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ORIGINAL ARTICLE

Basic and Translational Allergy Immunology

Allergen-specific immunotherapy induces the suppressive secretoglobin 1A1 in cells of the lower airways

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Abstract

Background: While several systemic immunomodulatory effects of allergen-specific immunotherapy (AIT) have been discovered, local anti-inflammatory mechanisms in the respiratory tract are largely unknown. We sought to elucidate local and epithelial mechanisms underlying allergen-specific immunotherapy in a genome-wide approach.

Methods: We induced sputum in hay fever patients and healthy controls during the pollen peak season and stratified patients by effective allergen immunotherapy or as untreated. Sputum was directly processed after induction and subjected to whole transcriptome RNA microarray analysis. Nasal secretions were analyzed for Secretoglobin1A1 (SCGB1A1) and IL-24 protein levels in an additional validation cohort at three defined time points during the 3-year course of AIT. Subsequently, RNA was extracted and subjected to an array-based whole transcriptome analysis.

Results: Allergen-specific immunotherapy inhibited pro-inflammatory *CXCL8*, *IL24*, and *CCL26*mRNA expression, while *SCGB1A1*, *IL7*, *CCL5*, *CCL23*, and *WNT5B*mRNAs were induced independently of the asthma status and allergen season. In our validation cohort, local increase of SCGB1A1 occurred concomitantly with the reduction of local IL-24 in upper airways during the course of AIT. Additionally, SCGB1A1 was identified as a suppressor of epithelial gene expression.

Conclusions: Allergen-specific immunotherapy induces a yet unknown local gene expression footprint in the lower airways that on one hand appears to be a result of multiple regulatory pathways and on the other hand reveals SCGB1A1 as novel anti-inflammatory mediator of long-term allergen-specific therapeutic intervention in the local environment.

Abbreviations: AIT, allergen-specific immunotherapy; CCL, C-C Motif Chemokine Ligand; CXCL, C-X-C Motif Chemokine Ligand; E2, type-2 primed epithelium; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; HDM, house dust mite; HNBE, human normal bronchial epithelial cell; Ig, immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; RQLQ, rhinitis quality of life questionnaire; SCGB, secretoglobin; Th, T helper cell; Tregs, regulatory T cells; Wnt, Wingless-type.

Schmidt-Weber and Chaker these authors equally contributed to the manuscript.

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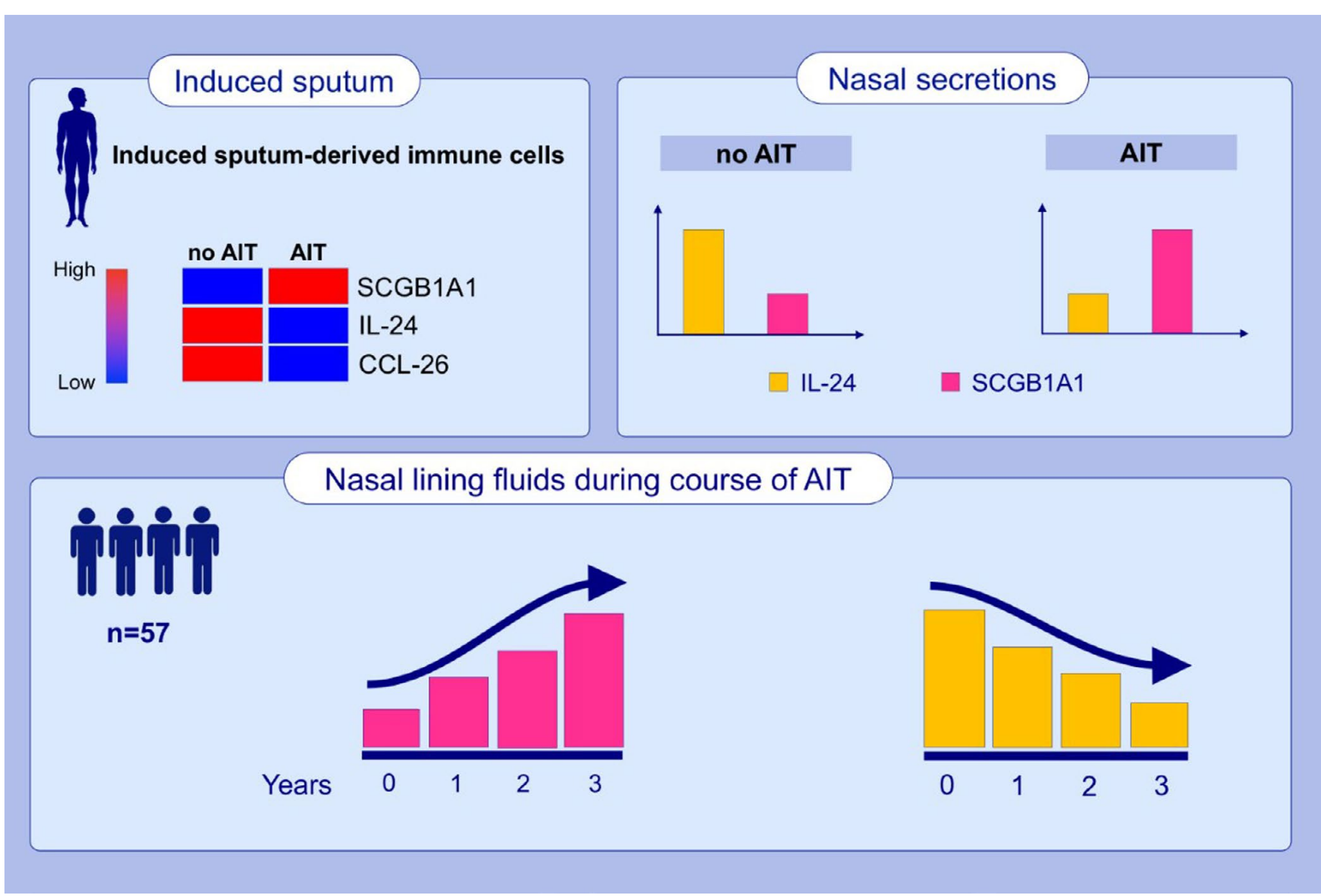
KEYWORDS
allergen-specific immunotherapy, allergic rhinitis, asthma, induced sputum, tolerance



GRAPHICAL ABSTRACT

Allergen-specific immunotherapy inhibited pro-inflammatory *CXCL8*, *IL24* and *CCL26* mRNA expression in alveolar sputum cells. *SCGB1A1* was induced in lower airway cells independently of the asthma status and allergen season. The epithelial type-2 (E2) cytokine IL-24 was reduced following 3 years of AIT-treatment, while SCGB1A1 was significantly increased and identified as a suppressor of epithelial gene expression.

1 | INTRODUCTION

Allergen-specific immunotherapy (AIT) for allergic airway diseases such as hay fever is the only causative treatment of underlying immune-pathology and has proven to be safe and effective on long-term. However, biomarkers with predictive value for therapy efficacy are not yet available. It can be considered as a model for tolerogenic vaccination and understanding its mechanisms may provide insight for rational vaccine design not only in the field of allergy but also in autoimmunity, or transplantation. Albeit largely driven by Th-cells, type 2 responses are mainly involved in the late phase reaction to allergens. These Th2 responses have been hypothesized to be reduced during AIT, probably via inhibition by *de novo* recruited Tregs, as was shown in biopsies of affected nasal mucosa.[1] Recent studies reported a shift in Th1/Th2 profiles under AIT, suggesting potentially competitive mechanisms being responsible for AIT-mediated reduction of Th2 cells and increased *IFN-γ*-expression of Th1 cells[2–4] and was discussed as a potential antagonistic mechanism of suppression.[2]

Increasing evidence indicates that tissue-resident cells are key regulators of critical elements in the development of immune tolerance, such as tissue-resident regulatory T cells[5] and myeloid regulatory cells.[6,7] We have recently suggested a 3-phase model (initiation, conversion, and tolerance-mounting phase) to describe changes in the lymphocyte compartment during allergen immunotherapy, characterized mainly by spatially separated populations.[4] While these phenotypic changes of undulating Th17 responses, increasing levels of B-/Tregs and Th1-cells with simultaneous decrease of Th2 cells were mainly observed in peripheral blood we were able to detect these patterns also in upper airway brushings pointing to an interplay of systemic and local epithelial mechanisms in the development of immunological tolerance during AIT. This is in line with earlier

published studies that were able to show reduced *IL-5* mRNA levels in the airways[8] and reduced number of sputum eosinophils.[9] In vitro transcriptomic data and samples from disease cohorts provide evidence that airway epithelium mirrors type1 and type2 responses, we thereby accordingly categorized E1 and E2: IL-4 primes epithelial cells toward an E2 phenotype characterized by the expression of *CCL26* and *IL24*, as well as a number of transcription factors, mucins, and anti-microbial peptides.[10] Therefore, CCL-26 and IL-24 can be considered as tissue-derived biomarkers for type-2 immune responses.[11] Th1 cells impede the E2-phenotype,[10] whereas IFN-γ has been reported to potentiate TNF-α release of alveolar macrophages.[12] Similar to Th1/Th2-epithelial cell interaction Tregs were shown to regulate epithelial repair functions by production of amphiregulin and restore tissue homeostasis.[13] In addition, Tregs support tissue regeneration by promoting basal stem cell growth.[14]

Respiratory tract macrophages represent the major population within sputum cells and do not only represent phagocytic scavengers and microbial sensors, but also exert tissue homeostatic functions. A recent murine study provided experimental evidence that depletion of alveolar macrophages favors development of type-2 dominated immune responses.[15] Alveolar macrophages and epithelial cells interact via CD200R and CD200 respectively, as CD200 expressed by epithelial cells is associated with tolerogenic and inhibitory functions,[16] thereby restoring epithelial homeostasis.[17] As a consequence of this interaction, we hypothesize that macrophage and epithelial cytokines may serve as markers for treatment efficacy and local tolerance induction.

2 | METHODS

2.1 | Clinical study

We investigated in this non-interventional observational study two (independent) cohorts of study participants. For the cross-sectional seasonal cohort, we recruited and induced sputa in 12 healthy controls and 40 grass pollen allergic participants with allergic rhinitis (AR) in (May-July) and off the pollen season (October-December). Within the group of grass pollen allergic patients, 21 received effective AIT since at least 1 year or longer (see Table 1). In the group of untreated AR patients, nine of them suffered from asthma comorbidity, while in the group of AIT-treated AR patients 12 showed asthma comorbidity. For details of all methods see Appendix S1.

2.1.1 | 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 ± s.e.m.

TABLE 1 Patients' characteristics and sputum cell differentiation of recruited patients

	Controls (*n* = 12)	Allergic rhinitis w/o AIT (*n* = 19)	Allergic rhinitis with AIT (*n* = 21)
Age [years]	26.6 (±0.93)	28.8 (±2.51)	31.05 (±2.20)
Sex (m/f)	7/5	10/9	12/9
Asthma [%]comorbidity	0	9/19 [47.3%]	12/21 [57.1%]
mRQLQ score	0 (±0.15)	2.37 (±0.34)[###]	1.5 (±0.26)[##, a]
Total IgE [IU/L]	87.46 (±57.14)	372.2 (±88.26)[###]	123.4 (±24.33)[###, a]
(PT/CAP)to			
Grass	0	19/19	21/21
Birch	0	16/12	16/15
HDM	0	12/9	13/8
Cat	0	11/7	5/7
Clinical symptoms to			
Grass	0	19	18
Birch	0	9	8
HDM	0	3	5
Cat	0	4	11
Initially unclear anamnesis	0	2	1
FEV1 [L]	4.56 (±0.44)	4.13 (±0.27)	4.32 (±0.25)
FVC [L]	5.31 (±0.68)	4.51 (±0.27)	4.84 (±0.26)
MEF25 [L]	3.52 (±0.36)	2.80 (±0.27)[#]	2.99 (±0.29)[#]

Note: Data are presented as mean ± SEM.

means significance in relation to healthy controls, # = $p < .05$; ## = $p < .01$; ### = $p < .001$; #### = $p < .0001$.

[a]significance in relation to untreated patients.

Non-parametric statistical test were chosen, as the data points were not normally distributed. Kruskal-Wallis tests were performed initially to avoid multiple testing, and, only when medians across patient groups varied significantly, multiple single comparisons were performed using two-tailed Mann-Whitney *U* tests. Statistically significant differences are depicted as $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$. For further details, see Appendix S1.

3 | RESULTS

To understand local mechanisms of airway inflammation and the impact of AIT, we analyzed sputum cells from patients with allergic rhinitis (Table 1) and from healthy controls and discovered a previously unknown footprint in sputum cells. Patients during AIT for at least 1 year were included into the AIT group.

3.1 | Sputum cell composition and patient characteristics

Differences were assessable for total IgE, RQLQ, and cell load (number of sputum cells per ml, Figure 1A) between treated and untreated patients and healthy controls as shown in Table 1. Of note, substantial reductions in total IgE are usually only visible in larger sample sizes and later phases of up to 3 years following initiation of AIT.[18] No differences were observed for lung function parameters (in season), assessed during the study. Some differences in composition of sputum cells became visible between untreated rhinitis patients and healthy controls, which were, however, in the range of previously reported sputum studies (Table 1).[19]

3.2 | Differential expression of genes encoding for secreted proteins

Sputum cells of AIT-treated and untreated patients revealed significant gene expression differences which were found at a 1.5-fold threshold and a *p*-value of ≤ .05 with 1,680 downregulated genes and 485 upregulated genes in the pollen season and 7,916 observed downregulated and 1,288 upregulated off-season (Table S1,2,3,and 4). Sputum leukocytes infiltrate and interact with the lung via soluble mediators, we focused further analysis of AIT-induced expression changes specifically on secreted gene products using Gene Ontology (GO) terms. For in season comparison, this group contained genes such as type-1 cytokine genes *IFN-γ* (2.90-fold increase) and *IL18* (1.62-fold increase), tissue remodeling factors (*FGF20*,*COL6A2*,*ACTA2*, *FGFR3*, *ANGPTL1*, *RSPO3*), enzymes (*MOV10*,*GPX3*), complement factors (*C3*,*C4B*,*Serpins*,*CFB*) and receptors that can be shedded or secreted (*CD40*,*IL15RA*; Figure 1F). In contrast, *CXCL2* (1.75-fold increase), *CXCL10* (3.75-fold increase), *CXCL11* (5.59-fold increase), and *CCL20* (1.99-fold increase) were exclusively found to be differentially regulated off-season (Figure 1G).

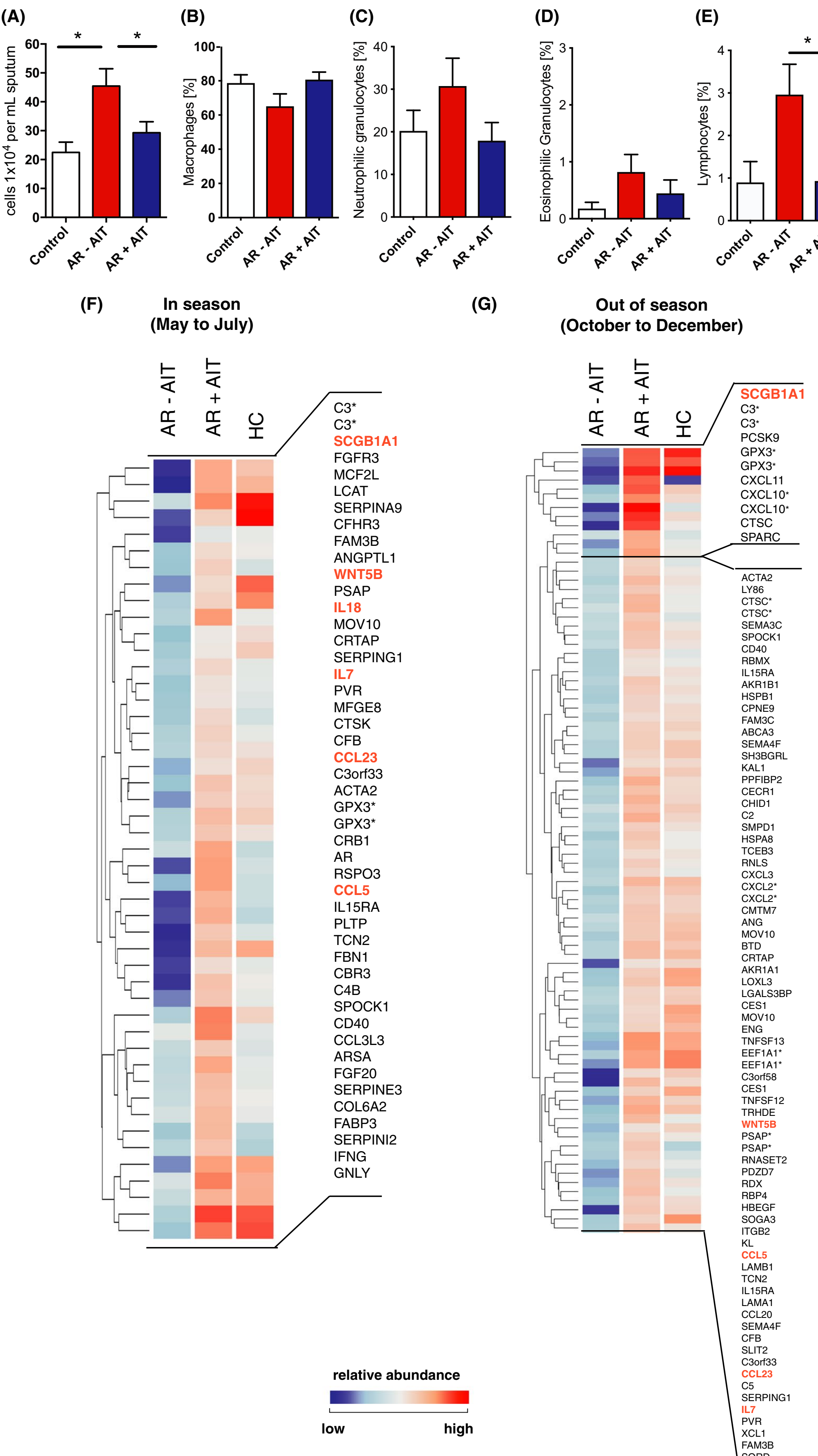
However, of particular interest are such genes that are differentially expressed in response to AIT independent of seasonal influences and pollen exposure as they may represent more stable and robust (bio)markers for a successful AIT treatment. Twenty-two genes encoding for secreted mediators were upregulated after AIT independently of the pollen season. Among these, we identified cytokines *SCGB1A1*, *IL7*, and *WNT5B* as well as the chemokines *CCL5* and *CCL23* (Figure 2A,C). Interestingly, the CCL-5 scavenger receptor *ACKR2* was downregulated by AIT in season (Table S1). Among them, *SCGB1A1*showed the most robust change over the whole observation period, with a 3.34-fold increase even off-season compared with untreated allergic rhinitis patients.

We identified SCGB1A1 positive cells in sputum leukocytes by confocal microscopy, including mononuclear cells such as macrophages (Figure 2E–H). Interestingly, SCGB1A1-positive lymphocytes were observed as well, which we currently investigate using a flow cytometric protocol. We verified the staining of SCGB1A1 in healthy turbinate tissues, which showed - as expected - SCGB1A1 expression in alpha-tubulin negative goblet cells, but also cells close to the basal membrane (Figure S1E–H). Furthermore, the expression of SCGB1A1 in airway epithelial cells was further confirmed in NHBE cells (Figure S1A,B) and *ex vivo* nasal polyp tissue (Figure S1C,D).

In a subgroup analysis, we investigated treated rhinitis patients who also suffered from asthma comorbidity and detected consistently higher expression of *SCGB1A1*, *IFNγ* (only in season), *CCL5* and *CCL23* compared with untreated patients in induced sputum transcriptomes (Figure S2).

In addition, we also found some important factors in the group of downregulated genes such as *CCL1*, *IL24*, *CSF1*, and *Pannexin-3* (*PANX3*; Figure 2D). In particular, the anti-microbial gene *CCL1* and the known type-2-related epithelial cytokine *IL24* are of great

FIGURE 1 Sputum cell distribution and upregulated secreted biomarkers identified by whole transcriptome analysis. Inflammatory cell load, determined by sputum cells per ml sputum, was significantly higher in untreated rhinitis patients compared with treated patients as well as healthy control subjects (A). Sputum cell differentiation revealed no differences (B–D) except for lymphocytes, which showed higher numbers in untreated rhinitis patients compared with treated rhinitis patients (E). Induced sputum cells were processed and subjected to RNA whole transcriptome analysis by array technology. Genes encoding for secreted proteins were identified by Gene Ontology-Terms (GO-Terms) 0007267, 0005125, 0008009, and 0005615. Gene symbols of members of the cytokine or chemokine family were highlighted in red color. Asterisks indicate two isoforms that were present in the analysis. The color code indicates the abundance of transcripts ranging from low (blue) to high (red) (F, G). 45 genes were identified to be upregulated by allergen-specific immunotherapy (AIT) during grass pollen season (F) while a total of 86 genes were shown to be upregulated by allergen-specific immunotherapy (AIT) out of the grass pollen season (G)


(A)
(B)
(C)
(D)
(E)
cells 1x10^4 per mL sputum
Macrophages [%]
Neutrophilic granulocytes [%]
Eosinophilic Granulocytes [%]
Lymphocytes [%]
Control
AR - AIT
AR + AIT
*
(F)
In season
(May to July)
(G)
Out of season
(October to December)
AR - AIT
AR + AIT
HC
C3*
C3*
SCGB1A1
FGFR3
MCF2L
LCAT
SERPINA9
CFHR3
FAM3B
ANGPTL1
WNT5B
PSAP
IL18
MOV10
CRTAP
SERPING1
IL7
PVR
MFGE8
CTSK
CFB
CCL23
C3orf33
ACTA2
GPX3*
GPX3*
CRB1
AR
RSPO3
CCL5
IL15RA
PLTP
TCN2
FBN1
CBR3
C4B
SPOCK1
CD40
CCL3L3
ARSA
FGF20
SERPINE3
COL6A2
FABP3
SERPINI2
IFNG
GNLY
SCGB1A1
C3*
C3*
PCSK9
GPX3*
GPX3*
CXCL11
CXCL10*
CXCL10*
CTSC
SPARC
ACTA2
LY86
CTSC*
CTSC*
SEMA3C
SPOCK1
CD40
RBMX
IL15RA
AKR1B1
HSPB1
CPNE9
FAM3C
ABCA3
SEMA4F
SH3BGRL
KAL1
PPFIBP2
CECR1
CHID1
C2
SMPD1
HSPA8
TCEB3
RNLS
CXCL3
CXCL2*
CXCL2*
CMTM7
ANG
MOV10
BTD
CRTAP
AKR1A1
LOXL3
LGALS3BP
CES1
MOV10
ENG
TNFSF13
EEF1A1*
EEF1A1*
C3orf58
CES1
TNFSF12
TRHDE
WNT5B
PSAP*
PSAP*
RNASET2
PDZD7
RDX
RBP4
HBEGF
SOGA3
ITGB2
KL
CCL5
LAMB1
TCN2
IL15RA
LAMA1
CCL20
SEMA4F
CFB
SLIT2
C3orf33
CCL23
C5
SERPING1
IL7
PVR
XCL1
FAM3B
SORD
relative abundance
low
high

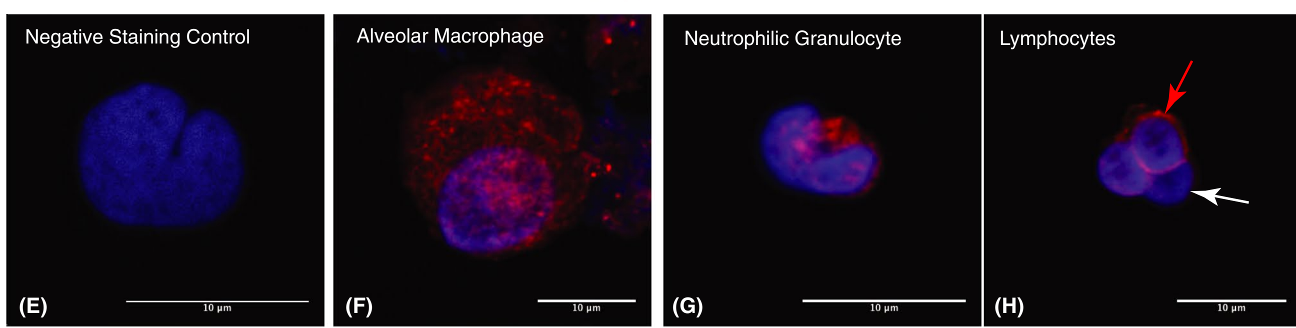
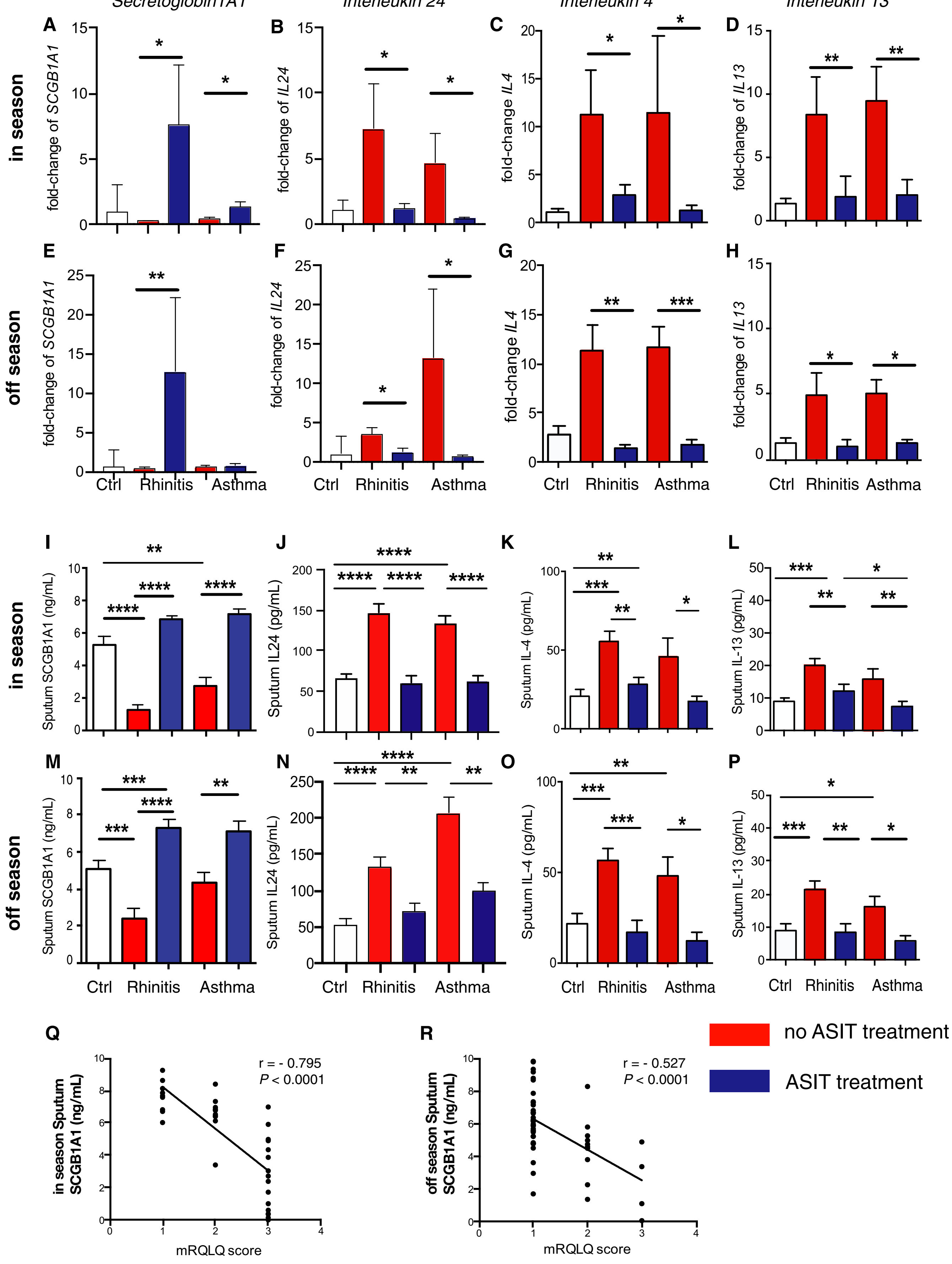


FIGURE 2 Cytokine-related factors differentially expressed independent of season. Distribution of the 45 AIT-induced genes in season, and the 86 AIT-induced genes out of season in a Venn diagram (A). An overlapping set of 22 genes was identified which includes *SCGB1A1*, *CCL5*, *CCL23*, *Interleukin-7*, and *WNT5B* (B). Asterisks indicate two isoforms that were present in the analysis. The color code indicates the abundance of transcripts ranging from low (blue) to high (red). Venn diagram distribution of genes expressed at reduced levels in response to AIT: 27 genes in season and 211 genes out of season (C). An overlapping set of 7 genes was identified, highlighting the type2-related factors *IL24*, *NFE2*, *CCL1*, and *CSF1* (D). Asterisks indicate two isoforms that were present in the analysis. The color code indicates the abundance of transcripts ranging from low (blue) to high (red). Representative confocal images of sputum-derived leukocytes demonstrate in red the expression of SCGB1A1 in negative control (E), sputum macrophages (F), sputum granulocytes (G), and sputum lymphocytes (H). Arrows in sub-figure H indicated SCGB1A1-positive (red) and SCGB1A1-negative (white) lymphocytes

FIGURE 3 Gene expression and protein levels for selected cytokines. Sputum cells of healthy controls and patients were subjected to RNA isolation, real-time quantitative reverse transcription–PCR (qRT-PCR) was performed, and gene expression changes were analyzed for *SCGB1A1* in (A) and out of the season (C) revealing increased levels in treated patients compared with untreated patients. Similar analyses were performed for the type-2 cytokines *IL24*, *IL4*, *and IL13* in (B-D) and out of the season (E-G) revealing decreased levels for treated patients compared with untreated patients. Sputum supernatant protein levels were validated for SCGB1A1 (I, M), IL-24 (J, N), IL-4 (K, O), and IL-13 (L, P) in subjects in and out of grass pollen season, respectively. In addition, protein levels of SCGB1A1 were correlated to symptom scores in (Q) and out of grass pollen season (R) using two-tailed Spearman's rank correlation. ****$p < .0001$, ***$p < .001$, **$p < .01$,*$p < .05$


Secretoglobin1A1
Interleukin 24
Interleukin 4
Interleukin 13
in season
off season
fold-change of SCGB1A1
fold-change of IL24
fold-change IL4
fold-change of IL13
Ctrl Rhinitis Asthma
Sputum SCGB1A1 (ng/mL)
Sputum IL24 (pg/mL)
Sputum IL-4 (pg/mL)
Sputum IL-13 (pg/mL)
in season Sputum SCGB1A1 (ng/mL)
off season Sputum SCGB1A1 (ng/mL)
r = - 0.795
P < 0.0001
r = - 0.527
P < 0.0001
mRQLQ score
no ASIT treatment
ASIT treatment

interest, as IL-24 represents an E2 cytokine that marks the type-2 polarization of epithelial cells independent of the infiltration of immune cells.

3.3 | Key cytokines confirmed on transcriptome and protein level in induced sputum

Selected genes, such as *SCGB1A1* and*IL24*, which were significantly regulated in the array analysis (in comparison with known type-2 cytokines *IL4* and *IL13*), were validated by real-time quantitative reverse transcription PCR (Figure 3A–H) and at protein level (Figure 3I–P). AIT-treated allergic patients showed significant differences in *SCGB1A1*expression levels compared with untreated patients suffering from rhinitis with asthma comorbidity. Up to sevenfold mRNA-expression and significant differences were found when comparing untreated (rhinitis: 0.24 ± 0.05; rhinitis+asthma: 0.42 ± 0.13) vs. AIT-treated rhinitis patients (7.64 ± 4.49; p = .041) or rhinitis patients with asthma comorbidity (1.36 ± 0.44; p = .048) in the pollen season (Figure 3A). For *IL24*, differences were shown in season for treated (1.16 ± 0.37) and untreated (7.27 ± 3.42; p = .03) rhinitis as well as for treated (0.41 ± 0.14) and untreated (11.31 ± 8.84; p = .026) rhinitis patients with asthma comorbidity (Figure 3B,F).

Protein levels for SCGB1A1 and IL-24 in- and off-season (Figure 3I,J,M,N) were validated using the MSD Mesoscale platform. Interestingly, SCGB1A1-levels in sputum supernatants were significantly lower in rhinitis (in and out of season) and rhinitis patients with asthma comorbidity (in season) as compared to healthy controls while IL-24 (as well as IL-4 and IL-13) levels were rather increased. Consistent with array and qPCR data, rhinitis patients receiving AIT treatment showed elevated protein levels of SCGB1A1 in sputum supernatant both in (7.24 ng/ml ± 0.46) and off-season (6.86 ng/ml ± 0.23) compared with untreated patients (in season: 2.38 ng/ml ± 0.60, p < .0001; off-season: 1.23 ng/ml ± 0.38, p < .0001; Figure 3I,M). The difference in asthma patients off-season is not visible on mRNA level of sputum cells (Figure 3E), and therefore, the protein level may represent SCGB1A1 (Figure 3M) originating from airway epithelial cells, which is excreted into airway lumen and collected during sputum induction. Similar changes were seen in treated (in season: 7.09 ng/ml ± 0.52; off-season: 7.22 ng/ml ± 0.31) and untreated rhinitis patients with asthma comorbidity (in season: 4.38 ng/ml ± 0.55, p = .002; off-season: 2.74 ng/ml ± 0.49, p < .0001). AIT-treated patients showed decreased levels of secreted IL-24 in sputum supernatants in (59.89 pg/ml ± 9.14) as well as out of season (71.07 pg/ml ± 11.43) compared with untreated patients (in season: 146.5 pg/ml ± 10.33, p < .0001; off-season: 133.1 pg/ml ± 12.98, p < .0001; Figure 3J,N). Similar changes became visible for AIT-treated (in season: 62.03 pg/ml ± 7.42; off-season: 98.95 pg/ml ± 12.49) and untreated rhinitis patients with asthma comorbidity (in season: 133.1 pg/ml ± 9.73, p < .0001; off-season: 206.6 pg/ml ± 22.03, p = .0011). SCGB1A1 protein levels were negatively strongly correlated with mRQLQ in season (r = −.795, p < .0001; Figure 3Q) and showed moderate negative correlation out of season (r = −.527, p < .0001; Figure 3R). Taken together, these data show that SCGB1A1 levels are inversely regulated by AIT compared with decreased levels of pro-inflammatory type-2 cytokines IL-24, IL-4, and IL-13.

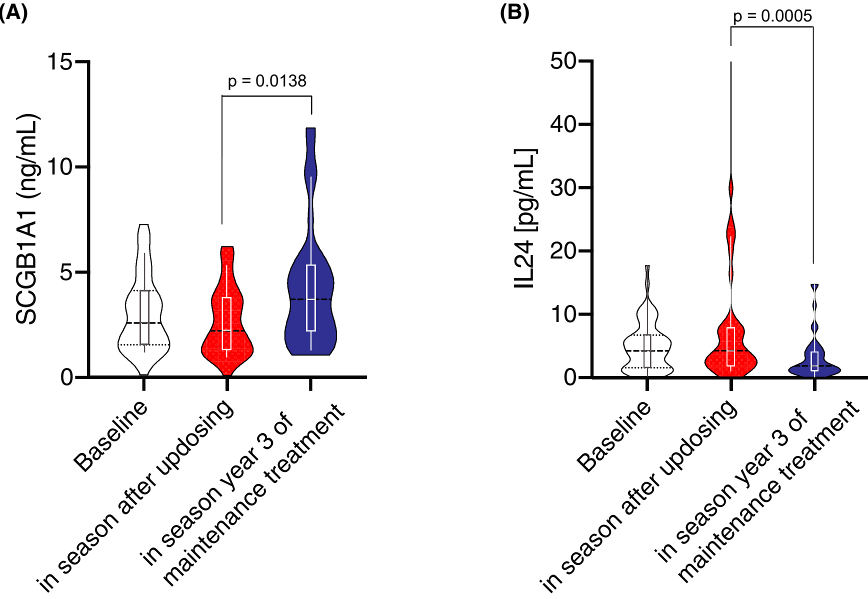
Unexpectedly, increased SCGB1A1 levels were not only observed in sputum, but also in serum in and out of grass pollen season (Figure S3A,B), however with lower differences in magnitudes between groups. In rhinitis patients receiving AIT treatment, elevated serum SCGB1A1 levels were observed in (2.25 ng/ml ± 0.09) as well as off-season (2.79 ng/ml ± 0.02) compared with untreated patients (in season: 1.91 ng/ml ± 0.01, p = .035; off-season: 2.00 ng/ml ± 0.01, p = .006). In rhinitis patients with asthma comorbidity, similar levels for SCGB1A1 in serum were detected for treated (in season: 2.55 ng/ml ± 0.01; off-season: 2.82 ng/ml ± 0.01) and untreated patients (in season: 1.67 ng/ml ± 0.01, p = .002; off-season: 2.22 ng/ml ± 0.01, p < .0001; Figure S3).

3.4 | Secretion of SCGB1A1 and IL-24 during the course of AIT detected in nasal lining fluids

In order to cross-validate SCGB1A1 and its regulation during AIT and to assess another, yet easy accessible airway sample, we measured SCGB1A1 (and IL-24) using non-invasive nasal sampling at baseline of grass specific immunotherapy in a cohort of 57 patients, after preseasonal up-dosing period[20] and after 3 years of AIT.[4] SCGB1A1 remained unchanged immediately after up dosing (2.68 ng/ml ± 0.26) compared with baseline (3.02 ng/ml ± 0.24; Figure 4A). However, after completion of 3 years AIT SCGB1A1 levels were significantly increased compared with the time point after up dosing (4.27 ng/ml ± 0.45, p = .0138), even though changes were not significant to off-season baseline. The epithelial type-2 (E2-) cytokine IL-24 was significantly reduced following 3 years of AIT (3.23 pg/ml ± 0.56) compared with the time point after up dosing (9.32 pg/ml ± 2.78, p = .0005; Figure 4B). IL-24 and SCGB1A1 showed a significant and reverse correlation (r = −.4818, p = .0016; Figure 4C).

3.5 | Function of SCGB1A1 as luminal immunotherapy target in vitro

To investigate the possible regulatory role of SCGB1A1 in AIT on respiratory tract epithelial function, recombinant human protein SCGB1A1 was added to primary epithelial cells in culture. Since we intended to demonstrate regulatory/suppressive effects of SCGB1A1, we needed to activate airway epithelial cells. While no intrinsic mechanisms of epithelial activation were so far described for grass pollen extracts, we decided to use house dust mite (HDM) extract, where intrinsic mechanisms of epithelial activation were previously described.[21,22] The concentration applied in the in vitro cultures was titrated ahead of the experiments in cell cultures (qPCR of *CCL26* and *IL8*; Figure S4) and confirmed the concentration range that is observed in nasal secretions. Both, resting and HDM-activated primary human bronchial epithelial cells (NHBE) were exposed to



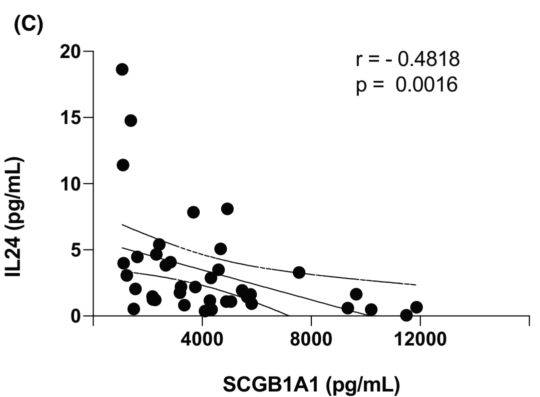


FIGURE 4 Secreted proteins regulated in nasal lining fluids of allergic rhinitis patients throughout the course of grass-pollen AIT. Levels for SCGB1A1 (A) and IL-24 (B) protein were measured at three time points in an independent validation cohort at baseline, in season after up dosing as well as after 3 years of completed grass pollen-specific immunotherapy. Reverse correlation between secreted levels of SCGB1A1 and IL24 in nasal lining fluid (C)

SCGB1A1. For this experiment, we used unprimed NHBEs and also applied natural HDM-extracts as they are used in the clinical routine for diagnosis and treatment of patients. Of note, HDM-extracts naturally contain lipopolysaccharide (LPS; controlled amounts); however, at these concentrations NHBEs did not exhibit an LPS-typical footprint (data not shown). No cytotoxic effects were observed and a live metabolic assay showed no effect on altered oxygen consumption rates or extracellular acidification rates (data not shown). In a whole transcriptome analysis, 75 genes in resting NHBEs were upregulated, while 539 genes were downregulated after addition of SCGB1A1 (Figure 5). HDM-primed NHBEs revealed 91 upregulated genes, while 650 genes were downregulated. Subsequent pathway analysis revealed no significant footprints that could uncover the signaling mechanism of SCGB1A1-mediated cellular suppression. Thus, both resting and HDM-activated NHBEs responded to SCGB1A1 with a generalized downregulation of gene expression. We analyzed the top 50 downregulated genes for SCGB1A1-stimulated and HDM-activated, SCGB1A1-stimulated NHBEs, revealing suppression of pro-inflammatory mediators and pathways, such as*CARD9* and *MEG3* respectively (Table S13 and S14). Interestingly, between only SCGB1A1-stimulation and HDM-activation followed by SCGB1A1-stimulation, 24 genes including *mucin-3a* were found to be downregulated thus independently of the activation status (Figure 5).

The effect of SCGB1A1 on immunoregulatory epithelial cytokine mRNA expression such as *IL8* and *IL24* revealed decreased levels, however did not reach statistical significance in the microarray experiment. In BEAS2B cells, SCGB1A1 displayed a significant difference for both genes, IL8 (HDM fold change: 1064.0-fold; HDM+SCGB1A1: 324.8-fold; $p = .0142$) and IL24 (HDM fold change: 58.8-fold; HDM+SCGB1A1: 22.67-fold; $p = .0016$; Figure S4).

Future studies need to further characterize epithelial genes and pathways to facilitate the molecular understanding of the SCGB1A1 induced silencing effect.

4 | DISCUSSION

This study reveals the effect of AIT on lower airway cells and identifies SCGB1A1 as mediator of AIT-induced anti-inflammatory mechanisms in the epithelium. This finding is novel in a sense that

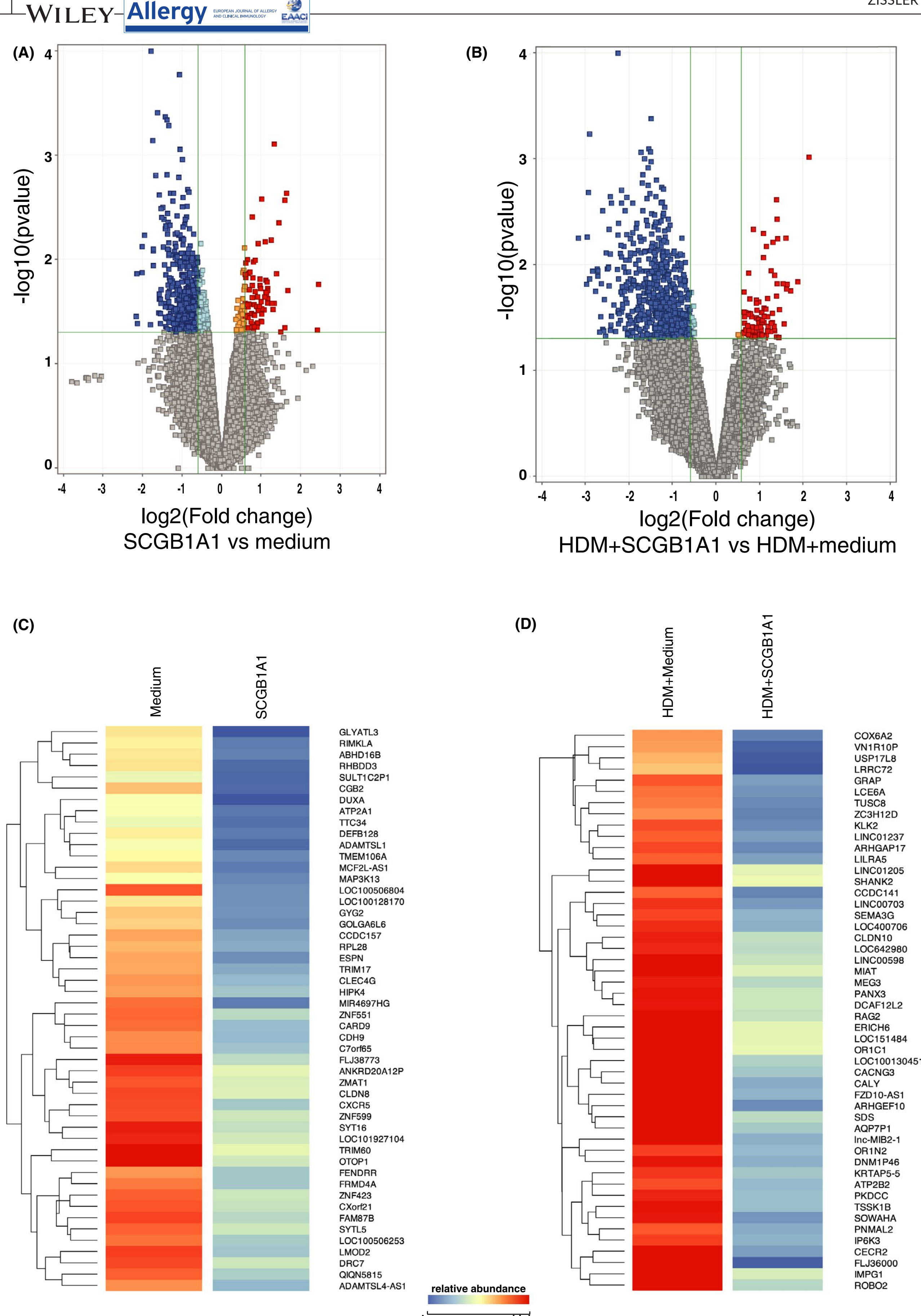

(A)
-log10(pvalue)
log2(Fold change)
SCGB1A1 vs medium
(B)
-log10(pvalue)
log2(Fold change)
HDM+SCGB1A1 vs HDM+medium
(C)
Medium
SCGB1A1
GLYATL3
RIMKLA
ABHD16B
RHBDD3
SULT1C2P1
CGB2
DUXA
ATP2A1
TTC34
DEFB128
ADAMTSL1
TMEM106A
MCF2L-AS1
MAP3K13
LOC100506804
LOC100128170
GYG2
GOLGA6L6
CCDC157
RPL28
ESPN
TRIM17
CLEC4G
HIPK4
MIR4697HG
ZNF551
CARD9
CDH9
C7orf65
FLJ38773
ANKRD20A12P
ZMAT1
CLDN8
CXCR5
ZNF599
SYT16
LOC101927104
TRIM60
OTOP1
FENDRR
FRMD4A
ZNF423
CXorf21
FAM87B
SYTL5
LOC100506253
LMOD2
DRC7
QIQN5815
ADAMTSL4-AS1
relative abundance
low
high
(D)
HDM+Medium
HDM+SCGB1A1
COX6A2
VN1R10P
USP17L8
LRRC72
GRAP
LCE6A
TUSC8
ZC3H12D
KLK2
LINC01237
ARHGAP17
LILRA5
LINC01205
SHANK2
CCDC141
LINC00703
SEMA3G
LOC400706
CLDN10
LOC642980
LINC00598
MIAT
MEG3
PANX3
DCAF12L2
RAG2
ERICH6
LOC151484
OR1C1
LOC100130451
CACNG3
CALY
FZD10-AS1
ARHGEF10
SDS
AQP7P1
lnc-MIB2-1
OR1N2
DNM1P46
KRTAP5-5
ATP2B2
PKDCC
TSSK1B
SOWAHA
PNMAL2
IP6K3
CECR2
FLJ36000
IMPG1
ROBO2

FIGURE 5 Effect of SCGB1A1 on airway epithelial cells. Normal human bronchial epithelial cells (NHBEs) were cultured in medium only, in the presence of SCGB1A1 (20 ng/ml) or house dust mite extract (HDM;40 μg/ml), and SCGB1A1 together with HDM extract for 6 h to study RNA whole transcriptome by array technology. (A) Comparison of SCGB1A1-related gene expression changes to medium only, revealing 75 upregulated and 539 downregulated genes. The analysis is based on cultures of six genetically independent NHBEs of healthy donors. (B) Comparison of treatment with SCGB1A1+HDM and HDM only to identify SCGB1A1-related gene expression changes, revealing 91 upregulated and 650 downregulated genes. Top 50 downregulated genes for SCGB1A1-stimulated (C) and HDM-activated, SCGB1A1-stimulated NHBEs (D) were extracted. The analysis is based on cultures of six genetically independent NHBEs of healthy donors.

anti-inflammatory mechanisms were so far mainly attributed to regulatory T- and B-cells[3,4] but not to alveolar macrophages.

4.1 | Type-2local responses suppressed by AIT

Substantial knowledge exists about AIT mechanisms in systemic lymphocyte compartments. Our current data reveal yet unknown mechanisms namely those represented by sputum cells generated from the airways, where exposure to allergen, inflammation, and symptoms locally occur. Consistent with previous studies, we observe suppressed *CXCL8* (*IL8*) expression, but also show the reduction of type-2-related mediators *IL24* and *CCL26*.[10,23]We have previously shown that both cytokines are induced by IL-4 or IL-13.[10] Therefore, the seasonal unresponsiveness of CCL26 and IL24 in the course of AIT could be the consequence of diminished IL4 and IL13 production. Interestingly, this effect was also observed not only in sputum samples of rhinitis patients with asthma comorbidity but also in patients limited to upper airway symptoms only. In fact, all analytes were principally indistinguishable between these two patients groups with the exception of SCGB1A1 that is generally higher in rhinitis patients compared with rhinitis patients with asthma comorbidity. This finding underlines the validity of the "*united airway*" concept, which claims that epithelial activation of the lower airways is mirrored by the upper airways.[24,25] Consequently, the inflammatory mediators in the lower airways of rhinitis patients observed in this study could therefore indicate for a form of "*latent*" asthma even in rhinitis patients, which requires further investigation in longitudinal cohorts.

4.2 | AIT-induced local changes in off-season

Of particular interest in understanding the local mechanisms of AIT in the luminal airways are genes that are induced independently of the allergen season, because they may indicate potential long-acting mechanisms independent of the seasonal influence. Interleukin-7 is an AIT-induced cytokine, as previously described as being important for the survival of tissue-resident B- and memory T-cells[26] and thereby also modulate the balance of regulatory T cells.[27] Two AIT-induced chemokines, CCL-5 (RANTES), and CCL-23 (MIP3), which are known to be involved in macrophage chemotaxis and were reported to be elevated in serum samples of patients receiving (insect venom) immunotherapy, were also detected in- and off-season.[28]CCL-5 is a major regulator of suppressive immune programs[29] and may contribute to antagonize pro-allergic type-2 immunity by recruiting Th1 cells as well as regulatory T cells.[30] The atypical chemokine receptor ACKR2,[30] the major CCL-5 scavenger receptor, is decreased through AIT and further increases the biological activity of CCL5. CCL23 was also described to enhance IL10 production in monocytes and T cells.[31]

4.3 | AIT-induced changes in tissue repair mechanisms

Largely unknown are mechanisms of immunotherapy that affect tissue repair and homeostasis. Among those, the epithelial differentiation cytokine WNT5B was induced by AIT in- and out of season and is known to be tightly regulated by the TGF-β signaling pathway.[32] Angioarrestin (ANGPTL1), also induced by AIT, can block the angiogenic cascade,[33] while AIT-induced FGFR3 (in contrast to other FGFRs) has been shown to negatively regulate epithelial cell proliferation and therefore has oncogenic capabilities.[34] These observations suggest that AIT promotes tissue homeostasis in combination with matrix remodeling processes.

4.4 | AIT-induced local expression in and off-season

The strongest AIT-mediated induction in and out of season was, however, observed for secretoglobin1A1 (SCGB1A1), a tetrameric glycoprotein of the secretoglobin family, with homologies toFel d1 and Fel d2. It has been previously described to be induced by glucocorticoids[35] and IFN-γ.[36] In fact, IFN-γ is induced by AIT, but becomes only visible in transcriptomes during the pollen season particularly in the subgroup of rhinitis patients with asthma comorbidity. Further, it has been shown that SCGB1A1 exerts immunosuppressive functions in the cyclooxygenase (COX)-2 pathway[37] and gene defects in the *SCGB1A1* gene are associated with susceptibility to asthma.[38] Also in SCGB1A1 knockout mice, anti-inflammatory and immunomodulatory functions of SCGB1A1 were observed, for example, these mice showed increased type-2 immune responses and exaggerated respiratory tract inflammation upon allergen challenge.[39] Further, SCGB1A1 knockout mice show increased airway infiltration by eosinophils upon *streptococcus pneumoniae* exposure.[40] In addition, SCGB1A1 has been shown to attenuate LPS- or IL-13-induced activation of airway epithelial cells.[41] Known sources of SCGB1A1 are airway and testicular club cells. Cross-talk between airway cells and macrophages in the conducting airways suggest that ANXA1 was involved in the immunoregulatory functions of SCGB1A1 via the regulation of the activity

of pro-inflammatory enzymes such as inducible nitric oxide synthase (iNOS), cPLA2, and potentially cyclooxygenase.[42] It is of particular interest that SCGB1A1 was found to be diminished in asthma patients in both BAL[43] and serum.[44] Serum levels of SCGB1A1 correlate with FEV1 and the Tiffeneau coefficient (FEV1/FVC).[44] Detection of SCGB1A1 in serum could originate from the lung or from SCGB1A1-producing lymphocytes, which were shown in this study. Due to well-controlled clinical symptoms, our patients showed no correlation between SCGB1A1 levels and lung function parameters. However, we were able to show a significant correlation to their symptom load assessed by mRQLQ in- and off-season. The mechanisms of AIT-induced SCGB1A1 reconstitution to levels of healthy homeostatic conditions are entirely unclear; however, it is known that IL4 and IL13 are downregulating SCGB1A1.[45] Therefore, it can be speculated that either the AIT-reduced expression of IL4 and IL13 or the increase of IFN-γ as natural antagonist of these type-2 cytokines could play an important role in underlying mechanisms of AIT.

4.5 | SCGB1A1 as local silencer of epithelial cells

In addition, our data demonstrate for the first time that SCGB1A1 gene expression of resting as well as HDM-activated primary respiratory epithelial cells. In this experiment, primary bronchial epithelial cells were activated with house dust mite (HDM)-extracts in order to use a physiological IgE-independent mechanism. We observed a decrease of *IL8* and *IL24*, which however did not reach statistical significance as using primary NHBEs; however, in BEAS2B cells a robust suppression was observed. SCGB1A1 downregulated expression of *CARD9*, an adaptor molecule in the NFκB pathway that can have pro- but also anti-inflammatory functions.[46,47] The SCGB1A1-mediated suppression of *CARD9* can suppress the NFκB and ERK pathways resulting in decreased levels of pro-inflammatory mediators, such as IL-6, IL-12, GM-CSF, TNF, and IL-1β upon activation, the latter of which also involves the RASGRF1–H-Ras pathway.[48] HDM-activation of epithelial cells resulted in downregulation of *MEG3*, a known target of *CARD9* downstream signaling,[49] which was described to be positively correlated with basal cell markers TP63, KRT5, KRT17, KRT14, and ITGB4.[50]Further, *MEG3* regulates basal epithelial cell identity[50] and inhibits *SOX2*,[51] which is related to *SCGB1A1* expression. In this study, transgenic deletion of *SOX2* in airway epithelial cells prevented SCGB1A1 expression, consistent with the requirement of SOX2 in differentiation of both club and ciliated cells.[51]SCGB1A1 is also known to bind and neutralize lipid mediators, which are also triggered by HDM-stimulation of NHBEs. As SCGB1A1 acted independently of activation, it appears unlikely that SCGB1A1 inhibits airway epithelial cells by neutralization of lipid mediators. Future studies need to clarify the role of SCGB1A1 in epithelial lipid turnover and signaling. Our findings on the role of SCGB1A1 in AIT-induced immune suppression are of particular interest, as SCGB1A1 is the first AIT-induced gene involved in epithelial biology which is not a typical immune regulator produced by infiltrating cells.[11] Overall, AIT-induced genes that do not underlie seasonal changes (*IL7*, *CCL5*, *CCL23*, *WNT5B*, and *SCGB1A1*) are associated with tolerogenic pathways that appear to cumulate in the lumen tissue. Future studies are needed to clarify, whether these changes persist after discontinuation of AIT as it is known for T cell–derived factors.

5 | CONCLUSION

In conclusion, the current study demonstrates that AIT is reducing features of local airway inflammation including a reduction in generic inflammatory genes such as *IL8*, but also a reduction of epithelial type-2 inflammation such as IL-24 and CCL-26. Secretoglobin1A1 is identified as a local mediator induced in the response to AIT. In addition, secretoglobin1A1 is shown to be a factor that can restore intact lung tissue homeostasis and may thus be of relevance for future therapeutic approaches.

CONFLICT OF INTEREST

UMZ received payment for manuscripts from Deutsches Aerzteblatt and funds for travel from the European Academy of Allergy and Clinical Immunology (EAACI) and Collegium InternationaleAllergologicum (CIA). CSW received support for research projects from PLS Design, LETI, Zeller AG, and Allergopharma and accepted honoraria for consultancy and seminars from LETI and Allergopharma. He also received travel support from EAACI. AMC has consultancy arrangements through Technical University Munich with Allergopharma, ALK-Abello, AstraZeneca, GSK, HAL Allergy, Immunetek, Lofarma, Regeneron, Sanofi Genzyme; has conducted clinical studies and received research grants through Technical University Munich from Allergopharma, Novartis, the German Federal Environmental Agency, Bencard/Allergen Therapeutics, ASIT Biotech, GSK, Roche, and Zeller AG; has received payment for lectures from Allergopharma, ALK-Abello, AstraZeneca, GlaxoSmithKline, Leti, Bencard/AllergenTherapeutics, and SanofiGenzyme; has received payment for manuscript preparation from Bayerisches Aerzteblatt; and has received travel support from the European Academy of Allergyand Clinical Immunology (EAACI) and DGAKI. The rest of the authors declare that they have no relevant conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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