

T regulatory cells in atopic dermatitis and subversion of their activity by superantigens

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Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease involving colonization by superantigen (SAg)—secreting *Staphylococcus aureus*. CD4⁺CD25⁺ T regulatory (Treg) cells are thought to play an important role in controlling inflammatory responses.

Objective: In this study we examined whether Treg cells might be deficient in patients with AD.

Methods: CD4⁺CD25⁺ and CD4⁺CD25[−] T cells were isolated from PBMCs by using immunomagnetic beads. Cells were cultured with anti-CD3 or SAg, staphylococcal enterotoxin B (SEB), for 72 hours. Proliferation was measured by means of tritiated thymidine incorporation. CD4, CD8, CD25, and cutaneous lymphocyte—associated antigen expression on PBMCs was assessed by means of flow cytometry. RNA was extracted from isolated subsets of T cells, and the results of real-time PCR for FoxP3 mRNA were determined.

Results: Surprisingly, CD4⁺CD25⁺ T cells were significantly ($P < .01$) increased in patients with AD ($6.68\% \pm 0.99\%$, $n = 15$) compared with in asthmatic patients ($3.42\% \pm 0.58\%$, $n = 12$) or nonatopic healthy control subjects ($3.34\% \pm 0.43\%$, $n = 14$). Patients with AD also had a higher expression of CD25⁺ in skin-homing, CD4⁺, cutaneous lymphocyte—associated antigen—positive T cells than asthmatic and nonatopic subjects, with values of 35.95% versus 22.44% versus 23.03%, respectively ($P < .006$). Only CD4⁺CD25⁺ cells expressed FoxP3, whereas CD4⁺CD25[−] T cells and CD4[−] cells did not. Consistent with known properties of Treg cells, CD4⁺CD25⁺ cells were anergic to anti-CD3 stimulation. When CD4⁺CD25⁺ cells from each study group were mixed with CD4⁺CD25[−] cells, proliferative responses were equally suppressed after anti-CD3 stimulation. In contrast, after SEB stimulation, CD4⁺CD25⁺ cells were no longer anergic. Furthermore, when CD4⁺CD25⁺ cells were mixed with CD4⁺CD25[−] cells and stimulated with SEB, the suppressive function of Treg cells was reversed.

Conclusion: Patients with AD have significantly increased

numbers of peripheral blood Treg cells with normal immunosuppressive activity. However, after SAg stimulation, Treg cells lose their immunosuppressive activity. These data suggest a novel mechanism by which SAg could augment T-cell activation in patients with AD. (J Allergy Clin Immunol 2004;113:756-63.)

Key words: T regulatory cell, atopic dermatitis, superantigen

Atopic dermatitis (AD) is a highly pruritic, chronic inflammatory skin disease.¹ More than 90% of patients with AD have *Staphylococcus aureus* colonization of their skin lesions.^{2,3} An important mechanism by which *S aureus* contributes to skin inflammation is the secretion of toxins known as superantigens (SAg).⁴ In human subjects more than 90% of the skin-infiltrating T cells express cutaneous lymphocyte—associated antigen (CLA), which acts as a skin-homing receptor.⁵⁻⁹ In addition, it has been demonstrated that peripheral blood CLA⁺ T cells of patients with AD express T_H2 cytokines,¹⁰⁻¹² thus mirroring the cytokine expression seen in early AD skin lesions.

In both mice and human subjects, CD4⁺CD25⁺ T regulatory (Treg) cells constitute 5% to 15% of peripheral CD4⁺ T cells.^{13,14} They can suppress immune responses through direct cell-cell contact in a process that is dependent on signaling through cytotoxic T-lymphocyte antigen 4 (CTLA-4), as well as secretion of TGF- β and IL-10.¹⁵ The forkhead-winged helix transcription factor gene FoxP3 is specifically expressed by CD4⁺CD25⁺ Treg cells and programs their development and function.^{16,17} Human CD4⁺CD25⁺ Treg cells have been identified in peripheral blood and thymus.¹⁸⁻²⁴ These cells are anergic and strongly suppress the proliferation responses to alloantigens or polyclonal activation with anti-CD3 stimulation. In mouse studies adoptive transfer of these Treg cells reduces the pathology of experimentally induced autoimmune diseases, such as thyroiditis, gastritis, insulin-dependent diabetes mellitus, and colitis.²⁵⁻²⁸ CD4⁺CD25⁺ Treg cells have been shown to inhibit the development of airway eosinophilia in animal models of asthma.²⁹⁻³² Patients with XLAAD/IPEX disease³³ specifically lack CD4⁺CD25⁺ Treg cells and have severe eczema, increased IgE levels and eosinophil counts, and food allergy. However, it is not known whether CD4⁺CD25⁺ Treg cells also play an important role in human allergic diseases, such as AD.

These studies prompted us to ask whether patients with AD have low numbers of CD4⁺CD25⁺ Treg cells or

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Abbreviations used

AD: Atopic dermatitis
APC: Antigen-presenting cell
CLA: Cutaneous lymphocyte-associated antigen
CTLA-4: Cytotoxic T-lymphocyte antigen 4
PE: Phycoerythrin
PerCP: Peridinin chlorophyll protein
SAg: Superantigen
SEB: Staphylococcal enterotoxin B
Treg cells: T regulatory cells

a deficient function of Treg cells. An advantage of studying AD is that the phenotype of peripheral blood CLA⁺ T cells reflects the T cells that infiltrate into the skin of patients with AD. Therefore we examined the expression of CLA marker within the Treg cell population as well. Furthermore, because the skin of patients with AD is colonized with SAg-producing *S aureus*, we studied the effect of SAg on CD4⁺CD25⁺ Treg cells.

METHODS

Patients and control subjects

Fifteen adult patients with moderate-to-severe AD (skin involvement of 15% to 80%) were studied. The mean age of the AD group was 42 years (range, 20-62 years; $n = 15$). Their diagnosis was based on the criteria of Hanifin and Rajka.³⁴ None of the patients with AD had received any systemic corticosteroids previously. All patients were no longer taking topical corticosteroids for at least 1 week before their blood drawing. Two age-matched control groups were involved in this study: 14 healthy subjects (age range, 23-60 years; mean, 37 years) with no history of AD, allergic rhinitis, or asthma and 12 patients with asthma (age range, 21-50 years; mean, 36 years). Asthmatic patients were not receiving any systemic treatment and were taking less than 800 μg per day of inhaled steroid. This study was approved by the Institutional Review Board at National Jewish Medical and Research Center. Informed consent was obtained from all subjects before the study.

Antibodies

All the following mAbs to human cell-surface molecules were purchased from Becton-Dickinson Immunocytometry Systems (San Jose, Calif): peridinin chlorophyll protein (PerCP)—conjugated anti-CD4, PerCP-conjugated anti-CD8, phycoerythrin (PE)—conjugated anti-CD69, PE-conjugated anti-HLA-DR, allophycocyanin-conjugated anti-CD25, and FITC-conjugated anti-CLA HECA-452. PerCP-conjugated mouse IgG1, allophycocyanin-conjugated mouse IgG1, PE-conjugated mouse IgG2, and FITC-conjugated rat IgM isotype controls were used.

Preparation of PBMCs

Heparinized venous blood from each donor was layered on a Ficoll-Plaque density gradient (Amersham Pharmacia Biotech) and centrifuged for 25 minutes at 400g at room temperature. The PBMC layer was collected, washed, and resuspended in HBSS (GIBCO BRL Life Technologies). The cell viability was greater than 95% viable, as determined by using a trypan blue exclusion assay.

Purification of CD4⁺CD25⁺ Treg cells

Human PBMCs were suspended in RPMI 1640 media (Bio-Whittaker) containing 10% heat-inactivated FCS, 40 $\mu\text{mol/L}$ L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 20

mmol/L HEPES buffer solution (GIBCO BRL Life Technologies). CD4⁺ T cells were purified by means of negative selection with immunomagnetic beads (by depletion of CD8⁺, CD11b⁺, CD16⁺, CD19⁺, CD36⁺, and CD56⁺ cells) with the Untouched CD4⁺ T cell isolation kit (Miltenyi Biotec). The purity of CD4⁺ cells was usually greater than 95%. Then CD4⁺CD25⁺ T cells and CD4⁺CD25⁻ T cells were separated by means of positive selection with immunomagnetic beads with a CD4⁺CD25⁺ T-cell isolation kit (Miltenyi Biotec). Starting with 1×10^8 PBMCs, typically 0.8 to 1.2×10^6 CD4⁺CD25⁺ T cells were isolated, with a purity range from 96% to 99%. The purity of CD4⁺CD25⁻ cells was always more than 90%. All steps were performed according to the manufacturer's instructions.

Proliferation and suppression of T cells

To analyze the T-cell proliferative capacity, CD4⁺, CD4⁺CD25⁺, and CD4⁺CD25⁻ T cells were plated at 5×10^4 cells per well with antigen-presenting cells (APCs; 5×10^4 irradiated 3000 rad PBMCs) in 96-well, round-bottom culture plates (Nunc, Nunc Brand Products) and stimulated with 0.5 $\mu\text{g/mL}$ anti-CD3 mAbs (OKT3, Orthoclone) or different concentrations (1 pg/mL to 100 ng/mL) of staphylococcal enterotoxin B (SEB; Toxin Technology) in a final volume of 200 μL of complete medium. CD4⁺CD25⁻ cells (5×10^4) were cultured with irradiated APCs (5×10^4) and indicated numbers of CD4⁺CD25⁺ T cells to analyze the suppressive capacity of CD4⁺CD25⁺ T cells. Cultures were incubated for 72 hours at 37°C in a humidified, 5% CO₂-containing incubator. Eighteen hours before harvesting, tritiated thymidine (ICN Biomedicals) was added at a final concentration of 1 μCi per well. Cells were then harvested (Tomtec Harvester 96) onto glass fiber filters (Wallac Oy) and placed into a liquid scintillation cocktail (Wallac, Milton Keynes), and incorporated tritium was counted in a liquid scintillation counter (1450 Microbeta Plus counter). For all proliferation assays, the experimental values reported represent the means of triplicate determinations.

RNA extraction and quantitative real-time PCR for FoxP3

Isolated subsets of T cells were placed into 350 μL of Buffer RLT (Qiagen RNeasy mini-kit). Total RNA was then extracted according to the manufacturer's protocol in the presence of DNase I (Qiagen). The primers and probes used in the real-time PCR of FoxP3 were selected with Primer Express software (Perkin-Elmer Applied Biosystems) as follows: forward, 5'-GAGAAGCTGAGTGCC ATGCA-3'; reverse, 5'-GGAGCCCTGTGCGGATGAT-3' and 6FAM-CCACCTGGCTGGGAAAATGGCAC-TAMRA. Primers and probes for GAPDH were purchased from Perkin-Elmer Applied Biosystems. One microgram of RNA was reverse transcribed in a 20- μL reaction containing Random Primers (Invitrogen) and Superscript II enzyme (Invitrogen). Each PCR reaction system (25 μL) contained 1 μL of cDNA, 1 $\times$ gold buffer II, 4 mmol/L MgCl₂, 500 $\mu\text{mol/L}$ dNTP, 900 nmol/L primers, 200 nmol/L probe (labeled with 6-carboxyfluorescein), and 1.25 U AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems). Real-time PCR was performed, and results were analyzed by means of the dual-labeled fluorogenic probe method with an ABI Prism 7700 sequence detector (Perkin-Elmer/Applied Biosystems). Relative expression levels were calculated by using the relative standard curve method, as outlined in the manufacturer's technical bulletin. A standard curve was then generated with the use of the fluorescence data from the 10-fold serial dilutions of total RNA from a sample that highly expressed that particular gene. This curve was used to calculate the relative amounts of target gene expression in test samples. Quantities of target gene expression in test samples were normalized to the corresponding GAPDH RNA transcript in the blood cell samples.

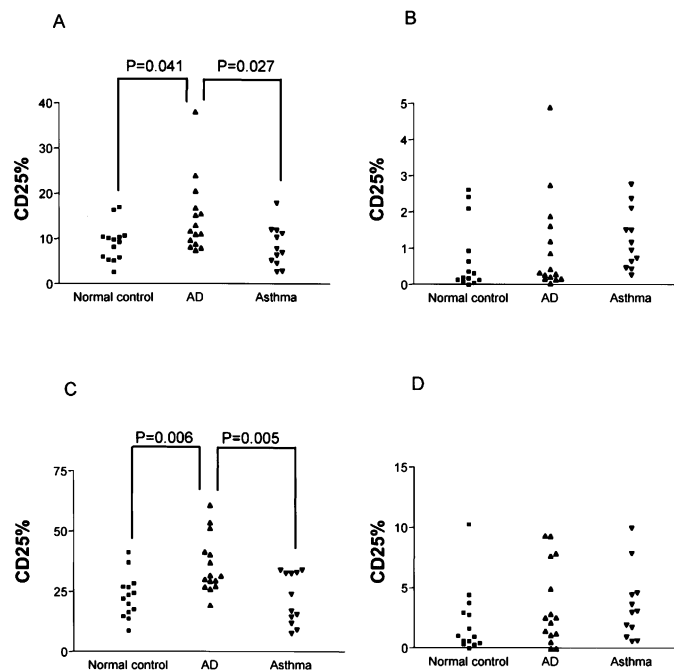


FIG 1. Surface expression of CD25 in CD4⁺ T cells (**A**), CD8⁺ T cells (**B**), CD4⁺CLA⁺ T cells (**C**), and CD8⁺CLA⁺ T cells (**D**). The PBMCs were stained with mAb to CLA, CD4, CD8, and CD25. The percentage of CD25⁺ cells was determined within gated CD4⁺, CD8⁺, CD4⁺CLA⁺, and CD8⁺CLA⁺ cell populations (AD group, 15 subjects; normal control group, 14 subjects; asthma group, 12 subjects).

Phenotypic characteristics of T cells

Four-color fluorochrome-conjugated mAb sets were used to stain T-cell surface markers. All antibodies were used at concentrations titrated for optimal staining. In brief, 5×10^5 PBMCs were incubated with the fluorochrome-conjugated mAbs at 4°C in the dark for 30 minutes, washed with PBS, and fixed in 1% paraformaldehyde. Cytofluorometry was performed with the Becton-Dickinson FACSCalibur system equipped with a 488-nm blue laser and a 635-nm red diode laser for multicolor fluorescence, in addition to forward-scattering and side-scattering measurements. Laser and photomultiplier parameters were kept constant for all experiments. All cytofluorometric data were subsequently analyzed and graphically displayed with CELL Quest software (Becton Dickinson). Each analysis measured a minimum of 20,000 cells.

Statistical analysis

Data were expressed as means $\pm$ SEMs unless otherwise specified. Statistical comparisons of flow cytometric data among 3 groups of subjects were performed with 1-way ANOVA with the Tukey HSD. Statistical comparisons of proliferation and suppression between 2 groups of subjects were performed with the Student unpaired *t* test. The proliferation, suppression changes, and FoxP3 real-time PCR expression in the same group were analyzed with a paired Student *t* test. A *P* value of less than .05 was considered statistically significant.

RESULTS

Patients with AD have increased CD4⁺CD25⁺ Treg cells

CD4⁺CD25⁺ T cells were considered as a phenotypic marker for Treg cells. Our data indicate that patients with

AD had a higher expression of CD25 in CD4⁺ T cells than asthmatic patients and nonatopic subjects, with values of $14.73\% \pm 2.08\%$ (range, 7.58% to 38.17%) versus $9.14\% \pm 1.09\%$ (range, 2.66% to 17.04%) versus $8.49\% \pm 1.29\%$ (range, 2.88% to 18.06%), respectively ($P < .05$; Fig 1, A). In contrast, there was no difference in expression of CD25 in CD8⁺ T cells (Fig 1, B). Furthermore, we found that patients with AD had a significantly higher expression of CD25 in CD4⁺CLA⁺ T cells than asthmatic patients and nonatopic subjects, with values of $35.95\% \pm 3.01\%$ (range, 19.55% to 61.02%) versus $22.44\% \pm 3.05\%$ (range, 7.98% to 34.25%) versus $23.03\% \pm 2.38\%$ (range, 8.85% to 41.4%), respectively ($P < .006$; Fig 1, C). The CD25 expression in the CD8⁺CLA⁺ T-cell population was not different between the groups (Fig 1, D). We also found that patients with AD had more CD4⁺CD25⁺ T cells ($6.68\% \pm 0.99\%$, $n = 15$) in PBMCs than healthy control subjects ($3.34\% \pm 0.43\%$, $n = 14$; $P = .006$) or asthmatic control subjects ($3.42\% \pm 0.58\%$, $n = 12$; $P = .01$). There was no difference in CLA distribution in the CD4⁺ and CD8⁺ T-cell populations in all study groups (data not shown).

The surface expression of other activation markers (CD69 and HLA-DR) on CD4⁺CD25⁺ T cells from PBMCs between the AD group ($n = 6$) and the healthy control group ($n = 6$) was compared. CD69 expression in CD4⁺CD25⁺ T cells was very low in both patients with AD and healthy control subjects, with values of 4.77% versus 3.48%, respectively ($P > .05$). The HLA-DR expression was also not different between the AD and

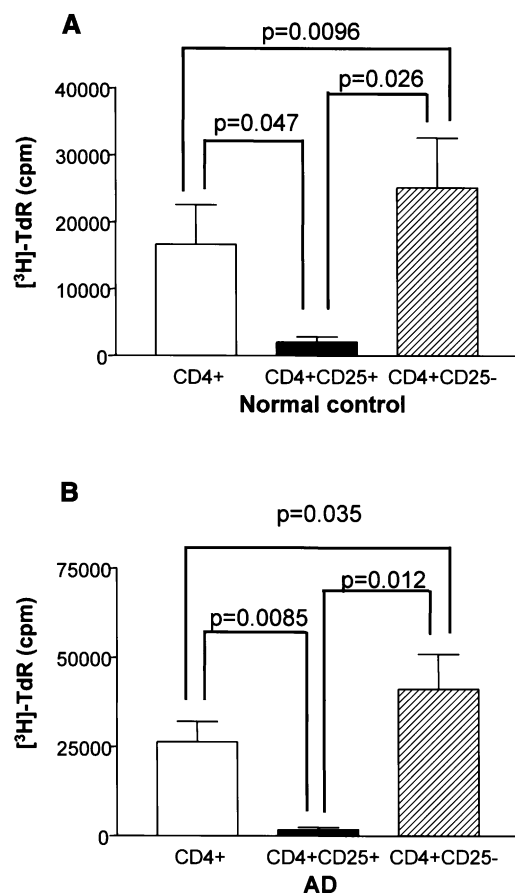


FIG 2. Human CD4⁺CD25⁺, as compared with CD4⁺CD25⁻, T cells (solid bar) are anergic in both patients with AD and healthy control subjects (A and B). Purified CD4⁺, CD4⁺CD25⁻, and CD4⁺CD25⁺ T cells were stimulated with 0.5 μ g/mL anti-CD3 in the presence of irradiated APCs for 72 hours. The proliferative response was measured by means of tritiated thymidine ([³H]-TdR) incorporation during the last 18 hours. Results (means $\pm$ SEMs cpm) are shown for patients with AD (n = 5) and healthy control subjects (n = 5).

normal groups, with values of 22.02% versus 21.26%, respectively ($P > .05$).

Ex vivo isolated CD4⁺CD25⁺ T cells have the properties of Treg cells

CD4⁺ T cells were purified from 5 patients with AD and 5 healthy control subjects by means of negative selection, which resulted in 95% to 98% purity. Then CD4⁺ T cells were separated by using immunomagnetic beads to obtain CD4⁺CD25⁺ T cells and CD4⁺CD25⁻ T cells with a CD4⁺CD25⁺ T-cell isolation kit.

The proliferative responses of the different isolated CD4⁺ T-cell subsets were investigated after stimulation with anti-CD3 mAb by using irradiated APCs. Consistent with previous studies of Treg cells,¹⁸⁻²¹ CD4⁺CD25⁺ T cells from patients with AD and healthy subjects were completely unresponsive-anergic on stimulation with anti-CD3 (Fig 2). These data confirmed that our ex vivo isolated CD4⁺CD25⁺ T cells have properties of Treg cells.

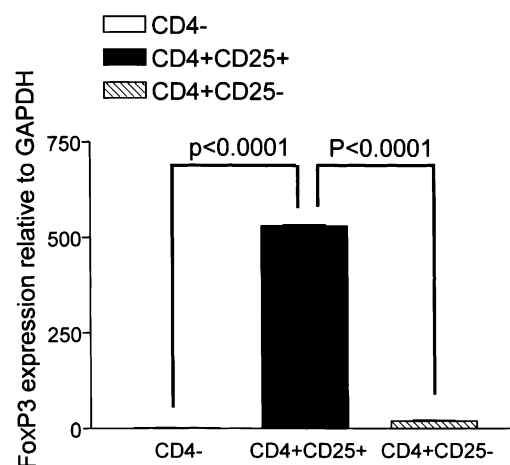


FIG 3. CD4⁺CD25⁺ Treg cells from peripheral blood of patients with AD express FoxP3. Real-time PCR analysis of FoxP3 gene expression relative to GAPDH expression in purified CD4⁻, CD4⁺CD25⁺, and CD4⁺CD25⁻ cells from 3 patients with AD.

In contrast, isolated CD4⁺ and CD4⁺CD25⁻ T cells from both groups proliferated significantly more than CD4⁺CD25⁺ Treg cells (Fig 2). When CD4⁺CD25⁺ T cells were removed from total CD4⁺ T cells, the T-cell response of the remaining CD4⁺CD25⁻ T cells significantly increased in both groups (Fig 2, A: $P = .0096$; Fig 2, B: $P = .035$). These results suggested that CD4⁺CD25⁺ T cells were not only anergic but were actively suppressing the proliferative responses of other CD4⁺ T cells in both groups. However, the degree of proliferation of these T-cell subsets was not different between patients with AD and healthy control subjects (Fig 2).

FoxP3 is preferentially expressed in CD4⁺CD25⁺ T cells

CD4⁺ T cells were purified by means of negative selection from 3 patients with AD and 3 healthy control subjects to examine the expression of FoxP3 in human cells. Then CD4⁺ T cells were separated by using immunomagnetic beads to obtain CD4⁺CD25⁺ T cells and CD4⁺CD25⁻ T cells with a CD4⁺CD25⁺ T-cell isolation kit. FoxP3 gene expression was ascertained by means of real-time PCR in purified CD4⁻ cells, CD4⁺CD25⁺ T cells, or CD4⁺CD25⁻ T cells. In the AD group CD4⁺CD25⁺ T cells expressed significantly higher levels of FoxP3 (529.6 ± 3.03) than CD4⁺CD25⁻ T cells (19.71 ± 1.73 , $P < .0001$) and PBMCs without CD4⁺ (CD4⁻) cells (1.00 ± 0.35 , $P < .0001$; Fig 3). Similar data were observed in healthy control subjects (data not shown).

CD4⁺CD25⁺ T cells can suppress the proliferation of CD4⁺CD25⁻ T cells

In these experiments CD4⁺CD25⁺ T cells from patients with AD or healthy control subjects were cocultured with CD4⁺CD25⁻ T cells (Fig 4). The response of CD4⁺CD25⁻ T cells to anti-CD3 mAb in the presence

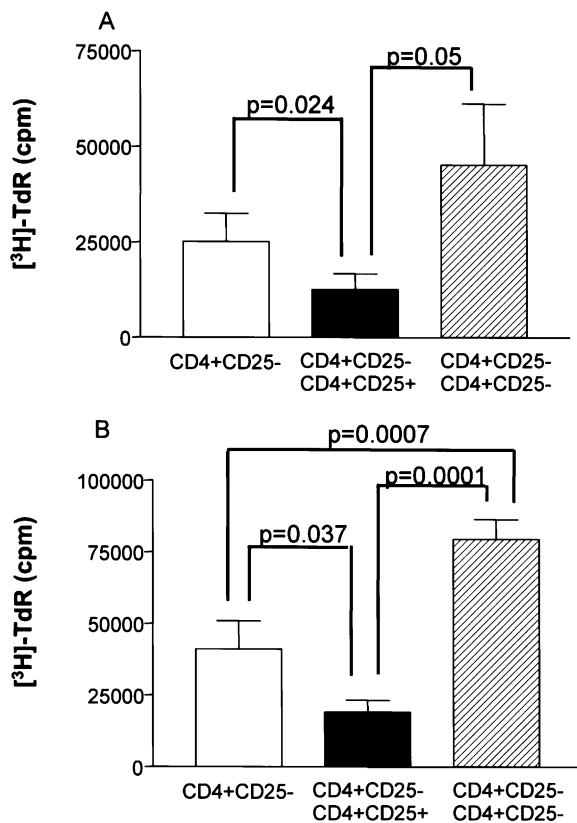


FIG 4. Direct suppressive effect of CD4⁺CD25⁺ T cells after anti-CD3 mAb stimulation in healthy control subjects (**A**) and patients with AD (**B**). Purified CD4⁺CD25⁻ T cells (responsive cells) from PBMCs were cultured alone (*open bars*) or cocultured with the same numbers of anergic-suppressive CD4⁺CD25⁺ T cells (*solid bars*) or nonanergic CD4⁺CD25⁻ T cells (*hatched bars*) and stimulated with anti-CD3 mAb in the presence of irradiated APCs for 72 hours. The proliferative response was measured by means of tritiated thymidine ([³H]-TdR) incorporation during the last 18 hours. Results (means ± SEMs cpm) are shown for patients with AD (n = 5) and healthy control subjects (n = 5).

of APCs was significantly suppressed in both groups by up to 74% when cocultured with CD4⁺CD25⁺ T cells (Fig 4, A: $P = .024$; Fig 4, B: $P = .037$). In contrast, when CD4⁺CD25⁻ T cells were cocultured with CD4⁺CD25⁻ T cells, the proliferation response was enhanced in both groups. Thus the suppression of T-cell proliferation by CD4⁺CD25⁺ Treg cells was not due to competition for space or nutrients at higher cell concentrations. However, there was no difference in the CD4⁺CD25⁺ T cell-suppressive effect between patients with AD and healthy control subjects.

After SEB stimulation, CD4⁺CD25⁺ T cells are not anergic and do not suppress the proliferative response of CD4⁺CD25⁻ T cells

In contrast to anti-CD3 mAb stimulation, CD4⁺CD25⁺ T cells from patients with AD were not anergic but actually responded to activation by 100 ng/mL SEB stimulation (1752 ± 701 cpm vs $40,524 \pm 9294$ cpm for

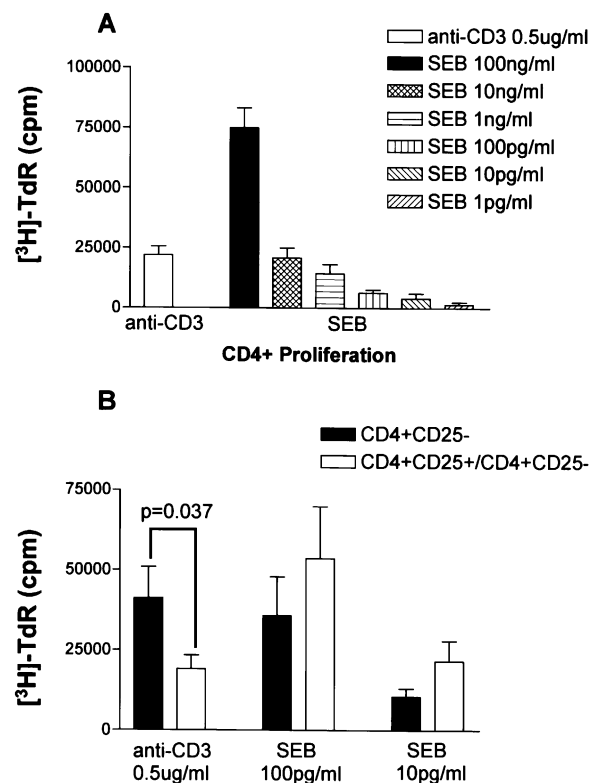


FIG 5. CD4⁺CD25⁺ Treg cells lose their suppressive function after SEB stimulation. **A**, Purified CD4⁺ T cells from patients with AD (n = 3) were stimulated with 0.5 μg/mL anti-CD3 or different concentrations (1 pg/mL to 100 ng/mL) of soluble SEB (100 ng/mL) in the presence of irradiated APCs for 72 hours. **B**, Purified CD4⁺CD25⁻ T cells were cultured alone or cocultured with the same numbers of anergic-suppressive CD4⁺CD25⁺ T cells and stimulated with 0.5 μg/mL anti-CD3 or SEB (100 pg/mL or 10 pg/mL) in the presence of APCs for 72 hours. The proliferative response was measured by means of tritiated thymidine ([³H]-TdR) incorporation during the last 18 hours. Results (means ± SEMs cpm) are shown for patients with AD (n = 5).

anti-CD3 vs SEB, respectively; n = 5; $P = .003$). Data from similar experiments in healthy control subjects (data not shown) showed the same results as found in the AD group. Because 100 ng/mL SEB stimulation is stronger than 0.5 μg of anti-CD3 stimulation, we diluted the concentration of SEB from 100 ng/mL to 1 pg/mL in a proliferation experiment by using cells from patients with AD. When the SEB concentration was less than 1 ng/mL (Fig 5, A), CD4⁺ T-cell proliferation was lower than that of CD4⁺ T cells stimulated with 0.5 μg of anti-CD3.

We then investigated the direct suppressive effect of CD4⁺CD25⁺ T cells by coculturing CD4⁺CD25⁺ T cells with CD4⁺CD25⁻ T cells from patients with AD and healthy control subjects after stimulation with 10 or 100 pg/mL SEB or 0.5 μg of anti-CD3 in the presence of APCs. In contrast to anti-CD3 mAb stimulation, when CD4⁺CD25⁺ T cells from patients with AD were added to CD4⁺CD25⁻ T cells in the presence of SEB, there was no suppressive effect (Fig 5, B) but rather a greater proliferative response. The data from the same experiment in

healthy control subjects (data not shown) were similar to those seen in the AD group. These results indicate that the CD4⁺CD25⁺ T cells were no longer anergic and had lost their suppressive function after SEB stimulation. Because 10 pg/mL SEB-induced T-cell proliferation is no stronger than 0.5 µg of anti-CD3 stimulation, the effect was not concentration dependent.

DISCUSSION

CD4⁺CD25⁺ Treg cells were described by Sakaguchi et al³⁵ as a small proportion (5% to 15%) of CD4⁺ T cells. These cells suppress immune responses and play a major role in controlling the expression of asthma and allergy.¹⁵ Recently, Francis et al³⁶ demonstrated that grass pollen immunotherapy can increase CD4⁺CD25⁺ Treg cell numbers, with concomitant significant improvement of overall symptoms for patients with hay fever.

The current study is the first to examine CD4⁺CD25⁺ Treg cells in patients with AD. Surprisingly, we found that patients with AD had more CD4⁺CD25⁺ Treg cells in peripheral blood than healthy control subjects and asthmatic patients. Patients with AD also had a higher expression of CD25⁺ in CD4⁺CLA⁺ skin-homing T cells than asthmatic patients and nonatopic subjects. Because CD25⁺ effector T cells also can be induced within CD4⁺CD25⁺ cells after stimulation, we also compared the surface expression of other activation markers (CD69 and HLA-DR) on CD4⁺CD25⁺ T cells from PBMCs between the AD group and the healthy control group. Previous studies have demonstrated that activated CD4⁺CD25⁺ effector T cells have high levels of CD69 expression.²⁴ In our study CD69 expression in CD4⁺CD25⁺ T cells was very low in both patients with AD and healthy control subjects (approximately 4%). HLA-DR expression was also not different between the AD and healthy control groups. These data suggest the CD4⁺CD25⁺ T cells we studied here were not activated memory effector T cells. Akdis et al¹² reported that CD3⁺CLA⁺ T cells in patients with AD had increased expression of CD25 as an activation marker. In the current study we found that CD4⁺CLA⁺ T cells had increased expression of CD25 in patients with AD but not CD8⁺CLA⁺ T cells. Because we were not able to get sufficient numbers of purified CD4⁺CLA⁺CD25⁺ T cells in our study, we could not evaluate the function of these cells directly. Future studies are needed to clarify whether the CD25 expression on CLA⁺ T cells represents an activation marker or the cells also have the Treg cell properties.

More than 90% of patients with AD have *S aureus* colonization on their skin lesions. SAg can induce CLA expression,³⁷ and approximately 90% of the T cells isolated from skin express CLA.^{8,9} Our study demonstrated that CD25 expression was increased in the CD4⁺CLA⁺ T-cell population of patients with AD, and SAg can subvert the suppressive function of CD4⁺CD25⁺ Treg cells. If SAg induce dysfunction of Treg cells in the

skin, it underscores the importance of treating and eradicating *S aureus* on the skin of patients with AD.

Previous studies have demonstrated that human CD4⁺CD25⁺ T cells proliferate poorly and are deficient in IL-2 secretion when stimulated with polyclonal activators, such as PHA, anti-CD3 antibodies, or allogeneic cells.³⁸ In our study the CD4⁺CD25⁺ Treg cells from study subjects had the capacity to suppress the proliferation of autologous CD4⁺CD25⁺ T cells in vitro with anti-CD3 stimulation. Furthermore, removal of CD4⁺CD25⁺ T cells from CD4⁺ T cells resulted in enhanced proliferative response to anti-CD3 (Fig 2).

The suppressive activity of CD4⁺CD25⁺ T cells is related to their ability to inhibit IL-2 production and might involve signals through CTLA-4,^{26,39} glucocorticoid-induced TNF receptor,⁴⁰⁻⁴² or both. The roles of IL-10 and TGF-β in the suppressive effects mediated by CD25⁺CD4⁺ Treg cells are highly controversial. CD4⁺CD25⁺ T cells isolated from mice genetically deficient for IL-10 or TGF-β retain their suppressive capacity.⁴³⁻⁴⁵ CTLA-4 and glucocorticoid-induced TNF receptor have limitations similar to those of CD25 because they both are expressed at a high level by activated CD4⁺CD25⁺ T cells.⁴⁶ FoxP3 is considered to be the most specific marker for Treg cells.⁴⁶ In mice FoxP3 has been shown to be expressed exclusively in CD4⁺CD25⁺ Treg cells,^{16,17} and FoxP3 is both necessary and sufficient for the development and function of CD4⁺CD25⁺ Treg cells.⁴⁷ In this report we show that human CD4⁺CD25⁺ Treg cells in patients with AD and healthy control subjects express FoxP3. In contrast, CD4⁺CD25⁺ T cells and CD4⁺ cells do not express FoxP3.

In contrast to anti-CD3 mAb stimulation, when the human CD4⁺CD25⁺ Treg cells were stimulated with SEB, these cells were no longer anergic but were responsive to the stimulation. Furthermore, the addition of CD4⁺CD25⁺ T cells to CD4⁺CD25⁺ T cells in the presence of SEB did not result in any suppressive effect. SAg are potent stimulators of T cells and macrophages,^{48,49} and signaling by SAg involves strong T-cell receptor engagement in conjunction with CD28 costimulation.⁵⁰⁻⁵² Some studies have shown that increasing the strength of signal with the addition of soluble anti-CD28 to plate-bound anti-CD3 stimulation can abrogate suppression by Treg cells.⁵³ The authors concluded that Treg cells selectively suppress target cells that received weak signals lacking costimulation while being unable to suppress responder T cells that had been induced by foreign microbial antigens to undergo clonal expansion through strong signals.⁵² However, in this study we found that low concentrations of SEB are also able to abolish the immunosuppressive properties of the CD4⁺CD25⁺ Treg cell population, and therefore the effect was not concentration dependent.

In conclusion, our study demonstrates for the first time that patients with AD have significantly increased numbers of peripheral blood Treg cells with normal immunosuppressive activity. However, after SAg stimulation, Treg cells lose their immunosuppressive activity, suggesting that Treg cells might not be functioning at the

local skin level in AD colonized with SAg-producing *S. aureus*. This observation suggests a novel mechanism by which SAGs can augment T-cell activation. Further studies are needed to examine the interaction between SAGs and Treg cells at the local skin level in patients with AD.

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