Regulation and cross-talk between environmental triggers of local immune responses in airway epithelial cells
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Citation for published version (APA):
Chapter 1
General introduction and scope of the thesis
INTRODUCTION

The human body is challenged on a daily base by a multitude of invading pathogens, allergens, and potentially immunogenic triggers. There are many routes for entry of a microbe or an immunogen into a human body, however most of them infect or trigger responses via inhalation or aerosol contact with the respiratory tract.

Since airway epithelial cells are located at the interface with the outside environment, they are the first line of defense against these potential threats [1]. Through functional pattern recognition receptors (PRRs), such as Toll-like receptors, airway epithelium is able to detect, recognize, and respond to invading immunogens by activation of trigger-dependent pro-inflammatory pathway networks. These activated signalling cascades induce a production and secretion of pro-inflammatory cytokines and chemokines that create a microenvironment in the local tissue that subsequently attracts and modulates functions of other immune cells [2, 3] (Figure 1).

Inflammatory responses of airway epithelium are very complex. Composition of an inflammatory cocktail produced by epithelial cells strongly depends on the nature of the trigger, its concentration within the local environment, pre-existing or on-going inflammatory processes in the tissue, or concurrent presence of other stimulants. What makes the inflammatory responses even more difficult to model are the intra-individual differences, where epithelial cells from different individuals may respond differently to the same trigger even if an exposure takes place in very similar setting [4-6]. For example, in some asthmatic individuals, a viral infection with human respiratory virus (HRV) or respiratory syncytial virus (RSV) will trigger a life threatening exacerbation, while in others only of a mild severity occurs [7-11].

Although the exact impact of epithelial cells on development, severity, and management of upper-airways diseases is not entirely clear, we do know that airway epithelium plays an important role in shaping of the local environment of affected tissues. Epithelium possesses intrinsic gene expression profile perhaps controlled at the epigenetic or autocrine level that may define the outcome of the trigger-epithelial cell interaction and production of downstream effector compounds [12, 13].

Allergic state and inflammation

Allergic inflammation is characterized by an influx of immune cells to allergen-challenged local tissues. A primary encounter between an allergen and mucosa triggers epithelial cells to produce and secrete cytokines and mediators that will activate and/or attract other immune competent cells such as dendritic cells (DCs) or innate lymphoid cells (ILCs) to the site of the allergen entry [14, 15]. Attracted dendritic cells sample vigorously the local environment for allergens, while activated ILCs produce archetypal type-2 cytokines (IL-5, IL-13, and perhaps IL-4) to further polarize immune responses [16]. Once DCs take up the allergen, they migrate to the lymph nodes where epitopes of the allergen are presented to naive T-cells. Such an event skews the T cell towards T helper 2 phenotype that in turn will activate B cells to produce allergen-specific antibodies. [17, 18]. Upon release of the antibodies into the bloodstream, they are loaded onto the effector cells: eosinophils and mast cells [19]. When the same allergen comes in contact with mucosa for the
second time, epithelial cells will again produce mediators that attract previously primed immune cells. This time, the allergen encounters mast cells or basophils that already possess the antigen (or allergen) specific IgE antibodies bound to the high affinity receptors FcεR on their surface. The symptomatic allergic reaction is triggered when the allergen binds to the surface-bound IgE followed by mast cell degranulation and histamine release [20].

**Figure 1.** Type 2 immune responses to allergens or parasites. Schematic overview of the processes that take place during the primary allergen/parasite encounter (sensitization) and elicitation of immune responses.

Despite the fact that only some individuals will develop fully blown type-2 allergic response, which in fact is a misused anti-parasitic immune response against a harmless allergen, airway epithelial cells from both allergic and non-allergic individuals do respond to an allergen challenge [21-26]. These responses differ tremendously however between the two groups. A very remarkable difference between epithelium obtained from healthy or allergic subjects is that the birch pollen allergen (bet v1) binds to epithelial cells only in allergic, but not in healthy individuals [27]. Until now, this unique observation has only been demonstrated for the birch allergen. Another noteworthy dissimilarity is based on the proteome analysis of nasal epithelium. It demonstrates that allergen-challenged healthy control subjects show protective and anti-inflammatory effector molecules production promoting mucus and mucosal homeostasis, whilst in patients with allergic rhinitis – a clear dysregulation of cysteine proteases expression is observed, which may affect the stability of the epithelial barrier [27]. Our research group studied extensively the differences of nasal epithelial cell responses from
healthy or HDM-sensitized individuals to HDM and our findings further explain the distinctness of the two groups. We have shown that airway epithelial cells from house dust mite (HDM) allergic individuals are in a constantly activated inflammatory state regardless the presence or absence of the allergen. Non-allergic epithelium turns on the inflammatory machinery (NFκB or genes from the AP-1 transcription factor family) only in the presence of HDM. Another group of genes that defines the differences between HDM allergic and non-allergic epithelium are transcription factors (among others: EGR-1 and DUSP-1) that are known for down-regulation of inflammatory responses. In non-allergic epithelium, an expression level of these negative regulators is triggered dramatically upon exposure to HDM, while in allergic individuals – the same transcription factors fail to be up-regulated when challenged with HDM. Moreover, the baseline expression of these negative regulators is lower in allergic epithelium [13]. Remarkably, the latest data suggest that the activated inflammatory state is also present in epithelium of Timothy grass pollen allergic individuals (personal communication A.M van Kuijen).

**Viral infection and allergy exacerbation**
Clinical links between viral infections of the respiratory tract and allergy are very well established. In allergic individuals, the clearance of a viral infection is somewhat prolonged and the manifestation of an infection may be more severe [28, 29]. Conversely, respiratory virus infection may contribute to allergic sensitization to allergens and the development of asthma and may also trigger a life threatening allergic exacerbation in asthmatic patients, since viruses may prevent the induction of tolerogenic responses [30-32].

The mechanism of anti-viral and allergic inflammatory responses interplay is not fully understood. We know that enhancing the access of aeroallergens and allowing them to penetrate mucosal tissues. In turn this facilitates an enhanced recruitment of dendritic cells to the mucosal sites and augment the possibility of allergic sensitization [33-35].

Recent studies demonstrate a molecular link between allergy and viral infections. Impaired release of IFN-family cytokines in response to a rhinovirus challenge is observed in individuals with chronic allergic inflammation [36]. Moreover, airway epithelial cell exposure to HDM results in a down-regulation of genes that are linked to the defense against viral infection: TLR-3, TICAM-1, and IVNS1ABP [12]. Importantly, TLR-3 triggering feeds into the transcription factor complex that is activated upon allergen exposure [12, 13, 37].

**Inflammatory responses of airway epithelium to microbial triggers**
Perpetual exposure of airway epithelium to variety of microbial and environmental stimuli leads to an activation of pattern recognition receptors mediated pro-inflammatory pathways. PRRs recognize conserved motives that are associated with bacteria, fungi, or viruses and among others, the most predominant group of PRRs are Toll-like receptors that are present on and in epithelial cells.

Despite the fact that in daily life airway epithelium is exposed to a multitude of triggers, most of available data focus on cell responses to a specific trigger that stimulates a single TLR [38-41]. These simplified models prevent us from understanding the impact of potential cross talks between
different PRRs upon simultaneous microbial challenges. We know that cell exposure to some single TLR agonists indirectly deregulates the expression of other TLR and from studies in bronchial epithelium we learned that epithelial cells exposed to viral RNA analogue (TLR-3 agonist) affects TLR-2 expression and further cell responses to bacterial cell wall components [42, 43]. It is unclear whether this functional collaboration between TLRs can be extended to nasal epithelium and what consequences for subsequent microbial challenges it may have.

Responses of airway epithelium to bacterial triggers are more complex than previously considered. Despite the presence of the LPS receptor complex (TLR-4, CD14, and MD-2) [44], prolonged exposure of nasal epithelium to a Gram negative bacterium *Pseudomonas aeruginosa* or to its major cell wall component LPS is not sufficient for induction of cell pro-inflammatory responses. On the other hand, similar challenge of epithelial cells from the lower airways triggers a rapid production of pro-inflammatory mediators. The immune system is able to discriminate pathogens from harmless microbes based on, among others, distinct molecular signature that triggers responses mediated through one or multiple PRRs [45]. Moreover, mechanisms of a pathogen recognition differ between tissues [46]. Potential immunogens may be tolerated in the nose, whereas in the lung a full host-defense immune response against the same microbe may be developed. This peculiar observation may explain why *P. aeruginosa* is usually well tolerated in the nose, but its rare presence in the lung initiates a full anti-bacterial innate response [47].

To explain why a stimulation of the TLR-4 complex alone in nasal epithelium by microbial challenges is insufficient for inflammation development, one has to keep in mind that the ultimate cell response to a trigger relies on the co-operative collaboration between multiple cell receptors. Synergistic responses to TLR agonists and immunoglobulin G (IgG) have been demonstrated to regulate inflammatory responses of dendritic cells and macrophages [48, 49]. Since active infections are characterized by the presence of IgG in the bloodstream and tissues, the potential role of the interaction between human IgG and bacteria in triggering the inflammatory responses of nasal epithelium may be of interest.

**Cross-talk of epithelium and innate lymphoid cells in CRSwNP**

Chronic rhinosinusitis (CRS) is a multifactorial inflammatory disease of upper airways that affects globally more than 200 million individuals and is defined by the presence of inflammation of the nose and paranasal sinuses [50, 51]. One of CRS variants, chronic rhinosinusitis with nasal polyps (CRSwNP), is a typical type-2 mediated disease and the local tissue affected by the disease is enriched with multitude of immune cells contributing to extensive inflammatory processes [52]. Remarkably, nasal polyps tissue is also significantly enriched with recently described type 2 innate lymphoid cells (ILC2), cells that origin from a common lymphoid progenitors, produce cytokines similar to those produced by lymphocytes, yet lack the T cell receptor, precluding any antigen specificity and emphasizing their innate immunological function [53-55]. ILC2 mediate parasite expulsion but also contribute to airway inflammation, strengthening the functional similarity between these cells and Th2 cells. These cells, similar to Th2 cells, produce IL-5, IL-13, and perhaps IL-4 and
rely on the GATA-3 transcription factor required for the archetypal type-2 cytokines production [56, 57]. Cytokines and mediators present in the local tissue environment of nasal polyps mediate activation of GATA-3. Whether the major source of these cytokines may be epithelial cells remained unclear.

SCOPE OF THE THESIS

The main goal of the work described in this thesis is to investigate the role of airway epithelial cells in the local regulation of the innate immune responses in relation to allergy and other airway diseases.

In chapter 2, we describe airway epithelium as a passive and active barrier when encountering an allergen and how this encounter triggers pro-inflammatory allergic responses.

In chapter 3, we seek to investigate to what extend the pro-inflammatory responses induced in airway epithelial cells by viral infections and by allergen exposure are similar. In this study, we established a temporal expression pattern of selected pro-inflammatory transcription factors and production levels of cytokines in a bronchial epithelial cell line in response to a house dust mite (HDM) allergen and a viral analogue and a TLR-3 agonist polyinosinic:polycytidylic acid [poly(I:C)]. We also seek to identify a molecular mechanism by which viral infections and responses to an allergen may affect each other.

In chapter 4, we investigate in greater detail the previously demonstrated activated allergic state and verify if the transcription factors EGR-1 and DUSP-1 act as negative regulators of an allergic inflammation. Since responses of epithelium to HDM and a viral analogue that are shown in the chapter 2 share a very similar molecular mechanism, we also investigate how dysregulated expression of EGR-1 or DUSP-1 contributes to anti-viral inflammatory responses. Additionally, we look into a potential clinical consequence of dysregulated expression of EGR-1 and DUSP-1 by measuring the effectiveness of steroid treatment in suppressing inflammatory responses.

In chapter 5, we continue to investigate epithelial cell responses to viral triggers, however, to mimic a real life constant exposure to variety of microbial challenges, we subsequently expose epithelial cells to viral and bacterial stimuli. Here, we explore the functional interaction between TLR-3 and TLR-2 in primary nasal epithelial cells and we seek whether the functional collaboration of TLRs can be extended to lower airways, in the lung epithelium cell line.

In chapter 6, we expand our repertoire of collaborative interactions between different epithelial receptors. In this part of the thesis, we seek to investigate how the tolerance of epithelial cells to Gram negative bacterium P. aeruginosa and/or a TLR-4 agonist LPS challenge is broken by co-stimulation with immunoglobulin G. We also explore the TLR and FcγR cross-talk in relation to Gram positive bacterium S. aureus challenge.

Since the local tissue environment consists of epithelial cells that cooperate directly or indirectly with other immune cells, we want to explore the importance of epithelium in polarization and activation of other immune cells in pathogenesis of upper airway diseases. Therefore, in chapter 7, we
explore the enrichment of nasal polyps of patients with chronic rhinosinusitis, a typical type-2 mediated disease with type 2 innate lymphoid cells (ILC2). We seek to explore the role of cytokines present in the nasal polyp local tissue environment, potentially produced by epithelium, in activation and polarization of ILC2. We also try to identify essential transcription factors for the function of ILC2.

In chapter 8, we expand the findings from the chapter 6 by verifying if epithelial cells are indeed the major source of the type 2 skewing cytokines necessary for ILCs activation. Here, we explore the reactivity of epithelial cells isolated from nasal polyps and healthy controls to broad range of TLR agonists in their ability to inducing the expression and production of TSLP, IL-33, and IL-25. As some of the nasal polyp individuals have concurrent allergies, we also seek to investigate whether EGR-1 and DUSP-1, negative regulators of pro-inflammatory responses demonstrated in the chapter 3, affect TSLP, IL-33, and IL-25 responses.

REFERENCE LIST


53. Miljkovic, D., et al., *Association between group 2 innate lymphoid cells enrichment, nasal polyps and allergy in chronic*


