiotic resistance among *S.* Typhi clones has dramatically increased in endemic regions including the emergence and spread of an extensively drug resistant (XDR) clone throughout Pakistan in 2018 (3, 4). This has sparked concern for continued spread of XDR *S.* Typhi internationally, therefore, vaccination efforts against typhoid fever have become the primary focus for prevention (5).

The live-attenuated Ty21a vaccine and the subunit Vi polysaccharide (Vi-PS) vaccine constitute two of the most widely used typhoid fever vaccines worldwide. Both Ty21a and Vi-PS exhibit moderate efficacy of ∼50–55% in the first year (6, 7), but neither is suitable for use in infants and young children. Ty21a is limited to use in children over 5 years of age due to formulation in large oral capsules that are difficult to swallow. Vi-PS is non-immunogenic in children under 2 years of age (8, 9) due to the nature of the Vi polysaccharide as a T-cell independent antigen that does not engage the T cell pool or stimulate germinal center reactions in young children (9, 10). Conversely, the Vi polysaccharide capsule confers bacterial resistance to complement deposition and phagocytosis (11–13), allowing the bacterium to disseminate systemically; therefore, Vi remains a major target for vaccination efforts against typhoid fever.

In light of this, typhoid protein-polysaccharide conjugate vaccines (TCVs) have been developed which may improve immune responses in early childhood and help combat this illness (14). In particular, a Vi-tetanus toxoid (Vi-TT) conjugate vaccine, which has recently undergone WHO prequalification, has been shown to be more immunogenic in adults than the Vi-PS vaccine (15). Vi-TT was evaluated for efficacy in the recent Vaccines against *Salmonella* Typhi (VAST) trial, in which healthy adult volunteers were vaccinated with either Vi-PS or Vi-TT and then orally challenged with live *S.* Typhi bacteria. Following challenge, participants exhibiting a positive *S.* Typhi blood culture and/or prolonged fever of ≥38°C for ≥12 h were defined as diagnosed. Interestingly, the attack rate, defined as the proportion of participants diagnosed with typhoid fever, was similar between vaccine groups, with 37% of Vi-PS vaccinees being diagnosed with typhoid fever and 35% of Vi-TT vaccinees being diagnosed (15). The lack of increased efficacy of the conjugate vaccine compared with the purified polysaccharide vaccine highlights the need for determining and better understanding the characteristics which confer protective immunity to typhoid fever.

For many years, research on immune mechanisms of protection against typhoid fever have been greatly hindered due to *S.* Typhi restriction to human hosts (11). However, regardless of similar attack rates between the Vi-PS and Vi-TT vaccines, the VAST trial, which used a controlled human infection model (CHIM) to measure efficacy, provides a direct approach to investigate the human immune response and identify immunological correlates of protection against typhoid fever. Despite higher immunogenicity of the Vi-TT vaccine, a significant difference in Vi total IgG titers between diagnosed and protected individuals was only observed in the Vi-PS group (15). Moreover, when comparing IgG1, IgG2, and IgG3, only Vi IgG2 subclass titers predicted protection in the Vi-PS group. While there were no correlations with Vi IgG subclass titer and protection in the Vi-TT group, Vi-TT vaccinees reported less severe clinical symptoms and most diagnosed participants had positive bacteremia with no reported fever whereas a higher proportion of diagnosed Vi-PS vaccinees had both fever and bacteremia (15). Given these differences in immune response and patterns of protection, we hypothesized that variability in the biophysical properties of antibodies induced by the Vi-PS and Vi-TT vaccines, including antibody subclass distribution and avidity, may impact protective outcomes. The Vi-PS vaccine, which exhibits moderate efficacy over 3 years, demonstrates that antibodies against Vi can impart protection in the absence of a cell-mediated response (16, 17). However, the critical classes, characteristics, and functional mechanisms of these protective antibodies against typhoid fever have not been identified.

It is well-established that different isotypes (IgA vs. IgG) and subclasses (IgG1 vs. IgG3) of antibodies have different distributions throughout the body as well as different biophysical characteristics that greatly influence antibody affinity (18, 19). These characteristics also determine success in antigen binding, recruitment of effector cells, and subsequent antibody Fc-mediated effector functions (20). Here we evaluate the maturation level and magnitude of the circulating antibody response to the Vi polysaccharide of *S.* Typhi and determine whether these measures correlate with protection in a typhoid fever CHIM. Determining the nature of the vaccine-elicited antibody response including proportion of Ig subclasses and affinity of those antibodies may be important in further identifying which functional mechanisms play a role in

protection. These insights on antibody biomarkers of protection will pave the way for advances in vaccine design toward more efficacious vaccines, allow comparative assessment of new Vi-conjugates, and inform the use of Vi-conjugate vaccines in new populations, with resulting improvements in health.

MATERIALS AND METHODS

VAST Trial

The VAST clinical trial (Clinicaltrials.gov ID: NCT02324751) was conducted as previously described (15). Briefly, participants provided written informed consent to receive either a single-dose control meningococcal-conjugate vaccine, a Vi polysaccharide (Typhim, Vi-PS) vaccine, or a Vi-tetanus toxoid conjugate vaccine (Typbar TCV, Vi-TT) intramuscularly at day minus 28 (D-28) (pre-vaccination/baseline). At 4 weeks post-vaccination, day 0 (D0), participants were challenged with *S.* Typhi by oral ingestion and monitored for development of *S.* Typhi bacteremia or persistent fever $\geq$38°C for 12 or more h (diagnosed) or lack thereof (protected). Immune responses to vaccination were assessed in the Vi-PS ($n = 35$) and Vi-TT ($n = 37$) groups at 4 weeks post vaccination (D0) as well as 3 and 6 months post-challenge (D90, D180).

Binding Antibody Multiplex Assay- Avidity Index (BAMA-AI)

The WHO international standard for Vi polysaccharide (*C. freundii*, NIBSC, UK, Product Code: 12/244) (21) was biotinylated by Innova Biosciences. Vi polysaccharide and tetanus toxoid binding assays were modified from the binding antibody multiplex assay (22, 23). Briefly, biotinylated Vi polysaccharide (ViBiot) was conjugated to neutravidin-coupled, magnetic, Luminex microspheres, and tetanus toxoid (Reagent Proteins, USA) was conjugated by amine coupling with EDC/NHS. Native Vi polysaccharide (nViPS) was conjugated to polystyrene microspheres using an adipic acid dihydrazine linker as previously described (24, 25). Antigen-coupled beads were incubated with diluted vaccinee serum or plasma, followed by incubation in PBS or dissociative pH=3 sodium-citrate (CIT) buffer. Detection reagents include R-Phycoerythrin-conjugated affiniPure goat anti-human IgA, alpha chain specific (Jackson Immunoresearch, USA), mouse anti-human IgA1-Biot (Southern Biotech, USA), mouse anti-human IgA2-Biot (SouthernBiotech, USA), mouse anti-human IgG1 antibody (BioLegend, USA), mouse anti-Human IgG2 (Biolegend, USA), and mouse anti-Human IgG3 (Invitrogen, USA) followed by goat anti-Mouse IgG, Human ads-PE (Southern Biotech, USA). IgG4 Vi and TT levels were below the limit of detection (data not shown). Fluorescence intensity (FI) was collected using the Bio-Plex 200 Platform. Plots are representative of $n = 2$ technical and experimental replicates. Positive controls included mouse anti-Vi IgG1 monoclonal (lot 188L-8; Statens Serum Institute Diagnostica A/S, DK) and WHO International Standard for anti-typhoid capsular Vi polysaccharide human IgG (16/138 WHO typhoid IS, NIBSC, UK, Product Code: 16/138). Normal human serum (NHS, Sigma, USA) and typhoid seronegative serum samples were used as negative controls, and non-specific binding to beads was controlled by subtracting FI reading of blank beads. IgA isotype assays were performed on IgG depleted serum or plasma. Magnitude of response was multiplied by dilution factor. Fold-change was calculated as the ratio of magnitude at D0, D90, or D180 to baseline (D-28). For MFI below 100, MFI was truncated to 100 for magnitude and fold-change calculations due to noise range of the instrument. Preset criteria for positive vaccine response were: MFI*Dilution > 95th percentile of baseline (D-28), MFI > 100, and MFI*Dilution >3-fold over subject-specific baseline (D-28) MFI*Dilution. Avidity Index (AI), expressed as a percentage, was calculated as $AI = \left[\frac{FI-Bkgd\ (CIT)}{FI-Bkgd\ (PBS)}\right]^{*} 100$. Subclass specific standard curves were included in every assay by conjugating the same primary detection antibodies that were used to detect the vaccine samples, including anti-human Ig (A, G2, G3), to beads via amine coupling followed by titration of known concentrations of purified Ig and detection with goat anti-human kappa-Biot (Southern Biotech, USA) and streptavidin-PE (BD Pharmingen, USA). The subclass standard curves utilized the exact same capture antibody that was used to detect the subclass-specific Vi antibodies in vaccinee sera/plasma, to ensure that the capture of polyclonal vaccine antibodies had equal sensitivity to that of the purified Ig capture in the subclass standard curves. Antibody concentrations were reported from serum dilutions in the linear range of the assays. For the IgG1 subclass standard curve, the goat anti-human kappa was amine coupled to bead, followed by titration of purified IgG1, and detected with Mouse anti-Human IgG1 (BioLegend, USA) and Goat anti-Mouse IgG, Human ads-PE (SouthernBiotech, USA), to detect vaccine specific responses (22). The μg/ml concentration of typhoid specific antibodies were calculated for positive vaccine responders only (defined as MFI*dilution >95th percentile of the baseline (D-28), MFI > 100, and MFI*Dilution >3-fold over the subject-specific baseline).

Human Isotyping Immunoassay

The total concentration of antibody within 16/138 WHO typhoid IS was quantified using the Bio-Plex Pro Human Isotyping Assay kit per manufacturer's instructions (26). Briefly, anti-human IgA, IgM, IgG1, IgG2, IgG3, and IgG4-conjugated magnetic microspheres were incubated with titrated 16/138 WHO typhoid IS serum followed by primary and secondary detection. Fluorescence intensity (FI) was collected using the Bio-Plex 3D instrument (Bio-Rad, USA). The isotype and subclass components of the international serum standard, 16/138 typhoid IS, were determined with this method (5.69 mg/ml IgG1; 6.84 IgG2; 0.57 IgG3; 0.58 IgG4; 1.38 IgM; 0.96 IgA mean mg/ml, of 6 technical replicate measurements). These concentration units were utilized to calculate antibody subclass concentration in μg/ml equivalents of 16/138 in vaccinee sera. The 16/138 Typhoid IS IgA equivalents are noted for the non-IgG depleted standard.

BioLayer Interferometry (BLI)

Polyclonal IgG fractions from vaccinees were purified from serum and plasma by Protein G HP MultiTrap™ plates (GE

Healthcare, USA) prior to analysis by BLI. Kinetics of polyclonal antibody responses to Vi polysaccharide were analyzed by BLI as previously described (27) with modifications. Briefly, nViPS (5 μg/mL) was immobilized to aminopropylsilane (APS) biosensors via hydrophobic interaction. nViPS loaded sensors were then washed with 10X Kinetics Buffer (ForteBio, USA) in order to coat unoccupied sensor area and minimize non-specific binding. A baseline time course was established in 1X Kinetics Buffer, and then Ag-loaded sensors were dipped into wells containing 100 μg/mL (in 1X Kinetics buffer) purified IgG from vaccine participants to monitor Ab association. The dissociation step was monitored by dipping polyclonal Ab-bound sensors back into the 1X Kinetics buffer wells used to collect the baseline. Non-specific interactions were subtracted out using parallel blank sensors that were also washed in 10X Kinetics Buffer and dipped into polyclonal samples. The subtracted binding curves were used to obtain antibody binding response (nm) and dissociation rates (k_d). Avidity score was calculated based on [binding response (nm)/dissociation rate (s^{-1})].

Statistical Analyses

Comparisons between diagnosis status or between vaccine groups (Vi-PS vs. Vi-TT) were conducted by non-parametric Wilcoxon rank-sum tests. Paired comparisons between time points were conducted using non-parametric Wilcoxon signed-rank tests. Comparisons between ViBiot and nViPS antigens for any measurement (magnitude, fold-change, avidity index) were conducted using non-parametric Spearman rank correlation. Multivariate analysis of all variables measured by BAMA and BLI was performed using Principle Components Analysis (PCA). Statistical analyses were performed using R statistical software (version 3.5.1; R Foundation for Statistical Computing, AT). All raw *p*-values were adjusted within isotype and antigen using the Benjamini-Hochberg method to account for multiple testing; adjusted *p*-values <0.05 were considered significant.

RESULTS

IgA Dominates the Vaccine-Elicited Antibody Response to Vi Polysaccharide

Assessment of subclass-specific Vi antibody concentrations in post-vaccination samples identified Vi IgG2 as the predominant subclass (antibody median concentration of ∼10–20 μg/ml) (**Figure 1A**). However, after factoring in baseline responses (D-28) to include only positive vaccine responders, as previously defined in the methods section, Vi IgA antibodies were induced most in response to vaccination (D0). Specifically, Vi IgA1 antibodies exhibited the highest fold-change from baseline to day of challenge for both vaccine groups, followed by Vi IgG2 (**Figure 1C**, **Table 1B**). IgG3 responses were almost exclusively elicited in the Vi-TT vaccine group; however, the concentration was very low (**Figure 1A**). No antibody subclass was significantly boosted following oral challenge (D90, D180) with live *S.* Typhi (**Figure 1A**). As expected, the binding response to TT was only boosted in vaccinees who received Vi-TT and boosting was primarily in the IgG1 subclass (**Figure 1B**, **Table 1A**). Response rate and magnitude of IgA and IgG2 response to Vi by day of challenge (D0) was significantly higher in Vi-TT individuals; however, IgA and IgG2 median avidity was not significantly different between vaccine groups (**Table 1B**, **Table 3**) despite a broader range of IgA and IgG2 avidity in the Vi-TT group. To determine the relationship between the immune responses elicited by Vi-PS and Vi-TT vaccines, a principle components analysis (PCA) was conducted (**Figure 1D**). Variables include binding response magnitudes by BAMA and BLI for Vi and TT antigens across all subclasses: total IgA, IgA1, IgA2, IgG1, IgG2, IgG3, and total purified IgG (**Figure 1D**). At all the time points measured, IgG4 subclass antibodies to Vi and TT were below the limit of detection. Vi-TT vaccinees clustered away from Vi-PS vaccinees on the basis of higher tetanus and higher Vi antibody responses (**Figure 1D**), indicating that response to tetanus was a substantial driver of the differences observed between vaccine groups.

Higher Vi Polysaccharide-Specific IgA Magnitude and Fold-Change in Protected Vaccinees

To examine the question of whether pre-existing immunity impacted vaccine responsiveness, we evaluated the levels of baseline responses (D-28) across all antibody measurements in this study. The only antibody responses with a detectable measurement at baseline that showed a difference between responders and non-responders was IgA to tetanus (median MFI values of 563 and <100 at a 1:50 dilution, respectively). However, there was no difference in baseline responses between diagnosed vaccinees and protected vaccinees. Both the magnitude and fold-change from baseline (D-28) of ViBiot IgA were significantly higher in Vi-TT vaccinees at day of challenge (D0) and 3 months post-challenge (D90) when compared with Vi-PS; however, there was no significant difference between groups by 6 months post-challenge (D180) (**Figures 2A,B**, **Supplementary Material**). ViBiot-specific IgA magnitude was higher in protected compared with diagnosed Vi-PS vaccinees (**Figure 2C**, **Table 2**), and IgA fold-change was higher in protected compared with diagnosed Vi-TT vaccinees (**Figure 2D**, **Table 2**), however these observations were not statistically significant (**Table 3**, FDR $p = 0.078$, FDR $p = 0.061$). In addition, anti-Vi IgA avidity was slightly higher in protected individuals in the Vi-TT group, however this was not significant (**Figure 2E**, **Table 2**, FDR $p = 0.231$).

To further characterize the antibody responses, we utilized two available forms of Vi polysaccharide (i.e., nViPS and ViBiot) with slightly different antigenicity profiles (**Supplementary Material**, **Figure 1**). Both magnitude (**Figure 3A**, **Table 2**, FDR $p = 0.01$) and fold-change (**Figure 3B**, $p = 0.02$) of nViPS IgA correlated with protection status in the Vi-PS vaccine group. Similar to ViBiot IgA, fold-change of nViPS IgA appeared higher in protected individuals of the Vi-TT vaccine group, however this was not statistically significant (**Figure 3B**, FDR $p = 0.12$). To determine whether there was a protective threshold concentration of Vi-specific IgA and to facilitate comparison across studies and trials, we calculated the concentration of Vi-specific IgA in μg/ml equivalents of the WHO typhoid Serum International Standard (NIBSC 16/138).

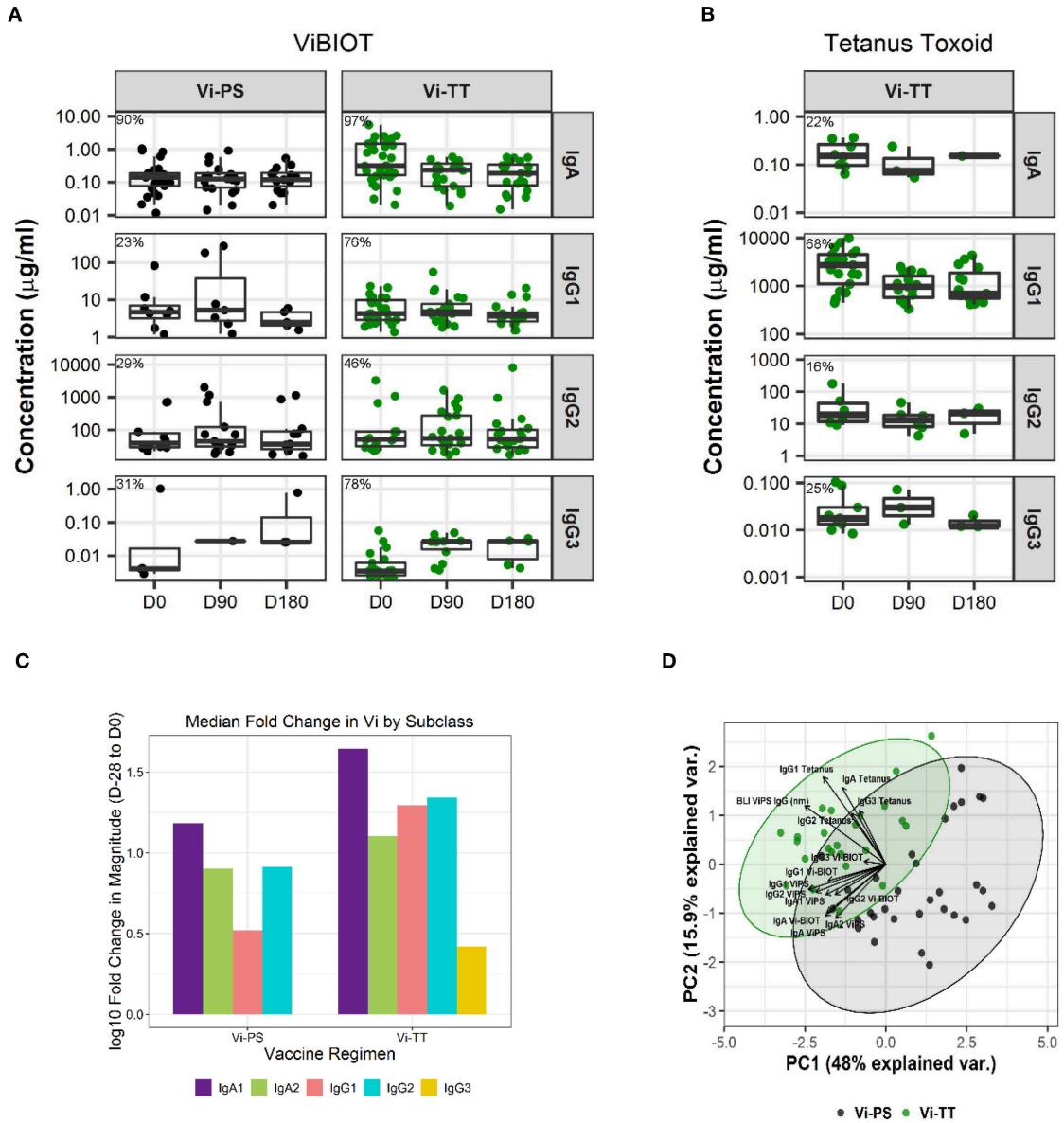

FIGURE 1 | IgA dominates the vaccine-elicited antibody response to Vi polysaccharide. Concentration of antigen-specific, vaccine-induced IgA, IgG1, IgG2, and IgG3 to ViBIOT **(A)** and tetanus toxoid **(B)** by vaccine group, Vi-PS in black and Vi-TT in green, of positive vaccine responders only. Percent positive responders indicated post-vaccination at D0. Vi-PS vaccinees exhibited no vaccine-induced tetanus toxoid response. Data points are representative of $n = 2$ independent experiments (each with $n = 2$ technical replicates). Fold-change in magnitude of the response to Vi from Baseline to Day of Challenge across subclasses by vaccine group **(C)**. A principal components analysis with all tetanus and Vi responses included **(D)** with a scatter plot of the first (PC1) and second (PC2) principal components is shown. Each measurement from a Vi-PS ($n = 35$ participants) or a Vi-TT ($n = 37$ participants) vaccinee is represented by a black or green dot, respectively. Ellipses represent 95% confidence regions.

The effect size of the difference in nViPS IgA antibody between protected individuals of the Vi-PS group (median = 504 μg/ml) compared with diagnosed (median = 227 μg/ml) was nearly 2-fold. In the Vi-TT group, the effect size of the difference in nViPS IgA between the protected Vi-TT group (median = 2,118 μg/ml) compared with diagnosed (median = 595 μg/ml) was 3.5-fold. However, there was no threshold above which 100% of individuals were protected (**Figure 3C**) suggesting that other immune mechanisms contribute to protection status. The maturation of the vaccine-elicited IgA response, as measured by IgA avidity index, was not significantly different between protected and diagnosed individuals for either vaccine group (**Figure 3D**).

Vi IgA1 and IgA2 Is Higher in Protected Individuals

Since nViPS IgA correlated with protection in Vi-PS vaccinees and was also higher in protected Vi-TT vaccinees, though not

TABLE 1A | Antigen-specific magnitude by vaccine group at Day of challenge (D0).

Subclass	Antigen	Vaccine arm	Response rate	Median mag[a]	Median mag[a] range
IgG1	ViBiot	Vi-PS	23% (8/35)	3.82E4	1.00E4–4.88E6
		Vi-TT	76% (28/37)	2.49E5	1.00E4–2.30E6
	TT	Vi-PS	0% (0/35)	2.60E5	5.30E4–5.72E6
		Vi-TT	68% (21/31)	8.51E6	5.21E5–5.30E7
IgG2	ViBiot	Vi-PS	29% (10/35)	6.25E4	4.00E3–5.44E6
		Vi-TT	46% (17/37)	1.59E5	4.00E3–2.35E8
	TT	Vi-PS	0% (0/35)	4.00E3	4.00E3–4.19E4
		Vi-TT	16% (6/37)	1.92E4	5.41E3–1.60E6
IgG3	ViBiot	Vi-PS	31% (11/35)	5.00E3	5.00E3–3.55 E6
		Vi-TT	78% (29/37)	1.31E4	5.00E3–3.17E5
	TT	Vi-PS	0% (0/34)	1.24E4	5.00E3–6.21E4
		Vi-TT	25% (9/36)	3.60E4	1.61E4–5.64E5
IgA	ViBiot	Vi-PS	88% (30/34)	4.71E5	2.43E4–5.78E6
		Vi-TT	97% (36/37)	1.05E6	9.78E4–1.86E7
	TT	Vi-PS	0% (0/35)	5.00E3	5.00E3–4.02E5
		Vi-TT	22% (8/37)	1.01E5	5.00E3–9.83E5

[a] *Magnitude calculated as MFI*Dilution.*

TABLE 1B | Antigen-Specific Responses by Vaccine Group at Day of Challenge (D0).

Subclass	Antigen	Vaccine arm	Median mag log fold change[a]	AI[b] median	AI[b] range
IgG1	ViBiot	Vi-PS	0.52	18.5	0–102
		Vi-TT	1.29	32	2–125
	TT	Vi-PS	0	0	0
		Vi-TT	1.17	37	11–70
IgG2	ViBiot	Vi-PS	0.91	27	2–74
		Vi-TT	1.34	47	9–91
	TT	Vi-PS	0	0	0
		Vi-TT	0.45	19.5	7–39
IgG3	ViBiot	Vi-PS	0	9	1–50
		Vi-TT	0.42	35	12–68
	TT	Vi-PS	0	0	0
		Vi-TT	0.64	74	16–88
IgA	ViBiot	Vi-PS	1.62	38.5	8–63
		Vi-TT	2.11	42	4–90
	TT	Vi-PS	0	0	0
		Vi-TT	0.78	53	31–70

[a] *Fold Change from Baseline (D-28) to Day of Challenge (D0).*
[b] *AI, Avidity Index.*

statistically significant, we determined whether this potentially protective mechanism was driven by IgA1 or IgA2 subclass responses. IgA1 magnitude (FDR $p = 0.0070$) and fold-change (FDR $p = 0.007$) were significantly higher in the Vi-TT group at day of challenge (D0) (**Figure 4A**). In contrast, nViPS IgA2 magnitude and fold-change were higher in the Vi-TT group compared with the Vi-PS group, but were not significantly different (**Figure 4B**). While there was no difference in IgA2 avidity between the vaccine groups, nViPS IgA1 avidity was higher in the Vi-TT group, though not statistically significant (**Figure 4C**, **Table 3**, FDR $p = 0.14$). Fold-change in nViPS IgA1 and IgA2 was higher in protected individuals of both vaccine groups; however, there was no significant difference (**Figure 4D**). Neither IgA1 nor IgA2 avidity index was higher in protected individuals of the Vi-PS vaccine group; however, for Vi-TT vaccinees, both nViPS IgA1 (FDR $p = 0.14$) and IgA2 (FDR $p = 0.14$) avidity index were higher in protected individuals, though not statistically significant (**Figure 4E**). In order to determine whether individuals with high IgA1 responses were associated with high IgA2 responses, we conducted a

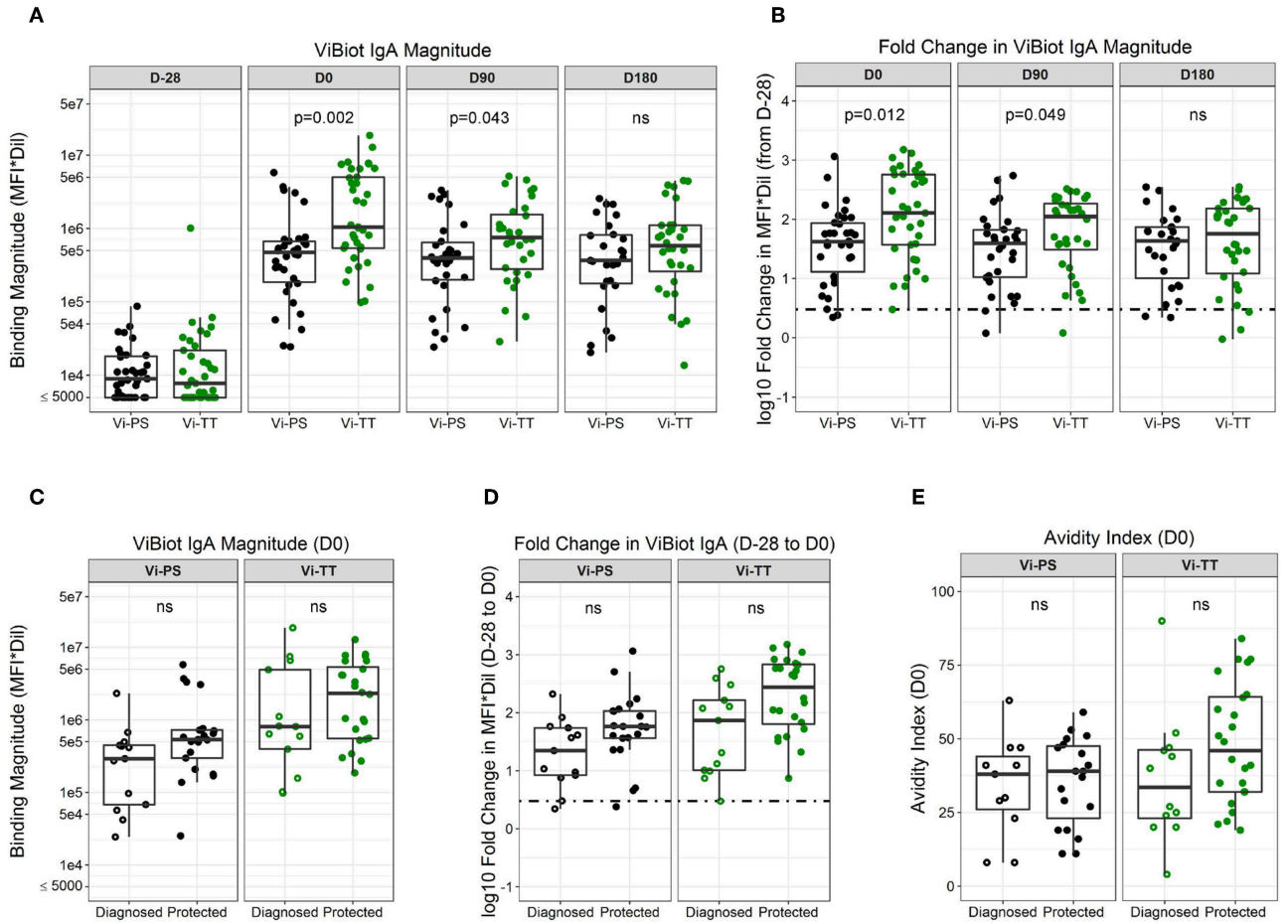

FIGURE 2 | ViBIOT-specific total IgA magnitude, fold change, and avidity higher in protected individuals of both vaccine groups. ViBIOT-specific IgA magnitude **(A)** and fold change **(B)** by vaccine group over time. ViBIOT-specific IgA magnitude **(C)** and fold change **(D)** by diagnosed/protected outcome at day of challenge. ViBIOT IgA avidity index **(E)** by diagnosed/protected outcome at day of challenge. Diagnosed and protected individuals are represented by open and closed circles, respectively. Data points are representative of $n = 2$ independent experiments (each with $n = 2$ technical replicates). n.s indicates non-significant FDR-corrected p values.

Spearman correlation of IgA1 and IgA2 response magnitude (**Figure 4F**). There was a moderate correlation between IgA1 and IgA2 magnitude (Spearman correlation=0.611, $p < 0.001$), but not all individuals with high IgA1 exhibited high IgA2 overall suggesting a greater degree of subclass specificity in the response to Vi.

IgG1 Avidity to Vi Polysaccharide Is Higher in Protected Individuals

Given that the addition of protein carriers, such as tetanus toxoid, to a polysaccharide vaccine allows for T cell engagement and avidity maturation (14, 28), we determined whether the Vi-TT vaccine improved Vi antibody avidity, and whether avidity maturation was associated with protection. Overall, the median avidity to Vi polysaccharide was higher in the Vi-TT group across all subclasses (IgA, IgG1, IgG2, IgG3); however, the only significant difference in avidity between vaccine groups was for ViBiot IgG3 (**Tables 1B**, **3**), where Vi IgG3 avidity was 4-fold higher in Vi-TT (median AI: 35%) vaccinees than Vi-PS vaccinees (median AI: 9%). However, IgG3 magnitudes in the Vi-PS group were very low. In the Vi-PS group, Vi IgG2 and IgG1 antibodies exhibited the highest avidity, with IgA1 and IgA2 in between, and IgG3 exhibiting the lowest avidity. In contrast, for the Vi-TT group, Vi IgG2 and IgG3 antibodies exhibited the highest avidity followed by IgG1, and the IgA subclasses exhibited the lowest (**Table 1B**). While there was no significant difference between Vi-PS and Vi-TT in ViBiot IgG1 avidity at any time point (**Figure 5A**), ViBiot IgG1 avidity was higher in protected individuals in the Vi-TT group, however this did not meet statistical significance at $p < 0.05$ (**Figure 5B**, FDR $p = 0.058$). The Vi-PS group was not tested for statistical differences among vaccine groups for IgG1 avidity due to low numbers of positive responders. To determine if the avidity of the vaccine-elicited IgG response further increased following oral challenge with live *S.* Typhi, we examined responses at 3 and 6 months post-challenge (D90, D180) compared to D0.

TABLE 2 | Vi Polysaccharide responses by protection status at day of challenge (D0).

Subclass	Vaccine arm	Protection status	Response rate	Median mag[a]	Median mag log fold change[b]	AI[c] median
IgG1	Vi-PS	Diagnosed	1/13 (8%)	3.54E4	0.43	10 (10–10)
		Protected	7/22 (32%)	4.09E4	0.61	27 (0–102)
	Vi-TT	Diagnosed	11/13 (85%)	2.63E5	1.09	22 (2–44)
		Protected	17/24 (71%)	2.38E5	1.33	46 (2–125)
IgG2	Vi-PS	Diagnosed	4/13 (31%)	8.62E4	0.87	25.5 (2–68)
		Protected	6/22 (27%)	4.98E4	0.98	35 (6–74)
	Vi-TT	Diagnosed	6/13 (46%)	1.59E5	1.34	26.5 (15–81)
		Protected	11/24 (46%)	1.77E5	1.38	50 (9–91)
IgG3	Vi-PS	Diagnosed	2/13 (15%)	5.00E3	0	3 (1–5)
		Protected	9/22 (41%)	5.00E3	0	18 (1–47)
	Vi-TT	Diagnosed	9/13 (69%)	1.09E4	0.34	35 (18–49)
		Protected	20/24 (83%)	1.36E4	0.44	37.5 (21–68)
IgA	Vi-PS	Diagnosed	11/13 (85%)	2.92E5	1.35	38 (8–63)
		Protected	19/21 (90%)	5.36E5	1.76	39 (11–59)
	Vi-TT	Diagnosed	12/13 (92%)	8.09E5	1.87	33.5 (4–90)
		Protected	24/24 (100%)	2.32E6	2.44	46 (19–84)

[a] *Magnitude calculated as MFI*Dilution.*
[b] *Fold Change from Baseline (D-28) to Day of Challenge (D0).*
[c] *AI, Avidity Index.*

There were no significant differences when compared with day of challenge (D0), of the median off-rate (s^{-1}) of vaccinee purified polyclonal IgGs to nViPS by BLI analysis (**Table 4**). However, the median IgG1 avidity index modestly increased in the diagnosed group but not in the protected group at 3 and 6 months post-challenge (**Figure 5C**).

Vi-PS and Vi-TT Vaccines Elicit Differential Immune Responses Associated With Protection

To better understand what factors are associated with protection in Vi-PS vs. Vi-TT vaccinees, we examined data collected across all antibody subclasses. In Vi-PS vaccinees, protection was significantly associated with Vi IgA magnitude and fold-change, and protected subjects had over 5-fold higher Vi IgA2 magnitude and fold-change compared with diagnosed vaccinees (**Figure 6A**). In Vi-TT vaccinees, there were no significant associations with protection, but distinct trends were observed for higher anti-Vi IgG1 avidity as well as total IgA fold-change, IgA1 fold change, and IgA2 avidity in protected individuals (**Figure 6B**).

DISCUSSION

Although moderately efficacious vaccines against *S.* Typhi have been licensed for decades, identification of mechanistic correlates of protection (mCoP), which could be used to improve vaccine design, have not been identified. This is because *S.* Typhi is a human restricted pathogen, thus limiting insights into the pathogenesis of typhoid fever and the host response (11). The establishment of a CHIM for the evaluation of the WHO prequalified Vi-tetanus tetanus (Vi-TT) conjugate vaccine has provided a platform with which to directly characterize vaccine-elicited Vi antibodies obtained from protected and diagnosed individuals. This is difficult to accomplish in field efficacy trials, as it requires post-vaccine blood samples being available from tens of thousands of participants and prolonged observation for cases to occur naturally. Following oral challenge with live *S.* Typhi, both the Vi-PS and Vi-TT vaccines exhibited similar attack rates at 37 and 35%, respectively, despite higher immunogenicity of the Vi-TT vaccine. In addition, a significant difference in anti-Vi IgG titers between diagnosed and protected individuals was only observed in the Vi-PS group, particularly for the IgG2 subclass, yet Vi-TT vaccinees exhibited attenuated clinical outcomes compared with Vi-PS vaccinees (15). Based on these data, since there are no currently known direct cell-mediated effector mechanisms generated by PS or conjugate vaccines, we hypothesized that the Vi-PS and Vi-TT vaccines may elicit antibody responses with distinct biophysical properties including subclass distribution and avidity that may influence protective outcomes.

In this study, we sought to evaluate the effect of the tetanus carrier protein on the quality of the immune response, which may play a role in affinity maturation and isotype switching of antibodies via engagement of the T cell pool. In addition, we aimed to identify characteristics of vaccine-elicited antibodies that correlated with protection in the CHIM study. To evaluate and compare host responses between vaccine groups and protection status, we characterized the antibody (IgA, IgA1, IgA2, IgG1, IgG2, and IgG3) response pre-vaccination, post-vaccination, and up to 6 months post-challenge. We observed that among antibody types tested, the highest concentration of antibody belonged to the IgG2 subclass, consistent with studies showing that IgG2 is the predominant IgG subclass response

TABLE 3A | Primary statistical analysis of IgA responses to Vi at day of challenge (D0).

Isotype	Measure	Analyte	Comparison	raw.p	FDR.p
IgA	Magnitude	Vi-Biot	Vaccine	**0.0002**	**0.0017**
			Diagnosis Vi-PS	*0.0241*	0.0785
			Diagnosis Vi-TT	0.3528	0.4992
		nViPS	Vaccine	**0.0016**	**0.0088**
			Diagnosis Vi-PS	**0.0021**	**0.0098**
			Diagnosis Vi-TT	0.1159	0.2314
	Fold Change	Vi-Biot	Vaccine	**0.0028**	**0.0118**
			Diagnosis Vi-PS	*0.0503*	0.1348
			Diagnosis Vi-TT	*0.0179*	*0.0610*
		nViPS	Vaccine	**0.0028**	**0.0118**
			Diagnosis Vi-PS	*0.0058*	**0.0230**
			Diagnosis Vi-TT	*0.0397*	0.1190
	Avidity Index	Vi-Biot	Vaccine	0.1087	0.2314
			Diagnosis Vi-PS	0.7465	0.7998
			Diagnosis Vi-TT	0.1108	0.2314
		nViPS	Vaccine	0.1018	0.2314
			Diagnosis Vi-PS	0.4562	0.6051
			Diagnosis Vi-TT	0.2359	0.3539
IgA1	Magnitude	nViPS	Vaccine	**0.0011**	**0.0066**
			Diagnosis Vi-PS	0.1363	0.2324
			Diagnosis Vi-TT	0.2106	0.3224
	Fold Change	nViPS	Vaccine	**0.0012**	**0.0072**
			Diagnosis Vi-PS	0.1363	0.2324
			Diagnosis Vi-TT	*0.0362*	*0.1132*
	Avidity Index	nViPS	Vaccine	*0.0498*	0.1348
			Diagnosis Vi-PS	0.6409	0.7395
			Diagnosis Vi-TT	0.0503	0.1348
IgA2	Magnitude	nViPS	Vaccine	0.1278	0.2314
			Diagnosis Vi-PS	0.1027	0.2314
			Diagnosis Vi-TT	0.1660	0.2594
	Fold Change	nViPS	Vaccine	0.1278	0.2314
			Diagnosis Vi-PS	0.1027	0.2314
			Diagnosis Vi-TT	0.1660	0.2594
	Avidity Index	nViPS	Vaccine	0.5349	0.6800
			Diagnosis Vi-PS	0.7379	0.7998
			Diagnosis Vi-TT	0.0547	0.1416

Raw p values <0.005 and FDR-corrected p values <0.05 bolded. Raw p values <0.05 italicized. Vi-BIOT antigen data not collected for IgA1 and IgA2 subclasses.

to polysaccharide antigens (29). However, IgA dominated the overall vaccine-elicited antibody response to Vi in both groups, with the largest fold-change in IgA1, when only positive responders were evaluated. This is consistent with results from one study that detected higher frequencies of IgA antibody secreting cells (ASCs) compared with IgG ASCs following vaccination with Vi-PS (30). In addition, vaccination with Vi-PS or another Vi-conjugate vaccine using *Pseudomonas aeruginosa* recombinant exoprotein A (Vi-rEPA) demonstrated that fold-change in total IgA was higher than total IgG, though this study did not further classify by antibody subclass (8). None of the subclasses examined were boosted in response to challenge with live *S.* Typhi bacteria. This may have been a result of challenging volunteers near the peak of their post-vaccination antibody response when antibody titers were very high. Instead, challenge likely induced immuno-dominant responses to alternative antigens such as LPS and flagellin as has been previously described, at least amongst those who developed clinical or microbiological evidence of infection (31).

In evaluating antibody correlates of protection against typhoid fever, we examined both antibody magnitude and fold-change from baseline, as well as antibody avidity to Vi antigen. We observed that magnitude (FDR $p = 0.01$) and fold-change (FDR $p = 0.02$) from baseline of nViPS IgA

TABLE 3B | Primary statistical analysis of IgG responses to Vi at Day of challenge (D0).

Isotype	Measure	Analyte	Comparison	raw.p	FDR.p
IgG1	Magnitude	Vi-Biot	Vaccine	**0.00008**	**0.0010**
			Diagnosis Vi-PS	1.0000	1.0000
			Diagnosis Vi-TT	0.6717	0.7632
		nViPS	Vaccine	**0.00002**	**0.0006**
			Diagnosis Vi-PS	0.1272	0.2314
			Diagnosis Vi-TT	0.2908	0.4276
	Fold Change	Vi-Biot	Vaccine	**0.0004**	**0.0032**
			Diagnosis Vi-PS	0.7056	0.7899
			Diagnosis Vi-TT	0.7417	0.7998
		nViPS	Vaccine	**0.000002**	**0.0001**
			Diagnosis Vi-PS	0.0571	0.1429
			Diagnosis Vi-TT	0.9875	1.0000
	Avidity Index	Vi-Biot	Vaccine	0.1427	0.2365
			Diagnosis Vi-PS	Not tested	Not tested
			Diagnosis Vi-TT	*0.0154*	*0.0576*
		nViPS	Vaccine	**0.000003**	**0.0001**
			Diagnosis Vi-PS	0.9303	0.9691
			Diagnosis Vi-TT	0.6141	0.7197
IgG2	Magnitude	Vi-Biot	Vaccine	*0.0162*	*0.0577*
			Diagnosis Vi-PS	0.9866	1.0000
			Diagnosis Vi-TT	0.4223	0.5759
		nViPS	Vaccine	**0.0003**	**0.0025**
			Diagnosis Vi-PS	0.1210	0.2314
			Diagnosis Vi-TT	0.4598	0.6051
	Fold Change	Vi-Biot	Vaccine	**0.0021**	**0.0098**
			Diagnosis Vi-PS	0.4682	0.6055
			Diagnosis Vi-TT	0.8384	0.8856
		nViPS	Vaccine	**0.0006**	**0.0049**
			Diagnosis Vi-PS	0.1296	0.2314
			Diagnosis Vi-TT	0.3695	0.5132
	Avidity Index	Vi-Biot	Vaccine	0.1451	0.2365
			Diagnosis Vi-PS	Not tested	Not tested
			Diagnosis Vi-TT	0.1191	0.2314
IgG3	Magnitude	Vi-Biot	Vaccine	**0.000047**	**0.0007**
			Diagnosis Vi-PS	0.5950	0.7084
			Diagnosis Vi-TT	0.5758	0.7079
	Fold Change	Vi-Biot	Vaccine	**0.000045**	**0.0007**
			Diagnosis Vi-PS	0.5950	0.7084
			Diagnosis Vi-TT	0.5758	0.7079
	Avidity Index	Vi-Biot	Vaccine	**0.0007**	**0.0049**
			Diagnosis Vi-PS	NA	NA
			Diagnosis Vi-TT	0.2993	0.4316

Raw p values <0.005 and FDR-corrected p values <0.05 bolded. Raw p values <0.05 italicized. nViPS antigen data not collected for IgG2 avidity or the IgG3 subclass. "NA" indicates that data was not collected. "Not tested" indicates insufficient data points for statistical testing.

correlated with protection status at day of challenge in the Vi-PS vaccine group. We also noted higher ViBiot IgA fold-change in the Vi-TT group (FDR $p = 0.06$), though not statistically significant. Protected individuals in both vaccine groups exhibited 2–3.5-fold higher concentrations of Vi-specific IgA compared with diagnosed individuals (in μg/ml equivalents of the 16/138 Typhoid IS Standard). Notably, the 16/138 Typhoid IS standard was non-IgG depleted therefore, IgA equivalents may be understated due to antigen-specific competition by IgG. However, the IgA correlation with protection status suggests that Vi IgA may serve as a surrogate marker that predicts protection from disease following vaccination. Studies of other infections have reported antibody titer and fold-rise as potential non-mechanistic correlates of protection (nCoP) that may aid

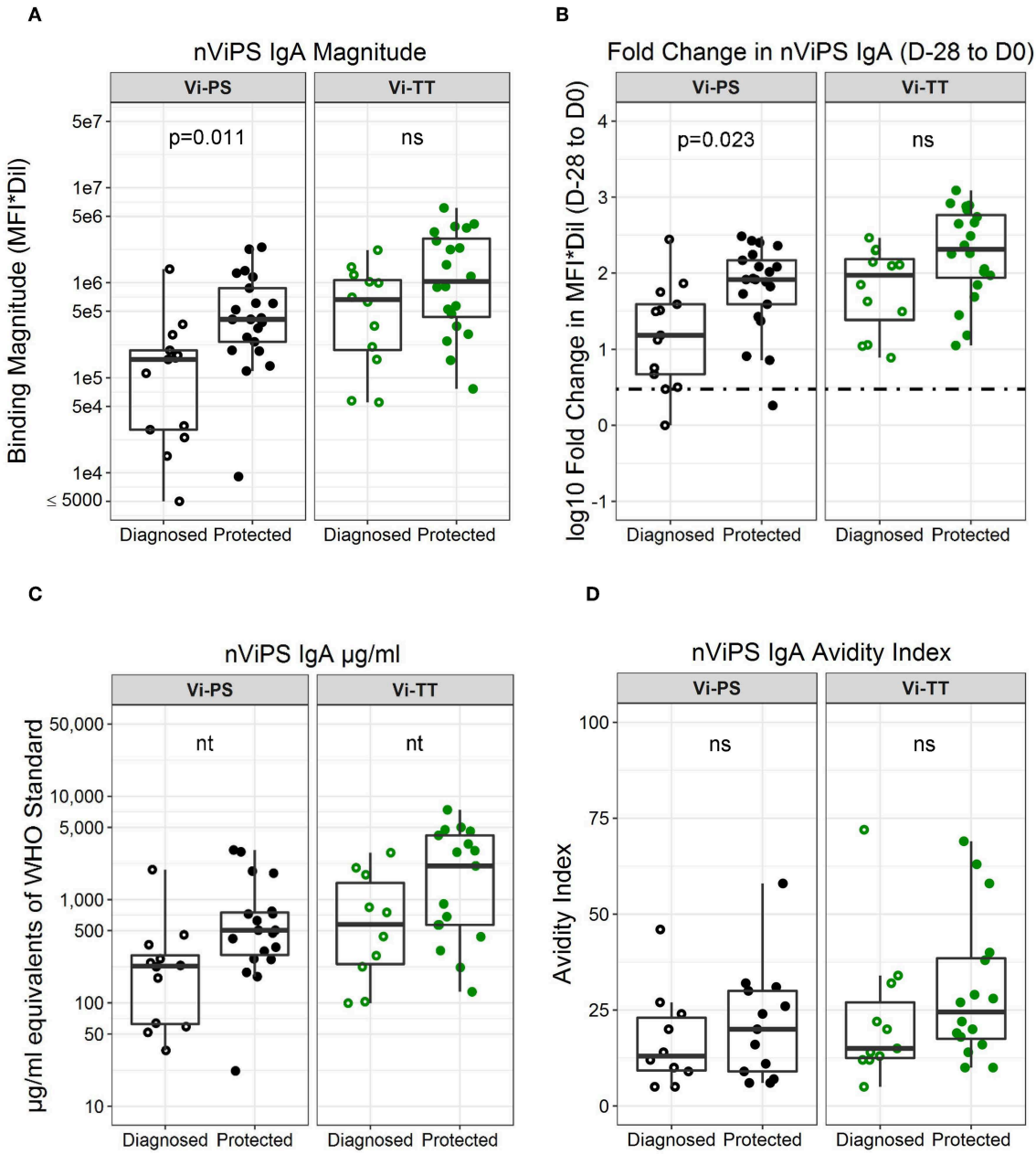

FIGURE 3 | nViPS-specific total IgA magnitude, fold change, and avidity higher in protected individuals of both vaccine groups. nViPS-specific IgA magnitude **(A)** fold change **(B)** µg/mL equivalents of WHO typhoid IS 16/138 **(C)** and avidity index **(D)** by diagnosed/protected outcome at day of challenge. Data points are representative of $n = 2$ independent experiments (each with $n = 2$ technical replicates). n.s indicates non-significant FDR-corrected p values. nt indicates not tested for statistical significance.

in development and evaluation of vaccines (32–34); however, these data may also be evidence of an underlying mCoP that has yet to be identified. The Vi polysaccharide capsule, as the main virulence factor of *S.* Typhi, limits complement deposition and neutrophil chemotaxis which are essential in the immune response against *S.* Typhimurium (11–13). The lack of these innate immune responses to *S.* Typhi are what ultimately allow the bacteria to establish systemic infection; therefore, pre-existing Vi IgA may provide protection against *S.* Typhi infection via Fc-dependent mechanisms. IgA may activate the complement cascade via the lectin pathway and exhibit bactericidal activity (35), or alternatively enhance neutrophil phagocytosis (ADNP), as neutrophils express the highest levels of FcαR among the phagocytic immune cells (20). Serum bactericidal activity and high affinity opsonophagocytic antibodies remain the accepted correlates of protection for meningococcal (36–38) and

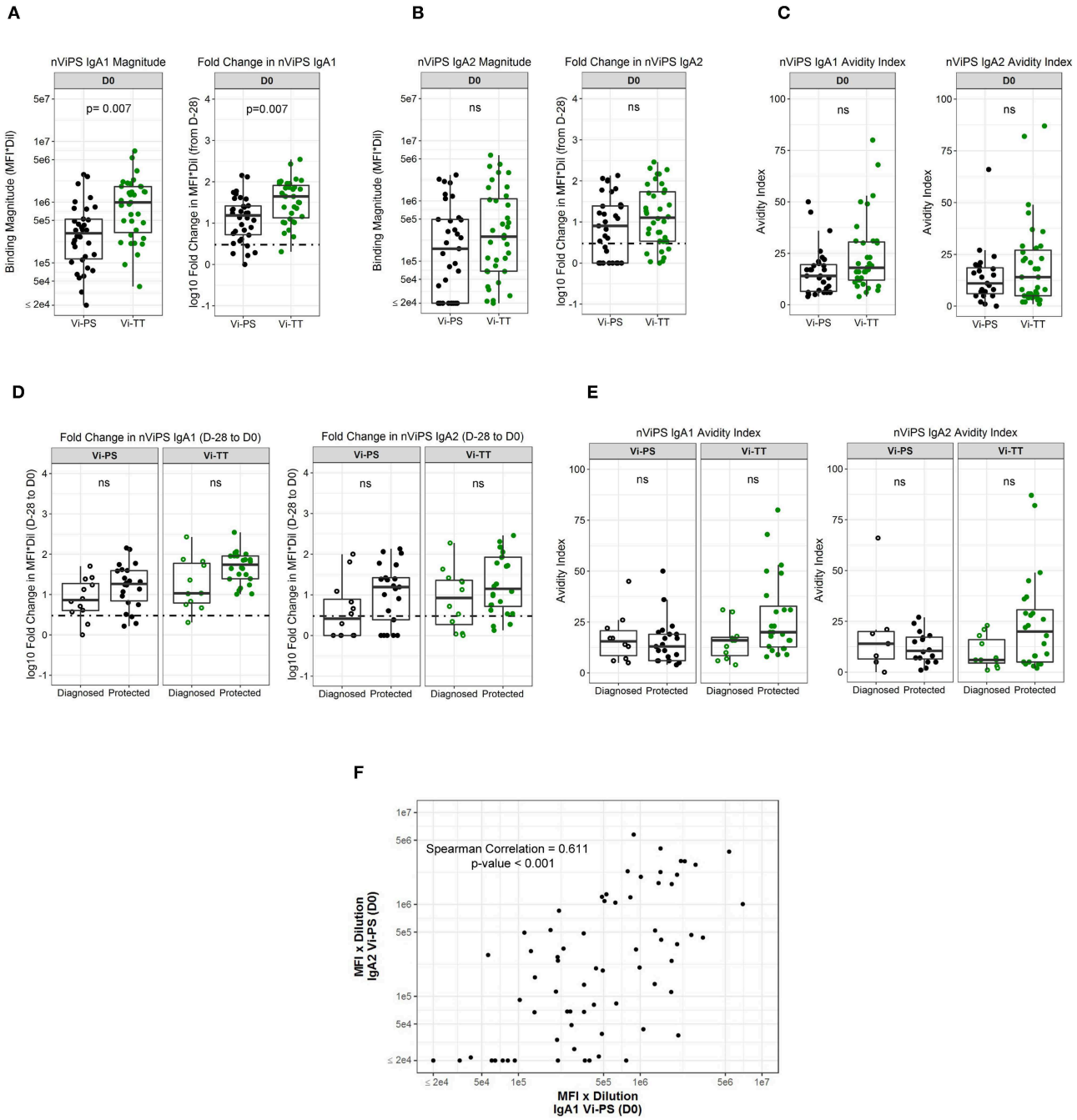

FIGURE 4 | nViPS IgA1 and IgA2 fold change higher in protected individuals in both vaccine groups. nViPS-specific IgA1 magnitude and fold change **(A)** and nViPS-specific IgA2 magnitude and fold change **(B)** by vaccine group from baseline to day of challenge. nViPS IgA1 and IgA2 avidity index **(C)** by vaccine group at day of challenge. IgA1 and IgA2 fold change from baseline to day of challenge by protected/diagnosed status **(D)** and IgA1 and IgA2 avidity index at day of challenge **(E)** by protected/diagnosed status. Data points are representative of $n = 2$ independent experiments (each with $n = 2$ technical replicates). Spearman correlation between nViPS IgA1 and IgA2 magnitude **(F)** at day of challenge. n.s indicates non-significant FDR-corrected p values.

pneumococcal (36, 39, 40) polysaccharide conjugate vaccines, respectively. For typhoid fever, studies have shown enhanced opsonophagocytosis of live *S.* Typhi using post-vaccination sera from various typhoid fever vaccine constructs (41, 42), yet none have done so with the Vi-PS or Vi-TT vaccines.

Further analysis of Vi IgA separated by IgA1 and IgA2 revealed that Vi IgA2 levels were 6-fold higher in protected vs. susceptible Vi-PS vaccinees. IgA2 is present in higher concentration in the mucosa than in serum (43). As such, this may suggest a role for IgA2 in mediating a protective

TABLE 4 | Dissociation rates of purified polyclonal IgGs to nViPS.

Days post challenge	Vaccine arm	Median Off-rate (s^{-1})	Range (s^{-1})
D0	Vi-PS	1.44E-03	2.63E-02–1.00E-06
	Vi-TT	8.13E-04	9.01E-03–1.00E-06
D90	Vi-PS	1.28E-03	2.21E-03–1.00E-06
	Vi-TT	1.04E-03	4.36E-03–1.00E-06
D180	Vi-PS	2.12E-03	3.70E-03–2.65E-04
	Vi-TT	1.28E-03	2.87E-03–1.20E-04

effect at the gut epithelium prior to bacterial entrance into intracellular niches within the lymphatic system. It may also suggest a functional role for Vi IgA2 in the serum, as many studies have shown that anti-polysaccharide IgA responses are associated primarily with IgA2 subclass unless conjugated to a protein carrier (44, 45). In the Vi-TT group, anti-Vi IgA1 rather than IgA2 fold-change was 5-fold higher in protected individuals. These diverging data suggest that although IgA appears to predict protection in both vaccine groups, either the functional mechanism of IgA-mediated protection differs or IgA1 and IgA2 play redundant roles. A previous study on the Vi-PS vaccine demonstrated that nearly 60% of Vi-specific antibody secreting cells (ASCs) from human peripheral blood expressed gut-homing marker $\alpha_4\beta_7$ while nearly 80% expressed systemic homing marker L-selectin (30). Therefore, it is still unknown whether Vi-specific ASCs mediate protective function at the mucosal or systemic level. Additional studies looking at plasmablast phenotypes and probing the mucosal compartment, including saliva Ig, may help determine whether the serum antibody pool is recapitulated at the mucosal level and may help to further elucidate the role of IgA, the most abundant class of antibody in the mucosa.

Previous studies have reported that tetanus toxoid as a carrier protein produces higher avidity antibodies against pneumococcal and *Haemophilus influenzae* type b (Hib) polysaccharides when compared with other licensed carrier proteins such as meningococcal outer membrane protein complex (OPMC) or diphtheria toxoid (28, 46, 47). Interestingly, the Vi-TT vaccine did not significantly increase the avidity of the response for any subclass other than IgG3 (FDR $p = 0.005$). However, though not statistically significant, avidity of each antibody subclass was higher in Vi-TT vaccinees compared with Vi-PS vaccinees. Despite lower median avidity compared with its IgG subclass counterparts, ViBiot IgG1 avidity was higher in protected Vi-TT vaccinees compared with diagnosed, though not statistically significant (FDR $p = 0.058$). IgG1 subclass antibodies have greater affinity for FcγRs compared with IgG2 (20), therefore, these data may suggest a role for IgG1 in Fc-mediated protection against typhoid fever. IgG3 antibodies, like IgG1, are also known to bind to FcγRs with higher affinity than IgG2 and IgG4; however, despite exhibiting higher avidity to Vi in the Vi-TT group, the magnitude of the IgG3 response was very low. In addition to Vi IgG1 avidity, Vi IgA2 avidity was 3-fold higher in protected vs. diagnosed Vi-TT vaccinees. Interestingly, IgA2 exhibited the lowest median avidity in the Vi-TT group yet showed the largest difference between protected and diagnosed individuals. This finding, along with the Vi IgG1 avidity, illustrates how antibody-mediated protection may encompass not only affinity for antigen but also distribution in the correct locations throughout the body as well as ability to engage available effector cells.

Unlike the Vi-TT group, there were no trends with avidity and protection status in the Vi-PS vaccinees. The increase in avidity of serum antibodies, expressed by avidity index and antibody off-rate, in Vi-TT vaccinees suggests that the tetanus carrier protein did stimulate germinal center reactions and affinity maturation. However, the avidity index of Vi antibodies did not exceed 50% post-vaccination, indicating that the antibodies exhibited low to medium avidity at best, even with the conjugate vaccine. In fact, most antibodies elicited by conjugate vaccines have low affinity to their polysaccharide ligands. Nearly all isolated polysaccharide monoclonal antibodies have micromolar binding affinities which is orders of magnitude lower than typical anti-protein antibodies (48–50). For example, in this study the off-rate (k_d) of purified polyclonal IgGs from vaccinated individuals was on the order of $1e^{-3}$ s^{-1}, whereas malaria-naïve subjects receiving the malaria RTS,S vaccine develop antibodies to circumsporozoite protein (CSP) with off-rates on the order of $1e^{-4}$ s^{-1} or less, measured using the same BLI method (27). However, these CSP antibodies underwent affinity maturation over the course of three doses of vaccine, whereas Vi-TT and Vi-PS vaccines were single dose. Additional studies examining the phenotype and functional properties of the TT-specific T cell pool may shed light on the low level of affinity maturation observed in this trial. Similar to the magnitude of the response, the avidity of Vi antibodies, across all subclasses, did not increase post-challenge with live *S.* Typhi. This could suggest that boosting at later time points is required for increased affinity maturation to Vi.

Examining how the biophysical properties of Vi antibodies relates to protection status has revealed that the Vi-PS and the Vi-TT vaccines elicit different protective antibody signatures. Trends in the protected group of Vi-PS vaccinees are dominated by total IgA and IgA2 levels whereas in the Vi-TT group, protected individuals appear to exhibit a signature consistent with high IgA levels and higher avidity responses for IgA2 and IgG1. Interestingly, although both the Vi-TT and Vi-PS vaccines exhibited a similar overall efficacy using stringent diagnosis criteria, fewer Vi-TT diagnosed vaccinees reported severe symptoms and fewer exhibited fever $\geq 38^{\circ}$C (15). In the VAST study, diagnostic criteria included prolonged fever of $\geq 38^{\circ}$C, *S.* Typhi bacteremia, or both together. In the Vi-PS group, more participants exhibited both criteria, whereas in the Vi-TT group, many exhibited bacteremia without fever that may have self-resolved (15). In fact, in a *post-hoc* analysis using the field definition of typhoid fever, which requires both fever and positive bacteremia, the Vi-TT vaccine efficacy was 87.1% and for Vi-PS only 52.3% (15). These improved clinical manifestations seem to suggest that a combined IgA response with higher affinity IgA and IgG1 antibody may provide

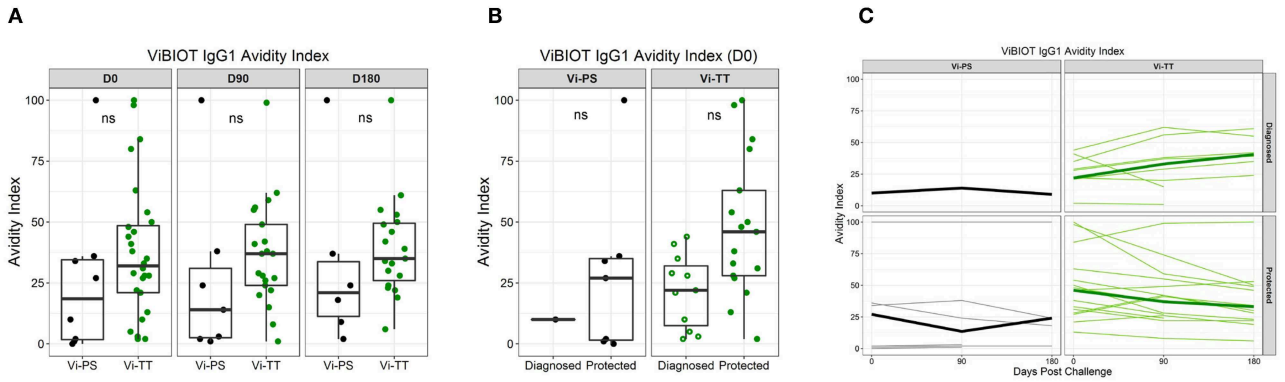

FIGURE 5 | Vi polysaccharide IgG1 avidity higher in protected individuals. ViBIOT IgG1 avidity index at pH 3.0 in positive vaccine responders by vaccine group over time **(A)** and by diagnosed/protected outcome at day of challenge **(B)**. Avidity index in positive vaccine responses of anti-ViBIOT IgG1 over time **(C)** from 4 weeks post-vaccination (D0) to 6 months post-challenge (D180) by vaccine group and protection status. Bolded lines indicate median avidity index and faint lines indicate individual level data. Data points are representative of $n = 2$ independent experiments (each with $n = 2$ technical replicates). nt indicates not tested for statistical significance.

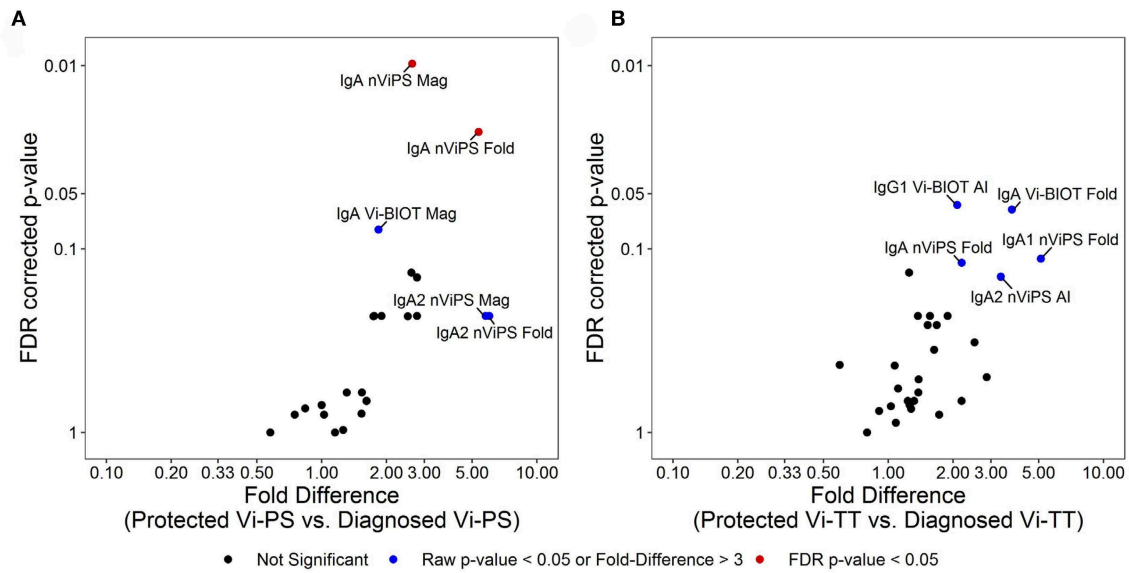

FIGURE 6 | Different immune signatures of protection status for Vi-PS and Vi-TT. Variables in analysis of immune signatures between vaccine groups include magnitude, fold-change, and avidity of IgA1, IgA2, IgG1, IgG2, and IgG3 to Vi-Biot and nViPS. Volcano plot of FDR-corrected *p*-value and fold difference by diagnosed/protected outcome for Vi-PS vaccinees **(A)** and Vi-TT vaccinees **(B)**. Red dots represent FDR-corrected *p*-values < 0.05, blue dots represent raw *p*-values <0.05 or fold difference >3 (trending).

better protection and attenuate disease outcome. In a typhoid endemic setting where pre-existing immunity, including IgA and IgG1 is likely to be present, the further boost in antibody maturation by vaccination (i.e., increasing antibody avidity), may enhance the protective response by vaccination, with a more pronounced boost in the Vi-TT group. Overall, the improved immunological properties of the Vi-TT conjugate vaccine are (i) higher IgG and IgA responses, (ii) immune responses in children under 2 (51), (iii) induction of immunological memory, and (iv) different pattern of antibody responses as shown in our studies. Additional studies are needed to determine if any of these responses relate to differences of VE in the field.

The analyses in this study were limited by statistical and ethical factors. Firstly, sample sizes in the CHIM were small, with merely 13 diagnosed individuals per group, and analysis of only positive vaccine responders and correcting for multiple comparisons affected the statistical power of the study.

Furthermore, in the context of the CHIM, participants were treated immediately after identification of positive bacteremia or prolonged fever. Therefore, this provides a model of infection which differs from field trials in endemic settings in which disease is detected, as only symptomatic patients present to healthcare settings. In addition, considerations such as challenge dose, timing of challenge, and challenge population may all differ from natural infection. Importantly, the study population in this CHIM was typhoid naïve adults, and therefore, validation of these results in the target population, children in typhoid endemic regions, will be critical. Finally, we observed modest differences in the antigenicity of ViBiot and nViPS antigens in which responses positively correlated yet differed in statistical outcome. We believe that non-targeted biotinylation of the nViPS molecule may result in epitope changes that impact antigenicity, therefore, careful consideration, and characterization of immunogens is necessary. Studies using newly generated human monoclonal antibodies could help to resolve the fine specificity of antibody forms to these antigens elicited by typhoid fever vaccines.

Despite these limitations, the findings of this study demonstrate the utility of CHIMs in understanding protective immune responses to human-restricted pathogens and suggest that higher magnitude and affinity IgA may be a potential target for induction by typhoid fever vaccine strategies. This study identifies Vi IgA as a biomarker of protective immunity against typhoid fever and quantifies concentration of Vi IgA in vaccinees using the 16/138 typhoid International Standard (NIBSC). This standard could be used to compare vaccine-induced IgA responses across studies of other TCVs currently in development, such as the Vi-CRM_{197} and Vi-diphtheria toxin TCVs (52, 53), aiding vaccine evaluation. Using standard measures to compare across studies will allow careful consideration of which TCVs to deploy for routine immunization in infants under 2 years of age. Future work is required to understand the functional role of Vi IgA and determine whether IgA represents a mechanistic correlate of protection or a surrogate marker of an underlying immune response. IgA is known to play a critical role in mucosal immunity, and as *S.* Typhi is an enteric pathogen, IgA may be providing protection at the site of infection. Further studies are needed to determine whether the mucosal antibody response recapitulates the systemic compartment. More importantly, determining the potential IgA Fc-mediated functions that correlate with protection as well as further characterizing the epitope specificities of protective antibodies will inform vaccine design for elicitation of a more targeted and protective immune response against typhoid fever.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The South Central Oxford A Ethics Committee (14/SC/1427). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LD, AP, and GT conceived and designed the work. CJ, JH, EJ, and AP carried out the clinical trial. LD, FF, and RM acquired data for the work. LD, CJ, SD, RS, LZ, KS, SA, AP, and GT analyzed and interpreted the work. KS, SD, SA, and GT designed experimental procedures and supervised. RS and LZ performed statistical analysis. LD, AP, and GT wrote the manuscript. CJ, RS, KS, and SD edited the manuscript. All other authors reviewed and approved the final document.

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NOMENCLATURE

Controlled human infection model (CHIM); Vaccines against *Salmonella* Typhi (VAST); typhoid conjugate vaccine (TCV).

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REVIEW
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Update on Fc-Mediated Antibody Functions Against HIV-1 Beyond Neutralization

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Antibodies (Abs) are the major component of the humoral immune response and a key player in vaccination. The precise Ab-mediated inhibitory mechanisms leading to *in vivo* protection against HIV have not been elucidated. In addition to the desired viral capture and neutralizing Ab functions, complex Ab-dependent mechanisms that involve engaging immune effector cells to clear infected host cells, immune complexes, and opsonized virus have been proposed as being relevant. These inhibitory mechanisms involve Fc-mediated effector functions leading to Ab-dependent cellular cytotoxicity, phagocytosis, cell-mediated virus inhibition, aggregation, and complement inhibition. Indeed, the decreased risk of infection observed in the RV144 HIV-1 vaccine trial was correlated with the production of non-neutralizing inhibitory Abs, highlighting the role of Ab inhibitory functions besides neutralization. Moreover, Ab isotypes and subclasses recognizing specific HIV envelope epitopes as well as pecular Fc-receptor polymorphisms have been associated with disease progression. These findings further support the need to define which Fc-mediated Ab inhibitory functions leading to protection are critical for HIV vaccine design. Herein, based on our previous review Su & Moog Front Immunol 2014, we update the different inhibitory properties of HIV-specific Abs that may potentially contribute to HIV protection.

Keywords: HIV-1, antibody functions, non-neutralizing antibodies, FcR-mediated inhibition, ADCC

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INTRODUCTION

Currently, sexual transmission is the major route for human immunodeficiency virus (HIV) infection and contributes to 80% of newly diagnosed cases worldwide. This statistic implies that the virus crosses the mucosal barrier to reach and infect HIV target cells (1) and that an effective vaccine needs to induce an immune response that acts rapidly at mucosal sites. Neutralizing antibodies (NAbs), which can be IgG or secretory IgA, are certainly desired for blocking HIV transmission and have been shown to be highly effective at preventing infection through this route (**Figure 1**) (2, 3). During the last decade, a whole new series of broadly neutralizing Abs (bNAbs), which are NAbs with exceptional potency and breadth, have been isolated (4–6) and have efficiently protected humanized mice and non-human primates (NHPs) from experimental challenge. Some of these bNAbs are undergoing testing in human clinical prevention and therapeutic trials (6–14).

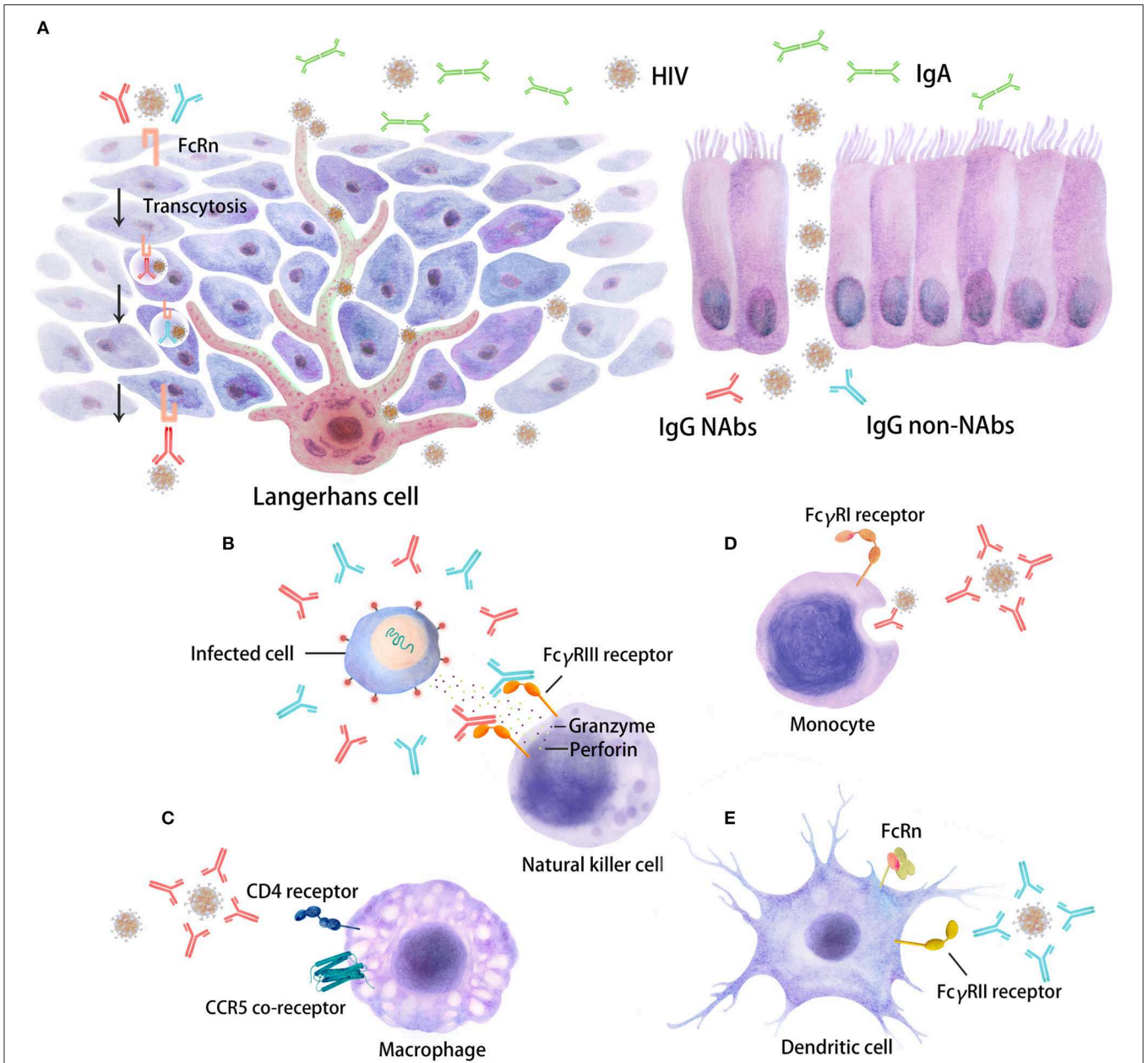


FIGURE 1 | Distinct inhibitory activities of Abs in HIV target cells. The interaction of Fc regions with Fc gamma receptors (FcγRs) on immune cells, such as dendritic cells (DCs), macrophages, and natural killer (NK) cells, will trigger several antiviral immune responses, including immune activation, which are able to restrain HIV replication. Five major Ab responses against HIV infection that can occur as a result of IgG neutralizing Abs (depicted in red), IgG non-neutralizing inhibitory Abs (depicted in blue) or IgA (in green) are shown. **(A)** There are two types of stratified epithelium and columnar epithelium. The binding of virus/Ab immune complexes to FcRn at the surfaces of Langerhans cells may lead to transcytosis through genitorectal mucosal surfaces; **(B)** FcγRIII-mediated antibody-dependent cellular cytotoxicity (ADCC); **(C)** neutralizing antibody (NAb)-mediated activity; **(D)** phagocytosis mediated by NAbs/non-NAbs through FcγRI binding; **(E)** FcγRII-mediated non-neutralizing inhibition; some FcγR/Fc interactions may also enhance HIV entry and infection. These different inhibitory activities can occur at mucosal sites beneath the mono- or pluri-stratified epithelial layers where HIV target cells reside.

However, bNAbs that display these features have very specific characteristics. Indeed, bNAbs exhibit uncommonly long complementarity-determining loops and extensive somatic hypermutation, which requires a long maturation process (6, 15–17). In turn, bNAbs are developed by only 10–30% of HIV-infected individuals (6, 17–20), and attempts to induce them by vaccination have encountered extreme difficulties (17, 21).

An increasing body of evidence suggests that Ab functions mediated by the Fc domain may play a role in protection against infections (22–27). Interestingly, the moderately protective effects observed in the RV144 HIV-1 vaccine trial were achieved in the absence of detectable NAbs, suggesting an important role for Fc-mediated functions in protection (28, 29). Fcγ receptor (FcγR)-mediated Ab responses, which lead to phagocytosis, aggregation, complement inhibition, Ab-dependent cellular cytotoxicity (ADCC), Ab-dependent cellular phagocytosis (ADCP), and Ab-dependent cell-mediated virus inhibition (ADCVI), have been shown to decrease HIV replication and may therefore substantially contribute to HIV protection (22–25, 27). This review focuses on the importance of Ab Fc-mediated functions in preventing HIV-1 infection and highlights their possible relevance for the development of new vaccine strategies.

ANTIBODY RESPONSES DURING HIV INFECTION

Significant efforts have been made over the past two decades to deepen the understanding of the role of the humoral immune response in HIV infection and to foster the development of vaccine strategies to control viral replication (30). The acquisition of HIV-specific IgG Abs appears within the first 2 months (31, 32) and evolves during the course of infection. In the neonatal period, the early serological response to infection in infants is obscured by the presence of transplacentally acquired maternal HIV Ab. The amount of immunoglobulin to HIV-1 and the number of HIV-1 antigens recognized increases with age (31, 33). The emergence of Ab responses to the viral envelope during HIV infection can generally be divided based on the timing of their appearance and their functions (34, 35). These responses include the following:

(1) A non-neutralizing inhibitory Ab (non-NAb) response directed at immunogenic epitopes that develops in all individuals soon after infection. Typically, this Ab response is directed first against viral gp41 following gp120/CD4 binding (34, 36) and soon thereafter against the V3 loop of gp120 (37). These Abs have a low impact on the virus and plasma viral load because they may have limited interactions with functional Env trimers (38). However, they may play an important role in protection via Fc-mediated effector functions, although the exact mechanism remains to be elucidated (24). Holl et al. reported that polyclonal sera with non-neutralizing activity inhibit HIV replication in macrophages by the phagocytosis of immune complexes bound to FcR expressed on cells (27). In a clinical trial called RV144, non-NAbs exhibiting *in vitro* inhibitory functions were described and associated with decreased HIV acquisition (39, 40).

(2) An HIV strain-specific NAb response targeting Env epitopes that are expressed on the native trimer, which is directed toward viruses present earlier in infection and is detected within the first year after seroconversion (20, 30). The NAbs recognizing Env epitopes via their Fab fragments may block HIV entry. An autologous NAb response is observed *in vitro* in the absence of additional factors, such as FcRs or complement, and is mainly a result of the blocking of virus-cell interactions (41). Such NAbs will encounter challenges, as they have to cope with a staggering level of viral diversity. Continuous viral escape from NAbs will occur as a consequence of single amino acid substitutions, insertions, and deletions, and through an "evolving glycan shield," in which shifting glycans prevent access by Abs to their cognate epitopes (38). Nonetheless, these autologous neutralizing responses may display additional effector functions involving Fc-mediated contributions to the decreases in the viral load detected within the first months after HIV acquisition (42).

(3) An Ab response capable of neutralizing a wide range of viral isolates that develops 2–5 years after seroconversion (43–46). However, this bNAb response occurs in only a minority of patients and is associated with increased HIV replication and diversity, although bNAbs can sometimes be detected in subjects that control HIV (16, 47, 48) or in chronically HIV-infected individuals (19). Ultimately, the virus will escape from bNAbs. Notably, bNAbs have also demonstrated efficient Fc-mediated inhibitory function in addition to neutralization.

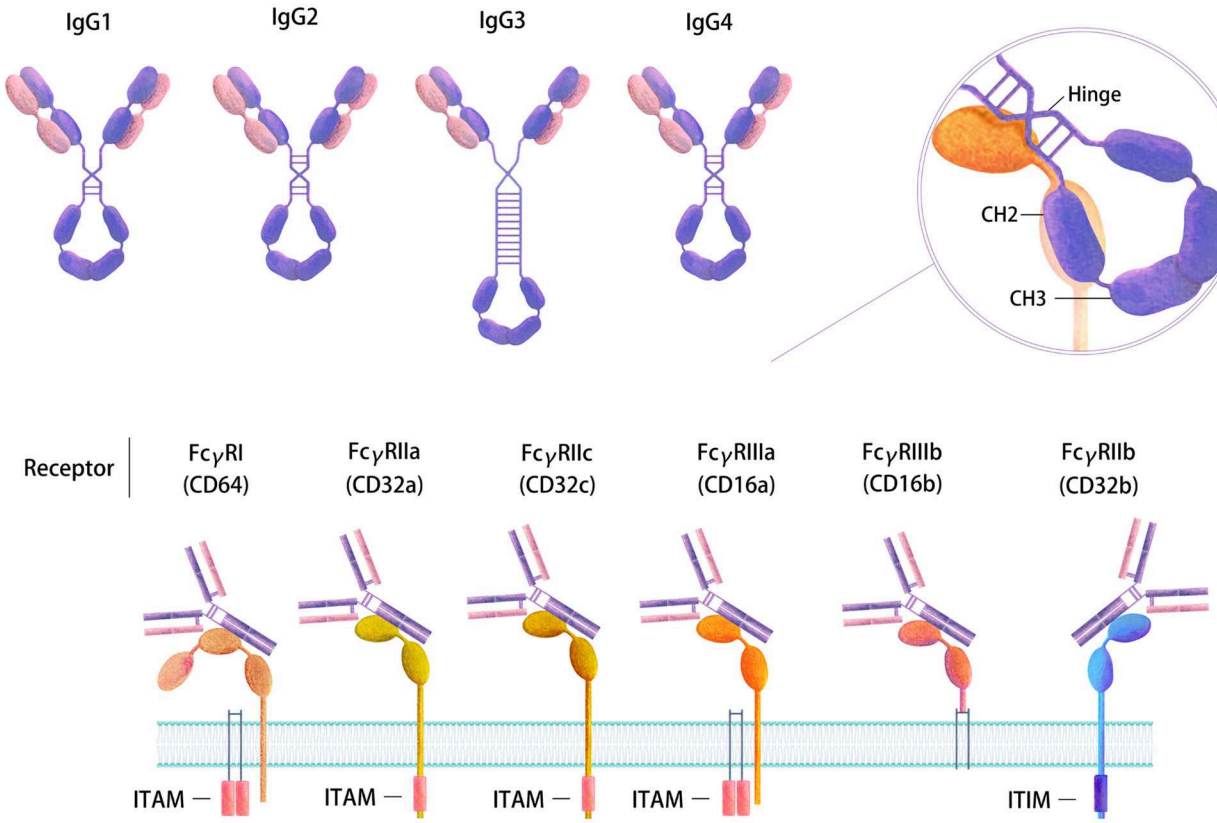
Therefore, during the course of HIV infection, the Ab response evolves, leading to complex polyfunctional activities that may certainly impact the course of HIV disease. The specific role of bNAbs in disease evolution and the potential contributions of other inhibitory functions have not been firmly demonstrated.

NEW GENERATION OF bNAbs

Thanks to major improvements in Ab isolation technologies, more than 100 HIV-1-specific bNAbs with remarkable potency against a wide variety of HIV subtypes have been developed (4, 6, 8, 9, 17, 43, 44, 49–53). The breadth of viral recognition and the antiviral potency of bNAbs can be classified according to their preferential target on the Env spike (4, 26, 49, 54, 55). Passive transfer of bNAbs performed in macaques has shown their remarkable capacity to protect non-human primates (NHPs) from experimental simian-HIV (SHIV) challenge when administered via different routes and modes (a single high dose or repeated low doses administered by the intravenous, rectal or vaginal route) (56–59). Interestingly, recent studies have demonstrated that such protection was not necessarily sterilizing, as was previously thought. Indeed, a few infected cell foci were detected 1–3 days after experimental challenge (60, 61), which intriguingly disappeared leading to complete protection. These results strongly suggest that protection is not solely due to neutralization of the virus particles and that Fc-mediated inhibitory function leading to the lysis of HIV-infected cells by bNAbs participate in this protection (62–64).

Although the newer bNAbs react with more than 90% of circulating HIV-1 strains when tested *in vitro*, at present, no single bNAb potently neutralizes all HIV strains. Therefore, a combination of two or more bNAbs would be desirable to cover the entire range of viral strains encountered *in vivo*

(6, 7, 65, 66). In this regard, bi-specific and even tri-specific Abs targeting multiple HIV-1 Env epitopes were developed recently to increase Ab breadth and potency (67–70). These new bNAbs were characterized following the sequencing of their Fab heavy and light chains and were further reconstituted with an IgG1 heavy chain to form the Ab Fc domain. Most bNAbs have high levels of somatic hypermutation, including amino acid in-frame substitutions (71), frequent in-frame insertions and deletions (72), and genetic bias in the Ig heavy chain variable region (IGHV) (73). Modifications of the FcR domain were introduced in some of these bNAbs to increase their stability and persistence *in vivo* and to potentially allow long-lasting activity and less frequent administration (23, 74–76).



Function	Activating	Activating	Activating	Activating	Activating	Inhibitory
Reference SNP	-	rs1081274	rs114945036	rs396991	rs5030738	rs1050501
SNP	-	H131R	126C>T	F158V	NA1/2	I232T
		H	C	F	NA1	T
Affinity Expression	High affinity	High affinity For IgG2	Decreased FcR expression	High affinity For IgG1,3,4	High affinity For IgG1,3	Decreased Inhibitory activity
Distribution	Mo,Mϕ,DC Neu,Eos	Mo,Mϕ,DC Neu,PLT	NK	Mo,Mϕ,DC NK	MC,Neu,Eos	Mo,Mϕ,DC MC,Neu,Bas,BC

FIGURE 2 | Human FcγR gene polymorphisms and the expression and affinity of IgG subtypes. Four IgG subtypes are present in human serum that have distinct structures and functions (top). FcγRs belong to the Ig receptor superfamily and comprise two or three extracellular Ig domains that mediate IgG binding. Differential immune regulatory effects are produced depending on binding to FcRs. Activating or inhibitory functions occur based on the presence of an intracellular cytoplasmic domain ITAM or ITIM motif that transduces an immunostimulatory or inhibitory signal, respectively, following receptor cross-linking. Binding of the Fc to the receptors is mediated at the CH2-CH3 interface following a conformational change (right). The diversity of FcγRs is further increased by SNPs in their extracellular domains, which in turn affect the expression of FcRs and their binding affinity and function (bottom). Mo, Monocyte; Mϕ, Macrophage; DC, Dendritic cell; MC, Mast cell; Neu, Neutrophil; Bas, Basophil; Eos, Eosinophil; NK, Natural killer cell; BC, B cell; PLT, Platelet.

ANTIBODY ISOTYPES AND THEIR SUBCLASSES

Upon B-cell activation by immune complexes, an HIV-specific Ab response will be generated that induces the production of immunoglobulins (Ig) of different types and isotypes (77, 78). These types and isotypes differ in their related Ig heavy chains, which therefore impacts the Fc-mediated function of the Abs. Four IgG subtypes are present in healthy adult serum: IgG1 (60–72%), IgG2 (20–31%), IgG3 (5–10%), and IgG4 (<4% of the total main IgG subclasses) (79) (**Figure 2**), whereas the gp120-specific IgG subclass distribution and IgA/IgM distribution are generally as follows: IgG1>IgG2 = IgG4>IgG3 and IgA>IgM (80). Indeed, diverse types (IgM, IgG, and IgA) and IgG subtypes will be induced by HIV in plasma and other body fluids, such as cerebrospinal fluid, saliva and genital secretions (80). The Ig subclasses will vary according to the type of infection, the related inflammatory stage and pathogen localization (81). Therefore, the isotype distribution following infection remains to be established. Some studies have proposed that Abs against gp41 are mainly IgG1 rather than other IgG subtypes or IgA or IgM classes (80, 82), whereas others reported gp41-specific Abs of other subclasses (83, 84). The functional role of these different isotypes also needs to be better characterized. It has been proposed that Env-specific gp41 IgM and IgG Ab responses have little effect on the control of the acute phase of viral replication (36). IgG1 and IgG3 Abs are highly active against viral infection, and IgG3 Abs appear first during the course of infection (85), whereas the role of the IgG2 subclass is not known. This isotype is mainly induced by bacterial capsular polysaccharide antigens (79, 86). Moreover, the IgG subclass prevalence has been reported to change over time. For example, gp120-specific IgG1 levels remain constant during the first 6 months after infection, whereas the level of gp120-specific IgG3 peaks after 1 month and then declines (84). Another example is gp140-specific IgG2 and IgG3 responses, which do not occur simultaneously in HIV-1-infected individuals (87). Isotype switching may largely impact Ab functionalities linked to Fc affinity toward Fc receptors (22, 78).

The Ab isotype/subclass recognition of Env epitopes is associated with certain disease courses, symptoms and responses to medication. Assessment of the IgG subclass distribution in the plasma of HIV-1-infected patients enrolled in a French prospective asymptomatic long-term (ALT) cohort showed that in contrast to the IgG1 titers, the IgG2 titers directed against HIV-1 Env gp41 and anti-p24 Abs were correlated with and were highly predictive of decreased viral loads and slower disease progression, especially long-term non-progression (88). This observation was confirmed recently (89, 90). Moreover, Martinez et al. found that in addition to HIV-1-specific $CD4^+$ Th1 cell responses, anti-gp41 IgG2 was the best predictor of long-term non-progression (91). In the RV144 vaccine trial (29, 92), the serum Env-specific IgA level was correlated with an increased risk of HIV-1 infection, which was potentially due to monomeric circulating IgA competing with IgG and interfering with the ability of IgG to mediate ADCC and ADCP (28). The presence of IgG3 against the Env V1V2 region was correlated with a lower risk of HIV-1 infection (29, 93). Interestingly, IgG3 appeared to neutralize HIV more efficiently *in vitro* than IgG1 (94). Moreover, the plasma IgG1 and IgG2 anti-HIV-1 p24 levels were inversely correlated with the plasma HIV RNA levels in viremic HIV patients (95).

In addition to isotype switching, the glycosylation profile of Ig changes during infection. Agalactosylation and afucosylation were more common in HIV-specific Abs among patient with spontaneous control of HIV and were linked to enhanced NK cell activity (96). The modifications of specific glycan groups determine the functional properties of Abs.

Taken together, these different results suggest that dynamic Ab subclasses/isotypes, posttranslational modifications, and glycosylation will impact disease progression. A comprehensive interrogation of the extensive biological diversity in naturally or experimentally protected subjects may provide insights critical for guiding the development of effective vaccines and Ab-based therapies.

Fc-RECEPTOR: THE CELLULAR COUNTERPART FOR ANTIBODY-MEDIATED RESPONSES

Each Ig isotype binds to specific Fc receptors, which in humans are the high-affinity receptors Fcα/μR for IgA and IgM, FcμR for IgM, FcαRI for IgA, FcεRI for IgE and FcγRI and neonatal Fc receptor (FcRn) for IgG and the low-affinity receptors FcεRII for IgE and FcγRII and III for IgG (97) (**Table 1**). Special attention has been paid to genes encoding the Fcγ receptors, since they bind the constant domain of IgG, which is the major Ab type induced by the host response following viral or bacterial infection (98). Human cells express three FcγRIIs (A–C) and two FcγRIIIs (A and B). All human FcγRs except FcγRIIB signal through an immunoreceptor tyrosine-based activating motif (ITAM), whereas FcγRIIB delivers inhibitory signals through an immunoreceptor tyrosine-based inhibitory motif (ITIM) (99) (**Figure 2**). The diversity of human FcγRII and III is further increased by the presence of single nucleotide polymorphisms (SNPs) in their extracellular domains, the most studied of which are H131R in the FcγR gene *FCGR2A* (100), 126C>T in *FCGR2C* (99), F158V in *FCGR3A* (101), and NA1/2 in *FCGR3B* (102) (**Figure 2**). FcγRIIC has an unusual structure and is generated by the unequal crossover of FcγRIIA and FcγRIIB (99, 103). *FCGR2C* in FcγRIIC (126C>T) shares the extracellular sequence of *FCGR2B* but signals through the ITAM, similar to *FCGR2A*.

Importantly, the different FcR polymorphisms in the host need to be taken into consideration when analyzing the FcR-mediated functions of Abs. FcγR SNPs will impact both binding to the complementary Fc portion of the Abs and on the other side the expression and activation state in cells (**Figure 2**). Indeed, increasing evidence suggests that FcγR SNPs impair receptor expression on DCs, which in turn influences the risk of HIV infection and vaccine efficacy (104). Similarly, the FcγRIIIA polymorphism appears to modify NK cell activation and, as a consequence, ADCC activity (105). Specific polymorphisms in the *FCGR2A* (encoding Arg at position 131) and *FCGR3A* (encoding Phe at position 158) gene loci have been associated

TABLE 1 | Fc receptors and their corresponding Ig binding in humans.

Receptor	Fcα/μR	FcμR	FcαRI	FcεRI	FcεRII	FcγRI	FcγRIIa	FcγRIIb	FcγRIIc	FcγRIIIa	FcγRIIIb
Human Ig	IgA/IgM	IgM	IgA	IgE	IgE	IgG	IgG	IgG	IgG	IgG	IgG
Affinity for monomer Ig	*N.D.	*N.D.	Low	High	Low	High	Low	Low	Low	Medium	Low

*N.D., not determined.

with decreased HIV acquisition (106). The latter SNP leads to the increased binding capacity of Abs for FcγRIIIA, which is the receptor involved in ADCC, suggesting that vaccine efficacy may be related to the increased efficacy of this function. More recently, Li et al. found that a tagged SNP (rs114945036) in *FCGR2C* (126C>T) was significantly associated with protection against infection with the HIV-1 AE subtype strain in the RV144 vaccine clinical trial. The direct effect of this SNP is not well-documented, although the authors proposed that it may lead to an FcR with an atypical FcR protein sequence, thereby modifying FcR expression or accessibility on cells (99). Interestingly, FcγRIIC has been reported to mediate ADCC and may play a role in anti-HIV-1 Ab neutralizing activity similar to that of FcγRIIB (103, 107–109).

Furthermore, although it has not yet been thoroughly investigated, there is evidence that FcγR polymorphisms are associated with mother-to-child transmission of HIV. Mothers with the FcγRIIIa-158V allele have enhanced binding affinity for IgG and ADCC capacity, which reduces the susceptibility of their fetuses to HIV infection and significantly reduces the chance of mother-to-child transmission during both the intrapartum and *in utero* periods compared with the FcγRIIIa-158F allele (110, 111).

These studies showing the significant role of FcγR polymorphisms strongly suggest that the Fc-driven function induced by vaccination may play a role in HIV protection. However, whether specific FcR polymorphisms are also involved in the control of HIV replication is not clear. One study did not detect significant differences when comparing the genotype profiles of *FCGR2A* and *FCGR3A* (polymorphisms H131R and V158F, respectively) in 73 patients in which HIV infection was controlled with those in patients who progressed to disease (112). Conversely, another study showed that the combination of these two SNPs was significantly associated with HIV progression in 53 patients who progressed compared with 43 patients in which HIV infection was controlled (113). Additional studies will be needed to define the roles of these SNPs in HIV replication and disease.

Importantly, the Fc regions of Abs contain a binding epitope for FcRn (neonatal FcR), which is responsible for the extended half-life, placental transport, and bidirectional transport of IgGs or immune complexes through the mucosal layer (**Figure 1**) (75, 114–116). FcRn is also expressed in myeloid cells, where it participates in both phagocytosis and antigen presentation together with the classical FcγR and complement. The relevance of this receptor in HIV infection has not been defined. However, this characteristic was largely exploited by modifying Fc regions in monoclonal Abs for use in the treatment of cancer or HIV infection.

Fc-MEDIATED Ab FUNCTIONS

Abs with Fc-mediated inhibitory activities, such as ADCC, ADCP, or aggregation, in addition to neutralizing activity have been detected at all stages of HIV disease. These inhibitory functions involve the Fc domains of Abs as well as the Fab domain. Therefore, both the Ab isotype and FcR expression on effector cells will be determinants of these functions (**Figure 1**).

Antibody-Dependent Cellular Cytotoxicity

ADCC is a complex but potent Fc-mediated effector function that is involved in the clearance of malignant or infected cells. In the latter process, ADCC eliminates virus-infected cells through mediating cooperation between innate and acquired immunity (117–119). Specifically, Abs act as a bridge between an infected target cell and an effector cell; the Fab domain binds to a specific viral antigen expressed by the infected cell, and the Fc domain binds to FcγR expressed on the surface of the effector cell (i.e., NK cells, monocyte/macrophages, and neutrophils) (120–122). As a result of this interaction, effector cells release perforin and granzymes, leading to death of the Ab-bound infected target cells. Several studies have shown an association between ADCC and slower disease progression in NHPs and humans (29, 123–126), highlighting the importance of ADCC *in vivo* (25, 26, 126–128). In the NHP model, ADCC was associated with protection from infection by a pathogenic virus (129). Interestingly, Abs directed against the V2 epitope were found to efficiently exhibit ADCC activity *in vitro* (130, 131). Similarly, non-NAbs targeting the V2 region of Env were associated with a decreased risk of HIV acquisition in the RV144 vaccine trial in Thailand (28, 29, 92). The relevance of ADCC was also demonstrated for mother-to-child transmission (MTCT), where the presence of ADCC-mediating Abs was associated with improved clinical status, delayed disease progression in infants (132) and a reduced risk of infection through breastfeeding (133).

In-depth studies are required to determine how Abs clear HIV-1-infected cells, including the investigation of epitopes recognized by ADCC-mediating Abs, naturally occurring Fc domains on ADCC-mediating Abs and Fc receptors on physiologically relevant effector cells (134). Further studies will be required to determine how to elicit the appropriate combinations of Abs and effector cells in the desired locations by vaccination. Because ADCC is a complex, multilayered process, the detection of this process using *in vitro* assays is challenging. Numerous assays have been developed to analyze ADCC activity *in vitro*. These assays differ in their use of various effector and target cell types (cell lines or primary cells), antigens (Env or whole virus), and read-outs (binding, effector cell activation, granzyme release or infected cell lysis). As a consequence, the

results obtained from each ADCC assay will reflect the different aspects and factors involved in each step (135).

First, Abs bind a specific epitope on target cells to mediate ADCC. This step can occur either during early events of virus binding to target cells or at a later step when viral epitopes are expressed on infected cells (121, 128, 136). As a consequence, different Env conformations can be involved according to the infection stage, and therefore the recognition of different specific epitopes by Abs may impact the ADCC results (137). Moreover, caution needs to be taken to ensure that the identified Abs effectively target infected cells and not uninfected cells that have captured HIV Env via their CD4 receptor (138–140). Therefore, identifying the viral epitopes on infected cells involved in ADCC is critical. The number of viral epitopes targeted by ADCC activity seems to be higher than that targeted by neutralization. Indeed, numerous non-NAbs recognizing non-functional spikes on the viral surface or specific conformations of epitopes expressed on infected cells were shown to mediate ADCC without displaying neutralizing activity. We may very well-propose that Abs mediating ADCC may be complementary to bNAbs in potentiating the inhibitory activity of an HIV vaccine.

Second, the Fc domain of an Ab binds to the FcR expressed on effector cells. This binding is dependent on one side of the Ab heavy chain sequence. Interestingly, the Fc domain sequence is influenced by B-cell activation, the recognized epitope and intrinsic donor variability and therefore varies according to the isotype and gene rearrangement. Notably, newly developed monoclonal Abs were generated by their reconstruction with a same IgG1 heavy chain therefore all expressing an identical Fc domain. These constructs provided the opportunity to analyze ADCC while maintaining a constant Ab Fc domain. However, we must remember that these bNAbs do not reflect the large spectrum of variability in the Ab Fc domain *in vivo*.

Third, the function of Abs is dependent on the polymorphisms and expression of the FcRs on effector cells. FcR expression is regulated in different cell populations according to their localization, maturation stage, and genotype. For example, NK cells from healthy donors are usually used as effector cells to test ADCC *in vitro*. However, little is known about how the distribution of FcR expression on NK cells varies in different tissues and among individuals. This phenomenon may impact vaccine responses, HIV transmission and disease progression. Moreover, the combination of specific HLA and killer immunoglobulin-like receptor (KIR) expression on NK cells was shown to play a role in protection against infection and elite control (141–145). Taken together, the results suggest that all primary NK cell variables may influence ADCC outcomes *in vitro* if not adequately standardized. This may be overcome by using an NK cell line, albeit at the expense of the physiological variability of FcRs *in vivo*.

Finally, the assays currently being developed in the field use distinct read-outs (24, 134, 146). The detection of the lysis of HIV-infected cells is the ultimate physiologically relevant read-out, but this outcome is technically highly challenging to measure. More straightforward read-outs, such as detection of FcR triggering, are being proposed, but whether these indirect detections methods effectively reflect ADCC function is not clear. Further investigations are urgently required to precisely define the important epitopes and to determine how to efficiently trigger effector cells to achieve the *in vivo* destruction of infected cells.

Antibody-Dependent Cellular Phagocytosis

Abs can also eliminate opsonized pathogens through phagocytosis via the engagement of FcRs expressed by cells of the innate immune system, including monocytes, macrophages, neutrophils, dendritic cells (DCs), and mast cells (27, 147–149). Phagocytosis is important for pathogen clearance by direct lysis or antigen presentation and innate immune cell activation with consequent pathogen elimination. The detection of ADCP *in vitro* leads to efficient inhibition of HIV replication in infected cells (27, 148, 149) and is associated with protection from repeated intrarectal challenge with SHIV-SF162P3 or SIVmac251 in immunized rhesus macaques (150–152). ADCP mediated by IgG3 Abs was elicited in recipients of the RV144 vaccine (153). However, the epitope specificity of Abs mediating ADCP still needs to be investigated. Musich et al. demonstrated that anti-V2 monoclonal Abs mediated ADCP activity in a dose-dependent manner similar to anti-V3 and CD4bs monoclonal Abs against clade B gp120 (154) but displayed increased activity against clade C gp120 compared to anti-V3 and anti-CD4bs monoclonal Abs, suggesting the broader recognition of exposed epitopes (154); this may also have been due to V2 epitopes being more conserved between clade B and C than V3 epitopes. Moreover, the role played by the cell type that mediates ADCP should also be defined. Current *in vitro* ADCP assays mainly use cell lines that may largely reduce the physiological relevance of these *in vitro* assays. However, recently, phagocytosis mediated by macrophages or activated neutrophils in human mucosal and lymphoid tissues was proposed to play a significant role in protection from infection (155). Thus, this type of assay requires further development to better define the *in vivo* role of ADCP function.

Antibody-Dependent Cell-Mediated Virus Inhibition

ADCVI involves a combination of different FcγR-mediated antiviral activities that occur when an Ab bound to a virus-infected target cell engages FcγR-bearing effector cells, such as NK cells, monocytes or macrophages (156). *In vitro* virus inhibition assays partly measure target cell death mediated by ADCC and partly measure non-cytolytic mechanisms of HIV inhibition due to β-chemokine release from effector cells or the phagocytosis of immune complexes (27, 42, 157).

Overall, Fc-mediated function was found to actively contribute to Ab inhibitory activities. The combination of these activities was found to be associated with protection (158).

The involvement of Fc-mediated activity in HIV protection was demonstrated in an experimentally challenged macaque model. The protection observed with the bNAb b12 was markedly reduced after the modification of the Fc domain, leading to impaired FcR binding (159). However, Fc-mediated effector functions might not be absolutely necessary to generate maximum protection, as was recently shown for the bNAb

PGT121. In this study, a mutation impairing FcγR binding of PGT121 did not modify the protective effect of the bNAb (160).

ANTIBODY CAPTURE OF INFECTIOUS HIV PARTICLES/AGGREGATION

The Fc domains of Abs can directly bind the virus, leading to the formation of virus/Ab aggregates. Ab inhibition by aggregation of a pathogen is a very basic inhibitory mechanism that results in a decrease in viral infectivity (161). The potential role of aggregation was reviewed recently (24, 146, 162).

Formation of the Ab/virus immune complex may hinder viral movement and impede viral replication by adhering the complex to mucus and restraining its transfer and transcytosis across mucosal epithelial cells (**Figure 1**). HIV aggregates will be trapped more efficiently than free virus particles (e.g., in the female reproductive tract, where there is abundant cervical mucus) (163). Moreover, immune aggregates may be retained efficiently in the mucus by binding of the Fc domain of IgG to mucins and specific binding in the vaginal tract to MUC16 (164). Analogous mechanisms may act on other mucosal surfaces, such as those in the gastrointestinal tract (165).

These findings suggest a direct inhibitory effect of HIV/Ab immune complex formation on HIV infectivity. Abs that retain HIV and hinder its diffusion through the epithelial barrier need to be better characterized to elucidate how they can be selectively induced at the mucosal site. We expect that these Abs recognize quaternary functional trimeric envelopes and non-functional Env spikes expressed on HIV particles. Therefore, the number of Abs able to form aggregates will be enlarged compared to that of NAbs, which will open up new opportunities to induce functional Abs with distinct epitope recognition by vaccination.

ANTIBODY-MEDIATED COMPLEMENT ACTIVATION

The complement system is key to both innate and adaptive immunity, where it exerts multiple functions. Complement activation occurs through three distinct pathways (classical, alternative and lectin), which result in several types of antimicrobial activity, such as opsonization, inflammatory cell recruitment, cell lysis and virolysis [see review (24, 26, 166, 167)].

HIV has developed a sophisticated defense to protect itself by failing to bind complement proteins (168). Indeed, the gp120 Env does not bind complement (168). Moreover, during budding, HIV incorporates glycosyl phosphatidylinositol (GPI)-anchored CD55 and CD59 as well as transmembrane CD46, which are downregulatory molecules that inhibit complement-mediated damage to the virus (169). HIV also captures serum complement factor H, which plays a central role in protecting cells from complement by downregulating complement binding and in turn increases virulence (170–172). For these reasons, the use of primary isolates produced by primary cells is absolutely mandatory for *in vitro* studies of complement-mediated responses.

Moreover, bNAbs engineered to lack complement binding activity do not lose their protective effect, as shown in macaques challenged with SHIV (159), which is in contrast to those engineered to lack FcR. This finding suggests that complement may not be required for optimal *in vivo* Ab protection against infection. Nonetheless, in the RV144 vaccine trial that showed modest efficacy, complement activation induced by V1V2-specific Abs was stronger and more frequently detected compared to that in two related trials, VAX003 and VAX004, in which no significant protection was observed (173).

In contrast, complement and Ab may contribute to the enhancement of infection, as first described for Dengue virus (174). Binding of IgGs to FcRs induces the enhanced transcytosis of the virus at mucosal sites, although the exact mechanism is still unclear (**Figure 1**) (175). Moreover, whether this phenomenon has physiological relevance during HIV infection is still under debate (176, 177).

Collectively, complement components and their interactions with their cognate receptors are key to controlling adaptive immune responses, which provides insight into the use of complement components as novel drug targets. However, the relative contribution of complement to virolysis vs. viral enhancement in tissues and the periphery needs to be further investigated to understand its role in protection against HIV.

ROLES OF Fc-MEDIATED ANTIBODY FUNCTIONS IN MUCOSAL TISSUES

Mucosal surfaces are the first entry site for HIV during transmission (**Figure 1**). Indeed, Langerhans cells (LCs), urethral macrophages and/or conventional DCs residing in mucosal tissues have been proposed to capture HIV (178–185) and further replicate or transfer the virus locally to potential HIV target cells, such as macrophages and $CD4^+$ T cells (**Figure 1**). NK cells, macrophages and DCs, which are the effector cells involved in Fc-mediated Ab functions, are present in different mucosal tissues at different levels. Accordingly, mucosal effector cells may act through an Fc-mediated Ab response and be the first cells that modulate the early events of HIV transmission. The relative contribution of any of the aforementioned Fc-mediated Ab responses will depend on the frequency and distribution of the cells present within a given tissue and their FcR expression levels (3, 26, 182, 184, 186). Overall, FcR expression on cells and their affinities and binding profiles for Abs will directly impact Fc-mediated functions. Indeed, a comparison of penile, cervical and intestinal tissues showed that the expression profile of FcR on mucosal effector cells was reduced compared to that on blood cells, although the overall cell frequency was substantially different (187). Specifically, $Fc\gamma RII^+$ DCs and macrophages were well-represented in all three tissues, whereas $Fc\gamma RIII^+$ NK cells were rare only in the intestinal mucosa. We may imagine that Fc-mediated Ab function(s) may be less relevant in the blood circulation, where infected $CD4^+$ T cells express very little FcγR on their surfaces (188).

Importantly, FcR expression varies according to the immune cell type and the localization and activation status. FcR-bearing cells, such as macrophages and DCs, predominate and tightly interact with tissues, which may facilitate their activity. Although resident NK cells express negligible levels of FcRs, mature circulating NK cells expressing high FcγRIII levels are rapidly recruited to the site of infection. Therefore, Fc-mediated inhibitory functions involving binding to FcγR on resident tissue cells may be of particular interest for the inhibition of mucosal transmission, although the exact mechanisms underlying FcR expression are not well-defined. Further studies on the potential role of Fc-bearing HIV targets and the involvement of Fc-mediated Ab inhibition at mucosal sites are needed to inform HIV vaccine strategies.

STRATEGIES TO INDUCE Fc-MEDIATED ANTIBODY FUNCTIONS BY VACCINATION

Antibodies have now been proven to contribute to HIV protection and are therefore a central component of new vaccine strategies (189). As Fc-FcR interactions are able to generate powerful extraneutralizing Fc functions, these additional functions should be defined (159).

Numerous vaccine trials have already reported the induction of Fc-mediated functions. Vaccination with the gp120/CD4 mAb immune complex was found to be more efficient in inducing Fc-mediated adjuvant activity than that with gp120 alone. However, highly variable elements in the gp120 sequence limit the breadth of the responses to immune complex vaccines to a few HIV-1 isolates (190, 191). Moreover, vaccination with the SIVmac239-ΔNef virus induced an FcR-mediated inhibitory response that prevented founder virus entry and avoided local expansion (192). These results suggest protective Fc-mediated Ab function against transmitted/founder viruses at the mucosa surface. Another study demonstrated robust polyfunctional non-NAb responses, such as ADCC and ADCP, associated with protection against SIVmac251 challenge following vaccination with adenovirus serotype 26 (Ad26) vector priming/purified envelope (Env) glycoprotein boosting strategy in rhesus monkeys (151). In addition, a robust non-NAb response to the V1V2 region of the gp120 Env glycoprotein, which has been shown to exhibit Fc-mediated functions, was associated with a decreased risk of HIV acquisition in the RV144 clinical vaccine trial (193, 194). These different vaccine trials indicate that Abs displaying Fc-mediated functions participate in protection against HIV acquisition. This protection may be particularly efficient when several Fc-mediated activities are combined (158). Fc polyfunctionality increases over time following HIV infection and has recently been correlated with the further induction of neutralizing activity (195).

Therefore, we hypothesize that the induction of immune responses leading to Fc polyfunctionality may require the presentation of multiple antigens, similar to what induces bNAb production. Indeed, multiple antigens appear to be required for induction of bNAbs, as they appear following long periods of high viral replication in which there is continuous mutation of the viral Env protein. The general concept that the induction of an efficient humoral response, i.e., with bNAbs, and possibly Fc polyfunctionality will require high viral replication is sometimes misleading within the field. The correlation between an efficient Ab response and a high viral load provides a confusing dichotomist message regarding the potential role of Abs in protection against HIV acquisition. Accordingly, we may need a complex combination of immunogens to induce efficient Fc polyfunctional Abs as for induction of bNAbs. A more systematic analysis of Fc polyfunctional activity induced following vaccination is mandatory to understand the dynamics of the induction of functionally relevant Abs and to avoid switching to an immunodominant non-functional decoy Ab response.

To define the correlates between Ab profiles and protective functions, an integrative approach analyzing the "humoral Fc fingerprints" of vaccines was proposed. According to these principles, Chung et al. developed a unique approach called "Systems Serology" to retrospectively examine recent vaccine trials in humans to reveal features of immune complex composition underlying protective immunity to HIV (196). Using the "Systems Serology" approach, the protective humoral response signatures in vaccinated or naturally infected individuals in HIV can be defined. Moreover, systematic data production and application of machine learning approaches may identify distinct immunogenic regimens and Fc effector functions, allowing the selection of promising vaccine candidates (197).

These different studies provide insight into how to potentially induce Fc-mediated functions able to protect or control HIV infection via Fc-mediated antiviral activity (198, 199). New vaccine strategies aimed at directly inducing Fc-mediated activity should now be designed to improve the induction of potential functional activity in addition to highly desirable neutralizing activity.

SUMMARY AND CONCLUSION

This review highlights the potential role of FcγR-mediated Ab immune functions besides neutralization in protection against HIV. Although the role of the Fc-mediated function of Abs lacking broadly neutralizing activity for HIV protection is still a matter of debate (160, 200), numerous independent studies now hint at their relevance for HIV inhibition. Above all, the specific Fc-mediated functions of non-NAbs are the only correlates of protection against infection observed in the RV144 vaccine trial conducted in Thailand (28, 29, 193, 201). However, no *in vivo* demonstration of the pertinence of non-NAbs for the prevention of HIV transmission is currently available. Dedicated experiments utilizing the NHP model may help dissect the role of Fc-mediated Ab responses and their relevance for the prevention of HIV infection. Nonetheless, to exert their function, these Abs must colocalize with the appropriate FcR-bearing cells at the site of infection. This scenario may occur during the early events of HIV mucosal transmission, when the virus crosses the epithelial barrier to infect the underlying target cells and further disseminate to other organs. Close contact between the

virus, infected cells, Abs and effector cells in mucosal tissues may provide an ideal environment for Fc-mediated Ab functions to occur. We can also imagine that the prevalent and relevant Fc-mediated Ab function(s) may differ according to the infection route and mucosal type involved (i.e., genital or intestinal mucosa). An important hurdle for HIV vaccine designers will be to determine how to induce high concentrations of Abs with Fc-mediated functions directly within the mucosal sites of exposure, which is a challenge that may equal or be greater than that of inducing bNAbs (202).

AUTHOR CONTRIBUTIONS

BS wrote the first draft of the manuscript. BS, SD, RC, GS, and CM revised the manuscript. All authors listed have made a substantial, direct and intellectual contribution to the work, read, and approved the final manuscript.

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