

Fox-P3-positive regulatory T cells are present in the skin of generalized atopic eczema patients and are not particularly affected by medium-dose UVA1 therapy

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Background: Regulatory T cells (T-reg cells) have been described as an important cell population in the UV treatment of inflammatory skin diseases.

Methods: We have treated five patients with generalized atopic eczema (AE) using medium-dose (15 cycles of 50 J/cm², total dose of 750 J/cm²) UVA1 therapy and have analyzed the skin-infiltrating T-cellular subsets before and after therapy. Skin biopsies were split for immunohistochemistry and Real-time PCR and analyzed for CD4, Fox-P3, GATA-3, and IL-10 transcription as well as for CD3, CD4, CD152, Fox-P3, and GITR staining.

Results: In all the investigated patients, we observed a good clinical response to UVA1. As described

previously, the number of epidermal T cells slightly declined after irradiation. However, we did not observe a general decrease in T cell numbers. Within the population of T cells, no specific difference in the kinetics of Fox-P3-positive cells and Fox-P3-specific mRNA was noted as compared with GATA-3 positive T cells. These results were paralleled by RT-PCR for IL-10 and staining for CD152, a surface molecule that has been described for T-reg cells.

Conclusion: In our hands, the clinical benefit of UVA1 treatment in AE patients does not seem to be due to a preferential survival/proliferation of T-reg cells.

UVA1 treatment is generally accepted as an effective therapy for adult atopic eczema (AE) patients with a generalized distribution of their skin lesions (1, 2). The clinical benefit results from different mechanisms including alterations of antigen-presenting cells, formation of reactive oxygen species, and the depletion of epidermal T cells (3). However, it has also been reported that UV irradiation of the skin leads to the induction of tolerance in a mouse model through the induction of IL-10. This IL-10 production might reflect the activation of regulatory T cells (T-reg cells) among other T cell populations (4–6). T-reg cells have the ability to suppress both TH1- and TH2-driven immune responses and are characterized by dominant transcription of FoxP3, a transcription factor of the forkhead/winged helix family (7) and by the constitutive expression of CD25, CTLA-4, and GITR (4, 6, 8). This cell type has been attributed a central role in the

control of various autoimmune but also allergic diseases (9).

To investigate the possible role of T-reg cells in AE patients undergoing UVA1 treatment, we have analyzed skin biopsies from five adult generalized AE patients before and after medium-dose (750 J/cm², 15 cycles of 50 J/cm²) UVA-1 therapy with regard to the composition of the T-cellular subsets before and after therapy.

Material and methods

Patients

Five patients with the diagnosis of AE were included in the study, of whom four showed characteristics of the extrinsic type, with high serum IgE levels and one patient with the intrinsic type, showing sIgE levels within normal ranges. All patients showed elevated SCORAD (10) values when starting the therapy and

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all patients gave informed consent for UVA1 treatment and for taking two distinct biopsies. Four millimeters of punch biopsies were taken before and immediately after UVA1 therapy from symmetrical areas of the extremities under local anesthesia. Biopsies were snap-frozen, and were submitted for immunohistochemistry and molecular biology. The detailed characteristics of the included patients are given in Table 1.

Table 1. Characteristics of the five included AE patients

Patient (years)	AE type	Total sIgE (IU/ml)	SCORAD BT	SCORAD AT
BS, 54 males	Extrinsic	4847	77.5	44.5
BM, 72 females	Intrinsic	30	76.5	42.5
ED, 27 females	Extrinsic	528	65.5	35.5
SS, 31 females	Extrinsic	3428	39.2	21.7
RK, 26 males	Extrinsic	3138	66.5	31.5

AE, atopic eczema.

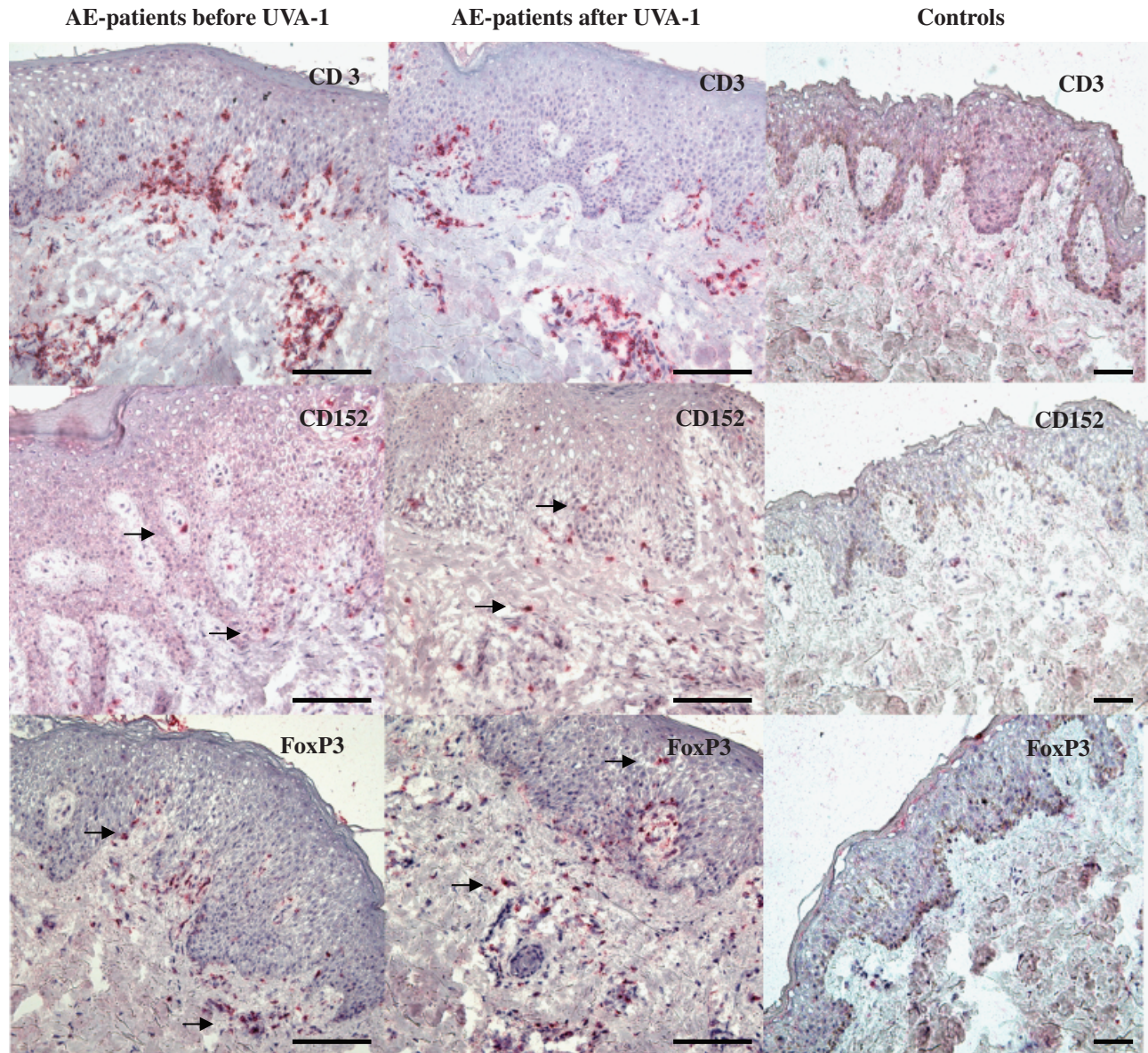


Fig. 1. (a, b) Staining pattern for CD3, CD152, and FoxP3 before and after (middle panel) UVA1 therapy. Control staining in normal skin is shown in the right panel. Biopsies were taken immediately before starting and after cessation of UVA1 irradiation. In Fig. 1a, the representative staining for patient BS is shown; in Fig. 1b, the percentage of positive cells among 100 mononuclear cells within the biopsies is represented. Cell counts did not change significantly between pre- and post-treatment biopsies. However, after irradiation cells were rather located in the upper dermis whereas pre-treatment biopsies also showed homing to the epidermis. Slides were incubated with the respective antibodies and positive reactivity was evaluated with phenol red as an indicator using alkaline phosphatase-coupled secondary antibodies (patient ED – blue line, patient BM – pink line, patient RK – yellow line, patient BS – light blue line, patient SS – purple line). Bars represent 50 μ m.

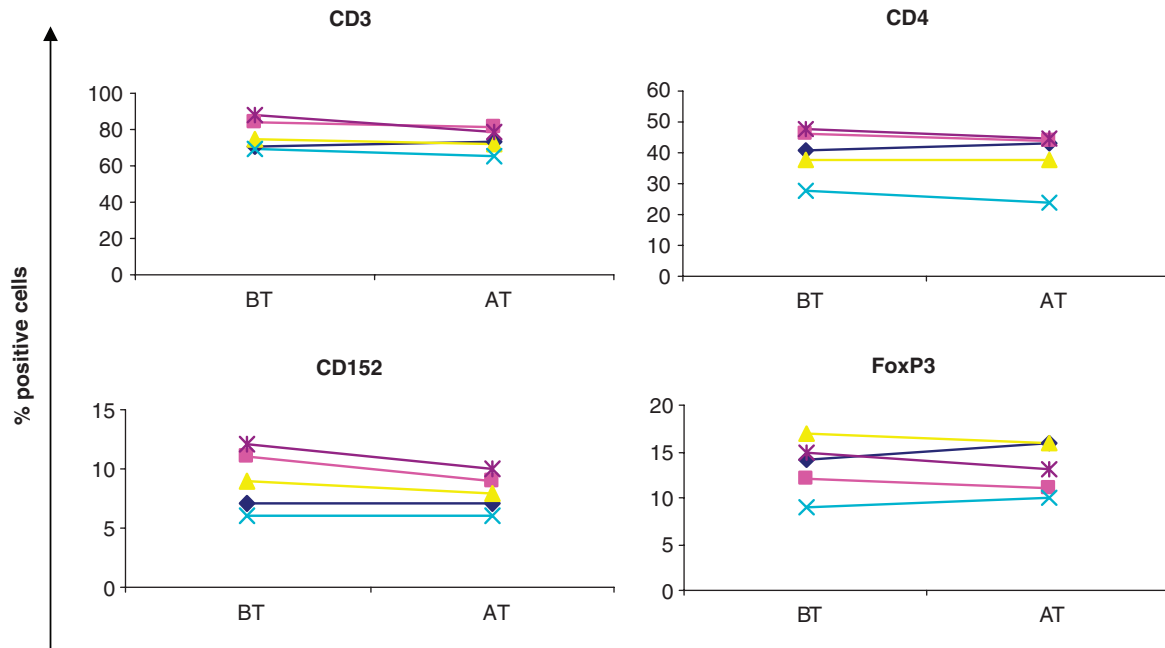


Fig. 1. (Continued)

All patients completed 15 cycles of 50 J/cm² UVA-1 (GP-24-H, Hönle, Kaufering, Germany) over a 3-week course therapy, resulting in a total dose of 750 J/cm².

The study was approved by the local ethic committee of the Technical University Munich.

Immunohistochemistry

We used standard staining conditions for CD3, CD4, CD8 (Dako, Hamburg, Germany), CD152 CTLA-4, and FoxP3 (Abcam, Cambridge, MA, USA), by fixing the cryo-sections in cold acetone for 10 min and incubation of the respective primary antibodies for 90 min in staining buffer (PBS containing 1% paraformaldehyde (Sigma, Deisenhofen, Germany), followed by three washes with PBS and a 60-min incubation with the secondary antibody (Dako) coupled to an alkaline phosphatase. Positive reactions were then evaluated with phenol red as the indicator agent.

Real-time PCR

Real-time RT-PCR reactions for CD4, GATA-3, T-bet, Fox-P3, IL-10, IL-4, IL-12, TNF- α , and IFN- γ were carried out using standard protocols. To this end, RNA was prepared from the obtained biopsy by homogenizing the biopsies with a PolytronTM homogenizer in 1 ml TRIZOL (Invitrogen, Carlsbad, CA, USA) followed by standard extraction procedures. RNA was then converted into cDNA by using Super-

script II, oligo dT primers, and RNAsin (all from Invitrogen) following the protocol supplied by the manufacturer.

Real-time PCR was carried out using the ABI Prism 7000 Sequence Detector System (Applied Biosystems, Foster City, CA, USA) and reagents from the same company.

All PCR results were normalized to the expression of GAPDH. In order to screen for changes in the T-cell-specific transcription factor mRNAs, the results for GATA-3, T-bet, and Fox-P3 were additionally correlated to CD4 transcripts. Real-time PCR primers have been published previously (11–13) or designed to span at least one intron (IL-4-forward: CACAGG-CACAAGCAGCTGAT, IL-4-reverse: CCTT CA-CAGGACAGGAATTCAAG, IL-4-probe: CGGC TCGACAGGAACCTCTGGG, IL-12p40-forward CATCTCTTGGTTTTCCCTG

GTT, IL-12p40-reverse: CATAAACATCTTT CTTCAGTTCCCATAT, IL-12p40-probe: TTCTGG CATCTCCCCTCGTGGC FoxP3-forward: ATGCC TCCTCTTCTTCCTTGAA, FoxP3-reverse: GGG CATCCACCGTTGAGA, FoxP3-probe: CCCATG CCACCATCGCAGCTG) (all primers and specific labelled probes from MWG, Ebersberg, Germany).

Statistical analysis

Results were analyzed for statistical significance using Student's *t*-test.

Results

Clinically, all five patients improved markedly after the UVA1 treatment (total dose 750 J/m²), leading to a substantial amelioration of clinical symptoms proving effectiveness of the UVA1-treatment (SCORAD values in Table 1).

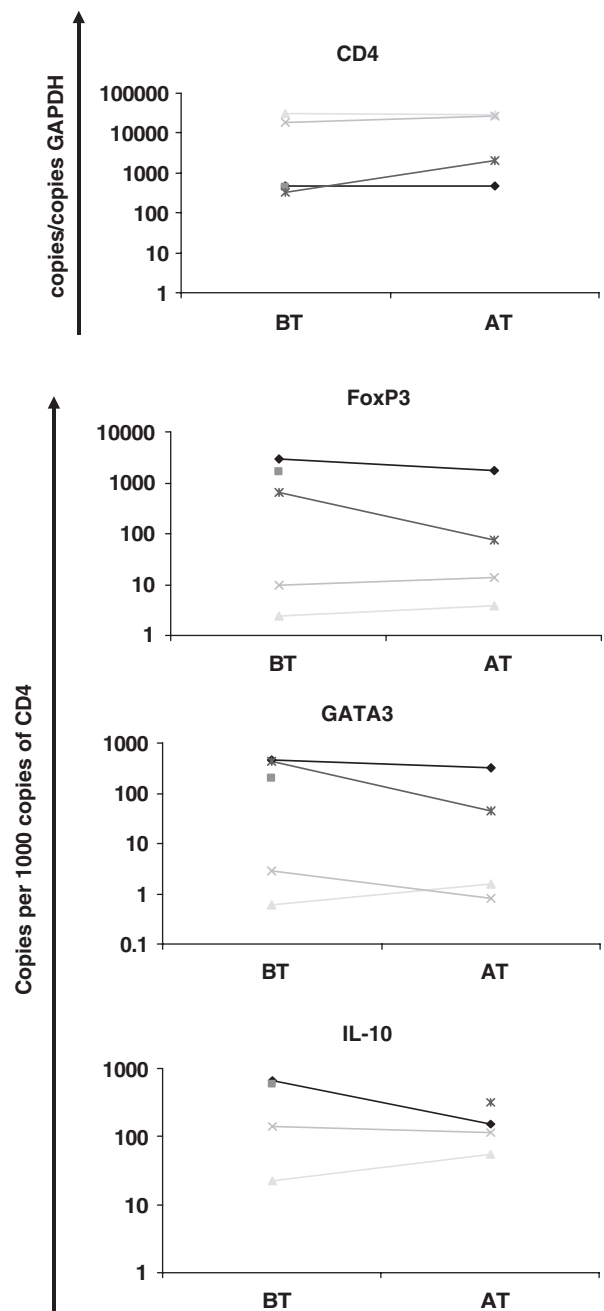
As a first approach to the possible mode of action, we stained the biopsies for changes of T-cell infiltrations (anti-CD3) which showed a stable infiltrate of T cells which in general was found in the epidermis and dermis before therapy, slightly changing after UVA1 treatment when a dominant dermal infiltrate was seen. Total CD3 numbers, however, did not differ between the two biopsies (Figs. 1a and b). As we preferentially sought for changes in the presence of T-reg cells, we screened for staining patterns of CD152 and FoxP3, the crucial transcription factor of this cell population. Staining for CD152 and FoxP3 repeatedly identified a subset of lymphocytes, which, however, did not change in quantity between the pre- and post-treatment biopsies (Figs. 1a and b).

We then decided to extend our studies to mRNA changes by Real-time PCR, targeting the TH1-transcription factor T-bet, the TH2-transcription factor GATA-3, and the T-reg transcription factor FoxP3. On analyzing these molecules, we found either no detectable mRNA (as for T-bet either before or after treatment) or a relatively stable expression pattern (as for GATA3 and also FoxP3), which did not substantially change between pre- and post-therapy biopsies (Fig. 2). However, in contrast to the staining pattern in immunohistochemistry, we observed a decrease in FoxP3 transcription in two patients. Of note, levels for IL-4-, IL-10-, TNF- α , and IFN- γ -mRNA did also not change for pre- and post-treatment biopsies.

Fig. 2. Real-time PCR results for CD4, GATA3, FoxP3, and IL-10. mRNA was reverse transcribed and analyzed for gene expression as indicated. While CD4 was normalized to GAPDH, transcripts of GATA3, FoxP3, and IL-10 were additionally correlated to CD4 expression and are expressed per 1000 copies of CD4. For all but GAPDH transcripts in the post-treatment biopsy of patient BM and for IL-10 analysis in the pre-treatment biopsy of patient SS, no substantial transcripts were found (patient ED – blue line, patient BM – pink line, patient RK – yellow line, patient BS – light blue line, patient SS – purple line). Values for pre- and post-treatment biopsies did not differ significantly for any of the analyzed markers. Biopsies were additionally screened for transcripts of T-bet (not detectable), IL-4, IL-12, TNF- α , and IFN- γ , for which also no changes were detected.

Discussion

T-reg cells have emerged as the focus of interest in the control of various allergic diseases (9) including AE (14). Little is known, however, about their role in the target organs of inflammation such as the naso-pharyngeal and bronchial mucosa and/or the skin. A recent paper (15) has shown that atopic patients lack a functional subset of T-reg cells in their skin and therefore cannot efficiently down-modulate allergen-driven immune responses. However, our experiments revealed the presence of FoxP3⁺ cells within the skin of AE patients that were not significantly influenced by UVA-1 treatment. Of note, our findings



are not suited to discuss the possible functionality of this subset. As a conclusion of our results, medium-dose UVA1 therapy did not induce substantial changes in T-cell infiltration and T-cell subsets in patients with atopic dermatitis. The induction of T-reg cells or the selective depletion of TH2-cells thus seems unlikely to be an operative mechanism in skin improvement of AD patients undergoing UVA1 therapy. The question remains as to why the number of depleted CD4 cells was relatively low despite the good clinical outcome. As a possible explanation, the relative resistance of CD4 cells of non-malignant origin (as is the case in atopic eczema) has to be taken into account. In a publication by Yamauchi et al., (16) a preferential induction of apoptosis has been reported in T cells arising from cutaneous T-cell lymphoma.

We can only speculate on the actual mechanism of skin improvement in our patients but as we did not screen for APC functions or changes in reactive oxygen metabolism, it is possible that one of these pathways plays a crucial role in the therapeutic effect. Owing to the relatively low number of patients, our study should be considered preliminary. Consequently, we think that the replication of the reported findings in larger patient numbers should follow to corroborate the results presented.

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References

1. Abeck D, Schmidt T, Fesq H, et al. Long-term efficacy of medium-dose UVA1 phototherapy in atopic dermatitis. *J Am Acad Dermatol* 2000; **42**: 254–257.
2. Tzaneva S, Seeber A, Schwaiger M, et al. High-dose versus medium-dose UVA1 phototherapy for patients with severe generalized atopic dermatitis. *J Am Acad Dermatol* 2001; **45**: 503–507.
3. Morita A, Werfel T, Stege H, et al. Evidence that singlet oxygen-induced human T helper cell apoptosis is the basic mechanism of ultraviolet-A radiation phototherapy. *J Exp Med* 1997; **186**: 1763–1768.
4. Schwarz A, Beissert S, Grosse-Heitmeyer K, et al. Evidence for functional relevance of CTLA-4 in ultraviolet-radiation-induced tolerance. *J Immunol* 2000; **165**: 1824–1831.
5. Schwarz A, Maeda A, Wild MK, et al. Ultraviolet radiation-induced regulatory T cells not only inhibit the induction but can suppress the effector phase of contact hypersensitivity. *J Immunol* 2004; **172**: 1036–1043.
6. Schwarz T. UV-induzierte regulatorische T-Zellen. *J Dtsch Dermatol Ges* 2005; **3**: 504–510.
7. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**: 1057–1061.
8. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 2002; **3**: 135–142.
9. Akdis M, Blaser K, Akdis CA. T regulatory cells in allergy: novel concepts in the pathogenesis, prevention, and treatment of allergic diseases. *J Allergy Clin Immunol* 2005; **116**: 961–968.
10. Kunz B, Oranje AP, Labreze L, Stalder JF, Ring J, Taieb A. Clinical validation and guidelines for the SCORAD index: consensus report of the European Task Force on Atopic Dermatitis. *Dermatology* 1997; **195**: 10–19.
11. Hafsi N, Voland P, Schwendi, et al. Human dendritic cells respond to *Helicobacter pylori*, promoting NK cell and Th1-effector responses in vitro. *J Immunol* 2004; **173**: 1249–1257.
12. Rad R, Gerhard M, Lang R, et al. The *Helicobacter pylori* blood group antigen-binding adhesin facilitates bacterial colonization and augments a nonspecific immune response. *J Immunol* 2002; **168**: 3033–3041.
13. Rad R, Dossumbekova A, Neu B, et al. Cytokine gene polymorphisms influence mucosal cytokine expression, gastric inflammation, and host specific colonisation during *Helicobacter pylori* infection. *Gut* 2004; **53**: 1082–1089.
14. Ou LS, Goleva E, Hall C, Leung DY. T regulatory cells in atopic dermatitis and subversion of their activity by superantigens. *J Allergy Clin Immunol* 2004; **113**: 756–763.
15. Verhagen J, Akdis M, Traidl-Hoffmann C, et al. Absence of T-regulatory cell expression and function in atopic dermatitis skin. *J Allergy Clin Immunol* 2006; **117**: 176–183.
16. Yamauchi R, Morita A, Yasuda Y, et al. Different susceptibility of malignant versus nonmalignant human T cells toward ultraviolet A-1 radiation-induced apoptosis. *J Invest Dermatol* 2004; **122**: 477–483.

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