



Improved Overall Survival, Relapse-Free-Survival, and Less Graft-vs.-Host-Disease in Patients With High Immune Reconstitution of TCR Gamma Delta Cells 2 Months After Allogeneic Stem Cell Transplantation

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T-cell receptor (TCR) $\gamma\delta$ cells are perceived as innate-like effector cells with the possibility of mediating graft-vs.-tumor (GVT) without causing graft-vs.-host disease (GVHD) in the setting of hematopoietic allogeneic stem cell transplantation (HSCT). We conducted a prospective study to assess the clinical impact of TCR $\gamma\delta$ cell immune reconstitution on overall survival, relapse-free-survival, relapse and GVHD. The impact of CD3, CD4, and CD8 T cells together with NK cells including subtypes were analyzed in parallel. A total of 108 patients with hematological malignancies transplanted with HLA-matched, T cell replete stem cell grafts were included for analyses of absolute concentrations of CD3, CD4, and CD8 positive T cells and NK cells together with a multi-color flow cytometry panel with staining for TCR $\alpha\beta$, TCR $\gamma\delta$, V δ 1, V δ 2, CD3, CD4, CD8, HLA-DR, CD196, CD45RO, CD45RA, CD16, CD56, CD337, and CD314 at 28, 56, 91, 180, and 365 days after transplantation. Immune reconstitution data including subsets and differentiation markers of T and NK cells during the first year after transplantation was provided. Patients with TCR $\gamma\delta$ cell concentrations above the median value of 21 (0–416) $\times 10^6$ cells/L 56 days after transplantation had significantly improved overall survival ($p = 0.001$) and relapse-free survival ($p = 0.007$) compared to patients with concentrations below this value. When day 56 cell subset concentrations were included as continuous variables, TCR $\gamma\delta$ cells were the only T cell subsets with a significant impact on OS and RFS; the impact of TCR $\gamma\delta$ cells remained statistically significant in multivariate analyses adjusted for pre-transplant risk factors. The risk of death from relapse was significantly decreased in patients with high concentrations of TCR $\gamma\delta$ cells 56 days after transplantation ($p = 0.003$). Also, the risk of acute GVHD was significantly lower in patients with day 28 TCR

$\gamma\delta$ cell concentrations above the median of 18×10^6 cells/L compared to patients with low concentrations ($p = 0.01$). These results suggest a protective role of TCR $\gamma\delta$ cells in relapse and GVHD and encourage further research in developing adaptive TCR $\gamma\delta$ cell therapy for improving outcomes after HSCT.

Keywords: allogeneic stem cell transplantation, innate immunology, gamma delta ($\gamma\delta$) T cells, transplant immunology, cell therapy

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially curative treatment for patients with hematological malignancies by elimination of recipient tumor cells by reactive immune cells from a donor (1). The graft-vs.-leukemia (GVL) effect is originally thought to be mediated by conventional CD4 and CD8 positive T cells expressing the $\alpha\beta$ T cell receptor (TCR) through alloreactivity caused by differences in human leukocyte antigens (HLA) and minor histocompatibility antigens (mHAg) between the recipient and the donor (2). When the alloreaction from the donor is aimed at non-malignant recipient tissue, graft-vs.-host disease (GVHD) with extensive morbidity and mortality to the patient can occur (3). The $\alpha\beta$ T cells, which constitute the majority of human T cells, raise an adaptive immune response including antigen-specific effector- and memory phases. In contrast, CD4/CD8 negative TCR $\gamma\delta$ bearing T cells, which normally constitute about 1–10% of the total T cell repertoire in peripheral blood, operate in an innate-like manner in the initiation phase of the immune reaction independently from HLA-antigen presentation (4). The $\gamma\delta$ TCR heterodimer is encoded by two distinct set of genes, γ and δ , with a restricted repertoire of V and J gene segments used for TCR rearrangement. In cord blood the V δ 1 subset constitutes the majority of TCR $\gamma\delta$ cells, but already by 1 year of life the V δ 2 subtype becomes the predominant subset in circulation. In adults, the TCR V δ 2 subtype constitutes the majority (>75%) of circulating TCR $\gamma\delta$ cells, while tissue- and epithelial associated TCR $\gamma\delta$ cells are mainly of the non-V δ 2 (primarily V δ 1) subtype (5). The peripheral V δ 2 subset expand clonally until about 10 years of age and is believed to be driven both by environmental changes and endogenous stimuli in the maturing immune system. TCR $\gamma\delta$ cells are capable of tumor-recognition through the $\gamma\delta$ TCR and activating receptors shared with natural killer (NK) cells (e.g., NKG2D) (6), and their anti-tumor effects are increasingly being documented in solid tumors as well as in leukemia (7, 8). In the setting of HSCT, the anti-leukemic properties together with the innate-like effector mechanisms in the absence of HLA-restriction suggest that TCR $\gamma\delta$ cells mediate GVL in the absence of GVHD (9, 10).

Early immune reconstitution is essential for clinical outcomes after transplantation (11), but recent results on the impact of TCR $\gamma\delta$ cell immune reconstitution are primarily from studies in pediatric, HLA-mismatched, and/or graft-manipulated HSCT settings (12–14). Here, we report the results from a prospective, clinical study on the impact of TCR $\gamma\delta$ T cell immune reconstitution on overall survival (OS), relapse-free

survival (RFS), relapse, and GVHD after HSCT in adult patients receiving HLA-matched, T cell replete stem cell grafts.

MATERIALS AND METHODS

Patients

From October 2015 to March 2017, 113 consecutive patients were transplanted at the Stem Cell Transplantation Unit, Department of Hematology, Copenhagen University Hospital, Rigshospitalet. Two patients declined entrance in the study, the remaining 111 were included. Only patients above 18 years receiving bone marrow (BM) or peripheral blood stem cells (PBSC) and undergoing their first transplantation were included. The study was approved by the Danish National Committee on Health Research Ethics, and all participants gave written informed consent prior to transplantation in accordance with the Declaration of Helsinki. Three patients with non-malignant diseases were later excluded from analyses, **Table 1** shows transplant characteristics for the remaining 108 patients.

Conditioning Regimen

Myeloablative regimens were cyclophosphamide 120 mg/kg plus 12 Gy total body irradiation (TBI); cyclophosphamide was replaced with Etophophos 1,800 mg/m² for acute lymphoblastic leukemia. Cyclophosphamide doses were calculated using adjusted ideal body weight if BMI was >27.5. Non-myeloablative conditioning was fludarabine 90 mg/m² plus 2 Gy TBI; TBI was increased to 4 Gy in patients not previously treated with chemotherapy. High risk MDS patients were conditioned with fludarabine 150 mg/kg plus treosulfan 42 g/mq. The conditioning regimen for haplo-identical transplantation was cyclophosphamide 29 mg/kg, fludarabine 150 mg/m² and 2 Gy TBI. Twelve patients received anti-thymocyte-globulin (ATG) as part of the conditioning regimen: two patients with antigen mismatch received Thymoglobulin 2.5 mg/kg at day –3, –2, and –1, 10 patients with matched unrelated donors and transplanted with PBSC after myeloablative conditioning received Grafalon 10 mg/kg at day –3, –2 and –1.

GVHD Prophylaxis and Diagnosis

Myeloablative patients received cyclosporine 6.25 mg/kg orally b.i.d. from day –1 combined with a short-course of intravenous methotrexate on days 1 (15 mg/m²), 3, 6, and 11 (10 mg/m²). Cyclosporine was tapered to stop at day 180, unless GVHD was present. Tacrolimus was used in patients receiving the fludarabine/treosulfan regimen. Non-myeloablative patients received tacrolimus 0.06 mg/kg orally b.i.d. from day –3

TABLE 1 | Patient and transplant characteristics.

N	108
Follow-up time, days, median, range	673 (386–913)
Age, years, median, range	58 (20–74)
Disease, n, percent	
AML	46 (43%)
ALL	14 (13%)
MDS	25 (23%)
Myelofibrosis	8 (7%)
Lymphoma	6 (6%)
Chronic leukemia	4 (4%)
Other	5 (4%)
Disease risk index, n, percent	
Low	10 (9%)
Intermediate	87 (81%)
High	11 (10%)
Graft source, n, percent	
BM	15 (14%)
PBSC	93 (86%)
Donor, n, percent	
MRD	26 (24%)
MUD	82 (76%)
Donor match, n, percent	
10/10 or 9/10	97 (90%)
1 Ag MM	9 (8%)
Haplo-identical	2 (2%)
CMV serological status, D/R	
+/+	41 (38%)
+/-	8 (7%)
-/-	26 (24%)
-/+	33 (31%)
Recipient-donor sex, n, percent	
M/M	52 (48%)
M/F	28 (26%)
F/F	17 (16%)
F/M	11 (10%)
Conditioning intensity, n, percent	
Myeloablative	51 (47%)
Non-myeloablative	57 (53%)
Conditioning regimen, n, percent	
TBI-Flu	53 (49%)
Flu-Treo	27 (25%)
TBI-Cy	21 (19%)
TBI-Etopophos	4 (4%)
Other	3 (3%)

AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; BM, bone marrow; PBSC, peripheral blood stem cells; MDR, matched related donor; MUD, matched unrelated donor; TBI, total body irradiation; Flu, fludarabine; Treo, treosulfan; Cy, cyclophosphamide; CMV, cytomegalovirus.

combined with mycophenolate mofetil 15 mg/kg b.i.d. from day 0 to days 27 in related transplants. In unrelated transplants, tacrolimus 0.06 mg/kg orally b.i.d. from day -3 was combined with mycophenolate mofetil 15 mg/kg orally t.i.d. from day 0

to day 30, then b.i.d. to day 40 and tapered to stop day 96 in the absence of GVHD. In the absence of GVHD, tacrolimus was tapered from day 56 to zero by day 180 in related non-myeloablative transplants, and from day 100 to zero by day 180 in unrelated non-myeloablative transplants. In mismatched non-myeloablative transplants, cyclosporine 5 mg/kg orally b.i.d. and sirolimus 2 mg orally q.d. from day -3 and mycophenolate mofetil 15 mg/kg t.i.d. from day 1 was administered. Cyclosporine was tapered to stop day 180 and mycophenolate mofetil day 150; sirolimus was tapered to stop day 365, unless GVHD was present. In haplo-identical transplants, cyclophosphamide 50 mg/kg was administered day 3 and 4; tacrolimus 1 mg q.d. and mycophenolate mofetil 15 mg/kg t.i.d. from day 0 to 35.

Acute and chronic GVHD (aGVHD, cGVHD) was diagnosed and graded from clinical symptoms and biopsies according to the modified Glucksberg-Seattle criteria (15, 16).

Relapse

Relapse was morphologically diagnosed in leukemia patients as more than 5% blast cells in the bone marrow or the appearance of extramedullary leukemic lesions, in MDS patients, recurrence of MDS by morphology, cytogenetics, or both. Relapse in lymphoma patients was defined by new or progressing foci in PET/CT scans.

CMV Monitoring and Treatment

Analyses for CMV DNA was done weekly on peripheral blood with quantitative PCR from day 21–100 after transplantation. Cut-off level was 300 CMV DNA copies/ml. In case of a positive CMV PCR, treatment with oral valganciclovir 900 mg b.i.d. was commenced. Patients not able to take oral medication were treated with intravenously ganciclovir 90 mg/kg b.i.d.

Patient Samples

Peripheral blood samples for evaluation of immune reconstitution were collected at 5 fixed time points within the first year after transplantation: day 28 (median 28 [23–39]), 56 (median 56 [48–76]), 91 (median 91[74–122]), 180 (median 181[148–239]), and 365 (median 364 [334–452]). Samples were collected on 2 mL ethylenediamine tetra acetic tubes and analyzed freshly at the Tissue Typing Laboratory, Department of Clinical Immunology, Copenhagen University Hospital, Rigshospitalet.

Flow Cytometry

Routine analyses for absolute concentrations of CD3, CD4, and CD8 positive T cells and NK cells were evaluated by flow cytometric analyses with BD™ Trucount tubes containing fluorescent beads as an internal standard according to the manufacturer's instructions (BD Biosciences, San Jose, California). Immunolabeling with CD4 FITC/CD8 PE/CD3 PerCP and CD3 FITC/CD16+CD56 PE/CD45 PerCP from BD™ Biosciences were used. Samples were analyzed on Navios™ (Beckman Coulter, Miami, Florida), and Navios™ software was used for software analyses. Residual blood volume was used for immune phenotyping in a 2-tube, stain-lyse, multi-color flow cytometry panel developed for the study. For multi-fluorochrome

staining, 100 μ L of whole blood was labeled for TCR $\alpha\beta$ -FITC, TCR $\gamma\delta$ -PE, CD4-PerCP-Cy5.5, CD45RA-PE-Cy7, CD197-APC, CD45RO-APC-H7, HLA-DR-V450, CD3-V500, and CD8-BV605 for tube 1, and TCRV δ 2-FITC, TCR $\gamma\delta$ -PE, TCRV δ 1-PE-Cy7, CD314-APC, CD16-APC-H7, CD56-V450, CD3-V500, and CD337-BV605 for tube 2. After 15 min incubation, red blood cells were lysed by the addition of 2 mL of lysing solution (EasyLyseTM) followed by 10 min of dark incubation at room temperature. This was followed by a 5-min wash procedure (300 g) with 2 mL BDTM FacsFlow and resuspension in 300 mL BDTM FacsFlow prior to immediate acquisition.

Acquisition and Data Analysis

Sample acquisition was performed using BDTM FACSCanto flow cytometer with the BDTM FACSDiva software which was also used for data analyses. Phenotype subset definition and gating strategies are shown in **Tables 1, 2** and **Figure 1** in **Supplemental Data**.

Immune Reconstitution Analyses

Analyzed lymphocyte subset were absolute concentrations of total TCR $\gamma\delta$ cells, TCR V δ 1, TCR V δ 2, CD3 T cells, CD4 T cells, CD8 T cells, total NK cells, CD16bright NK cells, CD16/56 NK cells, and CD56bright NK cells. In addition, we analyzed fractions of differentiation subsets in terms of naïve (CD45RA+CD197+), central memory (CD45RA-CD197+), effector memory (CD45RA-CD197-) and TEMRA (CD45RA+CD197-) cells of CD4, CD8, and TCR $\gamma\delta$ cells. The fractions of TCR $\gamma\delta$ cells of total CD3 cells, the V δ 1, V δ 2, and nonV δ 1-nonV δ 2 of total TCR $\gamma\delta$ cells, and the CD16bright, CD16/56, and CD56bright of total NK cells were also analyzed. The expression of HLA-DR as a marker of activation was analyzed on total TCR $\gamma\delta$ cells, CD4 T cells, and CD8 T cells. The expression of the activating receptor NKG2D was analyzed on total TCR $\gamma\delta$ cells, V δ 1, and V δ 2 cells. Throughout the text, “concentration” refers to absolute concentrations ($10^6/L$) and “percentages” or “fractions” refer to percent of the specified cell subsets of the specified cell populations. Cell concentrations were analyzed as continuous and categorical variables (high vs. low) dichotomized by the median value of the above-mentioned absolute cell concentrations.

Outcomes

The primary outcomes were overall survival (OS) and relapse-free survival (RFS) from day 56. OS was defined as the probability of survival from day 56 with death as an event. RFS survival was defined as the probability of survival without relapse from day 56 with an event defined as the composite of death and/or relapse. Day 56 after transplantation was selected for the primary outcome as this was the closest time point to relapse occurrence, which still preceded relapse in all patients. Secondary outcomes included death from relapse, aGVHD and cGVHD. For associations to aGVHD the earliest sample after transplantation (day 28) was used. Nine patients were diagnosed with aGVHD before their respective day 28 sample and were therefore excluded from the aGVHD analyses. Association to cGVHD was performed for both day 28

and day 56 immune reconstitution. Furthermore, associations between post-transplant CMV infection and TCR $\gamma\delta$ cell immune reconstitution (high vs. low median concentrations and fractions) were analyses at day 56, 91, 180, and 365 after transplantation. For each time point only patients with CMV infection diagnosed at least 1 week prior to their blood sampling were included for time to establish an immunological cellular response (for this reason associations with day 28 immune reconstitution were not analyzed, as only 3 patients had CMV infection more than 1 week before their respective day 28 sample). The associations between pre-transplant CMV status of the donor and TCR $\gamma\delta$ cell immune reconstitution were tested in all patients and for all time points after transplantation. For all outcomes, patients with graft rejection ($n = 1$) and graft failure ($n = 2$) were censored at the time of rejection or booster transplantation.

Statistical Analyses

Kaplan Meier survival analysis and Cox proportional hazards models were used to investigate the associations between immune reconstitution and OS and RFS. In addition to cell concentrations, pre-transplant factors thought to have a possible impact on OS and RFS were included in the analysis. Disease Risk Index was included for all patients after previously published criteria (17). Due to size of the dataset only factors distributed with more than 10% of patients in the smallest group were included. Pre-transplant factors with a $p < 0.1$ in univariate analysis were included in the multivariable models. The pre-transplant factors were analyzed in patients alive by day 28 after transplantation ($n = 106$) and these results were used for all multivariate analyses.

The cumulative incidence of aGVHD and death from relapse, was determined using Gray's competing risks analysis. Competing risk for death from relapse was death from transplant-related mortality (TRM). TRM was defined as death from all causes other than relapse. Competing risk for aGVHD was death from all causes other than aGVHD. Only grade II-IV aGVHD were included in the aGVHD analyses. Associations between CMV status of the donor/post-transplant CMV infection and TCR $\gamma\delta$ cell immune reconstitution were analyzed by the χ^2 -test. Differences between categorical and continuous variables were determined by the χ^2 and Student's T -test, respectively. Spearman correlation was used for non-parametric assessment of correlation between different cell subsets during immune reconstitution.

Sensitivity Analysis

In sensitivity analysis Cox-proportional hazards models were used to investigate the robustness of the results when cell concentrations were included as time-update variables. For these analyses all patients with a sample at day 28 were included from the date of that sample and followed until relapsed, death or the last follow-up date, depending on the outcome of interest.

Statistical analyses were performed using SPSS version 22 (SPSS, Chicago, IL) and R version 3.2.0 (R Foundation for Statistical Computing, Vienna, Austria) combined with the EZR platform (18) and SAS version 9.4.

TABLE 2 | T and NK cell concentrations and subset distribution 28–365 days after transplantation.

Cell subset concentration, 10 ⁶ /L, median (IQR)	Day 28 N = 106	Day 56 N = 104	Day 91 N = 99	Day 180 N = 86	Day 365 N = 66
T cells (CD3)	420 (225–553)	425 (260–738)	460 (240–750)	835 (458–1400)	1150 (680–2000)
CD4	190 (110–300)	190 (113–280)	210 (100–300)	280 (170–420)	395 (250–490)
CD8	150 (71–240)	175 (100–395)	230 (100–440)	440 (198–950)	650 (340–1425)
TCR $\gamma\delta$	18 (6.5–44)	21 (7.2–50)	21 (6.6–53)	36 (12–87)	62 (19–111)
V δ 1	1.5 (0.7–3.4)	1.6 (0.6–4.6)	1.6 (0.6–6.8)	4.4 (1.6–24)	7.9 (3.3–35)
V δ 2	13 (4.8–42)	17 (5.1–39)	14 (4.0–38)	16 (6.1–53)	24 (7.2–62)
NonV δ 1-nonV δ 2	0.4 (0.2–0.8)	0.3 (0.1–1.0)	0.5 (0.2–1.3)	0.8 (0.3–4.4)	1.2 (0.5–7.3)
NK cells (CD16/56)	280 (140–403)	190 (110–330)	160 (100–270)	240 (130–388)	235 (150–360)
CD16bright	26 (14–60)	25 (12–51)	24 (10–47)	25 (13–55)	27 (15–56)
CD16/56	158 (82–258)	113 (69–202)	106 (65–184)	152 (97–282)	179 (112–294)
CD56bright	57 (27–96)	31 (17–63)	23 (14–38)	17 (12–32)	17 (10–27)
Relative concentrations, %					
CD4/CD3	56 (44–65)	49 (28–61)	46 (25–57)	37 (22–50)	35 (23–48)
CD8/CD3	37 (31–52)	45 (36–65)	51 (34–68)	59 (46–74)	62 (47–75)
CD4/CD8, ratio	1.5 (0.9–2.1)	1.0 (0.4–1.8)	0.9 (0.4–1.5)	0.6 (0.3–1.1)	0.6 (0.3–1.0)
Naïve CD4/CD4	28 (15–37)	26 (12–37)	26 (15–35)	21 (12–32)	22 (13–29)
Central memory CD4/CD4	40 (34–49)	41 (33–49)	40 (34–48)	40 (31–48)	43 (34–48)
Effector memory CD4/CD4	25 (19–37)	29 (18–42)	27 (19–39)	32 (22–46)	31 (23–42)
TEMRA CD4/CD4	0.8 (0.4–1.8)	1.1 (0.6–2.5)	1.3 (0.6–2.5)	1.5 (0.9–4.8)	1.7 (0.9–4.7)
HLA-DR CD4/CD4	21 (13–40)	27 (17–51)	31 (21–49)	37 (24–51)	26 (20–37)
Naïve CD8/CD8	23 (7.6–37)	13 (4.4–33)	13 (5.8–26)	12 (5.9–22)	9.4 (4.4–19)
Central memory CD8/CD8	4.7 (2.9–7.9)	4.3 (2.3–8.4)	5.2 (2.9–9.2)	5.6 (3.1–9.5)	5.6 (2.6–9.2)
Effector memory CD8/CD8	39 (27–56)	40 (29–52)	36 (28–47)	32 (24–43)	37 (29–52)
TEMRA CD8/CD8	21 (13–38)	32 (18–45)	36 (24–52)	44 (33–57)	39 (23–51)
HLA-DR CD8/CD8	40 (22–61)	52 (26–75)	58 (40–74)	64 (50–78)	52 (35–62)
Naïve TCR $\gamma\delta$ /TCR $\gamma\delta$	1.8 (0.9–4.0)	1.6 (0.6–3.4)	2.1 (0.8–4.3)	2.0 (1.0–4.5)	2.1 (0.7–4.5)
Central memory TCR $\gamma\delta$ /TCR $\gamma\delta$	1.2 (0.4–2.3)	0.9 (0.3–2.6)	0.9 (0.2–2.2)	1.2 (0.3–3.5)	2.1 (0.6–4.0)
Effector memory TCR $\gamma\delta$ /TCR $\gamma\delta$	51 (37–67)	48 (32–61)	38 (23–54)	37 (22–52)	43 (27–65)
TEMRA TCR $\gamma\delta$ /TCR $\gamma\delta$	46 (29–61)	49 (35–65)	52 (40–69)	58 (38–72)	47 (25–63)
HLA-DR TCR $\gamma\delta$ /TCR $\gamma\delta$	44 (28–58)	40 (22–57)	45 (30–61)	47 (33–68)	36 (25–53)
TCR $\gamma\delta$ /CD3 T	4.6 (2.6–11)	4.5 (2.3–9.8)	4.1 (2.0–10)	3.7 (2.4–7.4)	4.0 (2.1–7.6)
V δ 1/TCR $\gamma\delta$	10 (3.3–24)	11 (3.7–22)	13 (3.8–30)	28 (8.5–48)	35 (10–56)
V δ 2/TCR $\gamma\delta$	88 (70–95)	86 (68–95)	82 (60–95)	67 (38–90)	61 (31–86)
NonV δ 1-nonV δ 2/TCR $\gamma\delta$	2 (0.8–5.8)	2.1 (0.6–5.2)	3.0 (0.9–7.8)	3.9 (0.9–9.6)	4.0 (1.1–12)
CD16bright/NK	11 (6.2–24)	14 (7.7–21)	15 (8–22)	12 (7.8–21)	13 (7.3–19)
CD16CD56/NK	59 (47–70)	68 (54–77)	68 (55–81)	76 (65–83)	80 (71–86)
CD56bright/NK	23 (16–33)	15 (10–24)	13 (8.3–22)	8.7 (5.1–14)	7.4 (3.7–13)

RESULTS

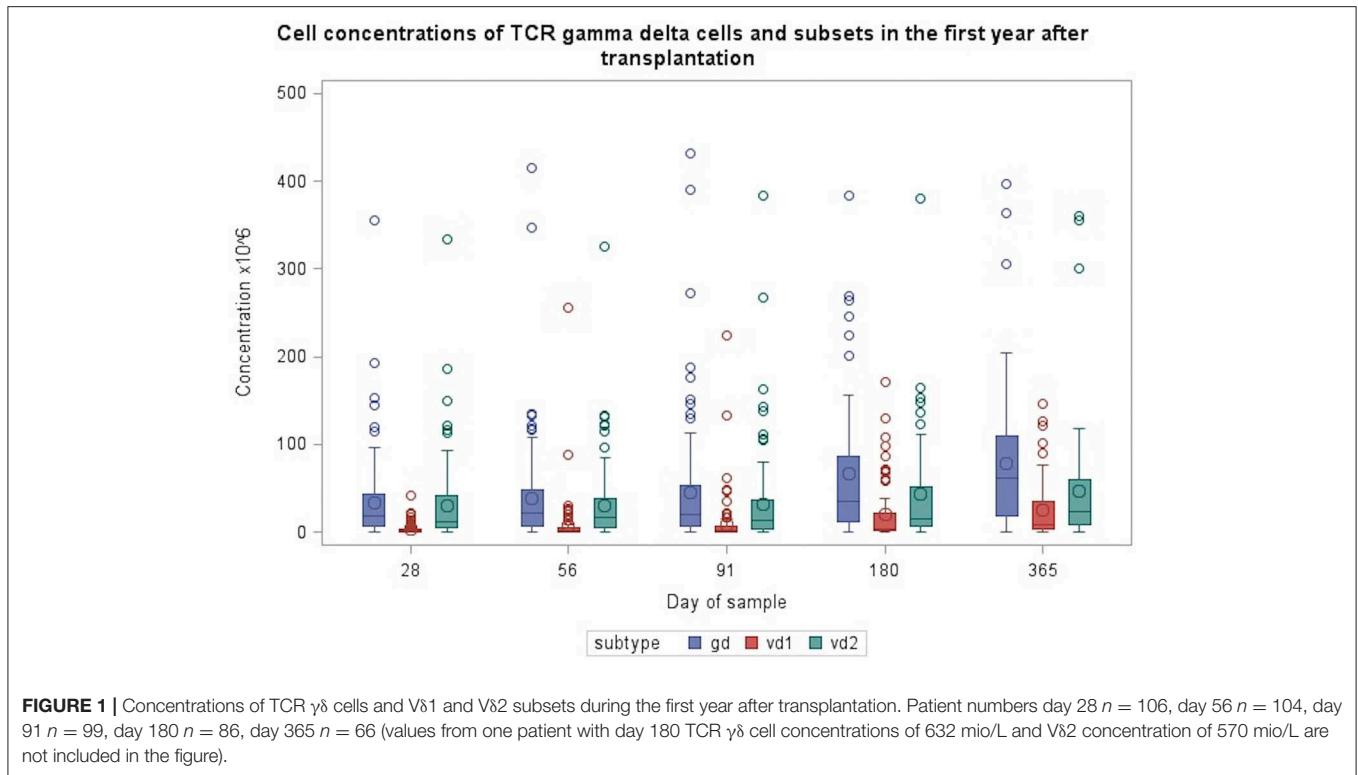
Patient Outcome

Patients were followed for a median of 673 (386–913) days after transplantation. At the end of follow-up 80 (74%) of the 108 patients were alive; 14 (13%) patients died from TRM and 14 (13%) died from relapse. Overall survival in all patients was 61% (95% CI 35–79%), RFS was 47% (95% CI 24–68%), and TRM was 23% (95% CI 4.7–50%). In the TRM group, the registered cause of death was aGVHD in four patients, cGVHD in one patient, infection in three patients, organ failure in three patients, toxicity in one patient, and lung embolism in one patient. One patient died from unknown causes other than relapse later than 2 years

post-transplant. Two and four patients died from TRM prior to their day 28/56 samples. A total of 24 (22%) experienced relapse during the follow-up time with a median time to relapse of 177 (56–778) days. Acute GVHD grade II-IV was diagnosed in 38 (35%) patients, and cGVHD in 54 (50%) patients. A total of 28 patients (26%) experienced CMV infection at a median of 42 (15–101 days) after transplantation.

Immune Reconstitution

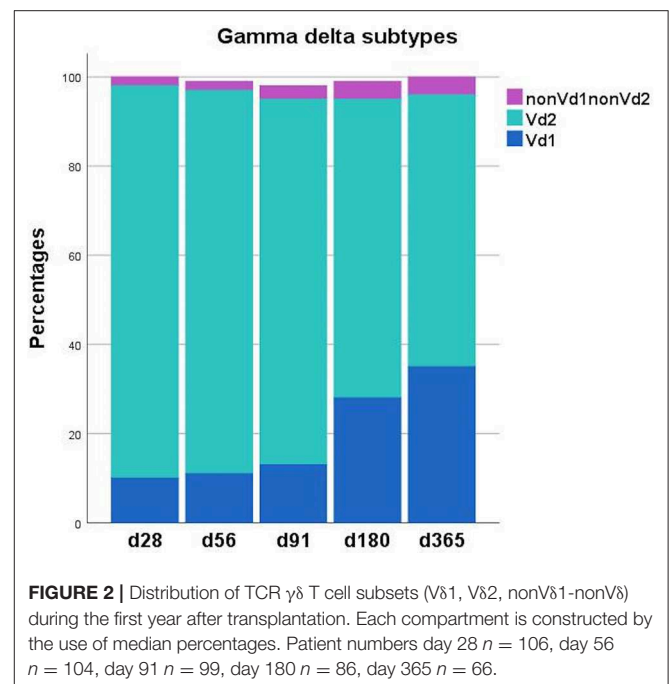
Table 2 shows concentrations and subset fractions of T cells and NK cells 28, 56, 91, 180, and 365 days after transplantation. Median concentrations of CD3 cells doubled after 6 months and



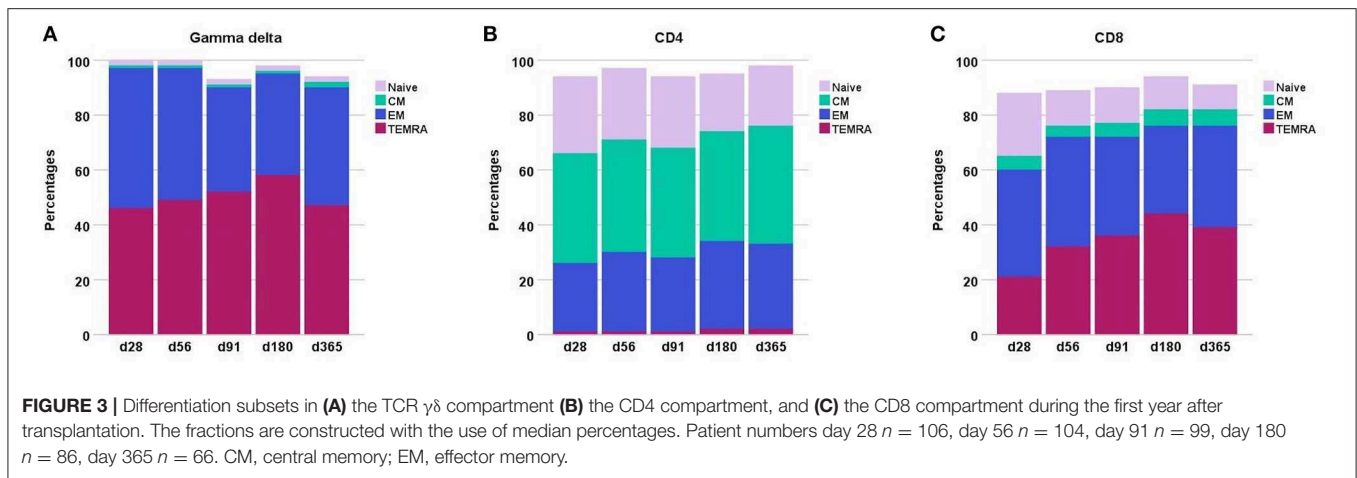
tripled after 1 year; the CD4/CD8 ratio shifted from 1.5 to 0.6 as the concentration of CD8 cells increased more than 4-fold during the first year post-transplant. The total NK cell concentration was highest early after transplantation (day 28) and decreased until 3 months after transplantation; hereafter it increased by 6 and 12 months post-transplant. The CD56bright NK subset peaked early after transplantation and declined steadily during the first year.

Table 2, Figures 1, 2 shows immune reconstitution of TCR $\gamma\delta$ cell concentrations and subset fractions during the first year after transplantation; median TCR $\gamma\delta$ cell concentrations remained relatively constant within the first 3 months, doubled after 6 months and tripled after 12 months. The fraction of TCR $\gamma\delta$ cells of the total CD3 T cell concentration remained stable below 5%, while the TCR V δ 1 fraction of the total TCR $\gamma\delta$ cell concentration increased, and the TCR V δ 2 fraction decreased during the first 12 months after transplantation, **Figure 2**.

Throughout the first year after transplantation the total TCR $\gamma\delta$ cell compartment consists mainly of effector memory and TEMRA cells, **Figure 3**. In contrast, the CD8 T cell and especially the CD4 T cell compartment is constituted by much higher fractions of naïve cells. Moreover, the CD4 compartment contains a substantially higher percentage of central memory cells compared to both the CD8 and TCR $\gamma\delta$ cell compartments. The distribution of the subsets remained relatively constant through the first year after transplantation in the CD4 T cells compartment while the most striking change occurred in the decreasing percentages of naïve cells in the CD8 compartment. The percentages of TEMRA cells increased during the first 6 months in both the TCR $\gamma\delta$ cell and CD8 T cell compartment.



Median TCR $\gamma\delta$ cell concentration 56 days after transplantation was 21 (IQR 7.2–50) $\times 10^6/L$, **Table 2**. A higher proportion of patients with high TCR $\gamma\delta$ cell concentrations at day 56 had donor age <30 years (67 vs. 56%), 9–10/10 allele



HLA-match (96 vs. 83%), AML/MDS disease type (75 vs. 56%), and received PBSC as a graft source (94 vs. 77%), as shown in **Table 3**. Of note, the use of ATG in the conditioning regimen was not significantly different in patients with high TCR $\gamma\delta$ cell concentration compared to low. The TCR $\gamma\delta$ cell concentration day 56 was significantly correlated to both CD3, CD4, and CD8 T cells and NK cell concentrations, and these were also significantly correlated to each other ($p < 0.01$ for all comparisons, data not shown).

Immune Reconstitution and Clinical Outcomes

Table 4 shows the results of the univariate analysis investigating the association between pre-transplant factors and death. Donor age >30 years (HR 2.12, 95% CI 0.98–4.57) and non-myeloablative conditioning regimen (HR 2.23, 95% CI 0.98–5.10) were associated with an increased risk of death. The same factors were associated with an increased risk of death or relapse and thus were included in the multivariate models investigating the association with cell concentrations and OS/RFS.

Overall Survival and Relapse-Free Survival

Figure 4 shows OS and RFS for patients with high vs. low day 56 median concentrations of TCR $\gamma\delta$ cells. In the T cell compartment, patients with low (below the median) concentrations of TCR $\gamma\delta$ cells, TCR V δ 1 cells, TCR V δ 2 cells, and CD4 cells 56 days after transplantation had an increased risk of death compared to patients with high concentrations, **Table 5A**. Within the NK cell compartment, low median concentrations of the CD56bright subset was significantly associated with increased risk of death. These cell subtypes remained significantly associated with death after adjustment for pre-transplant factors (donor age and conditioning regimen). Patients with low TCR $\gamma\delta$ cells at day 56 had over a 5 times greater risk of death compared to those with TCR $\gamma\delta$ cells above the median (HR 5.16, 95% CI 1.94–13.7, $p = 0.001$). Similar associations were observed for relapse free survival where patients with low concentrations of TCR $\gamma\delta$ cells at day 56 had a 2.7 (95% CI 1.32–5.53, $p = 0.007$) times greater risk of death or

relapse compared to patients with high concentrations, this also remained significant after adjustment for pre-transplant factors, as indicated in **Table 5A**.

When cell subset concentrations were included as continuous variables, **Table 5B**, in the T cell compartment, increasing concentrations of TCR $\gamma\delta$ cells, TCR V δ 1, and TCR V δ 2 cells were significantly associated with decreased risk of death while increasing concentrations of TCR $\gamma\delta$ cells and TCR V δ 2 cells were significantly associated with decreased risk of death or relapse. In the NK cell compartment, increasing concentrations of the CD56b subset were significantly associated with both outcomes. All significant cell subsets remained significant in the multivariate analyses adjusted for pre-transplant factors as indicated in **Table 5B**.

After including a further adjustment for other significant cell subtypes in the multivariable analysis, only median day 56 concentrations of TCR $\gamma\delta$ cells remained significant associated with an increased risk of death (HR 4.20, 95% CI 1.55–11.4, $p = 0.005$) and death or relapse (HR 2.26, 95% CI 1.08–4.77, $p = 0.03$), **Table 6**.

Death From Relapse

Patients with low TCR $\gamma\delta$ cell concentrations 56 days after transplantation had a significantly higher cumulative incidence of death from relapse compared to patients with low concentrations, $p = 0.003$ (**Figure 5A**), and $p = 0.003$ for TCR V δ 2 concentrations separately. High fractions of TCR $\gamma\delta$ T cells of the total CD3 T cell concentration was also significantly associated to lower risk of death from relapse, $p = 0.02$, **Figure 5B**. No significant association was observed between death from relapse and day 56 concentrations of total CD3 cells, CD4, or CD8 cells ($p = 0.67, 0.38, \text{ and } 0.66$, respectively). The only other cell subset concentration significantly associated with lower risk of death from relapse was the CD56bright NK cell subset for high vs. low concentrations ($p = 0.03$). The total median NK cell concentration, the CD16bright subset and the CD16CD56 subset were not associated to death from relapse ($p = 0.98, 0.96 \text{ and } 0.49$, respectively).

TABLE 3 | Distribution of pre-transplant factors associated with TCR $\gamma\delta$ cell concentrations 56 days after transplantation (χ^2).

	Total	Day 56 TCR $\gamma\delta$ high ($>21 \times 10^6/L$)	Day 56 TCR $\gamma\delta$ low ($<21 \times 10^6/L$)	P-value
N	104	52	52	
Age				
≤ 45	23	13 (57%)	10 (43%)	0.48
> 45	81	39 (48%)	42 (52%)	
Disease type				
AML/MDS	68	39 (57%)	29 (43%)	0.04
Other	36	13 (36%)	23 (64%)	
Donor age				
< 30	59	35 (59%)	24 (41%)	0.03
> 30	45	17 (38)	28 (62%)	
Disease stage				
Early	59	28 (47%)	31 (53%)	0.55
Intermediate/Late	45	24 (53%)	21 (47%)	
Recipient CMV serological status				
Positive	71	35 (49%)	36 (51%)	0.83
Negative	33	17 (52%)	16 (48%)	
Donor CMV serological status				
Positive	46	18 (39%)	28 (61%)	0.08
Negative	58	34 (59%)	24 (41%)	
Donor-recipient sex				
Female-male	10	4 (40%)	6 (60%)	0.51
Other	94	48 (51%)	46 (49%)	
Donor type				
MRD	21	11 (52%)	10 (48%)	0.36
MUD	81	41 (51%)	40 (49%)	
Haplo-identical	2	0 (0%)	2	
HLA-match				
9/10 or 10/10 allele match	93	50 (54%)	43 (46%)	0.03
Other (antigen MM+haplo-identical)	11	2 (18%)	9 (82%)	
Stem cell source				
BM	15	3 (20%)	12 (80%)	0.01
PBSC	89	49 (55%)	40 (45%)	
Conditioning regimen				
Myeloablative	48	26 (54%)	22 (46%)	0.43
Non-myeloablative	56	26 (46%)	30 (54%)	
ATG				
Yes	11	6 (55%)	5 (45%)	0.75
No	93	46 (49%)	47 (51%)	

MDR, matched related donor; MUD, matched unrelated donor; MM, mismatch; BM, bone marrow; PBSC, peripheral blood stem cells; ATG, anti-thymocyte-globulin. The italic values represent *p*-values equal to or below 0.05.

Sensitivity Analysis

In addition to analyses of the cell subsets concentrations at day 56 post-transplant, cells subsets were analyzed as continuous, time-updated variables. Results from these sensitivity analyses are presented in **Table 7** for the 106 patients with a day 28 sample. As shown, increasing concentrations of all cell subsets apart from the CD16b and the CD56b NK cells were significantly associated with a decreased risk of death in both univariate and multivariate analyses. The findings were consistent when relapse-free survival was the considered. Similarly, in univariate analyses of death from relapse, with death from TRM as competing risk, increasing concentrations of all T cell subsets and the NK CD16/56 subset were significantly associated with increased risk of death from relapse (data not shown).

Acute GVHD

Patients with high TCR $\gamma\delta$ cell concentrations 28 days after transplantation had significantly lower risk of aGVHD with death as competing event compared to patients with low concentrations, $p = 0.02$ for total TCR $\gamma\delta$ cells (**Figure 6A**) and $p = 0.02$ for the TCR V δ 2 subset separately. Association to the TCR V δ 1 subset showed the same trend ($p = 0.08$). Analyses of differentiation subsets of the total TCR $\gamma\delta$ population showed that it was specifically the concentration of effector memory TCR $\gamma\delta$ cells that was associated with aGVHD, as patients with values above the median of $8.4 \times 10^6/L$ had significantly lower risk of developing aGVHD compared to patients with values below this concentration ($p = 0.001$). The strongest association was, however, with high vs. low fractions of TCR $\gamma\delta$ cells of the total CD3 T cell concentration, $p < 0.001$, **Figure 6B**. There were no association between day 28 median concentrations of total CD3 cells ($p = 0.65$), CD4 cells ($p = 0.71$), or CD8 cells ($p = 0.84$) and aGVHD. High concentrations of the CD56bright NK cell subset and the CD16bright NK cell subset 28 days after transplantation were also significantly associated with lower risk of aGVHD, $p = 0.02$ and $p = 0.01$, respectively, while there was a non-significant association to the total NK cell concentration ($p = 0.06$) The CD16/56 subset was not associated with aGVHD ($p = 0.41$).

Chronic GVHD

TCR $\gamma\delta$ cell concentrations on day 28 and 56 were not associated to the risk of cGVHD with death as competing event, $p = 0.98$ and $p = 0.79$, respectively. High CD4 T cell concentrations on both day 28 and 56 were significantly associated with increased risk of cGVHD, $p = 0.006$ and $p = 0.04$, respectively; separate analyses of differentiation subsets of CD4 T cells showed that it was specifically the concentration of naïve CD4 T cells that was associated with cGVHD; patients with values above the median of $58 \times 10^6/L$ had a trend toward increased risk of developing cGVHD compared to patients with values below this concentration by day 28 ($p = 0.09$) and this association became significant by day 56 ($p = 0.03$) when median concentration of naïve CD4 T cell was $53 \times 10^6/L$. None of the other T cell or NK cell subsets showed any significant associations to cGVHD 28 or 56 days after transplantation.

TABLE 4 | Univariate analyses of pre-transplant factors and the impact on overall survival and relapse-free survival from day 28, $n = 106$.

Variable	N	Overall survival		Relapse-free survival	
		HR (95% CI)	P-value	HR (95%CI)	P-value
Recipient age					
Per 10 years older	–	1.15 (0.84–1.59)	0.39	1.04 (0.80–1.36)	0.76
Disease type					
AML/MDS	70	1.00	0.38	1.00	0.36
Other	36	1.42 (0.66–3.06)		1.37 (0.70–2.68)	
Donor age					
<30 years	60	1.00		1.00	
>30 years	46	2.12 (0.98–4.57)	0.06	2.58 (1.32–5.04)	0.01
Donor type					
Matched related donor	23	1.00		1.00	
Matched unrelated donor	83	1.50 (0.52–4.36)	0.45	0.83 (0.38–1.84)	0.65
Graft source					
PBSC	91	1.00		1.00	
BM	15	0.99 (0.34–2.87)	0.99	0.88 (0.34–2.26)	0.79
Conditioning regimen					
Myeloablative	49	1.00		1.00	
Non-myeloablative	57	2.23 (0.98–5.10)	0.06	1.80 (0.92–3.55)	0.09
ATG					
No	94	1.00		1.00	
Yes	12	0.69 (0.16–2.93)	0.62	1.51	0.40
CMV donor					
Negative	58	1.00		1.00	
Positive	48	1.60 (0.75–3.43)	0.22	1.43 (0.75–2.74)	0.28
CMV recipient					
Negative	33	1.00		1.00	
Positive	73	0.88 (0.40–1.97)	0.76	0.88 (0.44–1.76)	0.72
Disease risk index*					
Low	10			1.00	
Intermediate	85			5.05 (0.69–36.98)	
High	11	–		4.11 (0.46–36.92)	0.27

Hazard ratios are of death in overall survival and of death or relapse in relapse-free survival. AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; CMV, cytomegalovirus; DRI, Disease Risk Index. *Disease Risk Index, as there were no deaths in the low risk group during the observation time, no estimate of this variable is included for OS.

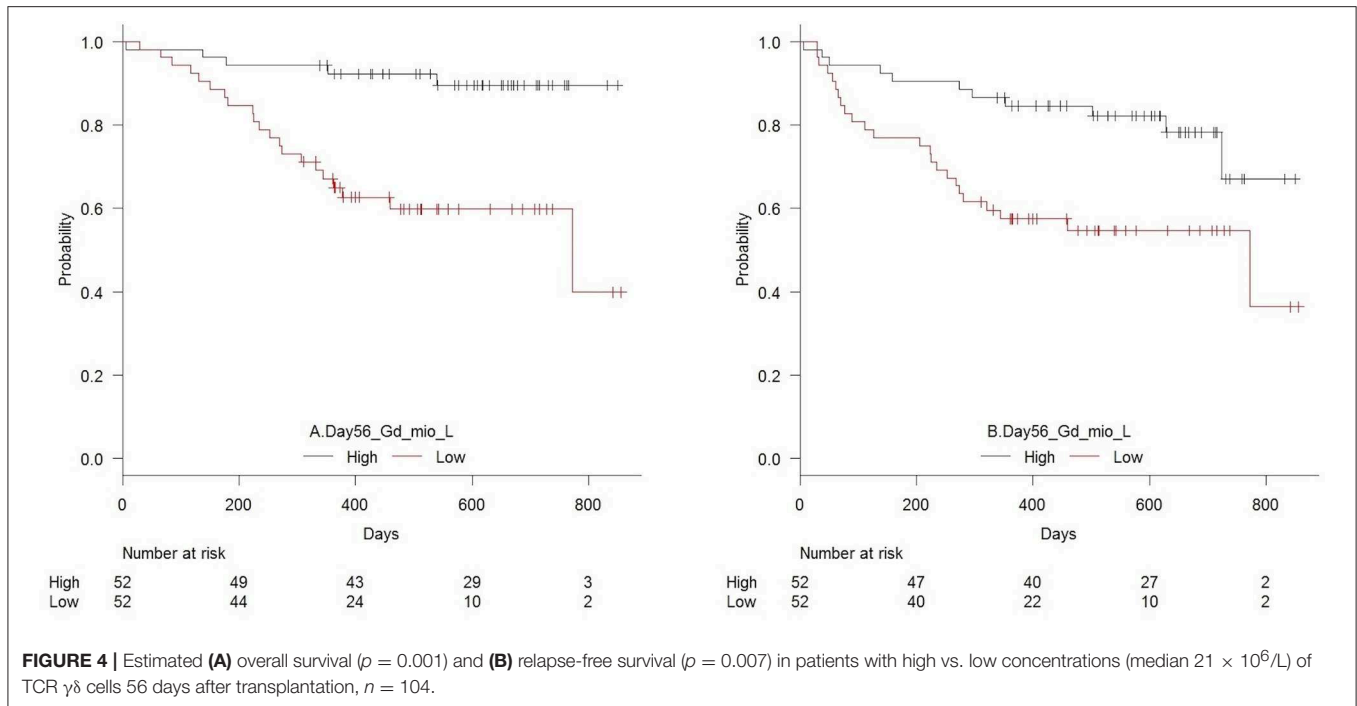
Differentiation and Activation Markers of TCR $\gamma\delta$ Cells Day 56

Since the day 56 TCR $\gamma\delta$ cell immune reconstitution was significantly associated with relapse and RFS, we further investigated the possible effect of differentiation and activation of the TCR $\gamma\delta$ cells at this time point. The TCR $\gamma\delta$ cell compartment 56 days after transplantation in all patients consisted of a median of 1.6% naive cells, 0.9% central memory cells, 48% effector memory cells, and 49% TEMRA cells, **Table 2, Figure 3**. A median of 40 (IQR 22–57) % of all TCR $\gamma\delta$ cells were HLA-DR positive, and >99% of both total TCR $\gamma\delta$ cells, V δ 1, and TCR V δ 2 cells were positive for the activating receptor NKG2D. We found no differences in concentrations or fractions of TCR $\gamma\delta$ naive, central memory, effector memory or TEMRA subsets in patients who relapsed compared to patients without relapse. The number of HLA-DR positive TCR $\gamma\delta$ cells and the median MFI values of NKG2D expression on total TCR $\gamma\delta$ cells, V δ 1, and V δ 2 cells were

also not statistically different in patients who relapsed compared to patients without relapse (data not shown). Since >99 percent of the total TCR $\gamma\delta$ cells, V δ 1, and V δ 2 cells were positive for the expression of NKG2D, we made no comparison of the numbers of percent positives cells of this value between the groups.

CMV and Association to TCR $\gamma\delta$ Cell Immune Reconstitution

We tested the association between post-transplant CMV infection and immune reconstitution of the TCR $\gamma\delta$ cell compartment for day 56, 91, 180, and 365 after transplantation. We saw no significant differences in immune reconstitution on day 56 or 91 after transplantation and previous CMV infection. Day 180 and 365 analyses (**Figure 7A**) however, showed that patients with CMV infection had significantly higher absolute concentrations of V δ 1 cells, significantly higher percentages of V δ d1/TCR $\gamma\delta$ cells and significantly lower percentages of



V δ 2/TCR $\gamma\delta$ cells compared to patients who had not had CMV infection. The percentages of nonV δ 1-nonV δ 2/TCR $\gamma\delta$ cells were also significantly higher in patients with CMV infection at 365 days after transplantation (data not shown). The concentration of total TCR $\gamma\delta$ cells was not associated with CMV infection at any time point after transplantation. Regarding differentiation subsets, patients diagnosed with CMV had significantly lower concentrations and percentages of central effector TCR $\gamma\delta$ of total TCR $\gamma\delta$ cells together with a non-significant trend toward higher concentrations and percentages of TEMRA TCR $\gamma\delta$ cells compared to patients without CMV at 180 and 365 days after transplantation (data not shown). One year after transplantation, patients with previous CMV had significantly lower concentrations of naive TCR $\gamma\delta$ cells and a trend toward higher concentrations and fractions of TEMRA TCR $\gamma\delta$ cells compared to patients without CMV. Subsequently, we tested if CMV infection was an independent factor associated with relapse incidence and RFS at a landmark analyses 100 days after transplantation (9 patients had events before day 100, one patient diagnosed with CMV day 28 died before day 100). Previously diagnosed CMV infection was not an independent factor associated with relapse incidence ($p = 0.49$ cumulative incidence,) or RFS ($p = 0.43$, KM, log rank) in all patients ($n = 99$). A subgroup of patients with MDS or AML ($n = 66$) was tested separately, also with no association to relapse incidence ($p = 0.28$) or RFS ($p = 0.51$), data not shown.

The serological status of the donor was also tested for associations to immune reconstitution of TCR $\gamma\delta$ cells the first year after transplantation. Recipients of CMV positive donors had significantly higher percentages of V δ 1/TCR $\gamma\delta$ cells and significantly lower percentages of V δ 2/TCR $\gamma\delta$ compared to recipients of CMV negative donors the first 3 months after

transplantation (time points day 28, 56 and 91), **Figure 7B**. The absolute concentrations of V δ 1 and V δ 2 cells were similarly significantly different in these patients 28 days after transplantation, but not at later time points. The percentages of nonV δ 1-nonV δ 2/TCR $\gamma\delta$ cells were significantly higher in recipients of CMV positive donors compared to recipients of CMV negative donors 28 and 56 days after transplantation (data not shown). By day 180 and day 365 after transplantation there was no longer associations to the serological status of the donor. The concentration of total TCR $\gamma\delta$ cells was not significantly associated with the serological status of the donor at any time point.

DISCUSSION

Following earlier, small-numbered studies of the clinical impact of the immune reconstitution of TCR $\gamma\delta$ cells with contrasting results (19–21), a 5-year follow-up study in 2007 confirmed improved disease-free survival and overall survival in patients with high concentrations of circulating TCR $\gamma\delta$ cells after HLA-mismatched, TCR $\alpha\beta$ cell depleted HSCT (12). Recently, a pediatric study demonstrated lower probability of infections and increased event-free survival in patients with high concentrations of TCR $\gamma\delta$ cells in a diverse HSCT-setting including both haplo-identical donors and the use of umbilical cord blood as graft source (13). In 2017, a pediatric study demonstrated how infusion of zoledronic acid in recipients of $\alpha\beta$ T- and B-cell-depleted HLA-haploidentical HSCT stimulates $\gamma\delta$ T-cell differentiation and cytotoxicity and may increase patient survival (22). Adult HLA-matched transplants, however, are by far the most common type of transplant (23), and we here report data from a prospective, clinical study in this setting.

TABLE 5 | Univariate analyses of the impact of day 56 cell subsets on overall survival and relapse-free survival in **(A)** cell subsets included with median cut-off values and **(B)** cell subsets included as continuous (\log_2 -transformed) values, $n = 104$.

(A)				
Cell subset	Overall survival		Relapse-free survival	
	HR (95% CI)	<i>P</i> = value	HR (95% CI)	<i>P</i> = value
CD3				
High	1.00		1.00	
Low	2.18 (0.97–4.90)	0.06	1.15 (0.77–2.96)	0.23
CD4				
High	1.00		1.00	
Low	2.73 (1.15–6.48)	0.02*	1.99 (0.99–4.00)	0.05*
CD8				
High	1.00		1.00	
Low	2.12 (0.94–4.76)	0.07	1.45 (0.74–2.84)	0.28
TCR $\gamma\delta$				
High	1.00		1.00	
Low	5.16 (1.94–13.7)	0.001**	2.70 (1.32–5.53)	0.007*
V δ 1				
High	1.00		1.00	
Low	2.26 (1.00–5.01)	0.05	2.20 (1.09–4.45)	0.03*
V δ 2				
High	1.00		1.00	
Low	5.02 (1.89–13.4)	0.001**	2.67 (1.30–5.50)	0.007*
NK				
High	1.00		1.00	
Low	1.55 (0.7–3.43)	0.28	1.98 (0.98–3.98)	0.06
CD16b				
High	1.00		1.00	
Low	1.96 (0.87–4.42)	0.11	1.35 (0.69–2.66)	0.38
CD16/56				
High	1.00		1.00	
Low	2.01 (0.89–4.54)	0.09	2.14 (1.06–4.32)	0.03*
CD56b				
High	1.00		1.00	
Low	2.31 (1.00–5.34)	0.05*	1.81 (0.91–3.61)	0.09
(B)				
Cell subset, per doubling value	Overall survival		Relapse-free survival	
	HR (95% CI)	<i>P</i> = value	HR (95% CI)	<i>P</i> = value
CD3	0.85 (0.64–1.42)	0.29	0.88 (0.67–1.14)	0.33
CD4	0.86 (0.64–1.16)	0.33	0.86 (0.67–1.12)	0.27
CD8	0.79 (0.59–1.05)	0.10	0.83 (0.65–1.07)	0.16
TCR $\gamma\delta$	0.63 (0.49–0.80)	<0.001**	0.76 (0.61–0.93)	0.008*
V δ 1	0.65 (0.44–0.96)	0.03*	0.81 (0.60–1.09)	0.16
V δ 2	0.65 (0.51–0.81)	<0.001**	0.75 (0.62–0.91)	0.003*
NK	0.75 (0.52–1.07)	0.11	0.81 (0.60–1.10)	0.18
CD16b	0.83 (0.63–1.09)	0.18	0.95 (0.75–1.19)	0.65
CD16/56	0.85 (0.60–1.18)	0.33	0.86 (0.6–1.13)	0.28
CD56b	0.61 (0.45–0.84)	0.002*	0.74 (0.56–0.97)	0.03*

Hazard ratios are of death in overall survival and of death or relapse in relapse-free survival. Cell subsets significant in multivariate analyses including adjustments for pre-transplant factors (conditioning therapy and donor age) are indicated by *, * $p \leq 0.05$, ** $p \leq 0.01$. The italic values represent p -values equal to or below 0.05.

We found a significantly increased OS and RFS with lower risk of relapse and aGVHD in patients with high TCR $\gamma\delta$ cell concentrations 56 days after HLA-matched, T cell replete transplantation. The studies by Godder et al. (12) and Perko et al. (13) used the cut-off limit of 150/175 cells/ μ L ($150/175 \times 10^6/L$) at two consecutive time points during the first year of transplantation, which categorized only about 10% of included patients as having high TCR $\gamma\delta$ cell reconstitution but in a wide time frame. In our study, clinical impact was shown by day 56 by a dichotomization of the median concentration values of $21 \times 10^6/L$ preceding relapse in all patients and suggesting a protective effect of early robust TCR $\gamma\delta$ cell immune reconstitution. TCR $\gamma\delta$ cells were the only T cell subsets significantly associated with OS and RFS in after adjustment for transplant related factors and other cell subsets, 56 days after transplantation. The overall day 56 CD3 cell concentration was not significantly associated with clinical outcomes, and thus the association of increasing concentrations of TCR $\gamma\delta$ T cells suggest a “dose-response” relationship to the absolute concentration of this subset particularly. Day 56 TCR $\gamma\delta$ cell concentrations were furthermore the only T cell subsets significantly associated with death from relapse. However, in sensitivity analysis, including cell subsets as time-updated variables, all T cell subsets together with the overall CD3 concentrations were significantly associated with both OS and RFS. This reflects how a more long-term robust T cell concentration in general is important for outcomes after transplantation. The specific effect of TCR $\gamma\delta$ cells early after transplantation may reflect the fact that, in opposition to CD4 and CD8 cells, this innate-like T cell type reconstitutes early and efficiently post-transplant (24, 25) and therefore might be protective of early relapse. Further studies are necessary to support this hypothesis.

Statistical associations for the TCR V δ 2 subtype closely followed those of the overall TCR $\gamma\delta$ cell concentrations. This was not surprising as TCR V δ 2 constitutes the majority of TCR $\gamma\delta$ cells in peripheral blood and is logically an important study subject for anti-tumor immunotherapy (26). Although low in fractions and concentrations, the TCR V δ 1 subset which also has reported graft-vs. leukemia effects (27, 28), was also significantly associated with RFS and OS by dichotomization of the day 56 median value in our study.

Several possible mechanisms may be involved in tumor recognition and elimination by TCR $\gamma\delta$ cells. Through their HLA-independent TCR $\gamma\delta$ receptor, TCR $\gamma\delta$ cells recognize small, non-peptide, phosphorylated antigens, “phospho-antigens” (pAgs), that are metabolites of metabolic pathways of practically all living organisms including humans (5, 29). Hypothetically, pAg-recognition allows discrimination between resting and malignant transformed cells facilitating TCR $\gamma\delta$ cell tumor recognition (30). Other possible mechanisms for tumor recognition are through receptors from the natural cytotoxicity (NCR) family like Nkp30 (31), the DNAM1/DNAM-1 receptor-ligand recognition (32, 33), and receptors from the NK receptor family like the mentioned NKG2D-receptor that recognized “stressed” or malignant transformed cells by activation through MHC-class I-related molecules MIC-A and MIC-B (6, 34) and UL16-binding proteins (ULBPs) (35, 36). As expected,

TABLE 6 | Multivariate analyses of the impact of day 56 TCR $\gamma\delta$ cell concentration (median cut-off values) including other significant cell subtypes, $n = 104$.

Cell subset	Overall survival		Relapse-free survival	
	HR (95% CI)	<i>P</i> -value	HR (95% CI)	<i>P</i> -value
TCR $\gamma\delta$ cell concentration				
High	1.00		1.00	
Low	4.20 (1.55–11.4)	0.005	2.26 (1.08–4.77)	0.03
CD4 T cell concentration				
High	1.00		1.00	
Low	1.82 (0.74–4.44)	0.19	1.36 (0.63–2.92)	0.42
CD16CD56 cell concentration				
High			1.00	
Low	–		1.64 (0.77–3.49)	0.20
CD56b cell concentration				
High	1.00		–	
Low	1.68 (0.72–3.95)	0.23		

The italic values represent p-values equal to or below 0.05.

the vast majority of our patient population (>99%) had TCR $\gamma\delta$ cells expressing NKG2D. Effector functions of TCR $\gamma\delta$ cell include direct cytotoxicity through perforin/granzyme secretion, death receptor ligands (Fas-L, tumor necrosis factor-related apoptosis-inducing ligand, TRAIL) and cytokine and interleukin production (4). Indirect effector mechanism involve antigen presentation (37, 38), regulation of the tumor microenvironment (39) and interactions with dendritic and stromal cells (4, 5). **Figure 8** shows possible mechanisms for tumor recognition and effector mechanisms mediated by TCR $\gamma\delta$ cells.

We observed a significantly lower risk of aGVHD in patients with high concentrations of TCR $\gamma\delta$ cells 28 days after transplantation. Other studies have investigated potential effects of TCR $\gamma\delta$ cell immune reconstitution on GVHD (12, 13, 19). Our study is, to our knowledge, the first to demonstrate clinical results suggesting a protective effect of early high TCR $\gamma\delta$ cell concentrations, especially by the effector memory subset. Possible immunoregulatory properties of TCR $\gamma\delta$ cells, together with the observation that high fractions of the TCR $\gamma\delta$ cells of the total CD3 T cell compartment was associated with a lower less risk of aGVHD, suggest a protective effect of a high ratio of “innate/alloreactive” T cells. Further studies are needed to uncover the possible cellular mechanisms involved and potentially tolerogenic properties of TCR $\gamma\delta$ cells.

While the immune reconstitution of TCR $\gamma\delta$ cells did not impact the incidence of cGVHD, we found a significantly association with high CD4 T cell concentrations at day 56 and cGVHD. The analyses of differentiation subsets showed, that is was high concentration of naïve CD4 T cells, which correlated

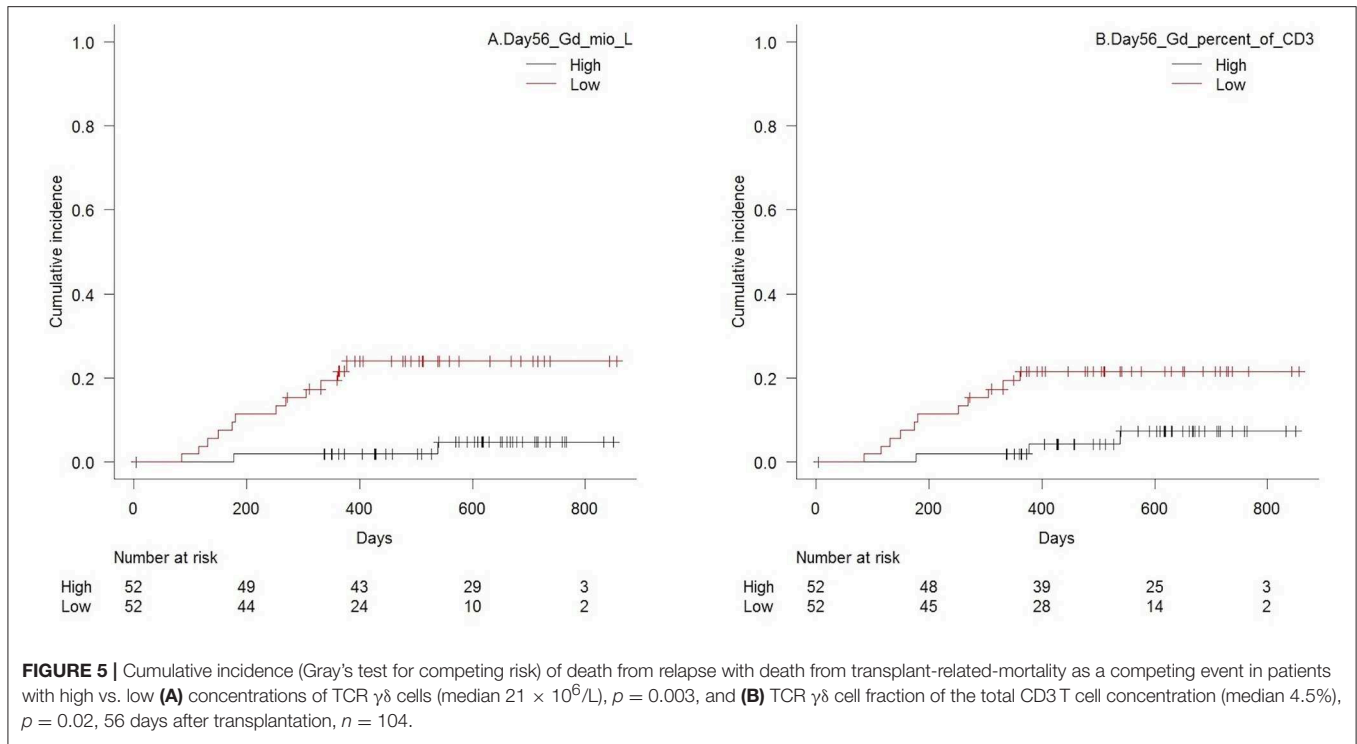


TABLE 7 | Univariate analyses of the impact of time-updated cell subset concentrations (\log_2 transformed continuous variables) on overall survival and relapse-free survival, $n = 106$.

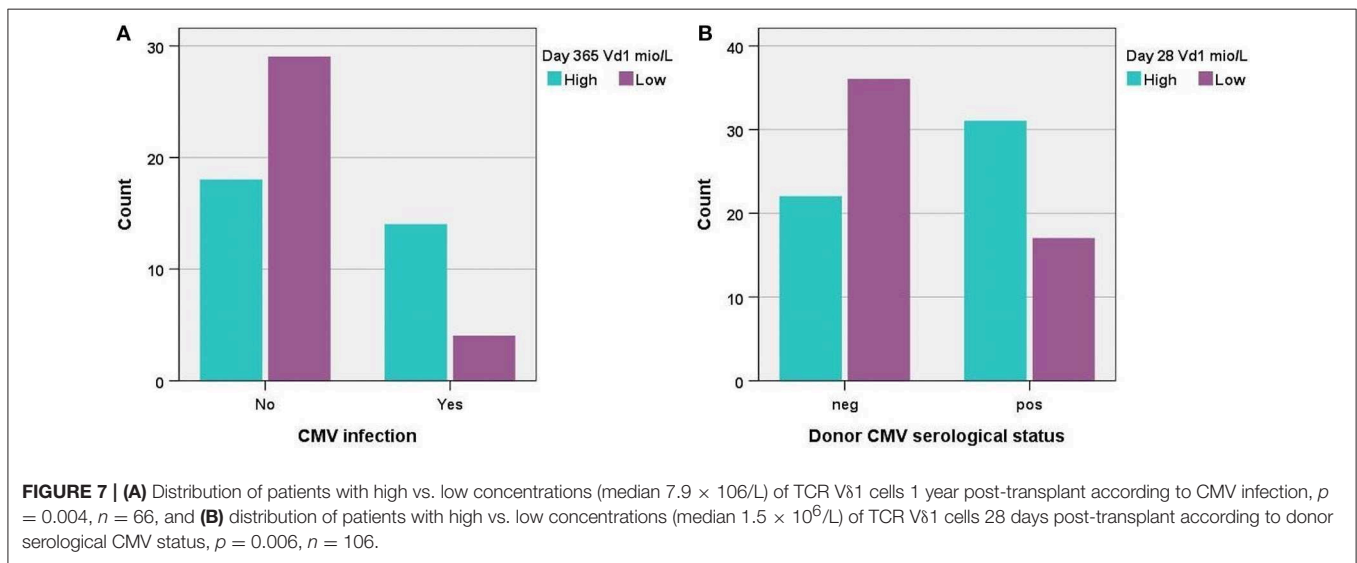
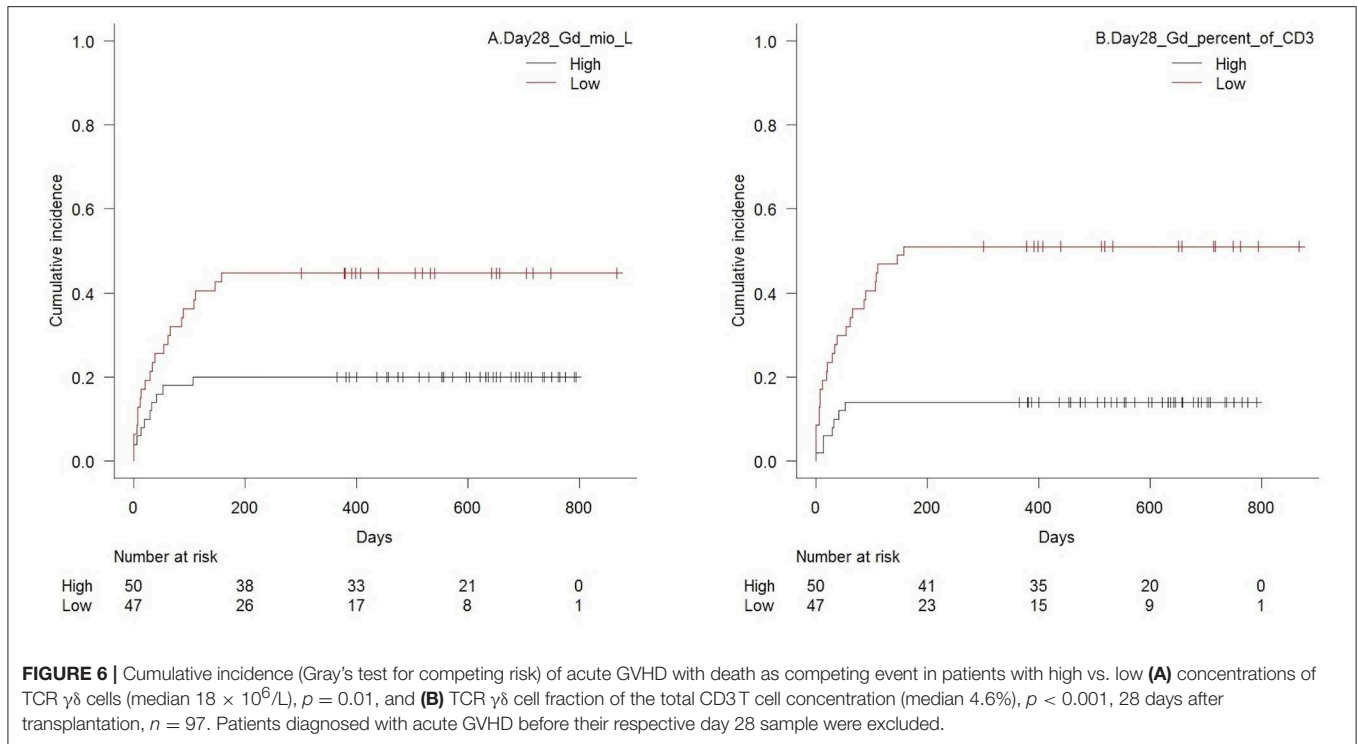
Cell subset	Overall survival		Relapse-free survival	
	HR (95% CI)	p-value	HR (95% CI)	p-value
CD3	0.56 (0.43–0.74)	<0.0001***	0.58 (0.45–0.74)	<0.0001***
CD4	0.50 (0.37–0.66)	<0.0001***	0.52 (0.40–0.68)	<0.0001***
CD8	0.64 (0.50–0.81)	0.0002***	0.64 (0.52–0.79)	<0.0001***
TCR $\gamma\delta$	0.66 (0.53–0.83)	0.0003***	0.72 (0.59–0.88)	0.001**
V δ 1	0.74 (0.59–0.93)	0.01*	0.83 (0.69–1.00)	0.04
V δ 2	0.68 (0.54–0.85)	0.0003**	0.74 (0.61–0.90)	0.002**
NK	0.61 (0.43–0.87)	0.007**	0.63 (0.46–0.87)	0.005**
CD16b	0.77 (0.58–1.04)	0.09	0.90 (0.70–1.16)	0.41
CD16/56	0.59 (0.42–0.84)	<0.0001***	0.59 (0.44–0.81)	0.0008***
CD56b	0.78 (0.55–1.10)	0.16	0.94 (0.69–1.29)	0.71

Cell subsets significant in multivariate analyses including adjustments for conditioning therapy and donor age are indicated by *, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. The italic values represent p-values equal to or below 0.05.

to increased cGVHD. We found no association to CD8 T cells, including the naïve subset; naïve T cells have, however, earlier been associated with increased risk of chronic GVHD (40).

A functional characterization of subsets and differentiation of TCR $\gamma\delta$ cells was provided in this study. Analysis of differentiation subsets during immune reconstitution showed how the TCR $\gamma\delta$ cell compartment consisted primarily of effector memory and TEMRA cells throughout the first year after transplantation. In this context, the TCR $\gamma\delta$ cell population resembled the CD8 T cell population, the latter however, having substantially higher percentages of naïve cells especially early after transplantation. The CD4 compartment exhibited a

relatively stable and high percentage of central memory cells which were not found in the CD8 or TCR $\gamma\delta$ cell compartment. It was previously described, how peripheral expansion of effector memory cells in the CD8 compartment exceeds that of the CD4 compartment, presumably due to the CD8 reaction to herpes and other viruses which is most likely also the reason for the inversion of the CD4/CD8 ratio during the first year post-transplant (41). In the TCR $\gamma\delta$ cell compartment, TEMRA cells expanded up to 6 months after transplantation, at which point this subset actually comprised more than half of the total TCR $\gamma\delta$ cell compartment. One year after transplantation the TEMRA and effector memory subsets were again equally large as early after HSCT. We found



CMV infection after transplantation to be associated with a trend toward higher percentages of TEMRA cells 6 months after transplantation which could be part of the reason for the expanding TEMRA subset. The expansion of the TEMRA subset was primarily at the expense of significantly lower percentages of the central memory subset, however this constitutes a minority of the total population in all patients (<5%) at all analyzed time points.

CMV infection was significantly associated with both higher absolute concentration and percentages of the $V\delta 1$ subset 6

and 12 months after transplantation; the fraction of non $V\delta 1$ -non $V\delta 2$ subset/TCR $\gamma\delta$ cells was also significantly higher in CMV-infected recipients 12 months after transplantation. This supports the conception of a role of non $V\delta 2$ cells in CMV infections as earlier described (25, 42). In 2011 the somewhat paradoxical association between CMV replication after transplantation and decreased relapse risk in 266 AML patients was demonstrated (43). Two years later, the study by Scheper et al. (44) demonstrating how TCR $\gamma\delta$ cells are capable of cross-recognizing CMV and leukemia cells lead to the

in GVHD (57), but our results support the view of a regulatory role, as high CD56bright NK cells were significantly associated with less risk of aGVHD in our patient population. In the analyses of cell subsets included as time-updated variables, the overall NK cell concentration and the CD16/56 subsets were significantly associated with outcomes, perhaps in accordance with a gradual development and maturation of this dominating subtype after transplantation.

The strength of this study was the prospective design, standardized treatment protocols of a single-center institution, fixed sample time points, and available clinical data on all patients at ended follow-up time. The limitations of our study are the relatively heterogenous patient population in terms of disease. Also, although we present a functional characterization of TCR $\gamma\delta$ cells, functional analyses of isolated TCR $\gamma\delta$ cells demonstrating a specific graft-vs.-tumor effect on a cellular level have not been performed in this study. Clearly, this would support the proposed possibility of a causal relationship between TCR $\gamma\delta$ cells immune reconstitution and relapse.

In conclusion, we report improved OS and RFS with less risk of relapse and aGVHD in patients with high concentrations of TCR $\gamma\delta$ cells 2 months after transplantation, suggesting an active role of TCR $\gamma\delta$ cells in the graft-vs.-tumor response after transplantation. Our clinical findings support further research of methods of improving the efficiency of post-transplant TCR $\gamma\delta$ cell adoptive immune therapy in order to prevent relapse and improve survival after HSCT.

ETHICS STATEMENT

The study was approved and carried out in accordance with the recommendation by the Danish National Committee on Health Research Ethics, and all participants gave written

informed consent prior to transplantation in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

LM performed the research, analyzed the data, and wrote the paper. HM and LR contributed with new reagent and analytic tools in the laboratory. NS, IS, LF, and EH performed the research. BK and SP performed the research, helped to write the paper, and analyzed the data. AF-N and HS designed the research. JR performed the statistical analyses and revised the manuscript as native-speaker. HS furthermore performed the research and analyzed the data.

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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.2019.01997/full#supplementary-material>

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