Endotoxin-induced Inflammation in Healthy Human Airways

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Gothenburg, Sweden, 2016

Cover illustration by Sara Tengvall.: Immunofluorescence of macrophages and IL-26 in paper III. Red represents CD68 and green IL-26.

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ISBN 978-91-628-9718-5 (print)
ISBN 978-91-628-9719-2 (PDF)

Printed in Gothenburg, Sweden 2016 by Ineko

To my family

Abstract

The aim of this thesis was to investigate the innate immune response in healthy human airways *in vivo* after simulation of a Gram-negative infection.

Intrabronchial exposure to the TLR4 agonist endotoxin was used as a model for the innate mechanisms in the immune response that are caused by cigarette smoke and by natural infection with Gram-negative bacteria. Endotoxin is part of the outer cell wall of these bacteria and is one of many components of cigarette smoke. Healthy volunteers were exposed to endotoxin and phosphate buffered saline in contralateral lung segments during bronchoscopy. Bilateral bronchoalveolar lavages (BAL) were then performed at different time points thereafter. Inflammatory cells and soluble mediators involved in the inflammatory response were analyzed in BAL samples.

The exposure of healthy airways to endotoxin led to a prompt increase in proinflammatory mediators as well as to an influx of inflammatory cells, a process that receded within days. In the first study, the proteolytic homeostasis of the healthy human lung was evaluated, where endotoxin induced a net activity of serine proteases, but not of gelatinases. In the second study, an endotoxin-induced increase of the neutrophil recruiting cytokine IL-17 and the presence and endotoxin-induced increase of IL-17-producing memory T-helper cells of a unique phenotype were shown. In the third study, the presence and endotoxin-induced increase of another cytokine, IL-26, was demonstrated. IL-26 was revealed to be expressed by macrophages and to exert chemotaxis on neutrophils. The fourth study analyzed effects of endotoxin on antimicrobial peptides (AMPs), possible candidates for options for new treatment of infectious diseases. Endotoxin did increase the levels of LL-37, but not those of Calprotectin.

In conclusion, the delicate balance of tissue degrading enzymes and their inhibitors is disrupted by a transient stimulus, resembling the initial phase of an inflammation. It is open to speculation as to whether repeated or continuous stimuli of this kind may contribute to the imbalance in proteolytic homeostasis that is a common denominator for chronic inflammatory lung diseases. It can also be concluded that interleukins that are integrated with the innate immunity are involved in the response to endotoxin in healthy human lungs. The findings on interleukins and AMPs may be used to target new drugs for inflammatory diseases and infections.

Keywords: LPS, bronchoalveolar lavage, neutrophils, human airways, innate immunity

Endotoxinorsakad inflammation i mänskliga luftvägar – på svenska

Lipopolysackarid (LPS), alltså en förening mellan fett (lipo) och flera (poly) sockerenheter (sackarider), är synonymt med endotoxin och är en beståndsdel av vissa bakteriers ytterhölje. Dessa bakterier, de gram-negativa, är många gånger orsaken till att patienter med vissa inflammatoriska sjukdomar i lungorna drabbas av försämringsepisoder (exacerbationer). Om endotoxinet kommer ut i blodbanan i samband med en infektion kan själva endotoxinet orsaka chock. Endotoxin finns också i cigarettrök. Sannolikt fanns endotoxin redan på tobaksbladen när de plockades. Då endotoxin är så starkt inflammationsgenererande (pro-inflammatoriskt) är endotoxin utmärkt att utnyttja till att på konstgjord väg efterlikna en inflammation, vilket är det som gjorts inom ramen för denna avhandling. Syftet var att kartlägga inflammationsprocesser i luftvägar på frivilliga försökspersoner (friska), en kartläggning som tidigare i huvudsak var gjord på patienter (sjuka), samt på djur. Planeringen var att senare göra samma sorts studier på patienter och jämföra resultaten.

Sammanlagt utsattes 34 försökspersoner för stimulering med renat koksalt i ena lungan och endotoxin i andra. Detta gjordes via bronkoskop, dvs ett 5-6 mm tunt böjligt fiberinstrument med optik. Bronkoskopet fördes ner i luftrören, en undersökning som tar mindre tid än en kvart att genomföra. Vid olika tidpunkter därefter, antingen 12, 24 eller 48 timmar, gjordes en ny bronkoskopi med koksaltsköljning i båda lungorna. I sköljvätskan suger man upp både celler och äggviteämnen (proteiner) från luftvägarnas periferi. På så sätt kan man se vad som hänt ute i de finaste luftrören när man utsatt dessa för koksalt eller endotoxin. Eftersom vi har två lungor och respektive lunga utsattes för antingen bara koksalt eller endotoxin så kunde vi jämföra resultaten mellan sidorna och på så sätt få försökspersonerna att bli sina egna referenser, d.v.s. de blev både kontrollperson och försöksperson samtidigt.

Sköljmaterialet analyserades nogsamt och digert, vilket resulterade i flera publikationer som alla avhandlade det medfödda (innate/naturliga) värdförsvaret i lungorna. Denna del av vårt immunförsvar är s a s portvakten till vår kropp och första linjens försvar, till skillnad från vårt förvärvade (adaptiva) värdförsvar, vilket bygger på att kroppen bildar minnesceller, när vi utsätts för olika skadliga ämnen. När vi nästa gång utsätts för dessa skadliga ämnen triggar det förvärvade värdförsvaret igång ett komplicerat försvar. Det medfödda försvaret

är på intet sätt okomplicerat. Både vita blodkroppar, fr a av två typer, neutrofiler och makrofager (=storätare), och ett flertal små proteiner som frisätts från dessa celler bidrar till detta första immunförsvar. Proteiner som bildas av och signalerar till immunförsvarets celler kallas cytokiner, varav en speciell undergrupp är interleukinerna, förkortas IL (inter=mellan & leukin=vit blodkropp). Av dessa analyserades bl a IL-17 och IL-26.

För att hålla oss friska är det viktigt att immunförsvaret är i balans. När vi rubbar den balansen, i detta fall genom exponering för endotoxin i luftvägarna, ser man en snabb och övergående aktivering av det medfödda värdförsvaret, med inströmning av massor av neutrofiler, men i viss mån också av makrofager. Dessa frisläpper proteaser (protein-nedbrytande ämnen), vilket gör att kroppen också frisätter anti-proteaser (som motverkar proteaserna). Dessa mättes i första arbetet och det blev tydligt att en viss typ av proteas var aktiv, som en reaktion på att lungan utsattes för endotoxin. I den andra lungan syntes inte detta. Sedan fortsatte vi med att mäta interleukiner och såg att IL-17 faktiskt var relaterad till en celltyp som förr räknats till det förvärvade immunförsvaret och att IL-17 delvis reglerade sig själv. Interleukiner sätter igång kaskader av reaktioner i cellerna och gör att andra interleukiner frisläpps och/eller att andra celltyper dras till den lokal där inflammationen sitter, som flugor på en sockerbit. Detta hände när IL-26 (som inte tidigare var påvisat i lunga) frisläpptes och bl a påverkade neutrofilernas rörelsemönster, vilket endast hände i den lunga som utsattes för endotoxin och inte i den andra. Sist, men inte minst, analyserades flera antimikrobiella peptider (AMPs), vilka har blivit omtalade som möjliga kandidater för den nya tidens antibiotika. Deras närvaro, och i viss mån aktivitet av åtminstone en av dem, belystes efter exponering för endotoxin.

Vad betyder nu alla dessa fynd? Vi kan se att det medfödda immunförsvaret innefattar många olika potentiellt nedbrytande och farliga ämnen. Dessa hålls dock i schack av ett fungerande immunförsvar. Vid sjukdomar i lungorna har man tidigare visat att immunförsvaret har vissa brister och därmed skulle dessa processer, som nu påvisats hos friska personer, kunna förvärra inflammatoriskt orsakade lungsjukdomar, vilket resultaten kan tänkas antyda. För att få svar på detta behöver patienter undersökas på samma sätt.

Därutöver kan flera av de ämnen vi undersökt vara möjliga måltavlor för nya läkemedel mot både infektioner och inflammation, vilket vore mycket spännande och utmanande att undersöka vidare. Största svårigheten i utveckling av nya läkemedel kan dock vara komplexiteten i den inflammatoriska processen, eftersom flera av de undersökta proteinerna påverkar varandra – och sig själva – i både positiv och negativ riktning. Det gäller att finna vilken del av processen som är mest betydelsefull vid inflammation hos patienterna (som dessutom ofta har ett påverkat immunförsvar) och som samtidigt är lättast att påverka med läkemedel, en utmaning så god som någon.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Smith ME, Bozinovski S, Malmhäll C, Sjöstrand M, Glader P, Venge P, Hiemstra PS, Anderson GP, Lindén A, Qvarfordt I.
Increase in net activity of serine proteinases but not gelatinases after local endotoxin exposure in the peripheral airways of healthy subjects. PLoS One. 2013 Sep 23;8(9):e75032. doi: 10.1371/journal.pone.0075032. eCollection 2013.

II. Glader P, Smith ME, Malmhäll C, Balder B, Sjöstrand M, Qvarfordt I, Lindén A. *Interleukin-17-producing T-helper cells and related cytokines in human airways exposed to endotoxin.* Eur Respir J. 2010 Nov;36(5):1155-64. doi: 10.1183/09031936.00170609. Epub 2010 Feb 25

III. Karlhans F. Che, Sara Tengvall, Bettina Levänen, Elin Silverpil, Margaretha E. Smith, Muhammed Awad, Max Vikström, Lena Palmberg, Ingemar Qvarfordt, Magnus Sköld, and Anders Lindén. *Interleukin-26 in Antibacterial Host Defense of Human Lungs: Effects on Neutrophil Mobilization.* Am J Respir Crit Care Med Vol 190, Iss 9 pp 1022–1031, Nov 1, 2014.

IV. Margaretha E. Smith, Marit Stockfelt, Sara Tengvall, Peter Bergman, Anders Lindén, Ingemar Qvarfordt *Endotoxin exposure increases LL-37 - but not Calprotectin - in healthy human airways.* In Manuscript

Contents

Abbreviations

AM	Alveolar macrophage
AMP	Antimicrobial peptide
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
BALf	Bronchoalveolar lavage fluid
CAP	Community acquired pneumonia
CCR	Chemokine receptor
CD	Cluster of differentiation, an identification of cell surface molecules
cDNA	complementary DNA
CF	Cystic Fibrosis
COPD	Cronic Obstructive Pulmonary Disease
CRP	C-reactive protein
CXCL	Chemokine ligand
CXCR	Chemokine receptor
DAMP	Damage-associated molecular pattern
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter (in which flow cytometry is performed)
GM-CSF	Granulocyte macrophage colony stimulating factor
HβD2	Human β-defensin 2
hCAP	Human cationic antimicrobial protein
HNL	Human neutrophil lipocalin (abbreviation NGAL sometimes used)
ICC	Immunocytochemistry (intracellular staining)
ICF	Immunocytofluorescence
IFN	Interferon
IL	Interleukin
LL-37	a 37-amino-acid antimicrobial peptide starting with two leucines (LL)
LPC	Leukocyte particle count, i. e number of white blood cells (WBC)
LPS	Lipopolysaccharide, also known as endotoxin
LT	Lymphotoxin
MHC	Major histocompatibility complex (for recognition on cell surface)
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
NE	Neutrophil elastase
NET	Neutrophil extracellular trap

NK	Natural killer (cell)
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMN	Polymorphonuclear neutrophil
PRR	Pattern recognition receptor
RIA	Radioimmunoassay
RAR	Retinoic acid receptor (a nuclear receptor)
ROR	RAR-related orphan receptor (a transcription factor)
SLPI	Secretory leucoproteinase inhibitor
STAT	Signal transducers and activators of transcription (a transcription factor)
TGF	Transforming growth factor
Th	T-helper cell, a specific type of lymphocyte
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF	Tumor necrosis factor

Introduction

Inflammation

The definition of inflammation dates back to ancient cultures. The word stems from the Latin word *infla´mmo*, which means ignite. Inflammation is initially beneficial in protecting the body from external attack by blows, bacteria or viruses or other noxious particles causing tissue injury. The description of the four cardinal signs of inflammation is credited to the Roman Aulus Celsus, who lived about the time of the birth of Christ and whose work "On Medicine" was printed soon after the printing was invented in the 15th century. The classical characteristics of inflammation, known to every medical student in the world, are *calor* (warmth), *dolor* (pain), *rubor* (redness) and *tumor* (swelling) (1). The warmth and redness are due to a hyperemia, which is an immediate response of the body allowing better blood supply to the area of inflammation (2), thereby leading to the occurrence of the processes described in this thesis. The fifth classical part of inflammation, *functio laesa* (impaired function) was not introduced until the 2nd century.

Host defence

The inflammation is an expression of the immunological response of the human body. The word immunity derives from the Latin word *immunis*, which means exempt. The premise of a well-functioning immune response is the ability to distinguish between foreign (non-self) and host (self). The host should provide protection against foreign attacks, but also tolerate all cells within itself.

The human host defence comprises several components working together, the first of them being the physical barrier, which in the lung is the airway epithelium with its cilia, mucus and surfactant, the latter containing proteins that affect pathogen uptake by immune cells (3). The airway epithelium is not just a barrier but an interface between the environment and the host (4), with the capacity to express receptors and produce antimicrobial compounds common to the cells usually included in the innate immunity (5). The epithelium also produces proinflammatory mediators that recruit immune cells, both neutrophils and T lymphocytes, and later more macrophages as well, to the site of inflammation.

The immune system is however traditionally divided into the innate and the adaptive immune response (6). In the general framework of this thesis the focus is

on the innate immunity; certain cells and some of the mediators of the adaptive immune system are however studied, and will be described accordingly.

Innate immunity

The innate immunity, formerly called the non-specific immunity, is the oldest component of our host defence, speaking in terms of evolution, and is found through all classes of plants and animals. The innate response should come into play immediately – or very soon – after a foreign attack on the body, whatever the cause may be. This response is essential for the detection of viruses, bacteria and other noxious agents and for initiating an inflammatory cascade leading to the disposal of the pathogen. The main effector cells in the innate response are the primary defenders macrophages and neutrophils, since they are able to dispose of the pathogens in a non-specific manner (6). These cells are easily mobilized to the site of inflammation due to the increased blood flow mentioned above. The cells and mediators in the innate immune system are crucial for the initiation of the immune response and subsequent activation of the adaptive immune response.

Macrophages

Both acute and chronic inflammation involve leukocytes (white blood cells) at different stages. The most abundant white blood cells in the alveolar space are the macrophages, which account for 90-95 % of the alveolar leukocytes in a normal healthy lung. These cells comprise the innate immunity together with the neutrophils (6). Their name stems from Greek and means "big eater". Macrophages originate from the bone marrow as monocytes, having one nucleus, classically considered as being bean-shaped. After a short period in the bloodstream, they migrate to different tissues, where they become dendritic cells or macrophages, i.e. the alveolar macrophages (AMs) in the alveolar space (7). The alveolar macrophages are phagocytes, but also antigen-presenting cells as well as cytokine producers. They engulf a foreign particle in a similar way to that of neutrophils described below.

The macrophages continue patrolling the tissue for months in search of noxious particles and change according to the stimuli of the surroundings (8). Since their granulae are only rudimentary compared to the neutrophils, their synthesis of mediators continues throughout their lifespan, in response to environmental signals. In normal healthy lungs they contribute to the balance of cells and mediators, by not presenting all possible pathogens to the adaptive immune cells and thereby avoiding inflammation (9). In fact, the AMs are poor antigen-presenting cells compared to macrophages elsewhere in the body. Macrophages are the first line of defence and

are replaced in the tissue by elicited monocyte-derived macrophages within 3-4 days of inflammation and these newer macrophages have slightly different properties compared to the first ones (6). The macrophages have been described as orchestrating both acute and chronic inflammation through their release of mediators, phagocytosis, and antigen-presenting properties, but they also orchestrate later repair processes. Like other cells, macrophages express different proteins on their surfaces that may be used in analyses for detecting specific cells; Cluster of differentiation (CD) and in this thesis CD68 is used to identify the alveolar macrophage.

Neutrophils

The most abundant white blood cell in the body is the easily recognized polymorphonuclear neutrophil, the PMN, which has a lobulated nucleus and an army of granulae, each containing different kinds of mediators (10) that can digest tissues and kill microbes (11). PMNs also contain intracellular feedback mediators that downregulate the proinflammatory signals, to keep the homeostasis (11, 12).

Neutrophils form the first line of host defence together with macrophages. The neutrophils are produced in the bone marrow and during their short lifetime, they circulate the body as dormant powerhouses until an injured tissue summon on them. Then they roll over the endothelium, in the lungs that happens in the small capillaries spanning the alveoli, adhere to the endothelium (13) and squeeze in between the endothelial cells out to the site of foreign exposure. In the lungs, the trans-endothelial process takes time, due to the velocity of the blood in the small capillaries being quite low. Consequently, there is a reservoir of leukocytes in the capillaries, the marginated pool, which is ready to move into the tissue instantly (14). In an acute inflammation, the neutrophils are recruited to the tissue, in the case studied here the airways or alveoli, by several interleukins, among them IL-1β, IL-6 and the chemokine IL-8, produced by epithelial cells and resident macrophages (15). The macrophages also express a degradation product of arachnoid acid that increases the vascular permeability and enables the migration of the neutrophils (15). Depending on the kind of foreign stimulus, other mediators enhancing the migration of neutrophils may be active. In a prolonged, or chronic, inflammation, as in disease states such as Chronic Obstructive Pulmonary Disease (COPD), the neutrophils tend to be recruited to the lung tissue or airways by mediators released from T-cells, like IL-17 (11)(see below).

When in the tissue, the neutrophils release their mediators in relation to the type of noxious stimuli, mainly by *degranulation* of their granulae or by *phagocytosis* of the foreign agents or by throwing out neutrophil extracellular traps (*NET*s) to capture microbes. It is not yet clear why the neutrophil choose a particular mechanism (10).

In *degranulation*, the granulae of the neutrophil fuse with the cytoplasmic membrane, open up to the surroundings, and release their mediators (16). The granulae contain different kinds of mediators, see table 1. The contents of the granulae differ according to the stage of maturation of which they were formed. Consequently, they have different thresholds for exocytosis (11), but may be released at the same time, depending on the nature of the foreign agent to which the neutrophil is exposed.

Phagocytosis is Greek for "the process of being devoured by a cell" and means that the neutrophil engulfs the noxious particle, incorporating it in an internal vesicle, the phagosome, where the particle is degraded by mediators released by the granulae into the phagosome, i.e. within the neutrophil. This rapid antimicrobial action depends on synergistic cooperation among the toxic mediators generated in the neutrophil, namely those that are delivered into the phagosome. After the toxic mediators have destroyed the foreign agent, the neutrophil itself commits suicide, i.e. the programmed cell death called apoptosis. To avoid release of toxic agents into the tissue the neutrophil is then engulfed by macrophages. The noxious particle is at first recognized by pattern recognition receptors (PRRs) before being incorporated with the neutrophil (10) (see further under the endotoxin section) or the phagocyte may recognize the foreigner by opsonins (17).

The formation of *NET*s was discovered more than ten years ago (18), but details of the mechanisms behind the formation have been described relatively recently (19). When the nucleus of the neutrophil swells, the chromosomes dissolve and the decondensated DNA is discarded in a web-like formation together with granular constituents and the NETs have been shown to contain several mediators from the neutrophil (20). It has been considered whether forming NETs is an alternative to apoptosis and necrosis, but the mechanism is not totally clear (21). The NETs are believed to capture the foreign particle in the net, like a fly in a spider´s web, and consequently consume it.

Table 1. Some of the contents of the neutrophil granulae.

Mediator in the PMN	Azurophil primary granulae	Specific secondary granulae	Tertiary granulae	Secretory vesicles	Cytoplasma	Other origins
Calprotectin					x	
hBD2	x					Airway epithelium
HNL		x				no
LL-37		x				
MMP-2			(x)			AMs and others
MMP-8		x				no
MMP-9			x			Eosinophils and others
MPO	x					Monocytes
NE	x					no
SLPI					x	Bronchial mucosa

The four types av granulae of the neutrophil, all of which may be emptied into the phagosome or extracellularly. The contents of the different granulae are not complete, but cover the mediators within this thesis. Notably, the secretory vesicles may contain serum albumin, but since that is not a mediator, it is not within the above list. hβD-2=human β-defensin-2, HNL=human neutrophil lipocalin, MMP=matrix metalloproteinase, MPO=myeloperoxidase, NE=neutrophil elastase, SLPI=secretory leukocyte proteinase inhibitor

Mediators of immune cells; Proteases and anti-proteases

Proteases, also called proteinases or peptidases, are enzymes that break peptide bonds between amino acids in proteins or peptides. There are different kinds of proteases depending on the target protein they are to cleave, for example, collagenases cleave collagen (the dominating structural protein in connective tissue), gelatinases cleave gelatin, and elastases cleave elastin (an elastic protein in the connective tissue responsible for keeping the small airways opened). This overview is far from complete, but the important mediators in this thesis are briefly described.

One collagenase was analyzed in this thesis, namely matrix metalloproteinase *(MMP)-8*, also called neutrophil collagenase, or formerly collagenase 2. MMPs are a family of different proteases sharing zinc-dependency, calcium requirement, delight in extracellular matrix, and secretion in a state of inactive pro-forms to be cleaved extracellularly to their active forms (22). MMP-8 is specific to the neutrophil (23) and has been shown in lung tissue (24) and BALf (25) in COPD.

The gelatinases analyzed in this thesis are *MMP-2* and *MMP-9*, also called Gelatinase A and B respectively. MMP-2 is constitutively expressed in several cell types, among them macrophages (22). MMP-9 is expressed in eosinophils and neutrophils, but may be activated in other cells in diseases. MMP-9 is released as a 92kD (kiloDalton) precursor to be cleaved and activated extracellularly by serine proteases, but exists also in multimers and complexes. After activation, the MMP-9 itself, in turn, may cleave interleukin (IL)-8 and the inactive membrane-bound form of tumor necrosis factor-alpha (TNF-α) into their active forms (22), thereby promoting inflammatory response. On the other hand, MMP-9 may inactivate the pro-inflammatory form of growth-related oncogene (GRO)-α, also known as chemokine ligand (CXCL)-1.

Malignant cells often produce both gelatinases and they have been shown to be expressed in lung cancer (26), and also in COPD (27) and in emphysema (28), all disease states in which the MMPs have contributed to the vascularization by initiating the degradation of the matrix and in cancer also to the angiogenesis (29). Altogether MMP-9 is a multi-domain enzyme with many different functions in biology and pathology (30).

For this thesis, one of the serine proteases, neutrophil elastase (*NE*), was analyzed, but other serine proteases, such as proteinase 3 and cathepsin G (31), were not. NE is specific to the neutrophil (see table 1) and one of the active degrading proteinases within the phagosome. After stimulation, e.g. by the tumor necrosis factor (TNF)-α, interleukin (IL)-8, or endotoxin, the neutrophil releases NE (32). In addition to degrading extracellular matrix, NE has been shown to degrade other

proteins, e.g. cytokines and chemokines (32), but also to upregulate human beta-defensin-2 (HβD-2) in epithelial cells (33), see the paragraph on antimicrobial peptides. NE also stimulate the production of mucus in human airways (34). Elastin, the substrate of NE, is essential in the structure of small airways, preventing them from collapse, and the ability of NE to degrade elastin has proved to be important in emphysema (35). Of the other elastases of the neutrophil, so far only proteinase 3 has been shown to induce emphysema in animal models (36), while cathepsin G has not.

Human neutrophil lipocalin (*HNL*), or neutrophil gelatinase-associated lipocalin (NGAL), is specific to the secondary granulae of the neutrophil (37), see table 1. HNL is a 24 kD peptide that may form a complex with MMP-9 (30), a complex that is secreted by the neutrophil and helps to avoid extracellular proteolytic cleavage of MMP-9. This complex is often used in research analyses to verify the presence of neutrophils. HNL has been considered as a prognostic factor in adenocarcinoma of the lungs (38) and is found in bronchoalveolar lavage (BAL) from subjects with emphysema verified with computer tomography (28, 39). The presence of neutrophils, and thereby HNL, in acute inflammation and infection is well established. Recently, an interesting method using HNL to distinguish acutely between bacterial and viral infections in clinic has been presented (40).

The anti-proteases counteract the proteases on different levels. Tissue inhibitor of matrix metalloproteinases (TIMPs) are four anti-proteases inhibiting the MMPs. TIMP-2 is inhibiting MMP-2 and *TIMP-1* is preventing the actions of MMP-9 by binding both to its precursor and active form (22). TIMP-1 is not produced by neutrophils, but after activation it can be produced by monocytes and released in an easily broken complex with MMP-9 (41). An imbalance of the ratio MMP-9/TIMP-1 has been suggested to be of importance in structural changes of the airways in smokers with asthma (42, 43).

The anti-proteases of serine proteases are mainly secretory leukocyte proteinase inhibitor (*SLPI*) that counteract NE in the airways (44) and α-1-antitrypsin that inhibits serine proteases in the peripheral airways and lung tissue (45). SLPI is now included in the group of antimicrobial peptides (see below). It is believed, among other functions, to downregulate the response of the macrophage to endotoxin. SLPI is produced by different epithelial cells, but also by neutrophils (32) where it resides in the cytosol (see table 1). Recent research has suggested that SLPI might control the formation of NETs by inhibiting NE, at least in skin disease (46). Its binding to NE is reversible, while the 1:1 binding between α-1-antitrypsin and NE is not.

The *a-1-antitrypsin* is produced in the liver and constitutes a circulatory source of anti-protease to be diffused into the lungs. It is also produced in neutrophils and mainly released from secretory vesicles upon stimulation, in tissues affected by inflammation (47). It is believed to contribute to more than half of the anti-elastinolytic effect in the airways, but interestingly it has been found mainly in its inactive form in sputa from patients with CF and COPD (32). The elastase inhibitory capacity of α-1-antitrypsin may be inhibited by cigarette smoke (48, 49), making the lungs more susceptible to elastase in smokers. Moreover, the defiency of α-1-antitrypsin is the classical route to emphysema leaving space for NE to cleave elastin unopposed (35). α-1-antitrypsin also counteracts proteinase 3 (36), but has a greater tendency to inhibit NE.

Myeloperoxidase (*MPO*) is described briefly under this heading though it is not included in the group of proteases. MPO functions as an icebreaker with a resulting antimicrobial effect. It is a neutrophil peroxidase that is stored in the primary granulae (see table 1) and released into the phagosome when needed (50). MPO is there responsible for the respiratory burst of the neutrophil, thereby creating better conditions for the other toxic mediators to break down the foreign particle in the phagosome.

Mediators of immune cells; Antimicrobial peptides (AMPs)

Up to date the antimicrobial peptide database (APD) contains more than 2600 AMPs, from all species, but only just over a hundred defined AMPs in humans (51). The AMPs are divided into different groups by their three-dimensional structure. This, together with different net charges and sequences give the AMPs a wide functional diversity. Most of them consist of less than 50 amino acids. For this thesis, four of them were analysed in different papers. The serine protease inhibitor *SLPI* described above has 107 amino acids and is strictly human.

Human beta-defensin-2 (*HβD-2*) is a 41-amino-acid salt-sensitive peptide residue with the capability of killing Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* but has a bacteriostatic effect on the Gram-positive *Staphylococcus aureus* (51). It has proved to be able to induce chemotaxis through interaction with chemokine receptors (CCR) 2 and 6, active in Alzheimer's disease (52) and gastrointestinal cancer (53) respectively. HβD-2 expression is upregulated by the interleukins IL-17 and IL-22 (see paper II). Moreover, HβD-2 and the AMP LL-37 have shown to have a synergetic effect on the disposal of *S. aureus* on skin (54).

The only human cathelidin *LL-37* (leucine-leucine-37) was discovered simultaneously by three laboratories in 1995. It is stored in the neutrophil as its precursor human cationic antimicrobial protein-18 kD (hCAP-18) and is cleaved extracellularly, by the elastase proteinase 3, into its active form. LL-37 is active against a wide range of microbes including *E. coli* and fungi and LL-37 also has other functions (55) like chemotaxis (for example to neutrophils and T-cells), cell differentiation, immune modulation and wound healing. The transcription of LL-37 is vitamin D dependent (56).

Calprotectin is a zinc-binding protein consisting of two subunits, S100 A8 and S100 A9 (formerly MRP 8 and 14 respectively), and is abundant in the neutrophil cytosol. It induces endothelial cell detachment and consequently triggers cell death, both by apoptosis and necrosis (57). Calprotectin stimulates the inflammatory response of phagocytes after exposure to endotoxin (58). It is also a widely used clinical marker for inflammatory bowel disease (59). In the airways, it could be useful as a marker of cystic fibrosis (CF) exacerbation (60) since the level of calprotectin after exacerbation could predict the timespan to the next exacerbation.

Adaptive immunity

The specific adaptive immunity, also named the acquired immunity, is the more complex and sophisticated part of our host defence, existing only in vertebrates. The term specific refers to antigen-specific and requires an initial exposure to a pathogen, referred to as the antigen. This first exposure makes the host process the information and create an army of programmed immune cells. These effector cells, mainly different types of lymphocytes, are designated to attack that specific antigen should the body be exposed to it again. The adaptive immune response also creates an immunological memory and remembers how to encounter that specific antigen, in creating antigen-specific antibodies, which is basically how vaccination works.

Lymphocytes

The lymphocytes are mononuclear leukocytes that originate in the bone marrow, like the other white blood cells. The lymphocyte is visually recognized through its big nucleus. There are three main types of lymphocytes; natural killer (NK) cells, B- and T-cells where B stands for bursa and T for thymus, giving a hint about the locus of differentiation (where B actually in humans is the bone marrow).

NK cells are actually included in the innate immunity and are able to recognize sick cells in the absence of labelling in the form of antibodies or when the usual

recognition pattern, the class I major histocompatibility complex (MHC), is downregulated as in a viral attack. In other words, they are able to distinguish self from nonself by other means than MHC recognition. They are primarily designed to recognize and kill virally infected and neoplastic cells and are recruited to the infected tissue by cytokines (61). They are also involved in the regulation of the T cell activity (62) as well as macrophage activity through considerable secretion of IFNγ (see below). NK cells differ from other lymphocytes with regard to recognition since in humans they are phenotypically defined as $CD56^+$ $CD3^-$.

B-cells are responsible for the humoral immunity by secreting antibodies. They also function as antigen-presenting cells and they generate memory cells just like the T-cells. B-cells are not analyzed in this thesis and will not be further discussed.

T-cells are defined phenotypically as $CD3^+$. They may be divided into two main subgroups: *T-helper (Th) cells*, which always are $CD4^+$ and *cytotoxic T-cells* that are $CD8^+$. There is also a smaller group of γδ-T-cells with a capacity for tumor antigen-presentation (63).

T-helper cells release cytokines and growth factors that regulate other cells like the innate immune cells neutrophils and macrophages, but also the B-cells. Thus, they play an essential role in orchestrating mainly the adaptive immune response, through their release of mediators. The cytotoxic T-cells are capable of lysing tumor cells, virally infected cells and other damaged cells; through identification of the MHC I complex in conjugation with a specific antigen on the cell surface of the infected cell. The foreign molecule inside the cell is bound to MHC I, transported to the surface and there becomes recognized by the cytotoxic T-cell, which subsequently destroys the infected cell. After exposure to its specific antigen, the naïve cells change another CD-molecule, the CD 45, from A to O. This can be used as a differentiation when analyzing whether the cells are memory cells or not.

Naïve $CD4^+$ T-helper cells may, depending on the pattern of signals they receive during their initial interaction with antigens, mature into different subtypes; Th1, Th2, Th17 and induced regulatory T (iTreg) cells, which can be distinguished from one another by different functions and specific cytokine productions (64).

Th1 cells are involved in the immune response to intracellular pathogens and in autoimmune disease. One important cytokine of the Th1 cell is the macrophage-activating interferon γ (IFNγ); others are lymphotoxin α (LTα) and interleukin (IL)-2, the latter of importance for memory cell formation.

Th2 cells mediate immune response to extracellular pathogens as well as being important in asthma and allergy. They produce several interleukins; IL-4, -5, -9, -10, -13, and -25, the three former being of importance particularly in asthma and allergy. IL-10 has different effects depending from which type of cell it is released, in this case, suppressing Th1 cells. IL-13 is active in helminthic infection, but interest-

ingly also plays a role in airway hypersensitivity. The last interleukin of the Th2 cell, IL-25, initiates and upregulates the whole Th2 cell response.

Th17 cells play a role in protecting against extracellular bacteria and fungi and have another role in autoimmunity. Th17 cells are stimulated to become exactly Th17 by Il-1β, -6, and -23 (65), which all use signal transducers and activators of transcription (STAT)3 for signal transduction (64) to activate the transcription factor retinoid-related orphan receptor (ROR)Cvar2, which is thought to be the master regulator of Th17 differentiation (65). The Th17 cells produce IL-17, -21, -22, all of which have been more closely examined in the second paper of this thesis. For a description of IL-17, see below. IL-21 exercises positive feedback on the cell in which it is produced and amplifies the Th2 reaction. In addition, the transcription factor STAT3 has been the subject of investigation for paper III.

iTreg cells are one type of Treg cells and the slightly different types have not been fully distinguished as to why they are treated as a single group in this thesis. They are active in controlling the lymphocyte homeostasis and in regulating the immune response and tolerance. They specifically produce IL-10 and -35 as well as transforming growth factor (TGF)β, which controls proliferation and differentiation of immune cells (see below). IL-10 in particular is important in suppressing inflammatory bowel disease and lung inflammation when it is released from Treg cells (64). Moreover, these $CD25^+$ cells are believed to play a role in the development of tolerance to inhaled allergens (9).

Mediators of immune cells; Cytokines

Cytokines are a rather loose category of small proteins that are released from cells to affect other cells. The name cytokine stems from the Greek words *cyto* and *kinesi* for cell and movement respectively. Cytokines usually include the groups of lymphokines, interleukins, chemokines, interferons, and tumor necrosis factors. They are produced by the cells directly involved in the immune response, but also by other cells in the body. Thus, one cytokine may be produced by more than one cell, just as in the case of proteases and AMPs. The cytokines show a great variety in function and activity and may even alter or reverse the effects of their fellow cytokines (66).

Lymphokines are always produced by lymphocytes and have an effect on other immune cells. In this group, one may find members from the other subgroups of cytokines, like interleukins, and interferon.

Interleukins (ILs) have already been mentioned several times due to the close interactions between this group of cytokines and different cells. The name of inter-

leukin stems from the word *inter*, meaning in between and *leukin*, derived from leukocytes. At first, they were considered to be produced only by leukocytes and most of them are produced by $CD4^+$ T-helper cells, i.e. lymphocytes. In addition, monocytes, macrophages and epithelial cells produce interleukins. The ILs both have up and down regulating effects on cells in the immune system and consequently affect other mediators in the immune cells. Two particular interleukins are described in detail here, since they are of special interest in this thesis.

First, *IL-17*, which is mainly produced by the $CD4^+$ T-helper cell Th17, but sometimes also by the γδ-T-cells or $CD8^+$ cytotoxic T cells. IL-17 is a family with members from 17A to F, but A is now considered as the archetype form of IL-17. IL-17, the name used in this thesis, is a conductor in the interface between innate and adaptive immunity. It induces neutrophil-mobilizing mediators, like the chemokine (CXCL) IL-8, and the neutrophil-activating IL-6. It also affects GM-CSF (Granulocyte Macrophage colony stimulating factor) among others (65). IL-17 might also, more generally, stimulate neutrophil activity by activating MPO, NE and MMP-9.

Secondly, *IL-26*(formerly AK155) is produced by Th17, and by other leukocytes under certain conditions (67). Like IL-17 it induces IL-8 in the target cells as well as IL-1β and TNF-α (see below), suggesting that IL-26 drives or sustains inflammation, but so far there have not been enough functional studies on IL-26 (67) to verify this suspicion. IL-26 belongs to the IL-10 family (together with IL-10, -19, -20, -22, and -24) and partly uses the same receptor as IL-10, but is its own entity (67).

Chemokines are *chemo*tactic cyto*kines*, meaning that they mediate chemoattraction leading to chemotaxis. Chemotaxis is the term used when nearby cells move towards a specific site, as in magnetism. Chemokines are usually very small (90-130 amino acids) and may be released by several cell types. Some are constitutively expressed and others are inducible by inflammation. Some interleukins may function as chemokines, like IL-8 mentioned above. There are several subfamilies of chemokines, such as CXC, CC, CX3C, and XC and their designation sometimes end with an extra "L", which stands for ligand. The chemokines exert their chemotactic capacity through transmembranous receptors specifically found on their target cells. These receptors get an "R" for receptor hooked on to the name of the chemokine.

Interferons (IFNs) are named after *interfere*, meaning interfering with viral replication. In addition, they are released in response to other pathogens like bacteria, parasites and tumor cells and in turn activate NK-cells and macrophages. Interferons upregulate the expression of the MHCs, thereby increasing the antigen presentation. The archetype interferon is IFNγ, which is produced for example by NK-cells (68) and T-lymphocytes, but also by neutrophils in response to a Gram-positive

stimulus (69). IFNγ is able to inhibit a viral attack directly and has immunomodulatory effects.

Tumor necrosis factors (TNFs) are cytokines that are able to induce cell death. The archetype TNF-α is a potent pyrogen and, together with IL-1, considered as "the proinflammatory cytokines"(66). It is produced by monocytes, T-cells, and recruits neutrophils locally. Systemically TNF-α stimulates the liver to produce acute phase proteins as well as regulating the fever reaction in response to endotoxin.

Transforming growth factor (TGF)-β is another cytokine that is released by, among other cells, macrophages in an inactive form to be cleaved and activated extracellularly. TGF-β is important in cell differentiation and proliferation, for example TGF-β is one of the factors which stimulate the naïve Th cell to become a Th17 cell (65). It suppresses the release of mediators from both alveolar macrophages and dendritic and epithelial cells of the lungs and stimulates collagen formation (9). It has thus an anti-inflammatory effect and a role in the resolution of inflammation, which it exercises together with IL-10.

Granulocyte Macrophage colony stimulating factor (GM-CSF) is a hematopoietic glycoprotein secreted by various cells, among them macrophages, after stimulation by IL-1, IL-6, and TNF-α or endotoxin (70). GM-CSF stimulates the growth of all granulocytes and monocytes in the immature immune system and is clinically used to treat neutropenia (70), as is the related G-CSF. In the mature immune system, GM-CSF activates macrophages that are essential for the clearance of surfactant in the alveoli. Thus, lack of (or blocked) GM-CSF leads to alveolar proteinosis (71). GM-CSF has many roles in different organ systems, but altogether the proinflammatory effect seems to be the most important role (72).

Endotoxin

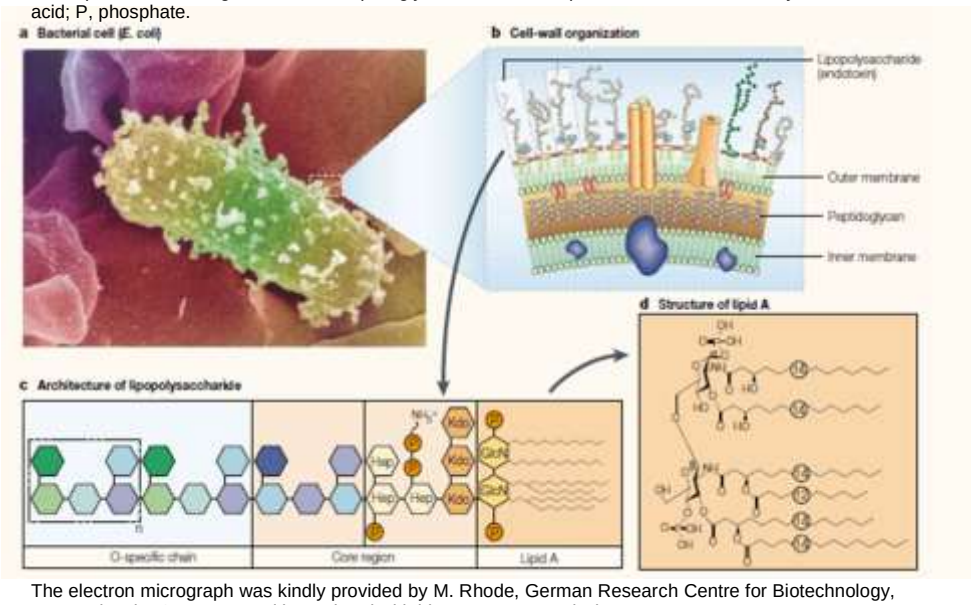
Endotoxin is equivalent to lipopolysaccharide, LPS, which is a constituent of the outer layer of Gram-negative bacteria and a potent proinflammatory agent. Both names; endotoxin and LPS, are used interchangeably throughout this thesis. Endotoxin got its name through a co-worker of Koch; Richard Pfeiffer, who discovered the heat-stable component of bacteria and labelled it as a "poison from within" (from Greek), i.e. *endo-toxin* (73). In the airways it has been connected to disease states both through the presence of Gram-negative bacteria in human airways and through the presence of endotoxin in tobacco smoke (74, 75). In fact, the presence of endotoxin has been proved both in regular cigarettes and in water pipe smoke,

but not yet in smoke or fluid from e-cigarettes (76). The hazard of inhaling dust has been known for a long time and was first discovered among cotton workers (77). Several studies in this area were carried out connecting endotoxin to swine dust (78). Endotoxin is ubiquitous (79) and is widely used as a research tool to simulate an infection or inflammation, especially in the airways (77). The most common model of exposing humans to endotoxin is through inhalation (80), but models of intravenous administration and intrabronchial instillation have also been evaluated as safe procedures (81, 82).

Endotoxin structure and the TLR4 response

The endotoxin molecule consists of three parts; an O-specific polysaccharide chain, a core region and lipid A, which is responsible for the toxic effect, see figure 1. All parts form a functional unit, in which all components are vital; the function changes if one of them is missing or changing its form (73). Endotoxin from different Gram-negative bacteria has great similarities, whereas the surface structure on Gram-positive bacteria, the LTA (lipoteichoic acid) differs considerably from the LPS both in appearance and in immunogenicity. In general, the LPS from *E. coli* is used as a research tool when exposing humans to endotoxin (77).

Figure 1. A gram-negative bacterium. a) Electron micrograph of *Escherichia coli* together with b) a schematic representation of the location of lipopolysaccharide (LPS; endotoxin) in the bacterial cell wall and c) the architecture of LPS d) Also shown is the primary structure of the toxic centre of LPS, the lipid A component.GlcN, D-glucosamine; Hep, L-glycero-D-manno-heptose; Kdo, 2-keto-3-deoxy-octulosonic acid; P, phosphate.



The electron micrograph was kindly provided by M. Rhode, German Research Centre for Biotechnology, Braunschweig, Germany, and is reprinted with his generous permission.
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Endotoxin binds to the plasma protein LBP (LPS-binding protein) that was identified at the end of the 1980s. Concomitantly, the LPS-sensing receptor CD14, the ligand-binding part of the endotoxin receptor complex, was discovered. The endotoxin signaling pathway through the intracellular nuclear factor (NF)-κB was discovered at about the same time, in 1990, but it was not until 1998 that the receptor of endotoxin was discovered; the toll-like receptor (TLR)4 (73). TLR4 is a transmembranous glycoprotein that requires a small protein myeloid differentiation (MD)-2, to which it is physically connected, to function (83, 84). When the body is exposed to Gram-negative bacteria, the LBP is upregulated as an acute-phase reactant and may, because of a size smaller than albumin, probably diffuse from the plasma compartment into the alveolar fluid (6). Moreover, it has been shown to be produced by human lung epithelial cells (85). LBP seems to be essential for the bacterial clearance in Gram-negative pneumonia (86). It binds to the lipid A of the endotoxin and presents it to TLR4 through interaction with CD14, which could be either membrane-bound or soluble. In addition, soluble CD14 may enhance the binding of endotoxin to high-density lipoproteins, thereby reducing the activity of endotoxin in plasma since this complex-binding seems to incapacitate endotoxin(6).

There are several TLRs, all specific to different microbial agents, as TLR4 is specific to Gram-negative bacteria or more specifically to the endotoxin itself (87). The majority of the Gram-negative bacteria express the lipopolysaccharide that binds to TLR4 specifically, but a few also express the lipoprotein-ligand specific to TLR-2 (5, 88, 89). The affinity to the TLR4 seems to differ between the species of bacteria, which could be of relevance in case of disease (90). The TLRs are part of the pattern recognition receptors, PRRs, which are expressed by point-of-entry cells and whose task is to recognize foreign invaders of the body. These cells could be the epithelial or dendritic cells as well as macrophages or neutrophils. The foreign invaders are either pathogen-associated molecular patterns (PAMPs) like microbial pathogens or damage-associated molecular patterns (DAMPs), like cell components from cell death. In other words, endotoxin is a PAMP, which is recognized by the PRR TLR4 (5).

After the binding of endotoxin to the TLR4, a cascade of reactions is triggered within the cell. This signaling may occur through the cytosolic adaptor myeloid differentiation primary-response (MyD)88 protein (91), which is an essential signal transducer in both TLR and IL-1 signaling. The cascade passes through several intracellular factors and finally translocates NF-κB to the nucleus of the cell, which induces the transcription and production of proinflammatory cytokines, like IL-1β, IL-6, IL-8, and TNF-α. They in turn trigger the inflammatory process (5). There is also an alternative non-MyD88-dependent pathway resulting in different responses (88). At the same time, the complement and coagulation pathways are stimulated, but these are not covered here.

The hazard of smoking – effect on human lungs

It is well established that smoking destroys the lungs in many different ways. Cigarette smoke contains such a large amount of toxic substances (75), including endotoxin, that the fact that cigarettes are still on sale is most depressing. Moreover, the newer e-cigarettes are definitely not atoxic (76). In mouse models, smoke vapor from e-cigarettes decreased the function of macrophages and consequently increased the susceptibility to infection, in the refereed case to infection with *S. aureus* (92), but an alteration in the differential count in bronchoalveolar lavage (BAL) did not occur, which would have been expected. E-cigarettes have clear similarities to conventional cigarettes. The extract of conventional cigarette smoke has been shown to increase the resistance of already resistant *S. aureus* by changing its surface charge, thereby impeding LL-37, among other factors, in helping to defeat the bacteria (93). This influence on the host response to bacteria by cigarette smoke is one explanation as to why smokers are more susceptible to infections than non-smokers.

Conventional cigarette smoke has been shown to activate the respiratory endothelial cells and rearrange the cytoskeleton, thereby causing disruption of the microvascular barrier of the lung, possibly through nicotine (94). The increased permeability of the alveolar-capillary membrane is verified through a higher total BAL protein level in smokers compared to non-smokers after inhalation of LPS (95). This mechanism likely paves the way for virus, endotoxin and whole bacteria to enter, as has been shown by upregulation of TLR4 (96-98). The mechanism seems dose-dependent on nicotine, which is why smoking e-cigarettes containing nicotine probably has the same effect as regular cigarettes, but to a lesser extent (94). This chronic damage of the epithelium, which leads to an inflammation in the airways and elicits the immune response to a constant readiness to act, is another explanation of the susceptibility to infections in smokers. Not only are smokers prone to infection, they are at a higher risk of developing acute respiratory distress syndrome (ARDS) when ill in sepsis (99), even if they are presumably healthier and definitely younger (100) than non-smokers with sepsis.

Conventional cigarette smoke not only affects the epithelial cells and macrophages (101), it also leads to an increase in bronchoalveolar cellularity and thus in the amount of proinflammatory cytokines (102). It leads to oxidative stress through enhancing production of free radicals in lung cells. Moreover, the upregulation of TLR4 in lung epithelial cells (96), leads to the release of, among others, IL-8, one of the chemoattractants of neutrophils, thereby amplifying the accumulation of neutrophils in the airways (102). There is also a concomitant increase of other immune cells, like the T-cells.

One can summarise by stating that smokers have an exaggerated inflammatory response. Not only does the smoking give rise to infections; eventually, the chronic inflammatory state induced by smoking leads to disease, as described below.

Health and inflammation of the human lungs

In healthy humans, the normal host defence maintains the homeostasis in lower airways and lungs despite daily inhalation of significant amounts of pathogens, pollutants and allergens (3). For a long time, the airways and lungs below the larynx have been considered sterile, a relative truth that has now been modified (103). Through bronchoscopy-mediated brushing and gene analyses, the healthy human airway has been shown to display traces of a genus of bacteria, Bacteroidetes, mainly the Prevotella-species, which may be considered as the microbiota of the lungs, i. e. the ecological community of bacteria that share our body compartments with the real body specific cells. In a healthy lung not affected by smoking, these anaerobic commensal bacteria are believed to protect the lungs from pathogens, since they directly inhibit the growth of other bacteria (104). Similar aspects of the microbiota have been shown in the guts (103).

Thus, in healthy airways the interaction between the airway epithelium with its surfactant and mucus, the resident macrophages and other immune cells containing their potential toxic mediators and the microbiota is balanced. However, excess external stimuli disrupt this homeostasis through different pathways, depending on the type of noxious stimulus (13, 105).

Acute inflammation of the lungs

In acute inflammation of otherwise healthy lungs, the innate immunity is alerted by disturbance of the delicate interplay between the surfactant proteins A and D, the constitutive suppressing TGF-β and the sensing of microbes by alveolar macrophages and dendritic cells, which forward the information to fellow immune cells and first of all recruit neutrophils (105). The acute lower respiratory tract infection is still an important disease entity and the outcome depends both on the defence capacity of the immune response, as indicated above, and on the virulence of the organism (105).

For over ten years, it has been known that the respiratory syncytial virus (RSV) increases the expression of TLR4 on airway epithelial cells and thus potentiates a subsequent infection with a LPS-containing bacterium (97). Virus infection is actually a cause of community-acquired pneumonia (CAP) that has probably been underestimated before molecular tests were available (106) and co-infection with

bacteria is common. The most common bacterium that causes CAP, in Europe, is the Gram-positive *Streptococcus pneumoniae* (pneumococcus in everyday speech), closely followed by the Gram-negative *Haemophilus influenzae* and atypical bacteria (107), at least when CAP is microbiologically verified. However, with an increasing ageing population, presumably with dysphagia, and with a population with increasing overweight, microaspiration must be taken into consideration. In a hospitalized population, and even in a community-based population with micro-aspiration, the bacterial spectrum looks different with a change towards Gram-negative and anaerobic species (108), all of which attack the host in slightly different ways. The bacteria have learnt to take advantage of the host signals, both in interfering with them, for example by destroying the NETs, and by responding to them, for example by increasing their own growth by stimulation of TNF-α (105).

The innate immune response that originally is responsible for ridding the airways and lungs of noxious and microbial agents may also injure the tissue, as described, and acute lung injury (ALI) may develop (105). ALI is the milder form of acute respiratory distress syndrome (ARDS) and is caused mainly by infections in the lungs or elsewhere, trauma or aspiration of gastric contents. There is a standard definition dividing these two interwined clinical conditions based on the degree of severity (109). ARDS was first described in 1967, and the underlying mechanism is a leakage of the alveolar epithelium-endothelium barrier that leads to a flooding of proteins into the alveolar space. The protein-leakage leads to pulmonary edema, which in turn results in hypoxia and hypercapnia, a condition which has a high mortality rate (109). The mortality risk increases with nonpulmonary organ failures and to survive these patients need careful positive pressure ventilation (110).

Recently, the alveolar epithelial layer has been assessed as the most important barrier for preventing the oedema in ARDS, and the influenza A virus has been shown to destroy tight junctions of the alveolar epithelial barrier in an *in vitro* model (111). The disturbance of the alveolar epithelial layer with the subsequent killing of the epithelial cells and the call for neutrophils through chemokines (released from the epithelial cells) was discussed a few years ago (112, 113) as an alternative to the more established hypothesis of endothelial destruction by mediators from neutrophils and platelets (109). The mechanism of alveolar epithelium destroyed by a viral infection may facilitate the invasion of secondary bacteria and it is possible that the degradation of the respiratory part of the alveolar barrier explains the ARDS caused by pneumonia, while in other origins the ARDS may develop through the classic model. Gram-negative and Gram-positive bacteria are in equally common sources of sepsis (2), but whether the type of bacteria is of relevance for the mechanism of barrier destruction is not clear. Gram-negative infection, or rather endotoxemia, has been shown to affect the T-cells with a relative increase of Tregs (114) and in a combined human- and mouse-model a subset of Th17 cells was identified after

endotoxin exposure. Their release of IL-17 further increased the permeability of the barrier (115). These recent findings indicate that the adaptive immunity might be involved in the process, but this mechanism remains to be clarified.

The resolution of the inflammatory state is essential but information about the healing process is limited (116). STAT-3 has tissue-protecting and anti-inflammatory effects, but the exact way of exercising these effects is unclear (105). IL-10-producing macrophages and Tregs have been suggested as being important, based on a non-human model (117) after priming of mouse lungs, as have TGF-α and IL-1β (116), but more information on humans is required.

Chronic inflammation of the lungs

Among the neutrophil related chronic diseases in human lungs, cystic fibrosis (CF) and COPD stand out as diseases where the known reasons for the prolonged exposure to neutrophils are not fully clarified. There are chemoattractants as well as bacteria present in the airways in both these disease entities (32).

COPD is an entity covering several phenotypes, in all of which chronic inflammation is a hallmark (118). Typical symptoms of COPD are shortness of breath, chest tightness and a cough, depending on whether the disease mostly affects the airways or lung parenchyma, symptoms that worsen during exacerbations.

The accumulation of neutrophils in human lungs of patients with COPD is kept at a high level by the inhibition of neutrophil cell death by cigarette smoke (102). In addition to the neutrophil dominance in COPD, macrophages are of importance. During the progression of the disease, the cellularity in the bronchoalveolar space changes towards an increase in T-lymphocytes and dendritic cells. Even if the T-cells are recruited to the airways there are hints that both the development of memory cells and the activation of the adaptive immunity are impaired in COPD patients (32), which is why they repeatedly suffer from exacerbations with the same bacteria (102). Moreover, a subgroup of patients with COPD has been identified, having submucosal eosinophilia correlating to levels of eosinophils in blood (119), and this particular group of COPD patients might benefit from another type of treatment.

A number of mediators of immune cells are of importance in the development of COPD. The components of cigarette smoke do cause a chronic inflammatory state with remodeling and subsequently repair with deposition of extracellular components like collagen, leading to chronic airway limitation and parenchymal destruction. Several mediators described and analyzed in this thesis may be of importance in this process. For example, LL-37 expression in airway epithelium of smokers with COPD has been shown to be correlated to the deposition of collagen (120). In

addition, the concentrations of LL-37 are higher in BAL in COPD patients, compared to healthy individuals, but the levels of LL-37 decrease as the stage of COPD increases (121). Macrophages from patients with COPD are more easily stimulated to release MMP-9 (122), compared to macrophages from other smokers and healthy individuals. MMM-9 is also increased in BAL from ex-smokers with COPD compared to other ex-smokers and healthy individuals (123), as are NE and its inhibitor α-1-antitrypsin, and they correlate with neutrophil counts, just as IL-8. Moreover, MMPs have been shown to correlate with signs of small airway disease on high-resolution computer tomography (HRCT) of the thorax (25) and serum levels of MMP-9 correlates to a decline of lung function in COPD (124). There is also relatively recent evidence for an COPD related upregulation of the TLR4 in both central and peripheral airways (divided on the basis of airway diameter) (98) as well as evidence for an upregulation of HβD-2 in the peripheral airways.

The neutrophil count of the peripheral airways of COPD patients correlates with the bacterial load in between exacerbations (123) and the COPD airways are colonized with proteobacteria, including the Gram-negative bacteria *H. Influenzae* and *E.coli*, inter alia, (103). These bacteria are presumably pathologic in contrast to the colonization of anaerobic species in healthy airways, but whether the stage of COPD is relevant for the presence of different species of bacteria is not yet clear (118). In addition, immunological connections with the intestinal microbiota have been considered. One rationale for this is an epidemiological correlation between COPD and inflammatory bowel disease (125) as well as a proved effect of changes in intestinal microbiota on the outcome of respiratory tract influenza in mice (126).

Even if the inflammatory cells and many of the neutrophil related cytokines have been evaluated and shown in higher levels in patients with COPD, no specific treatments have yet proved successful. The pathogenesis of COPD appears to be quite well studied, but even so, we do not know the whole truth about the development of the disease.

Patients with *CF* also have high numbers of neutrophils in their airways, but the underlying mechanism resulting in the inflammatory state is different from the one in COPD. CF is an inherited disease with impaired mucociliary clearance, which leads to an abundance of neutrophils and mediators in the peripheral airways, as well as a higher load of bacteria (127). Typical symptoms from the airways are a cough with a lot of mucus, frequent respiratory tract infections and sinusitis, and the development of bronchiectasies. Already in infancy one of the chemoattracts of neutrophils, IL-8, is elevated along with the neutrophils and NE (127). NE is released in such amounts from the neutrophils, which are not properly cleared out of the airways, that the anti-proteases are unable to cope with the quantity of NE. The surplus of NE leads to digestion of the extracellular matrix and NE is, in fact, a marker that predicts structural damage and decline of lung function, when meas-

ured in sputum (128) and BAL (129) in infancy. The degradation leads to the bronchiectasies that are typical for CF. Several other neutrophil related mediators seem of importance as well, like MMP-9 (130) and calprotectin, and the level of the latter in serum before and after exacerbation seems to have a predictive value for the time to the next exacerbation (60). Furthermore, higher levels of the AMP LL-37 in BAL correlate to higher levels of neutrophils and the deterioration of lung function, while HβD-2 is rather reduced in more severe CF (131). Moreover, IL-17 levels in BAL and IL-17+ cells in the bronchial submucosa from patients with CF are higher than in healthy individuals (132).

The inability of the CF airways to clear bacteria enhances the accumulation of neutrophils even more, which is why the inflammation of CF airways becomes self-perpetuating. The inflammatory response of the CF airway has also been shown to be disproportionate to the bacterial burden partly due to neutrophils upregulating their TLR4 (127). Furthermore, as in smokers, but not entirely due to the same mechanism, the exposition of the airways to viral infection seems to affect the immune response, in the CF case resulting in an aggravated outcome of exacerbations (133). The CF airway is chronically colonized with bacteria, from the Gram-positive *S. aureus* to the Gram-negative *H. Influenzae* and *P. Aeruginosa* inter alia. The two former do not affect the lung function of the patients with CF, while the latter, together with multiresistant staphylococcus (MRSA), does (134). Moreover, CF is a polymicrobial disease and these – and other – bacteria often co-colonize the airways of patients with CF, making the treatment a challenge. Therefore, it is important to evaluate the mechanisms of the inflammation in CF, to be able to find new ways of therapy.

Incentives for this thesis

Even if the knowledge of the innate immunity has increased during the last decade, much of the inflammatory processes and the physiology of normal lungs and airways remain unknown. In inflammatory diseases of the airways, such as COPD and CF, it is clear, however, that an excessive stimulation of the innate immune response and subsequent destruction of tissue are essential. Both these diseases are subjects of exposure to Gram-negative bacteria and, in the case of COPD, tobacco smoke, which in both cases means exposure to endotoxin. By exposing healthy lungs to the same proinflammatory substance, it is possible to increase the awareness of the inflammatory processes of normal lungs. With this improved understanding, a reference to the processes in inflammatory diseases in airways can be established. Thus, the purpose of this research project was to characterize the physiological response of healthy human lungs to the TLR4 agonist endotoxin.

Aims

The overall aim of this thesis was to characterize the innate mechanisms in the immune response in healthy human airways *in vivo,* in response to endotoxin.
The following specific research questions were posed:

- Are the activities of proteases and anti-proteases in healthy human airways balanced under normal conditions (the proteolytic homeostasis)? If so, can a single exposure of these airways to endotoxin induce an imbalance of the proteolytic homestasis towards an excess activity of serine proteases or gelatinases?

- Are IL-17-producing Th cells present in healthy human airways? Are IL-17 and other Th17-associated cytokines involved in the innate immune response to endotoxin in healthy human peripheral airways?

- Is the cytokine IL-26 involved in the antibacterial host defence of the human lungs? Which characteristic features does IL-26 have in healthy airways in response to endotoxin?

- Are antimicrobial peptides present in healthy human peripheral airways and do they take part in the innate immune defence against Gram-negative bacteria?

Study Population

This thesis is based on four cross-sectional studies on healthy human volunteers targeting various aspects of the immune response in healthy airways. All four studies are based on the same homogenous group of volunteers, with the exception of paper III, in which other healthy humans have also been brochoscopically examined without previous exposure to endotoxin. This part, without endotoxin exposure, took place at Karolinska University Hospital, Stockholm, Sweden and was approved by the Regional Ethical Review Board in Stockholm, Sweden. The main part of the studies with exposure to endotoxin all took place at Sahlgrenska University Hospital, Göteborg, Sweden and were approved by the Ethical Review Board for studies on humans at Göteborg University.

The participants were recruited by local advertising and by the "mouth-to-mouth" method. All volunteers were evaluated for inclusion at a first clinical visit, during which an interview and a medical examination took place, including lung function testing, an electrocardiogram and blood sampling. All the included participants had a non-atopic medical history, as well as no history of smoking. Inclusion criteria were also a normal ventilatory lung capacity defined as forced expiratory volume during one second (FEV_1) >80% of predicted value, a normal electrocardiogram and an unobjectionable physical status. Negative *in vitro* screening for the presence of specific IgE antibodies, the Phadiatop test, was also required, as well as a normal level of IgE in serum. No medication was allowed, with the exception of oral contraceptives. All participants gave their written consent after receiving both written and verbal information.

We recruited in total 34 healthy non-smoking non-allergic volunteers between the ages of 20 and 29, with equal distribution between men and women. The demographic data and distribution of participants between the four studies are recorded in table 2. Three participants were excluded; No 8 due to vomiting after the first bronchoscopy, no 22 due to a positive allergy test, i.e. positive Phadiatop (which did not arrive until after the bronchoscopies), no 31 due to taking antihistamine before the bronchoscopies (which she failed to mention before the bronchoscopy). Moreover, after the first analyses of bronchoalveolar fluid (BALf), the samples from no 15 were analyzed as a pilot in the second study, after which the

samples were unavaible due to human error. In the table below these volunteers are marked with red in the column on the far right.

Table 2. Demographic data on the study population

Time	No.	Gender	Age	P I	P II	P III	P IV	Excl
12 h	15	Q	24					x
	21	M	27		x	x	x	
	22	M	24					x
	23	Q	22		x	x	x	
	24	Q	23		x	x	x	
	25	M	24		x	x	x	
	26	M	25		x	x	x	
	28	M	24		x	x	x	
24 h	7	M	23	x		x		
	9	M	29	x		x		
	11	M	23	x		x	x	
	12	Q	26	x		x		
	13	M	25	x		x		
	14	Q	20	x		x	x	
	16	Q	21	x		x	x	
	17	Q	26	x		x	x	
	18	Q	21	x		x	x	
	19	M	22	x		x	x	
	20	M	21	x		x	x	
	27	M	23		x	x	x	
	29	Q	24		x	x	x	
	30	Q	20		x	x	x	
	31	Q	21					x
	32	M	24		x	x	x	
	33	Q	25		x	x	x	
48 h	1	M	27	x		x		
	2	M	25	x		x		
	3	M	21	x		x		
	4	Q	21	x		x		
	5	Q	20	x		x		
	6	M	23	x		x		
	8	Q	21			x		x
	10	Q	24	x		x		
No. in each study/excl.				**18**	**12**	**31**	**19**	**4**

Running numbers of participants (No.), sorted after the time interval between the first and second bronchoscopies. Gender: Q being women and M being men. Age in years at the time of the bronchoscopies. P I-IV refers to Paper I-IV in this thesis. For comments on exclusion, please see text above.

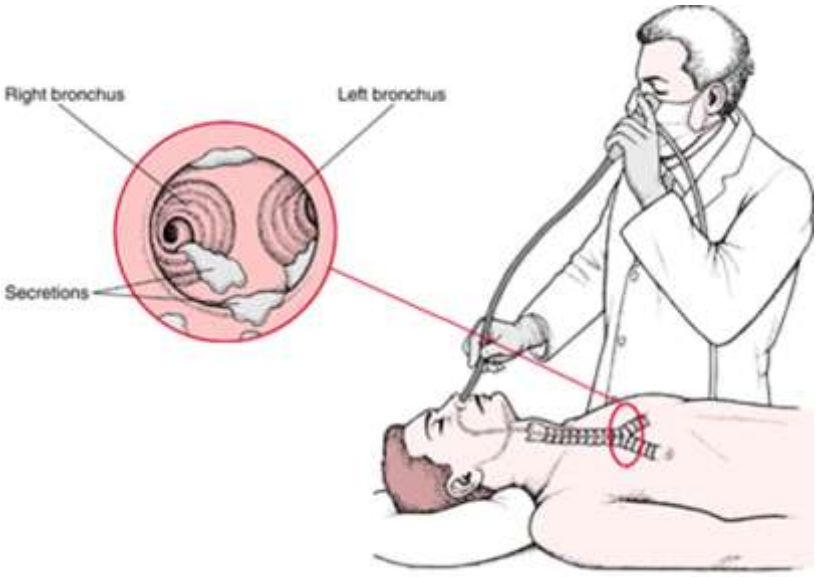

Figure 2. The bronchoscopy procedure is illustrated here by a very old and widely used figure. In this figure the bronchoscopy is performed transnasally by a man, while in this thesis it was performed transorally and by a female physician. Nowadays, a screen is used for a better view, instead of peering through the bronchoscope.

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Methods

Study design

Throughout the whole thesis, the study population described in detail above was assessed in a cross-sectional manner, retrieving bronchoalveolar lavage (BAL) samples bilaterally at one time point from each participant. Blood sampling was performed three times; the first time was during the first visit in preparation for the upcoming bronchoscopies and as a part of the inclusion. After inclusion, the participants were assigned to a time point of bronchoscopy with a pre-defined interval of 12, 24 or 48 hours (h) to the second bronchoscopy. The allocation of participants to either time point was performed on a practical basis, set both by the complex hospital logistics and by the volunteer's own schedule, but also as a result of the ambition to create similar groups with respect to gender distribution. All participants had to be healthy without any cold or other inflammations the month before the exposure to endotoxin. They were not allowed to consume any non-steroid anti-inflammatory drugs (NSAIDs) during the same period of time. The day before the first bronchoscopy, we were in contact with the participant to verify that he or she had been free from infection and inflammation during the last four weeks as well as being free from medication. On a few occasions, we had to substitute the defined volunteer, at short notice, with another person from the group of included volunteers waiting for the bronchoscopies.

Time line

All the participants went through three visits altogether, the first one described above. The participants then underwent two bronchoscopies, described as visits 2 and 3 on the time line below (figure 3). During the first bronchoscopy, the participant was exposed to intrabronchial endotoxin in one lung segment and to vehicle in the contralateral lung segment. The second bronchoscopy was performed 12, 24 or 48 h later, always in the morning between 8.00 and 10.00 a.m., and always during the winter season, between October and May. During this second bronchoscopy we performed bilateral bronchoalveolar lavages (BAL), starting with the vehicle-exposed lung. In 13 cases, we also obtained bronchial biopsies, which were needed for analyses in the second study. Blood samples were drawn immediately before both bronchoscopies; see time line in fig 3, where T stands for time, given in hours.

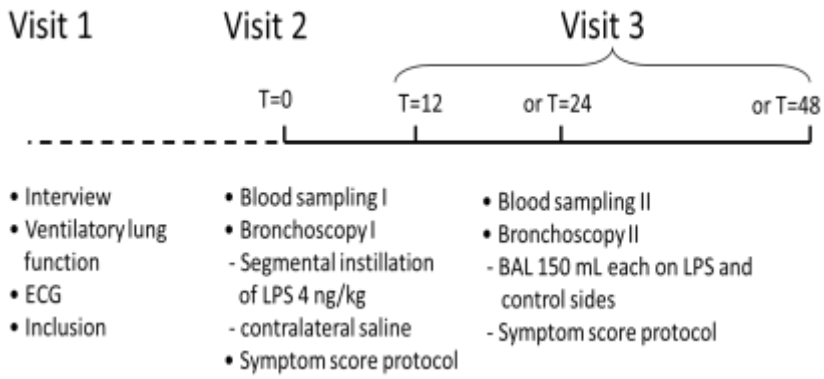


Figure 3. Time line for all studies.
The first visit was for inclusion after which the time interval to the second visit varied.
The second visit was always the first bronchoscopy with exposure to vehicle and endotoxin contralaterally. Blood samples were drawn immediately before the bronchoscopy procedure.
The third visit was always the second bronchoscopy with bronchoalveolar lavage bilaterally. It took place either 12, 24 or 48 hours after the first bronchoscopy. Blood samples were drawn immediately before the bronchoscopy procedure.
The PhD student performed all bronchoscopies, during a period of five years

Bronchoscopies

The first bronchoscopy took place in the morning, between 8.00 and 10.00 am in the 24 and 48 h-groups and in the evening, at 8.00 pm, in the 12 h-group. Consequently, the second bronchoscopy always took place in the morning, between 8.00 and 10.00 am. All the bronchoscopies were performed transorally with the participant in supine position. Olympus flexible fibreoptic bronchoscopes of several models were used (Olympus Co, Tokyo, Japan). The participant fasted according to clinical routine. In some cases, the participants in the 12 h-group got rehydration with intravenous fluid, due to the relatively long fasting period.

Intramuscular ketobemidonhydrochloride was given as premedication half an hour in advance, the dose depending on clinical condition, but it varied between 2.5 and 7.5 mg. Nebulized local anesthesia was sprayed into the oropharynx (xylocaine 10 mg/dose, 3x2 doses), followed by additional local anesthesia given through the bronchoscope (xylocaine 20 mg/mL, up to 14 mL). Endobronchial photographs were taken bilaterally during both bronchoscopies to ensure that the BAL sampling was performed in the very same bronchial segment that had previously been exposed to either vehicle or endotoxin, see figure 4.

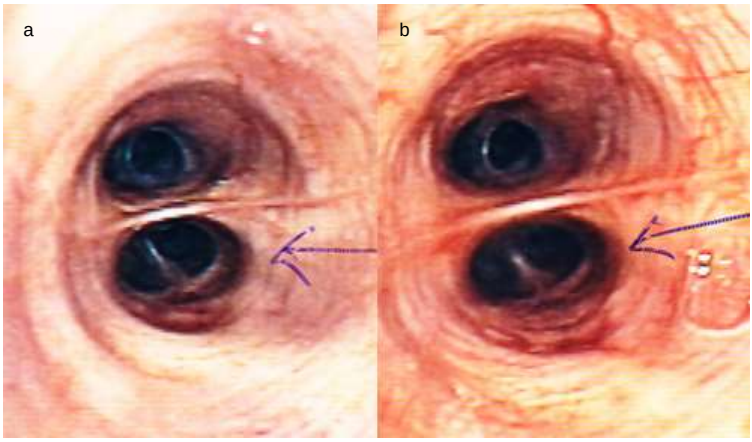


Figure 4. Endobronchial photographs from one volunteer, Right middle lobe segments are seen at a) first bronchoscopy just before exposure to endotoxin and b) 12 h post exposure. Arrows pointing to the actual subsegment of exposure and BAL.

Exposure to vehicle and endotoxin

The exposures took place during the first bronchoscopy. A balloon-tipped catheter was inserted through the bronchoscope and placed in the chosen segment, either lingula or right middle lobe (a change of sides was made for every other bronchoscopy), see figure 5. The balloon was inflated with air to seal off the chosen segment, after which the first segment was challenged with vehicle (10 mL of 0.9% phosphate-buffered saline, PBS) and subsequently with 10 mL of air. The bronchoscope was then retracted and moved over to the contralateral segment (either right middle lobe or lingula) where the sealing-off-procedure was repeated, this time followed by instillation of endotoxin (USP reference standard endotoxin from *Escherichia coli* 0113: H10, from USP, Rockville, MD, USA). The dose of endotoxin had previously been assessed to be both safe and efficacious (82). After bilateral exposures, the bronchoscope was withdrawn and the head of the operating table was elevated 30º for an hour, with the participant in place, to prevent the instilled fluid from the exposed segments from spreading throughout the bronchial tree.

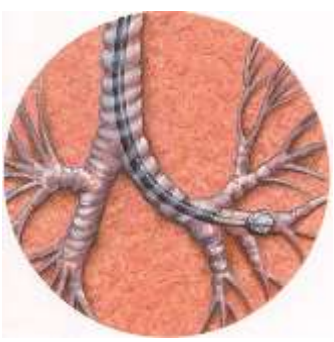


Figure 5. Intrabronchial balloon, wedged into a segment immediately before exposing the bronchial tree to either vehicle or endotoxin.

Bronchoalveolar lavage (BAL) sampling

The bronchoalveolar lavage (BAL) took place during the second bronchoscopy. The bronchial segments previously exposed to vehicle or to endotoxin respectively were re-identified. The bronchoscope was wedged in, starting with the vehicle-exposed segment, and BAL was performed (with the instillation of 3 x 50 mL of PBS at 37º C). After the aspiration of each 50 mL aliquot, the entire yield was collected in a siliconized glass container. The procedure was repeated contralaterally, in the endotoxin-exposed bronchial segment, and the BAL samples were instantaneously transported to our research laboratory.

Blood sampling

In addition to taking blood tests for the inclusion, venous blood was drawn immediately before both bronchoscopies. The blood samples were sent to the laboratory of Sahlgrenska University Hospital (accredited by the Swedish Board of Accreditation and Conformity Assessment, SWEDAC) for analysis of C-reactive protein (CRP), haemoglobin and leucocytes, both total (LPC) and differential counts. The instructions of the Sahlgrenska laboratory for the handling of samples and its reference values were used. Plasma and serum of additional blood samples were separated from blood cells, frozen and stored in -70º C for further analyses.

Symptom assessment

Clinical symptoms were assessed immediately before the premedication for both bronchoscopies and at three time points thereafter; at 1, 3 and 12 h, using a questionnaire. Both local pulmonary and systemic symptoms were recorded as well as their severity, graded by the subjects from zero=none, 1=mild, 2=moderate to 3=severe. At 3 h, the physician in charge also made a clinical evaluation of the participant. The 12 h-assessment was made by the participants themselves, at home, with the exception of the 12 h-group in which this assessment coincided with the evaluation immediately before the second bronchoscopy.

BAL samples

All BAL samples were immediately transported on ice to our research laboratory, where all the samples were initially treated in the same way. The recovery volume of BAL was measured and noted. The BAL sample was filtered for removal of

mucus and cell debris. The filtered BAL sample was then centrifuged (300 x G, 10 minutes at 4º C) and the supernatant, the cell-free BAL fluid (BALf), was separated and frozen at -80º C pending further analyses. The cell pellet from the centrifugation was re-suspended in PBS. Cell viability was then determined using trypan blue exclusion and a total cell count was carried out utilizing a haemocytometer (Bürker chamber). After counting 2 x 300 cells per sample using a conventional light microscope (Axioplan 2, Carl Zeiss®, Jena GmbH, Eching, Germany) differential counts for BAL cells were performed on the cytospin preparations according to standard morphological criteria.

Immunological analyses of BAL samples

The evaluation of the recovery of BALf and the viability of BAL cells, as well as differential counts were performed on all BAL samples from both sides in all participants, as described above. After the initial handling of the samples, the further analyses differed between the four studies.

Immunological analyses of BAL cells

Immunocytochemistry (ICC) was performed on BAL cells in papers I and III, and Immunocytofluorescence (ICF) in paper III. Flow cytometry was performed in both papers II and III, as was the real-time polymerase chain reaction (Real-time PCR). No further analyses on BAL cells were performed in paper IV.

Immunocytochemistry (ICC)

To determine the source of matrix metallo-proteinase (MMP)-9 in paper I, ICC was performed on BAL cells from all participants in the 48 h-group and the six participants who first underwent bronchoscopies in the 24 h-group. The method is further described in paper I, but the main principle is marking the cells with antibodies to MMP-9 and then visualizing the antibodies with staining substrate. The stained ICC samples may then be evaluated under a light microscope. The cells are first fixed, washed twice and air-dried, after which the samples are treated with donkey-serum to avoid unspecific binding before the biotin is blocked. After this preparation, the samples were marked with polyclonal goat anti-human MMP-9 antibodies and then with a second antibody against the first, after which the bound antibodies were visualized, in this case through a red alkaline substrate and counterstained with hematoxylin.

In paper III the same principle was used to detect the sources of interleukin (IL)-26, which had not been analyzed in human airways before, but with slightly different washing and blocking. To detect IL-26, a monoclonal mouse anti-human IL-26 antibody was used. Following the secondary antibody against the primary IL-26 antibody, another primary rabbit anti-human Cluster of Differentiation (CD) 68 antibody was used, followed by a secondary antibody against this one, resulting in a double ICC, not only detecting the IL-26 but also marking the type of cell, since CD68 is a human macrophage marker. The bound antibodies were visualized and final counterstaining was carried out with Methyl Green, for further information see the online supplement to paper III.

Immunocytofluorescence (ICF)

To verify the source of IL-26 in paper III, ICF was also used. The principle is similar to ICC. In paper III, again, the setting with antibodies against both IL-26 and CD68 was used, but both the secondary antibodies were marked with fluorescent dye, green and orange/red for IL-26 and CD68 respectively. The samples were then counterstained with a formula also preserving the fluorescence and imaged through a confocal light microscope. For further details, see the online supplement to paper III.

Flow cytometry

In order to investigate the subpopulations of lymphocytes expressing IL-17 and IL-26 (the target cytokines in paper II and III respectively), flow cytometry in a fluorescence-activated cell sorter (FACS) was performed for these two studies. The method is further presented in papers II and III respectively.

In paper II the BAL cells were stained with antibodies, conjugated with fluorochromes, against CD3, CD4, CD8 (surface antigens on T-lymphocytes, T helper cells and cytotoxic T cells respectively), CCR-6 (Chemokine receptor 6, typical for T helper cells), IL-23R (receptor of IL-23) and CD45RO (expressed on T memory cells). The cells were then fixed, permeabilized and stained with flourochrome-conjugated antibodies against the intracellular molecules IL-17, IFNγ and IL-22 (a member of the IL-10 family that was known to act together with IL-17), some of them double-stained as described in the paper, where details about the different fluorochromes can be found.

In paper III unsorted BAL cells from unexposed volunteers, i.e. not the endotoxin-exposed subjects included for this thesis, were pre-cultured *ex vivo* in media containing either vehicle or endotoxin, after which the cells were stained with fluo-

rochrome-conjugated antibodies against CD3, CD4, CD8 (N.B. not exactly the same fluorochromes as in paper II). After the cells had been permeabilized the intracellular molecules of RORCvar2, IL-17A, and IL-26 were stained with fluorochrome-conjugated antibodies. In both studies, matched isotype antibody controls were used and the gates were set manually to obtain the most specific results.

Real-time PCR

In paper II, assessment of mRNA encoding IL-17, IL-22 and transcription factors RAR-related orphan receptor alpha (RORα) and RORCvar2 (the transcription factor orchestrating the differentiation of Th 17 cells) in BAL cells and bronchial biopsies was made by using real-time PCR. Only BAL cells from the endotoxin-exposed bronchial segment were used, since the number of cells from the vehicle-exposed segment was too low. For specific details on purification, cDNA (complementary DNA) and devices, see paper II.

In paper III the same principle was used to assess mRNA encoding IL-26 in BAL cells from the same group of volunteers that donated their cells to the flow cytometry, but not from the exposed subjects included for this thesis. For details, see the online supplement of paper III.

Immunological analyses of cell-free BAL fluid (BALf)

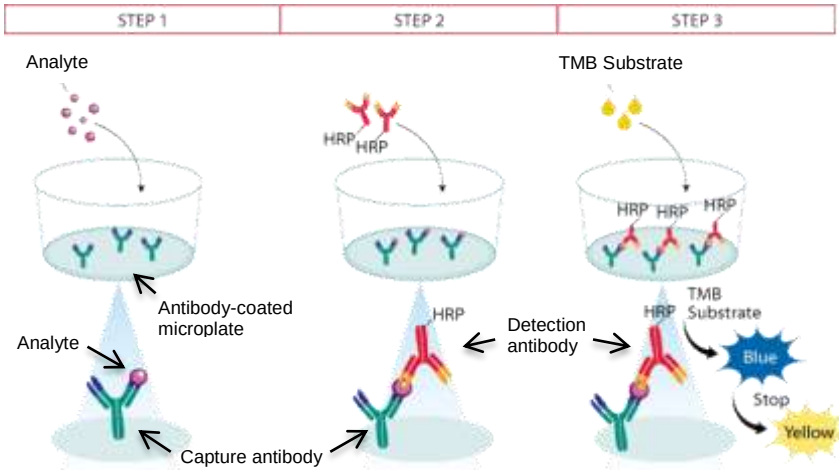
The supernatant after the centrifugation of the BAL samples was divided into several portions for different analyses, which were stored in -80º C in anticipation of the arrival of all samples.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used in all studies for assessment of soluble peptides and proteins in the BALf. The specific method for each protein is described, or referred to, in each paper but usually the samples and standards are incubated in a polystyrene microtiter plate with 96 wells pre-coated with antibodies recognizing the protein to be analyzed. The protein is captured by the antibody during incubation, whereafter unbound material present in the sample is removed by washing. The tracer or detection antibody, i.e. a second antibody against the protein is added and bound to a different epitope on the captured protein. After incubation, the excess antibodies are again washed away, and the process is repeated with a conjugate. The last step is to add a substrate to the wells leading to colour developing proportionally to the

amount of protein in the sample. The reaction is stopped, and the absorbance at a specific wavelength is recorded using a spectrophotometer. A standard curve is obtained by plotting the absorbance versus the corresponding concentrations of the known standards thereby determing the unknown concentrations of the samples.

Figure 6. ELISA method, as described on the homepage of R&D systems, used with the permission of R&D systems, a bio-techne brand



Step 1. A microplate pre-coated with capture antibody is provided. Samples or standards are added and any analyte present is bound by the immobilized antibody. Unbound materials are washed away

Step 2. A second HRP-labeled antibody (detection antibody) is added and bound to the captured analyte. Unbound detection antibody is washed away.

Step 3. Tetramethylbenzidine (TMB) substrate solution is added to the wells and a blue color develops in proportion to the amount of analyte present in the sample. Color development is stopped turning the color in the wells to yellow. The absorbance of the color at 450 nm is measured.

In paper I the concentrations of the proteinases MMP-9 and MMP-2, as well as the anti-proteinases tissue inhibitor of metalloproteinase (TIMP)-1 that is specific to MMP-9, secretory leukoproteinase inhibitor (SLPI) and α-1-antitrypsin (both inhibiting neutrophil elastase (NE) on different levels in the airways), were measured by ELISA. For further details, see paper I. The serine protease NE was determined using a solid-phase ELISA and was analyzed in The Netherlands.

In paper II all cytokines but IL-17 were analyzed by ELISA, i.e. IL-8/CXCL-8 (a neutrophil chemotactic factor, whose release is stimulated by IL-17 and which in turn upregulates MMP-8) IL-15, -21, and -23 (which were all known to regulate IL-17 in different ways), IL-22, as well as MMP-8 and hβD-2 that can be upregulated

by IL-17 and IL-22 in synergy. For the analyses of the interleukins, except IL-8, the BALf had to be concentrated 10-20 times but the results are presented as the concentrations of native BALf.

In paper III, ELISA was used to assess IL-26 and the neutrophil-mobilizing factors IL-1β, TNF-α, IL-8, and GM-CSF, as well as MPO, a neutrophil-derived enzyme of importance in killing microbes. A phospho tracer ELISA was also utilized to measure the intracellular phosphorylated signal transducer and activator of transcription (STAT)-1 and STAT-3 on a similar principle, but with fewer steps, since the samples and reagents could be added at the same time. This assay has to be read by a fluorescent reader.

In paper IV, the concentrations of both antimicrobial peptides (AMPs) LL-37 and Calprotectin were analyzed by ELISA.

Radioimmunoassay (RIA)

To determine the protease human neutrophil lipocalin (HNL) for paper I a double-antibody RIA was used (135). The principle is similar to that of an ELISA, but instead the antibodies are radiolabeled and special equipment is needed since the set-up is radioactive. The RIA in paper I was performed in Uppsala. For further details, see paper I.

Zymography

Zymography is based on gel electrophoresis, a method in which molecules are separated by charge and size. By applying an electric field to a matrix loaded with the substrate the negatively charged molecules are forced to move across the matrix. The migration, visualized by a band, corresponds to a reference level representing the molecular weight. Zymography was used in paper I to quantitify expression of MMP-2 and -9 and the results were compared to those detected by the ELISA. See figure 7, right below.

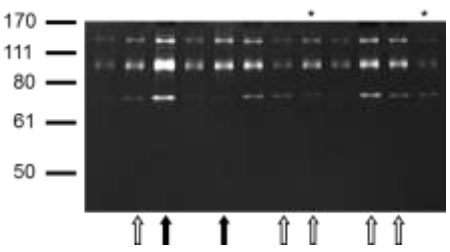


Figure 7. Photo of representative zymography gel, based upon analysis of BALf samples from endotoxin- and vehicle-exposed bronchial segments, showing three bands representing different molecular weights, corresponding to matrix metalloprotease (MMP)-2 (72kD), MMP-9 (92kD) and a complex bound form of MMP-9 (130kD). Black and white arrows designate BALf from endotoxin-exposed segments 24 and 48h after exposure, respectively. Bands not marked with arrows show lanes resulting from analysis of BALf from vehicle side. *Two lanes from the same subject.

Protease activity

In paper I, the activity of two main proteinases was measured through fluorescence assays. Since MMP-9 is a gelatinase, gelatin was fluorescently tagged and incubated with BALf samples leading to degradation of gelatin according to the amount of active MMP-9 in the sample. The fluorescence intensity was then measured.

The same principle was used to evaluate the activity of serine protease (NE) using a tagged substrate of serine peptides and measuring the relative absorbance intensity of the degraded peptides after incubation with the BALf.

Immuno-qPCR

To determine the concentration of IL-17 in BALf a sensitive method was needed and a specific IL-17 immuno-qPCR was developed for this purpose. For details on this specific assay, see paper II.

Migration assay

In paper III, migration assay was used to evaluate neutrophil chemotaxis. It was performed on blood neutrophils, but not on BAL cells from the exposed participants in our setting. Briefly, a 96-well plate was filled with the chemoattractants to be examined, in this case IL-26 in different concentrations with or without IL-8 and fMLP (formylmethionine-leucyl-phenylalanine, a potent PMN chemotactic factor). The fluorochrome-labelled neutrophils were loaded on an adjacent filter and the fluorescence was measured after incubation. The method is thoroughly described in the online supplement to paper III and its reference.

Western Blot

This method is based on gel electrophoresis just like the zymography. Western blot was used in paper IV to differ between the concentrations of the precursor and active form of LL-37 and is described in detail in paper IV.

Statistical methods

Mainly non-parametric statistical methods were used all through the thesis, since normal distribution of data could not be proved. The exception is paper III, in which parametric statistics were applied. See online supplement to paper III for details. With few exceptions, the SPSS 19.0 (SPSS Inc, Chicago, Ill, USA) software package was utilized for statistical analyses. Data are presented as median values and ranges unless otherwise stated. Differences between paired samples, i.e. comparisons within the same participant, were analyzed using the non-parametric Wilcoxon Signed rank test. Unpaired comparisons of the effect of endotoxin exposure between time points (samples from different volunteers) were made by calculating the difference between results from the endotoxin- and vehicle-exposed segment within each participant, at each time point, followed by a comparison of these differences between time points, using the Mann Whitney U-test. For correlations between individual data in different groups Spearman´s two-tail rank correlation test was applied. P-values<0.05 were accepted as significant.

Results

Clinical evaluation of participants (all papers)

Since endotoxin, which was used in all studies in this thesis, is such a strong pro-inflammatory agent, safety had to be taken into account when exposing healthy individuals to LPS and performing bronchoscopic procedures. A thorough evaluation was made of the participating volunteers, as previously described. The results of the symptom assessment is presented in table 3 below. The participants themselves generally described symptoms as mild or moderate.

Table 3. Side effects associated to the first bronchoscopy and endotoxin exposure

Symptoms and signs		N	%	Ratio q/m
No Symptoms		2	6	2/0
Systemic:	*Combinations*	21	68	9/12
	Nausea	13	42	6/7
	Headache	13	42	6/7
	Myalgia	11	35	4/7
	Fever	2	6	0/2
Local:	*Combinations*	4	13	2/2
	Cough	16	52	6/10
	Rhonchi	4	13	1/3
	Chest pain	4	13	1/3
	Increased mucus	20	65	9/11
	Dyspnea	1	3	1/0

Self-reported side effects associated with the first bronchoscopy, and intrabronchial exposure to endotoxin (and vehicle), in 31 healthy volunteers (age 20-29), equally women and men. N=number of individuals, %=percentage of total number of volunteers experiencing side effects, Ratio q/m= gender distribution, q being women and m being men. Symptoms may either reflect being exposed to endotoxin in the airways or be attributed to the bronchoscopy procedure itself.

Blood samples

The blood samples drawn immediately before the first bronchoscopy were all normal, see table 4. Due to technical reasons data could not be retrieved from two participants. In association with the second bronchoscopy, at 24 and 48 h, but not at 12 h, CRP was slightly increased compared to the baseline. LPC and PMN counts increased somewhat earlier, at 12 and 24 h compared to baseline.

Table 4. Systemic reaction after endotoxin exposure in all, but two, participants

Time point		CRP (mg/L)	LPC (x 10^9/L)	PMN (x 10^9/L)
Baseline	(N=29)	2.5 (2.5-6)	6.0 (3.9-9.2)	3.0 (1.5-6.4)
12 h	(N=8)	2.5 (all) δ	11.3 (9.9-13.3)* δ	8.8 (6.9-9.6)* δ
24 h	(N=15)	7 (2.5-65)**	7.0 (4.9-10.4)**	4.6 (2.1-7.6)**
48 h	(N=6)	8 (2.5-30)*	5.0 (3.9-8.8)	2.4 (2.0-5.7)

Analyses of serum and blood samples collected before and after endobronchial exposure to endotoxin and vehicle. CRP; C-reactive protein (reference value <5mg/L), LPC; leukocyte particle count (reference value 3.5-8.8 x 10^9/L), PMN; polymorphonuclear leukocyte count(reference value 1.8-7.5 x 10^9/L). Median values (range) are shown. *=p<0.05, **=p<0.01, comparing time points to baseline (paired analysis). δ=p<0.01, 24h vs 12h (unpaired analysis).

Bronchoalveolar lavage samples (all papers)

Generally, the recovery of BAL fluid was good, never less than 80 milliliters and usually more, see table 5. The viability of BAL cells were in line with other studies on BAL (136), and significantly higher in samples from the endotoxin-exposed segments compared to the vehicle-exposed ones.

Table 5. Recovery of BAL and viability of BAL cells in all included participants

	Recovery (mL)	Viability (% of cells)
Vehicle side	102 (91-120)	85 (62-95)
Endotoxin side	100 (80-113)**	95 (74-99)**

Recovery of and cell viability in bronchoalveolar lavage fluid (BALf) samples from lung segments exposed to vehicle (Veh) and endotoxin (E), respectively, in 31 healthy volunteers. Samples were harvested 12 (n= 8), 24 (n=16) and 48 (n=7) hours after exposure. Median values (range) are shown. **E vs V; p<0.01

BAL cells

The total concentrations of BAL cells were higher in the endotoxin-exposed bronchial segments compared to the vehicle-exposed segments at all time points. A dynamic pattern can be distinguished with a prompt influx of cells already at 12 h post-exposure on the endotoxin sides and the amount of cells receding over time thereafter. In table 6, only the total cell counts are shown. The differences in differential counts are reported separately in the papers.

Table 6. Total concentrations of BAL cells x 10^4/mL BALf.

Time point		Vehicle side	Endotoxin side	Increase x-fold
12 h	(N=8)	10 (4-42)	164 (75-297)	14.0 (3.6–66)
24 h	(N=16)	11 (5-26)	98 (37-354)	7.9 (2.2–57)
48 h	(N=7)	15 (11-23)	66 (20-127)	3.8 (1.5–7.5)

Total concentrations of BAL cells in BAL samples from all participating volunteers; comparing the vehicle-exposed segments to the endotoxin-exposed ones. Median (extreme values) are shown. All differences between the vehicle-exposed and endotoxin-exposed segments are significant, $p<0.05$.

Proteolytic homeostasis (paper I)

The analyses made for evaluation of the balance of proteases and anti-proteases included both immunological examinations on cells and on cell-free BALf, as described in the methods section. The study also included activity measures to give a more comprehensive understanding of the proteolytic homeostasis in healthy airways at 24 and 48 h after endotoxin exposure. At both these time points BAL cells in general, and neutrophils (PMNs) and alveolar macrophages (AMs) in particular, were higher on the endotoxin-exposed side, as described in the paper. In addition, lymphocytes and eosinophils were significantly affected at both time points. See figure 8, on page 54. All cell concentrations but the eosinophils are presented using a logarithmic scale.

The immunological analyses on BALf showed higher levels of matrix metalloproteinases (MMP)-2 and -9 after endotoxin exposure, at both the evaluated time points, 24 and 48 h, but with no significant difference between time points (figure 2 in paper I). Using zymography analyses, it was concluded that MMP-9 was the dominant gelatinase, see figure 7, on page 47, for a photographic example of a zymography gel. Comparison of the intensities of the bands of MMP-9 and MMP-2 verified MMP-9 as the most prominent gelatinase. On ICC-slides MMP-9 could be seen in neutrophils in all the samples, but visually ICC gave the impression of neutrophils with MMP-9 being engulfed by macrophages at 48 h. See figure 9, on page 54. The anti-protease TIMP-1 was also higher in the endotoxin-exposed segments compared to the vehicle-exposed ones, with no significant difference between time

points. The net activity of the gelatinases, i.e. predominantly MMP-9, was found to be about the same on both the endotoxin- and vehicle-exposed sides and at both time points and thus no significant gelatinase activity could be seen in healthy peripheral airways at 24 and 48 h after endotoxin exposure.

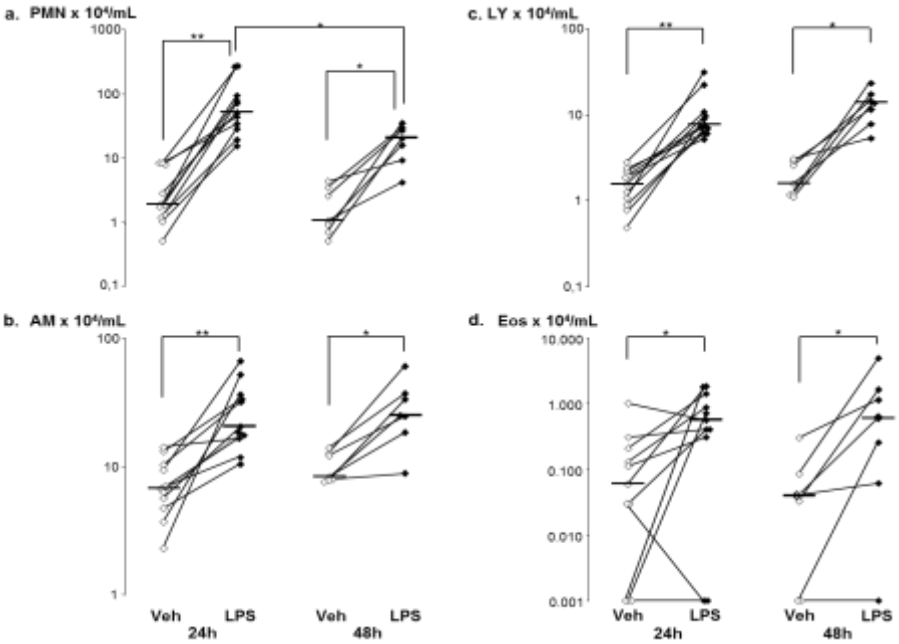


Figure 8. BAL cell concentrations of a) polymorphonuclear leucocytes (PMN), b) lymphocytes (Ly), c) alveolar macrophages (AM) and d) eosinophils (Eos) from vehicle (Veh) and endotoxin (LPS) exposed lung segments, harvested 24 (n=11) and 48 (n=7) hours after exposure. Median and individual values are shown. Significant differences between sides at each time point and between time points are indicated; *=p<0.05, **= p<0.01.

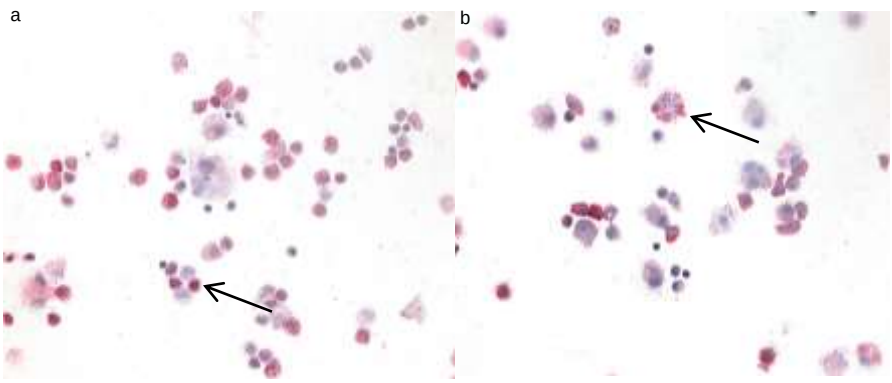
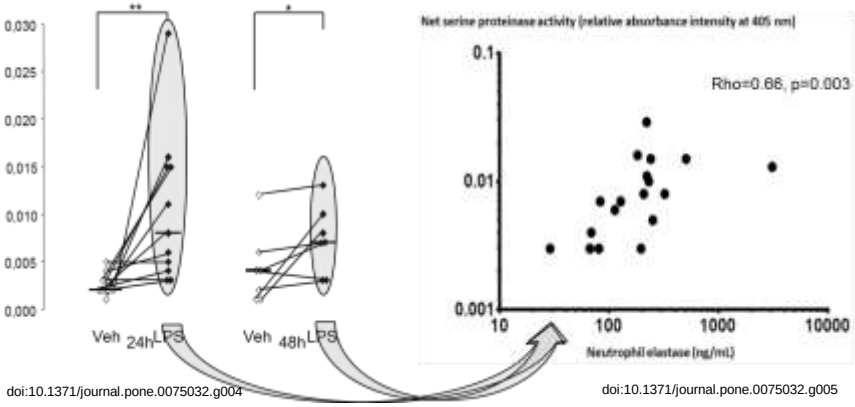


Figure 9. BAL cells from the endotoxin-exposed lung segments, stained with MMP-9 detecting antibodies, intracellular staining of MMP-9 is seen as red. a.) at 24 h, arrow points to a PMN, b.) at 48 h, arrow points to an AM.

The neutrophil specific human neutrophil lipocalin (HNL) concentration was higher in samples from the endotoxin-exposed sides at both time points, with no significant difference between the time points. The concentrations of neutrophil elastase (NE), also known as leucocyte elastase and one of the serine proteases, were higher in the endotoxin-exposed segments at both time points with no significant difference in between (figure 4, in paper I). The other two serine proteases of the neutrophil primary granulae, cathepsin G and proteinase 3 (137), were not analyzed in this study. The concentrations of NE itself showed a correlation to the concentrations of PMNs, the cell from which NE is presumably released, a correlation that was stronger at 48 h after endotoxin exposure (rho=0.82, $p<0.05$) than at 24 h. The anti-proteases of NE, which are SLPI, also known as antileukoproteinase, counteracting NE in the airways, and α-1-antitrypsin (formerly α-1-protease inhibitor) inhibiting NE in the alveoli, were both higher on the endotoxin side. α-1-antitrypsin was higher at both 24 and 48 h, with no significant difference between time points, although SLPI was only significantly affected by the endotoxin at 48h (figure 4 in paper I). In total, the net serine protease activity ended up significantly higher on the endotoxin side at both time points. The net serine protease activity correlated to the concentration of NE, see figure 10.



doi:10.1371/journal.pone.0075032.g004

doi:10.1371/journal.pone.0075032.g005

Figure 10. Net serine protease activity correlating to the concentrations of neutrophil elastase (NE) at both time points, in the endotoxin-exposed segments. The figure illustrates the selection of samples for the computation of correlations. Net serine protease activity at both time points are shown to the left, in arbitrary units.

Cytokines (paper II and III)

In papers II and III, the focus was on the cytokine signaling in the innate immunity. Two relatively new, but above all very interesting, interleukins, namely IL-

17 and IL-26, were both analyzed considering their up- and down-regulating properties in the innate immune response after endotoxin exposure.

Based on animal models and *in vitro* experiments, the Th17cells were to be explored in human airways after endotoxin-exposure at 12 and 24 h. In doing so, a sketch of a typical animal, or human blood, Th17 cell was drawn, see figure 11, for comparison with our results.

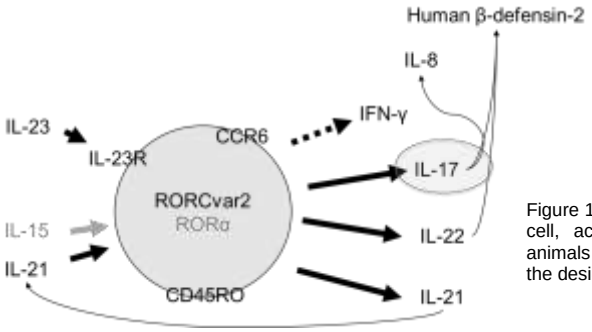


Figure 11. A sketch of a presumed Th17 cell, according to former studies on animals and blood cells, at the time of the design of study II

An assessment of the mediators included in the figure above showed higher levels of the interleukins that were supposed to stimulate the cell (138), IL-21 and IL-23. They were both significantly higher in the endotoxin-exposed lung segments compared with the vehicle-exposed ones at 12 h and tended to be higher at 24 h. IL-15, which had been shown to trigger the production of IL-17 in peripheral blood monocytes, *in vitro* (139), was significantly lower in the endotoxin-exposed segments at both time points.

The concentrations of IL-22 were higher in the endotoxin-exposed segments at both time points. IL-17 was only significantly higher at 12 h, although there was a tendency to higher levels also at 24 h. IL-22 and IL-17 had been shown to be functionally related when it came to stimulation of MMP-8 and the AMP hβD-2, as well as the subunits of calprotectin (140). The two former were analyzed for this paper, and MMP-8 was significantly higher in the endotoxin-exposed segments. The AMP hβD-2 was higher after endotoxin exposure at both time points, as shown in the paper.

The flow cytometry performed for paper II revealed that the IL-17 producing $CD3^+CD4^+$ (Th) cell expressed CD45O. The cells turned out to be $IL\text{-}23R^-$ and there was no trace of IFNγ-expression. On the contrary, the Th cells that did not produce IL-17 did express the IL-23 receptor. Moreover, the $IL\text{-}17^-$ cells stained positively for IL-22, although the $IL\text{-}17^+$ did not. On the other hand, mRNA in samples from the endotoxin side was positive for both IL-17 and IL-22 as well as for the transcription factors RORα and RORCvar2. IL-17 correlated strongly to the latter.

The other proinflammatory cytokine in focus, IL-26, had been shown in the human intestines and joints (141), but so far not in human airways. The concentrations of IL-26 in BALf were analyzed in BAL samples from all three time points; 12, 24 and 48 h. At all three time points higher levels of IL-26 could be seen in the endotoxin-exposed segments compared to the vehicle-exposed ones. BALf samples from healthy individuals without previous exposure to endotoxin *in vivo* were cultured and exposed to endotoxin *in vitro* after which a similar response was seen. See figure 1 a and b, respectively, in paper III. The mRNA analyses also displayed the same pattern.

IL-26 correlated to neutrophils and macrophages in BAL from healthy volunteers who had not been exposed to endotoxin *in vivo*, but IL-26 did not correlate to lymphocytes from the same individuals. Statistical calculation also revealed similar correlations to neutrophils and macrophages in the endotoxin-exposed study population. BAL cells from the endotoxin-exposed study population were analyzed with ICC and ICF to determine the intracellular expression of IL-26. The results of the ICF analysis may be seen on the cover of this thesis. Both analyses showed that the macrophages dominated in expressing the immunoreactivity of IL-26. This was verified by analyzing the unexposed BAL cells *in vitro*, both through FACS and mRNA.

The significant correlation between IL-26 and neutrophils was thus not attributed to neutrophils expressing IL-26. Therefore, migration analyses were performed. IL-26 proved to potentiate chemotaxis on blood neutrophils. Further *in vitro* analyses were made to evaluate the effect of IL-26 on neutrophils as well as on epithelial cells and IL-26 was shown to have opposite effects on the different cells when it came to neutrophil-mobilizing factors like IL-8, IL-1β, TNF-α and GM-CSF. The release of these mediators was increased in BAL cell medium, but decreased in epithelial cell medium, after stimulation with IL-26. Moreover, IL-26 inhibited release of MPO in a neutrophil-rich media of BAL cells. Last, but not least, IL-26 did not show any effect in a medium of bronchial epithelial cells on the AMPs SLPI and calprotectin while LL-37 and hβD-2 were not even detectable in the samples.

Antimicrobial peptides (mainly paper IV)

In Paper IV, antimicrobial peptides in BAL were determined, although two antimicrobial peptides, secretory leukoproteinase inhibitor (SLPI) and human β-defensin (hβD)-2 were analyzed for papers I and II respectively, but for other purposes. SLPI was analyzed at 24 and 48 h and was hardly affected until 48 h post-exposure, when it increased significantly in the endotoxin-exposed segments (figure 4 in paper I). In study II, hβD-2 was analyzed at 12 and 24 h after endotoxin expo-

sure as a marker of the release of IL-17 and IL-22. The AMP hβD-2 was increased at both these time points (figure 6 e and f, in paper II).

An increase of LL-37 at the same time points in the endotoxin-exposed segments could be proved. There was a significant difference in concentrations of total LL-37 between 12 and 24 h. The concentrations of the precursor of LL-37 (hCAP-18) were significantly higher at both time points on the endotoxin side, with no significant difference between time points. The concentrations of the active form of LL-37 was higher in the endotoxin-exposed segments at 24 h, but not significantly higher at 12 h. Calprotectin was present but remained unchanged irrespective of exposure. In addition to the initially performed differential counts on the BAL cells, no further analyses on cells were made to evaluate AMPs.

Discussion

The endotoxin model

The effect of the transient stimulus of endotoxin was used in all four studies in this thesis, as a simulation of a Gram-negative infection, or of acute exposure to cigarette smoke. Endotoxin is a very relevant stimulus, as has been pointed out, but appears to be even more appropriate since cigarette smoke upregulates the endotoxin receptor TLR4 in airway epithelial cells *in vitro* (96) as well as in smokers *in vivo* (98), regardless of whether the smokers suffer from COPD or not. Gram-negative bacteria in turn often colonize the airways of patients with COPD (103, 123). Moreover, Gram-negative bacteria are the cause of pneumonia more often than one may imagine (107, 108) and often cause the exacerbations in COPD as well as CF (90). Since the endotoxins in the outer layer of the different species of Gram-negative bacteria show greater similarities than differences, LPS from *E. coli* was used in this thesis, as it is generally used in studies of the effects of Gram-negative infections in airways (80, 81), irrespective of studies in mice or in men.

A model was chosen in which the participants were healthy, since the aim was to study the normal physiological response of the innate immune system to a single potent proinflammatory stimulus. Moreover, the model with the very compartmentalized exposure to endotoxin allowed the participants to serve as their own controls, an aspect that is obviously beneficial when analyzing the results and drawing conclusions.

We may never know whether the dose of endotoxin was adequate, or perhaps even excessive, to resemble a real pneumonia or mimic the exact inhalation dose of smoke from cigarettes, but the local endobronchial dose was adequate to elicit a clear and reproducible inflammatory response. The specific dosage we utilized had previously been tested and proved to be sufficient and efficient (82). Furthermore, only a slight impact on the serum data was percieved, suggesting a mild and limited systemic inflammation. The data confirm that the effect of endotoxin was certainly located in the airways and that the endotoxin did not affect the rest of the body to any significant extent. Moreover, the absence of severe symptoms in our symptom assessment reassured us of the safety of the model and supported the assumption of a strictly localized inflammation.

Inflammatory cells

In all the volunteers a great influx of inflammatory cells was found following local endotoxin exposure (table 6, page 53), whereas the total concentrations of cells in the vehicle-exposed segments were within normal levels for non-smoking young individuals (142). The viability of BAL cells was in general higher in samples from the endotoxin-exposed segments, suggesting that the inflammatory cells, mainly the macrophages and neutrophils, are early activated and primed to further stimuli as has been described after smoke injury to the lungs (143). This priming of cells and increased sensitivity to further stimulus can also be seen in patients with COPD exposed to LPS by inhalation (144).

Macrophages situated in the alveoli are long-lived sentinels of the alveolar space waiting for action and were indeed activated in our model, but the model of newly monocyte-derived macrophages is not likely to be applicable here, considering the time points measured (6). Furthermore, neutrophils are short-lived, but produced in such enormous amounts in young and healthy humans that they make up a pool, from which they may easily be mobilized. The PMNs are quickly in place, easily activated, and dominate both in terms of total cell counts and fractions of the differential counts (see paper I; figure 1 and table 1, and the online supplement of paper II; table 2). Notably the increases in neutrophils in papers I and II are similar to that seen in acute bacterial pneumonia, but the neutrophil dominance was not so pronounced in our studies (145). The concentrations of neutrophils in our studies peaked at 12 h. In an animal model of inhalation of endotoxin, PMNs were shown to peak at 4 h in lung tissue and at 24 h in airways (79). Presuming that the animal model is applicable in humans, also after intrabronchial exposure to LPS, there should be a faster neutrophil response to LPS in the very periphery of the airways (the lung tissue) and a slower response more proximally (sputum). In our setting the concentrations of neutrophils peaked at 12 h, which is in between, which is why it may be assumed that the LPS in our setting reaches peripherally in lungs, where it should; our intentions were to illustrate events in the alveoli. Moreover, an earlier time point was not assessed in our studies, which is why we do not know the neutrophil counts at 4 h. The fast response of neutrophil influx in the peripheral lung may be referred to the very thin epithelial-endothelial barrier in the alveoli.

In figure 8, on page 54, the concentrations of lymphocytes and eosinophils are also shown. The eosinophils are found in such low concentrations that they are probably not of importance, but it may be speculated on the relevance of the concentrations of lymphocytes, which were higher in the endotoxin-exposed segments at 24 and 48 hours. Comparing this figure with tables 2 and 3 in the online supplement for paper II, it can be seen that the absolute concentrations of lymphocytes on the endotoxin-exposed side are also higher at 12 h. The relative concentration,

i.e. the percentage in relation to the other leukocytes, is lower on the endotoxin side, depending on the relatively higher influx of neutrophils and macrophages. However, considering that more than half of the lymphocytes are Th cells (see table 3 in the online supplement for paper II) it may be assumed that a few might be Tregs, even if we did not analyze the BAL samples regarding Tregs. These findings might suggest that the regulatory system came into play in our setting even if the proof is very tentative (117). Nonetheless, the relation of macrophages and neutrophils, the latter also in absolute lower levels at later time points, supports the theory that the resolution of inflammation after a single inflammatory stimulus starts within a few hours after exposure (146).

Proteolytic homeostasis

The balance between the proteases and anti-proteases is essential for keeping the airways healthy. In paper I, the peripheral airways were examined at 24 and 48 h after endotoxin exposure, with the intention of exploring whether this balance, the proteolytic homeostasis was disrupted after a single exposure to endotoxin.

An influx of inflammatory cells was found and visually, through the ICC, MMP-9 was determined intracellularly. The location of the intracellular MMP-9 appeared to differ between time points, in contrast to the levels of MMP-9 in BALf that did not change significantly over time. In samples from the endotoxin-exposed segments, MMP-9 was observed predominantly within the neutrophils at 24 h. At 48 h these neutrophils, with their MMP-9, seemed to be engulfed by macrophages (see figure 9, on page 54), which is in line with the results in previous studies showing macrophages engulfing DAMPs (147, 148). This difference between time points is also in line with the peak and resolution of inflammation caused by the inhalation of endotoxin (146). In addition, at 48 h, the neutrophils were declining and the fraction of macrophages had increased in our setting. In the vehicle-exposed segments, intracellular MMP-9 could be demonstrated through the ICC in a similar way, but to a lesser extent. The conclusion that may be drawn from the ICC results is that a well-functioning cellular response to LPS may inhibit the release of MMP-9 in a physiological way, thus maintaining the proteolytic homeostasis, and that exposure to LPS leads to a transient inflammation already well on the way towards resolving within two days.

The absence of net gelatinase activity confirmed that the proteolytic homeostasis concerning gelatinases is actually maintained in healthy human airways, even after exposure to a single potent proinflammatory stimulus like endotoxin. The concentrations of MMP-9 and its anti-proteinase TIMP-1 changed in parallel on both the endotoxin- and vehicle-exposed sides in our setting. This balance is prob-

ably physiological, while the imbalance seen in patients with COPD and emphysema is detrimental (24, 124), although the exact mechanism behind the prolonged increase of MMP-9 in the airways of COPD patients in not completely clear. A correlation of the levels of MMP-9 (and levels of MMP-8 as well) in BALf and computer tomography signs of small airway disese in COPD has been found (25), without clarifying anything about the development of disease. Stimulated macrophages which release MMP-9 have been suggested (122) as well as a vicious circle with initially primed neutrophils releasing mediators leading to chemotaxis and the recruitment of more neutrophils (32). In other disease states, like ALI, the increase of MMP-9 is believed to be closely related to the influx of neutrophils. In these cases direct inhibition of MMP-9 could be of value (22). The knowledge that MMP-9 is balanced to its counterparts in healthy peripheral airways after a potent proinflammatory stimulus is perhaps an indication that additional factors, such as smoking, are necessary in developing chronic inflammatory disease in lungs.

In contrast, the balance between the proteases and anti-proteases was indeed disrupted when it came to serine proteinases, mainly NE in our setting. Even if NE and its strong anti-proteinase α-1-antitrypsin were both higher in the endotoxin-exposed segments at both time points compared with the vehicle-exposed segments, the other anti-proteinase SLPI was not. SLPI did not increase until 48 h post-exposure. This was unexpected considering the fact that SLPI is an antimicrobial peptide. The net activity of serine protease on the endotoxin side was significantly higher at both time points, more so at 24 h. This effect probably mirrors the lack of elevation of SLPI-levels at 24 h. This difference between time points shows that SLPI is of relatively greater importance than expected, considering that processes in the most peripheral airways are studied, where the inhibition by α-1-antitrypsin is presumed to dominate (149). On the other hand, in peripheral airways it could well be that the concentration of α-1-antitrypsin is not sufficient, resembling the situation in the CF airway where the response of the immune system is overwhelmed by the amount of NE (127), although naturally in our setting to a much lesser extent. Furthermore, the effect of α-1-antitrypsin might be impaired due to a downregulation by NE (149). There is also new evidence that NE even might play a role in the resolution of inflammation (150), and consequently that the imbalance towards excess serine proteinase activity found in our model may unexpectedly be physiological. In our setting, the net serine proteinase activity was correlated to the concentrations of NE, which is why it was speculated that the other serine proteinases cathepsin G and proteinase 3 could be of less relevance for the findings in this study.

In total, the results of paper I show that endotoxin exposure of healthy peripheral airways creates an imbalance in the proteolytic homeostasis with excess serine proteinase activity both at 24 and at 48 h post-exposure. The relevance of this find-

ing in relation to the development of chronic inflammatory lung diseases is however still unclear.

Cytokines

In papers II and III, we performed both descriptive and functional studies primarily on two cytokines, IL-17 and IL-26. Both had formerly been described in animal models and in other human tissues than the lung (139, 141), but the IL-17 releasing cell was not properly characterized in human airways. Our aims were to both demonstrate the Th17 cell in human lungs *in vivo*, and characterize the Th17 cell and it associated cytokines in relation to a strong proinflammatory stimulus. Moreover, the aim of paper III was to investigate the presence and role of IL-26 in the human immune defence of healthy airways *in vivo*, in response to the same stimulus.

The Th17 cell and IL-17

Through a segmental exposure to endotoxin, we were able to demonstrate the presence of IL-17-producing memory Th cells in human peripheral airways along with increased levels of IL-17 in BALf. The putative Th17-stimulating interleukins IL-21 and IL-23 were also higher in the endotoxin-exposed segments mainly after 12 h, just like IL-17. However, in our analyses we found that the IL-17-producing cells did not express the receptor of IL-23. This was in contrast to what had been shown earlier in murine models. In these, IL-23 had been shown to stimulate the $CD4^+$ T cells to produce IL-17 (151). Nonetheless, in another animal study *in vitro*, the TLR4-induced stimulation of IL-17 production could also be mediated through $CD8^+$ T cells (138). In our study the cytotoxic T cells were however hardly expressing IL-23 at all (online supplement of paper II; table 3). In addition to the specific Th17 cells in BAL, there is evidence from studies in mice of cells other than T lymphocytes producing IL-17, both in lung tissue and in BAL (152). This interpretation could be applicable in our study. Moreover, in former studies on humans, discrepant views on the effect of IL-23 on the mere production of IL-17 has been presented (153). Further studies have neither confirmed the specific Th17, nor revealed the fraction of Th17 of all IL-17-producing cells in human airways. A negatives feedback on IL-23, by IL-17, has been shown on human alveolar macrophages (154) which might have relevance in the differentiation of Th cells into IL-17-producing cells (155), although probably not on the release of IL-17 from the specific Th17 cell.

Further characterization of the Th17 cell in our setting revealed that it did not produce IL-22. The production of IL-22 might have been expected according to previous animal studies. On the other hand, IL-23 signaling had been shown to be crucial for the cooperative production of IL-17 and IL-22 from the same cell (140), which is probably why IL-17 and IL-22 were not produced within the same cells in our setting. Furthermore, IL-22 has been shown to be mainly released by other types of lymphocytes, as well as by a lineage of the relatively newly defined innate lymphoid cells (ILCs) (156). In humans, IL-22 has been demonstrated in alveolar macrophages after stimulation with LPS (157) and in epithelial cells *in vivo* (158). In addition, in our study IL-22 was higher in the endotoxin-exposed lung segments at both time points indicating that IL-22 and IL-17 would probably not have been released simultaneously.

Furthermore, in a study of patients with CAP, with BAL taking place within 24 hours of the onset of disease, T cells expressing both IL-17 and IL-22 were found, as well as single IL-17$^+$ and single IL-22$^+$ cells (159). However, in that study population there was a mix of non-smokers and smokers as well as patients with COPD. In these patients suffering from pneumonia of various etiology (Gram-positive as well as Gram-negative or unknown microbial origin), there was a tendency to relatively more IL-17$^+$ cells in BAL, compared with in the blood tests. The results from that study indicated that all three T cell types existed simultaneously in the airways in patients suffering from CAP. This might be expected, since it has been proposed that the Th17 cell has retained its plasticity and its ability to express divergent cytokines, thereby adapting its function on the basis of the signals it receives (155). Following this line of thought, the Th17 cells found in our study are suited to act on the LPS stimulus, but not to act on other bacteria lacking LPS as a structural component. Findings in the airways of patients with CF can be said to confirm this claim in that several other types of IL-17-producing cells may be found in these bronchiectasis-struck airways (132).

IL-17 has been shown to stimulate the production and release of several mediators, for example IL-8 (CXCL-8)(155), which was higher at 12 h after endotoxin exposure in our study, and likely contributes to the recruitment of neutrophils at that time point. In our setting, these processes occurred fast, and at 24 h, the concentrations of both IL-17 and IL-8 were no longer significantly higher on the endotoxin side. In contrast, when performing BAL on days 2-5 after the onset of the acute inflammation in ARDS patients, higher concentrations of IL-17 could be seen in BALf (115) compared with findings in BAL from healthy individuals, even at these later time points. Moreover, *in vitro,* in the same study, IL-17 increased the permeability of a human alveolar epithelial-endothelial barrier, which may potentiate the influx of inflammatory cells to the alveolar space. This change in permeability was not explored in our study, but it may be speculated as to whether a change

of permeability could also occur earlier than after 2-5 days as in the referred study. In that case, the change of permeability could have occurred in our study as well, considering the great influx of cells already at 12 h. On the other hand, IL-22, which was still higher at 24 h on the endotoxin side in our setting, has been shown to play a role in the regeneration of damaged tissue and to promote proliferation of epithelial cells (155, 160), partly independently from IL-17 (161). Thus, there are signs of resolution - and balance - also within the cytokine part of the immune system.

The accumulation and activation of PMNs increase MMP-8 and HβD-2, both of which were higher in the endotoxin-exposed segments. In a mouse model, the neutrophil specific MMP-8 has been shown to have a protective role in the development of inflammation in lungs during endotoxemia, through the inhibition of calprotectin (162). On the other hand, calprotectin may inhibit all MMPs through their zinc-dependency; at least *in vitro* (163). The role of HβD-2 will be discussed further in the paragraph on antimicrobial peptides.

Even if the characteristics of the Th17 cell identified in this project differed from those in previous studies, we can definitely say that the proinflammatory IL-17 is expressed in healthy airways after simulation of a Gram-negative infection and that IL-17 probably potentiates the recruitment of neutrophils, but with a transient effect. Considering the immunomodulatory effects of IL-17 and the other mediators, we may conclude that the release and effects of cytokines probably are balanced in healthy human airways after endotoxin exposure.

The interleukin IL-26

The other cytokine in focus of our research, IL-26, was evaluated in healthy human airways for the first time. The concentrations of IL-26 were higher in the endotoxin-exposed bronchial segments at all time points; 12, 24 and 48 h. In further analyses *ex vivo*, it was concluded that alveolar macrophages rather than lymphocytes were the main source of IL-26, which is well compatible with the fact that IL-26 has been found in (macrophage-like) synoviocytes in patients with rheumatoid arthritis (141).

The interleukin IL-26 was first discovered about 15 years ago, in virus-infected $CD8^+$ T cells (164). There is no equivalent to IL-26 in mice, which explains why it was discovered relatively recently and why the information about its functions is limited. IL-26 belongs to the IL-10 cytokine family (67) and has been shown to be expressed by the Th17 cell and more recently also by NK cells (165). In our study, we determined the source of IL-26 utilizing several methods and the main source was found to be the alveolar macrophages, but with a co-localization in T lympho-

cytes, mainly in Th cells but in cytotoxic T cells as well. The macrophages were shown to be able to both produce and release IL-26. This is a new discovery implying that the differentiation in the tissue, from monocytes to mature alveolar macrophages, is essential for the expression of IL-26, again in a parallel to the macrophage-like cells in the joints (141).

IL-26 proved to influence the chemotaxis of neutrophils, by potentiating the chemotactic effect of IL-8. Thus, both IL-26 and IL-17 contribute to the influx of neutrophils in our setting. By examining the concentrations of IL-26 at the different time points, it was found that the concentrations appear to be stable over time in the vehicle-exposed segments. However, in the endotoxin-exposed segments, the concentrations of IL-26 were highest at 12 h, lower at 24 h and then again higher at 48 h. At 48 h, the concentrations of neutrophils were lower and presumably, IL-26 did not exert the chemotaxis on neutrophils at that time point. The reason for this is unknown, and the effect of IL-26 at 48 h is shrouded in mystery.

A question is whether there is a second wave of released IL-26, at 48 h, and, in that case, whether IL-26 partly originates from lymphocytes. At 48 h post-exposure, the concentrations of lymphocytes on the endotoxin side were slightly higher than at 24 h (figure 8, on page 54), and it may be speculated as to whether these cells turned into IL-26-producing cells as time passed, in a similar way to the switch of the T cells into Th17 cells demonstrated in joints (141). The properties of the immune cells and of IL-26 itself to be poised to respond differently to different signals and change the mode of action depending on the surroundings could also explain why IL-26 stimulates the mediators IL-8, IL-1β, TNF-α and GM-CSF in BAL cells, but has the opposite effect on the same mediators in epithelial cells. Foreign agents enter through the alveolar space and there is where the innate immune system must be prepared to cope immediately.

In this context a new mechanism of the action of IL-26 has turned out be intriguing. Recently, IL-26 was shown to be able to form multimers, and through this property, and the strongly cationic quality of the multimer, capable to kill extracellular bacteria by pore formation (166). The concentrations of the IL-26 required for killing Gram-negative bacteria was similar to the concentrations required for bacterial killing by LL-37. From this perspective, IL-26 may be considered as an antimicrobial peptide, just like LL-37 and others. The antimicrobial effect was strictly shown on IL-26 derived from activated Th17 cells, while IL-26 derived from other T cells did not show the same effect. No comparisons were made with IL-26 derived from macrophages of any kind. Moreover, IL-26 was capable of presenting DNA from the killed bacteria to dendritic cells and promoted their activation (166). The affinity to LPS was less than to the whole bacterium, which complicates the assessment of the relevance of this process in our study.

The discoveries on IL-26 so far are intriguing, but further research is necessitated to clarify the biological functions of IL-26 in human airways, in both health and disease.

Antimicrobial peptides

Antimicrobial peptides have come into focus the last two decades and the database for all existing AMPs was created in 2002. In this thesis, four of the AMPs presumably involved in the innate immunity of the human lungs are discussed.

In paper I, SLPI was analyzed in the context of being an anti-protease to NE (44), and its concentrations were higher in the endotoxin-exposed segments mainly at 48 h post-exposure. SLPI was not analyzed at 12 h.

In paper II, the rationale for the analyses of hβD-2 was originally as a control of the effect of IL-17. hβD-2 was increased at both at 12 and 24 h after endotoxin exposure.

In paper IV, we determined the concentrations of LL-37 and calprotectin, the former of which was stimulated by endotoxin both at 12 and 24 h after exposure, and the latter mainly constitutively present.

All four AMPs analyzed for this thesis are released by neutrophils (20, 44, 55), and at least three of them are produced also by the airway epithelial cells and are present in the epithelial lining fluid (167). There are indirect hints that calprotectin is present in the airway epithelium as well, at least early in life and in mice (168). In the epithelial lining fluid the AMPs have been shown to act together in killing at least *E. coli*, *in vitro* (167), which is why it would be natural and important if they were released at the same time, in response to the same stimulus. In our setting, we could prove that hβD-2 and LL-37 were higher in the endotoxin-exposed compartments at the same time points and might then act together in the airways with an additive effect. In the fourth paper, we also confirmed that LL-37 was present in its active form at both these time points, but more so at 24 h. In skin lesions hβD-2 and LL-37 could act synergistically on the Gram-positive bacterium *S. aureus* (54), and considering this fact, together with the parallel increase in concentrations after endotoxin exposure, a synergetic effect could be assumed in healthy human airways as a response to LPS as well.

In this perspective, the delayed release of SLPI is puzzling. SLPI has emerged as multi-potent player of the innate immunity (169), not only inhibiting NE but controlling NFκB and exerting a direct antimicrobial effect. The exact mechanism of the microbiocidal effect is not clear, but since SLPI is cationic, like other AMPs, binding to the outer layer of bacteria could be an appropriate explanation. SLPI is very salt-sensitive and its direct effect on bacteria is impaired by higher salt concen-

trations as in the epithelial lining fluid of the airways of CF patients (167). SLPI has also been shown to be a regulator of the NET formation (46) and in addition to inhibiting NE in the formation of NETs, SLPI is found within the NETs (169). In our setting SLPI is, as mentioned, increased relatively late and in insufficient amounts to completely inhibit NE. Therefore, it is reasonable to presume that SLPI acts through inhibiting the formation of NETs as well as inhibiting the TLR4 induced activation of NFκB in our setting. The location of SLPI in the cytosol of neutrophils (44) would facilitate both these processes. SLPI would in that case contribute to the resolution of inflammation, which could be in line with former studies on the function in other human organs (169).

The concentrations of active LL-37 were slightly higher in the endotoxin-exposed segments already at 12 h post-exposure, but not significantly higher until the time point of 24 h. hCAP-18, i.e. the precursor of LL-37, is cleaved extracellularly for this activation, by serine proteases, presumably proteinase 3 (170). In paper I, the net serine protease activity was shown to be higher at 24 h than at 48 h, mainly reflecting the activity of NE. It may be speculated that the extracellular cleavage of hCAP-18 to LL-37 was due to the active serine protease that we had demonstrated. The time point of the significant increase in the concentrations of active LL-37 coincided with the highest level of net serine protease activity. However, the activity of serine proteases at 12 h post-exposure is not known, and therefore this assumption is unsure. Anyhow, LL-37 was cleaved and activated and in being so, one may reflect on its effect in our setting. *In vitro*, LL-37 has been shown to have diverse effects, depending on concentrations and surroundings (55).Moreover, the structure of LL-37 could be modified by smoking. These modifications could change the chemotactic effect of LL-37 mainly towards mononuclear leukocytes (171), which could be of relevance in COPD, but not in our setting. Adding to the synergetic effect with hβD-2 that is suggested above, LL-37 might exercise its chemotactic effect on neutrophils and/or T-lymphocytes in our setting (55, 172). The time aspect of activity for LL-37 suggests that T-lymphocytes might be the targets for this chemotaxis. Moreover, another effect of relevance in healthy airways might be the protective one; LL-37 may prevent Gram-negative bacteria to affect the epithelial cells (173) as well as be active in the reparative process (174).

However, these speculations are merely suggestions since we have not been able to exactly point to a specific function for LL-37 in healthy human airways. The question is what effects LL-37 really have *in vivo*, and in healthy airways. The impression is that the host defence strives to keep the homeostasis and we may only indirectly, with the knowledge of the levels and activities of the other mediators measured in this thesis, draw conclusions about the physiological effects of LL-37 *in vivo*.

Calprotectin was also present in human healthy airways, but was not affected by the endotoxin stimulus, in spite of the fact that there was an enormous influx of neutrophils and calprotectin is abundant in the cytosol of PMNs. It may be active in forming the NETs and thus not detectable, but the evidence for this is weak. It was shown that calprotectin is sensitive to oxidation (175), probably not only in the airways of CF patients, but its function in healthy airways needs to be further elucidated.

Closing remarks

Throughout this thesis, different aspects of the immune response to a single inflammatory stimulus in healthy human airways are in focus. The emphasis is on the innate pulmonary defence, a part of the immune response that could be thought to be active in between the first-line defence of the epithelium and the specific adaptive immune response. The interleukins that are explored in this thesis are usually incorporated in the innate immunity, and there is no clear evidence of the adaptive immunity being involved in the studies we have performed. In contrast, the airway epithelium, the surfactant and the mucus may come into play in the immediate immunological response of the healthy respiratory compartment and these parts seem intertwined with the innate immune response.

The results from the different studies in this thesis are also intertwined, in several ways. The cells communicate with each other in intricate ways, partly through mediators analyzed in this thesis. The different mediators interact with each other. The higher levels of MMP-9 on the endotoxin side might activate IL-8 extracellularly (22), but we can not be certain of this in our model since the analyses of MMP-9 and IL-8 were not made in samples exactly from the same time points. Moreover, IL-8 may be upregulated by IL-17 and IL-22 in synergy. The simultaneous increase of IL-8, IL-17 and IL-22 would rather speak for the latter alternative.

Other connections are that IL-17 stimulates MMP-8, which in turn may be inhibited by calprotectin and vice versa. NE is a key player that cleaves MMP-9 extracellularly, but NE also is able to kill *E. coli* directly (176). NE is counteracted by α-1-antitryspin and SLPI, the latter of which also being an antimicrobial peptide. Other antimicrobial peptides works in synergy, like LL-37 and HβD-2, which in turn is stimulated by NE. Calprotectin is stimulated by IL-17. In addition, there are more connections that have not been addressed in this thesis. Moreover, we do not know all the functions and roles of IL-26, a possible conductor in this orchestra, as a supplement to IL-17.

In evaluating the model of exposure to endotoxin intrabronchially, on may say that using such a strong, but still safe, stimulus turned out to be a key to clear re-

sults. The method of using the participating volunteers as their own controls, by exposing them to vehicle in one lung and endotoxin in the other, also proved propitious. Owing to the logistical difficulties of enrolling large numbers of subjects in a study with multiple bronchoscopy procedures, the number of participants may seem small, but it is a well-defined cohort. The safety data state that the bronchoscopical procedures with exposures and BAL were performed safely and the method is reproducible for further studies. It would have been interesting to evaluate the participants in a longitudinal way, but owing to the interventional design, this would not be possible since the effects of several bronchoscopies would affect the results.

In this model, we have focused strictly on examining conditions in the peripheral airways, but considering the adjacent compartments when assessing the results. Still there are more areas in the peripheral airways of healthy humans that need to be covered, or as one writer put it: "The mucous layer that bathes the epithelium works as a "killing field" that relatively few pathogens have evolved strategies to negotiate effectively" (155).

Conclusion

This thesis presents a cross-sectional investigation of the innate immunity in healthy human airways with emphasis on the innate neutrophil-related responses. The overall objective was to highlight the innate immune response after simulation of a Gram-negative infection. In doing this, we gain insight into a complex network of cells and mediators with several tasks and with a high level of flexibility depending on the task they are facing. As in a multinational company, the different parts have to be able to work together in changeable surroundings and accustom their behaviour to the incoming signals. The airways with a large interface to their external environments have an amazing ability to deal with every incoming signal even if they have to cover every inch of an area as big as a football ground. The desired outcome is to keep the human organism healthy, even after the exposition to a foreign agent. Considering all these specialized cells, mediators, and everything that could go wrong at every step, it is fascinating to note that human beings tend to be healthy rather than constantly suffer from some disease.

In this thesis, we have attempted to mimic an early development of an inflammatory disease and to point out a few processes that might go wrong and a few mediators that might be over-expressed when a real inflammation develops. Our results point to a surprisingly balanced response to a very potent stimulus of the healthy airways.

- The activities of proteases and anti-proteases are balanced in healthy human airways under normal conditions, but a single exposure to endotoxin changes the proteolytic homeostasis towards an imbalance with excess serine proteinase activity. The effect is transient and the conditions start to return to normal within two days.

- Memory Th17 cells are present in healthy human airways. Their archetype cytokine, IL-17, and other Th17-related cytokines, are involved in the innate immune response to endotoxin in healthy human peripheral airways.

- The interleukin IL-26 is involved in the innate immune response to endotoxin in healthy airways. Alveolar macrophages and Th17 cells emerge as prominent sources of IL-26 in the human airways and IL-26 exerts a chemotactic effect on the neutrophils. These facts support the involvement of IL-26 in the antibacterial host defence.

- The AMPs calprotectin and LL-37 are present in healthy airways under normal conditions and LL-37 is increased after stimulation with endotoxin, similar to what is observed for hβD-2 and SLPI, indicating that antimicrobial peptides take part in the innate immune response in healthy human airways.

Finally, the response of the innate immunity in lungs to microbes (and other noxious agents) is essential. The studies in this thesis reveal that the innate immune response to a very potent proinflammatory stimulus is remarkably well balanced, in healthy human airways, but with a readiness to come to alert. The information may be valuable when reflecting over the mechanisms in development of disease in human airways.

Future Perspective

Press release in May 2026:

"Breaking news!
The drug company of Gothenbourg has finally solved the huge problem of antibiotics against the multi-drug resistant bacteria that kill millions of people! The drug company claims at the same time to have found a super-drug for patients with COPD, which should be a concomitant finding in the development of the new antibiotics. Both drugs are already approved by the FDA (Food and Drug administration) in the US for clinical use from July 1st. They are expected to be approved by the European equivalent as well.
The antibiotic is based on endogenous antibiotics, which are released by the cells of the immune system. The company has been able to amplify these and steer them towards aggressive bacteria, without the development of new resistance. In doing so they have used endogenous molecules to split the bacteria into pieces, after which the body cells themselves could engulf them. We have certainly an intelligent body!
The drug for COPD, the widespread inflammation of the airways and lungs, was developed with the discovery of a small molecule that works as a conductor of the orchestra of inflammation. Finally, the company has evolved a specific antibody against this little "hireling" and has been able to improve the carrier of the antibody, thus making inhalation of the drug possible. In the phase III studies the drug has been shown to reduce the decline of lung function with more than 50%. This is really good news for all those ex-smokers who are now to be saved from an incurable disease!"

This press release may never occur, but the hope for the future is that further research on the neutrophil-related inflammation will help patients with neutrophil-related diseases and that the knowledge of a normal inflammatory process in healthy airways could serve as a good basis.

Acknowledgements

First, my thanks go to all participants in these studies. Without you, this research on inflammation in human healthy airways would not have been possible.

I wish to express my deepest gratitude to all those who have made it possible for me to accomplish this thesis, especially

Ingemar Qvarfordt, my main supervisor, for crucial and inexhaustible support, encouragement and endless patience. Your accuracy is admirable, as are your skills in diplomacy and statistics and the way of sharing your thorough knowledge. Thanks for all the discussions, not only on research but also on other things that make life enjoyable, like sailing, single malt, trips and ever more.
Anders Lindén, my co-supervisor, for introducing me to all these small mediators that I did not know existed before this long journey started into in the secrets of immunology. The way you reminded me of the keywords and structures will not be forgotten, nor will your high standard of ethics.
Gerdt Riise, my second co-supervisor, for many pep-talks and good-humoured and honest criticism and support.

Barbro Balder, my first research nurse, who was able to make the impossible possible – in memoriam.
Monica Crona, my research nurse, who replaced Barbro with such accuracy and energy and knowledge that it was a joy to continue the work.
The staff at the diagnostic ward at the Department of Respiratory Medicine at Sahlgrenska University Hospital, for your help with planning and performing all the bronchoscopies, both in mornings and evenings - and for all the coffee and pleasant moments together.
Carina Malmhäll and *Margareta Sjöstrand* at the Lung Immunology group for excellent laboratory work and encourage and support in my first stumbling steps in the laboratory world.
Pernilla Glader, with so much positive energy, thanks for introducing me in the world of interleukins. *Sara Tengvall* and *Marit Stockfelt*, and the rest of you who performed the laboratory work – without you there would not have been any thesis!
Stefan Berg for proof-reading this thesis, coming with valuable input and cleaning up my references – any remaining errors are totally my own.

Britt-Marie Eldh, for planning my schedule in a favourable way and for all small talks and chats about everything and anything, in our corner of the clinical department.
Cattis Dellborg, head of the Lung section, for letting me off the clinical world for a while and for practical and wise advice and encouragement
Mona Palmqvist, present head of the Department of Respiratory Medicine, for support and encouragement
All other research colleagues in the laboratory and clinical world, thanks.

All my friends who are so many that it would be unfair to mention anyone in particular. You make my life happy but you have also been a problem offering other things to do instead of working on my research – and you have often succeeded.
My sister with family, organizing the things I did not do, while writing
My father, Björn, who would have been proud of me - in memoriam.
My supportive and encouraging *mother Kerstin*, in general, but in particular for tip-offs on the layout and for helping me in all other ways during this intense period at the computer
Shelagh Warme, who has meticulously reviewed this thesis – all remaining errors are my own responsibility

Henrik, for love and endless support, and for keeping our home in a decent state during the last intensive phase of my thesis writing. And for organizing our social life and… everything else.
Erik and *Johan*, for encouraging support and reminding me to stick to my computer instead of doing other things. Love you guys! Now I will have more time for you – if wanted.

Moreover;
This thesis was made possible through generous support from the Swedish Heart-Lung foundation, the Göteborg Medical Society and through LUA/ALF in Göteborg, Region Västra Götaland, as well as Federal funding from Karolinska Institutet.

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