Original article

Reduced dermal infiltration of cytokine-expressing inflammatory cells in atopic dermatitis after short-term topical tacrolimus treatment

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Background: In several clinical studies, topical calcineurin inhibitors have been shown to be effective in the treatment of atopic dermatitis (AD). They target signaling pathways that control gene expression, particularly the expression of cytokines.
Objective: We examined the cellular infiltrate in skin lesions of 10 patients with AD and characterized the cytokine pattern expressed by the infiltrating cells before and after short-term topical therapy with tacrolimus 1% ointment.
Methods: Skin biopsies were examined for histologic alterations (hematoxylin and eosin staining), composition of the cellular inflammatory infiltrate (immunofluorescence), and cytokine expression (ribonuclease protection assay, ELISA, immunofluorescence) before as well as 1 and 3 weeks after initiation of tacrolimus therapy. For comparison, biopsies from nonlesional AD and normal skin were analyzed. Systemic immunologic effects were assessed by analyzing peripheral blood leukocytes (immunofluorescence) as well as *in vitro* stimulated pan–T-cell cytokine production (ELISA).
Results: All patients showed a significant improvement of their skin lesions associated with a marked regression of spongiosis, acanthosis, and density of the cellular infiltrate in the dermis. The last was a result of reduced infiltration of T cells, B cells, and eosinophils. In contrast, the numbers of mast cells did not change. Moreover, the expression of the T_H2 cytokines IL-5, IL-10, and IL-13 in $CD4^+$ T cells was reduced after therapy. Interestingly, tacrolimus therapy was also associated with a reduction of $CD8^+$ T cells expressing the T_H1 cytokine IFN-γ. Furthermore, the numbers of epidermal $CD1a^+$ dendritic cells increased after treatment. In the peripheral blood, a decrease of granulocytes (eosinophils and neutrophils) but no changes in the distribution of lymphocyte subpopulations were noticed.
Conclusion: Topical tacrolimus treatment has anti-inflammatory effects on AD skin as indicated by reduced infiltration of cytokine expressing inflammatory cells. No evidence for drug-induced systemic immunosuppression was obtained. (J Allergy Clin Immunol 2004;114:887-95.)

Key words: *Atopic dermatitis, cytokines, inflammation, tacrolimus, T cells*

Atopic dermatitis (AD) is characterized by chronic or relapsing eczematous lesions with typical morphology and distribution, as well as by pruritus.[1] In the dermis of AD lesions, there is a marked perivascular T-cell infiltrate,[2,3] in which both $CD4^+$ and $CD8^+$ T cells are present.[4] The cellular infiltrate may also contain variable numbers of eosinophils.[5] The infiltration of cells is associated with increased expression of cytokines, in particular of IL-5 and IL-13.[6,7] In chronic lesions, IFN-γ–producing cells have also been described,[8,9] despite a reduced capacity of PBMCs to generate this cytokine under *in vitro* conditions.[10,11]

Tacrolimus (FK506) is an immunosuppressive drug that inhibits intracellular signaling pathways leading to cytokine production in lymphocytes. Like cyclosporin and pimecrolimus, tacrolimus decreases the phosphatase activity of calcineurin, leading to reduced dephosphorylation of the transcription factor NF-AT.[12] Dephosphorylation of NF-AT, however, is required for its translocation to the nucleus. Therefore, this group of drugs prevents activation of or silences activated cytokine genes. Although topical tacrolimus has been shown to be effective in the treatment of AD,[13,14] little is known about its immunopharmacologic effects in the skin. The well documented effect of tacrolimus on isolated T cells indicates that it may also suppress T cells in the dermis of patients with AD. In this study, we investigated the effects of this drug on dermal inflammatory cells and their cytokine gene expression in patients with AD.

METHODS

Patients

Ten patients (7 female patients; 3 male patients; age, 18-58 years; average age, 34 years) with moderate to severe AD[15] fulfilling the diagnostic criteria of Hanifin and Rajka[16] were included. Patient characteristics are summarized in Table I. All patients had an acute exacerbation of their AD and were treated with tacrolimus 0.1%

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TABLE 1. Patient characteristics

Group	Initials	Age (y)	Sex	Duration of AD (y)	Score*	EASI at baseline	Allergic rhinitis	Bronchial asthma	Total IgE (kU/L)	Allergen-specific IgE†	Skin prick test‡
1	KS	22	F	22	7	6.8	+	+	4703	Dp, Df, c, g, b	+
	MO	18	F	18	7	12.6	+	−	19	g	+
	SB	23	F	2	8	10.8	+	+	258	Dp, Df, c, g, b	+
	GC	30	F	30	6	17.4	+	+	1227	Dp, Df, c, g, b	+
	HT	58	F	10	6	13.2	+	−	10	Negative	Negative
2	KH	51	M	47	9	55.2	+	+	8174	Dp, Df, c, g, b	ND
	DR	35	M	2	9	37.4	+	+	8516	Dp, Df, c, g, b	ND
	CG	32	F	32	9	54.9	+	−	931	c, g, b	ND
	FR	46	M	22	7	30.1	−	−	9824	Dp, Df, c, g, b	ND
	EL	23	F	21	7	20.8	−	−	111	Negative	Negative

*Grading score of AD, on the basis of the criteria proposed by Rajka and Langeland.[15]
†On a routine base, we determined specific IgE against *Dermatophagoides pteronyssimus* (Dp), *Dermatophagoides farinae* (Df), cat dander (c), grass pollen (g), and birch pollen (b).
‡Skin prick tests could not be done (ND) in case of skin lesions at the test sites and/or treatment with antihistamines.

Abbreviations used
AD: Atopic dermatitis
EASI: Eczema area and severity index
IDEC: Inflammatory dendritic epidermal cell
RNase: Ribonuclease

ointment twice daily for 3 weeks. Therapy with topical steroids was stopped at least 3 days, with systemic steroids 1 week, with nonsteroid immunosuppressives 2 weeks, and with UV radiation 6 weeks before the beginning of this study. None of these treatments was allowed during the study.

The eczema area and severity index (EASI)[17] was determined before as well as 1 and 3 weeks after starting tacrolimus therapy. According to the actual disease severity, patients were divided into 2 groups (Table I). Group 1 included 5 patients with moderate activity (EASI <20), whereas the 5 patients of group 2 had severe AD (EASI >20). The mean EASI levels between group 1 and 2 differed significantly ($P = .008$). Skin biopsies were taken from acute AD lesions at week 0 and from corresponding areas 1 week (group 1) or 3 weeks (group 2) after starting therapy. At the same time points, 10 mL ethylenediamine tetraacetic acid and 20 mL heparin anticoagulated blood were obtained for immunologic analysis. All patients signed a written informed consent agreement. For comparison, biopsies from nonlesional skin of 3 patients with AD and normal skin of 3 control individuals were analyzed. The study was approved by the local medical ethics committee.

Analysis of leukocytes and lymphocyte subsets

Blood leukocyte and differential counts were determined by automatic blood count analysis (Sysmex KX-21, Sysmex Corp, Kobe, Japan; Digitana SA, Yverdon-les-Bains, CH). To determine lymphocyte subsets, blood was analyzed by standard flow cytometric techniques[18,19] by using a FACS Calibur (Becton Dickinson, Heidelberg, Germany).

Ribonuclease (RNase) protection assay

Total RNA was isolated from skin biopsy tissues by using TRIzol solution (Invitrogen, Basel, Switzerland) according to the manufacturer's instructions. Multiprobe template set hCK-1 (DNA templates for IL-2, IL-9, IL-13, IFN-γ, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Becton Dickinson Biosciences (Basel, Switzerland), and the assay was performed as described previously.[20] The gel was visualized by using the imaging system Typhoon 9200 (Amersham Biosciences Europe GmbH, Otelfingen, Switzerland).

Immunoassays

PBMCs were separated from peripheral blood by Ficoll-Hypaque density gradient centrifugation. The cells were suspended at 1×10^6/mL in complete culture medium (RPMI 1640 supplemented with 2 mmol/L L-glutamine, 200 IU/mL penicillin, 100 μg/mL streptomycin, and 10% FBS; all from Life Technologies, Basel, Switzerland). PBMCs (1×10^6/mL) were also stimulated with PHA for 24 hours, and the supernatants were harvested. Skin biopsies were chopped into small pieces, transferred to 0.25 mL 0.9% NaCl solution, and homogenized for 5 minutes on ice. Suspensions were centrifuged for 10 minutes at 3000 rpm at 4°C. Supernatants from fresh and cultured PBMCs and tissue fluids were frozen at −80°C until cytokine analysis.

IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ were measured by using the T_H1/T_H2 Cytometric Bead Array Assay from Becton Dickinson Biosciences. IL-13 levels were determined by a commercial ELISA kit (Immunotech, Marseille, France). Cytokines in skin were given as ratios (ng/g total protein). Total protein concentrations were measured with a commercial kit (BCA Protein Assay Kit, Perbio Science Switzerland SA, Lausanne, Switzerland).

Histologic examination

Skin biopsy specimen were fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 6 μm were stained with hematoxylin and eosin and examined by light microscopy (Axiovert 35, Carl Zeiss, Heidelberg, Germany). Histologic criteria specific for AD such as acanthosis, hyperkeratosis, spongiosis of the epidermis, and intensity of the inflammatory infiltrate in the dermis were semiquantitatively analyzed by using a 4-point scale: absent, 0; minimal, 1; moderate, 2; and severe, 3.

Immunohistologic examination

Immunofluorescence staining was performed on 4-μm sections of paraformaldehyde-fixed and paraffin-embedded skin biopsies. To identify cell populations in the dermal infiltrate, sections were treated with monoclonal antibodies against CD4 (T_H cells), CD8 (T-effector cells), CD21 (B cells; all from Serotec, Oxford, United Kingdom), eosinophil cationic protein (eosinophils; Pharmacia Diagnostics AB, Uppsala, Sweden), and tryptase (mast cells; Dako, Zug, Switzerland) at 4°C overnight. To detect epidermal dendritic cells, we used an anti-CD1a monoclonal antibody (M3571, Dako, Zug, Switzerland).

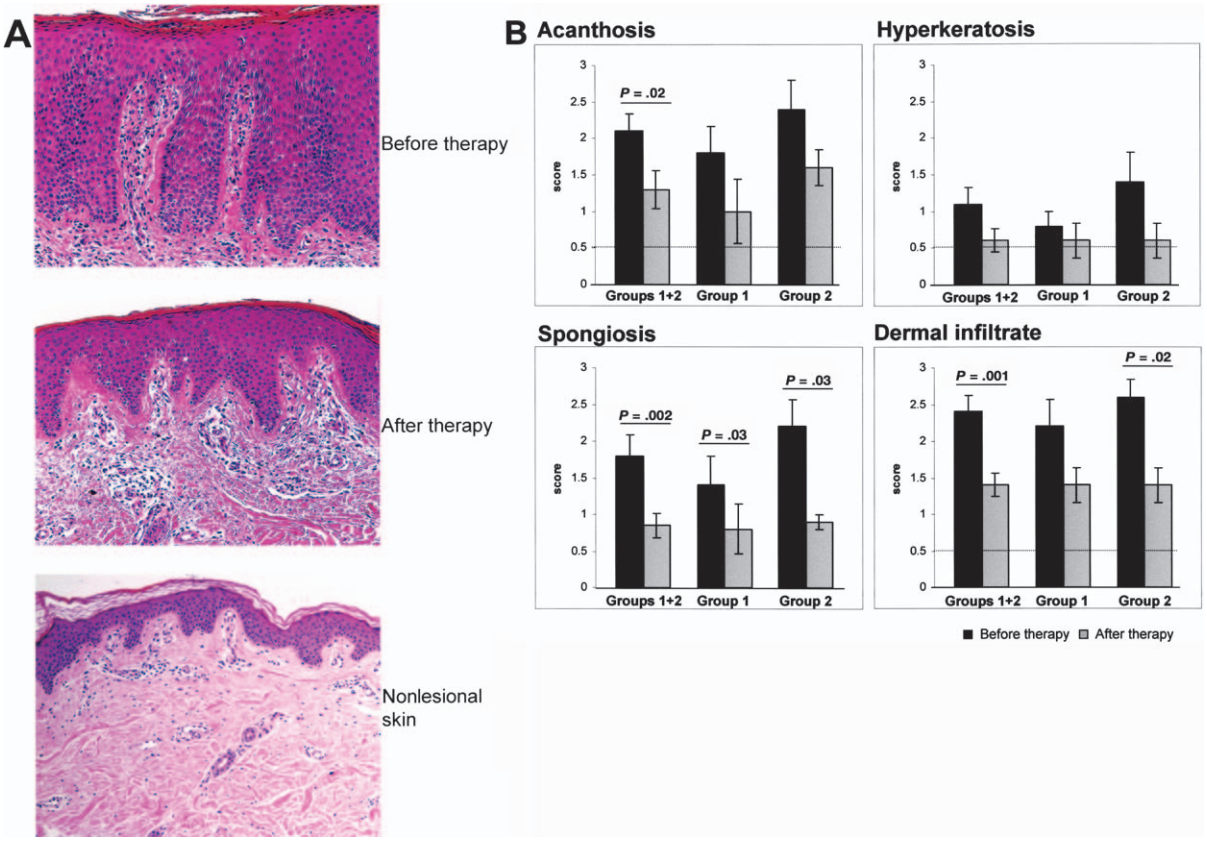

FIG 1. Histologic features of patients with AD before and after topical tacrolimus treatment compared to nonlesional AD skin. **A,** Hematoxylin and eosin–stained skin-biopsy specimens (×200). **B,** Statistical analysis of acanthosis, hyperkeratosis, spongiosis, and dermal infiltrate in all patients and separated for each patient group. The *dashed lines* represent the mean scores in nonlesional AD skin (n = 3). There was no evidence for spongiosis in nonlesional AD skin.

Cytokine expression was analyzed by using polyclonal antibodies recognizing rabbit antihuman IL-5, IL-13 (both from Santa Cruz Biotechnology, Santa Cruz, Calif), IL-10, and IFN-γ (both from R&D Systems, Abington, United Kingdom). Antibody binding was detected with appropriate antimouse and antirabbit rhodamine-tetramethyl rhodamine isothiocyanate–conjugated and/or fluorescein isothiocyanate–conjugated secondary antibodies (Milan Analytica AG, La Roche, Switzerland). Immunofluorescent staining was viewed by 2 independent investigators by using a confocal laser scanning microscope (LSM 510, Carl Zeiss). To calculate the number of immunofluorescent positive cells in the skin specimens, we used a 2-dimensional system.[21,22]

Statistical analysis

Data are presented as means ± SEMs. To compare the results before and after therapy as well as between group 1 and 2, the unpaired *t* test, paired *t* test, and Mann-Whitney *U* test were applied. A *P* value of <.05 was considered statistically significant. The *P* values of significant differences are indicated in the figures and Table I.

RESULTS

Tacrolimus treatment is associated with clinical improvement

To evaluate the clinical efficacy of the treatment with topical tacrolimus, the EASI was determined. Before treatment, the EASI ranged from 6.8 to 54.9 (mean, 25.7). All patients had a rapid improvement of their AD lesions within the first week of treatment. A further improvement within the next 2 weeks was achieved in 8 of the 10 patients. In group 1, the mean EASI was 12.2 before therapy and dropped down to 6.9 after 1 week ($P = .036$) and to 5.1 after 3 weeks ($P = .009$). In group 2, the decrease was more drastic, from 39.7 to 20.9 after 1 week ($P = .041$) and to 16.5 after 3 weeks ($P = .026$).

Normalization of the skin architecture and decreased numbers of skin-infiltrating inflammatory cells

In all skin biopsy specimens taken from lesional skin before therapy, histologic alterations characteristic for an acute exacerbation of AD such as spongiosis and dermal inflammatory infiltrate were observed. Moreover, signs of chronic AD such as hyperkeratosis and acanthosis were also present in all cases (Fig 1, *A*). To evaluate therapeutic effects on histologic parameters, we semiquantitatively calculated the degree of hyperkeratosis, acanthosis, spongiosis, and inflammatory cell infiltration. Statistically different improvements were seen regarding spongiosis,

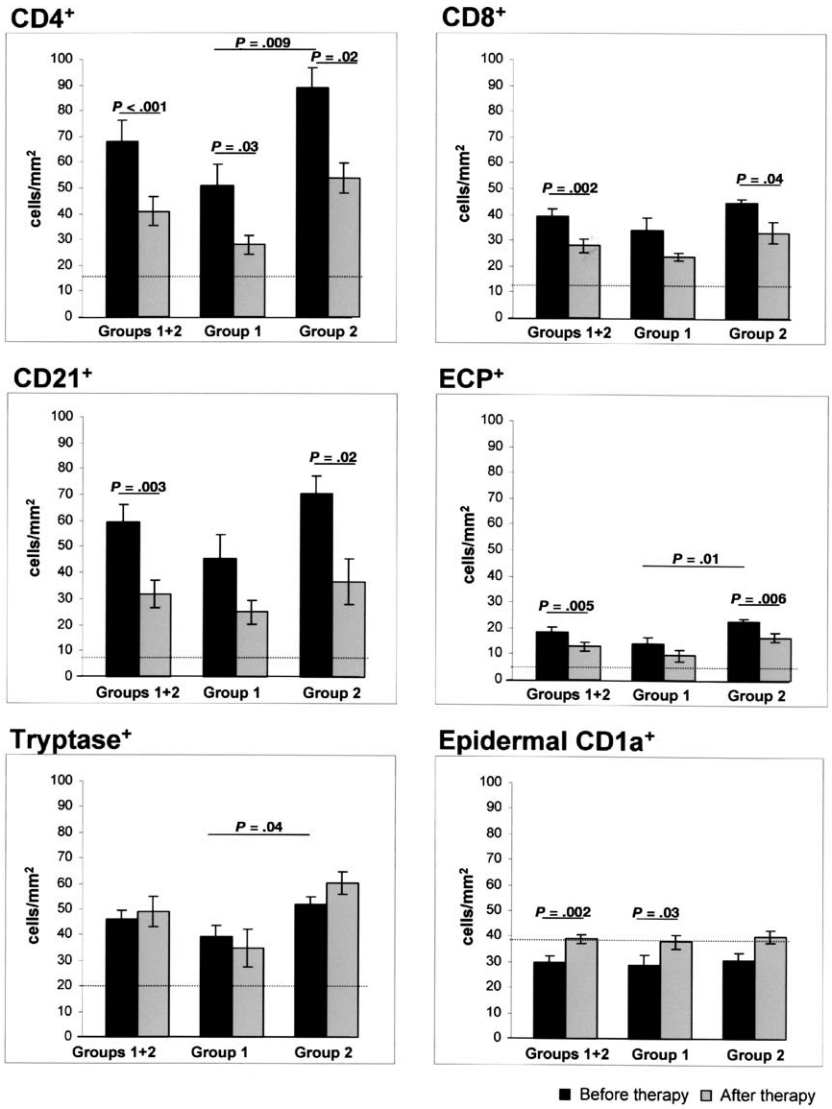

FIG 2. Statistical analysis on the numbers of inflammatory cells in skin lesions of patients with AD before and after tacrolimus treatment as assessed by immunofluorescence and confocal microscopy. The *dashed lines* represent the mean cell numbers in nonlesional AD skin (n = 3). Normal dermis contained only single lymphocytes and no eosinophils (data not shown). Representative original results of these experiments are shown in Fig 3.

dermal infiltrate, and acanthosis, although the after-treatment scores were still higher compared with those in nonlesional AD skin. Spongiosis was the most sensitive parameter, which significantly improved independent of disease severity and time of treatment (Fig 1, *B*).

Decreased numbers of dermal CD4$^+$, CD8$^+$, CD21$^+$, and eosinophil cationic protein–positive cells, but increased numbers of epidermal CD1a$^+$ cells

To identify the inflammatory cells infiltrating the dermis of lesional skin, we used a single immunofluorescence technique with antibodies against lineage-associated molecules. Patients in group 2 had significantly more CD4$^+$ but not CD8$^+$ cells compared with group 1 before treatment. Moreover, numbers of mast cells and eosinophils were significantly increased in group 2 compared with group 1 (Fig 2). We also observed infiltration of large numbers of B cells in both patient groups, a finding that has not previously been associated with AD. B-cell numbers were higher in group 2 than in group 1, but this difference was not statistically significant ($P = .058$). These data suggest that the disease severity of AD correlates with the numbers of infiltrating dermal CD4$^+$ T cells, mast cells, and eosinophils, and perhaps, also with dermal B cells.

When both patient groups were taken together, tacrolimus treatment was associated with a significant reduction of CD4$^+$ and CD8$^+$ cells as well as B cells and eosinophils (Figs 2 and 3). In contrast, the numbers of

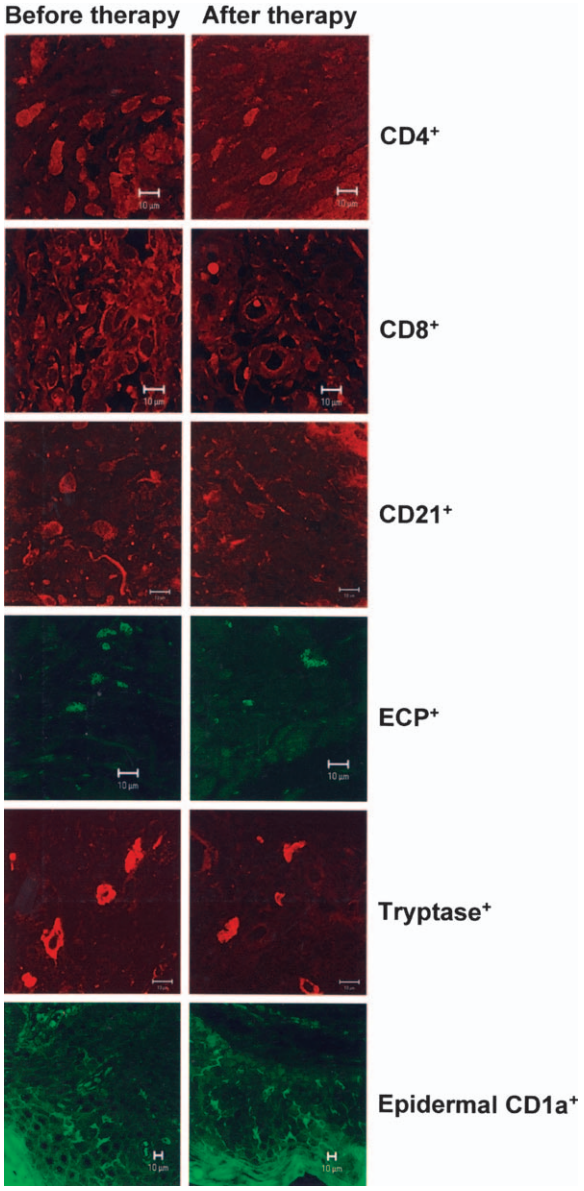

FIG 3. Single immunofluorescence staining of skin biopsy specimens with monoclonal antibodies against lineage-associated molecules of leukocytes. The *scale bars* represent 10 μm.

epidermal $CD1a^+$ cells increased after treatment, perhaps because of a repopulation with high CD1a-expressing Langerhans cells and a reduction of low CD1a-expressing inflammatory dendritic epidermal cells (IDECs).[24] The numbers of mast cells did not show any reduction after drug application. When groups 1 and 2 were compared, a more pronounced reduction of infiltrating lymphocytes and eosinophils was seen in patients of group 2. The numbers of inflammatory cells present in short-term tacrolimus-treated lesional skin were higher than those infiltrating into nonlesional AD skin. Interestingly, no difference was seen in the numbers of epidermal $CD1a^+$ cells between treated lesional and nonlesional AD skin.

Decreased cytokine expression in lesional skin

Atopic dermatitis has been associated with increased expression of both T_H1 and T_H2 cytokines at inflammatory sites.[6-9] We analyzed cytokine gene expression by RNase protection assay in skin tissues taken from lesional skin of 3 patients before and after tacrolimus treatment. We observed in all 3 patients detectable levels of IL-2, IL-9, and IL-13 mRNA in acute skin lesions (Fig 4). IFN-γ mRNA was seen in 2 out of 3 patients. Furthermore, we noticed a clear reduction in the expression of these cytokine genes after 1 week (patient MO) and after 3 weeks (patients KH and CG) of treatment, suggesting that tacrolimus targets both T_H1 and T_H2 cytokines.

To determine whether the difference in cytokine mRNA expressions is also seen at the protein level, we measured cytokine levels in crude tissue fluids obtained from skin biopsies before and after tacrolimus treatment. IL-5 and IL-13 were readily detectable by using the immunoassays described in the Methods section. IL-5 tissue levels (per gram tissue protein) decreased from 3.8 ± 1.7 ng to 1.5 ± 0.5 ng, whereas levels of IL-13 in skin decreased from 24.4 ± 12.1 ng to 6.2 ± 1.7 ng. The levels of IL-2, IL-4, IL-10, TNF-α, and IFN-γ were usually below the detection limit even before therapy (data not shown).

Decreased numbers of cytokine-expressing dermal $CD4^+$ and $CD8^+$ cells

Because many cytokines were not detectable by using regular immunoassays in tissue fluids, we analyzed the T_H1/T_H2 cytokine expression pattern of $CD4^+$ and $CD8^+$ cells by using a double immunofluorescence technique. We observed that at least a subpopulation of both cell types expressed IL-5, IL-10, IL-13, and IFN-γ. In general, the numbers of cytokine-expressing $CD4^+$ cells were higher than cytokine-expressing $CD8^+$ cells. Moreover, the numbers of cytokine expressing $CD4^+$ and $CD8^+$ cells were higher in group 2 than in group 1 patients (Fig 5, *A* and *B*).

The reduced expression of the T_H2 cytokines IL-5, IL-10, and IL-13 during the tacrolimus treatment occurred mainly in $CD4^+$ and less in $CD8^+$ cells (Fig 5 and 6). An additional interesting observation was that tacrolimus appeared to reduce the expression of IFN-γ in $CD8^+$ cells more effectively than in $CD4^+$ cells. In contrast to the numbers of inflammatory cells (Figs 2 and 3), the reduction of the numbers of cytokine-expressing cells were more pronounced in group 1 than group 2.

Reduced numbers of blood neutrophils and eosinophils but no changes in PBMC numbers or in the capacity of PBMCs to generate cytokines

The white blood cell count showed a decrease in the leukocyte numbers (from 8280 ± 530 to 6510 ± 500 per mm^3; $P = .001$) as a result of decreased numbers of neutrophils (from 4600 ± 490 to 3080 ± 300 per mm^3;

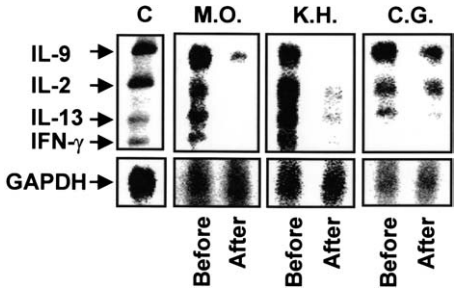

FIG 4. Expression of T_H1 and T_H2 cytokines in skin lesions of 3 patients with AD before and after tacrolimus treatment as assessed by RNase protection assay. *GAPDH,* Glyceraldehyde-3-phosphate dehydrogenase.

$P = .002$) and eosinophils (from 810 ± 330 to 620 ± 330 per mm^3; $P = .018$) after therapy. No difference between groups 1 and 2 was seen. No significant differences before and after tacrolimus treatment were observed regarding lymphocyte subpopulations ($CD4^+$ and $CD8^+$ T cells including activation markers, B cells, natural killer cells). However, when groups 1 and 2 were compared, patients in group 2 had higher levels of activated $CD4^+$ T cells, indicating that disease severity may correlate with peripheral blood T-cell activation. In particular, numbers of $CD4^+HLA\text{-}DR^+$ T cells were increased (group 1, 6.4 ± 1.1%; group 2, 18.6 ± 9.9%). Despite the obvious suitability of HLA-DR as indicator of T-cell activation, tacrolimus had no effect on the numbers of this T-cell subpopulation in blood (data not shown). Moreover, we noticed that only approximately 90% of the blood monocytes from group 2 expressed HLA-DR, perhaps because of IFN-γ deficiency.[10,11] In addition, the B cells of both AD patient groups expressed high levels of the low-affinity IgE receptor (CD23).[4] Tacrolimus corrected neither the decreased expression of HLA-DR on monocytes nor the increased expression of CD23 on B cells (data not shown).

We also analyzed the pattern of cytokine generation in PBMCs induced by PHA stimulation before and after tacrolimus treatment (data not shown). Group 2 patients appeared to have a more polarized T_H2 cytokine pattern compared with group 1 patients. Moreover, PBMCs had a slightly larger capacity in producing T_H2 cytokines and generated less IFN-γ *in vitro* after tacrolimus treatment, although these differences did not reach statistical significance. PHA-induced lymphocyte proliferation was not affected by tacrolimus treatment (data not shown).

DISCUSSION

Little is known about the therapeutic effect of tacrolimus on the inflammatory cells and cytokines in skin lesions of AD. Previous reports suggest that increased keratinocyte apoptosis is a key event in eczema formation[9] and that tacrolimus and other immunomodulators would stop the accelerated apoptosis in these cells.[23] Other investigators observed a decrease in the numbers of IDECs in skin lesions as well as a decrease in high-affinity IgE receptor expression on IDECs and Langerhans cells during treatment with tacrolimus ointment,[24] suggesting that dendritic cells represent 1 cellular target for the drug. We provide additional information demonstrating that tacrolimus ointment abolishes classical immunologic abnormalities associated with AD, such as the mixed cellular infiltration of the dermis and the high expression of proinflammatory cytokines by infiltrating cells.

In agreement with a previous study conducted in our laboratory,[4] we observed more infiltrating $CD4^+$ than $CD8^+$ cells in the skin of patients with AD. In addition, we detected high numbers of $CD21^+$ cells. Although plasma cells have not been described in hematoxylin and eosin–stained skin specimens from AD lesions,[25] the possibility exists that at least a subpopulation of these B cells represents a source of IgE production, which is increased in most patients with AD. Besides lymphocytes, we observed mast cells and eosinophils in the dermis of skin lesions.

By histology, no significant difference in the dermal infiltrate between group 1 and group 2 patients was detected. However, when we applied a highly sensitive and more specific immunofluorescence technique,[26] we found interesting differences. The numbers of all dermal inflammatory cells, in particular of $CD4^+$ cells, eosinophils, and mast cells, appeared to correlate with the EASI. Moreover, all of the various dermal infiltrating cell types decreased in the course of the treatment, except mast cell numbers, which did not change. This, however, does not exclude the possibility that tacrolimus inactivates skin mast cells.[27,28] Overall, the decrease in inflammatory cell infiltration during the tacrolimus treatment was more pronounced in group 2 compared with group 1, perhaps because of increased pretreatment numbers in group 2 and/or because of the longer treatment period. In spite of this decrease, the numbers of inflammatory cells present in tacrolimus-treated lesional skin were still higher compared with those determined in nonlesional AD skin.

We observed a high expression of the T_H2 cytokines IL-5 and IL-13 in acute lesions of AD. By using a double immunofluorescence technique, we identified $CD4^+$ and $CD8^+$ lymphocytes as important sources of these cytokines. In general, the frequency of T_H2 cytokine producing cells was higher among $CD4^+$ cells than $CD8^+$ lymphocytes. Besides T_H2 cytokines, smaller numbers of IFN-γ–producing $CD8^+$ lymphocytes were also observed after the tacrolimus treatment. Previously published work suggested that IFN-γ expression is induced in later phases of AD, in which a switch from T_H2 to T_H1 cytokine expression may occur.[8,29] Moreover, IFN-γ has been described as a mediator that sensitizes keratinocytes to undergo Fas receptor–mediated apoptosis.[9] Although the overall suppression of IFN-γ by topical tacrolimus therapy as assessed by immunofluorescence was not impressive, these findings were supported at the mRNA level with RNase protection assay. It is possible that the levels of IFN-γ fall below the threshold required for induction of keratinocyte apoptosis.

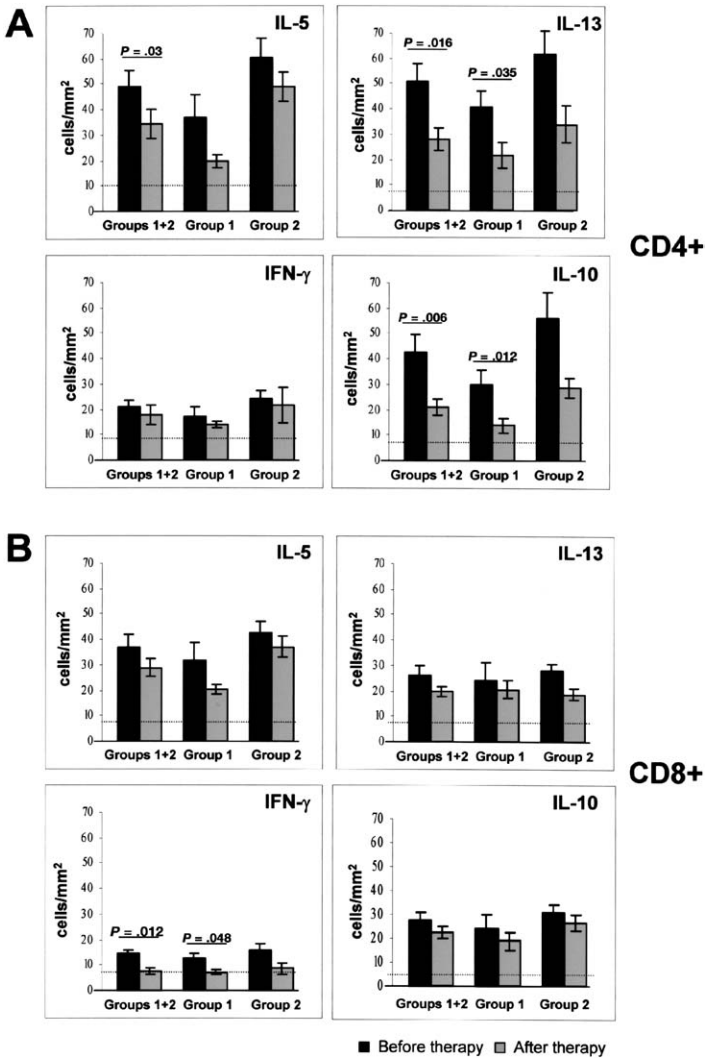

FIG 5. Expression of cytokines in skin lesions of patients with AD before and after tacrolimus treatment as assessed by double immunofluorescence analysis and confocal microscopy in $CD4^+$ **(A)** and $CD8^+$ **(B)** cells. The *dashed lines* represent the mean cell numbers in nonlesional AD skin (n = 3). Normal dermis did not contain cytokine-producing infiltrating cells (data not shown). Representative original results of these experiments are shown in Fig 6.

Several mechanisms, which all involve reduced cytokine gene expression, may be important for the dermal inflammatory cell decrease after tacrolimus treatment. One mechanism is suggested by the tacrolimus-associated reduction in granulocyte numbers in blood. It is possible that cytokines generated at the site of allergic inflammation reach via the circulation the bone marrow, where they stimulate the differentiation of granulocytes.[30] Thus, by decreasing cytokine levels in the skin, tacrolimus may influence cell generation in the bone marrow. But how can we explain reduced dermal lymphocyte numbers? Reduced TNF-α gene expression[31] likely results in reduced recruitment of inflammatory cells. Moreover, activated lymphocytes may undergo apoptosis in the absence of IL-2, IL-4, or IL-15, which represent T-cell survival cytokines.[32] Lymphocytes may also proliferate at the site of inflammation, and tacrolimus has been shown to inhibit both T-cell[12] and B-cell[33] proliferation under *in vitro* conditions.

Another possibility is that activated skin lymphocytes enter the blood. This idea is supported by 2 observations made in this study. First, we observed increased levels of $CD4^+HLA\text{-}DR^+$ cells in the blood of patients with severe AD. It is likely that these cells had been activated at the site of inflammation. Tacrolimus did not decrease the numbers of these cells. Second, freshly purified PBMCs from patients with AD have been shown to rapidly generate high levels of T_H2 cytokines.[10,11] This suggests that the promoter regions of these cytokines are easily assessable for transcription factors in many circulating T cells from patients with AD. Such cells have previously been suggested to be effector memory T cells.[34] Tacrolimus did not decrease the numbers of these cells in blood. In fact, a slight increase in the capacity of peripheral T cells in

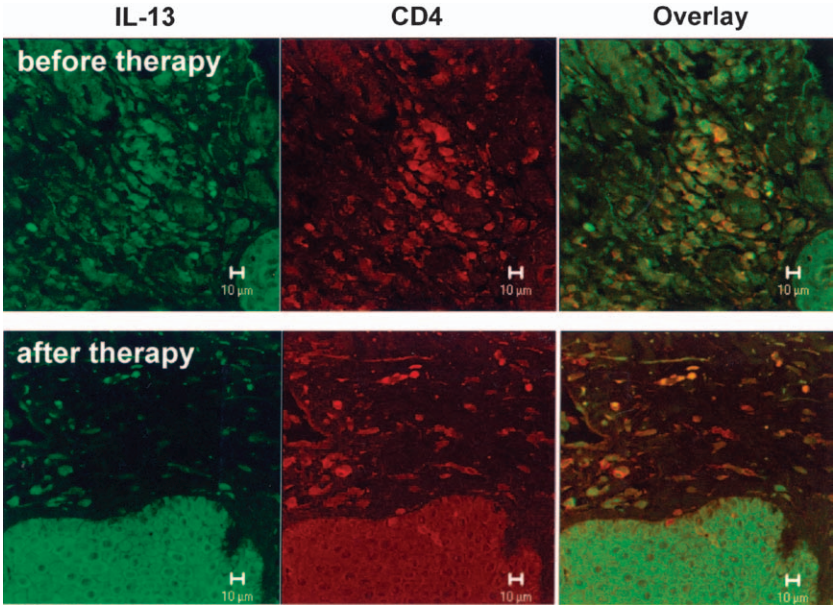

FIG 6. IL-13 production by $CD4^+$ cells that infiltrate the dermis of patients with AD before and after tacrolimus treatment as assessed by double immunofluorescence and confocal microscopy. The *scale bars* represent 10 μm.

generating T_H2 cytokines was observed after treatment, indicating that effector memory cells were generated in the skin and then released into the circulation, and that this process is not blocked by tacrolimus. Moreover, an additional conclusion may be drawn by these results. Because tacrolimus did not decrease cytokine generation by peripheral blood T cells, it is unlikely that significant amounts of the drug reach the circulation after topical treatment. Indeed, tacrolimus was almost undetectable in blood when applied topical.[35,36]

In conclusion, we provide evidence that many of the predicted mechanisms regarding the suppression of cytokine gene expression by tacrolimus indeed occur under *in vivo* conditions. Reduced cytokine expression was associated with reduced numbers of dermal infiltrating inflammatory cells in AD. These immunopharmacologic observations correlated with dramatic improvements of the clinical condition. Additional research is required to understand better the contribution of the various inflammatory cells and their products to the inflammation in AD.

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