Evaluation of the Potential Role of Cytokines in Toxic Epidermal Necrolysis

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Toxic epidermal necrolysis is a rare disease observed as a consequence of adverse reactions to drugs. It results in the widespread apoptosis of epidermal cells and has a high mortality rate. The mechanisms leading to this apoptosis are not yet elucidated. We investigated whether the cytokines present in the blister fluid, which accumulates under necrotic epidermis, originated from T lymphocytes and may play a role in the propagation of keratinocyte apoptosis. Interferon gamma (IFN-γ), soluble tumor necrosis factor alpha (TNF-α), soluble Fas ligand (sFas-L) were present in much higher concentration in the blister fluids of 13 toxic epidermal necrolysis (TEN) patients than in control fluids from burns. The results of RT-PCR studies, however, indicated that only IFN-γ and to a lesser extent interleukin (IL)-18 were produced by mononuclear cells present in the fluid. That suggests that the other cytokines also present (TNF-α, sFas-L, IL-10) rather originated from activated keratinocytes. Fas-L was indeed overexpressed on the membranes of keratinocytes in lesional skin *in situ*. The Th1 profile of T lymphocyte activation found in the blister fluid of patients with TEN is consistent with a key role for drug-specific cytotoxic T lymphocytes (CTL) as previously reported, the activation of keratinocytes by IFN-γ making them sensitive to cell-mediated cytolysis. We propose the hypothesis that the production of Fas-L, TNF-α, and IL-10 by keratinocytes could be a defense mechanism against CTL rather than a way of propagating apoptosis among epidermal cells.

Keywords: CD95/interferon gamma/interleukin-18/TNF-alpha/toxic epidermal necrolysis
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Toxic epidermal necrolysis (TEN) is an acute and severe adverse reaction to drugs, characterized by the widespread destruction of the epithelium of the skin and mucous membranes (Lyell, 1979; Becker, 1998). This destruction by massive apoptosis (Paul *et al*, 1996) leads to a clinical pattern resembling second-degree burns, with large sheets of necrotic epidermis detached from the underlying dermis. The death rate from TEN averages 30%. Even though very rare TEN is a disease of interest because of a high impact on the evaluation of the benefit/risk ratio of medicines and because the mechanisms of acute and extensive destruction of the skin are still poorly understood.

At the onset of TEN, blisters often develop from the fluid that accumulates in the gaps between the dead epidermis and the dermis. High concentrations of mononuclear cells are present in this blister fluid. In the first days of blister formation, these cells are principally CD8 T lymphocytes (Correia *et al*, 1993) often expressing natural killer (NK) markers (Le Cleach *et al*, 2000) and that may exhibit a drug-specific major histocompatibility complex (MHC) class I-restricted cytotoxicity against autologous cells (Nassif *et al*, 2002). These findings strongly suggest that these cells are not bystander lymphocytes but probably play a role in the destruction of epidermal cells. Anyhow, in skin biopsies of early lesions of TEN, there are rather low numbers of CD8 T lymphocytes within the epidermis (Miyauchi *et al*, 1991), suggesting that the intervention of soluble mediators may be necessary for amplifying the apoptosis of keratinocytes. Several pathways could be implicated in the apoptosis of keratinocytes in TEN. Tumor necrosis factor alpha (TNF-α) (Paquet and Pierard, 1998), granzyme B, and perforin (Yawalkar *et al*, 2000; Posadas *et al*, 2002) were found to be overexpressed in lesional skin, but it has been suggested that the Fas/Fas ligand (Fas-L) pathway was the key factor (Viard *et al*, 1998).

To better understand how the relatively few lymphocytes present within the skin lesions lead to the extensive apoptosis of epidermal cells we: (1) measured the concentration of a variety of soluble cytokines in the blister fluid of 13 patients with TEN in comparison with the blister fluids of burns; (2) evaluated the synthesis and expression of some cytokines by the mononuclear cells extracted from the blister fluids; and (3) looked at the effect of the blister fluid on autologous keratinocytes.

Abbreviations: CTL, cytotoxic T lymphocytes; DMSO, dimethyl sulfoxide; FACS, fluoresence activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; ICAM, intracellular adhesion molecule; IFN-γ, interferon gamma; IL, interleukin; PE, phycoerythrin; PBL, pheripheral blood lymphocytes; PMA, phorbol myristate acetate; sFas-L, soluble Fas ligand; TEN, toxic epidermal necrolysis; TGF-β, tumor growth factor β; TNF-α, tumor necrosis factor alpha

Results

Principal characteristics of the patients are summarized in Table S1 (see online supplement).

Concentration of soluble cytokines in the blister fluid Results of cytokine levels in the 13 blister fluids from TEN patients are shown in Table S1. In all, 13 cases interleukin (IL)-18 was present at a very high concentration. Interferon gamma (IFN-γ) was elevated in 12 cases, soluble tumor necrosis factor alpha (sTNF)-α in nine, and soluble Fas ligand (sFas-L) in nine. High concentrations of IL-10 were found in all 10 patients tested. On the other hand, IL-2 and IL-12 were within normal serum values or not detectable in all samples. IL-5 was slightly elevated in three of 13 cases, while tumor growth factor β (TGF-β) was moderately elevated in five of 13 and IL-15 in five of 13.

When we looked for possible correlation between cytokine concentrations, we found significant positive correlation (Spearman rank non-parametric test) between sTNF-α and sFas-L ($r = 0.59$, $p = 0.03$) on one hand and between IFN-γ and sFas-L on the other ($r = 0.66$, $p = 0.01$).

We did not observe any significant difference when we separated samples obtained early or late in the course of the disease ($\leqslant 4$ d *vs* > 4 d). The five cases without obvious drug cause did not differ from the eight cases with a probable drug origin. The serum of some of the 13 patients had been obtained at the same time than blister fluid. When tested for IL-10 (patient #12) and IL-18 (patients #5, #6, #7, #9, #10, #11, and #12), all contained normal concentrations (not detectable for IL-10 and always < 100 pg per mL—mean 46 ± 17—for IL-18). In addition, the serum concentration of IL-10 was also not measurable in six TEN patients not included in the present study.

In the control blister fluids from burned patients (Table I), we found high concentrations of IL-18, slight elevations of IL 10 in 2 cases, of sTNF-α in one, and normal levels of IFN-γ and sFas-L.

Expression and synthesis of cytokines by mononuclear cells contained in the blister fluid As shown in Fig 1, lymphocytes from the blister fluid of TEN patients were strongly marked by granzyme B antibody (mean 85% positive cells) in the five patients tested, but not by anti-Fas-L antibody (mean 3% positive cells) in the three patients tested, two of them (eight and 12) having high concentration of sFas-L in the supernatant of the same blister fluid.

Table I. Cytokine concentrations in the blister fluids of three patients with burns as measured by ELISA

Burn patients	IFN-γ N[a] < 20	TNF-α N < 5	sFas-L N < 250	IL-18 N < 150	IL-10 N < 10
1	1	9	18	1531	7
2	0	0	18	650	19
3	5	0	18	528	31

[a]N, upper limit of normal serum value. All results are expressed in pg per mL.
TEN, toxic epidermal necrolysis; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; sFas-L, soluble Fas ligand; IL, interleukin.

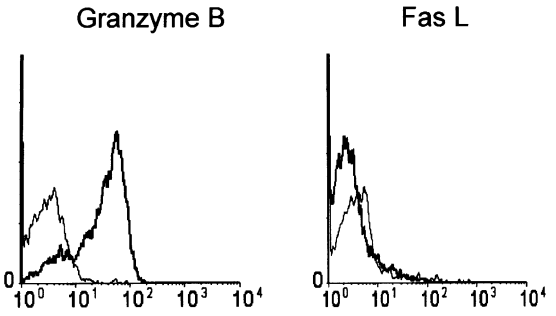

Figure 1
Granzyme B and Fas L expression by blister fluid lymphocytes. Blister fluid lymphocytes were stained with PE-labeled anti-granzyme B and with biotinylated anti-Fas L and analyzed by flow cytometry.

Table II shows the content of messenger RNA for a variety of cytokines in the mononuclear cells from the blister fluid of three TEN patients as compared with (1) the unstimulated blood mononuclear cells from five normal controls and (2) phorbol myristate acetate (PMA)-stimulated blood mononuclear cells from one normal control. Blister fluid cells differed from non-activated control cells only by higher contents in mRNA for IFN-γ and a lower content of mRNA for Fas-L. On the other hand, they were similar to non-stimulated control cells for the synthesis of IL-2, IL-10 and TNF-α. They differed from stimulated normal peripheral blood lymphocytes (PBL) by a lower production of all cytokines.

The production of IL-18 was evaluated in a separate experiment (see material and methods). Blister fluid cells differed from positive control cells (monocyte-derived dendritic cells) by a much lower content in IL-18 mRNA (100-

Table II. Reverse transcription real-time PCR mRNA analysis of various cytokine gene expression in blister fluid cells and in resting or activated control cells

	Fas-L	IL-2	IFN-γ	TNF-α	IL-10	IL-18[a]
Blister fluid cells (patient #8)	0	12.5	991	1381	8	0.09
Blister fluid cells (patient #11)	93.3	4.3	3779	1287	125	0.09
Blister fluid cells (patient #12)	0.3	1	2024	0	1.5	0.04
Control PBMC ($n = 5$)	169 ± 46	6.6 ± 5.3	241 ± 360	$8713 \pm 12{,}798$	47.8 ± 57.6	0.03 ± 0.01
PMA-stimulated PBMC	4233	11,607	31,178	17,994	667	ND

Results are expressed as mean of the ratio target gene AU per $\beta 2$ microglobulin AU $\times 10^6$.
[a]For IL-18, results are expressed in $\Delta\Delta C_t$ (see "Patients and Methods").
Fas-L, Fas ligand; IL, interleukin; IFN-γ, interferon gamma; IL, interleukin; PBMC, peripheral blood mononuclear cells; PMA, phorbol myristate acetate; TNF-α, tumor necrosis factor alpha, ND, not determined.

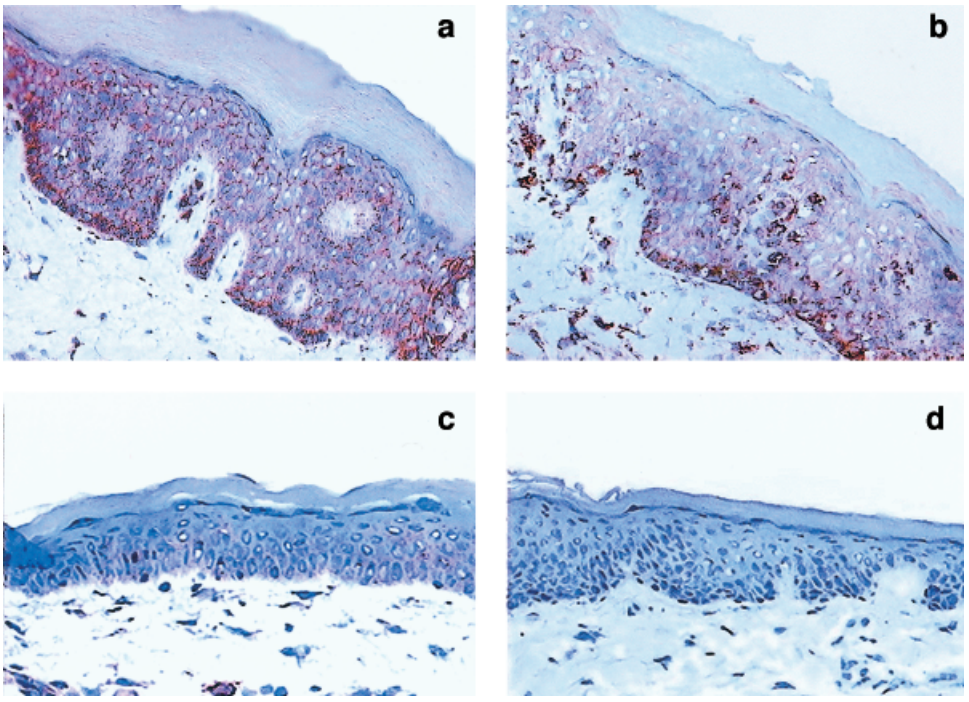

Figure 2
Immunohistochemical analysis of keratinocyte expression of Fas and Fas-L. Cryosections of biopsies of lesional skin from two patients were incubated with a rabbit polyclonal anti-human Fas-L and a mouse monoclonal IgG1 anti-Fas as described in Patients and Methods. One patient had a diffuse labeling of keratinocytes with anti-Fas-L (*a*) and labeling of basal keratinocytes with anti-Fas (*b*), the other patient had a faint labeling of keratinocytes with anti-Fas-L (*c*) and no labeling with anti-Fas (*d*).

fold less). Yet, IL-18 mRNA expression was 3-fold higher in blister fluid cells than in resting control PBL.

***In situ* expression of Fas and Fas-L by keratinocytes** As shown with two representative cases in Fig 2, keratinocytes of lesional skin with non-detached or partially detached epidermis expressed Fas-L on their membranes. On the other hand, Fas expression was mostly restricted on the basal cell layer, when it was strongly expressed on invading mononuclear cells both in the upper dermis and in the epidermis.

Blister fluid induced activation but not apoptosis of autologous keratinocytes Keratinocytes cultured from hair follicles, obtained after recovery in patient 12, were incubated for 48 h with cell-free supernatant of autologous blister fluid. As shown in Table S1, this fluid contained especially high amounts of IFN-γ, TNF-α, and sFas-L. Incubation resulted in activation of cultured keratinocytes as evidenced by expression of HLA-DR and CD54 (Fig 3), but we did not find any sign of apoptosis with annexin V labeling (data not shown).

Discussion

In 13 patients with TEN, we studied the cytokines present within the blister fluids. Our findings provided additional information on the mechanisms involved in the widespread destruction of the epidermis that characterize this disease.

Three cytokines were found at high concentrations in all tested samples: IL-18, IFN-γ, and IL-10. Variable amounts of TNF-α and sFas-L were found in about two-thirds of cases. Other evaluated cytokines were present at low concentrations in a minority of cases (IL-5, IL-15, TGF-β) or were not detectable in any sample (IL-2, IL-12).

To our knowledge, the presence of high amounts of IL-18 in the blister fluids of diseased skin has never been reported before. We had tested IL-18 in TEN because it is the principal IFN-γ-inducing factor produced by human keratinocytes (Stoll *et al*, 1997). Interestingly, IL-18 induces not only IFN-γ, but the Th1 cytokines TNF-α and IL-15, as well as Fas-L. It also stimulates cytotoxic T cells. We were anyhow surprised to find elevated concentrations of IL-18 also in the blisters from burned patients, since the mechanisms of the two diseases are quite different. Apoptosis has been established as the mechanism of cell death in TEN (Paul *et al*, 1996), whereas necrosis is predominant in burns (Matylevitch *et al*, 1998). These finding may suggest that IL-18 has been preformed in keratinocytes and released by dying cells, whatever the mechanism of death has been. As we found, however, that mononuclear cells present in the blister fluid also produced some amounts of IL-18, it seems likely that mononuclear cells also contribute to the high amounts found in the blister fluid. Nevertheless, IL-18 may enhance the production of IFN-γ in TEN cases, but not in burns because of a much lower concentration of T cells in burn fluids. Actually, in burn fluid the concentration of cells is 10 times lower than in TEN fluid and T lymphocytes account for less than 10% of these cells in contrast with more than 50% in TEN (Correia *et al*, 1993).

The presence of IL-10 in both types of fluids, although the concentration was higher in TEN as previously reported (Correia *et al*, 2002), also suggests that it is produced by keratinocytes. That IL-10 originated from keratinocytes in TEN patients was indirectly supported by the RT-PCR study of blister fluid cells. Actually, the mononuclear cells present in the blisters contained no more IL-10 mRNA than unstimulated PBL from normal controls. IL-10 is chemotactic for peripheral blood CD8$+$ T cells and may contribute to the recruitment of CD8$+$ T lymphocytes to the blister fluid of patients with TEN. In addition, IL-10 inhibits the production of inflammatory cytokines such as TNF-α or IL-6 and is a potent suppressor of the effector functions of macrophages, T cells, and NK cells. It may act as a downregulator of these cell functions and as a physiological defense

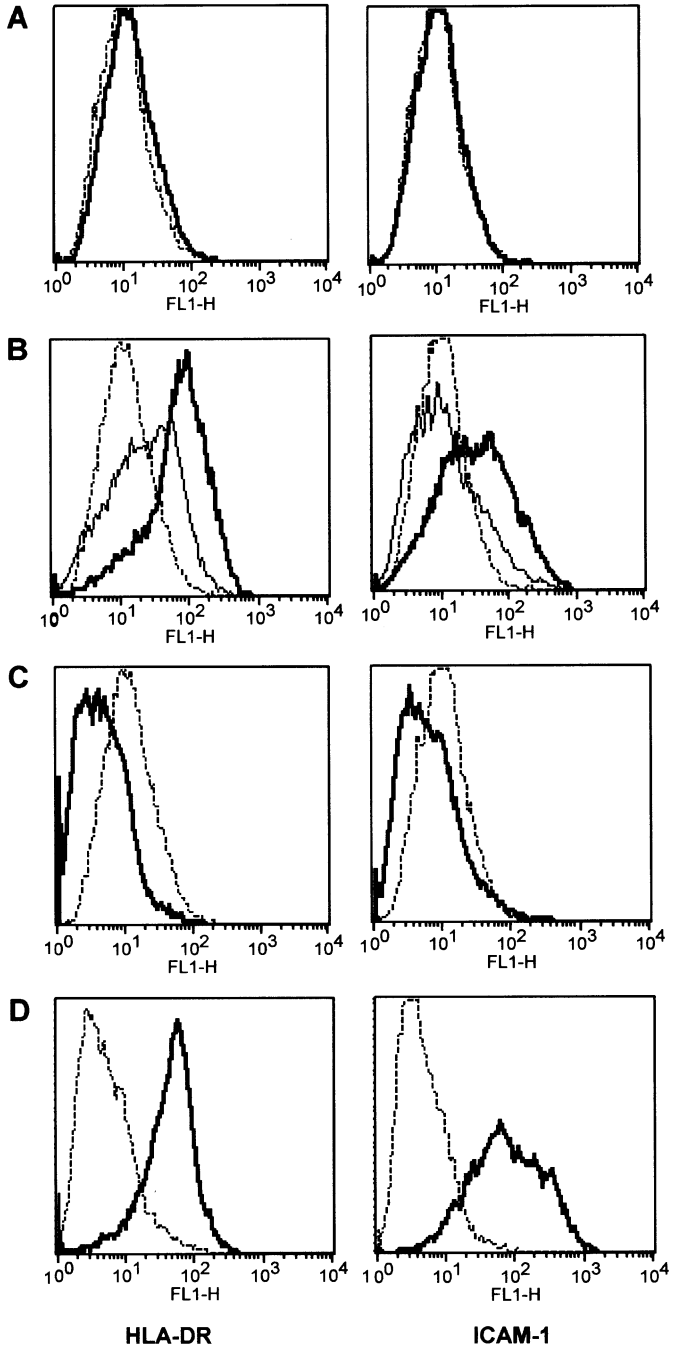

Figure 3
Activation of keratinocytes from one TEN patient by autologous blister fluid. Keratinocytes obtained from hair follicles of patient 12, after recovery, were cultured for 48 h with 1/2 or 1/10 dilutions in culture medium of autologous blister fluid. (*A*) After a 48 h incubation in culture medium alone, keratinocytes did not express HLA-DR and CD54/ICAM-1. (*B*) After 48 h incubation with autologous blister fluid, diluted 1/10 (*normal line*) or 1/2 (*bold line*), keratinocytes expressed HLA-DR and CD54/ICAM-1 (78 ± 10% and 85 ± 5%, respectively with 1/2 dilution). (*C*) After 48 h incubation with 1/2 dilution of blister fluid from a burned patient, HLA-DR and CD54/ICAM-1 were not expressed. (*D*) When incubated for 48 h with IFN-γ (500 U per mL) keratinocytes expressed as expected HLA-DR and CD54/ICAM-1 (90% ± 5% and 93% ± 5%, respectively).

mechanism against excessive tissue inflammation (Enk and Katz, 1992; Spits and Malefyt, 1992).

The absence of IL-2, confirmed by a functional test and by RT-PCR, may be considered surprising in a fluid where many activated T cells expressing CD25 are found (Le Cleach *et al*, 2000). But these cells are mostly CD8 cytotoxic T cells, which have been already shown to produce little or no IL2 (Chouaib *et al*, 1988; Fong and Mosmann, 1990).

The main characteristic of TEN fluid, compared to burn fluid, was the nearly constant presence in high concentrations of IFN-γ, sTNF-α, and sFas-L, with some correlation between the concentrations of these three cytokines. IFN-γ was the cytokine with the highest concentration, and always present in TEN when not found in burns. IFN-γ is not known to be produced by keratinocytes. Not surprisingly high levels of IFN-γ mRNA were detected in blister fluid mononuclear cells confirming that these cells were the source of IFN-γ. The high level of production of IFN-γ, contrasting with the inconstant presence and low concentrations of IL-5, points to a Th1 pattern of activation of T lymphocytes in TEN.

Significantly higher amounts of TNF-α have been previously found in TEN blisters than in burns (Correia *et al*, 2002). Both TNF-α and Fas-L can be produced by keratinocytes and their production is increased by IFN-γ (Arnold *et al*, 1999). By RT-PCR we found that blister fluid cells did not express more TNF-α and Fas-L transcripts than control lymphocytes. Furthermore, we did not find a significant expression of the Fas-L protein on these cells by fluoresence activated cell sorter (FACS) analysis. On the other hand, immunolabeling of keratinocytes in biopsy samples of lesional skin demonstrated an overexpression of membrane Fas-L. Taken together, these results suggest that sFas-L present in the blister fluid of TEN had been produced by activated keratinocytes. Conflicting results had been previously obtained on this point. One study showed that *in vitro* cytokine-activated keratinocytes expressing membrane Fas-L did not release measurable amounts of sFas-L (Arnold *et al*, 1999), when another one found sFas-L in the supernatant of cultured keratinocytes (Berthou *et al*, 1997).

When incubated for 48 h with cell-free supernatant of autologous blister fluid, cultured keratinocytes were activated but did not undergo apoptosis. This result suggests that there is enough IFN-γ to activate keratinocytes but not enough sFas-L to induce Fas-mediated apoptosis. This is not really a surprise considering that the concentration of sFas-L in the blister fluid was about 5 ng per mL, when human recombinant sFas-L induced apoptosis of keratinocytes at much higher concentrations ($IC_{50} = 89$–232 ng per mL) (Viard-Leveugle *et al*, 2003).

These results do not exclude that the over expression of membrane Fas-L could participate in the suicidal apoptosis of keratinocytes. This had been proposed (Viard *et al*, 1998) on the basis of experiments showing that keratinocytes from TEN lesions induced apoptosis on Jurkat cells. Whereas Jurkat cells express high levels of Fas we, after others (Paquet *et al*, 2002), found no gross overexpression of Fas/CD95 by keratinocytes of lesional skin of TEN. Since an overexpression of Fas by keratinocytes has been observed in many clinical situations where the apoptosis is much more limited than in TEN, e.g., UV irradiation (Bang *et al*, 2002), contact dermatitis or pemphigoid (Sayama *et al*, 1994), we consider that the hypothesis that dissemination of apoptosis in TEN is Fas mediated lacks definite support.

On the other hand, we have previously demonstrated that cells present in the blister fluid behave as drug-specific CTL (Nassif *et al*, 2002). The activation of keratinocytes by

IFN-γ is an important step in making them sensitive to lysis by CTL (Schnyder *et al*, 1998).

Taken together, these results support the following hypotheses on the mechanism of TEN: the key effectors are drug-specific CD8 CTLs (Nassif *et al*, 2002) engaged in a Tc1 pathway. The secretion of IFN-γ by these cells results in activation of keratinocytes, including the overexpression of MHC class I molecules, and makes them sensitive to MHC restricted, perforin, and granzyme B mediated apoptosis. The release of TNF-α and sFas-L by keratinocytes is a marker of activation and a part of innate defense mechanisms of epidermal cells. These cytokines might contribute to the dissemination of keratinocyte death. But we can also postulate that together with the production of IL-10, the release of TNF-α and the overexpression of Fas-L are part of a defense mechanism of epidermal cells against invading CTLs (Berthou *et al*, 1997).

Patients and Methods

Patients The blister fluids were collected from 13 patients with clinical and pathologic criteria of TEN. The study had been approved by an ethical committee (CCPPRB, Pitiè-Salpêtrière, Paris, France) and all patients (or relative in case of incapacity) provided informed consent to sampling of blood and blisters.

As controls, we studied the blister fluids obtained from three patients suffering from localized second-degree thermal burns.

Supernatants and cells from blister fluid Samples of blister fluid were obtained by puncture of several blisters within 2 days after hospitalization, i.e., 1–14 d after the onset of the skin reaction. It was not possible to collect adequate information on the duration of individual blisters before sampling. Fluid obtained by direct aspiration was centrifuged (1000 × *g*) and supernatants were collected and stored at $< -20^{\circ}$C until use. Cells from the pellet were resuspended in 20% fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO) containing medium (RPMI 1640 supplemented with penicillin 100 U per mL, streptomycin 100 mg per mL, glutamine 2 mM, sodium pyruvate, non-essential amino acids, HEPES buffer—Flow Laboratories Glasgow, UK), frozen and stored in liquid nitrogen. We know from prior studies that these cells are about 80% lymphocytes and 20% monocytes. (Le Cleach *et al*, 2000). From the distribution of cell size in FACS analyses in three patients included in the present study, the proportion of lymphocytes among living cells was estimated to 75%, above 95%, and 90%, respectively.

Cytokines measurement in supernatant of blister fluid We quantified the following cytokines by a sandwich ELISA according to standard protocols. Commercial ELISA kits from Immunotech (Marseille, France) were used for quantifying sTNF-α, IL-5, IFN-γ and sFas-L. Quantikine assays (R&D systems, Minneapolis, Minnesota) were used for TGF-β1, IL-12, IL-15, IL-18, and Diaclone used for measuring IL-2 and IL-10. All kits were used as specified by the manufacturers.

IL-2 measurement was also performed by a biological assay. Cells from the CTL-L2 cell line were washed in RPMI 1640 and suspended in RPMI 1640 completed with 10% bovine calf serum at 5×10^4 cells/well in a volume of 200 μL in a U-bottom microplate. Supernatants were added and incubated for 3 d at 37°C in 5% CO_2. After 3 d incubation, microcultures were pulsed with 1 μCi per well of methyl-[^{3}H]thymidine (20 μL) and cells harvested 7 h later and counted in a microplate β-counter. The IL-2 sample levels were determined by comparison to a standard curve using different concentration of IL-2.

Cytokine concentrations were expressed in pg per mL. Normal values were chosen as the upper limit of concentration in normal human serum according to figures provided by the manufacturers.

Culture of human keratinocytes About 20 hair follicles were plucked from the scalp and the bulk of the hair shafts cut off as described earlier (Limat and Noser, 1986). The follicles were immersed in the Dulbecco-modified Eagle's medium (DMEM) buffered with 25 mM HEPES and supplemented with 400 U per mL penicillin 400 μM per mL streptomycin. A single cell suspension was obtained by incubating the follicles for 30 min. at 37°C with 200 μl 0.1% trypsin (1:250) and 0.02% EDTA each in phosphate-buffered saline (PBS). The dissociated keratinocytes were centrifuged for 10 min. at 200 × *g*. The culture medium consisted of three parts of DMEM containing sodium pyruvate and 1 g per liter glucose, and 1 part of Ham's F-12 medium, supplemented with 10% FCS, 5 μg per mL insulin (Sigma, St. Quentin Fallavier, France), 0.10 nM cholera toxin (Sigma), 0.4 μg per mL hydrocortisone (Upjohn, St. Quentin en Yvelines, France), 2 nM 3,3′,5-triiodo-L-thyronine (Sigma), 0.135 mM adenine (Sigma), 10 ng per mL epidermal growth factor (Sigma), penicillin 50 U per mL, streptomycin 50 μg per mL, and amphotericine-B 2.5 μg per mL (Invitrogen, Cergy Pontois, France). The keratinocytes were seeded at a density of $2–3 \times 10^3$ cell per cm^2 on a 3T3 feeder layer (2×10^3 cell per cm^2). The 3T3 cell proliferation had been halted by a treatment with γ irradiation at 60 Gy. The culture was incubated at 37°C and 5% CO_2-humidified atmosphere. After 2 wk in primary culture, keratinocytes were detached by trypsin.

Immunostaining of blister fluid cells and keratinocytes Phenotypic analysis was performed on thawed cells, using a flow cytometer XL coulter after staining with fluorescent monoclonal antibodies against granzyme B (Caltag, San Francisco, California), Fas, Fas-L, CD54/intracellular adhesion molecule (ICAM)-1 (UB2-FITC (fluorescein isothiocynate), 4H9-biotin and H11-FITC, S45-FITC, respectively, Beckman Coulter, Mareseille, France) and HLA-DR (Pharmingen, San Diego, California).

Immunohistochemical analysis of keratinocyte expression of Fas and Fas-L After rehydratation and inhibition of endogenous peroxidases, 6 μm thin cryosections of biopsies of lesional skin were incubated with primary specific or isotype control irrelevant antibodies for 30 min, then incubated with a multi-species biotinylated second antibody for 10 min before the addition of streptavidin–peroxidase reagent for 10 min. (Ultratech HRP Streptavidin–Biotin, Immunotech, Universal Detection System, Marseille, France).

The amino-ethyl-carbazol (AEC) substrate-chromogen (Beckman-Immunotech, Marseille, France) was used to reveal antibody fixation. Sections were counterstained with Mayers hematoxylin before glycerol mounting.

A rabbit polyclonal anti-human Fas-L (N20, Santa Cruz, Tebu, France) diluted to 1/50, and a mouse monoclonal IgG1 anti-Fas (UB2, Beckman Coulter, Immunotech) diluted to 1/20 were used to detect Fas/Fas-L expression, respectively.

Isotype control antibodies were included in each experiment.

To assess the cell infiltrating area on the sections, a Larborlux S Leitz (Leica Microsystems, Rueil-Malmaison, France) microscope was used with a × 25 objective.

Study of keratinocyte activation and apoptosis with flow cytometry Subconfluent keratinocyte cultures derived from hair follicles of patient #12 were incubated for 48 h. with several dilutions in the culture medium (1/20 to 1/2) of autologous blister fluid (containing the highest concentration of sFas-ligand) or with IFN-γ 500 U per mL. After detaching the keratinocytes, membrane immunostaining was performed with specific antibodies against HLA-DR, CD54/ICAM-1, and annexin V-FITC.

RNA isolation and quantification of gene expression by real-time quantitative RT-PCR In the blister fluid cells of three selected patients, we investigated mRNA expression level of IL-2, IL-10, IL-18, IFN-γ, TNF-α, and Fas-L/CD95L. Total mRNA was purified using Rneasy kit (Qiagen SA, Courtaboeuf, France). The sequence of primers and probes and PCR parameters are indicated in Table S2. All nucleotide primers were targeted to separate exons of the genes of interest. The expression levels of tested cytokines were

analyzed in first-strand cDNA reaction (RT) samples, each containing: 1–10 μg total RNA isolated from blister fluid cells or control PBL, 16 U per μL M-MLV reverse transcriptase (Gibco-BRL, Life Technologies, Cergy-Pontoise, France), 4 μM Oligo-(dT) 12-18 (Amersham-Pharmacia Biotech, Saclay, France) and 0.8 mM mixed dNTP (Amersham-Pharmacia Biotech, Saclay, France). For IL-2, IL-10, IFN-γ, TNF-α, Fas-L/CD95L quantitative PCR was performed using the LightCycler System (Roche Diagnostics, Meylan, France) with hybridization probes that use the fluorescence resonance energy transfer (FRET) phenomenon (Wittwer *et al*, 1997). A standard curve was made by PCR of eight dilutions (10^8–10 copies) of the corresponding "quantitative DNA standard" (QDS), generated by using the "looped oligo" method (Sarkar and Bollander, 1994). The PCR products were cloned in plasmids for storage stability of the QDS. The relative value for amplification of each sample was evaluated using the Light Cycler analysis software using the C_t value ("Second Derivative Maximum", version 3.5; Roche). Amplification of the β2 microglobulin (β2μg) gene served to monitor the quality of the RNA isolation and the efficiency of the reverse transcription reaction. The β2μg gene was chosen as housekeeping control because its expression remained relatively constant. The magnitude of target gene expression was calculated as copies number of target gene cDNA per 10^6 copies of β2μg cDNA. We obtained the same relative results when the number of copies of the target gene cDNA was calculated per 10^3 copies of GAPDH cDNA (data not shown). IL18 mRNA expression was evaluated by relative quantification of real-time-PCR according to Gibson *et al* (1996) cDNA dilution series of calibrator cell were run to set up a standard curve for IL-18 and GAPDH (as HKG), and the C_t values were plotted against the log cDNA concentration added. Monocyte-derived dendritic cells were used as calibrator cells and compared either to blister fluid cells or control peripheral blood mononuclear cells of healthy donors. By using the obtained linear graphs, the differences in C_t values were determined for each sample and were expressed as relative percentage of mRNA present in the reference dilution of the calibrator cells, according to the $\Delta\Delta C_t$ method, after adjustment of PCR efficiency with the RelQuant software (Roche). Each PCR experiment was carried out in duplicates and at least two times.

Statistical analyses Analyses were performed with Student' *t* test and Spearman non-parametric correlation using InStat.2 software (GraphPad Software, San Diego, California).

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID23439/JID23439sm.htm

Table S1. Cytokine concentrations in the blister fluids of 13 patients with TEN as measured by ELISA

Table S2. Primer and FRET probe sequences used for RT real-time PCR

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