

Pimecrolimus leads to an apoptosis-induced depletion of T cells but not Langerhans cells in patients with atopic dermatitis

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Background: Apart from the long-used corticosteroids, topical calcineurin inhibitors (tacrolimus, pimecrolimus) represent novel therapeutic options for the treatment of atopic dermatitis.

Objective: This study was designed to investigate the pathophysiological target cells and mode of action of pimecrolimus in atopic skin.

Methods: Twenty-two patients were randomly assigned to treatment with pimecrolimus cream 1%, matching vehicle cream, or β -methasone-17-valerate (BMV) cream 0.1% in a randomized, double-blind, vehicle-controlled, parallel group clinical trial. Treatment was given twice daily for 3 weeks. Cryostat sections of skin biopsies were taken before as well as at selected time points after initiation of therapy. For certain experiments, healthy volunteers were topically treated with the creams described twice a day on 5 consecutive days. Epidermal sheets were prepared from suction blisters. For *in vitro* experiments, untreated, healthy human skin was obtained from patients undergoing plastic surgery. The effect of pimecrolimus and BMV on Langerhans cells (LCs), inflammatory dendritic epidermal cells, and T cells was investigated by using immunofluorescence and flow-cytometry techniques.

Results: While topical BMV treatment depleted LCs in healthy and atopic skin, pimecrolimus did not affect their number. Correlating with the disappearance of inflammatory cells, we observed a depletion of inflammatory dendritic epidermal cells and T cells on pimecrolimus and BMV treatment.

Furthermore, we show that pimecrolimus depletes T cells by the induction of apoptosis.

Conclusion: In summary, our data show that pimecrolimus reduces pathological T cells in atopic dermatitis skin via apoptosis, whereas LCs remain unaffected. (J Allergy Clin Immunol 2005;115:1276-83.)

Key words: Atopic dermatitis, Langerhans cells, T cells, apoptosis, corticosteroids, pimecrolimus

Abbreviations used

AD:	Atopic dermatitis
APC:	Allophycocyanin
BMV:	β -Methasone-17-valerate
CLSM:	Confocal laser scanning microscope
DC:	Dendritic cell
EASI:	Eczema area and severity index
EC:	Epidermal cell
EMP:	Extracellular matrix protein
FITC:	Fluorescein isothiocyanate
IDEC:	Inflammatory dendritic epidermal cell
LC:	Langerhans cell
PE:	Phycoerythrin
TUNEL:	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

Atopic dermatitis (AD) is one of the most common inflammatory skin disorders, characterized by pruritus, a chronic relapsing course, genetic background, and typically distributed lesions with dry skin, excoriations, and lichenification.^{1,2} Successful management of AD requires a multipronged approach involving skin care, identification as well as elimination of trigger factors, and anti-inflammatory treatment.³ Topically applied corticosteroids have been used for approximately 50 years and are considered a mainstay in therapy. For short treatment periods, such formulations are usually well tolerated, whereas long-term application is restricted.⁴ Recently, the topical calcineurin inhibitors tacrolimus (FK 506; Protopic) and pimecrolimus (SDZ ASM 981; Elidel) have been identified, and clinical trials have demonstrated their efficacy and safety in short-term as well as long-term treatment of AD.⁵⁻⁷

As part of the skin's indigenous immune system, Langerhans cells (LCs) play an important role in cutaneous immune surveillance. We and others have previously reported that topical application of pimecrolimus onto healthy murine skin does not grossly interfere with the integrity, survival, and immune function of resident LCs, in contrast to corticosteroids.^{8,9} To determine whether topical pimecrolimus interferes with the constituents of the human skin immune system, we first explored the effects of pimecrolimus on LCs in healthy and AD skin and compared these effects with those of the corticosteroid β -methasone-17-valerate (BMV).

Atopic dermatitis skin lesions harbor a variety of inflammatory cells, like mast cells, eosinophils, inflammatory

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Supported by a research grant from Novartis Pharma AG, Basel.

Disclosure of potential conflict of interest: Anton Stuetz is an employee of Novartis; all other authors state none to disclose.

Received for publication December 10, 2004; revised January 29, 2005; accepted for publication February 2, 2005.

Available online April 5, 2005.

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doi:10.1016/j.jaci.2005.02.011

dendritic epidermal cells (IDECs), plasmacytoid dendritic cells, and T cells.¹⁰⁻¹³ On topical treatment with calcineurin inhibitors, this inflammatory cell infiltrate is markedly decreased, as evidenced by a tacrolimus-induced reduction of IDECs, eosinophils, and T cells.¹⁴⁻¹⁶ However, the mechanisms leading to the disappearance of inflammatory cells have remained unclear so far. In this study, we show for the first time that the reduction of skin-infiltrating T cells on pimecrolimus treatment occurs through apoptotic cell death, as demonstrated by nuclear DNA breaks.

METHODS

Reagents

For flow cytometry and immunofluorescence stainings of cryostat sections, the mAb T6 (phycoerythrin [PE]–anti-CD1a; Coulter Clone, Miami, Fla), HI149 (fluorescein isothiocyanate [FITC]–anti-CD1a), SK7 (allophycocyanin [APC]–anti-CD3), ICRF44 (APC–anti-CD11b), M-A251 (FITC–anti-CD25), 5C3 (FITC–anti-CD40), L307.4 (FITC–anti-CD80), HB15e (PE–anti-CD83), 2331 (FITC–anti-CD86), G46-2.6 (FITC–anti-HLA-A,B,C), TÛ36 (FITC–anti-HLA-DR; all from BD PharMingen, San Jose, Calif), DCGM4 (anti-CD207/Langerin; Immunotech, Marseille, France), and M2120 (anti-CD1a; PeliCluster, Amersham, The Netherlands) were used. Irrelevant, isotype-matched antibodies were used as negative controls in each experiment. Second step reagents were FITC/PE–F(ab')₂ goat antimouse IgG (Caltag Laboratories, Burlingame, Calif). Pimecrolimus (SDZ ASM 981; Novartis Pharma AG, Basel, Switzerland) and BMV (Sigma-Aldrich, St Louis, Mo) were dissolved in dimethyl sulfoxide and added to cultures at final concentrations ranging from 10⁻¹⁰ mol/L to 10⁻⁶ mol/L.

Healthy volunteers

Pimecrolimus cream, the corresponding vehicle cream, and BMV cream were applied at their clinically used concentrations (pimecrolimus, 1%; BMV, 0.1%) to forearm areas (4 cm × 5 cm) of healthy volunteers (n = 4) twice a day on 5 consecutive days. Subjects were between the ages of 23 years and 56 years, of both sexes, not pregnant, and not using any medication. Twenty-four hours after the last application, epidermis was obtained by using the suction blister method¹⁷ and immediately fixed in acetone (10 minutes; room temperature). Sheets were washed 3 times in PBS and then subjected to immunofluorescence staining (PE-conjugated anti-CD1a mAb diluted in PBS containing 2% BSA; 2 hours; 37°C). Staining patterns observed were documented with a confocal laser scanning microscope (CLSM 510; Carl Zeiss, Jena, Germany). For quantification of LC numbers, sheets were read in a blinded fashion at 400× magnification by 2 independent investigators using a fluorescence microscope (Leitz/Diaphan, Wetzlar, Germany), and 20 to 40 fields per sheet (2 to 3 sheets could be obtained from each treated skin area) were counted.

Patients, therapeutic protocol, and tissue material

Twenty-two patients (17 female subjects, 5 male subjects; age, 18–60 years; average, 27 years) with mild to moderate AD (investigator's global assessment, 1–3) fulfilling the diagnostic criteria of Hanifin and Rajka^{18,19} participated in an explorative, double-blind, vehicle-controlled, observer-blind, monocenter clinical trial. Patients were randomly assigned to treatment with pimecrolimus cream (1%; 8 patients), the corresponding vehicle cream (6 patients), or BMV

cream (0.1%; 8 patients). Creams were applied twice daily to all affected areas of the skin, excluding the face, for 3 weeks. The patients' dermatitis was assessed at each study visit according to an adapted eczema area and severity index (EASI).^{7,20} Punch biopsies (4 mm) were taken from acute lesions before treatment (day 0; begin of study), and on days 7 or 14 and 21 (end of study) after initiation of therapy. With this protocol, we tried to cover 4 time points without the need to take 4 biopsies per patient. Half of the creams of each group were labeled with the numbers 7 or 14 before randomization. Before the beginning of the study, patients had to stop oral treatment with systemic corticosteroids or other immunosuppressive drugs for 2 weeks, treatment with UV radiation for 4 weeks, and topical application of corticosteroids or other immunosuppressive drugs for 2 weeks, and none of these treatments were allowed during the study. This study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the local ethics committee of Vienna University's Medical School (code number, EK303/2003). A written informed consent was obtained from all patients.

Preparation of epidermal cell suspensions and culture conditions

Normal human split skin was obtained from patients undergoing plastic surgery, and epidermal cell (EC) suspensions were prepared by means of trypsinization. Briefly, split skin was placed dermal side down on a 0.8% trypsin-PBS (pH 7.2) solution and incubated for 1.5 hours at 37°C. Epidermal sheets were peeled from the underlying dermis and incubated for 20 minutes at 37°C in RPMI 1640 containing deoxyribonuclease (200 µg/mL; Sigma-Aldrich). Cell aggregates were disrupted by repeated aspiration using a pipette, and the resulting cell suspension was filtered through a cell strainer. EC suspensions were washed twice and resuspended in culture medium (RPMI 1640 medium supplemented with 10% heat-inactivated FCS, PAA Laboratories GmbH, Linz, Austria; 25 mmol/L HEPES, 10 µg/mL gentamycin, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 50 µmol/L 2-mercaptoethanol, and 1× antibiotic-antimycotic solution, all from Gibco Life Technologies, Inc, Grand Island, NY). Viability was always ≥90% as determined by trypan blue exclusion. EC suspensions (1 × 10⁶ cells/mL) were cultured for 48 hours in 24-well plates with 1 mL culture medium in the presence of increasing concentrations (10⁻¹⁰ mol/L to 10⁻⁶ mol/L) of pimecrolimus, BMV, or the solvent alone.

Flow cytometry

For 2-color analysis, cells (5 × 10⁵/sample) were resuspended in cold PBS/1% FCS/0.1% NaN₃, and Fc-receptors were blocked by adding human IgG (30 minutes, 4°C; Beriglobulin P; Aventis Behring, King of Prussia, Pa). After 3 washing steps, cells were serially incubated with FITC-conjugated mAb directed against selected antigens and PE-conjugated anti-CD1a mAb (30 minutes, 4°C). Specificity of staining was confirmed by using isotype-matched control mAb. Fluorescence was measured by using a FACScan flow cytometer, and data were analyzed with Cell Quest software (both from Becton Dickinson, San Jose, Calif). Dead cells were excluded by 7-amino-actinomycin D (Sigma-Aldrich) uptake.

Immunofluorescence staining and evaluation of immunofluorescence results

Skin biopsies were embedded in OCT Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands), snap-frozen in liquid nitrogen, and stored at –80°C until further processing. Frozen tissue was cut into 5-µm-thick sections and mounted on capillary gap microscope slides (Dako, Glostrup, Denmark). Sections were air-dried for 20 minutes, fixed in ice-cold acetone for 10 minutes, and

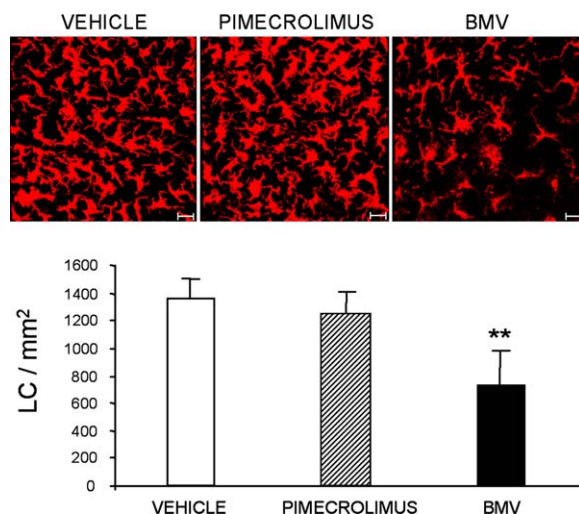


FIG 1. Epicutaneous treatment with BMV depletes LCs from healthy human skin, in contrast to pimecrolimus. Pimecrolimus (1%), BMV (0.1%), and vehicle cream were applied twice daily for 5 consecutive days onto the forearm of 4 healthy volunteers. Twenty hours after the last treatment, suction blisters of treated areas were prepared, and LCs were visualized with a PE-conjugated anti-CD1a mAb. Twenty to 40 randomly selected fields per sheet were counted, and results are expressed as means $\pm$ SEMs. ** $P < .01$ vs control. Scale bars, 20 μ m.

washed 3 times in PBS. To identify LCs, sections were incubated with a purified anti-CD207 mAb (12 hours, 4°C) and then reacted with a PE-conjugated goat antimouse IgG antibody (2 hours, room temperature). After several washes in PBS, the sections were incubated with 5% mouse serum and 2% BSA (30 minutes, room temperature) and then counterstained with a FITC-conjugated anti-CD1a mAb (2 hours, room temperature). To identify IDECs, sections were stained with a purified anti-CD1a mAb (12 hours, 4°C), followed by a FITC-conjugated goat antimouse IgG antibody (2 hours, room temperature). After blocking as described, slides were incubated with an APC-labeled anti-CD11b mAb (2 hours, room temperature). Immunostained sections were analyzed by using a CLSM. For quantification of cell populations, 5 randomly selected fields of the epidermis per section were scanned, and cells were counted in a blinded fashion by 2 independent investigators.

Identification of apoptotic dendritic cells and T cells

To detect DNA breaks *in situ*, cryostat sections were fixed with 4% paraformaldehyde (30 minutes, room temperature) and then washed 3 times in PBS. Permeabilization was performed by incubating with 0.1% Triton X-100 (Sigma) in 0.1% sodium citrate for 2 minutes on ice. After rinsing in PBS, sections were subjected to the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). After blocking (5% mouse serum in 2% BSA; 30 minutes, room temperature), sections were counterstained with an APC-labeled anti-CD3 mAb (2 hours, room temperature) and the nuclear dye Sytox Green (Molecular Probes, Eugene, Ore; 30 minutes, room temperature) or a PE-labeled anti-CD1a mAb (2 hours, room temperature). As a positive control, sections were first incubated with 1500 U/mL DNase I (10 minutes, room temperature) to induce DNA breaks in the nucleus and were then subjected to TUNEL/CD3/DNA or TUNEL/CD1a stainings. Data acquisition and image cytometry

were performed by using an automated fluorescence CLSM and the analysis software TissueQuest (TissueGnostics, Vienna, Austria), which permitted recognition of individual cells by a specific identification strategy.²¹ In brief, the whole dermis (25–75 fields per section; 1 field, 0.2375 mm²) under 40 $\times$ object lenses were scanned. Specificity of the staining procedure and accuracy of instrument settings were crosschecked by isotype-matched negative controls. Cellular subpopulations were defined, and the thresholds for statistical evaluation were set according to the negative controls.

Statistical analysis

One-way ANOVA was used to evaluate statistically significant differences ($P < .05$) between test and control groups.

RESULTS

Topical BMV but not pimecrolimus depletes human LCs in healthy skin

To determine the effects of topical pimecrolimus and BMV on the fate of human LC, creams were applied twice daily on 5 consecutive days onto the forearm skin of healthy volunteers. Quantification of LCs assessed 1 day after cessation of treatment revealed comparable numbers in pimecrolimus-treated and vehicle-treated skin (Fig 1). In contrast, a significant reduction (by 50%) of CD1a⁺ LCs was observed on BMV treatment.

LCs undergo similar phenotypic changes in pimecrolimus-containing and vehicle-containing EC cultures, in contrast to BMV

To investigate whether pimecrolimus and BMV influence the maturation capacity of human LCs *in vitro*, EC suspensions of healthy human skin were prepared and cultured with the compounds. The presence of BMV but not pimecrolimus resulted in a dose-dependent inhibition of the upregulation of MHC class I and II, CD40, CD86, and CD83 molecules on CD1a⁺ cells when assessed at and compared with controls after a culture period of 48 hours (Fig 2, A, left panel, and B, and data not shown). Furthermore, we observed reduced numbers of CD40⁺ LCs on BMV culture (Fig 2, A, right panel). Pimecrolimus marginally reduced the upregulation of the mean fluorescence intensity of anti-CD80 and anti-CD25 stainings on LCs and caused a dose-dependent inhibition in the increase of LCs positive for CD80 and CD25, although these differences did not reach statistical significance (Fig 2, A). Taken together, the phenotypic analysis revealed that, although both compounds affect LC maturation, BMV displays a much more pronounced and broader spectrum of effects than pimecrolimus.

Pimecrolimus has no effect on LC numbers but depletes IDECs in atopic skin

To study the effect of pimecrolimus on immunocompetent cells in atopic skin and to compare the effects with a corticosteroid, patients with AD were treated with pimecrolimus, the vehicle cream, and BMV for 3 weeks. The clinical efficacy of the treatment was determined by

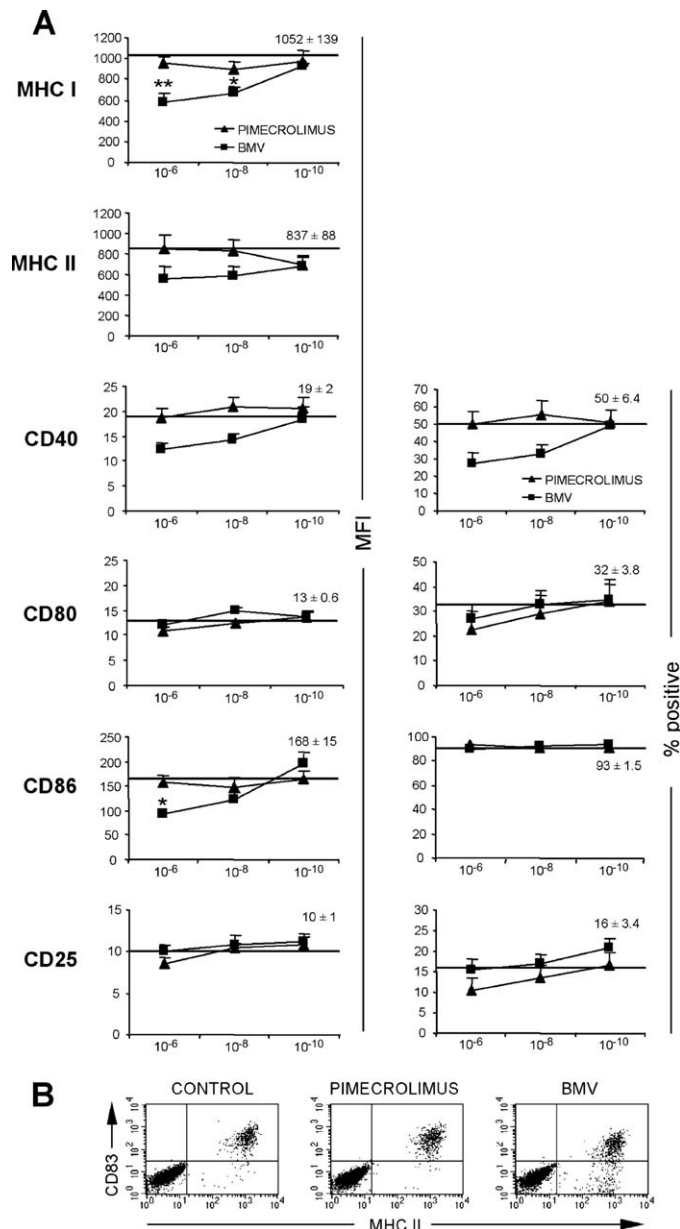


FIG 2. Pimecrolimus and BMV differentially affect the maturation process of human LCs *in vitro*. EC suspensions were cultured, stained, and analyzed as described in Methods. **A**, The *left and right panels* display the mean fluorescence intensity (MFI) of selected markers within CD1a⁺ cells and the percentage of LCs positive for these markers, respectively. *Horizontal lines and numbers* in the upper right corner of each graph represent the means $\pm$ SEMs of vehicle controls. Data shown are the means $\pm$ SEMs of 5 independent experiments using skin from 5 different donors. **B**, Expression of CD83 on MHC class II⁺ cells was analyzed by flow cytometry, and the *dot plot* of 1 representative experiment out of 3 is shown (molarity of compounds: 10^{-6}). ** $P < .01$ vs control; * $P < .05$ vs control.

EASI scoring. The median EASI decreased over the course of treatment in the pimecrolimus-treated and BMV-treated groups (Fig 3, A). The greatest decrease was seen in BMV-treated patients (77%), followed by the pimecrolimus group (59%). In contrast, patients receiving the vehicle cream did not benefit from treatment, as indicated by an increased EASI. To determine the effects of pimecrolimus and BMV treatment on the fate of

epidermal dendritic cell (DC) populations in atopic skin, sections from skin biopsies taken before and at selected time points during the treatment were stained for CD1a versus CD207 and CD1a versus CD11b to identify LCs and IDECs, respectively. LC numbers in atopic skin remained essentially unaltered during the whole treatment period with pimecrolimus compared with vehicle control, whereas a significant 73% reduction of LCs occurred after

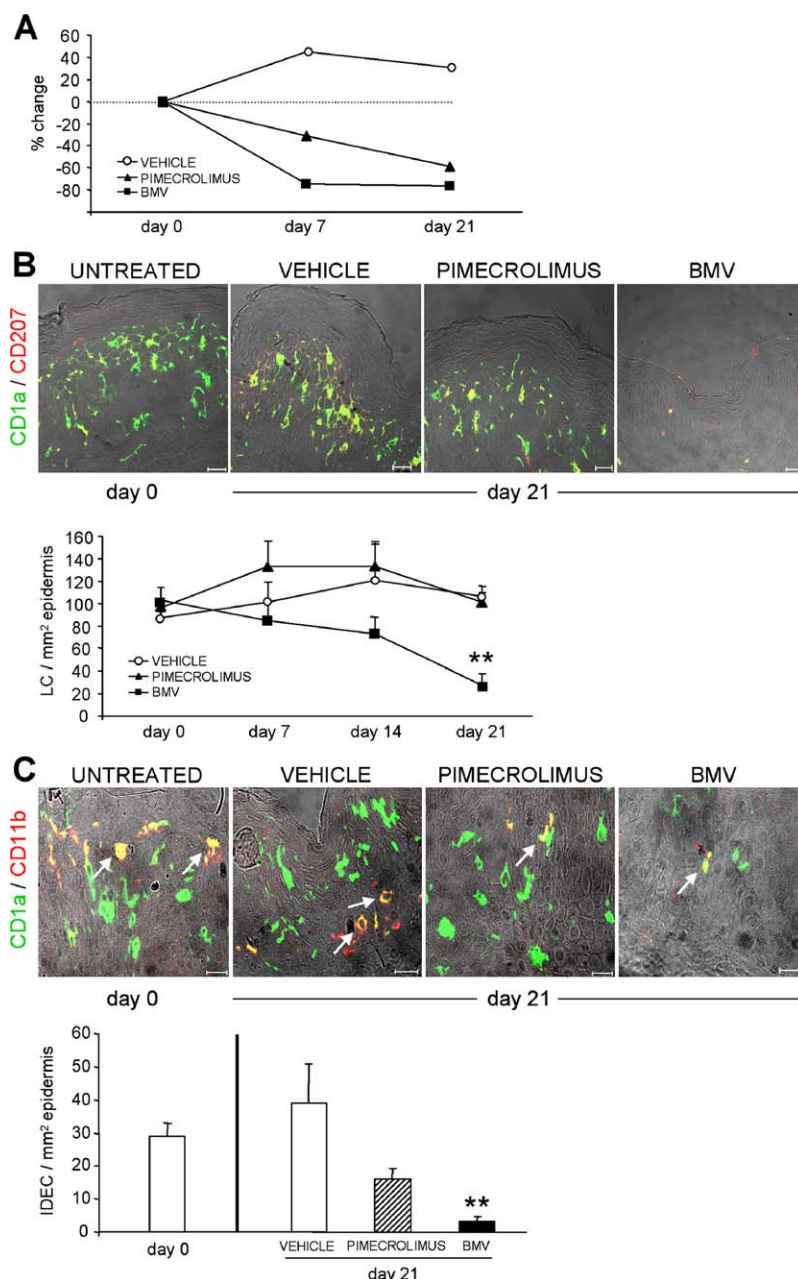


FIG 3. Pimecrolimus depletes IDECs from atopic skin but does not affect LCs, in contrast with BMV. **A**, Patients with AD were treated with pimecrolimus cream, the corresponding vehicle cream, or BMV cream. The patients' dermatitis was assessed at the indicated time points according to the EASI. Skin sections from untreated and treated patients with AD were exposed to PE-anti-CD207/FITC-anti-CD1a (**B**) and APC-anti-CD11b/FITC-anti-CD1a (**C**) double labeling to identify LCs and IDECs, respectively. Arrows in **C** denote double-positive IDECs. **A** and **B**, lower graphs, Kinetics of LCs and IDECs on pimecrolimus and BMV treatment. Biopsies at the indicated time points were subjected to immunofluorescent analysis. Results are expressed as the means $\pm$ SEMs of positive cells/mm² epidermis. ** $P < .01$ vs control. Scale bars, 20 μ m.

3 weeks of BMV treatment (Fig 3, B). In contrast to their diverse effect on LCs, both pimecrolimus and BMV induced a reduction in the cell number of IDECs by 45% and 90%, respectively (Fig 3, C). To test whether IDEC depletion is a result of apoptosis, cryostat sections were double-stained with the TUNEL kit and a DC-specific mAb and examined with a CSLM. At no time point of

investigation did we observe increased numbers of TUNEL⁺/CD1a⁺ DC on pimecrolimus treatment (Fig 4).

Pimecrolimus reduces T-cell numbers in atopic skin by apoptosis

Kinetics experiments revealed a significant reduction of dermal T cells after treatment for 3 weeks with pimecrolimus

and BMV relative to vehicle control (Fig 5, A). At day 7 of treatment, BMV but not pimecrolimus had already largely reduced the mean T-cell number per square millimeter of dermis. Slightly decreased numbers of T cells after pimecrolimus treatment compared with vehicle-treated skin were first observed at 14 days, and then progressively declined until day 21 (Fig 5, A). To investigate how topical pimecrolimus leads to a disappearance of lesional T cells, we used a combination of TUNEL and direct immunofluorescence methodologies. While few TUNEL⁺ T cells were seen in vehicle-treated skin, the decrease in T-cell number on pimecrolimus treatment was reflected by a continuous increase in the percentage of TUNEL⁺ T cells (Fig 5, B-D).

DISCUSSION

In this study, we have demonstrated that the treatment of AD skin with pimecrolimus cream (1) does not affect the number and viability of epidermal LCs, (2) leads to a depletion of IDECs and T cells, and (3) reduces T-cell numbers via apoptosis.

The effect of corticosteroids and calcineurin inhibitors on LCs seems to be divergent. With regard to corticosteroids, several studies, including the current one, have shown a depletion of LCs and an impairment of their immune functions on topical treatment.^{8,9,22-29} In line with this, 2 groups have demonstrated that steroids markedly inhibit the expression of CD80 and CD86 on epidermal DC *in vitro* and *in vivo*.^{15,30} The effect of calcineurin inhibitors on LCs is less clear. Tacrolimus supposedly affects the phenotype and function of LCs *in vitro*, leading to an inhibition of the upregulation of MHC I and II, CD25, CD40, and CD80 molecules and impairing their allostimulatory activity.^{30,31} *In vivo* studies demonstrated that topical tacrolimus does not affect LC numbers in atopic lesions^{14,15}; however, it inhibits the expression of the costimulatory molecule CD80 on epidermal DC and hampers the immunostimulatory capacity of *ex vivo* isolated LCs, suggesting that cutaneous antigen-presenting cells represent a cellular target for this drug.¹⁴ Our groups have recently reported that, in the murine system, topical treatment of healthy skin with pimecrolimus at therapeutic concentrations has no gross effect on number, maturation capacity, and function of murine LCs.^{8,9} Given the divergent reports on the effect of the 2 calcineurin inhibitors on cutaneous DC, we investigated whether human LCs are targets for pimecrolimus. In a first step, we demonstrated that topical application of steroids but not pimecrolimus onto normal skin depletes LCs. In line with this, we found that the addition of BMV but less so pimecrolimus to EC cultures interferes in a dose-dependent manner with the maturation process of human LCs. We showed that BMV significantly impairs MHC I and II expression on LCs and interferes with the *de novo* expression of the costimulatory molecules CD40, CD80, and CD86. In contrast, pimecrolimus only marginally inhibited the upregulation of MHC I, CD80, and CD25 at

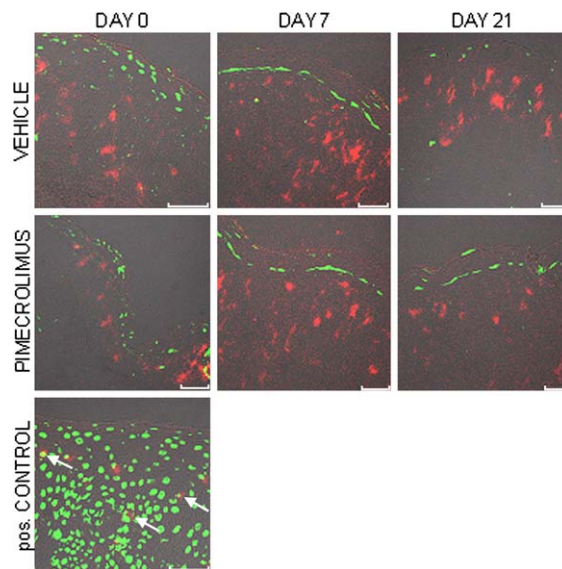


FIG 4. Reduction of IDECs on pimecrolimus treatment in atopic skin is not a result of apoptosis. Sections from untreated and treated patients with AD were exposed to TUNEL/anti-CD1a labeling. TUNEL⁺/CD1a⁺ cells in the positive (pos.) control are labeled with an arrow. Scale bars, 50 μ m.

the highest dose used. This is in agreement with a previous study by Kalthoff et al³² showing that the induction of CD80 and CD86 on monocyte-derived DC was inhibited by steroids but not by pimecrolimus when added during differentiation.

Clinical improvement on topical treatment with calcineurin inhibitors and corticosteroids is mirrored by a marked reduction of the inflammatory infiltrate in which T cells are predominantly present.¹⁰ Whereas it has been shown *in vitro* that tacrolimus and pimecrolimus mainly mediate their immunosuppressive mode of action on T cells by blocking the synthesis of cytokines,^{33,34} the mechanisms by which T-cell depletion is achieved *in vivo* have remained unclear so far. With regard to tacrolimus, it has been shown most recently that its ointment abolishes classical immunologic abnormalities associated with AD, such as the mixed cellular infiltrate of the dermis and the high expression of cytokines by infiltrating cells.¹⁶ We describe now for the first time that the reduction in T-cell numbers on pimecrolimus treatment of subjects with mild to moderate AD twice daily for 3 weeks occurs through apoptotic cell death, as demonstrated by nuclear DNA breaks in T cells. In line with this are results showing that systemic treatment of mice with tacrolimus has an immunosuppressive effect in an autoimmune pancreatitis model through the augmentation of T-cell apoptosis.³⁵ However, the effect of tacrolimus on cell viability and survival is still a matter of debate, because others have reported an antiapoptotic effect of tacrolimus on neuronal cells, which was associated with a downregulation of the c-jun protein.³⁶ With regard to corticosteroids, we have shown in the current study that BMV treatment largely reduced the T-cell

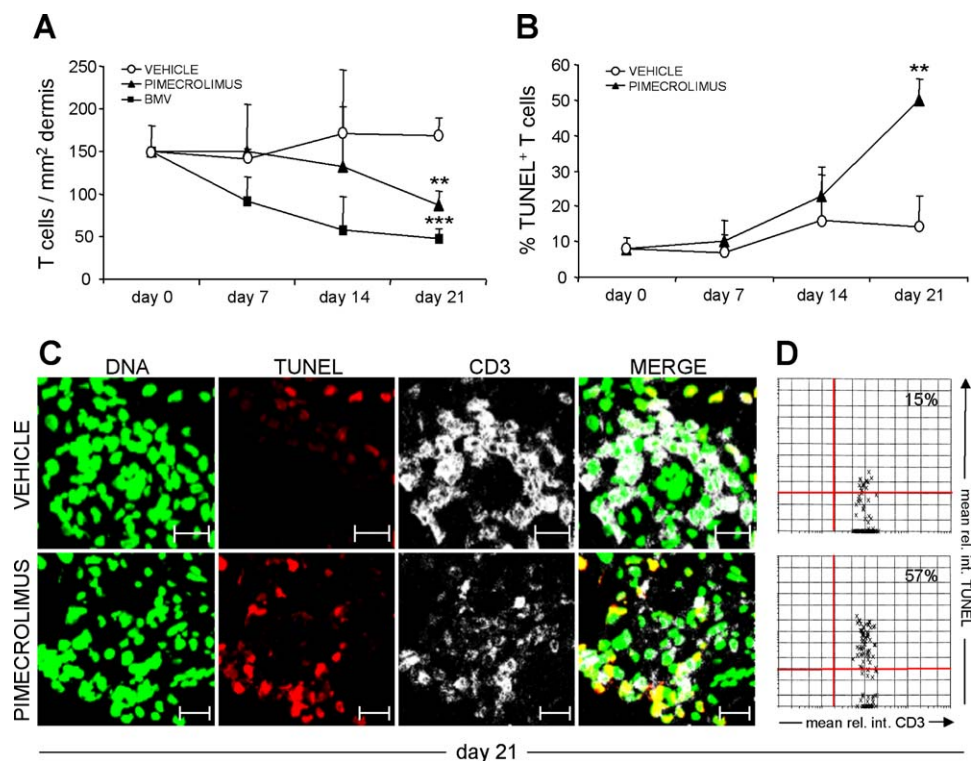


FIG 5. Pimecrolimus reduces infiltrating T cells in atopic skin by apoptosis. Kinetics of T cells/mm² dermis (**A**) and the percentage of TUNEL⁺ T cells (**B**) over the indicated time course are displayed as means $\pm$ SEMs. **C**, Sections from treated patients with AD were exposed to TUNEL labeling and counterstained with an anti-CD3 mAb and Sytox Green. **D**, Dot plots show the mean relative intensity (*mean rel. int.*) of apoptotic T cells. Cryostat sections were screened as described in Methods and the numbers of CD3⁺/TUNEL⁺ T cells evaluated. The cutoff values (*solid lines*) were determined by using isotype-matched control antibodies. On the basis of these cutoff values, the relative number of positive and negative events was calculated. ****P* < .001 vs control; ***P* < .01 vs control. Scale bars, 20 μ m.

density after as early as 7 days. Because no significant increase of TUNEL⁺ T cells was observed on BMV treatment at the various time points investigated (7, 14, and 21 days; data not shown), we assume that apoptotic events in BMV-exposed T cells have probably occurred before day 7. Indeed, in preliminary experiments, we found that even a 3-day treatment with BMV increases apoptosis in T cells (17.2%) compared with untreated lesions (8.1%; (Hoetzenecker, unpublished data, October 2004). In line with our results are data from O'Sullivan et al³⁷ showing that inhaled corticosteroids reduce T cells in the bronchial wall of patients with asthma via apoptosis. Which of the mechanisms known to induce apoptosis in activated T cells play a role in AD skin remains to be investigated. Several studies have shown that the withdrawal of certain cytokines (eg, IL-2, IL-7, IL-15) results in T-cell death.³⁸⁻⁴⁰ Apart from cytokines, extracellular matrix proteins (EMPs) such as fibronectin could play a role. Inflammatory cells, like activated T cells, reside in an EMP network, which exerts profound control over their survival, death, proliferation, and differentiation. The effects of EMP are primarily mediated by integrin-EMP ligation.⁴¹

Inflammatory dendritic epidermal cells are another inflammatory cell population present in lesional AD

skin.³ These dendritic cells are of particular interest because recent reports have assigned them an important role in the pathogenesis of AD. It is assumed that IDECs are highly efficient in antigen presentation of IgE-bound allergens to T cells via their Fc ϵ RI, and Novak et al⁴²⁻⁴⁴ reported recently that these cells contribute distinctly to the outcome of the T-cell response. Topical treatment with BMV almost completely depleted IDECs from lesional skin. Similar to what has been reported for tacrolimus,^{14,15} pimecrolimus led to decreased numbers of IDECs. Their depletion is not a result of an apoptotic process, because no increase of TUNEL⁺/CD1a⁺ dendritic cells was observed at any time point of investigation. This is in line with data provided by Schuller et al,¹⁵ showing that tacrolimus-induced diminution of IDECs is not a result of an increase of Annexin V⁺/TUNEL⁺ dendritic cells. Theoretical possibilities are that processes are induced that promote IDEC depletion by mechanisms like cell migration or differentiation into other cell types. Experiments in our laboratory are ongoing to explore the mechanisms behind this phenomenon.

We thank Drs N. Mothes (Department of Dermatology, Division of Immunology, Allergy and Infectious Diseases, Medical University of Vienna) and F. Kalthoff (Novartis Institutes for Biomedical Research

Vienna) for their advice and help during this study, and Mag S. Farokhnia from the Pharmacy of the General Hospital in Vienna for excellent technical assistance. We thank all of the volunteers who participated and without whom this study would not have been possible.

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