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On airway host defence during allergic inflammation

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2016

Document Version:
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Citation for published version (APA):
Gela, A. (2016). *On airway host defence during allergic inflammation*. [Doctoral Thesis (compilation), Respiratory Medicine, Allergology, and Palliative Medicine]. Lund University: Faculty of Medicine.

Total number of authors:
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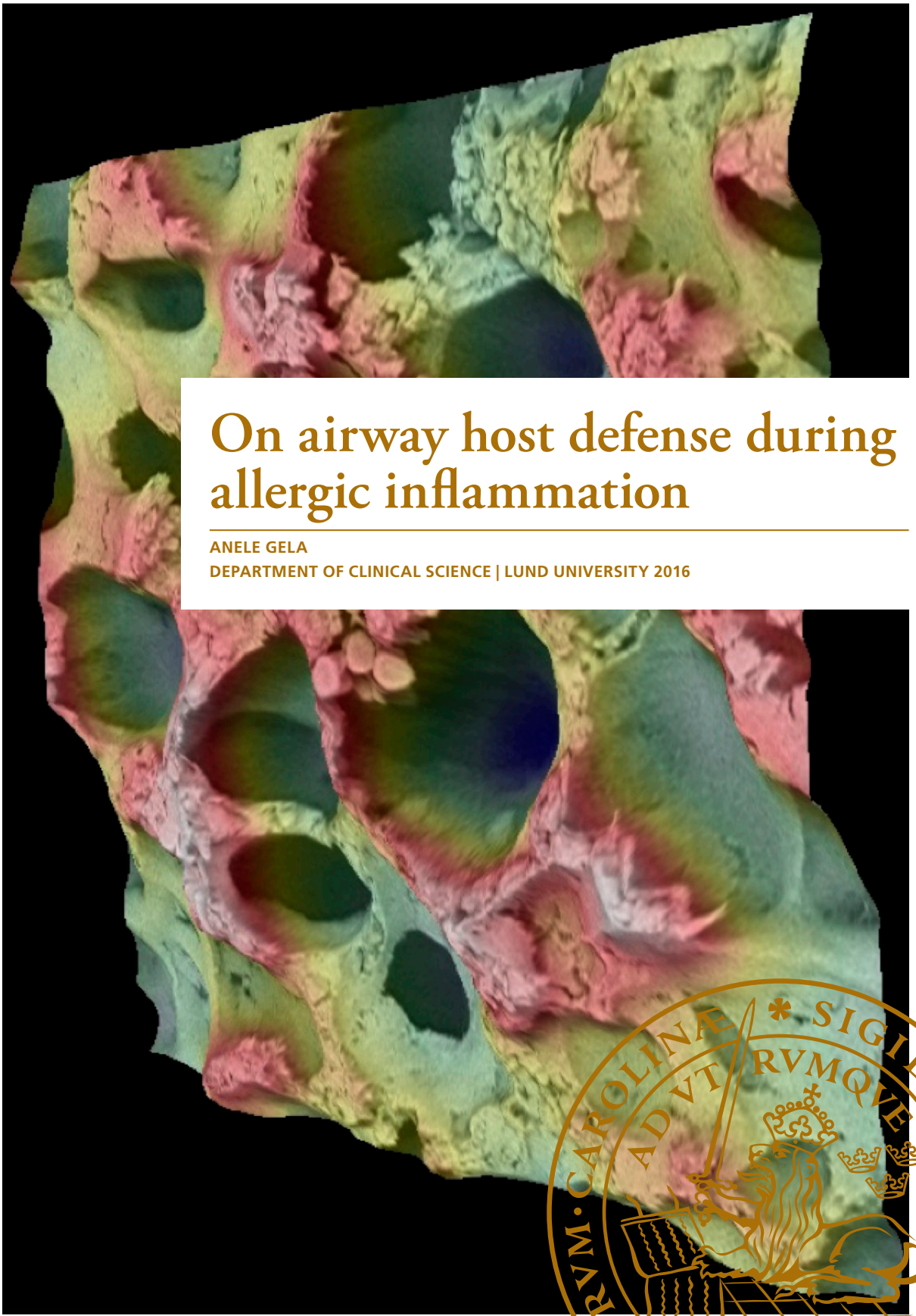

On airway host defense during allergic inflammation

ANELE GELA
DEPARTMENT OF CLINICAL SCIENCE | LUND UNIVERSITY 2016

On airway host defense during allergic inflammation

On airway host defense during allergic inflammation

Anele Gela

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To be defended at Segerfalksalen on September 3rd at 09:00.

Faculty opponent
Professor. Hans-Uwe Simon

Organization	Document name
LUND UNIVERSITY	DOCTORAL DISSERTATION
Department of Clinical Science Division of Lung medicine and allergology 22184 Lund, Sweden	Date of issue September 3rd, 2016
Author(s)	Sponsoring organization

Title and subtitle: On airway host defense during allergic inflammation

Asthma is a chronic inflammatory disease of the airways, affecting and disabling approximately 300 million people worldwide. The inflammatory profile is characterized by infiltration of eosinophils, which are a rich source of factors that are implicated in tissue remodeling. The chronic inflammatory response and the remodeled phenotype creates a hospitable environment for secondary bacterial infections. In recent years, systemic infections caused by *Streptococcus pneumoniae* in asthmatics have received global attention. The risk of acquiring pneumonia in patients suffering from asthma is 2-10 fold increased as compared to healthy individuals. The cause is not known and in this thesis we hypothesized that the dysregulated allergic response may impair innate host defenses. The mechanisms being investigated may help to explain how the prolonged and dysregulated inflammatory response increases the vulnerability of asthmatics to invasive pneumococcal disease. Initially, the regulation of chemokines, in particular eotaxins, by mast cell proteases was investigated. From this study, we were able to map the region of eotaxin-3/CCL26 that harbors antimicrobial (COOH-terminal) and anti-endotoxin (NH_2-terminal) activity following proteolytic cleavage with mast cell chymase and tryptase, respectively. However, the receptor activating properties (NH_2-terminal) were lost. In a separate study, the anti-endotoxin fragment derived from CCL26 conferred therapeutic benefits in a mouse model of LPS-induced inflammation. Furthermore, the interaction of chemokines, particularly Th-2 chemokines, with osteopontin (OPN) was investigated. OPN is an anionic glycoprotein that is upregulated in asthma and its expression increases with the severity of asthma. OPN bound to the COOH-terminal of chemokines and completely abolished their antimicrobial activity without affecting their NH_2-terminal localized functions, including LPS-neutralization and receptor activating properties. To ascertain if whether the effects of OPN are generic or specific for Th-2 chemokines, we investigated its interaction with the classical antimicrobial peptides that are constitutively and upregulated during COPD. Interestingly, OPN bound and neutralized their antimicrobial activity but did not interfere with the muraminidase activity and protease inhibitory function of lysozyme and secretory leukocyte protease inhibitor (SLPI), respectively. These studies suggest that chemokines and antimicrobial peptides can serve as host defense peptides but their actions are modulated by mast cell proteases and OPN. Therefore, there is an urgent need for studies focusing on modification of antimicrobial peptides to become resistant to proteolytic cleavage, altered pH and various salt conditions. Also, the elucidation of the novel roles of OPN during allergic inflammation could present potential pharmaceutical targets. Taken together, this thesis explains several mechanisms that impair innate host defenses during allergic inflammation.

Key words: Asthma, COPD, Th-2 chemokine, antimicrobial peptides, bacteria.

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language: English
ISSN and key title: 1652-8220		ISBN: 978-91-7619-310-5
Recipient's notes	Number of pages	Price
	Security classification	

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Cover photo: Scanning electron microscopy depicting lung tissue from mouse.
Courtesy-: Maria Baumgarten.

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Faculty of Medicine | Department of Clinical Science | Lund University

ISBN 978-91-7619-310-5
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University, Lund 2016

This work is dedicated to my late mother,

Xoliswa Gela.
Ndiyabulela ngothando nemfundiso zakho Sweetwords.

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Acknowledgements

I would like to express my heart-deep felt gratitude and appreciation to my supervisor, **Arne Egesten**. Your immeasurable wisdom for science and your compassion for mankind are impeccable. Not only were you a lab supervisor but also you extended your role beyond the lab. Just saying thank you for your mentorship and guidance will not repay your kindness and all that you have done for me. You made my PhD journey worthwhile and for that I am grateful.

To my co-supervisor, **Heiko Herwald**, you created a conducive working environment and made everyone feel welcomed at BMC. Above all, your friendly nature and insatiable appetite for different cultures is admirable.

To **Lars Björck**, you created a nice atmosphere in the floor and your scientific discussions in every platform are uplifting. It has been an experience working in the same floor with such great minds.

Pia Andersson, thank you for your tireless efforts, helping with experiments, and always there when needed. You have ensured smooth running of the lab.

Sandra Jovic, you taught me a lot of lab techniques when I first arrived in the lab and I learnt a lot from you. Thank you for your patience and guidance.

Gopinath Kassetty, thank your for the good working relations.

Ravi Bhongir, soft spoken and hardworking individual I have had the pleasure to work with.

Mohammed Ali, your eagerness to learn is admirable and thanks for the friendship, jokes and fun times.

Matthias Mörgelin, I have learnt to speak more Swedish and German from you than I could possibly ever learn in the classroom. Thank you for the jokes, smiles in the corridor and more importantly the beautiful microscopy images.

Maria Baumgarten, for your constant help and suggestions with microscopy.

I would also like to extend a very special thanks to **Eva Körner Muhrbeck** and **Anita Berglund** for helping with all administrative duties.

My sincere thanks to everyone I have had the privilege to work with during my years at BMC: **Ann-Charlotte Strömdahl, Sara Nordin, Ramesh Tati,Andreas Nägeli, Christofer Karlsson, Emmanuel Smeds, Inga Maria, Ingbritt Gustafsson, Malgorzata Berlikowski, Maria Allhorn, Maria E Johansson, Mariena van der Plas, Markus Roupe, Matthias Collin, Praveen Papareddy, Raja Kalluru and Oonagh Shannon**.

A special thanks to the '*fika'* colleagues: **Anas**, **Ariane, Azadeh, Erik, Finja, Frida, Helena, Jane, Jesper, Johannes, Lech, Malin, Madlen, Marta, Niklas, Sandra, Sinead, Suado, Tirthankar, Torgny,** and **Jonathan**.

To the **LUGI Lions** rugby family, I want to say it has been a life changing experience to be part of such an astonishing community. I hope you make everyone feel welcome at **LUGI** as much as I have.

To the **EUROSA scholarship**, thank you for granting me that first step towards realizing my PhD. I wouldn't have done it without **Lorna Holtman**.

I am deeply indebted to **Monique Bennett** and **Chontrelle Willemse**, otherwise my academic journey would not have gone beyond the year 2007.

To the long list of friends at home, thank you for the memories and I look forward to many adventures and also helping one another grow in our different career paths.

A word of appreciation goes out to my family for believing and supporting me throughout this PhD journey. I wouldn't be doing justice if I don't mention **Usanda, Aviwe, Lathitha, Xolisa, Solomon, Thandi, Kholosa, Mthawelanga, Khanya, Wongama, Wilson Gela, Chumani Gobizembe, Mlamli Malangabi**. Ndiyabulela Mkhuma, Xesibe, Mbathane, Nonzaba, Khandanyawana, Mbaca. Not forgetting the Nkota family: **Buyelwa Nosizwe, Zukiswa, Siyathemba, Nomzamo and Azola**. Enkosi nakuni Mpondo, Thahla, Ndayeni, Faku, Hlamba ngobende, Kadakhile, Ziqelekazi.

Abstract

Asthma is a chronic inflammatory disease of the airways, affecting and disabling approximately 300 million people worldwide. The inflammatory profile is characterized by infiltration of eosinophils, which are a rich source of factors that are implicated in tissue remodeling. The chronic inflammatory response and the remodeled phenotype create a hospitable environment for secondary bacterial infections. In recent years, systemic infections caused by *Streptococcus pneumoniae* in asthmatics have received global attention. The risk of acquiring pneumonia in patients suffering from asthma is 2-10 fold increased as compared to healthy individuals. The cause is not known and in this thesis we hypothesized that the dysregulated allergic response may impair innate host defenses. The mechanisms being investigated may help to explain how the prolonged and dysregulated inflammatory response increases the vulnerability of asthmatics to invasive pneumococcal disease. Initially, the regulation of chemokines, in particular eotaxins, by mast cell proteases was investigated. From this study, we were able to map the region of eotaxin-3/CCL26 that harbors antimicrobial (COOH-terminal) and anti-endotoxin (NH_2-terminal) activity following proteolytic cleavage with mast cell chymase and tryptase, respectively. However, the receptor activating properties (NH_2-terminal) were lost. In a separate study, the anti-endotoxin fragment derived from CCL26 conferred therapeutic benefits in a mouse model of LPS-induced inflammation. Furthermore, the interaction of chemokines, particularly Th-2 chemokines, with osteopontin (OPN) was investigated. OPN is an anionic glycoprotein that is upregulated in asthma and its expression increases with the severity of asthma. OPN bound to the COOH-terminal of chemokines and completely abolished their antimicrobial activity without affecting their NH_2-terminal localized functions, including LPS-neutralization and receptor activating properties. To ascertain if whether the effects of OPN are generic or specific for Th-2 chemokines, we investigated its interaction with the classical antimicrobial peptides that are constitutively expressed and upregulated during COPD. Interestingly, OPN bound and neutralized their antimicrobial activity but did not interfere with the muraminidase activity and protease inhibitory function of lysozyme and secretory leukocyte protease inhibitor (SLPI), respectively. These studies suggest that chemokines and antimicrobial peptides can serve as host defense peptides but their actions are modulated by mast cell proteases and OPN. Therefore, there is an urgent need for studies focusing on modification of antimicrobial peptides to become resistant to proteolytic cleavage, altered pH and various salt conditions. Also, the elucidation of the novel roles of OPN during allergic inflammation could present potential pharmaceutical targets. Taken together, this thesis explains several mechanisms that impair innate host defenses during allergic inflammation.

Preface

My journey as an aspiring scientist began when I was offered a scholarship to pursue PhD studies in Arne Egesten's research group. Having been trained (in my M.Sc.) in the field of molecular virology I knew I had a mammoth task of familiarizing myself with the new academic discipline(s). Luckily for me, even though not exactly the same but my background in working with respiratory viruses made the transition less challenging.

The thesis deals with dysregulated inflammatory responses in asthmatic patients and how it increases the vulnerability of these patients to acquire invasive pneumococcal infections. I am attempting to decipher some of the mechanisms that impair airway host defenses during chronic inflammation with the intention of devising new strategies for therapeutic intervention. I have structured the thesis in such a way that it will help the reader understand the homeostatic control of the immune system and how this balance is skewed during chronic inflammation, and the consequences therefore.

I have presented four papers that I hope will shed light on the mechanistic insights on how airway host defenses are impaired. In addition, I have also included data on how this research could yield potential therapeutic benefits *in vivo*. I have certainly enjoyed writing this thesis and I hope the reader would find it educational, thought provoking and insightful and hopefully learn something new.

Anele Gela

Lund, 2016

Chapter 1: The respiratory system

The respiratory system is an organ system consisting of specific organs and biological structures that are responsible for the process of respiration. The major respiratory structures span the nasal cavity and extend to the diaphragm. Their key function is the intake and exchange of gases, mainly O_2 and CO_2, between an organism and the environment.

Functionally, the respiratory system is divided into a conducting and respiratory zone. The conducting zone extends from the nose to the terminal bronchioles. The main functions are to provide a route for incoming and outgoing air, clear debris and pathogens from inhaled air, and warm and humidify the incoming air. In addition to providing a passageway for inhaled air, other structures, such as epithelium in the nasal passage have a secondary function. The epithelium in the nasal passage is essential for sensing odors (via specialized olfactory epithelium), whereas the bronchial epithelium can respond and, to some extent clear, airborne particles and toxins. The conducting component can be divided into the upper and lower respiratory tract (Figure 1). The upper respiratory tract spans from the nasal cavity into the larynx. The lower respiratory tract, on the other hand, begins with the trachea, which bifurcates at the carina, into two main bronchi. The latter divide repeatedly forming airways of decreasing diameter. The respiratory zone, on the other hand, is part of the respiratory system where gas exchange occurs. This zone begins where the terminal bronchioles join a respiratory bronchiole, which then lead to an alveolar duct, opening to a cluster of alveoli. In normal healthy subjects, the respiratory zone is structurally suited for efficient gaseous exchange (Figure 2):

- The blood-air barrier (formed by type 1 pneumocytes of the alveolar wall, the endothelial cells of the capillaries, and the basement membrane between the two cells) is thin, which allows quick diffusion of gases (e.g. O_2 and CO_2).
- The moist alveolar wall allows efficient diffusion of gases.
- The branched alveoli increase the surface area for diffusion of gases.
- The rich capillary network ensures efficient transport of gases to and from the lungs.

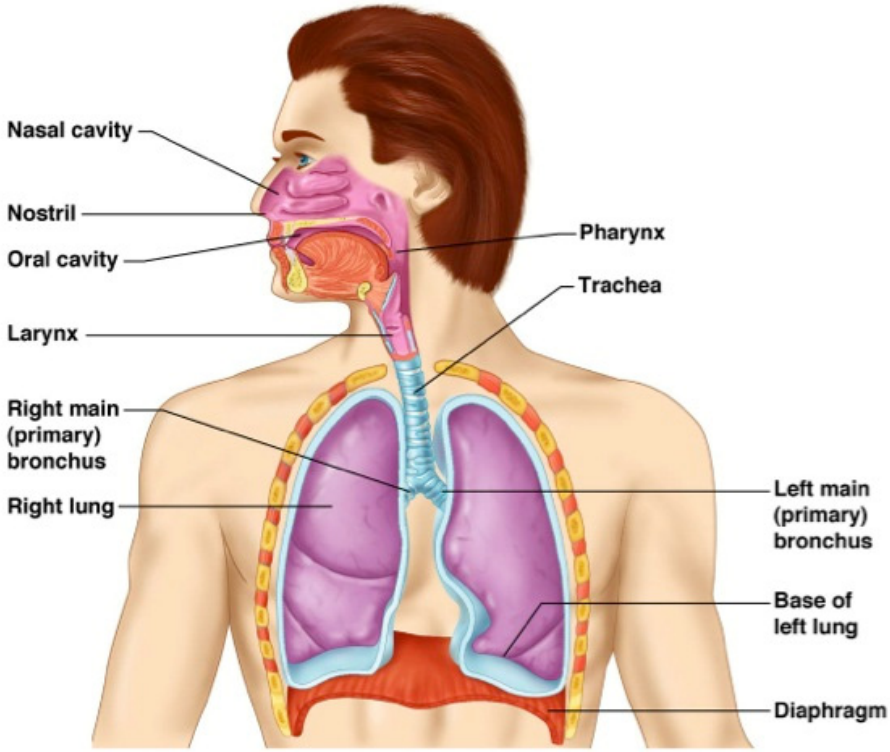

Figure 1
Anatomy of the respiratory system. The upper respiratory tract begins in the nasal cavity and end in the larynx. From the trachea down to the bronchioles is the lower respiratory tract (the picture is adapted from http://www.ncbi.nlm.nih.gov/pubmedhealth/PMHT0022181/).

In addition to their function in gas exchange, the respiratory system is constantly being exposed to foreign material. Therefore, a robust constitutive immune defense is essential to protect the host from invading microorganisms. These defense mechanisms include a complex mix of innate and adaptive immune systems to protect the lungs and the rest of the respiratory system against inhaled microorganism. Secretions from the epithelium, goblet cells and mucous glands form a viscous fluid layer overlying the respiratory epithelium. The mucus traps the invading microbes and the sweeping action of the cilia removes the invading microbe. This fluid is rich in mucins, ions, and antimicrobial peptides including lysozyme. Bacteria that invade or breach this first line barrier are dealt with by a second line of defense, which includes phagocytes as well as antimicrobial peptides that are secreted by the surface epithelium (1). Lastly, bacteria that escape the host defense peptides or phagocytes are eliminated by the adaptive immune responses.

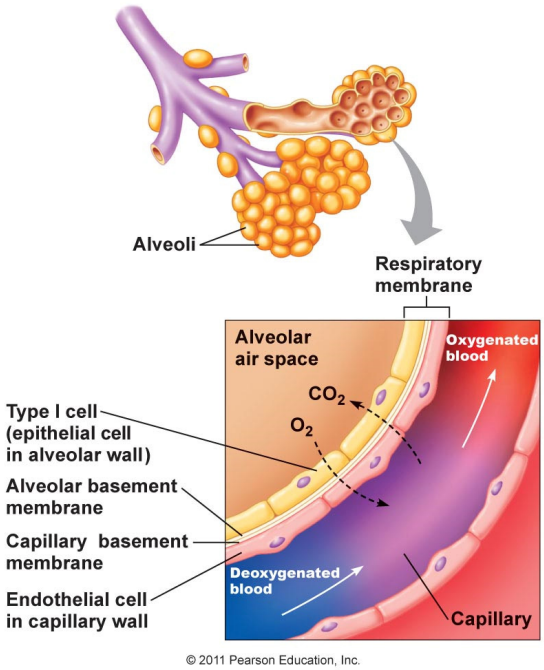

Figure 2
Schematic representation of the respiratory zone where exchange of gases take place. Also the different component of the blood-air barrier (the picture is adapted from Dr. Robert Droual's website http://droualb.faculty.mjc.edu).

Chapter 2: The immune system

The immune system is a collection of barriers, cells, tissues, and molecules that function to defend us against infectious microbes and other foreign substances. The vertebrate immune system is a product of years of evolutionary struggle between evolving pathogens and their much less rapidly producing, and hence less adaptable hosts. Traditionally, the immune responses are classified as innate and adaptive (2). The modern model of immune function is organized into three phases based on the timing of their effects (3). The three phases consist of the following:

Barrier defenses: these include mucous membranes, pH, salt, and skin, which act instantaneously to prevent any pathogenic or foreign invasion of body tissue.

Innate immune response: this wing of the immune system acts rapid but is non-specific, and consist of a variety of specialized cells and soluble factors.

Adaptive immune response: the slower but more specific and effective arm of the immune system, which involves many cell types and soluble factors, but is primarily controlled by white blood cells (lymphocytes), which help control immune responses.

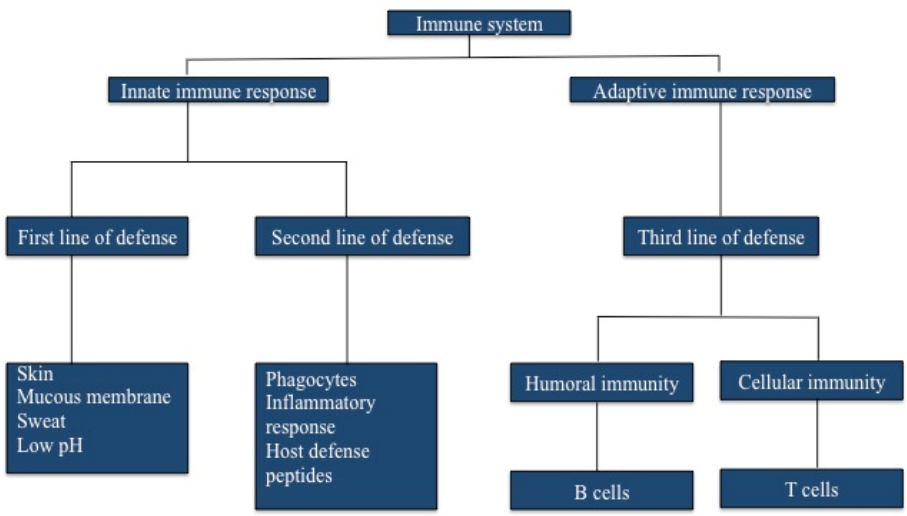

Figure 3
Schematic overview of the immune system depicting the divisions of the immune response and their subdivisions.

2.1 Innate immune response

Innate immune responses are phylogenetically far more ancient, being widely represented in multicellular phyla. They are based on recognition of common molecular signatures of potential pathogens (4). Pathogen recognition occurs when germ-line encoded pathogen-recognition receptors (PRRs) expressed by a variety of cells recognize and bind pathogen-associated molecular patterns (PAMPs). PAMPs recognized by PRR include lipids, lipoproteins, proteins and nucleic acids derived from a wide range of microbes, including bacteria, viruses, parasites and fungi (5). This recognition can occur in various cellular compartments, including plasma membrane, endosomes, lysosomes and endolysosome (5). These interactions activate signaling pathways that lead to the production of secreted factors that are involved in the inflammatory response.

2.1.1 Granulocytes

Granulocytes also known as polymorphonuclear (PMN) leukocytes, is a category of white blood cells characterized by the presence of granules in their cytoplasm. They are the most abundant leukocyte with $\approx 10^9$ PMNs produced per hour in the healthy adult, but have a short life span of ≈5 days in the circulation. They are classified by light microscopy into three types based on their staining characteristics. Neutrophils are the most abundant type, and the other types (eosinophils, basophils, and mast cells) are present at lower numbers but may accumulate in extra-vascular tissues (Figure 4).

2.1.1.1 Neutrophils

Neutrophils are the first line of innate defense. Elie Metchnikoff first discovered them and since their discovery they have been considered as potent inflammatory cells causing inflammatory tissue damage. Neutrophil possess an array of granules that contain a large number of cytotoxic and immune regulatory molecules. The cytoplasm of neutrophils contain four types of granules: azurophilic (primary), specific (secondary), gelatinase, and secretory (6).

- Azurophilic granules contain enzymes, such as proteinase 3, cathepsin G, and elastase, as well as α-defensins and bactericidal permeability increasing (BPI) protein.
- Specific granules contain lactoferrin and the pro-forms of cathelicidin peptides.
- Gelatinase granules are rich in gelatinase and a marker of terminal neutrophil differentiation.

- Secretory granules contain a variety of receptors that are inserted into a cell membrane upon activation and exocytosis, converting neutrophils into a cell responsive to many inflammatory stimuli.

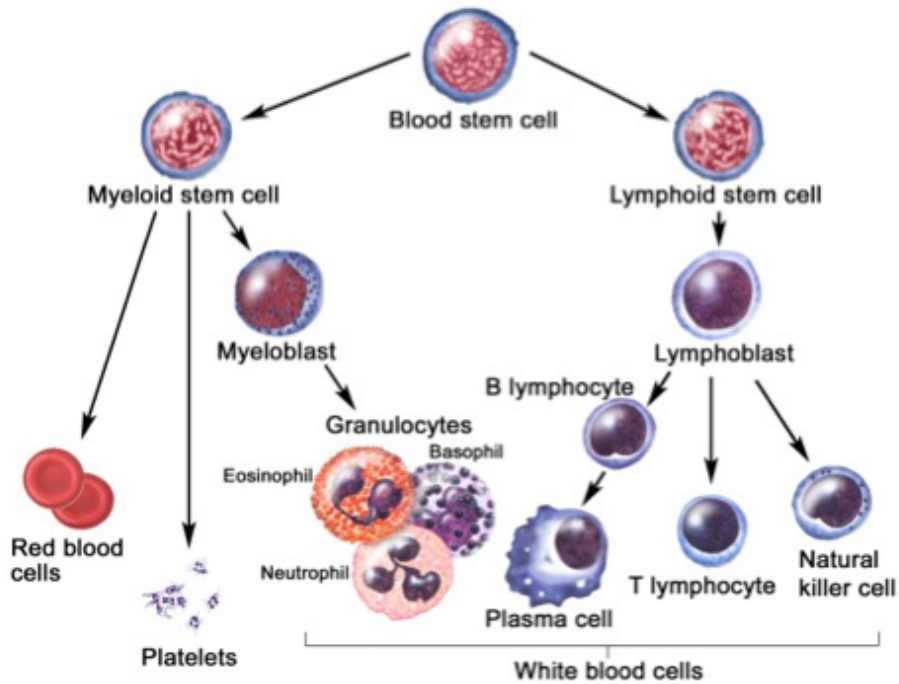

Figure 4
Flow diagram illustrating the process of hematopoiesis. The different cellular components are formed through differentiation of hematopoetic stem cells into mature blood cells (picture is adapted from http://www.ncbi.nlm.nih.gov/pubmedhealth/PMHT0022048/).

2.1.1.2 Eosinophils

Eosinophils are granulocytic leukocytes first discovered by Heinrich Caro in 1874 and described by Paul Ehrlich in 1879 (7). They are granulocytes that develop in the bone marrow from pluripotent progenitors and are released into peripheral blood in a phenotypic mature state. Their differentiation occurs under the influence of granulocyte-monocyte colony stimulating factor (GM-CSF) and IL-3 in early phases and IL-5 in the latter phases of differentiation (7). They are recruited into tissues in response to IL-5 and eotaxins. Human eosinophils are characterized by a bi-lobed nucleus with a highly condensed chromatin (Figure 4). They contain 4 major proteins in their granules: the eosinophil peroxidase, major basic protein (MBP), ribonucleases eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) (8). The release of the granule contents into the extracellular space is a prominent eosinophil function. It was previously

thought that this degranulation promoted immunity to helminthes but now this view has been called into question by results from animal studies (9, 10). Eosinophils have been reported to exhibit a protective role against RNA viruses, such as respiratory syncytial virus. In addition, MBP and ECP have potent antimicrobial activity *in vitro* (11).

Eosinophils express surface receptors for ligands that support growth, chemotaxis, adhesion, degranulation, and cell-to-cell interaction. Among the main receptors, IL-5 receptor (IL-5Rα), CCR3 and Siglec-8/Siglec-F are important for eosinophil function. Upon activation by IL-5 and the eotaxins, they are recruited into tissues. They are recruited and activated in lung tissue as part of the pathophysiology of asthma, and most recent evidence suggest that eosinophils contribute to airway dysfunction and tissue remodeling in asthma (10, 12). ECP has been found to attach to cell membrane altering permeability as well as increasing production of reactive oxygen species (13). MPB, on the other hand, was found to cause alveolar epithelium lysis allowing for penetration of inhaled antigen (13). These studies suggest that targeting eosinophils themselves or their migration should provide therapeutic benefits for the treatment of asthma.

2.1.1.3 Innate lymphoid cells (ILC)

Innate lymphoid cells constitute a family of lymphocytes that are distinct from T and B cells. The family comprises of NK cells, Rorγt-dependent ILCs, and type 2 ILCs (ILC2s). Three independent groups recently discovered ILC2s in 2010 (14). The identification of ILC2s, which produce large amounts of IL-5 and IL-13 in response to epithelial derived cytokines, implicated a new key player besides Th-2 cells in asthma. They seem to contribute to the initiation as well as the maintenance of the Th-2 response. Murine studies have shown that ILC2s are an early innate source of IL-5 and IL-13 after allergen exposure, which induce eosinophilia, mucus hyper-production, and airway hyper-responsiveness (15). These properties make ILC2 cells an interesting target for the development of new therapeutic strategies for asthma treatment.

2.1.1.4 Mast cells

Mast cells (MC) are effector cells of the allergic reaction. They develop from hematopoietic progenitors in response to stem cell factor (KIT ligand). Progenitors subsequently migrate from blood into various tissues, including the skin, lungs, and mucosal interface, where they acquire their tissue-specific phenotype. They are distinguished by their content of electron-dense secretory granules, which are filled with large amounts of preformed compounds. These preformed molecules include histamine, serotonin, TNF-α, and proteases (e.g. chymase, tryptase, and carboxypeptidase). For the purpose of our studies, focus has been drawn into the serine proteases, specifically chymase and tryptase. The MC proteases are

expressed at exceptionally high levels, with mRNA approaching and even exceeding those of classical housekeeping genes, and they are stored in high amounts (16). The proteases are stored in fully active form; and when MCs undergo degranulation, large amounts of enzymatically active proteases are released into the extracellular space and could probably have a profound impact during allergic reaction. MC degranulation is triggered through exposure to an antigen (allergen) that cross-links allergen-specific IgE, which is already bound to the high affinity Fc epsilon receptor I (FcεRI). Therefore, in paper 1, we set out to investigate the potential effects of MC proteases on the biological function and host defense properties of human eotaxins, especially eotaxin-3/CCL26.

2.1.1.5 Cytokines and chemokines

Cytokine is defined as a signaling molecule that allows cells to communicate with each other over short distances. They are secreted into the intercellular space, and their action induces the receiving cell to change its physiology. A chemokine, on the other hand, is a soluble chemical mediator similar to cytokine except that its function is to attract cells from longer distances. A broad range of cells, including immune cells as well as endothelial cells, fibroblasts and various stromal cells, produces them. They are redundant secreted proteins with growth, differentiation, and activation function that regulate and determine the nature of immune responses and control immune cell trafficking and the cellular arrangement of immune organs (17). The type of chemical messenger produced in response to an immune insult determines whether an immune response will be cytotoxic, humoral, or cell mediated.

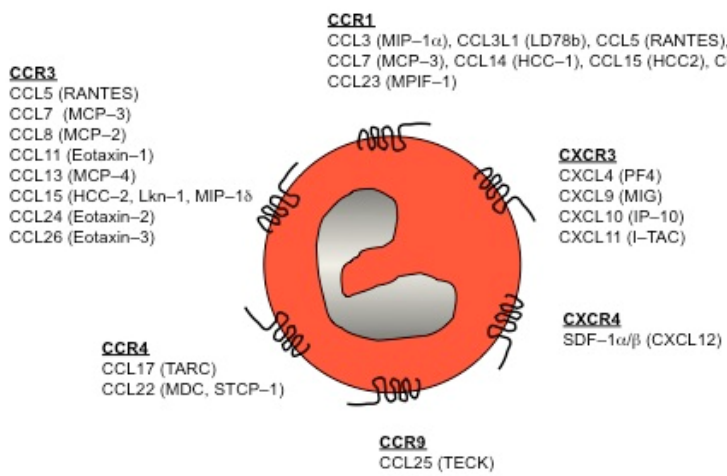

Figure 5
Schematic representation of chemokine receptors expressed on the surface of mature leukocytes and their corresponding ligands. One receptor may have more than one ligand and one ligand may bind several receptors. Courtesy- Arne Egesten.

Chemokines are defined as small proteins, approximately 8-13 kDa that exert their effect by binding to specific G-protein-coupled receptors (GPCR), which are present on mature cells, notably leukocytes (18). In humans, chemokine families consist of over 40 members divided into two major and two minor families. The classification is based on the location of the two N-terminal cysteine residues (18). In the CC family, these cysteine residues are adjacent to each other, whereas in the CXC family, they are separated by a single amino acid (Figure 6). Two other minor classes, CX3C and C chemokine families exist which have three members between them (Figure 6). Similar to chemokines, the corresponding receptors are defined based on the class of chemokine ligand they bind. For example, the prefixes CCR and CXCR are used to define receptors for CC and CXC chemokines, respectively. Most notably, one receptor can bind many different ligands and one ligand may bind several receptors (Figure 5). Eosinophils, for example, express several surface receptors, including CCR1, CCR3, CCR4, CCR9, CXCR3 and CXCR4. The principal receptor expressed by eosinophils, CCR3, binds the eotaxin family of chemokines comprising CCL11, CCL24, and CCL26, as well as members of the monocyte chemoattractant protein (MCP) family, including MCP-2, MCP-3, and MCP-4 (19-22).

Chemokine have recently been described, in addition to the above-mentioned functions, to possess antimicrobial activity. Antimicrobial activity for chemokines was first described in the year 2000 with the identification of truncated forms of CXCL7 as platelet microbicidal protein (23). Since chemokines can be induced during inflammatory conditions, they may act as the first line of defense against pathogens. Studies have shown that antimicrobial chemokines are effective at killing pathogens in micromolar concentrations. In general, naturally occurring chemokine concentrations are in the picomolar to nanomolar range (24). During infection and inflammation, chemokine concentration in biological fluids may be greatly increased. We have tested the activity of these Th-2 chemokines at micromolar range, because, even though our chemokines of interest occur in nanomolar range under normal physiological conditions, they can be greatly enhanced to micromolar concentrations during allergic inflammation. In addition to studying their antimicrobial activity, it is equally essential to map the domains that harbor such activity. Studying the domains that are important for antimicrobial activities might lead to development of more potent antimicrobial peptides (AMPs). So far, it is becoming increasingly clear that the cationic charge and amphiphatic helix are important for antimicrobial property. Although, there has not been an approved AMP as a drug, but interest for the development of this type of therapeutic is huge (25). They are fast acting and the development of resistance is relatively low.

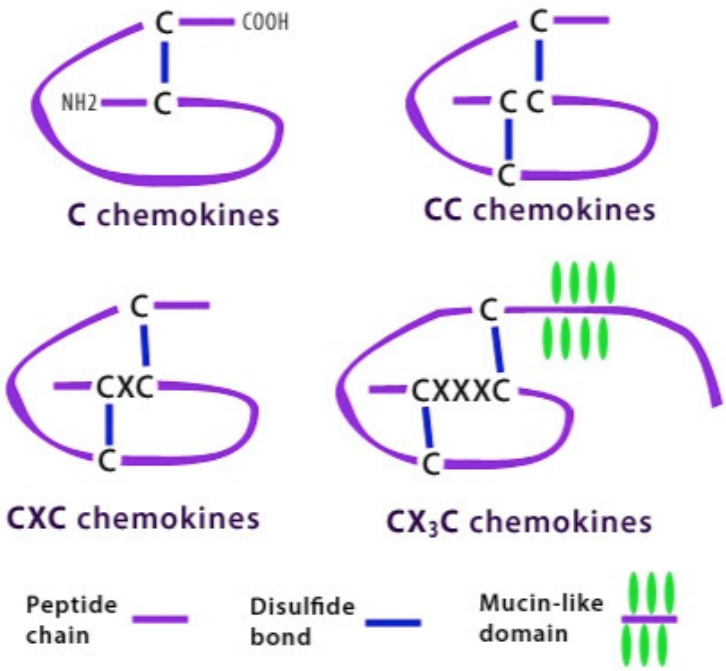

Figure 6
Illustration of the different classes of chemokine: They are classified into groups based on the spacing of their first two cysteine residues (pictured adapted from http://www.biolegend.com/chemokine_receptors).

2.1.1.6 Antimicrobial peptides

Antimicrobial peptides are effector molecules of the immune system containing approximately 15-45 amino acid residues and possess a broad-spectrum antibiotic activity. They can be grouped into five classes with different 3-D structures (26). However, interest in AMPs has been largely focused on three classes; linear peptides free of cysteines, peptides with 3 disulfide bonds, and peptides with unusual bias to certain amino acids. The first type of peptide without cysteines, known as cecropin, was discovered in insects, whilst the defensin type with disulfide bridges was found in rabbit granulocytes (27). For almost all the linear peptides investigated, the mechanism of action seems to involve disruption of the bacterial membrane. The proposed mechanism is that, when bacterium is exposed to a peptide, more and more peptide molecules dock onto the bacterial surface. The end result is collapse of the membrane when it is completely covered in peptide molecules, estimated at approximately 1-10 billion molecules. This is, however, not the only mechanism of bacterial killing. PR-39 has been reported to stop bacterial DNA synthesis, thereby killing the pathogen. Generally, AMPs,

specifically defensins, are cationic and flat triple stranded molecules held together by 3 intra-molecular disulfide bonds. These physiochemical properties are consistent with the prototypic chemokines (Figure 6). Chemokines consist of 30-130 amino acid residues and their molecular weight range from 8-13 kDa. Most of them are cationic at physiologic pH due to a relatively high content of arginine and lysine residues. Structurally, the majority contains anti-parallel β-sheets in the NH_2-terminal, while the amphiphatic COOH-terminal consist of a α-helix. The amphiphatic helix makes it possible for the peptide to associate with, and disrupt bacterial membranes. For this reason we have decided to investigate if Th-2 chemokines possess antimicrobial activity.

2.1.1.7 Osteopontin

Osteopontin (OPN) is a phosphorylated glycoprotein secreted by activated macrophages, leukocytes, and activated T lymphocytes, and present in extracellular fluids, at sites of inflammation, and in the extracellular matrix (ECM) of mineralized tissues (28, 29). It was first described as a secreted, 60 kDa transformation-specific phosphoprotein (Senger *et al*., 1979). More than a decade later, OPN was described independently together with bone sialoprotein (BSP), as a sialoprotein in the bone ECM (30, 31). Interest regarding OPN has grown since the late 1990s and OPN has moved from being a bone matrix protein to a complex cytokine that plays a role in regulating immunity. In the immune system, it plays a role in chemotaxis, leading to migration of macrophages and dendritic cells (DCs) to sites of inflammation. OPN is recognized as a Th-1 cytokine, but now there is increasing evidence to support its active role in Th-2 linked inflammation. In a study by Delimpoura (2010), OPN levels were measured and compared between normal subjects, patients with mild, moderate and severe refactory asthma (SRA). OPN levels were significantly elevated in patients with SRA (32). Therefore; there is an urgent need to investigate the active role of OPN in asthma.

As described above, OPN is highly anionic due to the level of phosphorylation and glycosylation. In paper 2 and 4, we sought to investigate if whether OPN could bind with the cationic AMPs through electrostatic interactions. More importantly, study the functional consequences of this interaction on the innate defense function of AMPs as well as the chemotactic properties of Th-2 chemokines

2.2 Adaptive immune response

The adaptive immune response is more specific and efficient in comparison to the innate immune response. This specificity can be attributable to the unique ability of the adaptive immune system to develop as many as 100 trillion different

receptors to recognize nearly every conceivable pathogen. The primary cells that control the adaptive immunity are the B and T lymphocytes. The essence of this immune arm is molecular distinction between self-constituents and potential pathogens. This discrimination is predominantly a responsibility of T lymphocytes. This is achieved through recognition of antigens by receptors on the surface of B and T lymphocytes. T cells are particularly important, as they do not only control a multitude of immune responses directly, but also control B cell immune responses in many cases. Thus, many of the decisions about how to attract a pathogen are made at T cell level.

2.2.1 B cells

B cells differentiate in the bone marrow and during the process of maturation; up to 100 trillion different clones of B cells are generated, which is similar to the diversity of antigen receptors seen in T cells. They can recognize native, unprocessed antigen and do not require the participation of major histocompatibility complex (MHC) molecules and antigen presenting cells (APCs). After activation of B cells by binding to their antigen, they differentiate into plasma cells. The latter often leave the secondary lymphoid organs, where the response is generated, and migrate back into the bone marrow, where the whole differentiation process started. After secreting antibodies for some time, they die, as most of their energy is devoted to making antibodies.

2.2.1.1 Antibodies

Antibodies are glycoproteins consisting of two types of polypeptide chains (heavy and light) with attached carbohydrate. In general, antibodies have two basic functions. They can act as the B cell antigen receptor or they can be secreted, circulate, and bind to a pathogen, often labeling it for identification by other forms of the immune response. All antibody molecules have two identical heavy chains and two identical light chains. The heavy chains coming together usually linked by disulfide bonds form the Fc region of the antibody. The Fc portion is essential in that many effector cells of the immune system have Fc receptors. Cells having these receptors can then bind antibody-coated pathogens, greatly increasing the specificity of the effector cells. There are five classes of antibodies viz IgM, IgA, IgG, IgD, and IgE. The latter is of interest for this thesis as it is one of the key players involved during allergic inflammation.

2.2.1.2 Immunoglobulin (Ig)-E

IgE is the immunoglobulin isotype that has the lowest abundance *in vivo* and its levels are tightly regulated. It is usually associated with type 1-hypersensitivity reactions, which includes both systemic and localized anaphylaxis. Inappropriate IgE mediated immune responses to environmental antigens can contribute to the pathogenesis of allergic disease such as asthma. IgE binds to IgE receptors on immune cells in the tissues and in the circulation: the high-affinity receptor for IgE (FcεRI) and the low-affinity Fc receptor for IgE (FcεRII) (33). Activation of these receptors can lead to mast cell degranulation, eicosaniod and cytokine production. Collectively, these events contribute to the inflammatory profile seen during allergic inflammation (detailed description in Chapter 2).

Chapter 3: Asthma and chronic airway inflammation

The word 'asthma' originates from the Greek meaning 'short of breath'; thus any patient who presented with shortness of breath was considered asthmatic. In the 19th century, Henry Hyde Salter (a London physician who suffered from asthma) refined the term and described asthma as '*Paroxysmal dyspnea of a peculiar character with intervals of healthy respiration between attacks*'. As a therapeutic strategy, Henry described black coffee, a drink with a high content of theobromine, a derivative of theophylline and theophylline itself, as a reliever of bronchospasm (34). He also was the first person to describe the importance of airway smooth muscle in asthma. The definition was later refined by the father of modern medicine in western world, William Osler, into what is described as today in modern medicine (35).

In the first half of the 20th century, asthma was regarded as a disease of bronchospasm and the treatment strategies relied mainly on bronchodilators. In the mid 1960s, an epidemic of asthma related deaths was reported in the UK, US, and Australia, and this was associated with over-reliance on bronchodilators (35). As a result, asthma research drew into sharp focus the shortfalls of asthma treatment and emphasized on the knowledge gap on the mechanism underlying bronchospasm. As research on asthma continued to receive global attention, more breakthroughs were made. In the 1970s, Turner-Warwick *et al* established, in clinical trials, that inhaled corticosteroids, notably beclamethasone dipropionate (BDP), was a highly effective controller drug for asthma when taken daily (36). This discovery shed light on the mechanistic reason for their efficacy in controlling day-to-day asthma. A decade later, in the 1980s, a clearer understanding of how allergen exposure triggered chemical mediator release from airway mast cells and this resulted in a cascade of events that led to the recruitment of other inflammatory cells (eosinophils, basophils, and mononuclear cells). A major breakthrough came with the identification of a special subset of T cells capable of secreting cytokines that selectively interacted with mast cells, basophils, and eosinophils. These Th-2 cells with cytokine repertoire (IL-4, IL-5, IL-8, IL-13 and GM-CSF) were responsible for the recruitment, priming, and survival of the primary effector cells of the allergic cascade (37). Most recently,

focus has been on the role of DCs, as APCs, on how they recognize allergens and communicate the specific sensitizing signal to naïve T cells involving MHC-II restricted peptide presentation to the T cell receptor (CD3) and engagement of co-stimulatory molecules (35).

3.1 Allergic inflammation

Asthma is a chronic inflammatory disorder of the airways, characterized by infiltration of mast cells, eosinophils and Th-2 $CD4^+$ T lymphocytes in the airway walls (37). The response is triggered when an atopic host inhales an allergen. The inhaled allergen activates sensitized mast cells by cross-linking surface-bound IgE molecules to release several bronchoconstrictor mediators, including cysteinyl leukotrienes and prostaglandin D2. These mediators stimulate airway smooth muscle cells, leading to bronchoconstriction. Epithelial cells release stem-cell factor (SCF), which is important for maintaining mucosal mast cells at the airway surface. Allergens are also processed by myeloid DCs, which are conditioned by thymic stromal lymphopoietin (TSLP) secreted by epithelial cells and mast cells to release the chemokine CC-chemokine ligand 17 (CCL17) and CCL22, which act on CC-chemokine receptor 4 (CCR4) to attract Th-2 cells. The latter have a central role in orchestrating the inflammatory response through the release of IL-4 and IL-13 (which stimulate B cells to synthesize IgE), IL-5 (necessary for eosinophilic inflammation) and IL-9 (stimulates mast cell proliferation). Epithelial cells release CCL11, which recruits eosinophils via CCR3 receptor.

Persistence of this chronic inflammation may alter the lung parenchyma due to airway remodeling. The Th-2 response, an in particular increased expression of IL-13 within the lungs of asthmatics, triggers an aberrant injury-repair mechanism leading to airway remodeling. IL-13 causes an increase in goblet cell differentiation, activation of fibroblasts and increase in bronchial responsiveness (38). Moreover, IL-13 induces bronchial epithelial cells to secrete periostin, which in turn enhances pro-fibrotic transforming growth factor (TGF)-β (38). Eosinophils produce a wide range of proteins involved in fibrogenesis and angiogenesis, as well as cytokines, which activate various mesenchymal cells and induce synthesis of extracellular matrix (ECM) proteins (pro-collagen III, proteogylcans, tenascins and lumicans). The remodeled phenotype in asthma, which is the consequence of excessive repair process following repeated airway injury, includes increased deposition of several ECM proteins in the reticular basement membrane and bronchial mucosa, as well as increase in airway smooth muscle (ASM), goblet cell hyperplasia and angiogenesis. These structural changes may impair the protective functions of the airway by disrupting mucociliary

clearance and thereby increasing the risk of infection. Furthermore, activated fibroblasts and ASM cells may release pro-inflammatory mediators, leading to amplification of subsequent tissue damage.

3.2 Asthma phenotypes

The above definition of asthma focuses on the traditional allergic, eosinophilic, and Th-2 mediated (corticosteroid-responsive) disease. About some decades ago, asthma was classified as either extrinsic or intrinsic. This classification quickly fell out of favor when studies revealed that the levels of Th-2 cytokines between the diseases were similar, and treatment with inhaled corticosteroid was found to be effective in the majority of mild to moderate cases (39, 40). So over the years, asthma has been considered a single disease. Approaches on treating the disease halted when Th-2 focused clinical trials yielded negative results. Simultaneously, a group of people with severe asthma was observed to have corticosteroid resistant asthma in the absence of eosinophils (41, 42). Moreover, studies have shown the heterogeneity of the disease that consist of multiple phenotypes or multiple diseases rather than a single disease. By understanding the molecular basis of these phenotypes, research on the field could lead to more targeted and personalized approaches to asthma therapy. To date, the proposed asthma phenotypes include early-onset allergic Th-2, late-onset eosinophilic, exercise induced, obesity related, and neutrophilic asthma. These are based on clinical characteristics, triggers or general inflammatory process.

3.2.1 Early-onset allergic asthma

This phenotype originates early in childhood, has an atopic and allergic component, but a specific age cut off has not been determined. Most people with asthma are likely to have this phenotype. However, the lack of responsiveness to corticosteroids and lower concentrations of IgE in some children suggest that not all early-onset asthma is Th2 associated (42, 43). Studies have shown that this phenotype is typically associated with other atopic disease, including rhinitis and atopic dermatitis (44-46).

3.2.2 Exercise induced asthma (EIA)

This phenotype refers to asthma whose symptoms are experienced primarily after exercise. It has been described for decades but very little is understood about its immunological and inflammatory underpinnings. EIA has been reported to be common in athletes with atopy and it's pathologically associated with high percentage of eosinophils in both sputum and tissue (47-49). Therapeutically, these patients respond to drugs that modify the cysteinyl leukotrienes. Importantly, monoclonal antibody blockade of the mast cell-promoting cytokine, IL-9, has also been shown to inhibit EIA (50), suggesting that the disease is partly associated with Th-2.

3.2.3 Non-Th2 asthma

This subgroup represents the large proportion of asthma phenotypes. Approximately 50 % of corticosteroid-naïve individuals are affected by non-Th2 asthma. Clinically, they present with less airway hyper-reactivity than people with Th-2 asthma (51, 52). Very little is understood about this subgroup, the phenotype underlying it and the molecular mechanisms controlling it. Studies have shown that people with no history of childhood allergic features and mild to moderate adult-onset asthma are likely to fall into this category (53).

3.2.4 Obesity related asthma

Obesity has been suggested to have a potential role in the development, control and severity of asthma. Some studies have proposed an association between obesity and a generalized pro-inflammatory state involving high expression of TNF-α, IL-6, and leptins (54). However, obesity is generally associated with low amounts of FeNO, less eosinophils and a diminished response to corticosteroid therapy (54-57). This phenotype seems to be associated with non-Th2 asthma. The poor clinical response to corticosteroid that have been reported may be attributed to the lack of association of this phenotype with Th-2 inflammation, which is traditional responsive to corticosteroids (51, 55, 56). Despite this, it is still unclear if whether obesity is the principal driving force in asthma development or a mere confounder. There are no known or specific biomarkers for this phenotype although adipokines have been proposed. As part of the treatment plan, weight loss programs are encouraged (57). Notably, the treatment is effective only for non-Th2 asthma. Studies have reported that weight loss in obese individuals with non-Th2 asthma improved quality of life and bronchial hyper-responsiveness (57).

3.2.5 Neutrophilic asthma

Neutrophilia has been inconsistently linked with asthma and severe asthma for several years (58, 59). This subgroup is generally seen in patients receiving corticosteroid therapy. It is thought that corticosteroids inhibit neutrophil apoptosis and, in some settings, directly activate neutrophils, suggesting that the treatment itself is likely to have some role in the development of neutrophilia (60, 61). Lung neutrophilia has been associated with lower lung function, more trapping of air, thicker airway walls and increased expression of matrix metalloproteinases, but it has not been linked with airway hyper-responsiveness. Neutrophilic asthma can also co-exist with eosinophilia, and this identifies people with the most severe asthma and emphasizes the immunobiology of severe asthma in which multiple innate and adaptive immune pathways and cells may have roles (62, 63). Also, Th-17 inflammation has been strongly linked with neutrophilia (64, 65). This subgroup represents the corticosteroid resistant phenotype, perhaps because of the absence of a Th-2 profiled inflammation. To treat neutrophilic asthma, macrolide antibiotics have been suggested. The treated patients show a reduction in the expression profile of neutrophilic marker and improvement in quality of life, but no improvements in asthma control and FEV_1. However, the lack of interventions that specifically target neutrophils undermine the prospects of determining whether neutrophilia is a biomarker or a target for therapy.

3.3 Chronic obstructive pulmonary disease (COPD)

COPD is a lung disease characterized by chronic obstruction of lung airflow that interferes with normal breathing and is not fully reversible. The primary risk factor for COPD is tobacco smoking, but biomass exposure, secondhand smoking, air pollution and work exposure to fumes and dust have also been proposed. The disease is characterized by destruction of lung parenchyma with loss of elastic recoil and infiltration of the wall of the small airways by inflammatory cells. It is the 4^{th} leading cause of death worldwide and its expected to be the 3^{rd} leading cause in the next 20 years (66).

Patients with COPD are prone to acute respiratory exacerbations, which can develop suddenly or subacutely over the course of several days. Exacerbations of COPD are heterogeneous with respect to inflammation and etiology. They are typically associated increased neutrophilic and to a lesser extent eosinophilic airway inflammation (67, 68). Eosinophil inflammation is associated with a favorable response to corticosteroid therapy, whereas neutrophilic inflammation has better clinical outcomes with antibiotic therapy (69). Respiratory viral and

bacterial infections have been implicated in causing most exacerbations (70-72), but how these infections alter the inflammatory profile of the lower airways remains unclear. Bacteria are considered to play a role in up to 50 % of exacerbation (72). Viruses, on the other hand, have been implicated as a major cause of COPD exacerbations and are detected in approximately half of severe exacerbations (70). This heterogeneity means that at present, clinicians have limited tools to phenotype COPD exacerbations.

3.4 Asthma-COPD overlap syndrome (ACOS)

The term has been applied to the condition in which a person has clinical features of both asthma and COPD (73). Asthma inflammation has previously been described as different from that in COPD. Asthma is characterized predominantly by eosinophilic inflammation driven by Th2 lymphocytes, whereas COPD is characterized by neutrophilic inflammation involving CD8 lymphocytes. Acute exacerbations in asthma and COPD are usually associated with the development of acute (in addition to the chronic) inflammation of the airways, with enhanced eosinophila in asthma and further neutrophilia in COPD. There is now increasing evidence that eosinophils might play an important role in 10-40 % of patients with COPD. A Th-2 inflammatory signature has also been reported in COPD. In a subgroup of patients with COPD, a Th-2 inflammatory-related gene-expression signature was upregulated in biopsy specimens of airway wall (74). Patients with asthma who have severe or late-onset disease or chronic infection or who smoke may also exhibit neutrophilic inflammation (75), and CD8 cells in the airways, both of which were previously thought to be hallmarks of COPD.

3.5 Therapeutic advances in asthma and COPD

Asthma and COPD are characterized by airflow obstruction, which is typically variable and reversible in asthma, but fixed and progressive in COPD. As discussed previously, eosinophilic inflammation can occur in both conditions. Studies have shown that treatment aimed at maintaining eosinophil counts < 2-3 % decrease the number of exacerbations and hospital admissions in both asthma and COPD (7). The presence of sputum eosinophilia (> 3 %) in asthma and COPD, and the Th-2 signature (described above) has shown to be a good predictor of the response to corticosteroid treatment in stable disease. Inhaled and systemic corticosteroids are the mainstay anti-inflammatory therapy for asthma and COPD

in stable disease and at exacerbations. They decrease eosinophilic bronchial mucosal inflammation and induced sputum differential count (76, 77).

Leukotrienes have been described as potent bronchoconstrictors, causing airway smooth muscle contraction and mucus hypersecretion. They are derived from arachidonic acid metabolism and released predominantly by mast cells and eosinophils. Therefore, leukotriene receptor antagonist (e.g. montelukast) is used to decrease eosinophilic airway inflammation in asthma, albeit to a lesser extent than inhaled corticosteroid (78). Unfortunately, there are no documented therapeutic benefits in COPD patients.

Theophylline is prescribed as add on treatment in poorly controlled asthma and COPD (79). This member of the xanthine family acts as a bronchodilator and anti-inflammatory agent. Similarly so corticosteroids, it plays a role in regulating expression of inflammatory genes. Theophylline has been reported to cause a significant reduction in the number of eosinophils in bronchial biopsies, bronchoalveolar lavage (BAL) and induced sputum in asthma (80). In COPD, low dose theophylline in combination with corticosteroid significantly decreased sputum eosinophil count.

Most recently, research has moved towards targeted therapies using humanized monoclonal antibodies as well as molecular antagonists. Omalizumab is, by far, the only biological therapy approved for the treatment of asthma. It specifically targets IgE antibodies that are predominantly expressed on the surface of mast cells. Omalizumab has been reported to show a reduction in sputum, epithelial and submucosal eosinophil count (81).

The use of humanized monoclonal antibodies has drawn tremendous interest. Research focus is now drawn towards the development of biological and small molecular therapies. Currently, monoclonal antibodies against IL-5 (mepolizumab), IL-5Rα (benralizumab), IL-13 (tralokinumab/lebrikizumab), IL-4Rα (dupilumab), and CCR3 antagonist are in clinical development. Even though most of these have not been approved yet but they are promising candidates for treatment of chronic inflammatory conditions.

3.6 Infections in asthma

Asthma affects approximately 300 million people worldwide, including both children and adults, and the incidence is more in developed countries. Epidemiologically, studies show strong association between asthma and infection with lower respiratory pathogens, including common respiratory viruses (e.g. respiratory syncytial virus (RSV) and rhinovirus (RV)), as well as bacteria (e.g.

Streptococcus pneumoniae) and fungi (e.g. *Aspergillus fumigatus*) (82). These microorganisms usually represent the pathogenic state in infected individuals. However, some microbes and their products have potential therapeutic effects in asthma. Some scientists argue that future strategies for management of asthma must take into account the role of microorganisms and their products. In the hygiene hypothesis, it is proposed that repeated exposure to diverse common infections (in particular with bacteria, food-borne and orofaceal parasite, and hookworms) and exposure to environmental microbiota during childhood are strongly associated with healthy maturation of the immune system and with protection from the development of asthma and allergies later in life (83, 84). The idea is that the immune system is geared to respond to antigens, and if pathogens are not present, it will respond instead to inappropriate antigens such as allergens and self-antigens. This is one explanation for the rising incidence of allergies in developed countries, where the response to non-pathogen like pollen causes allergic response while not serving any protective function.

3.6.1 Viruses

Viruses have long been linked to asthma and asthma exacerbations. There is evidence that point to recurrent infections of infants (< 2 years of age) with human RSV and/or RV in early life and development of recurrent wheeze followed by asthma diagnosis in later childhood. The link of RSV with asthma is most clearly demonstrated in a long-term (18 years) study of a cohort of Swedish children (85). The most recent update reports that, compared with control, children who have RSV-mediated bronchiolitis in infancy are more likely to be asthmatic at 18 years old. Evidence obtained from other studies; so far, indicate that by preventing such infections in early childhood, there could be long-term beneficial effects on asthma development.

3.6.2 Fungi

Fungal species have also been implicated in asthma development. In fact, severe asthma with fungal sensitization has been considered as an independent subgroup of severe asthma, and these patients may benefit from anti-fungal therapy. Filamentous fungal species of the *Aspergillus, Alternaria, Cladosporium, Penicillium,* and *Didymella* genera are known to produce spores that may act as allergens and initiate bronchial asthma in atopic individuals (82). The release and abundance of these spores is affected by environmental factors, including season, humidity, rainfall, and temperature (86), and many have been shown to increase in abundance in the summer along with high pollen levels. Pulimood *et al* (2007)

reported that fungal aeroallergens have been associated with asthma epidemics that occur during summer (87).

3.6.3 Bacteria

Amongst the airway pathogens that have been studied in asthma, several bacterial pathogens have been shown to be of clinical significance. For the purpose of this thesis, focus has been drawn into *S. pneumoniae, P. aeruginosa, non-typeable H. influenzae,* and *S. aureus*. Briefly, invasive pneumococcal disease (IPD), which is casued by *S. pneumoniae*, has been linked to asthma. There is a 2-10 fold increased risk of developing IPD in adults with stable asthma, and asthma has been shown to be a risk factor for IPD in both adults and children (88-90). The other bacterial strains, on the other hand, have been reported in neutrophilic asthma, with one study showing an association between airway neutrophils, increased bacterial load and neutrophil chemokine, IL-8.

3.6.3.1 Streptococcus pneumoniae

Gram-positive β-hemolytic cocci that normally colonizes the upper airways (Figure 7). The pathogen was first isolated in 1881. It is one of the leading causes of childhood pneumonia and meningitis. In spite of the availability of antibiotics, in developing countries, it is responsible for the death of a large number of children under the age of 5 years from pneumococcal pneumonia. Almost all isolates that cause infection are encapsulated, and to date 91 separate capsular serotypes have been identified (91). Although advances have been made in developing improved vaccines against *S. pneumoniae*, a better understanding of its virulence factors determining its pathogenicity is needed to cope with the devastating effects of *S. pneumoniae*. The pathogenicity of *S. pneumoniae* has been attributed to various structures, most of which are situated on its surface. The virulence factors include, amongst others, capsule, cell wall, pneumolysin, autolysin, PspA, and pneumococcal proteins (complement factor H-binding component, neuraminidase, peptide permease, hydrogen peroxide, and IgA1). One group of factors, such as capsule, provides resistance to phagocytosis and thus promotes the escape of pneumococci from the host immune response. Other factors, including cell wall components and the intracellular toxin pneumolysin, are involved mainly in the inflammation caused by infection. The inflammation ensues only once the bacteria have been lysed by autolysin.

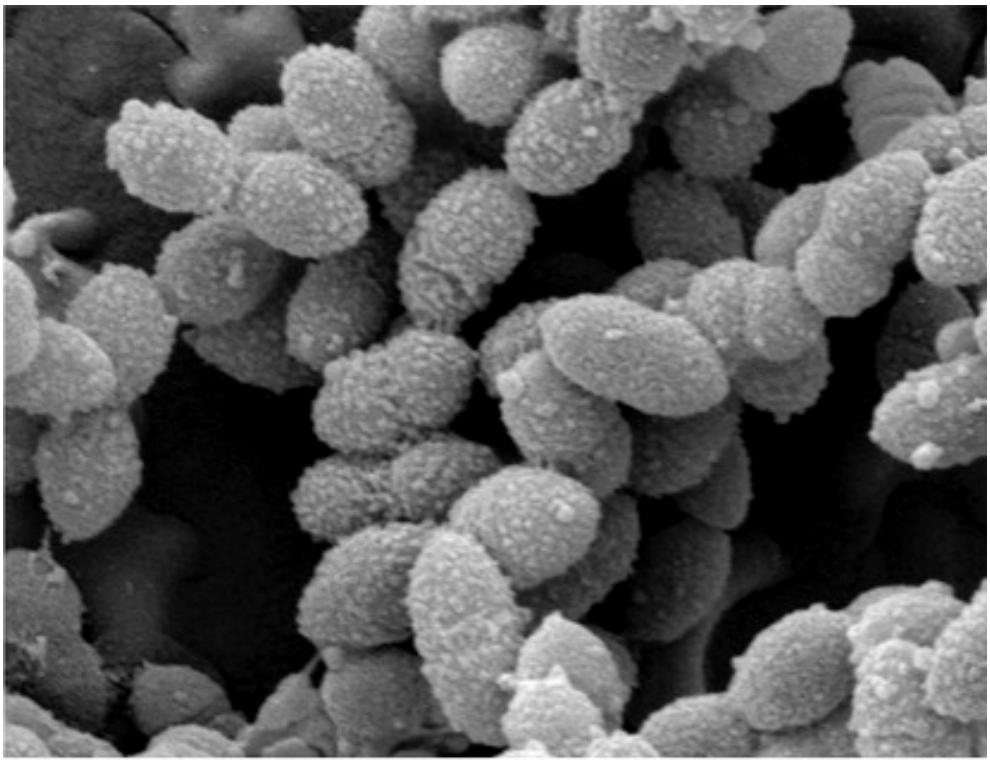

Figure 7
Scanning electron microscopy (SEM) picture of *Streptococcus pneumoniae* (TIGR4 strain). The bacteria appear in their characteristic cocci shape. Courtesy-Matthias Mörgelin.

3.6.3.2 Pseudomonas aeruginosa

Gram-negative, rod shaped, and monoflagellated bacterium that has versatile nutritional requirements (Figure 8). It's a ubiquitous microorganism found in environments such as soil, water, human, animals, plants, and hospitals. Within the genus *Pseudomonas*, there is more than 120 species that are known to exist. Amongst these, *P. aeruginosa* is most frequently associated with causing human infections (92, 93). The bacterium is regarded as an opportunistic pathogen, primarily causing infections in immunocompromised patients (93-96). In respiratory tract infections, *P. aeruginosa* is well known for establishing permanent residency in the airways of Cystic fibrosis as well as COPD patients; both conditions are characterized by impaired mucocilliary clearance (97, 98). The bacterium produces a number of toxins via the type III secretion system (T3SS), as well as secreting enzymes and proteins including elastases, phospholipase C and siderophores. The T3SS injects toxic effector proteins into the cytosol of eukaryotic cells to inhibit cellular function thereby allowing bacterial survival.

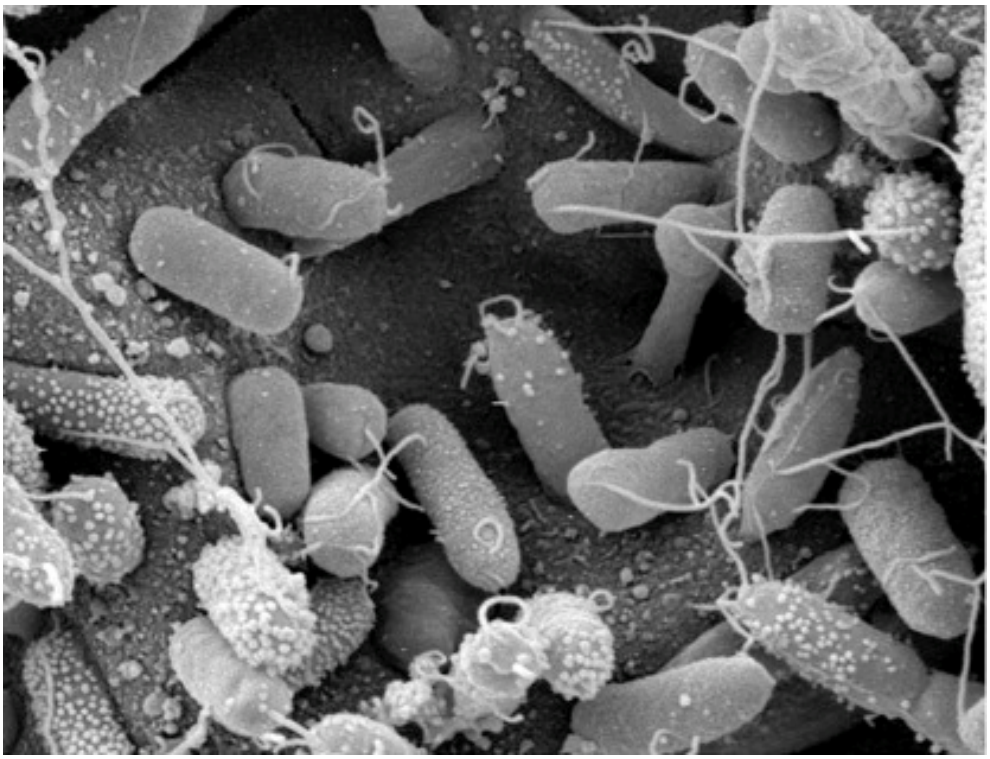

Figure 8
SEM of *Pseudomonas aeruginosa* (PA01 strain). The monoflaggelated rod shaped bacteria. Courtesy- Matthias Mörgelin.

3.6.3.3 Staphylococcus aureus

Gram-positive coccal bacterium that is frequently found in the nose, respiratory tract, and on the skin (Figure 9). The bacterium is one of the major pathogens in humans; it causes suppurative diseases, pneumonia, and toxic shock syndrome. Moreover, the methicillin-resistant strain has emerged as the major cause of nosocomial infections, including pneumonia and sepsis. *S. aureus* produces many virulence factors, such as hemolysin, leukocidins, proteases, enterotoxins, exfoliative toxins, and immunomodulatory factors (99, 100). The expression of these factors is tightly regulated by the quorum-sensing system. *S. aureus* has been reported to exacerbate atopic eczema and contributed to the development of chronic sinusitis via a Th-2 biased immune response to bacteria superantigen proteins such as staphylococcal enterotoxin (SE), suggesting that *S. aureus* could also affect Th-2 driven airway disease.

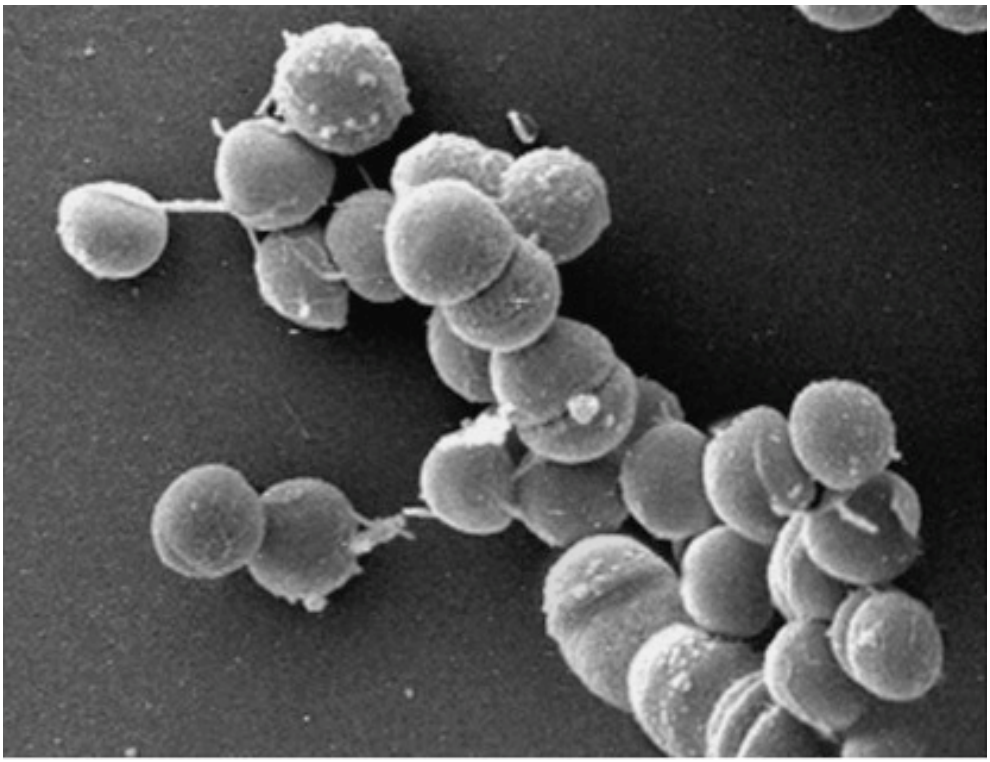

Figure 9
SEM of *Staphylococcus aureus* (5120 strain). The bacteria appear to grow in ther characteristic round and grape-like clusters. Courtesy- Matthias Mörgelin.

3.6.3.4 Non-typeable Haemophilus influenzae

Haemophilus spp are small fastidious Gram-negative coccobacilli that can be categorized by their requirement for haemin (X-factor) and NAD (V-factor), and other phenotypic characteristics, including capsular polysaccahrides (Figure 10). The non-capsulated NT*Hi* is a major cause of mucosal infections such as otitis media, sinusitis, conjunctivitis, and exacerbations in COPD. Recent studies have shown the increasing importance of NT*Hi* as a pathogen of the lower and upper respiratory tract, and in invasive diseases. NT*Hi* has been isolated from the airways of neutrophilic asthmatics (101, 102). The nature of the association between infection and the development of neutrophilic asthma is not well understood; whether the infection promotes the pathogenesis of neutrophilic asthma, or if neutrophilic asthmatics are predisposed to infection is not clear. The NT*Hi* colonization often causes recurrent respiratory disease in individuals with compromised airways (103-105).

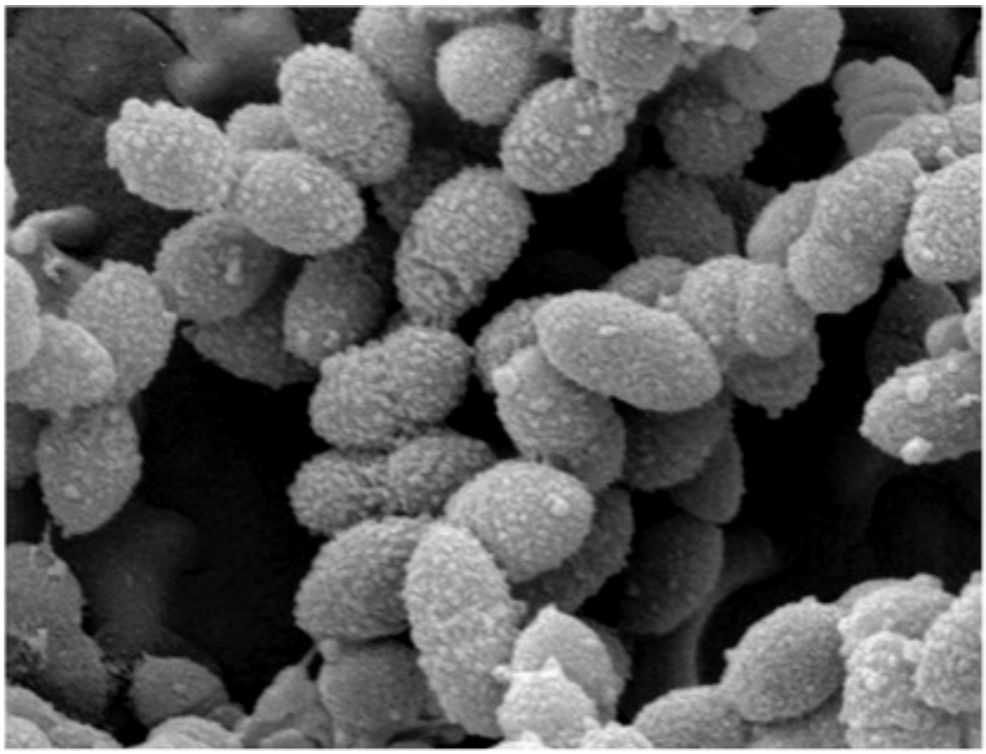

Figure 10
SEM of Non-typeable *Haemophilus influenza*. The bacteria appear in their characteristic rod or coccobacillus form. Courtesy- Matthias Mörgelin.

Chapter 4: Purpose of the thesis

Allergic asthma is a chronic inflammatory disorder of the airways involving several types of immune cells, the hallmark being the presence of eosinophils. The disease is characterized by the upregulation of multiple inflammatory proteins, including cytokine and chemokines, adhesion molecules, inflammatory enzymes, and receptors (82). Recent studies have shown an association between asthma and respiratory pathogens. This increased vulnerability can be attributed to a number of factors, including chronic inflammation, Th-2 profiled inflammation, inflammatory cells, and the treatment used for asthma.

The persistent inflammatory response may cause epithelial injury thereby compromising host defense factor and impairment of barrier functions. The excessive repair process following repeated airway injury may lead to tissue remodeling. Constant inflammation and remodeling are not conducive for normal airway function.

Asthma is characterized by an eosinophil, Th-2 mediated, allergic response. During Th-2 profiled inflammation, IFN-γ and IL-12 levels are decreased (106), and this may reduce their capacity to inhibit IgE synthesis and therefore allergic inflammation. The hallmark of allergic inflammation is the presence of eosinophils, which are a rich source of several factors that are implicated in tissue remodeling.

The mainstay of asthma therapy is corticosteroids. Corticosteroids suppress inflammation by inducing recruitment of the nuclear enzyme histone deacetylase (HDAC-2) to multiple activated inflammatory genes, which lead to deacetylation of the hyperactivated genes, thereby suppressing general inflammation. Therefore, the treatment itself may impair native host response.

Research on asthma therapy has now shifted to alternative therapeutic strategies, including the use of humanized monoclonal antibodies. In this thesis, we are exploring the use of antimicrobial peptides as the alternative innate antibiotics. Th-2 chemokines exhibit similar biophysical properties as the classical antimicrobial peptides, including cationicity, the 3-stranded β-sheet and an amphiphatic α-helix and two adjacent cysteine residues close to the NH_2-terminus. Therapeutically, these chemokines could potentially serve a host defense function and are constitutively expressed and upregulated during a Th-2 skewed inflammation.

Chapter 5: Present investigations

Paper 1

Eotaxin-3 (CCL26) exerts innate host defense activities that are modulated by mast cell proteases.

Background

Allergic asthma is chronic inflammation of the airways involving several types of immune cells. The disease is characterized by an up-regulation of a number of factors, including enzymes, receptors, chemokines and other chemotactic factors. In comparison to the classical antimicrobial peptides (e.g. defensins), these chemokines exhibit similar biophysical properties, including a typical chemokine fold, which includes a 3-stranded β-sheet and an amphiphatic alpha helix and two adjacent cysteine residues close to the NH_2-terminus. Immunologically, these chemokines could potentially serve as host defense peptides and are constitutively expressed and upregulated during a Th-2 profiled inflammatory response.

Aims

- To investigate if human eotaxins can serve as host defense peptides
- To map the regions of eotaxins that harbor host defense functions.
- To investigate the biological effects of mast cell proteases on eotaxins

Results

Eotaxin-3/CCL26 showed potent antimicrobial activity against Gram positive and negative bacteria, which was also retained at physiological salt concentration. The region holding the bactericidal activity was mapped to the cationic COOH-terminus. Proteolytic cleavage by chymase and typtase released two distinct fragments from the COOH- and NH_2-terminal regions, respectively. The COOH-terminal exhibited antimicrobial activity while the NH_2-terminal fragment had

potent LPS neutralizing activity. Functionally, both fragments had no receptor activating properties and did not interfere with full-length CCL26.

Discussion

In this study, we demonstrate that eotaxins have potent antimicrobial activity against Gram positive and negative bacteria. In addition, the three chemokines have potent anti-endotoxin ability that has not been demonstrated before. In most cases, host defense peptides lose their activity in the presence of salt and low pH. Interestingly, CCL26 had antimicrobial activity that was retained at physiologic salt concentration. Mast cell chymase and typtase cleaved CCL26 into distinct fragments where the NH_2- and COOH-terminal domains had LPS-neutralizing and antimicrobial activity, respectively. The current findings suggest that the eotaxins, in addition to recruiting and activating eosinophils, may play several roles in innate host defense of the airways during allergic inflammation.

Conclusion

Human eotaxins can contribute to host defense against common airway pathogens but their activities are modulated by mast cell proteases.

Paper 2

Osteopontin binds and modulate functions of eosinophil-recruiting chemokines

Background

Allergic asthma is characterized by eosinophil inflammation and airway obstruction. There is also an increased risk of pulmonary infection caused by *S. pneumoniae*, in particular during severe asthma where high levels of the glycoprotein, osteopontin, are present in the airways. Eosinophils can be recruited by Th-2 chemokines via CCR3 receptor. In addition to inducing chemotaxis, several of these chemokines have defensin-like antibacterial properties.

Aims

- To investigate whether osteopontin interferes with bactericidal properties of Th-2 chemokines.
- To investigate whether osteopontin interferes with the chemotactic ability of Th-2 chemokines via CCR3 signaling.

Results

The anionic phosphoglycoprotein (OPN) that is highly expressed in the airways during allergic asthma binds all eosinophil-recruiting chemokines with high affinity except for CCL5. These chemokines all displayed bactericidal activity against pneumococci, but only CCL26 and CCL28 retained high bactericidal activity in the presence of sodium chloride at physiologic concentrations. Preincubation of the chemokines with OPN at equimolar concentrations strongly inhibited their antibacterial activity but did not affect their ability to activate CCR3, a receptor highly expressed on eosinophils.

Discussion

In this study, we show that OPN binds all eosinophil-recruiting chemokines, impairing their defensin-like antibacterial properties against *S. pneumoniae* without interfering with their receptor-activating properties. Most of the cationic charge of the chemokines is localized to the amphiphatic and α-helical COOH-terminal, which is most likely to associate with anionic regions of OPN. The antibacterial activity of CCL26 has been mapped to the COOH-terminal region, while the NH_2-terminal harbors the LPS-neutralizing properties. This distribution of the functions of the NH_2-and COOH-terminal domains may also be the case for the other chemokines investigated and can explain why binding to OPN mainly impair their antibacterial properties. This interaction may, at least in part, explain the increased vulnerability to acquire pneumococcal infection during asthma, a risk that increases in parallel with increased OPN expression in the airways.

Conclusion

The study suggests that OPN may impair host defense activities of the chemokines without affecting their eosinophil-recruiting properties. This could be one mechanism explaining the increased vulnerability to acquire pneumococcal infection in parallel with sustained allergic inflammation in asthma.

Paper 3

A chemokine-derived fragment as a novel therapeutic against LPS-induced inflammation.

Background

Lipopolysaccharides (LPS) are part of the outer envelope of Gram-negative bacteria and their recognition as pathogen associated molecular patterns by TLR4 results in the development of adequate local inflammatory responses but may also develop into systemic inflammation or septic shock. Up to date, there is no efficacious therapy against septic shock and high mortality rates persist. Therefore, there is an urgent need to devise alternative strategies. Our recent study demonstrated that the human eotaxin-3/CCL26-derived fragment, GSD35, has LPS-neutralizing properties.

Aims

- To investigate whether GSD35 has anti-inflammatory effects *in vivo*.

Results

In a mouse model, GSD35 showed strong anti-inflammatory activity against sterile inflammation caused by LPS. The treatment significantly increased mouse survival. This was accompanied by an increase in anti-inflammatory cytokine expression (e.g. IL-10) as well as a decrease of pro-inflammatory cytokine production (e.g. IL-6, TNF, and IL-1β).

Discussion

In this study, we show that a fragment derived from the NH_2-terminal domain of CCL26 can be used to treat LPS-induced inflammation *in vivo*. What properties make the NH_2-terminal, not the COOH-terminal domain of CCL26 LPS-neutralizing? LPS has an anionic lipid core and hydrophobic properties. Thus, cationic as well as hydrophobic residues of GSD35 may be essential for interaction. We intend to investigate these further by using truncated forms of GSD35 as well as performing amino acid substitutions to define critical residues.

Conclusion

Taken together, this study demonstrates a novel peptide-based strategy to treat systemic inflammation. This approach can be applied in a broad set of clinical conditions that are difficult to treat with the therapies available today.

Paper 4

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

Background

Bacterial infections of the respiratory tract contribute to exacerbations and disease progression in chronic obstructive pulmonary disease (COPD). There is also an increased risk of invasive pneumococcal disease in COPD. The underlying mechanisms are not fully understood but include impaired mucocilliary clearance and structural remodeling of the airways. In addition, antimicrobial proteins that are constitutively expressed or induced during inflammatory conditions are an important part of the airway host defense. COPD patients have been reported to have high levels of osteopontin, and the levels increased with disease severity.

Aims

- To investigate whether OPN co-localizes with classical antimicrobial peptides in the airways of COPD patients.
- To investigate the functional consequences of OPN binding to the AMPs

Results

The data demonstrate that OPN co-localizes with several AMPs expressed in the airways during COPD. *In vitro*, OPN bound lactoferin, secretory leukocyte protease inhibitor (SLPI), midkine, human beta defensin-3 (hβD-3), and TSLP but showed low or no affinity for lysozyme and LL-37. Binding of OPN impaired the antibacterial activity against the important bacterial pathogens, *S. pneumoniae* and *P. aeruginosa*. Some AMPs have additional functions. However, the

muraminidase-activity of lysozyme and the protease inhibitory function of SLPI were not affected by OPN.

Discussion

In this study, we show that OPN binds and impair the bactericidal activity of several AMPs that are expressed in the airways during COPD. The nature of this interaction seems to be electrostatic, as deduced from surface plasmon resonance. The elevated levels of OPN can impair the activity of AMPs, promoting vulnerability to acquire bacterial infections.

Conclusion

OPN can contribute to the impairment of innate host defense by interfering with the function of AMPs, thus increasing the vulnerability to acquire infections in COPD and other chronic inflammatory airway diseases.

Chapter 6: Future perspectives

In pathologic conditions, such as asthma, the defense mechanisms of the airways are compromised. As a consequence, the vulnerability to acquire bacterial infections increases. Despite advances in asthma research, many patients have poorly controlled symptoms and frequent exacerbations. This could be attributed, in part; to the held misconception that asthma is a single disease; allergic, eosinophilic, corticosteroid-responsive, and Th-2 mediated disease. Recognizing and accurately classifying the different phenotypes could be one step closer to effective management. This could lead to more specific and personalized approaches, managing the disease.

Recent studies have shown an association between asthma and development of invasive pneumococcal infections (107). The problem is further aggravated by misdiagnosis of asthma, incorrect management plan, which in turn lead to frequent exacerbations. Moreover, the current therapeutic approach, which requires daily treatment with immunosuppressive macrolide antibiotic and glucocorticoids have adverse effects on individual's microbiota. For example, increases in bacterial pneumonia have been noted in patients with COPD who are treated with glucocorticoid therapy (108). Some of the bacterial pathogens pose challenges of drug resistance (109). Therefore, there is a need for alternative treatment strategies.

Can AMPs be used as drugs? Yes, that seems likely. In paper 1, we reported for the first that a Th-2 chemokine possess anti-endotoxin activity, in addition to receptor activating ability (110). There are, of course, some challenges with the use of AMPs as templates for drug development. They are susceptible to proteolytic degradation by host and bacterial proteases, resulting in impaired activity. The changes in salt and pH at sites of infection may further impair the activity. Lastly, the bacteria may circumvent their action by altering their lipid composition, thereby reducing the negative charge on their surface. Despite all these, modification of AMPs may be needed in order to obtain adequate protection. Bodelsson *et al.* (2007) demonstrated that truncation of LL-37 lead to improved LPS neutralization and less cytotoxicity.

A few bacteria known to produce proteolytic enzymes, like *Pseudomonas*, *Serratia*, and *Staphylococcus*, are generally found to be less susceptible or even resistant to AMPs. Resistance to proteolytic enzymes could be overcome by

making peptides with only D-amino acids (D-enantiomers) (111). Another approach to overcome proteolytic resistance would be to synthesize peptides with B-amino acids.

Although there is no licensed AMP as an antibiotic or immune-modulatory compound yet, they provide a promising alternative for future research and they can be immediately applicable to other pathologic pathogens, including septic shock.

Chapter 7. Populärvetenskaplig sammanfattning

Astma är en sjukdom som innebär att luftvägarna blir kroniskt inflammerade. Ungefär 300 miljoner människor är drabbade av sjukdomen globalt. Inflammationen vid astma karakteriseras av närvaro av så kallade eosinofila granulocyter vilka producerar faktorer som kan förstärka inflammationens svårighetsgrad men också orsaka bindvävsombyggnad (ärrskrumpning) av lungvävnaden. Tillståndet med långvarig luftvägsinflammation och förändrad anatomi i luftvägarna ökar risken för bakteriella luftvägsinfektioner. På senare år har man kommit att uppmärksamma att risken för svåra infektioner orsakade av en bakterieart som heter pneumokocker är ökad om man lider av astma. Risken för dessa infektioner är mellan dubbel och kanske upp till tio gånger så hög om man har astma jämfört med lungfriska individer. Orsakerna är inte klarlagda och i det här avhandlingsarbetet undersöks hur specifika molekyler som bidrar till den obalanserade allergiska inflammationen försämrar luftvägarnas immunförsvar. I synnerhet möjliga orsakssamband mellan den långdragna inflammationen och risken för svåra pneumokocksjukdomar hos astmatiker, i synnerhet lunginflammation. Först studerades hur signalsubstanser som styr rekryteringen av eosinofila granulocyter, så kallade eotaxiner, påverkas av enzymer frisatta från mast-celler. Mast-celler har en central roll vid allergisk inflammation då allergi-antikroppar som känner igen allergiframkallande ämnen sitter på deras yta och kan orsaka allergiska symptom. Våra resultat visar att en av eotaxinerna (eotaxin-3/CCL26) fungerar som ett kroppseget antibiotikum i ena halvan av molekylen, den så kallade karboxy-terminala delen. I den andra halvan fann vi att den hade egenskapen att neutralisera inflammation orsakad av så kallat endotoxin som frisätts från ytan av vissa bakterier. Denna uppdelning i egenskaper i två olika delar av molekylen ka orsakas av inverkan av två enzymer frisatta från mast-celler, kymas respective tryptas. I en annan studie kunde vi visa att den endotoxin-neutraliserande aktiviteten kunde användas som ett läkemedel för att förhindra inflammation i en sjukdomsmodell. Vidare kunde vi visa att flera signalerande molekyler som aktiverar eosinofila granulocyter (så kallade kemokiner) binder till osteopontin (OPN). OPN är ett äggviteämne med stark negativ laddning och det finns närvarande i luftvägarna i relativt stora kvantiteter vid astma. OPN visade sig binda till den delen av kemokiner som fungerar som ett kroppseget antibiotikum

och detta orsakade att den antibakteriella aktiviteten försvann utan att funktionerna som finns i den andra halvan av molekylerna påverkades (aktivering av mottagare för signalering in i de eosinofila granulocyterna respektive endotoxin-neutralisation). För att fastställa om denna egenskap hos OPN innefattar endast kemokiner eller också generellt omfattar kroppsegna antibiotika, undersökte vi former av de senare vilka produceras både när vi är friska men också sådana som ökar vid sjukdomstillstånd med kronisk luftvägsinflammation. Det visade sig att OPN påverkade flera av dessa men inte alla. Inte heller påverkade OPN-bindning den enzym-aktivitet som en del av dessa besitter. Sammanfattningsvis visar dessa studier att visa kemokiner kan fungera som kroppsegna antibiotika men att deras egenskaper påverkas av nedbrytande enzymer från mast-celler och OPN. I framtida forskning kan man försöka producera dessa molekyler syntetiskt med vissa modifieringar för förhindra deras nedbrytning och också för att öka deras antibakteriella aktivitet på platsen för infektioner. Fynden av de inflammationsrgelerande egenskaperna hos OPN kan också leda till strategier för utveckling av nya läkemedel.

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My journey as an aspiring scientist began when I was offered a scholarship to pursue PhD studies in Arne Egesten's research group. Having been trained (in my M.Sc.) in the field of molecular virology, the transition to lung immunology did not come without its challenges. Nonetheless, my PhD work has expanded the existing knowledge in asthma research and led to discoveries that provide new insights into chemokine immunology. Going forward, this knowledge can be used to devise new therapeutic strategies for asthma.

Printed by Media-Tryck, Lund University 2016
Nordic Ecolabel 3041 0903

LUND UNIVERSITY
Faculty of Medicine

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2016:84
ISBN 978-91-7619-310-5
ISSN 1652-8220