1997:23

Acute Cytokine Responses to Inhaled Swine Confinement Building Dust

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ARBETE OCH HÄLSA VETENSKAPLIG SKRIFTSERIE
ISBN 91–7045–445–0 ISSN 0346–7821

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ARBETE OCH HÄLSA
Redaktör: Anders Kjellberg
Redaktionskommitté: Anders Colmsjö
och Ewa Wigaeus Hjelm

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Arbetslivsinstitutet,
171 84 Solna, Sverige

ISBN 91–7045–445–0
ISSN 0346-7821
Tryckt hos CM Gruppen

献给

我的父母，
丈夫，
儿子。

To my family and my parents

List of original papers

This thesis is based on the following papers which will be referred to by their Roman numerals:

I. Zhiping Wang, Per Malmberg, Per Larsson, Britt-Marie Larsson, Kjell Larsson. **Time course of IL-6 and TNF-α in serum following inhalation of swine dust.** Am J Respir Crit Care Med 153:147-152 1996

II. Zhiping Wang, Kjell Larsson, Lena Palmberg, Per Malmberg, Per Larsson, Lennart Larsson. **Inhalation of swine dust induces cytokine release in the upper and lower airways.** Eur Respir J 10: 381-387 1997

III. Zhiping Wang, Per Malmberg, Britt-Marie Larsson, Kjell Larsson, Lennart Larsson, Anita Saraf. **Exposure to bacteria in swine house dust and acute inflammatory reactions in humans.** Am J Respir Crit Care Med 154: 1261-1266 1996

IV. Zhiping Wang, Alli Manninen, Per Malmberg, Kjell Larsson. **Inhalation of swine dust increases the concentrations of interleukin-1 beta (IL-1ß) and interleukin-1 receptor antagonist (IL-1ra) in peripheral blood.** (submitted to J Allergy & Clin Immunology)

V. Zhiping Wang, Per Malmberg, Lena Palmberg, Alexandra EK, Kjell Larsson. **Swine dust induces cytokine secretion from epithelial cells and human alveolar macrophages.** (submitted to Eur Respir J).

Abbreviations

AM	Alveolar macrophage
BAL	Bronchoalveolar lavage
BHR	Bronchial hyperresponsiveness
CD	Cluster of differentiation (e.g CD14 on macrophage)
CRP	C- reactive protein
CV	Coefficient of variation
EDTA	Ethylene diamine tetraacetic acid
EASIA	Enzyme amplified sensitivity immunoassay
ELISA	Enzyme-linked immunosorbeant assay
FEV_1	Forced expiratory volume in one second
FCS	Fetal calf serum
GC-MS	Gas chromatography mass spectrometry
GM-CSF	Granulocyte/macrophage colony-stimulating factor
G+	Gram-positive stain
G-	Gram-negative stain
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
ICAM-1	Intercellular adhesion molecule-1
IFNγ	Interferon-γ
kDa	Kilodalton
LAL	*Limulus* amebocyte lysate assay
LBP	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
MuAc	Muramic acid
NAL	Nasal lavage
ODTS	Organic Dust Toxic Syndrome
OD	Optical density
PAF	Platelet-activating factor
PBMC	Peripheral blood mononuclear cells
PD20 FEV1	Cumulated methacholine dose causing a 20% decrease in FEV_1
RANTES	Factor regulated upon activation in normal T-cell, expressed and secreted
SD	Standard deviation
SEM	Standard error of the mean
Swine dust	Swine confinement building dust
VC	Vital capacity
TNF-α	Tumor necrosis factor alpha
3-OH	3-hydroxylated

Content

1. Introduction

1.1. Survey of ODTS

Organic Dust Toxic Syndrome (ODTS) is a term applied to "mill fever, grain fever, inhalation fever, humidifier fever and toxic pneumonitis". In ODTS, there is typically an acute febrile condition, following inhalation of organic dust with transient fever, muscle aches, chest-tightness, dyspnoea and other influenza-like symptoms (1-3). The ODTS reaction does not require pre-sensitization. Diagnostic chest x-ray is usually normal. The symptoms usually disappear within 24 to 48 h and there are no remaining sequelae.

The dusts associated with ODTS include swine products, hay, straw, grain, wood chip, moldy silage and haylage, and other moldy material, mostly present in an agricultural environment (2, 4-9)

The cumulative prevalence of ODTS is 6 - 8 % among farmers (10). The occurrence of ODTS is more common among swine producers and grain workers, affecting up to 30% of the exposed subjects (11, 12). These rates are 30-50 times higher than that of allergic alveolitis (13).

Diagnosis of ODTS is usually based upon a number of factors, with an appropriate temporal exposure history being the most important. Symptoms are directly related to exposure levels. The syndrome is often misdiagnosed as acute hypersensitivity pneumonitis (HP, also called extrinsic allergic alveolitis) since symptoms are similar. HP is a pulmonary illness resulting from an immunologic reaction against a variety of inhaled environmental antigens. There are many differences between ODTS and HP: the chest x-ray does not show infiltrates; severe hypoxemia does not occur; prior sensitization to antigens in the organic dust is not required; and there are no known sequelae of physiological significance, such as the increased sensitivity to dust and pulmonary fibrosis. The differences are given in table 1 (14-16).

Exact agents and mechanisms associated with ODTS are still not clear; however, it has been suggested that the reaction can be caused by endotoxin, fungi, bacteria and possibly other agents in the dust (3, 15, 17).

Table 1. Similarities and differences between HP and ODTS

	HP	ODTS
Synonymous	farmers' lung mushroom workers' lung bark strippers' disease Allergic alveolitis	grain fever silo unloaders' disease inhalation fever toxic pneumonitis
Exposure level	low or high	high
Latency	4-8 hours	4-12 hours
Symptoms	fever, chills, cough, dyspnea	fever, chills, cough, myalgias, dyspnea
Occurs in cluster	No	Common
Duration of illness	Usually less than 24 hours	12-36 hours
White blood count neutrophilia	Leukocytosis, neutrophilia	Leukocytosis,
Pulmonary function	Restrictive defect may be severe	Normal or mild restrictive
Chest examination	Crackles	Clear (rarely rhonchi)
Chest radiograph	Often abnormal interstitial pattern	Usually normal
Serology	Usually positive	Usually negative
BAL	Mononuclear cells	Neutrophils
Lung histology	Lymphocyte and plasma cell reaction, granulomas	Polymorphonuclear reaction predominant
Pathogenesis	Hypersensitivity to fungal or bacterial antigens	Nonspecific inflammatory reaction to endotoxin
Treatment	Glucocorticoids	Supportive
Progressive disease	Can occur	None
Risk of recurrence	Likely on any exposure to antigen	Likely only after new heavy exposure to organic dust
Prevention	Absolute avoidance of exposure to causative antigen	Dust mask, avoidance of heavy exposure

1.2. Animal Confinement Building

Swine are usually bred in confinement buildings with many swine which generate high dust levels. Intensive swine housing began in Europe and Sweden in the early 1960's. The confinement building are self-contained structures that are fully enclosed and that usually have minimally effective ventilation systems. Swine are housed in individual pens, are fed through an automatic feeding system that generates respirable grain dust, and stand on a slotted floor so that their urine and faeces can collect in manure canals, leading to manure pits outsides the building. The pigs are therefor exposed directly manure gases like NH_3, H_3S and CH_4 also (18).

1.3. Swine Dust

Swine dust is a very complex substance containing components of both animal and plant origin, as well as microbial constituents and microbial metabolites.

Animals contribute to the composition of organic dust primarily by their shedding of skin, gut epithelium and microorganisms, or by their excretion of

faecal waste products (19). Material of plant origin in swine dusts comes from animal feed, and straw used as bedding material.

In the confinement house, there are also irritating gases, such as hydrogen sulphide, carbon dioxide, carbon monoxide, ammonia, and methane. In addition to having direct toxic or allergenic properties, these products also serve as a substrate for the growth of microorganisms. Airborne microorganisms in animal houses come from several different sources, including moldy feed, bedding, the animals themselves, and their excreta. The animals yield airborne skin scales, which may carry bacteria, while urine and faecal material may form aerosols also carrying Gram-negative bacteria (20-24). Microorganisms in pig houses are listed in table 3.

Table 2. Swine dust components (25, 26).

Swine dander
Animal hair
Urine, mites, or their parts
Bacteria
Bacterial endotoxin
(1-3) ß-D-glucan in feed
Microbial protease
Pollen grains
Particles of plants
Feed grains
Hay
Silage
Fungal spores
Hyphae or sporangia from decomposing organic material
Mycotoxins

Table 3. Pig house microorganisms (culturable)

Bacteria	**Molds**	**Yeasts**
Aerococcus viridans	*Acremonium*	*Candida*
Bacillus spp	*Aspergillus*	*Cryptococcus*
Escherichia coli	*Alternaria*	*Hansenula*
Klebsiella	*Circinella*	*Rhodotorula*
Micrococcus lylae	*Cladosporium*	*Trichosporon*
Pseudomonas	*Fusarium*	*Torulopsis*
Rhodococcus	*Geotrichum*	*Prototheca*
Staphylococcus lentus	*Mucor*	
S.hominis	*Paecilomyces*	
Xanthomonas	*Penicillium*	
Yersinia	*Rhizopus*	
	Scopulariopsis brevicaulis	
	Stemphylium	

Measurements of bacteria in swine houses were reported by Crook and Kiekhaefer (22, 24). In pig houses in southern Sweden, total bacteria were 2.3 to 3.6 * 10^5 cfu/m^3 air, Gram-negative bacteria were 6.5 to 11.0 *10^4 cfu/m^3, and fungi 95 to 410 cfu/m^3. The majority of the bacteria (68 - 96%) were Gram-positive enterococci. About 25% of Gram-negative bacteria were on particles with an aerodynamic diameter <5 μm (21).

1.4. Bacteria Cell Wall

The cell membrane is very similar in both G+ and G- bacteria. The external cell wall structure differs greatly between the two type of organisms (figure 1). G+ bacteria are distinguished by a cross-linked, large peptidoglycan at the surface, outside the lipid cell membrane. G- bacteria have only a thin peptidoglycan wall, but they have an additional outer cell membrane with endotoxin (LPS) on its surface. The LPS molecule consists of lipid A (which is highly conserved and is responsible for most of toxicity of LPS), the core of oligosaccharides (which are also well conserved) and the outer O-antigen or the surface of the oligosccharide (which is structurally and antigenically diverse between strains) (27, 28).

Gram-positive bacteria can cause inflammation in two ways: by secreting a toxin (e.g superantigens, streptolysin O, S.*aureus* a toxin), or via servial components of the cell wall. LPS is the primary initiator of Gram-negative inflammation. LPS stimulates various inflammatory cells (macrophages or polymorphonuclear) to produce cytokines, which in turn results in a cascade of events leading to inflammation or sepsis (29, 30).

Gas chromatography mass spectrometry (GC-MS) analysis has been developed for quantifying and characterising airborne microorganisms (31, 32). Muramic acid (MuAc) is a chemical marker for bacterial peptidoglycan and 3-hydroxylated fatty (3-OH fatty) acid is a marker for total LPS, which is found in lipid A, the toxic part of the endotoxin. The GC-MS gives quantitative results and enables the characterisation of the microbiological composition of the air filter sample (33).

1.5. Cytokines

Cytokines are a group of low-molecular weight (<80 kDa) regulatory glycoproteins secreted by white blood cells and a variety of other cells in response to a number of inducing stimuli. They are mediators of short range signals between cells. Cytokines are extremely biologically active compounds acting at concentrations as low as 10^{-15} to 10^{-10} mol/L in an autocrine, paracrine, or endocrine fashion via high affinity specific receptors to stimulate target cell functions (34).

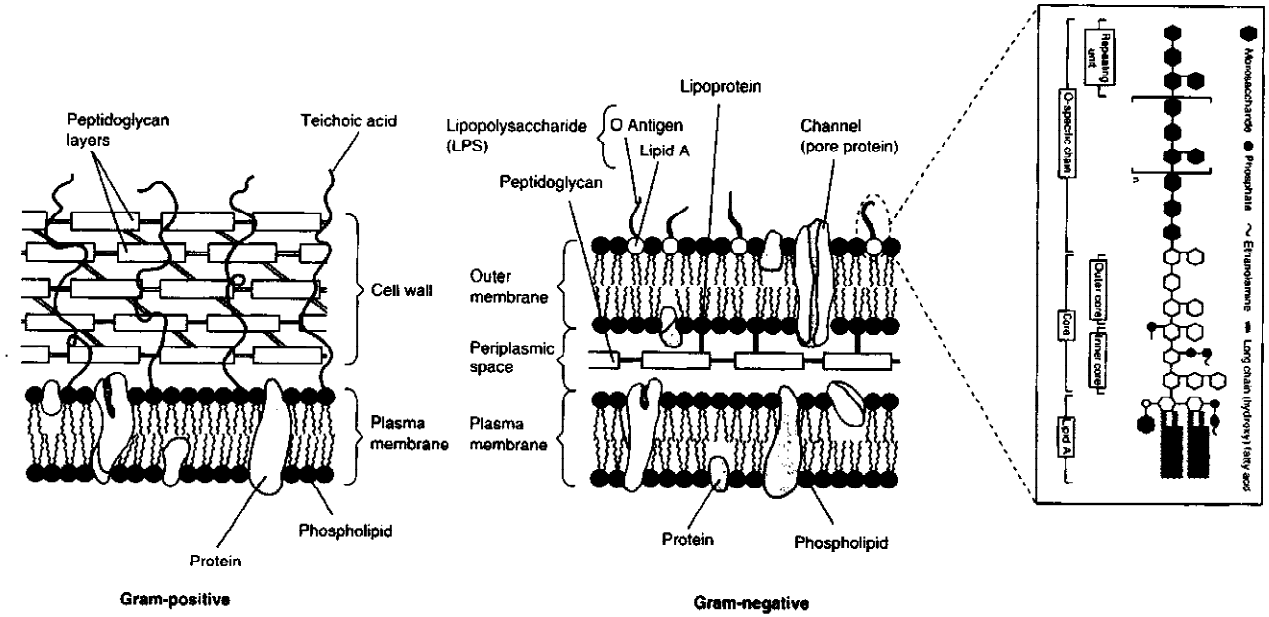

Figure 1. Comparison of the cell wall structures of Gram-positive and Gram-negative bacteria, with an inset showing the structure of lipopolysaccharide. Modified from Microbiology, Second Edition. Tortora et al. 1986, The Benjamin/Cummings Publishing Company.

Cytokines manifest an incredible array of biological effects which frequently overlap. A key feature of many of them is their pleiotropy, redundancy, synergy and antagonism, by which they can regulate cellular activity in a coordinated interactive way.

Cytokines may be roughly grouped into pro-inflammatory (i.e. IL-1, IL-6, IL-8, IL-10, IL-12, TNF-α, TGF-ß), immuno-regulatory (i.e. IL-1, IL-2, IL-4, r-IFN, IL-6, IL-7, IL-13, IL-14), and growth and differentiation function (i.e. IL-3, IL-5, GM-CSF).

Cytokines are also possible to classify according to structure, biological activity and the structure of their receptor (table 4) (34, 35).

Table 4 . Structural groups of cytokines and receptors

Cytokine structure	Receptor class	Shared chain	Cytokine
4-α-helical short chain	Haemopoietin domain	common γ	IL-2, IL-4, IL-7, IL-9, IL-13, IL-15
4-α-helical short chain	Haemopoietin domain	common ß	IL-3, IL-5, GM-CSF
4-α-helical short chain	IFN-γ R		IFN-γ
4-α-helical long chain	Haemopoietin domain	gp130	IL-6, IL-11
ß-sheet	serine/threonine kinase		TGFß
ß-sheet	Ig-like		IL-1α, IL-1ß
ß-sheet	TNFR p75, p55		TNF-α, TNF-ß CD40L,CD27L FASL

1.5.1. Inflammatory Cytokines

Inflammation is a physiologic response to a variety of stimuli. An acute inflammatory response exhibits rapid onset and is of short duration, involving both localized and systemic responses. The local inflammatory response is accompanied by a systemic response known as the "acute phase response" which is initiated by activation of tissue macrophage and the release of inflammatory cytokines. Inflammatory cells (36) with capability to produce the cytokines IL-1, IL-6, and TNF include monocytes-macrophages, granulocytes, B and T -lymphocytes, endothelial cells, epithelial cells, mast cells, fibroblasts, nerve-cells, astrocytes, synovial cells, and keratinocytes (37-39). Inflammatory mediators are released from the inflammatory cells, causing vasodilatation (e.g. prostaglandins and histamine) and vascular permeabilization (e.g. leukotrienes, prostaglandins, histamine and serotonin). The increased blood flow through the tissue (causing redness and heat), as well as the leakage of cells and fluid

(extravasation) into the tissue (swelling and pain). A short summary of inflammatory cytokine is shown in table 5.

A complete discussion of cytokines can not be included in this thesis. However, the properties of IL-1, IL-6 and TNF-α which are important and related to the present study are presented. Those cytokines are produced in high concentrations by monocytes and were previously called monokines. However, other types of cells can also produce these cytokines and these cytokines are influenced by one another (40-42). For example, IL-1 and TNF are potent inducers of IL-6, and IL-6 inversely regulates TNF expression. Although these cytokines are pleiotropic, they are able to regulate the immune response, hematopoiesis, and inflammatory reaction in specific manners *in vivo*. The action of these three cytokines is widely overlapping (figure 2), but each show a particular characteristic function through which it was discovered and identified (43). It is known that these three cytokines induce activate transcription factor e.g. NF-kB, which is a crucial signalling intermediate in the LPS response pathway, coordinating this inflammatory response (44, 45). The mechanism is shown in figure 3.

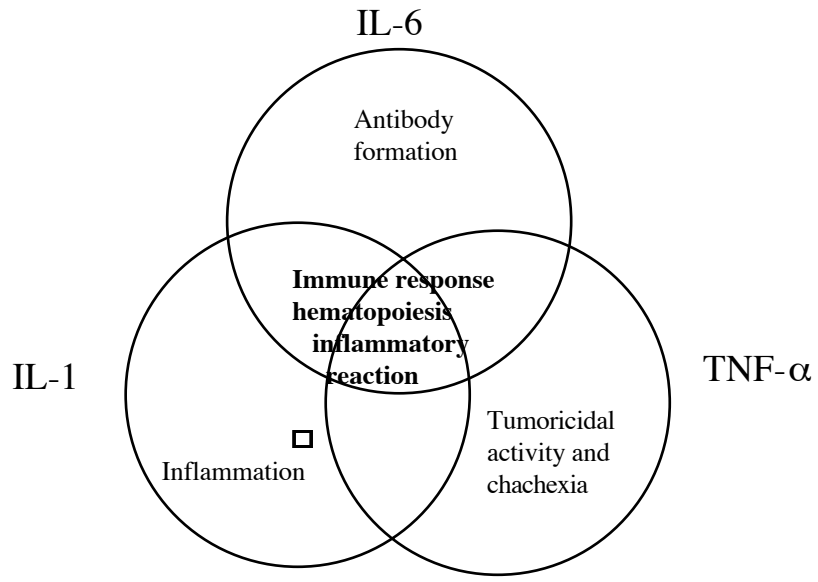

Figure 2. Cytokine Overlapping

Table 5. Inflammatory Cytokines, Principal Effects

1. Interleukin-1
   Antigen presentation to T-cell; Adhesion molecules; Granuloma formation; Lymphocyte proliferation; IL-2, IL-3, IL-6 production; Acute phase proteins; Fever.
2. Interleukin-6
   Differentiation of T-cell; IL-2 production; IgG synthesis; Acute phase proteins; Fever.
3. Interleukin-8
   Chemotaxis; Activate macrophages; Induces adhesion molecules.
4. Tumour necrosis factor-α
   Proliferation of T and B-cells; Stimulates IL-1a and ß; Acute phase proteins; Fever.
5. Interleukin-2 receptors
   Mediates: T-cell proliferation; B-cell proliferation; Macrophage activates; TNF, IL-1, LAK activity in T- cell.
6. Interleukin-10
   Macrophage activator and deactivator; IgA synthesis; Suppresses TNF-α release; Anti- inflammatory.
7. Interleukin-12
   Lymphocyte activation; Increased antibody production; Acute phase proteins; Fever.
8. Interferon γ
   Anti viral; Antiprotozoal; Up-regulate ICAM-1; MHC I and II expression.

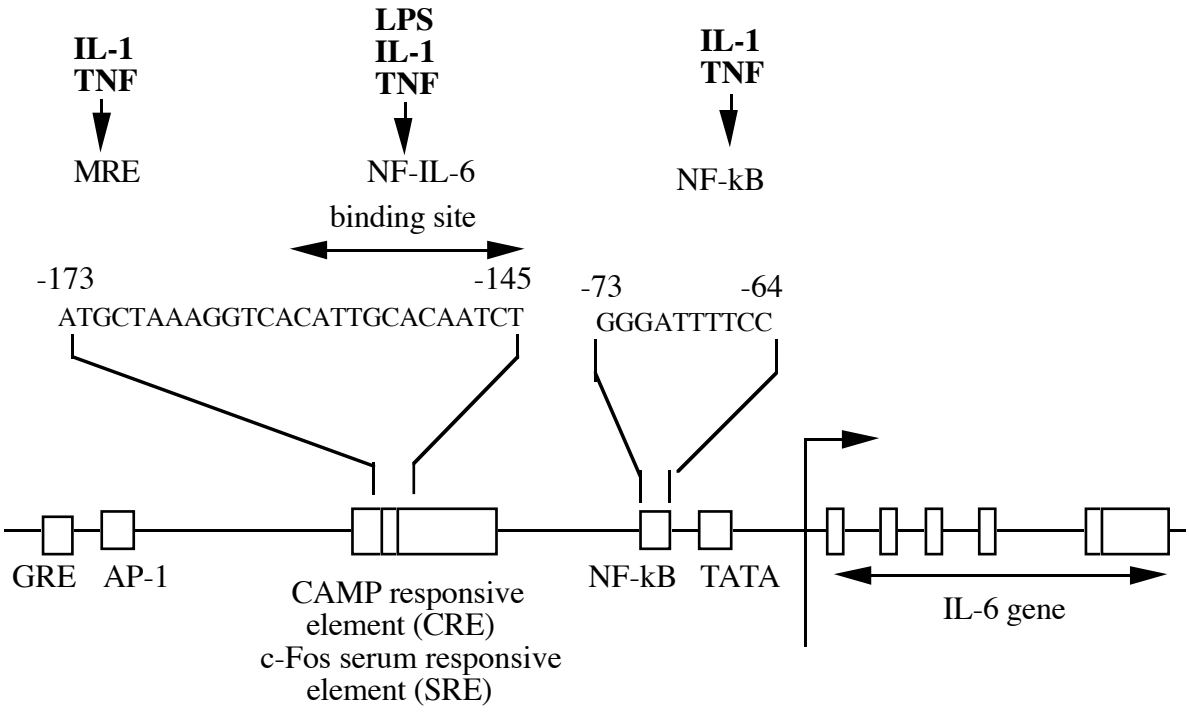

Figure 3. Three nuclear factor NF-IL-6, the multiresponse element MRE, and NF-kB are in the promoter region. These DNA-binding proteins, which are induced by IL-1 and TNF-α, stimulate transcription of the IL-6 gene. Thus, as IL-1 and TNF-α levels increase, production of IL-6 also increases.

1.5.1.1. IL-1 system

Interleukin was originally named "Lymphocyte Activating Factor"(LAF) (46) and was later called other names until "interleukin" was established in 1979 (47). Interleukin-1 refers to a group of three proteins, i.e. IL-1α, IL-1ß, and IL-1ra (receptor antagonist). Mature IL-1α and IL-1ß are both polypeptides with molecular weights of about 17.5 kDa. They have different isoelectric points and two distinct receptors binding both forms of them (48) and have the same biological functions.

The pro-IL-1ß is processed by an aspartate specific protease called IL-1 converting enzyme (ICE) (49). ICE does not cleave pro-IL-1α.

The type I receptor (IL-1RI) has a cytoplasmic domain of 213 amino acid residues, and type II (IL-1RII) has only a 29 amino acid residue (50). The IL-1RI binds IL-1α better than IL-1ß and the IL-1RII binds IL-1ß more strongly than IL-1α. IL-1RI can signal (51) and the IL-1RII could itself act as an IL-1 antagonist (52).

IL-1ra competes with and binds to the IL-1 receptor (53). IL-1ra has an affinity similar to that of IL-1 and its molecular weight is 22 kDa; but IL-1ra does not transduce any signal (54). IL-1ra occurs also in an intracellular form (icIL-1ra, as compared to the soluble IL-1ra (sIL-1ra)). icIL-1ra is thought to be important for blocking IL-1α binding to nuclear DNA (50).

Recently it has been suggested that there is a fourth member of the IL-1 family, tentatively designated as IL-1γ. This is based on amino acid sequence homology and functional similarities between IL-1α and ß and this cytokine has been called interferon-γ-inducing factor (55).

IL-1 can be produced by many different cells, but all nucleated cells are capable of making this protein under appropriate conditions (42, 56). IL-1 has a wide variety of effects in immune systems and in the inflammatory reaction to bacteria. IL-1ra can therefore act specifically to depress the effects of IL-1. Schematic view of the IL-1 system is shown figure 4. For the recent reviews on IL-1, see (42, 54, 57).

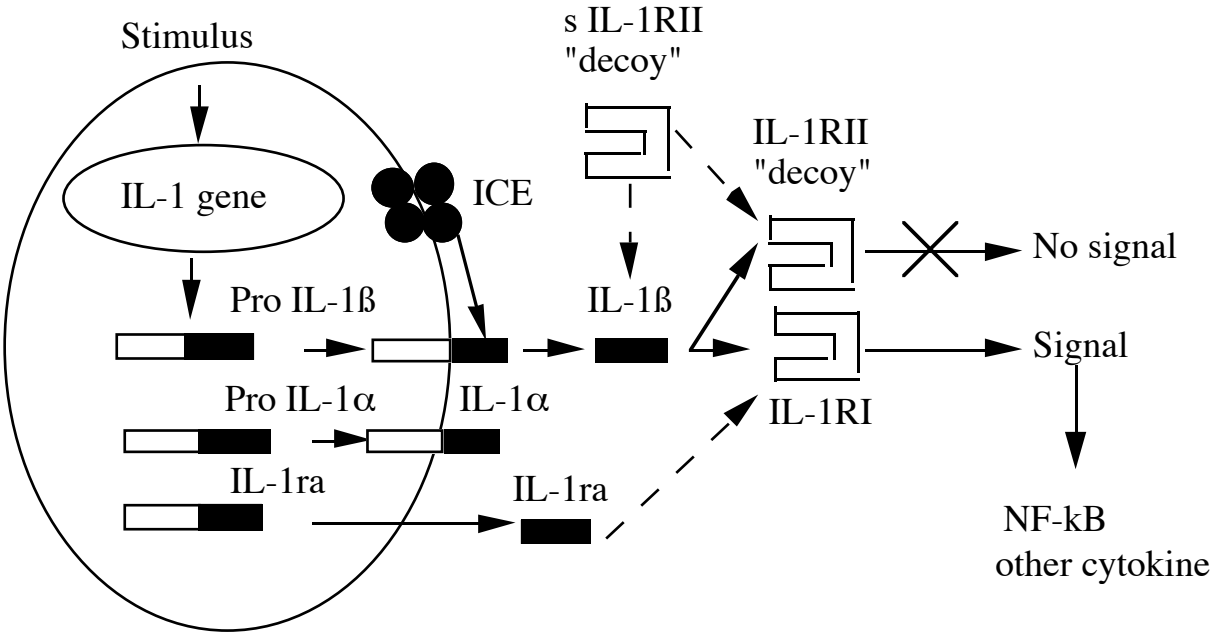

Figure 4. Schematic diagram of IL-1 structure and mechanism of action. Cell stimulation leads to gene expression of Pro IL-1α and ß, followed later by IL-1ra. Pro IL-1ß is then cleaved at the cell-surface by interleukin-1ß converting enzyme (ICE). The secreted mature IL-1ß can bind to the type I receptor (IL-1RI) and trigger a cellular level of gene expression and competition for binding to the IL-1RI (shown by the dotted lines) from the IL-1ra, surface-bound decoy IL-1RII, and soluble decoy IL-1RII.

1.5.1.2. IL-6 system

It is now well-known that IL-6 is a multifunctional cytokine acting on a wide variety of cells (41). IL-6 is a 22-29 kDa glycoprotein and was first described due to its ability to induce the production of immunoglobulin from B cells (58). IL-6 exerts its activity through binding to a high affinity receptor consisting of two membrane glycoproteins, an 80 kDa receptor protein (IL-6R, or a chain), and a 130 kDa signal transducing protein (gp130, ß chain) (59, 60). The presence of IL-6R together with gp 130 will result in the formation of high-affinity IL-6 binding and subsequent signal transduction (61, 62). Soluble IL-6R (sIL-6R; 55-60kDa; 15-150 ng/ml) and soluble gp130 (sgp130; 95kDa; 300-400 ng/ml) are present in the peripheral circulation in humans (63).

There are several cytokines that are closely related to IL-6. They share the gp130 ß-chain as part of their signal transducing receptor complex (table 6). This subfamily of cytokines also shares some biological activities, such as induction of acute phase protein response. OM, LIF and IL-6 induce the stasis of certain tumor types. IL-6 and IL-11 among other things induce megakaryocyte differentiation.

The effects of IL-6 on different cells are numerous and various. The major role of IL-6 is to mediate inflammation and immune response initiated by infection or injury. IL-6 have been reported to be associated with a variety of diseases (64),

including autoimmune diseases such as arthritis (65), Castleman´s disease, mesangial proliferative glomerulonephritis, psoriasis, inflammatory bowel disease and malignancies (66, 67).

Table 6. Interleukin-6-Type Cytokines (68, 69)

Interleukin-6 (IL-6)
Interleukin-11 (IL-11)
Leukaemia inhibitory factor (LIF)
Oncostatin M (OM)
Ciliary neurotrophic factor (CNTF)
Cardiotrophin-1 (CT-1)

1.5.1.3. TNF system

TNF was first identified in the serum of mice challenged with endotoxin after BCG inoculation (70). TNF-α and ß are members of a family of secreted and cell surface proteins that mediate immune and inflammatory responses. TNF-α, also called cachectin, is a 17 kDa polypeptide. In a human the TNF-α has 157 amino acids. TNF is produced by macrophages, neutrophils, activated T and B lymphocytes, NK cells, LAK cells, astrocytes, endothelial cell, smooth muscle cells, and some transformed cells (71). TNF-α shows high affinity with two receptors, TNFRII (Type A, 75kD) and TNF-I (Type B, 55kD) (72). Both receptors are members of the NGFR/TNFR (nerve growth factor receptor) surperfamily with four cysteine rich repeats in the extracellular domain (73). The two types of TNF receptors mediate both overlapping and nonoverlapping function (table 7). The family of TNF is shown in table 8 (40, 74).

TNF-α exists in a membrane anchored form on the surface of macrophage and/ or monocytes. The release of soluble TNF-α has cytotoxic activity and plays an important role in intercellular communication (75). Many of the actions produced by the TNF-α are functionally similar to those produced by IL-1. A number of pathological conditions, including Cachexia (76), septic shock following infection with Gram-negative bacteria (77), autoimmune disorders (78), and meningococcal septicaemia (79) have increased production TNF-α (80).

Table 7. A partial list of signals transmitted through each type of TNF receptor

TNFR 55	Induction of NF-kB; Induction of c-fos; Stimulation of protein kinase C; Stimulation of sphingomyelinase; Stimulation of phospholipase; Production of diacylglycerol; production of ceramide; Induction of IL-6; Induction of Mn superoxide dismutase mRNA; Prostaglandin E2 synthesis; Induction IL-2R; HLA class I & II Ag expression; Antiproliferaton/ cytotoxicity/ apoptosis; Growth stimulation; Endothelial cells adhesion; Generation of lymphocyte activated killer (LAK) cells; Proliferation of NK cells; Antiviral activities
TNFR75	Induction of NF-kB; Proliferation of thymocytes; Induction of IL-6 Generation of NK & LAK; DN fragmentation;Antiproliferation/ cytotoxicity/ apoptosis

Table 8. The TNF superfamily

Receptor	Cellular expression
TNFR55	Epithelial cells
TNFR75	Myeloid cells
CD27	Lymphocytes
CD30	Lymphocytes
CD40	B-cells & macrophages
Fas/Apo-1	Myeloid & Lymphoid cells
OX40	$CD4^{+}$Tcells
4-1BB	Activated T cells, thymocytes
NGF-R	Neurons, schwann cells & melanoma cells

Table 9. Comparison of the Biological Activities of IL-1, IL-6 and TNF-α (43, 81, 82)

Effect	IL-1	TNF-α	IL-6
Endogenous pyrogen fever	+	+	+
Synthesis of acute-phase proteins	+	+	+
No specific resistance to infection	+	+	+
Increased vascular permeability	+	+	+
Increased adhesion molecules on vascular endothelium	+	+	-
Increased fibroblast proliferation	+	+	-
Increased synovial cell collagenase and PGE2	+	+	-
Decreased albumin synthesis	+	+	-
Decreased lipoprotein lipase	+	+	-
Induction of IL-8	+	+	-
Induction of IL-6	+	+	-
Induction of IL-1 and TNF from monocytes	+	+	-
Platelet production	+	-	+
T-cell activation	+	+	+
B-Cell activation	+	+	+
Increased immunoglobulin synthesis	-	-	+
Stem cell activation	+	-	+
Endothelial cell activation	+	+	-

1.6. Mechanisms in ODTS

The exact mechanism of ODTS has not been discovered. The ODTS response is produced by agents which activate different cells, such as macrophages, to excrete inflammatory mediators (83). This results in leaking of plasma from the capillaries into the alveolar and pulmonary tissue as well as invasion of cells, particularly neutrophils through chemotaxis (84-86). There is an increase of leukocytosis in the BAL, as well as acute phase proteins in peripheral blood (9).

Dust exposure causes TNF-α secretion in the airways, in the lung tissue and the blood. The TNF-α is subsequently distributed via the blood. High level TNF-α causes cachexia, tissue destruction and fatigue symptoms. The AM cells produce an increase in lysosomal enzymes and release different cytokines such as IL-1, TNF-α, IL-6 and platelet activating factor (PAF) (17, 87-90). The airway epithelial cells also secrete IL-6. IL-1, IL-6 and TNF-α act on the central nervous system and induce fever (91-93)(Fig 5).

1.7. The Airway Epithelial Cells

The mature airway is a complex structure lined by a continuous layer of epithelial cells. The distribution of cell types within the epithelium varies along the airway. Table 10 shows epithelial cell types distinguished according to position (basal or luminal), presence of cilia and secretory granules, the non-epithelial and the neural component of mature airway epithelial. The surface layer of cells consists largely of ciliated cells with a few goblet cells (mucus producing) attached to the basement membrane. These fail to reach the luminal surface and lie sandwiched between the other varieties of cells. The specialized secretory epithelial of the submucosal gland is composed of serous cell and mucous cells. In the distal airway, Clara cells and basal cell are predominant and the epithelial has a more columnar appearance. At the alveolar levels, the columnar epithelial gives way to thin epithelial cells. Both are comprised of Type I cells, interspersed with Type II cells (94, 95). Cellular functions can subdivided into five steps: 1) mucus secretion; 2) ciliary beating; 3) leukocyte interactions; 4) permeability; and 5) bronchoconstriction.

The airway epithelial cell barrier induced by environment particles, viruses, bacteria and cigarette smoke may lead to adverse changes, resulting in airway inflammation. Specific mechanisms involved in this inflammation include (figure 6): a) increased synthesis and/or release of inflammatory mediators (e.g, arachidonic acid metabolites, chemotactic agents) or alternatively, decreased synthesis of protective mediators; b) synthesis of proinflammatory cytokines; c) modulation of cell adhesion molecules; and d) modulation of immunoregulatory processes (96-98).

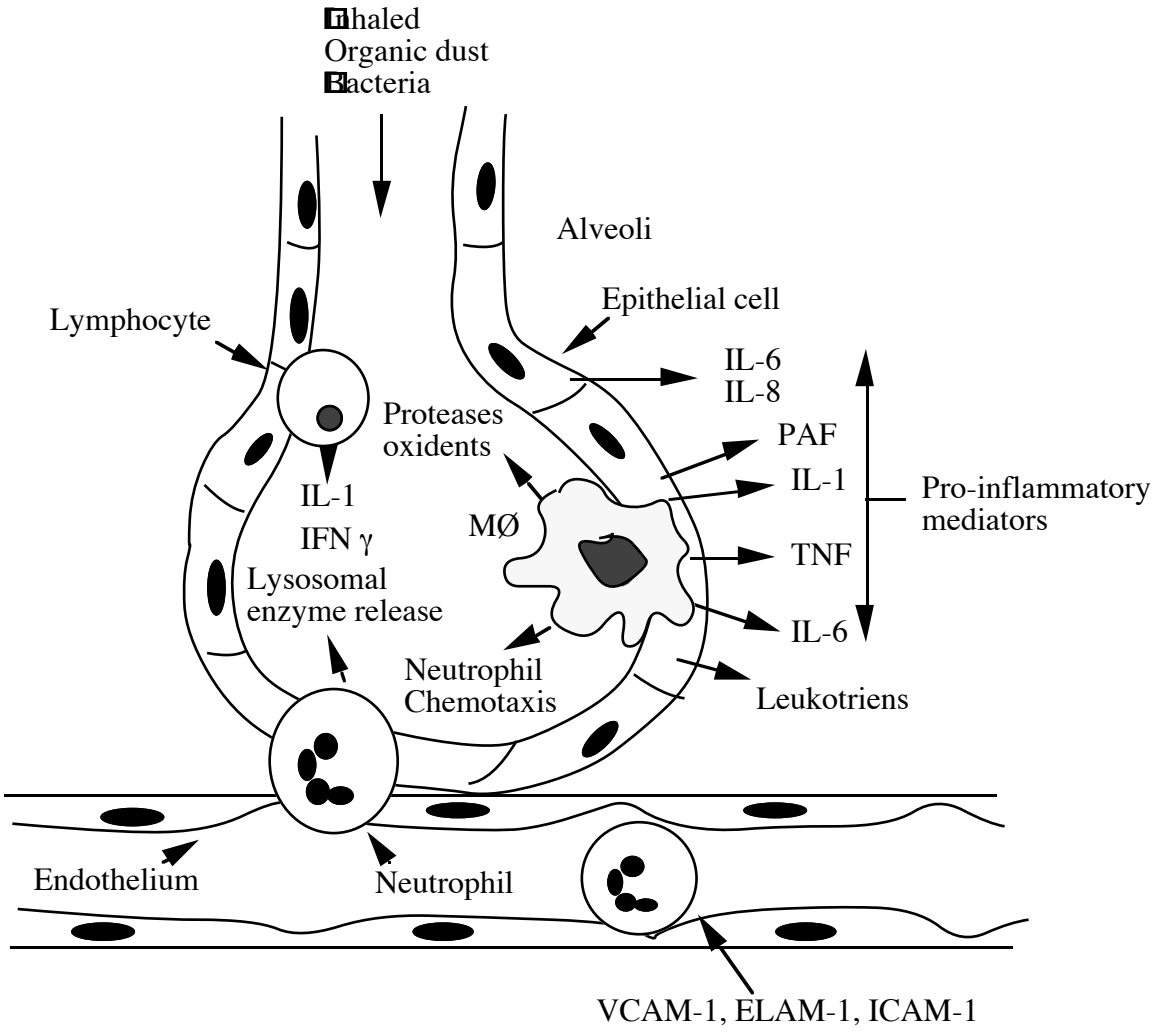

Figure 5. Mechanisms proposed in the ODTS. It appears that activation of alveolar macrophages, epithelial cells and lymphocytes with release of pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, IFN γ and chemotactic factors as well as leukotrienes, oxidants and protease sets the stage for development of ODTS.

Table 10. Mature airway epithelium

Epithelial cells	Ciliated epithelial cells; Basal cells; Dense-core granulated cells; Mucous secretory cells; Serous secretory cells; Clara secretory cells; Special-type cells; Brush cells
Migratory cells	Lymphocytes; Globule leukocytes; Mast cells
Neural component	Neuroepithelial bodies; Never terminals

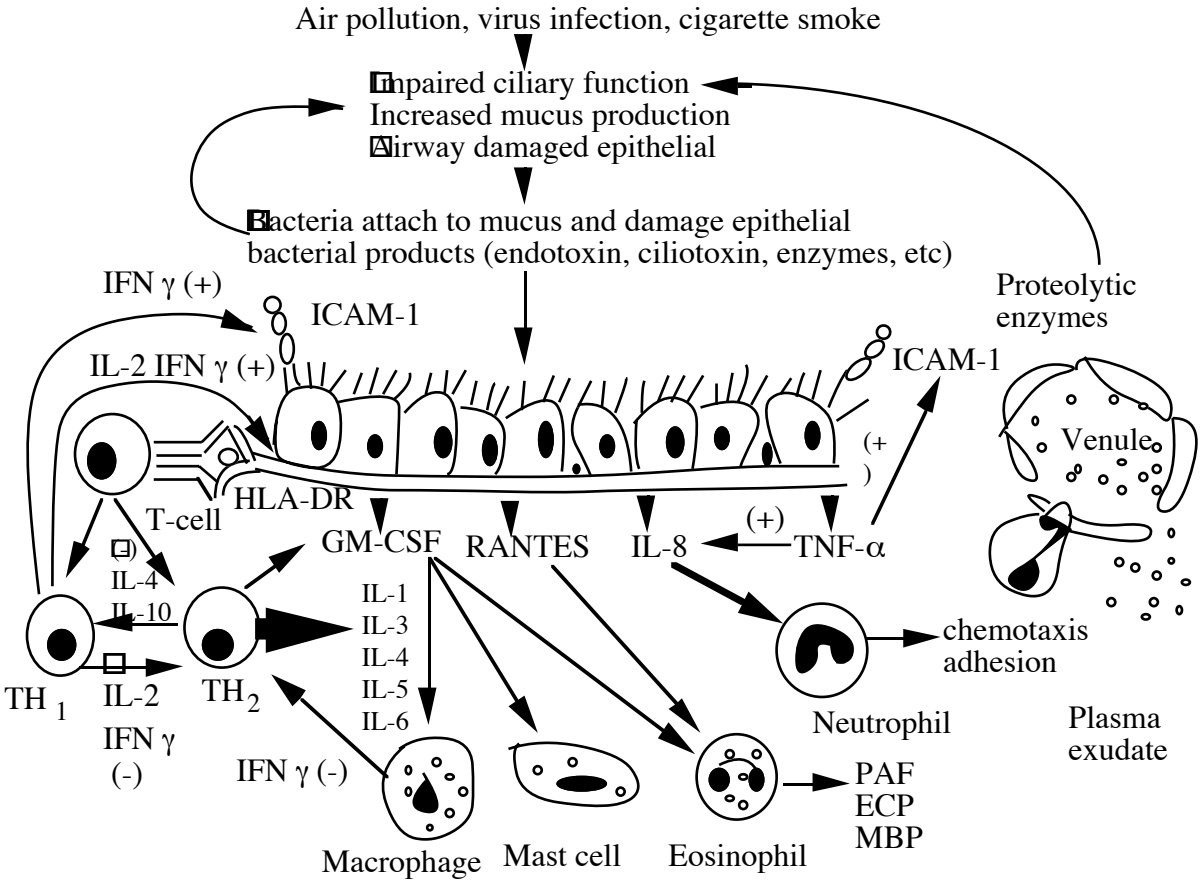

Figure 6. Schematic view of the role of epithelial cells in the modulation of airway inflammation. For abbreviations, see the section" Abbreviation". ECP: eosinophil cationic protein; MBP: major basic protein.

1.8. The Alveolar Macrophage

The lung tissue includes four different macrophages: the alveolar macrophage (AM); the interstitial macrophage; the intravascular macrophage and the dendritic cell (99). The alveolar macrophages have a unique localization in the body, since they are placed within the alveolar surfactant film, which is produced by type II alveolar lining cells and is composed of phospholipids and proteins. In addition, they are the only macrophages in the body which are exposed to air. They are the first line of defence against inhaled constituents of the air. They possess a high phagocytic and microbicidal potential. The total number of AM in the human lung is estimated to be $23x10^9$, with approximately 50-100 per alveolus (100).

AM have been reported to produce cytokine (101): IL-1, IL-6, IL-8, TNF, TGF-ß, IFNα ß γ, GM-CSF, MIP-1α and MIP-1ß (102-104). The production of individual cytokines is influenced to a great extent by the different stimulus. The

most widely used stimuli are bacterial LPS and viruses (105). Adhesion, endocytosis and secretion are three characteristics of AM. Membrane receptors and surface markers make AM different function (figure 7). AM has been shown to produce metabolites of arachidonic acid, both along the cyclo-oxygenase pathway (the thromboxances and prostaglandins) and the lipoxygenase pathway (the leukotrienes and hydroxyeicosatetraenoic acids HETEs)(106). AM produces the reactive oxygen intermediates (ROIs), such as superoxide anion (O_2, hydrogen peroxide (H_2O_2) and hydroxyl radical (OH-), in association with phagocytosis (107). AM also efficiently kills microbial and can clear certain bacteria, viruses and fungi (108).

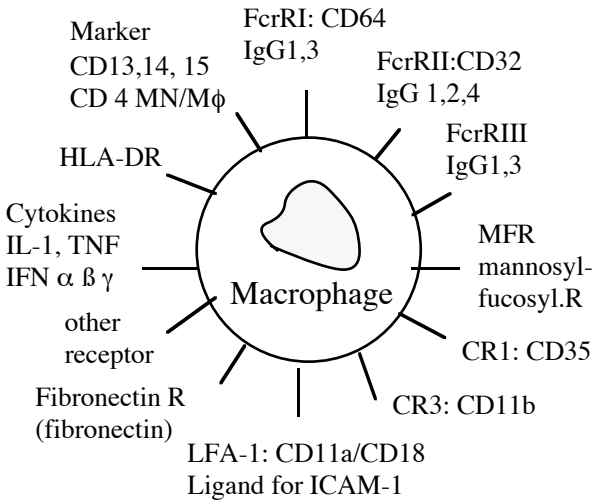

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Figure 7. Macrophage receptor and its ligands

2. Aim of the Present Study

The purpose of this thesis is to characterize the appearance of acute phase cytokines in ODTS and to correlate the acute health effects with markers for microbial contaminants in inhaled swine dust causing ODTS. The specific aims of the present study are:

1. To study the time course of changes in TNF-α and IL-6 in peripheral blood after acute exposure to swine dust (study I).

2. To evaluate the release of pro-inflammatory cytokines IL-1, IL-6 and TNF-α in the upper and lower airways following exposure to airborne swine dust (study II).

3. To investigate the correlation between markers for microbial contaminants in the dust, cytokine responses, and health effects (study III).

4. To study if the IL-1 family of cytokines increase in peripheral blood after inhalation of swine dust (study IV).

5. To study release of proinflammatory cytokine producing cells, from an epithelial cell line (A549) and human alveolar macrophages *in vitro* stimulated through exposure to swine dust or LPS (study V).

3. Materials and Methods

In this section a summary of the design of the experiment and the methods used is given. More details are given in the original papers.

All subjects gave written consent after being informed about the experiment, and the human studies were approved by the local Ethics Committee of Karolinska Institute (KI No 92:74, 93:3, 93:45, 93:223 and 95:347).

3.1. Swine Dust Exposure: Studies in Humans

3.1.1. Subjects

All participants were healthy non-smoking volunteers. They had no previous exposure to farm dusts. All denied present or past symptoms of allergy or asthma. None had experienced respiratory infections in the two weeks preceding the study. All subjects in study I were included in study III, but none were included in study II. All subjects in study II were included in study III and some of them were included in study IV. Details about subjects for the four experiments are given in table 11.

Table 11. Subjects in four human exposure studies

	I	II	III	IV
Number	14	22	38	36
Sex: F/M	8/6	9/13	22/16	16/20
Age (range)	29 (19-45)	30 (22-50)	30 (18-50)	31 (18-59)
overlapping			I+II	II (16)+others

3.1.2. Study Design

Each subject spent 3-4 hours inside a swine confinement building containing about 700 swine, with a body weight of about 100 kg. During this time the swine were guided through a weighing box, a procedure that causes considerable aerosolisation of settled dust. On testing occasions one or two subjects were present. On those testing occasions when two subjects were present, they worked in close proximity to each other. The study design process is shown in figure 9. All participants responded to a symptom questionnaire containing questions about shivering, headache, weakness, muscle pain, and nausea. Table 12 describes the tests used in the studies.

In paper I, we studies the time course and peak levels of cytokine concentration in peripheral blood after exposure to swine dust. Six subjects participated the study. The blood samples were taken at 2, 5, 7, 9, 11 and 24 h after the start of the

exposure for IL-6 analysis in serum. When the TNF high sensitivity kits were available, another eight subjects were involved and additional blood samples were taken at 3 h.

In paper II, cytokine (IL-1α, IL-1ß, IL-6 and TNF-α) concentrations in the BAL and NAL samples were measured. Albumin was also analyzed in BAL and NAL fluid. As a control experiment, BAL fluid from seven subjects, was concentrated 14-fold by lyophilization and was subsequently analysed in order to compare the results from Quantikine™ kits and high sensitivity Quantikine kits. The sensitivity of IL-1α assay was improved by adding the ELAST® amplification system.

In paper III, the quantity of inhaled dust and different markers of exposure to microorganisms were correlated to health effects including changes in lung function (BHR, FEV_1, and VC), serum IL-6, peripheral blood leukocytes and neutrophils, body temperature, and other symptoms.

In paper IV, different methods to analyse IL-1ß were compared. In one method isolated PBMC were studied. IL-1ß was measured in PBMC (n=16) and poor platelet plasma (n=8). Blood samples from eight subjects were taken at 3, 4, 7, and 24 h in order to study time course the release of IL-1ß. The percentage of mononuclear cells was measured in PBMC. Levels of IL-6, TNF-α and IL-1ra were analysed in the serum.

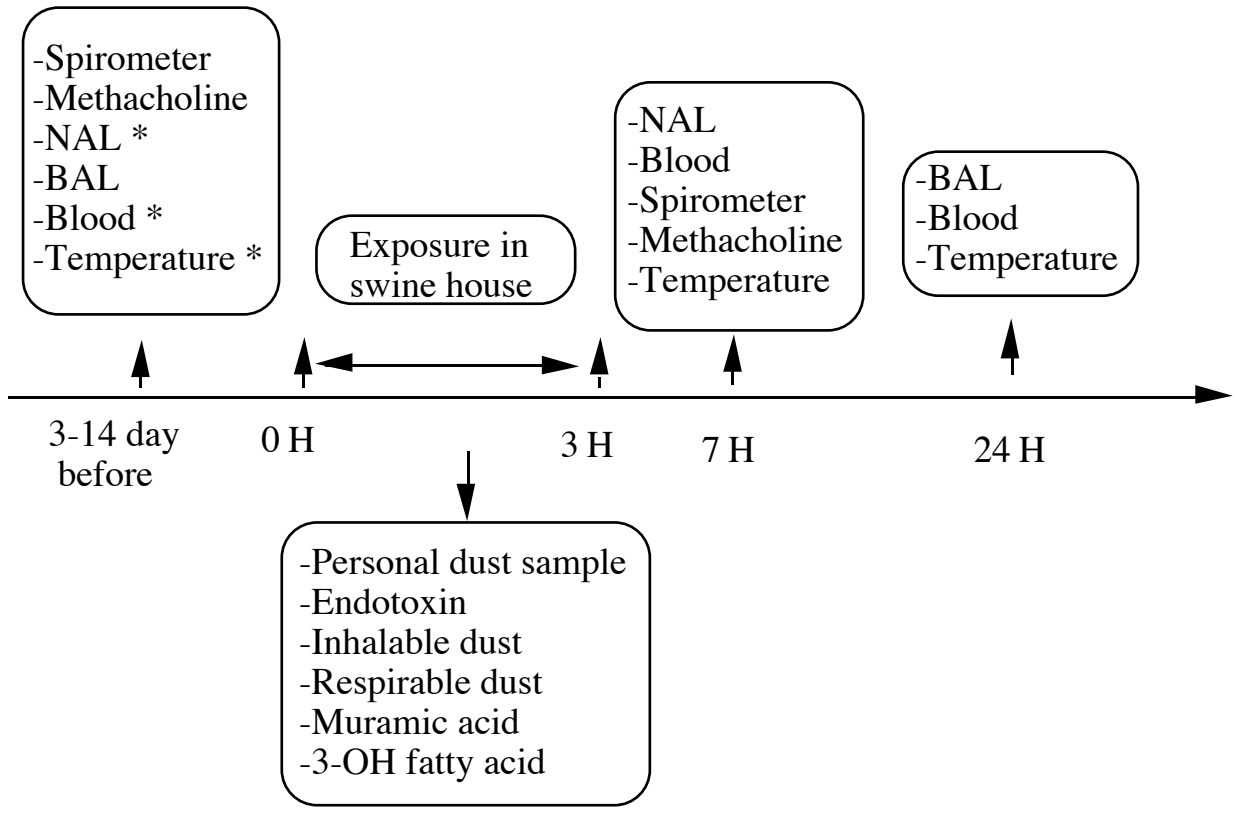


Figure 9. Measurement performed on the exposure day

* Performed 30 minute before exposure

Table 12. Tests performed in different studies

test	I	II	III	IV
Lung function	√	√	√	√
Methacholine test	√	√	√	√
BAL cytokine		√		
NAL cytokine		√		
BAL cells		√		
NAL cells		√		
Temperature	√	√	√	√
leukocytes in blood	√	√	√	√
Albumin in BAL		√		
Albumin in NAL		√		
CRP	√			
IL-1		√		√
IL-6	√	√	√	√
TNF	√	√		√
IL-1ra				√

3.1.3. Lung Function and Bronchial Challenge Tests

FEV_1 and VC were measured with a low-resistance rolling-seal spirometer (OHIO model 840, Airco, Madison, WI). The protocol followed the guidelines of the American Thoraxic Society (109). Local reference values were used (110, 111).

Bronchial responsiveness was measured with a methacholine bronchial provocation test. Inhalation of diluent was followed by doubling (study I, II, III) or four-doubling (study III, IV) concentrations of methacholine starting at 0.5 mg/ml until FEV_1 had decreased 20 % compared with the volume obtained after inhalation of the diluent or until the maximum concentration was reached. The $PD_{20}FEV_1$ was calculated. The method is standardized with control of inhalation flow (0.4 l/s), inhalation volume (0.8 l), and number of breaths. The output of the nebulizer (0.38±0.01 ml/min) was measured daily. The details of the procedure have been described elsewhere (112).

3.1.4. Exposure Measure

In the present study, two different dust fractions were measured. Dust is classified by size into two primary categories: (113)

* Respirable dust, size <5 μm, reaching the alveolar region.
* Inhalable dust, size ≤10 μm, most of which is trapped in the nose, throat and upper airway.

3.1.4.1. Inhalable dust, respirable dust and endotoxin

Inhalable dust and endotoxin were sampled at an airflow of 1.9 to 2.0 L/min during the exposure with personal samplers using 25 mm IOM head open-phase

filter cassettes and portable pumps (SKC Ltd, Dorset, UK). The airflow was calibrated before and after exposure. The cassettes were carried in the breathing zone and were equipped with 0.4 μm polycarbonate filters (Nuclepore®; Costar Corp., Cambridge, MA). The subjects also carried Cyclone® samplers (study I, IV) for measurement of the respirable dust fraction (Cyclone, cut-off 5μm; SKC). Inhalable dust and the respirable dust were measured by weighing after 24 h of conditioning, using a ME 22 Mettler® balance (Mettler, Greisensee, Switzerland) and reference filters.

Endotoxin was measured after suitable dilution with a chromogenic version of the *Limulus* amebocyte lysate assay (QCL-1000, Endotoxin with *Escherichia coli* 0111:B4 as standard; BioWhittaker, Walkersville, MD).

3.1.4.2. Microbial Markers

Samples were analyzed by gas chromatography-mass spectrometry (GC-MS) for peptidoglycan and lipopolysaccharide by using muramic acid (MuAc) and 3-hydroxylated fatty acids (3-OH FAs) respectively, as chemical markers. The filter extracts were transferred to test tubes equipped with Teflon-lined screw caps, dried, and heated overnight at 100 °C in 4M hydrochloric acid. Thereafter, 1 ml of hexane was added to each tube and after shaking, the hexane phase was transferred to a separate tube. The hexane phase was evaporated to dryness, heated overnight in 4M methanolic HCL at 100□°C, extracted, purified by using a disposable silica gel column, subjected to trimethylsilyl (TMS) derivatization, and analyzed for 3-OH fatty acids as described in detail previously (114). The aqueous (acidic) phase was evaporated, subjected to TMS derivatization, and analysed for MuAc (115)**.** Table 13 gives exposure measure in the four studies.

Table 13. Measurement of dust and microbial marker

Test	I	II	III	IV
Inhalable dust	√	√	√	√
Respirable dust	√ (n=7)			√ (n=8)
Endotoxin	√	√	√	√
muramic acid		√	√	
3-OH fatty acid		√	√	

3.1.5. Blood sampling

Samples for cytokine analyses were obtained in vacuatainer silicone tubes and samples for flow cytometry were obtained in EDTA tubes manufactured by Terumo Venoject (Terumo Europe, Belgium). Samples were allowed to coagulate for 1-1.5 h at room temperature and centrifuged using a Sigma 3K-2 refrigerated centrifuge (Axel Johnson, Helsingborg, Sweden). Supernatants were transferred to small glass tubes and stored in freezer at -70°C pending analysis. Each sample underwent only one freeze-thaw cycle before assay.

In paper IV, PBMC were obtained using Ficoll-Paque and Histopaque method and sterile tubes were used in the whole process. Poor platelet plasma sample and chloroform extraction in plasma were prepared.

Chloroform extraction was found to provide the best recovery of exogenous IL-1ß. 1 ml plasma and 2.0 ml of chloroform were agitated for 5 minutes, and spun for 5 min at 10,000 g, 4 °C (Beck Man Model J2-21). The aqueous phase was separated and recovered. The extraction procedure was repeated two times.

The blood tubes were centrifuged immediately after venipuncture (400 • g for 10 minutes). Plasma was removed without disturbing the buffy coat, aliquoted in 1.5 ml microfuge tubes and was spun at 1600 •g at 3000 RPM for 10 minutes to pellet the platelets. The platelet free plasma was transferred to new microfuge tubes and frozen until assay.

3.1.6. Flow Cytometry

This method was employed to examine total blood and the proportion of polymorphonuclear granulocytes, monocytes, and lymphocytes (Epics Profile II®; Coulter Corp., Hialeah, FL). The samples were prepared in a COULTER Q-PREP (Coulter Electronics Inc, Hialeah, FL, USA) and incubated for 10 minutes with CD14-CD45 monoclonal antibodies (Mo2-RD1/ Kc56-Fitc, Cytostat ®/Coulter Clone®, Coulter Corp, Hialeah, Florida).

3.1.7. Cytokine assays

The cytokines analyses were performed using the commercial enzyme immunoassays (R&D Systems Europe, Abingdon, UK) and according to the manufacturer's instructions. This test is based on a "Sandwich" assay, in which a monoclonal antibody specific for IL-1ß, IL-1ra, IL-6 and TNF-α has been pre-coated onto the microtier plate. Any cytokine present is bound by the immobilized antibody. After washing away any unbound proteins, an enzyme-linked polyclonal antibody specific is added the wells to "Sandwich" any IL-1ß, IL-1ra, IL-6 and TNF-α immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of cytokine bound in the initial step. The color development is stopped and the intensity of the color is measured. A curve is prepared, plotting the Optical Density (OD) versus the concentration of those cytokine in the standard wells. By comparing the OD of the samples to this standard curve, the concentration of the IL-1ß, IL-1ra, IL-6 and TNF-α in the unknown sample is then determined. The cytokines detection limits and CV showed in table 14.

To this method an amplification system was added to construct a high-sensitivity system. In this amplification system a reaction with alkaline phosphatase provides a co-factor activating a redox cycle leading to formation of a coloured product. IL-1α was analyzed using commercially available kits (R&D

Systems Europe, Abingdon, UK) with ELAST® amplification system (DuPont, Nen®, Boston, MA, USA). The principles of the two amplification systems are given in figure 10. The standard curve for IL-1α was between 0.49 and 31.3 ng/L. Table 15 gives reference values in healthy subjects from our laboratory samples.

Table 14. Detection limits and coefficients of variation (CV) intra- and inter assays for IL-1, IL-6 and TNF using normal Quantikine™ and high sensitivity EASIA™ kits

Cytokine	Detection limit ng/L	CV inter%	CV intra%	Detection limit ng/L	CV inter%	CV intra%
	Quantikine™			EASIA™		
IL-1a	3.9	< 8	< 8	0.49	< 10	< 7*
IL-1ß	3.9	< 6	< 3	0.125	< 9	< 9
IL-6	1.5	< 4	< 3	0.156	< 11	< 6
TNF-a 15.6	< 6	< 5		0.5	< 9	< 6
IL-1ra	46.9	< 5	< 5			

* IL-1α EASIA improvement by our laboratory.

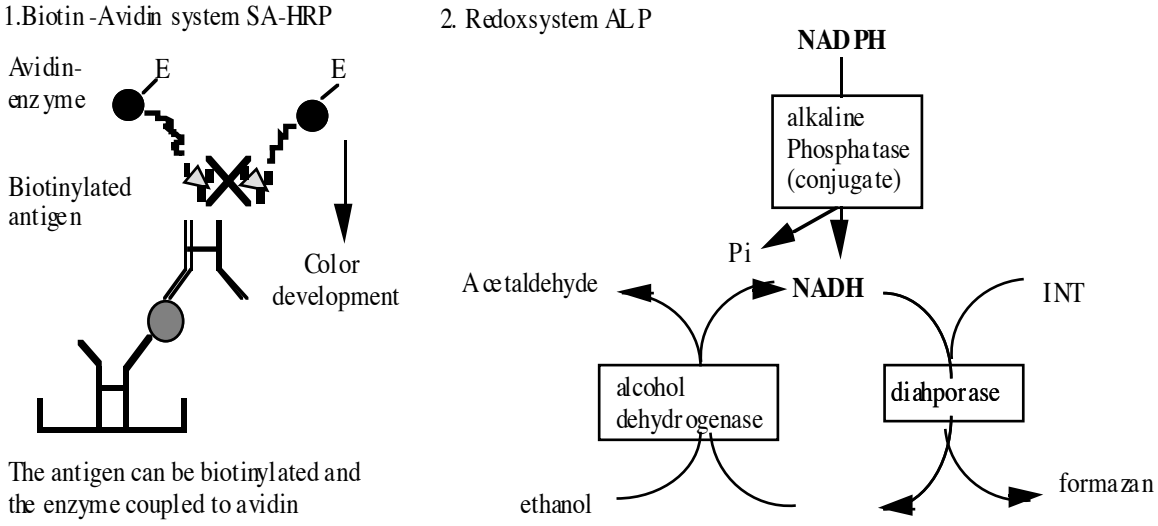


Figure 10. Amplified substrate system

Table 15. Reference values from our laboratory (IL-1, IL-6 and TNF) from healthy individuals using Quantokine™ (Q) and EASIA ™ (HS) kits

Cytokine	Sample type	N	Detectable Mean ng/L	Detectable Range ng/L
IL-1α (Q)	Serum	20	< 3.9	
IL-1ß (Q)	Serum	24	< 3.9	
IL-1ß (HS)	NAL	33	2.82	0.167-6.62
IL-1ra (Q)	Serum	40	250	95 - 880
IL-6 (Q)	serum	71	< 3.13	
IL-6 (HS)	serum	43	1.02	0.156-4.89
	NAL	124	1.9	0.11-14.53
	BAL	59	0.71	0.156-2.3
TNF-α (Q)	Serum	20	< 15.6	
TNF-α (HS)	Serum	56	1.93	0.25-8.93
	NAL	67	0.281	0.27-2.16
	BAL	65	0.298	0.25-1.37

3.1.8. Methodology

When measuring cytokines in biological fluids, some pitfall can be encountered. The factors that affect cytokine measurement also influence cytokine activity *in vivo*, thus they are important for methodological standpoint (116, 117)

It is important that the samples for cytokine analysis are taken in endotoxin free tubes (118). The blood evacuated collection tube was measured from random sample for endotoxin with the LAL assay in our laboratory.

In order to minimise the variability of the assay only R&D system kits were used in our test (119).

The minimum detectable concentration of IL-6 was determined by adding two standard deviations to the mean OD value of 20 zero standard replicates and calculating the corresponding concentration from the standard curve.

For both kits (EASIA™ and Quantikine™), the CV is less than 10 % in intra- and interassays with the acceptable value. IL-6 concentrations were determined in the same samples (n=30) using both EASIA™ and Quantikine™ kit.

3.1.9. Nasal Lavage

Using a syringe, five ml of room temperature isotonic saline solution (0.9 %) was instilled into each nasal cavity while the subject gently flexed the head backward (approximately 30˚ from the horizontal plane) while closing the soft palate. This position was maintained for 10 seconds, after which the subject leaned forward and expelled the nasal fluid into a plastic collection cup. The lavage fluid was immediately centrifuged at 200 g, +4˚C, for 10 minutes, and the supernatant was frozen in aliquots at -70 ˚C until analyzed.

3.1.10. Bronchoalveolar Lavage (BAL)

Bronchoscopy was performed through the mouth with a flexible fibreoptic bronchoscopy (Olympus Type 4B2) under local anaesthesia with 2% lidocaine (Xylocaine, Astra, Södertälje, Sweden) after premedication with benzodiazepine and atropine. The bronchoscope was wedged in a middle lobe bronchus and sterile saline solution at 37 ˚C was instilled in five aliquots of 50 ml. After each instillation, the fluid was gently aspirated and collected in a siliconized plastic bottle kept on ice. The cellular component was immediately centrifuged at 400 g for 5 minutes at 4 ˚C, and the supernatant was frozen in aliquots at -70 ˚C for subsequent analysis. The BAL technique has been described in detail elsewhere (120).

3.1.11. Nasal and BAL Cell Determination

Nasal and BAL cells were counted in a Bürkerchamber. A cell smear was stained with the May-Grünward-Giemsa stain, in order to make a differential cell count.

3.1.12. Protein Analysis

Albumin was analyzed using a sandwich ELISA developed by our laboratory. Each plate was calibrated with human serum protein (Dakopatts, Glostrup, Danmark). Orosomucoid was analyzed by immunoeletrophoresis using commercial antiserum (Dakopatts, Glostrup, Denmark). C-reactive protein (CRP) was analyzed using nephelometry with the NA Latex CRP reagent (Behring, Frankfurt am Main, Germany).

3.2. Swine Dust Exposure: Studies *in vitro*

3.2.1. Epithelial Cells

The human lung epithelial carcinoma cell line A549 (American Type Culture Collection, Rockville, Maryland, USA. CCL 185) was used in the study. The cells were cultured in Ham's F-12 supplemented with penicillin/streptomycin (1%), and heat-inactivated (56 ˚C 1H in oven) fetal calf serum (FCS). Frozen cells were taken from liquid nitrogen and were seeded onto 80 cm^2 plastic flasks and grown to confluence in humidified atmosphere with 5 % CO_2 at 37 ˚C for 5 to 7 days. The cells were removed from the flask through trypsin/EDTA treatment and seeded onto 6-well plates to a final concentration of 9 $*10^5$ cells per well. The cells were at subconfluency stimulated with LPS or swine dust of concentrations of 12.5, 25, 50 and 100 μg/ml in triplicate or quadruplicate and incubated for 8 hours in medium without serum. Control media were prepared from cell-free

dishes in the same manner. The supernatants were collected, centrifuged (1000 g, 10 min at 20 ˚C) and analysed using the ELISA method.

3.2.2. Alveolar Macrophages

The alveolar macrophages (AM) were obtained by BAL from six healthy volunteers. The lavage fluid was immediately centrifuged at 200 g for 10 minutes at 4 ˚C. The cells were then resuspended in RPMI medium supplemented with 5% FCS (heat-inactivated), 1% penicillin/streptomycin + 50 mg/ml gentamycin. A volume of 2 ml containing 10^6 cells was dispensed in each well of 6-well culture plates and incubated for 2 h at 37 ˚C in 5 % CO_2. Non-adherent cells were removed after 2 h through washing with RPMI and the adherent cells were incubated for 18 h. After about 18 hours of incubation, the cells were washed with RPMI medium and stimulated with LPS or swine dust at concentrations of 12.5, 25, 50 and 100 μg/ml, incubated for 8 h in serum-free RPMI medium. The supernatants were collected and centrifuged (1000 g, 10 min at 20 ˚C) and stored at -70 ˚C until assay. Control media were prepared from cell-free dishes in the same manner.

3.3. Statistics

Results are presented as mean± SD (paper I), mean± SEM (paper II, V) and medians of 25th - 75th percentiles (paper I, II, III). Comparisons were performed by the use of the Wilcoxon signed rank test (paper, I, II, III, IV). The differences were considered significant when $p<0.05$. The JMP statistics program was used for calculation of Pearson correlation coefficients and Spearman ρ (rho, non-parametric correlation, pairwise comparisons, as shown in paper II, III, IV). The Statview II software statistics program (Abacus Concepts, Inc.) was used for stepwise multiple regression and linear regression (paper III). In paper V, analysis of variance (ANOVA, Abacus Concepts, Inc) and a Fisher's PLSD test was used.

4. Results

4.1. Human exposure studies

4.1.1. Lung Function and Challenge Test of Subjects

As shown in table 16, the result of the lung function test before and after exposure is slightly different among the subjects. However, the changes are statistically significant. FEV_1 were 5-6% lower at 7 h after the start of the exposure. The lung function results are similar and P value was less than 0.01 since the subjects were overlapped in each study. VC changes were 2-3% lower in the four studies and $p< 0.05$. After exposure, PD_{20} for methacholine dose fell about 4-10 fold ($p< 0.001$) in the test .

4.1.2. Dust Levels

The dust levels were high in our studies. The average airborne inhalable dust concentration was 20-23 mg/m^3. The respirable dust levels were 0.7-1.0 mg/m^3. In paper I, seven subjects were analysed and in paper IV eight subjects were analysed. For the microbial marker, the result in paper III was slightly higher than that in paper II. (table 17).

Table 16. Details of the Subjects Lung function and challenge mean(SD) or median (Q_{25}-Q_{75})

		FEV_1 in % pred value	VC	PD_{20} mg/ml	FEV_1 L	VC L
I**	Before	101 (10)	101 (9)	2.5 (1.2-5.4)	4.2 (0.6)	5.2 (1.1)
	After			0.2 (0.1-0.7)*	3.9 (0.5)*	5.0 (1.1)*
II	Before	101 (11)	101 (10)	5.2 (1.3-24)	4.4 (0.9)	5.4 (1.0)
	After			0.7 (0.2-1.2)*	4.2 (0.9)*	5.3 (1.0)*
III	Before	102 (7)	101 (9)	4.4 (1.3-22)	4.2 (0.8)	5.3 (1.0)
	After			0.5 (0.2-1.0)*	4.0 (0.8)*	5.1 (1.0)*
IV	Before	99 (11)	97 (10)	2.7 (1.1-11)	4.1 (0.9)	5.1 (1.2)
	After			0.3 (0.1-0.7)*	3.9 (0.9)*	5.1 (1.0)*

* mean $P< 0.05$
** Eight subjects performed challenge test 24 hours after exposure and six subjects performed challenge 7 hours after exposure.

4.1.3. Systemic Health Effects

The participants responded to a symptom questionnaire and the symptoms were classified on a five-graded scale (1-5) where 1 denoted no symptom and 5 very strong symptoms. The following table shows symptoms after exposure to swine dust for each study. The subjects with symptoms graded 4 and 5 are counted and listed in table 18.

Oral temperature rose slightly after exposure and reached the highest levels at 7h. Comparing the non-exposure value for each person with the value after exposure, the temperature changes are between 0.6-0.9 ˚C. The results are given in table 19.

Table 17. Exposure levels median (25th -75th percentiles)

	paper I	paper II	paper III	paper IV
Inhalable dust (mg/m^3)	22.5 (20-24)	20.5 (14.6-30)	21 (16-25)	23 (20-30)
Respirable dust (mg/m^3)	0.7 (0.4-1.4)			1.0(0.1-1.2)
Endotoxin (μg/m^3)	1.2 (0.9-1.4)	1.2 (0.8-1.4)	1.2 (0.9-1.4)	1.1(0.8-1.4)
Muramic acid (μg/m^3)		0.9 (0.3-1.9)	1.0 (0.4-1.9)	
Peptidoglycan (μg/m^3)		6.0 (2.0-12.7)	6.5 (2.7-13)	
3-OH fatty acid (μg/m^3)		3.5 (2.2-4.5)	3.9 (2.5-4.9)	

Table 18. Symptoms in each study (according to the subject´s grading)

Symptom	I N=14	II N=21	III N=35	IV N=36
Fever	1 (37.9°C)	3 (>38°C)	4 (>38°C)	5 (>38°C)
Shivering	2	2	4	5
Headache	2		2	3
Malaise	5	4	9	10
Muscle pain	1	1	2	3

Table 19. Temperatures at different time points (Mean±SEM)

Time	I (˚C)	II (˚C)	III (˚C)	IV (˚C)
Before exposure	36.4 (0.12)	36.2 (0.09)	36.3 (0.07)	36.2 (0.09)
After 5 hour	36.3 (0.12)	36.5 (0.09)	36.4 (0.07)	36.5 (0.07)
After 7 hour	37.0 (0.10)	36.7 (0.07)	36.8 (0.06)	36.8 (0.08)
After 24 hour	36.7 (0.2)	36.2 (0.08)	36.3 (0.07)	
Δ temperature	0.6 (0.2)	0.9 (0.14)	0.8 (0.11)	0.9 (0.14)

4.1.4. Relationship between Temperature And Cytokines Changes

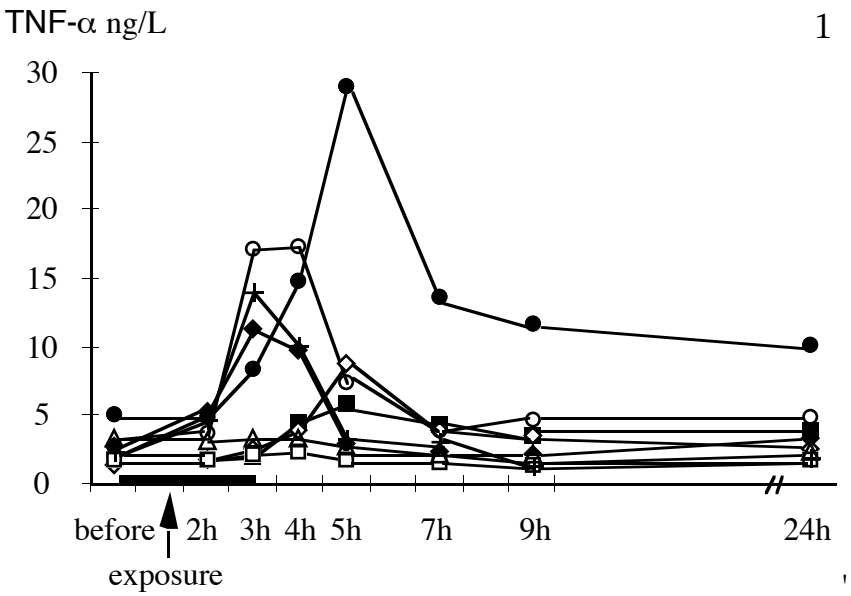
In paper III, nine subjects demonstrated temperature increases that were greater than 1.5 °C. These occurred seven to nine hours after exposure. Four of them had fever (T over 38°C). Two of the four fever subjects had the highest IL-6 levels in all of the exposure population. These two individuals had chill symptoms graded 4 and the temperatures were 38.2 °C and 38.1°C, respectively. IL-6 in serum was increased to 124 ng/L and 128 ng/L. The other two subjects experienced great fatigue and adynamia.

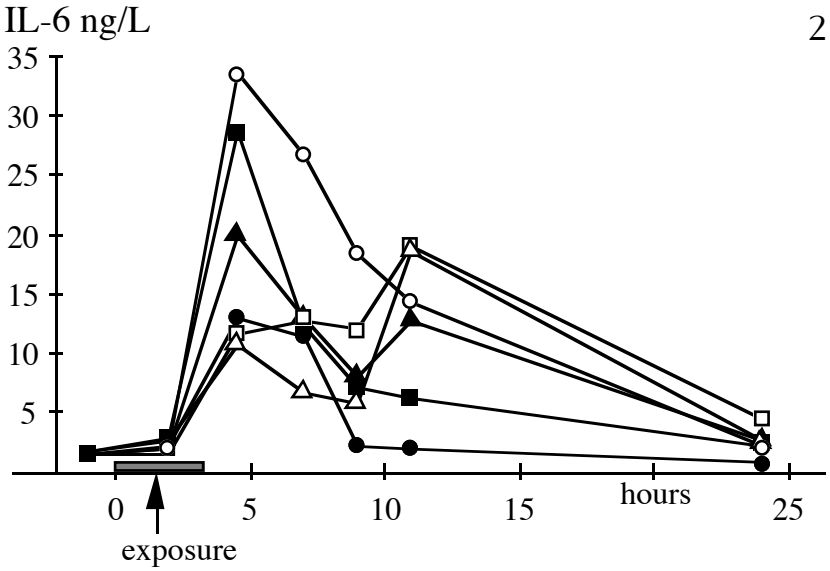
4.1.5. Time Course of Cytokine Release after Inhalation of Swine Dust (paper I and IV)

TNF-α in serum increased from 2.5 (1.8 - 3.1) ng/L (median, 25th-75th percentile) before exposure to maximum values of 10.0 (4.6 - 15.7) ng/L between 3 and 5 h after the start of exposure. IL-6 increased from less than 1.5 to 21.4 (18.6 - 33.6) ng/L 4 to 11 h after the start of exposure. Maximum IL-6 occurred 1 to 5 h after the maximum TNF-α However, in some cases an early increase in IL-6 parallel to the change in TNF-α was observed (the details are shown in paper I, figure 1). There was a significant correlation between maximum TNF-α and IL-6 values (r^2=0.48, P<0.01). TNF-α levels returned to baseline 7 hours after exposure. The levels of IL-6 were still significantly increased at 12 hours (P<0.01), but not 24 hours after the start of exposure (figure11- 1, 2).

Peak levels of TNF-α and IL-6 were reached at 4 (3-5) and 7 (4-9) hours after the start of the three hours period of exposure respectively. Although the TNF peak always preceded maximal IL-6 values, smaller increases in IL-6 parallel to the TNF change were observed. There was a considerable variability in the temporal relations between TNF and IL-6. Thus peak levels of TNF-α preceded the maximum IL-6 level by only a 1 1/2 hour difference in some cases and up to five hours difference in other cases.

IL-1ß in PBMC increased from 1.6 (1.3-2.5) ng/L (median, 25th-75th percentile) before exposure to 4.3 (2.7-5.2) ng/L at 7 h after the start of exposure. Maximum IL-1ß occurred at 4 to 7 h after the exposure. The level increased significantly at 3, 4 and 7 h, but not at 24 h (figure 11-3).


TNF-α ng/L
1
30
25
20
15
10
5
0
before
exposure
2h
3h
4h
5h
7h
9h
24h


IL-6 ng/L
2
35
30
25
20
15
10
5
0
exposure
5
10
15
hours
25

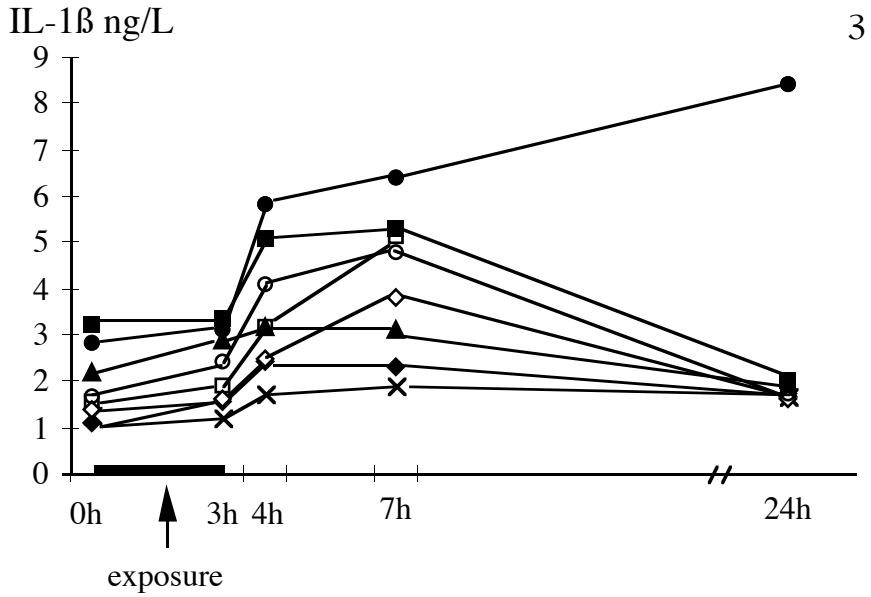


Figure 11. TNF-α (n=8), IL-6 (n=6) in serum and IL-1ß (n=8) in PBMC fraction in previously non-exposed subjects at different times before and after the start of the three hours exposure to swine dust.

4.1.6. Cytokine Release in Upper and Lower Airways (paper II)

IL-6 increased approximately 25-fold (from 0.43 (0.21-0.78) ng/L to 11.6 (8.1-21.0) ng/L, P<0.001) in BAL fluid and 15-fold (from 1.4 (0.6-2.6) ng/l to 21 (14-40) ng/L, P<0.001) in NAL fluid after exposure to swine dust. TNF-α in BAL and NAL fluid was below detection limits (0.25 ng/L) in most subjects before exposure and increased to 3.8 (2.4-5.7) ng/L and 1.3 (0.6-2.3) ng/L respectively, after exposure (P< 0.001). In BAL fluid there was a slight but significant increase in IL-1α and IL-1ß (P< 0.005), while the increases in NAL fluid were more pronounced; medians of 3.2 (2.2-5.8) to 6.0 (5.6-9.3) ng/l (P< 0.01) and 2.6 (0.8-3.9) to 8.5 (6.2-19.8) ng/L (P<0.001) respectively. See figure 12.

4.1.7. Cytokine Relationship in Different Body Fluids

In paper II IL-6 values in BAL fluid collected 24 hours after exposure were compared with serum values obtained at the same time point. IL-6 in NAL collected at 7 h after the start of the exposure were compared with serum collected 7h after exposure in spite of a marker dilution of the biological fluid collected in NAL and BAL fluids. The IL-6 values were higher than in serum (figure 13).

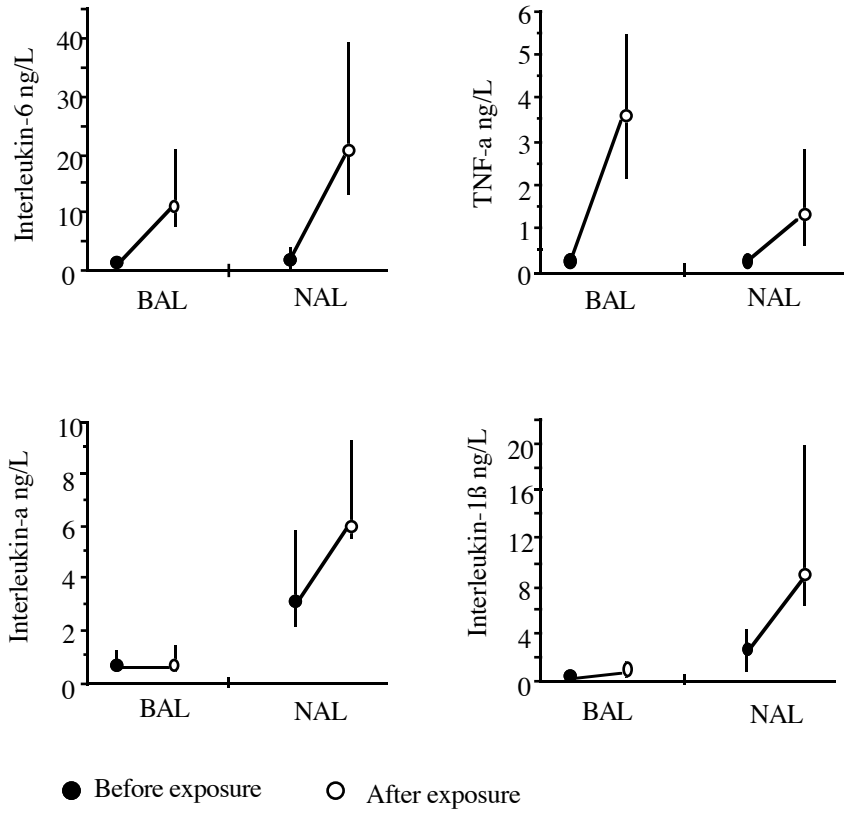


Figure 12. Cytokine concentration in BAL and NAL. BAL sample was taken at 24 h and NAL sample was taken at 7 h after the start of exposure. Median and 25th-75th percentiles are presented.

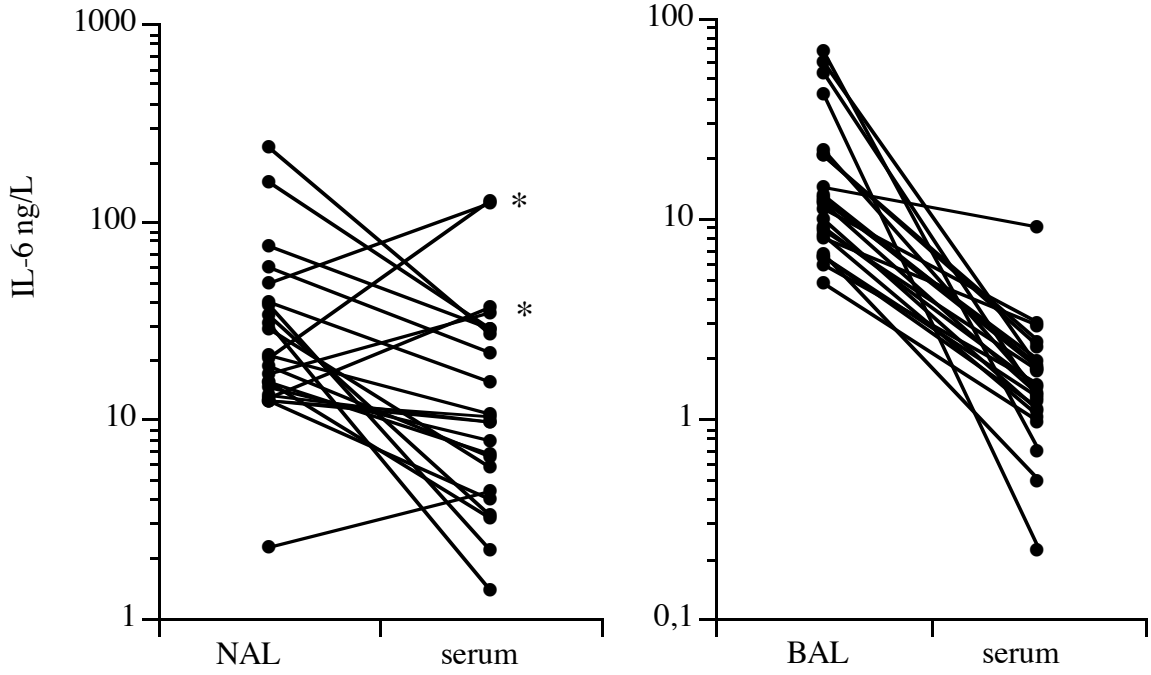


Figure 13. IL-6 derived from NAL and blood (paired data) at seven hours after exposure. IL-6 derived from BAL and blood (paired data) at twenty-four hours after exposure. * denote subject have the fever (4 cases)

4.1.8. Correlations

4.1.8.1. With microbial markers

All markers for exposure showed significant positive correlations with increases of IL-6 in serum according to the non-parametric Spearman test. The three markers for microbial exposure showed statistically significant correlations with at least one additional health effect, but the amount of inhalable dust did not correlate significantly with any other health effect according to the Spearman test (III table 2).

Endotoxin generally gave higher correlation coefficients than those of 3-OH Fatty acid, and demonstrated the highest linear correlation with IL-6. Endotoxin also correlated significantly with symptoms and the lung function measures VC and PD_{20} FEV_1. Muramic acid correlated with the temperature change at 7 hours and with changes in peripheral blood granulocytes (and IL-6 as described above), but did not correlate significantly with symptoms and lung function changes. There were moderate correlations between different markers for health effects within subjects (III table 2).

4.1.8.2. With health effect

Significant correlations were found for all combinations of change in VC, $PD_{20}FEV_1$, IL-6 and neutrophils, except for the change in VC and neutrophils, which did not quite reach significant levels (paper III, table 3). The 7 h temperature correlated with maximal IL-6 levels after exposure, $p<0.001$ (figure 14).

The peptidoglycan concentration in the filter sample also correlated with the temperature change at 7 h (paper III, figure 3).

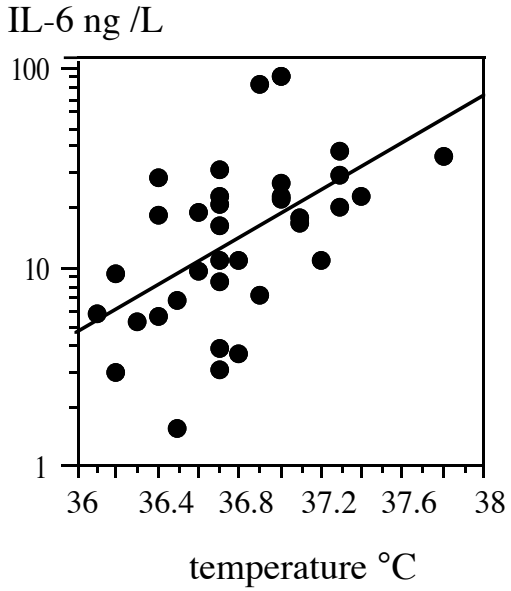


Figure 14. Relationship between maximum IL-6 value and 7 hour oral temperature (°C)
R^2 = .287 p< 0.01

4.1.8.3. In BAL fluids

There was a significant correlation between post-exposure IL-6 levels in BAL fluid and the endotoxin activity (Rho=0.49; P< 0.05) and 3-OH fatty acid concentrations (Rho=0.47; p< 0.05) of inhaled dust. No other significant correlations were found between exposure and the cytokine response in lower or upper airways. A weak but significant correlation was found between the increase in albumin in BAL fluid and the endotoxin activity in inhaled dust (Rho=0.48, p< 0.05). Post-exposure levels of IL-6 and TNF-α in BAL fluid were significantly correlated with the increase of granulocytes (Rho=0.55; p< 0.02 for both).

4.1.8.4. With IL-1 family

The endotoxin concentration correlated with IL-1ß in PBMC and the respirable dust concentration correlated with IL-1ß in plasma. IL-1ß also correlated with $PD_{20}FEV_1$, temperature change and leukocyte count (Paper IV, table 4 and figure 15).

The concentration of inhalable dust correlated with IL-1ra at 7 h. The TNF-α and IL-6 also correlated with IL-1ra (figure 16). IL-1ra was correlated with challenge test, temperature and white blood cell count. The details are given in paper IV, table 4.

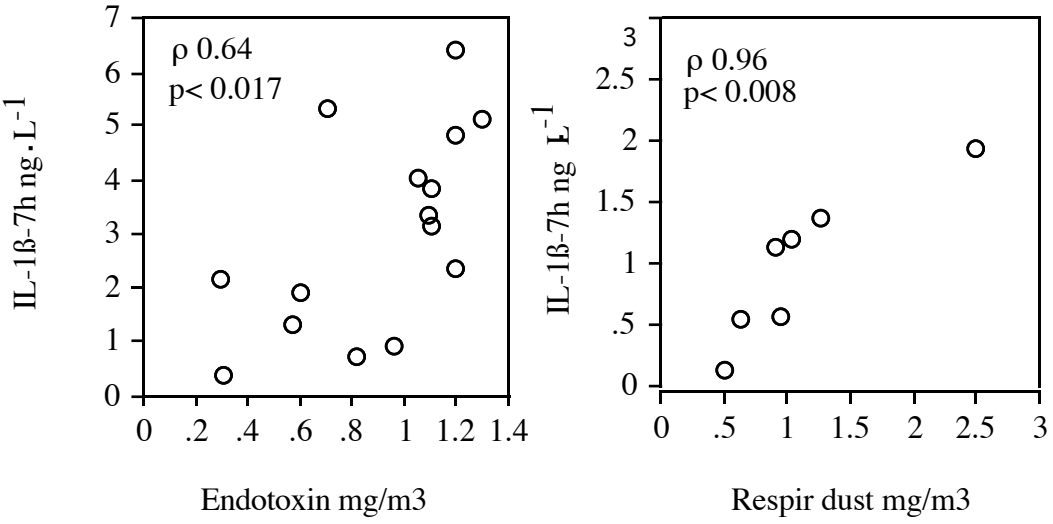


Figure 15. IL-1ß correlated with respirable dust and endotoxin

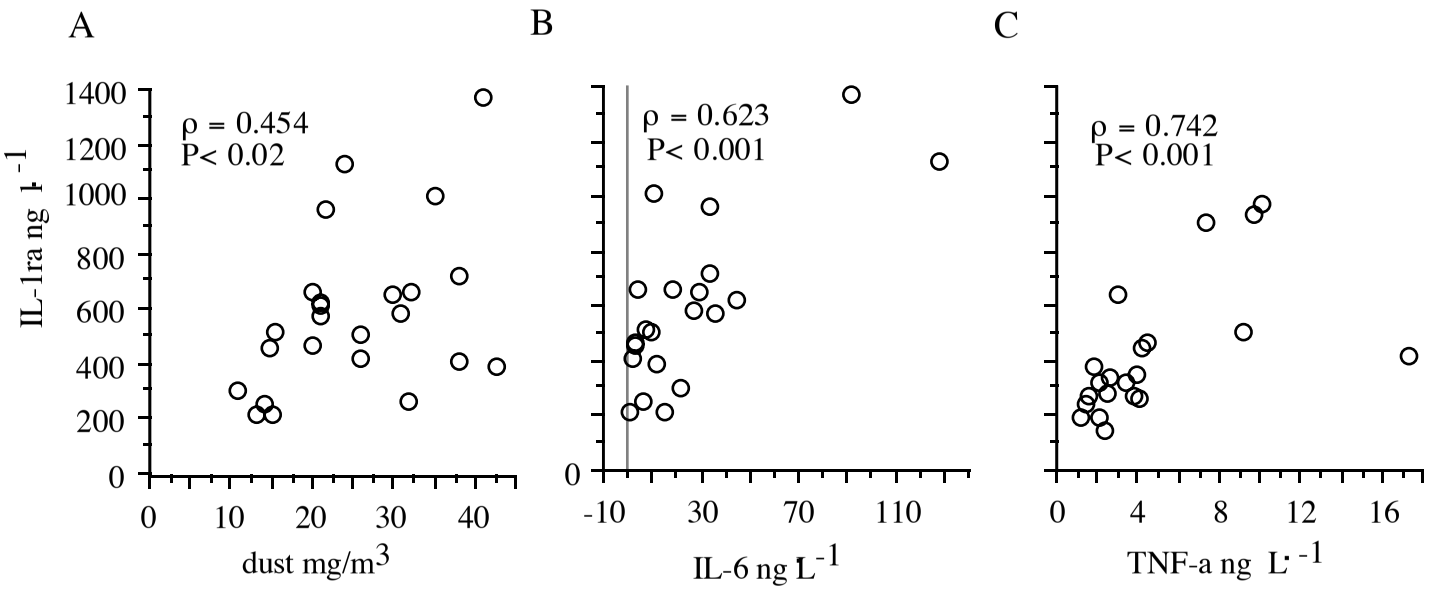


Figure 16. Correlation between the IL-1ra concentration and inhalable dust, IL-6 and TNF-a.

4.1.9. Acute Phase Proteins and Albumin

In the paper I, serum CRP, orosomucoid and haptoglobin levels were significantly elevated 12 to 24 hours after exposure. This is shown in table 20.

The albumin concentration in BAL and NAL fluid approximately doubled, respectively. See table 21.

4.1.10. Leukocytes Response in Peripheral Blood Cells, BAL and NAL.

The peripheral blood leukocyte count reached maximum levels 7 hours after the start of exposure, mainly due to a rise in the concentration of granulocytes (table 22).

The granulocyte concentration in BAL fluid increased more than 50-fold. The number of lymphocytes and monocytes was more than doubled following exposure, $p<0.01$ and 0.001, respectively. The total cells in NAL fluid increased after exposure. Prior to exposure, differential count NAL was possible only on 13 subjects. The number cells increased, see table 23.

4.1.11. Mononuclear Response in PBMC Fraction

In the mononuclear cell fraction (n=8), the lymphocytes decreased and the monocytes increased. The lymphocytes decreased from 2.3 ±0.2 (mean±SE) at 0 h, to $1.6(\pm0.2)*10^9$ cell /L at 7 h after the start exposure ($p< 0.01$). The monocytes increased from 0.5±0.1 at 0 h, to $0.7(\pm0.1)*10^9$ cell /L at 7 h after the start exposure ($p< 0.01$). The mononuclear percent, see also paper IV, figure 1.

Table 20. Measurement of CRP, orosomucoid, and haptoglobin in serum. Analyses before exposure time, 12 and 24 hours after exposure .

Time	C R P mg/L n = 14	Orosomucoid g/L n = 14	Haptoglobin g/L n = 14
before	0.90±1.10	0.74±0.13	1.15±0.23
12 hour	1.87±1.57 *	0.79±0.16 **	1.16±0.24
24 hour	10.0±8.42 **	0.87±0.15 **	1.38±0.21 **

* P< 0.05 compared with pre-exposure values.
** P< 0.01 compared with pre-exposure values.

Table 21. Albumin levels in BAL and NAL (paper II)

	Before mg.L -1	After mg.L -1
BAL n= 22	20.73 (±8.6)	40.6 * (±17.2)
NAL n= 16	24.5 (±24.2)	51.7 * (±72.5)

BAL sample is collected 24 hours after exposure. and NAL sample is collected 7 hours after exposure. * p< 0.001 compared with non-exposure value.

Table 22. The change of peripheral blood leukocyte count

		Granulocyte			Lymphocyte			Monocyte		
		0 h	7 h	24 h	0 h	7 h	24 h	0 h	7 h	24 h
		(L/10*9)			(L/10*9)			(L/10*9)		
Paper I n=12	Mean	**2.6**	**7.1**	**4.6**	**1.8**	**1.3**	**1.6**	**0.3**	**0.5**	**0.4**
	SD	(0.8)	(1.5)	(1.9)	(0.3)	(0.3)	(0.3)	0.1)	(0.1)	(0.1)
Paper II n=22		**2.4**	**6.3**	**3.8**	**1.8**	**1.3**	**1.4**	**0.3**	**0.5**	**0.4**
		(0.6)	(2.4)	(1.5)	(0.4)	(0.4)	(0.3)	(0.1)	(0.2)	(0.1)
Paper III n=38		**2.4**	**6.6**	**4.1**	**1.8**	**1.3**	**1.5**	**0.3**	**0.5**	**0.4**
		(0.7)	(2.0)	(1.6)	(0.3)	(0.4)	(0.3)	(0.1)	(0.2)	(0.1)
Paper IV n=32		**2.3**	**6.6**	**3.9**	**1.7**	**1.4**	**1.5**	**0.4**	**0.5**	**0.4**
		(0.7)	(2.0)	(1.4)	(0.5)	(0.5)	(0.5)	(0.1)	(0.2)	(0.1)

Table 23. Leukocyte count in BAL and NAL fluid before and after exposure (paper II)

	BAL		NAL	
	Cell count 10*6/L		Cell count 10*6/L	
	before	after	before	after
Granulocyte Median	1.9	107	2.7	121*
25-75	(1.0-2.4)	(53-199)	(0.3-13.1)	(45-229)
Lymphocyte	4.9	10.0		
	(3.1-8.7)	(6.3-19.9)		
Monocyte	85	188		
	(68-99)	(133-275)		
Total cell	91.8	306	3.6	79**
	(47-95)	(157-315)	(1.8-7.4)	(35-185)

* N=13, **N=22

4.2. *In vitro* experiment

Swine dust, but not LPS, induced a marked dose-dependent release of IL-6 from epithelial cells. There was no IL-6 activity in cell free control samples. TNF-α and IL-1ß were not detected in epithelial cell supernatants, following either swine dust or LPS exposure. After 8 or 24-hour incubation with rTNF-α or rIL-1ß, TNF-α recovery was 80-84 % and the IL-1ß recovery was 74-84 %.

Compared to the unstimulated control situation, swine dust induced a 500-fold increase, TNF-α induced a 200-fold increase and a combination of swine dust and TNF-α caused a 1700-fold of IL-6 (paper V, Fig 2). However, IL-1ß did not induce IL-6 production in epithelial cells and neither did a combination of IL-1ß and swine dust .

Swine dust and LPS caused a dose-related increase of IL-6, IL-1ß and TNF-α in AM (Fig 17). Control media without cells but identically incubated with swine dust or LPS did not show any cytokine activity. TNF-α was significantly increased at all the dose levels following swine dust and LPS exposure, LPS being significantly more potent than swine dust . At the two high dose concentrations, LPS was significantly more potent than swine dust at inducing IL-6 secretion from AM. Swine dust and LPS were equally potent induces of IL-1ß in alveolar macrophages.

4.3. Methodology

There was no endotoxin has been detected in evacuated blood collection tubes.
For our IL-6 assay the minimum detectable concentration using diluent RDF6 was found to be 1.5 ng/L (figure 18).

The relationship between the EASIA™ and Quantikine™ kits are given in figure 19. These two kits are made up of exactly the same protein in different amounts and the antibodies used in the kits are also the same. The only difference between the kits is the amplification stage, which is present in the HS kit to provide extra sensitivity (as referred to by R&D System). We made the analysis using the Quantikine™ kit for the after exposure high value .

Our result showed that the best IL-1ß signal was obtained by use of the PBMC method. The fresh PBMC, which gave the best signal after two cycles of freezing (to create intercellular IL-1ß release), was used. The values detected by use of chloroform extraction and poor-platelet plasma were identical (figure 20).

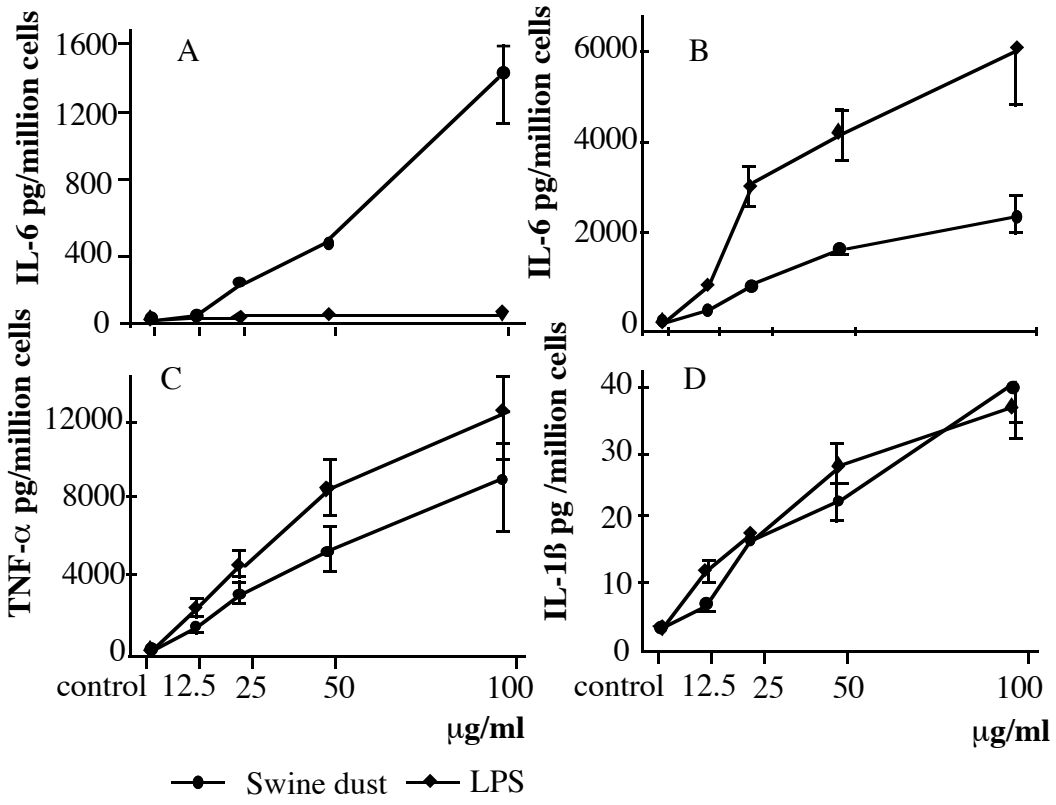
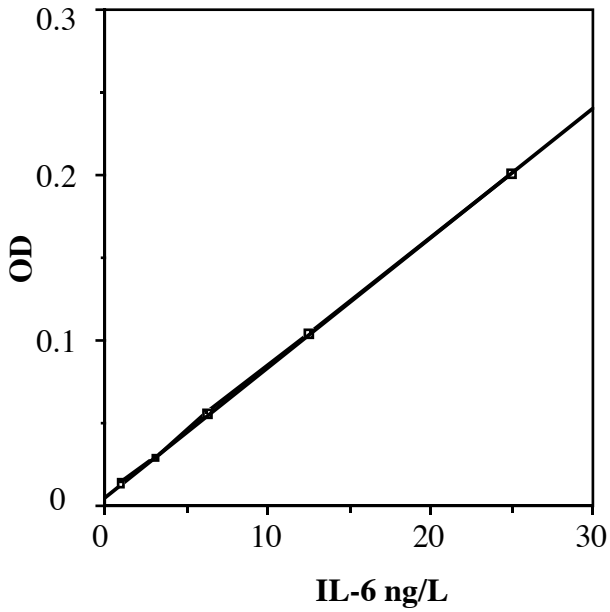


Figure 17. A) IL-6 produced in epithelial cells, B) IL-6 produced in alveolar macrophage, C) TNF-α produced in alveolar macrophages and D) IL-1ß produced in alveolar macrophage.



y = 5.8460e-3 + 7.8203e-3x R^2 = 1.000

Figure 18. IL-6 sensitivity in R&D system kit (n=20)

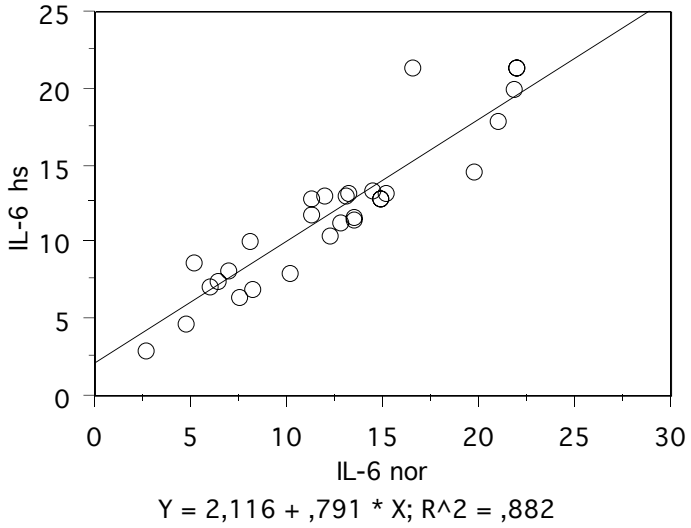


Figure 19. High sensitivity kit with Quantikine kit comparison

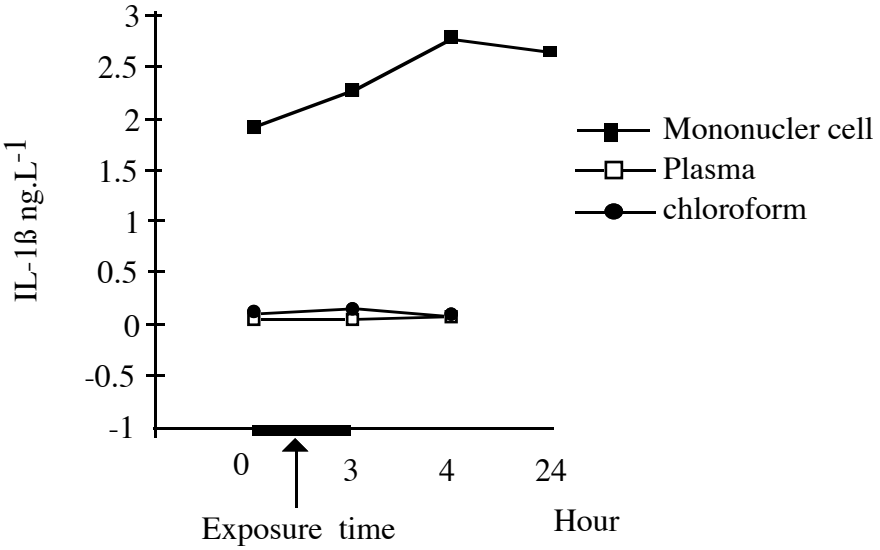


Figure 20. Comparison of three methods for IL-1ß measurement in blood following inhalation of swine dust. The samples were from the same subject. Mononuclear cells were isolated using the Ficoll-Paque method (close squares). Plasma was centrifugated by taking away the platelet (unfilled squares). Plasma was extracted using chloroform (close circles). IL-1ß assayed by ELISA. Results are expressed by mean value of the 8 samples.

5. Discussion

Inhalation of dust generated during the weighing of swine causes acute inflammatory changes in the airways and systemic effects such as malaise and fever. The condition is often called ODTS and is well known from other environments such as in cotton cardrooms ("mill fever"), and in grain handling ("grain fever") (121, 122). The present study was focused on the proinflammatory cytokines IL-1, IL-6 and TNF-α in this condition. The concentrations of these acute phase cytokines were studied in airway and alveolar lining fluid and in peripheral blood. and time course of appearance of the cytokines was studied in blood, as well as the relationship with exposure and with local and general symptoms. The role of endotoxin and other possible agents was studied both in human exposure studies and *in vitro*, and finally the production of these cytokines by alveolar macrophages and an epithelial cell line were compared after addition of swine dust in vitro.

In order to ensure reliable results, several measures were taken. Blood was collected using evacuated collecting tubes. Random and only selected tubes were analysed for endotoxin, and all were negative. The same pairs of monoclonal antibodies (R&D) were used throughout the studies. For some purposes an amplified method was used, however, using the same monoclonal antibody. Comparison of 30 samples analysed with the regular or the amplified method show good correlation. Methods to measure IL-1ß were only available in the late phase of the study. Three different methods were used. Analysis of chloroform extracted plasma, analysis of platelet poor plasma (123) and analysis of the PBMC fraction isolated (89, 124) using density-grade centrifugation of blood. Since clotting protein may inhibit IL-1ß (116, 118)., plasma was immediately separated for use. The chloroform extraction method and the fresh platelet poor plasma samples gave similar IL-1ß results, which however were below the detection limit of the method in pre-exposure blood samples. The PBMC method gave higher values, detectable also in pre-exposure blood.

In the present study the proinflammatory cytokines all increased in peripheral blood, NAL and BAL fluid, with much higher concentrations in BAL than in blood. The concentration of the cytokines correlated with exposure intensity and with local and general effects such as increase in acute phase proteins (CRP, orosomucoid and haptoglobin), a well known effect of the acute phase cytokines (125, 126). TNF-α, IL-6, IL-1ß and IL-1ra in peripheral blood increased in all subjects. Peak levels of TNF-α and IL-6 were reached respectively at 4 (3-5) and 7 (4-9) hours after the start of the 3 h period of exposure. Although the TNF peak always preceded maximal IL-6 values, there were signs of a smaller increase in IL-6 that was parallel to the TNF-α change. There was a considerable variability in the temporal relations between TNF-α and IL-6. Thus peak levels of TNF-α preceded the maximum IL-6 level, in some cases by only one and half hour, and

in others up to five hours (paper I). There was also an indication of a late phase of IL-6 release following swine dust exposure in several subjects. There are several sources that contribute to cytokine release following inhalation of swine dust. Alveolar macrophages and epithelial cells have been induced to produce and release cytokines (127). An early source might be release of pro-inflammatory cytokines in airway mast cells (128), and possibly cells recruited to the airways may contribute as late sources. TNF−α is a potent trigger for IL-6 release *in vivo* and *in vitro* (129). The positive correlation of the maximum values of IL-6 and TNF−α found in the present study are consistent with the observation that IL-6 can be induced by TNF−α. However, the apparent biphasic peaks of IL-6 and the variable temporal relationship between the peak values of TNF−α and IL-6 suggests that other mechanisms than TNF−α inducing IL-6 synthesis contribute to the release of IL-6 as well.

IL-1ra reached peak level at 3-6h and remained elevated at 24 h in administration of LPS in rabbits (130). Our experimental results show that IL-1ra levels are higher at 7 h than at 4 h after the start of exposure. Time course studies indicate that PBMC IL-1ß was elevated 3 h after exposure to swine dust, with peak values after 4 to 7 h (paper IV). IL-1ß time curve was similar with the kinetics observed for IL-1ß mRNA in purified monocytes and the whole blood culture (103). From the whole blood culture it is known that IL-1ß mRNA was present quite early after stimulation, peak levels were sustained at least 6 hours after stimulation, and mRNA still existed at 18 h (131).

Inhaled swine dust also causes a release of the pro-inflammatory cytokines, TNF-α, IL-6, IL-1-α and IL-1β in the upper and lower airways. There are many cell types such as monocytes, macrophages, lymphocytes, fibroblasts, neutrophils, epithelial and endothelial cells in the airways which are capable of IL-1 production (132). Both IL-1α and IL-1β could be produced by one cell type but there are also cells which are specialized to produce IL-1 of one or the other type. Thus monocytes/macrophages are the major sources for IL-1β (89, 133)(23) while T cells are predominantly IL-1α producers (134). IL-6 is produced by epithelial and endothelial cells, monocytes/ macrophages and lymphocytes (135). Alveolar macrophages are the major source of TNF-α in the lower airways (136).

It is not clear which cells are responsible for the cytokine production in the nose. Nasal epithelial cells may be one of the sources for IL-1 and TNF-α (127) In the present study an clear increase neutrophils was found in nasal lavage fluid following exposure. Other cells like macrophages, dendritic cells, lymphocytes and mast cells may also be of importance as sources of cytokines (137) in the present study.

The pro-inflammatory cytokine family, including IL-1, TNF-α and IL-6, share many overlapping biologic functions. High levels of IL-6 signals can contributes to the inflammatory reaction and reflect the production of biologically active IL-1 and TNF (138, 139). TNF and IL-1 were important in the reaction of inflammation and pathogenesis of airway disease. IL-1ra and IL-1 measurement

can explain the symptoms, for example, fever, chill, myalgia, and malaise. Elevated leukocyte count may increase serum cytokine concentrations, since leukocytes are major secretors of cytokines (140).

Swine farmers have an increased number of cells and an increased proportion of neutrophils in bronchoalveolar lavage fluid, indicating alveolitis (141). Similar but more pronounced cellular changes in bronchoalveolar lavage fluid were observed following exposure to dust in a swine confinement building also in subjects not previously exposed to swine dust (142, 143). The present study demonstrated a high levels of TNF-α and IL-1 in BAL and NAL These cytokines act synergistically on the stimulation of polymorphonuclear neutrophilic granulocyte migration (144) and stimulate vascular endothelial cells to promote transendothelial passage of neutrophils, albumin, and fluid in vitro and in animal experiments (145-147). TNF-α, IL-1 and LPS induce the expression of the adhesion molecules ICAM-1, VCAM-1 and E-selectin on endothelial cells promoting adhesion to granulocytes, monocytes and lymphocytes (148-150). The production of TNF-α and IL-1 in the upper and lower airways could thus contribute to the influx of inflammatory cells following exposure. We have also found high levels of IL-8 in NAL and BAL (151). IL-8 is a potent neutrophil chemotactic and activating factor(152, 153).

Inhalation of swine dust caused a marked increase in bronchical responsiveness (154). Our studies confirmed these results: post exposure PD $_{20}$ FEV$_1$ were < 8% of the pre-exposure value. The FEV$_1$ decreased approximately 5 % and VC 2 % in our studies. Pig-derived material, such as dander and faeces, some irritate gases and TNF–α may contribute to increase bronchial responsiveness, as well as for example mast cell products like cysteinyl leukotriene (155-157).

It is not clear which components in swine dust are responsible for the inflammatory reaction. Air sampled from pig confinement building contains grain dust, amonia, fungi and bacteria, mostly Gram positive but also Gram negative (22). Lipopolysaccharide (LPS) from endotoxin in Gram negative bacteria is a potent stimulus for cytokine production in alveolar macrophages and inhalation of endotoxin induces airway inflammation with a cellular reaction dominated by neutrophils (158) Although endotoxin could be responsible for the airway reaction there are findings indicating that other components probably are of importance. Studies in our laboratory have shown that swine dust is more potent than LPS in stimulating the release of IL-6 and IL-8 from epithelial cells (159) while LPS seems to be a more potent stimulus than swine dust inducing production of those cytokines in alveolar macrophages

The first report on possible etiologic agents was published by Neal and co-workers in 1942 (160). They studied fever among rural mattress workers and provided good evidence that toxic agents from Gram-negative bacteria, "endotoxin", could be a causative factor. Inhalation of endotoxin can reproduce most of the symptoms of ODTS (161).

The present studies dust exposure was very high, above the Swedish threshold limit value (5 mg/m^3) for organic dust. The procedure of weighing pigs causes

much agitation and results in the aerosolisation of dust from finely milled feed and faecal products. The respirable fraction of the dust, measured with the personal Cyclone sampler, constituted about 5 % of the inhalable dust fraction, suggesting that a significant proportion of the dust may reach the alveoli. However, measurement of BAL cytokines suggests considerable involvement also of the peripheral airways. Dust deposited in the airways may interact with airway epithelial cells, which are known to be important sources of inflammatory cytokines, and may contribute to induce and sustain inflammatory reactions (95).

The swine faeces contains Gram-positive and Gram-negative bacteria both of which contain peptidoglycans. Both Gram-positive and Gram-negative bacteria contain soluble cytoplasmic components, which may induce cell activation *in vitro*. Gram-positive bacterial cell walls contain many elements that may influence airway cells, including peptidoglycans, teichuronic acid and formyl-methionyl-leucyl-like peptides (fmlp) (162). The dose of inhaled endotoxin required to cause ODTS appears to be much higher than the amount of endotoxin inhaled in swine dust causing similar symptoms (163). In experiments where aerosols of endotoxin or Gram-negative bacteria are inhaled, the endotoxin dose must be in the order of 30-100 μg to produce symptoms. The aerosol size used in these experiments favors alveolar deposition and interaction between endotoxin and alveolar macrophages. In the present study, the average dose of inhaled endotoxin was about 4 μg (assuming a ventilation of 1 m^3) per hour. Even the estimate for the total mass of inhalable LPS as calculated from the 3-OH fatty acid content (about 16 μg) appears insufficient.

Endotoxin reflects "biological activity" of Lipopolysaccharides (LPS) in Gram-negative bacteria and 3-OH fatty acids is a marker for total amount of LPS. Muramic acid is a marker for peptidoglycans in cell walls of Gram-positive and Gram-negative bacteria. In the present study, markers of microbial contaminants present in μg-quantities possessed a stronger relationship with health effects than did the mass of inhaled dust in mg-quantities. Most of the mass of inhaled dust is probably made up by particles from finely milled barley, but numerous other constituents are also present. One such component is bacteria from, for example, swine faeces. These are both Gram-negative and Gram-positive, and probably many are present as non-viable bacterial fragments. Preliminary data indicate that the concentration of peptidoglycan in 9 bacterial strains (G- and G+) varied between 0.25-6.4 % of the dry weight. The concentration of peptidoglycans in swine dust samples varied between 0.01-0.23 % of the dry weight suggesting that bacteria could make up about 4 % of the swine dust mass. Muramic acid is a prominent component of Gram-positive bacteria, but is also present in Gram-negative bacteria, although in lesser amounts. The present study suggests that some of the health effects were related to mainly Gram-negative markers, while other effects were correlated with muramic acid, but not with endotoxin or 3-OH fatty acid. The correlation between exposure markers and health effects were generally rather weak, which reduces the strength of the evidence. Still, the results are more compatible with hypothesis that also other agents than LPS contribute to

the health effects, rather than supporting an hypothesis that LPS is the sole responsible agent. The data also suggests that different health effects may be influenced by different components in the dust, rather than one agent causing all effects.

Swine dust, but not LPS, induces a IL-6 release from an epithelial cell line (A549). The cytokine concentrations in the cell culture supernatant increased in a dose-effect manner. The surface epithelium plays an important role in the repair of tissue damage inflicted by infection, inhaled particles and inflammation. Secretions of cytokines have an important role to repair bronchial tissue damage. The cytokine response to LPS for different epithelial cells difference. Most carcinoma epithelial cells have no TNF-α production (164). A549 cell, derived from alveolar epithelial cells of a cancer patient, has been used as a model system for investigation of human alveolar epithelium.

We have found that both Gram-positive (*Bacillus subtil*is, *Staphylococcus hominis*, *S.Lentus*, *Micrococcus luteus*) and Gram-negative (E.coli) bacteria in swine dust induce IL-8 (165) and IL-6 secretion in epithelial cells. Swine dust contains different bacteria, both dead and cultivable. The cultivable flora is dominated by Gram-positive bacteria (22). In a human exposure study, bacterial markers showed better correlation with IL-6 changes in serum than did the inhalable dust concentration. Markers for both G+ and G- bacteria (muramic acid and 3-OH Fatty acids) correlated significantly with increase in serum IL-6 (166).

LPS is an abundant component of the outer membrane of Gram-negative bacteria (167). LPS triggers the production of TNF-α, IL-1ß, IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in monocytes, endothelial cells (168-171) and alveolar macrophages (172). In the present study, LPS was used as a reference agent. LPS did, however, not induce IL-6 release from the A549 epithelial cell line used in the present study. The lack of a LPS-induced effect on epithelial cells may be due to the absence of surface CD-14 receptor on these cells (173). In a previous study swine dust induced a 10-fold greater IL-8 production in normal human bronchial epithelial cells in primary culture compared with our A549 cell line. Thus the A549 epithelial cell line may not be representative of human epithelial cells in all respects. It is, however, interesting that swine dust was a powerful inducer of IL-6 in the A549 cell line.

The alveolar macrophage plays a central role in the defence against invading microorganisms (174). Alveolar macrophage produces chemokines that recruit neutrophils from the bloodstream. Alveolar macrophage releases IL-1, IL-6 and TNF-α when cultured together with swine dust or LPS in the present study. It is interesting to note that swine dust was almost equipotent on a weight basis compared with LPS. Since the endotoxin concentration in the swine dust extract was only 2.6 ng/100 µg, our findings cannot be explained by the endotoxin content in the dust. This suggests that one or more very potent agents must be present in the swine dust. These agents may be derived from bacteria or from other components in the dust.

6. Conclusions

• Healthy subjects who have no previous exposure to farm dust or swine dust display elevated serum IL-6 and TNF-α levels, following 3 hours inhale swine of dust.
• The IL-1 family participates in and modulates the inflammatory response together with IL-6 and TNF-α.

• The levels of the blood cytokines change correlated with the systemic effects, suggesting that the serum cytokine concentrations determined may explain the transient fever, malaise, myalgia and other symptoms in ODTS.

• The exposure to swine dust causes an intense airway inflammation which involves the pro-inflammatory cytokines IL-1, IL-6 and TNF-α in bronchoalveolar and nasal lavage.

• The serum IL-6 concentration correlates with exposure to the swine dust. The changes in cytokines and inflammatory parameters correlated better with markers of bacterial contaminants than with total dust.

• Both Gram-positive and Gram-negative bacteria may play a role in the induction of airway inflammation and systemic health effects.

• Swine dust activates a cytokine response in epithelial cells and alveolar macrophages. The epithelial cell has more limited cytokine response profile than have alveolar macrophages. These two type cells may contribute to the lower airways inflammatory reaction to swine dust.

• In the epithelial cell line swine dust was a more potent stimulus for IL-6 release than LPS.

7. Summary

Zhiping Wang. Acute Cytokine Responses to Inhaled Swine Confinement Building Dust. *Arbete och Hälsa* 1997; 23 1-61.

Inhalation of swine house dust (swine dust) may cause an acute inflammatory reaction known as Organic Dust Toxic Syndrome (ODTS). The reaction does not require sensitisation and is associated with airway inflammation, general symptoms, and slight spirometric changes. The agents causing this reaction are not known. The purpose of this study was to investigate the release of pro-inflammatory cytokines in the airways and peripheral blood following acute exposure to swine dust, the correlation between markers for microbial contaminants in the dust, cytokine responses and health effects, and the release of those cytokines from an epithelial cell line (EP) and from human alveolar macrophages (AM) stimulated by swine dust and LPS.

Healthy subjects, previously unengaged in farm work were exposed to swine dust for 3-4 hours. Interleukin (IL) -6, IL-1 receptor antagonist (ra) and tumour necrosis factor (TNF)-α in serum, and IL-1ß in peripheral blood mononuclear cell (PBMC) were measured prior to and after exposure. IL-1α, IL-1ß, IL-6 and TNF-α were also measured in nasal lavage (NAL) fluid and bronchoalveolar lavage (BAL) before and after exposure. The time curves were done for IL-6, TNF-α and IL-1ß. The mass of inhaled dust, the endotoxin concentration and two markers (muramic acid is a marker for total peptidoglycan content and 3-(OH)-fatty acid is a marker for total lipopolysaccharide content) for microbial exposure were quantified. Spirometry and a methacholine bronchial challenge were performed before and 7 h after exposure to swine dust. Granulocytes, monocytes, and lymphocytes were measured in blood with flow cytometry. NAL, BAL and PBMC cells were measured with colour stain. The oral temperature and general symptoms were recorded.

Exposure to swine dust caused fever, headache, malaise, increased bronchial responsiveness, and a slight decrease in FEV1 and VC. IL-1ra, IL-6 and TNF-α increased significantly in serum, IL-1ß increased significantly in PBMC and plasma, IL-1, IL-6 and TNF-α increased significantly in BAL and NAL fluids. There was a marked influx of inflammatory cells, especially of granulocytes. In peripheral blood, TNF-α, IL-6 and IL-1ß increased and peaked during 3-5 h, 5-7 h and 3-7 h respectively, after the start of exposure. The leukocytes doubled and monocytes increased slightly 6-8 h after exposure. Endotoxin, muramic acid, and 3-OH fatty acid correlated significantly with increase in serum IL-6. Inhalable dust correlated with increase in serum IL-1ra, and endotoxin correlated with increase IL-1ß in PBMC. Endotoxin and 3-OH fatty acids also correlated significantly with the IL-6 increase in BAL. Bacterial markers showed better correlation with IL-6 changes than with total dust concentrations. LPS correlated with symptoms and with lung function changes. Peptidoglycan correlated with increase in blood granulocytes and body temperature.

Swine dust caused a dose-dependent increase of IL-1ß, IL-6 and TNF-α in AM. No increase of IL-1ß or TNF-α, but a clear increase in IL-6 was found in EP. LPS caused a clear increase in all three cytokines in AM, but none of the cytokines in EP.

In conclusion, acute inhalation of dust contaminated with bacterial debris increased the concentration of pro-inflammatory cytokines in BAL, NAL, PBMC and peripheral blood. The changes in cytokines and inflammatory parameters correlated better with markers of bacterial contaminants than with total dust

amounts. A marker for bacterial peptidoglycan showed better correlation with some changes than endotoxin did, suggesting that several bacterial constituents may play a role in ODTS. The results of in vitro tests suggest that swine dust activated a cytokine response in EP and AM. The EP had more a limited cytokine response profile than AM. In EP, swine dust is a more potent stimulus for cytokine release than LPS. Both cells can contribute to inflammatory reaction after inhalation of swine dust, and several agents including LPS may play an important role in cytokine release.

8. Sammanfattning (Summary in Swedish)

Zhiping Wang. Akuta cytokinreaktioner efter inandning av damm från svinhus. *Arbete och Hälsa* 1997;23 1-63.

Inandning av svinhusdamm (svindamm) kan ge upphov till en akut inflammatorisk reaktion i form av allergisk alveolit och feberattacker(Organic Dust Toxic Syndrome, ODTS). Reaktionen förknippas med luftvägsinflammation, allmänna symptom och lätta spirometriska förändringar och kräver ingen föregående sensibilisering för att uppträda. Vilka ämnen som orsakar reaktionen är inte känt. Syftet med undersökningen var att undersöka frisättandet av inflammatoriska cytokiner i luftvägar och perifert blod som en följd av akut exponering för svindamm, korrelationen mellan markörer för kontaminanter i mikrobiellt damm, cytokinreaktion och hälsoeffekter, samt cytokinfrisättningen från en epitel cellinje (EP) och mänskliga alveolära makrofager (AM) som stimulerats av svindamm och LPS.

Friska försökspersoner utan tidigare erfarenhet av arbete med djur exponerades för svindamm under 3 - 4 timmar. Före och efter exponeringen uppmättes interleukin (IL) -6, receptorantagonist IL-1 (ra), nekrosfaktorn för tumörer (TNF) -α i serum, samt IL-1ß i mononukleära celler från perifert blod (PBMC). Även IL-1α, IL-1ß, IL-6 och TNF-α uppmättes i nasalt lavage (NAL) och bronkoalveolärt lavage (BAL) före och efter exponeringen. Tidskurvorna utfördes för IL-6, TNF-α och IL-1ß. Massan för det inhalerade dammet bestämdes, samt endotoxin koncentrationen och två markörer för mikrobexponeringen (muraminsyra, som är en markör för totalinnehållet av peptidoglykan, och 3-(OH)-fettsyra, som är en markör för totalinnehållet av lipopolysackarider). Före exponeringen för svindammet, och 7 timmar efter, utfördes spirometri och en bronkial metakolintest. Granulocyter, monocyter och lymfocyter uppmättes i blodet med flödescytometri. NAL-, BAL- och PBMC-cellerna uppmättes med hjälp av färgning. Orala temperaturer och allmänna symptom antecknades.

Exponering för svindamm orsakade feber, huvudvärk, lätt illamående, förhöjd bronkiell reaktivitet samt en liten minskning av FEV_1 och VC. IL-1ra, IL-6 och TNF-α ökade signifikant i serum, IL-1ß ökade signifikant i PBMC och plasma, och IL-1, IL-6 och TNF-α ökade signifikant i BAL- och NAL-vätskor. Det fanns ett markant inflöde av inflammatoriska celler, i synnerhet granulocyter. TNF-α, IL-6 och IL-1ß ökade och nådde toppvärden i perifert blod inom loppet av 3-5 tim, 5-7 tim respektive 3-7 tim efter exponeringens början. Antalet leukocyter fördubblades och en måttlig ökning av antalet monocyter inträffade 6-8 tim efter exponeringen. Endotoxin, muraminsyra och 3-OH-fettsyra korrelerade signifikant med ökningen av serum IL-6. Respirabelt damm korrelerade med ökningen av serum IL-1ra och endotoxin korrelerade med ökningen för IL-1ß i PBMC. Endotoxin och 3-OH-fettsyror korrelerade också signifikant med ökningen av IL-

6 i BAL. Bakteriella markörer visade bättre korrelation med förändringar för IL-6 än med totala dammkoncentrationer. LPS korrelerade med symptom och förändrade lungfunktioner. Peptidoglykan korrelerade med en ökning av blodgranulocyterna och kroppstemperaturen.

Svindamm orsakade dosberoende ökningar av IL-1ß, IL-6 och TNF-α i AM. Ingen ökning av IL-1ß eller TNF-α förekom, men en tydlig ökning av IL-6 konstaterades i EP. LPS orsakade en tydlig ökning av samtliga tre cytokineri AM, men inte för någon av cytokinerna i EP.

Sammanfattningsvis kan sägas att akut inandning av bakteriellt förorenat damm ökade koncentrationen av inflammatoriska cytokiner i BAL, NAL, PBMC och perifert blod. Ändringar av cytokiner och inflammatoriska parametrar korrelerade bättre med markörer för bakteriella kontaminanter än med total dammängd. En markör för bakteriell peptidoglykan uppvisade bättre korrelation med vissa förändringar än vad endotoxin gjorde, vilket ger anledning förmoda att flera bakteriella komponenter kan ha betydelse för ODTS. Resultaten av in vitro-testerna talar för att svindamm aktiverade en cytokin respons i EP och AM. EP hade en mer begränsad cytokin responsprofil än AM. I EP är svindamm en mer potent stimulant för cytokinfrisättning än LPS. Båda cellerna kan bidra till inflammatoriska reaktioner efter inandning av svindamm, och flera av ämnena - inklusive LPS - kan vara viktiga faktorer för cytokinfrisättningen.

9. Acknowledgements

I would like to express my sincere gratitude to **all** the people, who in various ways helped me throughout my study, and especially acknowledge:

Professor Per Malmberg, my supervisor, for giving me an opportunity to study at IMA, for his permanent encouragement, trust, help and patience, for sharing his vast knowledge of occupational medicine, for his constructive and critical advice, for putting his never - ending effort on my project, for his personal support always. Without Pelle, I would not finish my Ph.D training smoothly.

Associate Professor Kjell Larsson, my tutors, for always encouraging and understanding me, for his valuable guidance, support, enthusiasm and constructive discussion throughout all my research and studies.

Prof. S.G.O Johansson and Associate Prof. M. van Hage-Hamsten, my supervisor, for letting me study in the stimulating environment at the Department of Clinical Immunology.

My co-authors of the articles, that this thesis is based on, are worth a million thanks! Thank you, Britt-Marie Larsson, Lena Palmberg, Per Larsson, Alexandra Ek, Lennart larsson, and Anita Saraf. I am especially grateful to Associate Professor Lennart Larsson and Anita Saraf, for measuring dust bacterial markers and valuable discussion.

Professor Göran Hedenstierna, for inspiration and support in the beginning of my study and introducing me to Prof. Per Malmberg.

Everyone at the Division of Respiratory Allergy and Immunology is appreciated for forming the good atmosphere, and for adding to the colourful mixture of personalities. Especially my labmates are acknowledged, for making the lab a fun place to be. Britt-Marie Sundblad and Charlotte Müller-Suur, for generously helping me in the laboratory and their continuous display of good friendship. Anne-Renström, Siw Siljerud, Marie Hedrén, Anni-sophie Karlsson, Cecilia Bayard for excellent technical help and for their good company in the laboratory.

Lars Eklund, for help me with his extensive computer knowledge, friendship and technique support at all times. Mary Reuterdahl, for language correction of all my papers, for very kind help with whatever I needed and her friendship. Maud Kaddik, for helping me with all the practical things during the preparation of this thesis.

Associate Professor Bengt Robertson, for his inviting me to Sweden and his understanding.

Professor Zhang Jiou-shan and Yu Weixian, for being my excellent tutors during my M.D. and M.Sc. in lung medicine studies at The First Central Hospital, Tianjin. Thank also go to The First Central Hospital for being friendly and continuous support.

Old time friends, like Xiaoming, Yali, Yangwei, Jianping, Shuhua, Xujing and Kaixuen, are thanked for friendship and for reminding me of the life outside the Institute.

My Swedish friends outside the institute, Ingrid, Stefan, Åka, Helen, Lena, Michael for friendship and language help. Specially to Michael for reviewing and correcting my English of the thesis.

All my Chinese friends in Stockholm, for much fun we had together, for nice chatting, and inform many things.

My parents, my sister and my brothers, for their endless love and support.

My husband, Zhi, with special thanks for his continuous encouragement and scientific advice, for his always becomes my paper first viewer, for invaluable support. My dear son, Boyuan, for giving me great joy in life, for explaining the traditional Swedish word and life style for me and being my patient language teacher.

Colleagues of Division of Respiratory Allergy and Immunology, IMA.

The volunteers and farmers who made this work possible.

This work was supported by the Swedish Work Environment Fund, Swedish Farmers Foundation for Agricultural Research and Swedish Heart Lung Foundation.

This investigation was carried out at the Department of Occupational Medicine and Division of Respiratory Allergy and Immunology, National Institute for Working Life, Solna, and the Department of Clinical Immunology, Karolinska Hospital, Karolinska Institute.

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Submitted for publication 1997-10-24