

Original article

Low-dose cyclosporine A therapy increases the regulatory T cell population in patients with atopic dermatitis

Background: Atopic dermatitis (AD) is a T cell dependent chronic relapsing inflammatory skin disorder successfully treated with cyclosporine A (CsA). Clinical observations indicate that even low-dose CsA therapy is successful in severely affected AD patients. We studied the impact of low-dose CsA therapy on the ability of T helper cells to be activated, and examined whether regulatory T (Treg) cells are increased in these patients.

Methods: Peripheral T cells were activated in a whole blood sample and interleukin-2 producing cells were measured by intracellular cytokine staining. Regulatory T cells were analyzed by intracellular FoxP3 staining. Regulatory T cells (CD4⁺CD25⁺CD127^{low}) and effector T cells (CD4⁺CD25⁻CD127⁺) were sorted by flow cytometry and used for suppression assays.

Results: A group of AD patients treated with low-dose CsA had a significantly larger Treg cell population than a healthy control subject group. In individual patients, onset of low-dose CsA therapy reduced the ability of T cells to be activated to $42 \pm 18\%$ ($P < 0.005$) and significantly increased Treg cells, both in absolute numbers (1.6-fold change) and frequencies (1.7-fold change). Treg cells from AD patients showed similar suppressive capacities as Treg cells from healthy donors. Furthermore, Treg cells from AD patients had skin homing properties.

Conclusion: Our results indicate that the therapeutic effect of low-dose CsA therapy in AD patients might be not only mediated by the inhibition of T cell hyperactivity but also by an increased population of Treg cells.

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Key words: atopic dermatitis; cyclosporine A; FoxP3; interleukin-2; T cell activation.

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Atopic dermatitis (AD) is a chronic eczematous inflammatory skin disease. The clinical phenotype of AD is caused by complex interactions between susceptibility genes (1), the environment, defective skin-barrier function, and immunologic responses (2). It is suggested that AD is a cutaneous manifestation of a systemic disorder characterized by elevated serum immunoglobulin E levels and peripheral eosinophilia (2). The clinical outcome of AD is monitored by the severity scoring of atopic dermatitis (SCORAD) score (3). Atopic dermatitis skin lesions typically show infiltration of immune cells, particularly CD4⁺ T cells (4). More than 90% of infiltrating T cells present in cutaneous inflammatory sites express the skin-homing receptor cutaneous lymphocyte-associated antigen (CLA) (5).

Abbreviations: AD, atopic dermatitis; CLA, cutaneous lymphocyte-associated antigen; CsA, cyclosporin A; HC, healthy control; IL, interleukin; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; SCORAD, scoring of atopic dermatitis; Th, T helper.

Systemic cyclosporin A (CsA) therapy is an effective and very useful treatment for patients with severe AD refractory to conventional topical therapy (6). CsA is an immunosuppressive drug interfering with T cell receptor (TcR) signaling at a hub of T cell activation, the Ser/Thr phosphatase calcineurin. Thus, CsA inhibits the expression of cytokines such as interleukin-2 (IL-2) and proliferation of T cells (7, 8). It is well known that low-dose CsA therapy improves the clinical signs and symptoms of severely affected AD patients and is well tolerated and safe (6, 9).

The best-described regulatory T (Treg) cell population is characterized by the forkhead-winged-helix (FoxP3) transcription factor. FoxP3⁺ Treg cells have immunosuppressive capacity on effector T (Teff) cells and other immune cells (10). A role for Treg cells in AD has been suggested by the ubiquitous manifestation of AD in children with immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) and an AD-like phenotype in mice with a loss-of-function Foxp3 mutation (11). A recent study assumed an impact of superantigens

on Treg cell functionality and thereby on pathophysiology of AD (12).

The observation that severely affected AD patients respond well to systemic CsA treatment even at low doses prompted us to investigate whether Treg cells are functional and induced by low-dose CsA therapy.

Methods

Subjects

Subjects included in this study were 12 untreated AD patients, 20 AD patients under low-dose CsA therapy, and 17 healthy age- and sex-matched volunteers (Table 1). Atopic dermatitis patients aged from 23 to 65 years were moderate or severely affected and fulfilled the criteria of Hanifin and Rajka (13). The clinical severity during therapy was monitored by the SCORAD score (3). Two AD subjects were also affected by asthma. None of the patients had other skin diseases beside AD and they did not receive any systemic corticosteroids. Atopic dermatitis patients taking part in this study received a low-dose (2 mg/kg/day; 33–149 µg/l CsA serum concentration; own data) but not a high-dose CsA treatment (4–5 mg/kg/day; 165–501 µg/l CsA serum concentration; 14). The patients took CsA tablets p.o. in two divided doses with good tolerability and achieved CsA blood levels of 89.71 ± 36.1 µg/l (Table 1). During low-dose CsA therapy first samples were taken after 3 weeks of treatment. Usually, the standard low-dose CsA therapy lasts from several weeks up to several months. However, the clinical symptoms already improved after 2 weeks of treatment in all AD patients studied here. So far, the duration of CsA therapy in patients of our study ranged from 20 to 40 weeks and is still ongoing in the majority of the patients. For FACS analysis and suppression assay

Table 1. Clinical characteristic of study subjects

Subjects	HC	AD	AD + low-dose CsA
Number	17	12	20
Age (years)	33 ± 10	47 ± 10	43 ± 13
Sex, male/female	9/8	6/6	7/13
SCORAD	NA	53 ± 13	27 ± 15
Asthma	NA	1	1
CsA dose (mg/kg/day)	NA	NA	2
CsA serum concentration (µg/l)	NA	NA	89.71 ± 36.1
Performed test systems	Number of included subjects		
IL-2 (% abs. cell number)	17	12	20 (8)
FoxP3 (% abs. cell number)	17	12	20 (8)
CLA (% abs. cell number)	7*	8†	5‡ (5)
Suppression assay	4*	3†	3‡ (0)
CD3, CD4, CD8 (abs. cell number)	17	12	20 (8)
SCORAD	NA	11†	12‡ (7)
CsA serum concentration (µg/l)	NA	NA	14‡

Values within parenthesis indicate the number of patients, which are included in the AD group (before CsA therapy) and in the AD + low-dose CsA group (during CsA therapy).

HC, healthy control; AD, atopic dermatitis; SCORAD, scoring atopic dermatitis; CsA, cyclosporin A; IL, interleukin; CLA, cutaneous lymphocyte-associated antigen; NA, not applicable.

*Out of 17 HC donors.

†Out of 12 untreated AD patients.

‡Out of 20 low-dose CsA-treated AD patients.

experiments, 20 and 50 ml of peripheral blood, respectively, were collected directly before the next CsA intake in the morning. Study protocol was approved by the local ethics committee of the Charité.

Intracellular IL-2 expression in whole blood

Heparinized peripheral blood (1 ml) was stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 2.5 µg/ml ionomycin (Sigma, Munich, Germany) for 5 h at 37°C. At 1 h of incubation, 10 µg/ml Brefeldin A (Sigma) was added and at 5 h, 100 µl/ml cold 20 mM PBS/EDTA was added. After erythrocyte lysis with FACS Lysing Solution (Becton Dickinson, Heidelberg, Germany), and lymphocyte permeabilization with FACS Permeabilizing Solution 2 (Becton Dickinson), the lymphocytes were stained with anti-CD3-FITC (UCHT1) and anti-IL-2-APC (MQ1-17H12; Becton Dickinson). Typically, 20 000 CD3⁺ cells were analyzed using four-color flow cytometry (FACSCalibur, Becton Dickinson).

Flow cytometry of T cell subsets

Peripheral blood mononuclear cells (PBMC) were separated as described (15). Fixed and permeabilized cells (Human Regulatory T cell Staining Kit, eBioscience, San Diego, CA, USA) were stained with anti-FoxP3-FITC (PCH101, eBioscience, San Diego, CA, USA), anti-CD4-Cy5 (TT1), anti-CLA-PE (HECA-452; Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD25-APC, and anti-CD127-PE antibodies (2A3 and hIL-7R-M21; Becton Dickinson). The absolute cell numbers were determined with the TruCOUNT tube technology (Becton Dickinson) using the following antibodies: CD3-FITC (UCHT1), CD4-Cy5 (TT1), and CD8-PE (GNII/134.D7). Typically, 100 000–200 000 total events or 3000–5000 TruCOUNT beads were analyzed by flow cytometry (FACSCalibur; Becton Dickinson) with appropriate compensation settings and isotype controls to establish background fluorescence for each fluorochrome.

Isolation of CD4⁺CD25[−]CD127⁺ responder T cells and CD4⁺CD25⁺CD127^{low} Treg cells

Blood was collected into Na-heparin tubes and PBMC were separated. Positive selected CD4⁺ cells (MACS[®] Separation Reagents; Miltenyi Biotec, Bergisch-Gladbach, Germany) were stained with anti-CD25-APC and anti-CD127-PE antibodies (2A3 and hIL-7R-M21, Becton Dickinson) and sorted (Aria sorter, Diva software; Becton Dickinson). The CD25/CD127 gating was performed as shown in Fig. 5A. The purity of the sorted CD4⁺ populations was ≥ 98% (CD25[−]CD127⁺ FoxP3⁺) and ≥ 96% (CD25⁺CD127^{low} FoxP3⁺).

Suppression assay

To analyze the suppressive capacity, sorted CD4⁺CD25[−]CD127⁺ and CD4⁺CD25⁺CD127^{low} T cells were placed in 96-well plates (2.5×10^4 cells/well) and stimulated with 0.2 µg/ml anti-CD3 (OKT3; eBioscience) and 0.5 µg/ml anti-CD28 (15E8; Chemicon, Chemicon International, Billerica, MA, USA) monoclonal antibodies (mAbs) or 1 µg/ml SEB (Sigma) in the presence of 2.5×10^4 APCs in a final volume of 200 µl of complete RPMI medium. Cultures were incubated for 96 h at 37°C in a humidified, 5% CO₂-containing incubator. Sixteen hours before harvesting, tritiated thymidine (Hartmann, Braunschweig, Germany) was added at a final concentration of 1 µCi per well. Cells were then harvested

(FilterMate Harvester 96; Perkin Elmer, Milano, Italy) onto GF/B filters (Unifilter 96 GF7b; Perkin Elmer) and placed into liquid scintillation cocktail (Microscint 0; Perkin Elmer), and incorporated tritium was counted in a microplate scintillation counter (TopCount NXT; Perkin Elmer). For all suppression assays, the experimental values reported represent the mean values of triplicate from three independent experiments.

Statistical analysis

GRAPHPAD software (San Diego, CA, USA) was used for all statistical analysis. All data are expressed as means $\pm$ SD. Statistical analysis was performed with the Student's *t*-test if values were normally distributed, or otherwise by the Mann-Whitney test. A *P*-value < 0.05 was considered statistically significant.

Results

The ability of T cells to be activated is diminished but not blocked by low-dose CsA therapy

Low-dose CsA therapy (2 mg/kg/day) was applied to patients with severe AD and a SCORAD score (3) higher than 37. The SCORAD index was monitored before onset of CsA and during low-dose CsA treatment, and showed a significant reduction by CsA (53.1 vs 27.2; $P < 0.005$; Table 1).

Using the whole blood assay developed by us, we measured the potential of IL-2 cytokine production

after PMA/ionomycin (50 ng/ml/2.5 μ g/ml) stimulation of whole-blood samples. Therewith we investigated and compared the ability of peripheral T cells to be activated in blood samples of healthy control (HC) subjects and AD patients with or without low-dose CsA therapy. IL-2 producing CD3⁺ cells, determined by intercellular cytokine staining for IL-2, surface staining for CD3, and flow cytometric measurement, were significantly higher in patients with AD ($20.3 \pm 2.0\%$) compared with HC subjects ($13.8 \pm 1.1\%$; $P < 0.005$) and AD patients treated with low-dose CsA ($8.7 \pm 0.9\%$; $P < 0.0005$; Fig. 1A). TcR signaling in peripheral blood T cells of low-dose CsA-treated AD patients was reduced to $42 \pm 19\%$ compared with untreated AD subjects but was not totally blocked after the onset of low-dose CsA treatment.

Similarly, absolute numbers of IL-2 producing CD3⁺ T cells from AD patients under CsA therapy are lower compared with those from HC and AD subjects ($P < 0.005$; Fig. 1B). These blood samples were taken when CsA blood concentration was the lowest, i.e. in the morning directly before the next CsA intake. In addition, the IL-2 production of T cells was measured in blood samples from treated AD patients at the CsA peak concentration, 2 h after CsA intake in the morning ($3.4 \pm 0.7\%$; $P < 0.0005$, data not shown). The ability of T cells to be activated in AD patients 2 h after CsA

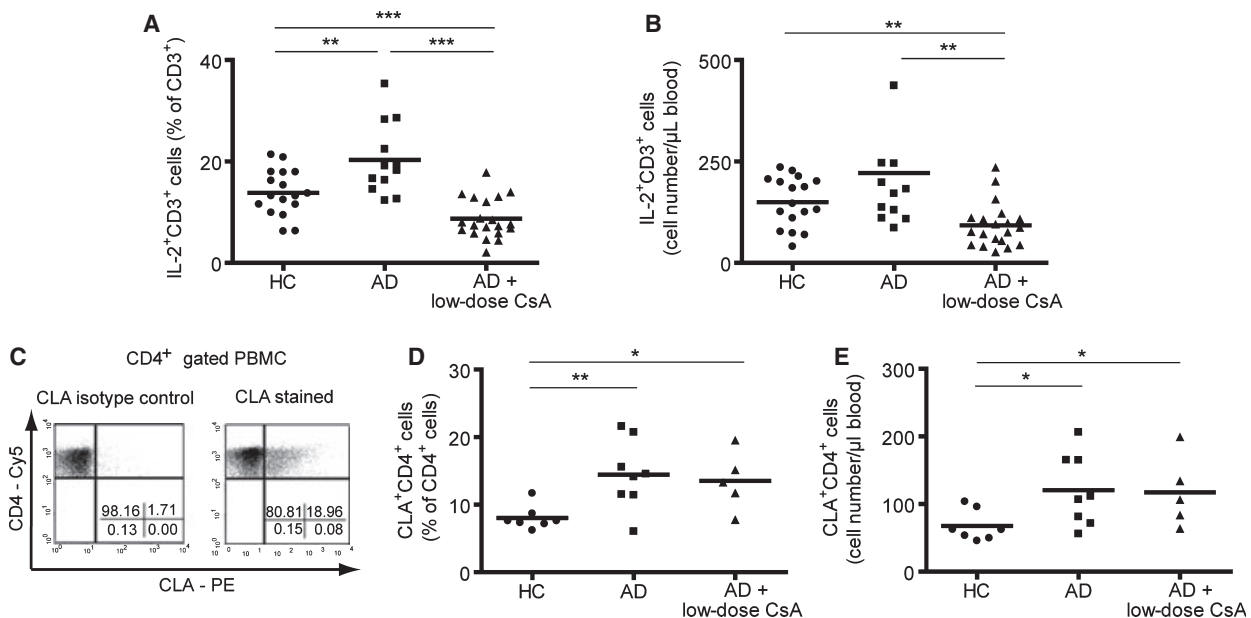


Figure 1. Low-dose CsA therapy diminished the IL-2 but not the CLA expressing lymphocytes. (A) Percentage and (B) absolute cell numbers of IL-2 expressing CD3⁺ T cells were examined in stimulated whole-blood samples of AD patients ($n = 12$), AD patients with CsA therapy ($n = 20$), and healthy subjects (HC; $n = 17$). (C) CLA expression within the CD4⁺ T cell population was measured by surface staining of CD4 and CLA (lower panel). The CLA unstained sample served as control (upper panel). (D) Percentage and (E) absolute cell numbers (calculated using TruCOUNT technology) of CLA expressing T cells were determined by gating the CD4⁺ population out of PBMC (HC, $n = 7$; AD, $n = 8$; AD with low-dose CsA therapy, $n = 5$). * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

intake is reduced to $17 \pm 11\%$ compared with untreated AD patients.

The range of absolute numbers and frequencies of IL-2⁺CD3⁺ T cells was stable in individuals from all groups measured repeatedly within several weeks.

AD patients have similar numbers of peripheral T cells but diminished numbers of cytotoxic T cells compared with HC subjects

To examine the effect of low-dose CsA treatment on the composition of different T cell subpopulations, we measured frequencies and numbers of peripheral CD3⁺, CD4⁺ and CD8⁺ T cells. Absolute cell numbers and frequencies of both CD3⁺ and CD4⁺ cells were not significantly different between the three groups studied (data not shown). In contrast, CD8⁺ cell number was lower in AD patients without CsA compared with HC subjects (data not shown). The numbers of CD3⁺, CD4⁺ and CD8⁺ cell population did not change within five individual subjects at different time points within 20 weeks (data not shown).

We measured the widely used surface marker CLA (4, 5) to determine skin-homing properties of peripheral T cells in AD patients (see Fig. 1C). We identified significant higher frequencies of CLA⁺ T cells in AD patients without ($14.4 \pm 1.8\%$; $P < 0.005$) and with low-dose CsA therapy ($13.5 \pm 1.9\%$; $P > 0.05$) compared with HC subjects ($8.0 \pm 0.7\%$; Fig. 1D). Similarly, we found that AD patients had significantly higher numbers of CLA⁺ T cells than HC subjects independent of CsA treatment (Fig. 1E).

AD patients with low-dose CsA therapy have higher numbers of Treg cells compared with HC subjects and AD patients without CsA therapy

The best characterized Treg cell subset expresses the forkhead transcription factor FoxP3. Our intracellular FoxP3 staining (Fig. 2A) demonstrated that AD patients treated with low-dose CsA for at least 3 weeks had significantly higher frequencies of FoxP3 expressing T cells ($9.6 \pm 0.7\%$) than HC subjects ($6.9 \pm 0.3\%$; $P < 0.005$) and AD patients without CsA therapy ($6.8 \pm 0.8\%$; $P < 0.05$; Fig. 2B). Similar results were obtained by comparison of absolute FoxP3⁺ cell numbers (Fig. 2C). To validate that our FoxP3⁺ cells are indeed Treg cells, we co-stained FoxP3 with an alternative antibody (clone: 206/C7; BD) and measured in addition CD25 and CD127. We showed that both anti-FoxP3 antibodies (clone PCH101, eBioscience; clone 259D/C7; Becton Dickinson) recognized nearly the same cells expressing FoxP3 (Fig. S1). Furthermore, the FoxP3 expressing Treg cells were consistently high in CD25 and low in CD127 expression (Fig. 2D,E) and showed almost no cytokine expression with or without stimulation in all the three groups studied (Fig. S2).

Onset of low-dose CsA therapy enhances the Treg cell population in single AD patients

To provide direct evidence that low-dose CsA treatment induces Treg cells *in vivo*, we measured FoxP3⁺ T cells in eight single AD donors before and after onset of CsA therapy. Single AD patients showed a statistically significant increase in frequency (AD $6.3 \pm 0.6\%$ vs AD + CsA $11.5 \pm 1.2\%$; $P < 0.005$; Fig. 3A) and absolute cell number (Fig. 3B) of FoxP3⁺ Treg cells 3 weeks after onset of CsA therapy.

The onset of low-dose CsA therapy nearly doubled the mean frequency and mean absolute cell number of FoxP3⁺ Treg cells in the AD patients studied. A follow up of FoxP3⁺ Treg cells measured up to 20 weeks in five of these patients showed a stable enlarged FoxP3⁺ Treg-cell population during low-dose CsA therapy (e.g. frequencies: 3 weeks CsA treatment $11.8 \pm 3.7\%$ vs 20 weeks CsA treatment $11.4 \pm 2.1\%$; $P > 0.05$; absolute cell number: 3 weeks CsA treatment 116.2 ± 28.5 cells/ μ l vs 20 wks CsA treatment 102.1 ± 23.4 cells/ μ l; $n = 5$; $P > 0.05$; Fig. S3).

Treg cells in AD patients are functional and have skin-homing properties

To investigate the skin-homing property and function of Treg cells from AD patients, we studied the expression of CLA on FoxP3⁺CD4⁺ T cells by flow cytometry and performed suppression assays using [³H] thymidine incorporation, respectively. In AD patients, there are not only more CLA⁺ cells in the CD4⁺ population (Fig. 1D) but also in the FoxP3⁺ Treg-cell subpopulation. Peripheral blood mononuclear cells gated on CD4⁺ cells after staining (Fig. 4A) showed higher frequencies (HC $20.1 \pm 1.3\%$ vs AD $60.9 \pm 4.7\%$; $P < 0.0005$; HC vs AD + CsA $55.4 \pm 2.2\%$; $P < 0.0005$; Fig. 4B) and absolute cell numbers (Fig. 4C) of CLA⁺FoxP3⁺ cells in AD patients compared with healthy control (HC) subjects independent of low-dose CsA therapy.

The average of CLA⁺FoxP3⁺ cells/ μ l in AD patients without and with CsA therapy was different. However, this difference was not statistically significant.

To analyze the suppressive capacity of Treg cells on Teff cells, we sorted these populations from AD patients and HC subjects by flow cytometry using the surface markers CD25 and CD127 (Fig. 5A). The resulting Treg (CD4⁺CD25⁺CD127^{low}) and Teff cells (CD4⁺CD25^{low}CD127⁺) had purity $>96\%$ (Fig. 5B). Co-culture experiments with antibody stimulation showed that the concentration dependent suppressive effect of Treg cells on autologous Teff cells was similar in HC subjects and AD patients with or without CsA therapy (Fig. 5C). The [³H] thymidine intake of stimulated T cells (soluble anti-CD3/anti-CD28 mAbs) was suppressed in all the three groups by up to 70% at a ratio of Teff to Treg cells of 1 : 1. Furthermore, the Teff cells

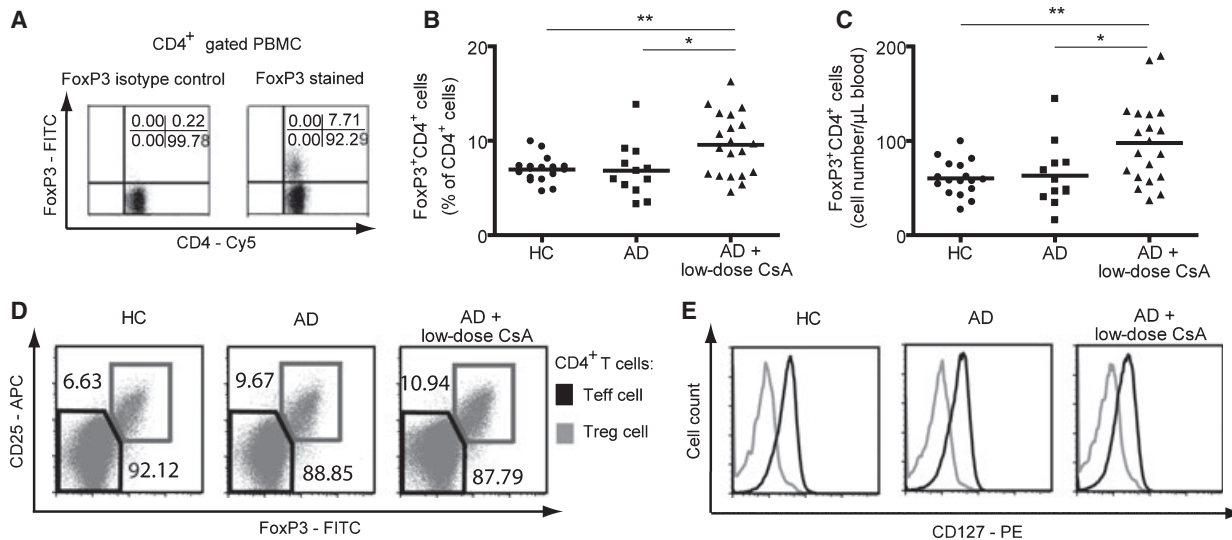


Figure 2. AD patients with low-dose CsA therapy have more FoxP3⁺CD4⁺ Treg cells. (A) FoxP3 expression within the CD4⁺ T cell population was measured by co-staining of surface CD4 and intracellular FoxP3 in PBMC (right panel). The FoxP3 isotype staining served as a control (left panel). (B) Percentage of FoxP3⁺CD4⁺ cells was determined by flow cytometry and (C) absolute cell number of the FoxP3⁺CD4⁺ T cell population was calculated using TruCOUNT technology (HC, $n = 17$; AD, $n = 12$; AD with low-dose CsA therapy, $n = 20$). * $P < 0.05$, ** $P < 0.005$. (D, E) Isolated human CD4⁺ T cells were stained for CD25, CD127 as well as intracellular FoxP3 and measured by flow cytometry. Treg and Teff cells were (D) gated according to their expression of CD25 and FoxP3 and (E) plotted as CD127 histograms. Data are representative for five independent experiments.

from AD patients were similarly susceptible to inhibition by autologous Treg cells and allogeneic Treg cells from a healthy donor (Fig. 5D). Using stronger stimulus (SEB and APC stimulation), higher numbers of Treg cells, namely a ratio of Teff to Treg cells of 1 : 4, were required to suppress Teff cell [³H] thymidine incorporation to 50% (Fig. 5E). Finally, AD patients with or without low-dose CsA therapy have functional Treg cells. In addition, Teff cells in all AD patients were found to be sensitive to Treg cell inhibition.

Discussion

Low-dose CsA therapy is an effective and good tolerable immunosuppressive treatment for severely affected AD patients (6, 9). Here, we showed that low-dose CsA leads not only to partial inhibition of T cell activation but also to an increased Treg-cell population. Both molecular mechanisms of low-dose CsA might contribute to the improvement of clinical symptoms in treated patients: CsA inhibits hyperactivation of T cells by allergens or superantigens directly by the inhibition of cytokine production as well as indirectly by enhanced numbers of functional FoxP3⁺ Treg cells.

Atopic dermatitis is characterized by sustained inflammatory responses within the skin caused by an amplification cycle of atopic skin inflammation. The amplification is driven by pruritus, pro-inflammatory cytokines and chemokines, leukocyte recruitment and

activation, suppressed production of antimicrobial peptides as well as colonization of inflamed skin with microbes (16). Within these immunopathogenic processes, T cells play a crucial role, in particular, the CLA⁺ subpopulation, which has a memory T cell phenotype and skin-homing properties (5, 17). Allergen- and superantigen-hyperactivated T cells produce chemokines and typical T helper (Th) 1 and Th2 cytokines and in addition other pro-inflammatory cytokines such as tumor necrosis factor- α and IL-31 (16, 18).

The amplification cycle of skin inflammation is successfully disrupted by systemic CsA therapy or topical therapy with tacrolimus or pimecrolimus interfering mainly with T cell activation. Here, we observed that low-dose CsA therapy diminished the ability of T cells to be activated but not totally blocked it. Therefore, we assume that either partial inhibition of T cell activity might be already sufficient to improve the SCORAD value in AD patients. In addition, other molecular mechanisms might take place. We identified increased frequencies and numbers of FoxP3⁺ Treg cells in low-dose CsA-treated AD patients, which might also contribute to the improvement of clinical signs and symptoms. This assumption is supported by the observation that Treg cells in AD patients with or without therapy are functional and have a similar suppressive capacity as Treg cells from HC subjects in our study. Furthermore, a role for FoxP3⁺ Treg cells in AD has been corroborated by several lines of evidence (11). Recent studies in animal models have shown that

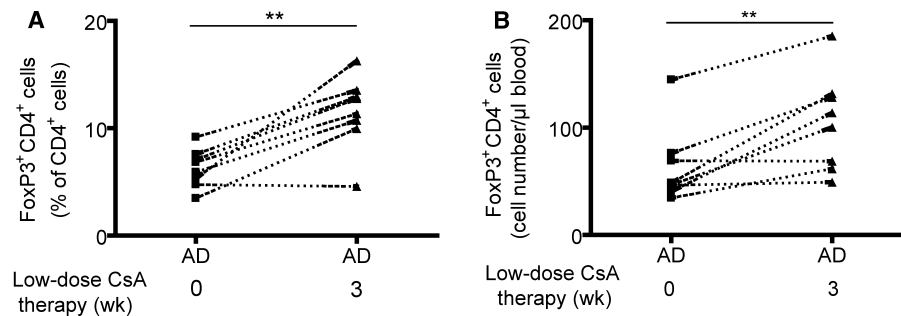


Figure 3. Onset of low-dose CsA therapy increased the FoxP3⁺CD4⁺ Treg cells in individual AD patients ($n = 8$). (A) Percentage of FoxP3⁺ cells within gated CD4⁺ T cell population and (B) absolute cell number of the FoxP3⁺CD4⁺ T cells before and 3 weeks after onset of CsA therapy. ** $P < 0.005$.

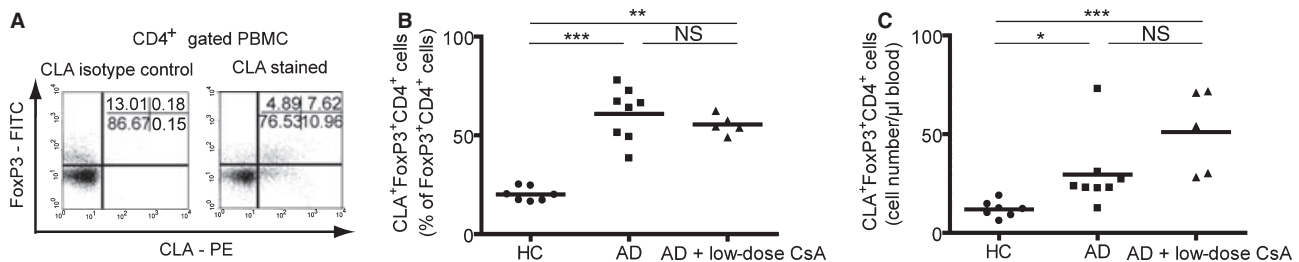


Figure 4. AD patients express more CLA on FoxP3⁺CD4⁺ Treg cells. (A) FoxP3 and CLA expression within the CD4⁺ T cell population was determined in PBMC by co-staining of surface CD4, surface CLA and intracellular FoxP3 and gating on CD4⁺ population (right panel). PBMC stained only for CD4 and FoxP3 served as a control (left panel). (B) Percentage of CLA⁺ cells was determined within gated FoxP3⁺CD4⁺ T cell population. (C) Absolute cell number of the CLA⁺FoxP3⁺CD4⁺ T cell population was calculated using true count technology (HC, $n = 7$; AD, $n = 8$; AD with low-dose CsA therapy, $n = 5$). * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

adoptive transfer of CD4⁺CD25⁺ Treg cells can prevent or even cure allergic diseases (19). It is suggested that abrogation of Treg-cell immunosuppressive function by stimulation with superantigen from *Staphylococcus aureus* is part of the molecular pathomechanism in AD (12). This implies that Treg cells could play a protective role in suppressing allergen-specific responses in general.

For atopic subjects, it has been suggested that a defect of allergen-specific Treg cells (20) and reduced numbers of Treg cells (21) are causal factors for allergic inflammation. The results of our study provide evidence that peripheral CD4⁺CD25⁺FoxP3⁺ Treg cells are neither absent nor functionally defective in AD patients in agreement with other recent reports (12, 22, 23). In addition, effector T cells from AD patients are not resistant to suppression because their Teff cells are prone to inhibition by autologous as well as allogeneic Treg cells.

We observed elevated frequencies and numbers of peripheral Treg cells with skin-homing properties (CLA⁺CD4⁺FoxP3⁺) in AD patients compared with HC subjects. Therewith, we confirmed a previous report showing a larger population of peripheral CLA⁺CD4⁺CD25⁺ cells in AD patients (24). All these data are in line with recent observations of higher numbers of FoxP3⁺ Treg cells in AD skin lesions (25, 26). Of potential therapeutic relevance is the finding that the

skin-homing CLA⁺CD4⁺FoxP3⁺ cell subpopulation remained elevated upon low-dose CsA treatment of the patients. However, we observed a much smaller population of CLA⁺ cells among the FoxP3 Treg cells in healthy subjects compared with other studies (27, 28). These varieties might be because of different CLA antibodies, different Treg markers and/or different gating strategies used.

Low-dose CsA treatment (2 mg/kg/day) increased the number and frequency of the peripheral Treg-cell subpopulation, but did not significantly change the CD3⁺, CD4⁺, and CD8⁺ cell populations in AD patients. The Treg cells from all the groups studied are characterized by consistently low expression of CD127 and high expression of CD25. Dual staining with two different antibody clones for Foxp3 confirmed our staining for Treg cells. Furthermore, cytokine staining of *ex vivo*-isolated CD4⁺ T cells revealed almost no cytokine expression in FoxP3⁺ cells without or with external stimulation. It remains unclear whether this increase in the Treg cell number is due to *de novo* induction or expansion of Treg cells by low-dose CsA.

In contrast to our own results, recent studies showed that CsA inhibits tolerance induction and even abrogates established tolerance (29–31). It is expected that CsA interferes with Treg-cell expansion and induction by

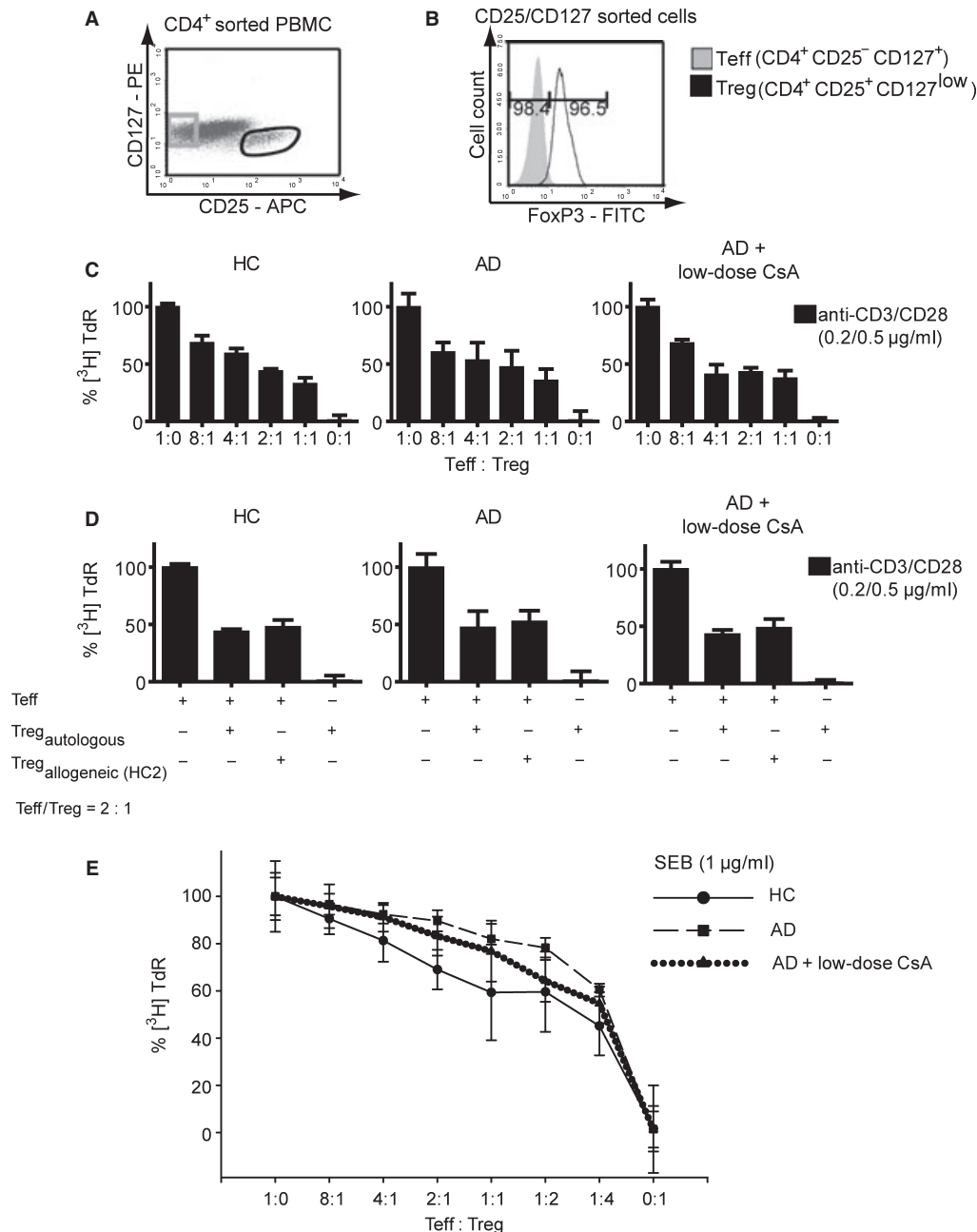


Figure 5. *Ex vivo*-isolated Treg cells from AD patients with or without low-dose CsA therapy are suppressive. Suppression assays were performed with flow cytometry-sorted Teff and Treg cells, which were stimulated with either anti-CD3/CD28 antibodies or SEB in the presence of APCs. (A) Freshly isolated CD4⁺ T cells stained with anti-CD25 and anti-CD127 antibodies were sorted for CD4⁺CD25^{low} CD127⁺ Teff cells and CD4⁺CD25⁺CD127^{low} Treg cells. (B) The FoxP3 expression was analyzed in these sorted fractions. (C, D, E) Autologous isolated Treg and Teff cells from HC and AD patients with and without CsA therapy were used in (C, E) different ratios or in (D) a Teff to Treg ratio of 2 : 1. In addition, (D) allogeneic isolated Treg cells from a healthy subject [the same healthy subject (HC₂) for all groups] were incubated with Teff cells from HC and AD patients with and without CsA therapy. (C, D, E) The proliferative response was measured by [³H] Thymidine incorporation ([³H]TdR). The presented data were normalized to Teff-cell proliferation without addition of Treg cells. Data are presented as mean ± SD of triplicate of three independent experiments.

blocking TcR signaling, which prevents, at high CsA concentrations, IL-2 production, and FoxP3 expression (29) as well as activation and differentiation of naïve Th cells and activation-induced cell death of alloreactive Th

cells (30, 31). However, these effects were observed *in vitro* and *in vivo* with high-dose CsA. Low-dose CsA, in contrast, showed positive and cumulative effects on tolerance in different transplantation models (32, 33)

and human kidney transplant patients (34). All these results are in line with our *in vitro* data on promotion or prevention of murine Treg-cell induction dependent on the CsA concentration used. Low or intermediate CsA concentrations (< 7 nM) promote the induction of Foxp3 expression in murine naïve T cells *in vitro*, whereas high CsA concentrations (> 10 nM) totally block this process (own unpublished data; 29). We hypothesize that low-dose CsA induces Treg cells by the simulation of a suboptimal activation of naïve T cells because of partial inhibition of TcR signaling, whereas high-dose CsA may block T cell activation and therefore differentiation. This is in agreement with the findings that a low or impaired stimulation of naïve T cells promotes efficient murine Foxp3 expression *in vitro* (35) and *in vivo* (36).

In summary, low-dose CsA therapy may represent an important strategy for the induction of tolerance *in vivo*, as it attenuates the immune response, selectively affects activated T cells and induces and/or expands peripheral Treg-cell population. With our results, we contribute to recent efforts to identify and develop novel Treg cell-based therapeutic strategies for allergic diseases (11, 37, 38).

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Dual staining of CD4⁺ cells with two different FoxP3 antibodies. Isolated human CD4⁺ T cells were stained using anti-FoxP3-Alexa488 (clone 259D/C7; BD) and anti-FoxP3-PE (clone PCH101; eBioscience) antibodies. The flow cytometry data are representative of two independent experiments.

Figure S2. Cytokine expression in FoxP3-expressing CD4⁺ Treg cells. Isolated human CD4⁺ T cells from (A) HC subjects, (B) AD patients without, and (C) with low-dose CsA treatment were stimulated with PMA/ionomycin, treated with brefeldin A 1 h after stimulation and cultured for further 4 h. Subsequently, the cells were fixed and stained for FoxP3. Intracellular cytokine production was determined by staining with anti-IL-10, -IL-17, -IL-4, -INF- γ , -IL-2 antibodies and by flow cytometric measurement. Data are representative of three independent experiments.

Figure S3. Frequencies and numbers of FoxP3⁺CD4⁺ Treg cells in AD patients did not change between 3 and 20 weeks of low-dose CsA therapy ($n = 5$). (A) Percentage of FoxP3⁺ cells within CD4⁺ T cell population and (B) absolute cell number of the FoxP3⁺CD4⁺ T cells 3 weeks after onset and 20 weeks of CsA therapy.

Data S1. Methods

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SUPPLEMENTAL MATERIAL METHODS

Intracellular cytokine expression in FoxP3⁺ CD4⁺ T cells

Peripheral blood mononuclear cells (PBMC) were separated as described (15). Positively selected CD4⁺ cells (MACS[®] Separation Reagents; Miltenyi Biotech) were stimulated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 µg/mL ionomycin (Sigma) for 5 h at 37°C. After 1 h of incubation 5 µg/mL Brefeldin A (Sigma) was added and after 5 h the cells were fixed (eBioscience kit). The cells were permeabilized (eBioscience kit) and subsequently stained with anti-FoxP3-FITC (PCH101, eBioscience) and different cytokine antibodies (anti-IL-10-Cy5 (19F11, own production); anti-INFγ-Cy5 (4SB3, own production); anti-IL-2-APC (MQ1-17H12, Becton Dickinson); anti-IL-17-Alexa647 (eBio64Dec17, eBioscience); anti-IL-4-APC (MP4-25D2, Becton Dickinson)). Typically 100.000-200.000 CD4⁺ cells were analyzed using 4-color flow cytometry (FACSCalibur, Becton Dickinson).

Dual staining for FoxP3

Peripheral blood mononuclear cells (PBMC) were separated as described (15). Positively selected CD4⁺ cells (MACS[®] Separation Reagents; Miltenyi Biotech) were fixed, permeabilized (eBioscience kit) and stained with anti-FoxP3-PE (PCH101, eBioscience, San Diego, USA) and anti-FoxP3-Alexa488 (259D/C7, BD). Typically 100.000-200.000 CD4⁺ T cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson) with appropriate compensation settings and isotype controls.

FIGURE LEGENDS

Figure S1. Dual staining of CD4⁺ cells with two different FoxP3 antibodies.

Isolated human CD4⁺ T cells were stained using anti-FoxP3-Alexa488 (clone 259D/C7, BD) and anti-FoxP3-PE (clone PCH101, eBioscience) antibodies. The flow cytometry data are representative for two independent experiments.

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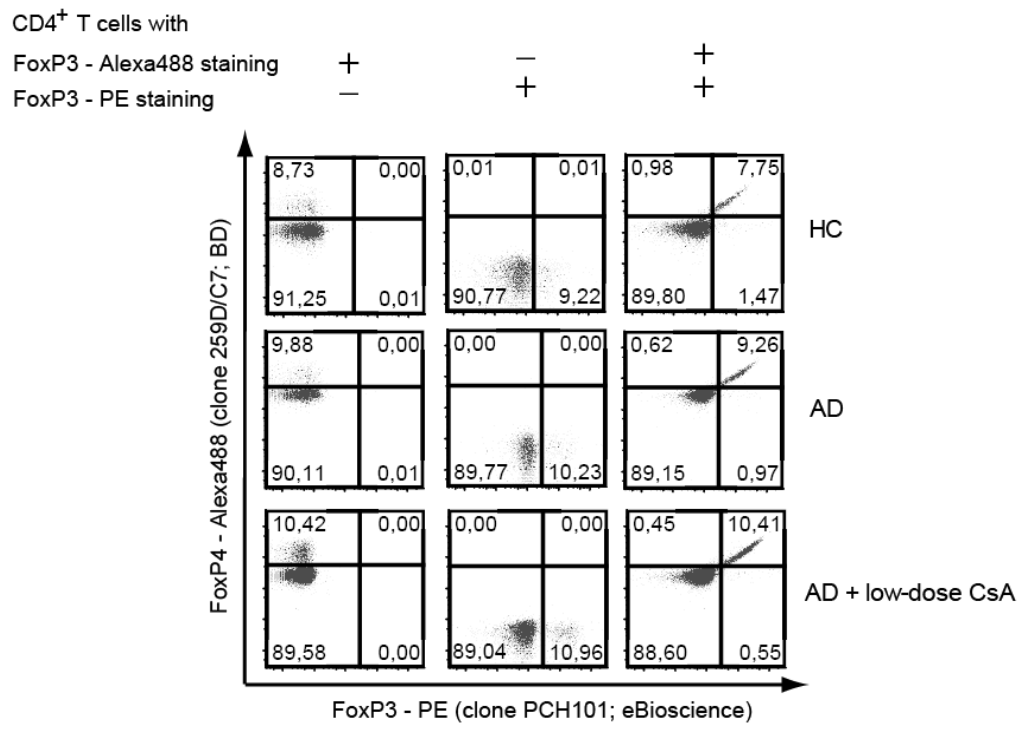


Figure S1

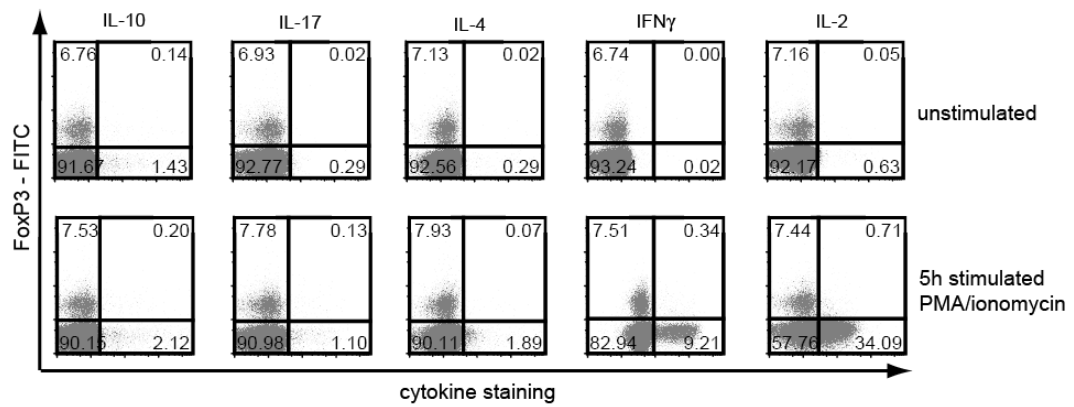
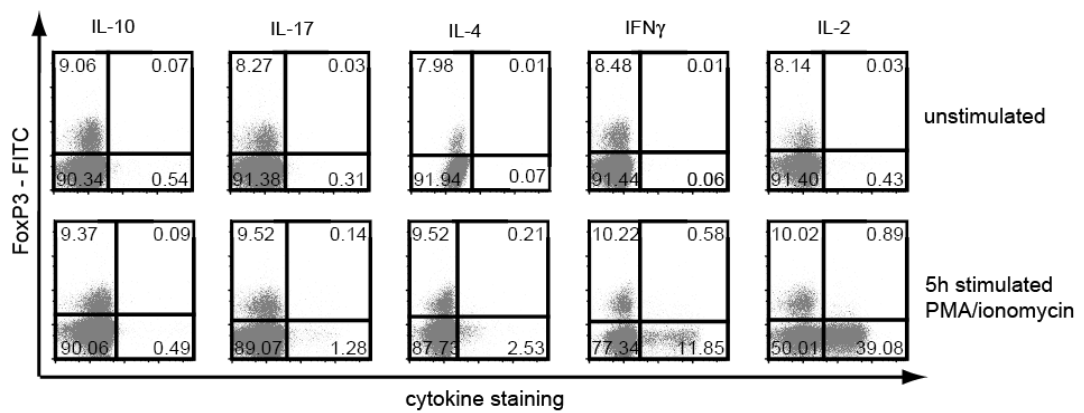
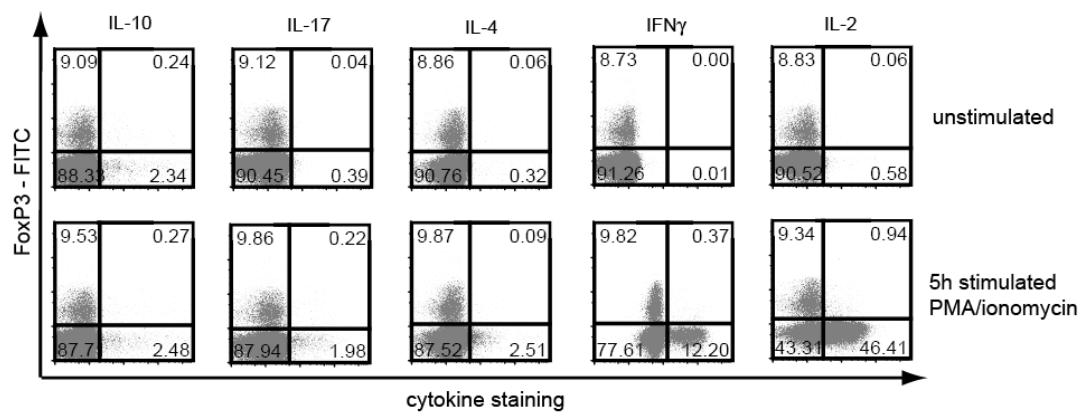
A HC (isolated CD4⁺ T cells)B AD (isolated CD4⁺ T cells)C AD + low-dose CsA (isolated CD4⁺ T cells)

Figure S2

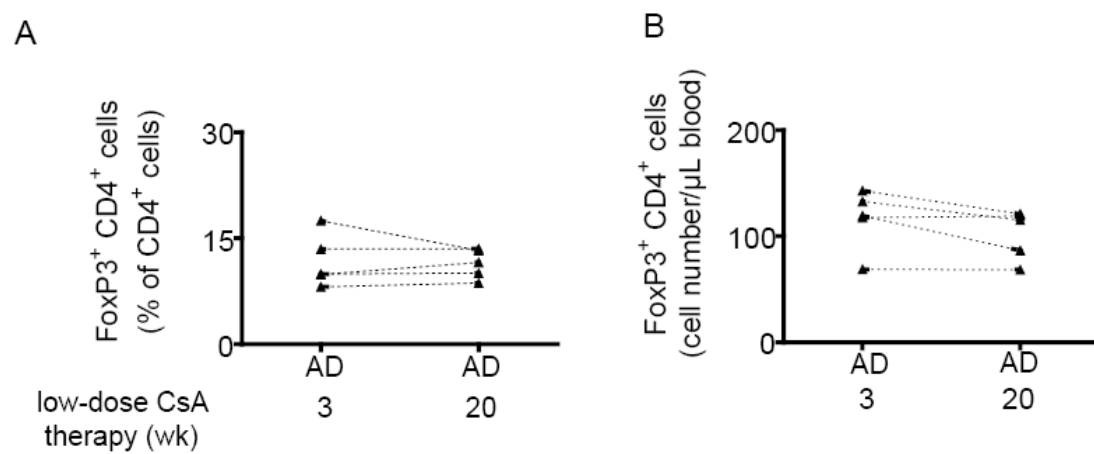


Figure S3