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ORIGINAL PAPER

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A modified *ex vivo* skin organ culture system for functional studies

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Abstract **To investigate the immunological function of cells in normal and diseased skin under conditions approximating the *in vivo* situation, it is necessary to maintain the structural integrity of the tissue. To achieve this, freshly isolated skin has to be cultured *ex vivo*, or an *in vitro*-constructed complete skin equivalent may be used. Different skin organ culture systems have been described. Basically two systems prevail: submerged or air-exposed skin organ cultures. The former model has been used for measuring cytokine secretion by skin cells in the medium, and the latter to study the expression of cell membrane proteins in situ and the kinetics of epidermal Langerhans cells. Here we present a modified *ex vivo* skin organ culture system which approaches the *in vivo* situation by maintaining the normal skin architecture without spontaneous induction of the regenerative maturation markers. This method allowed the expression of cell membrane proteins in situ to be measured, and the cytokine secretion by skin cells in the culture medium to be quantitated in the same experiment. In this system, both general and specific stimuli (LPS and IL-1β) upregulated the expression of skin-derived cytokines IL-8 and IL-6 in the medium and different markers (ICAM-1, CD40 and CD86) on cells in the tissue in a 24-hour culture-formed. Elevation of both cytokine and cell marker expression could be blocked by dexamethasone and by IL-1ra which acts specifically on IL-1β-mediated responses. The system presented here is both quick and simple and can be used as a model to study the behaviour of skin cells in their natural microenvironment.**

Keywords Psoriasis · LPS · IL-1 · Cytokine · Dexamethasone

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Introduction

After initiation of inflammation in the skin, a complex network of immune reactions come into effect. Cells interact with each other directly through cell-cell contact or indirectly through cytokine signalling. Interaction of the cells with the extracellular matrix also plays a crucial role during immune processes. Thus, the microenvironment of the skin dictates the outcome of these interactions. To investigate the interaction of cells in situ, an intact microenvironment is essential. One way to study the behaviour of skin cells in their microenvironment is by making use of the SCID-hu xenogeneic transplantation model (reviewed in reference 1). In this system human skin is transplanted onto SCID mice in order to investigate different parameters of the immune system in intact skin. Boehncke et al. [2] and Wrone-Smith [3] have shown that this model is a valid tool for investigating cellular immunity in skin. In addition, it has recently been shown that this model allows the screening of antipsoriatic drugs [4].

However, the SCID-hu xenogeneic transplantation model has some disadvantages. It is time-consuming, requires specific expertise and facilities, and furthermore, although the SCID mouse does not have functional T and B cells, it possesses other immunocytes that might have an effect. An alternative to this model is the culturing of total skin. Skin organ culture models have been used for a long time (reviewed in reference 5). Different methods have been used, varying from culture of skin biopsies in immersed medium [6, 7] to methods in which skin is cultured on metal grids covered by filter paper [8, 9]. The latter method was developed in 1959 by Trowell [10] and was later modified by Jensen et al. [11].

Several studies have been performed using an *ex vivo* culture system. These studies include analysis of the behaviour of epidermal Langerhans cells [12], differences in cytokine expression in normal and lesional psoriatic skin

[13], and the function of epidermal growth factor (EGF) in psoriatic skin [14]. These models were either used to investigate the modulation of certain cell markers in the tissue or secretion of cytokines in the medium. Moreover, in most studies the skin was cultured under normal conditions (atmosphere containing 5% CO_2 at 37 °C) in which diseased skin, for example psoriatic lesional skin, deteriorates. This makes monitoring of modulatory effects on diseased skin in these systems difficult.

We describe here a modified skin organ culture model based on the method presented by Trowell [10], which allows monitoring of skin cell activation and differentiation in situ and cytokine release into the culture medium. The effects of two immunostimulators and suppressors on the expression of different immunologically relevant parameters in both culture medium and skin tissue were monitored simultaneously using this system. Lipopolysaccharide (LPS) stimulates inflammatory reactions through different receptors [15, 16], whereas IL-1β acts in a more specific manner and signals specifically through the type I IL-1 receptor which is present on most cells in the skin, including keratinocytes [17, 18]. Using these stimuli we were able to stimulate secretion of IL-8 and IL-6 in the culture medium, as well as the expression of CD40, CD86 and ICAM-1 on cells in the tissue sample. In addition, we were able to block the elevation of these markers using pharmacologically relevant agents including dexamethasone, a general immunosuppressive agent, and IL-1 receptor antagonist (IL-1ra), a specific antagonist of IL-1. We also showed that IFN-γ secretion by psoriatic lesional skin biopsies was induced after stimulation of T cells through CD3.

This *ex vivo* culture system allows monitoring of different immunological parameters after stimulation or inhibition by different agents.

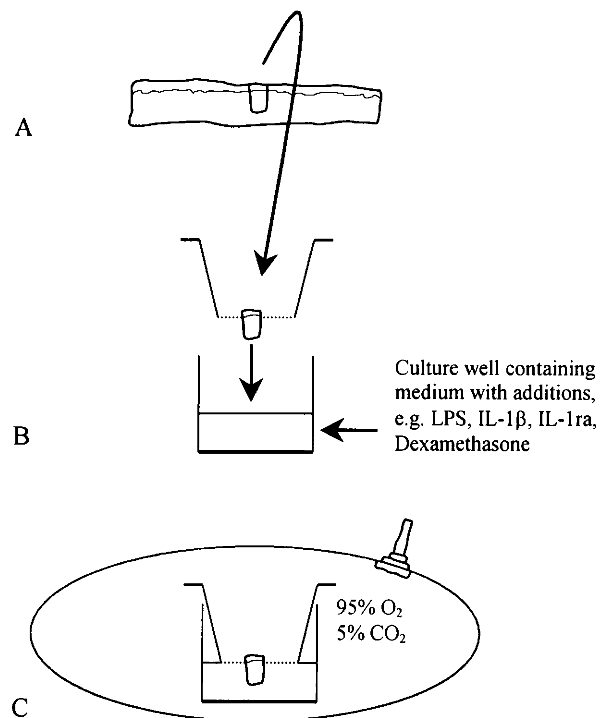

Fig. 1 A–C Schematic overview of the skin organ culture system. **A** A skin biopsy is isolated using a 3-mm diameter biopsy punch. **B** The biopsy is inserted through a 2-mm opening in the Transwell filter and placed in culture medium to which specific stimuli are added. **C** Biopsies are placed at 32 °C in a sealed bag containing an atmosphere of 95% O_2 and 5% CO_2

Material and methods

Skin biopsies

Normal skin biopsies were obtained from seven healthy patients undergoing breast reduction in the Department of Plastic Surgery of the Dijkzigt Hospital or Sint Franciscus Gasthuis Rotterdam. Psoriatic lesional skin biopsies were obtained from seven patients with plaque-type skin lesions. All patients provided informed consent. The biopsies had an average length of 3 mm and were taken with a 3 mm diameter biopsy punch (Stiefel, Leuven, Belgium). They were either snap-frozen in Tissue Tek (Bayer, Munich, Germany), or cultured (see below). After culture the biopsies were immersed in Tissue Tek, snap-frozen in liquid nitrogen and stored at –80 °C until use.

Organ culture conditions

The culture method presented here is based on the method described by Trowell [10]. Biopsies were cultured as follows: a 2-mm hole was punched in a Transwell filter (pore size 0.75 µm; Corning Costar, Corning, NY). The biopsy was inserted into the hole, and the filter containing the biopsy was placed in a 12-well culture plate (Corning Costar) containing 1 ml medium with or without stimulus with the epidermis facing upwards at the liquid-air interface and the dermis suspended in the culture medium. Culture plates containing the biopsies were placed in Tedlar culture bags (Pacwill Environmental, Fredericton, Canada). The bag was filled with an atmosphere of 5% CO_2 and 95% O_2 through a valve, sealed with a removable clamp, and placed in an incubator. The biopsies were cultured in Iscove's modified Dulbecco's medium (IMDM; Gibco-BRL, Paisley, UK) containing 1% heat-inactivated human serum (Sigma, St. Louis, MO), 100 U/ml penicillin and 100 µg/ml streptomycin (BioWhittaker, Verviers, Belgium) for 24 h under normal conditions (atmosphere containing 5% CO_2 at 37 °C) or special conditions (atmosphere containing 95% O_2 and 5% CO_2 at 32 °C). All experiments were done at least three times, with triplicate readings. A schematic drawing of the culture procedure is shown in Fig. 1.

Some biopsies were stimulated for 24 h with either 10 µg/ml LPS (Brunschwig, Amsterdam, The Netherlands) or 250 U/ml IL-1β (Glaxo, Research Triangle Park, NC). Normal skin biopsies were also cultured in the presence of dexamethasone (Sigma) at concentrations in the range 10^{-5} to 10^{-9} *M* or 1 µg/ml IL-1ra (Synergen, Denver, CO) with or without stimulation with 250 U/ml IL-1β or 2 µg/ml LPS. Psoriatic lesional skin biopsies were stimulated with 1500 ng/ml CD3-stimulating antibodies (CLB, Amsterdam, The Netherlands).

Immunohistochemistry

Skin biopsies were snap-frozen in liquid nitrogen and cryosections were cut using a cryostat (Jung Frigocut 2800 E; Leica, Rijswijk, The Netherlands) and stored in a sealed box containing silica gel at –80 °C prior to use. Sections were fixed in acetone for 10 min at room temperature (RT) and preincubated for 10 min with PBS (pH 7.4) containing 0.05% Tween 20 (Merck, Whitehouse Station, NJ) at RT. Subsequently sections were incubated for 18 h at 4 °C with anti-human CD86 (1G10, Tanox Pharma, The Netherlands) at a di-

lution of 1:750, anti-human CD40 (5D12, Tanox Pharma) at a dilution of 1:300 or biotinylated anti-HLA-DR (L243, Becton Dickinson) at a dilution of 1:3000, or for 1 h at RT with anti-human ICAM-1 (BBA4, Boehringer Mannheim, Mannheim, Germany) at a dilution of 1:250, anti-human keratin 17 (K17) (E3, DAKO, Carpinteria, Calif.) at a dilution of 1:100 or anti-human transglutaminase kinase (TGk) (BT-621, Biomedical Technologies, Stoughton, Mass.) at a dilution of 1:200, followed by an incubation for 30 min with a peroxidase-linked secondary rabbit anti-mouse polyclonal antibody (pAb) (DAKO) at a dilution of 1:400. Biotinylated anti-HLA-DR was directly detected with streptavidin-linked peroxidase (DAKO). Sections incubated with an antibody of the same isotype as the specific antibody but of irrelevant specificity served as a control. 3-Amino-9-ethylcarbazole (Sigma) was used as the chromogen. Staining intensity and numbers of positive cells were reproducibly ranked by two independent observers blinded to the treatment using a semiquantitative scoring scale.

Cytokine ELISA

Maxisorb ELISA plates (Nunc, Roskilde, Denmark) were coated for 18 h at 4 °C with 100 μl 0.5 μg/ml anti-human IL-8, anti-human IL-6 or anti-human IFN-γ monoclonal antibody (mAb) (Biosource, Camarillo, CA) followed by blocking with 0.5% bovine serum albumin (BSA, Sigma) for 2 h at RT. A 100-μl aliquot of recombinant cytokine standard or sample and 50 μl 0.2 μg/ml biotin-linked anti-human IL-8, anti-human IL-6 or anti-human IFN-γ pAb (Bioscource) detection antibody were simultaneously added to each well. The standards were diluted in PBS (pH 7.4) containing 0.5% BSA (Sigma) and 0.1% Tween 20 (Merck). Samples, standards and detection antibodies were incubated for 2 h at RT. Cytokines were detected using streptavidin-linked peroxidase (CLB) and TMB peroxidase substrate (Kirkegaard and Perry, Gaithersburg, MD). The optical density was measured at 450 nm.

Statistical analysis

The Wilcoxon signed ranks test was used to determine the significance of differences between treatment and no treatment, and $P < 0.05$ was considered to be significant.

Results

Culture of skin biopsies under different conditions

We observed that the morphology of psoriatic lesional skin was negatively affected by incubation under standard culture conditions (atmosphere containing 5% CO_2 at 37 °C; Fig. 2 A). Therefore we asked whether culture under different atmospheric conditions could reverse the deterioration in the morphology. As illustrated in Fig. 2 B, we found that in this system the degenerative effect on the morphology of lesional skin biopsies was largely abolished during culture at 32 °C in an atmosphere containing 95% oxygen. Deterioration of the morphology of normal skin did not occur in an atmosphere containing 5% CO_2 at 37 °C, but to allow adequate comparison with lesional psoriatic skin, normal skin was cultured under identical conditions.

A main drawback of an organ culture system is the occurrence of spontaneous activation, a wound-healing reaction, in the explanted skin sample characterized by the expression of regenerative maturation markers. Because this effect might be induced by a high serum concentration in the medium, we assessed the effect of human serum on the induction of regenerative markers in our *ex vivo* model. Culturing in IMDM without human serum was compared with

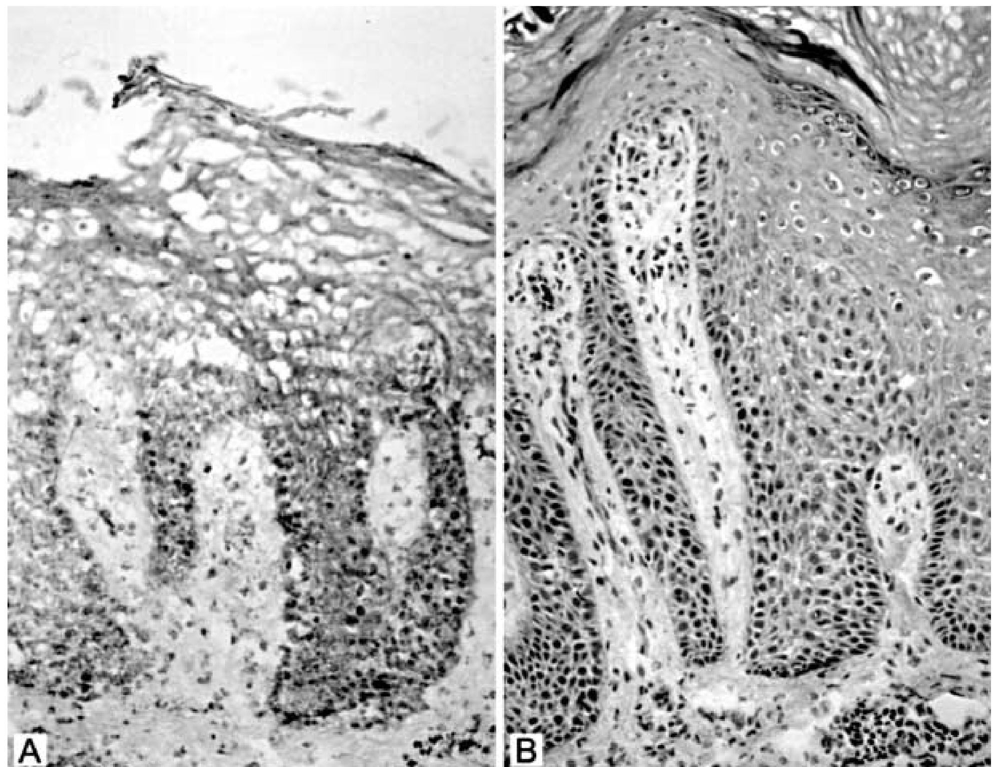

Fig. 2 A, B Standard atmospheric culture conditions affect the morphology of psoriatic skin. Haematoxylin and eosin staining of a psoriatic lesional skin biopsy specimen cultured (**A**) at 37 °C in an atmosphere containing 5% CO_2 or (**B**) at 32 °C in an atmosphere containing 95% O_2 and 5% CO_2 for 24 h (× 160)

culturing in medium supplemented with 1% human serum. To measure the spontaneous activation of certain markers in normal skin after culture, the secretion of IL-8 and IL-6 into the culture medium was compared. Differences in IL-8 or IL-6 secretion during culture in medium without human serum and medium supplemented with 1% human serum were not observed (data not shown). However, there was a slight upregulatory effect of human serum on the expression of K17 and TGk.

Intersample variation of cytokine secretion

To investigate the variability in cytokine expression between different biopsies, IL-8 secretion in the medium of four biopsies from the same donor, cultured under the same conditions was compared. The background secretion of IL-8 showed some variation, e.g. 487 to 1225 pg/ml. LPS stimulation always consistently increased IL-8 secretion, and variation in the secretion of this cytokine was also observed (range 4153 to 11143 pg/ml). IL-6 secretion showed a similar trend. Background IL-6 secretion ranged from 577 to 990 pg/ml and LPS stimulation increased IL-6 secretion to 7301 to 16090 pg/ml.

Variation in background expression of IL-8 was also observed among biopsies from different individuals (range 428 ± 96 to 2076 ± 831 pg/ml, $n = 7$, readings in triplicate). LPS stimulation consistently resulted in a 4- to 13-fold increase in IL-8 secretion by biopsies of different individuals compared to background secretion. Variation in IL-6 expression similar to the variation in IL-8 expression was also observed (data not shown).

Increased cytokine secretion upon stimulation with LPS or IL-1β

IL-8 levels in the culture medium were significantly increased after culturing skin for 24 h with LPS or IL-1β ($P < 0.05$; Table 1). Similar results were observed for IL-6 secretion ($P < 0.05$; Table 1). Compared to background levels of expression, LPS stimulation resulted in a 7 ± 2-fold increase in IL-8 secretion and a 12 ± 5-fold increase in IL-6 secretion. IL-1β stimulation resulted in a 7 ± 3-fold increase in IL-8 secretion of and a 7 ± 2-fold increase in IL-6 secretion on average.

Table 1 Modulation of IL-6 and IL-8 secretion after stimulation and suppression. Secretion of IL-6 and IL-8 by normal skin biopsies stimulated for 24 h with 250 U/ml IL-1β or 10 μg/ml LPS alone and in combination with 10^{-5} *M* dexamethasone or 1 μg/ml IL-1ra. IL-6 and IL-8 levels were measured in the culture supernatant by ELISA. The values are the means ± SEM from at least four experiments. Readings were done in triplicate

Stimulus	IL-6 (pg/ml)	IL-8 (pg/ml)
Medium	992 ± 177	1273 ± 252
IL-1β	6268 ± 1224*	7424 ± 1467*
IL-1β+IL-1ra	667 ± 74	754 ± 77
IL-1β+dexamethasone	4327 ± 424	3280 ± 528
IL-1ra	347 ± 108	389 ± 175
LPS	10134 ± 1299*	7131 ± 2049*
Dexamethasone	451 ± 53*	226 ± 45*

*$P < 0.05$

LPS- and IL-1β-induced cytokine secretion is suppressed after treatment with immunosuppressive mediators

Normal skin biopsies were pretreated with dexamethasone or IL-1ra for 1 h followed by stimulation with IL-1β or LPS. IL-8 secretion induced by IL-1β was partially suppressed by dexamethasone and was completely abolished to below background levels by IL-1ra (Table 1). This indicates that both induced and non-induced IL-1 signalling were blocked by this IL-1 antagonist. This was also the case for IL-6 secretion (Table 1). Similar results were obtained for LPS-induced IL-8 and IL-6 expression after dexamethasone treatment (data not shown). The effect of dexamethasone on IL-1β-induced cytokine expression was dose-dependently diminished at very low concentrations. For example, IL-1β-induced IL-6 secretion was suppressed by 62% after treatment with 10^{-5} *M* dexamethasone and by 18% after treatment with 10^{-9} *M* dexamethasone (data not shown).

Dexamethasone alone suppressed spontaneous IL-8 secretion to below background levels, indicating that dexamethasone suppressed medium-induced stimulation in this *ex vivo* system ($P < 0.05$; Table 1).

Modulation of the expression of CD86, CD40, ICAM-1 and HLA-DR

In freshly isolated skin, CD40 was expressed on keratinocytes, especially in the basal layer, and on cells with dendritic morphology in both the epidermis and dermis. CD86 and HLA-DR were present only on cells with dendritic morphology in the epidermis and dermis. ICAM-1 expression was observed mainly in the dermis, and sporadically on keratinocytes in the basal layer.

Spontaneous upregulation of the tested markers was observed after culture in medium alone (data not shown). CD86 expression on cells with dendritic morphology in both the dermis and epidermis was further enhanced by stimulation with LPS (Fig. 3 A versus Fig. 3 B; Table 2). LPS stimulation also resulted in an elevation of CD40 expression on basal keratinocytes and cells with dendritic morphology in the dermis and epidermis, and resulted in increased expression of ICAM-1 in the dermis and basal epidermal layer (Table 2). The expression of HLA-DR showed no further increase after application of the stimuli (Table 2).

Culturing of normal skin biopsies in medium containing 10^{-5} or 10^{-6} *M* dexamethasone inhibited the expression of CD86, CD40 and ICAM-1 to levels below medium-induced expression. The effects on the expression of CD86 are shown in Fig. 3 C. The effect of dexamethasone was

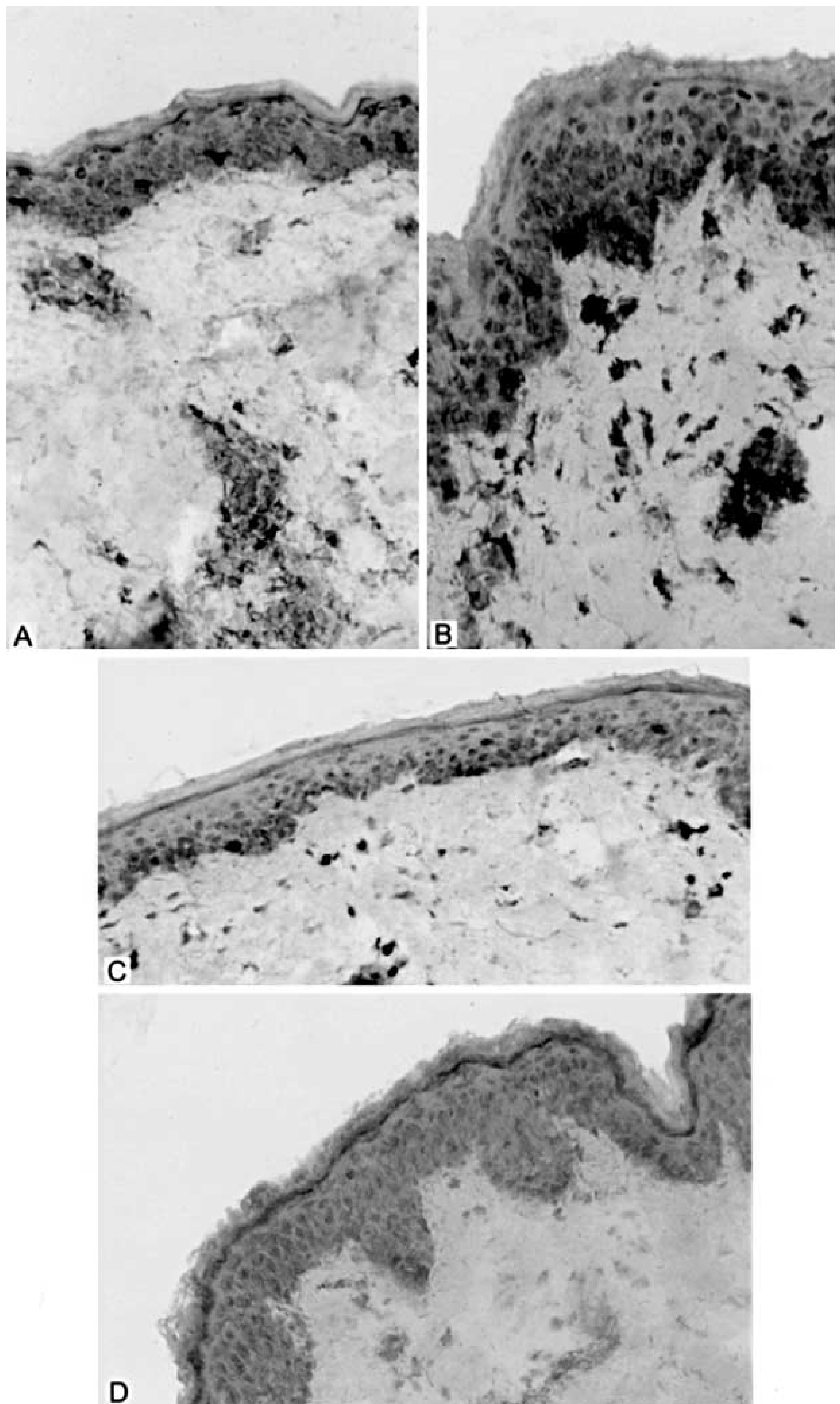

Fig. 3 A–D CD86 expression is increased after stimulation with LPS and downregulated after treatment with dexamethasone. **A** Medium control; **B** CD86 expression after LPS stimulation; **C** CD86 expression after culture in the presence of 10^{-5} *M* dexamethasone; **D** isotype control antibody staining (× 400). The results of one representative experiment out of four are shown

Table 2 Modulation of CD86, CD40, ICAM-1 and HLA-DR expression after stimulation and suppression. Expression of CD86, CD40, ICAM-1 and HLA-DR in normal skin biopsies stimulated for 24 h with 250 U/ml IL-1β, 10 μg/ml LPS or 10^{-5} *M* dexamethasone. Staining intensity and numbers of positive cells were reproducibly ranked by two independent observers blinded to the treatment using a semiquantitative scoring scale (range: 0 no staining to 3 high staining intensity). Values are the means ± SEM from at least four experiments. Readings were done in triplicate

Marker	No stimulus	LPS	IL-1β	Dexamethasone
CD86	2.0 ± 0.0	3.0 ± 0.0	2.5 ± 0.3	1.4 ± 0.7
CD40	2.2 ± 0.2	3.0 ± 0.0	2.5 ± 0.3	1.4 ± 0.4
ICAM-1	1.4 ± 0.7	3.0 ± 0.0	2.8 ± 0.3	0.6 ± 0.5
HLA-DR	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0

dose-dependently diminished at low concentrations (e.g. 10^{-7} *M* dexamethasone). IL-1β stimulation resulted in upregulation of CD40, CD86 and ICAM-1 similar to that seen after LPS stimulation (Table 2). IL-1β did not induce a further increase of HLA-DR expression. IL-1ra partially blocked the IL-1β-induced effects (data not shown).

IFN-γ induction by CD3 stimulation

Secretion of IFN-γ was detected in supernatants of psoriatic lesional skin biopsies stimulated with CD3 stimulatory antibodies (237 ± 97 pg/ml; mean ± SEM). No IFN-γ was detected in supernatants of unstimulated biopsies. Biopsies were stimulated for 24, 48 and 72 h but no significant differences in the amount of IFN-γ secretion was detected in the medium (305 ± 122 pg/ml at 48 h and 346 ± 107 pg/ml at 72 h).

Discussion

An *ex vivo* skin organ culture model is presented which allowed close monitoring of the events following activation and suppression of the skin immune system in healthy human skin. This was illustrated on the basis of the stimulatory effects of LPS and IL-1β on the levels of IL-6 and IL-8 released into the culture medium and on the expression of cell signalling and adhesion molecules in situ. These stimulatory effects of LPS and IL-1β were able to be inhibited by dexamethasone and IL-1ra. The system used here was based on the method developed by Trowell [10], but modified by using punctured Transwell filters such that the dermal part of the skin biopsy was immersed in the culture medium and the epidermis remained exposed to air. This system allowed data on the amount of cytokine released into the culture medium and on the expression of cell activation markers on the cells in the cultured skin samples to be collected simultaneously.

We showed that strong stimuli efficiently upregulated different markers in this system. However, strong stimulation could result in decay of the skin architecture which could interfere with monitoring of the modulation of specific inflammation markers using immunohistochemistry. Kondo and others have shown that the morphology of psoriatic lesional skin is preserved when it is cultured under special atmospheric conditions (atmosphere containing 5% CO_2/95% O_2 at 32 °C) [19–21]. These conditions might mimic the natural environment of human skin. In particular, inflamed skin needs a high oxygen supply to meet the demands of an enhanced tissue metabolism. We showed that in this system the morphology of psoriatic lesional skin remained intact when cultured under these conditions. We also showed that culturing under these specific conditions allowed upregulation and downregulation of certain markers of inflammation in the medium and skin to be monitored.

Previous experiments in our laboratory have shown that the regenerative maturation markers K17 and TGk are strongly induced during culture in medium containing a high serum concentration (e.g. 5% human serum). This hampers the assessment of stimulatory effects in cultured skin biopsies (Wei, unpublished data). The background induction of the regenerative maturation markers was limited in this system, mainly due to the lower concentration of human serum and probably the elimination of tight adhesion of the biopsy sample to the culture plate. Previous studies have shown similar effects [22].

Secretion of human skin cell-derived cytokines into the medium after culture has been shown previously [6] using an *ex vivo* skin explant system in which submerged skin biopsies were cultured. Some fluctuation in the levels of IL-6 and IL-8 secretion between several normal skin donors were observed. Variation in cytokine levels after culturing of biopsies from different donors occurred in this system as well, and we also observed variation in cytokine levels within a single individual. However, the level of cytokine induction after LPS or IL-1β stimulation and the degree of inhibition after treatment with immunosuppressive agents was significant.

Culturing with bacterial LPS and IL-1β resulted in a clear increase in IL-6 and IL-8 levels in the medium and of CD40, CD86 and ICAM-1 expression on skin cells. These effects were able to be suppressed by dexamethasone in a dose-dependent fashion and IL-1ra specifically antagonized the effects of IL-1β. The suppressive effect of dexamethasone in skin has also been observed by Furue et al. [23]. Furthermore we were able to induce INF-γ secretion in psoriatic lesional skin biopsies, showing that cytokines associated with the pathology of psoriasis can be assessed as well.

Additionally, the release of soluble IL-1 receptor type 2 (sIL-1RII) and sICAM-1 into the culture medium by skin biopsies cultured under standard conditions is enhanced by IL-1β and IFN-γ stimulation, respectively (Wei et al., submitted for publication; [24]). This indicates that secretion of soluble cell membrane proteins can also be assessed in this system.

We describe here a modified *ex vivo* skin organ culture model in which the different read out systems were combined. In addition, we used special culture conditions that preserved the morphology of diseased skin, so that this

model could be used to investigate modulation of different markers in diseases such as psoriasis (Wei et al., submitted for publication). In this model the modulation of cytokine secretion and membrane protein expression in response to immunostimulators or suppressors could be measured in the same experiment.

This system provides a fast and simple method for functional studies using readily available human skin and would also allow quick screening of drugs for treatment of a variety of skin diseases.

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