

Modulation of Lymphocyte Function *In Vivo* via Inhibition of Calcineurin or Purine Synthesis in Patients with Atopic Dermatitis

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TO THE EDITOR

In atopic dermatitis (AD), lymphocytes have an important role via regulation of the immune response, production of Igs, and sustaining cutaneous inflammation.

In AD, activated T cells can be found in blood, and most importantly in affected skin a marked increase of activated T cells is present (Akdis *et al.*, 2006). Serum levels of T-cell chemokines CCL17 (thymus and activation regulated chemokine, TARC) and CCL27, as well as their receptors on T cells, CCR4 and CCR10, are increased in patients with AD and are correlated with disease activity (Kakinuma *et al.*, 2001; Hijnen *et al.*, 2004). In a differential gene array analysis of CD4 cells, we demonstrated decreased expression of several genes related to activation of regulation, such as tumor necrosis factor- α inducible protein-3 (TNF- α IP3), also named A20, and GADD45a (Hijnen *et al.*, 2005). A20 is considered a negative regulator of inflammation (Sun, 2008). Decreased A20 expression level in T cells in AD patients may therefore be the primary cause of the activated state of these cells.

In patients with severe AD, treatment with the calcineurin inhibitor cyclosporine A (CsA) is effective. Recently, mycophenolic acid (MPA), an inhibitor of purine synthesis in both T and B lymphocytes, has been used in treatment of severe AD. In a recent study, we have evaluated the clinical

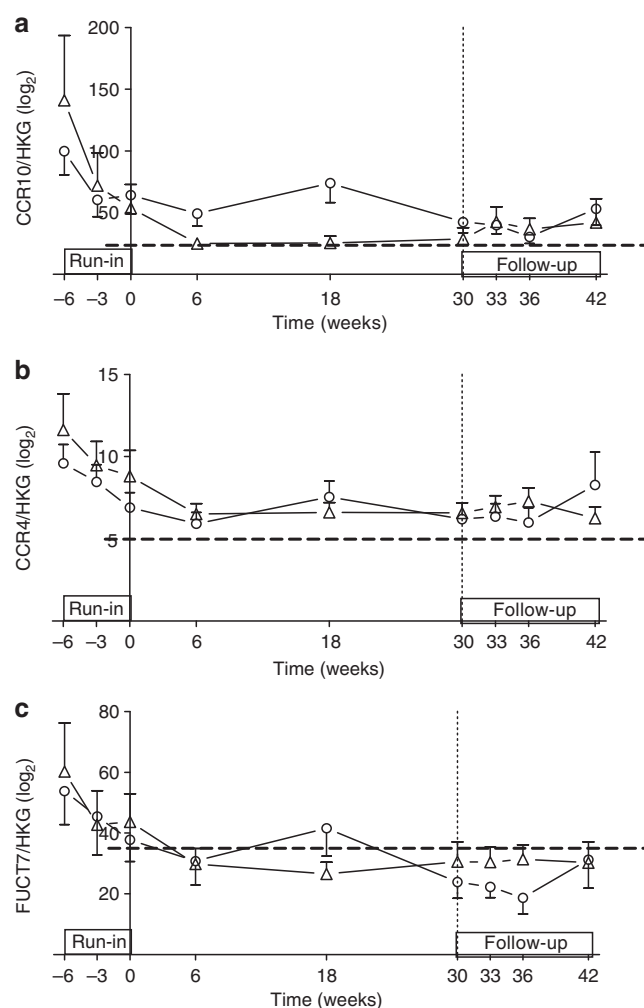


Figure 1. Changes in skin-homing marker expression. Relative mRNA levels were determined for CCR10 (a), CCR4 (b), and fucosyl transferase VII (FUCT-VII, c). Relative expression is determined as described in Materials and Methods. The horizontal line depicts the expression level in 20 nonallergic controls. These control values were CCR10 19.8 ± 2.6 /HKG; CCR4 4.69 ± 0.4 /HKG; and FUT7 31.9 ± 6.4 /HKG. O, cyclosporine A; Δ, enteric-coated mycophenolate sodium. Data are shown as mean $\pm$ SEM.

Abbreviations: AD, atopic dermatitis; CsA, cyclosporine A; EC-MPS, enteric-coated mycophenolate sodium; FUCT-VII, fucosyl transferase VII; MPA, mycophenolic acid; TARC, thymus and activation regulated chemokine; TNF- α IP3, tumor necrosis factor- α inducible protein-3

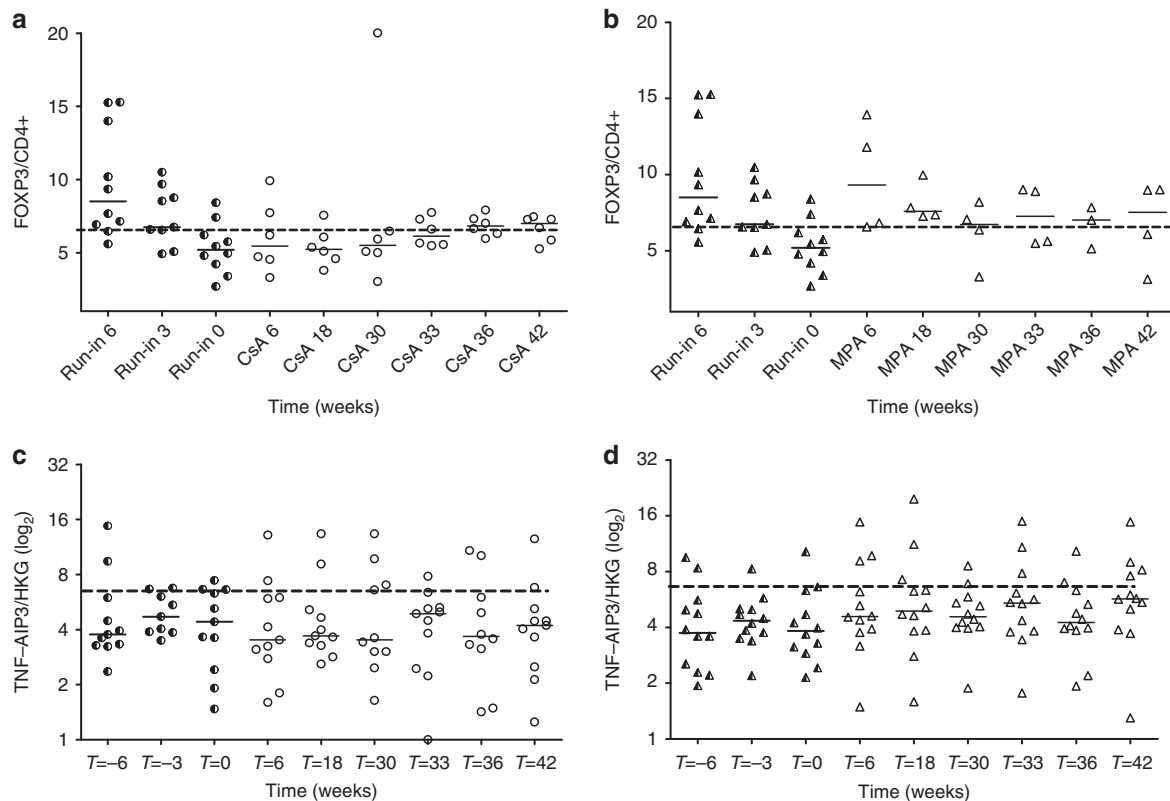


Figure 2. Expression of markers of regulation during treatment. The amount of regulatory T-cell markers was quantified at the level of FOXP3 protein expression in CD4⁺ lymphocytes determined by flow cytometry during cyclosporine A (CsA) treatment (a) and during enteric-coated mycophenolate sodium (EC-MPS) treatment (b). Expression of A20/tumor necrosis factor- α inducible protein-3 (TNF-AIP3) mRNA expression in CD4⁺ T cells during CsA treatment (c) and during EC-MPS treatment (d). Each symbol depicts the determination in an individual patient, and the horizontal mark depicts the median. The horizontal line depicts the expression level in 20 nonallergic controls. O, CsA; Δ, EC-MPS. MPA, mycophenolic acid.

effect of these immunosuppressive drugs in AD (Haecck *et al.*, 2011). After a run-in on 5 mg kg⁻¹ CsA, AD patients were treated with 3 mg kg⁻¹ CsA or 1,440 mg enteric-coated mycophenolate sodium (EC-MPS) for 30 weeks. Treatment resulted in reduction in disease severity as determined by SCORAD and serum TARC (CCL17) levels. The aim of the current study was to analyze the effect of treatment of AD patients with CsA ($n=11$) or EC-MPS ($n=12$) on immune cells to relate clinical improvement with immunological changes. Therefore, we analyzed T and B cells at the level of cell numbers, activation state, chemokine receptors, skin-homing molecules, and expression of FOXP3 and A20 in CD4⁺ T cells. Analyses were performed during run-in, and during study medication. CD4⁺ T-cell isolation and mRNA analysis was performed as described earlier (Hijnen *et al.*, 2005). In addition,

20 healthy nonallergic controls were included for a single leukocyte analysis. Differences between time points, as well as treatment groups, were tested using a paired *t*-test for normally distributed parameters and a Wilcoxon signed-rank test for nonnormally distributed parameters.

During run-in and maintenance, neither the percentage of CD3⁺ T cells nor the percentage of CD4⁺ and CD8⁺ within the CD3 population changed. In addition, the distribution of memory and naive CD4⁺ T cells, characterized by CD45RO vs. CD45RA, remained stable during treatment. In addition, CD19⁺ B-cell percentages did not change, nor was expression of the activation marker CD69 altered on B cells.

Expression of CCR10 and CCR4 mRNA did reduce during the run-in period with 5 mg kg⁻¹ CsA ($P=0.001$ and 0.005 for CCR4 and CCR10,

respectively; Figure 1a and b). The gene expression of fucosyl transferase VII (FUCT-VII) followed a similar pattern as the chemokine receptors (Figure 1c).

Owing to the setup of the study, we were unable to analyze functional responses of the regulatory T cells. Therefore, we used three different markers, namely CD4⁺CD25^{high} expression by flow cytometry, expression of FOXP3 mRNA (data not shown), and, for a limited group of patients, we analyzed FoxP3 protein expression in the CD4⁺ T cells (Figure 2a and b). Already at the 3-week run-in time point, there was a maximal decrease for all markers. There was no effect on the expression of A20 in both treatment regimes (see Figure 2c and d).

In this study, we showed that the clinically successful treatment of severe AD with CsA or EC-MPS results in

selective changes in lymphocyte parameters. Already during the run-in phase with 5 mg kg⁻¹ CsA, the reduction of skin-homing markers and regulatory T-cell (Treg) markers was achieved. This reduction was further sustained during the maintenance period with 3 mg kg⁻¹ CsA and EC-MPS.

The decrease in the three skin homing-related markers in CD4⁺ T cells indicates that during treatment peripheral blood T cells have a diminished capacity to enter the skin. Interestingly, when we analyzed changes in CCR8, related to Th2-type inflammation, and CXCR3, related to Th1-type inflammation (Islam *et al.*, 2011), we found a more delayed ($t \geq 6$ weeks, after randomization) or no decrease for CCR8 and CXCR3, respectively (data not shown). This indicates that the changes in CCR4, CCR10, and FUCT-VII are directly linked to the clinical improvement.

The markers for Tregs change in a manner similar to the clinical score of the eczema and serum TARC levels of the patients. *In vitro*, CsA inhibits Treg promoter activity (Mantel *et al.*, 2006). On the other hand, in another study, it was found that neither CsA nor MPA has an effect on the expression of Treg transcription marker FOXP3 (Abadja *et al.*, 2009). In our view, it is more likely that the decreased Treg levels are due to decreased inflammatory signals by treatment with CsA and EC-MPS. Unfortunately, we were not able to test the functional capacity of this Treg population, which is important.

No increase in the expression of A20 was shown, although the patients' condition clinically improved. One of the explanations is that A20 expression is not related to AD severity, but that its expression is more upstream in the regulation of T cells, as it is supposed to be important in several chronic

inflammatory diseases (Elder *et al.*, 2010). A20 is involved in termination of NF- κ B signaling. Therefore, we checked the expression of lymphotoxin- α , which is involved in NF- κ B activation (Schneider *et al.*, 2004), and could not find a changed expression (not shown). Another A20 family member is Sezanne (Ruland, 2011). Its expression was also not changed during treatment (not shown).

We conclude that treatment of patients with severe AD with drugs affecting lymphocytes have selective effects on lymphocytes. Mainly the expression of skin-homing molecules is diminished, limiting the potency of T cells to enter skin sites. On the other hand, the decrease in Treg markers might be the result of decreased inflammation, and together with the persistent decreased expression of A20/TNF-AIP3 might be important for the only transient improvement often seen after CsA treatment.

The study was approved by the local ethics committee and patients gave their written informed consent. This study was conducted according to the Declaration of Helsinki Principles.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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