

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

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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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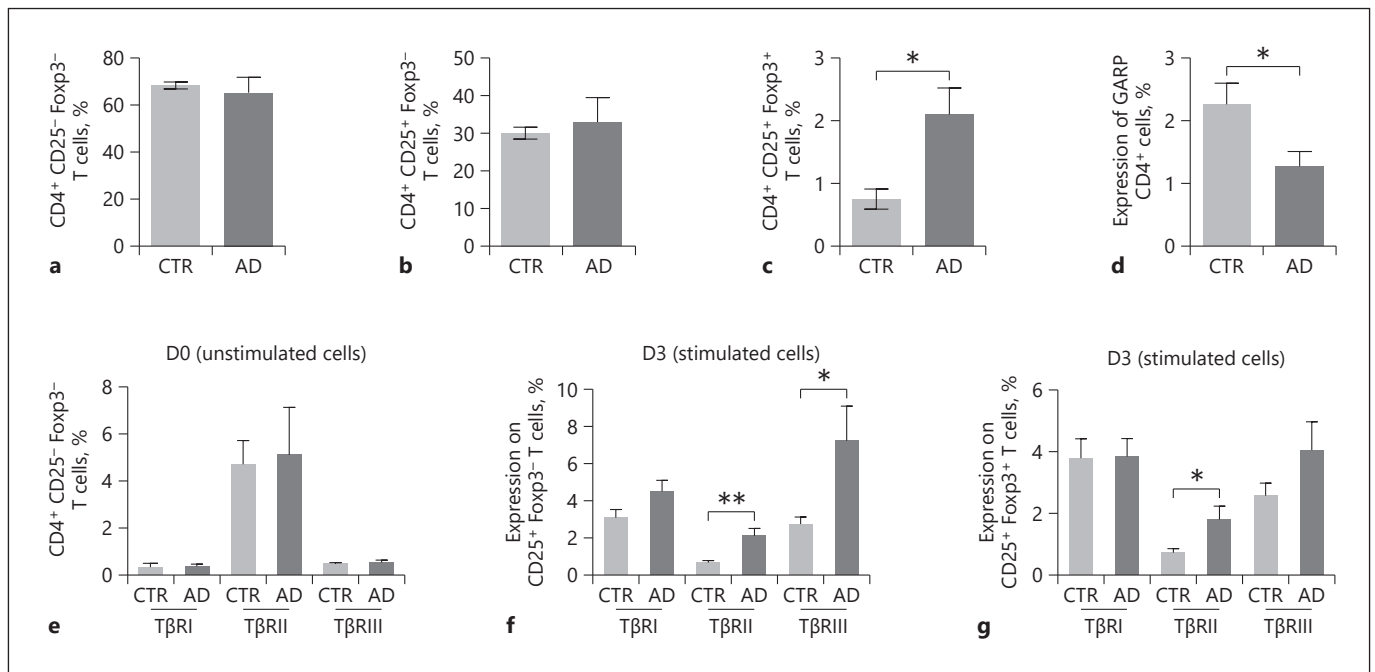


Fig. 1. **a–c** Frequency of CD4⁺CD25⁻Foxp3⁻, CD4⁺CD25⁺Foxp3⁻, and CD4⁺CD25⁺Foxp3⁺ T cells in the peripheral blood of AD patients and healthy donors (CTR), measured by flow cytometry. **a** CD4⁺CD25⁻Foxp3⁻ T cells in AD and CTR. **b** CD4⁺CD25⁺Foxp3⁻ T cells in AD and CTR. **c** CD4⁺CD25⁺Foxp3⁺ T cells 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

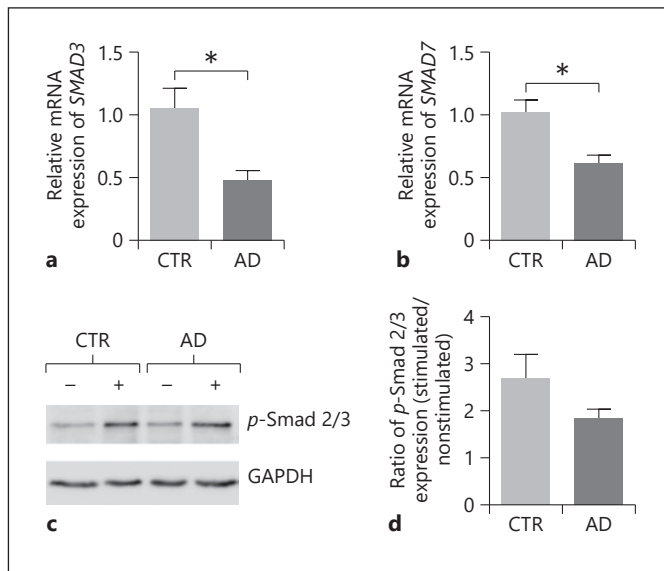


Fig. 2. **a** RNA expression of SMAD3 in CD4⁺CD25⁻ T cells of AD patients in comparison to CTR after isolation. * $p = 0.0059$. **b** RNA expression of SMAD7 in CD4⁺CD25⁻ T cells of AD patients in comparison to CTR after isolation. * $p = 0.0065$. **c** Phosphorylated (p-) Smad2/3 was detected with Western 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, T β RII 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 T β RI, as we identified its expression on CD25⁺Foxp3⁻ and CD25⁺Foxp3⁺ cells after stimulation [13]. Meanwhile, it has been described that overexpression of T β RIII is associated with a longer half-life of T β RII and T β RI on the cell membrane [13]. Interestingly, our results demonstrated a significant upregulation of T β RIII in AD-derived CD25⁺Foxp3⁻ cells compared to healthy donors. Therefore, AD inflammation might adapt to the trafficking mechanism of T β RII on CD25⁺Foxp3⁻ induced by the overexpression of T β RIII [14]. Furthermore, the altered expression of T β RII 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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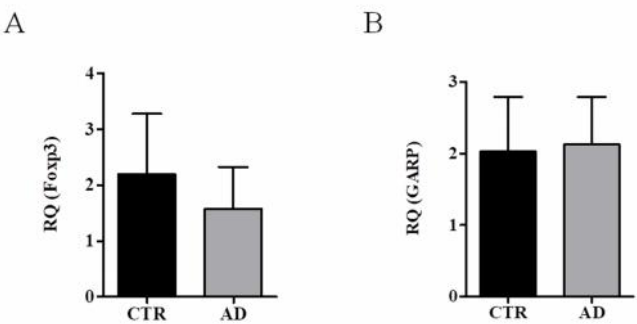
Author Contributions

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.

Supplement

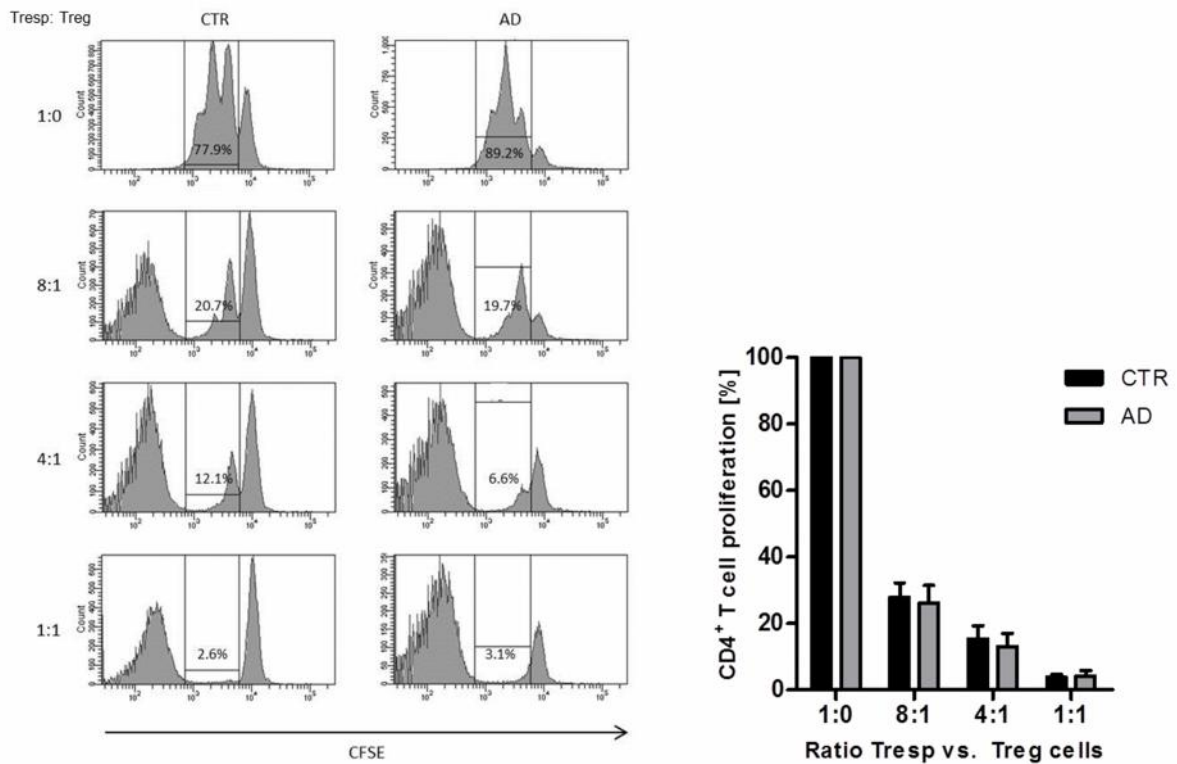
CELL POPULATION	HEALTHY CONTROL [%]	ATOPIC DERMATITIS [%]	P = VALUE
CD25 ⁻ FOXP3 ⁻	61.3 ± 5.0	70.7 ± 4.1	0.16
CD25 ⁺ FOXP3 ⁻	17.7 ± 3.6	17.7 ± 3.4	0.91
CD25 ⁺ FOXP3 ⁺	14.6 ± 2.0	8.5 ± 1.7	0.03

Frequency of CD25⁻Foxp3⁻, CD25⁺Foxp3⁻ and CD25⁺Foxp3⁺ cell subsets after 3 days of culture is shown (n = 12/10 (AD/CTR)).



RNA expression of Foxp3 and GARP of CD4⁺CD25⁺ T cells isolated from peripheral blood of AD patients and healthy donors.

RNA expression of Foxp3 and GARP of AD patients and healthy controls was measured by RT-PCR after isolation of CD4⁺CD25⁺ T cells from PBMC. A: RNA expression of Foxp3 (AD: n= 10; CTR: n = 9). B: RNA expression of GARP (AD: n = 12; CTR: n = 9). Data are shown as mean ± SEM. Statistical significance was determined by t-test.



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17 **Functionality of iTregs derived from AD is not reduced**

18 Peripheral CD4⁺CD25⁻ T cells were cultured on CD3-coated plates in the presence of IL-2 and TGF- β .
 19 Induced CD25⁺ cells were purified with anti-CD25-coated beads on day 3 and co-cultured for 3 days
 20 with autologous CFSE stained CD25⁻ T responder cells at ratios. Representative histograms are shown
 21 on the left. T cell proliferation of CD4⁺ T cells is shown on the right. Data are shown as mean $\pm$ SEM (n
 22 = 6/7 (AD/CTR)). Statistical significance was determined by t-test.

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Materials and Methods

Peripheral blood was obtained from 37 AD patients (mean age 37 years, range 18–66). Atopic dermatitis diagnosis was based on clinical history and physical examination. 38 healthy volunteers were used as controls (mean age 32 years, range 19–61). The patients did not receive any systemic therapy. Each donor gave written informed consent and the study was approved by the local Ethics Committee.

Cell purification and culture

Peripheral blood mononuclear cells (PBMCs) were prepared by a LymphoprepTM gradient centrifugation. Purification of CD4⁺CD25⁻ T cells was accomplished by two consecutive negative selections with a regulatory T cell isolation kit (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions. The purity of isolated CD4⁺CD25⁻ T cells was > 97 % and identical in cells from AD patients and healthy controls. CD4⁺CD25⁻ T cells were cultured on 5 µg/ml anti-CD3 (clone UCHT1) -coated plates in the presence of 100 U/ml recombinant human IL-2 (PeproTech, Rocky Hill, NJ) and 5 ng/ml recombinant human TGF-β (R&D Systems, Wiesbaden, Germany) at 1 Mio cells per ml in RPMI 1640 supplemented with 10 % inactive foetal calf serum (FCS), 1 % antibiotics/antimycotics, 1 % L-glutamine and 0.2 % (v/v) mercaptoethanol. iTregs were harvested after 1 or 3 days of culture at 37 °C in a 5 % CO₂ milieu in a humidified chamber.

Detection of phosphorylated Smad2/3 after in vitro stimulation with TGF-β in Western Blot

CD4⁺CD25⁻ T cells were cultured 16 hours in RPMI 1640 + 0.25 % FCS. Cells were then stimulated with 5 ng/ml TGF-β (R&D Systems, Wiesbaden, Germany) for 1 hour or left untreated. Subsequently, cells were washed with ice cold PBS and then lysed in RIPA buffer with 1 mM PMSF, 5 µg/ml aprotinin, 5 µg/ml leupeptin (all Santa Cruz Biotechnology, CA, USA) and 1 % phosphatase inhibitor cocktail (Thermo Scientific). After 30 min of extraction on ice, cell lysates were collected by centrifugation at 15000 xg for 15 min. Protein content was measured by Bradford-Assay and 10 µg of total protein was loaded onto 12 % SDS-PAGE. Proteins were blotted onto PVDF membranes (Millipore, Billerica, MA)

and blocked in 5 % BSA in Tris-buffered saline (20 mM Tris, pH 7.5, and 0.15 % M NaCl) containing 0.1 % Tween-20 for 1 h at room temperature. Rabbit anti-phosphorylated Smad2/3 (clone D27F4, Cell Signaling Technology, Inc., Danvers, MA, USA) was incubated overnight. Mouse anti-GAPDH (clone 0411, Santa Cruz Biotechnology, CA, USA) was used as loading control. Donkey anti-rabbit IgG-HRP (sc-2317) and goat anti-mouse IgG-HRP (sc-2005, both Santa Cruz Biotechnology) respectively was used for detection of the primary antibodies. Detection was performed using WesternBright Sirius (Advansta, Menlo Park, USA) on Fujifilm LAS 3000 analysis (Fujifilm Europe GmbH, Düsseldorf, Germany).

Immunofluorescence staining of surface markers

Mouse anti-CD25-PE (clone 4E3, Miltenyi Biotec GmbH) and mouse anti-CD25-FITC (clone M-A251, BD Biosciences Pharmingen) were incubated for 10 min at room temperature. Mouse anti-GARP-APC (clone 7B11, BD Pharmingen) was incubated for 20 min at 4 °C. Unconjugated anti-TGF- β RI (clone MM0015-8G33; abcam, Berlin, Germany), anti-TGF- β RII (clone MM0056-4F14; abcam) and anti-TGF- β RIII (clone 31349, R&D Systems) were applied for 20 min followed by 20 min of goat anti-mouse-APC (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) Cells were then incubated with 5 % mouse serum for 15 min followed by extracellular staining of mouse anti-CD25-FITC (clone M-A251, BD Pharmingen). Consecutive intracellular rat anti-FOXP3-PE staining was carried out as described beneath.

Immunofluorescence staining of intracellular markers

Intracellular rat-anti FOXP3-PE staining was carried out as described previously. Briefly, cells were first stained with extracellular markers and then fixed with Transcription Factor Staining Buffer Set for 30 min. Consecutive permeabilization was carried out for 10 min with permeabilization buffer (Fixation/Permeabilization Diluent eBioscience) + 2 % rat serum. Rat anti-FOXP3-PE (clone PCH101, eBioscience) was then added and incubated at room temperature for 30 min. All experiments were performed using isotype-matched controls of unrelated specificity.

Flow cytometric analysis

Cells were analyzed to determine the expression of phenotypic markers, TGF- β RI-III and FOXP3 on a FACScan flow cytometer using FACSDiva software (both Becton Dickinson GmbH, Heidelberg, Germany). To analyze the staining of cell-surface markers, the cells were gated on FSC-SSC dot plot, then the lymphocytes were gated in a SSC-CD4 dot plot. The CD4⁺CD25⁺ cells were selected, and a CD25 FOXP3 dot plot was created to get the double positive CD25⁺FOXP3⁺ T cells. Then, TGF- β RI-III positive cells were gated on the expression of CD4⁺CD25⁻, CD25⁺FOXP3⁻ and CD25⁺FOXP3⁺.

Quantitative real-time PCR

Total RNA was isolated from CD4⁺CD25⁻ T cells or from magnetically enriched CD4⁺CD25⁺ iTreg after 3 days of culture with a NucleoSpin RNA kit (Macherey-Nagel, Dueren, Germany). Complementary DNA synthesis was carried out with TaqMan reverse transcription reagents with random hexamers (Applied Biosystems, Darmstadt, Germany). The cDNA was amplified using TaqMan Gene Expression Master Mix and predesigned TaqMan Gene Expression Assays, according to the recommendations of the manufacturer's instructions. Expression levels of FOXP3, Smad3 and Smad7 were quantified with the given primers and 18S was used as an endogenous reference. Each reaction was performed in duplicate. Primers, including probes, were as follows: FOXP3 (Hs01085835_m1), Smad3 (Hs00969210_m1), Smad7 (Hs00998193_m1) and 18S (4310893E). All assays were performed according to the manufacturer's instructions. Relative quantification and calculation of the range of confidence was performed using the comparative CT method (1).

Statistics

Student's t-test was used to compare the data of patients with AD to those from healthy donors. The statistical analysis was performed using Graph Pad Prism software 5.01. Data are presented as mean $\pm$ SEM, and *P* values less than 0.05 was considered statistically significant.

103 Literature

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