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Absence of T-regulatory cell expression and function in atopic dermatitis skin

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Background: The role of regulatory T cells has been widely reported in the suppression of T-cell activation. A dysfunction in $CD4^+CD25^+$ T-regulatory cell–specific transcription factor FoxP3 leads to immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome, often associated with atopic dermatitis. Increasing the number and activity of regulatory T cells in affected organs has been suggested as a remedy in various inflammatory diseases, including allergy.
Objective: To determine the presence and function of regulatory T cells in atopic dermatitis.
Methods: Immunohistochemistry of lesional atopic dermatitis skin and control skin conditions was used to demonstrate regulatory cells and cytokines in situ. The role of effector and regulatory T cells as well as their specific cytokines in apoptosis in human keratinocyte cultures and artificial skin equivalents was investigated.
Results: Human T-regulatory type 1 cells, their suppressive cytokines, IL-10 and TGF-β, as well as receptors for these cytokines were significantly expressed, whereas $CD4^+CD25^+FoxP3^+$ T-regulatory cells were not found in lesional and atopy patch test atopic dermatitis or psoriasis skin. Both subsets of regulatory T cells suppress the allergen-specific activation of T_H1 and T_H2 cells. In coculture and artificial skin equivalent experiments, subsets of T-regulatory cells neither induced keratinocyte death nor suppressed apoptosis induced by skin T cells, T_H1 cells, IFN-γ, or TNF-α.
Conclusion: A dysregulation of disease-causing effector T cells is observed in atopic dermatitis lesions, in association with an impaired $CD4^+CD25^+FoxP3^+$ T-cell infiltration, despite the expression of type 1 regulatory cells in the dermis.

Abbreviations used
AD: Atopic dermatitis
APT: Atopy patch test
FasL: Fas ligand
HDM: House dust mite
NAD: Nonallergic type of dermatitis
Tr1: T-regulatory type 1
Treg: T-regulatory

Atopic dermatitis (AD) is a chronic relapsing skin disorder with an interplay of migrating lymphocytes and epidermal keratinocytes (KC).[1,2] Lesional AD skin is histologically characterized by dermal mononuclear infiltration and spongiosis in the epidermis. At the initial stages of inflammation, T_H2 cells migrate to the dermis, where they acquire a T_H0/T_H1 phenotype under the influence of IL-12, produced by antigen-presenting cells or activated keratinocytes.[3-5] These T_H0/T_H1 cells are characterized by the expression of Fas ligand (FasL) and secretion of significant amounts of the effector cytokines TNF-α and IFN-γ.[2,4,5] The secreted IFN-γ induces apoptosis of keratinocytes, leading eventually to the eczematous lesions characteristic of AD.[6,7] In response, keratinocytes upregulate the production of IFN-γ–inducible chemokines,[8] which in turn promotes the further infiltration of T cells into the epidermis, thereby augmenting the severity of inflammation and keratinocyte apoptosis.

After their initial discovery in the early 1970s, the concept of T-regulatory (Treg) cell–mediated immune suppression has been extensively explored. Two main groups of Treg cells have been defined. One comprises the natural Treg cells, which are characterized by their $CD4^+CD25^+$ phenotype. These cells have been suggested to develop under the control of the transcription factor FoxP3.[9] The other group of Treg cells, the adaptive Treg or T-regulatory type 1 (Tr1), are characterized by the secretion of high levels of IL-10 with or without TGF-β.[10-12] They develop in the periphery under the influence of presumably immature dendritic cells[13] and/or the presence of IL-10 and TGF-β, but also immunosuppressive drugs like glucocorticoids and vitamin D3,[14] and operate in a cytokine-mediated manner.

Most research on the inhibitory capacities of Treg cells has focused on their ability to suppress proliferation of effector T cells. It has been tempting to speculate that migration of increased numbers of Treg cells to the inflammation area, or the induction of their local proliferation, might be beneficial in the treatment of several

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inflammatory diseases, including allergy, autoimmunity, and transplantation rejection. Accordingly, we investigated the presence of Treg cells and their cytokines IL-10 and TGF-β, as well as their potential to suppress T-cell effector functions in AD skin. Here, we show that IL-10–secreting Tr1 cells, but not FoxP3$^+$CD4$^+$CD25$^+$ T cells, infiltrate lesional AD skin, demonstrating an imbalance in T-cell regulation in the affected organ.

METHODS

Subjects

PBMCs were isolated from peripheral blood of 15 healthy volunteers or patients with AD (aged 19-45 years) hypersensitive to house dust mite (HDM) or birch pollen allergens and then purified or cultured to provide the various types of T cells used in this study.

Twenty-four–hour positive atopy patch test (APT) biopsies were taken of 3 patients with AD at the University Medical Center Utrecht, The Netherlands, as previously described.[15] Three psoriasis biopsies were obtained from the ZAUM-Center for Allergy and Environment, Munich, Germany. Lesional skin biopsies were obtained from 3 patients with allergic contact dermatitis and 8 patients with AD diagnosed according to standard criteria[16] at the allergy unit of the department of dermatology, University of Zurich. Patients with AD and nonallergic form of dermatitis (NAD)[17] were included in the study. They did not receive any systemic therapy for at least 2 weeks before taking the biopsy. All studies were approved by the ethical commissions of the Canton of Graubunden, Switzerland, the Zurich University, Switzerland, the ZAUM, Munich, Germany, or the University Medical Center Utrecht, The Netherlands.

Purification and *in vitro* differentiation of T-cell subsets

Cytokine-secreting cells. PBMCs were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation of peripheral venous blood. Cells were washed 3 times and resuspended in RPMI 1640 medium supplemented as described.[12] Cells, 2.5 × 10^7, were stimulated with 0.3 μmol/L Der p 1 in 5 mL medium in 6-well plates (Corning-Costar Corp, Cambridge, United Kingdom). Secreting T cells were purified by immunomagnetic separation using the cytokine-secretion assay (AutoMacs; Miltenyi Biotec, Bergish Gladbach, Germany) for IL-4, IL-10, or IFN-γ as previously described.[12] Purified IL-4–secreting cells, IL-10–secreting cells, and IFN-γ–secreting cells were stimulated in complete culture medium with 1 nmol/L doses of growth factors: IL-2 (Roche, Basel, Switzerland) and IL-4 (Novartis, Basel, Switzerland), IL-2 and IL-15 (Peprotech, London, United Kingdom), and IL-2, respectively, and the following combination of mAbs to T-cell surface molecules: anti-CD2 (4B2 and 6G4, each 0.5 μg/mL), anti-CD3 (0.5 μg/mL), and anti-CD28 (0.5 μg/mL; all from CLB, Amsterdam, The Netherlands). Their cytokine profiles have been previously reported as T_H2-like, Tr1-like, and T_H1-like cells, respectively.[12]

CD4$^+$CD25$^+$ T cells. CD4$^+$CD25$^+$ T cells were purified from PBMCs of healthy donors by using the CD4$^+$CD25$^+$ regulatory T-cell isolation reagents (Miltenyi Biotec).[18] The expression of FoxP3 on CD4$^+$CD25$^+$ T cells was significantly higher than on CD4$^+$CD25$^-$ T cells, as previously reported.[19] Purified CD4$^+$CD25$^+$ T cells were cultured for 18 hours in complete RPMI 1640 medium containing IL-2 before being used in experiments.

T_H1 cells. CD45RA$^+$ T cells were purified by depletion of PBMCs using Pan T-cell isolation reagents (Miltenyi Biotec) and anti-CD45RO labeled microbeads (Miltenyi Biotec). Naive T cells (1 × 10^5 cells in 500 μL complete RPMI 1640, in 48-well plates; Corning-Costar Corp) were cultured with IL-2 (50 ng/mL), IL-12 (25 ng/mL), anti-CD2/3/28 mAb, and anti–IL-4 mAb (10 μg/mL) for 12 days to generate T_H1 cells.[20]

AD skin–derived T cells. T cells from the epidermis of lesional biopsies of patients with AD were isolated as previously described.[5] FoxP3 mRNA expression was analyzed as previously described.[19]

Keratinocyte cultures

Primary human keratinocytes (pooled normal human epidermal keratinocytes from neonatal skin) were purchased from BioWhittaker, Verviers, Belgium; PromoCell GmbH, Heidelberg, Germany; or Invitrogen, Basel, Switzerland, and grown in fully supplemented keratinocyte growth medium (KGM-2 bullet kit, BioWhittaker). During experiments, hydrocortisone was left out of the medium. Immortalized human HaCaT keratinocytes (a gift from Prof Dr N. E. Fusenig, Heidelberg, Germany) were grown in complete RPMI 1640 medium.

T-cell–keratinocyte cocultures

Keratinocytes were first seeded into 48-well or 96-well plates (Corning-Costar Corp) and were incubated to allow attachment and formation of a 75% to 90% confluent monolayer. After refreshing the medium, cytokines IFN-γ (Peprotech), soluble Fas ligand (Alexis Corp, Lausen, Switzerland), TNF-α (Alexis Corp; all 10 ng/mL), IL-10 (20 ng/mL; Peprotech), and TGF-β (2 ng/mL; R&D Systems Inc, Abingdon, United Kingdom) were used in different combinations. IL-10 and TGF-β were added at least 2 hours before if combined with other cells and/or cytokines. In cocultures with keratinocytes, T cells were incubated for only 24 hours and then washed away. If effector and IL-10–secreting Tr1 or CD4$^+$CD25$^+$ T cells were combined, regulatory cells were added at least 2 hours earlier. Of each type of T cell, 2.5 to 5 × 10^4 (96-well or 48-well plate, respectively) were used per well.

Artificial skin equivalents

Skin equivalents were cultured on dead de-epidermized dermis from human foreskin. A total of 4 × 10^5 third passage primary human keratinocytes were seeded onto the de-epidermized dermis and grown in an air-fluid interface for 10 days in modified Greens medium.[21] Fully differentiated artificial skin equivalents were grown for 4 days in a transwell system, while combinations of IFN-γ, soluble Fas ligand (sFasL), IL-10, and TGF-β were added directly to the medium below the insert. After 4 days, the skin pieces were snap-frozen in liquid nitrogen and embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands).

Viability and apoptosis detection

Keratinocyte viability was evaluated by means of ethidium bromide (25 μmol/L; Sigma Chemical Co, St Louis, Mo) exclusion and flow cytometry (EPICS XL-MCL flow cytometer; Coulter Corp, Hialeah, Fla). Hoechst staining was performed according to Norris et al[22] on cytospin samples and frozen sections of artificial skin equivalents. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining was performed on frozen sections of artificial skin equivalent by using the MEBSTAIN apoptosis kit II (MBL, Naka-ku Nagoya, Japan). Stained samples were mounted with Fluorescent Mounting Medium (DAKO, Glostrup, Denmark) and subsequently evaluated under an ultraviolet microscope (Axiovert 405M; Carl Zeiss AG, Feldbach, Switzerland).

Immunohistochemistry

Biopsy specimens were taken from lesions 3 to 6 days old and positive 24-hour APT of patients with AD, and from normal skin

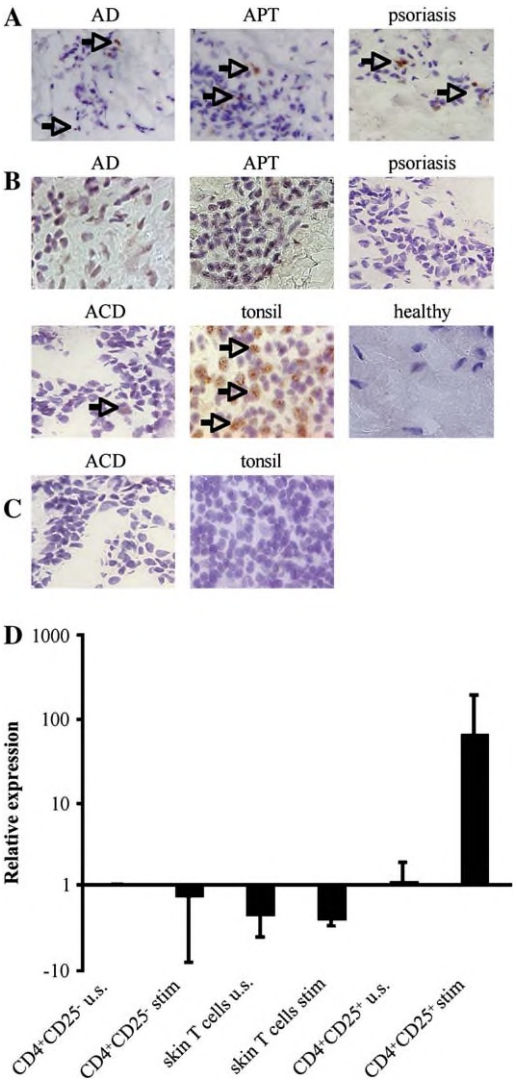

FIG 1. A, CD25 expression in the dermis of AD, APT, and psoriasis skin. **B,** FoxP3 staining in corresponding skin diseases (*ACD,* allergic contact dermatitis) and human tonsil. *Arrows* indicate positive cells. **C,** Blockage of FoxP3 staining with specific peptide. **D,** FoxP3 mRNA expression in skin T cells and blood $CD4^+CD25^+$ Treg cells relative to $CD4^+CD25^-$ T cells (*u.s.,* unstimulated; *stim,* anti-CD2, anti-CD3, and anti-CD28 mAbs stimulation for 2 hour). Same results obtained in ≥3 samples.

of healthy individuals. Frozen sections were stained by using the ready-to-use Vectastain Universal Elite Kit (Vector Laboratories, Burlingame, Calif). Primary antibodies were antihuman IL-10, antihuman TGFβ1 (both R&D Systems Inc), antihuman IL-10R1, antihuman TGFβRI, anti-human TGFβRII (all Santa Cruz Biotechnology Inc, Santa Cruz, Calif), antihuman FoxP3 ab10563 (Abcam, Cambridge, United Kingdom), antihuman CD25 (BD Biosciences Pharmingen, San Diego, Calif) and polyclonal rabbit or mouse IgG (Santa Cruz Biotechnology) as isotype controls. FoxP3 peptide ab14151 (0.125 μg/mL; Abcam) was used as a control to block anti-FoxP3 binding.

Statistical analysis

All data are expressed as means ± SDs. Statistical analysis was performed by using the Student *t* test and Mann Whitney *U* test for samples n < 6 (*$P < .05$).

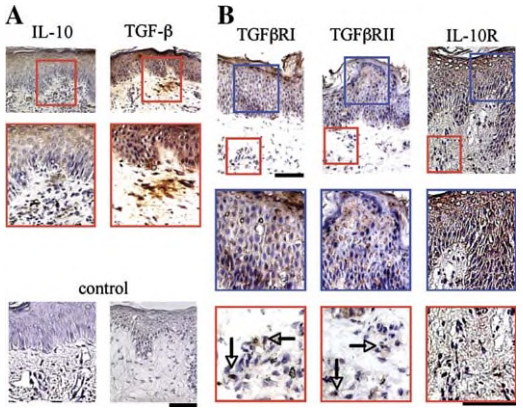

FIG 2. A, IL-10 and TGF-β expression in AD skin. **B,** TGFRI and TGFRII were expressed throughout the skin, IL-10R almost exclusively in the epidermis. *Arrows* indicate positive cells. *Highlighted* areas magnified below. Scale bar: 50 μm. Similar results obtained in ≥3 patients.

RESULTS

Expression of IL-10 and TGF-β as well as their receptors, but not FoxP3, in lesional AD skin

To investigate the expression and function of Treg cells in AD, we looked at whether the Tr1 cell-specific cytokines, IL-10 and TGF-β, or $CD4^+CD25^+$ Treg cell-specific transcription factor FoxP3 are expressed in lesional AD skin. Despite the presence of large numbers of $CD25^+$ cells (Fig 1, *A*), we did not detect any $FoxP3^+$ cells, neither in the dermal infiltrate of chronic lesional AD skin nor in acutely inflamed skin 24 hours after APT, psoriatic skin, or healthy skin (Fig 1, *B*). FoxP3 was detectable abundantly on T cells in the follicular area of the tonsil, and on approximately 1% of infiltrating T cells in allergic contact dermatitis skin by an exclusively nuclear staining. The binding of FoxP3 antibody could be blocked by preincubating the antibody with specific FoxP3 peptide (Fig 1, *C*). Analysis of mRNA levels confirmed that T cells isolated from AD skin express very low levels of FoxP3, comparable to $CD4^+CD25^-$ T cells. $CD4^+CD25^+$ Treg cells express relatively high levels of FoxP3 mRNA, which are upregulated on activation (Fig 1, *D*). Both IL-10 and TGF-β were abundantly present throughout epidermal keratinocyte layers as well as in dermal mononuclear cell infiltrate of affected skin (Fig 2, *A*). TGFβRI and TGFβRII were highly expressed in the epidermis and dermis of affected skin, whereas IL-10R was expressed in the whole epidermal layers, but only on a few cells in the dermal infiltrate (Fig 2, *B*).

IL-4–secreting, IL-10–secreting, and IFN-γ–secreting T cells are present in both AD and NAD skin lesions

Next, we isolated and characterized T cells from lesional skin biopsies of 4 patients with AD and 4 patients with NAD. Cytokine patterns determined by ELISA

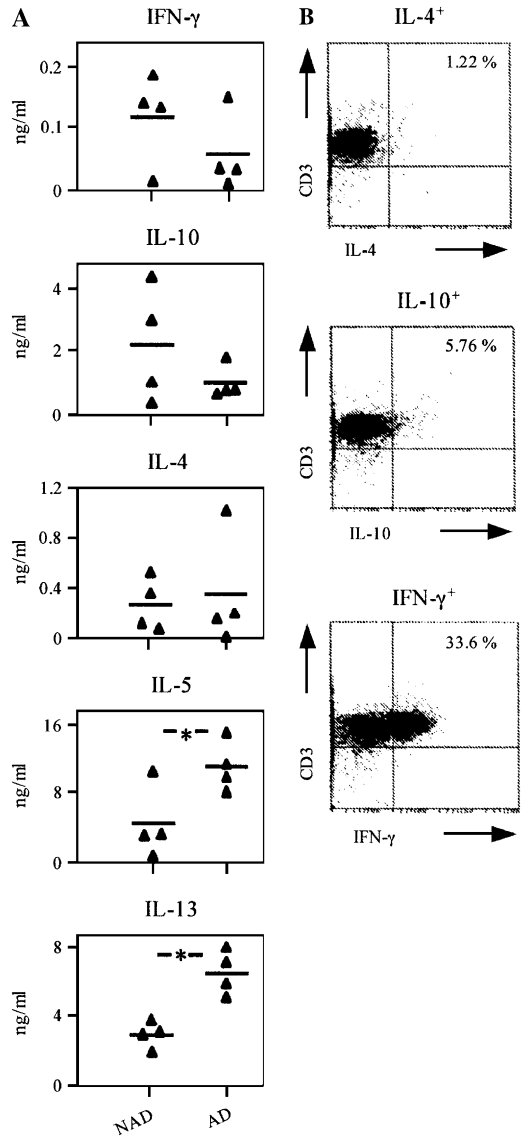

FIG 3. Cytokine profile of T cells isolated from AD skin. **A,** T cells isolated from AD and NAD skin restimulated with anti-CD2, anti-CD3, and anti-CD28 mAbs. Cytokines were measured after 72 hours in supernatants, by ELISA. *$P < .005$). **B,** Skin T cells were stimulated for 48 hours with anti-CD2, anti-CD3, and anti-CD28 antibodies. IL-4–secreting, IL-10–secreting, and IFN-γ–secreting $CD3^+$ T cells were demonstrated.

showed a significant increase in IL-5 and IL-13 in AD. IL-10, IFN-γ, and IL-4 secretion did not differ significantly between the 2 types of AD (Fig 3, *A*). In either type of AD, both IFN-γ as an effector cytokine and IL-10 as a regulatory cytokine were detectable. A quantitative determination of IL-4–secreting, IL-10–secreting, and IFN-γ–secreting T cells was possible by capturing the secreted cytokine on the surface of the T cell. The majority of T cells isolated from AD skin were IFN-γ–secreting cells. A considerable percentage of IL-10–secreting cells, but only a minor fraction of IL-4–secreting cells, were found among skin T cells (Fig 3, *B*).

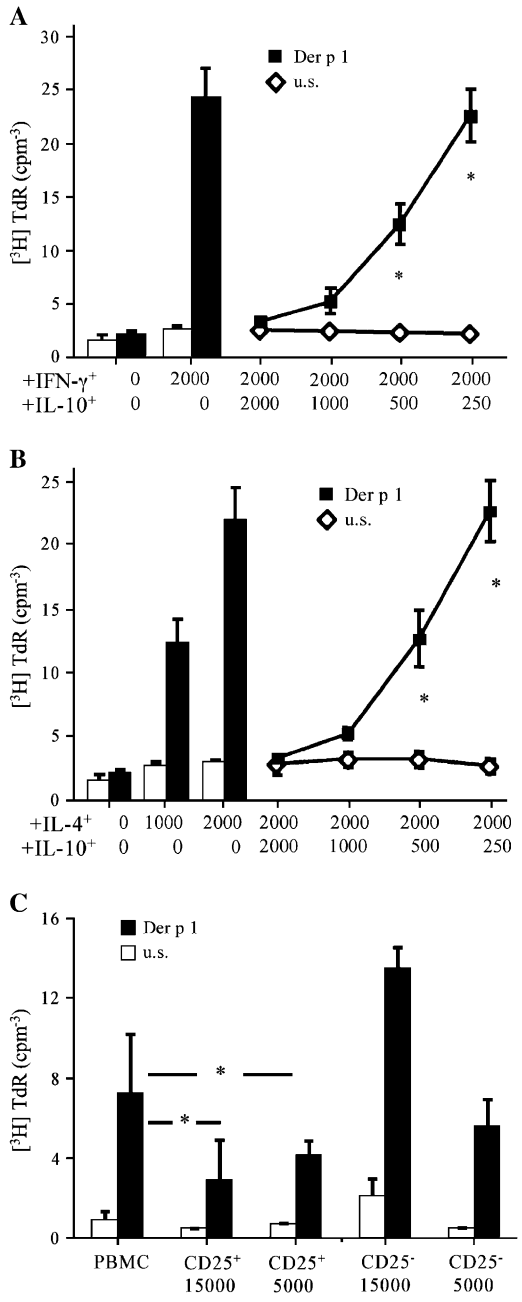

FIG 4. Tr1 and $CD4^+CD25^+$ T cells suppress allergen-specific T cells. Der p 1–specific IFN-γ–secreting, IL-4–secreting, and IL-10–secreting T cells were purified from peripheral blood. IFN-γ–secreting **(A)** and IL-4–secreting **(B)** T cells were added to PBMCs and stimulated with Der p 1 in the absence or presence of IL-10–secreting Tr1 cells. One experiment representative of 8 is shown. **C,** $CD4^+CD25^+$ T cells suppress the Der p 1–induced proliferation of PBMCs of donors allergic to HDM (n = 3). *u.s.,* Unstimulated. [^{3}H] Thymidine (TdR) incorporation determined after 5 days. *$P < .05$.

Allergen-specific IL-10–secreting Tr1 cells and $CD4^+CD25^+$ Treg cells inhibit activation of allergen-specific effector T cells

To establish the immune regulatory capacity of the IL-10–secreting Tr1-like cells used in this study, we

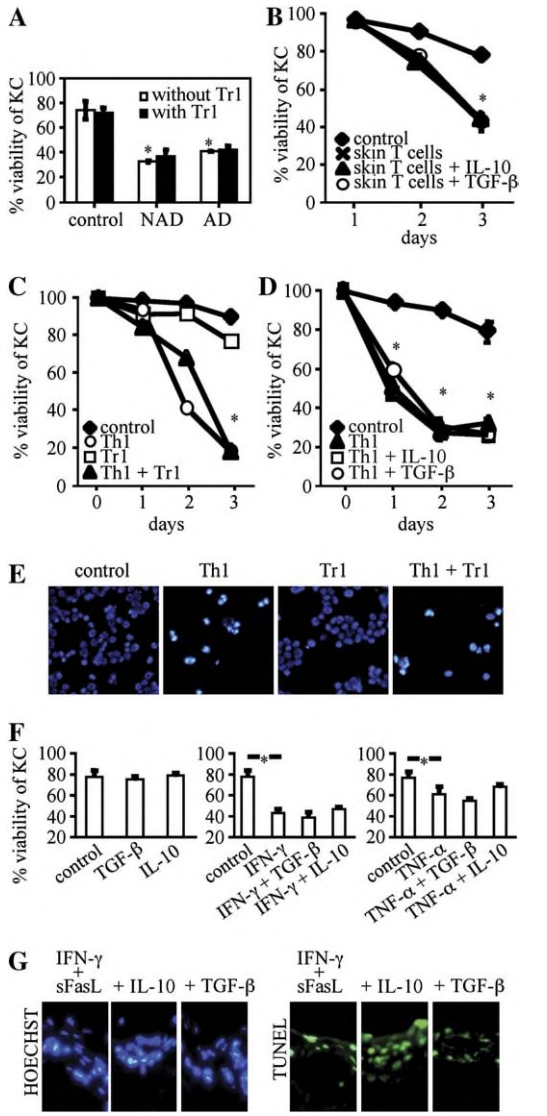

FIG 5. IL-10, TGF-β, and Tr1 cells do not influence keratinocyte (KC) apoptosis. **A,** Viability of KCs 3 days after coculture with T cells isolated from AD or NAD skin. IL-10–secreting Tr1 cells were added in equal amounts to skin T cells. **B,** IL-10 and TGF-β do not influence skin T-cell–induced KC death. **C,** KCs cocultured with Tr1 cells and T_H1 cells. **D,** IL-10 and TGF-β do not influence T_H1 cell–induced KC death. **E,** Staining of KCs 3 days after coculture with T_H1 cells and/or IL-10–secreting Tr1 cells with Hoechst 33342. **F,** KCs stimulated for 3 days with IFN-γ, TNF-α, IL-10, and TGF-β. *(A-F)* One experiment representative of 3 is shown. Viability determined by ethidium bromide exclusion. **G,** Artificial skin equivalents were cultured with IFN-γ and sFasL for 4 days, in the presence or absence of IL-10 (10 ng/mL) and TGF-β (2 ng/mL). $*P < .05$. *TUNEL,* Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

examined their effect on the antigen-specific activation of IL-4–secreting T_H2-like and IFN-γ–secreting T_H1-like T cells. Freshly purified T_H cells selected for their specificity against the HDM allergen Der p 1 showed increased proliferation upon encounter with this allergen when added to autologous PBMCs (Fig 4, *A and B*). An equal amount of IL-10–secreting Tr1 cells to that of IL-4–

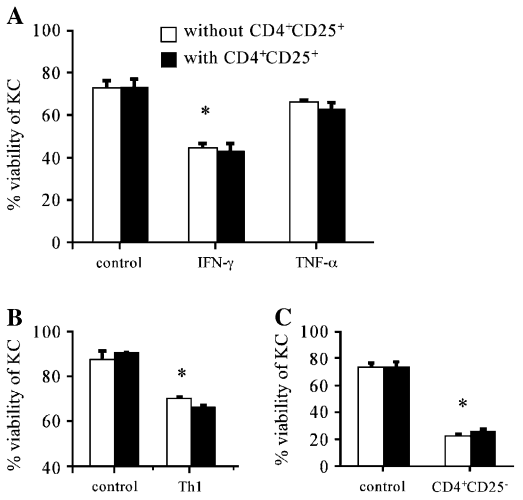

FIG 6. $CD4^+CD25^+$ Treg cells do not affect keratinocyte (KC) death induced by **(A)** IFN-γ and sFasL (72 hours), **(B)** *in vitro*–differentiated T_H1 cells (48 hours), or **(C)** $CD4^+CD25^-$ T cells, preactivated for 48 hours. Viability was measured by ethidium bromide exclusion 48 to 72 hours after coculture. Experiments were performed at least twice in triplicate cultures. $*P < .05$.

secreting or IFN-γ–secreting cells nearly abolished this response. This shows that IL-10–secreting Tr1 cells can suppress allergen-specific activation of both T_H2-like and T_H1-like cells. Similarly, $CD4^+CD25^+$ T cells showed significant suppression of Der p 1–specific proliferation of PBMCs from donors allergic to HDM (Fig 4, *C*).

Tr1 cells, their cytokines IL-10 and TGF-β, and $CD4^+CD25^+$ Treg cells do not suppress T-cell–induced keratinocyte apoptosis

T-cell–induced keratinocyte apoptosis plays an essential role in the development of eczematous lesions in AD. Accordingly, we investigated whether Tr1 cells or their suppressive cytokines IL-10 and TGF-β can suppress skin T-cell–induced, *in vitro* differentiated T_H1 cell–induced, or IFN-γ and sFasL–induced keratinocyte apoptosis in cocultures and artificial skin equivalents. Keratinocyte death induced by skin T cells did not show any difference between T cells isolated from AD or NAD biopsies. In both cases, keratinocyte death induced by preactivated skin T cells was not prevented by the addition of IL-10–secreting Tr1 cells in coculture (Fig 5, *A*). Moreover, skin T-cell–induced keratinocyte death was not blocked by the addition of IL-10 or TGF-β (Fig 5, *B*).

The suppressive capacity of IL-10–secreting Tr1 cells on IL-12–driven T_H1 cell–induced keratinocyte apoptosis was further analyzed in cocultures with human keratinocytes. IL-10–secreting Tr1 cells did not affect keratinocyte viability alone, nor did they suppress T_H1 cell–induced keratinocyte death in cocultures (Fig 5, *C*). Addition of IL-10 or TGF-β to keratinocytes during coculture again did not suppress T_H1-induced keratinocyte death (Fig 5, *D*). Bright, condensed, and fragmented staining of keratinocyte nuclei with Hoechst 33342 dye, 3 days after coculture with T_H1 cells, further confirmed these findings

and suggested that cell death was in the form of apoptosis, which was not inhibited by IL-10–secreting Tr1 cells (Fig 5, *E*).

Because direct T-cell–keratinocyte contact is not essential for the pathology of AD, we performed the same experiments based solely on cytokines. Keratinocytes were cultured with the effector cytokines IFN-γ and TNF-α. Again, a substantial reduction in the viability of keratinocytes was observed, mainly after culture with IFN-γ and to a lesser extent with TNF-α after 3 days. As in the experiments with T_H1 cells, the induced keratinocyte death was not suppressed by IL-10 and TGF-β (Fig 5, *F*). Similar results were obtained with HaCaT and primary human keratinocytes.

The differentiation status of keratinocytes was hypothesized to play a role in the observations with monolayer cell cultures. Accordingly, the effects of the aforementioned cytokines were studied in a model of artificial skin equivalents in a 3-dimensional structure that involves primary human keratinocytes, dermal fibrocytes, and extracellular matrix proteins. IFN-γ and sFasL induced severe cell death throughout the epidermis of skin equivalents after 4 days (Fig 5, *G*). Similar to monolayer keratinocyte cultures, IL-10 or TGF-β did not suppress the apoptosis of keratinocytes in artificial skin equivalents.

A direct interaction of $CD4^+CD25^+$ Treg cells and keratinocytes was investigated by the addition of $CD4^+CD25^+$ Treg cells to TNF-α–stimulated or IFN-γ–stimulated keratinocytes. The reduced viability of keratinocytes observed after 3 days was not affected by $CD4^+CD25^+$ Treg cells, similar to the findings with IL-10–secreting Tr1 cells (Fig 6, *A*). Like Tr1 cells, $CD4^+CD25^+$ Treg cells did not induce keratinocyte apoptosis. To assess whether $CD4^+CD25^+$ Treg cells suppress T_H1-induced or $CD4^+CD25^-$ T-cell–induced keratinocyte apoptosis, $CD4^+CD25^+$ Treg cells were added to a coculture of T_H1 or $CD4^+CD25^-$ T cells with keratinocytes. In both cases, $CD4^+CD25^+$ Treg cells did not inhibit keratinocyte death (Fig 6, *B and C*).

DISCUSSION

During the last decade, a significant amount of data has accumulated on the suppressive effects of Tr1 or $CD4^+CD25^+$ T-regulatory cells in models of autoimmunity, allergy, transplantation tolerance, tumor tolerance, and chronic infections.[12,23] The efficacy of various Treg cell subsets in the suppression of inflammation has tempted scientists to speculate that increasing Treg cell numbers may suppress inflammation and tissue injury in affected organs. In the current study, we show that $FoxP3^+CD25^+$ T cells are not present in AD skin, whereas Tr1 cells, their suppressive cytokines IL-10 and TGF-β, and receptors for these cytokines are abundantly expressed. Both $CD4^+CD25^+$ T regulatory cells and Tr1 cells can efficiently suppress activation of T_H1 and T_H2 cells stimulated with allergen/antigen. However, the effector function of preactivated T cells, namely keratinocyte apoptosis, is not affected either by $CD4^+CD25^+$ T cells or Tr1 cells and their suppressive cytokines IL-10 and TGF-β.

One difficulty in comparing the results of different studies with Treg cells is the variation in regulatory cell types. The IL-10–secreting Tr1 cells used in this study were selected for their allergen-induced IL-10 secretion as previously described.[12] $CD4^+CD25^+$ T cells were isolated from peripheral blood of healthy and donors allergic to HDM. Most studies with IL-10–secreting Tr1 cells and $CD4^+CD25^+$ T cells have focused on their ability to inhibit proliferation of responder cells. Here, we demonstrate that IL-10–secreting Tr1 cells can inhibit the allergen-specific proliferation of IL-4–secreting T_H2 as well as of IFN-γ–secreting T_H1 cells. In addition, a considerable percentage of T cells isolated from lesional AD skin are IL-10–secreting cells. Furthermore, we showed the presence of Tr1 cytokines, IL-10 and TGF-β, and their receptors in biopsies of AD skin. Supporting these findings, overexpression of IL-10 was previously described in AD,[24] and all isoforms of TGF-β have been described to be expressed in nonaffected skin, with an upregulation during wound repair.[25]

Previously, $CD4^+CD25^+$ Treg cells and $CLA^+CD4^+CD25^+$ T cells have been demonstrated to be elevated in peripheral blood of patients with AD compared with healthy controls or patients with asthma.[26] Although we found a high amount of $CD25^+$ cells in the dermal infiltrate of AD skin, we did not detect any FoxP3 expression. This shows that these $CD25^+$ cells present in the skin are activated T cells and not regulatory $CD4^+CD25^+$ T cells. Similarly, circulating CLA^+CD4^+ or $CD8^+$ T cells have been demonstrated to express CD25 highly and display effector functions by inducing IgE production by B cells and prolonged survival of eosinophils.[26] Mutations in the FoxP3 gene have previously been reported to play a critical role in the onset of immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome, an X-linked recessive immunological disorder. This rare disease is often associated with eczema (4 out of 5 patients in 1 study) and high levels of IgE.[27] Together, these data suggest that there might be an essential role for $CD4^+CD25^+FoxP3^+$ Treg cells in controlling inflammation of the skin, a system apparently malfunctioning in AD.

Both IL-10R and TGF-βR have been described in the epidermis of healthy skin, with a marked upregulation of the latter during wound repair.[28,29] In contrast to psoriasis,[28] IL-10R was abundantly expressed throughout affected epidermis in AD. Although no effect of IL-10 on keratinocytes has previously been reported, this expression of IL-10R suggests that there might be a role for IL-10 in the control of keratinocyte death in AD. Accordingly, we examined the role of IL-10–secreting Tr1 and $CD4^+CD25^+$ Treg cells in the control of keratinocyte apoptosis on both a cell-to-cell contact and a cytokine-mediated level. We found that T cells isolated from lesional skin of patients with AD induced apoptosis of keratinocytes, despite the presence of a considerable amount

of IL-10–secreting Tr1 cells. The addition of exogenous IL-10–secreting Tr1 cells isolated from peripheral blood of healthy donors did not prevent the apoptosis of keratinocytes in these cocultures. A defect in regulation has been suggested as a determinant in the ongoing effector functions of AD skin T cells. Superantigens, present in the skin of more than 90% of patients with AD, can induce strong proliferation of $CD4^+$ and $CD8^+$ T cells in the skin.[30] Strong binding of superantigens to the TCR in conjunction with CD28 costimulation[31] was shown to render T cells insensitive to suppression by $CD4^+CD25^+$ Treg cells[32] and IL-10.[33] Superantigens also abrogate immune suppression by corticosteroids,[34] which operate via the induction of Treg cells[19] This provides a possible explanation for the absence of inhibition of skin T-cell–induced keratinocyte apoptosis by Tr1 or $CD4^+CD25^+$ Treg cells. To exclude this phenomenon, the same experiments were repeated with Th1 cells and $CD4^+CD25^-$ T cells from healthy donors. The apoptosis induction by these cell types was comparable to that seen with skin T cells, but Tr1 and $CD4^+CD25^+$ Treg cells did not induce keratinocyte apoptosis. Because an inhibitory effect might be cell contact–independent and cytokine levels secreted by T cells may vary, similar experiments were performed with effector cytokines such as TNF-α and IFN-γ and suppressor cytokines like IL-10 and TGF-β. Again, no inhibitory effect of IL-10 and TGF-β was seen on IFN-γ–induced or TNF-α–induced apoptosis.

In perspective, 4 distinct stages play an important role in allergic inflammation of the skin. The first is the activation of T cells by allergens or superantigens, followed by organ-selective homing, whereby cells are influenced by the network of chemokines in the skin.[2,35] The third stage is classified by prolonged survival of inflammatory cells within the inflamed skin and reactivation, by allergens and/or superantigens. Finally, the effector role of T cells in the skin is characterized by the induction of keratinocyte apoptosis and development of spongiosis, all of which are important factors in AD. Thus, regulatory T cells of either Tr1 or $CD4^+CD25^+$ Treg phenotype can suppress antigen-specific activation of T cells (stage 1 and stage 3), but they cannot prevent activated effector T-cell–induced keratinocyte apoptosis (stage 4). In addition, taken together with AD and hyper-IgE in the phenotype of immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome, the absent expression of $CD4^+CD25^+FoxP3^+$ Treg cells in AD and psoriasis skin suggests a dysregulated control of inflammation, particularly by natural Treg cells.

REFERENCES

1. Leung DYM, Boguniewicz M, Howell MD, Nomura I, Hamid QA. New insights into atopic dermatitis. J Clin Invest 2004;113:651-7.
2. Akdis CA, Blaser K, Akdis M. Apoptosis in tissue inflammation and allergic disease. Curr Opin Immunol 2004;16:717-23.
3. Muller G, Saloga J, Germann T, Bellinghausen I, Mohamadzadeh M, Knop J, et al. Identification and induction of human keratinocyte-derived IL-12. J Clin Invest 1994;94:1799-805.
4. Thepen T, Langeveld-Wildschut EG, Bihari IC, van Wichen DF, van Reijsen FC, Mudde GC, et al. Biphasic response against aeroallergen in atopic dermatitis showing a switch from an initial TH2 response to a TH1 response in situ: an immunocytochemical study. J Allergy Clin Immunol 1996;97:828-37.
5. Akdis CA, Akdis M, Simon D, Dibbert B, Weber M, Gratzl S, et al. T cells and T cell-derived cytokines as pathogenic factors in the nonallergic form of atopic dermatitis. J Invest Dermatol 1999;113:628-34.
6. Trautmann A, Akdis M, Kleemann D, Altznauer F, Simon HU, Graeve T, et al. T cell-mediated Fas-induced keratinocyte apoptosis plays a key pathogenetic role in eczematous dermatitis. J Clin Invest 2000;106: 25-35.
7. Trautmann A, Akdis M, Schmid-Grendelmeier P, Disch R, Brocker EB, Blaser K, et al. Targeting keratinocyte apoptosis in the treatment of atopic dermatitis and allergic contact dermatitis. J Allergy Clin Immunol 2001;108:839-46.
8. Klunker S, Trautmann A, Akdis M, Verhagen J, Schmid-Grendelmeier P, Blaser K, et al. A second step of chemotaxis after transendothelial migration: keratinocytes undergoing apoptosis release IFN-gamma-inducible protein 10, monokine induced by IFN-gamma, and IFN-gamma-inducible alpha-chemoattractant for T cell chemotaxis toward epidermis in atopic dermatitis. J Immunol 2003;171:1078-84.
9. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057-61.
10. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997;389:737-42.
11. Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. J Clin Invest 1998;102:98-106.
12. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med 2004;199:1567-75.
13. Roncarolo MG, Levings MK, Traversari C. Differentiation of T regulatory cells by immature dendritic cells. J Exp Med 2001;193:F5-9.
14. Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, et al. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. J Exp Med 2002;195:603-16.
15. Langeveld-Wildschut EG, van Marion AM, Thepen T, Mudde GC, Bruijnzeel PL, Bruijnzeel-Koomen CA. Evaluation of variables influencing the outcome of the atopy patch test. J Allergy Clin Immunol 1995;96: 66-73.
16. Hanifin JM. Atopic dermatitis. J Am Acad Dermatol 1982;6:1-13.
17. Schmid-Grendelmeier P, Simon D, Simon HU, Akdis CA, Wuthrich B. Epidemiology, clinical features, and immunology of the ''intrinsic'' (non-IgE-mediated) type of atopic dermatitis (constitutional dermatitis). Allergy 2001;56:841-9.
18. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszcz M, Blaser K, et al. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol 2003;33:1205-14.
19. Karagiannidis C, Akdis M, Holopainen P, Woolley NJ, Hense G, Ruckert B, et al. Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. J Allergy Clin Immunol 2004;114:1425-33.
20. Sallusto F, Mackay CR, Lanzavecchia A. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. Science 1997;277: 2005-7.
21. Traidl C, Sebastiani S, Albanesi C, Merk HF, Puddu P, Girolomoni G, et al. Disparate cytotoxic activity of nickel-specific CD8+ and CD4+ T cell subsets against keratinocytes. J Immunol 2000;165:3058-64.
22. Norris DA, Middleton MH, Whang K, Schleicher M, McGovern T, Bennion SD, et al. Human keratinocytes maintain reversible anti-apoptotic defenses in vivo and in vitro. Apoptosis 1997;2:136-48.
23. Taylor A, Verhagen J, Akdis CA, Akdis M. T regulatory cells in allergy and health: a question of allergen specificity and balance. Int Arch Allergy Immunol 2004;135:73-82.
24. Ohmen JD, Hanifin JM, Nickoloff BJ, Rea TH, Wyzykowski R, Kim J, et al. Overexpression of IL-10 in atopic dermatitis: contrasting cytokine patterns with delayed-type hypersensitivity reactions. J Immunol 1995; 154:1956-63.
25. Frank S, Madlener M, Werner S. Transforming growth factors beta1, beta2, and beta3 and their receptors are differentially regulated during

normal and impaired wound healing. J Biol Chem 1996;271: 10188-93.
26. Akdis M, Akdis CA, Weigl L, Disch R, Blaser K. Skin-homing, CLA+ memory T cells are activated in atopic dermatitis and regulate IgE by an IL-13-dominated cytokine pattern: IgG4 counter-regulation by CLA-memory T cells. J Immunol 1997;159:4611-9.
27. Chatila TA. Role of regulatory T cells in human diseases. J Allergy Clin Immunol 2005;116:949-59.
28. Michel G, Mirmohammadsadegh A, Olasz E, Jarzebska-Deussen B, Muschen A, Kemeny L, et al. Demonstration and functional analysis of IL-10 receptors in human epidermal cells: decreased expression in psoriatic skin, down-modulation by IL-8, and up-regulation by an anti-psoriatic glucocorticosteroid in normal cultured keratinocytes. J Immunol 1997;159:6291-7.
29. Gold LI, Sung JJ, Siebert JW, Longaker MT. Type I (RI) and type II (RII) receptors for transforming growth factor-beta isoforms are expressed subsequent to transforming growth factor-beta ligands during excisional wound repair. Am J Pathol 1997;150:209-22.
30. Kappler J, Kotzin B, Herron L, Gelfand EW, Bigler RD, Boylston A, et al. V beta-specific stimulation of human T cells by staphylococcal toxins. Science 1989;244:811-3.
31. Saha B, Harlan DM, Lee KP, June CH, Abe R. Protection against lethal toxic shock by targeted disruption of the CD28 gene. J Exp Med 1996; 183:2675-80.
32. Ou LS, Goleva E, Hall C, Leung DY. T regulatory cells in atopic dermatitis and subversion of their activity by superantigens. J Allergy Clin Immunol 2004;113:756-63.
33. Joss A, Akdis M, Faith A, Blaser K, Akdis CA. IL-10 directly acts on T cells by specifically altering the CD28 co-stimulation pathway. Eur J Immunol 2000;30:1683-90.
34. Hauk PJ, Hamid QA, Chrousos GP, Leung DY. Induction of corticosteroid insensitivity in human PBMCs by microbial superantigens. J Allergy Clin Immunol 2000;105:782-7.
35. Sicherer SH, Leung DYM. Advances in allergic skin disease, anaphylaxis, and hypersensitivity reactions to foods, drugs and insects. J Allergy Clin Immunol 2005;116:153-63.