



# FasL and TRAIL signaling in the skin during cutaneous leishmaniasis – implications for tissue immunopathology and infectious control

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Cutaneous leishmaniasis (CL) is associated with chronic inflammation and ulceration of the skin. Tissue macrophages serve as host cells and immune activation is necessary for parasite clearance. The balance between immune-mediated tissue destruction and successful clearance of infection is delicate and ulceration has been proposed to be a result of infiltration of activated immune cells into the skin. FasL and TRAIL play a dual role in skin homeostasis through induction of apoptosis as well as proinflammatory signaling. During leishmaniasis, dysregulation of both FasL and TRAIL has been described by us and others but the resulting pathogenic effects in the skin during human leishmaniasis are not fully elucidated. Targeting disease specific immune deviations has proven to be a promising new approach for the therapy of autoimmune diseases. Potentially, targeting FasL or TRAIL in combination with microcidals could offer a future treatment strategy to reduce the disfiguring immunopathology associated with CL. In this mini review we will discuss how FasL and TRAIL-induced signaling may influence on the extent of tissue inflammation and the efficacy of parasite clearance in leishmaniasis.

**Keywords:** cutaneous leishmaniasis, FasL, TRAIL, ulceration, immunopathology, neutrophils, macrophages, keratinocytes

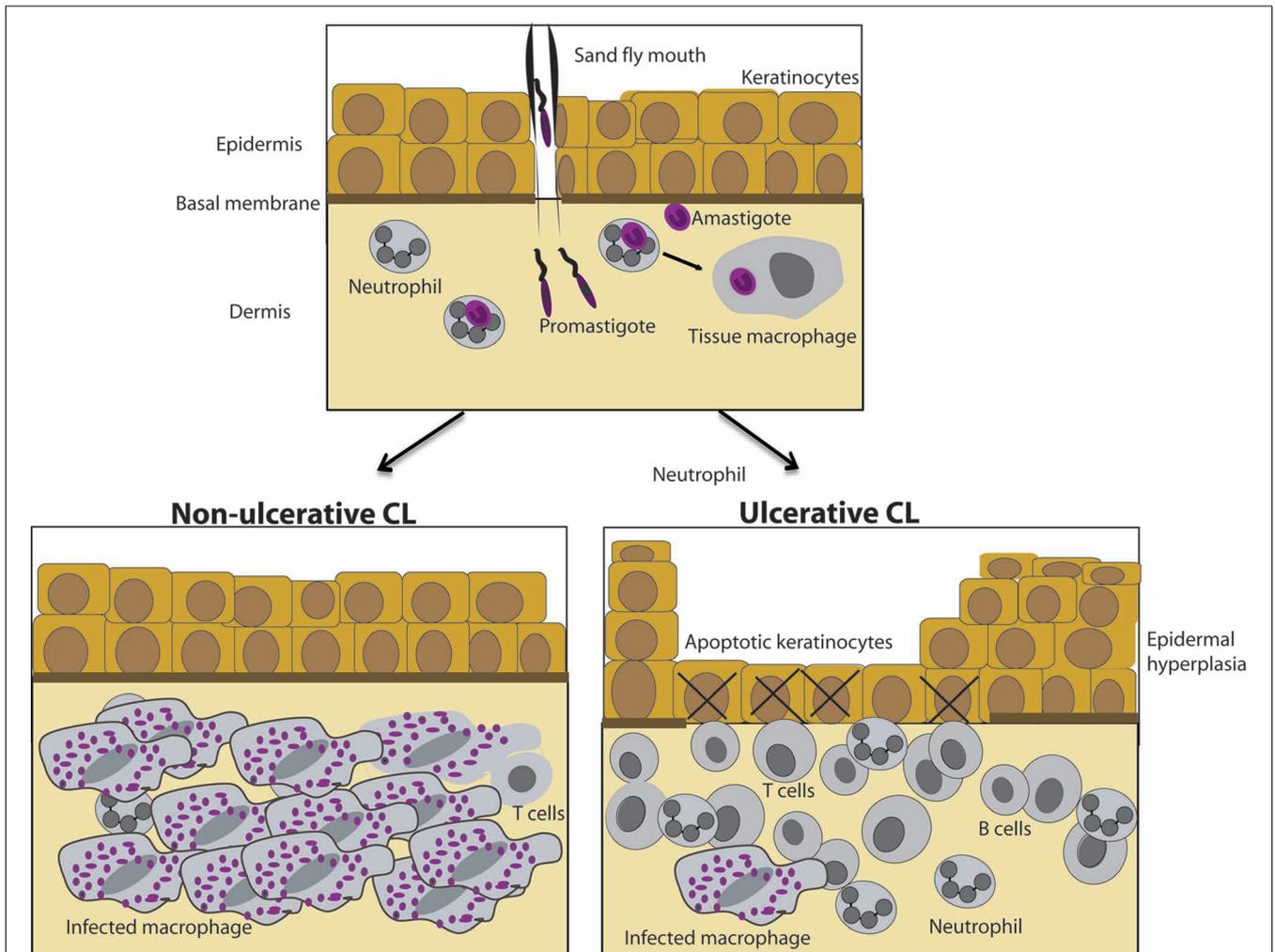
## LEISHMANIA INFECTION OF THE SKIN CAUSE CHRONIC TISSUE INFLAMMATION AND ULCERATION

Human leishmaniasis of epithelial surfaces presents with a plethora of clinical manifestations – from asymptomatic infection to chronic skin ulcers or devastating ulceration of mucous surfaces. Cutaneous leishmaniasis (CL) in the Old World is caused by *Leishmania major*, *L. tropica*, and *L. aethiopica* and careful histological studies have elucidated the patterns of inflammation in these lesions (Gaafar et al., 1995, 1999; Connor et al., 1997). Different subspecies of *Leishmania* are associated with heterogeneous cutaneous manifestations but large clinical variations are also noted in skin pathology caused by the same species of *Leishmania*. This is exemplified by *L. aethiopica* infection that induces localized CL (LCL) or diffuse CL (DCL; **Figure 1**). LCL is characterized by erosive ulcers and a strong T cell-mediated response (Akuffo et al., 1987) which typically results in spontaneous healing within a year, scar formation, and solid protection against re-infection (Lemma et al., 1969). In contrast, DCL is linked to non-ulcerative chronic nodular disease with abundant parasitic infiltration of the dermal compartment of the skin and antigen-specific T cell unresponsiveness (Nilsen and Mshana, 1987). In the Americas, *L. braziliensis* cause devastating mucocutaneous lesions as well as diffuse and ulcerating CL. *L. braziliensis* infection can also lead to disseminated CL with a high number of ulcerated lesions spread over the body at large devoid of detectable parasites (Costa et al., 1986; Carvalho et al., 1994; Turetz et al., 2002). Patients affected by disseminated cutaneous *L. braziliensis* infection show effective

antileishmanial T cell responses in circulating lymphocytes and histological studies proposed pronounced T cell infiltration into the affected skin (Leopoldo et al., 2006; Machado et al., 2011). Ulceration and tissue destruction in CL seems to be mainly a consequence of immune activation evoked by the infection rather than a direct effect of the infectious burden of parasites although definite proofs of this hypothesis has not been shown to date. Nevertheless, a low infectious burden in combination with an efficient antileishmanial T cell response is detected in disseminating or localized *L. braziliensis*-induced CL or *L. aethiopica*-induced LCL and is linked to ulcerative disease. On the other hand, *L. aethiopica*-induced DCL, with a high infectious burden and inefficient T cell responses, is linked to non-ulcerative disease. One obvious future treatment strategy would be to try to modulate the immune response to *Leishmania* so that the antileishmanial effects are maximized but tissue destruction minimized.

## FasL AND TRAIL SIGNALING LEAD TO APOPTOSIS OR PRODUCTION OF PROINFLAMMATORY MEDIATORS

FasL and TRAIL (tumor necrosis factor-related apoptosis inducing ligand) are death receptor ligands signaling through Fas, and TRAIL R1-4, respectively (**Figure 2A**). Fas, as well as TRAIL-R1 and TRAIL-R2 contain a functional intracellular death domain and may induce apoptosis but also alternative signals via NF- $\kappa$ B and MAPK activation (Falschlehner et al., 2007; Peter et al., 2007). TRAIL-R3 lack an intracellular cytoplasmic domain and TRAIL-R4 contains an incomplete intracellular death domain but may



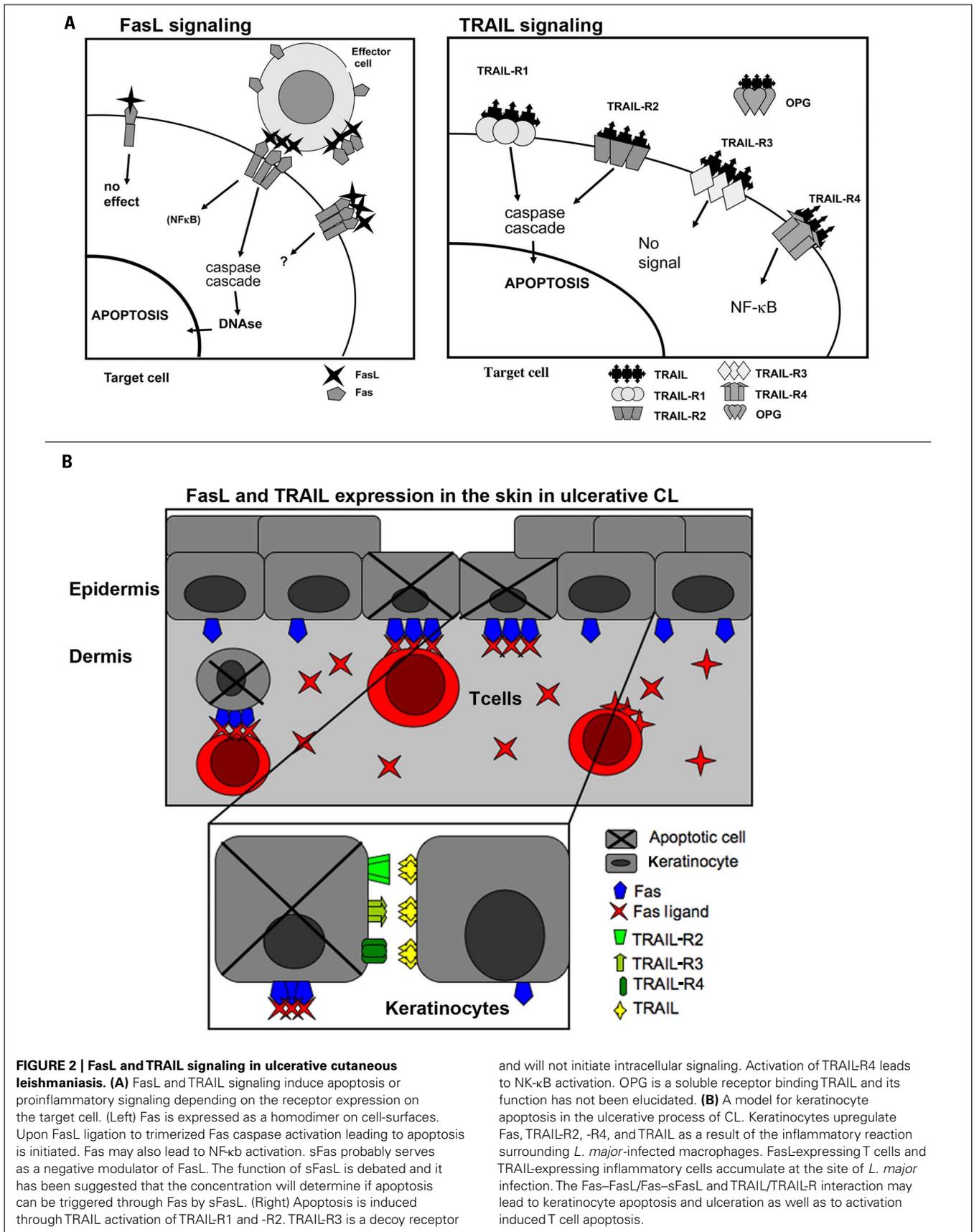
**FIGURE 1 | Histological hallmarks of diffuse and localized cutaneous leishmaniasis caused by *L. aethiops*.** (Top) Neutrophils, dendritic cells, and tissue macrophages are targets for infective *Leishmania* promastigotes and are present in the healthy skin. (Bottom) *L. aethiops* cause non-ulcerative diffuse cutaneous leishmaniasis (DCL) or ulcerative localized cutaneous leishmaniasis (LCL). In non-ulcerative DCL (left), epidermis is intact and a large number of tissue macrophages packed

with *Leishmania* amastigotes are present in dermis and the lymphocyte infiltrate is sparse as compared to ulcerative LCL (right). In ulcerative disease, apoptotic keratinocytes are present in the ulcerated epidermis. Epidermal hyperplasia and proliferating keratinocytes surround the ulcer and a dense inflammatory infiltrate is present in the dermal compartment. The number of infected tissue macrophages is low.

induce proinflammatory signaling through NF-κB (Falschlehner et al., 2007). Fas regulates immune homeostasis and tolerance via inducing apoptosis of activated T, B, and dendritic cells (DCs), as suggested by the lymphoproliferative diseases associated with Fas or FasL mutations and by studies using targeted Fas deletion on T, B, and DCs (Stranges et al., 2007; Hao et al., 2008; Mabrouk et al., 2008; Turbyville and Rao, 2010). By killing APCs, FasL might decrease bystander T cell activation and in addition Fas can serve as a molecular break for T cell responses by complementing the effects of Bim to eliminate activated T cells (Hughes et al., 2008). Death receptor-induced apoptosis thus regulates turnover of effector cells during immune responses and additionally, due to FasL and TRAIL being expressed by activated T, NKT, and NK cells, it can eliminate cells infected by various pathogens. Death receptor-induced apoptosis can contribute to bystander apoptosis

in inflamed tissues thereby increasing disease pathogenesis. Such activity is well studied during HIV infection where FasL, TNF, and TRAIL-mediated apoptosis have all been implicated in the depletion of non-infected T lymphocytes (Gougeon, 2003). FasL and TRAIL have also been shown to contribute to memory B cell apoptosis, possibly contributing to the decreased serological memory (Moir and Fauci, 2009; van Grevenynghe et al., 2011).

Non-apoptotic signaling activities of Fas stimulate T cell proliferation, induce proinflammatory responses in various immune cells, promote neuronal tissue remodeling, liver regeneration, and tumor progression (Peter et al., 2007). Apoptotic and non-apoptotic signals are probably occurring concomitantly in response to death receptor triggering, with the outcome determined by the activity of several other mechanisms influencing apoptosis sensitivity. Specifically, DISC localization at the cell



membrane, high c-FLIP expression or the activity of Toso have been shown to increase stimulatory signals from death receptors and the threshold for apoptosis (Lee et al., 1998; Peter et al., 2007; Nguyen et al., 2011). In addition, the same cell types can respond differently to death receptor triggering depending on the stage of activation. Fas triggering can induce early apoptosis among activated T cells and enhanced proliferation of the surviving T cells (Maksimow et al., 2003). Triggering Fas on non-activated T cells of HIV-infected individuals can lead to apoptosis, whereas the same signal contributed to strong activation of T cells that received weak, otherwise ignorable TCR stimuli (Rethi et al., 2008). In addition to activation or death decided at the level of single cells, death receptor-induced apoptosis of one cell can modulate the activities of others thereby playing a complex regulatory role during immune responses. As will be discussed later, Fas-induced apoptosis of neutrophils during *Leishmania* infection can lead to macrophage activation or inactivation depending on the host's genetic background, which strongly influence the immune response against the pathogen (Ribeiro-Gomes et al., 2004, 2005).

### FasL AND TRAIL SIGNALING IN SKIN HOMEOSTASIS AND INFLAMMATION

Epidermis is a stratified epithelium mainly composed of keratinocytes. It is separated from the underlying dermis by the basal membrane and basal keratinocytes are susceptible to FasL and TRAIL-induced apoptosis (Jansen et al., 2003; Vassina et al., 2005). IFN $\gamma$  renders keratinocytes more susceptible to death receptor-induced apoptosis (Konur et al., 2005). TRAIL, as well as TRAIL-R1, -R2, and -R4 are expressed in keratinocytes in the healthy skin (Leverkus et al., 2000; Stander and Schwarz, 2005). TRAIL induces apoptosis in IFN $\gamma$  stimulated keratinocytes and was shown to induce apoptosis in proliferating but not differentiating human keratinocytes (Jansen et al., 2003). To further show the complex role of TRAIL signaling in the skin, TRAIL was shown to induce caspase-dependent differentiation of primary keratinocytes (Wu et al., 2011).

FasL-mediated killing of IFN $\gamma$  sensitized keratinocytes by T cells has been shown in the skin during atopic dermatitis (AD). In this setting, apoptotic keratinocytes were shown to release T cell chemoattractants. Thus, a loop of signaling between infiltrating, FasL-expressing T cells and activated, Fas-expressing keratinocytes was suggested which ultimately increased the number of inflammatory T cells in eczematous skin (Trautmann et al., 2000). FasL signaling has also been shown to induce production of several proinflammatory cytokines such as TNF $\alpha$ , IL1 $\beta$ , and IL-6 and activation of the epidermal growth factor receptor in keratinocytes in AD (Iordanov et al., 2005). FasL driven production of proinflammatory cytokines could in part explain the increased proliferation of keratinocytes which has been reported in many dermatoses including CL. TRAIL-expressing CD4+ and CD8+ T cells are present in dermis during AD (Vassina et al., 2005). TRAIL-expressing dermal DCs (Zaba et al., 2010) and upregulation of TRAIL-R1 and -R2 has been shown in the psoriatic skin (Goto and Lindoso, 2010). Nickel renders basal keratinocytes sensitive to TRAIL-mediated apoptosis through upregulation of TRAIL-R1 and -R2 in contact dermatitis toward nickel

(Schmidt et al., 2010). Despite many descriptive studies showing the presence of TRAIL-expressing cells in the skin during inflammation, the consequences of such dysregulation are still at large unknown.

Alterations of Fas and FasL expression in keratinocytes (Viard et al., 1998) and in the effector T cell compartment (Abe et al., 2003) leading to keratinocyte death and ulceration has been described in toxic epidermal necrolysis (TEN). Recently, sTRAIL secreted by dermal DCs present in TEN lesions was shown to induce keratinocyte apoptosis (de Araujo et al., 2011). Thus, a synergistic killing of keratinocytes by FasL released from infiltrating T cells and TRAIL released by infiltrating DCs could occur in TEN. Intravenous immunoglobulins (IVIg) exhibits a broad spectrum of immunoregulatory activities and both activation and inhibition of Fas signaling (Prasad et al., 1998; Viard et al., 1998) has been ascribed IVIg and systemic treatment with IVIg-containing Fas-blocking antibodies limited the ulcerative process during TEN (Abe et al., 2003). To date, direct targeting of FasL signaling has not been tested in man during TEN.

### DYSREGULATION OF FasL AND TRAIL AND THEIR RECEPTORS DURING HUMAN LEISHMANIASIS

*Leishmania* promastigotes are rapidly ingested by neutrophils and DCs present in the healthy skin (Laskay et al., 2008; Ng et al., 2008; Peters et al., 2008). In visceral leishmaniasis (VL), the parasites spreads from the skin to draining lymph nodes and further on throughout the reticuloendothelial organs in the body including the spleen, the liver, and the bone marrow. VL leads to a systemic and potentially lethal infection. Fas and FasL are highly expressed on spleen-derived leukocytes but not on peripheral blood mononuclear cells during VL, suggesting localized increase in Fas/FasL expression only at the site of infection (Eidsmo et al., 2002).

In CL, the infection is mainly confined to the skin and within a few weeks after infection tissue macrophages serves as the main host cells. Localized chronic tissue inflammation arises and *Leishmania*-laden macrophages need to be activated through signals from infiltrating leukocytes, primarily T cells but also B cells and neutrophils, to clear infection. Keratinocyte proliferation and activation with an upregulation of activation markers such as HLA DR is noted together with marked epidermal hyperplasia, necrosis, and apoptosis (ElHassan et al., 1995; Gaafar et al., 1999).

We and others have shown that Fas and FasL is upregulated in the skin in *L. major* and *L. aethiopic*a-induced ulcerative CL (Mustafa et al., 2001; Eidsmo et al., 2005, 2007; Tasew et al., 2010; **Table 1**) and apoptotic keratinocytes surround ulcers (Eidsmo et al., 2005). In the case of *L. aethiopic*a-induced CL, we have shown increased number of dermal FasL-expressing cells in ulcerating as compared to non-ulcerative lesions. In ulcerative CL, the number of apoptotic keratinocytes correlates with the number of FasL-expressing dermal cells (Eidsmo et al., 2005; Tasew et al., 2010). In agreement with our findings, increased numbers of FasL-expressing cells were detected in *L. braziliensis*-induced LCL and DCL (Carneiro et al., 2009) and the number of apoptotic cells correlated to the number of FasL-expressing cells in ulcerative LCL. In addition, increased expression of Fas and FasL was recently shown in oral mucosal leishmaniasis caused by

**Table 1 | Increased expression of FasL, TRAIL, TRAIL-R2, and -R4 at the site of infection during ulcerative leishmaniasis.**

	Healthy skin	Ulcerative CL	Non-ulcerative CL
<b>Epidermis</b>			
Apoptosis	Absent	High	Low
Proliferation	Low	High	Low
FasL	Absent	Absent	Absent
Fas	Moderate	High	High
TRAIL	Low	High	Moderate
TRAIL-R1	Moderate	Moderate	Moderate
TRAIL-R2	Low	High	Moderate
TRAIL-R3	High	High	Not done
TRAIL-R4	Absent	Moderate	Not done
<b>Dermis</b>			
Apoptosis	Low	High	Low
Proliferation	Low	Low	Low
FasL	Absent	High	Low
Fas	Low	High	High
TRAIL	Absent	High	Moderate
TRAIL-R1	Absent	Low	Low
TRAIL-R2	Absent	Moderate	Low

Expression of FasL and TRAIL and their receptors together with detection of apoptosis was visualized in human skin biopsies obtained from *L. major*-induced CL in Iran and *L. aethiops*-induced CL in Ethiopia and compared to endemic healthy controls. A total of 51 biopsies were assessed: healthy individuals ( $n = 11$ ), ulcerative LCL induced by *L. major* ( $n = 8$ ), *L. aethiops* ( $n = 19$ ), and non-ulcerative DCL induced by *L. aethiops* ( $n = 13$ ; Eidsmo et al., 2005, 2007; Tasew et al., 2010).

*L. braziliensis* (Palmeiro et al., 2012). In ulcerative CL induced by *L. major*, TRAIL as well as the pro-apoptotic receptor TRAIL-R2 and the proinflammatory receptor TRAIL-4 were upregulated in infected skin as compared to healthy controls (Figure 2B; Eidsmo et al., 2007). In *L. aethiops*-induced CL, higher levels of TRAIL and TRAIL-R2 were detected in patients as compared to healthy control and ulcerative LCL had higher levels of TRAIL and TRAIL-R2 as compared to non-ulcerative DCL (Eidsmo et al., 2007; Tasew et al., 2010) and (unpublished observations; Table 1 and Figure 2B).

To test if the observed increased expression of FasL and TRAIL were indeed involved in ulceration during CL, we performed *in vitro* experiments using supernatants derived from *L. major* and *L. aethiops* stimulated PBMCs that contained high levels of sFasL and sTRAIL. Primary keratinocytes and the keratinocyte cell line HaCaT exposed to sFasL- and sTRAIL-containing supernatants underwent apoptosis and this could be prevented by adding FasL and TRAIL neutralizing antibodies to the keratinocytes prior to exposure to supernatants. Interestingly, when strains of *L. aethiops* isolated from patients with non-ulcerative DCL were used, keratinocyte apoptosis was not induced (Eidsmo et al., 2005, 2007; Tasew et al., 2010). To study the full consequences of FasL and TRAIL signaling during complex tissue infection and

inflammation, we turned to an animal model of ulcerative CL. *L. aethiops* inoculation in mice does not lead to productive infection or ulcerative disease. Murine CL can be obtained by *L. major* infection where in our hands the parasite strain Friedlin leads to ulcerative infection in BALB/c and the parasite strain LV39 leads to non-ulcerative infection. Thus, we have used *L. major* as a model organism to induce CL in mice as discussed below.

## REGULATION OF NEUTROPHIL CHEMOTAXIS BY Fas ACTIVATION IN LEISHMANIA INFECTION

During the chronic phase of CL, large numbers of neutrophils are present at the site of infection (Doerr and Seifert, 1995). Neutrophils age rapidly in the circulation and are constantly removed by macrophages in bone marrow, spleen, and liver (Furze and Rankin, 2008). Their activation leads to rapid apoptosis in which death receptors and Bcl-2 family member proteins play a role (Crocker et al., 2011). Neutrophils serve as a first host during *Leishmania* infection and it has been shown in several settings that their spontaneous apoptosis is delayed upon infection and that infected, apoptotic neutrophils can serve as Trojan horses allowing the parasite a silent entry into tissue macrophages serving as the definite host (Aga et al., 2002; van Zandbergen et al., 2004).

FasL contributes to massive apoptosis of macrophages exposed to *L. major* in BALB/c mice and to the production of the chemokines CXCL1 and CCL3, both with neutrophil chemoattractant activity (Ribeiro-Gomes et al., 2005). Similarly to macrophages, Fas triggering on the surface of DCs, keratinocytes, and a subset of NKT cells induced the production of neutrophil chemoattractants (Giroux and Denis, 2005; Guo et al., 2005; Farley et al., 2006). The Fas/FasL molecule pair can thus serve as an accelerator of inflammation in addition to inducing apoptosis in various cell types. The FasL-induced neutrophil infiltration has several consequences. In addition to increasing pathogen clearance, the phagocytic cells might be important for the removal of apoptotic cells that accumulate as the result of the increased Fas activation. Apoptotic neutrophils can also initiate tissue repair mechanisms in macrophages, partly by inducing TGF $\beta$  secretion, which, on the other hand, facilitate *Leishmania* replication in macrophages (Afonso et al., 2008). In FasL-deficient *gld* mice, both macrophage apoptosis and chemokine production was reduced as well as the extravasation of neutrophils (Ribeiro-Gomes et al., 2005). Similarly, FasL neutralization using antibodies during ulcerative *L. major* (strain Friedlin V1) infection in BALB/c mice resulted in decreased neutrophil infiltration into infected skin (Tasew et al., 2010). The number of lesional neutrophils was approximately sixfold lower in a non-ulcerative model of *L. major* infection induced by the parasite-strain LV39. FasL neutralization in LV39-induced infection further reduced the number of lesional neutrophils (Tasew et al., 2010).

## MODULATION OF MACROPHAGE RESPONSE TO LEISHMANIA BY APOPTOTIC NEUTROPHILS

Neutrophils eliminate infecting pathogens via phagocytosis or by secreting reactive oxygen species, inflammatory cytokines, and chemokines and thereby increasing tissue inflammation. In addition, activated neutrophils can release a network formed of DNA and various granule proteins, called neutrophil extracellular

traps, which can kill infecting pathogens including *Leishmania* (Guimaraes-Costa et al., 2009). Neutrophil responses are under tight regulation and the activation of these cells is rapidly followed by apoptosis, partly induced by Fas triggering. Apoptotic neutrophils can increase the activation of macrophages or DCs (Majai et al., 2010) and their phagocytosed material can be used as a source of pathogen-derived peptides for antigen presentation. It has been shown that apoptotic neutrophils regulate macrophage responses to *Leishmania* infection differently in BALB/c or C57BL/6 mice. In BALB/c mice, more sensitive to *Leishmania* infection, apoptotic neutrophils inhibit macrophage activation and facilitate pathogen replication in the cells via a PGE2 and TGF- $\beta$ -dependent pathway (Ribeiro-Gomes et al., 2005). Interestingly, the absence of FasL fundamentally changed the role of neutrophils in the regulation of macrophage responses to *Leishmania* infection. The absence of FasL altered kinetics and probably the mechanism of apoptosis, and in this case neutrophils increased parasite clearance by macrophages via a NO-dependent mechanism (Ribeiro-Gomes et al., 2005).

In C57BL/6 mice, more resistant to *Leishmania* infection, apoptotic neutrophils increased parasite killing by macrophages through a neutrophil elastase-dependent induction of TNF $\alpha$  secretion (Ribeiro-Gomes et al., 2004). PGE2 and TGF $\beta$ , the neutrophil-induced factors in BALB/c mice that promote parasite growth in macrophages, were not induced during neutrophil-macrophage interactions in the C57BL/6 model. Thus, FasL turns neutrophils to suppressors of *Leishmania* clearance in BALB/c mice which was reflected by the finding that *in vivo* depletion of neutrophils decreased parasite load in wild-type (WT) but increased parasite load in *gld* mice (Ribeiro-Gomes et al., 2005). In C57BL/6 mice, in line with the macrophage stimulatory functions of neutrophils, granulocyte depletion led to increased parasite load (Ribeiro-Gomes et al., 2004).

### Fas-MEDIATED MACROPHAGE APOPTOSIS MAY CONTRIBUTE TO PARASITE CLEARANCE

In contrast to BALB mice, Fas or FasL deficiency on MLR and C57BL/6 background led to impaired parasite clearance when a high number of parasites was inoculated subcutaneously (Conceicao-Silva et al., 1998; Huang et al., 1998). *Leishmania*-infected macrophages upregulated Fas and apoptosis sensitivity in response to the Th1 cytokine IFN $\gamma$  and to autocrine TNF $\alpha$  production. It was suggested that Fas-mediated elimination of *Leishmania*-infected macrophages play an important role in decreasing the parasite load (Conceicao-Silva et al., 1998). However, other studies have shown that *gld* mice on a C57BL/6 background control infection as efficient as WT control and that TNF, acting like another death receptor ligand, plays an essential role in pathogen

clearance (Wilhelm et al., 2001, 2005). In these studies, the results varied depending on the *Leishmania* strain used for infection, indicating that the genetic background of both the host and the pathogen can influence the contribution of death receptors in the *Leishmania*-specific immune responses (Wilhelm et al., 2001; Ritter et al., 2004).

### SHORT-TERM INHIBITION OF FasL AND TRAIL SIGNALING DECREASE ULCERATION IN BALB/c MICE INFECTED WITH *L. MAJOR*

BALB/c serves as an ulcerative, non-healing model of CL and C57BL/6 mice serve as a non-ulcerative but healing model of CL. Thus, the characteristics of human CL are not completely mimicked by the models available. To study the effect of FasL or TRAIL neutralization during ulcerative CL, we utilized a model of injecting a low-infectious dose of metacyclics into dermis in BALB/c mice. Neutralization of both FasL and TRAIL inhibited ulcer development and no effect on the infectious load in the skin or draining lymph nodes was noted (Tasew et al., 2010). Interestingly, FasL neutralization reduced neutrophil infiltration into the skin during established infection, suggesting an additional pro-inflammatory role of FasL in addition to direct keratinocyte killing. FasL signaling resulting in recruitment of activated neutrophils into dermis may lead to destruction of the basal membrane and thus allow direct FasL-mediated killing of exposed keratinocytes *in vivo*.

A majority of patients affected by CL will request treatment despite the benign prognosis of the disease. The current treatment regimes are primarily aimed at parasite elimination and many will result in exacerbation of inflammation, tissue destruction, and scar formation (Mishra et al., 2007; Goto and Lindoso, 2010). Targeting specific immune mechanisms has proven to be a promising new approach for the therapy of cancer and autoimmune diseases and could potentially be used to improve treatment regimes of neglected tropical diseases including leishmaniasis. The current revolution in the development of immunotherapeutics could lead to novel effective treatments of neglected tropical diseases. Skin diseases serves as an excellent model system to test novel treatment regimes as the pathology and disease development can be followed by the naked eye. It has been suggested that TNF $\alpha$ -blocking antibodies could reduce tissue pathology during CL (Oliveira et al., 2011) and that inhibition of tyrosine kinases during VL could improve treatment efficacy (Dalton and Kaye, 2010; Dalton et al., 2010). We suggest that inhibition of reducing FasL and TRAIL signaling at the site of infection in combination with leishmanicidals could in theory reduce the chronic inflammation and the ulcerative process and subsequent scar formation.

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