Received: 1 March 2019 | Revised: 2 October 2019 | Accepted: 3 October 2019

DOI: 10.1111/sji.12835

Scandinavian Journal of Immunology WILEY

Antagonism of the interleukin 4 receptor α promotes T_H1-signalling among T cells from patients with atopic dermatitis after stimulation

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Funding information
The study presented here was made possible by financial support from Sanofi-Aventis Denmark. Sanofi-Aventis was not involved in any portion of the study design, data collection, data analysis, manuscript preparation or review process, nor in the decision to submit the manuscript for publication.

Abstract

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease. Molecular characterization of AD shows an underlying inflammation with tissue infiltration of T helper (T_H) 2 cells and increased IL-4 and IL-13. The multifaceted roles of IL-4 and IL-13 in allergic disease development make IL-4Rα an attractive target for treatment strategies, and a neutralizing monoclonal antibody which antagonizes the effects of both IL-4 and IL-13 by blocking the interaction site found in the IL-4 receptor subunit α (IL-4Rα) has been successfully used to treat patients with moderate-to-severe AD. To elucidate the effects of IL-4Rα blockade on the cellular level, we used flow cytometry to examine cytokine production after antigen stimulation in human T cells from patients with AD (n = 12) and healthy controls (n = 6). The cells were stimulated with and without a neutralizing monoclonal antibody against IL-4Rα. Our results indicate that blocking IL-4Rα prohibits IL-4 signalling and IL-13 signalling and thereby T_H2 differentiation followed by an upregulation of interferon-γ-producing cells.

1 | INTRODUCTION

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease with a lifetime prevalence of 10%-20% worldwide.[1] The pathogenesis of AD is characterized by skin barrier impairment which promotes an increased interplay between exogenous stressors and an overactive immune system leading to eczema.[1] Molecular characterization of AD shows an underlying inflammation with tissue infiltration of T helper (T_H) 2 cells, IgE-producing B cells, eosinophils and mast cells.[2] The T_H2 cells specialize in mustering of type 2 immune responses, characterized by their production of cytokines IL-4, IL-5, IL-13 and IL-31, which are found in high concentrations in acute AD lesions compared with unaffected skin.[3] In addition to these immunological changes, a deregulation of skin barrier proteins induced by the T_H2 cytokine milieu is observed in keratinocytes from AD lesions. However, it remains unclear whether increased skin permeability leads to an overstimulated immune response or whether a dysregulated and T_H2-dominant immune system promotes a decreased skin barrier function.[4] Independent of primary factors leading to AD, it is generally recognized that IL-4 is key in augmenting the type 2 immune response during disease development by upregulating the transcription of genes encoding T_H2-associated cytokines, including IL-4, leading to an auto-augmenting loop while decreasing the expression of T_H1 cytokines like interferon γ (IFN-γ).[2,5] In active AD tissue, the T_H2 polarization plays a central role for the occurrence of pruritus and has been linked to the presence of IL-31 in murine skin, which is believed to stimulate sensory nerve endings.[6] Additionally, the IL-4, IL-13 and IL-31 activation pathways lead to an increase in epidermal permeability by decreasing gene expression of barrier proteins in keratinocytes.[7,8] It is very likely that the decrease in skin barrier function and the auto-augmenting effects of IL-4 both play important role

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in AD chronicity. However, chronic AD has been associated with a gradual increase in T_H1 and T_H22 cell populations following the morphological changes such as dermal lichenification and excoriated plaques.[9] The multifaceted roles of IL-4 and IL-13 in allergic disease development make IL-4Rα an attractive target for treatment strategies. Dupilumab (Dupixent®; Regeneron, Tarrytown, NY, USA), a neutralizing monoclonal antibody which antagonizes the effects of both IL-4 and IL-13 by blocking the interaction site found in the IL-4 receptor subunit α (IL-4Rα), has been successfully trialled in adult patients with moderate-to-severe AD.[10] Dupilumab treatment decreases the expression of T_H2-associated genes (eg *IL13*, *IL31*, *CCL17*, *CCL18* and *CCL26*) in the skin of patients with AD.[11,12] To elucidate the effects of IL-4Rα blockade on the cellular level, we used flow cytometry to examine cytokine production after antigen stimulation in human T cells cultivated with a neutralizing monoclonal antibody against IL-4Rα.

2 | MATERIALS AND METHODS

2.1 | Study overview

We included 12 adults with mild-to-moderate AD to the clinic, of whom six had a history of cat allergy as well as six healthy adults with no signs of atopy. AD was diagnosed according to the Hanifin and Rajka criteria. Peripheral blood was drawn from each subject, and peripheral blood mononuclear cells (PBMCs) were isolated immediately after each visit. Plasma was stored at −20°C and used to measure the concentration of IgE. The study was approved by the local ethics committee of the Capital Region of Denmark (H-18006810) and was carried out in accordance with the Declaration of Helsinki.

2.2 | PBMC preparation and activation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples with Lymphoprep™ (Fresenius Kabi), washed repeatedly in PBS (Sigma-Aldrich) supplemented with 0.2% human serum albumin (CSL Behring) and 2 mmol/L EDTA and once in PBS with 5% foetal bovine serum (Sigma-Aldrich), before they were stained with 5 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Thermo Fisher Scientific). The PBMCs were cultured in RPMI-1640 (Sigma-Aldrich) with 5% heat-inactivated human AB serum (Thermo Fisher Scientific) at a density of 2×10^6 cells per mL and with or without 0.2 μg/mL staphylococcal enterotoxin B (SEB; Sigma-Aldrich) for 3 days or 3 μg/mL natural Fel d 1 (LTN-FD1-1, Endotoxin < 0.03 EU/μg; Indoor Biotechnologies) for 7 days at 37°C and 5% CO_2. In parallel, all cultures were prepared with either 0.5 μg/mL neutralizing monoclonal antibody (mAB) against human IL-4Rα (#25463; R&D Systems) or 0.5 μg/mL IgG_{2A} isotype control (#20102; R&D Systems).

2.3 | Reactivation and flow cytometric analysis

After incubation for either 3 (SEB) or 7 days (Fel d 1), PBMCs were re-stimulated for 5 hours with 25 ng/mL phorbol myristate 13-acetate (PMA) and 1 μg/mL ionomycin with the addition of 10 μg/mL brefeldin A (Sigma-Aldrich) for the final 4 hours. The cells were then stained with FVS780 viability dye (BD Biosciences) for 15 minutes at 4°C and then prepared for intracellular cytokine staining with an IntraPrep kit (Beckman Coulter).

The staining mixture was composed of anti-CD3 BV711 (UCHT1), anti-CD4 BV510 (SK3), anti-CD8 PerCp-Cy5.5 (SK1), anti-IFN-γ AF700 (B27), anti-IL-5 PE (JES1-39D10), anti-IL-10 PE-CF594 (JES3-19F1), anti-IL-13 BV421 (JES10-5A2, all from BD), anti-IL-31 APC (31SNEZE, Thermo Fisher Scientific) and anti-IL-4 BV605 (MP4-25D2; Biolegend). Flow cytometry was performed with an LSR Fortessa instrument (BD) to identify the production of chosen cytokines in proliferating lymphocytes. To ensure comparability between separate runs, the instrument was tested using BD FACSDiva CS&T Beads (BD) before each analysis. The output files were subsequently analysed in FLOWJO v.10 (FlowJo).

2.4 | Measurement of specific IgE

Specific IgE towards recombinant Fel d 1 and cat extract were analysed by the Phadia ImmunoCAP immunoenzymatic assay (Thermo Fisher Scientific) from plasma.

2.5 | Statistical analysis

Flow cytometric data are presented with medians. Comparisons between the AD and healthy groups were

TABLE 1 Demographic and clinical characteristics of study participants at baseline

Feature	Healthy controls	AD patients
Participants, n	6	12
Age, years, median (range)	26 (24-63)	29 (23-57)
Sex, female, n (%)	4 (66.6)	4 (33.3)
AD, n (%)	0 (0)	12 (100)
Asthma, n (%)	0 (0)	2 (12.5)
Hay fever, n (%)	0 (0)	3 (25)
Cat allergy, n (%)	0 (0)	6 (50)
Positive sIgE to cat (>0.35 kUA/L), n (%)	0 (0)	2 (16.7)
Positive sIgE to Fel d 1 (>0.35 kUA/L), n (%)	0 (0)	2 (16.7)

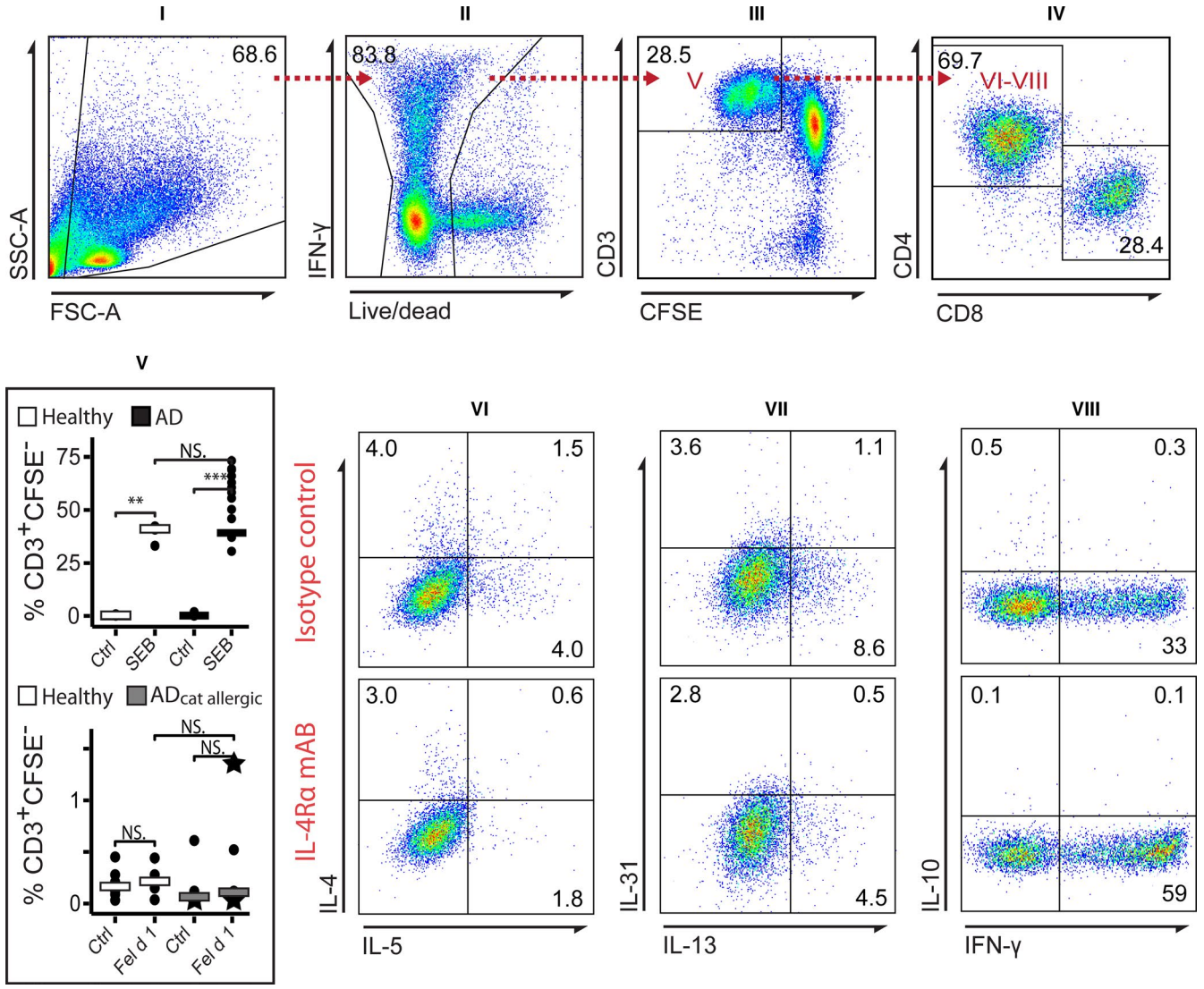

FIGURE 1 Identification of SEB-activated T helper cells. Panels I-IV show the gating strategy for identifying proliferating T helper cells after exclusion of debris and doublets (not shown): I, PBMCs were selected after plotting forward scatter (FSC) against side scatter (SSC). II, The fraction of viable PBMCs were identified by plotting live/dead signal against IFN-γ, because the panel is known to cause a spillover from strong IFN-γ signals into the live/dead channel, resulting in the characteristic "mushroom" density plot. III, Proliferating T cells were isolated by gating for $CD3^+$ events with a low CFSE signal. IV, The T helper subset was distinguished from cytotoxic T cells by plotting CD4 against CD8. Panel V summarizes the degree of proliferation induced among T cells from AD patients (n = 12) and healthy controls (n = 6) by SEB (upper panel) or among T cells from AD patients with cat allergy history (n = 6) or healthy controls by Fel d 1. The degree of proliferation is indicated by the fraction of events selected in panel III ($CD3^+CFSE^-$). Two cat allergic patients with high concentrations of IgE against rFel d 1 are marked by stars. Panels VI-VIII exemplify the cytokine production in $CD4^+$ T cells activated with SEB from one AD patient with or without IL-4Rα antagonism. Significance levels correspond to the following *P* values: $^{**}P < .01$ and $^{***}P < .005$

assessed with the Mann-Whitney *U* test, while comparisons within the same group were performed with the Wilcoxon signed-rank test. Statistical significance was considered for a two-sided *P* value below .05. Significance levels are indicated on figures corresponding to the following *P* values: $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .005$. Statistical analyses were performed using R STUDIO software, version 1.1.463 (RStudio: Integrated Development Environment for R).

3 | RESULTS

The characteristics of the patients and healthy controls are shown in Table 1. In total, 12 patients with AD were included; of the patients with AD, six had a history of cat allergy. However, only two had a positive (>0.35 kU/L) specific IgE towards the major cat allergen (Fel d 1) and cat extract (Table 1). None of the six healthy controls had a history of cat allergy and cat-specific IgE (<0.35 kU/L, Table 1). The flow

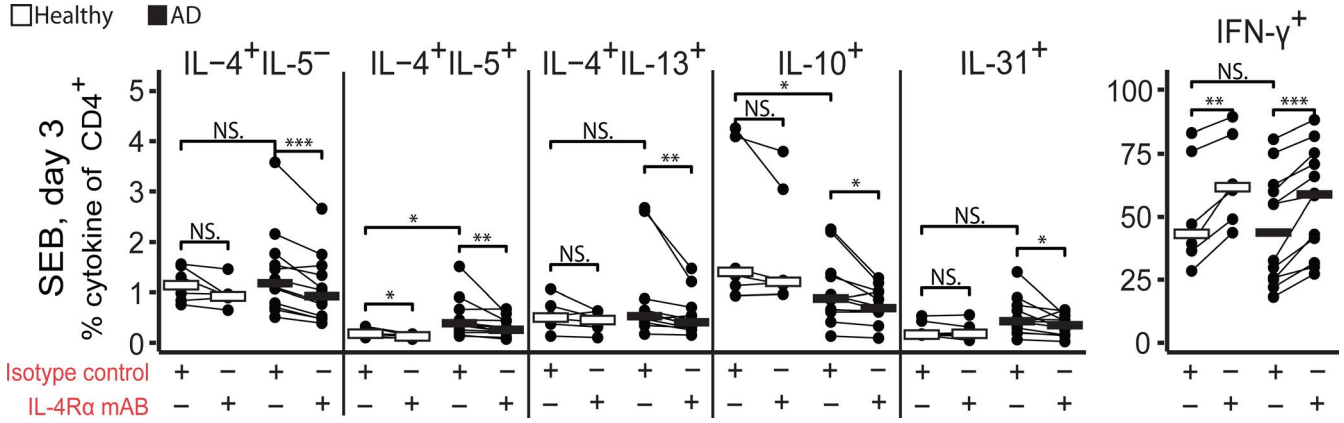

FIGURE 2 Cytokine production in SEB-activated T helper cells. Frequencies of immature T_H2 cells (IL-4^+IL-5^-), mature T_H2 cells (IL-4^+IL-5^+ or IL-4^+IL-13^+) and cells producing either IL-10, IL-31 or IFN-γ in response to SEB stimulation are presented for healthy controls (n = 6) and AD patients (n = 12). Comparisons are made between cells stimulated with the IL-4Rα antibody or an isotype control. Horizontal bars represent group medians and are marked in white for healthy controls and black for AD patients. Significance levels correspond to the following *P* values: $*P < .05$, $**P < .01$ and $***P < .005$

cytometry panel was designed to isolate and examine cytokine production in proliferating T helper cells. After exclusion of debris and doublets using forward and side scatter gating, the fraction of viable cells was isolated using the viability dye (Figure 1, I-IV). The degree of proliferation among T cells induced by either SEB or Fel d 1 was determined by the percentage of viable cells that stained positive for CD3 and negative for CFSE (CD3$^+$CFSE$^-$). SEB induced significant proliferation among T cells compared to un-stimulated cells (Figure 1, V, top panel). Fel d 1 could not induce enough proliferation in T cells after 7 days of incubation to distinguish them from un-stimulated cells as seen from the results for the six cat allergic patients with AD (Figure 1, V, bottom panel).

3.1 | IFN-γ-production is promoted in SEB-activated CD4$^+$ T cells during IL-4Rα antagonism

The graphs in Figure 1, VI-VIII show the frequencies of proliferating CD4$^+$ T cells from one patient with AD producing the cytokines of interest after 3 days of SEB stimulation with the IL-4Rα antibody (Top panel) or an isotype control (Bottom panel). Importantly, the frequencies of CD4$^+$ T cells producing IL-4, IL-5, IL-10, IL-13 or IL-31 were lower when stimulated with SEB in the presence of anti-IL-4Rα compared with the isotype control, while the opposite is true for IFN-γ-producing cells. The results from SEB stimulation and IL-4Rα neutralization are summarized for all subjects in Figure 2 and showed that SEB-activated CD4$^+$ T cell subsets were dominated by IFN-γ-producing cells (Figure 2, rightmost panel), while smaller populations of cells producing T_H2 cytokines were also present. Fractions of CD4$^+$ T cells which stained positive for IL-4, IL-5, IL-13 and IL-31 tended to be larger in four patients with AD (Figure 2, panel 1-5). Conversely, about half of the patients with AD had lower fractions of CD4$^+$ T cells that produced anti-inflammatory IL-10 with markedly higher fractions seen for two of the non-atopic adults. Notably, fractions of CD4$^+$ T cells producing type 2 cytokines were lowered in most subjects by the IL-4Rα antibody with effects being most prominent in patients with AD.

In particular, the antibody significantly reduced fractions of CD4$^+$IL-4$^+$IL-5$^-$ cells ($P < .001$, 1.20% vs 0.93%), which represent an immature subset of T_H2 cells. Significant reductions were also seen for CD4$^+$IL-4$^+$IL-5$^+$ cells ($P < .006$, 0.40% vs 0.27%) and CD4$^+$IL-4$^+$IL-13$^+$ cells ($P < .009$, 0.54% vs 0.42%). IL-4, IL-5 and IL-13 are typically co-expressed by T_H2 cells, and it can therefore be expected that many of these events overlap in our results. Inversely, the fraction of CD4$^+$IFN-γ$^+$ cells was highly increased by anti-IL-4Rα ($P < .0005$%, 43% vs 57.6%). A similar increase was observed for CD8$^+$T cells producing IFN-γ (data not shown).

3.2 | Anti-IL-4Rα reduces T_H2 cytokine production among Fel d 1 stimulated CD4$^+$ T cells

To investigate effects of IL-4Rα antagonism in a T_H2 environment, Fel d 1 was used to activate PBMCs from six of the AD patients with a history of cat allergy and the six healthy controls. When the CD4$^+$ T cells from the small Fel d 1-activated subsets (CD3$^+$CFSE$^-$) were analysed in detail, T_H2 cytokine-producing cells were identified from patients with AD allergic to cat and not from the non-atopic adults (Figure 3).As for SEB-activated cells, the proportion of these T_H2 cytokine-producing cells was reduced in the presence of anti-IL-4Rα. Specifically, notable reductions were reflected by the CD4$^+$ cells from patients with AD, which were IL-4$^+$IL-5$^+$ ($P < .03$, 4.33% vs 0.16%), IL-4$^+$IL-13$^+$ ($P < .03$, 5.74% vs 1.27%), IL-10$^+$ ($P < .03$, 8.00% vs 2.06%), IL-31$^+$ ($P < .03$, 7.01% vs 3.83%) or

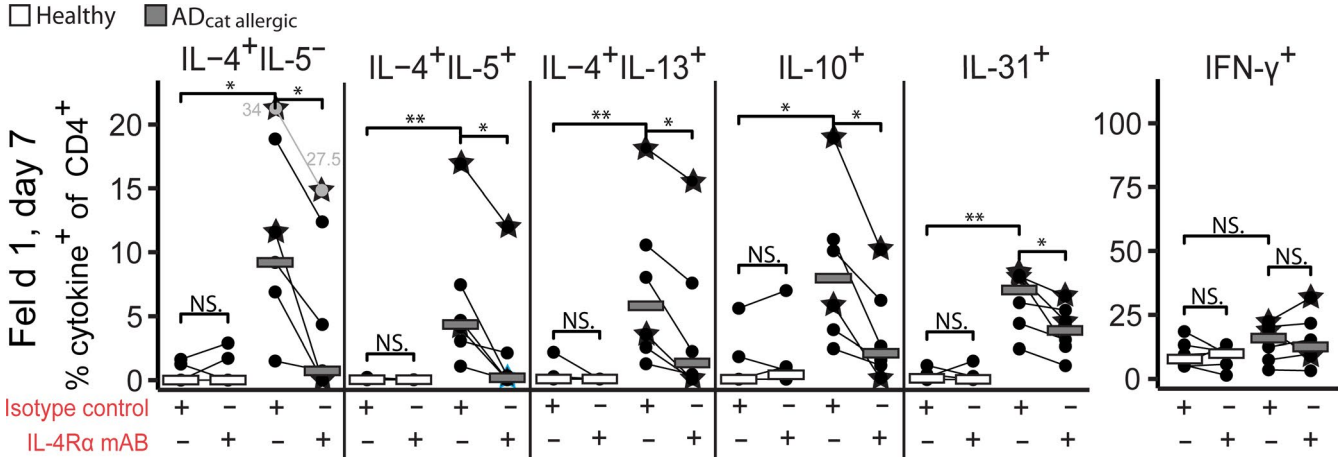

FIGURE 3 Cytokine production in Fel d 1-stimulated T helper cell. The frequencies of immature T_H2 cells (IL-4$^+$IL-5$^-$), mature T_H2 cells (IL-4$^+$IL-5$^+$ or IL-4$^+$IL-13$^+$) and cells producing either IL-10, IL-31 or IFN-γ in response to Fel d 1 stimulation are presented for healthy controls (n = 6) and AD patients with cat allergy (n = 6).Comparisons are made between cells stimulated with the IL-4Rα antibody or an isotype control. Horizontal bars represent group medians and are marked in white for healthy controls and grey for AD patients with cat allergy. Two cat allergic patients with high concentrations of IgE against rFel d 1 are marked by stars. Significance levels correspond to the following *P* values: **P* < .05, ***P* < .01

IL-4$^+$IL-5$^-$ (*P* < .03, 10.4% vs 2.54%). In contrast to SEB-stimulated cells, IFN-γ-producing cells made up a smaller fraction of the CD4$^+$ subset after Fel d 1 stimulation and were not promoted to the same extent by the addition of anti-IL-4Rα. All cultures were also prepared without antibodies and showed similar phenotypes to cells stimulated with the isotype control (data not shown), suggesting that effects of the IL-4Rα antibody are caused by its binding specificity.

4 | DISCUSSION

In this study, we have shown that IL-4 antagonism during SEB stimulation inhibits production of T_H2 cytokines and promotes IFN-γ production in CD4$^+$ T cells from both healthy controls and patients with AD. Stimulation with cat allergen induces T_H2 cytokine production in CD4$^+$ T cells from patients with AD and a history of cat allergy, but not in CD4$^+$ T cells from healthy controls and the cytokine production was inhibited by IL-4 antagonism. The data presented here suggest that a T_H2 cytokine milieu in activated PBMC cultures can be easily reverted by restricting IL-4 and IL-13 signalling, favouring the production of IFN-γ after SEB stimulation.

Though our data are generated from relatively small populations, our results are in agreement with previous findings, showing that recombinant IL-4 reduces the production of IFN-γ protein and mRNA[13] and that immunized lymph nodes from IL-4 knockout mice produced more IFN-γ,[14] suggesting that IL-4 has a strong influence on the T_H1/T_H2 balance. This could indicate that inhibitory effects of IL-4 on IFN-γ production have been neutralized by IL-4Rα antibody in our assays and supports the potential of the drug dupilumab as a treatment option for skin affected by AD, which is typically dominated by T_H2 cytokines. It could be that a shift towards T_H1 and increased IFN-γ production at least in theory contributes to increased colonization of *Staphylococcus aureus* and a risk for the development of adverse effects such as conjunctivitis or worsening of allergic contact dermatitis symptoms.[15-17] However, recent studies have shown that patients with AD who are treated with dupilumab experience a reduction in *S. aureus* counts and that the delayed hypersensitivity response observed in allergic contact dermatitis is not attenuated.[18,19] The same effect on IFN-γ production was not reached when blocking IL-4Rα in Fel d 1 cultures and might indicate that the IFN-γ level was too low to promote IFN-γ-production due to a lack of T_H1-promoting agents in these cultures. Similarly, treatment of AD patients with dupilumab led to no change or only a slight decrease in the expression of IFN-γ in the skin.[11,12] These differences in the regulation of IFN-γ production may indicate that the effects and adverse events observed for drugs such as dupilumab depend on the respective environment found in the skin of the patient. New ways to monitor the molecular environment of diseased skin in an easy and pain-free manner such as *tape stripping* are currently being developed and could help examiners to determine the most optimal treatment approach.[20] Collectively, our findings are in accordance with previous studies showing that IL-4Rα antagonism causes a decrease in T_H2 cytokines in the skin.

4.1 | Study limitations

The strength of this study is the inclusion of both patients with AD and a healthy control group. However, limitations of our study are present in the small number of healthy controls

and patients, the lack of data on IL-17 expression and the diversity between cat allergic patients.

CONFLICT OF INTEREST

P. Brøgger, L. Blom and S. Simonsen have no conflicts of interest. J. Thyssen is an investigator for Sanofi-Genzyme, Eli Lily & Co, LEO Pharma and AbbVie. He is an advisor for Sanofi-Genzyme, Eli Lily & Co. and Union Therapeutics. He has been a speaker for Sanofi-Genzyme and LEO Pharma. L. Skov has been a paid speaker for Pfizer, AbbVie, Eli Lilly & Co, Novartis and LEO Pharma, and has been a consultant or served on Advisory Boards with Pfizer, AbbVie, Janssen Cilag, Novartis, Eli Lilly & Co, LEO Pharma and Sanofi. She has served as an investigator for Pfizer, AbbVie, Eli Lilly, Novartis, Amgen, Regeneron and LEO Pharma and received research and educational grants from Pfizer, AbbVie, Novartis, Sanofi, Janssen Cilag and Leo Pharma. The authors do not have equity in pharmaceutical companies.

AUTHOR CONTRIBUTIONS

All authors have approved the submitted version of this manuscript. P. Brøgger drafted the paper, and the other authors contributed equally to revise it critically. Additionally, the authors have made substantial contributions to the following: Peter Brøgger contributed to the research design, patient visits, acquisition, analysis and interpretation of data; Lars H. Blom contributed to the research design, acquisition and interpretation of data; Stine Simonsen contributed to patient visits, acquisition and interpretation of the data; Jacob P. Thyssen contributed to research design and interpretation of the data; Lone Skov contributed to patient visits, research design, acquisition and interpretation of the data.

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How to cite this article: Brøgger P, Blom LH, Simonsen S, Thyssen JP, Skov L. Antagonism of the interleukin 4 receptor α promotes T_H1-signalling among T cells from patients with atopic dermatitis after stimulation. *Scand J Immunol*. 2020;91:e12835. https://doi.org/10.1111/sji.12835