In vitro Conversion into CD4⁺CD25⁺Foxp3⁺ Induced Regulatory T Cells Is Reduced in Atopic Dermatitis Patients

Eva-Maria García Jorge Galicia-Carreón Natalija Novak

Department of Dermatology and Allergy, University Hospital Bonn, Bonn, Germany

Keywords

Atopic dermatitis · In vitro conversion · Induced regulatory T cells · T cell activation · TGF-β signaling

Abstract

Background: Atopic dermatitis (AD) is one of the most common inflammatory skin diseases, with an increasing incidence in clinical practice. AD models have demonstrated that TGF-β signaling is compromised in regulatory T cells (Tregs). **Objectives:** This study aimed to investigate the TGF-β-dependent in vitro conversion of CD4⁺CD25[−] T cells derived from AD-patients into CD4⁺CD25⁺Foxp3⁺ induced Tregs (iTregs) in comparison to healthy controls. **Methods:** To analyze in vitro iTreg conversion, human CD4⁺CD25[−] T cells were cultured on anti-CD3-coated plates in the presence of TGF-β and IL-2 for up to 3 days. Consequently, the underlying mechanism of impaired CD4⁺CD25⁺Foxp3⁺ iTreg generation was explored by focusing on TGF-β signaling. Finally, the functionality of iTregs was investigated. **Results:** Conversion of CD4⁺CD25[−]Foxp3[−] into CD4⁺CD25⁺Foxp3⁺ iTregs was diminished in AD individuals. Impaired iTreg generation was accompanied by a reduced surface expression of GARP (glycoprotein A repetitions predominant), a marker for activated Tregs. A reduced expression of Smad3 mRNA was revealed in CD4⁺CD25[−] T cells. Interestingly, the sup-

pressive quality of iTregs was found to be equal in cells derived from AD and healthy donors. **Conclusion:** The signaling effect of TGF-β receptors on the suppressor quality of iTreg conversion is conserved. Impaired iTreg generation might be a reason for the lack of immune suppression in AD patients and contributes to the chronicity of the disease.

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disorder characterized by a dysregulated T cell response. As in many inflammatory diseases, regulatory T cells (Tregs) are thought to play an important role in AD pathogenesis [1]. However, several controversial reports have been published about the number and the in vitro conversion of circulating Tregs in AD [2, 3]. Activated Tregs are well known to anchor soluble latent TGF-β by the expression of GARP (glycoprotein A repetitions predominant) [4]. TGF-β-GARP binding leads to the formation of heterodimeric transmembrane receptor complex-

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Fig. 1. **a–c** Frequency of CD4⁺CD25⁻Foxp3⁻, CD4⁺CD25⁺Foxp3⁻, and CD4⁺CD25⁺Foxp3⁺ T cells in the peripheral blood of AD patients and healthy donors (CTR), measured by flow cytometry. **a** CD4⁺CD25⁻Foxp3⁻ T cells in AD and CTR. **b** CD4⁺CD25⁺Foxp3⁻ T cells in AD and CTR. **c** CD4⁺CD25⁺Foxp3⁺ T cells in AD and CTR (* *p* = 0.014). **d** Expression of GARP on CD4⁺ T cells after 1 day of culture (*n* = 13/10 [AD/CTR]; * *p* = 0.02). **e** Expression of TβRI–III measured on CD25⁻ cells directly after isolation. **f, g** Expression of TβRI–III on CD25⁺Foxp3⁻ and CD25⁺Foxp3⁺ cells after 3 days of stimulation. **f** * *p* = 0.021, ** *p* = 0.0013. **g** * *p* = 0.023.

es consisting of TGF-β receptor (TβR) I–TβRII and supported by TβRIII to induce phosphorylation of R-Smads (receptor-mediated Smads) [5, 6]. Nevertheless, the role of TGF-β in the induction of Tregs and its effect on the activation and functionality of circulating Tregs in AD conditions are not entirely understood. Therefore, this work investigated the effect of TGF-β receptors, and the Smad-signaling pathway involved in CD4⁺ T cell conversion into induced Tregs (iTregs), in AD-patients compared to healthy subjects.

Materials and Methods

Thirty-seven patients with AD (mean age 37 years, range 18–66) and 38 healthy volunteers (mean age 32 years, range 19–61) from the Department of Dermatology and Allergy, University Hospital Bonn, were studied. Peripheral blood mononuclear cells isolated from AD patients and healthy donors were used for negative selection of CD4⁺CD25⁻ T cells on AutoMACS. CD4⁺CD25⁻ T cells were cultured on anti-CD3-coated plates in the presence of recombinant human IL-2 and TGF-β at 1 million cells/ml in RPMI 1640. CD4⁺ T cells were harvested after 1 or 3 days of culture for the detection of CD25, GARP, FOXP3, and TβRI–III by flow cy-

tometry. Likewise, CD4⁺CD25⁻, CD4⁺CD25⁺, and iTregs were analyzed for the constitutive and TGF-β-induced expression of activating Smad3 and inhibitory Smad7 at the transcriptional and protein levels. Total RNA was isolated from CD4⁺CD25⁻ T cells or from magnetically enriched CD4⁺CD25⁺ iTregs after 3 days of culture with a NucleoSpin RNA kit (online suppl. material; see www.karger.com/doi/10.1159/000506285 for all online suppl. material). Student's *t* test was used to compare the data of patients with AD to those from healthy subjects. The statistical analysis was performed using GraphPad Prism 5.01 software. Data are presented as means ± SEM, and *p* < 0.05 was considered statistically significant.

Results and Discussion

Initially, we measured the frequency of CD4⁺CD25⁺Foxp3⁺ cells in circulating T cells (Fig. 1). Activation per se was not impaired in CD4⁺ T cells, and no difference was detected in the percentage of CD25⁻Foxp3⁻ and CD25⁺Foxp3⁻ cells (Fig. 1a–b). However, the amount of circulating Foxp3-expressing CD4⁺CD25⁺ cells (Tregs) was significantly higher in AD patients

Fig. 2. **a** RNA expression of SMAD3 in CD4⁺CD25⁻ T cells of AD patients in comparison to CTR after isolation. * $p = 0.0059$. **b** RNA expression of SMAD7 in CD4⁺CD25⁻ T cells of AD patients in comparison to CTR after isolation. * $p = 0.0065$. **c** Phosphorylated (p-) Smad2/3 was detected with Western blot. One representative experiment out of 10 is shown. **d** The ratio of p-Smad2/3 expression of stimulated and unstimulated cells evaluated by Western Blot (**c**) is shown (AD: 1.8-fold increase; CTR: 2.4-fold increase; $n = 10$; $p = 0.3$). Data are shown as means $\pm$ SEM. Statistical significance was determined by a t test.

(Fig. 1c). To evaluate the regulatory phenotype and the induction level of Tregs, we next investigated the in vitro conversion of peripheral blood CD4⁺CD25⁻ T lymphocytes into CD4⁺CD25⁺Foxp3⁺ T cells (iTregs) (online suppl. material). After 3 days of culture, we observed the highest frequency of iTregs (CD25⁺Foxp3⁺) in healthy controls (CTR). Conversely, the percentage of iTregs was significantly lower in AD-derived CD4⁺ cells (AD: 8.5% $\pm$ 1.7; CTR: 14.6% $\pm$ 2.0; $n = 12/10$; $p = 0.03$). Besides, our results show that the number of CD4⁺CD25⁺Foxp3⁺ T cells was the same in AD as in healthy donors (AD: 17.7% $\pm$ 3.4; CTR: 17.7% $\pm$ 3.6, $n = 12/10$).

To determine the in vitro activation level of iTregs, we examined the surface expression of GARP in both groups of donors. AD-derived cells revealed a lower expression of GARP after 16 h of stimulation (Fig. 1d). The low conversion of T cells into iTregs and the diminished expression of GARP suggest that AD-CD4⁺ cells were less able to be programmed towards an activated Treg-GARP⁺ phenotype. Furthermore, we investigated the RNA levels of Foxp3 and GARP in CD4⁺CD25⁺ T cells but there were no differences in the 2 groups of donors

(online suppl. material). These results are in accordance with other reports about the expression of Foxp3 in effector T cells and the absence of suppressor cell function [7–10]. Additionally, some authors have indicated that memory CD4⁺ T cells are resistant to TGF- β induced Foxp3 expression without conferring a regulatory phenotype [11].

We next investigated the involvement of TGF- β receptor expression on freshly isolated CD4⁺CD25⁻ versus CD25⁺Foxp3⁻ and CD25⁺Foxp3⁺ cells. T β RII expression was highest in directly isolated CD4⁺CD25⁻ cells (Fig. 1e). After 72 h of stimulation, T β RII expression was diminished in both groups of donors. Nevertheless, T β RII was significantly upregulated in CD25⁺Foxp3⁻ and CD25⁺Foxp3⁺ cells derived from AD patients. Interestingly, we observed a significantly higher expression of T β RIII on AD-CD25⁺Foxp3⁻ T cells (Fig. 1f–g).

After observing the TGF- β receptor expression in CD4⁺CD25⁻, CD4⁺CD25⁺, and iTregs, we analyzed the expression of activating Smad molecules at the transcriptional and protein levels. We found that SMAD3 mRNA expression was significantly reduced in CD4⁺CD25⁻ T cells from AD-patients (AD: 0.48; CTR: 1.05; $n = 6/5$; $p = 0.0059$) (Fig. 2a). Nonetheless, the Smad3 protein amount was detected by Western blot but no differences were observed. Smad2/3 proteins are directly phosphorylated by T β RI; hence we measured the phospho-Smad2/3 (p-Smad2/3) activity in CD4⁺CD25⁻ cells but the difference was not significant in the 2 groups of donors (AD: 1.8-fold increase; CTR: 2.7-fold increase; $n = 10/8$; $p = 0.13$) (Fig. 2c–d). On the other hand, it is well known that Smad7 can antagonize TGF- β signaling by competing with Smad2/3 for interaction with T β RI. Our results showed a reduced level of mRNA SMAD7 in AD-derived CD4⁺CD25⁻ T cells (AD: 0.61; CTR: 1.02; $n = 6/5$; $p = 0.0065$) (Fig. 2b).

Finally, we examined the inhibiting qualities of iTreg conversion derived from CD4⁺CD25⁻ T cells. We did not detect changes in the suppressive functionality of iTregs at different ratios in the 2 groups of donors (online suppl. material).

In summary, we found higher frequencies of circulating Tregs in AD patients. The conversion of CD4⁺CD25⁻Foxp3⁻ into CD4⁺CD25⁺Foxp3⁺ iTregs was diminished in AD donors. Impaired iTreg generation was accompanied by a reduced surface expression of GARP and different frequencies of T β RII and T β RIII on CD25⁺Foxp3⁻ and CD25⁺Foxp3⁺ cells. Additionally, AD patients revealed a low expression of mRNA SMAD3 and SMAD7 in CD4⁺CD25⁻ T cells. Lastly, the suppressive function of

iTregs was not altered in cells derived from AD and healthy individuals.

Our results are in line with previous studies in terms of the frequencies of circulating Treg cells and the low expression of GARP in AD patients [7–10]. This phenomenon could go along with a lower availability of GARP⁺ Tregs in AD able to control inflammation. Furthermore, TβRII may preserve the initial interaction on freshly isolated CD4⁺CD25[−] cells with TGF-β and diminish the conversion of iTregs [12]. This binding might be associated with the subsequent phosphorylation of TβRI, as we identified its expression on CD25⁺Foxp3[−] and CD25⁺Foxp3⁺ cells after stimulation [13]. Meanwhile, it has been described that overexpression of TβRIII is associated with a longer half-life of TβRII and TβRI on the cell membrane [13]. Interestingly, our results demonstrated a significant upregulation of TβRIII in AD-derived CD25⁺Foxp3[−] cells compared to healthy donors. Therefore, AD inflammation might adapt to the trafficking mechanism of TβRII on CD25⁺Foxp3[−] induced by the overexpression of TβRIII [14]. Furthermore, the altered expression of TβRII may modify the correct assembly of the receptor complex to get proper physiological signaling in AD-derived cells. Thus, we conclude that the signaling effect of TGF-β receptors on the suppressor quality of iTreg conversion remains conserved as we did not detect changes in functional assays in the 2 groups of donors.

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Statement of Ethics

All of the participants gave their written informed consent for blood sampling. The study protocol was approved by the local institute’s committee on human research.

Disclosure Statement

The authors declare no conflict of interests.

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Author Contributions

Eva Maria García collected the data, performed the analysis, and wrote this paper. Jorge Galicia-Carreón contributed analysis tools, performed the analysis, and wrote this paper. Natalija Novak designed the analysis, contributed analysis tools, and wrote this paper.