

From the Department of Clinical Science, intervention and Technology,
Division of Ear, Nose and Throat Diseases
Karolinska Institutet, Stockholm, Sweden

NEUTROPHIL SUBSETS IN AIRWAY INFLAMMATION AND HYPERREACTIVITY

Sandra Ekstedt



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Neutrophil subsets in airway inflammation and hyperreactivity

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By

Sandra Ekstedt

Principal Supervisor:

Professor Lars Olaf Cardell
Karolinska Institutet
Institutionen för klinisk vetenskap,
intervention och teknik
Enheten för öron-, näs-
och halssjukdomar

Co-supervisor(s):

PhD. Susanna Kumlien Georén
Karolinska Institutet
Institutionen för klinisk vetenskap,
intervention och teknik
Enheten för öron-, näs-
och halssjukdomar

Opponent:

Docent Madeleine Råding
Göteborgs universitet
Avdelning för invärtesmedicin och klinisk
nutrition vid Institutionen för medicin

Examination Board:

Professor Johan Grunewald
Karolinska Institutet
Institutionen för medicin

Docent Anna Smed Sörensen
Karolinska Institutet
Institutionen för medicin

Professor Torbjörn Ledin
Linköpings Universitet
Institutionen för klinisk och
experimentell medicin

All vetenskaps början är förvåningen över att tingen är som de är
Aristoteles

ABSTRACT

Neutrophils are part of the first lines of defence against invading microbes. They play an essential role in antimicrobial host defence by recognizing microorganisms through the various receptors that can be expressed on their surfaces. The known function of neutrophils in the airway inflammatory process has changed over time, from being an expendable cell limited to phagocytosis, releasing enzymes and other cytotoxic agents, to a dedicated cell that can release mediators with a more precise role in the defence and disease development. Identifying pathways that are active during inflammation and its resolution is central for understanding the mechanisms behind disease development and conjure a foundation for the development of new therapeutic strategies.

Novel subsets with different functions of already classified cells are continuously discovered. In line with this, four different neutrophil subsets have been identified based on their expression of CD16 and CD62L. These subsets reflect different stages of cell maturity and activity. The dim/high neutrophils are considered immature as they are recently derived from the bone marrow. The high/high neutrophils are considered mature and the high/dim neutrophils are activated. The dim/dim neutrophils are believed to be active in a stage just before apoptosis.

In paper I, we studied the distribution of neutrophil subsets in blood, nasal biopsies and nasal lavage in healthy controls and allergic patients. We saw that healthy subjects and allergic patients harboured high/high neutrophils in blood and nasal biopsies. High/dim neutrophils were found in the nasal biopsies and to some extent in the nasal lavage, whereas the dim/dim neutrophils were only found in the nasal lavage. However, among the allergic patients the distribution in nasal biopsies was skewed towards high/dim neutrophils. The high/high and high/dim neutrophils were used in functional assays to study their effects on T-cells and eosinophils. The high/dim neutrophils had the ability to prime the CD4⁺ T-cells and function as a chemotactic factor for the eosinophils. This shows that the high/dim neutrophil might have a role in the development of allergy.

In paper II, neutrophils from healthy subjects and allergic asthma patients were obtained and stimulated in order to study markers crucial for their clearance. Stimulated neutrophils from patients with asthma had much higher expression of “don’t eat me” markers than neutrophils from healthy subjects. The former neutrophils also had reduced production of CCL3, CCL4 and CLL20, which affected the migration rate of monocytes. The findings may explain the prolonged duration of an infection that allergic asthmatics often experience.

Paper III-IV characterised neutrophil subsets in blood before and after an inhaled allergen provocation. The fraction high/high neutrophils decreased and the high/dim neutrophils increased as a result of the challenge. To evaluate the effects of high/high and high/dim neutrophils on airways; human bronchi or mice trachea were co-cultured with the different subsets. The functional changes caused by the co-cultures were then evaluated in a myograph. The high/dim neutrophils increased the response towards bradykinin. They also enhanced contraction induced by nerve-mediated stimulation. The increase in the bradykinin response was related to a release of TNF α that subsequently upregulated the bradykinin receptor 2. The nerve-mediated airway hyperresponsiveness in conjunction with high/dim neutrophils was due to production of IL-1 β that caused an increase of substance P in the nerves via COX-2. These new findings may lead to a better understanding of the role of neutrophils in severe asthma, and potentially to new treatments.

In summary, the thesis demonstrates that the distribution of the neutrophil subsets differs between healthy subjects and allergic individuals and that the different subsets might have diverse effects on the airways.

LIST OF SCIENTIFIC PAPERS

- I. Arebro J, Ekstedt S, Hjalmarsson E, Winqvist O, Kumlien Georen S, Cardell LO. A possible role for neutrophils in allergic rhinitis revealed after cellular sub-classification. *Sci Rep*. 2017;7:43568.
- II. Sandra Ekstedt, Ellen Tufvesson, Bjerner L, Susanna Kumlien Georén and Lars Olaf Cardell. “Don’t eat me” markers indicate that neutrophils from asthmatic patients resist phagoptosis, explaining the occurrence of prevailing airway infections. – Manuscript
- III. Ekstedt S, Stenberg H, Tufvesson E, Diamant Z, Kumlien Georen S, et al. The potential role of CD16^{high}CD62L^{dim} neutrophils in the allergic asthma. *Allergy* 2019.
- IV. Ekstedt S, Safholm J, Kumlien Georen S, Cardell LO. Dividing neutrophils in subsets, reveals a significant role for activated neutrophils in the development of airway hyperreactivity. *Clin Exp Allergy*. 2019 Mar;49(3):285-291.
- V. Sandra Ekstedt, Olivia Larsson, Susanna Kumlien Georén and Lars Olaf Cardell. CD16^{high}CD62L^{dim} neutrophils induce nerve mediated airway hyper-reactivity – Manuscript

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LIST OF ABBREVIATIONS

AR	Allergic rhinitis
BK	Bradykinin
<i>dim/high</i>	CD16 ^{dim} CD62L ^{high}
<i>high/ high</i>	CD16 ^{high} CD62L ^{high}
<i>high/dim</i>	CD16 ^{high} CD62L ^{dim}
<i>dim/dim</i>	CD16 ^{dim} CD62L ^{dim}
LPS	lipopolysaccharide
IL-1 β	Interleukin 1 beta
IL-1Ra	Interleukin-1 receptor antagonist
TNF α	Tumor necrosis factor alpha
NGS	Normal goat serum
TLR	Toll-like receptor
SP	Substance P
BAL	Bronchoalveolar lavage
LPS	Lipopolysaccharide
FACS	Flow cytometry
AHR	airway hyperreactivity
FSC	Forward scatter
SSC	Side scatter
MFI	Medium fluorescence intensity
DTT	Dithiothreitol

1 AIMS

The overall aim of this thesis was to study neutrophils and different neutrophil subsets in allergic rhinitis and acute asthma.

The specific aims were to:

- Identify neutrophil subsets in the blood, nasal mucosa and nasal lavage fluid and characterise their role in allergic rhinitis
- Investigate the role of neutrophils in the resolution of inflammation
- Explore if neutrophil subsets shift during acute asthma
- Characterise the role of neutrophil subsets in lower airway hyperreactivity
- Evaluate the effect of neutrophil subsets on sensory nerve-mediated lower airway hyperresponsiveness.

2 INTRODUCTION

2.1 Immune system

The immune system has evolved to protect us from pathogens and is capable to produce a large number of cells and molecules that specifically are supposed to recognize and eliminate pathogens and invaders¹. The innate immune system is the rapid response to pathogens, while the adaptive immune system develops over time and is a more specific and effective defence. The two are often described as two separate systems; in fact, there is a close interaction between the two. They are synergistic parts of a system that mediates an effective defence against tissue injury and infections².

2.2 The united airways

The nose and the lungs are both parts of the respiratory tract and are divided by functionality: the conductive part and the part responsible for gas exchange. Conventionally, the respiratory tree is divided into upper and lower airways. The upper airways signify the nose and mouth to the larynx, whereas the lower airways reach from the larynx to the alveoli^{3, 4}. There is evidence that there is a link between asthma, allergic rhinitis and rhino sinusitis and these diseases may have a common pathogenic phenomenon, with the term “united airway disease” becoming a more accepted concept⁵.

2.3 Allergic rhinitis

The prevalence of Allergic rhinitis (AR) has increased and today AR is a common healthcare problem. AR is one of the most common chronic conditions, affecting approximately 400 million people around the world. The cost of lost productivity in Sweden for rhinitis has been estimated to be 2.7 billion euros a year⁶. Nasal itching, sneezing, rhinorrhea and nasal congestion¹ are common symptoms of the disease. The disease is believed to be initiated during a sensitisation phase. An antigen is taken up by dendritic cells, which migrate to the regional lymph nodes where the processed antigen is presented to a naïve T-cell. The naïve T-cells mature into T_H2 cells and interact with the B-cells. The B-cells then undergo a class switch, resulting in the production of allergen-specific IgE^{1, 7, 8}. The disease will then progress in two phases, the early and the late phase.

In the early phase of allergic rhinitis mast cells appear. A re-exposure of an antigen results in crosslinking of IgE-molecules on mast cells, which causes degranulation and release of histamine, bradykinin, prostaglandins, cytokines and leukotrienes. The early phase is categorised by bronchoconstriction of the smooth muscle, mucus

production, vascular leakage, enhanced airway hyperresponsiveness and recruitment of inflammatory cells^{1,9}. Neutrophils in the early phase are responsible for, among many things, the release of myeloperoxidase, elastase and lipid mediators¹⁰. The late phase is characterised by inflammation of the airways. In this phase, mediators such as IL-4, IL-5, IL-13, eosinophil chemotactic factor, platelet-activating factor and tumour necrosis factor alpha (TNF- α) are released in the airways. This leads to an upregulation of adhesion molecules on endothelial cells and increases the influx of inflammatory cells, such as eosinophils and neutrophils, to the airways¹.

2.4 Asthma

Currently, asthma affects 300 million people worldwide. The prevalence of asthma is increasing and it has been estimated that an additional 100 million people will develop asthma in 2025. Many asthmatics live with unmet needs related to their disease, which leads to poor asthma control¹¹. Asthma is a complex disease with symptoms such as coughing, wheezing and chest tightness and is characterised by chronic inflammation, airway obstruction and airway hyperresponsiveness^{1,12}. Asthma is a heterogenic and multicausal disease, is not an age- or income-biased disease, and is known to affect people in all countries. The disease has a multitude of phenotypes. However, the current treatments for all the phenotypes are bronchodilators and anti-inflammatory drugs. The inflammation in allergic asthma is usually T_H2 dominant and the immune response could be described as an interplay between the epithelial cells, the smooth muscle in the airways and the immune system. The inflammation and airway hyperreactivity (AHR) in the airway smooth muscle result, after some time, in structural and functional changes of the airways; which contribute to the symptoms¹. The phenotypes in asthma were often based on the presence of neutrophils and eosinophils. However, the number of phenotypes have increased beyond the neutrophilic and eosinophilic asthma. The treatment strategies are still often focused on eosinophils. That is despite the fact that blood neutrophils increase during the first hours after bronchial allergen challenge¹³ as well as in the late phase of asthma¹⁴. The corticosteroids, which work well on eosinophilic inflammation, do not affect the neutrophils or may even recruit neutrophils to the site of inflammation¹⁵. With the advent of phenotyping and cluster analysis, the “one size fits all” approach to asthma treatment is outdated¹⁶. As the patients with difficult-to-treat asthma present with a large number of neutrophils in the airways, focusing on the neutrophils may offer new treatment possibilities.

2.5 Asthma exacerbation

The main reason for hospital-related admissions and one of the central complications in asthma is exacerbation¹⁷. This phenomenon can be defined as an acute deterioration in lung function, resulting in heightened mucous production,

excessive increased inflammation and more severe airway hyperresponsiveness, ultimately resulting in a serious decline in quality of life¹⁸. The primary cause of asthma exacerbations is microbial or viral infections, where microbial-dependent exacerbations have been shown not only to extend the period of illness, but also to increase respiratory symptoms, such as airway hyperresponsiveness.

Bacterial infections, such as *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae* are present in 38% of exacerbations, but the exact role bacteria and viruses play is as of yet unknown¹⁹. Viral infections in children has been reported to be a trigger for asthma, where the respiratory syncytial virus (RSV) and human rhinovirus (HRV) are the most important pathogens. Exacerbations of the disease are correlated to viral infections in 80 % for children and 50-75 % for the adult²⁰. However, there is insufficient evidence to suggest that asthma patients have more colds than healthy individuals. Asthmatic patients that develop a cold have extended duration and increased severity of the illness, and often suffer from secondary bacterial infections^{21,22}. It seems that it is neither the delayed microbial or viral clearance nor the viral load that is the cause; rather it seems to be defects in the innate immune host defence pathways^{23,24}. One of the key steps to reach homeostasis is the need to regulate and remove the neutrophils from the tissue after an infection or inflammation. This process also leads to the release of anti-inflammatory mediators to stop the influx of new neutrophils into the tissue^{25,26}.

2.6 Airway hyperresponsiveness

Airway hyperresponsiveness is a characteristic feature of asthma and measurements of airway responsiveness are useful when diagnosing asthma. The definition of airway hyperresponsiveness is an exaggerated obstructive response of the airways to endogenous or exogenous chemical and physical stimuli, such as histamine, methacholine, bradykinin, adenosine 5'-monophosphate, fog and cold air. Airway hyperresponsiveness determines the need for therapy and the severity of respiratory symptoms and weakening of lung functions^{27,28}.

Airway hyperresponsiveness is sometimes divided into two different components that affect the feature of asthma: persistent or baseline and variable or episodic. The persistent feature can be ascribed to structural changes, such as airway remodelling. It can also be caused by increased expression of contractile proteins and genetic changes in the airway smooth muscle. The episodic feature can be ascribed to the inflammation in the airways mediated by various triggers such as: nitric oxide, substance P (SP), PGD₂, PGF₂ α , CCL28, IL-13 and IL-5. Persistent and variable is an easy way to describe airway hyperresponsiveness and the two are linked together²⁹. The contraction in the airways can be due to a direct smooth muscle stimuli or indirect neural stimuli.

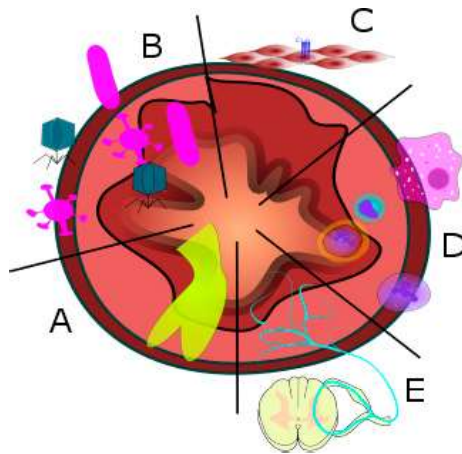


Figure 1. Schematic image of the inflammatory and remodeling processes in the airway that may contribute to airway hyperresponsiveness. Viral and bacterial infections lead to increased inflammation (D) and increased mucus production (A, B). Increased smooth muscle mass (C) together with altered nerve activation (E) create the foundation of asthma exacerbation and airway hyperresponsiveness.

2.7 Airway smooth muscle and sensory nerves in airways disease

Some of the hallmarks for asthma are increased airway smooth muscle mass and alterations in the neural phenotype. The smooth muscle is linked to airway hyperresponsiveness as it is responsible for airway narrowing, induced by mediators released from i.e. mast cells and nerves. Increased smooth muscle mass and exaggerated responses to contractile stimuli are often seen in asthma patients³⁰. Stimulation of bradykinin 2 receptors can induce airway constriction and bradykinin has been proposed as a sensitive indicator for AHR³¹. As the nervous system is intertwined with the smooth muscle and activation of sensory nerves may lead to a contraction of the muscle, the interplay between the smooth muscle and the nerves may be of importance for regulating the airways. However, any dysfunction may contribute to the pathogenesis of airway diseases. The sensory nerve fibres originate in the vagal ganglia³², characterised by their release of neuropeptides, including tachykinins such as substance P and neurokinin A and calcitonin gene related peptide (CGRP). Stimulation of a local axon in the lung may lead to activation of sensory fibres, which results in the release of substances which, via NK1, NK2 and NK3 receptors, induce a contraction of the smooth muscle³³. It is well established that neural mechanisms are involved in asthma. It has further been postulated that, upon infection, the sensory nerves in asthma may be primed, which leads to an exacerbation³⁴. In an animal model, studying RSV infections, levels of SP were elevated and treatment with an NK1 antagonist inhibited AHR development^{35,36}. In the human lung, the levels of SP are increased in patients suffering from asthma, as compared to the healthy lung. SP is higher in sputum³⁷, bronchoalveolar lavage (BAL) and even higher in BAL after an allergy challenge in the lung³⁸. IL-1 β , a cytokine largely produced by the neutrophil, has been shown to enhance the SP release from sensory nerves when triggered.

2.8 Inflammation and resolution in the airways

Inflammation has evolved to protect us from pathogens. It is a response of the immune system to non-self-antigenic stimuli, beneficial under normal circumstances³⁹. Acute inflammation is characterised by an influx of inflammatory cells followed by the release of pro-inflammatory cytokines. These cytokines can then recruit and activate leukocytes. Altogether, this leads to the classical symptoms of inflammation, namely heat, redness, swelling and pain.

Return to tissue homeostasis following inflammation is essential for the resolution of inflammation. Resolution is an active process that is necessary for the avoidance of tissue damage after clearance of the inflammatory site. Following exposure to antigens, an acute inflammatory response develops in the airways of allergic individuals. Later, in the absence of that antigen, this inflammation can either resolve or become chronic. The resolution depends on several pro-inflammatory, anti-inflammatory and pro-resolving mediators secreted by various cells. Induction of apoptosis and removal of pro-inflammatory cells by macrophages, particularly neutrophils, is a key to the resolution of inflammation³⁹. Removal and clearance of neutrophils by professional and non-professional phagocytes triggers specific downstream intracellular signal transduction pathways resulting in anti-inflammatory effects, such as a reduced influx of neutrophils, leading to resolution. If one of the steps fails, the inflammation could persist and become chronic⁴⁰.

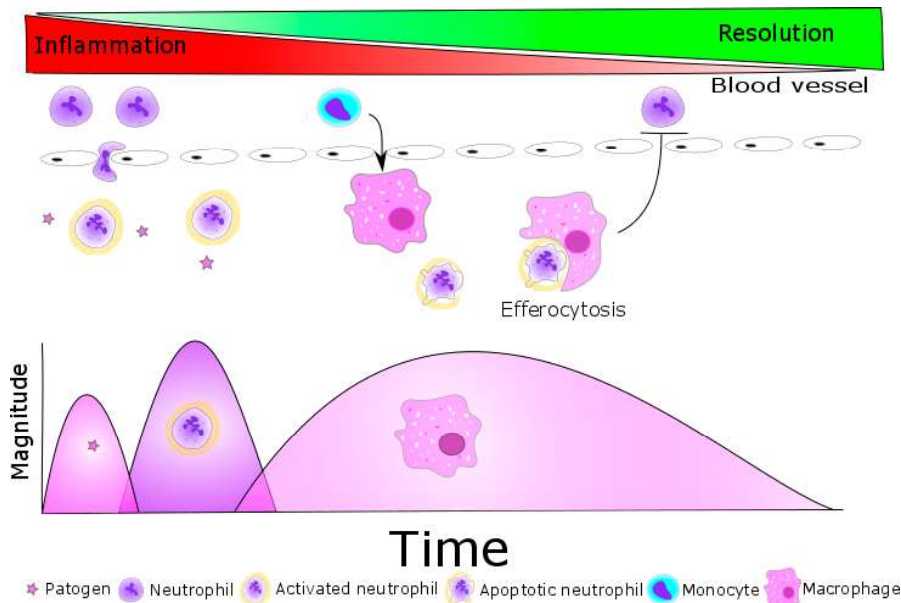


Figure 2. Schematic image of the inflammation, its resolution and the interplay between neutrophils, monocytes and macrophages.

2.9 Neutrophils and neutrophil subsets

Neutrophils constitute an initial part of the first line of defence against invading microbes. The known function of neutrophils in the inflammatory process has changed over time. Initially, the neutrophil was described as a cell limited to phagocytosis and the release of enzymes and cytotoxic agents.

Inflammatory research is continuously identifying novel subsets of already classified cells, with different functions⁴¹. In line with this, four variants of neutrophil subsets based on their expression of CD16 and CD62L have been identified. The different subsets can be seen as different stages of activity and maturity. The $CD16^{dim}CD62L^{high}$ (*dim/high*) neutrophil cannot be found in the blood of healthy donors. They can, also be found in patients suffering from severe injuries or given LPS. They have a banded nuclear morphology and are considered immature as they are recently derived from the bone marrow. The $CD16^{high}CD62L^{high}$ (*high/high*) neutrophil is considered mature and is the neutrophil we have in the circulation. The $CD16^{high}CD62L^{dim}$ (*high/dim*) neutrophil with a hyper-segmented nucleus is considered active. It has been shown to have an important role in systemic inflammation⁴², suggesting that the presence of specific neutrophil subsets can play a role also in asthma and allergy. The $CD16^{dim}CD62L^{dim}$ (*dim/dim*) neutrophil is the least defined subset and thought to be at the end stage right before apoptosis.

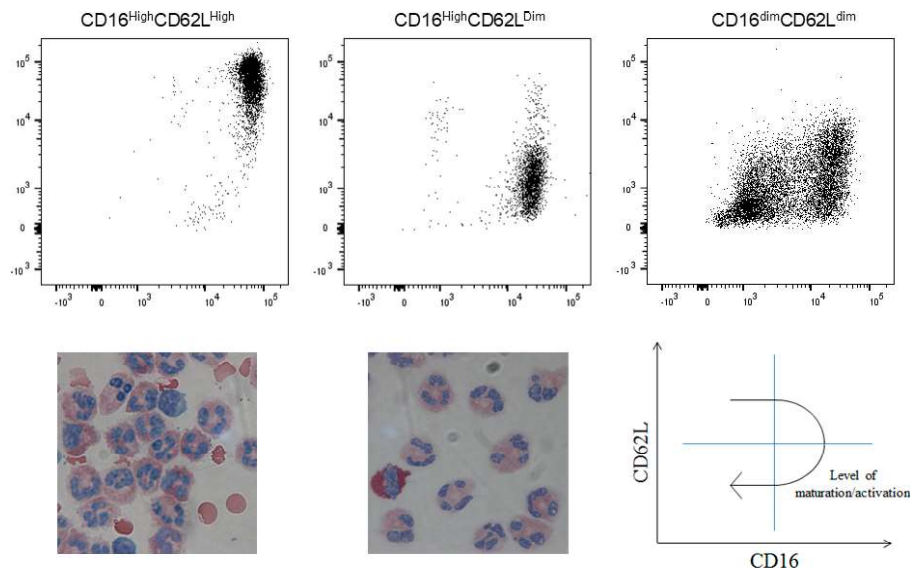


Figure 3. Dot plots of the expression levels of CD16 (X-axis) and CD62L (Y-axis) in the different stages of activation of neutrophils.

It is important to regulate neutrophil abundance and turnover. Failure in any step can directly cause or contribute to human diseases, with symptoms such as such as dyspnoea, hypoxia, neurologic deficits and chronic inflammation. Efferocytosis of neutrophils inhibits pro-inflammatory cytokine production and results in changes of macrophages towards a pro-resolution phenotype. If regulation or removal of apoptotic neutrophils fails, toxic intracellular components from neutrophils may damage healthy tissue^{1, 43}. There is an imbalance between apoptotic and live neutrophils in asthma. The neutrophils have an increased lifespan mediated via IgE⁴⁴. New research also indicates that living neutrophils can be the target of phagocytes, often referred to as phagoptosis. This is regulated by expression of “eat me”, CD43 /CD36 and “don’t eat me”, CD47 signals^{45, 46} on the surface of the neutrophils. There is evidence that “don’t eat me” ligands are associated with inefficient clearance of neutrophils and prolonged inflammation⁴⁷. However, “eat me” may facilitate phagocytosis of the neutrophils, promoting resolution of inflammation. The role of these neutrophil phenotypes in airway allergy, particularly in exacerbations, is unknown.

A common question is whether the neutrophil is good or bad. Is the neutrophil our friend or our mortal enemy? If we rephrase this question and instead ask *when* is the neutrophil good or bad? Is the friendly neutrophil the same neutrophil when it is our enemy? When is the neutrophil our friend and what changes are needed to make the neutrophil our enemy? This thesis aims to answer that question.

3 MATERIALS AND METHODS

This section contains a brief overview of the material and the methods used in the studies. More details can be found in the individual papers I-V.

3.1 Human subjects

The diagnoses of the AR patients (paper I) were based on clinical history and positive skin prick test and/or positive RAST for the relevant allergen.

The diagnoses of the allergic asthmatic patients (papers II and III) were based on clinical history and positive skin prick test and/or ImmunoCAP Rapid.

Healthy controls (papers I, III-V) had no history of sinus disease, asthma or allergy. None of the healthy controls had a history of steroid use and they all had a negative skin prick test or negative ImmunoCAP Rapid.

Paper I: Eight allergic rhinitis patients and six healthy controls were included. Nasal biopsies, nasal lavage fluid and blood were obtained from all patients in the study.

Paper II: Human lung tissues were obtained at the Departments of Cardiothoracic Surgery and Anesthesiology, Karolinska University Hospital, Stockholm and blood was collected from non-allergic healthy volunteers (age 18-65) recruited from the blood bank in Stockholm and at the Karolinska University Hospital, Stockholm.

Paper III: nine non-smoking subjects (aged 18-50) from Lund University Hospital with a diagnosed allergic asthma and with less than 40% of activated neutrophils in the blood at baseline. Inhaled allergen bronchoprovocation test was performed and blood was collected before and after the provocation.

Paper IV: Allergic asthmatic patients and healthy controls were recruited at the Karolinska University Hospital, Stockholm and at Lund University Hospital.

Paper V: Non-allergic or asthmatic healthy volunteers (age 18-65) were recruited from the blood bank in Stockholm and the Karolinska University Hospital, Stockholm. They were all tested with an ImmunoCAP Rapid to exclude allergy.

All studies with human samples were approved by the local ethics committee, 2014/260, 2010/181-31/2, 2014/299-13, 2013/2075-31/3 and 2016/823-31/2, and all patients provided written informed consent.

3.2 Neutrophil isolation, activation and stimulation

Neutrophils were purified using ficoll-paque according to the manufacturer's instructions (papers I, and IV). The granulocyte rich pellet was treated with ammonium chloride erythrocyte lysis solution (0.8 % NH_4Cl , 10 mM KHCO_3 0.1 mM EDTA). In paper I, one extra step was added in the isolation of the neutrophils, they were enriched with CD15 microbeads according to the manufacturer's instructions.

In papers I, IV and V, the neutrophils were isolated using MACSExpress kit with magnetic beads through negative selection. The beads were mixed with whole blood and incubated for five minutes during slow rotation. The neutrophils were then placed in a magnet for 15 min after which the neutrophils were collected. At this state, the neutrophils were contaminated with erythrocytes and an erythrocyte depletion kit was used. Then new magnetic beads were added for a 5 minutes incubation during slow rotation, allowing the erythrocytes to bind to the magnetic beads. The neutrophils were then placed one more time in the magnet. The neutrophils did not accumulate against the wall of the tube and were easily removed.

Neutrophils were suspended in complete medium (CM) consisting of RPMI 1640 supplemented with 20% autologous plasma, 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin. To achieve different subsets of neutrophils in clearly defined groups, high/high neutrophils were treated with 1 $\mu\text{g/mL}$ LPS or a combination of 1 $\mu\text{g/mL}$, 5 ng/ml $\text{TNF-}\alpha$ and/or 10 ng/ml IL-8 for 15 minutes, 1 or 2 h at 37°C in humidified 5% CO_2 in atmospheric air. IL-1RA was added to the neutrophils before lipopolysaccharide (LPS) stimulation to evaluate the effect of IL-1 β on the neutrophils (Paper IV).

3.3 *In vitro* model

3.3.1 Tissue preparation and organ culture

Human bronchi were obtained from patients undergoing lobectomy. The patients were recruited for the purpose of acquiring macroscopically healthy human lung tissue in an investigation approved by the regional ethical review board in Stockholm. Within 3 h of resection, human isolated small airways, with a diameter of 0.5–2 mm, were identified based on their visual appearance (thin and almost transparent wall, bone white colour, presence of air bubbles and mucus, as well as lack of blood) and dissected free under ice-cold Krebs-Henseleit buffer (KH, Sigma, St. Louis, MO, U.S.A) solution and cut into rings (paper II).

BALB/c mice (weight 19–30 g, 8-14 weeks old) were housed in plastic cages (papers II and V) or individually ventilated cages (paper V) with adsorbent bedding in temperature and light/dark cycle (12-hour/12-hour) controlled rooms.

Food and water were available ad libitum. Animals were handled in accordance with the Federation for European Laboratory Animal Science Associations guidelines. All animal experiments were approved by the local ethics committee at Karolinska Institutet (Stockholm. Ethical permit numbers: N258/13, N254/15 and 1155-2019). The animals were sacrificed by cervical dislocation, their lungs and tracheae were removed and the tracheae were dissected free from surrounding tissue. The tracheae were then cut into four rings for evaluation of direct smooth muscle contractions or two rings for evaluation of nerve-mediated smooth muscle contractions. Each segment, human or mice, was cultured for 24 hours at 37°C in humidified 5% CO₂ in atmospheric air. The segments were then moved to new wells containing *high/high* or *high/dim* neutrophils or CM alone as a control. To evaluate mechanisms initiated from the *high/dim* neutrophils different substances, antagonist or antibodies were added to the trachea 1 hour or 30 minutes before addition of the neutrophils.

3.3.2 In vitro pharmacology

Changes in smooth muscle contraction were evaluated in myographs (paper II). Murine and human airway ring segments were placed in a myograph (Organ Bath Model 700MO; DMT A/S, Aarhus, Denmark) containing 5 mL KH buffer solution at 37°C, continuously bubbled with carbogen (5% CO₂ in O₂) to maintain a stable pH of 7.4. Changes in the smooth muscle contraction were monitored and isometric forces were measured (ADInstruments, Hastings, United Kingdom). During an equilibration phase of 1 h for mice and 1.5 h for humans, the tension of the tissue was set to 0.8 mN for murine tissue and 1.5 mN for human tissue. To confirm tissue viability, contractile responses were induced by KCl (60 mM) followed by a wash and an additional 30-minute equilibration period. In presence of indomethacin (3 µM) and atropine (1 µM) bradykinin was cumulatively added and force changes registered.

3.3.3 Electric field stimulation

This was used together with the myograph to evaluate changes in nerve mediated smooth muscle contractions (paper V). Mice tracheae were mounted in a myograph (Organ Bath Model 700MO; DMT A/S, Aarhus, Denmark) as treated as describe above. During an equilibration phase for 1,5 h, the tension was set to 1.6 mN and to confirm tissue viability, contractile responses were induced by KCl (60 mM). EFS electrodes were then placed in the bath and KCl (60 mM) was added once more followed by a wash and an equilibration period. The segments were incubated with 3 µM indomethacin for 30 minutes and each segment was given five training impulses of 4 Hz, 55 mA (≈10 V) followed by a wash and an equilibration period and additional indomethacin. Later, segments were given a 2log EFS series of

0.2–25.6 Hz. Each impulse was 0.8 ms long with a duration of 1 min, followed by a 1.5 min resting period. The experiment ended with a contraction to 0.1 μ M carbachol to evaluate the maximal contraction of each segment.

3.4 Flow cytometry

Flow cytometry (FACS) was used in all studies. FACS analyses physical and chemical properties on individual cells in a suspension based on how they scatter light from a laser beam. Using different detectors, the flow cytometer can provide information about cell size (displayed as forward scatter, FSC), granularity (displayed by side scatter, SSC) and fluorescence intensity of fluorochrome-conjugated antibodies used for detection of intra- or extracellular antigens. The number of positively labelled cells and the medium fluorescence intensity (MFI) can be calculated.

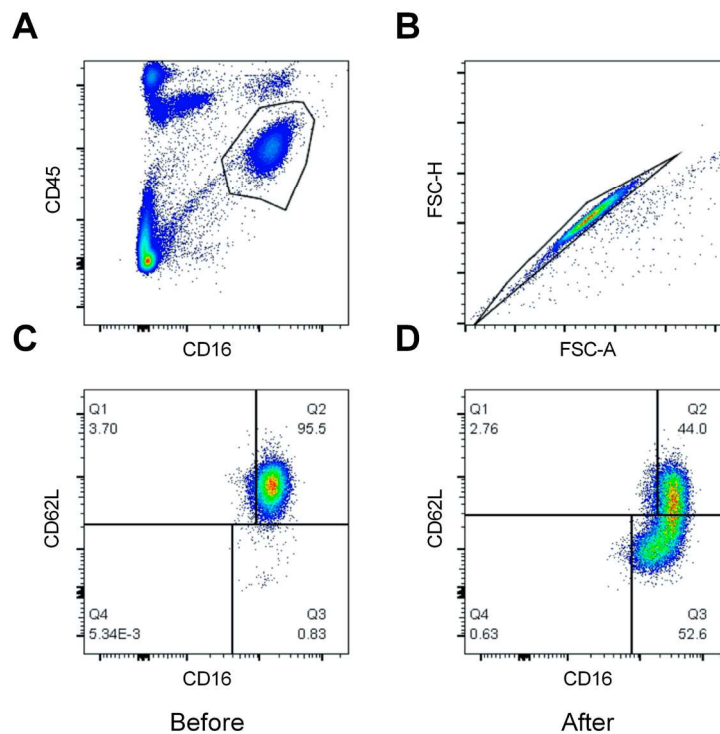


Figure 4. Gating strategy plots (A) first and (B) second gating steps from the blood samples obtained from patients before and after an inhaled allergen bronchoprovocation test. Gating strategy plot (C-D) from blood samples before (C) and after (D) the allergen provocation test (Paper III).

By gating cells on CD15 or CD16 on the Y-axes, neutrophils can be distinguished. Doublets can be excluded by gating on Forward-Height (FSC-H) and Forward-Area (FSC-A). By having CD62L on the X-axes and CD16 on the Y-axes can the different subsets be evaluated. The Vybrant Apoptosis Assay Kit was used to assess the percentage of viable cells.

All cells have to be in single-cell suspension before staining. Hence, biopsies used for flow cytometry were first placed through a 100- μ m cell strainer, into DMEM/F-12 containing FBS and incubated for 5 min. All types of samples with cells were washed and centrifuged, after which the supernatant was aspirated and discarded. Fluorochrome-conjugated antibodies were incubated for 20 minutes and all samples were then fixed in 1 % paraformaldehyde. All cells were gated based on forward and side scatter and events in the range of 10,000–100,000 were collected.

3.5 ELISA

ELISA can be used to detect and quantify proteins in suspensions, such as cell supernatants from stimulated cells. A sandwich ELISA uses a microplate pre-coated with an antibody meant to bind the antigen in question. When the sample is added to the microplate, the antigen binds to the immobilised antibody. After washing away unbound substances, an enzyme-linked antibody specific for the antigen of interest is added and upon adding a substrate solution, a colour develops in proportion to the amount of bound substrate. ELISA was used to measure IL-1 β , TNF α (paper II) and CCL20 and CCL4 (paper IV), respectively.

3.6 PCR

3.6.1 Tissue preparation and RNA extraction

Neutrophils were lysed using RLT-buffert with Dithiothreitol (DTT) before the mRNA was purified. RNA from neutrophils were isolated using the RNeasy Micro Plus Kit (Qiagen) according to the manufacturer's instructions. RNA yield and purity were assessed with a NanoDrop 1000 using the wavelength absorption ratio (260/280 nm) (papers IV and V).

3.6.2 cDNA syntheses and qPCR

cDNA was synthesised and amplified with the QuantiTect Whole Transcriptome Kit using the manufacturer's protocol for high-yield reaction (paper IV). Omniscript RT kit was used according to the manufacturer's instructions (paper V). All qPCR reactions used the QuantiFast Multiplex PCR Kit and were performed on a Stratagene mx3000p machine (Agilent Technologies).

3.7 Immunohistochemistry

Immunohistochemistry can be used to visualise an antigen in different fixed tissues. Tissues used in the myograph were stored in 4% paraformaldehyde, embedded in paraffin and sectioned into five μM thin slices using a microtome and mounted on glass. Before staining, the sections were deparaffinised, re-hydrated and treated with heat mediated antigen retrieval. The sections were permeabilised and a block solution was added to minimise unspecific staining. The sections were incubated at 4 °C overnight with a bradykinin 2 receptor antibody (paper II). DAB was used to visualise the expression of the bradykinin receptor.

3.8 Cell separation, and co-culture of eosinophils, t-cells and neutrophils

Eosinophils and T-cells were used in different co-cultures with different subsets of autologous neutrophils (paper I). Blood was collected in heparin coated tubes and density gradient isolation of neutrophils and T-cells was performed using ficoll-paque (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The granulocyte rich, neutrophil containing, pellet was treated with ammonium chloride erythrocyte lysis solution and to further enrich the neutrophils magnetic beads with CD15 attached were added. To activate the *high/dim* neutrophils, *high/high* neutrophils were treated with 1 $\mu\text{g/mL}$ LPS, 5 ng/mL TNF α and 10 ng/mL IL-8 for 15 minutes and then washed. The lymphocyte interface from the ficoll-paque isolation was collected and washed with PBS. Different subsets of neutrophils were co-cultured with the T-cells for 30 minutes before the T-cells activator CD3 was added. The co-culture was incubated for 90 minutes and the activation markers, CD69, was analysed using FACS. For mechanistic purposes, a transwell was used to block cell-cell contact.

As eosinophils play a major role in allergy, we were interested to see if the activated neutrophils could affect the migration and movement of eosinophils (paper I). Eosinophils were separated from whole blood using MACSExpress kit with negative selection. The *high/dim* neutrophils were achieved as above and added to the bottom of a plate. The eosinophils were then added to a transwell and the cells were allowed to migrate for three hours. The cell count in the lower compartment was analysed with FACS.

3.9 Migration

The human monocytic leukaemia cell line, THP-1 was cultured in RPMI Medium 1640 – GlutaMAX™-I (Invivogen, San Diego, CA, USA) supplemented with 10% heat-inactivated FBS (Invivogen, San Diego, CA, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Invivogen, San Diego, CA, USA) at 37 °C and with 5% CO₂ in air. The monocytes were suspended in FBS free RPMI Medium 1640 - GlutaMAX™-I overnight before the migration started. 200.000 cells were added to insert with a pore size of 5 µM and left to migrate towards the supernatant from the stimulated neutrophils for 2 and 4 hours. The migrated cells were then counted using trypan blue.

3.10 Statistics

Statistical analyses were performed using GraphPad Prism software (version 6.0, Graph Pad Software, La Jolla, CA). All data were shown as mean ± SEM. A p-value of <0.05 was considered statistically significant (*P <0.05, **P < 0.01, ***P < 0.001). A D'Agostino & Pearson omnibus or Shapiro-Wilk normality test was used to determine if the data were normally distributed. For more than two sets of data, a two-way ANOVA or one-way ANOVA with Bonferroni post-hoc or Tukey post-hoc test was performed. For two sets of data Mann-Whitney test or a student t-test was used.

4 RESULTS AND COMMENTS

4.1 Neutrophil subsets in the nose and their role in allergic rhinitis (paper I)

A major feature in AR is a rapid response of the innate immune system, originally designed to detect invading pathogens. Innate immune cells, like neutrophils, eosinophils and epithelial cells, create a foundation for nasal inflammation. Traditionally, eosinophils have been in focus when granulocytes are studied in AR. The neutrophil is well known for being the first cell at the site of inflammation and in an allergic reaction. We therefore thought that a study of neutrophil subsets may lead to novel insights in AR.

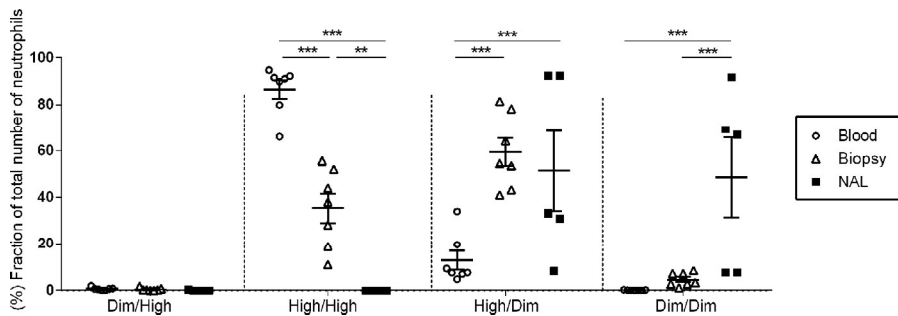


Figure 5. The distribution of neutrophil subsets in blood, nasal biopsies and nasal lavage in AR patients ($n = 5-7$).

4.1.1 Distribution of neutrophil subsets in the nose

Neutrophils from blood, nasal biopsies and nasal lavage were analysed based on the expression of CD16 and CD62L and divided into four different subsets. The subset *dim/high*, classified as immature neutrophils, was almost undetectable in the nose. The *high/high* subset, classified as mature but non-activated neutrophils, was predominant in the blood but also detectable in the nasal biopsies. The *high/dim* subset, classified as mature and activated neutrophils, was mainly located in the nasal biopsies but was also detectable in the NAL. The *dim/dim* subset, classified as the end-stage just before apoptosis, was found in the NAL. This pattern in subset distribution was the same for AR patients and healthy control patients (Figure 5). When only considering nasal biopsies, the distribution between the non-activated and the activated neutrophils was skewed towards the activated form, *high/dim* neutrophils, in AR patients (Figure 6). This was not seen in healthy patients.

4.1.3 High/dim neutrophils can affect eosinophil migration

Lastly, we studied the effect of neutrophil subsets on eosinophil migration using a transwell system where autologous eosinophils could migrate towards different neutrophil subsets. We found that the eosinophils migrated towards the *high/dim* neutrophils but not to the *high/high* neutrophils (Figure 8).

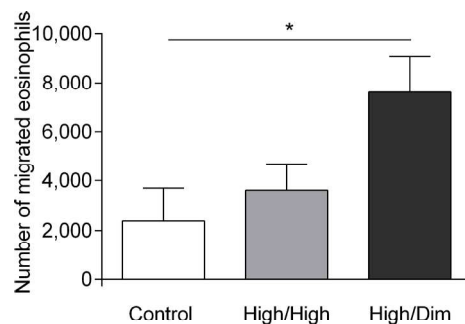


Figure 8. The capacity of neutrophil subsets to induce eosinophil migration in a transwell assay ($n = 4-5$). Control = no added neutrophils

4.1.4 Comments

There was an increased number of neutrophils in blood, nasal biopsies and NAL of the AR patients compared to the healthy control individuals during the pollen season (Figure 1 in paper I). This is in line with previous studies and, as the neutrophil is part of the first line of defence and the first cell to be recruited to an area of inflammation or infection, is not surprising by itself. However, when the neutrophils were characterised based on the expression of CD16 and CD62L an interesting pattern emerged. The *high/dim* neutrophil had a stronger presence in the nasal biopsies of the AR patients than in the healthy controls. It has been shown that microbiota⁴⁸, as well as pathogenic bacteria⁴⁹ and viruses⁵⁰ have the capacity to change the neutrophil subset. We now show that allergen has a similar ability, direct or indirect, to change the subsets in these patients.

The shift from one subset to another facilitates T-cell activation by priming the CD4⁺ T-cell. The presence of the *high/dim* neutrophil increases the expression of CD69 on the CD4⁺ T-cell after CD3-mediated activation. This stands in opposition to what was reported by Pillay and co-workers, where the *high/dim* neutrophil suppressed T-cell activation⁴². The discrepancy is most likely due to the time frames in the two different methods. Our study was focused on what effect a living *high/dim* neutrophil had on the T-cell, and we therefore used a shorter incubation time of 90 minutes. In comparison, Pillay may have focused on the long term effect of neutrophils and therefore used a longer incubation, 96 hours. Another group has shown that neutrophils characterised as CD11b^{high}, CD62L^{low} have the ability to migrate to the lymph nodes. Specific blocking of the neutrophils migration reduced T-cell proliferation, which would be in line with our findings⁵¹.

The present data indicate that *high/dim* neutrophils, have a strong presence in nasal biopsies of AR patients and that they can facilitate eosinophil migration. The role of the eosinophil in AR has been investigated in numerous studies, whereas the role of the neutrophils has been overlooked, despite there being a significant decrease in neutrophils in the nasal mucosa after sublingual immunotherapy⁵². The idea that the neutrophils are essential for increasing the influx of eosinophils is supported by their ability to be first on site in exacerbations of allergic asthma¹⁰.

4.2 Neutrophils in persistent inflammation and delayed phagoptosis (paper II)

The neutrophil is the first cell to be recruited to a site of inflammation but also a cell that has to be regulated and removed before the inflammation can be resolved and the tissue returns to hemostasis. The chemokines and cytokines produced by the neutrophil together with the expression of different surface proteins can delay the removal and thereby delay the resolution. The surface proteins CD36 and CD43 are named “eat me”, and CD47, “don’t eat me”. The type and the levels of these surface markers will determine if the neutrophils will be cleared from the tissue.

4.2.1 Neutrophil clearance may be impaired in the allergic asthma patients due to changes in the expression of “eat me” and “don’t eat me” markers

The expression of CD36, CD43 and CD47 were analysed with flow cytometry prior to stimulation with LPS and TNF α , as well as 60, 90 and 120 minutes after. We observed that neutrophils from the allergic asthmatic patients had a higher expression of CD47 at steady state than the healthy controls and that the expression increased with stimulation. This increase was not seen in healthy controls. The expression of CD36 and CD43 was lower on neutrophils from allergic asthmatic patients as compared to healthy control patients. In addition, the CD43 expression decreased in neutrophils from healthy controls upon *in vitro* stimulation (Figure 9 A-C).

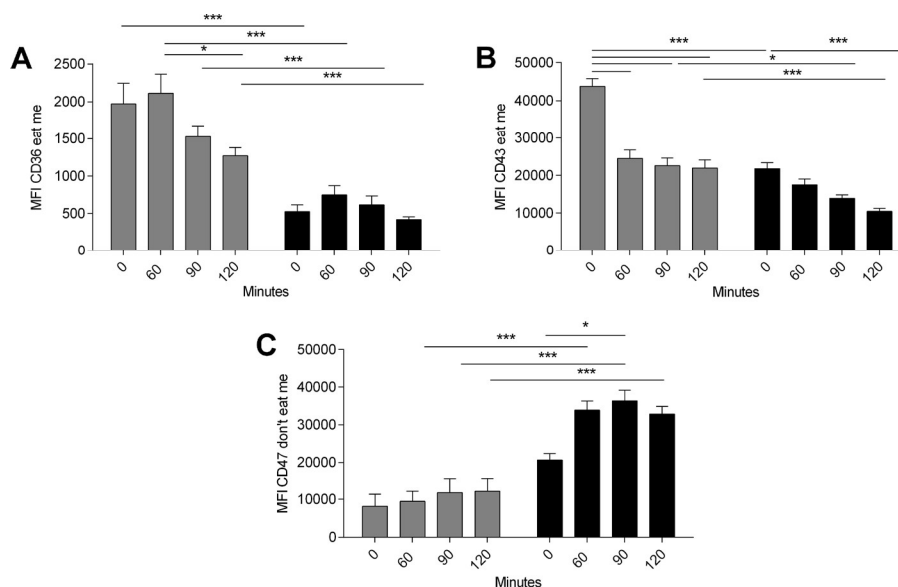


Figure 9. The expression of (A) CD36, (B) CD43 and (C) CD47 on neutrophils from healthy controls (grey, $n = 7$) and allergic asthmatics (black, $n = 7$).

4.2.2 The high/dim subset is more difficult to remove from the site of inflammation than the high/high subset

Stimulation of *high/high* neutrophils from healthy controls with LPS and TNF α to *high/dim* neutrophils revealed a decreased expression of CD43, whereas the expression of CD36 and CD47 was unaffected. Upon stimulation with LPS and TNF α , the levels of CD36 did not change in healthy controls and allergic asthmatic patients. The expression of CD36 on the neutrophils from the allergic asthmatic patients was lower in both subsets compared to the healthy controls. In neutrophils from the allergic asthma group, CD43 was low in both subsets, whereas CD47 was high in the *high/high* subset and increased further after stimulation in the *high/dim* subset (Figure 10 A-C).

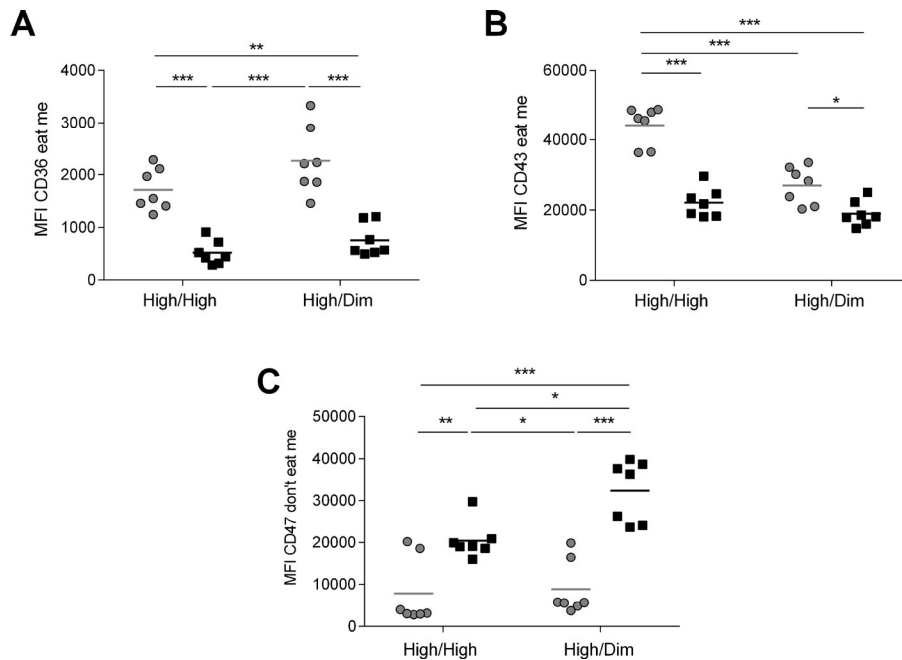


Figure 10. The expression of (A) CD36, (B) CD43 and (C) CD47 on *high/high* neutrophils and *high/dim* neutrophils from healthy controls (grey, $n = 7$) and allergic asthmatics (black, $n = 7$).

4.2.3 Neutrophils have an impaired production of CC-chemokines in allergic asthma patients leading to dampened monocyte migration

Neutrophils from blood of allergic asthmatic patients and healthy controls were stimulated with LPS and TNF α and the supernatants were collected. Investigation of ccl3, ccl4 and ccl20 at mRNA level, (Figure 11 A-C) and CCL4 and CCL20 at protein level (Figure 12), revealed lower levels of these cytokines in neutrophils from allergic asthmatic individuals, as compared to healthy individuals. The functional outcome related to the reduced CCL production was investigated with a monocyte migration assay. The supernatants, free from neutrophils, were used in the migration assay with a monocyte cell line (THP-1). A decreased migration of monocytes towards neutrophils from allergic asthmatic patients was seen (Figure 13 A, B).

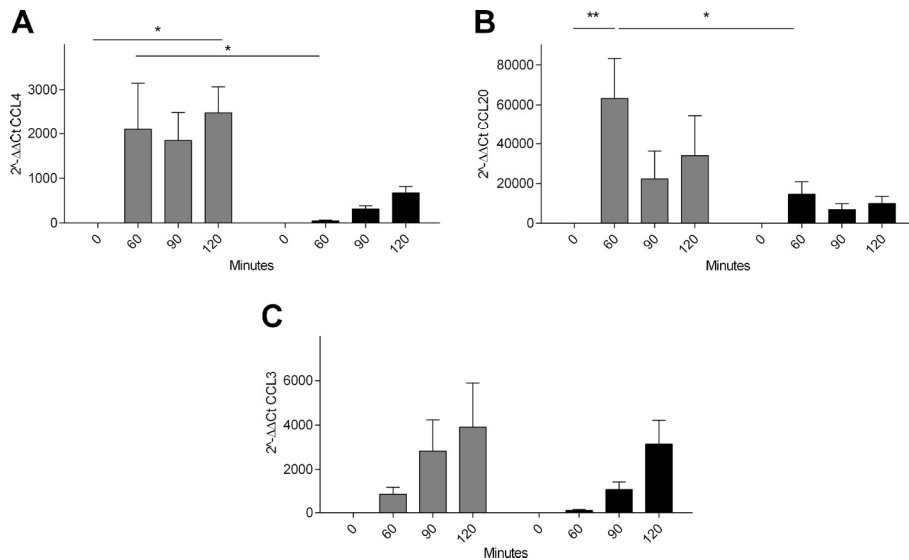


Figure 11. mRNA levels of (A) ccl4, (B) ccl20 and (C) ccl3 in neutrophils stimulated with LPS and TNF α from healthy controls (grey, n = 7) and allergic asthmatics (black, n = 8).

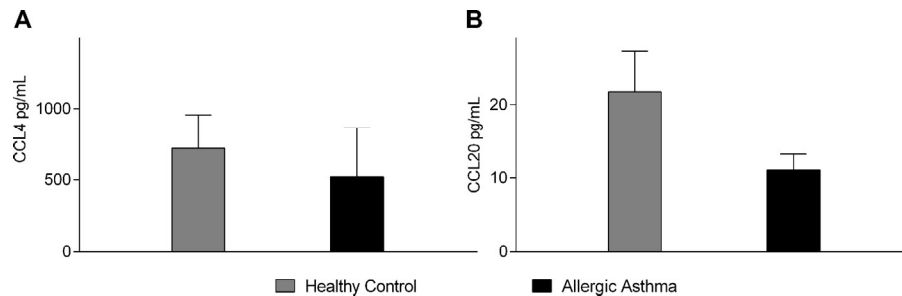


Figure 12. Protein levels for CCL4 (A) and CCL20 (B) from LPS and TNF α stimulated neutrophils from healthy controls (grey, $n = 7$) and allergic asthmatics (black, $n = 8$).

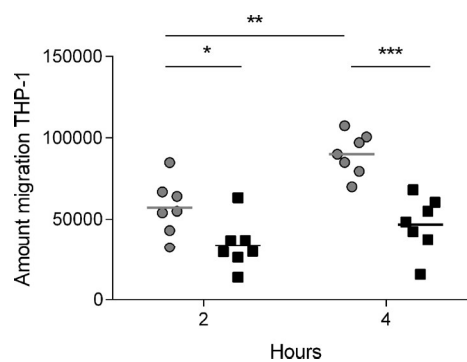


Figure 13. Monocyte migration towards supernatant from stimulated neutrophils from healthy controls (grey, $n = 7$) and allergic asthmatics (black, $n = 8$).

4.2.4 Allergic asthma patients might have an impaired TLR defence against secondary bacterial colonisation

Neutrophils from healthy individuals showed an upregulation of tlr2 and tlr4 at the mRNA level upon stimulation with LPS and TNF α . This upregulation was not seen in neutrophils from allergic asthmatic patients (Figure 14 A, B).

4.2.5 Comments

The need to regulate and remove live cells from inflamed tissue is important, as an imbalance between neutrophil apoptosis and survival among patients with allergic asthma is known to prolong the inflammatory duration^{44, 53}. “Eat me” markers and “don’t eat me” markers on neutrophils from allergic asthma patients demonstrated an expression pattern that may hinder phagoptosis. The levels of CD36 and CD43 were lower and CD47 tended to be higher in asthma than during healthy control conditions.

CD47 increased upon stimulation, an effect that was not seen in neutrophils from healthy controls. CD36 is important for phagocytosis in studies on healthy neutrophils⁵⁴. Increased CD47 expression on neutrophils in non-small-cell lung carcinoma patients has been shown to be associated with a delay in neutrophil apoptosis and with an impaired clearance. This may be one of the underlying mechanisms leading to neutrophilia in these patients⁵⁵. CD47 also inhibits macrophage phagocytosis of ovarian cancer cells, which can be reversed with a CD47 antibody⁵⁶.

High/dim neutrophils, found at the site of the inflammation in allergic rhinitis patients, seemed to be harder to remove from the site of inflammation than the *high/high* neutrophils. In line with our findings, another group has shown that a bacterial infection can promote the development of *high/dim* neutrophils in the blood. This activated subset has then been shown to migrate to the lung⁵⁰. In our BAL samples, we have also seen that this is the dominant subset of neutrophils.

Neutrophils from patients with allergic asthma showed a tendency towards a reduced production of CCL4 and CCL20, which may lead to slower monocyte migration. CC-chemokines are involved in the initiation of macrophage differentiation and it has been demonstrated that rhinovirus infection can impair the innate immune response in alveolar macrophages facilitating additional bacterial infections⁵⁷.

LPS and TNF α stimulation increased the tlr2 and tlr4 expression on neutrophils from the healthy controls. This was not seen on the neutrophils from the allergic asthmatic patients. To decrease the use of antibiotics and improve the quality of life for these patients the mechanisms behind recurrent secondary bacterial infections following viral infections has to be fully understood. tlr2 and tlr4 can be upregulated on neutrophils and other cells upon pro-inflammatory stimulation and has been associated with a defence against secondary bacterial infections or viral antigens⁵⁸. Patients with asthma have an altered regulation of TLRs against bacteria leading to an increased risk of colonisation and infections in the airways⁵⁹. Further studies of “Eat me” and “don’t eat me” markers in allergic asthma, as well as other asthma phenotypes, could generate a better understanding of the disease development.

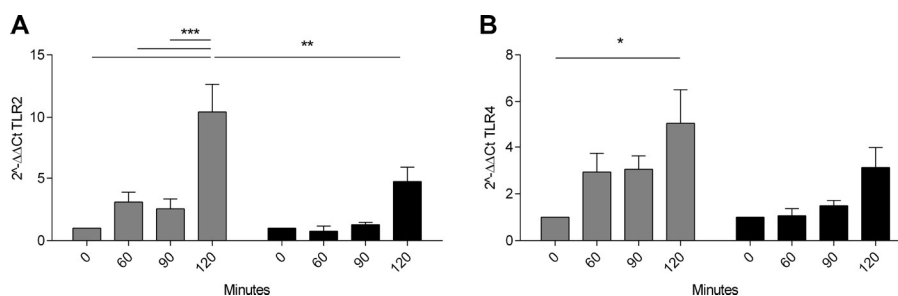


Figure 14. Levels of mRNA for (A) tlr 2 and (B) tlr4 in LPS and TNF α stimulated neutrophils from healthy controls (grey, n = 7) and allergic asthmatics (black, n = 8).

4.3 Activated neutrophils affect the direct and indirect contractile response in acute asthma (paper III, IV and V)

Traditionally, neutrophils have been associated with a chronic asthma phenotype. However, they might also contribute to the development of acute allergic airway inflammation and the airway hyperresponsiveness seen during asthma exacerbations.

4.3.1 Allergens may indirectly activate neutrophils in the blood

Blood was collected before and 24 hours after an inhaled allergen provocation. We observed a shift of the neutrophil subsets in the blood from the *high/high* neutrophils as being the largest fraction before provocation, to a situation where the *high/dim* fraction peaked after provocation (Figure 15).

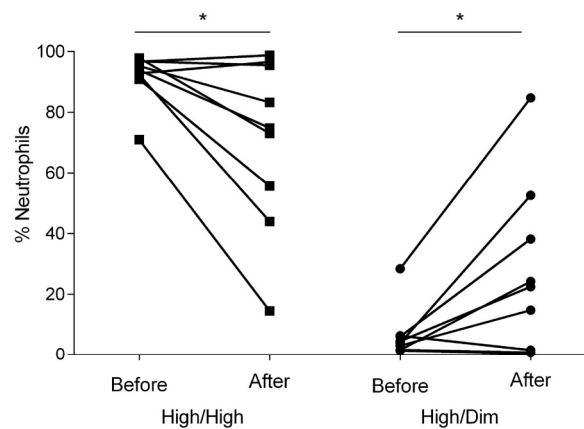


Figure 15. The presence of *high/high* neutrophils (■) and *high/dim* neutrophils (●) in the blood before and after inhaled allergen provocation ($n=9$) of allergic asthmatic patients.

4.3.2 A change in the neutrophil subgroup population of the lung can alter the direct and indirect airway reactivity

High/dim neutrophils were co-cultured with human bronchi or mice tracheae and changes in bradykinin-or nerve-induced hyperreactivity were evaluated. An increased contractile response to bradykinin was noted in both human and mouse airways (Figure 16 A, B) after co-culture with *high/dim* neutrophils. The same co-culture procedure also caused an augmented nerve-mediated hyperreactivity in the mouse trachea (Figure 16 C).

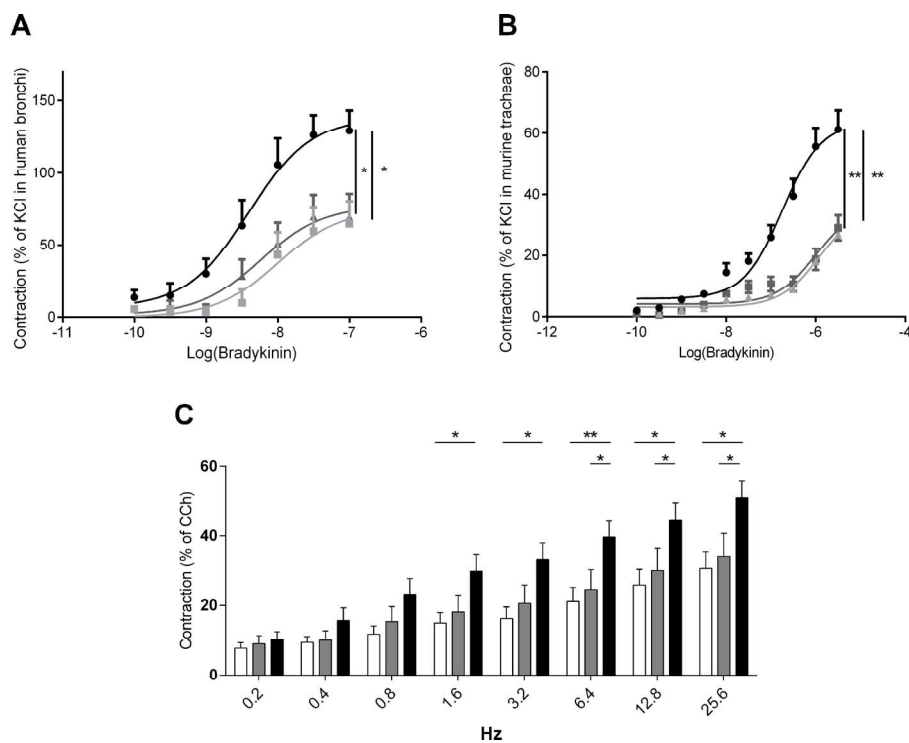


Figure 16. Neutrophil stimulation of the lower airways and airway contraction. (A) Isolated human lower airways segments ($n = 7$) and (B,C) isolated murine lower airway segments ($n = 8-10$) treated with high/high neutrophils (■, grey) or high/dim neutrophils (●, black) or control vehicle (▲, white). The reactivity in the lower airways was evaluated based on the (A, B) bradykinin induced or (C) nerve-mediated smooth muscle contraction.

4.3.3 $\text{TNF}\alpha$ and $\text{IL-1}\beta$ produced by High/dim neutrophils create a foundation for the direct and indirect nerve-mediated airway reactivity

Supernatants from co-cultures from tracheae and different subsets of neutrophils were analysed with ELISA. The results showed that the *high/dim* neutrophils produced more $\text{TNF}\alpha$ and $\text{IL-1}\beta$ than the *high/high* neutrophils (Figure 17 A, B). The use of $\text{TNF}\alpha$ inhibition did not affect the nerve-mediated hyperreactivity but partly inhibited the bradykinin-induced reactivity (Figure 17 C). In comparison, inhibition of $\text{IL-1}\beta$ had the opposite effect, blocking the nerve-mediated hyperreactivity but failing to change the bradykinin response (Figure 17 D). The blocking experiments were all performed on mouse tracheae. Additionally, we found that the $\text{TNF}\alpha$ -induced bradykinin response was the result of an upregulation of the bradykinin 2 receptor (Figure 18 A, B), whereas the $\text{IL-1}\beta$ -induced nerve-mediated hyperreactivity, the result of the NK-1 (Figure 19) and the cyclooxygenase-2 system (COX-2) (Figure 20 A, B).

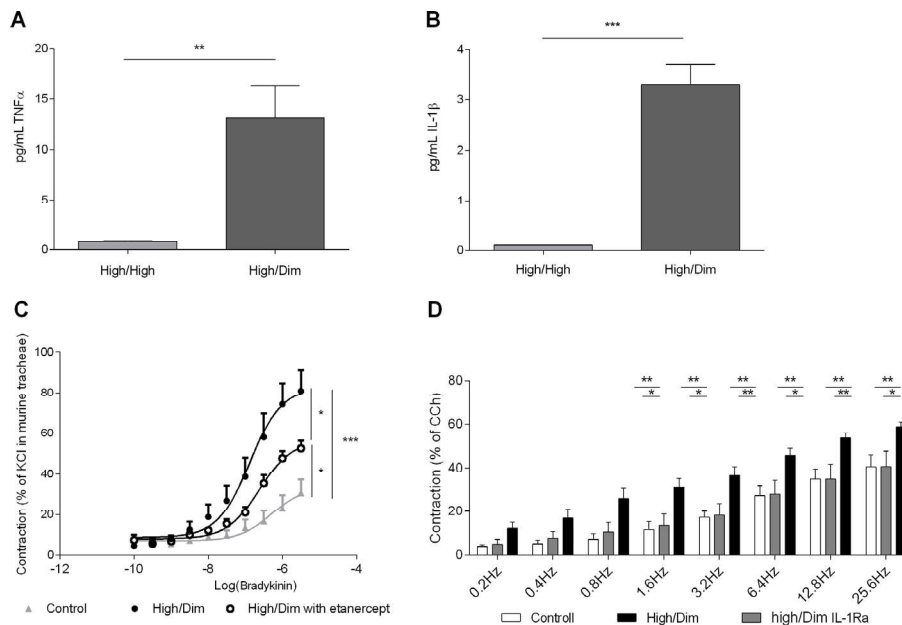


Figure 17. The production of (A) $\text{TNF}\alpha$ ($n = 4$) and (B) $\text{IL-1}\beta$ ($n = 4$) from *high/high* neutrophils and *high/dim* neutrophils from healthy controls. (C) Reactivity towards bradykinin was evaluated in control tracheae (▲, $n = 8$) and tracheae exposed to *high/dim* (●, $n = 8$) as well as *high/dim* exposed tracheae treated with etanercept (○, $n = 8$). (D) The nerve-mediated smooth muscle contraction was evaluated in control tracheae (white, $n = 5$), and in tracheae treated with *high/dim* neutrophils (black, $n = 6$), in together with IL-1Ra (grey, $n = 5$).

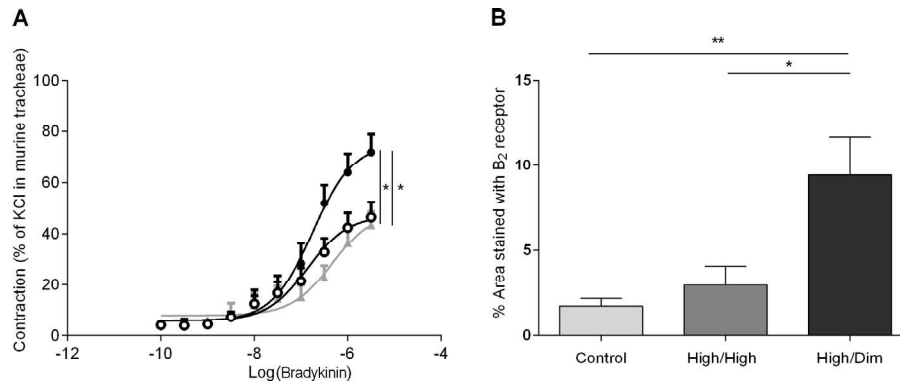


Figure 18. High/dim Neutrophils upregulated the bradykinin receptor 2. (A) Control tracheae (\blacktriangle , $n=8$), and tracheae exposed to high/dim (\bullet , $n=8$) as well as high/dim exposed tracheae treated with Actinomycin D (\circ , $n=8$). (B) Immunostaining of the tracheae ($n=13-15$) for bradykinin B₂ receptor in non-treated tracheal segments and treated with high/dim or high/high neutrophils.

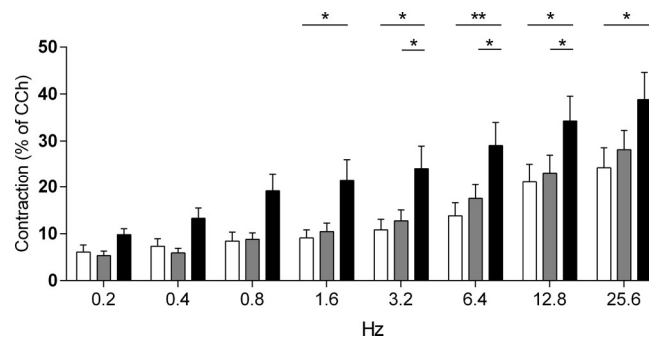


Figure 19. Evaluation of nerve-mediated smooth muscle contractions in mice tracheae after co-culture with (Black, $n=7$) or without (white, $n=7$) high/dim neutrophils in the presence of Aprepitant ($n=7$) (grey).

4.3.4 Comments

We found that the *high/dim* neutrophils increased in blood after an inhaled allergen provocation. It has been shown that the cytokines TNF- α , IL-1 α , G-CSF and IL-6 are increased in plasma after allergen challenges in the lung or after an allergic asthma attack⁶⁰⁻⁶². It is also well established that TNF- α , IL-1 α , G-CSF and IL-6 can activate neutrophils⁶³⁻⁶⁶. Thus, we believe that neutrophils present in the circulation change to an activated subtype. It has been demonstrated that allergen and bacteria can induce a change from one neutrophil subset to another⁴⁸⁻⁵⁰. It has also been shown that the *high/dim* neutrophils are overrepresented during bacterial infections and are able to migrate to the lung.

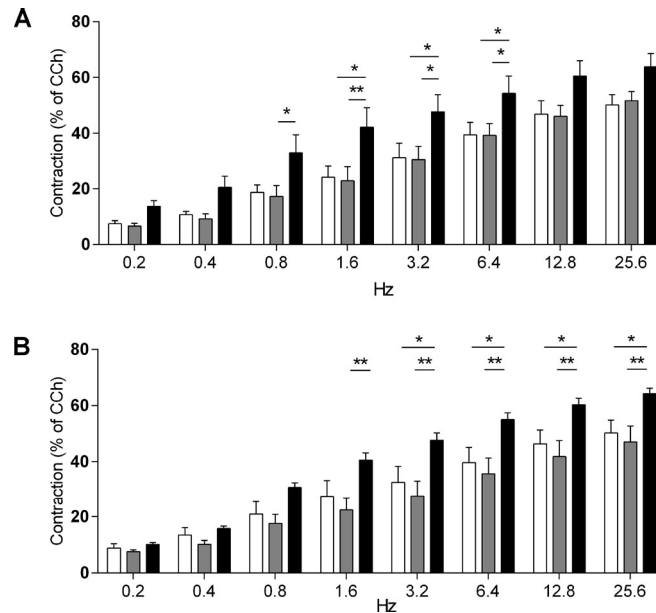


Figure 20. Evaluation of nerve-mediated smooth muscle contractions in mice tracheae after co-culture with (Black, $n = 6$) or without (white, $n = 6$) high/dim neutrophils in the presence of (A) indomethacin (grey, $n = 6$) and (B) Lumiracoxib (grey, $n = 7$).

High/dim neutrophils were found to upregulate the bradykinin response and nerve-mediated smooth muscle contractions. $\text{TNF}\alpha$ and $\text{IL-1}\beta$ were produced by the high/dim neutrophil. Blocking the $\text{IL-1}\beta$ receptor decreased the nerve-mediated hyperactivity, but did not decrease the bradykinin response in the smooth muscle. $\text{IL-1}\beta$ has been shown to increase the nerve-mediated hyperactivity, and this response was due to an upregulation of SP in the nerve caused by COX-2 ^{67, 68}. We could confirm the COX-2 involvement in our study, but could not completely rule out the involvement of COX-1 . The neutrophils itself produced less $\text{TNF}\alpha$ when blocking the $\text{IL-1}\beta$ receptor, which confirms our impression that $\text{IL-1}\beta$ can affect the TNF production⁶⁹. The use of a $\text{TNF}\alpha$ antagonist partly decreased the increased bradykinin response but did not affect the nerve-mediated hyperactivity. The tentative roles of $\text{IL-1}\beta$ and $\text{TNF}\alpha$ suggested by these experiments are well in line with previous reports on the role of these cytokines in the development of hypersensitivity^{67, 68, 70-72}.

We have demonstrated changes occurring within the neutrophils during allergic asthmatic disease and their different effects on asthma symptoms; from the inhaled allergens effect on changes of neutrophil subsets in the blood, to their effect of on the airway reactivity.

5 CONCLUSIONS

- Four neutrophil subsets were characterised in the nose and blood. The proportion of CD16^{high} CD62L^{dim} neutrophils was increased in the nasal mucosa from patients with AR as compared to controls. The CD16^{high} CD62L^{dim} neutrophils had the ability to facilitate eosinophil migration and to lower the threshold for T-cells activation at the site of inflammation. Hence, CD16^{high} CD62L^{dim} neutrophils might play a role in AR pathology.
- Neutrophils found in allergic asthma patients had an insufficient production of CCL4 and CCL20, leading to a reduced migration of monocytes. Neutrophils from allergic asthmatic patients displayed an altered exposure pattern of markers associated with neutrophil clearance, which could lead to impaired phagoptosis of neutrophils. Defects in neutrophilic cell signalling could be an underlying cause for the prolonged duration, increased severity and secondary bacterial infections seen in allergic asthma patients.
- 24 hours after an inhaled allergen provocation in patients with allergic asthma a shift from the CD16^{high} CD62L^{high} to the CD16^{high} CD62L^{dim} neutrophil subset was seen in the blood. This suggests that allergen causes an indirect activation of neutrophils in the blood.
- The CD16^{high} CD62L^{dim} neutrophils appeared to have a direct effect on airway smooth muscle via release of TNF α . Thus, CD16^{high} CD62L^{dim} neutrophils may contribute to the hyperreactivity, one of characteristic condition in asthma.
- The CD16^{high} CD62L^{dim} neutrophils induced hyperresponsiveness of sensory nerve fibres by secreting IL-1 β . This in turn induced a cyclooxygenase-2 dependent NK-1 contraction of the smooth muscle. The altered sensorineural activity may play a role in neurogenic inflammations and airway hyperresponsiveness.

6 GENERAL DISCUSSION

Neutrophils respond quickly to external and internal events like injury, infection, cancer and autoimmunity. They are part of a delicate balance between hypo- and hyperactivation of the innate immune system⁷³. Hence, a tight regulation of their presence and activity is necessary. Functional disturbances can cause infectious complications like sepsis. A hyperactive state may lead to severe pro-inflammatory conditions resulting in i.e. acute respiratory distress syndrome. Investigation of differences in the specific functions of these cells during various stages of acute inflammation, sepsis and trauma, paved the way for dividing these cells into four functionally diverse subsets⁴². These subsets have then been further studied during cancer^{74, 75}, acute inflammation, severe trauma⁴² and airway disease^{76, 77}. The *high/dim* subset increases in allergic rhinitis patients, where it drives the progress of the disease. The same subset can also affect the lung negatively by increasing AHR. On the other hand, an increased fraction of *high/dim* neutrophils has been shown to correlate with an increased survival rate in patients with head and neck squamous cell carcinoma⁷⁴. Thus, the neutrophil behaviour appears to depend on the context in which it appears.

It is believed that the balance of the neutrophil activation state is maintained by the microbiota. The microbiota in our gut and lung, affected by our lifestyle, can change the activation state on our neutrophils through TLRs and Myd88⁷⁸. Delayed gut microbiota development is a risk factor for asthma development in infants⁷⁹. Having the right bacteria present in the gut microbiota is correlated to a more efficient clearance of lung neutrophils at 3 days after infection, which is linked to a decrease in CD47⁸⁰. It would be interesting to evaluate if airway dysbiosis could lead to hyperactivation of neutrophils resulting in an increased presence of *high/dim* neutrophils and an increased expression of CD47 on neutrophils from allergic asthmatic patients. Along the same lines, caesarean delivery is correlated with a higher risk of asthma than vaginal delivery, particularly in children of allergic parents. Caesarean delivery also increases the risk for common allergies in children with non-allergic parents⁸¹. At the same time caesarean delivery is correlated to colonisation of opportunistic pathogens associated with the hospital environment, such as enterococcus, enterobacter and klebsiella species⁸². Evaluation of the effects of these pathogens on neutrophil activation could be of importance for understanding the disease development. In patients with allergic asthma the TLR2 and TLR4 regulations in the neutrophils seem to be dysfunctional. It is tempting to speculate that this malfunction interferes with the microbiota-derived signals in an attempt to decrease activation and reduce the presence of *high/dim* neutrophils. It could be a well-regulated TLR 2 and 4 that is responsible for the balance between subsets seen in nasal biopsies obtained from healthy individuals (paper I).

The presented thesis demonstrates that different subsets of neutrophils have diverse effects on T-cells, eosinophils and airway smooth muscle. As the neutrophil subsets seem to have different roles in their interaction with the innate immune system, future therapeutic manipulation of the subsets could tentatively be used to decrease the duration of infections. This could be achieved by promoting the *high/dim* neutrophils during a hypoinflammatory conditions or perhaps more relevant inhibiting *high/dim* neutrophils during hyperinflammatory conditions. Mitrephylline (MTP) has been suggested to reduce LPS dependent activation of neutrophils. The presence of *high/dim* neutrophils in the blood diminished after MTP administration and levels of TNF α , IL-6 and IL-8 were reduced. Reducing the presence of the *high/dim* neutrophils together with target therapies against CD47 should be the therapeutic goal. Further, the use of antibodies that could block the phagocytes to interact with CD47 on target cells may be a first step to decrease the duration of inflammation and infection in asthmatic patients.

TNF α , IL-1 β or NK-1 are all involved in the increased reactivity in the airways caused by the *high/dim* subset. Targeting TNF α , IL-1 β or NK-1 is of increasing interest in ongoing attempts to find new ways of treating allergy and asthma. Inhibition of TNF α has been shown to be effective in a relatively small subgroup of patients with severe asthma⁸³. IL-1b has the potential to be a new therapeutic target, as it is highly associated with many of the asthma phenotypes and IL-1 receptor type I-deficient mice show a suppressed AHR. There is a phase I trial with an IL-1R antagonist (originally developed for treatment of rheumatoid arthritis), in healthy volunteers without asthma.⁸⁴ There are studies on safety and tolerability for an anti-IL-1 β monoclonal antibody in mild asthmatics and in patients with a late asthmatic response (LAR). IL-1 β levels did decrease by >90% for a 14-week period and anti-IL-1 β appeared to diminish LAR as compared to pre-treatment values⁸⁵. NK-1 inhibitors may decrease airway responsiveness and improve lung function in patients with asthma; however, available evidence is limited⁸⁶.

The functions of “eat me” and “don’t eat me” markers are not restricted to neutrophils. CD47 is a major “don’t eat me” marker in the innate immune system and it is co-expressed with a programmed death-ligand 1 (PD-L1), a critical “don’t find me” signal of the adaptive immune system^{87, 88}. CD47 is overexpressed in cancer cells⁸⁹. Hence, targeting CD47 can activate innate immunity, promoting cancer cell destruction by macrophages⁹⁰. Interestingly, blockage of both PD-L1 and CD47 enhances immunotherapy against circulating tumour cells⁹¹. It would be interesting to study the metastatic cells in tumour-draining lymph nodes and crosscheck CD47 in functional setups with PD-L1.

The diversity of neutrophils is the main focus in all papers in this thesis, either via analysis of subsets characterised by CD16 and CD62L or in the light of “eat me” and “don’t eat me” markers. Four out of five papers describe how these subsets can induce functional changes in lungs with ongoing inflammation. It is obvious that the induced effects could both be beneficial and damaging for the patient depending on the situation so unspecific removal or incapacitation of these cells might be dangerous. However, a better understanding of the role of neutrophil subgroups in allergy and asthma might open new avenues for specific therapeutic targeting.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Neutrofila celler är en av del av vårt medfödda immunförsvar. De tillhör de celler som först anländer till en infektion eller inflammation och har förmågan att där känna igen virus och bakterier. Synen på neutrofilens roll i den inflammatoriska processen har genom åren utvecklats till att bli allt mer specifik och sjukdomsriktad. Ett led i detta har varit upptäckten av att neutrofila celler kan indelas i olika subgrupper, var och en med sin specifika funktion. Idag erkänner vi fyra subgrupper baserat på den neutrofila cellens uttryck av CD16 och CD62L på cellytan.

Det övergripande syftet med denna avhandling var att kategorisera förekomst och funktion hos dessa subgrupper. Hos friska försökspersoner och hos patienter med allergisk rinit och allergisk astma har vi även studerat likheter och skillnader hos neutrofila celler med avseende på förmodad inflammatorisk aktivitet.

Förekomsten och fördelningen av neutrofila subgrupper i blod, näsbiopsier och nässköljvätska har beskrivits hos friska försökspersoner och patienter med allergisk luftvägssjukdom. Näsbiopsier från friska individer karakteriserades av en jämn fördelning mellan aktiverade och oaktiverade subgrupper, medan näsbiopsier från patienter med allergisk rinit dominerades av en aktiverad subgrupp. För att utröna hur denna ökning av aktiverade celler kan tänkas påverka det inflammatoriska förloppet studerades hur olika neutrofila subgrupper interagerade med andra celler i immunförsvaret såsom T-celler och eosinofiler. Vi fann att den aktiverade subgruppen kan förbereda T-cellen för aktivering och samtidigt locka till sig eosinofiler. Detta ger den aktiverade neutrofila cellen en specifik och väl definierad roll i sjukdomsförloppet.

Under normala förhållanden bidrar vårt immunförsvar till att neutrofiler avlägsnas från inflammationsområdet när inträngande mikrober väl undanröjts. Så kallade "ät mig/ät mig inte" markörer på neutrofila cellers yta tros vara av betydelse för denna del av inflammationsupplösningen. Störningar i detta skeende kan leda till ett utdraget sjukdomsförlopp och utveckling av kronisk inflammation. Utryck och aktivitet av "ät mig inte" markörer på neutrofila celler isolerade från blod studerades. Vi fann att såväl ostimulerade som stimulerade neutrofila celler från allergiska astmatiska patienter hade fler "ät mig inte" markörer på sin cellyta än neutrofila celler från friska försökspersoner. De stimulerade neutrofila cellerna från allergiska astmatiska patienter hade också en otillräcklig produktion av vissa specifika mediatorer, vilket minskade kroppens möjlighet att få monocytter med "städfunktion" till platsen för den tilltänkta inflammationsupplösningen. Tillsammans kan dessa fynd bidra till att förklara den förlängda sjukdomsbild som ofta ses vid luftvägsinfektion hos patienter med allergisk astma.

Sedan studerades hur neutrofila subgrupper i blodet ändrades när patienter med allergisk astma fick inhalera ett allergen som utlöste en allergisk luftvägsreaktion. Vi kunde se att den inaktiverade subgruppen minskade samtidigt som den aktiverade gruppen ökade efter inhalationen. Avslutningsvis odlades isolerade luftvägar tagna från möss och människor tillsammans med de olika neutrofila subgrupperna. Vi fann att de luftvägar som samodlats med aktiverade neutrofila celler fick en ökad känslighet för såväl kontraherande ämnen som nervstimulering. Motsvarande hyperaktivitet sågs inte i luftvägarna när oaktiverade neutrofila subgrupper använts. Ökningen var relaterad till en frisättning av två cytokiner, TNF α och IL-1 β , från den aktiverade neutrofila cellen.

Sammantaget kan sägas att avhandlingens resultat kastar nytt ljus över neutrofila cellers roll vid allergisk luftvägssjukdom och att den öppnar vägar för potentiellt nya behandlingsformer vid dessa tillstånd.

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9 REFERENCES

1. Kindt TJ, Goldsby RA, Osborne BA, Kuby J. Kuby immunology. New York: W.H. Freeman; 2007.
2. Clark R, Kupper T. Old meets new: the interaction between innate and adaptive immunity. *J Invest Dermatol* 2005; 125:629-37.
3. Ciprandi G, Caimmi D, Miraglia Del Giudice M, La Rosa M, Salpietro C, Marseglia GL. Recent developments in United airways disease. *Allergy Asthma Immunol Res* 2012; 4:171-7.
4. Caimmi D, Marseglia A, Pieri G, Benzo S, Bosa L, Caimmi S. Nose and lungs: one way, one disease. *Ital J Pediatr* 2012; 38:60.
5. Passalacqua G, Ciprandi G, Canonica GW. The nose-lung interaction in allergic rhinitis and asthma: united airways disease. *Curr Opin Allergy Clin Immunol* 2001; 1:7-13.
6. Hellgren J, Cervin A, Nordling S, Bergman A, Cardell LO. Allergic rhinitis and the common cold--high cost to society. *Allergy* 2010; 65:776-83.
7. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature* 2008; 454:445-54.
8. Maggi E, Vultaggio A, Matucci A. T-cell responses during allergen-specific immunotherapy. *Curr Opin Allergy Clin Immunol* 2012; 12:1-6.
9. Bloemen K, Verstraelen S, Van Den Heuvel R, Witters H, Nelissen I, Schoeters G. The allergic cascade: Review of the most important molecules in the asthmatic lung. *Immunology Letters* 2007; 113:6-18.
10. Monteseirin J. Neutrophils and asthma. *J Investig Allergol Clin Immunol* 2009; 19:340-54.
11. ginasthma. 03/12-12.] Available from <http://www.ginasthma.org/uploads/users/files/AsthmaBkgrdr2012.pdf>.
12. Holgate ST. Pathogenesis of asthma. *Clin Exp Allergy* 2008; 38:872-97.
13. Upham JW, Denburg JA, O'Byrne PM. Rapid response of circulating myeloid dendritic cells to inhaled allergen in asthmatic subjects. *Clin Exp Allergy* 2002; 32:818-23.
14. Asman B, Strand V, Bylin G, Bergstrom K. Peripheral neutrophils after allergic asthmatic reactions. *Int J Clin Lab Res* 1997; 27:185-8.

15. Tien Nguyen L, Lim S, Oates T, Chung KF. Increase in airway neutrophils after oral but not inhaled corticosteroid therapy in mild asthma. *Respiratory Medicine* 2005; 99:200-7.
16. Walford HH, Doherty TA. Diagnosis and management of eosinophilic asthma: a US perspective. *Journal of Asthma and Allergy* 2014; 7:53-65.
17. Murray CS, Poletti G, Keadze T, Morris J, Woodcock A, Johnston SL, et al. Study of modifiable risk factors for asthma exacerbations: virus infection and allergen exposure increase the risk of asthma hospital admissions in children. *Thorax* 2006; 61:376-82.
18. Kurai D, Saraya T, Ishii H, Takizawa H. Virus-induced exacerbations in asthma and COPD. *Front Microbiol* 2013; 4:293.
19. Papadopoulos NG, Christodoulou I, Rohde G, Agache I, Almquist C, Bruno A, et al. Viruses and bacteria in acute asthma exacerbations--a GA(2) LEN-DARE systematic review. *Allergy* 2011; 66:458-68.
20. Jackson DJ, Johnston SL. The role of viruses in acute exacerbations of asthma. *Journal of Allergy and Clinical Immunology* 2010; 125:1178-87.
21. Gillissen A, Paparoupa M. Inflammation and infections in asthma. *Clin Respir J* 2015; 9:257-69.
22. James KM, Peebles RS, Jr., Hartert TV. Response to infections in patients with asthma and atopic disease: an epiphenomenon or reflection of host susceptibility? *The Journal of allergy and clinical immunology* 2012; 130:343-51.
23. Gill MA, Bajwa G, George TA, Dong CC, Dougherty, II, Jiang N, et al. Counterregulation between the FcεpsilonRI pathway and antiviral responses in human plasmacytoid dendritic cells. *J Immunol* 2010; 184:5999-6006.
24. van Elden LJ, Sachs AP, van Loon AM, Haarman M, van de Vijver DA, Kimman TG, et al. Enhanced severity of virus associated lower respiratory tract disease in asthma patients may not be associated with delayed viral clearance and increased viral load in the upper respiratory tract. *J Clin Virol* 2008; 41:116-21.
25. Prame Kumar K, Nicholls AJ, Wong CHY. Partners in crime: neutrophils and monocytes/macrophages in inflammation and disease. *Cell and tissue research* 2018; 371:551-65.
26. Marwick JA, Mills R, Kay O, Michail K, Stephen J, Rossi AG, et al. Neutrophils induce macrophage anti-inflammatory reprogramming by suppressing NF-κB activation. *Cell Death & Disease* 2018; 9:665.

27. Meurs H, Gosens R, Zaagsma J. Airway hyperresponsiveness in asthma: lessons from in vitro model systems and animal models. *Eur Respir J* 2008; 32:487-502.
28. Amrani Y, Panettieri RA. Airway smooth muscle: contraction and beyond. *Int J Biochem Cell Biol* 2003; 35:272-6.
29. Busse WW. The relationship of airway hyperresponsiveness and airway inflammation: Airway hyperresponsiveness in asthma: its measurement and clinical significance. *Chest* 2010; 138:4s-10s.
30. Black JL, Johnson PR. Factors controlling smooth muscle proliferation and airway remodelling. *Curr Opin Allergy Clin Immunol* 2002; 2:47-51.
31. Berman AR, Togias AG, Skloot G, Proud D. Allergen-induced hyperresponsiveness to bradykinin is more pronounced than that to methacholine. *J Appl Physiol* 1985; 78:1844-52.
32. Lee LY, Yu J. Sensory nerves in lung and airways. *Compr Physiol* 2014; 4:287-324.
33. Joos GF, Germonpre PR, Pauwels RA. Neural mechanisms in asthma. *Clin Exp Allergy* 2000; 30 Suppl 1:60-5.
34. Dakhama A, Park JW, Taube C, El Gazzar M, Kodama T, Miyahara N, et al. Alteration of airway neuropeptide expression and development of airway hyperresponsiveness following respiratory syncytial virus infection. *Am J Physiol Lung Cell Mol Physiol* 2005; 288:L761-70.
35. King KA, Hu C, Rodriguez MM, Romaguera R, Jiang X, Piedimonte G. Exaggerated Neurogenic Inflammation and Substance P Receptor Upregulation in RSV-Infected Weanling Rats. *American Journal of Respiratory Cell and Molecular Biology* 2001; 24:101-7.
36. PIEDIMONTE G. Neural Mechanisms of Respiratory Syncytial Virus-induced Inflammation and Prevention of Respiratory Syncytial Virus Sequelae. *American Journal of Respiratory and Critical Care Medicine* 2001; 163:S18-S21.
37. Tomaki M, Ichinose M, Miura M, Hirayama Y, Yamauchi H, Nakajima N, et al. Elevated substance P content in induced sputum from patients with asthma and patients with chronic bronchitis. *Am J Respir Crit Care Med* 1995; 151:613-7.
38. Nieber K, Baumgarten CR, Rathsack R, Furkert J, Oehme P, Kunkel G. Substance P and beta-endorphin-like immunoreactivity in lavage fluids of subjects with and without allergic asthma. *J Allergy Clin Immunol* 1992; 90:646-52.

39. Van Hove CL, Maes T, Joos GF, Tournoy KG. Chronic inflammation in asthma: a contest of persistence vs resolution. *Allergy* 2008; 63:1095-109.
40. Bratton DL, Henson PM. Neutrophil clearance: when the party is over, clean-up begins. *Trends Immunol* 2011; 32:350-7.
41. Zhang H, Kong H, Zeng X, Guo L, Sun X, He S. Subsets of regulatory T cells and their roles in allergy. *J Transl Med* 2014; 12:125.
42. Pillay J, Kamp VM, van Hoffen E, Visser T, Tak T, Lammers JW, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest* 2012; 122:327-36.
43. Kolaczowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* 2013; 13:159-75.
44. Saffar AS, Alphonse MP, Shan L, Hayglass KT, Simons FE, Gounni AS. IgE modulates neutrophil survival in asthma: role of mitochondrial pathway. *J Immunol* 2007; 178:2535-41.
45. Brown GC, Neher JJ. Eaten alive! Cell death by primary phagocytosis: 'phagoptosis'. *Trends Biochem Sci* 2012; 37:325-32.
46. Brown GC, Vilalta A, Fricker M. Phagoptosis - Cell Death By Phagocytosis - Plays Central Roles in Physiology, Host Defense and Pathology. *Curr Mol Med* 2015; 15:842-51.
47. Li W. Eat-me signals: keys to molecular phagocyte biology and "appetite" control. *J Cell Physiol* 2012; 227:1291-7.
48. Rosales C. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? *Frontiers in physiology* 2018; 9:113-.
49. Peñaloza HF, Salazar-Echegarai FJ, Bueno SM. Interleukin 10 modulation of neutrophil subsets infiltrating lungs during *Streptococcus pneumoniae* infection. *Biochemistry and biophysics reports* 2017; 13:12-6.
50. Cortjens B, Ingelse SA, Calis JC, Vlaar AP, Koenderman L, Bem RA, et al. Neutrophil subset responses in infants with severe viral respiratory infection. *Clinical Immunology* 2017; 176:100-6.
51. Hampton HR, Bailey J, Tomura M, Brink R, Chtanova T. Microbe-dependent lymphatic migration of neutrophils modulates lymphocyte proliferation in lymph nodes. *Nature Communications* 2015; 6:7139.
52. Passalacqua G, Albano M, Riccio A, Fregonese L, Puccinelli P, Parmiani S, et al. Clinical and immunologic effects of a rush sublingual immunotherapy to *Parietaria* species: A double-blind, placebo-controlled trial. *J Allergy Clin Immunol* 1999; 104:964-8.

53. Corne JM, Marshall C, Smith S, Schreiber J, Sanderson G, Holgate ST, et al. Frequency, severity, and duration of rhinovirus infections in asthmatic and non-asthmatic individuals: a longitudinal cohort study. *Lancet* 2002; 359:831-4.
54. Fadok VA, Warner ML, Bratton DL, Henson PM. CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor (alpha v beta 3). *J Immunol* 1998; 161:6250-7.
55. Barrera L, Montes-Servin E, Hernandez-Martinez JM, Garcia-Vicente MLA, Montes-Servin E, Herrera-Martinez M, et al. CD47 overexpression is associated with decreased neutrophil apoptosis/phagocytosis and poor prognosis in non-small-cell lung cancer patients. *Br J Cancer* 2017; 117:385-97.
56. Liu R, Wei H, Gao P, Yu H, Wang K, Fu Z, et al. CD47 promotes ovarian cancer progression by inhibiting macrophage phagocytosis. *Oncotarget* 2017; 8:39021-32.
57. Oliver BG, Lim S, Wark P, Laza-Stanca V, King N, Black JL, et al. Rhinovirus exposure impairs immune responses to bacterial products in human alveolar macrophages. *Thorax* 2008; 63:519-25.
58. Larsson O, Tengroth L, Xu Y, Uddman R, Kumlien Georén S, Cardell L-O. Substance P represents a novel first-line defense mechanism in the nose. *Journal of Allergy and Clinical Immunology* 2018; 141:128-36.e3.
59. Habibzay M, Saldana JI, Goulding J, Lloyd CM, Hussell T. Altered regulation of Toll-like receptor responses impairs antibacterial immunity in the allergic lung. *Mucosal Immunol* 2012; 5:524-34.
60. Yokoyama A, Kohno N, Fujino S, Hamada H, Inoue Y, Fujioka S, et al. Circulating interleukin-6 levels in patients with bronchial asthma. *Am J Respir Crit Care Med* 1995; 151:1354-8.
61. Kobayashi T, Hashimoto S, Imai K, Amemiya E, Yamaguchi M, Yachi A, et al. Elevation of serum soluble intercellular adhesion molecule-1 (sICAM-1) and sE-selectin levels in bronchial asthma. *Clin Exp Immunol* 1994; 96:110-5.
62. Pelikan Z. Delayed Type of Asthmatic Response to Allergen Challenge and Cytokines in the Peripheral Blood. *Respiration* 2012; 84:385-95.
63. Ferrante A. Activation of neutrophils by interleukins-1 and -2 and tumor necrosis factors. *Immunol Ser* 1992; 57:417-36.
64. Moore FD, Jr., Socher SH, Davis C. Tumor necrosis factor and endotoxin can cause neutrophil activation through separate pathways. *Arch Surg* 1991; 126:70-3.

65. Wright HL, Cross AL, Edwards SW, Moots RJ. Effects of IL-6 and IL-6 blockade on neutrophil function in vitro and in vivo. *Rheumatology* 2014; 53:1321-31.
66. Yan B, Wei JJ, Yuan Y, Sun R, Li D, Luo J, et al. IL-6 cooperates with G-CSF to induce protumor function of neutrophils in bone marrow by enhancing STAT3 activation. *J Immunol* 2013; 190:5882-93.
67. Barchasz E, Naline E, Molimard M, Moreau J, Georges O, Emonds-Alt X, et al. Interleukin-1beta-induced hyperresponsiveness to [Sar9, Met(O2)11] substance P in isolated human bronchi. *Eur J Pharmacol* 1999; 379:87-95.
68. Neeb L, Hellen P, Boehnke C, Hoffmann J, Schuh-Hofer S, Dirnagl U, et al. IL-1beta stimulates COX-2 dependent PGE(2) synthesis and CGRP release in rat trigeminal ganglia cells. *PLoS One* 2011; 6:e17360.
69. Bethea JR, Gillespie GY, Benveniste EN. Interleukin-1 beta induction of TNF-alpha gene expression: involvement of protein kinase C. *J Cell Physiol* 1992; 152:264-73.
70. Zhang Y, Adner M, Cardell L-O. IL-1 β -Induced Transcriptional Up-Regulation of Bradykinin B1 and B2 Receptors in Murine Airways. *American Journal of Respiratory Cell and Molecular Biology* 2007; 36:697-705.
71. Zhang Y, Adner M, Cardell LO. Up-regulation of bradykinin receptors in a murine in-vitro model of chronic airway inflammation. *Eur J Pharmacol* 2004; 489:117-26.
72. Cardell LO, Uddman R, Zhang Y, Adner M. Interleukin-1beta up-regulates tumor necrosis factor receptors in the mouse airways. *Pulm Pharmacol Ther* 2008; 21:675-81.
73. Tak T, Wijten P, Heeres M, Pickkers P, Scholten A, Heck AJR, et al. Human CD62L^{sup}<sup>dim</sup> neutrophils identified as a separate subset by proteome profiling and in vivo pulse-chase labeling. *Blood* 2017; 129:3476.
74. Millrud CR, Kagedal A, Kumlien Georen S, Winqvist O, Uddman R, Razavi R, et al. NET-producing CD16^{high} CD62L^{dim} neutrophils migrate to tumor sites and predict improved survival in patients with HNSCC. *Int J Cancer* 2017; 140:2557-67.
75. Podaza E, Risnik D, Colado A, Elias E, Almejun MB, Fernandez Grecco H, et al. Chronic lymphocytic leukemia cells increase neutrophils survival and promote their differentiation into CD16^(high) CD62L^(dim) immunosuppressive subset. *Int J Cancer* 2019; 144:1128-34.

76. Ekstedt S, Safholm J, Georen SK, Cardell LO. Dividing neutrophils in subsets reveals a significant role for activated neutrophils in the development of airway hyperreactivity. *Clin Exp Allergy* 2019; 49:285-91.
77. Arebro J, Ekstedt S, Hjalmarsson E, Winqvist O, Kumlien Georen S, Cardell LO. A possible role for neutrophils in allergic rhinitis revealed after cellular subclassification. *Sci Rep* 2017; 7:43568.
78. Zhang D, Chen G, Manwani D, Mortha A, Xu C, Faith JJ, et al. Neutrophil ageing is regulated by the microbiome. *Nature* 2015; 525:528-32.
79. Durack J, Kimes NE, Lin DL, Rauch M, McKean M, McCauley K, et al. Delayed gut microbiota development in high-risk for asthma infants is temporarily modifiable by *Lactobacillus* supplementation. *Nature Communications* 2018; 9:707.
80. Felix KM, Jaimez I, Nguyen T-V, Ma H, Raslan W, Klinger C, et al. Gut microbiota enhances neutrophil resolution in immunocompromised hosts to improve response to pneumococcal pneumonia. *The Journal of Immunology* 2018; 200:173.10.
81. Roduit C, Scholtens S, de Jongste JC, Wijga AH, Gerritsen J, Postma DS, et al. Asthma at 8 years of age in children born by caesarean section. *Thorax* 2009; 64:107.
82. Shao Y, Forster SC, Tsaliki E, Vervier K, Strang A, Simpson N, et al. Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. *Nature* 2019; 574:117-21.
83. Matera MG, Calzetta L, Cazzola M. TNF- α inhibitors in asthma and COPD: We must not throw the baby out with the bath water. *Pulmonary Pharmacology & Therapeutics* 2010; 23:121-8.
84. Gibeon D, Menzies-Gow AN. Targeting interleukins to treat severe asthma. *Expert Rev Respir Med* 2012; 6:423-39.
85. Dhimolea E. Canakinumab. *mAbs* 2010; 2:3-13.
86. Ramalho R, Soares R, Couto N, Moreira A. Tachykinin receptors antagonism for asthma: a systematic review. *BMC pulmonary medicine* 2011; 11:41-.
87. Yang Z, Xu J, Li R, Gao Y, He J. PD-L1 and CD47 co-expression in pulmonary sarcomatoid carcinoma: a predictor of poor prognosis and potential targets of future combined immunotherapy. *Journal of Cancer Research and Clinical Oncology* 2019.

88. Liu B, Guo H, Xu J, Qin T, Guo Q, Gu N, et al. Elimination of tumor by CD47/PD-L1 dual-targeting fusion protein that engages innate and adaptive immune responses. *MAbs* 2018; 10:315-24.
89. Wu L, Yu GT, Deng WW, Mao L, Yang LL, Ma SR, et al. Anti-CD47 treatment enhances anti-tumor T-cell immunity and improves immunosuppressive environment in head and neck squamous cell carcinoma. *Oncoimmunology* 2018; 7:e1397248.
90. Folkes AS, Feng M, Zain JM, Abdulla F, Rosen ST, Querfeld C. Targeting CD47 as a cancer therapeutic strategy: the cutaneous T-cell lymphoma experience. *Curr Opin Oncol* 2018; 30:332-7.
91. Lian S, Xie R, Ye Y, Lu Y, Cheng Y, Xie X, et al. Dual blockage of both PD-L1 and CD47 enhances immunotherapy against circulating tumor cells. *Scientific Reports* 2019; 9:4532.