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Atopy status affects innate immune receptor repertoire, physical and immunological barrier function and response to prebiotics and probiotics of primary epithelial cells

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Abstract

Background: The human epithelium represents the interface between the body and the environment. To achieve an effective defense against environmental insults a crosstalk between epithelial and immune cells as well as the microbiota has to be maintained. Dysregulation of this system either due to genetic, epigenetic or environmental factors may lead to development of chronic inflammation or allergy. To illustrate dysbiosis of commensals on the gastrointestinal tract, airways and skin in early childhood is thought to disturb the innate immune repertoire and subsequently lead to a deficiency in maintaining peripheral tolerance against self or harmless foreign antigens. Ultimately, this renders individuals more susceptible to the development of autoimmunity or allergy.

Prebiotics, probiotics or active microbial structures are assumed to not only influence host microbial homeostasis but also directly affect epithelial and immune cells. However, the underlying mode of action remains largely unclear and direct health benefits have to be proven.

Aim: The first aim of this study was to establish a new method for isolation and culture of primary nasal epithelial cells (HNECs) from well characterized non-atopic and atopic donors. The second aim was to subsequently gain deeper knowledge in the role of the innate immune receptor repertoire, physical and immunological barrier function and response of HNECs. Therefore differences between atopic and non-atopic donors in pattern recognition receptor (PRR) expression as well as reaction to exposure with microbial compounds and allergens were studied in HNECs.

Furthermore, it was investigated if there is a direct effect of short-chain galacto- and long chain fructo-oligosaccharides (scGOS/lcFOS) and lactic acid bacteria (LAB) or lactocepin on epithelial cell physical and immunological barrier function and response of human primary keratinocytes (KCs) and HNECs and whether it is affected by atopy status.

Methods: For experiments with HNECs a fast and minimal invasive method to isolate and culture HNECs from clinically and immunologically well characterized patients was established. Differentiated air-liquid-interphase (ALI) cultures were obtained from HNECs and tested for barrier integrity by transepithelial electrical resistance (TER) measurements, immunofluorescence and scanning electron microscopy. Cells derived from atopic or non-atopic donors were compared with respect to their barrier integrity, expression of toll-like receptors (TLRs), intracellular TLR adaptors, inflammasome components as well as immune responses in steady state and after exposure to aeroallergens and an array of TLR ligands using TER, immunofluorescence staining, qPCR, flow cytometry and ELISA readouts.

For experiments with pre- and probiotics, human primary KCs and HNECs from non-atopic or atopic (eczema) donors were stimulated in different cell culture systems with cytokines (IFN-γ, TNF-α, IL-4, IL-13), TLR ligands (PolyI:C) or pollen extracts (birch, timothy grass, common ragweed) and treated with lactocepin or a specific mixture of non-digestible scGOS/lcFOS and

lactic acid bacteria (*IMS1* or Lactobacillus *rhamnosus*). Pro-inflammatory cytokine and chemokine release as well as effects on barrier function were assessed via ELISA or TER.

Results: Freshly isolated and cultured HNECs showed all characteristics of epithelial cells with the presence of goblet and ciliated cells and the formation of a tight barrier. Hence, the newly created method to isolate und culture HNECs from well-defined donors was successfully established.

On mRNA level, unstimulated HNECs expressed TLR1-6 and 9. mRNA expression of TLR-4 and -9 and TLR-9 protein expression was lower in cells of allergic rhinitis (AR) donors than in cells of non-atopic donors. Chemokine responses to TLR ligand stimulation were similar between HNECs of AR and non-atopic donors. However, secretion of IL-18 and IL-33 was elevated under steady state conditions as well as after stimulation with TLR ligands, inflammasome activators and pollen extracts in HNECs of AR donors as compared to controls. Similarly, activation of the inflammasome resulted in higher IL-1β levels while transcript levels of the inflammasome-related genes NLRP-3, AIM-2 and Caspase-1 were decreased in HNECs of AR patients when non-atopic donor derived cells.

In HNECs of non-atopic donors, the presence of scGOS/lcFOS, both alone, or in combination with *IMS1* or Lactobacillus (L.) *rhamnosus* decreased the IFN-γ- and TNF-α-induced secretion of pro-inflammatory CXCL-10 and CCL-5 while this effect was only reproducible for CCL-5 in AR donor derived cells. Similarly, scGOS/lcFOS as well as *L. rhamnosus* decreased CXCL-10 and CCL-5 in supernatants of non-atopic but not atopic eczema (AE) donor derived KCs in this setup. No effect was observed in HNECs stimulated with PolyI:C or aqueous pollen extracts (APEs) or KCs stimulated with IL-4 and IL-13. Stimulation with the bacterial endopeptidase lactocepin, significantly decreased the IFN-γ- and TNF-α-induced secretion of CXCL-10 and CCL-5 in HNECs and KCs, irrespective of the donor atopy status. CCL-2 levels were only decreased in KCs from non-atopic donors. The combination of scGOS/lcFOS and *L. rhamnosus* or *IMS1* (only KCs) induced a temporary significant rise in TER in ALI cultures derived from non-atopic donor HNECs and KCs but not from AR or AE donors.

Conclusion: In summary, results of this study indicate differences in expression levels of innate immune receptors, adaptors and inflammasome components but also immunological responses in cells derived from atopic donors when compared to non-atopic donors. Thus, different expression patterns of TLR, -adaptor molecules or inflammasome components may be predisposing factors skewing the development of systemic and/or local immune function away from a balanced tolerogenic function towards a phenotype more susceptible to allergic sensitization and chronic inflammation.

Additionally, direct anti-inflammatory, microbiota independent, immune-modulatory properties of prebiotics and probiotics were observed but showed to be affected by atopy status. Therefore, prebiotics and probiotics may be a tool to restore immune homeostasis. However,

the here used substances may be more suitable for T helper (Th)1-response associated conditions and prebiotics and probiotics more fitted for a Th2-response associated conditions such as allergy may still have to be tested.

Zusammenfassung

Hintergrund: Das humane Epithel stellt die Schnittstelle zwischen dem menschlichen Körper und der Umwelt dar. Sowohl die Aufrechterhaltung der Homöostase als auch die erfolgreiche Immunabwehr beruhen auf einem ausgeklügeltem Zusammenspiel von Epithel- und Immunzellen sowie der körpereigenen Mikrobiota. Eine Deregulierung dieses Zusammenspiels, die durch genetische oder Umweltfaktoren hervorgerufen werden kann, kann zur Entwicklung von chronisch inflammatorischen Erkrankungen oder Allergien führen. Eine Dysbiose der körpereigenen Mikroflora könnte beispielsweise zu Störungen des innaten Immunrepertoires führen oder durch diese bedingt sein. Vermultich resultiert sie in einer verminderten Fähigkeit zur Aufrechterhaltung der peripheren Toleranz gegenüber Selbst- oder harmlosen Fremdantigenen, was eine verstärkte Neigung zu Autoimmunerkrankungen und Allergien bedingt.

Es wird vermutet, dass Präbiotika, Probiotika und aktive mikrobielle Strukturen hier Einfluss nehmen können, indem sie einer Dysbiose der Mikrobiota entgegenwirken oder direkten Einfluss auf Epithel- und Immunzellen ausüben. Allerdings ist der zugrundeliegende Mechanismus noch nicht geklärt, ebenso wie die Existenz direkter gesundheitlicher Nutzen.

Ziel: Das erste Ziel der vorliegenden Studie war humaner nasaler Epithelzellen (HNECs) von ausreichend charakterisierten Probanden zu isolieren und zu kultivieren. Um einen tieferen Einblick in die Rolle des innaten Immunrepertoires, der physikalischen und immunologischen Barriere Funktion und Antwort humaner primärer Epithelzellen zu gewinnen, wurden dann Unterschiede der HNECs von Spendern mit verschiedenem Atopiestatus im Bezug auf deren Mustererkennungsrezeptorenexpression und Reaktionen auf mikrobielle und allergene Stimuli untersucht.

Außerdem wurde untersucht, ob kurzkettige Galakto- und langkettige Fruktooligosaccharide (scGOS/lcFOS) und Milchsäurebakterien oder Laktozepin einen direkten Effekt auf die Immunantwort von Epithelzellen (Keratinozyten und nasale Epithelzellen) sowie auf deren Barrierefunktion haben können und ob dieser Effekt vom Atopiestatus abhängig ist.

Methoden: Für Experimente mit HNECs wurde eine schnelle und minimalinvasive Isolations- und Expansionsmethode entwickelt, um klinisch und immunologisch gut charakterisierte Patientenproben kultivieren zu können. Differenzierte Air-liquid-interface Kulturen (ALIs) wurden aus HNECs hergestellt und im Bezug auf die Integrität und Dichtigkeit ihrer Barriere mit Hilfe von transepithelialen elektrischen Resistenz (TER) Messungen, Immunfluoreszenzfärbungen und Rasterelektronenmikroskopaufnahmen verglichen. Zellen nichtallergischer Spender und Heuschnupfenpatienten wurden im Hinblick auf deren Barrierefunktion, Expression von Toll-like Rezeptoren und intrazellulären Adaptermolekülen, Inflammasomkomponenten sowie deren Immunantwort im Kontrollzustand und nach

Behandlung mit Aeroallergenen und TLR Liganden mit Hilfe von TER Messungen, Immunofluoreszenz Färbungen, qPCR, Durchflusszytometrie und ELISA verglichen.
Humane primäre Keratinozyten (KCs) und HNECs von gesunden Spendern sowie Patienten mit Heuschnupfen oder Neurodermitis wurden dann in verschiedenen Zellkultursystemen mit Zytokinen (IFN-γ, TNF-α, IL-4, IL-13), TLR Liganden (PolyI:C) oder Pollenextrakten (*Birke, Timothy Gras, Ambrosia*) stimuliert und gleichzeitig mit Laktozepin oder einer speziellen Mischung von scGOS/lcFOS oder Milchsäurebakterien (*IMS1* oder Lactobacillus *rhamnosus*) behandelt. Die anschließende Zytokinausschüttung und Effekte auf die Barrierefunktion wurden mittels ELISA oder TER gemessen.
Ergebnisse: Frisch isolierte und kultivierte HNECs zeigten alle Charakteristika epithelialer Zellen und die Präsenz von Becher- und ziliierten Zellen sowie die Ausbildung einer Barriere. Die Etablierung der neuen Methode zur Isolation und Kultivierung von HNECs war also erfolgreich.
Auf mRNA Ebene exprimierten HNECs im unstimulierten Zustand TLR-1 bis -6 und -9. Zellen von Heuschnupfen Spendern exprimierten weniger TLR-4 und -9 mRNA und TLR-9 Protein als Zellen von nicht-atopischen Spendern. Allgemein zeigte die Stimulation mit TLR Liganden ähnliche Chemokinantworten bei HNECs beider Spendertypen. Unterschiede wurden hingegen bei den Zytokinen IL-18 und IL-33 beobachtet, die sowohl im Kontrollzustand als auch unter Stimulation mit TLR Liganden, Inflammasom-aktivierenden Stoffen sowie Pollenextrakten in Heuschnupfen Spendern erhöht waren. Stimulation des Inflammasoms resultierte außerdem in erhöhten IL-1β Werten, wobei die Inflammasomgene NLRP-3, AIM-2 und Caspase-1 in Heuschnupfen Spendern im Vergleich zu Kontrollen verringert waren.
In mit IFN-γ und TNF-α vorstimulierten HNECs von nicht-allergischen Spendern konnte die Präsenz von scGOS/lcFOS und deren Kombination mit *IMS1* oder Lactobacillus (L.) *rhamnosus* die Ausschüttung von CXCL-10 und CCL-5 verringern, was in Heuschnupfen Spendern nur auf CCL-5 zutraf. In KCs verringerten scGOS/lcFOS und *L. rhamnosus* hier die Ausschüttung von CXCL-10 und CCL-5 auch nur in gesunden, nicht aber in Neurodermitis Spendern. Wurden die HNECs mit PolyI:C oder wässrigen Pollenextrakten, die KCs mit IL-4 und IL-13 stimuliert, konnte deren Chemokinausschüttung nicht durch die Prä- und Probiotika verringert werden. Laktozepin verringerte die IFN-γ- und TNF-α-induzierte Sekretion von CXCL-10 und CCL5 bei HNECs, sowohl in HNECs und KCs von nicht-allergischen und Heuschnupfen oder Neurodermitis Spendern. CCL-2 wurde nur in KCs gesunder Spender reduziert. Die Kombination von scGOS/lcFOS und *L. rhamnosus* oder *IMS1* (in KCs) bewirkte außerdem einen temporären signifikanten TER Anstieg, allerdings nur in HNECs und KCs von nicht-atopischen Spendern.
Fazit: Zusammenfassend weisen die Ergebnisse der hier beschriebenen Studie darauf hin, dass Unterschiede im Expressionslevel von innaten Immunrezeptoren, Adaptern,

Inflammasomkomponenten aber auch der Immunantwort in Spendern mit Heuschnupfen im Vergleich zu deren Kontrollen bestehen. Unterschiede in der Expression von TLRs, Adaptermolekülen und Inflammasomkomponenten könnten also zu einer Neigung zur Allergieentwicklung führen und durch unterschiedliche Ausbildung des Immunsystems zurück zu führen sein.

Außerdem konnte beobachtet werden, dass Präbiotika und Probiotika direkte anti-inflammatorische, Mikrobiota-unabhängige und immunmodulatorische Effekte hervorrufen können, die allerdings abhängig vom Atopiestatus sind. Daher könnten Präbiotika und Probiotika zwar ein Werkzeug zur Wiederherstellung der Immunhomöostase sein. Allerdings mögen die hier verwendeten Substanzen eher auf T Helfer (Th)1 Immunantwort assoziierte Erkrankungen zugeschnitten sein passende Präbiotika und Probiotika für Th2 Immunantwort assoziierte Erkrankungen wie Allergie müssen noch getestet werden.

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Abbreviations

AE	atopic eczema
AECM	airway epithelial cell medium
AIM-2	absent in melanoma 2
AJ	adherens junction
ALI	air liquid interface
ALR	AIM-2-like receptor
Amb-APE	APE of *Ambrosia artemisiifolia*
AMP	antimicrobial peptide
APC	antigen-presenting cell
APE	aqueous pollen extracts
AR	allergic rhinitis
ASC	apoptosis-associated speck–caspase recruit domain
ATP	adenosintriphosphat
B.	bifidobacterium
Bet-APE	APE of *Betula pendula*
BSA	albumin from bovine serum
CCR	CC chemokine receptor
cfu	colony forming units
CLR	C- type lectin receptor
DAMO	danger-associated molecular pattern
DC	dendritic cell
degree celsius	°C
EC	epithelial cell
FCS	fetal calf serum
FLG	filaggrin
FMO	fluorescence minus one
GABA	γ-aminobutyric acid
GALT	gut-associated lymphoid tissues
GITRL	glucocorticoid-induced TNF receptor–related protein ligand
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBD	human β-defensin
HDM	house dust mite
HMGB1	High Mobility Group Box 1
HMO	human milk oligosaccharide
HNEC	human nasal epithelial cells
HRV	human rhinovirus
IFM	interferon
IG	immunoglobulin
IL	interleukin
ILC-2	innate lymphoid cells 2
IRF-3	interferon regulatory factor 3
JAM	junctional adhesion molecule
KC	keratinocyte
L.	lactobacillus
LAB	lactic acid bacteria

LAL	limulous amoebocyte lysate
lcFOS	long-chain fructooligosaccharide
LDH	lactate-dehydrogenase
LPS	lipopolysaccharide
LTC4	leukotrienes C4
M S	magnetic activated cell sorting
MAPK	mitogen-activated protein kinases
MDF	myeloid differentiation factor-88
MFI	mean fluorescence intensity
MHC-TCR	major histocompatibility complex-T-cell receptor
MPPtype 2	multipotent progenitor type 2
MRS	deMan, Rogosa Sharpe
MyD88	TIR domain-containing adaptor-inducing interferon b
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NLRP	NOD-like receptor-pyrin-containing
NO	nitric oxide
NOD	nucleotide-binding-and-oligomerization domain
PAF	platelet-activating factor
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PF840	PromoFluor 840
PGI2	prostaglandin I2
Phl-APE	APE of *Phleum pratense*
PolyI:C	polyinosinic–polycytidylic acid sodium salt
PRR	pattern recognition receptor
RAST	radio-allergen-sorbent-test
RIG	retinoic acid-inducible gene
RLR	RIG-I-like receptor
ROS	reactive oxygen species
RT	room temperature
S.	staphylococcus
SCFA	short-chain fatty acid
scGOS	short-chain galactooligosaccharide
SEM	scanning electron microscopy
SEM	standard error of the mean
SIT	allergen specific immunotherapy
SMN	synthetic nasal medium
TARC	TNF-α induced thymus and activation-regulated chemokine
TER	transepithelial electrical resistance
Th1	T helper 1
Th2	T helper 2
TIR	Toll/interleukin-1 receptor
TIRAP	TRIF TIR domain-containing adaptor
TJ	tight-junction
TLR	toll-like receptor
TMB	tetramethylbenzidine

TNR	tumor-necrosis factor
TRAM	TRIF related adaptor molecule
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
VCAM-1	vascular cell adhesion molecule 1
ZO	zona-occluden

1 Introduction

1.1 Allergy- an environmental disease

Non-communicable diseases such as cancer, metabolic, neurodegenerative or autoimmune diseases, psychological disorders and allergies are on the rise. Especially the increase in prevalence of allergy and associated diseases to up to 30 % is attributed to a westernized life style and the disappearance of chronic infectious diseases. (Lambrecht & Hammad, 2017). The attempt to explain this rise in prevalence resulted in the creation of the “hygiene hypothesis”. Epidemiologic correlations between the modern lifestyle meaning more hygiene and less contact with pathogens that prime T helper 1 (Th1) responses were used to explain the increase in T helper 2 (Th2) cell activity which characterizes allergy and chronic inflammation (Rook & Brunet, 2005). However, only increased “hygiene” might not accurately explain the rise in diminished immune regulation. Therefore, as shown in figure 1, the “Old Friends” concept and the “Biodiversity hypothesis” were created which essentially state that rather the increase in westernized life style, thus a rise of urbanization, hygiene, diet changes and less physical activity result in decreased exposure to organisms which normally not only shape the Th1/Th2 balance but rather result in tolerance development, thus immune regulation (Hanski et al, 2012; Rook, 2012; Rook et al, 2003). Indeed, several studies could show that children at risk for allergy development and allergic patients show microbial dysbiosis in the gut, the nose and on the skin, not only meaning a lack of particular strains but also an excess of others and loss of diversity (Altunbulakli et al, 2018b; Chiu et al, 2017; Depner et al, 2017; Gong et al, 2006; Hua et al, 2016; Kobayashi et al, 2015; Song et al, 2016). In addition, psychosocial stress, an unhealthy diet or a lack of physical exercise were shown to induce epigenetic changes in genes related to allergy susceptibility (Corbo et al, 2008; Wright et al, 2004). Finally, climate change and increasing air pollution seem to not only affect the prevalence and severity of allergy spectrum diseases but also allergens themselves due to earlier and prolonged flowering and pollination periods, thus shorter allergen-free seasons (D'Amato et al, 2015b; Fotiou et al, 2011; Schiavoni et al, 2017; Ziello et al, 2012). There seems to be complex gene-gene and particularly gene-environment interactions, putting forth that allergy is not only a genetic but rather also an environmental disease (Gilles et al, 2018).

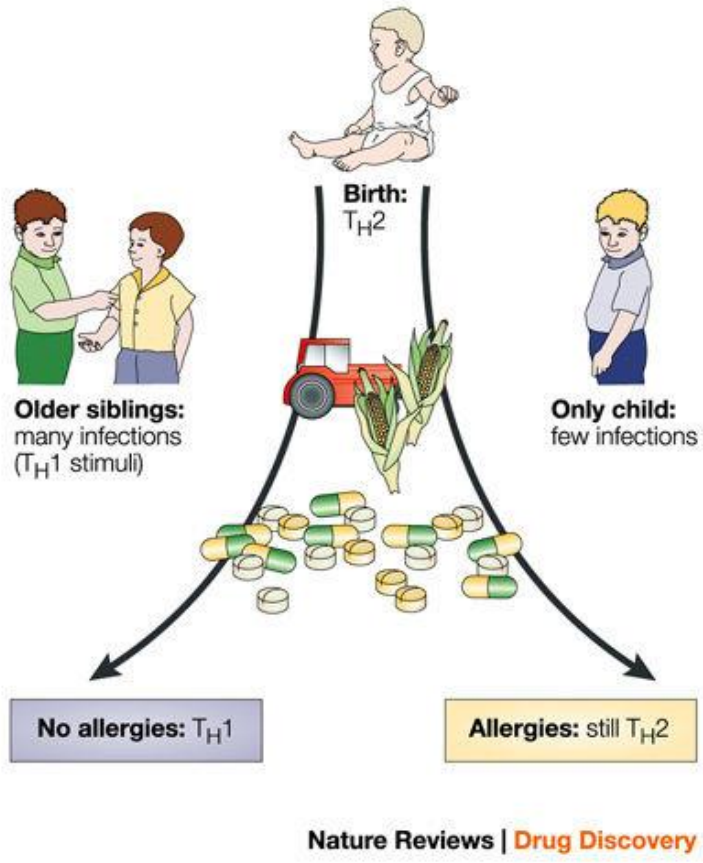

Figure 1: Scheme of factors able to influence the development of allergic sensitization after birth
Allergic sensitization is influenced by family size, socio-economic status, diet but also exposure to whole or parts of microorganisms such as not only pathogens but also commensals. Modernisation, urbanization and lifestyle change are therefore hypothesized to be the reason for a worldwide rise of allergic diseases (Holgate & Broide, 2003b).

Allergy is defined as a harmful immunologically mediated hypersensitivity reaction that occurs in response to normally innocuous substances such as from pollen, food and drugs. Allergens may be any environmental substances able to induce Immunoglobulin (Ig) E production or an adaptive immune response associated with local inflammation (Galli et al, 2008). According to Gell and Coombs this hypersensitivity reaction can be divided into four types. Type I is an immediate hypersensitivity or anaphylactic reaction mediated by IgE antibodies leading to mast cell and basophil activation as the major final effector mechanism. Type II and III hypersensitivity reactions are driven by antigen-specific IgG or IgM antibodies leading to complement activation (Type II) resulting in cell lysis or direct action of macrophages, neutrophils and eosinophils that are linked to Ig-coated FcR-bearing target cells (Type III). The final type, Type IV hypersensitivity, is mediated by cellular effectors such as lymphocytes and a variety of myeloid cell types in contrast to the other three types that are antibody-mediated (Descotes & Choquet-Kastylevsky, 2001).

Allergen exposure can sensitize a subject via induction of IgE production or IgE-independent induction of an adaptive immune response associated with local inflammation so that later re-

exposure to that allergen induces an acute reaction. Persistent and repetitive exposure to this allergen may then mediate the development of chronic inflammation. This chronic inflammation is characterized by the presence of innate and adaptive immune cells releasing inflammatory immune mediators, tissue alterations as well as changes in the function and phenotype of structural cells (Galli et al, 2008).

Barrier epithelial cells (ECs) such as skin keratinocytes (KCs), nasal or gut ECs mostly first encounter allergens and are therefore thought to play a crucial role in determining the outcome of an allergen or pathogen contact since they may control the subsequent immune reaction (Hallstrand et al, 2014). Pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs) or proteases from allergen sources or organisms coming in contact with ECs elicit a specific cytokine response in these cells. In case of the “Old Friends” concept these patterns would be recognized as harmless by ECs and lead to immune regulation rather than an aggressive immune response (Rook & Brunet, 2005). However, in an environment that fails to prime immune regulation, ECs would release cytokines after contact to allergens resulting in a harmful hypersensitivity reaction (Lambrecht & Hammad, 2017).

1.2 Epithelial cells – barrier but also immune function

The body´s barrier sites are not only a mechanical barrier but rather an active organ sensing danger signals and mounting defense mechanisms (Eyerich et al, 2018). Especially, barrier ECs do not only form a protective physical and chemical barrier between the body and the environment but are additionally the first line of immunological defense. An impaired epidermal barrier may be the primary cause of respiratory allergies or inflammatory skin diseases (Taieb, 1999).

1.2.1 Anatomy of the body´s barrier

The airway epithelium is composed of three different types of epithelial cells when compared for their function, ultrastructure and biochemical criteria: basal, ciliated and secretory ECs. The most frequent cells are ciliated cells, which arise either from basal or secretory cells. They possess cilia in order to constantly sweep mucus (acid mucins) which is produced by goblet cells and represents a chemical barrier for pathogens and noxious particles. Basal cells are important for the structure of the airway epithelium because they are ubiquitous and the only cells firmly attached to the basement membrane. Therefore, they play a role in the attachment of more superficial cells to the basement membrane via hemidesmosomal complexes. The basal cells seem to give rise to the goblet and ciliated cells similar to cells in the stratum basale in the skin (Knight & Holgate, 2003).

The epidermis of the skin can be divided into four strata. The stratum basale, which gives rise to renewed cells of the epidermis, contains undifferentiated, basal KCs. The stratum spinosum is the second layer, in which KCs begin their maturation process, but also divide to replenish

the basal layer. In the stratum granulosum KCs actively produce keratin proteins and lipids. The outermost layer, the stratum corneum, contains KC-derived dead cells that are matured and called corneocytes. They are responsible for the barrier function of the skin (Nestle et al, 2009). These epithelial cells form adhesive contact with adjacent cells in order to generate a tight barrier, whose function is to retain moisture and to prevent the penetration of allergens and microbes.

1.2.2 Levels of the body´s barrier

1.2.2.1 The microbiome – influenced early in life

The microbiome is the outermost layer of the body´s barrier to the environment. Similar to ECs and immune cells, it also shapes immune responses and homeostasis. Its influence on health and disease has been extensively studied and found to be important especially when shaping the host´s immune, metabolic, physiologic and even behavioral programming (Cryan & Dinan, 2012; Purchiaroni et al, 2013; Schwiertz et al, 2010; Trompette et al, 2014). Every body part such as particularly the intestine, the skin and the airways have been described to possess their own characteristic microbiota (Grice & Segre, 2011; Karczewski et al, 2014; Marsland & Gollwitzer, 2014). However, body surfaces are not only covered by bacteria but also by viruses and fungi (Abeles & Pride, 2014; Underhill & Iliev, 2014). The human microbiome, mycobiota and virome differs between body sites such as the nose, mouth, lung, stomach, intestine and the vagina. Actinobacteria and Firmicutes dominate in the nose and stomach while Bacteroidetes, Firmicutes and Bacteriophages are the predominant phyla and viruses in the lung.

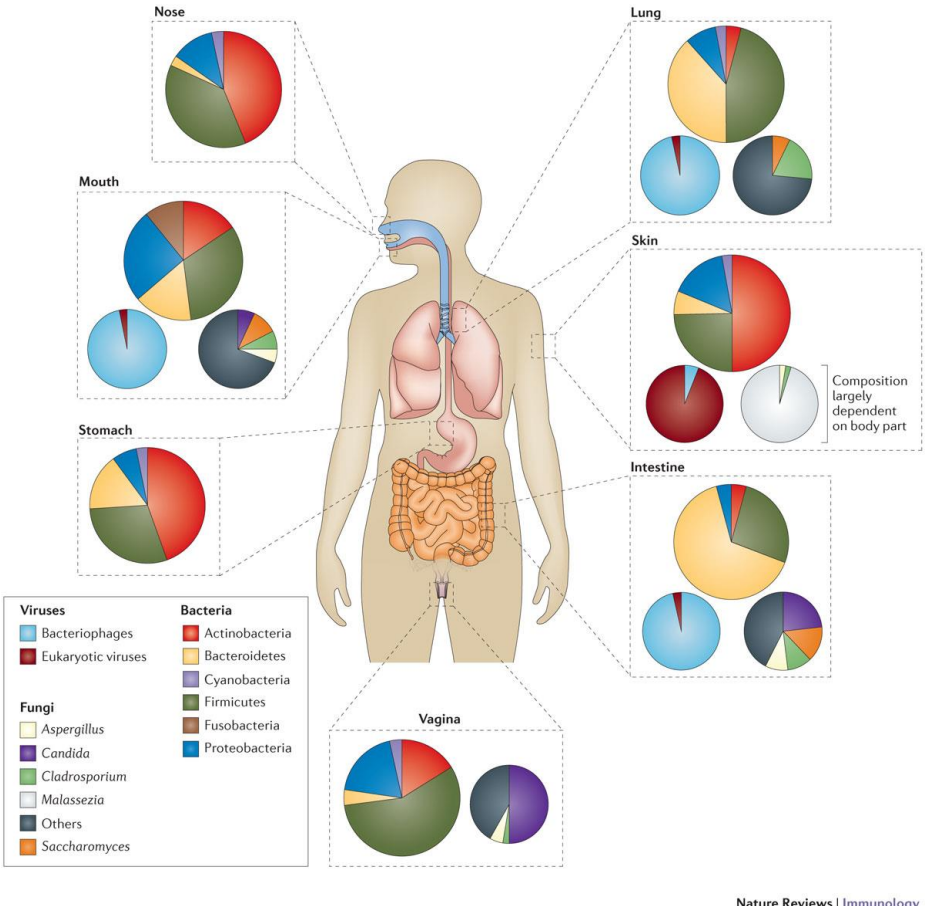

Figure 2: Relative abundance of bacterial, fungal and viral communities on the body. Different body sites which are exposed to the environment such as the nose, mouth, skin, stomach, intestinal tract, vagina and lungs harbor variable relative abundances of bacterial, fungal and viral communities. The bacterial composition is represented by the six most prominent phyla (Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria and Proteobacteria). The fungal composition comprises the most abundant genera (*Aspergillus*, *Candida*, *Cladrosporium*, *Malassezia* and *Saccharomyces).* Different types are summarized as "Others". Viral abundance is described as bacteriophages and eukaryotic viruses (Marsland & Gollwitzer, 2014).

In contrast, the skin microbiome composes mainly of Actinobacteria and eukaryotic viruses while the vagina is characterized by a high content of Firmicutes such as Lactobacilli and fungi such as candida (Byrd et al, 2018; Frank et al, 2010; Kim et al, 2009; Lazarevic et al, 2009; Marsland & Gollwitzer, 2014). The intestine is predominated by four phyla which are Actinobacteria such as Bifidobacteria, Bacteroidetes, Firmicutes and Proteobacteria while Bacteroidetes display the most common phylum (Turnbaugh et al, 2007).

The composition of the microbiota on different body surfaces, especially on the skin, is dependent on the microenvironment of the specific body part which may be explained by nutrient availability. It has been described that sebaceous, moist or dry body parts comprise different distributions of bacteria, fungi and viruses. On moist and dry areas viruses are more prominent than on oily parts while fungi do not differ as much between oily, dry and moist areas but vary on the foot. Similarly, especially the occurrence of bacteria on the foot and on oily areas seems to be different from moist and dry body parts. Moreover, the microbiota and

virome varies between individuals but seems to stay stable over time despite environmental perturbations (Byrd et al, 2018).

Microorganisms are not only able to educate the innate and adaptive immune system but additionally produce molecules that inhibit the colonization by other microorganisms or alter their behaviour. For instance, it was shown that the microbiome alters macrophage development and regulates its function, possibly via bacterial metabolites such as short-chain fatty acids (SCFA) (Luo et al, 2015). The SCFAs acetate, proprionate, and butyrate have been shown to inhibit the histone deacetylase activity in the FoxP3 promoter resulting in remodeling of murine regulatory T (Treg) cell expansion and dampening of macrophage immunity (Chang et al, 2014; Park et al, 2015). Moreover, reduced responsiveness to lipopolysaccharide (LPS) of monocytes was associated with altered cytokine promoter methylation (Sureshchandra et al, 2017). And finally, the microbiota also seems to affect the barrier. Expression of two tight-junction (TJ) proteins was shown to negatively correlate with *Staphylococcus* (S.) *aureus* frequency while especially in lesional atopic eczema (AE) skin *S. epidermidis*, *haemolyticus* and *hominis* were positively correlated to their expression (Altunbulakli et al, 2018b). *S. aureus* is known to overcolonize skin of AE patients. It therefore seems not surprising that altered microbial states such as dysbiosis and loss of diversity are often associated with many diseases (Altunbulakli et al, 2018a; Byrd et al, 2018; Clavel et al, 2014; Hoen et al, 2015; Pfefferle et al, 2013).

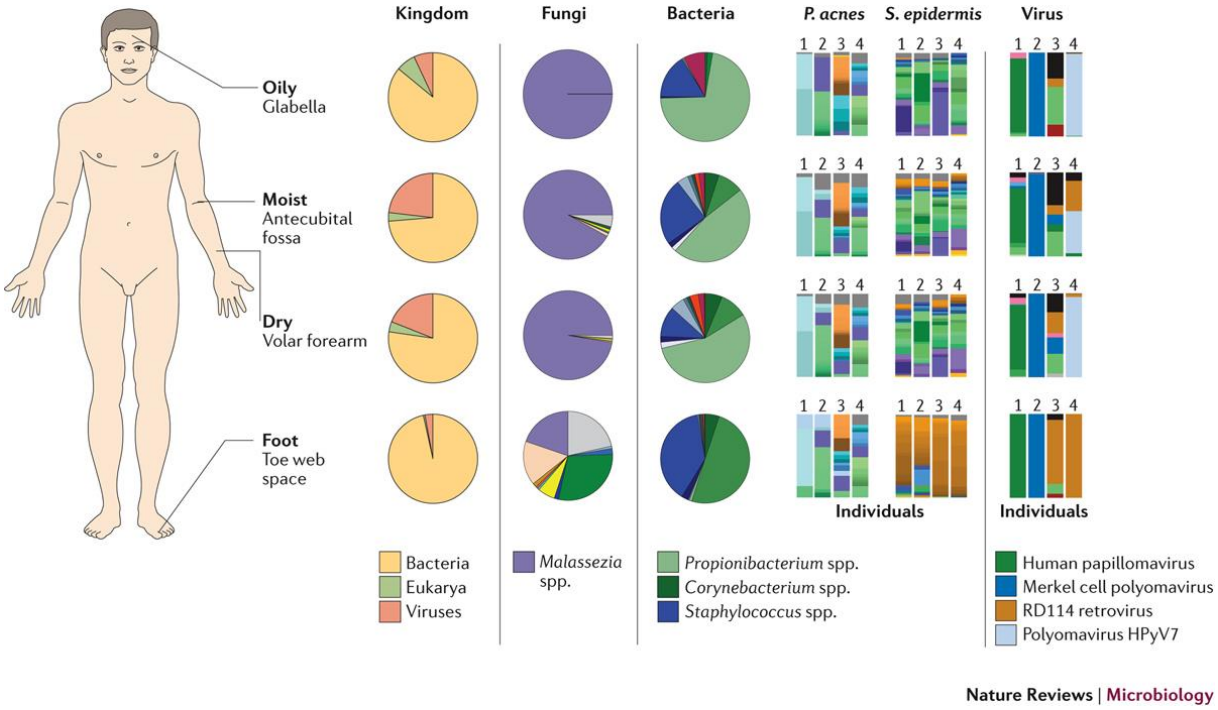

Figure 3: Relative abundance of bacterial, fungal and viral communities on four different, distinct body sites
This figure shows four body sites representing major skin conditions such as sebaceous, moist, dry and body spaces and their major bacterial, fungal and viral microenvironment. The pie charts show the relative abundance of bacteria and fungi respectively. Bar charts show the bacterial species *Propionibacterium acnes* and *Staphylococcus epidermidis* as well as eukaryotic DNA viruses in four reprentive individuals to highlight individuality between persons (Byrd et al, 2018).

Especially chronic inflammatory, non-communicable diseases (NCD), such as allergies or inflammatory bowel diseases have often been associated with a dysbalanced immune response and an altered gut microbiota (Haller & Hormannsperger, 2015; Kau et al, 2011; McLoughlin & Mills, 2011; West et al, 2015). It seems crucial to establish the intestinal microbiota early in life in order to achieve a fully matured immune system which has already been exposed to and provides immune reaction to foreign microorganisms and tolerance to commensals (Lathrop et al, 2011). As already mentioned, factors like genotype, maternal factors, birth mode, diet, weaning, environment and microbes impact the development of the immune system and bacterial colonization of the body (Dore & Blottiere, 2015; Penders et al, 2007; Sjogren et al, 2009; Thavagnanam et al, 2008). Therefore, it is of particular interest that exposure of children to a farm environment in early life results in a protective effect against asthma, allergic rhinitis (AR) and AE development (Debarry et al, 2007; Flohr & Yeo, 2011; Horak et al, 2014; Lluis et al, 2014; von Mutius, 2007). This beneficial farming effect has mostly been attributed to raw cow´s milk consumption (House et al, 2017). However, inhalation of plant-derived compounds from cowshed dust or non-pathogenic bacteria isolated from barns, were also shown to be immune-modulatory and possibly protective (Conrad et al, 2009; Stiehm et al, 2013). Interestingly, also the proximity to the nearest farm as well as a broader bacterial diversity in dust samples were found to be related to this protective effect against atopy in

children (Muller-Rompa et al, 2018). Similarly, it has been described that the prevalence of atopy and blood eosinophil counts were decreased in a population of Amish people that live close to their animals and practice a traditional way of farming (Stein et al, 2016). Dust samples collected in their houses contained high levels of bacterial LPS and were shown to have a protective effect against asthma in mouse models. In contrast, a population of Hutterites, who practiced industrial farming, showed higher rates of allergic sensitisation and dust collected in their homes was found to be less rich in bacterial LPS failing to produce this protective effect in mice.

Similarly, breastfeeding was shown to impact the development of the immune system and bacterial colonization of the body early in life. Breastfed children exhibit a different intestinal pattern which comprises more bifidogenic bacteria in their intestinal microbiota (Wang et al, 2015) and may be protective towards allergic diseases (Hoppu et al, 2001; van Odijk et al, 2003). This effect is thought to be mainly due to human milk oligosaccharides (HMOs) such as galactooligosaccharides, a key ingredient of human milk, which mechanism however, is still not fully understood. Galactooligosaccharides are a group of non-digestible, fucosylated or non-fucosylated oligosaccharides which are structurally and biologically diverse (Bode, 2006; Boehm & Stahl, 2007). Their quantity and structure varies between women (Stahl et al, 2001) just like the composition of breast-milk itself differs significantly among allergic and non-allergic women (Hettinga et al, 2015). As already mentioned, it is thought that HMOs are acting as prebiotics, shaping the gut microbiota and affecting immune responses (Bode, 2012; Yu et al, 2013). Prebiotics are “non-digestible substances that provide a beneficial physiological effect on the host by selectively stimulating the favorable growth or activity of a limited number of indigenous bacteria.” (Reid et al, 2003). Utilization of HMOs by bacteria results in the production of SCFA which additionally favor the growth of beneficial gut commensals and provide nutrition for ECs (Trompette et al, 2014). Furthermore, HMOs seem to directly modulate the host-epithelial responses leading to reduced binding of pathogenic bacteria to the epithelium (Houghteling & Walker, 2015).

Likewise, breast milk also contains beneficial bacteria such as lactobacilli and bifidobacteria which might convey a protective effect towards allergy development and are therefore called probiotics (Hendaus et al, 2016; Soto et al, 2014). Probiotics are “live micro-organisms administered in adequate amounts which confer a beneficial physiologic effect on the host” (Reid et al, 2003). Similarly to what was mentioned beforehand, probiotics seem to aid in establishing an effective immune tolerance early in life, thereby reducing the risk for the development of allergic and inflammatory diseases (McLoughlin & Mills, 2011). In germ-free mice, the development of gut-associated lymphoid tissues (GALT) is disturbed or absent and probiotic bacteria were shown to reestablish this development, thereby inducing tolerance (Mazmanian et al, 2005).

1.2.2.2 The epithelium mounts a chemical and physical barrier

The chemical barrier is tightly connected to the physical barrier which are both mostly mounted by barrier ECs. Especially, the airway and intestinal mucosa are covered with a semipermeable layer of mucus. Mucus is a highly ordered and well hydrated gel composed of high-molecular-weight glycosylated proteins called mucins. Its semi-permeability enables nutrient, water and gas exchange but mucus also contains antimicrobial peptides (AMPs), immunoglobulins, opsonins, cytokines, antioxidant substances and metabolites derived from the commensal microbiota in order to prevent entry of pathogens and allergens (Williams et al, 2006). The normal respiratory tissue expresses eight different mucin genes (MUC1, 2, 4, 5AC, 5B, 7, 8) (Rose et al, 2001). However, the most frequent reported mucins are MUC5AC and MUC5B because they are possibly upregulated during airway inflammation (Williams et al, 2006). The cutaneous chemical barrier consists of hygroscopic factors that contribute to the acidic pH and the natural moisturizing factor of the skin such as amino acids and their derivatives, lipids, lactate, urea and electrolytes (Ali & Yosipovitch, 2013; Verdier-Sevrain & Bonte, 2007). The acidic pH of the skin is important for antimicrobial activity and the diverse composition of the cutaneous microbiome (Elias, 2007; Korting et al, 1990). Moreover, it has a distinct function for the physical barrier of the skin. Neutralization of the pH may result in malfunction of proteases and enzymes involved in the generation of stratum corneum lipids and in increased barrier permeability and decreased barrier integrity (Hachem et al, 2003).

In order to achieve the body´s physical barrier, ECs form TJs and adherens junctions (AJs) or desmosomes. TJs are intracellular junctions which are located at the lateral membrane surface at the apical side of the cell. TJs regulate the passage of ions, water and molecules through the paracellular pathway. This function is generally referred to as gate function.
Additionally, TJs have a fence function, meaning they maintain the cell polarity by blocking free diffusion of proteins and lipids. Occludins, claudins and junctional adhesion molecules (JAMs) are integral proteins of the TJs. Occludins and claudins constitute the backbone of the TJ strands while JAM regulates T cell, neutrophil and dendritic cell (DC) trafficking. Zona-occludens proteins (ZO-1, ZO-2, ZO-3) are linked to the actin cytoskeleton of the TJs and are implicated as important scaffolding proteins (Gonzalez-Mariscal et al, 2003). AJs mechanically connect adjacent cells and induce proliferation and differentiation through homotypic transmembrane E-Cadherin adhesions, which are linked to the actin cytoskeleton via the α- and β-catenin adapter complex (Tunggal et al, 2005). Hemidesmosomes which consist of non-classical cadherins form adhesive bonds between the filamentous cytoskeleton of ECs and the lamina propria (Nawijn et al, 2011).

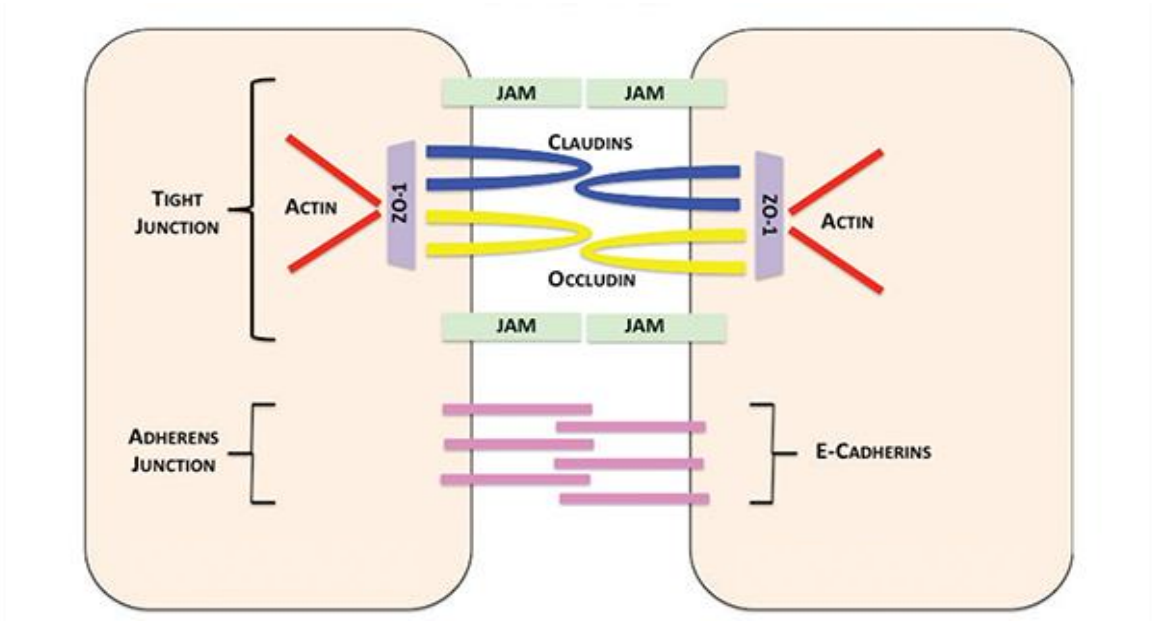

Figure 4: Scheme of epithelial cell junctions.
Epithelial cells form a tight barrier by apical tight-junction complex formation. Tight-junctions are mainly made of claudin proteins (here in blue) and regulated by occluding proteins (here in yellow) both of which are transmembrane proteins that are attached to an adaptor molecule. These adaptor molecules are called zonula-occluden proteins (here in violet). Anchor tight-junction proteins such as intracellular actins are marked in red. Junctional adhesion molecules (here in green) support tight-junction interactions while adherens molecules such as E-Cadherin (here in pink) also contribute to cell-cell contact (modified from Hammer et al, 2015)

Normal functioning of these mechanisms for physical and chemical barrier protection is of great importance since many pathogens are able to penetrate through tissues, e.g. via the production of proteases cleaving TJs and mucus. Moreover, many allergens are proteases themselves (Wan et al, 1999). Exposure of ECs to Der p1, the allergen derived from house dust mite (HDM), or to ragweed and white birch allergen can result in proteolytic degradation of epithelial intercellular adhesions leading to a rapid reduction in epithelial resistance with relocalisation of E-Cadherin and ZO-1 (Heijink et al, 2010; Runswick et al, 2007; Wan et al, 2001).

In this context, the increased air pollution may also play a role. Air pollutants may interact with the epithelial surfaces working as adjuvants which could cause detrimental immune reactions. O_3, NO_2, and other volatile organic compounds were shown to induce inflammation and disruption of the epithelial barrier (Saxon & Diaz-Sanchez, 2005; Traidl-Hoffmann et al, 2009). This disruption by air pollutants might be dependent on the activation of the ligand-activated transcription factor aryl hydrocarbon receptor (AhR) which is highly active in the epidermis and has many barrier-associated gene targets (Haas et al, 2016; Hidaka et al, 2017; Hidaka et al, 2016). For instance, in an AhR knock-out mouse model it could be shown that intercellular connectivity was lost due to the lack of the receptor, which resulted in increased transepidermal water loss, a parameter for skin barrier integrity (Haas et al, 2016).

1.2.2.3 The immune barrier

The fourth barrier of the body is the immune barrier which is often first induced by ECs. These cells elicit a highly complex cell-mediated immune response to all kinds of external factors in mucosal barriers and the skin which requires many cell types rapidly responding.

1.2.2.3.1 Innate immune receptors

In order to sense and respond to danger signals, ECs possess of pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) or RIG-I-like receptors (RLRs). These receptors recognise molecules from Gram-positive and –negative bacteria, DNA and RNA viruses, fungi and protozoa with high target specificity (McInturff et al, 2005). In contrast to antigen receptors of T and B cells, PRRs are germline-encoded and expressed constitutively. Following activation, PRRs activate specific signaling pathways that lead to robust but highly defined innate immune responses priming subsequent adaptive immune responses.

TLRs are a group of glycoproteins working as surface transmembrane receptors which are mostly located in cellular surface- or plasma membranes, intracellular compartments, endoplasmic reticuli or endosomes, enabling cells to recognize self from non-self (Hari et al, 2010; Valins et al, 2010). TLR-1, -2, -4, -6 recognise PAMPs like components of microbial, viral or fungal nature, TLR-5 bacterial flagellin, TLR-3, -7 and -8 recognise single stranded or double stranded viral RNA and TLR-9 unmethylated DNA CpG motifs which are common to bacterial DNA (Kawai & Akira, 2010). Activation of TLRs by its corresponding ligand results in interaction of the Toll/interleukin-1 receptor (TIR) domain of the TLR with an adaptor molecule (Miller & Modlin, 2007). TIR domain-containing adaptor molecules include Myeloid differentiation factor-88 (MyD88), TIR domain-containing adaptor-inducing interferon b (TRIF), TIR domain-containing adaptor (TIRAP) and TRIF related adaptor molecule (TRAM) which are recruited by different TLRs and activate distinct signaling pathways. MyD88 is used by all TLRs except for TLR-3 and activates the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and mitogen-activated protein kinases (MAPKs) to induce inflammatory cytokine release (Kawai & Akira, 2010). TLR-3 and -4 trigger the TRIF pathway resulting in activation of the transcription factors interferon regulatory factor 3 (IRF-3) and NFκB. TRAM and TIRAP function as sorting adaptors recruiting TRIF to TLR-4 and MyD88 to TLR-2 and -4 (Kawai & Akira, 2010; Miller & Modlin, 2007). Hence, TLR signaling pathways can be classified in MyD88-dependent, driving the induction of inflammatory cytokines or TRIF-dependent, driving the release of inflammatory cytokines and type I interferons.

Many allergens are ligands for TLRs or are able to activate these receptors. The allergens of Aspergillus, for instance, cleave fibrinogen into small fragments which then directly interact with TLR-4 (Millien et al, 2013). TLR-4 is also required for the recognition of HDM and cockroach allergen and the subsequent development of Th2-cell associated immunity

(Hammad et al, 2010; Ullah et al, 2014). Der p 2 is the main HDM allergen and shows structural homology with MD2, the LPS-binding component of TLR-4, enabling the allergen to directly interact with the receptor (Trompette et al, 2009). In contrast, cat and dog allergens were shown to bind to LPS, thereby interacting with TLR-4 (Herre et al, 2013). Similarly, *S. aureus* was shown to induce the TLR-2/-6 pathway which results in the release of thymic stromal lymphopoietin (TSLP) by KCs (Takai et al, 2014).

Again, increasing pollution influences innate immune recognition since pollution-derived reactive oxygen species (ROS) can favor the production of DAMPs by ECs inducing TLR or other PRR signaling, possibly even resulting in chronic inflammation (Maes et al, 2010). Activation of TLR signaling by pollutants is hypothesized to prime, particularly the airways, for pro-allergic responses or to alter PRR responses inducing different signaling cascades upon binding of ligands such as allergens (Bauer et al, 2012). This dysregulation of PRR signaling or more importantly mutations in PRRs have recently been related to susceptibility to, not only infectious but also chronic inflammatory diseases such as atherosclerosis, asthma, psoriasis and AE (Cook et al, 2004; Hari et al, 2010). On the other hand, the absence of TLR signaling by PAMPs may also favour an allergy-prone immune response. LPS, a ligand for TLR-4, was shown to promote T-helper 2 (Th2) prone immune responses in low doses whereas sufficient doses rather favoured Th1 priming (Eisenbarth et al, 2002).

1.2.2.3.2 Cytokine and chemokine network mounted by epithelial cells

ECs are able to produce a variety of cytokines and chemokines that allow the communication with immune cells. A type I hypersensitivity reaction is similar to a helminth infection or sterile inflammation. Upon activation, ECs release chemokines like CCL-17/TARC and CCL-22/MDC to recruit CC chemokine receptor (CCR) 4^+ innate lymphoid cells 2 (ILC2s), basophils, Th2, and regulatory T cells (Tregs) (Kataoka, 2014). Additionally, the immune mediator interleukin (IL) -33, IL-25, TSLP and CCL-5/RANTES are secreted to induce chemotactic migration and activate antigen-presenting cells (APCs) (Konig et al, 2015; Ozu et al, 2004; Scadding, 2014; Szegedi et al, 2015). Finally, eotaxins like CCL-11/ Eotaxin-1, -24/ Eotaxin-2, and -26/ Eotaxin-3 acting on $CCR3^+$ eosinophils and Th2 cells are also released. Especially, CCL-17 is overexpressed in many Th2-cell mediated diseases such as AE (Kataoka, 2014). Moreover, TSLP induces hematopoiesis in basophils (Siracusa et al, 2011). In mice, lung epithelial cell or KC over-expression of TSLP is sufficient to drive spontaneous development of an asthma- or AE - like disease (Li et al, 2005; Zhou et al, 2005). Interestingly, TSLP release is especially induced by proteolytic allergens, diesel-exhaust particles, cigarette smoke, and respiratory viruses (Bleck et al, 2010). Additionally, TSLP and IL-33 were shown to aggravate Th2-cell-mediated diseases like allergic airway inflammation or food allergy when over-expressed in the skin (Han & Ziegler, 2017; Noti et al, 2014) IL-25 was also shown to be elevated in patients suffering from asthma and was inversely correlated with airway hyperresponsiveness (Cheng

et al, 2014). Thus, elevated levels of these cytokines which were demonstrated in the skin and serum of AE patients might not only drive Th2- cell mediated diseases but additionally be a risk factor for atopic march.

IL-33, TSLP and IL-25 activate DCs to capture and process entered antigens, subsequently transporting them to draining lymph nodes (Lambrecht & Hammad, 2012). ECs additionally release CCL-20/ MIP-3α, a distinctive chemokine ligand for CCR6, inducing immature DCs to selectively migrate to the epithelium (Reibman et al, 2003; Sierro et al, 2001). It is still poorly understood, how exactly DCs induce Th2 immunity, but several studies could show that the major histocompatibility complex-T-cell receptor (MHC-TCR) interaction, the tumor-necrosis factor (TNF) ligand family member OX40L, the Notch ligand Jagged1, IL-6 as well as the absence of IL-12 production are of importance (Akbari et al, 2014; Amsen et al, 2004; Kleindienst & Brocker, 2005).

Moreover, IL-33 and IL-25 are able to induce different types of ILCs such as ILC2s but also multipotent progenitor type 2 (MPP^{type2}) cells, innate immune cells that promote Th2 type cell immunity (Halim et al, 2014; Saenz et al, 2013; Saenz et al, 2010; Schmitz et al, 2005). In addition, IL-25 and TSLP induce further CCL-17 and CCL-22 chemokine release in ECs, creating a feed forward loop to enforce the Th2 immune response (Saenz et al, 2008; Sebastian et al, 2008) µ. Th2 cells or ILC2s activated by IL-25 and IL-33 then produce IL-4, IL-5 and IL-13, the prototypical cytokines of a Th2 type immune response (Gour & Wills-Karp, 2015). In AE IL-4 and IL-13 were shown to decrease filaggrin, loricrin and involucrin gene expression (Gour & Wills-Karp, 2015; Howell et al, 2007) resulting in barrier disturbance. Additionally, these cytokines are thought to decrease the release of AMPs such as human β-defensin-3 (HBD-3) by KCs (Nomura et al, 2003). Especially, elevated levels of IL-13 were shown to result in an increase in goblet cell numbers and metaplasia leading to mucus hypersecretion and smooth muscle hyperreactivity in allergic asthma patients (Curran & Cohn, 2010; Stone et al, 2010). However, also IL-4 seems to induce bronchoalveolar lavage fluid eosinophilia, airway hyperresponsiveness, and goblet cell hyperplasia in mice (Perkins et al, 2006). This was shown to result in eosinophilic airway inflammation and further recruitment of ILC2s and DCs induced by EC-derived IL-33, TSLP and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Rajavelu et al, 2015) possibly regulated via interference with NF-kB (Janssens et al, 2014). GM-CSF additionally stimulates eosinophil survival and induces proliferation, maturation and function of DCs and macrophages (Shi et al, 2006). IL-5 was shown to induce the generation and activation of eosinophils in the bone marrow and in tissues (Stone et al, 2010). Furthermore, IL-4, IL-5 and IL-13 enforce further release of CCL-11, CCL-17 and CCL-22 by ECs, driving the differentiation into Th2 cells, resulting in activation and isotype class switching of B cells and hence allergen-specific IgE secretion (Lund et al, 2013; Paul & Zhu, 2010; Pulendran et al, 2010).

Activated B cells undergo heavy chain isotype switching and the produced IgE immune complexes bind to high-affinity IgE receptors (FcεRI) which are present on basophils and mast cells. Binding of IgE to the Ig-like domain of the α-chain of the polypeptide chain structure leads to signal transduction mediated via the β- and γ-chain and finally to activation and inflammatory mediator release of basophils and mast cells (Galli & Tsai, 2012). Recurrent activation of mast cells after repeated exposure to allergens leads to immediate hypersensitivity characterized by wheal and flare development. Especially, the inflammatory mediator histamine binds to its receptor on endothelial cells leading to the initiation of the so called late-phase reaction. Lipid mediators like prostaglandin I2 (PGI2), leukotrienes C4 (LTC4), nitric oxide (NO) and platelet-activating factor (PAF) are synthesized and released. Subsequently, vascular leakage, vasodilation and leukocyte extravasation occur. Additionally, further inflammatory cytokines and chemokines such as TNF-α, IL-8, CCL-2 are released by mast cells (Galli et al, 2008).
In addition to the release of cytokines, ECs are also able to release AMPs (Defensins). Defensins are small cationic peptides with their main function being the direct inhibition of pathogens and their products. Moreover, they modulate the innate and adaptive immune response acting as chemotactic agents for monocytes, macrophages, T cells and DCs (Doss et al, 2010). Moreover, ECs might regulate Th2- type immunity via DAMPs or alarmins. The exact cellular source of DAMPs has not been clearly identified but several studies show that ECs directly release ATP and uric acid in response to allergens such as grass pollen which seems to be crucial for the production of IL-25, IL-33, TSLP and GM-CSF (Hara et al, 2014; Kool et al, 2011; O'Grady et al, 2013). The High Mobility Group Box 1 (HMGB1), S100 family proteins, IL-33, IL-1α and IL-1β are alarmins and induced after EC exposure to allergens. HMGB1 and IL-1β are overexpressed in HDM-induced AE mouse models, their neutralization leading to disease improvement (Karuppagounder et al, 2015; Karuppagounder et al, 2014). Additionally, it was shown that the lack of IL-1α and HMGB1 leads to failure of Th2 cell immunity initiation (Ullah et al, 2014; Willart et al, 2012). The S100 protein family was shown to be increased in lesional AE skin. Especially, S100A7 is a KC-derived AMP highly expressed in skin of AE patients (Gittler et al, 2012). These proteins are intracellular differentiation markers amplifying inflammatory responses via promotion of IL-33 release (Jin et al, 2014). IL-1α, IL-1β, IL-33 but also IL-18 are members of the inflammasome-related IL-1 cytokine family which is thought to promote Th2 differentiation in the absence of a Th1 stimulus such as IL-12 (Akira, 2001; Komai-Koma et al, 2016; Santarlasci et al, 2013).

1.2.2.3.3 The inflammasome

TLRs are not the only PRRs ECs express. Besides, they also express nucleotide-binding-and-oligomerization domain (NOD) like receptors (NLRs), Retinoic acid-inducible gene (RIG)-I-like receptors as well as C-type lectin receptors (Takeuchi & Akira, 2010). NOD-like receptor-pyrin-containing (NLRPs) are a subgroup of NLRs and important for the formation of a multiprotein

complex called inflammasome. This intracellular multimolecular complex, also containing active caspase-1 results in the release of the pro-inflammatory cytokines IL-1 β, IL-18 and IL-33 (Eisenbarth & Flavell, 2009). To date three NLR-subset inflammasomes (the NLRP1/NALP1b inflammasome the NLRC4/IPAF inflammasome, the NLRP3/NALP3 inflammasome) and the absent in melanoma (AIM)-2-like receptor (ALR) family have been described. All of these inflammasome types are a multiprotein complex with a particular NLR or ALR protein, an apoptosis-associated speck–caspase recruit domain (ASC) and/or cardinal adaptor proteins, and pro-caspase-1 (Alcocer-Gomez et al, 2017). A comprehensive panel of different inflammasomes at different epithelial surfaces does still not exist but expression of NLRP1, NLRP3, NLRC4, and AIM-2 has been confirmed in normal and cancerous lung epithelial cells (Kong et al, 2015). Similar, the expression of NLRP3, AIM-2 and NLRP1 has been described in KCs (Sand et al, 2018). The different inflammasome types are characterized by their structure and the danger signals they respond to. Upon activation, the relevant NLR or ALR proteins serve as a scaffold to recruit the inactive zymogen pro-caspase-1. Oligomerization of pro-caspase-1 proteins results in its cleavage into active caspase-1. Subsequently, caspase-1, a cysteine-dependent protease, cleaves pro-forms of members of the pro-inflammatory IL-1 cytokine family into their active forms (Guo et al, 2015). To start and finish this cascade two activation signals are necessary one of which is the activation of TLR or TNF receptor triggering the NF-κB pathway and subsequently the expression of IL-1β and IL-18 pro-forms (Latz et al, 2013). For the activation of caspase-1 many different signals exist such as (microbial) toxins, asbestos, nucleic acids, however the exact mechanisms are still poorly understood (Strowig et al, 2012).

1.3 Allergic rhinitis

The most common forms of allergic diseases nowadays are bronchial asthma, AE, AR and food allergy. AR affects approximately 23 % of the European population leading to a reduced quality of life, increasing medical costs but also lower work productivity and school learning performance (Bauchau & Durham, 2004; Bousquet et al, 1994; Malone et al, 1997). AR is caused by hyperresponsiveness to environmental allergens, such as contained in plant pollen. It is characterized by acute, recurrent or chronic Th2- and IgE-mediated inflammation of the nasal mucosa, involving activation of resident mast cells and DCs as well as tissue infiltration with immune cells such as eosinophils, basophils, neutrophils, T- and B-lymphocytes. It was shown that genetic polymorphisms associated with collectins, surfactant proteins, mannose-binding lectin or even TLR-2 result in more severe AR (Kang et al, 2010; Kaur et al, 2006; Madan, 2007). Especially, prevailing tree, grass or weed pollen in the spring and summer months, but also indoor allergens, usually from HDM, animals and fungi are triggering symptoms such as rhinorrhea, sneezing, itching and nasal blockage. AR can be categorized into seasonal and perennial AR based on its timing and duration of allergen exposure. Approximately 20% of AR is strictly seasonal; its symptoms typically appearing during a defined season characterized by a high abundance of aeroallergens. Exposure is dependent on the geographic location and thus the on- and offset of symptoms. Exercise, temperature changes and triggers like tobacco smoke and noxious odors negatively influence the inflammatory reaction in AR and, therefore, hyperresponsiveness may endure after the pollen exposure (D'Amato et al, 2015a). In contrast, perennial AR most often persists for more than 9 months each year and is characterized by hypersecretion and nasal blockage caused by a swollen nasal mucosa. 80% of AR patients suffer from perennial AR with 40% undergoing seasonal exacerbations due to pollen exposure (Rudack, 2004).

1.3.1 Disturbance in the microbiome barrier

Site-specific microbial alterations may play an important role in disease pathophysiology. As already mentioned, Actinobacteria like Propionibacterium acnes and Firmicutes such as *S. epidermidis* and *S. aureus* together with Proteobacteria are the most prevalent and abundant phyla in the nasal microbiome (Ramakrishnan et al, 2013). Comparing the nasal microbiome in populations with different allergy prevalence demonstrated an alteration in diversity and composition. The nasal microbiota in the population showing a higher rate of allergic sensitisation was less diverse and consisted of less Actinobacteria but was enriched in Corynebacteria. Additionally, fungal communities were less diverse in the sensitised population (Ruokolainen et al, 2017). Similarly, it was shown that IgE levels correlate with microbial dysbiosis in the inferior turbinate of AR patients. Individuals with high IgE levels displayed a lower microbial diversity with a low relative abundance of Actinobacteria and a

high relative abundance of Firmicutes linked to an increase in signal transduction-related genes and a decrease in energy metabolism-related genes (Hyun et al, 2018).

1.3.2 Physical and chemical barrier dysfuntion

Barrier function and TJ and/ or adherence junctions expression is reduced in several Th2-cell mediated diseases such as AR, asthma and AE (de Boer et al, 2008; Gruber et al, 2015; Lee et al, 2016; Steelant et al, 2016) which is supposed to be part of the underlying pathology, especially, in AR. Particularly, E-Cadherin, Occludin and ZO-1 were shown to be reduced in the nasal epithelium of AR patients (Lee et al, 2016; Steelant et al, 2016). Additionally, stimulation of human nasal epithelial cells (HNECs) with IL-4 was shown to down-regulate E-Cadherin expression, only in cells of AR patients but not in non-atopic controls. A defective expression of E-Cadherin was hypothesized to activate DCs and ECs to express pro-inflammatory cytokines which in turn alters junction expression (Heijink et al, 2007; Schwarzenberger & Udey, 1996). E-Cadherin seems to suppress IL-5 and IL-13 release by ILC2s upon ligation (Salimi et al, 2013). Therefore, its down-regulation could represent an explanation for the resulting Th2 type cell sensitization. Moreover, it was shown that AR patients display a decreased transepithelial resistance and increased permeability which is related to symptom development. This epithelial barrier effect seems to be related to key inflammatory markers of allergy such as histamine and IL-4 and therefore not only due to proteases contained in allergens (Steelant et al, 2018).

Disturbance of the chemical barrier mounted by ECs such as mucus overproduction is another pathological feature of AR. Mucociliary clearance, a continuous flow of mucus, is impaired in AR patients (Schuhl, 1995) and elevated mucociliary clearance time was shown to be related to later development of sinusitis (Vlastos et al, 2009). Furthermore, activation of IL-13 receptors and epidermal growth factor receptor leads to activation of the γ-aminobutyric acid (GABA) system, possibly resulting in LPS-induced over-expression of MUC5AC (Shen et al, 2016; Xiang et al, 2007; Zhen et al, 2007). Over-expression of MUC5AC correlates closely with goblet cell hyperplasia/metaplasia of surface ECs in inflammatory diseases in the lung and therefore with mucus over-production (Zuhdi Alimam et al, 2000). Additionally, IL-4 results in EC differentiation towards a phenotype which releases more mucins (Dabbagh et al, 1999; Temann et al, 1997). Moreover, IL-8 was shown to be released by goblet cells in response to activation by IL-33 (Tanabe et al, 2014) which in turn increases levels of MUC5AC and MUC5B (Bautista et al, 2009).

1.3.3 Immune barrier dysfunction

1.3.3.1 Innate immune receptor repertoire

Recently, it was shown that AR displays an altered homeostasis of the innate immune system in the sinonasal cavity such as a different expression of PRRs. A decreased expression of TLR-9 in sinonasal epithelial cells from AR patients was observed (Melvin et al, 2011).

Additionally, TLR-9 was also found to be decreased by 50% in patients with chronic rhinosinusitis (Ramanathan et al, 2007). This finding is of particular interest because activation of TLR-9 is known to drive Th1-type differentiation and antagonise Th2 pathways, however, this effect is more pronounced in non-atopic subjects (Broide, 2009; Fonseca & Kline, 2009; Mansson et al, 2009). In contrast, a study investigating the expression of TLR-2 and -4 in 30 patients with chronic rhinosinusitis and 20 non-allergic adult donors observed the expression of both receptors in HNECs obtained by scraping but a higher expression in cells derived from the chronic rhinosinusitis patients (Dong et al, 2005). Interestingly, it has been described that PRR genes such as the gene for TLR-2, 4, 7 and -9 are part of several susceptibility genes involved in allergies and AR (Kang et al, 2010; Senthilselvan et al, 2008). Allergens such as ragweed pollen allergens seem to be able to interact directly with innate immune receptors, which is why a disturbance of their homeostasis appears to be crucial for the development of AR (Li et al, 2011).

Since microbial exposure was shown to affect immunity, especially prenatally and continuously after birth (von Mutius & Radon, 2008), there might be an association between the microbial interactions and alterations in the innate immune receptor repertoire in AR patients. Indeed, it was shown in animal models that maternal exposure to commensals such as *Lactobacillus* (L.) *rhamnosus* during pregnancy can attenuate allergic sensitisation and inflammation in the offspring which was associated with activation of TLR signalling and related epigenetic effects in immune genes (Blumer et al, 2007; Brand et al, 2011). Exposure to this Lactobacillus in late pregnancy of humans was additionally associated with an increase in TLR-4 and -5 in the fetal gut while TLR-2 was decreased and TLR-3 increased in the fetal intestine. Additionally TLR-1, -7, the TLR-4 co-receptor MD-2 and TIRAP were reduced while TLR-3 was induced in the placenta (Rautava et al, 2012). Furthermore, bacterial metabolic products such as SCFA may have systemic effects on the fetus, promoting regulatory immune response rather than Th2 immune responses and thereby protecting against airway diseases (Thorburn et al, 2015). Experimental studies demonstrate that early-life gut microbiota is responsible for select DNA methylation which underlines a potential epigenetic relationship between human-associated microbes and facilitation of postnatal epigenetic processes (Yu et al, 2015). These observations further support the argument that host-microbial interactions are well-established before birth and may have a persistent effect on phenotype and subsequent disease risk over the life course.

The innate immune system directs the adaptive immune system through providing early mediators and cytokines allowing lymphocytes to organise pathogen-specific or inflammatory immune responses (Wang et al, 2008). Defects in the innate immune receptor repertoire might therefore lead to immune disturbance such as seen in AR which is characterized by infiltration

of eosinophils, T cells, mast cells and basophils and their release of inflammatory mediators resulting in local and systemic inflammation (Naclerio, 1991).

1.3.3.2 Disturbance of cytokine and chemokine network

The infiltration of immune cells may be explained by elevated levels of TSLP, IL-25 and IL-33 which are found in AR patient´s epithelium and serum (Kamekura et al, 2012; Kim et al, 2017; Mou et al, 2009). Moreover, allergen contact results in increased CCL-5 levels in these patients (Semik-Orzech et al, 2009). Thus, ECs are activated and recruit immune cells such as eosinophils, basophils and mast cells. Especially, IgE-coated mast cells traverse the epithelium, recognize the mucosally deposited allergen, and release mediators that are hypothesized to act on endothelial cells in order to support the expression of molecules such as vascular cell adhesion molecule 1 (VCAM-1), ICAM-1 and E-Selectin. These molecules then aid the adhesion of circulating leukocytes to the endothelial cells. Moreover, mast cells release IL-4 and IL-13 in order to activate Th2 leukocytes (Toru et al, 1998). Once activated, these immune cells release further chemoattractant cytokines such as IL-5, thereby initiating the process of leukocyte tissue-cell recruitment involving endothelial cell activation as well as the activation, adherence and transendothelial migration of further eosinophils, neutrophils, basophils, T-cells and macrophages (Bascom et al, 1988). ECs release CCL-2/ MCP-1, CCL-3, CCL-4/ MIP-1β, CCL-5, CCL-11, CCL-13/ MCP-4 and CCL-17 as chemoattractants for these immune cells (Konig et al, 2015). Their infiltration and subsequent release of various inflammatory mediators such as major basic protein, eosinophils cationic protein and leukotrienes seems to be the predominant pathophysiologic factor for AR (Wang et al, 1995) since it has been shown that these mediators damage the epithelium, resulting in the clinical and histological pictures of chronic allergic disease (Gleich et al, 1988) which are mucus hypersecretion, edema, goblet cell hyperplasia, tissue damage and remodeling (Borish, 2003). Moreover, mast cells are abundantly found in the epithelial compartment of the nasal mucosa in AR (Bentley et al, 1992). These cells express elevated levels of very-late activation antigen-4 and antigen-5 and mast cell-extracellular matrix interactions result in an increased cytokine release by mast cells (Pawankar & Ra, 1996). Likewise, basophils are also elevated in the nasal mucosa of AR patients and their number correlates with disease severity (Winther et al, 1999). Infiltrating T cells are the principal factor for the adaptive immune response. In AR particularly $CD4^+$ T-cells, and $CD25^+$ activated T-cells migrate to the submucosa and the epithelium (Varney et al, 1992). Th2 cells release IL-3, IL-4, IL-5 and other cytokines aiding IgE production as well as further mast cell and eosinophil attraction and survival (Durham et al, 1992).

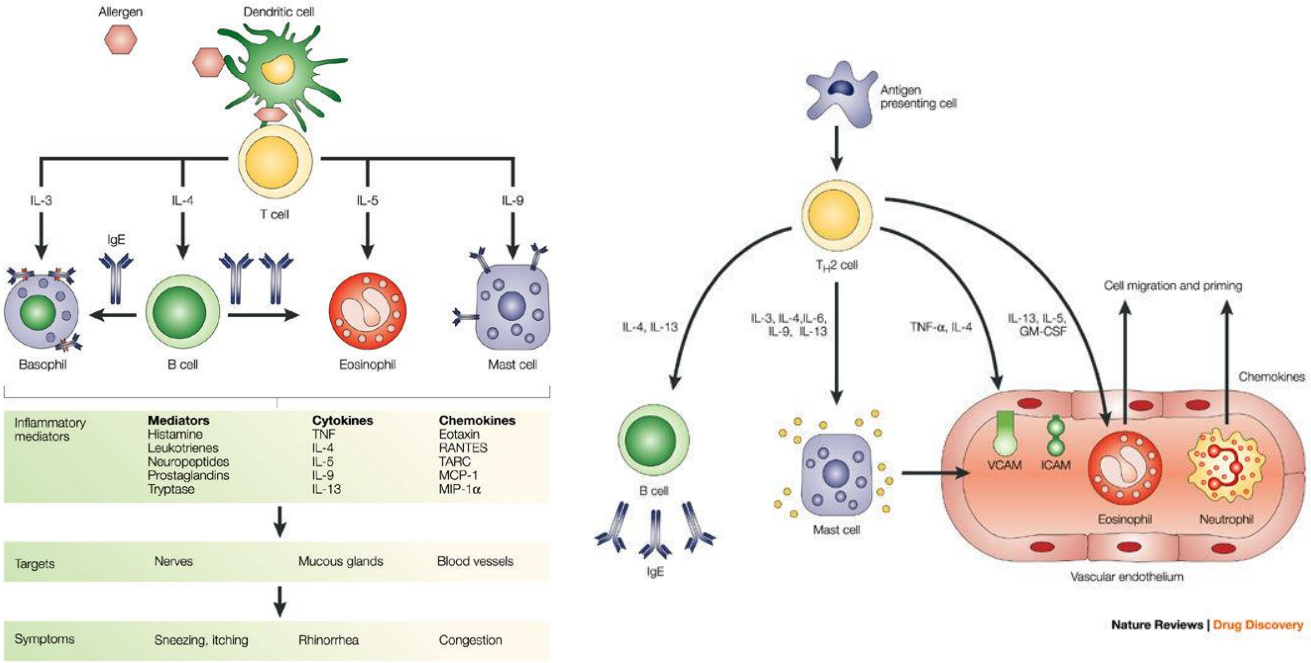

Figure 5: Allergic rhinitis is a disease induced by an interplay of several immune cells and mediators

Allergic rhinitis symptoms are the result of activation of resident and recruited inflammatory cells in the nasal mucosa leading to afferent nerve stimulation, glandular hypersecretion and increased vascular permeability. Chemokines direct te recruitment of circulating leucocytes such as eosinophils and basophils into the nasal mucosa. Release of cytokines like IL-4, IL-1 or TNF-α induce up-regulation of adhesion molecules on vascular endothelium stimulating the adhesion of leucocytes to mucosal blood vessels (modified from Holgate & Broide, 2003a).

1.4 Atopic eczema

AE is a chronically remitting inflammatory skin disease that is mainly presenting symptoms such as pruritus and epidermal barrier dysfunction. The prevalence of AE increased over the last years to 15-25 % in children of which 25 % (most often the severe cases) will persist into adulthood (Werfel et al, 2016). Complex interactions between genetic and environmental factors are responsible for the development of AE. AE is associated with other atopic diseases such as allergic rhinoconjunctivitis, allergic bronchial asthma, and food allergy.

AE is a complex disease with numerous distinct types which can be distinguished based on age of onset, race, chronicity, therapeutic response, triggers and even molecular and cellular characteristics which will have to be stratified for a personalized medicine approach in the future (Werfel et al, 2016).

1.4.1 Disturbance in the microbiome barrier

The skin is colonized with a tremendous number of microorganisms the most of which can be classified into four phyla namely Actinobacteria, Firmicutes, Bacteroides, and Proteobacteria (Grice et al, 2008). Occurrence of the microbiota differs between moist, dry or sebaceous locations on the body (Oh et al, 2014). Especially, at moist body areas Staphylococci and Corynebacteria predominate whereas sebaceous sites rather show propionic bacterial colonisation (Oh et al, 2014). The skin´s microbial composition seems to impact health status by interacting with the local immune system which seems to be particularly obvious in AE since lesional skin is heavily colonized with *S. aureus* and its frequency is significantly lower in non-lesional skin. This results in a loss of diversity, but especially a loss of *S. epidermidis* frequency at these sites, which seems to be restored after successful treatment (Altunbulakli et al, 2018a; Kong et al, 2012). But also occurrence of other Staphylococci such as *S. hominis*, *warneri*, *haemolyticus* or *caprae* are negatively correlated with *S. aureus* frequency on lesional AE skin sites (Altunbulakli et al, 2018a). Additionally, non-atopic individuals have a higher diversity of Gammaproteobacteria on their skin which appears to be related to anti-inflammatory IL-10 expression of peripheral blood cells (Hanski et al, 2012). It was shown, that especially in infants, the intestinal microbiota pattern has an impact on AE development and severity. The microbial diversity and particularly occurrence of butyrate-producing bacteria was inversely correlated with AE disease severity (Nylund et al, 2015). Thus, it seems that the composition of the skin microbiota has a complex role in the control of skin physiology and immunity. Gram-positive bacteria such as Lactococci, Streptococci and Streptomyces species produce bactericidal factors that inhibit the growth of other bacterial strains (Gallo & Hooper, 2012). Similarly, the commensal *S. epidermidis* generates a variety of molecules with antimicrobial activity displaying selective activity against *S. aureus* (Cogen et al, 2010) but also molecules that induce AMP production by KCs in a TLR-2-dependent manner (Lai et al, 2010). In contrast, *S. aureus* itself produces similar molecules but with limited antimicrobial activity which instead

show chemotactic activity for neutrophils (Wang et al, 2007). Commensal microbes seem to educate and prepare the immune system rather than activating it. *S. epidermidis*, for example, is able to control the activation of skin-resident T cells at steady state by production of IL-1α, facilitating IFN-γ and IL-17 release by dermal T cells (Naik et al, 2012). However, it is still unknown how the same innate signaling results in opposed immune responses such as defense or tolerance mechanisms. The combination of several different innate immune stimuli such as from an environment rich in microbes might therefore play an important role.

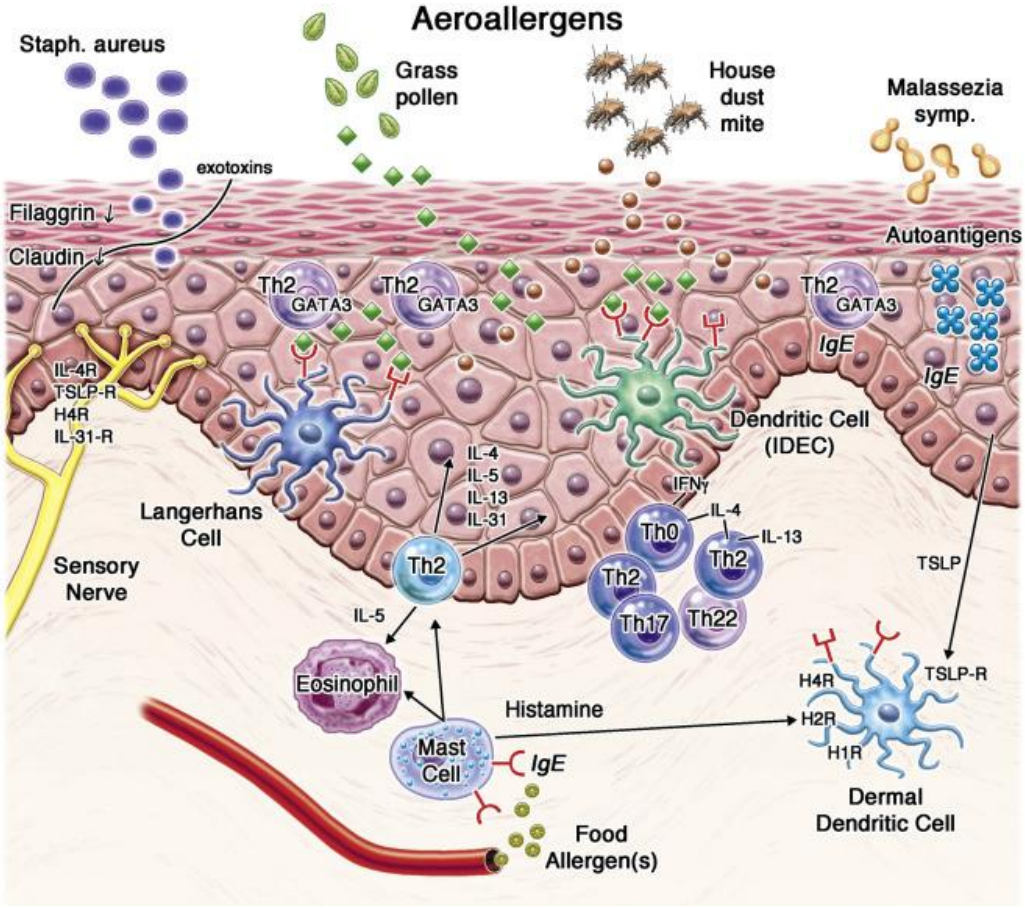

Figure 6 : Selected cellular and molecular pathways in lesional skin of patients with AE. Atopic dermatitis is characterized by a defective skin barrier which enables penetration of irritants, microbial products and allergens. The acute phase of AD is dominated by Th2 leucocytes while the chronic phase is rather governed by Th1 responses. Other immune cells and T cell subpopulations such as DCs, eosinophils are additionally increased in AD skin leading to release of inflammatory mediators and activation of other immune cells by their receptors (Werfel et al, 2016).

1.4.2 Physical and chemical barrier dysfunction in atopic eczema

In AE, the composition of the stratum corneum is altered, leading to xerotic (dry) skin, increased transepidermal water loss and a predisposition to a higher permeability to allergens, irritants and microbes (Di Nardo et al, 1998). Natural moisturizing factor, a breakdown product of Filaggrin (FLG) normally aids retaining moisture and hydrates the skin. FLG, the filament-associated protein, binds to keratin fibers in ECs in the stratum granulosum and is crucial for terminal KC differentiation. Mutations in the gene for *flg* were shown to lead to disruption of the

epidermal barrier due to decreased TJ protein expression (Lee & Lee, 2014; Nakai et al, 2012) and to be associated with AE (Debinska et al, 2017). However, only 15-30 % of the patients carry a *flg* null mutation (Carson et al, 2012; Margolis et al, 2012) leading to the conclusion that other skin barrier genes involved in barrier defects might exist.

Indeed, AE was shown to correlate with reduced expression of the TJ proteins Claudin-1, Claudin-4 and Claudin-23 (De Benedetto et al, 2011; Gruber et al, 2015). Moreover, breaking the tight barrier of ECs might be the cause for increased colonization with *S. aureus* in AE. As already mentioned, it was shown that the expression of the two TJ genes *CLDN4* and *TJP1* were negatively correlated with *S. aureus* frequency while especially in lesional AE skin *S. epidermidis*, *haemolyticus* and *hominis* were positively correlated to their expression (Altunbulakli et al, 2018b). However, the relationship between Staphylococci and barrier function is complex because many bacteria, but especially *S. aureus* produces high amounts of serine proteases which can degrade the skin barrier and thus may also be associated with barrier disruption in the first place (Koziel & Potempa, 2013; Nakatsuji et al, 2016). Especially since AE is additionally associated with reduced expression of HBD-2, an AMP that chemoattracts Th17 cells, which could possibly lead to a *S. aureus* super-infection (Howell et al, 2005).

Some studies suggest that the induction, release or mobilization of AMPs might not be sufficient to control microbial colonization in AE (Kopfnagel et al, 2013). On the other hand, there is evidence that AMPs may be overexpressed in the skin of AE patients in response to skin barrier disruption (Harder et al, 2010). Moreover, abnormalities in skin lipid composition (Yamamoto et al, 1991), claudin protein expression (De Benedetto et al, 2011), or inflammation leading to disturbed skin barrier protein expression may represent additional causes for barrier defects.

1.4.3 Immune barrier dysfunction

1.4.3.1 Innate immune receptor repertoire

Genetic analyses revealed that a TLR-2 polymorphism located within the intracellular part of the receptor correlates to *S. aureus* infections and to a more severe AE phenotype with elevated IgE levels to *S. aureus* superantigen and HDM allergens (Ahmad-Nejad et al, 2004; Lorenz et al, 2000). Additionally, lower TLR-2 expression was observed in AE skin and on macrophages of AE patients (Kuo et al, 2013; Niebuhr et al, 2009). This is of particular interest, since activation of TLR-2 enhances TJ function (Kuo et al, 2013). Thus, AE patients may develop a reduced responsiveness to TLR-2 ligands such as originating from *S. aureus* resulting in reduced TLR-2 functioning (Mrabet-Dahbi et al, 2008; Oh et al, 2009). Similarly, monocytes from patients with AE were observed to have a deficiency in TLR-2-mediated production of pro-inflammatory cytokines (Hasannejad et al, 2007). Furthermore, AE has been associated with a polymorphism resulting in higher promoter activity in the gene encoding for

TLR-9 (Novak et al, 2007). Therefore, it is obvious that innate sensing of microbial substances is crucial in the regulation of cutaneous inflammation.

1.4.3.2 Disturbance of cytokine and chemokine network

As a response to environmental signals sensed by PRRs such as *S. aureus*, KCs release IL-25, IL-33 and TSLP. These cytokines as well as GM-CSF are increased in AE (Hvid et al, 2011; Pastore et al, 1997; Savinko et al, 2012; Soumelis et al, 2002). They activate ILC2s which seem to play an important role in AE since they express skin-homing receptors and infiltrate the skin after contact to environmental factors (Salimi et al, 2013). Subsequently, ILC2s release IL-5 and IL-13 which was shown to further suppress FLG expression by KCs (Howell et al, 2009). E-Cadherin is down-regulated further contributing to ILC2 activity, which in turn is associated with insufficiency of FLG (Nakai et al, 2012; Salimi et al, 2013). The accumulation of ILC2s might act on DCs to promote Th2 phenotypes in T cells which further enhances activation of ILC2s, basophils, and mast cells (Hammad & Lambrecht, 2015). Furthermore, DCs affected by Th2 cytokines such as IL-4, lose the ability to produce anti-inflammatory IL-10 as reaction to bacterial stimulation which seems to further drive inflammation development into chronic cutaneous inflammation (Kaesler et al, 2014). Microbial cutaneous colonization such as in AE can further drive inflammation. Sensing of Gram-positive *S. aureus* by TLR-2-6 was shown to suppress immune responses via the induction of myeloid-derived suppressor cells (MDSCs) triggering the release of IL-6 (Skabytska et al, 2014) which blockage was shown to aid AE patients but increasing their susceptibility to infections (Navarini et al, 2011). DCs in the skin also include Langerhans cells and inflammatory dendritic epidermal cells which dispose of an increased expression of the high-affinity receptor for IgE (FcεRI) capturing allergens for antigen processing and presentation to Th2 cells (Wollenberg et al, 1996). Particularly under the influence of KC released TSLP, these cells activate T cells to produce decreased levels of IL-10 and IFN-γ while increased levels of IL-4, IL-13, TNF-α are released. Furthermore, LCs release more CCL-17 themselves (Ebner et al, 2007). IL-4 and IL-13, together with TNF-α, additionally, lead to an enhanced expression of glucocorticoid-induced TNF receptor–related protein ligand (GITRL) in KCs (Byrne et al, 2012) resulting in further release of CCL-17 attracting additional Th2 cells to AE lesions.

AE is characterized by a noticeable influx of T cells in lesions resulting in KC apoptosis and spongiosis (Verhagen et al, 2006). The expression of IL-4, IL-5 and IL-13 by skin T cells is significantly increased in acute as well as in chronic AE when compared to non-atopic individuals. However, with disease progression to a chronic type of AE IL-4 expression decreases while IL-5 and IFN-γ is further up-regulated (Grewe et al, 1998; Hamid et al, 1994). Recently, it was shown that AE might not be an exclusive Th2 polarized disease since IL-22 was observed to be consistently expressed in AE lesions (Nograles et al, 2009). It is speculated that AE onset is associated with IL-22 and IL-17 up-regulation leading to an increase of S100

proteins in the epidermis (Gittler et al, 2012). IL-22 as well as IL-4, IL-13, and IL-31 are hypothesized to directly affect KC differentiation and leading to epidermal hyperplasia (Cornelissen et al, 2012; Gittler et al, 2012). Epidermal hyperplasia is also associated with tissue eosinophilia which in turn is correlated with blood eosinophil levels and disease severity in AE (Kiehl et al, 2001). However, the exact role of eosinophils in AE remains still unknown. Eosinophils might support host defense, producing extracellular eosinophils traps after activation by TSLP which possibly regulate the immune response (Morshed et al, 2012). Mast cell numbers are increased in skin lesions of AE and it is hypothesized that their cytokine release might contribute to the pathogenesis of chronic inflammatory skin diseases (Mashiko et al, 2015). AE symptoms and inflammation are often worsened after contact to allergens (Werfel et al, 2015) leading to the hypothesis that IgE might play a role in skin inflammation. IgE mediates histamine release by cutaneous mast cells which could exacerbate symptoms through the itch-scratch cycle (Kawakami et al, 2009). However, degranulating mast cells are not always observed in AE lesions (Sugiura et al, 1992). Finally, it was shown that cutaneous inflammation and high IgE levels might be a consequence of diversity loss in the skin´s microbiome in a mouse model (Kobayashi et al, 2015).

1.5 Possibilities for prebiotics and probiotics in restoration of homeostasis

Since microbial exposure was shown to affect immunity, especially prenatally and continuously after birth (von Mutius & Radon, 2008), there are a lot of studies investigating the effect of prebiotics and probiotics on allergy development in infants. Especially, oligosaccharide structures displaying prebiotic effects are used to substitute HMOs in infant formulas. Indeed, infants nourished with these formulas demonstrate a microbiota similar to that of breast-fed infants (Knol et al, 2005) which might have a beneficial effect on AE development and severity (Gruber et al, 2010; Penders et al, 2013). Infants receiving those formulas exhibited increased fecal secretory IgA (Scholtens et al, 2008) while having a reduced total Ig response, modulating the immune response away from symptom-inducing IgE (van Hoffen et al, 2009). A meta-analysis of randomized controlled trials observed that administration of Lactobacilli or Bifidobacteria pre- and postnataly prevented or decreased the risk for AE development in children (Panduru et al, 2015). This effect could persist beyond infancy (Kalliomaki et al, 2003). It was shown that *L. reuteri* and *casei* are able to prime monocyte-derived DCs via DC-specific intercellular adhesion molecule 3-grabbing nonintegrin, driving IL-10 production by Tregs (Smits et al, 2005). Additionally, oral treatment seems to decrease skin inflammation in AE (Iemoli et al, 2012) reducing skin sensitivity while increasing barrier function recovery (Gueniche et al, 2014). However, a meta-analysis of trials studying a therapeutic effect of probiotics failed to show therapeutic efficacy (Lee et al, 2008).

However, there are only few studies investigating the cutaneous administration of non-pathogenic bacteria in order to restore microbial and immune homeostais directly. However, probiotics, when applied to skin directly may have a beneficial effect in AE reducing the SCORAD, *S. aureus* toxicity and skin colonization (Gueniche et al, 2008; Mohammedsaeed et al, 2014). This effect seems to be directly mediated via interaction with TLR-2 resulting in IL-10 production by DCs suppressing Th2 cell proliferation (Volz et al, 2014). Interestingly, IL-10 production by peripheral blood mononuclear cells (PBMCs) was shown to correlate with the abundance of Gram-negative Acinetobacter which is decreased on the skin of atopic individuals (Hanski et al, 2012).

Similarly to the findings for AE, birth cohort studies and findings from mouse models suggest that a lack of microbial exposure of the respiratory or gastrointestinal tract early in life may skew the immune function towards increased susceptibility to allergic sensitization (Lynch & Boushey, 2016). Therefore, it is also attempted to intervene in allergy using prebiotics and probiotics. Indeed, several studies report that the ingestion of probiotic bacteria or fermented milk products affects blood lymphocytes (Wheeler et al, 1997a; Wheeler et al, 1997b) and is able to increase IgA in the respiratory tract (Perdigon et al, 1999). Moreover, supplementation with the *L. casei* strain Shirota or *L. paracasei-33* contained in fermented milk improved the quality of life of AR patients (Wang et al, 2004) and tended to reduce symptom-medication scores (Tamura et al, 2007). A symbiotic, containing *L. acidophilus* NCFM, Bifidobacterium (B.) *lactis*, and fructo-oligosaccharides when used as adjuvant treatment, was able to improve symptoms, endoscopic feature, and cytology in patients with inflammatory non-allergic rhinitis (Gelardi et al, 2017). In a study giving *L. paracasei* orally to AR patients, immune markers such as IL-5 and IL-8 were down-regulated (Wassenberg et al, 2011). Similarly, *B. longum*, *L. rhamnosus* GG and *L. gasseri* were shown to decrease eosinophil numbers and affect Th2 balance in AR (Kandasamy et al, 2011). Furthermore, supplementation of sensitized mice with a prebiotic reduced sneezing, histamine production and inflammation in these mice (Yang et al, 2013). These observations seem promising, however, a far more comprehensive understanding of the multi-factorial environmental influences that shape site-specific microbiota and disease development is required to achieve a targeted, personalized therapy. Moreover, it should be considered that the efficacy of probiotics might be specific since mechanism of action vary between different kinds of probiotic bacteria.

2 Aim of the study

Allergy and atopy appear to have their origin in an insufficiency of exposure to a variety of environmental microbial stimuli early in life resulting in inadequate microbial colonization of different body sites such as the gastrointestinal but also the respiratory tract and the skin. This lack of microbial exposure and colonization seems to skew the development of systemic and/or local immune function away from a balanced tolerogenic function towards a phenotype more susceptible to allergic sensitization and chronic inflammation.

Therefore, the aim of this work is to investigate the differences of immune phenotypes and responses of primary human nasal epithelial cells (HNECs) between non-atopic and atopic donor derived cells. Therefore, a fast and minimal invasive method to isolate, culture and expand primary HENCs from well characterized donors of different atopy status will be established. Primary HNECs will then be compared with respect to their growth, barrier integrity, pattern recognition receptor expression and immune responses to different allergen sources such as house dust mite extracts or pollen, inflammasome stimulants such as nigericin but also an array of pathogen-associated molecular patterns such as TLR ligands.

A further objective of this study will be to explore if lactocepin, a PrtP-encoded protease of *L. paracasei* and *casei*, or non-digestible short-chain galacto- and long-chain fructo-oligosaccharides (scGOS/lcFOS) in presence or absence of two different strains of lactobacilli (*IMS1* and *L. rhamnosus*) harbor direct immune-modulatory potential on primary human nasal epithelial cells or keratinocytes.

Results from this project will provide more knowledge about differences in immune phenotypes and functions between non-atopic and atopic donors and therefore about allergy disease mechanism. This makes possible intervention targets for topic therapy strategies available, possibly enabling beneficial modulation of dysbalanced immunity in response to environmental triggers such as allergens.

Moreover, results from this study show if specific non-digestible galacto- and fructo-oligosaccharides and certain strains of lactic acid bacteria as well as active microbial structures may be useful for the restoration of immune or barrier homeostasis in such a setting.

3 Materials and Methods

3.1 Materials

3.1.1 Reagents

Table 1: Reagents

Reagent	Supplier
2-Mercapto-Ethanol	Sigma-Aldrich, München; Germany
ABTS	Roche Diagnostics, Mannheim; Germany
Adenine	Sigma-Aldrich, München; Germany
Adenosintriphosphat (ATP)	Merck Millipore, Darmstadt, Germany
Albumin from bovine serum (BSA)	Sigma-Aldrich, München; Germany
Airway Epithelial Cell Basal Medium	PromoCell, Heidelberg, Germany
Airway Epithelial Cell Supplement Pack	PromoCell, Heidelberg, Germany
Aqua ad injectabilia	Laboratori Diaco Biomedicali, Trieste; Italy
autoMACS rinsing solution	Miltenyi Biotech, Bergisch Gladbach; Germany
autoMACS running buffer	Miltenyi Biotech, Bergisch Gladbach; Germany
Calciumchloride	Merck, Darmstadt; Germany
Carbonate-Bicarbonate Buffer capsule	Sigma-Aldrich, München; Germany
CD14+ micro-beads (human)	Miltenyi Biotech, Bergisch Gladbach; Germany
Cytotoxicity Detection Kit (LDH)	Roche Diagnostics, Mannheim; Germany
DEPC treated water (pyrogen free)	Life technologies, Carlsbad, CA; U.S.A.
DermaLife Calcium-free basal medium	Lifeline Cell Technology, U.S.A.
DermaLife K Cell Culture Medium	Lifeline Cell Technology, U.S.A.
DermaLife K Cell Culture Medium Components	Lifeline Cell Technology, U.S.A.
DMEM high Glucose	Life technologies, Carlsbad, CA; U.S.A.
DMSO, cell culture grade	Applichem, Darmstadt; Germany
D-PBS w/o Ca/Mg	Life technologies, Carlsbad, CA; U.S.A.
EDTA (0,05 %, pH 8,0)	Life technologies, Carlsbad, CA; U.S.A.
Epidermal growth factor	Sigma-Aldrich, München; Germany
Ethanol absolute	Merck, Darmstadt; Germany
F12 + Glutamax Nutrient Mixture	Life technologies, Carlsbad, CA; U.S.A.

FcR-blocking Reagent, human	Miltenyi Biotech, Bergisch Gladbach; Germany
Fetal calf serum (FCS) Hyclone II	Perbio Science, Bonn; Germany
Flagellin from Salmonella typhimurium	Sigma-Aldrich, München; Germany
Fluoroshield™ with DAPI	Sigma-Aldrich, München; Germany
Formaldehyde (min 37%)	Merck, Darmstadt; Germany
Gentamycine	Life technologies, Carlsbad, CA; U.S.A.
Glutaraldehyde solution	Sigma-Aldrich, München; Germany
Goat serum	Sigma-Aldrich, München; Germany
Heparin-Natrium 250.000 U	Ratiopharm, Ulm; Germany
Hexamethyldisilazane	Polyscience, Niles, IL, U.S.A
Hydrochortisone	Sigma-Aldrich, München; Germany
Insulin	Sigma-Aldrich, München; Germany
iScript™ cDNA Synthesis Kit	Bio-Rad Laboratories, München, Germany
iTaq™ Universal SYBR® Green Supermix	Bio-Rad Laboratories, München, Germany
Keratinocyte-SFM (1X)	Thermo Fisher Scientific, Schwerte, Germany
Laemmli buffer	VWR International, Darmstadt, Germany
L-Glutamine	Life technologies, Carlsbad, CA; U.S.A.
Lipopolysaccharide	Invivogen, San Diego; U.S.A.
Lymphoprep	Axis Shield, Oslo, Norway
Mitomycine C	Sigma-Aldrich, München; Germany
Nigericin	Sigma-Aldrich, München; Germany
ODN 2006 (ODN 7909)	Invivogen, San Diego, CA, U.S.A
Osmium tetroxide	Polyscience, Niles, IL, U.S.A
Paraformaldehyde (PFA)	Sigma-Aldrich, München; Germany
Pam3-Cys-OH	Sigma-Aldrich, München; Germany
Penicillin-Streptomycin	Life technologies, Carlsbad, CA; U.S.A.
PerFix-nc Kit (non centrifuge assay kit)	Beckman Coulter, Brea, CA, U.S.A.
Pierce™ LAL Chromogenic Endotoxin Quantitation Kit	Thermo Fisher Scientific, Schwerte, Germany
Polyinosinic–polycytidylic acid sodium salt (PolyI:C)	Sigma-Aldrich, München; Germany
PromoFluor 840 reactive dye	PromoKine, Heidelberg; Germany
Retinoic acid	Sigma-Aldrich, München; Germany

RPMI 1640 + L-Glutamine	Thermo Fisher Scientific, Schwerte, Germany
rh GM-CSF	PromoKine, Heidelberg; Germany
rh IFN-γ	R&D Systems, Wiesbaden; Germany
rh IL-4	PromoKine, Heidelberg; Germany
rh IL-13	Thermo Fisher Scientific, Schwerte, Germany
rh TNF-α	R&D Systems, Wiesbaden; Germany
Progesterone	Sigma-Aldrich, München; Germany
RNA Isolation Kit	Qiagen, Venlo; the Netherlands
Sodium Cacodylate Buffer	Science Service GmbH, München, Germany
Sodium chloride (NaCl)	Carl Roth, Karlsruhe; Germany
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich (Fluka), München; Germany
Sodium hydrogen carbonate (NaHCO3)	Merck, Darmstadt; Germany
Sodium hydroxide (NaOH)	Merck, Darmstadt; Germany
Sodium-Pyruvate (C3H3NaO3)	Life technologies, Carlsbad, CA; U.S.A.
Streptavidin-horseradish peroxidase	R&D GE Healthcare UK limited, Wiesbaden
Sucrose	Sigma-Aldrich, München; Germany
Sulphuric acid (H2SO4)	Merck, Darmstadt; Germany
Tetramethylbenzidine (TMB)	Sigma-Aldrich (Fluka), München; Germany
Transferrin	Sigma-Aldrich, München; Germany
Triton X	Sigma-Aldrich (Fluka), München; Germany
Triiodothreonin	Sigma-Aldrich, München; Germany
Trypanblue 0.4% solution	Life technologies, Carlsbad, CA; U.S.A.
Trypsin 0.05% and 0.25% EDTA	Sigma-Aldrich, München; Germany
Tween 20 detergent	Merck Millipore

3.1.2 Antibodies and ELISA Kits

All used antibodies and assay kits are directed against human antigens.

Table 2: ELISA Kits

ELISA Kit	Targets	Supplier
OptEIA ELISA Kit	IL-1β; IL-10; GM-CSF; IL-8; IP-10, TNF-α, CCL-2	BD Biosciences, Heidelberg; Germany
Quantikine ELISA Kit	IL-1α; CCL-20, CCL-5, CCL-22; IL-33; CXCL-9;	R&D Systems, Wiesbaden; Germany

	CXCL-11; CCL-1; CCL-8; CCL-11; CCL-13	
TMB ELISA Development Kit	HBD-2	Peprotech, Hamburg, Germany
Paired Antibodies	IL-6; IL-12p70; IL-18	eBioscience, Alasdar Stewart; U.K.
Paired antibodies	Galectin-9	R&D Systems, Wiesbaden; Germany

Table 3: Primary antibodies for immunofluorescence stainings

Target	**Source**	**c(stock)**	**Dilution**	**Supplier**
Claudin-1	mouse	0.5 mg/ml	1:100	Thermo Fisher Scientific, Schwerte, Germany
Claudin-4	Rabbit	0.2 mg/ml	1:100	Abcam, Cambridge, U.K.
Cytokeratin anti-wide spectrum	mouse	not specified	1:100	Abcam, Cambridge, U.K.
Isotype control IgG	mouse	1 mg/ml	1 : 50	eBioscience, Alasdar Stewart; UK
Isotype control IgM	mouse	0.5 mg/ml	1 : 50	eBioscience, Alasdar Stewart; UK
Keratin 14	mouse	n.s.	1 : 50	Sigma-Aldrich, München; Germany
Mucin 5AC	mouse	0.2 mg/ml	1:100	Abcam, Cambridge, U.K.
Occludin	mouse	not specified	1:100	Thermo Fisher Scientific, Schwerte, Germany
Occludin labelled (AF488)	mouse	not specified	1:100	Life technologies, Carlsbad, CA; U.S.A.
Polyglutamylation Modification	mouse	not specified	1:200	Adipogen Life Science, San Diego, CA, U.S.A.
Tubulin anti-acetylated alpha	mouse	1 mg/ml	1 : 200	Abcam, Cambridge; U.K.
ZO-1	mouse	not specified	1:100	Thermo Fisher Scientific, Schwerte, Germany
ZO-1 labelled (FITC)	mouse	not specified	1:100	Life technologies, Carlsbad, CA; U.S.A.

Table 4: Secondary Antibodies for immunofluorescence stainings

Antibody	Source	c(stock)	Dilution	Supplier
α-mouse IgM PE	goat	0.4 mg/ml	1 : 100	Santa Cruz Biotechnology, Heidelberg; Germany
α-mouse IgG PE	donkey	0.4 mg/ml	1 : 100	Santa Cruz Biotechnology, Heidelberg; Germany
α-rabbit IgG FITC	goat	0.4 mg/ml	1 : 100	Santa Cruz Biotechnology, Heidelberg; Germany
α -mouse IgG AF546	goat	not specified	1:2000	Thermo Fisher Scientific, Schwerte, Germany
α -rabbit IgG AF488	goat	not specified	1:2000	Thermo Fisher Scientific, Schwerte, Germany
α -mouse IgG AF633	goat	not specified	1:2000	Thermo Fisher Scientific, Schwerte, Germany

Table 5: Primary antibodies for FACS stainings

Target	Source	Dilution	Supplier
CD45 PerCP	Mouse	1:100	BD Biosciences, Heidelberg; Germany
CD281 PE	mouse	1 : 20	eBioscience, Alasdar Stewart; U.K.
CD282 BV421	mouse	1 : 20	BD Biosciences, Heidelberg; Germany
CD283 PE	mouse	1 : 20	eBioscience, Alasdar Stewart; U.K.
CD284 AF700	mouse	1 : 20	BD Biosciences, Heidelberg; Germany
CD285 AF647	mouse	1 : 20	BD Biosciences, Heidelberg; Germany
CD286 FITC	mouse	1 : 20	Abcam, Cambridge, U.K.
CD289 APC	rat	1 : 20	eBioscience, Alasdar Stewart; U.K.
MyD88 AF405	mouse	1 : 20	R&D Systems, Wiesbaden; Germany
TRIF FITC	mouse	1 : 100	LifeSpan, Bioscience, Inc; Seatlle, WA, U.S.A.

3.1.3 Primer

All DNA Primer were ordered from metabion international AG. After delivery primers were reconstituted in RNase/DNase free DEPC water to a concentration of c = 100 pmol/µl and stored at -20°C. The final concentration per reaction well was 1,6 pmol/µl.

Table 6: DNA Primer for real-time qPCR

Primer		**Sequence from 5`-3`**
18S	forward	GTAACCCGTTGAACCCCA
	reverse	CCATCCAATCGGTAGTAG
AIM-2	forward	AGC AAG ATA TTA TCG GCA CAG
	reverse	GGA CTA CAA ACA AAC CAT TCA C
Caspase-1	forward	GAA TGT CAA GCT TTG CTC CCT AGA
	reverse	AAG ACG TGT GCG GCT TGA TGA CT
Claudin-1	forward	CAG TCA ATG CCA GGT ACG AAT TT
	reverse	AAG TAG GGC ACC TCC CAG AAG
Claudin-4	forward	TGT ACC AAC TGC CTG GAG GAT
	reverse	GAC ACC GGC ACT ATC ACC ATA
Elongationfactor 1α	forward	CTG AAC CAT CCA GGC CAA AT
	reverse	GCC CTG TGG CAA TCC AAT
GAPDH	forward	GAA GGT GAA GGT CGG AGT
	reverse	GAA GAT GGT GAT GGG ATT
Keratin 18	forward	TGAGACGTACAGTCCAGTCCTT
	reverse	GCTCCATCTGTAGGGCGTAG
MyD88	forward	CATATGCCTGAGCGTTTCGATG
	reverse	GGCAAGGCGAGTCCAGAACCA
NLRP1	forward	CC TGA TCC CAA GTG ACT GC
	reverse	TCT TCT CCA GGG CTT CGA TA
NLRP3	forward	CTT CTC TGA TGS GGC CCA AG
	reverse	GCA GCA AAC TGG AAA GGA AG
Occludin	forward	GAT GAG CAG CCC CCC AAT
	reverse	GGT GAA GGC ACG TCC TGT GT
S100A9	forward	TCA GCA TGA TGA ACT CCT CG
	reverse	GGA ATT CAA AGA GCT GGT GC
S100A8	forward	ACT TGT GGT AGA CGT CGA TGA T
	reverse	CAG CTG TCT TTC AGA AGA CCT G
SDHA	forward	AAA CCA AAC GCT GGG GAA GA
	reverse	CTG AGT CGC AGT TCC GAT GT
TLR-1	forward	GCCTTGTCTATACACCAAGT
	reverse	CCAATTGTTGCAGAGACTTC
TLR-2	forward	TCTCCCATTTCCGTCTTTTT

	reverse	GGTCTTGGTGTTCATTATCTTC
TLR-3	forward	TAAACTGAACCATGCACTCT
	reverse	TATGACGAAAGGCACCTATC
TLR-4	forward	CCGCTTCCTGGTCTTATCAT
	reverse	TCTGCTGCAACTCATTTCAT
TLR-5	forward	ACGGACTTGACAACCTCCAA
	reverse	AGTGGATGAGGTTCGCTGTA
TLR-6	forward	CCCAAGGAGAAAAGCAAAC
	reverse	TTCACCATCATCCAAGTAAAT
TLR-7	forward	CAGAGCTGAGATATTTGGACT
	reverse	TTGGTAAGTATCTGTTATCACCT
TLR-8	forward	CGGCAGAGTTATGCAAATAGT
	reverse	GTAAGAGCACTAGCATTATCA
TLR-9	forward	GGCCCTCCACGCATGAG
	reverse	CTTGTCCTTTTCTGCCCTTGTAG
TRIF	forward	AGCGCCTTCGACATTCTAGGT
	reverse	AGAACCATGGCATGCAGGA
Tubulin	forward	TTGGCCAGATCTTTAGACCAGACAAC
	reverse	CCGTACCACATCCAGGACAGAATC
ZO-1	forward	ACA GTG CCT AAA GCT ATT CCT GTG A
	reverse	TCG GGA ATG GCT CCT TGA G

3.1.4 Media and buffer

All media were sterile filtered and stored at 4°C.

Table 7: 3T3 medium

Ingredient	**Stock**	**ml of stock**	**Final**
DMEM high Glucose		395 ml	
FCS		100ml	20 %
Penicillin-Streptomycin	10 000 U/ml	5 ml	100 U/ml

Table 8: DermaLife K medium

Ingredient	**Stock**	**ml of stock**	**Final**
DermaLife basal		475.5 ml	
Penicillin-Streptomycin	10 000 U/ml	5 ml	100 U/ml

L-Glutamine LifeFactor	200 mM	15 ml	6 mM
Hydrocortisone	0.1 mg/ml	0.5 ml	100 ng/ml
rh Insulin LifeFactor	0.5 mg/ml	0.5 ml	0.5 µg/ml
Epinephrine LifeFactor	100 µM	0.5 ml	1 µM
Extract PTM LifeFactor		2 ml	4 %
Apo-Transferrin	0.5 mg/ml	0.5 ml	5 µg/ml
rh TGF-α LifeFactor	100 ng/ml	0.5 ml	100 ng/ml

Table 9: KSFM

Ingredient	**Stock**	**ml of stock**	**Final**
Keratinocyte SFM		497.9ml	
Penicillin-Streptomycin	10 000 U/ml	5 ml	100 U/ml
Bovine pituitary gland extract	25 mg	2 ml	20-30 µg/ml
Epidermal growth factor	2.5 µg	100 µl	0.1-0.2 ng/ml

Table 10: Keratinocyte/Feeder Medium

Ingredient	**Stock**	**ml of stock**	**Final**
DMEM high Glucose		300 ml	
Ham's F-12 GlutaMax Nutrient Mix		150 ml	100 U/ml
FCS		50 ml	10 %
Penicillin-Streptomycin	10 000 U/ml	5 ml	100 U/ml
Adenine	87.4 mM	1 ml	174.8 µM
L-Glutamine	200 mM	10 ml	4 mM
Hydrocortisone	7,7 mg/ml	1 ml	42 µM
Triiodothyronin (T3)	2 µM	0.5 ml	20 nM
Transferrin	5 mg/ml	0.5 ml	5 µg/ml
Choleratoxin	0.84 µg/ml	0.5 ml	0.84 ng/ml
Insulin (recombinant)	4 mg/ml	0.5 ml	4 µg/ml
Epidermal growth factor	1 mg/ml	0.5 ml	1 µg/ml

Table 11: HNEC/Feeder

Ingredient	**Stock**	**ml of stock**	**Final**
Airway epithelial cell medium		225 ml	

Ham's F-12 GlutaMax Nutrient Mix		225 ml	
FCS		50 ml	10 %
Penicillin-Streptomycin	10 000 U/ml	5 ml	100 U/ml
Epidermal Growth Factor	2.5 µg/ml	2 ml	10 ng / ml
Insulin	5 mg/ml	0.5 ml	5 µg / ml
Hydrocortisone	0.5 mg/ml	0.5 ml	0.5 µg / ml
Epinephrine	0.5 mg/ml	0.5 ml	0.5 µg / ml
Triiodo-L-thyronine	6.7 µg/ml	0.5 ml	6.7 ng / ml
Transferrin	10 mg/ml	0.5 ml	10 µg / ml
Retinoic Acid	0.1 µg/ml	0.5 ml	0.1 ng / m

Table 12: Medium Airway epithelial cell medium

Ingredient	**Stock**	**ml of stock**	**Final**
Airway epithelial cell medium		475.5 ml	
Penicillin-Streptomycin	10 000 U/ml	5 ml	100 U/ml
Epidermal Growth Factor	2.5 µg/ml	2 ml	10 ng / ml
Insulin	5 mg/ml	0.5 ml	5 µg / ml
Hydrocortisone	0.5 mg/ml	0.5 ml	0.5 µg / ml
Epinephrine	0.5 mg/ml	0.5 ml	0.5 µg / ml
Triiodo-L-thyronine	6.7 µg/ml	0.5 ml	6.7 ng / ml
Transferrin	10 mg/ml	0.5 ml	10 µg / ml
Retinoic Acid	0.1 µg/ml	0.5 ml	0.1 ng / m

Table 13: KC ALI medium

Ingredient	**Stock**	**ml of stock**	**Final**
Keratinocyte SFM		497.9ml	
DMEM high glucose		500 ml	
Penicillin-Streptomycin	10 000 U/ml	5 ml	100 U/ml
Bovine pituitary gland extract	25 mg	2 ml	20-30 µg/ml
Epidermal growth factor	2.5 µg	100 µl	0.1-0.2 ng/ml
Calcium chloride	18 M	0.05 ml	0.9 mM
Retinoic Acid	0.3 µg/ml	1 ml	0.3 ng / ml

Table 14: HNEC ALI medium

Ingredient	**Stock**	**ml of stock**	**Final**

Airway epithelial cell medium		475.5 ml	
DMEM high Glucose		500 ml	
Penicillin-Streptomycin	10 000 U/ml	5 ml	100 U/ml
Epidermal Growth Factor	2.5 µg/ml	2 ml	10 ng / ml
Insulin	5 mg/ml	0.5 ml	5 µg / ml
Hydrocortisone	0.5 mg/ml	0.5 ml	0.5 µg / ml
Epinephrine	0.5 mg/ml	0.5 ml	0.5 µg / ml
Triiodo-L-thyronine	6.7 µg/ml	0.5 ml	6.7 ng / ml
Transferrin	10 mg/ml	0.5 ml	10 µg / ml
Retinoic Acid	0.3 µg/ml	1 ml	0.3 ng / ml
Calcium chloride	18 M	0.05 ml	0.9 mM

Table 15: HU-DC medium

Ingredient	**Stock**	**ml of stock**	**Final**
RPMI 1640		450 ml	
FCS		50 ml	10 %
Gentamycine	10 mg/ml	1 ml	20 µg/ml
L-Glutamine	200 mM	5 ml	2 mM

Table 16: Freezing medium

Ingredient	**ml of stock**	**Final**
DMEM/F12	250 ml	50 %
FCS	200 ml	40 %
DMSO	50 ml	10 %

Table 17: FACS buffer

Ingredient	**Volume**
PBS w/o Ca/Mg	475 ml
FCS	25 ml
EDTA (0.5 M)	2 ml

3.1.5 Consumable material

Table 18: Consumable material

Material	**Supplier**
Adhesion slides SuperFrost® Plus	Thermo Fisher Scientific, Schwerte; Germany

Butterfly needles	Dahlhausen, Köln; Germany
Cover slips (24 x 60 mm)	Hirschmann Laborgeräte, Eberstadt; Germany
Cryotubes 1.8 ml	Nunc, Roskilde; Denmark
EDTA-Monovettes	Sarstedt, Nümbrecht; Germany
FACS tubes	Thermo Fisher Scientific, Schwerte; Germany
Maxisorp plates (96 well)	Nunc, Roskilde; Denmark
Microtubes 2 ml PP, sterile	Sarstedt, Nürnbrecht; Germany
Optifit Tips	Sartorius, Göttingen, Germany
PCR foil MicroAmp	Applied Biosystems (Life technologies) Carlsbad, CA; U.S.A.
PCR tubes	Eppendorf, Hamburg; Germany
Perfusor syringes	Braun, Melsungen; Germany
Pipettes (1, 5, 10, and 25 ml)	Greiner Bio-One, Frickenhausen; Germany
qPCR plates 384 well I	Bio-Rad, München; Germany
qPCR plates 384 well II	Thermo Fisher Scientific, Schwerte; Germany
Reaction tubes (0.5; 1.5; 2 ml)	Eppendorf, Hamburg; Germany
Reaction tubes (15 ml; 50 ml)	Sarstedt, Nürnbrecht; Germany
rhino-proR curette	Arlington Scientific, Springville, U.S.A.
RNeasy Mini Kit for RNA Isolation	Qiagen, Hilden; Germany
Safety Space™ Filter Tips	Sartorius, Göttingen, Germany
Sterile filter device (250 ml; 500 ml)	Sarstedt, Nürnbrecht; Germany
Syringe filter units (0.22; 0.45 µm)	Merck Millipore, Darmstadt; Germany
Tissue culture flask (25; 75 and 175 cm2)	Greiner bio-one, Frickenhausen; Germany
Tissue culture plates (96; 48; 24; 12 and 6 well)	Corning Incorporated (Falcon); Tewksbury, MA; U.S.A.
Transwell®plates with 0.4 µm Pore Polyester Membrane Insert	Corning Incorporated (Falcon); Tewksbury, MA; U.S.A.

3.1.6 Instruments

Table 19: Instruments

Device	**Supplier**
Absorbance Microplate reader Sunrise™	Tecan, Männedorf, Switzerland
autoMACS Pro Separator	Miltenyi Biotech, Bergisch Gladbach; Germany

BioDrop	BioDrop UK Ltd, Cambridge, U.K.
Centrifuge 5810	Eppendorf, Hamburg; Germany
Centrifuge 5418	Eppendorf, Hamburg; Germany
CFX384 Touch™ Real-time PCR Detection System	Bio-Rad, München; Germany
Cytoflex Flow Cytometer Platform	Beckman Coulter, Brea, CA, U.S.A.
Incubator	Binder, Tuttlingen, Germany
Magnetic stirrer RCT basic	IKA Werke, Staufen; Germany
Micro scale Quintix	Sartorius, Göttingen; Germany
Micro scale Quintix 2102S	Sartorius, Göttingen; Germany
Microplate washer 405 wash	Winoosky, VER, U.S.A
Microscope DM750	Leica, Wetzlar, Germany
Microscope DMi1	Leica, Wetzlar, Germany
Microscope DMi8	Leica, Wetzlar, Germany
Millicell-ERS-2-Voltohmmeter	Merck Millipore, Darmstadt; Germany
Multichannel Pipettes	Sartorius, Göttingen, Germany
pH meter S210	Mettler-Toledo, Columbus, OH, U.S.A
Pipettes with diposable tips	Sartorius, Göttingen, Germany
Thermo Mixer C	Eppendorf, Hamburg; Germany
Waterbath SW23	Julabo, Seelbach; Germany

3.2 Methods

3.2.1 Donors

Blood cells, KCs and HNECs were obtained from non-atopic individuals as well as from patients allergic to HDM, grass and birch pollen or AD patients. Non-atopic subjects were defined by a normal total IgE titer below 50 kU/l in the serum, a negative radio-allergen-sorbent-test (RAST) against common environmental allergens (D. *pteronyssinus*, cat danders, dog danders, wheat flour, celeriac, timothy grass, secale cereale, birch, hazel, ash, mugwort, buckhorn and fungal spores) and the absence of atopic diseases in history.

3.2.2 Isolation and cultivation of human primary keratinocytes

Primary human KCs were isolated from the epidermis using the suction blister method which was described earlier (Traidl et al, 2000). Briefly, single-cell suspensions of epidermal cells from suction blisters' roofs were prepared by trypsinization of the blister roof. Single cells were seeded on a feeder layer of murine 3T3 fibroblasts treated with 10 µg/ml mitomycine C for 2 h beforehand. First-passage KCs were cryopreserved in liquid nitrogen. Experiments were

performed in sterile 24 well tissue culture plates with second-passage KCs grown in DermaLife keratinocyte growth medium to 80-90 % confluence.

3.2.3 Isolation of human primary nasal epithelial cells from curettages or surgery specimen

HNECs were obtained by scraping the surface of the middle meatus bilaterally using a rhino-proR curette. The scraped cells were sterilized using antimycotic/antibiotic solutions of different concentrations consisting of gentamycin and 100x Antimycotics-Antibiotics solved in DMEM. Cells were washed with D-PBS once and then detached from one another by resuspending in 0.25% Trypsin-EDTA. Cells were added to mitomycine C arrested murine 3T3 fibroblasts as feeder cells in HNEC/Feeder medium. HNEC-feeder cell co-cultures were incubated at 37°C, 6.5% CO_2 for at least 4 – 5 days before the first medium change or passage.

Tissue specimens were obtained from conchotomic surgeries on otherwise non-atopic adults or AR patients of the ENT department of the hospital of Augsburg, Germany. Prior to surgery, patients gave their written informed consent. Conchotomic surgery specimen were sterilized using antimycotic/antibiotic solutions of different concentrations. Specimen were cut in pieces, washed with D-PBS once and HNECs detached from the tissue by resuspending in 0.25% Trypsin-EDTA. Finally, the resulting cell suspension and the residual tissue was squashed through a 30µm filter. The filtrate was spun and added to mitomycine C arrested murine 3T3 fibroblasts and cultured as mentioned above.

First-passage HNECs were crypopreserved in liquid nitrogen. For stimulation experiments, second-passage HNECs were grown in Airway epithelial cell medium (AECM) to 80-90% confluence and stimulated in sterile 24- or 12-well tissue culture plates.

3.2.4 Air liquid interface cultures of keratinocytes and nasal epithelial cells

KCs and HNECs were thawed from liquid nitrogen and seeded in T75 cell culture flasks in KSFM or AECM at 37°C, 5.0 % CO_2 for 7 days. Subsequently, cells were detached and seeded in sterile 24-transwell tissue culture plates at $1.5x10^5$ cells per well in basal KC or HNEC ALI medium (50 % KSFM + 50 % DMEM/ 50 % AECM + 50 % DMEM) at 37°C, 5.0 % CO_2 for 5 days. Medium was changed every other day. After 5 days, the cells were air lifted and medium was changed to KC or HNEC ALI medium. Cells were incubated at 37°C, 5.0 % CO_2 for further 21 days while medium was changed every other day.

3.2.5 Scanning electron microscopy

To characterize HNECs obtained from curettages and surgery specimen, scanning electron microscopy (SEM) was used. Therefore ALI cultures of HNECs were prepared and after 21 days of culture fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer. Subsequently, cells were dehydrated in 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.2) for 1h at 4°C, and finally washed twice with cacodylate buffer. Samples were then sputtered with 3 nm Au-coating in

order to avoid charging effects. Visualization of the samples' surface was realized by SEM using a Zeiss Merlin SEM with field emission gun. The SEM was operated with an acceleration voltage of 3 kV and a current of 100 pA. Imaging of the topography was done using an in-lens secondary electron detector with the help of Dr. Alexander Hartwig, Institute of Physics, University of Augsburg.

3.2.6 Immunofluorescence staining

For immunofluorescence staining sections, $1x10^4$ cells HNECs were seeded into ibidi 8-well slides in complete AECM and grown to 90-100 % confluence. Additionally, completed ALI cultures of HNECs were used. On the day of the staining, cells were washed twice with D-PBS and fixed with 4% paraformaldehyde (RT; 10 min). Subsequently, HNECs were treated with D-PBS + 0.1% Triton X + 0.02% SDS for 5 min. Afterwards, cells were blocked with 10% goat serum in D-PBS containing 1% BSA. Next, staining specimen were incubated with primary antibodies diluted in D-PBS + 1% BSA for 45 min, at RT and in a humid environment (see table 3). Secondary antibodies were applied diluted in D-PBS + 1% BSA and incubated for 45 min in the dark (see table 4). Finally, the cells were put on glass slides and mounting medium containing DAPI and cover slips were added. Between all steps washing steps with D-PBS ± Tween20 were conducted. Stainings were stored in the dark at -20 °C until picture taking using the Leica DMi8 and LAS X Life Science software.

3.2.7 Bacteria preparation

The *Lactobacillus rhamnosus* strain NutRes1 was kindly provided by Prof. Johan Garssen, Department of Pharmacology and Pathophysiology, Utrecht University. The Lactobacillus strain *IMS1* was obtained as glycerol cultures from Prof. Dr. Ing. Werner Back, Department of Brewery and Beverage Technology, Technical University München. *L. rhamnosus* and *IMS1* were grown at 37°C and 48°C, respectively, in deMan, Rogosa Sharpe (MRS) broth (Oxoid) under anaerobic conditions. Bacteria were harvested in the early stationary phase, washed and aliquoted for storage. Cell counts were determined by plating serial dilutions. *L. rhamnosus* was stored as 0.5 ml cultures of $5.9x10^9$ colony forming units (cfu), *IMS1* as 0.5 ml cultures of $2.8x10^9$ cfu at -80°C. To prepare bacterial strains for stimulation experiments, stocks were thawed, diluted appropriately in D-PBS (Gibco/Invitrogen) and concentrations of $1x10^4$ were generated by serial dilution in cell culture medium. Lactocepin was kindly provided by Prof. Dirk Haller and Dr. Gabriele Hörmannperger, Department of Nutrition and Immunology, Technical University München and stored at -20°C.To use Lactocepin for stimulation experiments, the bacterial supernatant was diluted 1:250 in cell culture medium.

3.2.8 Preparation of GOS/FOS

Short-chain galactooligosaccharide liquid (scGOS; Friesland Campina, 45% scGOS), containing 25% moisture, 15% lactose and 15% other carbohydrates (mainly glucose), long-

chain fructooligosaccharide powder (lcFOS, inulin, high performance, 96.5%, lcFOS, Orafti) containing 3.5% moisture were kindly provided by Prof. Johan Garssen, Department of Pharmacology and Pathophysiology, Utrecht University, in separate 50 ml tubes and stored at 4°C. To prepare a scGOS/lcFOS (9:1) mixture (final experimental concentration 5 mg/ml) 10 mg lcFOS powder and 388 mg scGOS liquid were dissolved in 9.7 ml warm cell culture medium. After 0.22 µm filter sterilization the scGOS/lcFOS mixture was ready to be used in the stimulation experiments. Oligosaccharide mixtures were freshly prepared in the morning before every experiment. Potential endotoxin contamination of the scGOS/lcFOS mixture was analyzed using a limulous amoebocyte lysate (LAL) assay (Thermo Fisher Scientific).

3.2.9 Preparation of aqueous pollen extracts (APE)

Pollen of *Betula pendula* and *Phleum pratense* were collected in the spring of 2015 and 2016 during flowering season in southern Bavaria. Catkins were sieved in order to extract the pollen with a 100 µm sieve followed by a 70 µm sieve. The pollen were then stored at −80 °C until further processing. Pollen from *Ambrosia artemisiifolia* was provided by Helmholtz Center München BIOP where ragweed plants were grown in fully air-conditioned greenhouse cabins under controlled conditions.

Aqueous pollen extracts (APE) were generated by incubation of pollen grains in D -PBS for 30 min at 37 °C. Subsequently, the suspension was centrifuged for 10 min at 4000 rpm, 4 °C. The supernatants were filtered using 0.22 µm syringe driven filter units. Endotoxin contamination was ruled out using a LAL assay.

3.2.10 Stimulation of keratinocytes and HNECs with scGOS/lcFOS and bacteria

Second passage KCs or HNECs were seeded into sterile 24-well flat bottom plates at a density of 4-4.5x10^4 cells/ml in complete DermaLife keratinocyte growth medium or complete Airway epithelial cell medium, respectively, and incubated for 4-5 days at 37°C and 5% CO_2. Upon reaching of 80-90 % confluence, cells were washed twice with D-PBS and changed to DermaLife keratinocyte growth medium or AECM without hydrochortisone. Subsequently, either, 10 ng/ml IFN-γ and 10 ng/ml TNF-α, 50 ng/ml IL-4 and 50 ng/ml IL-13, 10 µg/ml PolyI:C, 10 mg/ml pollen in common grass or birch pollen extracts or 2.5 mg/ml pollen in common ragweed pollen extracts were added to the cells. Simultaneously, KCs or HNECs were treated with scGOS/lcFOS mixture (9:1) with or without *IMS1* or *L. rhamnosus* at concentrations of 1x10^4cfu/ml or Lactocepin diluted 1:250 in cell culture medium. Untreated KCs or HNECs were used as negative control. After 24 hours of incubation at 37°C and 5% CO_2 supernatants were collected and further analyzed by ELISA or Pierce lactate-dehydrogenase (LDH) assay (Thermo Fisher Scientific).

Additionally, ALI cultures of KCs and HNECs were prepared as mentioned beforehand. After 21 days at the air-liquid interface, cells were treated with scGOS/lcFOS mixture (9:1) with or without *IMS1 or L. rhamnosus* at concentrations of $1x10^4$cfu/ml in KC/ HNEC ALI medium from the apical side. Untreated cells served as a control. Cells were incubated at 37 °C, 5 % CO_2 and transepithelial electrical resistance (TER) was measured at 0, 3, 6, 8, 24, 48 hours using the Millicell-ERS-2-Voltohmmeter.

3.2.11 Stimulation of HNECs with TLR ligands, APEs and inflammasome stimuli

HNECs passaged twice were prepared for stimulation experiments in sterile 12- or 24-well flat bottom plates seeding $1x10^5$ or $4.5x10^4$ cells, respectively, as mentioned beforehand. For stimulation with TLR ligands, HNECs were treated with 5 µg/ml flagellin from pseudomonas aeruginosa, 100 ng/ml LPS, 10 µg/ml PolyI:C, 200 ng/ml synthetic triacylated lipoprotein Pam3-Cys-OH or 2 µmol/l CpG-ODN2006. After identifying the most potent stimuli, HNECs were prepared for stimulation experiments and stimulated with 100 ng/ml LPS, 10 µg/ml PolyI:C for 24 h. Subsequently, 10 µg/ml nigericin were added to the cell culture for 30 min. Furthermore, HNECs were stimulated with 10 mg/ml pollen in common birch or grass APEs (Bet-APE, Phl-APE) or 2.5 mg/ml pollen in common ragweed APEs (Amb-APE). Untreated HNECs served as a negative control. For all stimulation experiments, HNECs were incubated for 24 h at 37°C and 5% CO_2 and supernatants were collected afterwards for being further analyzed by ELISA or LDH assay. Stimulated cells were trypsinized with 0.05 % EDTA trypsin and stored in RNAlater (Qiagen) until further analysis.

Additionally, ALI cultures of HNECs were prepared as mentioned beforehand. After 21 days at the air-liquid interface, cells were stimulated with 10 mg/ml pollen in common birch or grass APEs, 2.5 mg/ml pollen in common ragweed APEs or 100 µg/ml whole HDM extract in HNEC ALI or synthetic nasal medium (SNM) from the apical side. SNM was prepared as described previously (10.1371/journal.ppat.1003862). Untreated cells and cells treated with 100 mM EDTA served as controls. Cells were incubated at 37 °C, 5 % CO_2 and TER was measured at 0, 1, 2, 3, 4, 6, 24 hours using the Millicell-ERS-2-Voltohmmeter. After 24 hours, supernatants were taken and stored at -80 °C until further analysis.

3.2.12 RNA isolation and quantitative real-time PCR

Total RNA extraction from KCs and HNECs was performed using the RNeasy Mini kit with on-column DNase digestion according to manufacturer's instructions. RNA concentration was measured using the BioDrop spectrophotometer. Subsequently, cDNA was prepared with the iScript™ cDNA Synthesis Kit. Finally, mRNA expression was quantified with the CFX384 TouchTM Real-time PCR Detection System using the iTaq™ Universal SYBR® Green Supermix. Primers used for this experimental part are listed in table 6. Expression levels were normalized to the house-keeping gene 18S which was observed to be the most suitable one

among 18S, EF1a, GAPDH, K18, SDHA and Tubulin in establishment experiments beforehand. Relative changes in gene expression were analyzed using the comparative C(T) method (Schmittgen & Livak, 2008).

3.2.13 ELISA

For cytokine quantification ELISA kits were used according to manufacturer´s instructions (see table 2).

3.2.14 LAL Assay

Endotoxin contamination of pollen extracts and the scGOS/lcFOS mixture was quantified using the Pierce™ LAL Chromogenic Endotoxin Quantitation Kit from Thermo Fisher Scientific, Schwerte, Germany, according to manufacturer's instructions.

3.2.15 LDH Assay

Cell death was quantified using the cytotoxicity detection Kit from Roche, Basel, Switzerland, according to manufacturer's instructions.

3.2.16 FACS analysis

Freshly isolated, grown or TLR ligand stimulated HNECs were analyzed for their expression of extracellular TLR-1, 2, 4, 5, 6 and intracellular TLR-3, 9, MyD88 and TRIF using the flow cytometer CytoFlex. Therefore, HNECs were isolated or harvested, resuspended in D-PBS and distributed as approximately $1x10^5$ cells per well in a 96-well round bottom plate.

Freshly isolated and grown HNECs were divided in four wells in order to stain for unstained controls and extracellular expressed TLRs as well as for intracellular expressed TLRs and adaptor proteins.

For TLR ligand stimulated HNECs (in 12-well plates), HNECs of every stimulation condition were harvested and divided into two wells of a 96-well round bottom plate. Additionally seeded wells were used for unstained controls and fluorescence minus one (FMO) controls.

Cells were then stained with PromoFluor 840 (PF840) reactive dye for live/dead analysis for 15 min at 4 °C in a dilution of 1:1000 for extracellular TLR stainings while it was diluted 1:10000 for intracellular TLR and adaptor protein stainings. Wells with HNECs for extracellular TLR stainings were then washed with D-PBS and 5 µl FcR-blocking Reagent was applied for 5 min. Subsequently, cells were resuspended in 50 µl FACS buffer containing primary antibodies in appropriate dilution (see table 5). After further 60 min of incubation in the dark at 4 °C, cells were washed with FACS buffer and then analyzed. Wells with HNECs for intracellular TLR and adaptor protein stainings were washed with D-PBS after staining for PF840 and resuspended in 5 µl FcR-blocking Reagent for 5 min. Subsequently, the PerFix-nc kit was used and 12.5 µl of its fixation reagent were applied for 15 min at RT. Next, cells were resuspended in 50 µl of its permeabilization reagent containing primary antibodies in appropriate dilution (see table 5).

After 60 min of incubation at RT in the dark, cells were washed with FACS buffer and analyzed. Expression levels were calculated by difference in mean fluorescence intensity (MFI) to unstained or FMO controls.

3.2.17 Statistical analysis

Data was analyzed using full-factorial ANCOVA for dependent variables and covariates. Bonferroni was used to post-hoc group significant differences among factors. One-way ANOVA was also performed to investigate for differences between specific factors. Additionally, the Mann-Whitney-Test was used. Data of stimulation with pre-and probiotics were normalized so as to eliminate high variability among donors. Data transformations were also checked (natural logarithm, square root). Calculations for statistical analysis of pre- and probiotic stimulation and TLR and adaptor protein expression were made using GraphPad Prism version 6.0. Remaining analyses were performed using IBM SPSS software. Data are presented as mean ± standard error of the mean (SEM) or mean ± min/max. Significance levels (p) were estimated and data were considered significant at $p < 0.05$.

4 Results

4.1 Characterization of differences between nasal epithelial cells derived from non-atopic or atopic donors

A lack of microbial exposure early in life may result in insufficient education of the immune system and may skew the immune function towards increased susceptibility to allergic sensitization. ECs are the first line of defense against environmental insults to the body and prevent the penetration of foreign substances, sense their presence and inform the organism's immune system and might therefore play a major role in immune modulation and probably also in allergic sensitization. The investigation of differences of immune phenotypes and responses of primary human nasal epithelial cells (HNECs) between non-atopic and atopic donor derived cells will therefore deliver important insights in allergy development.

4.1.1 Establishment of an isolation method for primary human nasal epithelial cells

Therefore, a fast and minimal invasive method to isolate, culture and expand primary HNECs from well characterized donors of different atopy status was established.

4.1.1.1 Influence of cell-cycle arrested murine 3T3 fibroblasts on the growth of HNECs

Former expansion experiments with freshly isolated primary human KCs showed that the cells grow better and faster on mitomycine C cell-cycle arrested murine 3T3 fibroblasts. Therefore, this method was adapted for HNECs with the aim to optimise the expansion rate and to use the full capacity of the small amount of cells obtained from nasal curettages. Freshly isolated HNECs were grown with or without mitomycine C treated 3T3s and the development of the culture was observed via microscope. Indeed, during the observed time frame, HNECs grew much better in the presence of murine 3T3s, getting to the point that they only grew successfully in co-culture with the mouse fibroblasts (Figure 7).

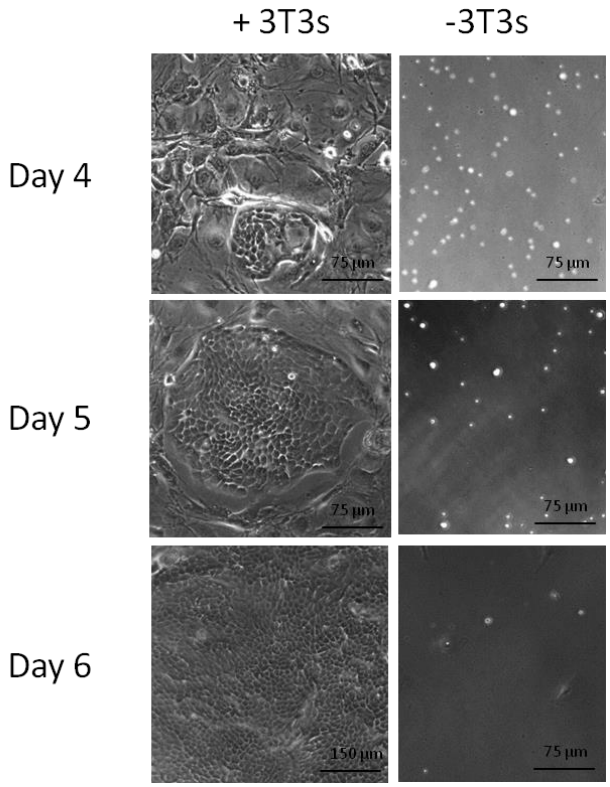

Figure 7: HNECs grow better and faster on mitomycine C treated 3T3 cells.
HNECs obtained from the curettages were seeded in two wells of a 6 well plate; one with and another without mitomycine C treated 3T3 cells. Cell growth was checked on days 4, 5, and 6 of culture.

In general, isolation of HNECs is endangered for contaminations. However, treatment with antimycotic/antibiotic agents was effective to prevent any fungal and/or bacterial contamination during cell growth. On day 1 of the culture HNECs were still floating in the medium while the fibroblasts were already adherent. HNECs became adherent and formed nest-like forms with a morphology typical of epithelial cells on day 3-5 of co-culture with mitomycine C treated 3T3s (Figure 7). After 5-7 days culture, HNECs had reached 70- 80% confluency and were able to be passaged. In contrast, HNECs grown without the murine fibroblasts seemed not to adhere and began to die after day 5-6.

4.1.1.2 Influence of different cell culture conditions on cell growth

To further optimize culture conditions for HNECs, different concentrations of FCS as well as cell culture flasks possibilities were tested. In comparison to 5% FCS in the cell culture medium, HNECs seemed to adhere faster, build nest-like forms easier and grow faster overall when the percentage of FCS in the medium was risen to 10% FCS (Figure 8).

Additionally, the cell culture dish used, made a difference regarding growth speed. Therefore, cultures of HNECs obtained by curettages were compared for their growth in either T25 cell culture flasks or 6-well cell culture plates. It was observed that the isolated NECs attached

slightly faster and formed larger nest-like forms when seeded into a 6-well cell culture plate compared to the T25 flasks (Figure 8).

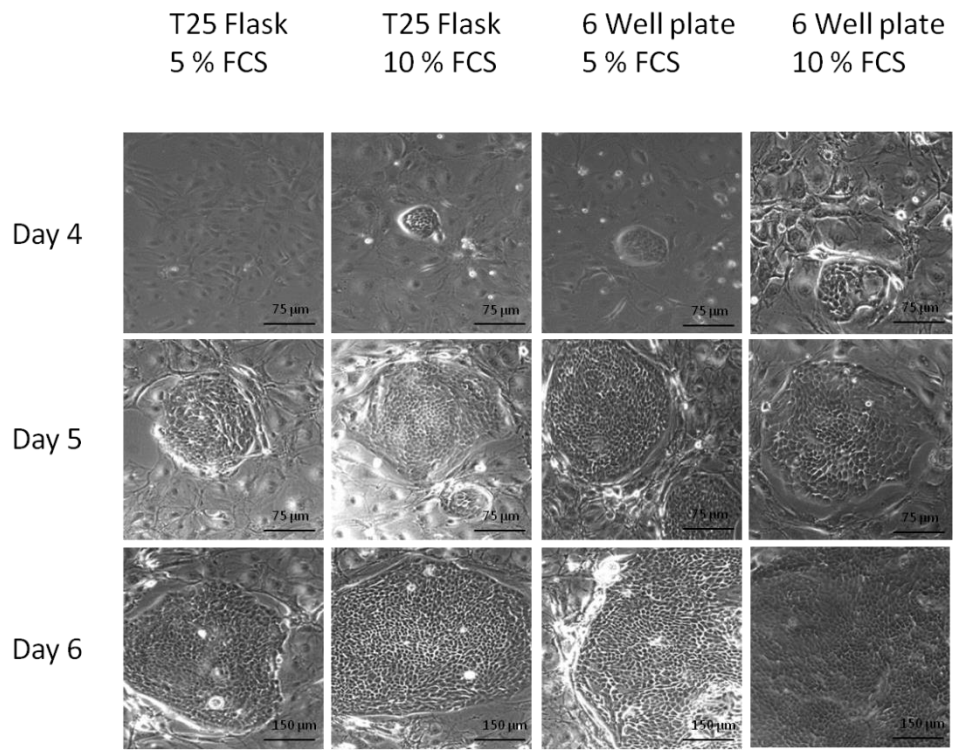

Figure 8: HNECs grow better and faster with more FCS and in 6 well plates.
HNECs obtained from the curettages were seeded in either T25 flasks or 6 well plates, either with 5 or 10 % FCS containing medium. Cell growth was checked on days 4, 5, and 6 of culture.

In the end, HNECs derived from both non-atopic and atopic donors were successfully cultured, took 7-13 days until they achieved 80-90% confluence and had a high survival rate (approximately $1.5x10^6$ live cells Figure 9A, C). Approximately, 1-2 million cells could be harvested for first passage with a very low percentage of dead cells ($1x10^5$ dead cells Figure 9B) which resulted in sufficient quantities and viability of cells to be used for up to four passages.

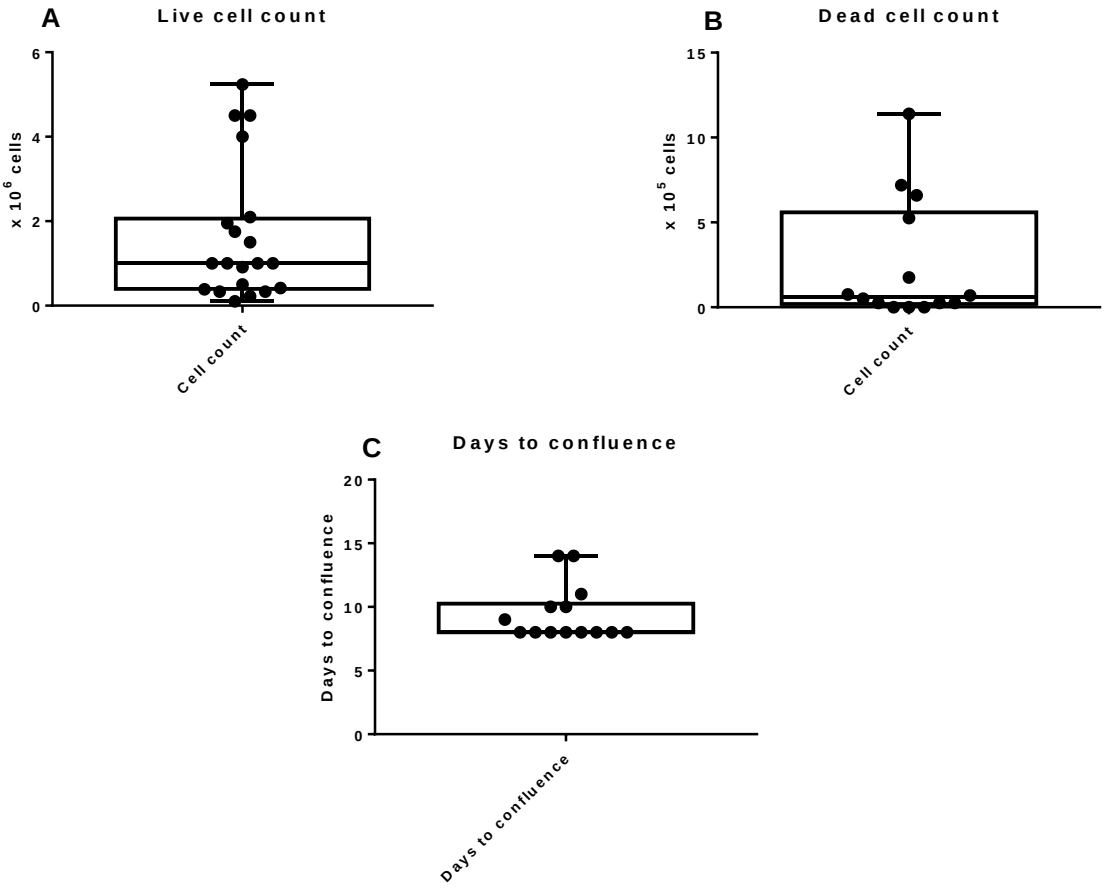

Figure 9: HNECs from curettages were successfully cultured.
Time for reaching confluency in cell culture after isolation of HNECs from curettages and cell count at this time point. n= 7 (dead cell count; days to confluence) n= 10 (live cell count). Data are depicted as mean ± min/max. *$p < 0.05$ (Mann-Whitney test).

4.1.1.3 Confirmation of epithelial cell type

To prove that the cells cultured from nasal curettages are of epithelial lineage, immunofluorescence staining for typical epithelial cell markers was carried out immunofluorescence staining for typical epithelial cell markers. It could be shown HNECs expressed pan-cytokeratin and cytokeratin-14, marking them as epithelial cells (Figure 10A, B). The expression of cytokeratins demonstrated the typical pattern, lacking a staining of the nuclei but showing an extensive network of keratin filaments distributed throughout the cytoplasm. Cytoskeleton was visualized by staining of tubulin (Figure 10C) and nuclei by staining with DAPI (Figure 10A-D). Additionally, we identified single cells staining positive for mucin 5AC, a marker of goblet cells were identified (Figure 10D). Mucin 5AC staining was restricted to a small fraction of the cells and was expressed in the cytoplasm which partly expanded to the plasma membrane.

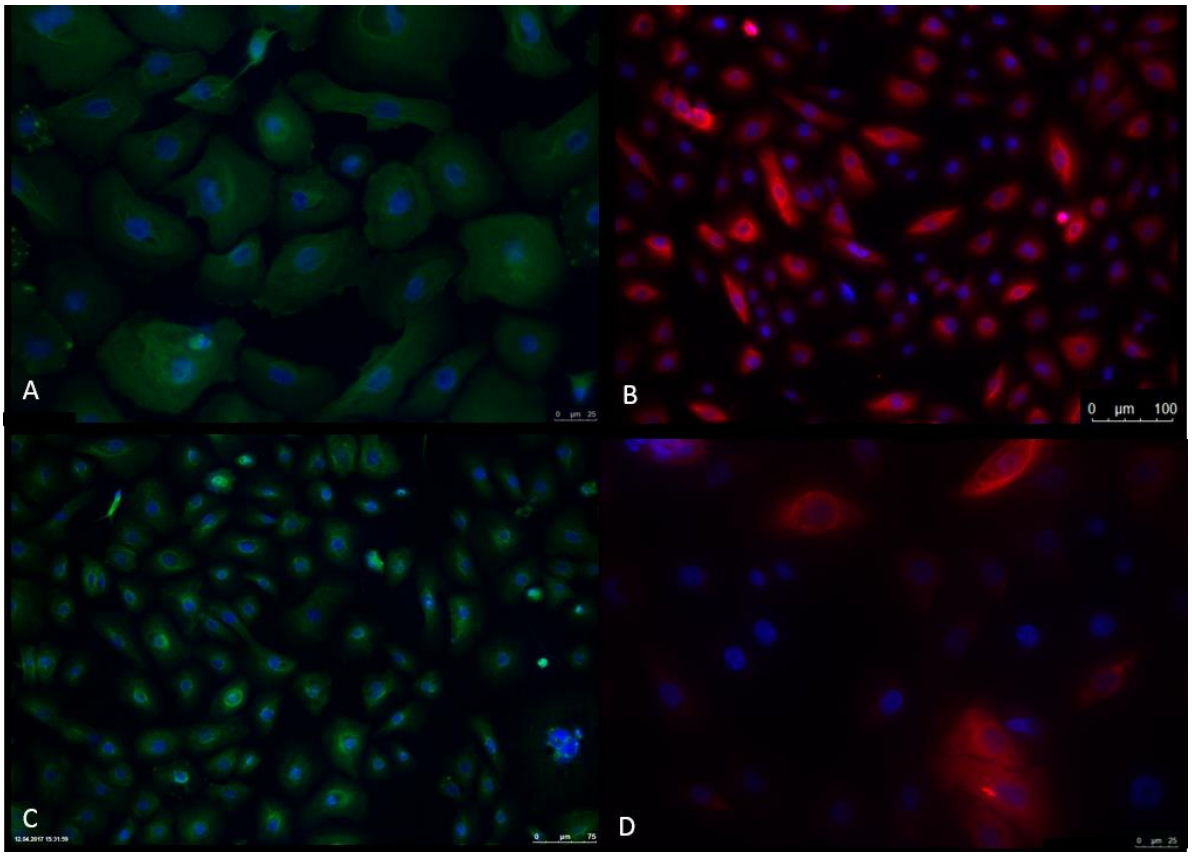

Figure 10: Cells from nasal curettages were identified as human nasal epithelial cells, containing goblet cells.
Exemplary immunofluorescence staining of HNECs for epithelial cell markers pan-cytokeratin (A) and cytokeratin-14 (B). Tubulin (C) was stained as cytoskeletal marker and nuclei were visualized by staining with DAPI. Goblet cells were visualized by the goblet cell marker mucin 5AC (D).

4.1.1.4 Ability of differentiation

To test if HNECs obtained by curettages were able to form a tight barrier as well as undergo mucociliary differentiation. ALI cultures of second-passage HNECs were established. Cells were grown for 21 days at the air-liquid interface in medium containing retinoic acid to induce mucociliary differentiation. SEM revealed the presence of tightly joined large polygonal non-ciliated epithelial-type cells with the typical cobblestone morphology but also polygonal cells with different sizes of microvilli (Figure 11). Additionally, goblet cells having droplets of mucus attached to their surface were observed (Figure 11).

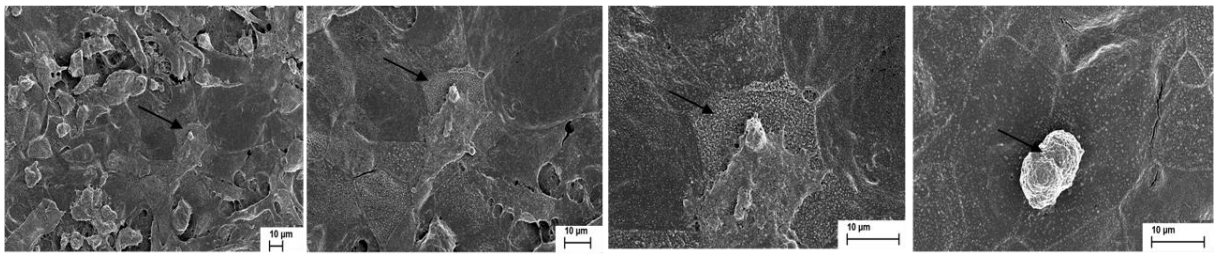

Figure 11: Scanning electron micrograph shows cells with microvilli and goblet cells.

Representative scanning electron micrograph of HNEC air-liquid interphase cultures. The cultures consisted mainly of large polygonal non-ciliated epithelial-type cells but also contained polygonal cells with different sizes of microvilli and several goblet cells.

Furthermore, TER was measured in these ALI cultures at 7, 14, and 21 days after confluence to confirm the formation of intact TJs, and normal-ranged TER values in the range of 1000 to 4000 Ω/cm^2 were observed from 7 days after confluence (Figure 12).

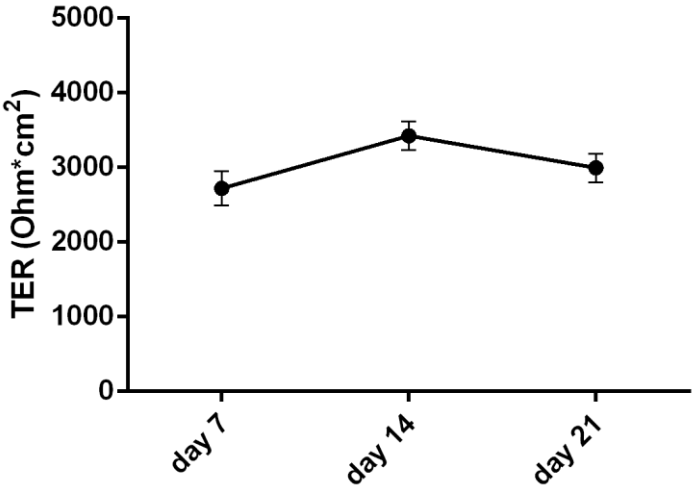

Figure 12: Development of transepithelial resistance in air-liquid interphase cultures of HNECs derived from nasal curettages.
Stability of transepithelial resistance (TER) over time in air-liquid interphase HNEC derived from curettages (*n* = 6, each). Data are expressed as mean ± SEM.

Cell adhesion via cell-cell junctions such as tight-junctions is a key regulator of cell differentiation. Therefore, HNECs were grown to nearly 100% confluence in ibidi slides and the expression of different TJ proteins was assessed by immunofluorescence staining (Figure 13). HNECs from nasal curettages stained positive for occludin (Figure 13A), claudin-1 (Figure 13B) and ZO-1 (Figure 13C), independent of the atopy status of donors. Additionally, ALI cultures stained positive for β-IV-tubulin, a marker for ciliated cells (Figure 13D).

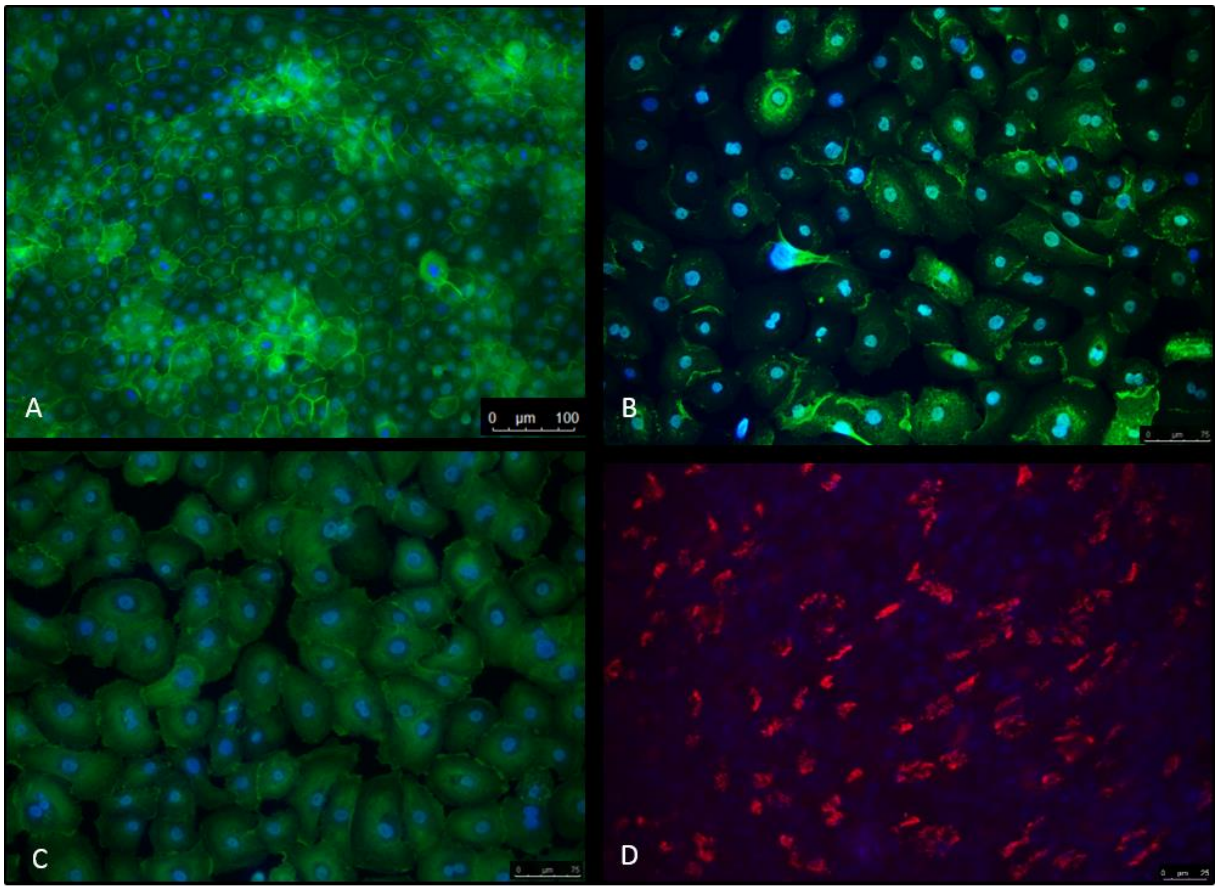

Figure 13: Cells from nasal curettages cultured in air liquid interface conditions differentiate and develop a tight barrier
Exemplary immunofluorescence staining of HNECs derived from nasal curettages, cultured in air-liquid interface conditions for tight-junctions occludin (A) claudin-1 (B) and ZO-1 (C). Ciliated cells were visualized by the ciliated cell marker β-IV-tubulin (D).

4.1.1.5 Differences in cell growth due to atopy status

Besides cytokine release and barrier resistance also differences in growth and survival between HNECs derived from non-atopic and from AR donors were observable. When first passage HNECs were harvested and counted microscopically, HNECs from non-atopic donors showed higher cell counts (Figure 14A). However, when counting the trypan blue stained dead cells, it was observed that this was not due to a higher death rate in HNECs derived from atopic donors (Figure 14C) but due to a general lower cell number. Additionally, HNECs from non-atopic donors were faster to achieve 80-90% confluence, still with similar survival rates (Figure 14B).

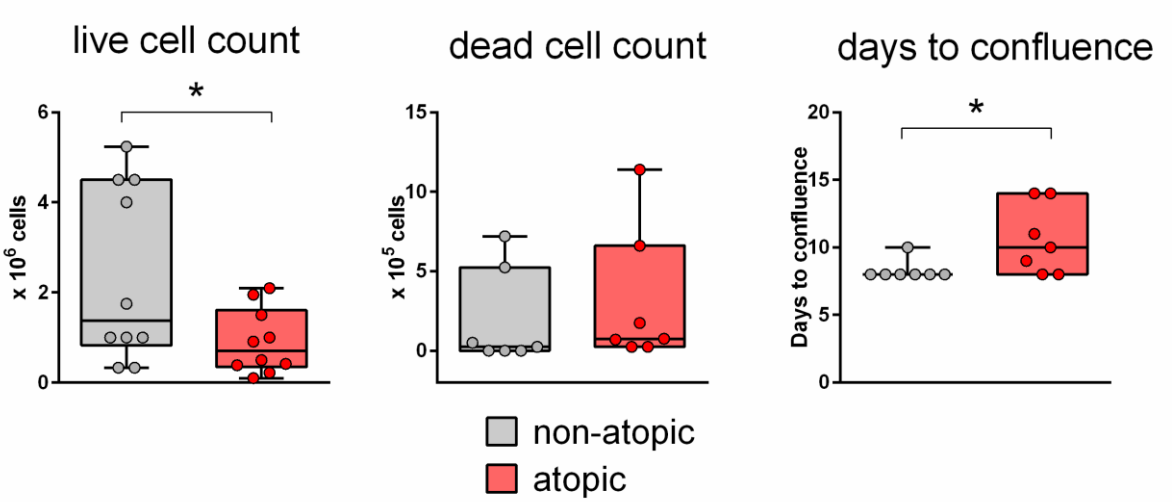

Figure 14: HNECs from non-atopic donors grow faster than HNECs from AR donors but have similar survival rates.
(A) Live cell count of HNECs obtained from the curettages from non-atopic and AR donors (B) Time for reaching confluency in cell culture after isolation. (C) Dead cell count of HNECs obtained from the curettages from non-atopic and AR donors. n= 7 (dead cell count; days to confluence) n= 10 (live cell count). Data are depicted as mean ± min/max. *$p < 0.05$ (ANCOVA and repeated measures ANOVA).

4.1.1.6 Expression of junction proteins in nasal epithelial cells derived from donors of different atopy status

When HNECs were investigated for their differentiation status, junction protein expression was also considered on mRNA level. Statistically significant differences in the expression of E-Cadherin as well as Occludin could be observed between HNECs derived from non-atopic donors and AR patients (Figure 15). E-Cadherin, but also Occludin showed a decreased expression in HNECs derived from atopic donors. In contrast, the opposite ZO-1 showed a tendency towards a higher expression in atopic donors. Finally, expression of Mucin 5AC was investigated (Figure 15). There was no statistically significant difference in Mucin 5AC

expression between cells from donors with different atopy status but HNECs from atopic donors showed a slight tendency of reduced expression.

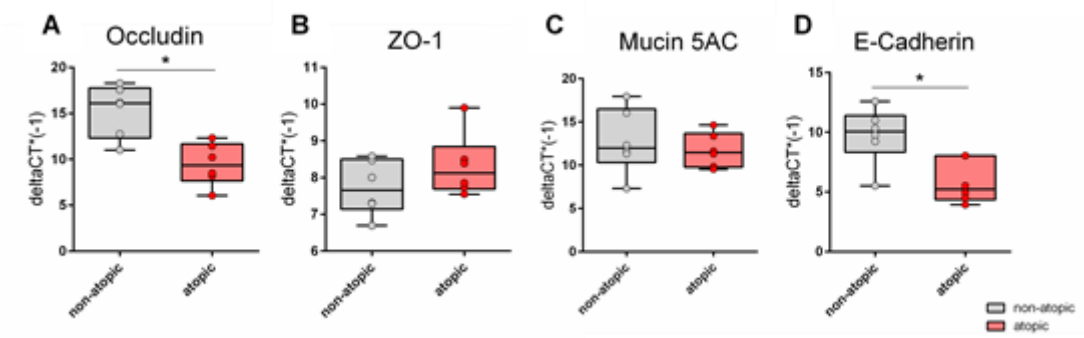

Figure 15: HNECs from atopic donors express less E-Cadherin and Occludin mRNA
Relative mRNA expression of (A) Occludin, (B) ZO-1, (C) Mucin 5AC and (D) E-Cadherin as determined by qPCR in HNECs from non-atopic and AR donors (n= 6 each). *$p < 0.05$ (Mann-Whitney test)

4.1.1.7 TER values in ALI cultures with nasal epithelial cells derived from non-atopic and atopic donors

Comparison of epithelial resistance development during ALI culture establishment showed that cells derived from atopic donors were not able to reach TER values as high as ALI cultures with cells from non-atopic donors (Figure 16). However, this effect was only significant after 14 and, especially, 21 days of culture at the air liquid interface.

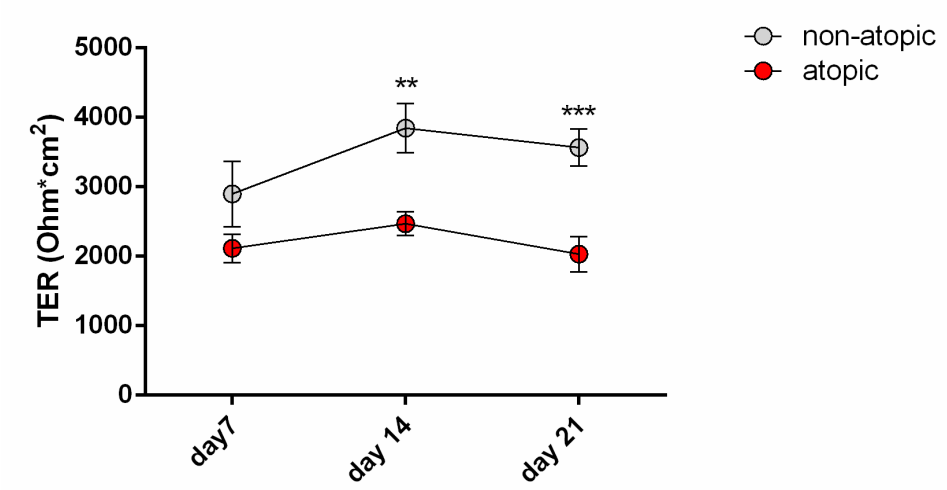

Figure 16: Transepithelial resistance differs between air-liquid interphase cultures of HNECs derived from non-atopic and AR donors.
Stability of TER over time in air-liquid interphase HNEC cultures of non-atopic and AR donors (n = 9, each). Data are expressed as mean ± SEM. **p < 0.01, ***p < 0.001 (ANCOVA and repeated measures ANOVA)

4.1.2 Barrier function in response to allergenic substances

Besides steady state conditions, development of TER values in response to different allergenic substances such as birch, ragweed and timothy grass pollen APEs and house dust mite (HDM) extract was investigated as well. In comparision to HNECs from non-atopic donors TER increased in ALI cultures with cells derived from AR patients after treatment with allergens, an effect that was statistically significant for HDM extract, Phl- and Amb-APE (Figure 17). Phl-

APE and HDM extract were the most potent stimuli of barrier resistance over time. Although Amb- and Bet-APE were less effective than HDM and Phl-APE, they also showed tendency to provoke a TER increase. This rise in TER was observed immediately after stimulation and became statistically significant after 2 hours for Phl-APE and after 4 hours for the HDM extract. The allergen-induced increase in TER was not observed in ALI cultures of HNECs from non-atopic donors. Only Phl-APE induced a rise in TER after 24 hours.

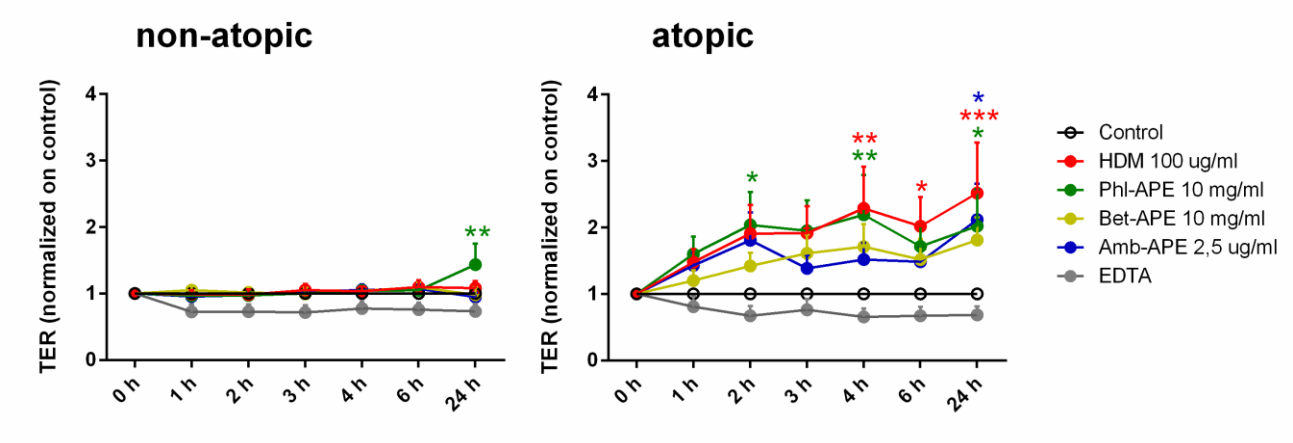

Figure 17: Transepithelial resistance differs between air-liquid interphase cultures of HNECs derived from non-atopic and AR donors stimulated with pollen extracts.
TER in cultures from non-atopic (*n*= 8) and AR donors (*n*= 7) in response to stimulation with different aeroallergen extracts EDTA was used as control to disrupt epithelial integrity. Data are expressed as mean + SEM. **p* < 0.05, ***p*< 0.01, ****p* < 0.001. (Two-way ANOVA with Bonferroni correction).

4.1.3 Innate immune receptor repertoire of nasal epithelial cells derived from donor with different atopy status

The innate immune receptor repertoire and the immunological barrier, factors possibly influenced by exposure to microbes early in life, were additionally studied in these cells in order to get a deeper insight in allergic disease pathology mechanisms. For this purpose, the expression of different Toll-like receptors (TLRs) in unstimulated HNECs was assessed. As RT-qPCR results related to 18S house keeper gene revealed, HNECs transcribe the genes of TLR 1-6 and -9, while expression of TLR-7 and -8 was absent (Figure 18). HNECs from AR donors expressed lower transcript levels of TLR-1-5 and -9 genes than HNECs from non-atopic donors. The difference in baseline mRNA expression between HNECs of AR and non-atopic donors was statistically significant for TLR-4 and TLR-9, and a tendency towards a statistically significant difference was observed for TLR-5 (p=0.06).

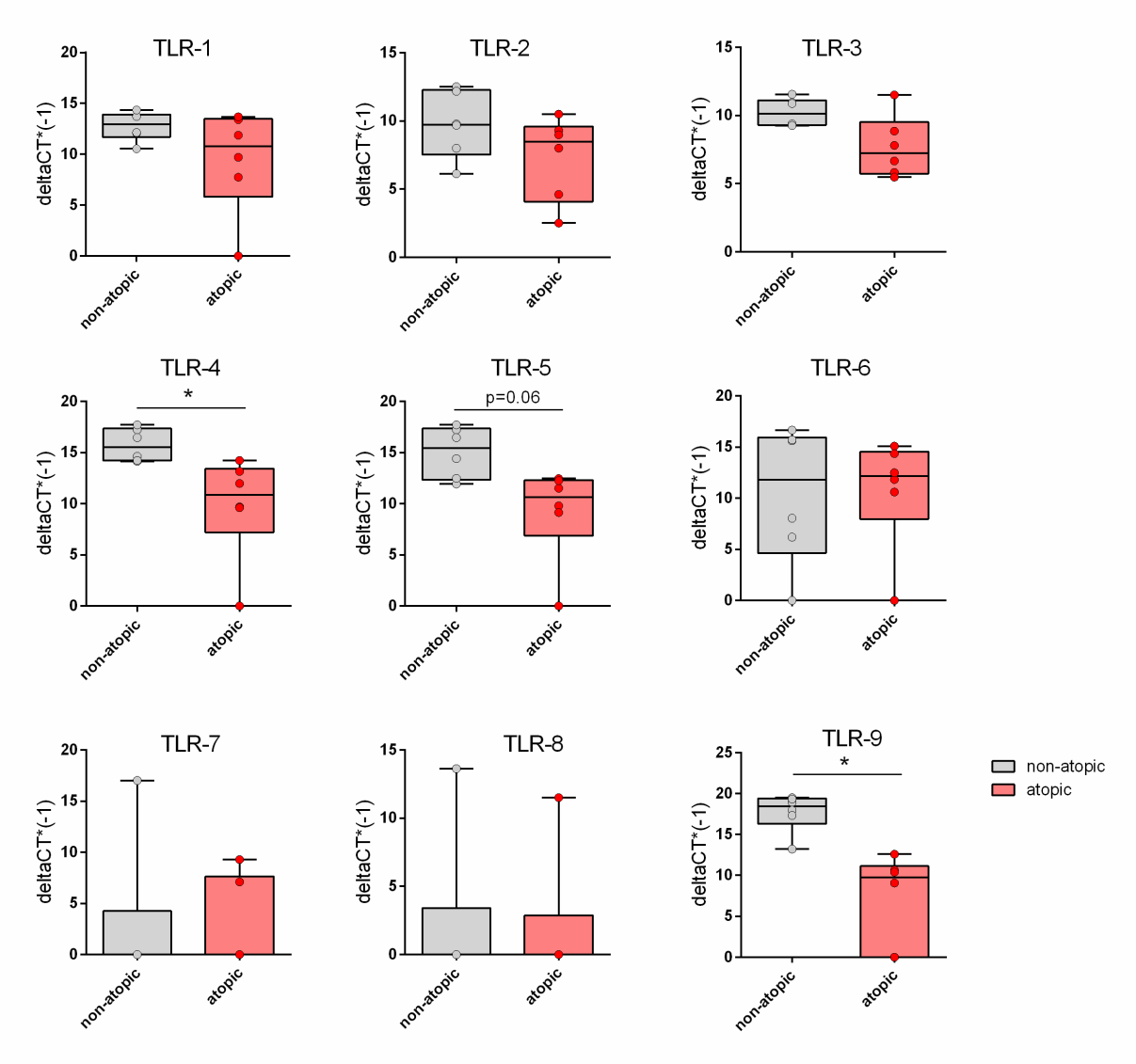

Figure 18: TLR expression differs in HNECs from non-atopic and AR donors.
Relative mRNA expression levels of TLRs in HNECs derived from non-atopic and AR donors (*n*= 6, each) after the first passage. Data are expressed as mean ± SEM. **p* < 0.05 (Mann-Whitney test; Bonferroni correction for multiple tests).

Furthermore, the expression of the TLR adaptor genes *myd88* and *trif* was compared between HNECs derived from non-atopic donors or AR patients on mRNA level (Figure 19). The expression pattern of both TLR adaptors differed between cells from AR and non-atopic donors. *myd88* was only expressed in some of the samples derived from AR donors and in one sample from one non-atopic donor, whereas *trif* showed a trend towards reduced expression in HNECs derived from atopic donors, as compared to AR donors (p=0.067).

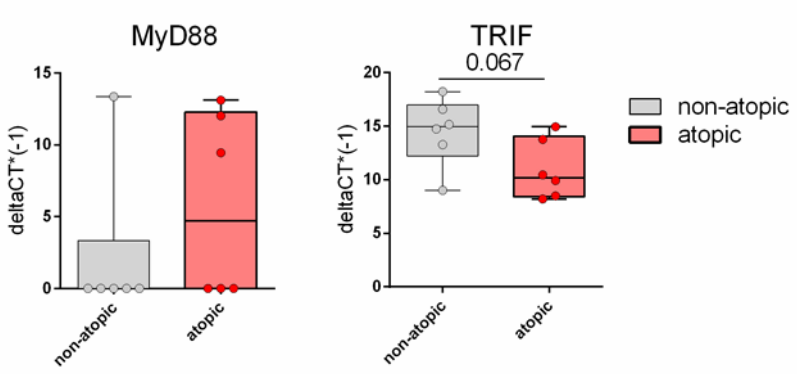

Figure 19: TLR expression differs in HNECs from non-atopic and AR donors.
Relative mRNA expression levels of TLRs in HNECs derived from non-atopic and AR donors (n= 6, each) after the first passage. Data are expressed as mean ± SEM. *p < 0.05 (Mann-Whitney test; Bonferroni correction for multiple tests).

The differences in TLR expression between HNECs of non-atopic and AR donors was confirmed on protein level via FACS staining and analysis (Figure 20). HNECs expressed TLR-1-4, -9. The expression of intracellular TLRs (TLR-3 and -9) was highest, followed by the extracellular TLRs -1, -2 and -4. Overall, there were only slight differences in TLR expression between HNECs derived from non-atopic and AR donors. However, TLR-9 showed a significantly decreased expression in HNECs derived from atopic donors. Whereas there were no differences in TLR-3 expression and only a slight tendency for diminished expression of TLR-4 between cells of different atopy status, the expression of the TLR-3 and -4-specific intracellular adaptor protein, TRIF, showed the same tendency of a reduced expression in HNECs from atopic donors as observed on mRNA level. MyD88 expression, in contrast, did not differ between cells derived from donors with different atopy status.

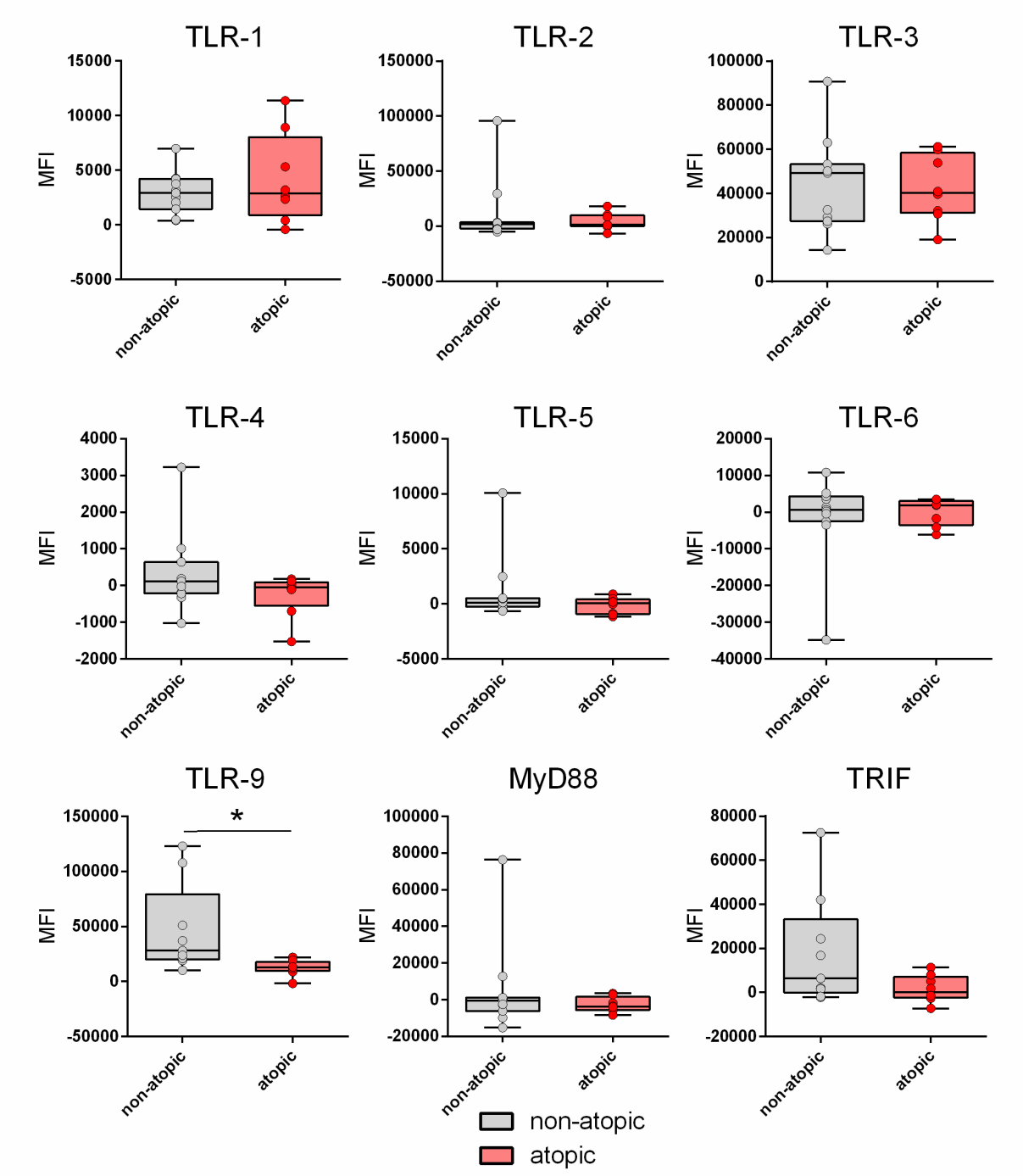

Figure 20: TLR and TLR adaptor molecule expression differs in HNECs of non-atopic and atopic donors.
Ex vivo TLR and TLR adaptor molecule expression in HNECs of non-atopic (*n*= 11) and AR donors (*n*= 8). Directly after isolation, HNECs from curettages and biopsies were stained against TLRs and TLR adaptor molecules and subjected to multi-color flow cytometry. For detection of intracellular proteins, cells were fixed and permeabilized before staining. Expression levels are given as mean fluorescence intensity (MFI). For each marker, the MFI of the respective FMO control was subtracted. *$p < 0.05$ (ANCOVA with post-hoc Bonferroni test).

4.1.4 Influence of atopy status on response to innate immune stimuli

We next assessed the expression of TLRs and TLR adaptor proteins in HNECs derived from non-atopic donors or AR patients in response to stimulation with Flagellin, PolyI:C, Pam3Cys, LPS and CpG as ligands for TLRs 1-9 (Figure 21). HNECs from AR donors maintained altered mRNA expression levels of TLR and TLR adaptor protein genes, even under stimulation. As revealed by full factorial ANCOVA, atopy status significantly affected the expression of TLR-1-4,-8 genes, with the expression of TLR-1 and -3 being particularly affected. TLR-9 gene expression was again reduced at baseline, though not statistically significant, but not affected by stimulation. Stimulation with PolyI:C significantly altered the expression of TLR-3 in HNECs derived from non-atopics and atopics. TLR-1, -2, -4-7 and -9 were not affected upon stimulation with TLR ligands while TLR-8 expression was up-regulated after stimulation with each TLR ligand in both HNECs from non-atopic and atopic donors.

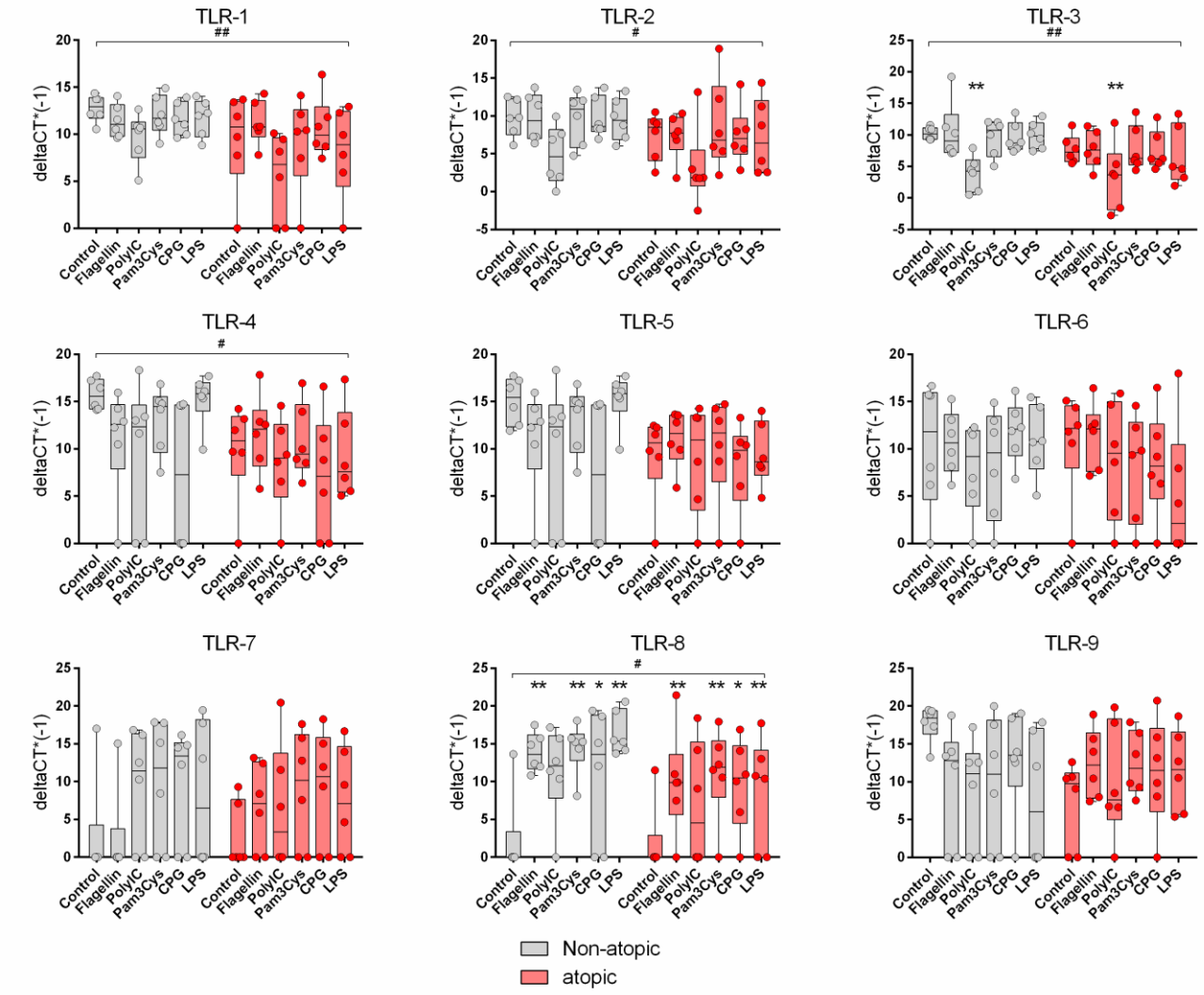

Figure 21: TLR expression differs in HNECs from non-atopic and AR donors stimulated with TLR ligands.
Relative mRNA expression of TLR-1-9 in HNECs after stimulation of cells derived from non-atopic and atopic donors (*n*= 6, each) stimulated with different TLR ligands. *: significant difference between stimulations; #: significant difference between cells of different atopy status. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ (ANCOVA with post-hoc Bonferroni test).

Furthermore, *trif* was highly significantly different between HNECs from non-atopic and atopic donors (Figure 22). In general, the expression of *trif* was reduced in HNECs from atopic donors and stimulation with TLR ligands did not alter its expression. In contrast, mRNA expression of *myd88* was induced by Flagellin in HNECs derived from non-atopic donors (Figure 22). However, atopy status did not significantly affect the expression of *myd88*. Still, there was a slight tendency for increased expression at baseline and after treatment with Pam3Cys, CPG and LPS in HNECs from AR patients while treatment with Flagellin and PolyI:C rather up-regulated *myd88* expression in non-atopics.

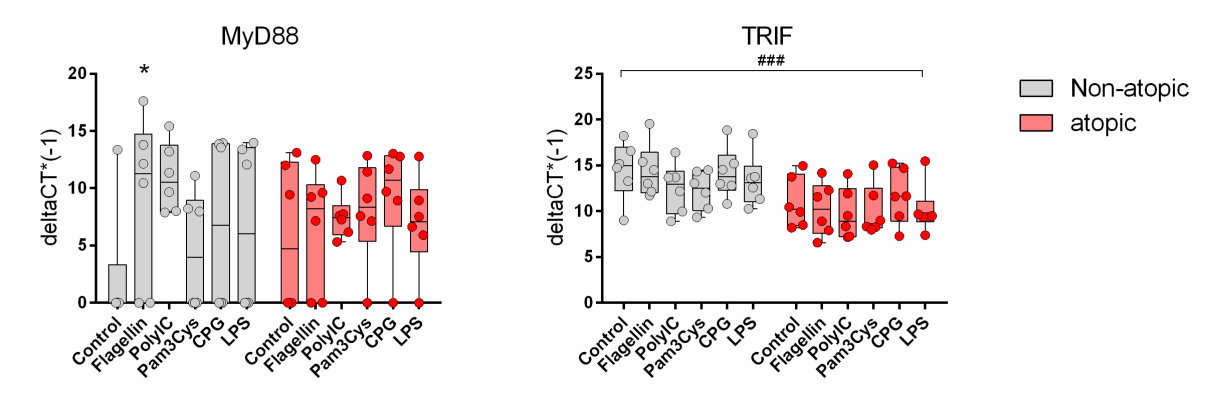

Figure 22: TLR adaptor molecule expression differs in HNECs from non-atopic and AR donors stimulated with TLR ligands.
Relative mRNA expression of TLR adaptor molecules in HNECs after stimulation of cells derived from non-atopic and atopic donors (*n*= 6, each) stimulated with different TLR ligands. *: significant difference between stimulations; #: significant difference between cells of different atopy status. *p < 0.05, **p < 0.01, ***p < 0.001; $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 (ANCOVA with post-hoc Bonferroni test).

4.1.5 Inflammatory cytokine release and inflammasome-related gene expression

4.1.5.1 Cytokine release in reponse to TLR ligand stimulation

To further investigate if differences in TLR repertoire affect the cytokine release of HNECs after stimulation with their respective ligands several cytokine levels were measured (GM-CSF, CCL-2, IL-8, CCL-20, CXCL10, CCL-5, HBD2, CCL22, IL-33, IL-18 and IL1-β). We observed an up-regulation of IL-1β, GM-CSF, CCL-2, IL-8, CCL-20, CXCL-10, CCL-5, IL-33, IL-18, HBD-2 and CCL-22 (Figure 23). Especially, PolyI:C and CpG were potent stimuli for cytokine and chemokine production by HNECs. Again, stimulation of HNECs with TLR ligands resulted in overall similar cytokine responses irrespective of donor atopy status. However, atopy status significantly affected levels of CCL-5, IL-33 and IL-18. IL-33 release was elevated at baseline in HNECs from AR patients while stimulation of HNECs with TLR ligands did not further alter the expression of IL-33. In cells from non-atopic donors, IL-33 release was lower at baseline in comparison to cells from atopic donors and stimulation with LPS, Flagellin, PolyI:C and Pam3Cys resulted in similar cytokine levels as compared to HNECs from AR patients. Release of IL-18 was generally increased in cells from AR patients in comparison to cells from non-

atopics, but stimulation with TLR ligands affected the IL-18 release of HNECs in a similar fashion, irrespective of donor atopy status.

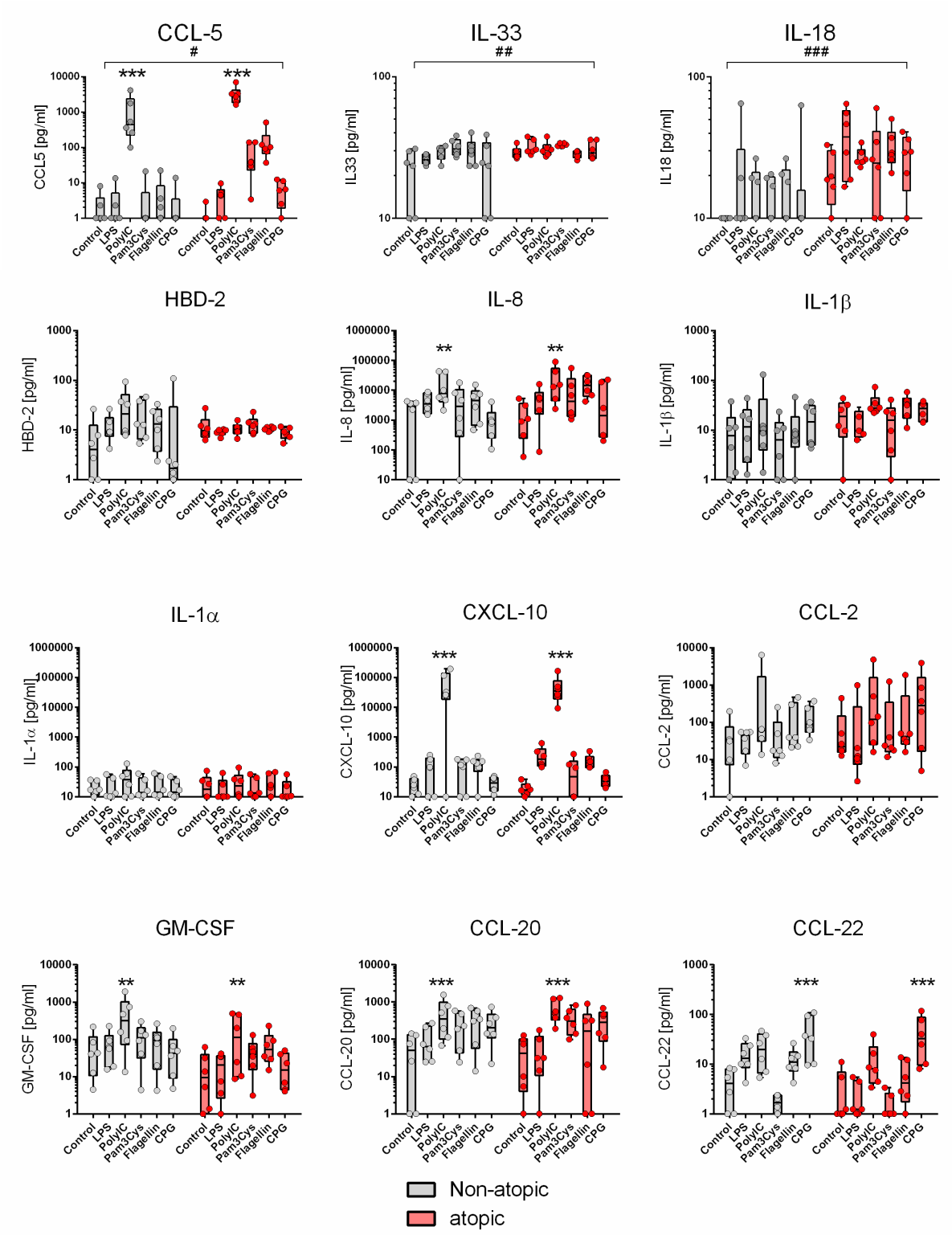

Figure 23: Patterns of TLR-ligand induced cytokine release differ in HNECs from non-atopic and AR donors.
Cytokine release of HNECs from non-atopic and AR donors stimulated with different TLR ligands (*n*= 6, each). *: significant difference between stimulations; #: significant difference between cells of different atopy status. **$p < 0.01$, ***$p < 0.001$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ (ANCOVA with post-hoc Bonferroni test).

4.1.5.2 Inflammasome-related gene expression

IL-18 and IL-33 are members of the IL-1 cytokine family which influenced by the inflammasome. Thus, these results prompted us to study inflammasome activation of HNECs more closely. We first assessed the expression of inflammasome-related genes in HNECs stimulated with TLR ligands (Figure 24). Cells from AR donors expressed significantly lower levels of mRNA for the inflammasome-related genes nlrp-3, caspase-1 and aim-2, whereas nlrp-1 mRNA expression was similar in cells of non-atopic and AR donors. The differences in inflammasome-related gene expression were already obvious at baseline and did not result from different responses to stimulation. Only stimulation with all PolyI:C showed a significant effect aim-2 expression in HNECs.

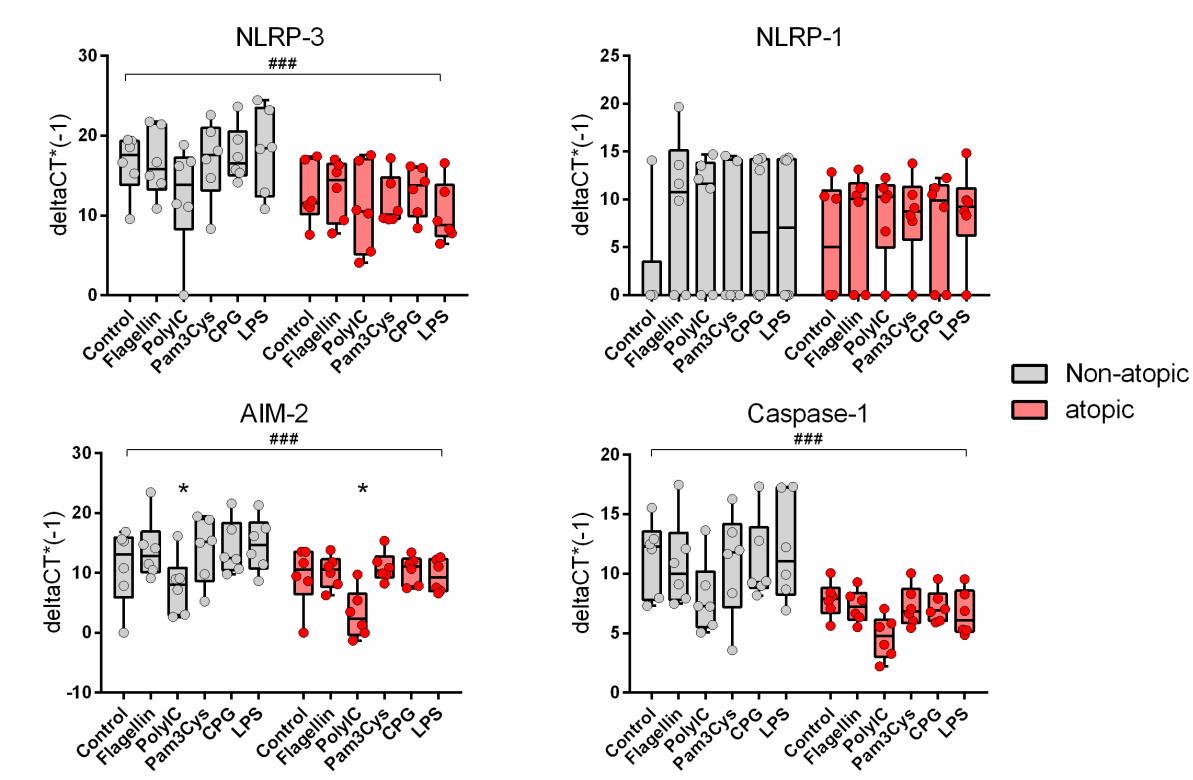

Figure 24: Expression levels of inflammasome-related genes differ in HNECs from non-atopic and AR donors.
Relative mRNA expression of the inflammasome-related genes NLRP-3, NLRP-1, AIM-2 and Caspase-1in HNECs derived from non-atopic and AR donors (*n*= 6, each) stimulated with different TLR ligands. *: significant difference between stimulations; #: significant difference between cells of different atopy status. $*p < 0.05$; $^{\#\#\#}p < 0.001$ (ANCOVA with post-hoc Bonferroni test).

4.1.5.3 Cytokine release in response to inflammasome stimulation

In a next step the effect of direct inflammasome activation was investigated and compared between HNECs from non-atopic and AR donors (Figure 25). Therefore, cells were primed with TLR ligands (PolyI:C, LPS), followed by stimulation with the inflammasome activator nigericin. LPS stimulation did not lead to significant cytokine induction in any of the cells, neither alone, nor in combination with nigericin. As already observed in previous experiments, both IL-18 and IL-33 were elevated at baseline in cells of AR donors as compared to cells of

non-atopic donors, but stimulation did not lead to significant further induction. IL-1β levels were low at baseline in cells of non-atopic and AR donors, and successive stimulation with PolyI:C and nigericin specifically induced IL-1β release ($p<0.05$) in cells of three out of six AR donors, however not in cells of non-atopic donors. IL-8 expression which was measured as a negative control, showed to be low at baseline in cells of all donors, but was induced by PolyI:C stimulation only in cells of AR donors ($p<0.001$), and additional stimulation with nigericin did not further enhance the IL-8 response to PolyIC.

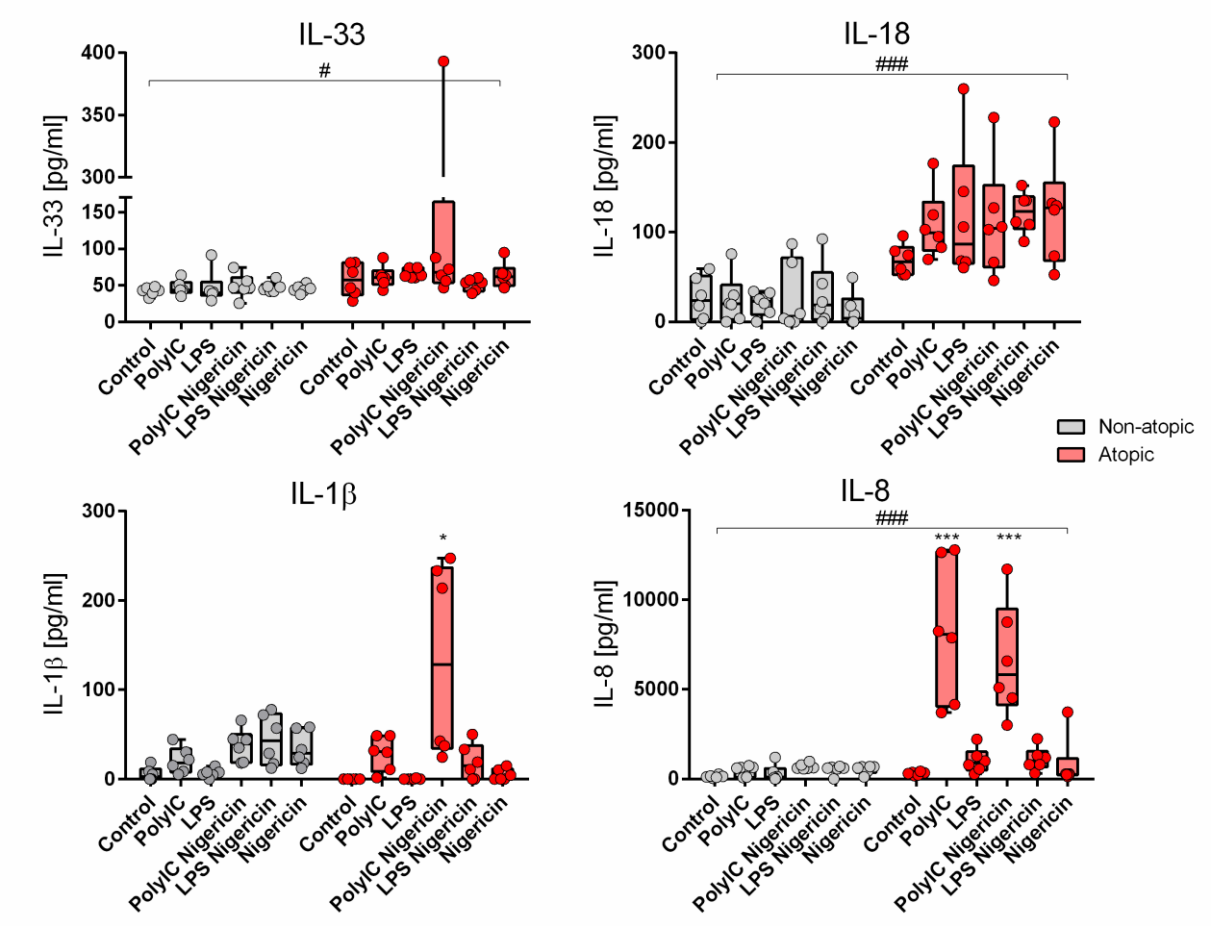

Figure 25: HNECs from non-atopic and AR donors differ in their responses to inflammasome stimulation.
Cytokine release of HNECs from non-atopic and AR donors (n= 6, each) stimulated with LPS, PolyI:C or with the inflammasome activator Nigericin, with or without prior stimulation with LPS or PolyI:C. *: significant difference between stimulations; #: significant difference between cells of different atopy status. $*p < 0.05$; $***p < 0.001$; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ (ANCOVA with post-hoc Bonferroni test).

4.1.5.4 Cytokine release in response to pollen stimulation

Finally we assessed whether stimulation of HNECs with pollen results in induction of the IL-1 family cytokines (Figure 26). Again, we observed overall elevated levels of IL-18 and IL-33 in cells of AR donors as compared to non-atopic donors. Whereas IL-33 release was not further affected by pollen stimulation, stimulation of HNECs with Phl-APE significantly induced the release of IL-18 in cells from non-atopic as well as AR donors. There were no significant

differences in pollen-stimulated IL-1β release. IL-8 was induced by stimulation with Phl-APE in cells of non-atopic donors, whereas cells of AR donors showed high inter-donor variability, and the effect of Phl-APE was not statistically significant.

In the same setup, levels of IL-1α, GM-CSF, CCL-2, CXCL-10 or CCL-5 did not differ significantly depending on atopy status or stimulation (Figure 26). Additionally, HBD-2 release was induced by stimulation with Amb-APE in HNECs from AR donors but not in cells of non-atopic donors. Moreover, CCL-20 and CCL-22 release was elevated in some non-atopic donors at baseline and after pollen stimulation when compared with cells from AR donors.

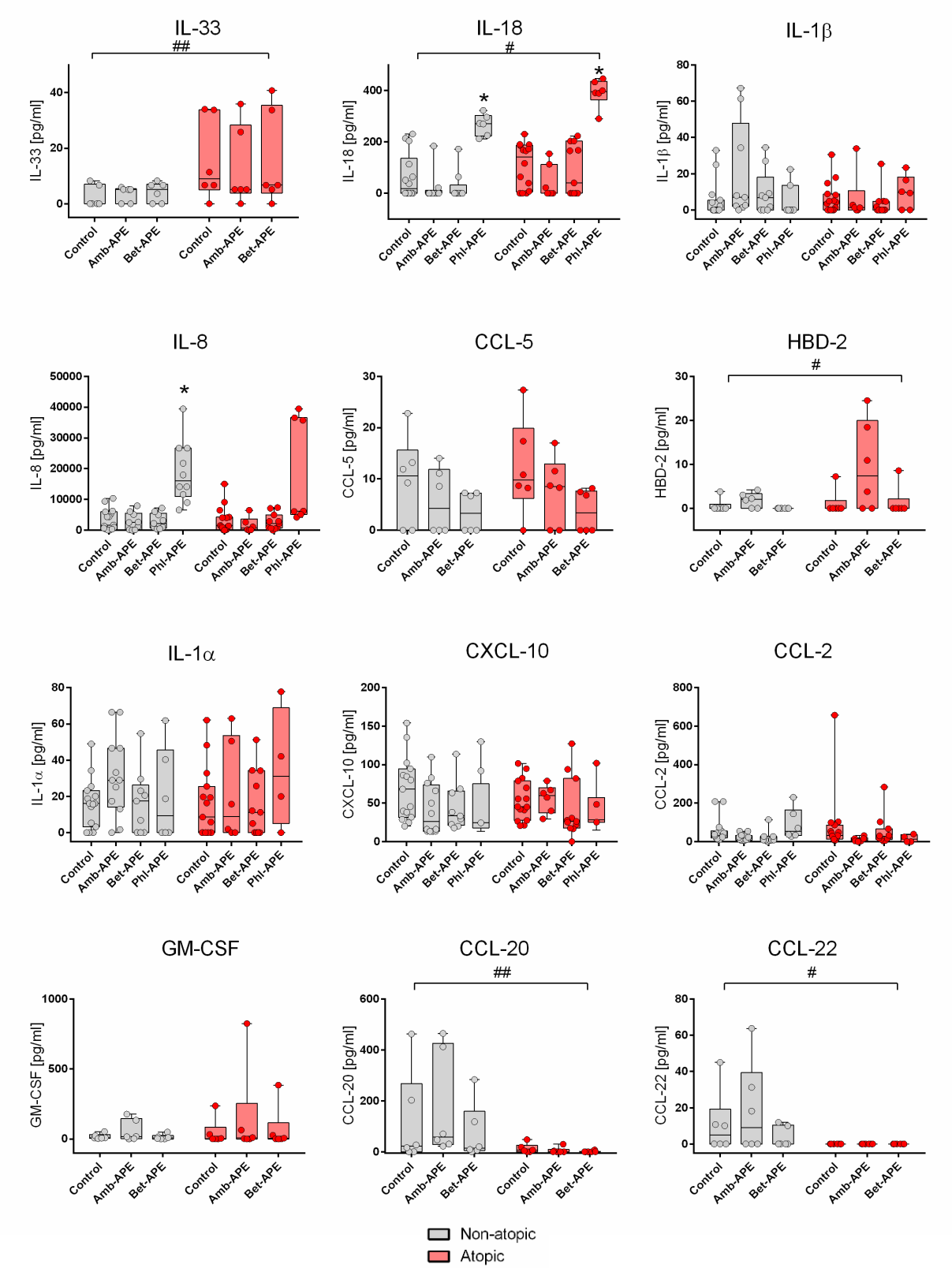

Figure 26: HNECs of non-atopic and AR donors show different cytokine responses to stimulation with pollen extracts.
Release of cytokines in HNECs from non-atopic and AR donors (n= 6-15) stimulated with different aqueous pollen extracts (Bet-APE: aqueous birch pollen extract; Phl-APE: aqueous grass pollen extract; Amb-APE: aqueous ragweed pollen extract). *: significant difference between stimulation; #: significant difference between cells of different atopy status. *$p < 0.05$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (ANCOVA with post-hoc Bonferroni test).

4.1.6 Differences in cell death

To rule out possible differences in cytokine release due to cell death LDH assays were carried out (Figure 27). When stimulating HNECs with TLR ligands, cell death did not change drastically between the differenct conditions (Figure 27A). TLR ligand treatment resulted in increase of cell death, which, however, was only statistically significant for CPG in HNECs derived from atopic donors. Atopy status did not affect cell death in this condition. In contrast, stimulation with different pollen extracts yielded decreased cell death rates (Figure 27B). Especially, treatment with Bet-APE of both donor types, but also treatment with Amb- and Phl-APE of HNECs derived from atopic donors led to a reduction of cell death. There was no difference in cell death when testing for an overall effect of atopy status.

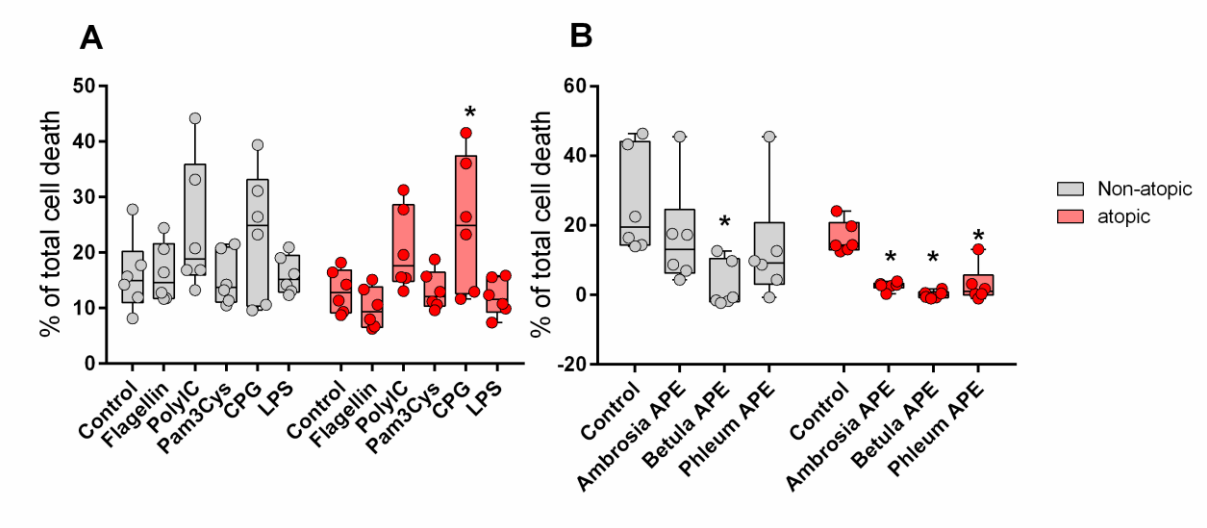

Figure 27: HNECs of non-atopic and AR donors show similar cell death rates in response to stimulation with TLR ligands and pollen extracts.
Percentage of total cell death in HNECs from non-atopic and AR donors (*n*= 6, each) stimulated with different TLR ligands (Flagellin, PolyI:C, Pam3Cys, CPG, LPS) and aqueous pollen extracts (Bet-APE, Phl-APE, Amb-APE). *$p < 0.05$ (ANOVA with post-hoc Bonferroni test).

4.2 Direct effect of non-digestible oligosaccharides, lactic acid bacteria and active microbial compounds on nasal epithelial cells

It is obvious that innate immune sensing of microbial and environmental substances is crucial in the regulation of epithelial inflammation. Commensal microbes seem to educate and prepare the immune system rather than activating it. Thus, it was investigated if intervention with prebiotics and probiotics or active microbial structures are able to restore the innate immune response or barrier function seemingly being disturbed in HNECs derived from atopic donors.

4.2.1 Cytokine release

4.2.1.1 Influence of non-digestible oligosaccharides and lactic acid bacteria on nasal epithelial cells during cytokine or TLR stimulation

To test the effect of the non-digestible oligosaccharides scGOS/lcFOS and the LAB *L. rhamnosus* and *IMS1* on chemokine release in stimulated HNECs from non-atopic donors and AR patients, HNECs were stimulated with IFN-γ, TNF-α, PolyI:C or aqueous extracts of different pollen (timothy grass, birch) and treated with scGOS/lcFOS ± *L. rhamnosus* or *IMS1*. In HNECs derived from non-allergic donors, stimulated with IFN-γ, TNF-α, there was a trend towards a reducing effect on chemokine release for scGOS/lcFOS, *IMS1* and *L. rhamnosus* (CCL-5, IL-8, CXCL-10; Figure 28). However, only the treatment with scGOS/lcFOS and its combination with *IMS1* and *L. rhamnosus* significantly reduced the secretion of both CCL-5 and CXCL-10 after stimulation with IFN-γ, TNF-α. When HNECs derived from AR patients were stimulated in the same way, treatment with scGOS/lcFOS and *IMS1* only reduced the release of CCL-5 while scGOS/lcFOS and *L. rhamnosus* only inhibited CXCL-10 secretion.

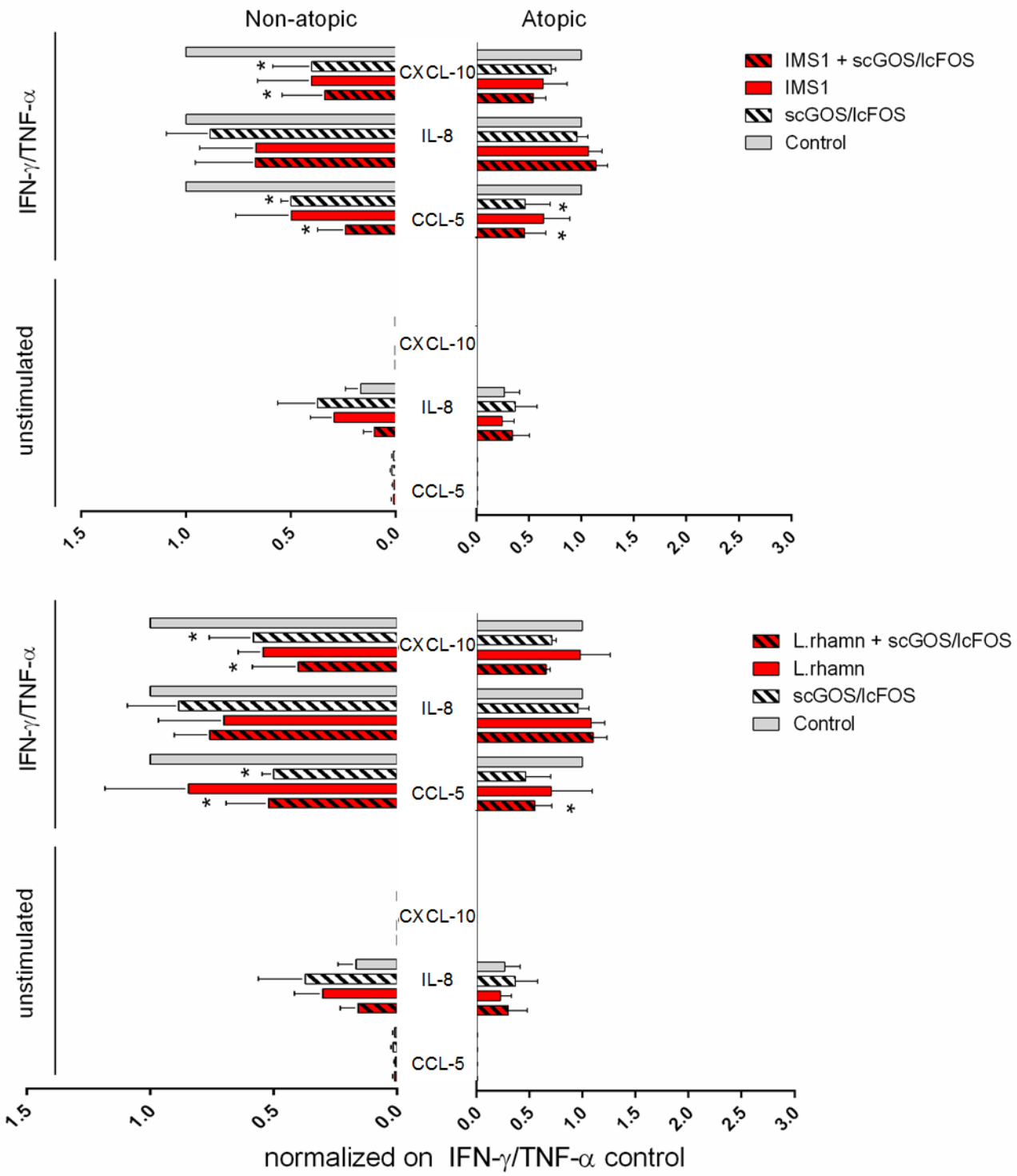

Figure 28: Chemokine release after stimulaton with IFN-γ/TNF-α and treatment with scGOS/lcFOS and LAB strains.
HNECs from non-atopic and AR donors were incubated with IFN-γ/TNF-α and treated with scGOS/lcFOS with or without *IMS1* or *L.rhamnosus* for 24 h. Only medium treated HNECs served as control. Supernatants were taken and analyzed by ELISA for CXCL-10, IL-8 and CCL-5. Results of 3 non-atopic and 3 AR donors are shown normalized on IFN-γ/TNF-α stimulated controls as mean ± SEM. *$p < 0.05$, one-way ANOVA with Bonferroni correction for multiple comparisons

In HNECs derived from non-atopic donors, stimulated with PolyI:C, treated with scGOS/lcFOS, the treatment did not lead to any significant effects. However, there was a trend to an anti-inflammatory effect of the oligosaccharide mixture. This trend was inversed for IL-8 release when HNECs derived from AR patients were stimulated with PolyI:C (Figure 29).

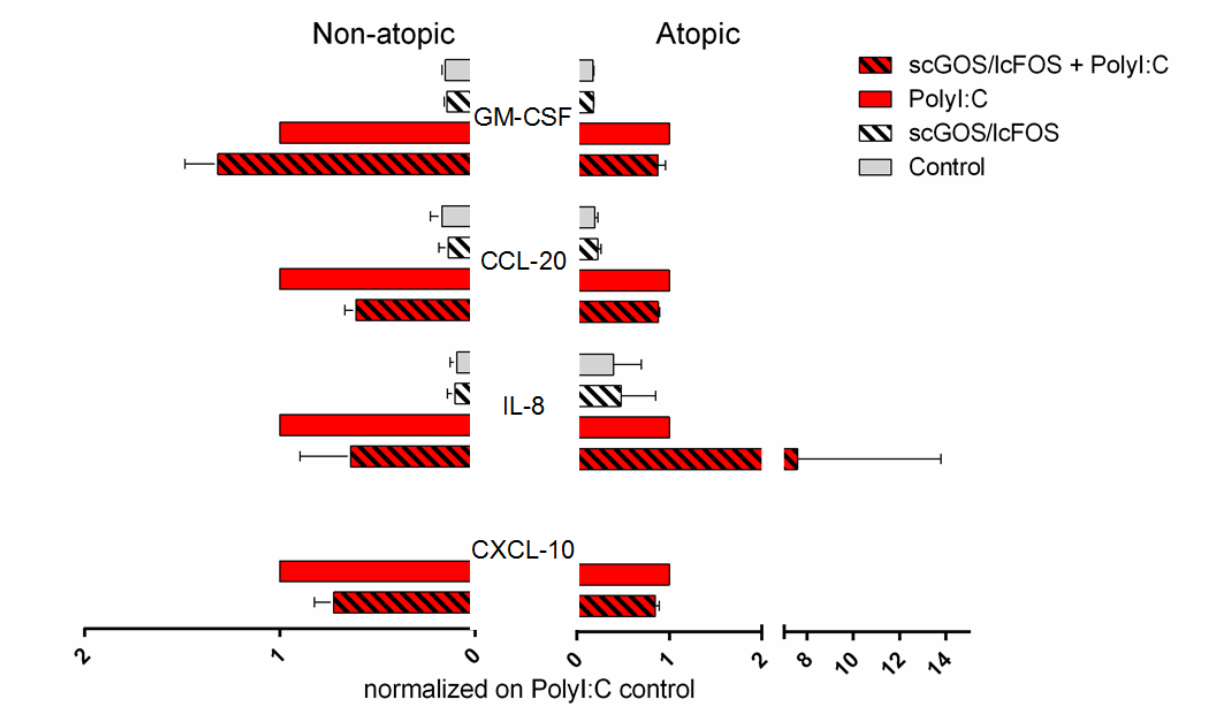

Figure 29: Chemokine release after stimulaton with PolyI:C and treatment with scGOS/lcFOS and LAB strains.
HNECs from non-atopic and AR donors were incubated with PolyI:C and treated with scGOS/lcFOS for 24 h. Only medium treated HNECs served as control. Supernatants were taken and analyzed by ELISA for GM-CSF, CCL-20, IL-8 and CXCL-10. Results of 4 non-atopic and 3 AR donors are shown normalized on PolyI:C stimulated controls as mean ± SEM. *$p < 0.05$, one-way ANOVA with Bonferroni correction for multiple comparisons

Stimulation of HNECs with different APEs showed that grass as well as birch pollen are able to induce a chemokine reaction in these ECs. IL-8 was up-regulated by stimulation with both Phl-APE and Bet-APE while CCL-20 was enhanced by Phl-APE in HNECs derived from non-atopic donors (Figure 30). CXCL-10 and GM-CSF were reduced after stimulation with both APEs in non-atopics. In contrast to stimulation of HNECs from non-atopics, there was a trend for increasd GM-CSF secretion in HNECs derived from atopic donors stimulated with both APEs. IL-8 seemed to be up-regulated and CXCL-10 down-regulated by stimulation with birch and grass pollen extracts. Bet-APE seemed to reduce CCL-20 release. Treatment of HNECs with scGOS/lcFOS while stimulating with grass or birch pollen extracts did not result in statistically significant changes of chemokine release.

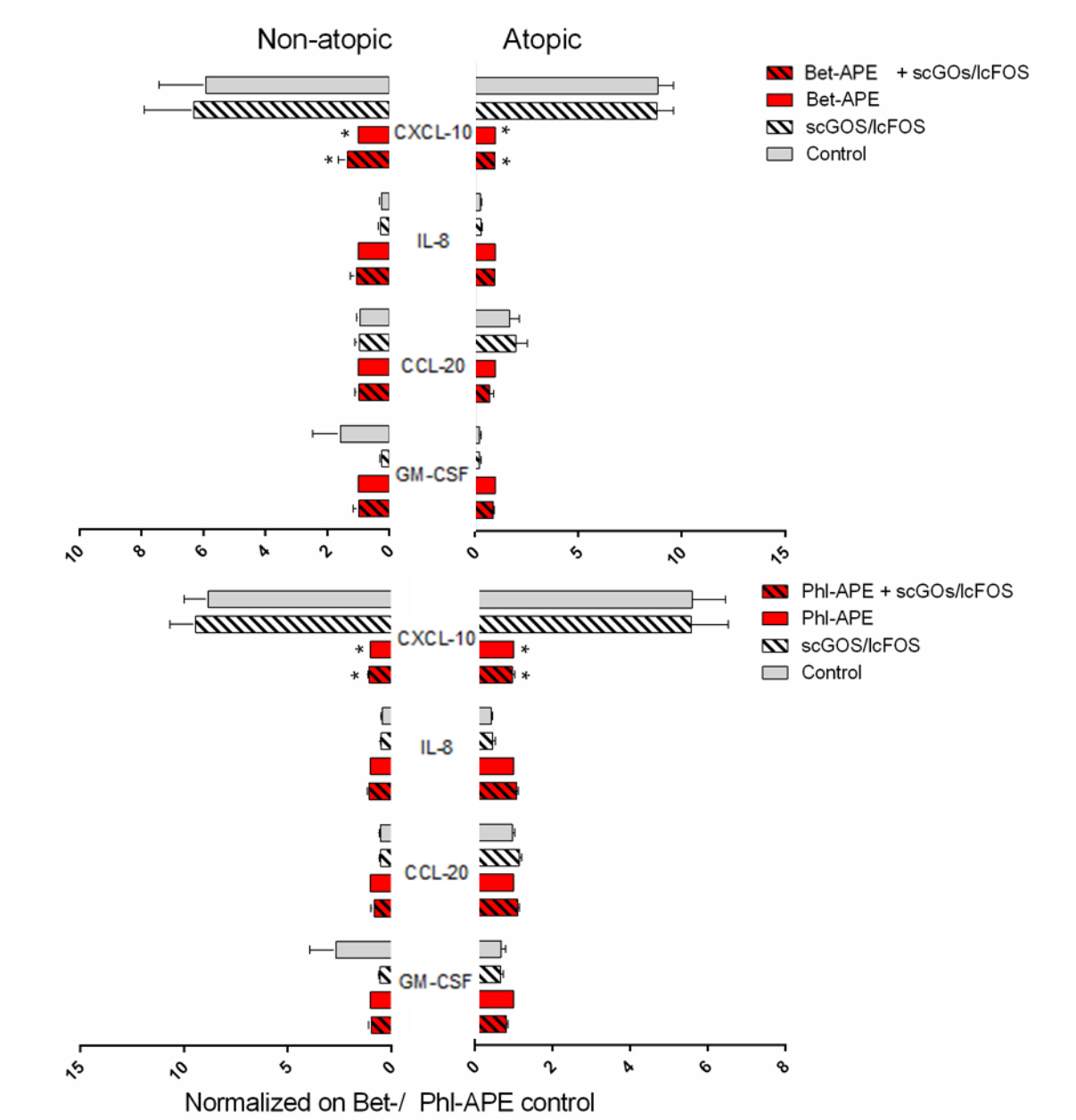

Figure 30: Chemokine release after stimulaton with pollen extracts and treatment with scGOS/lcFOS.
HNECs from non-atopic and AR donors were incubated with either Bet-APE or Phl-APE and treated with scGOS/lcFOS for 24 h. Only medium treated HNECs served as control. Supernatants were taken and analyzed by ELISA for CXCL-10, IL-8, CCL-20 and GM-CSF. Results of 5 non-atopic and 3 AR donors are shown normalized on either Bet- or Phl-APE stimulated controls as mean ± SEM. *$p < 0.05$, one-way ANOVA with Bonferroni correction for multiple comparisons

4.2.1.2 Effect of lactocepin on chemokine release in cytokine stimulated nasal epithelial cells

Treatment of HNECs derived from both non-atopic and atopic donors with the supernatant of *L. paracasei* and *casei* containing the endopeptidase lactocepin when stimulated with IFN-γ, TNF-α resulted in a significant reduction of the release of CCL-5 and CXCL-10 compared to control (Figure 31). IL-8 secretion however, was rather induced by treatment with lactocepin in HNECs derived from atopic donors. Simultaneous treatment of HNECs with lactocepin and

phenylmethanesulfonyl fluoride (PMSF), an inhibitor of lactocepin, abolished the effects of lactocepin.

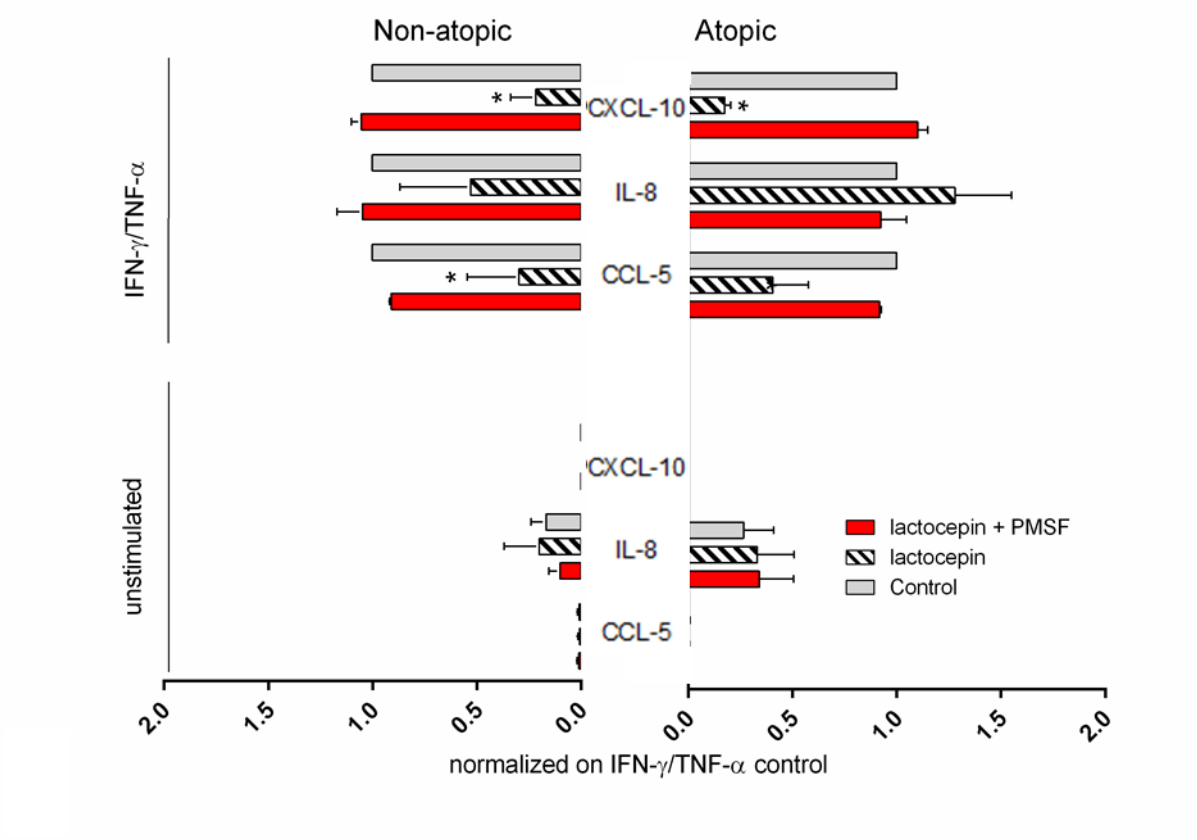

Figure 31: Chemokine release after stimulaton with IFN-γ/TNF-α and treatment with lactocepin. HNECs from non-atopic and AR donors were incubated with either IFN-γ/TNF-α and treated with lactocepin for 24 h. Only medium treated HNECs served as control. Supernatants were taken and analyzed by ELISA for CXCL-10, IL-8 and CCL-5. Results of 3 non-atopic and 3 AR donors are shown normalized on either IFN-γ/TNF-α stimulated controls as mean ± SEM. *, $p<0.05$, one-way ANOVA with Bonferroni correction for multiple comparisons

4.2.2 Barrier function

4.2.2.1 Influence of scGOS/lcFOS and lactic acid bacteria on transepithelial resistance in nasal epithelial cells

The influence on the barrier tightness of HNECs derived from non-atopic donors and AR patients was additionally examined. In HNECs from non-allergic donors, the combination of scGOS/lcFOS and *L. rhamnosus* were most beneficial in increasing the TER (Figure 32). This increase in TER was quickly established, showing the significant peak at 6 hours but then decreased again over time. When scGOS/lcFOS and/ or the different LAB strains were applied to cells derived from atopic donors, these effects were not observable. In contrast, *IMS1* in combination with scGOS/lcFOS rather decreased the barrier resistance while the other conditions did not result in any changes of TER.

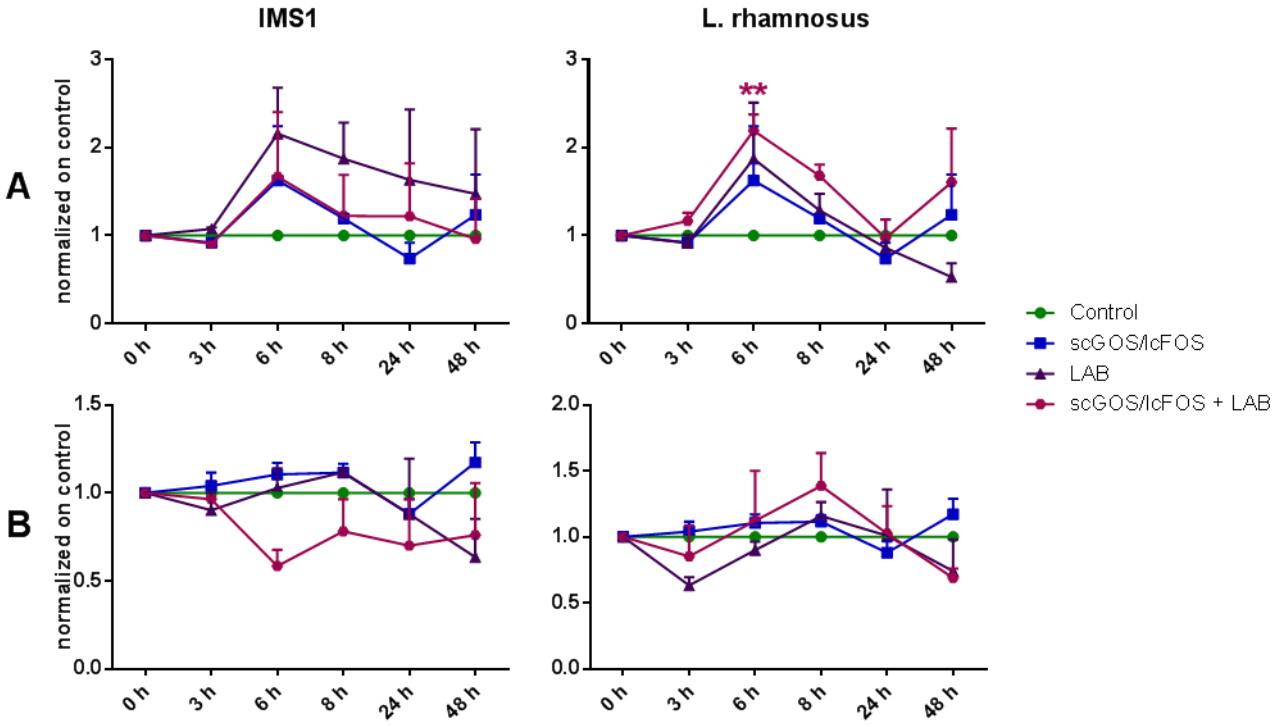

Figure 32: Development of transepithelial resistance in HNECs derived from non-atopic and AR donors after treatment with scGOS/lcFOS and lactic acid bacteria.
HNECs from non-atopic and AR donors were treated with scGOS/lcFOS with or without *IMS1* or *L. rhamnosus*. Only medium treated HNECs served as control. TER was measured after 0, 3, 6, 8, 24 and 48 hours. Results of 4 non-atopic (A) and 3 AR donors (B) are shown normalized on controls as mean ± SEM. $^{**}p < 0.01$, one-way ANOVA with Bonferroni correction for multiple comparisons

4.3 Direct effect of non-digestible oligosaccharides, lactic acid bacteria and active microbial compounds on keratinocytes

Since AE is known to be a disease of barrier disruption, immune disturbance and microbial dysbiosis, the effect of the here used prebiotics, probiotics and active microbial structures was additionally tested in this epithelial cell model in order to investigate if similar effect discrepancies between otherwise healthy and AE donors are observable.

4.3.1 Cytokine release

4.3.1.1 Effect of lactic acid bacteria and scGOS/lcFOS on chemokine release in KCs derived from non-atopic donors and atopic eczema patients

To test the effect of the non-digestible oligosaccharides scGOS/lcFOS and the lactic acid bacteria (LAB) *L. rhamnosus* and *IMS1* on chemokine release of KCs from non-allergic donors and AE patients, KCs were stimulated with IFN-γ, TNF-α or IL-4, IL-13 and simultaneously treated with scGOS/lcFOS ± *L. rhamnosus* or *IMS1* (Figure 33). In KCs derived from non-atopic donors, stimulated with IFN-γ, TNF-α, there was a tendency towards a decrease of chemokine release for all scGOS/lcFOS, *IMS1* and *L. rhamnosus*. However, only the combination of *L. rhamnosus* and scGOS/lcFOS significantly reduced the secretion of both CCL-5 and CXCL-10 after stimulation with IFN-γ, TNF-α. In contrast, KCs from AE patients rather showed a chemokine release inducing effect, when stimulated in the same way. Not only scGOS/lcFOS but also the LAB strains induced high levels of IL-8 alone and in combination. Similarly, the combination of scGOS/lcFOS ± *IMS1*/ *L. rhamnosus* induced CXCL-10, CCL-5, CCL-20 and CCL-2 secretion.

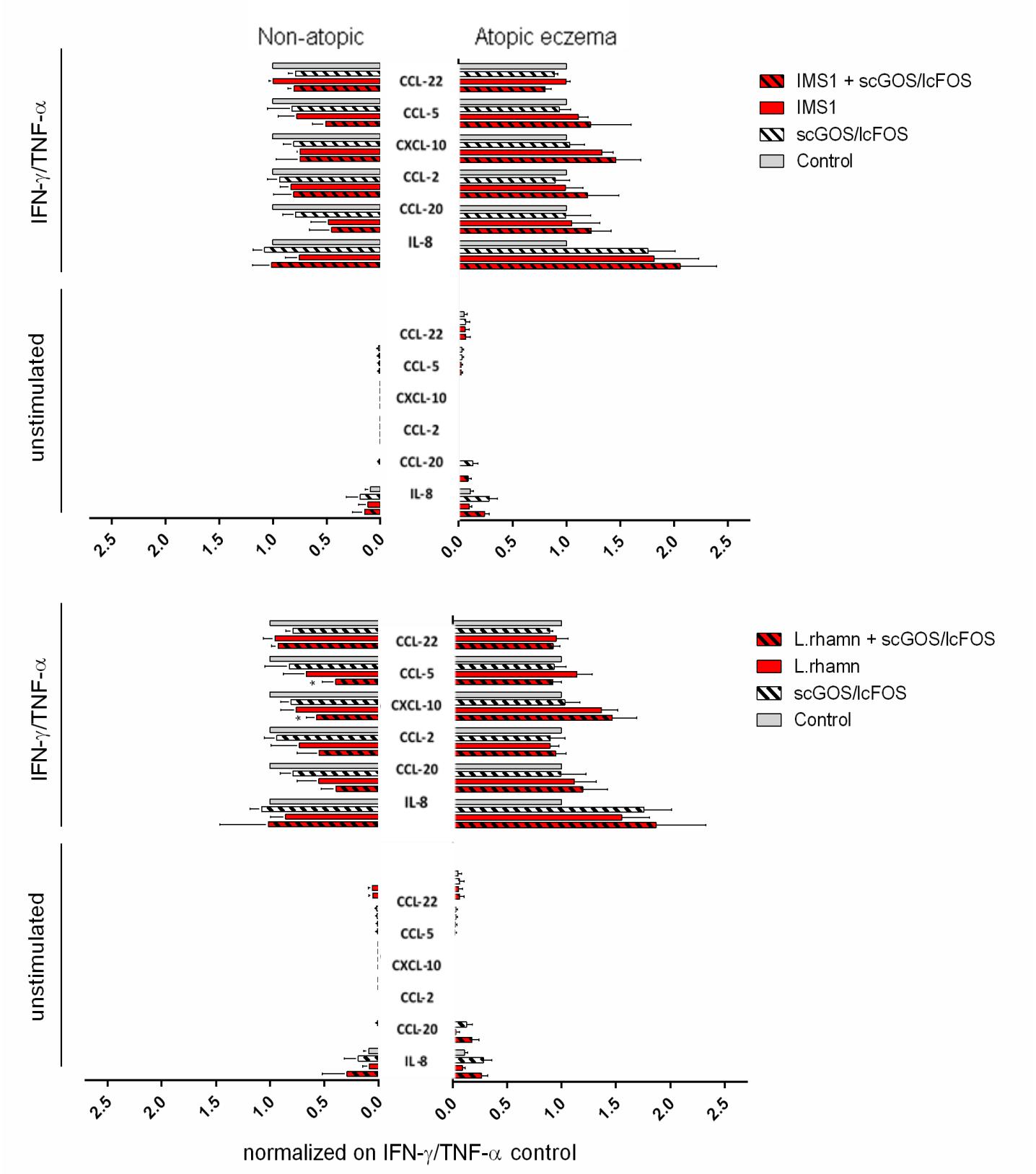

Figure 33: Chemokine release after stimulaton with IFN-γ/TNF-α and treatment with scGOS/lcFOS and LAB strains.

Human primary keratinocytes from non-atopic and AE donors were incubated with IFN-γ/TNF-α and treated with scGOS/lcFOS with or without *IMS1* or *L.rhamnosus* for 24 h. Only medium treated KCs served as control. Supernatants were taken and analyzed by ELISA for CCL-22, CCL-5, CXCL-10, CCL-2, CCL-20 and IL-8. Results of 4 non-atopic and 4 AE donors are shown normalized on IFN-γ/TNF-α stimulated controls as mean ± SEM. *$p < 0.05$, one-way ANOVA with Bonferroni correction for multiple comparisons

Similar results were obtained when KCs were stimulated with IL-4 and IL-13. As already described for the experimental setting with IFN-γ/TNF-α, there was a tendency for reduced release of CCL-2 in KCs derived from non-atopic donors when scGOS/lcFOS or its combination with *IMS1* or *L. rhamnosus* was applied. This was most likely due to scGOS/lcFOS alone (Figure 34). This effect was not observable for CCL-22. In contrast, in KCs derived from AE donors neither CCL-2, nor CCL-22 were affected by the treatment with scGOS/lcFOS or its combination with the two LAB strains.

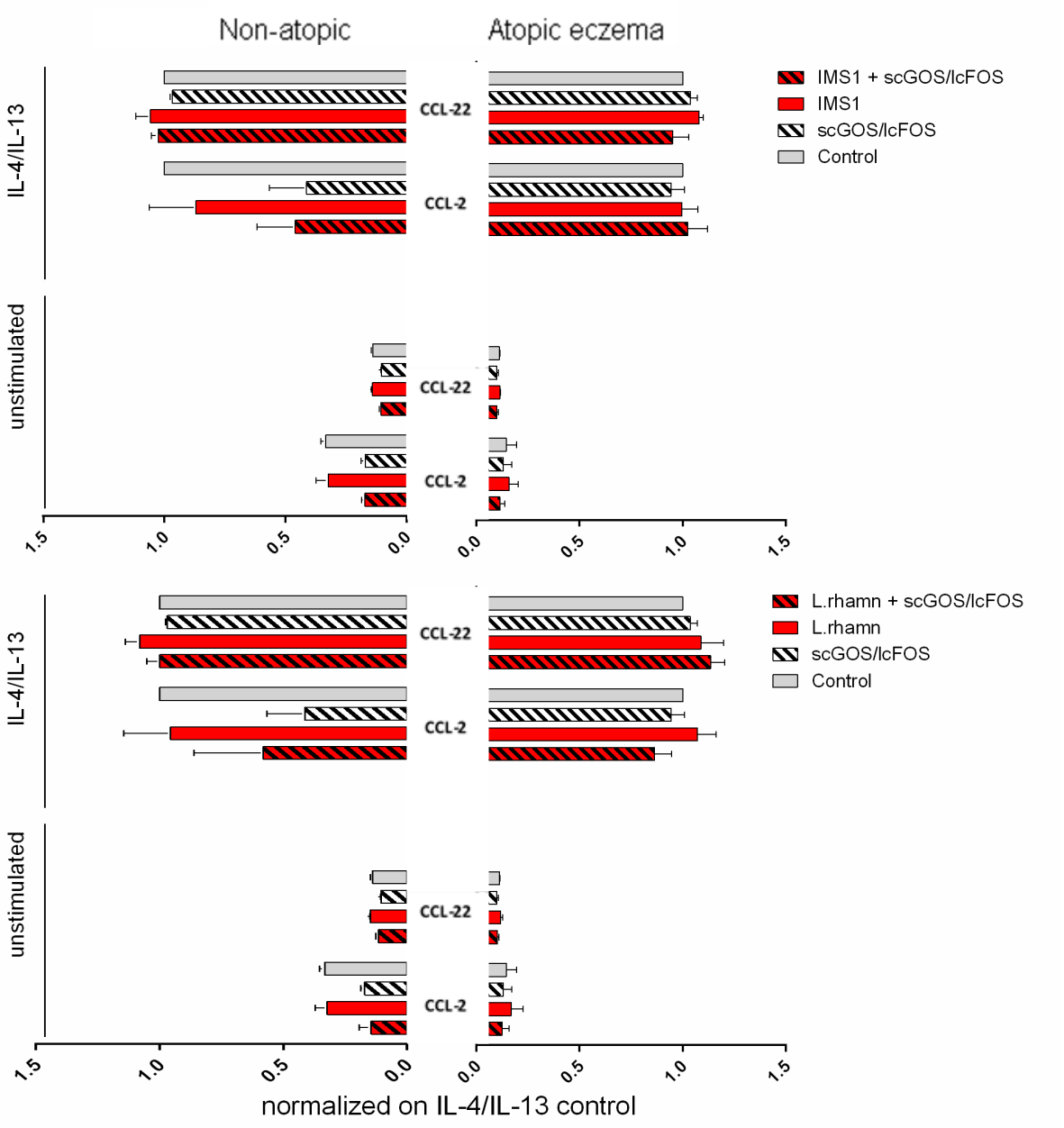

Figure 34: Chemokine release after stimulation with IL-4/IL-13 and treatment with scGOS/lcFOS and LAB strains.
Human primary keratinocytes from non-atopic and AE donors were incubated with IL-4/IL-13 and treated with scGOS/lcFOS with or without *IMS1* or *L.rhamnosus* for 24 h. Only medium treated KCs served as control. Supernatants were taken and analyzed by ELISA for CCL-22, and CCL-2. Results of 3 non-atopic and 3 AE donors are shown normalized on IL-4/IL-13 stimulated controls as mean ± SEM. *$p < 0.05$, one-way ANOVA with Bonferroni correction for multiple comparisons

4.3.1.2 Effect of lactocepin on chemokine release in KCs derived from non-atopic donors and atopic eczema patients

When stimulating KCs with IFN-γ, TNF-α while applying the supernatant of *L. paracasei* and *casei* containing the endopeptidase lactocepin simultaneously, the measureable amount of CCL-2 and CXCL-10 in KC supernatants of cells from non-allergic and AE donors was decreased when compared to the IFN-γ, TNF-α stimulation alone (Figure 35). However, similar to the results mentioned beforehand, the reduction in chemokine levels by lactocepin was more evident in KCs derived from non-atopic donors than in KCs from AE patients. Additionally, in KCs from non-allergic donors, CCL-5 levels were decreased by lactocepin which was not the case in KCs from AE patients. Simultaneous treatment of HNECs with lactocepin and PMSF, an inhibitor of lactocepin, abolished the effects of lactocepin.

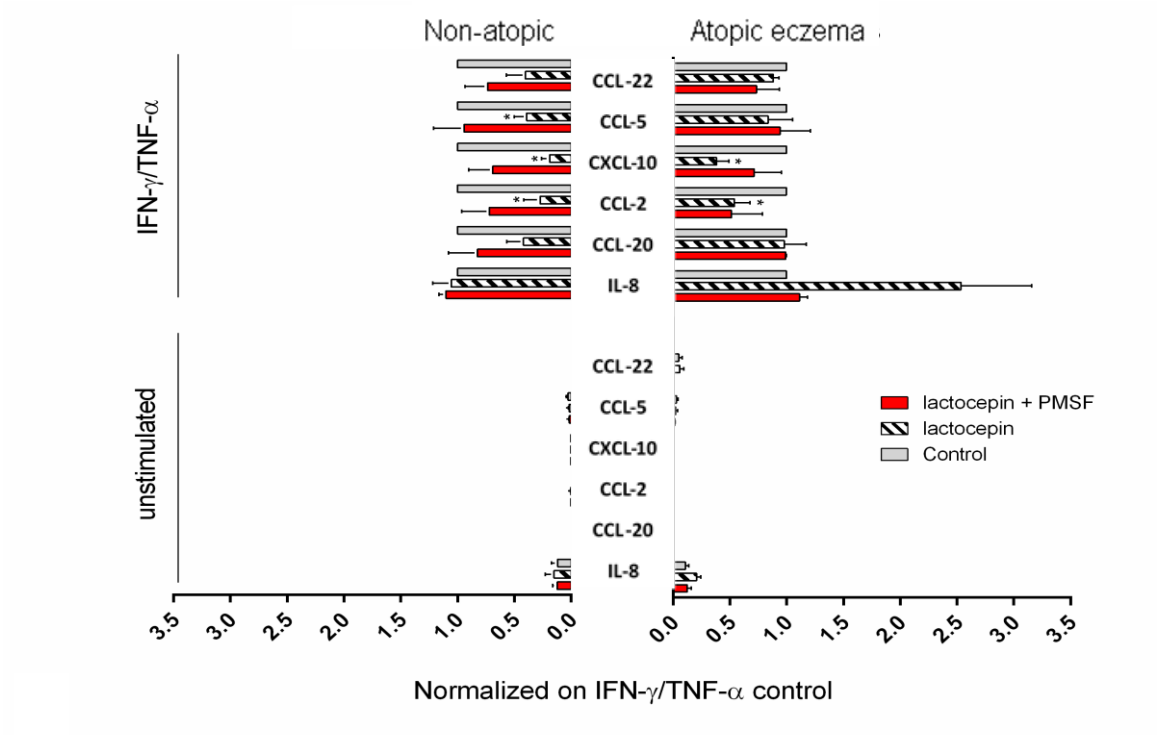

Figure 35: Chemokine release after stimulaton with IFN-γ/TNF-α and treatment with lactocepin. Human primary keratinocytes from non-atopic and AE donors were incubated with IFN-γ/TNF-α and treated with lactocepin or lactocepin in combination with its inhibitor PMSF for 24 h. Only medium treated KCs served as control. Supernatants were taken and analyzed by ELISA for CCL-22, CCL-5, CXCL-10, CCL-2, CCL-20 and IL-8. Results of 4 non-atopic and AE donors are shown normalized on IFN-γ/TNF-α stimulated controls as mean ± SEM. *$p < 0.05$, one-way ANOVA with Bonferroni correction for multiple comparisons

Similarly, when stimulated with IL-4, IL-13, lactocepin treatment of KCs derived from non-atopic and AE donors resulted in a slight tendency for reduced CCL-2 secretion in both donor types (Figure 36). In contrast, no effect on CCL-22 release was observed.

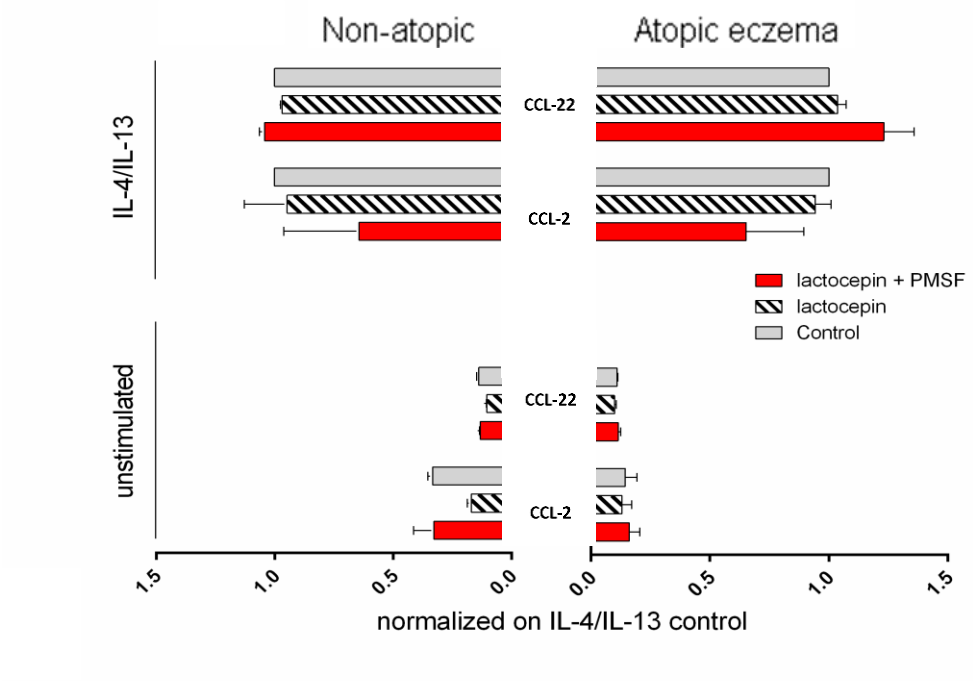

Figure 36: Chemokine release after stimulaton with IL-4/IL-13 and treatment with lactocepin. Human primary keratinocytes from non-atopic and AE donors were incubated with IL-4/IL-13 and treated with lactocepin or lactocepin in combination with its inhibitor PMSF for 24 h. Only medium treated KCs served as control. Supernatants were taken and analyzed by ELISA for CCL-22, CCL-5, CXCL-10, CCL-2, CCL-20 and IL-8. Results of 3 non-atopic and AE donors are shown normalized on IFN-γ/TNF-α stimulated controls as mean ± SEM. **p* < 0.05, one-way ANOVA with Bonferroni correction for multiple comparisons.

4.3.2 Barrier function

4.3.2.1 Effect of scGOS/lcFOS and lactic acid bacteria and the microbial substance lactocepin on transepithelial resistance in keratinocytes

To investigate further possible beneficial effects of non-digestible oligosaccharides and the LAB strains on KCs, the influence on the barrier function of KCs derived from non-atopic donors and AE patients was examined using TER measurement (Figure 37). In KCs from non-allergic donors, the combination of scGOS/lcFOS and both LAB strains was most beneficial in increasing the TER (Figure A). This effect was observable very quickly after treatment beginning. It increased three hours after application of the pre- and probiotics and stayed very stable for over 48 hours. scGOS/lcFOS alone rather decreased the TER in HNECs derived from non-atopic donors. In contrast, in KCs from AE patients, only scGOS/lcFOS alone slightly increased the TER (Figure B). In addition, the barrier tightness increase was slower in KCs from AE patients, reaching a maximum at 8 hours which was followed by a drop in TER at 24 hours being recovered at 48 hours.

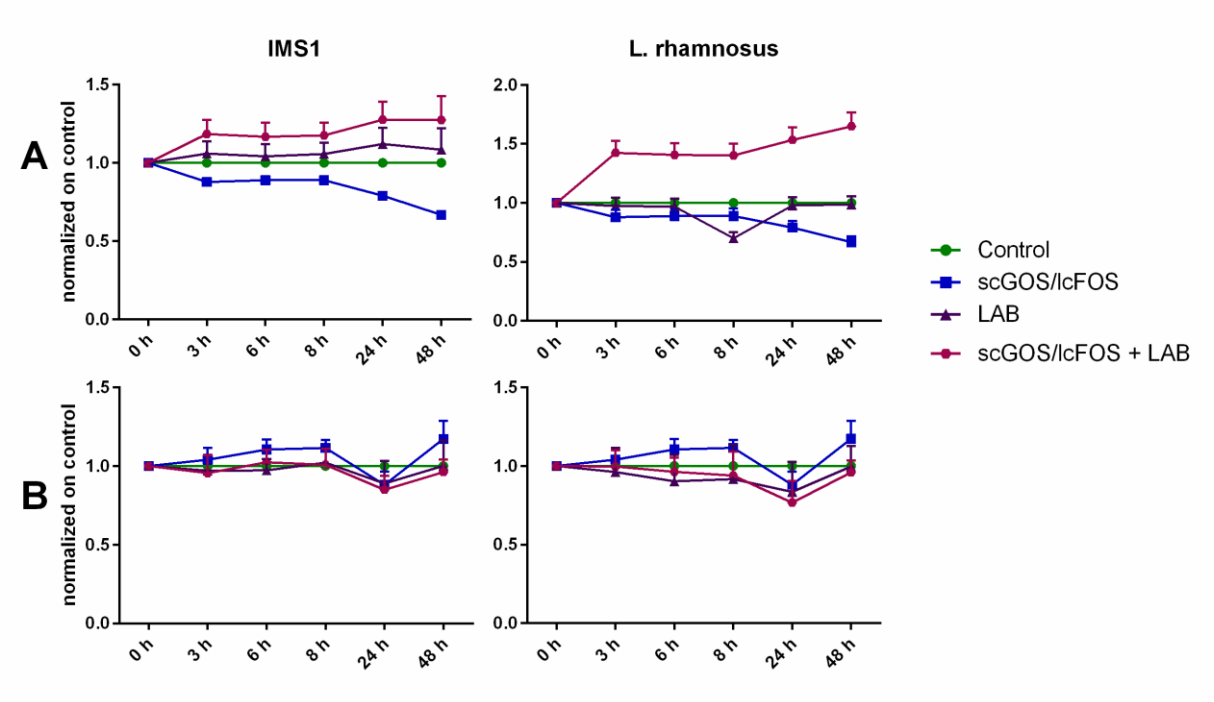

Figure 37: Development of transepithelial resistance in KCs derived from heatlhy and AE donors after treatment with scGOS/lcFOS and lactic acid bacteria
Human primary keratinocytes from non-atopic and AE donors were treated with scGOS/lcFOS with or without *IMS1* or *L. rhamnosus*. Only medium treated KCs served as control. TER was measured after 0, 3, 6, 8, 24 and 48 hours. Results of 3 non-atopic (A) and AE donors (B) are shown normalized on controls as mean ± SEM. *$p < 0.05$, ***$p < 0.001$, one-way ANOVA with Bonferroni correction for multiple comparisons

5 Discussion

Within the last decades, allergies and chronic inflammatory diseases such as atopic eczema (AE) or allergic rhinitis (AR) are increasing in their prevalence. In addition to possible genetic reasons or epigenetic mechanisms, environmental factors are taken into account (Martino & Prescott, 2010; Renz et al, 2011). The impact that environment and life style have on allergies is nicely demonstrated by the discovery that the formerly different prevalence of allergic diseases in eastern and western federal countries of Germany evened out within only 30 years after the German reunification. This growth in allergy occurrence cannot be attributed to genetic changes but has to be attributed to people′s environment (Gilles et al, 2018).

This means that, at least in part, improved hygiene and decreased exposure of the immature immune system to microbes in early life might lead to this increase in inflammatory and allergic diseases. But there are also beneficial environmental factors, common to which is that they favour the formation of a highly diverse microbiota (Gilles et al, 2018). This is also nicely illustrated by the fact that even later, sensitised and non-sensitised children show phylum and genus level differences in their microbiota in the gut but also on other body locations (Lehtimaki et al, 2017; Vebo et al, 2011). Early life interaction of the body and diverse microbes likely establishes immune homeostasis including the activation of pattern recognition receptors and downstream signaling pathways. This immune homeostasis influences the reaction capacity later in life. Taken together, a human´s genetic background may drive more or less susceptibility to the development of allergic diseases. However, this genetic background is subject to modification by environmental and life style factors via epigenetic changes in some of the many susceptibility genes for allergy which might then be transmitted to next generations (Gilles et al, 2018).

At least for altered composition of the microbiota which possibly leads to dysbalanced immune responses, intervention with pre- and probiotics has been shown to be beneficial in prevention and therapy of allergic diseases, especially when given orally (Kostadinova et al, 2017; Romeo et al, 2010; van Esch et al, 2016). Even direct effects on the immune system of especially prebiotics have been shown, e.g. activation of monocytes via interaction with TLR-2, inhibition of leucocyte rolling and adhesion and suppression of Th2-type cytokine production by T cells (Bode et al, 2004; de Kivit et al, 2011; Eiwegger et al, 2010; Eiwegger et al, 2004; Tsai et al, 2013).

Therefore, the objective of the present study was to characterise the epithelial cells (ECs) derived from non-atopic or atopic donors and find differences in their immune responses.

Moreover, the presented project aimed to investigate if non-digestible scGOS/lcFOS in presence or absence of the two LAB strains *IMS1* and *L. rhamnosus* may drive changes in immune responses possibly restoring immune disturbance of human primary keratinocytes

(KCs) and human primary nasal epithelial cells (HNECs) from both non-atopic and AR or AE donors.

5.1 Establishment of an isolation method for human primary nasal epithelial cells

The power of human primary cell culture models in allergy research is the potential to compare responses to stimulation between cells derived from donors of different atopy or disease status. Whereas it is rather easy to obtain high numbers of leucocytes, it remains difficult to obtain sufficient quantities of epithelial cells from well characterized donors. Therefore, it was assessed whether functional HNECs could be cultured in sufficient numbers from nasal curettages, a non-traumatic method for obtaining cell material from the nasal cavity. Then, HNECs from non-atopic and AR donors were compared in terms of growth rate, survival and immunological responses.

HNECs from nasal curettages from the nasal cavity of both non-atopic and atopic donors, when expanded on feeder cells and in medium containing FCS, result in sufficient quantities and viability to be used for up to four passages and cell culture experiments. Cells derived from donors allergic to birch or grass pollen were also readily cultivable despite slightly slower growth rates and cell numbers. Immunostaining confirmed that monocultures as well as ALI cultures display an epithelial cell phenotype and contain goblet cells.

Earlier work on this topic from Stokes *et al*. comparing three different isolation methods of HNECs and could show that nasal curettages are a suitable method to generate nasal epithelial cell cultures (Stokes et al, 2014). However, they also found that nasal brushes were the most successful isolation method because of the highest cell count obtained, and fastest growth rate. Yet, in the present study similar cell numbers using the nasal curettage technique were obtained when compared to those stated by Stokes *et al*., obtained with nasal brushes. Moreover, Stokes *et al.* found that cell viability was similar between the different methods used. Even though they also observed that nasal curettages were the most unpleasant method (Stokes et al, 2014), study participants of the present study did not report discomfort during the curettage procedure. It was concluded that even though the nasal curettage technique might be inferior to nasal brushing brushing in the setting used by Stokes et al., using mitomycine-arrested murine fibroblasts at least accounts or even improves the method suggested by Stokes *et al.*

Confimation of epithelial cell type

In order to confirm the epithelial cell type of the obtained cells, it was stained for pan-cytokeratin and cytokeratin-14. Both stainings demonstrated the typical pattern in all cells, lacking a staining of the nuclei but showing an extensive network of keratin filaments distributed

throughout the cytoplasm. Thus, the HNEC culture consisted of 100% epithelial cell type cells such as described after isolation via nasal brushing (Hussain et al, 2014).

Furthermore, immunofluorescence staining for β-IV-Tubulin, a marker for cilia on cells (Pathak & Drummond, 2009), as well as Mucin 5AC, a goblet cell marker (Inatomi et al, 1996) was carried out. Mucin 5AC staining was already carried out in monolayer cultures and found to be restricted to a small fraction of the cells and was expressed in the cytoplasm which partly expanded to the plasma membrane, marking them as goblet cells. β-IV-tubulin was stained in successfully established ALI cultures and showed a regular distribution which was apparent in 60-70 % of the cells marking them as ciliated cells in the ALI cultures.

Scanning electron microscopy of the ALI cultures, however, only showed tightly joined large polygonal non-ciliated epithelial-type cells with the typical cobblestone morphology and polygonal cells with different sizes of microvilli that did not seem to be long enough to be ciliated cells, even though the presence of various ciliated cells was shown previously (Li et al, 2014; Park et al, 2016). Additionally, goblet cells having droplets of mucus attached to their surface were observed. HNECs isolated by curettages, grown in ALI cultures showed the ability to develop a tight barrier, as demonstrated by stable transepithelial electrical resistance. Already after seven days in culture at the air liquid interface, ALI cultures or HNECs showed TER values above 2000 Ω/cm^2 which stayed somewhat stable until 21 days after air lift where experiments were carried out.

Ability of barrier formation

Moreover, the ability of HNECs to form a tight barrier was observed by expression of tight-junctions. HNECs expressed E-Cadherin as well as Occludin and ZO-1 mRNA. Immunofluorescence staining confirmed the expression of Occludin and ZO-1 in HNEC cultures while staining of Claudin-1 was additionally observed. In literature, it has already been shown that the human nasal mucosa expressed Occludin, JAM-A, ZO-1 and Claudin-1, -4, -7 (Kojima et al, 2013).

Furthermore, it was observed that E-Cadherin and ZO-1 were decreased in the nasal epithelium of AR patients (Lee et al, 2016). Somewhat in coherence with these results, decreased mRNA expression of E-Cadherin, but also Occludin in HNECs derived from atopic donors was observed in this study. However, expression of ZO-1 mRNA showed a tendency towards a higher expression in atopic donors which was not significant. A previous study conducted on HNECs from nasal brushings (Park et al, 2016) reported morphological differences between cultures from non-atopic and atopic donors, with cultures from HNECs of AR patients containing lower numbers of ciliated cells and elevated numbers of secretory cells. In our study, we did not observe differences in numbers of Mucin 5AC positive cells (data not shown) and expression levels of Mucin 5AC were comparable in HNECs from non-atopic and AR donors. However, it was observed that ALI cultures derived from HNECs from AR donors

showed decreased TER values at all time points which was expected since AR patients have been reported to suffer from increased barrier permeability (Fukuoka & Yoshimoto, 2018; Steelant et al, 2016; Toppila-Salmi et al, 2015).

In conclusion, this study shows that minimally-invasive nasal curettage is a suitable method to harvest nasal cytology specimen. Especially cultivation of these cells on mitomycinated mouse fibroblasts showed to be an efficient way in order to cultivate sufficient amounts HNECs of both non-atopic and atopic donors. Moreover, cells can be enough expanded to establish ALI cultures displaying the expected histologic morphologies and physiologic features of a 3D epidermal model which will expedite the clinical application of *in vitro* studies for the investigation of molecular mechanisms and the role of the epithelium in allergic disease development.

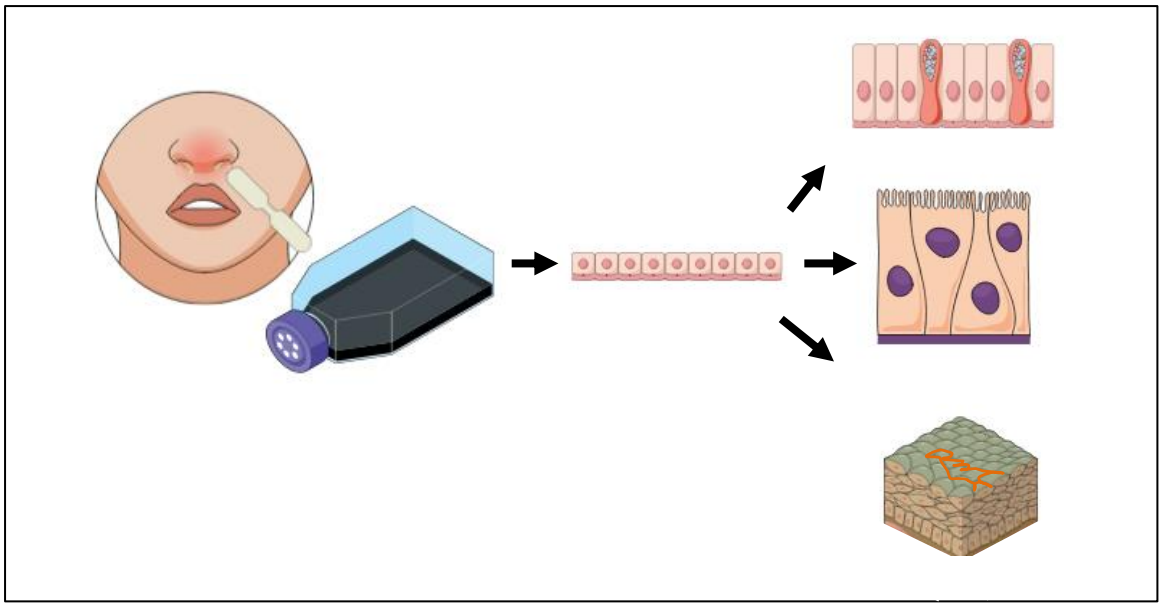

Figure 38: Cells isolated from nasal curettages are culturable HNECs and show differentiation ability
HNECs obtained from the curettages were shown to be of epithelial cell type and to be able to differentiate to goblet and ciliated cells while being tightly joined via tight-junctions.
Figure was created using mindthegraph.com

5.2 Characterization of nasal epithelial cells derived from donors with different atopy status

It becomes more and more obvious that exposure to microbes in early life is important to establish immune homeostasis which influences reaction capacity and allergy development later in life. ECs are the first cells to encounter these microbes and might therefore play a major role when it comes to allergic disease susceptibility, development and immune modulation. Therefore, the investigation of differences of immune phenotypes and responses of primary HNECs between non-atopic and atopic donor derived cells is important to gain insights for allergy development.

5.2.1 Barrier function in response to allergenic substances

Observation of TER levels of ALI cultures treated with birch, ragweed and timothy grass pollen extracts but also with HDM extract revealed that in cultures derived from atopic donors the TER increases over time in response to the named stimuli. Especially, HDM extract, Phl-and Amb-APE were potent inducers of epithelial resistance. In line with literature data, this was not observable in ALI cultures derived from non- atopic donors. Hence, cells from non-atopic donors seem not to react at all to pollen extracts while cells from AR patients, which should recognize pollen as danger signal, react to this signal with an increase of barrier integrity. Previous studies reported a disrupted barrier and barrier malfunction in allergic pollen and HDM allergic patients (Lee et al, 2016; Steelant et al, 2016). Moreover, especially studies using HDM extracts showed increased barrier permeability after treatment with HDM (Herbert et al, 1995; Winton et al, 1998). Different HDM extracts vary extensively in their biochemical properties which might be one explanation why the here used HDM extract rather induced barrier integrity than reducing it. However, Blume *et al.* reported comparable results in bronchial epithelial cells which were exposed to extracts of various pollen types, such as timothy grass, ragweed, mugwort, birch and pine. Similar to our results, all pollen types induced TER, with timothy grass pollen being the most potent of all pollen types. Adenosine, previously shown to be a major constituent of low molecular weight pollen fractions, contributed to the effect of pollen on barrier permeability, which was in part mediated by JNK (Blume et al, 2015).

5.2.2 Innate immune receptor repertoire of nasal epithelial cells derived from donor with different atopy status

As recent studies indicate innate immune recognition may play an important role in the development of allergic diseases, e.g. genome-wide association studies have shown that loci in TLR-1, -6, -10, STAT6, IL1RL1 regions are associated with allergic diseases (Bonnelykke et al, 2013; Hinds et al, 2013). In the current study, it is shown that HNECs express TLR-1-6 and -9 on mRNA level and especially TLR-3 and -9 on protein level, which is in concordance with previous reports (Renkonen et al, 2015; Tengroth et al, 2014; van Tongeren et al, 2015).

This expression pattern seems to be similar in epithelial cells of the lower airways. A study investigating the expression of TLRs in tracheal epithelial cells observed expression of TLR-1-7, -9 and -10, but also no expression of TLR-8. Immunohistochemistry revealed that TLR-3 was expressed on the apical and basolateral surface of the cells while TLR-1, -4, -5, -7, -9 and -10 were mostly expressed on the luminal surface. TLR-2 and -6 were basolaterally distributed. (Ioannidis et al, 2013).

In the present study, TLR -4 and -9 expression was lower on mRNA levels in HNECs from AR donors, while on protein level only TLR-9 expression was reduced in cells of AR patients when compared to non-atopic donors. Lauriello et al. also observed a reduced expression of TLR-4 and TLR-9 in the nasal mucosa of AR patients when compared to non-atopic donors. The authors concluded that this difference in expression of innate immune receptors might be related to type and severity of the disease (Lauriello et al, 2012). Hence, rather high donor variability might be due to different disease status of the patients which cells were used. Furthermore, in the present work, a tendency for a decreased expression of the TLR adaptor molecule TRIF was observed in HNECs derived from AR donors.

The combination of a reduced TLR-9 and TRIF expression might have further consequences. The TLR-9 ligand CpG is now being used as a therapeutic approach for allergies. Interestingly, it was shown that TLR-9 ligation by CpG results in physical association of TLR-9, TRIF, TRAF-6 and downstream activation of NF-κB, which, in turn, induces IRF-3 and TGF-β-dependent immune suppressive tryptophan catabolism, possibly protecting against allergic inflammation (Volpi et al, 2013) Hence, lack of TLR-9 and TRIF in AR patients might abrogate this protective effect. Moreover, usage of a ragweed pollen allergen coupled to a TLR-9 ligand during allergen immunotherapy was shown to improve several AR-related scores and clinical parameters (Creticos et al, 2006).

Furthermore, it was shown in literature that allergens influence the repertoire of innate immune receptors in allergic persons. For example, in nasal biopsies, obtained before and after allergy season, an increase of TLR-2, -3, and -4 was observed in patients during pollen season. However, this rise was only statistically significant for TLR-3 and, in addition, different sets of patients and subjects were investigated outside and inside the pollen season, and high inter-individual variation is likely (Fransson et al, 2005). Another study observed, that while non-atopic control and atopic donors showed similar expression of TLR proteins at baseline, off-seasonal intranasal birch pollen challenge down-regulated the expression score of TLR-1 and -6, only in atopic donors (Renkonen et al, 2015). In contrast to this data, in the present study, stimulation of HNECs with the respective TLR ligands did not strongly influence TLR and TLR adaptor protein expression. Only TLR-3 mRNA was significantly down-regulated by PolyI:C in both donor types and MyD88 mRNA by Flagellin in non-atopic donor derived HNECs.

5.2.3 Inflammatory cytokine release and inflammasome-related gene expression

TLR signaling in respiratory epithelial cells results in cytokine production, which promotes recruitment of phagocytic cells participating in pathogen clearance. Accordingly, it could be shown in the current study that stimulation with, particularly, the TLR ligand PolyI:C but also CpG resulted in up-regulation of GM-CSF, CCL-2, IL-8, CCL-20, CXCL-10, CCL-5, IL-33, IL-18 and CCL-22. Tengroth *et al.* observed similar results after stimulation of nasal epithelial cell lines with PolyI:C and CpG. PolyI:C up-regulated the release of IL-6 and GM-CSF while CpG was a potent inducer of IL-8 (Tengroth et al, 2014). In another study stimulation of primary HNECs with PolyI:C resulted in up-regulation of IL-4, IL-6, RANTES, IP-10, MIP-1β, VEGF, FGF, IL-1RA, IL-2R and G-CSF. Similar to the results obtained in the present study, LPS failed to induce statistically significant increases of cytokine levels. Of note, strong variation between TLR ligand-induced cytokine release was observed which is in concordance with our results (van Tongeren et al, 2015). Hewson *et al.* showed that blocking of TLR-3 abrogated the effect of PolyI:C on IL-6, IL-8 and CCL-5 release indicating that PolyI:C indeed induces this cytokine release via TLR-3 activation (Hewson et al, 2005).

5.2.3.1 CCL-5

CCL-5 was not only strongly up-regulated by PolyI:C but also stimulation with pollen extracts affected the release of CCL-5 by HNECs. Of note, however, Bet-APE significantly reduced the release of CCL-5 when compared to the control. These findings are in concordance with previously published work. Intranasal administration of PolyI:C was shown to induce CCL-5 in mice. The authors concluded that viral infections could contribute to exacerbation of respiratory diseases (Stowell et al, 2009). This if of interest, since it could be demonstrated that CCL-5 was elevated in the nasal wash of severe AR patients (El Sharkawy et al, 2011) but also in the serum of insect venom AR patients. Immunotherapy was shown to reduce these elevated levels in the insect venom AR patients (Gawlik et al, 2015). Moreover, it was shown that birch pollen extract significantly reduces the release of LPS- induced CXCL-10 and CCL-5, both Th1-favouring chemokines. On the other hand, Th2 favouring chemokines such as CCL-22 were up-regulated, thereby increasing Th2 cell recruitment after contact to pollen (Mariani et al, 2007). In other studies however, it was shown that grass pollen extract rather induces CCL-5, CCL-3 and CCL-4. Interestingly, this was only observable when the epithelial cell line was stimulated with the pollen extract; the purified allergen did not provoke this effect but rather stimulated the release of G-CSF, which was not seen with the pollen extract (Roschmann et al, 2012).

5.2.3.2 IL-8

In the current study, IL-8 was strongly induced in response to PolyI:C. Similar to CCL-5 induction, stimulation of IL-8 release by TLR-3 ligands such as viruses could contribute to exacerbation of allergy symptoms. Indeed, in an alveolar epithelial cell line, it was shown that

simultaneous stimulation with human rhinovirus (HRV), a natural TLR-3 ligand, and the HDM allergen Der f1 resulted in an additive effect on IL-8 secretion (10.4168/aair.2013.5.4.216) while allergen challenge after virus infection further increased IL-8 release in seasonal AR patients out of pollen season (Greiff et al, 1999).

Furthermore, this work shows that pollen extracts induce IL-8 secretion in HNECs, however, only Phl-APE in non-atopic donor derived HNECs. These results are in concordance with a previous study showing that Phl p1, one allergen of timothy grass pollen, is able to induce IL-8, but also IL-6 and TGF-β mRNA and release in respiratory epithelial cells (Roschmann et al, 2009). Furthermore, in asthma, it was shown that pollen contact to the airways results in TLR-4 and MD-2 activation leading to high levels of IL-8 and consequently recruitment of neutrophils (Hosoki et al, 2016). In a murine model of allergic asthma, this effect happened very early on, as one of the first events after allergen challenge. Only later, eosinophil infiltration was visible (Lommatzsch et al, 2006). Repetition of this effect seems to facilitate allergic sensitization and airway inflammation (Hosoki et al, 2016). Similarly, it was shown that knockout of TLR-4 in mice results in inhibition of CXCR signalling-linked neutrophil recruitment by inhibition of NFκB activation, ultimately leading to reduced allergic airway inflammation (Hosoki et al, 2014). Hence, activation of TLR-4 by pollen extracts and subsequent release of IL-8 could be one major factor of initiation of allergic sensitization or airway inflammation. Previous work from our institute is further supporting this hypothesis, since it was shown that nasal allergen challenges, especially, when combined with low molecular weight compounds of pollen, induce local release of IL-8 in nasal lining fluid of allergic patients. Additionally, repetitive challenge with this mixture even induced local nasal IL-8 secretion in non-atopic control persons (Gilles-Stein et al, 2016) which could possibly lead to sensitization. Of note, a study investigated several thousand transcripts of grass pollen treated cells and found that the most profound gene changes were related to the cytokines IL-8, IL-6, IL-1α and the transcription factor FOS (Roschmann et al, 2011).

Blume *et al.* also observed that bronchial epithelial cells released GM-CSF, CCL-20 and IL-8 in response to *Phleum pratense* pollen, however, particularly in response to pollen-derived flavonoid isorhamnetin. Furthermore, they showed that blockage of p38 and ERK1/2 pathways abrogated this effect while inhibition of PPARγ rather induced IL-8 and GM-CSF release, possibly via interaction of PPARγ with NF-kB activity (Blume et al, 2013; Blume et al, 2015).

5.2.3.3 IL-1 cytokine family

Furthermore, HNECs of atopic donors secreted significantly higher levels of IL-33 and IL-18 than HNECs of non-atopic donors. lL-33 and IL-18 were both elevated at baseline in HNECs from atopic donors. While stimulation with different TLR ligands did not affect IL-33 release, IL-18 showed the tendency to be induced. Still IL-18 release showed a similar pattern when comparing non-atopic and atopic donors. Lin *et al.* stimulated pulmonary mucoepidermoid

carcinoma cells with LPS, PolyI:C and human parechovirus type 1. Both PolyI:C and the virus induced IL-33 release and mRNA expression in these cells via interaction with IRF-3 and NF-κB. Of note, LPS did not result in IL-33 induction but rather down-regulation, especially after long-term exposure. The authors therefore suggested that early and long-term exposure to microbial stimulants such as LPS modulated innate immune signalling and mitigates allergic responses; a conclusion which is in coherence with the hygiene hypothesis (Lin et al, 2016). Thus, lack of microbial Th1 stimuli which was shown to favour allergy development in infants, could result in elevated levels of IL-33, in turn favouring Th2 immunity. Especially since IL-33 was shown to be crucial for the induction of both early- and late-phase AR responses in a HDM- and ragweed-induced murine AR models (Haenuki et al, 2012; Nakanishi et al, 2013).

Moreover, IL-33 is one of two growth factors for ILC2s and activates these immune cells to produce Th2 signature cytokines (Camelo et al, 2017; Monticelli et al, 2015; Rak et al, 2016). Additionally, IL-33 activates NF-κB and MAP kinases in Th2 cells driving the further production of Th2-associated cytokines such as IL-4, IL-5 and IL-13 (Schmitz et al, 2005), thus fostering an allergy-type immune response. Differentiation towards this immune response type is additionally thought to be driven by inflammasome-related cytokines such as IL-18 in the absence of a Th1 stimulus such as IL-12 (Nakanishi et al, 2001).

IL-33, IL-18, but also IL-1α and –β belong to the IL-1 cytokine family which is partly regulated by the inflammasome with important functions in immune regulation and inflammation. In keratinocytes, HDM extract but also pollen were shown to activate the inflammasome (Dai et al, 2011; Dittlein et al, 2016), thereby possibly exerting their effects. Thus, elevated inflammasome activation may be an important factor for allergen recognition. Moreover, IL-33 was not only found to be increased in the epithelium of AR patients, but also in the serum (Kamekura et al, 2012) and baseline IL-33 mRNA was shown to correlate with late-phase allergic responses (Leaker et al, 2017). Moreover, IL-33 was constantly expressed in HNECs of a ragweed-induced murine AR model and ragweed stimulation resulted in IL-33 release into nasal fluids (Haenuki et al, 2012).

Further in line with these observations, is the increase of IL-18 after stimulation with Phl-APE in both donor types which, again, is in accordance with results obtained in keratinocytes (Dittlein et al, 2016). Verhaeghe *et al.* observed elevated IL-18 levels in nasal secretions of AR patients outside of pollen season. Moreover, levels of IL-18 and IL-1α increased further parallel to the pollen season and stayed elevated for up to four weeks after the last pollen peak (Verhaeghe et al, 2002). IL-33 and IL-1β, however, were not affected by stimulation with pollen in the current setting. Only baseline levels of IL-33 of atopic donors were again elevated.

Additionally, it was shown that the late phase of an allergic reaction parallels changes of not only Th2-related cytokine genes but also complement and inflammasome-related genes such as IL-1β (Leaker et al, 2017). When stimulating HNECs from AR patients with the TLR-3 ligand,

PolyI:C, and nigericin, a particularly pronounced release of IL-1β in three of the six patients was observed in the current study. Unfortunately, no specific pattern could be found distinguishing these three patients from the others, except for a rather reduced IgE-level which would be in contrast to previously published data showing elevated IgE and IL-1β serum levels in allergics (Thomas & Chhabra, 2003).

Interestingly, stimulation with TLR ligands and/or inflammasome stimuli did not induce further IL-33 or IL-18 release but levels were unchanged, yet higher than in HNECs derived from non-atopics.

When the expression of inflammasome-related genes such as the NLRP inflammasomes 1 and 3, AIM-2 and Caspase-1 was furthermore investigated, differences in expression between non-atopic and atopic donor derived HNECs were observed. Of note, the NLRP-3 inflammasome as well as AIM-2 and Caspase-1 were significantly decreased in HNECs derived from AR patients while stimulation did not strongly affect the expression of these genes except for a reducing effect of Pam3Cys on AIM-2. Madouri *et al.* witnessed augmented airway inflammation with enhanced IL-33 secretion in Caspase-1-deficient mice which were exposed to HDM. Interestingly, IL-33 inhibition resulted in improvement of airway inflammation. Furthermore, mice deficient in NLRP-3 showed increased eosinophil influx and increased levels of Th2 cytokines and chemokines (Madouri et al, 2015).

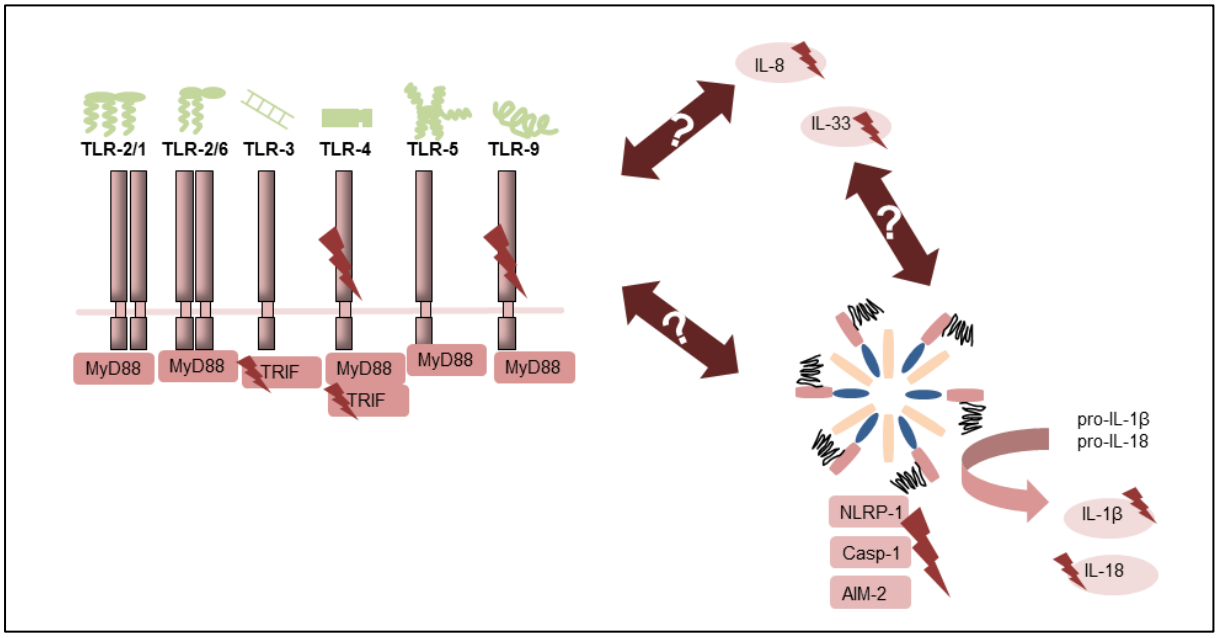

Figure 39: HNECs from atopic donors display different innate immune receptor repertoire and inflammatory response
HNECs obtained from the curettages derived from non-atopic and atopic donors were compared with respect to their TLR and inflammasome-releated gene expression. HNECs derived from atopic donors displayed reduced expression of TLR-9 and -4 (only on mRNA level) and reduced mRNA expression of NLRP-1, Caspase-1 and AIM-2. HNECs from atopic donors showed a different inflammatory immune response, especially for IL-8, IL-18, IL-33 and IL-1β release. This results in the hypothesis that possibly altered innate immune priming early in life may result in disturbed innate immune receptor signaling possibly leading to a dysbalanced immune homeostasis later in life.

5.3 Direct effect of non-digestible oligosaccharides, lactic acid bacteria and active microbial compounds on nasal epithelial cells

Early life interaction of the body and diverse microbes likely establishes immune homeostasis including the activation of pattern recognition receptors and downstream signaling pathways. This immune homeostasis influences the reaction capacity later in life by education of the immune system to induce tolerance.

In allergy, a human´s genetic background may drive more or less susceptibility but environmental and life style factors which also include contact to microbes may also induce modifications in susceptibility to the development of allergic diseases. It has been shown that the use of prebiotics and probiotics may have beneficial effects on AR disease symptoms (Vliagoftis et al, 2008), possibly by restoring dysbalanced immunity. Therefore, intervention with prebiotics and probiotics or active microbial structures was tested for the ability to restore the immune and/or barrier homeostasis in HNECs derived from AR donors when compared to non-atopic donors.

5.3.1 Cytokine release

Cytokine release at baseline

In the present study, HNECs were shown to release CCL-2, CCL-5, IL-8, CXCL-10, CCL-20 and GM-CSF at baseline. In line with this, Abdelaziz *et al.* previously observed constitutive release of CCL-5, IL-8 and GM-CSF in primary HNECs (Abdelaziz et al, 1998).

While levels of IL-8, CXCL-10, CCL-20 and GM-CSF were similar in supernatants of HNECs of both donor types, only HNECs of AR donors exhibited a baseline release of CCL-2 and CCL-5. Apart from that, however, no profound differences were found in baseline chemokine release from HNECs of non-atopic and AR donors.

A previous study showed that HNECs release distinct cytokine and chemokine profiles depending on atopy status. Specifically, HNECs from AR individuals released significantly higher levels of IL-8, GM-CSF, CCL-5 and TNF-α when compared to cells from non-atopic donors (Calderon et al, 1997). The high donor-to-donor variability observed in the present study might explain the absence of marked atopy status-specific differences in mediator release. However, we did observe increased IL-1 family cytokine production in HNECs of AR donors as compared to non-atopic donors (IL-18 and IL-33 at baseline and under TLR-stimulation; IL-1β after inflammasome stimulation). IL-1 family cytokines are associated with inflammasome activation and in the absence of a Th1 stimulus such as IL-12, these cytokines are thought to favour Th2 differentiation (Nakanishi et al., 2001).

Cytokine release after stimulation

Stimulation of the HNECs with the proinflammatory/Th1-cytokine cocktail, IFN-γ/TNF-α, resulted in up-regulation of CCL-2, CCL-5, IL-8, CXCL-10 and GM-CSF. This agrees well with

two previous studies demonstrating that TNF-α, IL-1β, IFN-γ as well as the combination of IFN-γ/TNF-α induced CCL-5 release in HNECs in a time-dependent manner (Altman et al, 1997; Terada et al, 1996).

Of note, TLR-3 appeared as the most potent stimulus for induction of cytokine- and chemokine release by HNECs in our study. This was demonstrated by PolyI:C treatment which resulted in increased secretion of GM-CSF and CCL-20 and strong increases of IL-8 and CXCL-10 release. In agreement with this, stimulation with PolyI:C was previously shown to result in increased release of IL-6 and GM-CSF by the nasal mucosa and by epithelial cells lines (Tengroth et al, 2014).

Stimulation of HNECs with aqueous pollen extracts (APEs) led to down-regulation of baseline CXCL-10, a weak increase in GM-CSF and a marked up-regulation of IL-8 levels. For these effects, there were no statistically significant differences between HNECs derived from non-atopic or AR donors. In the literature there is only one report on cytokine- and chemokine responses in HNECs derived from donors with defined atopy status on protein level (Calderon et al., 1997). In this study, cells were not subjected to *in vitro* stimulation with pollen. Instead, biopsies of AR donors, from which the HNECs were cultivated, were obtained either before or after the pollen season. Here, levels of IL-8, CCL-5, TNF-α and IL-1β were increased in cells cultured from AR explants, and cells from explants of pollen-exposed AR individuals showed even increased cytokine levels (Calderon et al., 1997).

There are several studies that analyzed nasal cytokine release in AR subjects under natural pollen exposure or upon experimental allergen challenge. For instance, in AR patients, nasal CCL-2 levels increased and IL-8 levels decreased within the pollen season as compared to out-of-season levels (Kuna et al, 1996). This seems to be in contrast to previous results from our own group. Specifically, repetitive nasal challenges of Bet-APE in birch pollen allergic AR patients and healthy control subjects resulted in enhanced nasal IL-8 release (Gilles-Stein et al., 2016). Likewise, stimulation of bronchial ECs, both in monocultures and in ALI models, with different pollen resulted in up-regulation of IL-8 levels (Blume et al., 2015; Wimmer et al., 2015). In these studies, IL-8 induction was shown to depend on the pollen-derived flavonoid isorhamnetin for grass pollen (Blume et al., 2015) and on adenosine for ragweed pollen (Wimmer et al., 2015).

Cytokine release after intervention with prebiotics, probiotics and active microbial structures

Treatment of HNECs from non-atopics with scGOS/lcFOS and/or *IMS1*, *L. rhamnosus* during stimulation with IFN-γ, TNF-α resulted in significant reduction of CCL-5 and CXCL-10. In contrast, only the incubation with *IMS1* could provoke the decrease of CXCL-10 release in HNECs from AR patients.

In ulcerative colitis patients, a bacteria mixture, VSL#3, consisting of eight LAB strains (*L. acidophilus, L. bulgaricus, L. casei, L. plantarum, Streptococcus thermophilus, B. breve, B.*

infantis and B. longum) was shown to reduce the levels of IL-1β, IL-8 as well as IFN-γ (Lammers et al, 2005). Additionally, this probiotic mixture was demonstrated to reduce CXCL-10 via post-translational degradation (Hoermannsperger et al, 2009). Later on, Hörmannsperger *et al.* found that the protective mechanism of VSL#3 in murine inflammatory bowel disease models was the *L. casei/paracasei*-produced lactocepin which selectively degrades pro-inflammatory chemokines (Hormannsperger et al, 2013; von Schillde et al, 2012). Since the authors also suggested an effect in allergic inflammation, lactocepin was investigated as possible beneficial factor in our EC models. Indeed, intervention with lactocepin when stimulated with IFN-γ/TNF-α resulted in reduction of the release of CCL-5, CXCL-10 and IL-8 in non-atopics and in HNECs from AR patients.

Addition of scGOS/lcFOS during stimulation of HNECs with PolyI:C or pollen extracts did not yield any significant changes. However, scGOS/lcFOS showed a tendency to increase cytokine production in non-atopics (GM-CSF) and HNECs from AR patients (IL-8) when treated with PolyI:C.

In conclusion, treatment of HNECs with scGOS/lcFOS and its combination with the two LAB strains or lactocepin seems to partially have the capacity of reducing the release of chemokines that provoke a Th1 response in response to some stimuli. Moreover, the reducing effect on CCL-5 and CXCL-10 was again more pronounced in HNECs derived from healthy subjects when compared to AR patients.

The partial lack of effectiveness of scGOS/lcFOS and the two LAB strains in cells derived from atopic donors and in response to some stimuli might arise from a lack of TLR-9 signaling. In a murine model of DSS-induced colitis; it was shown that mice deficient in MyD88 and TLR-9 did not respond to probiotics while colitis was significantly improved in mice deficient in TLR-2 and -4 (Rachmilewitz et al, 2004). Similarly, plain yogurt was shown to exhibit anti-inflammatory properties via decrease of TLR-4 and increase of TLR-9 expressing cells, thereby regulating T-cell expansion in a model of intestinal inflammation (Chaves et al, 2011).

There are only very little studies investigating the direct effect of prebiotics and probiotics on the nasal epithelium. However, there are clinical studies showing beneficial effects of prebiotics and probiotics on nasal immunity and allergy. One clinical trial giving a dairy drink containing *L. casei* Shirota to its patients daily, reported that the intervention resulted in increased levels of $CD86^+$ epithelial cells, sIL-1RII release and reduced IL-1β levels in the nasal mucosa of the patients when compared to the control group. Moreover, the intervention suppressed TGF-β production and increased IFN-γ levels in the blood (Ivory et al, 2013). Supplementation with *L. casei* DN-114 001 resulted in a prophylactic effect on the incidence of allergic rhinitis in children (Giovannini et al, 2007) while *B. longum* BB536 was shown to improve the symptoms of Japanese cedar-pollen allergy (Xiao et al, 2006). In contrast, however, in a European study

supplementation of *L. rhamnosus* GG did not yield any beneficial results in birch-pollen allergic patients (Helin et al, 2002).

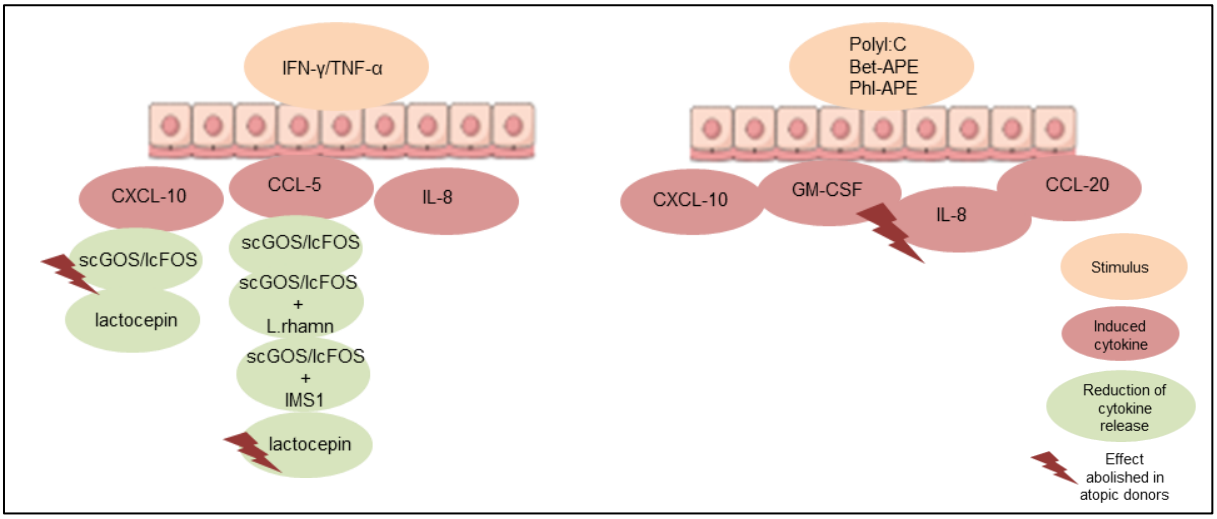

Figure 40: HNECs respond to treatment with prebiotics and probiotics
HNECs obtained from the curettages derived from non-atopic and atopic donors responded with reduced release of CXCL-10 and CCL-5 to treatment with prebiotics, probiotics and active microbial structures, however only after stimulation with IFN-γ/TNF-α. Treatment had no effect on Polyl:C or APE stimulated cells. The effect of scGOS/lcFOS and lactocepin on CXCL-10 and CCL5 were abolished in HNECs derived from atopic donors.

5.3.2 Barrier function

Evidence for barrier malfunctions in diseases of the upper and lower airways has only been recently found. In asthmatics, ZO-1, occludin and β-catenin expression is disturbed (de Boer et al, 2008; Xiao et al, 2011). In chronic rhinosinusitis with or without nasal polyps, the epithelium is also more permeable due to a lack of occluding and claudin-4 expression (Soyka et al, 2012). Finally, it was shown that AR patients and murine models of HDM induced allergic airway inflammation display a disturbed barrier function caused by decreased expression of ZO-1 and occludin (Steelant et al, 2016). Therefore in the present study, it was investigated if prebiotics and probiotics might have a beneficial effect on barrier tightness of HNECs.

Indeed, in HNECs from non-atopic donors, *IMS1* and the combination of scGOS/lcFOS and *L. rhamnosus* increased TER values. However, only the combination of scGOS/lcFOS and *L. rhamnosus* resulted in a significant rise of TER at 6 hours. In HNECs from atopic donors, scGOS/lcFOS and/ or the different LAB strains did not yield any increase in TER but rather a decrease in barrier resistance. In conclusion, scGOS/lcFOS and its combination with the here stated, specific LAB strains could reinforce barrier function in HNECs from non-atopic donors and might therefore be used for preventive approaches. However the compounds failed to show a therapeutic effect in cells derived from AR patients. Unfortunately these results cannot be compared to other evidence since no other literature resutls for the treatment of barrier malfunction in AR with prebiotics or probiotics were found.

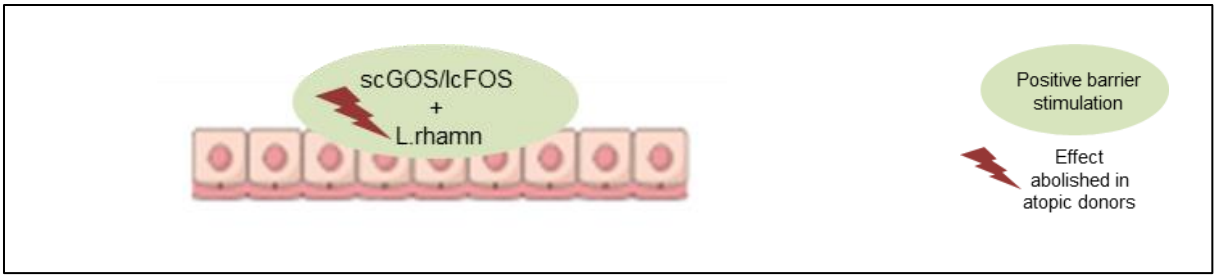

Figure 41: Only transepithelial electrical resistance of HNECs derived from non-atopic donors benefited from prebiotic and probiotic treatment
HNECs obtained from the curettages derived from non-atopic donors responded with improved TER to treatment with prebiotics and probiotics. This effect was not observable in HNECs derived from atopic donors.

5.4 Direct effect of non-digestible oligosaccharides, lactic acid bacteria and active microbial compounds on keratinocytes

Similar to AR, AE is also known to be a disease of barrier disruption, immune disturbance and microbial dysbiosis. Therefore it was tested if the here used prebiotics, probiotics and active microbial structures might induce the same effects in a similar epithelial cell model.

5.4.1 Cytokine release

Non-atopic individuals and AE patients differ in their cytokine milieu on the skin and blood. It has already been shown that AE patients have significantly increased levels of CCL-22, CXCL-12 and CCL-17 in their sera while CXCL-9, CXCL-10, CCL-17, and IL-18 were decreased when compared to non-allergic individuals (Narbutt et al, 2009). Furthermore, CCL-20 was abundantly found in the basal layer of lesional epidermis, induced by barrier disruption, but only weakly expressed in normal skin (Narbutt et al, 2009; Schmuth et al, 2002). Moreover, it was shown that the gene expression of IL-2R, IL-5, IL-.6, IL-8, IL-12B, IL-10, IL-23, IL-29 and TGF-β is increased in AE skin when compared to skin of non-atopic donors (Fedenko et al, 2011).

Cytokine release at baseline and after stimulation

In the present study, the release of CCL-2, CCL-5, IL-8, CXCL-10, CCL-20 and CCL-22 at baseline and after cytokine stimulation was investigated in human primary keratinocytes (KCs). KCs from AE patients do not seem to have an exaggerated response or lack of these chemokines when compared to KCs from non-atopic donors. However, it is possible that variation of the stimuli, the time points or the targets used in the present study would lead to a different outcome.

Moreover, KCs derived from non-lesional skin of AE patients were used which could possibly account for the lack of significant differences in chemokine response in comparison to KCs from non-atopic donors. It was shown that non-lesional skin differs from non-atopic healthy skin but is also distinct from lesional skin (Polanska et al, 2013); not only when it comes to physiological measures but also concerning its microbiome (Altunbulakli et al, 2018b; Matsui

et al, 2000). Therefore, it would be of interest for future experiments to take lesional skin derived KCs.

In contrast to the present results, a study from Giustizieri *et al.* measured IL-8 mRNA expression only at baseline in KCs derived from non-atopic donors. Furthermore, in their setting in KCs from non-atopic donors, stimulation with IFN-γ induced the expression of CCL-2 and CXCL-10 while TNF-α resulted in up-regulation of IL-8 and CCL-5. IL-4 treatment resulted in CXCL-10, CCL-5 and IL-8 expression while CCL-2 was not detectable. In KCs from AE patients, the chemokine responses were earlier and significantly higher, particularly for CCL-5, standing in contrast to the results observed in the current study. Additionally, mRNA of CCL-5 and CCL-2 was abundantly identified (Giustizieri et al, 2001). Giustizieri *et al.* also used non-lesional KCs but higher doses of the cytokines which might be a cause for the different results.

In agreement with the present results, however, another study showed the induction of CCL-22 by IL-13 in KCs derived from AE patients. CCL-22 release resulted in a higher chemotactic activity on T cells in migration assays with KCs from AE patients than non-atopic donors (Purwar et al, 2006).

Cytokine release after intervention with prebiotics, probiotics and active microbial structures

Treatment of KCs with scGOS/lcFOS and/or *IMS1*, *L. rhamnosus* during the cytokine stimulation induced a tendency for a reduced chemokine response in KCs from non-atopic donors for all conditions. Only IL-4/IL-13 induced CCL-22 release was not affected at all. The combination of scGOS/lcFOS and *L. rhamnosus* was most beneficial and reduced the IFN-γ/TNF-α induced release of CXCL-10 and CCL-5 significantly in KCs of non-atopic donors, while these effects were completely absent in KCs derived from AE patients. In contrast, with the exception of CCL-22, all of the measured cytokines were rather induced in AE KCs by the different treatment conditions after IFN-γ/TNF-α as well as IL-4/IL-13 stimulation. Thus, scGOS/lcFOS, especially in combination with the LAB, seems to have direct anti-inflammatory effects on KCs derived from non-atopic donors while in KCs derived from AE patients, scGOS/lcFOS and the LAB strains rather seem to induce an inflammatory chemokine response.

There are only very little studies investigating the effect of pre- and probiotics on skin and especially skin KCs. However, one experimental study could show that LAB strains reduce the TNF-α induced thymus and activation-regulated chemokine (TARC), a T helper 2 (Th2) cell chemokine important for the pathomechanism of AE, release in KCs. Particularly, the *L. reuteri* strain, Japan Collection of Microorganisms 1112, was most suppressive and dependent on TLR-2 in HaCaTs. Furthermore, the same study showed in a murine model that this LAB strain reduced the development of HDM-induced atopic skin lesions (Kawahara et al, 2018).

In the present study, the release of TARC was not observed but chemokines such as CCL-22, favoring a Th2 response, were not affected by treatment with our LAB strains.

Liu *et al.* observed that three different strains of *L. reuteri* (ATCC PTA4659, ATCC PTA 5289, and ATCC PTA 6475) suppressed the LPS-induced IL-8 production in cultured intestinal cells when given orally. Moreover, the strains induced IFN-γ and inhibited LPS-induced intestinal histological damage (Liu et al, 2010).

KCs derived from non-atopic donors treated with lactocepin while stimulated with IFN-γ/TNF-α decreased the measurable release of of CCL-2, CCL-5 and CXCL-10 while only levels of CCL-2 and CXCL-10 were reduced in supernatants of KCs from AE patients. Hörmannsperger *et al.* examined several cytokines, chemokines and junction proteins for cleavage sites for lactocepin and found that lactocepin demonstrated low cleavage site specificity but highly selective for an array of murine pro-inflammatory chemokines (CXCL-9, CXCL-10, CXCL-11, CXCL-12, CX3CL-1, CCL-11). Somewhat in accordance with the present data in non-atopic donor derived KCs, they also showed that IL-8 was not affected by lactocepin (von Schillde et al, 2012). However, the present study showed increased IL-8 levels in response to lactocepin in KCs derived from AE patients.

Moreover, Hörmannsperger *et al.* did not observe an effect of lactocepin on CCL-20. Nevertheless, in the present study, CCL-20 showed a tendency for effective reduction by lactocepin. CCL-5 was degraded in both KCs from non-atopic and AE donors while this chemokine shows no cleavage site or specificity for lactocepin. Thus, lactocepin could also act via other mechanism than post-translational cleavage of chemokines or affect human cytokines and chemokines differently.

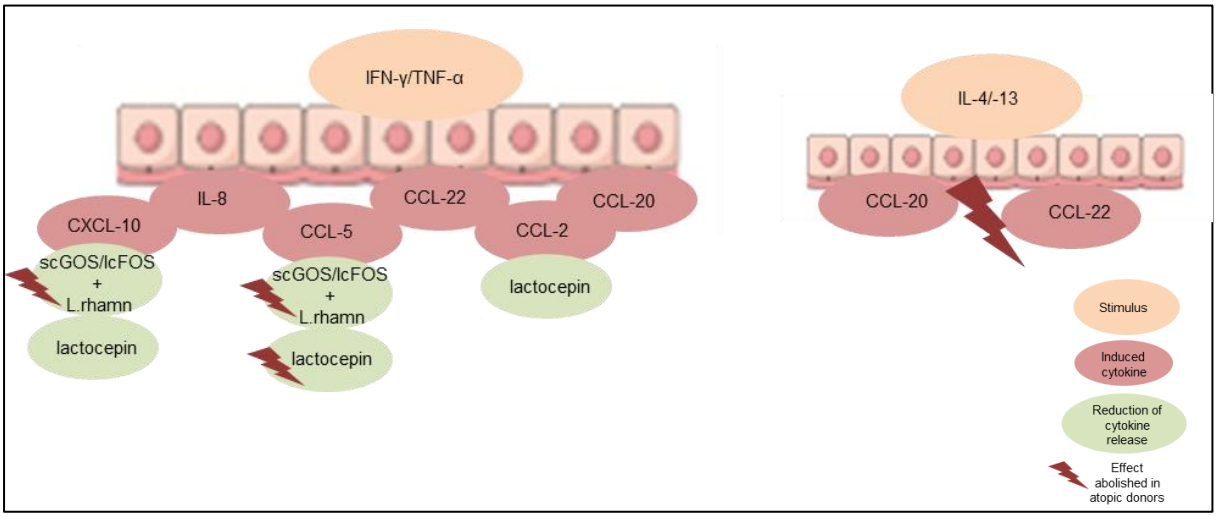

Figure 42: Keratinocytes respond to treatment with prebiotics and probiotics
Keratinocytes derived from non-atopic and AE donors responded with reduced release of CXCL-10, CCL-5 and CCL-2 to treatment with prebiotics, probiotics and active microbial structures, however only after stimulation with IFN-γ/TNF-α. Treatment had no effect on IL-4/-13 stimulated cells. The effect of scGOS/lcFOS plus *L. rhamnosus* and lactocepin on CXCL-10 and CCL5 were abolished in keratinocytes derived from AE patients.

5.4.2 Barrier function

Atopic eczema is a disease with barrier malfunction. Not only FLG mutations but also defects in TJ expression result in more permeability of the skin, leaving it unprotected to environmental and microbial insults. Therefore, especially probiotics have been used as alternative therapeutic approaches for barrier restoration.

In the present study, stimulation with the combination of scGOS/lcFOS and both *IMS1* and *L. rhamnosus* strains resulted in increased TER values in KCs derived from healthy donors. However, in KCs from AE patients, only scGOS/lcFOS slightly increased the TER and the effect was slower in comparison to the increase observed on KCs derived from healthy donors. In conclusion, scGOS/lcFOS and its combination with the here stated, specific LAB strains could reinforce barrier function and might be used for preventive approaches but does not seem to have a therapeutic effect. Lactocepin did not have any effects on TER development in the conducted experiments indicating that it has no direct effect on TJs or barrier influencing molecules under non-inflammatory conditions.

In agreement with our finding that probiotics exert potential positive effects on healthy skin barrier, it was previously shown that KCs derived from healthy subjects that had been experimentally scarred in an *in vitro* scratch assay showed increased the re-epithelialization and wound closure after treatment with *L. rhamnosus* and *reuteri* (Mohammedsaeed et al, 2015). Moreover, *Streptococcus thermophilus* increased ceramide production by HaCaTs *in vitro* and when topically applied as a cream (Di Marzio et al, 1999).

In contrast to our results, previous studies have demonstrated beneficial effects of probiotics in AE skin models. These conflicting results are most likely explained by differences in test model systems, modes of application, and readouts.

For instance, beneficial effects of *L. paracasei* were demonstrated for *in vitro* skin models of barrier function recovery, as well as by oral supplementation (Gueniche et al, 2010b; Philippe et al, 2011). Similarly, *B. longum*, applied in a cream, decreased the skin sensitivity score and strengthened the natural barrier in an *in vitro* inflammatory organ culture of human skin (Gueniche et al, 2010a). Furthermore, functional fermented milk was shown to improve the stratum corneum barrier function measured by transepidermal water loss after 6 weeks of oral consumption, an effect that was dependent on the body mass index of the patients (Puch et al, 2008).

To conclude, the pre-/pro- and symbiotic regimes tested in the present study, although largely ineffective on AE skin models under the conditions tested here, could prove to be beneficial in other test systems, e.g. in experimental wound assays. Alternatively, they could be effective if applied directly on AE skin *in vivo*, or supplemented in food.

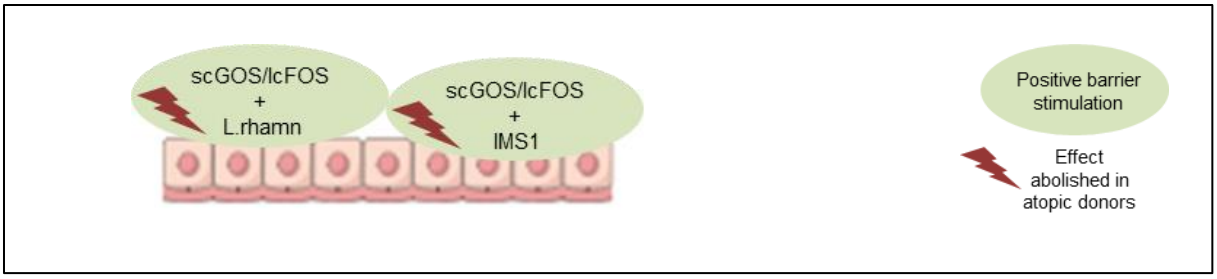

Figure 43: Only transepithelial electrical resistance of keratinocytes derived from non-atopic donors benefited from prebiotic and probiotic treatment
Keratinocytes derived from non-atopic donors responded with improved TER to treatment with prebiotics and probiotics. This effect was not observable in keratinocytes derived from AE patients.

5.5 Conclusion – may prebiotics, probiotics and synbiotics be a tool for immune-modulation in allergic diseases?

Prebiotics, probiotics and synbiotics have been shown to be beneficial in several studies of various diseases. However, it still needs to be stressed that the major problem of their usage is the lack of validated strains, protocols and methodologies to demonstrate direct health benefits or changes in the composition of the microbiota resulting in a health benefit. It seems to be wrong to sum up different prebiotics and probiotics under these terms instead of classifying them due to their molecular size and characteristics (Jeurink et al, 2012). Especially, studies investigating the effect of prebiotics and probiotics on allergic diseases were performed under strongly varying conditions with different cohorts and clinical protocols making it very difficult to draw conclusions (Gourbeyre et al, 2011). Different bacterial species were shown to exert different effects in various allergic diseases (Gruber et al, 2007; Hougee et al, 2010; Majamaa & Isolauri, 1997). This is why probiotic strains need to be selected due to their molecular and metabolic characteristics, interactions with matrix, diet, host and growth conditions (Kalliomaki et al, 2010). In the present study, scGOS/lcFOS and the two LAB strains were used due to previous beneficial results on dendritic cell (DC) immunity. However, this specific prebiotic mixture or its combination with these specific LAB strains might be inferior to other prebiotics and bacterial species with different molecular and metabolic characteristics when it comes to the proposed purpose of the present study.

For synbiotics it is even more important to find the right combination of prebiotics and a probiotic strain to induce beneficial health effects also taking into account the ideal doses and treatment option. For example, it was shown that specific prebiotics can stimulate the growth of specific probiotics only, thereby prolonging their survival and retention time in the gastrointestinal tract of mice (Su et al, 2007). Similarly, it was demonstrated that GOS increases the fecal concentration of *L. rhamnosus* and commensal bifidobacteria as well as lactobacilli in feces derived from *L. rhamnosus* treated children (Piirainen et al, 2008). Of note, in our experiments, the combination of scGOS/lcFOS and *L. rhamnosus* mostly appeared to

be more beneficial than the combination of scGOS/lcFOS and *IMS1* which could be due to a better utilisation of scGOS/lcFOS by *L. rhamnosus* than *IMS1*.

Moreover, the mechanisms of action are largely unknown. It is known that prebiotics, once utilised by probiotics or the host microbiome, are fermented into SCFA, mostly butyrate, propionate and acetate (Cummings, 1981). SCFAs decrease the pH, thereby inhibiting the growth of pathogenic bacteria (Blaut, 2002) which might be beneficial for known dysbiosis in allergy or especially *S. aureus* overgrowth in AE. Furthermore, propionate was shown to ease airway hypersensitivity in a mouse model of HDM induce asthma (Trompette et al, 2014) while both propionate and butyrate seem to induce Tregs (Arpaia et al, 2013). However, it is unclear whether prebiotics and probiotics only indirectly induce these effects or whether protection might also be granted via direct mechanisms which is of high importance since since some probiotic strains might mediate their effect via the activation of TLRs (Chaves et al, 2011; Grabig et al, 2006; Rachmilewitz et al, 2004) which expression pattern seems to be different in atopic individuals.

6 Conclusion

This thesis project gives insight into the complex role of epithelial cells (ECs) in innate immunity and how their response can be influenced by atopy status and environmental factors. Deeper analysis and characterization of primary human nasal epithelial cells (HNECs) showed that there are differences in innate immune repertoire and response in cells from donors with different atopy status.Furthermore, it was demonstrated that prebiotics, probiotics and active microbial structures indeed modulate barrier function and inflammatory responses of ECs of the airways and the skin which however seem to be different in cells derived from atopic donors.

In detail, results of this study indicate differences between HNECs derived from non-atopic or AR donors concerning the expression levels of innate immune receptors. Especially the expression of TLR-9 and TRIF was reduced in cells from AR donors. Moreover, AR donors´ cells respond to TLR ligation, treatment with aqueous pollen extracts but also stimulation of the inflammasome itself with an increased release of inflammasome-related cytokines, Th2 chemokines and a decrease of Th1 chemokines, which could create a Th2-promoting, thus allergy-prone micromilieu.

Direct anti-inflammatory, microbiota independent, immune-modulatory properties of especially lactocepin and scGOS/lcFOS in combination with lactic acid bacteria (particularly *L. rhamnosus*) could be observed on human primary epithelial cells of the skin and airways. However, this effect was more pronounced in cells derived from non-atopic donors. Moreover, the anti-inflammatory effect of pre- and probiotics was rather observable for Th1- than for Th2-promoting chemokines.

A disturbed TLR-9-TRIF response in in ECs from atopic donors could be an explanation for the lack of effectiveness of prebiotics and probiotics in otherwise healthy donor derived cells since anti-inflammatory and immune-suppressive effects were shown to be translated via this pathway. Since the two exemplary atopic diseases chosen as models in this study are rather Th2-dominated diseases, the prebiotics, probiotics and active microbial structure used here, have not been proven to be beneficial for immune dysbalance restoration.

In summary, these data show that atopics have a diminished innate immune repertoire and increased inflammation level which might be predisposing factors for increased susceptibility to allergy development or acquired due to allergy development. Prebiotics, probiotics and active microbial structures in general seem to have direct anti-inflammatory effects on epithelial cells and may therefore be a tool to either first educate the immune system early in life or restore immune homeostasis later in life. However, single strains or compounds and especially their combined used needs still to be distinctly validated for changes in the composition of the microbiota resulting in or direct health benefits in respect to different disease conditons.

Table of figures

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List of posters and talks

Oral presentations

Immuno-modulatory effects of prebiotics, probiotics and active microbial structures on human epithelial cells

Schlumprecht C., Dittlein D., Overbeek S., Hiller J., Lehmann S., van Bergenhenegouwen J., Knippels L. M. J., Garssen J., Hörmannsperger G., Haller D., Traidl-Hoffmann C.

8th Autumn School “Current Concepts in Immunology”
Merseburg, Germany, October 9th – 14th, 2016

Immuno-modulatory effects of prebiotics, probiotics and active microbial structures on human primary epithelial cells

Schlumprecht C., Dittlein D., Overbeek S., Hiller J., Lehmann S., van Bergenhenegouwen J., Knippels L. M. J., Garssen J., Hörmannsperger G., Haller D., Traidl-Hoffmann C.

EAACI (European Academy of Allergy and Clinical Immunology) Congress 2017
Helsinki, Finland, June 17th – 21st 2017

Poster presentations

Interaction of prebiotics and probiotics with human epithelial cells

Schlumprecht C., Dittlein D., Overbeek S., Hiller J., Lehmann S., van Bergenhenegouwen J., Knippels L. M. J., Garssen J., Traidl-Hoffmann C.

5. ADF (Arbeitsgemeinschaft dermatologische Forschung) Winter School “Targeted Therapies in skin diseases“

Zugspitze, Germany, January 28th -.30th 2016

Immuno-modulatory effects of probiotics, prebiotics and active microbial structures on human primary keratinocytes

Schlumprecht C., Dittlein D., Overbeek S., Hiller J., Lehmann S., van Bergenhenegouwen J., Knippels L. M. J., Garssen J., Hörmannsperger G., Haller D., Traidl-Hoffmann C.

The 46th Annual ESDR Meeting
Munich, Germany, September 7th – 10th 2016

Immunmodulatorische Effekte auf humane primäre Keratinozyten durch Präbiotika, Probiotika und active mikrobielle Strukturen

Schlumprecht C., Dittlein D., Overbeek S., Hiller J., Lehmann S., van Bergenhenegouwen J., Knippels L. M. J., Garssen J., Hörmannsperger G., Haller D., Traidl-Hoffmann C.

Allergiekongress 2016
Berlin, Germany, September 29th – October 1st 2016

Immuno-modulatory effects of prebiotics, probiotics and active microbial structures on human primary keratinocytes and human primary nasal epithelial cells

Schlumprecht C., Dittlein D., Overbeek S., Hiller J., Lehmann S., van Bergenhenegouwen J., Knippels L. M. J., Garssen J., Hörmannsperger G., Haller D., Traidl-Hoffmann C.

44th ADF Annual Meeting
Göttingen, Germany, March 8th – 11th 2017

Immuno-modulatory effects of prebiotics, probiotics and active microbial structures on human epithelial cells

Schlumprecht C., Dittlein D., Overbeek S., Hiller J., Lehmann S., van Bergenhenegouwen J., Knippels L. M. J., Garssen J., Hörmannsperger G., Haller D., Traidl-Hoffmann C.

DGfI (Deutsche Gesellschaft für Immunologie) Novel concepts in innate immunity
Tübingen, Germany, March 27th – 29th 2017

Innate immune responses to microbial and allergenic compounds in nasal epithelial cells

Schlumprecht C., Dittlein D., Mair S., Riepl R., Warzyniak P., Akdis C., Gilles S., Traidl-Hoffmann C.

16th EAACI Winter School
Saas-Fee, Switzerland, January 25th – 28th 2018

Nasal epithelial cells from allergic rhinitis patients show lower baseline TLR expression and altered cytokine responses to TLR ligands than cells from healthy individuals

Bergougnan C., Dittlein D., Damialis A., Wawrzyniak P., Akdis C., Traidl-Hoffmann C., Gilles S.

EAACI Congress 2018
Munich, Germany, May 26th – 30th 2018

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Appendix

Raw data for figures with normalized data

Figure 12			
Day	Ω_1	Ω_2	Ω_3
7	2940	11696	11533
7	3003	11690	11545
7	3240	11689	11534
7	6529	6696	6533
7	6535	6690	6545
7	6541	6689	6534
14	12212	12290	12508
14	13800	12458	13818
14	12230	12230	12233
14	7212	7290	7508
14	7226	7458	8818
14	7220	7230	7233
21	11500	11677	11680
21	11530	11588	11684
21	11500	11484	11650
21	5658	6677	6680
21	4652	6588	6684
21	5243	6484	6650

Figure 16				
Atopy status	Day	Ω_1	Ω_2	Ω_3
Non-atopic	7	2940	11696	11533
Non-atopic	7	3003	11690	11545
Non-atopic	7	3240	11689	11534
Non-atopic	14	12212	12290	12508
Non-atopic	14	13800	12458	13818
Non-atopic	14	12230	12230	12233
Non-atopic	21	11500	11677	11680
Non-atopic	21	11530	11588	11684
Non-atopic	21	11500	11484	11650
Atopic	7	6529	6696	6533
Atopic	7	6535	6690	6545
Atopic	7	6541	6689	6534
Atopic	14	7212	7290	7508
Atopic	14	7226	7458	8818
Atopic	14	7220	7230	7233
Atopic	21	5658	6677	6680
Atopic	21	4652	6588	6684
Atopic	21	5243	6484	6650

Figure 17							
Atopy status	Time point	Control	HDM 10 ug/ml	Birch 10 mg/ml	Ambrosia 2,5 ug/ml	Grass 10 mg/ml	EDTA
Non-atopic	0 h	4600	5000	4200	1600	2700	2200
Non-atopic	1 h	1300	3300	1830	1180	1770	1200
Non-atopic	2 h	1200	2300	1600	1100	1800	1100
Non-atopic	3 h	1200	2400	1700	1200	1800	1100
Non-atopic	4 h	1300	2700	2000	1500	2000	1300
Non-atopic	6 h	1500	2700	2500	1700	2200	1400
Non-atopic	24 h	3600	3900	3400	2600	3000	3200
Non-atopic	0 h	650	500	770	500	730	590
Non-atopic	1 h	1400	900	2100	1700	1000	260
Non-atopic	2 h	1700	1000	2400	2100	1500	190
Non-atopic	3 h	1900	1100	2600	2400	2000	190
Non-atopic	4 h	2000	1200	2900	2700	1800	270
Non-atopic	6 h	2000	1200	2900	2700	1800	270
Non-atopic	24 h	3600	1700	5700	5400	4700	240
Non-atopic	0 h	3000	2800	3200	2700	5000	3500
Non-atopic	1 h	5000	4400	4000	4300	6700	2000
Non-atopic	2 h	5500	4400	3500	2900	7300	1400
Non-atopic	3 h	3800	4300	3300	3000	5700	1400
Non-atopic	4 h	3100	3800	2500	2300	3600	1100
Non-atopic	6 h	3000	4100	2300	2100	4000	1200
Non-atopic	24 h	2820	4360	1980	2610	4040	1000
Non-atopic	0 h	1700	1400	1700	1700	1700	1600
Non-atopic	1 h	2000	2200	2500	3000	2800	3200
Non-atopic	2 h	3000	3700	3300	2800	2500	3300
Non-atopic	3 h	3000	3800	2700	2800	3600	4600
Non-atopic	4 h	3200	3200	3100	4000	2900	2300
Non-atopic	6 h	2400	3300	2900	2600	2900	2300
Non-atopic	24 h	2220	4560	2650	2920	2600	1800
Non-atopic	0 h	1700	1500	1500	1500	1600	1500
Non-atopic	1 h	4300	2720	3230	3160	3440	2770
Non-atopic	2 h	1800	2900	2600	2300	2300	3700
Non-atopic	3 h	1600	2800	2500	2400	2200	2300
Non-atopic	4 h	1600	3100	2200	1600	2000	1800
Non-atopic	6 h	1500	3400	1800	1900	1800	1900
Non-atopic	24 h	1500	3100	1900	1800	2100	1600
Non-atopic	0 h	2500	2500	2300	1650	1500	1700
Non-atopic	1 h	3210	6410	4310	4660	3180	3150
Non-atopic	2 h	3700	3100	3700	3000	2000	2700
Non-atopic	3 h	2900	2800	2600	3200	1900	2400
Non-atopic	4 h	2700	3300	2900	3300	2500	2700
Non-atopic	6 h	2600	2500	2300	2700	2100	2300
Non-atopic	24 h	2800	3000	3300	3500	2500	1500
Non-atopic	0 h	6300	3000	7700	5500	4800	3100
Non-atopic	1 h	5400	3000	6600	5200	4900	1800
Non-atopic	2 h	5300	2800	6800	4500	4000	1800
Non-atopic	3 h	5500	3100	7300	4700	4000	1800
Non-atopic	4 h	5600	4000	10200	5100	4400	2000
Non-atopic	6 h	4100	3200	5800	4100	3300	1800
Non-atopic	24 h	7000	3900	14000	3700	2500	1600
Non-atopic	0 h	1300	2300	3000	1600	850	1900
Non-atopic	1 h	1100	3200	3500	1300	830	170
Non-atopic	2 h	1080	2800	3400	1200	850	180
Non-atopic	3 h	1000	2900	3200	1200	820	170
Non-atopic	4 h	1820	3000	3600	1830	1280	160
Non-atopic	6 h	680	2900	2900	980	790	170
Non-atopic	24 h	740	3500	4700	740	570	430

Figure 17							
Atopy status	Time point	Control	HDM 10 ug/ml	Birch 10 mg/ml	Ambrosia 2,5 ug/ml	Grass 10 mg/ml	EDTA
Atopic	0 h	2300	2300	2200	3360	2780	3050
Atopic	1 h	5000	5000	3500	3812	3462	1425
Atopic	2 h	4500	3600	5900	3380	2980	1600
Atopic	3 h	5500	2700	5200	3140	2540	1317,5
Atopic	4 h	4300	3000	4700	3560	3080	1715
Atopic	6 h	5500	2700	5200	3500	2620	1150
Atopic	24 h	7500	4600	32000	3500	3560	1270
Atopic	0 h	1500	1400	1300	1500	1500	1300
Atopic	1 h	1800	1800	1700	2700	5000	2200
Atopic	2 h	3300	2100	2300	3400	2100	3100
Atopic	3 h	3000	2400	1700	3200	2800	2500
Atopic	4 h	3000	2500	1800	3600	2500	1500
Atopic	6 h	1800	1500	1800	3000	2800	1500
Atopic	24 h	3030	3500	1800	2500	1900	1600
Atopic	0 h	1900	2100	2300	2100	2100	2200
Atopic	1 h	4530	4070	4290	6060	3510	1200
Atopic	2 h	4200	3500	3300	2800	2900	1100
Atopic	3 h	2400	3600	2800	2700	2900	1100
Atopic	4 h	2400	3600	2100	2300	2700	1300
Atopic	6 h	2200	3900	2700	2400	2900	1400
Atopic	24 h	2700	5600	3500	2400	3100	3200
Atopic	0 h	4000	3400	3300	4000	3600	3300
Atopic	1 h	1800	3000	4100	1800	2700	300
Atopic	2 h	1000	3500	3700	2400	3400	200
Atopic	3 h	1100	3700	3800	3300	1400	170
Atopic	4 h	1000	4700	4300	3400	1600	160
Atopic	6 h	1700	6000	4500	5000	2500	200
Atopic	24 h	1400	7600	5700	5000	6200	180
Atopic	0 h	2300	2300	2900	3360	2780	2880
Atopic	1 h	3800	2600	5100	3812	3462	1380
Atopic	2 h	3800	2700	2800	3380	2980	1500
Atopic	3 h	2700	3000	3600	3140	2540	1274
Atopic	4 h	3800	3000	4800	3560	3080	1632
Atopic	6 h	3000	4000	4800	3500	2620	1200
Atopic	24 h	2300	3100	4600	3500	3560	1656
Atopic	0 h	1800	2400	3000	2200	2300	2600
Atopic	1 h	1500	2800	1800	1600	1900	1500
Atopic	2 h	1200	2700	1700	1800	1700	1300
Atopic	3 h	1300	2900	1400	500	1400	1200
Atopic	4 h	1200	2000	1000	1200	1300	1200
Atopic	6 h	1200	2000	1200	1200	1200	1100
Atopic	24 h	1000	1700	1300	1400	1200	1300
Atopic	0 h	7400	2300	5000	7000	4400	5000
Atopic	1 h	8500	3200	6300	6900	4200	1700
Atopic	2 h	7900	3000	5300	6500	4800	1800
Atopic	3 h	6200	3050	4700	6000	4200	1400
Atopic	4 h	6700	3100	6400	7300	7300	4000
Atopic	6 h	5300	3300	4600	5900	3700	1800
Atopic	24 h	14000	4350	8800	6200	5400	2000

Figure 28					
Atopy status	Stimulation	Condition	CCL-5	IL-8	CXCL-10
Non-atopic	Control	Control	0	4436,278	0
Non-atopic	Control	scGOS/lcFOS	0	5320,993	0
Non-atopic	Control	IMS1	0	7214,599	0
Non-atopic	Control	scGOS/lcFOS + IMS1	0	346,649	0
Non-atopic	Control	L. rhamn	0	5652,015	0
Non-atopic	Control	scGOS/lcFOS + L. rhamr	0	4357,475	0
Non-atopic	IFN-γ/TNF-α	Control	457,5835	14536,568	68637,525
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS	250,4379	6972,265	37565,685
Non-atopic	IFN-γ/TNF-α	IMS1	0	17123,683	0
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS + IMS1	0	4339,722	0
Non-atopic	IFN-γ/TNF-α	L. rhamn	320,1264	16459,48	48018,96
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS + L. rhamr	395,8822	6972,265	59382,33
Non-atopic	Control	Control	6,8545	2274,489	5,343
Non-atopic	Control	scGOS/lcFOS	4,5125	1546,754	0
Non-atopic	Control	IMS1	5,387	3811,716	15,885
Non-atopic	Control	scGOS/lcFOS + IMS1	7,2265	2764,5495	15,5275
Non-atopic	Control	L. rhamn	4,7285	2567,8795	18,107
Non-atopic	Control	scGOS/lcFOS + L. rhamr	7,458	2015,1785	22,9485
Non-atopic	IFN-γ/TNF-α	Control	266,5915	35849,876	601869,5
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS	107,9765	41902,595	373687,84
Non-atopic	IFN-γ/TNF-α	IMS1	155,627	19501,166	530664,66
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS + IMS1	124,7495	16777,456	425493,9
Non-atopic	IFN-γ/TNF-α	L. rhamn	91,3275	26897,193	347105,04
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS + L. rhamr	87,543	34297,309	377621,06
Non-atopic	Control	Control	0	2384,273	0
Non-atopic	Control	scGOS/lcFOS	6,534	12711,433	0
Non-atopic	Control	IMS1	0	5017,3665	0
Non-atopic	Control	scGOS/lcFOS + IMS1	0	3620,8595	0
Non-atopic	Control	L. rhamn	0	7950,7785	0
Non-atopic	Control	scGOS/lcFOS + L. rhamr	0	2088,225	0
Non-atopic	IFN-γ/TNF-α	Control	205,1695	18055,003	31389,029
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS	111,638	18143,537	1012,416
Non-atopic	IFN-γ/TNF-α	IMS1	186,231	5033,293	10078,447
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS + IMS1	50,2145	22322,951	9523,4535
Non-atopic	IFN-γ/TNF-α	L. rhamn	305,9185	3964,1485	10978,497
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS + L. rhamr	75,1405	15092,722	7943,925
Atopic	Control	Control	0	2861,67	47,6055
Atopic	Control	scGOS/lcFOS	0	4051,363	130,705
Atopic	Control	IMS1	0	2424,751	49,778
Atopic	Control	scGOS/lcFOS + IMS1	0	3464,392	75,3055
Atopic	Control	L. rhamn	0	2270,335	61,184
Atopic	Control	scGOS/lcFOS + L. rhamr	0	3395,305	78,5645
Atopic	IFN-γ/TNF-α	Control	842,04	5192,138	552085
Atopic	IFN-γ/TNF-α	scGOS/lcFOS	191,275	4815,922	396475
Atopic	IFN-γ/TNF-α	IMS1	327,805	6197,822	166725
Atopic	IFN-γ/TNF-α	scGOS/lcFOS + IMS1	211,355	6356,066	141475
Atopic	IFN-γ/TNF-α	L. rhamn	264,245	6056,442	831530
Atopic	IFN-γ/TNF-α	scGOS/lcFOS + L. rhamr	331,7	6352,696	406250
Atopic	Control	Control	0	1069,885	42,973
Atopic	Control	scGOS/lcFOS	0	1953,55	45,4265
Atopic	Control	IMS1	0	1701,75	52,2415
Atopic	Control	scGOS/lcFOS + IMS1	0	1841,4	56,3305
Atopic	Control	L. rhamn	0	1745,6	190,725
Atopic	Control	scGOS/lcFOS + L. rhamr	0	1656,3	96,9475
Atopic	IFN-γ/TNF-α	Control	842,04	12375,365	437780
Atopic	IFN-γ/TNF-α	scGOS/lcFOS	191,275	9916,76	275860
Atopic	IFN-γ/TNF-α	IMS1	327,805	9916,76	269730
Atopic	IFN-γ/TNF-α	scGOS/lcFOS + IMS1	211,355	11348,49	269320
Atopic	IFN-γ/TNF-α	L. rhamn	264,245	10191,835	241510
Atopic	IFN-γ/TNF-α	scGOS/lcFOS + L. rhamr	331,7	10426,44	276675
Atopic	Control	Control	19,218	1084,47	30,434
Atopic	Control	scGOS/lcFOS	21,548	1113,71	32,342
Atopic	Control	IMS1	23,6945	848,95	31,797
Atopic	Control	scGOS/lcFOS + IMS1	17,644	1419,1	37,794
Atopic	Control	L. rhamn	25,3345	652,395	37,2485
Atopic	Control	scGOS/lcFOS + L. rhamr	28,121	751,485	30,979
Atopic	IFN-γ/TNF-α	Control	1635,3	6754,4	256235
Atopic	IFN-γ/TNF-α	scGOS/lcFOS	1530,7	7753,4	200215
Atopic	IFN-γ/TNF-α	IMS1	1854,2	8127	179360
Atopic	IFN-γ/TNF-α	scGOS/lcFOS + IMS1	1415,7	8589,95	265635
Atopic	IFN-γ/TNF-α	L. rhamn	2428,05	8443,75	227200
Atopic	IFN-γ/TNF-α	scGOS/lcFOS + L. rhamr	1417,7	8370,65	154830

Figure 29						
Atopy status	Stimulation	Condition	CCL-20	IL-8	CXCL-10	GM-CSF
Non-atopic	Control	Control	105,041	5336,4	819,64	64,829
Non-atopic	Control	scGOS/lcFOS	116,755	4472,5	842,17	51,548
Non-atopic	Polyl:C	Control	1723,3	65783	208660	430,76
Non-atopic	Polyl:C	scGOS/lcFOS	883,34	8734,7	188950	505,58
Non-atopic	Control	Control	234,92	5500,1	1055,02	116,5095
Non-atopic	Control	scGOS/lcFOS	214,96	6260,15	1151,17	111,2
Non-atopic	Polyl:C	Control	784,79	34549	91178	648,93
Non-atopic	Polyl:C	scGOS/lcFOS	555,63	34134	73071	728,59
Non-atopic	Control	Control	204,83	1867,45	420,025	7,7301
Non-atopic	Control	scGOS/lcFOS	209,235	2679,65	445,125	8,6572
Non-atopic	Polyl:C	Control	2584,95	49505	188850	62,103
Non-atopic	Polyl:C	scGOS/lcFOS	1325,01	39150	83266	102,69
Non-atopic	Control	Control	281,35	4234,65	58,9375	63,02286667
Non-atopic	Control	scGOS/lcFOS	146,53	4470,7667	57,708	57,13506667
Non-atopic	Polyl:C	Control	1177,185	49945,667	163010	380,5976667
Non-atopic	Polyl:C	scGOS/lcFOS	833,445	27339,567	121025	445,62
Atopic	Control	Control	467,08	2850,1	467,02	73,441
Atopic	Control	scGOS/lcFOS	536,31	2913,5	462,14	68,149
Atopic	Polyl:C	Control	1183,3	3060	138510	383,96
Atopic	Polyl:C	scGOS/lcFOS	1082,9	61192	127270	279,89
Atopic	Control	Control	152,53	3060	354,6	176,19
Atopic	Control	scGOS/lcFOS	194	3759,5	361,63	201,51
Atopic	Polyl:C	Control	2711,4667	42165	160837	1094,1
Atopic	Polyl:C	scGOS/lcFOS	2363,2333	77326,667	137725,67	1092
Atopic	Control	Control	568,76667	4890,1	703,42	190,901
Atopic	Control	scGOS/lcFOS	665,64333	5419,8333	703,22667	202,489
Atopic	Polyl:C	Control	1922,6	40125	68497	1113,36
Atopic	Polyl:C	scGOS/lcFOS	1641,3	36532	52879	1007,89

Figure 30						
Atopy status	Stimulation	Condition	CCL-20	IL-8	CXCL-10	GM-CSF
Non-atopic	Control	Control	94,085	4929,5	819,64	64,829
Non-atopic	Control	scGOS/lcFOS	78,487	4014,4	842,17	51,548
Non-atopic	Phl-APE	Control	220,37	7675,8	75,912	70,175
Non-atopic	Phl-APE	scGOS/lcFOS	135,85	7858,8	77,039	55,96
Non-atopic	Bet-APE	Control	171,57	13601	190,82	167,82
Non-atopic	Bet-APE	scGOS/lcFOS	135,85	13809	168,29	144,25
Non-atopic	Control	Control	284,43	6854,4	1450,6	85,409
Non-atopic	Control	scGOS/lcFOS	273,96	7545,4	1563,7	69,1
Non-atopic	Phl-APE	Control	494,86	14427	118	149,58
Non-atopic	Phl-APE	scGOS/lcFOS	551,04	13709	149,69	124,54
Non-atopic	Bet-APE	Control	304,81	39883	329,72	365,02
Non-atopic	Bet-APE	scGOS/lcFOS	309,22	36898	636,81	391,95
Non-atopic	Control	Control	125,23	4145,8	659,44	158,07
Non-atopic	Control	scGOS/lcFOS	144,51	4974,9	738,66	170,42
Non-atopic	Phl-APE	Control	246,97	11636	91,973	305,43
Non-atopic	Phl-APE	scGOS/lcFOS	267,35	13571	104,42	310,78
Non-atopic	Bet-APE	Control	101,54	48672	329,72	2039,9
Non-atopic	Bet-APE	scGOS/lcFOS	137,9	46018	727,35	2021,3
Non-atopic	Control	Control	102,56	1982,1	442,99	71,29
Non-atopic	Control	scGOS/lcFOS	103,68	3210,6	455,54	4,8977
Non-atopic	Phl-APE	Control	154,11	6126,3	73,577	15,628
Non-atopic	Phl-APE	scGOS/lcFOS	49,158	5028,9	76,087	10,138
Non-atopic	Bet-APE	Control	96,863	10975	48,9	28,855
Non-atopic	Bet-APE	scGOS/lcFOS	56,028	6486,9	38,444	11,542
Non-atopic	Control	Control	111,8	1752,8	397,06	56,48
Non-atopic	Control	scGOS/lcFOS	110,83	2148,7	435,11	5,2784
Non-atopic	Phl-APE	Control	282,79	4876	48,9	28,855
Non-atopic	Phl-APE	scGOS/lcFOS	281,34	6551	38,444	11,542
Non-atopic	Bet-APE	Control	117,61	3967,3	40,34	12,015
Non-atopic	Bet-APE	scGOS/lcFOS	131,18	7072	37,803	18,848
Atopic	Control	Control	500,93	2850,1	467,02	73,441
Atopic	Control	scGOS/lcFOS	549,85	2913,5	462,14	68,149
Atopic	Phl-APE	Control	491,32	7701	101,85	86,205
Atopic	Phl-APE	scGOS/lcFOS	507,92	8461,9	86,724	74,917
Atopic	Bet-APE	Control	227,95	8156,4	46,702	193,8
Atopic	Bet-APE	scGOS/lcFOS	210,48	8075,1	45,726	153,23
Atopic	Control	Control	433,23	2927,2	478,4	65,856
Atopic	Control	scGOS/lcFOS	522,77	2844,8	463,74	64,269
Atopic	Phl-APE	Control	417,07	6898,1	97,692	93,613
Atopic	Phl-APE	scGOS/lcFOS	495,25	6774,5	97,203	77,386
Atopic	Bet-APE	Control	592,17	13768,8	64,25475	794,0325
Atopic	Bet-APE	scGOS/lcFOS	99,1425	13118,625	62,46975	739,395
Atopic	Control	Control	152,53	3060	354,6	197,48
Atopic	Control	scGOS/lcFOS	194	3759,5	361,63	216,85
Atopic	Phl-APE	Control	180,46	6290	48,451	408,87
Atopic	Phl-APE	scGOS/lcFOS	194,43	7244,4	50,908	306,24
Atopic	Bet-APE	Control	72,643	10202	38,971	864,91
Atopic	Bet-APE	scGOS/lcFOS	66,095	9416,4	37,567	832,63

Figure 31					
Atopy status	Stimulation	Condition	CCL-5	IL-8	CXCL-10
Non-atopic	Control	Control	0	4436,278	0
Non-atopic	Control	lactocepin	0	5978,444	0
Non-atopic	Control	lactocepin + PMSF	0	105,79055	0
Non-atopic	IFN-γ/TNF-α	Control	457,5835	14536,568	68637,525
Non-atopic	IFN-γ/TNF-α	lactocepin	0	24639,347	129632,74
Non-atopic	IFN-γ/TNF-α	lactocepin + PMSF	411,82515	16990,849	68974,123
Non-atopic	Control	Control	6,8545	2274,489	5,343
Non-atopic	Control	lactocepin	5,219	2304,2275	0
Non-atopic	Control	lactocepin + PMSF	0	175,39635	0
Non-atopic	IFN-γ/TNF-α	Control	266,5915	35849,876	601869,5
Non-atopic	IFN-γ/TNF-α	lactocepin	210,51	41578,479	251978,48
Non-atopic	IFN-γ/TNF-α	lactocepin + PMSF	239,93235	33252,237	604821,06
Non-atopic	Control	Control	0	2384,273	0
Non-atopic	Control	lactocepin	0	9652,6605	0
Non-atopic	Control	lactocepin + PMSF	0	0	0
Non-atopic	IFN-γ/TNF-α	Control	205,1695	18055,003	31389,029
Non-atopic	IFN-γ/TNF-α	lactocepin	20,289	7700,214	7287,0075
Non-atopic	IFN-γ/TNF-α	lactocepin + PMSF	190,30316	20582,703	36031,591
Atopic	Control	Control	0	2861,67	47,6055
Atopic	Control	lactocepin	0	3548,665	29,682
Atopic	Control	lactocepin + PMSF	0	1909,4153	0,0064934
Atopic	IFN-γ/TNF-α	Control	842,04	5192,138	552085
Atopic	IFN-γ/TNF-α	lactocepin	197,945	8399,89	120290
Atopic	IFN-γ/TNF-α	lactocepin + PMSF	757,836	6068,7526	554792,42
Atopic	Control	Control	0	1069,885	42,973
Atopic	Control	lactocepin	0	2028,659	22,256
Atopic	Control	lactocepin + PMSF	0	159,19418	32,6145
Atopic	IFN-γ/TNF-α	Control	842,04	12375,365	437780
Atopic	IFN-γ/TNF-α	lactocepin	197,945	9172,28	53011
Atopic	IFN-γ/TNF-α	lactocepin + PMSF	781,02679	9916,7599	502529,41
Atopic	Control	Control	19,218	1084,47	30,434
Atopic	Control	lactocepin	16,8805	991,895	20,075
Atopic	Control	lactocepin + PMSF	0	227,84726	25,2545
Atopic	IFN-γ/TNF-α	Control	1635,3	6754,4	256235
Atopic	IFN-γ/TNF-α	lactocepin	1221,7	9986,9	48513
Atopic	IFN-γ/TNF-α	lactocepin + PMSF	1516,8081	5412,5081	294133,18

Figure 32							
Atopy status	Time point	Control	scGOS/lcFOS	IMS1	scGOS/lcFOS + IMS	L. rhamn	scGOS/lcFOS + L. rha
Non-atopic	0	685	1900	2338	4100	5530	1798
Non-atopic	3	630	1475	2370	2856	4755	2190
Non-atopic	6	681	1060	2905	3155	2199	3360
Non-atopic	8	595	1838	2361	3424	2010	2970
Non-atopic	24	2749	3236	2343	5048	3833	4390
Non-atopic	48	4605	5681	3170	7233	6182	6642
Non-atopic	0	1860	872	765	2150	1298	777
Non-atopic	3	1756	816	759	2180	1079	741
Non-atopic	6	567	716	715	1950	1163	594
Non-atopic	8	882	527	937	1644	1250	536
Non-atopic	24	930	459	1155	1524	1469	519
Non-atopic	48	124	118	140	114	152	138
Non-atopic	0	1370	3800	4676	8200	11060	3596
Non-atopic	3	1260	2950	4740	5712	9510	4380
Non-atopic	6	1362	2120	5810	6310	4398	6720
Non-atopic	8	1190	3676	4722	6848	4020	5940
Non-atopic	24	5498	6472	4686	10096	7666	8780
Non-atopic	48	9210	11362	6340	14466	12364	13284
Non-atopic	0	3720	1744	1530	4300	2596	1554
Non-atopic	3	3512	1632	1518	4360	2158	1482
Non-atopic	6	1134	1432	1430	3900	2326	1188
Non-atopic	8	1764	1054	1874	3288	2500	1072
Non-atopic	24	1860	918	2310	3048	2938	1038
Non-atopic	48	248	236	280	228	304	276
Atopic	0	1803	1620	1725	1818	1725	2646
Atopic	3	1878	1404	1443	1080	1761	1704
Atopic	6	1158	948	1017	1125	750	1266
Atopic	8	897	735	945	1143	828	1500
Atopic	24	813	681	930	1116	750	1473
Atopic	48	519	360	423	513	525	579
Atopic	0	3606	3240	3450	3636	3450	5292
Atopic	3	3756	2808	2886	2160	3522	3408
Atopic	6	2316	1896	2034	2250	1500	2532
Atopic	8	1794	1470	1890	2286	1656	3000
Atopic	24	1626	1362	1860	2232	1500	2946
Atopic	48	1038	720	846	1026	1050	1158
Atopic	0	1026	2850	3507	6150	8295	2697
Atopic	3	945	2211	3555	4284	7131	3285
Atopic	6	1020	1590	4356	4731	3297	5040
Atopic	8	900	2757	3540	5136	3015	4455
Atopic	24	4125	4854	3513	7572	5749,5	6585
Atopic	48	6906	8520	4755	10848	9273	9963

Figure 33								
Atopy status	Stimulation	Condition	CCL-20	CCL-22	CCL-5	CCL-2	IL-8	CXCL-10
Non-atopic	Control	Control	0	0	8,313678	0	29,863	0
Non-atopic	Control	scGOS/lcFOS	62,01619	0	6,923189	0	489,48525	21,3725
Non-atopic	Control	IMS1	0	0	7,075825	0	28,24	0
Non-atopic	Control	scGOS/lcFOS + IMS1	21,68909	0	6,766834	0	71,6095	0
Non-atopic	Control	L. rhamn	0	0	8,623467	0	240,7995	0
Non-atopic	Control	scGOS/lcFOS + L. rhamn	0	0	8,623467	0	176,7675	0
Non-atopic	IFN-γ/TNF-α	Control	1765,6885	229,07	3316,2605	6779,0268	12268,279	844292,47
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS	1324,2111	158,07	2834,6285	7895,8293	10176,561	603701,9
Non-atopic	IFN-γ/TNF-α	IMS1	1045,927	213,31	2640,636	6721,957	6888,76	574863,3
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS + IMS1	417,5706	58,812	802,723	3230,2195	8045,3095	557891,79
Non-atopic	IFN-γ/TNF-α	L. rhamn	1653,699	223,535	4043,912	9775,461	9081,533	467905,73
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS + L. rhamn	743,018	125,27	1525,437	6394,4165	13362,644	405731,53
Non-atopic	Control	Control	0	0	0	7,834	262,9465	0
Non-atopic	Control	scGOS/lcFOS	0	0	0	0,457	212,27	0
Non-atopic	Control	IMS1	0	0	0	6,229	352,22483	0
Non-atopic	Control	scGOS/lcFOS + IMS1	0	0	0	3,452	460,16233	0
Non-atopic	Control	L. rhamn	0	0	0	0	385,14933	0
Non-atopic	Control	scGOS/lcFOS + L. rhamn	0	0	0	0	653,2335	3,618481
Non-atopic	IFN-γ/TNF-α	Control	279,6822	279,6822	1811,4725	15081,496	3640,4775	522098,55
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS	198,5596	198,5596	661,116	11298,216	3617,8635	467747,78
Non-atopic	IFN-γ/TNF-α	IMS1	102421,71	136371,9	571,153	9269,521	3750,7718	396095,4
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS + IMS1	33892,452	44969,104	662,4955	9713,9815	4784,8797	120408,45
Non-atopic	IFN-γ/TNF-α	L. rhamn	153672,92	204777,26	359,8915	3396,3735	4348,6178	606586,8
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS + L. rhamn	59570,595	79344,631	248,4855	3107,184	6111,1585	228815,55
Non-atopic	Control	Control	0	0	43,152	0	117,0495	25,8125
Non-atopic	Control	scGOS/lcFOS	0	0	56,0755	0	154,9005	35,6815
Non-atopic	Control	IMS1	0	0	41,158	0	108,1705	26,414
Non-atopic	Control	scGOS/lcFOS + IMS1	0	0	32,163	0	141,035	18,31
Non-atopic	Control	L. rhamn	0	0	48,9675	0	81,189	29,977
Non-atopic	Control	scGOS/lcFOS + L. rhamn	0	0	42,4125	0	139,553	22,4305
Non-atopic	IFN-γ/TNF-α	Control	1432,4995	259,625	14140,884	30023,922	2157,002	1190673,7
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS	810,21	205,02	11809,147	21448,033	2498,3335	1098670
Non-atopic	IFN-γ/TNF-α	IMS1	896,055	180,24	11767,277	29958,792	2161,1575	963193,35
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS + IMS1	910,005	193,18	11258,924	22809,934	2628,3895	933521,75
Non-atopic	IFN-γ/TNF-α	L. rhamn	971,17	205,9	10216,607	22349,34	2322,765	880217,29
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS + L. rhamn	870,3	171,69	10061,646	25459,919	3155,9225	1001090,4
Non-atopic	Control	Control	0	0	71,3955	0	724,788	19,024
Non-atopic	Control	scGOS/lcFOS	0	0	40,1545	0	1834,4585	18,637
Non-atopic	Control	IMS1	0	0	36,0835	0	920,264	7,687
Non-atopic	Control	scGOS/lcFOS + IMS1	0	0	47,5305	0	1167,8425	5,0765
Non-atopic	Control	L. rhamn	0	0	36,1515	0	833,4595	12,2865
Non-atopic	Control	scGOS/lcFOS + L. rhamn	0	0	43,849	0	1643,38	9,9645
Non-atopic	IFN-γ/TNF-α	Control	521,555	77,3955	1313,7875	4114,3815	3190,138	850299,14
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS	584,865	74,764	2141,0815	5174,3575	4232,034	864920,72
Non-atopic	IFN-γ/TNF-α	IMS1	371,32	67,5275	1518,2515	2963,6185	2202,398	627863
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS + IMS1	486,14	183,75	819,285	5532,389	3680,94	1113722,5
Non-atopic	IFN-γ/TNF-α	L. rhamn	303,72	27,836	674,975	2047,681	1641,5555	487995,14
Non-atopic	IFN-γ/TNF-α	scGOS/lcFOS + L. rhamn	279,035	17,5295	351,882	779,2005	1814,909	441392,1

Figure 33								
Atopy status	Stimulation	Condition	CCL-20	CCL-22	CCL-5	CCL-2	IL-8	CXCL-10
Atopic eczema	Control	Control	0	0	418,243	7,1424	7,1424	75,8775
Atopic eczema	Control	scGOS/lcFOS	0	0	494,891	4,9623	4,9623	50,265
Atopic eczema	Control	IMS1	0	0	394,661	4,52625	4,52625	119,31
Atopic eczema	Control	scGOS/lcFOS + IMS1	0	0	382,821	10,19475	10,19475	55,833
Atopic eczema	Control	L. rhamn	0	0	410,176	9,75875	9,75875	60,8445
Atopic eczema	Control	scGOS/lcFOS + L. rhamn	0	0	345,639	9,7587	9,7587	61,401
Atopic eczema	IFN-γ/TNF-α	Control	182,335	44,2035	6727,5	9565,35	9565,35	894720
Atopic eczema	IFN-γ/TNF-α	scGOS/lcFOS	164,67	42,265	7072,75	11266,5	11266,5	1140250
Atopic eczema	IFN-γ/TNF-α	IMS1	157,105	40,569	6108,55	14100,5	14100,5	972390
Atopic eczema	IFN-γ/TNF-α	scGOS/lcFOS + IMS1	183,85	36,208	6485,55	18198,5	18198,5	958195
Atopic eczema	IFN-γ/TNF-α	L. rhamn	222,705	46,626	7156,35	9565,75	9565,75	1100150
Atopic eczema	IFN-γ/TNF-α	scGOS/lcFOS + L. rhamn	184,855	45,1725	5617,95	8998,75	8998,75	767770
Atopic eczema	Control	Control	0	0	63,073	5,4797	5,4797	49,1515
Atopic eczema	Control	scGOS/lcFOS	44,059	0	50,3765	5,0905	5,0905	43,5835
Atopic eczema	Control	IMS1	0	0	45,289	0,80983	0,80983	45,254
Atopic eczema	Control	scGOS/lcFOS + IMS1	26,9	0	39,3495	0	0	43,0265
Atopic eczema	Control	L. rhamn	0	0	42,309	0	0	43,5835
Atopic eczema	Control	scGOS/lcFOS + L. rhamn	64,245	0	34,0715	0	0	46,9245
Atopic eczema	IFN-γ/TNF-α	Control	277,715	178,43	1245,1	2745,15	2745,15	282520
Atopic eczema	IFN-γ/TNF-α	scGOS/lcFOS	130,86	148,875	1463,15	2453,25	2453,25	356020
Atopic eczema	IFN-γ/TNF-α	IMS1	196,465	181,825	1687,4	2122,45	2122,45	386925
Atopic eczema	IFN-γ/TNF-α	scGOS/lcFOS + IMS1	323,64	169,465	2902,65	3990,4	3990,4	599060
Atopic eczema	IFN-γ/TNF-α	L. rhamn	237,845	205,565	1929,1	2842,45	2842,45	509700
Atopic eczema	IFN-γ/TNF-α	scGOS/lcFOS + L. rhamn	229,775	146,935	1413,95	2998,1	2998,1	483805
Atopic eczema	Control	Control	0	9,272	0	4,3984	4,3984	37,866
Atopic eczema	Control	scGOS/lcFOS	118,71	10,356	0	7,62175	7,62175	38,5485
Atopic eczema	Control	IMS1	0	9,272	0	6,382	6,382	41,9595
Atopic eczema	Control	scGOS/lcFOS + IMS1	72,861	11,983	0	4,15045	4,15045	37,866
Atopic eczema	Control	L. rhamn	0	8,1878	0	2,9107	2,9107	35,8195
Atopic eczema	Control	scGOS/lcFOS + L. rhamn	93,415	9,8141	0	4,3984	4,3984	39,913
Atopic eczema	IFN-γ/TNF-α	Control	494,215	120,405	6468,05	14303,5	14303,5	668795
Atopic eczema	IFN-γ/TNF-α	scGOS/lcFOS	486,31	100,89	4570,5	7695,6	7695,6	547010
Atopic eczema	IFN-γ/TNF-α	IMS1	396,98	114,17	7290,15	12394	12394	859145
Atopic eczema	IFN-γ/TNF-α	scGOS/lcFOS + IMS1	486,315	87,065	6298,35	10224,45	10224,45	841745
Atopic eczema	IFN-γ/TNF-α	L. rhamn	371,685	115,255	6070,55	9616,95	9616,95	777275
Atopic eczema	IFN-γ/TNF-α	scGOS/lcFOS + L. rhamn	536,905	98,992	6142,35	9753,55	9753,55	932830
Atopic eczema	Control	Control	0	9,272	17,638	6,50935	116,5	43,396
Atopic eczema	Control	scGOS/lcFOS	64,956	12,525	28,976	8,8523	190,205	44,158
Atopic eczema	Control	IMS1	0	11,983	31,3	7,8482	87,445	47,207
Atopic eczema	Control	scGOS/lcFOS + IMS1	55,469	12,525	37,5765	12,869	252,4444	56,353
Atopic eczema	Control	L. rhamn	68,118	11,44	28,976	7,8482	134,3	43,396
Atopic eczema	Control	scGOS/lcFOS + L. rhamn	153,5	13,067	29,601	8,8523	218	46,4445
Atopic eczema	IFN-γ/TNF-α	Control	529	74,3255	6020,5	82080	1305,8	188565
Atopic eczema	IFN-γ/TNF-α	scGOS/lcFOS	852,33	68,9045	4831,2	80579,95	2923,3	146265
Atopic eczema	IFN-γ/TNF-α	IMS1	967,75	81,644	6332,05	70413,3	3826,3	298320
Atopic eczema	IFN-γ/TNF-α	scGOS/lcFOS + IMS1	939,29	53,454	3839,3	58513,3	3571,45	261735
Atopic eczema	IFN-γ/TNF-α	L. rhamn	870,51	48,575	6150,55	71246,65	2275,2	241155
Atopic eczema	IFN-γ/TNF-α	scGOS/lcFOS + L. rhamn	983,56	77,036	4583,8	87913,3	4043,333	357770

Figure 34				
Atopy status	Stimulation	Condition	CCL-2	CCL-22
Non-atopic	Control	Control	63,470071	2,7744074
Non-atopic	Control	scGOS/lcFOS	28,746275	2,238995
Non-atopic	Control	IMS1	46,108961	2,6770599
Non-atopic	Control	scGOS/lcFOS + IMS1	29,535662	2,0929748
Non-atopic	Control	L. rhamn	47,686957	3,06645
Non-atopic	Control	scGOS/lcFOS + L. rhamn	12,962678	2,4823649
Non-atopic	IL-4/-13	Control	204,69	20,443
Non-atopic	IL-4/-13	scGOS/lcFOS	44,698196	19,469525
Non-atopic	IL-4/-13	IMS1	105,64745	21,416475
Non-atopic	IL-4/-13	scGOS/lcFOS + IMS1	29,460868	21,903223
Non-atopic	IL-4/-13	L. rhamn	120,88478	21,416475
Non-atopic	IL-4/-13	scGOS/lcFOS + L. rhamn	21,842204	21,416475
Non-atopic	Control	Control	64,102911	2,555375
Non-atopic	Control	scGOS/lcFOS	35,494147	2,1010866
Non-atopic	Control	IMS1	71,458671	2,9528769
Non-atopic	Control	scGOS/lcFOS + IMS1	38,76364	2,3850173
Non-atopic	Control	L. rhamn	68,189158	2,9528769
Non-atopic	Control	scGOS/lcFOS + L. rhamn	46,937812	2,6121616
Non-atopic	IL-4/-13	Control	123,39	24,361
Non-atopic	IL-4/-13	scGOS/lcFOS	62,07564	19,875138
Non-atopic	IL-4/-13	IMS1	188,32688	23,850173
Non-atopic	IL-4/-13	scGOS/lcFOS + IMS1	125,20126	21,010866
Non-atopic	IL-4/-13	L. rhamn	211,99907	24,418019
Non-atopic	IL-4/-13	scGOS/lcFOS + L. rhamn	219,88967	21,578731
Non-atopic	Control	Control	76,704896	3,1553321
Non-atopic	Control	scGOS/lcFOS	40,184168	2,0443
Non-atopic	Control	IMS1	80,906711	2,9775679
Non-atopic	Control	scGOS/lcFOS + IMS1	36,951621	1,9554173
Non-atopic	Control	L. rhamn	81,87557	3,022009
Non-atopic	Control	scGOS/lcFOS + L. rhamn	28,871791	1,9109761
Non-atopic	IL-4/-13	Control	184,36	20,6405
Non-atopic	IL-4/-13	scGOS/lcFOS	146,51123	19,998588
Non-atopic	IL-4/-13	IMS1	240,24056	19,554175
Non-atopic	IL-4/-13	scGOS/lcFOS + IMS1	127,12117	19,998588
Non-atopic	IL-4/-13	L. rhamn	253,17165	20,443
Non-atopic	IL-4/-13	scGOS/lcFOS + L. rhamn	114,19448	18,220936
Atopic eczema	Control	Control	30,456705	2,7921454
Atopic eczema	Control	scGOS/lcFOS	27,525269	2,2458569
Atopic eczema	Control	IMS1	29,967805	2,7314486
Atopic eczema	Control	scGOS/lcFOS + IMS1	20,684585	2,1244593
Atopic eczema	Control	L. rhamn	37,298044	2,7314486
Atopic eczema	Control	scGOS/lcFOS + L. rhamn	25,082079	2,3065557
Atopic eczema	IL-4/-13	Control	133,55	26,1005
Atopic eczema	IL-4/-13	scGOS/lcFOS	143,328	25,493512
Atopic eczema	IL-4/-13	IMS1	114,00965	29,13544
Atopic eczema	IL-4/-13	scGOS/lcFOS + IMS1	123,78243	20,637605
Atopic eczema	IL-4/-13	L. rhamn	123,78243	23,065557
Atopic eczema	IL-4/-13	scGOS/lcFOS + L. rhamn	104,23817	26,707493
Atopic eczema	Control	Control	19,822412	2,9920099
Atopic eczema	Control	scGOS/lcFOS	16,265081	2,7373708
Atopic eczema	Control	IMS1	23,055965	2,9920099
Atopic eczema	Control	scGOS/lcFOS + IMS1	15,295254	2,7373708
Atopic eczema	Control	L. rhamn	17,882158	2,9920099
Atopic eczema	Control	scGOS/lcFOS + L. rhamn	15,295254	2,61005
Atopic eczema	IL-4/-13	Control	106,45	25,9025
Atopic eczema	IL-4/-13	scGOS/lcFOS	114,14448	28,646891
Atopic eczema	IL-4/-13	IMS1	149,71783	27,373682
Atopic eczema	IL-4/-13	scGOS/lcFOS + IMS1	162,65375	26,737091
Atopic eczema	IL-4/-13	L. rhamn	165,88553	29,283482
Atopic eczema	IL-4/-13	scGOS/lcFOS + L. rhamn	104,44322	29,283482
Atopic eczema	Control	Control	7,922986	2,9000553
Atopic eczema	Control	scGOS/lcFOS	8,4850979	2,8033868
Atopic eczema	Control	IMS1	10,35811	3,0933921
Atopic eczema	Control	scGOS/lcFOS + IMS1	9,4216039	2,8033868
Atopic eczema	Control	L. rhamn	12,231297	3,4800658
Atopic eczema	Control	scGOS/lcFOS + L. rhamn	9,7961716	2,9967237
Atopic eczema	IL-4/-13	Control	147,1	35,4805
Atopic eczema	IL-4/-13	scGOS/lcFOS	120,43926	27,067184
Atopic eczema	IL-4/-13	IMS1	135,42371	28,033868
Atopic eczema	IL-4/-13	scGOS/lcFOS + IMS1	124,18494	27,067184
Atopic eczema	IL-4/-13	L. rhamn	139,16938	39,95295
Atopic eczema	IL-4/-13	scGOS/lcFOS + L. rhamn	137,29568	32,86729

Figure 35								
Atopy status	Stimulation	Condition	CCL-20	CCL-22	CCL-5	CCL-2	IL-8	CXCL-10
Non-atopic	Control	Control	0	0	8,313678	0	29,863	0
Non-atopic	Control	lactocpein	0	0	9,8137461	5,7366605	50,383501	0
Non-atopic	Control	lactocepin + PMSF	0	0	59,516927	15,734121	936,87941	1,6596257
Non-atopic	IFN-γ/TNF-α	Control	1765,6885	229,07	3316,2605	6779,0268	12268,279	844292,47
Non-atopic	IFN-γ/TNF-α	lactocpein	0	0	1251,0692	2327,0636	15033,39	68769,673
Non-atopic	IFN-γ/TNF-α	lactocepin + PMSF	2233,5041	220,70139	4017,2185	6807,7495	14709,164	833645,94
Non-atopic	Control	Control	0	0	0	7,834	262,9465	0
Non-atopic	Control	lactocpein	0	0	5,7188187	0	195,779	0
Non-atopic	Control	lactocepin + PMSF	0	0	3,6066417	0	319,77226	0
Non-atopic	IFN-γ/TNF-α	Control	279,6822	279,6822	1811,4725	15081,496	3640,4775	522098,55
Non-atopic	IFN-γ/TNF-α	lactocpein	156,7876	156,7876	1060,5302	3110,336	4460,995	172469,83
Non-atopic	IFN-γ/TNF-α	lactocepin + PMSF	269,46457	269,46457	2194,3634	15145,396	3655,9022	515514,89
Non-atopic	Control	Control	0	0	43,152	0	117,0495	25,8125
Non-atopic	Control	lactocpein	0	0	44,639	0	206,304	7,1885
Non-atopic	Control	lactocepin + PMSF	0	0	298,24537	0	429,96815	0
Non-atopic	IFN-γ/TNF-α	Control	1432,4995	259,625	14140,884	30023,922	2157,002	1190673,7
Non-atopic	IFN-γ/TNF-α	lactocpein	962,585	199,32	8278,8055	14267,831	2612,356	393326,69
Non-atopic	IFN-γ/TNF-α	lactocepin + PMSF	1414,4357	34,918784	1901,9064	0	2129,8022	96418,528
Non-atopic	Control	Control	0	0	71,3955	0	724,788	19,024
Non-atopic	Control	lactocpein	0	0	39,0975	0	954,25	0
Non-atopic	Control	lactocepin + PMSF	0	0	0	0	0	0
Non-atopic	IFN-γ/TNF-α	Control	521,555	77,3955	1313,7875	4114,3815	3190,138	850299,14
Non-atopic	IFN-γ/TNF-α	lactocpein	233,965	21,915	277,721	0	2316,292	128741,17
Non-atopic	IFN-γ/TNF-α	lactocepin + PMSF	42,234549	67,527496	1591,4828	3589,7937	3864,4375	590304,87
Atopic eczema	Control	Control	0	0	418,243	7,1424	7,1424	75,8775
Atopic eczema	Control	lactocepin	0	0	418,925	3,21817	3,21817	42,47
Atopic eczema	Control	lactocepin + PMSF	0	0	120,73844	22,201177	0	68326,188
Atopic eczema	IFN-γ/TNF-α	Control	182,335	44,2035	6727,5	9565,35	9565,35	894720
Atopic eczema	IFN-γ/TNF-α	lactocepin	241,38	42,5075	6826,2	8134,05	8134,05	300900
Atopic eczema	IFN-γ/TNF-α	lactocepin + PMSF	183,10755	42,588614	8149,4917	9444,7309	11587,178	1072732,6
Atopic eczema	Control	Control	0	0	63,073	5,4797	5,4797	49,1515
Atopic eczema	Control	lactocepin	0	0	31,1325	0	0	38,0155
Atopic eczema	Control	lactocepin + PMSF	0	0	2,4789941	0	0,0053961	24815,992
Atopic eczema	IFN-γ/TNF-α	Control	277,715	178,43	1245,1	2745,15	2745,15	282520
Atopic eczema	IFN-γ/TNF-α	lactocepin	180,825	132,64	1662,05	1869,55	1869,55	93771,5
Atopic eczema	IFN-γ/TNF-α	lactocepin + PMSF	267,56924	171,91142	1508,2768	2710,5337	222,29711	357373,11
Atopic eczema	Control	Control	0	9,272	0	4,3984	4,3984	37,866
Atopic eczema	Control	lactocepin	0	11,44	0	11,34075	11,34075	32,4075
Atopic eczema	Control	lactocepin + PMSF	0	0	136,41764	0	0	133314,92
Atopic eczema	IFN-γ/TNF-α	Control	494,215	120,405	6468,05	14303,5	14303,5	668795
Atopic eczema	IFN-γ/TNF-α	lactocepin	312,39	106,85	4384,85	5464,05	5464,05	113090
Atopic eczema	IFN-γ/TNF-α	lactocepin + PMSF	496,30899	16,194111	869,93332	0	12479,789	671628,68
Atopic eczema	Control	Control	0	9,272	17,638	6,50935	116,5	43,396
Atopic eczema	Control	lactocepin	0	10,356	40,389	20,232	360,5	43,396
Atopic eczema	Control	lactocepin + PMSF	0	0	82,338365	63,50256	0	22850,307
Atopic eczema	IFN-γ/TNF-α	Control	529	74,3255	6020,5	82080	1305,8	188565
Atopic eczema	IFN-γ/TNF-α	lactocepin	691,06	69,4465	1902,7	20146,65	5671,111	130260
Atopic eczema	IFN-γ/TNF-α	lactocepin + PMSF	522,32931	64,848924	7293,0531	6646,6849	906,52815	186187,2

Figure 36				
Atopy status	Stimulation	Condition	CCL-2	CCL-22
Non-atopic	Control	Control	62,849913	4,1315504
Non-atopic	Control	lactocpein	40,521157	2,545125
Non-atopic	Control	lactocepin + PMSF	72,833864	4,7292938
Non-atopic	IL-4/-13	Control	202,69	30,443
Non-atopic	IL-4/-13	lactocpein	129,39	20,361
Non-atopic	IL-4/-13	lactocepin + PMSF	194,36	30,6405
Non-atopic	Control	Control	28,465398	3,334233
Non-atopic	Control	lactocpein	22,436795	2,0926588
Non-atopic	Control	lactocepin + PMSF	38,156211	3,06405
Non-atopic	IL-4/-13	Control	166,57814	28,993335
Non-atopic	IL-4/-13	lactocpein	168,62972	19,795416
Non-atopic	IL-4/-13	lactocepin + PMSF	139,11731	29,974403
Non-atopic	Control	Control	43,313394	4,0590657
Non-atopic	Control	lactocpein	34,837235	2,8279169
Non-atopic	Control	lactocepin + PMSF	96,769628	3,7301469
Non-atopic	IL-4/-13	Control	36,717233	32,617513
Non-atopic	IL-4/-13	lactocpein	64,179277	21,492176
Non-atopic	IL-4/-13	lactocepin + PMSF	243,46136	30,6405
Atopic eczema	Control	Control	28,176158	4,9316794
Atopic eczema	Control	lactocepin	18,338143	5,2846939
Atopic eczema	Control	lactocepin + PMSF	7,3297261	5,1222773
Atopic eczema	IL-4/-13	Control	123,55	46,1005
Atopic eczema	IL-4/-13	lactocepin	96,45	28,9025
Atopic eczema	IL-4/-13	lactocepin + PMSF	167,1	30,4805
Atopic eczema	Control	Control	25,464223	3,9667871
Atopic eczema	Control	lactocepin	15,047179	4,8349328
Atopic eczema	Control	lactocepin + PMSF	7,849748	4,9515348
Atopic eczema	IL-4/-13	Control	132,59584	45,028396
Atopic eczema	IL-4/-13	lactocepin	105,59753	50,598111
Atopic eczema	IL-4/-13	lactocepin + PMSF	111,42097	47,807924
Atopic eczema	Control	Control	26,820493	4,61005
Atopic eczema	Control	lactocepin	23,722774	4,947372
Atopic eczema	Control	lactocepin + PMSF	9,409332	6,3174742
Atopic eczema	IL-4/-13	Control	91,0031	52,533133
Atopic eczema	IL-4/-13	lactocepin	126,5425	49,47372
Atopic eczema	IL-4/-13	lactocepin + PMSF	24,352372	68,297015

Figure 37							
Atopy status	Time point	Control	scGOS/lcFOS	IMS1	L. rhamn	scGOS/lcFOS + IMS1	scGOS/lcFOS + L. rhamn
Non-atopic	0	3130	2319	2869	3000	3389	3645
Non-atopic	3	3150	2074	2894	2975	4324	5285
Non-atopic	6	2547	2098	2843	2957	4262	5219
Non-atopic	8	2587	2100	2901	2140	4269	5201
Non-atopic	24	3598	1866	3000	2992	4722	5691
Non-atopic	48	3426	1576	2787	3016	4842	6119
Non-atopic	0	2509	2598	2264	2309	3278	3156
Non-atopic	3	3600	2207	2169	2175	3298	4347
Non-atopic	6	3100	2233	2131	2162	3240	4293
Non-atopic	8	2745	2235	2175	1565	3307	4278
Non-atopic	24	2760	1986	2249	2188	3419	4681
Non-atopic	48	3809	1678	2089	2206	3176	5033
Non-atopic	0	3567	3168	3592	3509	3218	2793
Non-atopic	3	3000	2826	4354	3471	4095	4039
Non-atopic	6	2343	2859	4291	3450	4036	3989
Non-atopic	8	2376	2862	4299	2497	4044	3975
Non-atopic	24	3509	2543	4754	3491	4472	4350
Non-atopic	48	2900	2148	4875	3519	4586	4677
Atopic eczema	0	1940	1780	2060	2050	2030	2230
Atopic eczema	3	2180	1910	1790	2000	2000	2230
Atopic eczema	6	1770	1700	1610	1670	1900	1930
Atopic eczema	8	1800	1700	1620	1680	1870	1800
Atopic eczema	24	2080	1810	1640	1890	1800	1550
Atopic eczema	48	2090	2050	1900	1830	1760	2050
Atopic eczema	0	2720	2480	2650	2300	2600	2750
Atopic eczema	3	2320	2520	2480	2490	2610	2820
Atopic eczema	6	2060	2320	2220	2140	2290	2350
Atopic eczema	8	1960	2150	2200	2240	2230	2440
Atopic eczema	24	1920	1720	2200	1900	1860	2020
Atopic eczema	48	2310	2200	1850	2410	2330	2150
Atopic eczema	0	2220	2150	2110	2950	2200	2400
Atopic eczema	3	2670	2520	2640	2760	2150	2590
Atopic eczema	6	2580	2590	2350	2320	2260	2270
Atopic eczema	8	2580	2780	2610	2370	2150	2000
Atopic eczema	24	2780	1910	1980	1850	1950	1840
Atopic eczema	48	2000	2720	2520	2580	2030	2390