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Molecular mechanisms of epithelial host defense in the airways

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CHAPTER 1

GENERAL INTRODUCTION AND SCOPE OF THE THESIS

INTRODUCTION

The human lungs form one of the largest surfaces that is in continuous contact with the outside world and constitute an estimated surface area of 100 m^2 that comes into contact with inspired air. Every day, approximately 10.000 liters of ambient air are inhaled, containing numerous potentially harmful pathogens such as bacteria, yeast, fungi, small parasites and viruses. Although the warm and humid environment within the lung creates favorable conditions for microbial growth, severe lung infections are rare in otherwise healthy individuals. This is thanks to the activity of an efficient host defense system that is operative in the lung. This chapter provides an overview of this host defense system, with a focus on the airway epithelium, and describes the scope of this thesis.

HOST DEFENSE AND THE IMMUNE SYSTEM

Elimination of pathogens is one of the main tasks of the host defense system. The effectiveness of this system is shown by the fact that the vast majority of pathogens are cleared by the host defense system before they can cause infection. The body's defense system consists of non-specific and specific components.

The epithelial tissues that constitute the interface with the external world form a physical barrier against infection and employ the innate immune system as a means of host defense. The physical barrier formed by these tissues is regarded as the first line of defense, and in the airways is complemented by the mucociliary clearance. Particles that are trapped in the mucus layer that covers the epithelium of the conducting airways are removed by the coordinated action of ciliated epithelial cells. The epithelial tissues also contribute to the second line of defense which is formed by the evolutionary ancient innate immune system. These lines of defense are relatively non-specific in nature, but provide the host with immediate and instant protection against microbes. The adaptive immune system forms a third line of defense that provides protection against pathogens that have not been effectively dealt with by the first lines of defense. The adaptive immune system is capable of recognizing and eliminating the causative pathogens with extremely high precision using antibodies and T cell receptors as antigen-specific receptors. The adaptive immune response is mediated by antigen presenting dendritic cells and lymphocytes and takes several days to develop. Under physiological conditions the combined actions of the host defense system provide sufficient protection to prevent the host from infection with encountered pathogens. Indeed, in healthy individuals host defense is very effective and most people only occasionally suffer from infectious diseases such as the common cold or the flu. However, when host defense mechanisms

are compromised, recurrent respiratory infections may occur. This is illustrated by the frequent occurrence of respiratory infections in transplant recipients that receive immunosuppressive drugs, cystic fibrosis patients and smokers.

Epithelial tissues play an important role in inflammatory and infectious diseases, because they form the first line of cells that encounter inhaled microorganisms, particles and gases and are now recognized as central regulators of inflammation and immunity [1]. Recent genetic studies also highlight the central role of these cells, not only in cystic fibrosis but also in atopic disease [2]. Therefore, studies into epithelial function in inflammatory and infectious lung disease are central to our understanding of the pathogenesis of these diseases.

THE AIRWAY EPITHELIUM: STRUCTURE

The conducting airways of the lungs are lined by a pseudostratified epithelium. This type of epithelium is predominantly formed by four cell types: ciliated epithelial cells, mucus-producing goblet cells, non-ciliated bronchiolar Clara cells and basal cells. A schematic representation of the airway epithelium structure is shown in ***Figure 1***. The ciliated cells of the conducting airways are columnar epithelial cells actively involved in the removal of inhaled microbes and other particles from the airways. Furthermore, ciliated epithelial cells produce host defense effector molecules that serve to prevent the airways from infection. The goblet cells and non-ciliated bronchiolar Clara cells are

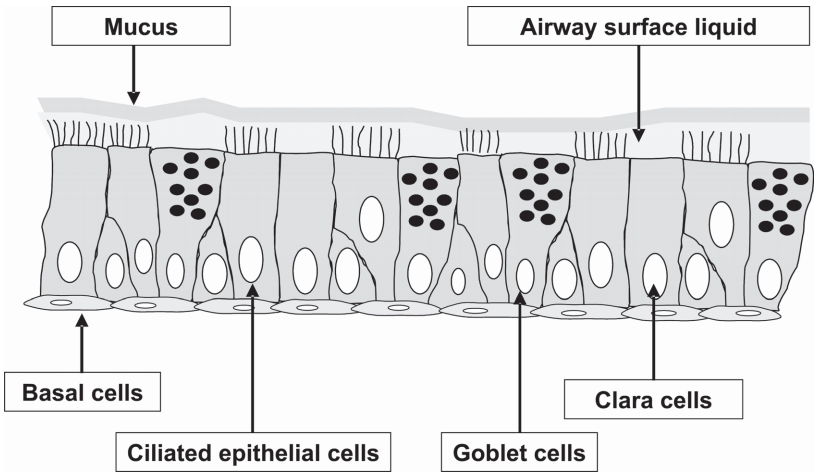

Figure 1: Schematic representation of the airway epithelium. The pseudostratified airway is mainly formed by four cell types: basal cells, ciliated epithelial cells, goblet cells and Clara cells. The airway epithelium is covered by a thin layer of airway surface liquid (ASL) composed of a periciliary layer around cilia and microvili, and an overlying mucus layer. This separation of the ASL into two layers allows the cilia to beat and to move mucus into which inhaled particles have been trapped [4].

two other main cell types of the airway epithelium of the lungs. Goblet cells secrete the mucus components of the extracellular airway surface liquid (ASL). The Clara cells also secrete components of the ASL and metabolize and detoxify inhaled toxic compounds. The ciliated epithelial cells, mucus-producing goblet cells, non-ciliated bronchiolar Clara cells and the basal cells are attached to a basal membrane.

The bronchial airway epithelium is covered by a dual-layered airway surface liquid of about 10 µm deep that is composed of a periciliary layer around cilia and microvili and an overlying mucus layer. This separation of the ASL into two layers allows the cilia to beat and to move mucus into which inhaled particles have been trapped [3,4]. Hydration of this layer is a main determinant of the effectiveness of mucus clearance, a process that is also regulated by ciliary activity and mucus production. Dehydration of the ASL in patients with cystic fibrosis results in impaired mucus clearance and recent studies show that this process can be reversed at least in part by inhalation of hypertonic saline [5]. ASL contains soluble factors that inhibit bacterial growth, amongst others lactoferrin, lysozyme and other antimicrobials [6]. Mucus is the upper and most viscous phase of the ASL that serves to trap inhaled microorganisms and other particles. In larger airways, mucus is predominantly produced by mucous glands and to lesser extent by goblet cells. The periciliary liquid layer forms the lower fluid phase that directly covers the epithelium on which the mucus layer rests and allows the cilia to move mucus [3,4]. This fluid phase is comprised of water, ions, and other molecules of which some are essential in host defense such as antimicrobial (poly)peptides and proteinase inhibitors [7].

THE AIRWAY EPITHELIUM: ROLE IN INNATE IMMUNITY

The airway epithelium provides an effective first line of defense in three ways: *(i)* physical barrier formation and mucociliary clearance; *(ii)* production of antimicrobial substances; and *(iii)* release of cytokines and chemokines. The primary function of the epithelium in the upper airways is to remove potentially harmful pathogens and particulate matter through mucociliary clearance [8]. Cilia on the surface of ciliated epithelial cells beat synchronically to remove inhaled particles from the airways. The vast majority of inhaled microorganisms and particulate matter are cleared through this mechanism.

Second, cells of the airway epithelium contribute to chemical barrier formation by producing host defense effector molecules such as proteinase inhibitors and antimicrobial (poly)peptides. Proteinase inhibitors protect against the proteolytic activity of proteinases released by microorganisms and inflammatory cells whereas antimicrobial peptides exert microbicidal activity [1]. Selected proteinase inhibitors such as secretory leukocyte proteinase inhibitor (SLPI) and elafin (SKALP/PI3) also display antimicrobial activity. Upon inhalation, the presence of microbes in the airways is sensed by epithelial

cells through pattern recognition receptors such as Toll-like receptors. These receptors enable to distinguish pathogens or microbial products based on of their chemical and structural properties [9]. Epithelial cells generally respond quickly to the invading pathogen by inducing effector mechanisms of the epithelial innate immune response: enhanced production of antimicrobial (poly)peptides, proteinase inhibitors and increased release of cytokines [1]. Antimicrobial (poly)peptides and proteinase inhibitors are released to the apical side of the cells into the ASL whereas cytokines are predominantly released at the basal side of epithelial cells.

A number of families of antimicrobial peptides has been identified, including defensins [10] and cathelicidins [11]. For a growing number of these molecules, multiple functions have been described including immune signaling, proteinase inhibitory activity and the capacity to enhance tissue regeneration [12]. Proteinase inhibitors are a second class of host defense effector molecules that is produced by epithelial cells. SLPI and elafin are major representatives of this class of effector molecules that serve to inhibit both microbial and host proteinases, thus restricting tissue injury and microbial colonization. Like defensins, proteinase inhibitors may also display multiple functions as demonstrated by the antimicrobial activity of both SLPI and elafin [13]. Some antimicrobial (poly)peptides such as lactoferrin and lysozyme are constitutively expressed in the epithelium and submucosal glands and thus provide a permanent protective shield. Expression of other antimicrobials, such as β-defensins [14,15], is specifically induced upon exposure to respiratory pathogens.

A third mechanism of host defense is provided by the production of immune signaling molecules such as cytokines and chemokines. Cytokines and chemokines are small proteins that mediate the communication between cells of the immune system. Cytokines function to alert the immune system whereas chemokines attract immune cells to the site of infection. The array of immune signaling molecules produced by epithelial cells includes the cytokine interleukin-1β (IL-1β) and the chemokine interleukin-8 (IL-8). IL-1β is essential in the onset of the inflammatory immune response [16], whereas IL-8 attracts neutrophils that frequently accumulate in the lungs during inflammation [17].

LARGE-SCALE GENE EXPRESSION PROFILING IN THE AIRWAYS

Although it is well recognized that airway epithelial cells fulfill central functions in host defense, our understanding of the molecular mechanisms that underlie the initiation of the epithelial host defense response is limited. Until recently, identification of (novel) molecules associated with epithelial host defense was achieved by hypothesis-driven research approaches. Candidate molecules were selected beforehand for subsequent functional analysis to confirm or disprove their contribution in epithelial host defense.

Recent advances in molecular biology have led to the development of experimental high-throughput technologies to determine gene expression at large scale. Genomics techniques such as DNA microarray analysis [18] and Serial Analysis of Gene Expression (SAGE) [19] enable the efficient assessment of the transcriptome (all genes expressed) within a single experiment. An interesting feature of these technologies is that they allow expression analysis of novel genes that were revealed by the human genome project [20,21]. Although the function of many novel genes is still unknown, their expression can be studied in biological models since their nucleotide sequence is known. This way, (novel) genetic pathways that are associated with cellular activities can be unraveled. A detailed overview of these technologies is provided in **Chapter 2** of this thesis.

As mentioned, epithelial-derived effector molecules such as antimicrobial (poly)peptides, proteinase inhibitors and immune signaling molecules are known to be involved in the epithelial host defense system in the human airways. We hypothesized that other (novel) molecules could also contribute to epithelial host defense, including genes that were previously not associated with this process. To make the initial "genetic inventory", we selected SAGE as high-throughput profiling technology. Despite the completion of sequencing the human genome, our knowledge of the repertoire of human genes is still incomplete. Since SAGE allows gene discovery, this method is highly suitable to identify the association of both known and unknown genes with epithelial host defense. An additional advantage is that SAGE data are ideally suited for comparative research as discussed in **Chapter 2**.

SCOPE OF THE THESIS

Epithelial cells are well-equipped with host defense mechanisms that can be either activated upon direct contact with inhaled pathogens or indirectly through activated macrophages ***(Figure 2)***. Macrophages that are activated by respiratory microbial pathogens release pro-inflammatory cytokines including IL-1β and tumor necrosis factor alpha (TNFα). These cytokines are potent stimulatory molecules that initiate the epithelial host defense response [22,23]. Epithelial cells respond to these alarm signals by the release of an array of antimicrobial peptides, proteinase inhibitors, cytokines and chemokines. Nevertheless, it is largely unknown which molecules are required for initiating the innate immune response. Insights into the molecular mechanism underlying the onset of the epithelial host defense may provide novel intervention targets for the treatment of recurrent pulmonary infections. The aims of the research presented in this thesis are *(i)* to delineate the early host defense response in epithelial cells upon microbial exposure using SAGE; and *(ii)* to identify (novel) epithelial-derived host defense effector molecules.

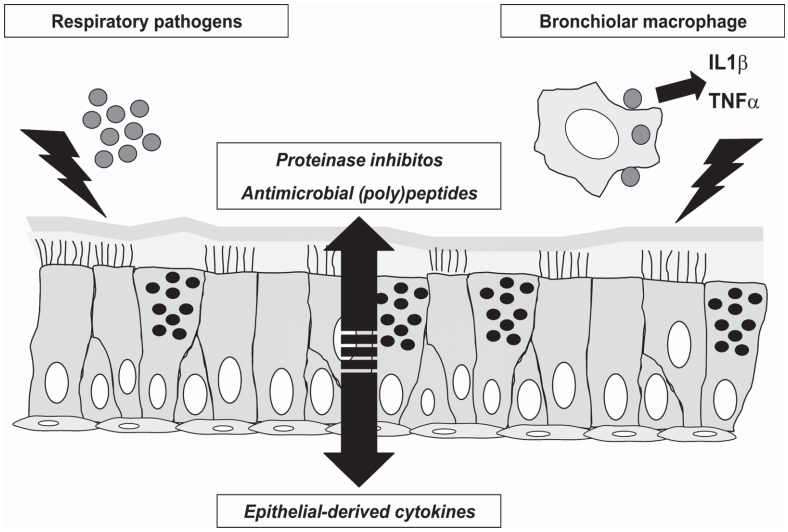

Figure 2: Mechanisms involved in the induction of the epithelial host defense response upon exposure to microbial respiratory pathogens. Microbial respiratory pathogens can induce an epithelial host defense response upon direct interaction with airway epithelial cells. Alternatively, pro-inflammatory cytokines released by bronchiolar macrophages that have recognized respiratory pathogens also effectively activate the epithelial host defense machinery. Upon activation, epithelial cells release host defense effector molecules such as proteinase inhibitors and antimicrobial (poly)peptides mainly at the apical side. Epithelial-derived cytokines are released mainly at the basal side of the epithelium

Application of SAGE and DNA microarray technology has been recently introduced in the area of host-pathogen research. As described, these technologies are powerful methods to identify and delineate (novel) genetic pathways associated with epithelial host defense. **Chapter 2** first provides an overview on available high-throughput gene expression profiling technologies and their application in the field of host-pathogen research. **Chapter 3** describes the SAGE analysis that was performed to delineate the early host defense response in epithelial cells upon microbial exposure. Briefly, *in vitro* subcultures of human bronchial epithelial cells were exposed to *Pseudomonas aeruginosa* and the pro-inflammatory cytokines IL-1β and (TNFα). After 6 hours of exposure, the repertoire of genes expressed was profiled using SAGE.

Chapter 4 describes the regulation of epithelial expression of the S100 calcium-binding proteins S100A8 and S100A9. These molecules were identified as two of the most transcribed and differentially expressed genes by bronchial epithelial cells upon exposure to *P. aeruginosa* as assessed by SAGE. The heterodimeric protein complex formed by S100A8 and S100A9 is a potential novel effector molecule in host defense. We hypothesized that S100A8/A9 contributes to the early epithelial host defense response. Therefore, we assessed the expression dynamics of S100A8 and S100A9 and investigated whether bronchial epithelial cells were able to synthesize and release the S100A8/A9 protein complex upon microbial exposure.

Because of the comparable functions in host defense, we hypothesized that different epithelia employ similar strategies to protect against microbial influences. Despite functional similarities of epithelia of different organs, only few epithelial-specific protective mechanisms are known. **Chapter 5** describes a comparative analysis of the patterns of antimicrobial (poly)peptides in mucosal secretions that were derived from various mucosal sites. We hypothesized that each mucosal secretion is characterized by a unique pattern of antimicrobial (poly)peptides, specifically tailored to defend its particular mucosal site. **Chapter 6** describes a comprehensive comparative analysis on SAGE data sets derived from two established culture models of epithelial inflammation using bronchial epithelial cells and keratinocytes. This chapter also demonstrates the advantages of the SAGE technology for comparative genomics. **Chapter 7** concludes this thesis with a summary and discussion of the described investigations, followed by future perspectives.

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