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On Dysregulated Inflammation and Airway Host Defense

RAVI KIRAN VARMA BHONGIR | FACULTY OF MEDICINE | LUND UNIVERSITY

On dysregulated inflammation and airway host defense

On Dysregulated Inflammation and Airway Host Defense

Ravi Kiran Varma Bhongir

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DOCTORAL DISSERTATION
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Abstract

Acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF) are characterized by dysregulated inflammation of the airways. The increased influx of immune cells and the accumulation of cytokines lead to cell death, tissue destruction and impaired pulmonary function. Intracellular proteins like histones and granule proteins of neutrophils, DNA fibers act as Danger-Associated Molecular Patterns (DAMPs), further promoting tissue damage. As a result, the lungs of such patients are at an increased risk of infection due to impaired host defense functions.

During inflammation, there is an increased expression of osteopontin (OPN), a highly anionic phosphoglycoprotein, in the airways and it is involved in cell recruitment, tissue remodeling, and repair. In this thesis we show that OPN can interact with many cationic proteins and peptides present in the extracellular milieu of the inflamed airways. In the first paper included in this thesis we show that OPN bound to extracellular histones have protective function against DAMPs-induced inflammation. In the second paper, we show that OPN binds to several common innate antibiotics and abrogate their antimicrobial activities. Taken together, these data suggest that OPN can modulate the host immune functions, thereby increasing the susceptibility of the patients with airway inflammatory diseases to acquire infections.

Use of anti-inflammatory drugs like roflumilast is a common treatment strategy in COPD to ameliorate severe exacerbations. In the third paper we highlight the adverse effects of roflumilast, in a murine acute airway infection model. The findings suggest that use of this drug can impair host defense functions of immune cells, thereby increasing the susceptibility of COPD patients to bacterial pathogens.

DNase I is used to clear the airways of CF patients from highly viscous, high molecular weight eDNA rich sputum. In the fourth paper of this thesis, we elucidated the molecular aspects of the fragmented DNA that are important to exhibit antimicrobial properties against the common CF lung pathogen, *i.e. P. aeruginosa*. The findings highlight a novel aspect of host defense that could be employed treating bacteria resistant against conventional antibiotics.

Key words: Aiway inflammation, chronic obstructive pulmonary disease, cystic fibrosis, *Pseudomonas aeruginosa*, DNase I

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Ravi Kiran Varma Bhongir

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"Nothing we achieve in this world is achieved alone. It is always achieved with others teaching us along the way."

- Lee J. Colan

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Abstract

Acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF) are characterized by dysregulated inflammation of the airways. The increased influx of immune cells and the accumulation of cytokines lead to cell death, tissue destruction and impaired pulmonary function. Intracellular proteins like histones and granule proteins of neutrophils, DNA fibers act as Danger-Associated Molecular Patterns (DAMPs), further promoting tissue damage. As a result, the lungs of such patients are at an increased risk of infection due to impaired host defense functions.

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List of Papers

This thesis is based on the following papers:

Paper 1

Bhongir RK, Papareddy P, Mörgelin M, Egesten A, Kasetty G.
Osteopontin is a key regulator of cytotoxic and proinflammatory activities exerted by extracellular histones. *Manuscript*

Paper 2
Gela A*, **Bhongir RK***[*], Mori M, Keenan P, Mörgelin M, Erjefalt J, Herwald H, Egesten A, Kasetty G.
Osteopontin that is elevated in the airways during COPD impairs the antibacterial activity of common innate antibiotics. *PLoS One*. 2016, 11(1):e0146192.

Paper 3
Kasetty G, Papareddy P, **Bhongir RK**, and Egesten A.
Roflumilast increases bacterial load and dissemination in a model of *Pseudomonas aeruginosa* airway infection. *J Pharmacol Exp Ther*. 2016, 357(1):66-72.

Paper 4
Bhongir RK, Kasetty G, Papareddy P, Mörgelin M, Herwald H, Egesten A.
DNA-fragmentation is a source of bactericidal activity against *Pseudomonas aeruginosa. Biochem J*. 2016, (doi:10.1042/BCJ20160706)

* These authors contributed equally to the study.

List of publications not included in this thesis.

1. Papareddy P, Kalle M, **Bhongir RK**, Mörgelin M, Malmsten M, Schmidtchen A.
   Antimicrobial effects of helix D-derived peptides of human antithrombin III. *J Biol Chem*. 2014 Oct 24;289(43):29790-800.

2. Papareddy P, Kasetty G, Kalle M, **Bhongir RK**, Mörgelin M, Schmidtchen A, Malmsten M.
   NLF20: an antimicrobial peptide with therapeutic potential against invasive *Pseudomonas aeruginosa* infection. *J Antimicrob Chemother*. 2016 Jan;71(1):170-80.

3. van der Plas MJ, **Bhongir RK**, Kjellström S, Siller H, Kasetty G, Mörgelin M, Schmidtchen A.
   Pseudomonas aeruginosa elastase cleaves a C-terminal peptide from human thrombin that inhibits host inflammatory responses. *Nat Commun*. 2016 May 16;7:11567.
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1. Introduction

1.1 The Immune System

Humans, like all other higher organisms, live constantly interacting with the environment and are colonized by a multitude of microorganisms that exceed the number of cells forming the human body (1). Most of these microorganisms are commensals but some are potential pathogens, which may cause infectious diseases (2). The first line of defense against the invading microorganisms is comprised of physical or anatomical, mechanical, biochemical and cellular barriers, and is known as innate immunity. This part of immunity provides a quick and non-specific response (3). Adaptive immunity is a complex and highly specific system that kicks in to action when the pathogens escape the innate immune defenses. The adaptive immunity is comprised of specialized cells that can recognize the pathogens and mount a directed response against it (4, 5). This system can also form a long-standing memory to a particular pathogen and provides protection against subsequent encounters with that same pathogen. In spite of these differences, the innate and adaptive immunities are interconnected and their combined effort is required for an effective immune protection (6).

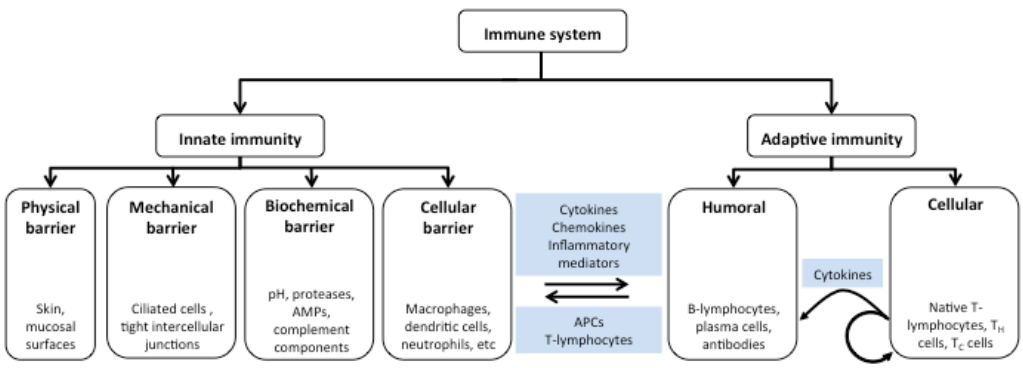

Figure 1. Schematic representation of the immune system

1.2 Innate Immunity

Innate immunity is a non-specific and an immediate response to the invading pathogen (3). The epithelial cell layer of the skin or the mucosal tissues (*e.g.* in the respiratory, gastrointestinal and genitourinary tracts), the glandular tissues (*e.g.* the salivary, mammary, and lacrimal glands) act as physical barriers to prevent infection. The ciliated cells from the respiratory tract and the tight junctions between the cells act as the mechanical barrier. The acidic pH, anti-microbial proteins and peptides on these surfaces and in sweat, tears, or saliva act as the biochemical barrier to infection. If the pathogen enters the body through a breach in the epithelial cell layer then it encounters the cellular components of the innate immune system that includes macrophages, neutrophils, dendritic cells, and natural killer (NK) cells.

The cells of the innate immune system have specific pattern recognition receptors (PRRs) that recognize conserved molecular motifs of pathogens called pathogen-associated molecular patterns (PAMPs). Upon pathogen recognition, these cells become activated and produce a wide range of pro-inflammatory cytokines and chemokines, which activates other parts of the innate immune system. The macrophages, neutrophils and monocytes can capture and ingest the pathogens by a mechanism called phagocytosis at the site of inflammation. The dendritic cells are also phagocytes that are present in the tissues in direct contact with the external environment such as in the skin and in the airways. They form a link between the innate and adaptive immune systems by presenting the antigens to the T cells, leading to their activation. NK cells are lymphoid progenitor cells of the innate immune system that can recognize and kill virus-infected cells.

1.2.1 Physical Barriers of the Airways

The mucosal surfaces of the nasal and respiratory passages act as a first line of defense against airborne foreign particles and pathogens. The epithelial cells of the respiratory tract are usually covered by thin hair-like structures called cilia. The cilia have a constant rhythmic sweeping motion which helps flush out inhaled foreign particles or pathogens that get trapped on the mucosal surfaces. Especially in the respiratory tract, a thin layer of fluid called the periciliary fluid liquid (PCL) covers the epithelial cells, keeping the cilia hydrated. Goblet cells, which are modified epithelial cells, secrete mucous which comprises the mucous layer (ML) on top of the PCL. The combination of the mucous and the PCL layers are called 'Airway Surface Liquid' (ASL). The volume of the PCL determines the proper functioning of the mucociliary transport. The ideal PCL height would be equal to the height of the cilia, *i.e.* approximately 7 μm. If the PCL volume is reduced, the

cilia will be trapped in the mucous layer and thus not able to carry out the movement. If the PCL volume on the other hand is too high, the ciliary movement will be ineffective in transporting the mucous layer. Apart from acting as a physical barrier to the pathogens the mucosal surfaces have a range of soluble biochemical factors that regulate the pH, ionic concentration, and proteases like lysozyme that can kill the pathogens or hinder their growth. If the pathogens breach these mucosal surfaces they can replicate and cause disease locally or, disseminate into deeper tissues or enter the blood stream, causing a systemic infection.

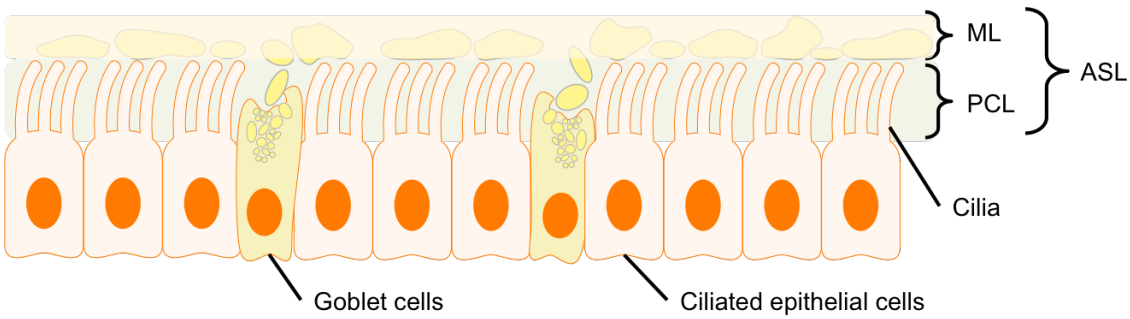

Figure 2. Airway surface liquid
The ciliated epithelial cells are covered with a thin layer of fluid called periciliary liquid (PCL) and specialized epithelial cells called goblet cells secrete the mucous layer (ML), together these two layers constitute the airway surface liquid (ASL).

1.2.2 Antimicrobial Proteins / Peptides

The antimicrobial peptides (AMPs) constitute an important and ancient arm of innate immunity. They are found in all living organisms and have potent antimicrobial activity against many Gram-positive and Gram-negative bacteria, yeast, and protozoans. Based on their structure, AMPs can be categorized into four groups: (i) those containing α-helical motifs; (ii) those containing β-sheets; (iii) cyclic peptides, and (iv) peptides rich in amino acids like proline, histidine, and arginine. The AMPs are usually short stretches consisting of 12-100 amino acids, including a high number of cationic amino acids and amphipathic regions (the latter containing combinations of hydrophobic and hydrophilic regions resulting in detergent-like activities). Apart from these four major classes of cationic peptides, it is interesting to note that also anionic antimicrobial peptides exist.

The hypothesis for the mode of action is that cationic AMPs associate and accumulate at the negatively charged bacterial membranes and once a threshold concentration is reached, they are incorporated into the lipid bilayers of the microbial membranes, resulting in disruption. Alternatively, they can exhibit

additional toxic effects like inhibiting the synthesis of DNA, RNA, and proteins, thereby killing the microbe. The sterical models of membrane permeabilization by cationic AMPs are the barrel-stave model, the toroidal pore model, and the carpet model (7).

Barrel-stave model

In the barrel-stave model, the peptides are thought to oligomerize on the bacterial membrane surface, forming transmembrane channels and pores. The hydrophobic part of the peptides interacts with the lipid core of the membrane and the hydrophilic part aligns towards the lumen of the pore. This leads to leakage of bacterial intracellular contents, resulting in death of the bacteria (7, 8).

Toroidal pore model

According to the toroidal pore model, the peptides get inserted perpendicularly into the membrane. The hydrophilic parts of the peptide interact with the polar headgroups of the lipids and induce the membranes to bend inwards to form pores (7, 9).

Carpet model

In this model, the peptides align parallel to the plane of the membrane and exert a detergent-like effect on the membrane. This causes the membranes to break off into micelles, causing membrane disruption (10).

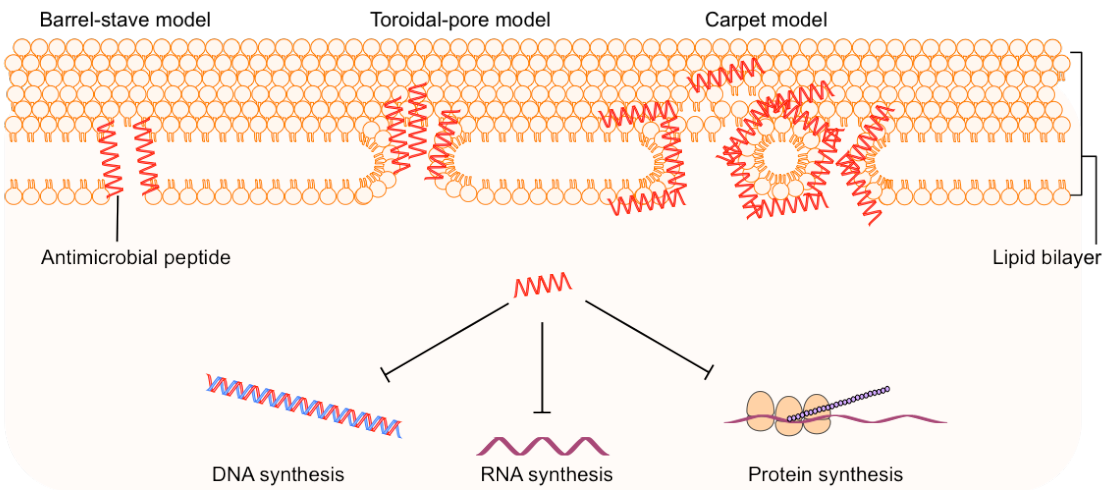

Figure 3. Proposed mechanisms of action of antimicrobial peptides (AMPs).

Microbial infections can be controlled with an assortment of AMPs, which can be found on a variety of exposed tissues or the mucosal surfaces such as in the eyes, mouth, airways, lungs, intestines and urinary tracts. The human cathelicidin, defensins, lysozyme, RNases, psoriasin, lactoferrin, dermcidin and some of the chemokines produced by the immune cells also constitute AMPs. Apart from the antimicrobial activities, these proteins and peptides may also have additional roles in promoting cellular growth *(e.g.* during angiogenesis), wound repair, and regulation of inflammation (11).

1.2.3 Cytokines

Cytokines are low molecular weight proteins that allow cells to communicate with each other. The cytokines are proteins with a broad range of functions including regulation of cellular proliferation, maturation, differentiation, and activation of effector functions. These molecules are produced by many cells, including immune cells and exert immunomodulating activities either by autocrine signaling (acting on the same cells that produce them), paracrine signaling (acting on the cells in close vicinity to the cell producing them), or endocrine signaling (acting on distant target cells). A cytokine may be produced by more than one cell type, can act on one or more cell types, or can have more than one function.

Cytokines orchestrate the evolution and resolution of inflammation by acting on cell-bound receptors, thereby playing an important role in regulating the host immune responses to infection and tissue-damage. Interleukin(IL)-1, IL-2, IL-17, IL-18, tumor necrosis factor (TNF), interferon-γ (IFN-γ), and granulocyte-macrophage colony stimulating factor (GM-CSF) are classified as pro-inflammatory cytokines while IL-4, IL-10, IL-13 and transforming growth factor-β (TGF-β) as anti-inflammatory cytokines. There are instances where a particular cytokine may behave as a pro- as well as an anti-inflammatory cytokine e.g., IL-6. The increased production and accumulation of the pro-inflammatory cytokines in tissues at sites of infection or trauma can aggravate the disease symptoms by inducing tissue destruction through excessive inflammation. The anti-inflammatory cytokines and specific cytokine inhibitors limit the potentially harmful effects of the prolonged or excess inflammatory response. If these anti-inflammatory mediators overcompensate and inhibit the immune responses, they can leave the host susceptible to systemic infection (12). Thus a dynamic interplay between the pro- and the anti-inflammatory mediators is maintained for the ideal functioning of the immune system.

Osteopontin

Osteopontin (OPN) is a highly anionic *O*-glycosylated phosphoprotein originally identified as a bone matrix protein (13). It is also known as bone sialoprotein. Initially it was described as a protein involved in normal bone calcification, resorption, and remodeling. Later on, this protein was found to be expressed in many other tissues and also in cells of the immune system. With the discovery of roles for OPN in cell recruitment, tissue repair, and inflammation; it has been classified as a cytokine. OPN acts as a macrophage and neutrophil chemoattractant and, as a consequence, it is linked to migration of these cells to sites of inflammation. OPN can also up-regulate other cytokines like IL-8 that in turn acts as an important chemotactic cytokine (chemokine) for neutrophils (14, 15). Generally, OPN is classified as a pro-inflammatory cytokine but in certain pathological conditions, this molecule seems to have anti-inflammatory properties. Dysregulation of OPN expression, resulting in either low or high expression has been related to and can be used as a biomarker for several inflammatory diseases including inflammatory bowel disease (16), cardiovascular disease (17), chronic obstructive pulmonary disease (COPD) (18) and asthma (19).

1.2.4 Neutrophils

Neutrophils are the most abundant cells of the immune system and are one of the first responders to external stimuli. They are also known as polymorphonuclear (PMN) leucocytes due to their multi-lobulated nucleus or granulocytes as the cytoplasm of these cells contain different types of granules and secretory vesicles that are equipped with a variety of AMPs (20). The primary or azurophilic granules contain myeloperoxidase (MPO), serine-proteases (*i.e.* neutrophil elastase, cathepsin G, and proteinase 3) and AMPs (*e.g.* α-defensins and azurocidin). The secondary granules (also know as specific granules) typically contain lactoferrin. The tertiary granules contain gelatinase, collagenase, and matrix metalloproteinases. Lysozyme is found in all three types of granules. In addition to the different types of granules, the cytoplasm of the neutrophils also contains secretory vesicles with a typical content of albumin (21).

Neutrophils are recruited to sites of infection where they recognize the microbes and kill them through both phagocytosis-dependent and phagocytosis-independent mechanisms. During phagocytosis, the microbes are ingested by the cells into a phagosome which acquire lysosomal charactersistics (becoming a phagolysosome) through fusion with primary and secondary granules leading to destruction of the microbe (22, 23). The phagocytosis-independent killing again can rely either on non-oxidative or oxidative mechanisms. Killing by non-oxidative mechanism is mediated by degranulation, where neutrophils release proteases (*e.g.* neutrophil

elastase, cathepsin G, proteinase 3, and gelatinase) and AMPs (*e.g.* lactoferrin and the cathelicidin hCAP-18) into the extracellular milieu where they can neutralize or kill the microorganisms. The oxidative burst or the rapid release of reactive oxygen species (ROS) through the activity of NADPH oxidase is one of the most important host defense mechanisms of the immune system. The oxidative burst also occurs in phagolysosomes to degrade internalized microorganisms.

Neutrophil Extracellular Traps (NETs)

Another important mechanism by which neutrophils can participate in the extracellular killing of microorganisms, is by the formation of neutrophil extracellular traps (NETs). These NETs are mainly comprised of DNA fibers that are studded with proteins of nuclear and cytoplasmic origin. The most abundant proteins to get associated with NETs are histones (core histones H2A, H2B, H3, and H4) and they account for around 70% of all NET-associated proteins. The second most abundant protein associated with NETs is neutrophil elastase. The remaining protein content of the NETs is composed of S100 class of proteins (S100A8 and S100A9), lactoferrin, azurocidin, cathepsin G, MPO, proteinase 3, lysozyme, actin, and catalase (24). The NETs trap the pathogens preventing dissemination and possibly kill them even before they are phagocytosed by the neutrophil. The process of formation of the NETs is called NETosis and is NADPH oxidase dependent and neutrophils lacking this enzyme are not capable of forming NETs (25). The process involves decondensation of the chromatin through the activity of peptidylarginine deiminase 4 (PAD4) on the histones (25-29). Furthermore, degradation of the nuclear membrane, mixing of nuclear and cytoplasmic contents followed by active expulsion of the protein studded DNA by the cells into the extracellular milieu (30). The process is regarded as a form of cell death that is independent of apoptosis and necrosis but recent studies have shown that neutrophils can release NETs without undergoing cell death, where only mitochondrial DNA is expelled, and the neutrophils retain some functions like migration and phagocytosis after the NETs release (31, 32).

A variety of stimuli can induce NET-formation. These stimuli include IL-8, IFN-γ, whole bacterial cells as well as cell wall components like lipoteichoic acid (LTA) of Gram-positive bacteria, lipopolysaccharide (LPS) of Gram-negative bacteria, some fungi and protozoan parasites (25, 32-35). Apart from external stimuli, certain NET components like neutrophil elastase and MPO are also important for the NET formation (30).

The generation of NETs is considered to be a double-edged sword. On a positive note, they can trap and kill the pathogen. The components, like neutrophil elastase, can degrade certain virulence factors of pathogens (36, 37). However, the NET components can cause injury to the epithelial and endothelial cells, leading to inflammation-induced tissue damage (38). The DNA fibers of the NETs and the

extracellular histones may serve as danger-associated molecular patterns (DAMPs) mediate inflammatory diseases including sepsis, acute lung injury, pancreatitis, and thrombosis (39-42).

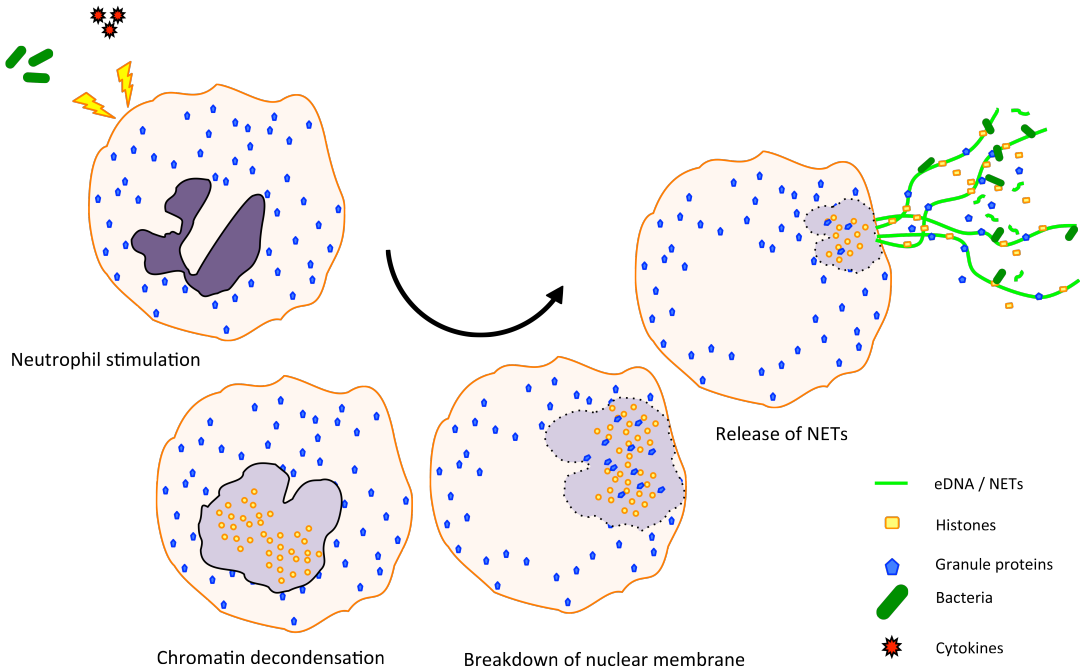

Figure 4. Stages involved in the release of neutrophil extracellular traps (NETs)
Neutrophils are activated with various stimuli leading to decondensation of the nuclear material, breakdown of nuclear membrane, mixing of the nuclear and cytoplasmic contents and finally expulsion of protein studded DNA fibers into the extracellular milieu.

Although neutrophils are equipped with an arsenal of antimicrobial mechanisms, many microbial pathogens have evolved different strategies to avoid killing by neutrophils (43). These evasive strategies include (i) production of oxidative stress proteins that neutralize the ROS and survive the oxidative burst, (ii) avoiding contact with neutrophils by producing neutrophil recruitment inhibiting proteins, (iii) prevent phagocytosis by producing capsules, interfere with opsonization, and inhibit actin cytoskeleton essential for engulfment, (iv) survive inside the neutrophils after phagocytosis by producing capsules or preventing phagolysosome fusion, (v) by producing toxins that can lyse the neutrophils, and (vi) avoiding killing in the NETs by producing nucleases to degrade the DNA and escape.

1.3 Adaptive Immunity

Adaptive immunity or the acquired immunity is comprised of specialized cells that develop immunological memory to a pathogen during its initial encounter. These cells can mount a boosted immunological response during subsequent exposure to specific pathogens. The important cells of the adaptive immunity are the B and T lymphocytes. Dendritic cells and B-lymphocytes encounter the pathogen, ingest it, and present the specific antigens on their surface receptors. This immunity is based on the recognition of the self and non-self antigens and the T-lymphocytes are particularly developed to perform this task. The T-lymphocytes that recognize foreign antigens on the presenting cells get activated and produce cytokines, which either initiates an inflammatory response by recruiting other immune cells or stimulate the proliferation of more B and T-lymphocytes or provoke the T-lymphocytes to become cytotoxic, thereby prompting them to track down and eliminate virus infected cells. The B-lymphocytes are involved in production of antibodies (immunoglobulins) that can specifically bind to the foreign antigens and neutralize them. Some of the activated B and T-lymphocytes become memory cells that can confer either passive short-term memory or active long-term memory (44).

2. Inflammation

Inflammation is a complex biological response of the host tissues and immune cells against harmful external stimuli like pathogens, irritants or internal stimuli like damaged host cell components (45). This is an important mechanism of host defense involved in clearing the source of infection, removing dead cells, and repair of damaged tissue. The inflammatory process involves several signaling molecules and activation of the immune cells through specific receptors. These signals include pathogen-associated molecular patterns (PAMPs), endogenous damage-associated molecular patterns (DAMPs), immune signaling receptors like pattern recognition receptors (PRRs), and protease activated receptors (PARs).

2.1 Pathogen-Associated Molecular Patterns (PAMPs)

PAMPs are molecular motifs associated with the pathogens and can be identified by the host immune cells through patter recognition receptors (PRRs) on their cell surface (46). They are usually highly conserved molecules associated with the pathogens. There are several types of molecules that act as PAMPs like the bacterial lipopolysaccharides (LPS), lipoteichoic acid (LTA), peptidoglycan (PGN), flagellin, CpG DNA, and double-stranded RNA (dsRNA) of viral pathogens. (47-51).

2.2 Damage-Associated Molecular Patterns (DAMPs)

Sterile inflammation or tissue-damage can also trigger inflammation through self or endogenous danger-associated molecules (52, 53). Injured tissues and cells release intracellular molecules into the extracellular milieu where they act as DAMPs. DAMPs include both protein and non-protein molecules. The proteins that act as DAMPs include heat shock proteins, high-motility group B1 (HMGB1) protein, S100 molecules, histones, and hyaluronan fragments. Extracellular DNA, ATP, uric acid, and heparan-sulfate are examples of non-protein DAMPs (54-56).

2.3 Pattern Recognition Receptors (PRRs)

PRRs are important receptor molecules of the innate immune system. They are evolutionarily highly conserved receptors that can recognize several exogenous PAMPs and endogenous DAMPs (54). The PRRs includes Toll-like receptors (TLRs), Nucleotide-binding domain proteins (NOD-like receptors; NLRs), and C-type lectin receptors. The TLRs and NOD-like receptors are membrane bound and are involved in signaling pathways leading to production of inflammatory cytokines. The TLRs detect specific PAMPs derived from bacteria, fungi, viruses, and protozoan parasites (57). The TLRs are usually present on the cell surface and recognize different PAMPs either individually, or by forming homodimer with similar receptor molecule, or by forming a heterodimer with other TLRs and initiate a signaling cascade.

2.4 Protease-Activated Receptors (PARs)

Many different cell types including immune cells, epithelial cells, endothelial cells, and platelets express PARs on their membranes. PARs are activated by the cleavage of amino-terminal sequence of their extracellular domain either to initiate or terminate signal transduction (58). Certain proteases like thrombin and trypsin can cleave the PARs and influence the release of pro-inflammatory cytokines, chemokines and growth factors, which in turn can promote cell activation, differentiation, and migration (59).

3. The Respiratory System

The respiratory system consists of specific organs and structures like the nasal passages, lungs, pulmonary vessels, and the diaphragm that are all involved in the process of respiration. Their main purpose is the intake of air into the lungs and uptake of oxygen and expulsion of carbon dioxide from the circulation. The respiratory tract can be divided into upper and lower respiratory tracts. The upper respiratory tract is comprised of the nasal cavity, the pharynx, and the larynx. The lower respiratory tract begins with the trachea, which bifurcates into two primary bronchi and further branches into secondary, tertiary bronchi and finally into bronchioles. The bronchioles end in the alveoli in the lungs. The alveoli are the air sacs in the lungs where the gaseous exchange takes place. These alveoli are richly supplied with blood capillaries where the red blood cells exchange the carbon dioxide with oxygen.

The upper respiratory tract is generally exposed to airborne foreign particles, pathogens and pollutants and, as a consequence, commonly prone to infections. The nasal passages and airways are lined with nerves, which upon irritation can induce sneezing, or chough as a reflex that can help remove the irritant from the airways. The ciliated epithelial cells and mucous produced by the goblet cells play a major role in trapping these contaminants and flushing them out either by expectoration or swallowing to the stomach. The lower respiratory tract is prone to infections when subject to trauma or due to impaired immune responses.

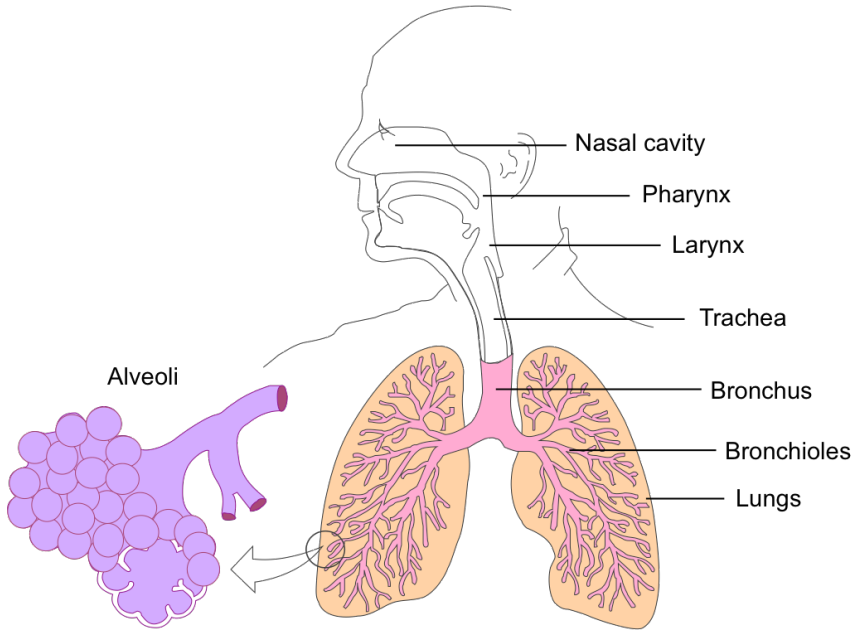

Figure 5. Respiratory system
Anatomy of the respiratory system showing the structures involved in the conductance and exchange of gases (the latter occurring in the alveoli).

4. Respiratory Diseases

Respiratory disease is a heterogeneous group of condition that affects the organs and tissues involved in the conductance and exchange of gases. The diseases can range from common cold to more serious lung diseases including acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF) lung disease. In particular COPD and CF are characterized by increased influx of inflammatory cells (neutrophils) and a vulnerability to acquire bacterial airway infections (*e.g.* caused by *Pseudomonas aeruginosa, Haemophilus influenzae, Streptococcus pneumoniae, and Staphylococcus aureus*).

4.1 Acute Respiratory Distress Syndrome

Acute respiratory distress syndrome is not caused by any particular disease but it is a condition that is triggered by different pathologies such as viral and bacterial pneumonia, severe trauma, and sepsis (60). ARDS can manifest within a few hours to days of the provoking event and has a high mortality rate, which depends on the severity of the underlying cause. ARDS is characterized by extensive injury to the cells in the alveoli, accumulation of neutrophils, increased inflammation, and impaired gas exchange (61-63). To some extent, the innate immune response mechanisms like the neutrophil extracellular traps (NETs) formation can aggravate the pathophysiology of ARDS as the histones and proteases released into the extracellular milieu can cause further tissue damage (64). The accumulation of the inflammatory cytokines and chemokines leads to increased vascular permeability resulting in build-up of fluid in the alveoli because of edema or can lead to collapsed alveoli due to decreased surfactant function. All this results in a hypoxic condition because of impaired gas exchange (61, 63, 65). To treat this clinical syndrome, mechanical ventilation is applied to increase oxygenation. Treatment of the underlying conditions is crucial like use of antibiotics against bacterial infections. In some animal studies, use of anti-inflammatory therapies have reduced the lung injury and mortality (66).

4.2 Chronic Obstructive Pulmonary Disease

COPD is characterized by a persistent and progressive restriction of airflow in response to chronic inflammation in the lower airways caused by long-time exposure to noxious gases (67). The most common symptoms of this disease include shortness of breath, chronic chough, and sputum production. This disease is caused majorly because of active tobacco smoking but other risks also have been identified, which include passive exposure to cigarette smoke, inhalation of toxic substances and gases through outdoor polluted air, indoor pollutants released by burning of biomass fuels used for cooking and heating, occupational dusts and chemicals (68-70).

The pathophysiology of the disease results in obstruction of the smaller airways (bronchiole) and destruction of alveoli due to emphysema. The obstruction of the smaller airways is caused by repetitive damage to the tissues, inflammatory repair and tissue remodeling resulting in thickening of the bronchial walls. In addition, excess mucous production plugs the lumen. The alveolar walls loose their elasticity and enlarge leading to emphysema which causes inefficient gas exchange, trapping of carbon dioxide, and impaired exhalation (71, 72). All these conditions lead to reduced lung function, increased risk of infections, and increased risk of death (73-75). COPD is diagnosed by assessing the airflow obstruction by using spirometry, which involves measurement of the forced expiratory volume in 1 second (FEV_1) and the forced vital capacity (FCV). In patients with airway obstruction the FEV_1 and FCV are reduced compared to healthy individuals. Based on the severity of the airway obstruction measured with spirometry, patients can be categorized into four stages according to guidelines set by Global initiative for chronic Obstructive Lung Disease (GOLD) (67). Stage I being less severe, characterized by mild airflow obstruction to stage IV characterized by very severe airflow obstruction and impaired quality of life.

The clinical consequence of COPD is a result of the abnormal inflammatory response in the lungs. Upon inhalation of toxic gases, epithelial cells and macrophages of the airways become activated. Alveolar macrophages migrate into the lumen of the airways where they secrete proteases, cytokines and chemokines, the latter resulting in recruitment of immune cells, in particular neutrophils, to flood the airways and lung parenchyma of COPD patients (76). Neutrophils along with NK cells cause severe injury to the alveolar lining due to their cytotoxic effector functions (77, 78). The accumulation of these immune cells increases with the severity of the COPD leading to emphysema and decreased lung function (79, 80).

Treating the symptoms and underlying conditions can delay the progression of COPD. Cessation of smoking, exercise, and bronchodilators can be used to

improve the lung capacity. Corticosteroids, long-term treatment with macrolides and phosphodiesterase 4 (PDE4) inhibitors can be used to regulate the immune responses and resolve inflammation.

Exacerbations can be defined as deterioration of respiratory function due to increased inflammation and are associated with increased mortality. There was a significant increase in mortality of COPD patients who have had an acute exacerbation requiring at least one hospital admission compared to patients without an exacerbation, implying their importance in COPD (81). Infectious agents including both viruses and bacteria trigger more than half of the exacerbations in COPD. Among bacteria, non-typeable *Haemophilus influenza* (Nt*Hi*), *Streptococcus pneumonia, Moraxella catarrhalis,* and *Pseudomonas aeruginosa* are important pathogens (82).

4.3 Cystic Fibrosis Lung Disease

Cystic fibrosis (CF) is a genetic disorder, which is caused by mutations in the *cystic fibrosis transmembrane conductance* (*CFTR*) gene. These mutations cause a defective chloride ion channel, which affects the secretory glands including sweat and mucous glands. The disease is characterized by a sticky and viscous mucous, which affects many organs of the body including the respiratory system, the digestive tract, and reproduction. The thick mucous layer in the digestive tract causes malabsorption of nutrients, leading to malnutrition and poor growth in some of the affected individuals. Internal organs like the liver and in particular the pancreas that are involved in the secretion of important digestive enzymes, are also severely affected. The thick mucous clogs the ducts of the glands secreting the enzymes, which leads to inflammation in these organs, development of cysts, and fibrosis of the damaged tissue.

Though this disease affects many different organs, the major cause of morbidity and mortality is the complications seen in the lungs. The CF lung disease is characterized by airflow obstruction due to thick viscous mucous, impaired mucociliary transport in the airways because of low PCL volume, persistent and chronic colonization of the airways with different bacteria during different stages of disease progression. The most common bacteria colonizing the lungs of CF patients are (Nt*Hi*), *Staphylococcus aureus*, and *P. aeruginosa*. The sustained bacterial infections of the airways leads to high levels of inflammation. The airways get flooded with immune cells like neutrophils, which leads to tissue damage to the airway epithelium because of the cytolytic effects of the inflammatory mediators. Because of the tissue damage there is an ongoing repair

and remodeling phase, which leads to permanent lung damage involving the formation of scar tissue (fibrosis).

The increased influx of neutrophils to the airways as a result of bacterial infection leads to tissue damage and cell death. The microbial components and cytokines present during these conditions regulate the neutrophil survival and death. The life span of the circulating neutrophils is usually few hours and they die by apoptosis but exposure to specific stimuli can induce neutrophil death either by necrosis or NETosis (83). The increased cell death at the sites of inflammation in the airways leads to accumulation of high concentration of extracellular DNA (eDNA), which becomes a major component of the mucous and causes high viscoelasticity of sputum in CF patients (84, 85). The highly viscous sputum causes airway obstruction and reduces the lung capacity. The mucociliary clearance is also impaired because of the thick mucous which bacteria can colonize and establish chronic infections in the airways.

Some of the commonly used treatment strategies for CF patients include nebulized hypertonic saline to clear the mucus form the airways by increasing the PCL volume, thereby restoring proper mucociliary clearance. Antibiotics are also commonly used to eradicate bacteria or decrease the bacteria in the airways of CF patients.

DNase I, an enzyme commonly found in body fluids (saliva, pancreatic secretions, and plasma), is responsible for the degradation of the eDNA. Thus, use of inhaled aerosolized DNase I of bovine origin was introduced for the management of airway clearance in CF patients. The DNase I degrades the eDNA into low molecular weight fragments thereby reducing the viscoelasticity of the sputum making it easier to mobilize the sputum from the airways. Later on recombinant human (rh) DNase I was developed to overcome adverse reaction to bovine protein (86, 87). The use of DNase I along with afore-mentioned treatment strategies in CF patients reduced the frequency and number of exacerbation, improved lung function, and quality of life (88).

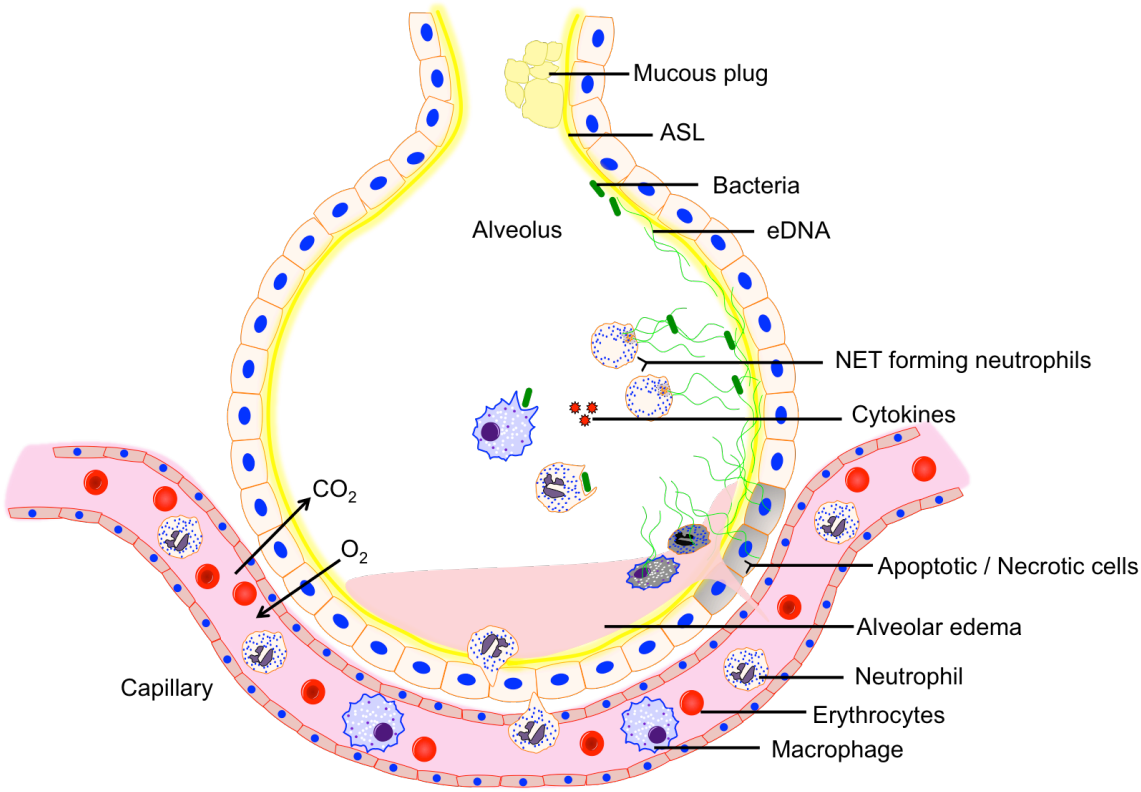

Figure 6. The consequences of infection and inflammation of the airways
Excess sputum production, reduced mucociliary clearence, colonization and infection, recruitment of immune cells, increased cytokine production, cell death, tissue injury, increased presence of eDNA resulting in high vicousity of the sputum, increased alveolar-capillary permeability, accumulation of fluid, and impaired gas exchange are some of the consequences of prolonged and excessive airway inflammation.

4.4 Bacterial Infections of the Lung

Some of the most common bacterial pathogens associated with the respiratory tract include *P. aeruginosa*, Nt*Hi, Streptococcus pneumoniae*, and *S. aureus*. The bacteria can gain entry to the lower respiratory tract when there is an injury, impaired host defense mechanisms, and dysregulated inflammation in the airways.

4.4.1 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative, rod shaped, monoflagellated, and ubiquitous bacterium found in soil, and water of the environment. These bacteria are also found as part of the normal flora of the skin and are considered to be an opportunistic pathogen that can infect individuals whose immune system is compromised (89-93). They can infect the airways of individuals with pre-existing disease conditions like COPD, and CF (92, 94). *P. aeruginosa* infections are usually difficult to treat due to its rapidly evolving resistance to antibiotics. They can also evade the host defense mechanisms by a variety of additional strategies, which include changing their phenotype from motile, non-mucoid to non-motile and mucoid types forming biofilms (95, 96). Furthermore, they can produce proteases like elastase, which can degrade several host defense molecules like complement proteins, coagulation proteins, cytokines, and chemokines (97, 98).

4.4.2 *Haemophilus influenzae*

H. influenzae is a Gram-negative coccobacilliary bacteria. There are two major categories of this bacterium, one is the encapsulated strains and the other is an unencapsulated strains. The unencapsulated strains are also known as nontypable *H. influenzae* (Nt*Hi*). Nt*Hi* is usually an opportunistic pathogen and can cause infections like sinusitis, conjunctivitis, and exacerbations in COPD. This bacterium produces beta-lactamases and hence has gained resistance against the penicillin family of antibiotics.

4.4.3 *Streptococcus pneumoniae*

S. pneumoniae is a Gram-positive β-hemolytic cocci. They can reside asymptomatically in the nasopharynx of healthy individuals and can manifest as an infection in immuno-compromised individuals, elderly and young individuals. It is the most common cause of community-acquired pneumonia. Once the lower respiratory tract gets colonized, there is an influx of inflammatory mediators and

immune cells leading to edema in the alveoli. This condition is called pneumonia. The pathogenicity of *S. pneumoniae* can be ascribed to its virulence factors, for example its capsule, cell-wall, pneumolysin, neuraminidase, and hydrogen peroxide to mention a few. The encapsulated bacteria are resistant to phagocytosis-mediated killing and can thus escape the host immune response.

4.4.4 *Staphylococcus aureus*

S. aureus is a Gram-positive coccus usually found as a commensal in the nose, respiratory tract, and skin. It is a common cause of infections like skin abscesses, food poisoning (through toxins), sinusitis, pneumonia and toxic shock syndrome. *S. aureus* produces various virulence factors which include enzymes like coagulase, DNase, beta-lactamase; toxins like staphylotoxin, exfoliative toxins, toxic shock syndrome toxin-1, enterotoxin to name a few. Many *S. aureus* have become resistant to most beta-lactam antibiotics and have become difficult to treat.

5. Present Investigations

5.1 Paper 1

Osteopontin is a key regulator of cytotoxic and proinflammatory activities exerted by extracellular histones.

Background

Osteopontin (OPN) is a multifunctional highly anionic phosphoglycoprotein that is categorized as a cytokine based on some of its functions including recruitment of neutrophils and matrix remodeling. OPN is expressed in the airways along with many other tissue and cell types, an expression that increases during inflammation and disease conditions like COPD, CF and asthma. During inflammation histones become present extracellularly as a result of abundant cell-death and formation of NETs. The extracellular histones aggravate the inflammation and can lead to further tissue damage.

Aims

- To investigate whether OPN co-localizes with extracellular histones in CF lung tissue.

- To investigate the functional consequences OPN interaction with extracellular histones.

Results and conclusions

Using immunohistochemistry, co-localization of histone H4 and OPN was found on the surface of bronchial epithelial cells of CF lung tissue. OPN interaction with individual subunits of histones (H1, H2A, H2B, H3.1, and H4) was confirmed using surface plasmon resonance and ELISA based binding assays. OPN inhibited the hemolytic, cytotoxic and NET inducing effects of histones. OPN knockout mice were susceptible to increased inflammation and higher mortality when exposed to extracellular histones in the airways compared to wild type mice.

Taken together, this study demonstrated that OPN has anti-inflammatory function and also acts as a cytoprotective molecule, protecting against the harmful effects of extracellular histones in the airways.

5.2 Paper 2

Osteopontin that is elevated in the airways during COPD impairs the antibacterial activity of common innate antibiotics.

Background

Bacterial infections of the airways contribute to exacerbations and disease progression in COPD despite the activation of innate immune mechanisms like accumulation of inflammatory cells, chemokines, and pro-inflammatory cytokines. The epithelial cells, submucosal cells and neutrophils are important sources of antimicrobial proteins that play an important role in airway host defense. A constitutively higher level of OPN has been reported in COPD patients, increasing with disease severity.

Aim

- To investigate whether OPN co-localizes with AMPs in the airways of COPD patients.

- To determine the functional consequences of OPN interaction with AMPs.

Results and conclusions

OPN was found to co-localize with several AMPs expressed in the airways during COPD. AMPs like lactoferrin, SLPI, midkine, hBD-3, and TSLP exhibited strong binding to OPN *in vitro* and also inhibited their antimicrobial activity against *S. pneumoniae* and *P. aeruginosa.* However, the muramidase activity of lysozyme and protease inhibitory function of SLPI were not affected by OPN.

This study demonstrated that OPN interacts with several AMPs, impairing their host defense functions and thus increasing the vulnerability of COPD patients to infections.

5.3 Paper 3

Roflumilast increases bacterial load and dissemination in a model of *Pseudomonas aeruginosa* airway infection.

Background

Exacerbations are a major clinical problem in many patients suffering from COPD. Roflumilast, an inhibitor of phosphodiesterase 4 (PDE4), has been beneficial in preventing exacerbations in severe COPD. Roflumilast is an anti-inflammatory molecule, which can interfere with potentially important host defense functions, including cytotoxic properties of neutrophils at the sites of inflammation. Structural and immunological changes in the airways of severe COPD increases the risk of chronic bacterial infections, in particular by *P. aeruginosa*.

Aim

- To investigate the possible effect from roflumilast treatment on the course of *P. aeruginosa* airway infection in mice.

Results and conclusions

Roflumilast increased the mortality of the mice infected with *P. aeruginosa* intranasally, increased bacterial load and also bacterial dissemination. There was a reduced influx of neutrophils in the BALF but an increased accumulation in lung tissue of roflumilast-treated infected mice. There was an increase in IL-6 and the anti-inflammatory cytokine IL-10, whereas other proinflammatory cytokines were downregulated. The airway host defense is compromised even with an unchanged or higher neutrophil numbers in the lung tissue suggests impairment in the cytotoxic functions of the neutrophils.

Although roflumilast-treatment has beneficial effects in patients with severe COPD, withholding the roflumilast treatment could benefit the patients suffering from acute and chronic bacterial infections of the airways.

5.4 Paper 4

DNA-fragmentation is a source of bactericidal activity against *Pseudomonas aeruginosa*.

Background

Cystic fibrosis (CF) is a genetic disorder that affects many organs but the lung disease is the major cause of morbidity and mortality in these patients. The characteristics of CF lung disease include chronic bacterial infections (especially *Pseudomonas aeruginosa)*, inflammatory exacerbations, and highly viscous sputum of the lower airways. Long-lasting and dysregulated inflammatory responses in CF airways leads to accumulation of immune cells like neutrophils, that eventually succumb, resulting in abundant extracellular DNA (eDNA). This eDNA is a major contributor to the viscous mucus seen in this disease and is the basis for use of recombinant human DNaseI as a treatment for reducing the viscoelasticity of the sputum for efficient clearance of the airway congestion.

Aim

- To investigate the effects of DNase I treatment in a murine model of acute *P. aeruginosa* airway infection.

- To determine the molecular properties of eDNA and possible roles for DNA-fragmentation, in executing bactericidal activity against *P. aeruginosa.*

Results and conclusions

DNase I treatment of *P. aeruginosa* infected mice enhanced their survival and decreased the bacterial load in the BALF and lung tissue. The treatment significantly decreased the pro-inflammatory cytokines IL-6 and TNF-α in the lung tissue.

The eDNA isolated form the BALF of the DNase I treated mice showed increased fragmentation with accumulation of smaller fragments of sizes around and below 200 bp.

By using synthetic DNA fragments of decreasing lengths, we showed that there is a size threshold for the DNA fragments to exhibit bactericidal activity and this activity was possibly because of the chelation of divalent cations from the surface of Gram-negative bacterial outer membrane.

The findings suggest a novel host defense strategy that could potentially be employed to treat *P. aeruginosa* infections, circumventing mechanisms involved in resistance against conventional antibiotics.

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