Regulation and cross-talk between environmental triggers of local immune responses in airway epithelial cells

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General discussion and a summary
GENERAL DISCUSSION AND A SUMMARY

The most important role of the immune system is to protect the host organism from invading microorganisms, since we coexist with a multitude of bacteria, viruses, and fungi that challenge us every day. Responses of the immune system should be rapid, with a balanced strength to prevent tissue damage, yet provide enough of resources for the eradication of a pathogen. Majority of us have a well-functioning immune system and are probably not even aware of having one. Nonetheless some individuals do suffer from its hyperresponsiveness, which manifests itself in an induction of unnecessary inflammatory responses against harmless particles, such as allergens [1]. These over-reactive immune responses require engaging many different types of immune competent cells and lead to production and secretion of cytokines and mediators that propagate the allergic inflammatory state even further, so that an encounter of an allergen results in a fully developed anti-parasitic immune response. Cytokines and mediators secreted by cells involved in these immune responses control local and systemic inflammatory processes and consequently influence the innate and even polarize the adaptive immune responses [2].

We cannot forget that, in everyday life, we are challenged not by a single, but by a multitude of potential immunogenic stimuli and/or microbes at the same time. These triggers are recognized by variety of receptors that subsequently activate numerous pro-inflammatory pathways in different types of immune competent cells. Additionally, it has become clear that the ultimate response of the host to exogenous stimuli is shaped by the interaction between different receptors from the same or different receptors family and/or different cell types [3]. Functional consequences of the collaborative interactions between pro-inflammatory pathways and immune cells, such as synergistic production of (pro-) inflammatory mediators may lead to, among others, a viral exacerbation of an allergic airway inflammation (chapter 3), an enhanced pro-inflammatory responses to bacterial triggers upon virus encounter (chapter 5), a shift from tolerance for Gram negative bacterium residing in the nasal cavity to inflammation triggered by the co-existence with IgG (chapter 6), and finally a collaboration between epithelium and innate lymphoid cells in nasal polyposis (chapter 7 and 8).

In this thesis, I wanted to better understand the role of epithelium in the complexity of the pro-inflammatory responses triggered by environmental stimuli and their consequences on the local tissue immunity.

Original laboratory studies based on variety of molecular biology techniques presented in this thesis resulted in the following findings:

- Pro-inflammatory responses of airway epithelium to the HDM-allergen and the viral analogue poly(I:C) resemble each other both at the gene expression and protein level (chapter 3)

- Pre-exposure of airway epithelium to the HDM-allergen dramatically reduces the production of anti-viral mediators IP-10, IL-12, and RANTES upon the subsequent cell exposure to a viral analogue (chapter 3)

- Dysregulated expression of the EGR-1 and DUSP-1 transcription factors plays an important role in maintaining of the perpetually activated allergic inflammatory state in house dust mite allergic individuals (chapter 4)
• There is a functional synergistic collaboration between TLR-3 and TLR-2 signaling in nasal epithelium. This receptor cross-talk was also demonstrated in lung epithelium (chapter 5)

• Activation of the pro-inflammatory responses of nasal epithelium to the Gram negative bacterium *P. aeruginosa* and/or LPS requires co-stimulation with immunoglobulin G (chapter 6)

• Nasal polyps from a majority of individuals suffering from CRSwNP are enriched with the type 2 innate lymphoid cells, defined as a Lin⁻, CD45⁺, CD127⁺, c-kit⁻, and CRTH2⁺ cell population. These cells produce massive amounts of the archetypal type 2 cytokines IL-5, IL-13, and perhaps IL-4 in response to a simultaneous stimulation with TSLP and IL-33 (chapter 7)

• Nasal epithelium from CRSwNP individuals is an important source of TSLP, required for ILC2s activation. This cytokine is produced by epithelium exposed to poly(I:C) and this enhanced production may be associated with DUSP-1 deficiency in epithelium from CRSwNP individuals (chapter 8)

**Molecular link between allergy and viral infection(s)**

Viral rhinitis or a common cold are leading causes of the infections in the respiratory track that are manifested by nasal blockage, rhinorrhea, cough, etc. An average adult with a properly functioning immune system will be affected by these viral infections from two to four times a year on average, whereas a child six to ten times a year [1, 4]. Seasonal or perennial allergic rhinitis (AR) is another common disease of upper airways with similar symptoms, that affects more than 30% of individuals from childhood through the senior years. The highest prevalence of AR is reported in the developed nations of the Western world, with 20-30% affected individuals in Europe and the USA. Much greater diversity in the prevalence is found in the non-Western world and it ranges from 2 to 54% in different countries. There are little inter-racial variations, adults suffer more often from perennial AR, while children and adolescents from seasonal AR [5-7]. Similar to viral infection of the airways, individuals with AR suffer from nasal congestion, headache, fatigue, and cognitive impairment. Both viral infections of the upper airway and AR significantly affect daily activities and business - related loss of productivity and are serious socio-economic burdens leading to a worldwide loss of billions of euros annually [8].

Links between viral infections and allergy are well described in literature. On top of the scientifically documented examples, some of the patients visiting a clinic in Amsterdam claim that their or their child’s allergy began with a very serious flu that would later on turn into a chronic but seasonal cold. Allergic patients do seem to suffer somewhat more from common colds and their recovery may be prolonged [9]. Foster and colleagues have shown that RSV triggered inflammation of upper airways can be modulated by biologically active indoor air contaminants [10]. In their study, pre-challenge of airway epithelium with Der p1 allergen enhanced the level of cell infection with a virus and modulated the expression of IL-6 and RANTES. Consequently, Chun et al demonstrated that epithelium infected with HRV changes
its cytokine expression profile after co-challenge with Der f1 [11]. In this thesis I have shown that the overlapping molecular mechanism of the allergic and anti-viral responses of epithelium may contribute to the existence of symptoms described by patients and further supports previous observations in the literature. In the chapter 3 we demonstrate the impaired anti-viral responses of epithelium challenged with the HDM-allergen. Diminished production of IP-10, IL-12, and RANTES, mediators necessary for virus clearance [12-14] in an allergy setting may result in defective anti-viral responses within the local tissue, hence weakening overall Th1 adaptive responses. Importantly, cytokines mediate immune cells recruitment to the inflamed tissues and have a potency to control the production of other mediators [15]. For example, Graham and colleagues have shown that during a respiratory tract infection, mast cells accumulating in the airway release cytokines and mediators that may enhance the co-existing allergic inflammation [16].

A part of this thesis is focused on the commonalities of responses of airway epithelial cells to an allergen and to a viral analogue. To understand how similar responses to an allergen and a virus are, we followed up a study of Vroeling et al. [17] that compared responses of epithelium from lower and upper airways to the HDM-allergen and revealed a common core consisting of transcription factors and mediators. Expression profiles of the genes from the core response indicated that allergen challenge of epithelium might affect cell responses to viruses, as HDM challenge resulted in a down-regulation of TLR-3 expression. The documented links between allergy and viral infections led us to study the commonalities of cell responses to those two triggers in a greater detail [4, 18-20]. In the chapter 3, we demonstrate that responses of airway epithelium to the HDM-allergen and a viral analogue poly(I:C) are indeed very similar, both for the common core transcription factors and a profile of (pro-inflammatory) mediators. More importantly, our data revealed a two-phase transcriptional response, overlapping for both triggers: rapid responders with among others, the EGR-1 and DUSP-1 factors described in the chapter 4 of this thesis, were induced quickly after the challenge and a delayed one with NFKB and AP-1 family members, defining the permanently activated allergic state in HDM allergic individuals [21] induced relatively late. The balance between the (inflammatory) immune responses and mechanisms that shut the inflammation down is critical for maintaining of a homeostatic state of the local tissue [22, 23]. The maximal gene expression level of the early up-regulated factors (EGR-1 and DUSP-1), whose role was previously reported [24] to down-regulate inflammatory processes takes place much earlier than of the peak up-regulation of ‘late phase’ (chapter 3) pro-inflammatory transcription factors and mediators (NFKB1 or IL6). This provides enough time for the translation of the early up-regulated genes, so that they would be able to down-regulate the potentially following NFKB responses and therefore balancing the inflammatory responses and preventing it from uncontrolled escalation.

A relation between a viral infection and allergy is described in literature as protective or promoting [25]. The protective effect of viral infections in early childhood on allergy development may be explained by the hygiene hypothesis [26]. Repeated exposures to viruses shape the immune responses towards Th1 rather than Th2, which may prevent from the uncontrolled sensitization to harmless particles [27]. On the other hand, viral infections, especially with RSV or rhinoviruses of the respiratory tract in older children and adults may aggravate the allergic sensitization or inflammation [28]. In this thesis, I show that expression of another pro-inflammatory mediator IL-8 was significantly enhanced in allergy setting. IL-8 production by airway epithelium is modulated by RSV [29], influenza virus [30], human rhinovirus [31], but
also by measles virus [32]. The data in the chapter 3 demonstrate the overall increased inflammatory responses to a virus within the local tissue, on the other hand, the anti-viral part of this response becomes significantly down-regulated. Our observations have been reflected by Rochlitzer and others. They showed that in a chronic asthma model, an infection with HRV does not lead to asthma exacerbation per se, but it is the impaired anti-viral response that results in defective inflammatory responses to the virus [33].

In the chapter 3 and 4 we have not attempted to investigate how epithelial cell responses affect downstream processes or what their overall contribution to a fully developed immune response is. However, the cytokine and gene expression profiles combined with the available literature do indicate or even identify the processes that they may shape.

**Control of the (allergic) inflammation in airway epithelium**

Related to inflammatory immune responses, most available data focus on the role and function of immune cells in the context of antigen recognition, activation, and contribution to the clearance of the antigen [34-36]. Somewhat less attention has been paid to epithelial cells, despite the fact that it is the outermost cell layer and the first one to encounter environmental triggers (reviewed in chapter 2 of this thesis). The major components of the innate immunity of the airways include: mucociliary apparatus, adherens-, gap-, and tight junctions, and anti-microbial peptides [3]. Disruption or impairment of any of these components enables the entrance of a microorganism or a potential immunogen through epithelium and penetration of local tissues [37].

Intracellular junctions of airway epithelium create structural adhesive forces between the neighbor cells and form an elastic tissue layer that separates the underlying tissue from the outer environment. Disruption of the airway epithelial barrier has been associated with pathology of respiratory track [38]. For instance, down-regulation of the expression of ZO-1, E-cadherin, and occludin in airway epithelium of asthmatic individuals has been shown in in vivo and in vitro models [39, 40]. The barrier damage may be directly caused by the proteolitic activity of the major allergen components, deregulated expression of junction proteins, or impaired E-cadherin membrane trafficking by down-regulation of caveoline-1 expression that regulates cell-cell contacts [41, 42]. Environmental pollutants may also contribute to disruption of cell junction and enhance the concomitant allergic inflammation. Cigarette smoke has been associated with an induction of epithelial cell gaps, decrease in trans-epithelial resistance, and an increase of an allergen uptake by epithelium [43]. Besides the barrier function, epithelium actively contributes to shaping of the immune responses after microbial or micro-particle challenge.

In this thesis, our experimental work focused on role of the epithelium as an active contributor to the (innate) immune responses (chapters 3-8). Additionally, I reviewed the literature for the dual role of epithelium acting as a passive or active barrier (chapter 2).

Numerous experimental setups utilize in vitro models of immune cells that are cultivated/expanded/challenged in (growth) medium without supplementation with tissue factors or mediators [44, 45]. Using these very simplified models is crucial for understanding the basics mechanisms of inflammatory responses and contribution of individual cell types to the inflammatory state, however oversimplified experimental settings may not reflect the in vivo situation of the pathology of a disease. In the chapter 4 I have demonstrated enhanced inflammatory responses of the EGR-1
and/or DUSP-1 airway epithelium mutant cell lines in response to HDM challenge with multitude of pro-inflammatory mediators as read-outs. These mediators may play an important role for shaping of the downstream processes in the local tissue upon the allergen challenge. The allergic inflammatory state of the nasal epithelium of HDM-allergic individuals is characterized by a perpetual activation of genes belonging to the NFKB transcription factors family regardless the presence or absence of the HDM-allergen. In contrary to this, the same group of genes in epithelium of non-atopic individuals is temporarily activated only after an exposure to the allergen [21]. The reasons for the persistent activation of the pro-inflammatory responses in the allergic epithelium are unclear. Since the experimental setup made use of cells cultured \textit{in vitro} for at least two weeks in a commercially available growth medium prior to the HDM challenge, cell responses to HDM were not directly affected by the local tissue environment. It is therefore very likely that an intrinsic cell feature rather than influence of the local environment are responsible for the dysregulated cytokine profile. Indeed, when we look further into gene expression profiles of epithelium, there is a group of genes that defines the differences between the allergic and non-allergic epithelium. Expression of, among others, EGR-1 and DUSP-1 is significantly enhanced by HDM-challenge in healthy epithelium, while in allergic epithelium the same group of genes fails to be amplified when exposed to HDM. The EGR-1 and DUSP-1 have previously been associated with negative regulation of inflammatory responses [24]. Therefore, the dysregulated expression of EGR-1 and/or DUSP-1 may be involved in maintaining of the activated transcriptional state of allergic epithelium.

It has become clear that allergy cannot be defined by the presence or absence of cell responses to an allergen, since both non-allergic and allergic individuals do respond to the allergen challenge [21, 46]. Instead, differences in these responses indicate the allergic status. For a development of the anti-parasitic type 2 immune responses that are directed against a harmless allergen, at a certain point of the response, an informed decision must be made that would instruct the body whether inflammation, rather than tolerance would be more suitable for a specific (potential) immunogen. What skews this decision towards the type 2 response remains unclear. Some data demonstrate that concurrent respiratory virus infections enhance sensitization to aeroallergens [47], while another study blames the impaired expression of epithelial tight junctions proteins [48] for easier allergen penetration. Other reports demonstrate that the imbalance between Treg and Th2 cells may trigger the hypersensitivity of our immune system [49, 50], while other demonstrate that healthy individuals do not develop the Th2 allergen-specific clones [51], which is somewhat contradictory to the study showing the presence of the allergen specific IgG4 in the bloodstream of non-allergic individuals [46]. Whether dysregulated EGR-1 and/or DUSP-1 expression that leads to a permanently activated inflammatory state in allergic epithelium should also be considered as a potential decision making factor, remains to be validated.

\textbf{Cross-talk of epithelial receptors and inflammation}

In everyday life we are exposed to a multitude of environmental triggers of bacterial, viral, or fungal origin. Therefore, the ultimate cytokine profile that shapes the downstream pro-inflammatory responses in the local tissue should be determined not by one receptor, but rather by the co-operative interaction between multiple receptors. The innate immunity plays a pivotal role in a rapid and non-specific pathogen eradication and its function strongly depends on multiple cells, such as

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macrophages, NK cells, innate lymphoid cell, or epithelial cells. Some of these cells can instantly kill pathogens, while other cells produce cytokines and mediators that attract more specialized immune cells to a local tissue and may modulate their function. Upon invasion of the human body, pathogens are rapidly recognized by multitude of microbial sensors present on epithelial cells and antigen presenting cells. The most predominant group of receptors involved in microbes’ recognition by airway epithelium are Toll-like receptors [52, 53].

In this thesis, I explored the role of the TLR-2, a receptor recognizing bacterial cell wall components, and TLR-3 – activated by viral dsRNA, on shaping of the innate immune responses. We are perpetually challenged by multiple microbes and responses to these triggers may affect each other. In the chapter 5 we demonstrated the functional cross-talk between TLR-3 and TLR-2. Stimulation of TLR-3 with a viral analogue leads to a significant up-regulation of the TLR-2 mRNA, which consequently is translated into an increased functionality of TLR-2 when this receptor is stimulated by a bacterial trigger. Such a synergistic collaboration of microbial sensors may imply in vivo consequences. Since bacterial co-infections during an on-going viral infection are relatively common [54], amplification of anti-bacterial responses by virus-triggered TLR-3 may be a protective mechanism acquired through out centuries by a human co-existing with surrounding microbiota. Whether this mechanism is sufficient for the protection against invading bacteria and if concomitant disease may impair these enhanced synergistic responses remains unclear. It has been demonstrated that a temporary breakdown of the airway epithelium barrier induced by a viral infection may enhance a susceptibility to bacterial transmucosal incursion, which in turn may further promote the on-going inflammatory condition [4, 55]. In this light, superantigen from S. aureus was reported to strengthen an allergic inflammation [56] that may explain the enhanced susceptibility to invasive pneumococcal disease in individuals with asthma [57]. It would therefore be interesting to verify if and how the atopic state affects the responses of epithelium to subsequent challenge with a virus and bacteria.

A single pathogen is recognized by multiple microbial receptors on epithelium and antigen presenting cells and activation of these sensors defines the ultimate immune response. In chapter 6 I demonstrate that inflammatory responses of epithelium upon bacterial challenge are not only attributed to the interaction of receptors belonging to the PRR family. TLR-4, together with CD14, and MD-2 form the LPS-binding complex which is necessary for the activation of pro-inflammatory responses to the major component of Gram negative bacteria LPS in majority of human cells [58]. Here, I show that FcγRIII, a high affinity receptor for immunoglobulin G, and TLR-4 co-operative activation plays a critical role for the induction of pro-inflammatory cytokines production upon P. aeruginosa and LPS exposure in nasal epithelium. Cross-talk between PRRs and FcγRs is not restricted to epithelium, but is a common feature of numerous antigen presenting cell subsets, such as dendritic cells [59], monocytes, and macrophages [60, 61]. Unlike PRRs, FcγRs does not bind PAMPs. Most likely, because of an on-going or previous bacterial infection, specific anti-bacterial IgG circulates in the bloodstream and resides in local tissues, for example in the nasal mucosa [62, 63]. Remarkably, exposure of nasal epithelium to P. aeruginosa or LPS alone is not sufficient for cell responses induction. Unresponsiveness to LPS has also been demonstrated in gut epithelium as a consequence of the absence of MD-2 [64], very low expression of TLR4 [65], or by the spatial regulation of TLRs expression with TLR4 expressed only on the basolateral surface of ileal crypt enterocytes in the human fetus [66]. Patients
with an active Crohn’s diseases display a high expression level of TLR4 on the apical side of colonic epithelial cells [67]. Only a combination of LPS or *P. aeruginosa* and IgG initiates the pro-inflammatory cytokine cascades, which suggests a relative tolerance of nasal epithelium to this pathogen and/or LPS that can be broken only by co-presence of IgG within the local tissue.

Whether IgG opsonization modulates epithelial cell responses to other bacterial species or other pathogens remains to be verified. IgG opsonization of pathogens requires that microbe-specific IgG are pre-existent in the blood or mucosal secretions at the time of infection [68]. The nasal cavity is colonized by numerous bacterial species [69]. It seems very likely that IgGs against many different bacterial species are present at the time of (re-)infection, which would allow a selective opsonization and triggering of PRRs and FcγRs. On the other hand, whether the colonizing or invading bacteria are continuously opsonized with IgGs remains unclear.

Consistent with the indiscriminative recognition of PAMPs by TLRs, nasal epithelium would be expected to be constantly activated or inflamed due to the presence of bacterial triggers in the nose. Given the fact, that the nasal flora is largely enriched with (opportunistic) pathogens, such responses would disadvantage the host and the mechanism relying on synergistic activation of TLR and FcγR might prevent fortuitous activation of inflammatory responses in the local tissues [70].

**Collaboration of epithelium with the type 2 innate lymphoid cells**

Next to the cross-talks between receptors, collaborative interactions of different cell types are crucial for shaping of the ultimate host responses to infections or immunogenic triggers challenges. These type of interactions maybe be beneficial for the host, on the other hand may as well strengthen pathological inflammatory processes. In chapter 7 we demonstrate that nasal polyps of CRS individuals are enriched with a novel immune cell – type 2 innate lymphoid cells. Innate lymphoid cells emerge as important players in innate immunity and lymphoid tissue formation [71, 72, 76]. They are characterized as innate equivalents of lymphocytes T, since they originate from the common lymphoid progenitor and their function is regulated by the same transcription factors and cytokines as in T cells [73]. The absence of T markers rules out any antigen specificity and highlights their functions within innate immunity. ILC2s possess the TSLP receptor and their function essentially depends on the GATA3 transcription factor [72]. TSLP drives the expression of GATA3 and thereby enhances the production of the archetypal type 2 cytokines. In the western world, CRSwNP, but also allergic rhinitis or asthma are typical type-2 mediated diseases where elevated TSLP have been reported to play a critical role in their pathology [1, 74]. TSLP was associated in promoting the polarization of naïve T cells into Th2 cells via enhanced expression of OX40L on dendritic cells [75], strengthening its role in atopy. We show that freshly isolated ILC2 produce high amounts of IL-5, IL-13, IL-9, GM-CSF, and perhaps IL-4 in response to *in vitro* TSLP and IL-33 challenge, without their prior activation. These cells are therefore capable of directly responding to TSLP, unlike Th2 cells, where T-cell receptor activation is required for GATA3 mediated cytokine production [73]. In an approach to identify the source of TSLP within the local tissue of nasal polyps, in chapter 8 I demonstrate that epithelial cells derived from polyps produce significant amounts of the TSLP protein after the viral analogue challenge. This finding could not be reproduced in epithelium from healthy controls, where some up-regulation of TSLP at the mRNA level could only be observed. Since TSLP is associated with Th2 skewing, its induction by a viral
analogue may seem somewhat counterintuitive. At the clinical level there are already many parallels between viral infections and allergy ranging from the induction of allergic exacerbations by viral infections [28] and in chapter 3 of this thesis we demonstrate molecular links between allergy and viral infection. In this light we can understand that a viral analogue can induce the Th2 skewing mediator TSLP. In chapter 8 we show more evidence strengthening the idea of common molecular pro-inflammatory responses to viral and allergen triggers. Just like in HDM allergic individuals, the baseline expression of the DUSP-1 transcription factor is significantly lower in polyp epithelium than in healthy control group. Impaired regulatory mechanism of inflammation control may contribute to enhanced TSLP production by nasal polyp epithelium and therefore reduce cell capability of tuning the viral-induced pro-Th2 inflammatory responses down.

What shapes the Th2 environment in the pathology of CRSwNP remains largely unexplored. When we think of ILC2 enrichment in the nasal polyp tissue and their activation by epithelial secretions, we may assume that ILC2 propagate and perhaps even polarize type-2 responses. On the other hand, CRSwNP in individuals born and residing in Asia displays Th1 of mixed Th1/Th2 phenotype, while symptomatically the disease remains comparable to those in the Caucasian race [1]. It remains unclear however, whether nasal polyps from Asian CRSwNP individuals are enriched with ILC2 or not.

**CONCLUDING REMARKS**

In this thesis we described and discussed the role of airway epithelium in the local innate immune responses. We have shown that epithelial cell responses to allergens, viruses, and bacteria are more complicated than previously assumed and simplistic models that study cell responses to only one trigger may not reflect the *in vivo* setting where tissue is exposed to multitude of stimulants. In this context, we demonstrated the importance of synergistic responses of viral and bacterial triggers, but also how responses to HDM-allergen and to viruses affect each other. Future studies are necessary to identify other cooperating and non-cooperating (families of) receptors, which may further help explain the cytokine responses and provide new perspectives for therapies or medication development.

We have also demonstrated the indirect collaboration of epithelium with ILC2s in pathology of CRSwNP resulting in shaping of the type-2 immunity in the local tissue of nasal polyps. Future identification of mediators that govern and maintain the ILC2 phenotype in polyps and also targeting ILC2s themselves and cytokines that they produce may provide us with better understanding of the pathology of CRSwNP and translate this knowledge into new therapy guidelines.

Lastly, we identified EGR-1 and DUSP-1 as important regulators of (allergic) inflammation. Reduced expression of these factors is implicated in maintaining of the constantly activated inflammatory state in HDM-allergic individuals and reduced baseline expression of DUSP-1 may play a role in enhanced TSLP production by individuals suffering from nasal polyposis. Identification of molecules that would enhance baseline expression of these two transcription factors would be an interesting target for a development of (personalized) medication for affected individuals.


33. Rochlitzer, S., et al., No exacerbation but impaired anti-


64. Lenoir, C., et al., MD-2 controls bacterial lipopolysaccharide
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