

Reno Debets<sup>1</sup>,  
Joost P. J. J. Hegmans<sup>1</sup>,  
Roger J. J. Troost<sup>2</sup>,  
Robbert Benner<sup>1</sup> and  
Errol P. Prens<sup>1</sup>

<sup>1</sup> Department of Immunology,  
Erasmus University Rotterdam,  
Rotterdam, The Netherlands

<sup>2</sup> Department of Dermatology,  
University, Hospital Rotterdam-  
Dijkzigt, The Netherlands

## Enhanced production of biologically active interleukin-1 $\alpha$ and interleukin-1 $\beta$ by psoriatic epidermal cells *ex vivo*: evidence of increased cytosolic interleukin-1 $\beta$ levels and facilitated interleukin-1 release

The expression of interleukin (IL)-1 is altered in psoriatic lesions. However, little is known about the actual production of IL-1 $\alpha$  and IL-1 $\beta$  by psoriatic epidermal cells (EC). We monitored IL-1 in the extracellular, the membrane and the intracellular compartment of freshly isolated EC from untreated lesional psoriatic (PP) and normal healthy (NN) skin during non-stimulated short-term cultures, representing a psoriasis model *ex vivo*. Cytokines were measured using bioassays combined with neutralizing antibodies and enzyme-linked immunosorbent assay in parallel. PP EC released significantly increased amounts of biologically active IL-1 $\alpha$  and IL-1 $\beta$  in a ratio of 3 : 1, whereas NN EC only released IL-1 $\alpha$ . Also, the release of IL-6, but not of TNF- $\alpha$ , by PP EC was significantly increased. Membrane-associated IL-1 activity, analyzed using glutaraldehyde-fixed EC, was low and not unique to PP EC. The cytosol of PP EC contained significantly increased levels of immunoreactive IL-1 $\beta$ . Furthermore, PP EC displayed loss of membrane integrity, as determined by trypan blue exclusion and release of cytosolic lactate dehydrogenase. This facilitated release of intracellular IL-1. Depletion of CD45<sup>+</sup> cells showed that intraepidermal leukocytes did not contribute to the production of IL-1. Our observations show that resident PP EC express enhanced IL-1 production *ex vivo*, which is due to an increased cytosolic IL-1 $\beta$  content and facilitated IL-1 release. This study provides the first evidence that PP EC can produce bioactive IL-1 $\beta$ .

### 1 Introduction

Human epidermal IL-1 is safely stored in the cytosol of the keratinocyte and stimuli (*e.g.* cell injury, T cell interaction) are required for its release [1]. Intracellularly stored IL-1 consists of pro-IL-1 $\alpha$  and pro-IL-1 $\beta$ , which both lack a signal peptide but differ in the way they are processed and modified after translation [2]. Pro-IL-1 $\alpha$  is fully biologically active, whereas pro-IL-1 $\beta$  has minimal biological activity [3]. Cultured normal human keratinocytes are unable enzymatically to cleave pro-IL-1 $\beta$  efficiently to the active mature form [4]. This implies that in normal human epidermis, all IL-1 activity is due to IL-1 $\alpha$  [5].

The epidermal expression of IL-1 is altered in psoriasis. IL-1 $\alpha$  protein is reported to be decreased in cytosolic extracts of psoriatic skin (PP) specimens and aqueous extracts of the stratum corneum, resulting in a considerable decrease of the total epidermal IL-1 activity [6–8]. IL-1 $\alpha$  levels were low but not decreased in suction blister

fluids from PP skin [9]. In contrast to IL-1 $\alpha$ , cytosolic extracts of PP skin contained increased levels of a processed, but non-functional form of IL-1 $\beta$  [6, 7]. IL-1 $\beta$  levels were negligible in extracts of the stratum corneum and in blister fluids [8, 9] and its expression seemed confined to the plasma membrane and the intracellular compartment of epidermal cells (EC) [10, 11]. The down-regulation of IL-1 $\alpha$  and up-regulation of IL-1 $\beta$  found with cytosolic skin extracts was not observed using immunohistochemistry [10]. Conflicting reports also exist on the expression of the IL-1-related cytokines IL-6 and TNF- $\alpha$  in psoriatic epidermis [8, 12, 13]. Taken together, available data are complex and inconsistent due to analysis of cytokines in different cellular compartments and at different levels of expression. To clarify the altered expression of epidermal cytokines in psoriasis, the focus of the present study was to characterize the actual production of IL-1, IL-6 and TNF- $\alpha$  by PP EC.

We investigated the spontaneous release of these cytokines during short-term cultures of freshly isolated EC from untreated PP and normal healthy (NN) skin, using a defined low-calcium basal medium. We considered these cultures to be an *ex vivo* model which is less prone to *in vitro* artefacts than cell lines. To study the epidermal IL-1 production in psoriasis, the extracellular, the membrane and the intracellular compartment of the same EC samples were analyzed for IL-1. Moreover, EC samples were assayed for several cytokines simultaneously and for different levels of protein expression in parallel. We tested for biologically active and immunoreactive IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$ .

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**Correspondence:** Reno Debets, Department of Immunology, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands (Fax: 31-10-4 36 7601)

**Abbreviations:** EC: Epidermal cell(s) NN: Normal healthy control PP: Lesional psoriasis

**Key words:** Interleukin-1 / Epidermis / Psoriasis / *Ex vivo*

## 2 Materials and methods

### 2.1 Patients and controls

Thirty-five otherwise healthy patients (Outpatient Department of Dermatology, University Hospital Rotterdam-Dijkzigt) with plaque-type psoriasis were studied after informed consent. Patients remained untreated for at least 3 weeks before entering the study. Thirty-five healthy volunteers without history or signs of skin disease, undergoing abdominal or breast plastic surgery (Sint Franciscus Hospital, Rotterdam) served as controls. Skin samples were collected after approval of the institutional medical ethical committee (MEC 104.050/SPO/1990/30).

### 2.2 EC suspensions

Split-skin specimens from PP and NN skin of 10–15 cm<sup>2</sup> were obtained using a portable dermatome (Davol, Cranston, RI). Preparation of single EC suspensions was based on standard methods [14]. Cells were resuspended in a chemically defined keratinocyte basal medium (KBM/unsupplemented MCDB 153, Clonetics, San Diego, CA). Cells were counted microscopically using phase-contrast illumination. Viability was determined by trypan blue exclusion.

### 2.3 EC-conditioned media

EC were seeded at 10<sup>6</sup> viable cells/ml in 2 ml KBM in 25 cm<sup>2</sup> vented culture flasks (Costar, Cambridge, MA). After culture for 24 h, the viability of the cells was checked and SN were collected by centrifugation, filter-sterilized, supplemented with 0.1 % BSA and stored at -80°C.

### 2.4 Fixation of EC

Freshly isolated EC or EC cultured for 24 h, were fixed with freshly prepared 0.08 % glutaraldehyde (Merck, Schuchardt, FRG) in 0.1 M sodium cacodylate buffer pH 6.9 for 10 s at 4°C. Subsequently, cells were washed twice with HEPES-buffered RPMI 1640 containing 0.5 % BSA. Next, cells were resuspended at 10<sup>6</sup>/ml in RPMI 1640 and incubated for 24 h at 37°C in a humidified atmosphere to release unfixed IL-1. SN and cells were collected separately and stored at 4°C.

### 2.5 Isolation of EC cytosol

EC were treated with digitonin as described for human monocytes [15]. Digitonin treatment perforates the plasma membrane without affecting intracellular organelles, resulting in leakage of pure cytoplasm. Digitonin and its solvent ethanol were removed from the cytosol by ultrafiltration of PBS diluted samples using a YM membrane with a 10-kDa cutoff (Centricon-10, Amicon, Danvers, MA). After filter-sterilization, samples were stored at -80°C. Lactate dehydrogenase (LDH) served as a marker for cytosol. Enzyme activity was determined by the rate of NAD<sup>+</sup> formation measured spectrophotometrically from the decrease in extinction at 340 nm.

### 2.6 Bioassays

IL-1 activity was measured using a subline of the murine T cell line D10.G4.1, designated D10(N4)M (D10) (kindly provided by Dr. S. J. Hopkins, Manchester, GB) [16]. Further optimization of the assay at our laboratory resulted in enhanced reproducibility of the test [17]. IL-6 activity was measured using the murine hybridoma cell line B9 (kindly donated by Prof. Dr. L. A. Aarden, Amsterdam, The Netherlands) [18]. Proliferation of the cytokine-dependent cell lines D10 and B9 was measured by [<sup>3</sup>H]dThd incorporation. TNF- $\alpha$  activity was measured using the murine fibroblast cell line WEHI 164.13 (kindly provided by Dr. W. A. Buurman, Maastricht, The Netherlands) [19]. The MTT cytotoxicity assay [20] served as an indirect measurement of necrosis of WEHI cells. Recombinant human (Hu)IL-1 $\beta$  (UBI, Lake Placid, NY), rHuIL-6 (Prof. Dr. L. A. Aarden) and rHuTNF- $\alpha$  (UBI) served as positive controls in the D10, B9 and WEHI assays, respectively. Cytokine activities were corrected for background activity of the culture medium and expressed in U per 10<sup>6</sup> viable cells, with 1 U/ml corresponding with the half-maximal response.

Neutralization experiments using the D10 assay were performed with sheep-anti-rHuIL-1 $\alpha$  and/or IL-1 $\beta$  Ab (Glaxo, Geneva, Switzerland). After addition of the Ab to the optimally diluted samples and incubation for 45 min at 37°C and 5 % CO<sub>2</sub>, the D10 cells were added. The anti-IL-1 $\alpha$  and IL-1 $\beta$  Ab, used at a titer of 15 000, neutralized about 10 U/ml natural NN EC-derived IL-1 $\alpha$  and PBMC-derived IL-1 $\beta$ , respectively. Neutralization results are given as the percentage residual IL-1 activity and were calculated using the formula:

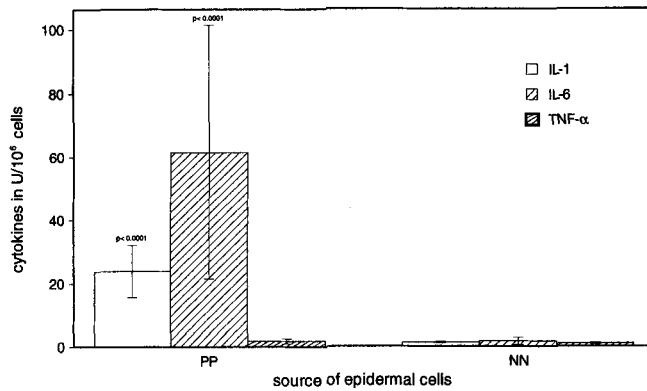
$$\% \text{ residual IL-1 activity} = \frac{\text{cpm neutralized sample} - \text{cpm background}}{\text{cpm non-neutralized sample} - \text{cpm background}} \times 100 \%$$

### 2.7 ELISA

Immunoreactive IL-1 $\alpha$ , IL-1 $\beta$  and IL-6 were measured with commercially available specific ELISA kits (D. Bergman, Eurogenetics, Tessenderlo, Belgium) using the protocols provided by the manufacturers. TNF- $\alpha$  was determined as described elsewhere [21], with minor adaptations to enhance the sensitivity. Recombinant HuIL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$  served as positive controls. The amounts of cytokine were corrected for nonspecific binding of the culture medium, and expressed in pg per 10<sup>6</sup> viable cells.

### 2.8 Depletion of CD45<sup>+</sup> cells from EC suspensions

Leukocytes were removed from freshly isolated PP EC by an immunomagnetic rosetting technique using the anti-CD45 mAb 2D1 (Becton Dickinson, Mountain View, CA) and goat-anti-mouse Ab-conjugated paramagnetic beads (Dyna, Oslo, Norway), as described [22]. Before and after depletion, the number of CD45<sup>+</sup> cells were determined using a two-step immunoperoxidase technique on cytocentrifuge preparations. The percentage of CD45<sup>+</sup> cells was calculated by counting the number of cells showing positive staining from a total of 1000 cells. The depletion efficiency ranged from 95% to 99%.



**Figure 1.** Release of biologically active cytokines by PP and NN EC in basal medium. Levels of IL-1, IL-6 and TNF- $\alpha$  in 24 h SN of KBM cultures were measured using bioassays. Results are given in mean  $\pm$  SEM U/10<sup>6</sup> viable cells. PP:  $n = 29, 20$  and  $8$ ; and NN:  $n = 32, 19$  and  $12$  for IL-1, IL-6 and TNF- $\alpha$ , respectively. The  $p$  values indicate significant differences versus NN samples. Human PBMC isolated by Ficoll-Hypaque density gradient centrifugation and cultured for 24 h in RPMI 1640 supplemented with 5% human serum were used as a control. PBMC released 52 U IL-1; 24 816 U IL-6; and 14 U TNF- $\alpha$  per 10<sup>6</sup> cells.

## 2.9 Statistical analysis

Results were analyzed with the Wilcoxon Rank Sum Test using STATA™ (Computing Resource Center, Los Angeles, CA). Significant differences are indicated by  $p$  values.

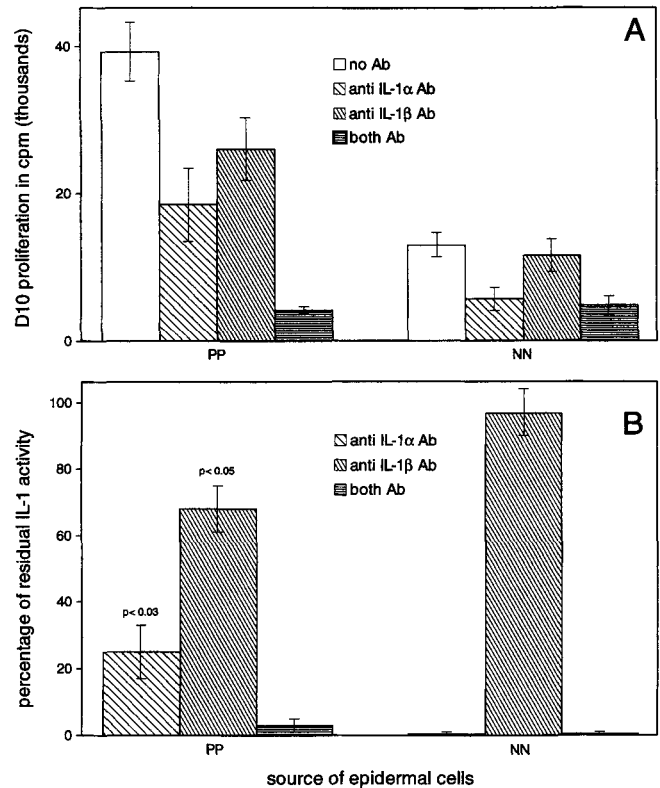
## 3 Results

### 3.1 PP EC release increased amounts of biologically active IL-1 and IL-6, but not TNF- $\alpha$

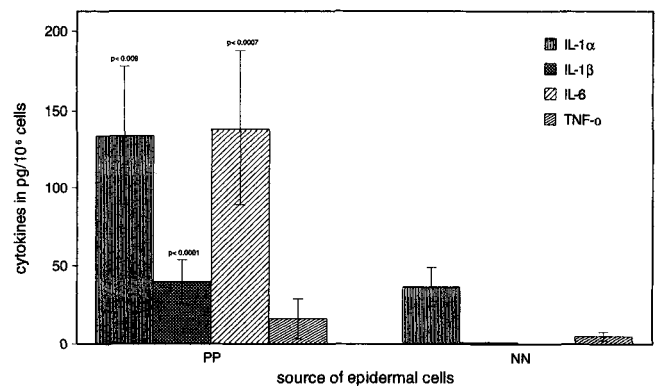
The amounts of cytokine released by EC from PP and NN skin are given in Fig. 1. It is evident that although NN EC release some IL-1, IL-6 and TNF- $\alpha$  under conditions *ex vivo*, PP EC release at least 15-fold more IL-1 ( $24 \pm 8$  U/10<sup>6</sup> EC,  $p < 0.0001$ ) and 30-fold more IL-6 ( $61 \pm 40$  U/10<sup>6</sup> EC,  $p < 0.0001$ ). However, PP EC did not release increased amounts of bioactive TNF- $\alpha$ .

### 3.2 PP EC release biologically active IL-1 $\beta$

The same samples were tested in the D10 assay using IL-1-neutralizing Ab as well as in IL-1 $\alpha$  and IL-1 $\beta$  ELISA. The effect of neutralizing Ab against IL-1 $\alpha$  and IL-1 $\beta$  on the IL-1 activity of EC SN is given in Fig. 2. When NN samples were used, anti-IL-1 $\alpha$  Ab reduced the [<sup>3</sup>H]dThd incorporation to background levels, in contrast to anti-IL-1 $\beta$  Ab that did not affect the D10 proliferation (Fig. 2A). Thus, the IL-1 activity of NN samples is completely due to IL-1 $\alpha$ . The IL-1 activity of SN of PP EC, however, could only be completely blocked when both Ab were used simultaneously. The residual IL-1 activity of SN of PP EC neutralized with either Ab differed significantly from those of NN EC ( $p < 0.03$  and  $p < 0.05$  for neutralization of IL-1 $\alpha$  and IL-1 $\beta$  activity, respectively) (Fig. 2B). Approximately 25% of the IL-1 activity of SN of PP EC was due to



**Figure 2.** PP EC release biologically active IL-1 $\beta$ . Neutralization of IL-1 activity of 24 h KBM SN of EC from PP and NN skin was determined with sheep-anti-rHuIL-1 $\alpha$  and/or IL-1 $\beta$  Ab using the D10 assay. Samples were diluted five times. Results are given in mean  $\pm$  SEM cpm (A). PP and NN groups consisted of 14 and 6 patients, respectively. Controls: rHuIL-1 $\beta$  (12.5 U/ml): 42 648 cpm; KBM: 6967 cpm; anti-IL-1 $\alpha$  Ab: 6874 cpm; anti-IL-1 $\beta$  Ab: 5671 cpm; and both Ab: 4316 cpm. Results are also expressed as the residual IL-1 activity after neutralization (B), calculated as described in Sect. 2.6. Shown are mean  $\pm$  SEM. Conditioned medium of PBMC was used as a control. See legend to Fig. 1 for more details. Neutralization of PBMC SN with anti-IL-1 $\alpha$  Ab and anti-IL-1 $\beta$  Ab resulted in 97 and 3% residual IL-1 activity, respectively.



**Figure 3.** Release of immunoreactive cytokines by PP and NN EC in basal medium. Levels of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were measured using ELISA. Results are presented as mean  $\pm$  SEM pg/10<sup>6</sup> viable cells. PP:  $n = 19, 24, 12$  and  $8$ ; and NN:  $n = 20, 19, 11$  and  $8$  for IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , respectively. Conditioned medium of PBMC contained 151 pg IL-1 $\alpha$ ; 137 pg IL-1 $\beta$ ; 70 950 pg IL-6; and 677 pg TNF- $\alpha$  per 10<sup>6</sup> cells.

**Table 1.** IL-1 activity of fixed PP and NN EC<sup>a)</sup>

	Psoriasis	Normal control	<i>p</i> value
Directly fixed	0.9 $\pm$ 0.9	1.3 $\pm$ 1.3	NS
Fixed after short-term culture	7.2 $\pm$ 4.1	9.0 $\pm$ 6.9	NS
Unfixed control	41.3 $\pm$ 19.5	4.5 $\pm$ 1.9	<0.05

a) Results are presented as mean  $\pm$  SEM U IL-1/10<sup>6</sup> treated EC. Each experimental condition was performed with five to ten samples of both PP and NN EC. PBMC used as control cells expressed 0, 121 and 267 U IL-1/10<sup>6</sup> cells when directly fixed, fixed after short-term culture and unfixed, respectively.

IL-1 $\beta$ . SN of NN EC contained negligible IL-1 $\beta$  activity. The use of anti-IL-6 Ab as a control had no effect in this system.

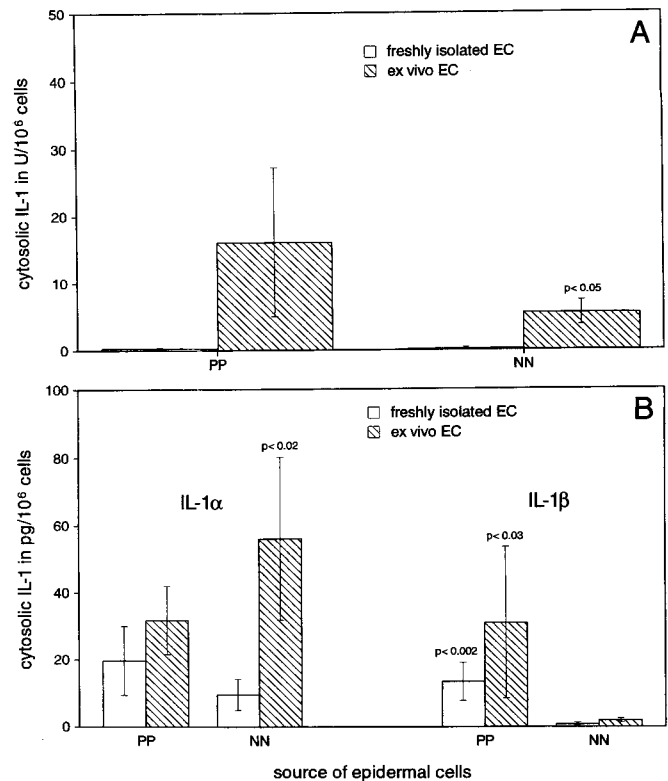
The release of IL-1 $\beta$  by PP EC was confirmed using IL-1 $\alpha$  and IL-1 $\beta$  ELISA. Fig. 3 shows that both EC types released predominantly IL-1 $\alpha$  under basal short-term culture conditions, with the amounts highest for PP EC (134  $\pm$  45 versus 37  $\pm$  12 pg/10<sup>6</sup> EC, *p* < 0.009). The amounts of immunoreactive IL-1 $\beta$  released by PP EC were about three-fold lower than the corresponding amounts of IL-1 $\alpha$  (40  $\pm$  14 pg/10<sup>6</sup> EC), but nevertheless significantly increased when compared to the amounts of IL-1 $\beta$  released by NN EC (*p* < 0.0001). These ELISA findings are in accordance with the IL-1 neutralization results, indicating that the increased basal release of bioactive IL-1 by PP EC is due to both IL-1 $\alpha$  and IL-1 $\beta$ , which are released in a ratio of 3:1. ELISA results for IL-6 and TNF- $\alpha$  are also given in Fig. 3, and are in agreement with the data on IL-6 and TNF- $\alpha$  activity presented in Fig. 1. Only levels of immunoreactive IL-6 in SN of PP EC were significantly increased (*p* < 0.0007).

### 3.3 PP EC do not express membrane IL-1 activity

To study the interrelationship between the increased release of IL-1 and the previously observed membrane expression of IL-1 $\beta$  on PP EC [11], the same EC samples were tested for membrane IL-1 activity. EC were fixed with glutaraldehyde and tested in the D10 assay. The possibility that the measured IL-1 had leaked out of the fixed cells instead of representing genuine membrane IL-1 activity was checked by testing the SN of the fixed cells. These SN contained less than 1 U per 10<sup>6</sup> cells. Results on IL-1 activity of fixed PP and NN EC samples are shown in Table 1. Fixation of freshly isolated PP and NN EC resulted in negligible IL-1 activities. Both types of EC slightly upregulated the expression of IL-1 activity during a 24 h culture in KBM. The levels of IL-1 activity found with unfixed PP EC were significantly increased due to release of IL-1.

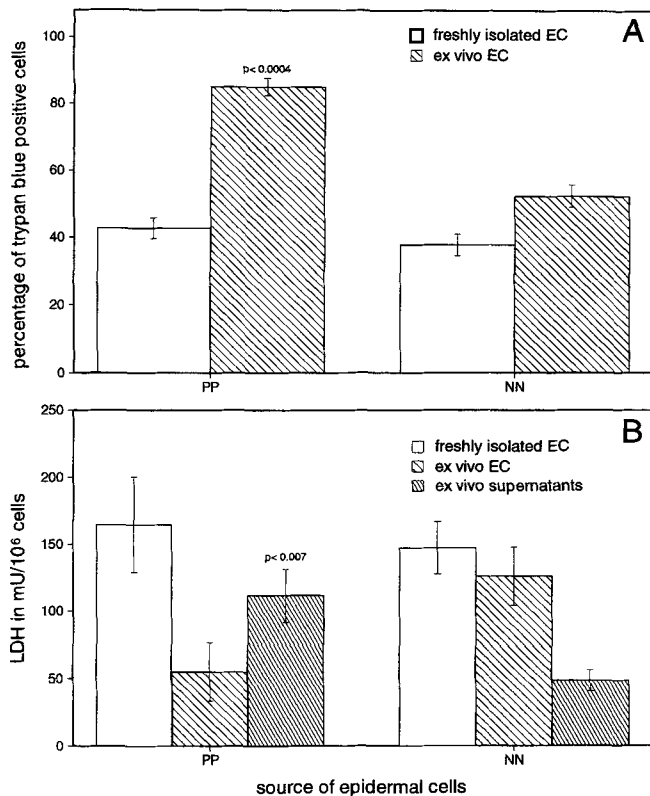
### 3.4 PP EC express increased levels of cytosolic IL-1 $\beta$

To investigate the intracellular compartment, cell samples were treated with digitonin. The cytosolic fractions were



**Figure 4.** PP EC express an increased cytosolic IL-1 $\beta$  content. Levels of biologically active IL-1 (A) and immunoreactive IL-1 $\alpha$  and IL-1 $\beta$  (B) in cytosolic fractions of freshly isolated EC and short-term cultured EC, termed *ex vivo* EC, from PP and NN skin are given in mean  $\pm$  SEM U or pg per 10<sup>6</sup> total cells, respectively. PP: *n* = 12 and 8; and NN: *n* = 6 and 6 for freshly isolated EC and EC *ex vivo*, respectively. Cytosolic fractions were obtained using digitonin. Samples were tested with the D10 assay, IL-1 $\alpha$  and IL-1 $\beta$  ELISA. The *p* values placed above bars of PP samples indicate significant differences between PP and the corresponding NN samples, whereas the *p* values placed above bars of NN samples *ex vivo* indicate significant differences between NN samples *ex vivo* and freshly isolated NN samples. PP samples *ex vivo* did not differ significantly from freshly isolated PP samples. Freshly isolated PBMC and PBMC *ex vivo* expressed 0 U IL-1; 11 pg IL-1 $\alpha$ ; and 2 pg IL-1 $\beta$ , and 109 U IL-1; 688 pg IL-1 $\alpha$ ; and 1655 pg IL-1 $\beta$  intracellularly per 10<sup>6</sup> total cells, respectively.

tested in the D10 assay and in the IL-1 $\alpha$  and IL-1 $\beta$  ELISA in parallel. The efficiency of the ultrafiltration procedure was more than 95% as evaluated by the recovery of IL-1 from PBS spiked with 10 U IL-1. Fig. 4A shows that the cytosolic IL-1 activity of freshly isolated PP and NN EC was negligible. Short-term culture resulted in an increase of intracellular IL-1 activity in both EC types. However, this increase was only statistically significant for NN EC (*p* < 0.05). Levels of immunoreactive cytosolic IL-1 $\alpha$  were significantly upregulated in NN EC during a 24 h culture period (*p* < 0.02) (Fig. 4B). The amounts of cytosolic IL-1 $\beta$  were negligible in NN EC. In freshly isolated PP EC, the cytosolic IL-1 $\alpha$  levels were somewhat higher than those of NN EC. The cytosolic IL-1 $\beta$  levels were significantly increased in freshly isolated (*p* < 0.002) and cultured PP EC (*p* < 0.03) when compared to NN EC. However, intracellular IL-1 $\alpha$  and IL-1 $\beta$  levels did not significantly increase in PP EC during short-term culture (Fig. 4B).



**Figure 5.** PP EC display enhanced cytosolic leakage. Loss of cellular viability during a 24 h culture of PP and NN EC in KBM was measured by trypan blue exclusion (A). Given are mean  $\pm$  SEM percentage of trypan blue positive cells of freshly isolated EC and EC *ex vivo*. PP:  $n = 11$  and 8; and NN:  $n = 10$  and 10 for freshly isolated EC and EC *ex vivo*, respectively. Part of these samples, together with the corresponding conditioned media (termed SN *ex vivo*) were used to measure cytosolic and released LDH activity (B). Cytosolic fractions were obtained by digitonin treatment of the cells. The LDH activity is given in mean  $\pm$  SEM mU/10<sup>6</sup> total cells. PP:  $n = 8, 5$  and 8; and NN:  $n = 5, 5$  and 10 for freshly isolated EC, EC *ex vivo* and SN *ex vivo*, respectively. PBMC did not show alterations in their viability using culture conditions as described in the legend to Fig. 1. LDH levels corresponding with freshly isolated PBMC, PBMC *ex vivo* and SN were 46, 100 and 7 mU/10<sup>6</sup> cells, respectively.

### 3.5 PP EC show facilitated release of IL-1 due to enhanced cytosolic leakage

Viabilities of freshly isolated EC from PP and NN skin, as determined by trypan blue dye exclusion, were similar. However, loss of viability during a 24 h culture in KBM was more than twofold higher for PP EC than for NN EC ( $p < 0.0004$ ), as shown in Fig. 5A. The loss of membrane integrity of PP EC correlated well with cytosolic leakage, monitored by release of LDH. The release of LDH from PP EC during a 24 h culture period was about twofold higher when compared to NN EC (Fig. 5B,  $p < 0.007$ ). This also applied to the concomitant decrease in cytosolic LDH. Release of cytosolic LDH correlated with that of IL-1.

### 3.6 Production of IL-1 by PP EC is not due to contaminating intraepidermal CD45<sup>+</sup> cells

Immunophenotyping of freshly isolated PP EC revealed that these cells contain about 5% CD45<sup>+</sup> cells as well as

**Table 2.** CD45<sup>+</sup> EC are the cellular source for IL-1 in psoriasis<sup>a)</sup>

	Experiment I	Experiment II
Total EC	57 550	36 444
CD45 <sup>+</sup> EC	54 325	32 110
Medium	6 530	5 420

a) D10 proliferation rates of two separate CD45 depletion experiments are given in cpm (mean of triplicate values with SEM less than 5% of mean), corresponding to five times-diluted samples. The depletion efficiency in both experiments was more than 95%.

HLA-DR<sup>+</sup> cells [14]. To determine whether intraepidermal leukocytes were responsible for the PP EC-derived IL-1, CD45<sup>+</sup> cells were immunomagnetically depleted before measuring the IL-1 production. From Table 2, it is evident that CD45<sup>+</sup> cells were not responsible for the enhanced production of biologically active IL-1. The presence of IL-1 $\beta$  in the SN of CD45-depleted EC was confirmed by neutralization studies as well as ELISA.

## 4 Discussion

In the present study, we show for the first time, using a large number of patients and controls, that PP EC release significantly increased amounts of bioactive IL-1. These results extend preliminary data reported by our group [17]. Other investigators, however, observed decreased epidermal IL-1 bioactivity in psoriasis [6–8]. The reported down-regulation of IL-1 $\alpha$  and up-regulation of a non-functional IL-1 $\beta$  *in vivo* [7] made us investigate the separate contributions of IL-1 $\alpha$  and IL-1 $\beta$  to the observed extracellular IL-1 activity. About 25% of the IL-1 activity of PP samples was due to IL-1 $\beta$ , while the IL-1 $\beta$  activity of NN samples was negligible. This corresponded nicely with the ELISA results. The capacity of PP EC to produce functionally active IL-1 $\beta$  is an unexpected and novel finding. Our observation that PP EC release equivalent amounts of bioactive and immunoreactive IL-1 does not point to the involvement of IL-1 receptor antagonist (IL-1ra) in psoriasis.

Since overexpression of IL-1 $\beta$  in the membrane and the intracellular compartment of PP EC has previously been reported [10, 11], we questioned whether PP EC express membrane-associated IL-1 activity. Membrane-associated IL-1 activity has been demonstrated on a variety of cell types, including keratinocytes [23]. Recently, it has been suggested that the assumed membrane IL-1 activity may be caused by the leakage of IL-1 from cells inadequately fixed with paraformaldehyde [24]. In our studies of membrane IL-1 activity, glutaraldehyde-fixed EC were tested in the D10 assay. Glutaraldehyde proved to be a more stringent fixative than paraformaldehyde, and abrogated leakage of intracellular IL-1. Our results do not provide evidence of a unique expression of membrane-bound IL-1 by PP EC.

Analysis of the intracellular compartment indicated that PP EC have a significantly increased cytosolic IL-1 $\beta$  content, and that newly formed IL-1 $\alpha$  and IL-1 $\beta$  are not retained intracellularly under conditions *ex vivo*. The

opposite is true for NN EC, which express negligible levels of cytosolic IL-1 $\beta$ , accumulate IL-1 $\alpha$  synthesized *de novo*, but do not release it *ex vivo* (Figs. 3 and 4B). Antibodies directed against the mature IL-1 isoforms often underestimate the actual intracellular pro-IL-1 levels due to incomplete cross-reactivity. This underlines the significance of the differences found at the cytosolic level with the IL-1 ELISA. Using freshly isolated EC, we could not confirm the decreased expression of cytosolic IL-1 $\alpha$  protein in extracts from PP skin as reported by others [6, 7]. Our findings of increased expression of intracellular IL-1 $\beta$  protein are in accordance with other reports [6, 7, 11]. In line with our results is the recent report of increased expression of IL-1 $\beta$  mRNA in lesional as well as non-lesional psoriatic skin [25].

Loss of membrane integrity may be associated with processing and secretion of IL-1. In this respect, ionic perturbation could be necessary for activation of the IL-1 converting enzyme [26]. The issue of whether membrane processes are involved in the production of biologically active IL-1 $\beta$  by PP EC was addressed. We demonstrated profound loss of membrane integrity and enhanced cytosolic leakage of PP EC under conditions *ex vivo* using trypan blue exclusion, FACScan analysis of propidium iodide-labeled cell samples (not shown), and measurement of LDH release. Thus, release of intracellularly stored proteins such as IL-1 is facilitated by cytosolic leakage in PP EC. For human monocytes, it has already been shown that the release of IL-1 paralleled that of LDH [27]. IL-1 $\beta$  processing may precede the secretion process, as previously suggested by Perregaux and others for monocytic cells [26]. Moreover, rapid apoptosis, as in macrophages that are targets for allospecific cytotoxic T cells, is also characterized by intracellular IL-1 $\beta$  processing [28]. The partial IL-1-neutralizing effect of anti-IL-1 $\beta$  Ab using cytosolic PP samples suggests that pro-IL-1 $\beta$  is processed intracellularly by PP EC. This is in accordance with the finding that intracellular levels of bioactive IL-1 correlate positively with the intracellular levels of immunoreactive IL-1 $\beta$  (not shown). In our laboratory, we are currently investigating to what extent necrosis and apoptosis contribute to the observed processing of cytosolic IL-1 $\beta$ .

The enhanced IL-1 production by PP EC could be attributed to contaminating intraepidermal leukocytes. To identify the potential contribution of bone marrow-derived cells, we tested PBMC and a variety of cell lines such as a B cell line (JY), a T cell line (HSB) and a myelomonocytic cell line (HL-60) using serum-containing media with and without LPS as a stimulus (not shown). These experiments showed that PBMC, as expected, are a prominent source of IL-1 in all cellular compartments. Nevertheless, results of the depletion experiments indicate that CD45<sup>+</sup> EC were responsible for the observed IL-1 production *ex vivo*. These results were confirmed by testing normal human PBMC for their IL-1 levels under similar conditions *ex vivo*. Extracellular as well as intracellular IL-1 levels were not detectable when PBMC were cultured in the basal medium KBM at 10<sup>4</sup>/ml and 10<sup>5</sup>/ml, reflecting 1% and 10% potential leukocyte contamination of EC samples, respectively. Our results suggest the existence of an IL-1 convertase associated with resident PP EC, which up to now is thought to be confined to myelomonocytic cells. This could be in line with reports on enzyme alterations in PP EC [29].

Bioassay and ELISA findings showed that PP EC also released increased amounts of IL-6, but not TNF- $\alpha$ . Our IL-6 results are in agreement with findings based on skin biopsies [12] and blister fluids [11], but in disagreement with findings based on extracts of the stratum corneum [8]. We found low, but consistent levels of biologically active and immunoreactive TNF- $\alpha$  in SN of both types of EC, while others could not detect TNF- $\alpha$  activity using extracts of the stratum corneum or blister fluids from either PP or NN skin [8, 13]. Our findings may be attributable to the use of viable EC to investigate cytokine production levels.

Cytokine production measured *ex vivo* is not merely based on the passive release of preformed cytosolic proteins (e.g. IL-1), and is, in our view, closely related to the cytokine production *in vivo* for the following reasons. First, up-regulation of cytosolic IL-1 proteins (Fig. 4, NN EC) and production of functionally active IL-1 $\beta$  during short-term culture (Fig. 2, PP EC) indicate active synthesis and processing of IL-1. Second, the release of increased amounts of IL-6, but not TNF- $\alpha$ , by PP EC point to selective cytokine synthesis *de novo*. Third, the reported up-regulation of cytosolic IL-1 $\beta$  by normal human keratinocytes *in vitro* [30] was not observed in our model, and the increased expression of cytosolic IL-1 $\beta$  in PP EC is in agreement with reported findings *in vivo* [7, 25]. Finally, treatment of psoriatic lesions *in vivo* with a class III topical corticosteroid resulted in normalization of the amounts of IL-1 and IL-6 released by PP EC under conditions *ex vivo* [17].

Taken together, these results provide clear evidence that PP EC show enhanced production of functionally active IL-1 $\alpha$  and IL-1 $\beta$  *ex vivo* due to enhanced release of IL-1 and activation of cytosolic IL-1 $\beta$  concomitant with its release. PP EC also release increased levels of IL-6, but not TNF- $\alpha$ . Others have shown that normal human keratinocytes need a stimulus to produce substantial amounts of inflammatory cytokines *in vitro* [31]. Furthermore, 48 h serum-free culture SN of PP EC, but not NN EC, potentiate T cell activation which was shown to be partly due to IL-1 [32]. The pathophysiological relevance of IL-1 *in vivo* is evidenced by transgenic mice in which EC-derived IL-1 induced scaly inflammatory skin lesions with marked erythema, crusting and psoriasis-like histological features such as hyperkeratosis and a dense inflammatory infiltrate [33]. Mice which express an IL-6 transgene in basal keratinocytes demonstrated a thickened stratum corneum, but IL-6 did not exert direct proinflammatory actions and probably acts synergistically with EC-derived IL-1 [34]. We speculate that PP EC display an activated state *in vivo* and are easily triggered to express an enhanced production of IL-1 and IL-6.

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