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In situ expression of the costimulatory molecules CD80 and CD86 on Langerhans cells and inflammatory dendritic epidermal cells (IDEC) in atopic dermatitis

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Abstract **The functional expression of costimulatory molecules on antigen-presenting cells may be a key event in the pathogenesis of atopic dermatitis (AD). Recently, the expression of CD86 (B7-2/B70) has been demonstrated on CD1a$^+$ epidermal dendritic cells (DC) in AD lesions by immunohistological and functional analysis. Therefore, we sought to further characterize the in situ expression of costimulatory molecules on these cells, considering the two subpopulations of (1) CD1a^{+++}/CD11b$^-$ Langerhans cells (LC) containing Birbeck granules and (2) CD1a$^+$/CD11b^{+++} inflammatory dendritic epidermal cells (IDEC), devoid of Birbeck granules, from AD and other inflammatory skin diseases. Flow cytometry, skin mixed lymphocyte reactions (SMLR) and immunohistological analysis were performed, and showed that IDEC and not LC are the relevant cells expressing the costimulatory molecules CD80 and CD86 in situ. This expression varied with the underlying diagnosis, with AD showing the highest expression of both CD80 and CD86 in situ. Furthermore, the expression of CD80, CD86 and CD36 were significantly correlated. With short-term culture, both CD80 and CD86 were further upregulated on LC and IDEC. Finally, anti-CD86 antibody reduced the stimulatory activity of epidermal DC. These results indicate that costimulatory molecules on LC and IDEC might play a role in the pathogenesis of AD.**

Keywords Costimulatory molecules · Langerhans cells · Inflammatory dendritic epidermal cells · IDEC · Atopic dermatitis

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Introduction

Atopic dermatitis (AD) is a common skin disease with an as-yet unclear pathomechanism, a characteristic distribution pattern of pruritic lesions and a chronic relapsing course. Skin lesions are histologically characterized by infiltrating activated T cells, but the mechanism of this activation remains unclear [28]. IgE-mediated facilitated antigen presentation of IgE-bearing dendritic cells (DC) to T cells may be a key event in the pathogenesis of AD [1, 4, 19].

Human epidermal Langerhans cells (LC), as described by Langerhans in 1868 [16], are the most extensively characterized antigen-presenting DC population of the human immune system. They are defined as epidermally located, dendritically shaped, suprabasal cells with antigen processing and presentation capacity. In response to contact with an antigen, LC migrate to the draining lymph node and simultaneously change their phenotype [21, 26]. This maturation process includes an upregulation of adhesion molecules such as ICAM-1, as well as costimulatory molecules of the B7 family [23, 24, 31]. Two different subtypes of human B7 molecules are known, namely CD80 (B7-1, B7) [18] and CD86 (B7-2, B70) [8]. Both functionally bind CD28 and CD152, but their expression is regulated differentially [17]. These two B7 molecules form a subgroup of an emerging new extended B7 family [12]. While LC from normal human skin do not express B7 molecules, these have recently been demonstrated on epidermal DC from AD lesions [14, 20].

LC have been identified during the past 18 years by immunostaining of the non-classical class Ib MHC molecule CD1a, a technique thought to be specific for LC [6]. More recently, it has become evident that two immunomorphologically and ultrastructurally distinct CD1a$^+$ cell populations are present in the inflammatory epidermis: the 'classical' human LC containing Birbeck granules (CD1a^{+++}, HLA-DR^{+++}, CD11b$^-$) and the inflammatory dendritic epidermal cells (IDEC) which lack Birbeck granules (CD1a$^+$, HLA-DR^{+++}, CD11b^{+++}) [27, 29]. In earlier studies of inflammatory skin, in which immunostaining for CD1a or

HLA-DR was used in the attempt to identify LC [3], the epidermal cell population identified was evidently a mixture of both LC and IDEC. This is also the case in the recent demonstration of B7 molecules on epidermal DC from AD lesions [20]. This raises the questions as to whether, in inflammatory skin diseases, LC or IDEC is the relevant cell population expressing B7 molecules in situ and whether the expression of the B7 molecules is in some way linked to the underlying diagnosis or is correlated with other surface molecules of epidermal DC.

Materials and methods

Reagents

The costimulatory molecules CD80 and CD86 were detected by mAb BB-1 (B7), L307.4 (Camfolio/Becton Dickinson, San Jose, Calif.) and mAb B70/B7-2, IT2.2 (Pharmingen, San Diego, Calif.). These mAbs do not show any cross-reactivity to CD74 as has been described for other B7 mAbs [9]. mAb IOT2b (IgG_1, Immunotech, Marseille, France) reacts with HLA-DR. mAb IOP36 (IgG_{2b}, Immunotech) was raised against CD36 which is the thrombospondin receptor and represents a putative collagen binding structure. The high-affinity receptor for IgE, FcεRI, was detected by mAb 29C6 and 22E7 (IgG_1, generous gift from Dr. J. Kochan, Hoffmann LaRoche, Nutley, N.J.) which is directed against the α-chain of FcεRI but does not interfere with the binding site for IgE on this molecule. T6RD1 labelled with phycoerythrin (PE) (IgG_1, Beckman Coulter, Krefeld, Germany) recognizes CD1a which, in normal skin, is expressed only by LC and related cells [6]. MOPC (IgG1), UPC10 (IgG_{2b}, Sigma, Deisenhofen, Germany) and IgG_1RD1 (Beckman Coulter) were used as appropriate isotype controls. Goat anti-mouse antibody conjugated with fluorescein-isothiocyanate (FITC) (GaM/FITC) was from Jackson Laboratories (West Grove, Pa.). Normal mouse serum for blocking purposes and 7-amino-actinomycin-D were from Sigma.

For the cell depletion experiments, mAbs against CD1a (BL6, Immunotech), CD3 (X35, Immunotech), CD11b (BEAR1, Immunotech), CD14 (RMO52, Immunotech), CD19 (J4.119, Immunotech) and HLA-DR (BL2, Immunotech) were coated to M280 Dynabeads (Dynal, Hamburg) at a ratio of 1 μg mAb per 500 μg bead suspension.

Preparation of epidermal cell suspensions

Punch or shave biopsies were obtained from 91 patients after obtaining signed informed consent, with the approval of the local ethics committee. After local anaesthesia, biopsies were taken from 21 patients with AD; chronic skin lesions which had not been treated for 2 weeks were sampled. AD was diagnosed according to the criteria defined by Hanifin and Rajka [11]. For comparison between skin diseases, biopsies taken from psoriasis vulgaris (n = 15), allergic contact eczema (n = 9), and normal human skin of patients undergoing cosmetic or melanoma surgery (n = 9) were used in the statistical analysis. Single-cell suspensions of epidermal cells were prepared from skin biopsies by trypsinization as described previously [30], washed with phosphate-buffered saline containing 1% fetal calf serum (FCS) and 0.1% sodium azide, filtered through a 50-μm nylon mesh and subjected to immunolabelling. Resistance of all receptor molecules to the limited trypsin digestion procedure had been established in preliminary experiments. Total serum IgE levels were determined by an ELISA technique (Enzygnost, Behringwerke, Marburg, Germany).

Immunolabelling and flow cytometric analysis

A standardized, indirect triple-staining for unfixed vital LC was performed as described in detail elsewhere [30]. In brief, about $2–5 \times 10^5$ epidermal cells were first incubated with the primary mAb, washed and further incubated with GaM/FITC. After washing, GaM/FITC was blocked with normal mouse serum. This was followed by washing and counterstaining with T6/RD1 and 7-amino-actinomycin-D. Finally, the washed cells were analysed on a FACScan (Becton Dickinson, Mountain View, Calif.). The vital LC population was gated out by a combination of forward and side scatters (FSC/SSC) and CD1a/7-amino-actinomycin D gate sets. Fluorescence parameters were collected using a built-in logarithmic amplifier and data from about 1×10^4 cells were obtained and analysed with the Cell Quest program (Becton Dickinson). For quantitative evaluation, the CD1a populations were gated out manually and the mean fluorescence intensity (MFI) was determined for each population of interest using Cell Quest software. Relative fluorescence indices (rFI) of all surface receptors were determined as follows:

$$rFI = (MFI_{receptor} - MFI_{control})/MFI_{control}$$

For statistical evaluation, the Wilcoxon Signed Ranks test, the Mann-Whitney U-test were used and Spearman's correlation coefficient determined. The results are shown as arithmetic mean±SEM.

Immunohistochemistry

Cryosections were prepared from the punch or shave biopsies, air-dried, fixed for 10 min in pure acetone, and then processed for immunohistochemistry using mAbs (all at 10 μg/ml) and the alkaline-phosphatase mouse anti-alkaline-phosphatase technique as described previously in detail [2].

Skin mixed lymphocyte reaction (SMLR)

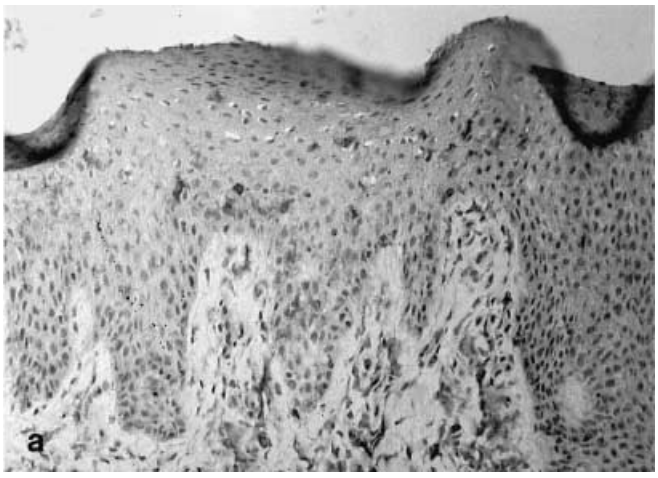
T-cell enriched peripheral blood mononuclear cells (PBMC) were prepared from heparinized whole blood according to the protocol of Ohki et al. [20] with slight modifications, as follows. Density gradient centrifugation was performed on Lymphoprep (Nycomed, Oslo) and the interphase cells were washed twice in RPMI medium (Seromed, Berlin) supplemented with 2 mmol/l L-glutamine, 1% antibiotic/antimycotic solution (Gibco, Paisley, UK) and 10% heat-inactivated FCS (complete medium). Sheep anti-mouse IgG-coated 280-nm immunomagnetic Dynabeads (Dynal, Hamburg) were coated for 30 min with antibodies against CD14, CD19 and HLA-DR and washed three times. PBMC were incubated for 30 min with the beads and the resulting T cells were negatively enriched in a magnetic field according to the manufacturer's protocol. The efficacy of the depletion was checked by immunostaining and flow cytometric analysis.

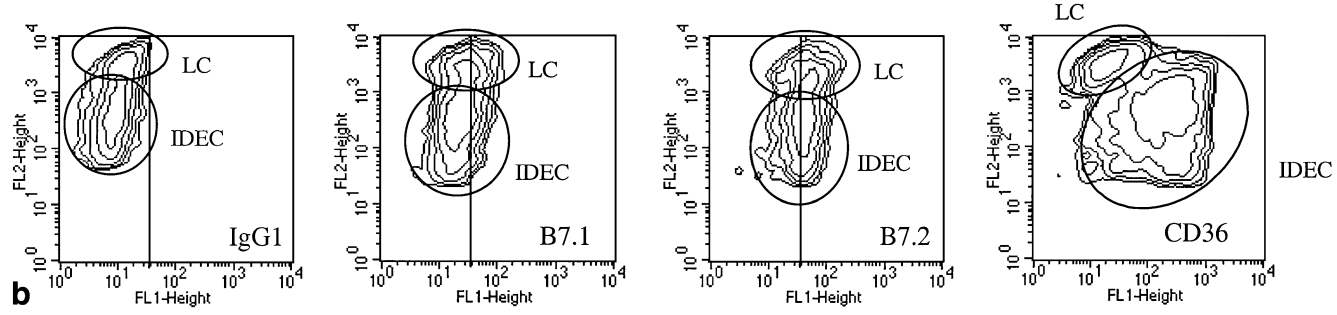
Epidermal cells were prepared under sterile precautions as described above and depleted from epidermal T cells by a similar 30-min incubation with anti-CD3 mAb-coated M280 Dynabeads. Epidermal cells (1.5×10^5 cells/well) were seeded as stimulator cells, and 5×10^4 enriched T cells were added as responder cells. The co-culture was performed in 96-well round bottomed plates at 37 °C in an atmosphere containing 5% CO_2 and at 100% humidity for 5 days in a volume of 200 μl complete medium. For inhibition of CD86 function, sodium azide-free B7-2 mAb was added at the optimal dose of 5 μg/ml at the beginning of the culture. Pulse labelling was done by adding 0.2 μCi/well ^{3}H-thymidine for 18 h, and the incorporated radioactivity was assessed in a beta counter. The results are given as counts per minute (cpm) for the respective culture conditions.

Results

Both epidermal DC types express costimulatory molecules in situ, but IDEC and not LC are the relevant B7-expressing cell population

Cryosections of inflammatory skin were immunostained to localize the $CD80^+$ and $CD86^+$ cells. $CD80^+$ as well as $CD86^+$ cells were identified in the lesional epidermis and





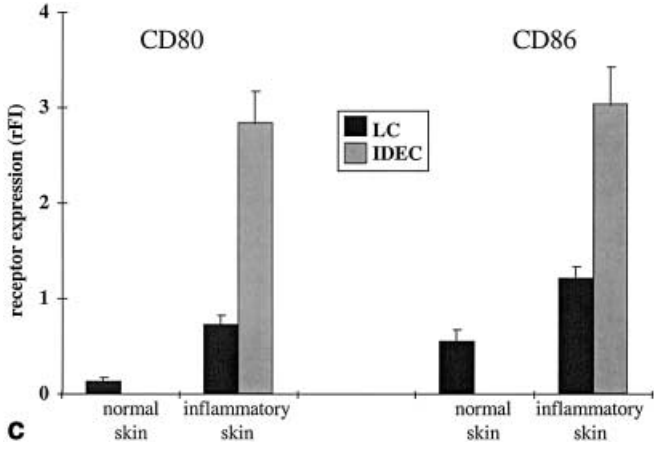
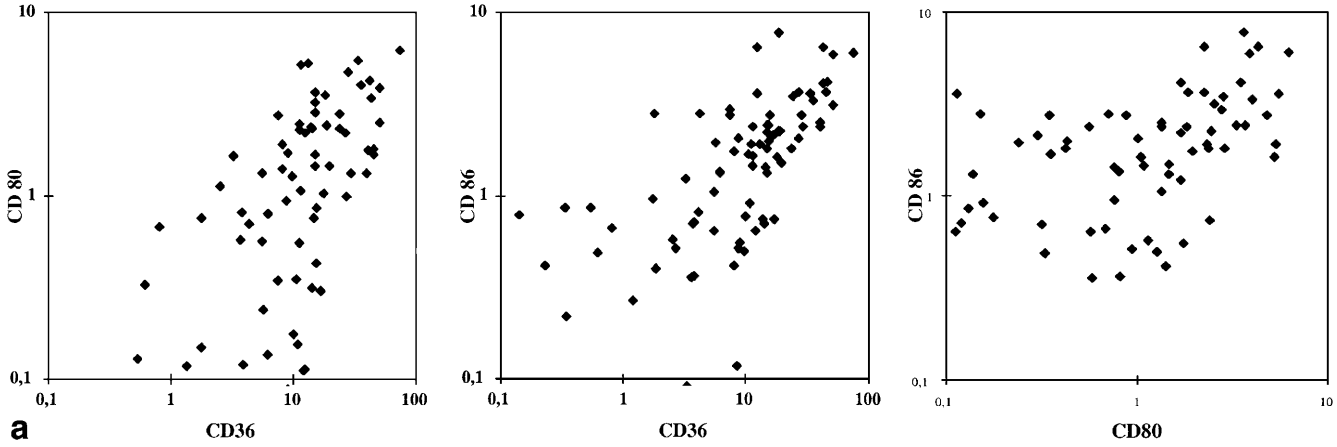


Fig. 1 a–c In situ detection of B7 molecules. **a** Immunohistochemical staining for CD80: cryosection of lesional AD skin, immunostained for CD80 with mAb BB-1 (APAAP). $CD80^+$ cells with a membranous staining pattern are visible in the basal and suprabasal regions of the epidermis, as well as in the dermis. The $CD80^+$ epidermal cells are dendritically shaped, suggesting that they are either LC or IDEC. **b** LC and IDEC express costimulatory molecules: flow cytometric contour plot analysis of an epidermal cell suspension from lesional skin of an AD patient (*FL1* control, CD80, CD86 or CD36; *FL2* CD1a), showing two $CD1a^+$ epidermal cell populations expressing costimulatory molecules. **c** CD86 expression in situ is mostly due to IDEC: flow cytometric analysis of the expression of CD80 and CD86 on $CD1a^+$ epidermal DC from normal and inflammatory skin. In inflammatory skin, IDEC and not Langerhans cells are the relevant cell population expressing CD80 and CD86 in situ

dermis. In the epidermis, cells expressing CD80 (Fig. 1 a) and CD86 (not shown) were found at a suprabasal as well as a basal level. The positive cells were scattered throughout the epidermis in an LC-like distribution pattern, were dendritically shaped and exhibited a membranous staining pattern, suggesting them to be either LC or IDEC. To clarify the nature of these cells, double immunofluorescence staining of isolated epidermal cells was performed.

Using flow cytometry, two distinct $CD1a^+$ DC types could be differentiated in lesional inflammatory epidermis irrespectively of the underlying diagnosis: $CD1a^{+++}/CD11b^-$ LC known to contain Birbeck granules, and $CD1a^+/CD11b^{+++}$ IDEC lacking this cytoplasmic organelle [29]. The expression of both costimulatory molecules could be attributed to the two respective $CD1a^+$ DC populations by flow cytometry (Fig. 1 b).

In general, only low amounts of both CD80 (rFI 0.13 ± 0.04) and CD86 (rFI 0.55 ± 0.13) were identified on freshly isolated LC from normal human skin. In contrast, LC from inflammatory skin expressed higher amounts of both CD80 (rFI 0.72 ± 0.11) and CD86 (rFI 1.21 ± 0.13). Most interestingly, in each of the biopsies, the IDEC showed significantly higher rFI values for both CD80 (2.84 ± 0.33 vs 0.72 ± 0.11, $P < 0.001$) and CD86 (3.03 ± 0.40 vs 1.21 ± 0.13, $P < 0.001$) than the corresponding LC (Fig. 1 c).



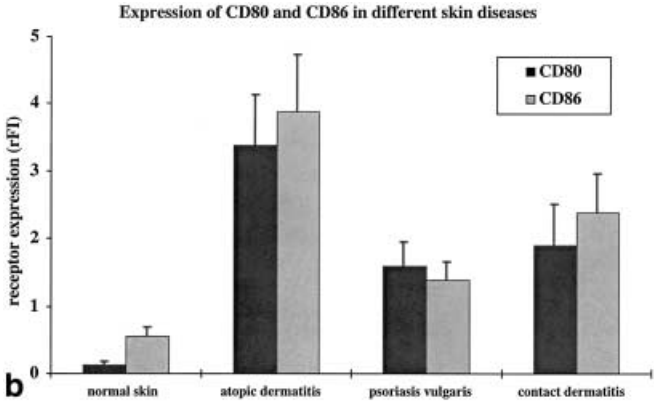


Fig. 2 a, b Statistical analysis of expression patterns. **a** Correlation of the expression of CD80, CD86 and CD36 on $CD1a^+$ epidermal DC. The expression of both costimulatory molecules CD80 and CD86 is significantly correlated ($P < 0.001$) on $CD1a^+$ epidermal DC. Furthermore, their expression is correlated with that of the thrombospondin receptor CD36. **b** Costimulatory molecule expression is diagnosis-dependent. The expression of costimulatory molecules varies with the underlying diagnosis. Means and SEM are shown for normal skin ($n = 9$), atopic dermatitis ($n = 21$), psoriasis vulgaris ($n = 15$) and contact dermatitis ($n = 9$). AD shows by far the highest in situ expression of both CD80 and CD86

The in situ expression levels of CD80, CD86 and CD36 are significantly correlated with each other and vary with the underlying diagnosis

A degree of correlation of the expression values for the thrombospondin receptor CD36 and the costimulatory molecules CD80 and CD86 was determined by calculating Spearman's correlation coefficient. Thereby, significant correlations between the expression levels of CD36 and CD86 ($r = 0.74$, $P < 0.001$), of CD36 and CD80 ($r = 0.72$, $P < 0.001$), and of CD86 and CD80 ($r = 0.67$, $P < 0.001$) were detected (Fig. 2 a). Since CD36 expression on epidermal DC is regarded as a marker of epidermal inflammatory activity present in the epidermal compartment [30], this correlation argues for an in vivo regulation of both B7 molecules depending on the inflammatory activity of the given skin lesion.

In a next step, the expression of costimulatory molecules was individually analysed for each skin disease. AD ($n = 21$) showed the highest expression of CD80 (3.37 ± 0.75) and CD86 (3.88 ± 0.84) on $CD1a^+$ cells of all the skin diseases examined. Psoriasis vulgaris ($n = 15$) exhibited lower amounts of both CD80 (1.59 ± 0.35) and CD86 (1.39 ± 0.26) than AD, the difference for CD86 being highly significant ($P < 0.003$; Fig. 2 b). In contact dermatitis ($n = 9$), the expression patterns showed intermediate results for CD80 (1.90 ± 0.60) and CD86 (2.38 ± 0.57).

Both LC and IDEC upregulate costimulatory molecules upon short-term culture

To compare the in vitro maturation of LC from normal human skin with the maturation of LC and IDEC from AD skin, epidermal cell suspensions were cultured under standard conditions (37 °C, atmosphere containing 5% CO_2, 100% humidity) for 24 h. Upregulation of CD80 and CD86 was observed in both the LC and IDEC populations, leading to an almost identical, strong expression of the respective costimulatory molecules in both cell populations. Figure 3 shows a representative experiment out of three.

CD86 signalling is essential for epidermal DC stimulatory function

To confirm the functional role of B7 molecules on the epidermal DC, SMLRs were performed with suspensions of epidermal cells from six patients and their respective autologous lymphocytes as described above (Fig. 4). Coculture of 5×10^4 T cells with 1.5×10^5 epidermal stimulator cells led to about a fourfold increase in ^{3}H-thymidine incorporation. Addition of a sodium azide-free anti-CD86 mAb resulted in a marked ($P = 0.075$) inhibition of T-cell proliferation (Fig. 4), whereas no such effect could be demonstrated following the addition of an isotype control antibody to the cell cultures in preliminary experiments

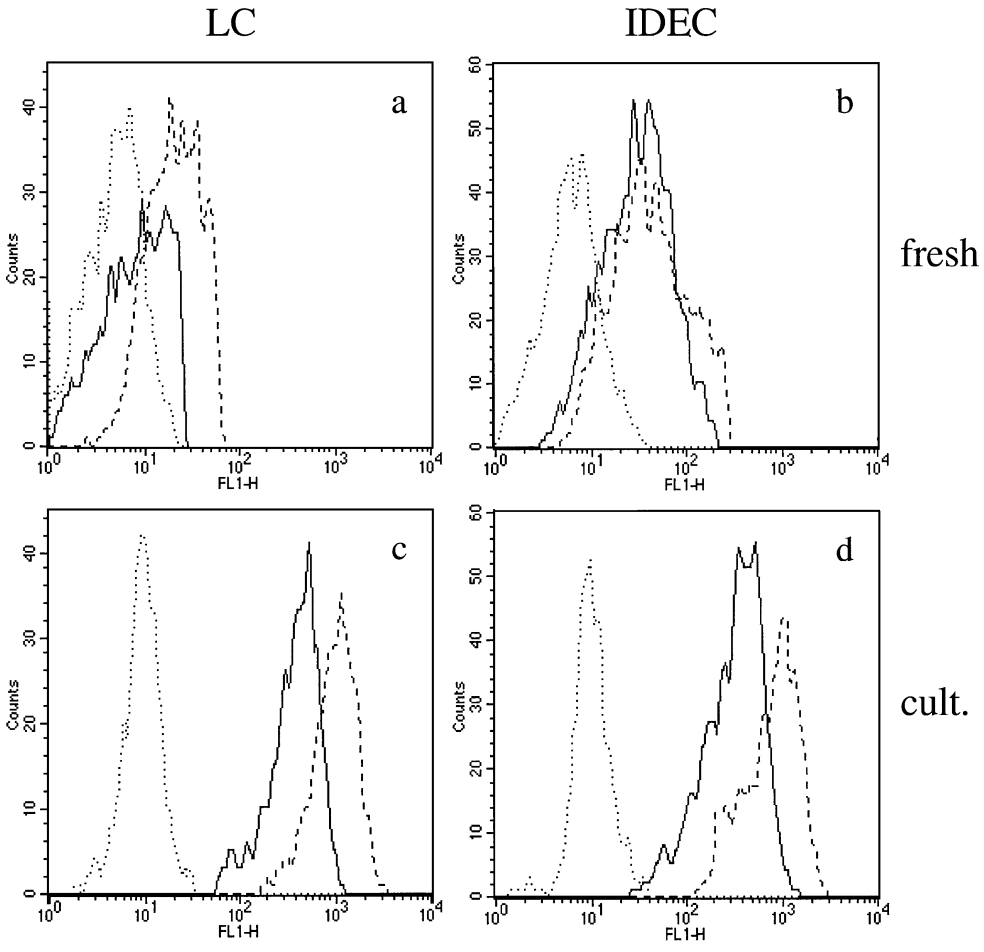


Fig. 3 a–d LC and IDEC upregulate costimulatory molecules upon short term culture. Flow cytometric analysis of freshly isolated and 24-h cultured CD1a$^+$ epidermal DC from inflammatory skin. On LC and IDEC, both CD80 *(dashed lines)* and CD86 *(solid lines)* are strongly upregulated during culture, as compared to the control antibody *(dotted lines)*. A representative experiment out of three is shown

(not shown). These results indicate a functional role of CD86 expression on epidermal DC derived from inflammatory skin in the SMLR.

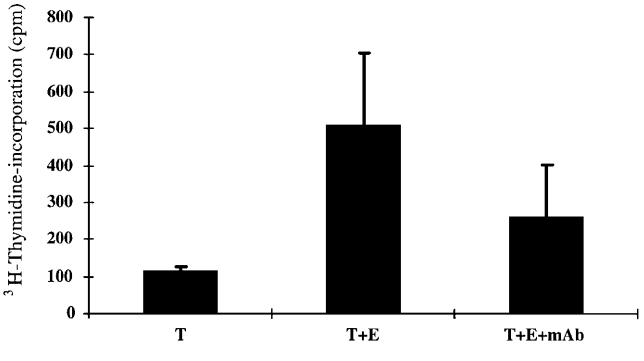


Fig. 4 CD86 signalling is critical for the stimulatory capacity of epidermal DC. Epidermal DC stimulate their autologous lymphocytes in the SMLR. The ^{3}H-thymidine incorporation in terms of counts per minute for purified T cells *(T)*, T cells cocultured with epidermal stimulator cells *(T+E)* and the latter reaction with the addition of a blocking CD86 antibody is shown as the means ± SEM from six independent experiments. Blocking of CD86 signalling reduced the stimulatory capacity of the epidermal DC

Discussion

Facilitated antigen presentation of aeroallergens has been proposed as a key event in AD [1, 19], and DC of the skin are likely to be the relevant antigen-presenting cell type for this process. Costimulatory molecules are an essential factor in the generation of an effective immune response, since failure to deliver costimulatory signals during antigen presentation leads to T-cell anergy [12, 22]. As well as the known upregulation of costimulatory molecules on LC during maturation in culture, the in situ expression of B7 molecules on epidermal DC may also be relevant to the pathogenesis of inflammatory skin diseases. In this study, we were able to demonstrate that IDEC and not LC are the major epidermal cell population expressing the costimulatory molecules CD86 and CD80 in situ, with AD showing the highest expression. We were also able to show a correlation between the surface expression of CD80, CD86 and CD36, as well as functional relevance of CD86.

The immunohistochemical analysis identified CD86$^+$ as well as CD80$^+$ cells in lesional epidermis in an LC-like distribution pattern. These results confirm previous in situ findings of B7-expressing cells in AD lesions [20]. The possibility that these results were a laboratory artefact resulting from passively attached shed B7 molecules was

excluded by (1) preliminary experiments showing a trypsin resistance of CD80 and CD86 and (2) our immunohistological demonstration of CD86 and CD80 in situ.

Flow cytometry was an essential technique for the further investigation of the contribution of the two recently delineated $CD1a^+$ cell populations [29] to B7 expression in situ. The in situ expression of CD86, as well as CD80, was mainly due to the IDEC population. Our quantitative data are in accordance with those of previous investigations based on the enumeration of $B7^+$ cells, which have shown a lower frequency of B7 expression in contact dermatitis than in AD or psoriasis [20]. A correlation of B7 expression with serum IgE levels, as has been shown for FcεRI [29], could not be established.

In our data set, CD86 and CD80 expression was correlated significantly with that of the thrombospondin receptor CD36. The latter molecule is already known to be induced by interferon-gamma (IFN-γ) in other cell systems [13]. Upregulation of CD80 and CD86 after incubation with IFN-γ has been shown on normal human LC [15]. Other investigators have demonstrated a differential effect of IFN-γ on CD86 and CD80 expression on LC [32]. Furthermore, flow cytometric analysis of other human DC has indicated that IL-10 does not modify the expression of CD80, but decreases CD86 expression on the cell surface [5]. Since IFN-γ is known to be expressed in chronic but not in acute lesions of AD [10], the enhanced expression of CD80 and CD86 may be due to the IFN-γ produced in situ.

We demonstrated that IDEC and not LC are the predominant B7-expressing epidermal DC population in inflammatory skin diseases, suggesting an active role in antigen presentation. However, the functional significance of IDEC in AD has not been shown conclusively. There is some indirect evidence of a hyperstimulatory activity of these cells. Taylor et al. conducted functional experiments with epidermal cell suspensions from AD [25] and demonstrated an increased autologous stimulatory capacity of epidermal DC. Their investigation clearly identified the lesional epidermal DC as the causative subpopulation for this hyperstimulatory activity as compared to the non-lesional epidermal DC. Furthermore, they demonstrated an increased expression of CD36 molecules on the $CD1a^+$ cells all of which they assumed to be LC [25]. Since they did not differentiate LC from IDEC in AD, they must have been dealing with a mixture of LC and IDEC in their epidermal cell suspensions. Hence, the hyperstimulatory activity of these epidermal cell suspensions could also have been attributed to the IDEC present in the lesional but not non-lesional skin of AD [29]. This assumption is further supported by the work of Ohki et al., who demonstrated an epidermal cell-induced T-cell proliferation which could be inhibited by an anti-CD86 mAb and, to a lesser extent, by an anti-CD80 mAb [20]. By double immunofluorescence staining, they attributed the expression of both CD80 and CD86 to $CD1a^+$ epidermal DC [20].

SMLRs are the mainstay of analysis of the antigen-presenting capacity of epidermal DC. However, this technique requires a cell culture step which is associated with a phenotypic and functional maturation of the DC [21], including the upregulation of CD80 and CD86 on LC [31] and IDEC (shown above). The in situ expression of low levels of CD80 and CD86 in AD might reflect a preactivation of epidermal DC in this disease, which is different from normal human skin, and might be relevant to the pathogenesis of AD. On the other hand, both LC and IDEC are still capable of further upregulation of costimulatory molecules, which might also be a relevant factor in the pathogenesis of AD.

In conclusion, we demonstrated that IDEC are the major cell population expressing costimulatory molecules in situ and showed that AD had the highest expression of these molecules. Keeping in mind the hyperstimulatory capacity of epidermal DC in AD [7], our findings support the concept of a role for IDEC in the presentation of antigens in AD skin.

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