Increased Expression and a Potential Anti-Inflammatory Role of TRAIL in Atopic Dermatitis

Ekatherina Vassina,* Martin Leverkus,† Shida Yousefi,* Lasse R. Braathen,‡ Hans-Uwe Simon,* and Dagmar Simon‡

*Department of Pharmacology, University of Bern, Bern, Switzerland; †Laboratory of Experimental Dermatology, Department of Dermatology, Otto-von-Guericke University Magdeburg, Magdeburg, Germany; ‡Department of Dermatology, University of Bern, Bern, Switzerland

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis of many transformed but also of non-transformed cells. In addition, TRAIL receptor activation has been reported to activate non-apoptotic signaling pathways. Here, we report an increased expression of TRAIL in peripheral blood T cells and monocytes from patients with atopic dermatitis (AD) compared with control individuals. High TRAIL expression was also observed in skin-infiltrating T cells of AD patients. Topical tacrolimus treatment reduced the total number of T cells in the skin, but the relative proportion of TRAIL-positive cells within both CD4+ and CD8+ cell populations did not change. TRAIL was demonstrated to induce the expression of interleukin-1 receptor antagonist (IL-1Ra) in keratinocytes in a caspase-independent manner *in vitro*. Moreover, increased expression of IL-1Ra was observed in keratinocytes of AD lesional skin. These data suggest that TRAIL-expressing inflammatory skin cells may contribute to the epidermal activation of the IL-1Ra gene in AD.

Key words: atopic dermatitis/interleukin-1 receptor antagonist/keratinocytes/monocytes/T cells/TRAIL
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Atopic dermatitis (AD) is characterized by chronic or relapsing eczematous lesions with a typical morphology and distribution, as well as by pruritus (Leung and Bieber, 2003). In the dermis of AD lesions, there is a marked perivascular T cell infiltrate (Braathen *et al*, 1979; Leung *et al*, 1983), in which both CD4+ and CD8+ T cells are present (Akdis *et al*, 1999). The infiltrating T cells express pro-inflammatory cytokines, in particular, interleukin (IL)-5 and IL-13 (Hamid *et al*, 1994, 1996). In chronic lesions, IFN-γ-producing cells have also been described (Grewe *et al*, 1994; Trautmann *et al*, 2000), despite a reduced capacity of peripheral blood mononuclear cells (PBMC) to generate this cytokine under *in vitro* conditions (Simon *et al*, 2002, 2003). Because T cells are believed to play a pivotal role in the pathogenesis of AD, they are considered as primary targets of current anti-inflammatory treatment strategies in this disease (Tomi and Luger, 2003; Simon *et al*, 2004).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor (TNF) superfamily, and has been implicated in the regulation of various physiological and pathological immune responses (Kimberley and Screaton, 2004). This might, at least partially, be because of its wide expression among cells of the immune system, including activated T cells (Mariani and Krammer, 1998; Thomas and Hersey, 1998; Kayagaki *et al*, 1999a; Mirandola *et al*, 2004), B cells (Mariani and Krammer, 1998; Kemp *et al*, 2004), monocytes (Griffith *et al*, 1999; Halaas *et al*, 2004; Tecchio *et al*, 2004), dendritic cells (Fanger *et al*, 1999), natural killer cells (Zamai *et al*, 1998; Kayagaki *et al*, 1999b; Mirandola *et al*, 2004), and neutrophils (Renshaw *et al*, 2003; Matsuyama *et al*, 2004; Tecchio *et al*, 2004). Most of these studies, however, were performed *in vitro* and little is known about TRAIL expression under *in vivo* conditions.

TRAIL originally received considerable attention following the observation that it selectively induced apoptosis in cancer but not in normal cells (Ashkenazi *et al*, 1999; Walczak *et al*, 1999). Therefore, TRAIL is considered as a promising future drug for cancer patients. But recent work suggests that TRAIL also has several physiological functions that are not limited to the killing of transformed cells. For instance, TRAIL has been shown to induce apoptosis in several primary cells, such as hepatocytes (Jo *et al*, 2000; Zheng *et al*, 2004), HIV-activated T cells (Miura *et al*, 2001), plasma cells (Ursini-Siegel *et al*, 2002), immature dendritic cells (Leverkus *et al*, 2000), and neutrophils (Renshaw *et al*, 2003; Matsuyama *et al*, 2004). Moreover, TRAIL has been shown to activate a caspase-independent signaling pathway leading to the activation of nuclear factor-κB (NF-κB) (Ehrhardt *et al*, 2003; Leverkus *et al*, 2003a).

The IL-1 family consists of five members, the pro-inflammatory cytokine IL-1 (IL-1α and IL-1β), two IL-1 receptors, and the IL-1 receptor antagonist (IL-1Ra). Whereas IL-1 exerts a broad spectrum of functional activities, IL-1Ra fails to activate cells after binding to the IL-1 receptor (Arend, 2002). The ratio between IL-1 and IL-1Ra seems to be crucial for the intensity of the inflammatory response in

Abbreviations: AD, atopic dermatitis; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

Table I. Relative surface expression of TRAIL on blood cells as assessed by flow cytometry

Cell type	Controls	AD patients	p
Monocytes	1.24 ± 0.09 (9)[a]	4.59 ± 1.45 (9)	0.0003
CD8+ T cells	1.37 ± 0.23 (5)	10.04 ± 5.27 (5)	0.0079
CD4+ T cells	1.02 ± 0.01 (5)	6.22 ± 1.77 (5)	0.0079
Eosinophils	1.07 ± 0.06 (6)	1.44 ± 0.16 (5)	0.0556
Neutrophils	1.03 ± 0.02 (6)	2.44 ± 0.47 (5)	0.0159

[a]Data represent the mean ratios (± SEM) of mean fluorescence intensities (anti-TRAIL mAb per control mAb) of (n) independent experiments. Representative original data are shown in Fig 1.
TRAIL, the tumor necrosis factor-related apoptosis-inducing ligand; AD, atopic dermatitis; mAb, monoclonal antibody.

many diseases. To lower this ratio, therapeutic trials with IL-1Ra have been carried out (Dinarello, 2003).

Here, we report a dramatically increased expression of TRAIL by several immune cells, in particular, CD4+ and CD8+ blood and skin T cells, in AD. The increased expression of TRAIL by immune cells was associated with increased expression of IL-1Ra by keratinocytes. A potential link between these two phenomena was shown by the observation that TRAIL-induced IL-1Ra gene expression in keratinocytes *in vitro*. Therefore, T cells may, besides their pro-inflammatory activities, also exert anti-inflammatory effects in AD.

Results

Leukocytes of AD patients express increased levels of TRAIL We examined the surface expression of TRAIL by flow cytometry using a specific anti-TRAIL monoclonal antibody (mAb). CD4+ and CD8+ T cells as well as CD14+ monocytes were identified using mAb against the corresponding lineage-associated molecules within PBMC populations. Neutrophils and eosinophils were analyzed after isolation. TRAIL expression in both CD4+ and CD8+ T cells as well as in monocytes was significantly higher in AD patients compared with normal controls (Table I), which usually demonstrated little TRAIL expression (Fig 1). In particular, CD8+ T cells expressed large amounts of TRAIL in AD. Interestingly, strong TRAIL expression was also often

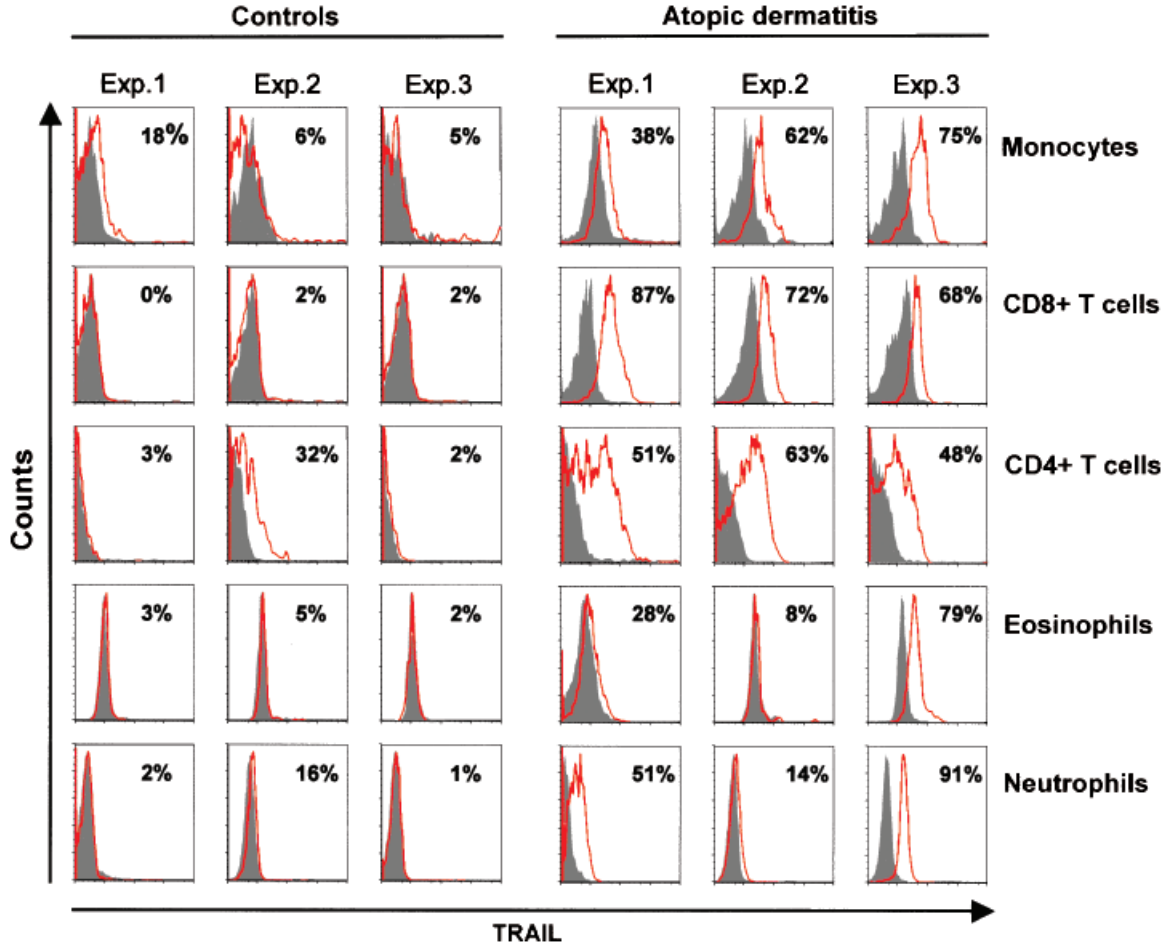


Figure 1
Expression of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in different subsets of blood cells. Cells were isolated from atopic dermatitis patients and normal donors. Monocytes, CD8+ cells, and CD4+ cells were analyzed within PBMC and identified using anti-CD14, anti-CD8, and anti-CD4 monoclonal antibody (mAb), respectively. TRAIL expression on neutrophils and eosinophils was analyzed using purified cell populations. TRAIL expression was measured by flow cytometry using mouse anti-TRAIL mAb (*red*). Control IgG1 mAb staining is shown in gray. Three representative and independent experiments for each cell type. Statistical analysis of these experiments is provided in Table I.

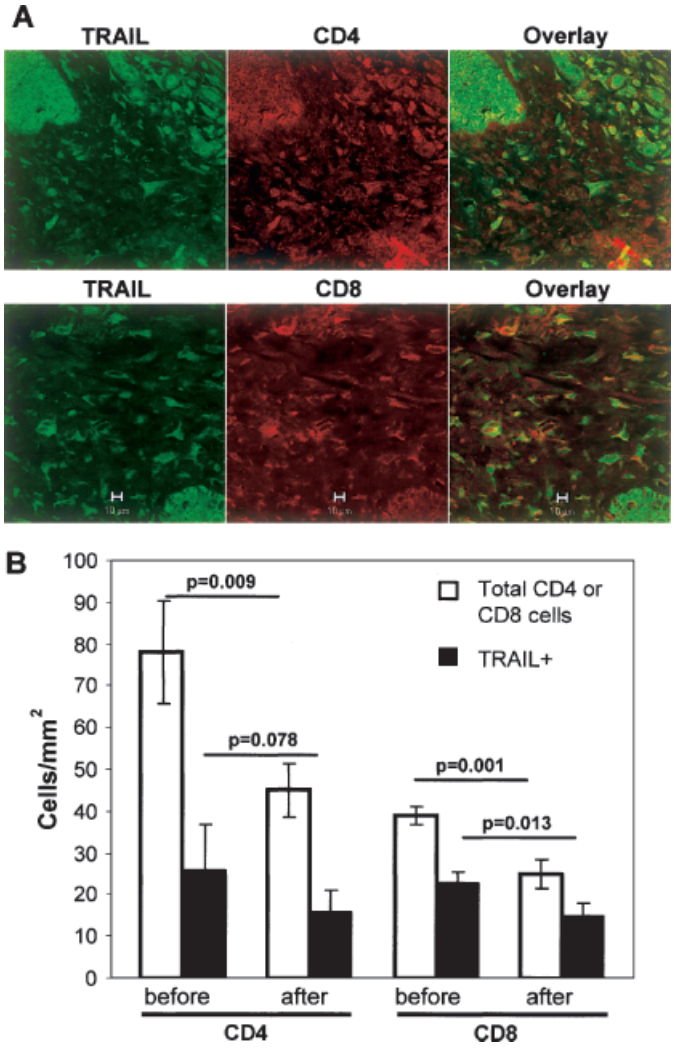


Figure 2
Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression in atopic dermatitis (AD). (*A*) Representative double immunofluorescence stainings of skin biopsy specimens from AD patients using CD4+, CD8+, and TRAIL mAb. *White bars*, 10 μM. (*B*) Bars indicate mean numbers of total and TRAIL-positive CD4+ and CD8+ cells of 10 patients in the dermal infiltrate of lesional skin before and after treatment with topical tacrolimus.

observed in eosinophils and neutrophils from AD patients, but only exceptionally in control individuals.

Dermal-infiltrating T cells express TRAIL in AD To investigate whether T cells express TRAIL under *in vivo* conditions, we examined biopsies from lesional skin of AD patients. As assessed by double immunofluorescence staining and confocal microscopy, we observed TRAIL expression in both CD4+ and CD8+ cells. As observed in blood T cells, the proportion of TRAIL-expressing cells was higher in CD8+ (mean: 60%) compared with CD4+ (mean: 33%) cells. Therefore, although less CD8+ than CD4+ cells infiltrated into the skin, the absolute numbers of TRAIL-expressing cells were approximately the same in both T cell subgroups (Fig 2). After topical treatment with tacrolimus 0.1% ointment, the numbers of infiltrating T cells significantly declined; however, the proportion of TRAIL-expressing cells in both CD4+ and CD8+ cells did not change (Fig 2). In contrast, other cytokines such as IL-5 and IL-13 were reduced as a consequence of this therapy (Simon *et al*, 2004).

The epidermis of AD lesions exhibits increased IL-1Ra expression Previously published work suggested that keratinocytes express IL-1Ra (Leverkus *et al*, 2003a). We compared the expression of IL-1Ra by keratinocytes in skin biopsies from AD patients and normal controls by immunofluorescence. The expression of IL-1Ra in the epidermis of AD lesions was much stronger compared with tacrolimus-treated AD skin and normal skin (Fig 3). The staining was localized intracellularly. Whereas in lesional skin, positive cells were distributed throughout the epidermis including the basal layer, in normal skin the positive cells were situated above the basal layer.

TRAIL induces IL-1Ra production in keratinocytes *in vitro* As TRAIL–TRAIL receptor interactions have recently been implicated as a negative regulatory mechanism in immune responses (Diehl *et al*, 2004), we analyzed IL-1Ra expression in HaCaT keratinocytes following stimulation with TRAIL. TRAIL-mediated IL-1Ra gene induction was analyzed by determining mRNA levels at different time points following TRAIL stimulation using multiprobe ribonuclease protection assays. We detected a 2–3-fold induction of IL-1Ra (Fig 4*A*), whereas the steady-state mRNA levels of IL-1α, IL-1β, or MCP-1 remained unchanged (data not shown), confirming our previous findings (Leverkus *et al*, 2003a). The pan-caspase inhibitor zVAD had no effect in this system, indicating that TRAIL-mediated transcriptional activation of IL-1Ra is caspase independent. To determine whether the expression of IL-1Ra mRNA correlates with protein expression, we also analyzed IL-1Ra protein expression in supernatants of HaCaT keratinocytes as well as primary keratinocytes by using an immunoassay. A 3–10-fold induction of IL-1Ra protein was found following TRAIL stimulation of these cells for 24 h (Fig 4*B* and *C*). In order to verify TRAIL-specific induction of IL-1Ra, the same experiments were performed in the presence of 10 μg per mL of TRAIL-R2-Fc (to block TRAIL) or control IgG as described (Leverkus *et al*, 2003a). Preincubation with TRAIL-R2-Fc fully abrogated TRAIL-induced IL-1Ra induction in both HaCaT and primary keratinocytes. Furthermore, additional control experiments were performed with TNF-R2-Fc, which was ineffective in blocking TRAIL-induced IL-1Ra (data not shown). Taken together, these data suggest that TRAIL induces IL-1Ra expression in human keratinocytes in a caspase-independent manner.

Discussion

Because of the crucial role of IL-1Ra in the prevention of massive tissue damage during inflammatory responses, its regulation has been investigated by many groups under both physiological and pathophysiological conditions. IL-1Ra is found in large amounts in the blood during inflammation and it is primarily generated in the liver (Arend, 2002). Many other cells, however, including monocytes, macrophages, and neutrophils produce IL-1Ra (Malyak *et al*, 1998). Moreover, structural cells such as epithelial

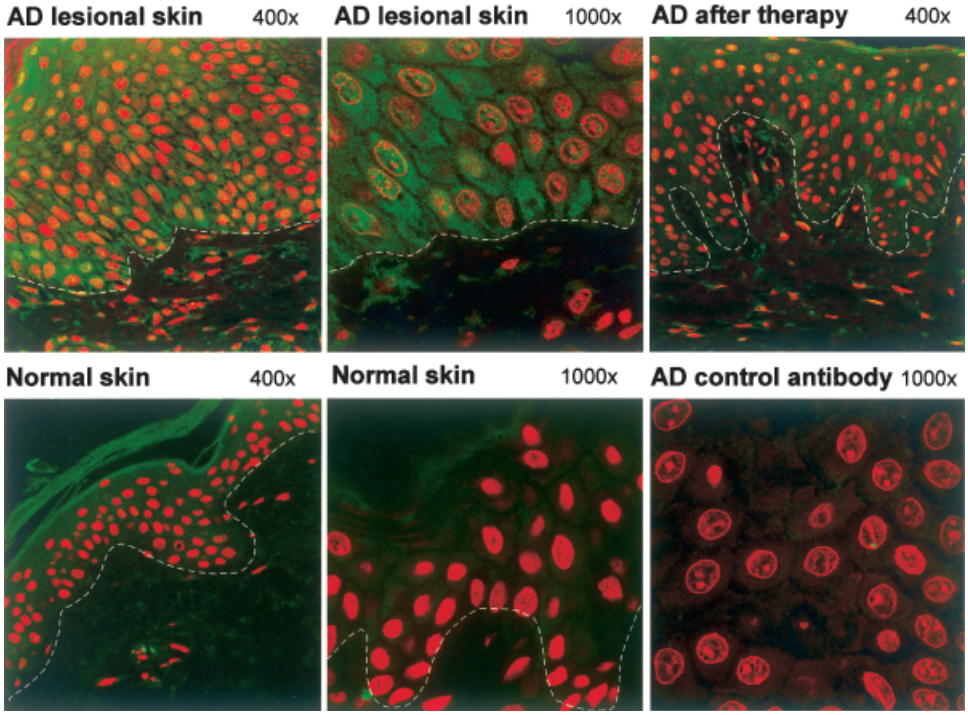
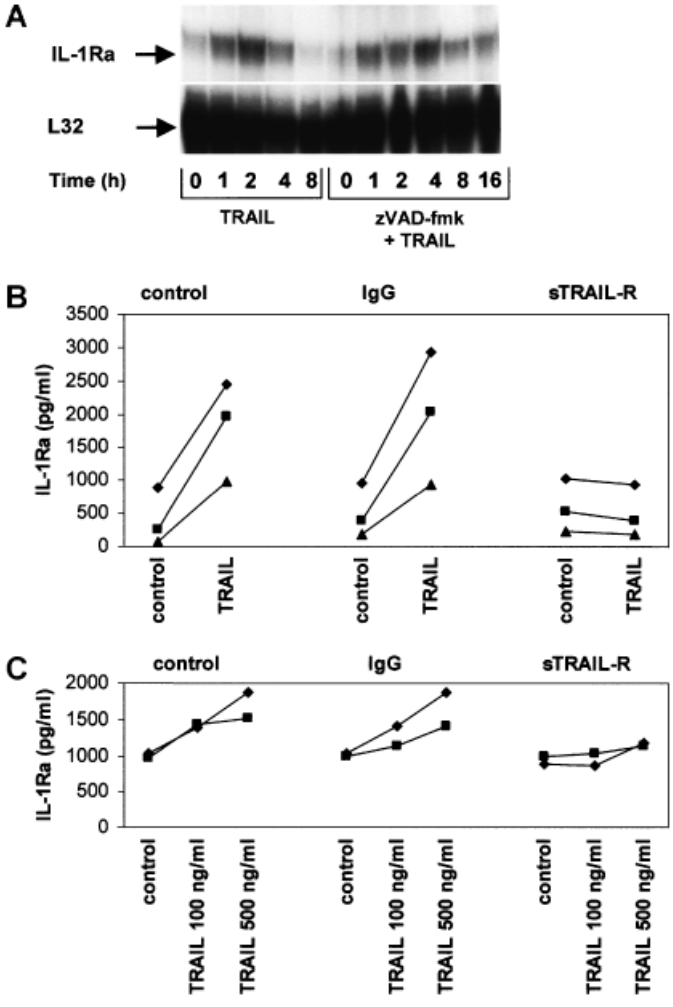


Figure 3
Representative immunofluorescence stainings of interleukin-1 receptor antagonist (IL-1Ra) in the skin. Staining with an anti-IL-1RA Ab reveals strong positive cells throughout the epidermis of lesional AD skin. Nuclei were counterstained with propidium iodide. After treatment, few keratinocytes in the suprabasal layers stained weakly positive. Weak staining was also observed in normal skin. The white dashed line indicates the border between the epidermis and the dermis. The original magnification is indicated in each panel.



cells, fibroblasts, and endothelial cells are also IL-1Ra producers (Haskill *et al*, 1991; Corradi *et al*, 1995).

In this study, we obtained evidence that keratinocytes express IL-1Ra under physiological conditions. This confirms previous work, suggesting IL-1 and IL-1Ra expression by keratinocytes (Leverkus *et al*, 2003a). In psoriasis, IL-1Ra expression by keratinocytes was elevated, resulting in an increased IL-Ra/IL-1 ratio (Hammerberg *et al*, 1992; Kristensen *et al*, 1992; Debets *et al*, 1997). Here we report an increased expression of IL-1Ra in the epidermis of AD patients, suggesting that the transcriptional activation of this anti-inflammatory gene is a general phenomenon in inflamed skin independent of the underlying pathomechanism. IL-1Ra may limit the inflammatory response in the skin and thereby skin damage.

The regulation of IL-1Ra gene expression in keratinocytes is unclear. It is possible that IL-1 induces IL-1Ra

Figure 4
Interleukin-1 receptor antagonist (IL-1Ra) expression following tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) stimulation of keratinocytes. (*A*) Following preincubation with either diluent alone or 40 μM zVAD-fmk for 1 h, HaCaT cells were treated with recombinant TRAIL (500 ng per mL) for the indicated times. Multiprobe RPA (RiboQuant) were performed using 5 μg of total RNA. Steady-state levels for IL-1Ra were determined by normalizing against the expression of the L32 housekeeping gene. (*B*) TRAIL-induced IL-1Ra secretion in HaCaT cells. Cells were preincubated with either diluent alone (control) in the presence or absence of 10 μg per mL of TRAIL-R2-Fc or control IgG for 1 h, and subsequently treated with 500 ng per mL recombinant TRAIL for 24 h. Cell-free supernatants were collected and assayed for IL-1Ra by ELISA. (*C*) TRAIL-induced IL-1Ra secretion in primary keratinocytes. Cells were preincubated with either diluent alone (control) in the presence or absence of 10 μg per mL of TRAIL-R2-Fc or control IgG for 1 h, and subsequently treated with the indicated concentrations of recombinant TRAIL for 24 h. Cell-free supernatants were collected and assayed for IL-1Ra by ELISA. Two independent experiments are shown.

expression in these cells as it does in other cell types (La and Fischer, 2001). To increase the IL-1Ra/IL-1 ratio, however, additional factors are probably involved. One such factor could be TRAIL, which, as demonstrated in this study, transcriptionally activated the IL-1Ra but not the IL-1 gene. It is possible that IL-1Ra gene induction involves the activation of NF-κB (Leverkus *et al*, 2003a).

TRAIL has been shown to be critical in the regulation of the immune response as it induced apoptosis in dendritic cells, regulating adaptive immune response (Fanger *et al*, 1999). The fact that TRAIL mediates apoptosis in neutrophils (Renshaw *et al*, 2003; Matsuyama *et al*, 2004; Tecchio *et al*, 2004) suggests that it also controls innate immune responses. Moreover, TRAIL-deficient mice showed increased susceptibility to experimentally induced autoimmune arthritis (Lamhamedi-Cherradi *et al*, 2003b) and diabetes (Lamhamedi-Cherradi *et al*, 2003a). On the other hand, TRAIL application improved clinical signs of experimental arthritis (Liu *et al*, 2003) and autoimmune encephalitis (Hilliard *et al*, 2001). Furthermore, *in vitro* activated PBMC from AD patients with poor responses to corticosteroids demonstrated low TRAIL levels compared with AD patients with good clinical efficacy (Heishi *et al*, 2002). Altogether, these data point to the possibility that TRAIL exerts anti-inflammatory activities, which may include the induction of apoptosis in inflammatory cells (Jo *et al*, 2000; Leverkus *et al*, 2000; Miura *et al*, 2001; Ursini-Siegel *et al*, 2002; Renshaw *et al*, 2003; Matsuyama *et al*, 2004; Zheng *et al*, 2004), blocking of the cell cycle (Song *et al*, 2000), activation of inhibitory phosphatases (Daigle *et al*, 2002), and, perhaps, as this report suggests, the inducible expression of IL-1Ra.

In spite of the recent progress in the field, the physiological role of TRAIL remains to be determined. In this study, we demonstrate increased TRAIL expression by several inflammatory cells under *ex vivo* and *in vivo* conditions in AD. To our knowledge, there are no previous reports on the expression of TRAIL under inflammatory conditions. In addition, we observed that anti-inflammatory therapy with tacrolimus did not decrease the relative proportion of TRAIL-expressing skin-infiltrating T cells in AD. To better understand TRAIL functions, expression studies in other diseases should be performed and controlled following pharmacological treatment. Further experimentation is required to determine more precisely the role of TRAIL in the regulation of the IL-1Ra/IL-1 system.

Materials and Methods

Subjects Ten patients (18–58 y) with moderate-to-severe AD fulfilling the diagnostic criteria (Hanifin and Rajka, 1980) were included. Skin biopsies were taken from acute lesions before therapy and 1–3 wk after starting therapy with tacrolimus 0.1% ointment twice daily. At the same time points, 20 mL heparin-anticoagulated blood samples were obtained. Nine healthy individuals served as controls. All patients signed a written informed consent. The study was approved by the medical ethical committee of the canton Bern. It was conducted according to the Declaration of Helsinki Principles.

Cells PBMC and neutrophils were purified by Ficoll–Hypaque centrifugation (Simon *et al*, 2002; Baumann *et al*, 2003; Altznauer *et al*, 2004). Eosinophils were isolated by immunomagnetic selection with anti-CD16 mAb-conjugated beads as described previously (Dibbert *et al*, 1998; Simon *et al*, 2003). The resulting cell populations contained more than 98% eosinophils and neutrophils, respectively. HaCaT cells (a kind gift from Dr N. Fusenig, Heidelberg, Germany) were maintained in DMEM (Invitrogen, Basel, Switzerland) containing 10% fetal calf serum.

Flow cytometry Purified PBMC, neutrophils, and eosinophils were stained with mouse anti-TRAIL mAb (clone 2E5, Alexis Corporation, Basel, Switzerland). Briefly, 1×10^5 cells were incubated with 10 μg per mL of anti-TRAIL mAb and IgG1 control mAb (Alexis Corporation), respectively, on ice for 45 min. After three washings with staining buffer (PBS, 0.4% of BSA), cells were incubated with anti-mouse PE-conjugated Ab for 30 min, washed, and analyzed by a standard flow cytometric technique using FACS Calibur (Becton Dickinson, Heidelberg, Germany). To determine lymphocyte and monocyte subsets, PBMC were additionally stained with FITC-conjugated anti-CD8 and anti-CD14 mAb, respectively, or with APC-conjugated anti-CD4 mAb. Collected data were analyzed by FlowJo software (Tree Star, Ashland, Oregon). Except anti-TRAIL and control IgG1 mAb, all Abs were obtained from Becton Dickinson Biosciences (Basel, Switzerland).

Confocal microscopy Immunofluorescence staining was performed on 4 μm sections of paraformaldehyde-fixed and paraffin-embedded skin biopsies as described previously (Simon *et al*, 2004). To identify TRAIL-expressing T cells in the dermal infiltrate, sections were treated with mAb against CD4 and CD8 (Serotec, Oxford, UK) at 4°C overnight. TRAIL expression was analyzed by using a polyclonal Ab against human TRAIL (Alexis Corporation). To identify IL-1Ra in the epidermis, a polyclonal Ab against IL-1Ra (Santa Cruz Biotechnology, Santa Cruz, California) was used. Ab binding was detected with appropriate tetramethyl rhodamine isothiocyanate-conjugated and fluorescein isothiocyanate-conjugated secondary Abs (Milan Analytica AG, distributed by La Roche, Basel, Switzerland). Nuclei were counterstained with propidium iodide (Sigma, Buchs, Switzerland). The immunofluorescent staining was viewed by two independent investigators using a confocal laser scanning microscope (LSM 510, Carl Zeiss, Heidelberg, Germany).

RNase protection assay (RPA) Total RNA was extracted using the Qiagen RNEasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. Five micrograms of total RNA was processed according to PharMingen's RiboQuant protocol for mRNA expression of IL-1Ra (San Diego, California). L32 mRNA was used as a loading control.

ELISA IL-1Ra secretion from keratinocyte cultures was analyzed by ELISA (R&D Systems, Wiesbaden, Germany) as described previously (Leverkus *et al*, 2003b). Briefly, HaCaT cells (in DMEM) as well as primary keratinocytes (in keratinocyte growth medium 2; PromoCell, BioConcept, Allschwil, Switzerland) from two independent donors (Euroderm GmbH, Leipzig, Germany) were seeded in 48-well plates at a density of 3×10^4 and maintained at 37°C for 24 h. Plates were washed, and stimulation with recombinant LZ-TRAIL (a kind gift from Dr H. Walczak, Heidelberg, Germany) was performed under serum-free conditions for an additional 24 h. Cell-free supernatants were analyzed by ELISA according to the recommendations of the manufacturer normalized to protein secretion present in control cultures.

Statistical analysis All statistical analyses were performed with Graph Pad Prizm (GraphPad Software, San Diego, California). Data are presented as means ± SEM. To compare groups as well as results before and after therapy, unpaired and paired *t* tests were applied. A probability (p) value below 0.05 was considered significant.

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Address correspondence to: Dr Dagmar Simon, Department of Dermatology, Inselspital, University of Bern, CH-3010 Bern, Switzerland. Email: dagmar.simon@insel.ch

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