

Immunomodulation in Patients with Chronic Hand Eczema Treated with Oral Alitretinoin

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Key Words

Alitretinoin · 9-*cis*-retinoic acid · Chronic hand eczema · T cells · Regulatory T cells · Interleukin-17 · B cells · Plasmablasts · Immunoglobulin

Abstract

Background: Oral alitretinoin (9-*cis*-retinoic acid; 9-*cis*-RA) has shown clinical efficacy in patients with chronic hand eczema (CHE). Herein, we investigated the impact of oral 9-*cis*-RA on the local and systemic immune response in patients with CHE. **Methods:** Twenty patients with CHE were treated with oral alitretinoin (10 or 30 mg/day) for at least 24 weeks. Blood samples were taken for flow cytometry, and serum samples were assessed by ELISA to determine immunoglobulin (Ig) levels. Skin biopsies from lesional skin were evaluated immunohistochemically. **Results:** Upon 9-*cis*-RA treatment, improvement of the CHE was observed in all patients. A significant decrease in plasmablasts in the peripheral blood and a significant reduction of serum IgE levels were determined. Furthermore, we detected a significant reduction of CD4⁺ cells and regulatory T cells in the peripheral blood upon treatment. By contrast, these cell subsets were significantly increased in the affected skin. Cytokine analysis of activated CD154-positive T cells showed a reduction of interleukin (IL)-17 but not of IL-4 or IFN- γ production.

Conclusions: Overall, our data indicate a disease-modifying effect of 9-*cis*-RA, including a systemic decrease in IL-17-positive cells, but decreased serum IgE and CD23 expression. The increased frequency of FoxP3-positive cells in the skin upon treatment may suggest a mechanism by which hand eczema is therapeutically targeted by 9-*cis*-RA, but this will need to be proven in the future studies.

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Introduction

Hand eczema is a common inflammatory skin disease with an estimated 1-year prevalence of 10% in the general population. Five to seven percent of these patients have severe chronic hand eczema (CHE) [1]. In CHE, the most relevant pathologic factors are irritant, allergic, and endogenous. Atopy is found in up to 50% of patients with CHE [2]. Irritants and an impaired skin barrier can promote allergic contact dermatitis. The etiology of hand eczema is complex in most patients and requires standardized management of the disease [1].

Oral alitretinoin (9-*cis*-retinoic acid; 9-*cis*-RA), a vitamin A derivate, has shown a convincing good clinical efficacy in the treatment of severe CHE refractory to potent topical corticosteroids [3]. To date, it has been licensed in

multiple countries in Europe and Canada. There are only a few reports directly addressing the impact of 9-*cis*-RA signaling on chronic inflammatory skin diseases [4].

Previous studies have shown that 9-*cis*-RA binds with a high affinity to the retinoid X receptor but also to the retinoid A receptor, each consisting of 3 subtypes: α to γ [5–7]. As a ligand of these receptors, 9-*cis*-RA regulates the expression of many cytokines, which are also important for CHE [8].

As previously published by us, retinoids can inhibit immunoglobulin (Ig) E production in vitro [9]. Moreover, several other reports from other groups indicate an immunomodulatory impact on T cell differentiation and cytokine production [10, 11].

Therefore, the aim of this study was to assess the peripheral immune response upon oral alitretinoin treatment in patients suffering from CHE. Based on our previous observations, we first analyzed the phenotype and function of human B cells in the alitretinoin-treated CHE group. As T cells are important players in the pathophysiology of at least certain subtypes of hand eczema, Th1 and Th2 effector cells were analyzed via multiparametric flow cytometry, but so were Th17 cells as they have been recently associated with the development and outcome of allergic diseases [12, 13].

Finally, as FoxP3+ regulatory T (Treg) cells are the best-described T cells with an immunosuppressive capacity on effector T cells and as a reduced number of Treg cells can be a causal factor for allergic inflammation [14, 15], we also studied the presence of Treg cells upon systemic alitretinoin treatment.

Materials and Methods

Study Design and Patients

Twenty subjects (13 females and 7 males) aged 32–66 years with CHE refractory to potent topical corticosteroids were recruited at the Department of Dermatology and Allergy, Charité – Universitätsmedizin Berlin, Germany. The exclusion criteria were identical to those described previously [3]. Nineteen patients received the standard dose of 30 mg/day of oral alitretinoin, and 1 patient received 10 mg/day because of elevated serum cholesterol values prior to this study. Clinical response was defined as a Physician's Global Assessment (PGA) score of 'clear' or 'almost clear'. The treatment was stopped at week 12 if at least 1 of these 2 scores had been achieved. All of the other subjects continued the therapy for up to 24 weeks in the same way as in the phase 3 pivotal trial for oral alitretinoin, which was the basis for marketing authorization [3].

The clinical severity of the hand eczema during therapy was monitored based on the Manuscore (MS) [16]. Blood samples were collected every 4 weeks for routine lab testing and for the study

analysis. Approval from the local ethics committee was obtained prior to the study initiation and all patients gave written informed consent. Five patients with severe CHE gave additional informed consent for skin biopsies to be performed before and after therapy with oral alitretinoin.

Histological Analysis

For histological analysis, 5- μ m sections from skin biopsies were stained with hematoxylin for 1 min. The thickness of the epidermis was determined by Axiovision measurement tools on an AxioPlan light microscope (Zeiss, Berlin, Germany) at a $\times 100$ magnification. Ten measurements were obtained for each slide and were expressed in micrometers; means and SEM were calculated.

Immunohistochemistry

Sections (1–2 μ m) of formalin-fixed, paraffin-embedded skin biopsies were cut, deparaffinized, and subjected to a heat-induced epitope retrieval step. Slides were rinsed in cool running water, washed in Tris-buffered saline (pH 7.4), and blocked with avidin/biotin (Dako, Hamburg, Germany) prior to incubation with monoclonal mouse anti-human CD4 antibody (clone 1F6; Novocastra, Newcastle Upon Tyne, UK) or rat anti-human Foxp3 antibody (clone PCH101; eBioscience, San Diego, Calif., USA) for 30 min followed by incubation for 30 min with biotinylated secondary antibodies (donkey anti-mouse and rabbit anti-rat, respectively, both from Invitrogen Carlsbad, Calif., USA). For detection, a streptavidin-alkaline phosphatase kit and an EnVision kit (Dako) were used. Nuclei were counterstained with hematoxylin and slides were mounted with gelatin (Merck, Darmstadt, Germany). Negative controls were created omitting the primary antibodies.

Images were acquired using an AxioImager Z1 microscope (Carl Zeiss MicroImaging, Inc., Jena, Germany). Positive cells were quantified per high-power field (hpf) (0.237 mm²) and 5 hpf were averaged in each case. All immunohistochemical evaluations were performed in a blinded manner.

Flow Cytometry

For T cell cytokine staining, heparinized blood was stimulated for 6 h with staphylococcal enterotoxin B (1 μ g/ml of staphylococcal enterotoxin B; Sigma-Aldrich, Steinheim, Germany) and 1 μ g/ml anti-CD28 (clone 28.2; BD Pharmingen, Heidelberg, Germany) at 37°C. For the last 4 h, brefeldin A (2 μ g/ml; Sigma-Aldrich) was added. Afterwards, cells were treated with lysing solution (BD Pharmingen) and Perm II solution (BD Pharmingen) followed by surface and intracellular staining with antibodies against CD154-FITC, human interleukin (IL)-4-PE (BD Pharmingen), human IL-17A-PE (eBioscience), human IFN- γ -APC, and CD4-PerCP (Miltényi Biotec, Bergisch Gladbach, Germany).

CD19+ B cells were characterized from whole blood using fluorochrome-conjugated monoclonal antibodies according to the surface expression of CD19 and CD23 (BD Pharmingen), CD38 (Beckmann Coulter GmbH, Krefeld, Germany), and CD27 (Miltényi Biotec).

Treg cell staining was performed using a human Treg cell staining kit (eBioscience) with anti-FoxP3 (PCH101), anti-CD4-PerCP, and anti-CD25 APC.

Absolute T and B cell numbers were determined using TruCount tubes (BD Pharmingen) and antibodies against CD4 (Miltényi Biotec) and CD19 (BD Pharmingen). All measurements were

performed using a flow cytometer (FACSCalibur; Becton Dickinson, Heidelberg, Germany) and analyzed using FlowJo 7.2.2 (Tree Star, Ashland, Oreg., USA).

Enzyme-Linked Immunosorbent Assay

The anti-human IgE clone HP6061 coated on 96-well MaxiSorp plates (Nunc) and biotinylated HP6029 (SouthernBiotech) were used, and the captured IgE was detected via the enzymatic activity of streptavidin-alkaline phosphatase (R&D Systems, Minneapolis, Minn., USA) and colorimetric analysis of the cleaved *p*-nitrophenyl phosphate substrate (Sigma-Aldrich) at 405 nm. Detection of IgA, IgG, and IgM was performed with matched antibody pairs (Jackson ImmunoResearch Laboratories) using alkaline phosphatase-conjugated detection antibodies and *p*-nitrophenyl phosphate substrate.

Statistical Analysis

For data interpretation, arithmetic mean values with SEM were calculated using GraphPad Prism (GraphPad Software, La Jolla, Calif., USA). Statistical analysis was performed using Student's *t* test if the values were normally distributed; otherwise, the Wilcoxon matched-pairs test was used. To assess whether the data were normally distributed or not, the Kolmogorov-Smirnov test was used. *p* < 0.05 was considered statistically significant.

Results

Clinical Efficacy Correlates with a Decrease in Epidermal Thickening

Twenty patients with CHE were enrolled into this study. The data of the subjects are shown in table 1. Clinical efficacy was assessed via a standardized severity system (MS) and the PGA before and during the therapy with 9-*cis*-RA. All patients improved upon treatment over time (fig. 1a, b). The average treatment time was 18 weeks (± 6). Data derived from skin biopsies (*n* = 5) indicated that the clinical response was associated with a significant reduction of the epidermal thickness (before treatment: 398 ± 111 μ m; after treatment: 205 ± 97 μ m; fig. 1c, d).

Upon Oral Alitretinoin Treatment, the Frequencies of Circulating Plasmablasts and the Ig Levels in the Peripheral Blood Decreased

Since we have previously demonstrated that 9-*cis*-RA inhibits IgE production in vitro [9] and because it is well known that retinoids are important for B cell differentiation [17], we next analyzed different B cell subsets and Ig levels before and after systemic alitretinoin treatment.

We identified equal absolute numbers of CD19+ cells after treatment with alitretinoin as shown in figure 2a. CD38 is a B cell surface marker that becomes upregulated when B cells differentiate towards antibody-producing

Table 1. Characteristics of the study patients

Characteristics	Values
Total, n	20
Female/male ratio	13/7
Mean age (range), years	53 (33–67)
Atopic HE	10
Hyperkeratotic HE	6
Pompholyx type MS	4
Before therapy	189 $\pm$ 100
At the end of therapy	57 $\pm$ 32
PGA severity rating	
Before therapy	20 severe
At the end of therapy	12 almost clear, 8 clear ¹
Alitretinoin	
30 mg/day	19
10 mg/day	1

HE = Hand eczema.

¹ Treatment was stopped after a PGA assessment of clear or almost clear.

plasmablasts. Interestingly, we observed that the percentage of circulating CD27++CD38++CD19+ plasmablasts significantly decreased upon alitretinoin treatment (*p* = 0.002; fig. 2b). Moreover, we determined a marginally significant reduction of the total IgE levels in serum by ELISA (*p* = 0.026) after treatment with 9-*cis*-RA (fig. 2c). Similar results were obtained with regard to the total IgA and IgM serum levels but not the total serum IgG (data not shown).

Accordingly, we observed significantly lower frequencies of CD23+CD19+ B cells after treatment with alitretinoin (*p* = 0.004; fig. 2d).

Alitretinoin Promotes a Decrease in CD4+ T Cells and Treg Cells in the Peripheral Blood

Next, we analyzed the frequency of CD4+ T cells from peripheral blood to investigate the phenotype and function of T cells subsets upon treatment with oral alitretinoin. Our data showed a significant reduction of the absolute numbers of CD4+ T cells after treatment with oral 9-*cis*-RA over 12 weeks (*p* = 0.003; fig. 3a). In contrast, the absolute numbers of CD8+ cells remained stable upon therapy (data not shown).

FoxP3 is the master regulator of natural and induced Treg and it is frequently used to identify Treg cells. To detect this cell subset, we performed surface CD4, CD25, and intranuclear FoxP3 staining. We observed a similar

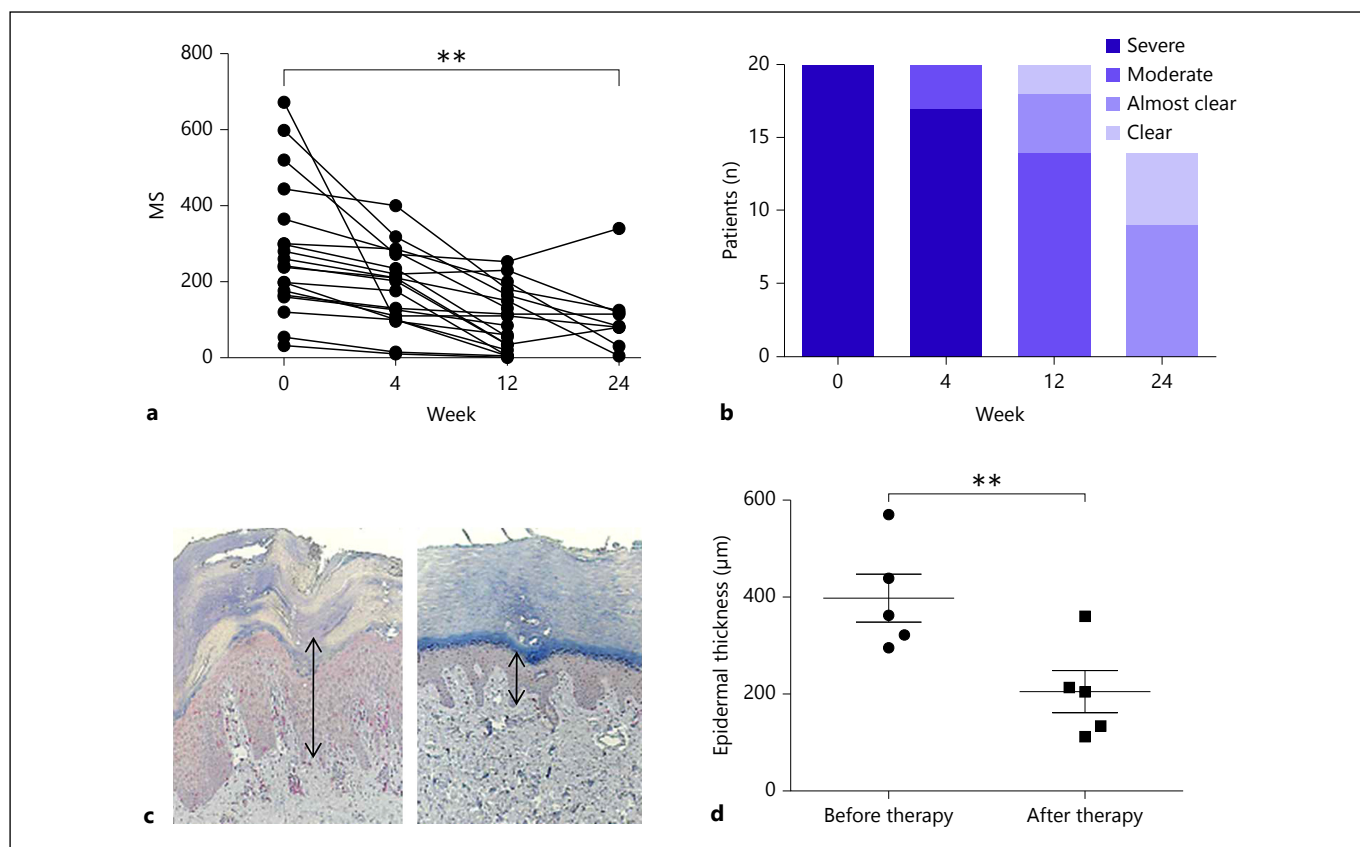


Fig. 1. Efficacy of oral alitretinoin in patients with CHE. Clinical improvement of hand eczema was defined according to the MS (**a**) and to the PGA (**b**) before (week 0) and 4, 12, and 24 weeks after therapy with 9-*cis*-RA ($n = 20$). **c** Skin biopsies from the affected skin area were taken before and after therapy ($n = 5$). Representative illustration of palm skin from a patient with CHE before (left)

and after (right) therapy. The double-headed arrows show epidermal thickness, which is reduced upon treatment. H&E. Original magnification $\times 100$. **d** There was a significant reduction of epidermal thickness before ($398 \pm 111 \mu\text{m}$) and after ($205 \pm 97 \mu\text{m}$) treatment ($n = 5$). ** $p < 0.01$.

result for FoxP3+ cells after treatment and detected a significant reduction of the absolute numbers of Treg cells in the peripheral blood as well ($p = 0.025$; fig. 3e). No changes in the percentage of CD4+ T cells and CD4+CD25+FoxP3+ T cells were observed (data not shown).

Increase in Treg Cells in Lesional Skin after Treatment of Hand Eczema

To determine whether the observations mentioned above were due to modulation of the skin-homing capacity of Tregs, we stained lesional skin from alitretinoin-treated patients with antibodies against CD4 and FoxP3 before (fig. 3c, g) and after treatment (fig. 3d, h). Interestingly, we observed a significant increase in the number of CD4+ cells ($p = 0.049$; fig. 3b) as well as an accumulation of FoxP3-expressing cells in lesional skin after treatment

with alitretinoin (fig. 3f). The median proportion of FoxP3+ from all CD4+ cells was 9.8% (9.1–27.5%) after treatment. In contrast, only few FoxP3+ cells in the lesional skin were detectable before therapy.

Alitretinoin Treatment Diminishes IL-17+ T Cells in the Peripheral Blood

Subsequently, we analyzed the effector cytokine profile in activated CD154+CD4+ T cells following restimulation with staphylococcal enterotoxin B and anti-CD28. As shown in figure 4b, the percentages of IL-17+ T cells were significantly decreased after treatment ($p = 0.029$). Moreover, the frequencies of IL-4- (Th2) and IFN- γ - (Th1) producing effector T cells were not decreased (fig. 5c, d). Of note, the percentage of activated CD154+CD4+ T cells remained stable during therapy with alitretinoin (fig. 5b).

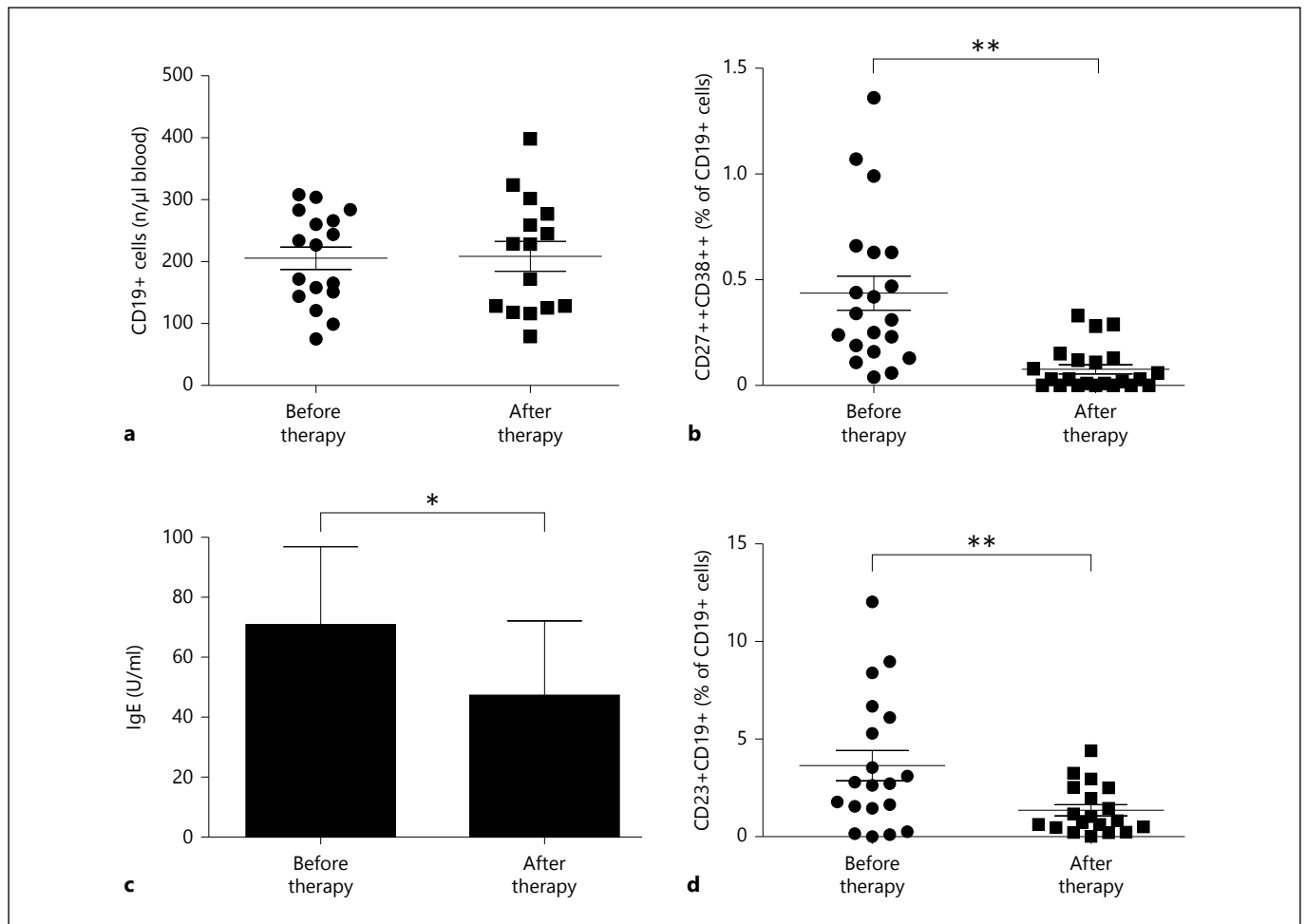


Fig. 2. Reduced frequencies of peripheral Ig-secreting plasmablasts, CD23+ B cells and IgE levels upon oral alitretinoin treatment. **a** Absolute number of CD19+ B cells calculated in whole blood of CHE patients using Trucount technology (n = 17). Percentages of CD27++CD38++CD19+ (plasmablasts; n = 20) (**b**)

and CD23+CD19+ B cells (**d**) were determined by flow cytometry (n = 19). **c** Total serum IgE was detected by ELISA before and after treatment with alitretinoin (n = 17). Data are shown as means ± SEM. * p < 0.05; ** p < 0.01.

Discussion

Our results confirm the data of several clinical studies reporting that oral alitretinoin is an effective and well-tolerable systemic therapy for several morphological and etiological types of CHE [3]. In this study, clinical improvement was reflected in a significant reduction of the MS and it was supported by histologic findings, with a significant decrease in epidermal thickness compared to baseline. In addition, clear or almost clear PGA response rates were achieved by all patients.

Moreover, we observed profound immunomodulatory effects upon systemic alitretinoin treatment in the T but also in the B cell compartment.

T-cell-mediated immune responses against common environmental antigens or allergens penetrating into the skin play a crucial role in the pathogenesis of CHE [18].

The initial phase of atopic eczema is predominantly characterized by IL-4-producing Th2 cells, which result in the activation of macrophages, eosinophil attraction, and enhanced IgE levels produced by B cells. Subsequently, IL-12 production of macrophages and eosinophils induces a Th1 response with increased IFN-γ expression during the chronic phase of eczema [19].

We showed that alitretinoin treatment not only results in a reduction of the total number of CD4+ T cells, but also leads also to a significant reduction of FoxP3+ Treg cells in the peripheral blood.

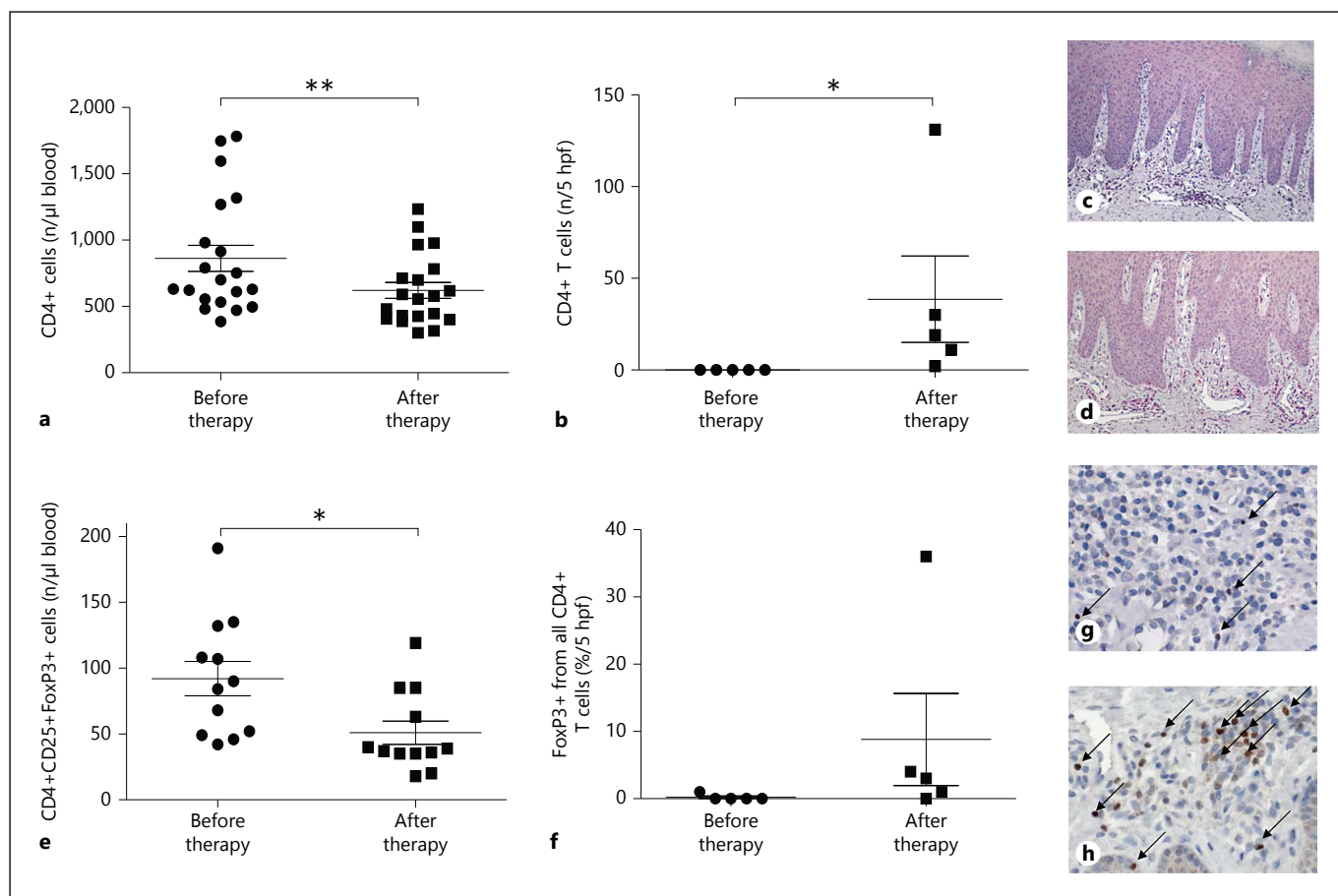


Fig. 3. Impact of oral alitretinoin treatment on peripheral and local T cells. **a** Decreased absolute numbers of CD4+ T cells were detected in whole blood of CHE patients after therapy with alitretinoin ($n = 20$). **b** Increased numbers of CD4+ T cells were found in palm skin after therapy with alitretinoin ($n = 5$). Representative illustrations of CD4 staining before (**c**) and after (**d**) therapy. Original magnification $\times 100$. **e** According to **a**, but absolute numbers

of CD4+CD25+FoxP3+ cells in whole blood ($n = 12$) are shown. **f** An increased proportion of FoxP3+ cells of CD4+ cells was found in palm skin after therapy with alitretinoin ($n = 5$). Representative examples of FoxP3 staining before (**g**) and after (**h**) treatment with alitretinoin. The arrows indicate FoxP3+ T cells. Original magnification $\times 400$. Data are shown as means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$.

These data suggest that systemic alitretinoin treatment may affect T cell homing. Indeed, we determined elevated T cell numbers in skin from our patients after treatment with alitretinoin compared to baseline. We identified approximately 9.8% of the skin T cells to be FoxP3 positive in clinically improved skin, which corresponds to the previously reported prevalence of FoxP3+ Tregs in normal human skin [20].

Recent data have demonstrated that retinoic acid enhances Treg cell differentiation while blocking differentiation towards Th17 cells in mice in vitro and in vivo [21]. Following these findings, we also observed a significant decrease in peripheral Th17 cells (IL-17+ cells). In addition to Th1 and Th2 cells, Th17 cells have

been associated with the development and pathophysiology of allergic diseases [12, 13]. Previous studies have demonstrated that Th17 cells are increased in AD patients and can be determined in eczematous skin lesions of AD [22]. Th17 cells are proinflammatory via stimulation of keratinocytes to produce cytokines, chemokines, and vascular endothelial growth factor. Moreover, we have previously shown that increased numbers of Th17 cells are detectable in AD patients and that IL-17 promotes IgE production in vitro [23, 24].

IL-4, an inflammatory cytokine produced by Th2 cells, and IFN- γ produced by Th1 helper cells remained stable upon alitretinoin treatment.

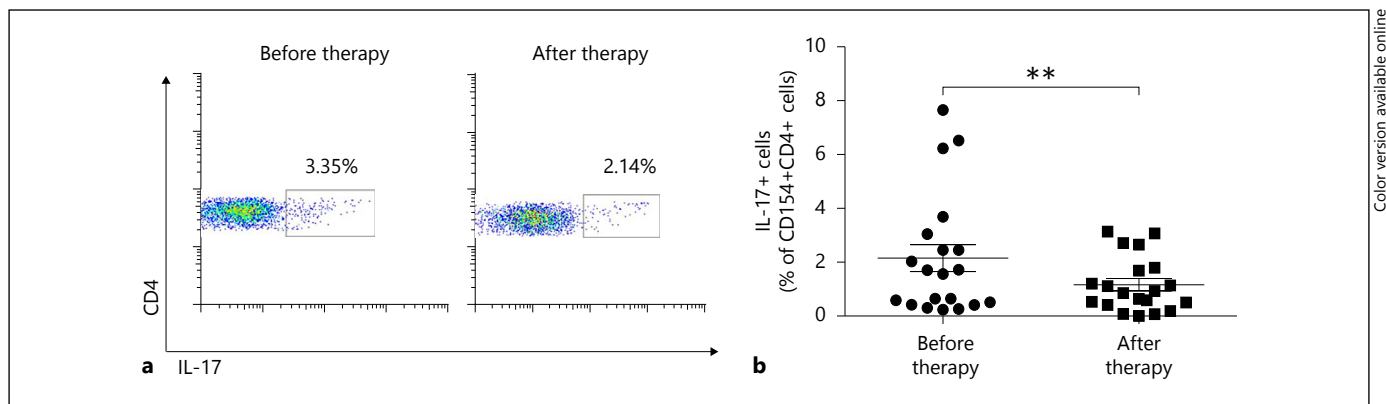


Fig. 4. Decreased frequencies of IL-17 producing T cells upon oral alitretinoin treatment. Intracellular cytokine expression was determined in stimulated peripheral CD4+ T cells before and after oral alitretinoin treatment. **a** Flow cytometric analysis of IL-17-producing T cells. Data represent cytokine-producing T cell

frequencies as a percent of CD154+CD4+ (activated) T cells. A dot plot from 1 representative donor is shown. **b** Frequencies of IL-17-producing T cells (n = 20). Data are shown as means $\pm$ SEM. ** p < 0.01.

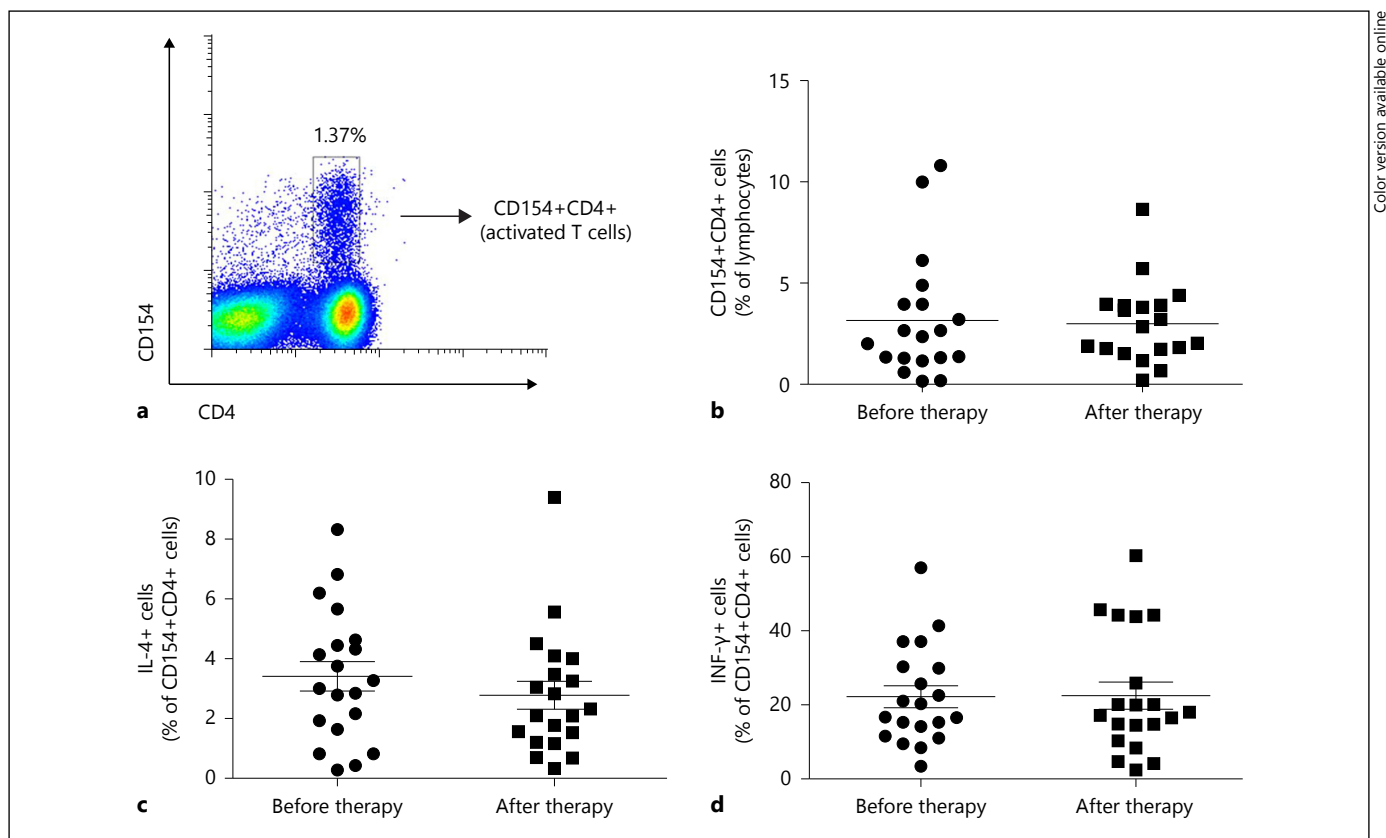


Fig. 5. No effects of oral alitretinoin treatment on T cell activation or IL-4 and IFN- γ production. Activation and intracellular cytokine expression were determined in stimulated peripheral CD4+ T cells before and after oral alitretinoin treatment. **a** Flow cytometric analysis of CD154+CD4+ (activated) T cells. A dot plot from 1 representative donor is shown. **b** Frequencies of CD154+CD4+

T cells. Results are summarized for all donors (n = 20). **c** Frequencies of IL-4-producing T cells (n = 20). Data represent frequencies of cytokine-producing T cells as a percent of activated T cells. **d** According to **c**, but frequencies of IFN- γ producing T cells are shown. Data are presented as means $\pm$ SEM.

However, several studies have previously described that vitamin A supplementation blocks the production of Th1 cell cytokines in mice in vitro [25]. Other groups have shown that 9-*cis*-RA increases the expression of IL-4 from human T cells in vitro [11]. Therefore, it remains to be clarified how exactly alitretinoin acts on differentiated T cells. It is important to emphasize that the percentage of activated CD154+CD4+ T cells remained stable before and during therapy with alitretinoin.

In contrast, our data showed significant modulation of the peripheral B cell compartment upon systemic treatment with alitretinoin.

The percentage of CD27++CD38++CD19+ plasma-blasts decreased during treatment with alitretinoin. Because of the strong reduction of the number of plasmablasts, we analyzed the Ig levels in the serum samples of our treated patients as well. Indeed, we detected significant diminishment of serum IgE levels. Although a tendency towards a reduction of IgA and IgM levels was also detectable, no significance was reached for these Ig subclasses. As RA is not only capable of inhibiting IgE synthesis but also reduces IL-6 production [26], which is an important factor for plasmablast differentiation [27], such mechanisms might be responsible for our results. Additionally, the reduction of the total IgE was associated with a reduction of the frequency of the IgE low-affinity receptor CD23 on B cells, which plays an important role in the regulation of IgE synthesis [28].

These data confirm our previous in vitro observations showing inhibition of IgE production with retinoids and suggest clinically an additional mechanistic effect of systemic alitretinoin in atopic hand eczema via diminution of serum IgE, which is elevated in a high percentage of atopic dermatitis patients [29]. IgE binds to cutaneous mast cells and dendritic cells and contributes, together with specific T cells, to the inflammatory response in the skin [30]. In

line with these observations, previous data have shown that the standard daily dose of 30 mg oral alitretinoin promotes a clinical improvement of extrapalmar lesions in patients with AD [31]. Whether or not systemic alitretinoin might be in principle a therapy for severe AD patients needs to be proven in prospective, placebo-controlled clinical trials.

Taken together, our results confirm the good clinical efficacy of oral alitretinoin seen both in the clinical development program and in clinical practice [32]. Moreover, our data indicates that 9-*cis*-RA is a potent modulator of inflammatory processes concerning the T cell but also the B cell compartment in patients with CHE. This conclusion is based on the impact of 9-*cis*-RA on the differentiation, proliferation, and migration of T and B lymphocytes. Previously published data suggest that RA inhibits costimulatory molecule expression on antigen-presenting cells [33], which results in a reduced efficacy of antigen presentation, lymphocyte activation, proliferation expansion, and consequently cytokine secretion [33, 34]. RA also shows effects on the differentiation and Ig production of B cells [9, 26]. Accumulated FOXP3+ Treg cells in the skin can regulate the activity of effector T cells, contributing to the reduction of proinflammatory responses. The histological data obtained from lesional skin support these observations, showing improvement of the epidermal integrity associated with an increase in regulatory FoxP3-positive cells in the skin after treatment. Further studies should focus on the long-term stability of these observations even after a reduction of the dose or discontinuation of the treatment.

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