Nasal secretions trace epithelial type 2 response to allergen-specific immunotherapy

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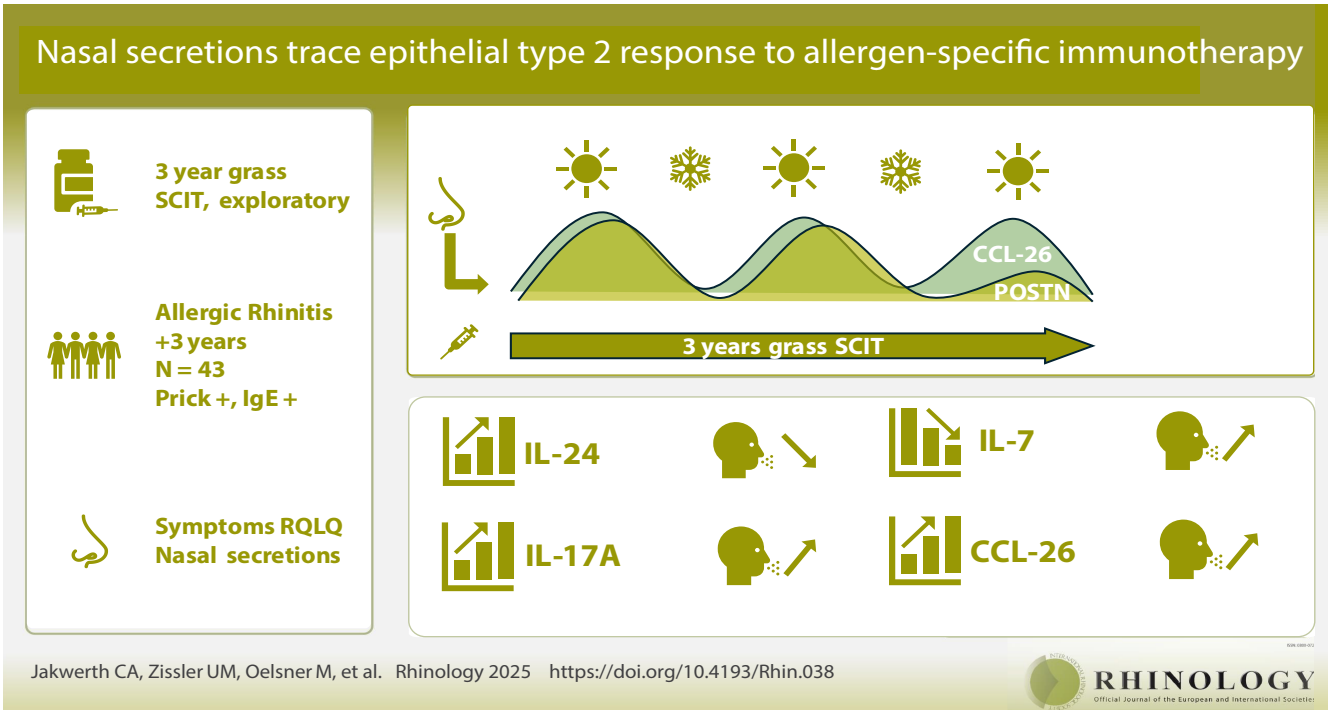
# equal contribution



Abstract

Background: Allergen-specific immunotherapy (AIT) is a disease-modifying therapy and is effective to reduce the symptoms of grass pollen-allergy. The airway epithelium of these patients releases inflammatory mediators including type-2 cytokines, which are associated with cellular processes involved in the symptomatic response of the affected tissue. Aim of the study was to identify epithelial biomarkers indicating AIT progress. **Methods**: In an exploratory, observational allergy cohort, we longitudinally phenotyped 56 grass pollen-allergic patients undergoing AIT for over three years and 18 controls using nasal secretions at critical time windows during therapy to assess peak-season responses along the course of therapy. Type-2 cytokine protein levels were analyzed using the high-sensitivity multiplex electrochemiluminescence mesoscale technique. **Results**: The type-2 cytokines CCL26 and POSTN oscillated seasonally, in contrast to TSLP and IL-33. However, only POSTN was reduced over the three-year AIT progression. In addition to POSTN, IL-24 and IL-37 levels were continuously reduced during AIT, while IFN-γ and CCL27 were increased. Compared to healthy individuals, AIT did not restore healthy secretion levels but rather induced a novel homeostasis **Conclusion**: Nasal secretions trace the epithelial response during different phases of AIT. We demonstrate that AIT only partially controls the epithelial type 2 cytokine CCL26, which also adapts to seasonal changes, while POSTN and IL-24 are potential indicators of therapy success. Therefore, nasal secretions represent a promising, non-invasive tool for monitoring seasonal progress of AIT.

Key words: allergic rhinitis, allergen-specific immunotherapy (AIT), airway epithelium, nasal secretions, non-invasive biomarker

Introduction

The local production of type 2 cytokines in the airway mucosa is considered crucial for the pathophysiology of allergic airway diseases [1] and can be triggered by allergen challenge [2]. Allergen-specific immunotherapy was reported to revert the type 2 response in response to allergens towards a type 1 [3] or regulatory immune response [4].

Repetitive administration of a defined tolerogenic dose of the causative allergen constitutes the therapeutic regimen of AIT and generally results in enhanced clinical unresponsiveness to the allergen of interest if therapy is continued for at least three years (reviewed in: [5]). Within these three years of treatment, we have previously observed that grass pollen-specific therapy can be divided into three different phases based on systemic changes in peripheral blood: In the initiation phase, we observed increased IL-10 expression in B cells, while the conversion phase in the second year was characterized by a trans-differentiation from T helper (Th) 17 to T regulatory 17 (Tr17) cells and the tolerance-mounting phase in the third year was characterized by a suppression of Th2 responses [6]. It has previously been hypothesized that regulatory T cells (Tregs) suppress Th2 cells by IL-10- and TGF-β-dependent mechanisms and thereby control the inflammatory cascade in AIT [7-9]. The decreased frequency of local T cells correlates with the number of tissue-infiltrating eosinophils following AIT [10], providing evidence that Th2 cell activity plays a crucial role in local symptom development. Our previous studies have shown that IL-4 is also expressed in the airways of non-symptomatic healthy individuals during the pollen season, but not during winter [6], raising the question of which factors besides IL-4 in the nasal mucosa may contribute to hay fever symptoms.

In the peripheral immune system, IL-4 plays an important role in priming towards type 2 responses involving IL-4-producing Th2 cells. In interaction with contact-dependent mechanisms (e.g. CD40/CD40L ligation), IL-4 is required for immunoglobulin class switch in allergen-specific B cells from IgM to IgE. IL-4 also inhibits T cell receptor-driven Treg differentiation [11-13] and affects non-lymphoid cells such as macrophages [14] and epithelial cells, which respond by producing type 2 cytokines themselves [15].

The current study focuses on local allergic inflammation that is assumed to be orchestrated by a variety of type 2 cytokines. Type 2 nasal mediators originate from infiltrating immune cells and local epithelial cells (reviewed in: [16]). Type 2 epithelial mediators such as CCL26 are more abundant than those secreted by infiltrating cells and are therefore better biomarker candidates. Notably, the upper and lower airways are in contact with each other (united airway concept [17]) and we have shown that changes in the lower airways correlate with those of the upper airways for many cytokines [18]. IL-33 and TSLP are known epithelial cytokines that promote type 2 immunity and can induce IL-4 and IL-13 directly in innate lymphocytes [19] and indirectly via Th2-promoting dendritic cells [20]. IL-4 in turn induces the epithelial type 2 cytokines CCL26 (Eotaxin-3) and periostin (POSTN) [21,22]. Based on the literature, these cytokines are the best characterized type 2 epithelial marker (reviewed in: [16]). The epithelium also mediates several other functions that are likely to play a role in the allergic inflammatory response and hence in AIT. Based on our genome-wide expression screening [6] we also included tissue homeostatic (IL-7) and anti-inflammatory factors (IL-37), chemokines that recruit specialized cells to the barrier (CCL27), and components of the complement system (Serpin G1). CSF1/M-CSF is also an epithelium-derived type 2 cytokine, as it was suggested to promote differentiation of macrophages into the M2 phenotype [23,24]. All these factors may contribute differently to AIT success, independent of the well-known effects of AIT on T and B cell subsets.

The aim of the study was to investigate the impact of AIT on type 2-related epithelial cytokines and thus shed light on the local mechanisms underlying the therapeutic efficacy. In the current study, we followed these type 2-related factors in nasal secretion through the updosing phase and over a three-year observation phase AIT in and out of grass pollen season. We reveal that the nasal type 2 factors illuminate all three phases of AIT as well as seasonal changes and may represent promising candidates for future biomarker-guided AIT. Thereby, this study could form the basis for future predictive and non-invasive nasal biomarkers.

Materials and methods

Clinical study

This exploratory study was designed as an open and observational real-life, long-term, case-controlled clinical cohort (Prospective Allergy and Clinical Immune Function Cohort study (PACIFIC, EudraCT 2015–003545-25) [6]. The study was approved by the Ethics Commission of the Technical University of Munich, Medical School (5534/12). Written and informed consent was obtained from all patients. Grass pollen-allergic patients (n=57; Table 1) with a history of moderate to severe and chronic-persistent allergic rhinitis according to ARIA criteria (Allergic Rhinitis and its impact on Asthma) were recruited for this study [25]. Two patients were excluded from nasal secretion analysis due to missing visits, resulting in data from 55 patients. In addition, 19 rhinitis patients were included as AIT-naïve controls along with 21 healthy controls (Table 1). From the PACIFIC cohort, nasal samples from 57 AIT-treated patients were included in this analysis. 43 patients attended the initial visits during the AIT updosing and top dose phases (T0-T5). During the follow-up phase, 36 patients attended four out of five visits except for the year 2 off season visit (T8). Further, inclusion criteria were a history of allergic rhinitis for more than two years, a positive skin prick test with a wheal of ≥3 mm in diameter and grass pollen-specific IgE levels above 0.70 kUA/L. Patients during pregnancy, lactation,

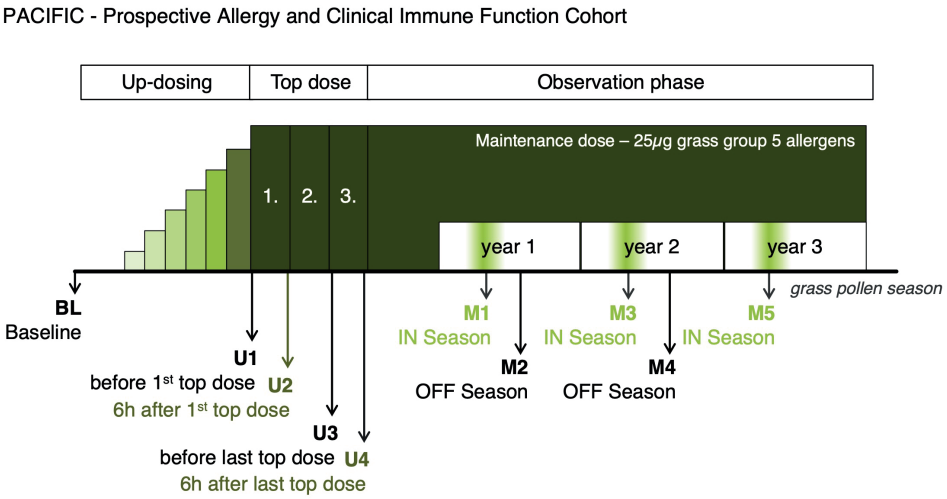


Figure 1. Study design scheme. Following a pre-seasonal updosing phase, grass pollen-allergic patients were treated biweekly with three top dose injections of a standard grass pollen-specific immunotherapy. Maintenance doses of 25 µg of grass group 5 allergens were administered every 4–6 weeks for a three-year observation phase, with doses adjusted seasonally according to symptom burden. Sampling: BL – Baseline (n=55), U1 – just before the first initial top dose (n=55), U2 – 6 hours after the first top dose (n=52), U3 – just before the last initial top dose (n=55), U4 – 6 hours after the last top dose (n=43), M1 – in the grass pollen season year 1 of the observation phase (n=40), M2 – out of grass pollen season (= off season) year 1 (n=37), M3 – in season year 2 of the observation phase (n=36), M4 – off season year 2 (n=12), M5 – in season year 3 of the observation phase (n=40).

minors (age <18 years), medication with systemic steroids, ACE inhibitors, beta-blockers, or immunosuppressive active substances, as well as patients with chronic rhinosinusitis with/out nasal polyps, severe systemic diseases, in particular cardiovascular or malignant diseases, were excluded from AIT treatment. Patients received a pre-seasonal, subcutaneous grass pollen-specific AIT with a licensed grass pollen allergoid (Allergovit®, Allergopharma GmbH & Co. KG, Germany) consisting of a 100% mixture of allergen extracts from six species of grass group 5 pollen (*Holcus lanatus, Dactylis glomerata, Lolium perenne, Phleum pratense, Poa pratensis*, and *Festuca pratensis*) that were chemically modified with formaldehyde and alum-absorbed. Consistent with our previous published studies, 25 µg of grass group 5 allergens were used per maintenance dose during the observation phase [26-28]. At predefined time points, nasal secretions were collected from the patients using an absorptive membrane and curettages were performed afterwards. Patients were allowed to take medication as needed, except for a seven-day washout period prior to sample collection. Time points ranged from baseline to the peak grass pollen season after three years of AIT. All patients had responded to AIT with a decreased frequency of Th2 cells in peripheral blood and locally in their nasal curettages. Clinical outcome was measured from M1 to M5 during the AIT observation phase using the validated, patient-assessed mini Rhinitis Quality of Life Questionnaire (mRQLQ; [29]) by assessing all relevant hay fever symptoms and the disease related quality of life. The mRQLQ is a validated questionnaire considering not only nasal but also ocular symptoms and other parameters and is therefore recommended for the assessment after allergen-specific immunotherapy [30].

Collection of nasal samples

Nasal secretions were collected for the analysis of secreted mediators. Baseline samples were taken out of grass pollen season, i.e. when there was neither tree nor grass pollen flight in the Munich area, samples during the AIT as indicated in Figure 1. All samples were stored and tested in one run, with no variations between runs. The analysis of nasal secretions was successfully performed on 55 patients who completed at least five initial visits. Filter paper strips (Leucosorb, Pall Inc., Port Washington, USA) were stored in an assigned 2 mL spinning tube (Costar® Spin-X® centrifuge tube filters cellulose acetate membrane, pore size 0.22 µm, Corning, Salt Lake City, UT, USA) for sampling. After a brief anterior rhinoscopy and, if necessary, crust cleaning of the nose, the membrane was removed from the spinning tube using a Halstead mosquito clamp. The membrane was carefully and atraumatically inserted into the left or right main nasal cavity using the clamp and fixed along the nasal septum at the level of the inferior turbinate, with the surface of the membrane pointing parallel to the nasal septum. The membrane was left in the nose for 45 seconds to absorb the nasal fluid. The membrane was then removed from the nose with the clamp and transferred to the portafilter tube. For elution purposes, 300 µL of sterile phosphate-buffered saline (Gibco, Carlsbad, CA, United States) was added to each tube. After one hour of gentle shaking at 4°C, the tubes were centrifuged for 5 minutes at 6.000 rpm at 4°C (Centrifuge 5702, Eppendorf, Hamburg, Germany). The portafilter tube was then carefully opened and the portafilter insert

Table 1. Patient characteristics in the PACIFIC Cohort (analysis subset).

	Immunotherapy Group n=57	Non-Allergic Controls n=21
Age (years)*	25.31 ± 5.47	26.95 ± 4.90
Male sex (%)	29 (50%)	8 (38%)
Sensitization (%)		
Grass	100%	0%
Birch	63%	0%
House Dust Mite	28%	0%
Total IgE	147.1 ± 167.1	18.63 ± 19.7
Grass-specific IgE	32.74 ± 27.88	0.00 ± 0.01
Allergic asthma (%)	18 (32%)	0 (0%)

Values are depicted as Mean ± S.D. * at informed consent procedure and inclusion to the study.

with the membrane discarded. The remaining supernatant was aliquoted into 0.5 mL tubes (LoBind;Eppendorf AG, Hamburg, Germany) and stored at -80°C.

Measurement of secreted mediators in nasal lining fluids

Protein levels of nasal IL-4, IFN-γ, IL-7, POSTN, IL-33, IL-24, CCL26 (eotaxin-3), Serpin G1, TSLP, IL-37, CSF-1 (M-CSF), CCL27 were measured in one run using high-sensitivity electrochemiluminescence detection on U-Plex and customized Prototype plates (Multi-Array technology; MSD Mesoscale, Rockville, MD, United States) according to manufacturer's instructions. The measurement was performed immediately on Sector Imager 6000 (MSD Mesoscale, Rockville, MD, United States).
Measurements were normalized to 100µg/mL total protein, quantified using the Bradford protein assay (Thermofisher). Lower limits of detection were for nasal IL-4 (0.012 pg/mL), IFN-γ (0.38 pg/mL), IL-7 (0.18 pg/mL), POSTN (19.31 pg/mL), IL-33 (8.46 pg/mL), IL-24 (4.25 pg/mL), CCL26 (1.41 pg/mL), Serpin G1 (19.31 pg/mL), TSLP (0.22pg/mL), IL-37 (0.99 pg/mL), CSF-1 (0.47 pg/mL), CCL27 (0.94 pg/mL). Upper limits of detection were observed for nasal IL-4 (1020.0 pg/mL), IFN-γ (12819.0 pg/mL), CCL-26 (9850.0pg/mL), Periostin (10000.0pg/mL), CSF-3 (500.0pg/mL), IL-24 (10000.0pg/mL), IL-33 (4000.0 pg/mL), IL-37 (2000.0 pg/mL), TSLP (1005.0 pg/mL), IL-7 (3210.0 pg/mL), CSF1 (9170.0 pg/mL), SCGB1A1 (30000.0 pg/mL), CCL20 (9500.0 pg/mL). Values reported below this limit were not imputed or adjusted.

Data acquisition and statistical analysis

All experimental procedures and analyzes of this exploratory study were conducted by blinded research personnel. Data are presented in parentheses throughout the results section as mean ± S.E.M. To avoid multiple testing and the need for corrections, a Kruskal-Wallis test was initially performed to identify global changes. If the null-hypothesis could be rejected ($p<0.05$) pairwise comparisons were performed ($\alpha=0.05$). Single comparisons were performed using Wilcoxon signed-rank tests. Correlations were performed for IL-24 immediately before the first top dose injection during the updosing phase at time point U1 and IL-17A six hours after the first top dose injection during the updosing phase at time point U2 with mRQLQ (categorial overall score) at the end of therapy in the third year of the pollen season (time point M5). Correlations of CCL26 and IL-7 during the updosing phase were calculated as delta (M1-BL) with mRQLQ at the end of therapy in during pollen season in the third year of AIT (time point M5). Spearman´s two-tailed rank correlation coefficient analyzes with 95% confidence intervals were used to calculate correlation coefficients between distinct cytokine levels and symptom scores. The cohort dataset has clear limitations due to the open study design with patients who did not complete all scheduled sample visits. Statistically significant differences are depicted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Results

Allergen-specific immunotherapy initially reduces secreted periostin and IL-33

To determine the effect of AIT on the nasal cytokine milieu, grass pollen-allergic patients were monitored in a longitudinal AIT cohort. The initial treatment phase consisted of a standard AIT updosing scheme, followed by three consecutive pre-seasonal injections of the top dose, which was then maintained every four to six weeks during the observation phase for a total of three years (Figure 1). Treatment was monitored by nasal lining fluid sampling, including time points immediately before and six hours after the pre-seasonal top-dose injections. In addition, samples were taken from patients throughout the course of therapy during and outside of grass pollen season to investigate the impact of natural allergen exposure. The characteristics of the analyzed cohort and controls are presented in Table 1.
The nasal response was analyzed during updosing (Figure 2) and consequently during the observation phase of three years in and out of grass pollen season (Figure 3). Already at the end of the updosing phase (U4), POSTN already decreased compared to baseline (BL) (Figure 2D; U4: 911.2 ± 209.6 vs BL: 1970.0 pg/ml ± 470.3; p=0.016). In the following years, POSTN showed a seasonal oscillation and a reduction in season comparing the last year of therapy with the first (Figure 3D). Similarly, IL-33 was reduced at the end of the updosing phase (U4: 9.12pg/ml ± 0.93 vs. BL: 13.17pg/ml ± 1.54; p=0.012, Figure 2E), while no significant changes were observed and the seasonal oscillation, seen for POSTN, was not observed for IL-33. IL-24 also tended to decrease during updosing, but this effect did not reach statistical significance. However, IL-24 steadily decreased during the observation phase of therapy independent of season and was lower after three years of therapy compared to baseline or seasonal values in the first year of therapy (M5: 3.20pg/ml ± 0.55

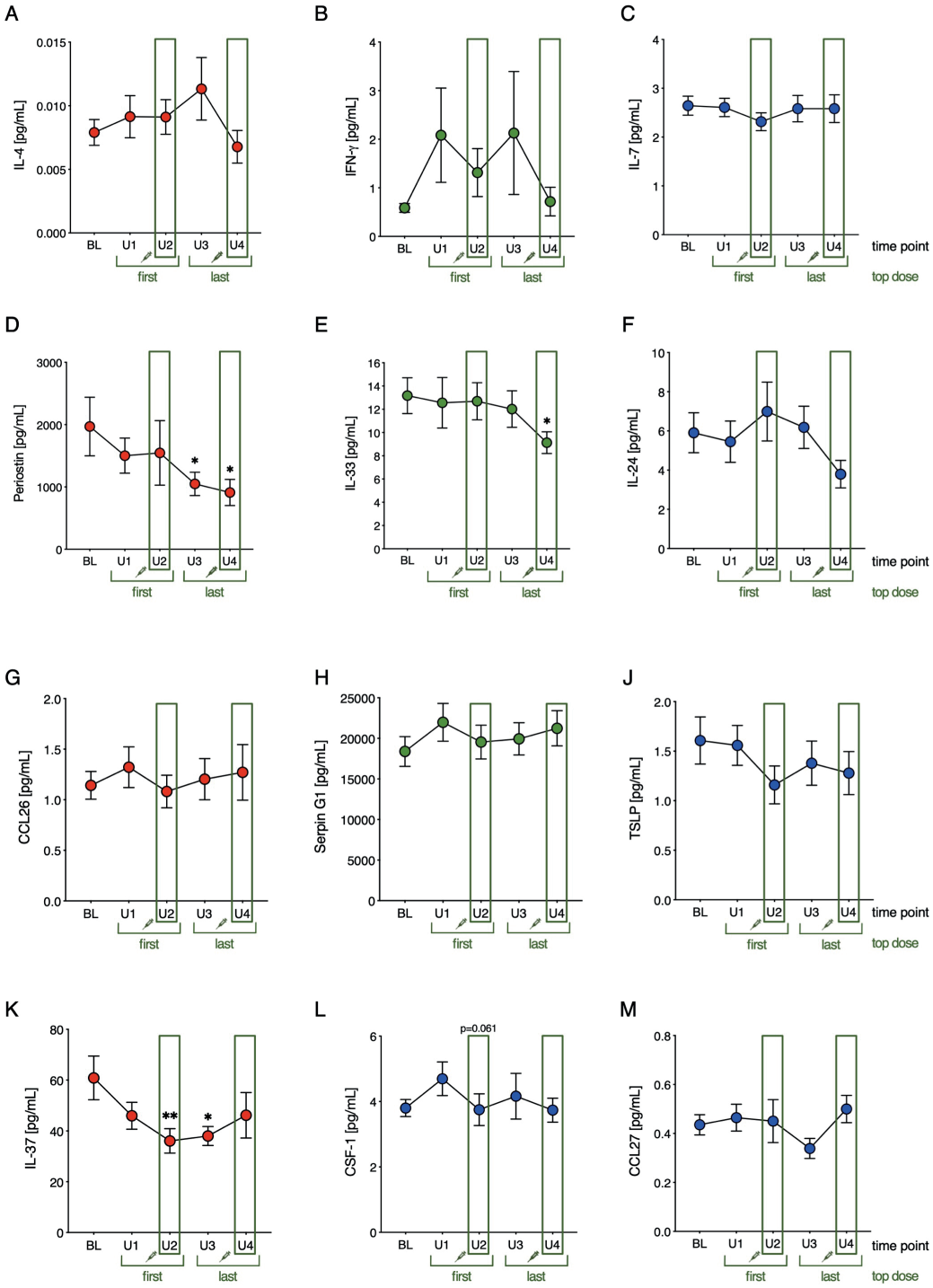


Figure 2. Fluctuations in nasal mediator protein levels in nasal secretions during the updosing phase of AIT. Nasal mediator protein levels of secreted (A) IL-4, (B) IFN-γ (C) IL-7, (D) POSTN, (E) IL-33, (F) IL-24, (G) CCL26, (H) Serpin G1, (J) TSLP, (K) IL-37, (L) CSF-1, (M) CCL27 were measured during the updosing phase: BL – Baseline (n=55), U1 – just before the first initial top dose (n=55), U2 – 6 hours after the first top dose (n=52), U3 – just before the last initial top dose (n=55), U4 – 6 hours after the last top dose (n=43). Dark green boxes indicate time points 6 hours after injections. Kruskal-Wallis tests and Wilcoxon signed-rank tests were performed for statistical evaluation. Significant differences are marked by asterisks compared to the baseline if not otherwise indicated; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

vs. M1: 9.32pg/ml ± 2.78; p<0.001; Figure 3F).

Seasonal oscillation of nasal cytokines unchanged by AIT

Similar to POSTN, CCL26 showed strong seasonal oscillation during the observation phase, however, in contrast to POSTN, no long-term AIT effect on this type 2 cytokine was observed (Fi-

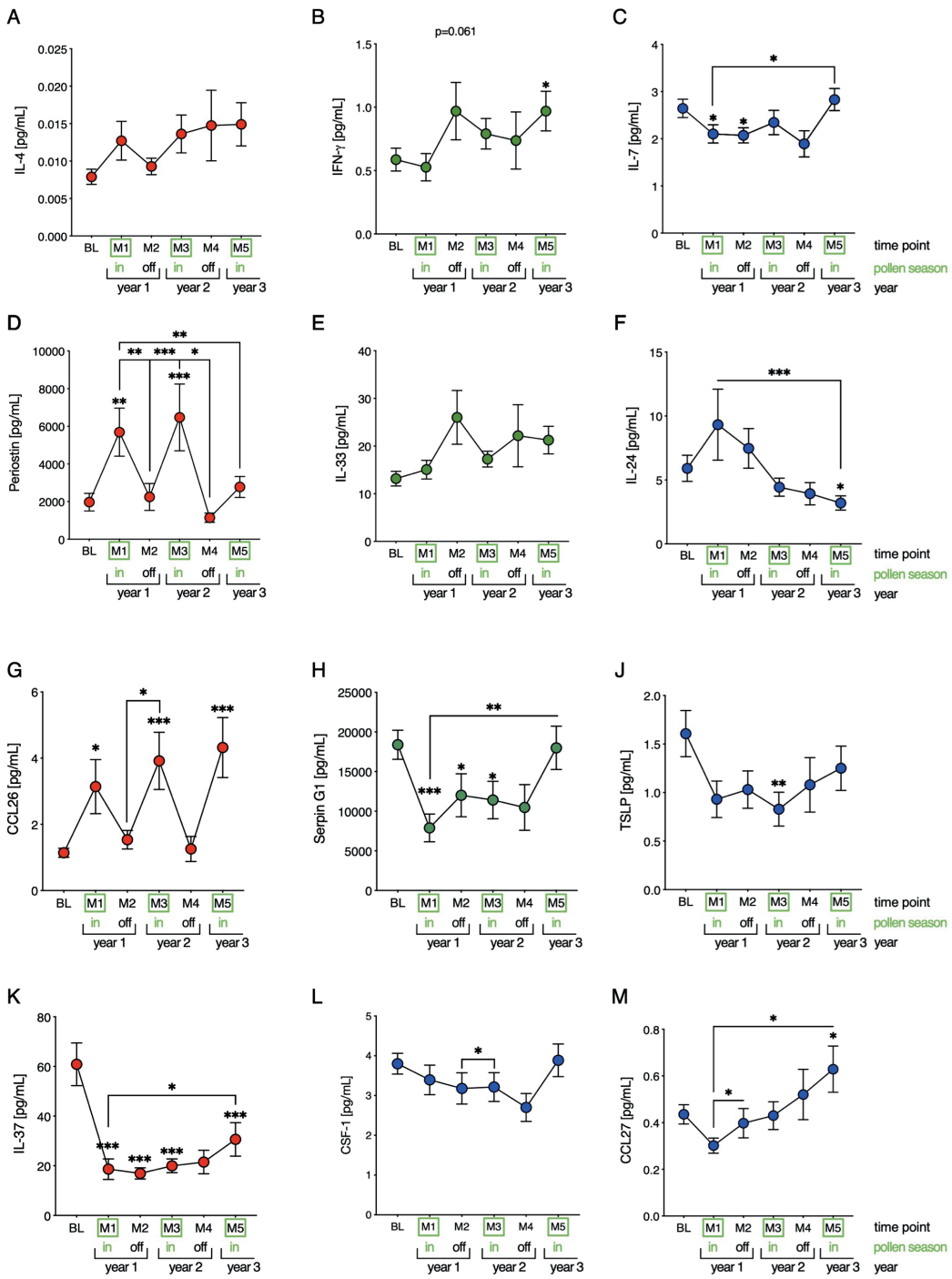


Figure 3. Nasal mediators in nasal secretions before and after AIT compared to healthy controls. Nasal mediator protein levels of secreted (A) IL-4, (B) IFN-γ (C) IL-7, (D) POSTN, (E) IL-33, (F) IL-24, (G) CCL26, (H) Serpin G1, (J) TSLP, (K) IL-37, (L) CSF-1, (M) CCL27 were measured at: BL – Baseline (n=55), M5 – in season year 3 of the observation phase (n=40) and in HC – healthy controls during pollen season. Wilcoxon signed-rank tests were performed for statistical evaluation. Significant differences are marked by asterisks; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

gure 3G). In contrast, Serpin G1 and TSLP showed reduced levels of secretion in the first and second year but recovered towards the end of therapy. During the updosing phase, no initial changes were observed for CCL26, SerpinG1 and TSLP (Figure 2G-J). While the cytokines IL-4, IFN-γ and IL-7 showed no secretion differences (Figure 2A-C), an increase of IL-4 could be observed, which missed significance in the group comparison at seasonal time at intra-individual levels (Figure 3A). While IFN-γ was initially not significantly increased (p=0.061) during the updosing phase (Figure 1B), a significant increase was observed in the third year of AIT compared to baseline (M5: 0.97 ± 0.16pg/mL; BL: 0.59 ± 0.09pg/mL p=0.036; Figure 3B).

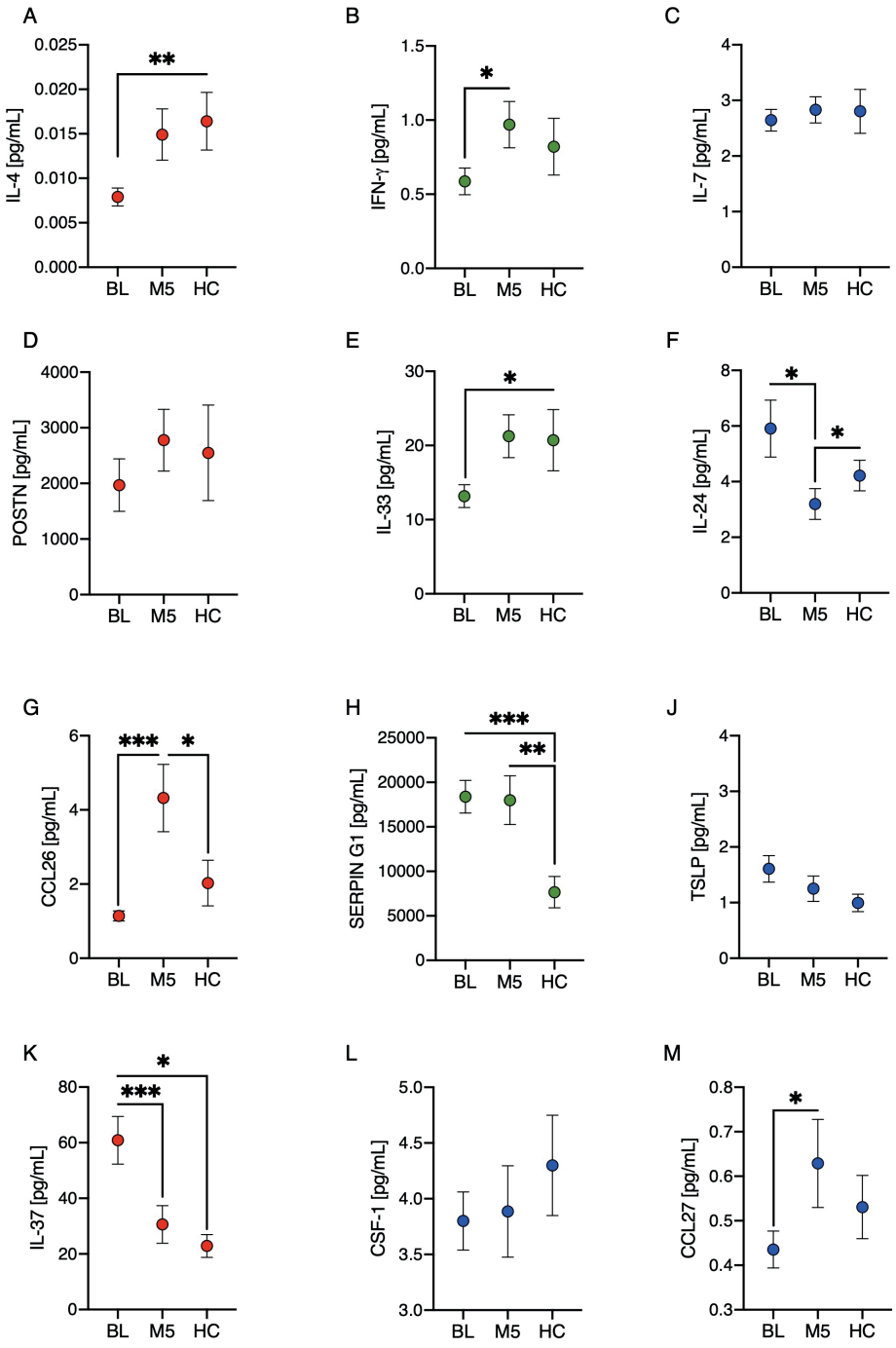


Figure 4. Nasal mediators in nasal secretions before and after AIT compared to healthy controls. Nasal mediator protein levels of secreted (A) IL-4, (B) IFN-γ (C) IL-7, (D) POSTN, (E) IL-33, (F) IL-24, (G) CCL26, (H) Serpin G1, (J) TSLP, (K) IL-37, (L) CSF-1, (M) CCL27 were measured at: BL – Baseline (n=55), M5 – in season year 3 of the observation phase (n=40) and in HC – healthy controls during pollen season. Wilcoxon signed-rank tests were performed for statistical evaluation. Significant differences are marked by asterisks; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Effects in the AIT conversion phase

Nasal IL-37 transiently decreased during updosing (Figure 2K) and showed remarkably reduced levels throughout the observation phase, recovering in the last year of therapy, although still remaining lower than baseline levels (Figure 3K). CSF-1/M-CSF showed only minor changes during the updosing and observation phase of AIT. In contrast, CCL27 remained unchanged during the updosing phase (Figure 2M), but showed steadily increasing levels of secretion, even exceeding baseline levels at three years (Figure 3M).

AIT does not restore healthy nasal cytokine levels

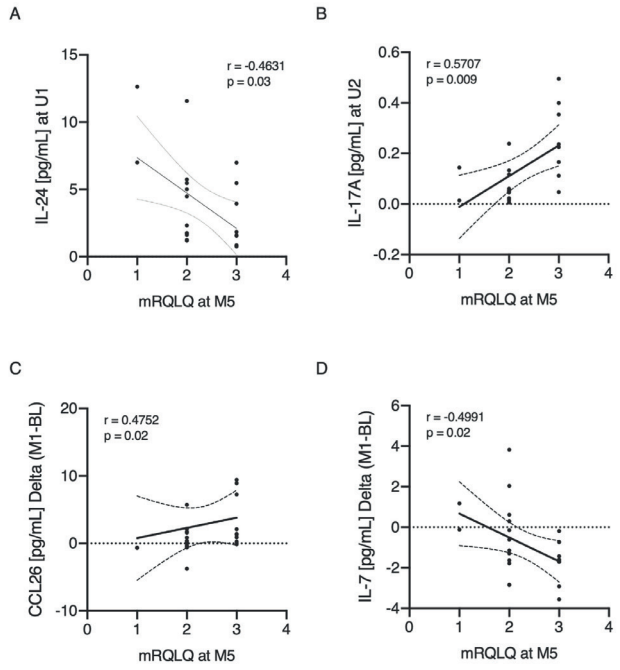


Figure 5. Correlation of the nasal cytokine levels during the updosing phase with total miniRQLQ (mRQLQ) score. Correlation of (A) IL-24 right before the first top dose injection during the updosing phase at time point U1 and (B) IL-17A six hours after the first top dose injection during the updosing phase at time point U2 with total mRQLQ at the end of therapy during the third year of the pollen season (time point M5). Correlations of the changes of the nasal type-2 marker CCL26 (C) and IL-7 (D), during the updosing phase calculated as delta(M1-BL) with mRQLQ at the end of therapy during the third year of the pollen season (time point M5). Two-tailed Spearman´s rank correlation coefficient analyses with 95% confidence intervals were used to compute coefficients of correlation between distinct cytokine levels and total categorial mRQLQ.

Nasal secretion of healthy individuals in grass pollen season were compared to those of patients before AIT at baseline (off-season therapy start) and after three years of AIT in season. While IL-4 only underwent seasonal changes and IFN-γ, IL-7, POSTN, IL-33, TSLP, CSF-1/M-CSF and CCL27 showed no changes (Figure 4B-E, J, L, M), only IL-37 levels indicated a restoration of baseline levels towards the secretion levels of healthy individuals (Figure 4K). IL-24, CCL26, and Serpin G1 still showed differences between healthy individuals and patients following 3 years of therapy (Figure 4F-H, K).

Association of nasal mediator levels with symptom score at end of AIT

Although, the PACIFIC clinical cohort was an exploratory study, which was underpowered to detect clinical efficacy, we analyzed the association of nasal cytokine levels during the updosing phase with the categorial mRQLQ score during the peak pollen season in the third year of AIT in these AIT treated patients compared to healthy controls. A higher mRQLQ indicates a higher symptom load and impaired disease-related quality of life of the patient. While secreted IL-24 levels at the first time point right before the first top dose of the updosing phase at U1 correlated negatively with increased symptoms at time point M5 (r = -0.4631; p = 0.03; Figure 5A), the levels of the proinflammatory cytokine IL-17A at time point six hours after the first top dose U2 correlated clearly positive with an increased mRQLQ at time point M5 (r = 0.5707; p = 0.009; Figure 5B). Furthermore, the increase in the nasal type 2 marker CCL26 during the updosing phase (Delta(M1-BL)) correlated positively with the mRQLQ at M5 (r = 0.4752; p = 0.02; Figure 5C), whereas IL-7 was negatively associated with increased symptoms (r = -0.4991; p = 0.02; Figure 5D). In summary, the higher the early levels of IL-17A and increase of CCL26, the more severe the symptoms at the AIT endpoint, while higher early levels of IL-24 and increase of IL-7 were associated with protection from allergic symptoms.

Discussion

Allergen-specific immunotherapy has a remarkable yet unexpected heterogenous impact on nasal type 2-related cytokines. In this study, we demonstrate that AIT has differential effects on the mucosal tissue and induces a new tissue homeostasis distinct from the mucosa of healthy individuals.

Seasonal oscillation of nasal cytokines

The biological relevance of nasal secretions can be seen from the very clear seasonal oscillation of the type 2 cytokines CCL26 and POSTN. Unexpectedly, CCL26 did not respond to AIT, although both CCL26 and POSTN are considered as prototypical epithelium-derived type 2 cytokines.

AIT-mediated reduction of nasal mediators

Nasal POSTN was reduced after three years of AIT treatment. In this context, it is important to note that POSTN is induced by many environmental stimuli [31,32] and plays a crucial role in tissue remodeling [33], especially in fibroblasts, cardiomyocytes and endothelium of the cardiovascular system. Therefore, we assume that the decrease in POSTN after AIT reflects improved airway inflammation control with resolution of type 2-mediated inflammation. This result is of particular interest since IL-4R-targeted pharmacotherapy has exactly the opposite effect on nasal POSTN and CCL26 in asthma patients: POSTN remains unchanged, while CCL26 is lowered by targeted anti-IL-4R therapy [34].

AIT-transition effects

We [6] and others [35,36] previously hypothesized that allergen-specific immunotherapy induces allergen tolerance across distinct phases of AIT and that the three-year duration of the therapy is necessary [5] to sufficiently elicit tolerogenic mechanisms including the decrease of Th2 frequency and the increase of Tregs at

the end of AIT. We previously proposed that the updosing phase is followed by a conversion phase, leading to the tolerance-mounting phase in the third year. The current data show that the anti-inflammatory cytokine IL-37 is reduced during the conversion phase. Since the conversion phase is characterized by the induction of Th17 cells, and since IL-37 has been negatively associated with Th17 immunity [37], possibly through suppression of Th17-inducing cytokines IL-1 and IL-6 [38], the drop in IL-37 may be indicative for the tolerogenic conversion during AIT. In addition, CCL27 also increased steadily in the conversion phase and into the tolerance-mounting phase. It could therefore represent an interesting biomarker, as it has been previously described to regulate mucosal immune homeostasis, specifically by orchestrating the migration of $CCR10^+$ (CCL27 receptor) $CD4^+$ and $CD8^+$ T cells [39]. The $CCR10^+$ T cells play an inflammatory role in the skin, and it has been suggested that redirection to other barrier organs could be important for a balanced immune homeostasis [39].

Two prototypical type 2 cytokines fall out of frame

AIT also induces an initial increase in IL-4 levels that did not reach statistical significance but was observed in most patients throughout the observation phase. Previous studies applied nasal allergen challenges to induce a synchronized IL-4 response and documented a decreased IL-4 secretion undergoing grass pollen-specific immunotherapy [40]. We have also previously shown this initial increase in circulating lymphocytes at local (transcriptome) and systemic levels [6] in relation to an exhausted Th2 phenotype [41]. This study confirms this IL-4 trend directly at the mucosal site of inflammation reflected by secreted protein levels in nasal secretions. While TSLP, another prototypical type 2 cytokine, is known to be triggered by environmental *Staphylococcus aureus* [42], it showed only a modest initial decrease during updosing, but no seasonal changes. This is consistent with experimental data showing that TSLP is dispensable for eosinophilic inflammation [43].

AIT does not restore nasal levels of healthy individuals

This study shows remarkably higher levels of SerpinG1, a complement C1 inhibitor, discussed in context of immune tolerance [44], in treated patients as compared to mucosal levels of healthy individuals. This finding is of particular interest as the complement system efficiently discriminates between self and non-self surfaces using soluble and membrane-bound complement regulators (reviewed in: [45]). Interesting is the lower level of mucosal IL-24, a member of the IL-10 family that we previously described as being inducible by IL-4 and to inhibiting the proliferation of tissue cells such as fibroblasts [46]. Since IL-24 at the first updosing visit also showed a negative correlation with the nasal symptom score (mRQLQ) at the AIT endpoint, we propose to conduct a randomized, double-blind, placebo-controlled trial to validate IL-24, IL-17A and updosing increase of IL-7 as early nasal biomarkers for therapy success. Another question is also whether the findings of this study apply also to other allergen sources. Since the discussed biomarkers are of epithelial origin and related to type 2 immunity, we speculate that they work for allergies that involve airway epithelium. In particular biomarker matrices that include both anti-inflammatory or homeostatic marker with those of tissue inflammation may represent useful tools for therapy monitoring.

Conclusion

Taken together, the current study underpins the different phases of allergen-specific immunotherapy, identifies CCL26 and therefore eosinophils as missed targets, as well as POSTN and IL-24 as potential markers for therapy success. Due to the non-invasive access to the nasal secretions, future therapies may benefit from the biomarkers provided in this study.

Abbreviations

AIT: Allergen-specific immunotherapy; CCL: C-C Motif Chemokine Ligand; CXCL: C-X-C Motif Chemokine Ligand; E2: type 2-primed epithelium; HDM: house dust mite; Ig: immunoglobulin; IL: interleukin; FEV1: forced exhaled volume; FVC: forced vital capacity; NHBE: normal human bronchial epithelial cell; PNECs: Pulmonary neuroendocrine cells; RQLQ: rhinitis quality of life questionnaire; SCGB: secretoglobin; Th: T helper cell; Tregs: regulatory T cells.

Authorship contribution

CAJ, UMZ, AMC and CSW developed the study and experimental layout. UMZ, MO, AE, MF, LP, LzB, MP, JK, MD, SB, and CAJ realized experimental cell culture models and measurements of protein levels. UMZ, AMC, UP, SB, BS, CAJ and CSW supported the study on ethical permissions and funding. AMC organized study part including human ethical approval and together with LP, LzB, MP, JK and MD the management of patient visits, patient information and sampling. The manuscript was written by CSW as well as CAJ and UMZ. All authors contributed to the article and approved the submitted version.

Conflict of interest

AMC reports grants from Allergopharma and German Center for Lung Research (DZL) during the conduct of the study; grants, speaker honoraria, consultancy or advisory fees and/or research support and other, all via Technical University of Munich from ALK-Abello, AstraZeneca, Bencard/Allergen Therapeutics, ASIT Biotech, GSK, Hippo Dx, Novartis, LETI, Roche, Sanofi, Regeneron, Zeller, grants from Federal German Ministry of Education and Research, grants from European Institute of Technology, all outside this submitted work. UMZ received payment for manuscripts from Deutsches Aerzteblatt and funds for travel

from the European Academy of Allergy and Clinical Immunology (EAACI) and Collegium Internationale Allergologicum (CIA). CSW received support for research projects from Zeller AG and Allergopharma and accepted honoraria for consultancy and seminars from Aimmune and Sanofi. JOM reports compensation for consulting services with Cellarity and Hovione. The rest of the authors declare that they have no relevant conflicts of interest.

Ethics

The studies involving human participants were reviewed and approved by ethics committee of the Klinikum rechts der Isar (5534/12). The patients/participants provided their written informed consent to participate in this study.

Data availability

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

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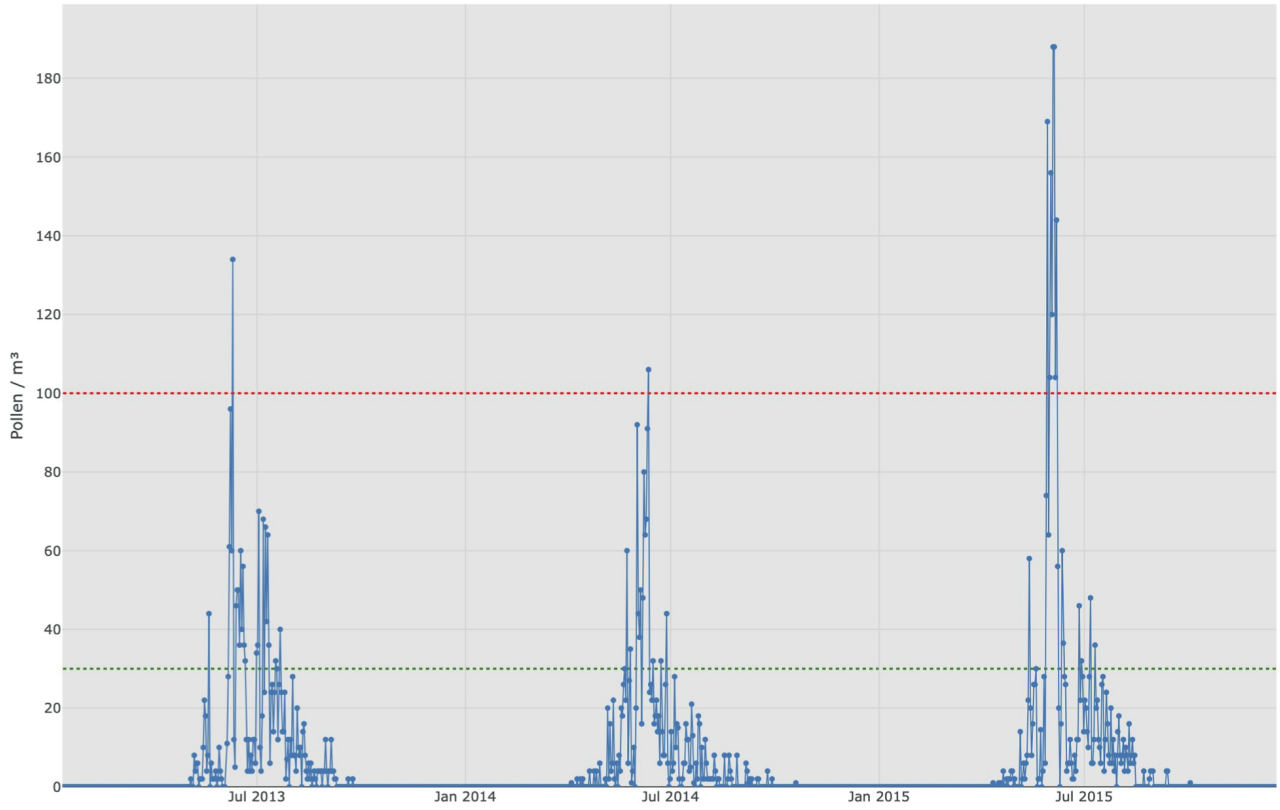

Supplementary Figure 1. Pollen count in the Munich region in the study years 2013-2015.