Elevated interleukin-18 protein expression in early active and progressive plaque-type psoriatic lesions

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ABSTRACT. Psoriasis is a T cell-mediated inflammatory skin disease characterized by an elevated IFN-γ and IL-12p70 expression in skin lesions. Interleukin-18 (IL-18) synergizes with IL-12 to induce IFN-γ production and a strong T-helper-1-mediated immune response, or to induce Th2 polarization depending on the immunological context. We have previously shown that keratinocytes in normal skin produce and store large amounts of pro-IL-18. In this study, we hypothesized that the expression of IL-18 in psoriatic lesional skin might be altered compared to normal skin. Therefore, IL-18 expression was assessed in psoriatic, stable, plaque-type lesions and early active and progressive lesions. IL-18 mRNA and protein concentrations were constitutively high, and did not differ between normal and stable, plaque-type epidermis. In active and progressive lesions an elevated expression of total IL-18 protein relative to normal and stable, plaque-type epidermis was detected using ELISA, while on Western blot, the differences in pro- or mature IL-18 were less clear. Our results indicate that the role of IL-18 in the pathogenesis of early phases of psoriasis may be more prominent than in established psoriatic lesions.

Keywords: IL-18, interferon-γ, psoriasis, skin inflammation

INTRODUCTION

An important feature of the inflammatory skin disease psoriasis is the infiltration of T cells in the dermis and epidermis [1], which have been shown to play a significant role in the pathogenesis of the disease [2, 3]. The T cell infiltrate in lesional psoriatic skin comprises $CD4^+$, $CD8^+$ as well as NK (T) cells, whereby the balance leans towards the T helper 1 (Th1) subset [1, 4, 5]. One of the hallmarks of Th1 cell activation is expression of interferon-γ (IFN-γ). Indeed an increased IFN-γ production in psoriatic lesional skin has been reported [2]. IFN-γ induces the expression of several markers that are characteristic for the psoriatic skin phenotype, such as ICAM-1, HLA-DR and CD40 [6, 7]. In addition, IFN-γ transgenic mice exhibit a clearly inflamed skin [8]. Hence, an important role for IFN-γ in psoriasis is apparent.

Synthesis of IFN-γ is induced by synergizing actions of IL-18 and IL-12 [9], which thereby act as Th1-inducing cytokines. IL-18 is expressed in many cell types, including keratinocytes. Stoll *et al.* [10] showed that murine keratinocytes produce IL-18 mRNA and functional protein after stimulation with contact allergens. Human keratinocytes were also shown to constitutively produce pro-IL-18 protein, which was processed into a functional protein after stimulation with pro-inflammatory mediators and dinitrochlorobenzene [11, 12]. In addition we showed that the production of IL-18 protein in human keratinocytes is, on average and on a per cell basis, a 100-fold higher than in leukocytes, peripheral blood mononuclear cells and monocytes [13]. This keratinocyte-derived IL-18 is predominantly produced as an inactive 24 kDa, leaderless protein, which needs post-translational proteolytic processing and extra cellular export to exert its biological function [14, 15]. Thus, as observed in dendritic cells (DC), it seems that the regulation of IL-18 in keratinocytes differs from that in monocytes in that synthesis, processing and secretion of bioactive IL-18 are separate processes in keratinocytes [14, 15].

Koizumi *et al.* [16] showed that IL-18 and the IL-18 receptor are simultaneously expressed by human keratinocytes in normal skin. They suggested that keratinocytes might be activated in an autocrine fashion by their own IL-18. In addition, the paracrine effects of keratinocyte-derived IL-18 on, for example, contiguous dendritic (Langerhans) and NK cells are also of importance [17]. These data indicate that the continuous availability of pro-IL-18 may have a major impact on the local cytokine milieu and on the outcome of immune responses in human skin [18].

Studies on the expression of IL-12 in keratinocytes, revealed that both IL-12p35 and IL-12p40 mRNA are constitutively expressed [19] and that IL-12p70, the active form of IL-12, is detectable in the supernatant of cultured keratinocytes. In psoriasis, an increase of IL-12p40 mRNA and IL-12p70 protein was reported [20], which indicates

the availability of IL-12 for facilitating the induction of IFN-γ in this disease.

However, the role of IL-18 in psoriasis remains ill-defined. Because IL-18 is a member of the IL-1 family, and the regulation of the IL-1 system in psoriasis is altered [21], we anticipated that the regulation of this cytokine might also be altered. The elevated IFN-γ expression in psoriatic lesional skin [5] might be an indication for this imbalance. Therefore, IL-18 mRNA expression in psoriatic lesional-, uninvolved and normal skin was investigated quantitatively together with the IL-18 protein levels.

The data presented here show that IL-18 mRNA and protein expression in stable plaque-type psoriatic skin is constitutively high as in normal skin. In active and progressive psoriatic skin however, a significantly elevated expression of total IL-18 protein was observed.

PATIENTS AND METHODS

Skin dermatome and biopsy specimens

Dermatome specimens for epidermal cell isolation or protein extraction were obtained (Padgett Instruments Inc., Kansas City, MO, USA) from 14 healthy donors (NN) and 14 patients with stable plaque-type psoriasis (stPP) (Dermatology Unit, Ziekenhuis Walcheren, Vlissingen, and Departments of Plastic Surgery, Erasmus MC and Sint Franciscus Gasthuis Rotterdam). Three-mm punch biopsies (Stiefel, Leuven, Belgium) were obtained from eight healthy donors and eight patients with stable, plaque-type psoriasis, snap frozen in Tissue Tek (Bayer, München, Germany) and stored at – 80 °C until use. Dermatome specimens and biopsies from the borders of active and progressive psoriatic lesions (apPP) were taken from six patients. All procedures were performed after written informed consent, and were approved by the Medical Ethical Committee of all hospitals. Patients received only mild topical treatment for at least three weeks, and did not undergo systemic or UV treatment for at least two months prior to skin sampling.

Epidermal cell suspensions

The epidermis was detached from the dermis of the dermatome specimens by trypsinisation. Epidermal cell suspensions (ECS) were prepared from epidermal sheets by incubating them for 45 min at 37 °C in trypsinisation buffer (0.025% (w/v) trypsin and 0.1% (w/v) EDTA in PBS), to which 0.25% DNAse was added for the last 15 min of the incubation (Roche, Basel, Switzerland). The cell suspension was filtered through a 30 μm mesh gauze and suspended in PBS containing trypsin inhibitors (5 mg/mL, Roche).

Epidermal protein extracts

The epidermis was detached from the dermis of the dermatome specimens after incubation in Hanks' Balanced Salt Solution (HBSS, Gibco BRL, Paisley, Scotland), containing 1 U/mL dispase (Roche) at 4 °C for 18 h. The epidermal sheets were cut with a scalpel and freeze-dried. The freeze-dried samples were homogenized in 1 mL of HBSS, supplemented with a broad mixture of protease inhibitors (Complete™ , Roche). To remove the cell debris, extracts were centrifuged (14 000 × g, 4 °C) and the supernatant was isolated. The total protein concentration was measured using the BCA-200 protein assay kit (Pierce, Rockford, IL, USA). Epidermal protein extracts were stored at – 80 °C until use.

Quantification of mRNA expression

RNA was isolated from 1 to 2×10^6 normal or psoriatic lesional epidermal cells from 7 normal donors and 12 psoriasis patients using RNAzol-B (Tel-Test Inc., Friendswood, TX, USA) and reverse transcribed into cDNA as previously described [22]. cDNA was analyzed using Taqman analysis (7 700 Sequence detector, Applied Biosystems, Foster City, CA, USA). The sequences of the primers and probes were as follows: IL-18-forward (Sigma Genosys, Cambridge, UK): 5'-GCT TTA CTT TAT AGC TGA AGA TGA TGA A-3'; IL-18-reverse (Sigma Genosys): 5'-CTC TAC AGT CAG AAT CAG TCA TAT CTT CAA ATA-3'; IL-18 (FAM labeled probe, Bioscource, Camarillo, CA, USA): 5'-TTC TCT TCA TTG ACC AAG GAA ATC GGC CT-3'. In each sample, cDNA of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was measured as a control. A commercially available kit (Applied Biosystems) was used for GAPDH mRNA detection according to the manufacturer's instructions. mRNA isolated from the HaCaT keratinocyte cell line [23] was used as a reference for IL-18 mRNA detection and mRNA isolated from the Th0 cell line B21 was used as a reference for GAPDH mRNA detection. The PCR signal detected for NN, stPP and apPP epidermal cell samples were correlated to the signals obtained for the HaCaT (IL-18) or B21 (GAPDH) control mRNA. Subsequently, the IL-18/GAPDH mRNA ratio was calculated to normalize for the variability in mRNA quality of the different samples.

Immunohistochemistry

Six μm cryosections were cut using a cryostat and stored in a sealed box containing silica at – 80 °C until use. Sections were fixed in acetone for 10 min at room temperature (RT) and pre- incubated for 10 min with PBS containing 0.05% Tween-20 at room temperature. Subsequently, sections were incubated for 18 h at 4 °C with anti-human-IL-18 (αhIL-18) (mAb MAB318, R&D Systems, Minneapolis, MN, USA, 2.5 μg/mL). This incubation was followed by incubation with biotin-linked secondary rabbit-anti-mouse polyclonal antibody (pAb) (DAKO, Carpinteria, CA, USA, dilution 1:400) and peroxidase-linked avidin (DAKO, dilution 1:200). Sections incubated with an antibody of the same isotype as the specific antibody but of irrelevant specificity served as a negative control. 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO, USA) was used as the chromogen. Staining intensity and numbers of positive cells were ranked in a blinded fashion by two independent investigators using a semiquantitative scoring scale as published before [7].

Cytokine ELISA

Maxisorb ELISA plates (Nunc, Roskilde, Denmark) were coated for 18 h at 4 °C with 100 μL of 0.5 μg mL^{-1} anti-human-IFN-γ (αhIFN-γ), αIL-12p40, αIL-1α, αIL-8 (Biosource, Camarillo, CA, USA) or 2 μg mL^{-1} αhIL-18 mAb (MAB318, R&D Systems) followed by blocking with 0.5% BSA (Sigma) for two hours at room temperature.

One hundred µL of the recombinant IFN-γ, IL-12p40, IL-1α, IL-8 (Biosource) or IL-18 (DNAX, Palo Alto, CA, USA) standard or sample and 50 µL of 0.2 µg mL^{-1} biotin-linked αhIFN-γ, αhIL-12p40, αIL-1α, αIL-8 (Bioscource) or αhIL-18 (BAF318, R&D systems) pAb detection antibody were simultaneously added to each well. The standards were diluted in PBS containing 0.5% BSA (Sigma) and 0.1% (v/v) Tween 20 (Merck). Samples, standards and detection antibodies were incubated for two hours at room temperature. Cytokines were detected using streptavidin-linked peroxidase (CLB, Amsterdam, The Netherlands, dilution: 1:10 000) and TMB peroxidase substrate (Kirkegaard, Perry, Gaithersburg, MD, USA). The substrate was incubated for a maximum of 20 min, and subsequently the OD was measured at 450 nm. Detection limits of the ELISA's used: IFN-γ and IL-1α: 1 pg/mL; IL-8: 0.5 pg/mL; IL-12p40: 2 pg/mL; IL-18: 20 pg/mL.

Western blotting and immunodetection

Proteins were separated using 15% SDS-PAGE gels according to Laemmli [24]. The proteins were blotted onto Hybond-C membranes (Amersham, Little Chalfont, UK) using an electroblot system (BioRad, Hercules, CA, USA). The membranes were blocked with Tris-buffered saline (TBS) containing 5% low fat milk and 0.05% Tween-20 for one hour at room temperature. Blots were stained with a primary antibody against IL-18 (MAB318, R&D systems, 0.062 µg/mL), followed by a secondary biotin-linked anti-mouse pAb (DAKO, dilution: 1:25 000) and streptavidin-linked peroxidase (CLB, dilution: 1:10 000). Incubation with an antibody of the same isotype as the specific antibody but of irrelevant specificity served as a negative control. IL-18-specific staining was detected using a chemoluminiscent substrate (Pierce).

Statistical analysis

The Mann-Whitney test was used to determine the significance of differences between patients and normal controls. A *P*-value equal to or less than 0.05 was considered to be significant.

RESULTS

Comparable high constitutive expression of IL-18 mRNA in normal and psoriatic lesional epidermis

The IL-18 and GAPDH mRNA levels in samples from seven healthy donors (NN = normal epidermis), seven stable, plaque-type psoriasis (stPP = psoriasis stable plaque-type lesional epidermis) and five active and progressive psoriatic lesion (apPP = active and progressive psoriatic lesions) were assessed using real time quantitative PCR. The IL-18/GAPDH mRNA ratio of NN and stPP epidermal cells did not differ significantly. Epidermal cells from active and progressive psoriasis lesions (apPP) displayed IL-18/GAPDH ratios similar to those found in normal and stable, plaque-type lesions *(Figure 1)*. These data show that both normal epidermal cells and psoriatic lesional epidermal cells express similar, high IL-18 mRNA levels.

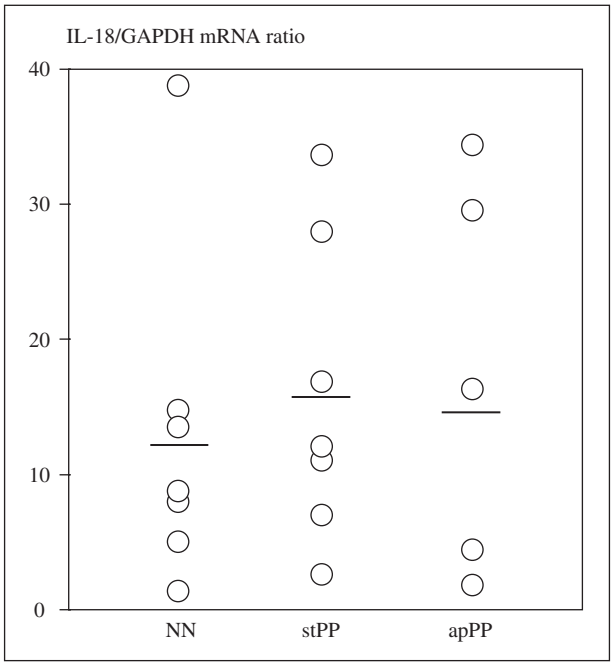

Figure 1
Similar IL-18 mRNA expression in normal *versus* psoriatic lesional skin.
IL-18 mRNA expression in seven normal (NN), seven psoriatic stable, plaque-type lesional (stPP) and five active and progressive lesional (apPP) epidermal cell suspensions was quantified using real-time quantitative PCR as described in the methods section. The IL-18/GAPDH mRNA concentration ratio was calculated to normalize for the variability in mRNA quality in the samples. The horizontal bars represent the mean and the open circles represent the individual IL-18/GAPDH ratios of the different samples.

IL-18 protein expression is increased in early active and progressive, plaque-type psoriatic epidermis

Skin sections stained for IL-18 showed a diffuse, granular staining pattern. In the epidermis, IL-18 expression was apparent in both keratinocytes and cells with a dendritic morphology, probably Langerhans cells (LC). Furthermore, the cellular infiltrate in the dermal papillae of psoriatic lesional skin clearly expressed IL-18 and included cells with dendritic and lymphocyte morphology. No difference in IL-18 staining intensity between NN and stPP epidermis was observed *(Figure 2a-c)*. In apPP epidermis however, a slightly, but not significantly increased staining intensity was observed using IL-18 specific mAb (not shown).

The total IL-18 concentration (processed and unprocessed) in the epidermal protein extracts was measured by ELISA. In accordance with the data obtained by immunohistochemistry, no significant difference in total IL-18 protein levels between NN and stPP epidermis was observed *(Table 1)*. However, in apPP epidermal extracts, higher IL-18 levels relative to NN and stPP were detected (on average a 3.5-fold increase, $P < 0.05$, *Table 1*).

To validate our protein extraction and detection methods, levels of expression of cytokines known to be differentially expressed in psoriasis were also measured using ELISA. In accordance with previous reports, in psoriatic skin we detected elevated levels of IL-8, IL-12p40 and IFN-γ, whereas IL-1α levels were reduced in both stPP and apPP epidermis *(Table 1)*.

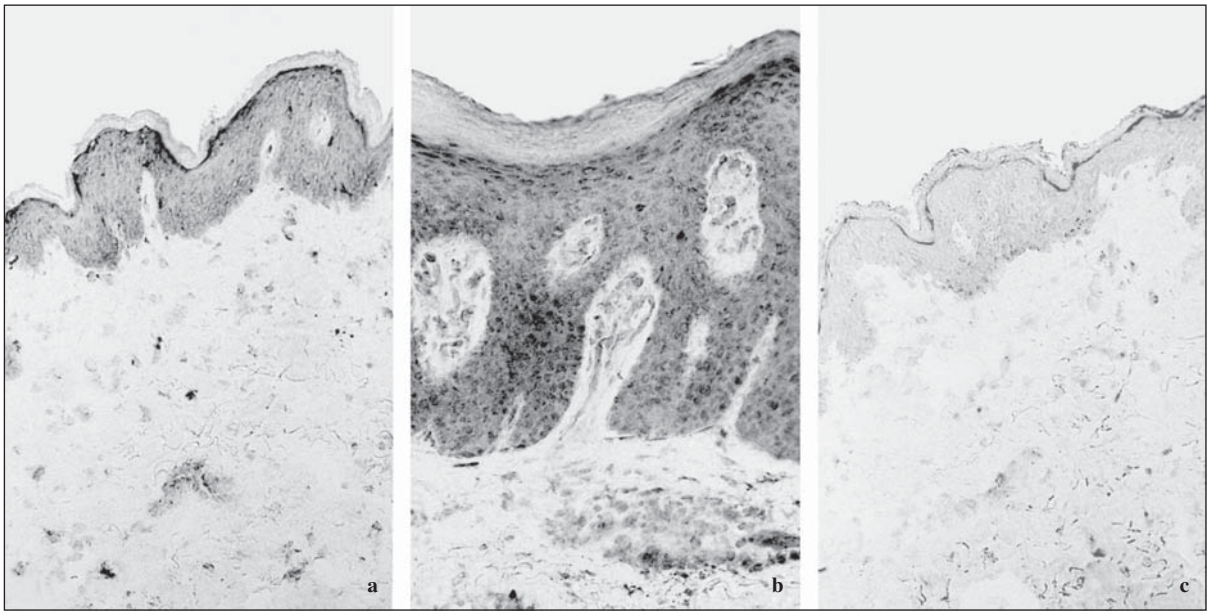

Figure 2
Similar IL-18 protein expression in normal and psoriatic lesional skin.
Acetone-fixed cryostat sections were stained with an IL-18-specific mAb as described in the methods section. (**a**) normal skin; (**b**) stable, plaque-type psoriatic lesion; (**c**) isotype control antibody staining on normal skin. Representative specimens out of 16 are shown.

Table 1
Total IL-18 protein expression in lesional psoriatic epidermis in comparison with other cytokines

Cytokine	NN	stPP	apPP
IL-18	6 838 ± 1 470	10 083 ± 1 691	24 481 ± 4 470[8]
IL-12p40	2.45 ± 1.73	205.03 ± 31[#]	215.92 ± 71[#]
IFN-γ	< 1.00	1.40 ± 0.39[#]	3.31 ± 0.89[8]
IL-1α	2 416 ± 343	145 ± 26[*]	243 ± 43[*]
IL-8	1.60 ± 0.47	747.83 ± 117[#]	4 974.97 ± 1 593[∞]

Cytokine concentrations were determined in normal (NN), psoriatic, stable, plaque-type lesional (stPP) and active and progressive lesional (apPP) epidermal extracts by ELISA and were normalized for the total protein concentration. Data represent the cytokine concentration in pg/mg total protein (mean ± SEM) of 7 NN, 7 stPP and 6 apPP epidermal extracts.
[#]: stPP or apPP > NN: $P < 0.01$;
[*]: stPP or apPP < NN: $P < 0.01$;
[∞]: apPP > stPP and NN: $P = 0.05$.

Together, these data show that total levels of IL-18 in psoriatic, stable, plaque-type epidermis are high, but comparable to levels in normal human epidermis. However, total IL-18 protein levels are increased 3.5 fold in active and progressive psoriatic epidermis when compared to normal and stable, plaque-type psoriatic epidermis.

IL-18 in psoriatic lesions is predominantly expressed in the unprocessed form

To become a biologically active molecule, pro-IL-18 needs to be processed by cysteine proteases. IL-18 processing was assessed by Western blotting and subsequent immunostaining. In SDS-PAGE, unprocessed IL-18 migrates at the 24 kDa level, whereas the bioactive mature form migrates at the 18 kDa level. Therefore, pro-IL-18 and processed IL-18 can readily be distinguished. The IL-18 protein detected by Western blot analysis, was predominantly present in the unprocessed (24 kD) form in extracts of stPP, apPP and NN epidermis. A clear but faint band at the 18 kD level was visible in most samples of NN, apPP and stPP epidermis. Although the expression of the 18 kD processed form of IL-18 in apPP epidermis seemed more pronounced in two cases, no consistent differences in the appearance and intensity of this band was observed between the other NN, apPP and stPP samples *(Figure 3)*.

DISCUSSION

Interleukin-18 has been implicated as a key inducer of IFN-γ and of Th1-mediated autoimmune and inflammatory diseases [9]. On the other hand, in the absence of IL-12, IL-18 has also been shown to facilitate Th2-mediated inflammation [25]. Recent data indeed show that transgenic mice with skin-targeted overexpression of IL-18, exhibit exacerbated and prolonged allergic (Th2) and non-allergic (Th1) skin inflammation [18]. The fact that pro-IL-18 is abundantly present in normal human keratinocytes [13], generally in the presence of bioactive IL-12, suggested a likely role for IL-18 in facilitating Th1-like skin inflammation such as occurs in psoriasis. However, our results showed similar high constitutive quantities of IL-18 mRNA and protein in normal (NN) and psoriasis, stable, plaque-type lesional (stPP) epidermis. We therefore additionally evaluated the possibility that IL-18 might only be transiently upregulated in early, active and progressive psoriatic lesions (apPP) rather than in stable, plaque-type psoriatic lesions.

Indeed, in active and progressive lesions an elevated expression of total IL-18 protein was detected by ELISA. Similar levels of IL-18 mRNA together with elevated IL-18 protein levels in apPP epidermis suggest higher translational activity or enhanced protein stability in apPP when compared to normal and stPP epidermis. Naik *et al.* [12] and Otha *et al.* [26] did observe an elevated IL-18

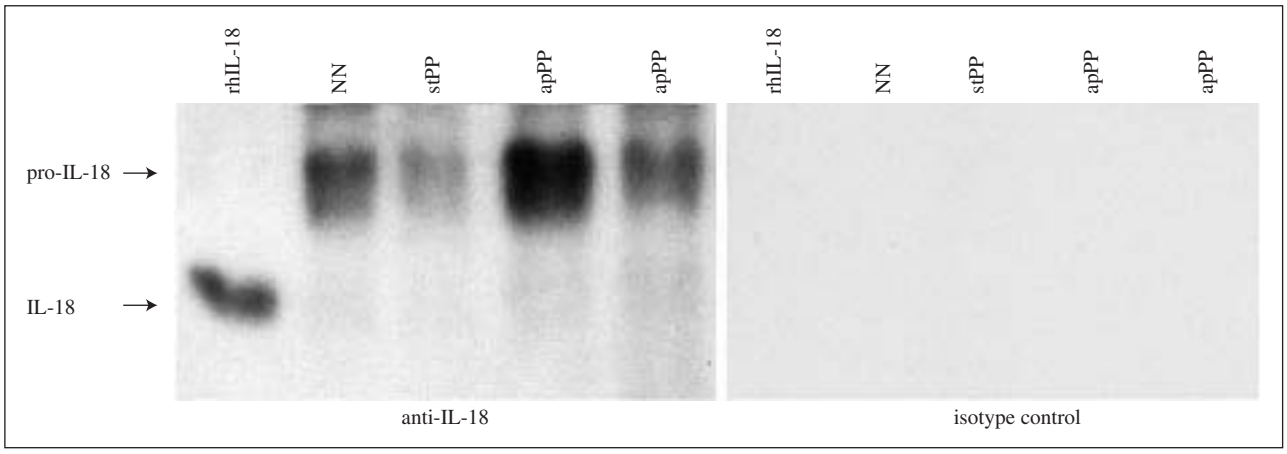

Figure 3
IL-18 in extracts of psoriatic lesional epidermis is predominantly in the unprocessed form.
Western blots of extracts from normal and psoriatic lesional epidermis were probed with an IL-18 specific mAb as described in the methods section. Recombinant human IL-18 was used as a positive control and an isotype matched antibody served as a negative control. Results from five extracts out of twelve are shown. rhIL-18: processed recombinant human IL-18; NN: normal epidermis; stPP: psoriatic, stable, plaque-type lesional epidermis, apPP: active and progressive psoriatic lesions. Pro-IL-18, 24 kDa; processed IL-18, 18 kDa.

protein and/or mRNA expression in psoriatic lesional skin. The differences between our results and these studies are probably due to methodological differences. In the case of the report by Naik *et al.*, a different primary antibody was used, whereas the tissue samples types used by Ohta *et al.* (whole skin biopsies and psoriatic scales) were different from the type used in our study (epidermal sheets).

In Crohn's disease, increased levels of processed, bioactive IL-18 were observed in extracts of lesional gut epithelium [27]. We therefore hypothesized that this might also occur in psoriatic lesional skin. Results from Western blot analysis showed that in all skin extracts, the expression of the 18 kDa form of IL-18 were comparable. As an alternative approach to assaying the presence of bioactive IL-18 in skin extracts, we used two different bioassays for IL-18, that both use the induction of IFN-γ as the read-out system. However, the assays used were not sensitive enough to detect bioactive IL-18 in extracts of normal, stPP or apPP epidermis.

The levels of IL-18 in psoriatic lesions as detected by Western blot are less clear than the differences observed using ELISA, and there could be several reasons for this. It might be the result of rapid degradation before addition of protease inhibitors during the extraction procedure of skin proteins. Degradation of pro- and mature IL-18, might explain the discrepancy between our ELISA results and Western blotting, since small fragments may not be detected in the blot.

The surplus of protease inhibitors in the extraction buffer might have prevented further processing *in vitro* of pro-IL-18, despite the presence in our skin samples of caspase-1 (data not shown) and other proteases that are able to cleave pro-IL-18 in normal and stPP epidermis [28-31].

Another possibility might be that in psoriatic skin *in vivo*, protease and caspase-1 activity in particular are inhibited via upregulation of natural inhibitors [32]. A natural inhibitor of caspase-1, the proteinase inhibitor (PI)-9, has indeed been demonstrated in human endothelial cells, cytotoxic cells and epithelial cells [33], which are all present in an activated state in psoriatic lesions. Whether other caspase-1 inhibitors such as CARD-8 [34], ICEBERG or pseudo-ICE [35] are expressed in human skin and are elevated in psoriatic lesional skin is presently unknown. Caspase-1 may also be inhibited is via S-nitrosylation by nitric oxide [36]. It is conceivable that this can occur *in vivo*, since nitric oxide is strongly induced in psoriatic lesions by IFN-γ.

Interleukin-18 can exert its pathological proinflammatory function(s) during early phases of psoriasis via various mechanisms. The most obvious mode of action of IL-18 is the induction of IFN-γ in synergy with IL-12, and, consequently, induction of Th1-mediated immune responses. This is illustrated in *Table 1*, which depicts a positive correlation between the increased levels of IL-8 (which is induced by IFN-γ), IL-12, IFN-γ and the IL-18 levels in apPP lesions.

In addition, IL-18 might exert its effects during early phases of psoriasis via IFN-γ independent routes such as: a) the induction of several chemokines in fibroblasts and neutrophils [37, 38]; b) increased T-cell adhesion to extracellular matrix ligands [39]; c) the induction of angiogenesis [40]; d) induction of specific chemotaxis in plasmacytoid DC [17], numbers of which are increased in psoriatic skin lesions [41]. Thus, IL-18 can be involved in the regulation of early inflammatory events by promoting the recruitment and adhesion of cells of the immune system to inflamed sites. The functional roles of specific proteases and anti-proteinases in normal and psoriatic skin at different stages of development of the disease require further investigation, since they seem to contribute to the amplification and the restriction of inflammation. Which of the abovementioned, IFN-γ-dependent or independent mechanisms of action of IL-18 is most relevant during the early initiation of psoriasis remains to be elucidated.

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