

Tacrolimus and TGF- β act synergistically on the generation of Langerhans cells

Bartłomiej Kwiek, MD,^{a,b*} Wen-Ming Peng, MSc,^{a*} Jean-Pierre Allam, MD,^a Andrzej Langner, MD,^b Thomas Bieber, MD, PhD,^a and Natalija Novak, MD^a Bonn, Germany, and Warsaw, Poland

Background: The proportion of dendritic cell subpopulations in the skin is important for the severity of atopic dermatitis because topical treatment with tacrolimus leads to rapid depletion of inflammatory dendritic epidermal cells, whereas Langerhans cells (LCs) predominate in cured sites.

Objectives: The effects of tacrolimus and TGF- β 1 on LC differentiation and the idea of tacrolimus skewing the differentiation of epidermal precursors to LCs were evaluated.

Methods: The presence of LC markers, MHC, and costimulatory molecules and stimulatory capacity toward T cells of monocyte-derived LCs were analyzed. Skin samples of patients with atopic dermatitis were assessed by means of immunofluorescence microscopy before and after tacrolimus treatment. TGF- β production of skin cells was analyzed.

Results: Tacrolimus and TGF- β 1 act synergistically on the generation of LCs and the expression of CD40, CD80, CD86, CD83, and MHC II; stabilize TGF- β receptor II expression; and decrease the stimulatory capacity of LCs toward T cells. *In vivo* the number of epidermal LCs in tacrolimus-treated skin increased.

Conclusion: The synergism between TGF- β 1 and tacrolimus leads to the generation of LCs, reduced expression of costimulatory and MHC II molecules, and reduced stimulatory activity. Shifting the balance of the dendritic cell population to LCs might be of major importance for the therapeutic effect of tacrolimus. (J Allergy Clin Immunol 2008;122:1000-1006.)

Key words: Langerhans cells, inflammatory dendritic epidermal cells, atopic dermatitis, TGF- β , tacrolimus, TGF- β receptor

Two different dendritic cell (DC) subtypes are the key players in the epidermal skin lesions of atopic dermatitis (AD): Langerhans cells (LCs), which are present in lesional and nonlesional skin, and inflammatory dendritic epidermal cells (IDECs), which

Abbreviations used

AD: Atopic dermatitis
DC: Dendritic cell
IDEC: Inflammatory dendritic epidermal cell
LC: Langerhans cell
PE: Phycocerythrin
RFI: Relative fluorescence intensity

are newly recruited into the skin at the initiation of AD and only present at inflammatory epidermal sites.¹ Recently, it has been shown that LCs and IDECs can exhibit different functions in the pathophysiology of AD.² LCs primarily induce T_H2 immune responses, which predominate in the acute phase of AD. Furthermore, LCs release chemotactic signals that contribute to the recruitment of a second proinflammatory DC type, the so-called IDECs, from precursor cells of the peripheral blood through the dermal compartments into the epidermis. In contrast to LCs, IDECs do not contain Birbeck granules, are Langerin (CD207) negative, and express the mannose receptor (CD206). IDECs produce high amounts of proinflammatory cytokines and chemokines after allergen challenge and most likely contribute to the switch of the initial T_H2-type immune response to a T_H1 polarization.² Considering the pathophysiology of AD, IDECs are regarded as the main amplifiers of immunogenic allergic inflammatory immune responses on the level of epidermal DCs, whereas LCs have been shown to be capable of inducing and maintaining some degree of immunotolerance in a constantly antigen-exposed epidermal environment.³⁻⁶ This leads to the hypothesis that LCs carry out rather tolerogenic functions, whereas IDECs, as prototypical proinflammatory DCs, perform rather immunogenic tasks in the skin. Data supporting the idea of a critical balance between LCs and IDECs in patients with AD in this context are still lacking. In the last decade, calcineurin inhibitors, such as tacrolimus (FK-506), have been introduced as an effective treatment of AD.^{7,8} Calcineurin inhibitors have been shown to downregulate the stimulatory capacity of skin DCs and suppress the proliferation of allogeneic and autologous T cells in mixed lymphocyte reactions.⁹⁻¹¹ Furthermore, it has been described that tacrolimus treatment reduces the number of IDECs in the epidermis, although the underlying mechanisms for this phenomenon remain unknown.¹¹ Several research groups have shown that the depletion of IDECs from the epidermis is not caused by tacrolimus-induced apoptosis of these cells.^{12,13}

TGF- β is a multifunctional cytokine playing a fundamental role in the differentiation of LCs.^{14,15} Tacrolimus was reported to increase levels of TGF- β 1 and its receptors in the kidneys of animals and patients who have undergone transplantation^{16,17} and was supposed to initiate TGF- β receptor signaling in *in vitro* studies.¹⁸⁻²⁰

From ^athe Department of Dermatology and Allergology, University of Bonn, and ^bthe Department of Dermatology, Medical University of Warsaw.

*These authors contributed equally to this work.

Supported by grants from the German Research Council (SFB704 A4; DFG NO454/1-4 and DFG NO454/2-3), a BONFOR grant of the University of Bonn, and a research grant of the Fujisawa/Astellas Pharma GmbH. N.N. is supported by a Heisenberg-Professorship of the DFG NO454/3-1 and NO454/5-1. B.K. was supported by a BONFOR grant and an Otto-Braun-Falco Fellowship.

Disclosure of potential conflict of interest: T. Bieber has received research support from Astellas. N. Novak is on the speakers' bureau for Astellas. The rest of the authors report that they have no conflict of interest.

Received for publication December 23, 2007; revised April 24, 2008; accepted for publication May 2, 2008.

Reprint requests: Natalija Novak, MD, Department of Dermatology and Allergology, Sig-mund-Freud-Str. 25, 53105 Bonn, Germany. E-mail: Natalija.Novak@ukb.uni-bonn.de. 0091-6749/\$34.00

© 2008 American Academy of Allergy, Asthma & Immunology
doi:10.1016/j.jaci.2008.05.005

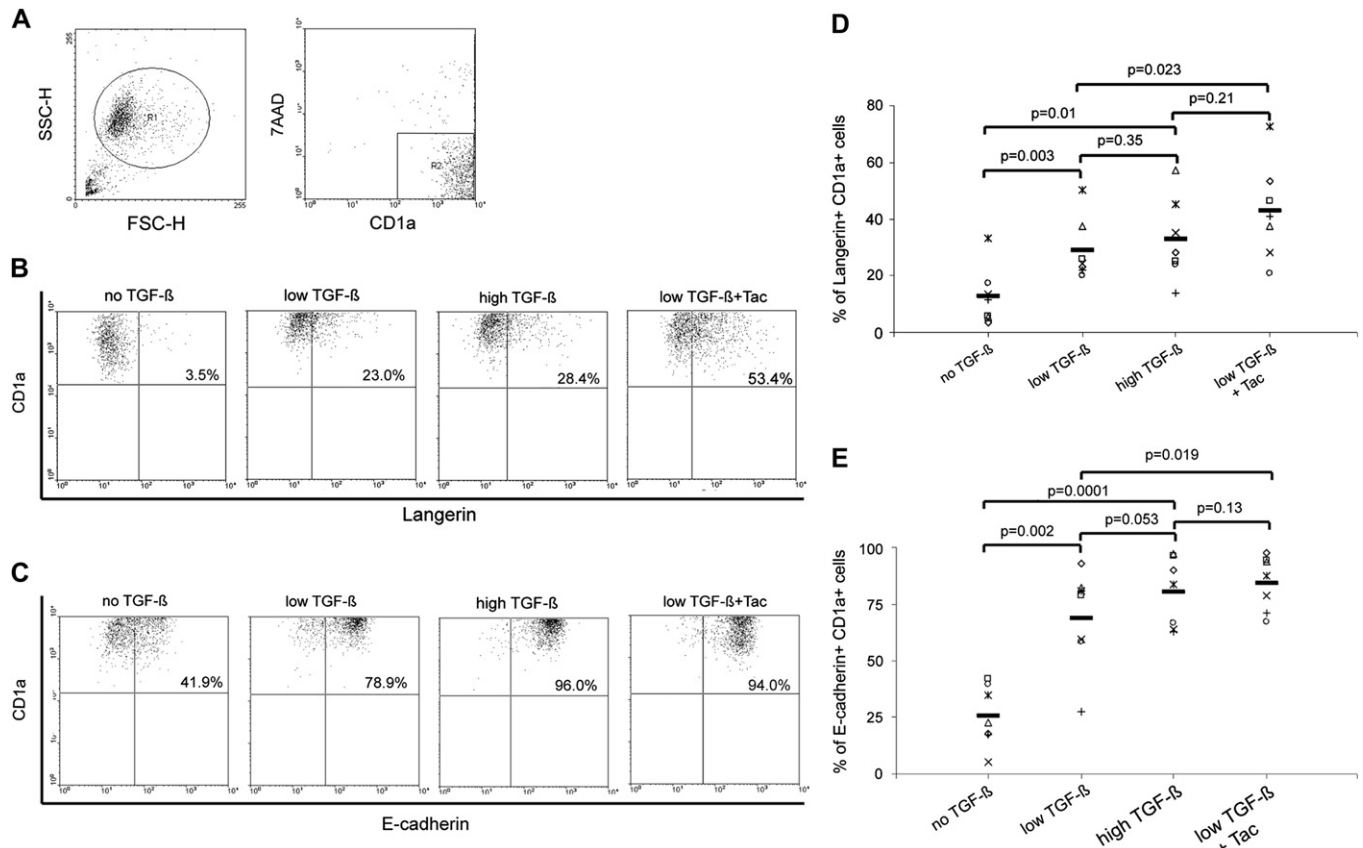


FIG 1. Tacrolimus enhances the effect of TGF- β 1 on LC-like DC generation *in vitro*. The gating strategy is shown in panel A. In the absence of exogenous TGF- β 1, the expression of Langerin (B) and E-cadherin (C) is low. Along with the increased concentration of TGF- β 1 (1 ng/mL TGF- β 1; 10 ng/mL TGF- β 1), the expression of E-cadherin and Langerin is augmented. Representative dot plots with the percentage of positive CD1a⁺ DCs depicted in the right corner are shown. CD1a⁺ DCs cultured with low-dose TGF- β 1 and tacrolimus expressed more Langerin (D) and E-cadherin (E) than LCs-DCs treated with a low dose of TGF- β 1 alone and resemble LCs treated with a high dose of TGF- β 1 ($n = 7$). SSC-H, Side scatter; FSC-H, forward scatter; 7AAD, 7-amino-actinomycin-D; Tac, tacrolimus.

Therefore the present study was designed to evaluate whether treatment of differentiating DCs with low doses of TGF- β 1 in combination with tacrolimus facilitates the generation of LC-like DCs from precursor cells, thereby moving the balance to “tolerogenic” LCs.

METHODS

Reagents

For more information, see the Methods section in the Online Repository at www.jacionline.org.

Patients and skin biopsies

Five patients (1 female and 4 male patients; age range, 25–33 years; average age, 28 years) with moderate-to-severe AD fulfilling the diagnostic criteria of Hanifin and Rajka and with an acute exacerbation of AD were treated topically with 0.1% tacrolimus twice daily for 7 days. The study was conducted in accordance with the principles of the Declaration of Helsinki. After obtaining written informed consent and local ethical committee approval, 4-mm skin punch biopsy specimens were obtained from the lesional skin of the flexor surface of the upper extremities before and after treatment from corresponding sites at the Department of Dermatology of the Medical University of Warsaw. Skin samples were immediately frozen and stored at -80°C until labeling. Only patients not treated with systemic therapies for at least 4 weeks and topical corticosteroids, topical calcineurin inhibitors (tacrolimus and pimecrolimus), or both for at least 2 weeks before the study were included.

Monocyte isolation and generation of DCs

After written informed consent and local ethical committee approval were obtained, the blood for monocyte isolation was obtained from healthy volunteers at the Department of Dermatology and Allergy of the University of Bonn. Monocytes were isolated from peripheral blood, as described previously,²¹ and cultured for up to 8 days in very low endotoxin medium (Biochrom, Berlin, Germany) with 1% antibiotics and antimycotics, 10% inactivated FCS with 500 U/mL GM-CSF (Berlex Laboratories, Inc, Richmond, Calif), and 500 U/mL IL-4 (Strathmann Biotec AG, Hannover, Germany) to yield immature control DCs or with 500 U/mL GM-CSF, 500 U/mL IL-4, and 10 ng/mL natural platelet-derived TGF- β 1 (R&D Systems, Wiesbaden, Germany) to yield immature control LC-like DCs. LC-like DCs cultured with 1 ng/mL TGF- β 1 only served as a low-dose TGF- β control to show TGF- β 1 concentration-dependent changes on DC phenotype and function. As an additional condition, 10^{-8} mol/L tacrolimus was added to cells treated with 1 ng/mL TGF- β 1. IL-4 was added only on day 0 to the cell culture to avoid any antagonistic effect of IL-4 on TGF- β 1. In addition, cells were fed every second day with half of the cytokine dosage $\pm 10^{-8}$ mol/L tacrolimus, and fresh medium was exchanged on day 4. Recombinant TNF- α (250 U/mL, R&D Systems) was added at day 6 of culture for an additional 40 hours to stimulate maturation of DCs.

Immunofluorescence staining and cell enumeration

For immunofluorescence staining, 7- μm cryostat sections of skin samples were air-dried and incubated with primary antibodies for 30 minutes at room temperature, washed 3 times for 10 minutes in PBS, and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse for another 30

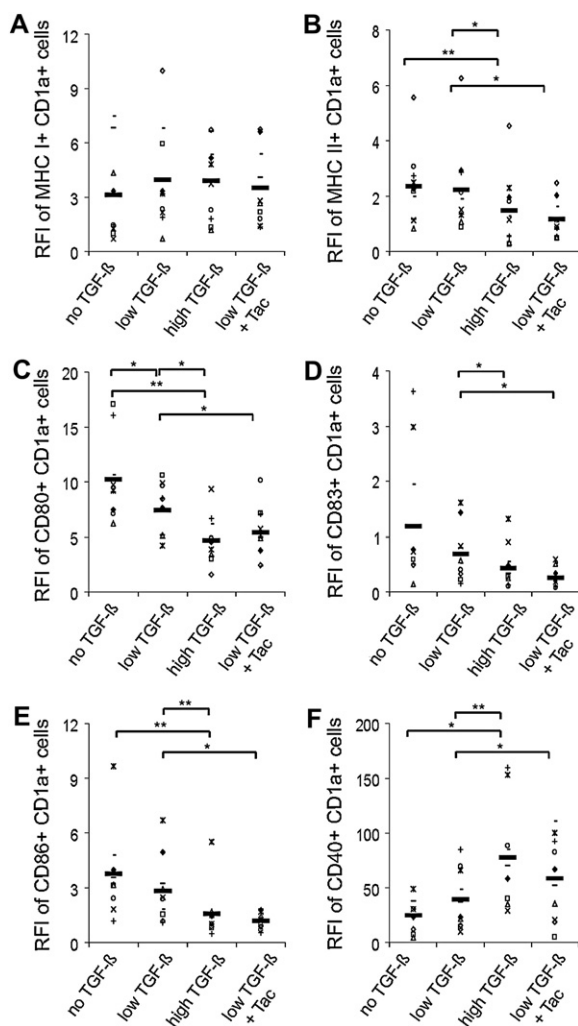


FIG 2. Tacrolimus amplifies the effect of TGF-β1 on the expression of costimulatory molecules of LC-like DCs. Tacrolimus synergized the effect of TGF-β1, upregulating the expression of CD40 (F) and downregulating the expression of costimulatory molecules and MHC II (B-E), but did not display any additional effect on MHC I expression (A). * $P < .05$, ** $P < .01$ ($n = 10$). Tac, Tacrolimus.

minutes. Negative controls were performed by replacing the primary antibody with PBS. Images were examined with the confocal microscope Radiance 2000 (Bio-Rad, Hercules, Calif) by using Lasersharpp2000 5.2 and Laser Pix software (Bio-Rad), and positive cells localized in the epidermis were counted in 10 high-power fields, as described previously.^{22,23}

Flow cytometric analysis

Immunolabeling for phenotyping was performed as previously reported.^{21,24} After washing with PBS, the cells were analyzed with a FACSCalibur and FACS Canto (Becton Dickinson, San Jose, Calif), as described in detail elsewhere.²¹ All incubations and washes were performed at 4°C. Results were expressed as the percentage of positive cells, and the relative fluorescence intensity (RFI) was calculated from the mean fluorescence intensity (MFI) as follows:

$$RFI = (MFI [of the parameter evaluated] - MFI [control]) / MFI (control).$$

T-cell proliferation assays

For the T-cell proliferation assays, DCs generated under the conditions mentioned above from the same donor were generated and assayed at the same time under identical conditions. Allogeneic T cells were obtained from healthy donors, as described previously.²⁵

For testing the stimulatory activity of DCs, cells were washed 3 times and seeded in triplicates of 1:100 (antigen-presenting cell/T-cell) cells per well in 96-well culture plates at 37°C for 96 hours. Then 20 μL of tritiated thymidine (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) was added to each well and incubated for another 16 hours. Culture plates were harvested, and the incorporated radioactivity was measured in a liquid scintillation counter. Relative stimulation indices (rSI) were calculated as follows:

$$rSI = cpm (mixed lymphocyte reaction) - cpm (T cell) / cpm (T cell).$$

TGF-β production in keratinocytes and epidermal DCs

For more information, see the Methods section in the Online Repository.

Statistical analysis

All results were expressed as means ± SEMs. The statistical analysis was performed by using the paired Student *t* test (SPSS for Windows 12.0; SPSS, Inc, Chicago, Ill). A *P* value of less than .05 was considered statistically significant.

RESULTS

Tacrolimus synergizes with TGF-β1 on the differentiation of DCs and promotes the generation of LC-like DCs

CD1a⁺ DCs were analyzed on day 7 of culture by means of flow cytometry for the expression of LC-specific markers, such as E-cadherin and Langerin; the gating strategy is shown in Fig 1, A. DCs cultured with 1 ng/mL TGF-β1 displayed higher expression of E-cadherin and Langerin than cells not treated with TGF-β1, whereas the highest expression of LC markers could be found on cells cultured with 10 ng/mL TGF-β1 (Fig 1, B and C). In the next step we evaluated potential synergistic functions of TGF-β1 and tacrolimus. For this purpose, tacrolimus was added to cells cultured with low concentrations of TGF-β1 (1 ng/mL). As a result, the number of E-cadherin-positive and Langerin-positive CD1a⁺ cells was higher on day 7 of culture in comparison with that seen in DCs cultured with 1 ng/mL TGF-β1 alone, reaching levels comparable with the expression of E-cadherin and Langerin on DCs cultured with 10 ng/mL TGF-β1 (Fig 1, D and E). Subanalysis of CD1a⁺/Langerin-positive DCs and CD1a⁺/Langerin-negative DCs revealed significantly higher expression of the LC marker E-cadherin on the CD1a⁺/Langerin-positive subpopulation, with the highest expression in DCs treated with low TGF-β1 plus tacrolimus (data not shown).

Tacrolimus synergizes TGF-β-driven MHC II and costimulatory molecule expression on DCs

The presence of MHC I and II antigens and costimulatory molecules represents a characteristic feature of DCs and was explored in a series of experiments. We could show that the expression of MHC II, CD80, CD86, and CD83 by DCs on day 7 of culture was diminished by TGF-β1 in a dose-dependent manner (data not shown). In contrast, CD40 expression increased with higher levels of TGF-β1 (data not shown). In the next set of experiments, we checked the expression of MHC I and II, CD40, CD80, CD86, and CD83 of DCs cultured with 1 ng/mL TGF-β1 together with tacrolimus in comparison with that seen on DCs cultured with low-dose TGF-β1 (1 ng/mL) and high-dose TGF-β1 (10 ng/mL) alone. Tacrolimus, together with low-dose

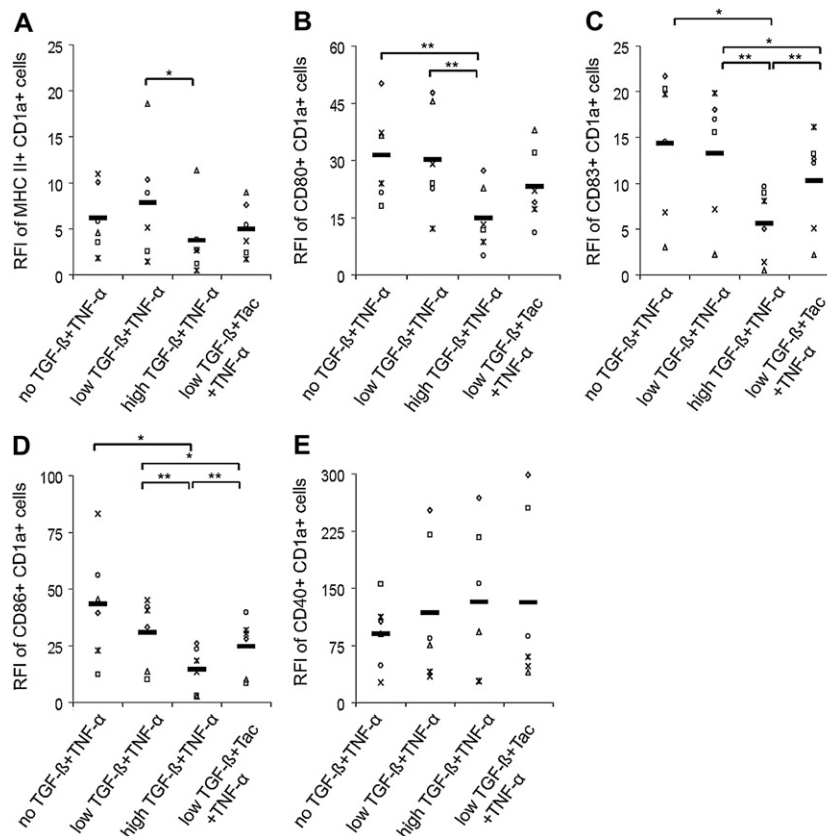


FIG 3. A-E, Tacrolimus synergizes the inhibitory effect of TGF- β 1 on DC maturation. Tacrolimus aggravates the TGF- β 1-mediated downregulation of CD80, CD83, CD86, and MHC II on mature DCs. * $P < .05$, ** $P < .01$ ($n = 6$). Tac, Tacrolimus.

TGF- β 1, reduced the expression of MHC II, CD80, CD86, and CD83 (Fig 2, B-E) on DCs after 7 days of *in vitro* culture, which is similar to effects seen with high doses of TGF- β 1, whereas no additional effect of tacrolimus on MHC I expression of DCs could be observed (Fig 2, A).

Tacrolimus enhances the inhibitory effect of TGF- β 1 on DC maturation

On antigen uptake, DCs mature and gain the capacity to efficiently present antigens to T cells. This process is characterized by the upregulation of MHC II and costimulatory molecule and CD83 expression and can be driven by TNF- α . It was shown previously that TGF- β 1 is able to inhibit TNF- α -related maturation of DCs, preventing the formation of CD83⁺ DCs.^{26,27} We were able to confirm this observation by adding TNF- α (250 U/mL) to the cell culture. DCs cultured with low TGF- β 1 plus tacrolimus expressed significantly less CD86 and CD83 than DCs cultured with a low dose of TGF- β 1 alone (Fig 3).

Tacrolimus synergizes the effect of TGF- β 1 and induces LC-like DCs, which display a reduced stimulatory capacity toward T cells

In the next step the functional significance of our phenotypic observations was verified with allogeneic T-cell proliferation assays. As a result, tacrolimus significantly reduced the

stimulatory capacity of immature and mature DCs generated with 1 ng/mL TGF- β 1 in comparison with DCs cultured with low TGF- β 1 alone (Fig 4).

Number of CD1a⁺/Langerin-positive/Lag⁺ cells in the epidermis of patients with AD increases after topical treatment with 0.1% tacrolimus ointment

To evaluate the clinical relevance of our *in vitro* findings and to support the hypothesis that IDECs could be replaced by a higher generation of LCs from precursor cells in the epidermis during topical treatment of AD with tacrolimus, we took sequential biopsy specimens from patients with AD before and after 7 days of topical treatment with 0.1% tacrolimus ointment twice a day. We found an increase in the number of CD1a-expressing epidermal cells in addition to an increase of Langerin-positive and Lag⁺ cells in the epidermis after 1 week of topical treatment (Fig 5).

Tacrolimus increases the expression of TGF- β receptor II on differentiating DCs

Extracellular expression of TGF- β receptor II, which binds TGF- β 1 and TGF- β 3 with high affinity and TGF- β 2 with low affinity, has been evaluated on differentiating DCs *in vitro* to evaluate whether tacrolimus modulates the capacity of DCs to respond to TGF- β . As a result, significantly higher expression of

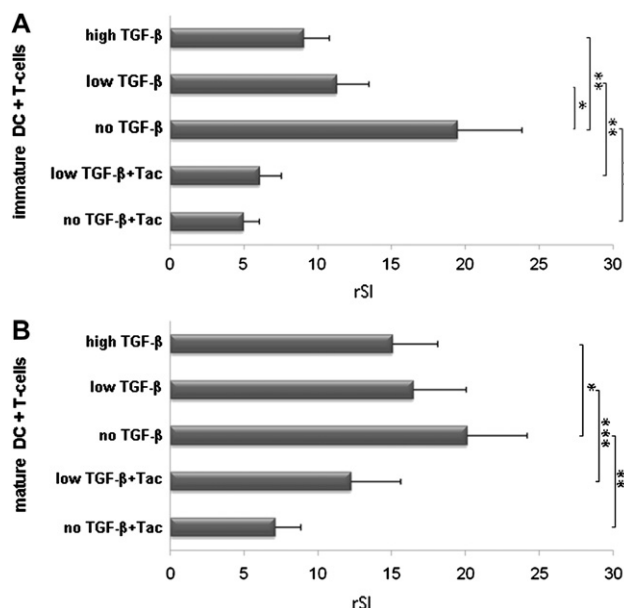


FIG 4. Tacrolimus enhances the suppressive effect of TGF-β1 on the stimulatory capacity of DCs toward T cells. Addition of tacrolimus to 1 ng/mL TGF-β1 (low TGF-β1) resulted in the reduction of the stimulatory capacity of DCs at an antigen-presenting cell/T-cell ratio of 1:100 in comparison with the condition with low TGF-β1 alone in both immature DCs (**A**) and mature DCs with the addition of TNF-α (250 U/mL; **B**). Averages of triplicates of 10 experiments are presented as means ± SEMs, and the relative stimulation index is depicted on the y-axis and calculated as follows: $rSI = \text{cpm (mixed lymphocyte reaction)} - \text{cpm (T cell)} / \text{cpm (T cell)}$. * $P < .05$, ** $P < .01$, and *** $P < .005$. Tac, Tacrolimus.

TGF-β receptor II on DCs generated with low-dose TGF-β and tacrolimus on day 2 of culture was observable (Fig 6, A).

Production of TGF-β1 on HaCaT cells has been evaluated to assess whether skin cells might represent a natural source for TGF-β *in vivo*. Baseline production of TGF-β1 was detectable in supernatants of HaCaT cells. However, no further increase of intracellular TGF-β1, 2, or 3 production, as well as TGF-β1 production in the supernatant of HaCaT cells incubated with tacrolimus, 10^{-7} to 10^{-9} mol/L, with and without stimulation with TNF-α (20 ng/mL), was detectable (Fig 6, B). Intracellular staining of crude cell suspension isolated from epidermal skin revealed that TGF-β1, 2, and 3 were produced mainly by CD1a⁺ epidermal cells, whereas no significant production of TGF-β1, 2, or 3 by CD1a⁺ epidermal DCs was detectable (data not shown).

DISCUSSION

Depletion of IDECs was shown to accompany the clinical improvement of AD and is one of the most striking events during topical treatment of AD with tacrolimus.¹¹ Previous studies revealed that this depletion is not simply based on apoptosis of IDECs.^{12,13} Therefore we analyzed whether skewing the differentiation of epidermal DCs to LCs could be a possible mechanism underlying this phenomenon. We observed that tacrolimus acts synergistically with TGF-β1 on LC generation *in vitro*, facilitating the development of CD1a⁺/Langerin-positive/E-cadherin-positive cells with phenotypic and functional features of regular LCs, and reduces the expression of costimulatory and MHC II molecules on both differentiating CD1a⁺/Langerin-positive DCs and CD1a⁺/Langerin-negative DCs.

In the natural course of AD, several factors, such as an impairment of the skin barrier and immunologic network triggered by increased microbial colonization together with intrinsic defects of keratinocytes, contribute to hyperresponsiveness toward normally harmless signals, which in AD are capable of breaking down immune tolerance and launching the immune response.²⁸⁻³⁰ Once the inflammation has started, inflammatory cells invade the skin and aggravate the disease. On the level of epidermal DCs, this scenario is conducted through a critical cross-talk of LCs and IDECs. LCs are the only antigen-presenting cells detectable in the epidermis of nonlesional skin from patients with AD. This exceptional position of LCs in a constantly antigen-exposed environment suggests that LCs primarily maintain tolerance rather than initiating inflammatory immune responses.^{6,15,31}

However, during the course of AD, this tolerance can collapse, and LCs might start to produce chemotactic signals that, together with signals from other skin cells, such as keratinocytes, might be responsible for the recruitment of IDEC precursors to the skin. IDECs are believed to aggravate the allergic inflammatory immune response and perpetuate the disease.² Therefore the idea to shift the balance from IDECs to LCs by means of therapeutic measures appears to be a promising concept. LCs might counteract the proinflammatory activity of IDECs in the chronic phase of AD and exert suppressive properties on T cells, as shown for LC-like DCs generated in the presence of low-dose TGF-β1 and tacrolimus in this study. Notably, tacrolimus acts on both myeloid DC subtypes in inflammatory AD skin because it not only reduces the activation state of CD1a⁺/Langerin-positive DCs but also CD1a⁺/Langerin-negative DCs, such as IDECs in our system, which, as an additive effect, most likely contributes to the improvement of skin lesions *in vivo*.

Because IDECs and LCs are different DC types originating from a common monocyte precursor,^{2,32-34} the restoration of the physiologic dominance of LCs could be explained by both rapid influx of precursor cells differentiating into LCs and fast depletion of IDECs in the high epidermal DC turnover during inflammation.³⁵ Whether the effect of tacrolimus is exerted mainly on the invading precursors of DCs is unclear. *In vitro* the effect of tacrolimus and TGF-β1 was only inducible at early time points (not later than day 2) of the DC culture (data not shown). This goes in line with higher surface expression of TGF-β receptor II maintained by tacrolimus, as shown in this study. Therefore one proposed mode of action of tacrolimus might be to increase the responsiveness of the differentiating DCs toward TGF-β1 and to support thereby the differentiation of LCs.

Because TGF-β1 is detectable in the supernatants of keratinocyte cell lines,³⁶ it is more than likely that TGF-β1 produced endogenously by several skin cells during inflammation synergizes with tacrolimus *in vivo*. Because we were not able to find a significant tacrolimus-related induction of TGF-β1, 2, and 3 in the supernatant or intracellular space of our *in vitro* cultured DCs (data not shown), autocrine secretion of TGF-β1 by DCs themselves is of less relevance than that of TGF-β produced by other skin cells. However, it has been found that autocrine/paracrine production of TGF-β1 by LCs might be responsible for LC differentiation in other systems.¹⁵

It cannot be excluded that increased numbers of CD1a cells observed in previous studies using microscopic analyses of skin samples after topical tacrolimus or pimecrolimus treatment³⁷⁻⁴⁰ might be partially based on the mechanisms described here.

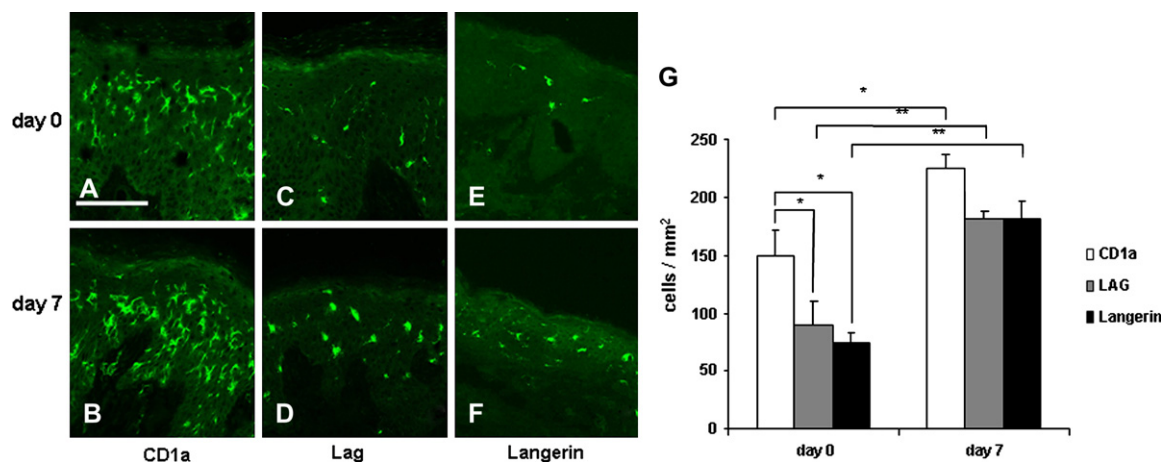


FIG 5. Treatment of AD with tacrolimus 0.1% ointment results in increased numbers of CD1a⁺/Langerin-positive/Lag⁺ cells in the epidermis. **A-F**, Images of microscopic evaluation of skin biopsy specimens for the expression of CD1a, as well as the LC markers Langerin and Lag, for 1 of 5 representative patients are presented. **G**, Results of a total of 5 experiments are shown. The number of CD1a⁺ epidermal cells before tacrolimus treatment significantly exceeds the number of Langerin-positive and Lag⁺ cells, indicating the existence of a CD1a⁺ non-LC population of DCs in the epidermis of patients with AD. After 1 week of tacrolimus application, the total number of epidermal CD1a⁺ DCs increased simultaneously with the increased amount of Langerin-positive and Lag⁺ LCs. Values are presented as means $\pm$ SEMs. * $P < .05$ and ** $P < .01$ ($n = 5$). Bar = 100 μ m.

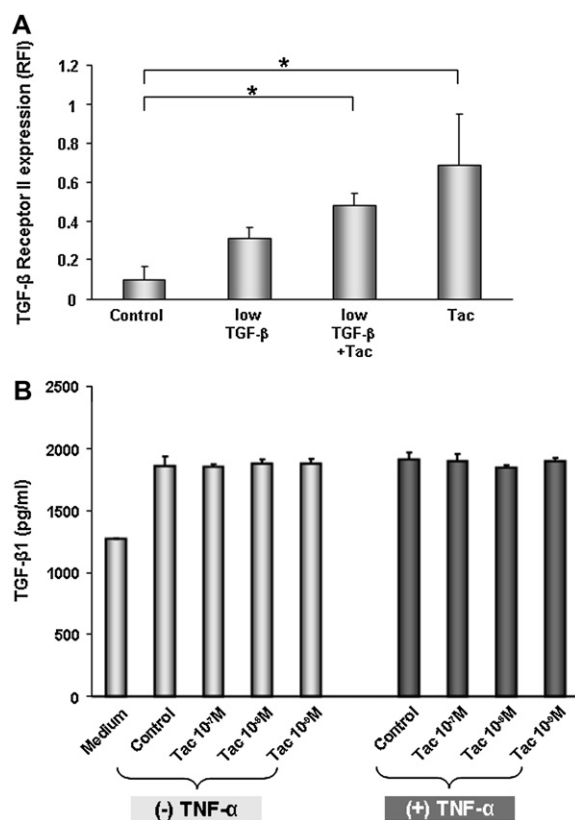


FIG 6. Higher TGF-β receptor II expression of DCs cultured with TGF-β and tacrolimus on day 2 of culture and TGF-β2 production of HaCat cells. **A**, Mean $\pm$ SEM of the RFI of the extracellular expression of TGF-β receptor II of differentiating DCs on day 2 of culture is shown ($n = 8$ experiments). **B**, Amount of TGF-β1 in the supernatant of HaCat cells stimulated with increasing amounts of tacrolimus, 10^{-9} to 10^{-7} mol/L, with or without TNF-α is shown as the mean $\pm$ SEM value of 3 experiments. Tac, Tacrolimus. * $P < .05$.

Current strategies of treating chronic inflammatory diseases of the skin, such as psoriasis or AD, are usually aimed at targeting T cells or soluble factors, such as cytokines.⁴¹ In recent years, concepts of immunoregulatory approaches were developed to find more physiologic ways of restoring immunologic homeostasis in a misbalanced disease state. In view of these data, the picture emerges that a promising strategy for the treatment of AD might be to increase the sensitivity of differentiating DCs to anti-inflammatory, tolerogenic mediators, such as TGF-β, to facilitate the function of LCs as potential natural silencers of allergic-inflammatory immune responses.

We thank Andreas Neubauer for critically reading this manuscript.

Clinical implications: TGF-β and tacrolimus act synergistically on LC development and function. This mechanism might underlie the restoration of physiologic LC dominance after tacrolimus treatment of AD.

REFERENCES

- Wollenberg A, Kraft S, Hanau D, Bieber T. Immunomorphological and ultrastructural characterization of Langerhans cells and a novel, inflammatory dendritic epidermal cell (IDEC) population in lesional skin of atopic eczema. *J Invest Dermatol* 1996;106:446-53.
- Novak N, Valenta R, Bohle B, Laffer S, Haberkost J, Kraft S, et al. FcεpsilonRI engagement of Langerhans cell-like dendritic cells and inflammatory dendritic epidermal cell-like dendritic cells induces chemotactic signals and different T-cell phenotypes in vitro. *J Allergy Clin Immunol* 2004;113:949-57.
- Steinman RM, Nussenzweig MC. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc Natl Acad Sci U S A* 2002;99:351-8.
- Probst HC, Lagnel J, Kollias G, van den BM. Inducible transgenic mice reveal resting dendritic cells as potent inducers of CD8⁺ T cell tolerance. *Immunity* 2003;18:713-20.
- Stoitzner P, Tripp CH, Douillard P, Saeland S, Romani N. Migratory Langerhans cells in mouse lymph nodes in steady state and inflammation. *J Invest Dermatol* 2005;125:116-25.
- Kissenpfennig A, Malissen B. Langerhans cells—revisiting the paradigm using genetically engineered mice. *Trends Immunol* 2006;27:132-9.

7. Ruzicka T, Bieber T, Schopf E, Rubins A, Dobozy A, Bos JD, et al. A short-term trial of tacrolimus ointment for atopic dermatitis. European Tacrolimus Multicenter Atopic Dermatitis Study Group. *N Engl J Med* 1997;337:816-21.
8. Novak N, Kwiek B, Bieber T. The mode of topical immunomodulators in the immunological network of atopic dermatitis. *Clin Exp Dermatol* 2005;30:160-4.
9. Homey B, Assmann T, Vohr HW, Ulrich P, Lauerma AI, Ruzicka T, et al. Topical FK506 suppresses cytokine and costimulatory molecule expression in epidermal and local draining lymph node cells during primary skin immune responses. *J Immunol* 1998;160:5331-40.
10. Panhans-Gross A, Novak N, Kraft S, Bieber T. Human epidermal Langerhans' cells are targets for the immunosuppressive macrolide tacrolimus (FK506). *J Allergy Clin Immunol* 2001;107:345-52.
11. Wollenberg A, Sharma S, von BD, Geiger E, Haberstok J, Bieber T. Topical tacrolimus (FK506) leads to profound phenotypic and functional alterations of epidermal antigen-presenting dendritic cells in atopic dermatitis. *J Allergy Clin Immunol* 2001;107:519-25.
12. Matsue H, Yang C, Matsue K, Edelbaum D, Mummert M, Takashima A. Contrasting impacts of immunosuppressive agents (rapamycin, FK506, cyclosporin A, and dexamethasone) on bidirectional dendritic cell-T cell interaction during antigen presentation. *J Immunol* 2002;169:3555-64.
13. Schuller E, Oppel T, Bornhove E, Wetzel S, Wollenberg A. Tacrolimus ointment causes inflammatory dendritic epidermal cell depletion but no Langerhans cell apoptosis in patients with atopic dermatitis. *J Allergy Clin Immunol* 2004;114:137-43.
14. Borkowski TA, Letterio JJ, Mackall CL, Saitoh A, Wang XJ, Roop DR, et al. A role for TGFbeta1 in Langerhans cell biology. Further characterization of the epidermal Langerhans cell defect in TGFbeta1 null mice. *J Clin Invest* 1997;100:575-81.
15. Kaplan DH, Li MO, Jenison MC, Shlomchik WD, Flavell RA, Shlomchik MJ. Autocrine/paracrine TGFbeta1 is required for the development of epidermal Langerhans cells. *J Exp Med* 2007;204:2545-52.
16. Bing P, Maode L, Li F, Sheng H. Comparison of expression of TGF-beta1, its receptors TGFbeta1R-I and TGFbeta1R-II in rat kidneys during chronic nephropathy induced by cyclosporine and tacrolimus. *Transplant Proc* 2006;38:2180-2.
17. Khanna A, Plummer M, Bromberek C, Bresnahan B, Hariharan S. Expression of TGF-beta and fibrogenic genes in transplant recipients with tacrolimus and cyclosporine nephrotoxicity. *Kidney Int* 2002;62:2257-63.
18. Wang T, Donahoe PK, Zervos AS. Specific interaction of type I receptors of the TGF-beta family with the immunophilin FKBP-12. *Science* 1994;265:674-6.
19. Chen YG, Liu F, Massague J. Mechanism of TGFbeta receptor inhibition by FKBP12. *EMBO J* 1997;16:3866-76.
20. Yao D, Dore JJ Jr, Leof EB. FKBP12 is a negative regulator of transforming growth factor-beta receptor internalization. *J Biol Chem* 2000;275:13149-54.
21. Novak N, Bieber T, Katoh N. Engagement of Fc epsilon RI on human monocytes induces the production of IL-10 and prevents their differentiation in dendritic cells. *J Immunol* 2001;167:797-804.
22. Baker BS, Swain AF, Griffiths CE, Leonard JN, Fry L, Valdimarsson H. Epidermal T lymphocytes and dendritic cells in chronic plaque psoriasis: the effects of PUVA treatment. *Clin Exp Immunol* 1985;61:526-34.
23. Bieber T, Ring J, Braun-Falco O. Comparison of different methods for enumeration of Langerhans cells in vertical cryosections of human skin. *Br J Dermatol* 1988;118:385-92.
24. Buckley CC, Ivison C, Poulter LW, Rustin MH. Fc epsilon R11/CD23 receptor distribution in patch test reactions to aeroallergens in atopic dermatitis. *J Invest Dermatol* 1992;99:184-8.
25. Novak N, Haberstok J, Kraft S, Siekmann L, Allam JP, Bieber T. Standardized extracts from Chinese herbs induce IL-10 production in human monocyte-derived dendritic cells and alter their differentiation in vitro. *J Allergy Clin Immunol* 2001;108:588-93.
26. Geissmann F, Revy P, Regnault A, Lepelletier Y, Dy M, Brousse N, et al. TGF-beta 1 prevents the noncognate maturation of human dendritic Langerhans cells. *J Immunol* 1999;162:4567-75.
27. Geissmann F, eu-Nosjean MC, Dezutter C, Valladeau J, Kayal S, Leborgne M, et al. Accumulation of immature Langerhans cells in human lymph nodes draining chronically inflamed skin. *J Exp Med* 2002;196:417-30.
28. Wollenberg A, Kraft S, Oppel T, Bieber T. Atopic dermatitis: pathogenetic mechanisms. *Clin Exp Dermatol* 2000;25:530-4.
29. Novak N, Bieber T, Leung DY. Immune mechanisms leading to atopic dermatitis. *J Allergy Clin Immunol* 2003;112(suppl):S128-39.
30. Boguniewicz M, Schmid-Grendelmeier P, Leung DY. Atopic dermatitis. *J Allergy Clin Immunol* 2006;118:40-3.
31. Grabbe S, Steinbrink K, Steinert M, Luger TA, Schwarz T. Removal of the majority of epidermal Langerhans cells by topical or systemic steroid application enhances the effector phase of murine contact hypersensitivity. *J Immunol* 1995;155:4207-17.
32. Geissmann F, Prost C, Monnet JP, Dy M, Brousse N, Hermine O. Transforming growth factor beta1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. *J Exp Med* 1998;187:961-6.
33. Novak N, Kraft S, Haberstok J, Geiger E, Allam P, Bieber T. A reducing microenvironment leads to the generation of Fc epsilon RIhigh inflammatory dendritic epidermal cells (IDEC). *J Invest Dermatol* 2002;119:842-9.
34. Ginhoux F, Tacke F, Angeli V, Bogunovic M, Loubreau M, Dai XM, et al. Langerhans cells arise from monocytes in vivo. *Nat Immunol* 2006;7:265-73.
35. Merad M, Manz MG, Karsunky H, Wagers A, Peters W, Charo I, et al. Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat Immunol* 2002;3:1135-41.
36. Lan CC, Kao YH, Huang SM, Yu HS, Chen GS. FK506 independently upregulates transforming growth factor beta and downregulates inducible nitric oxide synthase in cultured human keratinocytes: possible mechanisms of how tacrolimus ointment interacts with atopic skin. *Br J Dermatol* 2004;151:679-84.
37. Simon D, Vassina E, Yousefi S, Kozlowski E, Braathen LR, Simon HU. Reduced dermal infiltration of cytokine-expressing inflammatory cells in atopic dermatitis after short-term topical tacrolimus treatment. *J Allergy Clin Immunol* 2004;114:887-95.
38. Simon D, Vassina E, Yousefi S, Braathen LR, Simon HU. Inflammatory cell numbers and cytokine expression in atopic dermatitis after topical pimecrolimus treatment. *Allergy* 2005;60:944-51.
39. Belsito DV, Flotte TJ, Lim HW, Baer RL, Thorbecke GJ, Gigli I. Effect of glucocorticosteroids on epidermal Langerhans cells. *J Exp Med* 1982;155:291-302.
40. Hoetzonecker W, Ecker R, Kopp T, Stuetz A, Stingl G, Elbe-Burger A. Pimecrolimus leads to an apoptosis-induced depletion of T cells but not Langerhans cells in patients with atopic dermatitis. *J Allergy Clin Immunol* 2005;115:1276-83.
41. Gottlieb AB. Therapeutic options in the treatment of psoriasis and atopic dermatitis. *J Am Acad Dermatol* 2005;53(suppl 1):S3-16.

METHODS

Reagents

Phycoerythrin (PE)-labeled T6/RD1 (mIgG1) mAb (Coultertronics, Krefeld, Germany) was directed against CD1a. The mAbs against CD80 (L307.4; mIgG1), CD86 (FUN-1; mIgG1), CD83 (HB15e; mIgG1b), E-cadherin (67A4; mIgG1), MHC II (L243; mIgG2a), MHC I (HLA-A, B, C; G46-2.6; mIgG1), and CD40 (5C3; mIgG1) were from BD Biosciences Pharmingen (Heidelberg, Germany). The unconjugated mAb against CD207/Langerin (DCGM4; mIgG1) and the mAb against E-cadherin (67A4; mIgG1) were from Immunotech (Marseille Cedex, France). PE-labeled CD207/Langerin (DCGM4; mIgG1) was from Beckman Coulter (Fullerton, Calif), and the PE-Cy5-labeled mAb against CD1a (HI149; mIgG1) was from BD Pharmingen. PE-labeled antibody against TGF- β 1, 2, and 3 (1D11; mIgG1) and the TGF- β 1 and TGF- β 2 ELISAs were from R&D Systems. The unconjugated mAb against TGF- β receptor II (25508; mIgG1) was from R&D Systems. The PE-labeled IgG2b mAb against CD14 (MfP9) was obtained from BD Biosciences. Fluorescein isothiocyanate-conjugated goat anti-mouse antibody was from Jackson Laboratories (West Grove, Pa). Normal mouse serum for blocking purposes was obtained from Dianova (Hamburg, Germany), and 7-amino-actinomycin-D was from Sigma. All other reagents were obtained from Sigma Chemical Co (St

Louis, MO), unless otherwise indicated. Tacrolimus (FK506) was a kind gift from Shuhei Deguchi, PhD (Fujisawa Pharmaceutical Co, Ltd, Osaka, Japan).

Evaluation of TGF- β production in keratinocytes and epidermal DCs

The HaCat human keratinocyte cell line^{E1} was kindly provided by Professor Norbert E. Fusenig (German Cancer Research Center, Heidelberg, Germany). The HaCat cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, Calif) and supplemented with 10% FCS (Sigma) and 1% penicillin/streptomycin (Invitrogen). Cells were split and seeded in 24-well plates at 2×10^5 cells/well and cultured for 24 hours to allow adherence. Then HaCat cells were incubated with tacrolimus, 10^{-5} to 10^{-10} mol/L. After 48 hours, HaCat cells were stimulated with TNF- α (20 ng/mL). Cell-culture supernatants were collected to determine the content of TGF- β 1 and TGF- β 2 in ELISAs (R&D Systems).

REFERENCE

- E1. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* 1988;106:761-71.