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The unit of Lung and Allergy Research
Karolinska Institutet, Stockholm, Sweden

Occupational Exposure Alters Innate and Adaptive Immune Responses

Karin Sahlander

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Nu spelar vårens ljumma vind i myrens gula starr,
och sakta stiga sagorna kring ön i Berga fors.
Förlåt ett stänk av bitter fröjd, en visa till gitarr,
det starka oss till läkedom likt strandens unga pors.
”Till min syster” ur Svarta Ballader
Dan Andersson

Till nära och kära

ABSTRACT

The farming environment is contaminated with high levels of organic dust. Especially pig barn facilities are highly polluted with airborne inhalable organic dust containing high amounts of molecular patterns from bacteria and fungi known to activate cells of the innate immunity through pattern recognition receptors (PRRs). Some hours of exposure in pig barn environment leads to an intensive upper and lower airway inflammation with systemic influences in previously unexposed healthy subjects. In farmers, daily exposed in the pig barn environment, the immune response becomes attenuated. The effect of the attenuated response is not apparent but respiratory symptoms are very common among farmers, a group with higher prevalence of respiratory chronic inflammatory disorders such as asthma-like syndrome, chronic bronchitis and chronic obstructive pulmonary disease (COPD) than the population in general.

Working with laboratory animals is associated with high exposure to allergens but involves also exposure to molecular patterns such as lipopolysaccaride (LPS). Respiratory symptoms, allergic sensitisation against laboratory animals and development of occupational asthma are common health problem among personnel in biomedical research and industry.

In **paper I** the aim was to study influence of regular exposure to organic dust on expression of pattern recognition receptors (PRRs) on blood neutrophils and monocytes and the cytokine profile interleukin (IL)-2, IL-4, IL-13 and interferon (IFN)-γ of blood T-cells before and after exposure in pig barn environment and a bronchial LPS challenge. The study included one group of pig farmers, one group of smokers, who are regularly exposed to organic material and like farmers have an increased prevalence of chronic respiratory diseases, and in one group of non-farming non-smoking healthy subjects. Blood from farmers and healthy controls were also stimulated *ex vivo* with pro-inflammatory stimuli. Before exposure farmers and smokers had increased concentration of blood neutrophils compared to controls. Farmers also showed decreased expression of TLR2 on blood monocytes compared to controls. After *in vivo* and *ex vivo* exposure, the expression of TLR2 and release of IL-6 were attenuated in farmers compared to controls. Further, farmers and smokers had increased proportion of IL-4 and IL-13 producing T-helper cells (Th) compared to controls before exposure. After *in vivo* exposures the proportion of IL-4 and IL-13 producing Th cells increased in controls but not in farmers and smokers. This attenuation in PRR expression in farmers is probably due to repeated exposure to microbial components and might be involved in the attenuated response to pig barn exposure previously observed in pig farmers. Increased proportion of Th2 cells is also probably due to regular exposure to microbial components and may be involved in development of respiratory symptoms and airway disorders (i.e. chronic bronchitis) that are frequently occurring in these groups.

In **paper II** the aim was to investigate expression of PRRs, lymphocyte activating markers, T-cell cytokine profile and serum levels of soluble CD14 (sCD14) and sST2 in laboratory animal (LA) workers who experience respiratory symptoms while working with laboratory animals (LAs), one group with and one group without allergic sensitization to LA. Two control groups not exposed in LA facilities were included, one group with birch pollen allergy (run during season) and one group of non-atopic subjects. Laboratory animal workers, especially those without LA atopy, showed increased expression of CD14 on blood monocytes compared to the control groups. Further was the level of sST2 in serum elevated in birch pollen atopics and in the group of LA workers who experienced respiratory symptoms but without LA atopy. Increased expression of CD14 may be a marker for LPS exposure which seems to be associated with respiratory symptoms. Increased levels of sST2 in serum might be due to LPS exposure and may prevent allergic sensitization to laboratory animals. However, it might also be caused by exposure to allergens and being an early marker for allergic sensitization.

In **paper III** the aim was to elucidate the influence of regular exposure in pig barn facilities on expression of PRR, adhesion proteins on blood and sputum neutrophils, levels of soluble PRRs in blood and sputum and serum levels of sST2 before and after exposure in a pig barn and a bronchial LPS challenge. A further aim was to study release of pro-inflammatory cytokines after *ex vivo* stimulations of blood with PRR ligands in presence or absence of anti-ST2. Farmers had decreased expression of adhesion moleculer (CD62L and CD162) on blood neutrophils and CD14 on sputum neutrophils compared to controls. Farmers also had lower levels of sTLR2 and sCD14 in sputum compared to controls. Before exposure there was no difference in sST2 levels in serum but after *in vivo* exposures sST2 levels in serum increased only in the controls. Attenuated release of sST2 in serum is probably due to development of tolerance among pig farmers. Decreased expression of adhesion molecules might be involved in the reduction in cell recruitment after exposure in pig barn environment previously observed in farmers.

In **paper IV** the aim was to further investigate the influence of regular exposure to organic material on the cytokine profile of T-cells. Proportion and concentration of blood Th cells and cytotoxic T-cells (Tc) producing IL-2, IL-4, IL-13, IFN-γ were investigated in pig farmers, smokers and non-farming, non-smoking healthy subjects. Farmers and smokers had increased proportion and concentration of Th cells producing IL-4 and IL-13 compared to controls. Smokers also had increased proportion and concentration of IL-4 and IL-13 producing Tc cells and concentration of Tc cells producing IL-2 compared to controls and farmers. Farming environment seems to favor a Th2 profile, however, not to the same extent as does smoking. This increase in IL-4/IL-13 producing cells likely stimulate goblet cell metaplasia and might therefore be involved in development of chronic bronchitis, a frequently occurring condition in these groups.

In conclusion, occupational exposure in pig barn and in laboratory animal facilities alters expression of receptors and cytokines important for the inflammatory response. This alteration may be of importance in the development of chronic inflammatory airway disorders that are known to be common both in smokers and in farmers.

LIST OF PUBLICATIONS

I. Sahlander K, Larsson K, Palmberg L
Altered innate immune responses in farmers and smokers
Innate Immun. 2010 Feb;16(1):27-38.

II. Sahlander K, Larsson K, Palmberg L
Increased levels of soluble ST2 in birch pollen atopics and individuals working in laboratory animal facilities
J Occup Environ Med. 2010 Feb;52(2):214-8.

III. Sahlander K, Larsson K Palmberg L
Occupational exposure in pig farms alters innate immunity
Submitted

IV. Sahlander K, Larsson K, Sundblad BM, Palmberg L
T-cell cytokine profile in smokers and in farmers - two groups exposed to pathogen-associated molecular patterns on a daily basis
Submitted

Additional publications not included in the thesis:

Sundblad BM, Sahlander K, Ek A, Kumlin M, Olsson M, Larsson K, Palmberg L
Effect of respirators equipped with particle or particle-and-gas filters during exposure in a pig confinement building
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Effect of formoterol and budesonide on chemokine release, chemokine receptor expression and chemotaxis in human neutrophils
Pulm Pharmacol Ther. In press.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

APC	Allophycocyanin
APC	Antigen presenting cells
AP-1	Apetalia-1
BCR	B-cell receptors
BAL	Bronchial alveolar lavage
JNK	c-Jun N-terminal kinase
CLRs	C-type lectin receptors
CXCL8	Chemoattractants like C-X-C motif chemokine 8
CXCR	Chemoattractants like C-X-C motif receptor
CCL2	Chemokine (C-C motif) ligand 2
COPD	Chronic obstructive pulmonary disease
CD	Cluster of differentiation
CBA	Cytometric bead array
Tc	Cytotoxic T-cell
DC	Dendritic cell
ER	Endoplasmic reticulum
ECP	Eosinophilic cationic protein
EDTA	Ethylene diamine-tetra-acetic acid
FITC	Fluorescein isothiocyanate
FEV_1	Forced expiratory volume in one second
GINA	Global Initiative for Asthma
IRAK	IL-1R-associated kinase
Ig	Immunoglobulin
IKKs	Inhibitor of nuclear factor (NF)-κB (IκB) kinases
IFN	Interferon
IRF	Interferon (IFN)-regulatory factor
IL	Interleukin
IL-1Rs	Interleukin-1 receptors
KOL	Kroniskt obstruktiv lungsjukdom
LAs	Laboratory animals
LRRs	Leucine-rich repeats
LTB_4	Leukotriene B4
LPS	Lipopolysaccaride
LBP	Lipopolysaccaride binding protein
MCP-1	Macrophage cationic peptide 1
MHC	Major histocompatibility complex
MMPs	Matrix metalloproteases
MAP	Mitogen-activated protein
Mal	MyD88-adapter-like
MyD88	Myeloid differentiation primary-response protein 88
NAL	Nasal lavage
NK-cell	Natural killer cell
NKT-cells	Natural killer T-cells
NHBE	Normal human bronchial epithelial

NF-κB	Nuclear factor–kappa B
NLRs	Nucleotide binding and oligomerization domain-like receptors
ODTS	Organic dust toxic syndrome
Pam3Cys	tripalmitoyl-S-glycerylcysteine
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors
PerCp	Peridinin chlorophyll protein
PMA	Phorbol 12-myristate 13-acetate
PE	Phycoerythrin
PBEC	Primary bronchial epithelial cells
ROS	Reactive oxygen species
T_{reg}	Regulatory T-cell
RLRs	Retinoic acid-inducible gene 1- like receptors
SIGIRR	Single immunoglobulin and toll-interleukin 1 receptor (TIR) domain
SPT	Skin prick test
sCD14	Soluble CD14
sST2	Soluble ST2
sTLR2	Soluble TLR2
sTLR4	Soluble TLR4
ST2	Suppression of tumorgenicity 2
TCR	T-cell receptor
Th	T-helper cell
TRAF	TNFR-associated factor
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLRs	Toll-like receptors
TRIF	Toll-receptor associated activator of interferon
TIR	Toll/interleukin-1 receptor
TAK1	Transforming growth factor (TGF)-β-actvated kinase 1
TGF- β1	Transforming growth factor β1
TRAM	TRIF-related adaptor molecule
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
VC	Vital capacity

1 INTRODUCTION

We are constantly exposed to airborne foreign particles, substances and pathogens in our environment. Due to our need to breathe, we inhale thousands litre of air every day, which makes our airways extraordinary appointed to air pollutions.

Chronic inflammatory respiratory diseases such as asthma, chronic bronchitis and chronic obstructive pulmonary disease (COPD), are very common across the world, and cause millions of death yearly. Tobacco smoking is an important risk factor for developing chronic airway disorders but also environment exposure such as air pollution from traffic, industry, and indoor cooking contribute considerably (1).

One historic episode that clearly showed how air pollution may influence human health was the great smog that hit the city of London in 1952. Due to special weather conditions London was covered with thick smog for nearly a week leading to thousands of deaths (2, 3).

Already in the 17th century there were reports regarding respiratory illness among workers in specific occupations as mining (4) and, in the beginning of 18th century, the occupational physician Bernardino Ramazzini reported illness among several occupations including farming (5). Farming environment is associated with exposure to high levels of organic dust and since the middle of 20th century, when the meat industry expanded fast and the animal breeding became more indorsing, the work environment became even more polluted. Especially the pig barn facilities are highly contaminated with airborne inhalable organic dust that includes components from microorganisms known to activate our immune system through specific receptors described as pattern recognition receptors (PRRs). Respiratory symptoms are frequent among pig farmers (6), who have increased prevalence of chronic bronchitis (7-9) and there are data also indicating increased prevalence of COPD among pig farmers (10, 11).

Respiratory symptoms are common also in personnel working with laboratory animals within biomedical research and the industry (12). The laboratory animal facilities environment includes high levels of potent allergens from the animals but also microbial compounds, similar to pig barn facilities (13, 14). Allergic sensitization to laboratory animals is common among the personnel and they are also at high risk for development of asthma (12, 15).

In this thesis the influence of occupational exposure to organic dust on specific immune responses of the innate and adaptive immunity is elucidated. The work is focused on pattern recognition receptors (PRRs), cytokine profile of T-cell subsets and suppression of tumorgenicity 2 (ST2) in pig farmers and laboratory animal workers.

1.1 THE IMMUNE SYSTEM

Our first defense against foreign particles and invading pathogens is the physical barrier composed by the skin and mucosa. The mucosa covers the gastrointestinal tract and the airways and consists of various types of epithelial cells. The respiratory mucosa covers the oral and nasal cavity, pharynx, larynx, trachea and the bronchial tree. The respiratory mucosa is strengthened with ciliated epithelial cells that "sweeps up" foreign particles or pathogens trapped in the mucus, a viscous fluid produced by specific mucus producing cells within the mucosa (mainly by goblet cells in the large airways and Clara cells in the smaller airways), and glands in the submucosa. If this physical barrier fails to protect from invading pathogens the immune system has an enormous ability to recognize invading foreign microorganisms such as bacteria, viruses and parasites and a rapid process starts to obviate and eliminate the pathogen. The immune system consists of the innate and the adaptive immunity (16).

1.1.1 Innate immunity

The innate immunity is the first-line defense in recognizing invading pathogens and is active already at birth. It is promptly activated through either non-cellular response such as complement activation or in a cellular dependent manner. The cellular response is mediated by rapid recognition of invading pathogens by the epithelial cells in the epithelial layer building up the protecting mucosa together with other immune cells such as the leukocytes like monocytes/macrophages, neutrophilic granulocytes and mast cells. *Monocytes* are circulating mononuclear cells that differentiate into macrophages when they leave the blood and enter the tissue. The main function of *macrophages* is to rapidly recognize invading pathogens or foreign particles, start to eliminate the harmful agents by phagocytosis and call for help by secretion of specific proteins, cytokines and chemokines, that recruit other helping cells, e g neutrophilc granulocytes. Macrophages are also important in activating adaptive responses by bringing engulfed pathogens into lymphoid organs and present it to lymphocytes, the effector cells in the adaptive immunity system.

Neutrophilic granulocytes, also described as polymorphonuclear cells depending on their segmented nucleus. The neutrophilic granulocytes, shortly described as neutrophils, are like macrophages specialized in phagocytosis. They are further equipped with a large number of granules that contain toxic mediators such as oxygen radicals, histamine, lysozyme and collagenase that are released upon activation to eliminate harmful pathogens. The neutrophils, the most common cell in the blood, are short lived, produced in the bone marrow and circulates in the blood only for some hours, whereupon they migrate into the tissue and function as the first protector against pathogens that have succeeded in penetrating the skin or mucosa. There are also other existing granulocytes as the *eosinophils* and the *basophils*. They are also phagocytic cells equipped with granules, however, in contrast to neutrophils the granules of

eosinophils and basophils are loaded with toxic substances specific for elimination of parasite infections. The eosinophils and basophils also have important roles in allergic inflammation together with the granulocyte related mast cells. These immune cells including epithelial cells express special proteins, pattern recognition receptors (PRRs), highly conserved by evolution to rapidly recognize invading pathogens (17).

1.1.2 Pattern recognition receptors

Pattern recognition receptors comprise a group of receptor with an essential role in the innate immunity, but are also important in shaping the adaptive immune responses (18-20). The PRRs are involved in several vital immune functions such as phagocytosis, complement cascades, apoptosis, pro-inflammatory and type I interferon signaling pathways. They recognize and bind conserved molecular structures from microorganisms named pathogen-associated molecular patterns (PAMPs). The PRRs are divided into four families: toll-like receptors (TLRs), nucleotide binding and oligomerization domain-like receptors (NLRs), C-type lectin receptors (CLRs) and retinoic acid-inducible gene 1- like receptors (RLRs) (18, 19).

1.1.3 Toll-like receptors

The Toll receptors were originally identified in *Drosophila*. In the late 20th a homologue to the Toll receptor was identified in mammals and were consequently called toll-like receptor (TLR), today described as TLR4 (21). To day 12 TLRs have been identified in mammals, of which 10 are expressed in humans. They are broadly distributed in cells of the immune system, expressed on the cell surface or intracellularly. The TLR1, TLR2, TLR4, TLR5, TLR6 are expressed on the cell surface whereas TLR3, TLR7, TLR8 and TLR9 are expressed in intracellular vesicles as the endosomes and the endoplasmic reticulum (ER). The TLR are type I intergral membrane glycoproteins with one extracellular and one cytoplasmic domain. The cytoplasmic domain, which TLRs have in common with the interleukin-1 receptors (IL-1Rs), is called the toll/interleukin-1 receptor (TIR) domain. The extracellular domain that binds the ligand contains leucine-rich repeats (LRR) (18, 19). The TLRs bind a large number of conserved molecular patterns from pathogens such as bacteria, fungi, protozoa and viruses (table 1) but there are also a number of endogenous ligands identified (table 2) (19, 22). When the TLRs bind to their ligands they dimerize and undergo conformational changes. The most TLRs dimerize into homodimers but some, e g TLR2 can dimerize into heterodimers together with TLR1 or TLR6 depending on the ligand. TLR2 together with TLR1 binds diacyl lipopeptides (mycoplasma) and TLR2 together with TLR6 triacyl lipopeptides (bacteria) or lipoteichoic acid. Toll-like receptor 4 binds lipopolysaccharide (LPS) from Gram-negative bacteria in a receptor complex together with CD14, the adapter molecule MD-2 and lipopolysaccaride binding protein (LBP) (18, 19, 23-26). In the present work we have focused on expression of TLR2 and TLR4 and its co-receptor CD14.

	PAMPs	Species
TLR2	Peptidoglyglycans Phenol-soluble modulin Phospholipomannan Hemagglutinin	Gram-positive bacteria *S. Aures* (Gram-positive bacteria) *C. Albicans* (fungi) Measles virus
TLR2/TLR1	Triacyl lipoproteins	Bacteria
TLR2/TLR6	Diacyl lipoproteins Zymozan	Mycoplasma, bacteria *S. Cerevisiae* (fungi)
TLR3	dsRNA	Virus
TLR4	LPS Mannan F protein	Gram-negative bacteria *S. Cerevisiare* (fungi) *C. Albicans* (fungi) Respiratory syncytial virus (RSV)
TLR5	Flaggelin	Flagellated Bacteria
TLR7	ssRNA	Virus
TLR8	ssRNA	Virus
TLR9	CpG DNA Hemozoin	Bacteria *P. Falciparum* (protozoa)
TLR10	Unknown	Unknown

Table 1 TLRs and specific ligands (pathogen-associated molecular pattern PAMPs). Table modified from (19).

Endogenous ligands	TLRs
B-defensin 2	TLR4
Fibrinogen	TLR4
Fibronectin extra domain A	TLR4
High mobility group box 1 protein (HMGB1)	TLR2 and TLR4
Heat shock protein 60 (Hsp60)	TLR2 and TLR4
Hsp70	TLR2 and TLR4
Hyaluronan	TLR4
Surfactant protein-A	TLR4

Table 2 Endogenous TLR ligands. Table modified from (22).

1.1.4 TLR signaling

When TLRs bind their ligands and dimerize an activation of signal cascades leading to transcription of genes involved in inflammatory responses as pro-inflammatory cytokines and type I interferons starts. There are two main signaling pathways in the TLR signaling systems. The signaling occurs in myeloid differentiation primary-response protein 88 (MyD88)-dependent or MyD88-independent manner. All TLRs signaling except TLR3 and partly TLR4 are dependent of MyD88 to signal. Upon activation, MyD88 associates with the TIR domain whereupon IL-1R-associated kinase 4 (IRAK4) and IRAK1 are recruited. Further signaling through tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) and transforming growth factor (TGF)-β-activated kinase 1 (TAK1) occurs. This is followed by activation of mitogen-activated protein (MAP) kinase and inhibitor of nuclear factor (NF)-κB (IκB) kinases (IKKs). MAP kinase activates in its turn p38 and c-Jun N-terminal kinase (JNK) leading to activation of AP-1 and IKKs activates NF-κB. Activation of AP-1 and NF-κB results in induction of genes involved in inflammatory responses such as pro-

inflammatory cytokines and chemokines. To function, TLR2 and TLR4 signaling via MyD88 requires the adapter protein MyD88-adapter-like (Mal), also known as toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP) (18, 19, 24, 27, 28).

The MyD88-independent pathway (TLR3 and partly TLR4 signaling) is activated through toll-receptor associated activator of interferon (TRIF) and TLR4 also via TRIF-related adaptor molecule (TRAM), leading to phosphorylation of interferon (IFN)-regulatory factor-3 (IRF-3) and IRF-7 which together translocates into the nucleus and induces expression of type I interferons and interferon related genes (TLR signaling see figure 1). The signaling is, however more complex as described above, the pathway can interact resulting in ability by MyD88- dependent and -independent pathway to activate both NF-κB and IRFs (18, 19, 24, 27, 28).

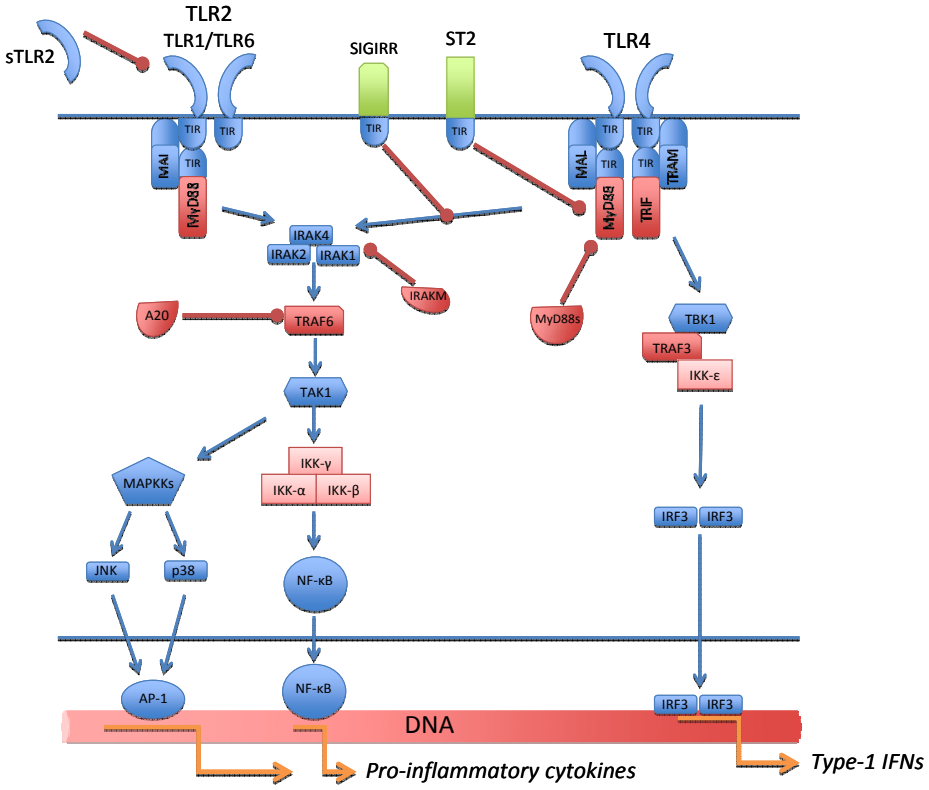

Figure 1 Myeloid differentiation primary-response protein 88 (MyD88)-dependent and MyD88–independent pathways in TLR-signaling. TLR2 and partly TLR4 are dependent on MyD88 and Mal for signaling. The MyD88-dependent pathway leads to activation of inhibitor of nuclear factor (NF)-κB (IκB) kinases (IKKs) and mitogen-activated protein (MAP) resulting in activation of nuclear factor (NF)-κB and AP-1 that leads to transcription of pro-inflammatory genes as pro-inflammatory cytokines and chemokines. TLR4 also uses MyD88-independent, the toll-receptor associated activator of interferon (TRIF) and TRIF-related adaptor molecule (TRAM) dependent pathway which leads to activation of interferon (IFN)-regulatory factor-3 (IRF-3) which translocates into the nucleus and induces expression of type I interferons and interferon related genes. Red markers indicate action positions of negative regulating proteins. Soluble TLR2 (sTLR2) work as an antagonist of membrane bound TLR2, MyD88s as an antagonist to MyD88, A20 inhibits TLR signalling by de-ubiquitylateing TRAF6, suppression of tumorigenicity 2 (ST2) sequesting MyD88 and Mal through its TIR domain and single immunoglobulin and toll-interleukin 1 receptor (TIR) domain (SIGIRR) by interacting with TRAF6 and IRAK. Figure modified from (27-29).

1.1.5 Negative regulating systems of the TLRs

Early recognition by TLRs and functional TLR signaling is crucial in innate immune responses and important in enhancing the adaptive immunity and thereby essential for human survival. However, too strong or uncontrolled signaling may be harmful and even lethal for the host and thereby the TLR signaling has to be strictly controlled. There are several negative regulating systems involved in TLR binding and signaling (figure 1) (28, 30). First there is soluble variants of TLRs identified, e.g. soluble TLR2 (sTLR2), probably working as an antagonist to the transmembrane receptor protein. Soluble TLR2 are constitutively present in human plasma and breast milk and have also been detected in amniotic fluid (31, 32). It has been demonstrated that monocytes release sTLR2 upon activation and this probably by post-translational modification of the transmembrane receptor (31). It has been shown that sTLR2 inhibits release of pro-inflammatory cytokines by monocytes stimulated with TLR2 ligands (31, 33). There are no study that have detected protein of sTLR4 but multiple TLR4 mRNA have been observed in mice (34) indicating that a soluble variant might also exist and be of importance in negative regulating TLR4 signaling. Soluble variants of the TLR4 co-receptor CD14 (sCD14) and LPS binding protein (LBP) has also been shown to have negative regulating capacities by transferring LPS from membrane bound CD14 to plasma lipoproteins (35, 36). The concentrations of sCD14 and LBP have shown to be of importance in their regulating capacities. In situations where the concentrations of sCD14 and LBP is high , such as in plasma during sepsis, sCD14 and LBP have a negative regulating function, probably to inhibit harmful systemic response (35). However, at lower concentrations sCD14 and LBP have been reported to enhance the response to LPS (37).

Suppression of tumorigenicity 2 (ST2) and single immunoglobulin and toll-interleukin 1 receptor (TIR) domain (SIGIRR) are transmembrane negative regulating proteins. ST2 and SIGIRR have, as the TLRs, a TIR domain and ST2 acts probably by sequestering MyD88 and Mal through its TIR domain and SIGIRR by interacting with TRAF6 and IRAK (38, 39).

Further, there are a number of intracellular negative regulating proteins such as A20, MyD88s and IRAK-M that inhibit the intracellular signalling pathway of TLRs. A20 inhibits TLR signalling by de-ubiquitylates TRAF6 and MyD88s work as an antagonist for MyD88. The function of IRAK-M is probably to inhibit phosphorylation of IRAK-1(40-43).

Several of these regulating proteins described above are induced in endotoxin (LPS) tolerance which is defined as reduced immune response to a repeated LPS challenge (29, 44).

1.1.6 ST2 and interleukin-33 (IL-33)

Suppression of tumorigenicity 2 (ST2) has previously been described as IL-1R4, T1 and lately as IL-33Rα. As described above ST2 has, similar to TLRs, an intracellular toll-interleukin 1 receptor (TIR) domain (45, 46). Alternate splicing of ST2 generates a soluble isoform of ST2 (sST2) that lacks the transmembrane region and the TIR domain (45). Increased serum levels of sST2 have been reported in several inflammatory diseases (47-49) and in myocardial infarction (50, 51) and correlation between serum levels of sST2 and disease severity and mortality have been observed in myocardial infarction and sepsis (47, 52). It has been observed that serum levels of sST2 strongly increase in healthy subjects following an intravenous LPS injection (53). The function of sST2 is not totally clarified but there are studies indicating a negative regulating function of sST2 as have been reported for membrane bound ST2 (54). The ST2 system is not only involved in pro-inflammatory responses it has also an essential role in allergic inflammation (55, 56). Serum levels of sST2 are increased during exacerbations in patients with allergic asthma (49) and the ST2 receptor expression is enhanced on several cell types important in allergic inflammation, e g mast cells, basophils, eosinophils, T-, NK- and NKT-cells (57-60). In 2005 the IL-1-like cytokine IL-33 was identified being the ligand for the ST2 receptor (61). Activated Th2 cells express ST2 and signaling through ST2 by IL-33 on Th2 cells leads to production of Th2 cytokines as IL-4, IL-5 and IL-13 (61), IL-33 is also shown to amplify Th1 responses by its activity on basophils and natural killer (NK) cells (57, 58). Cells producing IL-33 is mainly tissue-related cell types such as epithelial cells, smooth muscle cells, fibroblasts, and is usually not expressed by hematopoetic cells (61-64). Lung, gut and skin tissue are prominent IL-33 producers (63-65). Soluble ST2 is known to block the IL-33 signalling in allergic airway inflammation in mice (55).

1.1.7 Adaptive immunity

The adaptive immunity covers the function of B-cells and T-cells. Apart from innate immunity the adaptive immunity has a memory, and similar to the innate immunity it acts both in a non-cellular and a cellular manner.

B-cells are responsible for the antibody production, the non-cellular (humoral) response, in the adaptive immunity system. These cells are produced by the bone marrow from where it leaves as a mature B-cell that is immunologically naïve. B-cells produce antibodies composed by heavy and light immunoglobulin (Ig) chains, and are either surface bound, as the B-cell receptors (BCR) or secreted. An immunologically naïve B-cell express immunoglobulin M (IgM) but depending on activation the globulins differentiates and switch into other isotypes i.e. IgD, IgA, IgE, IgG and IgM with more specific affinity. The variation in how an immunoglobulin can be combined is enormous making the ability to recognize foreign peptides (antigens) almost infinite. B-cells do not secret immunoglobulin until they have bound an antigen and

differentiated into a plasma cell, a process for which T-cells are needed (i.e. T-helper cells) (16, 17).

T-cells are responsible for the cellular part of the adaptive immunity. The T-cells recognize antigens presented as peptides by major histocompatibility complex (MHC) class I and II via the T-cell receptor (TCR). First the T-cells are divided into $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T-cells. The T-helper cells recognize peptides presented by MHC I with its TCR together with CD4 whereas cytotoxic T-cells peptides presented by MHC II via its TCR and CD8. Pro T-cells are produced in the bone marrow and transported into the thymus where they undergo strong selection and mature into either T-helper cells or cytotoxic T-cells. At this stage the T-cells are inactivated but out in the system they become activated and differentiate into effector cells (16, 17). Depending on activation the T-helper cells, but also cytotoxic T-cells, differentiate into subgroups depending on what cytokines they produce. A T-helper 1 (Th1) cell produces interferon (IFN)-γ, IL-12 and tumor necrosis factor (TNF) that are important for activation of cytotoxic T-cell activation and B-cell class switch into IgG (i.e. IgG1 in human) (66, 67). A T-helper 2 (Th2) cell produces cytokines such as IL-3, IL-4, IL-5 and IL-13 important for instance in B-cell class switch into other isotypes such as IgE (16, 67). There are also other types such as the T-helper 17 (Th17) cells that produce IL-17, important in neutrophilic inflammation (68, 69).

A unique function of the adaptive immunity is the ability to remember. Activated B-cells and T-cells proliferate into effector cells but also into memory cells, that have a memory for pathogens. When infected a second time with the same pathogen the memory cells rapidly differentiate into effector cells and eliminate the pathogen more rapidly than at the first occasion (17).

Like innate responses adaptive responses also need to be controlled to maintain immunological tolerance and homeostasis to prevent autoimmunity and moderate inflammation primary induced by pathogens. Cells important in this controlling system are the regulatory T-cells (T_{reg}) (70). T_{reg}-cells have an essential role in preventing autoimmune diseases and are also known to be a regulator in inflammatory diseases such as asthma (71-73). There are different kinds of T_{reg}-cells with different functional mechanisms. One function of T_{reg}-cells is to produce suppressing cytokines such as IL-10 and transforming-growth factor (TGF)-β1, another mechanism is to suppress cellular functions via direct cell-cell contact (70). For instance are T_{reg}-cells capable of suppressing maturation and action of dendritic cells (DCs) (74). Another function by T_{reg}-cells is to induce apoptosis in effector cells (75). The T_{reg}-cells are also important in moderation of response to infections and IL-10 and TGF-β1, produced by T_{reg}-cells, induce immunological tolerance to bacterial and viral superantigens (76, 77).

1.1.8 Connection between adaptive and innate immunity

The adaptive and the innate immunity are cooperative partners in shaping each other's immune responses. For instance has the innate immunity an essential role in maturation

and development specific adaptive immune cell responses mediated by T-helper cells through activation by antigen presenting cells (APCs).

Monocytes, macrophages and dendritic cells are all working as APCs whereas the dendritic cell is believed to be the best in this respect. *Dendritic cells* (DCs) are phagocytosing mononuclear cells derived from bone marrow progenitors. The DCs circulate in the blood as immature precursors prior to migration into tissues to search for foreign pathogen through their pattern recognition receptors (PRRs) (17, 66). When the DCs take up pathogens they become activated and migrate into secondary lymphoid organs (lymphnodes) whereupon the DC present processed antigens from the pathogen to immunologically naïve T-helper cells that passes through the lymphnode on its way circulating around the blood and lymphatic system. The T-lymphocyte needs three signals from a pathogen-primed DC. The first signal is through the TCR that binds processed peptides presented by MHC class II on the DC. A second signal through CD28, triggered by CD80/CD86 on the activated DC is then required and the third signal, the polarizing signal, determines polarization, i.e whether the naïve T-helper will polarize into Th1 or Th2. This is mediated through soluble membrane bound factors such as IL-12 and chemokine (C-C motif) ligand 2 (CCL2) produced by the DC. This signal usually requires feedback stimulation by the T-cell through CD40L that binds CD40 on the DC. Simplified, if the polarizing factor is IL-12 the T-helper cell polarizes into a Th1 cell and if the polarizing factor is CCL2, also known as macrophage cationic peptide 1 (MCP-1), the T-helper cell polarizes into a Th2 cell. The kind of polarization factor released by the DC depends on what PAMPs that originally activated the DC (66, 67, 78, 79).

1.2 INFLAMMATION

1.2.1 Acute inflammation

Inflammation is a multifaceted protective response to stimuli such as invading pathogens or tissue damage caused by injury, toxic agents, radiation etc. Clinical signs of inflammation are characterized by *rubor* (redness), *calor* (increased temperature), *tumor* (swelling), *dolor* (pain) and *functio laesa* (loss of function).

The harmful stimuli are rapidly recognized by complement or cells already present in the tissue such as macrophages and dendritic cells. The activated cells start to produce pro-inflammatory mediators such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF) and chemoattractants like C-X-C motif chemokine 8 (CXCL8) also referred to as IL-8 and leukotriene B_4 (LTB_4). These inflammatory mediators are for instance accountable for the clinical signs of the inflammation as dilation of blood vessels leading to increased blood flow in the inflamed area (*rubor*) increase in heat, locally or systemically as fever (*calor*), increased permeability of the blood vessels resulting in leakage of plasma fluid and proteins into the tissue causing edema (*tumor*). These mediators are also important for recruitment of inflammatory cells, first neutrophils and later lymphocytes, into the inflamed area to support fighting and cleaning up dead cells

etc. Inflammatory mediators such as CXCL8 diffuse out in to the surrounding tissue and create chemotactic gradients with the highest concentration close to the infection or injury. Cells as neutrophils are equipped with chemokine receptors (CXCR1 and CXCR2) that easily sense the chemokines and the neutrophils start to migrate towards the inflamed area (17). The leukocyte recruitment is also dependent of adhesion molecules such as P-selectin (CD162), L-selectin (CD62L) and integrin αM (CD11b) (80). Adhesion proteins are expressed on inflammatory and vascular cells and are of importance for cell migration from the circulation to the site of inflammation. Adhesion molecules enable leukocytes to roll along the endothelial cells within and through the vessels to the site of action (17, 80).

An important step in inflammation is complete reinstatement of the inflamed tissue, so called resolution. This process may fail and inflammation becomes chronic. Chronic inflammation is almost always accompanied by tissue destruction/remodeling.

1.2.2 IgE mediated inflammation

Our immune system may react to non-harmful antigens described as allergens. An immunologic reaction against an allergen induces allergic inflammation and is usually immunoglobulin E (IgE) mediated, described as a type 1 hypersensitivity. Allergic individuals have a tendency to overproduce IgE by B-cells, a Th2 driven mechanism evolutionary formed to fight parasite infections. The first time an allergic individual is exposed to a potential allergen, B-cells undergo class switch mainly driven by Th2 cells producing Th2 cytokines such as IL-3, IL-4, IL-5 and IL-13, and differentiate into a plasma cells that start to secret IgE. Soluble IgE binds to high affinity receptors, FcεRIs expressed on the surface on mast cells and basophils. *Mast cells* do not exist in blood but are located in the tissue and in the protecting mucosa that covers the airways, eyes and gastrointestinal tract. When mast cells or basophils, with specific IgE bound on their surface, are exposed to the specific allergen, crosslinking of the allergen and membrane bound IgE occurs whereupon the mast cell or basophil degranulates and pro-inflammatory substances such as histamin, cytokines and prostaglandins are released. If the allergen is airborne, respiratory symptoms such as sneezing, runny nose and itchy eyes, occurs. The substances released by mast cells and basophils also recruit other inflammatory cells such as eosinophils and lymphocytes that are responsible for the late reaction that often occurs some hours after the first reaction. Allergic individuals may develop asthma, in which allergen is a potent trigger of asthmatic symptoms (16, 17).

The prevalence of allergic disorders has increased since the middle of the 20th century. The so called “hygiene hypotheses” have been widely discussed and might explain the rapid increase. This hypothesis states the lack of early childhood exposure to microbial compounds and bacterial infections (81) and several experimental and epidemiological studies strengthen the hypothesis. Growing up on a farm has been shown to prevent against allergy and atopic asthma later in life (82-84). Further, being the youngest sibling comprises protection against development of atopic disorders probably due to

high load of infections from the older siblings (85). However, in already allergic individuals there are studies indicating that exposure to microbial compounds worsen symptoms (86).

1.2.3 Chronic inflammatory airway diseases

Chronic bronchitis, chronic obstructive pulmonary disease (COPD) and asthma, are chronically inflammatory diseases affecting the airways.

Chronic bronchitis is an ongoing, progressive inflammatory disorder that affects the lower respiratory tract. Chronic bronchitis is defined as chronic (daily) productive cough during more than three months on two subsequent years (87). The disease is mainly caused by tobacco smoking but can also be caused by other airway irritants/pollutions (88) or recurrent airway infections (89). The inflammation in chronic bronchitis is mainly neutrophilic and mediators such as TNF are important (90-92). Chronic bronchitis is associated with tissue remodeling and characteristic features are goblet cell hyperplasia and metaplasia and thickening of the bronchial walls leading to airway obstruction (91, 92). The inflamed tissue and the strong increase in mucus production, caused by goblet cell hyperplasia and metaplasia, make it easier for pathogens to colonize the lung and frequent and severe infections are common (89). Chronic bronchitis is also a common feature in COPD (91, 93).

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease which is predicted to become the third most common cause of death in the world in 2020 (94). Tobacco smoking is a strong risk factor for development of COPD (95) but there are other risk factors such as age, indoors cooking over open fire, occupational exposure and air pollution (1, 69, 96). The disease is progressive and leads to airway obstruction that is poorly reversible (97). The inflammation mainly affects the airways but there is also a substantial systemic involvement (98, 99). Inflammatory cells of importance in COPD are neutrophils, macrophages, cytotoxic $CD8^+$ T-cells, airway epithelial cells, endothelial cells and fibroblasts (90, 100, 101). The disease leads to goblet cell hyperplasia and metaplasia, bronchiolitis, peribronchiolor fibrosis and emphysema (97, 101).

Important mediators maintaining the inflammation in COPD are TNF, LTB_4, MCP-1, CXCL8, reactive oxygen species (ROS) and mediators as transforming growth factor (TGF)-β, neutrophilic elastase, cathepsins and matrix metalloproteinases (MMPs) (100, 101).

Asthma is an airway disorder that affects around 300 million people worldwide and causes around 250000 deaths yearly. The prevalence of asthma differs from 1 to18% between countries (102).

According to the Global Initiative for Asthma (GINA) guidelines asthma is defined as "a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway

hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning"(102).

Asthma leads to airway obstruction (bronchoconstriction), but in contrast to COPD the obstruction is reversed and lung function is mostly normalized, either spontaneously or following treatment (102). Allergic asthma is triggered by an allergen through a type 1 reaction described above. Both allergic and non-allergic asthma can be triggered by irritants such as cigarette smoke, cold air and by respiratory infections. Inflammatory cells important in allergic asthma are Th2 cells, mast cells and eosinophils (103). Asthma also involves epithelial cells, smooth muscle and nerve cells (104, 105). Neutrophilic inflammation is a common feature in severe asthma and during asthma exacerbations (106, 107). Tissue remodeling is common in asthma and some asthmatics may develop chronic airflow obstruction. Goblet cell hyperplasia, thickening of basement membrane and the smooth muscle layer (hyperplasia and hypertrophia) and subepithelial fibrosis are characteristic remodeling features in asthma (108, 109). Mediators involved in these remodeling features are Th2 cytokines, TGF-β, MMPs and eosinophilic cationic protein (ECP) (103, 105, 110).

1.3 PIG FARMING ENVIRONMENT

Pig barn environment is highly contaminated with airborne inhalable organic dust. Concentrations up to 28.5 mg/m^3 of inhalable dust have been found in pig confinement buildings. The work in a pig barn is often intensive, as weighing pigs before slaughter, and the exposure levels are often around 10-20 mg dust/m^3 (111-114).

The pig barn dust is a complex composition with a large number of constitutents from hay, grasses, pollen, epithelial cells (from pigs), feedstuff, insect parts and mineral ash (115-117). The dust also contains high amounts of pathogen associated molecular patterns (PAMPs) from microorganisms as moulds, fungi and Gram-positive and Gram-negative bacteria (115-118) where the Gram-positive bacteria are the most frequent microbial in pig barn dust (115). Exposure levels of endotoxin (LPS), cell wall constituent of Gram-negative bacteria, usually diverge from 0.1 up to 1.4 μg/m^3 (119, 120) Whereas levels of peptidoglycan also known as muramic acid are higher (up to 6.6 μg/m^3) (119). Apart from organic particles, high levels of gases such as ammonia, methane, carbon monoxide and hydrogen sulphide are present in the barn and may contribute to the physiological and inflammatory response to exposure (121).

Characterization of pig barn dust	Culturable bacteria:	Culturable fungi:
Major components: Hay Animal feed Pig dander Pig feces Epithelial cells from the pigs Pollen grains Insect parts Mineral ash Microorganisms: Bacteria Fungi	*Alcaligenes* *Enterobacter* *Enterococcus* *Staphylococcus* *Micrococcus* *Bacillus* *Klebsiella* *Pseudonomas* *Escherichia* *Vibrio* *Pasteurella*	*Penecillium* *Alternaria* *Aspergillus* *Cladosporium* *Fucarium* *Verticillium* *Scopulariopsis* *Candida* *Hansenula* *Rhizopus*

Table 3 Components in pig barn dust. Table modified from (116-118).

1.3.1 Inflammatory response to pig barn dust

The dust in pig barn environment is a very potent pro-inflammatory stimulus. A few hours exposure in a pig barn leads to an intense upper and lower airway inflammation with systemic influences and flu-like symptoms such as chest-tightness, cough, headache, fever and muscle pain in healthy subjects, a condition also called organic dust toxic syndrome (ODTS) (114, 119, 122, 123). The exposure enhances bronchial responsiveness, induces airway neutrophilia with up to a 100-fold increase of neutrophils in bronchial alveolar lavage (BAL) fluid, a 20-fold increase of neutrophils in nasal lavage (NAL). The exposure leads to elevated release of pro-inflammatory cytokines and chemokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF), CXCL8 (IL-8) in nasal lavage fluid, BAL-fluid and in peripheral blood (111, 119, 123-126). Wearing a respirator with particle filter (which effectively reduces dust exposure) during work in the pig barn attenuates the inflammatory reaction but does not influence the increase in bronchial responsiveness (125).

The dust is also very potent in activating cells *in vitro*. Macrophages and airway epithelial cells (A549, primary bronchial epithelial cells (PBEC) and normal human bronchial epithelial cells (NHBE)) release high amount of pro-inflammatory cytokines, e.g IL-6 and CXCL8 (127-129) when stimulated with dust collected in pig barns. The dust is also a potent activator of T-cells, both *in vivo* and *in vitro* (130-132). Endotoxin (LPS) in the dust contributes to the inflammatory response but the responses are not exclusively due to LPS (119, 127, 133, 134).

1.3.2 Inflammatory airway diseases among pig farmers

It is known, since many years, that daily exposure in pig barn environment is harmful. Respiratory symptoms such as cough, increased phlegm and mucus production, chest

tightness and wheezing are more frequently observed among pig farmers than in non-farmers (135) and other kinds of farmers (8, 136) indicating that pig farming is particularly harmful. The prevalence of chronic bronchitis is increased in pig farmers (7-9) and there are studies indicating an increased prevalense of COPD compared to non-farmers (10, 11). An asthma-like syndrome has been reported to occur in approximately 11% of pig farmers and occurs mostly after weekends (137). Many of the components in pig barn dust are potential allergens (116) and reported airway symptoms are similar to symptoms observed in allergic individuals. However, several studies support that the symptoms observed in pig farmers are not IgE–mediated but rather associated with neutrophil inflammation (138-142). Previous studies have shown ongoing inflammation with increased concentration of neutrophils in the lower airways (BAL) also in farmers who do not experience respiratory symptoms (141). In symptomatic farmers there are reports indicating an increased thickness of the basement membrane of the bronchial wall compared to blue collar workers and farmers in other occupations than pig breeding (143). It has also been shown that pig farmers have an inflammatory reduction in the lower airway mucosa, with oedema and increased phlegm (142).

1.3.3 Tolerance to pig barn dust

Exposure to the pig barn environment induces an intensive airway and systemic inflammation in healthy, previously unexposed subjects. However, in pig farmers who have been working in this environment on a daily basis the immune response to the pig barn environment becomes attenuated. The farmers working in pig barn facilities seem to develop some kind of immunological tolerance similar to what is observed after repeated exposure to LPS. Pig farmers working for 5 years or longer develop ODTS less frequently than do farmers who have worked less than 5 years (144). Several studies clearly show that the airway inflammation following acute exposure in pig barn is dampened in pig farmers. After three hours exposure in a pig barn the increase of inflammatory cells in the upper airways (nasal lavage) and IL-6 levels in serum are attenuated compared to non-farming controls (145). Israël-Assayag *et al* have shown that pig farmers have increased levels of soluble L-selectin (sCD62) in serum compared to non-farming healthy controls. This might depend on increased shedding of membrane bound L-selectin and might be involved in the damped migration observed in farmers (145, 146). Further, bronchial responsiveness does not increase as much in farmers as in previously unexposed subjects after 3 hours in a pig barn (145). It is not clear whether this alteration of the inflammatory response is of importance for the farmer's health but respiratory symptoms and chronic inflammatory airway diseases are common among pig farmers (9, 143).

1.4 TOBACCO SMOKERS

Tobacco smoking constitutes a strong risk factor for development of respiratory disorders such as chronic bronchitis and COPD. There are studies indicating that up to 50% of smokers develop COPD (147). Cigarette smoke is a complex composition of large number of toxic constitutions. It have also been shown that tobacco smoke contains microbial components as LPS (148, 149) indicating that tobacco smokers are regularly exposed to pathogen-associated molecular patterns (PAMPs).

Tobacco smoke induces inflammation as shown in a number of studies (150, 151). The inflammation observed in symptomatic smokers is characterized by neutrophils, but there are studies showing that tobacco smoke also favour a Th2 profile in peripheral blood (152, 153). One common feature in tobacco smokers is increased mucus production due to goblet cell hyperplasia and metaplasia, tissue remodeling feature where Th2 cytokines, mainly IL-13 have shown to be of importance (154, 155).

1.5 LABORATORY ANIMAL HOUSE ENVIRONMENT

Allergic sensitisation against laboratory animals is a work environment problem within biomedical research and industry. Working with animals is associated with high exposure to allergens but involves also exposure to microbial compounds such as LPS (13, 14). Exposure to microbial components like LPS early in life seems to depress allergen sensitisation (156). However, in atopic subjects, exposure to LPS may worsen symptoms (86).

1.5.1 Occupational allergy and asthma in biomedical research

Respiratory symptoms have been reported to occur in a range from 20-60% among laboratory animal workers. Most common are symptoms from the nose and eyes but symptoms from chest and skin symptoms are also common (12, 157-159). Allergic sensitization with specific IgE or positive skin prick test (SPT) against laboratory animal (LA) allergens, mostly rat and mice, occurs in up to 20% or even more of LA workers (12, 160). Exposure in laboratory animal facilities also constitutes a significant risk factor for development of airway obstruction (15). It is known that the environment in animal house facilities contains endotoxin (LPS) and it is well known that LPS influences and induces airway symptoms in individuals with allergy to laboratory animals but also in individuals without LA atopy (13, 14).

2 AIMS

The general aim of the thesis was to elucidate influences of regularly occupational exposure to organic dust on innate and adaptive immunity with focus on expression of pattern recognition receptors (PRR) in blood and airways, the cytokine profile of blood T-cells and expression of suppression of tumorgenicity 2 (ST2).

Study I

The aim was to characterize the expression of pattern recognition receptors (TLR2, TLR4 and CD14) on blood neutrophils and monocytes and the cytokine profile of blood T-cells in pig farmers, smokers and healthy, non-smoking non-farmers before and after exposure in a pig barn, a bronchial LPS challenge and stimulation of blood *ex vivo*.

Study II

To characterize expression of PRRs on blood monocytes and neutrophils, activation markers on B- and T-cells, cytokine profile of blood Th cells and serum levels of sST2 and sCD14 in individuals (with or without allergy to LAs) who experience respiratory symptoms, while working in laboratory animal (LA) facilities compared with two non-LA exposed groups, one atopic group sensitized to birch pollen and one non-allergic control group.

Study III

To elucidate influence of regularly exposure in pig barn environment on surface expression of PRRs (TLR2, TLR4, CD14) and adhesion proteins (CD11b, CD62L and CD162) on blood and airway (sputum) neutrophils and levels sTLR2 and sCD14 in blood and sputum. Further, the aim was to investigate whether the influence of exposure in a pig barn and a bronchial LPS challenge on serum levels of sST2 differ between farmers and controls. A third aim was to find out whether blocking of the ST2 receptor influences the release of pro-inflammatory cytokines from peripheral blood cells stimulated with TLR ligands *ex vivo.*

Study IV

To investigate cytokine profile of Th cells and Tc cells and TGF-β1 levels in peripheral blood in pig farmers, smokers and healthy, non-smoking non-farmers.

3 MATERIAL AND METHODS

Material and methods are briefly summarized below. More detailed descriptions are provided in the papers and manuscripts.

Subjects

The studies were approved by the local ethics committee and all subjects gave their informed consent to participate in the studies.

In study I and IV non-smoking pig farmers, non-farming smokers and non-smoking, non-farming healthy subjects were included.

In study II individuals that experience respiratory symptoms while working with laboratory animal, one group with and one group without allergy to laboratory animals (rat or mice) were included. Two groups not exposed to laboratory animals, one group of individuals with birch pollen allergy and one group of non-atopic individuals were also included in study II.

In study III non-smoking pig farmers and non-farming non-smoking subjects were included. All subjects in study I, III and IV were non-atopic verified by negative skin prick test (SPT).

Lung function

In study I and IV vital capacity (VC) and forced expiratory volume in one second (FEV_1) were measured by using a wedge spirometer (Vitalograph®, Buckingham, United Kingdom) according to the criteria of the American Thoracic Society (161). Local reference values were used (162, 163).

Skin prick test

In study I-IV skin prick test (SPT) were performed using a panel of 17 common allergens. The SPT were performed on both forearms with histamine chloride (10 mg/mm, ALK and Allergopharma) as positive control and the diluent of the allergens (ALK and Allergopharma) as negative control.

Induced sputum

In study III sputum was induced after the inhalation of salbutamol by inhaling increasing concentrations of saline whereupon participants were asked to cough deeply and make an attempt to expectorate sputum. At least 1000 mg, macroscopically appeared free from saliva, was considered as sufficient sample. The samples were filtered and centrifuged for 10 minutes at 400*g* followed by cell count and a viability test (trypan blue). Slides were prepared by cytocentrifugation and stained with May-Grünwald Giemsa stain for differential cell count and remaining cells were stained for flow cytometry analyses. Sputum supernatants were divided into aliquots and stored in -70°C until analysed.

Exposure in a pig barn, bronchial LPS challenge

In study I and a part in study III the subjects underwent a 3 h exposure in a barn while helping a farmer weighing pigs. On a separate day a LPS challenge was performed by inhaling a LPS solution (*Escherichia coli* serotype 0111:B4 (SIGMA) dissolved in sterile saline (9 g/l) at a concentration of 1.25 mg/ml) with an inhalation dosimeter (SPIRA® Elektro 2, Hameenlina, Finland). The subjects inhaled 6 breaths from the dosimeter, corresponding to a LPS dose of 53.4µg. Blood was collected approximately 2 weeks before and 7 h after the start of both exposures which were performed in a randomized order.

Respiratory and inhalable dust measurements

To monitor inhalable and respirable dust and endotoxin levels, respectively, portable pumps with IOM filter cassettes (25 mm) (SKC Ltd, Dorset, UK) and plastic cyclones (25 mm) (Casella Ltd, London, UK) were used. The cassettes were equipped with Teflon filters (1.0 µ, Millipore, Sundbyberg, Sweden). The endotoxin concentration was analysed by the use of a kinetic technique version of *Limulus amebocyte* lysate assay (Limulus Amebocyte lysate, Endosafe® Endochrome-K™ U.S. Lisence No. 1197, Coatech AB, Kungsbacka, Sweden), with *E. coli* 0111:B4 as standard.

Blood sampling

Peripheral blood was collected in ethylene diamine-tetra-acetic acid (EDTA) vacutainer tubes (BD Bioscience, San Jose, California) for assessing cell surface markers (study I-III) and in heparinized tubes (BD Bioscience) for intracellular cytokine staining (study I, II and IV). For serum samples (study I-IV), blood was also collected in anticoagulant free tubes, left standing in room temperature for 1 h to allow blood to coagulate followed by centrifugation. Serum was divided into aliquots and stored in -70°C until analysed.

Isolation of blood neutrophils

Heparinized blood was sediment in PBS containing 2% dextran to separate leukocytes from red blood cells. Lymphoprep™ (Axis-Shield, Norway) was then gently added under the upper phase including leukocytes and centrifuged for 40 min. The fraction including polymorphonuclear cells were incubated with magnetically labelled MACS® anti-CD16 (Milteniy Biotech, Auburn, California) and isolated following the manufacturer's instructions using a magnet and LS column (MiniMACS, Milteniy Biotech). The $CD16^+$ cells were washed and suspended in RPMI 1640 culture media (Sigma–Aldrich).

Ex vivo stimulations of blood and purified neutrophils

In study I peripheral blood and purified neutrophils and in study III peripheral blood were stimulated *ex vivo* with pro-inflammatory stimuli. In study I blood and purified neutrophils were stimulated 2 h with LPS (*Escherichia coli* serotype 0111:B4 (SIGMA)) and dust collected in a pig barn on shelves and window ledges about 1.2 m

above the floor. In study III blood were stimulated for 4 h with LPS (*Escherichia coli* serotype 0111:B4 annat (SIGMA), dust from pig barn, the synthetic TLR2 ligand tripalmitoyl-S-glycerylcysteine (Pam3Cys) (EMC microcollections GmbH, Tübingen, Germany), TNF (R&D systems, Europe, Abingdon, UK), IL-33 (R&D systems). In study III *ex vivo* stimulations with LPS were also performed in presence of anti-ST2 (R&D systems) and matched isotype as negative control (R&D systems).

Flow cytometry

Expression of surface markers on blood (study I-III) and sputum cells (study III) and intracellular cytokines (study I and IV) were assessed with flow cytometry. Blood cells or sputum cells were stained on the surface with fluorochrome conjugated specific antibodies or matched isotype. Blood cells were stained at 20-22°C and sputum cells at 4°C. Red blood cells were lysed using PharM Lyse ™ (BD Bioscience Pharmingen). In study I blood monocytes and neutrophils were stained with anti-TLR2-PE (eBioscience), anti-TLR4-PE (eBioscience), and anti-CD14-FITC (eBioscience). In study II blood B-cells were stained with anti-CD23 PE, anti-CD45 PerCp and anti-CD19 APC, blood T-helper were stained with anti-CD25 PE, anti-CD3 FITC, anti-CD4 APC and anti-CD45 PerCp. Blood monocytes and neutrophils were stained with CD45-PerCp together with anti-TLR2-PE (eBioscience), anti-TLR4-PE (eBioscience) or anti-CD14-FITC (eBioscience). In study III blood and sputum neutrophils were stained with CD45-PerCp together with anti-TLR2-PE (eBioscience), anti-TLR4-PE (eBioscience), anti-CD11b-PE (BD Bioscience), anti-CD14-FITC (eBioscience), anti-CD62L-PE (BD Bioscience) or anti-CD162-PE (BD Bioscience). Unstimulated and stimulated (phorbol 12-myristate 13-acetate (PMA, 25ng/ml) and ionomycin (1μM, Sigma–Aldrich) in presence of GolgiPlug (Brefeldin A, BD Bioscience) for 4 h at 37°C) blood T-cells were stained with anti-CD4 APC (BD Bioscience) on the surface in study I and anti-CD3 PerCp (BD Bioscience) and anti-CD8 APC (eBioscience) in study IV. Red blood cells were lysed using PharM Lyse ™ (BD Bioscience Pharmingen) and leukocytes permeabilised using Cytofix/Cytoperm ™ (BD Bioscience Pharmingen). Intracellular cytokines were stained with anti-IL-4 PE, anti-IFN-γ FITC, anti-IL-2 FITC (BD Bioscience) or anti-IL-13 PE (BD Bioscience Pharmingen). To analyse cell distribution in peripheral blood (study I-IV) blood were stained with a four–colour antibody mixture (CD3FITC/CD8PE/CD45PerCp/CD4APC from BD Bioscience) in TruCOUNT ™ tubes containing a specified number of beads. The results are presented as relative median fluorescence intensity (rMFI = monoclonal antibody/ matched isotype control), proportion (%) of total population or as cells/volume blood. Samples were analysed using FACSCalibur ™ (BD Bioscience) flow cytometry and CELLQuest ™ or MultiSET ™ (BD Bioscience).

Measurements of soluble proteins

Soluble proteins in serum (study I-IV), sputum (study III) or supernatants (study I) were analysed with ELISA. In study I IL-6 was measured in serum by using high sensitive ELISA kit (R&D systems, Europe, Abingdon, UK). Soluble CD14 (sCD14)

and sST2 (study II-III) and sTLR2 (study III) were measured by using DuoSet ELISA kits (R&D systems, Europe, Abingdon, UK).

In study I IL-6 and IL-8 in supernatants were measured with an in-house ELISA method using commercially available antibody pairs (R&D systems, Europe, Abingdon, UK). In study III cytometric bead array (CBA, BD Bioscience) were used to measure IL-6 and TNF in supernatants from *ex vivo* stimulations and in study IV to measure transforming growth factor (TGF)-β1.

Statistics

Data are presented as scattergrams with median values, histograms, median values with 25^{th} and 75^{th} percentile or mean with range or 95% confidence interval or standard error of the mean (SEM). Data in study I and lung function in study IV were analysed by means of simple regression and ANOVA with Fisher's PLSD (between group comparisons) and paired t-tests (within group comparisons) as post hoc tests when appropriate. Data in study II-IV and serum levels of IL-6 in study I were analysed by using nonparametric tests. Friedman and Wilcoxon Signed Rank were used for within group comparison and Kruskal Wallis and Mann Whitney were used for between group comparisons. In all studies a P-value of <0.05 was considered significant.

4 RESULTS

Study I

Altered innate immune responses in farmers and smokers

In study I the aim was to investigate expression of PRRs, i.e. TLR2, TLR4 and CD14 on blood neutrophils and monocytes and cytokine profile interleukin (IL)-2, IL-4, IL-13 and IFN-γ) of blood T-helper cells were investigated in 11 non-smoking pig farmers, 12 smokers (at least 10 cigarettes per day during recent years) and 12 non-smoking, non-farming controls.

Further, the expression of TLR2, TLR4 and CD14 and release of IL-6 and IL-8 were investigated after *ex vivo* stimulations of peripheral blood with TLR ligands, LPS and dust collected in a pig barn, in the farmers (n=12) and the controls (n=12).

	Controls	Pig farmers	Smokers
Subjects (females/males)	**2/10**	**2/10**	**2/10**
Age Mean (range)	**33** (25-54)	**41** (22-61)	**41** (25-58)
Pack years Mean (range)	**0**	**0**	**21** (1.5-48)
Work in pig barns Mean (range) hours/day	**0**	**3.5** (0.5-8)	**0**
Years as a pig farmer Mean (range)	**0**	**13** (0.5-36)	**0**
Number of pigs in the barn Mean (range)	**0**	**1000** (65-3200)	**0**

Table 4 Subject characteristics.

Prior to exposures both pig farmers and cigarette smokers had signs of systemic inflammation with increased concentration of blood neutrophils (figure 2 A). Smokers also had increased concentration of blood monocytes (figure 2 B) and serum levels of IL-6 compared to control subjects (figure 3). After pig barn exposure and after bronchial LPS-challenge the concentration of blood neutrophils and monocytes increased in all three groups. The increase of neutrophils after LPS-challenge was higher in smokers compared to controls and farmers (Figure 2 A-B). The concentration of IL-6 in serum increased in controls and smokers but not in farmers after exposure in pig barn and the increase in controls was higher compared to farmers. After bronchial LPS-challenge concentration of IL-6 in serum increased in all groups, and the increase in smokers exceeded that of farmers and controls (figure 3).

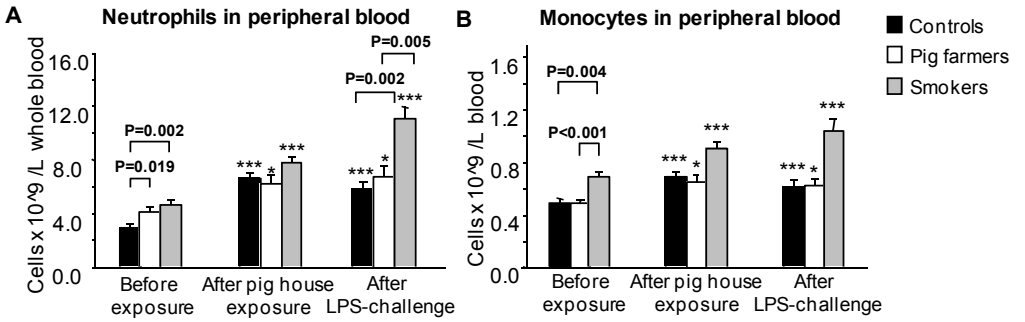

Figure 2 Concentration of neutrophils (A) and monocytes (B) in peripheral blood before and after exposure in a pig barn and a LPS challenge (n=11-12). * = P < 0.05, *** = P <0.001, compared with pre-exposure values. Data are presented as mean (+SEM). Brackets indicate pre-exposure differences between the groups or differences in exposure induced changes between the groups.

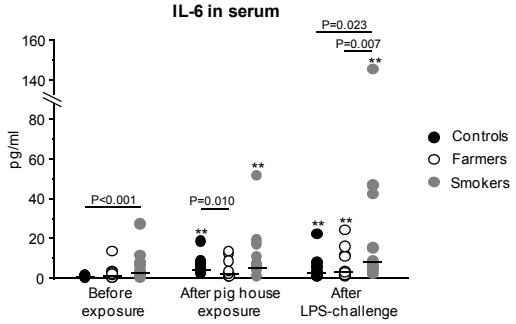

Figure 3 Scattergram with median showing concentration of IL-6 in serum before and after exposure in a pig barn and a LPS challenge (n=11-12). * = P <0.05, ** = P <0.01 *** = P <0.001, compared with pre-exposure values. Brackets indicate pre-exposure differences between the groups or differences in exposure induced changes between the groups.

Another observation was that farmers and smokers prior to exposure had increased proportion of blood T-helper cells producing IL-4 and IL-13 compared to the group of non-farming, non-smoking controls (figure 4 A-B). Further, the proportion blood Th cells producing IL-13 in the farmers correlated with the cumulative exposure, i.e. years working in pig barn facilities (figure 5). After pig barn exposure and after the bronchial LPS-challenge there was an increased proportion of IL-4 and IL-13 producing Th cells in the controls whereas the expression was elevated already prior to exposures and not further increased in farmers and smokers (figure 4 A-B). Controls also showed an increase in IFN-γ producing T-cells after LPS-challenge (figure 4 D).

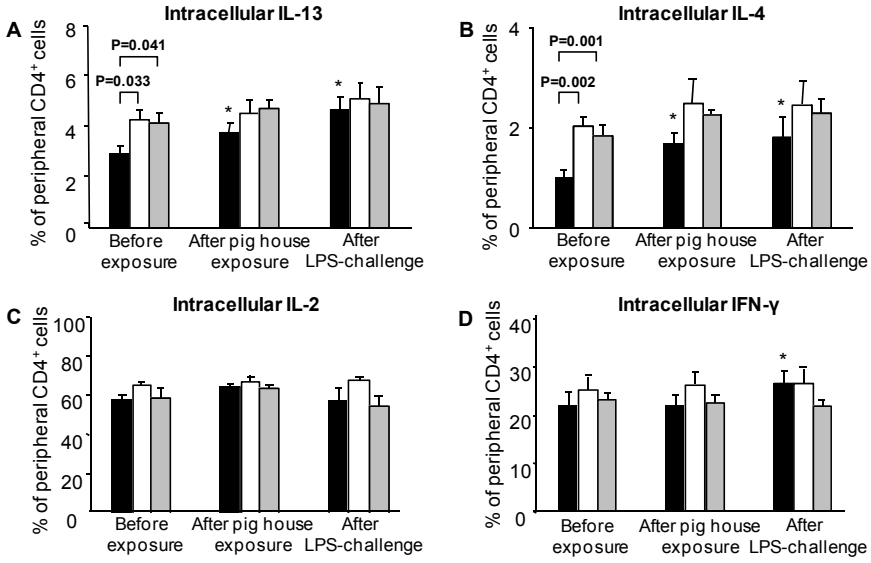

Figure 4 Proportion of peripheral T-helper cells producing intracellular IL-13 (A), IL-4 (B), IL-2 (C) and IFN-γ (D), before, after exposure in pig barn and after LPS challenge (n=10-12). Data are presented as mean (+SEM). * = P < 0.05, *** = P <0.001, compared with pre-exposure values. Brackets indicate pre-exposure differences between the groups.

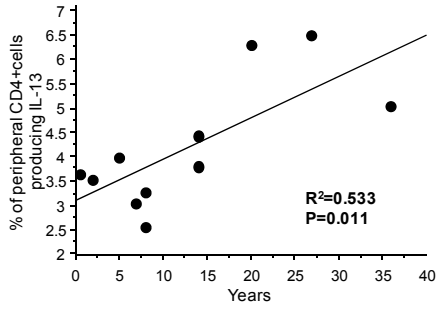

Figure 5 Correlation between percentage of T-helper cells producing IL-13 and cumulative exposure (number of years the farmers had been working in a pig barn) (n=11).

Prior to exposure farmers had lower expression of TLR2 on blood monocytes than smokers and non-smoking, non-farming healthy controls. After LPS-exposure there was an increase in TLR2 expression on blood neutrophils in the controls compared to pre-exposure but with no differences between the groups (figure 6 A-B). No further alterations in PRR expression was observed following *in vivo* exposures. The TLR2 expression on monocytes increased in controls in the lowest dose of LPS and in the highest doses of dust when blood was stimulated *ex vivo* (Figure 7 A-B). *Ex vivo* stimulations with LPS and pig barn dust increased CD14 expression dose-dependently in both farmers and controls. The observed increase in CD14 expression in farmers was significantly higher in control (culture media) and in the lowest and highest dose of dust compared to controls (figure 7 C-D).

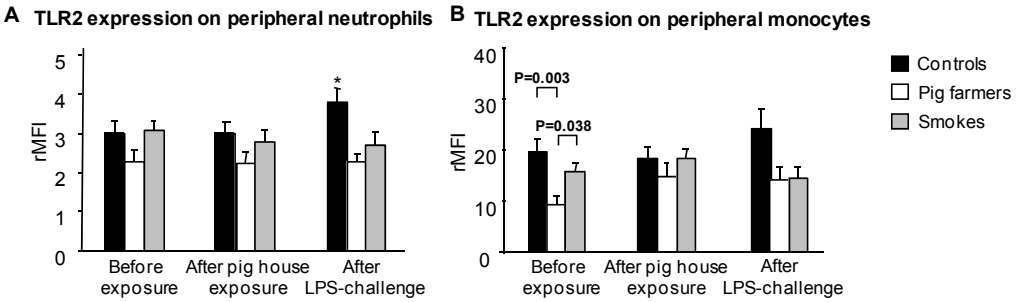

Figure 6 Surface expression of TLR2 on peripheral blood neutrophils (A) and blood monocytes (B), before and after exposure in a pig barn and a LPS challenge (n=10-12). Data are presented as mean (+SEM). * = P < 0.05, *** = P <0.001, compared with pre-exposure values. Brackets indicate pre-exposure differences between the groups or differences in exposure induced changes between the groups. rMFI = relative median fluorescence intensity.

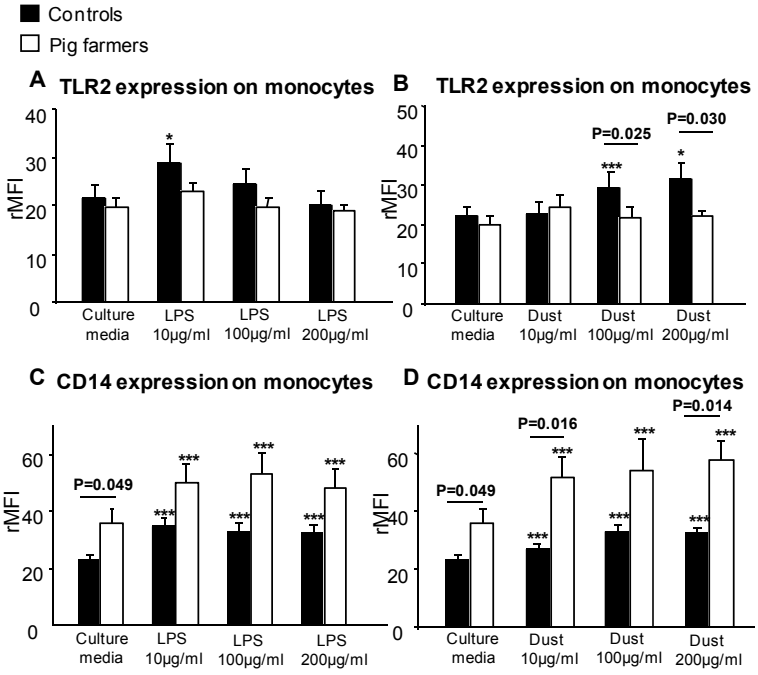

Figure 7 Surface expression of TLR2 (A-B), and CD14 (C-D) on peripheral blood monocytes after *ex vivo* stimulation with LPS (A, C) or dust collected in a pig barn (B, D). Data are presented as mean (+SEM). * = P < 0.05, *** = P <0.001, compared with media (n=10-12). Brackets indicate differences between the groups. rMFI = relative median fluorescence intensity.

After blood stimulation *ex vivo* there was an increased release of IL-6 at the lowest dust concentration in controls and the increase was higher than in farmers (figure 8 B). Stimulation of blood with LPS did not influence the IL-6 release in neither group (figure 8 A). All concentrations of LPS and dust increased the release of IL-8 in controls and farmers with no difference between the groups (figure 8 C-D).

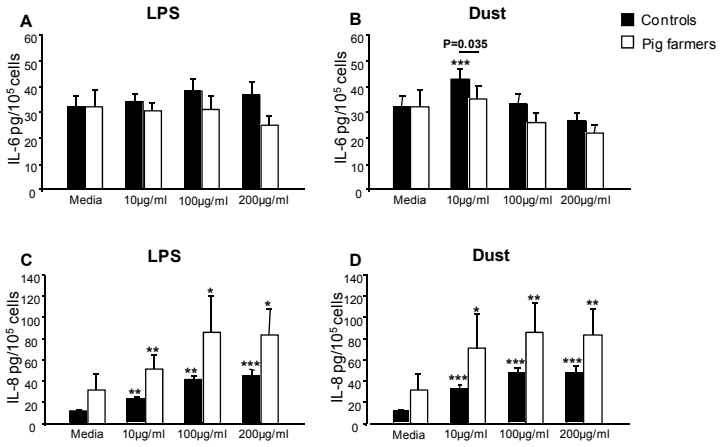

Figure 8 Release of IL-6 and IL-8 after LPS and dust stimulations *ex vivo* of peripheral blood. Data are presented as mean (+SEM). * = P < 0.05, ** = P <0.01, *** = P <0.001, compared with media (n=10-12). Brackets indicate differences between the groups.

Study II

Increased levels of soluble ST2 in birch pollen atopics and individuals working in laboratory animal facilities

In study II the aim was to investigate expression of TLR2, TLR4 and CD14 on blood neutrophils and monocytes, soluble CD14 and ST2, CD23 on blood B-cells and CD25 on blood T-cells and cytokine profile of blood T-helper cells. Twenty-seven subjects who experienced respiratory symptoms while working with laboratory animals (rat and/or mouse) were included of whom 19 were positive and 8 had negative skin prick test to rat and/or mouse. Two control groups, not exposed to laboratory animals, were also included, one group of 12 subjects with birch pollen allergy and one group of 11 non-allergic healthy subjects (table 5).

	Controls	***Birch pollen allergy***	***LA workers +SPT to LA***	***LA workers -SPT to LA***
Subjects (n)	11	12	19	8
Mean age (y [range])	36 (22-62)	31 (21-49)	35 (22-60)	32 (24-43)
Women (n [%])	10 (90.9)	8 (66.7)	15 (78.9)	6 (75.0)
+SPT Birch	0	12	4	1
+SPT Rat	0	-	6	0
+SPT Mouse	0	-	0	0
+SPT Rat and Mouse	0	-	13	0

Table 5 Subject characteristics.

In this study it was observed that the subjects who experience symptoms while working with laboratory animals (LAs), especially those with negative in SPT to Las, had higher expression of CD14 on blood monocytes compared to symptomatic, atopic subjects sensitized to birch pollen investigated during birch pollen season and healthy, non-LA-exposed control subjects (figure 9).

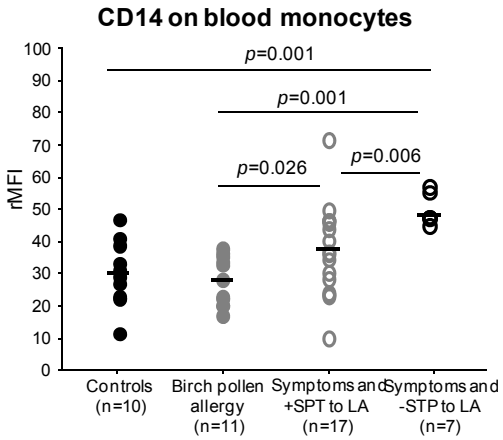

Figure 9 Scattergram showing surface expression of CD14 on peripheral blood monocytes. rMFI = relative median fluorescence intensity. Brackets indicate differences between groups and markers indicate median.

There were no differences regarding serum levels of sCD14 observed in study II (figure 10 A). However, blood levels of soluble ST2 were elevated during pollen season in subjects with birch allergy and in workers with laboratory animal induced symptoms and negative skin prick test to mouse or rat compared to a non-atopic non-exposed control group (figure 10 B).

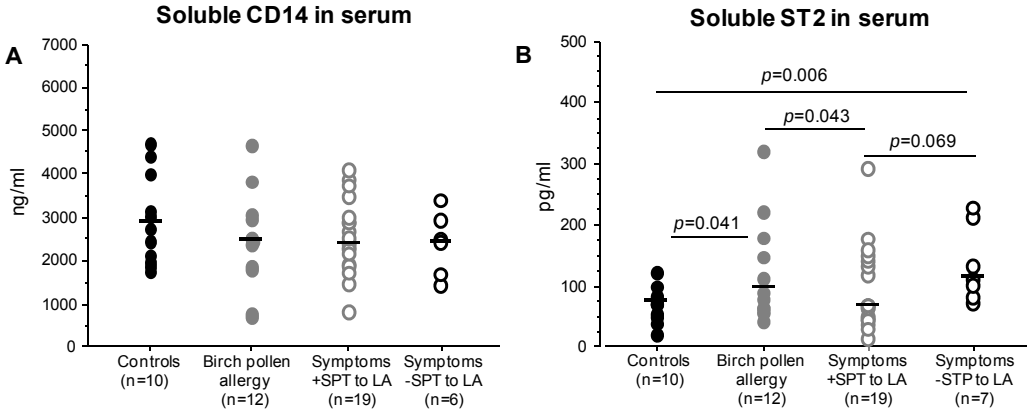

Figure 10 Scattergram showing concentration of sCD14 (ng/ml) (A) and sST2 (pg/ml) (B) in serum. Brackets indicate differences between groups and markers indicate median.

Study III

Occupational exposure in pig farms alters innate immunity

In study III the aim was to study surface expression and soluble variants of pattern recognition receptors (TLR2, TLR4, CD14, sTLR2 and sCD14) and surface expression of adhesion proteins (CD11b, CD62L and CD162) on blood and airway neutrophils (sputum) in 15 non-smoking pig farmers and 15 healthy subjects previously unexposed in pig barn environment (table 6). Blood samples were also collected in 11 farmers, and 12 controls (for subject characteristics, see table 4) to investigate whether serum levels

of sST2 differ between farmers and controls, before and after exposure in a pig barn and a bronchial LPS challenge. A third aim was to find out whether blocking of the ST2 receptor influences the release of pro-inflammatory cytokines from peripheral blood cells stimulated with TLR ligands *ex vivo*.

	Controls	Pig farmers
Subjects (females/males)	**2/13**	**2/13**
Age Mean (range)	**52** (26-64)	**45** (22-65)
Work in pig barn Mean (range) hours/day	**0**	**3.5** (0.5-8)
Duration of work in pig barn Mean (range) years	**0**	**16** (2-35)
Number of pigs in the barn	**0**	**2066** (60-6000)

Table 6 Subject characteristics.

In this study it was observed that farmers, exposed in pig barn facilities on a daily basis had higher concentration of blood monocytes (table 7), lower number of macrophages and higher proportion of neutrophils in sputum compared with naïve subjects (figure 11 A-D). Farmers also had lower levels of soluble CD14 (sCD14) and sTLR2 in sputum compared with the previously unexposed healthy subjects (figure 12 A-B)

	Controls *cells x10¨9/L*	***Farmers*** *cells x10¨9/L*	***P-value***
Neutrophils	2.99 (2.40;4.11)	3.57 (3.21;4.44)	0.221
Monocytes	0.44 (0.41;0.51)	0.55 (0.49;0.57)	0.024
Lymphocytes	2.07 (1.74;2152)	2.17 (2.00;2.40)	0.093
Basophils	0.05 (0.04;0.07)	0.06 (0.05;0.07)	0.254
Eosinophils	0.15 (0.65;0.20)	0.11 (0.09;0.19)	0.678

Table 7 Concentration (median) of neutrophils, monocytes, lymphocytes, basophils, and eosinophils in peripheral blood. Results are present as cells x10^9 /L blood.

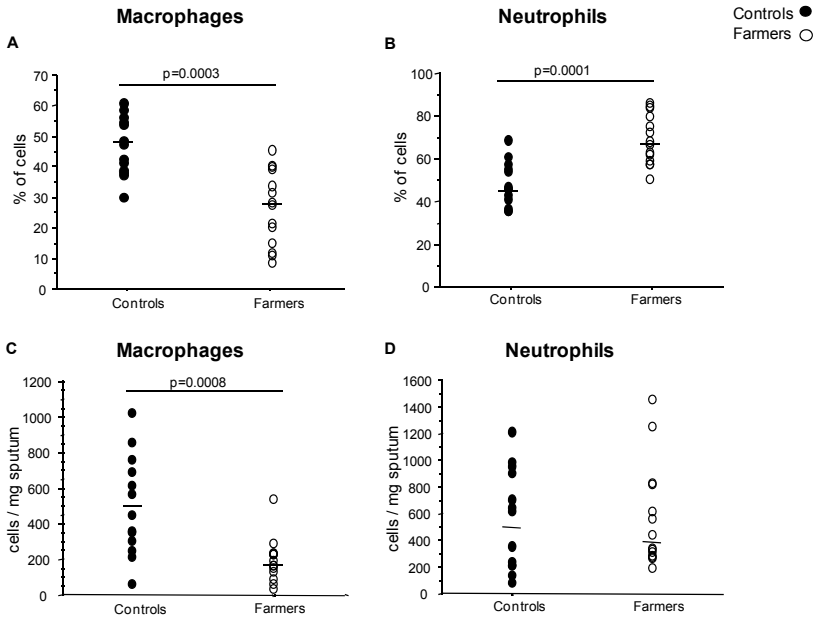

Figure 11 Scattergram showing proportion (%) and concentration (cells/mg of sputum) of macrophages and neutrophils in sputum. P-values indicate differences between the groups and markers indicate median.

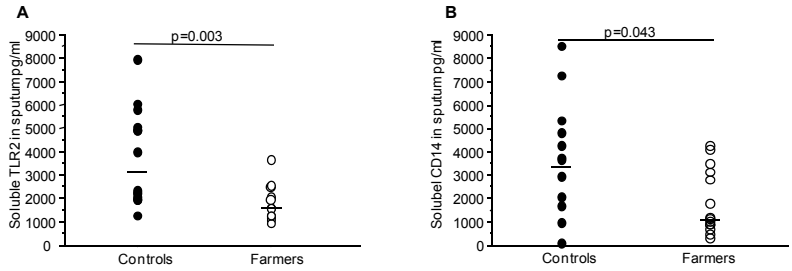

Figure 12 Scattergram showing concentration of sTLR2 in sputum (A) and concentration of sCD14 in sputum (B). P-values indicate differences between the farmers and controls and markers indicate median.

We also observed attenuated expression of CD14 on sputum neutrophils in farmers compared to controls. Further was the expression of adhesion molecules, i.e. CD62L and CD162 on peripheral blood neutrophils lower in farmers than in non-farming controls (figure 13).

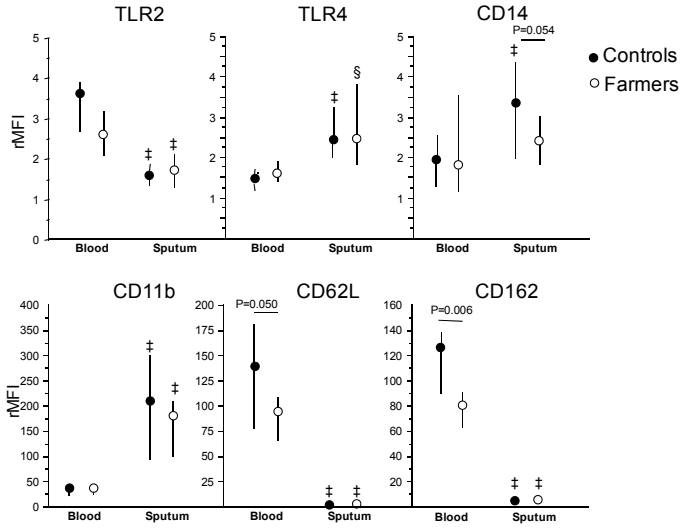

Figure 13 Expression of TLR2, TLR4, CD14, CD11b, CD62L, and CD162 on peripheral blood neutrophils and sputum neutrophils. P-values indicate differences between the groups. rMFI = relative median fluorescence intensity. Medians and 25th – 75th percentiles are presented. § indicates $p<0.05$ and ‡ indicates $p<0.01$ for within group comparisons between blood and sputum.

There was no difference in serum levels of sST2 prior to exposure (figure 14 A). However, exposure in a pig house and the bronchial LPS challenge increased sST2 levels in serum in the control subjects but not in the farmers (figure 14 B).

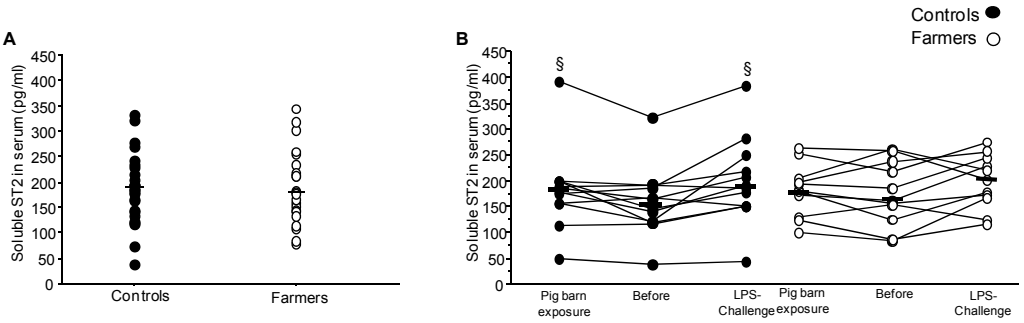

Figure 14 Concentration of sST2 in serum in the whole material (controls n=26 and farmers n=22, A). Concentration of sST2 in serum before and after exposure in a pig barn and a LPS challenge in 11 farmers and 12 healthy non-exposed control subjects (B). § indicate $p<0.05$ for pre- and post-exposure comparisons.

Release of IL-6 and TNF increased after ex vivo stimulation with both concentrations of LPS and the lipopeptide tripalmitoyl-S-glycerylcysteine **(**Pam3Cys) and in the highest concentration of pig barn dust in both farmers and smokers. Stimulation of peripheral blood with the lower pig barn dust concentration induced an increase of IL-6 in farmers and controls and an increase of TNF only in the farmers. However, TNF induced IL-6 increase only in the control group. There were no differences in IL-6 and TNF increase between the farmers and controls following LPS, Pam3cys and pig barn dust stimulations (figure 15).

Anti-ST2 antibodies induced a further increase of IL-6 and TNF release beyond the effect of LPS stimulation in controls (figure 16). However, release of IL-6 and TNF was not influenced by IL-33 in LPS stimulated blood in neither group with any differences between the groups (figure 16). Analyses of pooled data from both groups revealed that IL-33 induced a decrease in TNF release in LPS stimulated blood and a slight decrease in IL-6 (data not shown).

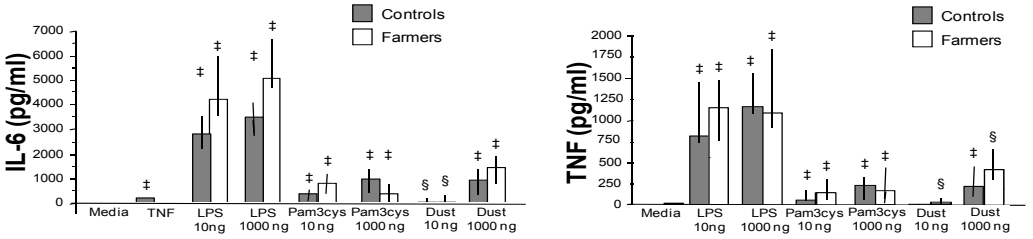

Figure 15 Release of IL-6 and TNF after *ex vivo* stimulation of peripheral blood with TNF, LPS, Pam3Cys or pig barn dust. Data are presented as median with 25^{th}-75^{th}. § indicates $p< 0.05$ and ‡ indicates $p<0.01$ compared with medium.

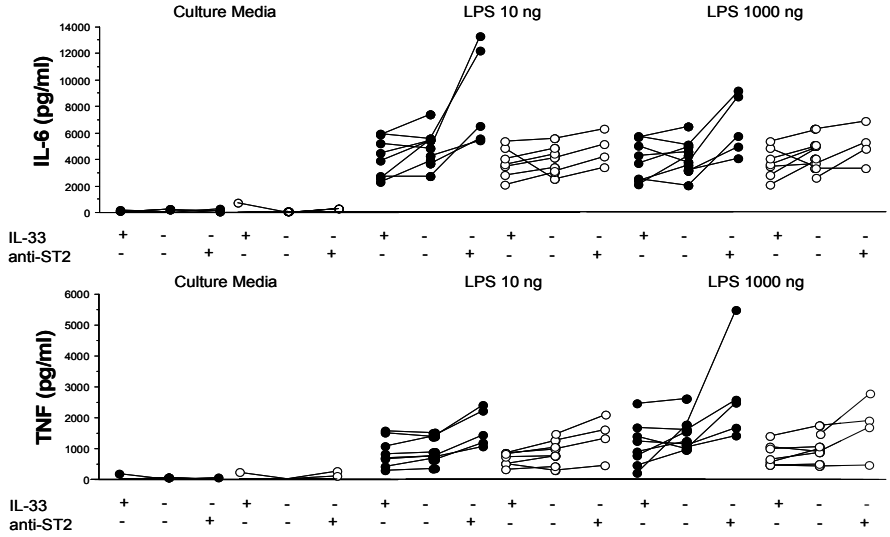

Figure 16 Release of IL-6 and TNF after *ex vivo* stimulations of peripheral blood with LPS in presence or absence of anti-ST2 or IL-33.

Study IV

<u>T-cell cytokine profile in smokers and in farmers - two groups exposed to pathogen-associated molecular patterns on a daily basis</u>

As we observed increased proportion of Th cells producing Th2 cytokines (IL-4 and IL-13) in farmers and smokers in study I, this study was designed to further investigate cytokine profile of Th and Tc cells. We also wanted to investigate if the levels of circulating TGF-β1 are altered in farmers.

In study IV proportion and concentration of blood Th and Tc cells producing IL-4, IL-13, IL-2, IFN-γ and serum levels of TGF- β1 were analysed in 15 non-smoking pig farmers, 15 non-farming cigarette smokers and 15 non-farming, non-smoking healthy controls (table 8).

	Controls	Farmers	Smokers
Female/male	**2/13**	**2/13**	**4/11**
Age Mean (range), years	**52** (26-64)	**45** (22-65)	**53** (41-64)
Duration of work in pig barns Mean (range), years	**0**	**16** (2-35)	**0**
Work in pig barns Mean (range) hours/day	**0**	**3.5** (0.5-8.0)	**0**
Number of pigs in the barn Mean (range)	**0**	**2066** (60-6000)	**0**
Pack-years Mean (range)	**0**	**0**	**32.5** (7-75)
VC (Litre) Mean (95 % CI)	**5.08** (4.70-5.47)	**4.87** 4.21-5.54	**4.69** 4.17-5.20
VC (% predicted value) Mean(95 % CI)	**95** (91-100)	**89** (82-97)	**91** (83-100)
FVC (Litre) Mean (95 % CI)	**5.02** (4.64-5.39)	**4.88** (4.22-5.54)	**4.54** (4.02-5.06)
FVC (% predicted value) Mean (95 % CI)	**96** (91-100)	**91** (83-98)	**90** (81-99)
FEV1 (Litre) Mean (95% CI)	**4.02** (3.69-4.36)	**3.93*** (3.38-4.47)	**3.38** (2.95-3.80)
FEV1 (% predicted value) Mean (95% CI)	**100** (96-105)	**94** (85-103)	**87** (80-95)
FEV1/VC	**0.79** (0.76-0.81)	**0.80** (0.77-0.83)	**0.72*#** (0.68-0.76)
% change after salbutamol Mean (95% CI)	**2.3** (0.9-3.7)	**2.9** (0.6-5.2)	**5.5** (2.2-8.7)

Table 8 Subject characteristics in controls, farmers and smokers. * indicates $p<0.01$ compared with controls. # indicates $p=0.001$ compared with farmers. Data are presented as mean (range) or mean with 95% CI. Reversibility is given as percent increase of pre-bronchodilator values.

Farmers and smokers had increased concentration of blood monocytes compared to controls. Smokers also had increased concentration of blood total leukocytes and lymphocytes compared to controls and increased concentration of blood eosinophils compared to farmers and controls (figure 17). Further smokers had increased concentration of T-cells ($CD3^+$), T-helper cells ($CD3^+CD4^+$) and cytotoxic T-cells ($CD3^+CD8^+$) compared to controls (figure 18). There were no differences in TGF-β1 in serum between the groups (figure 19).

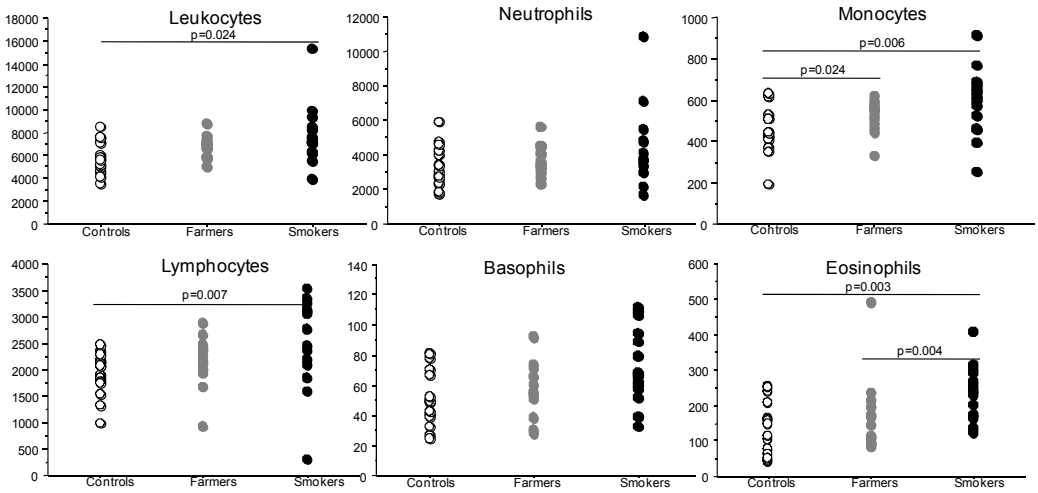

Figure 17 Concentration of blood leukocytes, neutrophils, monocytes, lymphocytes, basophils and eosinophils. Brackets indicate differences between the groups.

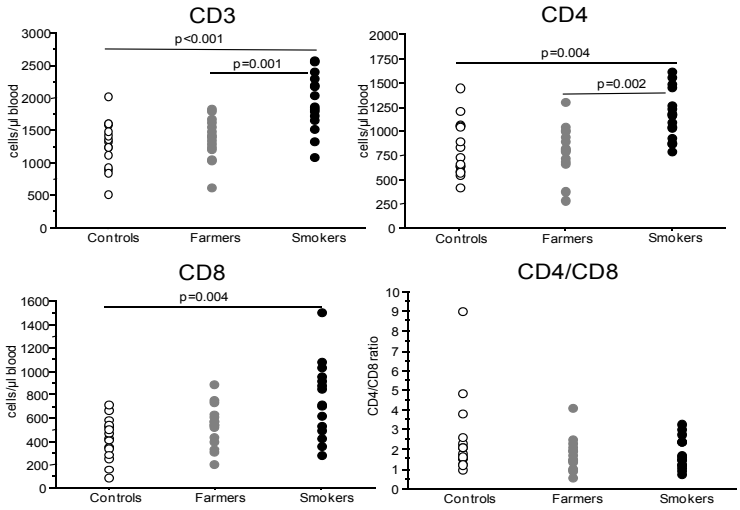

Figure 18 Concentration of blood T-cell subsets, T-cells $CD45^{+}CD3^{+}$, T-helper; $CD45^{+}CD3^{+}CD4^{+}$ and cytotoxic T-cells; $CD45^{+}CD3^{+}CD8^{+}$. Brackets indicate differences between the groups.

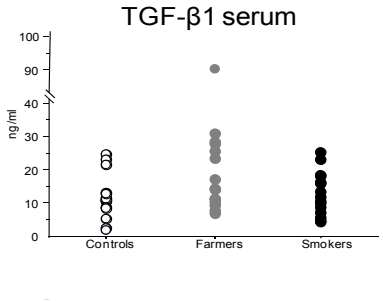

Figure 19 Concentration of TGF- β1 in serum.

Farmers and smokers showed increased proportion and concentration (cells/volume blood) of IL-4 and IL-13 producing T-helper cells in blood compared to non-smoking, non-farming controls (figure 20-21). Smokers also had increased proportion of cytotoxic T-cells producing IL-4 and IL-13 and increased concentration of cytotoxic T-cells producing IL-2, IL-4 and IL-13 compared to controls and farmers (figure 20-21).

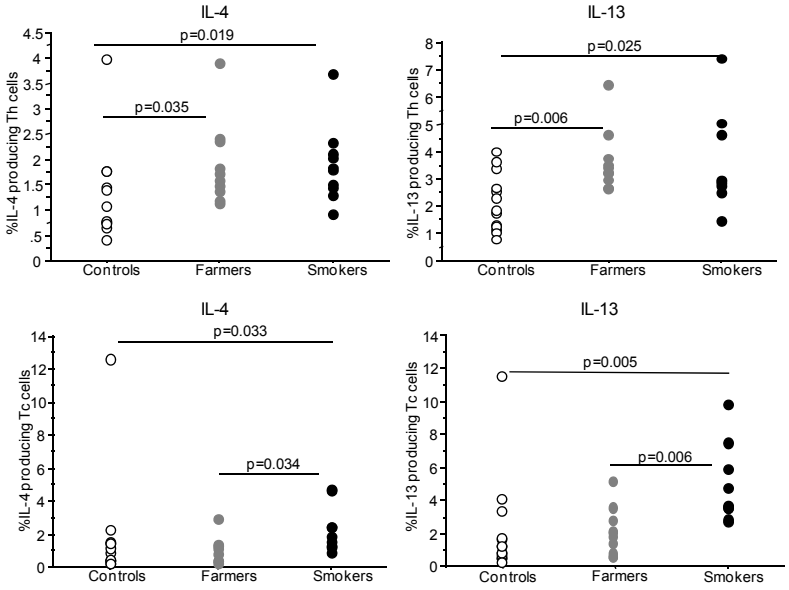

Figure 20 Percentage of peripheral T-helper cells and cytotoxic T-cells producing intracellular IL-4, IL-13. Brackets indicate differences between the groups.

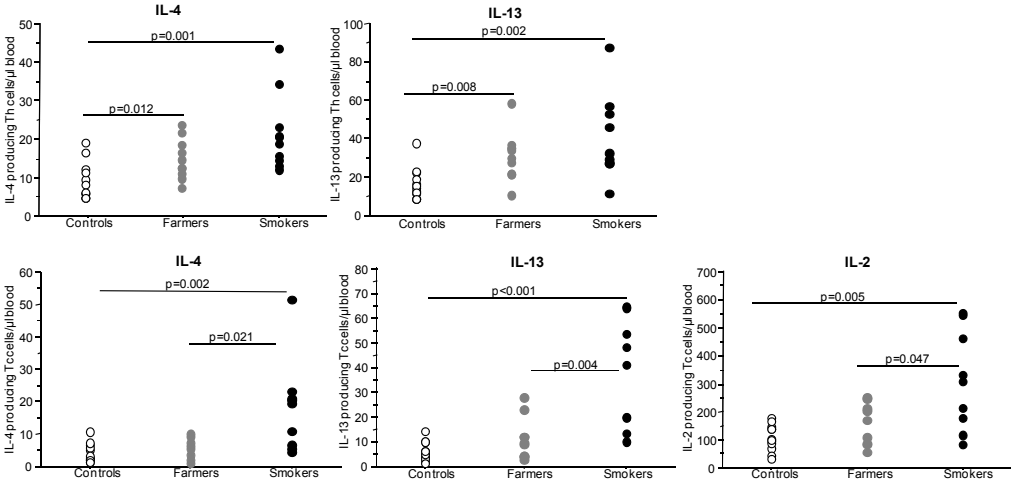

Figure 21 Concentration of peripheral T-helper cells producing intracellular IL-4, IL-13 and cytotoxic T-cells producing intracellular IL-4, IL-13 and IL-2. Brackets indicate differences between the groups.

5 DISCUSSION

There are many possible components in pig barn dust that may be responsible for the acute inflammation observed in healthy naïve subjects following acute exposure. The dust contains various microbial components from microorganisms such as molds, yeast and bacteria. Bacteria are abundant within the dust implying that the content of bacterial component, e g LPS is frequent (116, 117). Inhalation of LPS induces airway inflammation (164) and several occupations apart from pig breeding are known to involve LPS exposure (14, 165). Therefore the influence of LPS on human health is well characterized. LPS are also thought to be a pro-inflammation stimulus after exposure in pig barn facilities (166). Serum levels of IL-6, symptoms, bronchial responsiveness to methacholine ($PD_{20}FEV_1$) and reduction of vital capacity (VC) have shown to correlate with levels of inhalable LPS in healthy subjects exposed in pig barn environment (134). There is also a correlation between total leukocyte concentration, concentration of neutrophilic granulocytes, IL-6 in serum and increase in body temperature, increase of neutrophils in bronchiolar alveolar lavage (BAL) fluid and inhalable levels of measured peptidoglycan, derived from Gram-positive bacteria, in healthy subjects after pig barn exposure (119, 134). Wearing a respirator that reduces the LPS-exposure with 99% attenuates the inflammation but do not influence the increase in bronchial responsiveness in healthy subjects after exposure in a pig barn (125). This indicates that LPS is probably not the sole component causing the immunologic and physiologic responses to pig barn exposure. There are also *in vitro* studies indicating other components than LPS to be responsible for the effect of pig barn dust exposure. Normal human bronchial epithelial cells (NBHC) and macrophages stimulated with dust collected in pig barn facilities was a stronger stimulus for release of IL-8 compared to LPS (127). Similar results have been observed in another *in vitro* study where the response of human airway epithelial cells to pig barn dust exposure was unchanged when LPS was eliminated from the collected dust (133). Our results also indicate that the response to pig barn environment is probably not entirely due to LPS since we observed decreased expression of TLR2, that mostly binds ligands from Gram-positive bacteria, on blood monocytes in pig farmers who are exposed in pig barn facilities on a daily basis, compared to previously unexposed healthy subject and to cigarette smokers. Further, farmers showed attenuated expression of TLR2 after *ex vivo* stimulations with dust collected in a pig barn compared to controls.

It is not known if the decreased expression of membrane bound TLR2 on blood monocytes is involved in the attenuated response to pig barn dust observed in pig farmers (145). However, in the *ex vivo* stimulations with pig barn dust we also observed a decreased release of IL-6 in the farmers compared to controls indicating an adapted response by blood cells.

Muthukuru *et al* showed that monocytes, pre-stimulated with a TLR2 ligand, have decreased expression of membrane bound TLR2 and TLR2 mRNA when re-challenged

with the same ligand compared to not pre-stimulated monocytes (167). In study I we only elucidated the surface expression of TLR protein and it is thereby not clear whether the gene expression of TLR2 also is dampened in pig farmers.

Decreased surface expression may be caused by increased internalization of the receptor. Chai *et al* show that TLR2 expression is decreased on human mononuclear cells stimulated with *A. fumigatus* conidia due to internalization of membrane bound receptor. They further showed that mononuclear cells pre-treated with *A. fumigatus* conidia release less pro-inflammatory cytokines in response to the synthetic TLR2 ligand Pam3Cys compared to cells that were not pre-treated (168).

Decreased TLR2 expression might also depend on increased shedding of membrane bound TLR2 due to exposure. It is known that monocytes releases soluble TLR2 (sTLR2) upon activation and this is probably achieved by post-translational modification of the transmembrane receptor (31). This mechanism is also thought to be involved in negative regulation of TLR2 signaling by acting as an antagonist for the membrane bound receptor. Soluble TLR2 have been shown to attenuate the release of pro-inflammatory cytokines in monocytes stimulated with TLR2 ligands (31, 33) and in mice challenged with Gram-positive-derived components (33). If this is the case also in farmers, that the reduced surface expression of TLR2 is due to increased release of sTLR2, it might also influence the attenuated response in farmers to pig barn environment exposure. We have not yet been able to measure sTLR2 in serum but measurement of sTLR2 in sputum supernatants showed reduced levels of sTLR2 in the farmers compared to non-farming controls. This might be explained by high exposure to inhalable TLR2 ligands that bind to sTLR2 which make sTLR2 undetectable with our ELISA. It is also not clear whether macrophages releases sTLR2 to the same extent as monocytes and further it is not known whether sTLR2 has the same function systemically and locally, within the tissues. The function of sTLR2 is not as well studied as sCD14 that has a negative regulating capacity, in high concentrations (i.e. in plasma) but the opposite function at low concentration within different tissue (35, 37).

It has previously been shown that farmer's children have increased gene expression of TLR2 and CD14 compared to non-farmer's children (169). This is in contrast to what we observed in study I. It is likely that heavy exposure on a daily basis attenuates the expression of TLR2 while substantially lower exposure earlier in life enhances the expression. It would be interesting to elucidate if the adopted TLR2 expression and the attenuated inflammation is reversed when farmers have been absent from work for a period. It would also be interesting to follow PRR expression over time both in farmers and in naïve subjects, as we did not observe any further changes in TLR expression neither after exposure in the pig barn facilities nor after bronchial LPS challenge. We measured PRR expression only at one time point, 7 h after start of the exposure, a time point where it is known that a peak in release of pro-inflammatory cytokines occurs (170). Therefore we cannot exclude that further changes may occur at other time points.

It has been shown that TLR2 on blood monocytes is decreased during and after arterial surgery and reversed one day after the surgery. However, the attenuation observed regarding release of pro-inflammatory cytokines when stimulated *ex vivo* seems to maintain even when the surface expression of TLR2 is reversed and, interestingly, they observed even more attenuated TLR response due to cigarette smoking (171). Droemann *et al* have observed decreased expression of TLR2 on alveolar macrophages in smokers and COPD patients compared to healthy non-smoker, which might be associated with the increased colonization of bacteria commonly occurring in smokers and in patients with COPD (172). It has also been shown that alveolar macrophages from smokers stimulated *ex vivo* with TLR2 ligands, release pro-inflammatory cytokines to a lesser extent than alveolar macrophages from healthy non-smokers (173). This might be due to induction of tolerance caused by repeated exposure to microbial components (i.e. LPS) through the tobacco smoke (148).

However, this is in contrast to our results as we did not observe an attenuated response to pig barn exposure in the smokers and the response to LPS-challenge was even stronger in smokers compared to farmers and controls. However, there are results in line with our observation, showing a stronger response by smokers to LPS-challenge compared to non-smokers (174) indicating that smokers do not develop tolerance to the same extent as pig farmers.

In study I we observed alteration in TLR2 expression but no alteration in TLR4 expression, which might depend on lower exposure to TLR4 ligands. The levels of TLR4 ligands in inhaled pig barn dust may be too low to have any systemic influences. However it might also depend on different regulating mechanism regarding different TLRs. Lauener *et al* found increased gene expression of TLR2 and CD14 but no alteration in TLR4 expression in farmer's children compared to non-farmer's children indicating that TLR4 might be regulated differently than TLR2 and CD14 (169). Further, it is not clear whether membrane bound TLR4 is released from the cell surface as observed with TLR2. Soluble TLR4 protein have not been observed but multiple TLR4 mRNAs, including shorter variants of membrane bound TLR4 have been observed in mice and human (34, 175), indicating that a soluble variant exists but might be directly secreted and not through shedding of membrane bound protein. It has been shown that recombinant sTLR4 have similar properties as sTLR2 by attenuating the capacity to release pro-inflammatory cytokines in macrophages stimulated with LPS *ex vivo* (34). In study III we also observed different expression pattern of TLR2 and TLR4 on neutrophils in blood and sputum. The TLR4 expression on neutrophils was increased in sputum compared to blood whereas the expression of TLR2 was decreased on sputum neutrophils compared to blood neutrophils. This also indicates different regulating function between TLR4 and TLR2.

There are also several intracellular regulating proteins such as IRAK-M and sMyD88 that may influence the TLR response (29). In healthy subjects an intravenous LPS-challenge increased the levels of IRAK-M mRNA and protein in peripheral blood 2-6 h after the injection. At the same time point the TNF-release from peripheral blood cells

was decreased upon re-stimulation with LPS *ex vivo* (53). We cannot exclude that these regulating pathways might be involved in the attenuated inflammation with reduced release of pro-inflammatory cytokines and chemokines in upper airways and systemically observed in pig farmers (145). The reduced release of cytokines and chemokines might be of importance in the attenuated migration of cells observed in farmers after exposure in pig barn (145). However, this might also depend on the alteration in expression of adhesion molecules (i.e. CD62L and CD162) observed in study III. Decreased expression of adhesion molecules indicates an increased activation of blood neutrophils in farmers, as it is known that neutrophils release membrane bound adhesion proteins upon activation (176). It has previously been observed that pig farmers have increased serum levels of soluble L-selectin (sCD62L) compared to healthy subjects previously unexposed in pig barn facilities. The role of soluble adhesion proteins is not clear but it might inhibit migration of inflammatory cells (146).

A healthy workers effect cannot be excluded regarding the attenuated response in farmers, as there may be genetic differences that may influence the response to organic dust. Those who respond less already at the start of exposure in this occupation may be those who stay within the occupation for a longer time. Senthilselvan *et al* showed that non-farming subjects with a TLR4 polymorphism (i.e. TLR4 299/399) shows a less decrease in lung function (FEV_1) compared to non-farming subjects with wild-type TLR4 after exposure in pig confinement building (177). This is just one polymorphism out of several known polymorphisms in TLRs and CD14 (178-180) and these results indicate that the genotype might be of importance for the response to an environment contaminated with organic dust. Results from a study by Vogelzang *et al* indicate that it might be a healthy worker selection for asthma but not for chronic bronchitis. This may be explained by differences in the development of these disorders. Persons with asthma or atopy may not choose to work in a pig barn, and if they do, they may stop work at an early stage because of respiratory symptoms. The development of chronic bronchitis is mostly less dramatic than asthma, and develops gradually over a longer period of time and may thereby not be a cause for leaving the occupation in at early stage (9).

The soluble variant of ST2 (sST2) is elevated in several inflammatory disorders (49, 51, 181) and correlation between severity and morbidity has been observed in disorders such as myocardial infarction and sepsis (51, 52). It has also been shown that serum levels of sST2 strongly increases in healthy subjects challenged with an intravenous LPS injection (53). This response seems to be attenuated in pig farmers regularly exposed in pig barn facilities as increase in sST2 serum levels due to exposure in pig barn and a bronchial LPS challenge was only observed in healthy subjects previously unexposed in pig barn environment and not in pig farmers. It has previously been observed that the release of serum IL-6 is attenuated in pig farmers after exposure in a pig barn compared to previously unexposed subjects (145) and we now observed similar attenuation of sST2 release in serum after pig barn exposure in the farmers compared to controls.

The results with an increase of sST2 only in the controls after LPS-challenge indicate also an attenuated response to LPS and not only to pig barn dust in the farmers. Interesting results were recently reported by de Vos *et al* regarding cross-tolerance of blood cells to multiple TLR ligands after an intravenous LPS injection in healthy subjects (182). They show that blood cells stimulated *ex vivo* after the LPS injection showed attenuated response (release of pro-inflammatory cytokines) to ligands other than TLR4 specific. The blood cells also showed less response to ligands specific for TLR2, TLR5, TLR7 and interestingly also TLR3 that are MyD88 independent (182). This indicates that regardless which TLR ligands being the most potent in pig barn dust, this ligands might also influence the response by other TLRs.

The function of sST2 is not fully clarified but there are studies showing negative regulating functions of sST2 on TLR signaling (i.e. release of pro-inflammatory cytokines) similar to the membrane bound ST2 receptor (54). The results from study III indicate that this might be the case since *ex vivo* stimulations of blood cells from farmers and controls with LPS in the presence of anti-ST2 increased the release of IL-6 and TNF. There were no clear differences in the response between farmers and controls, but a tendency towards reduced response in farmers. This might be explained by the lack of difference in serum sST2 at a basal level between farmers and controls. However, it is known that inflammatory cells such as monocytes express membrane bound ST2 upon activation with pro-inflammatory stimuli such as LPS *in vitro* (53). There might be a difference in expression of membrane bound ST2 between farmers and controls due to regularly exposure to TLR ligands in the farmers but the result from study III indicates that this is probably not the case.

Soluble ST2 is involved in the response to pro-inflammatory stimuli and it has also been shown to be important in allergic inflammation (55, 56). The results from study II indicate that sST2 can be increased due to an acute allergic inflammation and not only to pro-inflammatory exposure since we observed increased serum levels of sST2 in birch allergic individuals, not regularly exposed to organic dust, during birch pollen season. This might be a negative regulating response of acute allergic inflammation since sST2 has been shown to block allergic inflammation in mice models (55). We further observed increased levels in laboratory animal workers that experience respiratory symptoms while working with laboratory animals (LAs) but without allergic sensitization to LA atopy. This increase might depend on high exposure to allergens as well, and might be an early marker for sensitization to LAs. However, it might also be caused by exposure to LPS that are common in LA facilities (13). Symptoms are most frequent among personnel with atopy to LAs but respiratory symptoms to LAs in workers without LA atopy are also common (13). In this context LPS exposure are thought to be the main inducer of the symptoms (13). If the increased levels of sST2 is due to endotoxin exposure it might prevent allergic sensitization depending on the blocking capacities sST2 have shown to have on allergic inflammation (55).

We did not observe increased levels of sST2 in LA atopic individuals as we did in birch allergic individuals and animal workers without LA allergy, which might depend on that the LA allergic individuals are conscious about their allergy, and thereby protect themselves to exposure to a higher extent than individuals without allergy. Animal workers are also regularly exposed to allergens compared to birch pollen atopics that are exposed during a quite short period, once a year. Regular exposure might reduce allergic inflammation by induction of tolerance in similar to what is observed in specific immunotherapy (SIT) to allergens (183).

The CD14 receptor are essential for LPS response (184) and as we did not observe any differences in CD14 expression between birch pollen atopics and controls the increased expression of CD14 on monocytes in personnel who experienced respiratory symptoms while working in laboratory animal facilities, especially among those without allergy to LAs further indicates LPS exposure. The results of induced CD14 expression on blood monocytes in animal workers were in contrast to the observation in farmers in whom no increase in CD14 was found at baseline. However, after exposure in pig barn there was a tendency towards increased CD14 expression in the farmers but not in the other two groups. Further, after *ex vivo* stimulations of blood with LPS there was a dose dependent increase in CD14 expression in both farmers and controls but the increase in farmers was significantly higher in the farmers compared to controls. This indicates a stronger response regarding CD14 surface expression in the farmers than in the controls, probably due to regular exposure to LPS.

The results from the present studies indicate that regular exposure in pig barn facilities as well as tobacco smoking have the ability to alter adaptive responses since we observed increased levels of T-cells producing IL-13 and IL-4 in smokers and pig farmers, in whom the proportion of IL-13 producing cells also correlated with the duration of work in pig barn facilities, compared to non-smoking non-farming controls. Is has previously been shown that cigarette smoking favors a Th2 profile in blood (152, 153). T-helper 2 cells and cytokines are known to be elevated and involved in patho-physiology of asthma (185, 186). It has also been shown that the proportion of cells expressing IL-4 and IL-13 is elevated in the airways of smokers with chronic bronchitis compared to asymptomatic smokers (187). The prevalence of chronic bronchitis is high in both farmers and smokers (7) and this airway disorder is, as asthma, associated with tissue remodeling and increased mucus production (103, 188, 189). Goblet cell hyperplasia and metaplasia are characteristic features in asthma and chronic bronchitis, and are an important cause for the increased mucus production seen in these diseases (103, 188-191). Interleukin-4 and especially IL-13 seems to have essential roles in the pathogenesis of goblet cell hyperplasia and metaplasia (189, 192). The most important pathway in transcription of mucus (i.e. MUC5AC the main mucus produced by goblet cells) seems to be IL-13 signaling through IL-4Rα (189). Interleukin-13 has been shown to induce goblet cell metaplasia and hyperplasia and increase transcription of MUC5AC in normal human bronchial epithelial (NHBE) cells (154, 193).

T-helper 2 cytokines have also been shown to be involved in fibrosis, another characteristic feature in tissue remodeling, and IL-13 seems to be more important than IL-4 also in this respect (155, 194, 195). Transgenic mice that overexpress IL-13 in the lung induce goblet cell hyperplasia but also subepithelial fibrosis and smooth muscle hypertrophy and hyperplasia (155).

Pig farming environment seems to favor a Th2 profile in blood, however not in the same extent as tobacco smoke. Smoking seems to be much more potent in favoring IL-4/IL-13 profile since increased levels of cytotoxic T-cells producing IL-4 and IL-13 was only observed in smokers and not in farmers. There are studies showing that the incidence of Th1 mediated hypersensitivity reaction to inhaled allergens (i.e. allergic alveolitis) is rare among cigarette smokers indicating an attenuated Th1 response as well (196, 197).

It is likely that farmers and smokers have increased levels of IL-13 producing cells also in the airways. This may be of importance in the pathogenetic mechanisms in development of chronic bronchitis in both farmers and smokers. To our knowledge there are no studies in which goblet cell hyperplasia or metaplasia in the airways have been investigated in pig farmers. However, in a study by Pedersen *et al* it was shows that pig farmers have increased inflammation in lower airway mucosa with oedema and increased phlegm compared with non-farmers (142). Further, it has been shown that the number of mucus producing cells in the bronchiolar wall is increased in rats after repeated exposure in pig barn facilities (198).

The mechanism behind the increased levels of T-helper cells producing IL-13 and IL-4 is not clear. It is known that DCs have an essential role in the development of Th1 and Th2 cells and it has been shown that DCs repeatedly exposed to TLR ligands express low or non-detectable levels of IL-12, an important cytokine for polarization of Th1 cells (199). This indicates that repeated exposure to TLR ligands might favor Th2 polarization of naïve T-helper cells. It has been shown that cigarette smoke extract also have this influence on DCs with decreased IL-12 production leading to induction of Th2 priming and polarization (200). This indicates that altered function of DCs might be of importance in pig farmers and smokers.

It is likely that repeated exposure to PAMPs also influences the phenotype of macrophages. It has been shown that macrophages from smokers and alveolar murine macrophages stimulated with cigarette smoke extract *ex vivo* releases pro-inflammatory cytokines to a less extent when further stimulated with TLR Ligands (173). This might be due to changed phenotype into more anti-inflammatory macrophages (M2s) known to be involved in wound healing and tissue remodeling (201). Increased number of M2 in the airways might further influence development of chronic airway disorders as chronic bronchitis. If proportion of M2 in the airways are increased in smokers and pig farmers is not known.

The inflammation caused by acute exposure in pig barn environment is dominated by neutrophils. Pig farmers who are exposed in a pig barn on a daily basis show a reduced

recruitment of neutrophils after exposure in pig barn compared to previously unexposed subjects (145). However, it has been shown that pig farmers have increased concentration of neutrophils in BAL-fluid (141) and in study I it was observed that pig farmers had increased concentration of neutrophils also in peripheral blood compared to previously unexposed healthy subjects. Activated neutrophils releases proteases such as neutrophilic elastase, cathepsin G and MMPs known to be tissue destructive (202). This indicates that neutrophils likely have an essential role in the development of respiratory symptoms and chronic airway diseases such as chronic bronchitis frequently occurring in pig farmers.

6 CONCLUSIONS

In the present studies it was demonstrated that occupational exposure to organic dust containing high levels of pathogen-associated molecular patterns (PAMPs) alters expression of PRR, release of sST2 in serum and cytokine profile of T-helper cells at basal levels but also in response to pro-inflammatory stimuli *in vivo* and *ex vivo*.

Pig farmers, who are, exposed in pig barn facilities on a daily basis with high levels inhalable organic dust including PAMPs and smokers, regularly exposed to LPS through the tobacco smoke, showed increased concentration of blood neutrophils and monocytes compared to non-smoking non-farming controls. Smokers also showed elevated levels of IL-6 in serum compared to controls. Farmers also had decreased number of macrophages in sputum compared to controls. This is probably sign of ongoing inflammation due to regular exposure to organic material.

After exposure in a pig barn and a bronchial LPS-challenge the concentration of monocytes and neutrophils increased in controls, farmers and smokers. However, the increase in neutrophils after LPS-challenge was higher in smokers compared to controls and farmers. Smokers also had higher increase of IL-6 in serum after LPS-challenge compared to controls and farmers. The results indicate that smokers do not develop tolerance according to the same mechanism as do pig farmers.

Farmers showed attenuated release of IL-6 both after *in vivo* exposure (pig barn exposure) and after *ex vivo* stimulations of blood with pig barn dust compared to healthy non-farming controls. Farmers also had attenuated release of sST2 in serum after exposure in a pig barn and after a LPS-challenge compared to non-farming healthy controls. This alteration in release of IL-6 and sST2 is probably due to development of tolerance to pig barn environment and in some extent to LPS.

Personnel experience symptoms while working in laboratory animal facilities showed increased expression of CD14 on blood monocytes, especially among personnel without allergy to laboratory animals compared to birch atopics and healthy controls not exposed in laboratory animal facilities. Increased CD14 expression might be caused by exposure to LPS in the animal facilities, indicating that LPS might cause or influence experienced respiratory symptoms.

Birch pollen atopics, not exposed in animal facilities, had increased levels of sST2 in serum compared to controls. This might be due to acute allergic inflammation. Increased levels of serum sST2 were also observed in laboratory animal workers

without allergy to laboratory animals. This might be due to exposure to high levels of allergens and may be an early marker for sensitization. It might also be a marker for LPS exposure, which may prevent allergic sensitization.

Pig farmers also showed decreased expression of TLR2 on blood monocytes compared to smokers and healthy non-farming controls and had attenuated TLR2 expression on blood monocytes when stimulated *ex vivo* with dust collected in pig barn facilities compared to non-farming controls. Controls showed increased expression of TLR2 on blood neutrophils after LPS-challenge, which was not observed in pig farmers. This might be caused by repeated exposure to TLR2 ligands known to be frequently occuring in pig barn environment and might be involved in the attenuated response to pig barn exposure observed in pig farmers exposed in pig barn facilities on a daily basis.

Farmers showed decreased expression of adhesion molecules (i.e. CD62L and CD162) compared to controls. The decreased expression of adhesion molecules might be involved in the reduced post-exposure recruitment of inflammatory cells previously observed in pig farmers.

Farmers had lower concentration of sTLR2 and sCD14 in sputum compared to controls. The expression of CD14 on sputum neutrophils was attenuated in farmers compared to controls.

Ex vivo stimulations of blood with LPS in presence of anti-ST2 increased the release of IL-6 and TNF in controls and farmers with a tendency towards even higher release in controls compared to farmers. *Ex vivo* stimulations in presence of IL-33 reduced the release of IL-6 and TNF in famers and controls. Suppression of tumorgenicity 2 seems not be involved in the attenuated response to pig barn exposure in pig farmers.

Farmers and smokers had increased proportion and concentration of blood Th cells producing IL-4 and IL-13 compared to controls. Proportion of Th cells producing IL-13 correlated with years working in pig barn facilities. After exposure in pig barn and a bronchial LPS challenge the proportion of Th cells producing IL-4 and IL-13 increased in controls but remained high in farmers and smokers. Farming environment and smoking thus seem to favor a Th2 profile. However, smoking seems much more potent in this respect as smokers also had increased proportion and concentration of blood cytotoxic T-cells producing IL-4 and IL-13 compared to farmers and controls. This increase of Th2 cells might be of importance in development of chronic bronchitis, a condition that is frequently occurring among farmers and smokers compared to the population in general.

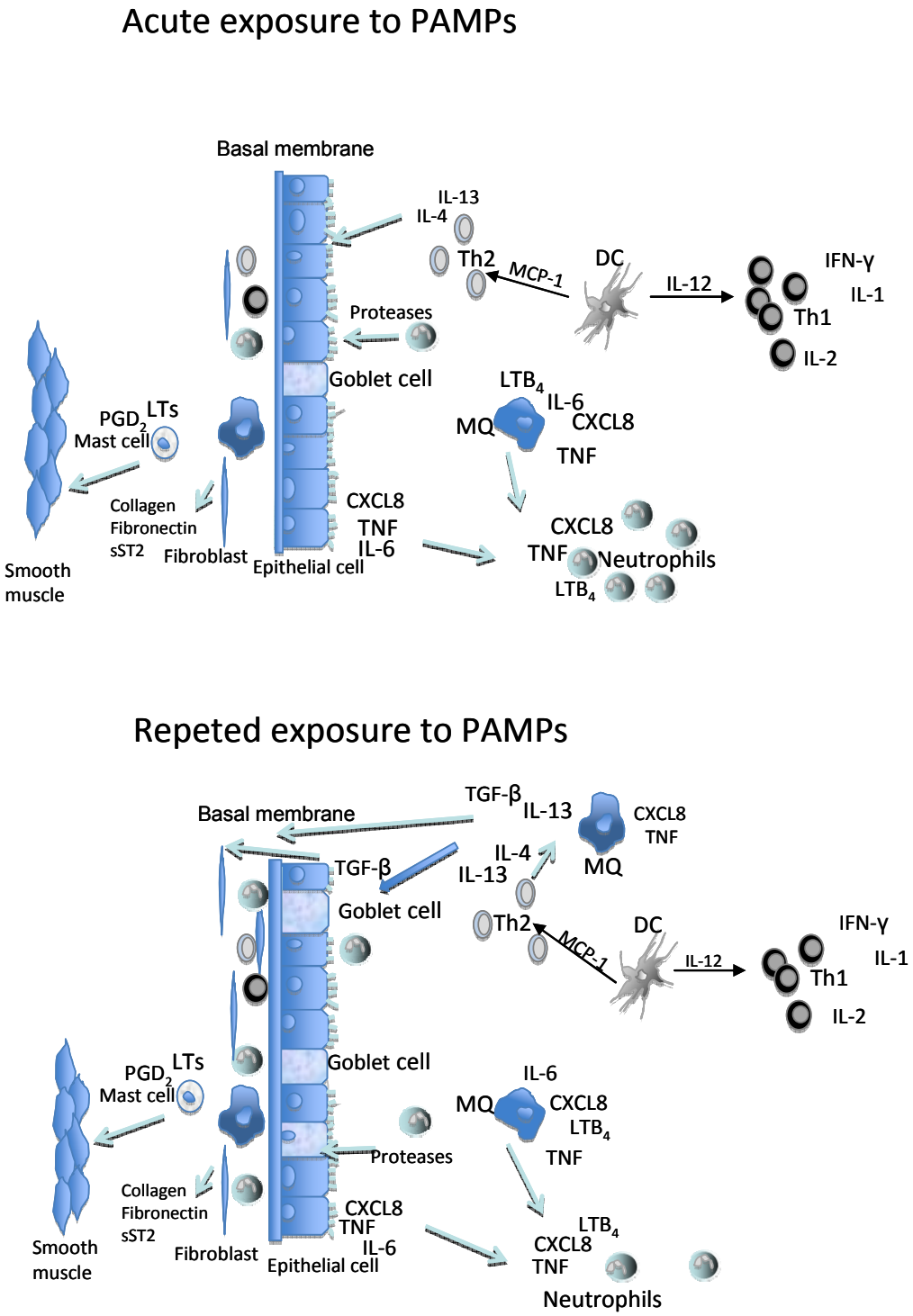

Figure 22 Possible inflammatory mechanisms due to acute or repeated exposure to pathogen-associated molecular patterns (PAMPs).

7 SVENSK SAMMANFATTNING

Flertalet arbetsmiljöer är kraftigt förorenade med organiskt damm. Detta gäller i hög grad svinstallar där grisarna går inne året runt, vilka är kraftigt nersmutsade med luftburet inhalerbart organiskt damm. Dammet är en komplex blandning av rester från hö, foder, avföring, hudavlagringar från grisarna och innehåller även stora mängder av mikroorganismer som bakterier och mögelsvampar samt produkter från dessa organismer. Dessa mikrobiella produkter aktiverar inflammatoriska celler via specifika receptorer kallade *pattern recognition receptors* (PRRs). Friska försökspersoner (kontroller) som tidigare aldrig exponerats i svinhusmiljö utvecklar en kraftig akut inflammation i övre och nedre luftvägar med systemisk påverkan efter några timmars vistelse i svinhus. Antalet inflammatoriska celler, framförallt neutrofila granulocyter ökar upp till 100 ggr i lungsköljvätska (BAL), 20 ggr i nässköljvätska (NAL) och omkring två gånger i blod som följd av exponeringen. Frisättning av inflammatoriska cytokiner som interleukin (IL)-1, IL-6 och tumörnekrotisk faktor (TNF) ökar i BAL, NAL och i perifert blod. Symtom i samband med exponering, t ex hosta, trånghetskänsla i bröstet, feber och muskelvärk, liknar de som är vanliga vid influensa. Hos bönder, som regelbundet exponeras i svinhus är denna kraftiga reaktion svagare än hos tidigare oexponerade kontrollpersoner. Bönderna tycks utveckla någon form av tolerans mot svinhusmiljön. Det är inte klarlagt hur denna adaption påverkar lantbrukarnas hälsa men symtom från luftvägarna som hosta och trånghetskänsla i bröstet är vanliga bland svinbönder och prevalensen av kronisk bronkit är högre hos bönder än hos befolkningen i övrigt. Det finns även flera studier i vilka en ökad prevalens av kroniskt obstruktiv lungsjukdom (KOL) visats hos bönder.

Att arbeta med försöksdjur inom biomedicinsk forskning och inom industrin innebär hög exponering för potenta allergener från försöksdjuren men även för mikrobiella agens som lipopolysackarider (LPS en beståndsdel i cellväggen på Gram-negativa bakterier) vilket är vanligt förekommande i svinhusmiljö. Luftvägssymptom förekommer i hög utsträckning bland försöksdjursarbetande personal och upp mot hälften av all personal upplever symtom från luftvägarna vid arbete med försöksdjur. Incidensen av försöksdjursallergi bland försöksdjursarbetande personal är cirka 20%.

Syftet med denna avhandling var att studera påverkan av regelbunden yrkesexponering av organiskt damm på det specifika och ospecifika immunförsvaret. Specifikt studerades uttrycket av PRRs, T-cellers cytokinprofil och frisättning av lösligt *suppression of tumorgenicity 2* (sST2).

I det första delarbetet var syftet att studera inverkan av regelbunden exponering för organisk damm på uttrycket av toll-lika receptorer (TLRs) som TLR2 och TLR4 och uttrycket av CD14 på neutrofila granulocyter och monocyter och T-hälparcellers cytokinprofil i blod, före och efter exponering i svinhus och efter en bronkialprovokation med LPS. En grupp av icke-rökande svinbönder, en grupp med rökare (som är regelbundet exponerade för LPS via tobaksröken) samt en grupp med icke-rökande friska kontroller tidigare oexponerade ingick i studien. Vidare var syftet att studera frisättning av inflammatoriska cytokiner och uttrycket av toll-lika receptorer på blodceller från bönder och kontroller efter stimulering *ex vivo* med damm samlat i svinhus och med LPS.

Bönderna och rökarna visade tecken på systemisk inflammation med ökad koncentration av neutrofila granulocyter i blod redan före exponering vid jämförelse med kontrollgruppen. Rökare hade även högre koncentration av IL-6 i blod i jämförelse med icke-rökande kontroller. Bönderna uppvisade ett nedsatt uttryck av TLR2 på monocyter i blod samt ett nedsatt uttryck av TLR2 och lägre frisättning av cytokiner efter *ex vivo* stimuleringar av blodceller med LPS och svinhusdamm i jämförelse med den kontrollgruppen. Vidare hade bönder och rökare ökad proportion av T-hjälparceller som producerar IL-4 och IL-13 utav den totala populationen av T-hjälparceller i jämförelse med kontroller. Efter exponering i svinhus och bronkialprovokation med LPS ökade proportionen IL-4- och IL-13-producerande celler hos kontrollerna men förblev oförändrat hos bönder och rökare.

I delarbete II studerades uttrycket av TLR2, TLR4 och CD14 på monocyter och neutrofiler i blod, aktiveringsmarkörer på B- och T-celler samt halten lösligt ST2 och CD14 i serum hos personal med upplevda luftvägssymptom vid arbete med försöksdjur, inkluderade en grupp med och en grupp utan försöksdjursallergi. En grupp med björkpollenallergi under björkpollensäsong och en icke-rökande frisk kontrollgrupp inkluderades även.

Djurarbetande personal, speciellt de utan försöksdjursallergi visade högre uttryck av CD14 på blodmonocyter i jämförelse med kontrollgrupperna. Björkpollenallergiker och djurarbetare utan allergi mot försöksdjur visade även högre halt av lösligt ST2 i blod i jämförelse med friska kontroller.

I delarbete III var syftet att studera hur regelbunden exponering i svinhusmiljö påverkar uttrycket av PRR (TLR2, TLR4 och CD14) och adhesionsmolekyler (CD11b, CD62L och CD162) på neutrofiler i blod och sputum. Syftet var även att studera halten av lösligt CD14, TLR2 i blod och sputum och lösligt ST2 i blod före och efter exponering i svinhus och efter bronkialprovokation med LPS. Ett tredje syfte var att studera frisättning av inflammatoriska cytokiner efter stimulering av blod med TLR ligander i frånvaro och närvaro av anti-ST2 och IL-33. I studien inkluderades en grupp med icke-rökande svinbönder och en grupp med icke-rökande kontroller som tidigare aldrig exponerade i svinhusmiljö.

Bönderna hade lägre uttryck av CD62L och CD162 på neutrofiler i blod och lägre uttryck av CD14 på neutrofila granulocyter i sputum i jämförelse med kontroller. Vidare hade bönderna lägre halter med lösligt TLR2 och CD14 i sputum i jämförelse med kontroller. Efter exponering i svinhus och efter bronkialprovokation med LPS ökade halten av lösligt ST2 i kontrollgruppen, något som inte kunde påvisas hos bönderna.

I delstudie IV var syftet att studera hur regelbunden exponering i svinhusmiljö påverkar cytokinprofilen hos T-hjälparceller och cytotoxiska T-celler och halten av tumörnekrotisk faktor (TGF)-β1 i blod i en grupp med icke-rökande svinbönder, en grupp med rökare och en grupp med icke-rökande friska kontroller tidigare oexponerade i svinhusmiljö.

Svinbönder och rökare påvisade högre proportion och koncentration av T-hjälparceller som producerar IL-4 och IL-13 i jämförelse med icke-rökande kontroller. Rökare hade även högre proportion av IL-4- och IL-13-producerande cytotoxiska T-celler och ökad

koncentration av IL-2-, IL-4- och IL-13-producerande cytotoxiska T-celler i jämförelse med bönder och icke-rökande kontroller.

Resultaten i dessa studier tyder på att kronisk exponering påverkar immunsystemet vad gäller uttryck av TLR och cytokinprofil hos T-celler. Detta förändrade inflammatoriska svaret kan vara av betydelse i utveckling av kroniskt inflammatoriska luftvägssjukdomar, framförallt kronisk bronkit, som är vanligt förekommande hos både rökare och bönder.

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