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# Immunologische Mechanismen an der epithelialen Barriere bei allergischen Entzündungen

## Habilitationsschrift

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**Meiner Familie**

Theorien sind nie mehr als kühne Vermutungen und Hypothesen, von uns gemachte Netze, mit denen wir die wirkliche Welt einzufangen versuchen.

*Sir Karl Raimond Popper*

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# 1. Zusammenfassung

Bei allergischen Entzündungen spielen Th2-assoziierte Immunantworten und deren Schlüsselmediatoren wie Interleukin-4 und IL-13 eine zentrale Rolle. Unter dem Einfluss dieser Typ 2-spezifischen Immunmediatoren reagieren die Epithelzellen der unteren und oberen Atemwege beispielsweise über die Regulation von Transkriptionsfaktoren und die Sekretion von weiteren Mediatoren, die zusätzlich in das Entzündungsgeschehen hineinwirken.
Das Ziel der vorliegenden Habilitation ist die Aufklärung der Mechanismen einer gestörten, mukosalen Immunität im Atemwegslumen und am Atemwegsepithel bei allergischen Erkrankungen. Im Besonderen sollen assoziierte Biomarker dieser aberranten Immunität erfasst werden. Im Weiteren sollen anti-inflammatorische Mechanismen, die durch die kausale Therapieoption einer Allergen-spezifischen Immuntherapie (AIT) induziert werden und der Inflammation entgegenwirken beleuchtet werden.
Der erste Teil dieser kumulativen Habilitationsschrift zeigt *in vitro* krankheitsspezifische Muster von primären Epithelzellen und *ex vivo* Immunzellen im Atemwegslumen bei Patienten mit allergischer Rhinitis und allergischem Asthma. Der zweite Teil dieser Habilitationsschrift fokussiert sich auf die therapeutische Interaktion und zeigt die therapeutische Modifikation der aberranten epithelialen Immunologie durch eine AIT.
In diesem Zusammenhang haben wir zunächst *in vitro* die Wirkung der prototypischen Mediatoren der Th1/Th2-Effektorzellen auf bronchiale Epithelzellen untersucht. Hier konnten wir erstmals ein IL-4-abhängiges, polarisiertes Transkriptom der Epithelzellen und die konsekutive Sekretion von Mediatoren wie IL-24 identifizieren. Zugrunde liegt eine epitheliale Aktivierung von Transkriptionsfaktoren wie GATA3, die üblicherweise bei Typ 2-Reaktionen in Immunzellen gefunden werden. Dies deutet auf eine verstärkende Rolle der Atemwegsepithelzellen im Entzündungsgeschehen hin. Eine pathogene Th2-assoziierte Signatur von distinkten sekretierten Proteinen konnten wir bei Asthmatikern in einer *ex vivo* Studie in nasalen Sekreten und Überständen des induzierten Sputums validieren und zeigen, dass die oberen Atemwege in diesem Falle die Pathophysiologie in den unteren Atemwege spiegeln. Ein Entzündungskontinuum, wie es nach der „United Airways Hypothese“ beispielsweise durch allergische Mechanismen abgebildet wird, kann dabei verschiedene Endotypen aufweisen und für therapeutisch relevante Phänotypen wichtig sein. Wir konnten lokale Analyte aus Sekreten der oberen und unteren Atemwege für das Vorliegen einer lokalen Th2-Antwort identifizieren, wobei wir zeigen konnten, dass IL-24 ein möglicher Marker für ein zugrundeliegendes Asthma sein kann. Dazu konnten wir in einer weiteren Arbeit *ex vivo* zeigen, dass in der allergischen Rhinitis und Asthma sowohl lokal als auch peripher durch eine AIT-Behandlung die Treg-Population numerisch und funktionell wieder induziert wird, während die Frequenzen an Th2- und Th9-Zellen sowie TGF-β-Spiegel während der Therapie abgesenkt wurden. Dieses Ergebnis weist darauf hin, dass eine AIT einen speziellen Zustand der Immunregulation induziert und die Wiederherstellung der natürlichen Allergentoleranz nicht bereits im ersten Jahr der Therapie erfolgt.
In einer Studie zu krankheits- und therapiebedingten Veränderungen auf Ebene kleiner, nicht-kodierender microRNAs (miRNAs) konnten wir beobachten, dass auch auf dieser Ebene eine aberrante Regulation im Kontext eines Th2-geprägten Zytokinmilieus zu finden ist, welches sich ebenfalls in veränderten ILC2-Frequenzen widerspiegelt. Wir konnten nachweisen, dass miR-3935 und ihre Ziel-mRNA, der EP3 Rezeptor gegenläufig reguliert sind. Im Kontext der Allergenimmuntherapie bewerten wir die erhöhte Expression der miR-3935 als Gewebe-protektiv, die reduzierte Expression des Rezeptors als Surrogat einer Besserung des Asthmas.

Mit dieser Studie konnten wir dokumentieren, dass die $PGE_2$-Spiegel im Sputum mit pro-entzündlichen zellulären und klinischen Parametern korrelieren und bei AIT-behandelten Patienten niedriger sind.
Eine epitheliale Typ 2-Signatur zeigen wir ebenfalls in einer *ex vivo* Studie, worin wir die Transkriptom-weite Untersuchung dieser Effekte gezielt im induzierten Sputum und den Überständen unter Berücksichtigung der Immunzell-Entitäten untersuchten. Während unter AIT proinflammatorische Mediatoren reduziert werden, zeigt sich im Atemwegsepithel ein Anstieg der Expression potentiell regulatorischer oder anti-inflammatorischer Mediatoren. Wir konnten Sekretoglobin1A1 als potentiell anti-inflammatorischen Indikator identifizieren. Dieser wurde durch die Therapie induziert, während die Expression pro-allergischer Th2-Mediatoren wie beispielsweise IL-24 reduziert wurde. Auch weitere prototypische Typ 2-assoziierte Mediatoren einer aberranten Immunität und ihre zellulären Ursprünge konnten wir im induzierten Sputum als Therapie-sensibel nachweisen.
Die von uns beschriebenen, pro-allergischen Biomarker haben wir in einer Langzeit-Beobachtungsstudie zur AIT über den Verlauf von drei Jahren nachverfolgt. Diese Untersuchungen wiesen Immunzellen und Mediatoren nach, die mit Typ 2-Immunreaktionen assoziiert sind, ebenso wie anti-entzündlich wirkende regulatorische T- und B-Zellpopulationen und deren Mediatoren. Sowohl während der Aufdosierung als auch im Verlauf der Erhaltungstherapie der subkutanen Gräser-AIT zeigen sich Veränderungen der prototypischen Th2-assoziierten Biomarker-Muster bis hin zur Regulierung der fehlgeleiteten Immunität und einer Reinduktion der tolerogenen Mediatoren und Zellpopulationen verfolgen.
Zusammengefasst zeigt die vorliegende Habilitationsschrift somit erstmals zugrundeliegendes epitheliales Transkriptionsfaktoren-Netzwerk in der fehlgeleiteten mukosalen Immunität der Atemwege, die über eine AIT als kausale Therapieoption in großen Teilen rückführbar ist und somit über die AIT therapeutisch greifbar ist. Für die Zukunft wäre zu klären, ob die mukosale Barrierefunktion, welche über die kausale Therapieoption der AIT rückführbar ist, auch im gleichen Maße über Antikörper-basierte Therapieoptionen möglich ist.

# 1. Summary

In allergic inflammation, Th2-associated immune responses and their key mediators such as interleukin-4 and IL-13 play a central role. Under the influence of these type 2-specific immune mediators, epithelial cells of the upper and lower airways respond, for example, by regulating transcription factors and secreting other mediators that also affect the inflammatory process.

The aim of this cumulative habilitation thesis is to elucidate the mechanisms of impaired mucosal immunity in type 2 driven diseases of the airway lumen and epithelium. In particular, the identification of biomarkers associated with this aberrant immunity was aim of the studies. Furthermore, anti-inflammatory mechanisms induced by the causal therapeutic option of allergen-specific immunotherapy (AIT) were studied.

The first part of this cumulative thesis shows *in vitro* disease-specific patterns of primary epithelial cells and *ex vivo* immune cells in the airway lumen of patients with allergic rhinitis and allergic asthma. The second part of this thesis focuses on the therapeutic interaction and shows the modification of aberrant epithelial immunology by an AIT.

In this context, we first investigated the effect of prototypical mediators of Th1/Th2 effector cells on bronchial epithelial cells *in vitro*. For the first time, we identified an IL-4-dependent polarized transcriptome of epithelial cells and the subsequent secretion of mediators such as IL-24. This is based on an epithelial activation of transcription factors such as GATA3, which are normally found in type 2 responses in immune cells. This suggests an amplifying role of airway epithelial cells in the inflammatory process. We were able to validate a pathogenic Th2-associated signature of different secreted proteins in asthmatics in an *ex vivo* study in nasal secretions and supernatants of induced sputum, showing that the upper airways in this case mirror the pathophysiology in the lower airways. An inflammation continuum, as mapped by allergic mechanisms according to the "United Airways Hypothesis", may show different endotypes and be important for therapeutically relevant phenotypes. We were able to identify local analytes from upper and lower airway secretions for the presence of a local Th2 response and showed that IL-24 may be a possible marker for underlying asthma. To this end, in another paper, we demonstrated *ex vivo* that in allergic rhinitis and asthma, both local and peripheral, AIT treatment numerically and functionally re-induces the Treg population, while the frequencies of Th2 and Th9 cells as well as TGF-β levels were decreased during therapy. This result suggests that AIT induces a specific state of immunosuppression and that restoration of natural allergic tolerance does not occur during the first year of therapy.

In a study of disease- and therapy-related changes at the level of small non-coding microRNAs (miRNAs), we observed that aberrant regulation also occurs at this level in the context of a Th2-dominated cytokine milieu, which is also reflected in altered ILC2 frequencies. We demonstrated that miR-3935 and its target mRNA, the EP3 receptor, are oppositely regulated. In the context of allergen immunotherapy, we evaluate increased expression of miR-3935 as tissue protective and decreased expression of the receptor as a surrogate for improvement of asthma. In this study, we demonstrated

that sputum $PGE_2$ levels correlate with pro-inflammatory cellular and clinical parameters and are lower in AIT-treated patients.

We also demonstrate the epithelial type 2 signature in an *ex vivo* study where we performed a transcriptome-wide investigation of these effects specifically in induced sputum and supernatants, considering immune cell entities. While proinflammatory mediators are reduced under AIT, an increase in the expression of potentially regulatory or anti-inflammatory mediators is observed in the airway epithelium. We identified secretoglobin1A1 as a potential anti-inflammatory indicator. This was induced by therapy, while the expression of pro-allergic Th2 mediators such as IL-24 was reduced. We were also able to show that other prototypical type 2 associated mediators of aberrant immunity and their cellular origin are sensitive to therapy in the induced sputum.

We followed the pro-allergic biomarkers we described in a long-term observational study of AIT over three years. These studies detected immune cells and mediators associated with type 2 immune responses, as well as anti-inflammatory regulatory T and B cell populations and their mediators. Changes in prototypical Th2-associated biomarker patterns, including regulation of misdirected immunity and reinduction of tolerogenic mediators and cell populations, were observed during both up-titration and maintenance treatment with subcutaneous grass AIT.

In summary, this thesis shows for the first time the underlying epithelial transcription factor networks in the aberrant mucosal immunity of the respiratory tract, which can largely be traced back to AIT as a causal therapy option. For the future, it remains to be clarified whether the mucosal barrier function that can be targeted by AIT as a causal therapeutic option can also be targeted by antibody-based therapeutic options to the same extent.

# 2. Einleitung

## 2.1 Mechanismen bei Th2-asssoziierten Atemwegserkrankungen

### 2.1.1 Allergische Atemwegserkrankungen

Aufgrund der funktionellen Beziehungen zwischen Nase und Bronchien kann die Allergie der Atemwege als eine Störung des gesamten Respirationstrakts betrachtet werden, die sich klinisch als Rhinitis und/oder Asthma manifestieren kann [1].

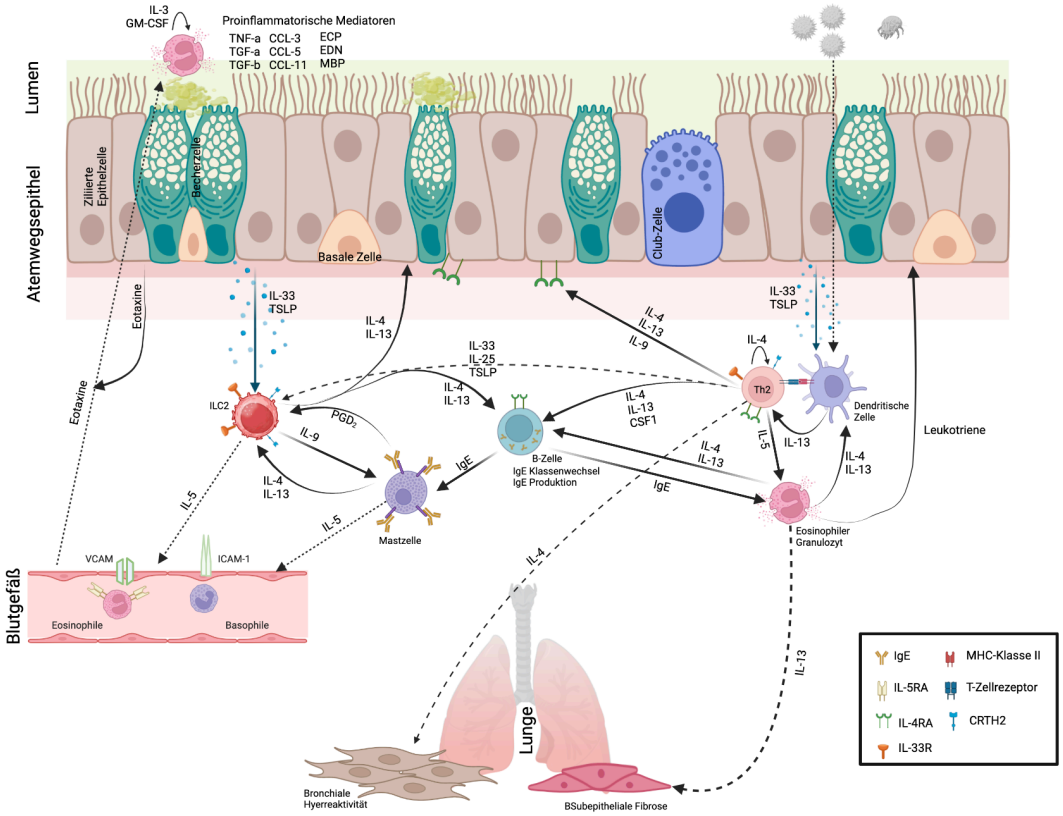


**Abbildung 1. Allergische Reaktionen am Atemwegsepithel.**

Allergische Immunreaktionen werden durch die Aufnahme und Verarbeitung von Allergenen durch dendritische Zellen, die professionellen antigenpräsentierenden Zellen, eingeleitet. Allergenpeptide werden naiven CD4+ T-Zellen präsentiert, die sich zu Th2-Zellen differenzieren. Th2-Zellen produzieren die Th2-Zytokine IL-4, IL-5, IL-9 und IL-13. B-Zellen sekretieren daraufhin IgE, dies bindet an spezifische Fc $\varepsilon$ -Rezeptoren auf Basophilen und Eosinophilen, den Effektorzellen der allergischen Entzündung. Bei wiederholtem Kontakt mit demselben Allergen führt die Degranulation dieser Effektorzellen zur Freisetzung und Produktion von Histamin und Leukotrienen. Neben anderen Funktionen bei allergischen Entzündungen wirkt IL-4 autokrin auf die Induktion von Th2-Zellen. IL-5 trägt zur Aktivierung, Rekrutierung und zum Überleben von Eosinophilen bei. IL-9 induziert eine erhöhte Schleimproduktion, während IL-13 und eosinophile Produkte Epithelschäden und eine undichte Barriere hervorrufen können. ILC2 trägt durch die Produktion von Zytokinen des Th2-Typs zur allergischen Entzündung bei. Die vom Epithel produzierten Zytokine IL-25, IL-33 und TSLP können auch von Th2-Zellen produziert werden und besitzen die Fähigkeit, ILC2 zu aktivieren. IL-25 induziert die DC-Aktivierung. Eosinophile produzieren beispielsweise Zytokine, die auf die Eosinophilen selbst wirken können.

Ein gemeinsamer Entzündungsprozess, der sich in den Atemwegen entwickelt, erklärt einige der komplexen Wechselwirkungen zwischen verschiedenen klinischen Erkrankungen wie Sinusitis, Rhinitis, Asthma, bronchialer Hyperreagibilität und Virusinfektionen [1]. Der Entzündungsprozess ist für die allergische Reaktion von zentraler Bedeutung, wie mehrere experimentelle Modelle, einschließlich nasaler und bronchialer Provokation, zeigen [2]. In induziertem Sputum zeigten Patienten mit Rhinitis während der Gräserpollen-Saison eine erhöhte Anzahl von Eosinophilen [3]. Die bronchiale Entzündungsreaktion nach Allergen-spezifischer Provokation mit Zellmigration und Basalmembranverdickung ist in Patienten identisch, die entweder nur an Asthma oder nur an Rhinitis leiden, unabhängig von den von der Krankheit betroffenen Atemwegen [4], was eine gemeinsame Entzündungsreaktion bei atopischen Patienten nahelegt.

Die allergische Rhinitis, eine IgE-vermittelte Entzündung des Nasen-Rachen-Raums als Reaktion auf eingeatmete Allergene [5, 6], ist eine weitverbreitete chronische Atemwegserkrankung bei ca. 30% der Erwachsenen [7] und ca. 10% aller Kinder mit altersabhängig steigender Tendenz [8, 9]. Eine Reaktionsabfolge einschließlich Früh- und Spätphase-Reaktionen kann durch eine wiederholte Allergenexposition ausgelöst werden, was zu den Leitsymptomen der allergischen Rhinitis wie Juckreiz, Niesen, Rhinorrhoe und einer Obstruktion der oberen Atemwege führt. IgE ist das Schlüsselmolekül für Überempfindlichkeitsreaktionen des Typs 1, welcher der allergischen Rhinitis zugrunde liegt [10]. Über Zellkontakt mit Th2-Zellen (Abbildung 1) und deren assoziierten Interleukine IL-4 und IL-13 , nach Aktivierung der Th2 Zellen produziert, wird ein Isotypenwechsel von IgM zu IgE in naiven B-Zellen induziert [11]. Antigenpräsentierende Zellen wie Dendritischen Zellen (DCs) in der Mukosa nehmen Allergenpeptide auf, prozessieren sie und präsentieren sie auf dem MHC-Klasse-II-Molekül [12]. Der Antigenkomplex und das MHC-Klasse-II-Molekül dienen als Liganden für T-Zell-Rezeptoren auf naiven $CD4^+$ T-Zellen, was zu einer Differenzierung naiver $CD4^+$ T-Zellen in allergenspezifische Th2-Zellen führt [12]. Die Quervernetzung des FcεR auf Mastzellen führt zur Freisetzung von allergischen Mediatoren [13], bestehend aus Histamin, Proteasen und Lipidmediatoren wie Leukotrien C4 ($LTC_4$) und Prostaglandin D2 ($PGD_2$), die Bronchokonstriktion, Entzündung und am Darm auch intestinale Hypermotilität verursachen [14]. Histamin ist dabei ein zentraler Mediator, da es die H1 Rezeptoren an den sensorischen Nervenenden und Niesen, Juckreiz und sekretorische Reflexe auslöst, häufige Symptome bei einer allergischen Rhinitis [15]. Weiter interagiert es auch mit H1 und H2-Rezeptoren an den Blutgefäßen der Schleimhäute, was zu nasaler Kongestion und Plasmaleckage führt [16]. Zwei bis sechs Stunden nach der Allergenexposition beginnt die Spätphase der allergischen Reaktion [17]. In dieser Phase

kommt es zu einer Entzündung der Nasenschleimhäute mit Akkumulation und Aktivierung einer Vielzahl von Entzündungszellen Zellen wie T-Zellen, Eosinophilen, Basophilen, Neutrophilen und Monozyten in der Nasenschleimhaut [18]. Die Rekrutierung dieser Entzündungszellen wird durch ein Zusammenspiel verschiedener Zytokine wie IL-4 und TNF vermittelt, welche die Expression von Adhäsionsmolekülen wie VCAM-1 auf Endothelzellen regulieren, was die Infiltration von Entzündungszellen erleichtert [19]. Die Aktivierung von Atemwegsepithelzellen induziert zusätzlich die Freisetzung von Chemokinen wie CCL-11 (Eotaxin-1) [20], CCL-5 (RANTES) [21] und CCL-17 (TARC) [22], welche zudem die Infiltration von Zellen aus dem peripheren Blut erleichtern.

Das allergische Asthma ist eine heterogene Erkrankung, typischerweise einhergehend mit chronischer Atemwegsentzündung, die neben respiratorischen Symptomen wie Pfeifen, Kurzatmigkeit und Hustenreiz durch bronchiale Hyperreagibilität und wiederkehrende, reversible Obstruktion der Atemwege gekennzeichnet ist [23]. Eine übermäßige Schleimproduktion in Zusammenhang mit einem Typ 2-Mikromilieu trägt zusätzlich zur Obstruktion der Atemwege bei [24]. Neben erhöhten IgE-Spiegeln ist bei allergischen Erkrankungen auch die Chronizität der Entzündungsreaktion elementar, die durch Th2-Schlüsselzytokine IL-4, IL-5 und IL-13 verursacht wird, wozu typischerweise eine eosinophile Entzündung nachgewiesen werden kann [25]. Die Th2-Schlüsselzytokine rufen wiederum Veränderungen in den strukturellen Komponenten der Atemwegswand wie glatten Muskelzellen und Fibroblasten hervor, außerdem kommt es zu einer Becherzellhyperplasie und, als direkte Folge, zu einer Schleimhypersekretion bei allergischem Asthma [26]. Es gibt mehrere Asthma-Phänotypen, welche wahrscheinlich mit unterschiedlichen Krankheitsmechanismen in Zusammenhang stehen [27, 28]. Dazu gehören allergisches, eosinophiles und neutrophiles Asthma [28]. Im Kindesalter findet sich überwiegend eine zugrundeliegende Th2-Entzündung mit allergischer Sensibilisierung und assoziierter Eosinophilie, während simultan erhöhte Neutrophilenzahlen mit einem erhöhten Schweregrad bei Asthma im Erwachsenenalter assoziiert ist [29]. Zusätzlich zu diesen allergischen Auslösern gibt es jedoch auch Risikofaktoren wie familiäre Vorbelastung [30], Atemwegsinfektionen mit Rhinoviren [31], Respiratorischen Synzytialviren oder Parainfluenzaviren [32], Rauch- oder Schadstoff-Exposition und Adipositas [33], welche im Zusammenspiel mit der genetischen Prädisposition zur Entwicklung von allergischem Asthma in der Kindheit beitragen [34]. So ist die Anfälligkeit für Asthma mit einer starken genetischen Komponente über Gene epithelialen Ursprungs wie *IL1RL1*, *IL33*, *TSLP*, *MUC5AC, KIF3A, EFHC1 und Gasdermin B (GSDMB)* assoziiert [35]. Aber auch Genregionen wie Chromosom 17q21 als der am stärksten assoziierte Asthma-Locus beim

Asthmabeginn in der Kindereit spielen eine wichtige Rolle [36]. Durch die systematische Kartierung der Auswirkungen von Einzelnukleotid-Polymorphismen (SNPs) auf das Auftreten von Asthma im Kindesalter durch genomweite Assoziation wurden zwei Gene, *ORMDL3* [36] und *GSDMB* [37] in den Fokus gerückt.

### 2.1.2 Th2-vermittelte Immunantworten

Eine Entzündung ist die physiologische Reaktion des Gewebes auf eine Schädigung sowie den Kontakt mit Krankheitserregern [38]. Bakterien oder Viren verursachen in der Regel eine "Typ-1-Entzündung", die durch Rekrutierung und Aktivierung von Neutrophilen, einer assoziierten Produktion von Interferonen sowie durch die Differenzierung von naiven T-Zellen in den "Th1"-Phänotyp gekennzeichnet ist [39]. Im Gegensatz dazu lösen multizelluläre Parasiten, Allergene sowie bestimmte Toxine eine „Typ-2-Entzündung“ aus, die durch eosinophile Granulozyten sowie eine erhöhte Produktion von Interleukin-4, -5 und -13 und erniedrigte Sekretion von IFN-γ charakterisiert werden kann [40]. Diese Art der Entzündung wird als "Typ 2-Entzündung" bezeichnet (Abbildung 1) und dominiert bei allergischen Erkrankungen wie allergischer Rhinitis, atopischer Dermatitis und allergischem Asthma [41]. Typ 2-Entzündungen werden ausgelöst, wenn Epithelzellen oder dendritische Zellen (DCs) auf Allergene sowie schädigungsassoziierte Moleküle mit der Produktion von Alarminen wie IL-25, IL-33 und TSLP oder Lipid-Mediatoren wie z.B. Leukotrienen reagieren [42]. Diese Mediatoren fördern die Differenzierung von angeborenen lymphoiden Zellen des Typs 2 (ILC2s) und deren Produktion von Mediatoren des Typs 2 [43]. Durch die Aufnahme von Allergenen durch DCs und die anschließende Übertragung endozytierter Antigene in Endosomen wird eine Immunreaktion eingeleitet, um die MHC-II-Antigenpräsentation sicherzustellen [44]. Die Folge ist eine Produktion von Typ 2-Zytokinen wie IL-4[45] und IL-13[46] durch ILC2s und Th2-Zellen, unter deren Einfluss B-Zellen vermehrt IgE produzieren. Die Antigen-induzierte Aggregation von FcεRI-gebundenem IgE stimuliert die Degranulation von Mastzellen und die Freisetzung von Mediatoren wie Histamin, $PGD_2$ und TNF, welche die Rekrutierung von Th2-Zellen, die Migration, Reifung und Aktivierung dendritischer Zellen und die Antigenpräsentation fördern [47]. IgE- und Antigen-IgE-Komplexe können das Epithel durch Transzytose durchqueren, die durch CD23 auf den Epithelzellen der Atemwege vermittelt wird, wodurch sie an FcεRI auf Mastzellen und dendritischen Zellen binden und diese aktivieren können [48]. Zudem können Leukotriene zu einer Aktivierung von innaten Lymphozyten des Typs 2 (ILC2) beitragen, wodurch die Entzündung bei Th2-Erkrankungen verstärkt wird [49].

Im Gegensatz zu primären Effektorzellen wie Mastzellen und Basophilen werden Eosinophile und Neutrophile als sekundäre Effektorzellen bezeichnet, die durch freigesetzte Mediatoren der primären Effektorzellen aktiviert werden können. Hierbei bilden DCs, ILCs und Basophile in einer Th2-vermittelten Immunkaskade ein enges Netzwerk der Immunreaktion. Daneben agieren die Atemwegsepithelzellen als physikalische Barriere gegenüber der Umwelt [50]. Die Atemwege sind sowohl im Lumen als auch an der Schleimhaut hohen Mengen an endogenen und exogenen Proteasen ausgesetzt [51], welche die Aktivität und Verfügbarkeit von Zytokinen und Wachstumsfaktoren regulieren und zu Epithelschäden sowie erhöhter Permeabilität beitragen können. Neben der Barrierefunktion des Epithels durch die bloße Anwesenheit der geschlossenen Zellschicht selbst werden zwei zusätzliche Barrierestrukturen über den Atemweg auf die luminale Oberfläche des Atemwegsepithels exportiert [52]. Sie bestehen aus einer statischen Komponente, der Glykokalyx (Periziliarschicht) sowie einer mobilen Komponente, die als Mucus (Schleim) bezeichnet wird [52]. Pathogene, die eine Typ 2-vermittelte Immunantwort verursachen, haben Mechanismen entwickelt, um das Epithel mittels spezifischer Proteasen, zu welchen auch einige Allergene wie z.B. *Dermatophagoides pteronyssinus* zählen, zu penetrieren [53]. Dies zeigt, dass Veränderungen am Epithel, sei es aufgrund genetischer Veranlagung, Umwelteinflüssen oder Allergenstrukturen, eine wichtige Rolle im Zusammenspiel mit dem Immunsystem in der Entstehung einer Th2-vermittelten Immunantwort spielen.

## 2.2 Rolle des Atemwegsepithels in Th2-assoziierten Erkrankungen

### 2.2.1 Interaktion von Epithel- und Immunzellen

Die Epithelzellen der Atemwege wirken in einer orchestrierten Weise mit lokalen und rekrutierten Immunzellen zusammen, um die Immunität in den Atemwegen zu regulieren. Während Untersuchungen des lokalen Transkriptoms anhand der extrazellulären Matrix, aber auch induziertem Sputum [54] oder bronchoalveolärer Lavage (BAL) [55], eine detaillierte Charakterisierung von Immunzellpopulationen im Atemwegslumen erleichtern, erhalten wir durch Bürstungen und Biopsien neben epithelialen auch die subepithelialen, stromalen Schichten, so dass auch Infiltrate über Immunzellen analysiert werden können. Involvierte epitheliale Alarmine, Mediatoren wie IL-25 (auch als IL-17E bekannt), IL-33 und TSLP, können über die Aktivierung von ILC2-vermittelten Antworten eine Typ 2-Reaktion auslösen, die individuellen Beiträge der einzelnen Zytokine zu deren Einfluss auf

Epithelzellen und die Regulation molekularer Mechanismen, die eine endogene Produktion dieser Zytokine verstärken, sind jedoch nur unzureichend verstanden [56]. Vor diesem Hintergrund ist auch das Konzept der „*united airways*“ von Interesse, welches eine anatomische und immunologische Beziehung über die Verbindung zwischen Erkrankungen der oberen und unteren Atemwege mittels epithelialer Zytokine und deren Transport entlang eines einheitlichen Atemwegs hypothetisiert [57]. Entsprechend häufig kommen Komorbiditäten vor, da sie Manifestationen einer einzigen Grunderkrankung an verschiedenen Stellen der Atemwege widerspiegeln [58]. Allergisches Asthma als Komorbidität von allergischer Rhinitis stellt die archetypische Erkrankung der „*united airways*“ dar, kann aber auch ein heterogener Zustand sein, der mehrere Phänotypen und zugrundeliegende pathobiologische Mechanismen in sich vereint.

IL-5 und IL-13 induzieren in der Schleimhaut der Atemwege die Rekrutierung von eosinophilen Granulozyten und Mastzellen, welche auf ihrer Oberfläche den IL-5R$\alpha$ exprimieren [59]. Ein weiteres Th2-assoziiertes Zytokin, IL-9, wird von klassischen Th9-Zellen sezerniert, die in Gegenwart von TGF-$\beta$ und IL-4 entstehen können [60, 61]. Die Fähigkeit der Th9-Zellen zur IL-9-Sekretion ist allerdings nicht von langer Dauer, da sie die IL-9-Expression schrittweise über mehrere Differenzierungsrunden verlieren, was auf eine entgegengesetzte Aktivierung des STAT3-Signalwegs zurückzuführen ist [62]. Das pleiotrope epitheliale Zytokin TGF-$\beta$ kann die Expression des Th9-assoziierten Schlüssel-Transkriptionsfaktors PU.1 induzieren, während IL-4 über STAT6 die Induktion des Transkriptionsfaktors IRF4 auslöst - beide Transkriptionsfaktoren gemeinsam fördern die Th9-Polarisierung [63]. Der Transkriptionsfaktor ETV5 kann unabhängig von PU.1 ebenfalls die IL-9-Produktion in Th9-Zellen regulieren [64]. Gehemmt werden kann die IL-9-Produktion in Th9-Zellen vor allem über IFN-$\gamma$ oder IL-27 [65]. Weiter erhöhen IL-9-vermittelte Umbauprozesse in den Atemwegen das Risiko lokaler Überempfindlichkeitsreaktionen, einschließlich Phänomenen wie einer Becherzell-hyperplasie [66].

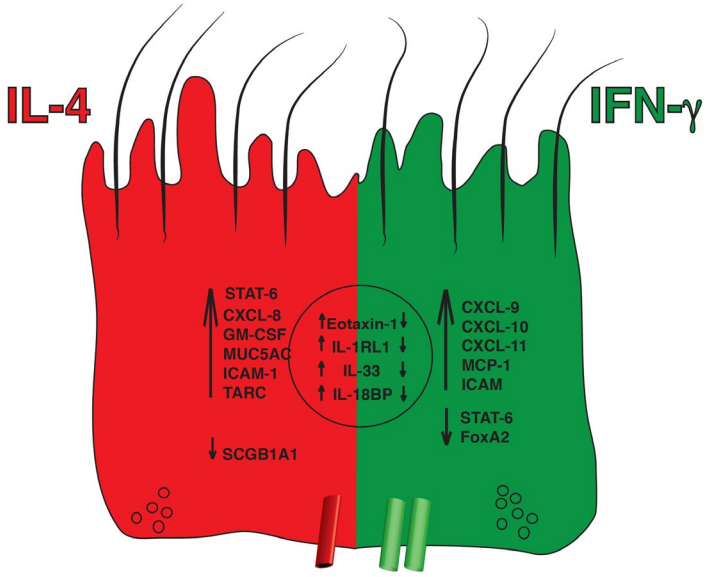


**Abbildung 2: Regulation der epithelialen Immunität durch IL-4 und IFN-γ.**

Unterschiedliche entzündliche Milieus, wie sie durch Typ 1- oder Typ-2-gerichtete Immunreaktionen charakterisiert sind, können unterschiedliche Auswirkungen auf die Biologie der Atemwege haben und führen zur erhöhten oder erniedrigten Expression von Schlüsselgenen.

Interessanterweise konnten wir in eigenen Arbeiten zur Immuntherapie auch regulatorische Moleküle wie die inhibitorisch wirkenden Rezeptoren PD-1 und CTLA-4 auf den Th2-Zellen hochreguliert beobachten [67]. Neben der Verschiebung hin zu einer Th2-Immunantwort tragen auch lokale Th17-Zellen, die über ihre Sekretion von IL-17 charakterisiert sind, in der Schleimhaut zu einer Neutrophilen-dominierten Atemwegsentzündung bei [68]. IL-17 induziert den polymeren Ig-Rezeptor als Schlüsselfaktor der mukosalen Immunität [69] und steigert die Chemotaxis von B-Zellen bei Asthma [70]. Lokale Th17-Zellen in der Darmschleimhaut exprimieren CCR6 als Reaktion auf seine Chemokin-Liganden CCL20 oder MIP-3α [71]. In Modellen für allergisches Asthma bei Mäusen wurde gezeigt, dass IL-17 bei allergischen Immunreaktionen seine proinflammatorischen Wirkungen auf das Epithel der Atemwege ausübt, indem es eine IL-33-induzierte neutrophile Entzündung fördert [72].

### 2.2.2 Prägung des Atemwegsepithels

Die Rolle der Epithelzellen wurde inzwischen in translationalen Studien und Tiermodellen hervorgehoben, die weit über die Rolle einer physischen Barriere und einer Komponente des angeborenen Immunsystems hinausgehen. Die vom Atemwegsepithel vermittelten Abwehrmechanismen reichen von Apoptose zur Eindämmung intrazellulärer Infektionen bis hin zu passiven Schutzreaktionen wie Membranverstärkung oder Schleimproduktion. Durch eine Interaktion von T-Zellen mit dem Atemwegsepithel können ebenfalls

Regenerations- oder sogar immunsuppressive Mechanismen (Abbildung 2) induziert werden [73]. Es wird vermutet, dass die verursachenden T-Zellen aus einer gemeinsamen Antigen-naiven Vorläuferzelle entstammen könnten, die sich entweder in Th1- oder Th2-Zellen oder in weitere T-Zell Subtypen in einem Prozess antagonistischer Regulation differenzieren, welche die phänotypische Polarisierung ermöglicht. Die von Lymphozyten sekretierten Schlüssel-Zytokine wie Th1-assoziiertes IFN-$\gamma$ und das von Th2-Zellen sezernierte IL-4 [74] werden auch von Epithelzellen der Atemwege erkannt und tragen über erhöhte Schleim- [75] und Matrixproduktion [76] zur Pathophysiologie von allergischen Atemwegserkrankungen bei. Andere bekannte, pro-allergische Typ-2-Zytokine des Epithels, wie IL-33 oder IL-1RL1, wirken auf innate Zellen und können wiederum die IL-4-Produktion selbst steigern [77]. IFN-$\gamma$ beeinflusst zudem die Induktion von CXCL9, CXCL10, CXCL11, MCP1, STAT1, ICAM, IL8, MHC-Klasse-I und MHC-Klasse-II in Atemwegsepithelzellen [73, 78]. Aufgrund der Induktion von Chemokinen und der Expression von Genen wie *ICAM* [79] und *MHC* [80] wird vermutet, dass das Atemwegsepithel in Verbindung mit Th1-assoziierten Zytokinen eine Entzündungsreaktion auslösen und antigenspezifische Abwehrmechanismen an der Epitheloberfläche induzieren kann. So induziert eine Virusinfektion des Atemwegsepithels hauptsächlich Interferone, welche zur initialen Unterdrückung der viralen Ausbreitung ohne Aktivierung einer Entzündungsreaktion angelegt ist. Demgegenüber vermitteln IL-4 und IL-13 maßgeblich an einer Typ-2-Antwort beteiligt, beide wirken über den Transkriptionsfaktor STAT-6, dessen Expression im Bronchialepithel ausgeprägt ist und bei schwerem Asthma weiter ansteigt [81, 82]. Die Expression der hochaffinen IL-13R$\alpha$2-Kette wurde ebenfalls auf Epithelzellen und Fibroblasten der Atemwege beobachtet und wird gemeinsam mit IL-13R$\alpha$1 und IL-4R$\alpha$ exprimiert [83]. Der IL-4-Signalweg kann über zwei Rezeptor-Heterodimere (Typ I und Typ II) vermittelt werden, bestehend aus entweder $\gamma$c/IL-13R$\alpha$ (Typ I) oder IL-4R$\alpha$/IL-13R$\alpha$ (Typ II), während die Bindung von IL-13 auf den Typ II beschränkt ist [84]. Resultierend daraus wurde auch eine verstärkte Freisetzung von CCL-11 aus asthmatischen Fibroblasten festgestellt, was eine Erklärung für die Akkumulation von Eosinophilen unter der *Lamina reticularis* bei Asthma sein könnte [85]. Jedoch konnte gezeigt werden, dass die IL-4 oder IL-13-abhängige Sekretion von CCL-11 aus glatten Atemwegsmuskelzellen über Inhibition der p42/p44-ERK- und p38-MAP-Kinase-Signalwege in STAT6-Antisense-Oligodesoxynukleotid-transfizierten Zellen verhindert wurde [86]. Über den JAK/STAT-Signalweg können IL-4 und IL-13 kann auch die epitheliale Produktion von CCL-26 (Eotaxin-3) in Atemwegsepithelzellen induziert [87, 88] und beispielsweise über Dexamethason inhibiert werden können [89]. Durch die

Stimulation der epithelial-mesenchymalen trophischen Einheit tragen IL-4 und IL-13 also zur chronischen Entzündung und zum Umbau der Atemwege bei [76]. Unabhängig von der Aktivierung von Th2-Zellen kann IFN-γ die Schleimsekretion, eosinophile Granulozyten sowie die Freisetzung von Chitinasen hemmen [90]. Die inhibitorische Rolle von IFN-γ auf Typ 2-assoziierte epitheliale Prozesse in der Asthma-Pathogenese deutet darauf hin, dass Typ 1-Reaktionen einen Gegenspieler von Typ-2-Signalwegen darstellen [91, 92]. Im Gegenzug kann die Hemmung des Th2-assoziierten Transkriptionsfaktors GATA-3 zu einer Erhöhung der Expression von Typ 1-assoziierten Faktoren *T-bet* und *IFN-γ* beitragen, was wiederum zur Suppression des allergischen Phänotyps führt [93]. Diese immunologischen Effekte der epithelialen Differenzierung bieten im Zusammenhang mit Sensibilisierung, aber auch bei Prozessen wie der Entzündungs-Resolution und dem Umbau der Atemwege neue Möglichkeiten zur Intervention und Prävention von.

### 2.2.3 Gestörte Barrierefunktion von Atemwegsepithelzellen

Es wird vermutet, dass aufgrund einer gestörten Barrierefunktion der Epithelzellen in den Atemwegen genetisch prädisponierte Personen anfällig für Virusinfektionen und die Aufnahme von Allergenen sind, was zu einer lokalen, allergenspezifischen Typ-2-Immunreaktion und damit zu einer Sensibilisierung führen kann [94, 95]. Die Allergenexposition und die anschließende Aktivierung von Mustererkennungsrezeptoren (PRRs) induzieren eine angeborene Immunreaktion durch die Epithelzellen des Respirationstrakts [96]. Infolgedessen werden die epithelialen Alarmine TSLP, IL-25 und IL-33 sowie M-CSF und GM-CSF innerhalb kurzer Zeit nach Allergenkontakt freigesetzt [97-99]. In Tiermodellen wurde die Neutralisierung eines oder mehrerer dieser epithelialen Zytokine als Merkmal der asthmatischen Atemwegsentzündung wie Eosinophilie, bronchiale Hyperreaktivität und Hyperplasie der Becherzellen nachgewiesen [100]. Zusätzlich wurde in verschiedenen murinen und humanen Studien der Einfluss von Faktoren wie Alter, Geschlecht, Saisonalität und epigenetischer Mechanismen auf die epitheliale Zytokinreaktion und die individuelle Amplitude der einzelnen Zytokine untersucht [101-103]. Dieser Einfluss wurde beispielweise in Studien über die Belastung durch Stallstaub auf Bauernhöfen gezeigt, in welchen eine Amish-Lebensweise zu einer geringeren Prävalenz und Prädisposition für Asthma führte [104]. Die Abundanz epithelialer Zytokine wie IL-33 und GM-CSF war ebenfalls reduziert, was unter anderem von einer Hochregulierung des negativen Regulators TNFAIP3 (A20) als Deubiquitinase des NFκB-Signalwegs abhängen kann [105]. Zusätzliche Stimuli, wie Luftverschmutzung

[106], Zigarettenrauch [107] oder Virusinfektionen [108] können die epitheliale Mediator-Expression ebenfalls beeinflussen. Da die epithelialen Zytokine können auch adaptive Th2-assoziierte Immunmechanismen aktivieren können, so können die epithelialen Mediatoren TSLP, IL-25 und IL-33 eine terminale Differenzierung und Aktivierung von Th2-Effektorzellen steuern [109]. Die epithelialen Mediatoren IL-25 und IL-33 stimulieren die Proliferation von Th2-assoziierten Zellen und beeinflussen deren OX40L-Expression [110-112]. Konventionelle Typ 2-DCs werden über eine Hochregulierung von OX40L und die Suppression von IL-12 aktiviert [113]. Studien in Tiermodellen haben gezeigt, dass die Produktion von Th2-assoziierten epithelialen Zytokinen eine spezifische Funktion von spezialisierten Epithelzellen ist. IL-25 wird von Tuftzellen im Epithel als Reaktion auf umweltbedingte und mikrobielle Reize freigesetzt [114]. Diese Zellen spielen über eine Induktions- und Rückkopplungsschleife eine zentrale Rolle bei der Aktivierung von ILC2-Zellen [115], die ihrerseits IL-13 sezernieren und damit wiederum die Expansion von Tuftzellen stimulieren [116]. In einem Allergenexpositions-modell bei Mäusen konnte die Bildung von Tuftzellen durch Knock-out von LTC4-Synthase oder des Rezeptors für LTE4 auf Epithelzellen abgeschwächt werden [117].

### 2.2.4 Einfluss von microRNAs auf das Atemwegsepithel

Biologische Prozesse können ebenfalls von kleinen, hoch-konservierten und nicht codierenden Nukleinsäuren, so genannten microRNAs (miRNAs), beeinflusst werden [118]. Die über Th2-assoziierte Mediatoren wie IL-13 veränderte Biosynthese oder Regulierung von miRNAs trägt zu pathologischen Prozessen bei, woran auch Epithelzellenüber die Sekretion kleiner extrazellulärer Vesikel beteiligt sind [119]. MiRNAs werden als potenzielle neue Biomarker in Betracht gezogen, da sie über Exosomen in den extrazellulären Raum abgegeben werden und daher in Körperflüssigkeiten wie Serum relativ stabil sind [120]. Daher haben sich die meisten Studien auf miRNAs im Serum konzentriert, da es relativ einfach zu gewinnen ist, aber nur wenige haben bisher ihre Expression in den Atemwegen untersucht [121]. Bei allergischen Erkrankungen, die durch ein Zytokin-Milieu über Th2-Mediatoren wie IL-4 und IL-13 geprägt ist, können Epithelzellen ebenfalls miRNAs exprimieren, was mit einer Verschlechterung der Lungenfunktions-parameter in Verbindung gebracht werden konnte [119]. Darüber hinaus können miRNAs Relevanz für den Umbau der Atemwege bei Asthma sowohl auf die Schleimbildung, Obstruktion als auch Hyperreaktivität der Atemwege besitzen, die möglicherweise durch kleine extrazelluläre Vesikel des Epithels über spezifische miRNAs reguliert werden [122].Verschiedene miRNAs mit funktioneller Bedeutung für die allergische Immunreaktion wurden in mehreren

unabhängigen Studien im Zusammenhang mit allergischen Atemwegserkrankungen untersucht. Eine erhöhte Expression von *miR-19a* in T-Zellen der Atemwege fördert einerseits die Produktion von Th2-Zytokinen [121, 123], andererseits führt eine verminderte *miR-19a*-Expression in den glatten Atemwegsmuskelzellen zu einem verstärkten Umbau der Atemwege [124]. Von besonderer Bedeutung ist wohl auch die *miR-155*, die bei Patienten mit atopischer Dermatitis [125] und in einem murinen Modell für allergische Atemwegsentzündungen untersucht wurde [126]. *MiR-155*-defiziente Mäuse wiesen reduzierte IL-33-Spiegel in den Atemwegen und geringere ILC2-Zahlen im Vergleich zu Wildtyp-Mäusen nach Allergenexposition auf [126]. Ein Augenmerk liegt bei der Betrachtung Th2-assoziierter Erkrankungen auch auf der *let-7* Familie, die als eine der ersten miRNAs mit hoher Abundanz in den Atemwegen beschrieben wurde [127]. Eine verringerten *let-7a*-Expression konnte in Lungenbiopsien schwerer Asthmatiker mit erhöhten IL-13-Spiegeln beobachtet werden [128]. Weiter konnte eine inverse Regulation *let-7a* und der IL-13-Expression in den Atemwegen gezeigt werden [129], wodurch über weitere Forschung die Verwendung der miRNAs als therapeutische Option in Betracht gezogen werden könnte.

## 2.3 Therapeutische Intervention bei Entzündungsprozessen in Epithelzellen

### 2.3.1 Verschiedenen Therapieoptionen und Einfluss auf die Gewebehomöostase

Das Hauptziel der Behandlung allergischer Atemwegserkrankungen sind die optimale Symptomkontrolle, die Verringerung des Exazerbationsrisikos und die Erhaltung der Lungenfunktion bei gleichzeitiger Minimierung der Nebenwirkungen von Medikamenten. Eine Entzündung der Atemwege als Hauptmerkmal bei Asthma, die mit einer Schleimhypersekretion einhergeht, kann mit Glukokortikoiden kontrolliert werden [23, 130]. Sie ist daher als entzündungshemmende Behandlung eine häufig eingesetzte Option bei der klinischen Kontrolle von Asthma [131]. Die zugrundeliegenden Mechanismen von Glukokortikoiden werden mutmaßlich durch Glukokortikoid-Rezeptoren vermittelt, die ubiquitär auf verschiedenen Zelltypen exprimiert werden, darunter Epithelzellen und Fibroblasten in den Atemwegen [132]. So verringern Glukokortikoide die Expression von MUC5AC in primären differenzierten normalen menschlichen Bronchialepithelzellen [133]. Epithelzellen zeigen auch eine intrinsische Fähigkeit zum Entzündungsgedächtnis, welches als Reaktion auf Veränderungen im pro- oder anti-inflammatorischen Zytokin-Milieu etabliert wird, kürzlich erst in der chronischen Rhinosinusitis nachgewiesen [134], einer chronischen Th2-assoziierten Erkrankung der oberen Atemwege, die durch eine anhaltende Entzündung der Nasenschleimhaut und der Nasennebenhöhlen gekennzeichnet ist. Gemeinhin kann

eine chronische Rhinosinusitis (CRS) anhand des Vorhandenseins von Nasenpolypen (NP) in einen Phänotyp mit Nasenpolypen (CRSwNP) und einen ohne Nasenpolypen (CRSsNP) unterteilt werden [135]. Die Erkrankung ist außerdem mit einer Epithelschädigung und Gewebedestruktion verbunden [136], die eine zusätzliche Anfälligkeit für Virusinfektionen fördern kann.

Da eine Basalzellhyperplasie auch ein Hauptmerkmal des Gewebeumbaus bei dieser Erkrankung ist, wurden unlängst Studien an Gewebeproben von Patienten mit CRS und CRSwNP durchgeführt, sowie an Nasenbürsten aus der unteren Nasenmuschel gesunder Probanden und Personen mit chronischer Rhinosinusitis mit Polypen [134]. Die auffälligsten krankheitsbedingten Veränderungen auf Ebene der Genexpression wurden hierbei in Basalzell-, Differenzial- oder Sekretionszell- und Drüsenzellpopulationen beobachtet. In Polypen wurde eine signifikante Basalzellenexpansion auf Kosten der Epithelzellvielfalt beschrieben [134]. Basalzellen regulieren die Genexpression von Transkriptionsfaktoren wie *ATF3*, *KLF5* und *FOSB* hoch, welche auf die Th2-Schlüsselzytokine IL-4 und IL-13 reagieren [134]. Daher könnten intrinsische Veränderungen auf epigenetischer Ebene für die Unterschiede im Basalzellstatus der Polypen verantwortlich sein und durch das lokale Typ-2 entzündlichen Milieu gesteuert werden [134].

### 2.3.2 Die Allergen-spezifische Immuntherapie

Das Ziel, die allergen-spezifische Immunantwort zu unterdrücken und in eine Gewebehomöostase zu bringen, begann im Jahre 1911 mit der Allergen-spezifischen Immuntherapie (AIT) und ihrer Beschreibung durch Leonard Noon anhand empirischer Erkenntnisse [137]. Sie hat sich seitdem laut Guidelines zu einer wichtigen Therapie-Option bei der Behandlung allergischer, IgE-vermittelter Erkrankungen entwickelt [138], einschließlich des allergischen Asthmas [139]. Es bleibt bis dato die einzige Krankheits-modifizierende Behandlungsoption, die Allergikern angeboten werden kann [138]. Ziel der Therapie ist es, eine periphere Toleranz aufzubauen, die Th2-Zellen nachhaltig zu supprimieren und über IL-10 den Immunglobulin-Klassenwechsel von IL-4-induziertem IgE in B-Zellen hin zu $IgG_4$ sowie zu mukosalem und systemischem IgA zu fördern [140]. Aufgrund einzigartiger besitzt $IgG_4$ anti-entzündliche Eigenschaften, darunter eine geringe Affinität zu den klassischen Fc$\gamma$-Rezeptoren und die Fähigkeit zur Bildung bispezifischer, funktionell monovalenter Antikörper [141]. Ähnliches gilt für IgA, wenn es während einer allergen-spezifischen Immuntherapie produziert wird. Es kann zelluläre Effekte durch verschiedene Rezeptoren vermitteln [142], die Bindung von Allergenen an IgE-Rezeptoren blockieren und die Freisetzung von Histamin aus Basophilen hemmen [143, 144]. Über den

Verlauf der Therapie verschiebt sich die Immunität in Richtung Allergen-spezifischer Th1-Immunantworten mit einem Anstieg von IFN-γ [145]. Ein weiterer Zusammenhang konnte zwischen CD27-negativen, Allergen-spezifischen Th2-Zellen [146] sowie follikulären T-Helferzellen [147] und der Synthese von Th2-Zytokinen sowie dem IgE-Switch in den B-Zellen hergestellt werden. Unter Allergen-spezifischer Immuntherapie werden die pro-allergischen Zellen durch IL-10-produzierende Zellen wie regulatorische B-Zellen [140] oder auch FoxP3-positive, regulatorische follikuläre T-Zellen [148] inhibiert. Aber auch B- und T-Gedächtniszellen könnten eine zentrale Rolle zum Erhalt des Therapieeffekts spielen, da sich diese Populationen bei Graspollen-spezifischer Immuntherapie über die Dauer von drei Jahren aufbauen und somit zur dauerhaften IgE-Blockierung beitragen könnten [149]. Mechanistische Studien der AIT (Abbildung 3) zeigen einen Zusammenhang der anti-allergischen Effekte mit einer Immunmodulation der innaten und der adaptiven Immunantwort [150]. So verdichten sich seit Jahren die Hinweise, dass ILC2s und auch basophile Granulozyten neben den Th2-Zellen weitere Quellen von Typ 2 Zytokinen sein können [151, 152]. Auch die epitheliale Antwort spielt hierbei eine wichtige Rolle. So unterstützen Dendritische Zellen unter dem Einfluss von epithelial sekretiertem TSLP und IL-33 die nachfolgende Induktion von ILC2s und Th2-Zellen [153].

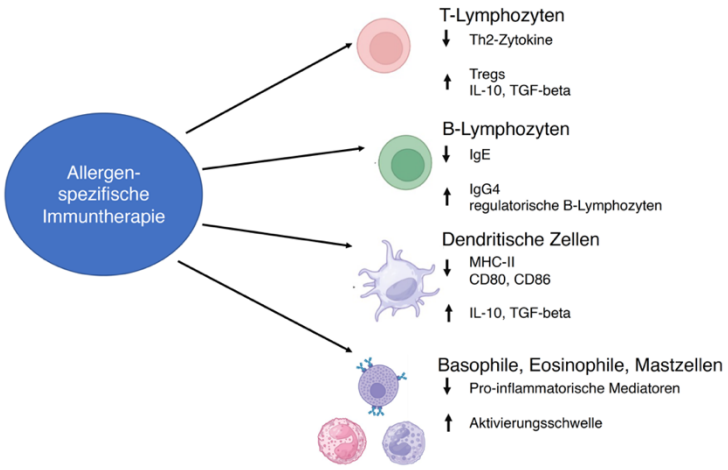


**Abbildung 3: Regulierung der allergischen Immunreaktion nach einer AIT.**

Allergenspezifische regulatorische T-Lymphozyten werden entweder als Folge einer erfolgreichen AIT induziert. Treg-Zellen sind in der Lage, die regulatorischen Zytokine IL-10 und TGF-β zu produzieren und exprimieren auch CTLA-4 und PD-1, die zur Immunsuppression von allergischen Reaktionen beitragen. Treg-Zellen unterdrücken Th2-Zellen, Eosinophile und Basophile und können auch allergenspezifische Breg-Zellen induzieren. Das suppressive Milieu schränkt die IgE-Produktion ein, während es die Produktion von IgG4 aus B-Zellen anregt.

# 3. Synopse

Das Epithel ist ein zentraler Angelpunkt, dessen Immunantwort auf Umwelteinflüsse und -belastungen im gesunden Körper angemessen ausfällt und im Gleichgewicht ist. Bei Patienten mit allergischen Atemwegserkrankungen ist dieses Gleichgewicht in Richtung einer beeinträchtigten angeborenen Abwehr, einer andauernden Typ-2-Entzündung und Gewebeumstrukturierung verschoben. In dieser Habilitationsschrift sollte das krankheitsspezifische epitheliale Reaktom in allergischen Erkrankungen aufgedeckt werden (Abbildung 4).

Wir zeigen erstmals, dass ein spezifisches Mikromilieu dieser prototypischen Th1- und Th2-assoziierten Zytokine IFN-$\gamma$ bzw. IL-4 eine phänotypische Polarisierung ermöglicht, in welcher die Epithelzellen vorab auf einen Th1- („E1“) oder Th2- („E2“) ähnlichen Phänotyp festgelegt sind. Diesen konnten wir *in vitro* aus einem Stimulations-Effekt ableiten, welcher ebenfalls zu einem epithelialen Gedächtnis bezüglich der fehlgeleiteten Immunantwort bei Atemwegsepithelzellen beitragen kann. Diese grundlegenden, unausgeglichenen Mechanismen und damit assoziierte epitheliale Mediatoren wie CCL-26 und IL-24, sowie deren Einfluss auf den Verlauf einer allergischen Erkrankung spielen eine zentrale Rolle: wir konnten in einer vergleichenden *ex vivo* Analyse von Nasensekreten allergischer Asthmatiker und gesunder Probanden anhand der Sekretion von IL-4, IL-5, IL-13, IL-24, IL-33 und Periostin die Zusammenhänge über bisher bekannte Muster hinaus erweitern. Weiter sind diese epitheliale Zytokine in die Regulation angeborener lymphoider Zellen des Typs 2 (ILC2) eingebunden, die neben epithelialen Alarminen auch über epitheliales TGF-$\beta$ aktiviert werden können. TGF-$\beta$ wird unter anderem von Epithelzellen im Rahmen einer allergischen Entzündung sezerniert und fördert somit das Zytokin-Milieu der allergischen Immunantwort am Epithel. Wir vermuten, dass die daraus folgende phänotypische Polarisierung und ihre Transkriptionsregulation einen zentralen Mechanismus in der Chronifizierung allergischer Entzündungen in Atemwegsepithelzellen darstellt. Aufgrund seiner pro- als auch anti-inflammatorischen Wirkweise war es eine wichtige Fragestellung, ob die AIT über einen anti-inflammatorischen Einfluss von TGF-$\beta$ vermittelt wird. Wir konnten zeigen, dass dies hier nicht der Fall war, da TGF-$\beta$ durch die Induktion über Th9-Zellen in ein pro-inflammatorisches Muster eingefügt hat. Zusätzlich zu diesen Mechanismen spielen auch nicht-kodierende microRNAs (miRNAs) als zentrale Regulatoren der posttranskriptionellen Genexpression im Atemwegslumen eine wichtige Rolle. Wir konnten zeigen, dass auch diese Regulatoren der posttranskriptionellen Genexpression ein unausgeglichenes mukosales Muster in Kontext einer Th2-abhängigen

Erkrankung aufweist, welches in direktem Zusammenhang mit Symptomen und sekretierten Mediatoren wie Prostaglandin $E_2$ ($PGE_2$) steht. Hierbei konnten wir erstmals die Verbindung zwischen einer Asthma-spezifisch hochregulierten miRNA, miR-3935 und der verringerten Expression ihres Zielgens, dem $PGE_2$-Rezeptor EP3, aufzeigen. Da der $PGE_2$/EP3-Signalweg zur Mastzell-Granulation beitragen und entzündungsfördernd wirken kann, könnte die miR-3935-vermittelte Suppression von EP3 einen Beitrag zur Mastzell-Desensibilisierung im Rahmen einer AIT darstellen. Die antientzündlichen Mechanismen der Desensibilisierung stellten einen weiteren wichtigen Schwerpunkt im Rahmen dieser Arbeiten dar. Das Atemwegsepithel selbst kann ebenfalls eine regulatorische Rolle im Sinne einer entzündungsauflösenden Eigenschaft zur Reversibilität der epithelialen Immunologie einnehmen. Hier konnten wir einen Beitrag mit der Identifizierung des lokalen, epithelialen Mediators Secretoglobin1A1 leisten, welcher im Rahmen einer allergischen Rhinitis reduziert ist. Wir konnten zeigen, dass Secretoglobin1A1 das immunologische Gleichgewicht der mukosalen Immunität positiv beeinflussen kann und daher für zukünftige therapeutische Ansätze bei allergischen Erkrankungen relevant sein könnte. Wie wir erstmals beschreiben konnten, bieten sezernierte, anti-inflammatorische Mediatoren des Epithels wie Secretoglobin1A1 möglicherweise eine Option, den Erkrankungsverlauf zu prognostizieren und eine Therapieoption wie die AIT hinsichtlich ihres individuellen Nutzens und Effektivität zu beurteilen. Im Verlauf der AIT wird das immunologische Gleichgewicht des Zytokinmilieus am Atemwegsepithel wie auch zwischen den spezifischen Immunzell-Subtypen wiederhergestellt. Mit dieser Arbeit konnten wir eine Kartierung des Immunzell- sowie des Epithelzell-Reaktoms während einer Gräserpollen-spezifischen AIT erstellen und die systemische mit der lokalen Immunantwort über einen Beobachtungszeitraum von mehr als drei Jahren verknüpfen. Tatsächlich waren die Veränderungen nicht auf das Immunzell-Reaktom beschränkt, da auch in Atemwegsepithelzellen Veränderungen beispielsweise bei IL-24, IL-17C, IL-37 und SCGB1A1 nachgewiesen werden konnten, so dass unsere Arbeiten die immunologischen Mechanismen sowie den nachhaltigen Einfluss der AIT auf das Epithel belegen. Zukünftige klinische Studien werden zeigen müssen, ob diese neuen diagnostischen Strategien eine Möglichkeit darstellen, den Patienten eine individuelle Behandlung ihrer jeweiligen Erkrankungen anbieten zu können.

Als Vision könnte auch eine Nukleinsäure-basierte Therapie entwickelt werden, die in das E1-E2-Gleichgewicht regulierend eingreift. Die folgenden sechs Originalarbeiten stellen die Grundlage für die epitheliale Polarisierung, die Vergleichbarkeit der oberen und unteren Atemwege, die Verknüpfung von Immun- und Epithelzell-Reaktom, AIT-induzierte Mechanismen sowie die Kartierung der Langzeitmechanismen.

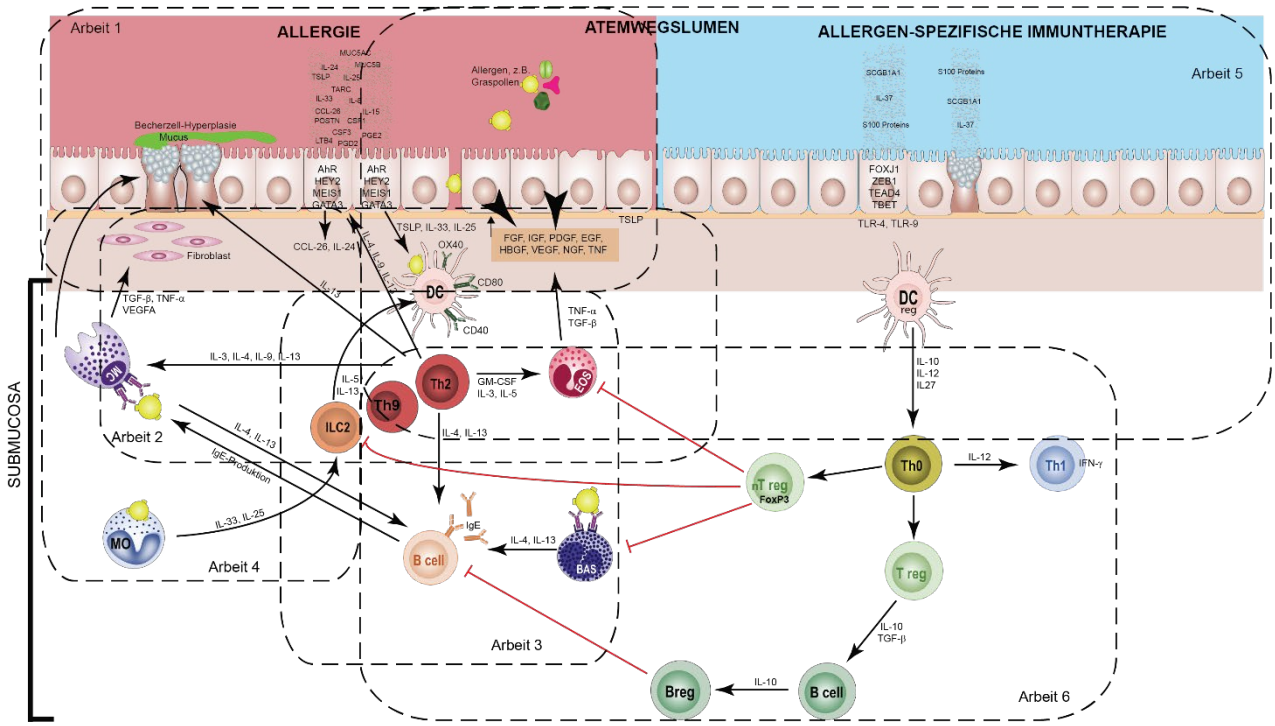


**Abbildung 4. Einordnung der eigenen Arbeiten in die pathophysiologischen Reaktionen der Atemwege bei allergischer Entzündung und therapeutischer Intervention.** Diese schematische Darstellung zeigt Mechanismen der epithelialen und lymphozytären Regulation in den Atemwegen während physiologischer und pathologischer Prozesse (linke Hälfte in rot) und die Auswirkungen einer allergen-spezifischen Immuntherapie (rechte Hälfte in blau). In der Arbeit 1 (Mucosal Immunology 2016, 9(4):917-26) zur Orchestrierung einer epithelialen Polarisierung über IL-4 und IFN-γ beschreiben wir transkriptionellen Unterschiede dieser antagonistischen Genregulation. Die Vergleichbarkeit der oberen und unteren Atemwege bei der lokalen, allergischen Atemwegsentzündung und der assoziierten Biomarker im Atemwegslumen haben wir in der Arbeit 2 (Journal of Allergy and Clinical Immunology 2018, 142(6):1980-1983) untersucht und mittels Korrelationsmatrices erfasst. In Arbeit 3 (Frontiers in Immunology, 2022 Jan 7;12:763243) lag der Fokus auf dem Zusammenhang auf dem Zytokin TGF-β und der Frage, ob die AIT über einen anti-inflammatorischen Einfluss von TGF-β vermittelt wird. In Arbeit 4 (Clinical and Experimental Allergy 2021, 51(12):1577-1591) konnten wir spezifisch in Sputen von Asthma-Patienten erstmals demonstrieren, dass die bei Asthma erhöhte miR-3935 durch die AIT herabreguliert wurde und einen direkten Zusammenhang von $PGE_2$ mit ILC2 sowie deren assoziierter Mediatoren wie IL-13 in Sputumproben von Rhinitis- und Asthmapatienten aufzeigt. In Arbeit 5 (Allergy 2021, 76(8):2461-2474) konnten wir nachweisen, dass die AIT wichtige Mediatoren mit anti-inflammatorischen Eigenschaften wie Secretoglobin1A1 induziert, als Gegenregulation zu pro-inflammatorischen Markern wie IL-24. Diesen Antagonismus haben wir in Arbeit 6 (EBioMedicine 2018, 36(2018):475-488) in Bezug auf den longitudinalen Effekt der AIT weiter herausgearbeitet.

# 4. Publikationen

Die Publikationen 4.1, 4.2, 4.3, 4.4, 4.5 und 4.6 beschreiben die Regulation von Immun- und Epithelzellen auf transkriptioneller und sekretorischer Ebene unter bekannten sowie neu beschriebenen Einflüssen wie Immuntherapie oder Zytokin-abhängiger Regulation.

So beschreibt die Arbeit „*Interleukin-4 and interferon-$\gamma$ orchestrate an epithelial polarization in the airways*“ (siehe 4.1) den Einfluss der Schlüsselzytokine der Th1- und Th2-Antwort, IFN-$\gamma$ und IL-4, auf die Regulation des Atemwegsepithels, woraus die Hypothese eines Th2-polarisierten E2-Epithels mit einem zugrunde liegenden Netzwerk beteiligter Transkriptionsfaktoren identifiziert wurde.

Die Arbeit „*Biomatrix for upper and lower airway biomarkers in patients with allergic asthma*“ (siehe 4.2) fokussiert sich auf die Vergleichbarkeit der oberen und unteren Atemwege bei der lokalen, allergischen Atemwegsentzündung und der assoziierten Biomarker im Atemwegslumen und vielversprechende Biomarker-Kandidaten identifizierten, welche zugrunde-liegende Asthma-Endotypen widerspiegeln könnten.

Die Arbeit „*TGF-$\beta$1 drives inflammatory Th Cell but not Treg cell compartment upon allergen exposure*“ (siehe 4.3) beleuchtet die Fragestellung, ob die AIT über einen anti-inflammatorischen Einfluss von TGF-$\beta$ vermittelt wird.

Die Arbeit zu „*Sputum microRNA-screening reveals Prostaglandin EP3 receptor as selective target in allergen-specific immunotherapy*“ (siehe 4.4) zeigt spezifisch in Sputen von Asthma-Patienten erstmals demonstrieren, dass die bei Asthma erhöhte *miR-3935* durch die AIT herabreguliert wurde und einen direkten Zusammenhang von $PGE_2$ mit ILC2 aufzeigt.

Im Weiteren befasst sich die Arbeit „*Allergen-specific immunotherapy induces the suppressive secretoglobin1A1 in cells of the lower airways*“ (siehe 4.5) mit den epithelialen Entzündungsmechanismen sowie wichtige Mediatoren mit anti-inflammatorischen Eigenschaften wie Secretoglobin1A1, welche durch eine AIT induziert werden.

Die Arbeit „*Early IL-10 producing B-cells and coinciding Th/Tr17 shifts during three year grass-pollen AIT*“ (siehe 4.6) befasst sich mit dem longitudinalen Effekt der AIT. Das Verhältnis von regulatorischen B- und Th17-Zellen nach Erstbehandlung erlaubt eine frühe Vorhersage der AIT-Wirksamkeit nach der Aufdosierungsphase auf der Ebene der peripheren Zellen.

## 4.1 Interleukin-4 and interferon-γ orchestrate an epithelial polarization in the airways

Ulrich M. Zissler, Adam M. Chaker, Renate Effner, Moritz Ulrich, Ferdinand Gürth, Guido Piontek, Katharina Dietz, Michael Regn, Bettina Knapp, Fabian J. Theis, Holger Heine, Kathrin Suttner and Carsten B. Schmidt-Weber

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Interferon-gamma (IFN-γ) and interleukin-4 (IL-4) are key effector cytokines for the differentiation of T helper type 1 and 2 (Th1 and Th2) cells. Both cytokines induce fate-decisive transcription factors such as GATA3 and TBX21 that antagonize the polarized development of opposite phenotypes by direct regulation of each other's expression along with many other target genes. Although it is well established that mesenchymal cells directly respond to Th1 and Th2 cytokines, the nature of antagonistic differentiation programs in airway epithelial cells is only partially understood. In this study, primary normal human bronchial epithelial cells (NHBEs) were exposed to IL-4, IFN-γ, or both and genome-wide transcriptome analysis was performed. The study uncovers an antagonistic regulation pattern of IL-4 and IFN-γ in NHBEs, translating the Th1/Th2 antagonism directly in epithelial gene regulation. IL-4- and IFN-γ-induced transcription factor hubs form clusters, present in antagonistically and polarized gene regulation networks. Furthermore, the IL-4-dependent induction of IL-24 observed in rhinitis patients was down regulated by IFN-γ, and therefore IL-24 represents a potential biomarker of allergic inflammation and a Th2 polarized condition of the epithelium.

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ARTICLES

# Interleukin-4 and interferon-γ orchestrate an epithelial polarization in the airways

UM Zissler[1], AM Chaker[1,2], R Effner[1], M Ulrich[1,2], F Guerth[1], G Piontek[2], K Dietz[1], M Regn[3], B Knapp[4], FJ Theis[4,5], H Heine[6], K Suttner[1] and CB Schmidt-Weber[1]

**Interferon-γ (IFN-γ) and interleukin-4 (IL-4) are key effector cytokines for the differentiation of T helper type 1 and 2 (Th1 and Th2) cells. Both cytokines induce fate-decisive transcription factors such as GATA3 and TBX21 that antagonize the polarized development of opposite phenotypes by direct regulation of each other's expression along with many other target genes. Although it is well established that mesenchymal cells directly respond to Th1 and Th2 cytokines, the nature of antagonistic differentiation programs in airway epithelial cells is only partially understood. In this study, primary normal human bronchial epithelial cells (NHBEs) were exposed to IL-4, IFN-γ, or both and genome-wide transcriptome analysis was performed. The study uncovers an antagonistic regulation pattern of IL-4 and IFN-γ in NHBEs, translating the Th1/Th2 antagonism directly in epithelial gene regulation. IL-4- and IFN-γ-induced transcription factor hubs form clusters, present in antagonistically and polarized gene regulation networks. Furthermore, the IL-4-dependent induction of IL-24 observed in rhinitis patients was downregulated by IFN-γ, and therefore IL-24 represents a potential biomarker of allergic inflammation and a Th2 polarized condition of the epithelium.**

## INTRODUCTION

The airway epithelium plays an important role as physical barrier to the environment, counteracts invasive microbes, and deals with harmless components such as pollen or other particles. Defense mechanisms range from apoptosis to limit intracellular infections down to passive protective reactions such as membrane reinforcement or mucus production.[1] By physical interaction of T cells with the epithelium, not only defense mechanisms but also regeneration or even immunosuppressive mechanisms are induced.[2] These T cells originate from a common antigen-naive precursor cell that differentiates either into T helper type 1 or 2 (Th1 or Th2) cells or other T-cell subtypes in a process of antagonistic regulation that allows the phenotypic polarization. Specifically Th1-derived interferon-γ (IFN-γ) inhibits Th2 cell differentiation, whereas Th2-derived interleukin-4 (IL-4) inhibits the fate in favor of Th1 differentiation. These key cytokines are also recognized by airway epithelial cells, but whether airway epithelial cells respond to IL-4 and IFN-γ by antagonistic gene regulation is only partially understood. The resulting polarization in transcriptional regulation of epithelial cells has been described for fate decision in pancreas cell development.[3] It is known that Th2-exposed epithelial cells can contribute to asthma by enhanced mucus production,[4] epithelial hyperplasia,[5] and enhanced matrix production.[6] Other well-known pro-allergic type 2 epithelial cytokines, such as IL-33, IL-18BP, or IL-1RL1, act on innate cells and can enhance IL-4 production.[7–9]

Th1 cells were previously described to play a role in multiple inflammatory airway disease such as cystic fibrosis,[10] sarcoidosis,[11] pulmonary tuberculosis,[12] bronchiolitis obliterans,[13] and hypersensitivity pneumonitis.[14] We and others have previously demonstrated that IFN-γ has substantial effect on airway epithelial cells such as the induction of *CXCL9*, *CXCL10*, *CXCL11*, *MCP1*, *STAT1*, *ICAM*, *IL8*, *MHCI*, and *MHCII*.[2,15] Because of the chemokine induction and expression of lymphocyte relevant genes such as *ICAM* and *MHC*, it is

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hypothesized that Th1-primed epithelium can trigger inflammation by recruitment and support antigen-specific defense mechanisms at the epithelial surface. However, it is not clear whether Th1/Th2 programs exist in airway epithelial cells, and hence the relevance of changed ratios of lung-infiltrating Th1 and Th2 cells is not known.[2] In fact, it could be speculated that the infiltration of Th1 cells in an otherwise Th2-affected epithelium is beneficial. In addition, epithelial Th2 responses could be easily diagnostically assessed in disease conditions with unclear case history such as wheezing in newborns. This unmet clinical need can be noninvasively addressed by nasal lining fluids that contain abundant amount of epithelial cytokines with biomarker potential for diagnosis of allergic inflammation such as allergic rhinitis and allergic asthma.

We previously showed that explants from allergic inflammation (skin, upper and lower airways) contain "plastic" T-cell phenotypes secreting multiple cytokines in parallel, including IL-4 and IFN-γ,[2] raising the question of the functional consequence on the side of airway epithelial inflammation.

Essential for the IL-4-mediated polarization is an intact IL-4 receptor (IL-4R) downstream signaling. Airway epithelial cells do not only recognize IL-4, but also express the receptor for its structural homolog *IL13*. Both cytokines share the IL-4Rα and the common γ chain (γc) or the IL-13Rα chain that activates the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways. As a consequence, IL-4 is able to bind to two receptor (type I and type II) heterodimers consisting of either γc/IL-13Rα (type I) or IL-4Rα/IL-13Rα (type II), whereas binding of IL-13 is restricted to type II.[16] The receptor complex that mediates IFN-γ signaling consists of two species-matched chains: IFN-γR1 and IFN-γR2. IFN-γR1 is the major binding subunit for IFN-γ, whereas IFN-γR2 increases the affinity of IFN-γR1 and is obligatory for transducing the IFN-γ signal.[17] Like the IL-4R complex, the IFN-γ receptors are expressed by airway epithelial cells and induce proinflammatory chemokines[18] and *MHCII* expression.[2]

The key hypothesis of this study was that IL-4 antagonizes IFN-γ-mediated gene regulation in airway epithelial cells. Therefore, we studied whether antagonistic gene regulation prevails over synergistic gene induction along with its associated transcriptional network.

This study shows for the first time that airway epithelial cells are subjected to a transcriptional program similar to the Th1/Th2 antagonism known in lymphocytes. Notably, airway epithelial cells express several IL-4- or IFN-γ-induced genes that are antagonized by IFN-γ and IL-4 respectively.

## RESULTS

To understand the pathological consequences of T-cell plasticity on the human airway tissue, we coexposed normal human bronchial epithelial cells (NHBEs) with IL-4 and IFN-γ at the same time and discovered that IL-4 and IFN-γ antagonize airway epithelial gene regulation.

### IL-4 and IFN-γ antagonistic and synergistic gene regulation

Airway epithelial cells were exposed to the cytokines alone or in combination for visualization of IL-4- and IFN-γ-induced antagonism. Although the response to IFN-γ is well established in epithelial cells, the response to IL-4 is more complex and was monitored before the whole genome transcriptome assessment. Immunocytofluorescence stainings were used as quality control to demonstrate the homogenous distribution of the IL-4Rα expression on the cell surface and cytoplasm of the majority of the cells (**Supplementary Figure S1** online). In order to extract Th1/Th2-driven antagonistic gene regulation, NHBE cultures were exposed to IL-4 or IFN-γ and coexposure was additionally analyzed. The cytokine-induced signaling resulted in 1,501 entities induced by IL-4 and 2,412 entities by IFN-γ (**Supplementary Figure S2**, gene list in **Supplementary Table S1**). The Venn diagram analysis revealed the genes that are induced by IL-4 and downregulated by IFN-γ and that still show changes in the presence of both cytokines (**Figure 1a**, center cut set). We found that the majority of genes were downregulated following cytokine treatments (**Figure 1a,c**). A cut set of 48 transcripts was regulated in the presence of both IL-4 and IFN-γ (**Figure 1b**; gene list in **Supplementary Table S2**). These genes were hierarchically grouped into three clusters (clusters 1–3; **Figure 1b**) of which cluster 1 represents the strongest upregulated genes by IL-4 (**Figure 1b**). The genes highlighted in **Figure 1b** are the result of a hypothesis-free ranking of literature citation numbers for each gene. Thus, the most cited genes (PubMed) are ranking highest, whereas less cited are ranking lower and noncited the lowest. Consequently, less studied genes show lower ranking. Using this visualization, it is possible to get an unbiased view of the nature of the cluster on the current state of knowledge. For cluster 1, these included *CCL26*, *ANO1* and *RUNX2* as most literature-cited genes. The cluster 2 contained particularly IL-4-enhanced and IFN-γ-neutralized genes and included the transcription factor *DLX5* (splice variant 1). Cluster 3 included IL-4-induced, IFN-γ-neutralized, or IFN-γ-antagonized genes containing *DLX5* (splice variant 2) and *MEX3B*.

The cut set of 70 genes is covering the genes (**Figure 1d**; gene list in **Supplementary Table S3**) that were IL-4 downregulated and IFN-γ upregulated as well as regulated in the presence of IL-4 and IFN-γ at the same time. Again, these genes were hierarchically grouped into two clusters (**Figure 1d**, clusters 4 and 5). For cluster 4, this included *IDO1 and LY96*. The cluster 5 carried IFN-γ-induced and IL-4 downregulated gene expression and covered *TNFAIP2* and *IL22RA1*.

In addition to antagonistic gene regulation, the synergistic induction of genes was investigated as well. For this purpose the cut set of genes was analyzed that are only induced in the presence of IL-4 and IFN-γ at the same time but not in the presence of only one of them. The cut set comprised 530 genes (**Supplementary Figure 3a**) in the case of IL-4- and IFN-γ-induced genes and 1,378 genes in the case of IL-4

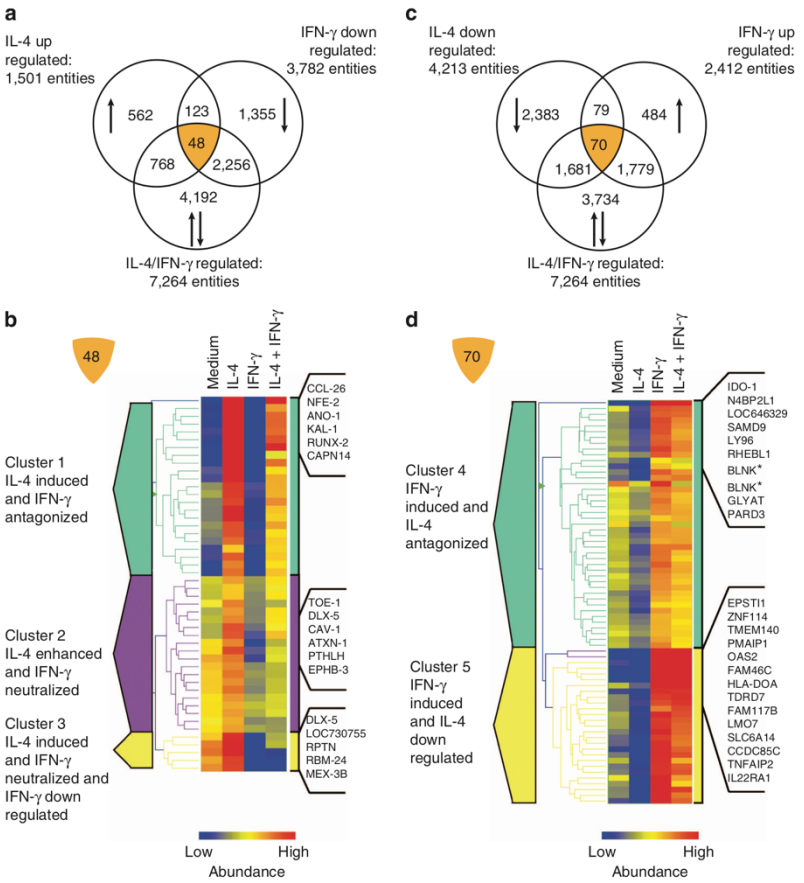


**Figure 1** Interleukin-4 (IL-4) and interferon-γ (IFN-γ) antagonistic gene regulation. Normal human bronchial epithelial cells (NHBEs) were cultured in the presence of IL-4 or IFN-γ or IL-4 and IFN-γ for 6 h to study RNA whole transcriptome by array technology. (**a**) Comparison of 1,501 IL-4-upregulated entities, 3,782 IFN-γ-downregulated entities, and 7,264 IL-4/IFN-γ-regulated entities in a Venn diagram. A cut set of 48 antagonistic regulated entities was identified. The analysis is based on cultures of six genetically independent NHBEs of healthy donors. (**b**) A hierarchic clustering analysis of genes upregulated in comparison with untreated cells (medium) was performed, resulting in three different clusters. Labeled are six most cited genes in each cluster. (**c**) Comparison of 4,213 IL-4-downregulated entities, 2,412 IFN-γ-upregulated entities, and 7,264 IL-4/IFN-γ-regulated entities in a Venn diagram. A cut set of 70 antagonistic regulated entities was identified. (**d**) A hierarchic clustering analysis of genes upregulated by IFN-γ in comparison with untreated cells (medium) was performed, resulting in two different clusters. Labeled are the most cited genes in each cluster, except cluster 3 that only contained five genes. 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.

and IFN-γ downregulated genes (**Supplementary Figure 3c**). However, a closer look reveals that both groups carry predominantly if not entirely those genes that escape the statistical thresholds of IL-4 or IFN-γ regulation, but that make it over these thresholds when an additive effect by the presence of both cytokines is generated. Among these entities are no genes with established immunoregulatory or epithelial function except SLC10A2 (sodium and bile channel) or RGS4 (G-protein regulator). Furthermore, a large number of not annotated genes were found (gene list in **Supplementary Tables S4** and **S5**).

### IL-4- and IFN-γ-induced secreted gene products

In order to extract specifically IL-4-inducible genes, all IFN-γ-regulated entities as well as all IL-4/IFN-γ-regulated entities were excluded from further analysis (**Figure 2a**). When IL-4-induced but not IFN-γ-regulated genes (540 entities) were filtered for specific secreted gene products, 22 entities were identified by Gene Ontology (GO) terms (**Figure 2a,b**; gene list in **Supplementary Table S6**). This group contained genes such as *Wnt5a*, *CCL6*, or *IL24* (**Figure 2b**). The group IL-4-induced and IL-4/IFN-γ-regulated genes (368 entities, **Figure 2a**) contained among others *TNFAIP6*, *CLDN5*, and *POSTN* (gene list in **Supplementary Table S7**). Conversely, to

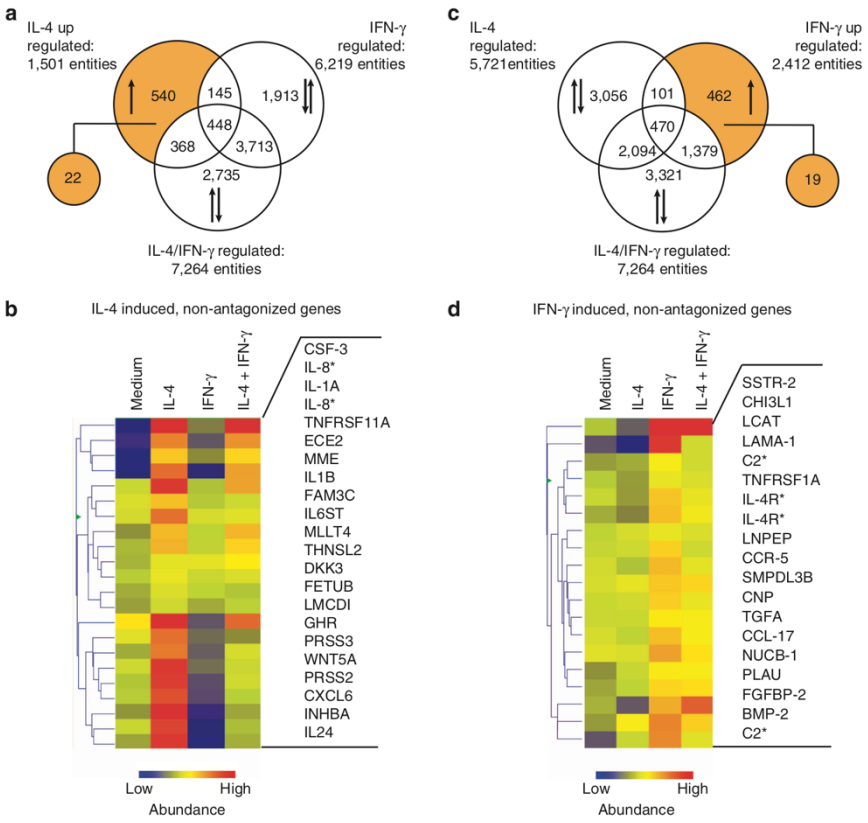


**Figure 2** Biomarker of T helper type 1/2 (Th1/Th2) imprinted airway epithelial cells. Normal human bronchial epithelial cells (NHBEs) were cultured in the presence of interleukin-4 (IL-4) or interferon-γ (IFN-γ) or IL-4 and IFN-γ for 6 h to study RNA whole transcriptome by array technology. The analysis is based on cultures of six genetically independent NHBEs of healthy donors. (**a**) Comparison of 1,501 IL-4-upregulated entities, 6,219 IFN-γ-regulated entities, and 7,264 IL-4/IFN-γ-regulated entities in a Venn diagram. A cut set of 540 IL-4 only-induced entities was identified within the groups of IL-4-upregulated entities. The identified genes were listed in a ranking according to current literature using the "Genomatix" software and Gene Ontology-Terms (GO-Terms) 0007267, 0005125, 0008009, and 0005615 for identification of biomarkers resulting in a list of 22 entities. (**b**) Hierarchical ranking of identified IL-4-induced secreted biomarkers. Asterisks indicate two isoforms that were present in the analysis. (**c**) Comparison of 5,721 IL-4-regulated entities, 2,412 IFN-γ-upregulated entities, and 7,264 IL-4/IFN-γ-regulated entities in a Venn diagram. A cut set of 462 IFN-γ only induced entities was identified within the groups of IFN-γ-upregulated entities. A filter for the functions of the identified genes was listed by GO terms for identification of biomarkers resulting in a list of 22 entities. (**d**) Hierarchical ranking of identified IFN-γ-induced secreted biomarkers. 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).

extract IFN-γ-specific genes induced in airway epithelial cells, all IL-4-regulated entities as well as all IL-4/IFN-γ-regulated entities were excluded from analysis (**Figure 2c**). The remaining 462 IFN-γ-induced entities were filtered for secreted gene products as described above and 19 entities were identified respectively by GO terms, including *CHI3L1 (YKL-40)* and *CCL17* (**Figure 2c, d**; gene list in **Supplementary Table S8**). The group of IFN-γ-induced and IL-4/IFN-γ-regulated genes (1,379 entities, **Figure 2c**) contained among others *CXCL9*, *CXCL10*, and *CXCL11* (gene list in **Supplementary Table S9**). Several genes found to be significantly regulated by the array technology were validated real-time quantitative reverse transcription-PCR (**Figure 3**).

### Transcriptional mechanisms underlying IL4 and IFN-γ antagonism in epithelial gene expression

Transcriptional antagonism and specifically the polarization of a transcriptional program are accompanied by direct counter-regulation of one transcription factor against the other as it is observed for *GATA3* and *TBX21* or *GATA3* and *FOXP3*.[19] As a result, a cellular phenotype may successively develop into either one or the other phenotype while sparing "in-between" phenotypes. To identify transcriptional regulators in this context, IL-4- or IFN-γ-induced genes were filtered for transcriptional regulators in conjunction with either IL-4- or IFN-γ-induced genes. Respectively, 80 and 102 entities were identified by GO terms and Genomatix curated

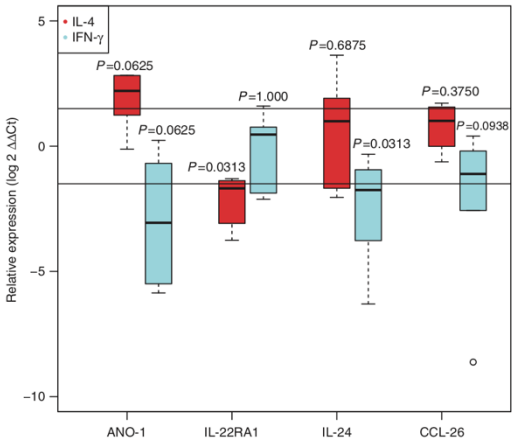


**Figure 3** Validation of *in vitro* gene expression changes. Normal human bronchial epithelial cells (NHBEs) were cultured in the presence of interleukin-4 (IL-4) or interferon-γ (IFN-γ) for 6 h to study RNA expression by real-time quantitative reverse transcription–PCR (qRT-PCR) confirming array results for *ANO1*, *IL22RA1*, *IL24*, and *CCL26*, expressed as log2 gene expression changes (ΔΔCt). The analysis is based on cultures of six genetically independent NHBEs of healthy donors. Data have been log2 transformed to get a normal distribution, and the Shapiro test for normal distribution was performed. The genes are normally distributed on a 1% significance threshold.

annotations (**Figure 4**; gene list in **Supplementary Tables S10 and S11**). In the group of IL-4-induced transcriptional regulators, a group of 15 entities was identified containing *NFE2*, *GATA3*, *RARB*, *FoxQ1*, and *AhR* (**Figure 4**). We therefore analyzed whether *GATA3* and *TBX21* could potentially account for the antagonistic gene expression. It is interesting to note that *TBX21* and *GATA3* are also expressed in airway epithelial cells, and IL-4Rα, STAT-6, and GATA-3 are present in a majority of cells (**Supplementary Figure S1 a–c**). We performed a bioinformatics analysis of all antagonized gene products and conducted a pathway analysis based on transcription factor binding site involving literature evidence as well as predicted binding sites in respective promoters (**Figure 5**). The resulting network visualizes that *GATA3* could be interlinked with 38 genes, and *TBX21* could only be connected with 10 gene promoters. However, other IL-4- or IFN-γ-induced transcription factors are frequently connected such as *Meis1*, *Hey2* that link with 23 or 38 IL-4-induced/IFN-γ-antagonized genes, respectively, and *TEAD4* connecting with 36 IFN-γ-induced/IL-4-antagonized genes (**Supplementary Table S12**).

### Th1/Th2-specific gene expression changes *in vivo*

In order to show the physiologic relevance of identified genes under well-known Th2 conditions during allergic inflammation in nasal mucosa, allergic subjects sensitized for grass pollen and healthy controls were recruited. Nasal airway epithelial cells were obtained through nasal scrapings from the inferior turbinates. We refrained to obtain bronchial cells for ethical reasons and instead used nasal epithelial cells (scrapings) on the basis of previous studies showing comparable regulation patterns of epithelial cells derived from upper vs. lower airways.[20] The scrapings were subjected to RNA isolation and real-time quantitative reverse transcription–PCR was performed for the prominent targets, in particular those that are secreted, and those known to be involved in transcriptional regulation in type 2 differentiation and disease: *IL7*, *IL24*, *IL18BP*, *IL22RA1*, *IL33*, *ANO1*, *IL1RL1 (sST2*; *IL33R)*, *AhR*, *GATA3*, *TBX21*, and *RUNX2* (**Figure 6**). Significant increased gene expression changes in allergic subjects in comparison with healthy control subjects were shown for *IL7* (log2 expression change: 1.98, 1.57 to 5.33; $P<0.05$), *IL24* (log2 expression change: 3.09, −1.12 to 4.02; $P<0.05$), *ANO-1* (log2 expression change: 1.56, −0.18 to 3.79; $P<0.01$), *IL18BP* (log2 expression change: 2.41, 1.84 to 3.23; $P<0.05$), *IL1RL1* (s*ST2, IL33R*; log2 expression change: 2.31, −0.42 to 4.78; $P<0.05$), *IL22RA1* (log2 expression change: 1.02, −0.27 to 4.20; $P<0.05$), *AhR* (log2 expression change: 1.08, 0.54 to 2.01; $P<0.05$), *GATA3* (log2 expression change: 1.64, 0.67 to 3.18; $P<0.05$), and *RUNX2* (log2 expression change: 1.35, 0.99 to 2.45; $P<0.05$). A trend for a decreased expression of *TBX21* was discovered in allergic subjects (log2 expression change: −1.33, −3.47 to 0.92) compared with healthy control subjects. No significant changes were shown for the expression of *IL33* in allergic subjects (log2 expression change: 1.57, 0.24 to 4.12) in comparison with healthy controls.

### Biomarker of Th2-primed epithelial cells

Among the identified IL-4-induced, secreted gene products, IL-24 has to be highlighted as potential Th2 biomarker, as this protein belongs to the IL-10 superfamily. Currently, it is not described to be regulated by other epithelial activation processes such as *IL8* that is not only induced by IL-4 but also by IL-17. Western blot analysis was performed for IL-24 in supernatants of *in vitro* culture of NHBEs after stimulation with IL-4, IFN-γ, and IL-4/IFN-γ for 6 and 24 h (**Figure 7a**). IL-24 was detected at 6 and 24 h in IL-4-stimulated samples but not in medium control or IFN-γ-subjected samples. As epithelial cells are able to secrete cytokines into the airway lumen, we hypothesized that IL-24 could also be detectable in nasal secretions. Therefore, secreted protein levels of nasal secretions of allergic asthma patients collected in and out of season were measured by electrochemiluminescence detection for IL-24 and compared with healthy controls. A significantly upregulated secretion of IL-24 was detected for patients in season (121.70 pg ml$^{-1}$, 61.91–242.80; $P<0.01$) in comparison with healthy controls (49.33 pg ml$^{-1}$, 31.98–71.13), whereas only a minor, nonsignificant increase of IL-24 was observed in the out-of-season group (93.85 pg ml$^{-1}$, 28.15–145.40) compared with healthy controls (**Figure 7b**).

## DISCUSSION

The objective of this study was to investigate whether IL-4 and IFN-γ are subject to antagonistic gene regulation in airway epithelial cells. The study reveals a large number of

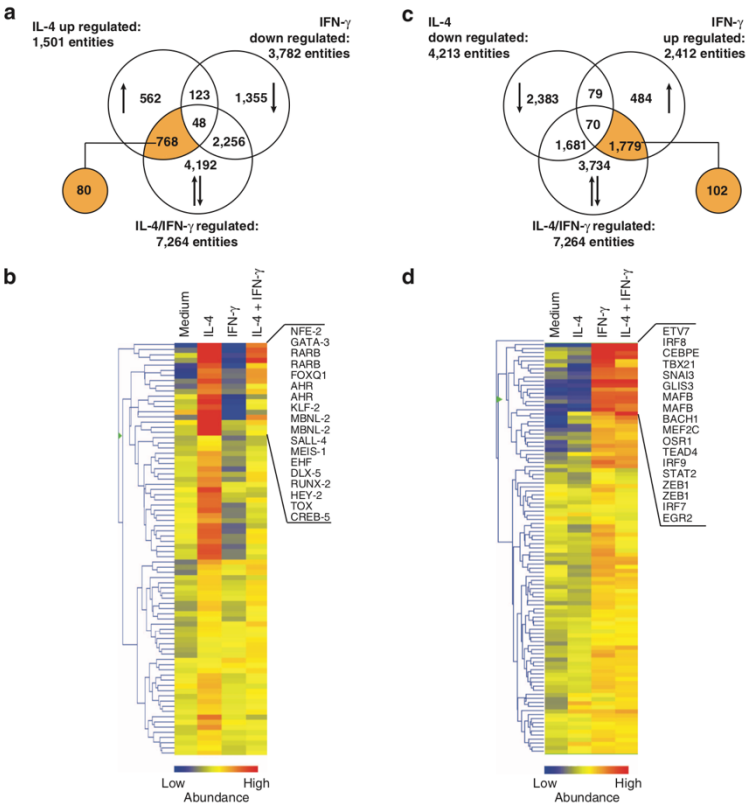


**Figure 4** Antagonistic transcriptional regulation. Normal human bronchial epithelial cells (NHBEs) were cultured in the presence of interleukin-4 (IL-4) or interferon-γ (IFN-γ) or IL-4 and IFN-γ for 6 h to study RNA whole transcriptome by array technology. The analysis is based on cultures of six genetically independent NHBEs of healthy donors. (**a**) Comparison of 1,501 IL-4-upregulated entities, 3,782 IFN-γ-downregulated entities, and 7,264 IL-4/IFN-γ-regulated entities in a Venn diagram. A cut set of 768 entities was identified within the groups of IL-4-upregulated and IL-4/IFN-γ-regulated entities. The identified genes were listed in a ranking according to current literature using the "Genomatix" software and Gene Ontology-Terms (GO-Terms) 0003676 and 0044212 for transcription factors resulting in a list of 80 entities. (**b**) Hierarchical ranking of identified IL-4-induced transcription factors, containing IL-4-master transcription factor GATA3. (**c**) Comparison of 4,213 IL-4-downregulated entities, 2,412 IFN-γ-upregulated entities, and 7,264 IL-4/IFN-γ-regulated entities in a Venn diagram. A cut set of 1,779 entities was identified within the groups of IL-4-upregulated and IL-4/IFN-γ-regulated entities. (**d**) Hierarchical ranking of identified IFN-γ-induced transcription factors, containing IFN-γ-master transcription factor TBX21. The color code indicates the abundance of transcripts ranging from low (blue) to high (red).

IL-4-induced and IFN-γ-inhibited genes. In fact, IL-4-inducible genes of cluster 1 such as *CCL26* (Eotaxin-3), *ANO1*, or *RUNX2* are dampened in the presence of IFN-γ. The example of *CCL26* is not only reproducing previous results that demonstrated that CCL-26 is an IL-4-inducible, proinflammatory chemokine in allergic rhinitis,[21] but also that epithelial chemokines can act as biomarkers for bullous pemphigoid and asthma with eosinophilic infiltration into the airways.[21,22] *ANO1* represents another example that has a major impact on basal and stimulated chloride conductance in airway epithelial cells[23] and serves as an indicator for epithelial proliferation and migration in the repair processes.[24] As this study shows that IFN-γ inhibits CCL-26-mediated chemotaxis and chloride conductance, it can be proposed that IFN-γ may ameliorate eosinophilic infiltration and tracheal cartilage deposition as well as repair processes. This study reveals 46 additional IL-4-inducible and IFN-γ-inhibited genes that are yet not known to be under control of this IL-4–IFN-γ antagonism. Most of these genes have not been implicated in Th2-driven diseases, except Calpain-14, a calcium-sensitive protease that together with TSLP is genetically associated with eosinophilic esophagitis.[25]

Conversely, genes of clusters 4 and 5 such as *IDO1* or *IL22RA1* were previously reported to be IFN-γ inducible and are downregulated by IL-4. The IL-4-mediated suppression of *IDO1*, a potential mediator of tolerance, was previously observed in house dust mite-stimulated dendritic cell cultures.[26] Similarly, *IL22RA1* has been shown to be related to potentially tolerogenic downregulation of *MHCI* and *MHCII* in airway epithelial cells, and thus IL-4-mediated downregulation can also contribute to break tolerance.[2,27] Taken together, it appears that important genes are subject to

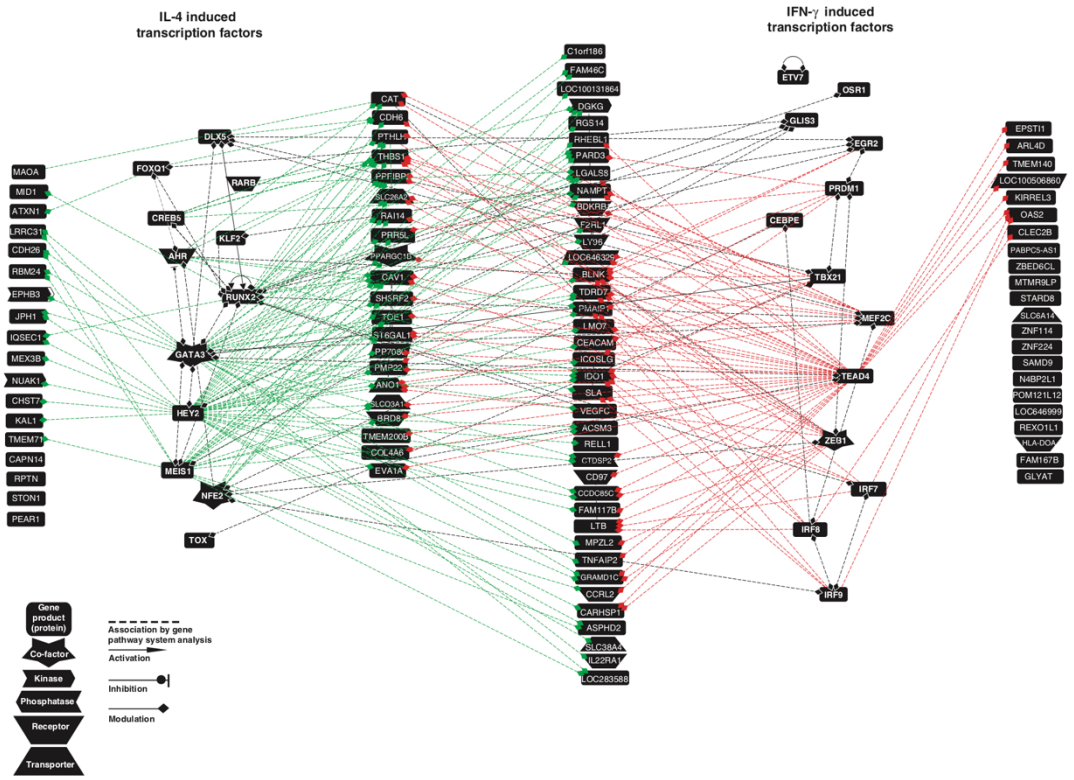


**Figure 5** Schematic illustration of the transcriptional network. Transcriptional network of interleukin-4 (IL-4; left)- and interferon-γ (IFN-γ)-induced transcription factors (right) and genes of the IL-4/IFN-γ antagonism of epithelial cells. The network connects genes that are known to be transcriptionally regulated or that contain transcription factor binding sites (Genomatix database) of the respective factor. Green lines originate from IL-4- and red lines from IFN-γ-induced transcription factors. Black lines show intertranscription factor connections. The figure shows numerous intertranscription factor lines that may be critical for polarized gene regulation. GATA3 appears to be connected to many type 2, and also several type 1 genes. On the side of IFN-γ-induced transcription factors it appears that TEAD4, MEF2C, and ZEB1 have hub character, whereas TBX21 is connected with only a few genes.

antagonistic regulation as it is known from T-cell differentiation and further substantiate the concept of IL-4 as tolerance breaking mediator that was previously demonstrated on the level of T cells.[19] Future studies are necessary to show that IL-4 counteracts tolerance also at the level of epithelial surfaces.

In contrast, the search for genes synergistically induced by IL-4 and IFN-γ, but not by isolated cytokines, did not reveal strongly induced clusters. However, it is clear that there are numerous genes that are insensitive to antagonistic regulation. These nonantagonized genes are particularly interesting, as they may provide robust indicators for Th2-mediated immune responses, even if other, IFN-γ-inducing inflammatory processes are disguising the pathology. Narrowing the view on genes that are secreted and detectable in secretions, 22 IL-4-induced genes were identified, including *CSF3*, *CXCL6*, *IL24*, and *WNT5a*. In addition, there is a small group of genes that is IL-4 induced and survives the presence of IFN-γ in the co-culture situation, such as *TNFAIP6*, *CLDN5*, and *POSTN*, and all of these genes are known to be IL-4 regulated.[28–30] Conversely, IFN-γ induced 19 genes with no influence of IL-4 such as *CHI3L1* (Chitinase-3-Like Protein 1 or YKL-40) that is known to be induced by IFN-γ nasal epithelial cells.[31] Of note, there is also a considerable number of genes that are induced by IFN-γ alone and regulated by the combination of IL-4 and IFN-γ. In this group, IFN-γ dominantly induces well-known IFN-γ-responsive genes such as *CXCL9*, *CXCL10*, and *CXCL11*, whereas IL-4 has a neglectable effect.[32]

The physiological relevance of these antagonized and nonantagonized genes in human disease conditions was assessed in nasal scrapings of in-season allergic rhinitis patients and healthy subjects. On the basis of previous studies showing comparable regulation patterns of epithelial cells derived from upper vs. lower airways,[20] we anticipate that nasal scrapings containing mainly nasal epithelial cells provide an ethically appropriate surrogate biomarker source for validation of the targets identified in bronchial epithelial cells. *IL24* and *ANO1*, the latter a biomarker known from cystic fibrosis, are showing distinct expression in allergic patients during the allergy season

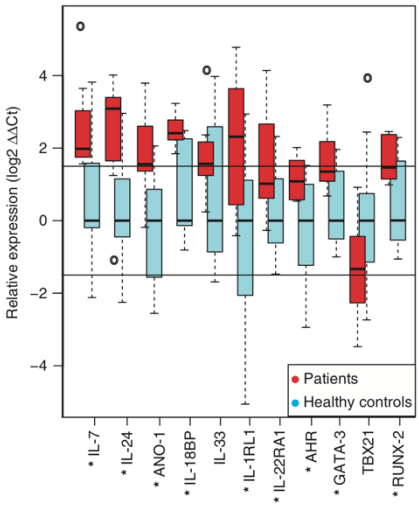


**Figure 6** Gene expression levels for selected transcripts. Nasal scrapings of asthmatic patients ($n = 8$) as well as healthy controls ($n = 12$) were subjected to RNA isolation and real-time quantitative reverse transcription–PCR (qRT-PCR) was performed. Gene expression changes (ΔΔCt) have been *log2*-transformed to get a normal distribution. Significant differences could be shown for *IL7*, *IL24*, *ANO1*, *IL18BP*, *IL1RL1* (*sST2*; *IL33R*), *IL22RA1*, *AhR*, *GATA3*, and *RUNX2*.

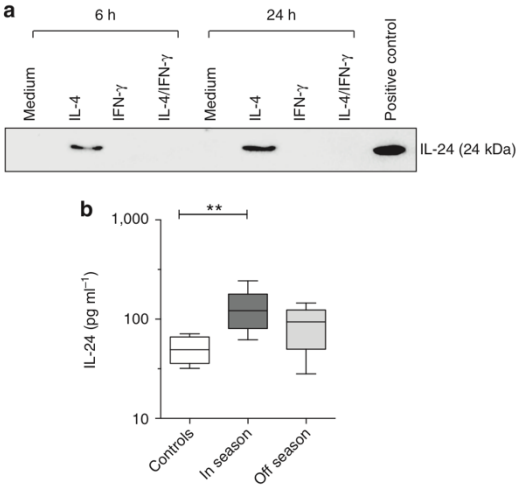


**Figure 7** Interleukin-24 (IL-24) protein secretion *in vitro* and *in vivo*. (**a**) A signal for IL-24 secretion *in vitro* was detected by western blot analysis after 6 and 24 h of stimulation with IL-4 but not for interferon-γ (IFN-γ) or IL-4/IFN-γ cultivation of normal human bronchial epithelial cells (NHBEs). There was no detectable signal in medium controls. (**b**) Significantly upregulated secretion of IL-24 of patients in season ($n = 13$) in comparison with healthy controls ($n = 14$) was detected. An increased secretion of IL-24 could be detected for both patient groups compared with healthy controls.

but not in healthy individuals. In addition, the secreted protein levels of IL-24 showed a significant increase for patients in season compared with healthy controls, whereas this difference is lost out of the allergen season. Current studies are initiated to validate the IL-10 family member IL-24 in large cohorts and may provide assistance to physicians to use immunomodulatory drugs in the treatment of rhinitis in anticipation of a Th2 response, for example, in wheezing newborns or in conditions where the allergic impact is not certain.

The antagonistic nature of the IL-4 and IFN-γ has been shown to depend on the crossregulation of *TBX21* and *GATA3* in lymphocyte differentiation. Both genes were previously detected in airway epithelial cells[33] and confirmed to be homogenously expressed in NHBEs in this study along with other essential signaling components IL4Rα and STAT6 (**Supplementary Information** and data not shown, respectively). Based on this finding we considered that a transcription network is underlying epithelial response profile of *GATA3* and *TBX21*. Strikingly, all IL-4-inducible, *GATA3*-co-clustered genes were downregulated by IFN-γ. *RUNX2* was previously described to be downregulated by IFN-γ in mesenchymal stem cells, whereas its inducibility by IL-4 is unknown so far.[34] As *RUNX2* enhances TGF-β-induced SMAD signaling, it appears possible that IL-4 induction of *RUNX2* promotes airway remodeling.[35] IL-4 signaling was also shown to synergize with TGF-β signaling that is enhancing the IL-4-inducible *FOXQ1* in alternatively (IL-4-) activated macrophages, also defined as "hidden type 2 inflammation".[36]

The IL-4-mediated induction of the *Aryl hydrocarbon receptor* (*AhR*) is confirming previous studies in B cells[37] and builds the bridge to the key function of *AhR* in inducing *IL22* (ref. 38,39) that in turn promotes epithelial repair mechanisms.[40,41] Furthermore, *AhR* is inhibiting *GATA3*,[42] and this could not only create a negative feedback loop, but also allow educt inhibition by IFN-γ-induced IDO metabolites such as kynurenine.[38,39,43]

Besides the connection of *GATA3* to the *AhR*, *GATA3* interconnects with many other IL-4- and IFN-γ-regulated genes, highlighting *GATA3* as hub gene, as it is known from the polarization network of T cells. *TBX21*, which is an equally important hub gene in T cells, is less interconnected in airway epithelial cells. In addition, the network analysis highlights that IL-4 also regulates hub genes *Meis1* and *Hey2* that are involved in transcriptional regulation of many genes, being a cofactor for *HOX* genes[44] and *HEY2* as a Notch-downstream target,[42] that in turn is known to regulate airway epithelial differentiation.[43,45] Future studies will have to confirm the role of these hub genes in the polarization of epithelial cells in Th2-driven diseases and airway remodeling.

Taken together, this study suggests that type 1-primed and type 2-primed airway epithelial cells exist and are pre-commissioned to respond along proallergic pathology. Future studies will need to define whether this behavior is epigenetically imprinted as it is the case for T cells and whether this knowledge can be used to exit proallergic disease conditions.

## METHODS

**Patients**. Healthy subjects ($n = 12$) and allergic patients in season ($n = 8$) aged between 20 and 65 years in good health (forced expiratory volume in one second 1% > 70%) with a history of clinically significant hay fever during the grass-pollen season since more than 2 years were included. Nasal scrapings were performed in the Allergy Section, Department of Otolaryngology, TUM School of Medicine (Munich, Germany). In addition, nasal secretions from healthy controls ($n = 14$) and allergic and asthmatic patients in and out of season ($n = 13$) were collected as previously described.[46] The full list of inclusion and exclusion criteria is available in the **Supplementary Information**. Each participant provided written informed consent. The study was approved by the local ethics committee.

**Cell culture**. Primary NHBEs (Lonza, Walkersville, MD) of six genetically independent donors were grown as monolayers in 100% humidity and 5% $CO_2$ at 37 °C in serum-free defined growth media (BEGM, Lonza). NHBEs (passage 3) were used at ~80% confluence in 6-well plates. To avoid gene expression changes or influences on the IL-4 or IFN-γ signaling induced by growth factors in the BEGM medium, cells were rested in basal medium (BEBM) for 12 h, then stimulated with recombinant human IL-4 at 50 ng ml$^{-1}$ (R&D Systems, Minneapolis, MN) and human IFN-γ at 10 ng ml$^{-1}$ (R&D Systems) in BEBM medium for 6 h at the indicated concentrations or medium alone or the same-time presence of both cytokines, IL-4 at 50 ng ml$^{-1}$ and IFN-γ at 10 ng ml$^{-1}$. For RNA analysis, harvested cells were lysed in RLT buffer (Qiagen, Hilden, Germany) containing 1% β-mercaptoethanol (Roth, Karlsruhe, Germany) directly in the cell culture well. For western blot analysis, cells were cultured in the same conditions, but for 6 h and an additional 24 h time point.

**RNA isolation and whole genome microarray**. Total RNA was extracted using RNeasy Mini Kit (Qiagen) with on-column DNase digestion (Qiagen) for avoiding DNA contaminations. RNA quantification and quality assessments were performed by ultraviolet–visible spectrophotometry (Nanodrop Technologies, Wilmington, DE) and the RNA 6000 Nano Chip Kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). The RNA quality of all samples reached a RNA integrity number (developed by Agilent Technologies) of 9.1. Total RNA was amplified and Cy3 labeled by using the one-color Low Input Quick Amp Labeling Kit (Agilent Technologies) according to the manufacturer's protocol. Hybridization to SurePrint G3 Human Gene Expression 8x60K Microarrays (Agilent Technologies) was performed with the Gene Expression Hybridization Kit (Agilent Technologies).

**Data analysis strategy**. Data analysis was performed using the Genespring Software GX 12.5 (Agilent Technologies) under minimal data reduction constraints (1.2-fold change and $P \leq 0.05$ cutoff). Upon data import a standard baseline transformation to the median of all values was performed, including log transformation and computation of fold changes ($\log2(A/B) = \log2(A) - \log2(B)$). Subsequently, a principle component analysis was conducted and revealed a homogenous component distribution. Compromised array signals (array spot is nonuniform if pixel noise of feature exceeds threshold or above saturation threshold) were excluded from further analysis. These threshold settings were verified looking at genes known to be regulated by the stimuli, e.g., HLADRs for IFN-γ or CCL-26 for IL-4.[37] Genes regulated more than 1.2-fold were further analyzed by using the paired Student's *t*-test and filtered for *P*-value ($P < 0.05$) that represents a rather permissive threshold. The significantly regulated genes were summarized in entity lists (see **Supplementary Tables**). These entity lists were analyzed for overlaps using Venn diagrams. Manhattan cityblock on entities (Ward's linkage) was used to cluster changes in gene expression. The functions of the identified genes were listed according to current literature using the Genomatix software (Munich, Germany) and GO terms 0003676 and 0044212 for transcription factors and GO terms 0007267, 0005125, 0008009, and 0005615 for identification of biomarkers. The gene expression network was generated using the Genomatix Pathway System (GePS) that integrates information extracted from public and proprietary databases to display canonical pathways or to create and extend networks based on literature data. Data displayed are focusing on binding sites of transcription factor families that were more than twofold regulated by IL-4 or IFN-γ extracted from the transcriptome data sets.

**Reverse transcription and quantitative real-time PCR**. Isolated total RNA was subjected to reverse transcription using a high-capacity cDNA kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Real-time PCR profiles were visualized using the commercially available FastStart Universal SYBR Green Mastermix (Roche, Basel, Suisse) and quantified by the ViiA 7 Real-Time PCR System (Applied Biosystems). The specific primers used in the real-time PCR are listed in **Supplementary Table S13**. The amount of *ANO-1*, *IL-22RA1*, *IL-24*, and *CCL-26* mRNA expression was normalized with endogenous control *18S*, *GAPDH*, and *β-actin* (housekeeping gene index, ΔCt values) and the relative quantification and calculation of range of confidence was performed using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method (relative gene expression) as previously described.[2] All amplifications were carried out at least in duplicate.

**Protein measurement by multi-array technology**. Protein levels of IL-24 in nasal secretions were detected in duplicates by MesoScaleDiscovery's (MSD) Multi-Array technology (MSD Mesoscale, Rockville, MD), a plate-based technology that enables the detection of biomarkers utilizing an electrochemiluminescence detection.

**Western blot analysis**. A Bradford assay was used to quantify amounts of protein concentrations of NHBE culture supernatants to ensure that equal amounts were loaded per lane of the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (for details see **Supplementary Information**).

**Immunofluorescent staining of NHBEs**. For immunofluorescent staining of NHBEs of three genetically independent donors, $15 \times 10^4$ cells were cultured on a sterilized, removable 12-well chamber (ibidi GmbH, Martinsried, Germany). At confluency of 50–60%, cells were fixed for 10 min with 4% paraformaldehyde at room temperature, followed by fixation with methanol, prechilled at − 20 °C and washed three times with phosphate-buffered saline. The primary antibody was diluted as 1:250 (IL-4Rα, R&D, Abdingdon, UK) and added overnight at 4 °C in a humid chamber. When incubation was finished, cells were washed again three times with phosphate-buffered saline and incubated with diluted secondary antibody (goat anti-mouse AlexaFluor 488, 1:300 (Life Technologies, Waltham, MA) with added DAPI D1306 (Life Technologies) for 90 min at room temperature in a humid chamber. After incubation, cells were washed three times and slides were covered with coverslips (Schott glass, Mainz, Germany) using VectaShield Mounting Medium (Vector Laboratories, Burlingame, CA). Slides were analyzed with LEICA confocal microscope (LEICA, Wetzlar, Germany).

**Statistical analysis**. Expression data of *in vitro* and *in vivo* gene expression have been log2 transformed. One-sample Wilcoxon rank-sum tests were used to determine significant changes in expression of *in vitro* samples of the real-time PCR analysis (R Statistical Programming Language, Vienna, Austria). For *in vivo* data, two-sample Wilcoxon-rank sum tests were used to test for significant differences between control and patient samples. Samples of each group are summarized taking the median. The fold change has been computed to get absolute differences. Mann–Whitney tests were used to determine significant changes of protein levels in nasal secretions. Statistically significant differences were defined as *P* values $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

**SUPPLEMENTARY MATERIAL** is linked to the online version of the paper at http://www.nature.com/mi

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**DISCLOSURE**
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## 4.2 Biomatrix for upper and lower airway biomarkers in patients with allergic asthma

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## Biomatrix for upper and lower airway biomarkers in patients with allergic asthma

*To the Editor:*

Although many biomarkers have been proposed for asthma monitoring, only the noninvasively accessible fraction of exhaled nitric oxide has been established into clinical routine, whereas implementation into guidelines is pending.[1] The upper and lower airways are considered a morphologic and functional unit and share air transport, physical barrier, mucociliary clearance, and immune interface as common features.[2] Furthermore, not only do allergic rhinitis and asthma share mechanisms of allergic inflammation, but also, transition of allergic rhinitis into asthma can represent a steady continuum depending on endotype. Therefore nasal secretions

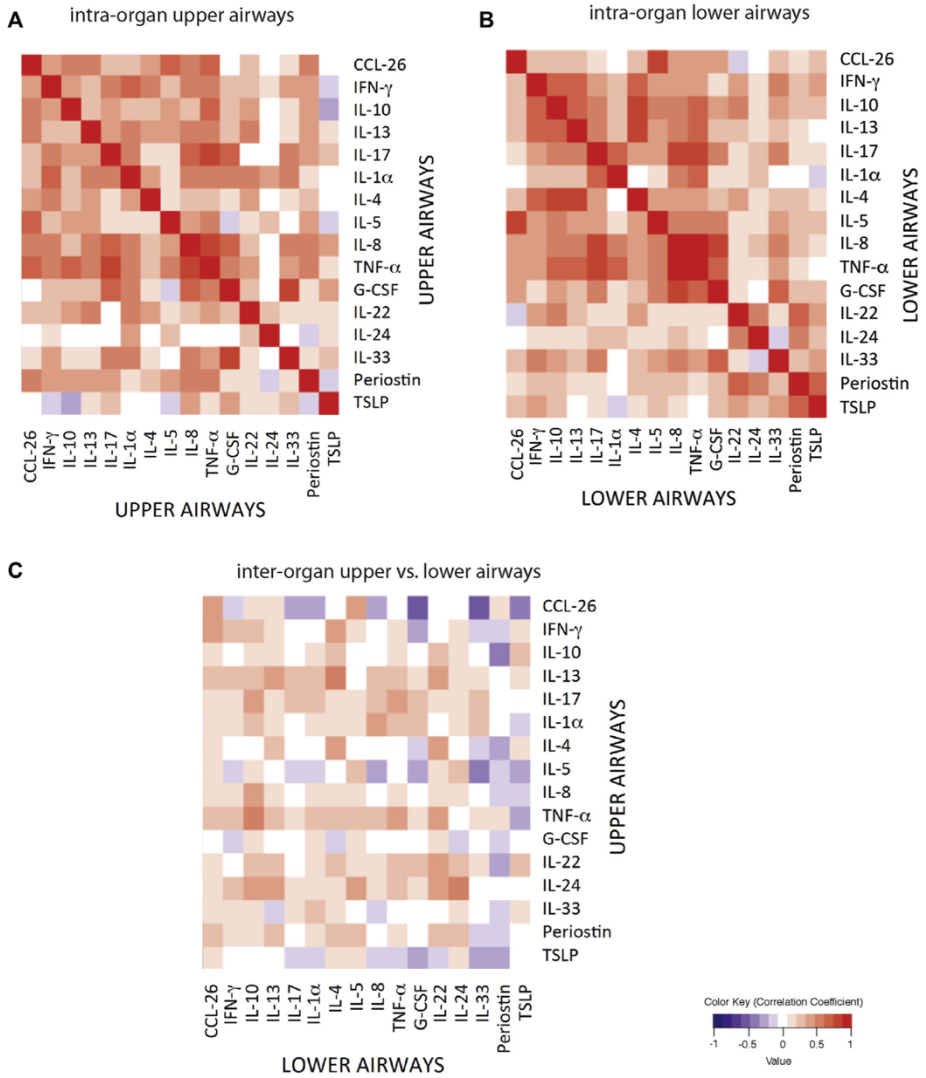


**FIG 1.** Biomarker grids reflecting correlations between upper and lower airways cytokine levels. Intraorgan correlations for upper **(A)** and lower **(B)** airways and interorgan correlations **(C)** were depicted by using respective correlation coefficients *(r)* from negative (dark blue) to positive (red) values. Intraorgan correlations seemed to be predominantly strong, whereas interorgan relations were most visible by using nasal and sputum IL-24 measurements. *G-CSF,* Granulocyte colony-stimulating factor; *TSLP,* thymic stromal lymphopoietin.

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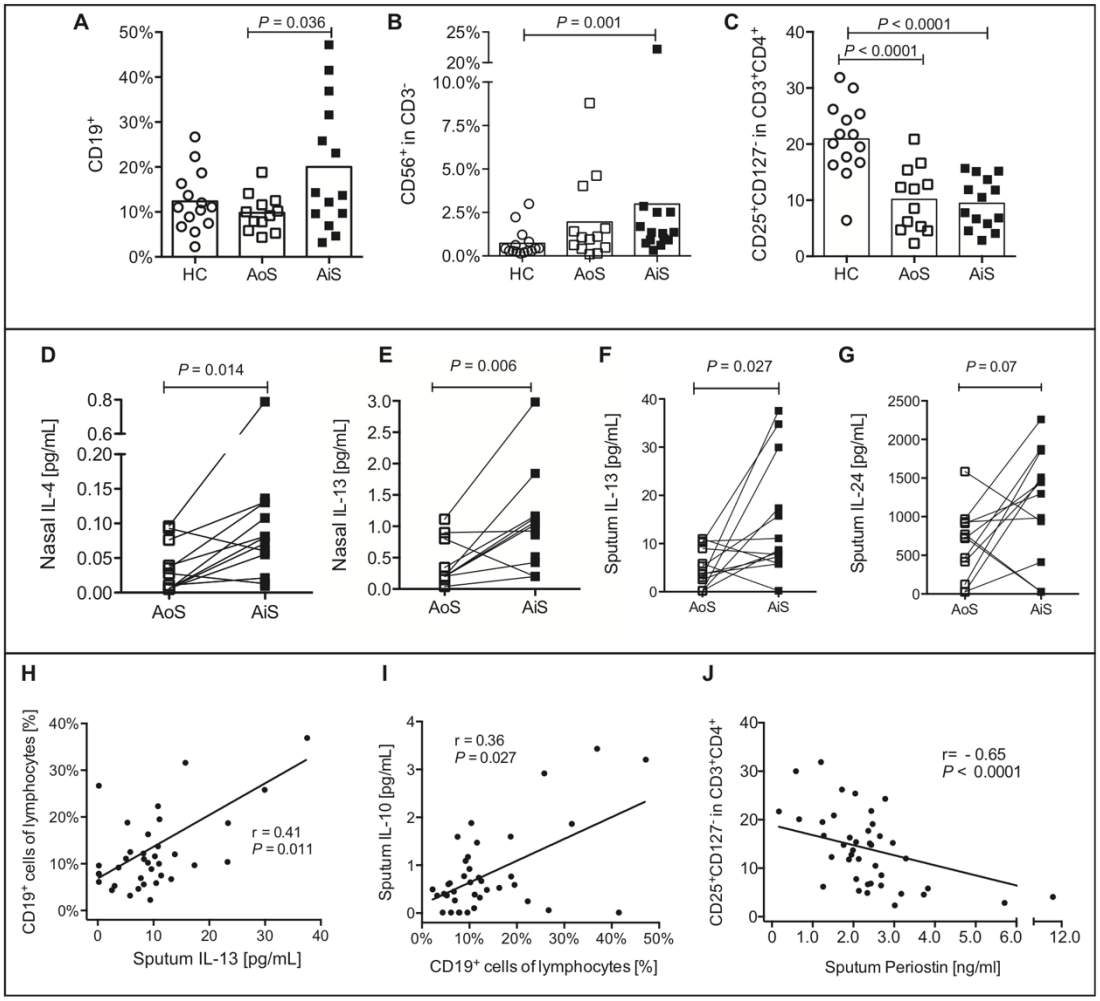


**FIG 2.** Airway cytokines and cell populations. **A-C,** Although numbers of B lymphocytes and natural killer cells were upregulated in asthmatic patients during the grass pollen season, in Treg cells there was only a difference between patients and healthy control subjects. **D-G,** Type 2 cytokines were differentially regulated in asthmatic patients out of the grass pollen season, as well as in the grass pollen season. **H-J,** Positive correlations were visible for B-cell counts and sputum IL-13 and IL-10 levels, respectively, whereas a strong negative correlation was seen for sputum periostin levels and Treg cell counts. *AiS,* Asthmatic patients in the grass pollen season; *AoS,* asthmatic patients out of the grass pollen season; *HC,* healthy control subjects.

of the upper airways can prove useful as noninvasive and easily accessible proxies mirroring lower airway inflammation.[3] The objective of the current study was to visualize the relationship of elementary protein biomarkers of the upper against the lower airways to identify potential nasal proxy candidates for the lower airways.

Nasal lining fluids and induced sputum were collected to find practical solutions to assess airway inflammation by using noninvasive methods because sputum opens another window to assess lower airway inflammation.[4] We included 15 patients with seasonal allergic asthma and 16 healthy participants, all of them nonsmokers, into the study (see Table E1 in this article's Online Repository at www.jacionline.org) and analyzed sputum supernatants and nasal secretions by using multiarray technology (see the Methods section in this article's Online Repository at www.jacionline.org). Furthermore, we assessed clinical scores as follows: Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ), Asthma Control Score (Global Initiative for Asthma [GINA]), and Perceived Stress Questionnaire (PSQ). Standard sputum differential cell counts (see Table E2 in this article's Online Repository at www.jacionline.org) and additional

sputum flow cytometry (fluorescence-activated cell sorting) were included.

To identify potential proxy biomarkers, we displayed the strength of the interaction of the biomarker signal of the upper and lower airways in a chessboard pattern (2-sided Spearman rank correlation; Fig 1). Known pathomechanisms, such as cellular source of biomarkers and sputum cell counts, were used to augment these patterns and enrich for best biomarker selection.

Levels of many cytokines and chemokines in sputum correlated moderately to strongly ($r > 0.5$) with other mediators of sputum (intraorgan comparison; Fig 1, *A* and *B*), whereas the correlation of these mediators between sputum and nasal secretions showed a low to moderate relationship (interorgan comparison, $r < 0.5$). These correlations are mainly seen diagonally (Fig 1, *C*), which displays the correlation of one cytokine in the lower airways to the same cytokine in the upper airways. IL-24 seemed to be of special interest as a proxy because it showed moderate correlations between the upper and lower airways ($r = 0.53$, $P = .001$; Fig 1, *C*, and see Table E3 in this article's Online Repository at www.jacionline.org). Interestingly, the GINA score showed a strong negative correlation to nasal IL-24 levels ($r = -0.85$, $P = .001$). Furthermore, sputum IL-24 levels were increased in asthmatic patients in season (1084.0 ± 199.6 pg/mL) compared with out of season (641.2 ± 131.7 pg/mL; $P = .07$; Fig 2, *G*). Flow cytometric analysis of cellular components in sputum revealed a moderate negative correlation of $CD25^+/CD127^-$ regulatory T (Treg) cell counts to sputum IL-24 levels ($r = -0.50$, $P = .002$). We speculate that this decrease in numbers of Treg cells could be caused by $T_H2$-mediated suppression at the site of inflammation. This interpretation is supported by the finding that numbers of Treg cells in asthmatic patients were diminished independently of season (Fig 2, *C*). From a mechanistic point of view, this result supports our previous findings showing that type 2 immune responses inhibit induction of regulatory cell.[5]

As expected, correlation of levels of one cytokine with those of one of the other mediators was observed frequently in the intraorgan correlation grid (Fig 1, *A* and *B*); for example, levels of the type 2 cytokine IL-5 correlated with levels of the type 2 IL-4–inducible CCL-26 (eotaxin-3; nasal: $r = 0.726$, $P < .0001$; bronchial: $r = 0.771$, $P < .0001$). A moderate correlation was also observed for nasal CCL-26 and nasal IL-13 levels ($r = 0.57$, $P < .0001$). It is well known that IL-13 is able to seasonally promote IgE and $IgG_4$ production.[6] In fact, a weak correlation of sputum IL-13 levels and sputum $CD19^+$ B-cell counts was observed ($r = 0.41$, $P = .011$; Fig 2, *H*). In general, the proportion of total B cells in sputum within asthmatic patients showed a significant increase during the grass pollen season (20.03% ± 3.85%) compared with off season (9.80% ± 1.18%; $P = .036$; Fig 2, *A*).

Levels of the regulatory cytokine IL-10 moderately correlated with TNF-α expression levels in both the upper and lower airways ($r = 0.38$, $P = .024$; Fig 1, *C*) and even more so in the intraorgan comparison (lower airways: $r = 0.61$, $P < .0001$; upper airways: $r = 0.61$, $P < .0001$; Fig 1, *A* and *B*). Sputum IL-10 levels correlated weakly with the presence of $CD19^+$ B cells ($r = 0.36$, $P = .027$) in sputum (Fig 2, *I*). IL-10 in airway lining fluid can originate from previously described IL-10–producing regulatory B cells, which are capable of regulating type 2 inflammation or promoting the IgE to $IgG_4$ switch.[6] Furthermore, regulatory B cells are known to play a pivotal role in allergen-specific immunotherapy.[7] Surprisingly, we observed weak to moderate correlation for nasal IL-10 expression with the perceived stress burden of asthma patients assessed by PSQ score ($r = 0.42$, $P = .01$; see Fig E1 in this article's Online Repository at www.jacionline.org), which showed a strong correlation with GINA score ($r = 0.78$, $P = .003$) and moderate correlation with RQLQ score ($r = 0.58$, $P < .0001$). Nasal IL-10 levels also negatively correlated with total sputum leukocyte count ($r = -0.65$, $P < .0001$), which is considered a marker for inflammation in the lower airways (see Table E2). Although IL-10 levels in the upper airways can display clinically relevant marker functions, it shows only few or even negative (eg, periostin) correlations between the upper and lower airways.

Periostin has been proposed as a biomarker for comorbid chronic rhinosinusitis in patients with asthma, revealing its relevance in $T_H2$-driven diseases also in patients with local tissue inflammation.[8] Expression pattern differed from markers because it was mostly correlated negatively with other mediators, including itself, when compared with the upper airways. This negative interaction was not observed in intraorgan comparison with one exception, namely IL-1α. The important role of periostin in the lower airways is implied by the finding that periostin expression in sputum negatively correlated with $CD25^+CD127^-$ Treg cell ($r = -0.65$, $P < .0001$; Fig 2, *J*) and positively with sputum eosinophil ($r = 0.36$, $P < .034$) counts, whereas RQLQ scores seemed to be associated to levels of lower airway periostin ($r = 0.38$, $P = .021$; see Fig E1). Taken together, local periostin might prove useful to assess disease progression in the lower airways, but our data suggest periostin to be a less favorable proxy in the upper airways because of its negative relationship to the lower airways.

In conclusion, the concept of united airways and consequently representative nasal proxy biomarker analytes holds particularly true for type 2 cytokines, specifically IL-24, which we have previously shown to be an epithelial type 2 cytokine.[1] Periostin and IL-10 levels correlate moderately with clinical symptoms and might be of limited use as proxy biomarkers because of the conflicting correlations between mediators of the upper and lower airways. These findings provide not only promising biomarker candidates but also correlation matrices, which might be reflective of underlying asthma endotypes. Larger clinical studies are needed to fully assess the validity and practability of cytokine measurements in nasal lining fluid to characterize asthmatic patients.

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## Genetically engineered cell factories produce glycoengineered vaccines that target antigen-presenting cells and reduce antigen-specific T-cell reactivity

*To the Editor:*

Allergen-specific immunotherapy is a promising approach to reduce or remove allergic symptoms by inducing tolerance.[1] As an alternative to current allergy vaccines derived from extracts of natural allergens, recombinant vaccines offer the opportunity for *de novo* antigen design,[2] with enhanced targeting to antigen-presenting cells (APCs) and potentially increased ability to induce tolerance.[3,4]

Using the type 1 allergen Bet v 1 as our model antigen, we evaluated the effects of combining a nonglycosylated antigen to a glycomodule[5] to target the APC-expressed family of C-type lectin receptors (CLRs). CLRs are known to recognize glycan structures and promote rapid internalization of antigens on binding (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org).[6,7] We used our recently developed glycoengineered cellular platform[8] to produce Bet v 1 with defined carbohydrates and evaluated APC uptake, efficacy using patient-derived T cells, and induction of tolerance in a murine model.

Currently, allergen-specific immunotherapy is primarily administered subcutaneously or sublingually. Focusing on human skin, we examined the CLR expression pattern in skin-localized APCs. We observed expression of the major CLRs, including mannose receptor (MR), dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin (DC-SIGN), and macrophage galactose-type lectin (MGL), in the dermal compartment of skin biopsy specimens (see Fig E1, *B*). Next, we examined expression of MR, DC-SIGN, and MGL in *in vitro*–differentiated human monocyte-derived dendritic cells (moDCs) and macrophages (M1 and M2) by means of transcriptomics and flow cytometry (see Fig E1, *C-E*). Expression of CLRs peaked after 3 days of differentiation, with simultaneous and significant expression of DC-SIGN, MR, and MGL in moDCs (see Fig E1, *D* and *E*). Cytokine measurements confirmed the nature of the APCs (see Fig E1, *F*) and their relevance as a cellular model.

To identify the optimal carbohydrate structure or structures for specific APC targeting, we evaluated uptake of chemically synthesized carbohydrate derivatives (N-acetylgalactosamine polyacrylamide [GalNAc-PAA], N-acetylglucosamine–PAA, Lewis X–PAA, and mannose-PAA) and a GalNAc glycosylated molecule with varying numbers of GalNAc residues (see Fig E2, *A* and *B*, in this article's Online Repository at www.jacionline.org). Both mannosylated and high-density GalNAc glycosylated structures were readily taken up by the APCs, and therefore GalNAc and mannose structures were selected for further studies.

We next designed Bet v 1 allergen constructs for the expression of full-length Bet v 1 fused to glycomodules, with consensus glycosylation motifs ensuring the incorporation of either O- or N-linked glycans. The fusion constructs were expressed in glycoengineered Chinese hamster ovary (CHO) cell lines and *Pichia pastoris* (Fig 1, *A*, and see Fig E2, *C-G*, and Table E1 in this article's Online Repository at www.jacionline.org). This strategy allowed us to generate fusion proteins decorated with high-mannose N-linked glycans from MGAT1 knockout CHO cells, linear α1-2 O-linked mannose residues from *P pastoris* (Pichia), or O-GalNAc residues from COSMC knockout CHO cells for efficient targeting of MR, DC-SIGN, and/or MGL on APCs (Fig 1, *A*, and see Fig E2, *C-G*).

Additionally, we expressed the O-glycan fusion construct in *Escherichia coli* and inserted GalNAc residues by means of *in vitro* glycosylation. Glycosylation profiles were verified by using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) of released glycans supplemented with lectin profiling (see Fig E2, *C-G*). As a control for glycan-mediated effects, we treated fractions of the glycoallergens with periodate, a treatment known to effectively destroy lectin recognition (see Fig E2, *E*).

Because moDCs were the only cell type to express high levels of DC-SIGN, as well as MR and MGL, we used moDCs to evaluate uptake of glycan-modified Bet v 1 fusion proteins. Internalization of all glycan-modified Bet v 1 fusion proteins was enhanced 1.5- to 6-fold compared with that of their

## 4.3 TGF-β1 drives inflammatory Th Cell but not Treg cell compartment upon allergen exposure

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TGF-β1 is known to have a pro-inflammatory impact by inducing Th9 and Th17 cells, while it also induces anti-inflammatory Treg cells (Tregs). In the context of allergic airway inflammation (AAI) its dual role can be of critical importance in influencing the outcome of the disease. Here we demonstrate that TGF-β is a major player in AAI by driving effector T cells, while Tregs differentiate independently. Induction of experimental AAI and airway hyperreactivity in a mouse model with inducible genetic ablation of the gene encoding for TGFβ-receptor 2 (Tgfbr2) on CD4+T cells significantly reduced the disease phenotype. Further, it blocked the induction of pro-inflammatory T cell frequencies (Th2, Th9, Th17), but increased Treg cells. To translate these findings into a human clinically relevant context, Th2, Th9 and Treg cells were quantified both locally in induced sputum and systemically in blood of allergic rhinitis and asthma patients with or without allergen-specific immunotherapy (AIT). Natural allergen exposure induced local and systemic Th2, Th9, and reduced Tregs cells, while therapeutic allergen exposure by AIT suppressed Th2 and Th9 cell frequencies along with TGF-β and IL-9 secretion. Altogether, these findings support that neutralization of TGF-β represents a viable therapeutic option in allergy and asthma, not posing the risk of immune dysregulation by impacting Tregs cells.

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# TGF-β1 Drives Inflammatory Th Cell But Not Treg Cell Compartment Upon Allergen Exposure

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TGF-β1 is known to have a pro-inflammatory impact by inducing Th9 and Th17 cells, while it also induces anti-inflammatory Treg cells (Tregs). In the context of allergic airway inflammation (AAI) its dual role can be of critical importance in influencing the outcome of the disease. Here we demonstrate that TGF-β is a major player in AAI by driving effector T cells, while Tregs differentiate independently. Induction of experimental AAI and airway hyperreactivity in a mouse model with inducible genetic ablation of the gene encoding for TGFβ-receptor 2 (*Tgfbr2*) on $CD4^+$T cells significantly reduced the disease phenotype. Further, it blocked the induction of pro-inflammatory T cell frequencies (Th2, Th9, Th17), but increased Treg cells. To translate these findings into a human clinically relevant context, Th2, Th9 and Treg cells were quantified both locally in induced sputum and systemically in blood of allergic rhinitis and asthma patients with or without allergen-specific immunotherapy (AIT). Natural allergen exposure induced local and systemic Th2, Th9, and reduced Tregs cells, while therapeutic allergen exposure by AIT suppressed Th2 and Th9 cell frequencies along with TGF-β and IL-9 secretion. Altogether, these findings support that neutralization of TGF-β represents a viable therapeutic option in allergy and asthma, not posing the risk of immune dysregulation by impacting Tregs cells.

**Keywords: TGF-beta, Th2, Th9, Th17, asthma, allergen-specific immunotherapy, induced sputum**

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## INTRODUCTION

The three isoforms of transforming growth factor-β (TGF-β), TGF-β1, TGF-β2 and TGF-β3 in mouse and human, encoded by separate genes, are involved in a plethora of biological processes during development, lineage commitment, wound healing, proliferation, migration and survival of cells (1). Each isoform and the TGF-β receptors (TGF-βR) are expressed in specific and temporal

**Abbreviations:** AA, allergic asthma; AAI, allergic airway inflammation; AHR, airway hyperreactivity; AIT, allergen-specific immunotherapy; AR, allergic rhinitis; BAL, Bronchoalveolar lavage; BALF, Bronchoalveolar lavage fluid; ICI, Inflammatory cell infiltrate; i.p., intraperitoneal; Mch, metacholin; n.d., not detectable; OVA, ovalbumin; PBMC, peripheral blood mononuclear cell; PBS, Phosphate-buffered saline; mRQLQ, Rhinoconjunctivitis Quality of Life mini Questionnaire; tIgE, total Immunoglobulin E; Tregs, regulatory T cells; Th, T helper cells.

patterns making their functions strongly context-dependent for various tissues and cell types (2). TGF-β was first described as suppressor of T cell proliferation (3). Within the immune system TGF-β signaling was later found to have essential roles in the T cell, B cell and phagocyte compartments, among others. Initially it was thought that TGF-β was a checkpoint molecule of T cell-mediated autoimmune inflammatory disease, mediated by Treg dependent (4) and independent (5) mechanisms. However, later it was found that this phenomenon was dependent on a lymphopenic environment (6, 7). TGF-β is now known to be involved, and in some cases essential, for the development of regulatory T (Treg) cells, for differentiation and lineage commitment of Interleukin-17 producing T helper (Th17) cells (mouse only), of follicular T helper (Tfh) cells (human only) and of IL-9 producing T helper (Th9) cells (8, 9). Furthermore, TGF-β is an important inducer of integrins, particularly of CD103 in $CD8^+$ resident T memory cells (10), Th1 cells (11), and Treg cells (12, 13), which makes it also a regulator of cell migration during and after inflammation. The various outcomes of TGF-β signaling are achieved through combinatorial sensing of additional cytokines (such as IL-6, IL-1, IL21 in combination with TGF-β for Th17 induction), hence result from specific local cytokine milieus (14).

Allergic diseases are characterized by an uncontrolled immune reaction towards harmless environmental antigens to which the body is exposed either *via* airways, as seen in allergic rhinitis (AR) and allergic asthma (AA), *via* skin (atopic dermatitis), gastrointestinal tract (food allergy) or by systemic exposure (anaphylaxis). In healthy individuals, allergen exposure is tolerated. Loss of T cell tolerance towards environmental antigens is a prerequisite for initiation of an allergic reaction and can lead to activation of Th2 cells, humoral (IgE) effector mechanisms as well as infiltration of inflammatory cells at the site of allergen exposure. At these tissue sites, increased mucus production (15), smooth muscle (16) and airway epithelial cell activation (17) are observed, subsumed as airway hyperreactivity (AHR).

In airway inflammation TGF-β1 is involved in tissue remodeling (18), yet all isoforms are expressed throughout the normal lung, including expression by bronchial epithelium, macrophages, vascular endothelium, smooth muscle and fibroblasts (19, 20). TGF-β is also a core inducer of the epithelial-mesenchymal transition process during fibrotic remodeling of airways (21). Both, TGF-β1 and TGF-β2 have been shown to be increasingly expressed during airway hyperreactivity (AHR), especially in eosinophils (22) and macrophages (23); in case of TGF-β2, in epithelium (24) and neutrophils. The amount of TGF-β1 in bronchoalveolar lavage (BAL) is increased in AA and both TGF-β1 and -2 levels are increased upon segmental allergen challenge of the lung (25). TGF-β is part of the regulatory mechanisms of Tregs (26), which can keep the potentially pathogenic IL-4-producing Th2 cells under control and thus avoid AAI (27). The production of IL-4 by Th2 cells is also observed in healthy individuals (28). Therefore, IL-4 cannot be alone acting as a disease checkpoint. In contrast, IL-9-producing T helper cells (Th9) were recently identified as critical subset in AA (29). In fact, increased IL-9-secretion was observed in BALF and lung tissue of AA patients (30). Allergen-induced-IL-9 was described to directly induce mucus production (31), support *de novo* mast cell generation and their proliferation *in situ* (32). It further serves as chemoattractant for recruitment of inflammatory cells to sites of inflammation (33). For Th9 cells both TGF-β-dependent (34) and –independent (35, 36) mechanisms of induction were described. TGF-β can directly induce Th9 cells without the need of another T-cell subset by epigenetic mechanisms (37, 38). Importantly, TGF-β primes together with IL-4 the production of IL-9 in $PPAR\gamma^+$ Th2 cells (39), as also shown for the induction of Th17 and Treg cells (40).

Beyond systemic autoimmune inflammation, TGF-β signalling was found to be essential for pathological T cell-mediated effects in a variety of diseases, such as experimental autoimmune encephalomyelitis, a multiple sclerosis model (7). Further, in lymphopenic disease induced by early abrogation of TGF-β signalling in T cells a pronounced involvement of the lung was a consistent observation (6, 7). Nevertheless, while this was connected to the extreme situation of lymphopenia, it remained an open question to which extent the TGF-β pathway would be involved in the homeostasis of T cells in AAI. To address this question, we used our system for inducible ablation of TGF-βRII in Th cells and interrogated the role of TGF-β signaling in a mouse model of AAI. While we confirmed a role of TGF-β signaling in T cells not only for the disease course but also in the development or recruitment of inflammatory T cells in AAI, we found astonishingly little involvement in Treg homeostasis. To test whether similar observation could be made in human rhinitis patients we took advantage of induced sputum as a non-invasive window to lung pathology in allergic patients and in context of allergen-specific immunotherapy (AIT). We confirmed opposite regulation of TGF-β with respect to IL-4 and IL-9 by disease and treatment and found no influence of local TGF-β signaling on Treg cells. Taken together, in contrast to our expectation, TGF-β signaling had a direct influence on pathogenic T cell compartments. This appeared to be directly responsible for the establishment of AIT mediated tolerance. In contrast, Treg cells did not show dependence on TGF-β in airway inflammation in mouse and human.

## RESULTS

### Reduced Airway Hyperreactivity and Lung Cell Infiltration in Allergic iCD4TGFBR2 Mice

We used OVA-induced AAI in a mouse model to address the question whether TGF-β is the cytokine that tips the balance between inflammatory or regulatory T cells upon allergen exposure. Because of the requirement of TGF-β for development of these pro and anti-inflammatory cell types it was an open question whether abrogation of TGF-β signaling would lead to an ameliorated or worse AAI disease course. We restricted our analysis to the time period after the sensitization

phase, to exclude the known effects of TGF-β signalling on T cell receptor signal strength during the priming phase of our experiment. In our setup we compared presence versus absence of TGF-β signaling by use of tamoxifen-facilitated Cre-loxP-mediated deletion of *TGFBR2* in $CD4^+$ cells (**Figure 1A**). These iCD4TGFBR2 mice (7) were compared to wildtype (WT) C57BL/6/J animals. Twenty-four hours after the last allergen challenge, we observed decreased AHR to methacholine in absence of TGF-β signaling (**Figure 1B**), showing a role of this pathway in T cells during the effector phase of AAI. The reduction of disease upon *Tgfbr2* ablation went along with significantly decreased number of leukocytes, specifically eosinophils, neutrophils, and lymphocytes but not macrophages in BAL, with the most prominent change in the eosinophil population (**Figures 1C, D**). PAS-staining of lungs revealed decreased perivascular and peribronchiolar inflammatory cell infiltration (**Figures 1E, H**) and lower mucus hypersecretion following AAI upon *Tgfbr2* ablation (**Figures 1E, I**), confirming the ameliorated clinical outcome. These local changes to the severity of AAI by abrogation of TGF-β signaling in $CD4^+$ T cells were reflected in serum, in which the levels of total IgE (**Figure 1F**) and OVA-specific IgE (**Figure 1G**) were drastically reduced. Without induction of AAI, induced Th cell specific *Tgfbr2* ablation did not result in any of the above-described outcomes, hence highlighting the critical role of TGF-β signaling during the challenge phase of established AAI.

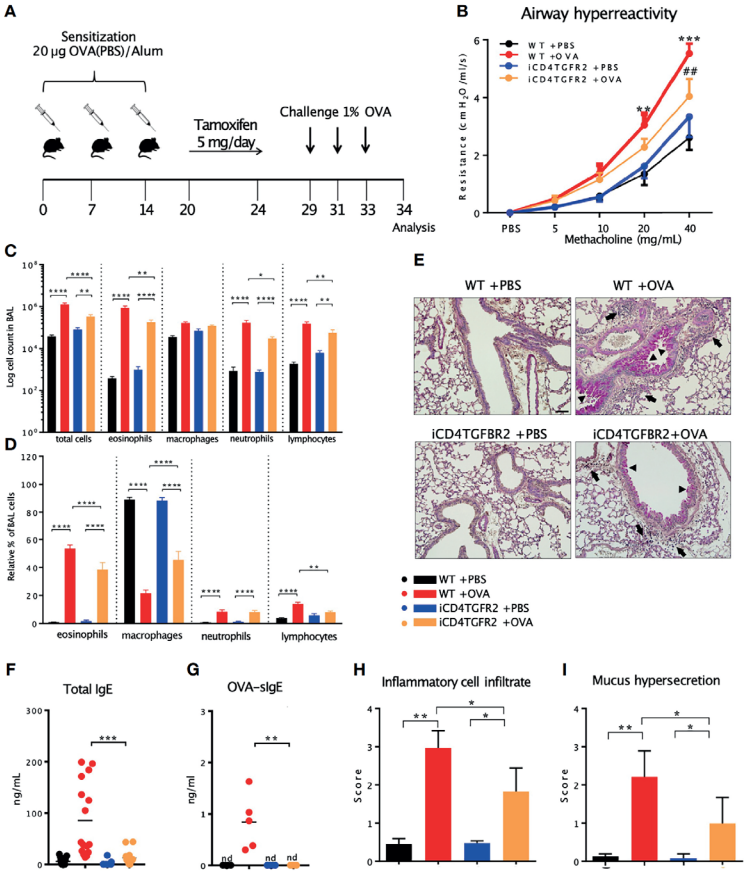


**FIGURE 1** | Impact of *Tgfbr2* ablation on lung inflammatory infiltrate and AHR. **(A)** Scheme of AAI induction, challenge and tamoxifen treatment. **(B)** Airway hyperreactivity measured in intubated, mechanically ventilated animals following methacholine provocation. n= 5 - 9/group; $^{**}p < 0.01$; $^{***}p < 0.001$ vs WT+PBS; $^{\#\#}p < 0.01$ vs WT+OVA at same methacholine concentrations (two-way analysis of variance (ANOVA) with Bonferroni's *post-hoc* test). **(C)** BAL total cell number and **(D)** relative population size of mice from the four experimental groups. **(E)** PAS-staining of lung sections from the four experimental groups. Arrows: inflammatory infiltrate; arrowheads: mucus hypersecretion; scale bar: 50 µm. **(F, G)** Levels of total and OVA-specific Immunoglobulin E (tIgE: n=11-16/group; OVA-sIgE: n=5/group) measured in plasma samples (two-tailed Mann-Whitney U test). **(H, I)** Histological scores of **(H)** Inflammatory cell infiltrates and **(I)** mucus production (mean ± SD; n=5/group). **(C, I)** Representative of three independent experiments each with 5 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, nd, not detected.

## Cytokine and T Cell Response Upon Ablation of *Tgfbr2* in CD4$^+$T Cells During AAI

To better understand how T cell-specific abrogation of TGF-β signaling before the challenge phase was ameliorating AAI, we determined cytokine levels in BAL fluid. As expected, AAI resulted in a local increase of all measured cytokines in WT animals (**Figure 2A** and **Table S1**). In absence of *Tgfbr2* signals within Th cells we observed significantly less IL-4, -5, -6, -9, -17A, IFN-γ (**Figure 2A** and **Table S1**), and TNF-α (**Figure S1A**), while IL-1β, CXCL-1, CXCL-2, CCL-2, and CCL-3 showed the same trend but did not reach statistical significance (**Figure S1A**). Similarly, serum levels of IL-9 appear lower following *Tgfbr2* ablation in allergic animals (**Figure S1C**), but this difference did not reach significance. IL-2, IL-10, and IL-13 levels in BAL remained unaffected by *Tgfbr2* ablation (**Figure 2A**).

Analysis of the specific cytokine response by splenocytes after *in vitro* restimulation with OVA revealed significantly reduced production of IL-2 and IL-5 after *Tgfbr2* ablation. IL-4, IL-6, and IL-9 appear to show the same direction of response (**Figures S1D–E**). The specificity of these immunological effects is supported by the unchanged TNF-α secretion (**Figure S1E**).

We next assessed whether the changes in the cytokine levels found in BAL and serum were associated with the numbers of the respective T cell types located in the lung. We found decreased frequencies of GATA3$^+$ Th2 cells and RORγt$^+$Th17 following *Tgfbr2* ablation in allergic animals (**Figure 2B**). Also, the number of T cells expressing IRF4, a transcription factor active in various T cell lineages but absolutely required for Th9 development (41, for review see: 42), was reduced. However, the number of IL-9$^+$ T cells was only slightly decreased. Both, AAI induction or *Tgfbr2* ablation alone led to an increased fraction of FOXP3$^+$ Tregs in the lung (**Figure 2B**), as would be expected from a previous report (7, 43, 44). Unexpectedly, we did not find an additive or synergistic effect of combined AAI induction and *Tgfbr2* ablation (**Figure 2B**) with respect to Treg cell numbers in the lung.

We also found the fraction of lung CD8$^+$ T cells to be increased in absence of TGF-β signaling in CD4$^+$ T cells, while CD4$^+$ T cells themselves were unchanged, as also the γδ T-cell percentages (**Figure S1F**).

Taken together, abrogated TGF-β signaling resulted in reduced responses of the Th2, Th9, and Th17 lineages during AAI while Treg cells expanded independently of AAI.

## Gene Network and Transcriptional Analysis

To further understand T cell differentiation in allergy, a gene interaction network was assembled (**Figure 3A**). Key

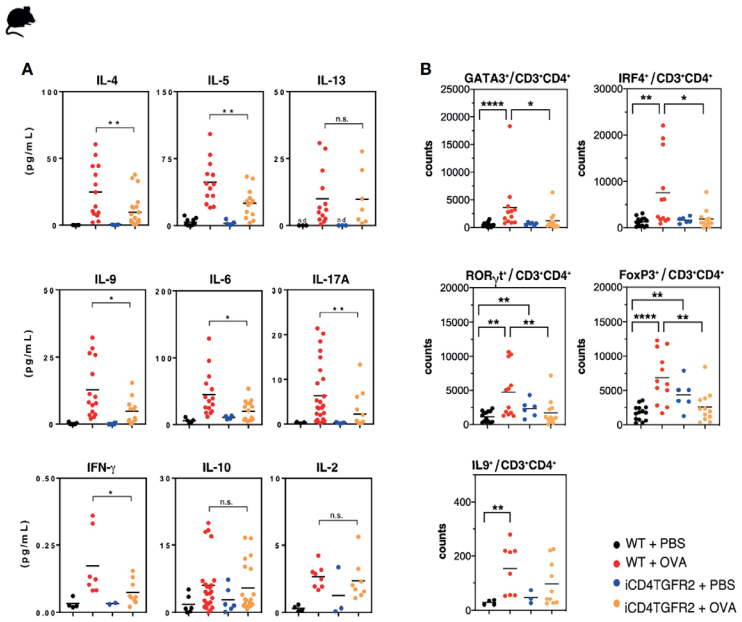


**FIGURE 2** | Impact of *Tgfbr2* ablation on lung cytokine production and T cell subsets. **(A)** BAL fluid of the indicated experimental groups was analyzed using electrochemiluminescence assay. Each data point represents an individual mouse. Data is compiled from one or two independent experiments (n=5-15/group). *p<0.05, **p<0.01, n.s. not significant (t-test). **(B)** Flow cytometric analysis of lungs focusing on distinct T cell subsets, performed 24h after the last OVA-challenge. Total Th2 (GATA3$^+$), Th9 (IRF4$^+$; IL-9$^+$), Th17 (RORγt$^+$) and Treg (FoxP3$^+$) cells within the CD3$^+$CD4$^+$T cell population in non-allergic WT+PBS and iCD4TGFBR2 +PBS mice and allergic WT+OVA and iCD4TGFBR2+OVA mice were assessed. Each data point represents an individual mouse. Data are representative of two independent experiments (n=6-13/group). *p < 0.05, **p < 0.01, ****p < 0.0001; n.s, not significant (two-tailed Mann-Whitney U test).

transcription factors of pro-inflammatory T cell subsets that directly or indirectly relate to TGF-β signaling are IRF-4 and PU.1, whereby IRF-4 is known to synergize with PU.1 (50). Th2, Th9 and Th17 are not only linked by IRF4 and SMAD3, but also FOXO1 and FOXO2 are regulating pro-inflammatory T cell subsets (55). *Pu.1* appears to be essential for *IL9* expression (49), and IRF4 can interact with NFATc2 to enhance *Il-4* gene expression (66) and with SMAD-3 to promote *Il9* expression (67). The IL-4-and TGF-β-inducible factors BLIMP-1 (not shown) inhibits IL-2 (68) and IL-9 (69). IRF4 also facilitates expression of the *IL-17* gene (52). To assess the different components of this network we performed expression analysis on CD4$^+$ T cells from lungs of animals of our four experimental groups. Allergic inflammation enhanced the protein levels of IL-4,

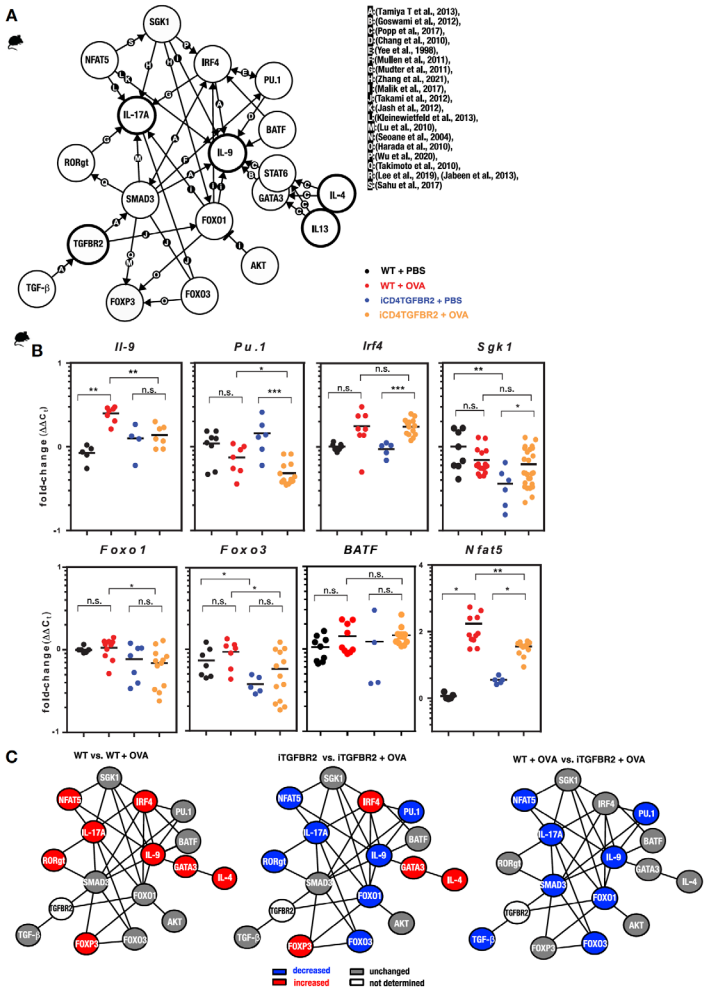


**FIGURE 3** | Impact of *Tgfbr2* ablation on Th-subset transcription factors and signal molecules. **(A)** Illustration of the complex interaction of genes in the regulation of T cell differentiation in allergy. The network indicates known molecular interactions or dependencies (either direct binding or gene regulation) and was inspired by the ImmunoNet (immunet.princeton.edu) (45) and extended manually by systematic literature research. The current knowledge was compiled in a gene regulation network of $T_H2$, $T_H9$ and $T_H17$ networks. The references for the network were manually added and are as follows: [a: (46), b: (47), c: (48), d: (49), e: (50), f:(51), g: (52), h (53), i: (54), j: (55), k: (56), l: (57), m: (58), n: (59), o: (60), p: (61), q: (62), r: (63), (64), s: (65)]. **(B)** CD4$^+$ T cells were isolated from digested lungs of non-allergic and allergic WT and iCD4TGFBR2 mice and analyzed for IL-9 expression as well as $T_H9$ and $T_H2$ relevant transcription factors and signal molecules using quantitative RT-PCR. Expression levels of *Il9, Pu.1, Irf4, Sgk1, Foxo1, Foxo3, Batf* and *Nfat5* were normalized to *Gapdh* house-keeping gene and relative changes were represented as $2^{-\Delta\Delta CT}$ ($\Delta\Delta C_T = \Delta C_T - \Delta C_{Control}$). Data is compiled from two independent experiments (n= 5-13/group). *p < 0.05, **p < 0.01, ***p < 0.001, n.s. not significant (t-test). **(C)** Comparisons of gene regulation network of T cells. Blue indicates reduced gene expression, while red indicates increased and gray unchanged gene expression of the respective factor for the respective comparison.

IL-9, IL-13 and IL-17 (**Figure 2A**) and mRNA levels of *Il9*, *Nfat5*, whereas *Foxo1* and *Foxo3*, *Spi1* (coding for PU.1), *Sgk1* and *Batf* remained unchanged (**Figure 3B**). Ablation of *Tgfbr2* decreased expression of *Il9*, *Pu.1*, *Foxo1*, *Foxo3* and *Nfat5* in diseased animals and of *Sgk1* and *Foxo3* in control animals. The current knowledge of gene regulation networks of pro-inflammatory Th2, Th9, and Th17 cells is overlapping or linked and some of these factors are connected to TGF-β signaling (**Figure 3C**). To obtain a schematic overview on gene regulation we assessed mRNA levels of these factors in the lungs with and without induction of AAI. Allergic inflammation in WT animals enhanced expression level of some Th2, Th9, Th17, and Treg and actors including IRF4, while the Th9 transcription factor PU.1 remained unaffected (**Figure 3C**, left). Allergic inflammation in *Tgfbr2*-ablated animals decreased the expression of Th17 and Th9 factors, while Th2 and Treg factors remained unchanged (**Figure 3C**, center and right) compared to WT (left). Interestingly, the IRF4 increase induced by allergic inflammation (left) is not affected by *Tgfbr2* ablation (center and right) despite the relationships of IRF4 to the TGF-β signaling.

Taken together, ablation of *Tgfbr2* seems to affect the transcriptional network of Th17 and Th9 cells, leaving the Th2 and Treg networks largely untouched.

## Effect of AIT on TGF-β and T Cell Lineage Specific Cytokines in Induced Sputum of Allergic Patients

In our AAI mouse model, we observed very specific effects of *Tgfbr2*-ablation on cytokine production and respective T cell lineages in the lung and proved the influence of TGF-β on local allergic airway reactions in established AAI. To confirm the relevance of these results for human allergy, we next investigated immune cells of the lower airways of rhinitis patient without and with asthma comorbidity in the context of AIT. Our cross-sectional cohort consisted of 26 healthy controls and 38 allergic rhinitis patients (AR), 19 of these with asthma comorbidity (AA; **Table S4**). Ten AR patients and nine AA patients received AIT, while nine untreated AR and ten untreated AA were assigned to the untreated groups.

In induced sputum samples from our patient group, we observed that AIT reduced back to baseline the elevated levels of TGF-β found both for AR and AA patients *in season* of natural pollen exposure (May-July, **Figure 4A** and **Table S5**). We also observed that sputum levels of TGF-β1 showed a positive correlation with total IgE (r=0.3488, $p$=0.0189; data not shown). Hence, allergic patients under AIT reveal a situation like the one artificially obtained by *Tgfbr2* ablation in the mouse model. AIT reduced back to baseline the cytokines IL-4, IL-5, IL-9, and IL-13, thus the classical mediators of Th2 and Th9 cells respectively (**Figure 4B** and **Table S5**). The levels of IL-2, IL-6, IL-10, and IFN-γ, hence pan-T cell, inflammatory, Treg, and Th1 cytokines, respectively, showed the opposite reaction with higher sputum levels upon AIT (**Figure 4B**). While the effect sizes were smaller, these observations were confirmed *out of season* (October-January, **Figure S2A**).

Taken together, following AIT in AR and AA patient airways TGF-β levels are reduced in parallel with those of Th2 and Th9 cytokines.

## Decreased Th2 and Th9 and Increased Treg Cells Upon AIT

Since TGF-β has distinct effects dependent on specific tissue context, we quantified the frequency of Th2 cells ($GATA3^+$ $CD3^+CD4^+$), Th9 cells ($IL9^+CD3^+CD4^+$), and Treg cells ($FoxP3^+$ $CD3^+CD4^+$) in induced sputum samples and PBMCs by flow cytometry (**Figure 5**). *In season*, frequencies of sputum Th2 and Th9 cells were strongly reduced in treated patients compared to untreated patients, both in sputum (**Figure 5C**) and in blood (**Figure 5E**). Percentages of Treg cells were, however, increased after AIT (**Figures 5C, E**). Hence, for all three T cell lineages frequencies were basically normalized by AIT. Similar effects, albeit less pronounced, could be observed *out of season* (October-January) of natural pollen exposure (**Figures 5D, F**). Surprisingly, *out of season* the amelioration of Th2 frequencies by AIT in sputum was very small in contrast to the Th9 frequency change (**Figure 5D**), while in blood the effect size remained large (**Figure 5F**). The size of the Th2 and Th9 subsets in sputum and blood presented only weak or no correlation to serum IgE levels (**Figures 5G, I**), whereas the symptom score mRQLQ, most relevant as therapy outcome parameter, was strongly positively correlated to the size of both Th subsets (**Figures 5H, J**). These results demonstrate that AIT modulates the local and systemic abundance of pro-allergic Th2 and Th9 cell subtypes, while Treg cells and local TGF-β were increased.

# DISCUSSION

The exact role of the TGF-β pathway during allergic airway disease awaits further delineation. With the available data TGF-β can be postulated to play contradictory roles, pro- and anti-inflammatory. This unclarity about TGF-β ´s role in AAI may be one reason why the pathway has so far been excluded from consideration as a drug target. Therefore, our study was aimed at dissecting these contradictory pro- and anti-inflammatory roles of TGF-β with a focus on inflammatory and regulatory T cells at the site of allergic airway inflammation. We started our investigation by using a murine model of AAI with induced *Tgfbr2* ablation in $CD4^+$ T cells (7). We demonstrate that mice with *Tgfbr2* ablation induced between sensitization and challenge phase presented reduced clinical features of AAI, confirmed by strongly ameliorated histological and functional disease parameters. Hereby, the study shows that during AAI TGF-β-signaling in $CD4^+$ T cells is an important factor for allergogenic Th2, Th9 and Th17 cell activity *in vivo*, while the Treg compartment remained largely unaffected. Th2, Th9 and Th17 cell recruitment upon allergen challenge (70) was reduced in absence of TGFBR2 along with respective transcription factors PU.1 (Th9), GATA3 (Th2), and NFAT5. NFAT5 is an osmosensitive transcription factor that negatively regulates

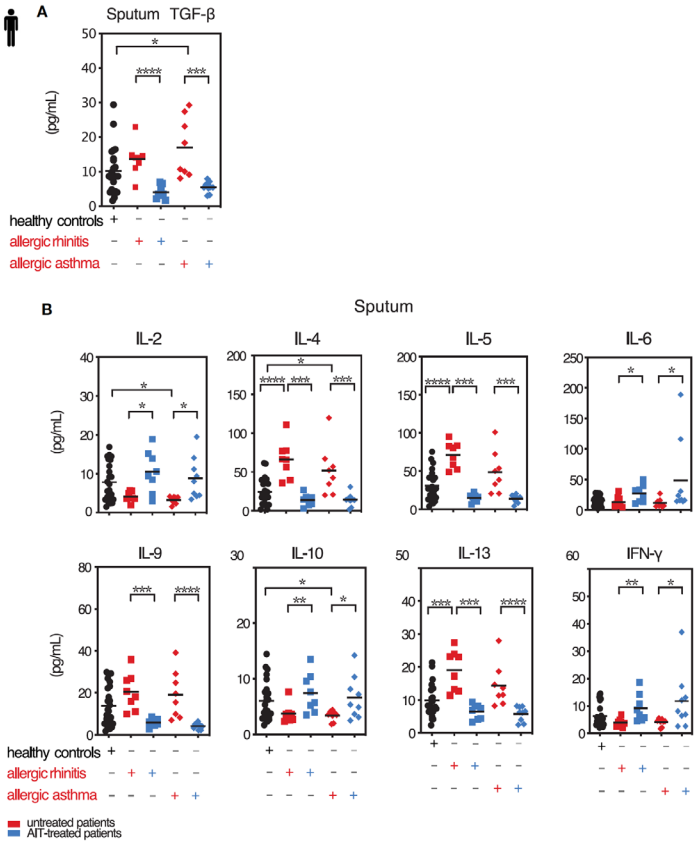


**FIGURE 4** | Local TGF-β is decreased along with pro-inflammatory cytokines upon AIT. Levels of selected cytokines in sputum of treated and untreated AR and AA patients and healthy controls. **(A)** Secreted TGF-β cytokine levels in induced sputum of allergic rhinitis and allergic asthma patients receiving (blue colored) or not (red colored) AIT as well as of control individuals were analyzed by LEGENDplex. Data presented by individual values and mean. **(B)** Levels of secreted cytokines IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 and IFN-γ detected in sputum supernatants of the groups as in **(A)** assessed by LEGENDplex. Data presented by individual values and mean. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; statistical significance was determined by Kruskal-Wallis tests and only when medians across patient groups varied significantly, multiple single comparisons were performed using two-tailed Mann-Whitney U tests.

IL-17 and IFN-γ gene expression (71) and enhances Th2 response (72). Moreover, the transcription factor FOXO1, which was recently described to be essential for Th9 cell differentiation and IL-9 production, was clearly reduced upon *Tgfbr2* ablation, correlating with reduced airway allergy (54, 73, 74). This specific impact of *Tgfbr2* depletion on T cell subsets during AAI was not limited to the respiratory system, but also extended to a systemic response measured in splenocytes. In line with these reduced sizes of pathologically relevant T cell lineages, disease activity was also ameliorated in terms of infiltrating myeloid cells and production of total and specific IgE. Treg cells were shown to be able to suppress AAI in various models (75, 76). Yet, in our model of induced ablation of TGF-β signaling on T cells we did not observe an effect on the size of the local and systemic Treg compartments during AAI. Hence, it appears likely that the Treg compartment size is not responsible for the reduced disease severity and associated cellular and molecular changes in *Tgfbr2*-ablated mice. Another example of such Treg independent suppression of AAI was reported for treatment with the TLR2 agonist Pam3CSK4, although it involved different mechanisms as seen by us (77).

Taken together, absence of TGF-β signaling by $CD4^+$ T cells after sensitization did not interrupt the immune control by the Treg cell compartment, while we clearly confirmed a major role for the Th9-, Th2-, and Th17-driven allergic response upon challenge. A limitation of our study is that that we could not restrict abrogation of TGF-β signaling to all the relevant allergogenic T cell lineages or Treg cells. Also, as we found first indications of reduced CD103 expression, an integrin regulating tissue-residency (10–13), this may reduce migration of T cells to

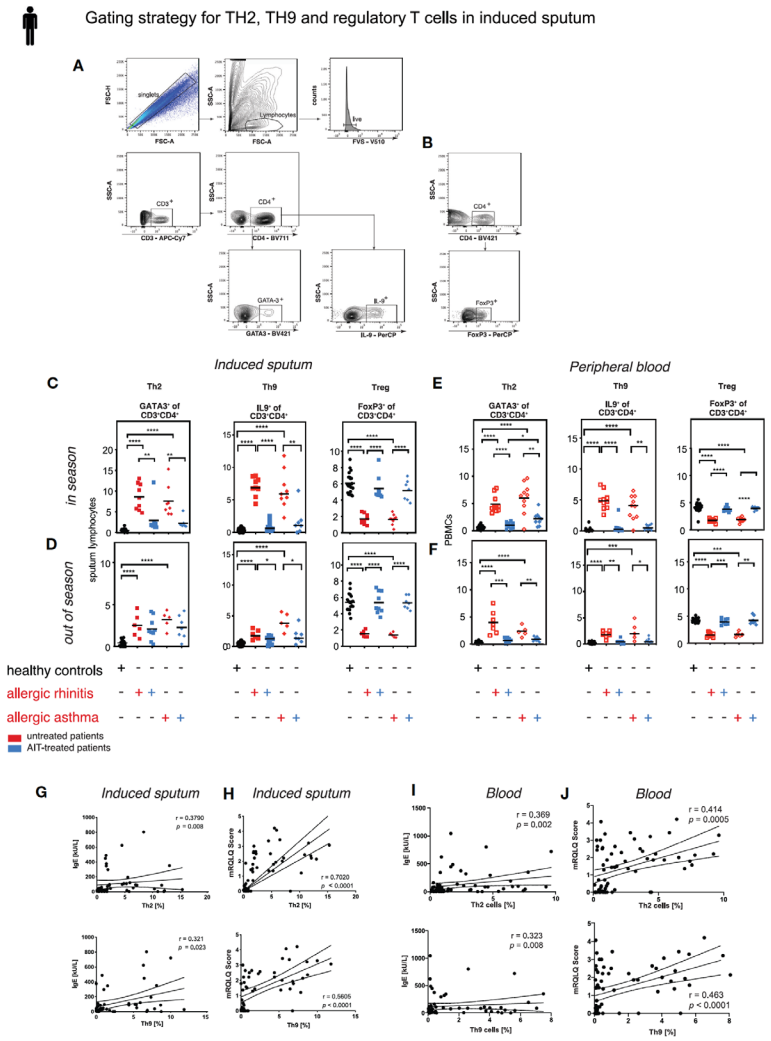


**FIGURE 5** | Treg cells increase and Th9 as well as Th2 cells decrease upon AIT. Th2, Th9, and Treg cell frequencies in sputum of treated and untreated AR and AA patients and healthy controls. **(A)** Gating strategy for Th2 and Th9 and **(B)** Treg cells in induced sputum. **(C, D)** Flow cytometric analysis of sputum derived T cell subpopulation Th2, Th9 and Tregs in healthy subjects, AR and AA patients as described in *in season* **(C)** and *out of season* **(D)**. **(E, F)** Flow cytometric analysis of peripheral blood derived T cell subpopulation Th2, Th9, and Treg cells in healthy subjects, AR and AA patients as described in *in season* **(E)** and *out of season* **(F)**. Data presented by single patient values and mean. Statistical significance was determined by Kruskal-Wallis tests and only when medians across patient groups varied significantly, multiple single comparisons were performed using two-tailed Mann-Whitney U tests. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. **(G, H)** Correlation of sputum $T_H2$ and $T_H9$ cell frequencies with total serum IgE **(G)** and symptom score mRQLQ **(H)**. **(I, J)** Correlation of peripheral blood derived $T_H2$ and $T_H9$ cell frequencies with total serum IgE **(I)** and symptom score mRQLQ **(J)**. Two-sided Spearman test was used to calculate the correlations.

the inflamed lung and their retention (78). Future studies are needed to explore the role of integrins in TGF-β dependent T cell populations and bone marrow chimaeras could dissect direct versus indirect effects of *TGFBR2* ablation on the differentiation of inflammatory T cell populations.

Similarly, to the murine AAI model, in human patients suffering from AR, treatment with AIT shows decreased levels of sputum TGF-β along with the pro-inflammatory type-2 cytokines. We took advantage of this treatment-induced TGF-β-modulation and monitored Treg, Th2, and Th9 cells. In the

AIT treated patients we demonstrate that the seasonally induced levels of Th2/Th9 cytokines were strongly decreased, locally and systemically, while Th1, general inflammatory cytokines, as well as the suppressive cytokine IL-10, were increased. These observations were mirrored by the respective population sizes, again locally and systemically. While local sputum cytokine levels were not yet investigated in context of AIT, it was previously shown that AIT controls IL-9 expression in the upper airways (79), as well as Th2 frequency in biopsies (79) and peripheral blood (28). Interestingly, Th2 cells were also detectable in sputum of health individuals both *in*- and *off-season*. This finding confirms our previous studies, in which we reported IL-4 mRNA and secreted IL-4 protein at low, but detectable levels in the airways of healthy individuals (28).

While the presence of Treg cells was previously detected in sputum (80) we were surprised that Treg cells constituted the most abundant T cell subpopulation in the sputum of healthy individuals *in* and *off season*. Furthermore, untreated AR and AA patients consistently displayed strongly reduced Tregs frequency compared to healthy individuals even *off-season*. In contrast, the AIT treatment restored Treg cells percentages, locally and systemically, even though TGF-β levels were reduced by AIT even below levels of healthy individuals. This result indicates that AIT induces a special condition of immunosuppression and is not restoring natural allergen tolerance in the first years of treatment. Overall, it appears that TGF-β plays a major role in the allergic response but not its suppression. Although the study was not powered to quantify treatment success, the symptom load was reduced by AIT treatment and correlated strongly with the local, and somewhat less with the reduced systemic percentages of Th2 and Th9 cells.

A reduction of Tr1 cells in the context of allergic airway disease has been reported previously and may be represented in our dataset by the reduction in IL-10 in induced sputum of allergic patients (81).

Taken together, our data demonstrate that TGF-β is a major player in allergic airway inflammation by reprogramming T cells into effector T cells. Since Treg cells remain unaffected (mouse) or are even increased (patients with AIT) by reduced levels of TGF-β signaling, targeting of the TGF-β pathway might therefore not only suppress tissue-remodeling processes but also target pro-inflammatory T cells, without the risk of affecting immune tolerance adversely.

# METHODS

## Animals and Murine Model of AAI

C57Bl/6J mice, originally obtained from Charles River, and CD4Cre$^{ERT2}$*Tgfbr2*$^{fl/fl}$ mice (iCD4TGFBR2) were bred at the animal facility at the Institute of Comparative Medicine of the Helmholtz Centre Munich. Generation and characterization of iCD4TGFBR2 mice was described elsewhere (7). All mice were co-housed under specific pathogen-free conditions in individually ventilated cages (VentiRack) and fed by standard pellet diet (Altromin Spezialfutter GmbH & Co. KG) and water *ad libitum*. Both female and male mice aged 6-8 weeks were used for the experiments. To induce AAI an established ovalbumin-sensitization model was used (82). Briefly, iCD4TGFBR2 and WT C57Bl/6J mice were sensitized by i.p.-injections of 20 µg ovalbumin (OVA; grade V; Sigma-Aldrich) in phosphate buffered saline (PBS) adsorbed to aluminum hydroxide (2.5 mg, ImjectAlum) on days 0, 7 and 14. Non-allergic control mice received the same volume of PBS in alum. The assignment to the two different groups occurred randomly. On days 29, 31 and 33 all mice were aerosol-challenged for 20 minutes with 1% OVA in PBS delivered by a Pari-Boy nebulizer (Pari), (82). The experimental protocol is depicted in **Figure 1A**. Blood samples were taken before sensitization and at the end of the experiment. Animals were sacrificed twenty-four hours after the last OVA challenge. The study was conducted under federal law and guidelines for the use and care of laboratory animals and was approved by the Government of the District of Upper Bavaria and the Animal Care and Use Committee of the Helmholtz Center Munich (approval number: 55.2-1-54-2532-75-2012).

## Tamoxifen Treatment

To ablate the subunit 2 of the heterodimeric TGF-β receptor, mice were treated with tamoxifen (TM, tamoxifen-free base, Sigma-Aldrich) after the sensitization phase. Tamoxifen was suspended in 100% ethanol to 1 g/ml, vortexed, and mixed with corn oil (Sigma-Aldrich) to a final concentration of 100 mg/ml. Before *in vivo* administration, the solution was heated to 37°C until it was properly dissolved. On day 20-24, 50 µl (5 mg) tamoxifen per day were administered by intra-gastric gavage in both iCD4TGFBR2 and WT control mice (7).

## Measurement of Airway Hyperreactivity

AHR to methacholine (Mch; Sigma-Aldrich) was measured 24 h after the last OVA challenge in intubated, mechanically ventilated animals (n = 6–10/group; Data Sciences International (DSI), as previously described (83). Briefly, animals were anesthetized by an intraperitoneal injection of Ketamine (100 mg/kg) and Xylazine (5 mg/kg) in PBS. After cannulation of the trachea and starting mechanical ventilation, the animals were challenged with increasing methacholine (Mch) concentrations, using an in-line nebulizer (5 µl Mch solution in PBS delivered for 30 seconds at the following concentrations: 0, 5, 10, 20 and 40 mg/ml). Data were recorded using the FinePoint software v2.4.6 (DSI). The highest values of respiratory system resistance (R) were recorded every 5 seconds during the data recording interval set at 3 min after each Mch level. The heart rate of each animal was continuously monitored using an ECG device connected with three subcutaneous electrodes throughout the entire experiment (DSI).

## Analysis of Bronchoalveolar Lavage, Lung Histology and Serology

BAL and evaluation of inflammatory cell infiltration were performed as described previously (82). Aliquots of cell-free BAL fluid were used to measure cytokines and chemokines *via* mesoscale technique using two different kits (V-Plex proinflammatory panel 1 mouse kit and V-Plex cytokine panel

1 mouse kit; MesoScaleDiscovery) according to manufacturer's instructions. Total and ovalbumin-specific IgE were measured in serum samples by ELISA as described previously (84). IL-9 was measured in serum samples using mouse-IL-9 ELISA (BioLegend). For lung histology, after BAL, the lungs were excised and the left lobe fixed in 4% buffered formalin and embedded in paraffin. Sections of 4µm thickness were stained with hematoxylin-eosin (H&E) and periodic acid Schiff (PAS). Mucus hypersecretion and inflammatory cell infiltration were graded in a blinded fashion on a scale from 0 to 4 (0=none, 1=mild, 2=moderate, 3=marked, 4=severe), reflecting the degree of the pathological alteration (82).

## Isolation and Analysis of Leukocytes From Lung Tissue

Lungs were excised, cut into small sized pieces and digested in RPMI medium supplemented with 100 µg/ml DNAse (Sigma-Aldrich) and 1 mg/ml Collagenase Type 1A (Sigma-Aldrich) at 37°C. Digested lungs were filtered through a 70 µm cell strainer, pelleted (400 G, 4°C, 5 min) and resuspended in 6 ml 40% percoll in RPMI (v/v) solution, which was underlayed with 4 ml 80% percoll solution (GE Healthcare-Life Sciences). Tubes were centrifuged (1600 G, RT, 15 min) with brake set to 0. Lymphocytes were collected from the interphase and analyzed by flow cytometry.

## Isolation and Restimulation of Splenocytes

Spleens were excised and single cell suspensions were obtained and re-stimulated as previously described (85). Cells were washed and re-suspended in complete medium [RPMI 1640 supplemented with 10% FCS, 1% glutamine, 1% penicillin-streptomycin, 1% Na-pyruvate, 1% non-essential amino acids (Gibco, Life Technologies GmbH) and 50µM 2-β-mercaptoethanol (Sigma-Aldrich)], plated in 96-well at a concentration of $2x10^5$ cells/well and cultured for 72 h with medium alone or with OVA V (5 µg/ml; Sigma-Aldrich). Their supernatants were analyzed for cytokine expression using the V-Plex proinflammatory panel 1 mouse and V-plex IL-9 kit (MesoScaleDiscovery) according to manufacturer's instructions.

## Patients

Specimen were taken from 26 healthy controls and 38 AR patients, of whom 19 received AIT (**Table S4**). All subjects completed a Rhinoconjunctivitis Quality of Life mini Questionnaire (mRQLQ), a lung function test and sputum induction. GINA scores were assessed from asthmatic subjects. Each participant provided written informed consent. The study was approved by the local ethics committee (5534/12). Among AIT-treated patients, 9 patients were additionally affected by asthma, 10 suffered of AR only. Asthmatic patients are represented as a subgroup of the rhinitis patients, if not otherwise indicated. Patients were considered as asthmatic based on previous physician's diagnosis and with a reported history of shortness of breath, cough, chest tightness during natural allergen exposure and/or earlier documented positive bronchodilation test. All patients were in good health (FEV1% >70%) with a history of clinically significant hay fever during the grass-pollen season since more than two years. Patients of the AIT-treated groups received at least one year of AIT treatment. Thereby, 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 since >2 years during the grass-pollen season, a positive skin prick test wheal >3mm in diameter and grass pollen specific IgE-level above 0.70kU/L underwent subcutaneous grass-pollen AIT.

In addition, total IgE was measured from sera of all individuals included in this study. Peripheral blood samples from all subjects included in this study were drawn at the same time points as sputum samples and were analyzed by flow cytometry. For flow cytometric analysis, CPT tubes (BD biosciences) were centrifuged according to manufacturer's instructions. Further, each sample was adjusted to $2.0x10^6$ cells and used for subsequent FACS staining. All procedures were performed in the Allergy Section, Department of Otolaryngology, TUM School of Medicine, Munich, Germany.

## Sputum Collection, Processing and Characterization

Collection and processing of sputum as well as differential cell counts was performed as previously described (86). Briefly, human participants first inhaled salbutamol and consecutively nebulized hypertonic saline at increasing concentrations of 3%, 4%, and 5% NaCl every 7 min. During this procedure, participants cleaned their noses and rinsed their mouth to reduce squamous epithelium cells in the samples. Sputum was processed within one hour of collection. The selected sputum plugs, which contained as little saliva as possible, were placed in a weighed Eppendorf tube and processed with 4x weight/volume of sputolysin working solution (Merck). Afterwards, 2x weight/volume of PBS was added. Samples were filtered through a 70 µm mesh and centrifuged for 10 min at 790 x g without break to remove the cells. Supernatants were stored at −80°C until further analysis. In addition, sputum cell slides were prepared for differential cell counts. Sputum samples were successfully collected from healthy controls (n=24; 92.3%) and allergic patients (n=34; 89.4%) once in and out of grass pollen season as previously described (86) and analyzed for secreted protein levels of TGF-β, IL-9, type-2 cytokines IL-4, IL-5, and IL-13, IL-10, type-1 cytokines IL-2, IFN-γ, and IL-6. Cytokine levels were analyzed by LEGENDplex TGF-β1 and multiplex human inflammation panel assay (BioLegend). All nine parameters were detectable in every sputum sample derived from patients with AR and AA and compared to healthy control subjects.

## Lung Function Testing in Humans

Baseline lung function was evaluated using a calibrated handheld pulmonary function testing device (Jaeger SpiroPro). The following parameters were recorded: vital capacity (VC), forced expiratory volume (FEV1), FEV1/VC, and maximum expiratory flow 25% (MEF 25%). Bronchodilator reversibility was tested after 400 µg of salbutamol.

## Total Serum IgE Measurement

Total serum IgE was assessed using diagnostic ImmunoCAP assays on a Phadia 100 device.

## Flow Cytometric Analysis of Human and Murine Samples

Human sputum cell or PBMCs samples were labeled without stimulation for flow cytometry with specific antibodies using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer's instructions.

For flow cytometric analyses of the murine samples, staining for transcription factors was performed by using Foxp3 Staining Buffer Set (eBiosciences), while cytokine staining was performed by using the fixation/permeabilisation solution Kit (BD Bioscience Cytofix/Cytoperm$^{TM}$) according to the manufacturer's protocols. For intracellular cytokine staining, cells were stimulated for 4h with 50 ng/ml PMA (Applichem), 500 ng/ml Ionomycin (Thermo Fisher Scientific) and 1:1000 GolgiPlug (BD Bioscience). Flow cytometric analysis was performed using a BD LSRII Fortessa flow cytometer (BD Bioscience). Flow cytometric data of both human and mouse samples were analyzed with FlowJo software (FlowJo). For gating strategy of human sputum cells ($0.5x10^6$ cells) or PBMCs ($2.0x10^6$ cells) samples see **Figures 5A**, **S2B**; the murine gating strategy is shown in **Figure S1**. Antibodies used for flow cytometry are listed in **Table S2** (murine) and **Table S6** (human).

## Real-Time Polymerase Chain Reaction

Total RNA was extracted either from mouse total lung tissue after homogenization using the RNeasy Mini Kit (Qiagen GmbH) or from enriched mouse lung $CD4^+$ T cells (CD4 T cell isolation kit, Miltenyi Biotec, Auburn, CA, USA), using RNeasy Micro Kit (Qiagen) according to supplier's instructions. RNA was reverse-transcribed directly (RevertAid H Minus First Strand cDNA Synthesis Kit, Thermo Scientific) and quantitative real-time PCR was performed using SYBR Green PCR Kit Master Mix (Qiagen) and the LightCycler®480 System (Roche) as previously described (83). The used primer sequences are listed in **Table S3**. Each reaction was performed in duplicate in 284-well plates (Applied Biosystems) and turned into mean. The expression levels were normalized to GAPDH house-keeping gene and relative changes were represented as $2^{-\Delta\Delta CT}$ ($\Delta\Delta C_T = \Delta C_T - \Delta C_{Control}$).

## Data Acquisition and Statistical Analysis

All experimental procedures and analyses were conducted by blinded research staff. For the mouse experiments, differences between the groups in AHR were evaluated with two-way analysis of variance (ANOVA) with Bonferroni's *post-hoc* test. Otherwise, differences between two data sets were evaluated using unpaired two-tailed Mann-Whitney test or t-test. Data were expressed as mean ± S.D., if not otherwise indicated. All statistically significant differences were depicted as *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Data were analyzed using Prism software version 6 (GraphPad software Inc.). The network map indicates known molecular interactions or dependencies (either direct binding or gene regulation) and was inspired by the ImmunoNet (immunet.princeton.edu) (45) and extended manually by systematic literature research. For the analysis of human samples, non-parametric statistical test were chosen, as the data points were not normally distributed. For **Figures 4**, **5**, 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. Two-sided Spearman correlation was used to correlate IgE or RQLQ with immune cell frequencies in **Figure 5**.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

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. The animal study was reviewed and approved by Government of the District of Upper Bavaria and the Animal Care and Use Committee of the Helmholtz Center Munich (approval number: 55.2-1-54-2532-75-2012).

## AUTHOR CONTRIBUTIONS

SM, FA, SH, TB, CS-W, and UZ developed the study and experimental layout. SM, FA, ES, BS, and JG realized experimental disease models and measurements of murine parameters. FA, SH, and TB supported the study on ethical permissions and funding. AC organized study part including human ethical approval and together with JK and MP the management of patient visits, patient information and sampling. Human samples were analyzed by IG, JU, JK, MP, FG, and UZ. The manuscript was written by SM, FA, TB, and CS-W as well as UZ. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.763243/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## 4.4 Sputum microRNA-screening reveals Prostaglandin EP3 receptor as selective target in allergen-specific immunotherapy

Constanze A. Jakwerth, Adam M. Chaker, Ferdinand Guerth, Madlen Oelsner, Lisa Pechtold, Lynn S. zur Bonsen, Julia T. Ullmann, Susanne Krauss-Etschmann, Anna Erb, Josephine Kau, Mirjam Plaschke, Marlene Winkler, Alexandra Kurz, Antonia Kloss, Julia Esser-von Bieren, Carsten B. Schmidt-Weber, Ulrich M. Zissler.

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Background: Several microRNAs (miRs) have been described as potential biomarkers in liquid biopsies and in the context of allergic asthma, while therapeutic effects on the airway expression of miRs remain elusive. In this study, we investigated epigenetic miR-associated mechanisms in the sputum of grass pollen-allergic patients with and without of allergen-specific immunotherapy (AIT).
Methods: Induced sputum samples of healthy controls (HC), AIT-treated and -untreated grass pollen-allergic rhinitis patients with (AA) and without asthma (AR) were profiled using miR-microarray and transcriptome-microarray analysis of the same samples. miR-targets were predicted in silico and used to identify inverse regulation. Local PGE2 levels were measured using ELISA.
Results: 259 miRs were upregulated in the sputum of AA patients compared to HC, while only one was downregulated. The inverse picture was observed in induced sputum of AIT-treated patients: while 21 miRs were downregulated, only 4 miRs were upregulated in asthmatics upon AIT. Of these 4 miRs, miR-3935 stood out, as its predicted target PTGER3, the prostaglandin EP3 receptor, was downregulated in treated AA patients compared to untreated. The levels of its ligand PGE2 in the sputum supernatants of these samples were increased in allergic patients, especially asthmatics, and downregulated after AIT. Finally, local PGE2 levels correlated with ILC2 frequencies, secreted sputum IL-13 levels, inflammatory cell load, sputum eosinophils and symptom burden.
Conclusions: While profiling the sputum of allergic patients for novel miR expression patterns, we uncovered an association between miR-3935 and its predicted target gene, the prostaglandin E3 receptor, which might mediate AIT effects through suppression of the PGE2-PTGER3 axis.

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ORIGINAL ARTICLE

Asthma and Rhinitis

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# Sputum microRNA-screening reveals Prostaglandin EP3 receptor as selective target in allergen-specific immunotherapy

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## Abstract

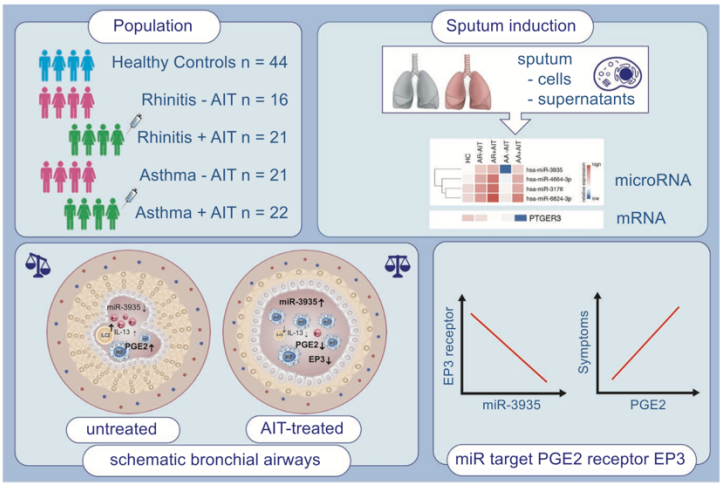
**Background:** Several microRNAs (miRs) have been described as potential biomarkers in liquid biopsies and in the context of allergic asthma, while therapeutic effects on the airway expression of miRs remain elusive. In this study, we investigated epigenetic miR-associated mechanisms in the sputum of grass pollen-allergic patients with and without allergen-specific immunotherapy (AIT).

**Methods:** Induced sputum samples of healthy controls (HC), AIT-treated and -untreated grass pollen-allergic rhinitis patients with (AA) and without asthma (AR) were profiled using miR microarray and whole-transcriptome microarray analysis of the same samples. miR targets were predicted *in silico* and used to identify inverse regulation. Local $PGE_2$ levels were measured using ELISA.

**Results:** Two hundred and fifty nine miRs were upregulated in the sputum of AA patients compared with HC, while only one was downregulated. The inverse picture was observed in induced sputum of AIT-treated patients: while 21 miRs were downregulated, only 4 miRs were upregulated in asthmatics upon AIT. Of these 4 miRs, miR-3935 stood out, as its predicted target *PTGER3*, the prostaglandin $EP_3$ receptor, was downregulated in treated AA patients compared with untreated. The levels of its ligand $PGE_2$ in the sputum supernatants of these samples were increased in allergic patients, especially asthmatics, and downregulated after AIT. Finally, local $PGE_2$ levels correlated with ILC2 frequencies, secreted sputum IL-13 levels, inflammatory cell load, sputum eosinophils and symptom burden.

**Conclusions:** While profiling the sputum of allergic patients for novel miR expression patterns, we uncovered an association between miR-3935 and its predicted target gene, the prostaglandin $E_3$ receptor, which might mediate AIT effects through suppression of the $PGE_2$-*PTGER3* axis.

**KEYWORDS**
allergen-specific immunotherapy, allergic asthma/rhinitis, induced sputum, microRNA, prostaglandin $E_2$




**GRAPHICAL ABSTRACT**

miR-3935 is upregulated in sputum cells of allergic asthma patients, who received allergen-specific immunotherapy treatement, while mRNA levels of its predicted target, the prostaglandin $E_2$ receptor, is downregulated as its ligand $PGE_2$ as well. $PGE_2$ is strongly upregulated in sputum supernatants of untreated allergic patients and is reduced in patients, who received allergen-specific immunotherapy. $PGE_2$ levels correlate with clinical parameters, like eosinophils and symptom score.

## 1 | INTRODUCTION

Allergic asthma (AA) comprises different disease forms that are classified either according to their clinical manifestations into phenotypes[1] or to their biological mechanisms into endotypes.[2,3] One of the best-described endotypes is the eosinophilic or type-2 endotype.[4] A number of sputum markers associated with type-2 endotypes were found to be elevated at both the protein and transcript levels, including secreted mediators of allergic inflammation such as IL-4, IL-5, IL-13, CCL17, CCL26,[5,6] IL-24[7] and periostin.[8] The concept of the "united airway" postulates that the pro-inflammatory mediator signature is also present and regulated in the airways of patients with allergic rhinitis without concomitant asthma (AR), suggesting that upper and lower airways are interdependently regulated.[6,9] This concept postulates that the upper and lower airways form a morphological and functional unit, sharing common features related to physical barrier, mucociliary clearance and immune processes. These mechanisms can be studied in the lower airways using induced sputum samples, which consists of components derived from the respiratory epithelium of the central airways.[10–12]

MicroRNAs (miRs) are small nucleic acid molecules that suppress gene expression post-transcriptionally and therefore via an epigenetic mechanism.[13] Individual miRs can have wide-ranging regulatory functions, as they can each target multiple mRNA transcripts along a particular signalling pathway.[14] As microRNAs are quite resistant to degradation, they can be easily be detected in body fluids including samples from the lower airways such as induced sputum. Along this line, deregulated microRNAs have been described in asthma as well as in allergic rhinitis (reviewed in[15]). For example, levels of miR-122-5p were increased in sputum and serum of severe, uncontrolled asthmatics in comparison to healthy controls,[16] and miR-223-3p was associated with increased neutrophil counts and T-helper cells of type 17 signalling in the sputum of asthmatics.[17] In allergic rhinitis, miR-206, −338-3p, −329 and −26a were found to be elevated in patients with AR, but not in healthy individuals or those with asthma.[18] Recent studies underscored the importance of miRNAs in the regulation of

**Key Messages**

- AIT induces miR-3935 in induced sputum cells of allergic asthmatics, while mRNA of its target, prostaglandin $E_3$ receptor, is downregulated.
- $EP_3$ ligand $PGE_2$ is reduced in sputum supernatants of AIT-treated patients.
- $PGE_2$ correlates with local clinical parameters, like eosinophils and inflammatory cell load.

airway epithelial responses in asthma. MiR-23a was postulated to target IL-4 in epithelial cells.[19] Finally, also airway epithelium-derived miRs (miR-34a, miR-92b and miR-210) were implicated to play a role in the early development of the local immune processes in asthma.[20]

Interestingly, miR-mediated expression inhibition also plays a role in eicosanoid biosynthesis.[21] Eicosanoids are key mediators of type-2 inflammation,[22–25] with miRs intervening at different steps on the eicosanoid pathway. In fact, it has been reported for the cyclo-oxygenase-2 (COX-2) that 28 miRs intervene in its gene regulation and thereby affect subsequent PGE2 production,[26,27] in particular, miR-155, the expression of which is increased in smooth muscle cells of the asthmatic airways.[28] In addition, miR-155 is systemically increased in severe asthmatics with cockroach allergy compared with non-asthmatics and has been shown to enhance COX-2 and the concomitant Th2/Th17-associated lung inflammation in asthmatic mice as well as in patients allergic to cockroaches.[29]

IL-13, produced by Th2 cells, but also by group 2 innate lymphoid cells (ILC2),[30–33] was found to increase neutrophil-derived $PGE_2$ levels through upregulation of COX-2 gene expression.[34] The suppressive effects of allergen-specific immunotherapy (AIT) on type-2 immune responses not only affect Th2 cells but also induce a shift from ILC2 to ILC1.[35]

The effect of allergen-specific immunotherapy on local miR expression and the corresponding gene transcript level, however, remains to be studied in patients, particularly in the mucosal context. In order to resolve local miR-related mechanisms including regulatory and anti-inflammatory mechanisms of allergic airway diseases, in this study, we profiled the genome-wide miR and simultaneously predicted miR-target mRNA signatures in induced sputum of patients with allergic rhinitis with (AA) and without (AR) concomitant asthma.

## 2 | METHODS

### 2.1 | Patients and IRB statement

All interventions were performed in the Allergy Section, Department of Otolaryngology, TUM School of Medicine, Munich, Germany. We used pooled data from two observational, non-interventional studies within three subsequent years, which were approved by the Institutional Review Board (IRB), namely the local Ethics Commission of the TUM School of Medicine, Munich, Germany (5534/12 and 5156/11). Each participant gave informed, written declaration of consent. Samples were taken from 44 healthy individuals and from 37 patients with grass pollen-related allergic rhinitis without concomitant asthma (AR), from whom, 21 had received grass pollen-specific AIT (patient characteristics; Table 1). In addition, 43 patients with grass pollen-allergic rhinitis who suffered from concomitant asthma (AA) were included, for whom the GINA scores were evaluated. In the group of 43 AA patients, 22 patients had received grass pollen-specific AIT. Further patient details can be found in the Supplemental Information. Asthma patients were classified as asthmatic based on a previous doctor's diagnosis and a reported history of shortness of breath, tightness in the chest and coughing during natural allergen exposure, that is during pollen flight, and / or a previously documented positive bronchodilation test. A GINA score was assessed from asthma patients. Patients with a grass pollen allergy with a history of moderate and chronic persistent allergic rhinitis according to the definition of allergic rhinitis and its effect on asthma (ARIA) criteria[36] for more than two years during the grass pollen season, a wheal with a diameter of more than three millimetres and grass pollen-specific IgE levels above 0.70kU/l, were included. To avoid effects of, for example, inhaled corticosteroids, patients were asked to stop medication intake seven days before sampling to have an adequate washout period. AIT treatment was not interrupted for the study. All patients had good lung function ($FEV_1\% > 70\%$). Patients in the AIT-treated groups received AIT for at least one year. All subjects completed a questionnaire on the quality of life in rhinoconjunctivitis (mRQLQ), received a pulmonary function test and underwent a sputum induction using the method described below.

### 2.2 | Data collection and statistical analysis

All experiments were performed by researchers blinded to the diagnosis. Significant miR/mRNA expression changes of sputum cells were identified in the microarray data analysis by an initial principal component analysis (PCA) with a homogeneous component distribution, and by subsequent filtration by means of Volcano plot analysis using the moderated t test with the cut-off criteria of FC ≥ 1.5 (fold change) and $p \leq .05$ using the GeneSpring Software GX 14.9 (Agilent Technologies). In the Genespring software, the miRNA microarrays were normalized using the percentile shift method. According to the manufacturer, it normalizes the microRNA array data using the 90th percentile. False-positive intensity values will most likely to fall into the lower percentile range of the array and are rated as noise, according to the manufacturer. Therefore, in this case, the 90th percentile is used as a robust intensity value to normalize the data. The details of the microarray analysis and all remaining techniques are described in detail in the supplemental information. The relative expression levels ($\log_2$ fold change) in the range from low in blue to high in red were shown as colour codes for the heat maps of the microarray analyses. Data in Figure 1A-G, 5A and B were tested statistically using Mann-Whitney U tests calculated with the GraphPad Prism software (GraphPad Software Inc.). In Figures 4G, 5C–F and in Figure S1A–C, Spearman correlation was used to correlated sputum $PGE_2$ levels with clinical parameters and sputum cell counts. All statistically significant differences are shown as $^*p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$, $^{****}p \leq .0001$.

## 3 | RESULTS

Induced sputum was obtained from allergic rhinitis patients with (AA) and without (AR) concomitant asthma, some of whom had received AIT for more than a year (AA+AIT; AR+AIT), and some of

**TABLE 1** Characteristics of the Sputum Cohort

| | Controls ($n = 44$) | Allergic rhinitis w/o AIT ($n = 16$) | Allergic rhinitis with AIT ($n = 21$) | Allergic asthma w/o AIT ($n = 21$) | Allergic asthma with AIT ($n = 22$) |
|---|---|---|---|---|---|
| Age [years] | 24.77 (±0.56) | 24.00 (±0.77) | 26.75 (±0.92) | 25.90 (±1.26) | 31.82 (±1.83) |
| Sex (m/f) | 27/17 | 8/8 | 10/11 | 10/11 | 16/6 |
| GINA score | n.d. | n.d. | n.d. | 1.17 ± 0.27 | 0.38 ± 0.16 |
| mRQLQ Score | 0.13 (±0.05) | 2.64 (±0.19) | 1.49 (±0.19) | 2.73 (±0.22) | 1.20 (±0.18) |
| Total IgE [IU/L] | 28.44 (±5.10) | 208.1 (±51.16) | 70.46 (±26.38) | 287.0 (±53.94) | 107.50 (±27.37) |
| sIge (kUA/l) | n.d. | 20.83 (±5.98) | 15.03 (±5.37) | 27.34 (±7.62) | 19.68 (±4.64) |
| (PT/CAP) to | | | | | |
| Grass | 0 | 16/16 | 21/21 | 21/21 | 22/22 |
| Birch | 0 | 14/13 | 15/12 | 17/15 | 15/14 |
| HDM | 0 | 10/9 | 8/9 | 9/9 | 6/6 |
| Cat | 0 | 5/3 | 8/5 | 13/11 | 10/8 |
| FVC [L] | 4.49 (±0.15) | 4.92 (±0.30) | 4.64 (±0.17) | 4.56 (±0.17) | 4.82 (±0.19) |
| FVC [%] | 98.60 (±1.77) | 103.40 (±2.69) | 104.40 (±3.16) | 101.40 (±3.01) | 102.60 (±2.69) |
| FEV [L] | 3.88 (±0.14) | 4.29 (±0.27) | 4.00 (±0.11) | 3.78 (±0.11) | 3.90 (±0.18) |
| $FEV_1$ [%] | 98.89 (±1.87) | 105.00 (±2.59) | 105.00 (±2.32) | 98.86 (±2.82) | 98.00 (±2.99) |
| $FEV_1$/FVC ratio | 85.93 (±1.08) | 87.06 (±1.23) | 87.00 (±1.46) | 82.99 (±1.74) | 77.66 (±3.48) |
| Sputum cells / ml ($x10^4$) | 89.34 (±12.62) | 250.5 (±47.42) | 71.29 (±11.72) | 282.3 (±61.99) | 80.46 (±10.52) |
| Macrophages (%) | 84.15 (±2.07) | 83.36 (±2.54) | 86.68 (±2.34) | 77.00 (±4.71) | 79.29 (±4.87) |
| Lymphocytes (%) | 1.09 (±0.21) | 1.43 (±0.23) | 0.95 (±0.18) | 2.81 (±0.97) | 0.93 (±0.31) |
| Neutrophils (%) | 14.79 (±2.05) | 12.21 (±1.98) | 11.68 (±2.21) | 15.38 (±4.48) | 18.57 (±4.45) |
| Eosinophils (%) | 0.23 (±0.11) | 3.36 (±0.78) | 0.79 (±0.33) | 5.13 (±1.23) | 1.50 (±0.69) |

*Note:* Data are presented as mean ± SEM.
Abbreviation: n.d., not determined.

whom did not (AA-AIT; AR-AIT) as well as from healthy controls (HC) in order to gain access to local mediators of the lower airways.

### 3.1 | Treatment-associated patient cohort characteristics and symptoms

The patients who were recruited for this study were identified based on clinical parameters, and the symptom score was assessed by the validated disease-related mini quality of life questionnaire (mRQLQ) (Figure 1A). Successful AIT was confirmed both in AR and AA patients by reduced total serum IgE levels (Figure 1B). The local inflammatory cell load, identified by sputum leucocytes per millilitre, showed a pattern similar to total serum IgE and mRQLQ, with untreated AR and AA patients having an increased IgE titre, but reduced quality of life compared to controls and to AIT-treated patients (Figure 1C). The composition of sputum macrophages and neutrophils did not differ among the groups (Figure 1D,E), except for the frequency of lymphocytes, which was reduced in AIT-treated AR patients compared with untreated and was slightly decreased in treated AA patients (AR+AIT and AA+AIT; Figure 1F). Sputum eosinophils were practically absent in healthy subjects, while their number was strongly increased in AR-AIT patients and even more so in AA-AIT patients (Figure 1G). As expected, these numbers were reduced in AR+AIT and AA+AIT compared with untreated patients. These data confirm that the patient cohort in this study was clearly defined not only on the basis of the symptom burden and the clinical picture, but also on the basis of local inflammation parameters.

### 3.2 | miR-target networks involve extracellular matrix organization, chemotaxis and antigen-presentation in allergic rhinitis with concomitant asthma

Since miRs are important regulators of airway epithelial responses, we focussed on the differential analysis of the expression of miRs in sputum cells from patients with allergic rhinitis with and without concomitant asthma. The cellular miR expression profile of induced sputum cells was measured using human microRNA microarray technology. Interestingly, 259 miRs out of 2549 miRs analysed were upregulated in sputum cells of AA-AIT patients, but only one miR was downregulated in comparison with healthy controls (Figure 2A; fold change (FC) ≥ 1.5; $p \leq .05$). Figure 2B shows the top upregulated miRs with the cut-off at FC ≥ 10 in AA-AIT samples compared with HC samples (Table S1; Figure S2; Table S27). Next, in order

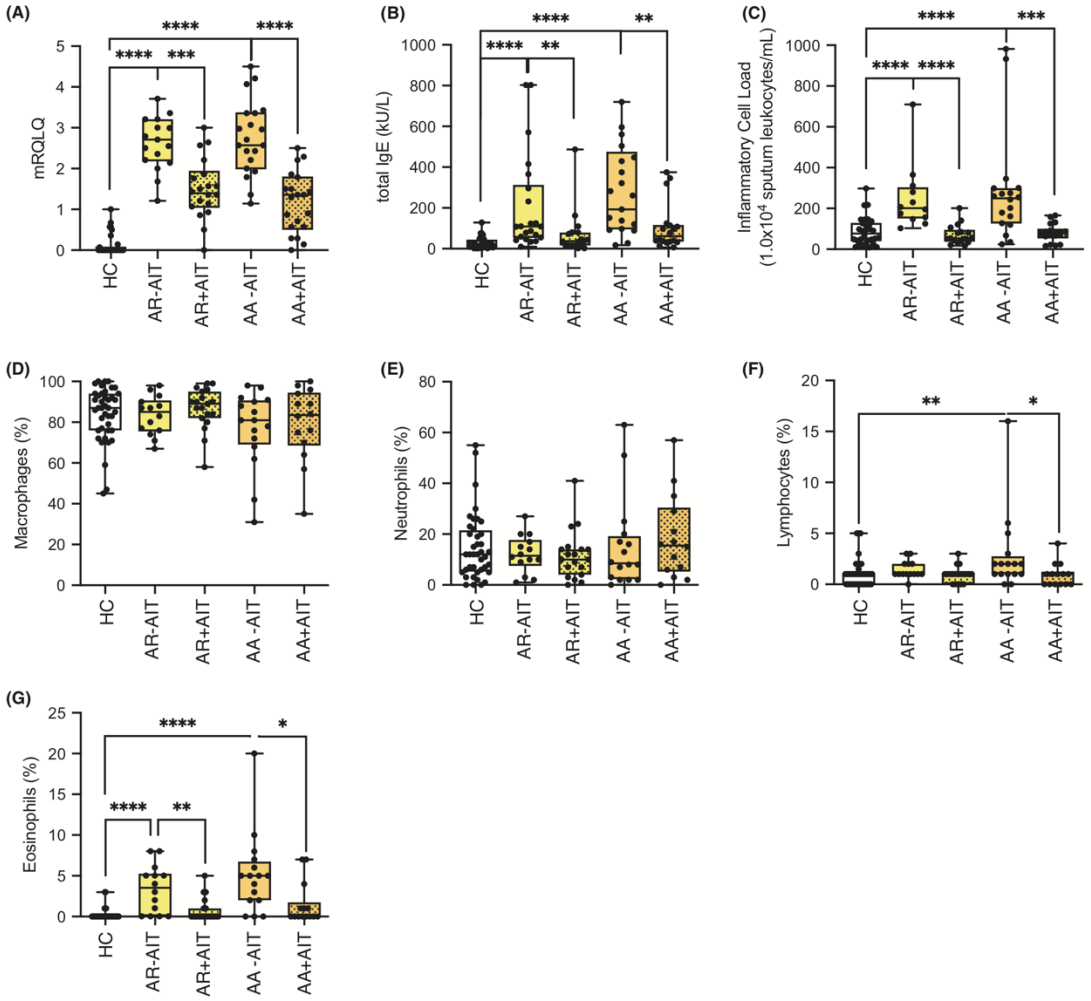



**FIGURE 1** Clinical parameters of patient cohort and AIT effectiveness. (A) Symptom score defined using mRQLQ questionnaire, (B) total serum IgE levels and (C) local inflammatory cell load measured by sputum cells per ml sputum of the five different patient groups. Sputum cell differentiation: percentage of (D) macrophages, (E) neutrophils, (F) lymphocytes and (G) eosinophils. Results are shown as median with range. Mann-Whitney U tests were used to calculate statistically significant differences, shown as $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$, $^{****}p \leq .0001$

to put the detected miRs into functional context in this disease background, we used an open-source miRNA database (mirdb.org) to predict transcript targets of the identified 259 cellular miRs. A whole-transcriptome microarray analysis of the same samples was performed, and the results from the miRdb database were inversely merged with significantly altered mRNA transcripts. The resulting cut set was mapped in order to define a potential miR-mRNA-network. 7552 mRNA transcripts were downregulated in AA-AIT patients compared with HC (FC≥1.5; $p \leq .05$), of which 2507 were predicted targets of the 259 upregulated miRs (Figure 2C, Table S2). Using a GO-term filter, 126 out of this cut set were considered as secreted factors, which were then subjected to string network analysis to identify enriched cellular mechanisms (Figure 2D, Table S3). In AA-AIT patients, target genes formed enriched networks that are involved in extracellular matrix organization, in antigen-processing/cross-presentation and in chemotaxis. All of those genes were significantly downregulated in sputum cells from AA-AIT patients compared with HC (Figure 2E; Table S4; Figure S3; Table S28; FC ≥ 1.5; $p \leq .05$) and 10 of them were predicted targets of miRs that were upregulated more than 10-fold.

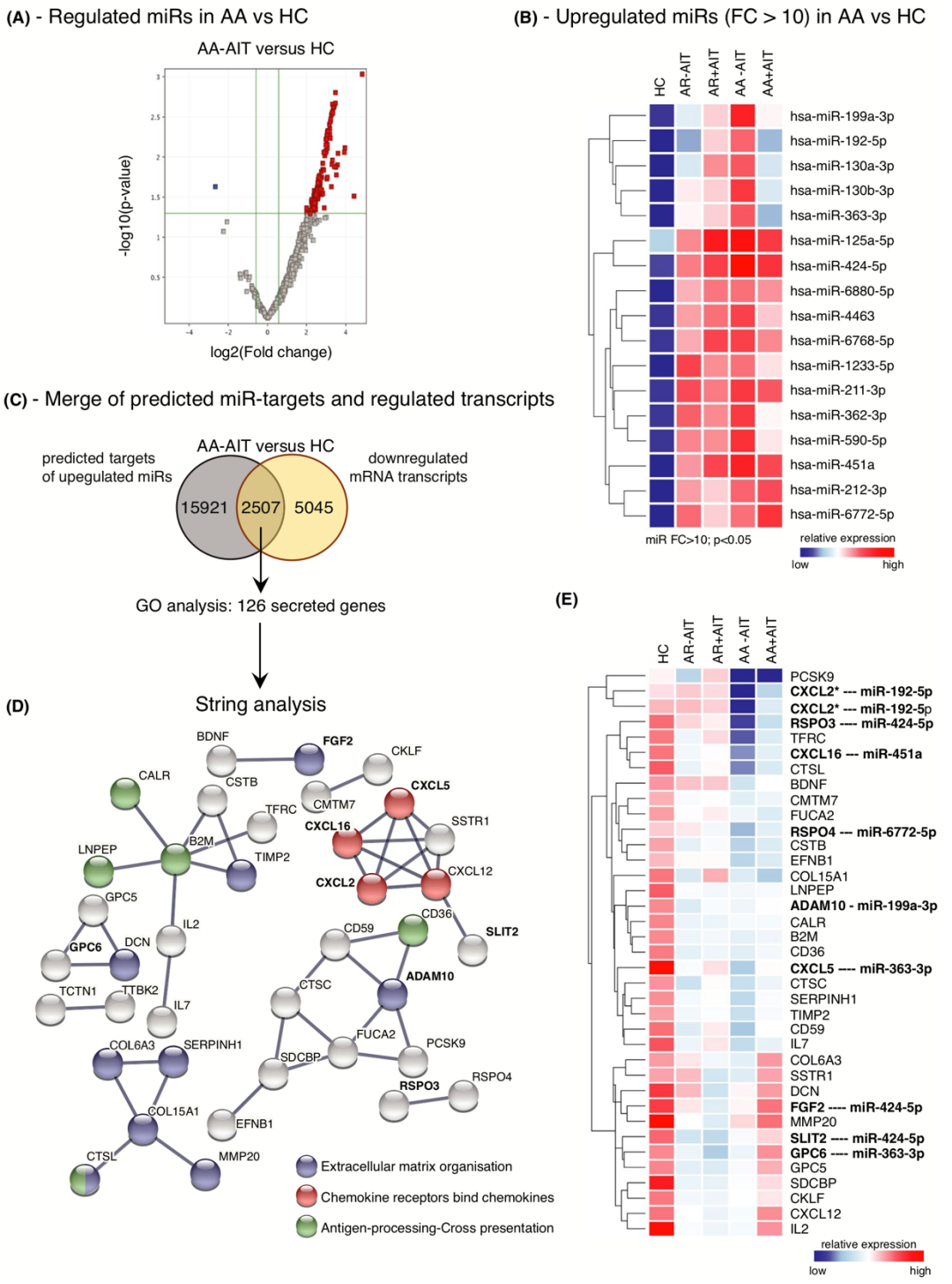

(A) - Regulated miRs in AA vs HC
AA-AIT versus HC
-log10(p-value)
log2(Fold change)
(B) - Upregulated miRs (FC > 10) in AA vs HC
HC
AR-AIT
AR+AIT
AA -AIT
AA+AIT
hsa-miR-199a-3p
hsa-miR-192-5p
hsa-miR-130a-3p
hsa-miR-130b-3p
hsa-miR-363-3p
hsa-miR-125a-5p
hsa-miR-424-5p
hsa-miR-6880-5p
hsa-miR-4463
hsa-miR-6768-5p
hsa-miR-1233-5p
hsa-miR-211-3p
hsa-miR-362-3p
hsa-miR-590-5p
hsa-miR-451a
hsa-miR-212-3p
hsa-miR-6772-5p
miR FC>10; p<0.05
relative expression
low
high
(C) - Merge of predicted miR-targets and regulated transcripts
predicted targets of upegulated miRs
AA-AIT versus HC
downregulated mRNA transcripts
15921
2507
5045
GO analysis: 126 secreted genes
(D)
String analysis
BDNF
FGF2
CKLF
CALR
CSTB
CXCL5
CMTM7
TFRC
SSTR1
CXCL16
B2M
LNPEP
TIMP2
CXCL12
CXCL2
GPC5
CD36
IL2
CD59
SLIT2
GPC6
DCN
ADAM10
TCTN1
TTBK2
CTSC
IL7
FUCA2
COL6A3
SERPINH1
PCSK9
SDCBP
RSPO4
RSPO3
COL15A1
EFNB1
CTSL
MMP20
Extracellular matrix organisation
Chemokine receptors bind chemokines
Antigen-processing-Cross presentation
(E)
HC
AR-AIT
AR+AIT
AA -AIT
AA+AIT
PCSK9
CXCL2* --- miR-192-5p
CXCL2* --- miR-192-5p
RSPO3 ---- miR-424-5p
TFRC
CXCL16 --- miR-451a
CTSL
BDNF
CMTM7
FUCA2
RSPO4 --- miR-6772-5p
CSTB
EFNB1
COL15A1
LNPEP
ADAM10 - miR-199a-3p
CALR
B2M
CD36
CXCL5 ---- miR-363-3p
CTSC
SERPINH1
TIMP2
CD59
IL7
COL6A3
SSTR1
DCN
FGF2 ---- miR-424-5p
MMP20
SLIT2 ---- miR-424-5p
GPC6 ---- miR-363-3p
GPC5
SDCBP
CKLF
CXCL12
IL2
relative expression
low
high

**FIGURE 2** Local miR induction in AA-AIT patients and corresponding target gene suppression. (A) Volcano plot of significantly changed miRs in sputum cells of AA-AIT patients compared with HC using human miR microarray technology. (B) Heat map of the highest upregulated miRs in AA-AIT patients compared with HC (FC ≥ 10; $p \leq .05$) across the five patient groups. (C) Venn diagram showing the cut set of predicted targets of all upregulated miRs in AA-AIT versus HC (FC ≥ 1.5; $p \leq .05$) and of downregulated mRNAs identified by mRNA microarray analysis in the same samples (AA-AIT; HC). Subsequently, cut set entities were filtered for secreted factors using GO-term analysis. (D) String protein interaction network analysis of 126 secreted genes from (C). Enriched RCTM pathways are highlighted: "extracellular matrix organization" (dark purple), "chemokine receptors bind chemokines" (red) and "Antigen-processing-Cross-presentation" (green). (E) Heat map of relative expression levels of members of the protein interaction network identified in (D) analysed by mRNA microarray analysis and significantly downregulated in AA-AIT compared with HC. Duplicate miR or gene names indicate the abundance of two or more isoforms of the same gene in the analysis

### 3.3 | Overlapping miR-associated gene regulation underpins the united airway concept in patients with allergic rhinitis

Similar to AA-AIT patients, in the sputum cells of AR-AIT patients, 97 miRs were increased and one miR was decreased compared with HC. Almost two-thirds of the elevated cellular miRs were also increased in asthmatic patients (Figure 3A, Tables S5–S6), although the main site of clinical manifestation of allergic rhinitis is the upper respiratory tract. The downregulated miR-6508 in the sputum of AR patients differed from the one downregulated miR in AA patients, miR-3935. We focussed on the miR targets, which showed a patient group-specific expression pattern, as well as on the genes that were synergistically downregulated in both patient groups. We identified 12630 predicted targets of locally upregulated miRs using the miRdb and 7089 downregulated mRNA transcripts in AR-AIT patients compared with HC (Figure 3B, Tables S7–S8). In this analysis, 2272 transcripts met both conditions. In a similar analysis in AA-AIT patients, 18428 predicted targets of upregulated were identified and 7552 mRNA transcripts were downregulated in the same samples, of which 2507 mRNA transcripts met both criteria. Next, a Venn analysis of the gene lists resulting from the analyses of AA-AIT versus HC and AR-AIT versus HC was performed to identify mRNA transcripts that are supposedly downregulated by miR (Figure 3C, Tables S9–S10), filtered for secreted genes and submitted to string network. The focus of this analysis was set on the above-mentioned regulated pathways in AA-AIT patients within the three subdivisions: targets only changed in rhinitis patients, targets changed in both patient groups and targets only changed in asthmatic patients (Figure 3D-I, Tables S11–S16; Figure S4–S6; Tables S29–S31).

### 3.4 | AIT-related induction of miR-3935 corresponds to suppression of transcripts of its target PTGER3

In comparison to miR regulation comparing allergic patients and healthy subjects, the analysis of the effect of AIT on cellular miR regulation revealed the opposite. Only four cellular miRs were locally upregulated, while 21 cellular miRs were downregulated in AA+AIT patients compared with AA-AIT patients (Figure 4A-C; Tables S17–S18; Figure S7–S8; Tables S32–33). In total, a number of 6677 genes were predicted as targets of the downregulated miRs (Figure 4D, Table S19; Figure S9; Table S34), of which 42 were significantly upregulated in AA+AIT patients compared with AA-AIT patients. On the other hand, 661 genes were predicted as targets of the upregulated miRs (Figure 4E, Table S20; Figure S10; Table S35), of which only one gene (PTGER3) was also significantly downregulated on the mRNA level in the same samples. PTGER3, the prostaglandin $EP_3$ receptor ($EP_3$), is one of four receptors for prostaglandin $E_2$ ($PGE_2$) and is involved in bronchoconstriction.[37,38] PTGER3 showed a counter regulation to miR-3935 as expected (Figure 4F, Figure S1E-F), which was underlined by the inverse correlation between miR-3935 and its predicted target *PTGER3* (Figure 4G; $r = -0.57$; $p = .0294$). Of the four known $PGE_2$ receptors $EP_1$, $EP_2$, $EP_3$ and $EP_4$, only $EP_3$ was significantly changed on the mRNA level upon AIT. Although $EP_2$ mRNA in sputum cells of asthmatics was slightly, but not significantly increased and $EP_4$ was significantly reduced in comparison to healthy controls, no significant regulation was found comparing AA+AIT with AA-AIT patient samples (data not shown). In addition, immunofluorescence microscopy of the $EP_3$ protein was performed on cytospin samples of the induced sputa of AA-AIT and AA+AIT patients, which revealed on the one hand that $EP_3$ was mainly detected in macrophages and on the other hand, however, mainly in specimens from AA-AIT patients, while the staining of samples of the treated asthma group (AA+AIT) was weak (Figure S11). This reflects the above-described results on the mRNA expression levels of *PTGER3* ($EP_3$) (Figure 4F, Figure S1E&F).

### 3.5 | Reduction of sputum protein levels of $EP_3$ ligand $PGE_2$ correlates with clinical parameters and symptoms in AIT-treated patients

Finally, we measured the levels of the $EP_3$ ligand $PGE_2$ in the sputum supernatants of the same samples using ELISA. $PGE_2$ was increased in the supernatants of AA-AIT patients compared with HC and was decreased upon AIT in AA and AR patients (Figure 5A). Since $PGE_2$ has been reported to be induced via IL-13 and COX-2, we examined the frequencies of IL-13-expressing ILC2s using flow cytometry (ILC2: $CD45^+Lineage^-CD127^+CRTh2^+IL\text{-}13^+$; Gating strategy: Figure S1D) and found that these cells were indeed increased in AR-AIT and AA-AIT patients compared with HC and decreased in AIT-treated compared with untreated AR and AA patients (Figure 5B). IL-13 and IL-22 protein levels in the sputum supernatants of the same samples also correlated positively with sputum $PGE_2$ levels (Figure 5C;

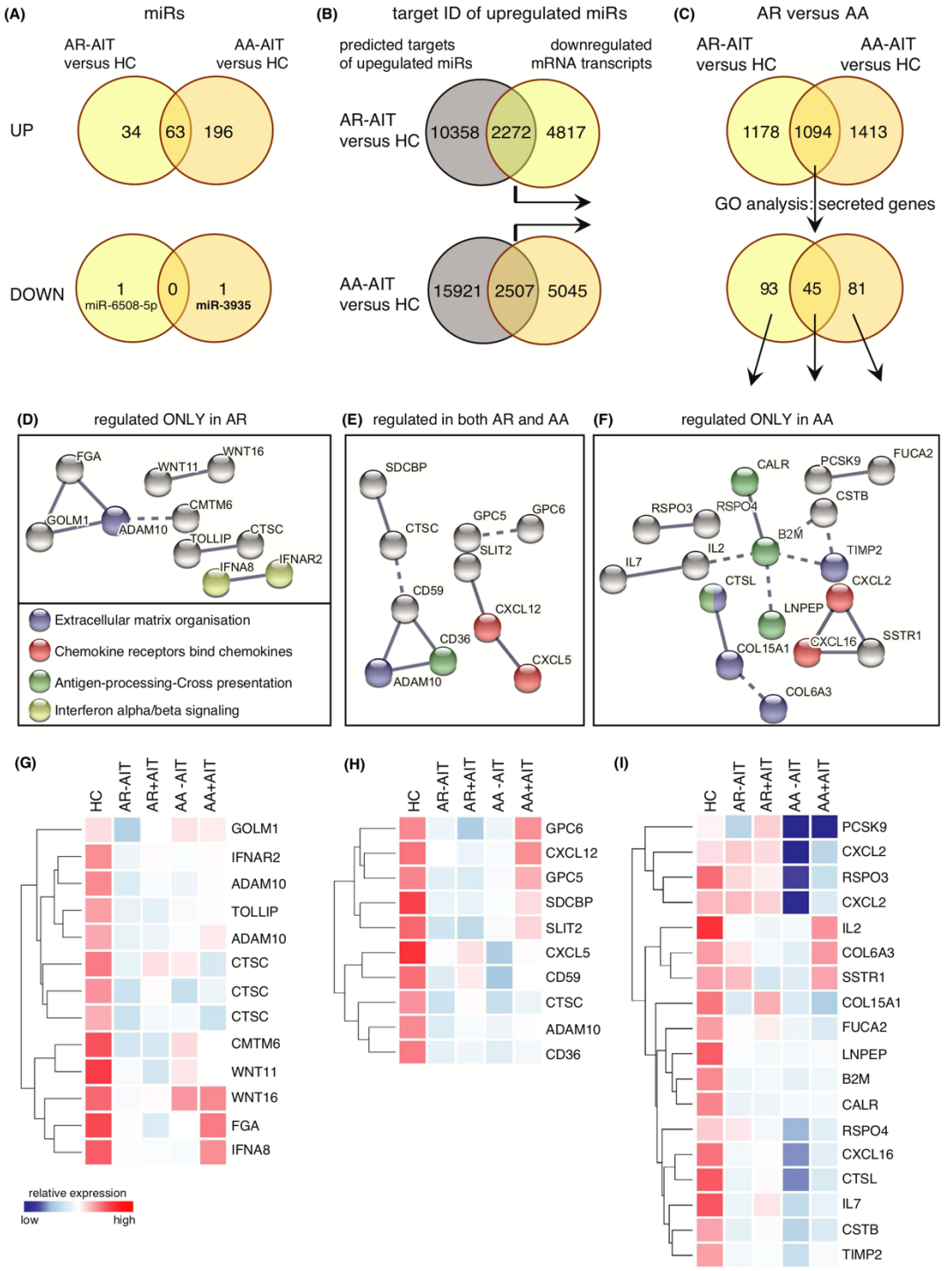

(A)
miRs
AR-AIT versus HC
AA-AIT versus HC
UP
34
63
196
DOWN
1
miR-6508-5p
0
1
miR-3935
(B)
target ID of upregulated miRs
predicted targets of upegulated miRs
downregulated mRNA transcripts
AR-AIT versus HC
10358
2272
4817
AA-AIT versus HC
15921
2507
5045
(C)
AR versus AA
AR-AIT versus HC
AA-AIT versus HC
1178
1094
1413
GO analysis: secreted genes
93
45
81
(D)
regulated ONLY in AR
FGA
WNT11
WNT16
CMTM6
GOLM1
ADAM10
TOLLIP
CTSC
IFNA8
IFNAR2
Extracellular matrix organisation
Chemokine receptors bind chemokines
Antigen-processing-Cross presentation
Interferon alpha/beta signaling
(E)
regulated in both AR and AA
SDCBP
CTSC
GPC5
GPC6
SLIT2
CD59
CXCL12
CD36
CXCL5
ADAM10
(F)
regulated ONLY in AA
CALR
PCSK9
FUCA2
CSTB
RSPO3
RSPO4
B2M
IL2
TIMP2
IL7
CTSL
CXCL2
LNPEP
CXCL16
SSTR1
COL15A1
COL6A3
(G)
HC
AR-AIT
AR+AIT
AA -AIT
AA+AIT
GOLM1
IFNAR2
ADAM10
TOLLIP
ADAM10
CTSC
CTSC
CTSC
CMTM6
WNT11
WNT16
FGA
IFNA8
relative expression
low
high
(H)
HC
AR-AIT
AR+AIT
AA -AIT
AA+AIT
GPC6
CXCL12
GPC5
SDCBP
SLIT2
CXCL5
CD59
CTSC
ADAM10
CD36
(I)
HC
AR-AIT
AR+AIT
AA -AIT
AA+AIT
PCSK9
CXCL2
RSPO3
CXCL2
IL2
COL6A3
SSTR1
COL15A1
FUCA2
LNPEP
B2M
CALR
RSPO4
CXCL16
CTSL
IL7
CSTB
TIMP2

**FIGURE 3** Comparison of locally regulated miRs and their target genes between AA-AIT and AR-AIT patients. (A) Venn diagrams showing the cut sets of predicted targets of all upregulated miRs in AA-AIT patients compared with AR-AIT (FC ≥ 1.5; $p \leq .05$) (upper part) and of all downregulated miRs in AA-AIT patients compared with AR-AIT (FC ≥ 1.5; $p \leq .05$) (lower part). (B) Identification of regulated miR targets: Venn diagram showing the cut set of predicted targets of upregulated miRs in AR-AIT vs HC (upper part) and AA-AIT vs HC (lower part) (FC ≥ 1.5; $p \leq .05$) and of downregulated miR targets on the mRNA level defined in mRNA microarray analysis of the same samples using the same comparisons (FC ≥ 1.5; $p \leq .05$). (C) Venn diagrams showing the cut sets of the two cut sets from (B) thereby depicting the cut set of downregulated targets of upregulated miRs between AR and AA patients. String network analysis of the results from the comparison depicted in (C), showing secreted factors regulated (D) only in rhinitis patients (AR-AIT vs HC), (E) in both, rhinitis and asthma patients (AR-AIT vs HC and AA-AIT vs HC), (F) only in asthmatic patients (AA-AIT vs HC). (G) Heat map of relative expression levels of members of the protein interaction network identified in (D) analysed by mRNA microarray analysis and significantly downregulated in AR-AIT versus HC. (H) Heat map of relative expression levels of members of the protein interaction network identified in (E) analysed by mRNA microarray analysis and significantly downregulated in AR-AIT and AA-AIT compared with HC. (I) Heat map of relative expression levels of members of the protein interaction network identified in (F) analysed by mRNA microarray analysis and significantly downregulated in AA-AIT versus HC (FC ≥ 1.5; $p \leq .05$). Duplicate miR or gene names indicate the abundance of two or more isoforms of the same gene in the analysis

Figure S1). Notably, the $PGE_2$ levels in sputum supernatants further correlated positively with local inflammatory cell load (Figure 5D; $r = 0.52$, $p < .0001$) and sputum eosinophil frequencies in the same sputum samples (Figure 5E; $r = 0.67$, $p < .0001$; for neutrophils and macrophages, please see: Figure S1) as well as with symptom burden assessed with the mRQLQ (Figure 5F; $r = 0.48$, $p < .0001$).

## 4 | DISCUSSION

The global profiling of miRs in local inflammation of the lower airways of allergic rhinitis and asthma reveals novel local miR expression patterns and confirms previously described disease-related miRs. These data were collected in the context of therapeutic interventions using allergen-specific immunotherapy.

### 4.1 | Known allergy-associated miRs detected in the lower airways

The miRs detected comprise previously reported miRs in the context of asthma, such as miR-15b-5p, whose systemic levels are associated with lung function,[39] miR-16-5p, whose circulating levels correlated with hyper-responsiveness of the airways,[18,40,41] miR-19a-3p, which has been reported to be expressed in local T cells,[42] and miR-21-3p, which was found in exhaled breath condensate from asthmatics, however in reduced levels compared to healthy controls.[43] In addition, we detected elevated levels of miR-191-5p, the serum levels of which also correlated with lung function, measured with $FEV_1$/FVC, in a recent paediatric study,[39] and miR-210-3p, which is involved in mast cell activation,[44] and, finally, miR-223-3p, which was increased in asthmatics compared to healthy controls and correlated with lung function parameters.[45] It has further been published that miR-223-3p is involved in the maturation and function of neutrophils.[46]

In order to understand the role of so far unknown miRs in the regulation of airway diseases, we also performed a transcriptome analysis on the same samples. This transcriptome analysis was then limited to predicted target transcripts of upregulated miRs, which were reversely regulated in the same sample. This analysis revealed a large number of genes whose mRNA levels were downregulated. Since the function of the luminal sputum cells is critically determined by their secreted factors, we focussed on the bioinformatic analysis of secreted and extracellular entities. The unsupervised network analysis revealed three principal mechanisms, namely those of extracellular matrix organization (including genes like *DCN*, *ADAM10*, *MMP20*, *TIMP2*, *LOXL3*, *FGF2*, *CTSK*, *ADAMTS3*, *COL6A3*, *CTSL*, *COL15A1*, *LAMA1*, *ADAM9*, *SERPINH1*), antigen-processing/cross-presentation (*LNPEP*, *CALR*, *CTSL*, *CD36*, *B2M*) and chemotaxis (*CXCL16*, *CXCL5*, *CXCL12*, *CXCL2*).

### 4.2 | miRs in upper and lower airways

MiR target genes involved in extracellular matrix reorganization were mainly repressed in asthmatic patients only, which was expected as asthma mainly manifests in the lower respiratory tract and has frequently been associated with extracellular matrix remodelling.[47–50] Similarly, the factors *CALR*, *B2M*, *CTSL* and *LNPEP* associated with antigen-processing/cross-presentation were only affected in sputum cells from AA-AIT patients, but not from AR-AIT patients, while CD36 (predicted target of miR-629-3p, miR-3180-5p, miR-485-3p and miR-3605-5p) was downregulated in both groups. However, this could be also due to inflammatory processes that are reflected by a shift from a macrophage-dominated towards leucocyte- and eosinophil-dominated in cell composition of the sputum. The scavenger receptor CD36 is known to be an important factor for macrophage recognition and phagocytosis of apoptotic fibroblasts[51] and to promote house dust mite allergy development.[52] Notably, CD36 was also increased in exosomes from asthmatics and associated with leukotriene production.[53] On the other hand, the reduction in the chemokine expression was common to both diseases. While *CXCL2* (predicted target of miR-192-5p, miR-215-5p, miR-7–1-3p and miR-582-5p) and *CXCL16* (predicted target of miR-451a and miR-7–1-3p) were only affected in asthmatics, *CXCL12* (predicted target of miR-141-3p, miR-200a-3p, miR-5100, miR-222-3p, miR-5587-5p, miR-3928-5p and miR-3605-5p) and *CXCL5* (predicted target of miR-25-3p, miR-363-3p and miR-3163) were also affected in AR-AIT patients. *CXCL2* is known to regulate airway

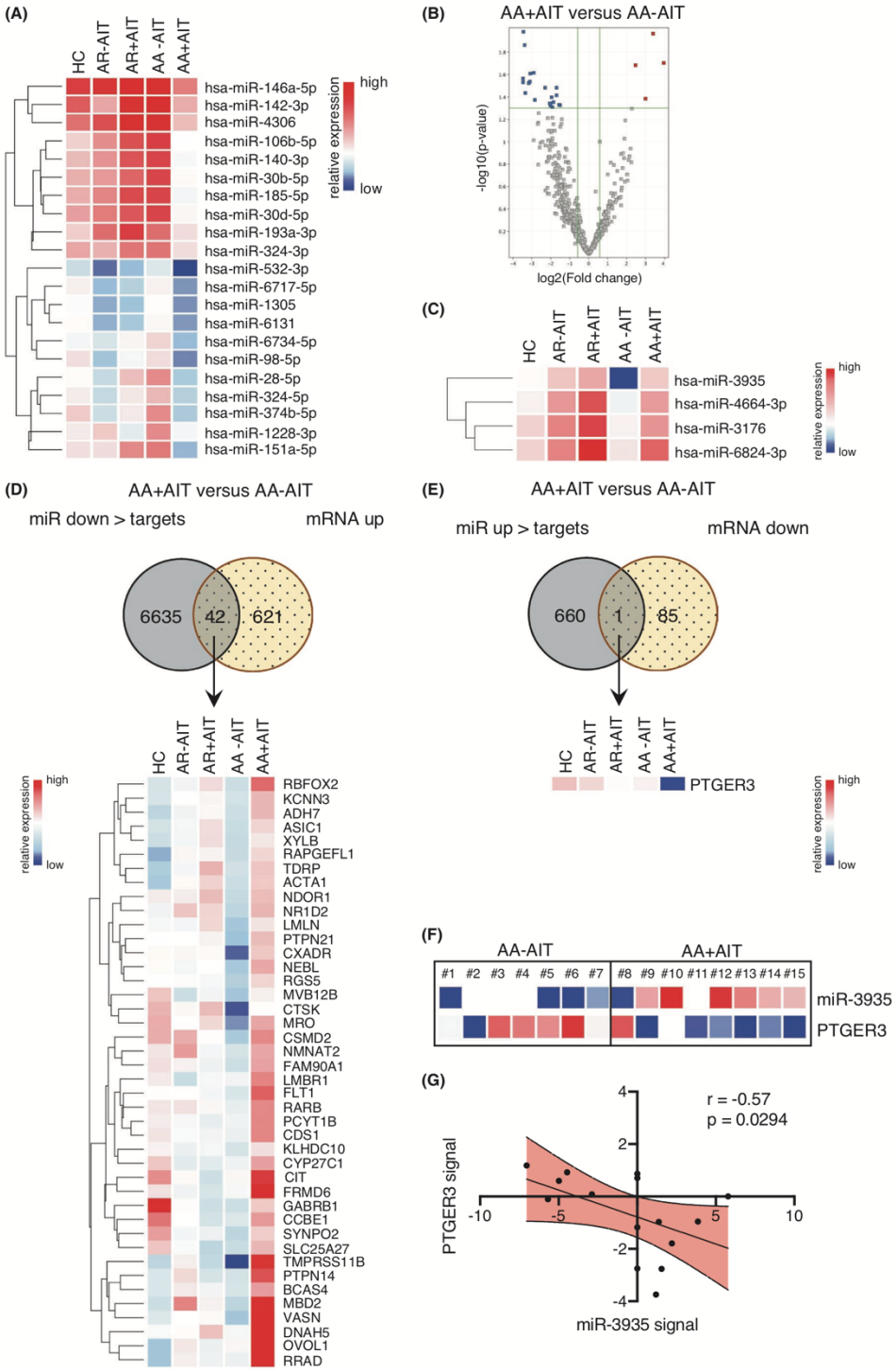

(A)
HC
AR-AIT
AR+AIT
AA -AIT
AA+AIT
hsa-miR-146a-5p
hsa-miR-142-3p
hsa-miR-4306
hsa-miR-106b-5p
hsa-miR-140-3p
hsa-miR-30b-5p
hsa-miR-185-5p
hsa-miR-30d-5p
hsa-miR-193a-3p
hsa-miR-324-3p
hsa-miR-532-3p
hsa-miR-6717-5p
hsa-miR-1305
hsa-miR-6131
hsa-miR-6734-5p
hsa-miR-98-5p
hsa-miR-28-5p
hsa-miR-324-5p
hsa-miR-374b-5p
hsa-miR-1228-3p
hsa-miR-151a-5p
relative expression
high
low
(B)
AA+AIT versus AA-AIT
-log10(p-value)
log2(Fold change)
(C)
HC
AR-AIT
AR+AIT
AA -AIT
AA+AIT
hsa-miR-3935
hsa-miR-4664-3p
hsa-miR-3176
hsa-miR-6824-3p
relative expression
high
low
(D)
AA+AIT versus AA-AIT
miR down > targets
mRNA up
6635
42
621
HC
AR-AIT
AR+AIT
AA -AIT
AA+AIT
RBFOX2
KCNN3
ADH7
ASIC1
XYLB
RAPGEFL1
TDRP
ACTA1
NDOR1
NR1D2
LMLN
PTPN21
CXADR
NEBL
RGS5
MVB12B
CTSK
MRO
CSMD2
NMNAT2
FAM90A1
LMBR1
FLT1
RARB
PCYT1B
CDS1
KLHDC10
CYP27C1
CIT
FRMD6
GABRB1
CCBE1
SYNPO2
SLC25A27
TMPRSS11B
PTPN14
BCAS4
MBD2
VASN
DNAH5
OVOL1
RRAD
relative expression
high
low
(E)
AA+AIT versus AA-AIT
miR up > targets
mRNA down
660
1
85
HC
AR-AIT
AR+AIT
AA -AIT
AA+AIT
PTGER3
relative expression
high
low
(F)
AA-AIT
AA+AIT
#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 #13 #14 #15
miR-3935
PTGER3
(G)
r = -0.57
p = 0.0294
PTGER3 signal
miR-3935 signal
4
2
-2
-4
-10
-5
5
10

**FIGURE 4** Comparison of locally regulated miRs and their target genes upon AIT in AA patients. (A) Heat map of upregulated miRs in sputum cells of AA+AIT versus AA-AIT (FC ≥ 1.5; $p \leq .05$) detected in human miR microarray technology. (B) Volcano plot of significantly changed miRs in AA+AIT versus AA-AIT. (C) Heat map of downregulated miRs in AA+AIT versus AA-AIT (FC ≥ 1.5; $p \leq .05$), detected in human miR microarray technology. (D) Venn diagram defining the cut set of predicted targets of downregulated miRs and upregulated transcripts detected in mRNA microarray analysis of the same samples in AA+AIT versus AA-AIT (FC ≥ 1.5; $p \leq .05$; upper part). Heat map of the 42 genes from the cut set above depicts significantly changed mRNA levels of miR targets of downregulated miRs comparing AA+AIT versus AA-AIT (FC ≥ 1.5; $p \leq .05$; lower part). (E) Venn diagram showing the cut set of predicted targets of upregulated miRs and of downregulated miR targets on the mRNA level detected in mRNA microarray analysis of the same samples in AA+AIT versus AA-AIT (FC ≥ 1.5; $p \leq .05$; upper part). Heat map of the one gene from the cut set above depicts significantly changed mRNA levels of miR-target *PTGER3* of upregulated miR-3935 comparing AA+AIT versus AA-AIT (FC ≥ 1.5; $p \leq .05$; lower part). (F) Heat map depicting the miR-3935 and *PTGER3*-mRNA expression levels of each patient sample individually. (G) Spearman correlation of the normalized total probe signal of miR-3935 and the normalized processed signal of *PTGER3*-mRNA. Spearman coefficient *r* and *p* values are depicted in the figure. Duplicate miR or gene names indicate the abundance of two or more isoforms of the same gene in the analysis

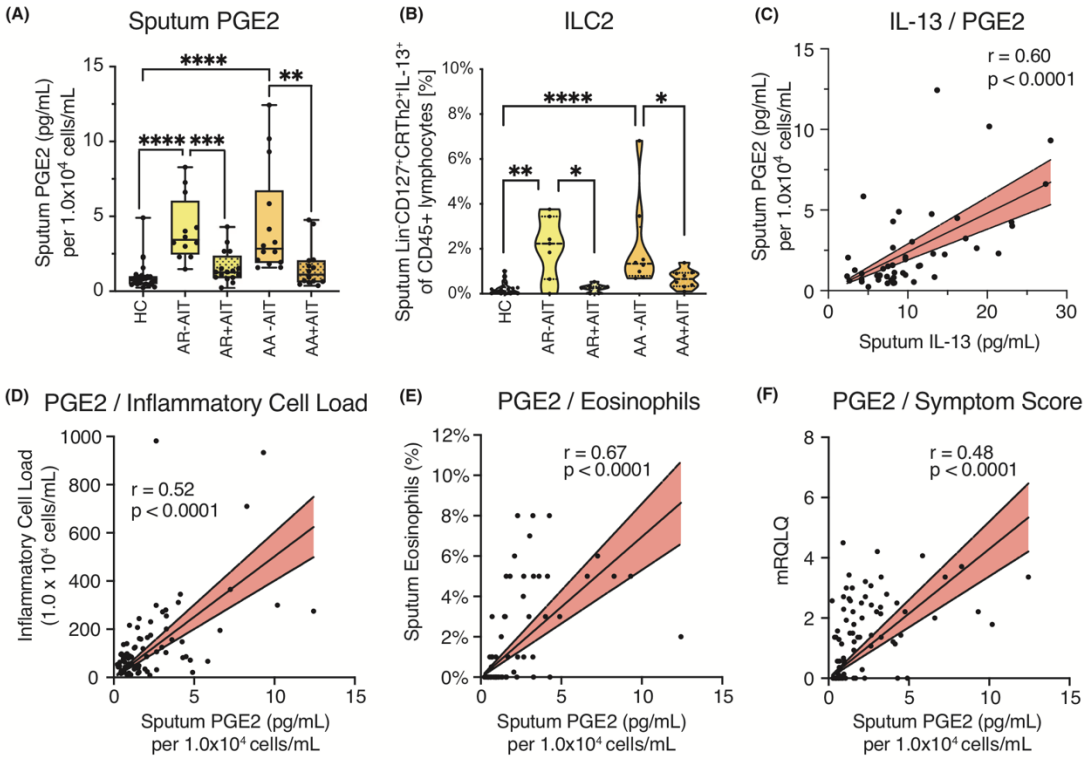



**FIGURE 5** Sputum $PGE_2$ levels are reduced in AIT-treated patients and correlate with clinical parameters and symptoms. (A) $PGE_2$ levels in sputum supernatants measured by ELISA and (B) sputum ILC2 frequencies (Lin-$CD127^+CRTh2^+IL\text{-}13^+$ of $CD45^+$ lymphocytes) analysed by flow cytometry are shown across a subset of the five patient groups (HC: $n = 22$; AR-AIT: $n = 7$; AR+AIT: $n = 7$; AA-AIT: $n = 8$; AA+AIT: $n = 8$). Results are shown as median with range. Mann-Whitney U tests were used to calculate statistically significant differences, shown as $^*p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$, $^{****}p \leq .0001$. Spearman correlation of (C) sputum IL-13 levels with sputum $PGE_2$ levels, (D) sputum $PGE_2$ levels with local inflammatory cell load, identified by sputum leucocytes per millilitre, (E) sputum $PGE_2$ levels with eosinophil frequencies, (F) sputum $PGE_2$ levels with symptom burden, measured by mRQLQ-Questionnaire. Spearman coefficient r and p values are depicted in each correlation figure

smooth muscle cell migration[54] and is a predicted target of multiple miRs, including miR-192-5p, which is upregulated more than 10-fold in this data set. *CXCL16* on the other hand exhibits a direct chemoattractant effect on ILC2,[55] while *CXCL12* levels were reported to correlate with decreased ILC2 numbers in blood of asthmatic patients.[56] Thereby, rhinitis patients share not only the reduction of an ILC2-attractant chemokine, but also of the scavenger receptor *CD36* and *ADAM10* with asthmatic patients, which underscores the

united airway concept mentioned above. In addition, our data show a decrease of *IFNA8* (predicted target of miR-642b-3p) and *IFNAR2* (predicted target of miR-6765-3p and miR-4286) in allergic rhinitis patients only, which is in line with the observation that allergic rhinitis is related to the dominance of type-2 cytokines, which is reversed by allergen-specific immunotherapy.[5,7] We have previously shown that in the upper airways of AR patients, the interferon expression levels (*IFNGR2*) were restored after more than three years of AIT.

### 4.3 | miR-3935-target PTGER3 and associated $PGE_2$ regulation

Studying miR expression in AIT-treated patients compared to untreated patients, we found that the AIT-associated upregulated miRs unexpectedly targeted structural genes rather than immunoregulatory factors. However, the AIT-associated downregulated miR-3935 targets prostaglandin $EP_3$ receptor (*PTGER3*), the receptor for prostaglandin $E_2$ ($PGE_2$). It was originally described to have bronchodilatory effects mediated through an inhibitory effect on smooth muscle cells[57–59] and mast cells that mainly depends on $EP_2$ signalling.[60] However, $PGE_2$ also influences the behaviour of several immune cells, namely macrophages,[61] T cells[62] and B cells,[63] thus suppressing type-2 inflammation. In this study, the levels of secreted $PGE_2$ in the sputum supernatant of the same samples resembled the mRNA levels of its receptor *PTGER3*. Depending on tissue and condition, $PGE_2$ has either pro- or anti-inflammatory effects.[64–71] In this study, sputum $PGE_2$ was associated with a pro-inflammatory state in patients with allergic asthma, since it correlated with pro-inflammatory clinical parameters like sputum inflammatory cell load and sputum eosinophil counts. Furthermore, sputum $PGE_2$ levels were decreased in patients who had undergone AIT. Further research is needed to elucidate the bilateral roles of $PGE_2$ in inflammatory processes in the airways in different diseases. It might even present itself as a critical factor in controlling inflammation in one versus the other direction. Future research needs to focus on the question, how lipid mediators can contribute to allergen tolerance and selective inhibitors can be utilized to promote tolerance re-induction.

As mentioned above, $PGE_2$ exerts its action via its four G-coupled protein receptors $EP_1$, $EP_2$, $EP_3$ and $EP_4$.[72] Previous studies have mainly focussed on the effect of $PGE_2$ in inflammatory processes exerted through the $EP_2$ and $EP_4$ receptors; however, its effect via the $EP_3$ receptor is not well understood and remains to be elucidated in further detail. Studies in human and guinea pig systems suggest a broncho-constrictive effect of $PGE_2$-$EP_3$ signalling,[37,38] thus suggesting that miR-mediated downregulation of $EP_3$ expression upon AIT may have beneficial effects on lung function in asthmatic patients.

The finding that the levels of $PGE_2$ were regulated synergistically to $EP_3$ leads us to believe that the pro-inflammatory, bronchoconstrictor effects of the $PGE_2$ via $EP_3$ are suppressed in AIT-treated patients upon upregulation of miR-3935 and downregulation of $EP_3$ and $PGE_2$. An indication of the pro-inflammatory characteristics of $PGE_2$ in this disease context is the finding that the sputum $PGE_2$ levels correlate positively with clinically assessed parameters such as sputum inflammatory cell load, sputum eosinophil infiltration and symptom burden. We therefore assume that this potential miR-3935-related mechanism suppresses the local pro-inflammatory effects of the $PGE_2$ signalling via $EP_3$, which in turn could represent one novel mechanism of AIT in allergic asthma. This hypothesis is supported by a recent publication describing reduced spontaneous release of $PGE_2$ by PBMCs a few months after venom-specific immunotherapy, which negatively correlated with the tolerogenic cytokine IL-10.[73] As $PGE_2$/$EP_3$ signalling can trigger mast cell degranulation and pro-inflammatory mediator release,[74,75] miR-3935-mediated downregulation of $EP_3$ may contribute to mast cell desensitization following AIT. Thus, future studies should assess potential correlations between miR-3935, $EP_3$ and markers of mast cell activation (e.g., histamine, CysLTs) in patients undergoing AIT.

In summary, this study shows a one-sided AA-associated upregulation of miR expression in sputum cells, whereas the AIT-associated miR expression revealed the opposite picture. These data provide first evidence for a negative regulatory association of miR-3935 and its predicted target gene, the $PGE_2$ receptor $EP_3$, as a potential AIT-mediated mechanism in the airways of AA patients. In this study, sputum $PGE_2$ correlates with pro-inflammatory cellular and clinical parameters. Sputum $PGE_2$ levels also drop in AIT-treated patients, which provides evidence for an involvement of the prostanoid $PGE_2$ in local inflammatory processes mediated by AIT.

This study therefore provides evidence that associates AIT with epigenetic processes in the lungs of allergic asthmatic patients. Future studies are needed to determine whether miR-3935 is a candidate biomarker for AIT efficacy.

#### CONFLICT OF INTEREST

Dr. Jakwerth has a patent "A ratio of immune cells as prognostic indicator of therapeutic success in allergen-specific immunotherapy: 17 177 681.8" that is pending. PD Dr. Chaker reports on grants and other from Allergopharma during the conduct of the study; grants and/or other from ALK Abello, Bencard / Allergen Therapeutics, ASIT Biotech, Lofarma, GSK, Novartis, LETI, Roche, Zeller, Sanofi Genzyme/Regeneron, European Institute of Technology, AstraZeneca, Immunotek, all outside of the submitted work. In addition, PD Dr. Chaker has a patent "A ratio of immune cells as prognostic indicator of therapeutic success in allergen-specific immunotherapy: 17 177 681.8" that is pending. Prof. Dr. Schmidt-Weber reports personal fees from Bencard and personal fees from Allergopharma during the conduct of the study. In addition, Prof. Dr. Schmidt-Weber has a patent on nasal secretions that is pending, and a patent "A ratio of immune cells as prognostic indicator of therapeutic success in allergen-specific immunotherapy: 17 177 681.8" that is pending. Dr. Zissler reports grants and personal fees from German Center for Lung research (DZL), grants and personal fees from German Research Foundation (DFG), grants from Helmholtz I&I Initiative, during the conduct of the study. In addition, Dr. Zissler has a patent "A ratio of immune cells as prognostic indicator of therapeutic success in allergen-specific immunotherapy: 17 177 681.8"

that is pending and Dr. Zissler 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). The rest of the authors declare that they have no relevant conflicts of interest.

## AUTHOR CONTRIBUTIONS

C.A.J., A.M.C., C.S.-W., and U.M.Z. involved in study design; C.A.J., F.G., M.O., L.P., L.G., J.U., J.K., M.P., M.W., A. Ku., A. Kl., and U.M.Z. conducted the experiments; C.A.J., A.M.C. F.G., M.O., L.P., L.G., J.U., J.K., M.P., M.W., A. Ku., A. Kl. and U.M.Z. collected the data; C.A.J., F.G., and U.M.Z. analysed the data; C.A.J., A.M.C., A.E., S.K.-E., J. EvB, C.S.-W., and U.M.Z. involved in data interpretation; C.A.J.,, A.M.C., A.E., S.K.-E., J. EvB, C.S.-W., and U.M.Z. involved in literature search; all authors wrote the article.

## DATA AVAILABILITY STATEMENT

The data discussed in this publication are accessible via the accession number GSE184382 deposited in NCBI's Gene Expression Omnibus (GEO). All other methods are described in the supplemental information.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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

Ulrich M. Zissler, Constanze A. Jakwerth, Ferdinand Guerth, Larissa Lewitan, Sandra Rothkirch, Miodrag Davidovic, Moritz Ulrich, Madlen Oelsner, Holger Garn, Carsten B. Schmidt-Weber, Adam M. Chaker.

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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 Secretoglobin 1A1 (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.

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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.

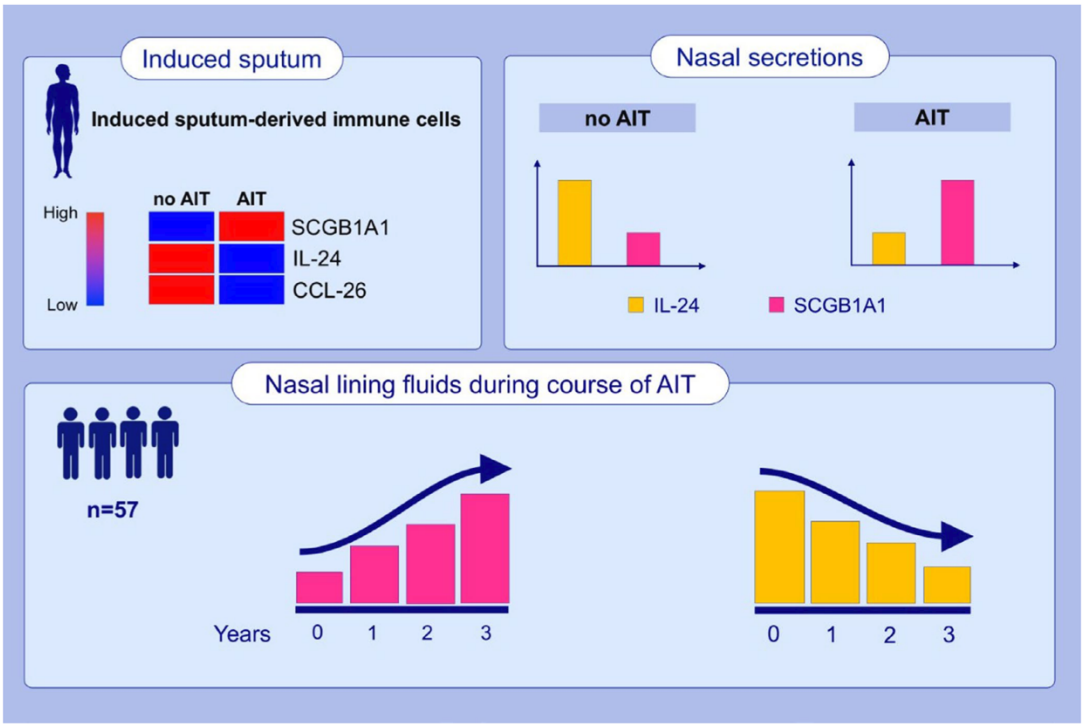
**Results:** Allergen-specific immunotherapy inhibited pro-inflammatory *CXCL8*, *IL24*, and *CCL26*mRNA expression, while *SCGB1A1*, *IL7*, *CCL5*, *CCL23*, and *WNT5B*mRNAs were induced independently of the asthma status and allergen season. In our validation cohort, local increase of SCGB1A1 occurred 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.

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).

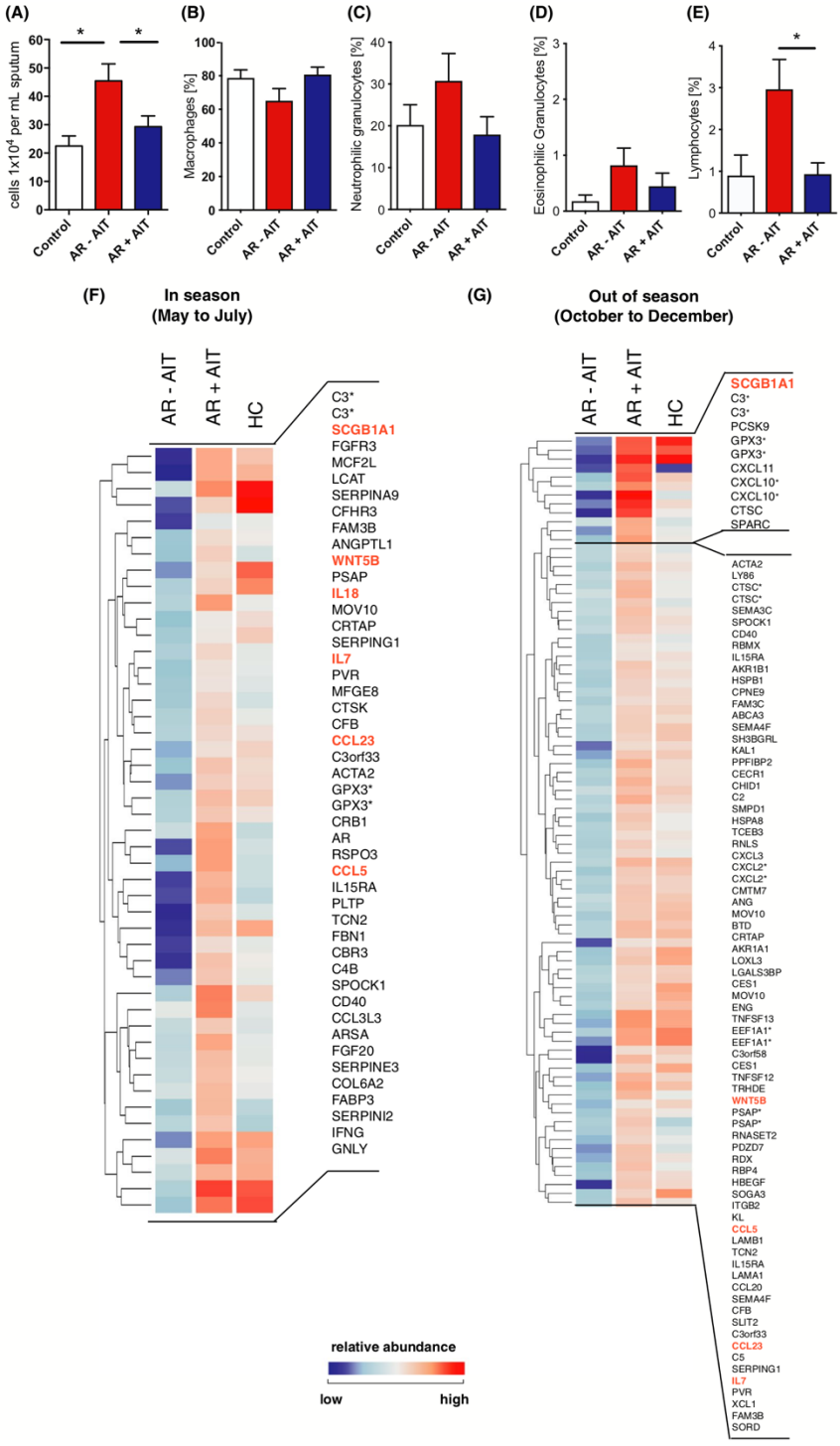
However, of particular interest are such genes that are differentially expressed in response to AIT independent of seasonal influences and pollen exposure as they may represent more stable and robust (bio)markers for a successful AIT treatment. Twenty-two genes encoding for secreted mediators were upregulated 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)
cells 1x10⁴ per mL sputum
Control
AR - AIT
AR + AIT
(B)
Macrophages [%]
(C)
Neutrophilic granulocytes [%]
(D)
Eosinophilic Granulocytes [%]
(E)
Lymphocytes [%]
(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
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

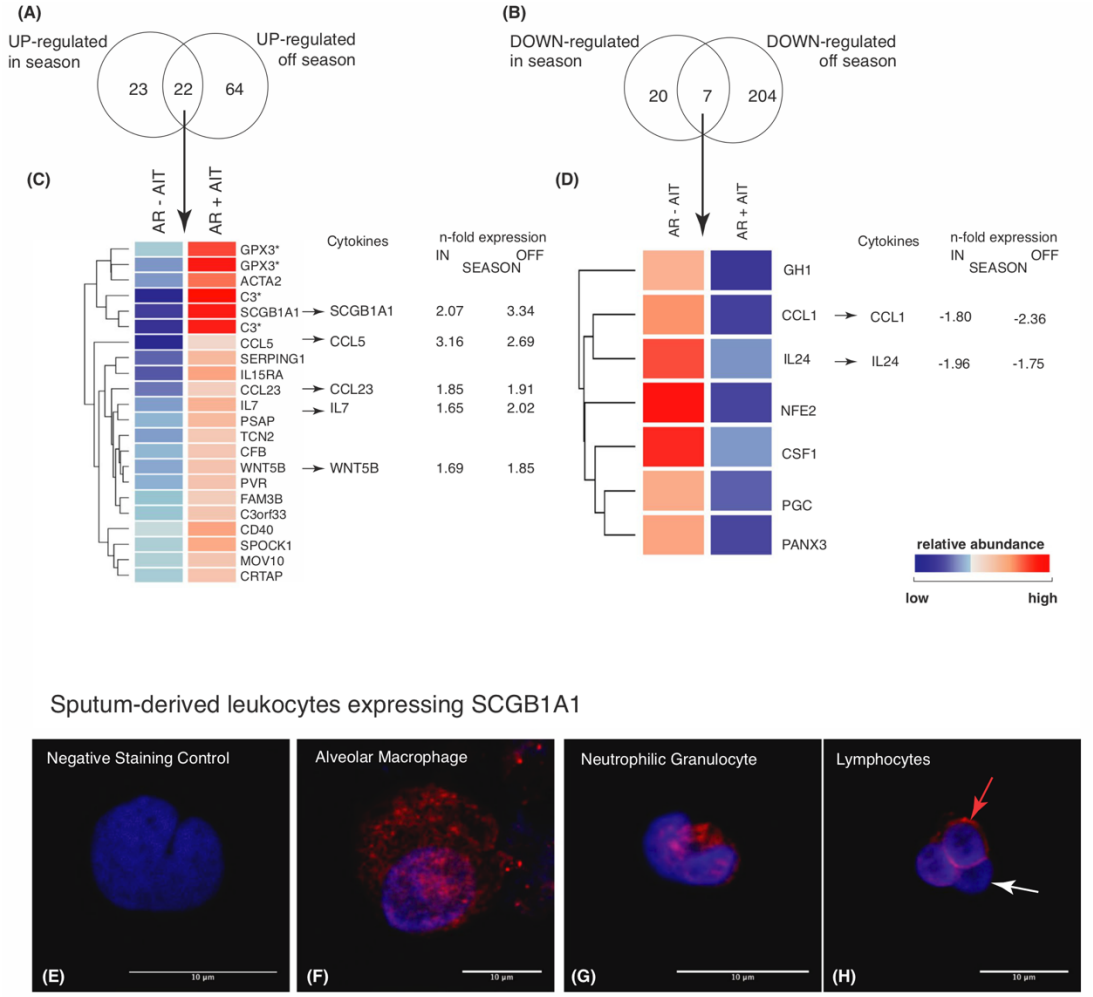
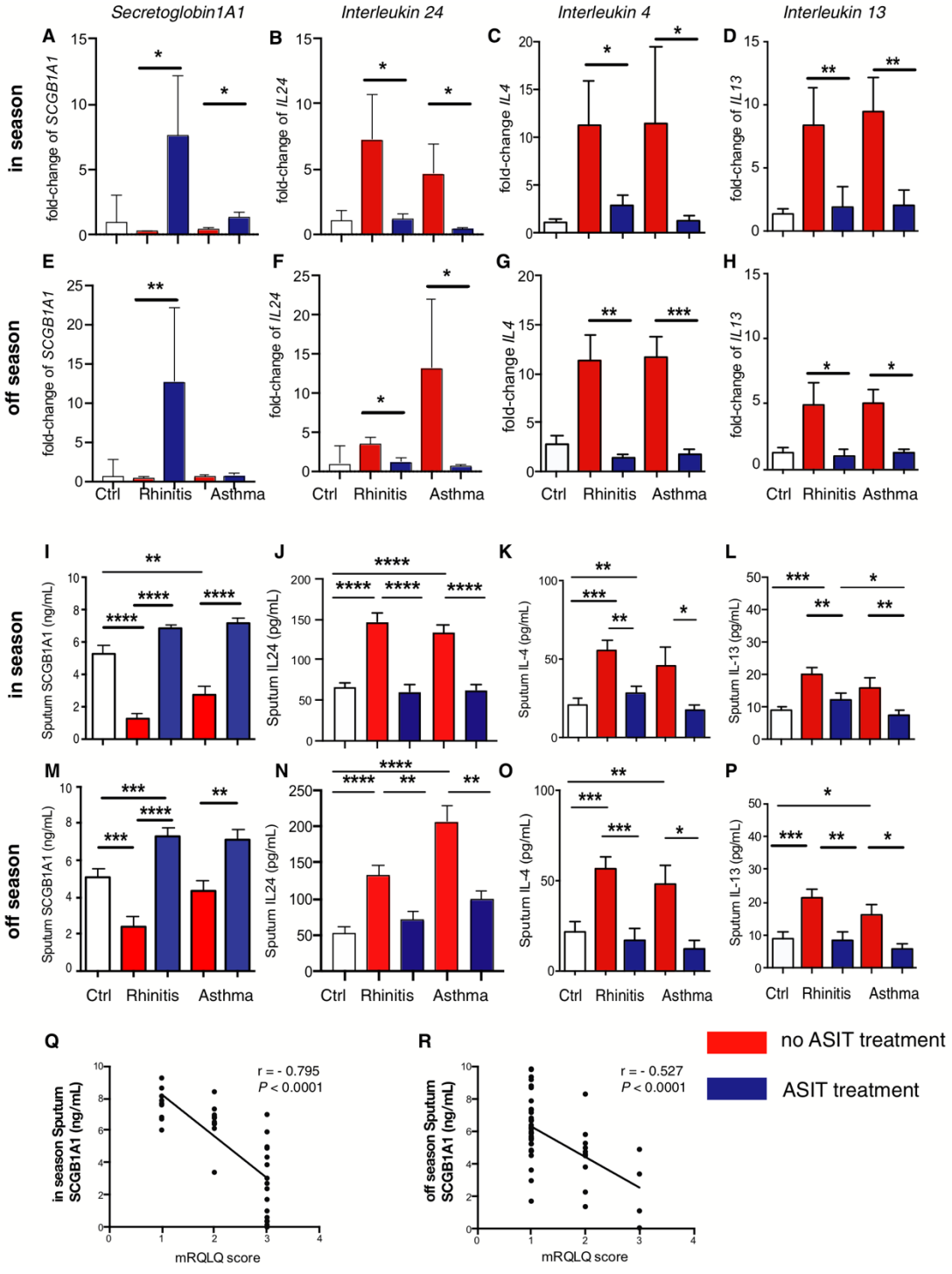






FIGURE 2 Cytokine-related factors differentially expressed independent of season. Distribution of the 45 AIT-induced genes in season, and the 86 AIT-induced genes out of season in a Venn diagram (A). An overlapping set of 22 genes was identified which includes SCGB1A1, CCL5, CCL23, Interleukin-7, and 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 of 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)
mRQLQ score
r = - 0.795
P < 0.0001
r = - 0.527
P < 0.0001
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 pre-seasonal 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

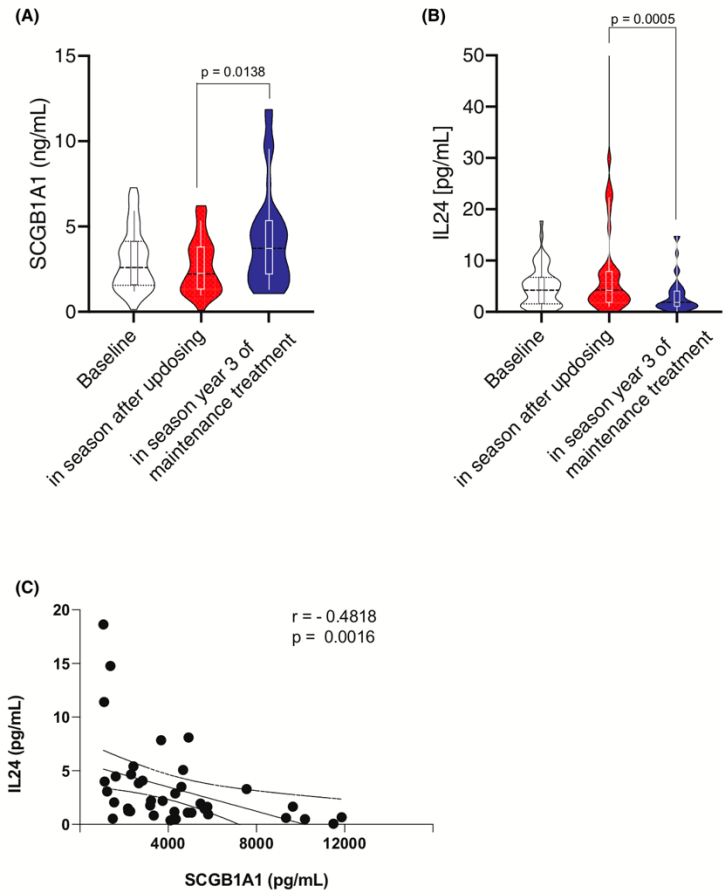



**FIGURE 4** Secreted proteins regulated in nasal lining fluids of allergic rhinitis patients throughout the course of grass-pollen AIT. Levels for SCGB1A1 (A) and IL-24 (B) protein were measured at three time points in an independent validation cohort at baseline, in season after up dosing as well as 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

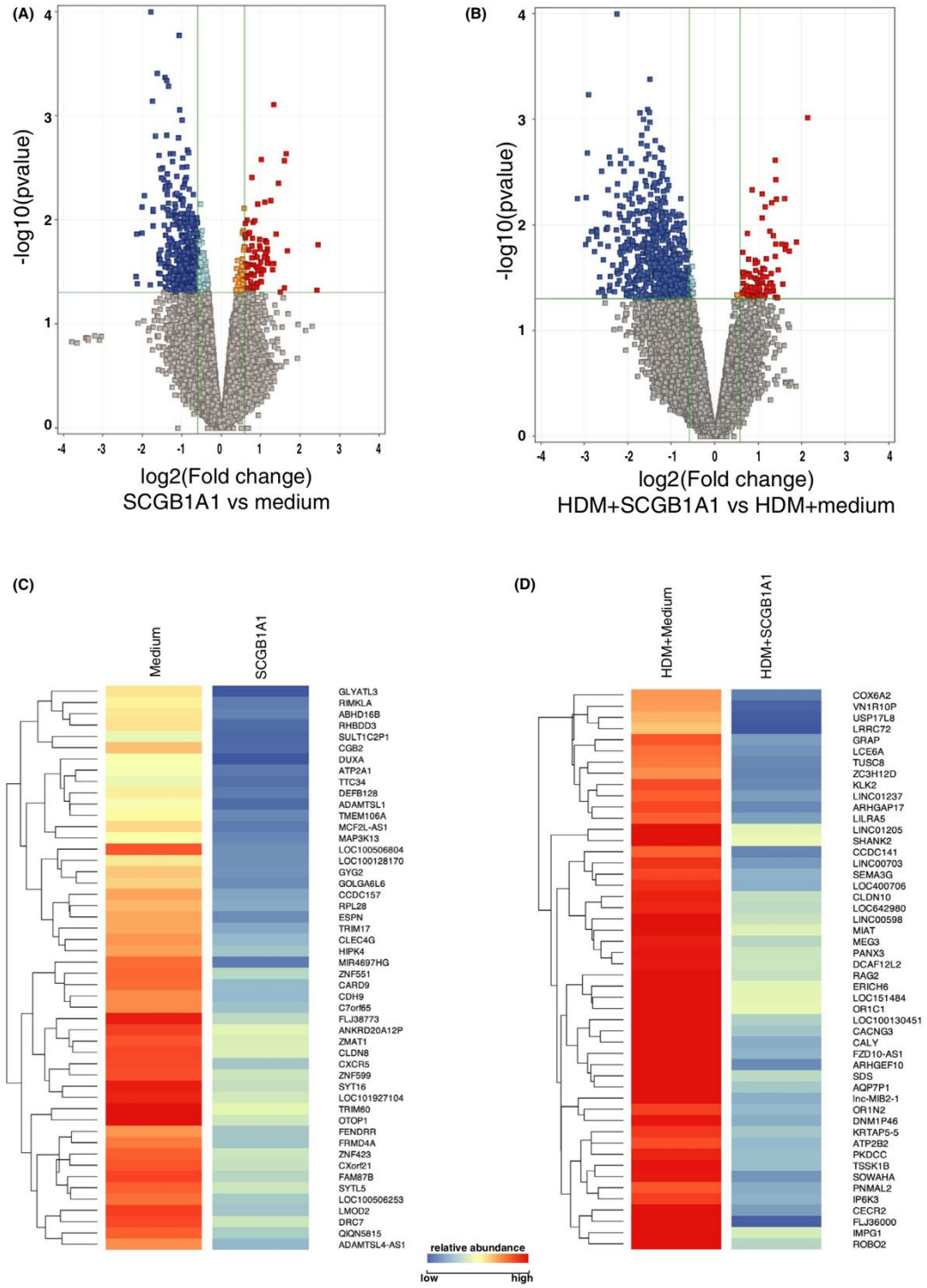

(A)
-log10(pvalue)
log2(Fold change)
SCGB1A1 vs medium
(B)
-log10(pvalue)
log2(Fold change)
HDM+SCGB1A1 vs HDM+medium
(C)
Medium
SCGB1A1
GLYATL3
RIMKLA
ABHD16B
RHBDD3
SULT1C2P1
CGB2
DUXA
ATP2A1
TTC34
DEFB128
ADAMTSL1
TMEM106A
MCF2L-AS1
MAP3K13
LOC100506804
LOC100128170
GYG2
GOLGA6L6
CCDC157
RPL28
ESPN
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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## 4.6 Early IL-10 producing B-cells and coinciding Th/Tr17 shifts during three year grass-pollen AIT

Ulrich M. Zissler, Constanze A. Jakwerth, Ferdinand Guerth, Lisa Pechtold, Juan Antonio Aguilar-Pimentel, Katharina Dietz, Kathrin Suttner, Guido Piontek, Bernhard Haller, Zuzana Hajdu, Matthias Schiemann, Carsten B. Schmidt-Weber, Adam M. Chaker.

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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 hay fever 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 hypothezise 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.

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

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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.
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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 surrogative 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 revertible 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].

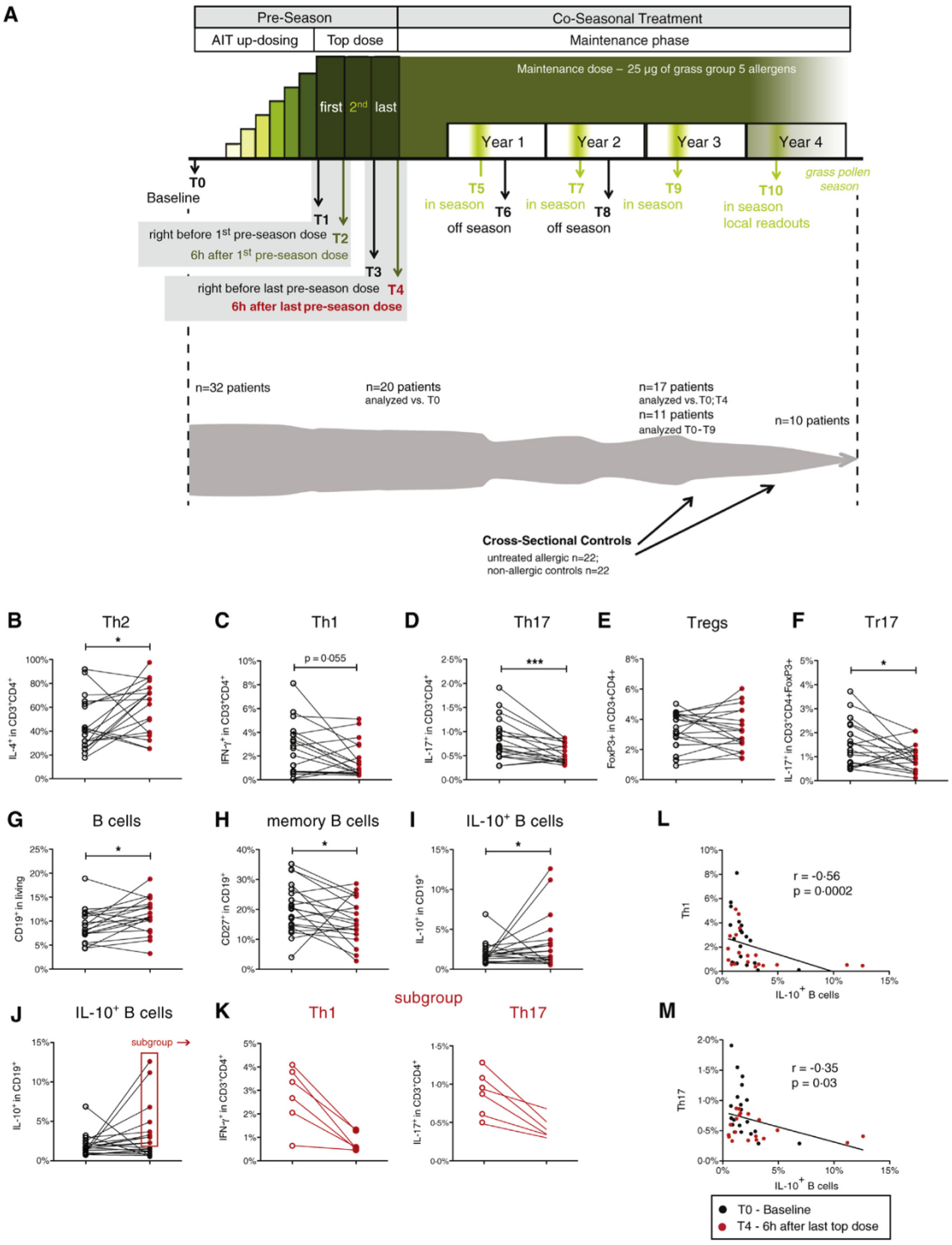
In this exploratory clinical cohort study with grass-pollen allergic patients undergoing AIT, we provide for the first time longitudinal data implicating the Breg/Th1/Th17 suppression axis early indicative for long-term therapy success, and demonstrating a shift of the Treg/Th2 balance late in tolerance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


A
Pre-Season
AIT up-dosing
Top dose
Co-Seasonal Treatment
Maintenance phase
Maintenance dose – 25 µg of grass group 5 allergens
first 2nd last
Year 1
Year 2
Year 3
Year 4
grass pollen season
T0 Baseline
T1
right before 1st pre-season dose
T2
6h after 1st pre-season dose
T3
right before last pre-season dose
T4
6h after last pre-season dose
T5 in season
T6 off season
T7 in season
T8 off season
T9 in season
T10 in season local readouts
n=32 patients
n=20 patients analyzed vs. T0
n=17 patients analyzed vs. T0;T4
n=11 patients analyzed T0-T9
n=10 patients
Cross-Sectional Controls
untreated allergic n=22;
non-allergic controls n=22
B Th2
C Th1
D Th17
E Tregs
F Tr17
p = 0·055
G B cells
H memory B cells
I IL-10+ B cells
L
r = -0·56
p = 0·0002
J IL-10+ B cells
subgroup
K Th1
Th17
M
r = -0·35
p = 0·03
T0 - Baseline
T4 - 6h after last top dose

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 $CD3^+CD4^+IL\text{-}17^+$ (Th17) cells, $CD3^+CD4^+FoxP3^+IL\text{-}17^+$ (Tr17) cells, $CD3^+CD4^+FoxP3^+$ (Tregs), $CD3^+CD4^+FoxP3^+IL\text{-}10^+$ ($IL10^+$ 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 $20 \times 10\text{^}6$/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 B™ 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% $CO_2$ in a fully humidified atmosphere for 7 days according to manufacturer's instructions. CTL Human B-Poly-SE™ 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 B™ 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% $CO_2$ 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, Millerica, MA, USA) and subjected to ELISA or MSD Mesoscale analysis.

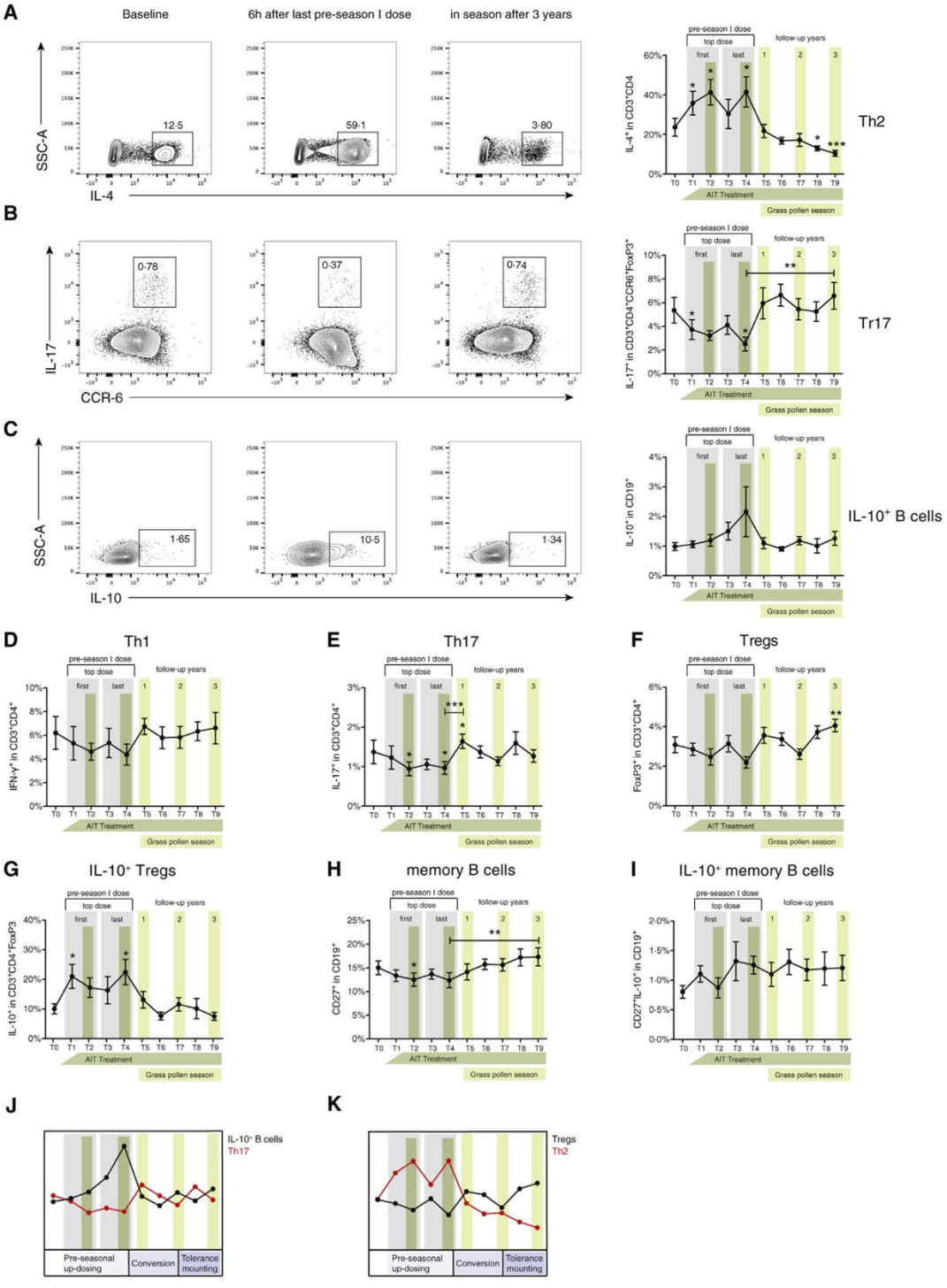
### 2.6. Flow cytometry

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) $IL\text{-}4^+$ $CD4^+$ Th2 cells; (C) IFN-$\gamma^+$ $CD4^+$ Th1 cells; (D) $IL\text{-}17^+$ $CD4^+$ Th17 cells; (E) $FoxP3^+$ $CD4^+$ Tregs; (F) $IL\text{-}17^+$/$FoxP3^+$-co-expressing $CD4^+$ Tr17 cells; (G) total $CD19^+$ B cell numbers; (H) $CD27^+$ memory B cells; (I) $IL\text{-}10^+$ 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 $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$.


A
Baseline
6h after last pre-season I dose
in season after 3 years
SSC-A
IL-4
12·5
59·1
3·80
B
IL-17
CCR-6
0·78
0·37
0·74
C
SSC-A
IL-10
1·65
10·5
1·34
Th2
Tr17
IL-10⁺ B cells
pre-season I dose
top dose
follow-up years
first
last
AIT Treatment
Grass pollen season
D
Th1
E
Th17
F
Tregs
G
IL-10⁺ Tregs
H
memory B cells
I
IL-10⁺ memory B cells
J
IL-10⁺ B cells
Th17
K
Tregs
Th2
Pre-seasonal up-dosing
Conversion
Tolerance mounting

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 μl. Subsequently, the one color spike-in mix was prepared and 2.0 μl were added to 1.5 μl of RNA on ice. In the next step, the T7-promoter primer mix was prepared and 1.8 μl 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 μl 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 μl 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 μl of nuclease-free water for a total volume of 100 μl and 350 μl 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 μg) as well as the specific activity (pmol Cy3 per μg cRNA). If the yield was below 0.825 μg or/and specific activity below 6.0 pmol Cy3 per μg 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 μl added to 600 ng cRNA in a volume of 19 μl. 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 ± 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 updosing 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 ± 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).

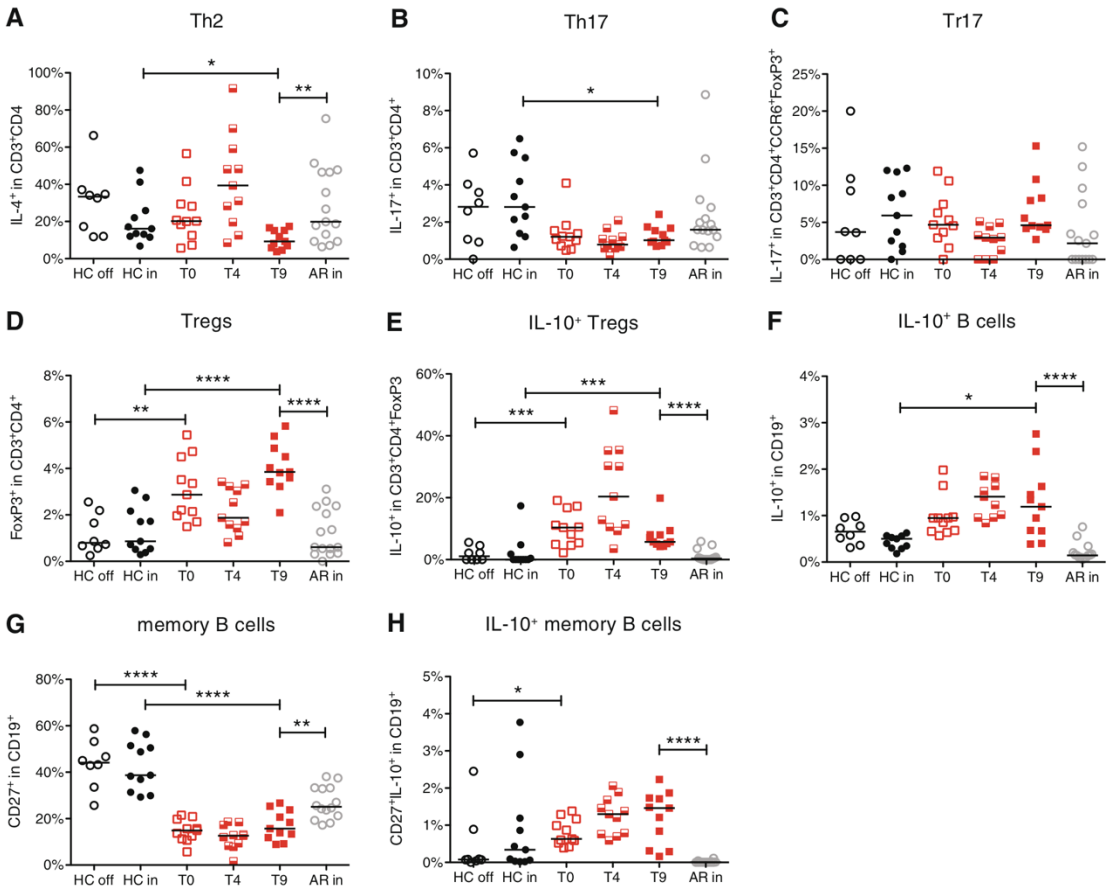



**Fig. 3.** Differential long-term systemic therapy effects on th17 and tr17 subsets. Intracellular flow cytometry analysis of circulating T and B cell subsets comparing healthy control subjects during off season (HC off; n = 8), in grass pollen season (HC in; n = 11), treated patients throughout course 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% ± 5·06 vs. T4:59·87% ± 5·08; $p = 0·049$; Fig. 1B) was observed, while Th1 (T0:2·62% ± 0·51 vs. T4:1·64% ± 0·34; $p = 0·055$; Fig. 1C) and Th17 (T0:0·85% ± 0·10 vs. T4:0·55% ± 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% ± 0·31 vs. T4:3·20% ± 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% ± 1·86 vs. T4:16·36% ± 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% ± 0·22 vs. T4:0·95% ± 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% ± 0·52 vs. T4:0·79% ± 0·17) and Th17 cells (T0:0·86% ± 0·12 vs. T4:0·43% ± 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% ± 4·50 vs. T9:10·43% ± 1·51; $p = 0·001$; Fig. 2A,K). Surprisingly, we found a significant decrease of Th17 cells (T0:1·37% ± 0·30 vs. T4:0·97% ± 0·16; $p = 0·04$; Fig.2E) and CCR6$^{+}$ Tr17 cells (T0:5·36% ± 1·08 vs. T4:2·52% ± 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% ± 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% ± 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% ± 0·59 vs. T9:6·58% ± 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% ± 0·40 vs. T9:4·06% ± 0·32; $p = 0·004$; Fig. 2F,K). During the up-dosing phase, IL-10$^+$ B cells increased progressively (single comparison T0:0·99% ± 0·13 vs. T4:2·16% ± 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% ± 1·71 vs. T4:22·46% ± 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% ± 1·45 vs. T2:12·52% ± 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% ± 1·58 vs. T9:17·33% ± 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% ± 0·11 vs. T4:1·26% ± 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% ± 5·35 vs. treated:10·43% ± 1·51; $p = 0·0043$; Fig. 3A) were significantly decreased, whereas Th17 displayed only a slight downward trend between groups (untreated:2·38% ± 0·56 vs. treated:1·27% ± 0·16; p = 0·12; Fig. 3B). However, AIT induced a significant increase of CCR6$^+$ Tr17 cells (untreated:3·75% ± 1·31 vs. treated:6·58% ± 1·13; p = 0·019; Fig. 3C) and Tregs (untreated:1·23% ± 0·26 vs. treated:4·06% ± 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% ± 0·50 vs. treated:7·51% ± 1·34; $p < 0·0001$; Fig. 3E) as well as in B cells (untreated:0·22% ± 0·05 vs. treated:6·09% ± 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% ± 1·88 vs. treated:17·07% ± 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% ± 0·01 vs. treated:1·21% ± 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 subets 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% ± 0·41, *phleum*(T0):13·22% ± 2·28; p < 0·0001; Fig. 4A). The antigen-specific induction of Th17 cells decreased strongly throughout course of therapy (*phleum*(T0):13·22% ± 2·28, *phleum* (T9):6·73% ± 0·66; p = 0·015). Tregs were also allergen-specifically induced, but only in cultures prior treatment (medium (T0):0·84% ± 0·30, *phleum*(T0):2·29% ± 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% ± 1·90, *phleum*(T0):36·65% ± 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% ± 7·24, *phleum*(T9):24·94% ± 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% ± 0·22, *phleum*(T0):10·37% ± 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% ± 1·26, *phleum*(T4):23·89% ± 2·88, *phleum*(T9):17·85% ± 1·75; Fig. 4E), which coincided with a minimal decrease of IgE levels from supernatants (*phleum*(T0):1·48% ± 0·24, *phleum*(T4):1·38% ± 0·19, *phleum*(T9):1·53% ± 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 (log2 FC > 1.5, $p < .05$; Fig. 5). To clarify, only mediators crucial to local airway immune processes are shown in Fig. 5B and

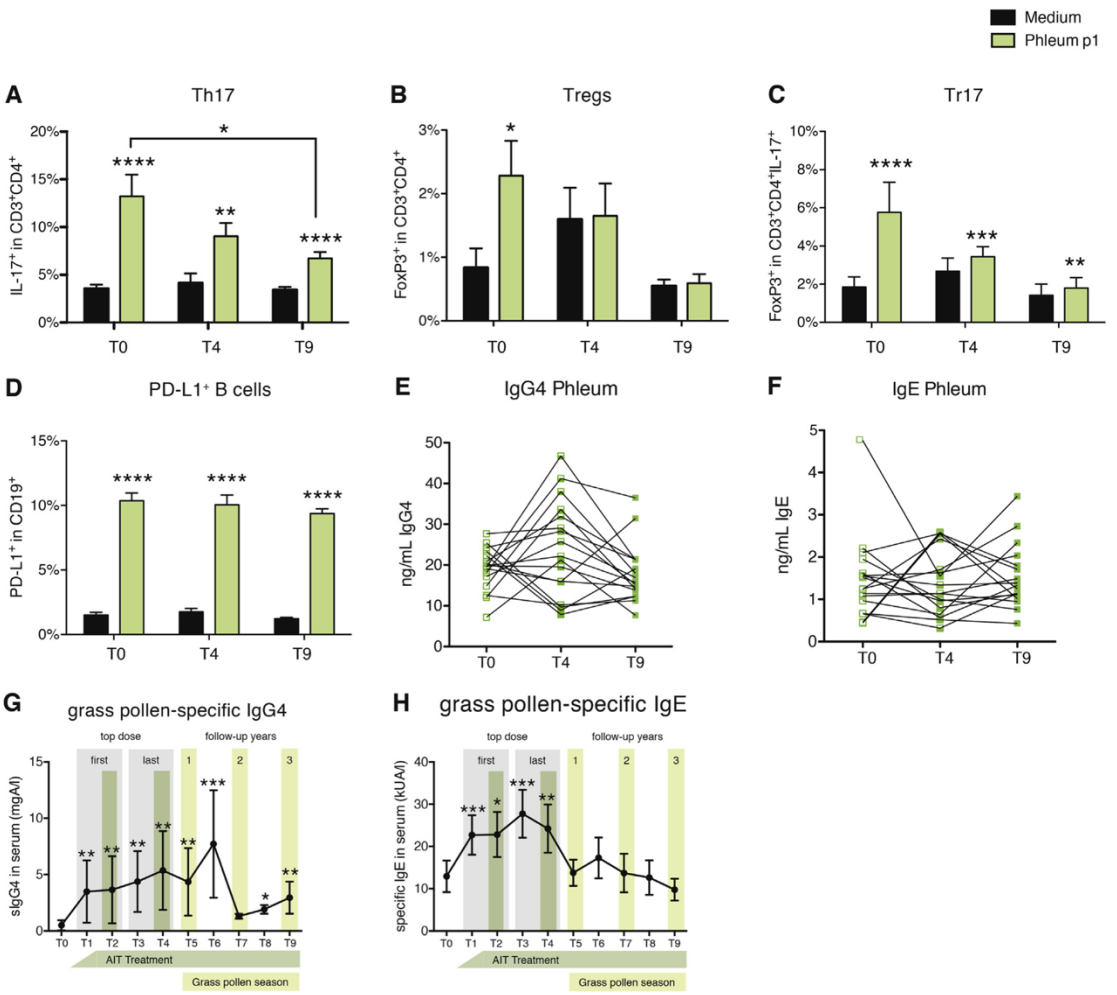



**Fig. 4.** In-vitro: allergen-specific lymphocyte activation in favor of immune regulation. PBMCs throughout therapy course time points T0, T4, and T9 ($n = 17$) were stimulated with medium (black) or 5 μg/ml phleum p1 (red) for 7 days in culture and T and B cells were analysed using intracellular antigen 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 ± 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 I $n = 8$). Results are depicted as mean ± 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

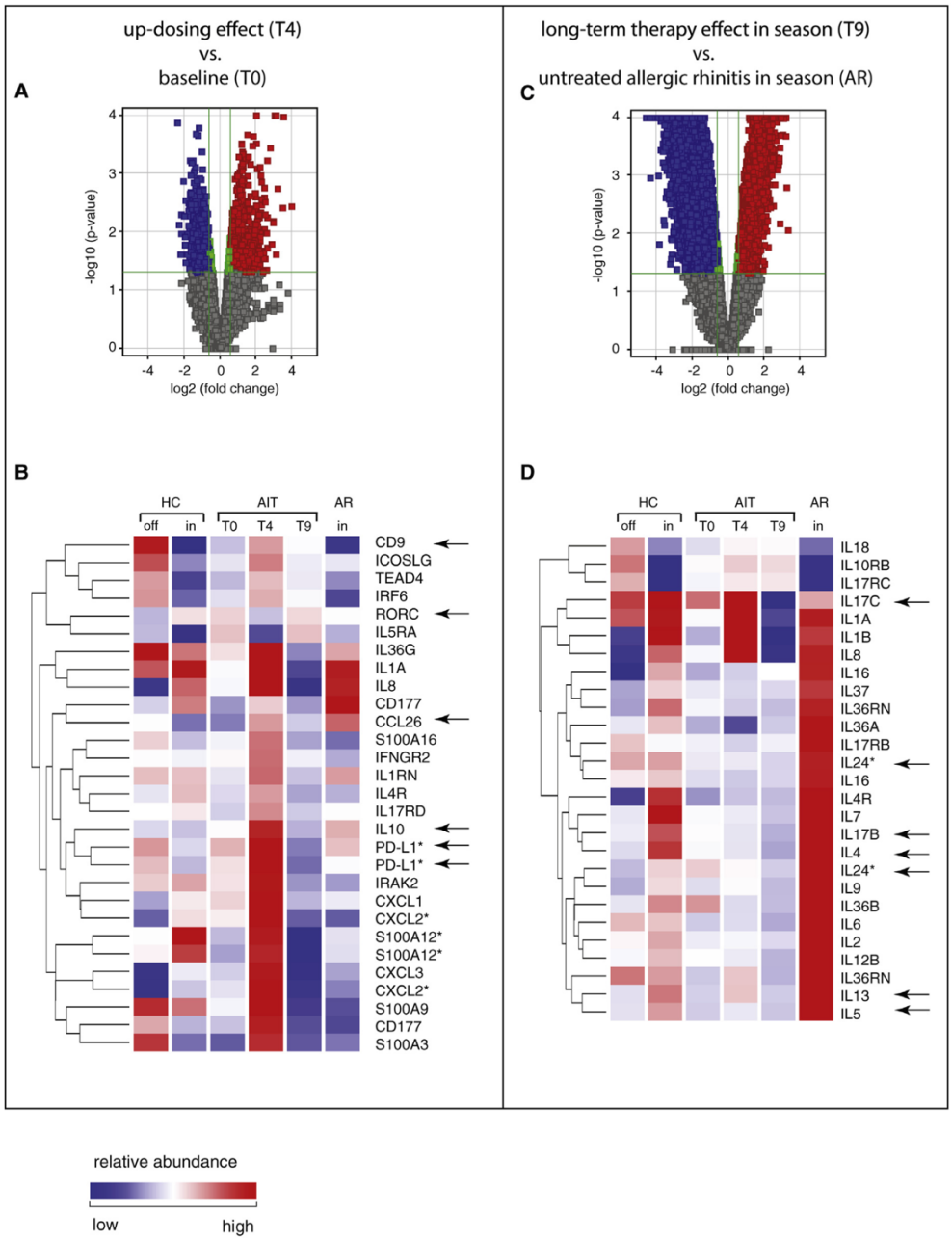



**Fig. 5.** Local gene expression changes indicate shifts in regulatory hierarchies in the nasal mucosa. Gene expression changes of nasal scrapings were taken from healthy control subjects during off season (HC off; $n = 3$), in grass pollen season (HC in; $n = 3$), treated patients throughout course 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 ≥ 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 ≥ 1.5). Selection of entities is shown, which are relevant for allergy. (C) Volcano plot of statistically significant entities ($p < 0.05$; FC ≥ 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.

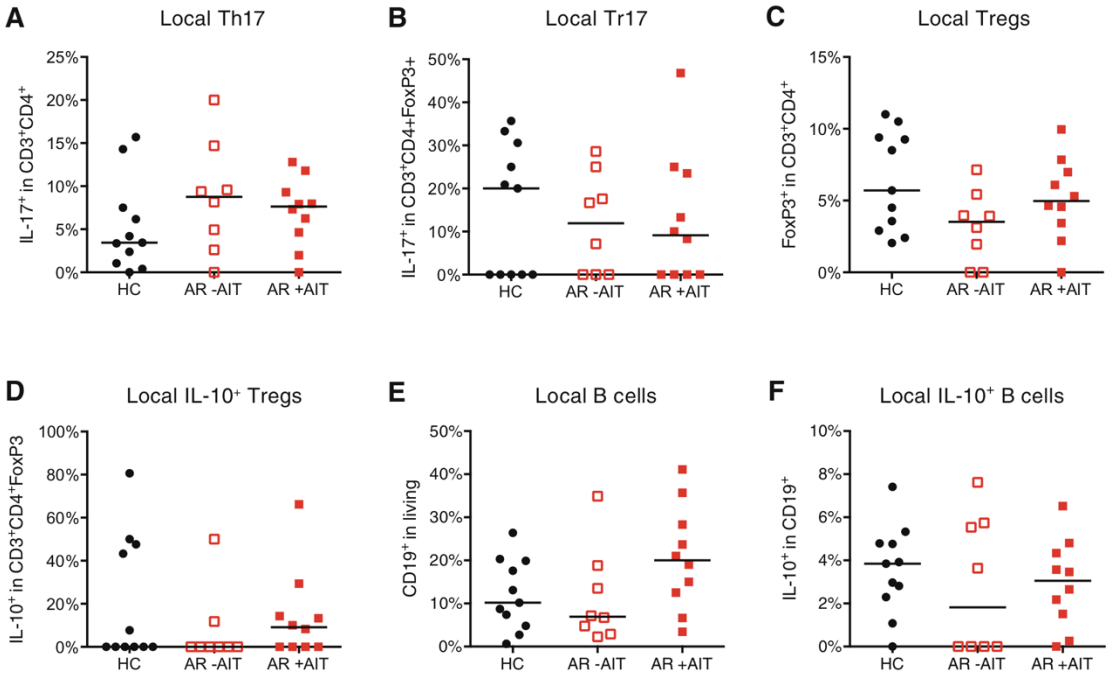



**Fig. 6.** Local shifts of immune cell compartments following AIT. Intracellular FACS staining of scraped nasal lymphocytes from healthy controls (HC; $n = 11$), allergic rhinitis patients without AIT (AR – AIT; $n = 8$), and allergic rhinitis patients with long-term AIT (AR + AIT; $n = 10$) in grass 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% ± 2·28, treated:7·43% ± 1·22; Tr17: untreated:11·88% ± 4·13, treated:12·61% ± 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% ± 0·88, treated:5·05% ± 0·82; Fig. 6C), and also displayed a higher frequency of IL-10 production (untreated:1·69% ± 1·69, treated:15·54% ± 6·04; Fig. 6D). Further, AIT-treated patients had by tendency higher numbers of local B cells (untreated:11·38% ± 3·90, treated:20·53% ± 3·45; Fig. 6E) as well as of IL-10$^+$ B cells compared to untreated patients (untreated:2·82% ± 1·13, treated:3·10% ± 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

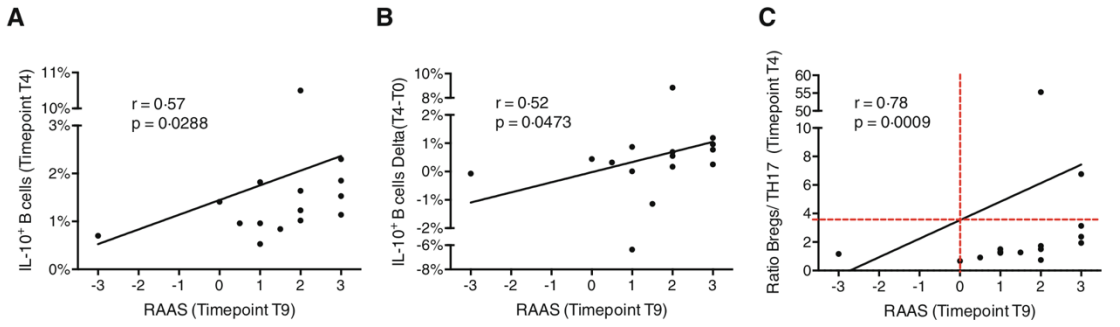



**Fig. 7.** Correlation of symptom score raas after 3 years of ait with early B and T cell frequencies. Spearman correlation analysis was performed comparing the RAAS at time point T9 with (A) total peripheral IL-10$^+$ B cell numbers at time point T4 ($n = 15$) or (B) with the in−/reduction of peripheral 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) ($r = 0{\cdot}57$, $p = 0{\cdot}0288$; $r = 0{\cdot}52$, $p = 0{\cdot}0473$; 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-$17^+$CD$4^+$ 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 ($r = 0{\cdot}78$; $p = 0{\cdot}0009$; 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-$17^+$FoxP$3^+$CCR$6^+$ 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 FoxP$3^+$ 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 FoxP$3^+$ 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.

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# 5. Diskussion

Die in dieser kumulativen Habilitationsschrift zusammengefassten Studien tragen zu einem besseren Verständnis der fehlgeleiteten, mukosalen Immunität der Atemwege der allergischen Rhinitis und Asthma, der Rolle des Atemwegsepithels in den zugrunde liegenden immunologischen Vorgängen und der Interaktion mit Immunzellen bei. Diese Arbeit bildet die Grundlage für neue Ansätze zur Verbesserung der Diagnostik und Therapie, die sich außerdem auf andere Bereiche der Atemwegsimmunologie übertragen lassen.
Bis vor wenigen Jahren wurde das Hauptaugenmerk bei der allergischen Entzündung vor allem auf allergen-spezifische Effektorzellen wie B- und T-Lymphozyten gelegt. In den vergangenen Jahren wurde die wichtige Rolle der Innaten Lymphoiden Zellen (ILCs) im Gewebe sowie an der epithelialen Barriere entdeckt, wodurch in diesem Bereich neue Mechanismen und Signalwege für die Entstehung der allergischen Entzündung erforscht werden konnten. Durch die gewebebasierten Entzündungsmechanismen entstehen auch die begleitenden Symptome, welche ein wichtiger Pfeiler bei der Diagnose der Erkrankungen sind, die Zusammenhänge mit dem Epithel und dessen aktive Rolle in der Immunregulation sind jedoch noch nicht lückenlos aufgeklärt.

## 5.1 Typ 2-Prägung in Atemwegsepithelzellen

Die Epithelzellen als erste Barriere unseres Körpers gegen die Umwelt wurden meist auf die Rolle als physische Barriere zurückgedrängt, Immunfunktionen wurden Epithelzellen bis vor wenigen Jahren allerdings nur eingeschränkt zugeschrieben, jedoch findet hier der erste Kontakt zu Umweltallergenen statt. Das Epithel verhindert nicht nur das Eindringen der Fremdstoffe in das Interstitium, sondern spürt auch ihre Anwesenheit auf, informiert das Immunsystem über einen bevorstehenden Angriff und transportiert Fremdstoffe intakt durch die Epithelzellschicht hindurch, wo sie dann im Weiteren von Antigen-präsentierenden Zellen aufgenommen und prozessiert werden können [154]. Diese Reaktion und Funktion des Atemwegsepithels als morphologische und funktionale Komponente des Immunsystems ihre Steuerung durch die jeweilige Stimulation des umgebenden Zytokinmilieus mit entsprechenden Schlüsselzytokinen der spezifischen Immunantwort standen im Mittelpunkt unserer Untersuchungen, welche durch lokale Immunzellen in das Gewebe sowie das Atemwegslumen sezerniert werden und im weiteren Verlauf die Entwicklung einer aberranten epithelialen Immunität fördern. Zu nennen sind hier im Besonderen IFN-$\gamma$ für die pro-inflammatorische Th1 sowie IL-4 und IL-13 für die pro-allergische Th2 Immunantwort [91]. Veränderungen im Zytokinmilieu erfolgen entsprechend

der Immunantwort in Richtung einer Th1-assoziierten Antwort der Atemwegsepithelzellen, von uns als E1-Typ bezeichnet, beispielsweise bei viralen Erkrankungen. Nasale Epithelzellen von allergischen Kindern, welche dem humanen Respiratorischen Synzytial-Virus (RSV) ausgesetzt waren, produzierten geringe Mengen des Typ 1 Interferons IFN-β und wiesen im Vergleich zu Epithelzellen von Gesunden eine höhere Freisetzung von Viren auf [155]. Wir konnten bei allergischen Erkrankungen eine Th2-assoziierte Antwort und Polarisierung der Atemwegsepithelzellen zeigen, von uns als E2-Typ bezeichnet [156]. IL-4 wie auch IL-13 können Veränderungen in der Struktur, Morphologie und Differenzierung des Epithels hervorrufen, die sowohl zur Entzündung als auch zu Strukturveränderungen in den Atemwegen, dem sogenannten Remodeling, beitragen können [157, 158]. Ein Merkmal der zellulären Differenzierung ist auch die epigenetisch geprägte Übertragung zellulärer Phänotypen einer Zellgeneration auf die nächste [159]. So zeigten wegweisende Arbeiten zur genomweiten Methylierung, dass selbst eine kurzzeitige Einwirkung von IL-13 langanhaltende Veränderungen bei der DNA-Methylierung induzieren konnte, welche spezifisch die Signalwege der Atemwegsepithelzellen verändert haben und zu Asthma-Phänotypen beitrugen [160]. Wir konnten in unseren eigenen Arbeiten zeigen, dass IL-4 zur Induktion von *WNT5A* beiträgt, welches wiederum eine wesentliche Rolle bei der epithelialen Differenzierung spielt [161]. Weiterhin ist bekannt, dass CCL-26 (Eotaxin-3), induziert von IL-4/IL-13, über eine CREB-vermittelte Histon-3-Acetylierung reguliert wird [162]. Es wurde auch gezeigt, dass IL-4 die Expression von CCL11 (Eotaxin-1) in Atemwegsepithelzellen durch Induktion von Arginin-Methyltransferase-1 erhöht [163]. Diese IL-4 induzierten Mechanismen zeigen, wie Epithelzellen unter dem Typ-2 bzw. E2 Einfluss spezielle Entzündungsmechanismen antreibt. Wir konnten erstmals ein transkriptionales Regulationsnetzwerk beschreiben, welches die spezifischen Transkriptionsfaktoren der E1- (TBX21, TEAD4, ZEB1, GLIS3, MEF2c) und der E2- (GATA3, NFE2, MEIS-1, HEY-2, AHR, RUNX2, CREB) Phänotypen in Atemwegsepithelzellen aufzeigte. Während GATA3 bisher nur als der Schlüssel-Transkriptionsfaktor der Th2-Antwort in Immunzellen beschrieben war, konnten wir auch zeigen, dass es ebenfalls von primären Lungenepithelzellen exprimiert wird. GATA3 mobilisiert im Besonderen die Chromatinzugänglichkeit von Gen-Loci wie dem IL-4-Cluster in T-Zellen, indem es mit dem NuRD-Komplex (*Nucleosome Remodeling and Deacetylase-Chromatin-Remodeling*) interagiert [164]. Darüber hinaus zeigen unsere Daten, dass sowohl unterschiedliche, Histon-modifizierende Enzyme wie die Klasse I-Histon-Deacetylase (HDAC)-2 durch IL-4 und Klasse II-HDAC-4 durch IFN-γ differentiell in E2 oder E1-polarisierten Zellen exprimiert werden, während HDAC-9, ebenfalls eine Klasse II-HDAC, durch beide Zytokine induziert wird. HDAC9 ist ein zentraler epigenetischer Faktor,

der die Expression von Entzündungsfaktoren reguliert [165]. Der Hochregulierung von Th2-Zytokinen folgt *in vitro* eine Hochregulierung der HDAC-1 und -9, die mit einer gestörten Integrität der Epithelbarriere *in vitro* korreliert ist [166]. Somit ist ein mehrstufiges Modell der Genaktivierung von IL-4- und IFN-γ wahrscheinlich, wobei die Locus-Zugänglichkeit die Zytokinexpression reguliert. So führt die T-Zell-Aktivierung unabhängig vom Zytokinmilieu zur Acetylierung und Transkription von IL-4 und in geringerem Maße von IFN-γ. Diese Phase wird mutmaßlich durch Signale vermittelt, welche durch TCR/CD28-Stimulation erzeugt werden.

Eine Frage ist auch die Natur der Gegenregulation in E1- und E2-polarisierten Zellen. Aus dem Antagonismus in Th1- und Th2-Zellen wissen wir, dass Zytokine und Transkriptionsfaktoren, welche die Differenzierung in Th1- oder Th2-Zellen fördern, auch Auswirkungen auf die jeweils andere Zellpopulation haben. Die positive und negative Regulation wird unter anderen durch STAT sowie GATA3 und TBX21 vermittelt [167-169]. GATA-3 kann die TBX21-Expression sowie die IFN-γ-Sekretion und damit die Differenzierung von naiven T-Zellen zu Th1-Zellen unterdrücken [167]. Auch die Zellfunktionen der Th1-Zellen können von STAT-6 [170] und c-MAF [171] unterdrückt werden, bei Letzterem sogar unabhängig von IL-4. Eine Differenzierung zu Th2-Zellen kann über Th1-assoziierte Mediatoren wie IFN-γ und IL-12 über STAT-1- [172] und STAT-4-Signale [169] inhibiert werden. Die Aufrechterhaltung und Erweiterung der Zugänglichkeit des relevanten Zytokin-Locus sowie supprimierende Signale, welche die Expression des antagonistischen Zytokins verhindern, verstärken die Polarisierung der jeweiligen Population [173]. Wir konnten ebenfalls eine Regulation von NF-E2 über IL-4 zeigen, welches im IL-4-abhängigen Signalweg bereits von anderen hinsichtlich seines zytoprotektiven Potentials untersucht wurde und eine starke Aktivität gegen Epithelzellschädigung beispielsweise durch Zigarettenrauch besitzt, was auf eine schützende Rolle in einem IL-4-dominierten Mikromilieu hinweisen könnte [174]. Zudem erzeugt die Inaktivierung von MEIS1, einem Co-Faktor der HOX-Gene, eine Zunahme der Masse der glatten Atemwegsmuskulatur und eine entsprechende Abnahme des Knorpelgewebes, was auf eine wichtige Rolle bei allergischen Atemwegserkrankungen hindeutet [175]. Der Verlust der Genfunktion von HOX schließt dagegen eine Atemwegsreparatur nicht kategorisch aus [176]. Der Verlust der HOXA5-Funktion in den Atemwegen führt zu einer Transdifferenzierung aus sekretorischen Zellen der distalen Atemwege, den Keulenzellen (ehemals Clara-Zellen) in Becherzellen, wodurch HOXA5 den Notch-Signalweg regulativ beeinflusst und zur Ausbildung einer Becherzellmetaplasie führt, welche mit einer erhöhten Aktivität des Notch-Signalwegs und dessen Effektor-Gen *HEY2*

verbunden ist [176]. In unserer Studie beobachteten wir eine Induktion von *HEY-2* über IL-4. Mehrere Studien haben zudem einen hemmenden Einfluss über IFN-γ und einen induzierenden Einfluss über IL-4 auf Notch-Signalwege gezeigt, welche eine Schlüsselrolle bei der Kontrolle der empfindlichen Homöostase zwischen ziliierten und sekretorischen Zellen sowie bei der Entwicklung einer Becherzellmetaplasie spielen [177-179]. Dementsprechend führte die Überexpression der *Notch-Intracellular Domaine* (NICD) im 3D-Modell humaner Atemwegsepithelzellen auch zu einer höheren Anzahl von Becherzellen und einer erhöhter Expression von *SCGB1A1* als Panmarker der Becherzellen und *Mucin5A/C* bei gleichzeitiger Verringerung der Anzahl ziliierter Zellen und der Expression assoziierter Gene [180]. Die IL-4-vermittelte Induktion des Arylhydrocarbon-Rezeptors (AhR), welcher ebenfalls einen wichtigen Beitrag bei der Epithelzelldifferenzierung leistet, schlägt indes eine Brücke in seiner Funktion als Schlüsselfaktor bei der Induktion von IL-22 [181, 182], welches seinerseits Mechanismen der Epithelreparatur fördern könnte [73]. Darüber hinaus kann GATA3 durch AhR gehemmt werden, was nicht nur eine negative Rückkopplungsschleife erzeugen kann, sondern auch eine Edukt-Hemmung durch IFN-γ-induzierte Indolamin-2,3-Dioxigenase (IDO)-Metaboliten wie Kynurenin erlaubt [182-184]. Als Mitglied der Interferon-stimulierten Gene (ISGs) besitzt IDO einen IFN-induzierbaren Promoter, welcher in Immun- und Epithelzellen zu einer erhöhten Genexpression führt [185]. Über den IDO-vermittelten Abbau von Tryptophan kann die T-Zellproliferation [186] inhibiert werden und stellt einen wichtigen Faktor bei der Vermittlung der T-Zell-Anergie bei Allergen-sensibilisierten Personen dar [187]. Die einzigartigen Funktionen von IL-4 selbst können durch die exklusive Expression des Typ-I-Rezeptors auf bestimmten Zelltypen erklärt werden, wie die Begrenzung der Typ-I-Rezeptorkomplexe auf T-Zellen das Ansprechen auf IL-4, nicht jedoch auf IL-13 vermitteln [84]. Analog zu den lymphozytären Mechanismen war STAT6 ebenfalls in IL-4-stimulierten Epithelzellen verstärkt exprimiert und liefert somit Hinweise darauf, dass STAT6 einer der Signalübermittler ist, welcher die epitheliale GATA3-Expression induzieren kann [188]. Unsere Studie zeigt somit, dass dieses antagonistische Netzwerk zwischen IL-4 und IFN-γ die epitheliale Genexpression und die epitheliale Differenzierung kontrolliert. Diese Ergebnisse unterstützen das in unseren Arbeiten untersuchte Konzept einer epithelialen Polarisation hinsichtlich ihres Kontakts mit den Th1- oder Th2-Schlüsselzytokinen IFN-γ oder IL-4. Dabei führt die Stimulation von respiratorischen Epithelzellen auch zur Freisetzung sekretierter Komponenten im Kontext des E2-Epithels. Im murinen Modell wurde die Fähigkeit von terminal differenzierten Atemwegsepithelzellen zur Dedifferenzierung in Vorläuferzellen nachgewiesen, so dass diese Zellen ein Potential als

basale Stammzellen besitzen könnten [189]. Eine epitheliale Vorprägung in Typ 2-abhäniger Weise, für deren Existenz unsere Daten eine Grundlage bilden, könnte über die Weitergabe dieses Musters an Tochterzellen einen wichtigen Einfluss auf die Chronifizierung von allergischen Atemwegserkrankungen besitzen.

### 5.2 Natürliche Allergenexposition induziert lokale Typ 2 Antworten

Die Interaktion zwischen Allergenen und Atemwegszellen beginnt mit der Erkennung eines Allergens durch Rezeptoren auf dem Atemwegsepithel, welche zur Freisetzung von Entzündungsmediatoren aus den Epithelzellen führt. Über Allergene aktivierte Epithelzellen rekrutieren nicht nur DCs an den Ort der Allergenexposition, sondern induzieren auch die Aktivierung von DCs, um Th2-Immunantworten zu induzieren, wenn sie in die Lunge migrieren [190]. Der genaue Ablauf der Induktion von T2-Immunantworten durch DCs in der Lunge ist unvollständig verstanden, beinhaltet aber eine Induktion von OX40-Liganden [113] und anderer costimulatorischer Moleküle auf DCs sowie ihrer löslichen Mediatoren wie Leukotrien C4, Zytokine wie IL-6, IL-33 und IL-1β sowie die Chemokine CCL17 und CCL22 [191, 192].

Wir konnten bei der Analyse der vorher beschriebenen, *in vitro* identifizierten, sekretierten Markern in induziertem Sputum und nasalen Sekreten von Patienten mit Asthma bronchiale zeigen, dass die Sekretion dieser identifizierten Th2-assoziierten Marker wie CCL-26, IL-24, Activin-A oder Periostin innerhalb der jeweiligen Matrix sehr gut mit einer E1- oder E2-Polarisation des Epithels und der jeweils zugrundeliegenden Entzündung assoziiert ist, aber auch zwischen oberen und unteren Atemwegen eine große Schnittmenge bildet [193-195]. Eine solche Übereinstimmung zeigte sich im Besonderen für Biomarker wie CCL-26, IL-24, Periostin, IL-4, IL-5 und IL-13, die eng mit einer Th2-Entzündung verbunden sind, aber auch für Marker wie IL-1α, TNF-α oder IFN-γ, die einer allgemeinen oder Th1-assoziierten Entzündung zuzuordnen sind. Interessanterweise war die Korrelation von TSLP mit Markern der Th-2 Entzündung innerhalb der oberen und unteren Atemwege dem jeweiligen Kompartment nicht eindeutig zuzuordnen. Dies könnte daran liegen, dass TSLP ebenso wie IL-33 als epitheliales Alarmin hauptsächlich von basalen Epithelzellen der Atemwege in einem Typ-2 Kontext gebildet und sekretiert wird [134], wohingegen die Halbwertszeit in einer Typ-1 Entzündung über die Hochregulation des LMP2-Proteasoms über IFN-γ Caspase-unabhängig reduziert ist, wie in Fibroblasten gezeigt werden konnte [196]. TSLP zählt mit zu den zeitlich am schnellsten freigesetzten Mediatoren der allergischen Entzündung und kann die Rekrutierung der Effektorzellen orchestrieren [97]. Pro-inflammatorische Zytokine wie IL-1α und TNF-α, welche die Th2 Schlüsselzytokine IL-4 und

IL-13 sowie einige Toll-like Rezeptoren einschließlich TLR2 aktivieren können, sind ebenfalls an der Induktion von TSLP im Epithel beteiligt [197]. Die Bindung von TLR-3 durch doppelsträngige RNA (dsRNA) und eine Infektion mit Rhinoviren sind ebenfalls starke Reize zur epithelialen Produktion von TSLP, die über eine Aktivierung von NF-κB und IRF-3 in Epithelzellen funktioniert [198, 199]. Zusätzlich kann IL-4 synergistisch die dsRNA- oder Rhinovirus-induzierte Produktion von TSLP in Epithelzellen verstärken [198]. Dies trifft im gleichen Maße auf Mediatoren wie IL-33 zu, welches als zentralen Regulator der immunologischen Aktivität bei allergischen Erkrankungen und Asthma beschrieben wurde [200]. Eine unregulierte IL-33-Aktivität führt zur Aktivierung von Th2-Zellen [200], Mastzellen [201], dendritischen Zellen [190], eosinophilen [202] und neutrophilen Granulozyten [203], was letztendlich zu einer erhöhten Expression von Mediatoren führt, welche prototypisch vor dem Typ-2 Kontext sind.

Die Dominanz der Typ 2-assoziierten Zellpopulationen bei natürlicher Allergenexposition ist seit Langem bekannt, wenn auch viele Publikationen auf peripheren Mechanismen aufbauen. Daher wollten wir in unserer Studie die Rolle des Zytokin TGF-β in der AIT untersuchen, welches sowohl pro- als auch anti-inflammatorisch wirken kann. Daher war es eine wichtige Fragestellung, ob die AIT über einen anti-inflammatorischen Einfluss von TGF-β vermittelt wird. Zudem waren Frequenzen von Th2- und Th9-Zellen sowie die Konzentrationen von relevanten Th2/Th9-Zytokinen lokal und systemisch bei Rhinitis- und Asthma-Patienten während der Allergen-Saison stark erhöht und zeigten sich im Gegensatz dazu bei AIT-behandelten Patienten lokal und systemisch stark verringert. Die lokalen Verschiebungen auf Zytokin-Ebene im Sputum konnten wir in der AIT erstmals zeigen. Überraschenderweise konnten wir zeigen, dass die AIT nicht über den anti-inflammatorischen Effekt von TGF-β vermittelt wurde, das es sich durch die Induktion über Th9-Zellen in ein pro-inflammatorisches Muster eingefügt hat. Bisher war lediglich bekannt, dass sich die IL-9-Expression in den oberen Atemwegen über eine AIT [204], sowie die Th2/Th9-Zellfrequenzen in peripherem Blut verändern [205]. TGF-β spielt eine Schlüsselrolle im Asthma, da es die Leukozyten-Chemotaxis zum Lungengewebe vermittelt, ein entscheidender Schritt bei der Genese und Aufrechterhaltung einer Entzündungsreaktion [206]. Daneben ist das pleiotrope Zytokin ein immunmodulatorischer Faktor, welcher eine entscheidende Rolle bei strukturellen Veränderungen der Atemwege bei Asthma spielt, jedoch auch anti-entzündliche Mechanismen induzieren kann [207]. Andererseits besitzt es chemoattraktive Eigenschaften und führt zu einer raschen Akkumulation von Makrophagen, Granulozyten und anderen Zellen am Ort der Entzündung [208]. Auch die Th17-Zelldifferenzierung kann über TGF-β induziert werden, wodurch Zellen

produziert werden, die in der Lage sind, große Mengen an IL-17 zu produzieren und einen akuten Entzündungsprozess aufrechtzuerhalten [209, 210]. Schließlich ist TGF-β entscheidend für die Entwicklung und Differenzierung regulatorischer T-Zellen [61], daneben auch ein starker Stimulus für die Proliferation von Fibroblasten und die Ablagerung von extrazellulärer Matrix [211]. Die extrazelluläre Matrix (ECM) als dynamische Überstruktur besteht aus selbstaggregierenden Molekülen, darunter Fibronektin, Kollagen und Proteoglykane [212]. TGF-β1 fördert die ECM-Ablagerung und stimuliert gleichzeitig die Zellen, ihre Produktion von Matrixkomponenten zu erhöhen, während die Freisetzung von Proteasen verringert wird, welche die ECM-Struktur abbauen können [213]. Die kontinuierliche Produktion von TGF-β als Ergebnis einer Gewebeverletzung, eines Defekts in der TGF-β-Regulation oder beidem führt zu einer unausgeglichenen ECM-Ablagerung, die beispielsweise einer Gewebefibrose bei chronischem Asthma zugrunde liegt [214]. Diese Umgestaltung der Atemwege ist das Ergebnis eines Reparaturprozesses in den Atemwegen nach einer anhaltenden Entzündung und impliziert eine komplexe Reihe von Ereignissen, einschließlich Epithelverletzung, Hyperplasie von Becherzellen, subepithelialer Fibrose, Hypertrophie und Hyperplasie glatter Muskelzellen sowie vaskulärem Remodeling, wobei jede dieser Komponenten zu einer Beeinträchtigung der Lungenfunktion beitragen kann [215]. Bei der Entwicklung eines asthmatischen Zustands kommt es zur Infiltration von Entzündungszellen und zur Sekretion von Zytokinen, einschließlich erhöhten TGF-β-Spiegeln, welche den Umbauprozess der Atemwege reguliert [216] und lokal direkt mit dem Schweregrad des Asthmas korrelieren können [217, 218]. Während der Fibrotisierung induziert TGF-β1 die Expression von epithelialen Zielgenen, einschließlich CTGF [219] und α-SMA [220, 221]. Diese induzieren die Chemoattraktion, Proliferation und Differenzierung von Fibroblasten in Myofibroblasten, die ECM-Proteine wie Fibronectin und Kollagen synthetisieren, die wiederum zur ECM-Kontraktion führen [219]. Fibroblasten spielen eine entscheidende Rolle bei der Regulation der Fibrose und bei der pulmonalen Immunantwort nach TGF-β1-Aktivierung [222]. Zudem wurde TGF-β1 als Hauptschalter bei der Induktion des epithelial-mesenchymalen Übergangs (EMT) identifiziert, der zur unterschiedlichen Umwandlung von Epithelzellen in Fibroblasten und Myofibroblasten führt. Daher bedingt die ECM die Zunahme von Fibroblasten und Myofibroblasten in der Schleimhaut sowie die Kollagenüberproduktion, welche bei Asthma und anderen Lungenerkrankungen wie der COPD beobachtet werden [223]. Die Expression von TGF-β nimmt in den Atemwegen von Asthmapatienten aufgrund sowohl struktureller als auch entzündlicher Zellinfiltrate zu [217, 218]. Interessanterweise wurde eine Korrelation zwischen erhöhter TGF-β1-Expression und

erhöhten Frequenzen von EG2-positiven Eosinophilen [214] und Makrophagen [224] festgestellt, was darauf hindeutet, dass TGF-β1 eine Rolle bei den fibrotischen Veränderungen in den Atemwegen von Asthmatikern spielt. Ebenso wurde eine Zunahme der TGF-β2-Expression im asthmatischen Epithel [225] gezeigt, dessen Kollagenschichtdicke mit einer Zunahme der Granulozytenzahl bei Patienten mit schwerem und leichtem Asthma korreliert [218, 226]. Im Epithel von Asthmatikern konnten in Vorarbeiten anderer Forschergruppen ebenfalls aberrante Expressionsmuster für microRNAs wie *miR-19a* zwischen verschiedenen Schweregraden sowie gesunden Kontrollen nachgewiesen werden, welche jedoch nicht reversibel auf die Gabe von Kortikosteroiden reagierten [227]. Eine verringerte Expression von *miR-19a* verstärkt die Phosphorylierung von *SMAD3* durch die TGFBR2-Signalübertragung und vermindert dadurch die Proliferation von Atemwegsepithelzellen [227]. Da eine Reduktion des SMAD3-Expressionsniveaus eine ausreichende Übertragung bestimmter TGF-β-Signale bewirkt, könnte hierüber eine Veränderung in der TGF-β-Signalkaskade über SMAD3 bewirkt werden [228]. Auch ein direkter Zusammenhang zwischen TGF-β und ILC2s konnte unlängst gezeigt werden, da TGF-β-aktivierte ILC2 Leukotrien C4 (LTC4) sezernieren und somit zu profibrotischen Prozessen beitragen können [229]. Im Vergleich zur typischen T-Zell- und B-Zell-vermittelten allergischen Reaktion ist die ILC2-vermittelte Reaktion schneller und unabhängig von der Antigenstimulation. ILC2s können zur Verstärkung lokaler Entzündungen und der Immunantworten über Freisetzung von Zytokinen beitragen, die direkt auf Schleimhautepithelien, Blutgefäße und Nerven wirken oder die Reaktionen von T-Zellen und DCs fördern. Die Aktivierung von ILC2s ist jedoch stark von Th2-assoziierten Zytokinen abhängig, einschließlich der epithelialen Alarmine IL-25, IL-33, TSLP [230], aber auch IL-9 [231], welche unter anderem für Aktivierung, Proliferation und Aufrechterhaltung von ILC2s erforderlich sind. Ebenso wie in unserer vorherigen Arbeit gezeigt, konnten wir hier sowohl die AIT-bedingte Reduktion Th2-assoziierter Zytokine wie IL-13 sowie reduzierter Frequenzen eosinophiler Granulozyten, aber auch ILC2-Zellen in Sputum zeigen.

Ebenfalls wurde bereits gezeigt, dass die epitheliale *miR-19a* als Induktor die Expression von IL-5 und IL-13 in ILC2s fördern kann [123]. Die Expression von *miR-19a* ist nicht nur in Atemwegsepithelzellen von Asthmapatienten, wie auch in unserer Studie beobachtet, sondern auch in CD4-positiven T-Zellen erhöht [121]. *MiR-19a* verstärkt die Th2-assoziierte Zytokinproduktion über eine direkte Beeinflussung der Inositolphosphatase *PTEN*, des Signaling-Inhibitor *SOCS1* und der Deubiquitinase *A20* (TNFAIP3) [121, 123]. Neben einer erhöhten *miR-19a*-Expression bei unbehandelten Asthmatikern konnten wir in unserer

Arbeit ebenfalls die *mir-221-3p* erhöht beobachten, ebenso wie eine erhöhte Frequenz an IL-13-produzierenden ILC2s und erhöhte Spiegel an sekretiertem Sputum-IL-13. Von anderen Forschungsgruppen wurden im induzierten Sputum von Patienten mit eosinophilem Asthma bereits eine erhöhte Expression von *miR-221-3p* gezeigt, welche die Expression entzündungshemmender Zytokine wie *CXCL17* inhibiert und die Expression von Typ 2-assoziierten Markern wie *CCL26* und *Periostin* über den p38-MAPK-Weg reguliert [232]. Für die Expression der *miR-221-3p* konnte eine Korrelation mit E2-Genen sowie ein Zusammenhang mit fraktioniertem exhaliertem NO (FeNO), systemischen Eosinophilen, sowie einer Verringerung der Lungenfunktion in Asthmapatienten gezeigt werden [232]. Eine Überexpression von *miR-221-3p* in den Atemwegen steigerte die eosinophile Entzündung bei Provokation mit Hausstaubmilben-Extrakt, wohingegen eine Inhibition von *miR-221-3p* die Expression von Eotaxin-2 (*CCL24*) und Eotaxin-3 (*CCL26*) sowie *Periostin* supprimierte [232]. Eine erhöhte Expression von *miR-221* und *miR-485-3p* konnte bei Kindern mit Asthma bei gleichzeitig erniedrigter Expression von *SPRED2* als Zielgen dieser miRNAs gezeigt werden [233]. SPRED-2 ist ein negativer Regulator des Ras/Raf/ERK/MAPK Signalwegs und konnte in Zusammenhang mit der leichten chronischen Entzündung bei Adipositas gebracht werden, welche mit einer Typ-2 Tendenz ebenfalls im Sputum nachweisbar ist [33, 234]. Für *miR-485-3p* konnten wir in unserer Studie ebenfalls erhöhte Expressionen in Sputumzellen der Asthmatiker in unserer Kohorte im Vergleich zu gesunden Kontrollen beobachten. Bei atopischer Dermatitis konnte eine systemische Regulation für *miR-151a* gezeigt werden, die zu einer aberranten Typ 2 Immunität führen könnte [235]. Wir konnten in unserer Studie beobachten, dass Expressionslevel von *miR-151a* bei AIT-behandelten Patienten mit allergischem Asthma signifikant reduziert waren. Im Kontext der AIT konnten wir auch zeigen, dass es Therapie-assoziierte Regulationen auch auf Ebene der miRNAs gibt, die auf strukturelle Gene wie auch immunregulatorische Faktoren abzielen. Die von uns in der Studie zur Veränderung von Expressionsprofilen von miRNAs im induzierten Sputum unter AIT-Behandlung erstmals beschriebene, AIT-assoziierte Herabregulierung der *miR-3935*-Expression zielt auf den Prostaglandin-EP3-Rezeptor (PTGER3) ab, einem Rezeptor für Prostaglandin $E_2$ ($PGE_2$). Über $PGE_2$ können Atemwegsepithelzellen spezifisch zelluläre Subtypen wie Dendritische Zellen modulieren [236]. $PGE_2$ wurde bisher eine bronchodilatatorische Wirkung zugeschrieben, die über eine hemmende Wirkung auf die glatte Muskulatur vermittelt wird [237-239]. Es beeinflusst jedoch auch das Verhalten mehrerer Immunzellen wie Makrophagen [240], T-Zellen [241] und B-Zellen [242], welche eine Typ 2-Entzündung unterdrücken können. In unserer Studie haben wir die Spiegel von sezerniertem $PGE_2$ sowie

der Expression seines Rezeptors PTGER3 im induzierten Sputum untersucht. Kontextabhängig kann $PGE_2$ je nach Gewebe und Zustand entweder pro- oder anti-inflammatorische Effekte steuern [243-247]. Interessanterweise konnte kürzlich gezeigt werden, dass IL-13 eine Veränderung im Signaling-Potential von Tuft-Zellen fördern kann, so dass auch den Tuft-Zellen eine zentrale Rolle bei der epithelialen Entzündung zukommen könnte [248]. Die Spiegel von IL-13 waren in den Sputumüberständen unserer unbehandelten Patienten gegenüber den behandelten Patienten signifikant erhöht. Zudem war das Sputum $PGE_2$ in unserer Studie mit einem pro-inflammatorischen Profil bei Patienten mit allergischem Asthma assoziiert, da es mit pro-inflammatorischen, klinischen Parametern wie der Zell-Last im Sputum, einem Marker für eine lokale Entzündungssituation, und erhöhten Eosinophilen-Frequenzen korrelierte. Dem gegenüber waren die $PGE_2$-Spiegel im Sputum bei Patienten erniedrigt, die sich einer AIT unterzogen hatten. Da die $PGE_2$-vermittelten Effekte der Bronchokonstriktion über $EP_3$ mittels Hochregulierung von *miR-3935* und Herunterregulierung von $EP_3$ und $PGE_2$ bei AIT-behandelten Patienten unterdrückt wurden, könnten die pro-inflammatorischen $PGE_2$-Spiegel synergistisch zu $EP_3$ reguliert werden. Einen Hinweis auf die pro-inflammatorischen Eigenschaften von $PGE_2$ im Kontext allergischer Atemwegserkrankungen liefert auch die erhöhte Frequenz von ILC2s im induzierten Sputum und deren Korrelation mit klinischen Symptomen sowie der lokalen Zell-Last und der Infiltration eosinophiler Granulozyten ins Atemwegslumen. Daher ist anzunehmen, dass dieser Mechanismus über *miR-3935* die lokalen, pro-inflammatorischen Effekte von $PGE_2$ unterdrückt. Diese Vermutung wird ebenfalls von aktueller Literatur gestützt, die eine Reduzierung der $PGE_2$-Spiegel von PBMCs nach Insektengift-AIT sowie einen negativen Zusammenhang mit der Expression von IL-10 zeigt [249]. Da die $PGE_2$/$EP_3$-Achse auch die Mastzellen-Degranulierung und die Sekretion pro-inflammatorischer Mediatoren beeinflussen kann, könnte die verminderte, *miR-3935*-abhängige Expression von $EP_3$ zu einer AIT-induzierten Mastzell-Desensibilisierung beitragen.

### 5.3 Anti-inflammatorische Regulation in Atemwegsepithelzellen

Mit den epithelialen Entzündungsmechanismen in Th2-getriebenen Erkrankungen wie allergischer Rhinitis und allergischen Asthmas geht als kompensatorischer Mechanismus oftmals auch eine Sekretion von anti-inflammatorischen Proteinen einher. Diese können regulatorisch in das Entzündungsgeschehen eingreifen, da sie im gesunden Organismus eine homöostatische Aufgabe erfüllen, im Kontext der allergischen Entzündung jedoch teilweise unterdrückt werden. Ein solches Beispiel sind neben dem bereits seit vielen

Jahren untersuchten tolerogenen Zytokin IL-10 auch neuere Mediatoren wie den vorwiegend epithelial-sekretierten Proteinen IL-37 [250] und Secretoglobin1A1 (SCGB1A1) [251]. Wie wir in einer Literaturübersicht zusammengefasst haben, wird die Familie der Sekretoglobine, alternativ als Uteroglobin bezeichnet, von sekretorischen Zellen der mukosalen Barrieren exprimiert und kann dabei in immunregulatorische und entzündungshemmende Prozesse bei Atemwegserkrankungen vermitteln [252]. Unsere Arbeit zur Induktion von SCGB1A1 in Abhängigkeit einer AIT sowie die Arbeiten anderer Arbeitsgruppen haben gezeigt, dass in Typ 2-assoziierten Krankheiten wie allergischem Asthma die Expression von *SCGB1A1* supprimiert wird [253], während eine therapeutische Interaktion mittels AIT die Expression von *SCGB1A1* in den oberen und unteren Atemwegen induzieren kann [195]. Im Allgemeinen wird angenommen, dass die AIT über ihren Verlauf Th2-Immunantworten unterdrückt und SCGB1A1 diesen Effekt mit beeinflusst, da SCGB1A1 Potential zur Unterdrückung des Zytokins Osteopontin besitzt, welches seinerseits die Th2-Entzündung fördern kann [254]. Aufgrund der antagonistischen Beteiligung von SCGB1A1 an einer Typ 2-Immunregulation müssen die involvierten Signalwege identifiziert werden, worüber der SCGB1A1-Promotor aktiviert werden kann. So können die Transkriptionsfaktoren FOXA1, auch bekannt als Hepatozyten-Kernfaktor-3 (HNF-3), FOXA2 sowie CAAT/enhancer-binding proteins (C/EBP) die *SCGB1A1*-Expression induzieren [255, 256]. Zudem wurden für SCGB1A1 anti-inflammatorische Eigenschaften auf die PLA2-Aktivität [257], die $PGD_2$-Spiegel und die Eosinophilen-Infiltration [258] sowie die Chemotaxis und die Zytokinproduktion im murinen Modell [259] beschrieben. Verringerte SCGB1A1-Spiegel wie beispielsweise bei Asthma wurden daher einer verringerten *FoxA2*-Expression zugeschrieben [260]. Th2-Zytokine wie IL-13 können *FoxA2* in Atemwegsepithelzellen hemmen und dadurch die *SCGB1A1*-Expression unterdrücken [261, 262]. SCGB1A1 wiederum inhibiert über die Bindung and FPR2 die Th2-Immunantwort durch Suppression der Expression von *SOCS3* und der STAT1-Aktivierung [263], welche eine wichtige Rolle bei der Differenzierung von Th2-Zellen sowie Initiierung und Aufrechterhaltung der Th2-vermittelten Immunantwort spielen [264]. In Secretoglobin-IL-4-transgenen Mäusen induzierte IL-4 eine erhöhten Zellinfiltration, Epithelhypertrophie, Schleimzellhyperplasie, Mucus-Sekretion und erhöhte Spiegel an Surfactant-Protein A und B [265]. Während dieses Modell alle Kennzeichen einer chronischen Allergenexposition zeigte, lieferte es jedoch keinen Hinweis darauf, ob IL-4 selbst die Differenzierung von Basalzellen induzieren kann oder ob Sekundäreffekte eine epitheliale Differenzierung auslösen. Diverse Differenzierungseffekte konnten in Abhängigkeit von Th2-Zytokinen bereits in humanen primären Epithelzellen der oberen

Atemwege beobachtet werden, wo IL-13 die Differenzierung von ziliierten hin zu sekretorischen Zellen modulierte [266]. Während des durch Luftexposition induzierten epithelialen Differenzierungsprozesses [267] und durch die Behandlung mit IL-13 und IL-4 wird eine Metaplasie induziert, welche in einer Suppression des Prostaglandin-Signalweges mündete [268]. Darüber hinaus wurde gezeigt, dass IL-4 und IL-13 durch Hemmung der *TLR3*-Expression und -Signalgebung über IRF3 die Immunantwort auf eine virale Infektion beeinträchtigen [269]. In unserer Studie zur Induktion von SCGB1A1 unter AIT konnten wir diese Abhängigkeit in nasalen Sekreten einer Immuntherapie-Kohorte über einen Behandlungszeitraum von drei Jahren verfolgen, insbesondere monitoriert über eine gegenläufige Sekretion von SCGB1A1 und des Typ2-assoziierten Zytokins IL-24. SCGB1A1 bindet auch $Ca^{2+}$, einen Cofaktor der Phospholipase A2 (PLA2), worüber die PLA2-Aktivität gehemmt werden kann [270], wodurch Typ 2-assoziierte Lipidmediatoren wie Arachidonsäurederivaten freigesetzt werden können [271]. PLA2 seinerseits kann die Membran von Atemwegsepithelzellen schädigen und korreliert direkt mit einem Lungenversagen [272]. Darüber hinaus unterdrückt SCGB1A1 die *COX2*-Genexpression, welches ein Schlüsselenzym bei der Synthese von pro-inflammatorischen Lipidmediatoren ist [258]. Auf diese Weise können anti-inflammatorische Mediatoren wie SCGB1A1 regulierend in die Immunantworten eingreifen.

## 5.4 Einfluss der Allergen-spezifischen Immuntherapie

Die Induktion solcher homöostatischer Immunmechanismen ist eine Folge der Toleranzinduktion über therapeutische Interventionen wie beispielsweise der allergen-spezifischen Immuntherapie (AIT). Diese haben wir erstmals über den gesamten Verlauf in einer longitudinalen, observationalen Studie zu IL-10-produzierenden B-Zellen, Th17-Zellen sowie IL-17 produzierenden regulatorischen T-Zellen (Tr17) bei Gräserpollen-Allergikern untersucht [273]. Der Fokus lag hier auf den Effektorzellen und ihrer Produkte, speziell der Induktion von IL-10-produzierenden B- und T-Zellen über den Therapieverlauf hinweg. Wir konnten in unserer Arbeit zeigen, dass mit der prä-saisonalen Aufdosierungsphase zu Beginn der AIT ein vorübergehender Anstieg der IL-4-positiven Th2-Zellen sowie des Graspollen-spezifischen IgE zu verzeichnen ist. Diese fallen jedoch schon in der ersten Graspollen-Saison nach Beginn der Therapie wieder auf das Ausgangsniveau in Verbindung mit einem Anstieg der IL-10-produzierenden B-Zellpopulation zurück. Bisher war es jedoch nicht möglich, die Früh- und Spätphase einer AIT anhand geeigneter Marker zu monitorieren. Eine solche Beobachtung ermöglichte uns in einem post-hoc Ansatz, den

Therapieerfolg zu einem möglichst frühen Zeitpunkt, am Ende der Aufdosierungsphase, vorherzusagen. Unsere Studie zeigt in Übereinstimmung mit anderen Publikationen, dass ein frühes Ansprechen auf die AIT mit einer systemischen wie auch lokalen IL-10-Induktion mit einer erniedrigten IgE-Produktion in CD27-positiven *memory* B-Zellen einhergeht [274]. Die Colokalisation von IL-10 in T-Zellen ist konsistent mit der Induktion regulatorischer T-Zellen (Tregs) in der AIT [275, 276]. Die Synergie der IL-10-positiven Tregs mit Th2-Effektorzellen, insbesondere während der Aufdosierungsphase, deutet auf einen möglichen Mechanismus der AIT hin, der IL-10 neben der $IgG_4$-Hochregulation involviert [275]. Für Allergen-bindende $IgG_4$-Spiegel konnten wir über den Verlauf der AIT ein Plateau oder sogar eine leichte Verminderung beobachten, welche eine ausgeprägte inhibitorische Aktivität gegenüber IgE besitzen und zur Langzeit-Etablierung der klinischen Toleranz beitragen könnten [277, 278]. Weiter fördert IL-10 aus regulatorischen B-Zellen die Bildung von $IgG_4$ in B-Zellen allergischer Patienten durch Kompetition mit IgE-Bindungsstellen auf Allergenen und verhindert gleichzeitig die Fc-Rezeptoraktivierung [279, 280]. B-Zellen können zudem die Unterdrückung der Th1- und Th17-Differenzierung durch IL-10-Sekretion vermitteln [281], welche der inversen Dynamik der zirkulierenden Th17- und Breg-Zellen zugrunde liegen kann. Die von uns beschriebene Phase der Konversion kann durch den Th17-Phänotyp abgegrenzt werden, da die Anteile der Th17-Zellpopulation nach einer initialen Reduzierung während der Aufdosierungs-Phase wieder ansteigen. Darüber hinaus könnte man spekulieren, dass die Induktion einer intermediären IL-$17^+$FoxP$3^+$CCR$6^+$ Tr17-Untergruppe als Übergangsstatus fungieren, wie in einem murinen Arthritis-Modell gezeigt wurde [282]. Diese können über regulatorische T-Zellen die Differenzierung hin zu einem pro-inflammatorischen Phänotyp fördern, welche IL-17 induzieren können [282]. Andererseits können sie aber auch eine entzündungshemmende Transdifferenzierung von Th17-Zellen in regulatorische T-Zellen induzieren [283]. Wir konnten die Bedeutung dieser transdifferenzierten Th17-Zellen ebenfalls in unserer Studie nachweisen [273]. Wir konnten zeigen, dass eine Untergruppe von T-Zellen, die sowohl das proinflammatorische Zytokin IL-17 und den regulatorischen Transkriptionsfaktor FoxP3 exprimiert, während einer AIT induziert wird [273]. Diese Zellen werden als eine Übergangsuntergruppe zwischen Th17-Zellen und Tregs postuliert, da beide Populationen sowohl in die eine als auch in die andere Richtung transdifferenzieren können Richtung transdifferenzieren können [282, 283]. Nach Stimulation können naive T-Zellen *Foxp3* exprimieren und in Gegenwart von IL-2 sowie TGF-β zu Tregs differenzieren [284]. Die Genese dieser „induzierten“ regulatorischen T-Zellen (iTregs) kann durch zusätzliche Stimuli weiter moduliert werden, insbesondere durch Retinolsäure [285] und AhR [286]. Obwohl TGF-β an der Entwicklung von Tregs und Th17-

Zellen beteiligt ist, ist die endgültige Rolle und Funktion von TGF-β bei der Differenzierung von IL-17$^{+}$FoxP3$^{+}$ T-Zellen nur unzureichend geklärt [284, 287, 288]. So könnte die Entstehung IL-17-produzierender Tregs von TGF-β abhängig sein [289] und die IL-17-Produktion mit einem TGF-Inhibitor reduziert, aber nicht vollständig eliminiert werden [290, 291]. Im Speziellen scheint jedoch die Kombination aus TGF-β und IL-2 die humanen IL-17$^{+}$FoxP3$^{+}$ T-Zellen in Gegenwart von Antigen-präsentierenden Zellen zu stimulieren [292]. In Mausmodellen konnten Tregs bei Zugabe von IL-6 eine ausreichende Menge an TGF-β produzieren, um ohne exogenes TGF-β zu IL-17-produzierenden Zellen zu konvertieren und darüber hinaus die IL-17-Produktion effektiver zu induzieren als exogenes TGF-β [290]. Demgegenüber kann jedoch die IL-17-Sekretion humaner IL-17$^{+}$FoxP3$^{+}$CCR6$^{+}$HLA-DR$^{neg}$ Tregs durch TGF-β blockiert werden [293]. Auch weitere Studien beobachteten keine Wirkung auf die IL-17-Produktion durch TGF-β [294]. Ein Grund für die Pleiotropie von TGF-β könnte die inhibitorische Wirkung hoher Konzentration exogenen TGF-βs auf die IL-17-Produktion sein, wodurch wiederum die *FoxP3*-Expression begünstigt werden könnte [295]. Ein weiterer möglicher Grund ist, dass die Umwandlung von Tregs in IL-17-produzierende Zellen durch andere dominante Faktoren neben TGF-β beeinflusst wird [296]. Während unsere Daten die Vermutung zulassen, dass zirkulierende IL-10-positive Tregs hauptsächlich in der anfänglichen Aufdosierungsphase induziert werden, konnten wir in Übereinstimmung mit vorherigen Publikationen lokal in den oberen Atemwegen sowohl IL10-positive als auch FoxP3-positive Tregs über den gesamten Verlauf der AIT messen [280, 297, 298]. In der Klimax der Aufdosierung zeigten sich klare Trends in unserer Studie für erhöhte Frequenzen von lokalen IL-10-positiven Tregs und lokalen IL-10-positiven B-Zellen in der nasalen Mukosa im Vergleich zu unbehandelten Rhinitis-Patienten und gesunden Kontrollprobanden, welche mit den entsprechenden Daten aus dem peripheren Blut übereinstimmen. Auch eine systemische Verschiebung zugunsten der Treg-Zellen verdeutlicht die suppressive Wirkung auf verschiedene Zytokine, nicht nur auf die Th2-Schlüsselzytokine IL-4, IL-5 und IL-13, sondern auch auf pro-inflammatorische Zytokine wie IL-1$\alpha$, IL-1$\beta$, IL-6 und IL-8. Diesen Effekt konnten wir nicht nur in Bezug auf lymphozytäre, sondern auch hinsichtlich epithelialer Zytokine in den oberen Atemwegen beobachten. So zeigen sich unter der AIT auch Veränderung am respiratorischen Epithel der behandelten Patienten in Bezug auf zentrale Mechanismen besonders in der Aufdosierungsphase anhand der deutlichen Reduktion von IL-24 unter gleichzeitigem Anstieg von SCGB1A1. Aber auch bereits beschriebene Mediatoren wie CSF-2 (GM-CSF) waren über den Verlauf der AIT reduziert, besonders aber im Vergleich von unbehandelten Patienten mit 3-jähriger Therapiedauer. CSF-2 wurde in Modellen mit Allergen-induzierter Atemwegsentzündung

stark exprimiert beschrieben, was zum Fortschreiten der asthmatischen Entzündung beitragen kann [299]. Wir konnten aber auch Veränderungen für die Transkriptionsfaktoren der aberranten E2-polarisierten, epithelialen Immunität über den Verlauf der AIT zeigen. Besonders zeigten sich die Expressionslevel von *MEIS1*, *RUNX2* und *AHRR* nach 3 Jahren AIT im Vergleich zu unbehandelten Patienten stark verändert. Wie bereits im Kontext des E2-Epithels erwähnt, erzeugt die Inaktivierung von MEIS1 eine Zunahme der Masse der glatten Atemwegsmuskulatur und eine Abnahme des Knorpelgewebes, was die Rolle von MEIS1 bei allergischen Atemwegserkrankungen unterstreicht [175]. Der *Aryl Hydrocarbon Receptor Repressor* (AhRR) als Downstream-Gen von des IL-4-induizierten Arylhydrocarbon-Rezeptors (AhR) verbindet die Wiederherstellung der epithelialen Homöostase, welche ihrerseits Mechanismen der Epithelreparatur über IL-22 fördern [300]. Darüber hinaus kann GATA3 über AhRR gehemmt werden, was auch eine Edukthemmung durch IFN-$\gamma$-induzierte Indolamin-2,3-dioxygenase-Metabolite wie Kynurenin erlaubt [182-184]. Das durch IFN-$\gamma$-induzierbare Enzym IDO stellt einen wichtigen molekularen Schalter dar, welcher das weitere Schicksal von Tregs beeinflusst [301]. So konnten nach TLR9-Ligation und CpG-Behandlung in plasmacytoiden DCs, welche als Reaktion auf Virusinfektionen große Mengen von IFN-$\alpha$ sekretieren, die IDO-Expression stimuliert werden [301]. Aber auch Th17-abhängige Signalwege wie das epitheliale IL-17C zeigten eine stringente Veränderung über den Verlauf der AIT bei den behandelten Patienten unserer Kohorte. IL-17C bindet an den IL-17RE/IL-17RA-Rezeptorkomplex, der sich hauptsächlich auf Epithelzellen befindet [302], um nachgeschaltete Signalwege auf autokrine Weise zu aktivieren. Es konnte ebenfalls eine wichtige Rolle für IL-17C bei der Rekrutierung von Immunzellen gezeigt werden, dazu induzierte es auch die Chemoattraktion für Neutrophile Lin der Haut [303]. Zudem verstärkt es die Expression innater Zytokine und Chemokine sowie antimikrobieller Peptide [303]. Eine deutliche Regulation zeigte sich jedoch auch für IL-24, welches vermutlich über die IL4R/STAT6/GATA3-Achse vermittelt wird. Sowohl die genannten Transkriptionsfaktoren, aber auch IL-17C, IL-24 und SCGB1A1 zeigen, dass die AIT über den Verlauf der Therapie Veränderungen der prototypischen Muster bis hin Auflösung der fehlgeleiteten Immunität und ihre zellulären Ursprünge vermitteln kann. Daher wird dem Austausch zwischen Epithel- und Immunzellen bei der Pathogenese allergischer Erkrankungen eine immer größere Bedeutung beigemessen. Erst die eng vernetzten Wechselwirkungen zwischen den residenten Immunzellen der Atemwege und den Epithelzellen sorgen für die Aufrechterhaltung eines homöostatischen Zustands der Immuntoleranz gegenüber

harmlosen Reizen wie Allergenen, gefolgt von schützenden Reaktionen auf eingeatmete Krankheitserreger mit effektiver Gewebereparatur.

## 5.5 Fazit

In der Originalarbeit (siehe 4.1) zur Orchestrierung einer epithelialen Polarisierung über IL-4 und IFN-γ beschreiben wir transkriptionellen Unterschiede dieser antagonistischen Genregulation. Da die Schlüsselzytokine der Typ 1- und Typ 2-Antwort, IFN-γ und IL-4, auch beim Epithel zugrunde liegen, formulierten wir daraus die Hypothese eines Allergie-assoziierten, Th2-getriebenen „E2-Epithels“ und seiner assoziierten sekretierten Biomarkern. Das innovative Potential bei dieser Arbeit liegt auch auf dem zugrunde liegenden Netzwerk beteiligter Transkriptionsfaktoren in Abhängigkeit von der jeweiligen epithelialen Prägung. Dieses Netzwerk ermöglicht die Entschlüsselung der regulatorischen Mechanismen im Immunnetzwerk der entzündlichen Erkrankungen in den Atemwegen. Darüber können die Hintergründe einer Beteiligung der oberen und unteren Atemwege unter chronisch-inflammatorischen Bedingungen und ihrer assoziierten Biomarker untersucht werden.

Die Vergleichbarkeit der oberen und unteren Atemwege bei der lokalen, allergischen Atemwegsentzündung und der assoziierten Biomarker im Atemwegslumen haben wir in der Arbeit 4.2 untersucht und mittels Korrelationsmatrices erfasst. Das Konzept der „*United Airways*“ und repräsentativer nasaler Biomarker zeigt sich insbesondere für Typ 2-Zytokine, im Speziellen für IL-24. Diese Ergebnisse lieferten vielversprechende Biomarker-Kandidaten, welche zugrunde-liegende Asthma-Endotypen widerspiegeln könnten und derzeit in weiteren Studien validiert werden.

In Arbeit 4.3 lag der Fokus auf dem Zusammenhang auf dem Zytokin TGF-β, welches sowohl das Epithel als auch Immunzellen beeinflussen und von beiden produziert werden kann. Da TGF-β sowohl pro- als auch anti-inflammatorisch wirken kann, war es eine wichtige Fragestellung, ob die AIT über einen anti-inflammatorischen Einfluss von TGF-β vermittelt wird. Überraschenderweise war dies nicht der Fall, da sich TGF-β durch die Induktion über Th9-Zellen in ein pro-inflammatorisches Muster eingefügt hat.

Den Einfluss von microRNAs auf die fehlgeleitete Immunregulation in allergischen Atemwegserkrankungen konnten wir in Arbeit 4.4 aufzeigen. Wir konnten spezifisch in Sputen von Asthma-Patienten erstmals demonstrieren, dass die bei Asthma erhöhte *miR-3935* durch die AIT herabreguliert wurde. Die *miR-3935* ist spezifisch für den Prostaglandin $EP_3$ Rezeptor und zeigt einen direkten Zusammenhang von $PGE_2$ mit ILC2

und deren assoziierter Mediatoren wie IL-13 in Sputumproben von Rhinitis- und Asthmapatienten auf. In Arbeit 4.5 konnten wir epitheliale Mechanismen in Th2-vermittelten Erkrankungen näher beleuchten. Wir konnten nachweisen, dass die AIT wichtige Mediatoren mit anti-inflammatorischen Eigenschaften wie Secretoglobin1A1 induziert, als Gegenregulation zu pro-inflammatorischen Markern wie IL-24. Diesen Aspekt der Gegenregulation haben wir in Arbeit 4.6 in Bezug auf den longitudinalen Effekt der AIT herausgearbeitet. Das Verhältnis von regulatorischen B- und Th17-Zellen nach Erstbehandlung erlaubt eine frühe Vorhersage der AIT-Wirksamkeit nach der Aufdosierungsphase auf der Ebene der peripheren Zellen.

In den hier zusammengefassten sechs Originalarbeiten konnten wir zeigen, dass bei allergischen Atemwegserkrankungen eine spezifische epitheliale Polarisation existiert und die assoziierten immunologischen Antworten der oberen und unteren Atemwege auch in Bezug die Entzündungssignatur vergleichbar sind. Diese Immun-Antworten können mittels moderner Immuntherapien modifiziert werden und beeinflussen sowohl die Zellmorphologie wie auch die Genaktivität. Die Ergebnisse verdeutlichen besonders, dass allergische Erkrankungen nicht nur die Immunzellen betreffen, sondern auch die Epithelzellen der oberen und unteren Atemwege, welche eine wichtige Rolle bei allergischen Entzündungen spielen und wertvolle Informationen für die Diagnose und Behandlung liefern können. Diese Erkenntnisse haben bereits Einfluss auf weitere klinische und experimentelle Forschung.

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# 7. Beiträge zu den Publikationen

Der Autor erklärt, dass er zu allen in Abschnitt 4 aufgeführten Publikationen wesentliche Beiträge geleistet hat. Spezifische Beiträge zu Manuskripten waren für die Veröffentlichung Nummer 1 (Zissler UM et al., PMID 26577568) die Durchführung der experimentellen Arbeiten, der Sammlung der ex-vivo-Proben, die anschließenden Datenanalysen sowie die Erstellung der Abbildungen und des Manuskripts.

Bei Veröffentlichung Nummer 2 (Zissler UM et al., PMID 30121290) war der Autor maßgeblich an der Sammlung des humanen Proben beteiligt, führte die experimentellen Arbeiten durch, analysierte die Daten in Zusammenhang mit der Quantifizierung von Proteinen der oberen und unteren Atemwege, der Durchflusszytometrie und erstellte das Manuskript.

Bei Veröffentlichung Nummer 3 (Musiol S et al., PMID 35069535) war der Autor maßgeblich an der Probensammlung der humanen Proben beteiligt, führte die experimentellen Arbeiten und die anschließenden Analysen der humanen Proben durch und erstellte das Manuskript.

Bei Veröffentlichung Nummer 4 (Jakwerth CA et al., PMID 34514658) war der Autor an der Probensammlung sowie an der Durchführung der experimentellen Arbeiten maßgeblich beteiligt und führte die anschließenden Datenanalysen durch, ebenfalls war er wesentlich an der Erstellung des Manuskripts beteiligt.

Bei Veröffentlichung Nummer 5 (Zissler UM et al., PMID 33528894) führte der Autor die aufwändigen experimentellen Arbeiten und die anschließende Datenanalysen sowie die Erstellung des Manuskripts durch.

Bei Veröffentlichung Nummer 6 (Zissler UM et al., PMID 30318182) führte der Autor einen Großteil der Probensammlung aus, war maßgeblich an den aufwändigen experimentellen Arbeiten beteiligt, führte anschließende Datenanalysen durch und war wesentlich an der Erstellung des Manuskripts beteiligt.

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