



Research paper

Early IL-10 producing B-cells and coinciding Th/Tr17 shifts during three year grass-pollen AIT



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ARTICLE INFO

Article history:

Received 28 June 2018

Received in revised form 10 September 2018

Accepted 10 September 2018

Available online 11 October 2018

Keywords:

Allergic Rhinitis

Allergy

Specific Immunotherapy

Biomarker

Prediction

Surrogate

Tolerance

Regulatory B-cells (Bregs)

Regulatory T-cells (Tregs)

ABSTRACT

Background: Allergen-specific immunotherapy (AIT) is a causative treatment in allergic airway disease, comprising long-term allergen administration and requiring three years of treatment. Mechanisms and biomarkers that translate into clinical efficacy remain urgently needed.

Methods: In an exploratory observational allergy cohort we phenotyped 32 grass-pollen allergic patients with hayfever undergoing AIT for over three years and controls using local and systemic samples for ex vivo FACS, nasal transcriptomes and in vitro *phleum*-stimulation at critical time windows six hours after therapeutic allergen administration and during peak-season responses.

Findings: The up-dosing phase is marked by increased IL-10⁺ B-cells with allergen-specific PD-L1 up-regulation, while effector Th1/Th17 cells and CCR6⁺IL-17⁺FoxP3⁺T-cells decrease. The conversion phase exhibits Th17 recovery in the absence of Th2 cells. The tolerance-mounting phase after three years of treatment is characterized by induction of Tregs while Th2 and *phleum*-specific Th17 responses decrease. Notably, high ratios of circulating Breg/Th17 following initial AIT correlate significantly with clinical improvement after three years.

Interpretation: Our exploratory data hypothesize differential shifts in the hierarchy of tolerance in three distinct phases of AIT characterized by conversion of regulatory against pro-inflammatory mechanisms, of which the Breg/Th17 ratio after initial treatment emerges as potential early prediction of AIT efficacy.

Fund: This study was partially funded by Allergopharma GmbH & Co. KG, intramural funding and the German Center for Lung Research (DZL).

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1. Introduction

Allergen immunotherapy (AIT) for allergic airway disease has been applied since more than a century [1]. Clinical efficacy and safety have been demonstrated in multiple sponsored studies, systemic reviews and meta-analyses [2–4], further in interventional academic trials and few long-term studies [5,6]. Allergy is characterized by the IgE-dependent allergen-specific degranulation of mast-cells in the early phase and predominant Th2 memory in the late phase response, where

T-cells produce IL-4, IL-5 and IL-13. The mechanisms of AIT have been dissected in different models, hierarchies and compartments and include B-cell derived shifts from IgE to IgG4 [7,8], the induction of IL-10 producing T-regulatory cells [9–11], reduced Th2 responses [12] and the presence of Foxp3⁺ regulatory T-cells in the upper airway mucosa [13]. However, the understanding of underlying Th2-suppressive mechanisms inducing tolerance towards allergens remains fragmentary and has yet to be translated into clinical applications. Mechanistic insight can improve our options for effective monitoring of therapeutic responses and prediction of therapy success [6,14]. A balance of allergen-specific Th2 and in particular Th2A cells against Th1 or Treg cells was hypothesized as therapy relevant mechanism, while Th17 cells were not yet considered in this equation [15,16]. Th17 cells are elevated in allergic patients, systemically and locally in upper and lower airways during pollen season, however, they have not been implicated in

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Research in context

Evidence before this study

With almost every fourth adult and every third child affected, allergic airway disease is an increasing global health concern. Allergen immunotherapy (AIT) with the aim to restore tolerance to allergen has proved clinically useful, effective and safe. However, despite great advances in the understanding of mechanisms, reliable molecular or cellular biomarkers that indicate restoration of tolerance to allergen and correlate with therapeutic efficacy of the vaccine are not available.

We performed a search in PubMed for clinical studies in human without language restriction with terms “specific immunotherapy” AND “allergy” AND “biomarker” AND “tolerance” OR “surrogate” or “prediction” for articles published between 1st of January 1999 and 1st of May 2018. We found heterogeneously designed a) efficacy studies with new allergen immunotherapy investigational products or new dosage regimens using conventional clinical endpoints, b) observational or interventional cohorts under standard treatment with related mechanistic data, using predominantly cultured T-cells, immune-phenotyping, cytokine-pattern or serum IgG4 responses, c) an increasing number of upcoming studies with targeted immune-modulation with e.g. biologics combined with allergen immunotherapy. Very few studies suggested surrogate or predictive endpoints using generic inflammatory serum biomarker or shifts in IgE, IgG4, IgA and IgE facilitated antigen binding, however mostly not reflecting the interaction of T- and B- cells in the induction of tolerance to allergen.

Added value of this study

This exploratory clinical cohort study hypothesizes an amended understanding of tolerogenic dynamics during AIT *in vivo*, suggesting three phases, characterized by an initiation, a conversion, and a tolerance mounting phase. In this cohort the ratio of IL-10⁺ B-cells and Th17 cells during the early initiation phase corresponded to symptom improvement after three years of treatment, representing a potential decision point for treatment adjustment prior to long-term therapy.

Implications of all the available evidence

There is an increasing demand for accurate surrogacy, prognostic and early decisive markers in AIT, ideally to identify those patients who benefit most and those who do not. Further, long-term immunological data for the rational application of booster AIT are required. Validation of these promising new exploratory data shall enable us to apply more precise personalized AIT, as this treatment is still time-consuming and expensive with however proven long-term beneficial effects.

AIT-driven responses [17–19]. Recently, it has been shown that Th17 cells can not only differentiate into regulatory T cells (Tregs) via an intermediary subset expressing FoxP3 and IL-17 simultaneously, but also, that Tregs carry the ability to transdifferentiate “back” into Th17 cells in experimental autoimmune animal models [20,21].

Current data has attributed an essential role not only to T cells but also to B cells in limiting excessive immune reactivity [22], while in allergic diseases evidence is rare. B cells with regulatory capacities are able to inhibit allergen-specific T cell proliferation mediated by IL-10 secretion [23,24]. This regulatory capacity was also demonstrated in mice lacking IL-10⁺ B cells, which exhibit aggravated allergic skin

inflammation reversible by adoptive transfer of these cells [25,26]. Furthermore, IL-10⁺ B cells are known to restrict Th1 and Th17 differentiation [27–29]. In line with this finding, regulatory B cells mediate induction of pulmonary regulatory T cell infiltration in the murine lung, controlling airway inflammation [30]. Not only regulatory B cells but also transitory IL-17⁺FoxP3⁺ T cells were implicated to suppress autoimmune diseases, like myasthenia gravis, multiple sclerosis and autoimmune thyroid disease [20,31,32].

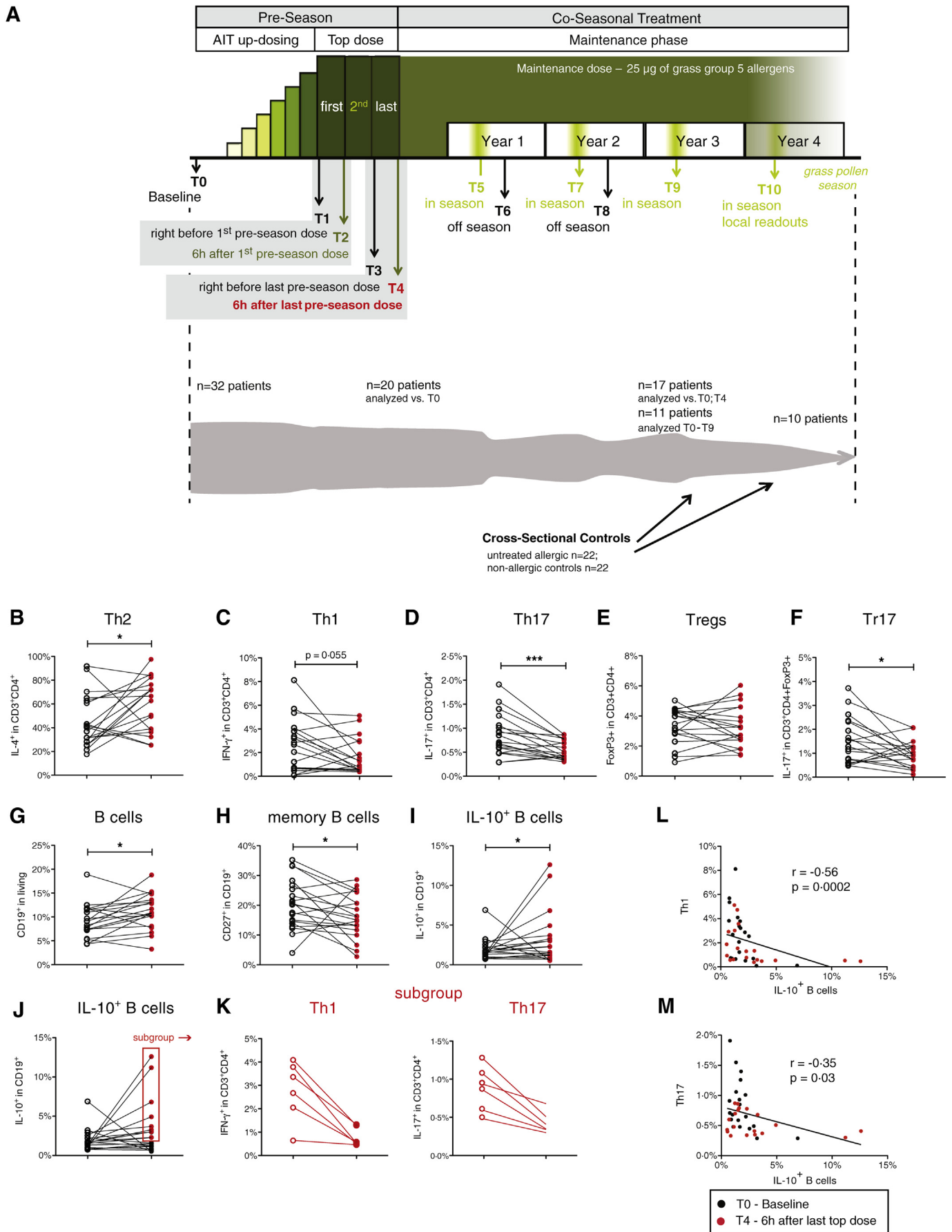
In this exploratory clinical cohort study with grass-pollen allergic patients undergoing AIT, we provide for the first time longitudinal data implicating the Breg/Th1/Th17 suppression axis early indicative for long-term therapy success, and demonstrating a shift of the Treg/Th2 balance late in tolerance induction during AIT.

2. Materials and methods

2.1. Study design and patients

This exploratory study was an open and observational real-life, case-controlled, long-term clinical cohort, aiming on (*Holcus lanatus*, *Dactylis glomerata*, *Lolium perenne*, *Phleum pratense*, *Poa pratensis*, and *Festuca pratensis*) mechanistic resolution of the allergen-specific immunotherapy effects, named *Prospective Allergy and Clinical Immune Function Cohort study* (PACIFIC, EudraCT 2015–003545–25). STROBE criteria were checked and respected throughout this manuscript. Thirty-two grass-pollen allergic patients with a history of moderate-severe and chronic-persistent allergic rhinitis as defined by ARIA (Allergic Rhinitis and its impact on Asthma) criteria [49] since >2 years during the grass-pollen season, a positive skin prick test wheal >3 mm in diameter and grass-pollen specific IgE-level above 0.70kU/l underwent subcutaneous grass-pollen AIT with a licensed grass-pollen allergoid (Allergovit®, Allergopharma GmbH & Co. KG, Germany) consisting of a 100% mixture of allergens from six grass pollen species (*Holcus lanatus*, *Dactylis glomerata*, *Lolium perenne*, *Phleum pratense*, *Poa pratensis*, and *Festuca pratensis*) chemically modified with formaldehyde and alum absorbed. 25 µg of grass group 5 allergens per maintenance dose was used in accordance to SPC and earlier published studies [50–52]. Twenty-two patients with defined grass-pollen allergy as described above but without AIT treatment and twenty-two non-allergic controls without a clinical history of chronic rhinosinusitis, a negative skin prick test and negative specific IgE-screening were recruited as controls. The study was approved by the ethics commission of the Technical University of Munich (5534/12). After written and informed patients' consent and in accordance with the Helsinki declaration, peripheral blood was obtained from patients at specific time points – at baseline levels, right before and 6 h after the first and the last pre-seasonal top dose injection in year one of AIT. All laboratory tests were conducted with blinded study personnel. Following the initial treatment phase, patients were treated with follow-up AIT injections every 4–8 weeks over a period of three years with reduction of the maintenance dose during grass-pollen season depending on symptom burden. Further blood samples were taken twice a year, once in (May – July) and once out of grass pollen season (November – March). This observational study used an open design to enable immune monitoring of as many patients at as many visits as possible: to reduce the rate of drop-outs and to make long-term monitoring feasible for the patients not every study visit was mandatory. This study was an exploratory biomarker study, but subjective outcome was measured using patient-assessed *Retrospective Assessment of seasonal Allergic Symptoms* (RAAS) by scoring overall hayfever symptoms in comparison to the season before and in year 3 in comparison to prior to treatment on a scale between +3 (much better), to 0 (no change) to –3 (much worse) as described recently [45].

Out of the PACIFIC cohort, peripheral and local samples from overall 32 patients were included in this study (Fig. 1A). 20 patients attended at least the initial visits during the AIT up-dosing and top dose phases and were used to analyse early treatment effects in Fig. 1. 17 patients



attended the initial visits and in addition visit T9 and were therefore eligible for the analysis of long-term treatment effects in Fig. 4A–F. Data from 15 patients were used for RAAS correlations in Fig. 7. 11 patients attended at least 9 out of all 10 visits and were therefore subjected to analysis of immune mechanisms during time trajectories in Fig. 2, Fig. 4G and H, and Supplementary Fig. 2, as well as to comparison with cross-sectional control groups in this longitudinal study in Fig. 3 (healthy controls $n = 13$, allergic rhinitis patients in grass pollen season without AIT $n = 14$). For Fig. 6 11 healthy controls, 8 untreated allergics and 10 patients, who had received at least 3 years of AIT, were included to analyse fresh local nasal samples. Longitudinal disposition of patients is displayed in Fig. 1 and patient characteristics including controls are summarized in supplementary Table 1.

2.2. Primary human nasal samples

Were taken during the peak-pollination season 2016 using nasal scrapings for nasal flow cytometric analysis and stained according to protocol. Frequencies of local T cell subsets of CD3⁺CD4⁺IL-17⁺ (Th17) cells, CD3⁺CD4⁺FoxP3⁺IL-17⁺ (Tr17) cells, CD3⁺CD4⁺FoxP3⁺ (Tregs), CD3⁺CD4⁺FoxP3⁺IL-10⁺ (IL10⁺ Tregs) as well as frequencies of local B cell subsets of CD19⁺ cells (B cells) and CD19⁺IL10⁺ cells (IL-10⁺ B cells) were assessed by flow cytometry using specific antibodies (Table 1.)

2.3. Freezing conditions

For cryopreservation, PBMC were resuspended at a concentration of $20 \times 10^6/\text{ml}$ in CTL-Cryo ABC Kit freezing medium A (Cellular Technology Limited, Cleveland, OH) at room temperature, according to manufacturer's instructions. An equal volume of freezing medium B, also at room temperature, was added dropwise, while gently mixing. The resulting cell suspension was pipetted in 1 ml aliquots into 1.8 ml cryovials (Sarstedt, Nümbrecht, Germany). Then, the tubes were placed into a room temperature Nalgene Cryogenic Controlled-Rate Freezing Container (Merck Chemicals GmbH, Darmstadt, Germany) that was directly placed into a -80°C freezer. After 24 h, the samples were transferred to a liquid nitrogen tank (Air Liquide, Paris, France) for indefinite storage until testing.

In Figs. 1, 2, 3, 4, 5, and 7, we used frozen samples from each patient, which were thawed and analysed simultaneously. In Fig. 6, we used fresh nasal samples, which were analysed immediately after isolation without culturing.

2.4. Culture conditions

For most experiments, cryopreserved primary PBMCs were used. Briefly, for intracellular stains cells were incubated in RPMI and treated according to protocol for 4 h including brefeldin A and then labeled for flow cytometry. For T cell ex vivo analyses in Figs. 1, 2, 3, and 7, we stimulated the cells for 4 h with PMA/ionomycin/BFA in order to allow

intracellular cytokine staining. For B cell ex vivo analyses in Figs. 1, 2, 3, and 7, we cultured the cells in RPMI supplemented with 10% human serum for 4 h in order to maximize intracellular IL-10 staining. For Fig. 4, we cultured the PBMCs in serum-free CTL-Test BTM Medium (Cellular Technology Ltd., Shaker Heights, OH, USA) supplemented with 2 mM L-glutamine at a density of 4 million PBMCs/ml and stimulated with either 5 µg/ml phleum (recombinant phleum p1, Allergopharma GmbH & Co. KG, Reinbek, Germany) or a specialized B-Poly-SE B cell stimulant (Cellular Technology Ltd., Shaker Heights, OH, USA) at 37°C and 9% CO₂ in a fully humidified atmosphere for 7 days according to manufacturer's instructions. CTL Human B-Poly-SETM is a stock solution containing recombinant human IL-4 and anti-CD40, which is used for generating IgE secreting memory B cells. According to manufacturer, seven-days prestimulation cultures were recommended.

2.5. Stimulation with phleum major antigen

PBMCs were cultured in serum-free CTL-Test BTM Medium (Cellular Technology Ltd., Shaker Heights, OH, USA) supplemented with 2 mM L-glutamine at a density of 4 million PBMCs/ml and stimulated with either 5 µg/ml phleum (recombinant phleum p1, Allergopharma GmbH & Co. KG, Reinbek, Germany) or a specialized B-Poly-SE B cell stimulant (Cellular Technology Ltd., Shaker Heights, OH, USA) at 37°C and 9% CO₂ in a fully humidified atmosphere for 7 days according to manufacturer's instructions. Cells were subjected to flow cytometric analysis, supernatants were concentrated to a 10-fold concentration by centrifugation through protein-binding Amicon columns (Merck Millipore, Billerica, MA, USA) and subjected to ELISA or MSD Mesoscale analysis.

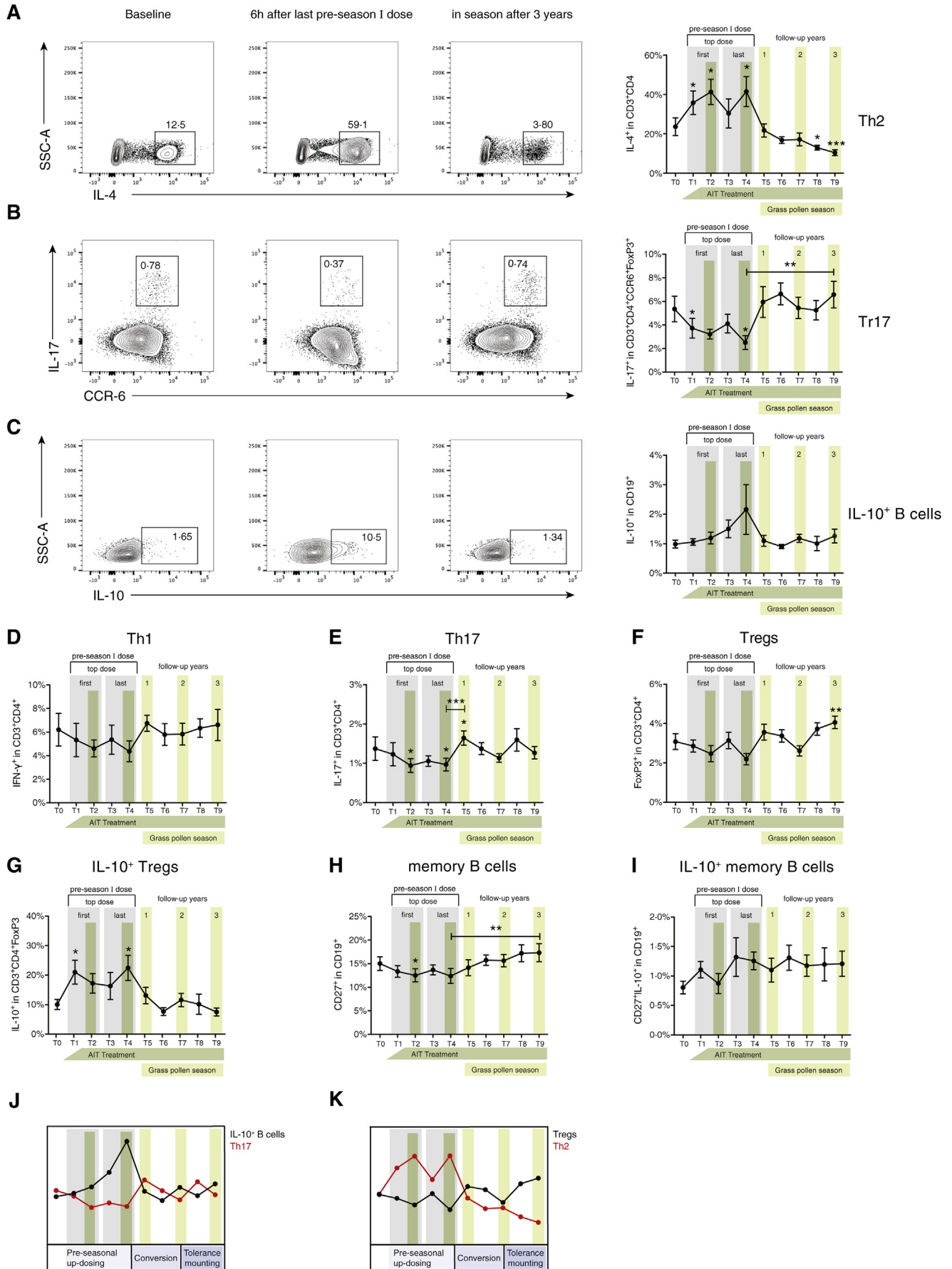
2.6. Flow cytometry

Following specific stimulation regimes, PBMCs or nasal samples were labeled for flow cytometry with specific antibodies using the Foxp3 / Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA, USA) according to manufacturer's instructions. Flow cytometric analysis was performed using a BD LSRII Fortessa flow cytometer (BD, Franklin Lakes, NJ, USA). Flow cytometry data were analysed with FlowJo software (FlowJo, Ashland, OR, USA). Antibodies used for flow cytometry are listed in Table 1.

2.7. RNA isolation and whole genome microarray

Total RNA was extracted using RNeasy Mini Kit (Cat.-No. 74104, Qiagen, Hilden, Germany) with on-column DNase digestion (Cat.-No. 79254, DNase-Free DNase Set, Qiagen) for avoiding DNA contaminations [47]. RNA quantification was performed by ultraviolet-visible spectrophotometry (Nanodrop Technologies, Wilmington, DE, USA), for assessment of the RNA integrity by the RNA 6000 Nano Chip Kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn,

Fig. 1. Increase of IL-10⁺ B cells following initial ait coincides with reduction of Th1 and h17 abundances during pre-seasonal up-dosing in year 1. (A) Study design scheme. Following a pre-seasonal weekly up-dosing phase, grass pollen-allergic patients were treated biweekly with three top dose injections of a standard grass pollen-specific immunotherapy. During the follow-up treatment phase, maintenance dose of 25 µg of grass group 5 allergens was applied 4–6 weekly with seasonal dosage adaption according to symptom burden throughout an observation period of three years. Sampling: T0 – Baseline, T1 – right before the first initial top dose, T2 – 6 h after the first initial top dose, T3 – right before the last initial top dose, T4 – 6 h after the last initial top dose, T5 – in grass pollen season year 1 of follow-up treatment phase, T6 – out of grass pollen season (= off season) year 1, T7 – in season year 2 of follow-up treatment phase, T8 – out of season year 2, T9 – in season year 3 of maintenance treatment and follow-up phase. At T10 (year 4) only local nasal samples were taken. Longitudinal disposition: peripheral and local samples from overall 32 patients were compared at distinct timepoints with cross-sectionally recruited controls. 20 patients attended at least the initial visits during the AIT up-dosing and top dose phases and were used to analyse early treatment effects until T4. 17 patients attended the initial visits and in addition visit T9 and were eligible for the analysis of long-term treatment effects. 11 patients attended at least 9 out of all 10 visits and were therefore subjected to analysis of immune mechanisms during time trajectories. 10 patients from the PACIFIC cohort, who had received at least 3 years of AIT, were analysed using local nasal samples at T10 in year 4. Analysis of systemic T and B cells using flow cytometry at time points T0 and T4 ($n = 20$ PBMC patient samples per group): (B) IL-4⁺ CD4⁺ Th2 cells; (C) IFN-γ⁺ CD4⁺ Th1 cells; (D) IL-17⁺ CD4⁺ Th17 cells; (E) FoxP3⁺ CD4⁺ Tregs; (F) IL-17⁺/FoxP3⁺-co-expressing CD4⁺ Tr17 cells; (G) total CD19⁺ B cell numbers; (H) CD27⁺ memory B cells; (I) IL-10⁺ B cells. Data are shown as median. (J) Identification of a subgroup of patients, which up-regulates Breg cell numbers following the initial treatment phase. (K) Changes in Th1 and Th17 frequencies of the selected subset from (J). (L) Correlation of Bregs with Th1 cell numbers of all data points (A in black and E in red). (M) Correlation of Bregs with Th17 cell numbers of all data points (A in black and E in red). Two-tailed Wilcoxon signed-rank tests were used to test for differences between data points. Statistically significant differences are depicted as * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.



Germany). All reagents (one-color Low Input QuickAmp Kit, Cat.No. 5190–2305; Gene Expression Hybridization Kit, Cat.No. 5188–5242; SurePrint G3 Human Gene Expression 8x60K Microarrays, Cat.No. G4851C) used for RNA preparation for whole genome microarray were supplied by Agilent Technologies, Waldbronn, Germany. Total RNA was adjusted to 17 ng in a final volume of 1.5 μ l. Subsequently, the one color spike-in mix was prepared and 2.0 μ l were added to 1.5 μ l of RNA on ice. In the next step, the T7-promoter primer mix was prepared and 1.8 μ l were added to the RNA spike-in mix and incubated in a thermocycler at 65 °C for 10 min. Immediately after run, the samples were put on ice for five minutes. In the meantime, the cDNA master mix (5xFirst Strand Buffer, 0.1 M dithiothreitol, 10 mM dNTP mix, AffinityScript RNase Block Mix) was prepared and kept on ice. Afterwards, 4.7 μ l cDNA master mix were added to the RNA T7 promoter mix and samples were placed in the thermocycler in a first step at 40 °C for two hours, followed immediately by a second step at 70 °C for 15 min. Hereupon, 6 μ l of Transcription master mix (Nuclease free water, 5xTranscription buffer, 0.1 M dithiothreitol, NTP mix, T7 RNA polymerase, Cy3-CTP) were prepared protected from light and added to the cDNA, before incubation in the thermocycler at 70 °C for 15 min. Ensuing, the resulting labeled and amplified cRNA was purified using the RNeasy mini spin columns (Cat.-No. 74104, Qiagen) by adding 84 μ l of nuclease-free water for a total volume of 100 μ l and 350 μ l of RLT buffer (Qiagen). Further, the RNeasy mini standard protocol was used and the purified cRNA was stored on ice. Afterwards, a quantification of cRNA was performed using NanoDrop ND-1000 ultraviolet–visible spectrophotometry (Nanodrop Technologies, Wilmington, DE, USA) to calculate the cRNA yield (in μ g) as well as the specific activity (pmol Cy3 per μ g cRNA). If the yield was below 0.825 μ g or/and specific activity below 6.0 pmol Cy3 per μ g cRNA, the cRNA was not used for hybridization and the procedure was repeated. Hybridization to SurePrint G3 Human Gene Expression 8x60K Microarrays (Agilent Technologies) was performed with the Gene Expression Hybridization Kit. Afterwards, the fragmentation mix was prepared and 6 μ l added to 600 ng cRNA in a volume of 19 μ l. This mixture was incubated in the thermocycler at 60 °C for 29 min and 30 s. Immediately after finished incubation, the mix was loaded onto the array slides, which was hybridized for 17 h at 65 °C. After hybridization, the slide was washed with the wash buffer I and II according to the Agilent's one-color platform, which has to be performed of necessity in a chamber where ozone levels are 50 ppb or less. Finally, the slide was covered with the ozone barrier cover slide. The slides were scanned immediately to minimize impact of environmental oxidants on signal intensities. Microarray experiments were performed by MIAME criteria. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [53] and are accessible through GEO Series accession number GSE118243 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE118243>).

2.8. Microarray data analysis strategy

Upon data import a standard baseline transformation to the median of all values was performed, including log transformation and computation of fold changes. Subsequently, a principle component analysis (PCA) was conducted and revealed a homogenous component distribution. Compromised array signals (array spot is non-uniform if pixel noise of feature exceeds threshold or above saturation threshold) were excluded from further analysis. Genes with an absolute log2 fold change larger than 1.5 and a *p*-value smaller than the testing level of 0.05 by using the Moderated *T*-Test were defined as significantly

differentially expressed hits. Based on previous statistical analysis strategies [54], plausibility testing of anticipated regulated genes was performed and is illustrated by e.g. *CCL-26*, which responds to seasonal pollen load. The significantly regulated genes were summarized in entity lists (supplementary Tables 2–5). Manhattan cityblock on entities (Ward's linkage) was used to cluster changes in gene expression.

2.9. Enzyme-linked Immunosorbent Assay (ELISA) and ImmunoCAP tests

ELISA was performed on conditioned supernatants from PBMC cultures using Human IgG4 or IgE ELISA Ready-SET-Go! Kits (eBioscience, San Diego, CA, USA) according to manufacturer's instructions. Absorption was visualized using an ELISA reader (Epoch™ spectrophotometer, BioTek Instruments, Inc., Winooski, VT, USA).

Grass pollen-specific IgE and IgG4 serum levels were analysed by the standardized diagnostic ImmunoCAP test (Phadia Thermo Scientific, Uppsala, Sweden), which determines antigen-specific IgE and IgG4 antibodies in the serum. The test principle is based on a sandwich immunoassay with high binding capacity of relevant immunoglobulins.

2.10. Data acquisition and statistical analysis

All experimental procedures and analyses of this exploratory study were conducted by blinded research staff. Data are included in parenthesis throughout the results section as mean \pm s.e.m. The cohort dataset has clear limitations given the open study design with patients, who did not comply with all scheduled sampling visits. Therefore a statistical analysis plan was predefined and addressed the problem of multiplicity in the following analysis hierarchy: data were not normally distributed and therefore non-parametric tests were chosen. For single comparisons, two-tailed Wilcoxon signed-rank tests were used to test for differences between data points from the same patient for Figs. 1, 2, 4A (*phleum*(T0) versus *phleum*(T9)), 4E-H, and supplementary Fig. 2. Two-tailed Mann-Whitney *U* tests were used to test for differences between different patient groups for Figs. 3, 4A-D and 6A-F. For Fig. 2, Fig. 4E-H, and supplementary Fig. 2, Friedman tests were performed initially, and, only when differences in means across time points were considered significant, multiple single comparisons were performed using two-tailed Wilcoxon signed-rank tests. For Figs. 3 and 6, Kruskal-Wallis tests were performed initially, and, only when medians across patient groups varied significantly, multiple single comparisons were performed using two-tailed Mann-Whitney *U* tests. Spearman correlation was used to correlate RAAS at the last time point (T9) with immune cell frequencies at initial time points (T0, T4) in Fig. 7. Statistically significant differences are depicted as **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

3. Results

3.1. Increase of IL-10⁺ B cells following initial AIT coincides with reduction of Th1 and Th17 cells

In this study, grass pollen-allergic patients were prospectively monitored in a longitudinal AIT cohort in order to characterize the immediate and long-term immune response in the periphery and in the upper airways. Following an initial treatment phase using a standard AIT up-dosing scheme and three subsequent top dose injections, patients obtained maintenance shots every four to six weeks over a period of three years (Fig. 1A). A complex sampling procedure during treatment

Fig. 2. Longitudinal changes in immune subsets during course of treatment: initial ait induces il-10-producing B and T cells. Subsets were analysed by intracellular flow cytometry including all time points (*n* = 11 patients; except time point T8 *n* = 8) for (A) IL-4+ CD4+ Th2 cells, (B) IL-17+/FoxP3 + –co-expressing CCR6 + CD4+ Tr17 cells, (C) B cells producing IL-10, (D) IFN- γ + CD4+ Th1 cells, (E) IL-17+ CD4+ Th17 cells, (F) FoxP3+ regulatory CD4+ T cells, (G) IL-10-producing FoxP3+ Treg cells, (H) CD27+ memory B cells, and (I) IL-10-producing CD27+ memory B cells. Results are shown as mean \pm s.e.m. Friedman tests were performed initially, and, only when considered significant, single comparisons were performed using two-tailed Wilcoxon signed-rank tests. *P* values are presented for comparisons to baseline, if not otherwise indicated. Statistically significant differences are depicted as **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < .0001. A schematic overlay of the populations is shown in (J) IL-10+ B cells (black) versus Th17 cells (red) and (K) of Tregs (black) versus Th2 cells (red).

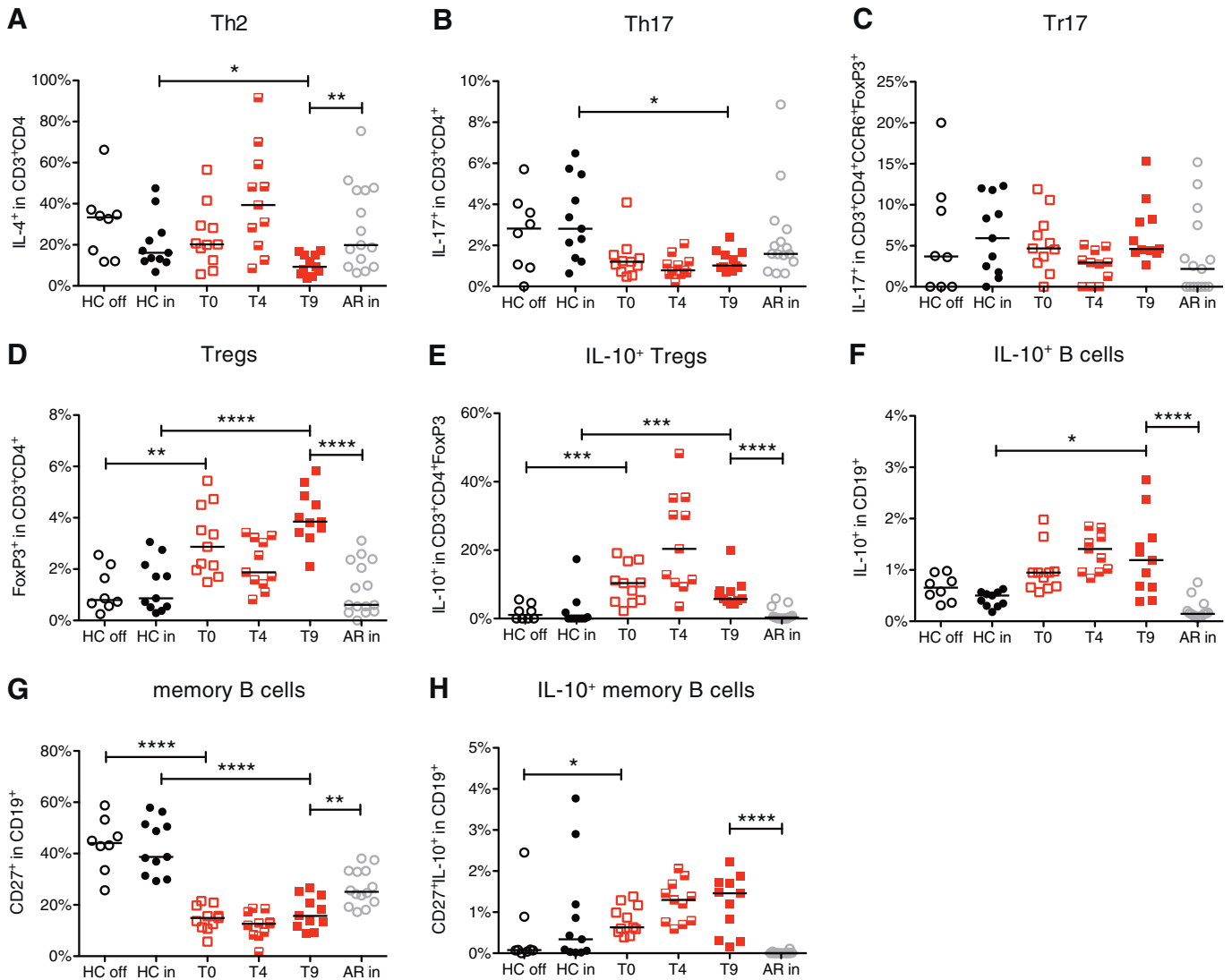


Fig. 3. Differential long-term systemic therapy effects on th17 and tr17 subsets. Intracellular flow cytometry analysis of circulating T and B cell subsets comparing healthy control subjects during off season (HC off; $n = 8$), in grass pollen season (HC in; $n = 11$), treated patients throughout course of therapy at time points T0, T4, and T9 ($n = 11$), untreated allergic rhinitis patients in grass pollen season (AR in; $n = 15$ for T cell analysis; $n = 14$ for B cell analysis): (A) IL-4⁺ CD4⁺ Th2 cells, (B) IL-17⁺ CD4⁺ Th17 cells, (C) IL-17⁺/FoxP3⁺ – co-expressing CCR6⁺ CD4⁺ Tr17 cells, (D) FoxP3⁺ Treg cells, (E) IL-10-producing FoxP3⁺ Treg cells, (F) IL-10⁺ B cells, (G) CD27⁺ memory B cells, and (H) IL-10-producing CD27⁺ memory B cells. Data points are depicted with median. Kruskal-Wallis tests were performed initially, and only when considered significant, single comparisons were performed using two-tailed Mann-Whitney *U* tests. Statistically significant differences between HC's and AR to treatment are depicted as * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$. Differences of treatment (in relation to baseline) are shown in Fig. 2.

course was chosen to assess allergen-induced immediate local and systemic immune reactions, including time points right before and six hours after pre-seasonal top dose injections. In addition, samples were analysed in and out of grass pollen season throughout the therapy course to identify the natural allergen-stimulating effect of grass pollination. The characteristics of the analysed cohort and controls are listed in supplementary Table 1. The analyses were performed in a subgroup of 32 patients out of the cohort, who completed at least five initial visits.

During the early treatment phase an initial induction of a Th2 phenotype (T0:45.02% \pm 5.06 vs. T4:59.87% \pm 5.08; $p = 0.049$; Fig. 1B) was observed, while Th1 (T0:2.62% \pm 0.51 vs. T4:1.64% \pm 0.34; $p = 0.055$; Fig. 1C) and Th17 (T0:0.85% \pm 0.10 vs. T4:0.55% \pm 0.05; $p = 0.001$; Fig. 1D) cells were significantly reduced systemically. Whereas the FoxP3⁺ Treg compartment remained unchanged at this early time point, we found a significant increase of IL-10-producing B cells (T0:1.90% \pm 0.31 vs. T4:3.20% \pm 0.75; $p = 0.036$; Fig. 1E,G,I) while, simultaneously, an opposing regulation in the memory B cell subset was observed (T0:20.30% \pm 1.86 vs. T4:16.36% \pm 1.64; $p = 0.036$; Fig. 1H). We further discovered a significant decrease of circulating IL-

17-expressing CD4⁺FoxP3⁺ T cells following initial AIT (Tr17 cells; T0:1.50% \pm 0.22 vs. T4:0.95% \pm 0.13; $p = 0.029$; Fig. 1F). IL-10 induction in B cells correlated with a simultaneous decrease of peripheral Th1 ($r = -0.56$; $p = 0.0002$) and Th17 cells ($r = -0.35$; $p = 0.03$; Fig. 1L, M). In particular, a patient subgroup characterized by an increase of IL-10⁺ B cells displayed a significant decrease of Th1 (T0:2.78% \pm 0.52 vs. T4:0.79% \pm 0.17) and Th17 cells (T0:0.86% \pm 0.12 vs. T4:0.43% \pm 0.06; Fig. 1J,K).

3.2. Initial immunotherapy induces IL-10-producing B and T cells

Following the initial boost, the Th2 response continuously decreased throughout the three years reaching statistical significance only during the last year of AIT (T0:23.67% \pm 4.50 vs. T9:10.43% \pm 1.51; $p = 0.001$; Fig. 2A,K). Surprisingly, we found a significant decrease of Th17 cells (T0:1.37% \pm 0.30 vs. T4:0.97% \pm 0.16; $p = 0.04$; Fig. 2E) and CCR6⁺ Tr17 cells (T0:5.36% \pm 1.08 vs. T4:2.52% \pm 0.59; $p = 0.027$; Fig. 2B) in the periphery following initial AIT, thus depicting inverse dynamics in comparison to Th2 cells (T0:23.67% \pm 4.50 vs.

Table 1
Fluorochrome labelled antibodies used in flow cytometry analyses.

Antigen	Fluorochrome	Company	Clone	Dilution
CD1d	PercP-Cy5.5	BioLegend	51.1	1:200
CD3	PercP-Cy5.5	BD	UCHT1	1:200
CD3	APC-Cy7	BioLegend	HIT3a	1:200
CD4	V450	BD	RPA-T4	1:200
CD4	PE0Dazzle&594	BioLegend	A161A1	1:200
CD5	FITC	BioLegend	UCHT2	1:200
CD19	Brilliant&Violet&605	BD	SJ25-C1	1:200
CD19	APC-Cy7	BioLegend	HIB19	1:200
CD24	PE-CF594	BD	ML5	1:200
CD27	Brilliant&Violet&711	BioLegend	O323	1:200
CD27	Brilliant&Violet&605	BioLegend	O323	1:200
CD38	Brilliant&Violet&605	BioLegend	HB-7	1:200
CD45RA	eFluor780	ebioscience	HI100	1:200
PD-L1&(CD274)	Brilliant&Violet&650	BD	MIH1	1:200
IFN- γ	APC	BioLegend	4S-B3	1:100
IL-4	Alexa&Fluor&488	BD	8D4-8	1:100
IL-10	PE	ebioscience	JES3-9D7	1:100
IL-13	APC	BD	JES10-5A2	1:100
IL-17A	Brilliant&Violet&711	BioLegend	BioLegend168	1:100
TNF- α	V450	BD	MAB11	1:100
FoxP3	APC	ebioscience	PCH101	1:50
FoxP3	PE	ebioscience	PCH101	1:50

T4:41.50% \pm 7.64; $p = 0.032$; Fig. 2A,K). The frequencies were restored in the first subsequent grass pollen season. The Tr17 phenotype was stabilized over time in comparison to initial treatment (T4:2.52% \pm 0.59 vs. T9:6.58% \pm 1.13; $p = 0.005$; Fig. 2B). Interestingly, Th17 cells showed an analogue pattern to Tr17 cells during up-dosing with a tendency to seasonal decrease (Fig. 2E). Further, a significant increase of tolerogenic FoxP3⁺ Tregs was observed, reaching significance after three years (T0:3.09% \pm 0.40 vs. T9:4.06% \pm 0.32; $p = 0.004$; Fig. 2F,K). During the up-dosing phase, IL-10⁺ B cells increased progressively (single comparison T0:0.99% \pm 0.13 vs. T4:2.16% \pm 0.84; $p = 0.032$; Fig. 2C,J), but vanished in the first pollen season. The induced B cell subset was characterized by the surface markers CD1d and CD5, while the proportion of CD24⁺CD27⁺ IL-10⁺ B cells decreased throughout initial AIT (see Supplementary Fig. 2). Similar effects were observed in IL-10-producing Tregs (T0:10.06% \pm 1.71 vs. T4:22.46% \pm 4.27; $p = 0.02$; Fig. 2G). In addition, we observed a slack of CD27⁺ memory B cells following the up-dosing phase (T0:15.02% \pm 1.45 vs. T2:12.52% \pm 1.38; $p = 0.032$; Fig. 2H) with subsequent build-up. A statistically significant difference to initial treatment effects was only observed after three years (T4:12.38% \pm 1.58 vs. T9:17.33% \pm 1.92; $p = 0.007$). Finally, we found an increasing trend of IL-10⁺ memory B cells initially during the pre-season (T0:0.80% \pm 0.11 vs. T4:1.26% \pm 0.15; Fig. 2I) with maintenance of higher levels throughout the three years of AIT.

Generally, following injection of the allergen (T2 versus T1 and T4 versus T3) we observed an immediate downward trend for Th1, Th17/Tr17 and Tregs, while IL-10⁺ B cells and Th2 cells rather increased. Seasonal changes had heterologous effects on most populations except of Th17 cells, which showed a zigzag trend with decreased frequencies during grass pollen flight (Fig. 2A–I).

3.3. Differential long-term systemic therapy effect on Th17 and Tr17 subsets

Comparing long-term treated (T9) and untreated (AR in) allergic patients in season, as expected, circulating Th2 levels (untreated: 28.77% \pm 5.35 vs. treated:10.43% \pm 1.51; $p = 0.0043$; Fig. 3A) were significantly decreased, whereas Th17 displayed only a slight downward trend between groups (untreated:2.38% \pm 0.56 vs. treated:1.27% \pm 0.16; $p = 0.12$; Fig. 3B). However, AIT induced a significant increase of CCR6⁺ Tr17 cells (untreated:3.75% \pm 1.31 vs. treated:6.58% \pm 1.13; $p = 0.019$; Fig. 3C) and Tregs (untreated:1.23% \pm 0.26 vs. treated:4.06% \pm 0.32; $p < 0.0001$; Fig. 3D) were detectable compared

to untreated controls. Additionally, a strong systemic increase of IL-10 production in Tregs (untreated:1.13% \pm 0.50 vs. treated:7.51% \pm 1.34; $p < 0.0001$; Fig. 3E) as well as in B cells (untreated:0.22% \pm 0.05 vs. treated:6.09% \pm 0.71; $p < 0.0001$; Fig. 3F) was monitored comparing treated to untreated patients. Reduced memory B cells were observed during AIT, but neither in untreated patients nor in healthy individuals (untreated:26.84% \pm 1.88 vs. treated:17.07% \pm 1.92; $p = 0.0034$; Fig. 3G), while IL-10⁺ memory B cells were significantly increased throughout AIT if compared to untreated patients (untreated:0.01% \pm 0.01 vs. treated:1.21% \pm 0.21; $p < 0.0001$; Fig. 3H). This pattern of exclusively treatment-dependent alterations of cell populations was also observed for Th17 and memory B cells, which were below levels of controls or even untreated allergic rhinitis patients. Interesting to note is that we observed also unexpected differences off-season between healthy controls and AR patients at baseline (T0): Both Treg subsets and Breg subsets were lower in healthy individuals than in AR patients prior treatment, which might be caused by a compensation mechanism antagonizing seasonal inflammation.

3.4. Allergen-specific lymphocyte activation in favor of immune regulation

Using in vitro stimulation assays of PBMCs for seven days with major grass pollen allergen *phleum p1*, we found a significant induction of IL-17-producing CD4⁺ T cells at baseline (medium (T0):3.56% \pm 0.41, *phleum*(T0):13.22% \pm 2.28; $p < 0.0001$; Fig. 4A). The antigen-specific induction of Th17 cells decreased strongly throughout course of therapy (*phleum*(T0):13.22% \pm 2.28, *phleum*(T9):6.73% \pm 0.66; $p = 0.015$). Tregs were also allergen-specifically induced, but only in cultures prior treatment (medium (T0):0.84% \pm 0.30, *phleum*(T0):2.29% \pm 0.55; $p = 0.046$; Fig. 4B). Similarly, we observed an even stronger effect in the Tr17 compartment, which was also antigen-specifically induced at baseline (medium(T0):6.74% \pm 1.90, *phleum*(T0):36.65% \pm 7.24; $p < 0.0001$; Fig. 4C). However, comparably to Th17 cells, the allergen-driven boost of Tr17 cells faded throughout the course of immunotherapy (*phleum*(T0):36.65% \pm 7.24, *phleum*(T9):24.94% \pm 6.16).

Further, we observed, that B cells from grass pollen-allergic patients up-regulate the tolerance-inducing surface marker PD-L1 (CD274) significantly after seven days of in vitro allergen stimulation and that this induction is maintained throughout the treatment course (medium (T0):1.51% \pm 0.22, *phleum*(T0):10.37% \pm 0.60; $p < 0.0001$; Fig. 4D). In addition, here, we report elevated levels of IgG4 following the initial up-dosing period in the supernatant of in vitro allergen challenge cultures (*phleum*(T0):19.09% \pm 1.26, *phleum*(T4):23.89% \pm 2.88, *phleum*(T9):17.85% \pm 1.75; Fig. 4E), which coincided with a minimal decrease of IgE levels from supernatants (*phleum*(T0):1.48% \pm 0.24, *phleum*(T4):1.38% \pm 0.19, *phleum*(T9):1.53% \pm 0.18; Fig. 4F). Both effects were abolished after three years of ongoing therapy. As our stimulation protocol did not include secondary stimulants in order to keep the protocol as physiologic as possible, IgG4 and IgE data might reflect production from B memory cells or plasma cells in culture. However, serum levels of grass-pollen specific IgG4 (sIgG4) were analysed throughout treatment course and support the data described above, as serum sIgG4 significantly increased during the first year of therapy (Fig. 4G). On the other hand, grass pollen-specific IgE (sIgE) serum levels increased significantly during the initial up-dosing phase (Fig. 4H) and were subsequently reduced back to baseline levels throughout the follow-up period and further fell below baseline levels after three years of AIT.

3.5. Local gene expression changes in the nasal mucosa

Whole transcriptome analysis of nasal scrapings identified several essential gene expression changes in the up-dosing phase and after long-term treatment (\log_2 FC > 1.5, $p < .05$; Fig. 5). To clarify, only mediators crucial to local airway immune processes are shown in Fig. 5B and

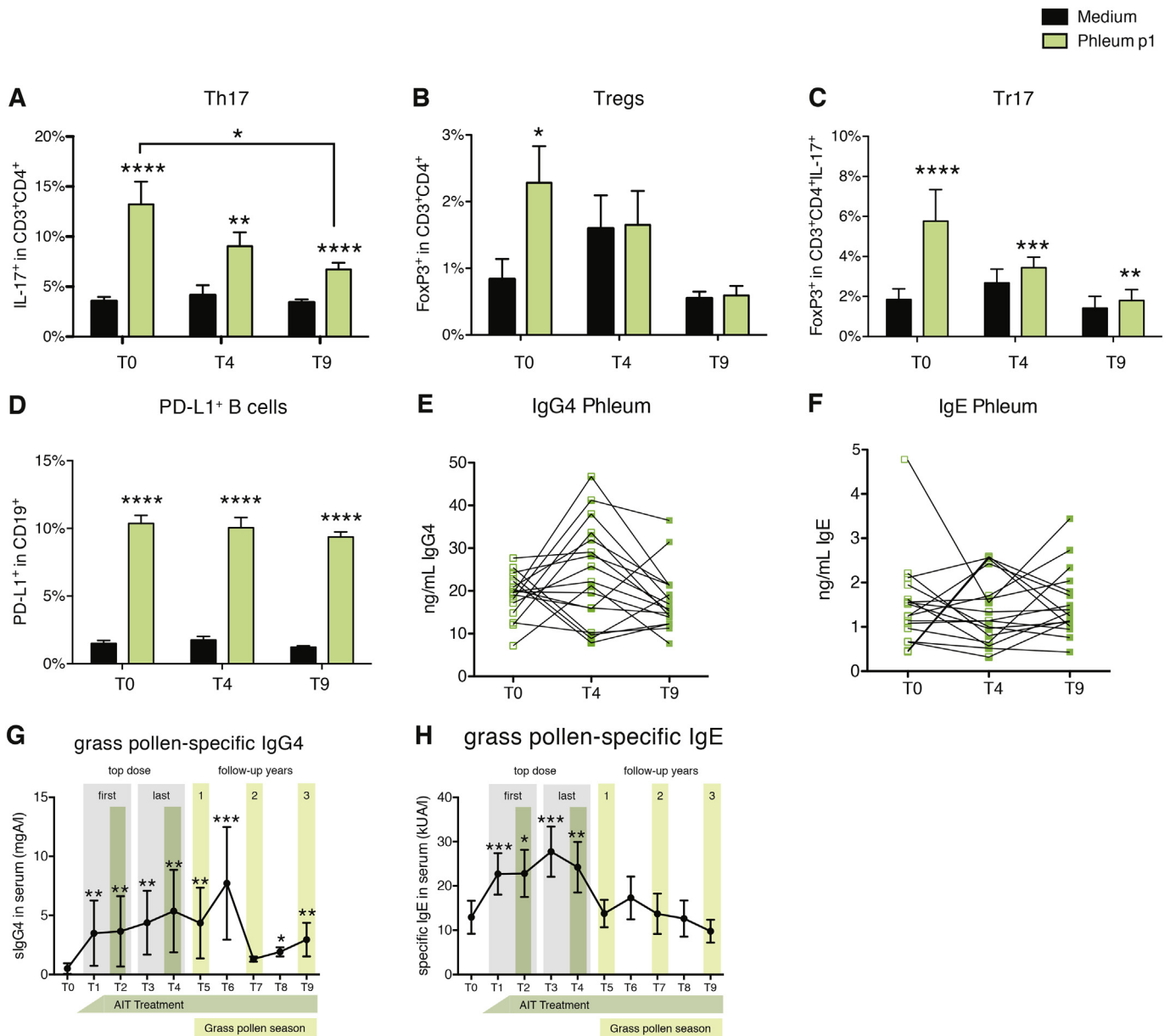


Fig. 4. In-vitro: allergen-specific lymphocyte activation in favor of immune regulation. PBMCs throughout therapy course time points T0, T4, and T9 ($n = 17$) were stimulated with medium (black) or 5 $\mu\text{g}/\text{ml}$ phleum p1 (red) for 7 days in culture and T and B cells were analysed using intracellular antigen staining: (A) IL-17 + CD4 + Th17 cells, (B) FoxP3 + Tregs, (C) IL-17 + FoxP3 + co-expressing CD4 + Tr17 cells, and (D) PD-L1 + B cells. Results are depicted as mean \pm s.e.m. Test results are shown for comparisons to medium/untreated, if not otherwise indicated. Two-tailed Mann-Whitney U tests were used to test for differences between patient groups. In (A), a two-tailed Wilcoxon signed-rank test was used to test for differences between *phleum*(T0) and *phleum*(T9). Immunoglobulin secretion was determined by supernatant analysis using total IgG4 (E) and IgE (F) ELISA following medium (data not shown) or phleum p1 stimulation for 7 days at the same time points T0, T4, and T9 ($n = 17$). Longitudinal changes of (G) grass pollen-specific serum IgG4 (sIgG4) and (H) grass pollen-specific serum IgE (sIgE) levels were analysed throughout treatment course by ImmunoCAP test ($n = 11$ patients; except time point 1 $n = 8$). Results are depicted as mean \pm s.e.m. Friedman tests were performed initially, and, only when considered significant, single comparisons were performed using two-tailed Wilcoxon signed-rank tests. Statistically significant differences are depicted as * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

D, a full list of genes is enclosed in the supplement. Hierarchical clustering was used to compare the short-term AIT effects (T4 versus T0), which involved changes of immune- and epithelial-cell origin in the nasal mucosa (Fig. 5A,B). The maximum expression of these genes was detected following initial up-dosing treatment (T4) with a strong decrease following long-term AIT (T9), where *IL10* and *CD274* fell below levels of untreated allergic patients. *CCL26*, a well-known IL-4-inducible epithelial marker, follows the systemic Th2 response over time, however on epithelial level in the upper airways. Notably, *RORC* expression resembles the contrary effect and therefore aligns with the systemic course of Th17 cells throughout AIT.

Long-term treatment effects were characterized by decreased local expression levels of multiple cytokines of the Th2 response, *IL4*, *IL5*, *IL13* and epithelial type 2 (E2) response, *IL24* (Fig. 5C,D). *IL17B* and *IL17C*, the latter a pro-inflammatory IL-17 isoform produced by the epithelial cells, and further, the up-regulation of *IL10RB*, were significantly reduced upon AIT.

3.6. Local shifts of immune cell compartments following AIT

In the local compartment, we observed changes in effector and regulatory immune cell subsets in the upper airways of allergic patients

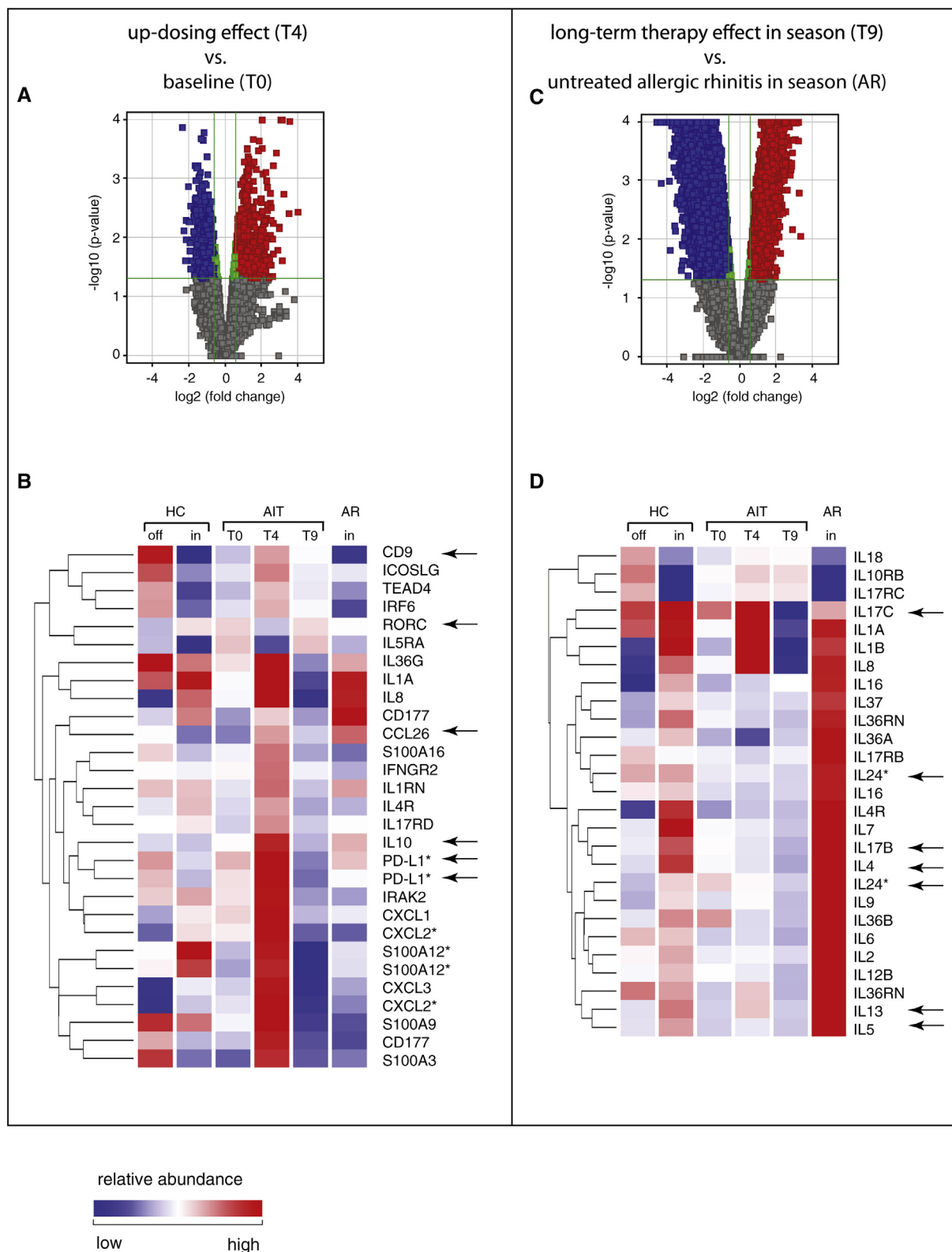


Fig. 5. Local gene expression changes indicate shifts in regulatory hierarchies in the nasal mucosa. Gene expression changes of nasal scrapings were taken from healthy control subjects during off season (HC off; $n = 3$), in grass pollen season (HC in; $n = 3$), treated patients throughout course of therapy at time points T0 ($n = 6$), T4 ($n = 5$), and T9 ($n = 9$), untreated allergic rhinitis patients in grass pollen season (AR in; $n = 5$) and subjected to RNA whole transcriptome microarray analysis. (A) Volcano plot of statistically significant entities ($p < 0.05$; $FC \geq 1.5$) comparing time point T4 and T0. (B) Comparison of T4 versus T0 depicts an extract of significant changes following initial AIT ($p < 0.05$; $FC \geq 1.5$). Selection of entities is shown, which are relevant for allergy. (C) Volcano plot of statistically significant entities ($p < 0.05$; $FC \geq 1.5$) comparing time point T9 and AR in. (D) Comparison of T9 versus AR in mirrors therapeutic effects on significant gene expression changes of interleukin family in nasal transcriptome. The color code indicates the abundance of transcripts ranging from low (blue) to high (red). *Asterisks indicate two isoforms that were present in the analysis.

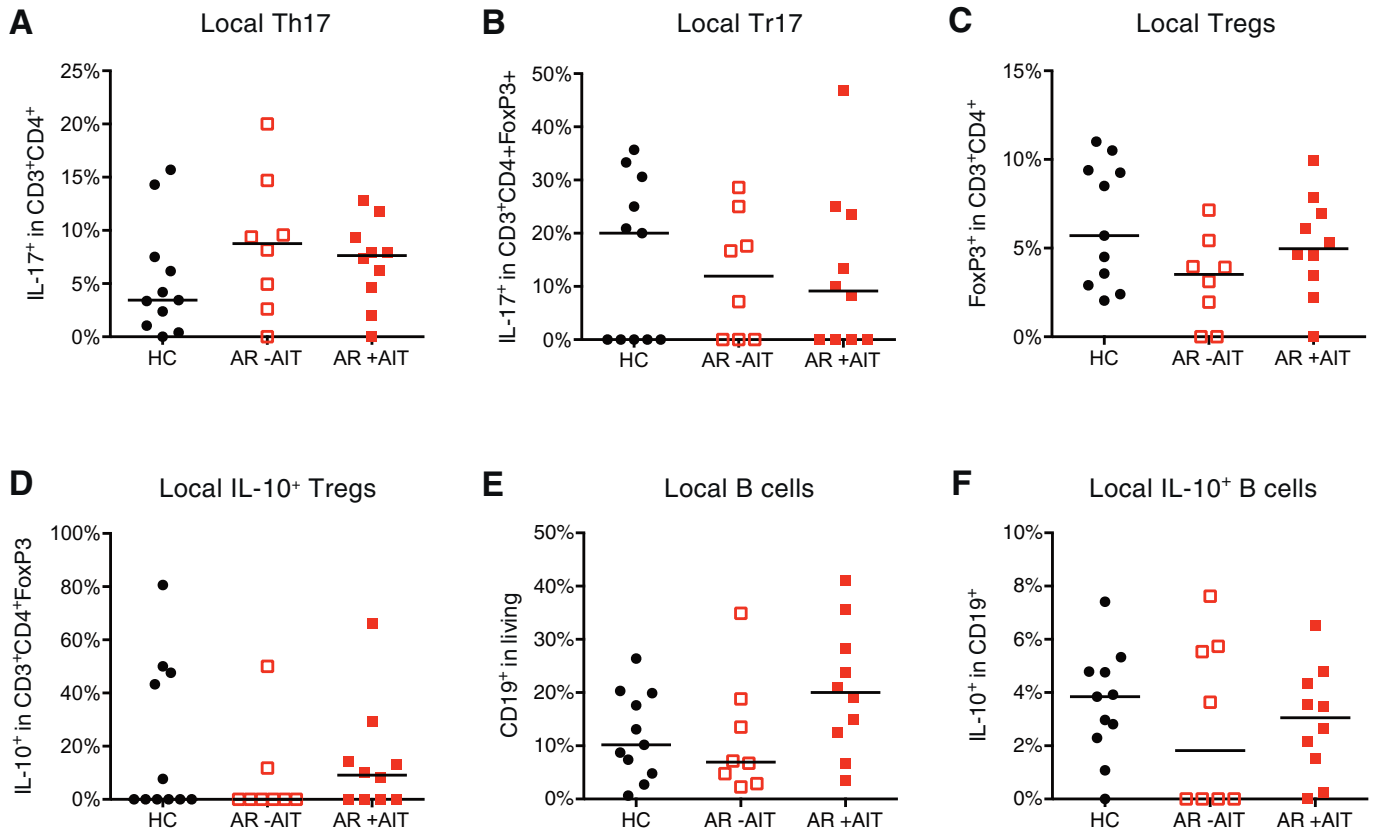


Fig. 6. Local shifts of immune cell compartments following AIT. Intracellular FACS staining of scraped nasal lymphocytes from healthy controls (HC; $n = 11$), allergic rhinitis patients without AIT (AR - AIT; $n = 8$), and allergic rhinitis patients with long-term AIT (AR + AIT; $n = 10$) in grass pollen season (A–F). Gating strategy for nasal samples; (A) IL-17+ CD4+ Th17 cells, (B) IL-17+/FoxP3+ –co-expressing CD4+ Tr17 cells, (C) FoxP3+ Tregs, (D) IL-10-producing FoxP3+ Tregs, (E) total B cells, and (F) IL-10+ B cells. Data are shown with median. Kruskal-Wallis tests were performed initially, and, only when considered significant, single comparisons were performed using two-tailed Mann-Whitney U tests.

using intracellular stainings in flow cytometry on nasal scrapings for the first time. Decreased Th17 and increased Tr17 populations became visible comparing treated with untreated patients during pollen flight (Th17: untreated: $8.67\% \pm 2.28$, treated: $7.43\% \pm 1.22$; Tr17: untreated: $11.88\% \pm 4.13$, treated: $12.61\% \pm 4.37$; Fig. 6A,B). As Kruskal-Wallis tests rendered non significant, we did not perform single comparison statistics. FoxP3+ Tregs tend to increase upon AIT (untreated: $3.19\% \pm 0.88$, treated: $5.05\% \pm 0.82$; Fig. 6C), and also displayed a higher frequency of IL-10 production (untreated: $1.69\% \pm 1.69$, treated: $15.54\% \pm 6.04$; Fig. 6D). Further, AIT-treated patients

had by tendency higher numbers of local B cells (untreated: $11.38\% \pm 3.90$, treated: $20.53\% \pm 3.45$; Fig. 6E) as well as of IL-10+ B cells compared to untreated patients (untreated: $2.82\% \pm 1.13$, treated: $3.10\% \pm 0.61$; Fig. 6F).

3.7. Early B- and T-cell responses correlate with retrospective symptom assessment

Total frequencies of IL-10+ B cells and the differential frequencies of the Delta (IL-10+ B cells (T0) - IL-10+ B cells (T9)) were positively

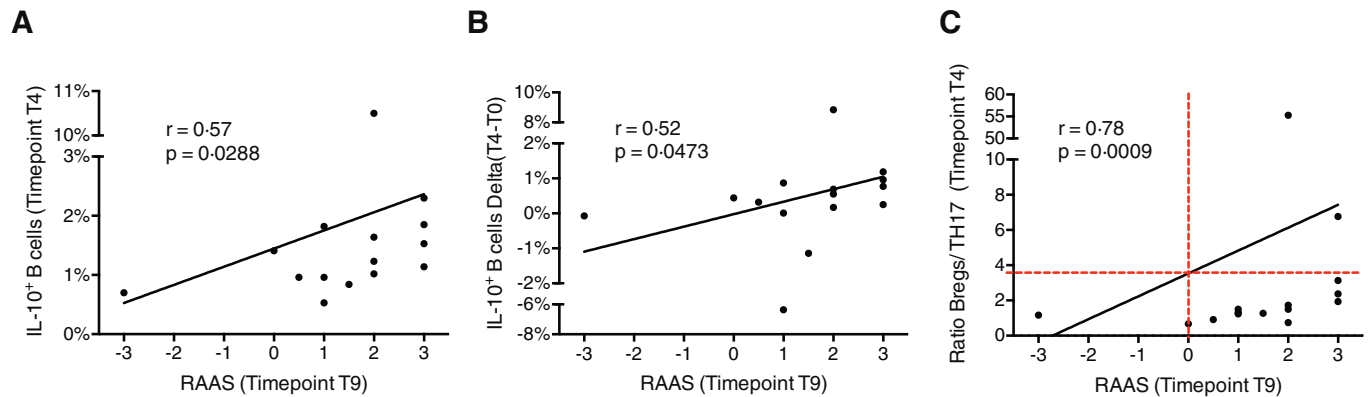


Fig. 7. Correlation of symptom score raas after 3 years of ait with early B and T cell frequencies. Spearman correlation analysis was performed comparing the RAAS at time point T9 with (A) total peripheral IL-10+ B cell numbers at time point T4 ($n = 15$) or (B) with the in-/reduction of peripheral IL-10+ B cells of time point T4 minus T0 ($n = 15$). (C) Spearman correlation of RAAS at time point T9 with the ratio of peripheral IL-10+ B cell percentage to peripheral Th17 percentage (Ratio Bregs/Th17) at time point T4 ($n = 15$). One patient was excluded from all correlations Due to clinically significant polysensitization after three years of treatment. Statistically significant differences are depicted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

associated with the Retrospective Assessment of seasonal Allergic Symptoms Score (RAAS), reflecting allergy symptoms at T9 in year 3 in comparison to baseline on a scale between +3 (much better), to 0 (no change) to −3 (much worse) ($r = 0.57$, $p = 0.0288$; $r = 0.52$, $p = 0.0473$; Fig. 7A,B). In order to quantify the suppressive potential of IL-10⁺ B cells towards the effector Th17 cell compartment, we computed the ratio of cell frequencies of IL-10⁺ B cells to IL-17⁺CD4⁺ T cells (Breg/Th17 ratio). Strikingly, the Breg/Th17 ratio at time point T4 following the initial up-dosing phase correlated strongly with RAAS after three years of AIT ($r = 0.78$; $p = 0.0009$; Fig. 7C), indicating a positive coherence of early induction of circulating IL-10⁺ B cells coinciding with a downregulation of circulation Th17 effector cells with a successful clinical outcome.

4. Discussion

Early prediction of therapy success would represent a breakthrough for AIT. In this exploratory study, we provide evidence for the first time that a shift in lymphocyte subsets is related to and possibly indicative for therapy success. Empiric evidence indicates that therapy success requires long-term treatment over several years [6]. AIT increased the Th2 cell population and specific IgE during initial treatment steps, which is an expected but unwanted side effect of the allergen exposure during up-dosing. We hypothesized that the initial phase as observed in the six hour time window after subcutaneous administration of allergen is characterized by a desensitization of the innate immune system (e.g. mast cells), while the specific immune system remains allergen-responsive [33,34]. In frame of this concept, immunological changes during AIT can be segregated in an early phase, characterized by IgG4 increase [35,36], which converts into a late tolerance mounting phase with suppression of Th2 responses. Functional IgG4 antibodies measured by the FAB assay have been shown to correlate with clinical responses after eight months of grass pollen AIT [37]. Allergen-binding IgG4 levels during AIT have been observed to plateau or slightly dampen [38,39], while we observed a decrease in year 2 in our cohort, that may be explained with prolonged treatment intervals between maintenance injections. We did not assess FAB or avidity of IgG4. However the IgG4 results fit the course of IgE levels and the significantly higher IgG4 levels at T9 compared to baseline undermine this known protective mechanism triggered by AIT.

Until now, it was not possible to connect the early and late phases of AIT, which, however, is essential to predict therapy success at an earliest possible timepoint. This study indicates that the early response to AIT involves IL-10 induction in B cells [40] as well as in trafficking T cells [10,41] both systemically and in the upper airways. Surprisingly, there are reports that hint to an effect of AIT prior to IgG4 changes [38]. The synergy of frequencies of IL-10⁺ Tregs with frequencies of Th2 effector cells, especially during the up-dosing phase, hints to a possible AIT mechanism involving IL-10 apart from IgG4 upregulation.

In addition, the regulatory B cell-derived IL-10 has been described to promote IgG4 expression by B cells of allergic patients by competing with IgE binding sites on allergens, at the same time preventing Fc receptor activation [23,35]. However, Breg-derived IL-10 does not only regulate IgG4, but was further described to interfere with MHC-dependent antigen presentation via downregulation of co-stimulation and thereby suppresses T cell proliferation in an antigen-specific manner [23,40]. In this context, it is important to note that this study exhibits for the first time restriction of allergen-specific Th17 and Tr17 responses during AIT using in vitro allergen stimulation experiments. This restriction aligns with the picture we see six hours after AIT injection during the pre-seasonal up-dosing period (T4), at which time point we expected to catch the immediate allergen-triggered systemic immune response by analysing peripheral blood lymphocytes. Total frequencies of unstimulated Th17 cells as well as Tr17 cells decreased significantly during this initial treatment phase and these data therefore support our assumption to truly catch the immediate systemic response at T4. The recovery of these cells during the conversion and the tolerance

mounting phase does not reflect direct responses to AIT injections, however, the in vitro stimulation data support that Th17 and Tr17 cells render unresponsive to grass pollen allergen throughout the maintenance phase. This novel finding supports the involvement of Th17 and Tr17 cells as pathogenic players in allergic rhinitis and hint to an important treatment effect of AIT.

In addition, it is interesting to note that the current study describes that the suppressive co-stimulator PD-L1 [42] was allergen-specifically up-regulated on circulating B cells and was also detected in the local gene expression analysis. Therefore, it could be speculated that the induction of PD-L1 could be one of the initial mechanisms of suppression, which is induced by the repetitive allergen administration.

Regulatory B cells mediate suppression of Th1 and Th17 differentiation by IL-10 secretion as shown in autoimmune disorders [22,27], which we presume to underlie the inverse dynamics of circulating Th17 and Breg cells observed in the current study. Notably, the recovery of the Th17 phenotype demarcates the conversion phase, as the Th17 subset surprisingly increases following reduction in the initial up-dosing phase. In addition, our data imply for the first time the induction of an intermediary IL-17⁺FoxP3⁺CCR6⁺ Tr17 subset with previously described transitory character [20]. On one hand, a pro-inflammatory differentiation arising from regulatory T cells inducing IL-17 was described in a murine arthritis model [20], while an anti-inflammatory transdifferentiation of Th17 cells into regulatory T cells was reported in experimental encephalitis and infection models [21]. We anticipate that this population demarks a fragile phase of conversion into allergen tolerance.

The current data propose that this conversion finally culminates in a significant increase of FoxP3⁺ regulatory T cells and strong reduction of Th2 cells after long-term treatment, which marks the final tolerance mounting phase. This therapy-induced shift of the Treg/Th2 balance is a well-established phenomenon following 24–36 months of AIT, which is assumed to lead to the suppression of seasonal re-induction of Th2 cells by Tregs [43–45]. However, this study implies that circulating IL-10⁺ Tregs are mainly induced during the initial up-dosing phase, while higher frequencies of both, IL-10-producing Tregs and total FoxP3⁺ Tregs were found in the upper airways following long-term AIT, which confirms previous reports [8,13,46]. On top of the early increase, the clear trends of higher local frequencies of IL-10⁺ Tregs and IL-10⁺ B cells we observed in the nasal mucosa after long-term therapy compared to untreated allergic patients and healthy controls match the data from the peripheral blood.

Further, the systemic Treg/Th2 shift during the tolerance mounting phase shows multiple suppressive effects on different cytokines, not only on Th2 cytokines like IL-4, IL-5, and IL-13, but also on pro-inflammatory cytokines like IL-1A, IL-1B, IL-6, IL-8, and IL-12B. Interestingly, the effect was not only observed regarding lymphocyte- but also epithelium-derived cytokines like IL-24 and IL-17C. These two cytokines are believed to play a role in the Th2-associated [47] as well as in the Th17-associated local epithelial response [48], respectively.

In conclusion, here, we hypothesize three sequential phases of AIT and, for the first time, link the late tolerance mounting phase to the initial up-dosing phase, as the ratio of circulating IL-10⁺ B cells to Th17 cells at the time point following the last top dose injection during the AIT up-dosing period correlates strongly with self-assessed allergic symptom score RAAS at the time point after three years of therapy. Future studies are needed to substantiate the Breg/Th17 ratio as clinical prediction marker of AIT success, also in other common allergies and dosage schemes. This study provides insight into novel AIT mechanisms already early during treatment, which may help to improve future antigen-specific interventions.

Author contributions

A.M.C. conceived of the project; C.S.W. and A.M.C. directed the research; U.M.Z., C.A.J., C.S.W., and A.M.C. designed the experiments and

evaluated the data; A.M.C. initiated the trial, A.M.C. and Z.H. collected samples from longitudinal cohort subjects; U.M.Z., C.A.J., F.G., L.P., K.S. and K.D. performed the experiments; U.M.Z. and C.A.J. prepared the figures; U.M.Z., F.G. and C.S.W. performed gene expression profiling studies; U.M.Z., C.A.J., C.S.W., and A.M.C. wrote the manuscript; J.A.A-P., M.S., B.H. and G.P. helped with the manuscript. A.M.C. confirms that he had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Sponsor

This was an investigator initiated observational cohort study, with the faculty of medicine of the TUM Medical School as acting sponsor.

Funding source

This study was partially funded by Allergopharma GmbH & Co. KG, intramural funding and the German Center for Lung Research (DZL). The funding sources had no influence with regards to sample analysis, interpretation of data or submitting this research for publication, but approved the manuscript prior to publication.

Declaration of interests

U.M.Z. received payment for manuscripts from Deutsches Aertzblatt, funds for travel from the European Academy of Allergy and Clinical Immunology (EAACI) and Collegium Internationale Allergologicum (CIA). C.S.W. received support for research projects from PLS Design, LETI, Zeller AG and Allergopharma and accepted honoraria for consultancy and seminars from LETI, Allergopharma and Leo Pharma. A.M.C. has consultant arrangements via Technical University Munich with Allergopharma, ALK-Abello, HAL Allergy, Mundipharma, and Lofarma; has conducted clinical studies and received research grants via Technical University Munich from Allergopharma, Novartis, the German Federal Environmental Agency, Bencard/Allergen Therapeutics, ASIT Biotech, GlaxoSmithKline, Roche and Zeller AG; has received payment via Technical University Munich for lectures from Allergopharma, ALK-Abello, and GlaxoSmithKline; has received payment for manuscript preparation from Bayerisches Aertzblatt; and has received travel support from the European Academy of Allergy and Clinical Immunology, DGAkl, and SMI. The other authors declare no conflict of interest.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2018.09.016>.

Acknowledgments

We thank Mrs. Christiane Hofmeier and Mrs. Jana Hartmann specialty and study nurses in allergy, Mr. Peter Strzelczyk, Mrs. Christine Weil and Mr. Michael Siegel for their excellent technical assistance. We also thank the study patients who supported us during this study with high commitment and personal dedication.

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