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INVESTIGATIVE REPORT

Regulatory T-cell Subsets with Acquired Functional Impairment: Important Indicators of Disease Severity in Atopic Dermatitis

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Our aim was to assess whether the presence of highly active effector T cells in atopic dermatitis (AD) is associated with changes in the number and/or function of regulatory T cells (Tregs). Flow cytometry was utilised to determine the percentage of $CD4^+CD25^{bright}CD127^{-/low}FOXP3^+$ and skin-homing $CLA^+CD4^+CD25^{bright}FOXP3^+$ Tregs in healthy controls and AD patients. The correlation between disease severity and Treg percentages was estimated. Treg suppressor activity and cell proliferation were measured after T-cell stimulation. Significantly increased percentages of Tregs were found in AD patients compared to healthy individuals, and significant correlation between the frequency of Tregs and disease severity was also detected. The otherwise normal suppressor activity of Tregs decreased in the presence of *Staphylococcus* enterotoxin B (SEB). In conclusion, the continuous presence of SEB can trigger an acquired functional impairment of Tregs in AD patients and the correlation between the increased frequency of Tregs and disease severity supports their important role in AD pathogenesis. *Key words: atopic dermatitis; cutaneous lymphocyte-associated antigen; regulatory T cells;* Staphylococcus *enterotoxin B*.

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Regulatory T cells are important contributors to peripheral immune tolerance. Three main subgroups of these cells have been identified: T helper (Th) 3 cells, T regulatory type 1 (Tr1) cells, and $CD4^+CD25^{bright}FOXP3^+$ T regulatory (Treg) cells (1). The latter Tregs participate in the regulation of the allergen-specific immune response, as well as suppressing antigen-presenting cells (APC), effector T cells, and mast cell function (2–4).

Atopic dermatitis (AD) is a chronic inflammatory skin disease with a complex pathogenesis. The combination of genetic and acquired skin barrier dysfunctions as well as distorted innate and adaptive immune responses lead to the development of the characteristic clinical picture (5–9). The well characterised dysregulation of the adaptive immune system can be the result of altered immune suppression (3, 9–11). For this reason, the role of Tregs has been investigated intensely in AD (1, 12). The functional disequilibrium of Tregs may also be predicted by the clinical evidence of AD-like symptoms (severe eczema, eosinophilia, elevated IgE levels, and food allergy) in IPEX syndrome (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome), where Tregs are absent due to genetic alterations in the FOXP3 gene (13). Recent studies have found evidence of an increase in the number of Tregs in AD, although only a small number of investigations have been conducted to functionally characterise these cells (14–16). Furthermore results show a discrepancy in characterising Treg functions that necessitate further studies in this regard.

The aim of the present study was to determine quantitative and functional changes of Tregs as well as skin-homing Tregs in AD, and to investigate whether these parameters could correlate with clinical severity.

METHODS

Patients

Peripheral blood was obtained from 27 AD patients (mean age: 24 years, range 5–41) with moderate or severe clinical symptoms and high (>1,000 U/ml) serum IgE levels (mean IgE level 10,321 kU/l, range 1,122–65,463). All patients fulfilled the diagnostic criteria for Hanifin & Rajka (17). The severity of disease was determined by the Scoring Atopic Dermatitis (SCORAD) index (mean SCORAD: 44, range 18–68). Hyper IgE Syndrome (HIES) was excluded in all patients using the HIES clinical scoring system (18). Patients who were enrolled in the study had not been treated with oral glucocorticosteroids or other immunomodulatory agents for at least 4 weeks and had not received antihistamines and topical corticosteroids for at least 4 days prior to sampling. Blood samples from 11 healthy age-matched volunteers served as controls. All participants provided their written informed consent according to the Declaration of Helsinki principles. The study was approved by the local ethics committee of the University of Debrecen.

Measurement of serum IgE level

Serum IgE levels were measured by using an enzyme-linked immunosorbent assay (Radim SpA, Pomezia (Rome), Italy) according to the manufacturer's instructions.

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Cell preparations

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (Sigma-Aldrich, Munich, Germany) gradient centrifugation from heparinised blood. $CD4^+CD25^+$ Treg cells were separated by positive selection with a regulatory T-cell isolation kit (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

Flow cytometric analysis of $CD4^+CD25^{bright}CD127^{-/low}FOXP3^+$, and $CLA^+CD4^+CD25^{bright}FOXP3^+$ Tregs in peripheral blood

Flow cytometry was utilised to determine cutaneous lymphocyte-associated antigen positive (CLA^+) $CD4^+CD25^{bright}FOXP3^+$ Tregs in the blood samples of AD patients and healthy controls. Because of the limitation of employable antibodies (regarding colours), first we proved that the gated $CD4^+CD25^{bright}$ cells were almost exclusively FOXP3 positive and $CD127^{-/low}$ (97.3% ± 2.0%) (Fig. 1A). These proportions were calculated as the percentage of gated $CD4^+CD25^{bright}$ cells. Next, the anti-CD127 antibody was replaced by anti-CLA antibody. CLA positivity was investigated on the gated $CD4^+CD25^{bright}FOXP3^+$ T cells (Fig. 1B, C). Cell surface and intracellular staining was carried out according to the manufacturer's instructions, as described previously (19). Briefly, after PBMC preparation, cells were washed in PBS 3 times and cell surface staining was performed using CD4-APC or CD4-FITC, CD127-APC, CLA-FITC (all Becton Dickinson, BD Pharmingen, Heidelberg, Germany), and CD25-PC5 (Immunotech, Marseille, France) antibodies. After cell surface staining, cells were washed in cold Flow Cytometry Staining Buffer and re-suspended in Fixation/Permeabilisation working solution (eBioscience, San Diego, CA, USA). Samples were incubated at 4°C for 30 min. After cells were washed with 1X Permeabilisation Buffer (eBioscience, San Diego, CA, USA) anti-human FOXP3-PE (clone PCH101; eBioscience, San Diego, CA, USA) antibody was added to the cell suspension. Cells were incubated at 4°C for 30 min in the dark. Then, cells were washed with 1X Permeabilisation Buffer and re-suspended in Flow Cytometry Staining Buffer. Samples were analysed on a Becton Dickinson FACS-Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Data were analysed by CellsQuest software (Becton Dickinson, BD Pharmingen, Heidelberg, Germany).

Suppression assay

The $CD4^+CD25^+$ Regulatory T-Cell Isolation Kit (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) was used to obtain Tregs from peripheral blood, as previously described in detail (20). The purity of isolated cell populations was above 95% in the case of $CD4^+CD25^-$ cells and 85–95% in the case of $CD4^+CD25^+$ cells. To detect the suppressor activity of $CD4^+CD25^+$ Tregs, 1×10^5 $CD4^+CD25^+$ T cells were co-cultured with 1×10^5 $CD4^+CD25^-$ effector T cells in 200 ml RPMI-1640 in a 96-well microtitre plate. For T-cell stimulation, anti-CD3/CD28 microbeads (Invitrogen Dynal AS, Oslo, Norway) were applied alone or together with *Staphylococcus* enterotoxin B (SEB) (Sigma-Aldrich, Munich, Germany). For the basic experiment, one anti-CD3/CD28 microbead per cell and a concentration of 1 pg/ml SEB were used. To prove a selective response of Tregs for suppressing SEB-stimulated effector T cells, dose-response experiments were utilised. The tests were performed first in the presence of 1 pg/ml SEB with increasing Treg:Teffector cell ratios (1:2, 1:1, 3:1), and then on Treg:Teffector 1:1 cell ratio with increasing concentrations of SEB (1 pg/ml, 500 pg/ml, 5 ng/ml). Cell proliferation was measured by EZ4U colorimetric cell proliferation assay (Biomedica, Vienna, Austria). All of the measurements were performed in triplicate and averages were calculated. Suppressor activity was determined by an index calculated from the optical density (OD) values. The OD of mixed $CD4^+CD25^-/CD4^+CD25^+$ lymphocyte culture was corrected with the OD of $CD4^+CD25^+$ T cells as the background. The suppressor activity index was equal to $OD_{CD25-}/(OD_{MLR}-OD_{CD25+})$, where MLR stands for mixed lymphocyte reaction (20).

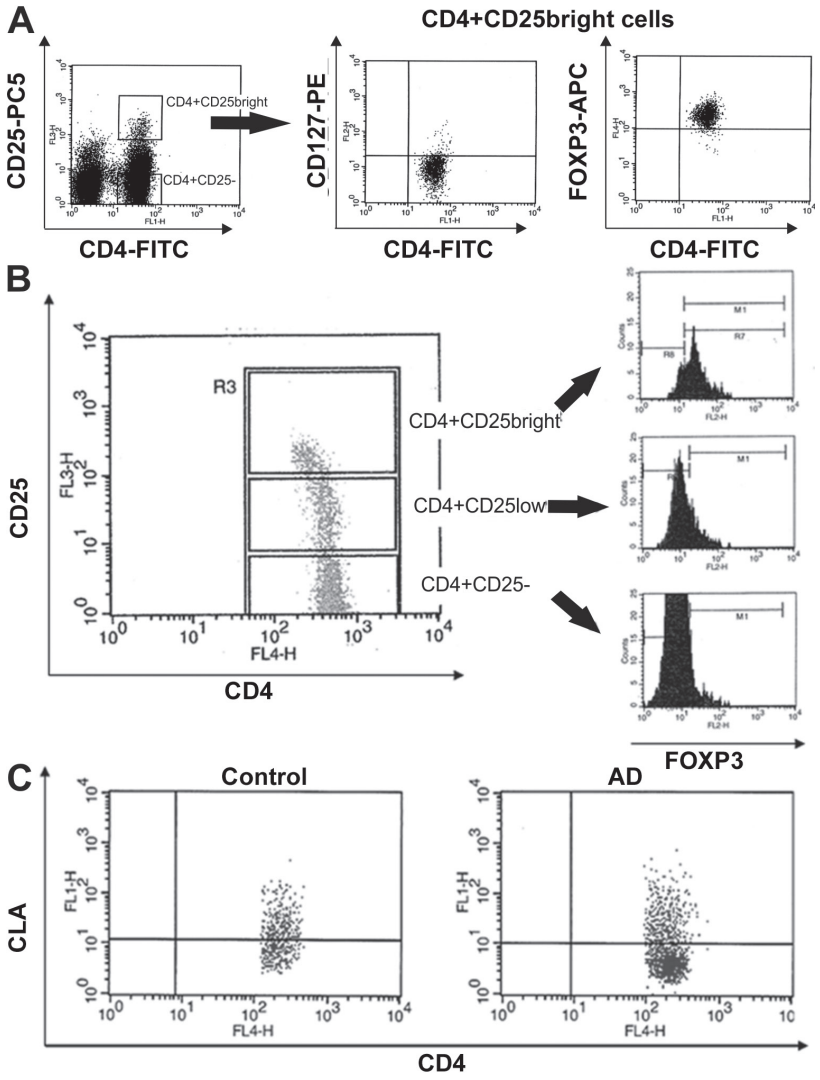

Fig. 1. The identification of regulatory T cells by flow cytometry. CD4 and CD25 cell surface molecules were stained with monoclonal antibodies. FOXP3 was quantitated in the $CD4^+CD25^{bright}$ and $CD127^{-/low}$– populations (A). CD4 and CD25 cell surface molecules were stained with monoclonal antibodies. FOXP3 was quantitated in the $CD25^{bright}$, $CD25^{low}$ and $CD25^-$ populations (B). A representative dot-plot from one AD patient and one healthy control displaying the percentage of $CLA^+CD4^+CD25^{bright}FOXP3^+$ Tregs (C).

Statistical analysis

Statistical analysis was performed using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). The normal distribution of data was tested using the Kolmogorov-Smirnov test. Student's *t*-test or the nonparametric Mann–Whitney test and Kruskal-Wallis test were used to compare the data of patients with AD to those from healthy controls. The correlation between the percentage of $CD4^+CD25^{bright}FOXP3^+$ Tregs and $CLA^+CD4^+CD25^{bright}FOXP3^+$ cells as well as IgE levels and the SCORAD index was assessed by Pearson's correlation. Data are presented as mean ± SD, and $p < 0.05$ was considered statistically significant.

RESULTS

First, $CD4^+CD25^{bright}CD127^{-/low}FOXP3^+$ Tregs were determined by flow cytometry in a small number of AD patients (n=3). We proved that the gated $CD4^+CD25^{bright}$ cells were almost exclusively FOXP3 positive (95.3 ± 3.4%), or $CD127^{-/low}$ (93.4 ± 4.1%) (Fig. 1A). The proportion was calculated as the percentage of gated $CD4^+CD25^{bright}$ cells (97.3 ± 2.0% of the $CD4^+CD25^{bright}$ cells were $FOXP3^+$ and $CD127^{-/low}$). The cells with $CD4^+CD25^{bright}FOXP3^+$ phenotype were examined in further analysis.

Flow cytometric analyses were performed to evaluate the frequencies of $CD4^+CD25^{bright}FOXP3^+$ Treg cells among $CD4^+$ T cells in the peripheral blood of AD patients (n=27) with high IgE levels and age- and sex-matched healthy controls (n=11). After staining for CD4, CD25, and CLA cell-surface molecules, FOXP3 positivity was evaluated in the $CD25^{bright}$, $CD25^{low}$ and $CD25^-$ populations (Fig. 1B, C). Our results showed that the percentage of $CD4^+CD25^{bright}FOXP3^+$ Tregs in total $CD4^+$ T cells was significantly elevated in patients with AD compared to healthy controls (AD: 3.62 ± 1.55%; control: 2.19 ± 0.84%; p=0.014) (Fig. 2A). To address whether the phenotype of Tregs could reflect the ability of cells to migrate to the skin, we determined the proportion of CLA expressing $CD4^+CD25^{bright}FOXP3^+$ Tregs in the $CD4^+$ T cells as well as among the peripheral Treg population. The frequency of $CLA^+CD4^+CD25^{bright}FOXP3^+$ Tregs was significantly higher in patients with AD compared to healthy controls (AD: 0.78 ± 0.46%; control: 0.43 ± 0.17%; p=0.048) (Fig. 2B). The fluorescence intensity of CLA on $CD4^+CD25^{bright}$ Tregs was higher in AD patients as compared to healthy controls (Fig. 1C), while there was no difference in the proportion of CLA^+ Tregs in the Treg population found in AD patients and healthy controls (data not shown).

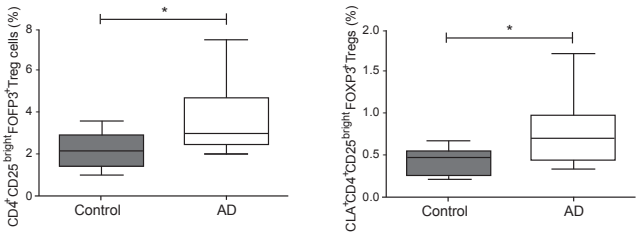

Fig. 2. Percentage of $CD4^+CD25^{bright}FOXP3^+$ Treg cells (**A**) and $CLA^+CD4^+CD25^{bright}FOXP3^+$ Treg cells (**B**) of total $CD4^+$ T-cells in AD patients (n=27) compared to healthy controls (n=11). *p<0.05, non-parametric Mann-Whitney and Kruskal-Wallis tests.

A statistically significant correlation was found between the percentage of $CD4^+CD25^{bright}FOXP3^+$ Tregs and serum levels of IgE (r=0.514, p=0.041, Fig. 3A) or SCORAD index (r=0.584, p=0.018, Fig. 3C). We also detected a significant correlation between $CLA^+CD4^+CD25^{bright}FOXP3^+$ Tregs and IgE level (r=0.561, p=0.024, Fig. 3B) as well as SCORAD (r=0.789, p=0.0003, Fig. 3D).

We compared the functional activity of Tregs obtained from randomly selected AD patients and healthy controls. Since $CD4^+CD25^+$ cells can be either effector or regulatory cells, to investigate the suppressor activity of $CD4^+CD25^+$ T cells, magnetically isolated $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were cultured alone and together in the presence of anti-CD3/CD28 microbeads, and the effect of exotoxin SEB was also tested. In the culture of mixed lymphocyte reaction of $CD4^+CD25^-/CD4^+CD25^+$ cells, the presence of $CD4^+CD25^+$ cells caused a significant decrease in the proliferation of $CD4^+CD25^-$ effector T cells (Fig. 4A.). The suppressor function of these cells was characterised using a suppressor activity index. The suppressor activity of $CD4^+CD25^+$ T cells that were not subjected to SEB stimulation was higher in patients with AD than in healthy controls (AD: 4.30 ± 1.79; control: 2.71 ± 0.72), although the dif-

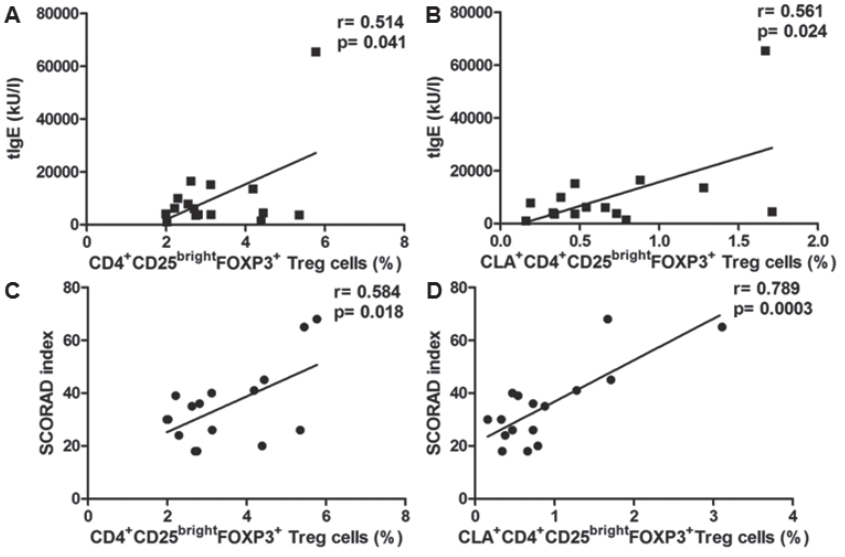

Fig. 3. Correlation between percentage of Tregs [$CD4^+CD25^{bright}FOXP3^+$ (A), percentage of $CLA^+CD4^+CD25^{bright}FOXP3^+$ cells (B)] and IgE serum level. The correlation between percentage of Tregs [$CD4^+CD25^{bright}FOXP3^+$ (C), $CLA^+CD4^+CD25^{bright}FOXP3^+$ (D)] and SCORAD index in AD patients (n=16). *p<0.05, non-parametric Mann-Whitney and Kruskal-Wallis tests.

ference was not significant. However, in the presence of SEB, the suppressor activity of $CD4^+CD25^+$ T cells was significantly decreased in both AD patients and healthy controls (AD: 1.79 ± 0.09; control: 1.49 ± 0.10) (Fig. 4B). The selective, dose-response reaction of Tregs was proved first when an increasing Treg:Teffector cell ratio (1:2, 1:1, 3:1) was studied with constant SEB concentration (1 pg/ml). The higher the Treg:Teffector ratio was increased, the more the Tregs suppressed effector T cells (20–60% decrease in the proliferation of effector T cells) (Fig. S1A[1]). When the effect of increasing concentrations of SEB (1 pg/ml, 500 pg/ml, 5 ng/ml) was analysed together with an equal ratio of Treg:Teffector cells (1:1 ratio), a high concentration of SEB (> 500 pg/ml) diminished the suppressor activity of Tregs, causing increased proliferation of effector T cells (Fig. S1B[1]).

DISCUSSION

Regulatory T-cells have an important control function in immunologic processes (2, 3, 21). Certain T-cell populations with regulatory potentials (TGF-β-producing Th3 cells, IL-10-producing Tr1 cells, and $CD4^+CD25^{bright}FOXP3^+$ Tregs) have been described (22); however, their proper functions in AD are not yet fully elucidated (21).

Allergic, autoimmune, and inflammatory diseases are characterised by derailed effector T-cell-mediated immune responses, partly as a consequence of the quantitative and/or functional alteration of Tregs. However, because of the heterogeneity of these cells and the lack of specific cell markers, the results of different studies regarding the characterisation of Tregs in such pathological conditions are difficult to compare (1, 12, 23–26). There is evidence indicating that the percentage of Tregs is either normal or increased in the peripheral blood in patients suffering from AD; however, there have only been a few studies investigating both the number and function of Tregs simultaneously (14, 15).

We have depicted the complex quantitative and functional characterisation of Tregs as well as specific skin-homing Tregs in AD patients with moderate or severe clinical signs and high IgE levels. Furthermore, for the first time we also clarified the correlation between CLA^+ skin homing Tregs and disease severity, which may indicate a more complex pathogenic role of Tregs in AD development.

Previously we have identified high numbers of $CD4^+CD25^{bright}FOXP3^+$ Tregs in close connection to dendritic cells in lesional AD skin and in atopy patch test positive biopsies (27). However, elevated numbers of $CD4^+CD25^{bright}FOXP3^+$ cells in AD patients do not necessarily mean that these cells have regulatory functions only, since FOXP3 alone is insufficient to determine the $CD4^+CD25^{bright}$ T-cell population with regulatory properties in humans (28). In AD, a proportion of $CD4^+CD25^{bright}FOXP3^+$ and $CLA^+CD4^+CD25^{bright}FOXP3^+$ cells exhibit Th2-like effector function after activation by bacterial superantigens (15, 16). To overcome the difficulty of the inappropriate characterisation of Tregs, we first verified the $CD4^+CD25^{bright}CD127^{-/low}FOXP3^+$ T-cell population. As a calculated proportion, almost the entire percentage of gated $CD4^+CD25^{bright}$ cells were $FOXP3^+$ and $CD127^{-/low}$ ($97.3\% \pm 2.0\%$). Henceforth, we identified the Treg population as $CD4^+CD25^{bright}FOXP3^+$ cells and detected significantly increased percentages of these cells in the peripheral blood of AD patients, in line with a previous result (29). In the next phase, we quantified the specific subset of $CLA^+CD4^+CD25^{bright}FOXP3^+$ skin-homing Tregs, and described them as also significantly increased in the peripheral blood, in accordance with previous results (30).

A significant correlation of $CD4^+CD25^{bright}FOXP3^+$ Tregs and SCORAD was previously shown, yet serum IgE levels and skin-homing Tregs in association with disease severity have never been assessed (4, 15, 27). Our current report is the first to demonstrate significant correlations between the percentage of both Tregs and skin-homing Tregs and disease severity, as assessed by SCORAD and serum IgE levels in a relatively larger AD population.

Previously, conflicting results have been published on Treg responses to antigenic stimuli in AD. Tregs remained anergic after stimulation by PPD or Der p1 antigens in both normal controls and AD patients (31).

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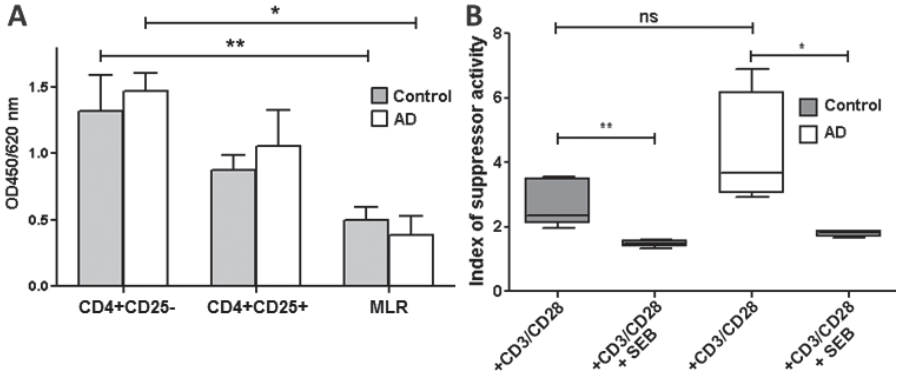

Fig. 4. Proliferations of $CD4^+CD25^-$, $CD4^+CD25^+$ and mixed lymphocyte reaction (MLR) of $CD4^+CD25^-/CD4^+CD25^+$ cells in the presence of anti-CD3/CD28 (in the case of MLR, the $CD4^+CD25^+$-corrigated optical density is displayed) (A). Suppressor activity index of $CD4^+CD25^+$ regulatory T cells stimulated with anti-CD3/CD28 only or together with *Staphylococcus* enterotoxin B (1 pg/ml) in patients with atopic dermatitis (AD) ($n = 11$) and in healthy subjects ($n = 11$) (B). $^*p < 0.05$, $^{**}p < 0.01$ Non-parametric Mann-Whitney and Kruskal-Wallis tests.

However, another study concluded that Tregs were no longer anergic after prior SEB stimulation (14). Our functional tests revealed that Tregs, stimulated with anti-CD3/CD28, maintained their suppressor activity in the peripheral blood of AD patients. However, when stimulated with additional SEB, Tregs lost their suppressive ability in both AD patients and normal controls, indicating that the primary functional characteristics of Tregs in AD are not different from those in healthy controls. Thus, we conclude that the main distinctive factor in AD is the surrounding environment in the skin due to the nearly continuous presence of SEB.

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