

**The Institute of Environmental Medicine
Division of Physiology
The unit of Lung and Allergy Research
Karolinska Institutet, Stockholm, Sweden**

**TOLL-LIKE RECEPTORS IN
AIRWAY INFLAMMATION
*IN VITRO AND IN VIVO***

Ida von Schéele



**Karolinska
Institutet**

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Cover: Primary bronchial epithelial cells (photo: Ida von Schéele and Lena Palmberg)

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Till min familj

All models are wrong, but some are useful!

George Edward Pelham Box.

ABSTRACT

During evolution a variety of solutions has been developed by different living organisms in order to protect itself from invading potential pathogens and particles. Protection of the host is essential for its survival and involves efficient recognition and elimination of potential pathogens. The recognition is often mediated by pathogen recognition receptors (PRRs). Activation of PRRs is mainly driven by exogenous pathogen associated molecular patterns (PAMPs) or endogenous danger associated molecular patterns (DAMPs) and result in secretion of multiple pro-inflammatory cytokines. Toll-like receptors (TLRs) are members of the PRR family and the importance of these receptors during pro-inflammatory conditions is addressed in the present thesis, both *in vitro* and *in vivo*.

In Paper I we showed that farmers and smokers, two groups that are continuously exposed to organic material through daily work at the farm or through tobacco smoke, have an ongoing inflammation in the respiratory tract. It was shown that chronically exposed subjects develop an adaptation to the effects of acute exposure to inhaled organic material. Further, it was demonstrated that exposure in the swine barn was a much stronger pro-inflammatory stimulus than inhaled pure lipopolysaccharide (LPS), *in vivo*.

In Paper II, the gene expression of TLR2 on primary bronchial epithelial cells was demonstrated. This expression was synergistically enhanced by co-stimulation with pro-inflammatory stimuli and glucocorticosteroids. Dust obtained from the swine barn was a more potent pro-inflammatory stimulus than pure LPS or pure peptidoglycan (PGN), *in vitro*, as already shown *in vivo*. The secreted pro-inflammatory cytokines from the epithelial cells were diminished by blocking of the TLR2 and TLR4 with monoclonal antibodies, indicating that the pro-inflammatory stimulation was at least partly dependent on TLR2 and TLR4.

In paper III we found that TLR2 on blood neutrophils was down-regulated by pro-inflammatory stimuli, whereas the expression TLR4 and CD14 were unaffected by the pro-inflammatory stimulation, *in vitro*. The expression of TLR4 and CD14 were increased by the presence of epithelial cells, irrespective of stimulation. Moreover, we showed synergistically enhanced secretion of CXCL8 (IL-8) and sCD14 during co-culture compared to single culture condition and a strong positive correlation between CXCL8 and sCD14 in LPS-stimulated co-cultured cells. These findings strongly suggest an active bidirectional cross-talk between alveolar epithelial cells and neutrophils.

In paper IV we confirmed what we already had shown *in vitro*, that TLR2 was down regulated by pro-inflammatory conditions on neutrophils, this time *in vivo*. We also showed the presence of soluble TLR2 (sTLR2) in BAL and sputum and that this expression was altered in COPD compared to healthy subjects. Moreover, CD14 expression on sputum neutrophils was enhanced compared with blood neutrophils and that the gene expression of CD14 on alveolar macrophages in BAL-fluid was increased in smokers compared with non-smokers. These findings indicate that PRRs expression

is altered by smoking per se, but also that the disease, COPD, contributes. This is likely of importance in COPD patho-physiology, in particular for exacerbations, which often are caused by microorganisms.

Overall, these studies have shown the involvement of PRRs in several immunological active cell types during pro-inflammatory conditions. A better understanding of the mechanisms behind PRRs regulation and outcome would potentially benefit drug development and in the end many patients with inflammatory diseases.

LIST OF PUBLICATIONS

The thesis is based on the following papers, referred to by their Roman numbers

- I. B.M Sundblad, I. von Scheele, L. Palmberg, M. Olsson, K. Larsson
Repeated exposure to organic material alters inflammatory and physiological airway responses
Eur Respir J 2009; 34: 80-88
- II. I. von Scheele, K.Larsson, L.Palmberg
Budesonide enhances Toll-like receptor 2 expression in activated bronchial epithelial cells
Inhal Toxicol 2010; 22(6):493-499
- III. I. von Scheele, K.Larsson, L.Palmberg
Interactions between alveolar epithelial cells and neutrophils during pro-inflammatory conditions
Submitted
- IV. I. von Scheele, K.Larsson, B. Dahlén, B. Billing, M. Skedinger, A-S. Lantz, L.Palmberg
Toll-like receptor expression in smokers with and without COPD
Respir Med 2011; Mar 23, [Epub ahead of print]

CONTENTS

1	Introduction	8
1.1	The respiratory system	8
1.2	Inflammation.....	9
1.2.1	Acute inflammation.....	9
1.2.2	Chronic inflammation.....	10
1.3	The immune system	11
1.3.1	Innate immune system.....	11
1.3.2	Pattern Recognition Receptors	13
1.3.3	Regulatory mechanisms of Toll-like receptors	15
1.3.4	Pro-inflammatory cytokines and chemokines.....	16
1.4	Glucocorticosteroids.....	17
2	Aims of the study.....	18
3	Material and Methods	19
3.1	Material.....	19
3.1.1	Human study population.....	19
3.1.2	Exposures (<i>In vivo</i>).....	19
3.1.3	Sample collection and processing.....	20
3.1.4	Cell-culture procedures and experiments (<i>in vitro</i>).....	22
3.2	Methods	23
3.2.1	Analysis of mRNA.....	23
3.2.2	Analysis of proteins.....	23
3.3	Statistics	24
4	Results	25
4.1	Paper I.....	25
4.2	Paper II.....	27
4.3	Paper III	31
4.4	Paper IV	33
5	General Discussion.....	38
6	Conclusions	43
7	Populärvetenskaplig sammanfattning (in swedish)	45
8	Acknowledgements	47
9	References	49

LIST OF ABBREVIATIONS

A549	Human lung adenocarcinoma epithelial cell line
APC	Allophycocyanin
BAL	Bronchoalveolar lavage
BMI	Body mass index
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CXCL8	C-X-C motif chemokine 8
DAMPs	Danger associated molecular patterns
DC	Dendritic cell
DLCO	Diffusing capacity of the lung for carbon monoxide
EDTA	Ethylene diamine-tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EpC	Epithelial cells
FCS	Fetal calf serum
FEV ₁	Forced expiratory volume in one second
FITC	Fluorescein isothiocyanate
FVC	Forced vital capacity
HMGB1	High mobility group box-1
HSP	Heat-shock proteins
IFN	Interferon
IL	Interleukin
IRAK	IL-1R associated kinases
IRF	Interferon regulating factor
KOL	Kroniskt obstruktiv lungsjukdom
kPa	Kilopascal
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response protein 88
MyD88s	Splice variant of myeloid differentiation primary response protein 88
NAL	Nasal lavage
NF- κ B	Nuclear factor-kappa B
NK-cell	Natural killer cell
NLRs	Nucleotide binding and oligomerization domain-like receptors
NO	Nitric oxide
ODTS	Organic dust toxic syndrome
Pam3Cys	Tripalmitoyl-S-glycerylcysteine
PAMPs	Pathogen associated molecular pattern
PBEC	Primary bronchial epithelial cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEF	Peak expiratory flow

PerCp	Perdinin chlorophyll protein
PGN	Peptidoglycan
PRRs	Pathogen recognition receptors
RLRs	Retinoic acid-inducible gene 1-like receptors
sCD14	Soluble CD14
SIGIRR	Single immunoglobulin and toll-interleukin 1 receptor (TIR) domain
SOCS	Suppressors of cytokine signaling
ST2	Suppressor of tumorigenicity 2
sTLR2	Soluble TLR2
sTLR4	Soluble TLR4
TIR	Toll-IL-1 receptor
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TOLLIP	Toll interacting protein
TRIF	Toll-receptor associated activator of interferon
VC	Vital capacity

1 INTRODUCTION

It has been known for centuries that our lungs are influenced by the air we breathe. In 1555 the Danish bishop Olaus Magnus warned about the dangers of inhaling grain dust. He suggested that: “In separating the grain from the chaff, care must be taken to choose a time when there is a suitable wind which will sweep away the grain dust, so that it will not damage the vital organs of the threshers” (1).

Rural living conditions are declining due to the urbanization of both developing and western countries. One consequence of these changes is that less people are exposed to the farming environment. The growing industrialization will, however, lead to increased air pollution which implies increased regular human exposure to inhalable potential pathogens. These factors will likely influence global health.

This thesis focuses on two groups with high airway exposure to environmental agents; farmers who work in a highly contaminated environment and smokers who are exposed to a mixture of airborne particles in the inhaled tobacco smoke.

1.1 THE RESPIRATORY SYSTEM

Depending on the physical activity, an adult person breathes 10-20 m³ of air each day. During inhalation the air passes through the nasal cavity down into the bronchial tree and ending up in the alveolar ducts and alveolar sacs where the gas exchange takes place.

Inhalation of air allows deposition of particles and microorganisms on the surface of the respiratory tract, an ideal environment for bacterial growth. Airflow, pharyngeal anatomy and the size of the particle are factors that affect the level of particle deposition in the respiratory tree. It is common to distinguish between inhalable and respirable particles, where inhalable particles are < 10µm and respirable particles are <5µm.

The ciliated epithelial cells, lining the respiratory tract, represent the first line of defence in the lungs and function as a mechanical barrier. Synchronized beating cilia works in viscous fluid where inhaled foreign particles get trapped. Mucus and particles are then transported towards pharynx by the coordinated beating of the cilia. This mucociliary clearance is one of the mechanisms that keeps the lower respiratory tract clean and prevent mucus accumulation in the lungs. Infections, tobacco smoke and pollutants reduce mucociliary clearance and increase the risk of recurrent respiratory infections. In the alveolar ducts the ciliated epithelium is replaced by non-ciliated epithelium with direct contact with the capillaries. Airway epithelial cells are multifunctional cells that serve as a barrier, but are also providing the host with an inflammatory response to threatening organisms by release of pro-inflammatory cytokines and chemokines (2).

The next line of defence of the host is the phagocytes. Alveolar macrophages are found peripherally of the lungs and together with neutrophils they provide the host with innate protection of the respiratory tract by identification and phagocytosis of foreign particles (3).

1.2 INFLAMMATION

Inflammation constitutes a defense against invading organisms and contributes to healing of wounds and infections. The cells and mediators involved in the immune response are powerful, which makes it critical to regulate the intensity and the duration of the inflammatory response, thereby diminishing potential harmful effects.

1.2.1 Acute inflammation

Acute inflammation is the initial response to harmful stimuli and consists of increased number of leukocytes, mainly granulocytes, which have been migrating from the bloodstream to the “site of action”. This will result in the five cardinal signs of inflammation; dolor (pain), calor (heat), rubor (redness), tumor (swelling) and functio laesa (loss of function). The players of the acute inflammation are rather short-lived and the acute inflammation requires constant stimulation to be sustained. An active termination of the inflammation is needed to prevent unnecessary tissue damage (4).

The working environment has potential health influences, e.g. among farmers whose daily work involves respiratory health hazards, due to exposure to airborne particulate material, organic dust. The content of the dust is heterogeneous and contains high amounts of pathogen-associated molecular patterns (PAMPs) such as fungi, endotoxins and peptidoglycans. The main contents of the dust obtained from the swine confinement buildings are microorganisms, animal dander, urine and feces (5). Inhalable dust and endotoxin concentrations up to 28.5 mg/m³ and 1.2µg/m³, respectively, have been reported (6, 7).

Organic dust is a potent pro-inflammatory stimulus. After a few hours of exposure in the swine confinement building healthy, naïve, non-allergic volunteers develop increased bronchial responsiveness, intense airway inflammation, dominated by neutrophils and increased levels of pro-inflammatory cytokines (IL-1β, IL-6, CXCL8 (IL-8) and TNF) in BAL-fluid (7, 8) and IL-6 and CXCL8 (IL-8) in sputum and NAL (9). Increased pro-inflammatory cytokines in serum (10) demonstrate that the reaction involves systemic engagement, often called organic dust toxic syndrome (ODTS) with flu-like symptoms such as fever, chills, malaise, headache, dyspnea and muscle pain (11). These symptoms (ODTS) normally disappear within 24 hours after exposure. Farmers who are exposed to this highly contaminated environment on a daily basis develop inflammatory and physiological attenuated responses to acute exposure when compared with healthy non-farmers (12). It has also been shown that farmers, despite lack of respiratory symptoms, have an ongoing lower respiratory tract inflammation, with increased numbers of inflammatory cells and elevated CXCL8 in BAL and sputum (9, 13). It is intriguing that children who grow up on farms have lower

prevalence of asthma, hay fever and allergy than children who grow up in urban areas (14, 15).

1.2.2 Chronic inflammation

Neutrophils are one of the first immuno-active cell types to arrive to the site of injury. A persistent acute inflammation may lead to a progressive shift in the type of cells at the site of injury. In general, chronic inflammation is often dominated by mononuclear cells, such as monocytes and lymphocytes, whereas in asthma by eosinophils. However, in severe asthma as well as in COPD, the dominating cell type is neutrophils.

1.2.2.1 Chronic bronchitis in farmers

As most farmers live on the farm where they work, they are continuously exposed to the farming environment and potential pathogens derived from organic dust. Farmers have an increased risk of respiratory morbidity, such as chronic bronchitis (16) and the duration of farming is associated with reduction in lung function (17).

Chronic obstructive pulmonary disease (COPD)

Tobacco smoking is the most common cause of COPD, but other types of exposure e.g. occupational exposure may be of relevance. Miners and tunnel workers, and never-smoking animal farmers have increased prevalence of COPD (5, 18-21). The prevalence of COPD has increased and is expected to be the third most common cause of death by 2020 (22, 23)

Chronic obstructive pulmonary disease is a condition which is characterized by irreversible airway obstruction caused by small airway disease (obstructive bronchiolitis) and parenchymal destruction (emphysema).

The severity classification of COPD into four stages is based on spirometry data (table 1)

<i>Spirometric classification of COPD severity(23)</i>	
All stages of COPD has a $FEV_1/FVC < 0.70$	
Stage I: mild	$FEV_1 \geq 80\%$ predicted
Stage II: moderate	$50\% \leq FEV_1 < 80\%$ predicted
Stage III: severe	$30\% \leq FEV_1 < 50\%$ predicted
Stage IV: very severe	$FEV_1 < 30\%$ predicted <i>or</i> $FEV_1 < 50\%$ predicted plus certain negative prognostic factors. *

Table 1 The GOLD guidelines.

FEV_1 : forced expiratory volume in one second, FVC : forced vital capacity

*** arterial partial pressure of O_2 less than 8.0 kPa with or without arterial partial pressure of $CO_2 > 6.7$ kPa, BMI < 22, mucus hypersecretion. All lung function values are obtained after bronchodilatation.**

The chronic inflammatory process in the lungs involves several cell types and neutrophils, macrophages, eosinophils, T-lymphocytes (especially CD8⁺) are reported to be increased in numbers in COPD-patients (24).

COPD is associated with systemic inflammation, inflammatory markers including CRP, leptin, IL-6, CXCL-8, IL-1 β and TNF- α , among others, are elevated in plasma from COPD-patients (25).

Co-morbidities are frequent in COPD-patients and are unfortunately, often undiagnosed. The prevalence of heart failure, lung cancer, depression and diabetes mellitus type II are increased in COPD, compared to the population in general (23, 26).

COPD is a chronic disease with irreversible destruction of the lungs. The overall aim of COPD treatment is to relieve the symptoms, slowing the progress of the disease and to prevent exacerbations. In all stages of the disease, from mild to very severe COPD, physiotherapy plays an important role in the treatment of the patient. Patients with COPD usually benefit from treatment with bronchodilators such as anticholinergics and β_2 -agonists. Frequent exacerbations contribute to the decline in lung function, impaired quality of life and increased mortality in patients with moderate and severe COPD (27-29). Therefore it is important to reduce the exacerbation rate in COPD-patients.

Pharmacotherapy with long acting β_2 -agonist and glucocorticosteroid in combination as well as treatment with roflumilast reduce the rate of exacerbations (30-32).

Anticholinergic therapy has also been shown to have positive effects on the rate of exacerbation, hospitalization and mortality in patients with moderate to very severe COPD, but not on the rate of decline in FEV₁(33, 34).

Smoking cessation is however the only measure that conclusively alters the negative trend of a rapid decline in FEV₁, which is connected to the progression of the disease.

1.3 THE IMMUNE SYSTEM

The immune system is a collection of both cellular and humoral component and its most important role is to discriminate between self and non-self. Traditionally the immune system is divided into innate and adaptive immune system. The innate immune system operates in all plants and animals; it is fast, fixed and effective in stopping most microbial threats at an early stage. The adaptive immune system is slowly activated, but powerful and highly specific in the mode of action. It is only present in vertebrates and is often induced by the innate immune system. The adaptive immune system is an evolving process within the lifetime of the host and is rapidly reactivated on future challenge with the same pathogen (35, 36).

1.3.1 Innate immune system

Already at birth the innate immune system provides a non-specific immediate defense against potential pathogens. The innate immune system has no immunological memory, implying that this system does not confer long-lasting immunity against pathogens.

Major functions of the human innate immune system are:

1. Identification and removal of potential pathogens accomplished by specialized leukocytes (white blood cells).
2. Recruiting other immune cells to the site of action by producing cytokines and chemokines.
3. Activation of the complement cascade, identifying bacteria and promote clearance of dead cells.
4. Activation of the adaptive immune system, through antigen presentation.

1.3.1.1 Cells participating in the defense of the organism

Different specialized cell types are involved in the protection of the host. *Epithelial cells* (EpC) are lining the respiratory tract and serve as the host first line of defense. The epithelial phenotype differs depending on localization and function. Thus, bronchial epithelial cells, alveolar type I and type II epithelial cells do not just constitute a physical barrier but are also active in the recognition of potential pathogens and hold an immunological response by release of several pro-inflammatory cytokines. Moreover, the ciliated bronchial epithelium is responsible for mucociliary clearance helping the lower respiratory tract to stay clean.

Monocytes (macrophages), neutrophils, dendritic cells, basophils, eosinophils, mast cells and lymphocytes, collectively known as leucocytes lack the ability of reproduction and are products of a multipotent hematopoietic stem cell, produced in the bone marrow. They are not associated with a specific organ but patrol the organism using the bloodstream as their main highway.

Alveolar *macrophages* are derived from blood monocytes and are multifunctional cells, highly involved in the defense of the airways. Phagocytosis of cell debris and foreign particles is one of the main functions, which is also orchestrating the immune response and function between innate and adaptive immune system. They are also producers of the pro-inflammatory cytokine TNF- α and CXCL8, an important chemoattractant for *neutrophil granulocytes*. The neutrophils, *eosinophils* and *basophiles* form together a family of granulated polymorphonuclear cells. The neutrophils are the most abundant type of white blood cells and are normally found in the blood stream. During the acute phase of inflammation neutrophils are migrating in large numbers to the site of injury. Neutrophils engulf and kill extracellular pathogen through release of an assortment of proteins during degranulation; a process with the main purpose to destroy invading microorganisms. The *mast cells* also have the ability to degranulate and release their histamine rich granule, they are distributed in tissues that are exposed to the external environment and are associated with allergy and anaphylaxis. *Dendritic cells* (DCs) are professional antigen presenting cells with a branched dendritic morphology. They serve as a bridge between the innate and the adaptive immunity, through stimulation of T-cell responses. *Lymphocytes* are divided into small (*T-cells* and *B-cells*) and large (*natural-killer cells*, NK-cells). T-cells and B-cells are both members of the adaptive immune system. T-cells are involved in the cell-mediated defense of the host, whereas the B-cells are antibody producing cells and play a key role in the humoral immunity. NK-

cells are part of the innate immune system and have the ability to distinguish between infected cells and “normal” cells and destroys/kills altered cells (36, 37).

1.3.2 Pattern Recognition Receptors

In 1989 Janeway suggested “that the actual detection of infection was mediated by the receptors of the innate immune system, rather than the antigen receptors” which later were called pattern recognition receptors (PRRs) (38). The PRRs are evolutionary conserved proteins that are divided into different classes: Toll-like receptors (TLRs), nucleotide binding and oligomerization domain-like receptors (NOD or NLRs) and retinoic acid-inducible gene 1-like receptors (RLRs). PRRs are expressed by non-immune and immune cells and can be differentially localized within the cells, both membrane bound and cytoplasmic PRRs occur (39). These receptors make it possible for the cell to identify pathogen-associated molecular patterns (PAMPs), which together with the endogenous alarmins, which are associated with cellular stress or death, form a larger category termed: damage associated molecular patterns (DAMPs) (40).

Toll-like receptors (TLRs) are type I transmembrane glycoproteins and consist of an extracellular leucine-rich repeat (LRRs) domain, the transmembrane domain and a cytoplasmic TIR-domain (Toll-IL-1 receptor). Toll-receptors were first discovered in *Drosophila melanogaster*, the first human homologue was reported in 1997 by Janeway and Medzhitov (41), and was consequently called Toll-like receptor. Since then, another ten human TLRs have been identified (TLR1-TLR11) (42). Each TLR recognizes specific PAMPs and exists in homo- and heterodimers, which increase the diversity of receptor specificity. TLR signaling can be classified as either MyD88- or TRIF-dependent pathways, both lead to induction of pro-inflammatory cytokines, but the TRIF-dependent pathway also leads to type I interferon response (42). Toll-like receptors have been considered to have a key-role in the discrimination between “self” and “non-self”, which is indispensable for a functional defense of the human body.

1.3.2.1 TLR2

Toll-like receptor 2 (TLR2) is located on the cell surface and has the capability to recognize a wide range of ligands such as Gram-positive bacteria, cell wall components like LTA (lipoteichoic acid), peptidoglycan and lipoproteins. The great variation of ligands is at least partially explained by the ability of TLR2 to form heterodimers with TLR1 or TLR6 (42). Not only exogenous ligands bind to TLR2. During stress or injury endogenous derived ligands, induce sterile inflammatory responses through TLR interaction. Heat-shock proteins (HSP) and high mobility group box-1 (HMGB1) are both examples of host derived proteins that interact with TLR2. The broad role and the potential of the endogenous ligands in a variety of pathological processes are still unclear (40, 42, 43). Signaling through the TLR2 pathway is MyD88 dependent and will lead to activation of NF- κ B and subsequently to release of various pro-inflammatory cytokines and chemokines.

Up to six different isoforms of soluble toll-like receptor 2 (sTLR2) are present in human plasma, breast milk, saliva, BAL fluid and sputum (44, 45). It is believed that sTLR2 establishes a regulatory function as a decoy receptor during inflammation (46).

1.3.2.2 *TLR4*

Toll-like receptor 4 (TLR4) was one of the first TLRs to be identified and are also one of the most extensively studied. TLR4 forms a complex together with MD2 on the cell surface serving as the main LPS (Lipopolysaccharide) binding component (47). LPS are one of the major constituent of the outer membrane of Gram-negative bacteria. An additional protein that also is involved in the TLR4-signaling pathway is LPS-binding protein (LBP). LBP is present in plasma and binds LPS and CD14. The LPS-LBP complex is delivered to the receptor TLR4-MD2. TLR4 is the only TLR that activates both the MyD88- and TRIF-dependent pathway, which is the reason why stimulation of the TLR4-pathway may lead to both early and late NF- κ B activation as well as release of type I interferons.

The response to LPS exposure is dependent on the presence of CD14, but also whether LPS is designated as rough or smooth (rLPS, sLPS). LPS classification as smooth or rough depends of the presence of an O-polysaccharide chain, if the LPS molecule has the O-polysaccharide chain it is classified as sLPS otherwise as rLPS. This affects the colony morphology of the LPS. The CD14-independent pathway can only bind rLPS and is limited to the MyD88-dependent pathway, resulting in activation of NF- κ B and transcription of TNF, IL-6, CXCL8 etc. In presence of CD14 the TLR4-MD2 complex binds both rLPS and sLPS. This complex does not only signal through the MyD88-dependent pathway but also through TRAM and TRIF, leading to IRF-3 activation and IFN- β transcription (48, 49).

Several endogenous ligands of TLR4 have been identified, most of them associated with cell damage and tissue injury, such as HSPs (Heat-shock proteins) and HMGB1 (High mobility group box 1). The soluble form of TLR4 (sTLR4) has been shown to function in mice as an antagonists to LPS-stimulation, inhibiting the binding of the ligand to the membrane bound receptor, serving as an important regulator of the innate immune response of the host (50). Soluble TLR4 mRNA has been found in human. Naturally occurring sTLR4 has been detected in human saliva and four different polypeptides of sTLR4 has been shown to be up-regulated during chronic inflammation of the oral mucosa. The largest of these forms suppress LPS activation in THP-1 cells (51, 52).

1.3.2.3 *CD14*

In 1990, almost a decade before the first Toll-like receptor was characterized. CD14 was the first PRR to be discovered (53). CD14 was first identified as a differentiation marker on the surface of monocytes and macrophages, but later on it became clear that CD14 together with LBP play an important role in binding of LPS. CD14 is expressed in a soluble form (sCD14) in plasma, sputum, BAL fluid etc. and is regarded as an acute phase protein (54). The LPS/LBP/CD14 complex binds to TLR4 which leads to pro-inflammatory cytokine production. The sCD14 are known to mediate LPS-

activation in cells with low expression of CD14, such as epithelial cells. However, at high concentrations of LPS, sCD14 are also known to function as a decoy receptor and thereby downregulate the LPS induced response. It has also been shown that CD14 contributes to amplified response of other TLRs, than TLR4, e.g. TLR2 (55, 56). The level of sCD14 increases in plasma during infection and inflammation and are elevated in BAL fluid from smokers. A strong correlation between neutrophil numbers, total protein and sCD14 are found in BAL fluid, indicating that CD14 are an important player in the complex system of inflammation in the lungs (57, 58).

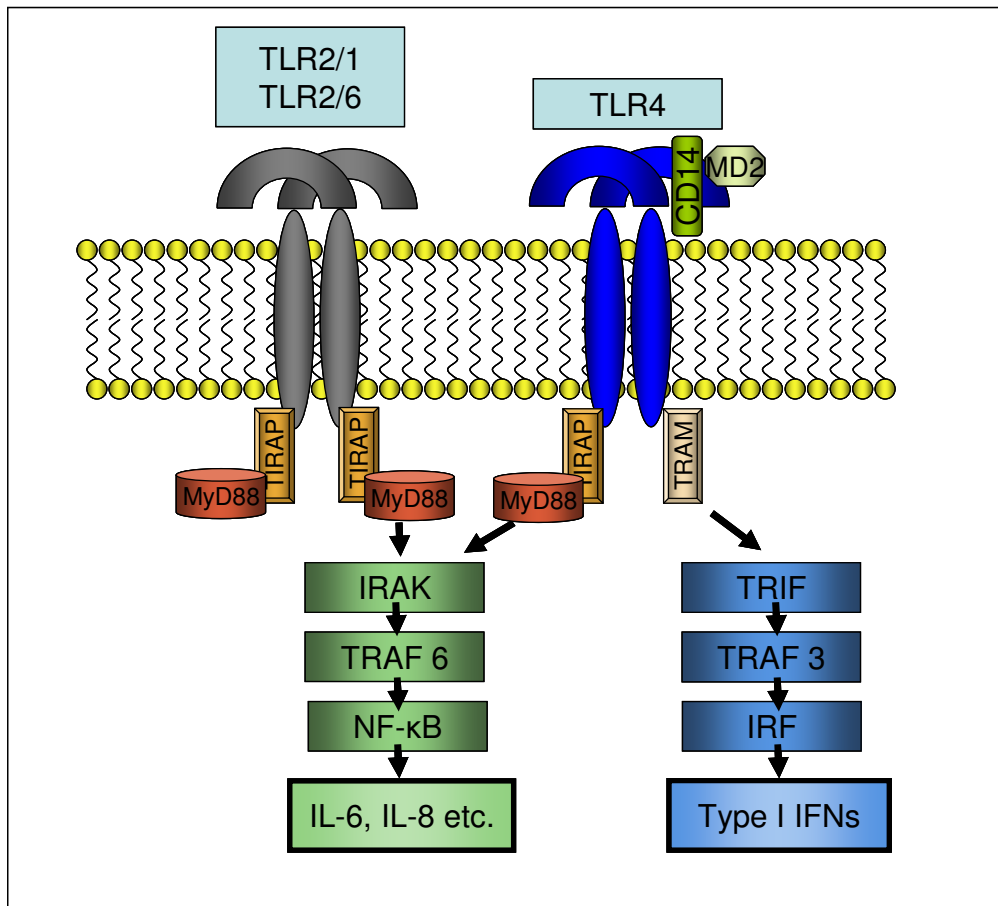


Figure 1 TLR2 and TLR4 signaling pathways; transmembrane and intracellular Toll-like receptor regulators

1.3.3 Regulatory mechanisms of Toll-like receptors

Toll-like receptor signaling is essential for a functional defense of the host against invading microorganisms and pathogens. Since it was discovered that Toll-like receptors not just respond to exogenous ligands but also endogenous ligands produced as a result of tissue damage, it has been an emerging interest of studies focusing on Toll-like receptors and chronic inflammation.

On one hand TLRs serves as regulators of the innate and adaptive immune responses and, on the other, excessive activation of the TLR signaling pathway have been

implicated in the development of several severe diseases such as sepsis, atherosclerosis, diabetes mellitus, rheumatoid arthritis, inflammatory bowel disease, asthma and COPD (59-61). Additionally, it has previously been shown that continuously exposed farmers have an ongoing inflammation in the respiratory tract and higher prevalence of chronic bronchitis than the population in general (17, 62). Recent findings have shown attenuated TLR2 expression on circulation blood monocytes in farmers compared to smokers and healthy controls, indicating systemic effects dependent of the exposure (63). In common for many of these diseases is the initiation of chronic inflammatory condition.

A tight negative regulation of TLR signaling and function seems to be crucial and several regulatory levels occurs. Soluble receptors (TLRs, CD14) have a first line of negative regulatory function as extracellular decoy receptors. Next level of regulating mechanisms is the transmembrane regulating proteins (SIGIRR, ST2L), but also degradation, deubiquitination and competition (SOCS, MyD88s) are general intracellular regulating mechanisms that all are present in the regulation of TLR signaling (51, 61, 64, 65). The field focusing on negative regulatory mechanism of TLR are growing fast and new therapeutics based on TLR activation are already in development were as some have progressed to clinical trials (66).

1.3.4 Pro-inflammatory cytokines and chemokines

The complex actions of cytokines are the host response to harmful exposure (infection and disease) and serve as regulators via receptor interactions. Cytokines are generally divided into two major groups' pro- or anti-inflammatory cytokines depending on the biological activities (67). Cytokines have a variety of functions and may signal in many different ways: Endocrine signaling, will affects target cells on a distance from the secreting cell. Hormones are a typical example of this kind of signaling, but also cytokines can act this way (IL-6, IL-1, TNF- α). Paracrine-signaling, affects cells that are near the cytokine secreting cell (IL-1, TNF- α) and autocrine-signaling cytokines affects the same cell that secreted the cytokine (IL-2). Chemokines such as CXCL8 (IL-8) function as a guide for migration of inflammatory cells to the site of action and are thereby important mediators during inflammation.

Signaling through toll-like receptors leads to transcription, translation and release of pro-inflammatory cytokines. Some of them are briefly described below:

IL-1 β is secreted by multiple cell types but mainly by monocytes/macrophages. *IL-1 β* activates the vascular endothelium and lymphocytes. It mediates local tissue destruction and increases the access of effector cells. Systemic effects derived by *IL-1 β* are fever and increased production of *IL-6*.

IL-6 is secreted by multiple cell types but mainly by monocytes/macrophages and epithelial cells. *IL-6* plays an important role in mediating fever, acute phase reactions and activates lymphocytes to increase antibody production. It has mainly pro-inflammatory properties but possess also anti-inflammatory capacity.

CXCL8 (IL-8) is a member of the chemokine family. It is potent activator and chemoattractant for neutrophils, but also for T-cells, basophils and eosinophils. Macrophages, neutrophils and epithelial cells are the main producers.

TNF- α (Tumor necrosis factor alpha) is produced by a wide variety of immune cell types and epithelial cells. It promotes inflammatory responses and is a key-cytokine in many inflammatory disorders. *TNF- α* binds to at least two receptors, TNF-receptor 1 and 2 (TNFR1 and TNFR2). More or less all cell types express TNFR1, whereas TNFR2 expression is limited to hematopoietic cells.

Interferons (*IFN*) are divided into two subgroups; type 1 and type 2, according to their receptor specificity and sequence homology. *IFN- α/β* is type 1 interferons and is expressed by almost all cell types and is early components of the immune activation, mainly induced by virus. *IFN- α/β* induces dendritic cell maturation, cytotoxicity of natural killer cells, promote half life of activated T-cells and stimulate B-cell differentiation into anti-body-producing plasma cells.

IFN- γ , the only type 2 interferon, has immuno-stimulatory and an immuno-regulatory effect such as, increasing antigen presentation and lysosome activity in macrophages, suppression of Th2 cell activity and promotion of leukocyte migration. *IFN- γ* is mainly produced by NK-cells, Th1-cells, cytotoxic T-cells and dendritic cells. Aberrant *IFN- γ* production is associated with a number of autoimmune diseases (36, 68).

1.4 GLUCOCORTICOSTEROIDS

In 1950 the Nobel Prize in Medicine and Physiology was awarded to Kendall and Reichstein, for isolation and synthetisation of cortisol and adrenocorticotrophic hormone (ACTH), which later on was developed and improved to be the glucocorticosteroids used today. Glucocorticosteroids are used in the treatment of inflammatory and immune diseases and are among the most widely used drug in the world.

Glucocorticosteroids cross the cell membrane, into the cytoplasm, where it binds to the intracellular glucocorticoid receptor (GR). The predominant effect of glucocorticosteroids is to switch of multiple inflammatory genes encoding for pro-inflammatory cytokines and chemokines, such as IL-6, *CXCL8* and *TNF- α* , counteracting some of the important inflammatory features (69). Glucocorticoids and the bronchodilating β_2 -agonists are often given together in asthma and COPD, two disorders characterized by chronic inflammation in the airways and lungs. A potential beneficial effect of the steroids is its ability to modulate the effect and function of β_2 -receptors thereby possible preventing tolerance development induced by β_2 -agonists (70). The mechanisms of glucocorticosteroids are complex, and not completely understood. In 2004 Homma et. al. proposed another function of glucocorticosteroids in airway inflammatory disorders, as they, showed that TLR2 was upregulated by glucocorticosteroids in stimulated respiratory epithelial cells *in vitro* (71).

2 AIMS OF THE STUDY

The general objective of these studies was to elucidate how pattern recognition receptors, with focus on TLR2, TLR4 and CD14, are affected during inflammation in the lungs. The specific aims were:

- To elucidate whether chronic exposure to organic material on a daily basis (smokers and farmers) alters physiological and inflammatory responses to acute exposure to organic material.
- To study the regulation of TLR2 and TLR4 in primary bronchial epithelial cells during pro-inflammatory conditions, and to explore possible effect of the interaction between glucocorticosteroids and these receptors.
- To elucidate if there is an active cross-talk between neutrophils and alveolar epithelial cells, regarding pattern recognition receptors (PRRs) and if this cross-talk is altered in COPD.
- To characterize the expression of PRRs and their soluble form on/from immuno-active cells from different compartments, in patients with chronic inflammation (COPD) as well as in smokers and non-smokers.

3 MATERIAL AND METHODS

The following section contains a brief description of the methods used. Detailed information is found in each publication and manuscripts.

3.1 MATERIAL

3.1.1 Human study population

All subjects gave informed consent and the studies were approved by the ethics committee of Karolinska Institutet.

Controls

All controls were non-smokers, had normal lung function and no airway hyper-responsiveness. None had a history of asthma or allergy and had no other chronic diseases. (Paper I, III, IV)

Farmers

Farmers were included in the study if they had been exposed in the pig barn on a daily basis for the past 6 month. (Paper I)

Smokers without COPD

The smokers without COPD had normal lung function (FVC>80% of predicted value) with post-bronchodilator FEV₁/FVC > 0.70 and had no history of allergy or asthma. The smokers without COPD were in age matched with farmers, (Paper I) and matched with regard to age and pack-years with COPD-patients. (Paper IV)

COPD

All subjects with COPD had a post-bronchodilator FEV₁/FVC < 0.70 and FEV₁ of 40-70 % of predicted value and arterial oxygen saturation (SaO₂) > 90%. All were current smokers. (Paper III, IV)

3.1.2 Exposures (*In vivo*)

The experimental design of the exposure in a pig confinement building and exposure to pure LPS is described below (Figure 2). The pig house exposure lasted for three hours while weighing pigs. The LPS (53.4µg) exposure was performed by inhalation of six breaths of a LPS-solution in saline (*E. Coli* 0111:B4) using an inhalation dosimeter, (SPIRA® Elektro 2), corresponding to 53.4µg LPS. FEV₁ was measured before, 30 min and 60 min after, and then every hour for 6 hours after the provocation. The subjects were exposed in randomized order, with a three weeks period between the exposures.

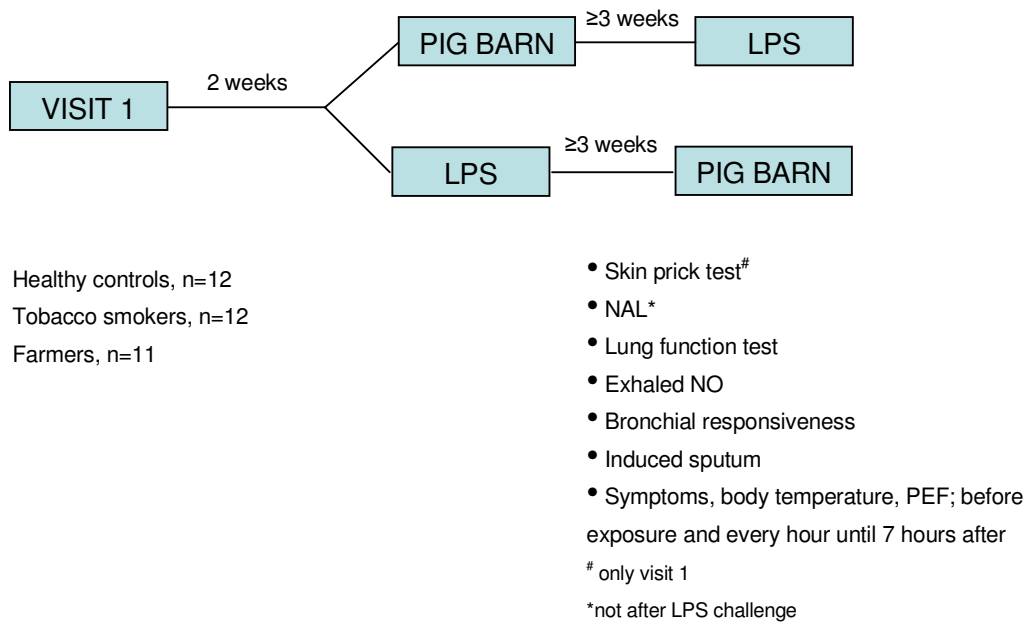


Figure 2, Experimental design and measured parameters (paper I)

Lung function and bronchial responsiveness were measured before and 7 hours after the start of each exposure. (Paper I)

3.1.3 Sample collection and processing

Lung function and bronchial responsiveness

Vital Capacity (VC) and FEV₁ were measured using a wedge spirometer according to ATS guidelines (72). PEF was measured with a peak flow meter (mini-Wright®). (Paper I)

Inhalation of doubling concentrations of methacholine up to 32 mg/ml (starting at 0.5 mg/ml) was used to test bronchial responsiveness. The result was expressed as the cumulative dose causing a 20% decrease in FEV₁ (PD₂₀FEV₁) (73). (Paper I)

Exhaled nitric oxide (NO)

Nitric oxide in exhaled air was measured during a single-breath exhalation, with a flow rate of 50mL/s, according to ATS guidelines (74). (Paper I)

Skin-prick test

Skin-prick test were performed on the forearms of each subject using a panel of 10 common allergens, with histamine chloride as a positive control. (Paper I)

Respiratory and inhalable dust measurement

Inhalable dust sampler (IOM) and plastic cyclones were used to monitor inhalable and respirable dust levels, respectively. The samplers were placed in the breathing zone on two subjects at each exposure occasion. (Paper I)

Nasal lavage (NAL)

Sterile 0.9% NaCl (5ml) was instilled into one nostril and 10 seconds later, expelled and collected and then repeated in the other nostril. The samples from the two nostrils were pooled and centrifuged, the cells were then counted in a Bürker chamber and the supernatant was stored in -70° C until further analysis. (Paper I)

Sputum induction and processing

After inhalation of salbutamol (0.4mg) sputum was induced by inhalation of saline in increasing concentrations, starting at 0.9%, using an ultrasonic nebulizer. Each concentration was followed by FEV₁ measurement. The subject made then an attempt to expectorate sputum. A sample that macroscopically appeared to be free from saliva and had a weight > 1g was accepted. The sputum sample was processed with dithiothreitol, filtered and centrifuged. The supernatant was stored in -70° C until further analysis. The cell pellet was resuspended and cell concentration, viability and differential cell counts were isolated. (Paper I, IV)

Bronchial lavage fluid (BAL)

Bronchoscopy was performed after pre-medication with morphine or pethidine and scopolamine and local anesthesia with xylocain® during the procedure. The bronchoscope was wedged in a middle lobe segmental bronchus and isotonic saline (5 x 50ml), was instilled into the airway tree and gently sucked back. The lavage fluid was centrifuged and the supernatant was stored in -70° C until analysis. The cell pellet was resuspended and the cells were cultured *ex vivo*. (Paper IV)

Blood sampling

Peripheral blood was collected in ethylene diamine-tetra-acetic acid (EDTA) vacutainer tubes for assessing cell surface markers using flow cytometry (FACS). (Paper IV)

For isolation of blood neutrophils, blood was drawn into heparinized tubes. (Paper III)

Symptoms

General and airway specific symptoms were recorded before and after exposure on a visual analogue scale 0–100 mm. The subjects were requested to put a cross on a scale where 0 indicated none, while 100 indicated unbearable symptoms. (Paper I)

3.1.4 Cell-culture procedures and experiments (*in vitro*)

Culture of Primary bronchial epithelial cells (PBEC)

Primary bronchial epithelial cells (PBEC) that had been established from patients who underwent thoracic surgery (lobectomy) were cultured as previously described (75). The cells were provided with Keratinocyte Serum-Free Medium (KSFM, Gibco, UK) that was supplemented with epidermal growth factor (EGF, 5 ng/ml, Gibco, UK), bovine pituitary extract (BPE, 50 µg/ml, Gibco, UK), pen/strep every second day. Test for *Mycoplasma* contamination (SVA, Uppsala, Sweden), were tested negative and in order to distinguish epithelial origin of the cells, immunostaining for cytokeratin was performed and was tested positive. (Paper II)

A549

The human alveolar epithelial type II cell line A549 was purchased from ATCC (American Type Culture Collection, Rockville USA). The cells were cultured in cell-culture flasks and provided with HAM's F-12 cell media supplemented with penicillin/streptomycin (1%) and heat inactivated fetal calf serum (10%) every second day. At confluence the cells were detached by trypsin/EDTA and re-cultured. Passages 6-10 were used in the cell experiments. (Paper III)

Neutrophils, isolation and cell culture

Whole blood was mixed with an equal volume of PBS-Dextrane (2%), and left for sedimentation. The leukocyte containing dextrane-blood was gently put on top of an equal volume of lymphoprep (Lymphoprep®) and then centrifuged. The cell pellet was then resuspended in PBS, washed and the red blood cells were lysed with distilled water. The neutrophils were washed twice with D-PBS and resuspended in RPMI supplemented with fetal calf serum (10%), L-glutamin (1%) and penicillin/streptomycin (1%). Cell concentration and viability were established with Türk solution and trypan blue, respectively and calculated in a Bürker chamber. (Paper III)

Co-culture of A549 and neutrophils

Freshly isolated neutrophils were added to cell-culture inserts with underlying A549 cells in the bottom of the 24-well plate. The cell culture experiments were performed in supplemented RPMI. (Paper III)

Alveolar macrophages

The cells obtained from BAL-fluid were seeded and after two hours the macrophages were presumed to be adhered. The supernatant was discarded and PBS was added to the macrophages in order to perform mRNA preparation. (Paper IV)

3.2 METHODS

3.2.1 Analysis of mRNA

mRNA preparation and Real-time PCR

Preparation of mRNA was performed from primary bronchial epithelial cells (Paper II) and from alveolar macrophages (Paper IV). Total mRNA was isolated by PureLink™ Micro-to-Midi Total RNA Purification System. DNase I, amplification grade was used to remove genomic DNA. First-strand cDNA was synthesized from 0.5µg of total RNA. Real-time PCR was performed using an ABI Power SYBR Green Master mix, 1µl of cDNA was amplified in 25µl PCR reaction to identify the products of interest. Beta-actin or GAPDH was used as internal control genes. The primers (beta-actin, GAPDH, IL-6, IL-8, TLR2, TLR4, CD14) were exon-exon spanning and designed using the software Primer3.

Data were analyzed using 7500 Software v.2.0.1, the results were then calculated and expressed as $2^{-\Delta Ct}$.

3.2.2 Analysis of proteins

Flow cytometry

To analyze cell distribution in peripheral blood a four color antibody mixture (CD3FITC/CD8PE/CD45PerCp/CD4APC) was used in TrueCOUNT™ tubes.

Flow cytometry has also been used to identify and quantify surface markers (TLR2, TLR4 and CD14) on blood neutrophils (Paper III, IV) and sputum neutrophils and blood monocytes (Paper IV). The cells were incubated with monoclonal fluorochrome conjugated or matched isotype antibodies for 30 minutes, washed and analyzed using FACSCalibur™ flow cytometry and CELLQuest™. Data are presented as relative median fluorescence intensity (rMFI : monoclonalantibody / matched isotype control). (Paper III, IV)

ELISA

IL-6 and CXCL8 (IL-8)

Interleukin-6 and CXCL8 (IL-8) have been measured in the supernatants from NAL, sputum and cell-culture experiments using an in-house ELISA method. Commercially available antibody pairs were used (76). (Paper I, II, III)

sCD14

Soluble CD14 (sCD14) has been measured in the supernatants from sputum, BAL fluid and cell-culture experiments using a purchased DuoSet ELISA CD14 kit. The analysis was performed according to the manufactures protocol. (Paper III, IV)

sTLR2

Soluble Toll-like receptor 2 (sTLR2) has been measured in the supernatants from BAL and sputum with a purchased DuoSet ELISA sTLR2 kit. The analysis was performed according to the manufactures protocol. (Paper IV)

Endotoxin measurement

Endotoxin concentration was analyzed using a kinetic technique version of *Limulus amoebocyte* lysate assay (Limulus Amebocyte lysate, Endosafe® Endochrome-K™ U.S. Lisence No. 1197), with *E. coli* 0111:B4 as standard. (Paper I, IV)

3.3 STATISTICS

Data are presented as scatters with median, with 25th-75th percentile, as mean ± SEM (standard error of the mean) or as 95% confidence intervals, depending on the distribution of the data.

When data was normally distributed, parametric test, such as, ANOVA for repeated measurements followed by paired t-test (within group comparisons) and ANOVA followed by Fisher's PLSD (between-group comparisons) were used.

When data was considered not to be normally distributed, non-parametric analyze methods were used. Within group comparisons were performed using Friedman's test (if more than two groups) followed by Wilcoxon signed rank-sum test as *post hoc* test. Between-group comparisons were analyzed by Kruskal-Wallis if more than two groups, followed by Mann-Whitney U-test as a *post hoc* test.

All data were analyzed by Statview version 5.0.1 (SAS Institute Inc., Cary NC). A value of P<0.05 was considered significant.

4 RESULTS

P-values are not stated in the text, but can be found in the figures and/or in the corresponding captions.

4.1 PAPER I

REPEATED EXPOSURE TO ORGANIC MATERIAL ALTERS INFLAMMATORY AND PHYSIOLOGICAL AIRWAY RESPONSES

The aim of this study was to find out whether the response to inhalation of organic dust and lipopolysaccharide (LPS) is altered in chronically exposed individuals i.e. smokers and farmers.

Baseline FEV₁ measurement were significantly lower in farmers and smokers compared to controls (data not shown).

The control subjects significantly decreased in VC and FEV₁ after pure LPS exposure. No such effect was observed in the continuously exposed groups, farmers and smokers. No significant changes in lung-function post-exposure compared to pre-exposure values were found between the three groups (controls, farmers, smokers). (Table 2)

	TABLE 2 Changes in lung function in healthy nonsmoking, nonfarming controls, smokers and farmers							
	ΔVC L LPS		ΔVC L dust		ΔFEV ₁ L LPS		ΔFEV ₁ L dust	
	Mean (95% CI)	p-value	Mean (95% CI)	p-value	Mean (95% CI)	p-value	Mean (95% CI)	p-value
Controls	-0.12 (-0.21– -0.04)	0.008	-0.22 (-0.32– -0.13)	0.0004	-0.10 (-0.18– -0.02)	0.02	-0.26 (-0.35– -0.17)	<0.0001
Farmers	-0.01 (-0.13–0.10)	0.8	-0.10 (-0.17– -0.03)	0.01	-0.05 (-0.18–0.08)	0.4	-0.14 (-0.26– -0.03)	0.02
Smokers	-0.09 (-0.23–0.05)	0.2	-0.21 (-0.39– -0.03)	0.02	0.02 (-0.11–0.15)	0.8	-0.12 (-0.25–0.003)	0.06

Differences (Δ) between pre-exposure and 7 h after lipopolysaccharide (LPS) challenge and exposure in a pig barn. The influence of LPS and pig barn exposure did not differ significantly between the three groups. CI: confidence interval; VC: vital capacity; FEV₁: forced expiratory volume in 1 s.

Table 2, Changes in lung function after exposure to dust or pure LPS

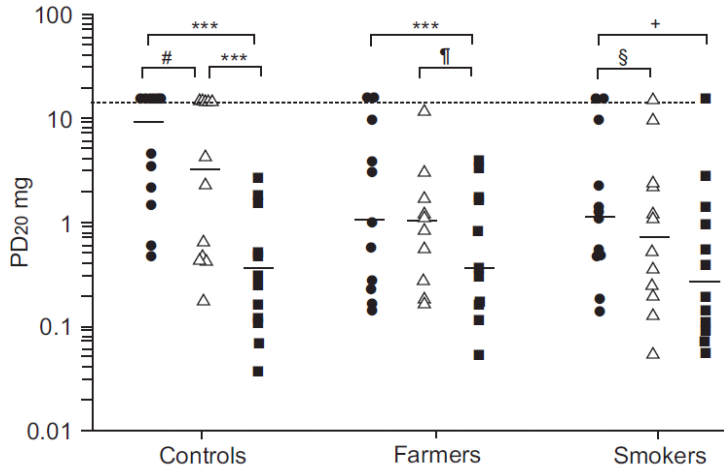


Figure 3, Bronchial responsiveness to methacholine, (as measured by the cumulative dose causing a 20% decrease in FEV₁ (PD₂₀)). At baseline (●), after LPS challenge (△) and after exposure in the swine barn (■). Horizontal lines indicates medians. P-values for within group comparisons are as follows: #P=0.01, ¶P=0.02, +P=0.003, §P=0.04, *P<0.001. Pre-exposure and post LPS-exposure PD₂₀ did not significantly differ between the groups. After exposure to the swine house bronchial responsiveness increased to a greater extent in controls compared to farmers and smokers P<0.001.**

Interleukin-6 (IL-6) and CXCL8 (IL-8) were measured in the supernatants from NAL (Figure 4) and sputum (Figure 5).

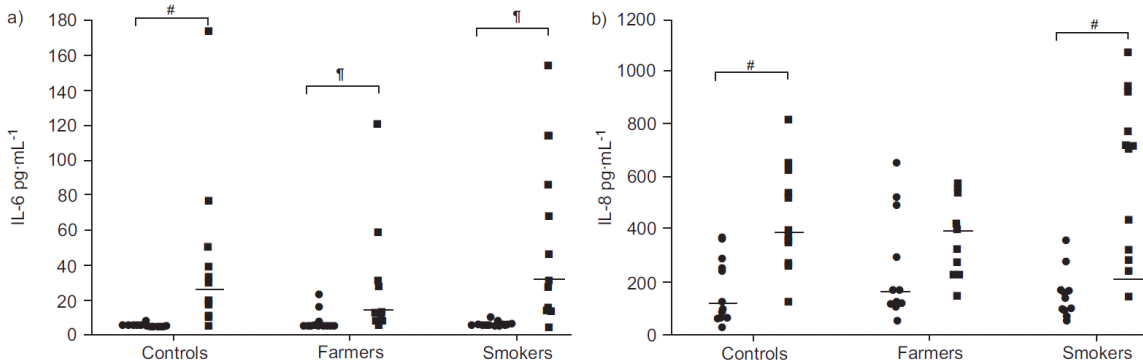


Figure 4, Concentration of IL-6 (a) and CXCL8 (IL-8) (b) in nasal lavage. At baseline (●) and after exposure in the swine barn (■). Horizontal lines indicates medians. P-values for within group comparisons are as follows: #P=0.002 and ¶P=0.004. No significant differences were observed in the baseline between the groups. After swine barn exposure: no differences between the groups regarding IL-6, whereas CXCL8 (IL-8) increased significantly less in farmers compared to the other groups.

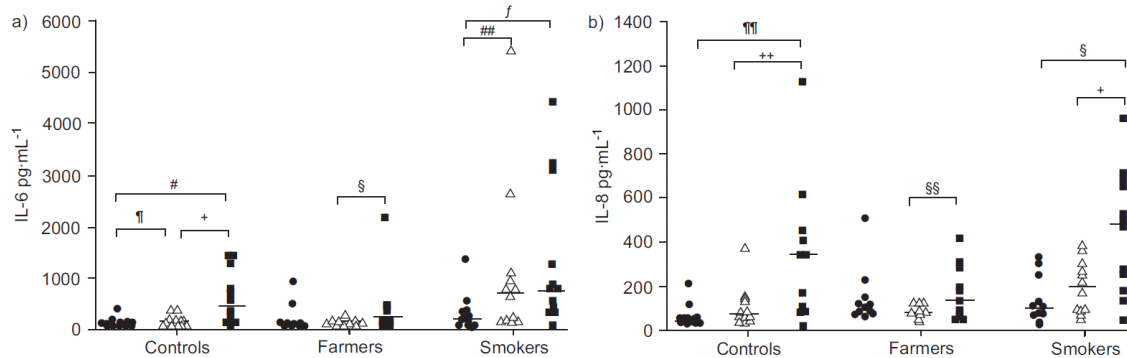


Figure 5, Concentration of IL-6 (a) and CXCL8 (IL-8) (b) in sputum supernatants. At baseline (●), after LPS challenge (Δ) and after exposure in the swine barn (■). Horizontal lines indicates medians. P-values for within group comparisons are as follows: #P=0.003, ¶P=0.05, +P=0.008, §P=0.005, fP=0.002, ##P=0.02, ¶¶P=0.006, ++P=0.01 and §§P=0.03. At baseline were IL-6 elevated in smokers compared to controls and CXCL8 (IL-8) were higher in both smokers and farmers compared to controls.

To conclude, we found that the two continuously exposed groups to organic material i.e. farmers and smokers have signs of an ongoing airway inflammation in the central but not in the upper airways. Smokers seem to have an increased response to LPS-exposure, compared to both farmers and controls, regarding pro-inflammatory cytokines in sputum (IL-6, IL-8). We also found that exposure in the swine barn was a much stronger pro-inflammatory stimulus than inhalation of pure LPS. The most important finding was that the response to exposure in a swine barn differed between the groups. Both physiological outcomes like bronchial responsiveness, but also markers of airway inflammation like exhaled NO and pro-inflammatory cytokines were attenuated in farmers compared to controls.

4.2 PAPER II

BUDESONIDE ENHANCES TOLL-LIKE RECEPTOR 2 EXPRESSION IN ACTIVATED BRONCHIAL EPITHELIAL CELLS

The aim of this study was to elucidate if Toll-like receptor (TLR) 2 and TLR4 expression were altered by exogenous and/or endogenous pro-inflammatory stimuli and to what extent glucocorticosteroids alter TLR expression. The experiments were performed on primary bronchial epithelial cells (PBEC). The mRNA expression and the released amount of the pro-inflammatory cytokines interleukin-6 (IL-6) and CXCL8 (IL-8) were measured and regarded as outcome variables.

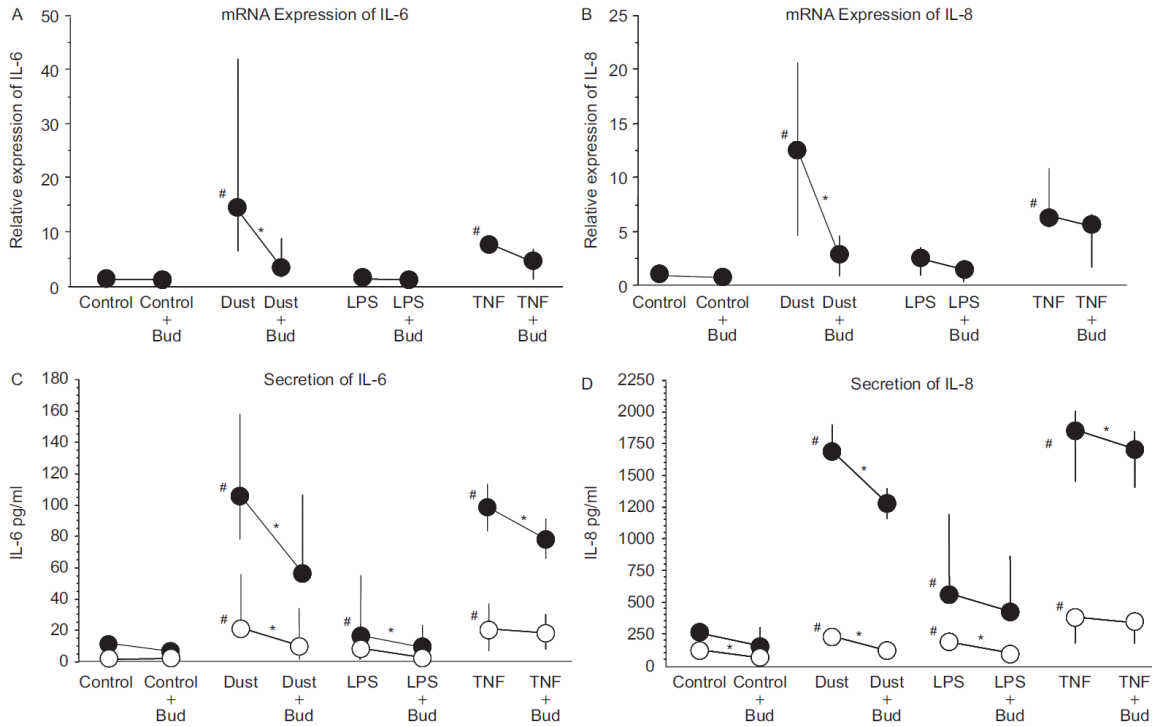


Figure 6, Stimulation of PBEC with dust, LPS, TNF with or without budesonide. mRNA expression (A, B) and secreted protein (C, D) of IL-6 and IL-8 respectively. IL-6 and IL-8 were measured after 1.5 hours (open circles) and 6 hours (filled circles). Data are presented as medians and interquartile ranges. #p<0.05 compared with the unstimulated control and *p<0.05 compared with the corresponding budesonide treated sample.

We found that the mRNA expression and release of IL-6 and IL-8 were increased by pro-inflammatory stimuli. This increase in cytokine secretion was reduced by budesonide both after endogenous and exogenous stimulation (Figure 6).

To elucidate if pro-inflammatory stimulation and/or glucocorticosteroids alter TLR2 expression, TLR2 mRNA was measured 6 hours after pro-inflammatory exposure with or without budesonide.

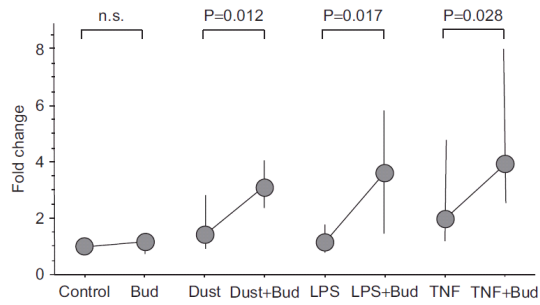


Figure 7, Expression of TLR2 mRNA after 6 hours exposure of pro-inflammatory stimuli, with or without budesonide. Data are presented as medians and interquartile ranges.

TLR2 mRNA was synergistically increased after 6 hours exposure to all tested pro-inflammatory stimulus only in the presence of a glucocorticosteroid (Figure 7).

Finally, we performed blocking experiments of TLR2 and/or TLR4 with antibodies to elucidate to what extent the released IL-6 and IL-8 was a result of ligand binding to TLR2 and TLR4.

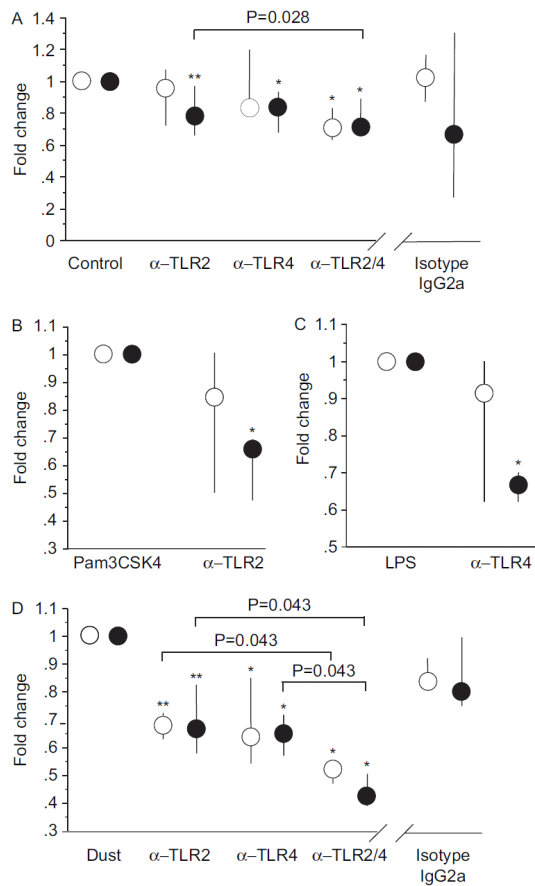


Figure 8, PBEC cells unstimulated (A) or stimulated with Pam3CSK4 (B), LPS (C) or dust (D). Blocking of TLR2 and/or TLR4 followed by stimulation with corresponding ligand. Secreted IL-6 and IL-8 were measured in the supernatant. Open circles represent IL-6, filled circles IL-8. Data are presented as fold change of the corresponding control as medians and interquartile ranges. *P<0.05 **P<0.01 compared to unstimulated control. Significant differences between treatments are indicated with brackets.

In general, blocking of TLR2 and/or TLR4 with monoclonal antibodies resulted in a greater inhibition of CXCL8 (IL-8) release than IL-6 release. A statistically significant blocked release of CXCL8 (IL-8) was obtained in use of stimulation with specific TLR2 and TLR4 agonist, Pam₃CSK₄ and LPS, respectively. During dust stimulation both IL-6 and CXCL8 (IL-8) release was significantly inhibited by either of the two TLR-antibodies and in the combination of them with some additive effect (Figure 8).

To conclude, IL-6 and CXCL8 (IL-8) released from stimulated bronchial epithelial cells are partly TLR2/4 dependent. TLR2 mRNA expression was increased and IL-6 and IL-8 production reduced when budesonide was added to stimulated primary bronchial epithelial cells.

4.3 PAPER III

INTERACTIONS BETWEEN ALVEOLAR EPITHELIAL CELLS AND NEUTROPHILS DURING PRO-INFLAMMATORY CONDITIONS

In paper III, an active cross-talk between alveolar epithelial cells (A549) and neutrophils during normal and pro-inflammatory conditions was studied. We also wanted to find out if this possible cross-talk is altered in COPD. Neutrophils and A549 cells were stimulated with TNF, PGN or LPS in single culture and during co-culture experiments. The neutrophils were obtained from subjects with or without COPD. After pro-inflammatory stimulation the expression of TLR2/4 and mCD14 on neutrophils were assessed with FACS.

After pro-inflammatory stimulation the expression of TLR2/4 and mCD14 on neutrophils were assessed with FACS.

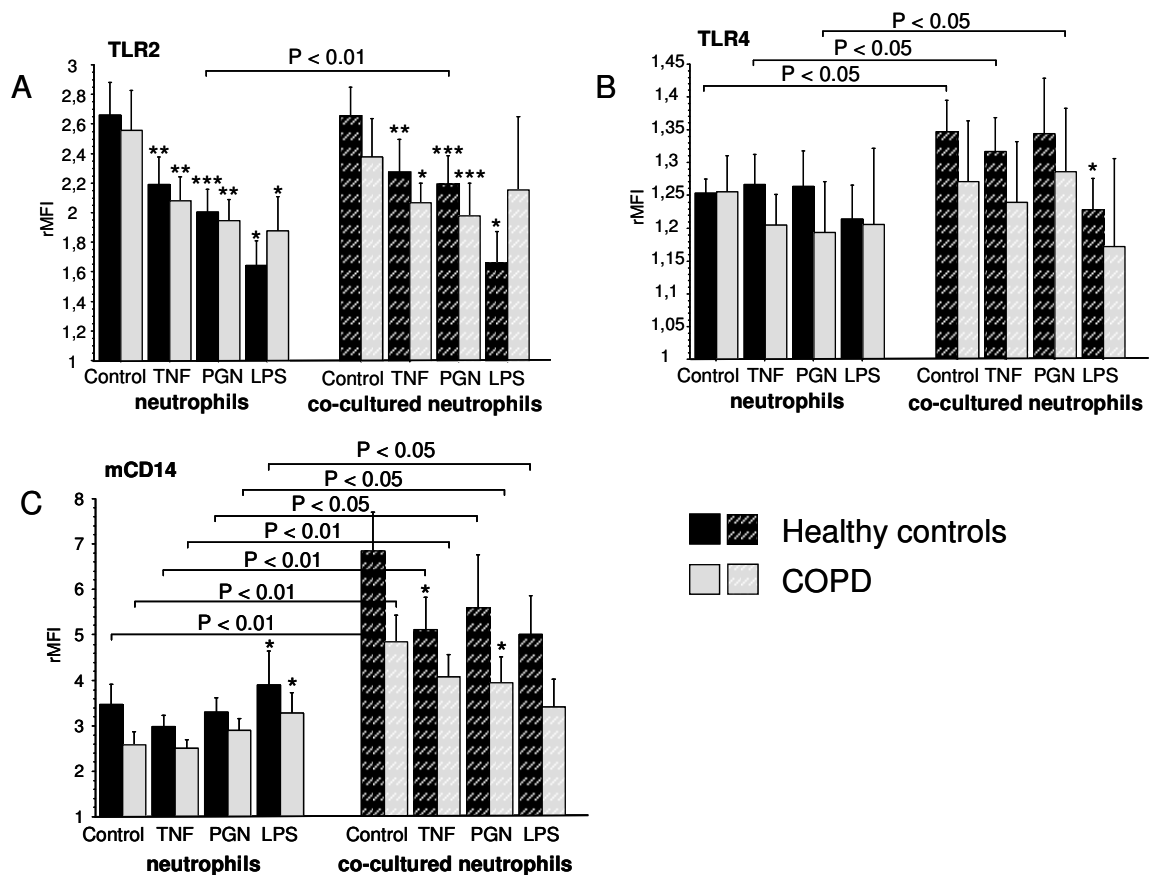


Figure 9, Cell surface expression of TLR2 (a), TLR4 (b) and mCD14 (c) in single and co-cultured neutrophils obtained from healthy non-smokers and smokers with COPD. Data are presented as mean±SEM. *P<0.05 **P<0.01 and ***P<0.001 compared to corresponding unstimulated control. Significant differences between single cultured and co-cultured neutrophils are indicated with brackets.

We found that TLR2 expression on the cell surface on neutrophils was down regulated by all tested pro-inflammatory stimuli. On the other hand, mCD14 was increased on the cell surface by co-culture conditions independent of pro-inflammatory or normal cell culture conditions (Figure 9).

As outcome variable, CXCL8 (IL-8) was measured in the supernatant, together with the soluble form of CD14 (sCD14).

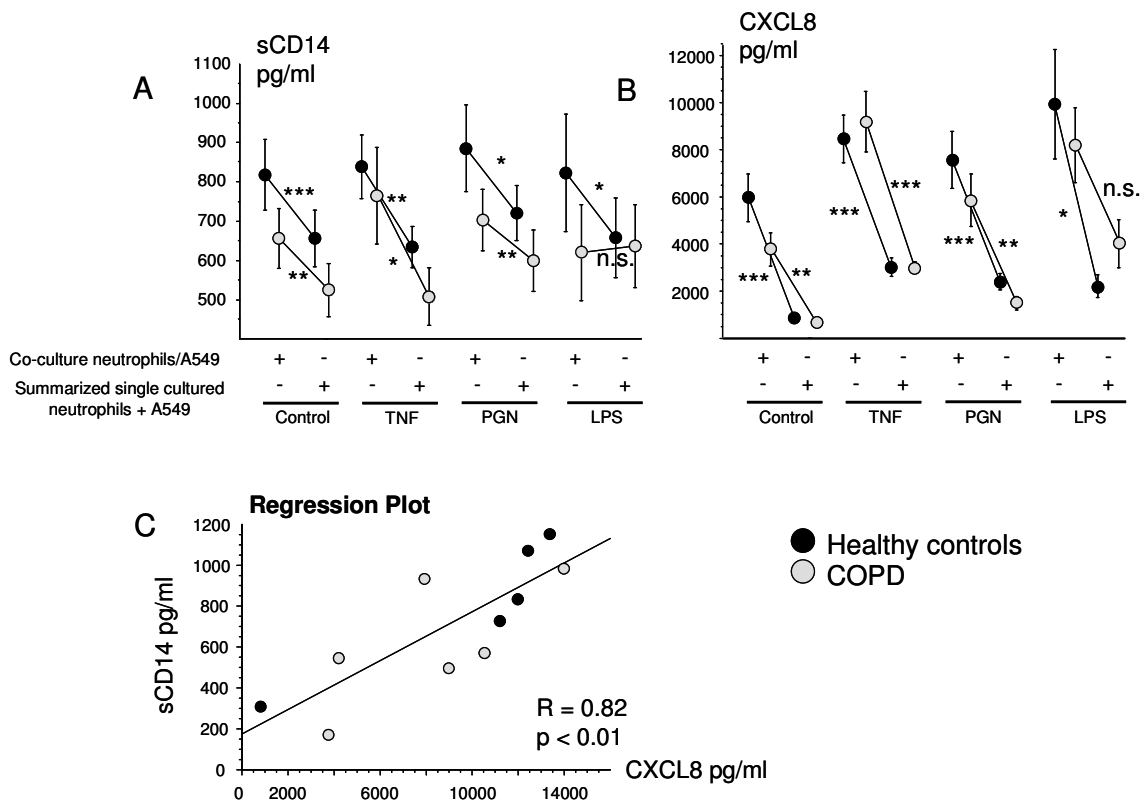


Figure 10, Co-cultured neutrophils/A549 and summarized single cultured neutrophils + A549. Soluble CD14 (A) and CXCL8 (B) were measured in the supernatant. *P<0.05 **P<0.01 *P<0.001. Regression plot of sCD14 and CXCL8 in LPS stimulated co-cultured neutrophils and A549 cells (c).**

We found that sCD14 and CXCL8 (IL-8) were synergistically enhanced during co-culture conditions compared to summarized single culture cells. Irrespective of normal or pro-inflammatory conditions (Figure 10A, B). We also found a strong correlation between sCD14 and released CXCL8 (IL-8) in LPS-stimulated co-cultured cells (Figure 10C).

To conclude we found an active cross-talk between alveolar epithelial cells and neutrophils, regarding regulation of pattern recognition receptors (PRRs) and one of their down-stream effector CXCL8 (IL-8). The cross-talk was observed both during normal and pro-inflammatory conditions. A strong correlation between sCD14 and

CXCL8 (IL-8) in LPS-stimulated co-cultured cells, indicate a bidirectional cross-talk where sCD14 are donated by neutrophils to epithelial cells facilitating LPS-signaling. This will subsequently lead to increased CXCL8 (IL-8), a potent chemo attractant for neutrophils, released by the alveolar epithelial cells. No statistical significant differences were observed between controls and COPD subjects.

4.4 PAPER IV

TOLL-LIKE RECEPTOR EXPRESSION IN SMOKERS WITH OR WITHOUT COPD

In paper IV we wanted to elucidate if innate immune receptors are altered in smokers with or without COPD and if this possible alteration is present in circulating cells. Inflammatory cell expression of TLR2, TLR4 and CD14 and soluble TLR2 and CD14 were assessed in blood, bronchoalveolar lavage fluid (peripheral airways) and induced sputum (central airways) from smokers with and without COPD and in healthy non-smokers.

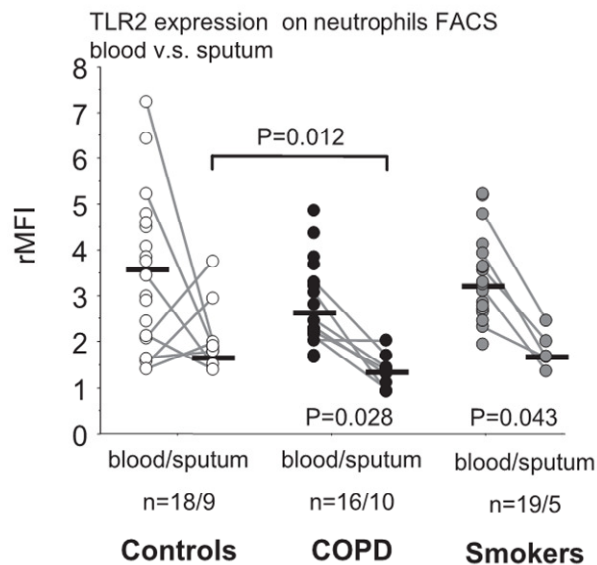


Figure 11, TLR2 expression on blood and sputum neutrophils. Data obtained from the same subject are connected with a line. Significant differences between compartments are stated with a P-value and between groups with a bracket.

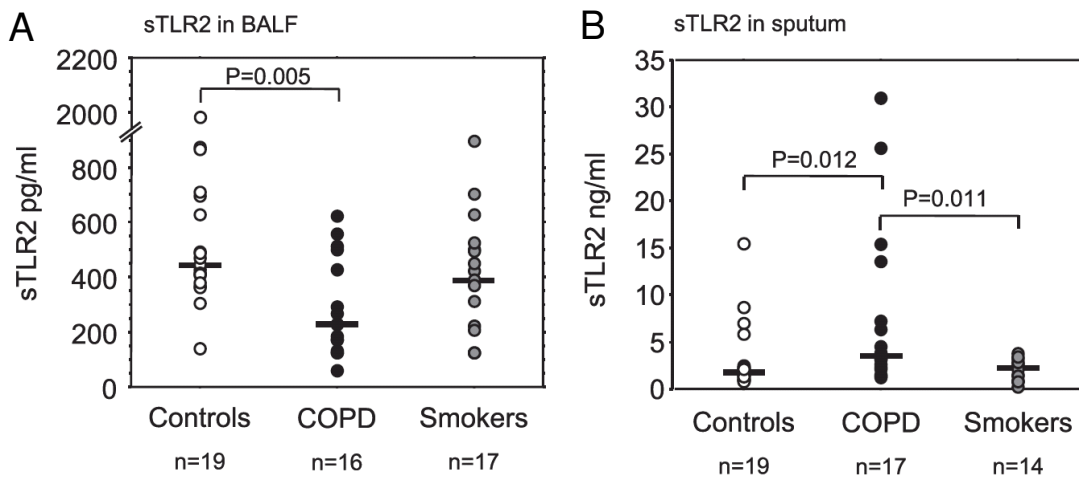


Figure 12, Soluble TLR2 (sTLR2) in BAL-fluid (A) and sputum (B) supernatants. Significant differences between groups are stated with a bracket and P-value.

We found that TLR2 on the surface on neutrophils were down regulated on sputum neutrophils compared to blood neutrophils. This was only significant in smokers irrespective of airway obstruction (Figure 11). Further, COPD-patients express less TLR2 on their sputum neutrophils compared to non-smoking controls. In line with that finding sTLR2 was elevated in sputum supernatants in the COPD group compared to both healthy smokers and controls. In BAL-fluid sTLR2 was about a tenth of the concentration in sputum supernatants with the lowest concentration were found in the COPD-group (Figure 12).

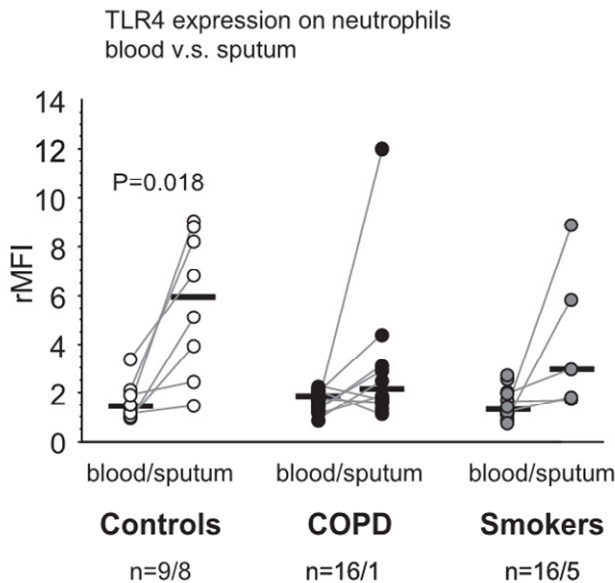


Figure 13, TLR4 expression on blood and sputum neutrophils. Data obtained from the same subject are connected with a line. Significant differences between compartments are stated with a P-value.

We found that TLR4 expression on neutrophils was upregulated on sputum neutrophils compared to circulating neutrophils. This was only observed in the non-smoking control group. No significant differences between the groups were observed (Figure 13).

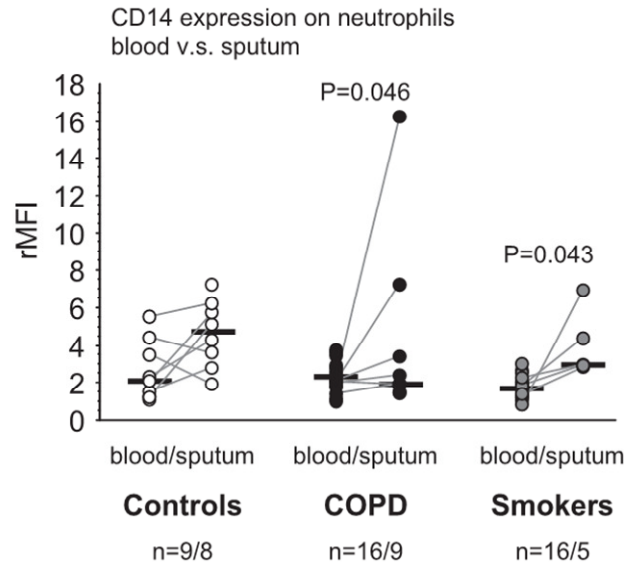


Figure 14, CD14 expression on blood and sputum neutrophils. Data obtained from the same subject are connected with a line. Significant differences between compartments are stated with a P-value.

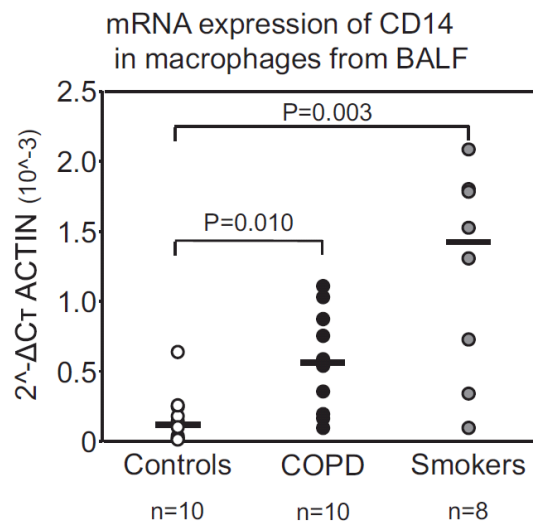


Figure 15, Gene expression of CD14 on alveolar macrophages. Significant differences between groups are stated with a P-value.

We found that CD14 are upregulated on sputum compared to blood neutrophils in smokers. No statistical significant differences were observed between the groups (Figure 14). We also found an increased gene expression of CD14 on alveolar macrophages obtained from smokers, irrespective of airway obstruction, compared to the control-groups (Figure 15).

To conclude, our studies showed that different PRRs are differently regulated during pro-inflammatory conditions. Further, we found that PRRs expression is affected by tobacco smoking, but also by COPD per se, as we found differences between smokers with and without airway obstruction, but the same cumulative exposure to tobacco smoke. Moreover, we showed for the first time the presence of the soluble form of TLR2 (sTLR2) in sputum and BAL fluid supernatants. We hypothesize that PRRs are affected and might even be involved in the development of the chronic inflammatory disease.

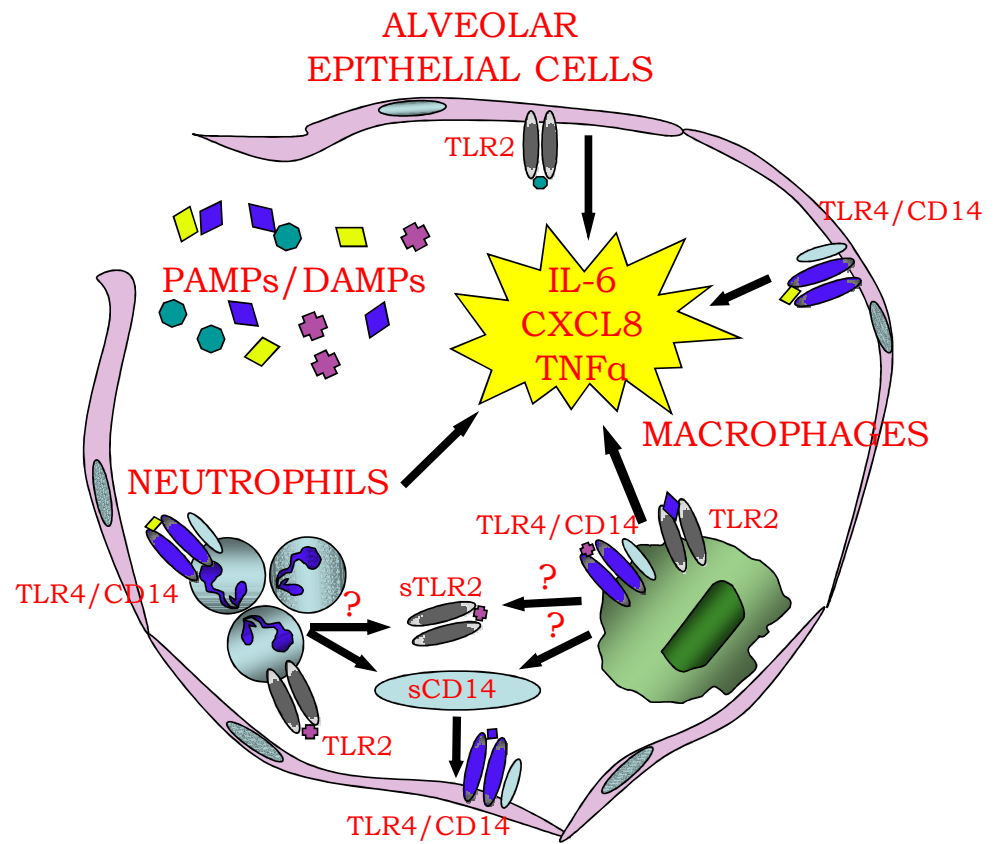
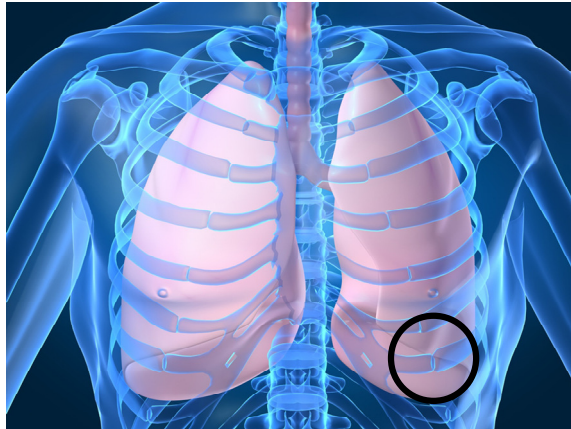


Figure 16, Schematic overview of PRRs during pro-inflammatory conditions in the alveoli.

5 GENERAL DISCUSSION

Continuous exposure to inhalable particles affects our immune system and our lungs. There are several characteristics of the exposure that determine the biological consequences, such as, the content, size and origin of the particles, the subject's age and the duration of the exposure.

The main objective of this thesis was to characterize the inflammatory airway response to chronic exposure of organic material. Therefore we studied farmers, who are exposed on a daily basis in their work environment, and smokers with and without chronic obstruction (COPD) exposed to organic material through tobacco smoke. The focus was on PRRs (Pattern Recognition Receptors), especially Toll-like receptors (TLR), and how they are regulated in immunologically active cell types as well as soluble receptors in different compartments *in vivo* and *in vitro*.

Children who grow up on a farm or have parents who are active in the agricultural sector have a lower prevalence of asthma and allergy, than children who grew up in an urban environment (14, 77, 78). On the other hand, the adult farmers have a higher prevalence of respiratory diseases such as chronic bronchitis and reduced lung function than the population in general (17).

However, children who grow up in the farming environment have an increased gene expression of TLR2, TLR4 and CD14 on circulating blood cells, and there is a positive correlation between the number of different animal species to which the mother was exposed to during pregnancy and the expression of these genes in the child. The same pattern of increased PRRs gene expression, although less pronounced are found in children exposed to the farm environment, in early life (79, 80). On the other hand, adult farmers have a reduced expression of TLR2 on circulating blood monocytes (63). The observed differences, with up-regulation of PRRs in young children and down regulation of PRRs in their parents, as an effect of daily exposure are intriguing. Importantly, these studies also demonstrate systemic effects of the chronic organic dust exposure. To summarize, it seems likely that the PRR expression on circulating cells are affected by the continuous exposure to organic dust and that these receptors are differently regulated in children and adults. This might be of importance for the outcome in the long run as the farmers have higher prevalence of chronic bronchitis and are also known to be colonized with bacteria in their airways.

In paper I it was shown, that the farmers had elevated levels of exhaled nitric oxide (NO) and CXCL8 (IL-8) in the sputum supernatant, compared with a naïve control group. This indicates an ongoing inflammation in the respiratory tract. Acute exposure in the swine barn showed that farmers and smokers had an attenuated immune response compared with healthy controls. The control group showed a significantly greater increase in bronchial responsiveness compared with the chronically exposed groups (farmers and smokers) after three hours exposure in the swine barn. This suggests some kind of tolerance, in the chronically exposed groups, a finding that supports earlier results (12, 81, 82). It is possible that this tolerance is not beneficial in the long

perspective, but may cause disease development, such as chronic bronchitis later in life. Although, no significant differences were observed in bronchial responsiveness prior to exposure, it was clear that the exposed groups had a somewhat higher bronchial reactivity than the controls. It can not be excluded that the difference in bronchial responsiveness ($PD_{20}FEV_1$) after exposure in the swine barn between controls and chronically exposed (smokers/farmers) is due to the difference in baseline values. This means that, farmers and smokers had a somewhat increased bronchial responsiveness already at baseline, which may have resulted in less pronounced reduction of $PD_{20}FEV_1$ after exposure in the swine barn. This has previously been shown to be of relevance (83).

Susceptible individuals may have symptoms that make them more prone to leave the farming environment. This health-based selection process has been suggested to occur in swine farmers for asthma, but not for chronic bronchitis, as the symptoms often appear later in life (16). Farms are often inherited and stay within the family. This might lead to a more pronounced healthy-worker effect within the farming families, than in other occupations. There are studies showing several inherited active relevant polymorphisms in the receptor complex that binds Lipopolysaccharide (LPS), i.e. both TLR4 and CD14. These polymorphisms have been shown to affect both the inflammatory response as decrease in FEV_1 (84-86). The polymorphisms in PRRs might be of relevance in the health-based selection process that occurs in farmers.

Lipopolysaccharide (LPS) is just one out of many potent components in the farming environment. The organic dust found in the swine barns where we have exposed the subjects are highly heterogeneous and contains various microbial components from microorganisms, molds, yeast and bacteria (5). It has been shown that wearing a respirator during the three hour visit in the swine barn reduces the LPS-exposure by 99%, measurements were performed behind the mask, and attenuates the inflammation, but not the increase in bronchial responsiveness in the exposed subjects (87, 88). Several *in vitro* and *in vivo* studies, including the first and second paper of this thesis, confirm these data; that dust from the swine barn was a more potent pro-inflammatory stimulus, than LPS and peptidoglycan (PGN) were etc. (89-91). This indicates that it might be other potent agents in the dust than LPS or PGN and/or several simultaneous occurring stimulations that will result in a synergistic immunological response.

In line with the findings of attenuated TLR2 expression on blood monocytes in farmers (92) a lower TLR2 expression on alveolar macrophages in patients with COPD has been demonstrated (93). Subjects with COPD are chronically exposed to organic material through inhaled tobacco smoke. In paper IV it was shown that sputum neutrophils have an attenuated expression of TLR2 in COPD-patients, compared to age matched non-smoking controls. Moreover, the concentration of soluble TLR2 (sTLR2) was higher in the sputum supernatant in the COPD-group compared to both the non-smoking and smoking controls. This suggests that the receptor is probably regulated by shedding but also that this effect is not directly dependent of tobacco smoke exposure, but is more specifically involved in the patho-physiological mechanism of COPD as the smokers with and without COPD were matched regarding age and cumulative exposure to tobacco smoke.

Bacterial colonization in the respiratory tract is commonly occurring in farmers and COPD-patients; this might lead to an increased risk for exacerbations (94, 95). It seems likely that neutrophils exposed to a pro-inflammatory environment, induces release of TLR2 as a negative regulatory mechanism. The soluble form of the receptor will compete for the ligand with the membrane bound form of the receptor. Soluble receptors usually retain their ligand binding capacity and it is known that sTLR2 attenuates the release of pro-inflammatory cytokines in monocytes that are stimulated with TLR2 ligands (44, 46).

This reasoning is further confirmed by results from paper IV, where it was shown that neutrophils, at transfer from the sterile blood stream into the lungs; show a down-regulation of TLR2 expression. This is previously shown in farmers (92) and seems to be a general occurring mechanism and is likely a sign of receptor shedding when entering a pro-inflammatory environment. Additional signs of this phenomenon are results from paper III, where blood neutrophils and alveolar epithelial cells have been co-cultured *in vitro* with or without pro-inflammatory stimulus. Regardless of endogenous or exogenous stimulation of the cells, unequivocal results showed significantly lower expression of TLR2 receptor. *In vivo* studies showed similar pattern, where TLR2 expression on blood neutrophils in patients with septic shock was down-regulated compared with healthy controls (96). While the results in this thesis are unanimous regarding TLR2 regulation during pro-inflammatory stimulation, the literature on TLR2-regulation is not entirely easy to interpret, as there are several above all *in vitro* studies showing controversial results (97-99).

Soluble TLR4 (sTLR4) are so far only found in human saliva and it has been shown to suppress LPS activation *in vitro* (52). It has also been shown in mice that recombinant sTLR4 had similar properties as sTLR2, by inhibiting the production inflammatory response to LPS-stimulation (50). Unlike TLR2, which appears to be down regulated by a pro-inflammatory environment, result from paper IV prove an up-regulation of TLR4 on sputum neutrophils compared with neutrophils from the blood in healthy controls. This pattern is also observed in farmers (92). What is clear is that different PRRs are differently regulated.

Ex-vivo stimulation with TNF, peptidoglycan (PGN) or LPS of blood neutrophils did not affect the expression of TLR4 (paper III). However, during co-culture with alveolar epithelial cells (A549) TLR4 was significantly up-regulated independently of pro-inflammatory stimulation. These findings are intriguing and indicate an interaction between epithelial cells and neutrophils, which involves regulatory mechanism of the signalling pathway of TLR4, irrespective of pro-inflammatory conditions. Endogenous TLR ligands released by epithelial cells can be of relevance in this aspect.

It could be assumed that CD14 has limited opportunities to interact in the inflammatory host response as CD14 lacks a transmembrane domain and therefore the potential to initiate intracellular-signalling by itself (55). It is however known that CD14 acts as a pattern recognition receptor and is one of the main players involved in the signalling pathway of LPS via the TLR4/MD2 receptor complex. Furthermore, CD14 is associated with and seems to be of importance for the TLR2/TLR1 recognition of Pam₃CSK₄, although this is less studied than the co-receptor function of CD14 in the

TLR4 signalling pathway (100, 101). This might explain the rather weak effect of Pam₃CSK₄ stimulation on epithelial cells, observed in paper II.

It has recently been found that the MyD88-independent pathway of TLR4-signaling requires CD14 for IFN-secretion. Moreover, murine macrophages lacking CD14, express the same amount of TNF upon stimulation with rough LPS as do CD14-positive macrophages. The macrophages failed to release TNF after stimulation with smooth LPS, an effect that was reversed after sCD14 was added (48), indicating that the signaling pathway of smooth LPS is dependent of CD14. Furthermore, stimulation with LPS in paper II resulted in a weak response in the measured outcome variables IL-6 and CXCL8 (IL-8). Epithelial cells are known to express very little CD14 and the affect on PBEC upon LPS-stimulation paper II was rather weak. But still we observed an increase in cytokine release as a result of stimulation, which probably was an effect of the CD14 independent pathway of TLR4, which bound rough LPS (rLPS).

Soluble CD14 are found in serum, bronchoalveolar lavage, sputum, etc (102, 103). Neutrophils, monocytes and macrophages are proposed to be the main producers of sCD14.(104, 105) Soluble CD14 is known to increase in serum during pro-inflammatory conditions, and is regarded as an acute phase protein. Moreover, sCD14 are also known to be elevated in bronchoalveolar lavage (BAL) fluid from smokers with or without COPD. (54, 57, 58) We were not able to confirm these data in paper IV. A possible explanation for this discrepancy could be that smokers which are exposed to LPS from the tobacco smoke (106), have less unbound sCD14 in their supernatants. It is possible that the ligand (LPS) will compete with the antibodies of the ELISA through sterical or competitive inhibition, which might result in false negative results. The significantly lower concentration of endotoxin in BAL fluid obtained from smokers compared to non-smoking controls might also be explained by the occurrence of a sCD14/endotoxin complex.

Soluble CD14 has, at least, a dualistic effect. On one hand it mediates the activation of CD14 negative cells or cells with very low expression of CD14, such as alveolar epithelial cells (107, 108). On the other hand sCD14 function as a decoy receptor, by competing with mCD14 for LPS and thereby reduce LPS-driven activities (109, 110). In paper III, communication between alveolar epithelial cells and neutrophils were examined. One of the main findings in that paper was that mCD14 was strongly up-regulated on the surface on neutrophils, when co-cultured with alveolar epithelial cells, irrespective of pro-inflammatory activation. Moreover, the soluble form of CD14 was synergistically enhanced during these conditions and when LPS was added to the co-culture CXCL8 (IL-8) and sCD14 showed an almost perfect correlation. Thus paper III confirms that sCD14, released by neutrophils, facilitate LPS-driven stimulation of epithelial cells, which in the end leads to increased CXCL8 (IL-8) production.

The positive effect of inhaled steroids in patients with COPD is less beneficial than the effect observed in asthma in general, although the beneficial effect of steroids in severe asthma, often dominated by neutrophils, are less pronounced (111, 112). It has been shown that steroid treatment reduces the number of exacerbations and that the combination of an inhaled steroid and long-acting β_2 -agonist reduces mortality in COPD (30). In paper II it was demonstrated that TLR2 mRNA was synergistically

enhanced by budesonide on activated primary bronchial epithelial cells. This effect was only observed when both a pro-inflammatory stimuli and the steroid were present. This is regardless of whether the pro-inflammatory stimulus was endogenous (TNF) or exogenous (LPS, PGN). The release of the pro-inflammatory cytokines IL-6 and CXCL8 (IL-8) was inhibited significantly by TLR2/TLR4 antibodies, suggesting that the pro-inflammatory response was TLR-mediated. One possible and even probable explanation to the lack of difference between ligand and non-ligand stimulation, regarding IL-6 and CXCL8 (IL-8) secretion is, that the epithelial cells release endogenous TLR2 and TLR4-ligands, DAMPs, that compete with the exogenous TLR-ligands (113). This might also explain why the release of IL-6 and CXCL8 (IL-8) was inhibited in un-stimulated cells with the blocking of the TLR antibodies.

To conclude, research focused on regulatory mechanisms of toll-like receptors has just started and it is clear that the mechanisms of TLR-regulation are many and complex (51, 64). We have found that chronically exposed subjects have altered PRRs expression on cells involved in the immunological response of the host. We have also showed that TLR2 expression on bronchial epithelial cells increases in the presence of a glucocorticosteroid, a common treatment in inflammatory diseases. Last but not least we found a strong positive correlation between the secreted soluble CD14 and CXCL8 (IL-8) from neutrophils and epithelial cells in co-culture during LPS-stimulation. This strongly suggests an up-take of sCD14 of the epithelial cells, which will facilitate the TLR4 signalling pathway. A better understanding of the mechanisms of chronic inflammation and host defence, might lead to development of new therapies that would potentially benefit patients with inflammatory diseases.

6 CONCLUSIONS

In summary, the main results obtained from this thesis suggest the following:

Paper I:

- Individuals who are repeatedly exposed to organic material develop an adaptation to the effects of acute exposure to inhaled organic material.
- Exposure to dust in the pig barn was a much stronger pro-inflammatory stimulus than inhalation of pure endotoxin (LPS).

Paper II:

- Exogenous stimuli (organic dust and LPS) and an endogenous stimulus (TNF) induced increased expression and release of IL-6 and CXCL8 (IL-8) in primary bronchial epithelial cells
- TLR2 expression synergistically increases in primary bronchial epithelial cells by co-stimulation with pro-inflammatory stimuli and steroids.
- Release of pro-inflammatory cytokines was attenuated by blocking of the TLR with monoclonal antibodies, indicating that the pro-inflammatory stimulation was at least partly dependent on TLR2/TLR4.

Paper III:

- Surface expression of TLR2 on neutrophils was down-regulated by pro-inflammatory stimuli.
- Neutrophil surface expression of membrane bound CD14 and TLR4 were increased when neutrophils were co-cultured with alveolar epithelial cells, irrespective of pro-inflammatory stimulation.
- Soluble CD14 (sCD14) and CXCL8 were synergistically enhanced when neutrophils and alveolar epithelial cells were co-cultured, *in vitro*. Soluble CD14 was strongly correlated to CXCL8 release during LPS-stimulation of co-cultured cells, whereas no such effect was observed in the absence of epithelial cells. This indicates an active cross-talk between A549 cells and blood neutrophils.

Paper IV:

- Toll-like receptor 2 expression was reduced on sputum neutrophils from patients with COPD compared with non-smokers, and the expression of TLR2 on neutrophils was higher in blood than in sputum, irrespective of the presence of airway obstruction.
- We show the presence of sTLR2 in sputum and BAL-fluid and that COPD was associated with higher levels of soluble TLR2 (sTLR2) in sputum and lower levels of sTLR2 in BAL-fluid compared with non-smokers.
- The expression of CD14 on neutrophils was enhanced in sputum compared with blood and the gene expression of CD14 on alveolar macrophages in BAL-fluid was increased in smokers compared with non-smokers.
- This indicates that pattern-recognition receptors (PRRs), are differently regulated in smokers with COPD compared with smokers without airflow limitation and non-smokers. This is likely of importance in COPD patho-

physiology, in particular for exacerbations, which often are caused by microorganisms.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING (IN SWEDISH)

Kontinuerlig exponering för luftburna inhalerbara partiklar påverkar vårt immunförsvar och våra lungor. Typ av partiklar, exponeringens duration samt när i livet denna exponering sker, är av betydelse för konsekvenserna av exponeringen.

Flertalet oberoende forskargrupper har visat att barn som växer upp på en lantgård eller har föräldrar som är aktiva inom jordbruksnäringen har ett visst skydd mot att utveckla astma och allergi. Intressant i detta avseende är att föräldrarna tycks löpa en ökad risk att utveckla kroniska luftvägssjukdomar t.ex. kronisk bronkit och nedsatt lungfunktion, jämfört med barnen.

Denna avhandling fokuserar på toll-lik receptorer (TLR), en receptorfamilj som uttrycks av de flesta av kroppens celler, men framförallt hos de celler som är aktiva inom immunförsvaret. TLR2 och TLR4, två receptorer inom denna familj, binder bakteriefragment och startar ett immunologiskt svar. Det finns flera studier som visar att dessa receptorer är påverkade lokalt i lungan, men även systemiskt i blod vid kontinuerlig inhalation av organiska partiklar. Barn som kontinuerligt exponerats tidigt i livet, har ett uppreglerat TLR-receptoruttryck på cirkulerande blodceller medan exponerade vuxna har nedreglerat receptoruttryck på motsvarande celler. Detta skulle kunna medverka till den skyddande effekten gällande prevalens av astma och allergi, hos barn som växer upp i lantbruksmiljö. Det är också möjligt att detta bidrar till förklaringen att vuxna som arbetar inom lantbruk på grund av det nedreglerade immunsvaret har en ökad risk för sjukdomsutveckling senare i livet.

Både bönder och rökare är kontinuerligt exponerade för organiskt material, bönderna genom sin arbetsmiljö och rökarna genom tobaksröken. Studie I visar att bönder och rökare har en pågående inflammation i de centrala luftvägarna och att de vid akut exponering i svinhuset har ett minskat immunsvaret jämfört med friska kontroller. Detta tyder på en toleransutveckling, vilket tidigare visats hos lantbrukare. Studien visade även att toleransutveckling hos rökare inte sker i samma utsträckning som hos bönder.

Många rökare utvecklar med tiden kroniskt obstruktiv lungsjukdom (KOL) en sjukdom som karaktäriseras av luftvägsobstruktion som inte normaliseras av behandling. KOL beror på inflammatoriska förändringar i de små perifera luftrören (bronkiolit) samt av emfysem i lungvävnaden. Det effektivaste sättet att stoppa denna destruktiva process är att avbryta den skadliga exponeringen vilket i de flesta fall innebär rökstopp.

Avhandlingens andra studie fokuserar på epitelceller i luftvägarna. Epitelceller är en celltyp som täcker insidan av luftrören och fungerar som en barriär mellan kroppens inre miljö och den yttre miljön. Epitelceller är aktiva i vårt immunförsvar och uttrycker flera olika typer av TLR. I denna studie visar vi att steroider (inflammationshämmande läkemedel) ökar TLR2-uttrycket på epitelcellerna när cellerna stimulerats med exempelvis bakteriefragment. Dessa resultat bidrar till en ökad förståelse för TLR-

reglering, samt kan eventuellt vara av betydelse gällande den positiva anti-inflammatoriska effekt som erhålls vid steroidbehandling.

Den tredje studien i avhandlingen studerar kommunikationen mellan alveolära epitelceller och neutrofila granulocyter. Avsikten med studien var att undersöka förekomst av aktiv kommunikation med avseende på regleringen av TLR och inflammatoriska markörer mellan epitelceller och neutrofiler. Vår hypotes var även att denna kommunikation skulle vara påverkad vid KOL. Neutrofilen är en relativt kortlivad cell som patrullerar vår kropp via blodbanan. Vid en infektion eller annan skadlig påverkan lämnar neutrofilen blodet och vandrar ut i vävnaden. Neutrofilen arbetar sedan aktivt med att eliminera för kroppen farliga ämnen. Resultaten från den tredje studien visar på att en aktiv kommunikation mellan epitelceller och neutrofiler pågår med avseende på uttryck av TLR och för kroppen viktiga inflammatoriska mediatorer. Vi kunde inte påvisa någon signifikant skillnad mellan friska kontroller och personer med KOL. Men vi fann ett tydligt mönster där KOL-patienterna uppvisar ett lägre uttryck av de studerade receptorerna. Det är troligt att skillnader mellan grupperna visats om materialet varit större. Resultaten i denna studie visar på en dubbelriktad kommunikation mellan epitelceller och neutrofiler med avseende på receptorreglering samt bildning och frisättning av inflammatoriska mediatorer. Förmodligen är detta samspel av stor relevans när det gäller kroppens försvar mot potentiellt patogena agens.

I den fjärde studien har vi inkluderat kontroller samt rökare med och utan KOL. Målsättningen med denna studie var att studera uttryck av toll-liknande receptorer från olika nivåer i luftvägarna, i bronkoalveolärt lavage (BAL) som speglar de nedre luftvägarna och sputum som representerar mer centrala luftvägar. Eftersom KOL är en systemsjukdom undersöktes även receptoruttryck på blodceller. Vi fann dels att olika TLR är beroende av olika regleringsmekanismer. Vi visade även på att den lösliga formen av TLR2 (sTLR2) finns i BAL och sputum, något som tidigare ej har visats. Dessutom, skilde sig mängden av denna lösliga receptor åt mellan våra studerade grupper. Förutom detta visar vi på att uttrycket av TLR skiljer sig åt mellan rökare som har utvecklat KOL och de som inte har det. Det är troligt att detta fynd är av betydelse, fram för allt med tanke på att akuta mer eller mindre långvariga försämringsperioder (exacerbationer) ofta orsakas av bakteriella infektioner där TLR har en nyckelfunktion i det inflammatoriska svaret hos individen.

Sammanfattningsvis visar denna avhandling att kroniskt exponerade, bönder och rökare, har en generell påverkan lokalt och systemiskt vad gäller det inflammatoriska svaret vid exponering för pro-inflammatoriska agens. Mer specifikt visas att regleringen av de för immunförsvaret viktiga toll-liknande receptorerna är påverkade vid KOL och vid rökning, men även av steroider. Regleringsmekanismerna för dessa receptorer är många och komplexa och forskningen på detta område är i sin linda. Detta är ett forskningsfält som kommer att växa och vara av betydelse för framtida läkemedelsutveckling.

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