

Tacrolimus ointment causes inflammatory dendritic epidermal cell depletion but no Langerhans cell apoptosis in patients with atopic dermatitis

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Background: The topical immunomodulators tacrolimus and pimecrolimus are novel therapeutic options for atopic dermatitis (AD). The inhibition of nuclear factor of activated T cell–dependent proinflammatory cytokine production in cutaneous lymphocytes is an established effect of topical immunomodulators, which additionally influence mast cells, eosinophils, and dendritic cells (DCs). The latter include a reduced expression of the high-affinity IgE receptor FcεRI, a reduced stimulatory capacity of lesional DCs, and a selective depletion of the inflammatory dendritic epidermal cells (IDECs) but not of Langerhans cells (LCs) from the lesional skin.

Objective: Because induction of apoptosis in lymphocytes is a reported tacrolimus effect, we asked whether tacrolimus ointment induces apoptosis of LCs or IDECs in AD lesions. **Methods:** Epidermal single-cell suspensions were prepared from AD lesions of 9 tacrolimus-treated and 5 hydrocortisone butyrate–treated patients with AD before and after 1 week of treatment. Cell numbers, apoptosis rate, and immunophenotype were assessed by using the standardized FACS technique with terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling, Annexin V, and 3-color immunophenotyping. Freshly isolated LCs and monocyte-derived DCs served as *in vitro* controls.

Results: Tacrolimus and steroid ointment induced a selective depletion of IDECs from the epidermis and reduced the expression of the costimulatory molecules CD80 and CD86. Tacrolimus ointment did not increase the rate of apoptotic DCs, whereas steroid ointment did so. The isolation-induced high apoptosis rate of freshly isolated LCs was unaffected by both drugs.

Conclusion: Tacrolimus ointment selectively depletes IDECs and alters the immunophenotype of epidermal DCs in AD lesions, but there is no evidence for tacrolimus-induced DC apoptosis in this phenomenon. (*J Allergy Clin Immunol* 2004;114:137–43.)

Key words: Epidermal dendritic cells, flow cytometry, atopic eczema, topical immunomodulators, steroid ointment

Although atopic dermatitis (AD) is a very common chronic inflammatory skin disease, its pathogenesis is still poorly understood.^{1,2} Among the therapeutic agents, the novel immunomodulatory macrolactam ointments have been shown to be highly effective in AD.³ These topical immunomodulators inhibit the transcription of proinflammatory cytokine genes, such as *IL2*, which are dependent on the nuclear factor of activated T cells. Lymphocytes are well-known target cells of these substances, but there is also evidence for an effect on mast cells, endothelial cells, and dendritic cells (DCs).³ In addition to Langerhans cells (LCs), a second population of epidermal DCs, the inflammatory dendritic epidermal cells (IDECs), has been described in AD lesions. These IDECs are characterized by their CD1a expression, as well as their high CD11b and high-affinity IgE receptor (FcεRI) expression, but they do not contain Birbeck granules.⁴ The term *epidermal DC* will be applied in this study to any group of LCs and IDECs if both cell populations either behave in the same way or could not be separated by means of the respective method used in this part of the study. Previous studies with tacrolimus ointment demonstrated a downregulation of FcεRI on epidermal DCs in the lesional skin of patients with AD and a depletion of IDECs from the epidermis in a smaller number of patients, but the cell biologic mechanism of this depletion remained unclear.⁵ Because the proapoptotic effects of tacrolimus have been described in T cells,^{6,7} the present study focused on the effects of tacrolimus versus glucocorticosteroid on the apoptosis rate and immunophenotype of LCs and IDECs *in vitro* and *in vivo*. Time-kinetic *in vitro* studies with epidermal cell suspensions were performed to analyze LC apoptosis induction by tacrolimus. *Ex vivo*, epidermal DCs were analyzed by using Annexin V and terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling (TUNEL) techniques before and 1 week after treatment with tacrolimus ointment.

METHODS

Cell suspensions: *In vitro* studies

For *in vitro* analysis of LC apoptosis induction by tacrolimus, crude epidermal cell suspensions were prepared from normal human skin by means of trypsinization with a standard technique, as previously described.⁸ The cell suspensions were cultured for 24 and

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Abbreviations used

7AAD: 7-Amino-actinomycin-D
AD: Atopic dermatitis
FSC: Forward light scatter
IDEC: Inflammatory dendritic epidermal cell
LC: Langerhans cell
MFI: Mean fluorescence intensity
MoDC: Monocyte-derived dendritic cell
rFI: Relative fluorescence index
SSC: Side light scatter
TUNEL: Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling

48 hours with either 10^{-9} , 10^{-7} , or 10^{-5} mol/L tacrolimus; 10^{-7} mol/L prednisolone; or nil as a control. Immature monocyte-derived dendritic cells (MoDCs) were prepared by means of the dextran sedimentation technique and short-term culture in GM-CSF and IL-4, as previously described,⁹ to serve as a control for effects of isolation-induced apoptosis in LCs.

Cell suspensions: *In vivo* studies

For analysis of the *in vivo* effects of tacrolimus ointment on the number of epidermal DCs, DC apoptosis, and DC immunophenotype, epidermal cell suspensions were prepared from shave biopsy specimens of AD lesions by using standard techniques, as previously described.¹⁰ The patients with AD participated in a double-blind, corticosteroid-controlled clinical trial with tacrolimus ointment.¹¹ After approval by the local ethics committee and after obtaining written informed consent, each patient had one lesional biopsy specimen taken before treatment and one lesional biopsy specimen taken after 1 week of treatment. Epidermal cell suspensions of 14 patients, 9 of them randomized to tacrolimus ointment and 5 randomized to hydrocortisone butyrate ointment, were included in this study.

Immunophenotyping of epidermal cell suspensions

Approximately 2 to 5×10^5 epidermal cells were incubated in a polypropylene tube with primary mAbs in a final concentration of $2 \mu\text{g/mL}$ for 30 minutes at 4°C in the dark. The costimulatory molecules CD80 and CD86 were detected by using mAb BB-1(B7), L307.4 (Camfolio/Becton Dickinson) and mAb B70/B7-2, IT2.2 (Pharmingen); these mAbs do not show any cross-reactivity to CD74, as has been described for other B7 mAbs.¹² The high-affinity receptor for IgE, Fc ϵ RI, was detected by using mAb 22E7 (IgG1; a generous gift of Dr J. Kochan, Hoffmann LaRoche Co, Nutley, NJ), which is directed against the α -chain of Fc ϵ RI but does not interfere with the binding site for IgE on this molecule. The low-affinity IgG receptor CD32 was detected by using VI.3 (IgG2b; Medarex).

After washing once in washing buffer, a second-step FITC-conjugated goat anti-mouse antibody (Jackson Laboratories) was added in a final dilution of 1:200 for 30 minutes at 4°C in the dark. After washing and blocking of the second-step antibody with normal mouse serum in a final dilution of 1:30 for 20 minutes, the washed cells were counterstained with the phycoerythrin-labeled anti-CD1a mAb T6RD1 (IgG1; Beckman Coulter) in a final dilution of 1:300 for 30 minutes at 4°C in the dark. For dead cell exclusion, 7-amino-actinomycin-D (7AAD) was added to the tube during the last antibody incubation, resulting in a final concentration of $1 \mu\text{g/mL}$.¹⁰

Flow cytometric analysis

The washed cells were analyzed with a FACScan (Becton Dickinson). A life gate based on the physical properties of the cells (forward light scatter [FSC] vs side light scatter [SSC]) was set to exclude cell multiples from acquisition. Typically, 5×10^3 events were acquired from a tube containing 2×10^5 cells. Vital CD1a⁺ epidermal DCs were gated out from the acquired events by means of a combination of a physical single-cell gate (FSC vs SSC) with a vital DC gate (CD1a-FL2 vs 7AAD-FL3).¹⁰ Epidermal DC subsets were determined by staining with the CD11b-specific mAb BEAR1 (IgG1; Immunotech) and manual gating for LCs (CD1a⁺⁺⁺, CD11b⁻) and IDECs (CD1a⁺, CD11b⁺⁺⁺). For quantitative evaluation of receptor expression, 2-dimensional dot-plot analysis was performed, and the CD1a⁺ cells were gated out manually. The mean fluorescence intensity (MFI) of the FL-1 signal was determined for the cells of interest by using LysysII software (Becton Dickinson). Relative fluorescence indices (rFIs) of all surface receptors were determined as follows:

$$rFI = (MFI[\text{receptor}] - MFI[\text{control}]) / MFI(\text{control}).$$

Flow cytometric Annexin V technique for analysis of apoptotic cells

For Annexin V analysis of apoptosis, 5×10^5 epidermal cells were preincubated with T6RD1 in a final dilution of 1:300 for 30 minutes at 4°C in the dark, washed, and incubated with $5 \mu\text{L}$ of Annexin V-FITC (Pharmingen) in a $100\text{-}\mu\text{L}$ cell suspension for 15 minutes at room temperature in the dark. After adding binding buffer (10 mmol/L Hepes/NaOH, pH 7.4; 140 mmol/L NaCl; and 2.5 mmol/L CaCl_2), cells were analyzed by means of flow cytometry immediately. In addition, 7AAD was added to the tube in a final concentration of $1 \mu\text{g/mL}$. Epidermal DCs were gated out by using a combination of a physical single-cell gate (FSC vs SSC) and CD1a expression, and quadrant gates were set to determine the percentages of vital (Annexin⁻, 7AAD⁻) cells, early apoptotic (Annexin⁺, 7AAD⁻) cells, and late apoptotic/dead (Annexin⁺, 7AAD⁺) cells.

Flow cytometric TUNEL technique for analysis of apoptotic cells

For TUNEL analysis of apoptosis, 5×10^5 epidermal cells were prepared, prestained with T6RD1, and washed as described above and then fixed in paraformaldehyde, washed, permeabilized with Triton X100, washed, incubated with TUNEL reaction mixture, washed, and analyzed on a FACScan with a commercial TUNEL kit (Boehringer), according to the manufacturer's instructions. Pure label solution was used as a control. In addition, 7AAD was added to the tube at a final concentration of $1 \mu\text{g/mL}$. On analysis, quadrant gates were set to determine the percentages of vital (TUNEL⁻, 7AAD⁻) cells, apoptotic (TUNEL⁺, 7AAD⁻) cells, and dead (7AAD⁺) cells.

Statistical analysis

Results show arithmetic means $\pm$ SEMs. The Wilcoxon signed-rank test was performed for statistical analysis.

RESULTS

Tacrolimus does not alter the isolation-induced apoptosis of LCs

Because epidermal LCs are known to spontaneously undergo apoptosis after isolation, the *in vitro* analysis of potential apoptosis-inducing tacrolimus effects on epidermal LCs must focus on the potentially increased apoptosis

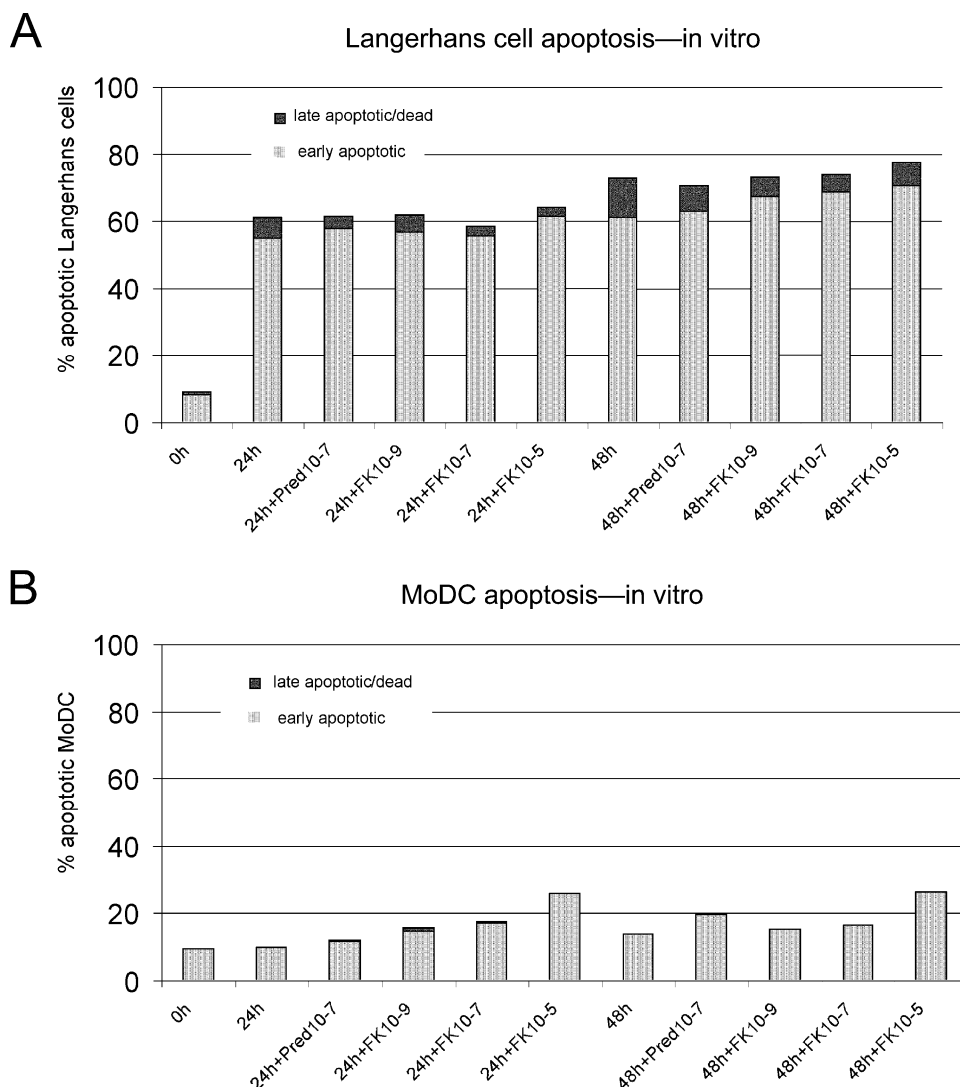


FIG 1. Apoptosis detection in epidermal LCs and MoDCs *in vitro*. The percentage of early apoptotic (Annexin V⁺, 7AAD⁻) and late apoptotic (Annexin V⁺, 7AAD⁺) DCs identified by using the Annexin V technique is shown. **A**, Epidermal cell suspensions prepared from normal human skin cultured for 24 or 48 hours with nil, 10^{-7} mol/L prednisolone, or 10^{-9} , 10^{-5} , or 10^{-7} mol/L tacrolimus. Langerhans cells are gated out by CD1a expression. **B**, Immature MoDCs are gated out by CD1a expression. Pred, Prednisolone; FK, tacrolimus.

rate compared with the spontaneous rate. Therefore epidermal cell suspensions from normal human skin were cultured for 24 and 48 hours with either tacrolimus, prednisolone, or nil as a control. Annexin-7AAD staining was performed and calculated for the different culture conditions, as previously described. A significant rate of spontaneous LC apoptosis was seen already at 24 hours after LC isolation from normal human skin, which increased further after 48 hours (Fig 1, A). Cell culture with different concentrations of tacrolimus or addition of prednisolone did not influence this spontaneous apoptosis rate (Fig 1, A). All LCs constantly expressed low amounts of CD95/Fas molecules (not shown).

Analogous investigations in MoDCs, which are not subject to spontaneous apoptosis, showed a much lower rate of apoptosis for prednisolone, tacrolimus, and

controls compared with LCs (Fig 1, B). The increased apoptosis rate seen in the concentration of 10^{-5} mol/L tacrolimus represents an approximately 1000-fold excess compared with the serum concentration established for oral tacrolimus use in transplant medicine.

Tacrolimus ointment depletes IDECs but does not deplete epidermal LCs *in vivo*

Ex vivo studies were performed in patients with AD participating in a double-blind, hydrocortisone butyrate—controlled clinical trial with tacrolimus ointment to assess potential tacrolimus effects in an *in vivo* setting in which the complex interactive mechanisms of the skin immune system are active. Epidermal cell suspensions of AD lesions were prepared before and 1 week after

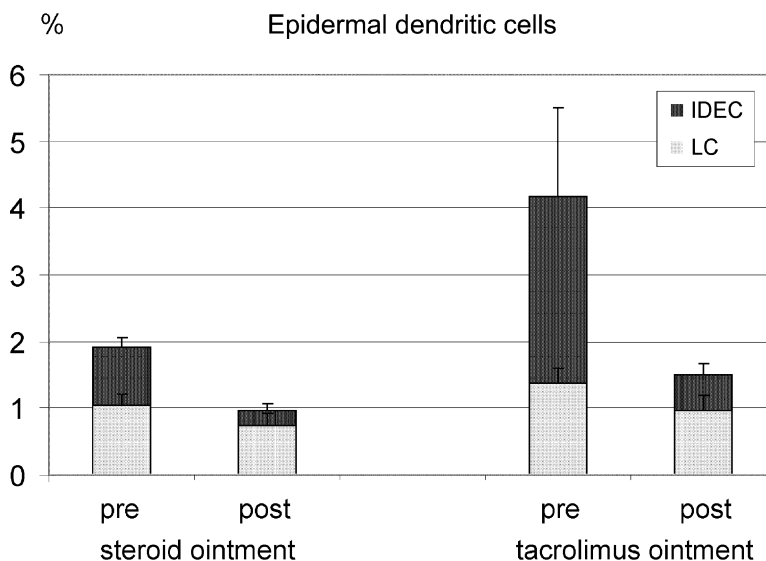


FIG 2. Tacrolimus ointment does not deplete epidermal LCs *in vivo*. Enumeration of LCs (CD1a⁺⁺⁺, CD11b⁺) and IDECs (CD1a⁺, CD11b⁺⁺⁺) in epidermal cell suspensions of AD lesions before and after 1 week of topical treatment with hydrocortisone butyrate ointment (n = 5) and tacrolimus ointment (n = 9). The IDEC population was depleted significantly from the epidermis by using tacrolimus ointment ($P < .01$) and steroid ointment ($P < .05$), whereas the LC population essentially remained unaltered.

initiation of therapy and processed for flow cytometric enumeration and epidermal DC phenotyping.

Epidermal DC numbers, which are known to be increased in AD lesions, returned to normal values during treatment with both tacrolimus and hydrocortisone ointment (Fig 2). Thereby the LC population essentially remained unaltered, whereas a significant 75% reduction in the cell number of IDECs occurred, thus confirming the results obtained in an earlier pilot study.⁵ The observed differences in epidermal DC numbers between both groups before treatment (Fig 2) probably reflect the low number of patients in the hydrocortisone group.

Tacrolimus ointment does not induce apoptosis of epidermal DCs *in vivo*

Apoptosis rates of epidermal DCs were examined with 2 different techniques (Annexin V and TUNEL) to control for their individual weaknesses. Early apoptotic DCs were detected by means of Annexin V staining in all our epidermal cell suspensions (Fig 3). The rate of (early) apoptotic DCs *in situ* was increased in the hydrocortisone-treated AD lesions, but this did not become significant because of the small number of steroid-treated control patients (n = 5). In contrast, neither technique showed any increase in the percentage of apoptotic epidermal DCs in the tacrolimus-treated patients (n = 9).

Tacrolimus ointment and steroid ointment reduce *in situ* expression of costimulatory molecules during AD treatment

Because an *in vitro* effect of tacrolimus on the maturation-associated upregulation of the costimulatory molecules CD80 and CD86 has been described in LCs

isolated from normal human skin,¹³ we investigated the effects of tacrolimus on the *in situ* expression of CD80 and CD86 on epidermal DCs in patients with AD *in vivo*. Topical treatment with tacrolimus ointment and hydrocortisone butyrate ointment led to a clinical improvement of the skin lesions.¹¹ The *in situ* expression of the costimulatory molecules CD80 and CD86 on epidermal DCs was reduced during treatment (Fig 4, A and B). The clinical improvement was observed through a significant reduction of the diagnostic expression ratio of FcεRI/FcγRII (Fig 4, C), which decreased to less than the threshold value of 1.5 for an inflammatory AD micromilieu.¹⁴ Again, the pretherapeutic differences regarding the FcεRI/FcγRII ratio (Fig 4, C) between the tacrolimus and hydrocortisone groups are an effect of the low number of patients in the hydrocortisone group because they indicate the differences between human subjects and the diseases they acquire. Working with skin biopsy specimens of living patients usually requires facing the problem of small sample sizes. Because we analyzed matched pairs of biopsy specimens, we were able to gain as much data from this limited number of patients as possible.

DISCUSSION

In a first step we demonstrated that the addition of tacrolimus or prednisolone did not influence the spontaneous apoptosis rate in LCs *in vitro* and that therapeutic concentrations of tacrolimus did not induce significant apoptosis in MoDCs. This is different from hydrocortisone-treated AD lesions, in which we detected an increased rate of early apoptotic DCs. In tacrolimus-treated

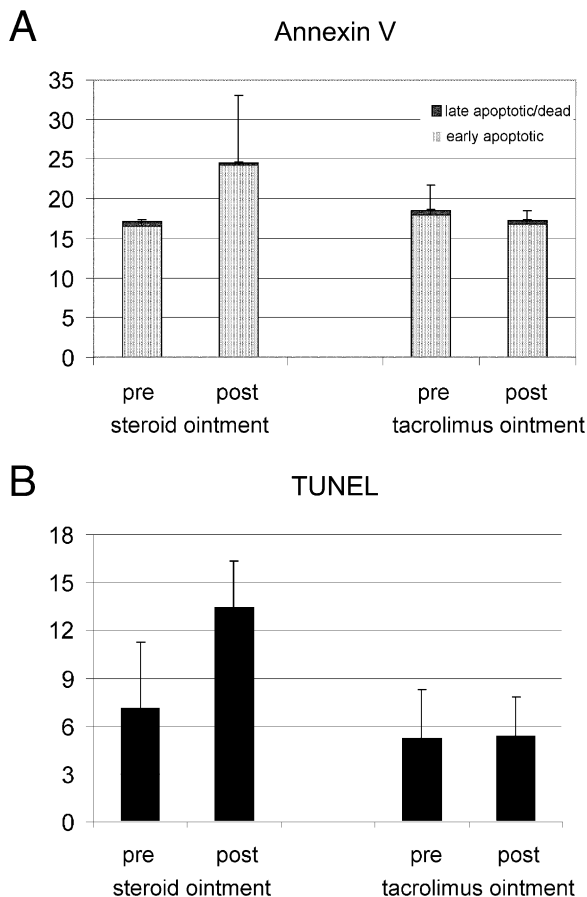


FIG 3. Tacrolimus ointment does not induce apoptosis of epidermal DCs *in vivo*. Analysis of epidermal DC apoptosis by Annexin V (**A**) and TUNEL (**B**) techniques is shown. The percentage of early apoptotic (Annexin V⁺, 7AAD⁻) and late apoptotic (Annexin V⁺, 7AAD⁺) cells (Fig 3, **A**) and the percentage of apoptotic (TUNEL⁺) cells (Fig 3, **B**) is determined from epidermal cell suspensions of AD lesions before and after 1 week of topical treatment with hydrocortisone butyrate ointment (n = 5) and tacrolimus ointment (n = 9).

AD lesions, there was no such increase of apoptotic epidermal DCs. The induction of apoptosis by hydrocortisone did not become statistically significant in our experiments, which was probably caused by the low number of patients in the hydrocortisone group. Published data about a steroid-mediated apoptotic effect include an induction of apoptosis of airway DCs in rat tracheal mucosa.¹⁵ When apoptosis was measured by determining Annexin V–positive cells after stimulation with dexamethasone, DC apoptosis occurred in a dose- and time-dependent manner.¹⁶ In contrast, the drug mechanisms of tacrolimus are still a matter of debate because some researchers have reported that tacrolimus induces apoptosis,⁷ whereas others suggest an antiapoptotic action of tacrolimus.¹⁷ Data from a mouse model for investigating autoimmune pancreatitis suggested an immunosuppressive effect of tacrolimus through augmentation of T-cell apoptosis.⁶ Tacrolimus-induced features of apoptosis

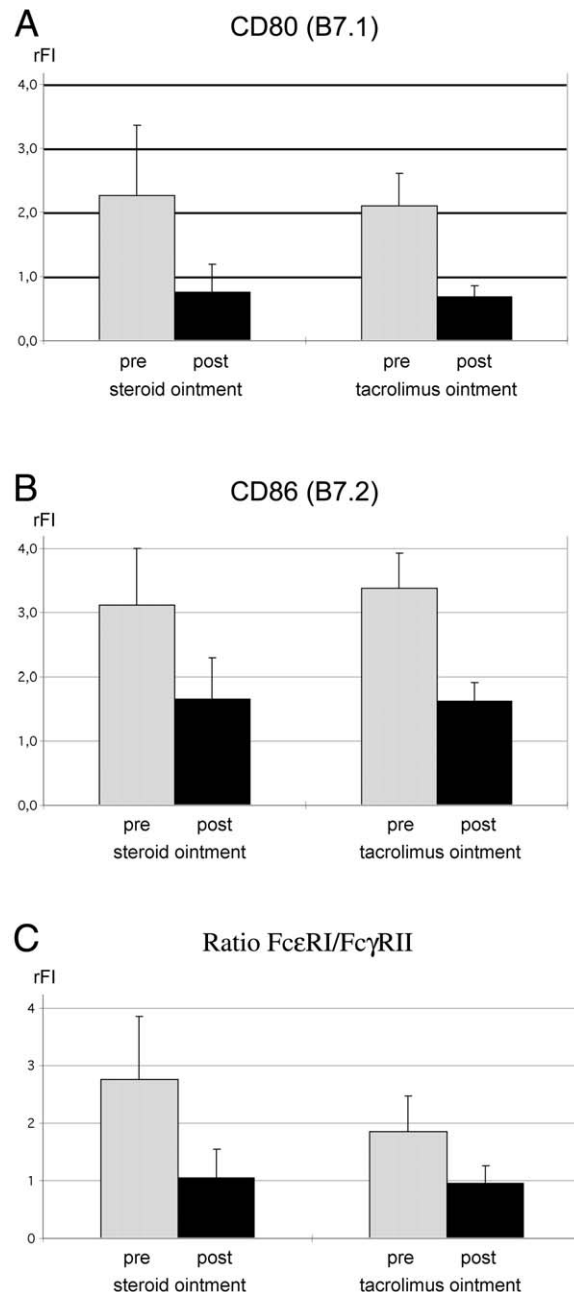


FIG 4. Tacrolimus ointment and steroid ointment reduce costimulatory molecule expression during AD treatment. The expression of the costimulatory molecules on epidermal DCs of AD lesions before and after 1 week of topical treatment with hydrocortisone butyrate ointment (n = 5) and tacrolimus ointment (n = 9) is shown. Tacrolimus ointment reduced the expression of CD80 (B7.1; **A**) and CD86 (B7.2; **B**) significantly ($P < .01$), and similar effects were observed for steroid ointment. Treatment success for both ointments is observed through a significant reduction ($P < .01$) of the FcεRI/FcγRII ratio (**C**) to less than the cutoff level of 1.5, an established marker for AD lesions.

(cell shrinkage, chromatin condensation, and DNA fragmentation) were also found in a murine capillary endothelial cell line model.⁷ Yardin et al¹⁷ detected an antiapoptotic effect of tacrolimus in a cell-culture model of

neuronal cells, which was associated with a downregulation of the c-jun protein expression. The authors concluded that the downregulation of c-jun might actually play a major role in the observed antiapoptotic effects of tacrolimus.¹⁷ In our epidermal DCs, neither an apoptosis induction nor an antiapoptotic tacrolimus effect could be detected. Because of limitations in technique and sample size, we could not differentiate LCs from IDECs. The minute amount of epidermal cells would not allow running of multiple apoptosis assay tubes for each patient, and the difference in CD1a expression alone did not allow the separation of LCs and IDECs with our FACScan flow cytometer. However, if there is no increased apoptosis rate in the entire DC population of the tacrolimus-treated skin samples, this is valid information in terms of cell biology. These results correspond nicely to a recent demonstration that pimecrolimus does not induce apoptosis of MoDCs if added during differentiation.¹⁸

In a second step we demonstrated that the number of epidermal DCs, which are known to be increased in AD lesions, decreased during topical tacrolimus and hydrocortisone treatment. This effect was largely due to a 75% depletion of IDECs, whereas LC numbers were essentially unaffected. These IDECs are present only in inflammatory skin lesions and not in normal skin.¹⁰ The results of a smaller pilot study that did not allow statistical analysis⁵ were now confirmed at a high level of significance ($P < .01$) in this larger group of patients. The 9 patients described in our first report were treated with 3 different concentrations of tacrolimus ointment or placebo for different periods of time,⁵ whereas the 14 patients of this report received uniform treatment with either tacrolimus ointment ($n = 9$) or placebo ointment base ($n = 5$) for 1 week. Although the total number of patients in this study is not much higher than in our pilot study, the statistical power of this report is much higher.

Tacrolimus-induced clinical improvement of epidermal hyperplasia and lichenification, which takes considerably longer than erythema reduction,³ does not explain this phenomenon because it is essentially based on keratinocyte reduction. It is an important aspect that tacrolimus ointment does not deplete LCs from the normal epidermis because this is a known side effect of potent steroid ointments.¹⁹ Because the selective depletion of IDECs is apparently not mediated by apoptosis, the underlying mechanism for this phenomenon remains to be identified. These other mechanisms might include cell migration after a yet-to-be-defined chemotactic gradient built up by lymphocytes or differentiation of IDECs into other cell types.

In a third step we demonstrated that tacrolimus ointment did not increase the apoptosis rate of epidermal DCs *in vivo*, whereas increased apoptosis was seen with steroid ointment. The apoptosis induction observed by steroids was an expected effect. Our results are in good accordance with those of a recent study showing that pimecrolimus cream does not deplete LCs in a mouse model,¹⁹ but these results differ from the finding of proapoptotic effects of tacrolimus described for T cells.⁶

Finally, we observed a reduced *in situ* expression of the costimulatory molecules CD80 and CD86 for topical tacrolimus and topical steroids. In another study tacrolimus had no significant effect on the expression of CD80 and CD86 in MoDCs,²⁰ whereas a tacrolimus effect has been observed on CD80, but not CD86, expression on LCs *in vitro*.¹³ Thus we identified a downregulation of CD80 on epidermal DCs in AD lesions *in vivo*, which corresponds to the previously reported effects of tacrolimus on freshly isolated LCs *in vitro*.¹³ Investigating the modulation of the antigen-presenting capacity of LCs from normal human skin, a marked inhibition of CD80 and CD86 expression on these LCs with dexamethasone was seen, whereas tacrolimus slightly inhibited the expression of CD80, but not CD86, on LCs.²¹ The induction of CD80 and CD86 on MoDCs was inhibited by coincubation with steroids but not with pimecrolimus.¹⁸ In our patients 1 week of treatment with topical steroids decreased the expression of CD80 and CD86 on epidermal DCs. In contrast, one *in vitro* study described an increased expression of CD80 and CD86 after steroid treatment.¹³ Another study with hydrocortisone-treated MoDCs showed a decreased expression of CD86.²² The embedding of LCs and IDECs in the complex network of the distorted skin immune system in AD will probably account for the differences between the results from our study and published *in vitro* data. Because the *in situ* expression of costimulatory molecules is a key feature of AD, costimulatory molecule expression might actually be important for the generation and perpetuation of the skin lesions in AD.²³

In conclusion, we observed tacrolimus-induced changes in both number and immunophenotype of epidermal DCs in AD lesions, but there is no evidence for a role of tacrolimus-induced DC apoptosis in this phenomenon.

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