On Mechanisms Modulating Pulmonary Innate Immunity and Tissue Injury

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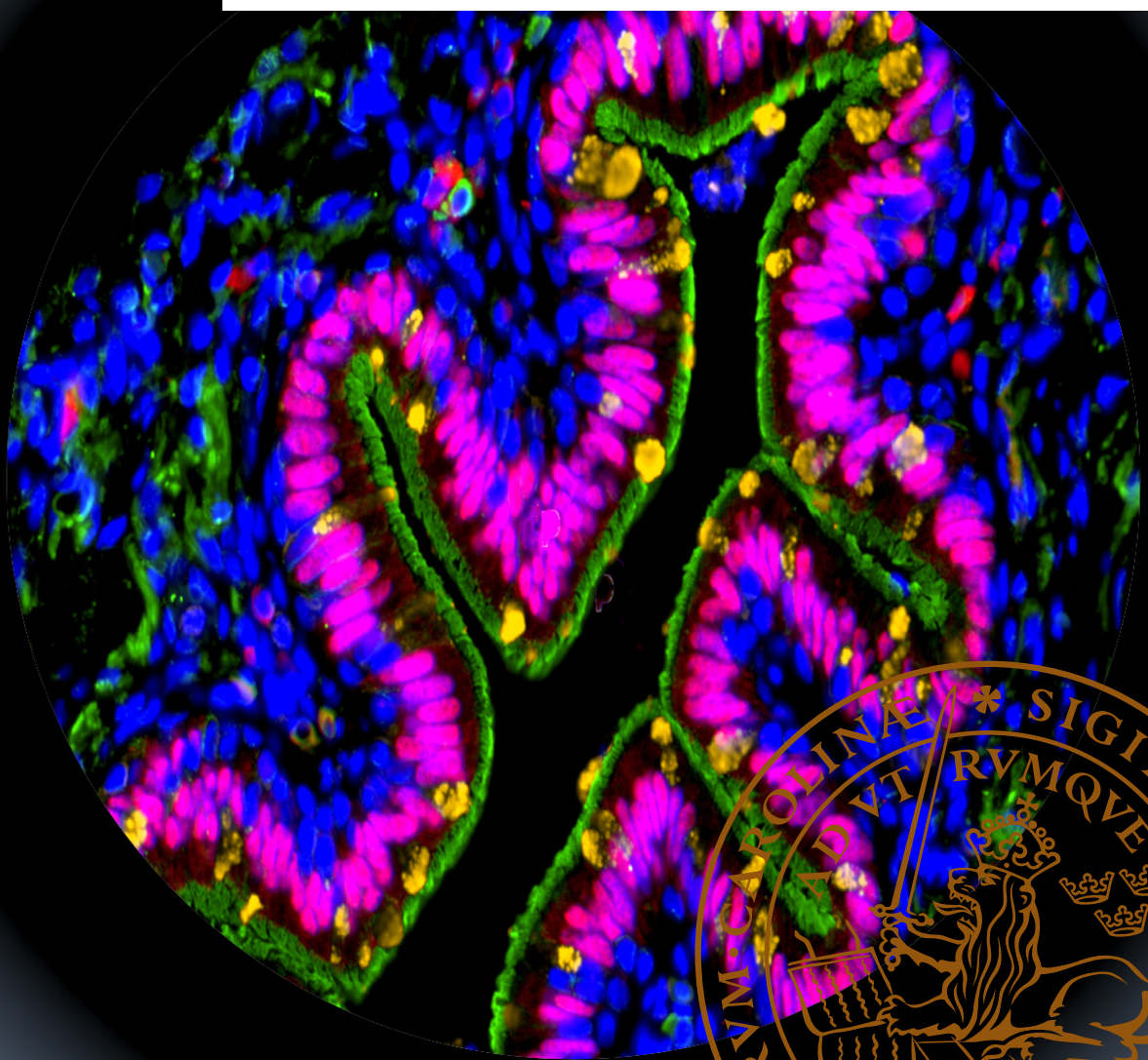

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Mohamad N. Ali

LUND
UNIVERSITY

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Abstract

The innate immune system is essential to keep us healthy in an environment that constantly present threats to our physical integrity and homeostasis. It consists of many branches, including granulocytes, epithelial barriers, and antimicrobial peptides. Not least the respiratory tract is subject to continuous challenge by harmful agents, for example pathogenic bacteria, viruses, and harmful agents such as those of cigarette smoke. To meet the assaults, inflammatory responses are mounted by the immune system. However, modulating mechanisms and resolution are important since longstanding and excessive inflammation cause tissue injury and remodeling. If not, inflammation may lead to a dysregulated host defense and impaired respiratory function as seen in diseases such asthma, COPD, and cystic fibrosis (CF).

Here, we investigated innate host responses and how these could be regulated and dampened to avoid damage to host tissues. In particular with a focus on osteopontin (OPN), a protein that is abundantly expressed in the airways. First, the source of OPN, i.e. the distribution of OPN-producing cells, in small airways was investigated. An increased number of OPN-expressing cells was seen in COPD. The production was confined to basal cells, goblet cells, as well as club cells but not ciliated cells. Furthermore, cigarette-smoke up-regulated OPN-production and promotion of goblet cell differentiation by IL-13 increased the production. OPN is heavily phosphorylated, a feature important for its activities, for example interaction with integrin-bearing immune cells. Tartrate-resistant acid phosphatase (TRAP) 5 is an enzyme having a preference for OPN as a substrate. We could show a redistribution of TRAP5 in CF airways where it co-localized with OPN in small airways and it was detected in sputum of both healthy individuals and CF patients. However, in sputum of CF patients it was subject to proteolytic degradation. During inflammation, tissue-damaging cationic histones are released to the extracellular environment in the airways. Using *in vitro* and *in vivo* models of airway inflammation, it was demonstrated that OPN has an important role, protecting host tissues during inflammation. The results also suggested that the phosphorylation status of OPN is important. Gram-negative bacterial infections, often caused by *Pseudomonas aeruginosa,* are commonly seen in CF and severe COPD. In another set of experiments, it was demonstrated that tissue factor pathway inhibitor-2 (TFPI-2), an endogenous anticoagulant, exerts antibacterial activity against *P. aeruginosa* through binding of immunoglobulins, in particular IgG, and subsequent activation of complement. Correspondingly, *TFPI-2*$^{-/-}$ mice were more susceptible to pulmonary *P. aeruginosa* infection, demonstrating the *in vivo* importance. Taken together, this thesis shows important aspects of airway innate immunity and its modulation.

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To my family for their love and support

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List of papers

Paper I **Ali MN**, Mori M, Mertens TCJ, Siddhuraj P, Erjefält JS, Önnerfjord P, Hiemstra PS, Egesten A. Osteopontin Expression in Small Airway Epithelium in Copd is Dependent on Differentiation and Confined to Subsets of Cells. *Scientific Reports,* 2019; 9: 15566.

Paper II **Ali MN**, Clausson CM, Andersson G, Egesten A. Tartrate Resistant Acid Phosphatase 5 is Increased in Cystic Fibrosis Airways and Co-localize with the Phosphoglycoprotein Osteopontin. (Manuscript)

Paper III Kasetty G, Papareddy P, Bhongir RKV, **Ali MN**, Mori M, Wygrecka M, Erjefält JS, Hultgårdh-Nilsson A, Palmberg L, Herwald H, Egesten A. Osteopontin Protects Against Lung Injury Caused by Extracellular Histones. *Mucosal Immunology,* 2019; 12: 39-50.

Paper IV **Ali MN**, Kasetty G, Elven M, Alyafei S, Jovic S, Egesten A, Herwald H, Schmidtchen A, Papareddy P. TFPI-2 Protects Against Gram-negative Bacterial Infection. *Frontiers in Immunology*, 2018; 9: 2072.

Abstract

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Introduction

The Anatomy of the Airways

The respiratory system consists of various organs that work with each other to support our bodies with oxygen. The air flows in through the nasal cavity all the way down to the alveolar sacs, where the exchange of gases, mainly O_2 and CO_2, takes place. The respiratory tract can be divided into the upper and the lower airways. The upper airways begin from the nasal cavity and extends over the nasopharynx, the oropharynx and the larynx. Whereas the lower airways consist of the trachea which bifurcates at the carina into two main bronchi, each supplying one lung with air. The bronchi subdivide many times as it enters the lung, creating a multibranched system of tubules resembling an inverted tree. This results in a decreased diameter of the airways as it goes from the main bronchus to the conducting bronchioles, terminal bronchioles and respiratory bronchioles that eventually gives rise to alveolar ducts from which the alveolar sacs bud (Figure 1).

Structure of the Airway Epithelium

The adult human airways are covered with a continues epithelial sheet of cells that are pseudostratified in the large airways, and are columnar and cuboidal in the small airways [1]. The cells can be classified into three main categories, i.e. basal, ciliated, and secretory cells, the latter including goblet and club cells (Figure 1) [2].

Columnar ciliated cells

Ciliated cells dominate and make up over 50% of the epithelial lining. The primary role of these cells is the transportation of mucus from the lungs to the throat with the help of the beating movement of the cilia present on the cells' surfaces [3, 4].

Goblet cells

Goblet cells are mucus secreting cells that are responsible of maintaining the correct viscoelasticity of mucus on the apical cell epithelial lining to ensure efficient mucociliary clearance [5]. In healthy individuals, the epithelium undergoes a short-term goblet cell hyperplasia during inflammation, resulting in a temporary increased mucus production. This will facilitate the clearance of a pathogen through the mucociliary movement. In contrast, in chronic airway inflammatory diseases such

as chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and asthma, goblet cell hyperplasia is usually a characteristic of the disease that is accompanied with an excessive and persistent mucus secretion [6]. Although the mechanism of goblet cell hyperplasia in these inflammatory diseases has not been fully understood, there are several key signalling pathways that have been suggested to be involved, such as epidermal growth factor receptor (EGFR), interleukins (TH2 cytokines), signal transducer and activator of transcription 6 (STAT6), Notch and WNT signalling [7, 8].

Club cells

Initially described in 1937 by the Austrian anatomic pathologist Max Clara that was a Nazi supporter, and he named the cells after him "Clara cells". But because of his controversial history, the name has been changed and these cells are now named "club cells".

Club cells are non-mucus secretory cells that contain granules made up of proteins, glycoproteins and lipids. They represent the major secretory cells of the small airways in human, and have a distinctive smooth, dome-shaped apical surface [9]. The primary secretory product of club cells is uteroglobin (UTG), which harbors a hydrophobic cavity that binds and sequester potent lipid mediators of inflammation, such as prostaglandins (PG)D_2 and $PGF_{2\alpha}$ [10]. Moreover, UTG works as a substrate of transglutaminase, protecting the embryos from the maternal immunity assault [11], as well as having anti-chemotactic properties where it inhibits both the adherence and migration of neutrophils and monocytes [12]. In addition, club cells have substantial amounts of cytochrome P-450, through which it participates in the detoxification of many harmful and toxic compounds [13].

Basal cells

Basal cells are attached to the basement membrane of the epithelial lining [14]. These cells express the cytoskeletal proteins, cytokeratins 5 (KRT5) and 14 (KRT14), as well as high levels of transcription factor transformation-related protein 63 (p63), which is important for the development of the cells [15]. Basal cells are thought to have stem cell characteristics, giving rise to other cellular phenotypes after differentiation. Upon epithelial injury, basal cells initiate a rapid proliferation process to repair the damaged tissue [16]. Thus, these multipotent stem cells control the homeostasis of the normal epithelium. On the other hand, disruption of the normal balance between proliferation and differentiation of basal cells, might contribute to the initiation and progression of a disease [17].

Functions of the Airway Epithelium

The mammalian respiratory system is a dynamic organ that requires the full collaboration of all its cell types and tissues in order to maintain normal homeostasis in the lungs. Depending on the insult and the nature of the condition, epithelial cells can be activated and signal through several pathways. For example, the epithelium can expand a specific cell population that would be important to eradicate the threat, like increasing the release of interleukin-33 (IL-33) which promotes type 2 immune responses, that is characterized by the production of IL-13. This in turn, remodels the airway epithelium to a disease-specific phenotype, including goblet cell hyperplasia and mucus hypersecretion, that will facilitate the clearing of inhaled pathogens or particles from the lungs [18]. Moreover, the tumor suppressor *Trp*53 (Tp53) can maintain quiescence of club cell by regulating its expression upon epithelial stress. Previous studies suggested that high Tp53 expression generates more club cells, while low expression facilitates the club cell to ciliated cell differentiation [19]. Another aspect of the epithelium, is the activation of the adaptive immunity that will increase the production of B-cells, T-cells and T-helper type 17 (TH17) cells, the latter being a major source of the cytokine IL-17, that can enhance airway smooth muscle contraction and proliferation [20]. In addition, the epithelium can respond to an injury by recruiting and activating a variety of inflammatory cells, including neutrophils, macrophages and innate lymphoid cells [4, 21, 22]. Thus, the airway epithelium plays a major role in maintaining the wellbeing of the host, and the dysregulation of its balance, might greatly contribute to the pathogenesis of major lung disorders, including chronic obstructive pulmonary disease (COPD), asthma and cystic fibrosis (CF) [3, 21, 23].

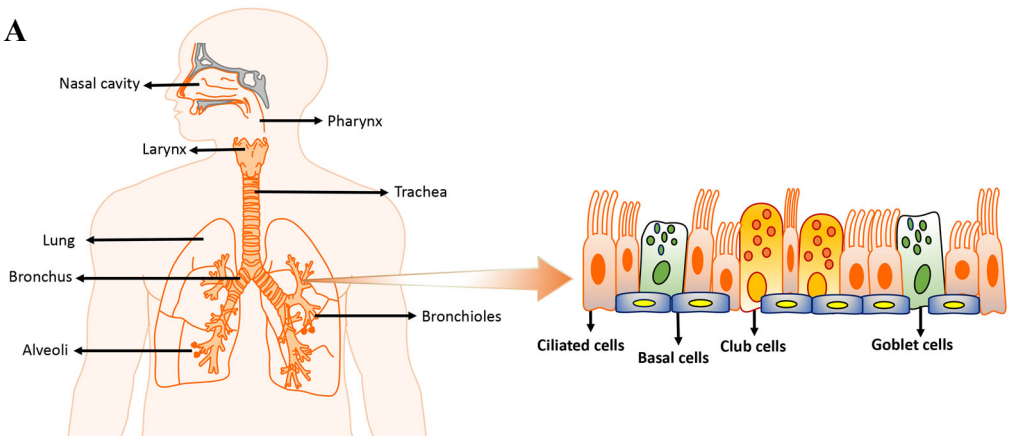

Figure 1. (A) Anatomy of the respiratory system. The arrow represent a section in adult epithelial airways where it shows the four major cell types (ciliated, basal, club and goblet cells).

B

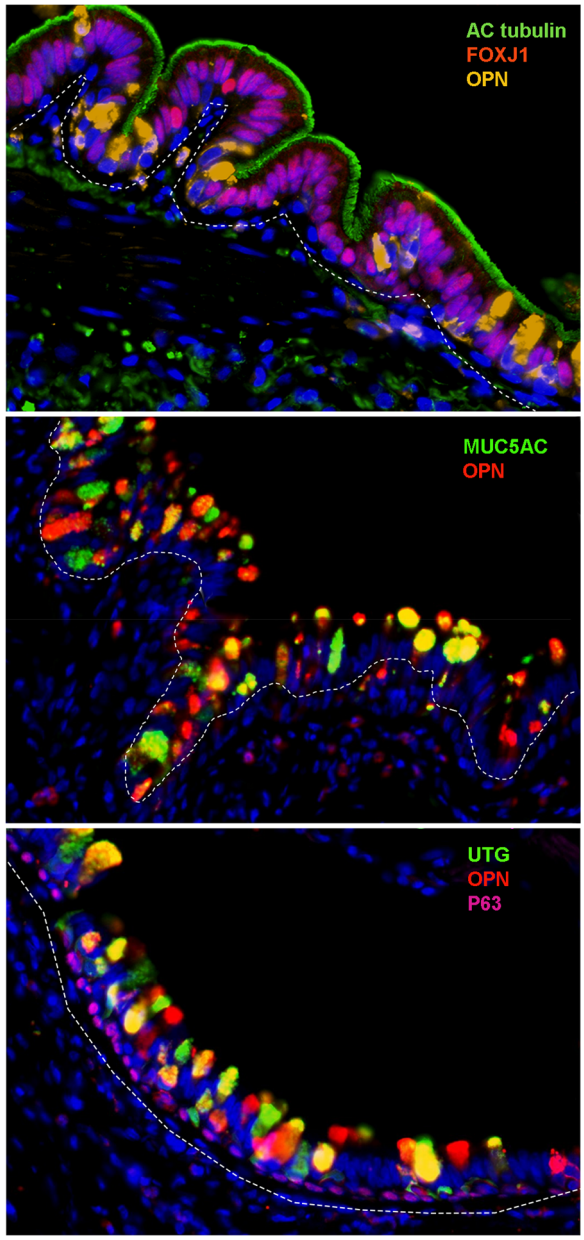

Figure 1. (B) Immunoflourescence microscopy of airway epithelium. Double or tripple staining of osteopontin (OPN) with the cell markers AC tubulin and FOXJ1 (ciliated cells), MUC5AC (goblet cells), uteroglobin (UTG) (club cells) and p63 (basal cells). Dotted lines represent basal membrane. A yellow color indicates presence of OPN in goblet and club cells respectively.

The Immune System

The immune system is a network of several organs, tissues and cells that orchestrate with each other to ensure the host protection from any invading bacteria, viruses and other harmful agents. Once a threat is detected, the immune system will initiate a scanning process where it evaluates the danger imposed by the invader and acts upon accordingly. The reactions are determined depending on which part of the immune system is engaged. Two main divisions lie under the umbrella of immunity, the innate and the adaptive, each of which can be further subdivided into humoral and cellular immunities (Figure 2) [24, 25].

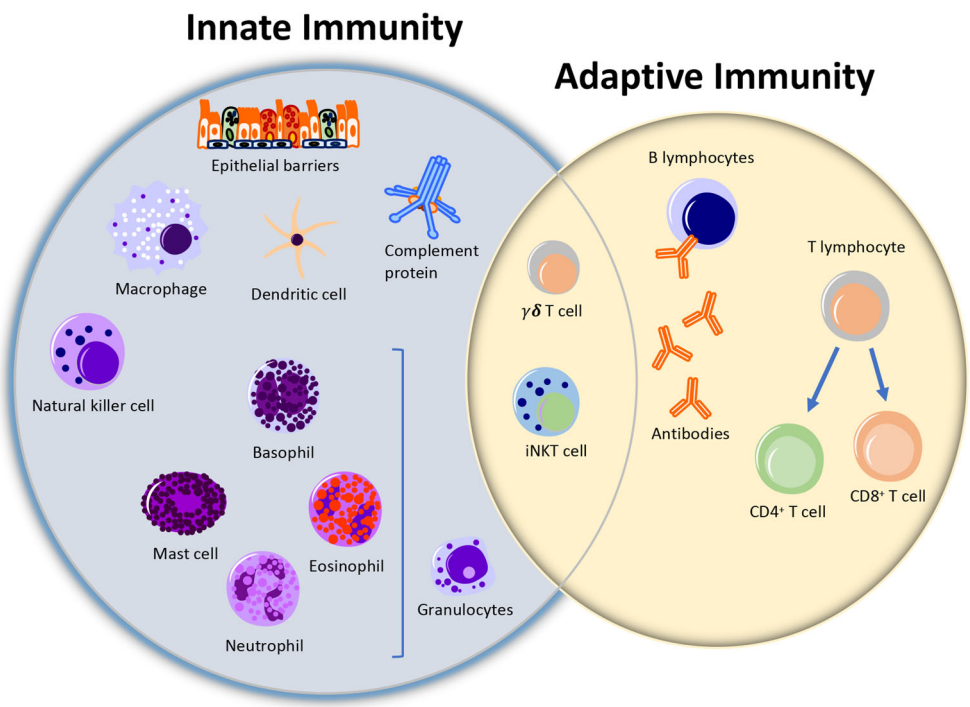

Figure 2. Illustrative figure presenting the components of innate and adaptive immunity, as well as the immune cells ($\gamma\delta$ T cell and Natural killer T cell) that bridge between these two system.

Innate Immunity

The innate immune system consists of physical barriers, humoral and cellular components, and is defined as the host's first line of defence against pathogens and other potential invaders. It is non-specific and provides a rapid response ranging from minutes to hours after the microbial invasion. It discriminates between self and non-self-molecules (e.g. pathogens), playing a critical role in optimizing the adaptive responses.

In the airways, the threats are recognized via a variety of receptors, including pattern recognition receptors (PRRs), such as Toll-like receptors (TLR), protease activated receptors (PAR), Nod-like receptors (NLR) and C-type lectin receptor. TLRs, such as TLR2 and TLR4 are expressed by epithelial cells and alveolar macrophages throughout the respiratory tract. The receptors are triggered by signals released by the invading pathogens, known as pathogen-associated molecular patterns (PAMPs) or from dying cells, i.e. damage-associated molecular patterns (DAMPs) [26, 27]. For example, TLR2 responds to a variety of microbial components, including lipoproteins from Gram-negative bacteria and peptidoglycan and lipoteichoic acid from Gram-positive bacteria [28]. TLR4 recognizes lipopolysaccharides (LPS), which is a major component of the outer membrane of Gram-negative bacteria [29]. These innate immune responses initiate a series of signalling cascades, which activates downstream genes encoding a broad range of pro-inflammatory cytokines, chemokines, antimicrobial peptides and complement factors [30, 31].

The Complement system

The complement system is evolutionary old and is a key component of the innate immune reaction. The system is activated in a cascade sequence, and has several important functions in innate immunity, through which lysis of bacteria, opsonisation of microorganisms, recruitment of inflammatory cells and microbial clearance, are the main ones. There are many components, composed of plasma and membrane bound proteins (designated as complement C1-C9) that take part in complement activation. These proteins usually circulate in the blood in their inactive form, and become activated due to specific cleavage (e.g. C3 to C3a and C3b; C5 to C5a and C5b). The complement activation is initiated via three major routes, the classical pathway, which is driven by antigen-antibody interactions, the alternative pathway by foreign cell surface constitutes, for example LPS, and finally the mannose-binding lectin pathway (MBL) which is stimulated by pathogen membrane containing carbohydrates like mannose. The end result of this activation, is the formation of membrane attack complex (MAC) composed of complement components (C5b, C6, C7, C8 and C9) that penetrates the microbial cell membrane and forming pores, leading to cell lysis and death. (Figure 3) [32].

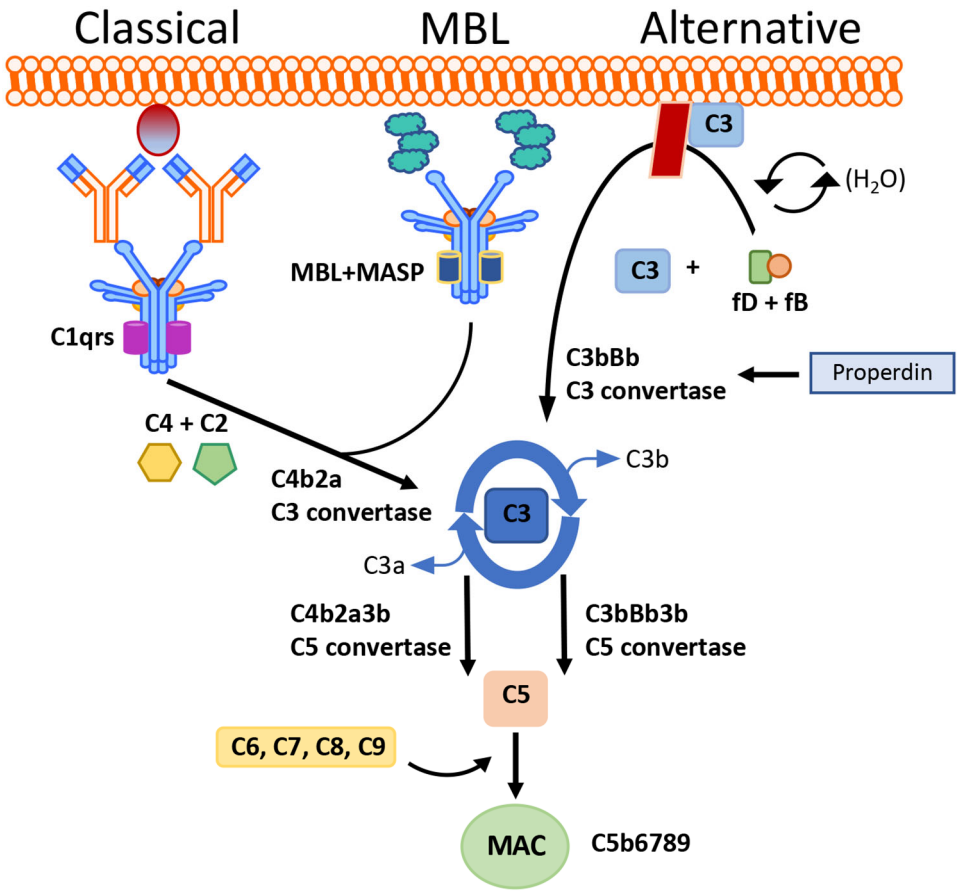

Figure 3. Overview of the complement cascade. (1) The classical pathway, activated when C1q binds to the Fc regions of IgG or IgM. (2) The mannose-binding lectin (MBL) pathway, activated when binding to bacterial carbohydrate motifs (including mannose). (3) The alternative pathway, initiated when C3b molecule binds to a foreign body. The end result is the formation of membrane attack complex (MAC), which induces cell lysis by making pores in the cell membrane. Membrane associated serine proteases, MASP; fD, factor D; fB, factor B.

Antimicrobial peptides

Antimicrobial peptides (AMPs) is one, among a variety of mechanisms, protecting the lungs against the continuous exposure of pathogens that are brought in by inhalation. These peptides belong to humoral immunity and are highly conserved in vertebrates. AMPs can be categorized into several subgroups depending on their structure (amphipathic α-helical, β-sheets, anionic, etc.), as well as on their amino acid composition. They are usually short cationic peptides consist of 15-100 amino acids, and include amphipathic regions [33]. The major AMPs found in the lungs include the neutrophil α-defensins (HNPs), human β-defensins (e.g, hBD-1, hBD-

2, hBD-3) and the cathelicidin LL-37 which is primary secreted by neutrophils during inflammation. In addition, lysozyme, lactoferrin and secretory leukocyte proteinase inhibitor are produced in the lungs [34]. These AMPs are expressed either constitutively or are induced by some kind of a challenge, for example microbial pathogens, allergens, injury or any stimuli resulting in expression of cytokines through activation of the transcription factor NF-κB [35]. AMPs display several types of defenses such as antibacterial, antifungal and antiparasitic activities through a variety of mechanisms including destabilization and pore formation in the bacterial membrane, protein aggregation and target blocking. Moreover, AMPs can play a critical role in shaping the immune responses, having pro- and anti-inflammatory properties, chemotactic activities and boosting the complement activation through recruiting its components [36-38].

Granulocytes

Granulocytes, also known as polymorphonuclear leukocytes (PMN), are white blood cells that are characterized by the presence of granules in their cytoplasm. They have a distinguishable multi-lobed shape nucleus. PMNs are the most abundant leukocytes in healthy adults, and can be classified into four types:

Neutrophils, constitute approximately 70% of leukocytes in the peripheral blood. These granulated cells are produced in the bone marrow and have a short life cycle. The neutrophilic granules can be divided into, primary granules (containing cationic defensins and myeloperoxidase), secondary granules (lactoferrin and serine proteases), and tertiary granules (gelatinase, collagenase and matrix metalloproteinases). Neutrophils play a critical role in the host responses against invading pathogens, where they are one of the first inflammatory cells to migrate from the circulating blood to infected tissue. They kill and clear microbes through several functions, including phagocytosis of a pathogen forming a phagosome, followed by the fusion of lactoferrin (iron chelator) and lysozyme (peptidoglycan degradation). In addition, neutrophils can trigger oxidative granules and initiate respiratory burst to produce reactive oxygen species (ROS) that limit bacterial growth [39]. Another important mechanism is the release of neutrophil extracellular traps (NETs), that occurs primarily through NETosis (a specific cell death process). These NETs are large web-like structures that are mainly composed of nucleic DNA, containing cytosolic and granule proteins. They have the ability to neutralize and kill bacteria [40], fungi [41], viruses [42] and parasites [43].

Eosinophils, in contrast to neutrophils, represent a minor component of circulating leukocytes and have longer life cycle. They are differentiated in the bone marrow and distributed in several organs and tissues including the lung, the gastrointestinal tract, the spleen and the adipose tissues [44]. Eosinophils are involved in managing a variety of immune responses, as they modulate lymphocyte recruitment, can serve as antigen-presenting cells, promote TH2 polarization, and many other functions [45]. Upon stimuli, the type 2 inflammatory responses are initiated, and eosinophils

are recruited from blood circulation to the inflammatory site through eosinophil-specific chemokines (eotaxins), which are induced by the cytokine IL-13 [46]. Moreover, the differentiation, priming and survival of eosinophils are promoted by IL-5, an eosinophil-specific cytokine produced by Type 2 T helper cells [47].

Basophils are short-lived cells (their life-span is less than one week) that constitute below 1% of the circulating leukocytes. They can act as antigen presenting cells, and have antiparasitic functions. Basophils play a role in immediate hypersensitivity and inflammatory reactions through their ability to bind immunoglobulin E (IgE) and, upon cross-linking of IgE, release the content of cytoplasmic granules including histamine and proteolytic enzymes. They also have the ability of secreting IL-4 and IL-13, thereby contributing to Th-2 immune responses [48].

Mast cells are derived from bone marrow progenitors that are in common with basophils. Mast cells accumulate at sites of inflammation associated with atopy, wound healing and at mucosal surfaces, for example in the eye, and in the respiratory and gastrointestinal tracts. They differentiate with the help of growth factors, including IL-9, IL-10, IL-3 and IL-33. Mast cell granules contain a variety of specific mediators such as, histamine, heparin, serine proteases, tryptase and chymase. They also express receptors that can bind IgE, IgG, and complement, that upon activation trigger release of granule contents [49].

Adaptive Immunity

The adaptive immunity has evolved to provide a more specific and efficient responses in comparison with the innate immunity. In contrast to the innate immunity, the adaptive immune responses are slower, typically taking 4-10 days, but are more accurate and provides an immunological memory that protects the host against re-occurring infections. The primary immune cells are the B and T lymphocytes. These cells are involved in a complex interplay with the antigen-presenting cells, facilitating pathogen-specific reaction. Once a pathogen is ingested by the antigen-presenting cells, a custom-tailored receptor for this specific antigen will be presented on the cell's surface. This will soon be recognized by T lymphocytes that will get activated, and initiate downstream inflammatory responses, including cytokine recruitment or stimulating B lymphocytes to produce antigen-specific antibodies [50].

In the Lungs

The respiratory system contributes to host defence by a variety of mechanisms, including the barrier function of epithelium, production of antimicrobial peptides and proteins, as well as a range of cytokine and chemokines. These actions can contribute in host responses directly, and/or through augmenting responses via the

recruitment of a variety of immune cells, like dendritic cells and alveolar macrophages which are the first cells to encounter inhaled antigens, recruitment of effector cells (e.g. neutrophils and eosinophils) and chemokines, as well as activation of complement factors (Figure 4) [51-53]. Also, immunoglobulin (Ig)A is locally secreted at the mucosal surface and provides protective mechanisms against bacterial invasion and inhaled antigens [54]. Moreover, B and T lymphocytes can be found in the airway lamina propria and in submucosa, and might play a significant role in local immunological homeostasis in the respiratory tract [55, 56].

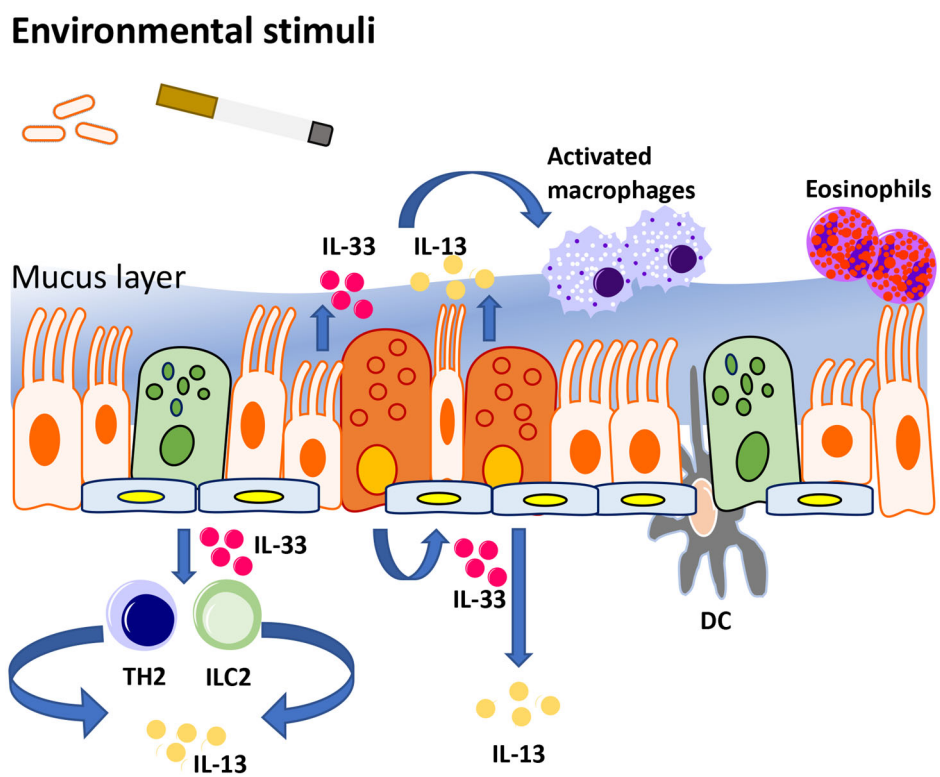

Figure 4. Schematic figure of type 2 airway inflammation. Upon stimulation with enviromental stimuli, such as allergens or smoke, epithelial cells, macrophages and dendritic cells (DC) will be activated. IL-33 is released from activated epithelial cells, leading to an autocrine activation, as well as activating Th2 cells and type 2 innate lymphoid cell (ILC2). This will result in the release of IL-13 and other type 2 cytokines that will shape the allergic airway inflammation characterized by airway eosinophilia, goblet cell hyperplasia and mucus hypersecretion

Alveolar macrophages

Alveolar macrophages (AM) are the most abundant immune cells in the distal lung. They have a long lifespan and relatively low turnover rate [57]. Although there is no definite proof whether AM originates from one source, many studies suggest that circulating monocytes give rise to AM [58]. Their differentiation from monocytes to AM is facilitated by granulocyte-macrophages colony-stimulating factor (GM-CSF) secreted by alveolar type II cells [59]. AM are the first to sense and respond to any possible threat, and help initiating and managing the immune responses in the lung. In addition, AM have other non-immune functions, like maintaining the homeostasis, clearing apoptotic cells and cellular debris [60, 61]. Despite of the numerous studies on characterizing AM, these cells could not be completely placed in either of the two main macrophages subtypes, which are M1 macrophage and M2 macrophage. During lung inflammatory diseases, it seems that AM share many of the M2 type features, like being induced generally by interleukin IL-4 and IL-13, contributing to wound healing, work as an anti-inflammatory agent, express the mannose receptor (CD206) and tumour necrosis factor-β (TGFβ) [62, 63].

Alveolar macrophages express, among other proteins, osteopontin (OPN) and tartrate resistant acid phosphatase 5 (TRAP5) [64-66], which are of particular importance in our studies. Both proteins have shown to be elevated in airway inflammatory diseases such as in COPD, CF and asthma. It has been suggested that these proteins play roles in the dysregulated inflammation seen in these diseases [67, 68, 66, 69].

Osteopontin

Osteopontin (OPN) is a large (314 amino acids) highly anionic phosphorylated glycoprotein which is secreted by a variety of cells, including epithelial cells, activated T cells, B cells, natural killer (NK) cells, dendritic cells and activated macrophages [70, 65, 69]. It is a member of the SIBLING (small integrin-binding ligand N-linked glycoproteins) family, and its sequence contains calcium binding sites, a thrombin cleavage site and two consensus heparin binding domains [71-73]. Initially, OPN was described as an extracellular matrix protein and studied almost exclusively in association with bone homeostasis and disease, but later on, it was found to be widely expressed in human tissues, and in particular at luminal epithelial surfaces [74]. Later studies demonstrated that OPN is a multifunctional protein, playing an important role in the host immune responses. Its functions ranging from being pro-inflammatory, driving the chemotaxis of macrophages and dendritic cells to the site of infection, to acting as an anti-inflammatory agent such as inhibiting the expression of inducible nitric oxide synthase in macrophages and reducing lung injury by binding histones [75-77].

Tartrate resistant acid phosphatase 5

Tartrate resistant acid phosphatase 5 (TRAP5) is a cationic glycoprotein, having metalloenzyme properties. It has been isolated from many mammalian sources, including bone, spleen, placenta and lungs [78-80]. TRAP5 is synthesized as a 35 kDa monomeric polypeptide (the 5a isoform) with low enzymatic activity, which undergoes posttranslational cleavage giving rise to a high enzymatic activity two-subunit structure (the 5b isoform), consisting of NH_2-terminal derived 23 kDa fragment and a COOH-terminal derived 15 kDa fragment linked by disulphide bonds [81]. TRAP5 has been extensively studied in bones, especially in the context of bone resorption and remodelling, because of its ability to dephosphorylate OPN. Other functions have been suggested for TRAP5, *e.g.* regulating macrophage migration [66], participating in iron transport [82] and acting as a growth and differentiation factor for hematopoietic and osteoblastic cells [83]. Interestingly, high expression of TRAP5 has been detected in AM, in particular the 5a isoform. Its expression was shown to be upregulated in smokers and COPD patients [66]. Although its function has not been completely clarified in AM, it is suggested that it might have an antimicrobial effect through generating reactive oxygen species (ROS) [84, 85].

In this work, we sought to elucidate the relation of TRAP5 to OPN in the lungs, and the role these proteins might play in the host immune responses.

The Coagulation System

The coagulation system is composed of a group of proteins that circulate in blood in their inactive form (zymogens; Factor I – Factor XIII). During an injury or damage to a blood vessel wall, these proteins will be activated (Proteases; Factor Ia – Factor XIIIa), leading to the formation of a fibrin clot which stops bleeding and facilitates tissue repair. This healing process is triggered via two pathways, (i) the intrinsic, through contact activation and (ii) the extrinsic, activated by tissue factor (TF) (Figure 5) [86-88]. Initially, it was though that the coagulation process takes place in the vascular compartment, but later on it was found that the coagulation and anticoagulation mechanisms can be initiated and regulated in the airways [89]. Activation of coagulation can be triggered by the high presence of TF in the lungs during inflammatory diseases, such as acute respiratory distress syndrome (ARDS), asthma and pulmonary fibrosis, as well as induced by the plasma proteins that are leaked into the bronchioalveolar space as a consequence of capillary leakage [89-91].

The intrinsic pathway

This pathway is activated when blood is exposed to collagen or to other stimuli like bacterial cell surfaces and bacterial products. The exposure triggers the reciprocal activation of kallikrein and Factor XIIa, mediated by high molecular weight kininogen (HK). This in turn activates a cascade of coagulation factors that eventually lead to fibrin formation [87, 88].

The extrinsic pathway

The extrinsic pathway is mainly initiated by TF, that binds and activates Factor VII, converting it to Factor VIIa. TF production is stimulated by environmental or bacterial products, such as LPS or by inflammatory mediators where its expression will be upregulated on the surface of monocytes, epithelial and endothelial cells. The binding of TF to Factor VII, forms a complex TF/FVIIa, which activates Factor Xa. This leads to a downstream activation of other clotting factors which eventually results in the formation of fibrin clot [88, 92].

The coagulation pathway is tightly regulated by a number of proteins, in order to maintain homeostasis and prevent excessive blood clotting. These anticoagulants are known as serpins (serine protease inhibitors), and include tissue factor pathway inhibitor-1 (TFPI-1) and -2 (TFPI-2), Antithrombin III (ATIII), Heparin cofactor II (HCII) and Protein C [93].

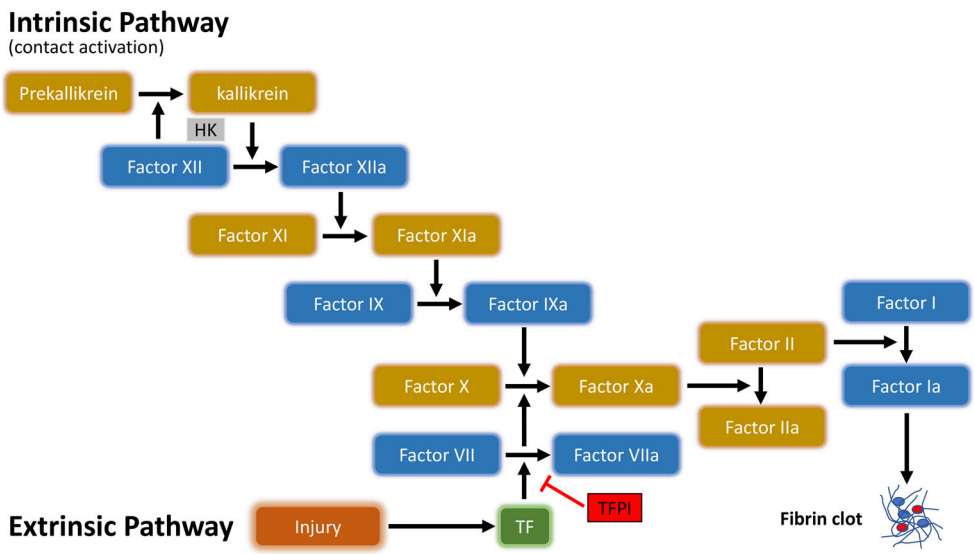

Figure 5. Schematic representation of the coagulation system

Tissue factor pathway inhibitor-2

Tissue factor pathway inhibitor-2 (TFPI-2), also known as matrix-associated serine proteinase inhibitor (MSPI) and placental protein 5 (PP5), is a 32-33 kDa serine protease inhibitor that shares approximately 50% homology with its sister protein TFPI-1. It consists of three Kunitz-type serine proteinase inhibitor domains, a highly negatively charged NH_2-terminus, and a highly positively charged COOH-terminus where our peptide of interest (EDC34) is derived. TFPI-2 is synthesized and secreted by a variety of cells, such as endothelial cells, smooth muscle cells, monocytes and macrophages, and has a critical role in coagulation, angiogenesis, apoptosis and wound healing. It inhibits the protease-activity of trypsin, chymotrypsin, cathepsin G, and plasma kallikrein. In addition, TFPI-2 inhibits the factor VIIa-TF complex, maintaining the balance required for normal homeostasis. In an LPS-infection model, TFPI-2 expression was upregulated in several organs, including spleen, lungs, brain and kidneys [94, 95, 38]. TFPI-2 exerts antimicrobial activity against Gram-negative bacteria, and its COOH-terminal region has shown to be up-regulated in acute and chronic wounds. Moreover, the COOH-terminal region derived peptide (EDC34) has proven to play an important role in amplifying the complement activation by targeting the complement factor C3a [96, 38].

Bacterial Infection

A bacterial infection is when a harmful strain of bacteria enters a host and proliferate. Bacteria can infect any part of the body which can have a detrimental effect at the entry point, or in a worse scenario, access the blood stream and circulate through all over the body causing damage to multiple organs or tissues. Upon bacterial infection, the immune system is activated by released toxins, resulting in an immediate and unspecific response to resolve the threat, a process which is usually driven by neutrophil, macrophages and dendritic immune cells. To further ensure the complete clearance of the pathogen, B and T lymphocytes which are the troops of the adaptive immunity, will interfere and work synergistically with innate cells to limit pathogen dissemination [97, 98].

In Respiratory Tract

The respiratory tract can be infected by a variety of bacteria, both Gram-positive and Gram-negative, causing several diseases that can vary in their severity. Some of the most common bacteria are described below.

Pseudomonas aeruginosa

Pseudomonas aeruginosa (PA) is a Gram-negative, rod-shaped bacterium that is found in soil, water and skin flora. It is an opportunistic pathogen that can cause serious infection, in particular in immunocompromised patients, or during existing diseases, such as in CF and COPD [99, 100]. This is underlined by the fact that PA is the most common bacteria isolated in severe COPD. It is usually very hard to treat as it is highly resistant against first line antibiotics used to treat exacerbations [101, 102]. PA have several strategies to protect its self from host immunity. It has the ability to form antimicrobial-tolerant biofilms, it rapidly undergoes several mutations to change its phenotype, and/or produce proteases like elastases, which can fight the immune system by degrading some of its molecules, including complement and coagulation proteins [103-105].

Streptococcus pyogenes

Streptococcus pyogenes is a Gram-positive bacterium that appears as chains of cocci. It commonly infects the upper respiratory tract. *S. pyogenes* release a variety of toxins and enzymes, such as hyaluronidase, collagenase and streptokinase that can damage the mucosal membranes of the pharynx and degrade connective tissues [106].

Streptococcus pneumoniae

Streptococcus pneumoniae is a Gram-positive β-haemolytic coccus that regularly colonize the upper respiratory tract, thus often appearing as a commensal. Besides being the most common cause of pneumonia and meningitis, *S. pneumoniae* is associated with several airway diseases, such as otitis media, sinusitis and bronchitis. Immunodeficient patients, young children and elderly are the most vulnerable targets of acquiring pneumococcal infections [107-109].

Haemophilus influenzae

Haemophilus influenzae is a Gram-negative coccobacillus, that can be divided into two categories, encapsulated and unencapsulated strains respectively, the latter also known as nontypable *H. influenzae* (Nt*Hi*). Nt*Hi* is one of the most frequently isolated pathogens from sputum samples in COPD, and a major contributor of bacterial exacerbations [110, 102].

Staphylococcus aureus

Staphylococcus aureus is a Gram-positive coccus, appearing as a human commensal that colonizes the nose, skin and respiratory tract. Through the release of toxins and exo-enzymes*, S. aureus* can cause acute infections, such as bacteraemia and skin abscesses. On the other hand, chronic infections are associated with biofilm formation that can enhance infection of bone and heart valves, resulting in osteomyelitis and endocarditis respectively [111, 112].

Chronic Airway Inflammation

In healthy individuals, the airways are covered by a mucus layer that contains a mixture of components including proteins, lipids and mucins secreted by the epithelial cells. This mucus layer entraps a wide range of harmful pathogens such as bacteria and viruses as well as environmental irritants, that are constantly deposited in the airways during the breathing process. All of these noxious stimuli are then escalated to the upper part of the respiratory tract to be removed from the lung. This process is coordinated by the movement of the beating cilia, which is referred as the mucociliary clearance [113, 5]. In chronic lung diseases including asthma, CF and COPD, the mucociliary clearance is reduced and mucus secretion is often increased, leading to a thick dense mucus on the epithelial layer, causing obstruction and plugging of the airways, that in turn affect lung function and homeostasis [114-116]. Another characteristic of chronic airway inflammation, is the accumulation of activated neutrophils and T cells in the small airways causing the destruction of their walls [111]. Moreover, antioxidants, such as superoxide dismutase, catalase and glutathione fail to respond against oxidative stress caused by, for example cigarette smoke, which will increase the expression of genes involved in inflammation, leading to imbalanced immune responses [117].

Respiratory Diseases

The lungs are continually exposed to infections agents. The disruption of homeostasis caused by harmful inhalants leads to a wide range of diseases, varying from mild common cold to a more severe cases, such as CF, COPD and ARDS.

Cystic Fibrosis

Cystic fibrosis (CF) is a rare genetic disease caused by mutations in the *cystic fibrosis transmembrane conductance regulator (CFTR)* gene, which encodes a chloride ion channel that is largely present in the apical membranes of epithelial cells. This channel is responsible for conducting chloride ions across the membrane of sweat and mucus producing cells, after which water follows to dilute the mucus. This gene defect leads to an inborn dysregulated fluid transport in the lungs with airway mucus plugging and air trapping [118-120]. In addition to the respiratory system, the disease affects other organs including the gastrointestinal tract, where the thick mucus obstruct the canaliculi of pancreas and gall bladder duct, preventing enzyme and bile flow, resulting in malabsorption and digestion abnormalities [121]. In CF disease, the dysregulated inflammation starts in the first months of life, and are characterized by the increase in neutrophil numbers and excessive secretion of

pro-inflammatory mediators, including TNF-α, IL-1β, IL-6, IL-8, IL-17, IL-33, GM-CSF, G-CSF, and HMGB-1 [122]. The inflammatory responses become excessive and persistent, and the host immunity will be burdened with continues bacterial infections. This in turn will impair host defenses, damage airway walls and ultimately leads to loss in lung functions [123, 124, 99].

Chronic Obstructive Pulmonary Disease

COPD is characterized by irreversible airflow limitation due to chronic inflammation. The disease has a high prevalence and is expected to become the third leading cause of death worldwide [125]. Tobacco smoking is one of the main causes for development and progression of COPD. However, other risk factors such as air pollution, including noxious gases and ambient particulate matter can significantly contribute to the pathogeneses of the disease [126, 127].

In COPD, the inflammatory responses are often persistent and progressive, resulting in different clinical phenotypes where chronic bronchitis, bronchiolitis, and emphysema may be seen. Chronic bronchitis is an inflammation of the lining of the bronchial tubules accompanied with goblet cell hyperplasia and increased mucus secretion in the small conducting airways, leading to airflow limitations. Emphysema is characterized by the remodelling of the small airway compartment and destruction of the parenchyma (Figure 6). This leads to the loss of elastic recoil or inadequate lung emptying on expiration, resulting in the entrapment of the air inside the lungs. COPD can be categorized, depending on its severity, into four stages (I, II, III and IV). This is based on the Global Initiative for Chronic Obstructive Lung Disease (GOLD) categorisation. GOLD stage I being the mildest while GOLD stage IV is the most severe condition, being based on the spirometry measurement of forced expiratory volume (FEV1). This measures the amount of air that can be forcefully exhale in the first second [128, 129].

The chronic inflammation in COPD is characterized by elevated levels of the main inflammatory cells including neutrophils, monocytes/macrophages and lymphocytes, that infiltrate the airways and lung tissues, and can be detected in sputum and bronchoalveolar lavage fluid [129]. The recruited neutrophils secrete several proteases including neutrophil elastase (NE) and matrix metalloproteinases (MMPs) which contribute to alveolar destruction and play a key role in the pathogeneses of COPD [130, 131]. Despite the canonical classification of COPD as a neutrophil disease, there is a large number of COPD patients with eosinophilia-derived inflammatory responses [132]. In fact, studies have suggested that eosinophilic COPD patients are prone to an increased risk of exacerbations [133]. Eosinophils produce a wide range of mediators, including eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), leukotrienes and a variety of chemokines and cytokines, such as IL-13. IL-13 in particular, has shown to contribute to the pathogeneses of the COPD through mediating several pathologies

including bronchoconstriction, fibrosis, mucus production and the remodelling of the airways resulting in emphysema [134-136].

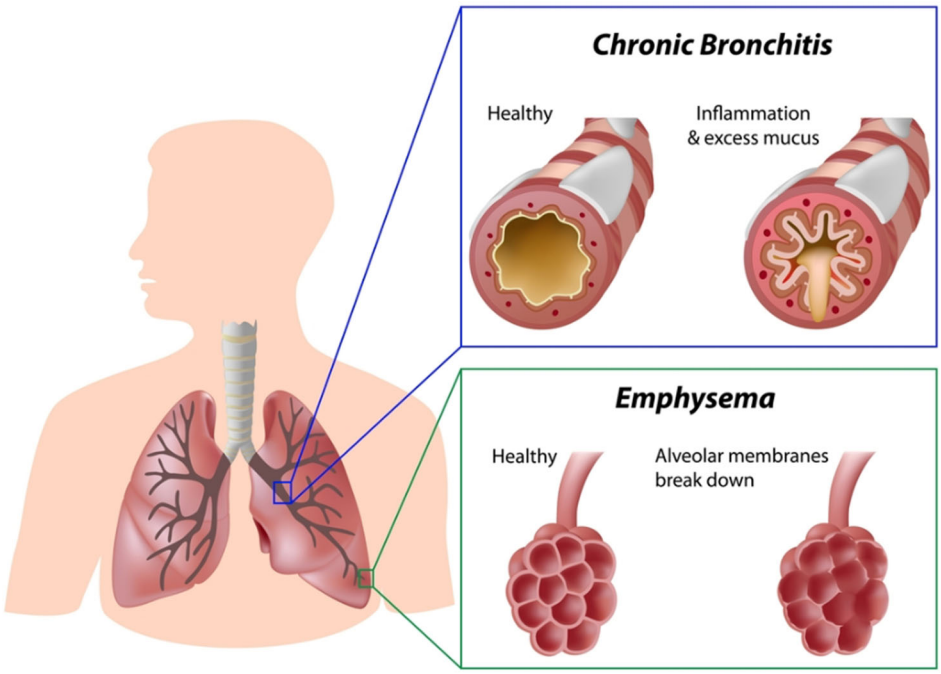

Figure 6. Chronic obstructive pulmonary disease (COPD). Schematic showing the difference between healthy lungs and lungs in COPD where chronic bronchitis and emphysema respectively may be present (picture is adapted from https://lungdiseasenews.com/2015/08/03/aclidiniumformoterol-therapy-reduces-copd-patients-exacerbations/)

Acute Respiratory Distress Syndrome

Acute respiratory distress syndrome (ARDS) is a severe clinical condition that has proven to be very challenging to treat because of its heterogeneity. It is not caused by a particular disease, but rather triggered by different pathologies, including viruses, bacteria, severe trauma, and sepsis. ARDS is characterized by a prolonged and vigorous inflammation accompanied with increased neutrophil influx to the lungs, resulting in pronounced release of neutrophil extracellular traps (NETs). This leads to tissue injury and cell death, followed by the release of intracellular molecules, i.e. DAMPs, including the highly cationic extracellular histones. This in turn leads to further release and accumulation of immune cells creating a self-destructive loop of inflammatory responses. As a result, the alveoli will be obstructed with fluid because of edema causing impaired gas exchange [137, 138].

Present Investigation

Paper I

Osteopontin expression in small airway epithelium in COPD is dependent on differentiation and confined to subsets of cells

Osteopontin is a large multifunctional protein, shown to have a critical role in a variety of physiological processes, such as biomineralization, cancer metastasis and wound healing. In airway inflammatory diseases, such as in COPD, high levels of OPN were detected in sputum and bronchioalveolar lavage. In addition, cigarette smoke which is a major cause for COPD, has shown to up-regulate OPN production in the lungs of asthmatic patients. Therefore, OPN is an interesting molecule to investigate, where several lines of evidence suggest that it might contribute to disease progression [64, 139, 67].

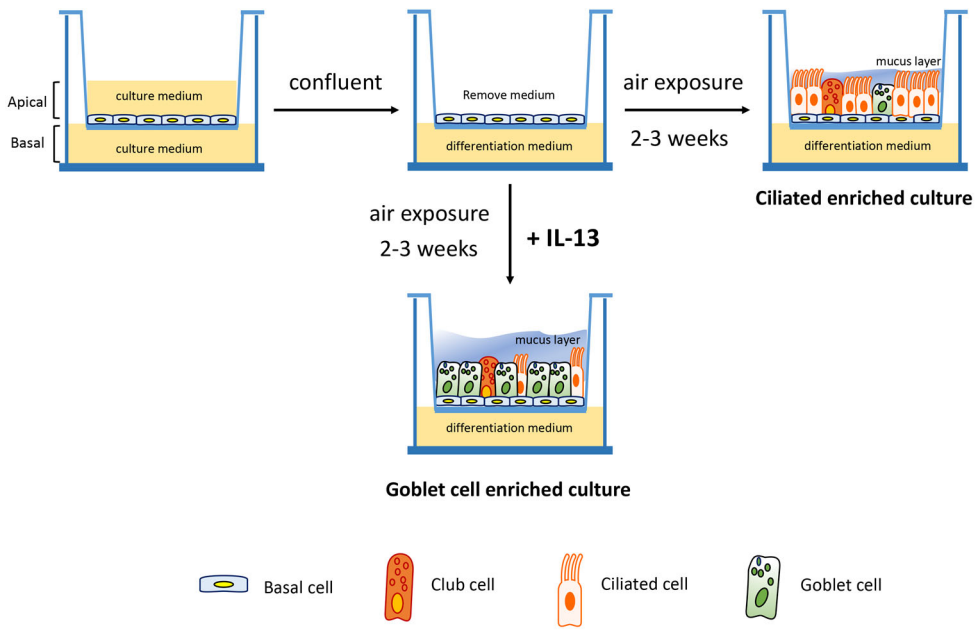

Figure 7. Schematic illustration of Air Liquid Interface culture. Human bronchial epithelial cells are cultured in culture medium until conflent. The medium is substituted (only in the basal compartment) with differentiation medium. The cells are either exposed to air alone for 2-3 weeks (ciliated cell enriched culture) or in the presence of IL-13 cytokine, added in the basal compartment (goblet cell enriched culture).

Despite that OPN has been studied extensively for the past decades, and many cells have shown to express OPN, such as T cells, NK cells and dendritic cells, the source of this protein in the airway epithelium has not been fully elucidated.

In this study, we showed that OPN expression levels peaked in severe COPD (GOLD stage II-III) compared to other conditions, and that *in vivo*, OPN was mainly expressed by goblet and club cells, but not ciliated or basal cells. Moreover, we found that *in vitro*, cigarette smoke induced OPN expression and release from basal cells, suggesting that these cells do not store but rather continuously release the OPN produced. In order to examine the effect of epithelial cell differentiation and chronic cigarette smoke exposure on OPN expression, we used Air Liquid Interface (ALI) culture (Figure 7). We cultured human bronchial epithelial cells (HBEC) and stimulated them with the cytokine IL-13 to promote goblet cell differentiation. Goblet cell hyperplasia is a characteristic of COPD disease, accompanied with an excessive secretion of mucus in the small airways. Our results demonstrated increased OPN secretions in goblet cell enriched cultures in response to cigarette smoke, suggesting that goblet cells are the primary producers of OPN. Taken together, the phenotype of the disease might determine the expression and production of OPN in the lungs. Whether this will contribute in the progression or resolving the disease, is something remains to be elucidated.

Paper II

Tartrate-resistant acid phosphatase 5 (TRAP5) displays altered levels and co-localize with osteopontin in cystic fibrosis

Tartrate resistant acid phosphatase 5 (TRAP5) is a metalloenzyme possessing phosphatase-activity that is expressed by osteoclasts, alveolar macrophages, dendritic cells and a number of other cells types. It is present in two isoforms, i.e. TRAP5a with low enzymatic activity and TRAP5b with high enzymatic activity. TRAP5 plays an important role in many biological processes including cytokine production by macrophages and dendritic cells, osteoblast regulation, macrophage recruitment, as well as it exhibits potent phosphatase activity towards osteopontin (OPN), a process needed to ensure normal bone development. Both TRAP5 and the highly phosphorylated glycoprotein OPN, levels are increased in patients suffering from chronic inflammatory diseases. However, their levels and expression have not been investigated in the context of cystic fibrosis (CF).

In the current study, significant differences of TRAP5a and OPN levels were found comparing healthy controls and CF patients. In serum, TRAP5a and OPN protein levels were significantly higher in CF than in controls. However, in sputum on the contrary, TRAP5a levels were significantly lower in CF patients compared to healthy donors. This observation was in line with western blot data, where control

sputum only showed presence of intact TRAP5a, while CF TRAP5a was seen both as an immunoreactive band corresponding to the full length protein as well as degraded fragments. This could be explained by the increased elastase activity in CF lungs, where we found that both elastase of *Pseudomonas aeruginosa* and neutrophils, but not aureolysin protease of *Staphylococcus aureus*, were able to cleave TRAP5 after 18 hours of incubation *in vitro*. In addition, immunohistochemical staining of TRAP5a and OPN was detected in the lungs of controls and CF, and their co-localization was seen on the airway epithelial lining, in alveolar macrophage and in the lumen of blood vessels of CF patients.

In conclusion, our data suggests that TRAP5 and OPN might interact in the lungs and blood, and their levels are associated with CF disease.

Paper III

Osteopontin Protects Against Lung Injury Caused by Extracellular Histones

Histones are important structural components of the chromatin of the cell nucleus, playing significant roles in the regulation of gene transcription [140]. In contrast, upon cell death, extracellular histones can have detrimental effects on host tissues where they may evoke toxic and immunostimulatory effects. During an infection or inflammation, as seen in acute respiratory distress syndrome (ARDS), the prolonged inflammatory responses and tissue injury cause cell death, including necrosis, apoptosis and release of neutrophil extracellular traps (NETs). This cell death results in the release of the highly cationic extracellular histones that triggers innate immune responses, including activation of Toll-like receptors (TLR) and inflammasomes [141-143].

Osteopontin (OPN) is a multifunctional phosphoglycoprotein that plays important roles during inflammation, where it is involved in cell-mediated immunity, neutrophil recruitment, regulation of dendritic cell activity, inducing Th1 type immune responses, as well as promoting fibrosis in lung injury. OPN expression is increased in the airways of several inflammatory airway diseases, including COPD, cystic fibrosis and asthma [144, 69]. In this study, we set out to investigate whether the highly anionic molecule, OPN, can bind to and sequester the cationic extracellular histones at the airway mucosal surface.

Our results demonstrate that OPN protects the airways in murine models of histone- as well as LPS-induced acute lung injury. Moreover, OPN bound to histones in bronchioalveolar lavage (BALF) of ARDS patients *in vivo*, and inhibited their cytotoxic and hemolytic effects *in vitro*, where these OPN-histone complexes depend on the phosphorylation degree of OPN. Finally, we showed that OPN inhibits histone-induced formation of NETs. In conclusion, OPN that is increased

in the airways during states of disease characterized by pulmonary inflammation might serve as a host protective mechanism against the cytotoxic effects of danger associated molecular patterns (DAMPs), as examplified by extracellular histones.

Paper IV

TFPI-2 Protects Against Gram-negative Bacterial Infection

Tissue factor pathway inhibitor-2 (TFPI-2), is a Kunitz-type serine proteinase inhibitor which is structurally similar to tissue factor pathway inhibitor-1 (TFPI-1). TFPI-2 plays, among other physiological functions, an important role in coagulation and wound healing, and is secreted by a variety of cells, such as endothelial cells, epithelial cells, smooth muscle cells, monocytes and macrophages. It consists of a highly negatively charged N-terminus and a highly positively charged COOH-terminus [95, 145]. *In vivo*, the TFPI-2 COOH-terminal region is present in fibrin slough from chronic wounds and associated with bacteria. *In vitro*, a TFPI-2 COOH fragment (EDC34) bind bacteria and exert antimicrobial activity. Moreover, EDC34 promotes complement activation through up-regulating the complement protein C3a on bacterial surfaces [96].

In the present study, we investigated the EDC34 mode of action in the context of infectious diseases. Our results revealed that EDC34 binds to immunoglobulin G (IgG), more specifically to the Fc region where this interaction boosted the classical complement activation through the assembly of the complement protein C1q at the bacterial surfaces. In addition, we show that TFPI-2 expression was increased in spleen, brain, lungs, kidney, large and small intestine in murine models of endotoxin-exposure as well as in an *E. coli* infection model. In humans, the COOH-terminal fragment was detected in association with immunoglobulins present in sputum from COPD patients. Finally, the importance of EDC34 and the corresponding VKG24 of mice, was shown in a murine model of systemic *E. coli* infection, where they had a protective effect.

Taken together, this work present evidence that TFPI-2 may exert important host defence functions during infectious diseases.

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