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FasL and TRAIL Induce Epidermal Apoptosis and Skin Ulceration Upon Exposure to *Leishmania major*

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Receptor-mediated apoptosis is proposed as an important regulator of keratinocyte homeostasis in human epidermis. We have previously reported that Fas/FasL interactions in epidermis are altered during cutaneous leishmaniasis (CL) and that keratinocyte death through apoptosis may play a pathogenic role for skin ulceration. To further investigate the alterations of apoptosis during CL, a keratinocyte cell line (HaCaT) and primary human epidermal keratinocytes were incubated with supernatants from *Leishmania major*-infected peripheral blood mononuclear cells. An apoptosis-specific microarray was used to assess mRNA expression in HaCaT cells exposed to supernatants derived from *L. major*-infected cultures. Fas and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mRNA and protein expression were significantly up-regulated, and apoptosis was detected in both HaCaT and human epidermal keratinocyte cells. The keratinocyte apoptosis was partly inhibited through blocking of Fas or FasL and even more efficiently through TRAIL neutralization. Up-regulation of Fas on keratinocytes in epidermis and the presence of FasL-expressing macrophages and T cells in dermis were previously reported by us. In this study, keratinocytes expressing TRAIL, as well as the proapoptotic receptor TRAIL-R2, were detected in skin biopsies from CL cases. We propose that activation of Fas and TRAIL apoptosis pathways, in the presence of inflammatory mediators at the site of infection, leads to tissue destruction and ulceration during CL. ***(Am J Pathol 2007, 170:227–239; DOI: 10.2353/ajpath.2007.060068)***

Cutaneous leishmaniasis (CL) caused by *Leishmania major* is endemic throughout the semidesert and dry-silt valleys of the Middle East and North Africa. This intradermal infection causes a vigorous inflammatory reaction, typically leading to skin ulceration within a few weeks, which is followed by a chronic skin ulcer that lasts for up to a year. Although primary infection with *L. major* is painless, secondary infections with bacteria often cause discomfort and pain. In addition, scar formation that evolves after healing is often considered disfiguring. At the site of the ulcer, in the dermis of the skin, *L. major*-infected macrophages and infiltrating, activated T cells, monocytes, neutrophils, and plasma cells are found. We[1] and others[2,3] have suggested a role for Fas apoptosis mediated by its natural ligand (FasL) in ulcer formation and parasite clearance during CL.

Regulation of apoptosis is pivotal during immune responses in human, and the complexity of this homeostatic regulation is illustrated by the involvement of several distinct receptor pathways in this process. Among these pathways, the best characterized is the Fas/FasL signaling pathway.[4] In the context of the human skin, it has been demonstrated that activated keratinocytes up-regulate Fas and become susceptible to FasL-induced apoptosis.[5] Fas-mediated apoptosis has been ascribed a role in the pathology of several skin disorders, including chronic inflammatory diseases such as eczematous dermatitis[6] and dermatological manifestations of drug reactions such as toxic epidermal necrolysis[7,8] and skin malignancies.[9] Although Fas expression is altered, keratinocytes are resistant toward FasL-induced apoptosis during psoriasis,[10,11] and recent data suggest a proinflammatory role of FasL[12] leading to proliferation of FasL-targeted keratinocytes.

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TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) was initially proposed to induce apoptosis only in immortalized or malignant cells, but several recent studies proposed a role for TRAIL in the physiological regulation of cell survival during HIV infection[13,14] and listeriosis.[15] In support of the important role of TRAIL *in vivo* is that TRAIL-deficient mice show impaired thymic apoptosis as well as increased susceptibility to autoimmune diseases such as collagen-induced arthritis.[16] Five different TRAIL receptors (TRAIL-Rs) have been described, TRAIL-R1 to -R4, and the soluble receptor osteoprotegrin. TRAIL-R1 and TRAIL-R2, once activated, signal through caspase 8 to mediate apoptosis, whereas TRAIL-R3 and TRAIL-R4 are decoy receptors lacking intracytoplasmic domains linked to caspase 8. TRAIL-R4 may induce nuclear factor (NF)-κB activation and resistance toward apoptosis.[17] It has been suggested that the expression pattern of the four TRAIL-Rs determines whether apoptosis occurs in the presence of TRAIL. Thus, a cell expressing predominantly TRAIL-R1 and -R2 will be susceptible to TRAIL-mediated apoptosis. On the other hand, because of competition of the receptors, a cell expressing an abundance of TRAIL-R3 and -R4 will be resistant toward TRAIL-mediated apoptosis even if TRAIL-R1 and -R2 are expressed at low or intermediate levels. TRAIL, as well as TRAIL-R1 and -R4, are expressed in normal skin,[18] and the expression pattern of TRAIL-R2 and -R3 in healthy skin is yet poorly characterized. In primary keratinocytes, TRAIL-R1 to -R4 are constitutively expressed, and the expression pattern may be altered by external stress signals such as UV radiation.[19] The role of TRAIL-induced apoptosis in skin homeostasis remains to be fully evaluated, although recent publications have proposed that TRAIL induces apoptosis in HaCaT[20] and primary keratinocytes.[21] In this context, undifferentiated cells are much more susceptible to apoptosis than mature keratinocytes. HaCaT cells have been proposed to be more susceptible to TRAIL-induced apoptosis[22] compared with human epidermal keratinocytes (HEKs). A role for interferon (IFN)-γ in TRAIL-induced apoptosis[23] of primary keratinocytes has recently been reported.

Previously, we have suggested a role for Fas/FasL-mediated apoptosis of keratinocytes in the development of ulcers during CL.[1] However, some of the results obtained in this context indicated that Fas pathway may not be the only player involved in apoptosis of keratinocytes during CL. For example, when soluble FasL (sFasL)-containing supernatants from restimulated CL peripheral blood mononuclear cells (PBMCs) were used to induce apoptosis in human keratinocytes, blocking of the Fas/FasL pathway inhibited apoptosis only in two thirds of the analyzed supernatants. Furthermore, many apoptotic keratinocytes observed in the epidermis of CL cases were found in the superficial part of the sample, and all of the FasL-expressing, infiltrating macrophages and T cells were consistently found in dermis. Thus, in the present work we investigated the possible involvement of other pathways in apoptosis of keratinocytes during CL. HaCaT cells were exposed to cell culture supernatants obtained from a *L. major* infection model of human PBMCs. The mRNA expression of 96 genes involved in apoptosis regulation was evaluated in HaCaT cells by a commercially available microarray and verified at the protein level. Furthermore, because HaCaT cells have been shown to differ from primary keratinocytes in their apoptotic responses,[24–26] protein levels of Fas, TRAIL, and TRAIL-R1 to -R4 were verified in HEKs. The levels of apoptosis on exposure to supernatants obtained from infected PBMCs and blocking experiments aimed at assessing the role of FasL and TRAIL in apoptosis induction were performed in both HaCaT and HEK cells. The expression of Fas, TRAIL, and TRAIL-R1 to -R4 was evaluated in skin biopsies from CL cases. The results indicate that TRAIL, in addition to the Fas pathway, is involved in keratinocyte apoptosis and skin ulceration during CL.

Materials and Methods

Patient Material

Skin biopsies were donated by Iranian CL patients and healthy volunteers. CL was diagnosed clinically and by detection of parasites in direct smears and/or culture of skin scrapings. Promastigotes were propagated by culture on Novy-Nicolle-McNeal-blood agar[27] from some of the smears and were identified as *L. major* by isoenzyme technique[28] and monoclonal antibodies.[27] The CL patients were all male military recruits who moved from nonendemic areas to *L. major* hyperendemic foci before the onset of disease. CL patients had a 1- to 7-month history of ulceration. Control skin was obtained from three healthy Iranian volunteers undergoing cosmetic surgery and processed in the same way as the biopsies from CL patients. Informed consent was obtained from all sample donors for the usage of biological material. This study has received ethical approval from both Swedish and Iranian ethical committees. Biopsies were taken under sterile conditions after local anesthesia from the indurations lining the ulcers in eight CL patients. The biopsies were split and either frozen in OCT (TissueTek, Zoeterwoude, The Netherlands) or fixed in 4% formalin and embedded in paraffin.

In Vitro *Infection of Human PBMCs with* L. major

PBMCs obtained from healthy donors were prepared as previously described.[29] *L. major* (Friedlin) (gift of Dr. D. Sacks, National Institutes of Health, Bethesda, MD) was cultured in Medium 199 (Gibco BRL, Life Technologies, Paisley, UK) with 20% heat-inactivated fetal calf serum (Sigma-Aldrich, Stockholm, Sweden), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/L L-glutamine. Stationary phase cultures of *L. major* promastigotes (4 to 5 days old) were added at 1×10^6 cells/ml (cell/parasite ratio, 1:1) to PBMCs in RPMI supplemented with 10% normal human serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/L L-glutamine (Gibco BRL) in six-well plates, 25-mm^2 flasks, or in chamber slides (Nunc, Heresford, UK). For assessment of infectivity,

cells were incubated overnight in an atmosphere of 5% CO_2 in air at 33°C and then moved to 37°C, 5% CO_2 in air. Infectivity was assessed after 72 hours by staining adherent cells with 5 μg/ml propidium iodide (Becton Dickinson, Stockholm, Sweden). Five to 7 days after infection, supernatants were harvested, centrifuged at 1200 $\times$ *g* for 10 minutes, and either ultracentrifuged or aliquoted and immediately transferred to −70°C. Supernatants from two different donors were ultracentrifuged at 30,000 $\times$ *g* for 30 minutes, followed by 100,000 $\times$ *g* for 1 hour. The ultracentrifuged fractions were transferred to −70°C.

Human Epidermal Keratinocytes

Primary normal HEKs (Cascade Biologics, Portland, OR) were cultured in EpiLife medium (Cascade Biologics) supplemented with human keratinocytes growth supplement (Cascade Biologics) and 1000 U of penicillin-streptomycin (Roche, Penzberg, Germany). Cells were grown to ~80% confluence before experiments were performed (5 to 7 days after seeding).

HaCaT—Human Keratinocyte Cell Line

HaCaT, immortalized human keratinocytes,[30] kindly provided by Prof. N.E. Fusenig (Heidelberg, Germany), were plated on sterile permanox culture slides (Nunc) or in 25-mm^2 cell culture flasks and incubated at 37°C, 5% CO_2 in air, until 70 to 80% confluence was reached (2 to 3 days after seeding).

cDNA Expression Array

Approximately 2 $\times$ 10^6 HaCaT cells in a 70 to 80% confluent monolayer covering 25 mm^2 were incubated with 5 ml of Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal calf serum, penicillin-streptomycin, and L-glutamine (Gibco BRL) (Dulbecco's modified Eagle's medium) or Dulbecco's modified Eagle's medium diluted 1:1 with infected or control supernatants for 6 or 24 hours. Nonadherent, apoptotic cells were washed off with phosphate-buffered saline (PBS) and total RNA isolated using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Approximately 40 to 50 μg of RNA was purified from each sample. Size chromatography of RNA was performed with an Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany). cDNA expression microarray analysis was performed using the nonradioactive human apoptosis GEarray Q Series (SuperArray Inc., Bethesda, MD), according to the manufacturer's instructions. In brief, 1.5 μg of total RNA was reverse-transcribed with reverse transcriptase (Invitrogen AB, Stockholm, Sweden) and GEAprimer mix (Invitrogen) in the presence of biotin-16-dUTP (Roche). Amplification of cDNA was performed during 30 cycles using the LPR Amplolabeling kit (SuperArray).

The biotinylated cDNA probes were denatured and added to the hybridization solution. GEArray Q Series membranes dotted with tetra-spot cDNA fragments of 96 human apoptosis genes were prehybridized at 60°C for 2 hours and then hybridized overnight with the cDNA probes. Membranes were then washed, blocked, and incubated with alkaline phosphatase-conjugated streptavidin. The labeled biotin on the membrane was detected by chemiluminescence, according to the manufacturer's instructions. RNAs extracted from HaCaT cultures incubated with three sets of infected or control supernatants from three different PBMC donors were used.

Analysis of cDNA Microarray

The luminescence intensities of hybridized cDNA probes were analyzed by GEArray software (SuperArray). Local background was subtracted for each point. Each signal intensity was normalized against the housekeeping gene ribosomal protein L13a. The ratio value of expression in HaCaT cells exposed to supernatants was obtained from the intensity value of individual genes relative to its median intensity in RNA obtained from untreated HaCaT. For the analysis of gene expression changes after incubation with supernatants, the ratio of relative expression between samples incubated with supernatants or HaCaT cells alone was analyzed. Genes were selected if the median ratio was changed at least twofold in all three samples analyzed for the different conditions described compared with untreated HaCaT.

Measurement of sTRAIL, sFasL, and IFN-γ in Culture Supernatants

Levels of sTRAIL, sFasL, and IFN-γ were assessed in duplicates in supernatants from *L. major*-infected PBMC cultures or supernatants from HaCaT cultures using commercial enzyme-linked immunosorbent assays (ELISAs) (TRAIL and IFN-γ: R&D Systems, Amersham, UK; sFasL: Nordic BioSite, Täby, Sweden).

Detection of Membrane-Bound TRAIL, Fas, and TRAIL-R1 to -R4 on HaCaT and HEK Cells

HaCaT and HEK cells were incubated with supernatants from *L. major*-infected or control PBMCs (supernatants: medium, 1:1) in six-well plates for 20 hours and thereafter washed with PBS, and adherent HaCaT cells were detached with 0.05% ethylenediaminetetraacetic acid in PBS and HEK cells with trypsin/ethylenediaminetetraacetic acid (Gibco). After an additional wash with PBS containing 1.5% bovine serum albumin [fluorescence-activated cell sorting (FACS) buffer, Sigma Aldrich] the samples were incubated with fluorescein isothiocyanate-conjugated mouse anti-human Fas monoclonal IgG (Dakopatts, Stockholm, Sweden), phycoerythrin-conjugated mouse anti-human TRAIL monoclonal IgG (R&D Systems), or fluorescein isothiocyanate- or phycoerythrin-conjugated mouse isotype control IgG (Dakopatts) in FACS buffer for 20 minutes. TRAIL-R1 to -R4 were stained by 30-minute incubation in mouse anti-human TRAIL-R1

to -R4 (20 μg/ml) (kindly provided by Immunex, Seattle, WA) followed by a 20-minute incubation with goat anti-mouse RPE (Dakopatts).

Induction of Apoptosis in HaCaT and HEK Cells

HaCaT or HEK cells were plated on sterile permanox culture slides (Nunc) and incubated in 37°C air with 5% CO_2 until 70 to 80% confluence was reached (2 to 3 days for HaCaT cells and 5 to 7 days for HEK cells). Apoptosis was assessed after 20 hours of incubation with 1 μg/ml Fas-activating monoclonal antibody (CH-11; MBL, Nagoya, Japan), 250 ng/ml recombinant TRAIL (R&D Systems) or supernatants obtained from either *L. major*-infected or control PBMCs (diluted 1:4 in medium). In an attempt to block Fas-mediated apoptosis of HaCaT and HEK cells induced by supernatants from *L. major*-stimulated CL PBMCs, the Fas-blocking monoclonal antibody ZB4 (MBL) was added to the cultures at 1 to 2 μg/ml or the FasL-blocking antibody was added at 2.5 μg/ml (R&D Systems). TRAIL was blocked[31] by adding 2.5 μg/ml of TRAIL-blocking antibody 2E5 (Alexis, KeLab, Gothenburg, Sweden) or 5 μg/ml sTRAIL-R2 (Alexis). These antibody concentrations had previously been optimized for blocking experiments (results not shown). The samples were washed twice in Annexin V-binding buffer and incubated in the dark for 25 minutes with Annexin V and propidium iodide. After incubation, the cells were fixed in 1% paraformaldehyde (Sigma) for 10 minutes at room temperature, mounted with anti-fading solution (Vector Laboratories Inc., Burlingame, CA), and immediately analyzed. Apoptosis was assessed by counting 10 to 20 fields under ×40 ocular magnification and expressed as the number of apoptotic cells per 10 fields.

Visualization of TRAIL and Fas in Skin Biopsies from CL Cases

Paraffin-embedded skin biopsies from eight CL cases and three healthy controls were sectioned in 5-μm sections. Deparaffination and rehydration were performed as previously described.[32] Sections were incubated with mouse anti-TRAIL monoclonal (R&D Systems) at 12.5 μg/ml, mouse anti-Fas monoclonals (Dakopatts) at 10 μg/ml, or isotype controls (Jackson ImmunoResearch Europe Ltd. Soham, Cambridgeshire, UK) for 15 minutes at room temperature. Streptavidin-avidin enhancement was performed according to the manufacturer's instruction (Dakopatts). The antigens were visualized with VIP (Vector) or DAB (Vector), and hematoxylin staining (Sigma-Aldrich) counterstaining was performed. Fas expression during CL has been published previously[1] and was shown in the present work to compare the expression pattern of Fas and TRAIL in different donors.

Visualization of TRAIL-R1 to -R4 in HaCaT Cells and Skin Biopsies from CL Cases

To visualize the expression of TRAIL-R1 to -R4 in relation to cellular compartmentalization in HaCaT cells, immunofluorescence and FACS were used. HaCaT cells were grown to 70 to 80% confluence on sterile glass chamber slides, washed, and fixed in acetone. Frozen skin biopsies from four CL cases and three healthy controls were sectioned in 12-μm-thick sections, briefly dried, and fixed in acetone. Sections from two donors were processed in duplicate with similar results. The sections or HaCaT cells were incubated in protein block (Dakopatts) for 10 minutes, followed by a 1-hour incubation with the primary antibody (mouse anti-human TRAIL-R1 to -R4 IgG; Immunex) at 40 μg/ml, followed by a 1-hour incubation with Cy3-conjugated goat anti-mouse (Jackson ImmunoResearch) at 1:400. Isotype antibody controls were used in parallel. The sections were washed, mounted, and counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Vector) and analyzed with ×25 ocular magnification in a Nikon Optiphot 2 UV microscope (Melville, NY).

Statistical Analysis

Mann-Whitney signed or unsigned rank test, the Wilcoxon signed rank test, or linear regression test using Statview 2.3 (Eurodex, Stockholm, Sweden) was applied for statistical analysis.

Results

Supernatants from in Vitro*-Infected PBMCs Contain High Levels of IFN-γ, sFasL, and sTRAIL*

On the addition of stationary phase *L. major* promastigotes to PBMCs, 50 to 80% of macrophages were infected with 1 to 15 *L. major* amastigotes 72 hours after infection. Supernatants from such cultures contained high levels of sFasL [median, 3065 pg/ml; interquartile range (IQR), 2373 to 4198; Figure 1a] and moderate amounts of sTRAIL (median, 259 pg/ml; IQR, 232 to 353; Figure 1b) compared with supernatants originating from uninfected PBMCs (sFasL median, 172 pg/ml; IQR, 105 to 254; sTRAIL median, 121 pg/ml; IQR, 86 to 167). To exclude the possibility that sFasL and sTRAIL could be associated with small membrane vesicles,[33] two sets of supernatants were ultracentrifuged to remove cellular membranes. Because the levels of sFasL and sTRAIL in the supernatants were comparable before and after ultracentrifugation, we concluded that sFasL and sTRAIL were not associated to remaining cellular components (results not shown). No sFasL was detected in medium containing normal human sera or fetal calf serum and 38 pg/ml TRAIL was measured in medium containing normal human sera but not fetal calf serum. All supernatants from *L. major*-infected PBMCs contained >4000 pg/ml IFN-γ, whereas the levels of IFN-γ in medium and supernatants from uninfected cultures were lower than 50 pg/ml.

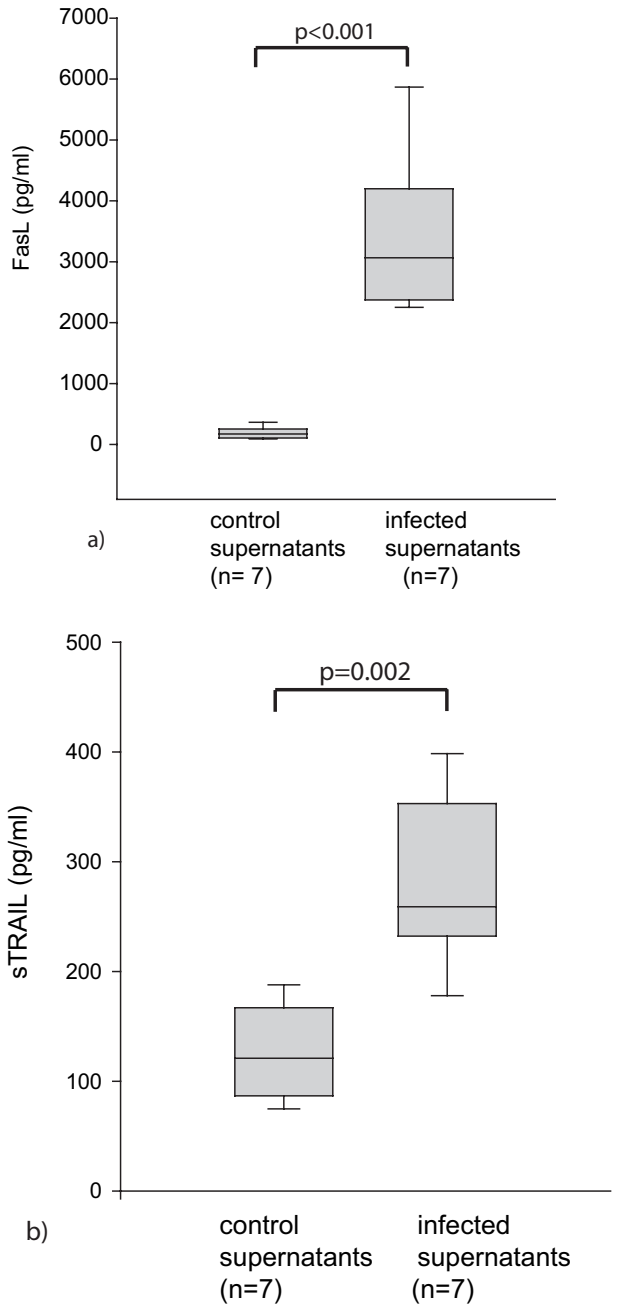


Figure 1. High levels of sFasL and sTRAIL were measured in supernatants from *L. major*-infected PBMCs. sFasL (**a**) and sTRAIL (**b**) were measured by commercially available ELISAs in supernatants from *L. major*-infected PBMCs and uninfected control culture. The horizontal and vertical lines in each box represent the median and the range (95% confidence interval), respectively. Differences were calculated by the Mann-Whitney test.

Gene Expression of Fas, TRAIL, and TRAIL-R2 Is Increased in HaCaT Cells on Exposure to Supernatants from L. major*-Infected PBMCs*

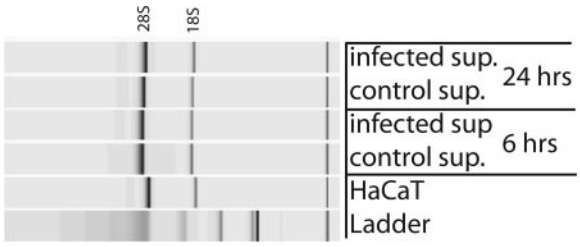
A commercially available microarray measuring the mRNA expression of apoptosis-related genes was used to characterize changes in gene expression of human HaCaT cells on 6 and 24 hours of exposure to supernatants from *L. major*-infected cells. The array (Q-series human apoptosis array, Superarray; MedProbe Sweden Office, Lund, Sweden) measures 96 genes belonging to the tumor necrosis factor (TNF) ligand (n = 14), TNF receptor (n = 15), Bcl-2 (n = 14), caspase (n = 12), IAP (n = 6), TRAF (n = 8), CARD (n = 6), death domain (n = 5), death effector domain (n = 3), and CIDE domain (n = 4) families, and the p53 and ATM pathways (n = 9). Total RNAs obtained from three different sets of HaCaT cells incubated at two different time points with supernatants from *L. major*-infected or uninfected PBMCs from three different donors were analyzed by size chromatographic separation. The intensity of the 28S band was approximately twofold the intensity of the 18S band in all samples prepared (Figure 2a). RNA (40 to 50 μg) was obtained from $\sim 2 \times 10^6$ HaCaT cells grown in a 25-mm^2 monolayer as indicated above. Although a large number of apoptotic HaCaT cells were detected after 16 to 18 hours of incubation with supernatants from *L. major*-infected PBMCs, the majority of cells from which intact RNA was purified were alive and adherent. The relative expression of apoptosis-related genes compared with the housekeeping gene RPL13A for all donors and genes investigated can be found in supplemental material, Table S1, at *http://ajp.amjpathol.org*.

When mRNA levels of the individual genes were altered more than twofold in three of three donors, the mRNA expression was considered as significantly dysregulated. No genes were significantly down-regulated in this study. The mRNA expression of Fas, TRAIL R1-, R2, and -R4 was detected in untreated HaCaT cells (Figure 2, b and c). On exposure to supernatants from *L. major*-infected PBMCs, the mRNAs for Fas and TRAIL were up-regulated in HaCaT, both at 6 and 24 hours after exposure (Figure 2, b–e) and the proapoptotic TRAIL-R2 (Figure 2, b and c) at 6 but not at 24 hours. TRAIL-R2 mRNA was also up-regulated in two of three samples exposed to uninfected supernatants from human PBMCs.

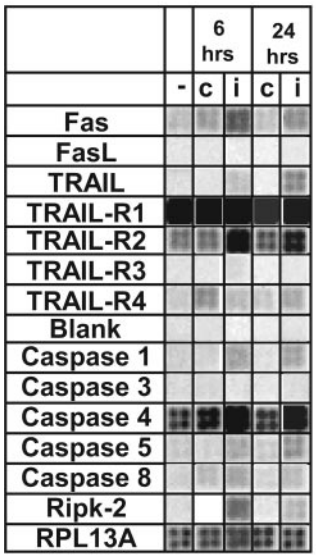
The levels of TRAIL-R1 mRNA were high in all samples investigated. Interestingly, a transient up-regulation of the expression of caspase 8 mRNA was detected at 6 hours but not at 24 hours, indicating that several components of the apoptotic pathway are affected by the supernatants from *Leishmania*-infected PBMC cultures. The mRNA of the receptor-interacting protein kinases-2 (Ripk-2) was up-regulated in HaCaT cells exposed to supernatants from infected or uninfected PBMCs at 6 hours, but this up-regulation was even higher when infected supernatants were used, and the increased gene expression persisted after 24 hours of incubation in the case of infected supernatants (Figure 2, b and c).

Membrane-Bound Fas and TRAIL Protein Expression Is Increased on HaCaT Cells after Exposure to Supernatants from L. major*-Infected PBMCs*

To determine whether up-regulation of TRAIL and Fas mRNA correlated with an increased accumulation of



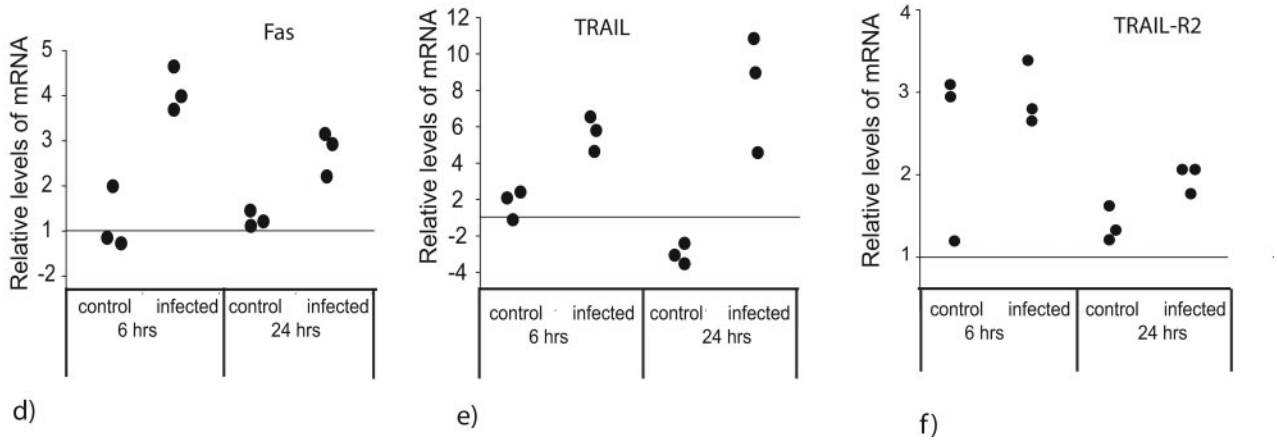
a)



b)

		6 hrs				24 hrs			
Genes	HaCaT	control supernatants (n=3)		infected supernatants (n=3)		control supernatants (n=3)		infected supernatants (n=3)	
		mean	(s.d.)	mean	(s.d.)	mean	(s.d.)	mean	(s.d.)
Fas	0,24	0,30	(0,15)	**0,99**	**(0,10)**	0,30	(0,05)	**0,67**	**(0,12)**
FasL	<0,01	0,06	(0,03)	0,04	(0,03)	0,01	(0,00)	0,02	(0,01)
TRAIL	0,05	0,09	(0,04)	**0,29**	**(0,05)**	0,03	(0,01)	**0,43**	**(0,17)**
TRAIL-R1	2,20	2,57	(0,31)	2,37	(0,51)	2,10	(1,28)	2,33	(0,68)
TRAIL-R2	0,68	1,64	(0,72)	**2,00**	**(0,26)**	0,94	(0,14)	1,33	(0,12)
TRAIL-R3	0,03	0,16	(0,11)	0,22	(0,16)	0,03	(0,00)	0,06	(0,05)
TRAIL-R4	0,17	0,83	(0,21)	0,94	(0,62)	0,35	(0,17)	0,62	(0,51)
Blank	0,09	0,10	(0,03)	0,06	(0,05)	0,10	(0,03)	0,11	(0,05)
Caspase 1	0,19	0,18	(0,03)	0,34	(0,08)	0,13	(0,03)	0,37	(0,03)
Caspase 3	0,10	0,13	(0,04)	0,13	(0,04)	0,10	(0,02)	0,11	(0,01)
Caspase 4	1,10	1,16	(0,56)	2,00	(0,46)	1,23	(0,15)	1,97	(0,29)
Caspase 5	0,18	0,37	(0,18)	0,68	(0,33)	0,19	(0,04)	0,41	(0,15)
Caspase 8	0,13	0,23	(0,05)	**0,36**	**(0,03)**	0,16	(0,01)	0,25	(0,02)
Ripk-2	0,07	**0,27**	**(0,13)**	**0,73**	**(0,29)**	0,06	(0,05)	**0,28**	**(0,03)**
RPL13A	1,00	1,00	(0,00)	1,00	(0,00)	1,00	(0,00)	1,00	(0,00)

c)



d) e) f)

Figure 2. Changes of mRNA expression of FasL- and TRAIL-associated genes in HaCaT cells exposed to supernatants from *L. major*-infected PBMCs. HaCaT cells were incubated with supernatants from three different healthy donors for 6 or 24 hours, and mRNA expression of 96 apoptotic-related genes were compared with the mRNA expression in untreated keratinocytes. **a:** Size chromatographic separation of RNA. **b:** Representative examples of signal intensity for genes associated with FasL- or TRAIL-induced apoptosis after cDNA hybridization from untreated HaCaT cells (−), HaCaT cells exposed to supernatants from control (c), or *L. major*-infected (i) PBMCs. **c:** Mean signal intensities after mRNA normalization against RPL13A are shown from three experiments, performed with supernatants from three different donors infected with *L. major* (infected supernatants) or uninfected PBMCs (control). SDs are shown within parentheses. More than a twofold difference in gene expression in three of three sets of RNA (compared with untreated HaCaT) was marked in bold. **d–f:** Individual data for Fas, TRAIL, and TRAIL-R2 mRNA alterations in HaCaT cells on exposure to supernatants from infected and uninfected cultures compared with untreated cells.

membrane-bound Fas and TRAIL, protein expression was analyzed by FACS on HaCaT cells exposed to infected supernatants for 20 hours. Both Fas and TRAIL are constitutively and homogenously expressed on the surface of HaCaT cells (Figure 3, a and b), and their surface expression was not altered by the addi-

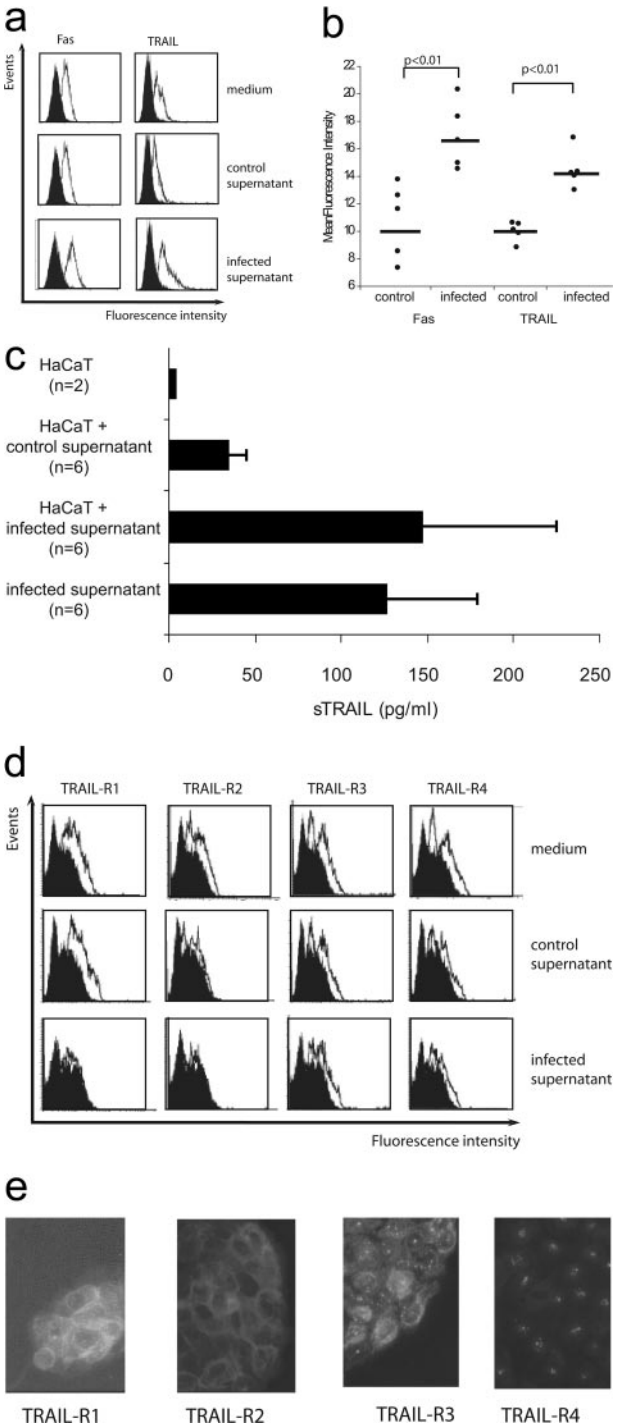


Figure 3. Expression of membrane-bound Fas, TRAIL, and TRAIL-R1 to -R4 and sTRAIL in HaCaT cells before and after exposure to supernatants from *L. major* or uninfected cultures. HaCaT cells were incubated with supernatants from *L. major*-infected or control PBMC cultures for 20 hours, and membrane expression of Fas and TRAIL was analyzed by flow cytometry. Filled histograms represent staining with isotype control and open histograms the specific Fas or TRAIL expression. The histograms shown are representative of five experiments. **a:** Fas was constitutively expressed on HaCaT cells and was up-regulated on incubation with supernatants from infected but not uninfected cultures. TRAIL is constitutively expressed at low levels on HaCaT cells. The addition of supernatants from *L. major*-infected PBMC cultures, but not from uninfected cultures, induced up-regulation of TRAIL. **b:** Supernatants obtained from five different donors were added to HaCaT cells. An up-regulation of Fas and TRAIL was noticed after exposure to all infected supernatants tested. **c:** sTRAIL was measured by ELISA in medium from HaCaT cells after incubation with infected supernatants. Although the levels of sTRAIL were higher in HaCaT cells exposed to infected supernatants, these levels were not higher than sTRAIL measured in the supernatants added to the cultures. **d:** Expression of TRAIL-R1 to -R4 was measured on HaCaT cells at baseline and after exposure to supernatants from control or *L. major*-infected PBMC cultures. Filled histograms represent staining with isotype control and open histograms the specific TRAIL-R expression. The histograms shown are representative of three experiments using different sets of supernatants. No significant alteration of TRAIL-R1 to -R4 was noted. **e:** TRAIL-R1 and -R2 is expressed on the cell membrane of HaCaT cells, whereas TRAIL-R3 is expressed both on the cell surface and in intracellular vesicles. TRAIL-R4 was predominantly expressed in cytoplasmatic vesicles.

tion of uninfected PBMC supernatants (Figure 3, a and b). However, both Fas and TRAIL were significantly and uniformly up-regulated on HaCaT cells on exposure to infected supernatants [Fas median MFI, 17 (IQR, 15 to 19) versus 12 (IQR, 8.6 to 13), $P = 0.01$ ($n = 5$); and TRAIL median MFI, 14 (IQR, 14 to 15) versus 10 (IQR, 9.6 to 11), $P = 0.01$ ($n = 5$); Figure 3, a and b].

Levels of Soluble TRAIL Are Not Increased in HaCaT Cultures after Exposure to Supernatants from L. major*-Infected PBMCs*

Increased levels of sTRAIL were measured in supernatants from HaCaT cells incubated with infected supernatants compared with HaCaT cells alone or incubated with control supernatants [median, 116 (IQR, 79 to 123), $n = 6$, versus 3 (IQR, 3.2 to 3.5), $n = 2$, and 38 (IQR, 31 to 39), $n = 6$]. However, when comparing to the levels of sTRAIL in the supernatants from *L. major*-infected PBMCs that were added to HaCaT cells 20 hours before the analysis [median, 115 pg/ml (90 to 173), $n = 6$], it seems that very low levels of sTRAIL, if at all, were secreted by HaCaT cells under these conditions (Figure 3c).

TRAIL-R1 to -R4 Expression on HaCaT Cells Is Not Significantly Altered on Exposure to Supernatants from L. major*-Infected PBMCs*

The expression of TRAIL-R1 to -R4 on the surface of HaCaT cells before and after exposure to supernatants was determined (Figure 3d). All four receptors were expressed on untreated HaCaT cells, and the expression pattern was not significantly altered after exposure to control supernatants or to *L. major*-infected supernatants. On untreated HaCaT cells, TRAIL-R1 and -R3 were predominantly expressed, but TRAIL-R2 was also weakly expressed (Figure 3d). TRAIL-R4 was present in intracellular vesicles in a majority of samples investigated and only weakly on the surface of these cells. The membrane expression of both TRAIL-R1 and -R2 suggested that HaCaT cells were sensitive to proapoptotic signaling through TRAIL under these culture conditions.

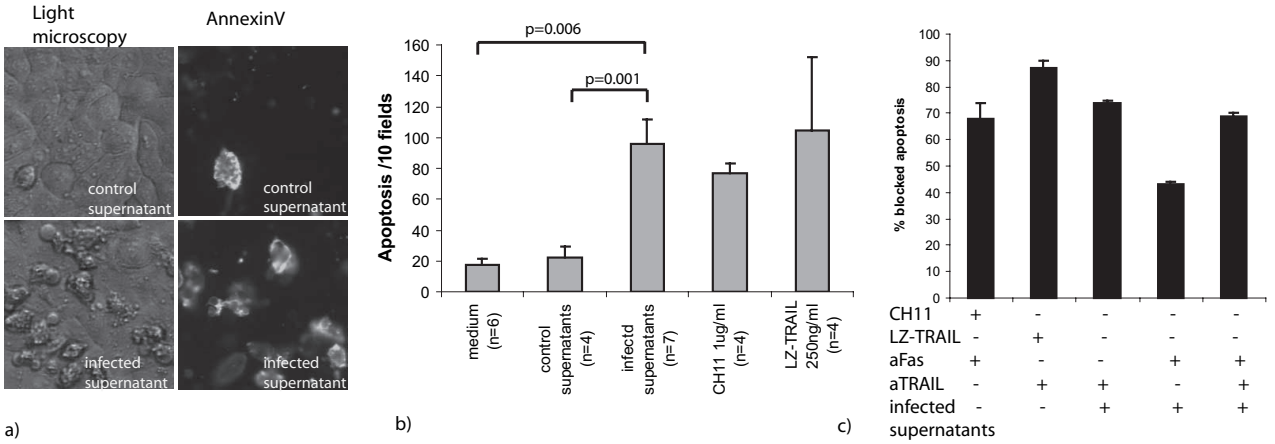


Figure 4. The apoptosis induced in HaCaT cells by supernatants from *L. major*-infected cultures can be blocked by the addition of neutralizing anti-Fas and anti-TRAIL antibodies. Apoptosis was quantified by Annexin V staining of HaCaT cell monolayers in chamber slides after 20 hours of incubation with supernatants from *L. major*-infected cultures or with Fas agonistic antibody (CH-11) or TRAIL. **a:** Phase-contrast photographs of HaCaT cells incubated with medium alone or supernatants from infected cultures. In white, Annexin V-positive cells (apoptotic). HaCaT cells incubated with supernatants from *L. major*-infected PBMC cultures (infected supernatants) show morphological signs of apoptosis after 18 hours incubation with an increased number of shrunk, detached cells, and Annexin V-positive, apoptotic cells. **b:** Both the Fas agonistic antibody CH-11 and recombinant, biologically active LZ-TRAIL induce apoptosis in HaCaT cells. HaCaT cells incubated with supernatants from infected cultures showed similar levels of apoptosis as HaCaT cells treated with LZ-TRAIL or CH11 and significantly higher levels of apoptosis compared with untreated cells ($P = 0.001$) and HaCaT cells treated with control supernatants ($P = 0.006$). **c:** Blocking of HaCaT cell apoptosis induced by CH-11 or LZ-TRAIL by the addition of TRAIL- or Fas-neutralizing antibodies. Duplicates from an experiment representative of three blocking experiment performed at different time points are shown. Original magnifications, ×40.

The Impact of Blocking Fas/FasL and TRAIL/TRAIL-R1 and -R2 Interactions on HaCaT Cell Apoptosis Induced by Supernatants from L. major*-Infected PBMCs*

We have previously reported that supernatants from *L. major* restimulated PBMCs from CL patients induce apoptosis in HaCaT cells.[1] We show here that supernatants from *L. major*-infected PBMCs induce apoptosis in HaCaT cells (Figure 4a), as did ultracentrifuged supernatants. CH-11, a Fas agonistic monoclonal antibody, induces apoptosis in HaCaT cells in a similar way to the supernatants from infected cultures (Figure 4b). Apoptosis induced by CH-11 could be blocked up to 70% by adding the blocking anti-Fas monoclonal antibody ZB4 (Figure 4c). When using supernatants from *in vitro*-infected PBMCs, blocking Fas only partly (40%) inhibited HaCaT cell apoptosis (Figure 4c). This picture was not ameliorated by using increasing concentration of anti-Fas or anti-FasL monoclonal antibodies up to 5 μg (results not shown). TRAIL-induced apoptosis (Figure 4b) was initiated in HaCaT cells by 250 ng of LZ-TRAIL, and up to 90% of apoptosis could be blocked by adding the TRAIL-neutralizing antibody 2E5 at 2.5 μg/ml (Figure 4c). Adding 2E5 to supernatants from *L. major*-infected PBMCs before incubation with HaCaT cells blocked up to 75% of apoptosis (Figure 5c). A similar level of apoptosis blocking was obtained by sTRAIL-R2 (results not shown). No additive or synergistic effect of the combination of monoclonal antibodies against FasL and TRAIL was noted (Figure 4c).

Expression of Fas, TRAIL, and TRAIL-R1 to -R4 on HEK Cells

The immortalized cell-line HaCaT differs from primary keratinocytes in the expression of several different apoptotic mediators.[22,24–26] Thus, HEK cells were exposed to supernatants from *L. major-infected* PBMCs, and the membrane expression of Fas and TRAIL and TRAIL-R1 to -R4 were analyzed. Fas was uniformly expressed to a higher degree by HEK cells compared with HaCaT cells [median MFI, 26 (IQR, 21 to 41), $n = 3$, versus 10 (IQR, 9 to 11), $n = 6$], and no further up-regulation of Fas was noted after incubation with infected supernatants. In contrast, TRAIL was expressed uniformly at similar levels on HEK cells compared with HaCaT cells [median MFI, 9 (IQR, 8 to 24), $n = 3$, versus 12 (IQR, 8 to 14), $n = 6$] (Figures 3 and 5) and was up-regulated on incubation with infected supernatants (Figure 5a). TRAIL-R1 to -R4 were expressed by HEK cells, and TRAIL-R1 and -R2 were partially down-regulated on exposure to infected supernatants (Figure 5a).

Induction of Apoptosis in HEK Cells

Despite the high surface expression of Fas, HEK cells were more resistant to Fas-induced apoptosis compared with HaCaT cells (Figures 3 and 5b); on the contrary, and despite the low expression of TRAIL on HEK cells, HEK cells were more susceptible to TRAIL-induced apoptosis compared with HaCaT. Supernatants from *L. major*-infected PBMC cultures induced apoptosis in HEK cells, albeit to a lower degree compared with HaCaT cells [mean, 64 (SD 14, $n = 7$) versus 96 (SD 15, $n = 7$)

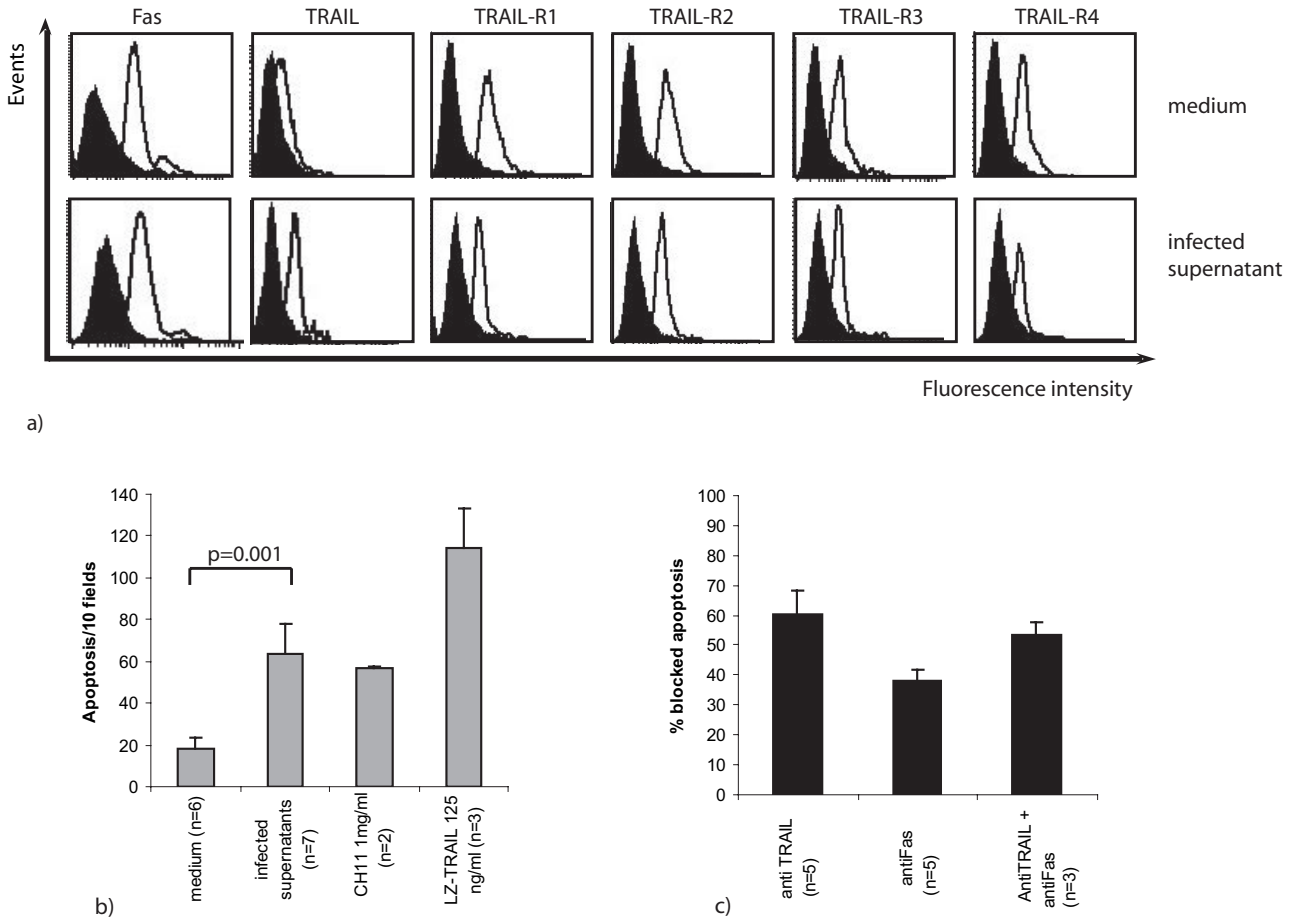


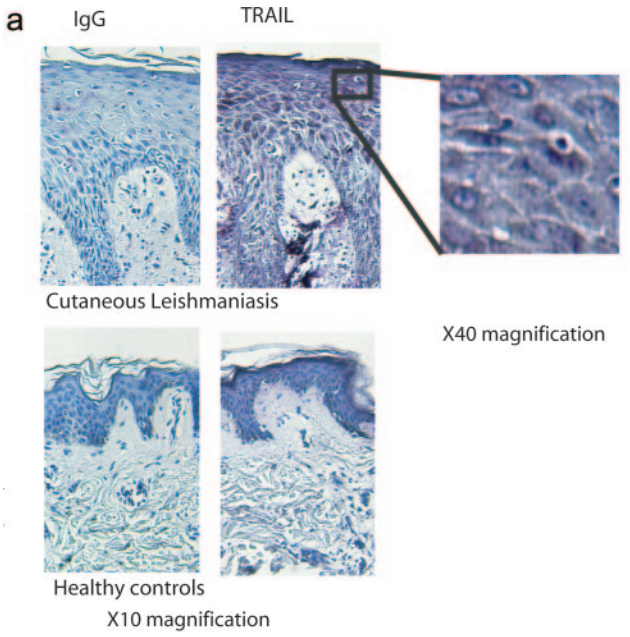
Figure 5. Supernatants from *L. major-infected* PBMC cultures induce up-regulation of TRAIL and apoptosis in primary keratinocytes. HEK cells were incubated with supernatants from *L. major*-infected or control PBMC cultures for 20 hours and membrane expression of Fas and TRAIL was analyzed by flow cytometry and apoptosis quantified by Annexin V staining. **a:** Fas was constitutively expressed on HEK cells, and the expression of this protein was not altered on incubation with infected supernatants. TRAIL is constitutively expressed at low level on HEK cells. The addition of supernatants from *L. major*-infected PBMC cultures induced up-regulation of TRAIL. Filled histograms represent staining with isotype control and open histograms the specific Fas or TRAIL expression. The histograms shown are representative of three independent experiments. **b:** Both Fas agonistic antibody CH-11 and recombinant, biologically active LZ-TRAIL induce apoptosis in HEK cells, at levels inferior to apoptosis induced in HaCaT cells. HEK cells incubated with supernatants from infected cultures showed similar levels of apoptosis as keratinocytes treated with CH-11 and significantly higher levels of apoptosis compared with untreated cells ($P = 0.001$). **c:** Partial blocking of HEK cell apoptosis induced by infected supernatants was obtained by the addition of TRAIL or Fas neutralizing antibodies. Data obtained from five experiments performed in duplicates are shown.

apoptotic cells per 10 fields]. Partial blocking was obtained by TRAIL-blocking (60%, SD 8, $n = 5$) or Fas-blocking antibodies (38%, SD 4, $n = 5$), but again, no additive or synergistic effect was noted in HEK cells as already reported for HaCaT.

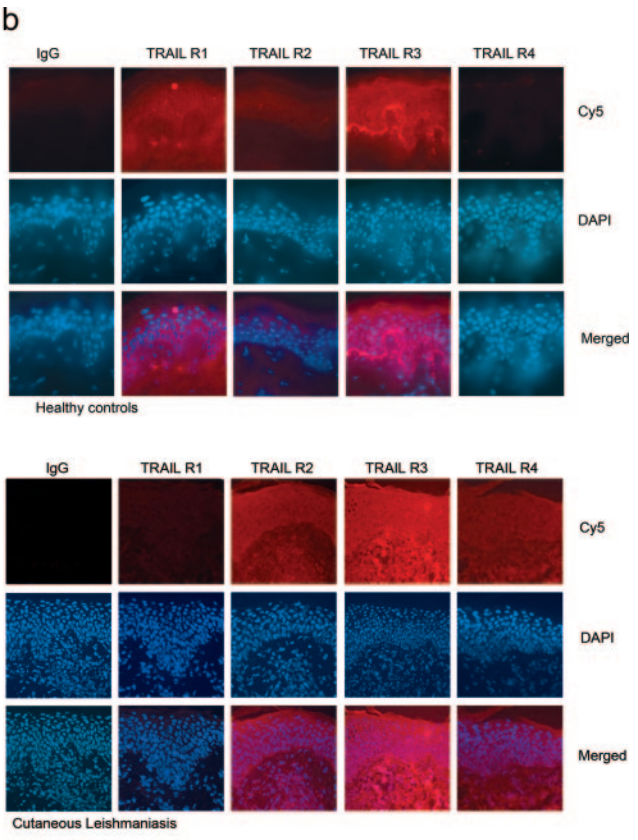
TRAIL, Fas, and TRAIL-R1 to -R4 Are Expressed in Epidermis Surrounding Ulcers Caused by L. major *Infection*

Previously, we have shown up-regulation of Fas on keratinocytes and an increased number of apoptotic keratinocytes during CL. The biopsies investigated in this study have morphologically been designated as active, active-healing, or healing.[1] In epidermis surrounding CL lesions, TRAIL and Fas were both expressed in samples designated as active and healing (Figure 6a). The expression of TRAIL was less homogenous in epidermis compared with Fas expression, with some cells very high in TRAIL expression (Figure 6a). Less TRAIL was expressed in skin biopsies from healthy controls compared with the TRAIL expression in epidermis during CL (Figure 6a).

Although both Fas and TRAIL expression were increased on CL skin compared with healthy controls, the expression did not follow the same pattern in the individual samples investigated (Figure 6a). TRAIL-R1 to -R4 were visualized in four biopsies from CL cases and in three healthy control biopsies. In the CL skin biopsies, TRAIL-R1 was not expressed adjacent to *L. major*-caused skin ulcers (Figure 6b). However, more TRAIL-R2 was expressed in CL specimens than in healthy epidermis in which a few TRAIL-R1- and -R2-expressing cells were noted. TRAIL-R3 was abundantly expressed in superficial epidermis and around the basal layer. TRAIL-R4 was weakly expressed throughout the epidermis in CL biopsies but not expressed in healthy skin.



Sample	Morphology	TRAIL	Fas
CL11	Active	++	++
CL31	Active	+++	+++
CL30	Active	+++	++
CL9	Active	+++	+
CL25	Active-healing	+	++
CL26	Active-healing	+++	+
CL1	Healing	++	+
CL8	Healing	++	+
Control1	Healthy skin	+	+
Control2	Healthy skin	++	+
Control3	Scar	+	+



Sample	IgG	TRAIL-R1	TRAIL-R2	TRAIL-R3	TRAIL-R4
CL11	-	-	+	+++	++
CL31	-	-	++	+++	+
CL9	-	-	+	+++	-
CL26	-	-	++	+++	++
Control1	-	+	(+)	+++	-
Control2	-	++	(+)	+++	-
Control3	-	+ (+)	+	+++	-

Figure 6. TRAIL, TRAIL-R1 to -R4, and Fas are expressed in epidermis surrounding *L. major*-caused ulcers. Skin biopsies from the borders of ulcers caused by *L. major* were used to visualize the expression of TRAIL, Fas, and TRAIL R1 to R4 during CL. TRAIL expression was photographed at ×25 and ×40 magnification. TRAIL-R1 to -R4 were photographed at ×25 magnification. All sections were assessed blindly, and all analyses were performed independently from each other and from morphological assessment. **a:** TRAIL was visualized with VIP staining (purple) on formalin-fixed and paraffin-embedded skin tissue. TRAIL and Fas were present on epidermis from CL cases and from healthy controls. **b:** TRAIL-R1 to -R4 were visualized with Cy3 staining (red) on frozen biopsies from CL cases and counterstained with DAPI (blue). The expression of TRAIL-R1 to -R4 was evaluated using an arbitrary scale ranging from − (no fluorescence) to +++ (very strong expression). In some cases there was very weak expression of TRAIL-R2, and these were designated (+).

Discussion

The mechanism underlying ulceration during *L. major*-induced CL remains unclear. We have proposed a role for receptor-mediated apoptosis of keratinocytes following the observation that Fas and FasL are up-regulated at the site of infection in concomitance to the presence of apoptotic keratinocytes.[1] Apoptosis can be induced through extracellular signals in cells that express one or several of the eight death receptors known to date, namely Fas, TRAIL-R1, TRAIL-R2, TNF-R1, DR-3, DR-6, EDA-R, and NGF-R.[4] The immortalized human keratinocyte cell line investigated in this study, HaCaT, has been shown to constitutively express the membrane-bound death receptors Fas and TRAIL-R1 and -R2 as well as the decoy receptor -R4.[34] However, HaCaT cells differ from primary keratinocytes in apoptotic behavior, partly because of reduced expression of p53.[35] By mapping alterations of apoptosis-related gene expression in HaCaT cells exposed to soluble inflammatory mediators secreted during *in vitro L. major* infection, alterations of mRNA levels of Fas, TRAIL, and TRAIL-R2 were detected. The mRNA levels of caspase 8 and ripk-2 were transiently up-regulated in keratinocytes exposed to supernatant from *L. major*-infected PBMC cultures. Caspase 8 is involved in apoptotic signaling through both Fas and TRAIL-R1 and -R2. The receptor-interacting protein kinases (ripk) are involved in signaling of extracellular stress transmitted through a number of membrane-bound receptors, including Fas.[36] Ripk 1 to 7 have a broad, and still not completely characterized, role in between innate and adaptive immune responses, leading to the activation of factors such as NF-κB, proinflammatory signals such as IFN-γ, and apoptotic pathways.

The alterations of Fas and TRAIL mRNAs detected by gene profiles were verified through the expression of membrane-bound proteins and in blocking experiments on HaCaT and HEK cells. Apoptosis was induced in both HaCaT and HEK cells on exposure to infected supernatants. Interestingly, keratinocyte apoptosis was more efficiently blocked by neutralizing TRAIL antibodies as compared with Fas-blocking antibodies. Furthermore, TRAIL and TRAIL-R2 were detected in skin biopsy sections from CL cases.

In many contexts it has been shown that the Fas/FasL pathway is involved in homeostasis of immune responses and that the Fas/FasL pathway is altered during several different inflammatory and infectious diseases. On the other hand, the TRAIL/TRAIL-R1 to -R2 apoptotic pathway has been suggested to be mainly proapoptotic in malignant cells.[37] *In vivo* experiments in which animals have received sTRAIL have not led to induction of apoptosis in liver and thymus,[38] whereas systemic treatment with Fas-agonistic agents leads to liver failure and death.[39] Thus, great hope has been laid on cancer treatment with TRAIL agonistic agents and clinical trials testing such drugs are being performed.[40] This preferential involvement of TRAIL in apoptosis of malignant cells has recently been challenged by several reports showing that TRAIL can induce apoptosis in nonmalignant cells such as hepatocytes[41] and primary keratinocytes.[21] In addition, TRAIL was shown to be expressed on T cells (both CD4 and CD8) infiltrating the skin and in circulation during atopic dermatitis.[42]

It has been suggested that in the presence of IFN-γ, the proapoptotic activity of TRAIL is greatly enhanced in nonmalignant keratinocytes.[23] IFN-γ or TRAIL alone induced apoptosis in human keratinocytes, but a strong synergistic effect in the ability to induce keratinocyte death was noted in the presence of both IFN-γ and TRAIL.[23] In the case of CL, in which local accumulation of infected macrophages in the dermis leads to inflammation, T-cell infiltration, and IFN-γ secretion, keratinocytes activated by the inflammatory microenvironment may become susceptible not only to FasL- but also to TRAIL-mediated apoptosis. In the present study, although IFN-γ-blocking antibodies reduced HEK cell apoptosis up to 30%, this reagent did not synergize the blocking ability of TRAIL blocking antibody *in vitro* (results not shown).

The aim with the visualization of TRAIL receptors in this study was to determine whether TRAIL could affect apoptosis of keratinocytes under the conditions used, and proapoptotic receptors were visualized in HaCaT cells, HEK cells, and skin biopsies. HaCaT and HEK cells down-regulated TRAIL-R1 and -R2 on incubation with infected supernatants. A possible explanation for this *in vitro* finding is that the keratinocytes remaining in the cultures after incubation with infected supernatants (and induction of apoptosis) were low in TRAIL-R1 and -R2 expression. TRAIL-R1 was down-regulated, whereas TRAIL-R2 and Fas were up-regulated in skin biopsies from CL patients. The two nonapoptotic receptors TRAIL-R3 and -4 were also present in the skin biopsies. The ligands show a different expression pattern, where FasL is expressed on infiltrating inflammatory cells such as macrophages and T cells,[1] and TRAIL is expressed in keratinocytes *in vitro* and also in epidermis in skin biopsies from CL.

Several different scenarios can be envisaged from the fact that two receptor-mediated apoptotic pathways are up-regulated in the skin during CL. The Fas/FasL pathway, which is activated during immune activation, is important to contract and terminate immune responses. In this regard, FasL killing of keratinocytes by activated infiltrating cells may be accidental because of the fact that infected macrophages are found in the proximity of epidermis. However, Fas up-regulation is considered as a sign of activation of cells, and keratinocytes adjacent to *Leishmania*-infected macrophages show increased expression of HLA-DR and ICAM-1 as further proof of their activated state.[43,44] Activated keratinocytes are capable of secreting high levels of chemoattractants and cytokines, and thus FasL killing of keratinocytes may be important to dampen local immune activation. On the other hand, FasL signaling may lead to inflammatory responses in keratinocytes resistant to FasL-mediated apoptosis as described during psoriasis.[12] Blocking of FasL during *L. major* infection of murine macrophages led to blockade of MIP1α secretion by infected macrophages, suggesting a proinflammatory role of FasL signaling in these cells.[45] It is possible that FasL acts in a similar manner on keratinocytes during CL.

TRAIL is expressed on normal keratinocytes in combination with TRAIL-R1 and high levels of TRAIL-R3. During CL both TRAIL and TRAIL-R2 were up-regulated, and it was suggested that signaling through TRAIL-R2 is more efficient in apoptosis induction compared with TRAIL-R1 signaling.[46] Activated keratinocytes could up-regulate the expression of both the ligand and its proapoptotic receptor (TRAIL-R2), again to dampen local inflammatory responses. On the other hand, TRAIL-R4 signaling has been suggested to lead to activation of NF-κB and inflammatory responses. Thus the expression of TRAIL receptors during the course of an inflammatory response may influence the fate of the keratinocyte, in that early during the response a majority of cells will express TRAIL-R3 to -R4 and later switch receptor expression to the proapoptotic TRAIL-R1 to -R2.

Neither FasL nor TRAIL could be shown to be completely responsible for the apoptosis measured in the *in vitro* experiments shown in this manuscript. No additive or synergistic effects were noted when blocking both FasL and TRAIL in the presence or absence of IFN-γ blocking antibodies. *L. major* infection in macrophages leads to the induction of a variety of cytokines in infected cells and by standard immune cells. It is likely that this complex cytokine mixture, containing for example TNF-α, TGF-β, IFN-γ, IL-4, IL-12, and IL-10 is cytotoxic and may affect apoptosis of keratinocytes. However, we present clear evidence for the role of Fas and TRAIL pathways for keratinocyte apoptosis during CL.

We here report that TRAIL and TRAIL-R2 mRNA expression is up-regulated in HaCaT cells on exposure to supernatants from *L. major*-infected PBMCs. In the case of TRAIL, the altered gene expression was validated by increased protein expression of this ligand on keratinocytes and through blocking experiments. To elucidate

further what effect the expression of TRAIL and FasL (and respective receptors) has on skin pathogenesis during *L. major*-induced CL, kinetic studies should be performed in animal models, following the pattern of receptor expression during different phases of the infection. Inhibition of TRAIL could also clarify the role of this ligand during CL; however, because of the local production of TRAIL in the skin, such studies would probably have to be performed in knock-out models of TRAIL or its receptors.

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