

Effects of Combined Exposure: *Fungal
Fragments and Air Pollution Particles on THP-1
Monocytes and Macrophages*

Fikirte Debebe Zegeye



Master thesis in Toxicology
Department of Molecular Biosciences
Faculty of Mathematics and Natural Sciences

UNIVERSITY OF OSLO
May 2016

Effects of Combined Exposure: *Fungal
Fragments and Air Pollution Particles on THP-1
Monocytes and Macrophages*

© Fikirte Debebe Zegeye

2016

Effects of Combined Exposure: *Fungal Fragments and Air Pollution Particles on THP-1 Monocytes and Macrophages*

Fikirte Debebe Zegeye

<http://www.duo.uio.no/>

Universitetet i Oslo

ACKNOWLEDGEMENT

This master thesis was conducted at the Norwegian Institute of Public Health (NIPH) under the supervision of main supervisor Dr. Jørn A. Holme (Chief Scientist), and co-supervisors Dr. Johan Øvrevik (Senior Scientist), Elisabeth Øya (PhD stipendiat), Prof. Ketil Hylland, and with the approval of director of the department Dr. Per E. Schwarze.

First of all, I would like to give a special thanks to Dr. Jørn A. Holme for being supportive, enthusiastic, positive, and for giving me all the guidance I needed. You gave me unreserved help and supported me in all the decisions I needed to make during the long process.

I am very grateful to my co-supervisors Dr Johan (for helping me on the analysis and critical comments on my thesis), Prof. Ketil Hylland (for introducing me to such a talented scientific community, for your valuable suggestions on the analysis and important comments on my thesis), Dr. Per E. Schwarze (for giving me this amazing opportunity and warm greetings on the hall way) and Elisabeth Øya (for being a co-supervisor, a friend, and for teaching me all the introductory skills at the lab, and integrating me with the lab work). Elisabeth Øya, you deserve thousand thanks!

I would also like to give big thanks to all the engineers at NIPH for their unreserved help and warm smiles on the hallways. In particular, I am thankful to Tonje S. Skuland (for teaching me RT-PCR), Leni Ekeren (for helping in the cell culture), Edel Lilleaas (for teaching me how to culture BEAS-2B cells) Hans Jørgen Dahlman (for teaching me ELISA).

My brother, Dr. Ephrem Debebe Zegeye, thank you for all your ideas and critical comments on my thesis. I would also like to thank Bendik C. Brinchmann (MD, PhD stipendiat), for the pleasant and helpful discussions on my project and helping out on RT-PCR result analysis.

Thank you to all the instructors and coordinators at the department of biosciences in University of Oslo for this opportunity.

Special thanks goes to my beloved husband, Yohannes B. Mekonnen, for not only being a caring and loving husband, but also for all your support, suggestions and comments throughout the process. My lovely son Yafet, you were my efficient medicine when I am feeling down. I love you dears!

Thank you my dad, you are my rock! I wouldn't have made it without your encouragement and prayers. Thank you all my sisters back home for supporting me in these intensive years. Mom, this is dedicated to you. I wish you were alive to see this one!

Above all, I would like to praise and thank you God for helping me accomplish!

Oslo, May, 2016

Fikirte Debebe Zegeye

ABSTRACT

Epidemiological studies have demonstrated that exposure to air pollution is associated with increased mortality and morbidity due to respiratory and cardiovascular disease. The majority of the effects have been related to exposure to various air pollution particles. Effects of air pollution particles and bio-aerosols *in vitro* are well characterized, however, less is known regarding possible interactions as a result of co-exposure. The aim of this study was to assess the co-exposure effect of *Aspergillus fumigatus* hyphae (AFH) and air pollution particles: Diesel Particulate Matter (DPM) or silica particles (Min-U-Sil), co-exposure of Lipopolysaccharide (LPS) and DPM or Min-U-Sil.

The human monocytic cell line THP-1 and Phorbol 12-myristate 13-acetate (PMA) differentiated THP-1 macrophages were exposed to AFH, LPS, DPM and Min-U-Sil both individually and in co-exposure. They were assessed for cytokine secretion (IL-1 β and TNF- α) by using Enzyme Linked Immunosorbent Assay (ELISA) and mRNA expression by using real time Reverse Transcriptase Polymerase Chain Reaction (real time RT-PCR). Cell viability was assessed using fluorescence microscopy after staining with Propidium Iodide/ Hoechst 33342.

We have shown that the exposure of AFH and LPS induce increased secretion of IL-1 β and TNF- α in THP-1 monocytes and PMA THP-1 macrophages. DPM and Min-U-Sil induced TNF- α and IL-1 β secretion, respectively only in PMA THP-1 macrophages. In our co-exposure set ups, AFH co-exposed with Min-U-Sil as well as LPS co-exposed with Min-U-Sil showed significant and a possible synergistic effect in IL-1 β secretion in both THP-1 monocytes and PMA THP-1 macrophages. These amplified secretions of IL-1 β were not shown in the IL-1 β gene expression. Furthermore, the co-exposure of AFH and Min-U-Sil, LPS and Min-U-Sil appear not to have any marked effects on TNF- α either on its mRNA expression or its secretion in the two cellular models.

In conclusion, primed cells gave increased pro-inflammatory reaction to selected air pollution particles. Notably, cells primed with AFH/LPS and co-exposed to Min-U-Sil showed significantly ($P < 0.05$) higher IL-1 β in both THP-1 monocytes and PMA THP-1 macrophages. These increased secretions were not reflected by a correspondingly increased mRNA expression

of the genes, suggesting Min-U-Sil primarily increased AFH/ LPS induced secretion of IL-1 β by acting on step two (activation of the inflammasome).

TABLE OF CONTENTS

AKNOWLEDGEMENT.....	II
ABSTRACT.....	III
LIST OF FIGURES.....	VIII
LIST OF ABBREVIATIONS AND TERMINOLOGIES.....	X
1 INTRODUCTION	1
1.1 Background	1
1.1 The Respiratory System	3
1.1.1 The anatomy and function	3
1.1.2 Immunity of the respiratory system	5
1.1.3 Monocytes and macrophages	5
1.1.4 Lung microbiota	6
1.2 Cell viability (Cytotoxicity)	7
1.3 Cell receptors and signaling pathways	8
1.3.1 Toll Like Receptor (TLR)	9
1.3.2 NOD Like Receptors (NLRs)	9
1.3.3 Nuclear Factor-Kappa B (NF-κB)	9
1.4 Inflammation	10
1.4.1 Cytokines	11
1.5 Fungi	13
1.6 Lipopolysaccharides (LPS)	15
1.7 Particulate Matter (PM)	15
1.7.1 Particle Deposition in the lung	16
1.7.2 Diesel exhaust (DE)	18
1.7.3 Silica	19
1.8 Combined Effects	20
2 AIM OF THE STUDY	21
3 MATERIALS AND METHODS	22
3.1 Cell culture	22
3.2 Fungal samples and preparation	22
3.3 LPS	23
3.4 Particles	23
3.4.1 Diesel Particulate Matter	23

3.4.2	Min-U-Sil 5	23
3.4.3	Particle samples and preparation	24
3.5	Exposures.....	24
3.5.1	Single exposures	25
3.5.2	Co-exposure I	25
3.5.3	Co-exposure II.....	26
3.6	Cytotoxicity.....	26
	Fluorescence Microscopy	26
3.7	Cytokine measurement.....	27
	Enzyme Linked Immunosorbent Assay (ELISA).....	27
3.8	Gene expression.....	28
	Real-time Reverse Transcriptase Polymerase Chain Reaction (real-time RT-PCR)	28
3.9	Statistical Analysis	30
4	RESULTS	32
4.1	Single and co-exposure induced cytotoxicity to THP-1 monocytes	32
4.2	Cytokine release of THP-1 monocytes after single and co-exposure.....	38
4.3	mRNA expression of THP-1 monocytes after co-exposures.....	42
4.4	Single and co-exposure induced cytotoxicity to PMA THP-1 macrophages.....	44
4.5	Cytokine release of PMA THP-1 macrophages after single and co-exposure	44
4.6	mRNA expression of PMA THP-1 macrophages after co-exposures	48
5	EXPERIMENTAL AND METHODOLOGICAL CONSIDERATIONS.....	50
5.1.1	Cellular models.....	50
5.1.2	Exposure agents	50
5.1.3	Methods.....	51
6	DISCUSSION.....	54
6.1	The effect of single and co-exposures on cytotoxicity and cytokine release in THP-1 monocytes	54
6.2	The effect of single and co-exposures on cytotoxicity and cytokine release in PMA THP-1 macrophages.....	57
6.3	NF- κ B and inflammasome involvement in IL-1 β synthesis	59
6.4	mRNA expression of TNF- α in THP-1 monocytes and PMA THP-1 macrophages after co-exposures	61
7	CONCLUSION	62
8	POSSIBLE IMPLICATIONS AND FURTHER STUDIES	63

9	FUNDING AND COLLABORATORS	65
10	REFERENCES	66
11	SUPPLEMENTARY	76

LIST OF FIGURES

Figure 1 Schematic diagram of the respiratory tract.....	3
Figure 2 Schematic diagram showing cilia move the mucus layer towards the pharynx removing trapped pathogens and PM.....	4
Figure 3 Illustration of apoptosis and necrosis	8
Figure 4 Schematic diagram of NF- κ B activation	10
Figure 5 Schematic diagram of IL-1 β secretion pathway.....	13
Figure 6 Life cycle of <i>A. fumigatus</i>	14
Figure 7 Particulate matter size illustration compared to other materials	16
Figure 8 Predicted inhaled particle deposition in the respiratory tract with respect to particle size.	17
Figure 9 The physico-chemical composition and structure of diesel particulate matter.	19
Figure 10 Induction of apoptosis and necrosis in THP-1 monocytes after exposure to DPM and Min-U-Sil determined by fluorescence microscopy.	33
Figure 11 Induction of apoptosis and necrosis in THP-1 monocytes that were exposed to four different concentrations of LPS and AFH determined by fluorescence microscopy. ...	34
Figure 12 Induction of apoptosis and necrosis in THP-1 monocytes that were primed with LPS or AFH and co-exposed with DPM determined by fluorescence microscopy.....	36
Figure 13 Induction of apoptosis and necrosis in THP-1 monocytes that were primed with LPS or AFH and co-exposed with Min-U-Sil determined by fluorescence microscopy.	37
Figure 14 Effects of DPM and Min-U-Sil on cytokines secretion of THP-1 monocytes determined by ELISA.	39
Figure 15 Effects of LPS and AFH on cytokine secretion of THP-1 monocytes determined by ELISA.....	40
Figure 16 Effects of co-exposure of LPS with DPM/Min-U-Sil and AFH with DPM/ Min-U-Sil on cytokines secretion of THP-1 monocytes determined by ELISA.	41
Figure 17 Effects of priming with LPS or AFH and co-exposure with Min-U-Sil on gene expression of THP-1 monocytes.	43
Figure 18 Effects of DPM and Min-U-Sil on cytokine secretion of PMA THP-1 macrophages determined by ELISA.....	45

Figure 19 Effects of LPS and AFH on cytokine release of PMA THP-1 macrophages determined by ELISA.....	46
Figure 20 Release of cytokines in PMA THP-1 macrophages after co-exposure of AFH with DPM or Min-U-Sil and co-exposure of LPS with DPM or Min-U-Sil determined by ELISA.	47
Figure 21 Effects of priming with LPS or AFH and co- exposure with DPM or Min-U-Sil on gene expression of PMA THP-1 macrophages.	49

LIST OF ABBREVIATIONS AND TERMINOLOGIES

%	Percentage
$\mu\text{g}/\text{cm}^2$	Microgram per square centimetre
μl	Microliter
μm	Micro meter
AFH	<i>Aspergillus fumigatus</i> hyphae
AFS	<i>Aspergillus fumigatus</i> spores
AIDS	Acquired Immune Deficiency Syndrome
AM	Alveolar Macrophage
ANOVA	Analysis Of Variance
ATP	Adenosine triphosphate
BaP	benzo[<i>a</i>]pyrene
BSA	Bovine Serum Albumin
CD	Cluster of differentiation
cDNA	Complementary DNA
cm^2	Square centimetre
CO ₂	Carbon dioxide
CYP1A1	Cytochrome P450 1A1
DE	Diesel Exhaust
DEP	Diesel Exhaust Particles
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleoside triphosphate
DPM	Diesel Particulate Matter
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
g	Gram
h	Hour
HRP	Horseradish Peroxidase
IFN	Interferon
IKK	I kappa B kinase

IL-1 β	Interleukin-1beta
IRAK	IL-1 receptor associated kinase
I κ B	Inhibitor kappa B
kD	Kilo Dalton
kGy	Kilogray
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
min	Minute
Min-U-Sil	Fine Ground Silica product
ml	Milliliter
mm	Millimeter
mRNA	Messenger RNA
MTT	Methyl thiazolyl tetrazolium
MyD88	Myeloid differentiation primary response gene 88
NF- κ B	Nuclear Factor kappa light chain enhancer of activated B cells
ng	Nano gram
ng/ml	Nano gram per millilitre
NLR	NOD-like Receptor
NLRC	NOD-like receptor family CARD domain containing
NLRP	NACHT, LRR and PYD domain containing
NOD	Nuclear binding Oligomerization Domain
NRC	No Reverse Transcription Control
NTC	No Template Control
°C	Degree Celsius
PAH	Polycyclic Aromatic Hydrocarbons
PAMP	Pathogen Associated Molecular Patter
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
Pg/ml	Pico gram per millilitre
PI	Propidium Iodide
PM	Particulate Matter

PMA	Phorbol 12-myristate 13-acetate
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
rpm	Revolution per minute
RT-PCR	Reverse Transcriptase - polymerase chain reaction
SiO ₂	Silicon dioxide
SR	Scavenging receptor
TACE	TNF- α converting enzyme
THP-1	Human leukaemia monocyte cell line
TLR	Toll-like Receptor
TNF- α	Tumor Necrosis Factor- alpha
TRAF	TNF receptor associated factor
UV	Ultraviolet

1 INTRODUCTION

1.1 Background

Air pollution remained one of the hottest issues within the field of environmental medicine for more than half a century (Brunekreef et al. 2002; Brimblecombe 2011). It is a contamination of the ambient air with heterogeneous mixture of particulate matter, bio-aerosols and other harmful materials (Brook et al. 2004). Among important sources, traffic emission is found to be a major contributor to the particle pollution (Weinhold 2002). Furthermore, activities such as mining industry, farming and construction also take a considerable share in pollution caused by particles (WHO 2000).

In the last few decades health effects of air pollution have been subjected to intense epidemiological and experimental studies. Epidemiological studies have demonstrated that exposure to particulate matter (PM) affect human health (Weinhold 2002; Pope III et al. 2006). It is found to be associated with increased mortality and morbidity due to respiratory and cardiovascular disease (Chan-Yeung 2000; Brunekreef et al. 2002; Brook et al. 2004). Experimental studies have substantiated these findings and found that exposure to PM damage the respiratory system through genotoxic, cytotoxic and pro-inflammatory effects (van EEDEN et al. 2001; de Kok et al. 2006; Gualtieri et al. 2010).

Bio-aerosols on the other hand, include airborne virus, bacteria, fungi and other biological fragments such as fungal spores and hyphae constituting up to 80% of the ambient aerosols (Lee 2011). They exist both indoor and outdoor and hence, we are exposed to them on daily basis. Among all the bio-aerosols, airborne fungal spores and hyphae have obtained widespread attention as possible causal agents for adverse health effects (Waegemaekers et al. 1989; Górný 2004). Studies have reported that fungi may adversely affect human health not only by causing infection, but also through allergic and non-allergic airway inflammation (Fung et al. 2003). Furthermore, hyphae have been reported to be more reactive than that of spores (Górný 2004). On the contrary, some airborne bacteria seem to contribute more in constituting the microbiota of the lung and play a crucial role in developing and maintaining the lung immune system

(Charlson et al. 2011; Ichinohe et al. 2011; Gollwitzer et al. 2014). Thus, most likely the lung cells are naturally exposed and primed with airborne bacteria.

Several studies have documented the individual health effects of these agents (air pollution particles and bio-aerosols). Although lung cells are either naturally primed with bacteria, or often are primed as a result of an ongoing low degree of infection, very little is known about their co-exposure effects. Therefore, this thesis evaluates the inflammatory potential of the co-exposure of *Aspergillus fumigatus* hyphae (AFH) and air pollution PMs namely, diesel particulate matter (DPM) and silica particles (Min-U-Sil), as well as the co-exposure of lipopolysaccharide (LPS) (a component in the outer membrane of Gram negative bacteria) and air pollution PMs (DPM and Min-U-Sil) in an *in vitro* model.

1.1 The Respiratory System

1.1.1 The anatomy and function

The respiratory tract is divided into the upper and lower respiratory tract. The upper respiratory tract includes the nasal and oral cavities, pharynx, larynx and trachea and the lower tract includes the bronchi and alveoli as illustrated in Fig. 1 (Sara Hickin 2013).

The air gets in through the nose and mouth and passes to the pharynx and further in to the larynx and trachea. It goes to the main bronchi and branches out to the small bronchi and bronchioles. Finally it reaches to the alveolar sacs and alveoli where the gas exchange takes place. When the air passes in these airways, it will be warmed, humidified and filtered (Sara Hickin 2013; Silverthorn et al. 2013).

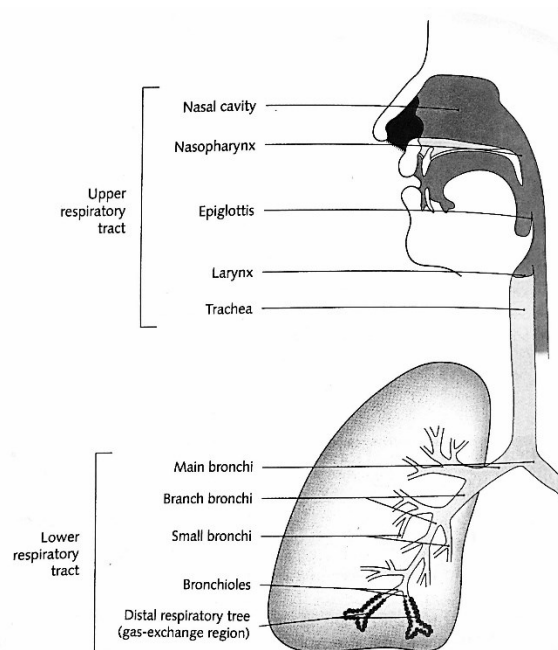


Figure 1 Schematic diagram of the respiratory tract. Figure from (Sara Hickin 2013)

Gas exchange is the major function of the lungs and it occurs down in the alveoli. The alveoli are composed of type I and type II alveolar cells. Type I alveolar cells perform the gas exchange and Type II alveolar cells synthesise and secrete surfactant, a chemical that aids the lungs to expand during breathing (Silverthorn et al. 2013).

In addition to the well-known function of exchanging gas between the body and the environment, the respiratory system has functions such as metabolism, excretion, regulation of pH and temperature, speech and clearance of inhaled pathogens and particles (Velden et al. 1998; Sara Hickin 2013). In order to perform these functions a number of distinct cells are involved in a unified manner. The air ways are lined with epithelium forming an interface between the respiratory system and inspired air. This layer lies on a connective tissue substratum consisting of a basement membrane, lamina propria and submucosa, and smooth muscle, glands and cartilage (Velden et al. 1998). The layer is mainly made of non-ciliated, ciliated and goblet cells and mucus layer. The non-ciliated (Clara) cells are involved in detoxifying inhaled agents through phase-1 metabolism involving cytochrome P450 isoenzymes (Widdicombe et al. 1982). The ciliated cells and the mucus secreted by the goblet cells remove inhaled particles larger than $2\mu\text{m}$ and microorganisms by a means of mucociliary stairway as shown in Fig. 2 (Velden et al. 1998; Silverthorn et al. 2013). Once the mucus carrying particles/ microorganism reaches the pharynx, it can be spit out or swallowed. When swallowed, stomach acid and enzymes will digest the mucus and particles (Silverthorn et al. 2013).

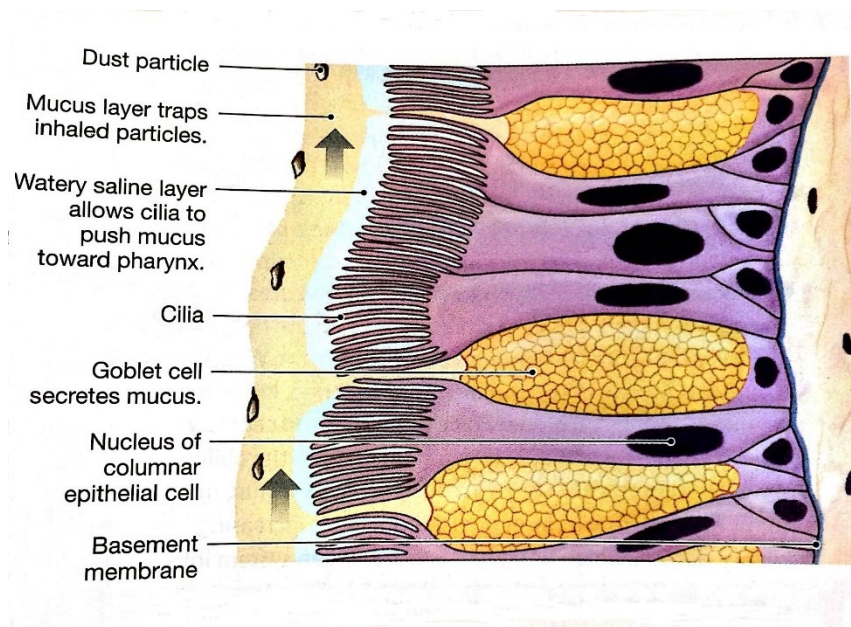


Figure 2 Schematic diagram showing cilia move the mucus layer towards the pharynx removing trapped pathogens and PM. Figure from (Silverthorn et al. 2013)

1.1.2 Immunity of the respiratory system

As described in section (1.1.1), many lung cells provide protection to the airways and lung through barrier formation, mucociliary clearance and secretion of mediators. However, optimal protection cannot be acquired without the involvement of the immune system. The immune system is composed of innate and adaptive immunity. The innate immunity operates nonspecifically at the early stage of an infection and mediated by pattern recognition receptors (PRRs). It includes complement proteins and diverse cell types including macrophages, monocytes, basophils, eosinophils, mast cells, dendritic cells and natural killer cells (Elgert 2009; Parham 2014). Adaptive immunity is specific, takes time to develop, has memory and is mediated by antigen specific cells and antibodies. Adaptive immunity includes T lymphocytes (T cells) and B lymphocytes (B cells) (Elgert 2009; Parham 2014). Innate and adaptive immunity are linked; cellular and soluble components of the former influences antigen processing, selection and presentation as well as the magnitude of response of the later (Ulrich 2007). Typical lung resident immune cells include a population of activated T cells (e.g. CD4, CD8 T cells) and B cells that secrete specific antibodies as well as macrophages and monocytes. They are sparsely distributed in the air ways (Ulrich 2007). Other immune cells (lymphocytes) recirculate between blood, tissue and lymph node. However, once monocytes and granulocytes (e.g. neutrophil) leave the blood, they do not recirculate (Ulrich 2007). The resident macrophages and T cells sample their environment continuously for a foreign antigen. Upon antigen encounter, other lymphocytes will be recruited to the infected site through cytokines and chemokines, and further cell proliferation takes place (Ulrich 2007).

1.1.3 Monocytes and macrophages

Monocytes are cells originating from hematopoietic cells in the bone marrow (Iwasaki et al. 2007). Monocytes are round shaped and constitute 2-10% of the blood leukocytes (Elgert 2009; Parham 2014). They express CD14, CD16 (Fc receptor) and chemokine receptors on their surface (Elgert 2009). They secrete a range of cytokines and chemokines including IL-1, IL-6, IL-8, IL-18, IL-1 β and TNF- α (Vignola et al. 1998). In addition to their well-known function as precursor to macrophage and dendritic cells, monocytes are involved in surveillance of the vascular surface and blood vessel using their high motility (Yona et al. 2010).

Macrophages are phagocytic cell. They measure 10 to 40 μm in diameter and their morphology varies depending up on their state of activity (Elgert 2009). Macrophages are distributed though out the entire body (Weiss 1972) and are known to have differentiated from monocytes with few exceptions (Sieweke et al. 2013). The process of the differentiation involve changes in morphology, number of organelles, expression of specific membrane receptors, phagocytic capacity and an increased concentration of transcription factor Nuclear factor-kappa B (NF- κ B) in the cytoplasm (Takeuchi et al. 2010; Sieweke et al. 2013). A typical matured macrophage expresses CD14, Fc receptors (CD16, CD23, CD32, and CD64), C3b receptors (CD11b), CD68 and class I and II Major Histocompatibility Complex (MHC) molecules. The major function of macrophages is to phagocyte foreign agents such as particles, pathogens and to eliminate apoptotic cells (Tauber 2003). Particularly, alveolar macrophage (AM) submerged in surfactant layer of the alveoli play a crucial role in host defence by removing inhaled particles and fungal spores from the alveolar lumen, thereby preventing further allergic reaction and proliferation of hyphae (Ulrich 2007; Margalit et al. 2015). In addition to their phagocytic action, macrophages secrete a range of cytokines, chemokines, hydrolytic enzymes, complement components, oxygen metabolites according to their micro environmental stimuli. They also play a key role in regulating the adaptive immune response through cytokine secretion, antigen processing and presentation (Elgert 2009).

1.1.4 Lung microbiota

The lung was classically believed to be sterile. However, recent studies have identified diverse communities of commensal microbiota in healthy individuals (Charlson et al. 2011; Dickson et al. 2013). Even though some studies suggest the existence of fungal commensal microbiota in the lungs such as *Aspergillus* spp. and *Scedosporium* spp. (Underhill et al. 2014), the most known and well-studied member of the lung microbiota are bacterial communities. These bacterial communities are shown to be similar in composition though out the air ways. However, their biomass decrease from the upper to the lower respiratory tract (Charlson et al. 2011). The presences of this microbiota are crucial to develop and maintain the lung immune system (Ichinohe et al. 2011; Gollwitzer et al. 2014)

1.2 Cell viability (Cytotoxicity)

There are three well known modes of cell deaths: apoptosis, autophagy and necrosis (Lockshin et al. 2004; Green et al. 2015). Even though apoptosis and autophagy are considered as two different modes, they do not have a clear distinction and at times the cell death begins with autophagy and end up with apoptosis (Lockshin et al. 2004). Therefore, in this thesis only apoptosis and necrosis will be focused.

Apoptosis is a cascade of events that lead to suicide of cells which commonly known as programmed cells death. Apoptosis is a mechanism for elimination of unwanted cells. It requires ATP and occurs as normal part of development. After apoptosis occurred, the apoptotic bodies are recognized and removed by macrophages without resulting any inflammation around the dying cells (Edinger et al. 2004). Necrosis, on the other hand, is an accidental or catastrophic cell death due to ATP depletion (Williams 1991; Schulze-Osthoff et al. 1998; Strasser et al. 2000). It is also known to induce inflammation around the dying cells by releasing cellular contents and pro-inflammatory molecules (Edinger et al. 2004).

The two types of cell death are distinguished by their morphology and their bio chemical manifestations: apoptosis is characterized by condensation and segregation of chromatin and cytoplasm with preservation of the integrity of organelles, nucleus fragmentation, whereas necrosis is characterized by irregular clumping of chromatin, swelling of mitochondria, dissolution of ribosomes, and focal rupture of membranes and disintegration of cellular components as shown in Fig. 3 (Kerr et al. 1995). These types of cell death can occur through natural causes and as a result of exposure to cytotoxic agents (Van Cruchten et al. 2002).

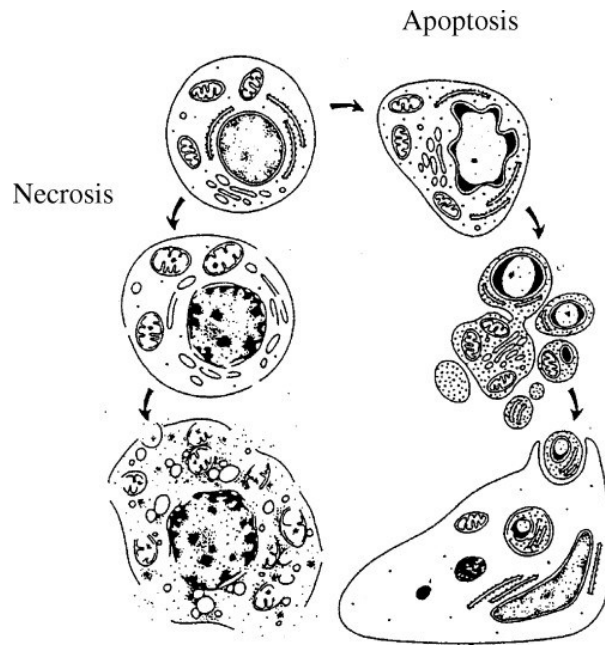


Figure 3 Illustration of apoptosis and necrosis. Figure from (Kerr et al. 1995)

1.3 Cell receptors and signaling pathways

Signal reception from the environment and other cells depends up on the existence of receptor proteins usually on the cell surface and sometimes inside the cell (Alberts et al. 1994). The extracellular receptors include Toll Like Receptors (TLR), mannose receptor (MR) and scavenger receptors (SR). While intracellular receptors include NOD Like Receptors (NLRs) (Yan et al. 2007). Macrophages and other related myeloid cells express these receptors abundantly (Taylor et al. 2005). These receptors are activated, when the signal molecule binds with them. They, in turn, activate one or more intracellular signaling pathways though intracellular signaling proteins. The intracellular signaling proteins transmit the signals further to the intracellular target. Depending on the signal received, change in metabolism, cell shape and or alteration of gene expression through activation of transcription factor might occur (Alberts et al. 1994; Ishii et al. 2008). The cellular receptors and signaling pathways relevant to this study are further discussed as follows.

1.3.1 Toll Like Receptor (TLR)

TLR is a family of cell receptor that recognize specific molecular patterns of microbial pathogens such as Lipopolysaccharides (LPS), peptidoglycan, bacterial lipoproteins bacterial DNA and viral RNA (Alexopoulou et al. 2001; Lien et al. 2002). Activation of TLR results an inflammatory response through production of pro-inflammatory cytokines and subsequent activation of the innate immune system (Lien et al. 2002; Takeda et al. 2003). TLR was first identified in *Drosophila* in 1988 (Hashimoto et al. 1988) and currently, 10 members of TLR (TLR1- TLR10) are identified in humans. Among them, TLR 2 and TLR 4 are mostly expressed in cells that respond to LPS such as macrophages and monocytes (Brightbill et al. 1999; Lien et al. 1999; Moresco et al. 2011). Moreover, *in vitro* studies have documented that TLR 2 and 4 also recognize fungal pathogens such as *Cryptococcus neoformans*, *Aspergillus fumigatus* and *Candida albicans* (Levitz 2004).

1.3.2 NOD Like Receptors (NLRs)

NLRs are intracellular receptors that belong to the innate immune system. NLR recognizes pathogen associated molecular patterns (PAMPs) and other danger signals in the cytosol (Kanneganti et al. 2007; Chen et al. 2009). The process is mediated by a multiprotein complex called inflammasomes. The inflammasomes include NLRs namely, NLRP1, NLRP3, NLRC4 and an adaptor protein ASC (Franchi et al. 2009; Guo et al. 2015). Up on activation, NLRs induce mitogen-activated protein kinase (MAPK) signaling or activate casepase-1 (Guo et al. 2015). Casepase-1 activation is important in secretion of pro-inflammatory cytokines such as interleukin (IL)-1 β and IL-18 (Dinarello 1998; Franchi et al. 2009).

1.3.3 Nuclear Factor-Kappa B (NF- κ B)

NF- κ B is a dimeric family of transcription factors (usually RelA/p65:p50) that has essential role in immune response, inflammation and apoptosis (Ghosh et al. 1998; Ghosh et al. 2002). NF- κ B is expressed abundantly as inactive form in the cytoplasm and it can be activated with a wide variety of stimulus such as UV radiation, inhaled particles, pro-inflammatory cytokines, oxidative stress, viral, fungal and bacterial products (Ghosh et al. 2002). However, its activation is tightly regulated by Inhibitor of κ B (I κ B). NF- κ B, in turn, regulates the encoding of genes including those that encode cytokines and pro-inflammatory mediators (de Martin et al. 1999; Ghosh et al. 2002; Oeckinghaus et al. 2009). When stimulated, I κ B will be ubiquitinated and

degraded through I kappa B kinase (IKK)-mediated phosphorylation releasing active NF- κ B (Fig.4). In the classical pathway, the activated NF- κ B contains two proteins p65 (relA) and p50. These proteins translocate to the nucleus and induce transcription of genes such as pro-inflammatory cytokines (Epstein et al. 1997; Oeckinghaus et al. 2009).

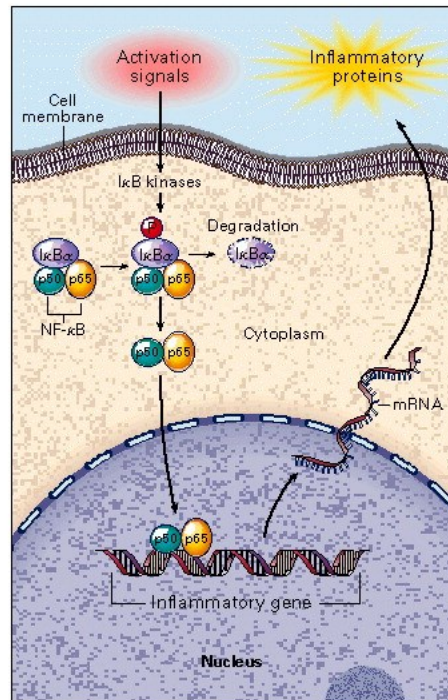


Figure 4 Schematic diagram of NF- κ B activation. Figure from (Epstein et al. 1997).

1.4 Inflammation

Inflammation is an important defensive body reaction to remove invading pathogens. It is traditionally characterized with redness, swelling, heat, pain and loss of tissue function (Rocha e Silva 1978; Scott et al. 2004). Inflammatory response is generally protective through elimination of the cause of injury, removing necrotic cells and initiation of repair process. Nevertheless, chronic inflammation is destructive and a possible contributor for a number of diseases including asthma, lung cancer, multiple sclerosis, cardiovascular disease, Alzheimer's disease and bowel disorders (Feghali et al. 1997; Klaassen 2013). At a cellular level, inflammation is mainly synthesis and release of a range of signaling molecules such as cytokines, chemokines, leukotrienes and adhesion molecules (Schwarze et al. 2013). These signaling molecules recruit

different immune cells to the infected site and optimize phagocytosis primarily with macrophages to remove invading pathogens (Ulrich 2007).

1.4.1 Cytokines

Cytokines are low molecular weight (<30kD) molecules. The term “cytokine” includes interleukin (IL) family, chemokine family, tumor necrosis factor (TNF) family, interferons (IFNs), mesenchymal growth factors and adipokines (Dinarello 2007). Cytokines are produced by both immune and nonimmune cells with a general function of mediating cell to cell communication (Elgert 2009). They are regulators and effector molecules that act on target cells with specific cytokine receptors. Cytokines are involved in signal transduction particularly in activating genes for growth, differentiation, and cell activity (Elgert 2009). Over 100 separate genes encode for cytokines: however, their function overlaps with one another (Dinarello 2007). Even though many of these cytokines are useful, there are some cytokines that have the potential to worsen an ongoing infection by causing fever, inflammation, tissue destruction, and in some cases sepsis and death (Dinarello 2000). Cytokines known to activate inflammation are called pro-inflammatory cytokines. Among the pro-inflammatory cytokines are IL-1 α , IL-1 β and TNF are key regulators of other cytokines (Dinarello 2000).

Cytokine secretion begins with the recognition of danger signal through PRRs such as TLRs, MR and SR. These receptors are expressed in many innate immune cells and usually have overlapping roles in recognizing various agents. Among all, TLRs are well- studied and they recognize various agents including LPS, and AFH and *A. fumigatus* spores (AFS) (Levitz 2004; Moresco et al. 2011). Moreover, AFH is also recognized by fungal receptor dectin-1 (Steele et al. 2005; Brown 2006; Gersuk et al. 2006). This recognition leads to a subsequent activation of NF- κ B commonly through MyD88 and secretion of pro-inflammatory cytokines such as IL-1 β (Ishii et al. 2008).

1.4.1.1 IL-1 β

IL-1 β is a cytokine that belong to the IL-1family. This family, in addition to IL-1 β , contains at least 10 other members. The gene encoding IL-1 β (IL1B) is located on chromosome 2 (Elgert 2009). IL-1 β is an important pro-inflammatory cytokine; it contributes to host defense by recruiting and activating immune cells and increasing body temperature (Dinarello 2010; Chen et

al. 2013). Hence, it is often used as an endpoint to study inflammation. IL-1 β is produced and secreted by a variety of cell types, but mainly from monocytes and macrophages.

Unlike most other cytokines, IL-1 β is expressed in the cytosol as inactive precursor protein called pro IL-1 β (Schwarze et al. 2013). Pro IL-1 β is amongst others produced as a response to recognition of PAMPs through the pattern recognition receptors (PRRs), mainly the TLRs (e.g. TLR2 and TLR4). The signal received by the TLRs is transmitted through adaptor protein TIRAP/Mal to MyD88. MyD88 interacts with IL-1 receptor associated kinase (IRAK) followed by another interaction to downstream protein TRAF6 resulting in the activation of NF- κ B and production of pro IL-1 β (Takeuchi et al. 2010; Chen et al. 2014). However, production or expression of pro IL-1 β is an insufficient stimulus for secretion, and it is generally referred as priming. Thus, the primed cell must encounter a second signal (PAMPs or danger associated molecular patterns (DAMPs)) in order to cleave pro IL-1 β to active IL-1 β (Lopez-Castejon et al. 2011). The cleavage or maturation of pro IL-1 β is controlled by inflammasomes (NLRPs) in the cytosol (Schroder et al. 2010; Owen et al. 2013). The activation of these inflammasomes, particularly NLRP3, is due to particle phagocytosis (Hornung et al. 2008). Upon recognition of the presence of PAMPs and DAMPs, the inflammasomes activate pro-caspase-1 to caspase-1 that further cleaves pro IL-1 β to active IL-1 β (Fig. 5) (Monteleone et al. 2015). Since IL-1 β secretion lacks the secretory signal sequence and does not follow the classical endoplasmic reticulum (ER)/Golgi secretion route, the secretion pathway has remained controversial for decades. Nevertheless, studies have proposed secretory lysosomes, macrovesicle shedding, exosome release and secretory autophagy as a possible IL-1 β secretion mechanism (Andrei et al. 1999; Piccioli et al. 2013; Monteleone et al. 2015).

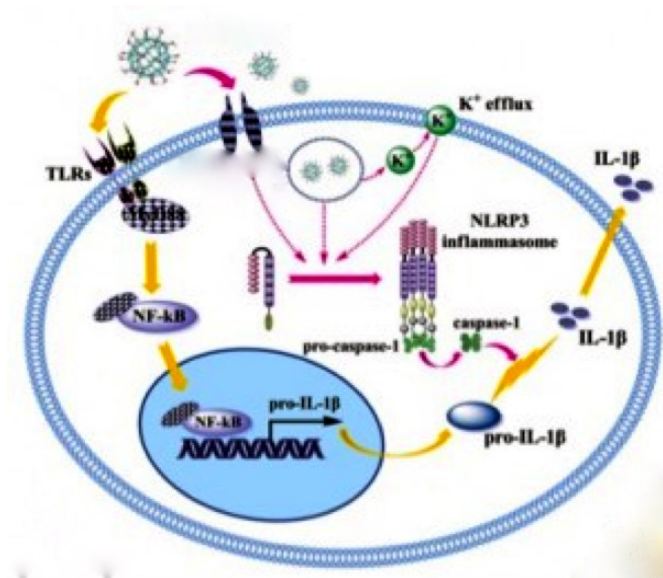


Figure 5 Schematic diagram of IL-1 β secretion pathway. Figure adapted from (Chen et al. 2014)

1.4.1.2 *TNF α*

TNF- α is a well-known pro-inflammatory and immunomodulatory cytokine. Prolonged and increased production of this cytokine implicated serious diseases such as rheumatoid arthritis, septic shock, and multiple sclerosis. It is mainly secreted by activated macrophages but other cells such as natural killer cells and mast cells also secrete it to a lesser extent. TNF- α plays a major role in the early stage of immune response in regulating other cytokines and increasing blood stream to the site of infection (Elgert 2009). TNF- α is initially expressed as type II membrane-anchored protein (pro- TNF- α) and which may be released via proteolytic cleavage by the metalloprotease TNF- α converting enzyme (TACE) (Gearing et al. 1995; Wajant et al. 2003). The overall synthesis pathway involves the activation of NF- κ B and transcription and production of pro-TNF- α (Schwabe et al. 2006).

1.5 Fungi

Fungi are heterotrophic organisms that include molds, mushrooms and yeasts. They reproduce through sexual and asexual spores, and comprise approximately 25% of global biomass (Miller 1992; Sorenson 1999). Their existence in nature is advantageous due to their role in recycling

nutrients. They are also used as food and drug production. However, they cause a number of diseases to plants and animals.

Among many fungal species, *Aspergillus fumigatus* is the most abundant as an airborne (Mullins et al. 1984). It is a saprophytic fungus with worldwide distribution. Their common habitat is in the soil and organic debris, with an essential role of recycling carbon and nitrogen. *A. fumigatus* is a filamentous fungus which grows branched multicellular filament structures called hyphae. Hyphae are a vegetative form and are collectively known as mycelium. The hyphae or its fragments give rise to conidiophores that release thousands of conidia to the atmosphere (Pady et al. 1963; Mullins et al. 1984). Both hyphal fragments and conidia can readily become airborne (Fig. 6). Furthermore, *A. fumigatus* is known to be the most prevalent airborne fungal pathogen in developed countries (Latgé 1999). Their small size gives them the ability to reach small air ways and alveolar spaces resulting in a series of lung cells reactions such as inflammation and activation of different immune cells (Rylander 1986; Latgé 1999; Bozza et al. 2002; Warris et al. 2002). Humans and animals inhale several hundreds of conidia per day; normally these conidia can readily be eliminated by the innate immune system (Goodley et al. 1994; Hospenthal et al. 1998). However, in immune compromised individuals (e.g. organ transplant recipient and AIDS patients), inhaling *A. fumigatus* conidia or hyphal fragments causes fatal invasive infection (aspergillosis) (Denning 1998; Latgé 2001). Moreover, exposure to *A. fumigatus* has been associated to worsen asthma and sinusitis (Zureik et al. 2002).

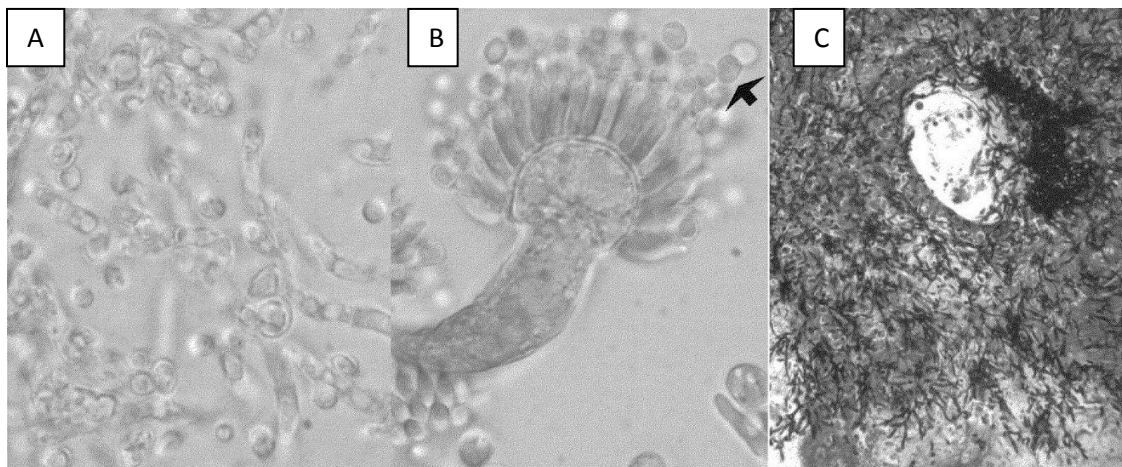


Figure 6 Life cycle of *A. fumigatus*. (A) Hyphae under light microscope after growth in Sabouraud's dextrose agar *in vitro*. (B) Conidiophores, the arrow shows conidia that can easily be aerosolized. (C) Hyphal growth in the tissue, Gomori Methenamine silver stained lung section of an immunosuppressed mouse two days after infection. Figure from (Feldmesser 2005)

1.6 Lipopolysaccharides (LPS)

LPS is an outer surface membrane component of most Gram-negative bacteria. It is a macromolecule with three components; Lipid A, a core oligosaccharide, and a repeating O-specific side chain (Rietschel et al. 1994). Its vital function is to protect the bacterium (bacterium viability). LPS is a potent activator of different mammalian cells leading to a cascade of events through the activation of NF- κ B. LPS is well known endotoxin to animals through its lipid A component. It is a prime target to be recognized by the immune system leading to a strong immune response in animals as well as causing a septic shock (septicemia) at a high dose (Schletter et al. 1995; Alexander et al. 2001; Natarajan et al. 2010).

1.7 Particulate Matter (PM)

PM is a mixture of compound that includes carbon centered combustion particle, secondary inorganics and crustal derived particles, nitrates, ammonia (Schwarze et al. 2006). They are heterogeneous components and are from various sources such as industrial process, biomass combustion, road abrasion, traffic emissions and wood burning (Chow et al. 2006; Schwarze et al. 2006). PM are commonly categorized into three groups with regard to their aerodynamic size as illustrated in Fig. 7, coarse fraction PM₁₀ ($PM \leq 10-2.5\mu\text{m}$), fine fraction PM_{2.5} ($PM \leq 2.5\mu\text{m}$) and ultrafine fraction PM_{0.1} ($PM \leq 0.1\mu\text{m}$) (Murphy et al. 1999; Schwarze et al. 2006).

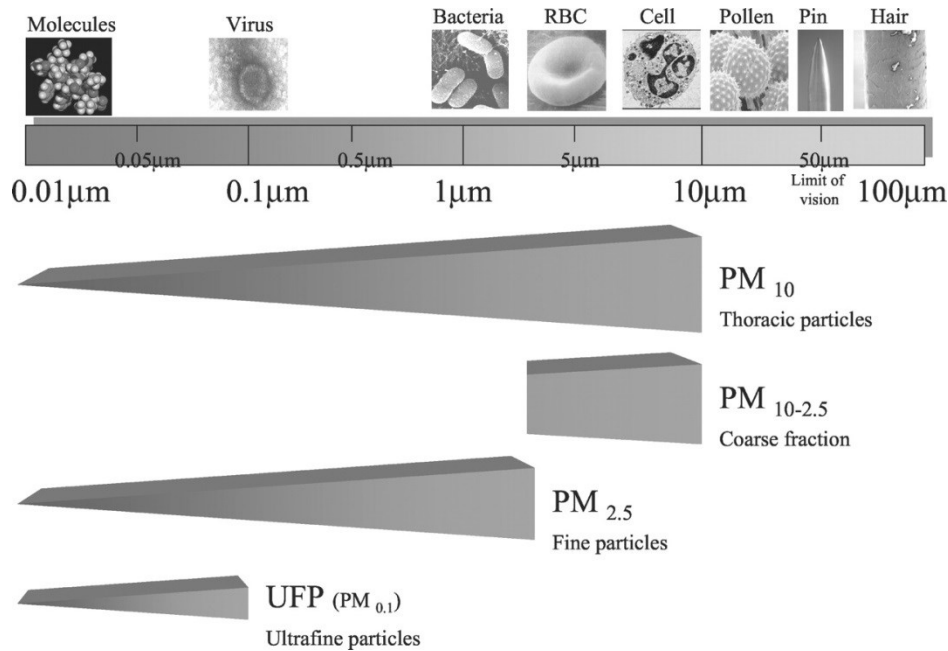


Figure 7 Particulate matter size illustration compared to other materials. Figure from (Brook et al. 2004).

Studies have documented that long-term exposure to high concentration of PM is associated to a higher risk of lung cancer, chronic respiratory diseases, arteriosclerosis and cardiovascular diseases whereas, acute exposures may cause or exacerbate respiratory diseases such as asthma and bronchitis (Samet et al. 2000; Sørensen et al. 2003; de Kok et al. 2006; Drummond 2014). The biological mechanism for these associations is unclear; however, *in vitro* studies have documented that exposure to PM induces several types of adverse cellular effects, such as DNA damage, cytotoxicity, mutagenicity and induction of Pro-inflammatory cytokine production (van EEDEN et al. 2001; de Kok et al. 2006; Gualtieri et al. 2010). Most of the adverse health effects are associated with PM_{2.5} (PM \leq 2.5 μ m), this mainly due to their ability to penetrate and deposit in the lung alveoli (Squadrito et al. 2001; de Kok et al. 2006). Furthermore, airborne particles are documented to be linked to high mortality and morbidity from cardiovascular disease, respiratory diseases and cancer (Pope III et al. 1995; Miller et al. 2012).

1.7.1 Particle Deposition in the lung

Particle size is one of the major factors that determine where in the airways inhaled particles end up. Inhaled particles are carried with the tidal air and deposited on the respiratory tract surface by diffusion, sedimentation, impaction, interception and electrostatic precipitation (Heyder et al.

2001). Larger particles ($>5 \mu\text{m}$) are mostly trapped in the upper respiratory tract (nasopharyngeal region and large conducting airways) whereas smaller particles ($0.2\text{--}5 \mu\text{m}$) are often deposited in smaller air ways and alveoli. Moreover, the smallest particles also tend to be deposited in the nose and throat as illustrated in Fig. 8 (Klaassen 2013). Particles may also accumulate unevenly in certain *hot spots* mostly in the branching area. In general the deposition sites are where the particles have the highest impact (Nemmar et al. 2013). However, a fraction of nanoparticles might have a tendency to translocate to other organs by penetrating the air-blood tissue barrier to the alveolar capillaries after deposition on the alveolar wall (McQueen 2010).

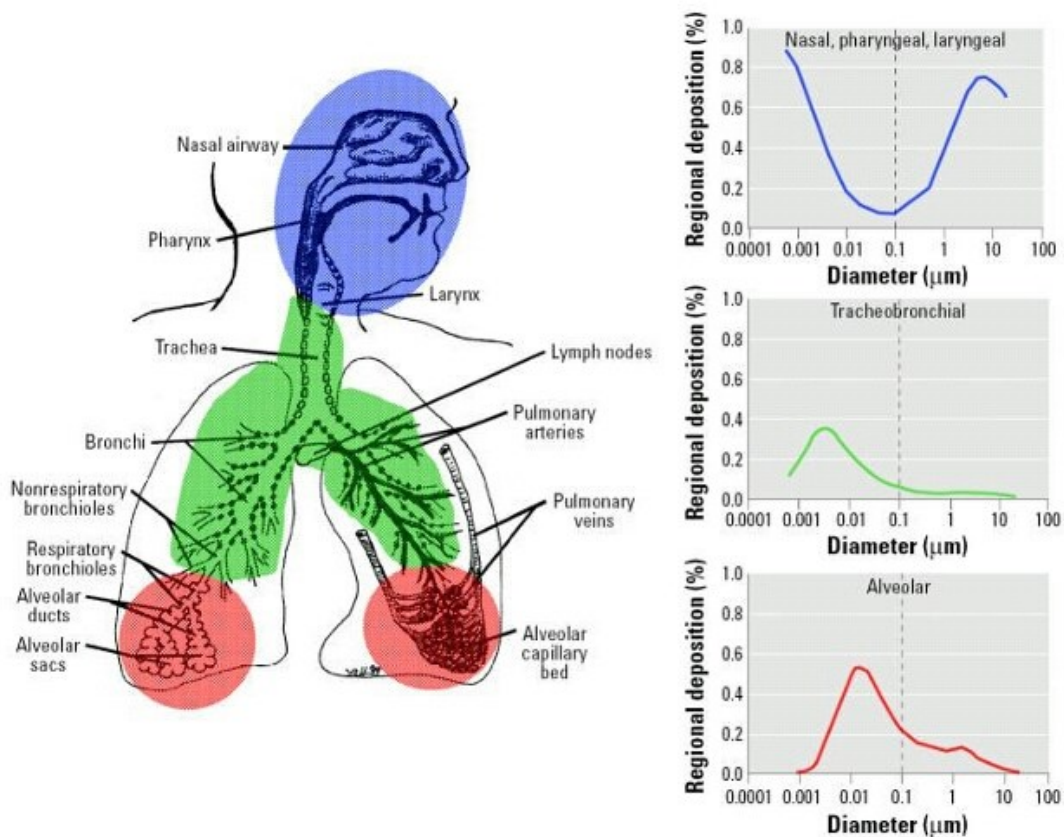


Figure 8 Predicted inhaled particle deposition in the respiratory tract with respect to particle size. Figure from (Klaassen 2013)

1.7.2 Diesel exhaust (DE)

Diesel exhaust is an exhaust from diesel engines such as motor vehicles, power generators diesel trains and ships. DE is a complex mixture of thousands of compounds. It is a carbon core compound adsorbed with Polycyclic Aromatic Hydrocarbons (PAHs), nitrates, metals, quinones and other trace elements (de Kok et al. 2006; Wichmann 2007). They can be in gaseous form or particulate form. The particulate form is known as Diesel Exhaustive Particle (DEP) or Diesel Particulate Matter (DPM). DPM from different engine types may vary in particle size and chemical composition. A typical DPM composition and structure is illustrated in Fig. 9. Each component diameter ranges from $2.5\mu\text{m} \leq 0.1\mu\text{m}$ (Wichmann 2007). DPM contribute a considerable amount to PM pollution, has been categorized as carcinogen by the International Agency for Research on Cancer (Salvi, Blomberg, et al. 1999; Ulrich 2007; (IARC) 2012). Human exposure to DPM is mainly due to occupational and ambient air exposure. Several studies have documented that DPM is associated to various health effects such as lung cancer, inflammation of respiratory tract, cardiovascular diseases, and susceptibility to infection and allergens (Bhatia et al. 1998; Salvi and Holgate 1999; Nordenhäll et al. 2001; Sydbom et al. 2001). Moreover, DPM has been documented to upregulate pro-inflammatory cytokines such as IL-8 and TNF- α in various immune cells as well as involved in developing asthma and tumor (Chung et al. 1999; Vogel et al. 2005).

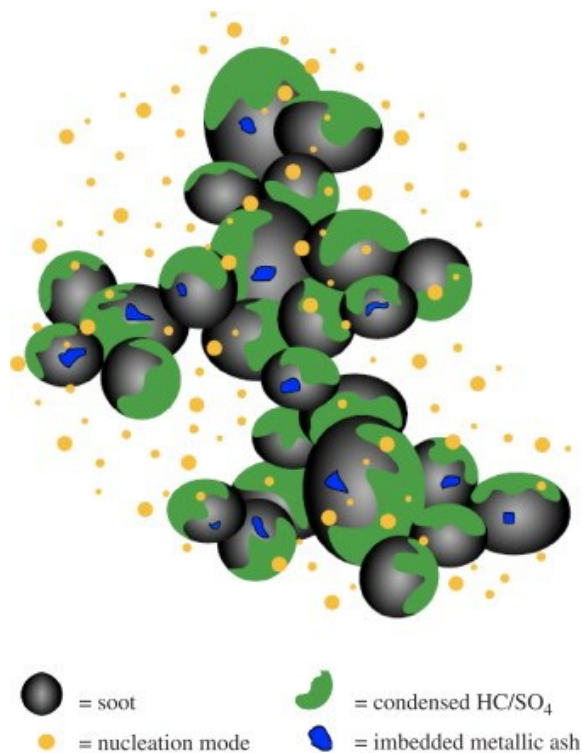


Figure 9 The physico-chemical composition and structure of diesel particulate matter.
 Figure from (Maricq 2007).

1.7.3 Silica

Silica is silicon dioxide (SiO₂) and it can occur either as crystalline or non-crystalline (amorphous) form. Silica exists abundantly in nature in rocks, soils, sands but it can also be created in industrial processes such as ceramic manufacturing, foundry processes and carbide manufacturing (WHO 2000). The major sources of human exposure to silica are occupational exposures (e.g. mining, farming, construction and other industrial process that utilize silica containing products) and ambient air exposure (e.g. road abrasions) (WHO 2000). The concentration of silica particles is documented to be higher in urban area due to the road abrasions. Studies have documented long term exposure to crystalline silica may lead to silicosis, lung fibrosis and cancer (Oxman et al. 1993; WHO 2000). *In vivo* and *in vitro* studies have showed that exposure to crystalline silica results cellular inflammations (Warheit et al. 1995; Herseth, Refsnes, et al. 2008).

1.8 Combined Effects

Our environment is filled with a number of air pollution particles both organic and inorganic. When we breathe, we are potentially exposed to a mixture of these compounds (agents). Hence, knowledge and understanding of the interaction of combined effect is important. When two or more agents combined, their effect might be additive, synergistic or antagonistic. It is called additive, when the combined response is equivalent to the sum of the individual effect, synergistic, when the combined response is more than the sum of the individual effect and antagonistic, when one of the substances decreases the effect of the other (Timbrell 2009). In the present study, we have studied the combined effect of *A. fumigatus* hyphae and particles (DPM or silica) as well as LPS and particles (DPM or silica).

2 AIM OF THE STUDY

The main aim of this study was to assess whether *A. fumigatus* hyphae (AFH) and LPS would prime the cells to give increased inflammatory reaction towards air pollution particles.

As part of the normal body defense against pathogens, exposure to AFH or LPS results an induction of inflammatory response. On the other hand, air pollution PM has been reported to give unnecessary pro-inflammatory reactions. Therefore, we hypothesize that cells exposed to AFH or LPS will have an increased cytotoxic and inflammatory reactions when co-exposed with air pollution PM such as Diesel Particulate Matter (DPM) or Min-U-Sil.

To elucidate our hypothesis, we tested out AFH, LPS, DPM and Min-U-Sil in single and co-exposure set ups using THP-1 monocytes and PMA differentiated THP-1 macrophages as experimental models to answer the following questions.

- How is the individual exposure effect of AFH, LPS, DPM and Min-U-Sil in cytokine (IL-1 β and TNF α) secretion? Are any of these agents cytotoxic?
- Which concentrations are suitable for the co-exposure with respect to cytokine response and cytotoxicity?
- Does the co-exposure of AFH and particles (DPM or Min-U-Sil), LPS and particles (DPM or Min-U-Sil) have increased (synergistic) effect on cytokine secretion compared to the individual exposures? Are these co-exposures cytotoxic?
- Do these co-exposures induce increased or decreased mRNA expressions of cytokines (IL-1 β and TNF α) compared to the individual exposures (controls)?

3 MATERIALS AND METHODS

3.1 Cell culture

In our experiments we used cell line model system called human monocyte THP-1 cell. THP-1 cells were extracted from an acute leukemia patient (Tsuchiya et al. 1980). The cell line has most of the distinct features of primary monocytes including morphology, secretory products, oncogene expression, Fc and C3b receptors; But it lacks surface and cytoplasmic immunoglobulins (Tsuchiya et al. 1980). In general, cell line models have relatively low cost, are easy to culture and grow perpetually. Unlike other cell lines THP-1 monocyte cell line has the ability to differentiate in to macrophages when treated with Phorbol 12-myristate 13-acetate (PMA) (Auwerx 1991). The PMA treated cells exhibit most of macrophage features and also adhere to the plastic surface. We used both the THP-1 cells (monocytes) and PMA differentiated THP-1 cells (PMA THP-1 macrophages) in our experiments.

The THP-1 cells were purchased from American Tissue Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI 1640 medium with L-glutamine (BioWhittaker, Lonza, Basel, Switzerland) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Sigma Aldrich, St. Louis, MO, USA), 10 mM HEPES (Gibco, Life Technologies, Grand Island, NY, USA), 1 mM pyruvic acid (Sigma–Aldrich, St. Louis, MO, USA) and 0.1% gentamicin (Gibico, Life Technologies, Grand Island, NY, USA). They were kept at concentration of 5×10^5 cells/ml in 75 cm² cell culture flasks (Sarstedt AG & Co, Numbrecht, Germany) and were grown in humidified atmosphere at 37 °C with 5% CO₂. The cells were passaged every week and refreshment of medium was done every second day. Cells are in suspension (do not adhere to plastic dishes or flask).

3.2 Fungal samples and preparation

Here after *A. fumigates* hyphae (*A. fumigates* hyphal fragments) is represented with AFH unless otherwise stated. AFH was grown on agar plate covered with cellophane membrane and aerosolized by air jets at the National Institute of Occupational Health in Norway (NIOH). Particles were next collected onto polycarbonate filters and characterized by field emission scanning electron microscopy quantified by mass content (µg/ml dry weight) as also described

(Afanou et al. 2014). The fungal fragments were inactivated on ice with x-ray (17,45 Gy/min, 225 kV, 13 mA, no filter, 5 cm distance to the source) from X-RAD 225 (Precision X-ray Inc., North Branford) receiving a total dose of 5 kGy to avoid destruction of important epitopes/enzymatic activity needed to trigger inflammatory related responses. The hyphae were dissolved in LHC-9 culture medium equivalent to a concentration of 31.8 mg/ml (stock solution). Many concentrations of AFH hyphae have been tested at the department. Based on that information, we chose final concentrations of 1 ng/ml, 10 ng/ml, 100 ng/ml, 500 ng/ml, 1 µg/ml, 10 µg/ml, 50 µg/ml and 100 µg/ml.

3.3 LPS

Lipopolysaccharide (LPS) (Sigma Aldrich St. Louis MO, USA) is extracted from Gram negative bacteria. It is a potent inducer of Pro-inflammatory cytokines including TNF- α and IL-1 (Pålsson-McDermott et al. 2004). Thus, LPS was used as a positive control in our experiments. The stock concentration of LPS was 1mg/ml. The final concentrations used were 0.05, 0.5, 5 and 25 ng/ml.

3.4 Particles

3.4.1 Diesel Particulate Matter

Diesel Particulate Matter (DPM) (Industrial Forklift) or Standard Reference Material (SRM) ®2975 was obtained from U.S. Department of commerce, National institute of standards and technology (Gaithersburg, MD). It is a diesel particulate matter collected from an industrial diesel powered forklift. It contains particles with sizes ranging < 2.5 µm (NIST 2013). It is manufactured to be used for evaluating analytical method for determination of selected PAHs and nitro substitute PAHs (nitro-PAHs) and other research purposes.

3.4.2 Min-U-Sil 5

Min-U-Sil 5 quartz is high purity natural crystalline silica particle that is composed of SiO₂ (99 %) and few other compounds (Al₂O₃, Fe₂O₃ and TiO₂) constituting less than 1%. Min-U-Sil has a diameter of \leq 5µm and it was purchased from U.S. Silica Company, Berkeley Springs, WV, USA.

3.4.3 Particle samples and preparation

DPM and Min-U-Sil were suspended in FBS free RPMI1640 medium at a concentration of 2 mg/ml and 5 mg/ml, respectively. They were stirred overnight at room temperature (RT), sonicated for 30 min in an ultrasonic water bath (Sonorex RK 100, Transistor) and stored at 4 °C (maximum 3 weeks) as also described elsewhere (Herseth, Refsnes, et al. 2008; Bach et al. 2014). The particle suspensions were vortexed for 30 s before added to the cells. The final concentrations of DPM tested were 6 $\mu\text{g}/\text{cm}^2$ (25 $\mu\text{g}/\text{ml}$), 12 $\mu\text{g}/\text{cm}^2$ (50 $\mu\text{g}/\text{ml}$), 24 $\mu\text{g}/\text{cm}^2$ (100 $\mu\text{g}/\text{ml}$) and 48 $\mu\text{g}/\text{cm}^2$ (200 $\mu\text{g}/\text{ml}$). Whereas, Min-U-Sil tested were 20 $\mu\text{g}/\text{cm}^2$ (83.5 $\mu\text{g}/\text{ml}$), 40 $\mu\text{g}/\text{cm}^2$ (167 $\mu\text{g}/\text{ml}$), 80 $\mu\text{g}/\text{cm}^2$ (333 $\mu\text{g}/\text{ml}$) and 160 $\mu\text{g}/\text{cm}^2$ (667 $\mu\text{g}/\text{ml}$). DPM and Min-U-Sil concentrations were adapted from other studies (Herseth, Refsnes, et al. 2008; Bach et al. 2015).

3.5 Exposures

In order to test our hypothesis, first we made a dose response curve of the individual exposure agents that we call it as “single exposure”. Based on the single exposure cytokine and cytotoxicity result, we selected few concentrations that gave low positive response in cytokine secretion and low cytotoxicity (< 10% cell death) and used them for co-exposures.

Table 1. Exposure agents and concentrations

Cell types	Exposure Agents	Concentrations of exposure agents
THP-1 Monocytes	LPS	0.05, 0.5, 5 & 25 ng/ml
	AFH	0.5 , 1, 10 & 50 $\mu\text{g}/\text{ml}$
	DPM	6, 12 , 24 & 48 $\mu\text{g}/\text{cm}^2$
	Min-U-Sil	20, 40 , 80 & 160 $\mu\text{g}/\text{cm}^2$
PMA-THP-1 Macrophages	LPS	0.05, 0.5 , 5 & 25 ng/ml
	AFH	1, 10, 50 & 100 ng/ml
	DPM	6, 12 , 24 & 48 $\mu\text{g}/\text{cm}^2$
	Min-U-Sil	20, 40 , 80 & 160 $\mu\text{g}/\text{cm}^2$

The concentrations in bold are concentrations selected for the co-exposure experiments.

3.5.1 Single exposures

3.5.1.1 THP-1 monocytes

THP-1 monocytes were seeded out in 9.6 cm² 6 well culture dishes (Corning Incorporated Costar, NY, USA) at a concentration of 8 x10⁵ cells per well in 1 ml fresh medium and were left for 2.5 h to adapt to the new medium before the exposures. The cells were exposed to four different concentrations of LPS or hyphae or Min-U-Sil or DPM for 24 h as shown in Table 1. Medium was added to the control. After 24 h the exposure was terminated, and both the supernatant and the cells were harvested by 10 min centrifugation at 1200 rpm and the supernatant was kept at -70 °C until it was analyzed for different cytokine secretion. The cells were stained as will be described in later section (3.6) to determine their viability.

3.5.1.2 PMA THP-1 macrophages

PMA THP-1 macrophages were seeded out in 1 ml medium at a concentration of 2x10⁵ cells/well in 3.7 cm² 12 well culture plates (Corning Incorporated Costar, NY, USA) and they were treated with 200 nM PMA (Sigma Aldrich, St. Louis MO, USA) for 72 h to differentiate them to macrophages. The PMA treatment makes the cells to adhere to the plastic surface and change their morphology from round to “fried egg” like shape. The adherence feature of the PMA THP-1 macrophages made it possible to change medium without touching the cells. Thus, after 72 h, the PMA treatment was stopped and fresh medium was added and left for 2 h to rest. After 2 h, four different concentrations of the exposures (i.e. LPS or hyphae or Min-U-Sil or DPM) as shown in Table 1 were added with 0.5 ml fresh medium. Then the cells were exposed for 24 h. At the end of the exposure, the supernatant was harvested and centrifuged for 10 min at 1200 rpm to remove dead cells and debris and kept in -70 °C. The cells were stained while in the plate to determine their viability as will be described in later section (3.6).

3.5.2 Co-exposure I

Few concentrations were selected for the co-exposure as described in table 1. Both types of cells (THP-1 monocytes and PMA-THP-1 macrophages) were seeded out as earlier (3.5.1). The cells were primed with either LPS or AFH for 3 h except for the controls. When the priming is done they were co-exposed with DPM or Min-U-Sil for 18 h. The control groups (the non-pretreated

groups) had just medium or LPS or AFH or DPM or Min-U-Sil. When the exposure is over, both types of cells were harvested as described in the previous sections (3.5.1.).

3.5.3 Co-exposure II

Based on the cytokine analysis result, fewer concentrations were chosen to test for gene expression (cytokine synthesis). Since the mRNA level curve flattens out in longer hours, we conducted a pilot study on time curve to find out the optimal time for co-exposure and harvest (Fig. S4). Then we decided to prime both types of cells for 3 h and co-expose them for 3 h.

The THP-1 monocytes were seeded out as described in section (3.5.1.1). They were primed either with LPS (25 ng/ml) or AFH (500 ng/ml) and co-exposed with Min-U-Sil (20 or 40 $\mu\text{g}/\text{cm}^2$). Exposure was terminated at 6 h. The cells were centrifuged and re-suspended in ice cold phosphate Buffered Saline (PBS) without Ca and Mg (Lonza, Verviers, Belgium), centrifuged again and finally kept at -70°C until analyzed for gene expression.

PMA THP-1 macrophages were seeded out in 1.5 ml at a concentration of 2×10^5 cells per well along with PMA in 6 well plates to increase the number of cells per well. Cells were treated with PMA for 72 h. When the treatment was over, the old medium was discarded and fresh medium was added and were left for 2 h to rest. Then they were primed with either LPS (0.5 ng/ml) or AFH (50 ng/ml) for 3 h and co-exposed with either DPM (12 $\mu\text{g}/\text{cm}^2$) or Min-U-Sil (40 $\mu\text{g}/\text{cm}^2$) for additional 3 h. After 6 h, the supernatant was discarded and the cells were rinsed with ice cold PBS twice while they were in the plates. The plates were stored at -70°C until analyzed for gene expression.

3.6 Cytotoxicity

Fluorescence Microscopy

Double stain apoptosis detection kit Hoechst 33342/ PI is a staining kit that enables to differentiate live, apoptotic and necrotic cells. As the name indicates the kit employs two dyes: Hoechst 33342 and Propidium Iodide (PI). They both bind to cell's DNA and fluoresce. Hoechst 33342 is a blue fluorescence dye (excitation/ emission maxima at $\sim 350/461$ nm), that stains apoptotic cell's chromatin more brightly than the chromatin of normal cells. PI is red

fluorescence dye with excitation/emission maxima at ~535/617 nm, it binds to dead cell's DNA and the cells exhibit high-red fluorescence (GenScript ; Invitrogen 2009; Atale et al. 2014).

In our experiments, the exposed THP-1 monocytes were harvested through centrifugation. The cells were re-suspended with 0.5 ml of a mix made of 20% FBS and 80% PBS and centrifuged at 1200 rpm for 10 min. Then 0.5 ml of culture medium was added to the pellet with 10 µl of PI/Hoechst 33342 mixed at 1:1 ratio and incubated for 30 min at RT. The suspension was centrifuged again, medium was discarded and 20 µl FBS was added to the pellet. Cells were fixed on microscope slides and scored under a fluorescence microscope (Nikon Eclipse E 400). The fractions of the stained cells were scored with a minimum of 400 cells per sample. The scoring of the cells were according to nuclei staining and plasma membrane integrity as viable, apoptotic and necrotic.

Exposed PMA THP-1 macrophages were harvested by removing the supernatant. Since the PMA THP-1 macrophages adhere to the plastic, we tried to detach them using Accutase® solution (Sigma Aldrich, St. Louis, MO, USA). However, it appeared to kill all the cells. Thus, we stained them while they were in the 12 well plates. First 10% FBS was mixed with medium, 250 µl of the mix was added to each well. Then 10 µl of PI/ Hoechst 33342 mixed at 1:1 ratio was added and incubated for 30 min at RT. The mix was discarded and 100 µl FBS was added and dried. The stained cells were examined under Nikon Eclipse E 400 fluorescence microscope.

3.7 Cytokine measurement

Enzyme Linked Immunosorbent Assay (ELISA)

The cytokine secretion was assessed using a widely used technique named Enzyme Linked Immunosorbent Assay (ELISA). ELISA detects and quantifies biological substances such as proteins, antibodies, antigens and hormones (Engvall et al. 1971). It is the most recommended assay to measure the concentration of cytokines in a supernatant (Leng et al. 2008). The technique generally involves specific interaction between antigen and antibodies linked to an enzyme or fluorescent compound. By adding a substrate, coloration will be developed and hence, detecting the antigen-antibody interaction. The intensity of the color corresponds to the amount of protein in the solution. Among the different types of ELISAs, “Sandwich” ELISA is known to

be highly specific and powerful. It is so called Sandwich ELISA because the antigen of interest is bound between two layers of antibodies (i.e. capture antibody and detection antibody).

In our study, we performed Sandwich ELISA to measure the concentrations of IL-1 β and TNF- α in the supernatant of exposed THP-1 monocytes and PMA THP-1 macrophages as described under (3.5.1) and (3.5.2).

In order to measure IL-1 β and TNF- α concentration in the supernatants, Human IL-1beta/ IL-1F2 DuoSet (R&D system Europe, Ltd UK) and Human TNF- α CytoSet (Nowex, Life Technologies, Camarillo, CA 93012, USA) kits were used. The two kits have similar protocols except IL-1 β has 2 h incubation after the addition of samples whereas, no incubation in the TNF- α kit. It was performed on 96 well microplates (Sigma Aldrich, St. Louis, MO, USA). The microplates were coated with capture antibody. They were blocked for 1 h at RT to avoid unspecific binding. Samples were diluted according to the manufacturer's recommendations and samples were diluted up to 40 times. Next, diluted standards and samples were added and incubated for 2 h with continuous shaking at 700 rpm. Detection antibody was added and incubated for further 2 h. Streptavidin conjugated with Horse Radish Peroxide (HRP) was added and incubated for 20 min. Aspiration and washing using wash buffer (0.05% Tween 20 in PBS) was done between each of the previous steps to remove unbound substances. Finally, 3,3',5,5'tetramethylbenzidine (TMB) was added, incubated for 20 min and stopped using sulfuric acid. The absorbance was read at 450 nm using a plate reader (TECAN sunrise, Austria GmbH) employing Magellan V1.10 software. The concentrations (pg/ml) were determined relative to the standard samples.

3.8 Gene expression

Real-time Reverse Transcriptase Polymerase Chain Reaction (real-time RT-PCR)

Real-time RT-PCR was performed to assess the gene expression of IL-1 β and TNF- α in both THP-1 monocytes and PMA THP-1 macrophages after the exposures described under section 3.5.3. Real-time RT-PCR is a quantitative method that measures the amount of target genes present in a sample relative to a control or untreated sample. In general, the process involves isolation of total RNA, preparing complementary DNA (cDNA) from the RNA through the process called reverse transcription and amplification of DNA.

In our experiments, we used TaqMan gene expression assay (Applied Biosystems, Life Technologies Corporation, CA, USA). The assay uses gene specific primer-probe interaction to detect specific amplification of genes as the PCR cycle occurs. A TaqMan probe is an oligonucleotide probe constructed with a fluorescent reporter dye bound to the 5' end and a quencher on 3' end. If the target sequence is present, the probe will be cleaved by 5' nuclease separating the reporter dye from the quencher, which increases the reporter dye fluorescent signal. The fluorescent signal is directly proportional to the accumulation of the PCR products (Bustin et al. 2005; Applied Biosystems 2010). The values were normalized against a house keeping gene actin beta (ACT β) which is a gene normally expressed in all cells.

Isolation of RNA

Isolation of RNA was performed using PerfectPure RNA cultured cell kit-50 (GmbH, D-22767 Hamburg, Germany). The harvested cells (both floating THP-1 monocytes in Eppendorf tubes and adherent PMA THP-1 macrophages on a dish) were lysed for 5 min by adding 200 μ l of lysis buffer and it is used to eliminate RNAase activity. Since the PMA THP-1 macrophages are in the plates, they were scraped off from the dish after the 5 min and added to Eppendorf tubes. Then they both were run through a 26 g brown cannula (BD, NJ, USA) to homogenize them. The lysate was transferred to purification column and centrifuged. It was washed using wash solution 1 and centrifugation to remove proteins from the samples. DNase was added, incubated for 15 min and centrifuged to remove the bound DNA fragments. The previous step was followed by a couple of washing using wash solution 2 and centrifugations to remove different salts. Finally elution buffer was added and centrifuged collecting down the purified RNA from the purification columns. The concentration and purity of the RNA was determined by using a Nanodrop (DeNoVIX DS-11 spectrophotometer). The RNA samples were stored at -70 $^{\circ}$ C until used for cDNA synthesis.

cDNA Synthesis

cDNA synthesis is a synthesis of cDNA from an RNA template using an enzyme named reverse transcriptase. The isolated RNA was reverse transcribed to cDNA using High Capacity cDNA Archive Kit (Applied Biosystems, Life Technologies, CA, USA). The transcription process generally involves preparation of cDNA master mix (2X master mix), dilution of the isolated RNA with Nuclease-Free water and running it in a PCR machine. The 2X master mix was made

of 10X reverse buffer, 25X dNTP, 10X random primers, multi-scribe reverse transcriptase and nuclease-free water giving a total volume of 12.5 μ l for each sample. Then 500 ng of the purified RNA was diluted with nuclease-free water and mixed with the 2X master mix giving a total volume of 25 μ l. Two control samples were made as a negative control for DNA and reagent contamination; they were named No Reverse Transcription control (NRC) and No Template Control (NTC), respectively. The NRC sample had no reverse transcriptase, whereas, the NTC sample had no RNA. We used a Gene Amp PCR system 2400 (PerkinElmer, Waltham, MA, USA) thermocycler using the following temperature/ time settings: 25 °C/10 min, 37 °C/120 min and 85 °C/5 min. The synthesized cDNA samples were stored at -70 °C until used for real time RT-PCR.

Real time RT-PCR

In order to assess the gene expression of IL-1 β and TNF- α , the cDNA samples were diluted to a ratio of 1:9 with nuclease-free water. Real time master mix was prepared by mixing each primer (IL-1 β , TNF- α and ACT β) with TaqMan Universal PCR Master Mix (Applied Biosystems, Life Technologies Corporation, CA, USA) with 1:10 ratio. Then 9 μ l of the cDNA and 11 μ l of the real time master mix giving a total volume of 20 μ l were added to 96-well plate in parallels. It was covered with optical adhesive strip and centrifuged for 1 min at 1000 rpm. The samples were run using a thermocycler 7500 Fast Real Time PCR machine (Applied Biosystems, Life Technologies Corporation, CA, USA) adjusted to the following temperature/ time setting: 50 °C/2 min, 95 °C/10 min for 40 cycles and 95 °C/ 15 sec and 60 °C/ 1 min. The gene expression data were normalized to the expression of an endogenous control (ACT β), and analyzed relative to the untreated (control) sample using 7500 fast system SDS V1.3 software using the formula below.

$$RQ = 2^{-(\Delta\Delta Ct)}, \Delta\Delta Ct = (Ct_{mRNA} - Ct_{ACT\beta})_{exposed} - (Ct_{mRNA} - Ct_{ACT\beta})_{control}$$

3.9 Statistical Analysis

Graph Pad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA) was used to analyze the data. Individual experiments was repeated at least three times (N=3). Statistical significance was assessed using one-way analysis of variance (One-way ANOVA) followed by Dunnett's post-test. ANOVA is a suitable analysis method to determine whether there is a significant

difference between the control and exposed groups. However, it has strict requirements to fulfill. Two requirements are having a normal (Gaussian) distribution and homogenous variance especially, for small data sets such as ours. Thus, after assessing the data for these requirements, the single exposure data were transformed to logs and co-exposure data were normalized. At the end of the analysis p-values were obtained and P values ($P < 0.05$) were considered as significant.

4 RESULTS

4.1 Single and co-exposure induced cytotoxicity to THP-1 monocytes

In the present study we were mainly interested in understanding the pro-inflammatory potential of co-exposures of cells primed with AFH or LPS and co-exposed with DPM or Min-U-Sil (AFH+DPM, AFH+Min-U-Sil, LPS+DPM and LPS+Min-U-Sil). However, characterizing the cytotoxic potential of these exposure agents both individually and together are needed to insight in to the whole process. Therefore, both single and co-exposed THP-1 monocytes were examined for cytotoxicity measured as induction of apoptosis and necrosis by using fluorescence microscopy after PI/Hoechst 33342 staining.

Cytotoxicity of THP-1 monocytes to single exposures

THP-1 monocytes exposed to four concentrations (6, 12, 24 and 48 $\mu\text{g}/\text{cm}^2$) of DPM for 24 h shows little cytotoxicity as judged by the level of apoptosis and necrosis (Fig.10A). On the other hand, THP-1 monocytes exposed to four concentrations (20, 40, 80 and 160 $\mu\text{g}/\text{cm}^2$) of Min-U-Sil for 24 h showed very low apoptosis and dose dependent increase of necrosis. All the four concentrations tested showed significantly high necrosis compared to the control (Fig. 10B). A similar dose dependent death pattern was observed on cells exposed to concentrations (0.5, 10, 50 and 100 $\mu\text{g}/\text{ml}$) of AFH for 24 h as that of Min-U-Sil (Fig.11B). THP-1 monocytes exposed to a range of concentrations (0.05, 0.5, 5 and 25 ng/ml) of LPS for 24 h did not show any significant apoptosis or necrosis except, for the cells exposed to 5 ng/ml LPS. This concentration caused significantly ($P < 0.05$) less apoptosis compared to the control (Fig.11A).

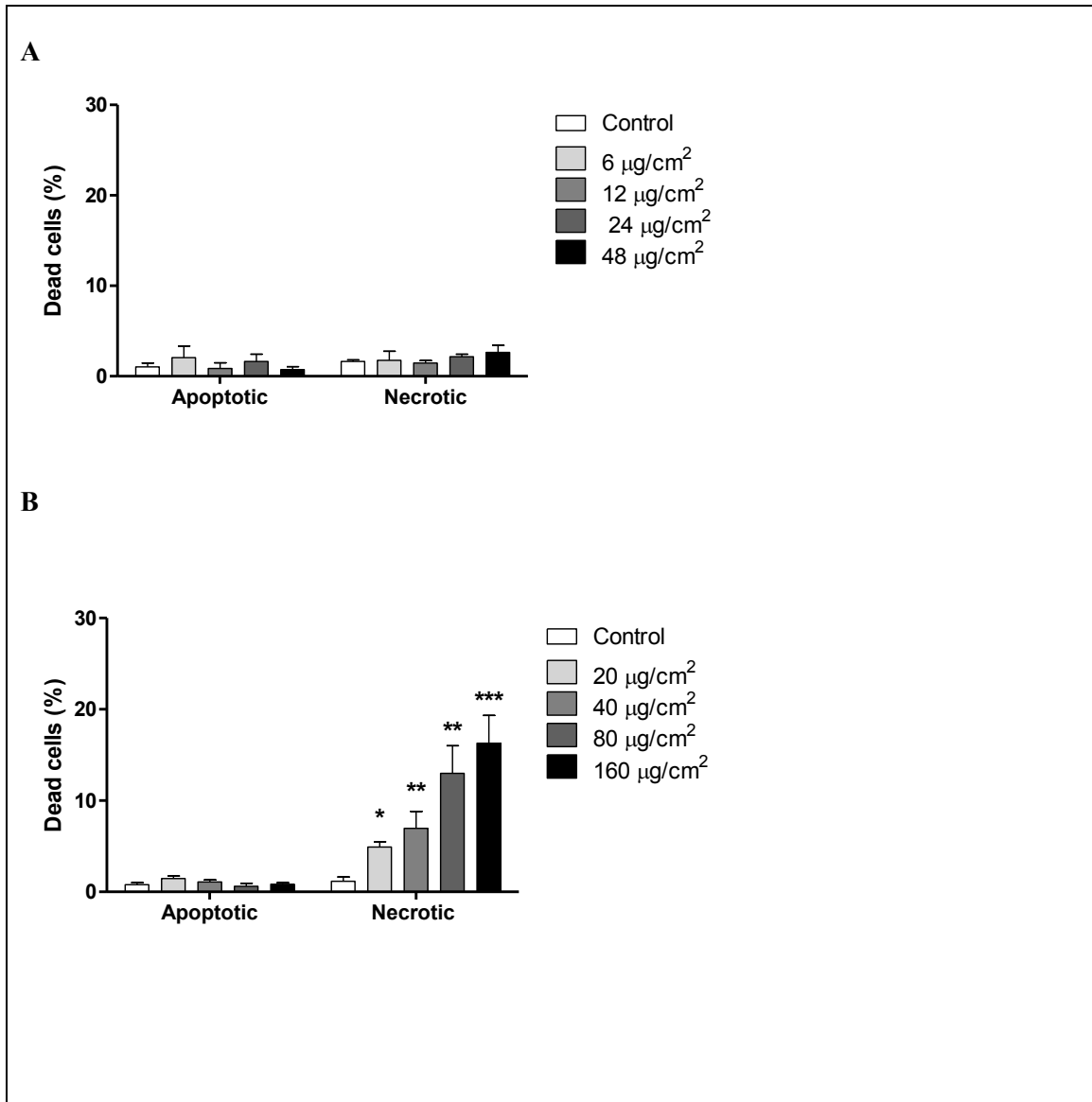


Figure 10 Induction of apoptosis and necrosis in THP-1 monocytes after exposure to DPM and Min-U-Sil determined by fluorescence microscopy. (A) Apoptotic and necrotic THP-1 monocytes after exposure to four different concentrations of DPM for 24 h. (B) Apoptotic and necrotic THP-1 monocytes after exposure to four different concentrations of Min-U-Sil for 24 h. Cells were stained with PI/ Hoechst and subsequently analyzed for necrosis and apoptosis with fluorescence microscope. At least 400 cells were counted per experiment per concentration. Bars represent percentages of means \pm SEM of 3 independent experiments. Statistical analysis was based on q log transformed data (One way ANOVA, Dunnett post-test). The asterisks denote significant difference of exposed cells compared to the control. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

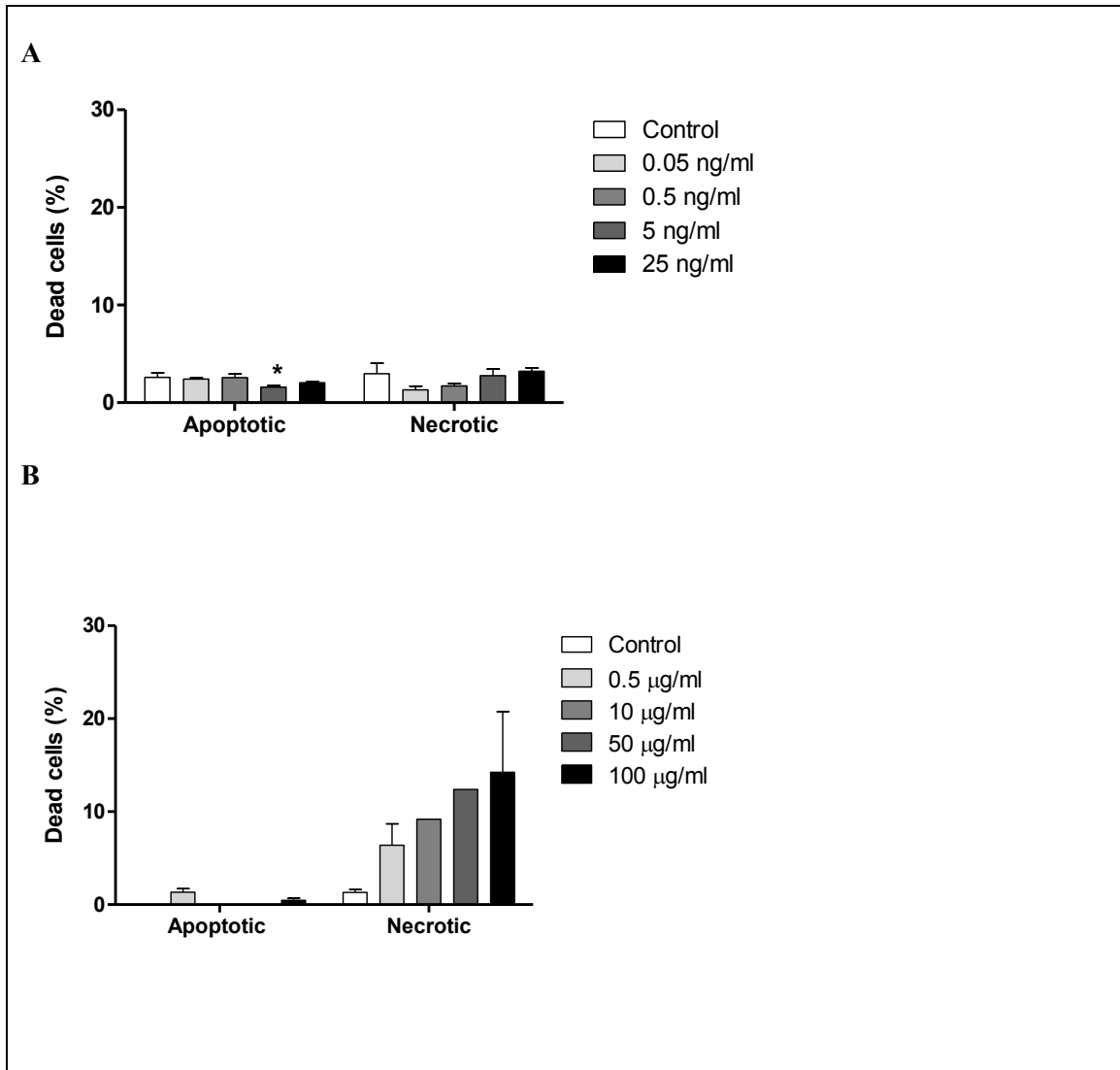


Figure 11 Induction of apoptosis and necrosis in THP-1 monocytes that were exposed to four different concentrations of LPS and AFH determined by fluorescence microscopy. (A) Apoptotic and necrotic THP-1 monocytes after being exposed to four different concentrations of LPS for 24 h. (B) Apoptotic and necrotic THP-1 monocytes after being exposed to four different concentrations of AFH for 24 h. Cells were stained with PI/ Hoechst 33342 and subsequently analyzed for necrosis and apoptosis with fluorescence microscope. At least 400 cells were counted per experiment per concentration. Bars represent percentages of means \pm SEM for N=1-3 separate experiments. Statistical analysis was based on a log-transformed data (One way ANOVA, Dunnett post-test). The asterisk (* $P < 0.05$) denote significant difference of cells exposed to LPS compared to the control. Statistical testing was not performed for the cells exposed to AFH as the number of experiment was < 3 for some of the AFH concentrations.

Cytotoxicity of THP-1 monocytes to co-exposures

THP-1 monocytes primed with either LPS (25 ng/ml) or AFH (500 ng/ml) for 3 h and co-exposed with DPM (6 or 12 $\mu\text{g}/\text{cm}^2$) for 18 h did not show any significant apoptosis or necrosis (Fig.12A and B), However, necrosis that reached up to 6% was observed in cells exposed to AFH (500 ng/ml) alone and cells co-exposed with AFH (500 ng/ml) and DPM (6 $\mu\text{g}/\text{cm}^2$). THP-1 monocytes primed with LPS (25 ng/ml) or AFH (500 ng/ml) for 3 h and exposed to Min-U-Sil (20 or 40 $\mu\text{g}/\text{cm}^2$) for 18 h also did not show any significant apoptosis (Fig.13A). On the contrary, there was significantly ($P < 0.05$) high necrosis observed in cells co-exposed to LPS (25 ng/ml) and Min-U-Sil (40 $\mu\text{g}/\text{cm}^2$) compared to (LPS (25 ng/ml) alone (Fig. 13B). In addition, cells co-exposed to AFH (500 ng/ml) and Min-U-Sil (40 $\mu\text{g}/\text{cm}^2$) also have showed highly significant ($P < 0.001$) increase in necrosis compared to its control i.e AFH (500 ng/ml) alone (Fig.13B).

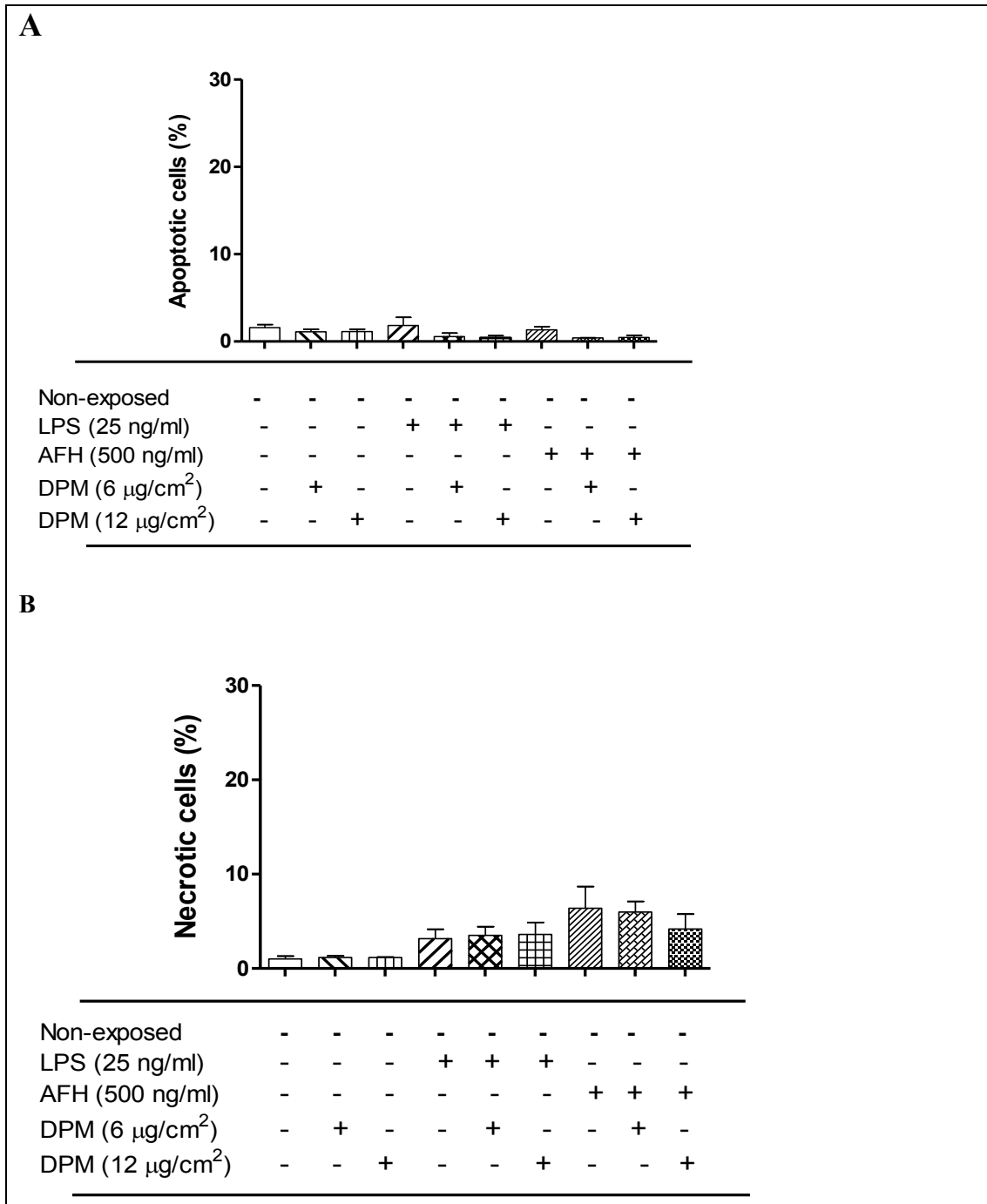


Figure 12 Induction of apoptosis and necrosis in THP-1 monocytes that were primed with LPS or AFH and co-exposed with DPM determined by fluorescence microscopy. THP-1 monocyte were primed with LPS (25 ng/ml) or AFH (500 ng/ml) for 3 h and co-exposed to DPM (6 or 12 $\mu\text{g}/\text{cm}^2$) for 18 h and was stained with PI/Hoechst and subsequently analyzed for apoptosis and necrosis with fluorescence microscope. (A) Apoptotic cells. (B) Necrotic cells. At least 400 cells were counted per experiment per sample. Bars represent percentages of means \pm SEM of 3 independent experiments + treated - untreated. Statistical analysis was based on normalized data (One way ANOVA, Dunnett post-test). None of the co-exposed showed significant apoptosis or necrosis compared to their individual control (non-exposed, LPS 825 ng/ml) and AFH (500 ng/ml).

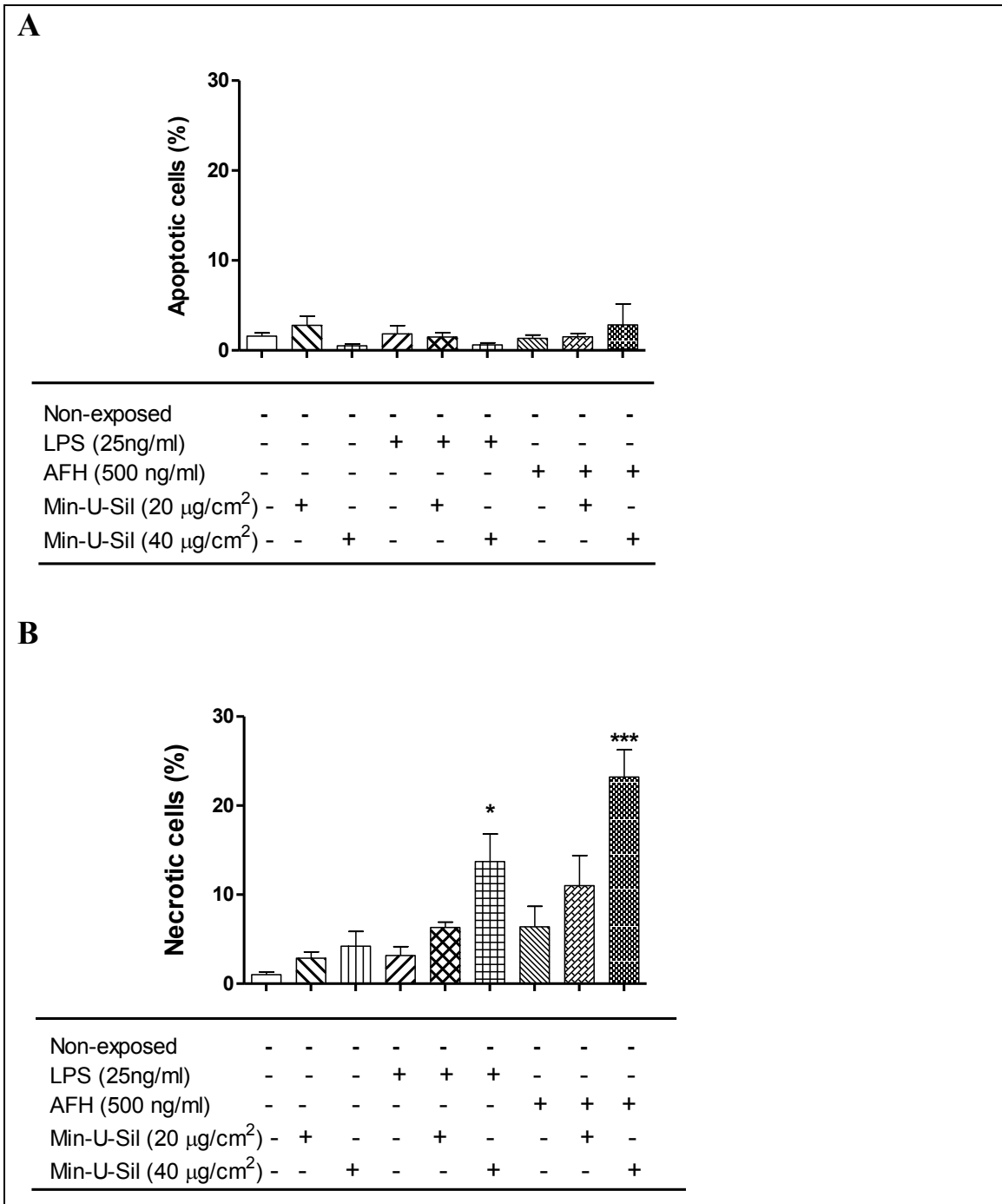


Figure 13 Induction of apoptosis and necrosis in THP-1 monocytes that were primed with LPS or AFH and co-exposed with Min-U-Sil determined by fluorescence microscopy. THP-1 monocytes were primed with LPS (25 ng/ml) or AFH (500 ng/ml) for 3 h and co-exposed to Min-U-Sil (20 or 40 $\mu\text{g}/\text{cm}^2$) for 18 h and were stained with PI/Hoechst and subsequently analyzed for apoptosis and necrosis with fluorescence microscopy. (A) Apoptotic cells. (B) Necrotic cells. At least 400 cells were counted per experiment per sample. Bars represent percentages of means \pm SEM of 3 independent experiments. Statistical analysis was based on normalized data (One way ANOVA, Dunnett post-test). Significant difference of the co-exposed cells compared to their individual control (Non-exposed LPS (25 ng/ml), AFH (500 ng/ml)) is denoted by asterisks * $P < 0.05$, ** $P < 0.001$, + treated, - untreated.

4.2 Cytokine release of THP-1 monocytes after single and co-exposure

Cytokines play a major role in regulating immune response and inflammation. Therefore, we measured the two important pro-inflammatory cytokines; IL-1 β and TNF- α in THP-1 monocytes exposed to single and co-exposures of AFH, LPS, DPM and Min-U-Sil by using ELISA.

Cytokine secretion to single exposures

THP-1 monocytes that were exposed to four different concentrations of each agent as follows: LPS (0.05, 0.5, 5 and 25 ng/ml), AFH (0.5, 10, 50 and 100 μ g/ml), DPM (6, 12, 24 and 48 μ g/cm²) and Min-U-Sil (20, 40, 80 and 160 μ g/cm²) for 24 h individually as described under materials and methods (3.5.1), and were evaluated for release of IL-1 β and TNF- α . The result revealed that cells exposed to various concentrations of DPM or Min-U-Sil was not significantly different from the control with respect to secretion of IL-1 β and TNF- α (Fig.14A&B). In contrast, THP-1 monocytes exposed to different concentrations of AFH showed highly significant release of IL-1 β and TNF- α in all concentrations tested (Fig.15C&D). LPS induced significantly ($p < 0.01$) higher release of IL-1 β and TNF- α only at the higher concentration (25 ng/ml) tested. Lower concentrations of LPS were not different from the control (Fig.15A and B).

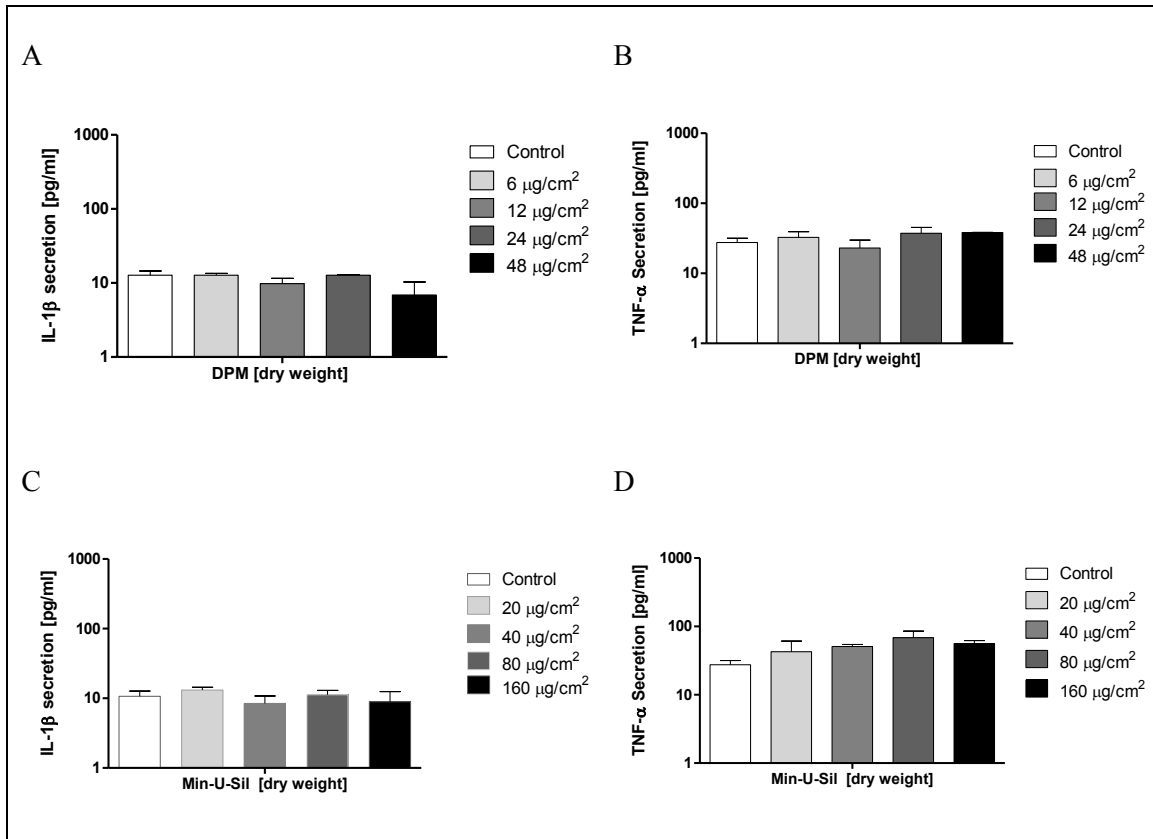


Figure 14 Effects of DPM and Min-U-Sil on cytokines secretion of THP-1 monocytes determined by ELISA. (A) IL-1 β and (B) TNF- α secretion of THP-1 monocytes exposed to indicated concentrations of DPM for 24 h. (C) IL-1 β and (D) TNF- α secretion of THP-1 monocytes exposed to indicated concentrations of Min-U-Sil for 24 h. Bars represent mean \pm SEM concentrations of 3 independent experiments. Statistical analysis was done on log transformed data (One way ANOVA, Dunnett post-test). There was no significant difference between the exposed vs control.

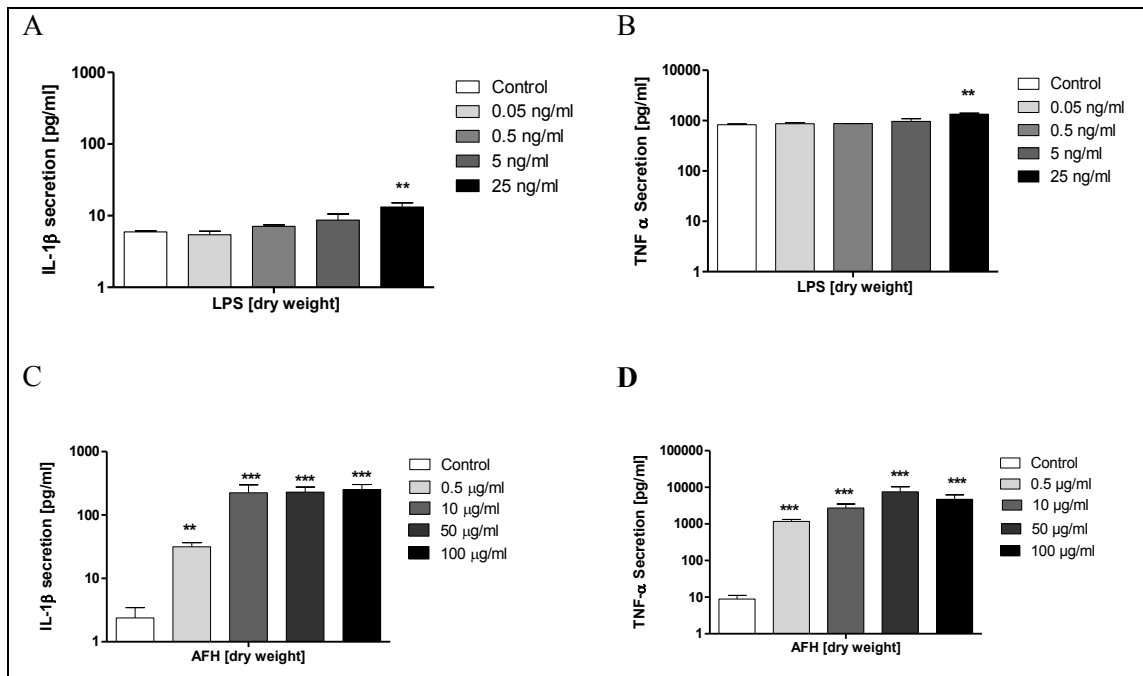


Figure 15 Effects of LPS and AFH on cytokine secretion of THP-1 monocytes determined by ELISA. (A) IL-1 β and (B) TNF- α secretion of THP-1 monocytes exposed to different concentrations of LPS for 24 h. (C) IL-1 β and (C) TNF- α secretion of THP-1 monocytes exposed to four different concentrations of AFH for 24 h. Statistical analysis was done on a log transformed data (One way ANOVA, Dunnett post-test, mean \pm SEM, N=3-4). Significant difference compared to the control denoted by asterisks * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Co-exposure induced cytokine secretion

THP-1 monocytes primed with either LPS or AFH for 3 h and co-exposed with DPM or Min-U-Sil for 18 h were assessed for IL-1 β and TNF- α secretion. Cells that were primed with LPS (25 ng/ml) or AFH (500 ng/ml) for 3 h and co-exposed to DPM (6 or 12 $\mu\text{g}/\text{cm}^2$) for 18 h did not show any significant difference in both IL-1 β and TNF- α secretion from their individual controls (non-exposed, LPS alone and AFH alone) (Fig. 16A & B). Cells primed with LPS (25 ng/ml) or AFH (500 ng/ml) for 3 h and exposed to Min-U-Sil (20 or 40 $\mu\text{g}/\text{cm}^2$) for 18 h showed a significant IL-1 β secretion. Notably, cells primed with AFH (500 ng/ml) and co-exposed with Min-U-Sil (20 $\mu\text{g}/\text{cm}^2$ or 40 $\mu\text{g}/\text{cm}^2$) were significantly different from AFH (500 ng/ml) alone exposure with P values $P < 0.05$ and $P < 0.001$, respectively (Fig 16C). Moreover, cells primed with LPS (25 ng/ml) and co-exposed to Min-U-Sil (20 or 40 $\mu\text{g}/\text{cm}^2$) also induced significantly higher secretion of IL-1 β compared to LPS (25 ng/ml) alone with P values $P < 0.01$ and $P <$

0.001, respectively (Fig 16C). No significant difference was found in the TNF- α secretion in cells primed with AFH or LPS and exposed to two concentrations of Min-U-Sil (Fig.16 B & D).

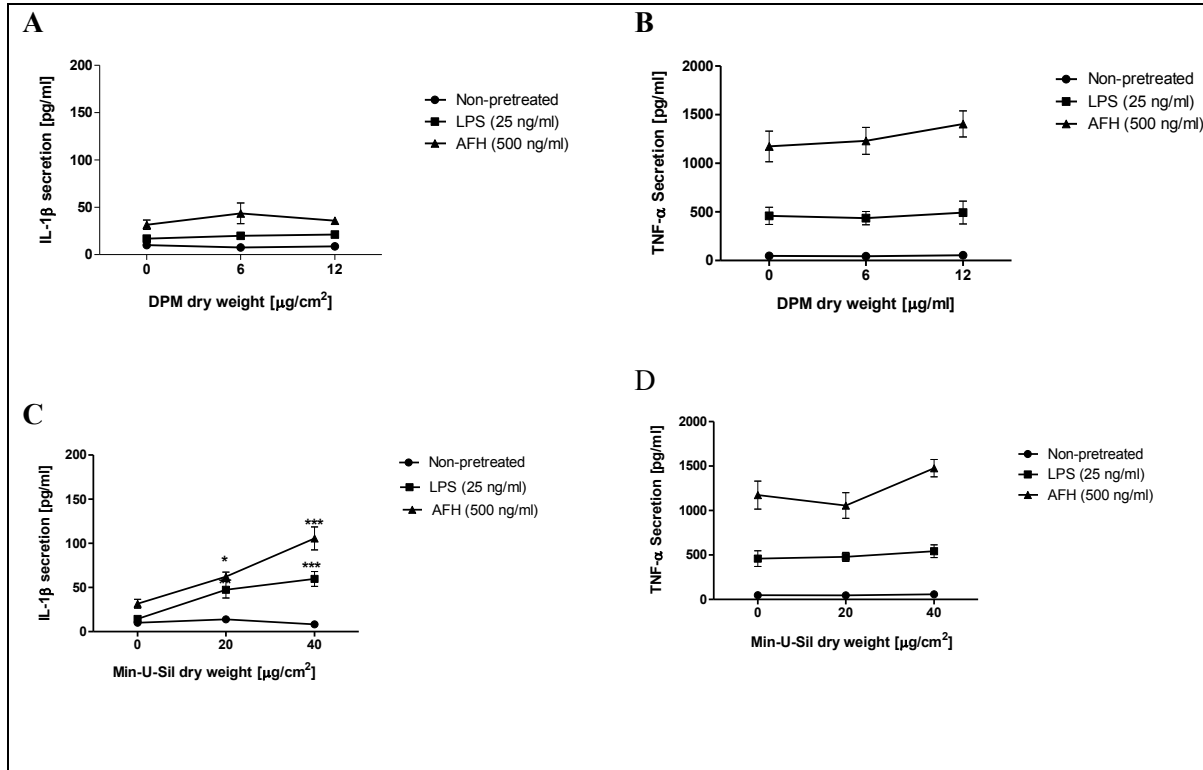


Figure 16 Effects of co-exposure of LPS with DPM/Min-U-Sil and AFH with DPM/Min-U-Sil on cytokines secretion of THP-1 monocytes determined by ELISA. (A) IL-1 β and (B) TNF- α secretion of THP-1 monocytes primed with AFH or LPS for 3 h and co-exposed with DPM for 18 h. (C) IL-1 β and (D) TNF- α secretion of THP-1 monocytes primed with AFH or LPS for 3 h and exposed to Min-U-Sil for 18 h. Lines represent mean \pm SEM concentration of 3 independent experiments. Statistical analysis was based on normalized data (One way ANOVA, Dunnett post-test). Significant difference between co-exposed cells vs individual controls (Non-exposed, LPS (25 ng/ml), AFH (500 ng/ml)) is denoted by asterisks * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.3 mRNA expression of THP-1 monocytes after co-exposures

We assessed the expression of the genes IL1B and TNF-A on THP-1 monocytes primed with LPS (25 ng/ml) or AFH (500 ng/ml) for 3 h and exposed to Min-U-Sil (20 or 40 $\mu\text{g}/\text{cm}^2$) for 3 h by using real time RT-PCR. AFH (500 ng/ml) alone exposure showed 70 fold expression of IL-1 β and 25 fold expression of TNF- α compared to non-exposed cells (Fig.17A&B). However, none of the co-exposures yield an expression that is significantly different from their individual controls (non-exposed, LPS (25 ng/ml) alone and AFH (500 ng/ml) alone) in IL-1 β expression (Fig. 17A). TNF- α expression was also not different from the controls except, for the cells primed with AFH (500 ng/ml) and co-exposed with Min-U-Sil (20 $\mu\text{g}/\text{cm}^2$). These cells showed significant ($P<0.01$) down regulation of TNF- α expression by 18 fold compared to AFH (500 ng/ml) alone (Fig.17B).

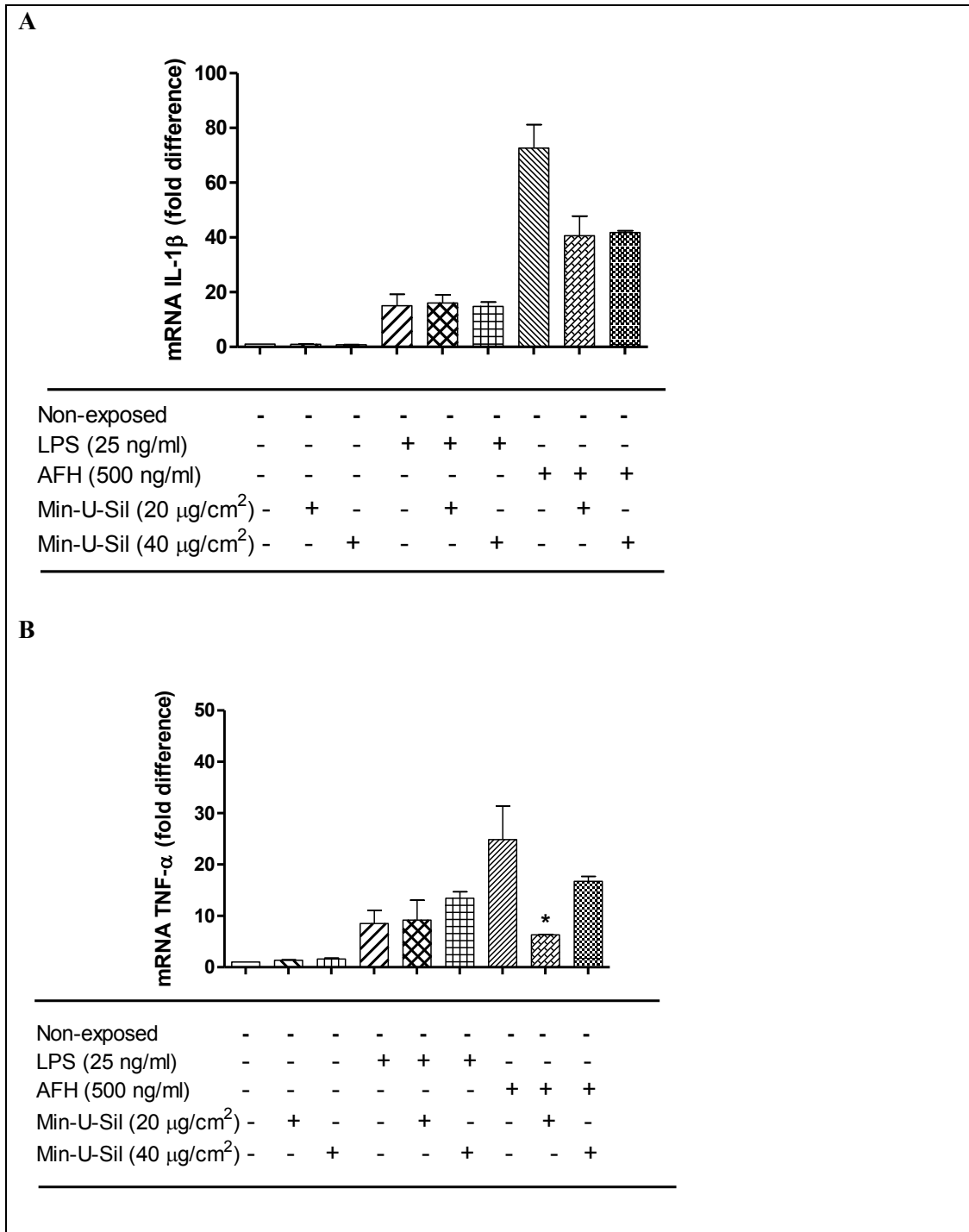


Figure 17 Effects of priming with LPS or AFH and co-exposure with Min-U-Sil on gene expression of THP-1 monocytes. Cells were primed with LPS (25 ng/ml) or AFH (500 ng/ml) for 3 h and co-exposed with Min-U-Sil (20 or 40 $\mu\text{g}/\text{cm}^2$) for further 3 h. (A) IL-1 β and (B) TNF- α expressions was assessed by using real time RT-PCR. Bars represent means \pm SEM of the fold increase relative to non-exposed cells. Statistical analysis was based on normalized data (One way ANOVA, Dunnett post-test, N=3). Significant difference between the individual controls (non-exposed, LPS (25 ng/ml), and AFH (500 ng/ml)) compared to the co-exposed cells is indicated by asterisks * P < 0.05, + treated, - untreated

4.4 Single and co-exposure induced cytotoxicity to PMA THP-1 macrophages

PMA THP-1 macrophages exposed to single and co-exposures were examined for cytotoxicity (apoptosis and necrosis) by staining them with PI/Hoceust and scoring them under a fluorescent microscope.

Cytotoxicity of PMA THP-1 macrophages to single exposures

PMA THP-1 macrophages exposed to a range of concentrations of DPM (6, 12, 24 and 48 $\mu\text{g}/\text{cm}^2$) or Min-U-Sil (20, 40, 80 and 160 $\mu\text{g}/\text{cm}^2$) or AFH (50 ng/ml, 10 $\mu\text{g}/\text{ml}$ and 50 $\mu\text{g}/\text{ml}$) for 24 h did not show any significant cytotoxicity compared to the control (Fig.S1A,B&D). PMA THP-1 macrophages exposed to increasing concentrations of LPS (0.05, 0.5, 5 and 25 ng/ml) also did not show significant cytotoxicity except for the LPS concentration (0.05 ng/ml). This concentration showed a slight increase in necrosis (Fig.S1C)

Cytotoxicity of PMA THP-1 macrophages to co-exposures

PMA THP-1 macrophages primed with AFH (50 ng/ml) or LPS (0.5 ng/ml) and co-exposed to DPM (6 or 12 $\mu\text{g}/\text{cm}^2$) for 18 h did not show any significant necrosis compared to their individual controls i.e. non-exposed, LPS (0.5 ng/ml) and AFH (50 ng/ml) (Fig.S2A) PMA THP-1 macrophages primed with AFH (50 ng/ml) or LPS (0.5 ng/ml) and co-exposed to Min-U-Sil (20 or 40 $\mu\text{g}/\text{cm}^2$) for 18 h also did not show any significant necrosis (Fig.S2B).

4.5 Cytokine release of PMA THP-1 macrophages after single and co-exposure

IL-1 β and TNF- α release of PMA THP-1 macrophages exposed to single and co-exposures of DPM, Min-U-Sil, AFH and LPS was assessed by using ELISA.

Cytokine secretion to single exposure

PMA THP-1 macrophages exposed to four concentrations of DPM did not secret significant IL-1 β (Fig.18A). However, TNF- α release was significantly ($P<0.05$) induced at all the concentrations tested except for 6 $\mu\text{g}/\text{cm}^2$ (Fig.18B). IL-1 β secretion was induced in an increasing manner in cells exposed to four concentrations of Min-U-Sil. Particularly, the IL-1 β release was significantly higher in cells exposed to 80 $\mu\text{g}/\text{cm}^2$ and 160 $\mu\text{g}/\text{cm}^2$ of Min-U-sil

compared to the control with a significant P values $P < 0.05$ and $P < 0.001$, respectively (Fig 18C). No significant increase in TNF- α release was noted in cells exposed to various concentrations of Min-U-Sil (Fig.18D). PMA THP-1 macrophages exposed to increasing concentrations of LPS showed a significant increase in both IL-1 β and TNF- α secretion. Three of the LPS concentrations (0.5, 5 and 25 ng/ml) resulted in significantly higher release of IL-1 β and TNF- α compared to the control with P values $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively (Fig.19A&B). Exposure of PMA THP-1 macrophages to a range of concentrations of AFH gave highly significant ($p < 0.001$) release of IL-1 β and TNF- α in all concentrations tested, except for the cells exposed to 1 ng/ml (Fig.19C&D).

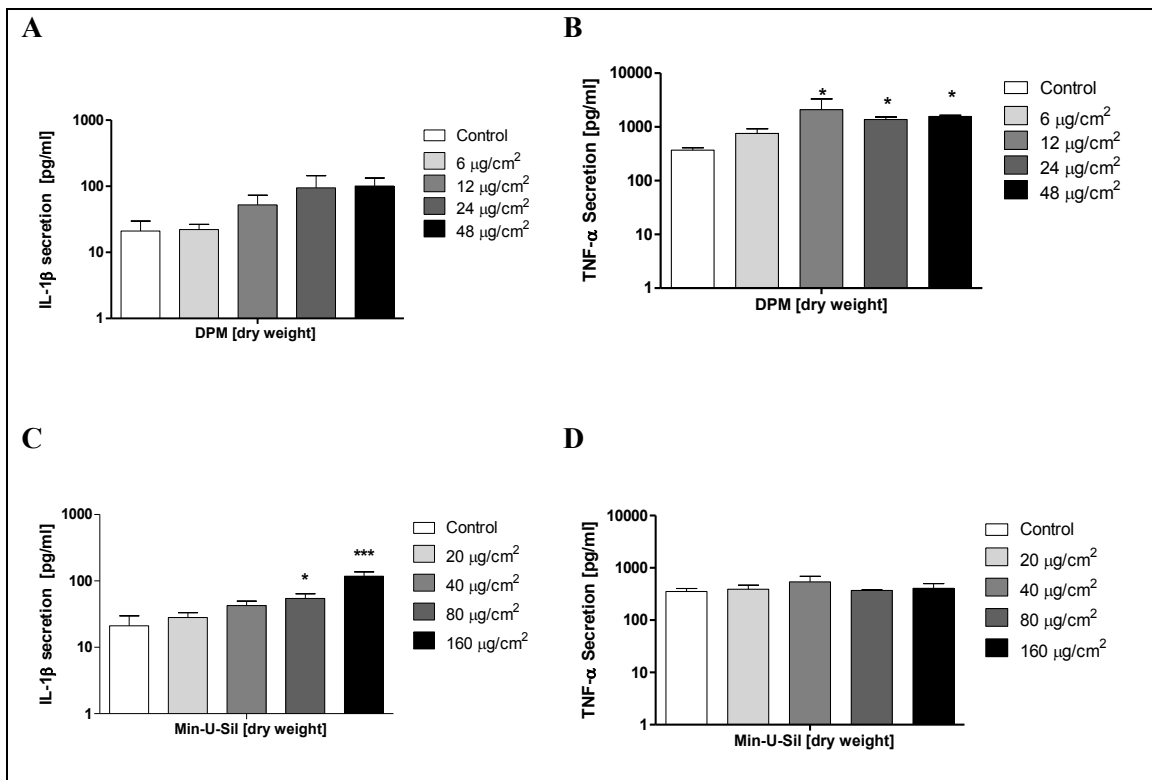


Figure 18 Effects of DPM and Min-U-Sil on cytokine secretion of PMA THP-1 macrophages determined by ELISA. (A) IL-1 β and (B) TNF- α secretion of PMA THP-1 macrophages exposed to various concentrations of DPM for 24 h. (C) IL-1 β and (D) TNF- α secretion of PMA THP-1 macrophages exposed to indicated concentrations of Min-U-Sil for 24 h. Statistical analysis was based on a log transformed data (One way ANOVA, Dunnett post-test, mean \pm SEM N=3). Significant difference compared to the control is indicated with asterisks * $P < 0.01$, *** $P < 0.001$.

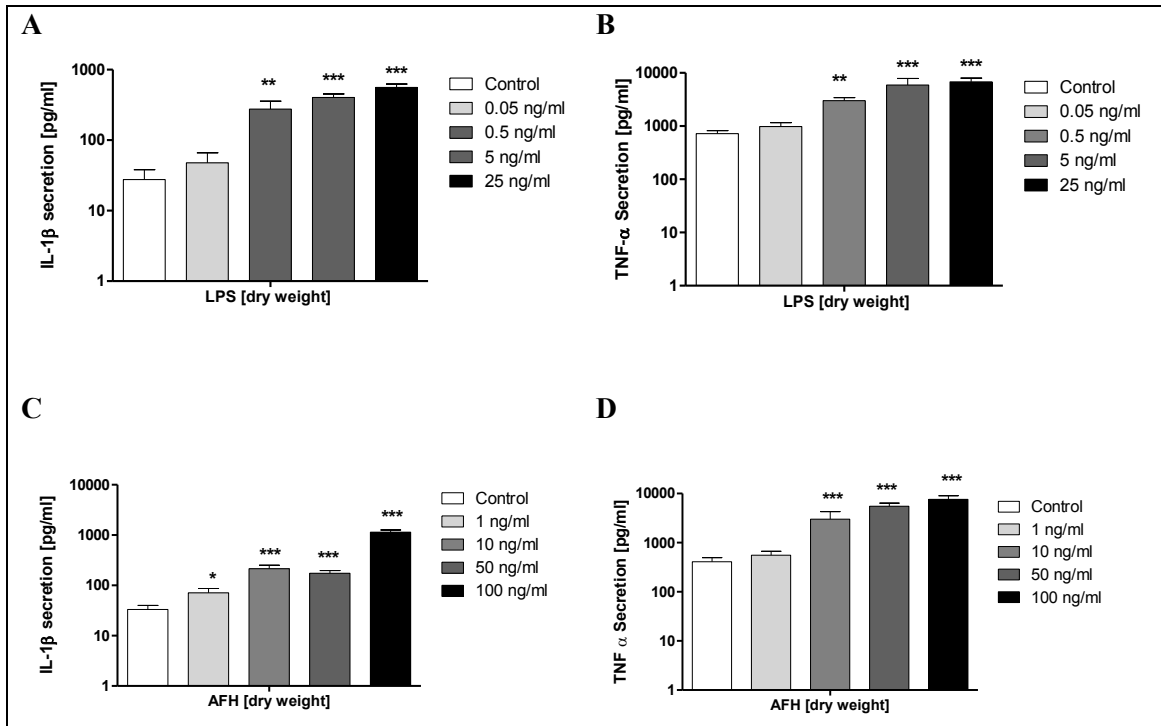


Figure 19 Effects of LPS and AFH on cytokine release of PMA THP-1 macrophages determined by ELISA. (A) IL-1 β and (B) TNF- α secretion of PMA THP-1 macrophages exposed to indicated concentrations of LPS for 24 h. (C) IL-1 β and (D) TNF- α secretion of PMA THP-1 macrophages exposed to four different concentrations of AFH for 24 h. Statistical analysis was based on a log transformed data (One way ANOVA, Dunnett post-test, mean \pm SEM N=3-4). Significant difference compared to the control is indicated by asterisks * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Co-exposure induced cytokine secretion

PMA THP-1 macrophages primed with either LPS or AFH and co-exposed with DPM or Min-U-Sil were examined for IL-1 β and TNF- α secretion (Fig 20). Cells that were primed with LPS (0.5 ng/ml) or AFH (50 ng/ml) and co-exposed with DPM (6 or 12 $\mu\text{g}/\text{cm}^2$) did not show any significance increase in secretion of IL-1 β and TNF- α compared to their corresponding controls (non-exposed, LPS (0.5 ng/ml) alone, AFH (50 ng/ml) alone) (Fig 20A & B). However, in an earlier experiment set up of co-exposure of DPM (12 $\mu\text{g}/\text{cm}^2$) with LPS (25 ng/ml) and a co-exposure of DPM (12 $\mu\text{g}/\text{cm}^2$) with AFH (10 ng/ml) apparently showed statistically significant ($P < 0.001$) increase in releasing IL-1 β (Fig.S3A). Cells primed with LPS (0.5 ng/ml) or AFH (50 ng/ml) and co-exposed with Min-U-Sil (20 or 40 $\mu\text{g}/\text{cm}^2$) also showed an increase in IL-1 β secretion (Fig 20C). Especially, the co-exposure of AFH and Min-U-Sil at both concentrations showed significant increase in IL-1 β release compared to the cells exposed to AFH (50 ng/ml)

alone with P values $P < 0.05$ and $P < 0.001$, respectively. The co-exposure of LPS (0.5 ng/ml) and Min-U-Sil (40 $\mu\text{g}/\text{cm}^2$) also induced significantly ($P < 0.05$) higher secretion of IL-1 β (Fig. 20C). A similar trend was observed on a different co-exposure set up of Min-U-Sil (20 or 40 $\mu\text{g}/\text{cm}^2$) with a higher concentration of LPS (25 ng/ml) and co-exposure of Min-U-Sil (20 or 40 $\mu\text{g}/\text{cm}^2$) with a lower concentration of AFH (10 ng/ml) tested (Fig. S3B). On the contrary, no significant difference was noted on TNF- α secretion in PMA THP-1 macrophages primed with AFH or LPS and co-exposure to Min-U-Sil (Fig. 20D).

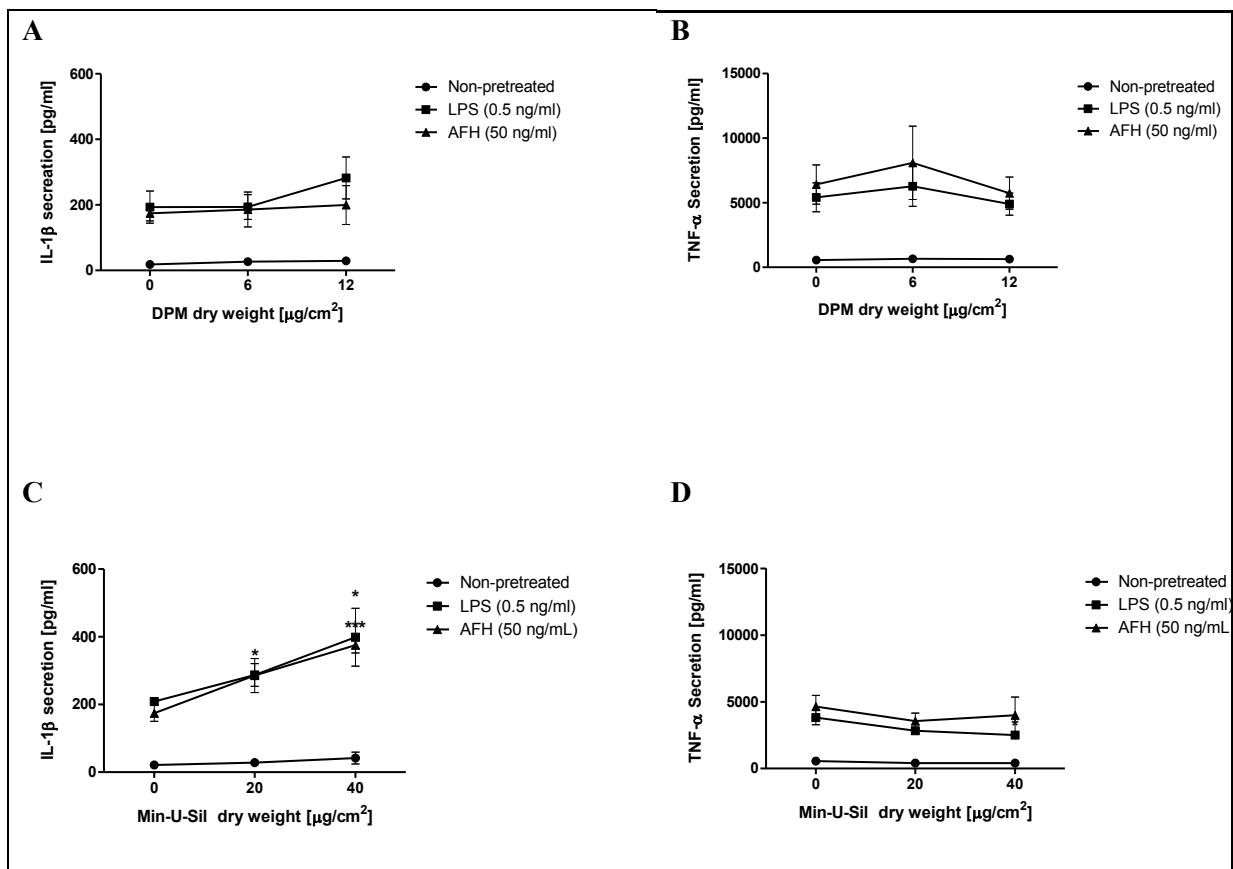


Figure 20 Release of cytokines in PMA THP-1 macrophages after co-exposure of AFH with DPM or Min-U-Sil and co-exposure of LPS with DPM or Min-U-Sil determined by ELISA. (A) IL-1 β and (B) TNF- α secretion of PMA THP-1 macrophages primed with LPS (0.5 ng/ml) or AFH (50 ng/ml) for 3 h and co-exposed with DPM (6 or 12 $\mu\text{g}/\text{cm}^2$) for 18 h. (C) IL-1 β and (D) TNF- α secretion of PMA THP-1 macrophages primed with LPS (0.5 ng/ml) or AFH (50 ng/ml) for 3h and co-exposed with Min-U-Sil (20 or 40 $\mu\text{g}/\text{cm}^2$) for 18 h. Lines represent mean \pm SEM concentration of 3 independent experiments. Statistical analysis was based on normalized data (One way ANOVA, Dunnett post-test). Significant difference compared to the individual control is indicated by asterisks * $P < 0.05$, *** $P < 0.001$.

4.6 mRNA expression of PMA THP-1 macrophages after co-exposures

PMA THP-1 macrophages primed with LPS (0.5 ng/ml) or AFH (50 ng/ml) and co-exposed with DPM (12 $\mu\text{g}/\text{cm}^2$) or Min-U-Sil (40 $\mu\text{g}/\text{cm}^2$) were assessed for the expression of IL-1 β and TNF- α in mRNA level (Fig. 21). The maximum IL-1 β expression observed was only about 5 fold and it was on cells exposed to LPS (0.5 ng/ml) alone and cells co-exposed with AFH (50 ng/ml) and Min-U-Sil (40 $\mu\text{g}/\text{cm}^2$) (Fig. 21A). Similar gene expressions were noted on TNF- α expression (Fig. 21B). However, none of the co-exposures showed statistically significant difference in IL-1 β and TNF- α expressions compared to their individual controls (non-exposed, AFH (50 ng/ml) alone and LPS (0.5 ng/ml) alone) (Fig. 21A&B).

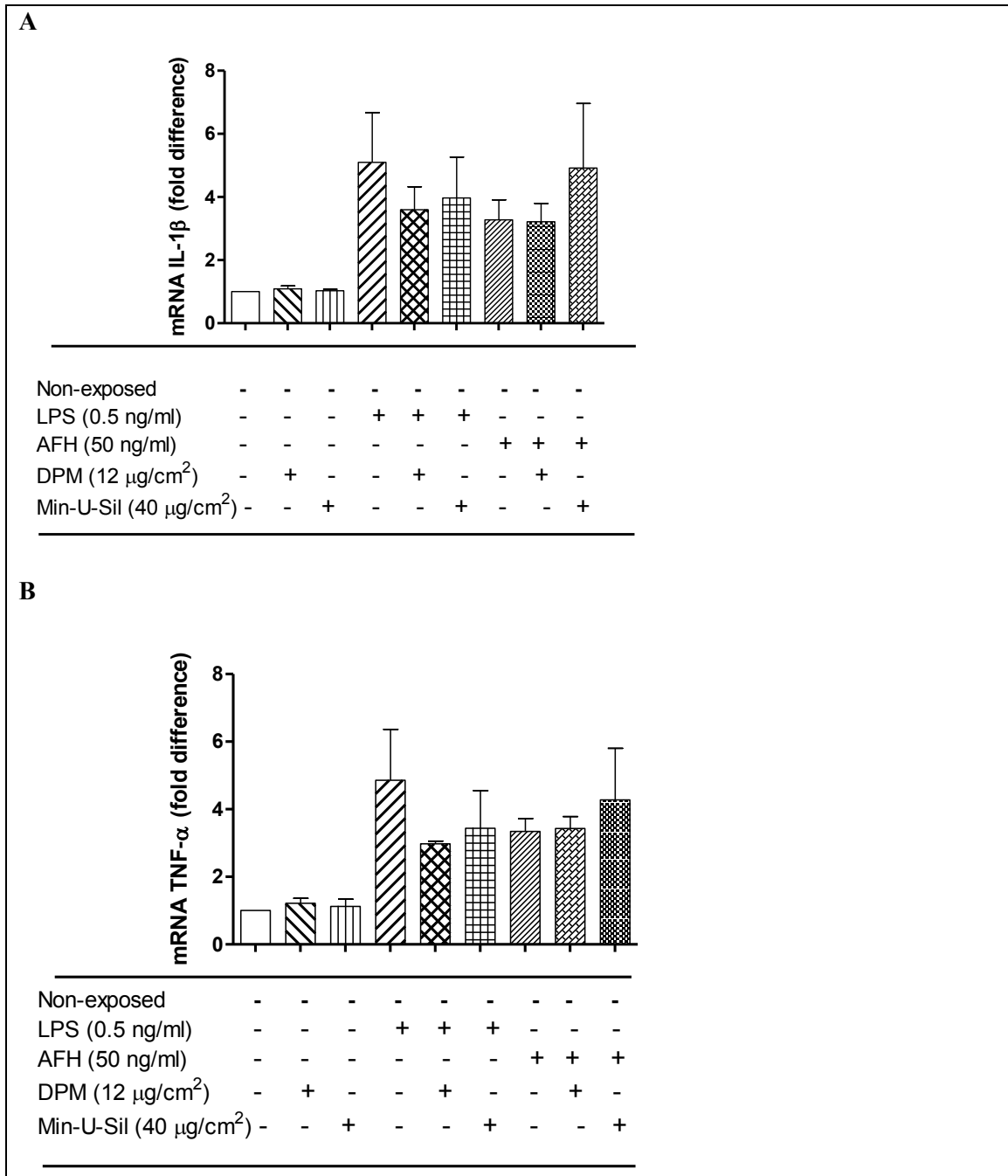


Figure 21 Effects of priming with LPS or AFH and co-exposure with DPM or Min-U-Sil on gene expression of PMA THP-1 macrophages. PMATHP-1 macrophages were primed with LPS (0.5 ng/ml) or AFH (50 ng/ml) for 3 h and exposed to DPM (12 $\mu\text{g}/\text{cm}^2$) or Min-U-Sil (40 $\mu\text{g}/\text{cm}^2$) for further 3 h. (A) IL-1 β and (B) TNF- α mRNA expression was measured by real time RT-PCR. Bars represent means \pm SEM of the fold increase relative to non-exposed cells. + treated, - untreated. Statistical analysis was based on normalized data (One way ANOVA, Dunnett post-test). There was no significant difference between the individual controls (non-exposed control, LPS (0.5 ng/ml), AFH (50 ng/ml)), and co-exposed cells.

5 EXPERIMENTAL AND METHODOLOGICAL CONSIDERATIONS

5.1.1 Cellular models

Alveolar macrophages (AM) are considered to be one of the primary targets in clearing out inhaled PM through phagocytosis. Monocytes on the other hand, are recruited during inflammation (Ulrich 2007; Margalit et al. 2015). More specifically, they have been found to accumulate in the alveoli of the lung following exposure to PM. Here the monocytes may differentiate into lung macrophage (Goto, Hogg, et al. 2004; Das et al. 2015). They are also been reported to participate in lung surveillance and particle clearance in a steady-state without differentiating to macrophages (Goto, Ishii, et al. 2004; Rodero et al. 2015). Thus, both of these cell types seem to be relevant when examining pro-inflammatory potential of PMs.

Human THP- 1 monocyte is a widely used cell line and is able to differentiate to macrophage like cells with PMA treatment (PMA THP-1 macrophages). The PMA-differentiated THP-1 macrophage behaves like primary human macrophages compared to other myeloid cell lines such as HL-60, U937, KG-1, or HEL (Auwerx 1991; Qin 2012). PMA has been shown to be the best differentiating agent compared to other agents such as 1, 25-dihydroxyvitamin D3 (VD3) in providing the desirable effect including phenotype, loss of proliferation, adherence and expression of receptors (Schwende et al. 1996). However, it is difficult to know if the PMA treatment, besides inducing the macrophage phenotype, additionally also interferes in the cytokine measurement. As we have seen in our experiments, the basal cytokine concentration is higher in PMA THP-1 macrophage compared to the THP-1 monocytes. However, since the cytokine secretion is calculated relative to the basal concentration (control) this problem can possibly be avoided.

In general, cell lines such as THP-1 are relatively cheap and easy to use in an experimental setting. However, results obtained employing these cell lines requires further verification using primary cells and/or *in vivo* models to generalize the findings.

5.1.2 Exposure agents

We may be exposed to both AFS and AFH fragments through inhalation. So far the spores have been investigated most extensively (Latgé 1999; Górny 2004; Park et al. 2009). Many phagocytic cells, including THP-1 cells, have been shown to ingest both spores and hyphae

efficiently (Marr et al. 2001; Gersuk et al. 2006). However, the inflammatory responses, as measured by the release of cytokines, vary. According to the result from an ongoing study in our laboratory and other studies, hyphae are found to be potent activator to different immune cells when compared to the spores (Wang et al. 2001; Górny 2004). Furthermore, hyphae represent the invasive stage of the fungi (Hohl et al. 2005). Therefore, we decided to investigate the immune response of AFH.

Bacteria are the primary member of lung microbiota and hence, lung cells are may be naturally primed with microbiota (Charlson et al. 2011). Thus, LPS (a component of Gram negative bacteria) was included in this study to represent the microbiota in the lung and, to obtain possibly a model that better reflect the *in vivo* situation. LPS is widely used to activate different immune cells (Imrich et al. 1999; Sawyer et al. 2009). This scenario may also be considered as a model mimicking ongoing infection.

A wide variety of DPM materials have been used in experimental studies of air pollution particles. There are no clear answer to which one is the best choice as the specific composition of DPM-material depends up on engine types used. We decided to use DPM (SRM 2975) in this study. DPM is predominantly used in DE related health effects (Schwarze et al. 2013). It is also well standardized and characterized PM, which makes the results obtained more easy to reproduce for other scientist. Moreover, it is found to be genotoxic and causes pulmonary inflammation (Kaewamatawong et al. 2009; Jantzen et al. 2012).

Silica particles vary in shape, size, constituent and source. Among other silica particles, Min-U-Sil 5 crystalline silica is well characterized and used very often to study lung response to silica (Gambelunghe et al. 2006; Antognelli et al. 2009). Moreover, Min-U-Sil 5 appears most relevant in size (i.e. 5 μm) to represent road abrasions due to traffic compared to other available sizes of Min-U-Sil (10, 15, 30 and 40 μm).

5.1.3 Methods

Cell viability

Cell viability assessment provides important and necessary information to studies examining inflammatory potential. We employed fluorescence microscopy of the cells, following staining with PI/Hoechst. This is a simple and most reliable technique in particle exposure studies and

also enables to differentiate apoptotic and necrotic cells. However, there are a number of other techniques available such as methyl thiazolyl tetrazolium (MTT), lactate dehydrogenase (LDH) test as well as more advanced tests using flow cytometry. These techniques are considered to be fast and accurate. However, as experienced in the laboratory they often seem to lose their accuracy when used to assess cells exposed to PM (Holme et.al unpublished). The reason is that, these techniques use absorbance and some remaining particles in the sample seem to absorb part of the light which may lead to inaccurate measures. Moreover, some of these techniques do not allow differentiating apoptosis and necrosis or are very costly. Thus, in this thesis we decided to use microscopic examination.

Cytokine measurement and cytokine types

One of the major objectives in this study was to examine the inflammatory potential, which *in vitro* is assessed as cytokine secretion. Cytokines can be measured using various techniques such as bioassays, immunoassays, molecular biology and flow cytometry (House 2001). Each of them have advantages and disadvantages (House 2001). Choosing the relevant technique is mainly determined by the sample type, time, accuracy and cost. Immunoassays are simple, cost effective and are often used. Among the available immunoassays, ELISA is shown to be the most validated method for measuring individual cytokines (Leng et al. 2008). Thus, we used sandwich ELISA to measure cytokine release.

Macrophages and monocytes are major sources of cytokines and chemokines (Cavaillon 1994; Chawla et al. 2011). They release different pro-inflammatory cytokines and chemokines. Among all, IL-1 and TNF- α are known pro-inflammatory cytokines in regulating other cytokines (Dinarello 2000). IL-1 β , on the other hand, is known to be pro-inflammatory via engagement through IL-1 receptor 1 (IL-1R1) (Dinarello 2000). Furthermore, macrophages and monocytes also release IL-8, a potent neutrophil chemoattractant during infection (Yoshie et al. 2001). Thus, we decided to assess IL-1 β , TNF- α and IL-8 in this study. However, our pilot study showed that IL-8 results were less interesting and unstable (data not shown). Therefore, we continued with only IL-1 β and TNF- α .

Gene expression

Following the assessment of cytokine secretion, we performed gene expression analysis to assess the possible signaling pathway in these cytokines synthesis by using real time RT-PCR. Although there are other techniques available, including northern blotting and DNA micro arrays, real time RT-PCR is the most used. It is known to be accurate, sensitive and easy to perform but requires normalization and proper interpretation (Bustin 2002).

Particle exposures with serum vs without serum

Many *in vitro* studies that involve particle exposure are done without serum or serum is added after few hours. This is mainly because the presence of serum during particle exposure somehow reduces the cell response, possibly by covering the particles and altering the direct interaction. However, one can argue that the increased response can be due to the lack of serum which may add another stressor to the cells. In addition to that, during inhalation, the particles will be humidified with mucus and surfactant components. Thus, the presences of serum in particle exposures possibly imitate the natural scenario. Furthermore, particles such as DPM has previously documented to bind to cytokines and interfere cytokine measurement (Kocbach, Totlandsdal, et al. 2008), The same study stated that the presence of serum could prevent the binding partly or completely (Kocbach, Totlandsdal, et al. 2008). Moreover, the presence of serum has been suggested to be important for PMA THP-1 macrophages and human primary monocytes to give a proper response to AFH (Marr et al. 2001; Wang et al. 2001). Therefore, all our exposures were done with the presence of serum. Nevertheless, it would still be interesting to perform these experiments without serum and compare the findings.

6 DISCUSSION

In the current study, we investigated the effects of AFH, LPS, DPM and Min-U-Sil, alone and in co-exposures with a special emphasis on IL-1 β and TNF- α synthesis and secretion using THP-1 monocytes and PMA THP-1 macrophages as experimental models. Our results showed that alone exposure of AFH and LPS induced increased secretions of IL-1 β and TNF- α in both cellular models. DPM and Min-U-sil resulted in an induction of TNF- α and IL-1 β secretion, respectively only in PMA THP-1 macrophages. In our co-exposure set up, both AFH and LPS with Min-U-Sil showed a possible synergistic effect in IL-1 β secretion in both cellular models. In addition to that, co-exposure of AFH or LPS with DPM showed no effects in the THP-1 monocytes and a variable apparent synergistic effect in IL-1 β secretion in PMA THP-1 macrophages. Moreover, we show that the apparent synergistic effect observed on the release of IL-1 β following co-exposure were not reflected by a correspondingly increased mRNA expression of the genes. There was no or rather decreased level of mRNA of IL-1 β and TNF- α were seen, suggesting an effect on the second step of the activation of the inflammasome.

6.1 The effect of single and co-exposures on cytotoxicity and cytokine release in THP-1 monocytes

In this study, we focused on testing concentrations that give no or low degree of cytotoxic effects in order to avoid non-specific effects of cytotoxicity interfering with cytokine secretion. Thus, we assessed the cytotoxicity (apoptosis and necrosis) of THP-1 monocytes following exposure to AFH, LPS, DPM and Min-U-Sil alone and in co-exposure by using fluorescent microscopy. Our data revealed that none of these agents caused apoptosis to THP-1 monocytes. However, AFH and Min-U-Sil, alone and in co-exposure and co-exposure of LPS and Min-U-Sil elicit increased necrosis to the THP-1 monocytes.

The present study showed that THP-1 monocytes exposed to DPM (6-48 $\mu\text{g}/\text{cm}^2$) for 24 h did not cause any apoptosis or necrosis (Fig.10A). This finding is in accordance with two other studies where they assessed cytotoxicity using LDH in THP-1 monocytes exposed to similar range of concentrations of DPM as ours (Kobach, Namork, et al. 2008; Danielsen et al. 2009).

Studies have shown that Min-U-Sil exposure is cytotoxic (Herseth, Volden, et al. 2008; Øvrevik et al. 2015). Similarly, here we demonstrate that Min-U-Sil is cytotoxic to THP-1 monocytes by causing significant necrosis in all concentrations (20-160 $\mu\text{g}/\text{cm}^2$) tested (Fig.10B). Furthermore, studies conducted in other lung cell lines such as Calu-3 and BEAS-2B reported similar cytotoxicity at concentrations 20 and 50 $\mu\text{g}/\text{cm}^2$, respectively at 24 h exposure (Journeay et al. 2008; Antognelli et al. 2009). Although these studies used different cell lines and cell viability measurements (LDH and Trypan blue), they all agree that Min-U-Sil is cytotoxic. On the contrary, (Herseth, Refsnes, et al. 2008) reported no or less cytotoxicity observed in THP-1 exposed to same concentration range of Min-U-Sil as ours by using LDH measurement. This discrepancy is possibly due to the different cell viability measurements used in our and their study and a possible interference of Min-U-Sil in absorbing the light in LDH measurement.

We have assessed cytotoxicity of THP-1 monocytes exposed to AFH (0.5-100 $\mu\text{g}/\text{ml}$) for 24 h. Our data showed that AFH caused increased necrosis in a dose dependent manner that reached up to 20% in the highest concentration (100 $\mu\text{g}/\text{ml}$) tested (Fig.11B). Cytotoxicity of AFH has also been shown previously in murine neutrophils (Bellocchio et al. 2004). We demonstrated Min-U-Sil and AFH are cytotoxic to THP-1 monocytes, when exposed alone. Therefore, we selected concentrations that gave relatively low cytotoxicity (i.e. AFH (0.5 $\mu\text{g}/\text{ml}$) and Min-U-Sil (20 or 40 $\mu\text{g}/\text{cm}^2$) and used them for our co-exposure set up. Our results showed that AFH co-exposed with the highest concentration of Min-U-Sil (40 $\mu\text{g}/\text{cm}^2$) caused an increased cytotoxic response (23% necrosis) (Fig. 13B). On the other hand, THP-1 monocytes co-exposed to AFH and DPM did not show any cytotoxicity. There are no studies examined this type of co-exposures previously.

The current study has showed that THP-1 monocytes exposed to concentrations (0.05-25 ng/ml) of LPS for 24 h did not elicit any significant increase in apoptosis or necrosis (Fig. 11A). A similar finding has been reported in other study that used THP-1 cells exposed to higher concentration up to 1000 ng/ml for 24 h and they also did not observe cytotoxicity (Wu et al. 2008). However, when we co-exposed LPS (25 ng/ml) with Min-U-Sil (40 $\mu\text{g}/\text{cm}^2$), significant necrosis was observed. Based on this result one can speculate that primed cells have a tendency to die easily.

There is substantial evidence that production of pro-inflammatory cytokines play a crucial role in host defense. The current study showed that THP-1 monocytes exposed to DPM for 24 h did not induce significant IL-1 β secretion compared to the control (Fig.14A). On the contrary, (Kocbach, Namork, et al. 2008) reported increased secretion of IL-1 β at DPM concentrations $\geq 35 \mu\text{g}/\text{cm}^2$ in 12 h exposure. Regarding DPM induced cytokines there are two factors to consider. The first one is DPM has a potential to bind to cytokines hence, it might interfere with cytokine measurement (Kocbach, Totlandsdal, et al. 2008). Another possible factor is the involvement of serum. Thus, in the study by Kocbach and co-workers, the cells were allowed to interact in serum free medium the first 2 h, while in our experiments the cells were with serum during the whole exposure time. Regarding TNF- α secretion, our data shows exposure of DPM to THP-1 monocytes did not induce TNF- α secretion (Fig.14B). This finding is in agreement with (Kocbach, Namork, et al. 2008).

The current study shows that THP-1 monocytes exposed to Min-U-Sil for 24 h did not induce significant release of IL-1 β and TNF- α in all concentrations (20-160 $\mu\text{g}/\text{cm}^2$) tested (Fig.14 C&D). Similarly, (Herseth, Refsnes, et al. 2008) reported no significant induction of both cytokines in all concentrations except at the highest concentration (160 $\mu\text{g}/\text{cm}^2$). Their finding may suggest that exposure to higher concentration of Min-U-Sil for longer time (43 h) may induce significant cytokine release.

Several studies have reported that monocytes are involved during exposure of AFH (Walsh et al. 2005; Cramer et al. 2011). Accordingly, the present study shows that THP-1 monocytes exposed to AFH for 24 h induced very high and concentration dependent release of IL-1 β and TNF- α (Fig. 15C&D). All AFH concentrations (0.5-100 $\mu\text{g}/\text{ml}$) tested showed significantly increased cytokine secretions compared to controls. On the other hand, exposure of AFH also seemed to cause increased cell death as measured by necrosis to a certain degree. Normally, one would assume that when the viability of cells decline the cytokine secretion will also decrease. The possible explanation could be that the increase in necrosis is first seen after the main production of cytokines has taken place. An additional explanation could be that as some of the cells die with necrotic pathway, this could have given pro-inflammatory signals to the rest of the cells. Particularly, the release of alarmins from the dead cells, that are essential antimicrobial immunity

may further induce the secretion of these cytokines from the live cells around (Edinger et al. 2004; Yang et al. 2009)

We have just demonstrated that AFH is a potent inducer of both cytokines (IL-1 β or TNF- α) to THP-1 monocytes in single exposure. However, cells primed with AFH didn't seem to be more sensitive to DPM as co-exposure showed no or reduced effect in secretion of both IL-1 β and TNF- α (Fig. 16A&B). However, AFH primed THP-1 monocytes seemed to be more sensitive to Min-U-Sil, as an increased release of IL-1 β could be seen (Fig. 16C). As the increase is more than just additive response seen after AFH and Min-U-Sil alone, the effect appeared to be synergistic. However, this suggestion needs follow up studies looking at the effects over broader concentration ranges. No additional/ synergistic effects were seen regarding TNF- α .

LPS has been reported to induce increased release of IL-1 β and TNF- α in THP-1 monocytes (Li et al. 2015). Similarly, our data shows that THP-1 monocytes exposed to LPS for 24 h released significant IL-1 β and TNF- α at the highest concentration (25 ng/ml) tested (Fig.15A&B). However, it is interesting to note that both the cytokine responses as well as the cytotoxicity of LPS were less marked than that seen for AFH, in accordance with a possible role of alarmins. In our co-exposure set ups, THP-1 monocytes co-exposed to LPS and DPM, showed no additional effect regarding the release of both IL-1 β and TNF- α . However, the co-exposure of LPS and Min-U-Sil showed an apparent synergistic effect in releasing IL-1 β (Fig.16C). This finding suggests that primed or already activated cells may be more sensitive to certain particles such as Min-U-Sil.

6.2 The effect of single and co-exposures on cytotoxicity and cytokine release in PMA THP-1 macrophages

Macrophages play the major role in clearing out inhaled particles (Ulrich 2007; Margalit et al. 2015). This process is reported to cause high production of Reactive Oxygen Species (ROS), which usually leads to cell death (Fubini et al. 2003; Øvrevik et al. 2015). Therefore, we assessed the cytotoxicity of PMA THP-1 macrophages exposed to DPM, Min-U-Sil, LPS and AFH alone and in co-exposures. None of the DPM concentrations tested were cytotoxic (Fig. S1A). This finding is in agreement with another study conducted in primary macrophages assessing DPM

(Mundandhara et al. 2006). Furthermore, neither of the Min-U-Sil concentrations (20-160 $\mu\text{g}/\text{cm}^2$) was cytotoxic in PMA THP-1 macrophages following 24 h exposure (Fig.S1B). On the contrary, (Xu et al. 2003) tested a wide variety concentration of Min-U-Sil in the same cell line and reported cell death at concentrations ($\geq 25 \mu\text{g}/\text{cm}^2$) using MTT. This variation could be attributed to the longer exposure time (48 h) or the difference in cell death assessment techniques (i.e. MTT).

The current study showed that PMA THP-1 macrophages exposed to AFH alone as well as in co-exposure with DPM or Min-U-Sil did not show cytotoxicity (Fig. S1C & Fig. S2A&B). Furthermore, the exposure of LPS (0.05-25 ng/ml) did not cause any cytotoxicity to PMA THP-1 macrophages (Fig.S1D). This finding is in agreement with another study where they used the same cell line and tested LPS concentrations (5 ng/ml and 5 $\mu\text{g}/\text{ml}$) (Landry et al. 2012). Moreover, the co-exposure of LPS and DPM or Min-U-Sil caused no cytotoxicity to PMA THP-1 macrophages in the present study (Fig. S2A&B). Similar observations were reported on human AM (Mundandhara et al. 2006; Sawyer et al. 2009).

Macrophages are abundant source of cytokines and secret wide variety of cytokines according to their micro environment stimuli (Elgert 2009). Thus, PMA THP-1 macrophages exposed to Min-U-Sil released significant IL-1 β in the two highest concentrations (80 and 160 $\mu\text{g}/\text{cm}^2$) tested (Fig.18C). Furthermore, we showed that exposure of Min-U-Sil for 24 h did not induce TNF- α secretion in all the concentration tested (20-160 $\mu\text{g}/\text{cm}^2$) (Fig 18D). Conversely, (Xu et al. 2003) found significant induction of TNF- α at Min-U-Sil concentration (25 $\mu\text{g}/\text{cm}^2$) after 48 h exposure. This inconsistency might be attributed to the difference in length of exposure time.

Our study showed that PMA THP-1 macrophages exposed to DPM alone for 24 h released negligible amount of IL-1 β (Fig. 18A). However, in our first set of experiments, when DPM (12 $\mu\text{g}/\text{cm}^2$) was co-exposed with the LPS (25 ng/ml), a significant induction of IL-1 β release was observed (Fig. S3A). Thus, we hypothesized that the LPS pre-treatment made the cells more sensitive to the secondary particle exposure giving an additive/ possible synergistic effect. However, in our next set of experiments when testing lower concentration of LPS (0.5 ng/ml) no such effects were seen (Fig. 20A). The reason for this discrepancy is still not known, but is probably due to factors such as difference in cell passage and/or serum batch. Further studies are needed in order to clarify any possible synergistic action with DPM.

We demonstrated that PMA THP-1 macrophages exposed to DPM for 24 h showed increased secretion of TNF- α (Fig. 18B). Others have found that DPM had no effect in TNF- α release in primary macrophages (Sawyer et al. 2009). This inconsistency may be due to the difference in cell model used.

The present study has demonstrated that PMA THP-1 macrophages exposed to AFH induced highly significant ($P < 0.05$) release of IL-1 β and TNF- α at 24 h exposure (Fig. 19C&D). This finding is in agreement with a study conducted in primary mouse AM showing induction of TNF- α secretion after exposure to AFH (Taramelli et al. 1996). Furthermore, in our co-exposure set up, PMA THP-1 macrophages primed with AFH and co-exposed to Min-U-Sil showed an additive possibly synergistic effect in the release of IL-1 β (Fig.20C). This finding indicates that cells pre-exposed to AFH are particularly sensitive to exposure to particles such as Min-U-Sil. As the priming represents a model of cells that are exposed to microbiota and/or that have an ongoing low inflammation these results may imply that these cells are more sensitive and give an increased response when co-exposed with Min-U-Sil. However, the co-exposure of AFH and DPM showed no effect with regard to IL-1 β and TNF- α secretion (Fig. 20A&B). To my knowledge, this is the first study examining the co-exposure of AFH and particles.

Our data revealed that PMA THP-1 macrophages exposed to LPS (0.05-25 ng/ml) for 24 h induced marked release of IL-1 β and TNF- α in the three higher concentrations (Fig. 19C&D). Similarly, (Landry et al. 2012) reported significant induction of IL-1 β at concentration (5 ng/ml and 5 μ g/ml) and TNF- α induction at 5 μ g/ml. Furthermore, the present study, PMA THP-1 macrophages co-exposed to LPS and Min-U-Sil showed an apparent synergistic effect in IL-1 β secretion (Fig. 20C). However, when LPS is co-exposed with DPM no significant induction was observed with respect to TNF- α release (Fig. 20D). Even though it is difficult to compare PMA THP-1 macrophages and primary AM, studies conducted on primary AM showed that DPM suppresses the effect of LPS with respect to TNF- α release (Mundandhara et al. 2006; Sawyer et al. 2009).

6.3 NF- κ B and inflammasome involvement in IL-1 β synthesis

Several studies reported that IL-1 β secretion requires two-step activation (Saïd-Sadier et al. 2010; Lopez-Castejon et al. 2011; Monteleone et al. 2015). These steps are NF- κ B activation (step one) and inflammasome activation (step two). Different foreign agents or pathogens are

reported to be involved in one or both steps. LPS is reported to activate both NF- κ B as well as the inflammasomes, while, particles are reported to activate the inflammasomes (NLRP3) (Covert et al. 2005; Dostert et al. 2008; Hornung et al. 2008). AFH is suggested to be involved in both activations (Saïd-Sadier et al. 2010).

Our study showed that both AFH and LPS exposed to THP-1 monocytes as well as PMA THP-1 macrophage alone, increased the release of IL-1 β . Based on these and the above studies, we hypothesize that AFH and LPS are involved in both step one and step two, whereas DPM and Min-U-Sil primarily are involved in step two. In order to test this hypothesis, both THP-1 monocytes and PMA THP-1 macrophage were primed with either AFH or LPS, and co-exposed to DPM or Min-U-Sil, and assessed for increased levels of IL-1 β mRNA (Fig.17A and 21A). Any increased level of IL-1 β mRNA possibly reflect increase expression caused by the activation of NF- κ B signaling pathway through the TLR2/4 pathway (Epstein et al. 1997; Oeckinghaus et al. 2009). Supporting our hypothesis, exposure of AFH or LPS alone showed an increased level of IL-1 β mRNA in THP-1 monocytes and PMA THP-1 macrophages (Fig.17A and 21A). On the contrary, no significant IL-1 β mRNA expression was observed in both THP-1 monocytes and PMA THP-1 macrophage that were exposed to DPM or Min-U-Sil alone. In the co-exposed groups (AFH+DPM, AFH+Min-U-Sil, LPS+DPM and LPS+Min-U-Sil), the mRNA expression of IL-1 β was not significantly different from the individual control (i.e. alone exposure of AFH or LPS). In spite of that, the co-exposed groups showed significantly ($P < 0.05$ or $P < 0.001$) higher release of IL-1 β compared to the controls (i.e. AFH/LPS alone) (Fig. 16C & 20C). To elaborate it further, co-exposure of Min-U-Sil with AFH or LPS showed an apparent synergistic effect in IL-1 β secretion in both cellular models (THP-1 monocytes and PMA THP-1 macrophages). This implies that the increased release of IL-1 β is presumably due to the cooperation of LPS and AFH in activating both steps and the increased involvement of Min-U-Sil in activating the NLRP3 (step two).

In one set of experiments, PMA THP-1 macrophages co-exposed to DPM and AFH or LPS also resulted in an apparent synergetic effect in IL-1 β secretion (Fig. S3A), in a similar manner as seen in Min-U-Sil. However, as these effects were not observed in the next set of experiments, this possibility needs to be further clarified in a follow up study.

6.4 mRNA expression of TNF- α in THP-1 monocytes and PMA

THP-1 macrophages after co-exposures

THP-1 monocytes exposed to AFH alone showed increased mRNA level of TNF- α (Fig.17B). This also has been reported in other studies conducted in THP-1 cells as well as in primary monocytes (Wang et al. 2001; Simitsopoulou et al. 2007). However, when we co-exposed AFH with Min-U-Sil, the mRNA expression of TNF- α was down regulated markedly (Fig.17B). In addition, LPS exposed THP-1 monocytes showed 10 fold increase in TNF- α expression and when co-exposed with Min-U-Sil a slight, but insignificant upregulation of TNF- α was observed (Fig.17B).

PMA THP-1 macrophages co-exposed to AFH and DPM did not show any effect in TNF- α mRNA expression. However, when AFH was co-exposed with Min-U-Sil a slight, but insignificant upregulation of TNF- α expression was observed (Fig.21B). Furthermore, exposure of LPS to PMA THP-1 macrophages showed a moderately increased mRNA expression. When PMA THP-1 macrophages were co-exposed to LPS and DPM, the LPS induced mRNA expression appears to be suppressed (Fig.21B). Similar finding has been reported elsewhere (Sawyer et al. 2009).

Finally, even though comparison of THP-1 monocytes and PMA THP-1 macrophages was not part of our objective, we had the opportunity to assess and compare the biological responses (without direct statistical comparison) of the two cells with regard to cytotoxicity, cytokine release and mRNA expression of (IL-1 β and TNF- α) following exposure to AFH, LPS, DPM and Min-U-Sil. According to our observation, PMA THP-1 macrophages were more resistant towards cytotoxicity and more responsive regarding cytokine release after exposures than the THP-1 monocytes. In contrast, the induced gene responses (fold increase) were higher in the THP-1 monocytes when compared to PMA THP-1 macrophages. This is probable due to the fact that the PMA THP-1 macrophage had a much higher basal level of the cytokine production (and correspondingly mRNA levels).

7 CONCLUSION

In the single exposures, THP-1 monocytes exposed to AFH and Min-U-Sil were moderately cytotoxic to THP-1 monocytes and none of the agents (AFH, LPS, DPM and Min-U-Sil) were found to be cytotoxic to PMA THP-1 macrophages. AFH and LPS are potent inducers in both IL-1 β and TNF- α to both cellular models. DPM and Min-U-Sil induce TNF- α and IL-1 β , respectively, but only in PMA THP-1 macrophages. While THP-1 monocytes seemed to be more sensitive to cytotoxic effects as judged by the effects following exposure to AFH and Min-U-Sil, PMA THP-1 macrophages appeared to show inflammatory responses at lower concentrations (AFH and LPS) compared to the THP-1 monocytes.

Our study supported the hypothesis that sensitized or primed cells may have increased pro-inflammatory reaction to selected air pollution particles. This was elucidated by experiments with co-exposure of AFH and Min-U-Sil as well as LPS and Min-U-Sil using THP-1 monocyte and PMA THP-1 macrophage models. These experiments revealed increased pro-inflammatory reactions through IL-1 β induction in both THP-1 monocytes and PMA THP-1 macrophages. However, these amplified secretions of IL-1 β were not shown in the IL-1 β gene expression, suggesting that Min-U-Sil primarily increased AFH/ LPS-induced secretion of IL-1 β by acting on step two (activation of the inflammasome). Accordingly, the co-exposure of AFH and Min-U-Sil, LPS and Min-U-Sil appeared not to have any marked effects on TNF- α mRNA expression nor its secretion in the two cellular models. In contrast to what was observed with Min-U-Sil, co-exposure of AFH/LPS with DPM showed no effect in THP-1 monocytes and variable amplified effect in PMA THP-1 macrophages with regard to IL-1 β . Thus, further experiments are needed to clarify this hypothesis.

8 POSSIBLE IMPLICATIONS AND FURTHER STUDIES

One of the important issues to consider when extrapolating from *in vitro* studies on cell lines is that these cells lack tissue communication and may behave differently than cells in the tissues. Apart from that, we have seen different cellular models may give different results and sometimes the results obtained are difficult to reproduce. Keeping that in mind, this study has indicated cells primed with bio-aerosols (AFH and LPS) appeared to have increased pro-inflammatory reaction towards air pollution particles. As the human lung is naturally exposed to microbiota, one possible implication could be that cells with LPS priming should represent the best model to screen for inflammatory effects of air-pollution particles. Another possible implication could be that individuals that already have low grade inflammation might have stronger pro-inflammatory reaction when exposed to silica particles. If so, this could represent a situation for increased inflammatory reaction involved in lung related diseases including asthma. However, these highly speculative suggestions need to be verified with the further studies:

- Examining the mechanisms involved in the synergistic responses. Possibly by inhibiting inflammasomes, caspase-1, NF- κ B pathway using specific inhibitors.
- Studying the receptors involved in initial triggering reactions by using antibody locking receptor activation and/or specific inhibitor and siRNA.
- Assessing whether the dose/concentration used are relevant to the real life situation.
- DPM has previously shown to bind with cytokines and interfere with the ELISA measurement (Kocbach, Totlandsdal, et al. 2008). Thus, measuring the intracellular cytokine level might possibly avoid that problem.
- Measuring the effect (in particular mRNA-levels) on different time points.
- Following up this study with whole blood assay, blood derived monocytes and macrophages and sputum derived macrophages and compare the findings.
- Possibly use co-cultures of different cell types, e.g. lung epithelial cells and macrophages
- Conducting similar type of study *in vivo* would provide further information on the complex interactions occurring between various type of cells (intercellular communication)
- Conducting human experimental studies would also substantiate these experimental studies. Most ideally, by taking representative sample individuals that are healthy and

people with respiratory diseases such as asthma and expose them to various particles and particle combinations and analyze their responses. More realistically, examine if e.g. people having an ongoing infection are more sensitive to air pollution than healthy people.

9 FUNDING AND COLLABORATORS

This master thesis is part of an ongoing project titled Fungal particles in indoor air: occurrence and toxic properties of viable and non-viable spores, hyphae and fragments which is funded by the Research Council of Norway (RCN).

Collaborators: other scientists at NIPH and NIOH (Prof. Wijnand Eduard, head of the RCN-project; and PhD-student Anani Johnny Komlavi Afanou).

10 REFERENCES

- (IARC), International Agency for Research on Cancer. 2012. 'IARC: Diesel Engine Exhaust Carcinogenic', http://www.iarc.fr/en/media-centre/pr/2012/pdfs/pr213_E.pdf.
- Afanou, Komlavi Anani, Anne Straumfors, Asbjørn Skogstad, Terje Nilsen, Ole Synnes, Ida Skaar, Linda Hjeljord, Arne Tronsmo, Brett James Green, and Wijnand Eduard. 2014. 'Submicronic fungal bioaerosols: high-resolution microscopic characterization and quantification', *Applied and environmental microbiology*, 80: 7122-30.
- Alberts, Bruce, Dennis Bray, Julian Lewis, Martin Raff, Keith Roberts, and James D Watson. 1994. *Molecular Biology of the Cell 6E: p.814*.
- Alexander, Christian, and Ernst Th Rietschel. 2001. 'Invited review: bacterial lipopolysaccharides and innate immunity', *Journal of endotoxin research*, 7: 167-202.
- Alexopoulou, Lena, Agnieszka Czopik Holt, Ruslan Medzhitov, and Richard A Flavell. 2001. 'Recognition of double-stranded RNA and activation of NF- κ B by Toll-like receptor 3', *Nature*, 413: 732-38.
- Andrei, Cristina, Cecilia Dazzi, Lavinia Lotti, Maria Rosaria Torrisi, Giovanna Chimini, and Anna Rubartelli. 1999. 'The secretory route of the leaderless protein interleukin 1 β involves exocytosis of endolysosome-related vesicles', *Molecular biology of the cell*, 10: 1463-75.
- Antognelli, Cinzia, Angela Gambelunghe, Chiara Del Buono, Nicola Murgia, Vincenzo N Talesa, and Giacomo Muzi. 2009. 'Crystalline silica Min-U-Sil 5 induces oxidative stress in human bronchial epithelial cells BEAS-2B by reducing the efficiency of antiglycation and antioxidant enzymatic defenses', *Chemico-biological interactions*, 182: 13-21.
- Applied Biosystems, Life Technologies. 2010. 'Introduction to gene expressions, Getting started guide'.
- Atale, N, S Gupta, UCS Yadav, and V Rani. 2014. 'Cell-death assessment by fluorescent and nonfluorescent cytosolic and nuclear staining techniques', *Journal of microscopy*, 255: 7-19.
- Auwerx, Johan. 1991. 'The human leukemia cell line, THP-1: a multifaceted model for the study of monocyte-macrophage differentiation', *Experientia*, 47: 22-31.
- Bach, Nicolai, Anette Kocbach Bølling, Bendik C Brinchmann, Annike I Totlandsdal, Tonje Skuland, Jørn A Holme, Marit Låg, Per E Schwarze, and Johan Øvrevik. 2015. 'Cytokine responses induced by diesel exhaust particles are suppressed by PAR-2 silencing and antioxidant treatment, and driven by polar and non-polar soluble constituents', *Toxicology letters*, 238: 72-82.
- Bach, Nicolai S, Marit Låg, and Johan Øvrevik. 2014. 'Toll like receptor-3 priming alters diesel exhaust particle-induced cytokine responses in human bronchial epithelial cells', *Toxicology letters*, 228: 42-47.
- Bellocchio, Silvia, Silvia Moretti, Katia Perruccio, Francesca Fallarino, Silvia Bozza, Claudia Montagnoli, Paolo Mosci, Grayson B Lipford, Lucia Pitzurra, and Luigina Romani. 2004. 'TLRs govern neutrophil activity in aspergillosis', *The Journal of Immunology*, 173: 7406-15.
- Bhatia, Rajiv, Peggy Lopipero, and Allan H Smith. 1998. 'Diesel exhaust exposure and lung cancer', *Epidemiology*, 9: 84-91.
- Bozza, Silvia, Roberta Gaziano, Antonio Spreca, Angela Bacci, Claudia Montagnoli, Paolo di Francesco, and Luigina Romani. 2002. 'Dendritic cells transport conidia and hyphae of *Aspergillus fumigatus* from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus', *The Journal of Immunology*, 168: 1362-71.
- Brightbill, Hans D, Daniel H Libraty, Stephan R Krutzik, Ruey-Bing Yang, John T Belisle, Joshua R Bleharski, Michael Maitland, Michael V Norgard, Scott E Plevy, and Stephen T Smale. 1999. 'Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors', *Science*, 285: 732-36.
- Brimblecombe, Peter. 2011. *The big smoke: a history of air pollution in London since medieval times* (Routledge; P.8).
- Brook, Robert D, Barry Franklin, Wayne Cascio, Yuling Hong, George Howard, Michael Lipsett, Russell Luepker, Murray Mittleman, Jonathan Samet, and Sidney C Smith. 2004. 'Air pollution and

- cardiovascular disease A statement for healthcare professionals from the expert panel on population and prevention science of the American Heart Association', *Circulation*, 109: 2655-71.
- Brown, Gordon D. 2006. 'Dectin-1: a signalling non-TLR pattern-recognition receptor', *Nature Reviews Immunology*, 6: 33-43.
- Brunekreef, Bert, and Stephen T Holgate. 2002. 'Air pollution and health', *The lancet*, 360: 1233-42.
- Bustin, SA. 2002. 'Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems', *Journal of molecular endocrinology*, 29: 23-39.
- Bustin, Stephen, and Reinhold Mueller. 2005. 'Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis', *Clinical Science*, 109: 365-79.
- Cavaillon, JM. 1994. 'Cytokines and macrophages', *Biomedicine & pharmacotherapy*, 48: 445-53.
- Chan-Yeung, MN. 2000. 'Air pollution and health', *Hong Kong Medical Journal*, 6: 390-98.
- Charlson, Emily S, Kyle Bittinger, Andrew R Haas, Ayannah S Fitzgerald, Ian Frank, Anjana Yadav, Frederic D Bushman, and Ronald G Collman. 2011. 'Topographical continuity of bacterial populations in the healthy human respiratory tract', *American Journal of Respiratory and Critical Care Medicine*, 184: 957-63.
- Chawla, Ajay, Khoa D Nguyen, and YP Sharon Goh. 2011. 'Macrophage-mediated inflammation in metabolic disease', *Nature Reviews Immunology*, 11: 738-49.
- Chen, Grace, Michael H Shaw, Yun-Gi Kim, and Gabriel Nuñez. 2009. 'NOD-like receptors: role in innate immunity and inflammatory disease', *Annual Review of Pathological Mechanical Disease*, 4: 365-98.
- Chen, Kaiwen W, and Kate Schroder. 2013. 'Antimicrobial functions of inflammasomes', *Current opinion in microbiology*, 16: 311-18.
- Chen, Zhiyun, Ying Liu, Baoyun Sun, Han Li, Jinquan Dong, Lijuan Zhang, Liming Wang, Peng Wang, Yuliang Zhao, and Chunying Chen. 2014. 'Polyhydroxylated Metallofullerenols Stimulate IL-1 β Secretion of Macrophage through TLRs/MyD88/NF- κ B Pathway and NLRP3 Inflammasome Activation', *Small*, 10: 2362-72.
- Chow, Judith C, John G Watson, Joe L Mauderly, Daniel L Costa, Ronald E Wyzga, Sverre Vedal, George M Hidy, Sam L Altshuler, David Marrack, and Jon M Heuss. 2006. 'Health effects of fine particulate air pollution: lines that connect', *Journal of the air & waste management association*, 56: 1368-80.
- Chung, KF, and PJ Barnes. 1999. 'Cytokines in asthma', *Thorax*, 54: 825-57.
- Covert, Markus W, Thomas H Leung, Jahlionais E Gaston, and David Baltimore. 2005. 'Achieving stability of lipopolysaccharide-induced NF- κ B activation', *Science*, 309: 1854-57.
- Cramer, Robert A, Amariliz Rivera, and Tobias M Hohl. 2011. 'Immune responses against *Aspergillus fumigatus*: what have we learned?', *Current opinion in infectious diseases*, 24: 315.
- Danielsen, Pernille Høgh, Steffen Loft, Anette Kocbach, Per E Schwarze, and Peter Møller. 2009. 'Oxidative damage to DNA and repair induced by Norwegian wood smoke particles in human A549 and THP-1 cell lines', *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 674: 116-22.
- Das, Amitava, Mithun Sinha, Soma Datta, Motaz Abas, Scott Chaffee, Chandan K Sen, and Sashwati Roy. 2015. 'Monocyte and macrophage plasticity in tissue repair and regeneration', *The American journal of pathology*, 185: 2596-606.
- de Kok, Theo MCM, Hermen AL Driecce, Janneke GF Hogervorst, and Jacob J Briedé. 2006. 'Toxicological assessment of ambient and traffic-related particulate matter: a review of recent studies', *Mutation Research/Reviews in Mutation Research*, 613: 103-22.
- de Martin, Rainer, Johannes A Schmid, and Renate Hofer-Warbinek. 1999. 'The NF- κ B/Rel family of transcription factors in oncogenic transformation and apoptosis', *Mutation Research/Reviews in Mutation Research*, 437: 231-43.
- Denning, David W. 1998. 'Invasive aspergillosis', *Clinical infectious diseases*: 781-803.

- Dickson, Robert P, John R Erb-Downward, and Gary B Huffnagle. 2013. 'The role of the bacterial microbiome in lung disease', *Expert review of respiratory medicine*, 7: 245-57.
- Dinareello, Charles A. 1998. 'Interleukin-1 β , Interleukin-18, and the Interleukin-1 β Converting Enzyme', *Annals of the New York Academy of Sciences*, 856: 1-11.
2000. 'Proinflammatory cytokines', *Chest Journal*, 118: 503-08.
2007. 'Historical insights into cytokines', *European journal of immunology*, 37: S34-S45.
2010. 'IL-1: Discoveries, controversies and future directions', *European journal of immunology*, 40: 599-606.
- Dostert, Catherine, Virginie Pétrilli, Robin Van Bruggen, Chad Steele, Brooke T Mossman, and Jürg Tschopp. 2008. 'Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica', *Science*, 320: 674-77.
- Drummond, M Bradley. 2014. 'Obstructive airway disease in urban populations', *Current opinion in pulmonary medicine*, 20: 180.
- Edinger, Aimee L, and Craig B Thompson. 2004. 'Death by design: apoptosis, necrosis and autophagy', *Current opinion in cell biology*, 16: 663-69.
- Elgert, Klaus D. 2009. *Immunology: understanding the immune system 2E* (John Wiley & Sons: p. 9).
- Engvall, Eva, and Peter Perlmann. 1971. 'Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G', *Immunochemistry*, 8: 871-74.
- Epstein, Franklin H, Peter J Barnes, and Michael Karin. 1997. 'Nuclear factor- κ B—a pivotal transcription factor in chronic inflammatory diseases', *New England journal of medicine*, 336: 1066-71.
- Feghali, Carol A, and Timothy M Wright. 1997. 'Cytokines in acute and chronic inflammation', *Front Biosci*, 2: d12-d26.
- Feldmesser, Marta. 2005. 'Prospects of vaccines for medically important fungi', *Medical mycology*, 43: 571-87.
- Franchi, Luigi, Tatjana Eigenbrod, Raúl Muñoz-Planillo, and Gabriel Nuñez. 2009. 'The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis', *Nature immunology*, 10: 241-47.
- Fubini, Bice, and Andrea Hubbard. 2003. 'Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis', *Free Radical Biology and Medicine*, 34: 1507-16.
- Fung, Frederick, and William G Hughson. 2003. 'Health effects of indoor fungal bioaerosol exposure', *Applied occupational and environmental hygiene*, 18: 535-44.
- Gambelunghe, A, C Antognelli, C Del Buono, R Piccinini, B Ugolini, N Murgia, VN Talesa, and G Muzi. 2006. '[Crystalline silica can induce oxidative stress by inhibiting glyoxalase system in bronchial epithelial cells]', *Giornale italiano di medicina del lavoro ed ergonomia*, 29: 397-99.
- Gearing, AJ, P Beckett, M Christodoulou, M Churchill, JM Clements, M Crimmin, AH Davidson, AH Drummond, WA Galloway, and R Gilbert. 1995. 'Matrix metalloproteinases and processing of pro-TNF-alpha', *Journal of Leukocyte Biology*, 57: 774-77.
- GenScript, The Biology CRO. 'Double Stain Apoptosis Detection Kit (Hoechst 33342/PI) ', http://www.genscript.com/tech_guide/TM0361.pdf.
- Gersuk, Geoffrey M, David M Underhill, Liqun Zhu, and Kieren A Marr. 2006. 'Dectin-1 and TLRs permit macrophages to distinguish between different *Aspergillus fumigatus* cellular states', *The Journal of Immunology*, 176: 3717-24.
- Ghosh, Sankar, and Michael Karin. 2002. 'Missing pieces in the NF- κ B puzzle', *Cell*, 109: S81-S96.
- Ghosh, Sankar, Michael J May, and Elizabeth B Kopp. 1998. 'NF- κ B and Rel proteins: evolutionarily conserved mediators of immune responses', *Annual review of immunology*, 16: 225-60.
- Gollwitzer, Eva S, Sejal Saglani, Aurélien Trompette, Koshika Yadava, Rebekah Sherburn, Kathy D McCoy, Laurent P Nicod, Clare M Lloyd, and Benjamin J Marsland. 2014. 'Lung microbiota promotes tolerance to allergens in neonates via PD-L1', *Nature medicine*, 20: 642-47.
- Goodley, JM, YM Clayton, and RJ Hay. 1994. 'Environmental sampling for aspergilli during building construction on a hospital site', *Journal of Hospital Infection*, 26: 27-35.

- Goto, Yukinobu, James C Hogg, Beth Whalen, Chih-Horng Shih, Hiroshi Ishii, and Stephan F van Eeden. 2004. 'Monocyte recruitment into the lungs in pneumococcal pneumonia', *American journal of respiratory cell and molecular biology*, 30: 620-26.
- Goto, Yukinobu, Hiroshi Ishii, James C Hogg, Chih-Horng Shih, Kazuhiro Yatera, Renaud Vincent, and Stephan F van Eeden. 2004. 'Particulate matter air pollution stimulates monocyte release from the bone marrow', *American Journal of Respiratory and Critical Care Medicine*, 170: 891-97.
- Green, D. R., and F. Llambi. 2015. 'Cell Death Signaling', *Cold Spring Harb Perspect Biol*, 7.
- Gualtieri, Maurizio, Johan Øvrevik, Jørn A Holme, M Grazia Perrone, Ezio Bolzacchini, Per E Schwarze, and Marina Camatini. 2010. 'Differences in cytotoxicity versus pro-inflammatory potency of different PM fractions in human epithelial lung cells', *Toxicology in vitro*, 24: 29-39.
- Guo, Haitao, Justin B Callaway, and Jenny PY Ting. 2015. 'Inflammasomes: mechanism of action, role in disease, and therapeutics', *Nature medicine*, 21: 677-87.
- Górny, Rafał L. 2004. 'Filamentous microorganisms and their fragments in indoor air—a review', *Ann Agric Environ Med*, 11: 185-97.
- Hashimoto, Carl, Kathy L Hudson, and Kathryn V Anderson. 1988. 'The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein', *Cell*, 52: 269-79.
- Herseth, Jan I, Vivi Volden, Per E Schwarze, Marit Låg, and Magne Refsnes. 2008. 'IL-1beta differently involved in IL-8 and FGF-2 release in crystalline silica-treated lung cell co-cultures', *Particle and fibre toxicology*, 5: 1.
- Herseth, JI, M Refsnes, M Låg, G Hetland, and PE Schwarze. 2008. 'IL-1 β as a determinant in silica-induced cytokine responses in monocyte-endothelial cell co-cultures', *Human & experimental toxicology*, 27: 387-99.
- Heyder, JOACHIM, and MAGNUS U Svartengren. 2001. 'Basic principles of particle behavior in the human respiratory tract', *Lung biology in Health and Disease*, 162: 21-46.
- Hohl, Tobias M, Heather L Van Epps, Amariliz Rivera, Laura A Morgan, Patrick L Chen, Marta Feldmesser, and Eric G Pamer. 2005. 'Aspergillus fumigatus triggers inflammatory responses by stage-specific β -glucan display', *PLoS Pathog*, 1: e30.
- Hornung, Veit, Franz Bauernfeind, Annett Halle, Eivind O Samstad, Hajime Kono, Kenneth L Rock, Katherine A Fitzgerald, and Eicke Latz. 2008. 'Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization', *Nature immunology*, 9: 847-56.
- Hospenthal, DR, KJ Kwon-Chung, and JE Bennett. 1998. 'Concentrations of airborne Aspergillus compared to the incidence of invasive aspergillosis: lack of correlation', *Medical mycology*, 36: 165-68.
- House, Robert V. 2001. 'Cytokine measurement techniques for assessing hypersensitivity', *Toxicology*, 158: 51-58.
- Ichinohe, Takeshi, Iris K Pang, Yosuke Kumamoto, David R Peaper, John H Ho, Thomas S Murray, and Akiko Iwasaki. 2011. 'Microbiota regulates immune defense against respiratory tract influenza A virus infection', *Proceedings of the National Academy of Sciences*, 108: 5354-59.
- Imrich, Amy, Yao Yu Ning, Henry Koziel, Brent Coull, and Lester Kobzik. 1999. 'Lipopolysaccharide priming amplifies lung macrophage tumor necrosis factor production in response to air particles', *Toxicology and applied pharmacology*, 159: 117-24.
- Invitrogen. 2009. 'Chromatin Condensation/Dead Cell Apoptosis Kit with Hoechst 33342 and PI for Flow Cytometry', <https://tools.thermofisher.com/content/sfs/manuals/mp13244.pdf>.
- Ishii, Ken J, Shohei Koyama, Atsushi Nakagawa, Cevayir Coban, and Shizuo Akira. 2008. 'Host innate immune receptors and beyond: making sense of microbial infections', *Cell host & microbe*, 3: 352-63.
- Iwasaki, Hiromi, and Koichi Akashi. 2007. 'Myeloid lineage commitment from the hematopoietic stem cell', *Immunity*, 26: 726-40.

- Jantzen, Kim, Martin Roursgaard, Claus Desler, Steffen Loft, Lene Juel Rasmussen, and Peter Møller. 2012. 'Oxidative damage to DNA by diesel exhaust particle exposure in co-cultures of human lung epithelial cells and macrophages', *Mutagenesis*: ges035.
- Journey, W Shane, Sarabjeet S Suri, Jesus G Moralez, Hicham Fenniri, and Baljit Singh. 2008. 'Low Inflammatory Activation by Self-Assembling Rosette Nanotubes in Human Calu-3 Pulmonary Epithelial Cells', *Small*, 4: 817-23.
- Kaewamatawong, Theerayuth, Akinori Shimada, Takehito Morita, Wijit Banlunara, and Anong Bintvihok. 2009. 'Acute and subacute pulmonary effects of diesel exhaust particles in mice: Pathological changes and translocation pathways to the circulation', *Thai J Vet Med*, 39: 311-18.
- Kanneganti, Thirumala-Devi, Mohamed Lamkanfi, and Gabriel Núñez. 2007. 'Intracellular NOD-like receptors in host defense and disease', *Immunity*, 27: 549-59.
- Kerr, JF, Glenda C Gobé, Clay M Winterford, and Brian V Harmon. 1995. 'Anatomical methods in cell death', *Methods in cell biology*, 46: 1-27.
- Klaassen, Curtis D. 2013. *Casarett and Doull's toxicology: the basic science of poisons (6th edition)* (McGraw-Hill New York (NY): p. 610).
- Kobach, A, AI Totlandsdal, M Låg, M Refsnes, and PE Schwarze. 2008. 'Differential binding of cytokines to environmentally relevant particles: a possible source for misinterpretation of in vitro results?', *Toxicology letters*, 176: 131-37.
- Kobach, Anette, Ellen Namork, and Per E Schwarze. 2008. 'Pro-inflammatory potential of wood smoke and traffic-derived particles in a monocytic cell line', *Toxicology*, 247: 123-32.
- Landry, Russell P, Valerie L Jacobs, Edgar Alfonso Romero-Sandoval, and Joyce A DeLeo. 2012. 'Propentofylline, a CNS glial modulator does not decrease pain in post-herpetic neuralgia patients: in vitro evidence for differential responses in human and rodent microglia and macrophages', *Experimental neurology*, 234: 340-50.
- Latgé, Jean-Paul. 1999. 'Aspergillus fumigatus and aspergillosis', *Clinical microbiology reviews*, 12: 310-50.
2001. 'The pathobiology of Aspergillus fumigatus', *Trends in microbiology*, 9: 382-89.
- Lee, Byung Uk. 2011. 'Life comes from the air: a short review on bioaerosol control', *Aerosol Air Qual. Res*, 11: 921-27.
- Leng, Sean X, Janet E McElhaney, Jeremy D Walston, Dongxu Xie, Neal S Fedarko, and George A Kuchel. 2008. 'ELISA and multiplex technologies for cytokine measurement in inflammation and aging research', *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 63: 879-84.
- Levitz, Stuart M. 2004. 'Interactions of Toll-like receptors with fungi', *Microbes and Infection*, 6: 1351-55.
- Li, Shuangshuang, Xiangli Gao, Xiaoxin Wu, Zhigang Wu, Linfang Cheng, Lifan Zhu, Dan Shen, and Xiangmin Tong. 2015. 'Parthenolide inhibits LPS-induced inflammatory cytokines through the toll-like receptor 4 signal pathway in THP-1 cells', *Acta biochimica et biophysica Sinica*, 47: 368-75.
- Lien, Egil, and Robin R Ingalls. 2002. 'Toll-like receptors', *Critical care medicine*, 30: S1-S11.
- Lien, Egil, Timothy J Sellati, Atsutoshi Yoshimura, Trude H Flo, Georges Rawadi, Robert W Finberg, J David Carroll, Terje Espevik, Robin R Ingalls, and Justin D Radolf. 1999. 'Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products', *Journal of Biological Chemistry*, 274: 33419-25.
- Lockshin, Richard A, and Zahra Zakeri. 2004. 'Apoptosis, autophagy, and more', *The international journal of biochemistry & cell biology*, 36: 2405-19.
- Lopez-Castejon, Gloria, and David Brough. 2011. 'Understanding the mechanism of IL-1 β secretion', *Cytokine & growth factor reviews*, 22: 189-95.
- Margalit, Anatte, and Kevin Kavanagh. 2015. 'The innate immune response to Aspergillus fumigatus at the alveolar surface', *FEMS microbiology reviews*, 39: 670-87.

- Maricq, M Matti. 2007. 'Chemical characterization of particulate emissions from diesel engines: A review', *Journal of Aerosol Science*, 38: 1079-118.
- Marr, Kieren A, Michael Koudadoust, Michele Black, and S Arunmozhi Balajee. 2001. 'Early Events in Macrophage Killing of *Aspergillus fumigatus* Conidia: New Flow Cytometric Viability Assay', *Clinical and diagnostic laboratory immunology*, 8: 1240-47.
- McQueen, Charlene. 2010. *Comprehensive toxicology (2nd edition)* (Newnes: pages 59-91).
- Miller, J David. 1992. 'Fungi as contaminants in indoor air', *Atmospheric Environment. Part A. General Topics*, 26: 2163-72.
- Miller, Mark R, Catherine A Shaw, and Jeremy P Langrish. 2012. 'From particles to patients: oxidative stress and the cardiovascular effects of air pollution', *Future cardiology*, 8: 577-602.
- Monteleone, Mercedes, Jennifer L Stow, and Kate Schroder. 2015. 'Mechanisms of unconventional secretion of IL-1 family cytokines', *Cytokine*.
- Moresco, Eva Marie Y, Diantha LaVine, and Bruce Beutler. 2011. 'Toll-like receptors', *Current Biology*, 21: R488-R93.
- Mullins, J, and RG Slavin. 1984. '*Aspergillus fumigatus* spore concentration in outside air: Cardiff and St Louis compared', *Clinical & Experimental Allergy*, 14: 351-54.
- Mundandhara, Sailaja D, Susanne Becker, and Michael C Madden. 2006. 'Effects of diesel exhaust particles on human alveolar macrophage ability to secrete inflammatory mediators in response to lipopolysaccharide', *Toxicology in vitro*, 20: 614-24.
- Murphy, SA, KA Berube, and RJ Richards. 1999. 'Bioreactivity of carbon black and diesel exhaust particles to primary Clara and type II epithelial cell cultures', *Occupational and environmental medicine*, 56: 813-19.
- Natarajan, Sudha, Jiyou Kim, and Daniel G Remick. 2010. 'Chronic pulmonary LPS tolerance induces selective immunosuppression while maintaining the neutrophilic response', *Shock (Augusta, Ga.)*, 33: 162.
- Nemmar, Abderrahim, Jørn A Holme, Irma Rosas, Per E Schwarze, and Ernesto Alfaro-Moreno. 2013. 'Recent advances in particulate matter and nanoparticle toxicology: a review of the in vivo and in vitro studies', *BioMed research international*, 2013.
- NIST, National Institute of Standards & Technology. 2013. 'Standard Reference Material 2975 Diesel Particulate Matter (Industrial Forklift)', <https://www-s.nist.gov/m-srmors/certificates/2975.pdf>.
- Nordenhäll, C, Jamshid Pourazar, MC Ledin, Jan-Olof Levin, Thomas Sandström, and Ellinor Ädelroth. 2001. 'Diesel exhaust enhances airway responsiveness in asthmatic subjects', *European Respiratory Journal*, 17: 909-15.
- Oeckinghaus, Andrea, and Sankar Ghosh. 2009. 'The NF- κ B family of transcription factors and its regulation', *Cold Spring Harbor perspectives in biology*, 1: a000034.
- Øvrevik, Johan, Magne Refsnes, Marit Låg, Jørn A Holme, and Per E Schwarze. 2015. 'Activation of proinflammatory responses in cells of the airway mucosa by particulate matter: oxidant-and non-oxidant-mediated triggering mechanisms', *Biomolecules*, 5: 1399-440.
- Owen, Judith A, Jenni Punt, Sharon A Stranford, and Patricia P Jones. 2013. *Kuby immunology* (WH Freeman New York; 7th edition; P.35).
- Oxman, Andrew D, David CF Muir, Harry S Shannon, Susan R Stock, Eva Hnizdo, and HJ Lange. 1993. 'Occupational dust exposure and chronic obstructive pulmonary disease: a systematic overview of the evidence', *American Review of Respiratory Disease*, 148: 38-48.
- Pady, SM, and PH Gregory. 1963. 'Numbers and viability of airborne hyphal fragments in England', *Transactions of the British Mycological Society*, 46: 609-IN20.
- Pålsson-McDermott, Eva M, and Luke AJ O'Neill. 2004. 'Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4', *Immunology*, 113: 153-62.
- Parham, Peter. 2014. *The immune system 3E* (Garland Science: p.15).

- Park, Stacy J, and Borna Mehrad. 2009. 'Innate immunity to *Aspergillus* species', *Clinical microbiology reviews*, 22: 535-51.
- Piccioli, Patrizia, and Anna Rubartelli. 2013. "The secretion of IL-1 β and options for release." In *Seminars in immunology*, 425-29. Elsevier.
- Pope III, C Arden, and Douglas W Dockery. 2006. 'Health effects of fine particulate air pollution: lines that connect', *Journal of the air & waste management association*, 56: 709-42.
- Pope III, C Arden, Michael J Thun, Mohan M Nambodiri, Douglas W Dockery, John S Evans, Frank E Speizer, and Clark W Heath Jr. 1995. 'Particulate air pollution as a predictor of mortality in a prospective study of US adults', *American Journal of Respiratory and Critical Care Medicine*, 151: 669-74.
- Qin, Zhenyu. 2012. 'The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature', *Atherosclerosis*, 221: 2-11.
- Rietschel, Ernst T, Teruo Kirikae, F Ulrich Schade, Uwe Mamat, Gunter Schmidt, Haardald Loppnow, Artur J Ulmer, U Zähringer, Ulrich Seydel, and F Di Padova. 1994. 'Bacterial endotoxin: molecular relationships of structure to activity and function', *The FASEB Journal*, 8: 217-25.
- Rocha e Silva, M. 1978. 'A brief survey of the history of inflammation', *Inflammation Research*, 8: 45-49.
- Rodero, Mathieu P, Lucie Poupel, Pierre-Louis Loyher, Pauline Hamon, Fabrice Licata, Charlotte Pessel, David A Hume, Christophe Combadière, and Alexandre Boissonnas. 2015. 'Immune surveillance of the lung by migrating tissue monocytes', *Elife*, 4: e07847.
- Rylander, Ragnar. 1986. 'Lung diseases caused by organic dusts in the farm environment', *American journal of industrial medicine*, 10: 221-27.
- Said-Sadier, Najwane, Eduardo Padilla, Gordon Langsley, and David M Ojcius. 2010. 'Aspergillus fumigatus stimulates the NLRP3 inflammasome through a pathway requiring ROS production and the Syk tyrosine kinase', *PLoS one*, 5: e10008.
- Salvi, S, and ST Holgate. 1999. 'Mechanisms of particulate matter toxicity', *Clinical and experimental allergy*, 29: 1187-94.
- Salvi, Sundeep, Anders Blomberg, Bertil Rudell, Frank Kelly, Thomas Sandstrom, Stephen T Holgate, and Anthony Frew. 1999. 'Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers', *American Journal of Respiratory and Critical Care Medicine*, 159: 702-09.
- Samet, Jonathan M, Francesca Dominici, Frank C Currier, Ivan Coursac, and Scott L Zeger. 2000. 'Fine particulate air pollution and mortality in 20 US cities, 1987-1994', *New England journal of medicine*, 343: 1742-49.
- Sara Hickin, James Renshaw, Rachel Williams. 2013. *Respiratory System 4E* (p.3-4).
- Sawyer, K, S Mundandhara, AJ Ghio, and MC Madden. 2009. 'The effects of ambient particulate matter on human alveolar macrophage oxidative and inflammatory responses', *Journal of Toxicology and Environmental Health, Part A*, 73: 41-57.
- Schletter, Jens, Holger Heine, Artur J Ulmer, and Ernst T Rietschel. 1995. 'Molecular mechanisms of endotoxin activity', *Archives of Microbiology*, 164: 383-89.
- Schroder, Kate, and Jurg Tschopp. 2010. 'The inflammasomes', *Cell*, 140: 821-32.
- Schulze-Osthoff, Klaus, Davide Ferrari, Marek Los, Sebastian Wesselborg, and Marcus E Peter. 1998. 'Apoptosis signaling by death receptors', *European Journal of Biochemistry*, 254: 439-59.
- Schwabe, Robert F, and David A Brenner. 2006. 'Mechanisms of liver injury. I. TNF- α -induced liver injury: role of IKK, JNK, and ROS pathways', *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 290: G583-G89.
- Schwarze, PE, J Øvreik, M Låg, M Refsnes, P Nafstad, RB Hetland, and E Dybing. 2006. 'Particulate matter properties and health effects: consistency of epidemiological and toxicological studies', *Human & experimental toxicology*, 25: 559-79.
- Schwarze, Per E, Annike Irene Totlandsdal, Marit Låg, Magne Refsnes, Jørn Andreas Holme, and Johan Øvreik. 2013. 'Inflammation-related effects of diesel engine exhaust particles: studies on lung cells in vitro', *BioMed research international*, 2013.

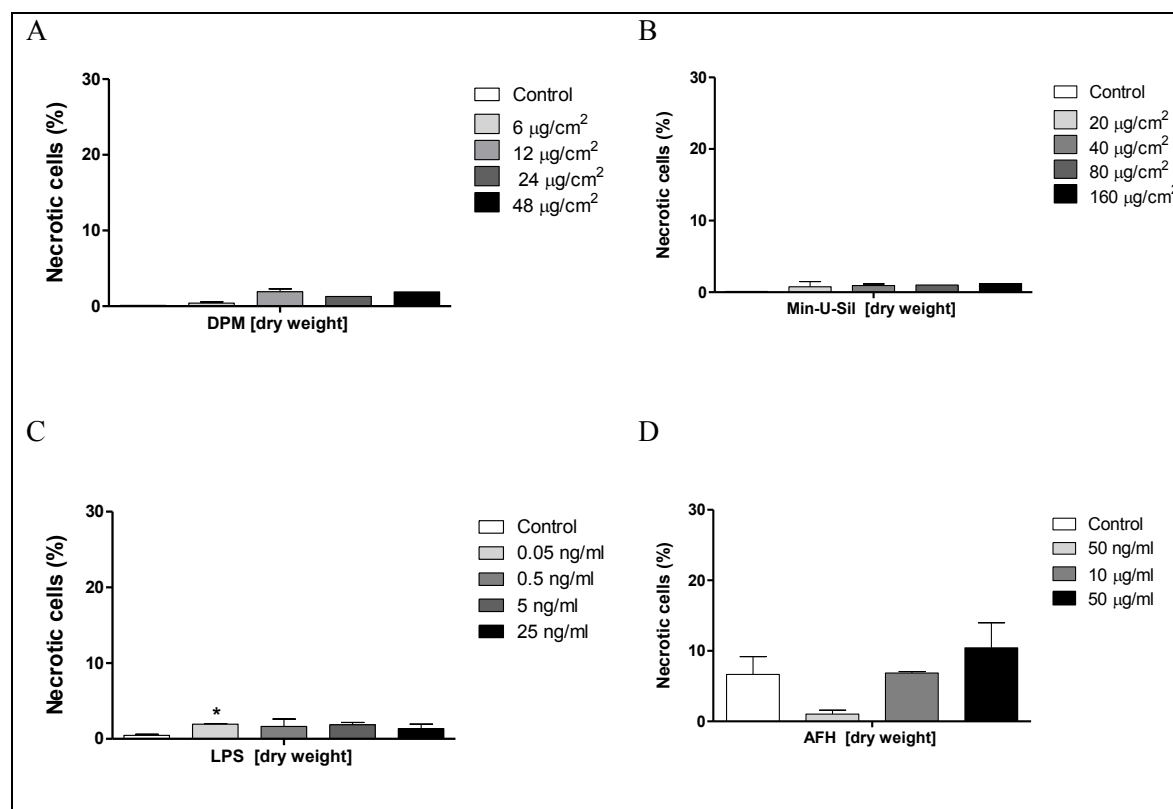
- Schwende, Heike, Edith Fitzke, Petra Ambs, and Peter Dieter. 1996. 'Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1, 25-dihydroxyvitamin D3', *Journal of Leukocyte Biology*, 59: 555-61.
- Scott, A, KM Khan, JL Cook, and V Duronio. 2004. 'What is "inflammation"? Are we ready to move beyond Celsus?', *British journal of sports medicine*, 38: 248-49.
- Sieweke, Michael H, and Judith E Allen. 2013. 'Beyond stem cells: self-renewal of differentiated macrophages', *Science*, 342: 1242974.
- Silverthorn, Dee Unglaub, William C Ober, Claire W Garrison, Andrew C Silverthorn, and Bruce R Johnson. 2013. *Human physiology: an integrated approach 6E* (Pearson/Benjamin Cummings San Francisco, CA, USA: p.571).
- Simitsopoulou, M, E Roilides, C Likartsis, J Ioannidis, A Orfanou, F Paliogianni, and TJ Walsh. 2007. 'Expression of immunomodulatory genes in human monocytes induced by voriconazole in the presence of *Aspergillus fumigatus*', *Antimicrobial agents and chemotherapy*, 51: 1048-54.
- Sørensen, Mette, Herman Autrup, Peter Møller, Ole Hertel, Steen Solvang Jensen, Peter Vinzents, Lisbeth E Knudsen, and Steffen Loft. 2003. 'Linking exposure to environmental pollutants with biological effects', *Mutation Research/Reviews in Mutation Research*, 544: 255-71.
- Sorenson, WG. 1999. 'Fungal spores: hazardous to health?', *Environmental health perspectives*, 107: 469.
- Squadrito, Giuseppe L, Rafael Cueto, Barry Dellinger, and William A Pryor. 2001. 'Quinoid redox cycling as a mechanism for sustained free radical generation by inhaled airborne particulate matter', *Free Radical Biology and Medicine*, 31: 1132-38.
- Steele, Chad, Rekha R Rapaka, Allison Metz, Shannon M Pop, David L Williams, Siamon Gordon, Jay K Kolls, and Gordon D Brown. 2005. 'The beta-glucan receptor dectin-1 recognizes specific morphologies of *Aspergillus fumigatus*', *PLoS Pathog*, 1: e42.
- Strasser, Andreas, Liam O'Connor, and Vishva M Dixit. 2000. 'Apoptosis signaling', *Annual review of biochemistry*, 69: 217-45.
- Sydbom, A, Anders Blomberg, S Parnia, Nikolai Stenfors, Thomas Sandström, and SE Dahlen. 2001. 'Health effects of diesel exhaust emissions', *European Respiratory Journal*, 17: 733-46.
- Takeda, Kiyoshi, Tsuneyasu Kaisho, and Shizuo Akira. 2003. 'Toll-like receptors', *Annual review of immunology*, 21: 335-76.
- Takeuchi, Osamu, and Shizuo Akira. 2010. 'Pattern recognition receptors and inflammation', *Cell*, 140: 805-20.
- Taramelli, D, MG Malabarba, G Sala, N Basilio, and G Cocuzza. 1996. 'Production of cytokines by alveolar and peritoneal macrophages stimulated by *Aspergillus fumigatus* conidia or hyphae', *Journal of medical and veterinary mycology*, 34: 49-56.
- Tauber, Alfred I. 2003. 'Metchnikoff and the phagocytosis theory', *Nature Reviews Molecular Cell Biology*, 4: 897-901.
- Taylor, Philip R, Luisa Martinez-Pomares, Martin Stacey, Hsi-Hsen Lin, Gordon D Brown, and Siamon Gordon. 2005. 'Macrophage receptors and immune recognition', *Annu. Rev. Immunol.*, 23: 901-44.
- Timbrell, John. 2009. *Principles of biochemical toxicology 4E* (Informa healthcare USA, Inc: p.14-15).
- Tsuchiya, Shigeru, Michiko Yamabe, Yoshiko Yamaguchi, Yasuko Kobayashi, Tasuke Konno, and Kei-ya Tada. 1980. 'Establishment and characterization of a human acute monocytic leukemia cell line (THP-1)', *International journal of cancer*, 26: 171-76.
- Ulrich, Charles E. 2007. *Toxicology of the Lung Edited by Donald E. Gardner Publisher: Taylor & Francis Group, New York. 2006. ISBN: 0-8493-2835-7* (International Journal of Toxicology: p.354).
- Underhill, David M, and Iliyan D Iliev. 2014. 'The mycobiota: interactions between commensal fungi and the host immune system', *Nature Reviews Immunology*, 14: 405-16.
- Van Cruchten, Steven, and Wim Van Den Broeck. 2002. 'Morphological and biochemical aspects of apoptosis, oncosis and necrosis', *Anatomia, histologia, embryologia*, 31: 214-23.

- van EEDEN, STEPHAN F, Wan C Tan, Tatsushi Suwa, Hiroshi Mukae, Takeshi Terashima, Takeshi Fujii, Diwen Qui, Renaud Vincent, and James C Hogg. 2001. 'Cytokines involved in the systemic inflammatory response induced by exposure to particulate matter air pollutants (PM10)', *American Journal of Respiratory and Critical Care Medicine*, 164: 826-30.
- Velden, VH, and HF Versnel. 1998. 'Bronchial epithelium: morphology, function and pathophysiology in asthma', *European cytokine network*, 9: 585-97.
- Vignola, Antonio M, Marco Gjomarkaj, Bernard Arnoux, and Jean Bousquet. 1998. 'Monocytes', *Journal of allergy and clinical immunology*, 101: 149-52.
- Vogel, Christoph Franz Adam, Eric Sciuillo, Pat Wong, Paul Kuzmicky, Norman Kado, and Fumio Matsumura. 2005. 'Induction of proinflammatory cytokines and C-reactive protein in human macrophage cell line U937 exposed to air pollution particulates', *Environmental health perspectives*: 1536-41.
- Waegemaekers, M, N van Wageningen, B Brunekreef, and JSM Boleij. 1989. 'Respiratory symptoms in damp homes', *Allergy*, 44: 192-98.
- Wajant, H, K Pfizenmaier, and P Scheurich. 2003. 'Tumor necrosis factor signaling', *Cell Death & Differentiation*, 10: 45-65.
- Walsh, TJ, E Roilides, K Cortez, S Kottlil, J Bailey, and CA Lyman. 2005. 'Control, immunoregulation, and expression of innate pulmonary host defenses against *Aspergillus fumigatus*', *Medical mycology*, 43: 165-72.
- Wang, JE, A Warris, EA Ellingsen, PF Jørgensen, TH Flo, T Espevik, R Solberg, PE Verweij, and AO Aasen. 2001. 'Involvement of CD14 and toll-like receptors in activation of human monocytes by *Aspergillus fumigatus* hyphae', *Infection and immunity*, 69: 2402-06.
- Warheit, David B, Tim A McHugh, and Mark A Hartsky. 1995. 'Differential pulmonary responses in rats inhaling crystalline, colloidal or amorphous silica dusts', *Scandinavian journal of work, environment & health*: 19-21.
- Warris, Adilia, Mihai G Netea, Jacob E Wang, Peter Gaustad, Bart-jan Kullberg, Paul E Verweij, and Tore G Abrahamsen. 2002. 'Cytokine release in healthy donors and patients with chronic granulomatous disease upon stimulation with *Aspergillus fumigatus*', *Scandinavian journal of infectious diseases*, 35: 482-87.
- Weinhold, Bob. 2002. 'Fuel for the long haul: diesel in America', *Environmental health perspectives*, 110: A458.
- Weiss, Leon. 1972. *The cells and tissues of the immune system: structure, functions, interactions* (Prentice-Hall Englewood Cliffs, NJ: p.107).
- WHO, World Health Organization. 2000. 'Crystalline silica, quartz'.
- Wichmann, H-E. 2007. 'Diesel exhaust particles', *Inhalation Toxicology*, 19: 241-44.
- Widdicombe, JG, and RJ Pack. 1982. 'The Clara cell', *European journal of respiratory diseases*, 63: 202-20.
- Williams, Gwyn T. 1991. 'Programmed cell death: apoptosis and oncogenesis', *Cell*, 65: 1097-98.
- Wu, Weidong, Neil E Alexis, Xian Chen, Philip A Bromberg, and David B Peden. 2008. 'Involvement of mitogen-activated protein kinases and NFκB in LPS-induced CD40 expression on human monocytic cells', *Toxicology and applied pharmacology*, 228: 135-43.
- Xu, Haiyan, David Dinsdale, Benoit Nemery, and Peter HM Hoet. 2003. 'Role of residual additives in the cytotoxicity and cytokine release caused by polyvinyl chloride particles in pulmonary cell cultures', *Toxicological sciences*, 72: 92-102.
- Yan, Zhong-qun, and Göran K Hansson. 2007. 'Innate immunity, macrophage activation, and atherosclerosis', *Immunological reviews*, 219: 187-203.
- Yang, D, and JJ Oppenheim. 2009. 'Alarmins and antimicrobial immunity', *Medical mycology*, 47: S146-S53.
- Yona, Simon, and Steffen Jung. 2010. 'Monocytes: subsets, origins, fates and functions', *Current opinion in hematology*, 17: 53-59.

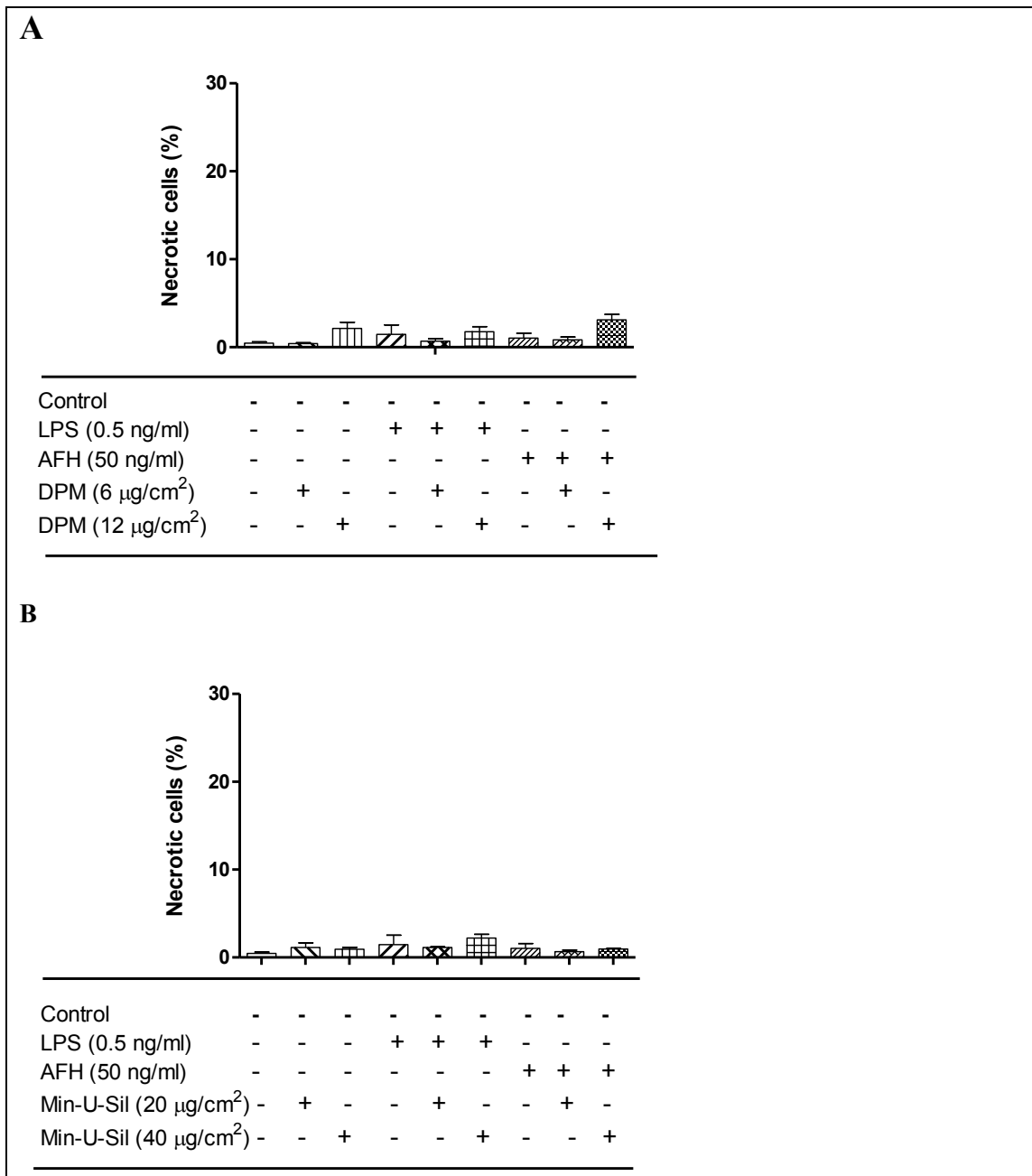
- Yoshie, Osamu, Toshio Imai, and Hisayuki Nomiya. 2001. 'Chemokines in immunity', *Advances in immunology*, 78: 57-110.
- Zureik, Mahmoud, Catherine Neukirch, Bénédicte Leynaert, Renata Liard, Jean Bousquet, and Françoise Neukirch. 2002. 'Sensitisation to airborne moulds and severity of asthma: cross sectional study from European Community respiratory health survey', *Bmj*, 325: 411.

11 SUPPLEMENTARY

Appendix 01: supplementary results

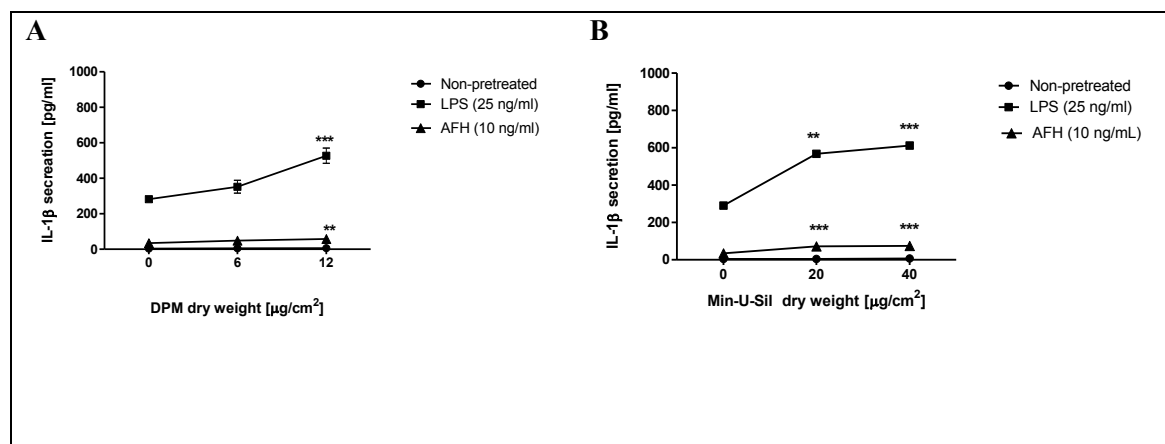


Supplementary figure S1 Induction of necrosis in PMA THP-1 macrophages exposed to DPM, Min-U-Sil, LPS and AFH determined by fluorescence microscopy. PMA THP-1 macrophages exposed to 3-4 different concentrations of (A) DPM, (B) Min-U-sil, (C) LPS and (D) AFH for 24 h. Cells were stained with PI/ Hoechst and subsequently analyzed for necrosis with fluorescence microscope. At least 400 cells were counted per experiment per concentration. Bars represent percentages of means \pm SEM of 1-3 independent experiments. Statistical analysis was based on a log-transformed data (One-way ANOVA, Dunnett post-test). The asterisks denote significant difference of exposed cells compared to the control (* $P < 0.05$). Statistical testing was not performed for the cells exposed to DPM and Min-U-sil as the number of experiment was < 3 for some of the tested concentrations.

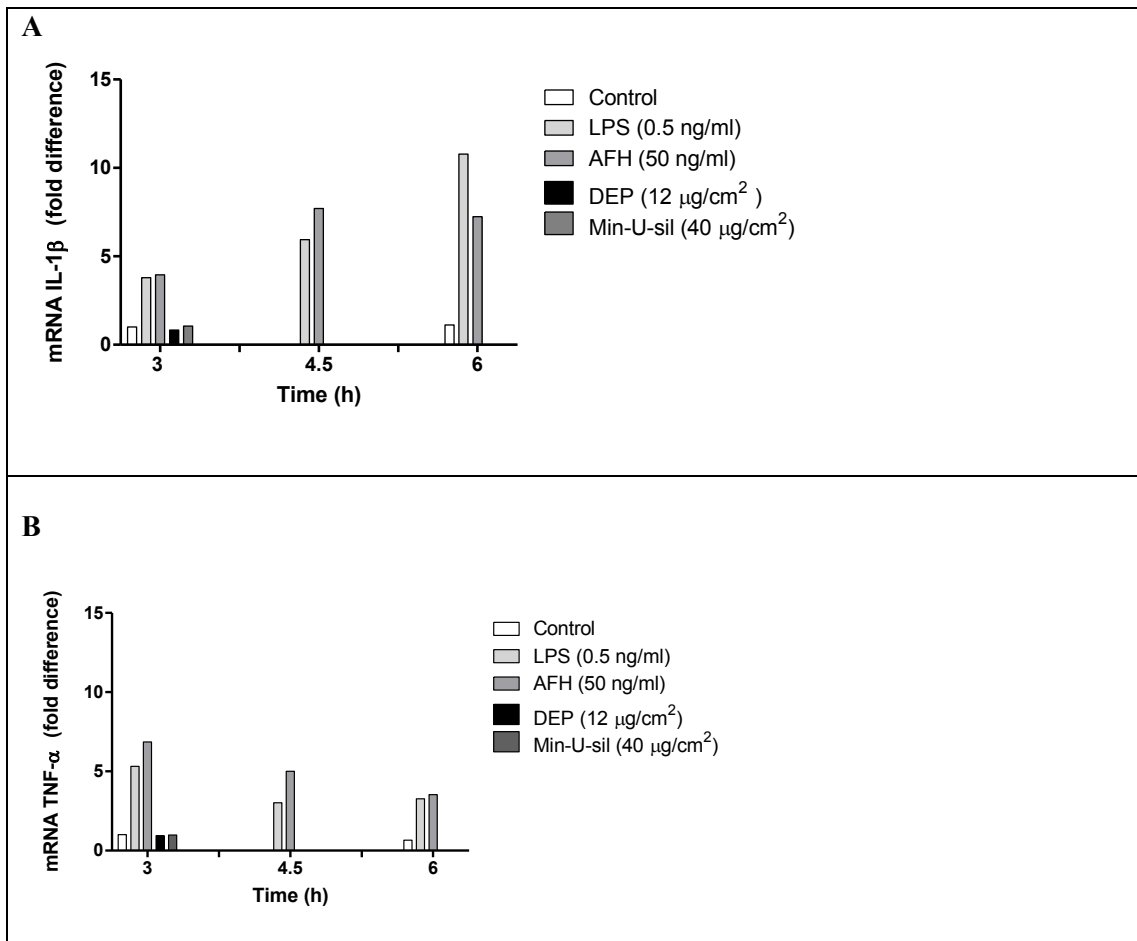


Supplementary figure S2 Induction of necrosis in PMA THP-1 macrophages that are primed with LPS or AFH and co-exposed with DPM or Min-U-sil determined by fluorescence microscopy.

(A) PMA THP-1 monocyte cells were primed with LPS (0.5 ng/ml) or AFH (50 ng/ml) for 3 h and co-exposed to DPM (6 or 12 $\mu\text{g}/\text{cm}^2$) for 18 h. (B) PMA THP-1 monocyte cells were primed with LPS (0.5 ng/ml) or AFH (50 ng/ml) for 3 h and co-exposed to Min-U-sil (20 or 40 $\mu\text{g}/\text{cm}^2$) for 18 h. After the termination of the exposure, cells stained with PI/Hoechst and subsequently analyzed for necrosis with fluorescence microscope. At least 400 cells were counted per experiment per sample. Bars represent percentages of means \pm SEM of 3 independent experiments. + treated; - untreated. Statistical analysis was based on normalized data (One-way ANOVA, Dunnett post-test). None of the co-exposed cells showed significant necrosis compared to their individual control (non-exposed, LPS (0.5 ng/ml) and AFH (50 ng/ml)).



Supplementary figure S3. Release of cytokines in PMA THP-1 cells after co- exposure of AFH/LPS and DPM/ Min-U-Sil determined by ELISA. (A) IL-1 β and TNF- α secretion of PMA THP-1 cells primed with LPS (25 ng/ml) /AFH (10 ng/ml) for 3h and exposed to DPM (6 or 12 $\mu\text{g}/\text{cm}^2$) for 18 h. (B) IL-1 β and TNF- α secretion of PMA THP-1 cells primed with LPS (25 ng/ml) or AFH (10 ng/ml) for 3h and exposed to Min-U-Sil (20 or 40 $\mu\text{g}/\text{cm}^2$) for 18 h. Lines represent mean \pm SEM concentrations of 3 independent experiments. . Statistical analysis was based on normalized data (One-way ANOVA, Dunnett post-test). Significant difference between co-exposed vs the individual control is indicated by asterisks ** $P < 0.01$ *** $P < 0.001$.



Supplementary figure S4. IL-1 β and TNF- α expression of PMA THP-1 cells exposed to either LPS, or AFH or DPM or Min-U-Sil for 3 h, 4.5 h and 6 h assessed by real time RT-PCR. Bars represent the fold increase relative to the control.

Appendix 02: solutions used in the study

Media preparation for THP-1 cells

Inactivated serum (FBS)	55 ml
Sodium pyruvate	55 mg
HEPES	5.5 ml
Gentamicin	550 µl
RPMI 1640	500 ml

Solutions for ELISA

Diluent buffer (Invitrogen, Life technologies)

BSA	5 g
Tween	20 l ml
Dulbeccos PBS	1000 ml

Blocking/diluent buffer (R&D systems)

BSA	10 g
Dulbeccos PBS	1000 ml

Blocking solution (Invitrogen, Life technologies)

BSA	5 g
Dulbeccos PBS	1000 ml 96

Citrate buffer

Sodium acetate trihydrate	3 g
Distilled water	200 ml
Citric acid is used to adjust pH to	5.5.

TMB

Citrate buffer	11 ml
TMB 6 mg/ml	200 µg
30 % H ₂ O ₂	2.2 µl

Stop solution

H ₂ SO ₄	50 ml
Distilled water	1000 ml

Solutions for Real time PCR

Mastermix for cDNA synthesis

Samples and NTC

10 x reverse buffer	2.5 μ l
25x dNTP	1 μ l
10x random primers	2.5 μ l
Multiscribe reverse transcriptase	1.25 μ l
Nuclease free water	5.25 μ l

NRC

10 x reverse buffer	2.5 μ l
25x dNTP	1 μ l
10x random primers	2.5 μ l
RNase free water	6.5 μ l

The total volume for the mastermix in each PCR tube is 12.5 μ l