Nano-sized silica particles: Cytotoxicity and cytokine responses in lung cell models involving differentiated THP-1 cells

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UNIVERSITY OF OSLO

April 2016

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Master thesis in Pharmacy for the grade Master in Pharmacy at

Deparment of Pharmaceutical Bioscience
School of pharmacy
Faculty of Mathematics and Natural Science
University of Oslo

The thesis is carried out at

Department of Air Pollution and Noise
Division of Environmental Medicine
Norwegian Institute of Public Health

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2016

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

Trykk: Reprosentralen, Universitetet i Oslo

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Acknowledgements

From June 2015 to May 2016, this thesis was carried out at the Norwegian Institute of Public Health, Department of Air pollution and Noise. This Master thesis was an assignment I had wished for a long time, and when I first was called in for an interview, I was overwhelmed of happiness. Therefore, I would, first and foremost, like to thank Jørn A. Holme for taking a risk and giving me the opportunity to be part of this study. To my other supervisors, Magne Refsnes and Marit Låg, whom I have spent more time with, I would like to thank for the support, for both the positive, but also the constructive feedbacks and for always being available for questions and discussions. I would also thank my co-supervisor Tonje Skuland for the constant enthusiasm, no matter how tiresome I could be at times. Thank you for helping me with experiments and also for understanding and supporting me during a challenging time of my life. I appreciated every advice, and comment. Furthermore, I would like to thank the whole gang at MILS. A special thanks to Anette Kochbach Bølling, Leni Ekeren, Edel Lilleaas and Hans J. Dahlman for always helping me out in the laboratories.

To family and friends, who have supported me during this last year: Thank you for encouraging me, for keeping me motivated and sending me positive feedbacks during the most stressful and frustrating moments these last weeks.

A special thanks is extended to Rannveig Damlien from the police department of Oslo and Akershus and Linda Pettersen at the emergency center of Oslo, who made it possible for me to reach my goals, finish this degree and most importantly for keeping me safe and giving me the opportunity to have a future.

April 2016, Lavin Kadir

Abstract

Nanoparticles are available in a wide range of products, like pharmaceutical product and cosmetics and in the last decade it has been an increasing use of silica nanoparticles. Nanoparticles are defined as particles with a diameter range from 1 to 100 nm in at least one dimension. They have unique physicochemical properties compared to larger-sized particles of similar chemical composition. This is mostly explained by their small size, larger surface area and surface reactivity. Since there still is some lack of knowledge on their effects on human health and environment, further research on their chemical properties and potential toxicity is required. Previously, silica nanoparticles (SiNPs) of 10 and 50 nm size have been shown to give inflammatory (cytokine) responses in epithelial lung cells. In this master thesis we focus on the ability of different sized silica particles to induce cytokine response in, both, macrophages (differentiated THP-1 cells) and in epithelial cells (BEAS-2B).

The aims in this project include deciding the best exposure conditions in which the cells respond to the Si-particles by cytokine release, without giving too much cytotoxicity. This is then followed by comparing different sized amorphous silica particles: 10, 12 and 500 nm, for cytotoxicity and cytokine release. Finally, the effects of one of the nanoparticles are analyzed, with regard to transfer of conditioned medium from SiNP-exposed differentiated THP-1 cells to another cell culture.

THP-1 cells cultured in RPMI-1640 with 10% FBS were differentiated to more macrophage-like cells with PMA (50 ng/ml), for 48 h in advance of experiments, while the BEAS-2B cells were cultured in LHC-9 medium. The cells were then exposed to the Si-particles, for 6 or 20 h. The cytokine release (IL-1β, TNF-α and IL-8) was measured by ELISA, while gene expression was measured by Real time PCR and the cytotoxicity was investigated by the AlamarBlue assay and by release of lactate dehydrogenase (LDH).

Cells exposed in medium without FBS gave increased cytotoxicity and a significantly higher release of cytokines, compared to the medium with 10% FBS. When comparing different Si-particles, the differentiated THP-1 cells responded markedly with release of IL-1β, TNF-α and IL-8, with Si12 and Si500 as most and least potent, respectively. The gene expression of IL-1β and TNF-α due to Si10 exposure indicated a time-dependent increase. The gene

expression of IL-1β did not have a significant increase before the 6th h of exposure, while the gene expression of TNF-α reached maximum after 4,5 h. The gene expressions were accompanied by an approximately similar time-course for release of the respective cytokines. Considering, the airways consist of several cells interacting, an investigation of possible inetracting reactions between different cell cultures, were done. The transfer of conditioned medium from Si10-exposed differentiated THP-1 cells to unexposed cell cultures showed a possible stimulatory effect on the release of IL-1B from the BEAS-2B cell, but no significant effect on TNF-a and IL-8. The differentiated THP-1 cell mostly seemed unaffected.

The model system of differentiated THP-1 cells seems suitable for further mechanistic studies, but using different batches of a cell-culture seems to influence the results. Furthermore, studies about interactions between cell cultures and cell types have to be explored more.

Abbreviations

BEAS-2B	Human bronchial epitheial cell line
BEGM	Bronchial epithelial growth medium
BSA	Bovine serum albumin
cDNA	Coplementary DNA
CD14	Cluster of differentiation 14
DMEM	Dulcobecco's Modified Eagle Medium
EIA	See ELISA
ELISA	Enzyme-linked immunosorbent assay
ENPSS	Engieneried Nano particles
FBS	Fetal Bovine serum
HLA	Human leukocyte-antibody
HRP	Horeseradish peroxidase
IL-8	Interleukin-8
IL-1α	Interleukin-1a
IL-1β	Interleukin-1B
LDH	Lactate dehydrogenase
LHC-9	See BEGM
LPS	Lipopolysaccaride
mRNA	Messenger ribonucleic acid
NPSS	Nanoparticle

PAF	Platelet-activating factor
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
RPMI	Roswell Park Memorial Institute medium
Si	Silica
SiNPSS	Silica nanoparticle
Si10	Silica nanoparticle 10 nm
Si12	Silica nanoparticle 12 nm
Si50	Silica nanoparticle 50 nm
Si500	Silica nanoparticle 500 nm
SiRNA	Small interfering RNA
THP-1	Human monocytic cell line
TLR	Toll-like receptor
TMB	3, 3', 5, 5' -tetramethylbenzidine
TNF-α	Tumor necrosis factor-a

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1. Introduction

1.1 Background

Nanoparticle (NPs) are defined as particles with a diameter range from 1 to 100 nm (nanometers) in at least one dimension, and exhibit unique features, compared to its bulky counterparts [2, 3]. NPSS is naturally occurring in the environment and generated incidentally as by-products of anthropogenic processes, e.g. vaporization, combustion, welding fumes and from diesel-and petrol-fueled vehicles [2, 4]. Due to its unique properties, NPs are also produced as the result of a manufacturing process, so called engineered nanoparticles (ENPs) [4]. Thus, in the more recent years, nanotechnology is increasingly used in a wide range of consumer products, such as food, cosmetics, and electronics. Of special interest, nanotechnology and the materials science are used for medical purposes such as diagnostic (*in vivo* imaging) and therapeutic purposes (targeted drug delivery) [5-7].

Previous research has shown that NPs can cross the blood-brain barrier and blood-testis barrier in mice and rats and that they are taken up by the cells in these tissues [5, 8, 9]. The mechanisms for uptake are still often not specifically known, and vary often between cell types. Their physicochemical properties in particular their small size, will allow to design particles to reach specific targets in the human body [2, 10]. Site-specific particles are of great interest in medicine, as this is desirable for a drug-delivery system. Accordingly, ENPs have been produced and are now used as drug-carriers for treating diseases such as asthma, but particular in cancer treatments [11].

Silica nanoparticles (SiNPs) have some advantagous properties to be used in medicine. However, SiNPs also have disadvantagous properties. SiNPs are not easily broken down in the body, furthermore SiNPs have been found to suppress cellular growth and proliferation, as well as triggering cell death at higher concentrations [12, 13].

Nevertheless, we may regard SiNPs as a model NPs also for medical use. A more relevant scenario is, however, potential advers effects induced by occupational exposure of SiNPs. Miners and traders are exposed to large–sized silica particles (quartz) exposure in these professions have been associated with different lung diseases [14, 15]. Presumably, smaller-sized SiNPs might not induce similar health effect in humans, but more knowledge is needed.

Some studies of SiNPs have reported that the size of these particles are directly linked to their toxic potency [7, 13, 16]. Nevertheless, it is important to further characterize the chemical properties and potential toxicity of various SiNPs. However, in this thesis the main subject will be the cytotoxic and pro-inflammatory effects of the SiNPs.

1.2 Nanoparticles

1.2.1 Particle characterization

Nanoparticles may be divided in naturally occurring nanoparticles, anthropogenic nanoparticles and engineered nanomaterials [17]. The physicochemical characterization of nanoparticles (e.g. size, shape, surface charge, chemical properties, crystal structure, solubility and degree of agglomeration) is important to determine, for the evaluation of the toxicological effects and thus safety of [6, 18, 19].

Particle size and surface area

Nanoparticles are often defined by the size range. The definition is a particle with a diameter range from 1 to 100 nm in at least one dimension [2]. In Figure 1, we see the comparison of a nanoparticle with e.g. molecules, viruses, bacteria, cancer cells and a tennis ball. The Figure is not drawn to scale, but a nanoparticle has a size of 10^{-9} nanometers, which makes them quite invisible next to a tennis ball.

The small size of the particles has both its advantages and its disadvantages. Because of the particle size and the large surface area per unit weight, the rate of release of compounds from the surface, may be rapid. This is a key property in e.g. pharmaceutical industries for drug delivery. The small size also makes it possible for these particles to be taken up easily, by epithelial cells [2, 12].

If we inject nanoparticles in the blood, they would not affect the flow in the capillaries because of the size. These particles would also not lead to embolization. At the same time, they clearly have an advantage of the size when it comes to surface erosion, adsorption of

ligands, endocytosis and extravasation; because all of these mechanisms are size-dependent and a small size is preferable. The size also gives these particles a physical colloid stability [2].

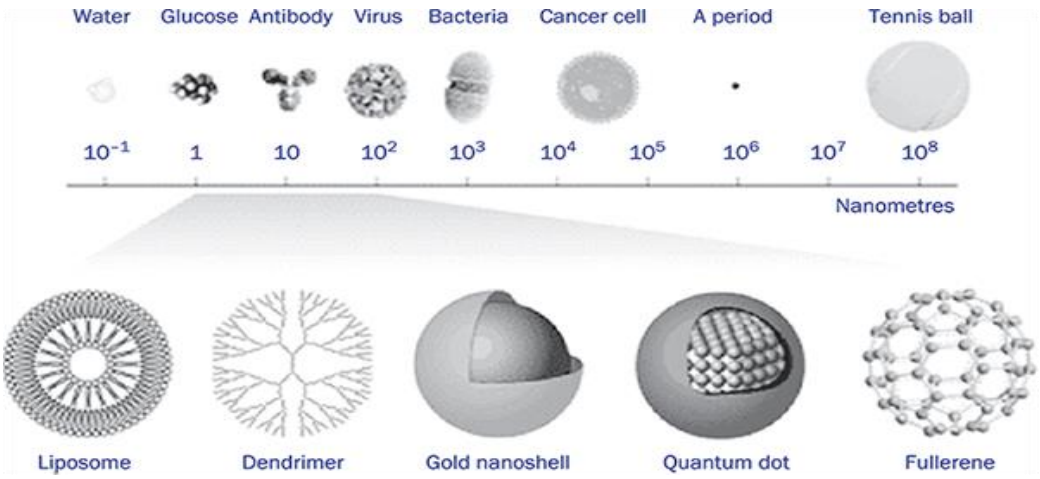

Figure 1: Actual size of a nanoparticle (https://www.medicinescomplete.com/mc/ftpp/current/c11afg001.png)

Particle shape

Nanoparticles that are engineered may be formed into different shapes, such as spheres rods, fibers, sheets and wires [13]. This is important, because previous studies have shown that the shape of a nanoparticle affects their biological responses. The studies have also shown, that when the particle is a fibrous micro-sized material, they induce long-term effects on cytotoxicity, inflammation, proliferation and in some instances genotoxicity. In other words, they increase the risk for lung fibrosis and lung cancer [20]. Examples of such materials are multiwalled carbon nanotubes (MWCNT)[21].

Another problem is that some of these nanofibers, like spherical particles, are more resistant to biodegradation and thereby persistent. These nanofibers will therefore stay in contact with the cells for a longer time, resulting in stronger biological effect, which is critical for their effects on lung fibrosis and cancer [22, 23].

Surface charge

The cell membrane is comprised of an anionic (negatively charged) and hydrophilic outer surface[24]. Therefore, when it comes to distribution in the cells, the surface charge of nanoparticles is of consideration. This property would again impact the interaction between the particle surface and the biological compartments. If, e.g. the nanoparticle is cationic (positively charged) they may be attracted to the cell surface. This would give internalization, while neutral charged nanoparticles will have a longer duration since an interaction with the cell surface is less possible [4, 17].

There are several types of interaction forces that may occur for nanoparticle, including van der Waals, electrostatic, solvation and depletion forces. These interactions may, although dissociate and thereby break up the interaction. This is, however, dependent on the chemical composition of the particle [17, 25].

Protein corona

During manufacturing or when adsorbed by lipids and protein, e.g. when transported or translocated in tissues, the surface of nanoparticles may by coated. This can impact the interaction between the nanoparticles and the cell [26-28]. It may also alter the effect and the biokinetics of the nanoparticles. This surface coating can initially form a protein corona, a dynamic layer of protein that covers the particle surface. A protein corona leads to negative net charge and zeta potential of the nanoparticles at physiological pH, which means higher hydrophobicity and therefor altered biodistribution and toxicity [26, 27, 29].

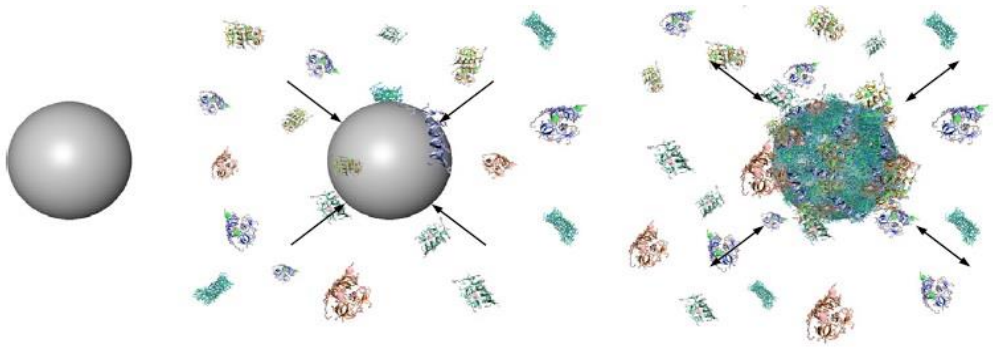

Figure 2: Formation of a protein corona on the surface of a nanoparticle (http://mappingignorance.org/fx/media/2013/06/Figureura-2.jpg)

Agglomeration and aggregation

Agglomeration and aggregation are two distinct terms, but seem to be used interchangeably without any apparent consideration for their meanings [30]. Both terms are mostly used to describe assemblages of particles that are found in dry powders and powders in liquid suspensions. Their definitions have been discussed throughout the years, but seem to be crossing. While agglomeration is used exclusively when particle assemblages are described, aggregation is confined to the association of molecules. Aggregates may again turn into agglomeration. This distinguishing is important for the discussion of nanoparticle assemblage.

An agglomeration will change the hydrodynamic size of the particles, decrease their diffusion, restrict extravasation, and reduce the effective surface area for interactions with receptors [29]. Because of their volume/weight ratios, which gives a larger surface, nanoparticles are prone to agglomerate [1]. The particles adhere because of the the surface tension. If electrolytes are present in biological solution, this will give repulsion of surface charges. The type of solvent or media used will also have an impact on the level of agglomeration [31].

The interactions which give these aggregations are van der Waals forces, electrostatic interactions or surface tension [2, 32]. These type of interaction are weak forces and can therefore be dispersed by ultrasonic [33, 34] treatments or sometimes simply by hand shaking the biological systems [10].

1.3 The respiratory system

The respiratory system is main organs responsible for the oxygen and carbon dioxide exchange in the body. The human body needs oxygen to sustain itself. The respiratory system is briefly divided into the upper and the lower respiratory tracts, where the lower tract again can be divided into the conducting airways and the respiratory airways.

The air streams into the mouth and nose, and is then moved through the trachea, continues to the bronchi and bronchioles and end in the alveolar ducts and sacs (see Figure 3 below). As the oxygen is transported further down the lower tract, both the diameter and the length of the tubules decrease. At the same time, the surface area is higher as a result of increased number of airways. This means that there is a higher probability for uptake in these parts of the lungs. Oxygen is penetrating to the circulation and transported by the red blood cells [24].

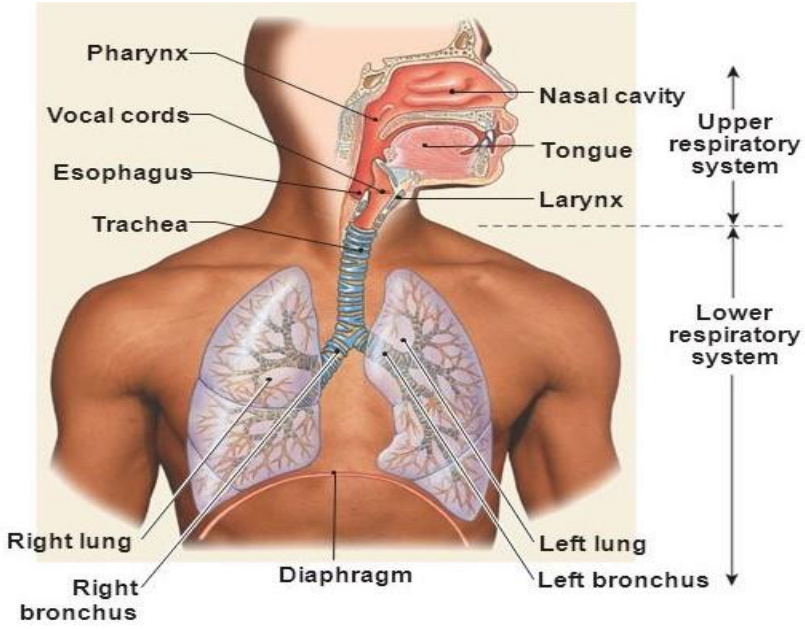

Figure 3: The respiratory system

(http://images.slideplayer.com/13/4156879/slides/slide_7.jpg)

1.4 Particle deposition in the respiratory tract

Nanoparticles can potentially penetrate various parts of the respiratory tract, because of their small size, but also because the lungs have a thin air-blood tissue barrie [19]. In the respiratory system we have an epithelium, which is protected by a thick layer of mucus. The mucus will immediately catch the particles and remove it from the airstream. In the same system, we have a barrier between the alveolar wall and the capillaries where the exchange of oxygen and carbon dioxide happens. It is at this point that particles can penetrate into the capillaries, if they have deposited on the alveolar wall [24, 35, 36]. However, particle deposition may be impacted by several factors [37].

Deposition seems to increase with how deep the breathing is and the duration of breathing. When holding the breath or quietly breathing, a larger volume is inhaled, which will result in greater peripheral distribution of particles. While exercising (heavily breathing), the inhalation and deposition increases [35, 37, 38]. If a patient has a lung disease, their condition in the lungs may modify the diameter of the conducting airways, thus the breathing pattern may be changed and this may result in altered particle deposition. A patient with e.g. chronic bronchitis has a thickened mucous layer, which may block the airways and result in altered airflow and therefore lead to higher risk of particle deposition in the respiratory tract [36, 39].

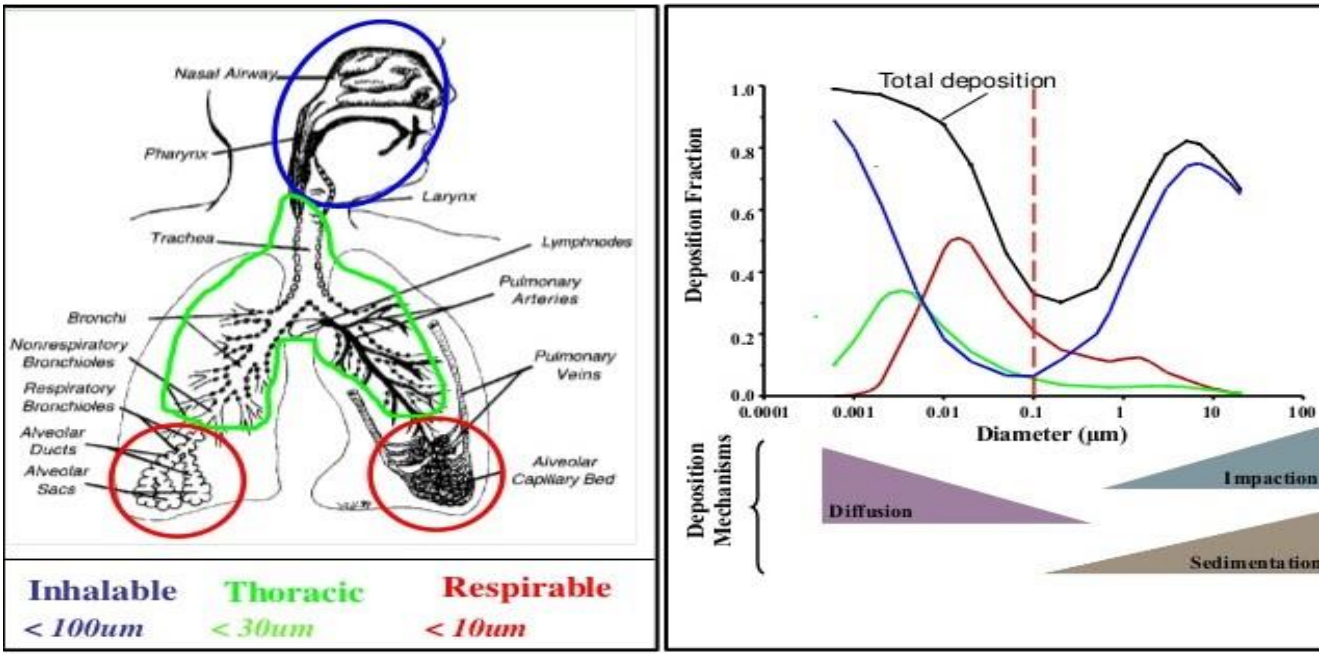

Figure 4: Deposition of different sized particles in the respiratory system [1]

As seen in the Figure, deposition of inhaled particles is dependent on particle size. The larger particles (>20 µm) will, through impaction and sedimentation, distribute to the upper parts of the respiratory tract. Smaller particles (2-20 µm) will settle in the bronchioles, whereas very fine particles (<0.5 µm), i.e. nanoparticles, diffuse and deposit on the walls of the smallest airways, the alveolar sacs These kinds of particles may trigger an inflammatory response in the airways and also diffuse over to the circulation system and give adverse responses there. The smallest nanoparticles (1-10 nm) will also deposit in the nasal area.

1.4.1 Mechanism of particle deposition

Depending on the particle size, there are different deposition mechanisms of particles in the respiratory tract. Among the mechanisms there are impaction, interception, sedimentation and Brownian diffusion [37, 40].

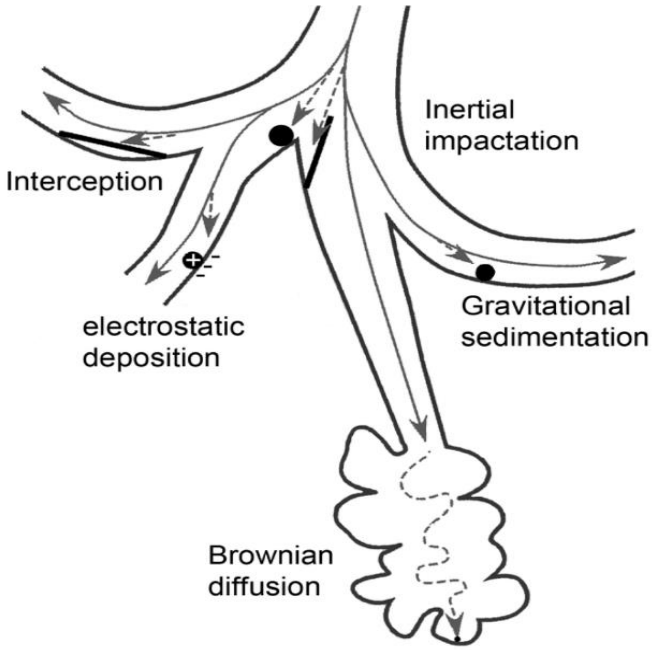

Figure 5: Different processes of particle deposition in the respiratory tract (http://www.mdpi.com/ijms/ijms-15-04795/article_deploy/html/images/ijms-15-04795f6-1024.png)

Impaction occurs mainly in the upper respiratory tract and the conducting airways. It is basically a change in direction of the air stream that causes particles with high velocity to impact on the wall of the airways, instead of changing direction [19]. This mechanism occurs for different particles sizes, but mostly for particles $\leq$ 10 µm. If the particle size is > 10 µm they will also impact, but in the upper airways and then be removed by coughing and swallowing through mucociliary mechanism. For smaller particles (0.5-5 µm), that escape the upper respiratory tract without impaction because of the small size, impaction and sedimentation occurs in the lower airways. **Interception** forces are, on the other hand,

relevant for particles that can attach to the wall in the airways, as they pass through the respiratory system.

Sedimentation is relevant for particles in the size range 0.5-3 µm. These particles will reach the bronchioles and alveoli, but their deposition is dependent on several factors, such as size, density and their residence time in the airways. During slow and deep breathing this mechanism is relevant.

Particles that are < 0.3 µm, will deposit by diffusion in the alveoli and distal airways. During a collision of small particles by gas molecules, a **Brownian movement** occurs. The principle is a movement from higher to lower concentration, which will cause a change of direction for the particles. The particles will move from the aerosol to the walls of the airways. At this point the diffusion is only influenced by the particle size. However, particles of 0.5 µm are too small for impaction or sedimentation and also too large for diffusion. They are thereforedirectly exhaled [19, 37, 40].

Because of the size, nanoparticles will naturally deposit by Brownian diffusion in both the upper and lower respiratory tract. When agglomerated, nanoparticles may change size from a nano-size to a larger size, approximately up to 100 nm [19, 37, 40]. Therefor agglomerated SiNPs are believed to deposit in the alveolar region. On the other hand, smaller sized particles (10-40 nm) will deposit in the bronchioles, in the nose, trachea, throat, pharynx and the alveoli. However, the overall deposition of nanoparticles is estimated to be in the bronchi and the bronchioles [19, 37, 40].

1.5 The immune system of the respiratory organs

The human immune system consists of cells, tissues, and organs that together provide care to the body during an infection or even to prevent an infection. Mainly the immune system works to distinguish pathogens, unknown agents, from the organisms own healthy tissue and also to remove the unknown and keep order in the body. The system is strictly divided into the innate immune system and the adaptive immune system, where the innate immune system, also defined as the first line of defense, are non-specific and the defense has a short-term period of protection. While the innate immune system usually is triggered when microbes are

identified by pattern recognition receptors, the adaptive immune system is based on previous exposure from the same kind of pathogen. The adaptive immune system has a memory and can take of stronger infections [41].

The first response of the immune system, when tissues are damaged or when we have an infection, is an inflammation with several symptoms. There are many different celltypes involved in the immune system, and these cell types have evolved mechanisms to erase the pathogen. Such mechanisms include phagocytosis, anti-microbial peptides called defensins and the activation of the complement system. If, on the other hand, the immune system fails or there is a disorder, this might result in autoimmune diseases, inflammatory diseases or cancer [42].

1.5.1 Immune cells in the airways

As a result of a large surface area in the airways and also inhalation, the lungs are exposed to numerous of airborne pathogens or pollutants. Therefore, it is important with an efficient defense system.

The inner surface of the airways is covered by a cell layer, named the epithelium. The epithelium is protected by a thick layer of mucosa. This is the first line of defense in the lungs and is important for recognition of the microbial exposure. The recognition is monitored by several cell types in the lungs, such as epithelial cells and immune cells, e.g. macrophages. Some of these cells act through the toll-like receptors, which then will stimulate the cells to secret cytokine, chemokines and peptides. The cytokine will further lead to stimulation and upregulation of the immune system, starting an inflammation, where some chemokine also will have the ability to activate chemotaxis [42]. The peptides are, on the other hand, effector molecules of the innate immune system. These are important in the phagocytosis of the microorganism, but also as regulators of inflammation, immunity and wound repair [35].

1.5.2 Defense through different cell types in the lung system

There are many cells contributing to the defense system of the lungs. These may have structural ability to remove pathogens, e.g. epithelial cells and fibroblast, or they may be immune cells, like leukocytes, macrophages, mast cells, dendritic cells and lymphatic cells, which can migrate through the circulation system and serve a critical function in the immune system [43, 44].

The **epithelial cells** serve as a structural barrier, that clears the lungs of inhaled particles through the mucosa, a process involving removal of these pathogens by the "muco-ciliary escalator". The epithelial cells also form intercellular junctions (tight and adherens junctions), that restrict the passive movement across the epithelium [45]. In addition, the epithelial cells can be stimulated by different bacterial components (e.g. LPS or different cytokines like TNF-α and IL-1β) and secrete cytotoxic and anti-microbial peptides, which can start an immune response and even kill microbial pathogen [46].

Next to the epithelial cells, the **endothelial cells**, also play a key role in modulation of particle movement in the lungs. These cells also form intercellular junctions that prevents passive movement of different molecules. When activated the endothelial cells trigger the surface expression of adhesion molecules and platelet activation factor (PAF), which is an agonist that binds to leukocytes and activates them. At the same time, the endothelial cells synthetize and secrete several mediators that regulate inflammatory responses. Among these mediators are e.g. cytokines and chemokines, prostaglandins and growth factors. These mediators, from the endothelial cells, can also affect the leukocyte transmigration and vascular permeability during an inflammatory response [43-45].

The **fibroblasts** also have a structural function, but they mainly synthetize extracellular matrix proteins, like collagen, which are important in the maintenance of structural integrity and repair of infections and injuries. They also express factors, including cytokines, which are important for activation of immune cells [43-45].

The **immune cells** in the lung are divided mainly in those that are normally resident in the lung (mast cells, macrophages, dendritic cells) and cells that are recruited when an inflammation occurs (neutrophils, monocytes, and lymphocytes) [43-45].

Mast cells are known for their ability to recognize environmental change and communicate the following state to other cells. These cells are located at the epithelial surfaces, near blood vessels, nerves and glands. The action of these cells is through activation of a number of receptors. **Macrophages,** on the other hand, are present on the mucosal surface, and also within alveolar spaces of the lung. The precursors of macrophages, monocytes, are recruited from the blood circulation and migrate to specific targets, attracted by chemokines and other factors in the tissues. Upon arrival, the monocyte will be influenced by the environment and thereby develop specific functions, which will result in a differentiation into macrophages. The macrophages are involved in the surveillance of the immune response and function as a bridge between the innate and adaptive immune system. The **dendritic cells**, in conjunction with human leukocyte antigen (HLA) bind antigens and display them on the surface for alarming and activation of the innate immune system. They are also responsible for the activation signals necessary for the activation of the adaptive immune system. **Eosinophils**, are mostly known for their role during an allergic inflammation, but also for their function to eliminate parasites. When an allergic response occurs, the eosinophils, release inflammatory mediators, which is meant to injure other cells. **Neutrophils,** are primarily meant to kill pathogens, through phagocytosis, which is a rapid mechanism for cellular uptake of the pathogen, release different mediators to destruct the pathogen and then remove them from the body [45].

1.5.3 Inflammation

When injured the body will responed with an inflammation for its own protection. An inflammation is triggered by the immune system, also known as the defense system of the body [47]. By an inflammation the cells of the immune system will remove harmful stimuli, including damaged cells, irritants, or pathogens and thereby begin the healing process. It is important to know the difference between an inflammation and an infection.

An infection is caused by a bacterium, virus or fungus and can also cause an inflammation, while an inflammation is a response to this stimulus [48]. It’s is also common to categorize an inflammation in acute and chronic inflammation. Acute inflammation develops quickly and the symptoms will last for only a few days or in worst case a few weeks, whereas a chronic

inflammation stands for a long-term inflammation, that can last for months or years and is often a result of failure in the immune system [35].

It is until now known that nanoparticles do affect the immune system and thereby can start an inflammation. *In-vivo* studies have shown that nanoparticles may cause an inflammation, but also that this is dependent on the dose given and the exposure time. The inflammation seems to be greater compared to larger-sized particles of the same composition [49].

Previous examination of the epithelium exposed to nanoparticles, at high doses, has revealed severe damage to the epithelium, such as holes in the cell membrane. It has also been detected cell detachment, cytotoxicity and apoptotic cell death. This was compared to nanoparticles of bigger size, ~100 nm, which did not cause membrane damage and were also less reactive [50]. Based on studies like this, the determination of hazard potential of nanoparticles for the human body is necessary to determine.

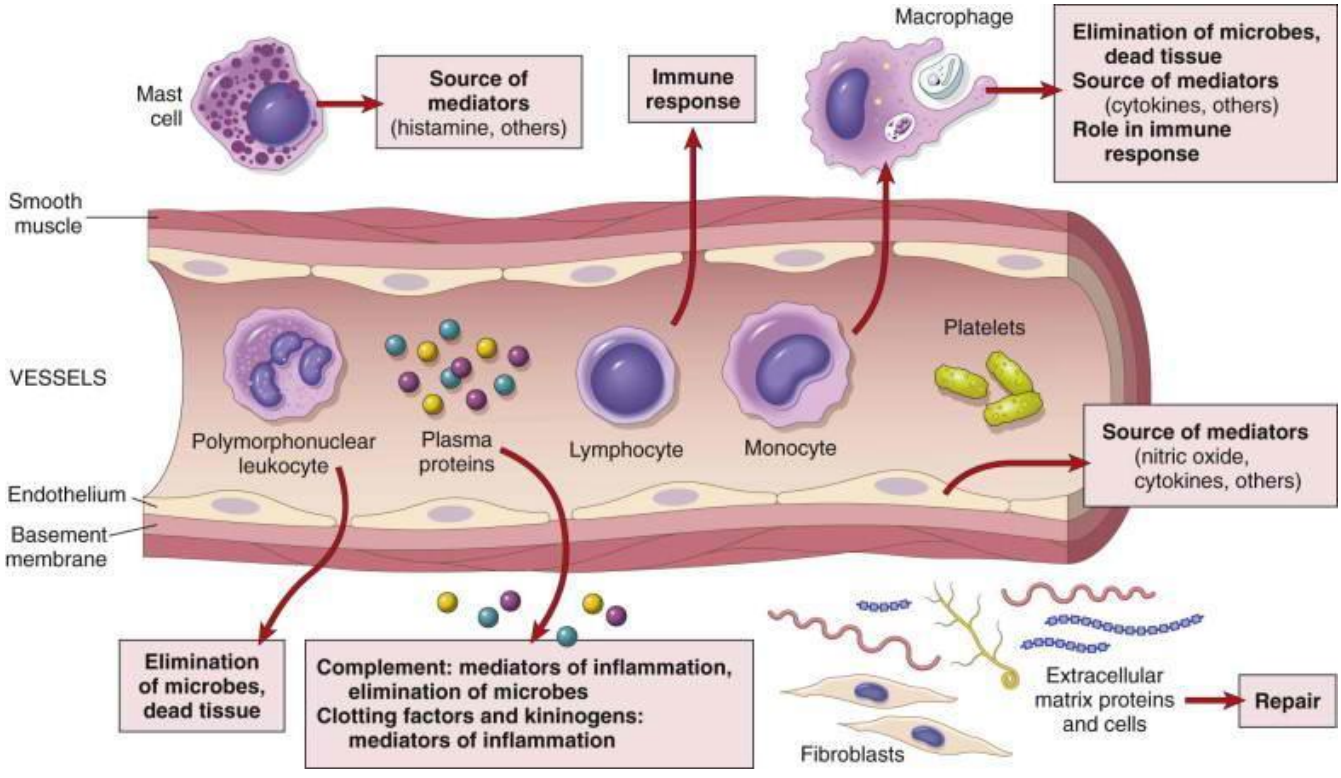

Figure 6: Components of the acute and chronic inflammation and their principal functions

(https://www.studyblue.com/notes/note/n/robbins-cha-3-/deck/11708138)

Acute inflammation

An acute inflammation starts a short time after the tissue is injured and occurs either as a physical damage or caused by an immune response. During the acute inflammation several important processes will appear. First the arterioles that supply blood to the damaged area, will dilate, which then will give increased blood flow. The capillaries in this area will become more permeable, so fluid and blood proteins can move into interstitial spaces (spaces between cells). The first line of defense will be activated, resulting is several reactions; The neutrophils and possibly some macrophages will migrate out of the capillaries and venules and into the interstitial spaces. The neutrophils, which are a type of white blood cells (granulocytes) contain enzymes that will digest microorganisms or toxicant substances. The macrophages have many of the same properties as the neutrophils [48].

When an inflammation has been triggered there are some signs to recognize the process. These are divided into the following categories, given in both latin and English; dolor (pain), calor (heat), rubor (redness), tumor (swelling) and functio laesa (immobility). The pain comes from the release of chemicals that stimulate nerve endings, while redness and heat appear because the capillaries are filled up with blood. Because of loss of some function, immobility may occur and swelling is caused by the accumulation of fluids. However, these signs are not that easy to detect when the inflammation occurs deep inside the body and further away from the skin [35, 42].

Chronic inflammation

When an inflammation has a long duration it is called a chronic inflammation. This kind of inflammation may have a delayed onset and can last for up to many years. The leading factors of a chronic inflammation is through different cytokines, chemokines and enzymes (see 1.5.4). The immune system is, during a chronic inflammation, Figurehting non-degradable pathogen, a viral infection or an autoimmune reaction, like rheumatoid arthritis or multiple sclerosis. Autoimmune is the term used when the immune system no longer can distinguish between healthy and damaged tissue. It attacks itself. Usually an acute inflammation mostly only involves neutrophils, but sometimes also eosinophil, whereas there are other major cell types involved in a chronic inflammation. We divide these cells into mononuclear cells, involving monocytes, macrophages, lymphocytes and plasma cells, and the fibroblast. The

mononuclear cells are responsible for the destruction or removal of the pathogen and because of these inflammatory cells there will also be an infiltration of these cells, and also tissue destruction. After the pathogen is destructed and removed, the fibroblasts are involved in repair of the damage [40, 48].

Besides an autoimmune disease there could be other reasons for why a chronic inflammation appears. It could be a persistent injury or infection like tuberculosis or a prolonged exposure to a toxic agent such as silica (nanoparticles in the lungs). Although, the same way a chronic inflammation can be a response of different diseases, it can also cause other diseases [51].

What starts as a healing-process, will lead to further inflammatory triggers, if not repaired properly. At a certain point, the inflammation will lead to altered cellular function and give defects in the healing process, which again can affect internal organs. This state of inflammation, has also been linked to mental and emotional imbalance, digestive disorder, skin problems, musculoskeletal condition and in worst cases, also cancer [42, 47].

When associated with the respiratory tract and inhalation, often a chronic inflammation can cause damages in the lung system[39, 52]. Structural damages [39]could be features to asthma. Asthma is a disease of long-term inflammation and is thought to be a genetic disease, and may occur as a response of environmental factors, such as air pollution and allergens (factors triggering an allergic inflammation) [39, 41].

1.5.4 Cytokines

During an inflammation the cells will secrete small proteins that mainly effect the communication and the interaction between cells. These proteins are called cytokines, and are divided into several group based on their origin. We have lymphokines, monokines, chemokinse and interleukins, respectively made by lymphocytes, monocytes, or by leukocytes. The cytokines may have autocrine, paracrine or endocrine actions, thus either acting on cells that secrete them, acting on nearby cells or cells distant from the origin cell. The cytokines are also pleiotropic, meaning similar functions can be stimulated by different cytokine. They can also act synergistically or antagonistically [45, 53].

These proteins are usually produced in a cascade reaction, where one cytokine stimulates a cell to make additional cytokines. The cells that make these cytokines are mostly the helper T cells (Th) and macrophages, but they can also be produced by mast cells, endothelial cells, epithelial cells, macrophages and Schwann cells during physiological and pathological processes or be synthesized in different tissue such as the spinal cord, the inflamed skin. Certain cytokine, such as IL-1α, IL-1β, IL-8, and TNF-α seem to initiate different inflammation processes [53].

During cell injury, infection, invasion and inflammation, **IL-1α and IL-1β** are released by monocytes and macrophages, but also by cells that are not part of the immune system, including fibroblasts, epithelial and endothelial cells. These two cytokines are from the interleukin-1 family and are also the most known cytokines from this family, based on well established studies. The studies around these two cytokines have confirmed that they are very potent in initiating a pro-inflammatory process. Both of these cytokines are synthetized as a precursor protein, which means that they have to be proteolytically cleaved for activation. Their site of action is through binding to the receptor molecule called type I IL-1 receptor (IL-1RI). The receptor binding will lead to recruitment of a co-receptor, which is necessary for signal transduction and activation of the chemokines (see below).

IL-1α seems to be stored in the cytoplasm of the cells as a precursor. They are affecting transcription in the nucleus, apart from interaction with a receptor and give receptor-mediated responses. They also stimulate monocytes to transcribe and secrete IL-1β. In this way IL-1α seems to be the initiator of an immune response, while **IL-β** is an amplifier of inflammation. IL-1β is synthesized via a dual pathway. Thus, synthesis of IL-1β precursor is mediated via stimulation of Toll-like receptors (TLRs) [54]. Notably, IL-1β precursor also needs to be cleaved before secretion (see below).

TNF-α, tumor necrosis factor alpha, is also one of the cytokines that make up the acute phase reaction of an inflammation. It is produced by activated macrophages, but can also be produced by other cells, such as mast cells and lymphocytes. This cytokine acts through several signaling pathways by binding to the TNF receptor 1 or 2, where TNFR1 (TNF receptor 1) is expressed in most tissues, while TNFR2 (TNF receptor 2) only are found in cells of the immune system. The receptor binding leads to regulation of apoptotic pathways, NF-kB activation of inflammation and activation of stress-activated protein kinases (SAPKs)

[55]. Studies have shown that dysregulation of this cytokine has effect on diseases like major depression, psoriasis, inflammation diseases and cancer [35, 42].

Activation of IL-1β through the inflammasome

IL-1β is one of the cytokines that is mostly associated with induction of pro-inflammatory responses. Studies have shown that this cytokine also plays a key role in activation of other types of inflammatory cytokines and chemokine, through activation of monocytes [41].

The activation of IL-1β consists of two signals, where the primary signals induce the expression of pro– IL-1β mediated by activation of toll-like-receptors (TLRs), while the second signals activate the NALP3 (or Nlrp3) inflammasome [56]. The NALP3, is an intracellular signaling complex composed of NALP3, procaspase-1, and the adaptor protein apoptosis-associated speck-like protein (ASC). When this complex is formed, it will lead to the proteolytic cleavage of procaspase-1 to the active form, caspase-1. This active form will then cleave pro- IL-1β and produce mature and active IL-1β, which when released extracellularly will bind in a paracrine manner and result in amplification of pro-inflammatory response. This process of activation of the IL-1β seems to be cell-type specific.

For some cells, such as blood monocytes, only the TLR stimulation is required for activation of the NALP3 inflammasome. This is because these types of cells have constitutively active NALP3 inflammasome. The same principal seems to be for dendritic cells [54, 56].

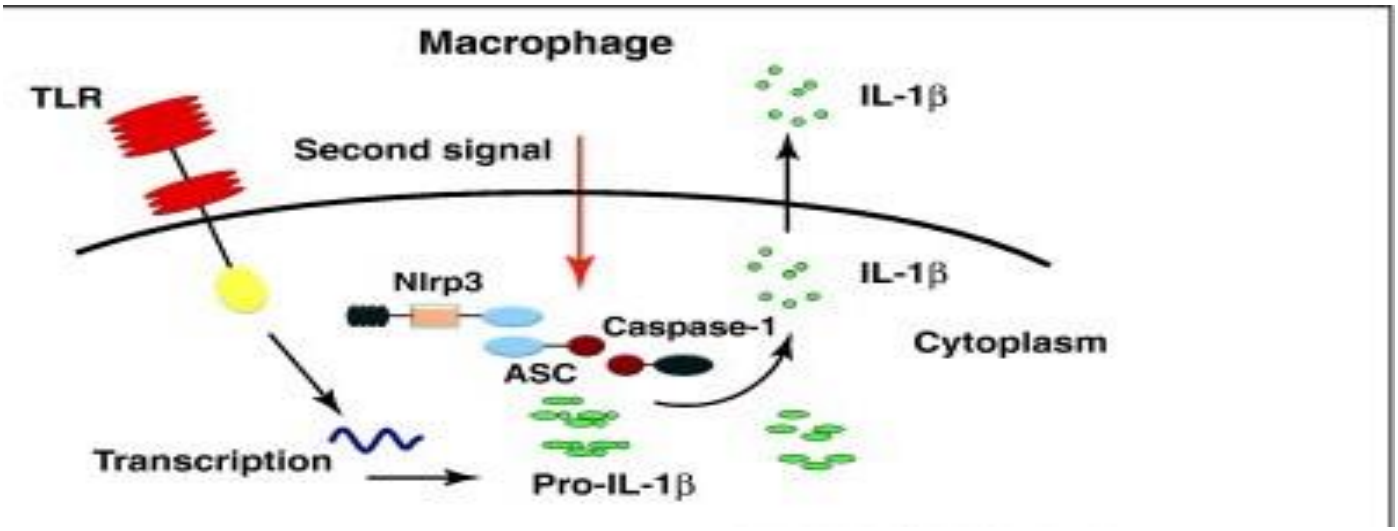

Figure 7: Inflammasome induced activation of the IL-1β through TLR

(http://www.cell.com/cms/attachment/610240/4878246/gr2.jpg)

Chemokines

A type of cytokines is called chemokines [41]. These are small cell-signaling molecules and are secreted to induce chemotaxis of nearby cells. Chemotaxis occurs as a response to nearby chemicals and is basically a process involving movement of a cell. A chemotaxis process can be elicited by a microbe or any other pathogen, that will when acting on epithelial cells, endothelia cells or macrophages trigger the production of mediators that will direct immune cells towards the site of injury and start an immune response [45].

The chemokines are small molecules with a weight not more than 10 kDa. They all consist of 4 cysteine residues, and thave herefore a three dimensional shape. Some of them are considered pro-inflammatory, while others have a homeostatic role, controlling the cells to sustain normal tissue growth and maintenance. Chemokines act through the interaction with a chemokine receptor, G protein-coupled receptor (GPCR) [41], which are transmembrane receptors coupled to intracellular G-proteins. These receptors are involved in the stimulation of signal transduction pathways inside the cells. The chemokines are found on the surface of

leucocytes and immune cells. There are so far 19 types of them. One of these is the IL-8, which has been examined in this study.

IL-8, interleukin 8, is a chemokine produced mainly by epithelial cells and macrophages, but also by endothelial cells and airway smooth muscle cells. The major cleaved form of the precursor is secreted by macrophages. The secretion is due to interaction with a toll-like receptor. When secreted, IL-8 bind to several surface receptors, such as G-protein-coupled serpentine receptors (CXCR1 and 2) and are thereby involved in mediation of the innate immune responses. IL-8 is also known as neutrophil chemotactic factor, because of the ability to induce chemotaxis in targeted cells, like neutrophils and granulocytes. When these cells have migrated to the site of infection, the IL-8 induces phagocytosis, by e.g. increasing the level of intracellular Ca^{2+}. IL-8 also is believed to play role in the pathogenesis of inflammation in the bronchioles [45, 53].

1.6 Lung Model systems

Different model systems are used for *in-vivo* and *in-vitro* studies [57]. A model system is based on a particular species of animal, which has been developed through many years of study and is therefore experimentally powerful to answer particular hypothesises. The models are quantified and modified for the specific study. A model system should reflect the organism which is of relevance for assessing the hazard to the chemical. In this study the organism of interest is human beings and their risk of exposure to air pollution by inhalation, more specific nanoparticle exposure.

A model system which is used in this thesis is macrophages, which are known to be present in the respiratory tract. A monocyte cell line (THP-1 cells) from humans can be differentiated into macrophages [58]. These THP-1 cells resemble monocytes, except for the fact that they lack surface and cytoplasmic immunoglobulins. They also grow in suspensions and do not adhere to the glass or plastic surface. They will, however, attach upon treatment with phorbol esters like phorbol 12-myristate 13-acetate (PMA) and then mature into cells with functional characteristics of mature macrophages [58].

Another respiratory cell model system is the epithelial cell line (BEAS-2B), which has been used in the research of nanoparticles in our laboratory. This cell line is a suitable transfection host. The cells are attached to the well surface and have the ability to undergo squamous differentiation and can be used to screen chemical and biological agents. Previous studies have shown significant response of cytotoxicity when exposed to silica nanoparticles. The BEAS-2B cell line have similar function as human epithelial cells and where therefor used for parts of our study [59].

Comparing the two cell lines describes above and studying interactions between them, gives a model system which can be described as a co-culture. Such model system mimics more the lung's complex mixture of various cells *in vivo*. This way, we will have a better understanding of how particles exert their effects in the respiratory tract. Therefore, compared to a single-cell system, a co-culture might be more important for the development of better in vitro studies when it comes to risk assessment.

1.7 Silica nanoparticles and inflammatory responses

The use of amorphous silica nanoparticles has increased in a range of product, such as in drugs, cosmetics, food and most importantly in biomedicine and biotechnological application (e.g. transplantations, cancer therapy and drug delivery). The main reason for the tremendous use is, first of all, their abilities, which makes it easier to handle and secondly, these nanoparticles seem to be safer or have less chronic effects compared to larger-sized crystallic particles. However, when exposed to higher doses of silica nanoparticles over time, it can cause epithelial damage, which can lead to a chronic inflammation. Over time the SiNPSSs may be involved in the development of asthma, but this has to be further assets. A condition of a chronic inflammation can further on develop to cancer. Therefore, it is until now concluded that the effects of these nanoparticles appear to be transient and dependent on their structure and concentration. Although, compared to crystalline micro-sized particles, which have been well studied, these nano-sized amorphous particles have shown to be less harmful. Nevertheless, the knowledge of their toxicity is limited and therefore it is risky to eliminate the possibility of toxic effects of amorphous silica nanoparticles [2, 19].

Amorphous silica nanoparticles have been shown to induce an inflammatory response, through the stimulation and release of different pro-inflammatory cytokines, IL-8, IL-1β and TNF-α [5, 13]. This stimulation of cytokine has been studied in several previous cell cultures and thereby confirmed that the amorphous silica nanoparticles have the potential to induce marked pro-inflammatory cytokine responses. Along with the cytokine release, previous studies have also demonstrated the potential of amorphous silica nanoparticles with size of 10 nm, 12 nm and 50 nm to induce cytotoxicity and pro-inflammatory response in human bronchial epithelial cells (BEAS-2B) [49, 55, 60].

2 Aims

The purpose of the study is to gain better understanding of the pro-inflammatory and cytotoxic response of silica nanoparticles in human macrophages (differentiated THP-1 cells) and also to find out more about the potency of different particles in this cell model. The pro-inflammatory and cytotoxic effects of the SiNPs of 10 nm (Si10) and 12 nm (Si12) were compared to a larger particle with a size of 500 nm (Si500), in the differentiated THP-1 cells. Furthermore, the cytotoxicity and cytokine release of human bronchial epithelial cells (BEAS-2B) were measured after exposure to transferred conditioned medium from Si10 exposed differentiated THP-1 cells. In order to implement this study, we raise the following questions:

- What are the most suitable exposure conditions in which the SiNPSSs induce cytokine responses, without giving too much cytotoxicity?

- When comparing amorphous silica particles of different sizes, Si10, Si12 and Si500, with respect to cytotoxicity and cytokine responses, what is the potency of each particle size?

- What is the time-course relationship of cytokine release from differentiated THP-1 cells after exposure to SiNPs?

- What is the relationship between cytokine release and cytokine gene expression for differentiated THP-1 cells exposed to SiNPs?

- To what degree does exposure of differentiated THP-1 cells to SiNPSSs release mediators that affect BEAS-2B cells, regarding cytotoxicity and cytokine release?

- To what extent do BEAS-2B cells respond differently than differentiated THP-1 cells to SiNPSSs?

3 Materials and methods

3.1 Materials

3.1.1 Silica Nanoparticles

Two different commercially produced amorphous silica nanoparticles and one micro-sized silicaparticle were used in this study. Particles used in this study were:

Si10	Amorphous silica nanoparticle, 10 nm from Kisker Biotech
Si12	Amorphous silica nanoparticle, 12 nm from Sigma-Aldrich
Si500	Amorphous silica micro particle, 500 nm from Kisker Biotech

The particle properties for Si10, Si12 and Si500 were characterized by Skuland and Pham et al and by ‘Anna Yu Godymchuk by analyzing the particle working solution of the particles (as prepared according to section 3.3.1), with respect to zeta-potentials [49, 60, 61]. Table 1 summarizes the measured values for amorphous silica nanoparticles and microparticle with nominal seizes of 10 nm, 12 nm and 500 nm.

Table 1: Characterizations of the particle properties for Si10, Si12 and Si500

Particle characterization	Si10	Si12	Si500
Surface area, m^2/g (BET data)	243.607	121.297	9
Average particle size, nm (TEM data)	10.8±1.6	15.8±1.2	369±20
Average particle size, nm (BET data)	9.3	18.6	546
Particle shape (TEM data)	Spherical	spherical	Spherical
Element composition, Si : O wt.%	47.9 : 52.1	36.6 : 63.4	
Zeta potential in water	-41,6 mV	-39.3	-38,7 mV

Table 1 summarizes the measured hydrodynamic size and zeta -potential for amorphous silica particles with a nominal size of 10 nm, 12 nm and 500 nm. The table is based on data from Skuland et al, and Godymchuk (personal communication).

3.1.2 Cell lines

In this study, THP-1 cells (monocytes) and BEAS-2B cells (epithelial), cell line were used. The cells were bought from European collection of cell culture (ECACC) in Salisbury, United Kingdom, while the medium (RPMI and LHC-9) was bought from life Technologies (a Gibco Thermo Fisher brand) in Grand Island, USA

3.1.3 Materials used in the study

See Appendix 1.

3.1.4 Solutions used in the study

See Appendix 2.

3.2 The principals

3.2.1 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay or also known as ELISA is an assay used to detect and quantify substances such as peptides, proteins, antibodies and hormones. ELISA can also have other names such as enzyme immunoassay (EIA). The principle of the ELISA method is that an antigen must be immobilized to a solid surface and then complexed with an antibody. The complex will then, through enzyme activity, produce a measurable product. The main step of the assay is the specific antibody-antigen interaction.

This method is mainly divided into direct and indirect ELISA strategies. The immobilization of the antigen in the sample can either be accomplished by direct adsorption to the assay plate or indirectly via a capture antibody that has been attached to the plate. The following detection will then also be direct or indirect, respectively through labeled primary antibody or labeled secondary antibody. A third ELISA method is the “sandwich” assay, which includes two primary antibodies, mainly the capture antibody and the detection antibody. In this assay,

the sample is bound between the two antibodies. This format is used because it is sensitive and robust. The direct detection method is quick, because there are fewer steps involved, but has minimal signal amplification, can be expensive and has no flexibility in choice of primary antibody labeled from one experiment to another. The indirect method is more commonly used because of increased sensitivity, maximum immunoreactivity is retained and many primary antibodies can be used. At the same, time cross-reactivity can occur and give nonspecific signals and also an extra incubation steps are required.

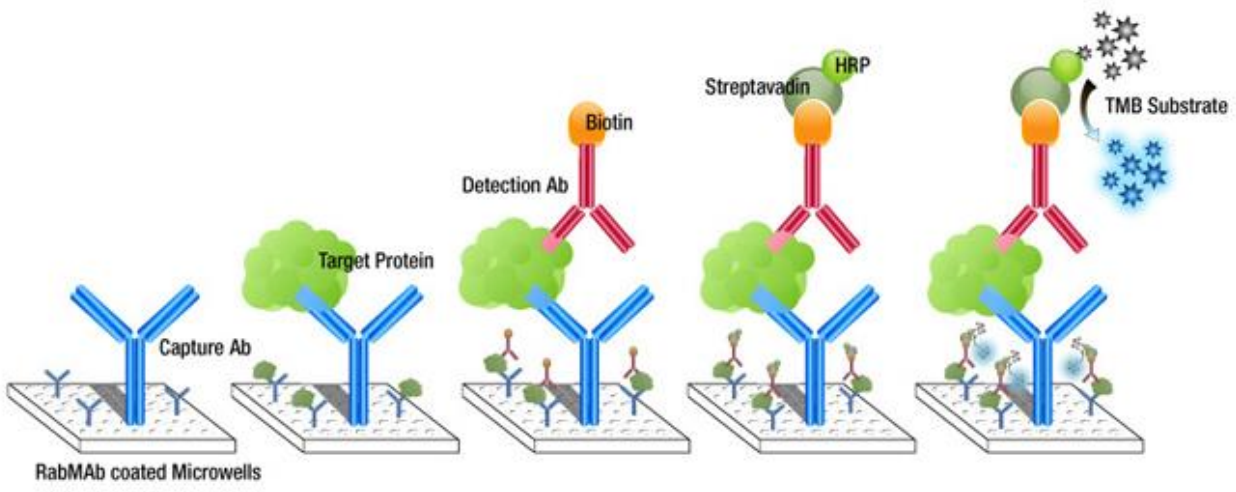

Figure 8: Common ELISA formats

(http://www.epitomics.com/images/products/sandwich.jpg)

Usually ELISA is performed in 96-well plate made of polystyrene. This kind of plate is positively charged and can therefore bind antibodies and proteins. For the sandwich ELISA, the bottom of each well is first coated with -an antibody for the protein of interest (coating antibody). This antibody will bind to the antigen (IL-8 etc.) in the sample. Simultaneously with the sample, a detection antibody is added. The detection antibody will bind to another epitope on the antigen. After incubation with sample and detection antibody, a buffer is used to rinse the plate for weakly adherent antibodies, according to the kit. There are also enzymes involved in this procedure, and one can use a wide range of enzymes, such as β-galactosidase,

acetylcholinesterase and catalase, but most of them have limited substrate options and therefor the most commonly used enzyme labels is horseradish peroxidase (HRP) and alkaline phosphates (AP). This is again dependent on what kind of assay sensitivity that is required and the instrument that is used (spectrophotometer, fluorimeter or luminometer). The chosen enzyme in our assay is HRP which will bind to the secondary antibody, and can metabolize chromogens into colored products. The enzyme solution is added after incubation with sample/detection antibody. The last step is the production of the colored products where streptavidin-HRP (horseradish peroxidase) oxidizes the chromogenic substrate tetramethylbenzidine (TMB), which will transform the colorless substrate blue. The blue color is dependent on the amount of the HRP bound to the detection antibody. As a last step, a stop solution of sulfuric acid is added. This will again change the color from blue to yellow and we can measure the color intensityby a spectrophotometer. The color produced is proportional to the amount of antigen in the samples and therefore the concentration of the unknown samples can be determined via a standard curve.

3.2.2 Colorimetric lactate dehydrogenase (LDH) assay

Lactat dehydrogenase (LDH) is an assay which measures the activity of the enzyme called lactate dehydrogenase. This enzyme catalyzes the conversion of pyruvate to lactate. After tissue damage or red blood cell hemolysis, the cells release LDH, which again makes this assay well established for analysis of toxicity of tissues and cells. In the Kit, the LDH reduces NAD^+ to NADH and H^+. This is followed by NADH reducing a yellow tetrazolium salt (INT) to a red formazan product, which then is detected by colorimetric (450 nm) assay. The assay is performed on a micotiter plate.

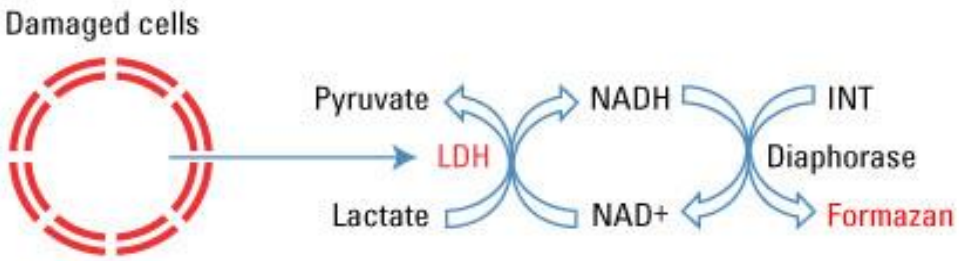

Figure 9: ***The principal of LDH assay***

(https://www.lifetechnologies.com/order/catalog/product/88953)

The amount of produced formazan is directly proportional to the amount of released LDH in the medium; therefor this method is a good indicator of possible membrane damage and toxicity of tissues or cells [62].

3.2.3 AlamarBlue

AlamarBlue is a cell viability assay that contains resazurin, which is a cell permeable, non-toxic and weakly fluorescent blue indicator dye. This assay quantitatively measures mitochondrial function in human, animal, bacterial, fungal and mycobacterial cells. It is used for in vitro cytotoxicity determination and cell growth monitoring.

The resazurin is a redox indicator (oxidation-reduction reaction), which will undergo colorimetric change to resorufin, in response to cellular metabolic reduction. Resorufin has a pink color and is highly fluorescent. As the number of living cell decrease, the intensity of the fluorescence also decreases.

The assay is ideally based on detecting the oxidation of the electron transport chain. The assay is very simple, the AlamarBlue solution can be added directly into the cell-culture, and the results can either be measured spectrophotometric or by fluorescence directly in the culture after 1 h.

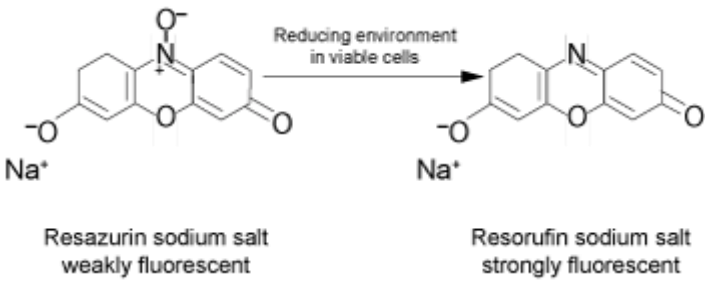

Figure 10: The principal of AlamarBlue assay

(http://www.bmglabtech.com/media/35707/1061886.gif)

3.2.4 Real time PCR

Real time PCR, standing for polymerase chain reaction, is a technique used to quantify amounts of cDNA or RNA. It can also be used to monitor the progress of PCR. The method is based on detecting fluorescence produced as the reaction proceeds. The fluorescence is caused by accumulation of the PCR product during the amplifications. The assay has high sensitivity and is very specific and therefore used a lot.

The amount of gene expression in a target cell is measured by the number of mRNA transcript. The first step of a real time PCR process when measuring gene expression is the cDNA synthesis where the enzyme reverse-transcriptase transcribes mRNA to cDNA. The cDNAs are then mixed with a mastermix made prior to the PCR assay. A mastermix consists of forward and reverse primers, a fluorogenic probe, buffer, a thermo-stable DNA polymerase, dNTPs and water. This cDNA is then used as a template during the PCR process. The fluorogenic probe (like TaqMan) was used for the detection of the specific PCR product as it accumulates during PCR cycling. Using Real time PCR makes it possible to measure the generation of amplified products at each PCR cycle, compared to standard PCR where the product is detected at the end. The relative quantification is based on the Ct value for each sample normalized against a housekeeping gene. The Ct value is the cycle threshold which describes the number of cycles required for the fluorescent signal to cross the threshold (see Figure 11). This is determined by the amount of material at the starting point. The more material at the start, the faster the fluorescence signal crosses the threshold. In other words, when the Ct value is low, it means that there is a greater amount of target nucleic acid in the sample than in another sampler were the Ct value is higher.

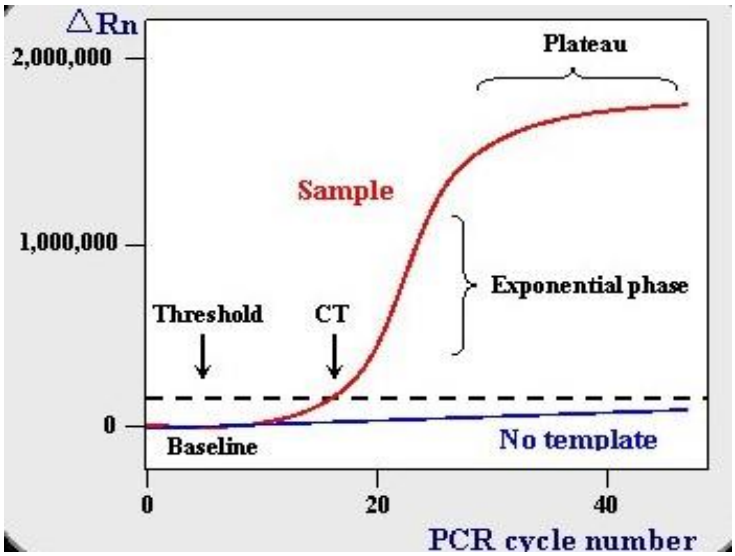

Figure 11: Model of a Real time PCR plot

(http://www.ncbi.nlm.nih.gov/genom/probe/doc/techQPCR.shtml)

A standard PCR process consists of a cycling process divided into 3 steps. The cycling process is often 30 cycles (Figure 10):

Step 1: Denaturation of cDNA at 95 ºC, where hydrogen bonds between complementary strands and single -stranded molecules are interfered with

Step 2: Annealing of primers, for complementary binding of the primers to the targeted sequencing (often at 50 degrees)

Step 3: DNA synthesis (at 74 degrees) mediated by DNA polymerase

The annealing and DNA synthesis are merged into one single step in real time PCR and the annealing and DNA extension occurs at approximately -60 ºC, depend on the primer and probes compositions.

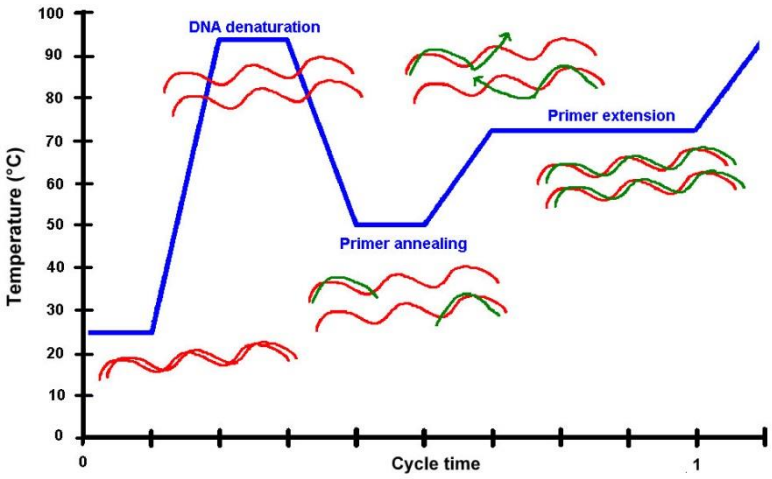

Figure 12: Thermal cycles during a PCR process

(http://biosistemika.com/workshop/qprc-basics/)

It is also important to remember that the Real time PCR method can be performed differently, but that the method used is in focus, in this thesis. Real time PCR can be divided in two based on the molecule used for detection. In each method, the probes will degrade and thereby release fluorophore, a product which then is used for fluorescence detection. The detected fluorescence of fluorophore is directly proportional to the amount of DNA template present in the PCR.

3.3 Methods: The Procedures

3.3.1 Preparation of the particle solution

Stock solutions of the Si10 and Si500 (25 mg/ml) particles were dispersed in sterile water to 2.3 mg/ml and sonicated for approximately 2 min on ice (until specific ultrasound energy of 420J was given to the nanoparticles). Bovine serum albumin (BSA, final concentration 0.15%) and phosphate buffed saline (PBS, final dilution 1x) were then added to the particle solution, according to the method by Bihari and co-workers [63]. This gave a "particle working solution" with a final particle concentration of 2 mg/ml

The "particle working solution" of Si12 was made by dissolving the nanoparticle to a concentration of 2.0 mg/ml in 0.15% BSA/PBS. The solution was then mixed well and sonicated for 19 min on ice-water on 50% amplitude, the given energy to this particle was 7056 J, which is according to Nanoregs recommendations (referance- personal comminications with skuland).

3.3.2 Cell culture

For this in vitro study a THP-1 cell line (monocytes), differentiated to cells characteristic of macrophages, and BEAS-2B cells (epithelial) were used as model systems. The THP- cells were cultured in RPMI-1640 culture medium with L-glutamine, supplemented with 10% heat inactivated foetal bovine serum (FBS) (10 mM), 1 mM sodium pyruvate and 0.1% gentamycine as described in Danielsen et al., (2009). The BEAS-2B cell, were on the other hand, cultured in a serum-free medium, supplied with gentamycine (see appendix for full overview).

The THP-1 cells were treated with phorbol 12-myristate 13-acetat (PMA), 48 h in advance of the experiments. From a stock solution of PMA (50 ng/ml). The PMA is responsible for the maturing of the THP-1 cells into cells with functional characteristic of mature macrophages.

BEAS-2B cell were maintained in LHC-9 medium in collagen-coated flasks, in a humified atmosphere, at 370 C with 5% CO2. The medium was refreshed every second day. Two days

in advance of the exposures, BEAS-2B cells were plated on 6-well culture plates, in LHC-9 medium. The day before the exposure, the cell culture medium was changed to RPMI-1640

For the detailed procedure culturing THP-1 and BEAS-2B cell see protocols.

3.3.3 Exposing of cells for analysis of cytotoxicity, cytokine release and gene expression.

The differentiated THP-1 cells were exposed to different concentrations of Si10, Si12 and Si500 (0-100 µl /ml) up to 6 h in RPMI-1640 without FBS. In some experiments only and Si10, the cells were exposed in RPMI-1640 with FBS, without FBS and with addition of FBS after 15 min of exposure.

When investigating the effect of transfer conditioned medium to unexposed cells (differentiated THP-1 and BEAS-2B), the differentiated THP-1 cells were exposed for 6 h, before the transfer of conditioned medium to either differentiated THP-1 or BEAS-2B cells, followed by another incubation for a total of 20 h.

To exclude impact of other factor, three other control were measured simultaneously. The controls included Si10 exposure of differentiated THP-1 cells for 6 h, centrifuged Si10 added medium with no cell contact, differentiated THP-1 cells exposed to Si10, for 20 h and BEAS-2B cells exposed to Si10, for 20 h. The Si10 exposure of differentiated THP-1 cells for 6 h, was measured for comparing with a possible significant increase of the cytokine release, after transfer of conditioned medium. The centrifuged Si10 added medium with no cell contact, was to make sure that any possible significance was not caused by Si10, rather then the transfer of conditioned medium. The latter control, which included exposure of differentiated THP-1 cells and BEAS-2B cells for 20 h, was measured for comparing of any possible cytotoxic or cytokine release after 20 h.

Harvesting cells to cytokine, LDH, AlamarBlue and mRNA analysis.

The cell culture medium was harvested for cytokine and LDH release after 6 or 20 h of particle exposure. The remaining cells were incubated with newly added medium with AlamarBlue (100 µl/ml) for 1 h, before detection of viability. The cell culture media were centrifuged at 300 g for 10 min to remove dead cells and thereafter at 8000 g for 10 min to remove particles. For the investigation of mRNA expression, the cell culture plates were put directly on ice, and then washed three times with Dulbeccos PBS before transferring to -70^{o}C for storing until further analysis.

When measuring the cytokine release/cytotoxicity over time the medium was harvested at different times points (0, 5-1, 5-3-4, 5-6 h), while the cells were frozen at -20^{o}C until for further analyzing (Figure13). -

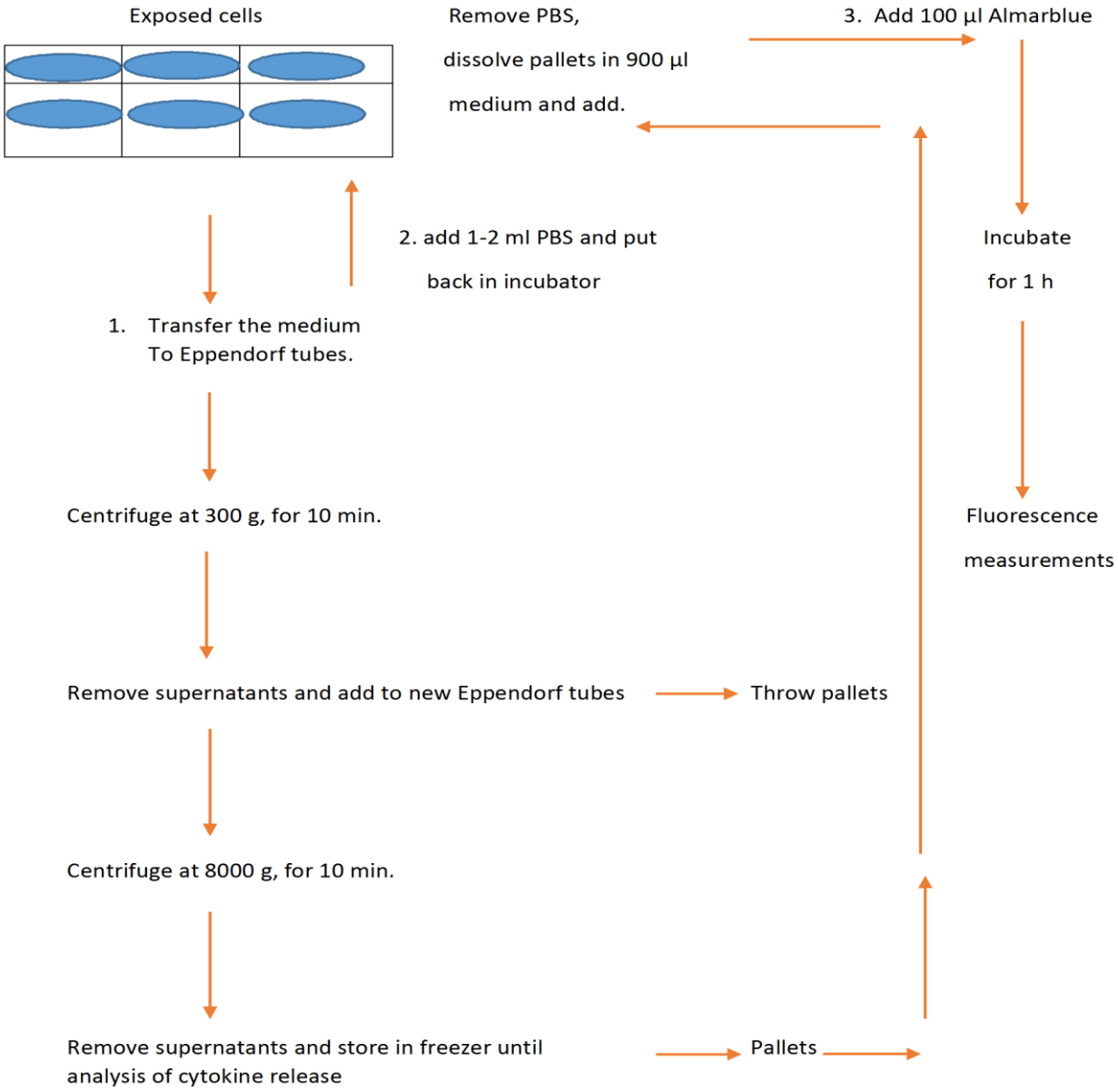

Figure 13: Schematic illustration of the harvesting process. *After each exposure experiment, the cells were harvested and thereby prepared for further analyzes of cytotoxicity and cytokine release.*

3.3.4 Sandwich ELISA

A 96-well microplate was pre-coated with a capture antibody solution for the IL-1α, IL-1β, IL-8 and TNF-α, one day before the analysis. The plates were then washed with a washing buffer Dulbeccos PBS med Tween and then treated with a blocking solution for 1 h at room temperature. After blocking, duplicates of the standard solution and the supernatants (sometimes also diluted) were added to the plates. The plates were also added a solution of detection antibody and then incubated for 2 h, in a shaking device. When the incubation was done, the plates were washed 4 times with the washing buffer to remove unattached antibodies and proteins, and then incubated with a Streptavidine-HRP solution for 30 min, while shaking. The plates were washed again and incubated with a mixture of 3, 3', 5, 5' - tetramethylbenzidine (TMB), citrate buffer and 30% H_2O_2. When the color development occurred, the stopping solution, 5% H_2SO_4, was added and the absorbance was measured by a spectrophotometer at 450 nm (Sunrise Absorbance Reader, TECAN) (Figure 13).

The IL-1β due set had some distinguished steps from the above. First the samples and the standard solution were incubated alone for 2 h and then washed with the washing buffer, before adding the detection antibody and incubation for further 2 h. The Streptavidine-HRP was then added to the plates and incubated for 20 min before the TMB was added. The rest of the procedure is similar to the procedure above.

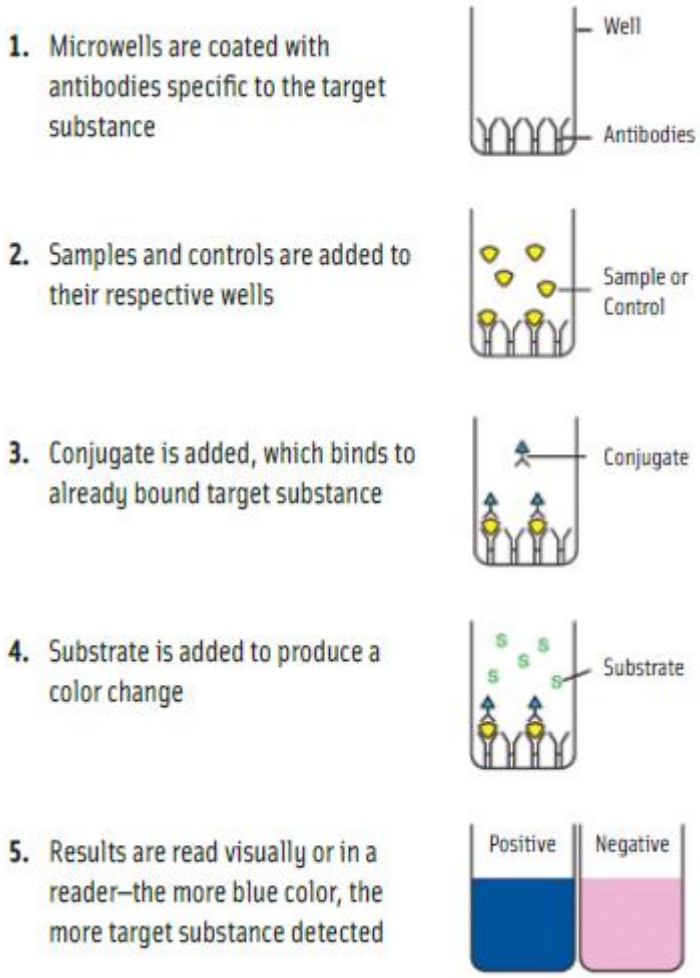

Figure 14: A simple illustration of the procedure of ELISA

(http://www.ift.org/~/media/food%20technology/column%20images/2011/02/food%20safety%20and%20quality/test.jpg)

3.3.5 AlamarBlue assay

After exposure for 6 h and harvesting, the remaining cells attached to the plates were used for determining whether the silica nanoparticles induced reduction in the cell viability. The cells were incubated for 1 h with 900 µl of medium and 100 µl of the AlamarBlue dye. With these also a blank well containing only medium and AlamarBlue, was incubated. 100 µl of each sample and the blank was then added to a 96-well plate in duplicates. The fluorescence was measured by in a Monochromator Multimode Microplate Reader; CLARIOstar (from BMG LABTECH)

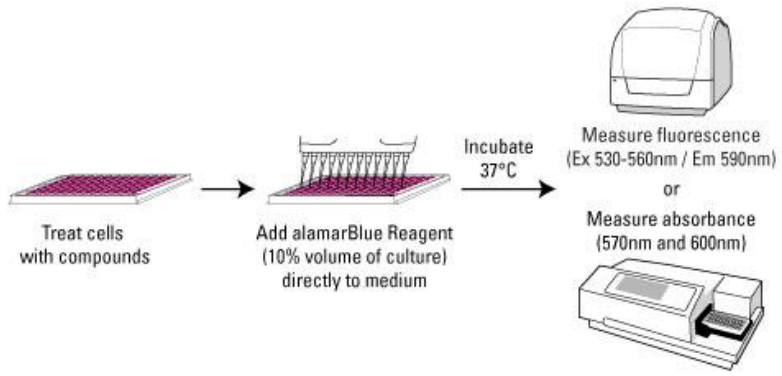

Figure 15: The steps in the AlamarBlue assay

(https://tools.thermofisher.com/content/sfs/gallery/high/88951-003-alamarBlue.jpg)

After detection the percentage of cytotoxicity was calculated with the following formula:|

Real Fluorescence= fluorescence of the sample- fluorescence of blank

Viability (%) = (Real Fluorescence/fluorescence control) *100

3.3.6 The LDH assay

The amount of LDH in the cell supernatants was quantified according to the supplier's recommendations with a LDH assay detection kit (Roche). In order to relate the amount of LDH to maximum cytotoxicity (% necrosis), two wells with THP-1 cells were exposed to 1% Triton x-100 in medium for 5-30 min to give 100% necrosis. This was a reference sample and cytotoxicity was confirmed under microscopic investigation, where it was clear that the cells lysed and the content floated around in the media. The sample was then centrifuged at 300 g for 10 min and the supernatant was diluted for analysis.

100 µl of the reference and samples were added to a 96-well plate, followed by 100 µl reaction mixture of catalyst and dye solution. The plate was then incubated in the dark for 5-30 min, until a color development appeared and the absorbance was measured at 490 nm by the Magellan V 1.10 software (TECAN Sunrise Absorbance Reader).

The percentage of the cytotoxicity was calculated with the following formula:

$$Cytotoxicity\ (\%) = \frac{\mathrm{ABSsample-ABSblank}}{\mathrm{ABSmaxCtr-ABSblank}}$$

3.3.7 Real time Polymerase Chain Reaction (PCR)

RNA Isolation

Using a RNA isolation kit (PerfectPure), cellular RNA was isolated from the cells. Lysis buffer (200 µl) was added to the cells for 5 min, before the wells were scraped for cells. Further on a cannula with 26 G was used to cleave the RNA and DNA molecules so that viscosity in the sample was reduced. Subsequently, the cell lysates were added to purification columns and washed with wash 1 solution (see appendix 2). Thereafter the columns were treated with DNase for 15 min to destroy bound DNA. This was followed by the column filters washed with DNase wash and the Wash 2 solution. Finally, the RNA was eluted with

an elution buffer for 1 min. Between each step the column filters were centrifuged at 8000 g for 1-2 min and the filters were added to new columns, before adding a new solution. Approximately 2 µl of each sample were used to determine the purity and the concentration of RNA on a micro spectrophotometer (DS-11), while the rest of the samples were stored in the freezer at -70^0C.

cDNA synthesis

Before the detection and quantification of the targeted mRNA in a Real Time PCR process, the reverse -transcriptase was used to transcribe mRNAs to cDNA. 1000 ng of RNA was distributed to 0.2 ml thin walled tubes (PCR tubes). The mixture of reverse transcriptase, random primers, dNTP mix, buffer and RNase free water was added to the RNA. In addition to the samples, a tube without reverse transcriptase (called NRC) and a tube without RNA (called NTC) were also made. These two additional tubes are controls, respectively to maneuver the presence of RNA and DNA contaminants in the reagents (NTC) and the contaminants from DNA in the RNA (NRC). The cDNA synthesis was performed in a gene Amp PCR System 2400 machine (Perkin Elmer) at 37ºC for 2 h. After the synthesis the cDNAs were stored in the refrigerator overnight or in the freezer for a longer time.

REAL TIME PCR

For the analyses of IL-1β, IL-8 TNF-α and ACTB mRNA, the cDNA solution was first diluted 1:9 with RNase free water. The cDNA was then mixed with a master mix containing of TaqMan universal master mix and TaqMan primers/probes for the gene of interest; cDNA in an optical Fast Real Time PCR 96 well plate, covered with an optical adhesive cover. Before the Real Time PCR the plate was centrifuged at 3000 g for 1 min. The Real Time PCR were performed in a 7500 Fast System Real Time PCR machine (Apllied Biosystems) with a standard 7500 PCR run mode with 1min extension at 60ºC. The analysis was performed in a 7500 Fast System SDS software.

The expression of each gene of interest (GOI) in each sample was normalized against house-keeping genes (ACTB) and expressed as fold change compared to the untreated control as calculated by the ΔΔCt-method:

$\Delta Ct = Ct(GOI) - Ct(\beta\text{-actin})$

$\Delta\Delta Ct = \Delta Ct(treated) - \Delta Ct(control)$

$Fold\ change = 2(-\Delta\Delta Ct)$

3.4 Statistical considerations

For our study, we used the software GraphPad prism (version 5 for windows) for the statistical analysis. We had at least 3 independent experiment and the data for these are given as mean $\pm$ standard error of the mean (SEM). We assumed that the datasets were normally distributed and therefore we choose parametric test for our experiment. We also had experiment where different groups were compared. To see if there was a significant difference between these datasets, we used one-way or two-way analysis of variance (ANOVA) with Dunnet's or Turkey's multiple comparison post-test. For statistically significant experiment we use a P-value < 0. 05 as an indicator. This value confirms that there was a 95% chance for significant difference between compared groups. The log transformed data was used to confirm a normal distribution for larger variation in the datasets.

4 Results

4.1 Importance of various exposure conditions for cytokine release and cytotoxicity in differentiated THP-1 cells.

Different culture media can affect the activity and responses of the cells [49, 64]. Addition of FBS and proteins, as BSA, to the medium can affect nanoparticles characteristics and impact, giving the nanoparticles a protein corona [26, 28, 64]. Thus, to find a medium condition in which the cells respond maximally to Si10, the cells were exposed to different concentrations (0-100 μg/ml) of Si10 for 6 h, in medium with (10%), without and with FBS added 15 min after Si10 exposure. The viability was observed in the microscope and measured by two different cytotoxicity kits (AlamarBlue and LDH). The cytokine release of IL-1β, TNF-α and IL-8 were evaluated, using the ELISA method.

Cytotoxicity

When comparing Si10-induced cytotoxicity using the AlamarBlue method, the viability was reduced with approximately 40% in medium without FBS, at the two highest concentrations, while in the cells exposed in medium with FBS or with FBS added after 15 min the viability was only reduced with up to 20%, after 6 h exposure. The viability for cell exposed in medium without FBS was significantly reduced, compared to both the control and the cells exposed to medium with FBS. When using the LDH-assay a similar pattern was observed, but the reduction in viability without FBS seemed to be less, compared to the AlamarBlue method (Figure 16 A and B).

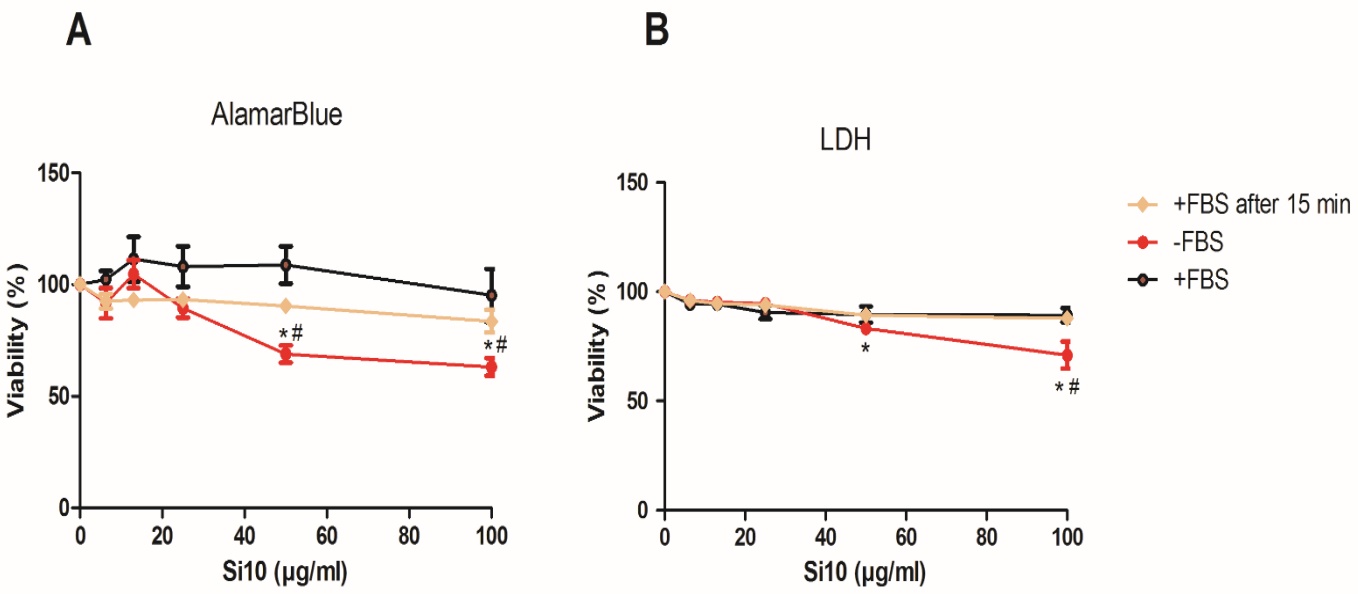

Figure 16: Viability of differentiated THP-1 cells in various exposure conditions after Si10 exposure. *Viability of the cells was measured after exposing the differentiated THP-1 cells to Si10 (0-100 µg/ml) in various exposure conditions (+FBS, -FBS and +FBS after 15 min). Following the total exposure time of 6 h, the measurements were done by AlamarBlue (A) or by LDH (B). The data are presented as mean values ± SEM of five experiments. * Significant difference relative to control, $p < 0.05$, # significant difference relative to the incubation with (+) FBS added, $p < 0.05$, for two-way ANOVA with Dunnett's Multiple comparison test.*

Microscopic evaluations

To determine any possible phenotypical change of the cells following exposure, microscopic evaluations were made throughout the study. The structural changes of the cells and also the density in each well were studied before and after exposure to Si10, with different concentrations (0-100 µg/ml). The density was simply analyzed through microscope analysis.

Figure 17 shows the change in the differentiated THP-1 culture, following exposure to Si10 (100 µg/ml) for 6 h. The cells were exposed to Si10 in the three different media, with FBS, without FBS and with FBS added after 15 min of exposure. In all three media, the apparent picture of the cell cultures was changed after exposure. Many of the cells were round and

detached, but there were an apparent number of flat and elongated cells that survived. When comparing all three media conditions, it is clear that in the plates with FBS (B and D), the cells were less detached than the plate without FBS (C).

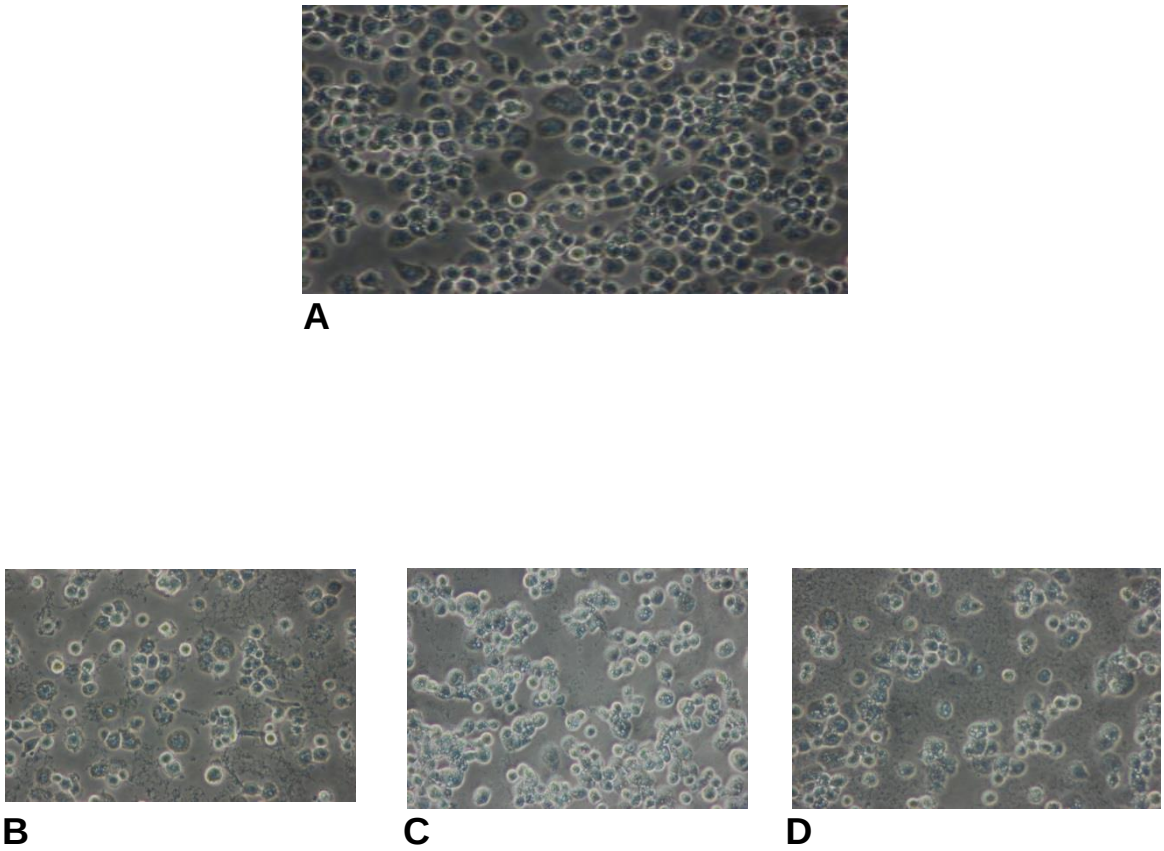

Figure 17: Microscopic pictures of the differentiated THP-1 cells, before and after exposure to Si10 (100 µg/ml), in different media. *The cells were plated on 6-well plates in RPMI-1640 with 10% FBS, for 2 days. They were exposed to Si10 for 6 h in three different media, with FBS, without FBS and with FBS added after 15 min of exposure. Microscopic pictures were taken, right before exposure (A) and 6 h after exposure for Si10, with FBS (B), without FBS (C) and FBS added after 15 min (D).*

Cytokine release

The possible induced release of cytokines, following Si10, was also analyzed. The levels of pro-inflammatory cytokines, IL-1α, IL-1β, TNF-α and IL-8 in culture media, were measured. The cytokine release would also be influenced by the effects seen on cellular viability.

As seen in Figure 18, the cells in medium without FBS induced a release of IL-1β which was significantly higher than the control, and also significantly higher compared to the cells exposed in medium with FBS. The release of TNF-α was more than twice as high from the cell in medium without FBS, than from the cells in medium with FBS. The overall response of the cells, in all three medium conditions, increased with increasing concentrations of Si10. An exception was the release of IL-1α and IL-8. The level of released IL-1α was below the detection level, while the release of IL-8 (in this set of experiments) showed no effect of the exposure (data are not shown).

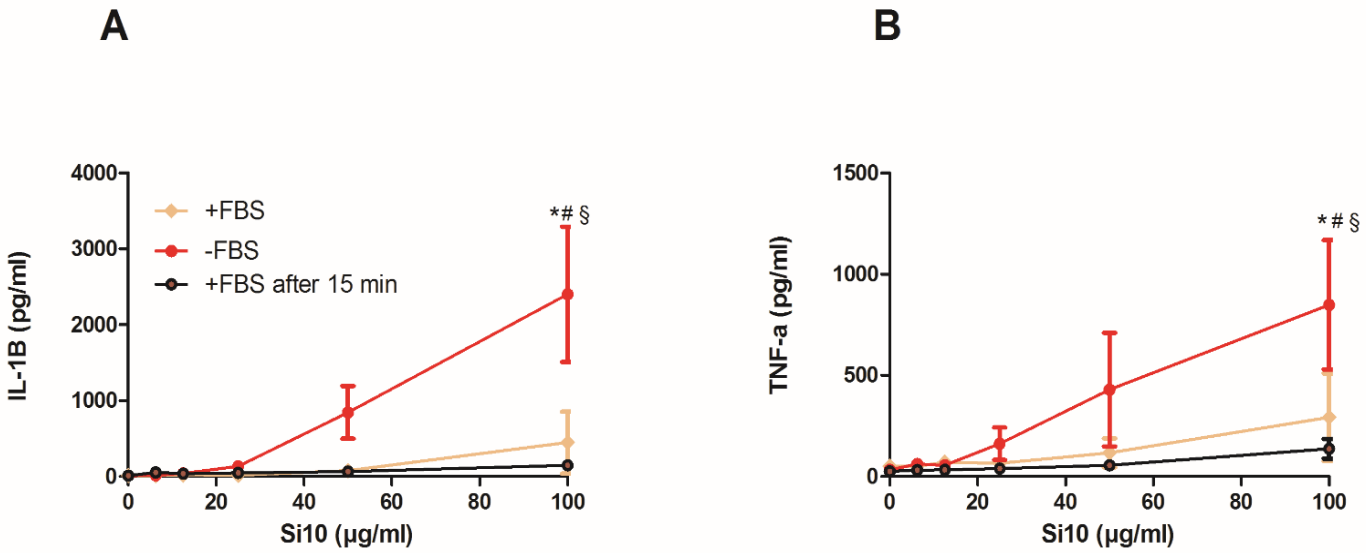

Figure 18: Release of cytokines IL-1β (A) and TNF-α (B) after Si10 exposure in various exposure conditions. *IL-1β and TNF-α were measured after exposing the differentiated THP-1 cells to Si10 (0-100 µg/ml) in various exposure conditions (+FBS, -FBS and +FBS after 15 min), for 6 h. The data are presented as mean values ± SEM of five experiments.* ***** *Significant difference relative to control, $p < 0.05$, # and § are significance difference relative to +FBS and +FBS after 15 min, $p < 0.05$ for two-way ANOVA with Dunnett's Multiple comparison test.*

Based on these experiments, medium without FBS seemed to most suitable for studying responses in the differentiated THP-1 cells exposed to Si10. The medium without FBS did not give too high cytotoxicity, compared to medium with FBS and the cytokine release was significantly higher (Figure 17 and 18). Therefore, this condition was chosen for future experiments.

4.2 The effects of different sized nanoparticles on the release of cytokines and cytotoxicity

After determining the suitable exposure condition, the effects of Si10 were compared to that of other amorphous silica particles, one nanoparticle; Si12 (12 nm) and one micro-sized particle Si500 (500 nm), in exposure-medium without FBS, for 6 h.

Cytotoxicity

Both viability assays showed that the toxicity of Si10 and Si12 were significantly higher than that of the Si500 particles. The cells exposed to Si500 seemed nearly unaffected as judged by the AlamarBlue method, but had a significant reduction with the LDH method. The Si10 and Si12 gave a reduction in viability near 50% or more at the highest concentration, with the Si12 as most potent (Figure 19). A significant difference between all three particle exposures was observed, at the highest concentrations.

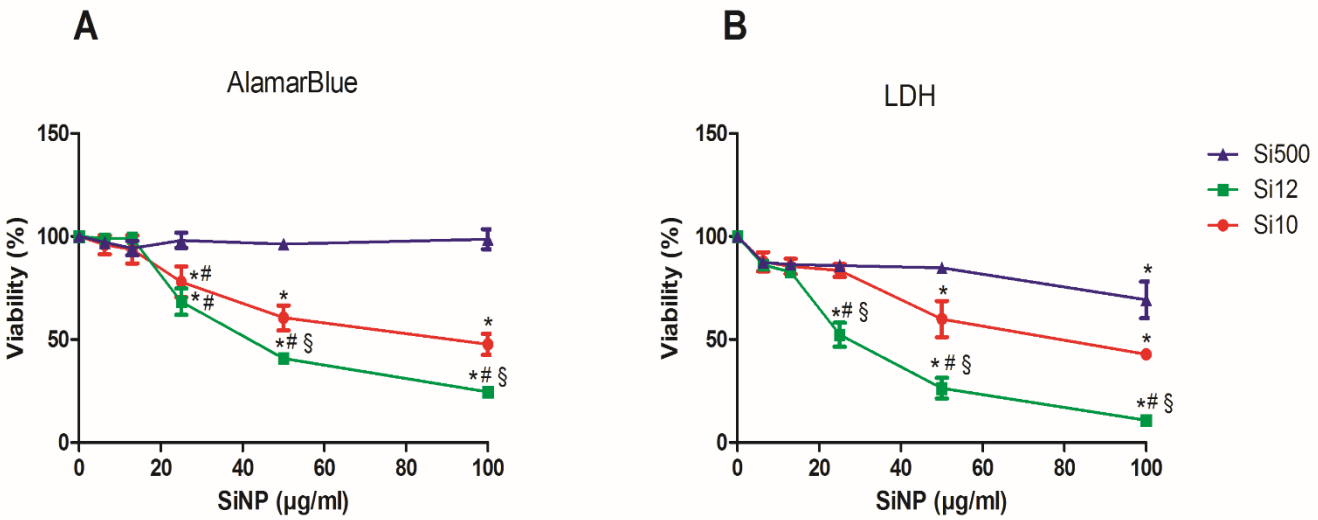

Figure 19: Viability of the differentiatedTHP-1 cells exposed to different sized particles. *Viability of the cells was measured by AlamarBlue (A) and LDH (B) after exposing the THP-1 cells to Si10, Si12 and Si500 in RPMI medium without FBS and at different concentrations (0-100 µg/ml) of particles, for 6 h.* ***** *significant difference relative to control, p < 0.05, § and # significance difference relative to Si10 and Si500, p < 0.05, for two-way ANOVA with Dunnett's Multiple comparison test.*

Cytokine release

The release of the pro-inflammatory cytokines, IL-1β, TNF-α and IL-8, were also measured after exposure to the different sized Si-particles. For the release of IL-1β, Si10 and Si12 were quite similar in potency, with a fold increase of approximately 30, at 100 µg/ml of the nanoparticles. The response to Si10 were possibly slightly more potent than Si12, but not statistically significant (Figure 20A). However, with regard to TNF-α, Si10 was significantly more potent than Si12 (Figure 20B). The release of TNF-α was increased 15-20-fold for the cells exposed to Si10 and 4-fold for the cells exposed to Si12. Exposure to Si500, giving only a minimal release of IL-1β, seemed to induce similarly levels of TNF-α as that of Si12.

The particle-induced release of IL-8 (Figure 20C) was very different compared to TNF-α and IL-1β responses. Si12 gave significant highest fold of IL-8 increase at 50 µg/ml. All five parallels of this experiment showed very varying results of IL-8 release. At low concentrations (12.5-25 µg/ml), we observed more similar patterns as for the release of IL-1β and TNF-α. Si500 was less potent, compared to Si10 and Si12.

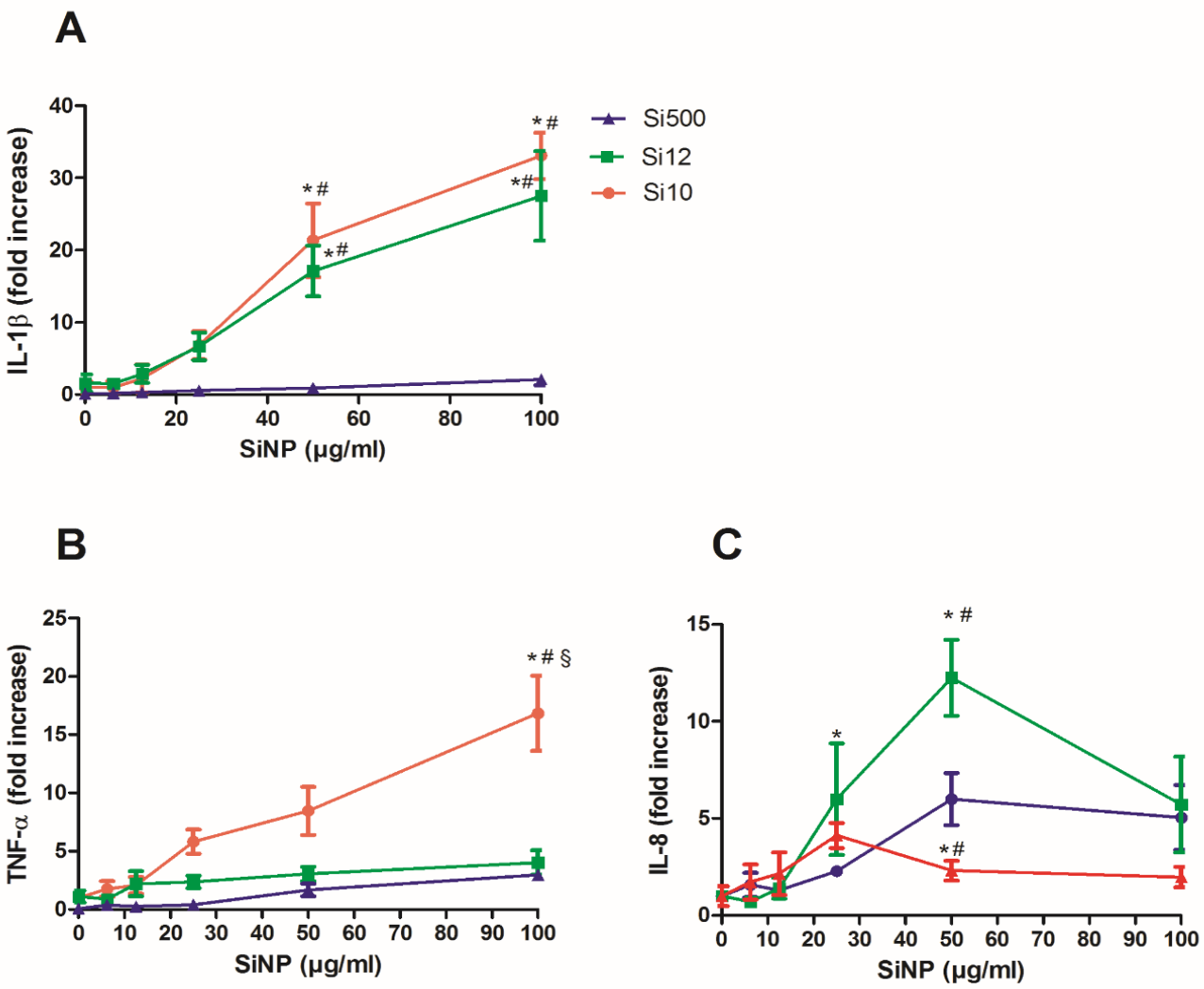

Figure 20: Release of IL-1β (A), TNF-α (B) and IL-8 (C) by different silica particles. *Cytokine release after exposing differentiated THP-1 cells to different nanoparticles (Si10, Si12) and Si500, at concentrations from 6.25 to 100 µg/ml, for 6 h. The data are presented as mean values ± SEM of five experiments. * Significant difference relative to control, $p < 0.05$, § and # significance difference relative to Si12 and Si500, respectively $p < 0.05$, for two-way ANOVA with Dunnett's Multiple comparison test.*

Since both the results of viability and cytokine release showed that Si10 and Si12 seemed most potent, compared to Si500, these particles were chosen for further investigations.

4.3 The time-course relationship of cytokine release from differentiated THP-1 cells following exposure for Si10 and Si12

The next step of the study was to determine the time-course for the cytokine responses. The cells were exposed to Si10 and Si12, with a concentration which gave a marked cytotoxicity, however also a high cytokine release. The chosen concentrations, which were based on previous studies, were 50 µg/ml for Si10 and 25 µg/ml for Si12,

The release of the cytokines, IL-1β and IL-8, induced by Si12, was low after 3 h of exposure, but a significant effect occured at 4,5 h. The highest release of Si12 appeared at 6^{th} h, for all 3 cytokines. The responses induced by Si10, showed a similar pattern, but with less significant responses than Si12. However, the releases of all three cytokines induced by Si10 and Si12, were time-dependent (Figure 21).

Compared to the control and Si10 exposure, Si12 was significantly more potent. This pattern was observed in these experiments for all three cytokines. However, in the first set of experiments presented in the thesis we had opposite observations, with Si10 more potent than Si12. Thus, as we have contradictory observations of the two SiNPs, further experiments of comparing these two particles are needed, in order to clarify this apparent discripancy.

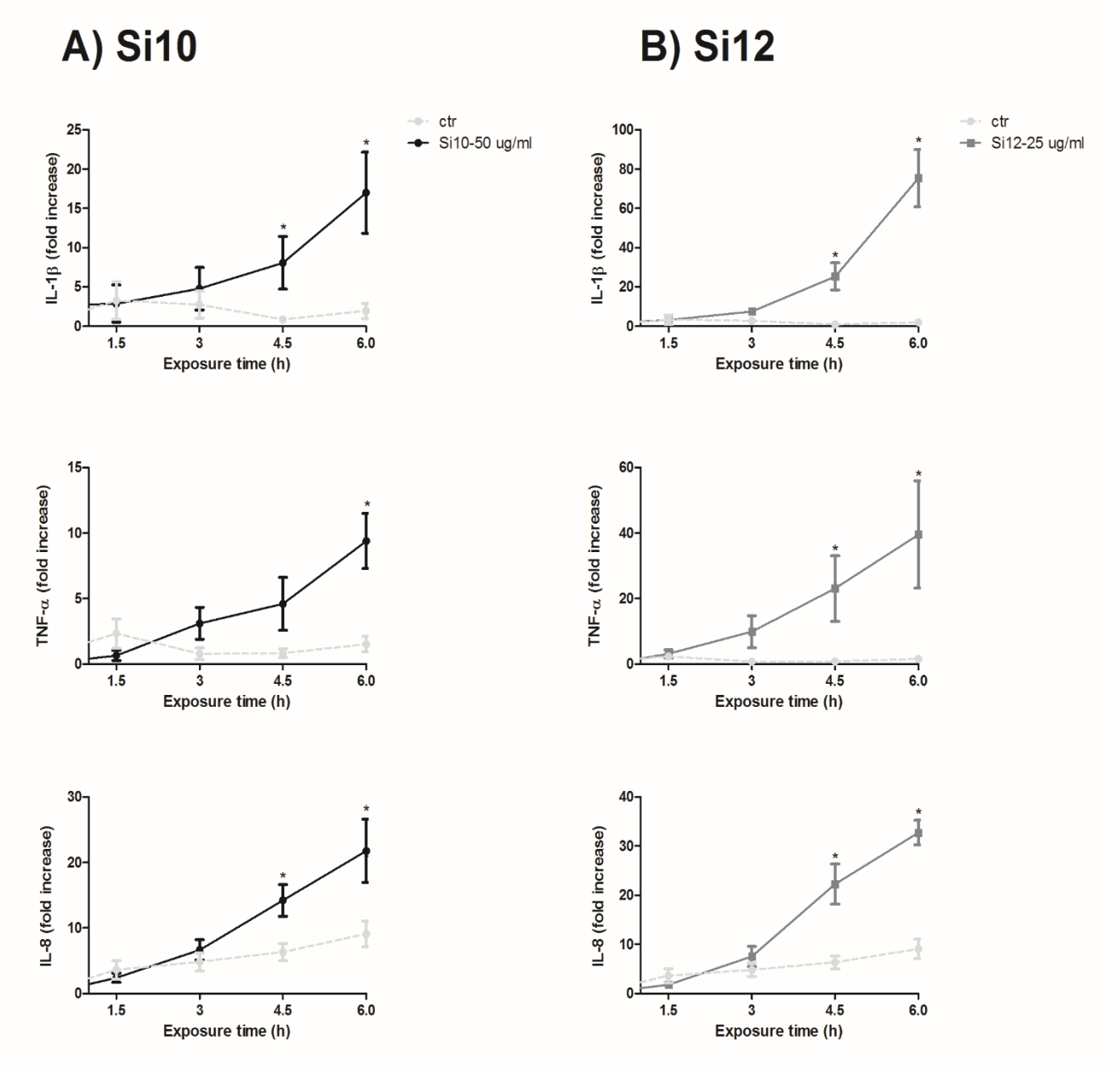

Figure 21: Release of cytokines IL-1β, TNF-α and IL-8 at different exposure times. *The cytokines were measured in differentiated THP-1 cell exposed to Si10 (50 µg/ml) (A) and Si12 (25 µg/ml) (B) at different time points (0.5, 1.5, 3, 4.5,6 h). The data are presented as mean values ± SEM of four experiments.* ***** *Significant difference increase, compared to the control, $p < 0.05$, for two-way ANOVA with Dunnett's Multiple comparison test.*

For the examination of how the Si-particles affect the gene expression of the pro-inflammatory cytokines, mRNA analysis was performed using Real Time PCR after 0.5, 1.5, 3, 4.5 and 6 h of exposure, with Si12 (25 µg/ml). As Si12 induced higher responses when compared to Si10 in the last experiment, we decided to continue with Si12. The exposure concentration (25 µg/ml) was chosen from earlier experiments.

Only two of the cytokines were examined, IL-1β and TNF- α. The chemokine IL-8 had shown such variable result that it became hard to interpret the results and therefor this cytokine was left out in the following experiments.

The induced gene expression of IL-1β due to S12 exposure was time-dependent, showing an increase following 4.5 h and 6 h exposure; which was similar to the cytokine release (Figure 21 and 22). For TNF-α, the gene expression was increased already after 3 h and reaching a maximum level after 4.5 h.

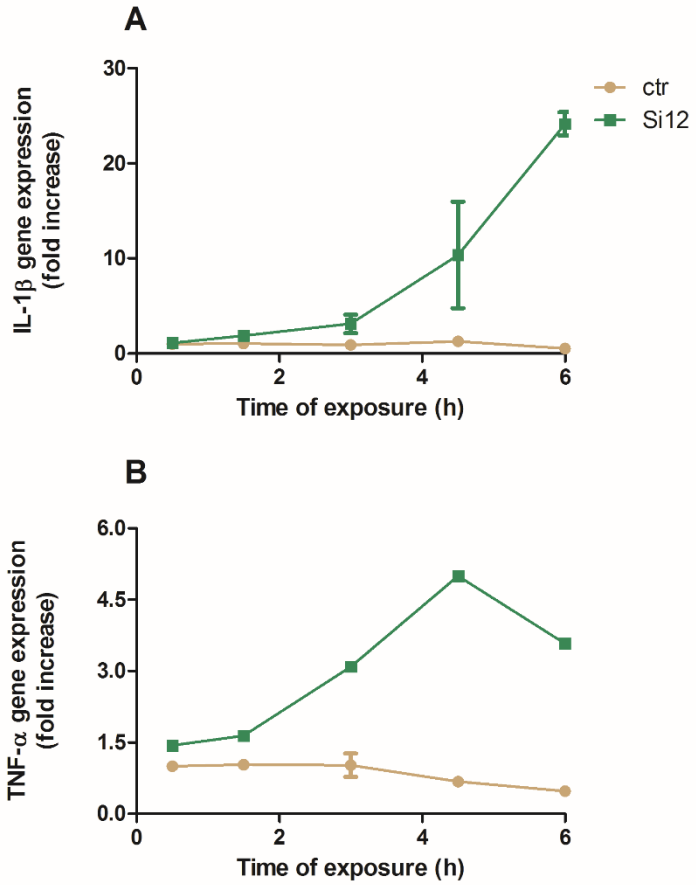

Figure 22: Gene expression of IL-1β (A) and TNF-α (B) after exposure to Si12. ***The*** *differentiated THP-1 cells were exposed to 25 µg/ml Si12 for 0.5, 1.5, 3, 4.5 and 6 h in RPMI medium without FBS. Cellular RNA was isolated from the cells with a RNA isolation kit and mRNA was then measured by reverse transcription followed by Real Time PCR with primers for IL-1β and TNF-α. The data are presented as mean values ± RANGE of two experiments.*

4.5 Effects of transfer of conditioned media from Si10-exposed differentiated THP-1 cells to unexposed cells

Since the airways consist of both immune cells and epithelial cells, it is of interest to investigate how silica nanoparticles might induce effects in culture models involving such cell types. Several controls were used to make sure other factors did not impact a possible effect of the transfer of conditioned medium [43, 45].

4.5.1 Effects of transfer conditioned medium to unexposed differentiated THP-1 cells on cytokine release and cytotoxicity

Cytotoxicity

Exposing the differentiated THP-1 cells to Si10 and transfer of the conditioned medium to unexposed differentiated THP-1 cells following centrifugation, did not seem to reduce cell viability (Figure 23). Both, the exposure to transferred conditioned medium and exposure to centrifuged Si10-containing medium, seemed to give similar cytotoxic effects. If a possible interaction had occurred during the transfer, the response of centrifuged Si10-containing medium should be different from the differentiated THP-1 cells exposed to transfer conditioned medium.

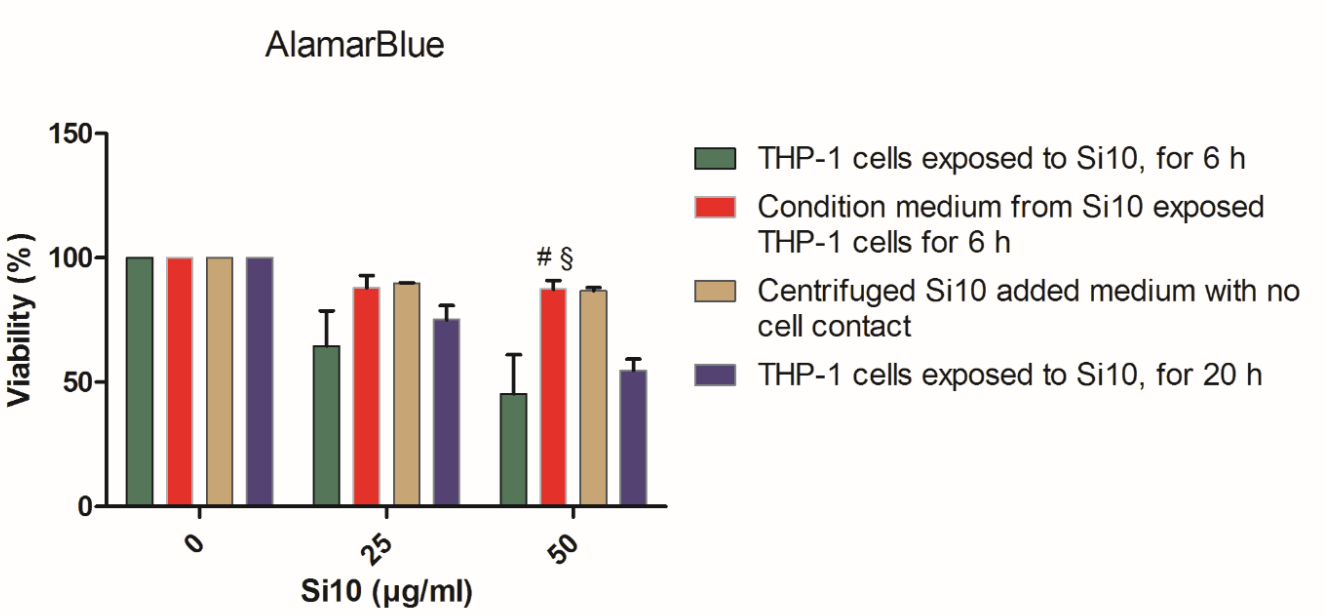

Figure 23: Viability of differentiated THP-1 cell after various types of exposures. *The cells were exposed to Si10 (0, 25 and 50 µg/ml) for 6 h, before the conditioned medium was transferred to unexposed differentiated THP-1 cells, which was incubated for a total of 20 h. After 6 or 20 h the cells were added the AlamarBlue dye and measured. The data are presented as mean values ± SEM of three experiments. # and § significant different relative to differentiated THP-1 cells exposed to Si10 for 6 h and differentiated THP-1 cells exposed to Si10 for 20 h, $p < 0.05$, for two-way ANOVA with Dunnett's Multiple comparison test.*

Cytokine release

The release of the inflammatory cytokines, IL-1β, TNF-α and IL-8 were measured by ELISA. The results are plotted in different colors, in Figure 24.

The transfer of conditioned medium to unexposed differentiated THP-1cells, resulted in an increased, but highly variable and not significant release of IL-1β after 20 h, compared to the different controls. This apparent effect was observed at a concentration of 25 µg/ml of Si10, however, the effect was marked lower at a concentration of 50 µg/ml. Nevertheless, the overall effect of transferring of medium on IL-1β release, could indicate a possible increase.

The transfer of medium from Si10 exposed cells (conditioned medium) to unexposed differentiated THP-1 cells, resulted in only a minor and not statistically significant increase in

the TNF-α levels, when comparing to the controls (the levels at 6 h, and with centrifuged Si10 in cell-free medium). With respect to IL-8, no significant effects were observed upon transfer of conditioned medium, although at the highest concentration, a trend of increased response appeared (Figure 24 C).

Overall, the data show no conclusive effect of transfer of Si10-exposed medium from differentiated THP-1 cells to unexposed THP-1 cells.

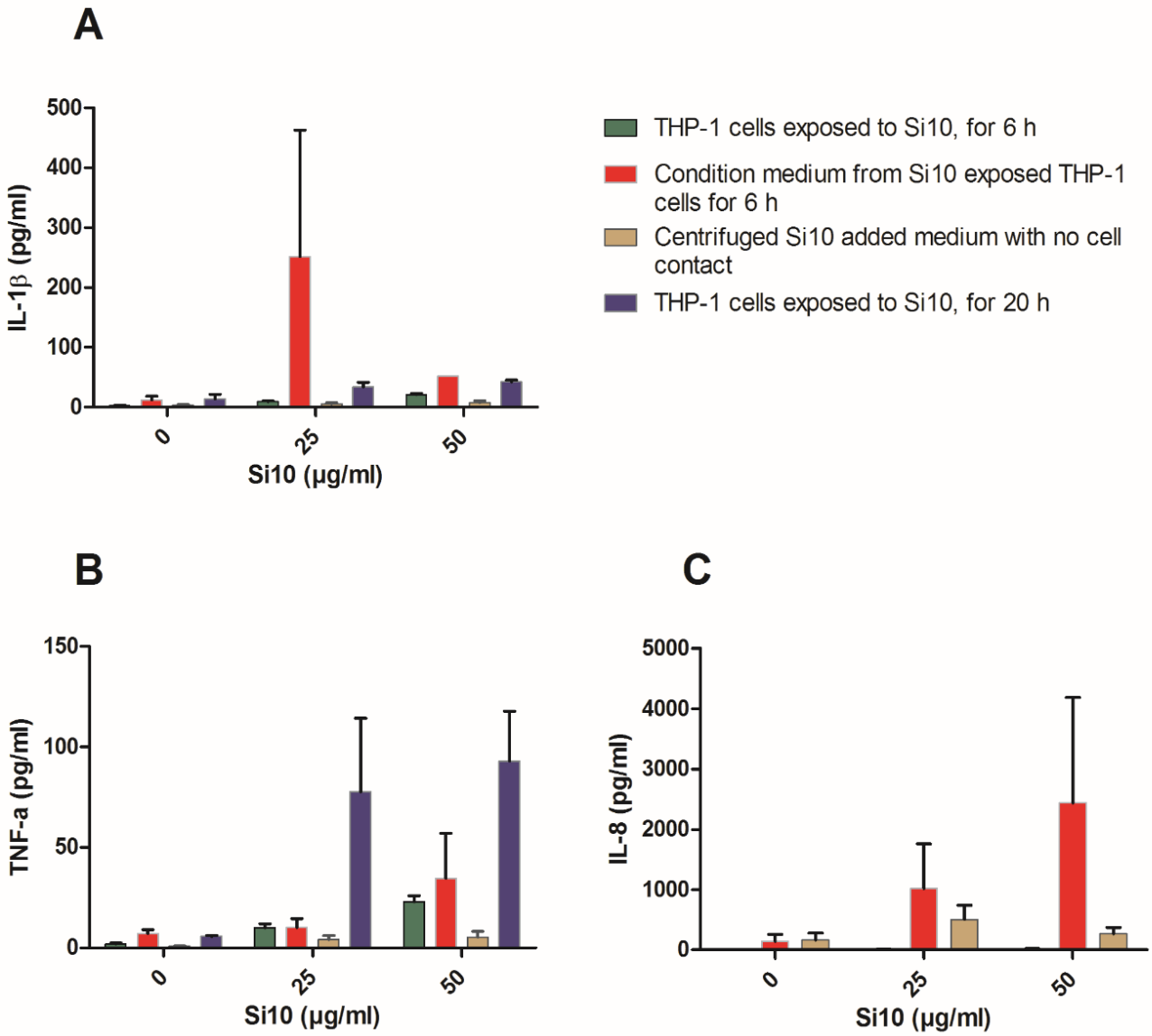

Figure 24: Release of pro-inflammatory cytokines, IL-1B (A), TNF-a (B) and IL-8 (C), from the differentiated THP-1 cells after various types of exposures. *The cells were exposed to Si10 (0, 25 and 50 µg/ml) for 6 h, before the conditionedl medium was transferred to unexposed differentiated THP-1 cells, which was incubated for a total of 20 h. After exposure of 6 and 20 h the cytokine release was measured by ELISA. The data are presented as mean values ± SEM of three experiments.*

4.5.2 Effects of transfer conditioned medium from SiNPSS-exposed differentiated THP-1 cells to epithelial cells (BEAS-2B)

Since the model system with BEAS-2B was well established at the institute, these cells were chosen for experiment on epithelial lung cells [43, 45, 59]. The question was whether mediators released from exposed differentiated THP-1 cells would give enhanced cytotoxicity or cytokine release when transferred to other cell systems such as BEAS-2B.

Microscopic evaluations:

The BEAS-2B cells in cultures are formed more like tubes, when compared to differentiated THP-1 cells. However, exposure to 25 µg/ml Si10, seemed to decrease cell density and increase the number of detached cells, when compared to control cultures.

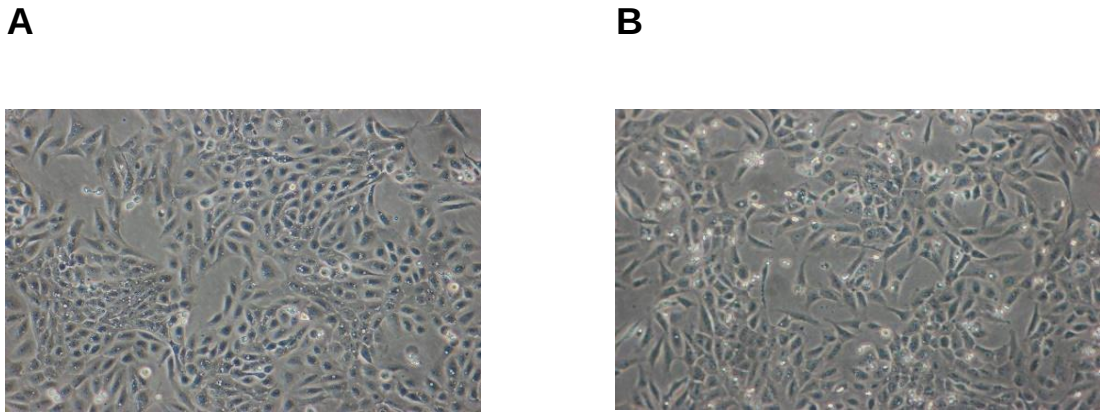

Figure 25: Microscopic picture of BEAS-2B cells, before and after exposure to Si10. *The BEAS-2B cells were plated on 6-well plates in LHC-9 medium and cultured for 2 days before exposed to Si10 (25 µg/ml) for 20 h, in DMEM medium as described in materials and method. Microscopic pictures of the control (A) and after 20 h of Si10-exposure (B).*

Cytotoxicity

The transfer of conditioned medium from Si10-exposed differentiated THP-1 cells, to unexposed BEAS-2B cells, showed that the viability of the BEAS-2B cell was almost not affected at all. Compared to this, the viability of the BEAS-2B cells exposed to Si10 for 20 h, seemed to be reduced significantly, by approximately more than 30% at the highest concentration (Figure 26).

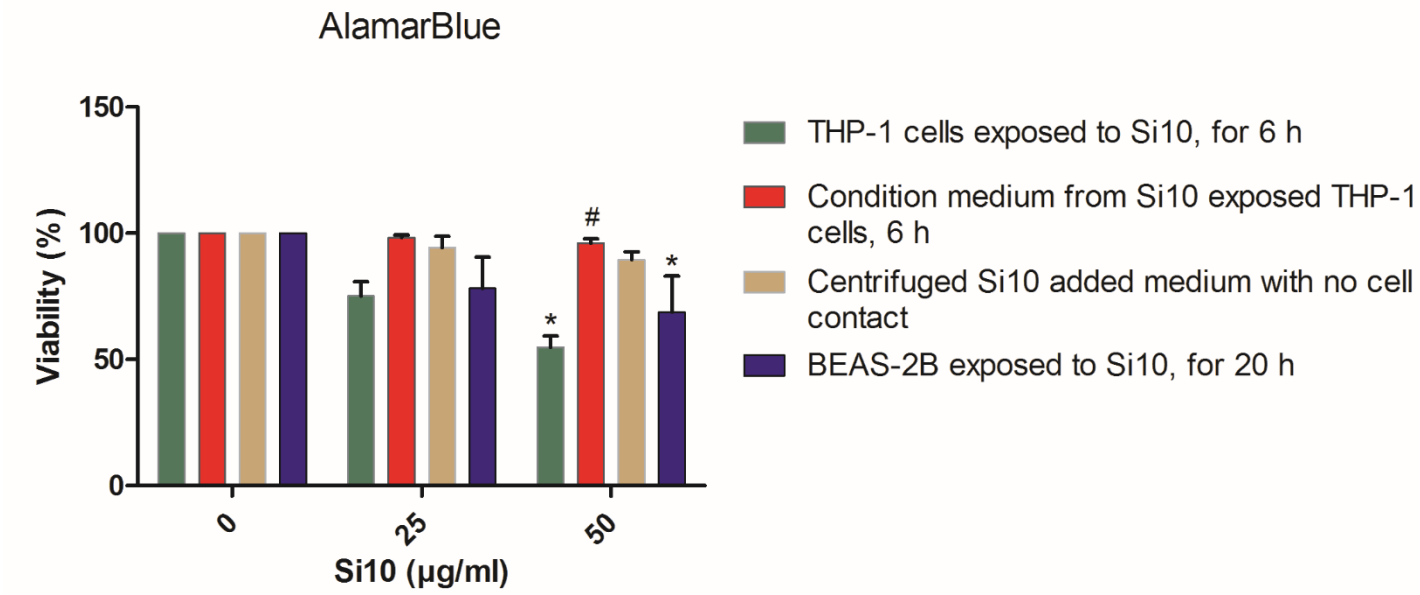

Figure 26: Viability of the BEAS-2B cells after various types of exposures. *The differentiated THP-1 cells were exposed to Si10 (0, 25 and 50 µg/ml) for 6 h, before the medium were transferred to new plates with BEAS-2B cells and incubated for additional 20 h, or BEAS-2B cells were exposed to Si10 for 20 h. After 20 h the cells were measured by AlamarBlue, as describes under material and methods. The data are presented as mean values ± SEM of five experiments.* ***** *Significant difference relative to control, $p < 0.05$, # significant difference relative to the differentiated THP-1 cells exposed to Si10 for 6 h, $p < 0.05$, for two-way ANOVA with Dunnett's Multiple comparison test.*

Transfer of conditioned medium, show significantly increased levels of IL-1β released from BEAS-2B cells at the highest concentration, 50 µg/ml Si10, compared to the respective controls (levels in the differentiated THP-1 cells at 6 h, centrifuged Si10- medium). A similar pattern appeared for the induced effect of TNF-α, at a concentration of 50 µg/ml but since we only have 2 parallells, possible significance was not calculated (Figure 27 A and C). The IL-8 result showed no significant effect of the transfer. However, the BEAS-2B cells responded significantly, after 20 h exposure to Si10.

Overall, it was observed significant effects for the transfer of conditioned medium on the release of IL-1β for BEAS-2B cells, but not for the release of TNF-α and IL-8.

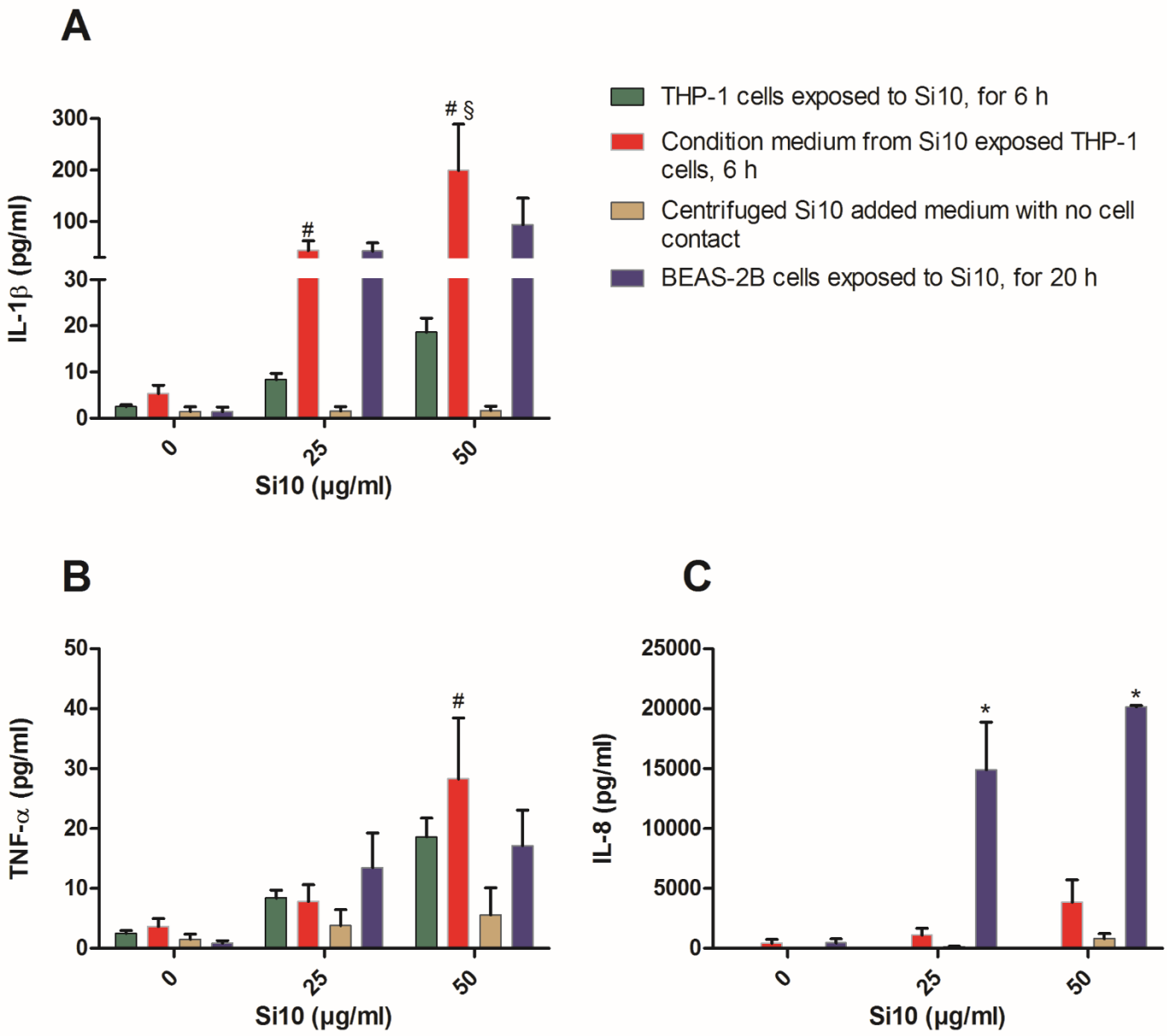

Figure 27: Release of IL-1B (A), TNF-a (B) and IL-8 (C) from the BEAS-2B cells after various types of exposures. *The differentiated THP-1 cells were exposed to Si10 (0, 25 and 50 µg/ml) for 6 h, before the conditioned medium was transferred to BEAS-2B cells and incubated for additional 20 h, or BEAS-2B cells were exposed to Si10 for 20 h. After exposure 20 h the cells were harvested and the cytokine release was measured by ELISA. The data are presented as mean values ± SEM of five experiments.* ***** *Significant difference relative to control, p < 0.05, # and § significant different relative to cytokine release in conditioned medium from differentiated THP-1 cells exposed to Si10 fo 6 h; and centrifuged Si10 added medium with no cell contact, p < 0.05, for two-way ANOVA with Dunnett's Multiple comparison test.*

Cytotoxicity

Figure 28, shows a significant decrease in the cell viability to almost half at the highest concentration (100 µg/ml), for all four cell batches. However, below a concentration of 25 µg/ml the cell seemed unaffected. This concentration appeared to be increasing gradualy for the cytotoxic effect. The overall results show that the four batches are quite similar in viability measurements, and are even equally significant at the two highest concentrations, compared to the control.

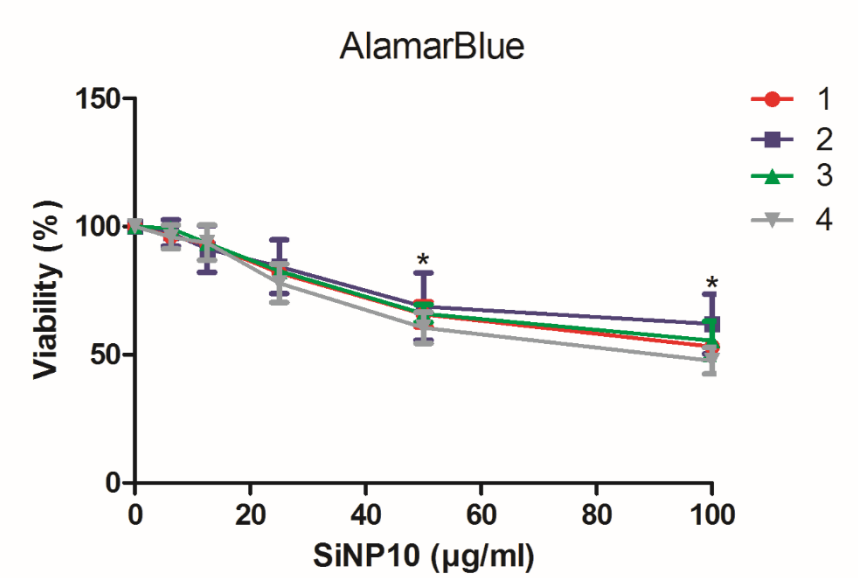

Figure 28: Viability of four different batches of differentiated THP-1 cellsafter exposure to Si10. *Comparison of four cell line batches of THP-1 cells, used at different times during the thesis. Batch 1 and 3 were used in the fall, while batch 2 and 4 were used from January to April. The cells were exposed to increasing concentration of Si10 (0-100 µg/ml), for 6 h. The viability was detected with a cytotoxicity detection kit, involving the AlmarBlue fluorescence dye. The data are presented as mean values ± SEM of 2-4 experiments. * Significant difference relative to control, p < 0.05, for two-way ANOVA with Dunnett's Multiple comparison test.*

Cytokine release

Simultaneously to viability analysis, the release of IL-8, IL-1β and TNF-α were measured with sandwich ELISA (Figure 29). The results were plotted similarly to the viability data. The variability in cytokine release from the different cell batches following exposure to Si10 was high.

Figure 29 showes the difference in cytokine release after 6 h of exposure, between the cell-batches. Batch 2 seemed to be more responsive to Si10 than the other batches when it comes to the release of IL-1β and TNF-α. At 50 µg/ml it is a significantly difference between batch 2 and 3, but at 100 µg/ml there is significantly differense between batch 2 and batch 1-3-4. The release of IL-8 on the other hand is much stronger (significantly) in batch 3 and 4.

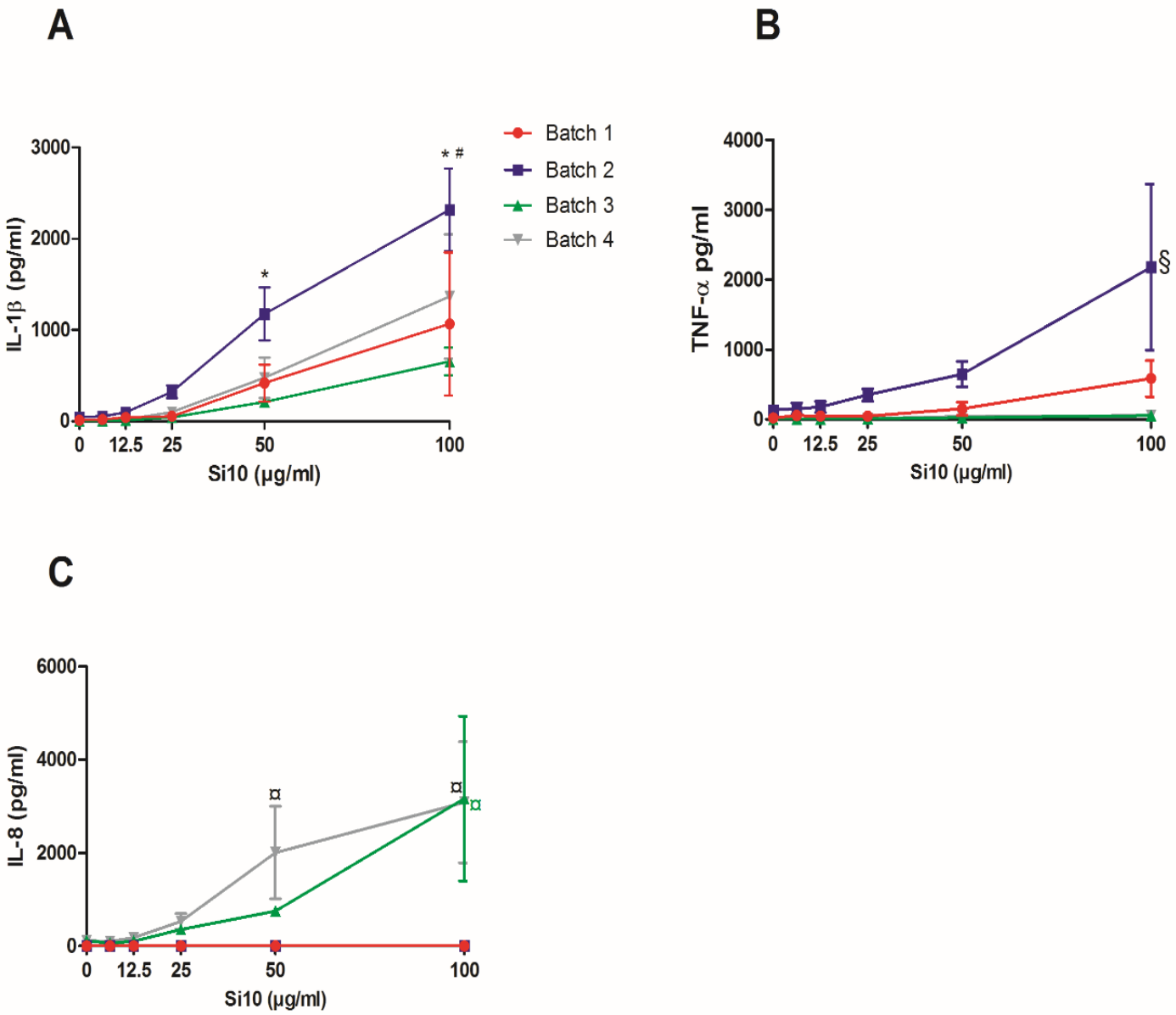

Figure 29: Comparison of four batches of differentiated THP-1 cell lines used throughout the thesis and their cytokine release after exposure to increasing concentrations of Si10 (0-100 µg/ml). *The cells were seeded on 6-well plates in medium with FBS and substituted with RPMI without FBS right before the exposure to Si10 (0-100) for 6 h. IL-1B (A), TNF-a (B) and IL-8 (C) responses were measured by ELISA ad the data are presented as mean values ± SEM of 2-4 experiment. * Significantly different from batch 3, # significantly different from batch 1 and 4, § significantly different from batch 1-3-4, ¤ significantly different from batch 1-2, p < 0.05, for two-way ANOVA with Dunnett's Multiple comparison test*

5 Discussion

In this thesis, the potentials of amorphous silica nanoparticles of different sizes to induce cytotoxicity and cytokine response have been compared with larger silica particles, with main focus on differentiated THP-1 cells. This cell culture has similarities with macrophages. The exposure medium was critical for the responses of Si10 in the differentiated THP-1 cells. Thus, exposure medium containing FBS gave less effects on cytotoxicity and cytokine responses, than medium without FBS. Exposure medium without FBS was chosen for the further experiments in this thesis.

The smaller sized nanoparticles (Si10 and Si12) were shown to be more potent than larger silica particles (Si500) of similar composition. All the particle sizes induced concentration-dependent pro-inflammatory responses. The release of TNF-α was less than for IL-8 and IL-1β Result from Alarmarblue and LDH measurments suggested a time-dependent cytotoxicity, depending on size, with Si12 being the most potent.

Comparing cytotoxicity and release of cytokines in differentiated THP-1 macrophages and the bronchial epithelial cells (BEAS-2B cell), revealed that BEAS-2B cells were less sensitive to Si10 than the differentiated THP-1 cells. Transfer experiments with conditioned medium from Si10-exposed differentiated THP-1 cells to differentiated THP-1 cells or to BEAS-2B cells (without any direct Si-exposure) showed a significant stimulatory effect upon transfer to the BEAS-2B cells, for one of the cytokines, IL-1B. The differentiated THP-1 cells were not significantly affected for any of the cytokines. The cytotoxicity seemed even less affected. Finally, a substantial variation was observed between different “batches” of differentiated THP-1 cells.

5.1 Methodological considerations

For *in vitro* study it is important that the cell line resembles the phenotype of the original primary cells, including having specific characteristics [57]. The cell-specific characteristics depend on whether the cell line is macrophages (differentiated THP-1 cells) or epithelial cells (BEAS-2B cells). For the study of toxicological effects of air pollution, including cytotoxicity and induction of pro-inflammatory cytokine signaling, *in vitro* experiments of immortalized

and normal cells have been broadly used [59]. These kinds of studies are essential for identifying the potential of various pollutants to induce cytotoxicity and inflammatory responses. A critical point is what are the advantages/disadvantages with primary cells versus cell lines. This is described below for the two cell lines used in this thesis.

5.1.1 Is the differentiated THP-1 cell line a suitable model for studying inflammatory responses in the airways?

The first cell line used was the THP-1 cell line, which is derived from an infant suffering of leukemia. After stimulation, *in vivo* monocytes emigrate from the blood stream into the tissue where the cells differentiate into mature macrophages. Macrophages are phagocytic cells in the immune system and play a key role in many diseases [41]. One can use pure human primary monocytes as a model system for the investigation of the biological function of human monocytes and macrophages, but the amount of primary monocytes or monocyte-derived macrophages for functional studies are limited. This is due to the missing proliferation of monocytes. Therefore, many *in vitro* studies have used immortalized proliferating cell lines, such as the human THP-1 cells which is a well-established and broadly used as a model in toxicology [58].

From early on, studies have indicated that THP-1 cells resemble the morphology and differentiation properties of primary monocytes and macrophages. When differentiated with PMA the monocytes undergo morphological changes, likes developing an amoeboid shape with well-developed Golgi apparatuses, rough endoplasmic reticula, and large numbers of free ribosomes in the cytoplasm [58, 65]. The morphological changes, appearing after differentiation, makes the THP-1 cells more similar to human macrophages, because they will have the ability of adherence, have a suitable rate of proliferation, phagocytosis and also cell-surface expression of certain regulation factors such as CD14 [35, 58]. However, if also stimulated with LPS, the THP-1 cells will induce the production of higher levels of TNF-α [46].

Compared to the human primary monocytes and macrophages, THP-1 cells have some advantages. For example, for the study of biological functions of chemicals with high variability, the THP-1 cells seem suitable based on their genetics. The THP-1 cells have

homogeneous genetics, compared to the primary monocyte, a characteristic which controls the degree of cell phenotype variability. At the same time, THP-1 cells have proven to be more desirable in response of genetic modification [58].

The availability of primary human monocytes-macrophages is limited, compared to the THP-1 cells which can be stored indefinitely in liquid nitrogen if the appropriate procedure is followed. The cell line can be recovered without providing any harmful effect on the monocytes, nor the macrophages features or cell viability. Considering this property, the THP-1 cells guarantee sufficient amount of cells for various studies. Another point is that under proper culture conditions, the immortalized THP-1 cell line can grow and divide indefinitely *in vitro*, while human primary monocytes require inflammatory mediators, such as IL-1β, TNF-α, and LPS, functioning as survival factors to prevent apoptosis [58].

5.1.2 How well is a BEAS-2B cell culture model suitable for studying inflammatory response in the airways?

As the study continued, we also included the BEAS-2B cell line, which is derived from normal bronchial epithelium [66].

This cell line was obtained from autopsy of non-cancerous individuals. First, they were immortalized by transfecting them with a SV-40 adenovirus hybrid and then cloning them. The immortalization gives possibilities for evaluation of for example cell transformation in the respiratory tract. After inhalation, the epithelial cells are the first cells to be in contact with pathogens, such as the particles used in this thesis. When it comes to *in vitro* toxicology testing of tobacco, pollutants and nanomaterials, the BEAS-2B cells have provided relevant characteristic and are therefore considered suitable for testing compounds that are inhaled. Another advantage of BEAS-2B cells is that the highest deposition of nanoparticles has been reported in the bronchi and bronchioles [59, 66].

The BEAS-2B cells have several advantages as a model system. First and foremost, these cells are stable and well defined, properties which will result in similar responses over a certain time period. The BEAS-2B cells have also been compared to other bronchial cells,

such as primary Human bronchial epithelial cells. The results showed that the BEAS-2B are more suitable for toxicological experiment, due to a constant supply of cells, and as they maintain their original differentiation characteristics [67]. This property is due to their prolonged lifespan. Another advantage is that the BEAS-2B cells have a relatively good ability to reflect the biological aspects of primary cells, which is of great use because it usually is difficult to resemble the effects in the respiratory tract *in vivo*. At this point, these cells can be used as a model for examination of toxicity and pathogenicity in the lungs. Simultaneously, extrapolation between humans and animals is not necessary, because this is a human cell line [68].

As we have seen in previous studies, the BEAS-2B cells behave like primary epithelial cells, when it comes to the induction of pro-inflammatory response and also expression of adhesion-molecules [69]. Nevertheless, the BEAS-2B cells are immortalized, resulting in altered phenotype which again makes it difficult to extrapolate the responses of toxicants, such as nanoparticles. Earlier studies have shown that the BEAS-2B cells seem to give significantly different amount of cytokine expression, compared to primary bronchial epithelial cells [70].

5.1.3 Difference in Si10-induced cytokine response in cells cultured in various media

In this thesis, the first assignment was to determine how cellular responses of differentiated THP-1 cells were affected by the exposure medium and decide the condition which was most suitable for the differentiated THP-1 cells. Since, the producents of the THP-1 cells have tested the quality of the cells and determined that RPMI 1640 medium with 2 mM L-glutamine is preferable, the testing started with this medium. However, THP-1 cells are growing best in RMPI 1640 containing 10% fetal bovine serum [64, 71] Serum is added to the medium because it has several advantages. It contains growth factors and hormones which stimulate cell growth and function, it helps in attachment of the cells, functions as a binding proteins, maintains the pH of the culture since it acts as a buffer and it also controls the viscosity and thereby reducing damage of the cells. Although it is advantegous to use serum for culturing cells, serum added to the medium might alter the responses to some toxicants.

Presence of serum in the medium has many drawbacks too and can therefor lead to misinterpretations in immunological studies. This can be, lack of uniformity, possible presence of growth inhibiting factors, increased risk for contamination and interfering with purification and isolation of cell culture products. It has also been advised to examine the responses of each batch before using the FBS [49, 64].

The serum may be more of a disadvantage, rather than a positive supplement, since FBS may coat the nanoparticle surface and impact on the reactivity of for example Si-particles by blocking active sites of the particles [72, 73]. This may interfere with the contact between particles and the cells and result in lower responses. Even in absence of cells, serum might lead to reduced formation of SiNP-induced acellular ROS (Reactive oxygen species), a predictor of cellular toxicity [74].

The coating may also lead to less formation of agglomarets [31, 72], and non-agglomareted particles will sediment slower than agglomareted particles and therefor also interact slower with the cells [75]. Notably, serum also coates the silica particles, resulting in less agglomeration and interaction between the particles and the cell [72, 73]. In line with this, medium containing FBS gave less cytotoxicity and also reduced release of the pro-inflammatory cytokines compared to the medium without FBS. The medium without FBS gave a 40-50% reduction in viability, while in the medium with FBS the cells seemed unaffected.

Another disadvantage of using FBS in the culture is the fact that there normally is no serum in the respiratory tract. Serum is a growth factor promoter and therefore FBS has impacts on the activity of cells [73]. However, in the airways there are other proteins, like surfactant protein A (SP-A) [76], that might affect the particle responses. SP-A has more recently been shown an ability to modulate host response to microbes [76, 77].

The fetal bovine serum is produced from the blood of cows. After centrifuging to remove red blood cells, the remaining blood fraction is used as serum for cells [73]. The FBS contain globular proteins, the same category as serum albumin in humans, but the FBS also contains growth factors and growth inhibitors [64, 73], which may impact the responses. It is because of the growth-promoting properties that the bovine serum is used in our cell culture. Therefore, when seeding and maintaining in culture, the cells were cultured in medium with 10 % FBS, but at the exposure day and during the harvesting the cells were to be handled in

medium without FBS. This was due the disadvantages of FBS on the induced effects of Si10. To make sure that the seeded cells did not contain any FBS, the old medium in each well was removed and then cleaned with PBS, before adding medium without FBS.

5.1.4 Difference in effect of several batches of THP-1 cells.

This thesis was performed during the fall and spring. Between these two periods, there was approximately 2-3 weeks where no experiments were done. As soon as the experiments were started again, the results were different from the result produced during the fall. These experiments gave responses quite lower than the earlier experiments and also with qualitative difference for Si10 and Si12. To explain to what extent the cell batches variability could explain the different results, a new THP-1 cell line was seeded and used and then compared with the three older batches used.

According to a couple of previous studies, the use of differentiated THP-1 cell as a model system should give similar results, regardless of the THP-1 cell line batch. The cell line can be recovered without impacting the THP-1 cells in such a way that it affects macrophages features or cell viability [58]. This has also been confirmed by the production company, which have a preferable procedure for the seeding of these cells after storage. This procedure is included in the delivered package. However, the method for recovering these cells does not seem to be optimal and therefor there may not be a guarantee for replicable experiments.

The problem could, however, also be the freezing and thawing of the batches [61]. Only if stored under proper culture conditions, the cell line can grow and divided indefinitely *in vitro*, guaranteeing sufficient amount of cells for various studies. In addition, variation in the PMA differentiation [65] of the THP-1 cells, could be the cause of these variating result.

A previous thesis, from our research group, has shown that the cell density in cultures seems to affect the release of pro-inflammatory cytokines differently, after exposure to Si10 [55]. However, the cytotoxicity and LDH release was little affected by cell density, which is corresponding to this thesis.

5.2 Size-dependent response in cells exposed to Si10, Si12 and Si500

When comparing the different particles sizes on a mass basis, Si12 induced a greater level of cytotoxicity than Si10 and Si500. Our research group has compared particles in other types of cell cultures and the conclusions seemed to be that the cytotoxicity and the cytokine release induced by nanoparticles, is dependent on the size of the particle [25, 38, 49, 78]. This is, to a certain point, in accordance with the result shown in this thesis, because the potency of Si10 and Si12 was markedly higher than the induced response of Si500. However, Si10 seemed to be less potent than Si12, in a set of mesurements.

Upon exposure to Si500 the cell viability was rather unaffected, even at the highest concentration tested. Also when comparing the release of the pro-inflammatory cytokines, IL-1β and TNF-α, Si500 induced low levels. However, with regard to the IL-8 response, Si500 seemed more potent than Si10. On the other hand, we noticed that the total release of IL-8, at this early time-point in the cell culture, was very variable and therefore the interpretation of these result seemed complex and has to be further studied before any conclusion is drawn. For the release of IL-1β, Si10 and Si12 were similar in potency. But, the release of TNF-α showed quite larger responses to Si10 than to Si12. The cytotoxicity of the cells induced by the different particles, clearly affected the cytokine and chemokine release. The higher magnitude of cell death, the greater release of the pro-inflammatory cytokine IL-1β. However, for TNF-α and IL-8, the responses were opposite.

Although, the particle size seemed to be the most important factor for inducing an inflammatory response, through different cytokines and chemokines, other characteristics may also be of importance [60]. Generally, it is known that the shape, morphology, crystallinity, composition, surface chemistry and surface area of the particles, may be factors affecting the biological response. This is shown in an article by Fruijtier-Pölloth, who states that particle shape and surface characteristics seem to be related to the biological activity of synthetic amorphous silica particles [79]. While Si-particles of larger sizes, such as Si500, are not agglomerated in water, and in different media, Si10 and Si12 were agglomerated. Si10 agglomerated to some extent, whereas Si12 showed a more complete agglomeration. Presumably, the agglomeration does not seem to affect the surface area very much, because of the gaps between agglomerated particles. The general view is that surface area rather than

particles size, seems to influence the biological activity of the particles [31, 75]. Another determining factor is sedimentation, which Teeguardeen et al links to the agglomeration [32]. Lison et al describes that particles of larger size (150-500 nm), sediment while particles in nano-size come in contact with the cells through Brownian motion [80]. During exposure, sedimentation is a main force leading to contact between particles and the cells. This gravitational force is affected by the agglomeration state which increases the size of the particle and thereby a larger amount of agglomerated particle reaches the cells. In our studies we have seen a greater cellular response in cells induced by the particle most prone to agglomerate, Si12. Since Si12 can agglomerate more than Si10, this would explain why Si12 of a larger size than Si10, still has a higher cytotoxic effect. It is, however, important to remember that also other factors have been discussed regarding to the biological effects of nanoparticles.

5.3 The relationship between gene expression and cytokine release of pro-inflammatory cytokines induced by Si12

In this thesis the Si12 was shown to induce a time-dependent up-regulation in the expression of pro-inflammatory genes, such as IL-1β and TNF-α in differentiated THP-1 cells. The mRNA expression was dependent on the exposure time, as demonstrated in previous studies, but this did not comprehend with both the cytokines of interest [81, 82]. The two cytokines measured showed different pattern of gene expression and release of cytokines, although they are both pro-inflammatory cytokines. Earlier studies have shown that the pro-form of IL-β is expressed in advance, stored, activated and ready for release [83]. This hypothesis could be the reason for why IL-1β and TNF-α showed different pattern. The gene expression of IL-1β, did not have a significant increase before the third h, which is similar to when the cytokines were released. This may indicate that the amount of stored pro-IL-1β might not be very high. However, the cytokine responses may also be impacted by the variation in different “batches” of THP-1 cells. A similar pattern accured for the gene expression and cytokine release of TNF-α.

5.4 Effect of conditioned medium from Si10 exposed differentiated THP-1 cells.

The purpose of this study is to analyze any possible adverse effects of SiNPs on human immunological cells and thereby determine the risk hazard of the particles. In addition, two different types of cell cultures were compared. The immunological cells (differentiated THP-1) and the epithelial cells (BEAS-2B) were both exposed similarly, and then compared regarding to cytotoxicity and the cytokine release induced by Si10. The differentiated THP-1 cells were shown cells to be most sensitive. The cell lines were chosen based on the fact that the airways consist of both immune cells and epithelial cells. Therefore, it is of interest whether the mediators from these two cell lines could affect each other. This was approached by transferring conditioned medium from Si10-exposed differentiated THP-1cells to unexposed differentiated THP-1cells and BEAS-2B cells.

The result showed that the two cell cultures reacted slightly different to the conditioned medium. The measurements of viability showed no significant effect of the transfer of conditioned medium, to the differentiated THP-1 cells, nor to the BEAS-2B cells. With respect to cytokine responses, BEAS-2B cells might be somehow affected by the transfer, based on the significant release of IL-1β, after exposure to transferred conditioned medium. Compared to this and the controls, the differentiated THP-1 cells showed no significant release of the cytokines. In conclusion, although there were some indications, that the differentiated THP-1 cells release mediators that exert pro-inflammatory response in the BEAS-2B cells, the data should be carefully interpretated and comfirmed by further studies. Notably, previous studies, on BEAS-2B cells, have reported that the magnitude of cytokine expression may not be optimal, because of the immortalization of the cell line [70].

Some studies on interaction between cell in co-culture [84, 85], have also shown greater effect, compared to data in this thesis. However, in this thesis, the cell cultures were not cultured together, which therefore reduces possibilities of interactions between the cells, possibly resulting in the less remarkable responses. Furthermore, different “batches” of differentiated THP-1 cells were used throughout this thesis and the release of IL-1β from the differentiated THP-1 cells was rather low in experiments with transfer of conditioned medium.

In a publication where the activation of the inflammasome and thereby IL-1β were studied, the activity of the differentiated THP-1 and BEAS-2B cells lines were compared. The experiments showed that the differentiated THP-1 cells exposed to SiNPSSs induced approximately a 22-fold more of IL-1β than the BEAS-2B cells do, although both the exposure and the cell culture number was similar when exposed [86]. The activation of IL-1β is induced through two signals. The first one is the interaction with the Toll-like receptors (TLR), which results in gene expression and accumulation of pro-IL-1, while the second signal is through NOD-like receptor (NLR) signaling, which results in caspase-1 activation and subsequently resulting in cleavage of pro-IL-1β to mature IL-1β [56, 87]. However, some myeloid cells, such as the epithelial cells, do not always use signals for releasing IL-1β. This indicates that the BEAS-2B cells may have had a lower level of cytokine release.

The responses, in this thesis, have been quite similar to earlier experiment done by our research group, for the BEAS-2B cells. The THP-1 cells have not been studied earlier with regard to nanoparticle exposure in our laboratory, and it is therefore necessary with further studies [49, 55, 60].

Comparing result of Si10-induced effects in THP-1 cells vs BEAS-2B cells

Exposing differentiated THP-1 cells and BEAS-2B cell to Si10, for 20 h, seems to indicate that the differentiated THP-1 cells are more effected, than the BEAS-2B cells. Considering a significantly higher reduction in viability of the differentiated THP-1 cells, compared to the BEAS-2B cells, it seems that the differentiated THP-1 cells are more sensitive to Si-particles exposure. Also, the pattern of released cytokines was different for these two cell cultures. The levels of both IL-1β and IL-8 appeared remarkably higher in the BEAS-2B cells, than in the differentiated THP-1 cells. However, the TNF-α release was opposite. Notably, BEAS-2B cells have been reported to induce variation in cytokine release [88], which makes interpretation of cytokine release harder and more uncertain for the BEAS-2B cells.

The pro-inflammatory cytokines measured were IL-1β and TNF-α. IL-1α did not give any increase in the differentiated THP-1 cells at the beginning of this thesis and was therefore not measured. Presumably, previous studies have shown that the BEAS-2B cell have a higher release of IL-1α [55]. Therefore, further studies, including the measurement of released IL-1α, is necessary.

It is also important to keep in mind that the two cell lines represent cells which have different properties and abilities [43, 44, 53]. Macrophages are present on the mucosal surface, are recruited by other factors such as chemokines and mainly act through phagocytosis. These immune cells are involved in the surveillance of the immune response and function as a bridge between the innate and adaptive systems. However, epithelial cells protect the organism through different functions, such as the "muco-ciliary escalator", which physically removw pathogens. They also form intercellular junctions and serve as a structural barrier that restrict the passive movement across the epithelium. In addition, the epithelial cells can be stimulated by different bacterial components and secrete cytotoxic and anti-microbial peptides, which can start an immune response and even kill microbial pathogen. Both of these cell types have important functions in protection of humans against particles, including nanoparticles.

6 Conclusions

- The medium in which the differentiated THP-1 cells were exposed to Si10, affected release of pro-inflammatory cytokines. Thus, Si10 induced lower levels of pro-inflammatory cytokines in medium with 10% FBS and medium with FBS added after 15 min, compared to the same exposure in medium without FBS.

- Compared on a mass basis, the smaller silica nanoparticles (Si10, Si12) caused a higher release of all pro-inflammatory cytokines and significantly greater levels of cytotoxicity of the differentiated THP-1 cells, compared to the larger-sized Si-particle (Si500).

- The experiments showed a time-dependent effect of Si10 and Si12 exposure on cytokine responses in differentiated THP-1 cells. The mRNA expression of IL-1β and TNF-α showed a marked time-dependent increase. Notably, the gene expression did not seem to be up-regulated ahead of the cytokine release.

- Upon transfer of conditioned medium from Si10-exposed differentiated THP-1 cells, no cytotoxicity was observed in neither the BEAS2B cells nor THP-1 cells. The conditioned medium induced a significant increase in release of IL-1β after transfer to BEAS-2B cells, but not to the THP-1 cells. Although, significance was reached, the results should be carefully interpreted. Further studies are needed.

- The differentiated THP-1 cells seem more sensitive than the BEAS-2B cells to Si10 exposure, with respect to cytotoxicity and cytokine responses.

- In our differentiated THP-1 cells, the responses varied among different cell line batches, therefore the interpretation of the results from different time periods were challenging. Different batches of cell cultures seemed to affect the cytokine release induced by Si10. Contrary, the cytotoxicity seems relatively similar between the batches.

7 Further studies

In this study we have shown that cytotoxic and pro-inflammatory potential of SiNPs of 10, 12 and 500 nm is affected by their size. The cells clearly react to amorphous silica nanoparticles, by an inflammatory response through the release of the cytokines IL-8, IL-1β and TNF-α. More specifically, smaller-sized nanoparticles seem to be more potent than silica particles of larger size, such as Si500. Notably, we have used high concentrations for these exposures, much higher than the concentration in the air we breathe in real-word scenarios. However, since these particles seem to induce a pro-inflammatory response and also have cytotoxic effect, at the same time as the use of silica nanoparticles has been suggested in medicine, the need for further investigation is important for future risk assessments. In particular, there is a need for studies of long-term effects of nanoparticles. Furthermore, it is important to develop model systems which are more sensitive and is relevantto the human body. In the present thesis I studied the possible interaction between two cell lines, respectively differentiated THP-1 cells and BEAS-2B cells. Although, our studies were not conclusive, they seem to indicate possibility for an interacting effect between the cell lines. Therefor, investigation of interacting cell co-cultures is needed. Perhaps, even other cell lines should be included, for a better underdstanding of the interactions.

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Appendix 1: Materials used in the study

Particles	**Manufacturer**
Amourphous Silica nanoparticle, 10 nm	Kisker Biotech Gmbh & Co, KG, steinfurt Germany
Amourphous silica nanoparticle, 12 nm	Kisker Biotech Gmbh & Co, KG, steinfurt Germany
Amourphous silica nanoparticle, 500 nm	Kisker Biotech Gmbh & Co, KG, steinfurt Germany

Kits	**Manufacturer**
IL-1α Duo set	Applied Biosystems, Life Technologies Corporation, California, USA
IL-8 cytoset	Invitrogen, Life Technologies Ltd, UK
Cytotoxicity Detection Kit (LDH)	Roche Diagnostics Deutschland Gmbh 68305 Mannheim, Germany
Cell viability reagent (AlamarBlue)	Molecular Probes, Invitrogen detection technologies, Eugene, UK
PerfectPure RNA cultured Cell Kit-50	5PRIME Gmbh, 40724 Hilden, Germany
High Capacity cDNA Archiev Kit	Applied Biosystems, Life Technologies Corporation, California, USA

SiRNA	**Manufacturer**
SignalSilence Control SiRNA	Cell signaling, technology, Inc., Danvers, MA 01923, USA

Chemicals, reagents and solutions	**Manufaturer**
LHC-9 medium	Gibco, Life Technologies, Grand Island, NY 14027, USA
DMEM/F-12 medium	Gibco, Life Technologies, Grand Island, NY 14027, USA
RPMI	Gibco, Life Technologies, Grand Island, NY 14027, USA
BSA (albumin, bovine)	
H_2O_2	Merck, Whitehouse Station, NJ, USA
H_2SO_4	
3,3',5,5' -tetramethylbenzidine	Merck, Whitehouse Station, NJ, USA
Glycerol	Merck, Whitehouse Station, NJ, USA
Sodium dodecyl sulphate (SDS)	Fluka, Sigma Aldrich, St. Lous, MO, USA
Sodium chloride	Merck, whitehouse station, NJ, USA
Sodium Pyrophosphate	Sigma Aldrich, st Louis, MO, USA
Triton-X 10	Sigma Aldrich, st Louis, MO, USA
Albumin Bovine Serum (BSA)	Sigma Aldrich, st Louis, MO, USA
Methanol	Poch S.A, Sowinskiego 11, Poland

Tween-20	Sigma Aldrich, st Louis, MO, USA
1 % bromophenol blue/ Tryptan blue Dye 0.4 %	Bio-RAD Laboratories, Inc., Hercules, CA 94547 USA
HiPerfect	Qiagen Gmbh, Hilsen, Germany

Primers and reagents for Real Time PCR	**Manufacturer**
IL-1β primer/probe	Applied Biosystems, Life Technologies Corporation, California, USA
TNF-α primer/probe	Applied Biosystems, Life Technologies Corporation, California, USA
RNase free water	Gibco, Life Technologies, Grand Island, NY 14072, USA
Universal PCR mastermix (Taqman)	Applied Biosystems, Life Technologies Corporation, California, USA

Instruments	**Manufacturer**
Sunrise Absorbance Reader	TECAN Austria Gmbh, 5082 Grödig, Austria
Ultrasonic sonicator	Sonics & Materials, Inc., Newton, USA
Chemi-Doc	Bio-Rad Laboratories, Inc., Hercules, CA 94547 USA
Bioruptor	Diagenode, Denville, NJ, 07834 USA

NanoDrop2000	Thermo Fisher Scientific Inc., Rockford, USA
7500 Fast System Real Time PCR machine	Applied Biosystems, Life Technologies Corporation, California, USA
Microscope, Zeis LSM510 Mea confocal	Carl Zeiss S.p.A, Italy

Gene Amp PCR System 2400	Perkin Elmer, Waltham, Massachusetts 02451 USA
TC10 Automated Cell counter Biorad	Bio-Rad Laboratories, Inc., Hercules, CA 94547 USA
CLARIOstar, High performance Monochromator Multimode Microplate Reader	BMG LABTECH, Allmendgruen 8, Ortenberg 777799, Germany

Equipments	**Manufacturer**
Microtiter plate	Nunc A/S, Roskulde, Denmark
Cell culture bottle	Nunc A/S, Roskulde, Denmark
6-well plate	Corning, Lowell, MA 01851 USA
10 cm dishes	Corning, Lowell, MA 01851 USA
Nitrocellulose Membrane 0,2 um	Bio-Rad Laboratories, Inc., Hercules, CA 94547 USA
Counting Slides	Bio-Rad Laboratories, Inc., Hercules, CA 94547 USA

Appendix 2: Solutions used in the study

Medium

DMEM/F12

DMEM/F12	6 g
$NaHCO_3$	1.2 g
Dstilled warer	500 ml

Antibiotics (add to 500 ml DMEM/F12 medium)

AMP	5 ml
Penicillin-streptomycin	12.5 ml
Amphotericin B	5 ml

RMPI-1640

Pyruvic acid	55 mg
Hepes	5,5 ml (1 M ph stabilized with 10 M NaOH)
FBS	55 ml

Antibiotics

Gentamicine	550 µl

Solutions for ELISA

Diluent buffer (Invitrogen, Life Technologies)

BSA	5 g
Tween	1 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

Citrat buffer

Sodium acetat 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_2O_2	2.2 µg

Stop Solution

H_2SO_4	50 ml
Distilled water	1000 ml

Solutions for AlamarBlue

Alamarblue dye	100 µl

Solutions for Real Time PCR

Mastermix for cDNA synthesis

Samples and NTC

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

NRC

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

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

Appendix 3: Protocols used

Preparations of particle stem solutions from Kisker:

Always work in a sterile hood except when sonicating the particle solution

- Dissolve the nanoparticle to a concentration of 2.3 mg/ml in sterile distilled water, mix well. (920 µl av 25 mg/ml + 9.08 ml sterilt vann)
- Store in refrigerator, use within a month or two.

Preparation of particle solution with BSA

Solutions required: 10 x PBS and 50 mg/ml BSA (5 %)

- Put the 10 x PBS on water-bath 37 degree for 5-10 min
- Make up a 1050 µl of 2.3 mg/ml solution of your particle solution
- Put it on ice
- Sonicate the solution on ice. Settings on the sonicator should be :

Fervency 50%

until 420 Joules

- The solution is ready for adding BSA/10XPBS. Do it like this:

1000 µl particle

34.5 µl BSA- mix

115 µL 10 X PBS-mix well

The final concentration of the particles will be 2.0 mg/ml and the BSA concentration will be 1.5 mg/ml;0.15 %

The solution will be stable for at least one week. We use it for several weeks☺

Adjust the pH to 7.4. Sterile filter the solution

Preparations of particle stem solution from powder (Si12):

Use the weight in the hood for Carcinogenic*. Use special gloves prefer two layers and extra arm-protection. Put in cell-paper with water beside the weight. Weigh up a small amount for example 6-10 mg in a small glass tube, weigh the tube before and after adding the powder. The powder is very static. Wash with a lot of water when finished and through the paper in the risk container.

In a biohazard bench dissolve the nanoparticle to a concentration of 2.0 mg/ml in 0.15 % BSA/PBS, mix well and sonicate for 19 min on ice-water on 50 % amplitude. Change ice-water after 10 min. NB. The probe should be as close as possible one third to one half into the solution.

*First-time users must be trained by experience users (eg Tonje or Leni)

Store in refrigerator, use within a month.

PREPARERING AV PARTIKKELLØSNING TIL BRETT-CYTOKINER (Wiggos metode 1.5 % BSA finalt)

- Lag opp 5 mg/ml partikkelløsning, sett på is og soniker til 420 KJ
- Bland deretter partikler, vann , BSA og PBS på følgende måte:
- 400 µl Partikkelløsning + 400 µl H_2O + 100 µl 10 x BSA + 100 µl 10 x PBS
    - Dette gir en konsentrasjon på 2 mg/ml
    - 10 x BSA = 15% BSA= 150 mg/ml

THP-1 cells: OMSETTING OG DIFFRENSIERING AV THP-1 CELLER

(Anettes metode)

Du trenger: RPMI 1640 *med natriumpyrovat, gentamycin og FKS* til cellekulturen og *PMA (2 mM)* til diffrensieringen

Hvis du ønsker å starte på tirsdag i stedet, så ut flasker med 800.000-1 mill/ml på mandag

Dag 1 (Mandag): (i 8-9 tiden) Differensiering til «makrofager»:

Cellene sås ut på 6 brønners brett a 1 mill/ml, 2 mill pr brønn i RPMI-1640 tilsatt PMA til en konsentrasjon på 81 nM (50 ng/ml).

Lag en 1:10 fortynning av PMA i medium, tilsett 16.2 µl av denne fortynningen til 40 ml medium

Dag 2 (Tirsdag): (i 14-15 tiden):

Cellene får nytt forvarmet RPMI-1640, 2 ml pr brønn.

Dag 3 (Onsdag): Cellene eksponeres for partikler, total volum i brønnen 1 ml. Cellene høstes etter ønsket tid (se egen prosedyre)

Dyrking av THP- 1 celler

NB! THP-1 cellene tar ca 14 dager for å bli forsøks klare etter opptining.

THP-1 cellene dyrkes i suspensjon i 75 cm^2-flasker. Cellene trives best mellom 400.000-1.000.000 celler/ml medium, totalt 12-20 ml. Cellene splittes mandag, onsdag og fredag, og passasjetallet økes for hver splitting.

Noter passasje og celletallet før og etter splitting i permen!

Mandag:

- Sentrifugeres ned, telles og fortynnes til 500.000 celler/ml. Såes ut i ny flaske.
- Forvarm 20 ml medium per flaske som skal såes ut
- Overfør cellene fra flasker til rør
- Sentrifuger i 4 min på 1.0 rcf
- Resuspander pellet i 10 ml forvarmet medium (eller 10 mL/flaske du overførte til røret)
- Tell celler (10 ul cellesuspensjon + 10 ul trypanblått)
- Sår ut i en nye flasker på 500.000 celler/mL i 15-20 ml (denne skal gå videre i kultur) og i 2 flasker a 800.000/ml til differensiering

Onsdag:

- Cellene telles og fortynnes til ca 500.000 celler/ml.
- Sett medium til varming (10-15ml per flaske)
- Bland cellesuspensjonen i flasken vha en 10 ml pipette
- Overfør < 1ml til et eppendorfrør
- Bland og overfør 10 ul til et nytt eppendorfrør, og tilsett 10 ul trypanblått.
- Bland og overfør til slide for telling
- Beregn fortynning, ta ut cellesusp og tilsett nytt medium slik at celletettheten blir 500.000 / ml (= 10 mill per flaske i 20 ml).

Fredag:

- Cellene telles og fortynnes til ca 500.000 celler/ml.
- Sett medium til varming (10-15ml per flaske)
- Bland cellesuspensjonen i flasken vha en 10 ml pipette
- Overfør < 1ml til et eppendorfrør
- Bland og overfør 10 ul til et nytt eppendorfrør, og tilsett 10 ul trypanblått.
- Bland og overfør til slide for telling
- Beregn fortynning, ta ut cellesusp og tilsett nytt medium slik at celletettheten blir 500.000 / ml (= 10 mill per flaske i 20 ml).
- Hvis cellene vokser for fort (opp mot 2 mill før splitting) eller for sakte (ned mot 700.000 før splitting, kan man så ut noen flasker med hhv 3-400.000/ml eller 6-800.000 for å påvirke celleveksten i den retningen man ønsker.

Tillaging av medium, THP-1

Basismedium: RPMI 1640 medium (500 ml, Med.Probe)

I dette tilsettes:

- Vei opp 55 mg natriumpyrovat (Pyruvic acid, ligger i isboks i kjøleskap) i sterilt begerglass.
- Overfør 5,5 ml pH justert Hepes (1 M Hepes pH- justeret til 7,3 med 10M NaOH) i avtrekk.
- Sterilfiltrer over på flaska med RPMI 1640 medium (0,5liter) (skyll sterilfilteret med medium etterpå)
- Tilsett 550 ul Gentamicineeee.

- Tilsett 55 ml FBS.

Telling av celler:

- Skru på maskinen, på høyre side, nederst.
- Ta ut 10 µl cellesusp og bland likt volum trypanblå
- Tilsett 10 µl på telleglasset, putt i maskin
- Maskinen detekterer trypanblå så telletallet som kommer ut tilsvarer tallet i cellesuspensjonen

BEAS-2B cell: SPLITTINGAV BEAS – 2BCELLER

(mandag/torsdag)

1. Sett trypsinløsningen og LHC – 9 medium tilsatt fettfri albumin (stoppløsning) i vannbad 5-10 min før splitting. Begge løsninger finnes nedfrosset ved -20 °C

2. Flaska skylles med temperert PBS 1-2 ganger. Sug godt av !

3. Trypsin helles/pipetteres på og flaska vippes frem og tilbake,----- trypsinen helles tilbake i røret. La litt bli igjen på flaska.

4. Sett på korken og la flaska stå ca 8 - 10 min. på benken. Bank forsiktig innimellom og se i mikroskop at alle cellene har løsnet!

5. Tilsett 10 ml LHC – 9 medium tilsatt fettfri albumin (2,25 mg / ml), som er med på å stoppe trypsineringen. Bruk 2 rør med «stoppmedium» ,hvis mer enn 2 flasker skal trypsineres !! Pipetter godt slik at du får med deg alle cellene, vask evt etter med LHC-9 medium.

6. Sentrifuger i 4 min ved 1000 rpm i den lille bordsentrifugen på cellelabben.

7. Resuspender pelleten i medium, ca 5 ml

8. Bland 20 µl cellesusp med 20 µl trypanblå i ett eppendorfrør, sett på 10 µl i tellekammer og les av i celletelleren

9. Cellene sås ut på collagencoatede flasker/skåler/brett. Collagen coatede flasker/brett/skåler finnes nedfrosset (cellelab eller fryser i gangen). Mandagsceller 900.000 pr flaske (75 cm^2), torsdagsceller 500.000 pr flaske (75 cm^2) i 15 ml LHC-9.

10. Cellene skal ha mediumskift dagen etter utsåing deretter hver annen dag.

11. For enkelte eksperimenter vil det være ønskelig å bytte til DMEM:F12 i stedet for LHC-9. Disse cellene får LHC-9 utsåingsdagen, men DMEM:F12 fra dag 2, husk å vaske med PBS

12. 1 gang, før nytt medium tilsettes.

13. Se i BEAS boka ang celletall for diverse skåler/ brett.
14. Husk alltid medieskift dagen etter splitting !!!!!
15. Cellene liker seg ikke hvis de blir for tette !!!!
16. **VIRKON** helles i flaskene/ brettene/ skålene som det har vært celler i, «korkes», puttes i plastpose og kastes i de gule avfallsboksene.
17. Sug igjennom VIRKON i Vacutainersystemet når du er ferdig i benken.

BUFFERE, MEDIUM mm TIL BEAS-2B

LHC-9: Ferdig laget medium, finnes lagret i fryser. Etter opptining oppbevares mediet pakket inn i al-folie i kjøleskap

DMEM:F12:

12 g DMEM:F12 pulver

2.4 g $NaHCO_3$

1000 ml sterilt vann

pH justeres til 7.2

HBS: (brukes til coatingen)

2,38g Hepes

3,5 g NaCl

0,1 g KCl

0,97g NaH2PO4

0,85g Glucose

500 ml sterilt vann

Juster pH til 7,2. Sterilfiltrer og fordel på 100 ml flasker

LHC-9 med albumin (stoppløsning)

200 ml LHC-9

450 mg fettfri BSA (står i kjøleskap 2469)

Add BSA, stirr and sterilfilter. Add 10 ml pr tube and put into the freezer

Sterilfiltrer. Add 3 ml pr tube and put into the freezer

ELISA; 1L-1a (Duo set)

Coating og detektion antistoff og standard løses i følge pakningsvedlegg, og lagres i kjøleskap for opptil 1 mnd, for lengere tid lagring i fryser.

NB: Skjekk alltid at mengdene av antistoff og HRP som skal brukes mot pakningsvedlegg/innside lokk, da dette varierer fra batch til batch

Dag 1: Coating med capture antibody

- Tilsett 83.3 µl (se innsiden av boksen) med capture antibody til 11 ml PBS. Tilsett 100 µl capture antiboby/pr brønn
- La platen stå i 24 timer i romtemperatur eller i kjøleskap over lengere tid

Dag 2: Blokkering

- Hell av coating løsningen
- Sett i platevasker. Vask 4 x med vaskebuffer
- Blokker med 300 ul blokkingbuffer/pr brønn (DD). Blokkingen skal stå minst 1 time i romtemratur og eventuelt lengre tid i kjøleskap.

ELISA assay:

- Merk 7 eppendorfrør: 0-7.8-15.6-31.25-62.5-125-250.
- Fordel 500 µl diluentbuffer (DD) i hvert av rørene
- Lag standard på 500 pg/ml ved å tilsette 7.14 µl standard til 993 µl DD. Lag deretter en fortynningsrekke fra 250-3.9 pg/ml ved å fortynne de 1:2 hele veien i l DD
- Fortynn prøvene i l DD etter ønsket konsentrasjon: *Ufortynnet, 1:10 (50 µl prøve + 450 µl DD), 1:40 (100 µl 1:10 + 300 µl DD)*
- Hell av blokkingløsningen. Bank brettet mot cellevatt.
- Sett på 100 ul standard eller prøve til brønnene. Inkuber i 2 time romtempratur.

- Sett i platevasker. Vask 4 x med vaskebuffer
- Lag Detektion antibody: 11 ml DD + 166.7 µl detektion antibody
- Tilsett 100 ul detection antibody/pr brønn. Inkuber i 2 timer i romtempratur.
- Sett i platevasker. Vask 4 x med vaskebuffer.
- Fortynn HRP; 300 µl HRP til 11 ml DD. Tilsett 100 ul HRP/pr brønn. Inkuber i 20 minutter i romtempratur. Sett platen mørkt.
- Sett i platevasker. Vask 4 x med vaskebuffer.
- Lag TMB løsning
- Tilsett 100 ul Substrate Diluent (TMB). La stå mørkt, men følg godt med på farge utviklingen, inkuber i 5-20 minutter
- Når blåfargen er "fin" ,tilsettes 50 ul stopp løsning (0,8 M H_2SO_4) .
- Les av platen 450 nm.
- DD : 1 g BSA til 100 ml PBS (BSA i kjøleskap på 2469)
- Stamløsning TMB: 6 mg TMB stokk/ml DMSO (TMB i kjøleskapet på ELISA lab, DMSO i brannskap på 2469)

ELISA; IL-2B (Duo Set)

- Coating og detektion antistoff og standard løses i følge pakningsvedlegg, og lagres i kjøleskap for opptil 1 mnd, for lengere tid lagring i fryser.
- NB: Skjekk alltid at mengdene av antistoff og HRP som skal brukes mot pakningsvedlegg/innside lokk, da dette varierer fra batch til batch

Dag 1: Coating med capture antibody

- Tilsett 83.3 µl (se innsiden av boksen) med capture antibody til 11 ml PBS. Tilsett 100 µl capture antiboby/pr brønn
- La platen stå i 24 timer i romtemperatur eller i kjøleskap over lengere tid

Dag 2: Blokkering

- Hell av coating løsningen
- Sett i platevasker. Vask 4 x med vaskebuffer
- Blokker med 300 ul blokkingbuffer/pr brønn (DD). Blokkingen skal stå minst 1 time i romtemratur og eventuelt lengre tid i kjøleskap.

ELISA assay:

- Merk 7 eppendorfrør: 0-3.9-7.8-15.6-31.25-62.5-125.
- Fordel 500 µl diluentbuffer (DD) i hvert av rørene
- Lag standard på 250 pg/ml ved å tilsette 4.5 µl standard til 994 µl DD. Lag deretter en fortynningsrekke fra 250-3.9 pg/ml ved å fortynne de 1:2 hele veien i l DD
- Fortynn prøvene i l DD etter ønsket konsentrasjon.
- *ufortynnet*
- *1:10 (50 µl prøve + 450 µl DD) eller 1:40 (100 µl 1:10 + 300 µl DD)*

- Hell av blokkingløsningen. Bank brettet mot cellevatt.
- Sett på 100 ul standard eller prøve til brønnene. Inkuber i 2 time romtempratur.
- Sett i platevasker. Vask 4 x med vaskebuffer
- Lag Detektion antibody: 11 ml DD + 166.7 µl detektion antibody
- Tilsett 100 ul detection antibody/pr brønn. Inkuber i 2 timer i romtempratur.
- Sett i platevasker. Vask 4 x med vaskebuffer.
- Fortynn HRP; 300 µl HRP til 11 ml DD. Tilsett 100 ul HRP/pr brønn. Inkuber i 20 minutter i romtempratur. Sett platen mørkt.
- Sett i platevasker. Vask 4 x med vaskebuffer.
- Lag TMB løsning
- Tilsett 100 ul Substrate Diluent (TMB). La stå mørkt, men følg godt med på farge utviklingen, inkuber i 5-20 minutter
- Når blåfargen er "fin" ,tilsettes 50 ul stopp løsning (0,8 M H_2SO_4) .
- Les av platen 450 nm.
- DD : 1 g BSA til 100 ml PBS
- Stamløsning TMB: 6 mg TMB stokk/ml DMSO
- TNF-α and IL-8 (cytosett

ELISA; IL-1α/IL-8 (cytoset)

Coating/capture antibody (1) Detection antibody(2) og HRP (3) oppbevares i kjøleskap. Standarden er frosset ved -70

Coating (Dag 1)

- Tilsett x µl *coating/capture antibody* (coating) til 11 ml PBS. *(Se innsiden av eskelokket for mengde capture)*
- Tilsett 100 µl av den fortynnede coating- løsningen til hver brønn på brettet.
- Forseil med plastfole
- Sett brettene i kjøleskap og la de stå der i minst 24 timer (over natt).

Blokkering (Dag 2).

- Tom ut coating- løsningen og bank brettet mot cellevatt.
- Vask 1-3 x
- Brettene må aldri stå lenge tørre.
- Tilsett 300 µl blocking- løsning til hver brønn. La brettene stå i en-to timer med blocking- buffer i romtemperatur eller lengre tid i kjøleskap (et døgn eller mer), med lokk.

Tillaging av standardrekke:

- Standardene finnes nedfrosset i -70ºC ferdig fordelt (100 µl).
- 900 µl Diluentbuffer (DC) tilsettes standard ⇒ kons 1000 pg/ml.
- Fordel 500 µl DC i hvert av da andre rørene som skal brukes til standardkurve (7 rør, 8 rør med utgangsstandaren)
- Overfør 500 µl fra utgangsstandaren til det neste (merket 500).

- Bland godt.
- Overfør til neste rør.
- Gjenta prosedyren til siste nør. NB: rør merket med 0 skal bare inneholde 500 µl diluentbuffer. Rør merket med 7.8125 vil da inneholde 1000 µl til sammen. Standarrekken blir :1000-500-250-125-62,5-31,25-15,625-0
- Fortynning av prøver: Ofte må prøvene fortynnes før måling. Fortynnes gjøres i DC. F.eks

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	1	1	9	9	17	17	25	25	33	33
B	15.6	15.6	2	2	10	10	18	18	26	26	34	34
C	32.25	32.25	3	3	11	11	19	19	27	27	35	35
D	62.5	62.5	4	4	12	12	20	20	28	28	36	36
E	125	125	5	5	13	13	21	21	29	29	37	37
F	250	250	6	6	14	14	22	22	30	30	38	38
G	500	500	7	7	15	15	23	23	31	31	39	39
H	1000	1000	8	8	16	16	24	24	32	32	40	40

- Fortynn *detektion antibody*: x µl *detection*- løsning/6 ml diluentbuffer. *(Se innsiden av eskelokket for mengde detektion)*
- Tilsett 50 µl av detection- løsning til hver brønn (til standard og prøvene). Inkuber brettet med plastfolie i to timer med forsiktig bevegelse.

- Fortynn strept-HRP *x µl strept- HRP/11 ml Diluent* (streptavidin-Horseradish Peroxidase Conjugate) til hvert brett. (*se lokket for mengde HRP*)
- Tøm ut innholdet i brønnene. Vask 4 ganger med vaske-buffer. Bank av på cellevatt.
- Tilsett 100 µl Strept. HRP til hver brønn. La det stå i 30 minutter i romtemperatur mørkt. Ved forsiktig bevegelse
- Vask 4- 5 ganger
- Lag TMB (tetramethyl benzidine)
- Tilsett 100 µl TMB- substrat i hver brønn, blå farge utvikles sett mørkt under farge utvikling.
- Tilsettes 50 µl stopp solution (0.9 M H_2SO_4) til hver brønn
- Mål absorbansen på spektrofotometer (450nm).