

Evidence for a role of Langerhans cell-derived IL-16 in atopic dermatitis

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Background: The factors controlling infiltration of inflammatory cells into atopic dermatitis (AD) lesions remain to be fully explored. Recently, epidermal cells in lesional AD were reported to contain increased messenger (m)RNA levels of IL-16, a cytokine that induces chemotactic responses in CD4⁺ T cells, monocytes, and eosinophils.

Objectives: We sought to determine the expression of IL-16 in epidermal cells in normal skin and skin from AD lesions and to investigate whether Langerhans cell (LC)-derived IL-16 may contribute to the initiation of atopic eczema.

Methods: The cutaneous expression of IL-16 was investigated by in situ hybridization and immunohistochemistry. Expression of IL-16 was also investigated in freshly isolated LCs and in keratinocytes by intracellular cytokine staining, quantitative real-time RT-PCR, and ELISA.

Results: Low levels of IL-16 mRNA, but no stored IL-16 protein, were detected in keratinocytes and LCs isolated from normal skin. Synthesis, storage, and secretion of IL-16 could be induced in LCs, but not keratinocytes, by activation with phorbol ester and ionomycin. In normal skin (n = 10) neither keratinocytes nor LCs expressed IL-16. In contrast, IL-16 was contained in approximately 40% of CD1a⁺ LCs in patients with active AD (n = 16). IL-16 expression in LCs in patients with AD correlated with the number of infiltrating CD4⁺ cells ($r = .72$, $P = .0017$) and was completely downregulated parallel to the clinical response of AD lesions to topical treatment with FK506.

Conclusion: LC-derived IL-16 may participate in the recruitment and activation of inflammatory cells in AD. (*J Allergy Clin Immunol* 2002;109:681-7.)

Key words: Atopic dermatitis, Langerhans cells, keratinocytes, IL-16, chemotaxis, CD4⁺ cells, real-time RT-PCR, tacrolimus

Atopic dermatitis (AD) is characterized by eczematous skin lesions that harbor increased numbers of CD4⁺ T cells, monocytes, and eosinophils.¹ The mechanisms controlling

Abbreviations used

AD: Atopic dermatitis

LC: Langerhans cell

MoDC: Monocyte-derived dendritic cell

PMA: Phorbol 12-myristate 13-acetate

infiltration of inflammatory cells into AD lesions and the complex network of signals involved is an area of active investigation.² Much interest has focused on factors that are capable of inducing eosinophil migration. Of these, RANTES, monocyte chemotactic protein 4, and eotaxin have been reported to be increased in AD lesions.^{3,4}

IL-16 is a natural soluble ligand to the CD4 molecule that induces chemotactic responses in CD4⁺ cells, such as CD4⁺ T cells, monocytes, and eosinophils,⁵ and activates CD4⁺ T cells synergistically with IL-2 or IL-15.⁶ IL-16 is synthesized by CD4⁺ and CD8⁺ T cells and released in response to antigen, mitogen, histamine, and serotonin.⁵ It is most likely synthesized as a nonbioactive precursor protein, termed *pro-IL-16*, which is encoded from a 2.6-kb transcript.⁷ The released 20-kd IL-16 is cleaved from *pro-IL-16* and biologically active as a multimer.^{7,8}

Recent studies suggest a role for IL-16 in the pathogenesis of allergic asthma, including recruitment of inflammatory cells to the bronchial mucosa, production of allergen-specific IgE, and development of airway hyper-responsiveness.⁹⁻¹¹ In contrast, there is only limited information on the expression and function of IL-16 in atopic skin inflammation. Increased numbers of IL-16 messenger (m)RNA-containing cells have been detected in the epidermis of AD lesions and found to correlate with the density of the CD4⁺ infiltrate.¹² However, the exact origin of epidermal IL-16 expression and the cutaneous distribution of IL-16 protein have not yet been determined.

We investigated the expression of IL-16 mRNA and protein in AD lesions compared with that in normal skin and other inflammatory skin conditions and attempted to more precisely determine the cellular sources of epidermal IL-16. Because epidermal Langerhans cells (LCs) play a fundamental role in the initiation of atopic eczema¹³ and because dendritic cells have recently been identified as a source of bioactive IL-16,¹⁴ we were particularly interested in exploring whether LC-derived IL-16 may contribute to skin inflammation in AD.

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METHODS

Subjects and skin biopsy specimens

Four-millimeter skin biopsy specimens were obtained from representative lesions of 10 patients with confirmed diagnosis of AD and from 10 age- and sex-matched healthy donors. All patients had long-standing moderate-to-severe disease with typical eczematous skin lesions, elevated total serum IgE levels, and specific serum IgE antibodies to aeroallergens (CAP; Pharmacia, Freiburg, Germany). Six specimens were collected from acute papular lesions of less than 3 days' onset, and 10 specimens were obtained from chronic lichenified lesions of greater than 2 weeks' duration. In 7 patients with AD, biopsy specimens were taken from the same chronic lesions after topical therapy with 0.1% FK506 ointment.¹⁵ For comparison, specimens were also collected from the active border of chronic psoriatic plaques (n = 6 patients; age of the lesions, >4 weeks), plaque-type lesions of mycosis fungoides (n = 5), and typical papular eruptions of lichen ruber planus (n = 4). Specimens were fixed in 4% buffered formalin, routinely processed, and embedded in paraffin. None of the patients had received any topical treatment (except emollients) within 2 weeks or any systemic therapy, including UV light therapy, within at least 4 weeks before study entry. Written informed consent was obtained in all cases.

Isolation and culture of LCs and keratinocytes

LCs were isolated from the foreskin of individuals undergoing circumcision (n = 3) by high-gradient magnetic sorting, as previously described,¹⁶ by using directly labeled anti-CD1a-conjugated colloidal superparamagnetic MicroBeads (incubation for 1 hour on ice) in combination with Minimacs separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Keratinocytes were prepared from individuals undergoing plastic surgery, cultured, and phenotypically characterized, as previously described.¹⁷ LCs were cultured in RPMI-1640 medium supplemented with 10% FCS (lot No. 371S), 12 mmol/L HEPES, 2 mmol/L L-glutamine, 100 U/mL penicillin G, and 100 µg/mL streptomycin (all purchased from Biochrom, Berlin, Germany). Freshly isolated LCs (>95% CD1a⁺, as determined by flow cytometry; see below) and keratinocytes derived from the second passage of culture (>95% cytokeratin positive, <1% CD1a⁺) were incubated with phorbol 12-myristate 13-acetate (PMA; 25 ng/mL) and ionomycin (1 µmol/L; both from Sigma, Deisenhofen, Germany) for 16 hours. At the indicated time points, cells were harvested and subjected to RNA extraction for quantitative real-time RT-PCR or flow cytometry for analysis of intracellular IL-16, and cell-free supernatants were analyzed for IL-16 protein by ELISA.

Flow cytometry

For the dual analysis of CD1a surface expression and intracellular IL-16, approximately 1×10^5 LCs were washed in ice-cold PBS containing 20% human AB serum (Sigma) and incubated for 30 minutes at 4°C with 5 µg/mL FITC-conjugated mouse mAb to CD1a (Caltag, Burlingame, Calif). Cells were fixed with 4% paraformaldehyde (15 minutes at 4°C), permeabilized by incubation with PBS/20% human AB serum/0.1% saponin (Sigma; 1 hour at 4°C), and incubated with 10 µg/mL phycoerythrin-conjugated anti-IL-16 mAb 14.1 (Pharmingen, San Diego, Calif; 30 minutes at 4°C) diluted in PBS/0.1% saponin. Cells were analyzed on a PAS III flow cytometer (Partec, Münster, Germany) by using the Winlist software (version 3.0; Varsity Software House, Topsham, Me). For control, mAbs were replaced with isotype-matched mouse immunoglobulins. As indicated, the IL-16 mAb was preincubated with 10 µg/mL recombinant human (rh)IL-16 (Peprotech, London, United Kingdom) to confirm specificity.

Quantitative real-time RT-PCR for IL-16

Quantitation of IL-16 mRNA was performed on the Light Cycler (Roche Diagnostics, Mannheim, Germany), as previously described.¹⁸ Values obtained for IL-16 were standardized for β -actin values. Quantitation was performed in duplicate (variation typically below 10%), and negative control reactions without template were always included.

ELISA for IL-16

Levels of IL-16 protein were determined in supernatants of LC cultures by using a commercially available ELISA (Biosource, Solingen, Germany; minimum detectable concentration, <5 pg/mL).

Immunohistochemistry

Pairs of 2-µm paraffin-embedded mirror sections were prepared from each biopsy specimen, dewaxed in xylol (Merck, Darmstadt, Germany), rehydrated in serial dilutions of ethanol, and used to detect CD1a⁺ and IL-16⁺ cells, respectively. Staining for IL-16 was performed by using mouse IgG₁ anti-IL-16 mAb LCF-1¹⁹ (kindly provided by H. J. Schluesener, Eberhard-Karls-University, Tübingen, Germany) in combination with an indirect immunoperoxidase method, as previously described.²⁰ 3-Amino-9-ethylcarbazole (Dako) or 3,3'-diaminobenzidine tetrahydrochloride (Sigma) served as the chromogen. Mirror sections were stained with anti-CD1a mAb (Immunotech, Marseille, France) by using an alkaline phosphatase-antialkaline phosphatase technique. CD4⁺ cells (mAb from Novocastra, Newcastle, United Kingdom) were analyzed in adjacent sections. Control reactions included substitution of the primary antibody with isotype-matched mouse immunoglobulins (Dako) and preincubation of LCF-1 with rhIL-16 (10 µg/mL).

Cell-counting procedures

Total numbers of CD1a-reactive cells per square millimeter of epidermis were obtained in biopsy specimens from normal skin, active AD lesions, and AD lesions after treatment with FK506 by using the Quantimet automated image analysis system equipped with the QWin and QProdit software (Leica, Bensheim, Germany). An overlay was produced of the microscopic image of the section stained with anti-CD1a and the corresponding mirror section stained with LCF-1 mAb to colocalize CD1a⁺ cells and IL-16⁺ cells in the epidermis of AD lesions. Double-positive epidermal cells were only counted if their nucleus could be clearly identified in both sections (compare Fig 4, A and B). The number of CD4⁺ cells per square millimeter of upper corium was also determined in adjacent sections by image analysis. All cell numbers were obtained as averages from triplicate determinations.

In situ hybridization for IL-16

In situ hybridization for IL-16 was performed essentially as previously described.²¹ Sense probes were always included as a negative control.

Western analysis

PBMCs were isolated by Ficoll/Hypaque gradient centrifugation and cultured for 24 hours in RPMI-1640 medium supplemented with 10% FCS, 5 µg/mL PHA, and 100 U/mL IL-2 (Strathmann Biotech, Hamburg, Germany). Cell extracts of activated PBMCs were subjected to Western analysis for IL-16 by using LCF-1 mAb, as described previously,²⁰ except that a nitrocellulose membrane (0.45 µm; Schleicher und Schüll, Dassel, Germany) was used.

Statistical analysis

Data are given as means and SEM and were analyzed by using nonparametric analyses (Prism 2.01 software; GraphPad, San

Diego, Calif). Two-group comparisons of paired and unpaired data were performed by using the Wilcoxon signed-rank test and the Mann-Whitney *U* test, respectively. Kruskal-Wallis ANOVA was used for multiple comparisons. If the overall *P* value was less than .05 for multiple comparison, the Mann-Whitney *U* test was then used to compare individual groups. Correlations were performed by using Spearman rank correlation coefficients. Two-sided *P* values of less than .05 were regarded as significant.

RESULTS

Anti-IL-16 mAb LCF-1 recognizes pro-IL-16 and mature IL-16

Western analysis was performed to test the reactivity of the LCF-1 mAb with different forms of IL-16. Cellular extracts of PBMCs activated by incubation with PHA and IL-2 were separated with SDS-PAGE, blotted, and probed with LCF-1 (Fig 1). A prominent band was detected at around 80 kd, and several smaller bands were found between 40 and 80 kd (Fig 1, *left lane*), resembling pro-IL-16 and partially processed IL-16, as previously demonstrated in activated PBMCs.⁷ In addition, LCF-1 mAb detected rhIL-16 (Fig 1, *right lane*) that migrated at approximately 20 kd, which is similar to the migration point of mature IL-16.^{7,22} Therefore LCF-1 mAb recognizes pro-IL-16 and mature IL-16, similar to mAb 14.1,²² which was used for the detection of intracellular IL-16 by flow cytometry in this study.

Epidermal cells in normal human skin do not constitutively contain IL-16 protein, but IL-16 is inducible at the mRNA and protein level in isolated LCs

Immunohistochemistry of biopsy specimens from normal skin (*n* = 10) with LCF-1 mAb showed a cytoplasmic reactivity of some mononuclear cells located mainly around blood vessels in the upper corium, whereas epidermal cells were clearly negative (Fig 2, *A*). In situ hybridization revealed a similar pattern of IL-16 mRNA expression, with no signals detectable in the epidermis (Fig 2, *B*). The absence of IL-16 in normal epidermis was despite the presence of multiple CD1a⁺ LCs (Fig 2, *C*).

Highly purified LCs and keratinocytes were prepared from normal skin (*n* = 3 experiments each) to further elucidate *IL16* gene expression in epidermal cells, and IL-16 mRNA was investigated by quantitative real-time RT-PCR. With this more sensitive method compared with in situ hybridization, low levels of IL-16 mRNA were detected in freshly isolated LCs, and very low levels were also observed in keratinocytes (Fig 3, *A*). However, neither LCs (Fig 3, *B*) nor keratinocytes (data not shown) constitutively contained intracellular IL-16 protein, as determined with flow cytometry.

The effect of activation on the production of IL-16 in isolated LCs is shown in Fig 3. Incubation with PMA and ionomycin upregulated *IL16* gene expression starting at 2 hours after stimulation. The highest levels of IL-16 mRNA were observed at 8 hours (Fig 3, *A*). Subsequent to the induction of IL-16 mRNA, activated LCs accumu-

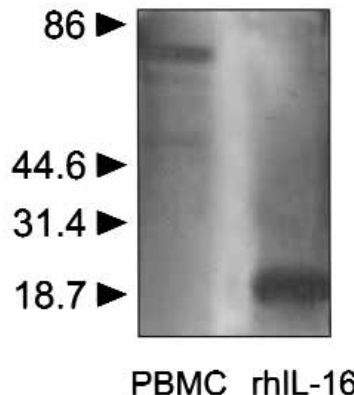


FIG 1. Reactivity of LCF-1 mAb determined by Western analysis. A total of 200 ng of rhIL-16 and 50 μ g of cellular extracts derived from activated PBMCs was separated with SDS-PAGE, blotted, and probed with LCF-1 mAb. Molecular mass standards (in kilodaltons) are indicated by arrows. Results are representative of 3 independent experiments.

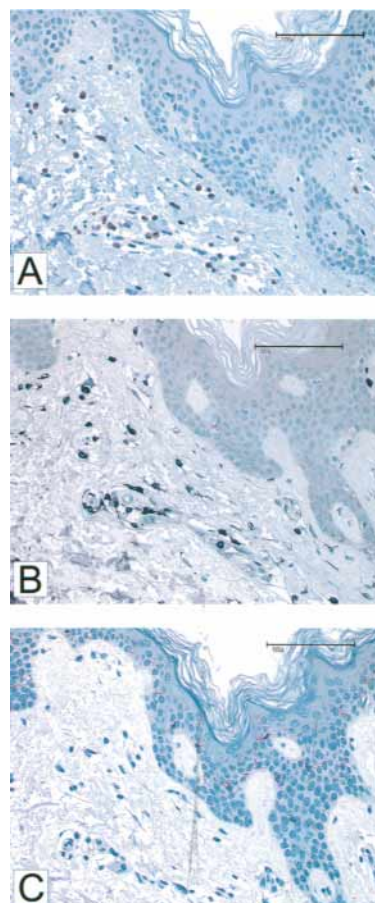


FIG 2. Expression of IL-16 in normal human skin. **A**, Immunohistochemistry. **B**, In situ hybridization. **C**, CD1a⁺ LCs within the epidermis. Scale bars represent 100 μ m.

lated IL-16 protein. Approximately 20% at 8 hours and approximately 70% at 16 hours of cultured LCs contained IL-16 protein (Fig 3, *B*). Moreover, IL-16 protein became detectable in the supernatant by ELISA (Fig 3,

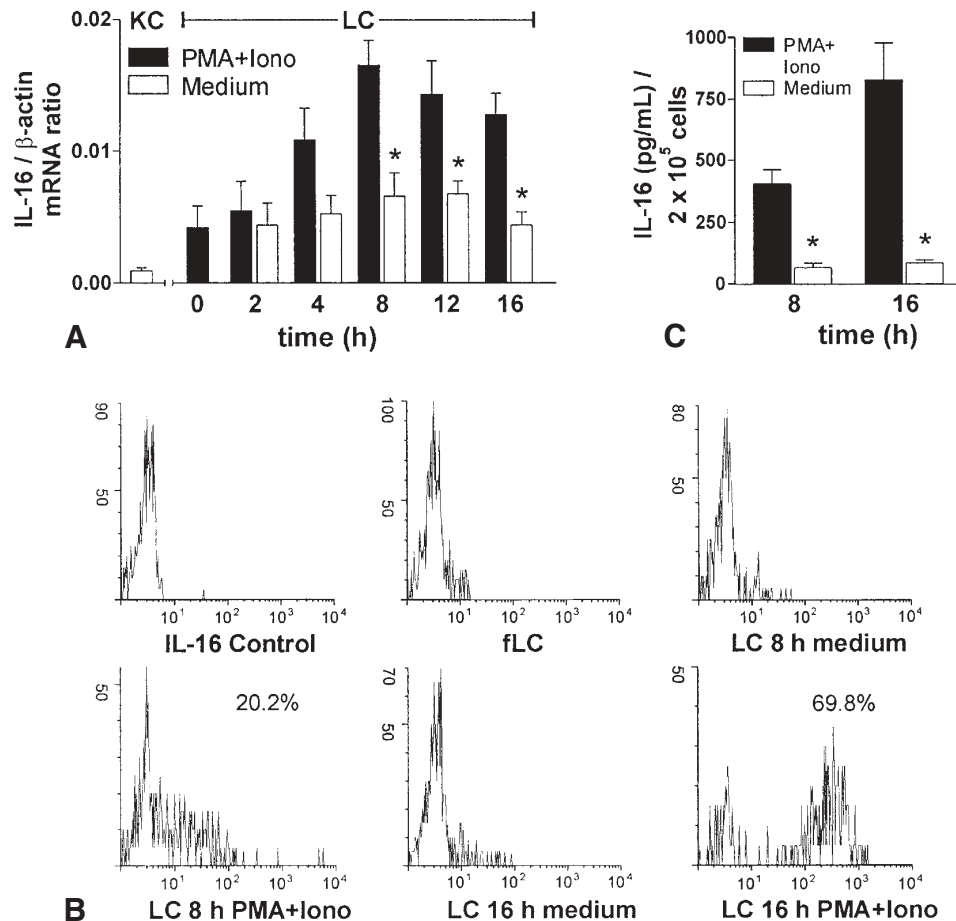


FIG 3. A, IL16 gene expression in keratinocytes (KC) and LCs isolated from normal skin. Freshly isolated LCs (time point 0) were incubated with PMA and ionomycin (filled bars) or cultured in medium alone (open bars). * $P < .05$ versus activated LCs (Mann-Whitney U test after Kruskal-Wallis ANOVA, $P < .0001$). **B, Expression of intracellular IL-16 protein in CD1a-gated freshly isolated (fLC) and cultured LCs.** For control, anti-IL-16 mAb was blocked with rhIL-16. **C, ELISA of supernatants from activated LCs (filled bars) and control cultures (open bars).** * $P < .05$ versus activated LCs. In **A** and **C**, bars and error bars represent mean $\pm$ SEM of 3 experiments.

C), which is consistent with a release of IL-16 from activated LCs. Therefore activation induced synthesis, storage, and secretion of IL-16 in isolated LCs. In contrast, incubation of cultured keratinocytes with PMA and ionomycin did not induce IL-16 mRNA or protein (not shown).

Expression of IL-16 in LCs in active AD

The expression of IL-16 was investigated in biopsy specimens of patients with active AD ($n = 16$) by immunohistochemistry and in situ hybridization. Findings were compared with those in normal skin ($n = 10$), psoriasis vulgaris ($n = 6$), lichen ruber planus ($n = 4$), and mycosis fungoides ($n = 5$). A high percentage of skin-infiltrating mononuclear cells in all inflammatory dermatoses contained IL-16. The majority of positive cells were morphologically lymphocytes located in the upper corium. Keratinocytes were negative in all cases. Examples for the expression of IL-16 in AD and psoriasis are

shown in Fig 4. The expression pattern of IL-16 in mycosis fungoides has been published earlier.²¹

IL-16⁺ epidermal cells with dendritic morphology were detected in all biopsy specimens from acute and chronic AD lesions but not in any of the other inflammatory dermatoses tested. Pairs of 2- μ m mirror sections were prepared from paraffin-embedded tissue and stained with anti-CD1a and LCF-1 mAbs, respectively (Fig 4, A and B), to confirm that these cells were LCs. Numbers of total CD1a⁺ cells and of IL-16-expressing CD1a⁺ cells in these sections were obtained per square millimeter of epidermis by image analysis (for details, see "Methods" section). The total number of CD1a⁺ LCs in acute lesions ($101 \pm 13/\text{mm}^2$, $n = 6$) was not significantly different from that found in chronic AD lesions ($95 \pm 5/\text{mm}^2$, $n = 10$) but was higher than that found in normal skin ($65 \pm 6/\text{mm}^2$; overall $P = .001$). In acute lesions the number of IL-16⁺ LCs was $42 \pm 5/\text{mm}^2$ (43% $\pm$ 4% of LCs), which is similar to that obtained in chron-

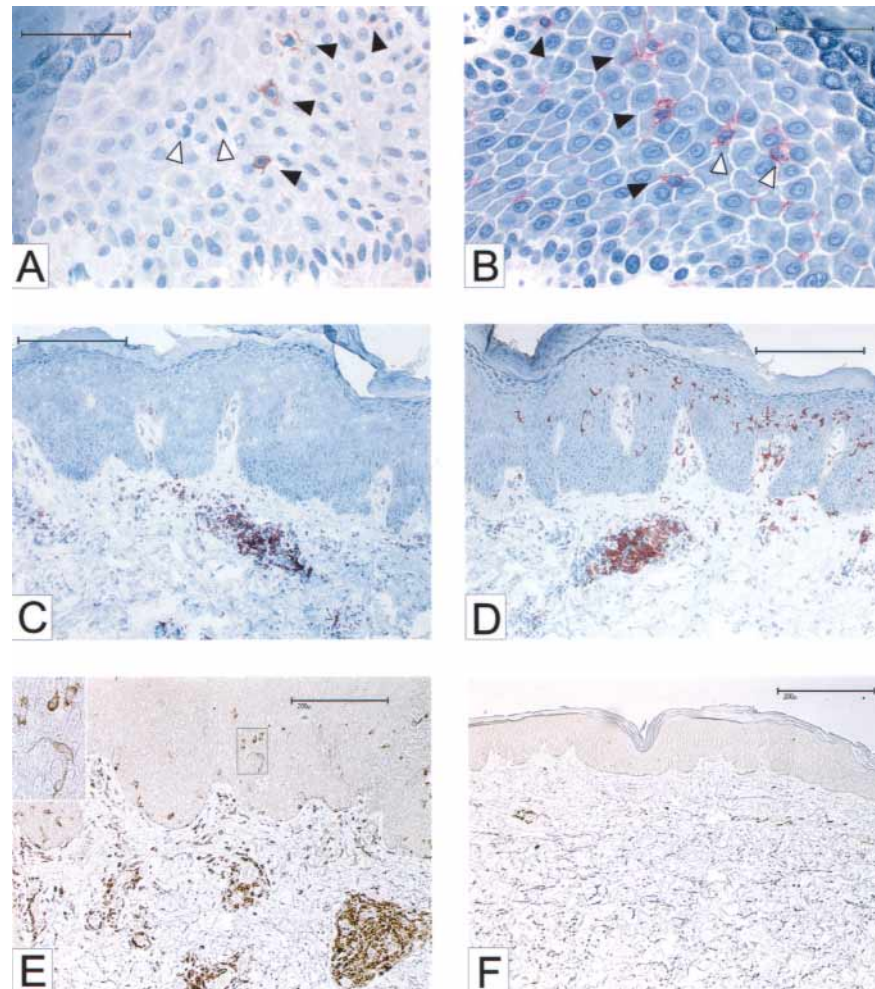


FIG 4. Mirror sections of active AD (**A** and **B**) and psoriasis (**C** and **D**) lesions were stained for IL-16 (**A** and **C**) and CD1a (**B** and **D**): filled arrowheads, LCs containing IL-16; open arrowheads, LCs lacking IL-16. Expression of IL-16 protein in active AD before (**E**) and after (**F**) treatment with 0.1% FK506 ointment is shown. Scale bars represent 50 μ m in **A** and **B** and 200 μ m in all other panels.

ic lesions ($35 \pm 5/\text{mm}^2$, $37\% \pm 5\%$). In contrast, LCs in normal skin did not contain IL-16.

Numbers of CD4⁺ cells infiltrating the upper corium were determined in sections adjacent to those used for quantification of IL-16-expressing LCs to compare the expression of IL-16 in epidermal LCs with the density of the CD4⁺ infiltrate in AD lesions. There was an excellent correlation between the number of IL-16⁺ LCs and the density of the CD4⁺ infiltrate ($r = 0.72$, $P = .0017$). Because this finding suggested that the production of IL-16 in LCs may contribute to the severity of AD lesions, we also investigated whether treatment with FK506 would affect the expression of IL-16 in these cells. Seven chronic lesions of similar clinical appearance were selected for treatment with FK506 ointment twice daily over 3 weeks, and posttreatment biopsy specimens were analyzed for the expression of CD1a and IL-16, as described. Clinical improvement induced by FK506 was associated with a marked downregulation of IL-16 in the epidermal

and dermal compartment at the protein (Fig 4, **E** and **F**) and mRNA level (data not shown). In 5 of 7 patients treated, application of FK506 ointment induced a complete clearing of marker lesions, and in these lesions the expression of IL-16 in epidermal dendritic cells became undetectable at the end of treatment (Fig 4, **F**).

DISCUSSION

An increased epidermal expression of IL-16 mRNA has been described in active AD lesions compared with that seen in normal skin.¹² In this previous study it appeared that the epidermal expression of IL-16 mRNA in AD occurs mainly in keratinocytes, but the exact cellular sources were not determined. Moreover, because posttranscriptional regulation is important in the control of IL-16 production,⁸ analysis of IL-16 mRNA expression alone may not be sufficient to assess the biologic relevance of IL-16 in AD.

By using *in situ* hybridization and immunohistochemistry, we failed to detect IL-16 mRNA or protein in the epidermis in normal skin, although IL-16 was present in some mononuclear cells in the upper corium. Absence of IL-16 protein in epidermal cells was confirmed by intracellular cytokine staining of keratinocytes and LCs isolated from normal skin, and only low levels of IL-16 mRNA were detected in these cells by using a highly sensitive real-time RT-PCR method. However, synthesis, storage, and release of IL-16 could be induced in isolated LCs by activation with PMA and calcium ionophore, suggesting that human epidermal LCs are a source of IL-16. Constitutive production of IL-16 has been described in dendritic cells generated by 6-day culture of monocytes in GM-CSF and IL-4 (monocyte-derived dendritic cells [MoDCs]).¹⁴ These cells lack CD83 and are usually regarded as immature dendritic cells. Because LCs isolated from normal skin required activation to upregulate IL-16 production, it may be speculated that immature MoDCs are in a different state of activation compared with immature (resting) LCs within normal epidermis. This is supported by earlier observations that dendritic cells generated from peripheral blood cells in the presence of cytokines constitutively synthesize a variety of mediators that are not detectable in normal epidermis.²³

In contrast to normal skin, a substantial fraction of epidermal LCs in active AD contained IL-16. Immunohistochemically, IL-16 was present in approximately 40% of CD1a⁺ epidermal LCs, with no significant difference detectable between acute and chronic lesions. Because the counting procedure of CD1a/IL-16 double-positive cells in pairs of mirror sections included only cells with a detectable nucleus in both sections, the given absolute number of double-positive cells (approximately 40/mm² of epidermis) may underestimate the actual number present in AD lesions.

The expression of IL-16 in LCs underlines their importance in atopic skin inflammation. According to current pathogenetic concepts, LCs carry the high-affinity IgE receptor FcεRI and may use this structure for the selective uptake of allergens and the subsequent induction of specific T-cell responses.¹³ We have recently shown that engagement of FcεRI upregulates IL-16 production in LCs derived from atopic donors.²⁰ Therefore the presence of IL-16 in a substantial fraction of LCs in lesional AD may indicate activation of these cells by allergen contact. Of 2 different subpopulations of epidermal dendritic cells recently identified in AD lesions, classical LCs and so-called inflammatory dendritic epidermal cells, the latter subset shows the highest expression of FcεRI and may preferentially respond to IgE-dependant activation.²⁴ Whether expression of IL-16 in active AD is restricted to inflammatory dendritic epidermal cells remains to be investigated in future studies.

Because IL-16 is a strong inducer of migratory responses in CD4⁺ T cells,^{5,14,25} LC-derived IL-16 may contribute to the recruitment of CD4⁺ T cells into AD lesions. In support of this concept, we found a good correlation between the number of IL-16-containing LCs and the number of CD4⁺ cells in active AD lesions. Inter-

pretation has to be done with caution because the correlation between cell numbers and the expression of chemotactic factors does not necessarily reflect causation. For example, it can not be excluded that another factor that recruits CD4⁺ cells is also inducing IL-16 expression in epidermal LCs. Furthermore, our study does not contain information about the relative contribution of IL-16 to the influx of CD4⁺ T cells into AD lesions compared with other chemoattractant factors. The migratory response induced in CD4⁺ T cells by supernatants of MoDCs *in vitro* has been shown to almost completely depend on the presence of IL-16,¹⁴ suggesting that IL-16 is a major chemotactic signal from dendritic cells toward CD4⁺ T cells.

Topical application of FK506 (tacrolimus) inhibits LC-dependant cutaneous T-cell responses²⁶ and is highly effective in AD. Therefore we were interested to determine whether FK506 would affect the expression of IL-16 in AD. We found that the clinical improvement of AD lesions induced by FK506 ointment was associated with a complete downregulation of IL-16 in the epidermal compartment. This is despite the fact that epidermal LCs, although significantly reduced in number (from $95 \pm 7/\text{mm}^2$ before to $59 \pm 7/\text{mm}^2$ after therapy, $P = .02$), were still present after treatment. These preliminary findings suggest that suppression of IL-16 in LCs is part of the anti-inflammatory effects of FK506.

Our data indicate that IL-16, in addition to its proposed role in allergic asthma, may also contribute to the influx of inflammatory cells in AD, supporting the concept of common inflammatory pathways in atopic diseases. Whereas respiratory tract epithelial cells represent a major source of IL-16 in allergic asthma,⁵ we obtained evidence that epidermal LCs are important producers of IL-16 in AD. In view of the cell types identified as targets of IL-16 activity, release of this cytokine from epidermal LCs may specifically control recruitment and activation of CD4⁺ T cells, monocytes, and eosinophils, which are all important components of the cellular infiltrate in AD.

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