Fakultät für Medizin

Allergies in a Changing World – The Impact of Environmental Influences on Pollen Allergenicity and Host-Microbe Interactions

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Dedicated to my family.

Table of Contents

List of tables

List of Figures

Abbreviations

(d)LN	(draining) lymph node
9-OTrE	9-Oxo-OTrE
AD	Atopic dermatitis
ADO	Adenosine
ADO	Adenosine
AJ	Adhesion junction
ALI	Air liquid interface
Amb a 1	major *Ambrosia artemisiifolia* allergen 1
AMP	Anti-microbial peptide
APC	Antigen presenting cell
APE	Aqueous pollen extract
AR	Allergic rhinitis
BC	B cell
BCR	B cell receptor
Bet	*Betula pendula*
Bet v 1	major birch allergen 1
Cat	Catalposide
CCL	CC chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CLR	C-type lectin receptor
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CXCR	C-X-C chemokine receptor
DAMP	Damage-associated molecular pattern
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing nonintegrin
DEP	Diesel exhaust particles
Der p	*Dermatophagoides pteronyssinus* allergen
EC	Epithelial cell
FACS	Fluorescence activated cell sorting

HDM	House dust mite
HNEC	Human nasal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
ITS	Internal transcribed spacer
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LTB_4	Leukotriene B_4
Lumi	Lumichrome
MACS	Magnetic activated cell sorting
Mal	Malvidin
MHC	Major histocompatibility
MI	Migration index
moDC	Monocyte derived dendritic cell
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor κ B
NGS	Next generation sequencing
NKT	Natural killer T cells
NLR	NOD-like receptor
NO_2	Nitrogen dioxide
O_3	Ozone
OTU	Operational taxonomic unit
PALM	Pollen-associated lipid mediator
PAMP	Pathogen-associated molecular pattern
PAR	Protease-activated receptor
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pC4OG	p-Coumaryl alcohol 4-*O* glucoside
PDA	Potato Dextrose Agar
Pel	Pelargonidin

PGE_2	Prostaglanding E_2
Phl	*Phleum pretense*
Phl p 1	major *Phleum pratense* allergen 1
Phl p 5	major *Phleum pretense* allergen 5
PM	Particulate Matter
PMN	Polymorphonuclear leukocytes
PPE_1	E_1 Phytoprostane
PR	Pathogen related
PRR	Patter recognition receptor
Q3OS	Quercetin-3-*O*-sophoroside
ROS	Reactive oxygen species
RT	Room temperature
RWE	Aqueous ragweed pollen extract
SNM	Synthetic nasal medium
TER	Trans-epithelial electrical resistance
Tfh	Follicular T helper cells
Th	T helper cell response
TJ	Tight junction
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
ZO	Zona occludens

Abstract

Background: The past decades have seen a rise in the prevalence of allergic diseases. A third of the worlds' population is already suffering from allergies, and the numbers are expected to grow, further increasing their socio-economic burden. Airborne pollen from wind pollinating plants are one of the main causative agents of allergies. The process of sensitization to allergens is highly complex and depends on an intricate interplay between genetics and environmental factors of both, the susceptible individual but also on the allergen source. The allergenic potential of pollen depends not only on the allergen itself, it is also influenced by endogenous adjuvant substances. In humans, these adjuvants skew immune responses and presumably promote allergic sensitization to pollen proteins. The composition of allergenic and adjuvant factors of pollen is likely influenced by climate change and air pollution.

A healthy microbiome provides protection against allergic diseases, but is constantly subjected to environmental influences. Exposure to microbes and microbial products is also suspected to influence the allergic sensitization process.

Objective: The aim of this work was to elucidate the influence of environmental stressors, such as climate change related CO_2 levels and air pollution on the allergenic potential of ragweed and birch pollen. In addition, the role of exposure to pollen and microbes under natural conditions and their interaction with the human immune system and influence on allergic inflammation was analyzed.

Methods: To investigate the influence of elevated CO_2 or ozone levels on the allergenicity of ragweed pollen, ragweed plants were grown in climate chambers and exposed to elevated CO_2 (700 ppm, CO_2-RWE, according to an intergovernmental panel on climate change (IPCC) scenario RCP4.5) or ozone (80 ppb, O_3-RWE, WHO threshold) levels during the whole vegetation period. The effect of urbanization and air pollution on birch pollen allergenicity was analyzed in birch pollen from rural (rural-BPE) and urban (urban-BPE) sampling sites in Augsburg and Munich, Germany. Allergenicity parameters (allergen content, LPS, $PALM_{PGE2}$, $PALM_{LTB4}$ and adenosine) were analyzed in the different pollen samples. Furthermore, for pollen from plants grown under elevated CO_2 levels, the metabolome was dissected to identify one or more substances contributing to increasing allergenicity. Mouse models for allergic sensitization (performed by F. Alessandrini, Helmholtz Zentrum München) to evaluate the

allergenic potential of the pollen samples were supplemented by this thesis with *in vitro* stimulations of human nasal epithelial cells (HNEC), dendritic cells (DC), murine and human B cells, and neutrophils from non-atopic cell donors, with aqueous pollen extracts to translate the findings from mouse to human. ELISA was performed to measure levels of secreted cytokines and chemokines as well as IgE. Maturation and differentiation markers of DCs stimulated with pollen extracts were measured by flow cytometry, as was the direct chemotaxis of neutrophils to the different pollen samples.

To study the effect of natural pollen exposure on the nasal microbiome of eight non-atopic subjects and eight allergic rhinitis patients with sensitization to pollen, a study was set up in 2015/2016, following the study participants over the course of a whole year. Nasal swabs were taken on a monthly basis and bi-weekly during the birch pollen season to analyze changes to the microbiome. 16S rRNA sequencing of the variable regions V1-V3 was performed and the data was analyzed in the context of atopy status and pollen exposure. Because pollen harbor a specific microbiome, the microbial composition of birch pollen from five pooled sampling sites was investigated using 16S sequencing.

Within the main grass (Poaceae) pollen and fungal spore season, in June and July 2018, eight volunteers stayed in a flowering meadow five times, one hour each, to be naturally exposed to airborne grass pollen and fungal spores. To quantify the Phl p 5 content from ambient air one group wore nasal filters for 1 hour before and during the outdoor exposure. Another group received nasal swabs directly before and after the exposure time for ITS sequencing. Simultaneously, airborne pollen and fungal spores were monitored by portable Hirst-type volumetric traps, both indoors and outdoors. Similar traps using PDA agar plates were set up to culture airborne fungi. Human nasal epithelial cells (HNEC) from non-atopic donors were stimulated with timothy grass pollen allergens (Phl p 1, Phl p 5) either alone or in combination with spores of fungal isolates. Immunological and physical barrier functions were assessed by cytokine ELISA and trans-epithelial electrical resistance measurements, respectively.

Results: Ragweed pollen from plants grown under elevated CO_2 levels caused stronger allergic lung inflammation in a mouse model, characterized by increased inflammatory cell infiltrate, mucus hypersecretion and elevated IgE levels *in vivo* and *ex vivo*. This was reflected by increasing inflammatory and reduced regulatory responses of DCs stimulated with CO_2-RWE or CO_2-RWE conditioned HNEC supernatants *in vitro*. In contrast, inflammatory responses of

HNECs to ragweed pollen stimulation did not differ with respect to plant treatment. Although slightly higher in CO_2-RWE, the measured allergenicity parameters were not significantly different between the treatments. The metabolome analysis revealed a plethora of differentially regulated secondary pollen metabolites for CO_2-RWE. In combination, but not when used by themselves, these metabolites elicited similar inflammatory responses in DCs as whole RWE.

Pollen from ragweed plants grown under elevated ozone levels were overall less immune-stimulatory and less immune-modulatory than pollen from control conditions. This was characterized by lower cytokine secretion in immature DCs and weaker cytokine inhibition in LPS-primed DCs. Neutrophil chemotaxis was induced more strongly by O_3-RWE, which was likely due to elevated $PALM_{LTB4}$ levels in the aqueous pollen extracts.

Birch pollen extracts (BPE) from urban sampling sites in Augsburg elicited strong allergic responses *in vivo* compared to a rural-BPE, which induced more regulatory responses. Similar effects were observed *in vitro*. Rural-BPE elevated the pro-inflammatory cytokine responses of HNECs more than urban-BPE. In DCs, the regulatory response was higher after stimulation with rural-BPE, whereas urban-BPE were more immune-modulatory. These effects could be ascribed to higher $PALM_{PGE2}$ and LPS levels in rural-BPEs.

Although the alpha diversity of the nasal microbiome within individual participants of the panel study appeared to be rather stable over the course of a whole year, cross-sectional analysis revealed differences in the microbial composition in relation to the atopy status and pollen exposure. Especially species of the *Corynebacteriaceae* family were generally more abundant in non-atopic than atopic individuals as well as during the pollen season, pointing towards a possible protective role of these bacteria against allergy.

During the study period of the 'picnic study' in 2018, the amount of Phl p 5 in nasal filters paralleled the concentration of grass pollen in the air. Recombinant Phl p 1 or Phl p 5 did not induce inflammatory responses in HNECs. In contrast, spores of *Cladosporium*, *Fusarium* and *Penicillium* induced expression of IL-8 and IL-1β, either alone or (to a slightly higher extent) in combination with the grass pollen allergens. The nasal mycobiome of the study participants differed significantly pre- and post-exposure. Human commensal *Malassezia* species were less

abundant in the nasal mycoflora after 1 hour outdoors, while *Cladosporium* and other environmental fungi were enriched.

Conclusion: In summary, these studies highlight the importance of the allergen matrix, which is species-specific and consists of various pollen-derived immunomodulatory substances and associated microbes in the form of, but not restricted to, bacteria and fungi. Together they shape the immune response towards pollen allergens and are yet themselves subject to environmental influences. Lastly, the individual microbial environment likely plays an important role as well by providing a protective milieu in healthy individuals and inflammatory stimuli in allergic patients. Understanding this complex interplay between environmental and host factors can be used to improve treatment for atopic diseases in the future.

Zusammenfassung

Hintergrund: Allergische Erkrankungen sind seit den letzten Jahrzehnten immer weiter auf dem Vormarsch. Weltweit ist in etwa ein Drittel der Bevölkerung von einer Allergie betroffen, mit steigenden Tendenzen und daraus folgenden hohen Belastungen für die Gesundheitssysteme. Die Entstehung einer Allergie hängt von einer Vielzahl an Faktoren ab, einschließlich genetischer Vorbelastung und Umwelteinflüssen. Pollen von Windbestäubern sind häufig Auslöser von allergischen Reaktionen, wobei die Allergenität nicht nur von den Allergenen selbst, sondern auch von zusätzlichen Pollen-assoziierten Substanzen abhängt. Diese Substanzen können die Immunreaktion des Menschen verändern und somit eine allergische Sensibilisierung ermöglichen. Nicht nur der Mensch, sondern auch Pflanzen sind vom Klimawandel und von Umweltverschmutzung betroffen. Das hat zur Folge, dass sich die Zusammensetzung der Pollen-assoziierten Substanzen von einigen Pflanzen verändert, wodurch diese stärker allergen wirken.

Das menschliche Mikrobiom hat großen Einfluss auf unsere Gesundheit, so auch auf die Entstehung von Allergien. Es ist jedoch gleichzeitig vielen Umwelteinflüssen ausgesetzt, die negative Auswirkungen haben können. Zudem wird vermutet, dass die Ko-Exposition mit Allergenen und mikrobiellen Produkten wie z.B. bakterielle Lipopolysaccharide oder Pilzsporen die allergische Sensibilisierung fördert.

Diese Doktorarbeit befasst sich mit den Auswirkungen von biotischen und abiotischen Umwelteinflüssen, wie z. B. Klimawandel, Umweltverschmutzung und verschiedenste Mikroben, auf die allergische Sensibilisierung gegen Pollen vom beifußblättrigen Traubenkraut (*Ambrosia artemisiifolia*), der Birke (*Betula pendula*) und von Gräsern (*Phleum pratense*). Zudem wird untersucht, wie sich die natürliche Pollenexposition auf das Nasenmikrobiom von Gesunden und Allergikern auswirkt.

Methoden: Um die Auswirkung von Umweltfaktoren auf die Pollenallergenität zu erforschen, wurde *A. artemisiifolia* in Gewächshäusern angepflanzt und während der Vegetationsperiode mit erhöhtem CO_2 (700 ppm, CO_2-RWE, entsprechend einem Klimwandelszenario der IPCC) oder Ozon (80 ppb, O_3-RWE, entsprechend WHO Richtwerten) begast. Außerdem wurden Birkenpollen aus städtischen (urban-BPE) und ländlichen (rural-BPE) Gebieten in und um Augsburg und München gesammelt. Allergenitätsparameter (Allergengehalt, LPS, Adenosin,

$PALM_{PGE2}$, $PALM_{LTB4}$) wurden in wässrigen Pollenextrakten der jeweiligen Proben gemessen. Die wässrigen Extrakte wurden in einem Mausmodell für allergische Sensibilisierung untersucht (Experimente durchgeführt von F. Alessandrini am Helmholtz Zentrum München) und die Ergebnisse wurden *in vitro* anhand humaner Immunzellen überprüft. Hierbei wurde besonders die Zytokinantwort von Nasenepithelzellen und dendritischen Zellen nach Stimulation mit den Pollenextrakten mittels ELISA analysiert. B-Zellen von Maus und Mensch wurden auf ihre IgE Produktion unter allergischen Bedingungen und in Verbindung mit Pollen untersucht. Außerdem wurde die Chemotaxis von Neutrophilen gegenüber den jeweiligen Extrakten betrachtet.

Um zu verstehen, wie sich natürliche Exposition gegenüber Pollen auf das Nasenmikrobiom auswirkt, wurden über den Zeitraum eines Jahres monatliche Abstriche der Nasenschleimhaut von acht Gesunden und acht Allergikern genommen („Panelstudie“). Während der Birkenpollensaison wurden die Abstriche zusätzlich alle zwei Wochen genommen. Aus den Abstrichen wurde DNA isoliert und daraus die bakterielle DNA der variablen Region V1-V3 des 16S rRNA Gens sequenziert. Zusätzlich wurde das Pollen-spezifische Mikrobiom von fünf zufällig ausgewählten Birkenpollenproben untersucht.

Die Auswirkungen der Ko-Exposition gegenüber Gräserpollen und Pilzsporen wurde in einer weiteren Studie („Picknickstudie“) untersucht. Hierfür saßen während der Gräserpollensaison 2018 sieben Probanden einmal wöchentlich für eine Stunde in einer blühenden Wiese. Eine Gruppe trug jeweils eine Stunde vor und während dem Aufenthalt im Freien einen Nasenfilter, aus dem der eingeatmete Allergengehalt bestimmt wurde. Von der zweiten Gruppe wurde jeweils vor und nach dem Außenaufenthalt Nasenabstriche genommen, um Veränderungen des Nasenmykobioms mittels ITS Sequenzierung zu bestimmen. Gleichzeitig wurden Pollenfallen aufgestellt, um die Pollen und Pilzsporenbelastung zu messen. Außerdem wurden modifizierte Pollenfallen, die mit Agarplatten ausgestattet waren, aufgestellt um Pilze aus der Umwelt zu kultivieren. Diese Pilze wurden dann, zusammen mit Gräserpollenallergenen, dazu verwendet, Nasenepithelzellen zu stimulieren. Deren Zytokinantwort sowie die Barrierefunktion wurden unter Ko-Expositionsbedingungen analysiert.

Ergebnisse: Wachstum unter erhöhten CO_2 Leveln, wie für das Jahr 2100 vorhergesagt, verändert die allergenen Eigenschaften von *Ambrosia* Pollen. Im Mausmodell war dies besonders durch erhöhte Anzahl von entzündlichen Zellen in der Lunge, erhöhte

Schleimproduktion und höhere IgE Level erkennbar. Im *in vitro* System war bei dendritischen Zellen, die mit CO_2-RWE-konditionierten Überständen von Nasenepithelzellen stimuliert wurden, vor allem die Produktion von entzündungsfördernden Zytokinen erhöht. Die in den verschiedenen Pollenproben gemessenen adjuvanten Kandidatensubstanzen ($PALM_{PGE2}$, $PALM_{LTB4}$, LPS, Adenosin) waren in CO_2-RWE nur leicht höher konzentriert als in Kontroll-RWE, und der Gehalt des Hauptallergens (Amb a 1) in den Proben war identisch. Durch eine differentielle Metabolomanalyse der verschiedenen Extrakte wurde festgestellt, dass in CO_2-RWE mehrere sekundäre Pflanzenmetaboliten stärker exprimiert waren als in Kontroll-RWE. Diese Metaboliten lösten schließlich ähnliche Zytokinantworten in dendritischen Zellen aus wie die Gesamtpollenextrakte, allerdings nur in Kombination miteinander und nicht alleine.

Pollenextrakte von Pflanzen, die unter erhöhten Ozonlevel wuchsen, waren generell weniger immunmodulierend. Jedoch zeichnete sich eine erhöhte Eigenschaft zur Neutrophilenrekrutierung ab, die wahrscheinlich von erhöhten $PALM_{LTB4}$-Werten stammt und somit zur allergischen Sensibilisierung beitragen könnte.

Wässrige Extrakte aus Pollen von städtischen Birken (urban-BPE) lösten im Mausmodell stärkere allergische Reaktionen aus als Extrakte aus Pollen von ländlichen Birken (rural-BPE). Dies ließ sich in Zellkulturversuchen insofern bestätigen, dass die Zytokinantwort von dendritischen Zellen, die mit urban-BPE stimuliert wurden, weniger regulatorisch und mehr proinflammatorisch war. Diese Befunde lassen sich auf einen geringeren $PALM_{PGE2}$- und LPS-Gehalt in den Extrakten zurückführen. Der Gehalt an Bet v 1, dem Hauptallergen der Birke, war auch hier bei urban-BPE und rural-BPE nicht unterschiedlich. Weitere Metabolomanalysen sind unverzichtbar, um genauere Einblicke in die Zusammensetzung und Allergenität der Pollen zu erhalten.

Die Alphadiversität des Nasenmikrobioms von Gesunden und Allergikern war über den Studienzeitraum der „Panelstudie“ stabil. Das Vorkommen einzelner Bakterien war jedoch unterschiedlich je nach Allergiestatus und Pollenexposition. Bakterien der *Corynebacteriaceae* Familie wurden besonders häufig bei Gesunden entdeckt und waren während der Pollensaison angereichert. Dies könnte auf einen potentiellen protektiven Effekt dieser Bakterien für die Allergieentstehung hinweisen.

Bei einem Außenaufenthalt von einer Stunde während der „Picknickstudie“ folgte der Allergengehalt in der Nase der Pollenbelastung. Gleichzeitige Stimulation von

Nasenepithelzellen mit Gräserpollenallergenen und Pilzsporen, die aus der Umgebung isoliert wurden, führte zu einer erhöhten proinflammatorischen Zytokinantwort. Dies könnte besonders relevant sein, da das Nasenmykobiom bereits nach einem Aufenthalt von einer Stunde im Freien verändert war und allergene Schimmelpilze nachgewiesen werden konnten, welche symbiotische Pilze des Menschen, wie z.B. *Malassezia,* verdrängten.

Schlussfolgerung: Zusammengefasst heben diese Studien vor allem den Einfluss der Allergen-Matrix hervor, welche spezifisch für das jeweilige Allergen ist und aus verschiedenen pollenassoziierten immunmodulierenden Substanzen besteht. Hierzu zählen auch pollenassoziierte Mikroben, wie z.B. Bakterien und Pilze. Im Zusammenspiel verändern sie die gegen Pollenallergene gerichtete Immunantwort und fördern die allergische Sensibilisierung und Entzündung. Die Zusammensetzung dieser Matrixbestandteile ist abhängig von Umwelteinflüssen wie Klimawandel und Umweltverschmutzung. Außerdem könnte das individuelle nasale Mikrobiom von Gesunden zum Schutz von allergischer Sensibilisierung beitragen, während es bei Allergikern entzündliche Signale sendet. Diese Erkenntnisse könnten in der Zukunft genutzt werden, um die Behandlung von allergischen Erkrankungen zu verbessern.

Chapter 1 – Introduction

1.1. Allergic diseases – a global health concern

Hay fever was first described by John Bostock in 1819, who reported annually recurring itching and redness of the eyes accompanied by mucus discharge, sneezing, tightness of the chest and difficulty breathing. The symptoms occurred during the second half of June and early July, coinciding with the pollen season of grasses (Bostock, 1819). This study was the first scientific report of what now is called “allergy”, a term encompassing a spectrum of diseases such as hay fever, or allergic rhinitis (AR), atopic dermatitis/eczema (AD), allergic conjunctivitis, allergic asthma, food and drug allergy and anaphylaxis.

Simply put, an allergy is a maladaptive immune response where the immune system overreacts to an otherwise harmless environmental protein, the so-called allergen. In immediate-type (type-I) allergies, sensitization to allergens leads to the production of specific antibodies (IgE), which are crucial for clinical manifestations of type-I allergies (C.A. Akdis, Agache, Allergy, & Immunology, 2014). The presence of IgE in blood provides information of the allergic sensitization status (atopy) of patients. Subsequent encounters with allergens via inhalation, ingestion or skin contact cause mucus hypersecretion or nasal congestion, sneezing, eye symptoms such as swelling or redness, airway hyperresponsiveness and constriction, skin rashes, eczema or itching, or gastro-intestinal symptoms (Bousquet et al., 2008). Often, atopic dermatitis in infants precedes the development of AR and is thought to be a gateway for allergic sensitization. While AD can subside with age, the incidence of AR increases throughout childhood into adulthood (Hill & Spergel, 2018).

Sources of airborne allergens are manifold and include pollens and outdoor fungal spores causing seasonal allergies, while house dust mites (HDM), cockroaches, animal dander, indoor fungi, various foods and insect venoms are perennial allergens (Bousquet et al., 2008).

The prevalence of AR caused by inhalant allergens, so called aeroallergens, is estimated at 40 % worldwide. There are regional differences especially between the Northern Hemisphere, particularly Europe with 20-30 % and North America with 12-30 %, and the Southern Hemisphere, where the affected population ranges between 2.9-54.1 % depending on country of origin (Cezmi A. Akdis, Hellings, & Agache, 2015; Bieber et al., 2016; Passali et al., 2018; Sterner et al., 2019).

Pollen from *Poaceae* (grasses) are among the most common aeroallergen sources worldwide. In Europe and the US, between 10-30 % of the population are sensitized to grass pollen allergens (Newson et al., 2014; Salo et al., 2014), while worldwide sensitization rates vary between 2-60 %. In Australia, up to 84 % of the population are sensitized to Johnson, Bahia or Bermuda grass pollen (C.A. Akdis et al., 2014; Davies, 2014).

In temperate climate zones of the northern hemisphere, pollen from trees of the *Betulaceae* (birch, hazel) and related *Fagaceae* (alder, and oak among others) families are some of the major contributors to AR. Their overlapping pollen seasons from early to late spring prolong suffering for the 8-16 % affected tree-allergic AR patients in Europe (Biedermann et al., 2019).

In North America, 26 % of the population are sensitized to common ragweed (*Ambrosia artemisiifolia L*). *A. artemisiifolia* has been spreading as a neophyte in Europe in the past decades, where, by 2016, 33 million Europeans were already sensitized. These sensitization rates are projected to double by 2041-2060 (Lake et al., 2018).

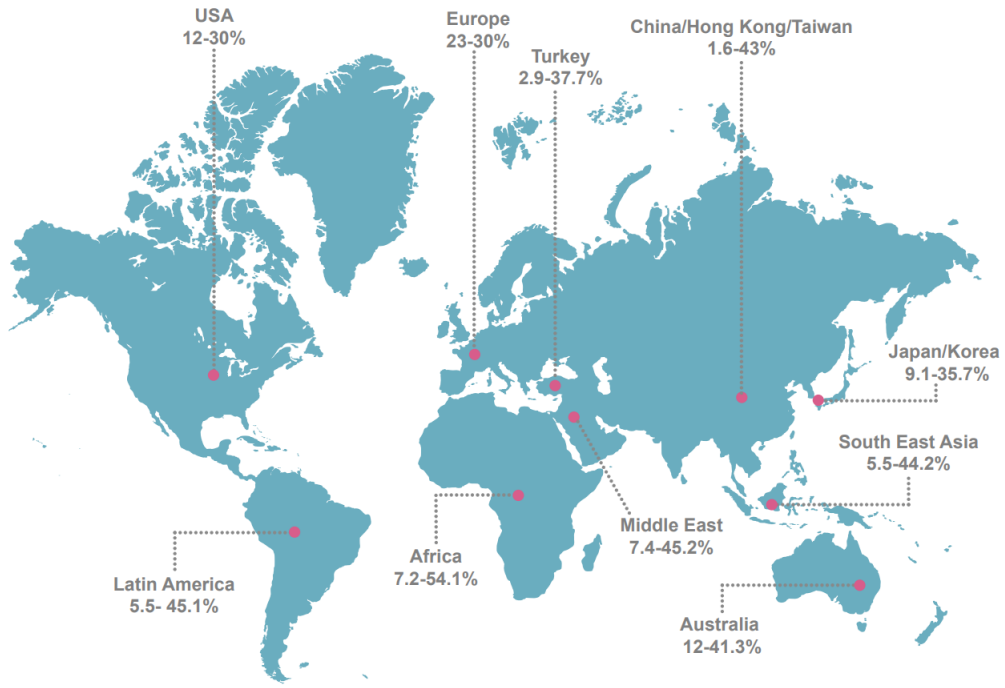

***Figure 1 Prevalence** of allergic rhinitis worldwide* (Cezmi A. Akdis et al., 2015).

Long before the terms hay fever and allergy were coined, adverse reactions to fungi were described (Floyer, 1698). Fungi are ubiquitous in nature and the built environment, and indoor molds in damp buildings are often cause for respiratory illness upon continued exposure. The most common allergenic fungal species belong to *Alternaria, Penicillium, Cladosporium* and *Aspergillus* genera (Bousquet et al., 2008). Sensitization to fungal allergens has been linked to

the development of AR and occurs in approximately 25 % of severe forms of asthma (Black, Udy, & Brodie, 2000; Boulet et al., 1997; Mari, Schneider, Wally, Breitenbach, & Simon-Nobbe, 2003).

In many cases, IgE against inhaled allergens cross-reacts with homologous food proteins, posing another risk for patients suffering from respiratory allergies and leading to pollen-food syndrome (PFS). Common examples for PFS are birch and apple, cypress and peach, or pollen contaminations, especially of the *Asteraceae* (mugwort, ragweed) family in food sources such as honey (Popescu, 2015).

The lifetime prevalence of allergic diseases has been increasing in recent decades, especially in low and middle income countries (Bjorksten et al., 2008). Because the rising burden of allergy is related to loss of quality of life, but also has far reaching economic and public health impact (Lamb et al., 2006; Schoenwetter, Dupclay, Appajosyula, Botteman, & Pashos, 2004; Zuberbier, Lotvall, Simoens, Subramanian, & Church, 2014), research has been focused on the "how" and "why" of allergic diseases.

1.2. Allergic diseases – Nature or nurture?

1.2.1. Nature – Genetic predisposition

The question why some people develop allergies has been studied extensively. Heredity of allergic diseases has been discussed as early as 1916 by Cooke and VanderVeer, who argued that the potential for allergic sensitization is passed down from parents to their children, even if the parents did not have clinical manifestations (Cooke & Veer, 1916). More recent studies confirmed an increased allergy risk for children of at least one parent with a history of allergic diseases (Dold, Wjst, von Mutius, Reitmeir, & Stiepel, 1992; Michel, Bousquet, Greillier, Robinet-Levy, & Coulomb, 1980; Palmer, Burton, James, Musk, & Cookson, 2000). Moreover, age, sex and ethnicity are risk factors for atopy (Bousquet et al., 2008). Technological advances, such as genome wide associated studies, whole genome sequencing or transcriptomics, have led to the discovery of many genes associated with asthma, AR or AD, many of them associated with type 2 immune response (Th2) cytokines and chemokines. This knowledge may pave the way for better and personalized treatments in the future (Gilles et al., 2018; Ortiz & Barnes, 2015).

1.2.2. Nurture – Environmental factors

While there is a clear genetic predisposition for allergic sensitization, epigenetic and twin studies have shown that environmental factors influence the risk to develop allergies (Durham, Chou, Kirkham, & Adcock, 2010). These environmental factors are mostly related to a "Westernized life style" of post-industrialization. Dietary habits, psychosocial factors such as stress or depression (Harter et al., 2019) or maternal smoking habits (Keil et al., 2009) have been linked to the rise of the prevalence of allergic diseases. Progressively more urban living conditions cause higher prevalence of allergic diseases in children compared to their peers from rural farm environments (Majkowska-Wojciechowska et al., 2007). This is congruent with the "hygiene hypothesis" suggesting improved living conditions lower the exposure to pathogenic and non-pathogenic microbes, leading, on the downside, to a higher risk for allergic sensitization. Indeed, children growing up in farm environments and born to mothers exposed to farm environments, have a lower risk to develop AD, asthma or AR, which has been linked to exposure to highly diverse environmental microbiomes (von Mutius, 2016).

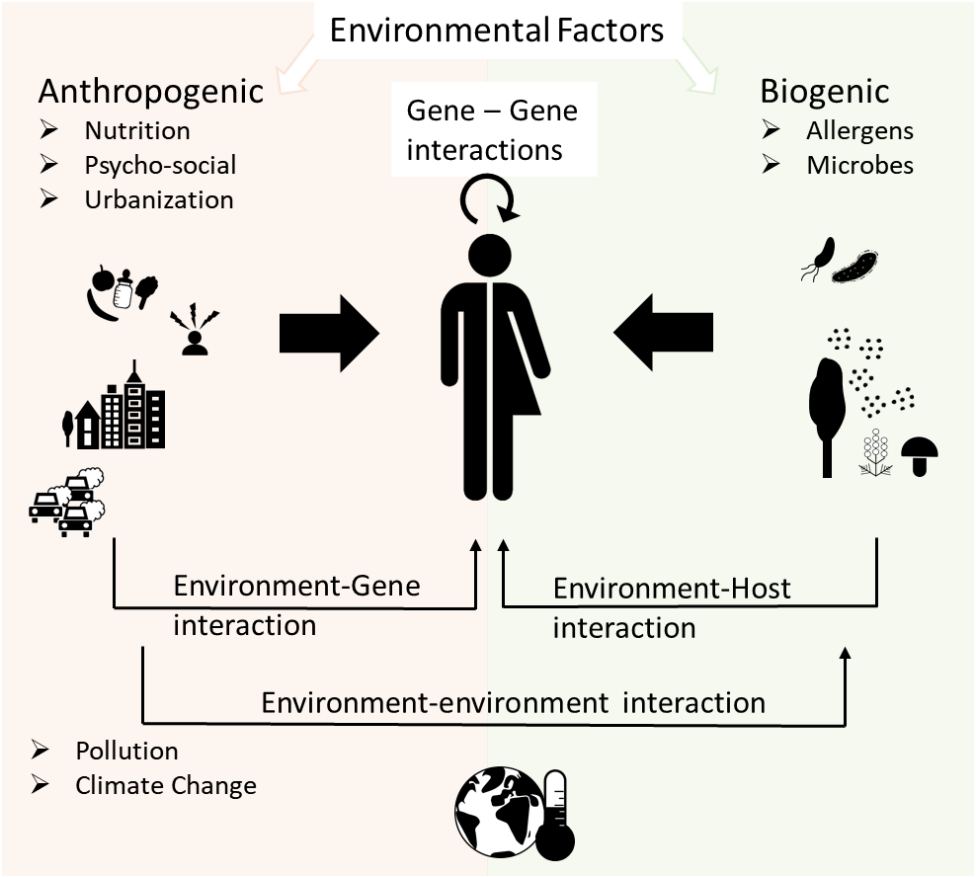

Figure 2 Allergies are multi-factorial diseases. An intricate interplay of genetics, biotic and abiotic environmental factors is involved in the process of allergic disease. Adapted from (Gilles et al., 2018).

Microbes – they're all around us

Not only the exposure to exogenous microbes but also the development of the individual microbiome, especially in infancy and early childhood, has been linked to the allergy risk later in life (Durack & Christophersen, 2020; Man, de Steenhuijsen Piters, & Bogaert, 2017; Stiemsma & Turvey, 2017). Commensal microbes, such as bacteria, fungi, viruses and protozoa, colonize the human body on the skin, urogenital tract, gut, mouth, nose and bronchial surfaces, where they shape and train the immune system (Belkaid & Harrison, 2017). Overall, the microbial composition on the barrier sites of the body is rather stable, although it changes with age and is formed by environmental exposure (Dimitri-Pinheiro, Soares, & Barata, 2020; Integrative, 2014).

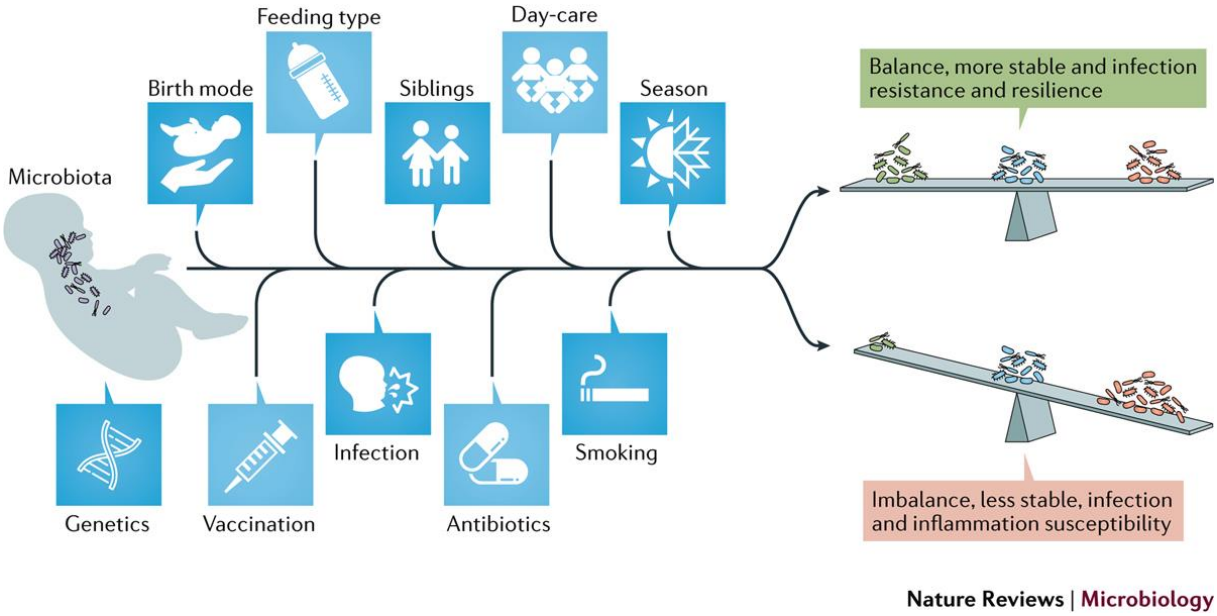

Figure 3 The microbiome in early life is highly dynamic and shaped by exposure to environmental influences. Together with host factors, these environmental stimuli prepare the foundation for a healthy microbiome or tilt the balance towards increasing susceptibility for infections, inflammatory diseases and allergy. Adapted from Man, de Steenhuijsen Piters, & Bogaert, 2017.

Drastic environmental changes such as changes in pH or use of antibiotics cause dysbiosis with dire consequences, allowing harmful pathogens to take over. In AD, skin resident *Staphylococcus aureus* plays a role in disease flare ups although it is not yet fully understood if *S. aureus* overgrowth plays a causative role or is an effect thereof (Blicharz, Rudnicka, & Samochocki, 2019). Microbiome research has mostly focused on the gut, and while the skin microbiome has received an exceeding amount of attention in recent years in the light of atopic skin diseases, fewer studies have focused on the nasal microbiome, especially in allergic diseases (Dimitri-Pinheiro et al., 2020). In asthma, the composition of the nasal microbiome

has been linked to disease severity, especially the *Moraxella* genus, which is correlated with severe asthma exacerbations and eosinophil activation (McCauley et al., 2019; Zhou et al., 2019). Asthma risk is also associated with early *Streptococcus* colonization of the nasopharynx in the first years of life (Teo et al., 2015). Higher microbial diversity in seasonal AR patients was detected during the tree and grass pollen season and correlated to stronger symptoms and higher nasal eosinophil counts (C. H. Choi et al., 2014). This is in contrast to another study, where AR patients were characterized by lower nasal biodiversity, although measurements were taken irrespective of the pollen season (Lal et al., 2017).

Moving from culture-based methods to NGS has advanced the field of fungal studies and enabled a more detailed insight into the human mycobiome, although research on its impact on health and disease is still in its infancy (Cui, Morris, & Ghedin, 2013; Tiew et al., 2020). In a culture-based approach, lower abundances of environmental fungi were detected in allergic patients compared to healthy controls (Sellart-Altisent, Torres-Rodriguez, Gomez de Ana, & Alvarado-Ramirez, 2007), while higher fungal diversity was detected in AR patients in a culture-independent approach. Interestingly, changes in the gut mycobiome upon antibiotic treatment were able to induce allergic airway responses to *Aspergillus fumigatus* spores in immunocompromised mice without prior allergen sensitization, highlighting a complex interplay between the mycobiome in different body sites and the importance of a balanced microbiome-mycobiome composition (Noverr, Falkowski, McDonald, McKenzie, & Huffnagle, 2005).

1.2.3. Climate Change and air pollution

Air pollution in high traffic or heavy industry areas has been linked to increasing numbers of allergic patients. Children growing up close to busy streets have a higher risk to develop allergies (Deng et al., 2016; D. Y. Jung et al., 2015). This is partly due to airborne particulate matter (PM), especially when made up of ultrafine particles, which penetrates the lower airways, inducing inflammatory responses and exacerbating asthma symptoms (D'Amato, Cecchi, D'Amato, & Liccardi, 2010). Ultrafine particles act as adjuvant factors in allergic lung inflammation in mouse models by inducing oxidative stress (Alessandrini et al., 2009; Alessandrini et al., 2006; Alessandrini et al., 2010). Likewise, diesel exhaust particles (DEP) have been shown to act as adjuvants in the sensitization phase to timothy grass pollen in a mouse model of allergic sensitization (Steerenberg et al., 2003). Nitrogen dioxide (NO_2) and

ozone (O_3) are oxidant pollutants, and their levels in ambient air are inversely correlated to each other. Damage to the nasal epithelium, as well as influx of inflammatory cells and increasing pro-inflammatory interleukins have been linked to exposure to high O_3 levels (D'Amato et al., 2010; Khatri et al., 2009). AR and sensitization rates to outdoor allergens were also increased in children with long term exposure to O_3 (B. J. Kim et al., 2011).

Climate change, which is closely linked to air pollution and caused by increasing emissions of anthropogenic greenhouse gases, is also a considerable risk factor for the increase of allergic diseases worldwide. Rising temperatures and changed precipitation patterns will allow the spread of allergenic neophytes such as ragweed, which is projected to shift its distribution from Central and Eastern Europe towards Northern European countries (Cunze, Leiblein, & Tackenberg, 2013; Rasmussen, Thyrring, Muscarella, & Borchsenius, 2017). The abundance of airborne allergenic pollen is already impacted by climate change, especially by higher average temperatures, which led to earlier flowering periods and higher pollen abundance, reducing the pollen-free window for allergic patients (Athanasios Damialis, Damialis, Fotiou, Halley, & Vokou, 2011; Ziello, Böck, Estrella, Ankerst, & Menzel, 2012; C. Ziello et al., 2012; L. Ziska et al., 2011; L. H. Ziska et al., 2019). Moreover, atmospheric carbon dioxide (CO_2) naturally contributes to plant growth and elevated CO_2 levels have already resulted in higher ragweed biomass, including pollen production (Wayne, Foster, Connolly, Bazzaz, & Epstein, 2002; L. H. Ziska et al., 2003). Elevated CO_2 levels during a whole ragweed vegetation period also changed the metabolic composition of the pollen (Rauer et al., 2020). This resulted in stronger allergic lung inflammation in mice sensitized, and exposed, to pollen from ragweed plants grown under elevated CO_2 levels and increasingly inflammatory responses of dendritic cells *in vitro* (Rauer et al., 2020).

A nine-year field experiment on the effect of climate change and fungal communities in soil has also shown that global warming will result in higher abundances of potential pathogenic or allergenic fungal species (Delgado-Baquerizo et al., 2020). Experimental exposure to higher temperatures as expected by 2100 have also shown to favor the growth of allergenic fungi and it is likely that *Cladosporium* spore production will also increase in the future (A. Damialis, Mohammad, Halley, & Gange, 2015). Already, climate related changes have shown earlier and longer fruiting periods of fungi, prolonging the risk of suffering for atopic patients (Boddy et al., 2014). These findings are alarming, since grass pollen and allergenic fungal spores of

Cladosporium and *Alternaria* are present at the same time (Grewling et al., 2019). A negative effect of co-exposure to fungal spores and grass pollen has already been observed: *Alternaria* spores alone or in combination with grass straw have been shown to exacerbate asthma symptoms in timothy grass (*Phleum pratense*) sensitized mice (Hernandez-Ramirez et al., 2020).

Extreme weather conditions, such as heatwaves, draught, heavy rainfalls, floods and thunderstorms have become more frequent in recent years. There is increasing evidence that thunderstorms induce severe asthma attacks requiring hospitalization or even causing death (Davidson, Emberlin, Cook, & Venables, 1996; Forouzan et al., 2014; Packe & Ayres, 1985; Thien et al., 2018). The so-called "thunderstorm asthma" was found correlated to higher ground-level concentrations of especially grass pollen and fungal spores, and higher release of allergens (Thien et al., 2018).

1.3. Allergic sensitization

Allergic sensitization is a process where the immune system reacts to an otherwise innocuous substance in an ill-directed manner, resulting in the induction of Th2 cells and IgE antibodies. A host of different immune cells of the innate and adaptive immune system is involved in developing type 2 immune reactions, which are thought to have evolved originally as protection against parasites (e.g. helminthic worms) and against reptile-, arthropod- or insect venoms (C. A. Akdis et al., 2020).

1.3.1. Epithelial cells – the stronghold of innate immunity

Airway epithelial cells (EC) are the first line of defense and an integral part of the innate immune response of the upper airways (Holtzman, Byers, Alexander-Brett, & Wang, 2014). The airway epithelium continuously lines the respiratory tract from the nasal cavity to alveoli in the lung and is mainly made up of three cell types: basal cells, secretory cells and ciliated cells (Whitsett, 2018).

Epithelial barrier – an impenetrable wall?

Epithelial cells form cell-cell contacts to protect the underlying tissue from the environment and to preserve physical barrier integrity. Tight junctions (TJ), adhesion junctions (AJ) and desmosomes comprise these intercellular junctions. The transmembrane part of TJs consists of claudins, occludins and junctional adhesion molecules (JAM) which are connected to the

cytoskeleton via zona occludens proteins (ZO-1, -2, -3) (Ohkuni et al., 2009; Takano et al., 2005). E-cadherins are integral in forming AJs and connect the cytoskeletons of adjacent cells (Ivanov & Naydenov, 2013; Nelson, 2008). ECs are connected to the lamina propria via non-classical cadherins forming desmosomes (Garrod & Chidgey, 2008). Downregulation of these junction molecules has been shown to be present in epithelial cells of asthma patients as well as nasal polyps in children suffering from asthma (H. J. Lee et al., 2016; Steelant et al., 2016), highlighting the importance of intact physical barrier functions to sustain a healthy airway epithelium.

The airway epithelial barrier function is not only mechanical, it is also supported by a liquid layer. This mucus layer removes inhalant particles from the airways by mucociliary transport dependent on cilia and their size and density (Khelloufi et al., 2018). A major component of the mucus layer are mucins, large glycoproteins, which polymerize to give the mucus layer its gel-like structure (Atanasova & Reznikov, 2019). MUC5AC and MUC5B are the most commonly secreted mucins and are critical factors in pathogen clearance by mediating immune responses and increasing airway hyperreactivity (Evans et al., 2015; Roy et al., 2014). Mucin hypersecretion is associated with the severity of multiple airway diseases such as asthma, cystic fibrosis and COPD (Atanasova & Reznikov, 2019; S. H. Kim et al., 2019).

To enforce the physical epithelial barrier, secretory ECs also secrete antimicrobial peptides, complement factors, IgA antibodies, cytokines and chemokines into the mucus layer (Kesimer et al., 2009). Furthermore, increased numbers of neutrophils and eosinophils have also been observed in sputum of asthmatic patients (Fahy, 2009).

Epithelial barrier disruption by exposure to pathogens, toxins and other stressors, e.g. allergen carriers, leads to the secretion of alarmins, interleukin-25 (IL-25), thymic stromal lymphopoietin (TSLP) and IL-33, which are responsible for activating downstream effector cells of the innate and adaptive immune system (C. A. Akdis et al., 2020).

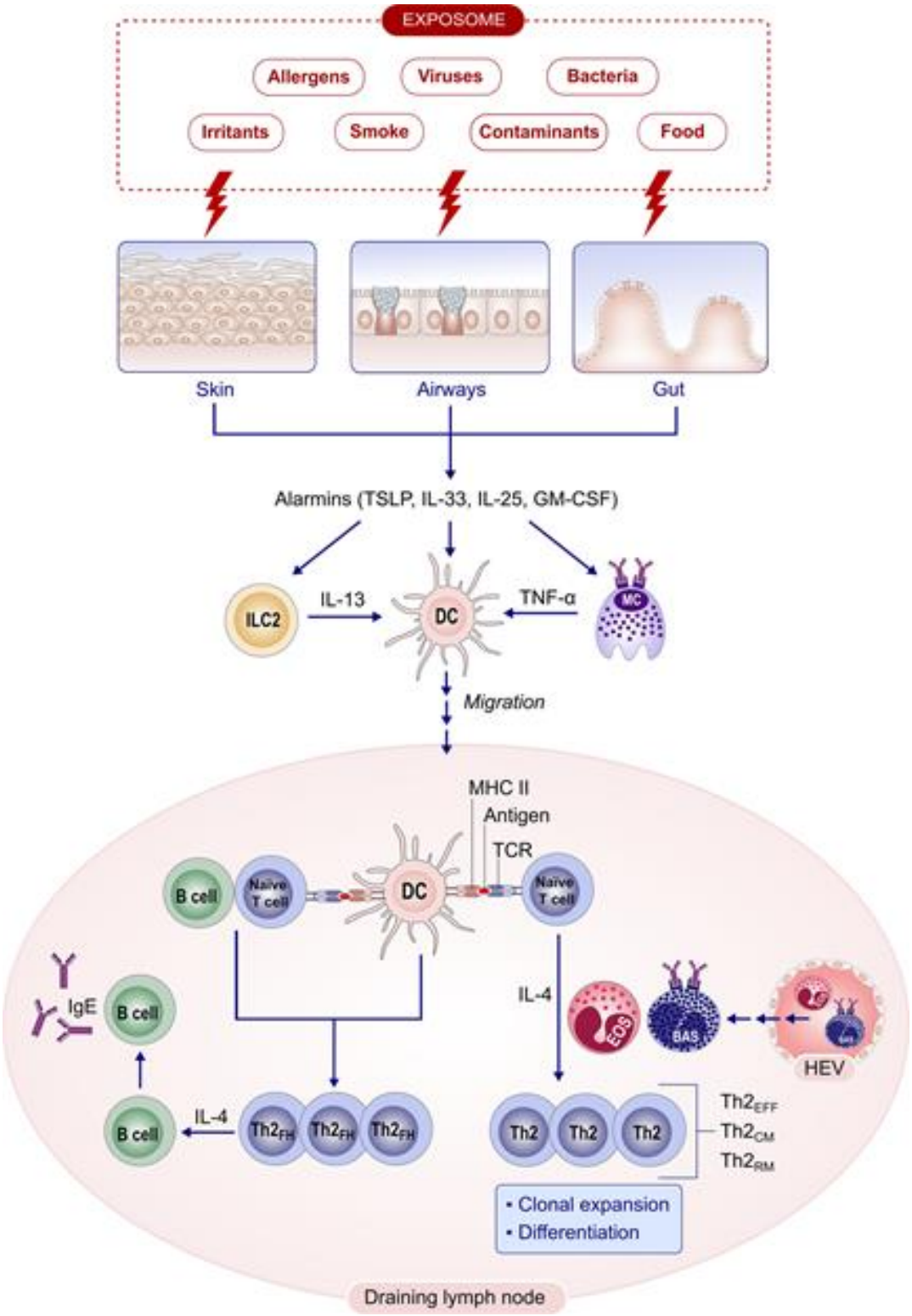

Figure 4 Allergic sensitization. Disruption of the epithelial barrier by allergens, bacteria, virus or fungi among other environmental stimuli, allows allergens to intrude in the tissue. Damaged epithelial cells secret alarmins which subsequently leads to recruitment and activation of ILC2 and dendritic cells (DC). Mature dendritic cells migrate to the draining lymph nodes, where they encounter naïve $CD4^+$ T cells. Presentation of processed allergen peptides on MHCII and secretion of IL-4 leads to the differentiation of Th2 cells. Th2 follicular helper cells interact with B cells in germinal centers and secretion of IL-4 and IL-13 induces the production of IgE. Secreted IgE binds to mast cells (MC) and basophils (BAS), thereby sensitizing them. IL, interleukin; TSLP, thymic stromal lymphopoietin; GM-CSF, granulocyte-macrophage colony-stimulating factor; MHC, major histocompatibility complex; TCR, T-cell receptor; HEV, high endothelial venules (C. A. Akdis et al., 2020).

Pattern recognition receptors – airways on the lookout

Epithelial cells sense their environment by expressing germline-encoded pattern recognition receptors (PPR), enabling them to detect conserved pathogen-associated molecular patterns (PAMPs) or cell damage-associated molecular patterns (DAMPs) and to induce an appropriate immune response (Leiva-Juarez, Kolls, & Evans, 2018). PRRs are subdivided into four distinct classes: Toll-like receptors (TLR) and C-type lectin receptors (CLR) are transmembrane receptors, whereas Retinoic acid-inducible (RIG)-1 like receptors (RLR) and NOD-like receptors (NLRs) are present in the cytosol. These PRRs are not restricted to ECs and are most commonly found in immune cells such as dendritic cells (DC) and macrophages. Activation of PRRs triggers intracellular signaling cascades regulating the transcription and translation of inflammatory cytokines (Mogensen, 2009).

To date, ten TLRs have been identified in humans. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are bound to the plasma membrane. Their ligands include lipoproteins, lipopolysaccharide (LPS), flagellin and profilin-like molecules from bacteria, viruses, protozoa, fungi and self-proteins. TLR3, TLR8, TLR9 and TLR10 are found in intracellular endolysosomes and bind to double and single stranded RNA, bacterial CpG DNA motifs and endogenous nucleic acids, respectively (Takeuchi & Akira, 2010). Dimerization of TLRs upon ligand binding leads to conformation changes in the intracellular toll/interleukin-1 receptor (TIR), which activates either myeloid differentiation factor 88 (MyD88) or TIR domain-containing adaptor inducing IFN-β (TRIF) dependent pathways (McClure & Massari, 2014). MyD88 signaling induces the production of pro-inflammatory cytokines and chemokines, type-I and -III interferons (IFNs), anti-microbial peptides (AMPs) and mucin. This pathway is shared by all TLRs except TLR3 which acts in a MyD88 independent manner and activates TRIF signaling, leading to type-I IFN and IL-10 secretion (McClure & Massari, 2014). *In vitro,* TLR1 to 6 and TLR9 are expressed by nasal ECs, with TLR4 and TLR9 expressed at lower levels in allergic rhinitis patients (Bergougnan et al., 2020).

Inflammasomes are important mediators of immune responses by cleaving pro-IL-1β and pro-IL-18 into their active forms IL-1β and IL-18, which are then secreted (Guo, Callaway, & Ting, 2015). Inflammasomes are multi-protein complexes consisting of a subfamily of NLR proteins, the Nod-like receptor family pyrin domain-containing proteins (NLRP). They form a multi-molecular complex with apoptosis-associated speck-like protein containing CARD (ASC) and

caspase-1 (Xiao, Xu, & Su, 2018). In the context of allergic diseases, the NLRP3 inflammasome has been well studied as a mediator for Th2 differentiation and inflammation contributing, to asthma exacerbation (Bruchard et al., 2015). Recent evidence points to NLRP3 involvement in the development of AR by impairing airway epithelial barrier through epithelial pyroptosis and increased IL-1β secretion in AR patients, as well as in an *in vivo* mouse model (Z. Yang et al., 2020).

CLRs are best described in antigen-presenting cells (APCs), where they are responsible for recognizing complex carbohydrate structures, such as fungal allergens. Activation of Dectin-1, a β-glucan recognizing CLR, was shown to be protective against HDM induced Th2 polarization (Gour et al., 2018). This process was mediated by inhibiting EC IL-33 secretion and was impaired in atopic individuals (Gour et al., 2018). Glycans in HDM allergen extracts are also recognized by Dectin-2 on dendritic cells, which is critical for airway inflammation and Th2 cytokine production by inducing cysteinyl leukotrienes in a murine HDM sensitization model (Barrett et al., 2011). Moreover, expression of a dendritic cell specific CLR, DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN), was reduced in HDM sensitized asthma patients, which translated to higher IL-6 and lower IL-12 secretion upon HDM stimulation (H. J. Huang, Lin, Liu, Kao, & Wang, 2011).

Protease-activated receptors (PAR) are seven-transmembrane G-coupled receptors. To date, four PARs have been discovered and characterized by their sensitivity to cleavage by trypsin and thrombin (Cocks & Moffatt, 2001). PAR2 is of special interest in the context of allergic diseases, as it is expressed on airway epithelial cells, mast cells, macrophages and neutrophils (Cocks & Moffatt, 2001). Activation of PAR2 has been shown to be involved in the re-location of ZO-1 and PAR2 activation has been shown to be enhanced in asthma patients (Enjoji, Ohama, & Sato, 2014; Knight et al., 2001).

1.3.2. Polymorphonuclear leukocytes – the first and late responders

Neutrophils, eosinophils, basophils and mast cells are innate immune cells contributing to the allergic inflammatory response and possibly allergic sensitization. Neutrophils are recruited to the airways early on during allergic sensitization to pollen allergens dependent on TLR-4 and C-X-C chemokine receptor 2 (CXCR2, also known as IL-8RB) pathways (Hosoki, Aguilera-Aguirre, et al., 2016). During infections their functions are tri-fold: degranulation to release AMPs or metalloproteases (MMPs), phagocytosis, and NETosis, the release of neutrophil

extracellular traps (NETs) to immobilize pathogens (Rosales, 2018). NETs have been observed in asthmatic patients (Dworski, Simon, Hoskins, & Yousefi, 2011; Wright et al., 2016) and likely contribute to disease pathology by destroying epithelial tissues (Saffarzadeh et al., 2012).

In allergic diseases, eosinophils are known for their role as effector cells (C. A. Akdis et al., 2020). IL-5, and to a lesser extend IL-13, production by Th2 cells as well as epithelial cell-derived IL-33 are key to recruit eosinophils to the source of inflammation (Jacobsen, Lee, & Lee, 2014). They contribute to inflammatory processes by secreting IL-13, TGFβ and leukotrienes, and induce tissue damage by releasing granule proteins (e.g. eosinophilic cationic protein, ECP) and extracellular traps (Dworski et al., 2011; Jacobsen et al., 2014). In turn, eosinophils release cytokines to recruit Th2 cells to the airways upon allergen challenge, creating a vicious cycle of inflammation (Jacobsen et al., 2014).

Basophils are important mediators of late phase and chronic allergic inflammation, which is mediated by FcεR1 bound IgE crosslinking and the resulting release of histamine and proteases (Stone, Prussin, & Metcalfe, 2010). Because basophils are able to secrete IL-4 and express CD86 on their surface, they are potential drivers of Th2 priming of naïve $CD4^+$ T cells while acting as APCs, although this is debated controversially (Perrigoue et al., 2009). This process appears mediated by TSLP (Perrigoue et al., 2009), which is up-regulated after allergen stimulation in allergic rhinitis patients (Arai et al., 2018).

Mast cells are the most prominent effector cells of allergic reactions. Cross-linking of membrane-bound IgE by allergens induces the release of histamine, proteases, prostaglandins and cytokines, which cause airway hyperresponsiveness and remodeling, and lymphocyte recruitment to the site of inflammation (Amin, 2012).

1.3.3. Innate lymphoid cells – a missing link?

The discovery of innate lymphoid cells (ILCs) has brought upon a paradigm shift in the interaction of innate and adaptive immunity (Eberl, Colonna, Di Santo, & McKenzie, 2015). ILCs are mainly tissue resident innate immune cells. Even though they share a common lymphoid progenitor with B and T cells, their role in immunity is not mediated by antigen-specific receptors but rather as a general response to tissue damage and infection (Eberl et al., 2015; Panda & Colonna, 2019). ILCs induce cytokine profiles similar to $CD4^+$ T helper cell subsets, making them the innate counterparts to T cells of the adaptive immune response.

ILC1s are similar to Th1 cells and respond to IL-12, IL-15 and IL-18 induction by intracellular bacteria and viruses by secreting IFNγ and TNFα (Eberl et al., 2015; Panda & Colonna, 2019). Helminth, epithelial damage and allergen- induced IL-25, IL-33, TSLP release, as well as basophil derived IL-4, activate ILC2s to secrete IL-4, IL-5 and IL-13 similar to Th2 cells, shown to contribute to Th2 polarization by inducing migration of $CD40^+$ activated DCs (Eberl et al., 2015; Halim et al., 2014). ILC2s were found in higher abundance in sputum of asthmatic patients, as well as in nasal polyps of AR and CRS patients (Poposki et al., 2017; Shaw et al., 2013). Allergen challenge induced ILC2 influx to the nasal mucosa of AR patients, which was accompanied by higher numbers of neutrophils and eosinophils especially in patients with high serum IgE (J. Dhariwal et al., 2017). Lastly, ILC3 production of IL-22, IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF) and lymphotoxins in response to fungal and extracellular bacterial infections corresponds to Th17 reactions (Eberl et al., 2015). ILC3s were detected in the bronchoalveolar lavage fluid (BALF) in an obesity induced murine asthma model and are thought to contribute to disease severity via the NLRP3 inflammasome (H. Y. Kim et al., 2014).

1.3.4. Dendritic cells – coordinating the immune response since 1868

Dendritic cells (DC) play a central role during allergic sensitization. As APCs they are responsible for antigen uptake, proteolytic processing and presentation to naïve T cells (Humeniuk, Dubiela, & Hoffmann-Sommergruber, 2017). Immature DCs are found in the skin and airways, as well as the gastrointestinal tract, where they are in constant exchange with the environment to scout for antigens. By forming tight junctions with epithelial cells they are able to extend dendrites into the lumen to sample their environment (Chieppa, Rescigno, Huang, & Germain, 2006; Jahnsen et al., 2006; Sung et al., 2006). Uptake of extracellular proteins, such as allergens, in combination with the activation of PRR leads to DC maturation, upon which DCs migrate to draining lymph nodes (dLN). Here, they activate naïve T cells (Kool, Hammad, & Lambrecht, 2012; Vermaelen, Carro-Muino, Lambrecht, & Pauwels, 2001). Extracellular antigens are presented to naïve $CD4^+$ T cells via class 2 major histocompatibility (MHC-II) molecules, subsequently leading to activation of Th cell polarization. Depending on the induction of cytokines and maturation markers upon allergen uptake, DCs are the crossroads between allergic sensitization and tolerance induction. Steady state DCs constitutively express MHC-II (HLA-DR) molecules and upregulate MHC as well as co-

stimulatory molecules CD40, OX40L, ICOS, CD80, CD83 and CD86 upon activation (Hellman & Eriksson, 2007).

DC subsets and their role in allergy

Currently, five DC subsets are defined: plasmacytoid DCs (pDCs), conventional DCs (cDCs), which are of myeloid origin and are further separated into cDC1 and cDC2, Langerhans cells (LC) and monocyte-derived DCs (moDC) (Eisenbarth, 2019). pDCs are characterized by expression of cluster of differentiation (CD) 123, CD303 and CD304 surface markers and TLR7 and TLR9 (Kadowaki et al., 2001), which are involved in the regulation of interferon production upon viral infections. Lung pDCs have been shown to protect against allergic sensitization to inhalant allergens in a mouse model of asthma (de Heer et al., 2004). Lower pDC numbers and impaired pDC function contribute to persistent asthma in patients with occupational allergies and predisposition to allergic sensitization in an IFNα dependent manner (Froidure, Vandenplas, D'Alpaos, Evrard, & Pilette, 2015; Wu et al., 2020).

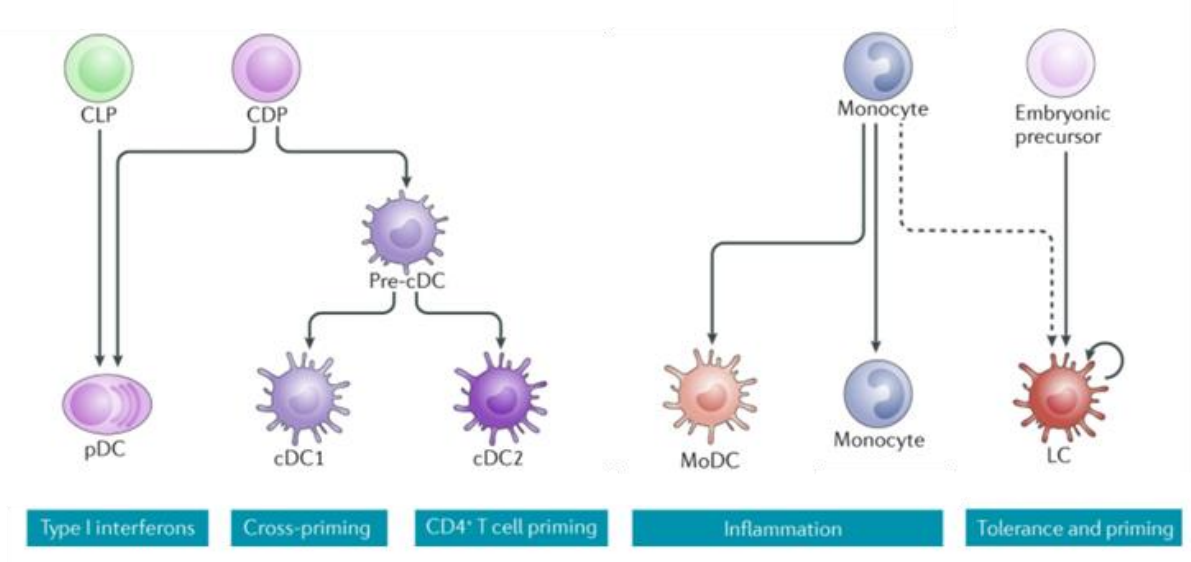

Figure 5 Dendritic cell subsets and their functions. Plasmacytoid dendritic cells (pDCs) are derived from common lymphoid progenitors, although some of them also share a common dendritic cell precursor (CDP) with conventional DCs (cDCs). pDCs are poor antigen-presenting cells (APCs) for naïve T cells, but produce type-I interferons. Impaired pDC function has been also been linked to asthma. cDCs are APCs and their main function is to prime naïve T cells. While cDC1 are able to cross-present extracellular antigens to $CD8^+$ T cells, cDC2 are responsible for $CD4^+$ T cell priming and play an important role in the development of allergies. Monocyte-derived DCs (moDCs) comprise a heterogeneous group of cells. Under inflammatory conditions they migrate to tissues, where they present antigens to effector T cells, clear pathogens and secrete cytokines. Langerhans cells (LC) stem from embryonic precursors, but can also be generated from monocytes under inflammatory conditions. LCs are able to self-renew in tissues and perform similar functions as cDCs. Adapted from Eisenbarth, 2019.

cDC1 and cDC2 are two distinct myeloid DC subsets and while both express CD13 and CD33, cDC1 are $CD141^+$ and $CLEC9^+$ (Collin & Bigley, 2018). cDC1 express TLR3, TLR9 and TLR10 and

are able to cross-present extracellular antigens to $CD8^+$ T cells via MHC-I (del Rio, Rodriguez-Barbosa, Kremmer, & Forster, 2007; Gutierrez-Martinez et al., 2015; Wohn et al., 2020). They play a major role during infections with intracellular pathogens. On the other hand, cDC2 can be identified by their CD1c, CD11b, and CD11c expression (Collin & Bigley, 2018). Tissue resident cDC2 also express low levels of CD1a and Langerin (De Monte et al., 2016; Melum et al., 2014). Similar to monocytes, cDC2 express a variety of PRRs (CLRs, TLRs, NLRs and RLRs) (Villani et al., 2017). Lung and dermal cDC2 subsets are the main contributors to allergen induced Th2 differentiation, which is dependent on transcription factors interferon-regulatory factor 4 (IRF4) and Krüppel-like factor-4 (KLF4) (Gao et al., 2013; Tussiwand et al., 2015). Migration of these Th2 inducing DC subsets to the dLN is also tightly regulated by a two-factor authentication. While homing to the dLN is mediated by C-C chemokine receptor 7 (CCR7), entrance into the lymph node has been shown to depend on CCL8-expressing resident macrophages in a papain sensitized mouse model (Sokol, Camire, Jones, & Luster, 2018). Subsequent positioning along the border between the T cell zone and B cell follicles is facilitated by CXCR5-CXCL13 signaling (León et al., 2012). Allergen challenge of AR patients leads to an increase in cDC2 in the upper airways, where they modulate Th2 responses in the tissue (Melum et al., 2014).

Langerhans cells were first discovered in 1868 by Paul Langerhans and are the forefathers of dendritic cell biology. Similar to cDC2, they express CD1a and Langerin, although at higher levels, while also expressing low levels of CD11b, CD11c and CD13 (Collin & Bigley, 2018). As residents of the epidermis, they are pivotal actors in skin inflammation in atopic dermatitis and psoriasis whereas their role in allergic sensitization is still under debate and insufficiently understood (Deckers, Hammad, & Hoste, 2018).

During inflammation, monocytes have been shown to differentiate into DCs (Leon, Lopez-Bravo, & Ardavin, 2005). This subset is sometimes hard to distinguish from cDC2 or macrophages and has been identified in various studies to contribute to allergic sensitization and allergic diseases such as atopic eczema, allergic rhinitis and psoriasis (Collin & Bigley, 2018; Eguiluz-Gracia et al., 2016; Jenner et al., 2014; Wollenberg, Kraft, Hanau, & Bieber, 1996; Wollenberg et al., 2002; Zaba et al., 2009). The abundance of monocytes in peripheral blood makes artificially generated monocyte-derived DCs perfect candidates for *in vitro* studies of infection and allergic diseases. They are distinctly different in healthy and asthmatic patients

(van den Heuvel, Vanhee, Postmus, Hoefsmit, & Beelen, 1998). In atopic individuals they have been shown to upregulate co-stimulatory molecules (CD80, CD83, CD86, HLA-DR, Jagged-1 and OX40L) upon stimulation with the major birch (Bet v 1) and grass (Phl p 5) pollen allergens and increase proliferation of allergen-specific T cell populations as well as Th2 cytokine release (Ashjaei et al., 2015).

1.3.5. T cells – building bridges

T cells are crucial in mounting a cellular immune response during allergic sensitization and allergic immune responses. MHC-II mediated allergen presentation of DCs to naïve DC4$^+$ T helper cells leads to activation of the T cells in the lymph nodes (Roche & Furuta, 2015). This process is antigen specific and involves ligation of co-stimulatory molecules CD80/86 on DCs with CD28 on T cells and DC secreted polarizing cytokines (Jutel & Akdis, 2011).

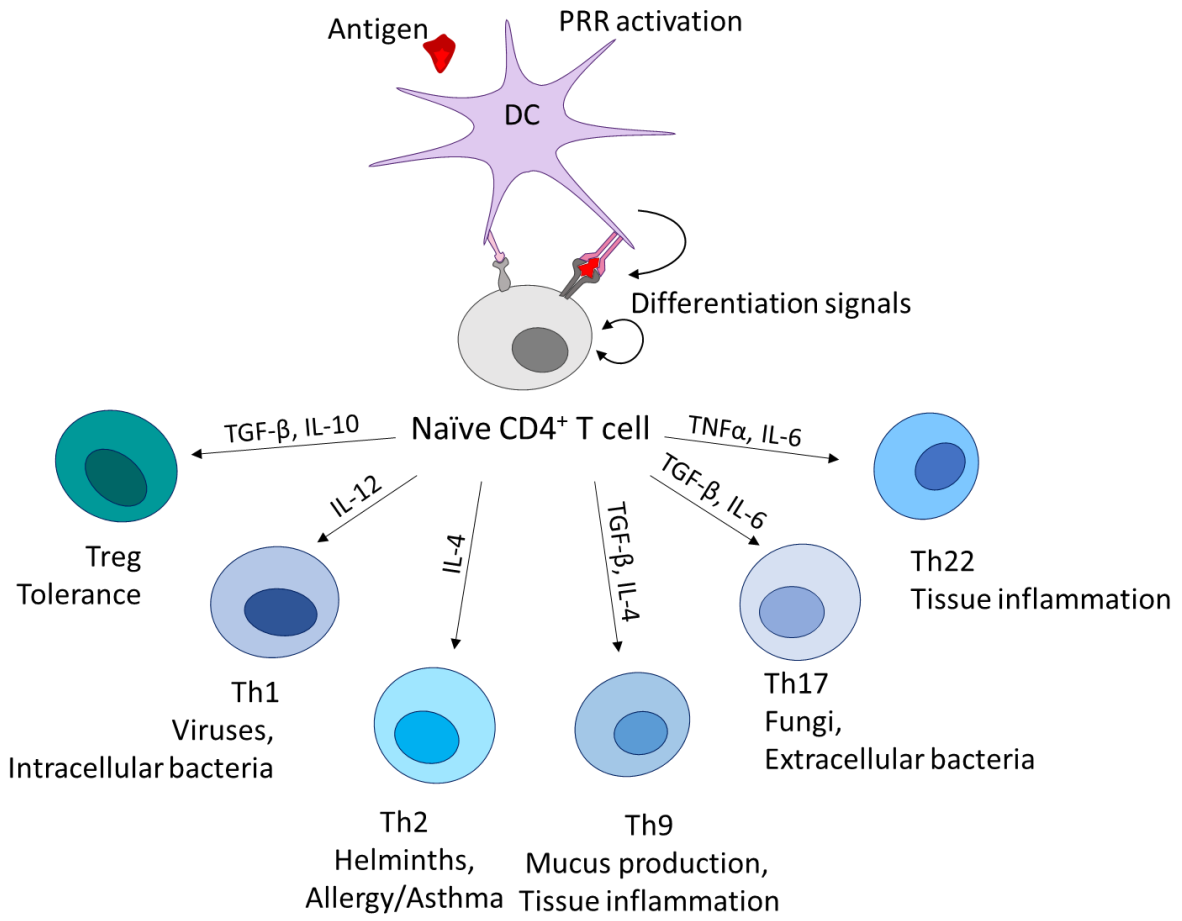

Figure 6 Differentiation of naive CD4 T cells. Antigens are taken up and processed by DCs before they are presented to naïve CD4$^+$ T cells. Depending on stimulatory signals by DCs, CD4$^+$ T cells secrete cytokines and differentiate into the respective T-helper (Th) subtypes. These subpopulations have specific effector functions, leading to different types of inflammatory responses. IL: interleukin, PRR: pathogen recognition receptor, TGF-β: transforming growth factor β, TNFα: tumor necrosis factor α, Treg: regulator T cell. Adapted from Jutel & Akdis, 2011.

During allergic sensitization, naïve T cells are activated to secrete Th2 cytokines IL-4, IL-13 and IL-5 via STAT6 mediated GATA3 activation (Amsen et al., 2007; Zheng & Flavell, 1997). GATA3 is also induced by IL-4 stimulation independently of STAT6, leading to a sustained Th2 response (Ouyang et al., 2000; Seki et al., 2004). Although IL-4 is a major driver of Th2 polarization, the origin of "primary" IL-4 has long been a paradox in Th2 immunity and puzzled scientists. Nowadays, potential candidates to provide IL-4 during Th2 polarization include basophils (Min et al., 2004), natural killer T cells (NKT) (Yoshimoto, 2018), mast cells (Ierna, Scales, Saunders, & Lawrence, 2008), follicular T helper cells (Tfh) (King & Mohrs, 2009), ILC2s and even naïve $CD4^+$ T cells (Yagi et al., 2002). Recently, IL-4 has also been detected in extracellular vesicles of DCs (Schierer et al., 2018). Increased IL-4 secretion of Th2 cells leads to isotype switching in B cells, which will then secrete allergen specific IgE. Tfh cells also play a role in this process by providing IL-4 and IL-21 to B cells to induce IgE secretion, as shown in a mouse model of allergic sensitization to *Alternaria* (Kobayashi, Iijima, Dent, & Kita, 2017).

Tregs are an important T cell subset and downregulate immune responses. Allergen-specific Tregs are found in higher frequencies in non-atopic individuals, while allergen-specific Th2 cells are the predominant $CD4^+$ T helper cell subset in atopic patients after challenge with their relevant allergen (M. Akdis et al., 2004). Tregs are committed to specific tolerance induction. Upon allergen recognition in non-atopics, their immunosuppressive capacities range from inhibition of IL-4 secretion by ILC2 (Rigas et al., 2017) and mast cell degranulation (Gri et al., 2008) in the innate immune response, to inhibition of IgE secretion in B cells (Meiler, Klunker, Zimmermann, Akdis, & Akdis, 2008), blocking of Th2 polarization of naïve $CD4^+$ T cells (Girtsman, Jaffar, Ferrini, Shaw, & Roberts, 2010; Wing et al., 2008) and restraining T effector cells (Noval Rivas & Chatila, 2016; Ring, Schäfer, Mahnke, Lehr, & Enk, 2006). Functional exhaustion of Tregs has been linked to strong inflammatory and increased Th2 responses, possibly contributing to allergic immune reactions (K. Yang et al., 2017).

1.3.6. B cells – The snipers of adaptive immunity

B cell maturation is a tightly regulated process. Upon encounter of specific antigens, circulatory naïve B cells migrate to primary follicles in LNs or the spleen via CXC chemokine receptor 5 (CXCR5) and CXCL13 interaction (Rasheed, Rahn, Sallusto, Lipp, & Müller, 2006), where they interact with T helper cells and form germinal centers (Crotty, 2015). Co-stimulation by CD40 and Tfh-expressed CD40L initiates B cell proliferation (Armitage et al.,

1992; Noelle et al., 1992). Somatic hypermutation to increase antigen specificity of the B cell receptor (BCR) is induced by Tfh derived IL-4 and IL-21 (Zotos et al., 2010), after which B cells interact with follicular DCs and Tfh (Crotty, 2015). High antigen affinity B cells are selected upon which isotype switching from IgM to IgE is induced by Tfh derived IL-4 and IL-21 (McGuire et al., 2015). Then, B cells further differentiate into long-lived Ig secreting plasma cells or memory cells (Basso & Dalla-Favera, 2015; Crotty, 2015; Mesin, Ersching, & Victora, 2016).

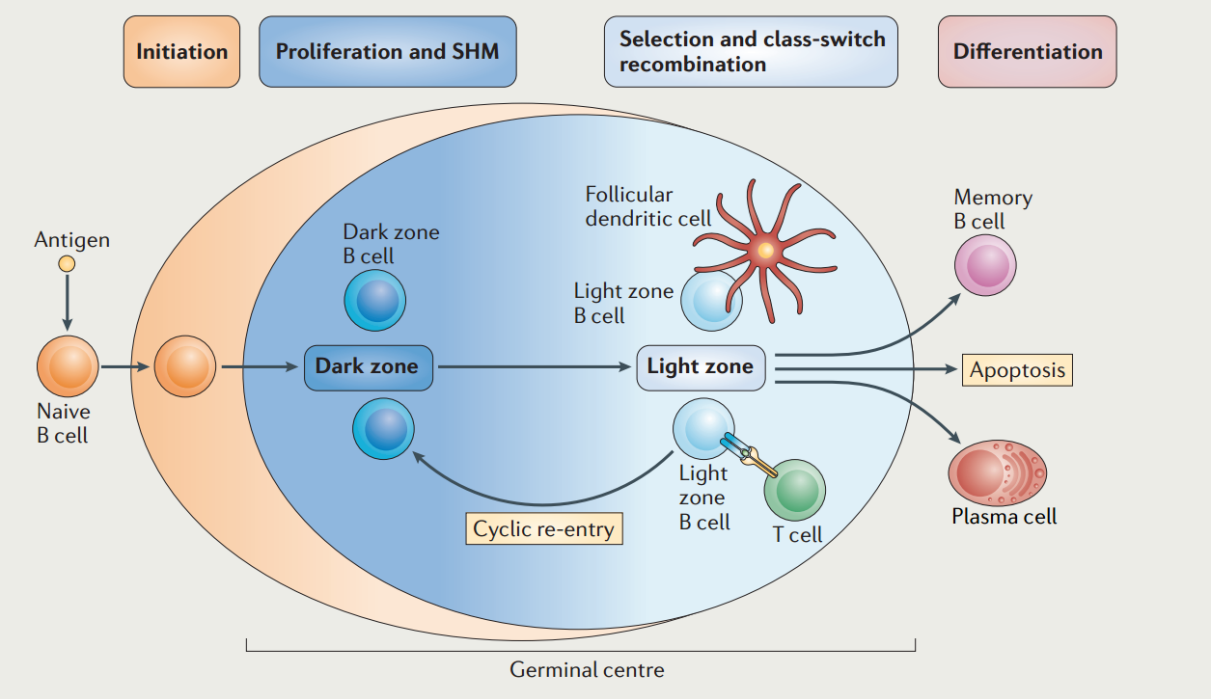

Figure 7 The Germinal center is a structure where B cells undergo immunoglobulin somatic hypermutation (SHM) and class-switch recombination upon antigen encounter. In the dark zone, B cells proliferate and then migrate to the light zone, where they interact with follicular dendritic cells and follicular T helper cells. B cells expressing high affinity-antibodies are selected and class-switch recombination is initiated. Then the B cells start to differentiate into plasma cells or memory B cells (Basso & Dalla-Favera, 2015).

Immunoglobulins in allergic diseases

B cells have the potential to secrete a variety of antigen specific immunoglobulins: IgM, IgA, IgD, IgG and IgE. In naïve B cells, IgM is expressed as membrane bound B cell receptor. There are four types of IgG antibodies: IgG_1, IgG_2, IgG_3 and IgG_4. Allergen exposure leads to isotype switching from IgM to IgE in the nasal mucosa of atopic patients and IgG_4 in non-atopic individuals (Takhar et al., 2005). IgG_4 induction is mediated by IL-10 secreting regulatory B cells (C. A. Akdis, Blesken, Akdis, Wuthrich, & Blaser, 1998). In this line, compelling evidence suggests that IgG memory cells act as precursors for IgE secreting B cells in aeroallergen sensitized children (Aalberse et al., 2018; Jenmalm & Bjorksten, 1999). Increased allergen specific IgG_4 has also been observed in patients after successful allergen immunotherapy,

further reinforcing the importance of the IgG-IgE balance in immune tolerance vs allergy (James & Till, 2016; Stylianou et al., 2016). Furthermore, pollen exposure affects the Ig profile of atopic and non-atopic individuals in nasal secretions and serum (Gökkaya et al., 2020). While IgA was generally higher in atopic individuals overall, Bet v 1 specific nasal IgA tended to be increased in healthy volunteers during the birch pollen season. Simultaneously, specific IgG_4 and IgE were increased in atopic patients and were negatively correlated in the non-atopic cohort outside the pollen season, further broadening the understanding of differential humoral immune responses to pollen in atopic and non-atopic individuals and the resulting clinical outcomes (Gökkaya et al., 2020). IgE is the hallmark immunoglobulin (Ig) of helminth infections and allergic diseases. Mast cells expressing FcεI receptor bind IgE, and cross-linking of receptor-bound IgE by allergens induces mast cell degranulation, effectively inducing allergic symptoms from mild reactions in the nasal mucosa to severe anaphylaxis (C. A. Akdis et al., 2020).

1.4. Characteristics of allergens

Aeroallergens such as pollen, house dust mite (HDM) and fungal spores are causative agents of respiratory allergic diseases. While the immune system reacts to the allergen in sensitized patients, it is often not fully understood how these otherwise innocuous proteins induce allergic sensitization. The question *"what makes an allergen"* has been studied by numerous research groups all over the world and yet the mystery remains. Characteristics of allergens include proteolytic activity, stability towards degradation, ligand binding and mimicking ligands for pattern recognition receptors (PRR) (Scheurer, Toda, & Vieths, 2015).

Cysteine and serine proteases are common allergens of HDM, pollen and fungi and have been shown to activate the innate immune system (Matsumura, 2012). Epithelial barrier permeability is increased by disrupting tight junction complexes involving occludin, claudin-1, E-cadherin and ZO-1, leading to higher probability of allergen-uptake by DCs in the respiratory epithelium (Gaspar et al., 2020; Tai et al., 2006; Vinhas et al., 2011). Proteases acting on protease activated receptor (PAR) 2 also destabilize E-cadherin (Cho et al., 2010; Heijink, van Oosterhout, & Kapus, 2010) and induce secretion of pro-inflammatory cytokines IL-6 and IL-8 and subsequent neutrophil recruitment (Gaspar et al., 2020; Kauffman, Tomee, van de Riet, Timmerman, & Borger, 2000; Shin, Lee, & Jeon, 2006). Furthermore, protease activity cleaves DC-SIGN and directly impairs Th1 polarizing capacity of DCs (Furmonaviciene et al., 2007).

The grass pollen allergen Phl p 1, although belonging to the family of cysteine proteases, contributes to airway inflammation independent of protease activity (Röschmann et al., 2009). An alternative route for allergens to pass through the epithelial barrier to the subepithelium has been suggested for grass pollen allergen Phl p 1 via transcytosis in non-acidic vesicles in airway epithelial cells (Blume et al., 2009). Post-translational glycosylation of Phl p 1 may enhance c-type lectin receptor (CLR)-dependent allergen uptake by keratinocytes, where the allergen is processed in lysosomes, underlining the importance of the route of allergen exposure via skin or respiratory epithelia (Blume et al., 2009). In oral Langerhans cells, Phl p 5 is also suspected to bind CLRs and subsequently enhance their migration (Allam et al., 2010).

Amb a 1, the major allergen of ragweed, belongs to the pectate lyase protein family. Allergenicity is conferred by three major epitopes that are recognized by T cells due to promiscuous MHC-II binding (Jahn-Schmid et al., 2010). Additionally, susceptibility towards endolysosomal degradation is associated with ragweed allergenicity (Wolf et al., 2018).

Structural analyses led to the discovery of ligand binding properties of allergens such as the HDM allergens or birch Bet v 1. Der p 2, a HDM allergen, has been found to be structurally homologous to MD-2, a lipopolysaccharide (LPS) binding protein interacting with toll-like receptor (TLR) 4. In the absence of MD-2, Der p 2 induced allergic airway sensitization in an allergic asthma mouse model in a TLR4 dependent manner (Trompette et al., 2009). Likewise, Der p 5 and Der p 13, minor HDM allergens, are presumed to have lipid-binding capacities and were able to induce TLR2 dependent IL-8 and GM-CSF secretion in airway epithelial cells (Pulsawat et al., 2019; Satitsuksanoa et al., 2016).

Bet v 1, a pathogen-related 10 (PR-10) protein, contains hydrophobic cavities opening up a range of possible ligands, mainly flavonoids, fatty acids and phytohormones (Aglas et al., 2020). Ligand binding to Bet v 1 protects against proteolytic processing by APCs and affects MHC-II peptide presentation to Th cells, thus favoring Th2 polarization (Freier, Dall, & Brandstetter, 2015; Machado et al., 2016; Soh et al., 2019). Although TLR4 activation by a complex of Bet v 1 and LPS or lipoteichoic acid (LTA) has been discussed (Bublin, Eiwegger, & Breiteneder, 2014), Soh *et al.* (2019) detected high dissociation constants for these ligands, suggesting Bet v 1 sensitization is not directly mediated by TLR interactions (Aglas et al., 2020).

1.5. Matrix matters

Interestingly, even though some inhalant allergens elicit pro-inflammatory responses, many pollen allergens are unable to induce sufficient Th2 polarization via DCs. For example, the major grass pollen allergen Phl p 5 enhances migratory capacities of oral LCs, but inhibits maturation upon allergen uptake and induces IL-10 secretion, a useful characteristic for sublingual immunotherapy (Allam et al., 2010). Bet v 1 seems similarly inert and, while upregulating DC maturation markers, fails to induce IL-4 and IL-13 expression in induced moDCs as well as T cell proliferation and Th2 polarization of non-atopic donors (Smole et al., 2015; Smole et al., 2010). In the absence of extrinsic adjuvants like alum, recombinant or isolated highly purified native pollen allergens (e.g. Amb a 1, Bet v 1) also do not readily induce sensitization in mice (Wimmer et al., 2020; L. Aglas, personal communication, December 17, 2018).

1.5.1. The Pollen Matrix

In recent years, research focused on investigating intrinsic adjuvant factors contributing to pollen allergenicity. Pollen are more than just allergen carriers, they also release thousands of low molecular weight compounds, as well as proteases (as discussed above) and NADPH oxidases into the airways.

Pollen contain eicosanoid-like lipids homologous to mammalian prostaglandin E_2 (PGE_2) or leukotriene B_4 (LTB_4), which induce inflammatory immune responses (Behrendt et al., 2001). E_1 phytoprostanes (PPE_1) are one class of such lipids and have been detected in aqueous pollen extracts, contributing to DC mediated Th1/Th2 skewing by inhibiting LPS-induced IL-12 secretion, a process regulated by peroxisome proliferator-activated receptor gamma (PPAR-γ) and NFκB (Gilles, Mariani, Bryce, Mueller, Ring, Jakob, et al., 2009; Traidl-Hoffmann et al., 2005). IgE production of memory BCs *in vitro* was also enhanced upon stimulation with PPE_1 compared to Amb a 1 alone (Oeder et al., 2015). Although PPE_1 seemed a promising candidate for the immunomodulatory effect of pollen extracts, follow-up studies have shown that migration and Th2 licensing potential of birch pollen extract stimulated DCs is independent of PPE_1 (Mariani et al., 2007), as is DC maturation (Gilles et al., 2010). Other contributors to allergic sensitization are PALMs of the linoleic acid pathway, which act as chemoattractants for granulocytes similar to mammalian leukotrienes (Traidl-Hoffmann et al., 2002). Cutaneous

reactions and nasal allergic inflammation to pollen allergens is also enhanced by low molecular weight compounds and lipid mediators (Gilles-Stein et al., 2016).

Furthermore, adenosine (ADO), commonly known for its role in immune regulation and suppression, was detected in pollen of ragweed, birch and timothy grass (Gilles et al., 2011). Adenosine in pollen extracts is a double-edged sword, since it appears to be protective for non-atopics by inducing Treg responses while failing to induce the same reaction in atopic individuals (Gilles et al., 2011). It also contributed to enhanced barrier function in bronchial epithelial cells similar to grass pollen extract (Blume, Swindle, Gilles, Traidl-Hoffmann, & Davies, 2015). *In vivo*, ADO acts protective during early intranasal sensitization to pollen, but is a necessary co-factor in inducing allergic inflammation in already sensitized mice (Wimmer et al., 2015).

NADPH oxidases have been detected in pollen of a variety of allergenic plants including ragweed, grasses and birch (Boldogh et al., 2005; X. L. Wang et al., 2009). Reactive oxygen species (ROS) generated by pollen-derived NADPH oxidases augment airway inflammation and lead to enhanced recruitment of inflammatory cells (Bacsi, Dharajiya, Choudhury, Sur, & Boldogh, 2005; Boldogh et al., 2005). Ragweed-derived NADPH oxidases have been shown to elevate intracellular ROS levels and subsequently trigger IL-1β production via the NLRP3 inflammasome in monocyte-derived macrophages (Varga et al., 2013).

Taken together, these findings highlight the complexity of pollen allergenicity.

1.5.2. Environmental factors and allergenicity

Throughout the year, plants are exposed to environmental influences of biotic and abiotic origins to which they need to respond accordingly. Therefore, it stands to reason that these environmental factors also influence the allergenic potential of pollen. Especially air pollution has been discovered to affect pollen allergenicity in many ways.

Allergen release has been shown to be increased by structural instability of pollen grains due to interactions with pollutants (Sedghy, Varasteh, Sankian, & Moghadam, 2018; Shahali, Pourpak, Moin, Mari, & Majd, 2009). Exposure to gaseous pollutants (NO_2, SO_2, NH_3) and vehicle exhaust particles as found in urban areas increased the allergen content of *Platanus orientalis* pollen (Lu et al., 2014) and increased allergenicity has been observed for DEP exposed timothy grass, cypresses (Cortegano et al., 2004; Suarez-Cervera et al., 2008) and

ragweed (Ghiani, Aina, Asero, Bellotto, & Citterio, 2012). Allergens belonging to the PR family often seem to be affected and are expressed as part of the plants defense to air pollution or soil contamination. In the case of Cup a 1, a PR-5 protein in *Cupressus arizonica*, traffic related pollution even appears to induce expression of this allergen compared to non-polluted areas (Cortegano et al., 2004; Suarez-Cervera et al., 2008). Nitration of Bet v 1 caused by exposure to NO_2 and O_3 in polluted urban areas changed the allergen conformation, leading to enhanced binding of specific IgE of birch allergic patients and Th1/Th2 skewing of DCs (Ackaert et al., 2014; Gruijthuijsen et al., 2006). Higher levels of O_3 have also been positively correlated to increased Phl p 5 and grass pollen levels (Ščevková, Vašková, Sepšiová, Dušička, & Kováč, 2020).

Elevated levels of allergens have also been observed in pollen from ragweed plants from urban areas (Ghiani et al., 2012), and *in vitro* exposure of ragweed plants to NO_2 or CO_2 combined with draught stress have led to an increase in allergen content (El Kelish et al., 2014; Zhao et al., 2017).

Not only is the allergen content influenced by the environment, but also the total protein and metabolite content of pollen has been reported to be changed. For example, high O_3 concentrations affected the lipid content in birch pollen, leading to higher chemotactic activity of neutrophils *in vitro*, as well as increased cutaneous immune responses (Beck et al., 2013). Exposure to high O_3-levels during the vegetation period has also been observed to change the lipid and wax composition of the pollen wall of ragweed pollen, possibly altering the allergen availability (Kanter et al., 2013).

1.5.3. Pollen microbiome

Just like animals, plants are exposed to and colonized by microbes. Bacterial and fungal communities have been detected on pollen from both wind and insect pollinating plants, which were found to be species-specific, but also pollination-specific (Manirajan et al., 2018; Obersteiner et al., 2016). Obersteiner et al. (2016) also found differences according to sample site. The bacterial diversity was lower in birch trees from urban collection sites with high NO_2 levels, indicating that abiotic stress influences bacterial diversity (Obersteiner et al., 2016). On the parameters of pollen allergenicity, the same study also reported that Bet v 1 content was positively correlated to higher bacterial richness, while low abundance species negatively correlated to $PALM_{PGE2}$ and $PALM_{LTB4}$ content. These findings are of particular interest,

because bacterial products, like LPS or flagellin, have been shown to induce ROS generation as part of the plants immune defense, which in turn upregulates PR-family proteins, some of them known allergens (Khodai-Kalaki, Andrade, Fathy Mohamed, & Valvano, 2015). Other than possibly increasing allergen content in the pollen themselves, bacteria isolated from birch pollen also provide pollen-independent protease activity, which potentially contributes further to allergic sensitization (McKenna et al., 2017). In addition, microbial products from pollen might serve as "adjuvant" factors during allergic sensitization to allergenic proteins by providing pathogen-associated molecular patterns (PAMPs). Without these adjuvants, which stimulate innate and adaptive immune responses, sensitization to many "allergenic" proteins would likely not occur (Eisenbarth et al., 2002; Hammad et al., 2009; Heydenreich et al., 2012; Ito et al., 2017; Oteros et al., 2019).

1.6. Hypothesis

The rising incidence and prevalence of allergic diseases in the past decades is a global health concern. At least one third of the global population suffers from allergies, which poses a tremendous socio-economic burden. Climate change will likely favor the spread of allergenic plants across the globe. Rising temperatures will change the course of the pollen seasons, causing overall prolonged pollen seasons and suffering for allergic patients.

The process of allergic sensitization is complex and influenced by an intricate interplay of genetic and environmental factors in both the patient and the allergenic plant. As plants are exposed to biotic and abiotic stressors, this leads to a change in the metabolic composition of allergenic pollen, which could, in turn, influence their allergenic potential.

The aim of this work was to elucidate the influence of increasing CO_2 levels, a driving force of climate change, on the allergenic potential of ragweed pollen. Special focus was on the metabolic changes induced by exposure to high levels of CO_2 during plant growth and how this influences allergic sensitization *in vitro*. Furthermore, as global temperatures rise, so will surface levels of O_3. It is possible that, similar to birch pollen, ragweed pollen allergenicity will be changed by prolonged exposure to elevated O_3 levels. For this purpose, a set of ragweed plants were grown under elevated CO_2 or O_3 levels and the pollen were analyzed for their allergenic potential *in vitro*.

Birch pollen are a major allergen source during spring in central and northern Europe. High degrees of urbanization and urban air pollution are abiotic stressors for plants and also affect the allergenicity of pollen. To analyze the effects of urbanization on birch pollen, pollen from trees in an urban area in Augsburg, Bavaria, were collected during the pollen season in 2015 and 2017, and compared to pollen from trees in rural areas in the greater Augsburg region. The molecular composition of pollen (major allergens, PALMs, LPS) from the different sampling sites and how the pollen act on human immune cells *in vitro*, were of special interest. This could provide novel insights into the potential of pollen to cause allergic sensitization and inflammation.

A healthy microbiome provides protection against allergic diseases, but is constantly subjected to environmental influences. How pollen exposure shapes and affects the nasal microbiome of healthy or allergic persons, and the implications thereof, is insufficiently

studied. Therefore the microbiome of eight AR patients and eight healthy volunteers was analyzed over the course of one year in 2016 and alterations during the pollen season were identified. Additionally, studying the pollen microbiome of birch trees will give insight into changes introduced to the nasal microbiome associated with pollen exposure.

Lastly, fungal spore and pollen co-exposure was assessed during the grass pollen season in 2018. Little is known on how pollen and outdoor fungal exposure directly influence the nasal mycobiome and how this affects nasal immune responses. Therefore, the co-exposure situation was mimicked *in vitro* and changes to the nasal mycobiome *in vivo* were assessed by next generation sequencing.

Taken together, the findings aim to improve our understanding of the interactions of environmental influences, such as climate change, anthropogenic air pollution or microbial exposure, on pollen allergenicity, as well as host-factors, such as the microbiome and mycobiome.

Chapter 2 – Material and Methods

2.1. Material

2.1.1. Instruments

Table 1. Instruments

Instrument	Manufacturer
Absorbance Microplate reader Sunrise™	Tecan, Männedorf, Switzerland
Analytical Balance New Classic MS	Mettler-Toledo GmbH, Gießen, Germany
AutoMACS Pro Separator	Miltenyi Biotech, Bergisch Gladbach; Germany
Big Squid, magnetic stirrer	IKA®-Werke GmbH & Co. KG, Staufen, Germany
BioDrop	BioDrop UK Ltd, Cambridge, UK
Bio-Plex Pro™ Wash Station	Bio-Rad Laboratories, Hercules, CA, USA
Bio-Plex® 200 System	Bio-Rad Laboratories, Hercules, CA, USA
Centrifuge 5810 R / 5418 R	Eppendorf, Hamburg, Germany
CO_2 Incubator CB160	Binder, Tuttlingen, Germany
CytoFlex LX	Beckmann Coulter GmbH, Krefeld, Germany
Duomax 1030 (10° tilt angle), shaker	Heidolph Instruments GmbH & CO. KG, Schwabach, Germany
Falcon® Pipet controller	Corning GmbH, Wiesbaden, Germany
GEL iX20 Imager Windows Version	INTAS Science Imaging Instruments GmbH, Göttingen, Germany
Microplate Washer 405 TS	BioTek Germany, Bad Friedrichshall, Germany
Microscope DMi-1	Leica, Wetzlar, Germany
Microwave	Severin, Wessling-Berzdord, Germany
Mini Sub-Cell GT Gel Caster	Bio-Rad Laboratories, Hercules, CA, USA
Mini-Sub Cell GT UV-Transparent Gel Tray, 7 x 10 cm	Bio-Rad Laboratories, Hercules, CA, USA
Mini-Sub® Cell GT Cell	Bio-Rad Laboratories, Hercules, CA, USA
Minitron incubation shaker	Infors HT, Bottmingen, Switzerland
New Classic MS Analytical Balance	Mettler-Toledo GmbH, Gießen, Germany
PCR Thermocycler Biometra TADvanced	Analytik-Jena AG, Jena, Germany
PowerPac™ Basic Power Supply	Bio-Rad Laboratories, Hercules, CA, USA
Precellys Evolution Homogenizer	Bertin Instruments, Montigny-le-Bretonneux, France
Quintix® Analytical Balance 60 | 120 g x 0.01 | 0.1 mg	Sartorius AG, Göttingen, Germany
Quintix® Precision Balance 3,100 g x 10 mg	Sartorius AG, Göttingen, Germany
Safety workbench Eco safe comfort plus	ENVAIR Deutschland GmbH, Emmendingen, Germany
Safety workbench HERASafe KS, 120 cm	Thermo Fisher Scientific
SevenCompact pH meter S210	Mettler-Toledo GmbH, Gießen, Germany

Sub-Cell GT Cell	Bio-Rad Laboratories, Hercules, CA, USA
Sub-Cell GT UV-Transparent Gel Tray, 15 x 10 cm	Bio-Rad Laboratories, Hercules, CA, USA
Thermo Mixer C	Eppendorf, Hamburg, Germany
Titramax 101 shaker	Heidolph Instruments GmbH & CO. KG, Schwabach, Germany
V-32 Multi-vortex	BioSan, Riga, Latvia
Waterbath SW22	Julabo, Hamburg, Germany
ZX3 Advanced Vortex Mixer	VELP Scientifica, Seelbach, Germany
Integra Vacusafe Vacuum pump	QIAGEN, Usmate, Italy
LGex 3410 -20°C freezer	Liebeherr MEDline, Feldkirchen, Germany
Systec DC-65 (autoclave)	Systec, Wessling-Berzdord, Germany
Ultra-freezers series UF V -80°C freezer	Binder GmbH, Bulle, Switzerland

2.1.2. Consumables

Table 2. Consumables

Consumables	**Manufacturer**
24-well Transwell® plates with 0.4 µm Pore Polyester Membrane Insert	Falcon®, Corning Inc., Tewksbury, MA, USA
48-well plates, cell culture treated, flat bottom	Falcon®, Corning Inc., Tewksbury, MA, USA
50 ml Spritzen Luer Lock	B. Braun Melsungen AG, Melsungen, Germany
Amicon® Ultra 15 ml Centrifugal Filters, 3 kDa	Merck KGaA, Darmstadt, Germany
Cell culture flasks (T25, T75, T175)	Greiner bio-one, Frickenhausen, Germany
ChemoTx® Disposable Chemotaxis System, 3.2mm dia. Sites, 5 µm pore size, 30 µL 96-well plate	Neuro Probe, Inc., Gaithersburg, MD, USA
Conical centrifuge tubes (15 ml, 50 ml)	Falcon®, Corning Inc., Tewksbury, MA, USA
Easy strainer (70 µm)	Greiner bio-one, Frickenhausen, Germany
F96 Cert. Maxisorp NUNC-Immuno Plate	Thermo Fisher
Inocculation loop sterile, 10 µl	Carl Roth, Karlsruhe, Germany
Micro tubes (1.5 ml, 2.0 ml)	Sarstedt Eppendorf, Newton, USA
Millex-GP Syringe Filter Unit, 0.22 µm, polyethersulfone, 33 mm, gamma sterilized	Merck KGaA, Darmstadt, Germany
Non-tissue culture plate 96-well flat/U-bottom	Falcon®, Corning Inc., Tewksbury, MA, USA
Omnifix Spritze steril (1 ml, 5 ml, 10 ml, 20 ml)	B. Braun Melsungen AG, Melsungen, Germany
Optifit pipette tips (10 µl, 120 µl, 200 µl, 350 µl, 1000 µl)	Sartorius, Göttingen, Germany
Optifit safety SpaceTM Filter tips	Sartorius Göttingen, Germany
PCR tubes	Eppendorf, Hamburg, Germany

RHINIXPRO™ NASAL FILTER	Indoor Biotechnologies, Inc., Charlottesville, VA, USA
Serological pipettes (1 ml, 5 ml, 10 ml, 25 ml, 50 ml)	Greiner bio-one, Frickenhausen, Germany
Sterile filter device (250 ml, 500 ml)	Sarstedt AG & Co. KG, Nümbrecht, Germany
Syringe filter unit (0.22 µm)	Merck Millipore, Darmstadt, Germany
Screw Cap Micro Tubes	Sarstedt AG & Co. KG, Nümbrecht, Germany

2.1.3. Kits and Reagents

Table 3. Reagents

Reagent	Manufacturer
Acetic Acid 1M	Merck, Darmstadt, Germany
0.25 % Trypsin-EDTA (1x), Phenolrot	Gibco™, Thermo Fisher Scientific, Darmstadt, Germany
0.05 % Trypsin-EDTA (1x), Phenolrot	Gibco™, Thermo Fisher Scientific, Darmstadt, Germany
1x TMB substrate solution	Invitrogen™, Thermo Fisher Scientific, Darmstadt, Germany
2-Mercaptoethanol	Gibco™, Thermo Fisher Scientific, Darmstadt, Germany
9-OxoOTrE	CaymanChemical, Ann Arbor, MI, USA
2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS™ Roche Molecular Systems)	Merck KGaA, Darmstadt, Germany
peqGOLD Universal Agarose	PEQLAB Biotechnologie GmbH, Erlangen, Germany
Airway Epithelial Cell Basal Medium (+ Supplement Pack)	PromoCell, Heidelberg, Germany
Ampuwa (Aqua ad injectibilia)	Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany
AutoMACS running/wash buffer	Miltenyi Biotech, Bergisch Gladbach; Germany
Bovine Serum Albumin (BSA)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Catalposide	Merck KGaA, Darmstadt, Germany
CytoFlex Daily IR QC Fluorospheres	Beckman Coulter GmbH, Krefeld, Germany
CytoFlex Daily QC Fluorospheres	Beckman Coulter GmbH, Krefeld, Germany
CytoFlex Sheath Fluid	Beckman Coulter GmbH, Krefeld, Germany
Dulbecco's Modified Eagle Medium (DMEM), high glucose	Gibco™, Thermo Fisher Scientific, Darmstadt, Germany
Ham's F-12 GlutaMax™ Nutrient Mixture	Gibco™, Thermo Fisher Scientific, Darmstadt, Germany
DMSO cell culture grade	Applichem, Darmstadt, Germany
DMSO for PCR	New England Biolabs, Frankfurt, Germany
Deoxynucleotide (dNTP) Solution Mix	New England Biolabs, Frankfurt, Germany

Dulbecco's Phosphate Buffered Saline, D-PBS	Gibco™, Thermo Fisher Scientific, Darmstadt, Germany
EDTA (0.05 %, pH 8.0)	Gibco™, Thermo Fisher Scientific, Darmstadt, Germany
DEPEC-Treated Water	Invitrogen™, Thermo Fisher Scientific, Darmstadt, Germany
Ethanol absolute	VWR, Bruchsal, Germany
FcR blocking reagent, human	Miltenyi Biotech, Bergisch Gladbach; Germany
Fetal Calf Serum (FCS)	HyClone™, Fisher Scientific, Darmstadt, Germany
Fetal Bovine Serum (FCS)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
FlowClean Cleaning Agent	Beckman Coulter GmbH, Krefeld, Germany
Gel Loading Dye Purple (6x)	New England Biolabs, Frankfurt, Germany
Gentamycin	Gibco™, Thermo Fisher Scientific, Darmstadt, Germany
HD Green Plus DNA Stain	Intas, Göttingen, Germany
Citric Acid monohydrate	Merck Millipore, Burlington, MA, USA
HEPES buffer solution 1 M	Gibco™, Thermo Fisher Scientific, Darmstadt, Germany
Hydrogen peroxide solution, 30 %	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Heparin (5000 U/ml) stabilizer-free, lyophilized	Merck KGaA, Darmstadt, Germany
Histopaque 1055	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Histopaque 1119	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Kanamycin	Gibco™, Thermo Fisher Scientific, Darmstadt, Germany
L-Glutamine 200 mM (100x)	Gibco™, Thermo Fisher Scientific, Darmstadt, Germany
Lumichrome	(Sigma/Merck) (Schubert&Weiss)
Lymphoprep™	STEMCELL Technologies, Inc., Vancouver, BC, Canada
Malvidin chloride	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
MEM non-essential amino acids (MEM-NEAA)	Gibco™, Thermo Fisher Scientific, Darmstadt, Germany
MEM sodium pyruvate	Gibco™, Thermo Fisher Scientific, Darmstadt, Germany
Mitomycin C	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
MEM vitamin solution (100x)	Gibco, Thermo Fisher Scientific, Darmstadt, Germany
NEBNext® Ultra™ II Q5 Master Mix	New England Biolabs, Frankfurt, Germany

NEBNext® Ultra™ Mastermix II	New England Biolabs, Frankfurt, Germany
p-Coumaryl alcohol 4-O-glucoside	Merck KGaA, Darmstadt, Germany
Pelargonidin chloride	Merck KGaA, Darmstadt, Germany
Penicillin-Streptomycin (10.000 U/ml)	Gibco™, Thermo Fisher Scientific, Darmstadt, Germany
Potato Dextrose Agar (PDA)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
PureCol® Type I Collagen Solution, 3 mg/ml (Bovine)	Advanced Biomatrix, Inc., Carlsbad, CA, USA
Q5 Hotstart High Fidelity DNA Polymerase	New England Biolabs, Frankfurt, Germany
Quercetin-3-O-spophoroside	Kindly provided by L. Aglas, University Salzburg
RBC Buffer	
Reagent DX	QIAGEN, Hilden, Germany
Recombinant human IL-21	PeproTech, Rocky Hill, NJ, USA
Recombinant human IL-4	PeproTech, Rocky Hill, NJ, USA
Recombinant human GM-CSF	PromoCell GmbH, Heidelberg, Germany
Roswell Park Memorial Institute RPMI-1640	Gibco, Thermo Fisher Scientific, Darmstadt, Germany
Silica/zirconium beads (100 µm)	Carl Roth GmbH, Karlsruhe, Germany
Stool Stabilizer Solution	Invitek Molecular, Berlin Germany
Streptavidin-horseradish peroxidase conjugate	Cytavia, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Tween20	Merck Millipore, Burlington, MA, USA
Proteinase (10 mg/ml)	AppliChem GmbH, Darmstadt, Germany
Q5® Reaction Buffer Pack	New England Biolabs, Frankfurt, Germany
Retinoic Acid	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Triton X-100	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Trypanblue 0.4 % solution	Invitrogen™, Thermo Fisher Scientific, Darmstadt, Germany
Trizma® base, Tris	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Sodium bicarbonate ($NaHCO_3$)	Merck KGa, Darmstadt, Germany
Disodium phosphate (Na_2HPO_4)	Merck KGa, Darmstadt, Germany
Sodium chloride (NaCl)	Carl Roth GmbH, Karlsruhe, Germany
Potassium phosphate (KH_2PO_4)	Merck KGa, Darmstadt, Germany
Potassium chloride (KCl)	Merck KGa, Darmstadt, Germany
Sodium carbonate (Na_2CO_3)	Merck KGa, Darmstadt, Germany
PromoFluor 840, Reactive Dyes	PromoCell GmbH, Heidelberg, Germany

Table 4. Kits

Kit	Manufacturer
CD14 MicroBeads, human	Miltenyi Biotech, Bergisch Gladbach; Germany
QIAamp UCP Pathogen Mini Kit	QIAGEN GmbH, Hilden, Germany
B Cell Isolation Kit, mouse	Miltenyi Biotech, Bergisch Gladbach; Germany
Monarch® PCR & DNA Cleanup Kit	New England Biolabs, Frankfurt, Germany
Bio-Plex Pro™ Human IgE Isotyping Assay	Bio-Rad Laboratories, Hercules, CA, USA
Phl p 5 Custom 1-plex Multiplex Array for Indoor Allergens (MARIA) with Magnetic Beads	INDOOR Biotechnologies, Inc., Cardiff, UK
B cell isolation Kit II, human	Miltenyi Biotech, Bergisch Gladbach; Germany
Pierce™ LAL Chromogenic Endotoxin Quantitation kit	Fisher Scientific, Darmstadt, Germany
QIAamp UCP Pathogen Mini Kit (50)	QIAGEN, Hilden, Germany

Table 5. ELISA kits. Lyophilized antibodies were reconstituted and stored according to manufacturer's instructions.

ELISA		Manufacturer
Bet v 1 ELISA		ALLERGOPHARMA GmbH & Ko.KG, Reinbek, Germany
Human Adenosine ELISA kit		MyBioSource, Inc., San Diego, CA, USA
Human IL-10 ELISA Set		BDBioscience Pharmingen, San Diego, CA, USA
Human IL-1β ELISA Set II RUO		
Human IL-4 ELISA Set RUO		
Human IL-8 OptEIA ELISA		
Human MCP-1/CCL2 ELISA		
Human TNF OptEIA ELISA		
Human CCL17/TARC DuoSet		R&D Systems, Wiesbaden, Germany
Human MDC/CCL22 ELISA		
Human IL-6 ELISA	IL-6 monoclonal antibody (MQ2-13A5), *capture antibody*	eBioscience, San Diego, CA, USA
	IL-6 monoclonal antibody (MQ2-39C3, Biotin), d*etection antibody*	eBioscience, San Diego, CA, USA
	Recombinant human IL-6 (RUO) *Standard*	BDBioscience Pharmingen, San Diego, CA, USA
Human IL-12 ELISA	Purified Rat Anti-Human IL-12 p70, *capture antibody*	BDBioscience Pharmingen, San Diego, CA, USA
	Biotin anti-human IL-12/IL-23 p40 antibody, *detection antibody*	eBioscience, San Diego, CA, USA
	Recombinant Human IL-12 (p70) (RUO), *Standard*	BDBioscience Pharmingen, San Diego, CA, USA

Murine IgE ELISA	Purified rat anti-mouse IgE, (Clone R35-72), *capture antibody*	BDBioscience Pharmingen, San Diego, CA, USA
	Biotin rat anti-mouse IgE (Clone R35-118), *detection antibody*	BDBioscience Pharmingen, San Diego, CA, USA
	Purified Mouse IgE K Isotype Control, *Standard*	BDBioscience Pharmingen, San Diego, CA, USA
	Streptavidin-Peroxidase	Calbiochem®, Merck Millipore, Burlington, MA, USA
PGE_2 ELISA		Enzo Life Sciences GmbH, Lörrach, Germany
LTB_4 ELISA		Enzo Life Sciences GmbH, Lörrach, Germany

Table 6. Antibodies for flow cytometry and cell culture.

Antibody	Manufacturer
CD40 Monoclonal Antibody (5C3), PE	eBioscience, San Diego, CA, USA
Mouse Anti-Human CD80, Clone L307.4 (RUO), APC-H7	BDBioscience Pharmingen, San Diego, CA, USA
Mouse Anti-Human CD83, Clone HB15e (RUO), APC	BDBioscience Pharmingen, San Diego, CA, USA
CD86	
Mouse Anti-Human HLA-DR, Clone G46-6 (RUO), PE-CF594	BDBioscience Pharmingen, San Diego, CA, USA
CD1a monoclonal antibody (HI149), PE-Cyanine-7	eBioscience, San Diego, CA, USA
FITC Mouse Anti-Human CD14 Clone M5E2	eBioscience, San Diego, CA, USA
LEAF™ purified anti-mouse CD40 antibody	BioLegend, Inc., San Diego, CA, USA

2.1.4. Buffers and media

Table 7 Buffers

Buffer	Reagents	Concentration
Fungal lysis buffer	H_2O	1 l
	TritonX-100	1 %
	Tween 20	0.5 %
	Tris-HCL (pH 8)	10 mM
	EDTA	1 mM
MARIA Buffer, pH 7.3-7.5	PBS	1x
	Tween20	0.02%
	BSA	1%
PBS (20x) for MARIA Buffer, pH 7.4	H_2O	1 l
	NaCl	640 g
	KH_2PO_4	16 g
	Na_2HPO_4	92 g
	KCl	16 g
PBS (20x) for ELISA, pH 7.2	H_2O	1 l

	Na_2HPO_4	2.88 g
	NaCl	160 g
	KH2PO4	4 g
	KCl	4 g
Tris Buffer (10x), 500 mM, pH 7.4, murine IgE ELISA	H_2O	1 l
	Tris	60.55 g
Coating Buffer BD OptEIA ELISA, pH 9.5	H_2O	500 ml
	$NaHCO_3$	4.2 g
	Na_2CO_3	1.78 g
FACS Buffer	D-PBS	
	FCS	5 %
	EDTA	2 mM

Table 8. Cell culture media. Cell culture media were sterile filtered and stored at 4°C or -20°C.

Medium	Reagents	Concentration
huDC medium	RPMI-1640	90 %
	FCS (HyClone)	10 %
	L-Glutamine	2 mM
	Gentamycin	20 µg/ml
	2-Mercaptoethanol	50 µM
Migration medium	RPMI-1640	90 %
	FCS (HyClone)	10 %
	L-Glutamine	1 %
	MEM-NEAA	1 %
	MEM sodium pyruvate	1 %
	Penicillin-Streptomycin	1 %
HNEC/Feeder medium	Airway Epithelial Cell Basal Medium	45 %
	Ham's F-12 GlutaMax™ Nutrient Mix	45 %
	FCS (HyClone)	10 %
	Penicillin-Streptomycin	100 U/ml
	Epidermal Growth Factor	10 ng/ml
	Insulin	5 µg/ml
	Hydrocortisone	0.5 mg/ml
	Epinephrine	0.5 mg/ml
	Triiodo-L-thyronine	6.7 ng/ml
	Transferrin	10 µg/ml
	Retinoic acid	0.1 µg/ml
HNEC medium	Airway Epithelial Cell Basal Medium	45 %
	Penicillin-Streptomycin	100 U/ml
	Epidermal Growth Factor	10 ng/ml
	Insulin	5 µg/ml
	Hydocortisone	0.5 mg/ml
	Epinephrine	0.5 mg/ml
	Triiodo-L-thyronine	6.7 ng/ml
	Transferrin	10 µg/ml

	Retinoic acid	0.1 µg/ml
ALI medium	Airway Epithelial Cell Basal Medium	40 %
	DMEM high Glucose	50 %
	Penicillin-Streptomycin	100 U/ml
	Epidermal Growth Factor	10 ng/ml
	Insulin	5 µg/ml
	Hydocortisone	0.5 mg/ml
	Epinephrine	0.5 mg/ml
	Triiodo-L-thyronine	6.7 ng/ml
	Transferrin	10 µg/ml
	Retinoic acid	0.7 µg/ml
Murine B cell medium	RPMI 1640	89 %
	FCS (HyClone)	10 %
	L-glutamine,	2 mM
	Penicillin-Streptomycin	1 %
	2-Mercaptoethanol	50 µM
	MEM sodium pyruvate	1 mM
	MEM nonessential amino acids	0.1 mM
	HEPES	20 mM
Human B cell medium	RPMI-1640	88 %
	FCS (Sigma)	10 %
	L-Glutamine	2 mM
	MEM-NEAA	1 %
	MEM sodium pyruvate	1 mM
	Pen/Strep	1 %
	2-Mercaptoethanol	50 µM
L-cell medium	RPMI-1640	87 %
	FCS (HyClone)	10 %
	Penicillin-Streptomycin	100 µg/ml
	Kanamycin	1 %
	MEM-NEAA	1 %
	MEM sodium pyruvate	1 mM
	MEM vitamin solution	1 %
3T3 medium	DMEM high Glucose	80 %
	FCS (HyClone)	20 %
	Penicillin-Streptomycin	100 U/ml
Synthetic nasal medium (SNM)	SNM medium was prepared as described by (Krismer et al., 2014)	

2.1.5. Primers

Table 9 Primer sequences *for Illumina sequencing and plastid exclusion PCR*

Application	**Name**	**Adapter +** Sequence	**Region**
Microbiome nasal	27F-YM forward	**5' TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG –** AGAGTTTGATYMTGGCTCAG **3'**	16S rRNA V1-3
	534R Reverse	**5' GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G** AT TAC CGC GGC TGC TGG **5'**	16S rRNA V1-3
Plastid exclusion	799f forward	**5'** AAC MGG ATT AGA TACC CKG **3'**	16S rRNA V5-V9
Pollen microbiome	Illum115f forward	**5' TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** CAA CGA GCG CAA CCC T-**3'**	16S rRNA V7-9
	Illum1492r Reverse	**5' GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**GT TAC CTT GTT ACG ACT T **3'**	16S rRNA V7-9
Nasal mycobiome	18S-F forward	**5' TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** GTA AAA GTC GTA ACA AGG TTT C 3'	ITS 18S
	5.8S-R reverse	**5' GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**GT GTT CAA AGA YTC GAT GAT TCA C **3'**	ITS 5.8S

2.1.6. Software

Table 10 Software *used for statistical and bioinformatic analyses*

Software	**Version**	**Company**
Kaluza Analysis	2.1	Beckman Coulter GmbH, Krefeld, Germany
Graphpad Prism	8.4.3	GraphPad Software, Inc., San Diego, CA, USA
R	3.6.2	R Core Team (2019)
microbIEM	v0.4	Institute of Environmental Medicine
MetaboAnalyst	4.0	(Chong, Wishart, & Xia, 2019)
MicrobiomeAnalyst		(Chong, Liu, Zhou, & Xia, 2020; A. Dhariwal et al., 2017)
MassTrix	v.3	http://masstrix3.helmholtz-muenchen.de/masstrix3/

2.2. Methods

2.2.1. Study Designs

2.2.1.1. Birch panel study 2016

Over the course of one year, monthly or bi-weekly (during the birch pollen season 2016) swabs of the middle nasal meatus of eight allergic rhinitis patients and eight non-atopic volunteers were taken. From those, 16S rRNA hypervariable regions V1-V3 were sequenced using the Illumina MiSeq sequencing platform to study the microbial composition. Visit 1, 14 and 15 were used as a baseline, since pollen concentration were lowest during these time points. The pollen season was defined as Visit 2-13. During the birch flowering season 2016, pollen from 60 characterized sampling sites in Augsburg, Germany, were collected for 16S sequencing of pollen-associated microbes (Figure 8).

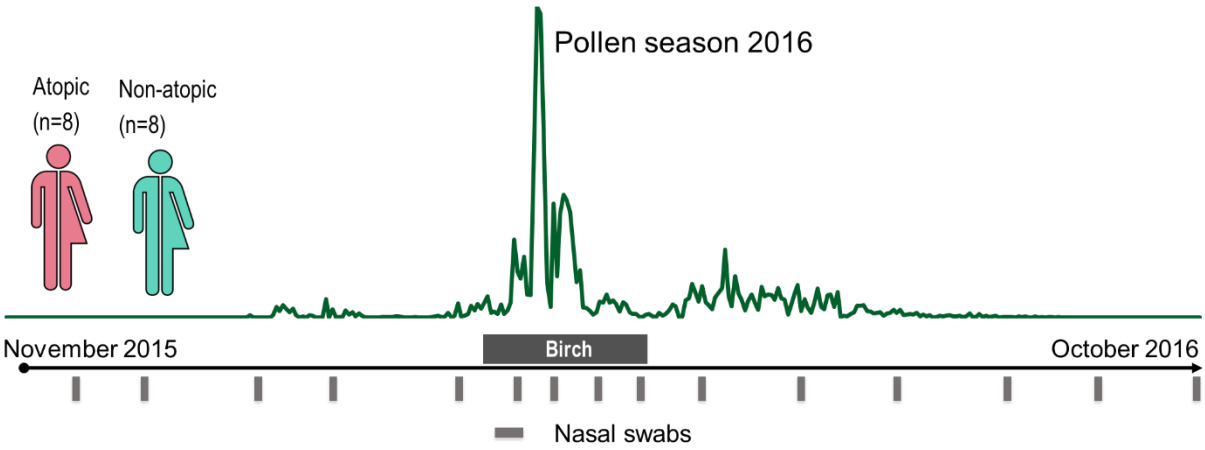

Figure 8 Study Design birch panel study. From November 2015 to October 216, the nasal microbiome swabs of the middle meatus of atopic (n=8) and non-atopic (n=8) study participants were taken during a total of 15 visits. During the birch pollen season in 2016, swabs were taken bi-weekly.

2.2.1.2. Picnic study 2018

During the grass and fungal spore season in June-July 2018, seven healthy volunteers sat in a flowering meadow for one hour after spending at least three hours indoors on five weekly visits. Swabs of the middle nasal meatus of four participants were taken immediately before and after the outdoor exposure for ITS sequencing of the nasal mycobiome. Three of the participants wore nasal filters 1 h indoors and 1 h during the outdoor stay. Simultaneously, portable pollen traps were set up indoors and outdoors to measure the pollen and fungal spore content. Additionally, traps were set up with PDA plates to collect cultivable fungal species (Figure 9).

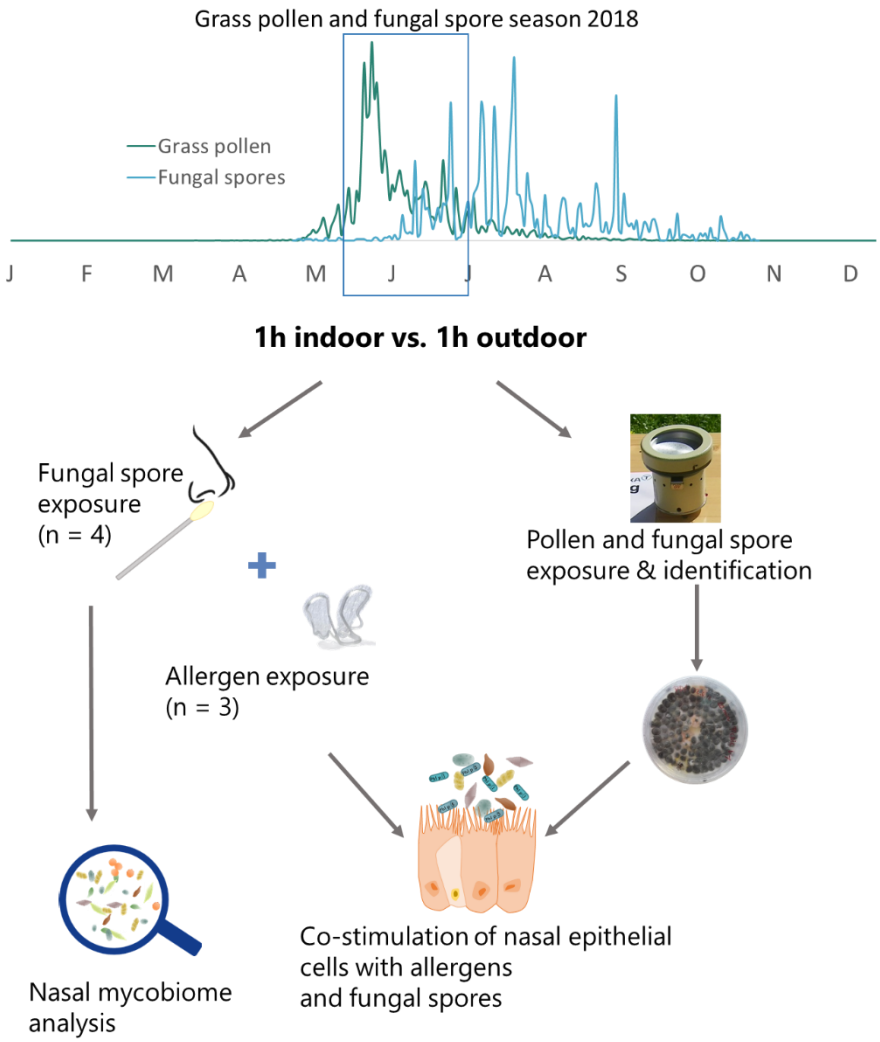

Figure 9 Study Design Picnic Study 2018. During the grass pollen and fungal spore season 2018, 7 volunteers spent one hour outdoors in a flowering meadow on 5 weekly visits. Inhaled allergens were measured in nasal filters worn 1 h before and during the outdoor exposure, while swabs of the nasal mycobiome were taken directly before and after the exposure. Simultaneously, pollen and fungal spore traps were set up indoors and outdoors to measure the pollen load and identify fungi from the environment for subsequent co-stimulation experiments of HNECs *in vitro*.

2.2.2. Sample collection and preparation

2.2.2.1. Pollen

Betula pendula (Bet) pollen were collected during the flowering periods in 2013 (Munich and surrounding area), 2015 and 2017 (Augsburg and surrounding area) and were kindly provided by Isabelle Beck (Chair and Institute of Environmental Medicine, Augsburg) and Franziska Kolek (Chair and Institute of Environmental Medicine, Augsburg). *Phleum pratense* (Phl) pollen were collected during the flowering season in 2015 and kindly provided by Stefanie Gilles (Chair and Institute of Environmental Medicine, Augsburg). *Ambrosia artemisiifolia* pollen were grown in phytotron walk-in chambers under control conditions (control-RWE, 380 ppm

CO_2 or 40 ppb O_3) and elevated CO_2 (CO_2-RWE, 700 ppm) or O_3 (O_3-RWE, 80 ppb) levels at the Institute of Biochemical Plant Pathology (BIOP), Helmholtz Zentrum München, and kindly provided by Ulrike Frank.

2.2.2.2. Aqueous pollen extracts

Aqueous pollen extracts (APE) were prepared by suspending pollen grains in either D-PBS or cell culture media. The suspension was incubated for 30 min at 37°C in a water bath with continuous shaking and vortexing every 10 min. Then, the extracts were centrifuged for 10 min at 4000 rpm, 4°C, and the supernatants were sterile filtered using a 0.22 µm filter. In the following, APE concentrations used in *in vitro* experiments correspond to the amount of pollen originally used for the extraction.

2.2.2.3. Nasal swabs

Samples for the nasal microbiome of the *meatus medius* were obtained by swabbing approximately 3 cm deep in both nostrils of the study participants. The swabs were stored in micro tubes prepared with 500 µL Stool Stabilizer and 0.5 mg silica beads at -80°C.

2.2.2.4. Nasal filter extracts

Study participants spent both one hour indoors and outdoors wearing nasal filters. The nasal filter devices were used as described by the manufacturer, allowing the participants to breathe normally for the duration of the indoor and outdoor exposure. The filter devices were collected in 5 ml D-PBS + 0.05 % Tween20 and washed for 2 h at RT and 300 rpm on an Eppendorf shaker. Finally, 1 mL aliquots of the extracts were stored at -80°C until further use.

To measure the allergen content in the extracts, the aliquots were thawed and diluted 1:10 in D-PBS to obtain a final concentration of 0.05 mM Tween20. The diluted extracts were then concentrated using 15 ml Amicon®Ultra filters with a 3 kDa cutoff according to the manufacturer's manual. Briefly, 4 ml diluted extract were added to the filter and centrifuged at 4000 x g at RT. The filtrate was discarded and another 4 mL of extract were added on top of the concentrate and centrifuged again. This process was repeated until the whole nasal extract was concentrated to a final volume of 200-500 µl. The concentrated extract was stored at -80°C.

2.2.3. Cell donors and ethics

Isolation, culture and stimulation of human primary nasal epithelial cells and dendritic cells, as well as microbiome sampling was approved by the ethical committee of the Medical Faculty of the Technical University Munich (ethics statement code: 54/17 S) and the consultative commission of the Augsburg University Medical School (ethics statement code: 2016-7). Blood samples, human nasal epithelial cells from turbinoplastic surgery or nasal microbiome swabs were collected after written informed consent. Atopy status of blood or nasal cell donors was determined by measuring total serum IgE and allergen specific IgE by serum ImmunoCAP (ThermoFisher, Massachusetts, USA). Non-atopic donors were defined by total IgE <100 kU/L and negative specific IgE against common aeroallergens (tree mix, grass mix, mugwort, ragweed, house dust mite, fungal spores). The panel study 2015/2016 was approved by local ethics committee of the Technical University of Munich (internal code 19/15).

2.2.4. Cell culture based methods

2.2.4.1. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy donors via density gradient centrifugation. Whole blood collected in sodium heparin coated syringes was diluted 1:2 in D-PBS and then overlaid onto 10 ml LymphoPrep. After 15 min centrifugation at 2200 rpm at RT with the break turned off, the PBMC layer was collected in a fresh tube and washed with D-PBS + 5 mM EDTA, followed by 10 min at 1600 rpm. The supernatant was aspirated and the pellet was suspended in D-PBS + 5 mM EDTA. Following centrifugation for 10 min at 1200 rpm, the pellet was suspended in either D-PBS + 2 mM EDTA (for MACS) or D-PBS + 2 mM EDTA (for freezing of cells) and the cell number was determined. After an additional wash step at 1200 rpm for 10 min, the cells were either suspended in freezing medium for storage in liquid N_2 or prepared for MACS.

2.2.4.2. Cultivation and differentiation of monocyte-derived dendritic cells

$CD14^+$ MACS sorted monocytes were seeded in a T75 flask at 10^6 cells/mL in huDC medium. To promote differentiation into monocyte-derived dendritic cells (moDCs), GM-CSF (500 U/ml) and IL-4 (500 U/ml) were added to the culture. After two days, half of the medium was changed and fresh cytokines were added. The cells were cultured at 37°C, 5 % CO_2 for a total of 5 days and then the differentiation was assessed by flow cytometry staining for CD14 and CD1a surface markers and adding PromoFluor810 for live-dead discrimination.

2.2.4.3. Stimulation of dendritic cells

Mature moDCs were seeded in a flat bottom 96-well plate (10^5 cells/well) and stimulated with huDC medium (unstimulated control), LPS (100 ng/ml or LPS concentrations corresponding to APEs), BPE (1 mg/ml, 3 mg/ml or 10 mg/ml), and Amb APE (0.3 mg/ml, 0.6 mg/ml, 1.25 mg/ml, 2.5 mg/ml). Pelargonidin, Malvidin, Catalposide, 9-Oxo-OTrE, Q3OS, p-Coumaryl alcohol-4-O glucoside, Lumichrome, CTRL-Mix and CO_2-Mix (Table 11) were used at $3*10^{-6}$ M. Supernatants of Amb-APE stimulated HNECs were pooled across all donors with concentrations corresponding to 0.5 mg/ml and 1.8 mg/ml pollen for moDC stimulation. After 24 h at 37°C, 5 % CO_2, supernatants were collected for chemokine and cytokine measurements via ELISA and the cells were harvested to analyze the maturation and differentiation markers (CD40, CD80, CD83, CD86, HLA-DR) via flow cytometry.

Table 11 ***Putative substances identified in CO_2-RWE and control-RWE***, their compound class and corresponding compound mix for moDC stimulation.

Compound	Compound class	Compound-Mix
Pelargonidin (Pel)	Anthocyanidins	CO_2-Mix
Malvidin (Mal)	Anthocyanidins	
Catalposide (Cat)	Terpenoids	
9-Oxo-OTrE (9-OTrE)	α-Linolenic acid metabolites	
***p*-Coumaryl alcohol 4-*O* glucoside (pC4OG)**	Phenylpropanoids	Control-Mix
Lumichrome (Lumi)	Riboflavins	
Quercitin-3-*O*-sophoroside (Q3OS)	Flavones and flavonols	

2.2.4.4. Isolation of polymorphonuclear cells

Polymorphonuclear leukocytes (PMNs) were isolated from blood of healthy donors via double density gradient centrifugation. Whole blood collected in 0.5 M EDTA coated syringes was diluted 1:2 in D-PBS and then overlaid onto a double density gradient consisting of 13 ml Histopaque 1077 overlaid on 13 ml Histopaque 1119. After centrifugation (15 min, 1900 rpm, RT, brake off), two fluffy bands form and the bottom band containing PMNs is collected in a fresh tube and washed with D-PBS + 5 mM EDTA (10 min, 1400 rpm, RT). The supernatant was discarded and the pellet washed in D-PBS + 5 mM EDTA (10 min, 1400 rpm, RT). Erythrocytes present in the pellet were lysed by suspending the cells in 2 ml cold RBC lysis buffer for 5 min at RT. The cells were washed with D-PBS (10 min, 1400 rpm, RT) and the cell number was determined.

2.2.4.5. Neutrophil migration assay

Freshly isolated PMNs were used to determine the chemotactic potential of aqueous pollen extracts. 30 µL of migration medium, LTB_4 (10^{-7} M), BPEs (10 mg/ml) or Amb-APEs (2.5 mg/ml) were added to the wells of a 96-well migration plate. A 5 µm pore membrane was placed on top of the plate and 5 x 10^4 cells were added in 50 µl drops to the membrane. The cells were incubated at 37°C, 5 % CO_2 for 1 hour. Non-migrated cells were removed from the membrane by D-PBS and the plate was shortly spun down to collect the cells. Migrated cells were moved to a fresh 96-well plate containing 200 µL FACS buffer and counted by flow cytometry for 45 s using a flow rate of 60 µl/min.

2.2.4.6. Culture of murine 3T3 fibroblast cell line

Murine 3T3 fibroblasts were used as feeder cells for primary HNECs. The cells were cultured in 12 ml 3T3 medium in T75 flasks at 37°C, 6.5 % CO_2 and passaged every 2-3 days. For co-culture with primary HNECS, the 3T3s were treated with DMEM + 2 % Mitomycin C for 2 h at 37°C, 6.5 % CO_2. Then the cells were washed with D-PBS and detached using 0.05 % Trypsin-EDTA and seeded at a density of 0.8 x 10^6 cells/T75 flask in feeder medium.

2.2.4.7. Isolation of primary human nasal epithelial cells (HNEC)

Primary human nasal epithelial cells (HNECs) were isolated from turbinoplastic surgery specimens of non-allergic donors as recently described (Bergougnan et al., 2020). Briefly, the tissue samples were treated twice with gentamycin and antimycotics-antibiotics solution for 10-30 min and cut into 1 cm pieces. To obtain a single cell suspension, the tissue pieces were repeatedly digested with 0.25 % Trypsin-EDTA and then passed through a 30 µm cell strainer. After centrifugation (300 x g, 10 min, RT), the cells were co-cultured with Mitomycin C arrested murine 3T3 fibroblasts in HNEC/Feeder medium. After 4-5 days (37°C, 6.5 % CO_2), the medium was replaced and from then on, every 2 days half of the medium was replaced until cells reached 80-90 % confluency. Then the cells were either passaged onto freshly Mitomycin C arrested 3T3s or frozen and stored in liquid N_2. Passage 1 cells were either kept in liquid N_2 or used directly for downstream assays.

2.2.4.8. Monolayer culture of HNECs

Second passage HNECs were seeded in tissue culture flat bottom 48-well plates at a density of 2 x 10^4 cells/well in complete HNEC medium and incubated at 37°C, 5 % CO_2 for five days. At 80 % confluence, the medium was changed to HNEC medium without hydrocortisone and

cells were stimulated with medium, LPS (corresponding to LPS content in pollen extracts), BPE (1 mg/ml – 10 mg/ml), RWEs (0.3 mg/ml – 2.5 mg/ml), Phl p 1 (1 µg/ml), Phl p 5 (1 µg/ml), or fungal spores of *Fusarium sp, Cladosporium cladosporioides* or *Penicillium manginii* (10^5 spores) or combinations of fungal spores and Phl p 1/5. After 24 h, supernatants were collected for cytokine and chemokine measurements.

2.2.4.9. Air liquid interface culture and stimulation of HNECs

Second passage HNECs were cultured in a T75 flask in complete Airway Epithelial Cell Growth Medium until they reached 80-90 % confluency. Then the cells were transferred to collagen coated transwells (0.3 mg/ml in Aqua ad inj., 30 min, 37°C) at a density of 1-1.5 x 10^5 cells/well in a 24-well plate, and cultured in ALI-medium for 5 days. The medium was changed in the apical (200 µl) and basolateral (600 µl) well on day 2. On day 5, the medium was changed in the basolateral well and aspirated in the apical well (airlift). For the next 21 days, the basolateral medium was changed every 2 days until the trans-epithelial electrical resistance (TER) reached a value of >2000 Ω. Then the cells were stimulated with medium, *Phl p 1* (1 µg/ml), fungal spores (3.3 x 10^4 spores of Fusarium or Cladosporium) or combinations with fungal spores and allergens. TER measurements were performed at baseline, 1 h, 6 h, 24 h and 48 h after stimulation.

2.2.4.10. Isolation and stimulation of murine B cells

Naïve murine B cells were isolated from spleens of 12 week old female Balb/c mice. The spleens were cut in small pieces and passed through a 70 µm cell strainer to obtain a single cell suspension. The cells were washed with D-PBS for 10 min at 300 x g at RT. Erythrocytes were lysed by adding 2 ml ice cold RBC buffer for 2-5 min. The reaction was stopped by 40 ml D-PBS. After washing the cells (10 min at 300 x g at RT), the number of cells was determined and naïve B cells were isolated via MACS. 10^5 cells/well were plated in murine B cell medium in a 96-well F-bottom plate and stimulated with anti-CD40 antibody (5 µg/ml) and murine IL-4 (25 ng/ml) as positive control or in the presence of RWE (0.3 mg/ml – 2.5 mg/ml) or BPE (1 mg/ml – 10 mg/ml). Unstimulated cells were used as negative control. After 8 days at 37°C, 5 % CO_2, supernatants were collected for IgE measurements.

2.2.4.11. Culture of murine L-cells

Murine L-cells are a murine fibroblast cell line expressing human CD40L (kindly provided by W. van de Veen, Swiss Institute of Allergy and Asthma Research). Then, 10^5 cells were seeded

in 25 ml L-cell medium in a T175 and cultured at 37°C, 5 % CO_2. Confluent cultures were passaged every 2-3 days. For co-culture with naïve human B cells, L-cells were treated with DMEM + 2 % Mitomycin C for 2 h at 37°C, 5 % CO_2. Then the cells were washed with D-PBS and detached using 0.05 % Trypsin-EDTA and seeded in a 96-well plate.

2.2.4.12.Isolation and stimulation of naïve human B cells

Naïve human B cells were isolated from the CD14 negative PBMC fraction from the DC isolation via untouched MACS isolation. Per well, 750 B cells were co-cultured with 10^4 L-cells per well in a flat bottom 96-well plate in human B cell medium and stimulated with IL-4 (50 ng/ml) + IL-21 (50 ng/ml) in the presence or absence of 3 mg/ml BPE. After 15 and 21 days, supernatants were collected to measure IgE content.

2.2.5. Molecular methods

2.2.5.1. Extraction of microbial DNA from nasal swabs

Microbial DNA from nasal swabs was extracted following the instructions of the QIAamp UCP Pathogen Mini Kit with the following modifications. 650 µl of ATL buffer containing 4.3 µl DX buffer were added to swabs in tubes containing 500 mg zirconia beads and 500 µl Stool stabilizing solution. Mechanical pre-lysis was performed by shaking at 5600 rpm, 2x 90 s with 15 s break in between on a Precellys Evolution bead beater. The volumes of the reagents were adjusted to the supernatant obtained from step 7 of the pre-lysis protocol. Finally, the DNA was eluted twice with 2x 50 µL elution buffer and stored at -20°C.

2.2.5.2. Extraction of microbial DNA from pollen

A pool of 200 mg birch pollen collected in 2016 was suspended in 500 µl Stool Stabilizer Solution and vortexed. The pollen solution was centrifuged for 10 min at 10,000 x g at RT. The supernatant was collected and transferred to a fresh tube containing 0.5 mg silica beads. Microbial DNA from pollen was extracted using the QIAamp UCP Pathogen Mini Kit following the same instructions as described above.

2.2.5.3. Extraction of fungal DNA for species identification

Pure strain fungal colonies that were incubated for 4-5 days were suspended in 200 µl fungal lysis buffer and vortexed vigorously. The samples were incubated for 10 min at 95°C and shaking at 600 rpm. After centrifugation for 2 min at 10,000 x g, the supernatants were collected and species were identified by Illumina Sequencing (ITS primers see **Table 9**).

2.2.5.4. Illumina Sequencing of nasal microbiome

For Illumina Sequencing of the 16S rRNA V1-V3 region of the nasal microbiome, DNA samples were sent to the TUM Core Facility Microbiome at the ZIEL Institute for Food and Health in Freising, Germany.

2.2.5.5. Illumina Sequencing of nasal mycobiome (ITS)

A first step PCR to amplify the ITS sequence of the nasal mycobiome and to add adapters for sequencing is described below (Table 12, Table 13). Subsequent barcoding and sequencing was carried out at the TUM Core Facility Microbiome at the ZIEL Institute for Food and Health in Freising, Germany.

Table 12 Mastermix for ITS adapter PCR.

Component	**Stock**	**final**	**1x**
NEBNext Ultra II Q5 Master Mix (2x)	2x	1x	12.5 µl
Primer 18S-F + 5.8S-R	10 µM each	0.2 µM each	1 µl
DEPC H_2O			6.5 µl
DNA		30 ng	5 µl
Total volume			25 µl

Table 13 PCR protocol for ITS sequencing.

Initial Denaturation	**Denaturation**	**Annealing**	**Extension**	**Extension**	**store**
98°C	98°C	58°C	72°C	72°C	4°C
10 min	30 s	30 s	60 s	10 min	∞
	25 cycles				

2.2.5.6. Illumina sequencing of pollen microbiome

After excluding pollen-associated chloroplast DNA from the samples (see below), the first step adapter PCR was performed and the samples were further processed at the Core Facility Microbiome at the ZIEL Institute for Food and Health in Freising, Germany.

Table 14 Master mix for 16S rRNA V7-V9 adapter PCR for sequencing of pollen microbiome.

Component	stock	final	1x
Q5 Buffer	5x	1x	5 µl
Q5 Enhancer	5x	1x	5 µl
dNTPs	10 mM	200 µM	0.5 µl
Primer illum1115f + illum1492r	10 µM each	0.4 µM	1 µl
Q5 HotStart Polymerase		0.02 U/µl	0.25 µl
dH_2O			8.25
DNA template		5 ng	5 µl
Total volume			25 µl

Table 15 PCR protocol for pollen microbiome adapter PCR.

Initial Denaturation	Denaturation	Annealing	Extension	Extension	store
98°C	98°C	60°C	72°C	72°C	4°C
30 s	10 s	30 s	20 s	5 min	∞
	15 cycles				

2.2.5.7. Plastid exclusion PCR of pollen-associated microbiome

To reduce contamination with chloroplast DNA for 16S rRNA V7-V9 regions of the pollen microbiome, 16S rRNA region V5-V7 was pre-amplified (**Table 16, Table 17**), resulting in two fragments. The smaller fragment, containing bacterial DNA, was extracted from a 2 % agarose gel using the Monarch Nucleic Acid Purification kit according to the manufacturer's manual.

Table 16 Master mix for plastid exclusion PCR.

Component	Stock	final	1x
NEBNext Ultra II Q5 Master Mix (2x)	2x	1x	12.5 µl
BSA		1 %	0.25 µl
Primer-Mix 799f + illum1492r	(10 µM each)	0.4 µM	1 µl
dH_2O			6.25 µl
DNA template		100 ng	5 µl
Total volume			25 µl

Table 17 PCR Protocol for plastid exclusion PCR.

Initial Denaturation	Denaturation	Annealing	Extension	Extension	store
94°C	95°C	53°C	72°C	72°C	4°C
3 min	20 s	40 s	40 s	7 min	∞
	25 cycles				

2.2.6. Immunology-based methods

2.2.6.1. Flow cytometry

For flow cytometry of moDC surface markers, 10^5 cells/well were suspended in 200 µL D-PBS in a round-bottom 96-well plate and spun down (short centrifugation, max. 2000 rpm, 15 s). The supernatant was discarded and the pellet re-suspended in 100 µL PromoFluor 840 live/dead stain (1:1000 in D-PBS). After 15 min at 4°C, the cells were washed with FACS buffer (short centrifugation, max. 2000 rpm, 15 s) and the pellet was suspended in 5 µL FcR blocking reagent for 10 min in the dark at RT to inhibit unspecific binding of the antibodies. Then, 50 µL antibody-mix (**Table 18**) were added for 30 min at 4°C, followed by a wash step (FACS buffer, short centrifugation, max. 2000 rpm, 15 s). The pellet was re-suspended in 200 µL FACS buffer to obtain a single cell suspension and the differentiation and maturation maker expression was measured on a CytoFlexLX. $50*10^4$ cells were recorded within 3 min at a flow rate of 60 µL/min. The data was analyzed using Kaluza 2.1 software.

Table 18. FACS antibodies for the analysis of surface DC maturation and differentiation markers. The optimum concentrations for each antibody were pre-determined in a titration experiment.

Antibody	Dilution in FACS-Buffer	Channel CytoFlexLX
Live/Dead PF840	1:1000	IR840-A790
CD14-FITC	1:100	B525-FITC
CD40 – PE	1:100	Y585-PE
CD1a-PE-Cy7	1:200	Y763-PC7
HLA-DR – PE-CF594	1:200	Y610-mCherry
CD83 – APC	1:100	R660-APC
CD80 – APCH7	1:200	R763-APCA750
CD86 – Pacific Orange	1:200	V610

2.2.6.2. Enzyme-linked immunosorbent assay – ELISA

To measure cytokines and chemokines in cell culture supernatants, and allergen content and PALMs in pollen extracts, ELISAs were performed according to the manufacturer's instructions. Amb a 1 measurements were provided by L. Aglas, University Salzburg.

2.2.6.3. Magnetic activated cell sorting – MACS

CD14 positive monocytes were isolated from PBMCs using positive selection and naïve human B cells were isolated from the resulting CD14 negative population. Naïve murine B cells were isolated via untouched selection. All isolations were carried out on an AutoMACS Pro cell separator following the manufacturers' instructions.

2.2.6.4. Multiplex Array for Indoor Allergens – MARIA®

Phl p 5 content in nasal filter extracts was analyzed using a single-plex array for indoor allergens according to the manufacturers' instructions and measured on a Bio-Plex 200™.

2.2.6.5. IgE BioPlex assay

IgE in cell culture supernatants of human B cells stimulated with BPE was analyzed using Bio-Plex Pro™ Human IgE Isotyping Assay according to manufacturer's instructions on a BioPlex 200™.

2.2.7. Microbiology

2.2.7.1. Isolation and identification of fungi

Indoor and outdoor fungi were collected on PDA plates using a portable volumetric Burkard pollen and spore trap. The plates were placed in a common area for 1 h indoors and next to the study participants for 1 h outdoor exposure. Exposed plates were kept at RT for 5 days and then colonies were picked with a single use inoculation loop and streaked on fresh agar plates. After another 4-5 days, single colonies were picked for DNA extraction.

2.2.7.2. Cultivation of fungi and spore collection

Subcultures of pure strain fungal cultures were obtained by cutting 1 cm^2 pieces of the original culture and placing it on a fresh PDA plate. The plate was incubated at RT (*Fusarium sp*) or 25°C (*Cladosporium cladosporioides, Penicillium manginii*) for 1-3 weeks until spores were produced. A swab was used to scrape the spores of the culture dish and placed into 1 ml HNEC or synthetic nasal medium (SNM). The spores were counted and adjusted to 10^6 spores/ml for cell culture experiments.

2.2.8. Metabolome Analysis

2.2.8.1. FT-MS

The metabolome of control-RWE and CO_2-RWE was analyzed by Constanze Mueller at the Research Unit Analytical BioGeoChemistry, Helmholtz Zentrum München, using ultra high-resolution mass spectroscopy (ICR-FT/MS) as previously described (Gilles et al., 2011). Broad band mode was used to acquire spectra which were externally calibrated to arginine clusters, as well as internally calibrated to fatty acids and phthalate diesters at a maximum error of 100 ppb. The signal/noise ratio threshold was set to 3 and peaks exceeding it were exported to peak lists and converted into corresponding C (carbon), H (hydrogen), N (nitrogen), O (oxygen) and S (sulfur) elementary compositions.

2.2.8.2. Analysis and identification of compounds

To annotate metabolites, the peak lists were submitted to MassTRIX v.3 (http://masstrix3.helmholtz-muenchen.de/masstrix3/). Retrieved masses were annotated against *Arabidopsis thaliana* compounds found in the Kyoto Encyclopedia Genes and Genome chemical compound database (KEGG database). The annotated data of the differently treated plant pollen were compared using the "*compare compounds*" function in MassTRIX v.3 and the list was used for further statistical analysis using MetaboAnalyst 4.0.

2.2.9. Data Analysis

2.2.9.1. Statistical analysis of *in vitro* data

The data were normalized to either unstimulated cells or LPS-stimulated positive controls due to donor-to donor variability. TER measurements were normalized to baseline and subsequently to unstimulated conditions for each time point. Repeated-measures two-way ANOVA with Sidak's correction was used for multiple comparisons of paired data. The area under the curve (AUC) was calculated for stimulations with multiple concentrations and ordinary one-way ANOVA or Student's t-Test were used to compare between treatments. The data are represented as mean ± standard deviation (SD), mean showing all points from min-max or heat maps and statistical significance was determined at $p < 0.05$. Statistical analysis and graph design were done in GraphPad Prism version 8.4.3.

2.2.9.2. Analysis of Illumina Sequencing data

Demultiplexing, sequence merging, filtering and removal of chimera artifacts of sequencing data of the microbiome and mycobiome was performed using the Integrated Microbial Next Generation Sequencing (IMNGS) platform (Lagkouvardos et al., 2016). Subsequently, quality filtering to remove contaminants was done using microbIEM v0.41. Filtered data were further analyzed using MicrobiomeAnalyst. To compare the different groups of interest, paired and unpaired t-test or one-way ANOVA were performed and statistical significance was determined as $p < 0.05$. Statistical analysis was performed in R 3.6.2 using the "*ggpubr*" package and graphs were prepared in GraphPad Prism version 8.4.3.

Chapter 3 – Effect of elevated CO_2 levels during ragweed growth on immune modulatory potential of ragweed pollen

Elevated CO_2 levels are a driving force of climate change. Under the IPCC "business as usual" scenario, CO_2 levels will reach 700-1000 ppm by the year 2100. Although plants naturally fixate CO_2, it has not been studied in depth how elevated CO_2 levels will influence the allergenic potential of ragweed pollen. The following data are an excerpt from Rauer et al. (2020) and represent my own work on the effect of elevated CO_2 during ragweed growth on pollen allergenicity.

3.1. Results

3.1.1. Cytokine and chemokine response of human nasal epithelial cells stimulated with control- and CO_2-RWE

Nasal epithelial cells are at the frontline of the immune system and the first point of entry for pollen. To analyze the allergenic potential of ragweed pollen from plants grown under elevated CO_2 levels, HNECs were stimulated for 24 h with control- and CO_2-RWE and cytokines and chemokines were measured in the supernatants. Compared to unstimulated control, IL-8 (control-RWE: $p < 0.01$ and $p < 0.001$; CO_2-RWE: $p < 0.01$), IL-1β ($p < 0.01$, $p < 0.001$) and IL-6 (control-RWE: $p < 0.01$; CO_2-RWE: $p < 0.05$) secretion of HNECs was significantly increased by stimulation with either control- and CO_2-RWEs (Figure 10 B, D-E). CCL2 and CCL22 secretion was unchanged (Figure 10 A, C). Low concentrations of CO_2-RWE and high concentrations of control-RWE increased TNFα differentially between the two treatment groups ($p < 0.001$, $p < 0.01$ vs control-RWE and $p < 0.01$ vs CO2-RWE, Figure 10 F).

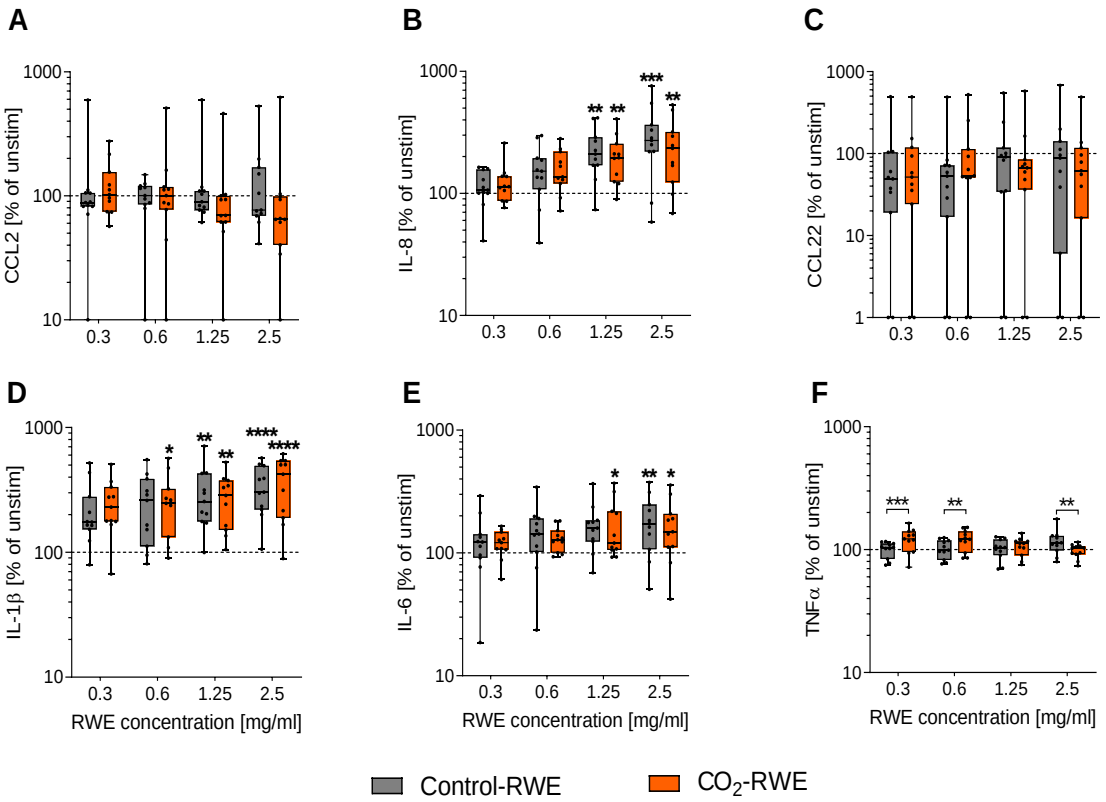

Figure 10 Cytokine and chemokine profile of HNEC stimulated with RWE. HNECs were stimulated with control- or CO_2-RWE (0.3mg/ml - 2.5 mg/ml). After 24h, the cytokines and chemokines were measured in the supernatants. n = 11 independent experiments. Dashed line: unstimulated control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs unstimulated unless indicated otherwise.

3.1.2. Stimulation of dendritic cells with supernatants from RWE-stimulated HNECs

Epithelial cells are important modulators of immune responses in the respiratory airways, therefore the effect of HNEC supernatants after RWE stimulation downstream of the nasal epithelium was investigated.

Therefore, RWE-conditioned HNEC supernatants were used to stimulate immature moDCs. HNEC supernatants were pooled in RWE-low (corresponding to 0.5 mg/ml RWE) and RWE-high (corresponding to 1.8 mg/ml RWE), and the cytokine and chemokine profile of the DCs was analyzed after 24 h exposure to the conditioned HNEC supernatants. Across all treatments, secretion of IL-6 and CCL17 was significantly higher than the baseline of unstimulated DCs (Figure 11; C, IL-6: $p < 0.001$; E, CCL17: $p < 0.001$ RWE-high, $p < 0.0001$ RWE-low, $p < 0.05$ unstimulated HNEC supernatants). Compared to unstimulated HNEC supernatants, stimulation with CO_2-RWE-conditioned HNEC supernatants increased IL-6 ($p < 0.05$) and CCL17 ($p < 0.01$) secretion of DCs. Both, unstimulated- and CO_2-RWE-conditioned HNEC supernatants ($p < 0.01$) increased IL-10 production of DCs (Figure 11 A), which was also

strongly increased by high control-RWE-conditioned HNEC supernatants ($p < 0.0001$). TNFα (Figure 11 B) secretion of DCs was higher upon stimulation with CO_2-RWE conditioned HNEC supernatants than upon stimulation with control-RWE-conditioned HNEC supernatants ($p < 0.01$). IL-1β was only increased by supernatants from unstimulated or CO_2-RWE-stimulated HNECs ($p < 0.05$, Figure 11 D). Overall, the cytokine profile induced by CO_2-RWE-treated HNEC supernatants was strongly pro-inflammatory (Figure 11 F).

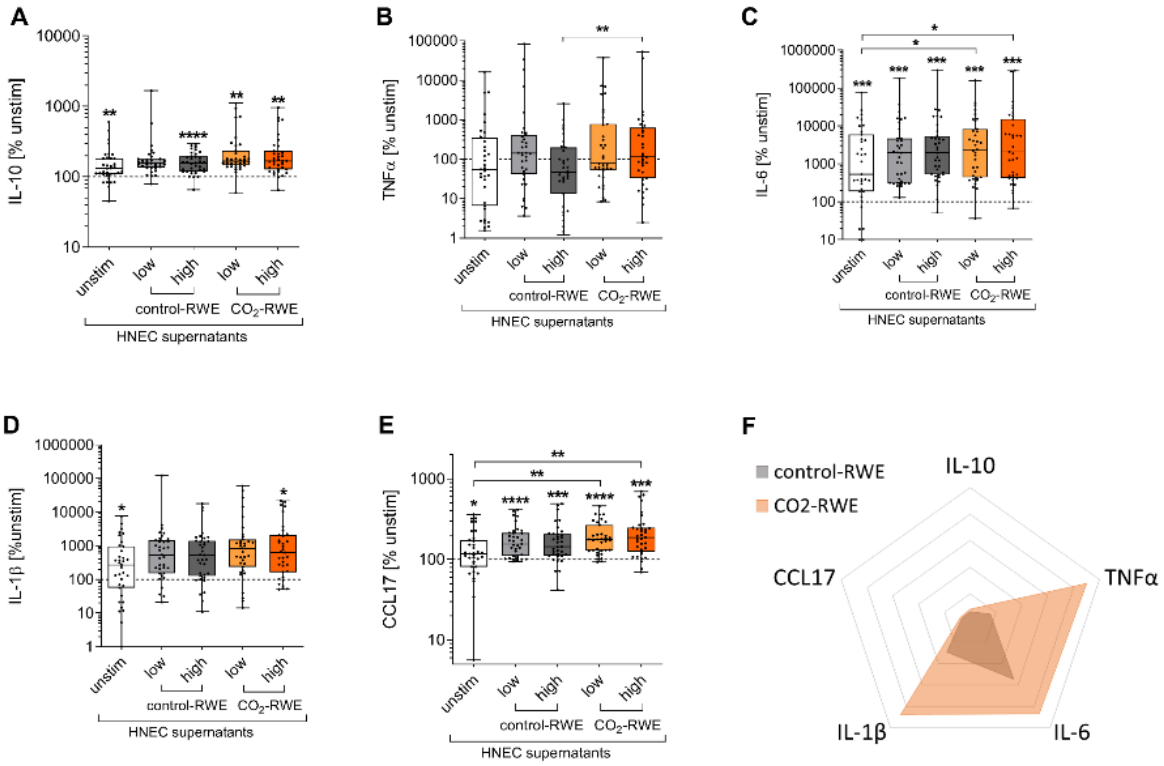

Figure 11 Co-culture of moDCs with supernatants of CO_2-RWE stimulated HNECs elicits pro-inflammatory cytokine profile. (A-E) IL-10, TNFα, IL-6, IL-1β, CCL17/TARC secretion and (F) cytokine profile of moDCs after 24 h stimulation with RWE-stimulated HNEC supernatants (corresponding to 0.5 mg/ml and 1.8 mg/ml RWE). Dashed line indicates baseline cytokine production of moDCs normalized to unstimulated cells (100 %). n = 35 independent experiments; **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. baseline unless indicated otherwise.

The effect of RWE-conditioned HNEC supernatants on the maturation of DCs was also studied. Supernatants from CO_2-RWE-stimulated HNECs increased CD80 and CD86 ($p < 0.05$, $p < 0.0001$, Figure 12 B, D) whereas CD40 and HLA-DR expression was unchanged (Figure 12 A, E). CD83 (Figure 12 C) was elevated by unstimulated HNEC supernatants ($p < 0.01$) and reduced by CO_2-RWE-stimulated HNEC supernatants compared to unstimulated ($p < 0.01$).

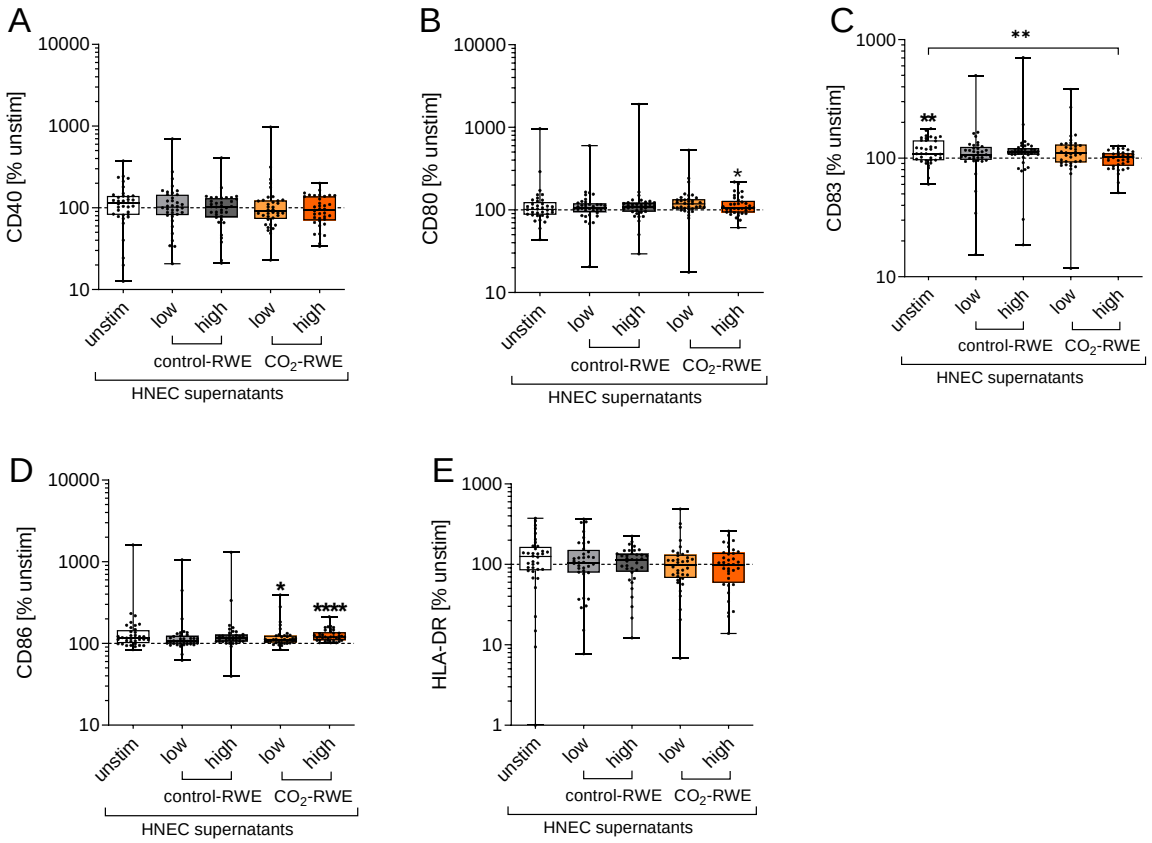

Figure 12 Maturation markers of moDCs stimulated with HNEC supernatants. MoDCs were stimulated with pooled supernatants from HNEC stimulations (pooled across all donors) corresponding to 0.5 mg/ml (low) and 2.5 mg/ml (high) RWE. Maturation markers were measured after 24 h. n = 35 independent experiments. Dashed line: unstimulated moDCs. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ vs unstimulated or as indicated.

3.1.3. Cytokine and maturation marker profile of DCs stimulated with CO_2-RWE

Antigen presenting cells, such as dendritic cells, are crucial for allergic sensitization, so the direct effect of RWEs on dendritic cell cytokine/chemokine secretion and surface marker expression was analyzed. MoDCs stimulated with CO_2-RWE secreted significantly less IL-10 than moDCs stimulated with control-RWE ($p < 0.05$, Figure 13 A). In contrast, CO_2-RWE significantly increased TNFα levels ($p < 0.05$, Figure 13 B). IL-1β, IL-6 and CCL17/TARC were not differently secreted upon stimulation with either RWE (Figure 13 C-E). Similar to the above described co-culture experiments, CO_2-RWE induced a pro-inflammatory cytokine profile in DCs (Figure 13 F).

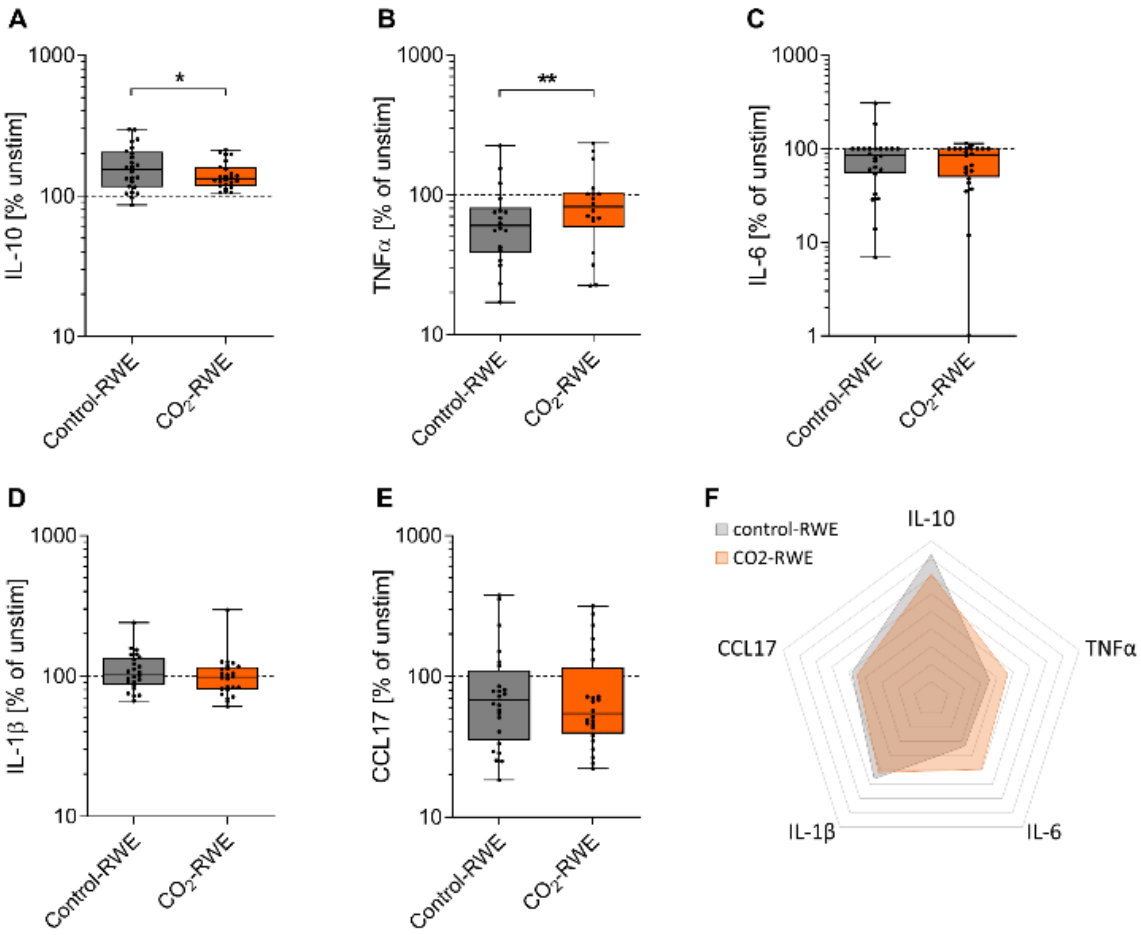

Figure 13 Pollen of ragweed plants grown under elevated CO_2 levels induce pro-inflammatory cytokine profile in moDCs. (A-E) IL-10, TNFα, IL-6, IL-1β, CCL17/TARC were measured in cell culture supernatants after 24 h stimulation with 2.5 mg/ml control- or CO_2-RWE and the results were summarized in a profile (F). Dashed line indicates unstimulated control. n = 24 independent experiments; *p < 0.05, **p < 0.01 comparison between treatment groups.

Both RWEs induced maturation profiles distinct from the unstimulated control, but similar between the treatments (Figure 14, bottom). Expression of CD86 was increased by both RWEs ($p < 0.05$), while CD80 expression was only induced in control-RWE-treated DCs ($p < 0.05$), (Figure 14, top).

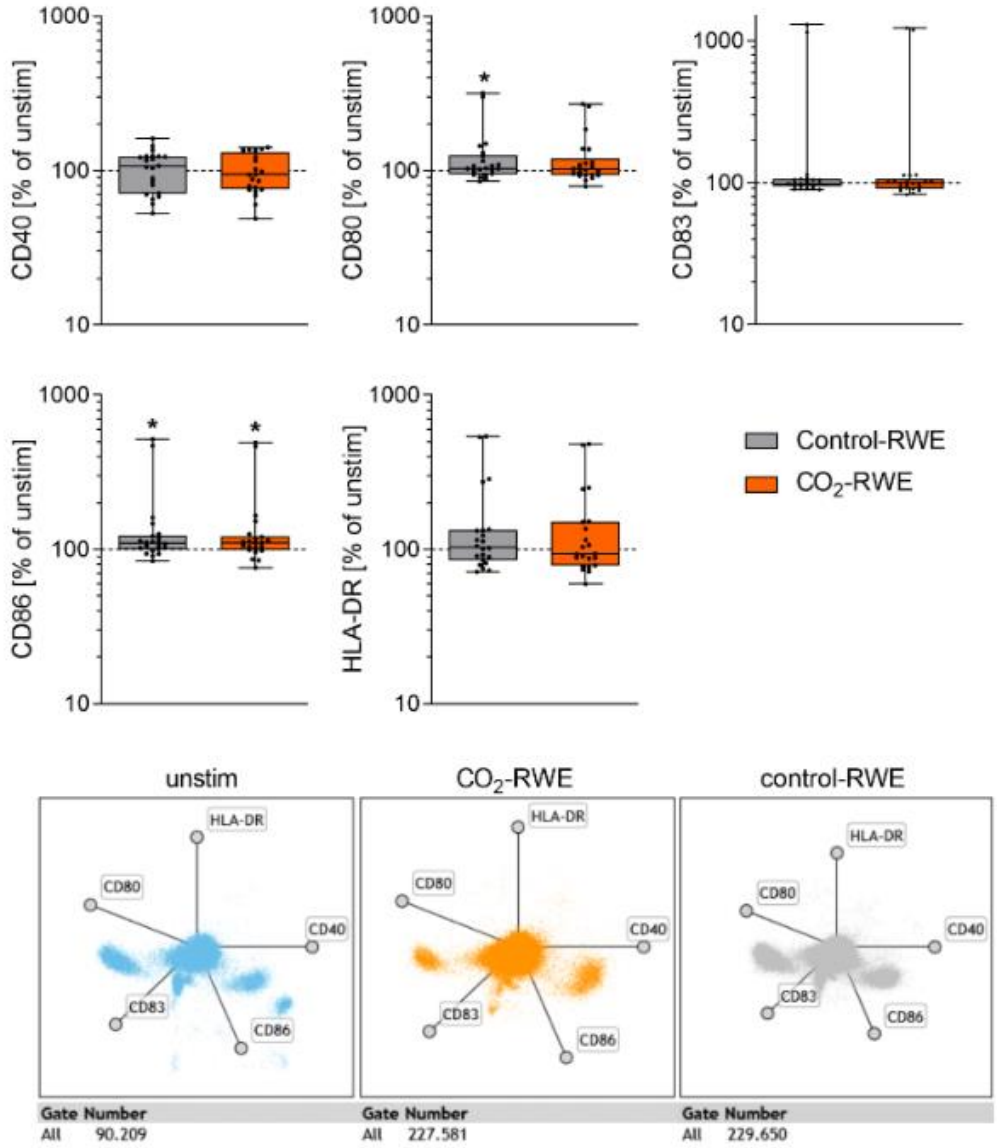

Figure 14 Maturation markers of moDCs stimulated with RWE. MoDCs were stimulated with RWEs (2.5 mg/ml) and maturation marker expression was measured after 24 h (top). Radial plots of maturation markers of pooled donors (bottom). N = 24 independent experiments. Dashed line: unstimulated moDCs. * $p < 0.05$ vs. unstimulated.

3.1.4. Chemotactic potential of CO_2-RWE

Neutrophils are attracted by chemokines as well as by pollen-associated lipid mediators. To investigate the chemotactic potential of control- and CO_2-RWE neutrophils migrated directly for 1 h towards the pollen extracts or LTB_4 as positive control. Here, control-RWE induced significantly higher neutrophil migration than CO_2-RWE (Figure 15, $p < 0.01$).

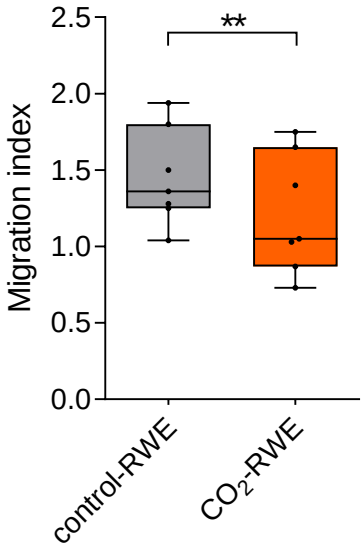

Figure 15 Neutrophil migration assay. Neutrophils migrated directly towards RWEs (2.5 mg/ml) for 1 h and migrated cells were counted by flow cytometry. n = 7 non-atopic donors. ** $p < 0.05$

3.1.5. IgE secretion of murine B cells

IgE is the hallmark immunoglobulin of Th2 immunity. IgE secretion after Th2 priming of naïve splenic B cells and subsequent stimulation with RWEs for eight days was measured to analyze the immune modulatory potential of RWEs. Unstimulated B cells did not produce IgE, whereas both RWEs increased IgE production under Th2 simulating conditions (i.e. priming with CD40L and IL-4). CO_2-RWEs induced higher IgE levels after CD40L and IL-4 priming of B cells than control-RWEs (Figure 16, $p < 0.05$).

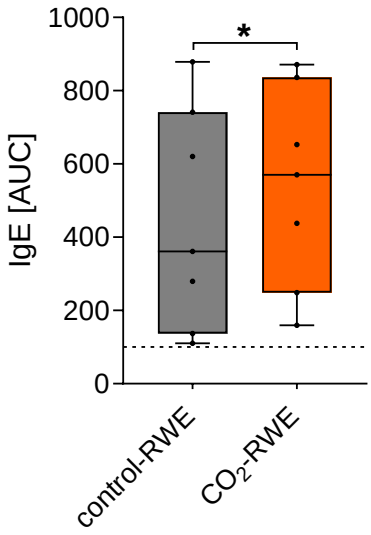

Figure 16 IgE secretion of murine naive splenic B cells. B cells were stimulated for 8 days with either CD40L and IL-4 alone (indicated by dashed line) or combined with RWEs (0.3 mg/ml – 2.5 mg/ml). Area under the curve (AUC) was calculated for statistical analysis. n = 7 independent experiments. * $p < 0.05$.

3.1.6. Metabolome analysis of CO_2-RWE

Pollen-derived substances act as immune-modulators or have pro-inflammatory properties. As such, $PALM_{LTB4}$, $PALM_{PGE2}$, adenosine and LPS were slightly, although non-significantly higher in CO_2-RWE than in control-RWE. The content of the major allergen Amb a 1 did not differ between control- and CO_2-RWE (Figure 17 A). Untargeted mass spectroscopy was used to gain insight into secondary metabolites present in the different RWEs. Clusters of substances upregulated in either control- or CO_2-RWE were observed in Figure 17 B. The metabolite profile of the extracts was distinctly different as revealed by principal component

analysis (PCA) (Figure 17 C). Six candidate substances were present only in control-RWE and 13 candidate substances were present only in CO_2-RWE (Figure 17 D). From those, commercially available or analogue substances and combinations thereof were chosen to stimulate moDCs (Table 11).

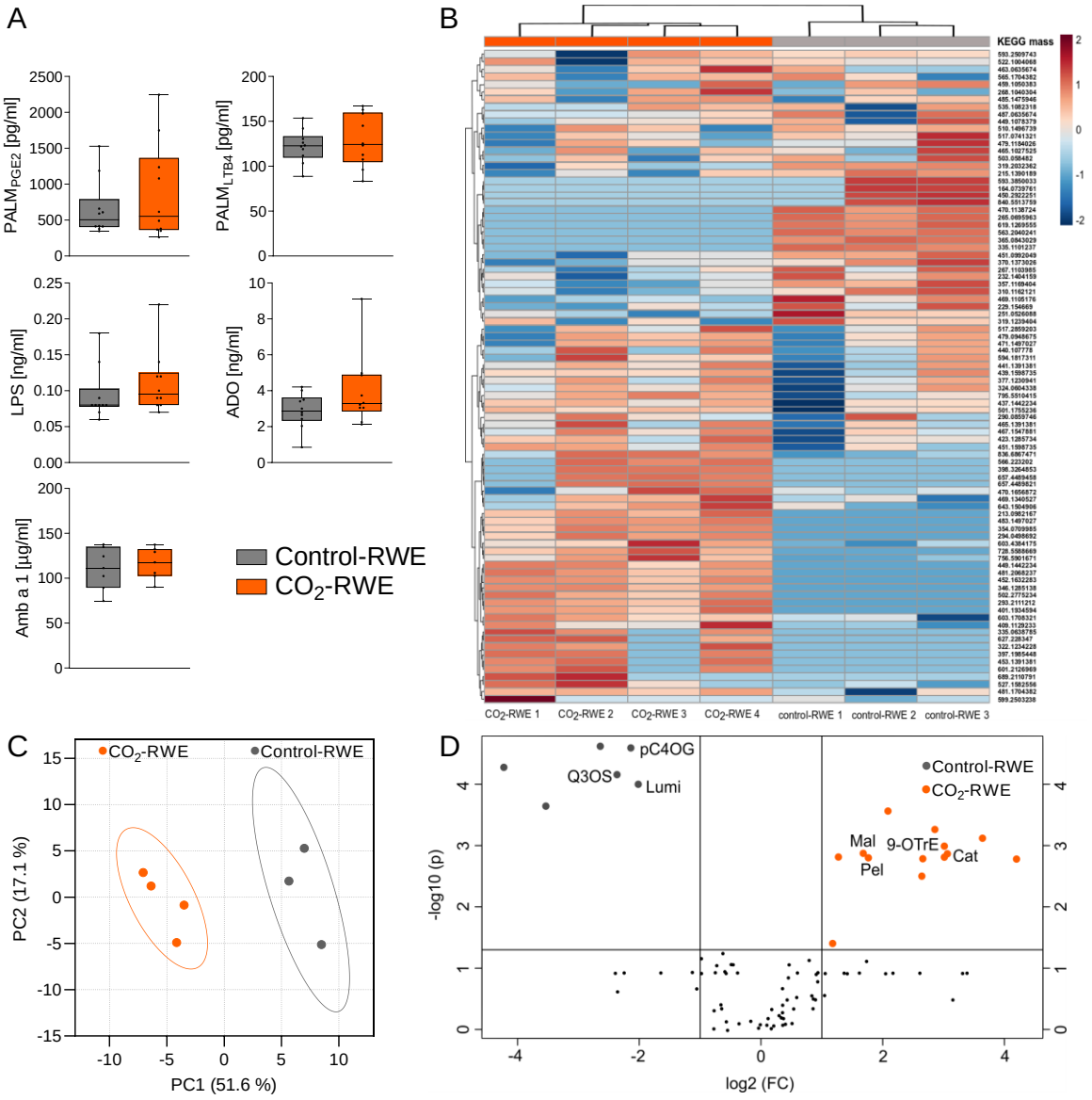

Figure 17 Pollen-associated mediators of allergenicity. (A) PALMs, LPS, adenosine and Amb a 1 measurements in RWEs. n = 10 single plants. (B) Heat map of secondary metabolites present in RWE are clustered using Euclidean distance measure and Ward's linkage-clustering algorithm. (C) (C) Principal component analysis (PCA). (D) Univariate volcano plot analysis of all metabolites. n=3 control-RWEs and n=4 CO2-RWEs for metabolome analysis.

3.1.7. Cytokine response of DCs to identified active substances

To identify substances responsible for the cytokine response of RWE stimulated moDCs, the compounds were used either alone or in combinations corresponding to control- or CO_2-RWE for moDC stimulation (Table 11). Pelargonidin and malvidin enhanced IL-10 secretion ($p < 0.0001$ and $p < 0.01$ vs. unstimulated), whereas pC4OG decreased IL-10 secretion ($p < 0.05$ vs. unstimulated) (Figure 18 A). Malvidin ($p < 0.001$) and 9-OTrE ($p < 0.05$) increased IL-1β secretion (Figure 18 A), and lumicrome decreased IL-6 secretion ($p < 0.05$ vs. unstimulated, Figure 18 A). Compared to a relatively low response to single substances, DCs stimulated with a compound pool mimicking CO_2-RWE secreted less IL-10 ($p < 0.05$, Figure 18 B) and more IL-1β ($p < 0.01$, Figure 18 B) than with the control-RWE compound mix. TNFα and IL-6 secretion did not differ between the two compound mixes.

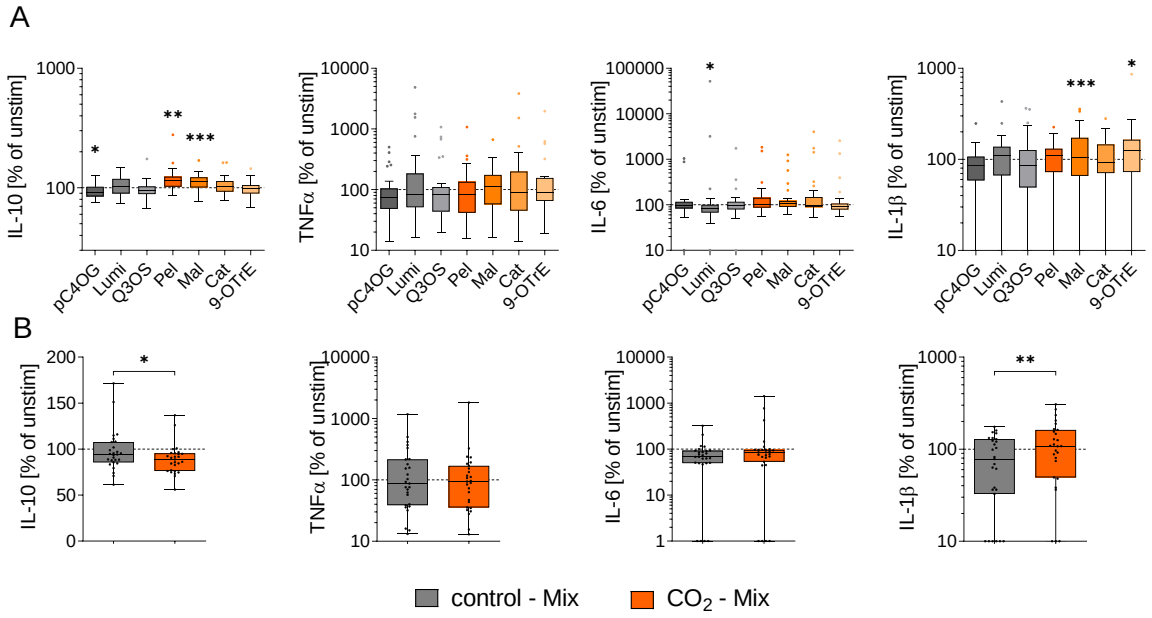

Figure 18 Cytokine response of moDCs stimulated with identified pollen metabolites. MoDCs were stimulated with either single substances (A) or compound mixes corresponding to the RWE compositions (B) (concentration $3x10^{-6}$ M). Cytokines and chemokines were measured in the supernatants after 24 h. n=24 independent experiments. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. unstimulated DCs or as indicated. Dashed line represents unstimulated control.

3.2. Discussion

Main findings

Exposure to elevated CO_2 levels (700 ppm) during the vegetation period of ragweed plants leads to

- Stronger *in vivo* allergic lung inflammation in mice sensitized with CO_2-RWE,
- Stronger *in vitro* pro-inflammatory responses in DCs stimulated with either CO_2-RWE-conditioned HNEC supernatants or CO_2-RWE directly,
- The differential expression of secondary-plant metabolites which elicit responses in DCs similar to whole RWE when they are applied as a mixture *in vitro*.

In the foreseeable future, climate change will pose a considerable threat to global health and increase the burden of allergic airways diseases by favoring the spread of allergenic plants (Lake et al., 2018; Storkey, Stratonovitch, Chapman, Vidotto, & Semenov, 2014; Watts et al., 2019). A major driving force of our changing climate are elevated CO_2 levels (Meehl, 2007). Even though CO_2 is a natural contributor to plant growth and physiology, increasing CO_2 levels have been shown to increase leaf thickness, negatively impacting on the climate by reducing carbon fixation (Kovenock & Swann, 2018). Additionally, doubling CO_2 levels have led to increased pollen production of ragweed plants (Rogers et al., 2006; Wayne et al., 2002), raising their impact on allergic patients (Ariano et al., 2015; Jones et al., 2019; Schmidt, 2016).

Part of this thesis aimed to investigate whether doubling ambient CO_2 levels from currently ~400 ppm to 700 ppm affects the allergenic potential of pollen by exposing ragweed plants to CO_2 levels proposed by a rather conservative ICPP scenario (RCP 4.5) during their growth. The following excerpt from Rauer et al. (2020) represents my own work for the publication and has partly been published as such.

Rauer et al. (2020) reported a stronger allergic phenotype in a murine sensitization model when mice were sensitized with pollen extracts from plants grown under 700 ppm CO_2. Intranasal instillations of CO_2-RWE led to elevated inflammatory cell infiltrates and mucus hypersecretion in the lung, higher inflammatory mediators in BAL fluid as well as higher serum IgE levels. Acute and chronic ragweed pollen exposure also led to elevated numbers of dendritic cells and ILC2s in lung and cervical lymph nodes, all hallmark characteristics of allergic inflammation (Rauer et al., 2020).

Translating these findings from mouse to human, different *in vitro* models were used to simulate the pollen turnover from inhalation and first contact with the nasal mucosa to IgE production by B cells. The nasal epithelium provides the first barrier aeroallergens encounter and plays a key role in allergic sensitization. Airway epithelial cells (ECs) respond to pollen stimulation with inflammasome-related cytokines IL-18 and IL-1β (Bergougnan et al., 2020). In keratinocytes, stimulation with RWE also activated the inflammasome by IL-1β secretion and caspase-1 activation (Dittlein et al., 2016). Here, both CO_2- and control-RWE induced IL-1β together with pro-inflammatory cytokines in HNECs. IL-1 family cytokines have been shown to promote Th2 in the absence of IL-12 (Caucheteux et al., 2016; Xu et al., 2000) and Th9 differentiation in the presence of TGF-β (Uyttenhove, Brombacher, & Van Snick, 2010) as well as proliferation of Th2 clones (Lichtman, Chin, Schmidt, & Abbas, 1988; Taylor-Robinson & Phillips, 1994). Allergen specific Th2 cell activation and airway inflammation has also been shown to require IL-1 in a mouse model of asthma (Nakae et al., 2003). Therefore it is likely that secretion of IL-1β in RWE-stimulated HNECs contributes to a Th2 promoting effect downstream of the nasal epithelium, as has similarly been observed *in vivo*.

Elevated CO_2 levels during plant growth also had an impact on DCs, downstream effector cells of ECs, activated with RWE-conditioned HNEC supernatants. Upon incubation with supernatants from CO_2-RWE-stimulated HNECs, DCs produced more pro-inflammatory cytokines and chemokines, especially Th2-cell attractant CCL17 and pro-inflammatory IL-6 and TNFα, compared to DCs exposed to control-RWE stimulated HNEC supernatants.

DC-induced Treg differentiation is mediated by IL-10, the hallmark cytokine for regulatory immune responses (C. A. Akdis & Akdis, 2014). Direct stimulation of DCs with CO_2-RWE or the CO_2 compound mix induced less IL-10 compared to control-RWE or the control compound mix. In line with this, pulmonary Treg numbers were reduced in mice sensitized with CO_2-RWE (Rauer et al., 2020). These findings are supported by a recently published study indicating impaired IL-10 signaling on dendritic cells fails to induce tolerance accompanied by an influx of neutrophils into BAL fluid (Dolch et al., 2019).

TNFα was consistently induced in CO_2-RWE stimulated DCs and is another critical factor in allergic sensitization (Bachus et al., 2019; J. P. Choi et al., 2012). It has been shown to act as an adjuvant in house-dust mite allergic sensitization (Lambert, Selgrade, Winsett, & Gilmour, 2001) and contributes to allergic asthma exacerbation (Kips, 2001). IL-6 secretion was upregulated in DCs stimulated with CO_2-RWE conditioned HNEC supernatants. Activation of

NFAT and up-regulation of SOCS-1 expression in naïve $CD4^+$ T cells by DC derived IL-6 is implicated in facilitating Th2 polarization and simultaneously inhibiting Th1 (Diehl & Rincon, 2002).

An important factor for Th2 differentiation is the expression of CD80 and CD86 on antigen-presenting cells (Li et al., 2016). DCs exposed to either CO_2-RWE-stimulated HNEC supernatants or both RWEs directly increased expression of both markers. Although CD83 expression appears to be important for $CD4^+$ T cell activation (Aerts-Toegaert et al., 2007) the role of its expression on DCs is discussed controversially (Prazma & Tedder, 2008). Compared to stimulation with unstimulated HNEC supernatants, CD83 was downregulated on DCs exposed to CO_2-RWE-stimulated HNEC supernatants. This finding, combined with the expression of CD80/CD86, emphasizes the importance of the mode of DC stimulation, either by RWE directly or indirectly via HNEC supernatants.

Not only are RWEs activating epithelial-DC cross-talk, but they have also been shown to act directly on B cells, increasing IgE secretion under Th2-mimicking conditions (Oeder et al., 2015). Here, an increased IgE response was demonstrated for under CO_2-RWE *ex vivo* as well as *in vivo* compared to control-RWE (Rauer et al., 2020). By acting on several levels of the immune response, RWE contributes to the clinical phenotype of ragweed allergy. Especially DC-mediated sensitization and B cell-mediated IgE production were both enhanced under exposure to CO_2-RWE.

Pollen-associated lipid mediators (PALMs) are known to have immune modulatory properties and were analyzed to identify one or more substances responsible for the observed CO_2-RWE-induced increased allergic response (Gilles, Mariani, Bryce, Mueller, Ring, Behrendt, et al., 2009; Traidl-Hoffmann et al., 2005). During allergic sensitization, pollen-derived adenosine appears to be protective and has been shown to induce regulatory responses in dendritic cell-primed T cells *in vitro* (Gilles et al., 2011). In contrast, adenosine also mediates exacerbation of allergic lung inflammation *in vivo* (Wimmer et al., 2015). Lipopolysaccharide (LPS) is often present in allergen extracts in low doses and has also been reported to facilitate allergic sensitization to house dust mite via TLR4 signaling in the airways (Eisenbarth et al., 2002; Hammad et al., 2009).

The increased inflammatory response upon CO_2-RWE exposure is only partly explained by the slightly elevated PALM, LPS and adenosine levels in CO_2-RWE. Therefore, the analysis was broadened to investigate the pollen metabolome.

The metabolome analysis revealed a plethora of secondary plant metabolites in pollen which were regulated differentially by the plant's CO_2 treatment. Malvidin, pelargonidin, 9-oxo-OTrE and catalposide were identified in CO_2-RWE, and quercetin-3-*O*-sophoroside, lumichrome and *p*-Coumaryl-alcohol-4-*O*-glucoside were found exclusively in control-RWE. Flavonoids are responsible for the color and aroma of fruits and flowers and can be divided into six subclasses: anthocyanidins, chalcones, flavanones, flavones, flavonols, and isoflavonoids. Their role in immune modulation and as potential therapeutic targets has been studied for a long time. Flavonoids have been shown to possess anti-inflammatory, anti-oxidative, anti-bacterial, anti-viral, anti-cancer and hepatoprotective properties (Kumar & Pandey, 2013) and have also been indicated to be anti-allergic by inhibiting basophil mediated histamine release, as well as IL-4, IL-13 and CD40L expression (Kawai et al., 2007). **Malvidin**, an anthocyanidin found in red wine grapes, has been shown to exhibit anti-inflammatory and antioxidant effects in a macrophage cell line *in vitro* by inhibiting LPS induced NF-κB signaling and ROS production, possibly contributing to the protective effects of red wine on cardiovascular disease, hypertension and diabetes (Bognar et al., 2013). **Pelargonidin**, another anthocyanidin identified in our study, is known to reduce nitric oxide (NO) production in macrophages *in vitro* by inhibiting NF-κB activation (Hämäläinen, Nieminen, Vuorela, Heinonen, & Moilanen, 2007).

The flavonol quercetin has long since been shown to play an important role as an immunosuppressor by interfering in the NF-κB cell signaling pathway (Nam, 2006). Quercetin was of special interest for in this study, because not only does it inhibit DC activation (R. Y. Huang et al., 2010) but its glycosylated form, **quercetin-3-*O*-sophoroside (Q3*O*S),** has also been found to be a natural ligand of the Bet v 1, the major birch pollen allergen (Seutter von Loetzen et al., 2014). Q3*O*S has also been shown to induce IL-10 secretion in DCs of atopic patients (Soh et al., 2019).

The anti-inflammatory effect of Chinese herbal medicines has been attributed to iridoid glycosides, secondary plant metabolites responsible for the bitter taste of some plants. One such iridoid glycoside, **catalposide**, has been shown to inhibit LPS induced TNFα, IL-6 and IL-1β secretion of macrophages *in vitro* in an NF-κB dependent manner (An et al., 2002).

Oxylipins, such as **9-Oxo-OTrE**, are synthesized from plant poly-unsaturated fatty acids by lipoxigenases in response to pathogenic stimuli and exhibit antimicrobial properties (Prost et al., 2005). Their role in allergy and allergic asthma has been studied extensively (Haeggstrom

& Funk, 2011; Holgate, Peters-Golden, Panettieri, & Henderson, 2003), marking 9-oxo-OTrE as yet another a prime suspect in this study.

So far, few studies investigated the biological role of ***p*-Coumaryl-alcohol-4-*O* glucoside**, which is a precursor of lignin (Jansen et al., 2014), a major component of plant cell walls. Lignin is found in most plant-derived food sources and lignin-carbohydrates have been identified as novel TLR4 ligands on DCs, depending on the degree of polymerization (Tsuji, Ikado, & Fujiwara, 2017). Therefore a potentially immune-modulatory role cannot be excluded. Additionally, lignin-carbohydrates appeared to increase mucosal IgA and serum IgG secretion, which makes them a promising novel candidate as mucosal vaccine adjuvant (Tsuji et al., 2017).

Lumichrome is a derivative of Riboflavin (Vitamin B2), which is an essential human nutrient and can be found in a variety of foods. Riboflavin is important for the generation of reactive oxygen species (ROS) and therefore plays an important role in the pathogen defense (Schramm et al., 2014). Even though it is beneficial and riboflavin deficiency has been linked to an impaired macrophage function (Mazur-Bialy, Buchala, & Plytycz, 2013), cases of riboflavin allergy have been reported previously (Ou, Kuo, & Huang, 2001). In addition, lumichrome and riboflavin have been shown to act as quorum sensing molecules, possibly playing an important role in the regulation of the gut microbiota (Rajamani et al., 2008).

Summarizing the literature, the compounds identified in CO_2 pollen (Malvidin, pelargonidin, catalposide, 9-Oxo-OTrE), were expected to be mostly anti-inflammatory, whereas substances from control pollen (lumichrome, Q3OS and p-Coumaryl-alcohol 4-*O* glucoside) were expected to be more tolerogenic. When DCs were stimulated with pelargonidin or malvidin alone, anti-inflammatory responses were observed, whereas p-Coumaryl-alcohol 4-*O* glucoside induced pro-inflammatory responses and the other substances did not have any effects. However, there appeared to be a synergistic effect of the compound mixtures which induced a cytokine profile that was comparable to the whole pollen extracts. Contrary to the expectations, the substances reported as anti-inflammatory were eliciting pro-inflammatory reactions when combined with each other. Especially the secretion of IL-10 in DCs was reduced, in addition to increased IL-1β levels by the CO_2-RWE-mix.

These results have to be interpreted with caution, however, because metabolic screening was performed in a non-targeted, semi-quantitative manner. This provides a global overview of the pollen metabolome without insight into absolute quantities of the significantly modulated compounds. Also, annotation of the substances was done according to their exact mass and elemental composition, leading to ambiguous multi-annotations. Finally, the compounds used for the subsequent stimulation experiments were chosen in a hypothesis-driven manner according to their immunological properties and commercial availability. Nevertheless, the results further add to the hypothesis that allergic sensitization is much more than just the reaction of the immune system to a single allergen or substance, but that the allergen matrix as a whole plays a crucial role in the transmission of integrated signals via DCs to downstream effector cells of the adaptive immune response, namely T and B cells.

Interestingly, neutrophil migration towards control-RWE was higher than towards CO_2-RWE, which is in contrast to the *in vivo* data (Rauer et al., 2020). However, instead of direct effects of ragweed extracts on chemotaxis the mouse model reflects sensitization and allergy, which is characterized by neutrophil and eosinophil migration towards the lung, a process mediated by chemokine secretion of epithelial cells and alveolar macrophages. Lipid-mediators and adenosine found in pollen have been known to induce neutrophil and granulocyte chemotaxis *in vitro* (Plotz et al., 2004; Traidl-Hoffmann et al., 2002; Wimmer et al., 2015), although they were not regulated differentially in the present study. Possibly, subtle changes in the pollen matrix are responsible for the increased direct chemotactic potential of control-RWE, which may be the reason the substances were not detected in the metabolome analysis.

The climate change scenario applied for this study was based on the respective pathway scenario (RCP) 4.5, which assumes that CO_2 emissions will peak between 580-720 ppm by 2040 and then start to decline, likely leading to an increase in global temperatures between 2-3°C (Edenhofer, 2014). This is a rather conservative scenario. If current efforts to reduce carbon emissions fail or business continues as usual (according to RCP8.5 scenario), we might expect CO_2 levels above 1000 ppm by 2100 (Meehl, 2007). So far, the effect of such an increase in atmospheric CO_2 on allergenic plants has not been studied, although it is common practice to increase CO_2 levels to 540-1200 ppm to improve the quality of cultivated crops (Dong, Gruda, Lam, Li, & Duan, 2018). This seems to mainly affect flavonoid, phenol and antioxidant pathways in some plant species (Dong et al., 2018). In addition, global warming will lead to

more and longer dry and hot periods. Elevated CO_2 levels in combination with draught stress have already been shown to alter ragweed pollen on the transcriptome level, potentially enhancing their allergenic potential (El Kelish et al., 2014). Combined with an increased ragweed pollen production under elevated CO_2 concentrations (Rogers et al., 2006), it is possible that pro-inflammatory metabolites will further increase, while pollen exposure will simultaneously surge under a more pessimistic IPCC scenario. Because this is mostly speculation, further research should focus on exposure to higher CO_2 levels during the vegetation period of allergenic plants in combination with targeted metabolome analyses. In this line, metabolic labeling of ragweed plants would be especially interesting and provide the opportunity to finally show which substances are increased under elevated CO_2 levels. Subsequent *in vivo* and *in vitro* experiments, similar to those performed by Rauer et al. (2020), will then show which substances are taken up by tissue cells, offering insights into their relevance for allergic sensitization.

To conclude, pollen from ragweed plants grown under elevated CO_2 levels induced stronger allergic immune responses compared to control pollen. Additionally, pollen allergenicity was not confined to a single pollen-derived substance, but rather depended on an intricate interplay of different substances. This study, among others, demonstrates the effect of climate change on plants and pollen allergenicity, further emphasizing the dire implications of climate change for human health.

Chapter 4 – Effect of elevated O_3 levels during ragweed growth on immune modulatory potential of ragweed pollen

Gaseous and particulate air pollutants in the atmosphere are known to affect the allergenicity of pollen (Sénéchal et al., 2015). For example, exposure of birch trees to high O_3 levels during their flowering season influences the immune-modulatory capacity of birch pollen extracts (Beck et al., 2013). O_3 is a radical-forming pollutant and especially elevated during the summer months. Many allergens are susceptible to abiotic stress, such as elevated ground-level O_3, which induces defense mechanisms in plants (Sandermann, Ernst, Heller, & Langebartels, 1998). To investigate this effect, ragweed plants were grown in climate chambers and exposed to either low (40 ppb O_3, control-RWE) or high (80 ppb O_3, O_3-RWE) levels of O_3. The pollen extracts were tested for their allergenic potential in various *in vitro* cell culture systems.

4.1. Results

4.1.1. Cytokine response of human nasal epithelial cells to O_3-RWE

To analyze the effect of O_3 during plant growth on the immune stimulatory potential of pollen, HNECs were stimulated with control- or O_3-RWE for 24 h. Cytokines and chemokines were measured in the supernatants. While both RWEs induced IL-1β and IL-8 secretion compared to unstimulated, there was no difference between the two treatments (Figure 19 , $p < 0.01$ and $p < 0.001$ respectively). Increased IL-6 levels were observed for treatment with 2.5 mg/ml control-RWE ($p < 0.01$) and 0.6 mg/ml and 2.5 mg/ml O_3-RWE ($p < 0.05$ and $p < 0.01$ vs unstimulated respectively) (Figure 19). CCL2 was significantly higher upon treatment with low concentrations of O_3-RWE ($p < 0.01$, $p < 0.001$ vs unstimulated) and 0.6 mg/ml control-RWE ($p < 0.05$ vs unstimulated) (Figure 19). O_3-RWE also enhanced CCL2 secretion at high concentrations (Figure 19 F, $p < 0.05$ vs unstimulated), while TNFα was unaffected by either treatment (Figure 19).

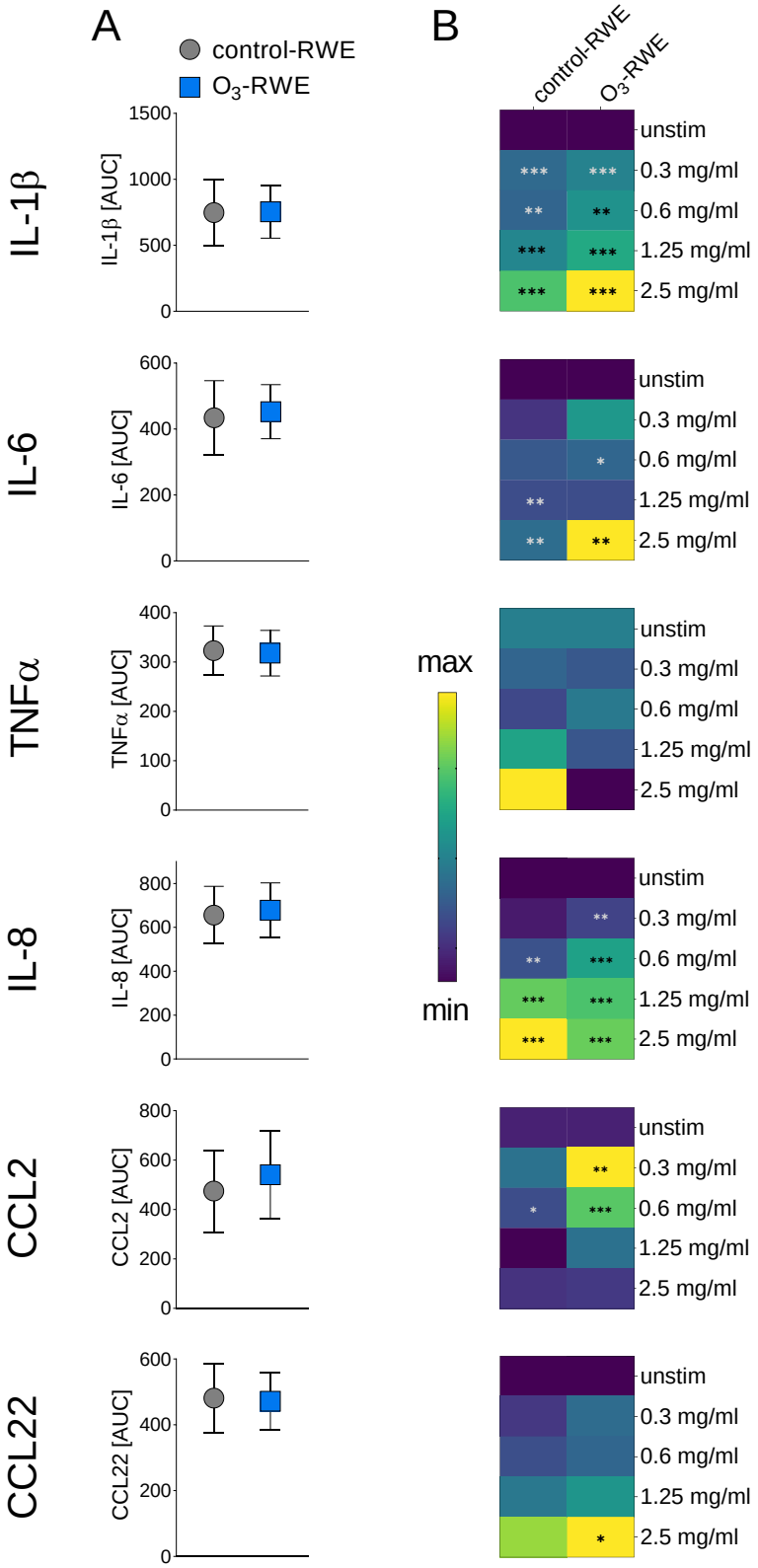

Figure 19 Cytokine and chemokine release of HNECs upon stimulation with control- and O_3-RWE. HNECs were stimulated with RWEs and cytokines and chemokines were measured in the supernatants after 24 h. n = 4 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to unstimulated (B) or as indicated (A). AUC: area under the curve.

4.1.2. Response of immature DCs to O_3-RWE stimulation

To investigate the potential of O_3-RWE to induce allergic sensitization, moDCs were stimulated with the different RWEs. Cytokine and chemokines released into the supernatants were measured (Figure 21) and the cells were analyzed for their maturation and differentiation marker expression (Figure 20). CD40, CD83, CD86 and HLA-DR were expressed higher by moDCs treated with control-RWE at the highest concentration compared to the unstimulated control ($p < 0.05$). O_3-RWE and control-RWE both significantly increased CD80 expression compared to unstimulated ($p < 0.001$), but there was no difference detected between the treatment groups in the analyzed cell surface markers (Figure 20).

IL-10 ($p < 0.001$), IL-6 ($p < 0.01$), IL-1β ($p < 0.01$) and IL-4 ($p < 0.01$) were induced more by control-RWE compared to O_3-RWE. CCL17 was increased by O_3-RWE ($p < 0.01$) (Figure 21 A). This was mirrored in the patterns observed in the heat maps, and while the treatments induced different cytokines compared to each other, there was no difference compared to unstimulated cells depending on RWE concentrations (Figure 21 B).

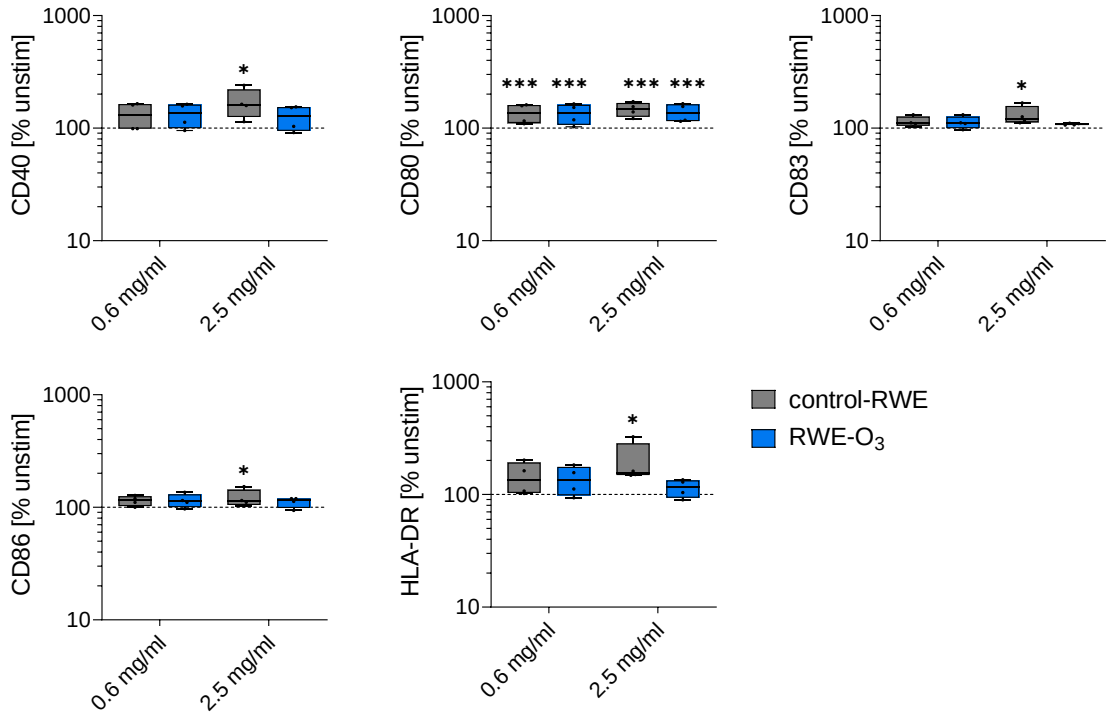

Figure 20 Cell surface marker expression of moDCs. Expression of maturation markers after RWE stimulation (0.6 mg/ml and 2.5 mg/ml) was measured after 24 h. n = 4 independent experiments, * $p < 0.05$, *** $p < 0.001$ vs unstimulated. Dashed line represents unstimulated control.

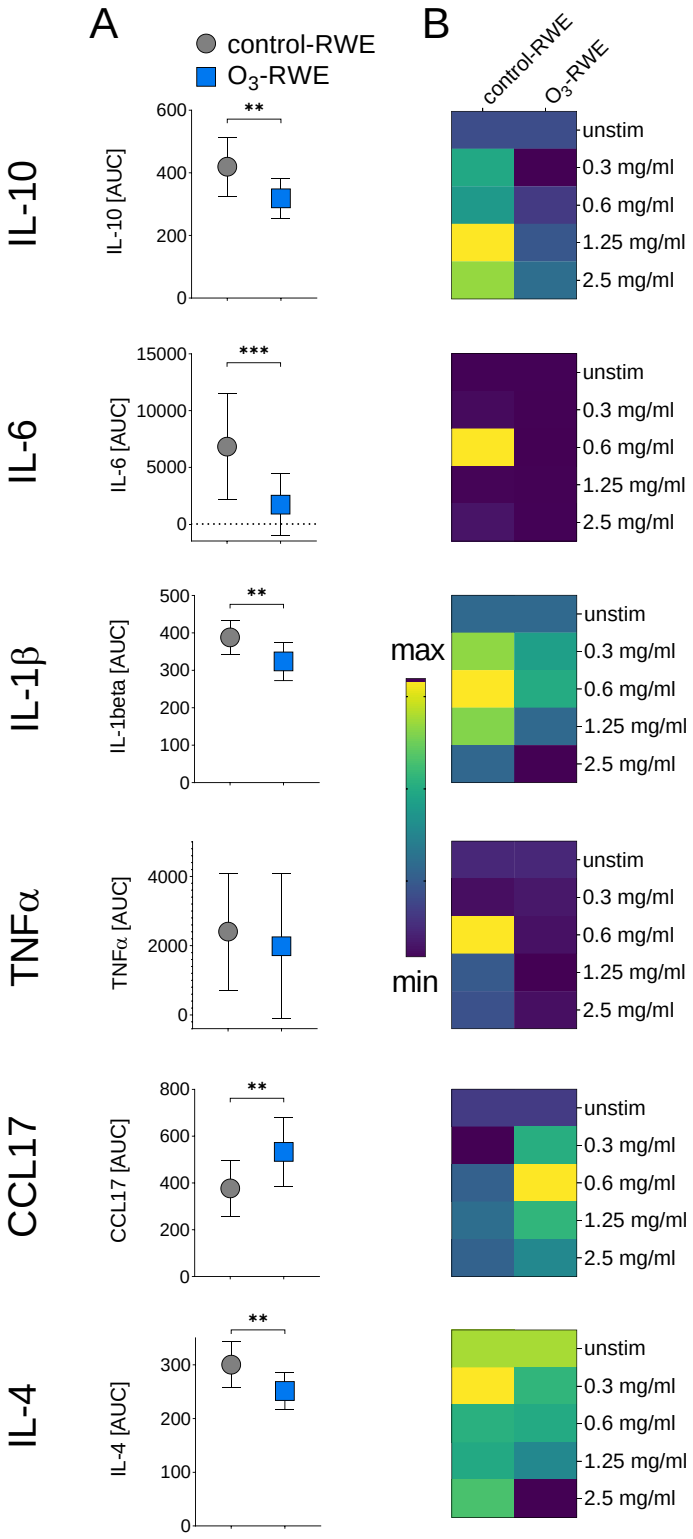

Figure 21 Cytokine and chemokines secreted by RWE stimulated moDCs. MoDCs were stimulated with 0.3 mg/ml – 2.5 mg/ml control- or O_3-RWE and cytokines and chemokines were measured in the supernatants after 1 h. (A) Area under the curve (AUC) and heat map (B) of cytokines/chemokines. n = 4 independent experiments. ** $p < 0.01$, *** $p < 0.001$ comparisons as indicated. AUC: area under the curve.

4.1.3. Response of LPS-primed DCs to O_3-RWE stimulation

Exposure of birch trees to high ozone levels during growth has been shown to skew LPS induced Th1 differentiating cytokines of moDCs upon stimulation with birch pollen extracts. To analyze the Th1 differentiating potential of ragweed pollen grown under elevated O_3 levels, moDCs were stimulated with LPS in absence or presence of RWEs and cytokines/chemokines in the supernatant and cell surface markers were analyzed after 24 h. While both RWEs strongly inhibited LPS-induced IL-12 secretion (Figure 22 A, $p < 0.05$, $p < 0.01$ and $p < 0.001$ vs LPS control), the observed effect was stronger upon control-RWE stimulation (Figure 22 A, $p < 0.001$ vs O_3-RWE). TNFα secretion was also inhibited more strongly by control-RWE ($p < 0.05$), although this was an overall effect and not seen for the single concentrations (Figure 22). While high concentrations of both, control- and O_3-RWE ($p < 0.05$ and $p < 0.01$ vs LPS respectively) significantly reduced LPS-induced IL-6 secretion, there was no difference between the two treatments (Figure 22, control-RWE: $p < 0.05$ and O_3-RWE: $p < 0.01$, vs LPS control). Neither IL-10 nor CCL17 secretion were inhibited significantly by control- or O_3-RWE (Figure 22).

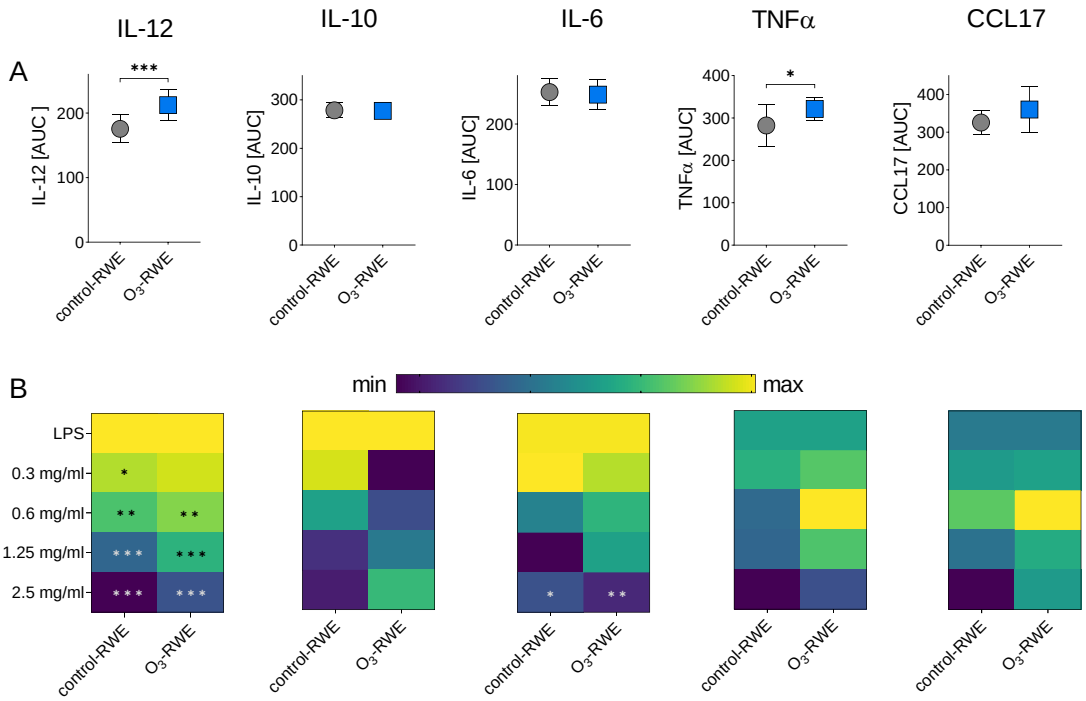

Figure 22 O_3-RWE inhibits LPS-induced Th1 cytokine secretion less than control-RWE. Cytokines secreted by moDCs stimulated with LPs in the presence or absence of RWEs (0.3 mg/ml – 2.5 mg/ml) were measured in the supernatants. (Top) Area under the curve was calculated to compare between treatments. (Bottom) Heatmaps represent inhibition of LPS induced cytokines/chemokines. n = 4 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ either in comparison to LPS stimulation control or as indicated. AUC: area under the curve.

Although inhibition of cell surface marker expression was rather low upon RWE treatment, both control- and O_3-RWE reduced the LPS-induced expression of CD40, CD80, CD83, CD86 and HLA-DR (Figure 23, $p < 0.05$, $p < 0.01$ and $p < 0.001$ vs LPS control). Control-RWE also decreased CD40 and CD86 stronger than O_3-RWE (Figure 23, $p < 0.05$).

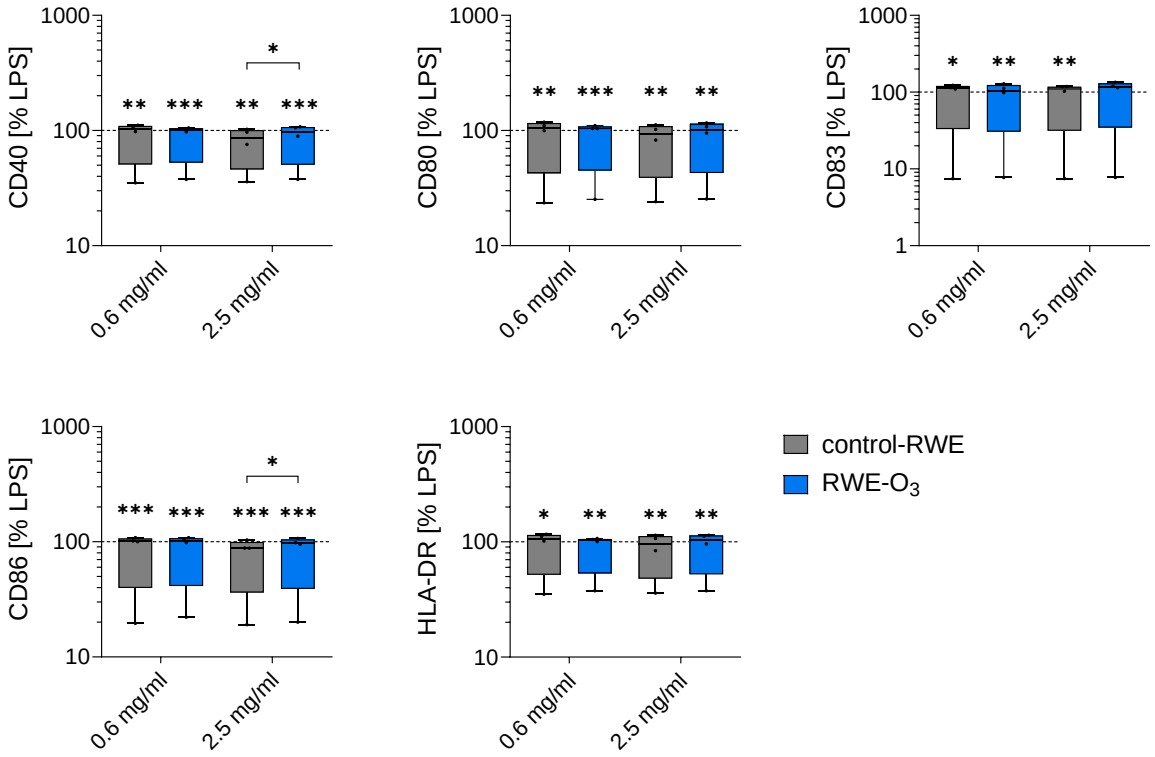

Figure 23 LPS induced maturation marker expression on moDCs was reduced by RWE stimulation. Expression of maturation markers after RWE stimulation (0.6 mg/ml and 2.5 mg/ml) was measured after 24 h. n = 4 independent experiments, * $p < 0.05$, *** $p < 0.001$ vs unstimulated or as indicated. Dashed line represents unstimulated control.

4.1.4. Neutrophil migration towards O_3-RWE

Pollen from birch trees collected in areas with high O_3 exposure have been shown to be more potent in inducing neutrophil chemotaxis *in vitro*. To assess if this is also true for ragweed pollen, RWEs from plants grown under different O_3 levels were investigated for their chemotactic potential. Neutrophils were exposed to 2.5 mg/ml RWE or LTB_4 as positive control for one hour and a migration index was calculated. While the migration index was not very high compared to LTB_4, O_3-RWE significantly increased neutrophil migration compared to control-RWE (Figure 24 A, $p < 0.05$). Because pollen-associated lipid mediators (PALMs) have been indicated to act as chemo-attractants for granulocytes, LTB_4- and PGE_2-like substances were measured in the pollen extracts by ELISA. $PALM_{LTB4}$ levels were higher, while $PALM_{PGE2}$ levels were lower in O_3-RWE than in control-RWE (Figure 24 B).

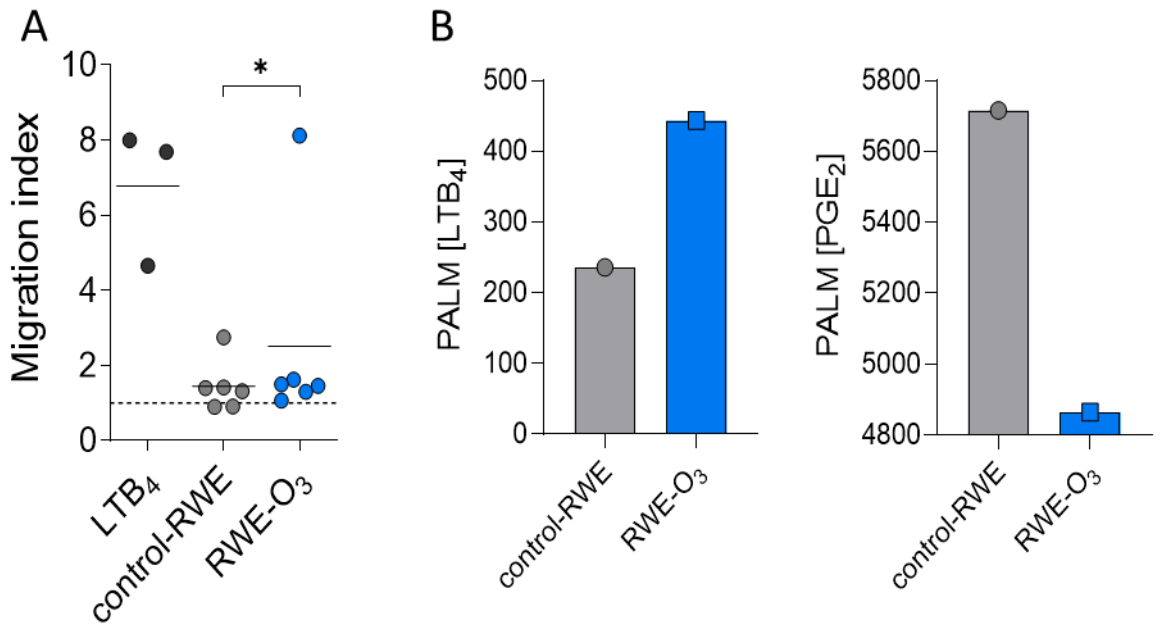

Figure 24 O_3-RWE acts as chemo-attractants for neutrophils in vitro. (A) Neutrophil migration towards 2.5 mg/ml RWE or LTB_4 (10^{-7} M). n = 7 independent experiments. * $p < 0.05$ as indicated. Dashed line represents unstimulated control. (B, C) Levels of PALMs in RWEs.

4.1.5. IgE secretion of murine B cells in response to control- and O_3-RWE

The effect of elevated O_3 levels during ragweed growth on IgE secretion was investigated by stimulating murine naïve splenic B cells for 8 days with CD40L and IL-4 to prime for Th2-mediated IgE secretion, in addition to RWE stimulation. Low concentrations of both RWEs significantly increased CD40L/IL-4 induced IgE secretion ($p < 0.05$). Overall, there was no difference detected between control- and O_3-RWE (Figure 25).

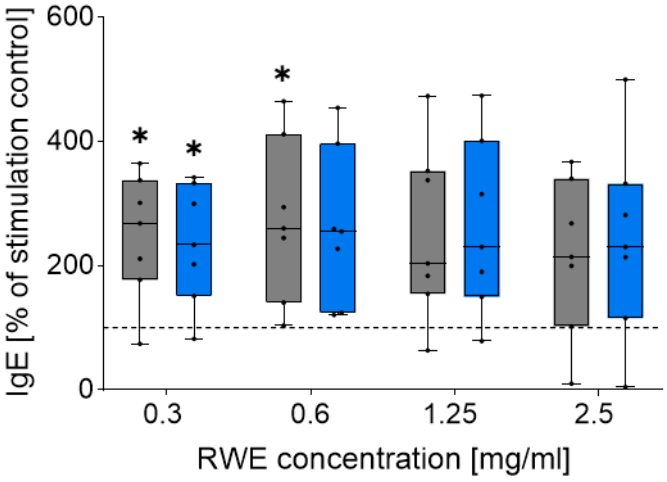

Figure 25 control and O_3-RWE both increase IgE secretion under Th2 simulating conditions. Naïve murine splenic B cells were stimulated with CD40L and IL-4 to mimic Th2 conditions in addition to RWE stimulation (0.3 mg/ml – 2.5 mg/ml) for 8 days. Secreted IgE was measured in the supernatants. n = 7 independent experiments. * $p < 0.05$ vs CD40L/IL-4 control (indicated by dashed line).

4.2. Discussion

Main findings

Exposure to elevated O_3 levels (80 ppb) during the vegetation period of ragweed plants leads to:

- Higher direct neutrophil chemotaxis towards O_3-RWE *in vitro*,
- Less cytokine secretion in immature DCs stimulated with O_3-RWE *in vitro* and
- Lower cytokine inhibition in LPS-primed DCs stimulated with O_3-RWE *in vitro.*

Tropospheric ozone is a secondary pollutant and is the byproduct of a photochemical reaction between nitrogen oxides (NO_x) and volatile organic compounds. O_3 levels fluctuate seasonally and are generally increased in summer months because higher temperatures favor O_3 generation. Since O_3 measurements were started in the 1970s, a trend has been observed for increased overall surface O_3 levels (Monks et al., 2015). Although this appears to be mitigated in some areas in Western Europe in recent years due to efforts in reducing emissions of O_3 precursors, an increasing trend persists for Southeast Asia and central Africa (Ziemke et al., 2019). Nevertheless, climate change projections estimate an increase in high ozone days by the early and mid-century (Archer, Brodie, & Rauscher, 2019). Not only will this lead to an increase in cardiovascular diseases, respiratory allergies, and asthma, but plants will also be influenced by prolonged exposure to elevated O_3 levels. O_3 and associated ROS are phytotoxic for plants by inducing metabolic changes and reducing photosynthesis among others, leading to injury, reduced carbon assimilation and premature leaf senescence (Emberson et al., 2018). Exposure to O_3 also appears to affect the allergenicity of pollen, although in a species specific manner (Frank & Ernst, 2016). Although most studies focus on the allergen and protein content of allergenic pollen, Beck et al. (2013) also showed altered immunological responses in birch allergic patients towards birch pollen from sampling sites exposed to high ozone levels during the flowering period.

Building on these findings, this part of the thesis aimed to understand how ragweed allergenicity is affected by prolonged exposure to elevated O_3 levels. Therefore during the whole vegetation period, ragweed plants were exposed to ambient O_3 levels (40 ppb) and double the amount (80 ppb) (Kanter et al., 2013), which is considered a dangerous threshold for human health by the world health organization (World Health, 2006). Aqueous pollen

extracts from these plants were used for *in vitro* experiments simulating the process of allergic sensitization.

Similar to the above discussed CO_2-RWE experiments, stimulation with RWE led to increased IL-1β, IL-6 and IL-8 secretion in HNECs independent of plant treatment. A common mechanism for ragweed pollen appears to be the activation of the NLRP3 inflammasome in epithelial cells, leading to increased IL-1β secretion, which has been shown to be caspase-1 dependent in keratinocytes (Dittlein et al., 2016). Additionally, RWE-intrinsic NADPH oxidase activity likely contributed to elevated cytosolic ROS in LPS-primed macrophages and dendritic cells and subsequent IL-1β secretion (Varga et al., 2013). Furthermore, IL-1β was elevated in bronchial epithelial cells and bronchoalveolar lavage fluid (BALF) of ragweed sensitized asthma patients as well as non-atopic study participants after RWE inhalation (Hastie et al., 1996). Pro-inflammatory responses of HNECs towards ragweed pollen extracts were also reported by Rauer et al. (2020) which are rather unaffected by plant treatment and appear to represent a general ragweed specific effect (Rauer et al., 2020).

Immature DCs stimulated with O_3-RWE secreted less regulatory IL-10, pro-inflammatory IL-6 and IL-1β and the Th2 cytokine IL-4. IL-10 secretion by DCs is the hallmark of Treg differentiation and essential for efficient tolerance induction (C. A. Akdis & Akdis, 2014; Dolch et al., 2019). Th1 differentiation of naïve $CD4^+$ T cells has been shown to be inhibited by IL-6, thereby facilitating Th2 differentiation (Diehl & Rincon, 2002). Corresponding to lower IL-6 levels, IL-4 secretion was also weaker in DCs stimulated with O_3-RWE. IL-4 is the most important Th2 cytokine and induces IgE production in B cells, and has recently been identified in extracellular vesicles of mature DC (Schierer et al., 2018).

Both RWEs induced CD80 expression, but only control-RWE induced CD40, CD83, CD86 and HLA-DR expression. These co-stimulatory molecules are important to mount a DC mediated T cell response and a lack thereof in O_3-RWE stimulated DCs, together with the lower cytokine response, corresponds to the generally lower immune-stimulatory potential of O_3-RWE.

The allergenic potential of pollen is not only characterized by immune-stimulatory properties, but Th1/Th2 skewing characteristics have also been observed. LPS-primed DCs generally secrete high levels of IL-12, which induces Th1 and inhibits Th2 responses (Kuipers et al., 2004). Modulation of the IL-12 response of DCs is therefore a good marker to measure the

immune modulatory potential. Overall, O_3-RWEs were less immunomodulatory: Weaker IL-12 and TNFα inhibition was observed in LPS-primed DCs co-stimulated with O_3-RWE. Likewise, although both pollen extracts inhibited LPS-induced maturation marker expression, CD40 and CD86 inhibition was less pronounced in DCs co-stimulated with O_3-RWE.

Overall, pollen from plants grown under 80 ppb O_3 were less immune-stimulatory and less immunomodulatory than their counterparts grown under 40 ppb O_3. Kanter et al. (2013) detected lower transcript levels for secondary metabolite pathways corresponding to the flavonoid synthesis in the same pollen used for this work. Reduced transcripts of the flavonoid synthesis pathways have also been observed in beech leaves exposed to O_3 (Olbrich, Gerstner, Welzl, Winkler, & Ernst, 2009). Flavonoids are known for their anti-inflammatory properties and a possible candidate, quercetin, has been shown to increase Th1 cytokines and to reduce IL-4 production in PBMCs (Nair et al., 2002). Various other flavonoids have also been observed to inhibit LPS-induced pro-inflammatory cytokine production (Leyva-López, Gutierrez-Grijalva, Ambriz-Perez, & Heredia, 2016), providing another explanation for the weak inhibition of LPS-induced cytokine secretion compared to control-RWE.

Neutrophil migration towards O_3-RWE was higher than towards control-RWE. This is supported by observations in birch pollen. A stronger chemotactic potential was seen for birch pollen from locations with high O_3 levels during the sampling period (Beck et al., 2013). LTB_4 is a critical factor in neutrophil recruitment (Lämmermann et al., 2013) and homologous substances detected in birch pollen have been correlated to increased neutrophil chemotaxis towards pollen from high ozone sampling sites (Beck et al., 2013; Traidl-Hoffmann et al., 2002). Products of the linolenic acid pathway have been shown to play an important role as adjuvant factors in pollen allergies (Gilles, Behrendt, Ring, & Traidl-Hoffmann, 2012; Traidl-Hoffmann et al., 2002; Traidl-Hoffmann et al., 2005). Phytoprostanes, fatty acids derived from α-linolenic acid by auto-and photooxidation, are indicators of oxidative stress in plants. PPE_1 has been shown to be an adjuvant substance found in pollen that is able to suppress Th1 responses initiated by DCs (Traidl-Hoffmann et al., 2005). Hydroxy-octadecadienoic acid/hydroxy-linoleic acid (HODE) and -hydroxy-octadecatrienoic acid/hydroxy-linolenic acid (HOTE) are also products of the linolenic acid pathway and are produced by lipoxygenases (LOX), metabolizing linolenic acid. These lipids are chemotactic for neutrophils (Traidl-Hoffmann et al., 2002; Traidl-Hoffmann et al., 2005). O_3 has been shown to upregulate

lipoxygenase transcription (Maccarrone, Veldink, Vliegenthart, & Finazzi Agro, 1997), which could explain why O_3 pollen are more chemotactic than their controls, but do not act as strong IL-12 suppressors.

Under IgE priming conditions, both RWEs increased IgE secretion of naïve murine B cells. This has been shown to be allergen independent and the PALM PPE_1 has been identified to directly contribute to IgE production of B cells (Oeder et al., 2015).

Detailed insight into the metabolome of ragweed pollen grown under elevated O_3 levels will help elucidate the effects seen in this work in the future. To speculate, lipoxygenases will likely be upregulated in pollen from plants grown under elevated O_3 levels, whereas levels of phytoprostanes and flavonoids might be higher in pollen from control plants, contributing to their stronger Th1 suppressing characteristics.

It should be mentioned that cell culture experiments with 24 h stimulations only allow for a snapshot in the process of allergic sensitization, which in reality is a long lasting process resulting from repeated allergen exposure. The full extent of long-term pollen exposure on the allergic sensitization will only be understood if the *in vitro* data is supplemented with data from a mouse model for allergic sensitization. Wimmer et al. (2015) established such a model using intranasal instillations and were able to distinguish between sensitization to ragweed after three to five consecutive instillations compared to established allergic asthma after eleven instillations. Especially early stages in allergic sensitization were characterized by increased neutrophil influx (Wimmer et al., 2015). So, even though in this thesis O_3-RWE was less immune-stimulatory and –modulatory, the increased chemotactic potential suggests that elevated ozone levels during plant growth could contribute to allergic sensitization by enhanced early neutrophil recruitment.

Because the *in vitro* experiments shown here were conducted using non-atopic cell donors, the effects are likely to differ in cells from ragweed sensitized patients. O_3-RWE might not necessarily enhance allergic sensitization, although it is possible that changes to the cell wall of ragweed pollen grown under elevated O_3 levels as observed by Kanter et al. (2013) lead to an increased availability of allergens and possibly stronger allergy symptoms.

Chapter 5 – Influence of air pollutants and urbanization on the allergenic potential of birch pollen

Levels of air pollutants such as NO_2 and O_3 differ between rural and urban areas. This could impact on the allergenic potential of wind pollinating plants. The effect of increasing air pollution on pollen allergenicity is of great importance for public health measures and city planning. To study this effect, pollen from birch trees in urban or rural areas in Augsburg and Munich, and the surrounding rural regions collected in 2010 (Munich), 2015 (Augsburg) and 2017 (Augsburg) were chosen according to their NO_2 and O_3 exposure. Both air pollutants are associated with urbanization: NO_2 levels are generally higher in heavy traffic urban areas, while O_3 accumulates in rural areas. Since BPEs from Augsburg from 2015 elicited the strongest immune responses across all *in vitro* experiments, they will be the focus of this work. Comparisons to Augsburg 2017 and Munich 2010 BPEs will be briefly touched upon.

5.1. Results

5.1.1. Characterization of birch pollen extracts

Aqueous birch pollen extracts (BPE) were characterized by the levels of NO_2 and O_3 from the collection sites. Sample sites for BPE #22 in Augsburg (2015 and 2017) and BPE #33 from Munich (2010) had higher O_3 and lower NO_2 levels during the respective study period compared their counterparts BPE #5 (Augsburg, 2015 and 2017) and BPE #18 (Munich, 2010) (Figure 26 E). Bet v 1 content in pollen from those sites was also higher than in pollen from sites with low O_3 and high NO_2 (Figure 26 A). LPS content in Augsburg BPE #22 and BPE #18 was higher than in BPE #5 and BPE #33 (Figure 26 B). Adenosine was higher in BPE #22 2017 and BPE #33 than BPE #5 2017 and BPE #18, while the opposite was observed for BPEs from 2015 (Figure 26 C). Based on their $PALM_{LTB4}$ and $PALM_{PGE2}$ profile, BPEs #22 were closely grouped together during both years, as well as BPEs #5. The PALM profile of BPE #33, even though from a rural sampling site, was closer to BPE #5, while BPE #18 hat the highest concentrations of $PALM_{LTB4}$ and $PALM_{PGE2}$ (Figure 26 D).

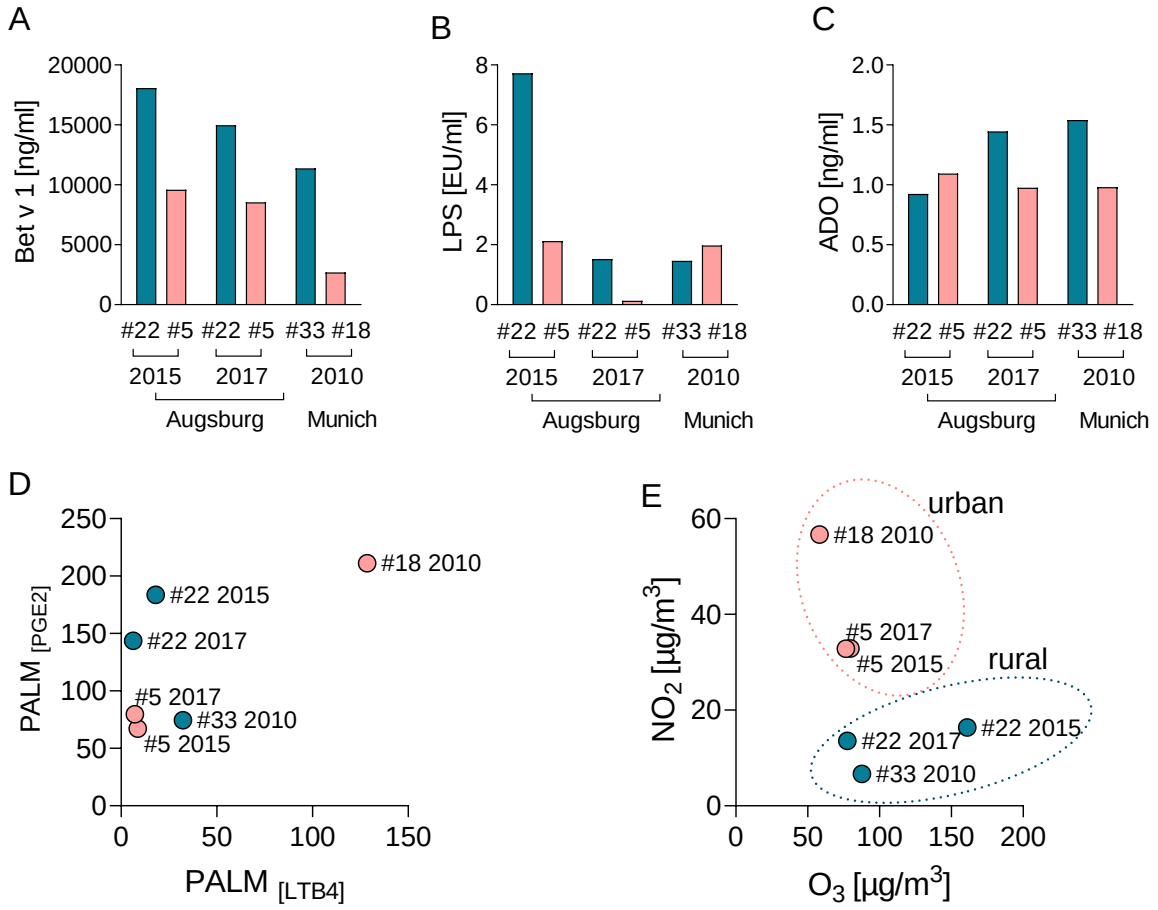

Figure 26 Determinants of allergenicity in BPEs. BPEs from Augsburg were collected from sampling site #22 and #5 in 2015 and 2017. BPEs from Munich sampling sites #18 and #33 were collected in 2010. (A-C) Measurements of Bet v 1 content, LPS and ADO concentration in BPEs. (D) PALM profile and (E) environmental parameters of BPEs. BPE = aqueous birch pollen extract.

5.1.2. Cytokine and chemokine response of HNECs stimulated with BPE

The potential of BPEs from low NO_2/high O_3 (BPE #22 and #33, from now on referred to as ***rural-BPE***) and BPEs from high NO_2/low O_3 (BPE #5 and #18, from now on referred to as ***urban-BPE***), from sites in Augsburg and Munich, respectively, to affect innate immunity was investigated by stimulating HNECs for 24 h with BPEs or LPS controls corresponding to 10 mg/ml BPE.

IL-1β ($p < 0.001$), IL-8 ($p < 0.001$) and TNFα ($p < 0.01$) were increased by stimulation with rural-BPE compared to urban-BPE (Figure 27 A, D, E). IL-6 release was not affected by either BPE (F) or their corresponding LPS controls.

There was no statistically significant difference in secretion of CCL2 and CCL22 when comparing between the two BPEs (Figure 27 B, C), but CCL22 was reduced by both BPEs compared to unstimulated control ($p < 0.001$). LPS controls corresponding to 10 mg/ml BPE

inhibited CCL22 less than the pollen extracts ($p < 0.001$) (Figure 28). LPS controls corresponding to 10 mg/ml BPE reduced IL-1β ($p < 0.001$) and IL-8 ($p < 0.001$ and $p < 0.01$) and CCL2 ($p < 0.5$ and $p < 0.01$) secretion (Figure 28).

Stimulation with BPEs from Augsburg 2017 elicited similar cytokine and chemokine patterns to the corresponding BPEs from 2015. BPEs from Munich birch trees from similar locations elicited the opposite cytokine and chemokine patterns compared to their Augsburg counterparts.

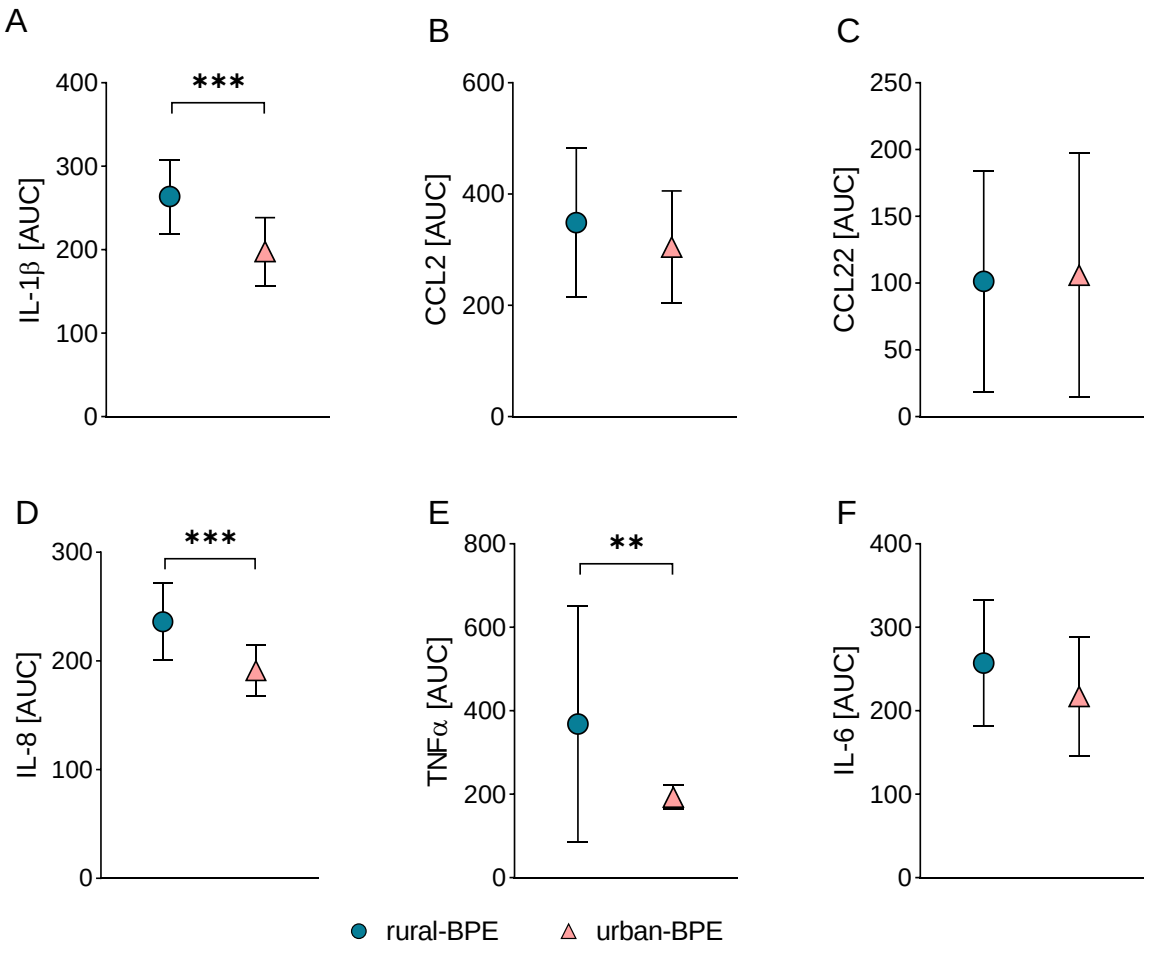

Figure 27 Rural-BPE 2015 elicits pro-inflammatory cytokine response in HNECs. After 24 h stimulation with 1 mg/ml – 10 mg/ml BPE, cytokines were measured in supernatants and an AUC was calculated. n = 9 independent experiments. ** $p < 0.01$, ***$p < 0.001$. AUC: area under the curve.

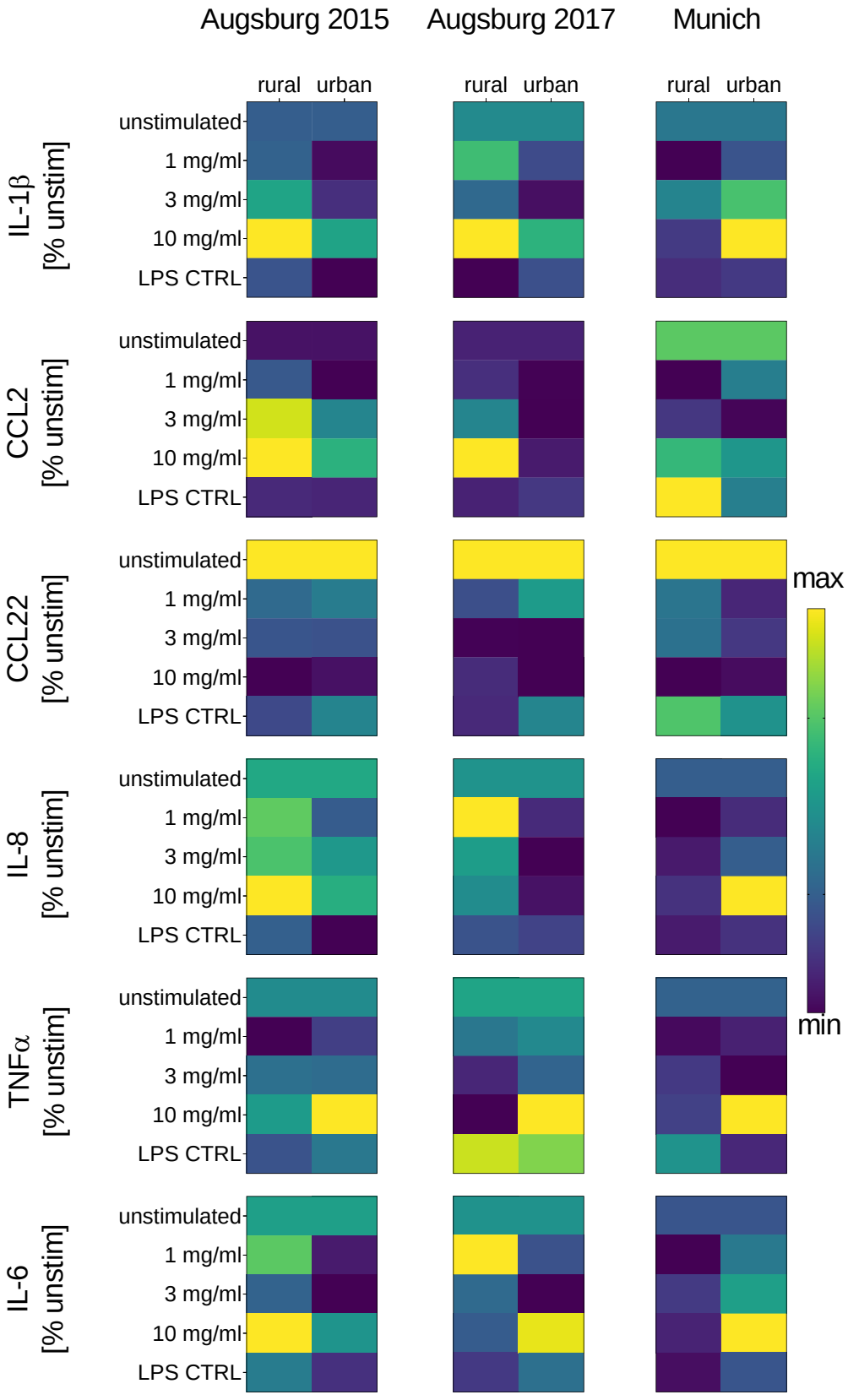

Figure 28 Heat maps of HNECs stimulated with Augsburg and Munich BPEs. HNECs were stimulated with BPEs or LPS corresponding to the LPS content in 10 mg/ml BPE and cytokines were measured after 24. The data were normalized to the unstimulated control. n = 9 independent experiments.

5.1.3. Response of immature DCs to BPE stimulation

The allergenic potential of BPEs from different sampling sites in Augsburg was investigated by treating immature moDCs with either BPE or LPS controls corresponding to LPS concentrations found in the BPEs. After 24 hours, cytokines were measured in the supernatants and cell surface markers were analyzed.

5.1.3.1. Cytokine Secretion

IL-10 and IL-1β secretion was increased upon stimulation with rural-BPE compared to urban-BPE and the corresponding LPS control (Figure 29 A, C; $p < 0.001$ vs urban-BPE or LPS control). Rural-BPE and the corresponding LPS control both induced higher IL-6 secretion compared to their BPE/LPS counterparts ($p < 0.001$). This effect was not different between the BPEs and their corresponding LPS controls (Figure 29 B). TNFα was increased by rural-BPE compared to urban-BPE ($p < 0.001$), but not to its LPS control (Figure 29 D). CCL17 was reduced in moDCs stimulated with urban-BPE compared to rural-BPE ($p < 0.01$), as well as its LPS control ($p < 0.001$). Both BPEs reduced IL-4 secretion compared to their LPS controls ($p < 0.001$), which was more pronounced by rural-BPE compared to urban-BPE (Figure 29 F, Figure 30).

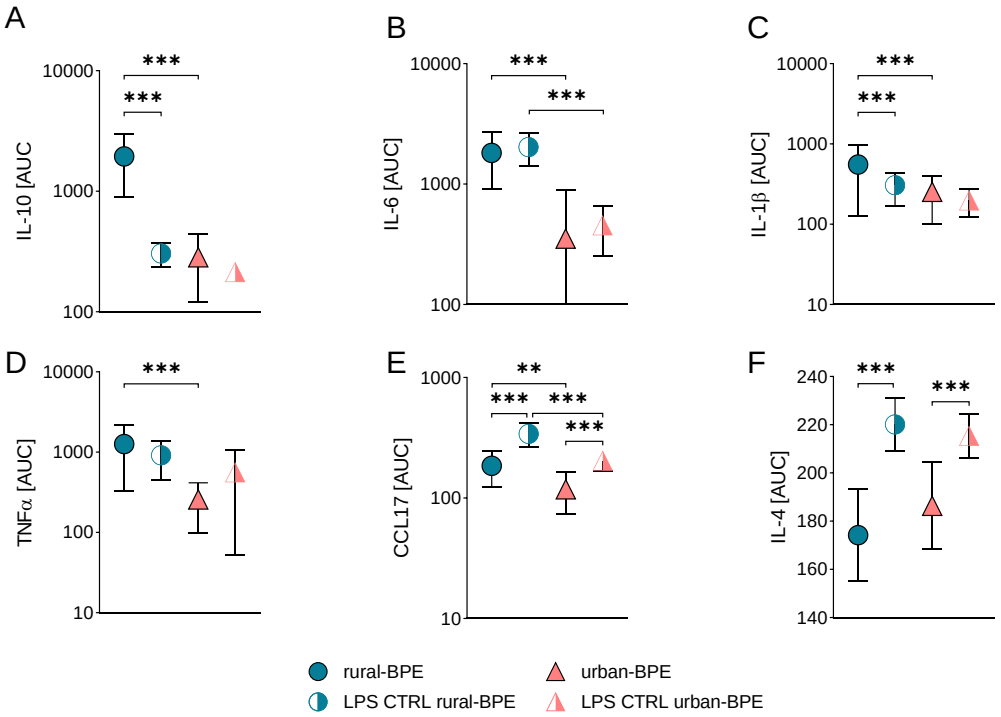

Figure 29 Rural-BPE 2015 elicits immune regulatory cytokine response independent of LPS control. MoDCs were stimulated with BPEs (1 mg/ml -10 mg/ml) or LPS (corresponding to LPS concentration in BPEs). After 24 h, cytokines and chemokines were measured in supernatants and AUC was calculated for comparison between BPEs and LPS controls. n = 13 independent experiments** $p < 0.01$, *** $p < 0.001$ as indicated. AUC: area under the curve.

Similar patterns for IL-10, IL-6 and IL-4 secretion were observed for stimulations with BPEs from 2017, while IL-1β was reduced by both BPEs compared to their LPS controls. The LPS control of rural-BPE 2017 increased TNFα compared to the pollen extract and the opposite was observed for urban-BPE 2017 and its LPS control. Both 2017 BPEs increased CCL17 secretion, while it was reduced by their LPS controls. IL-10, IL-6, IL-4 and CCL17 secretion patterns were reversed when moDCs were stimulated with corresponding Munich BPEs. Release of IL-1β and TNFα was similar between Augsburg and Munich BPEs stimulation (Figure 30).

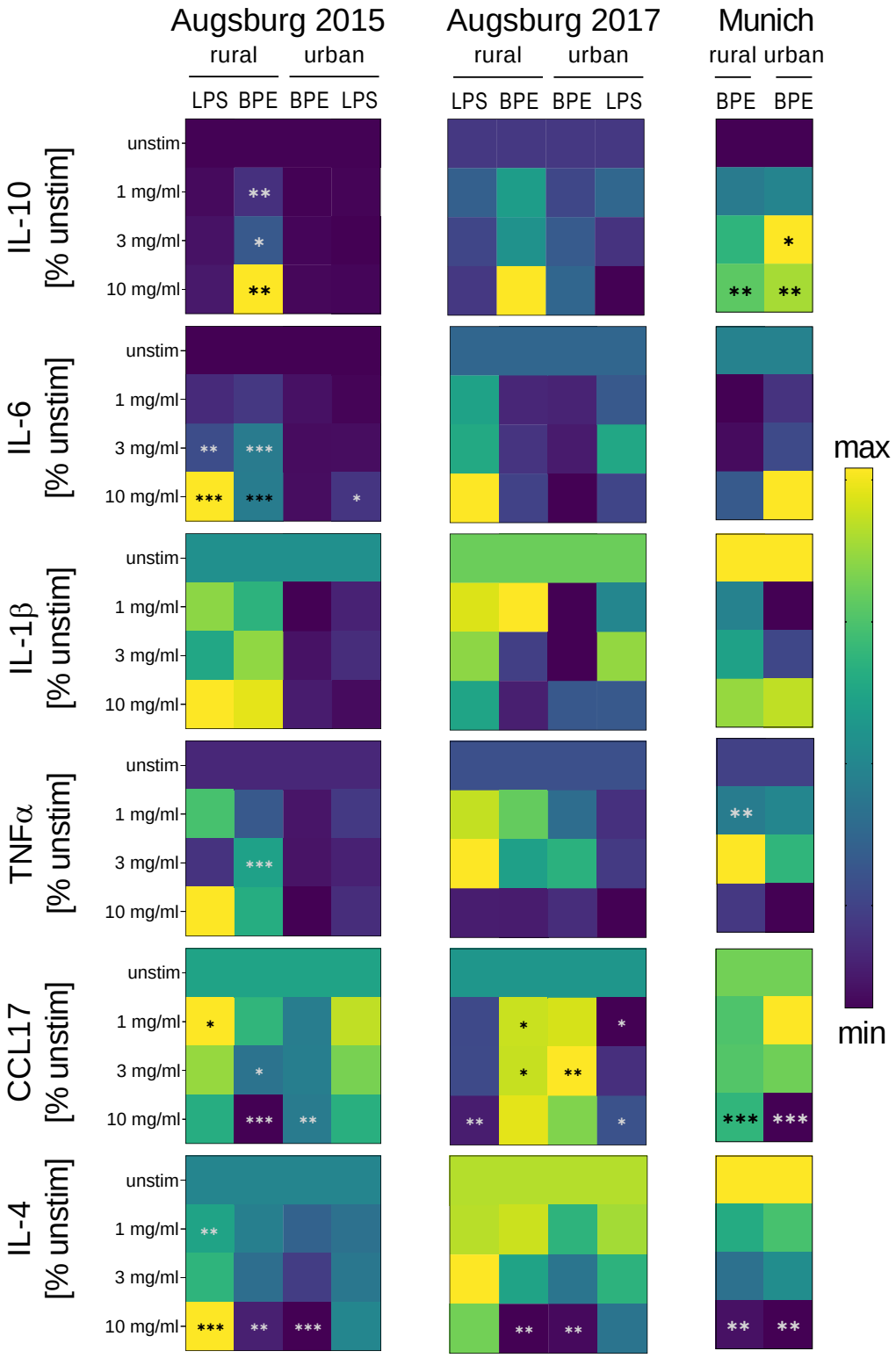

Figure 30 Heat maps of cytokine and chemokine secretion of DCs stimulated with Augsburg and Munich BPEs. MoDCs were stimulated with BPEs (1 mg/ml -10 mg/ml) or LPS (corresponding to LPS concentration in BPEs). After 24 h, cytokines and chemokines were measured in supernatants and concentrations were normalized to unstimulated controls (unstim). Mean of n = 13 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to unstim.

5.1.3.2. Maturation markers

CD40 expression of steady state moDCs stimulated with urban-BPE and its LPS control was reduced compared to rural-BPE and its LPS control (Figure 31 A $p < 0.001$ between BPEs or LPS controls). Expression of CD80 (Figure 31 B) was increased by rural-BPE stimulation compared to its LPS control and urban-BPE ($p < 0.001$), as well as between LPS-control rural-BPE and LPS-control urban-BPE ($p < 0.001$). CD83, CD86 and HLA-DR were expressed more strongly by rural-BPE treated moDCs compared to the LPS control or urban-BPE (Figure 31 C-E, $p < 0.001$ and $p < 0.01$ for rural-BPE vs LPS control urban-BPE).

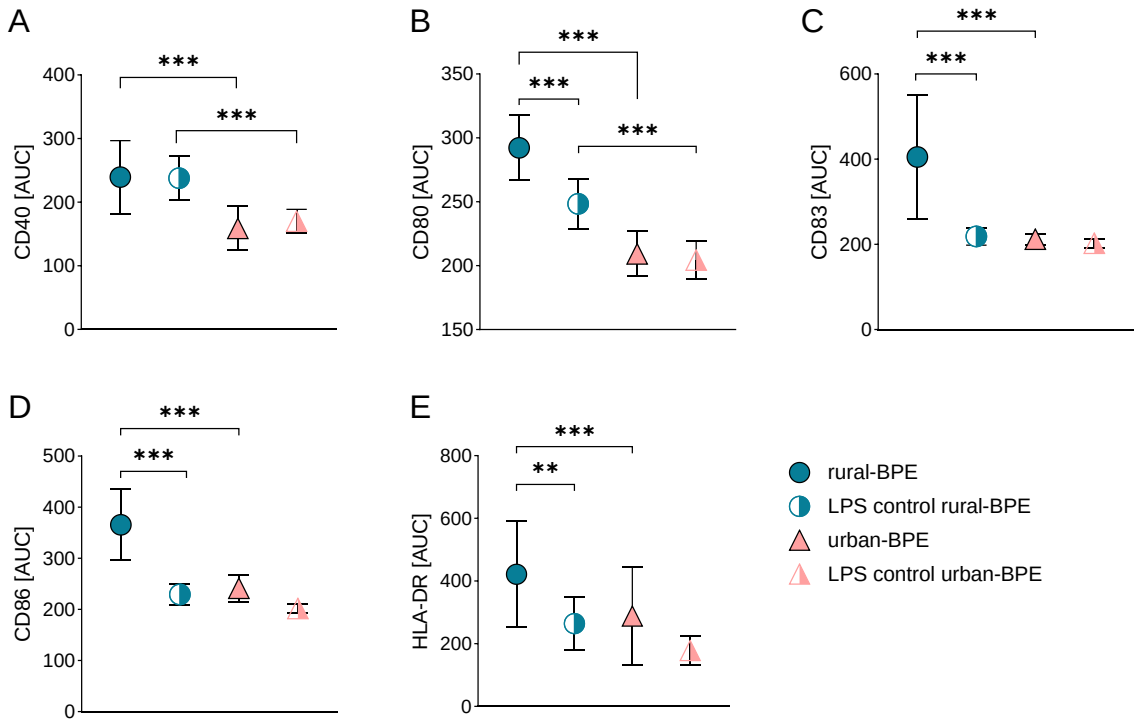

Figure 31 Maturation markers of steady state moDCs are increased by Rural-BPE 2015. MoDCs were stimulated with BPEs (1 mg/ml -10 mg/ml) or LPS (corresponding to LPS concentration in BPEs). After 24 h, cell surface marker expression was analyzed and AUC was calculated for comparison between BPEs and LPS controls. n = 13 independent experiments. ** $p < 0.01$, *** $p < 0.001$ as indicated. AUC: area under the curve.

Similar expression patterns were observed for stimulation with Augsburg 2017 and Munich BPEs for all cell surface markers (Figure 32).

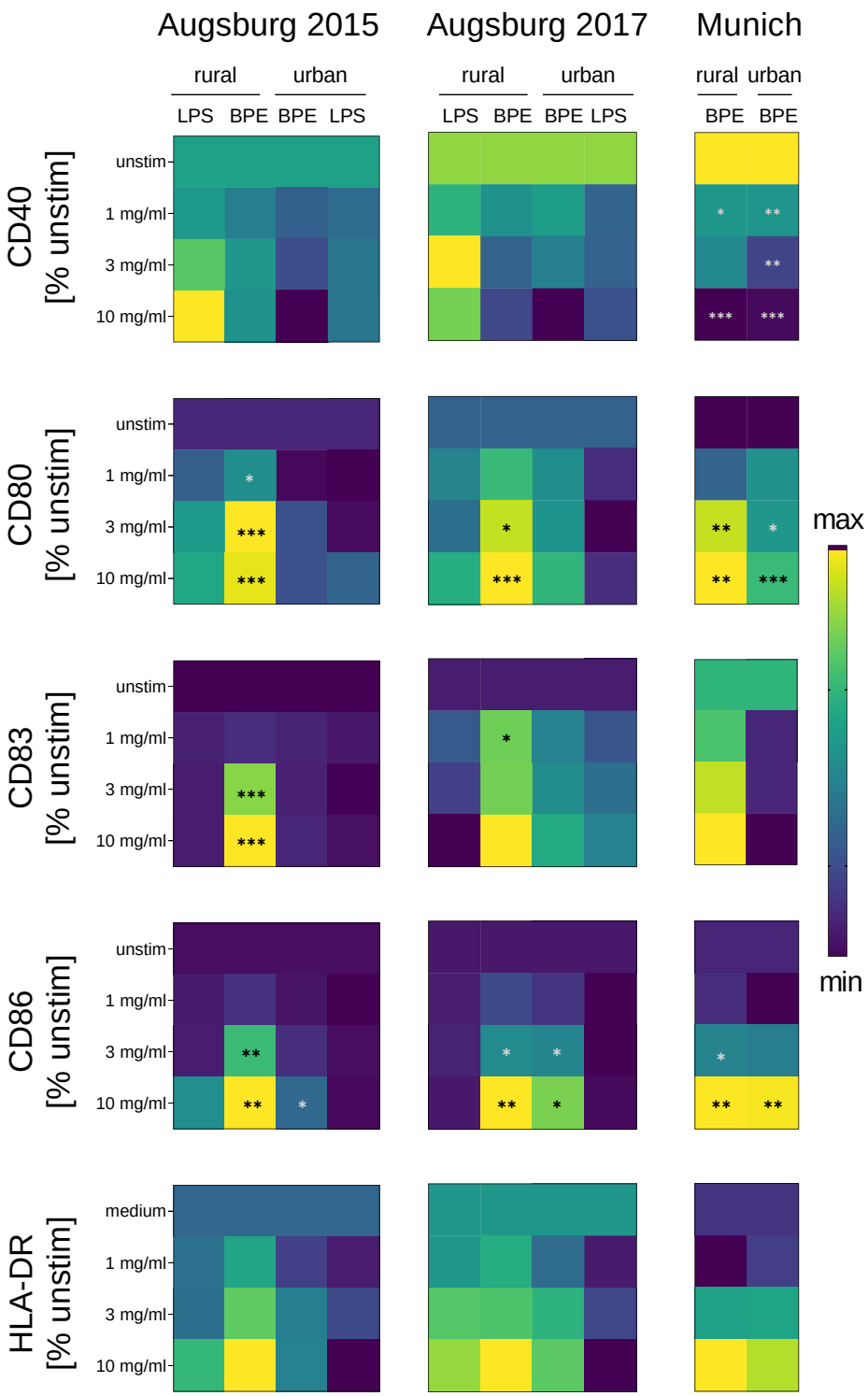

Figure 32 Heat maps of maturation marker expression of DCs stimulated with Augsburg and Munich BPEs. MoDCs were stimulated with BPEs (1 mg/ml -10 mg/ml) or LPS (corresponding to LPS concentration in BPEs). After 24 h, expression of maturation markers was measured. The data were normalized to unstimulated controls (unstim). Median of n = 13 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs unstim.

5.1.4. Response of LPS-primed DCs to BPE stimulation

To investigate the Th1 skewing potential of birch pollen extracts, moDCs were primed with LPS and additionally stimulated with BPEs. Cytokines and chemokines in supernatants and maturation marker expression were measured after 24 h.

5.1.4.1. Cytokine Secretion

LPS-induced expression of IL-10 (Figure 33 A, $p < 0.01$) and CCL17 (Figure 33 E, $p < 0.001$) was reduced by stimulation with urban-BPE compared to rural-BPE (Figure 33 $p < 0.01$). No differences were detected for LPS-induced IL-6 and TNFα secretion upon stimulation with both BPEs (Figure 33), although BPE generally reduced secretion of these cytokines (Figure 34). LPS-induced IL-12 was reduced slightly more in rural-BPE stimulated moDCs than urban-BPE stimulated moDCs (Figure 33 F). Secretion of IL-1β was slightly exacerbated by BPEs compared to the LPS positive control (Figure 34), but not differentially influenced by the two BPEs (Figure 33 C).

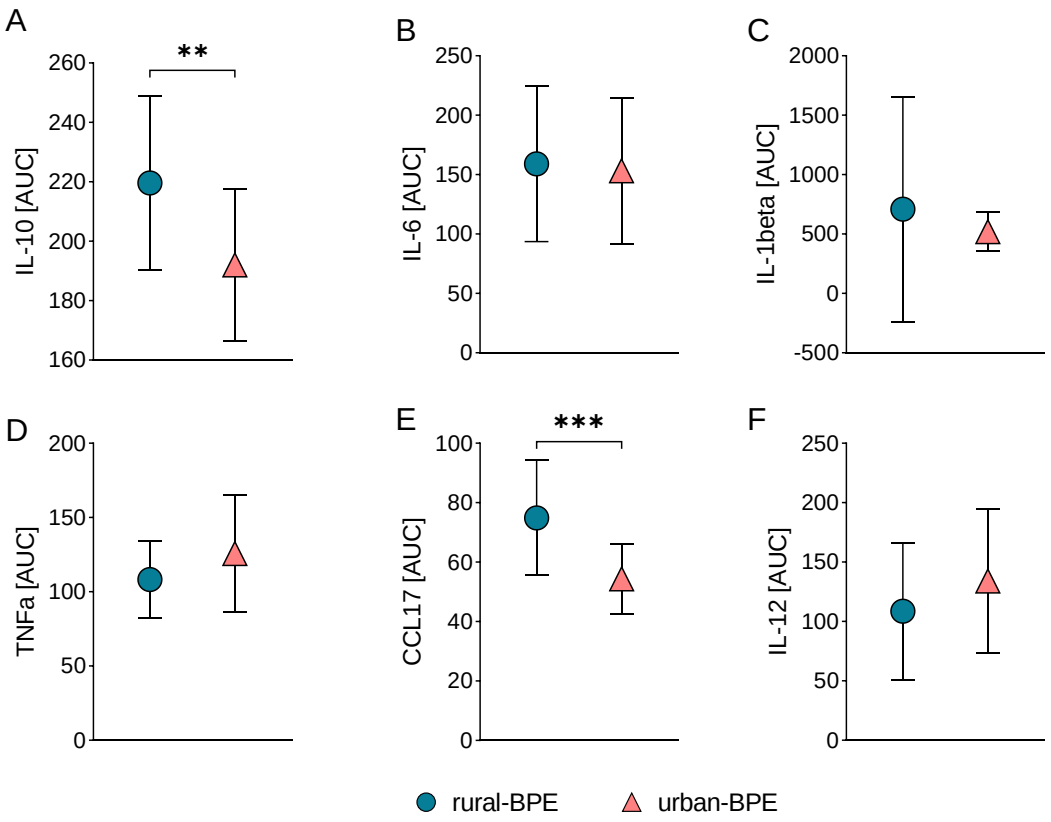

Figure 33 urban-BPE reduces LPS-induced regulatory cytokine/chemokine secretion in moDCs. MoDCs were stimulated with LPS (100 ng/ml) in addition to BPEs (1 mg/ml -10 mg/ml). After 24 h, inhibition of LPS-induced cytokines was measured in the supernatants and AUC was calculated for comparison between BPEs. n = 13 independent experiments. ** $p < 0.01$, *** $p < 0.001$ as indicated. AUC: area under the curve.

Overall, LPS-induced cytokine secretion was reduced by stimulation with BPEs (Figure 34). Only IL-1β was increased. Secretion patterns for LPS-induced IL-10, IL-6 and IL-1β were similar in moDCs stimulated with Augsburg 2015 and Munich BPEs, while the pattern was the opposite in BPE 2017 stimulated moDCs. Stimulation with Augsburg 2015 and 2017 BPEs led to similar patterns in LPS-induced TNFα, CCL17 and IL-12 release, which were in contrast to stimulation with Munich BPEs.

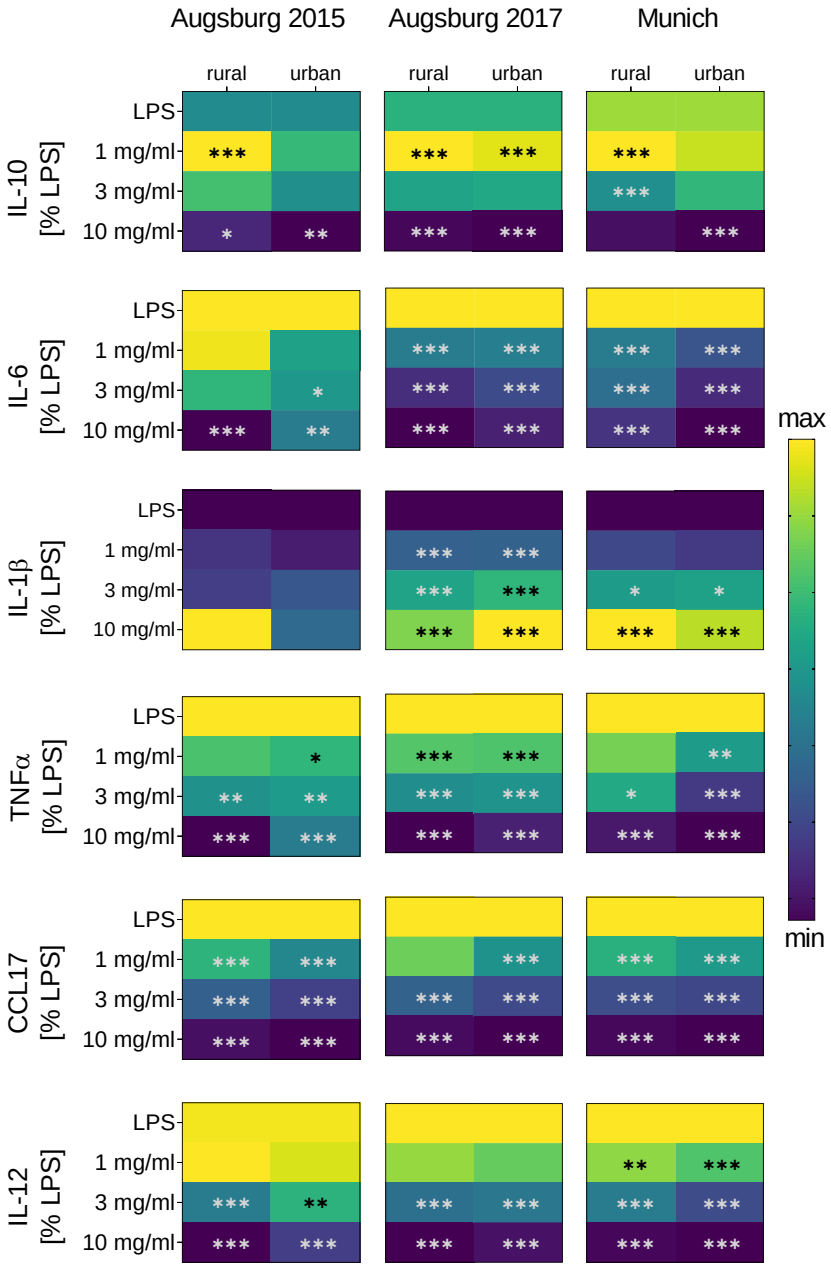

Figure 34 Heat maps of LPS-induced cytokine and chemokine secretion of DCs stimulated with Augsburg and Munich BPEs. MoDCs were stimulated with LPS (100 ng/ml) in addition to BPEs (1 mg/ml -10 mg/ml). After 24 h, inhibition of LPS-induced cytokines was measured in the supernatants and the data were normalized to the LPS positive control. n = 13 independent experiments. ** $p < 0.01$, *** $p < 0.001$ vs LPS control.

5.1.4.2. Maturation markers

LPS-induced CD40, CD80 and HLA-DR expression of urban-BPE stimulated moDCs was lower than of rural-BPE stimulated moDCs (Figure 35 A, B, E; CD40, CD80: $p < 0.05$, HLA-DR: $p < 0.001$). Expression of LPS-induced CD83 and CD86 was not different upon stimulation with either BPE (Figure 35 C, D).

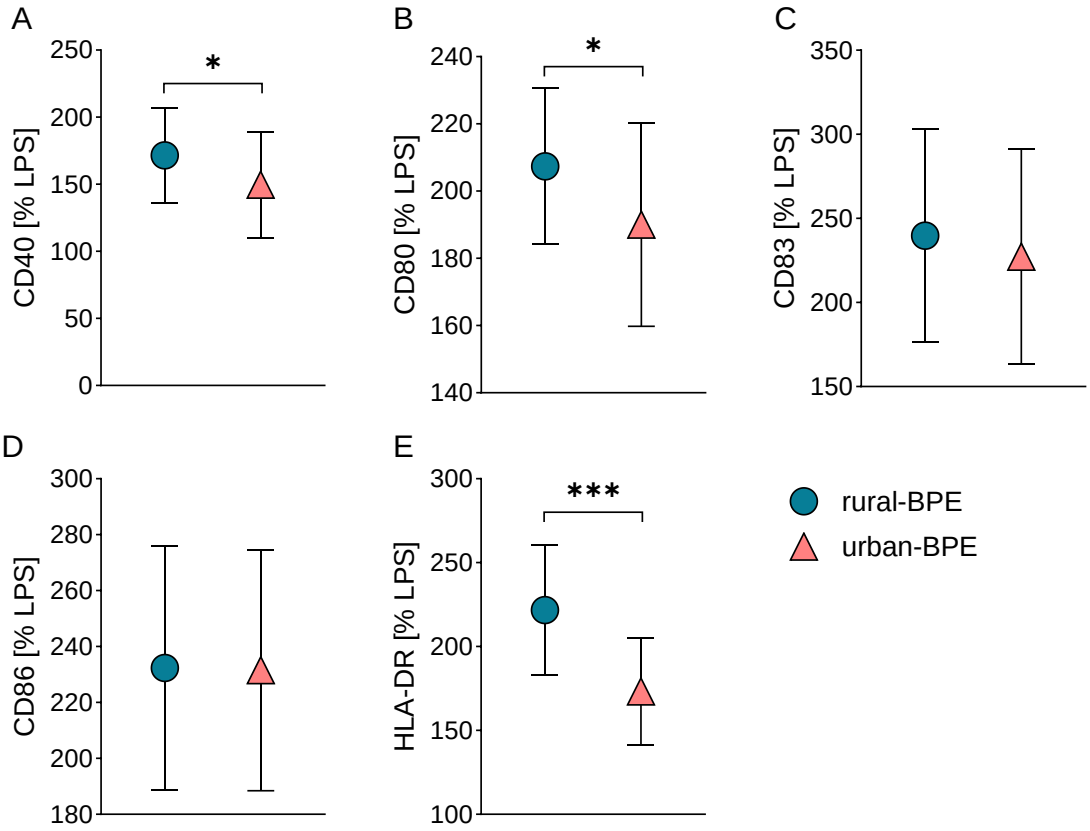

Figure 35 urban-BPE inhibits LPS-induced maturation marker expression. MoDCs were stimulated with LPS (100 ng/ml) in the presence or absence of BPEs (1 mg/ml – 10 mg/ml). After 24 h, maturation markers expression was analyzed and AUC was calculated for comparisons between the two treatments. n = 9 independent experiments. * $p < 0.05$, *** $p < 0.001$ as indicated. AUC: area under the curve.

Upon stimulation with BPEs from Augsburg and Munich, LPS-induced CD40 was reduced (Figure 36). The patterns were similar between Augsburg BPEs 2015 and Munich BPEs when analyzing LPS-induced CD80 and CD83 expression and was reversed in Augsburg 2017 BPE stimulated moDCs. CD86 expression was similar in moDCs stimulated with Augsburg BPEs, but not Munich BPEs. While HLA-DR expression was increased by Augsburg 2015 rural-BPE and

decreased by urban-BPE, BPEs from Augsburg 2017 and Munich both reduced LPS-induced HLA-DR expression.

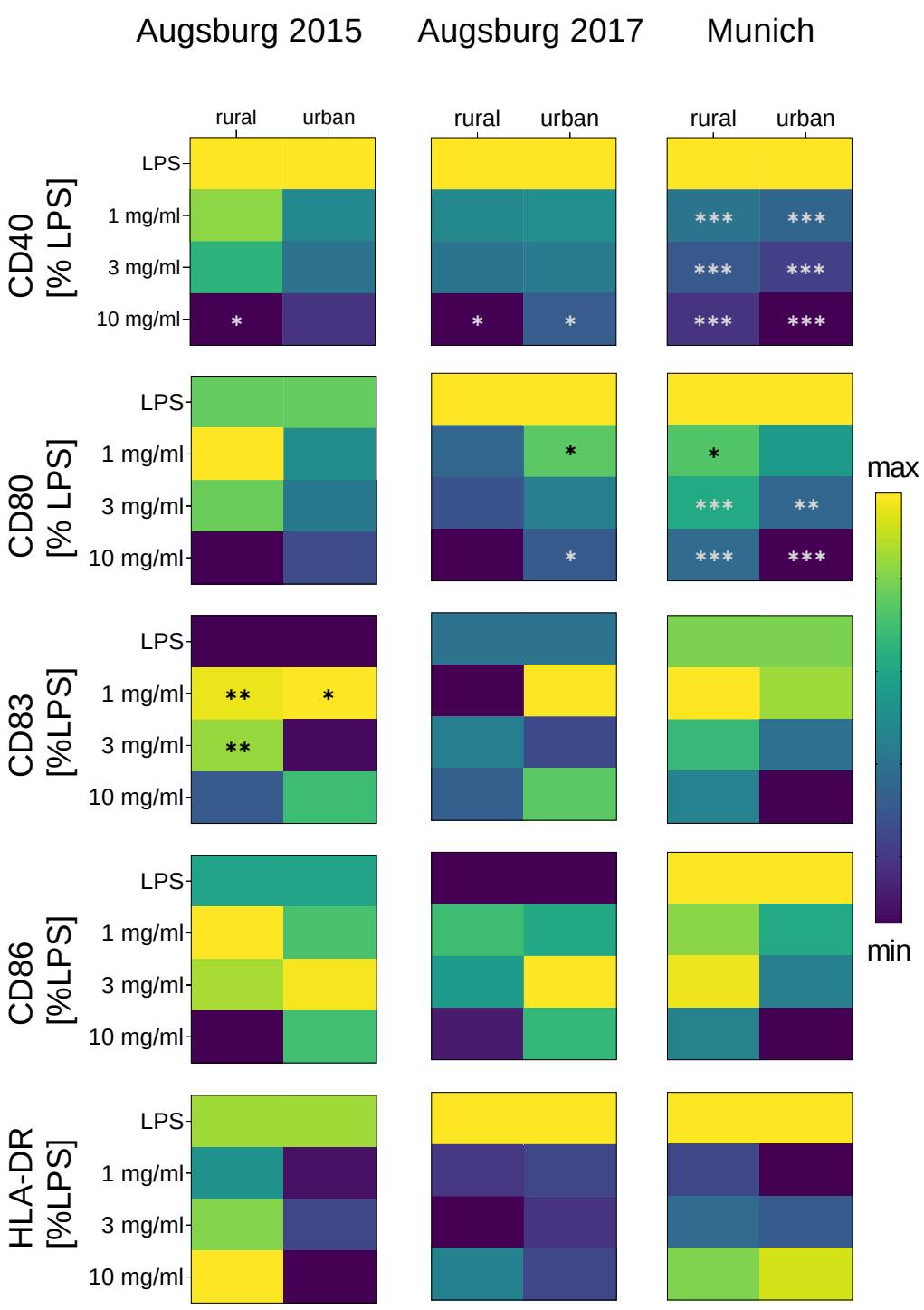

Figure 36 Heat maps of LPS-induced maturation marker expression of DCs stimulated with Augsburg and Munich BPEs. MoDCs were stimulated with LPS (100 ng/ml) in the presence or absence of BPEs (1 mg/ml – 10 mg/ml). After 24 h, expression of maturation markers was measured. The data were normalized to LPS. Median of n = 9 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs LPS control.

5.1.5. Neutrophil migration towards BPE

Neutrophil recruitment plays an important role in allergic sensitization (Hosoki, Itazawa, Boldogh, & Sur, 2016). The direct chemotactic potential of birch pollen extracts was analyzed by exposing neutrophils to 10 mg/ml BPE or 10^{-7} M LTB_4. After 1 h, significantly more neutrophils migrated towards Augsburg 2015 rural-BPE compared to unstimulated control (Figure 37 A, $p < 0.01$). Neutrophil migration was higher when exposed to Augsburg 2017 urban-BPE compared to unstimulated (Figure 37 B $p < 0.05$). There was no difference between the BPEs from Munich or Augsburg (Figure 37).

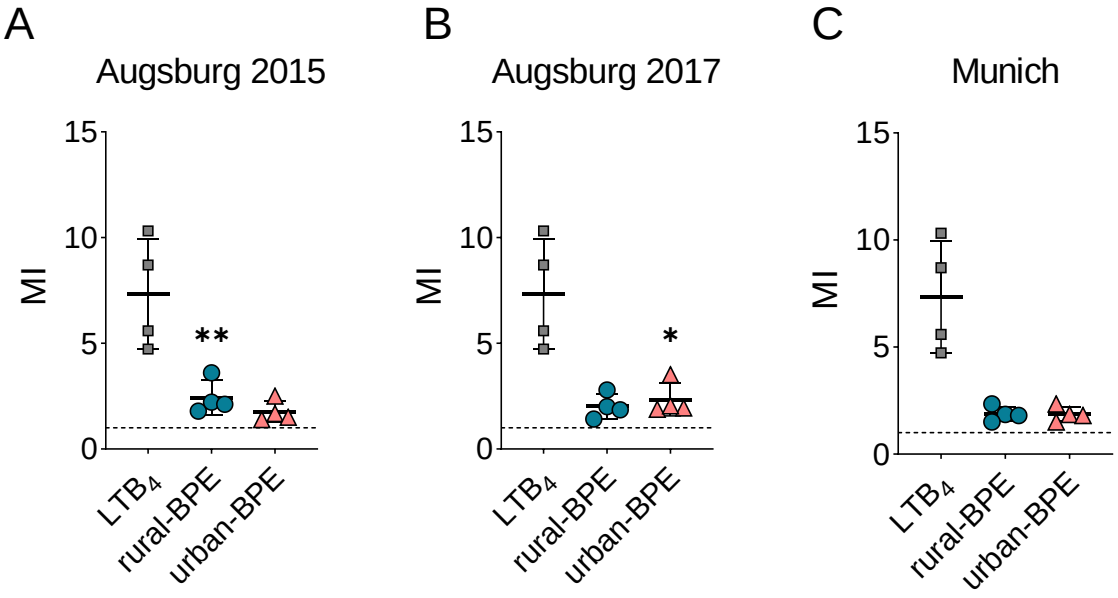

Figure 37 BPEs act as chemo-attractants for neutrophils. (A-C) Neutrophil migration towards 10 mg/ml BPE or LTB_4 (10^{-7} M) after 1h. n = 4 independent experiments. * $p < 0.05$, ** $p < 0.01$ vs unstimulated. Dashed line represents unstimulated control. AUC: area under the curve.

5.1.6. IgE secretion of murine B cells in response to BPEs

The different BPEs were tested for their IgE inducing potential. Murine naïve splenic B cells were stimulated for 8 days with CD40L and IL-4 to mimic Th2-mediated IgE secretion, in the presence or absence of Augsburg BPEs. IgE secretion was increased by all BPE concentrations used for stimulation ($p < 0.01$ and $p < 0.001$ vs CD40L/IL-4), except the highest concentration of Augsburg 2015 rural-BPE (Figure 38 B). There was no overall difference between Augsburg 2015 BPEs, whereas Augsburg 2017 rural-BPE promoted IgE secretion more strongly than urban-BPE (Figure 38 A, $p < 0.01$). Munich BPEs were not tested.

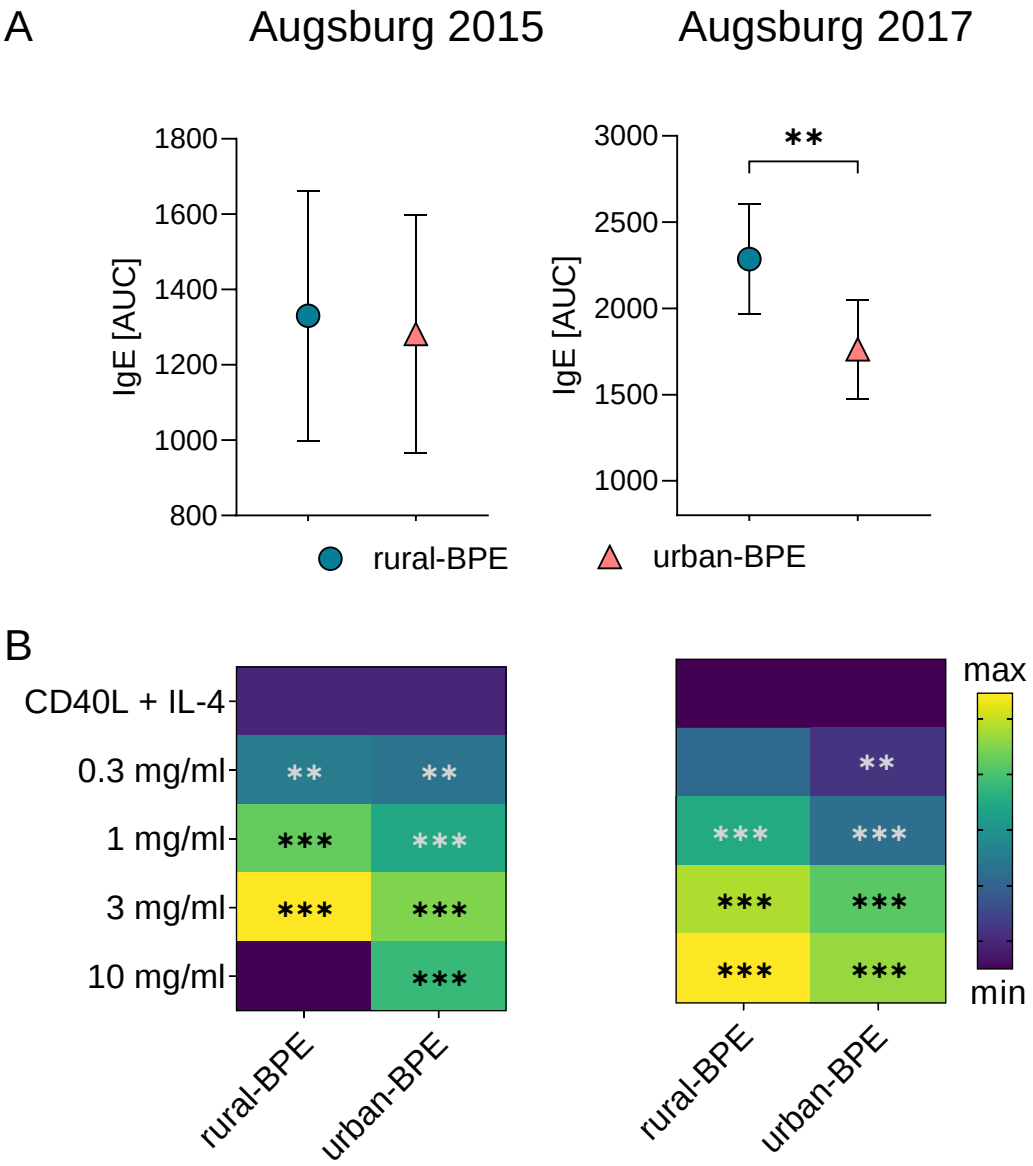

Figure 38 Rural-BPE exacerbates Th2 mediated IgE secretion. Murine naïve splenic B cells were stimulated with BPEs under Th2 mimicking conditions (CD40L + IL-4) for 8 days and IgE was measured in supernatants. (A) AUC to compare treatments. (B) Heat maps of data normalized to CD40L + IL-4. Augsburg 2015: n = 6, Augsburg 2017: n = 3. ** $p < 0.01$, *** $p < 0.001$ vs unstim or as indicated. AUC: area under the curve.

5.1.7. IgE secretion of human B cells

To complete the set of human *in vitro* measurements for pollen allergenicity, a model of IgE secretion by human B cells was established. Naïve B cells isolated from PBMCs of one atopic and one non-atopic donor each in co-culture with CD40L expressing murine L-cells were stimulated with IL-4 + IL-21 to mimic Th2 conditions in the presence or absence of Augsburg 2015 rural-BPE for 15 or 21 days. Unstimulated B cells and B cells stimulated only with BPE did not secrete any IgE. After 15 and 21 days, IL-4/IL-21 stimulated B cells from atopic and non-atopic donors secreted IgE, which was induced in the presence of BPE, especially in the atopic donor. Overall, IgE secretion was higher in B cells from the atopic donor and after 21 days (Figure 39).

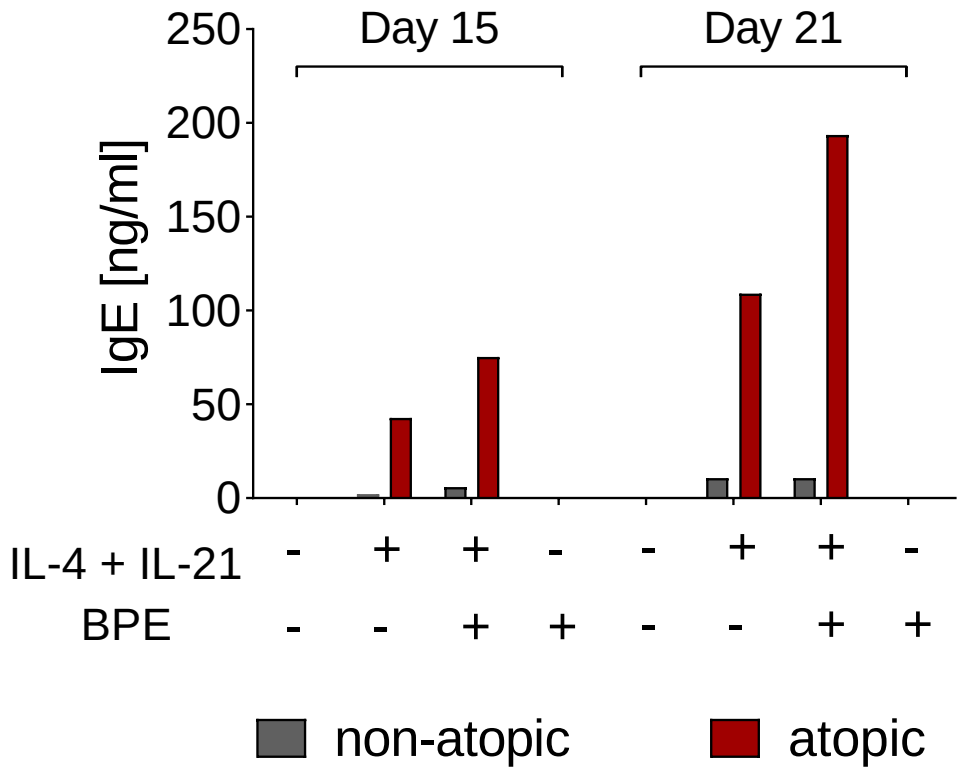

Figure 39 Pilot experiment to induce IgE switch in human naive B cells. Human naïve B cells were co-cultured with CD40L expressing murine L-cells and IL4 + IL-21 (50 ng/ml each) in the presence or absence of Augsburg 2015 Rural-BPE (3 mg/ml). After 15 and 21 days, IgE was measured in the supernatants. n = 1 atopic and 1 non-atopic donor.

5.2. Discussion

Main findings

Pollen from birch trees in urban locations in Augsburg

- Induce less regulatory responses in immature DCs and
- Induce more immunomodulatory responses in DCs *in vitro*

Compared to pollen from rural locations in Augsburg, which appears to be partly attributed to differing concentrations of pollen-derived LPS and $PALM_{PGE2}$.

Air pollution not only affects human health directly, but also has indirect influences by affecting pollen-producing plants and the allergenic potential of their pollen (Sénéchal et al., 2015). Especially between rural and urban areas there is a gradient of pollutants. Simply stated, urban areas are characterized by high NO_2 and low O_3 concentrations, while the opposite is observed in rural areas. This part of the thesis aimed to understand the influence of rural or urban sampling sites, characterized by their NO_2 and ozone levels during the sampling period, on the allergenicity and sensitization properties of birch pollen. For this purpose, pollen samples from two sampling sites representing high and low urbanization in Augsburg, as well as Munich, were chosen.

A mouse model was used to assess the sensitizing and allergenic potential of the different pollen samples (F. Alessandrini, unpublished data). Here, urban birch pollen extracts (urban-BPE) from Augsburg induced a stronger allergic phenotype as characterized by increased serum IgE and higher eosinophils counts in BAL fluid in mice receiving intranasal BPE instillations. In contrast, serum IgE and BAL eosinophils were lower in mice sensitized with rural-BPE, while Tregs were increased. Opposite effects were observed for rural- and urban BPEs from Munich. Here, rural-BPE was a stronger allergy inducer compared to urban-BPE (F. Alessandrini, unpublished data).

To account for these differences, several allergenicity parameters were measured in the pollen extracts. The major birch pollen allergen (Bet v 1) content was higher in all rural-BPEs compared to urban sampling sites. Bet v 1 content has previously been shown to be elevated in birch trees exposed to high O_3 levels, as is characteristic for rural areas (Beck et al., 2013). Even though the Bet v 1 content is also correlated to higher bacterial diversity (Obersteiner et al., 2016), a higher concentration of pollen-associated LPS was only detected in rural-BPE from Augsburg. In contrast, LPS levels were higher in urban-BPE from Munich. Pollen-associated

lipid mediators (PALMs) are immune-modulatory substances found in pollen (Behrendt et al., 2001; Gilles-Stein et al., 2016; Gilles, Mariani, Bryce, Mueller, Ring, Jakob, et al., 2009; Mariani et al., 2007; Traidl-Hoffmann et al., 2005) and were higher in rural-BPEs from Augsburg and urban-BPEs from Munich, while urban-BPE from Augsburg and rural-BPE from Munich contained similar amounts of $PALM_{PGE2}$ and $PALM_{LTB4}$. Rural-BPE from Augsburg 2017 and Munich contained higher pollen-derived adenosine levels compared to urban-BPEs, while the opposite was measured in Augsburg 2015 birch pollen extracts. Adenosine is another immune-modulating substance found pollen and plays a role in allergic sensitization and aggravation (Gilles et al., 2011; Wimmer et al., 2015). Combining these measurements, it appears unlikely that the Bet v 1 or adenosine content are responsible for the different allergic responses in the mouse model, which are rather caused by other pollen-associated mediators like LPS or PALMs.

To translate the findings from the *in vivo* mouse model to a human *in vitro* model and to further elucidate the role of the different pollen-associated mediators, the same BPEs were used to stimulate nasal epithelial cells, dendritic cells and B cells to simulate the allergic sensitization process.

As a first barrier encountered by pollen, nasal epithelial cells (HNEC) play an important role in initiating an allergic immune response (Mattila, Joenväärä, Renkonen, Toppila-Salmi, & Renkonen, 2011). The secretion of pro-inflammatory cytokines IL-1β, IL-8 and TNFα was higher when HNECs were stimulated with rural-BPE than urban-BPE. IL-8 is an important factor to recruit neutrophils to sites of infections or cell damage and is upregulated in airway epithelial cells upon stimulation with birch pollen extracts independently of protease activity (Tomee, van Weissenbruch, de Monchy, & Kauffman, 1998). LPS has been shown to upregulate IL-8 by translocating TLR4 to membrane lipid rafts microdomains (Abate, Alghaithy, Parton, Jones, & Jackson, 2010) and there is evidence that the major birch allergen Bet v 1 is transported through the epithelium in lipid rafts in atopic patients (Renkonen et al., 2009). Although low LPS concentrations, corresponding to levels measured in the pollen, did not significantly increase IL-8, possibly the combination of elevated Bet v 1 and LPS, a possible natural ligand of Bet v 1, in rural-BPE could contribute to the increased inflammatory response of HNECs. The airway epithelium is generally viewed as a modulator of downstream immunological processes and as such provides a pro-inflammatory milieu and chemotactic signals to immune

cells (Mayer, Bartz, Fey, Schmidt, & Dalpke, 2008). While already predisposing to either Th1 or Th2/Th17 favoring conditions, pollen derived substances other than the allergens themselves are released into this microenvironment and influence the cytokine response and maturation of antigen presenting cells.

Dendritic cells are important modulators of T and B cell responses to pollen allergens by secreting cytokines and chemokines and expressing co-stimulatory molecules. In the mouse model, Augsburg urban-BPE was a strong inducer of allergic inflammation, while Augsburg rural-BPE mainly induced regulatory immune responses. *In vitro*, this is reflected by elevated IL-10 secretion in immature DCs stimulated with rural-BPE compared to urban-BPE. TNFα and IL-6 were both also more strongly induced by rural BPE, although at lower levels than IL-10.

To supplement this data, the effect of BPEs on LPS-matured DCs was also analyzed. Stimulation of DCs with high levels of LPS leads to IL-12 production, which promotes Th1 polarization of $CD4^+$ T cells while preventing Th2 sensitization (Eisenbarth et al., 2002; Kuipers et al., 2004). In atopic individuals, IL-12 secretion is impaired, which is associated with the risk to develop Th2 immunity to allergens (Prescott et al., 2003; Reider et al., 2002; van der Pouw Kraan et al., 1997). These properties are a good model to study DC-mediated Th1/Th2 skewing.

Both Augsburg rural- and urban- BPEs inhibited the cytokine secretion in LPS-primed DCs, while the secretion of regulatory IL-10 was more strongly inhibited by urban-BPE, further contributing to the strong allergy-inducing phenotype of this pollen extract.

The expression of all measured maturation markers was higher in DCs stimulated with Augsburg rural-BPE compared to urban-BPE. Likewise, LPS-induced maturation markers CD40, CD80 and HLA-DR were less expressed on urban-BPE stimulated DCs.

Low levels of LPS have been associated with airborne allergens such as pollen and have been indicated to induce Th2 sensitization towards inhalant allergens in a murine allergy model (Oteros et al., 2019). This TLR4 mediated immune response was also dependent on LPS doses, e.g. low levels resulted in Th2 and high levels in Th1 priming (Eisenbarth et al., 2002). In murine alveolar macrophages, both TNFα and IL-4 were induced by low-level LPS exposure. Secretion of these cytokines requires TLR stimulation and downstream activation of MyD88 and, in the case of IL-4, also TRAM activation (Mukherjee et al., 2009). Transferring these findings to DCs,

stimulation with LPS doses corresponding to BPE were responsible for the enhanced TNFα secretion by rural BPE compared to urban BPEs, whereas IL-4 secretion induced by low-level LPS was strongly inhibited by rural and, less so, by urban BPEs. Birch pollen from locations with high ozone levels during the sampling period and high $PALM_{PGE2}$ content had a higher bacterial diversity (Obersteiner et al., 2016), which is mirrored by higher LPS levels in the rural pollen used for this study, providing a possible explanation for the observed effects.

Tolerance-induction to allergens is dependent on IL-10 (Dolch et al., 2019). DC derived IL-10 has been known to be Th2 suppressive and offers a promising strategy to treat allergic diseases (Schülke, 2018). Reduced DC-derived IL-10 production upon contact with allergens is suspected of skewing immune responses towards pro-allergic Th2 cells (Gentile et al., 2004), as is mirrored in urban-BPE stimulated DCs and corresponding to stronger allergy in the mouse model. IL-10 acts by inhibiting IL-1β, IL-6 and TNFα, thereby effectively reducing effector cell function (Armstrong, Jordan, & Millar, 1996; de Waal Malefyt, Abrams, Bennett, Figdor, & de Vries, 1991; Moore, de Waal Malefyt, Coffman, & O'Garra, 2001).

In this work, IL-10 was increased independently of LPS content in the pollen extracts, so it is possible that other compounds in the extracts augment the cytokine response to rural-BPEs. Interestingly, PGE_2-like PALMs were elevated in rural-BPEs. PGE_2 is an eicosanoid induced by LPS-dependent cyclooxygenase-2 (COX-2) in bone marrow derived DCs, which in turn leads to endogenous IL-10 induction and IL-12 inhibition (Harizi, Juzan, Pitard, Moreau, & Gualde, 2002). Even though in this work IL-10 induction appears to be independent of LPS content, endogenous PGE_2-like stimuli from the pollen extracts themselves might have an aggravating effect overriding the LPS-dependent signal. This is further supported by lower levels of $PALM_{PGE2}$ in urban-BPE, which already induced less regulatory cytokines in steady state DCs and inhibited IL-10 less in LPS-primed mature DCs in this study.

Rural-BPE from Augsburg collected in 2015 induced neutrophil chemotaxis, which is in line with the higher IL-8 levels secreted by nasal epithelial cells. Although the opposite effects were observed for pollen from Augsburg 2017, generally, neutrophil chemotaxis was rather low to either of the pollen extracts. Caution needs to be applied when analyzing these assays, because they are only a measure of the direct effect of pollen on neutrophils, which may not commonly be observed *in vivo*.

Both, rural-and urban-BPE enhanced murine IgE secretion under Th2 priming conditions, which was even more pronounced in rural-BPE from 2017 compared to urban-BPE. This appears to be in contrast to the in *vivo data*. In the mouse model, serum IgE was higher in mice instilled with urban-BPE than rural-BPE. *In vitro* B cells were stimulated directly with pollen extracts, whereas the elevated serum IgE levels *in vivo* are the result of a complex interaction of many different immune cells. The direct effects on B cells *in vitro* are possibly due to low molecular weight substances found in the pollen extracts. For example E_1 phytoprostane, a prostaglandin-like phytoprostane has been shown to enhance IgE production in naïve B cells under Th2 mimicking conditions (Oeder et al., 2015) and could contribute to the augmented IgE secretion. Similarly, IgE secretion was also augmented by rural-BPE in human B cells in a pilot experiment. Unfortunately, more data is missing to gain deeper insight into the direct influence of BPE on human B cells.

Overall, rural- and urban-BPEs from Augsburg from 2015 or 2017 induced similar immune responses *in vitro*. In contrast, rural and urban Munich BPEs induced inverse responses compared to pollen from Augsburg, especially on the DC level, which imitated the observations *in vivo*. Allergic sensitization was stronger when mice received nasal instillations with urban-BPE from Augsburg, whereas higher responses were induced by nasal instillations with rural-BPE from Munich. While some of the *in vitro* effects observed upon stimulation with Augsburg birch pollen could be explained by differences in PALM content, sometimes Munich rural pollen resembled urban Augsburg pollen and vice versa. Therefore, the settings of the sampling sites have to be carefully considered. Munich is generally considered a more urban city than Augsburg, with higher levels of air pollution. This has already been reported to induce changes in the proteome of pollen from urban birch trees making them more chemotactic (Bryce et al., 2010), although this was not observed in the present work. On the level of pollutants, NO_2 in urban-BPEs from Munich is especially high compared to the pollen from Augsburg and the rural Munich site and could therefore impact the pollen allergenicity (Cuinica, Abreu, & Esteves da Silva, 2014). Other environmental parameters such as temperature, precipitation, nutrient content and water availability in the soil or pathogen exposure were not measured in this study, but could also very likely influence the allergenicity of pollen. Furthermore, analysis of the pollen metabolome of the different birch pollen samples could shed some light on the differences observed between Munich and Augsburg

pollen and further help to detect secondary metabolites which might contribute to allergic sensitization.

In summary, rural birch pollen extracts induce a more tolerogenic immune profile compared to urban birch pollen extracts, which were more allergenic *in vivo* and *in vitro*. Some of these effects were mediated by LPS and $PALM_{PGE2}$, which were observed in Augsburg sampling sites exposed to higher ozone levels. To gain further mechanistic insights into the interplay between environmental factors and their influence on pollen-derived substances contributing to allergenicity, future studies should observe additional environmental parameters and include metabolic profiling of the pollen, similar to the one conducted by Rauer et al. 2020.

Chapter 6 – Panel Study: Time series analysis of the nasal microbiome under natural pollen exposure

The microbiome is an important player in the balance of respiratory health and disease (Man et al., 2017). Exposure to diverse microbial communities early in life has been shown to be protective against allergies and specific microbiome compositions are indicative of disease status. Because we are constantly exposed to environmental influences, we can assume that this also influences our own microbiome. To study how natural pollen exposure influences the nasal microbiome of atopic and non-atopic individuals (n = 8 in each group), a study was set up in 2015/2016 to follow the participants for one year. The nasal microbiome was analyzed on a monthly, or during the birch pollen season bi-weekly, basis and the microbial composition was assessed.

6.1. Results

6.1.1. Alpha diversity of nasal microbiome

To compare the variance of the nasal microbial composition of the study participants, different diversity indices (richness, evenness, Shannon, Simpson, and inverse Simpson) were calculated. Biodiversity is associated with efficient use of resources (Cardinale et al., 2012) and in the context of the respiratory tract, a loss of diversity has been associated with poor respiratory health (Abreu et al., 2012; Man et al., 2017). Alpha diversity analyzes the variance of the microbial composition in a given sample using different indices. Richness describes the total observed operational taxonomic units (OTUs) in one sample, while evenness describes the homogeneity of a sample. Shannon Diversity Index combines richness and abundance of rare species, while Simpson Diversity Index is influenced by the distribution of highly abundant species. Overall, the diversity of atopic and non-atopic nasal microbiomes, combined for the whole study period, did not differ (Figure 40 A). To further differentiate, a baseline microbiome was calculated using visits with low pollen counts (V1, V14, and V15). Likewise, the composition did not change from baseline to pollen season (V2-V13) for either atopic or non-atopic participants (Figure 40 B). Comparing the baseline and pollen season nasal microbiome irrespective of atopy status, inverse Simpson index was increased during the pollen season (Figure 40 C $p < 0.05$). The diversity of the microbiome of atopic patients and non-atopic volunteers was stable throughout the whole study period (Figure 40 D).

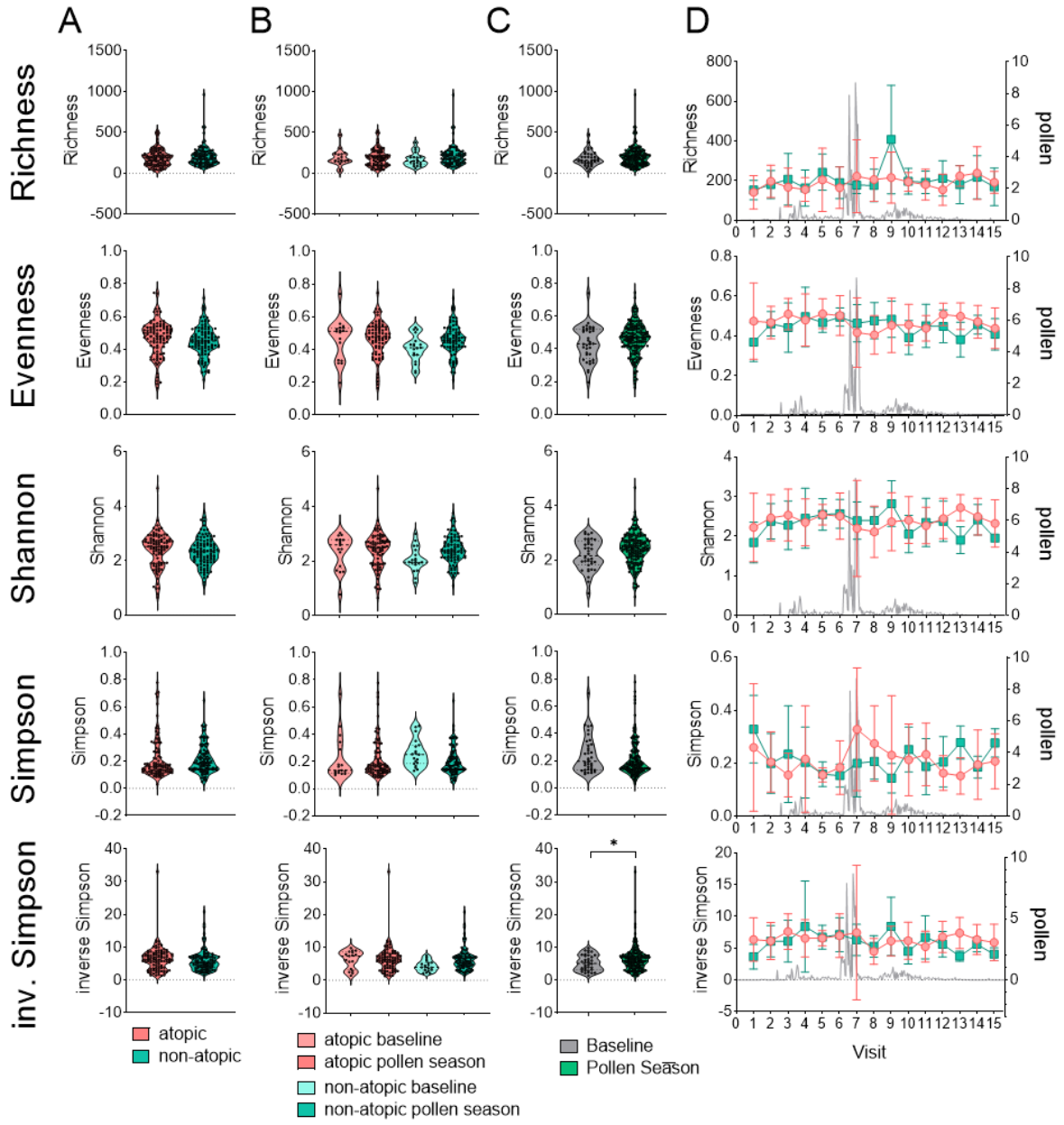

Figure 40 Microbial composition does not differ between atopic and non-atopic study participants and is stable throughout the study period. Richness, evenness, Shannon, Simpson and inverse Simpson diversity indices were calculated and compared between (A) atopic and non-atopic (n = 8 participants each, over 15 visits), (B) atopic vs non-atopic at baseline (visits 1, 14, 15) and during the pollen season (visits 2-13) and (C) composition at baseline and during the pollen season irrespective of atopy status. (D) Time series of alpha diversity indices of atopic and non-atopic participants.

6.1.2. Taxonomic overview of nasal microbiome

The top 10 most abundant families, genus and species were compared between atopic and non-atopic study participants to assess compositional changes during the study period (Figure 41). On all analyzed taxonomy levels, Propionibacteria seemed to be most abundant in atopic patients, while Corynebacteria were more abundant in non-atopic volunteers. *Moraxellaceae* were mainly present in atopic patients and Staphylococci were slightly higher in non-atopics.

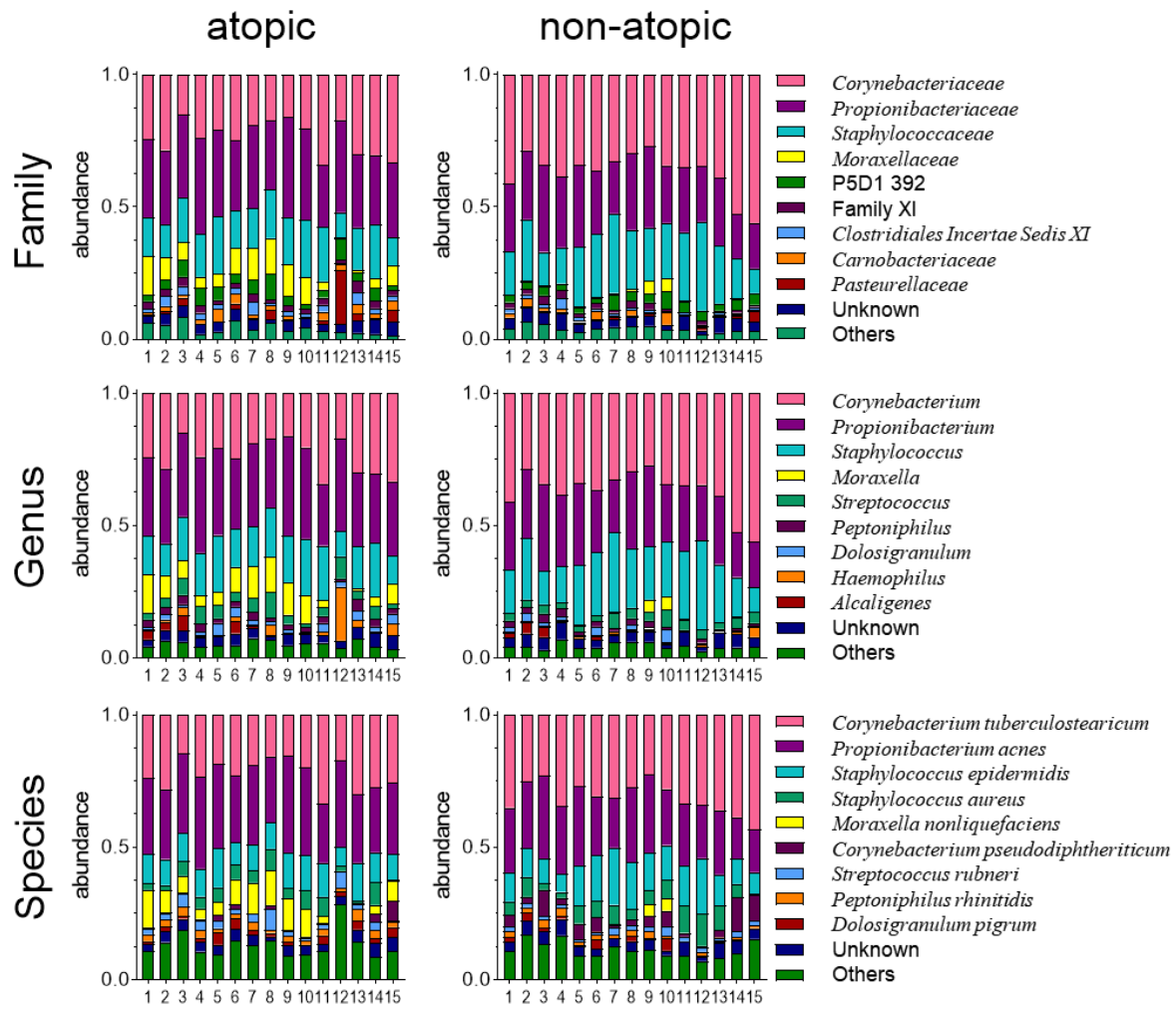

Figure 41 Taxonomic overview of top 10 bacterial families, genus and species. The abundance for each taxonomic level was combined for atopic and non-atopic donors and plotted across all visits.

Because the overall abundance of *Propionibacteriaceae* and *Corynebacteriaceae* was approximately 50 % for all participants during all visits, these two families were further analyzed and a ratio of *Corynebacteriaceae* to *Propionibacteriaceae* was calculated. The ratio was higher in non-atopic participants than in atopics (Figure 42 A, B $p < 0.05$). Especially at the beginning and at the end of the pollen season, this ratio differed the most between atopics and non-atopics but did not reach statistical significance (Figure 42 C).

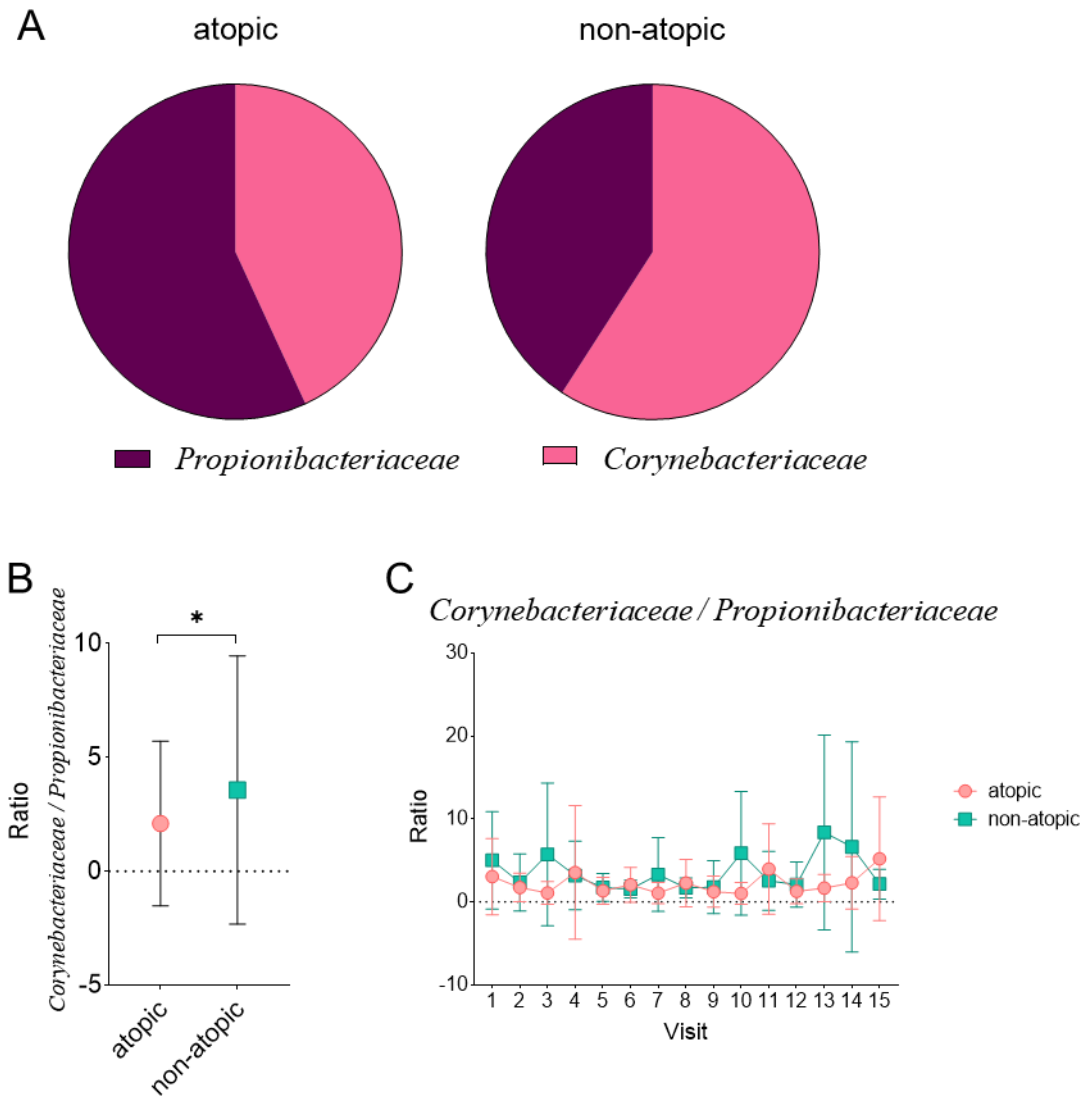

Figure 42 Propionibacteriaceae are more abundant in atopic study participants. (A) Pie chart comparing the abundance of *Propionibacteriaceae* and *Corynebacteriaceae* of atopic and non-atopic donors. (B) Ratio of Corynebacteriaceae vs Propionibacteriaceae of atopic and non-atopic participants. * $p < 0.05$.
(C) *Corynebacteriaceae*/*Propionibacteriaceae* ratio over all visits for atopic and non-atopic participants.

6.1.3. Differences of nasal microbial communities on family level

All families were further analyzed to identify those most abundant in either atopic or non-atopic participants, at baseline or during the pollen season, or comparing atopic and non-atopic at baseline and during the pollen season. Out of 30 bacterial families, 22 were not differentially abundant in atopic or non-atopic participants (Figure 43 A). *Corynebacteriaceae* ($p < 0.0001$), *Burkholderiaceae* ($p < 0.01$), Bacillales ($p < 0.01$) and *Micrococcaceae* ($p < 0.001$) were most abundant in non-atopic volunteers, while *Propionibacteriaceae* ($p < 0.05$), *Campylobacteraceae* ($p < 0.01$), Clostridiales ($p < 0.05$) and *Moraxellaceae* ($p < 0.001$) were higher in atopic patients (Figure 43 A). The abundance of 23 families did not differ between

baseline and pollen season. During the pollen season, Clostridiales ($p < 0.05$), Bacillales ($p < 0.05$), *Comamonadaceae* ($p < 0.05$), *Porphyromonadaceae* ($p < 0.05$), *Prevotellaceae* ($p < 0.01$), *Neisseriaceae* ($p < 0.01$) and *Streptococcaceae* ($p < 0.05$) were most abundant (Figure 43 B).

Comparing the baselines of atopic and non-atopic participants (Figure 43 C), there were no differences on the family level. During the pollen season, Burkholderiaceae ($p < 0.05$), Bacillales ($p < 0.01$), Corynebacteriaceae ($p < 0.0001$) and Micrococcaceae ($p < 0.001$) were higher in non-atopic volunteers and Moraxellaceae ($p < 0.01$), Campylobacteraceae ($p < 0.01$) and Clostridiales ($p < 0.01$) were increased in atopic patients.

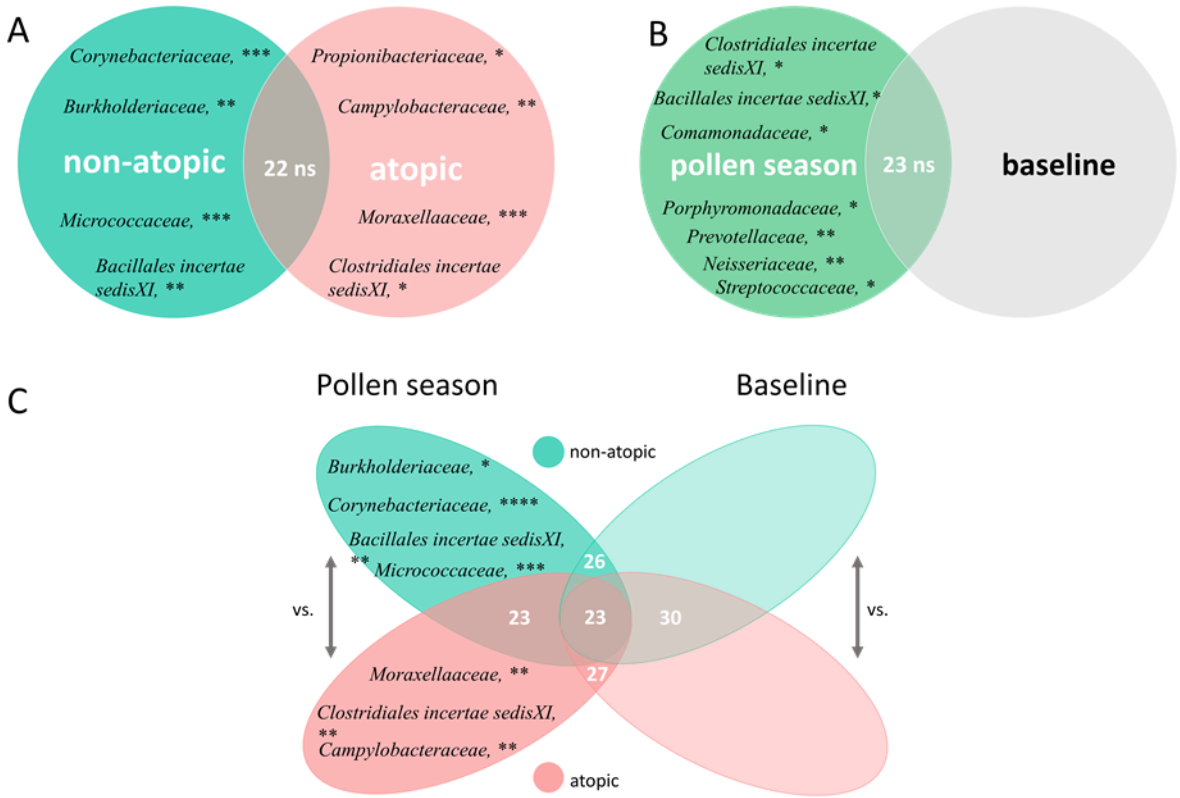

Figure 43 Analysis of differences of nasal community on family level. Total abundances were analyzed for differences between (A) atopic and non-atopic participants (n = 8 atopic / non-atopic, all visits), (B) baseline (visit 1, 14, 15) and pollen season (visits 2-13); pooled across all study participants or (C) atopic vs non-atopic at baseline and during the pollen season. Families with significant differences are reported in the Venn diagram.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

6.1.4. Differences of nasal microbial communities on species level

For more detailed insight into the nasal microbial communities of the study participants, the different species were further analyzed.

6.1.4.1. Atopic vs non-atopic study participants

Analysis of the atopic and non-atopic nasal microbiome revealed 11 species that were more abundant in non-atopic volunteers (Figure 44): *Corynebacterium accolens* ($p < 0.001$), *Corynebacterium pseudophtheriticum* ($p < 0.01$), *Corynebacterium tuberculostearicum* ($p < 0.001$), *Corynebacterium durum* ($p < 0.05$), *Gemella sanguinis* ($p < 0.05$), *Haemophilus influenza* ($p < 0.05$), *Kocuria palustris* ($p < 0.05$), *Lautropia mirabilis* ($p < 0.01$), *Propionibacterium mucilaginosa* ($p < 0.05$), *Rothia dentocariosa* ($p < 0.001$), and *Streptococcus dentisani* ($p < 0.05$).

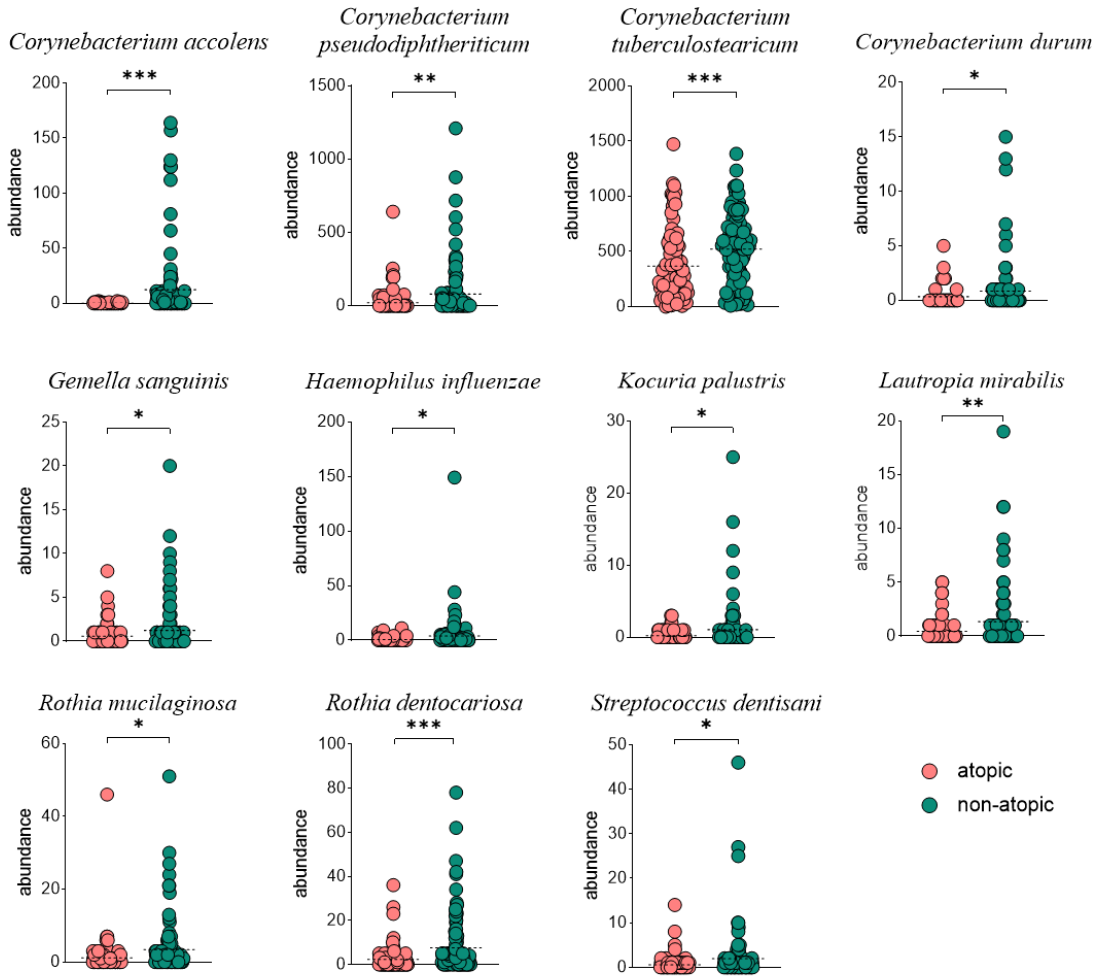

Figure 44 Species more abundant in non-atopic volunteers compared to atopic patients. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Nine species were more abundant in atopic compared to non-atopic study participants (Figure 45): *Anaerococcus provenciensis* ($p < 0.0001$), *Anaerococcus SPP* ($p < 0.0001$), *Campylobacter ureolyticus* ($p < 0.01$), *Finegoldia SPP* ($p < 0.01$), *Haemophilus parainfluenzae* ($p < 0.05$), *Moraxella nonliquefaciens* ($p < 0.001$), *Prevotella timonensis* ($p < 0.01$), *Propionibacterium acnes* ($p < 0.05$) and *Propionibacterium granulosum* ($p < 0.05$).

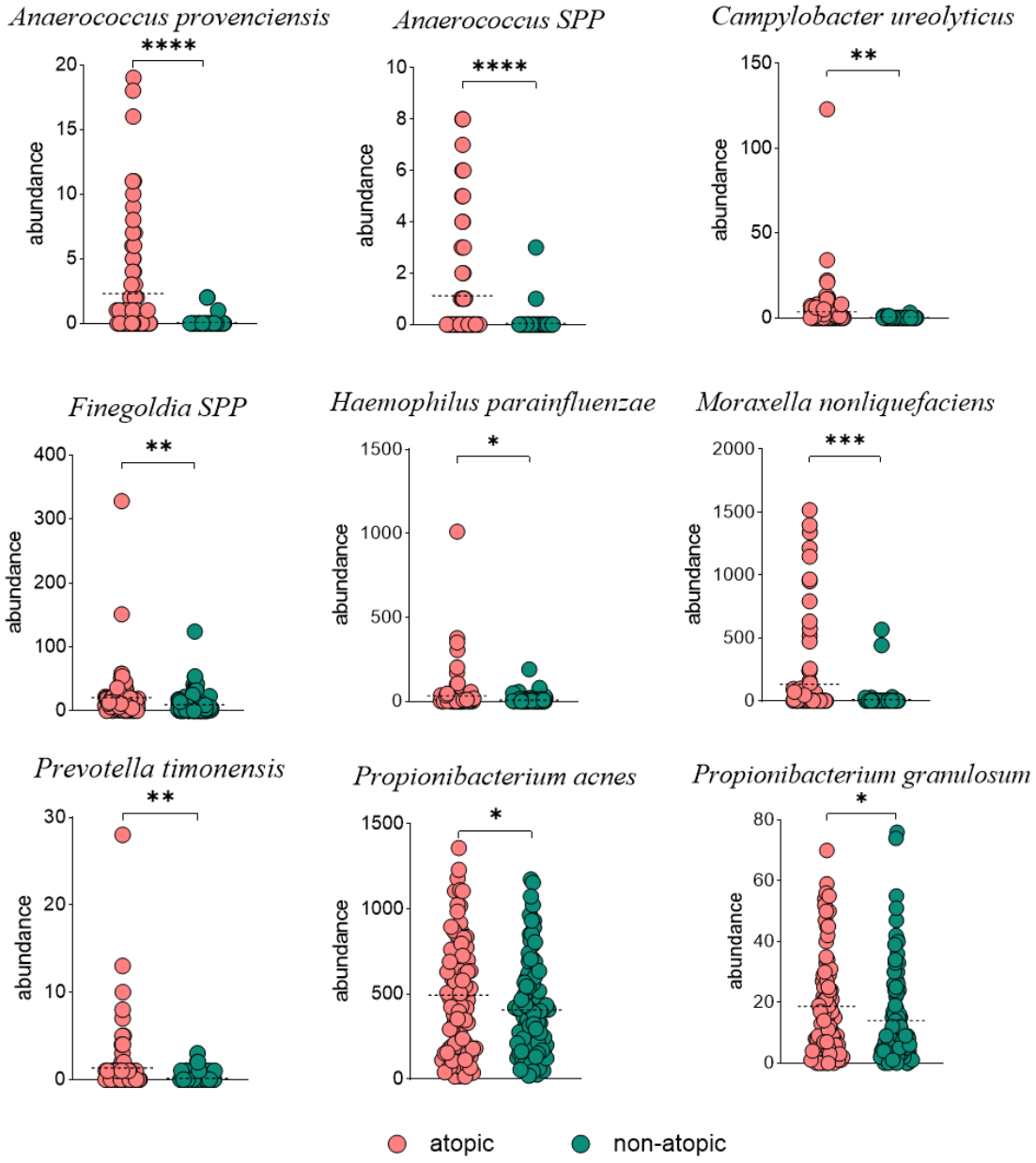

Figure 45 Species more abundant in atopic patients compared to non-atopic volunteers. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

6.1.4.2. Differences under pollen exposure

Natural pollen exposure potentially changed the abundance of certain species in the nasal microbiome. Twelve species were identified to be more abundant during the pollen season (visit 2-13) compared to baseline (visit 1, 14, 15) (Figure 46): *Corynebacterium accolens* ($p < 0.01$), *Corynebacterium durum* ($p < 0.05$), *Corynebacterium singulare* ($p < 0.01$), *Gemella haemolysans* ($p < 0.05$), *Acidovorac defluvii* ($p < 0.05$), *Finegoldia spp* ($p < 0.01$), *Haemophilus sputorum* ($p < 0.05$), *Granulicatella elegans* ($p < 0.05$) *Kocuria palustris* ($p < 0.05$), *Neisseria waeveri* ($p < 0.01$) *Propionibacterium granulosum* ($p < 0.05$), *Porphyromonas pastori* ($p < 0.05$) and *Peptoniphilus indolicus* ($p < 0.001$).

Further stratification by atopy type revealed 6 species that were more abundant in atopic patients during the pollen season than in non-atopic volunteers (Figure 47): *Anaerococcus provenciensis* ($p < 0.0001$)*, Anaerococcus SPP* ($p < 0.0001$), *Campylobacter ureolyticus* ($p < 0.05$)*, Finegoldia SPP* ($p < 0.05$)*, Moraxella nonliquefaciens* ($p < 0.01$) and *Prevotella timonensis* ($p < 0.01$). There were no differences between baseline and pollen season in atopic patients.

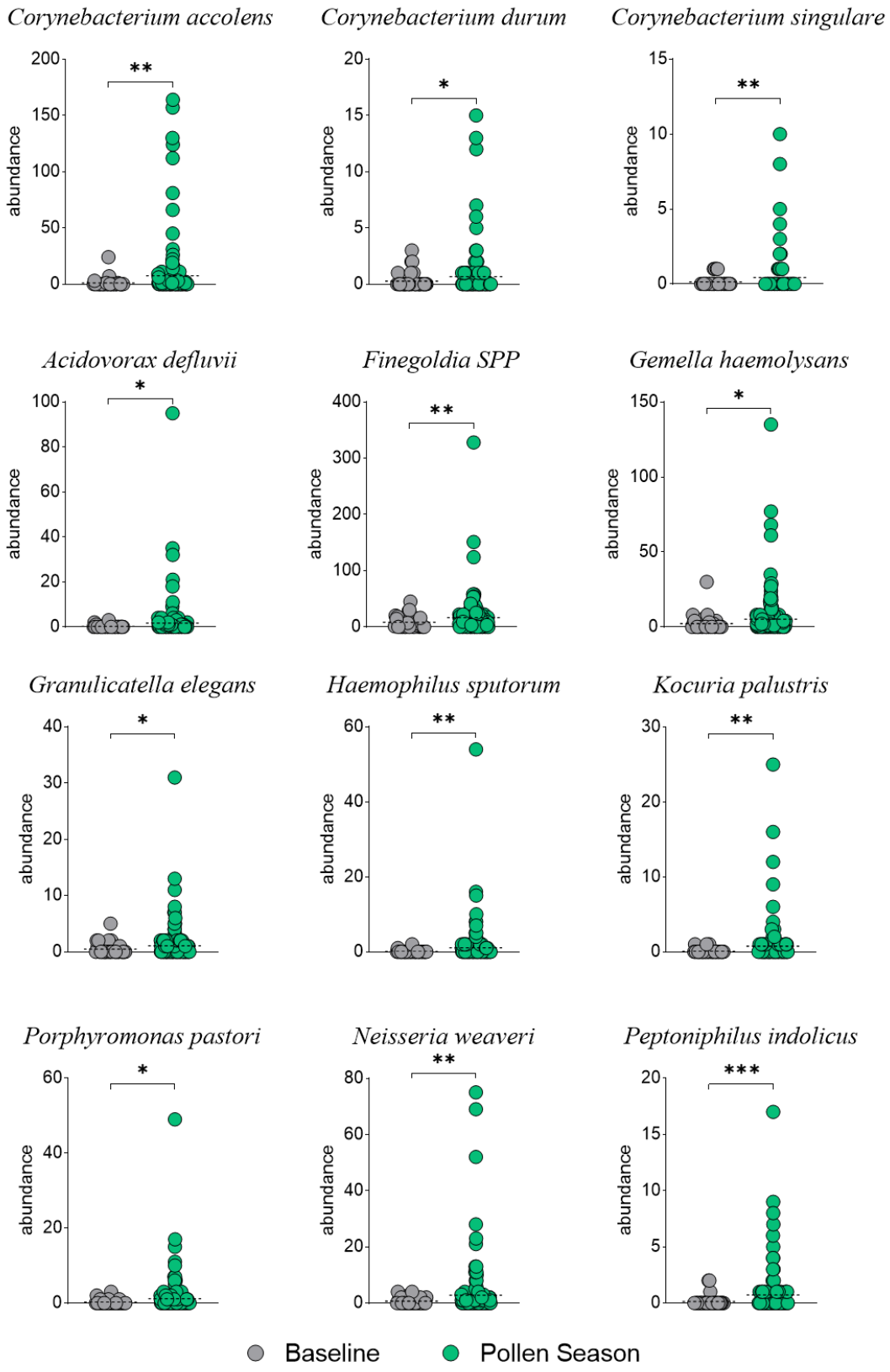

Figure 46 Species more abundant during pollen season compared to baseline. The pollen season was defined as visit 2-13, and the baseline included visits 1, 14 and 15. The data were pooled across all study participants ignoring atopy status. * $p < 0.05$, ** $p < 0.01$.

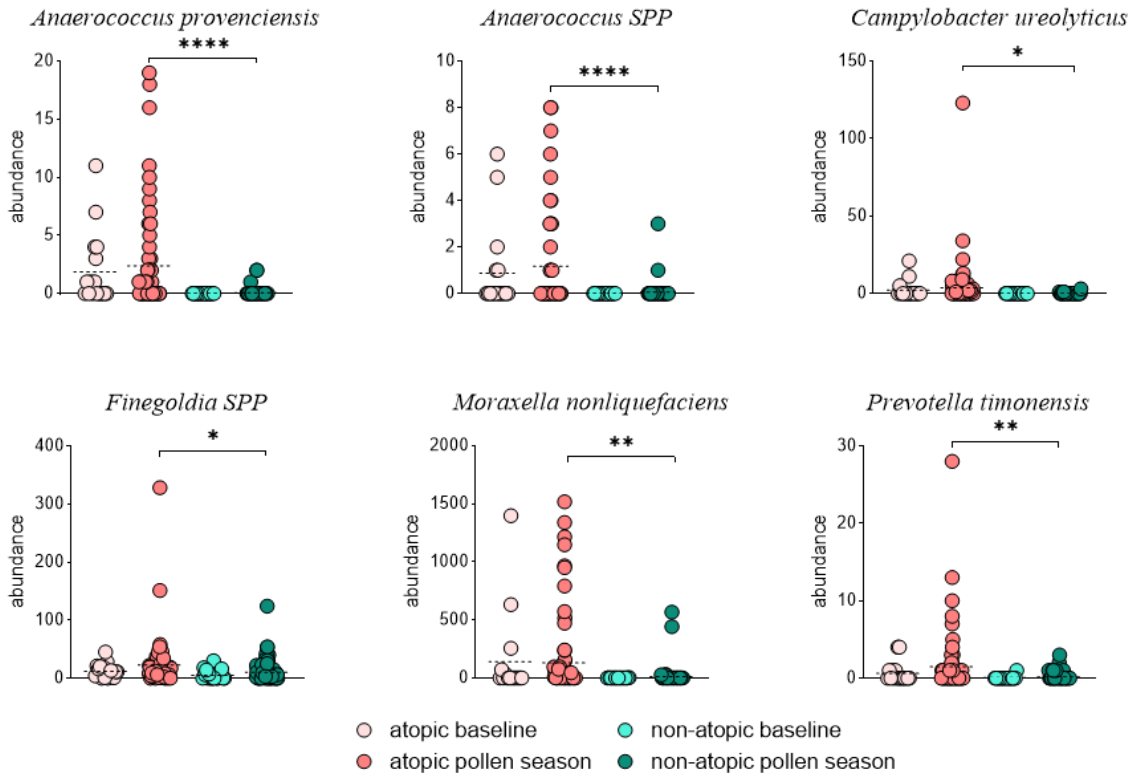

Figure 47 Species more abundant in atopic patients during pollen season compared to non-atopic. The pollen season was defined as visit 2-13, and the baseline included visits 1, 14 and 15. The data were pooled across all study participants ignoring atopy status. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

While no differences were detected for non-atopics between baseline and pollen season, another 7 species were more commonly found in non-atopic volunteers during the pollen season (Figure 48): *Corynebacterium accolens* ($p < 0.001$), *Corynebacterium pseudophtheriticum* ($p < 0.01$), *Corynebacterium tuberculostearicum* ($p < 0.01$), *Gemella haemolysans* ($p < 0.05$), *Kocuria palustris* ($p < 0.05$), *Lautropia mirabilis* ($p < 0.05$) and *Rothia dentocariosa* ($p < 0.01$) were identified.

A summary of the above mentioned comparisons of species abundances is shown in Figure 49.

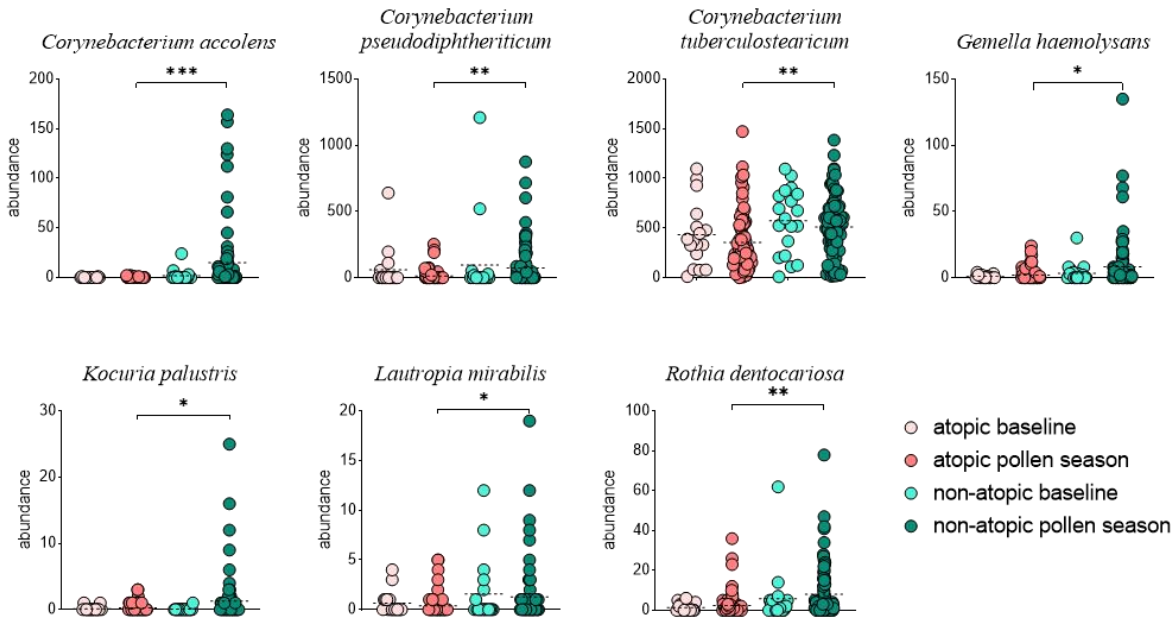

Figure 48 Species more abundant in non-atopic participants during pollen season compared to atopic. The pollen season was defined as visit 2-13, and the baseline included visits 1, 14 and 15. The data were pooled across all study participants ignoring atopy status. * $p < 0.05$, ** $p < 0.01$.

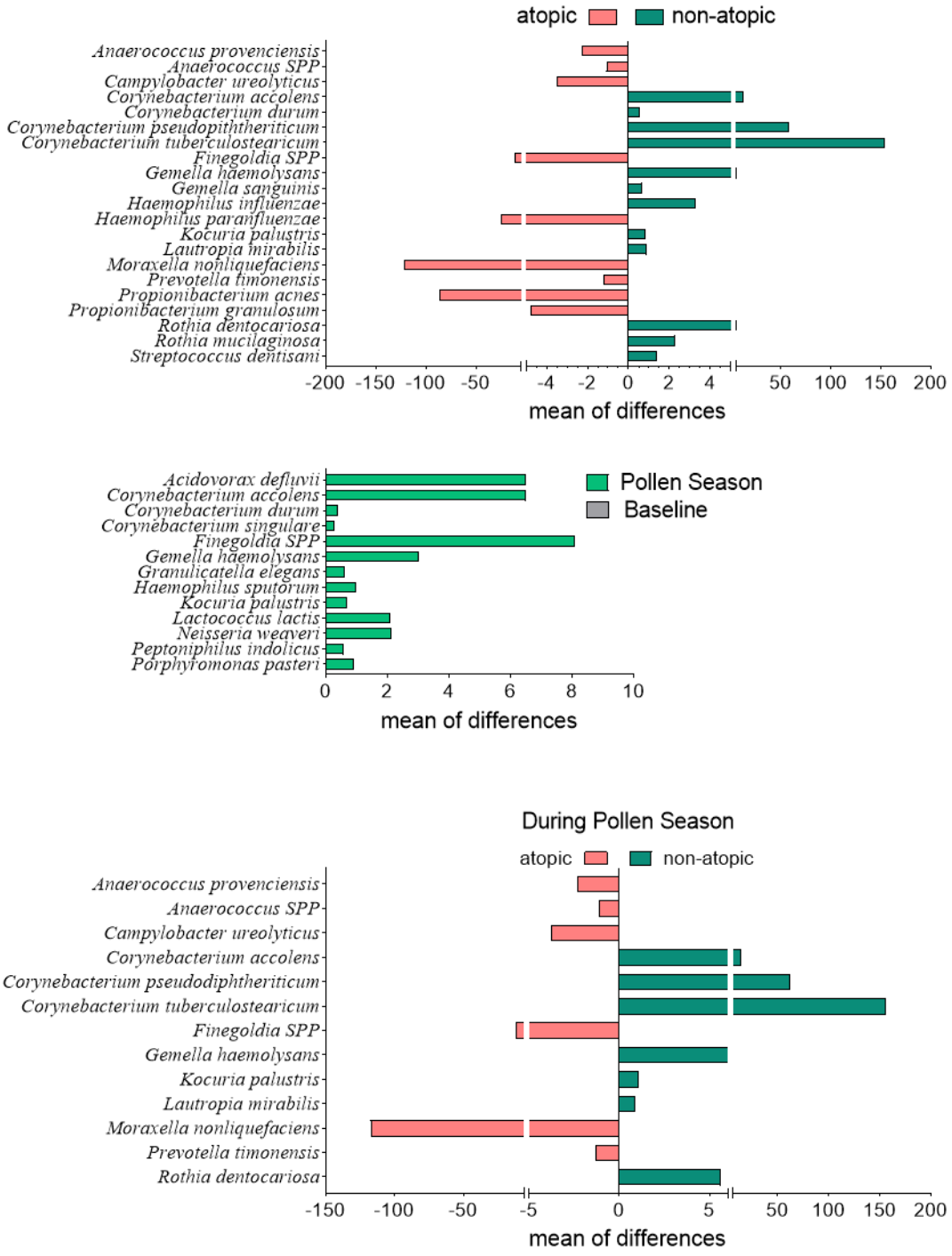

***Figure 49 Overview of significantly different species*.** Difference of mean abundance of (A) atopic vs non-atopic, (B) baseline vs pollen season and (C) atopic vs non-atopic during pollen season.

6.1.5. Pollen microbiome

Pollen harbor a specific microbiome. To identify bacterial species present on birch pollen, the DNA extraction method of was modified to reduce contamination with pollen-derived chloroplast DNA. Chloroplast DNA was successfully reduced from ~ 90 % to < 40 % and the top 10 family, genus and species were visualized in Figure 50.

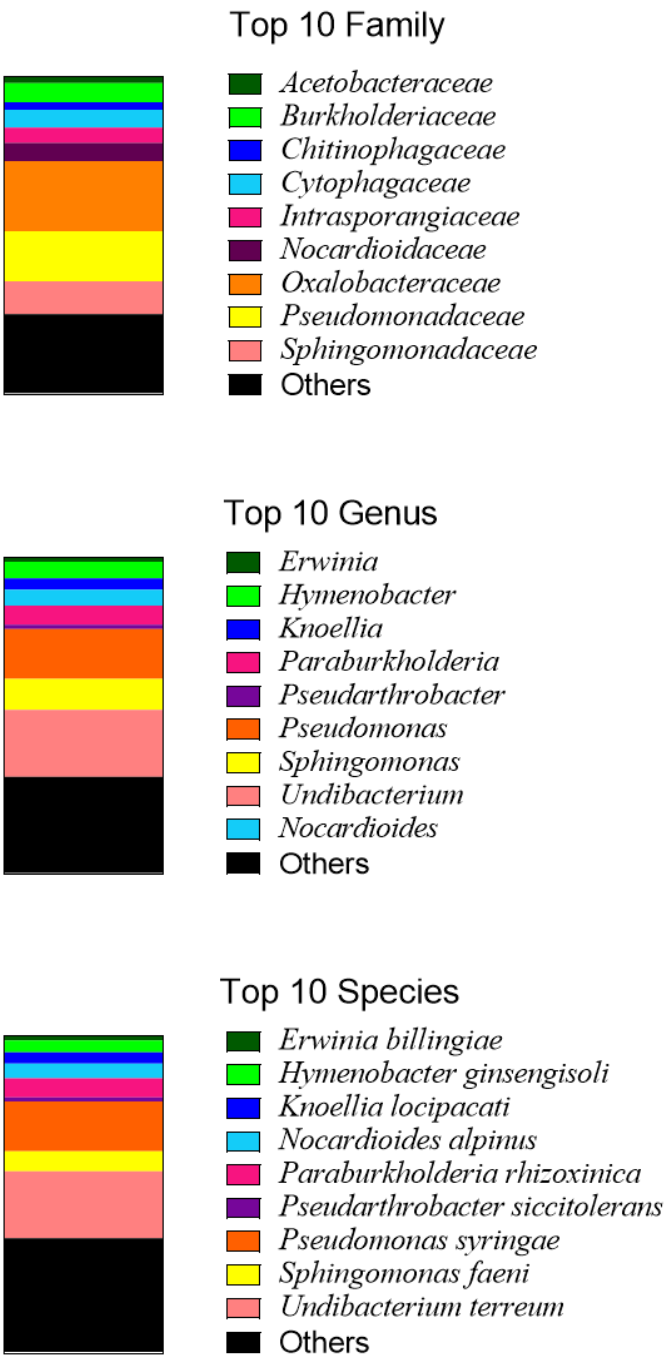

Figure 50 Taxonomic overview of pollen microbiome on family, genus and species level.

6.2. Discussion

Main findings

The nasal microbiome

- Of atopic and non-atopic study participants is stable over time (α-diversity),
- Changes composition during the pollen season,
- Of non-atopics is characterized by higher abundance of *Corynebacteria* species throughout the study period and especially during the pollen season, suggesting a potential protective role for the presence of *Corynebacteria* in the nasal microbiome of non-atopics during the pollen season, possibly paving the way to future treatment strategies.

In recent years, the nasal microbiome has been indicated to play an important role in disease outcomes of allergic asthma patients (Mahdavinia et al., 2018; Man et al., 2017). Imbalances and changes in mucosal microbiota parallel allergic disease outcome, the *effect-cause* question being unsolved. The microbiome is also influenced by environmental exposures (Dimitri-Pinheiro et al., 2020; Integrative, 2014). The impact of natural pollen exposure on the nasal microbiome and the implications for allergic patients are rather understudied. To gain insight into the effect of natural pollen exposure on the nasal microbiome of atopic and non-atopic individuals (n = 8 in each group), a study was set up in 2015/2016 to follow the participants for one year.

Overall, the alpha diversity of the nasal microbiome did not differ between atopic or non-atopic study participants, neither at baseline nor during the pollen season or the course of the whole study period, suggesting that the nasal microbiome is rather stable over a long time span. Only the inverse Simpson index was higher during the pollen season across all participants. Similar results were observed analyzing the nasal microbiome of chronic rhinosinusitis (CRS) and AR patients, and healthy controls (Gan et al., 2020; Mahdavinia et al., 2018), although these studies did not take the pollen season into account and analyzed only single time points. In contrast, a 2-week study conducted before and during the pollen season showed increased alpha diversity in atopic subjects compared to healthy during the pollen season (C. H. Choi et al., 2014). However, this study did not investigate the effects of long-term pollen exposure, as the total pollen season lasts from early spring to (late) summer. They used a fingerprinting method to determine the bacterial diversity instead of 16S rRNA

sequencing. Fingerprinting methods mostly account for the most dominant species of any given sample and do not provide detailed information about changes of species, their taxonomy or function (Hamady & Knight, 2009).

Even though the overall diversity was stable during the study period, differences were observed at species level. Taking a closer look at the taxonomy, bacterial species belonging to the *Corynebacteriaceae* family are more prominent in non-atopic participants, whereas the abundance of *Propionibacteriaceae* was higher in atopic patients. This difference was also stable throughout the whole study period. Interestingly, members of the *Moraxellaceae* family were only present in atopic patients. In contrast, *Corynebacterium accolens, Corynebacterium pseudodiphtheriticum, Corynebacterium tuberculostearicum, Gemella haemolysans, Kocuria palustris, Lautropia mirabilis and Rothia dentocariosa* were predominantly found in non-atopic study participants, especially during the pollen season.

Corynebacteria belong to the most abundant bacteria found in the skin and nose (Grice et al., 2009; Uehara et al., 2000). The presence of *Corynebacterium pseudodiphtheriticum* is negatively correlated to the presence of *S. aureus*, an opportunistic pathogen with a critical role in atopic eczema, while a positive correlation has been reported for the presence of *Corynebacterium accolens* and *S. aureus* (Yan et al., 2013). Yan et al (2013) also suggested that there was a competitive relationship between *S. aureus* and *C. pseudodiphtheriticum,* which was indicated by growth inhibition *in vitro*. This might potentially protect *C. pseudodiphtheriticum* carriers from over-colonization with *S. aureus*. On the other hand, *C. accolens* and *S.aureus* appear to have established a beneficial relationship in the nasal cavity (Yan et al., 2013). Similar synergistic relationships have been shown between *C. accolens* and *S.aureus. S. aureus* colonization has also been correlated with enhanced Th2 immune responses in AR patients (Refaat, Ahmed, Ashour, & Atia, 2008). Therefore the competitive interplay between Corynebacteria and *S. aureus* is studied intensively with respect to novel treatments (Hardy et al., 2019; Uehara et al., 2000), although this needs to be considered carefully, because Corynebacteria, e.g. *C. accolens,* are able to enhance inflammatory responses in an already inflamed setting (Ridaura et al., 2018). Nevertheless, Corynebacteria are found more commonly in non-atopic individuals, and are associated with a positive outcome after surgical removal of nasal polyps in CRS patients (Mahdavinia et al., 2018). In a birth cohort study, reduced abundance of Corynebacteria was associated with early onset of

rhinitis and allergic sensitization (Dzidic et al., 2018; Ta et al., 2018). Also, increased abundance of Corynebacteria reduced the risk for respiratory viral infections and asthma exacerbation (McCauley et al., 2019). Combining the literature and the findings of this study suggests a potential protective role for Corynebacteria, especially during the pollen season, and offers a tentative outlook on future treatment strategies for allergy sufferers.

Species belonging to the *Gemella* genus of the Bacillales order, which were more abundant in non-atopics, are commonly found in the upper respiratory tract and the oral cavity and were first detected in measles patients (Tunnicliff, 1917). On rare occasions, *Gemella* causes endocarditis, meningitis, endophthalmitis, pharyngeal abscess, thorax empyema, spondylodiscitis and brain abscesses in immunocompromised and, even rarer, immunocompetent patients (M. R. Lee et al., 2004). In contrast to the study presented here, *Gemella haemolysans* was previously found to be more abundant in atopic children at 7 years of age in a longitudinal birth cohort study (Dzidic et al., 2018). *Gemella* are hard to distinguish from Streptococci (Kilpper-Bälz & Schleifer, 1988), which could be the cause for this discrepancy, as well as the use of different sequencing and annotation methods.

Members of the *Micrococcaceae* family were more abundant in non-atopic overall, as well as during the pollen season. *Kocuria* species were found in low abundance in the nasal cavity (Kaspar et al., 2016), but are present in diverse environmental niches as well (Kandi et al., 2016; Park, Kim, Roh, Jung, & Bae, 2010). *Kocuria palustris* has been isolated from the rhizoplane of narrow-leaved cattail (*Typha angustifolia*) (Kovács et al., 1999), an herbaceous plant common to wet-lands. Although there are increasing reports of *Kocuria* species causing human illness (Kandi et al., 2016), this is the first report connecting low abundance of *Kocuria palustris* to atopy. Higher abundance in non-atopic study participants during the pollen season is possibly caused by behavioral differences, e.g. spending time outdoors.

Colonization with *Rothia,* another *Micrococcaceae* family member found overrepresented in non-atopics, in the gut microbiome during infancy is thought to be protective against childhood asthma and was found to reduce airway inflammation in a mouse model (Arrieta et al., 2015). Accordingly, the present study reported lower *Rothia dentocariosa and R. mucilaginosa* abundance in the nasal microbiome of atopic patients in general, but also during the pollen season, further supplementing proof for the protective role of *Rothia* species.

Burkholderiaceae is a diverse bacterial family of environmental bacteria and human commensals, including *Lautropia mirabilis*. *L. mirabilis* has been detected in oral cavities (Gerner-Smidt et al., 1994) and is generally associated with healthy status compared to patients suffering from periodontitis (Shaddox et al., 2012). To my knowledge, this study provides the first evidence of its occurrence in the nasal cavity. Even though in low abundance, *L. mirabilis* was more abundant in non-atopics, but the relevance of this bacterial species in the nasal microbiome and the development of allergies remains to be seen.

Compared to the control group, during the pollen season the nasal microbiome of atopic patients was dominated by *Anaerococcus provenciensis, Anaerococcus SPP, Campylobacter ureolyticus, Finegoldia SPP, Moraxella nonliquefaciens* and *Prevotella timonensis.*

Anaerococcus species belong to the order of Clostridiales and are commensals of the skin, oral cavity and gut. In asthma patients, the abundance of *Anaerococcus* was increased after treatment with azithromycin, a macrolide antibiotic with possible therapeutic use to reduce airway hyper-responsiveness (Slater et al., 2014). Likely, this increase in *Anaerococcus* contributes to the improved clinical outcomes and in the case of the present study could provide a potential protective agent in the nasal mucosa of atopic patients in the absence of other beneficial microbes observed in non-atopic patients.

Finegoldia are opportunistic commensal anaerobic Gram-positive bacteria, found in the skin and mucosal surfaces. They are found in chronic skin lesions, where they are able to successfully colonize by degrading collagen in the basement membrane of the dermis. Additionally, *Finegoldia* has recently been observed to directly interact with neutrophils and inducing NET formation and pro-inflammatory cytokine release (Neumann, Bjorck, & Frick, 2020). Additionally, protein L, a cell wall protein of *Finegoldia* is known to bind to human immunoglobulins and potentially binds IgE (Bjorck, 1988), which could lead to basophil activation and histamine release. Together, this potentially contributes to airway inflammation in atopic patients and might aggravate symptoms during the pollen season.

Longitudinal birth cohort studies have shown an increased risk for allergy and respiratory tract infections in children whose nasal cavity was colonized by *Moraxella* (McCauley et al., 2019). McCauley et al. (2019) also showed increased epithelial barrier damage as well as inflammatory responses caused by *Moraxella* cell free supernatants, providing mechanistic insights into the clinical relevance of *Moraxella* colonization.

Interestingly, *Prevotella* species are commensals associated with healthy airways. Although some studies point towards the potential to induce inflammatory processes in the setting of various inflammatory diseases, *Prevotella* abundance in the airways was even reduced in COPD and asthma patients compared to the abundance of proteobacteria such as *Haemophilus* (Larsen, 2017), which was increased during the pollen season in this thesis. This is emphasized by a lower capacity of *Prevotella* to induce pro-inflammatory processes in DCs (Larsen et al., 2012). On the other hand, co-stimulation of DCs with *Prevotella* and *Haemophilus* seemed to reduce *Haemophilus*-induced IL-12 production (Larsen et al., 2012). Thus, co-colonization of *Prevotella* and *Haemophilus* in atopic airways may lead to a reduced Th1 immune response upon pollen exposure, further paving the way for allergic sensitization or aggravating symptoms.

In summary, the overall diversity of the nasal microbiome of atopic and non-atopic study participants does not differ and is stable over time. Nevertheless, natural pollen exposure changes the microbial composition, both, in atopic and non-atopic patients. Corynebacteria species are of special interest, because they are more abundant in non-atopics during the pollen season, potentially playing a protective role. This opens further investigations into their role as therapeutic agents and future research should include mechanistic *in vitro* and *in vivo* studies.

Birch pollen microbiome

Not only are pollen allergen carriers, but they are also colonized by microbes (Obersteiner et al., 2016), which led to the hypothesis that pollen-associated microbes may very well end up in the nasal mucosa during the pollen season, especially considering the presence of environmental bacteria such as *Kocuria*. Unfortunately, none of the species detected on pollen were identified in the nasal microbiome of the study participants. However, this is not surprising since the study did not include a controlled exposure to birch pollen. In addition, the birch pollen used in this thesis were pooled from different locations and it is not certain that the study participants were even exposed to similar pollen grains at all. Future work should include the microbiome analysis of more trees from different locations, providing insight into sample site specific microbiomes which could possibly be correlated to the patients. A controlled exposure study, e.g. using a pollen exposure chamber, could add more profound insights in to the role of environmental microbes in allergic airway diseases.

Chapter 7 – A walk in the park: Influence of co-exposure to fungal spores and grass pollen allergens on the nasal inflammatory response and the nasal mycobiome

The grass pollen season in Germany usually last from spring to late summer. At the same time, fungal spores have also been observed (Grewling et al., 2019). Ultimately, fungal spores will be inhaled together with grass pollen and might impact the nasal immune response towards pollen allergens as well as inflict changes to the nasal mycobiome. To study this co-exposure effect, the Picnic study was set up. Over the course of 5 weeks in June-July 2018, 7 volunteers spent one hour per week in a flowering meadow. During this time, the fungal spore and pollen burden was measured and fungal species were isolated to further study the effect of co-exposure to fungal spores and pollen allergens *in vitro*.

7.1. Results

7.1.1. Indoor and outdoor pollen and allergen exposure

Three out of the 8 participants wore nasal filters to measure the content of a marker allergen (Phl p 5) in the nose during the outdoor exposure. They additionally wore the filters for 1 h indoors before sitting in the flowering meadow. To further comprehend the pollen burden, pollen counts were measured for 1 h indoors and during the outdoor exposure. The grass pollen count was approximately half of the total pollen count during the 1 h outdoors. Indoor pollen counts were generally lower than outdoor counts. On days with high pollen exposure, the nasal Phl p 5 content was higher than on days with lower pollen counts. No Phl p 5 was detected in nasal filters worn inside (Figure 51).

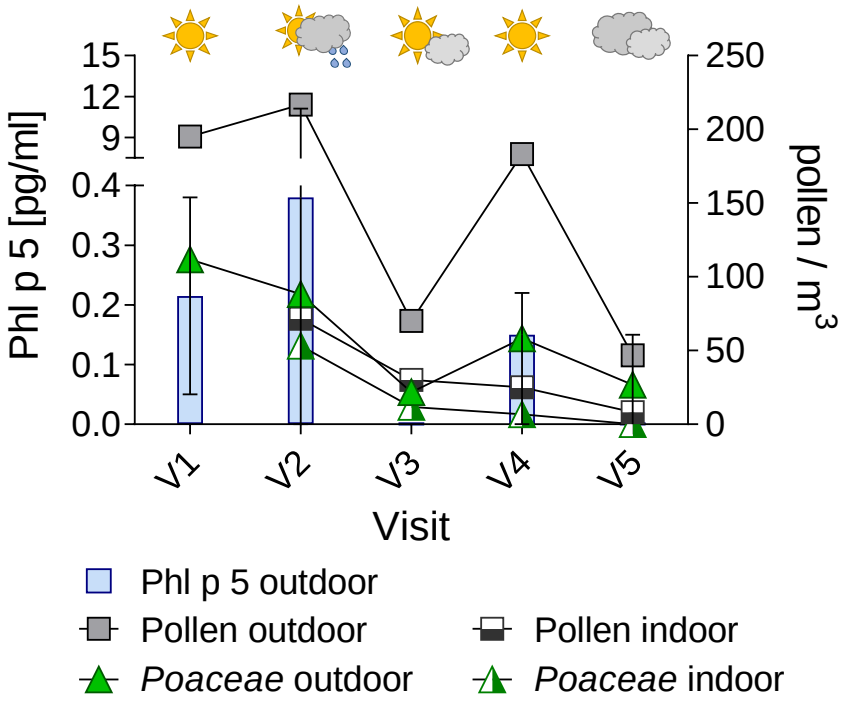

Figure 51 Nasal Phl p 5 content coincides with grass pollen exposure. Participants sat in a flowering meadow for 1 h. Before and during this time, they wore nasal filters to measure Phl p 5 content. At the same time, portable pollen traps were set up to determine the pollen load during the study periods. n = 3 participants, median + SD. Left y-axis = Phl p 5 measurements [mg/ml], right y-axis = pollen counts [pollen/m^3].

7.1.2. Indoor and outdoor fungal species

Fungal species are ubiquitous in nature and are present outdoors as well as indoors. Portable fungal traps containing PDA agar plates were set-up 1 h indoors and 1 h outdoors in the flowering meadow where the participants sat. Afterwards, fungi collected on the PDA plates were cultivated for 5 days, isolated and identified. The most commonly found fungal species are represented in Table 19. Because *Cladosporium cladosporioides, Fusarium sp* and *Penicillium manginii* were the most abundant present species, they were chosen for cell culture assays, representing the species mostly encountered during the grass pollen season.

Table 19 Most commonly found fungal species identified in indoor and outdoor samples over the whole study period.

Species	Location
Cladosporium cladosporioides	indoor & outdoor
Cladosporium tenuissimum	indoor & outdoor
Epicoccum nigrum	outdoor
Fusarium sp	indoor & outdoor
Penicillium chrysogenum	indoor
Penicillium commune	outdoor
Penicillium manginii	indoor
Trichoderma koningiopsis	outdoor

7.1.3. Cytokine response of HNECs stimulated with fungal spores and grass pollen allergens

Monolayer cultures of HNECs from non-atopic donors were stimulated either with fungal spores alone, recombinant grass pollen allergens (Phl p 1, Phl p 5) or spores and allergens combined to assess the effect of co-exposure on the nasal inflammatory response (Figure 52). Stimulation with *Fusarium* spores increased IL-1β ($p < 0.05$), IL-8 ($p < 0.05$) and IL-6 ($p < 0.05$) secretion of HNECs compared to unstimulated cells. *Cladosporium* spores also increased IL-6 secretion ($p < 0.01$). Neither Phl p 1 nor Phl p 5 stimulation induced IL-1β, IL-8 and IL-6 release. IL-1β secretion was higher when cells were stimulated with combinations of Phl p 1 + *Fusarium* or *Cladosporium* (both $p < 0.05$ vs unstim) or Phl p 5 + *Fusarium* ($p < 0.01$ vs unstim), *Cladosporium* ($p < 0.01$ vs unstim) or *Penicillium* ($p < 0.05$ vs unstim). Likewise, IL-8 secretion was higher when cells were co-stimulated with Phl p 1 + *Fusarium* ($p < 0.05$ vs unstim), or Phl p 5 + *Fusarium* ($p < 0.05$ vs unstim), *Cladosporium* ($p < 0.01$ vs unstim) or *Penicillium* ($p < 0.05$ vs unstim). Only *Fusarium* increased IL-6 release when HNECs were co-stimulated with Phl p 5 ($p < 0.01$). While all tested fungal spores increased cytokine secretion, *Penicillium* appeared to reduce IL-6 secretion, either when cells were stimulated with spores alone or combined with allergens (Figure 52 C).

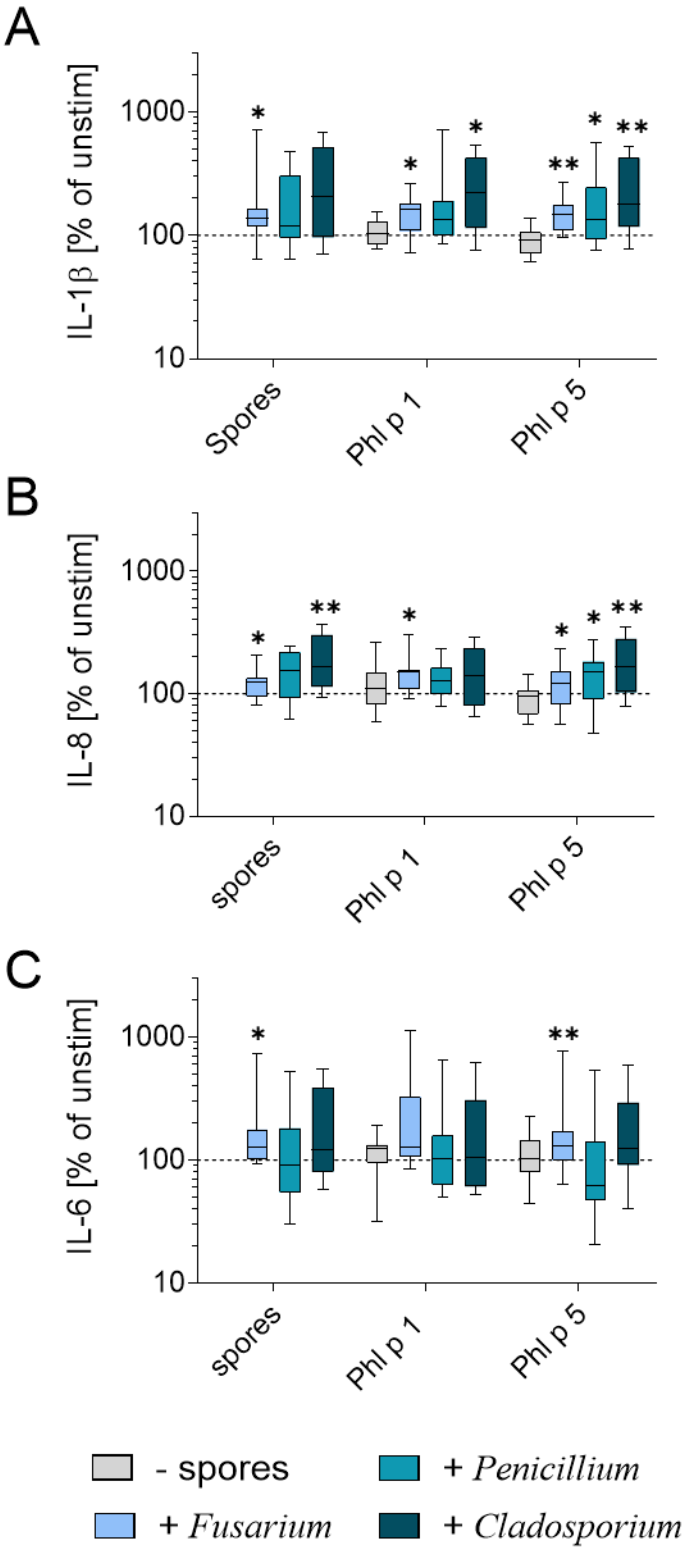

Figure 52 Stimulation with fungal spores leads to increased inflammatory cytokine release of HNECS. Fungal spores were used either alone or in combination with Phl p1 or Phl p 5 to stimulated HNECs. After 24 h, cytokines were measured in supernatants. n = 10 independent experiments. * $p < 0.5$, ** $p < 0.1$. Fusarium = *Fusarium sp.*, Cladosporium = *Cladosporium cladosporioides*, Penicillium = *Penicillium manginii*. Dashed line indicates unstimulated control.

7.1.4. Barrier function of HNECs stimulated with fungal spores and grass pollen allergens

The influence of fungal spores in combination with grass pollen allergens on the barrier integrity of the nasal epithelium was investigated in air liquid interface cultures of HNECs from non-atopic donors. Trans-epithelial electrical resistance (TER) upon spore and allergen stimulation was monitored after 1 h, 6 h, 24 h and 48 h. Stimulation with both, *Fusarium* and *Cladosporium* slightly reduced TER after 24 h and increased TER after 48 h (Figure 53 A). TER was not changed by stimulation with Phl p 1, but was again increased by Phl p 1 and fungal spore co-stimulation after 48 h. (Figure 53 B).

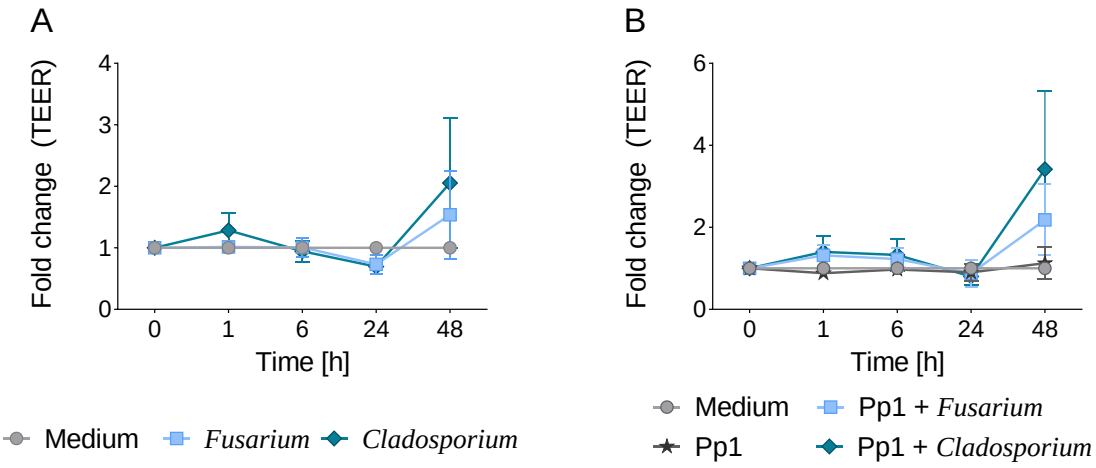

Figure 53 Trans-epithelial resistance is increased by fungal spore stimulation after 48 h. Air liquid interface cultures of HNECs were stimulated with (A) fungal spores, (B) Phl p 1 or a spore and allergen combinations for 48 h and TER was measured at 1 h, 6 h, 24 h and 48 h. n = 7 independent experiments (A, B), n = 4 for spore + Phl p 1 co-stimulation. Pp1 = Phl p 1, Fusarium = *Fusarium sp,* Cladosporium = *Cladosporium cladosporioides*.

Changes in nasal mycobiome after 1 hour outdoor exposure

To investigate whether the nasal mycobiome is influenced by spending time in a flowering meadow during the grass pollen season, the nasal mycobiome of 5 study participants was analyzed before and after spending 1 h outdoors. As baseline they had to spend at least 3 h indoors.

7.1.5.1. Alpha diversity

Alpha diversity indices were calculated for each sample and compared between pre and post exposure for all participants combined. While the richness was not different after spending 1 h outdoors, evenness ($p < 0.001$), Shannon-diversity ($p < 0.001$) and inverse Simpson ($p < 0.001$) indices were higher after outdoor exposure, while Simpson-diversity index was reduced

($p < 0.001$) (Figure 54 A). Shannon-Diversity index was increased post exposure on visit 2 ($p < 0.01$) and visit 5 ($p < 0.05$). While there were no significant differences detected for the other diversity indices during the single visits, diversity was increased after spending time outdoors on each visit (Figure 54 B).

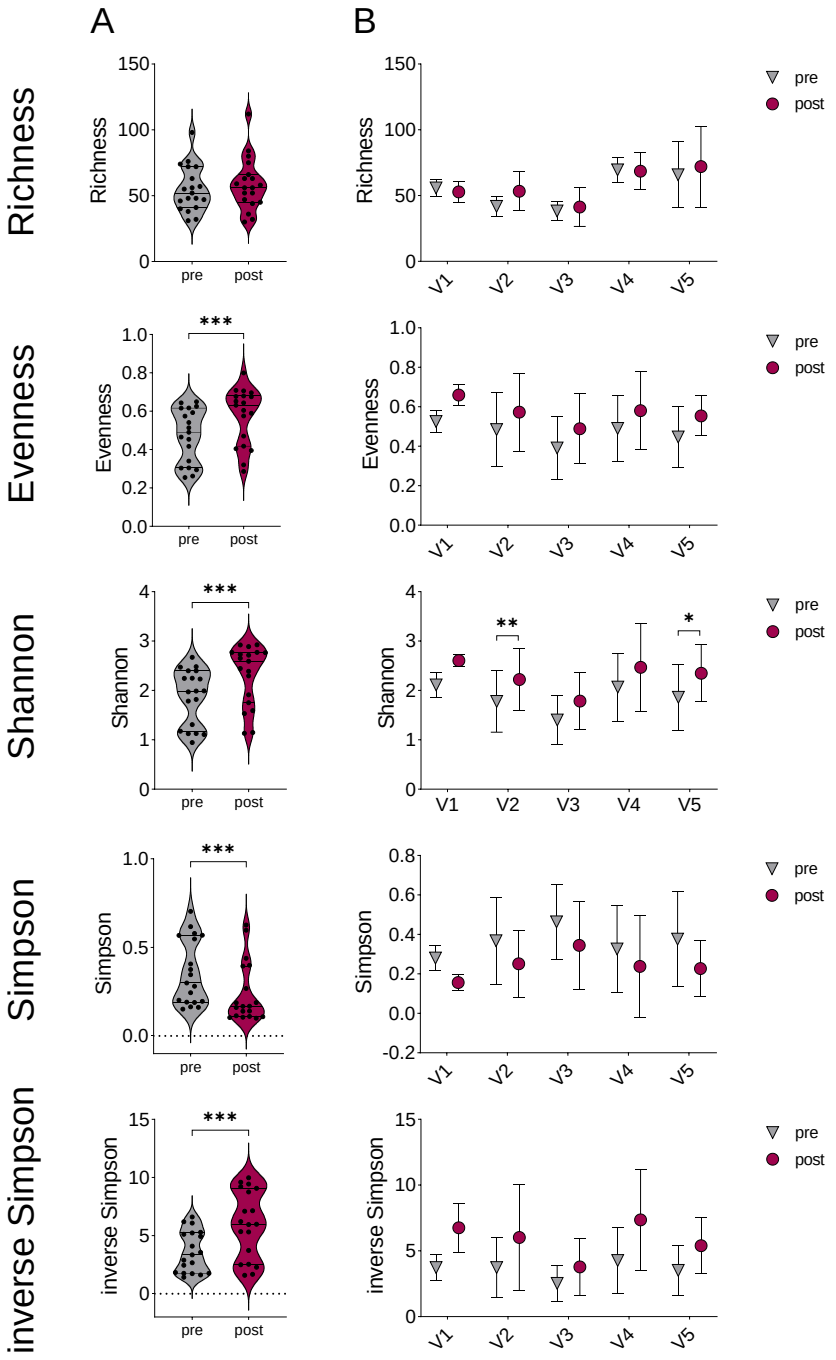

Figure 54 Diversity of nasal mycobiome is increased after spending 1 h in a flowering meadow. Richness, evenness, Shannon, Simpson and inverse Simpson diversity indices were calculated and compared between pre and post exposure for the whole study period (A) or for each specific visits (B) across all participants. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as indicated.

7.1.5.2. Taxonomic overview before and after 1 h outdoor exposure

The abundance of the top 10 families, genera and species was visualized to compare between pre- and post-exposure for each visit (Figure 55). Abundance of *Malassezia*, an integral part of the human mycobiome, was decreased after outdoor exposure on all visits, while outdoor fungi were increased after staying in the flowering meadow. This was observed especially for *Cladosporium*, *Neoerysiphe* and *Russula.*

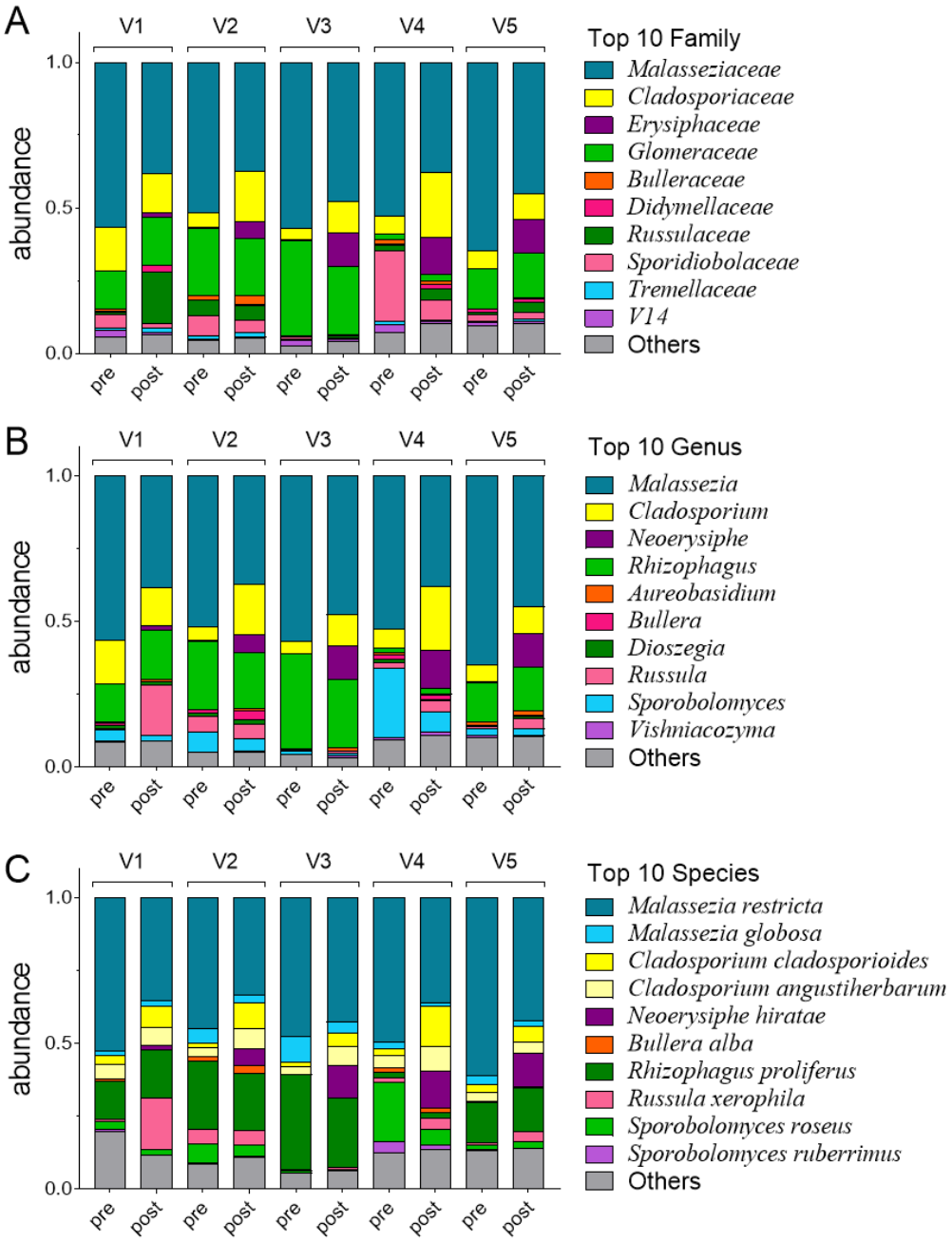

Figure 55 Abundance of outdoor fungi in the nasal mycobiome increased after staying outdoors for 1 h. The abundance for each taxonomic level was combined for the study participants and plotted for each visit comparing pre and post exposure. n = 4 participants.

7.1.5.3. Differences of nasal fungal communities after 1 hour outdoor exposure on family and species level

Because the alpha diversity and abundance of certain families and species was increased after spending 1 h outdoors, these changes were investigated in more detail. First, families were compared between pre and post exposure. Out of 48 families, 39 were not significantly different after the outdoor exposure. Before staying in a flowering meadow, *Malasseziaceae* ($p < 0.0001$), *Globuleviaceae* ($p < 0.05$) and *Sporidiobolaceae* ($p < 0.05$) were more abundant. After 1 hour outdoors, *Cladosporiaceae* ($p < 0.01$), *Erysiphaceae* ($p < 0.01$), *Didymellaceae* ($p < 0.05$), *Phaeosphaeriaceae* ($p < 0.05$), *Dotioraceae* ($p < 0.05$) and *Russulaceae* ($p < 0.05$) were increased in the nasal mycobiome (Figure 56 A).

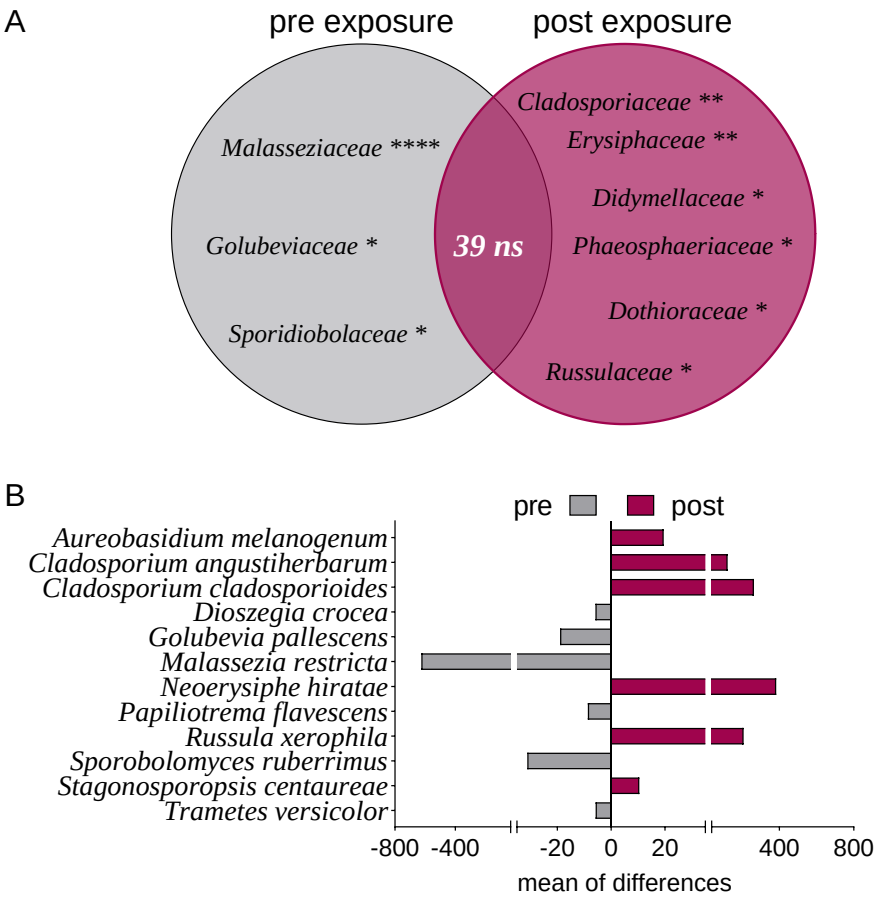

Figure 56 Comparison of the nasal mycobiome before and after 1 h outdoors. (A) Venn diagram of families significantly increased either pre or post exposure. (B) Overview of species significantly different between pre and post exposure, mean of difference in abundance. * $p < 0.05$, ** $p < 0.01$, * $p < 0.0001$.

Taking a closer look on the species level, *Malassezia restricta* ($p < 0.001$), *Dioszegia crocea* ($p < 0.05$), *Golubevia pallescens* ($p < 0.05$), *Papiliotrema flavescens* ($p < 0.05$), *Sporobolomyces ruberrimus* ($p < 0.05$) and *Trametes versicolor* ($p < 0.05$) were decreased in the nasal

mycobiome after spending 1 h outdoors (Figure 57 A). On the other hand, *Aureobasidium melanogenum* ($p < 0.05$), *Cladosporium angustiherbarum* ($p < 0.01$), *Cladosporium cladosporioides* ($p < 0.001$), *Neoerysiphe hiratae* ($p < 0.01$), *Russula xerophila* ($p < 0.05$) and *Stagnosporopsis centaureae* ($p < 0.05$) were increased post exposure (Figure 57 B), (Figure 56 B).

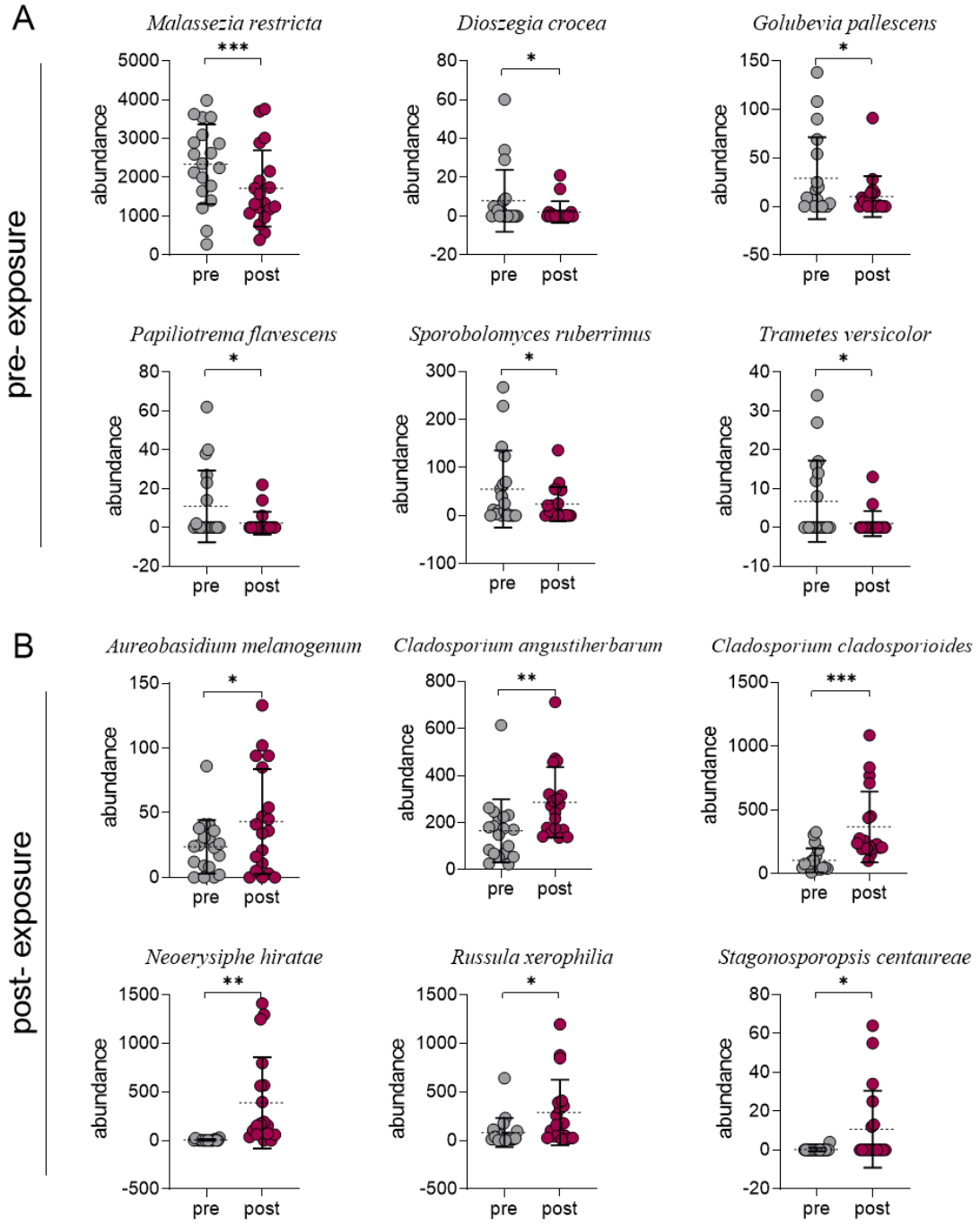

Figure 57 Species more abundant (A) before or (B) after spending 1 h outdoors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

7.2. Discussion

Main findings

The co-occurrence of grass pollen allergens and fungal spores during the grass pollen season

- Enhances pro-inflammatory responses of nasal epithelial cells *in vitro* and
- Leads to short-term changes in the composition of the nasal mycobiome after spending one hour outdoors, reducing the commensal fungal species *Malassezia*, while environmental fungi, e.g. *Cladosporium*, are enriched.

Fungal spores are ubiquitous biological components of indoor and outdoor air. Exposure to fungal allergens increases the risk to develop allergic rhinitis and asthma and exacerbates symptoms (Delfino et al., 1997; Kolodziejczyk & Bozek, 2016). The diversity and abundance of fungal spores are seasonally influenced and spore concentrations of common allergenic fungi coincide with the pollen season of allergenic grass and weed plants (Grewling et al., 2019). To investigate how the co-occurrence of grass pollen and fungal spores, which intensifies allergen exposure for susceptible individuals, influences the nasal innate immune responses to grass pollen allergens, a study was set up during the grass pollen season 2018. While participants spent one hour in a flowering meadow on a weekly basis for five weeks, the amount of pollen, pollen allergens and fungal spores they were exposed to was measured and nasal swabs were taken before and after the exposure.

The amount of outdoor *Poaceae* pollen and Phl p 5 collected in the nasal filters followed the same patterns during the study period. No Phl p 5 was detected in filters worn indoors, even though low amounts of pollen were present inside. This suggests that the amount of pollen in the air relates to allergen exposure in the nose.

At the same time, common environmental fungi were isolated from the outdoor air, so one might safely assume that grass pollen allergens and fungal spores would be inhaled and interact with the nasal epithelium simultaneously.

The fungal species chosen for *in vitro* stimulation of HNECs were *Fusarium, Penicillium* and *Cladosporium*, all of which are known allergenic fungi (Zukiewicz-Sobczak, 2013).

Fungal spores of *Fusarium* and *Cladosporium* induced inflammatory responses, namely increased IL-8, IL-6 and IL-1β, in nasal epithelial cells *in vitro*. *Cladosporium* extracts have been

shown to increase IL-8 secretion in nasal epithelial cells isolated from nasal polyps, and to induce IL-8 dependent neutrophil and RANTES-dependent eosinophil migration (Shin et al., 2006). This was found to be protease-activated receptor (PAR) independent, suggesting it was independent of the protease-like *Cladosporium* allergens (Shin et al., 2006). Other fungal allergens include β-glucans, known dectin-1 ligands, which induce IL-6 and IL-8 secretion in nasal epithelial cells. Binding of β-glucan and dectin-1 induces Th17 responses and neutrophilic inflammation *in vivo* (Mintz-Cole et al., 2012). In contrast, the β-glucan content on the surface of *C. cladosporioides* spores appears to be too low to sufficiently mount a dectin-1 and IL17A dependent immune response, rather leading to enhanced Th2 responses with elevated IL-4 and IL-13 levels, increased AHR and eosinophilic inflammation an asthma mouse model (Mintz-Cole et al., 2012). *Fusarium* species are plant pathogens and opportunistic human pathogens, causing disease in immunocompromised patients. They induce local infections of the eye, skin, sinuses and nails, as well as systemic infections, especially in the lung (Hof, 2020). Although clinical manifestations have been described in the literature, this is one of the first reports providing insights into the interaction of *Fusarium* spores with the innate immune system. So far, upregulated cytokines at mRNA level were only observed in porcine jejunum epithelial cells stimulated with *Fusarium* toxins (Wan, Woo, Turner, Wan, & El-Nezami, 2013). Although toxins were not analyzed in the present study, they cannot be excluded as possible causes for the effects observed.

Previously it was shown that recombinant Phl p 1 enhanced mRNA levels and secretion of IL-6, IL-8 and TGFβ in a bronchial epithelial cell line (Röschmann et al., 2009). In contrast, stimulation of HNECs from non-atopic donors with recombinant grass pollen allergens Phl p1 and Phl p 5 failed to induce cytokine responses on the protein level. These differences could stem from the use of a cell line by Röschmann et al. (2009) vs primary nasal epithelial cells. Although cell lines are an invaluable research tool, they often end up being genetically and phenotypically different from their primary cell counter parts, which represent physiological conditions better (Alge, Hauck, Priglinger, Kampik, & Ueffing, 2006; Pan, Kumar, Bohl, Klingmueller, & Mann, 2009). Because the allergens were directly isolated from grass pollen, it is also possible that the allergens used by Röschmann et al. were contaminated with bioactive pollen-associated compounds or bacterial products. Non-allergenic pollen-associated mediators have been shown to be important in mounting Th2 responses and inhibiting Th1 polarization, while allergens themselves often do not elicit any immune

response (Ashjaei et al., 2015; Gilles et al., 2012; Gilles et al., 2010; Mariani et al., 2007; Traidl-Hoffmann et al., 2005).

Co-exposure to fungal spores and Phl p 1 or Phl p 5 induced similar cytokine profiles as fungal spore exposure alone. Combined with the results from single stimulations, fungal spores are able to provide a pro-inflammatory milieu independent of grass pollen exposure, which is enhanced by co-exposure, thereby providing signals to the tissues downstream and possibly contributing to allergic sensitization to grass pollen allergens.

The epithelial barrier was neither impaired by fungal spores, allergen or spores and allergen co-stimulation. A possible explanation is the passage of grass pollen allergens through the nasal epithelial barrier by transcytosis, leaving tight junctions largely intact and preserving barrier functions (Blume et al., 2009). Fungal extracts of *Alternaria alternata* with known protease activity reduced TER in airway epithelial cells of asthma patients and in an airway epithelial cell line, but not in cells of non-asthmatic donors (Leino et al., 2013). This has been associated with the induction of ROS in a bronchial epithelial cell line (Zaidman et al., 2017). Interestingly, in asthmatic patients suffering from allergic bronchopulmonary aspergillosis (ABPA) a mutation in the zinc finger family transcription factor ZNF77 in bronchial epithelial cells has been associated with higher *Aspergillus fumigatus* colonization. This mutation caused epithelial cell desquamation and the deposition of extracellular matrix proteins, facilitating *A. fumigatus* adhesion to the epithelium (Gago et al., 2018). In line with the results presented here, this shows the importance of a functional epithelial barrier, which is not easily disrupted in non-atopic individuals as observed in the air liquid interface cultures. In this respect, it would be interesting to study the response of HNECs from atopic or asthma patients, which respond differently to allergen stimulation (Bergougnan et al., 2020), to fungal spore and allergen exposure to determine the role of co-exposure on the allergic population.

The nasal microbial composition of healthy adults is quite stable over a long period of time, although seasonal differences have been observed for both bacterial and fungal species which are not part of the core microbiome (Wagner Mackenzie et al., 2019). Studies investigating the direct changes to the mycobiome upon outdoor pollen and fungal spore exposure are lacking. By defining and measuring exposure to fungal spores and pollen, the current study provides evidence that direct pollen/outdoor exposure immediately alters the nasal microflora. Except for the number of species (richness), the composition and diversity of

dominant and low abundance species were impacted by outdoor exposure. This suggests that the nasal microflora is a dynamic niche easily influenced by the environment, although the long-term effects and stability of these differences were not within the scope of this work. These dynamic changes could have effects on allergic sensitization to airborne (pollen) allergens especially because microbial products from the environment are not only allergen sources themselves but also suspected to play a role in the development of allergic diseases. For example, there is a link between co-exposure to low levels of LPS and house dust mite (HDM) allergens, which facilitates HDM sensitization (Eisenbarth et al., 2002; Hammad et al., 2009). Bacterial species isolated directly from grass pollen have also been shown to act as adjuvants and enhanced dendritic cell and Th2 responses in atopic patients after *in vitro* allergen-bacteria co-exposure (Heydenreich et al., 2012). Also, pollen from *Artemisia* containing high levels of LPS were induced allergic sensitization and enhanced allergic lung inflammation *in vivo* (Oteros et al., 2019). Similar experiments using co-exposure to grass pollen and fungal spores in a mouse model should be conducted in the future to fully understand the role of fungal spores during allergic sensitization or exacerbation of allergic symptoms.

Environmental influences also include meteorological parameters such as humidity and atmospheric pressure (Wagner Mackenzie et al., 2019), which were not measured in the present study.

In the nasal mycobiome of healthy adults, *Malassezia* species, among them *M. restricta*, are most abundant (Jo et al., 2016; Jung, Croll, Cho, Kim, & Lee, 2015). Their role in (allergic) skin diseases is already well studied, while there is no clear indication that *Malassezia* plays a role in respiratory allergic diseases (Gelber, Cope, Goldberg, & Pletcher, 2016; W. H. Jung et al., 2015). Rather, they are commensal yeasts and, at least in the skin, *Malassezia* contributes to microbiome and immune homeostasis by interacting with CLRs and TLR2, thereby maintaining the IL-23/IL-17 axis (Baroni et al., 2006; Sparber et al., 2019; Sparber, Ruchti, & LeibundGut-Landmann, 2020). Reduced abundance of *Malassezia* as observed after one hour outdoors could upset the delicate balance and potentially pave the way for allergenic or pathogenic fungi and bacteria to induce immune responses in the nasal epithelium.

The abundance of other fungal species was also negatively affected by one hour of natural fungal spore and pollen exposure. *Dioszegia* is a yeast fungus known to colonize plants and

interconnects with the bacterial community of the phyllosphere (Agler et al., 2016). Although *Dioszegia* has not been detected in the nasal mycobiome, the findings of Agler et al. (2016) provide interesting insights into microbe-microbe interaction. Transferring their findings of negative correlations between *Dioszegia* and bacteria colonization to humans could indicate that exposure to the outdoor environment causes dysbiosis on the bacterial-fungal axis, potentially inducing inflammatory processes in the nasal epithelium. Other fungal species previously not detected in humans but negatively influenced by outdoor pollen and fungal spore exposure include: *Golubevia pallescens* (also known as *Tilletiopsis pallescens*), found in apples inflicted with "white haze" (Richter, Yurkov, Boekhout, & Stadler, 2019); *Papiliotrema flavescens*, which is found in bird excrements and is a possible opportunistic pathogen in humans (Brito et al., 2019); *Sporobolomyces ruberrimus*, identified in soil and air but belonging to a family of opportunistic human pathogens (Bergman & Kauffman, 1984); and *Trametes versicolor*, a medicinal mushroom with immune-activating properties (Benson et al., 2019).

During the grass pollen season in June and July, *Cladosporium*, together with *Alternaria*, is one of the most common fungal taxa (Grewling et al., 2019). *Cladosporium* is commonly found in the outdoor environment, but has been detected in homes as well. Since the indoor spore levels correlated to high outdoor spore levels, it is considered that *Cladosporium* is introduced from external sources and not naturally found indoors (Rosenbaum et al., 2010). Similarly, *Cladosporium* was detected in nasal swabs taken before the outdoor exposure. Because the study participants had to spend three hours indoors before the "experiment" this either indicates high levels of indoor *Cladosporium* spores or is possibly due to previous inhalation on the way to the study site. Furthermore, this would indicate that *Cladosporium* persists in the nasal cavities for at least three hours. Due to the allergenic properties of *Cladosporium* this is especially troubling as it further prolongs allergen exposure, increasing the risk to develop allergies in susceptible individuals, but also enhancing symptoms in allergic patients.

Aureobasidium melanogenum, also known as *Aureobasidium pollulans*, is a black yeast found in a variety of habitats similar to *Cladosporium* (Gostincar et al., 2014). This species has been identified as opportunistic pathogen (M. Wang et al., 2019) and its β-glucan has been shown to bind to IgG in human serum as a possible immunostimulatory agent for medical purposes (Tada et al., 2009). *A. melanogenum* is also of agricultural value, because it produces antifungal and antibacterial compounds and is used as a biocontrol agent (Gostincar et al.,

2014; Takesako et al., 1991). The different fungal community observed after outdoor exposure could partly be caused by this antifungal activity and furthermore, long-term exposure to *A. melanogenum* might upset the delicate balance of the nasal mycobiome with adverse health effects.

For the first time, the current study detected enriched *Neoerysiphe hiratae, Russula xerophila* and *Stagnosporopsis centaureae* in the nasal mycobiome after 1 hour spent outdoors. These fungal species are commonly found either as plant pathogens in the case of *N. hiratae* and *S. centaureae* or, like *R. xerophila,* as mushrooms. *N. hiratae* has been found to infect plants of the *Asteraceae* family (Heluta, Takamatsu, Harada, & Voytyuk, 2010) and because the study participants sat in a flowering meadow, it is possible they encountered this fungal species there. Likewise, *Russula* are widespread mushrooms and spores are in all likelihood easily picked up in meadows (Elliott & Trappe, 2018; Vidal et al., 2019).

While this study reports changes to the nasal mycobiome after spending one our outdoors in a flowering meadow, it does not measure the long-term effects of exposure to environmental fungal spores. Future work should include follow up analyses of the mycobiome for several hours and the next day, as well as successive outdoor stays to assess the stability and variability of the mycobiome.

In conclusion, the exposure to grass pollen, fungal spores and grass pollen allergens, as is encountered during late spring, caused pro-inflammatory responses in nasal epithelial cells *in vitro* which was not observed after exposure to major grass pollen allergens alone. Additionally, even though the nasal bacterial microflora was found to be stable over the course of a whole year, short term mycobiome changes were observed after only one hour outdoors, enriching especially allergenic *Cladosporium* species, while at the same time repressing commensal fungal species such as *Malassezia*. Taken together, this imbalance could cause an aggravation of allergic symptoms or facilitate allergic sensitization to grass pollen.

Chapter 8 – Conclusion

The rising incidence of allergic diseases is a cause for public health concern. Not only are allergic diseases caused by genetic factors, but are now generally considered an environmental disease. The process of allergic sensitization is influenced by many environmental stimuli: the allergens themselves and their matrix, air pollution increasing susceptibility to inflammatory immune responses and simultaneously affecting the allergen carrier, as well as microbial presence in the allergen carrier or the individual.

The aim of this thesis was to elucidate the effect of biotic and abiotic environmental factors on allergic sensitization to pollen allergens in a rather holistic approach combining controlled growth conditions during ragweed growth with metabolomics and *in vitro* and *in vivo studies*, as well as studying the direct effects of pollen exposure on the nasal epithelia and microflora.

Based on climate change scenarios, the influence of elevated CO_2 or ozone levels on the sensitizing and allergenic potential of ragweed pollen was investigated. Rauer et al. (2020) found exposure to elevated CO_2 levels during the vegetation period led to an increased inflammatory reaction towards ragweed pollen extracts and also changed the composition of secondary metabolites. While ragweed pollen grown under elevated ozone levels were less immune-modulatory, they were more chemotactic due to elevated $PALM_{LTB4}$ levels. Comparing pollen from urban and rural birch trees, especially pollen derived LPS and $PALM_{PGE2}$ were responsible for the more tolerogenic immune responses initiated by rural birch pollen extracts. Concluding from these studies, a myriad of pollen-derived substances, which is subjected to environmental changes, plays an important role in the intricate interplay of allergen and immune system to induce either allergic or anti-inflammatory responses.

Throughout the year, the nasal microbiome is exposed to varying levels of allergenic pollen. While the overall microbial diversity is stable even under long-term pollen exposure, the composition of the microbial community changes, especially between atopic and non-atopic individuals, further deepening our understanding of the protective properties of commensal microbes. Additionally, short term changes of the nasal fungal composition were observed under natural grass pollen (allergen) and fungal spore exposure, which also provided pro-inflammatory stimuli for nasal epithelial cells.

In summary, these studies highlight the importance of the allergen matrix, which is species specific and consists of various pollen-derived immunomodulatory substances as well as associated microbes in the form of, but not restricted to, bacteria and fungi. Together they shape the immune response towards pollen allergens and are yet themselves subject to environmental influences. Lastly, the individual microbial environment plays an equally important role by providing a protective milieu in healthy individuals and inflammatory stimuli in allergic patients.

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African proverb

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List of Posters and Talks

Oral Presentations

- *„A walk in the park: Exposure to fungal spores increases nasal inflammatory responses to grass pollen and changes the nasal mycobiome."*
  Rauer D, Herrmann S, Kolek F, Schwierzeck V, Skottke F, Leier-Wirtz V, Plaza M, Traidl-Hoffmann C, Damialis A, Gilles S
  7th European Symposium on Aerobiology, virtual edition, November 16-20, 2020

- *„A walk in the park: Exposure to fungal spores increases nasal inflammatory responses to grass pollen and changes the nasal mycobiome."*
  Rauer D, Herrmann S, Kolek F, Schwierzeck V, Skottke F, Leier-Wirtz V, Plaza M, Traidl-Hoffmann C, Damialis A, Gilles S
  EAACI Digital Congress 2020, June 6-8, late breaking oral abstract session

Poster Presentations

- *"Influence of elevated ambient CO_2 on the sensitizing potential of ragweed (Ambrosia artemisiifolia) pollen"*
  Rauer D, Frank U, Müller C, Gilles S, Aglas L, Ferreira F, Ernst D, Alessandrini F, Traidl-Hoffmann C
  II Joint Meeting of the German Society for Immunology (DGfI) and the Italian Society of Immunology, Clinical Immunology and Allergology (SIICA)
  Munich, Germany, September 10-13, 2019

- *"Nasal microbiome under natural pollen exposure conditions: a time series analysis in allergic rhinitis patients vs. health subjects"*
  Rauer D, Hülpüsch C, Reiger M, Nussbaumer T, Obersteiner A, Rothballer M, Häring F, Damialis A, Traidl-Hoffmann C, Neumann A, Gilles S
  46th ADF Annual Meeting
  Munich, Germany, March 13-16, 2019
  Winner of ADF-ECARF Award 2019

- *"Time-series assessment of nasal microbiome in allergic rhinitis patients and healthy subjects under natural pollen exposure"*
  Rauer D, Hülpüsch C, Reiger M, Nussbaumer T, Obersteiner A, Rothballer M, Häring F, Damialis A, Traidl-Hoffmann C, Neumann A, Gilles S
  16th EAACI Winter School
  Saas-Fee, Switzerland, January 25-28, 2018
  Poster Prize

- *"Influence of CO_2 and ozone on the allergenic potential of ragweed (Ambrosia artemisiifolia) pollen"*
  Rauer D, Fischbeck A, Frank U, Gilles S, Damialis A, Alessandrini F, Ernst D, Traidl-Hoffmann C
  13th Spring School on Immunology (DGfI)
  Ettal, Germany, March 5-10, 2017
- *"Differential impact of CO2 and ozone on the immune-modulatory potential of ragweed (Ambrosia artemisiifolia) pollen"*
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„Pollen induces reactivation of latent herpesvirus and differentially affects infected and uninfected murine macrophages."

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2018 Platzer A, Polzin J, Rembart K, Han PP, **Rauer D**, Nussbaumer T.
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Appendix

Cell Donors and Study Participants

Table 20 Overview Cell Donors. Non-atopic donors for HNEC and DC stimulation. <100kU/ml IgE and/or RAST class 0 for measured aeroallergens were considered non-atopic.

Donor	Sex	Age	Total IgE [kU/ml]	Aeroallergens (RAST class; HDM/Cat/Dog/Oat/Grasses/ Rye/Penicillium/Cladosporium/ Aspergillus/Alternaria/Botrytis/Alder/ Birch/Hazel/Ash/Mugwort/Buckhorn)
46	female	58	34	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
54	female	57	55	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
61	female	55	38	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
74	male	67	490	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
219	male	50	34	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
222	female	54	17	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
227	male	65	12	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
232	female	53	3.7	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
235	male	51	3	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
237	female	34	20	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
243	female	52	1.3	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
331	female	28	44	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
351	male	34	3.2	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
367	N/A	N/A	N/A	N/A
393	male	42	25	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
412	female	27	1.6	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
430	female	40	15	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
448	female	61	64	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
450	male	47	16	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
454	female	31	200	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
461	male	35	180	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
496	male	57	22	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
503	female	54	1.3	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
539	male	36	62	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
585	male	37	7.9	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
591	female	39	52	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
606	female	37	8.6	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
615	male	36	34	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0

9005	female	29	0	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9010	female	46	52	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9016	female	27	18	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9017	female	28	46.8	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9020	female	28	20	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9024	male	32	5.1	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9026	female	38	60	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9027	female	56	7.44	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9035	female	38	39	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9036	female	39	11	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9040	female	40	7.6	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9047	female	36	19	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9049	female	20	57	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9051	female	41	0.67	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9060	female	28	78	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0

Table 21 Study participants for Picnic Study

Donor	**Sex**	**Age**	**Atopy status**	**Total IgE [kU/ml]**	**Aeroallergens (RAST class; HDM/Cat/Dog/Oat/Grasses/Rye/ Penicillium/Cladosporium/ Aspergillus/Alternaria/Botrytis/Alder/ Birch/Hazel/Ash/Mugwort/Buckhorn)**
9008	male	34	atopic	233	3/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9041	female	34	atopic	360	4/0/0/0/3/0/0/0/0/0/0/1/2/2/0/0/0
9017	female	29	non-atopic	46.8	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9005	female	29	non-atopic	0	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9016	female	27	non-atopic	18	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9020	female	28	non-atopic	20	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0
9036	female	39	non-atopic	11	0/0/0/0/0/0/0/0/0/0/0/0/0/0/0/0

Table 22 Study participants for Panel Study. Atopy status was determined by total IgE and specific IgE levels indicating sensitization. AR: atopic rhinitis patients, NA: non-atopic participants.

Donor	Sex	Age	Atopy status	Total IgE [kU/ml]	Specific IgE (*D. pteronyssinus*; Cat; Wheat; Celery; *Phleum pratense*; Rye; Birch; Hazel; Artemisia)
AR-1	male	25	atopic	63.7	0.2; 0.06; 0.06; 0.04; 2.13; 0.86; 1.25; 0.97; 0.08
AR-2	female	26	atopic	24.8	0; 0; 0.03; 0.19; 0.41; 0.35; 9.45; 4.45; 0.01
AR-3	male	28	atopic	403	0.01; 0.06; 0.54; 1.57; 101; 79.5; 5.33; 6; 4.83
AR-4	female	53	atopic	29	0.02; 0.04; 0.05; 0.09; 0.02; 0.03; 4.67; 2.43; 0.02
AR-5	female	54	atopic	71.2	0.03; 1.43; 0.08; 0.24; 2.27; 1.06; 3.99; 3.43; 0.04
AR-6	female	25	atopic	52.4	0.01; 0.46; 0.02; 0.66; 0.24; 0.17; 7.3; 3.58; 0
AR-7	female	39	atopic	37.2	0.08; 0; 0.03; 0.19; 0.12; 0.07; 5.48; 3.91; 0
AR-8	male	31	atopic	159	0.02; 0.06; 0.04; 0.34; 4.8; 0.99; 41.6; 29; 0.53
NA-1	female	36	non-atopic	46.8	0.01; 0; 0.06; 0; 0.02; 0.02; 0; 0; 0
NA-2	female	26	non-atopic	21.6	0.02; 0; 0.05; 0.03; 0.04; 0.05; 0.03; 0.03; 0.03
NA-3	female	56	non-atopic	7.44	0.01; 0.02; 0.02; 0; 0; 0.01; 0; 0; 0.02
NA-5	male	29	non-atopic	5.62	0.01; 0.01; 0.02; 0; 0.13; 0.1; 0; 0.01; 0.01
NA-6	female	21	non-atopic	37.8	0.08; 0; 0.02; 0; 0; 0.01; 0; 0; 0
NA-8	female	51	non-atopic	17.9	0.01; 0; 0.05; 0; 0.01; 0.02; 0; 0; 0.01
NA-9	female	27	non-atopic	12.2	0; 0; 0.02; 0; 0; 0; 0; 0; 0
NA-10	female	23	non-atopic	152	0.02; 0; 0.03; 0.01; 0.01; 0.03; 0; 0; 0