**Supplement**

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| --- | --- | --- | --- |
| 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)).

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

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

**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/antimycotis, 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-βRI (clone MM0015-8G33; abcam, Berlin, Germany), anti-TGF-βRII (clone MM0056-4F14; abcam) and anti-TGF-βRIII (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.

*Immunoflourescence 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-βRI-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-βRI-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 ± SEM, and *P* values less than 0.05 was considered statistically significant.

Literature

1. Schmittgen T, Livak K. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008;**3**:1101–1108.