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ELINA RYDMAN

Inflammatory Effects of Nanosized Titanium Dioxide and Carbon Nanotube Pulmonary Exposure

DEPARTMENT OF BIOSCIENCES
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DOCTORAL PROGRAMME IN INTEGRATIVE LIFE SCIENCE
UNIVERSITY OF HELSINKI
Inflammatory effects of nanosized titanium dioxide
and carbon nanotube pulmonary exposure

Elina Rydman
(née Rossi)

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki in the Main Building, Auditorium XII, on October 15th at 12 o’clock noon.
We animals are the most complicated and perfectly-designed pieces of machinery in the known universe. It is hard to see why anyone studies anything else.

-Richard Dawkins-
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II. Elina M. Rossi, Lea Pylkkänen, Antti J. Koivisto, Heli Nykäsenoja, Henrik Wolff, Kai Savolainen, Harri Alenius. Inhalation exposure to nanosized and fine TiO$_2$ particles inhibits features of allergic asthma in a murine model. Particle and Fibre Toxicology 7:35 (2010)


*Equal contribution


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Author’s contributions to the publications

I. EMR participated in the design of the study, performed the *in vivo* experiments, participated in the *in vitro* experiments, carried out sample collection and conducted most of the analyses, participated in interpreting the results and drafted the manuscript.

II. EMR participated in the design of the study, performed the *in vivo* experiments, carried out sample collection and conducted most of the analyses, participated in interpreting the results and drafted the manuscript.

III. EMR participated in the design of the study, performed the *in vivo* experiments, carried out sample collection (together with MI) and part of the analyses (qualitative assessment of histological samples, AHR, BAL cell counts), participated in interpreting the results and in drafting the manuscript.

IV. EMR participated in the design of the study, performed the *in vivo* experiments, carried out sample collection and conducted most of the analyses, participated in interpreting the results and drafted the manuscript.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ABTS</td>
<td>2, 2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)</td>
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<tr>
<td>AHR</td>
<td>airway hyper-responsiveness</td>
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<td>AM</td>
<td>alveolar macrophage</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCLn</td>
<td>chemokine (C-C motif) ligand n</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>cnTiO₂</td>
<td>silica coated nanosized titanium dioxide</td>
</tr>
<tr>
<td>CNT</td>
<td>carbon nanotubes</td>
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<tr>
<td>CXCLn</td>
<td>chemokine (C-X-C motif) ligand n</td>
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<tr>
<td>DAMP</td>
<td>danger associated molecular patterns</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>DTS</td>
<td>dispersion technology software</td>
</tr>
<tr>
<td>ECP</td>
<td>eosinophil cationic protein</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDS</td>
<td>energy dispersive spectroscopy</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ENM</td>
<td>engineered nanomaterials</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulated protein kinases 1 and 2</td>
</tr>
<tr>
<td>FBAG</td>
<td>fluidized bed aerosol generator</td>
</tr>
<tr>
<td>FBGC</td>
<td>foreign body giant cells</td>
</tr>
<tr>
<td>FOXP3</td>
<td>forkhead box P3</td>
</tr>
<tr>
<td>fTiO₂</td>
<td>fine-sized TiO₂</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GIT</td>
<td>gastrointestinal tract</td>
</tr>
<tr>
<td>HARN</td>
<td>high-aspect-ratio nanoparticle</td>
</tr>
<tr>
<td>4-HBA</td>
<td>4-hydroxy benzoic acid</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HLA</td>
<td>human leucocyte antigen</td>
</tr>
<tr>
<td>HCL</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HNP</td>
<td>human neutrophil peptide</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>ICE</td>
<td>auto-activated caspase-1 enzyme</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>inductively coupled plasma-mass spectrometry</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>IgA</td>
<td>immunoglobulin A</td>
</tr>
<tr>
<td>IgE</td>
<td>immunoglobulin E</td>
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<tr>
<td>IL-n</td>
<td>interleukin n</td>
</tr>
<tr>
<td>ILC2</td>
<td>type 2 innate lymphoid cell</td>
</tr>
<tr>
<td>IL-1R</td>
<td>interleukin-1 receptor</td>
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<tr>
<td>IM</td>
<td>interstitial macrophage</td>
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<tr>
<td>IrIA</td>
<td>irritant-induced asthma</td>
</tr>
<tr>
<td>iTregs</td>
<td>Foxp3-positive regulatory T cells</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out, genetically modified mouse</td>
</tr>
<tr>
<td>LARN</td>
<td>low-aspect-ratio nanoparticle</td>
</tr>
<tr>
<td>LN</td>
<td>lymph nodes</td>
</tr>
<tr>
<td>LOAEL</td>
<td>lowest-observed-adverse-effect level</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MCh</td>
<td>methacholine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>MGG</td>
<td>May Grünwald-Giemsa</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MWCNT</td>
<td>multi-walled carbon nanotubes</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride, salt</td>
</tr>
<tr>
<td>NEDO</td>
<td>the new energy and industrial technology development organization</td>
</tr>
<tr>
<td>NETs</td>
<td>neutrophil extracellular traps</td>
</tr>
<tr>
<td>n/nanoTiO₂</td>
<td>nanosized titanium dioxide</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NIOSH</td>
<td>the National Institute for Occupational Safety and Health, USA</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NOAEL</td>
<td>no observed adverse effect level</td>
</tr>
<tr>
<td>OCT</td>
<td>oxalcalcitriol compound</td>
</tr>
<tr>
<td>·OH</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>OPS</td>
<td>optical particle sizer</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PAS</td>
<td>periodic acid-Schiff</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>Penh</td>
<td>enhanced pause</td>
</tr>
<tr>
<td>PEST</td>
<td>penicillin–streptomycin</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptors</td>
</tr>
<tr>
<td>PSR</td>
<td>picrosirius red</td>
</tr>
<tr>
<td>RADS</td>
<td>reactive airways dysfunction syndrome</td>
</tr>
<tr>
<td>REL</td>
<td>recommended exposure limit</td>
</tr>
<tr>
<td>R/rCNT</td>
<td>rigid CNT, mitsui-7</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RORα</td>
<td>RAR-related orphan receptor alpha</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SiO₂</td>
<td>silicon dioxide</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SWCNT</td>
<td>single-walled carbon nanotubes</td>
</tr>
<tr>
<td>Tc</td>
<td>CD8 cytotoxic T cells, killer T cells</td>
</tr>
<tr>
<td>tCNT</td>
<td>tangled CNT</td>
</tr>
<tr>
<td>T/CNT</td>
<td>tangled CNT</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscope</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>Th1</td>
<td>type 1 T helper cell</td>
</tr>
<tr>
<td>Th2</td>
<td>type 2 T helper cell</td>
</tr>
<tr>
<td>TiO₂</td>
<td>titanium dioxide</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TSLP</td>
<td>thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>TTIP</td>
<td>titanium tetraisopropoxide</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WT</td>
<td>wild type, normal phenotype mice</td>
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ABSTRACT

Common materials acquire new properties when manufactured in the nanoscale. The same properties that are responsible for the exciting new possibilities are also a cause for some concern. The high surface area to volume ratio is a feature of engineered nanomaterials (ENM) that causes the amount of surface area to dominate their possible effects. In the case of carbon nanomaterials and other fibers, also the shape and length of the particle play important roles. The two ENM studied in this thesis, nanosized titanium dioxide (TiO₂) and carbon nanotubes (CNT), are among the most widely used ENM in the world which means that they hold a high potential of occupational and possible customer exposure.

Inhalation is the most likely exposure route in occupational settings. For this reason, inhalation exposure of mice was the main route of administration. The study settings were chosen to mimic occupational exposure as far as possible. Different immunological parameters were examined in the lungs of the exposed mice, such as the influx of different leucocytes, expression of cytokine and chemokine messenger molecules and changes in the lung tissue.

The results from TiO₂ studies indicated that even a normally inert material when nanosized may become inflammogenic. In addition, even small changes in the structure, or even a coating, may modify radically the nature of a material. Exposure to most nanosized TiO₂ caused only modest to no inflammation, whereas a silica (SiO₂) coated TiO₂ triggered an inflammation characterized by pulmonary neutrophilia and mRNA expression of neutrophil chemoattractant CXCL1 and proinflammatory TNF-α. In tissues and bronchoalveolar lavage (BAL), TiO₂ was readily engulfed by macrophages. In a model of allergic asthma, it was found that exposure to both nanosized and larger TiO₂ seemed to prevent asthmatic symptoms. This underlines the importance of bearing in mind the heterogeneity of the human population when assessing the toxicity of ENM.
CNT have raised some serious concerns in the scientific community and the media due to their similarity in structure to asbestos. Here, a remarkable new type of eosinophilic inflammation was observed in long rigid CNT exposed mice after a week of inhalation to these particles. This inflammation was characterized by strong eosinophilia, goblet cell hyperplasia, Th2 type cytokines and increased airway hyper-responsiveness to methacholine. All of the above symptoms have previously been described as symptoms of classical asthma. Transcriptomic analyses revealed radical up-regulation of innate immunity and cytokine/chemokine pathways. There were also roles found for mast cells and alveolar macrophages in orchestrating the inflammation.

Lastly in the only exposure conducted by aspiration, mice were exposed to two long CNT (rigid/R and tangled/T) and to crocidolite asbestos. From a few hours to 28 days after a single exposure, there was a striking inflammatory cascade starting with macrophages and neutrophils, progressing to eosinophilic inflammation and eventually terminating as granulomas, goblet cell hyperplasia, Charcot-Leyden-like crystals and the mRNA expression of IL-1β, TGF-β, TNF-α and IL-13. The most dramatic inflammation was seen in the R/CNT group, followed by asbestos with T/CNT being clearly more weakly inflammogenic.

In summary, inhalation exposure especially to certain fibrous nanomaterials seems to cause strong pulmonary inflammation. This may put exposed individuals at risk of developing lung diseases. In addition to the material of the nanoparticle, two important factors in risk evaluation are the shape of the particle and the possible modification made (e.g. coating) to the particle. The model of allergic asthma demonstrated that an underlying inflammatory condition can greatly affect the inflammatory outcome seen after nanoparticle exposure. The results of this thesis help to understand the underlying mechanisms in nanoparticle induced pulmonary inflammation.
1 INTRODUCTION

1.1 Engineered nanomaterials and nanotechnology

At the beginning of this millennium, nanotechnology, the engineering of functional systems at the molecular scale, was just about to take off. Around that time, the first studies about the safety of engineered nanomaterials (ENM) were published and a gradual arousal swept through the science community (Ferin et al. 1991, Oberdorster et al. 2005). At first, there was no consensus on what exactly would be best to investigate with regard to nanotoxicology. In fact, at that time, it was not even clear what should be categorized as an ENM. Eventually, as more and more studies emerged, the area of nanosafety research became more focused. Even so, still to this day, researchers have not succeeded in identifying a single characteristic that could be used as a way of assessing the toxicity of a single ENM.

In 2011 the European Commission described a nanomaterial as "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm – 100 nm." (Commission 2011). Nanomaterials, as such, are not new and they exist in nature all around us. They can be found in ocean spray, fine sand, clay, dust, clouds and volcanic ash. They are also produced during human activities e.g. running car engines, burning wood, smoking a cigarette, peeling an orange, making paper copies, mining or even while frying food. Nanomaterials are also present in biological materials, e.g. as viruses or parts of a cell. On the other hand, ENM are materials that have been deliberately manufactured, have one dimension not more than 100 nm and possess carefully designed characteristics such as size, shape, surface properties and possibly attached functional groups. The behavior of ENM often depends more on their surface area than on the actual composition of the particle. It is this high surface area to volume ratio that makes nanomaterials so special in enhancing their potency, electrical properties and reactivity (Lohse 2013). In a 500 nm particle, only about 5% of its atoms are on the surface of the particle, but with a 50 nm particle every second atom is located
INTRODUCTION

on the surface. This large proportion of atoms on the surface means that many atoms are able to react with their environment. In addition to the special properties conferred by their small size, ENM are also more likely to react with cells as they are in the same size range as crucial biological compounds, e.g. proteins and DNA. In fact, nanomaterials can be considered as a link between bulk materials and molecular structures (Dobrovolskaia et al. 2013).

The nanomaterials studied in this thesis, titanium dioxide (TiO2) and carbon nanotubes (CNT), were chosen in the light of their extensive use, leading to substantial potential for human exposure. These nanomaterials belong to two distinctive groups: CNT is categorized as a high-aspect-ratio (HARN) whereas TiO2 is grouped into the low-aspect-ratio nanoparticles (LARN). HARN in general include tubes and wires whereas LARN tend to be spherical, oval, cubic, prism-, helical-, or pillar-shaped ENM. (Editors 2012) In addition to direct exposure to humans, ENM may pose dangers to the environment and eventually humans if not recycled or otherwise managed appropriately after their use. It has proved difficult to assess their effects on wild animals, livestock and eco-systems in general. All in all, it is recommended to acknowledge the entire life cycle of created materials.

1.1.1 Titanium dioxide

Titanium dioxide (TiO2) nanoparticles are widely used in many applications and manufactured worldwide in large quantities. TiO2 exhibits no absorption in the visible region providing it with a very white color. TiO2 also has a very high refractive index, meaning that it scatters light strongly. These properties make it the most widely used white pigment. TiO2 is incorporated into paints, coatings, plastics, papers, inks, pharmaceuticals, food products, cosmetics (especially sunscreens), and toothpaste. In fact, it is in the top five nanoparticles used in consumer products. When particles are nanosized, they possess different physicochemical properties than larger particles of the same composition. This influences their bioactivity and has resulted in a change of opinion about nanosized TiO2. Previously TiO2 was considered to be very inert, in fact it was commonly used as a negative control in many studies (Shi et al. 2013). TiO2 occurs in three crystal structures: anatase, rutile and brookite, (figures 1 and 2), of these, rutile is the most stable. Anatase
functions as a photocatalyst when excited by UV light, and thus is able to hydrolyze water. In addition to these naturally occurring forms, TiO₂ also has eight other modifications: three metastable phases and five high pressure forms (Finnegan et al. 2007, Hashimoto et al. 2005, Winkler 2003).

**Figure 1.** Rutile (www.jejaringkimia.web.id), anatase (www.museumwales.ac.uk) and brookite (www.mindat.org) crystal forms of titanium dioxide.

**Figure 2.** Unit cells of (A) rutile, (B) anatase and (C) brookite. Grey and red spheres represent oxygen and titanium, respectively. http://reuniz.com/introduction-to-titanium-dioxide/

Many research articles have been published on TiO₂ nanoparticles and their toxicology (figure 3). Researchers have been able to demonstrate that the physicochemical characteristics including size, surface properties, shape and the crystal form of nanosized TiO₂ particles have different degrees of toxicity to different organism groups under different conditions (Zhang et al. 2015). It was proposed in a study by (Li et al. 2010), that nanosized (3nm) TiO₂ might pass through the blood–brain barrier (BBB), and induce brain injury through the oxidative stress response. Another study showed that intra-nasally instilled nanosized TiO₂ could potentially be translocated into the central nervous system via the olfactory nerves and there cause potential brain lesions mainly
in the hippocampus (Wang et al. 2008). (Ferin et al. 1992) showed already in 1992 that migration of particles to the interstitium was related to the particle size, the delivered dose, and the delivered dose rate. They also showed that nanosized TiO₂ (20 nm) access the pulmonary interstitium easier than larger finesized TiO₂ (250 nm). A more recent study shows that long-term exposure to nanosized TiO₂ results in its deposition in pulmonary tissue and possibly great alveolar cells (pneumonocytes) (Li et al. 2013). Pulmonary exposure to TiO₂ has been shown to cause at least inflammation, oxidative stress, apoptosis, biochemical dysfunction, cytotoxicity and genotoxicity in murine lungs. Also ecotoxicity, phototoxicity and phytotoxicity has been observed (Sha et al. 2015, Zhang et al. 2015). General remarks are difficult to make because of the vast amount of different materials and unfortunate insufficiency of characterization in many cases.

In addition to exposure by inhalation TiO₂ has vast potential for dermal exposure due to its use in cosmetics and sunscreens. (Wu et al. 2009) showed in their study that nanosized TiO₂ was able to penetrate the skin and to travel to different tissues inducing diverse pathological lesions in hairless mice.

Figure 3. Number of publications listed in PubMed during the years 2000-2016/03. Search words "titanium dioxide, nanoparticles, toxicity" and "carbon nanotubes, toxicity" were used on 29.3.2016.
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However, (Adachi et al. 2013) did not find evidence of skin penetration in the hairless rat. A third dermal exposure study demonstrated an increase in ear swelling, suggesting that extremely high concentrations of nanosized TiO$_2$ may cause dermal irritation (Auttachoat et al. 2014). More research in this area is urgently needed.

TiO$_2$ exposure has also been studied together with asthma on several occasions. (Jonasson et al. 2013) used mice with ovalbumin (OVA)-induced airway inflammation to show that exposure to nanosized TiO$_2$ modulates the inflammation depending on the inflammatory status of the mice. They conclude that exposure to nanosized TiO$_2$ may aggravate respiratory diseases. TiO$_2$ nanoparticles were shown to significantly increase the inflammatory response in toluene di-isocyanate-sensitized animals in a study by (Hussain et al. 2011) while (Larsen et al. 2010) showed that nanosized TiO$_2$ may act as an adjuvant. In another study it was shown that exposure to TiO$_2$ nanoparticles during a critical window of vulnerability in lung development may lead to a higher risk of developing asthma (Scuri et al. 2010). An interesting study done on OVA-sensitized rats showed that allergic pulmonary inflammation is not up-regulated by inhalation of nanosized TiO$_2$, but on the contrary decreases lung inflammation (Scarino et al. 2012).

All in all, the traditional negative control particles are no longer considered valid and the International Agency for Research on Cancer (IARC) has even classified TiO$_2$ as a Group 2B carcinogen (possibly carcinogenic to humans) (Baan et al. 2006).
1.1.2 Carbon nanotubes

Graphene was first produced in a lab in 2004 and in 2010, the Nobel Prize in Physics was awarded “for groundbreaking experiments regarding the two-dimensional material graphene”. Graphene is a one atom thick sheet of pure carbon, with atoms bound in a hexagonal lattice structure. It is the basic building block for carbon nanotubes (CNT). Graphene can also exist as a single layer of graphite. CNT are formed from graphene sheets that are rolled into cylindrical tubes (figure 4). CNT have many unique mechanical and electrical properties, an unusual thermal conductivity and unique tensile strength, which make them attractive materials for a variety of applications. For example, CNT are increasingly appearing in new composite materials, electronics, heating elements and biomedical applications (e.g. bone grafting, dental implants and drug delivery systems). CNT can be divided into single-walled or multi-walled carbon nanotubes (SWCNT or MWCNT) depending on how many layers of graphene they possess. SWCNT usually have an outer diameter of 1-3 nm, whereas MWCNT are 10-200 nm in diameter. CNT vary from a few hundred nanometers to several tens of micrometers in length. It is difficult to evaluate their potential health effects (figure 3) because of the wide range of physical and chemical properties that vary depending on the production techniques and possible functionalization (De Volder et al. 2013, Grosse et al. 2014, Oberdörster et al. 2015, Wang et al. 2013).

Figure 4. Graphene and carbon nanotubes as (A) single wall carbon nanotube (SWCNT) and (B) multi-wall carbon nanotube (MWCNT) structures. (Vidu et al. 2014)
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The dangers that CNT pose are likely to be a sum of features of both ENM and conventional fibers. In general, fibers have been defined by the World Health Organization (WHO) in 1997 to have a length greater than 5 μm, a width less than 3 μm and a length to width ratio (aspect ratio) greater than 3:1 (WHO 1997). When evaluating effects of a CNT, the first property to be considered that can influence the toxicity is the length of the fiber, since this not only affects the deposition of the CNT but also determines whether or not they can be effectively phagocytized and cleared by the macrophages. The critical size according to the paradigm for long fibers is longer than 15-20 μm. The paradigm (figure 5) also states that the composition does not affect the pathogenicity. However features such as reactive surface or the capacity to release biologically active ions, may also contribute to the material’s toxicity. Long fibers are described as those being significantly larger than macrophages (10 μm), thus posing problems for these cells as they try to engulf these fibers. Secondly the biopersistence and the solubility of the CTN affects whether or not the fibers can be broken down and cleared or dissolved completely or if they will remain intact. Biopersistent long fibers result in incomplete/frustrated phagocytosis, cell activation and failed clearance, leading to inflammation, fibrosis and eventually even to the development of a cancer (Boyles et al. 2015, Donaldson et al. 2006, Donaldson et al. 2010, Donaldson et al. 2010, Liu et al. 2012, Oberdörster et al. 2015, Osmond-McLeod et al. 2011).

Figure 5. Diagram illustrating a pathogenic fiber according to the pathogenicity paradigm and the role of particle characteristics. Adapted from (Donaldson et al. 2010) and www.jamesheberg.com.
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In general, studies so far show that pulmonary exposure of both mice and rats to MWCNT causes inflammation, granulomas and interstitial fibrosis (Aiso et al. 2010, Oberdörster et al. 2015, Pauluhn 2010). A study by (Sargent et al. 2014) e.g. indicated that exposure to MWCNT strongly promotes the formation of lung tumors. It has also been shown that MWCNT are able to travel to the pleura and diaphragm once deposited in the lungs (Mercer et al. 2010, Mercer et al. 2013, Ryman-Rasmussen et al. 2009). Evidence also points to qualitatively similar results with both inhalation and bolus (e.g. aspiration) exposure studies as well as both commonly used rodents, mice and rats (Kasai et al. 2015, Mercer et al. 2013).

The authors of a recent paper (Kolosnjaj-Tabi et al. 2015) claimed that humans are routinely exposed to anthropogenic CNT. They claimed that CNT are the main component of fine particulate matter (PM) in air pollution thus contributing to adverse health effects and responsible for deaths all around the world. The effects caused by PM$_{2.5}$ (PM with diameter of less than 2.5$\mu$m) have been ranked as one of the leading causes of death and disability across the world (Lim et al. 2012). The participants in that study were asthmatic Parisian children and whether or not their asthma was a cause or an effect of the CNT exposure remains to be clarified (Kolosnjaj-Tabi et al. 2015).

Exposure to MWCNT and asthma have been linked in a couple of studies. (Ronzani et al. 2013) showed that when mice were exposed to house dust mite and MWCNT, the MWCNT dose-dependently increased the systemic immune response, airway allergic inflammation and remodeling induced by house dust mite exposure alone. In another study by (Mizutani et al. 2012) exposure to MWCNT and an antigen (OVA) demonstrated a biphasic increase in airway resistance, airway inflammation, goblet cell hyperplasia, and the production of antigen-specific antibodies. (Ryman-Rasmussen et al. 2009) studied whether inhaled MWCNT would increase airway fibrosis in mice with allergic asthma and concluded that individuals with pre-existing allergic inflammation may be susceptible to airway fibrosis from inhaled MWCNT.
1.2 Occupational safety

Nanosized TiO$_2$ and CNT are materials to which many workers may come into contact. There are some scenarios where occupational exposure levels have been assessed e.g. processes where these materials are produced, transported, weighted, blended, mixed, inserted into applications or researched (Bello et al. 2008, Bello et al. 2010, Cena et al. 2011, Dahm et al. 2012, Erdely et al. 2013, Han et al. 2008, Johnson et al. 2010, Lee et al. 2010, Maynard et al. 2004, Methner et al. 2010, Tsai et al. 2009). Occupational exposure is most likely to happen during production and handling of the material and in the cleaning of the production reactor. In these settings, CNTs usually occur as entangled respirable agglomerates. Exposure to CNT during other parts of their life cycle is possible, but regarded as being low according to the available data (Grosse et al. 2014, Kingston et al. 2014, Nowack et al. 2013).

There has been effort expended on the occupational safety front to set safe limits for exposure to different nanomaterials. Although this is a far-from-easy task, attempts have been made to produce critical values like no-observed-adverse-effect-levels (NOAEL) and lowest-observed-adverse-effect-levels (LOAEL), which are widely used in risk assessment and by agencies defining safe levels of exposure or use. It is therefore crucial that these levels are derived from research-based findings, and this means that experiments need to be conducted to gain more information which can then be applied in the exposure assessment. A recommended exposure limits (REL) of 1μg/m$^3$ has already been set for CNT by the US National Institute for Occupational Safety and Health (NIOSH Current Intelligence Bulletin 65, 2013; (Zumwalde et al. 2013)). Also Pauluhn et al. (Pauluhn 2010) set a limit of 0.05mg/m$^3$ for MWCNT (Baytubes) using a NOAEL from a 13-week subchronic inhalation study on rats. Both levels indicate a low safe exposure limit for CNT. NIOSH has also given a REL for ultrafine (including engineered nanoscale) TiO$_2$ which is 0.3mg/m$^3$. This is 10 times lower than the REL for fine TiO$_2$ (Dankovic et al. 2011). They also have assessed ultrafine TiO$_2$ as a potential occupational carcinogen. The New Energy and Industrial Technology Development Organization (NEDO) Project of Japan determined the
acceptable exposure concentration of titanium dioxide to be 1.2 mg/m$^3$ (Morimoto et al. 2010).

In addition to REL, other strategies exist to help protect workers. These include worker education and training, good working practices and work hygiene, personal protection (protective clothing and respirators) and in some cases medical screening and surveillance. It is believed that these kinds of combined strategies can help to control and minimize workplace exposures to ENM (FIOH 2015).

1.3 Immunity

Humans have three layers of protection against environmental pathogens: the physical barrier followed by non-specific and specific defenses. The physical barrier consists of the epithelial layers that reside underneath skin and mucous membranes. Each human being is born with an innate immunity system that matures during childhood and mounts non-specific responses to pathogens. These responses consist of cytokines, antimicrobial substances, fever and phagocytosis and they are aided by many white blood cells, including macrophages, neutrophils, mast cells, eosinophils, basophils and natural killer cells (Medzhitov et al. 2000). Recent evidence shows that innate immunity possesses a nonspecific immunological memory mediated through epigenetic reprogramming in myeloid cells or NK cells termed “trained immunity” (Netea et al. 2016). The adaptive immune system evolves throughout our lives and is altered every time a new antigen is encountered. It produces antibodies that are targeted towards certain pathogens and therefore generates pathogen-specific immunity that sometimes lasts for a lifetime (Medzhitov et al. 2000).

1.3.1 Pulmonary immunology and inflammation

Nanomaterials can enter the body through the gastrointestinal (GI) tract (via digestion), the skin (via penetration), blood vessels (via intravenous injection) or through the lungs (via inhalation) which is most critical exposure route for humans (Krug et al. 2011). Because of the critical role of inhalation exposure especially in the occupational context, the present research has focused on the lungs and their responses to the presented ENM.
During respiration, the human lungs are continuously exposed to a huge load of airborne pathogens and particles. As much as 10,000 liters of air pass through our largest epithelial surface daily, and therefore the lungs must possess robust mechanisms to house an efficient host defense system. As many entering pathogens have the potential to cause lethal infection in the fragile tissue designed for gas exchange, the body must mount an appropriate and well-regulated immune response in order to clear infections rapidly and prevent the development of chronic inflammation. Up to $10^{10}$ particles a day reach the alveolar region and the immune system has to process them without causing inappropriately excessive inflammatory responses that could potentially alter the function and architecture of the airways (Bals et al. 2004, Boyton et al. 2002, Crapo et al. 2000).

The lungs have many lines of defense against the entry of foreign material. The first line of defense is located in the upper respiratory tract where most of the inhaled particles are removed. In that region, most of the inhaled particles are deposited due to turbulent airflow on the mucus that coats the surface of the airways. The mucociliary clearance system then acts to move most particles to the posterior pharynx, where they are swallowed and then moved through the GI tract (Bals et al. 2004, Boyton et al. 2002, Crapo et al. 2000).

**Figure 6.** Predicted total and regional deposition probabilities of inhaled particles in nasopharyngeal (upper airways), tracheobronchial, and alveolar region of the human respiratory tract. Adapted and printed with permission from (Koivisto 2013).
Figure 6 demonstrates that the larger particles (over 1 μm) are mostly trapped in the upper part of the respiratory tract, the smallest (under 10 nm) in the tracheobronchial region and particles between 10 and 100 nm penetrate into the deepest parts of the lungs, to the alveoli. In the field of occupational safety, two size fractions are considered relevant: inhalable (below 10 μm) and respirable fractions (below 4.5 μm aerodynamic diameter). The inhalable fraction is considered to be able to enter the body and the respirable fraction to be capable of penetrating beyond the ciliated airways (Donaldson et al. 2006).

Particle deposition onto respiratory tract depends on many factors such as aerodynamic diameter, hygroscopic properties, and charge of the particles. Particles over 1 micrometer are deposited mainly by impaction and particles below 10 nm by diffusion in the upper airways (blue region in figure 6). The main deposition mechanisms in the tracheobronchial region are diffusion, impaction and interception whereas in the alveolar region, the predominant mechanisms are diffusion and gravitational settling due to air residence time (Asgharian et al. 2007).

Particles that manage to escape the mechanical barriers may travel through airways and airspaces, the interstitium and the vasculature. In all these compartments, they may undergo processing and may be further presented to the extensive lymphoid tissue surrounding the lungs.

1.3.2 Innate immunity in the lung

The next level of defense after the mechanical barrier is the innate immunity system. Many innate responses have evolved to recognize and respond to conserved structures in micro-organisms, such as lipopolysaccharide (LPS), and these have been conserved through evolution. These pathogen-associated molecular patterns (PAMP) as well as danger associated molecular patterns (DAMP) are recognized by the pattern recognition receptors (PRR) present on many immune cells. DAMP, e.g. heat-shock proteins, are released in cases of
tissue injury and they also participate in the clearance of damaged or apoptotic host cells (Medzhitov et al. 2000).

Particles that enter the airway surface fluid first encounter a range of soluble mediators. The lavage fluid and sputum contain substantial amounts of lysozyme; this enzyme acts as an important part of the antimicrobial defense and it is made by surface epithelial cells, macrophages and glandular serous cells. Lactoferrin is another mediator present in the airway; it is a component that kills and agglutinates bacteria and is produced by serous cells and neutrophils. Other important components include the α- and β-defensins, the collectins and immunoglobulin A (IgA). Human α-defensins or human neutrophil peptides (HNP) are present in abundance within neutrophils whilst β-defensins are located within the tracheal epithelia. They are both peptides with broad antibiotic activity against bacteria, fungi, mycobacteria and enveloped viruses and therefore their function is to eliminate or prevent the colonization of pathogenic organisms (Fang et al. 2003). The collectins are humoral molecules that recognize PAMPs present in plasma and on mucosal surfaces. They can implement effector mechanisms like direct opsonization, neutralization, agglutination, complement activation and phagocytosis to restrain microbial growth. They can also modulate inflammatory and allergic responses and apoptotic cell clearance. In all, the collectins limit infection and as a result, they attempt to modulate the adaptive immune responses (Gupta et al. 2007). IgA is the major class of antibody present in the mucosal secretions and it mediates a variety of protective functions via its interaction with specific receptors and immune mediators (Woof et al. 2006). A direct, rapid and autonomous response that can be made by epithelial cells and macrophages is the release of type I interferons (IFNs) which leads to a release of factors that can interfere with viral replication (Bals et al. 2004, Boyton et al. 2002, Crapo et al. 2000).

The lungs contain many immune cells with a variety of functions that deal with those particles that succeed in passing through its outer layers. There is a substantial population of dendritic cells (DCs) in the lungs that have an important role in defense, especially as antigen-presenting cells (APC). Furthermore, in some cases, macrophages and B cells can act as APC. Alveolar macrophages are however usually recognized as poor APC and it has
been postulated that they more commonly function to inhibit further amplification of immune pathways and quietly clear away any antigens that they come across. When macrophages encounter intruding foreign matter, their first response is to attempt to phagocytize the material. Alveolar macrophages have an important role in maintaining airway immune homeostasis, host defense and tissue remodeling. They are very flexible cells and can be specifically modified to fit the special needs of the lungs at any given time; this property is dependent on both the macrophages’ state of differentiation and the status of the micro-environment. Alveolar macrophages communicate with other cells and molecules via specific surface receptors and by releasing many secretory products. They also exhibit many PRRs used to recognize PAMPs and are involved in the phagocytosis of apoptotic and necrotic cells. Macrophages also express a multi-protein complex called the inflamasome, which controls the activation and maturation of interleukin-1β (IL-1β), a major pro-inflammatory cytokine (Hussell et al. 2014). Interstitial macrophages (IMs) are macrophages that reside inside the lung tissue. This cell type and its function have remained somewhat unknown and not yet fully characterized. (Bedoret et al. 2009) stated that IMs have a major role in maintaining immune homeostasis in the respiratory tract. The IMs were able to inhibit lung DC maturation and migration upon OVA + LPS stimulation. This is vital in preventing sensitization to inhaled antigens. The lung DCs may be paralyzed by the IL-10 produced by IMs, therefore allowing harmless antigens to pass without T cell–dependent responses.

Sometimes when the infectious, irritant or antigenic burden becomes too massive for the resident cells to handle, they call for help by releasing mediators that attract inflammatory cells to the site of attack. Neutrophils are recognized as major players in acute inflammation and they are usually the first cells to be recruited to the infected or damaged site. Neutrophils are continuously generated in the bone marrow from myeloid precursors in a process that is controlled by granulocyte colony stimulating factor (G-CSF). In times of inflammation, there is an increase in the number of neutrophils in tissues. Neutrophils usually live only for some hours, but when activated, they can endure for some days. Under normal conditions, these cells can be found in the bone marrow, spleen, liver and especially in the lungs. There is some
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evidence (Kolaczkowska et al. 2013) that there are distinct neutrophil subsets with distinct functions, but this theory needs to be confirmed. Neutrophils migrate to the lungs from the vasculature following a chemokine gradient along the endothelium. These chemokines and the increase in the permeability of local blood vessels are attributable to the actions of resident macrophages and mast cells. When neutrophils have been activated, they become extremely effective in phagocytizing and neutralizing bacteria. They have distinct killing mechanisms and can act both intra- and extracellularly. Phagocytized pathogens are eliminated inside phagosomes by the many antibacterial proteins released from granules. Neutrophils can also release these proteins as well as antimicrobial histones and proteases into the extracellular milieu to target extracellular pathogens. Another extracellular mechanism immobilizes pathogens using neutrophil extracellular traps (NETs). Activated neutrophils can also further release many other factors such as IL-6, IL-17, TNF-α, IFN-γ, defensins and IL-1β. Most neutrophils die in the infected tissue while performing their function and are cleared away by macrophages. In addition to these pro-inflammatory tasks, the neutrophils have been shown to possess anti-inflammatory and healing roles and also participate in adaptive immunity (promoting humoral and suppressing cellular response) (Kolaczkowska et al. 2013).

Eosinophils develop in the bone marrow from haematopoietic stem cells and their natural role is to defend the body against parasites. Eosinophils contain granules which contain enzymes that are released during infections, allergic reactions, and asthma. They normally represent less than 5% of leucocytes in the blood, but if their numbers should suddenly increase, this can be a symptom of many disorders such as allergies, asthma, atopic dermatitis, metabolic disorders and the hypereosinophilic syndrome. Recently, roles in malignancy and in regulating antibody production as well as participating in tumor formation have been proposed. When activated, eosinophils are recruited from the blood into the tissues where they act by releasing several different products that can also be toxic to airway epithelial cells and may contribute to tissue damage, organ dysfunction and tissue remodeling or result in a diverse biological activity of eosinophils in infection and inflammation. In asthma, the eosinophils are involved in airway hyper-reactivity, elevated
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mucus production, airway remodeling and asthma exacerbations (Fulkerson et al. 2013, Gleich 2000).

There is one final line of defense in the innate response; the natural killer (NK) cells. These cells scout through the body searching out cells with altered expression of human leucocyte antigen (HLA) class I tissue antigens. This altered expression is caused either by viral infection or transformation and if detected by NK-cells, it leads to their activation and to the lysis of the infected cells and also the release of interferon-gamma (IFN-γ). IFN-γ in turn may recruit other cells to this site.

The mast cells are nowadays considered a link between the innate and adaptive immunity. They were first described by Ehrlich (Ehrlich 1956) already in 1878 and have been considered major players especially in the early and acute stages of allergic reactions. Mast cells are derived from haematopoietic progenitor cells and they circulate in the blood in an immature form. They are distributed widely in the body, but are found particularly in the skin, respiratory mucosa and the GI track. After migrating to the vascularized tissues, the mast cells undergo final differentiation and maturation assisted by the stem-cell factor and other cytokines secreted by endothelial cells and fibroblasts. When activated, the mast cells undergo rapid degranulation releasing a variety of potent inflammatory mediators present within their cytoplasmic granules: histamine, proteases tryptase and chymase, chemotactic factors, cytokines (such as pre-formed TNF-α) and metabolites of arachidonic acid. These mediators then act on the vasculature, smooth muscle, connective tissue, mucous glands and inflammatory cells. Some hours after activation, the transcriptional up-regulation of cytokines and chemokines, including TNF-α and interleukin-4, can be observed. In all, the mast cells are capable of eliciting a wide array of responses that may occur alone or in combination depending on the stimulus.

The mast cells also play a central role in the initiation of the allergic reaction as they sometimes respond in an inappropriate manner to innocuous antigens. They act as the main effector cell responsible for IgE-mediated allergic reactions. Mast cells are also capable of processing and presenting antigens
through MHC I and MHC II complexes, thus playing an important role in sensitization. Furthermore, they provide signals that induce IgE synthesis by B-lymphocytes and also induce Th2 lymphocyte differentiation. Because of their crucial location, their evident plasticity, and the various mediators they are able to produce, the mast cells prove to be important immune effector and modulatory cells that bridge the innate and adaptive immunity (Amin 2012, da Silva et al. 2014, Urb et al. 2012).

1.3.3 **Adaptive immunity in the lung**

If the innate immune defenses are unable to terminate an attack, the adaptive immune system is activated. The adaptive response can be mediated by the antibodies that are produced by B lymphocytes. In this case, the response is often called humoral. In the cell-mediated immunity, the main players are called T lymphocytes. The B cells use antibodies to attack invading bacteria, viruses, and toxins. The T cells on the other hand target the body’s own cells that have themselves been infected by viruses or become cancerous.

Just as in other tissues of the body, the adaptive immune response starts with immature DCs at the site of inflammation ingesting the pathogen and travelling to the regional lymph nodes (LN). While they travel to the LN, the DC mature and start expressing co-stimulatory molecules in addition to breaking down the pathogen and displaying its pathogen peptide fragments on their surface. Exogenous antigens are broken down and presented in the MHC class II complexes while the endogenous antigens, self- or viral proteins, are cleaved by proteasomes and turned into MHC class I peptide complexes (Lesterhuis et al. 2004). When DC reach the LN, they encounter naïve T cells that possess T cell receptors (TCR) that are able to recognize specific antigenic pathogen peptides. When the mature DC encounters a naïve T cell that recognizes and binds the specific antigenic peptide, the T cell becomes activated not only via the signal of the antigen recognition but also by the co-stimulatory molecules expressed on the mature DC. Once the naïve T cell is activated, it differentiates into an effector T cell and makes multiple copies of itself.
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Effector T cells are classically divided into three classes: CD8 cytotoxic (killer, Tc) T cells, CD4 T helper 1 (Th1) cells and CD4 T helper 2 (Th2) cells. Tc cells kill target cells, often infected by intracellular pathogens such as viruses, that display antigens bound to major histocompatibility complex (MHC) class I molecules on the cell surface. Th cells, which both express the CD4 co-receptor, respond to antigens displayed on MHC class II molecules. Recently, also other lineages of Th cells have been described: e.g. Th17, regulatory T cells (Treg), Th22, Th9 and follicular helper T cells (Tfh).

1.3.3.1 Th1 vs Th2

The oldest known classes of Th cells, types 1 and 2 respectively, represent two very distinctive aspects of the adaptive immunity. Whether T cells develop into Th1 or Th2 can depend on many factors that influence the polarization e.g. the route of antigen entry, type of the APC, cytokine microenvironment and the expression of co-stimulatory molecules. Th1 cells are produced in response to intracellular pathogens, apoptosis of tissue cells and autoimmune diseases. Th2 cells, on the other hand, respond to extracellular parasites and allergic inflammation. IL-12, IFN and transcription factor T-bet have been associated with the Th1 inducing pathway, whereas IL-4 and transcription factor GATA3 have been linked to the Th2. Once activated, Th1 cells produce many different bioactive compounds, e.g. IFN-γ, IL-2 and TNF whereas Th2 cells synthesize IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Th2 cytokines are able to induce all of the hallmarks of allergic inflammation and asthma: IgE antibody production in B-cells, airway remodeling, mast cell growth, eosinophil accumulation, mucus hyper-production and airway hyper-responsiveness (Romagnani 2000). Th1 cells are specialized in activating macrophages, whereas Th2 cells mainly target B cells. (figure 7) Th1 and Th2 cells compete by inhibiting the differentiation of naïve CD4 T cells into each other, i.e. Th2 produced cytokines inhibit Th1 differentiation and vice versa (Boyton et al. 2002, O’Garra 2000, Rincón et al. 1997).
**Figure 7.** Different factors in Th1 and Th2 differentiation, the cytokines produced and the target cells.
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1.3.4 Cytokines

Different immune cells communicate with other cells by releasing small proteins called cytokines. The responding cells have specific receptors on their surfaces to which the cytokines bind. Cytokines are a very diverse group of biomolecules that is able to provoke a variety of responses in sensitive cells. Cytokines act to ensure that there is coordination of the appropriate immune responses. For example as described before, the subtypes of T cells are partially determined by the cytokine microenvironment in which they are maturing and the cytokines they produce upon activation are used to describe the different T cell subtypes.

Interleukins are a group of cytokines first found to be secreted by leucocytes to communicate with other leucocytes. Later they were found to be expressed by several other cells as well. IL-1β belongs to the IL-1 family and is a potent pro-inflammatory cytokine that acts as an endogenous pyrogen. IL-1β is one of the most important cytokines involved in the initiation and persistence of inflammation and for this reason, its induction is stringently controlled. IL-1 is produced mainly by macrophages and monocytes, but also by bronchial and alveolar epithelial cells, neutrophils, T-cells and fibroblasts. The pro-IL-1β is a precursor of the active form of IL-1β and it lacks biological activity. Auto-activated caspase-1 enzyme (ICE) or alternatively proteases cleave pro-IL-1β into its active form (Dinarello 1996, Lappalainen et al. 2005, Martinon et al. 2002). The effects on the immune functions of IL-1 family members are indirect, but they are able to provoke fever, reduced pain threshold, vasodilatation and hypotension. IL-1β can increase the expression of adhesion molecules and induce chemokines. It is also an angiogenic factor and in this respect, it plays a role in tumor metastasis and blood vessel formation (Dinarello 2009). IL-33 also belongs to the IL-1 family; it is a strong inducer of Th2 responses. IL-4, IL-5 and IL-13 are considered Th2 type cytokines that are usually expressed during IgE-mediated allergy. They are known to mediate IgE production, eosinophilia and immunity against helminth infection. IL-4 belongs to the γ-chain family, regulates allergic conditions and is a major stimulus of Th2-cell development. IL-5 is a strong promoter of eosinophilia and an inducer of hyper-reactivity in asthmatic patients. IL-13, on the other hand, has been linked to fibrosis, induction of IgE production, regulation of
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NF-kB activation and related cytokine/chemokine generation, activation and recruitment of mast cells and eosinophils while promoting their survival as well as some anti-inflammatory properties. IL-10 is a classical member of the family to which it gave its name (the IL-10 family) and acts as an anti-inflammatory factor regulating the inflammatory response (Akdis et al. 2011, Lentsch et al. 1999).

IFN-γ is an important link between the innate and adaptive systems; this cytokine is secreted by cell populations belonging to both immunity systems. IFN-γ has numerous roles in addition to activating macrophages, for example it can interfere with viral infection mainly by inducing antiviral enzymes (Akdis et al. 2011).

A member of the TNF superfamily, TNF-α, is first produced in a transmembrane form that is expressed by activated macrophages and lymphocytes and perhaps by other cells as well. The soluble form is then cleaved from the membrane. There is recent evidence suggesting that both forms are involved in inflammatory responses. TNF-α has been described as one of the most important inflammatory mediators and its activation is a crucial component of the innate immune system (Bradley 2008, Hehlgans et al. 2005).

Transforming growth factor-β (TGF-β) is considered to be anti-inflammatory, but it has a strong pleiotropic role - its effects can be different, or even opposite, depending on the cell type and the conditions. It has an important role in establishing immunological tolerance, but also has pro-inflammatory roles. For example, normally TGF-β induces Foxp3-positive regulatory T cells (iTregs), but when IL-6 is present, it induces the creation of the pathogenic IL-17 producing Th17 cells (Massague 2012).

Chemokines are a specific group of cytokines that chemically attract more immune cells to the site of inflammation to help combat invaders or repair
tissue damage. Many of these agents have additional homeostatic or housekeeping functions. Chemokines possess three to four conserved cysteine residues and are subdivided into 4 families based on the position of the N-terminal cysteine residues. The C-X-C-family and C-C family can also be distinguished by their primary target cell, with the C-X-C subfamily targeting neutrophils and the C-C family targeting eosinophils, monocytes, and T cells (Commins et al. 2010). The specific chemokines examined in this thesis are listed below in Table 1.

Table 1. Chemokines appearing in this thesis listed with their physiologic features and functions. Adapted from (Commins et al. 2010).

<table>
<thead>
<tr>
<th>CC chemokine/receptor family</th>
<th>Physiologic features</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2</td>
<td>inflammation</td>
<td>contributes to AHR and cellular emigration in asthma, promotes TH2 phenotype, role in wound healing</td>
</tr>
<tr>
<td>CCL3</td>
<td>inflammation, homeostasis</td>
<td>promotes development of IFN-γ TH1 cells, role in asthma</td>
</tr>
<tr>
<td>CCL7</td>
<td>inflammation</td>
<td>promotes TH2 phenotype, role in asthma</td>
</tr>
<tr>
<td>CCL11</td>
<td>inflammation, homeostasis</td>
<td>contributes to AHR and cellular emigration in asthma, most important eosinophil chemoattractant in allergic inflammation</td>
</tr>
<tr>
<td>CCL17</td>
<td>inflammation, homeostasis</td>
<td>promotes TH2 phenotype</td>
</tr>
<tr>
<td>CCL24</td>
<td>inflammation</td>
<td>role in asthma</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CXC chemokine/receptor family</th>
<th>Physiologic features</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL1</td>
<td>inflammation, homeostasis</td>
<td>angiogenic, neutrophil chemoattractant</td>
</tr>
<tr>
<td>CXCL2</td>
<td>inflammation</td>
<td>angiogenic, chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells</td>
</tr>
<tr>
<td>CXCL5</td>
<td>inflammation</td>
<td>angiogenic, neutrophil chemoattractant</td>
</tr>
<tr>
<td>CXCL8</td>
<td>inflammation</td>
<td>role in wound healing, asthma and allergic diseases, one of the most potent neutrophil chemoattractants</td>
</tr>
<tr>
<td>CXCL9</td>
<td>inflammation</td>
<td>T-cell chemoattractant, induced by IFN-γ</td>
</tr>
</tbody>
</table>
1.3.5 Transcription factors

Transcription factors are proteins that bind to specific DNA sequences. When binding to DNA, they either increase or decrease gene transcription. FOXP3 (forkhead box P3), a member of the forkhead transcription factor family, is mainly expressed in a subset of CD4+ T-cells that act as suppressors in the immune system. FOXP3 also functions as a transcription activator for many genes. It is known that FOXP3 plays a crucial role in the development and function of regulatory T cells and is used as a marker for regulatory T cells (Kim 2009).

1.4 Asthma

Asthma and allergies have become everyday acquaintances of the citizens in the industrialized countries, where these disorders are distressingly common. It has been estimated that 300 million people worldwide suffer from asthma and 250,000 people die annually because of the disease. About 70% of asthmatics also have allergies (WHO 2007). The development of asthma in an individual is a product of a genetic predisposition and environmental conditions. Children in wealthy countries are more likely to develop allergy-related asthma than children in poorer nations (Weinmayr et al. 2007). The reasons for increasing asthma occurrence are still to be established, but they have been linked to improved hygiene, antibiotics, changes in the diet, vaccinations and increased exposure to novel chemicals and environmental particulate matter (Holtzman 2012, Pandya et al. 2002, Rook 2012, Silverman et al. 2010, Stieb et al. 2009).

Asthma is a multifaceted chronic inflammatory disorder of the airways that causes pulmonary obstruction. Symptoms, such as mucus production and airway hyper-reactivity (AHR), that vary extensively between individuals can be triggered by exposure to allergens or irritants such as chemicals (e.g. tobacco smoke) or by exercise or infection (Lloyd et al. 2010, William W. Busse et al. 2007). The complexity of asthma has recently become more evident and it seems that rather than a single disease, asthma is a combination of different disease processes with variable etiologies and pathophysologies. Allergic asthma is considered to be a so-called endotype of asthma that is
INTRODUCTION

categorized by airway exposure to an allergen that triggers a hypersensitivity response in a sensitized individual involving an influx of T lymphocytes and eosinophils into the lungs (Afshar et al. 2008, Lötvall et al. 2011). There are many immune and inflammatory cells involved in asthma (Barnes 2008), which are presented in more detail in figure 8.

Figure 8. Immune and inflammatory cells involved in asthma.

Epithelial cells release stem cell factor (SCF), which keeps mucosal mast cells at the airway surface. These cells also release thymic stromal lymphopoietin (TSLP) together with mast cells to condition myeloid dendritic cells and in addition, secrete the eosinophil chemoattractant CCL11. Allergens are processed by dendritic cells, which react by secreting CCL17 and CCL22, which in turn attract T helper 2 (T\textsubscript{h}2) cells. Allergens also activate sensitized mast cells through surface bound IgE molecules. Mast cells react by releasing cysteinyl leukotrienes and prostaglandin D\textsubscript{2} that in turn act to evoke bronchoconstriction. T\textsubscript{h}2 cells act as central mediators of the inflammatory response by releasing interleukin-4 (IL-4), IL-13, IL-5 and IL-9. IL-4 and IL-13 stimulate B cells to produce IgE, IL-5 stimulates eosinophilic inflammation and IL-9 stimulates mast-cell proliferation. Regulatory T (T\textsubscript{Reg}) cells are known to malfunction in asthmatic patients, which may help T\textsubscript{h}2 cells to proliferate. Adapted from (Barnes 2008).
INTRODUCTION

In 1985 (Brooks et al. 1985) Brooks et al. proposed a new type of occupational asthma called Reactive Airways Dysfunction Syndrome or RADS. This asthma-like syndrome develops quickly, within hours, after a single exposure to high levels of irritating fume, smoke, vapor or aerosol. RADS differs from traditional asthma in particular due to its acute nature with no latency period. The initial respiratory symptoms are followed by asthma-like symptoms and airway hyper-responsiveness. Irritant-induced asthma (IrIA) is another more recent term used to describe an asthmatic syndrome that results from a single or multiple high dose exposures to irritant products. There is little information available on these conditions, but they have been claimed to include significant upper airway symptoms, nonspecific inflammation, neurogenic inflammation, primarily lymphocytic cellular infiltrate, macrophage activation, mast cell degranulation and epithelial desquamation (Brooks 2013, Shakeri et al. 2008, Tarlo 2013, Varney et al. 2011).

1.4.1 The mouse model of asthma

Allergic asthma is mostly studied using mouse models, because ethical reasons forbid carrying out mechanistic studies on asthmatic individuals. Due to the very complex nature of allergic asthma, the models cannot give answers to all crucial questions, but they can be used to model specific features of the disease. In this thesis we used an asthma model to study reactions to ENM exposure in mice owing an already burdened immunological status. A variety of different models and protocols have been employed in the studies of allergic asthma and naturally the findings and conclusions may be influenced by the choice of mouse strain, the allergen, as well as the type of sensitization and challenge protocol.

The basic and commonly used model (Bates et al. 2009), that was also used in this thesis, begins with protein sensitization (usually with ovalbumin) which is followed by an airway challenge. Sensitized mice are typically injected intraperitonally twice with a combination of the protein and an adjuvant (e.g. alum). The injections are given one to two weeks apart. Approximately one week after the last injection, the mice are challenged three days in a row to the allergen by inhalation or alternatively by intranasal or intratracheal
INTRODUCTION

administration. The inflammatory parameters are measured two days after the last challenge. This model reproduces many key elements of clinical asthma, e.g. elevated levels of IgE, airway inflammation, goblet cell hyperplasia, epithelial hypertrophy, high concentrations of Th2 type cytokines and chemokines as well as AHR and eosinophilia (Nials et al. 2008).
AIMS OF THE STUDY

2 AIMS OF THE STUDY

When this thesis work was started, the world of nanotoxicology research was an uncharted one. As more and more people potentially became in contact with ENM in their workplaces, concerns arose about the safety of these materials. From the occupational safety point of view, this thesis aimed to contribute to the debate of whether or not ENM would pose a risk to individuals working with these materials. In general, it was decided to examine whether, and if so how, these nanoparticles could evoke pulmonary inflammation. This thesis also tackles asthma from the point of view of a part of the population that might be more sensitive to ENM exposure. In addition, in vitro experiments were used, whenever they could add value to the research.

The specific aims were:

1. To investigate the direct effects of repeated nanoparticle exposure on the development of lung inflammation in vivo.

2. To examine how exposure to nanoparticles modulates the development of allergic asthma in vivo.
3 MATERIALS AND METHODS

3.1 Materials (I, II, III, IV)

The materials and their characteristics are listed in table 2. When purchased materials are aerosolized, they tend to form agglomerates (Nichols et al. 2002). This same phenomenon would however also take place in occupational settings and thus presents no problem to this research. In aerosol characterizations done during exposures we could see that the aerosols contained agglomerates and primary particles alike. (I, supplementary figure 2; II, figure 2; IV, figure 7).

3.1.1 Aerosol and particle characterization for nanoparticle powders (I, II)

In the TiO2 exposures, the aerosol size distributions were measured from 10 to 1000 nm with a scanning mobility particle sizer consisting of a 63Ni bipolar aerosol neutralizer, a Vienna type differential mobility analyzer with a length of 28 cm, and a TSI model 3010 condensation particle counter (Wang et al. 1990). Aerosol aerodynamic size distribution was measured from 15.9 nm to 10 µm with an electronic low-pressure impactor (Dekati Ltd., Tampere, Finland) (Marjamäki et al. 2000). The aerosol mass concentration was determined gravimetrically after collection on nitrocellulose filters (Millipore, Billerica, MA). The particle concentration inside the particle dispenser chamber was also monitored using a personal DataRAM (online mass concentration meter, pDR1000AN; Thermo Scientific, Waltham, MA).
### MATERIALS AND METHODS

**Table 2.** The materials used in the experiments of this thesis.

<table>
<thead>
<tr>
<th>Name</th>
<th>nano TiO₂</th>
<th>nano TiO₂</th>
<th>nano TiO₂</th>
<th>coarse/fine TiO₂</th>
<th>nano SiO₂</th>
<th>tangled CNT</th>
<th>rod-like CNT</th>
<th>asbesotos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer and tradename/product number</td>
<td>Sigma-Aldrich 637262</td>
<td>Sigma-Aldrich 637254</td>
<td>Nano Amor 4850MR</td>
<td>FIOH, reactor-generated</td>
<td>Sigma-Aldrich 224227</td>
<td>Nano Amor 5485HT</td>
<td>Cheap Tubes Inc., MWCNTs 8-15nm OD</td>
<td>Mitsui &amp; Co. Ltd., XNRI MWNT-7</td>
</tr>
<tr>
<td>Particle size</td>
<td>10x40nm</td>
<td>&lt;25nm</td>
<td>30-40nm</td>
<td>~21nm</td>
<td>&lt;5μm</td>
<td>10nm</td>
<td>OD 8-15nm, length 10-50μm</td>
<td>OD &gt; 50nm, length ~13μm</td>
</tr>
<tr>
<td>Specific surface area</td>
<td>132 m²/g</td>
<td>222 m²/g</td>
<td>23 m²/g</td>
<td>61 m²/g</td>
<td>2 m²/g</td>
<td>51.5 m²/g</td>
<td>&gt;233 m²/g</td>
<td>26 m²/g</td>
</tr>
<tr>
<td>Form/type</td>
<td>rutile</td>
<td>anatase</td>
<td>rutile: anatase (9:1)</td>
<td>anatase: brookite (3:1)</td>
<td>rutile</td>
<td>amorphous</td>
<td>multiwalled</td>
<td>multiwalled</td>
</tr>
<tr>
<td>Composition</td>
<td>Ti, O, SiO₂ – coating (&lt;5%)</td>
<td>Ti, O</td>
<td>Ti, O</td>
<td>Ti, O</td>
<td>Ti, O</td>
<td>Si, O</td>
<td>C, residues of Ni and Fe in total &lt;1wt%</td>
<td>C, residue content below EDS detection limit 0.1wt%</td>
</tr>
<tr>
<td>Publication</td>
<td>I, II</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>II</td>
<td>I</td>
<td>III, IV</td>
<td>III, IV</td>
</tr>
</tbody>
</table>

**Notes:**
- **Composition** for **nano TiO₂** includes SiO₂ coating (<5%).
- **Publication** references are I, II, III, IV.
MATERIALS AND METHODS

The size and morphology of the nanopowders were characterized by electron microscopy (Zeiss ULTRAplus FEG-SEM, Carl Zeiss NTS GmbH, Oberkochen, Germany and JEM 2010 TEM, Jeol Ltd., Tokyo, Japan) and their composition by energy dispersive spectroscopy (EDS; Thermo-Noran Vantage, Thermo Scientific, Breda, The Netherlands). The crystallinity and phase composition of the nanopowders were determined by powder X-ray diffraction analysis (Siemens D-500, Siemens AG, Kahrlruhe, Germany) and specific surface area of nanopowders was measured by nitrogen adsorption, using the Brunauer-Emmett-Teller method (Coulter Omnis-orp 100CX, Florida, USA).

3.1.1.1 In situ synthesized nanoparticles (I)

The synthesized aerosol particles were collected with a particle collector (fabric filter), from which the properties of the particle were characterized. The rest of the analyses were performed as described in 3.1.1. In the transmission electron microscope (TEM) analysis, a small volume of aerosol was drawn through a 200-mesh copper grid coated with lacey carbon film (SPI Supplies, West Chester, PA).

3.1.1.2 Hydroxyl Radical (·OH) Formation Capacity (I, II)

The hydroxyl radical (·OH) formation capacity was determined using the benzoic acid probe, which captures hydroxyl radicals forming the reaction product, 4-hydroxybenzoic acid (4-HBA) (Jung et al. 2006). Powder samples were suspended in 10mM phosphate-buffered deionized water with 10mM benzoic acid to achieve particle concentrations of 2, 5, 10, and 15 mg/ml. The vortexed samples were placed on an orbital shaker (IKA VibraX, IKA Werke GmbH & Co. KG, Staufen, Germany, 1000 rpm) in an incubator at 37°C for 24 or 90 h. After incubation, the suspensions were centrifuged for 30 min at 4°C (20,000 × g relative centrifugal force) to remove the particles and 250 µl was transferred to a glass vial with the addition of 10 µl 5M HCl to ensure protonation prior to chromatographic separation and UV detection of 4-HBA.
MATERIALS AND METHODS

In the analysis, 50 μl of the acidified sample was injected into a chromatographic system consisting of a Waters W600 pump and controller, a two serially mounted Waters XTerra MS 5 μm C18 (3.9 × 150 mm) columns, a Waters 996 PDA detector, and a Waters 717 autosampler (Waters HPLC, Milford, MA). The mobile phases consisted of water adjusted to pH 2 with HCl (A) and acetonitrile (B) with the following eluant program: 30% B for 1 min, 30%–80% B in 4 min, and 80% B for 1 min. The flow rate was 1 ml/min, and the 4-HBA was detected at 280 nm. The ·OH radicals were measured via the 4-HBA concentrations were converted to ·OH concentrations based on the 21.5% 4-HBA yield from the reaction between benzoic acid and ·OH (Bivas-Benita et al. 2005). The ·OH formation capacity was then calculated from a simple linear regression analysis of the ·OH radicals (nanomole ·OH per milliliter) as a function of the dose (milligram powder per milliliter). The standard deviation (SD) of the slope was determined as the SD between the predicted and observed y-value. All slopes were determined based on doublet analysis. All chemicals were obtained from Aldrich (St. Louis, MO).

3.1.1.3 Z-Potential (I)

The Z-potential of TiO2 was measured at pH 1–6. TiO2 particles were dispersed in 0.1M NaCl, and pH was adjusted with HCl. The Z-potential was measured with Zetasizer Nano Series particle characterization system (Malvern Instruments, Worcestershire, UK, 2005), and the analysis program was DTS (dispersion technology software, Malvern Instruments). Each dispersion was measured three times. The pH was determined at which Z-potential was zero.

3.1.2 Aerosol and particle characterization for fibrous materials (III)

In the CNT exposures, the aerosol concentrations were measured with an optical particle sizer (OPS, Grimm Dust Monitor 1.109) by applying the instrument calibration refractive index and a particle density of 2.6 g/cm³. Gravimetric samples were collected onto nitrocellulose filter (Millipore) and weighed on an analytical scale (RADWAG XA 110/Y; RADWAG Wagi Elektroniczne, Radom, Poland).
The morphology of the CNT was characterized by electron microscopy (Zeiss ULTRAPlus FEG-SEM, Carl Zeiss NTS GmbH, Oberkochen, Germany; Jeol JEM 2010 TEM, Jeol Ltd., Tokyo, Japan) and their composition was analyzed in an X-ray energy dispersive spectroscope (EDS ThermoNoran Vantage, Thermo Scientific, Breda, The Netherlands) attached to Jeol JEM 2010 TEM. Elemental composition data were expressed as the average of three separate analyses.

3.1.3 Assessment of bacterial lipopolysaccharide content (I, III)

Both nanosized TiO$_2$ samples that were investigated in detail in the present study (cnTiO$_2$ and nanoTiO$_2$ rutile/anatase) were tested for endotoxins to ensure that the detected inflammatory effects were not a response caused by possible endotoxin artifacts in the test materials. The concentrations of endotoxins were assayed in the Kuopio unit of the Finnish Institute of Occupational Health, which is accredited as a testing laboratory T013 (EN ISO/IEC 17025) by the Finnish Accreditation Service. Measurements were made using a standard (SFS-EN 14031:2003) kinetic chromogenic method based on the *Limulus amebocyte* lysate.

Levels of biologically active endotoxin in MWCNT were measured using a kinetic chromogenic *Limulus amebocyte* lysate assay (Kinetic QCL, Lonza, Walkersville, MD, USA) according to the instructions provided by the manufacturer. Endotoxin levels of the material samples were always <0.022 EU/mg.
3.2 **In vivo exposure methods**

Table 3 summarizes the main features of all the in vivo exposures described in details in the following chapters.

**Table 3.** Main features of all the *in vivo* exposures in the experiments of this thesis.

<table>
<thead>
<tr>
<th>Publication</th>
<th>Mouse strain</th>
<th>Exposure method</th>
<th>Exposure times</th>
<th>Doses</th>
<th>Sacrificed</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>balb/c</td>
<td>inhalation</td>
<td>2h, 2h on 4 consecutive days, 2h on 4 consecutive days on 4 consecutive weeks</td>
<td>10 mg/m³</td>
<td>4h and 24h after 1 and 4 days of exposure and 24h after 4 weeks of exposure</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>balb/c</td>
<td>inhalation</td>
<td>2h on 3 days a week for 4 weeks</td>
<td>10 mg/m³</td>
<td>after AHR measurement on day 28</td>
<td>OVA (asthmatic) vs. PBS (healthy)</td>
</tr>
<tr>
<td>III</td>
<td>c57bl/6, balb/c, KitW-sh /HNihrJa eBsmJ</td>
<td>inhalation</td>
<td>4h, 4h on 4 consecutive days</td>
<td>6.2-8.2 mg/m³ for rCNT and 17.5-18.5 mg/m³ for tCNT</td>
<td>immediately or 24 h after single exposure, 24h after 4 days</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>c57bl/6</td>
<td>aspiration</td>
<td>once</td>
<td>10 or 40 μg/mouse</td>
<td>4 and 16h, or 7, 14 and 28 days after exposure</td>
<td></td>
</tr>
</tbody>
</table>
3.2.1 Animals

All female mice (6-8 weeks old) listed in table 4 (7-8 weeks old) were quarantined for one week before the experiments and randomized into groups of eight. The mice were housed in groups of four in stainless steel cages bedded with aspen chip and were provided with standard mouse chow diet (Altromin no. 1314 FORTI, Altromin Spezialfutter GmbH & Co., Germany) and tap water *ad libitum*, when not in the exposure chamber. The environment of the animal room was carefully controlled, with a 12-h dark/light cycle, temperature of 20-21 °C, and relative humidity of 40-45 %. The experiments were performed in agreement with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg March 18, 1986, adopted in Finland May 31, 1990). All studies were approved by the Animal Experiment Board and the State Provincial Office of Southern Finland.

*Table 4.* The mouse strains used in the experiments of this thesis. (Gueders *et al.* 2009, Watanabe *et al.* 2004)

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>C57BL/6</th>
<th>BALB/c/Scarb</th>
<th>B6.129S7-Il1r1tm1Imx/J</th>
<th>Kit&lt;sup&gt;W-sh&lt;/sup&gt;/HNihrJaesmJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vendor</td>
<td>Scanbur AB, Sollentuna Sweden</td>
<td>Scanbur AB, Sollentuna Sweden</td>
<td>Jackson Laboratory, Bar Harbor, Maine</td>
<td>Scanbur AB, Sollentuna Sweden</td>
</tr>
<tr>
<td>Special characteristics</td>
<td>Th1/Th2 phenotype</td>
<td>Th2 dominant phenotype</td>
<td>IL-1 receptor deficient</td>
<td>mast cell deficient</td>
</tr>
<tr>
<td>Publication</td>
<td>III, IV</td>
<td>I, II, III</td>
<td>IV</td>
<td>III</td>
</tr>
</tbody>
</table>
3.2.2 Inhalation exposure to nanoparticle powders (I, II)

Inhalation exposure is considered the golden standard when attempting to model occupational exposure. Its only negative feature in mice is that there can be some contamination of the fur resulting in some additional gastrointestinal tract exposure due to preening. The nanoparticles were delivered into the whole-body inhalation exposure chamber using a solid particle dispenser (Palas Rotating Brush Generator, RBG 1000, Karlsruhe, Germany). The operating principle of the dispenser is based on feeding of the powdered material at a constant rate to the rotating brush, from where the powder is blown out by a high-velocity air stream and carried into the inhalation chamber supply air inlet. The stream of aerosol from the disperser is mixed with air before entering the inhalation chamber due to the turbulence in the air duct.

3.2.3 Inhalation exposure to \textit{in situ} synthesized TiO$_2$ nanoparticles (I)

A TiO$_2$ particle generator was attached to a whole-body inhalation exposure chamber to facilitate direct exposure to freshly made TiO$_2$ nanoparticles. The particle generator is a laminar flow reactor where TiO$_2$ particles were produced by thermal decomposition of titanium tetraisopropoxide (TTIP, Titan(IV)-Isopropylat 97%) vapor (Miettinen \textit{et al.} 2009, Okuyama \textit{et al.} 1986). Inside the reactor, the overall reaction stoichiometry was Ti(C$_3$H$_7$O)$_4$ $\rightarrow$ TiO$_2$ + 4C$_3$H$_6$ + 2H$_2$O (Kirkbir \textit{et al.} 1987). The primary TiO$_2$ particles were produced by homogenous nucleation, condensation, and coagulation from the TiO$_2$ vapor. Secondary particles were formed by agglomeration of primary particles and condensation of TiO$_2$ vapor on the particle defined the quantities of reactor by-product gas levels at different reactor settings. Gas levels were at maximum: 38.4 ppm propane (C$_3$H$_6$), 23.2 ppm carbon monoxide (CO), 4.1 ppm methane (CH$_4$), 2.1 ppm nitrogen oxides (NO$_x$), and additional oxygen and water vapor.

3.2.4 Inhalation exposure to fibrous materials (III)

MWCNT were aerosolized with a fluidized bed aerosol generator (FBAG; TSI Model 3400A, (Marple \textit{et al.} 1978)) for which the materials were used without any pre-treatment. In the generator, a chain transports CNT to the fluidizing bed where any material agglomerates are mechanically broken by 200-μm
bronze pellets and continuous air flow. To increase the CNT feed to the fluidizing bed, additional air flow was used. Since the density of CNT was very low, the material reservoir of the FBAG was not large enough to accommodate material for a whole 4-h exposure. Thus, the reservoir was filled after two hours from the beginning of each exposure. During the experiments, the pressure of the exposure chamber differed from the atmospheric pressure ±200 Pa. The pressure in the chamber was dependent on the experiment flow values and on the air intake, whether it was drawn from a room through a HEPA filter or from a generator.

3.2.5 Pharyngeal aspiration (IV)

The study material was suspended in PBS containing 0.6 mg/ml bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO) and sonicated for 20 min in Elmasonic S 15 H water bath sonicator (37 kHz, 35 W; Elma GmbH & Co. KG, Singen, Germany). The mice were anesthetized with vaporized 4.5% isoflurane and suspended by their incisors on a thin wire on a custom made mouse support at approximately 66 degrees angle. A coldlight source was placed against their throat to provide optimal illumination of the trachea. The tongue was pulled out using blunted forceps and pressed down using a small spatula to prevent the mouse from swallowing. A volume of 50 μl of the particulate suspension was delivered onto the vocal folds under visual control using an extended pipette tip (Finntip 200 Ext, Thermo Fisher Scientific Inc., Waltham, MA). Immediately after delivery, the mouse nostrils were covered forcing the mouse to inspire the instilled suspension.
3.3 *In vivo* experiment protocols

3.3.1 Exposure protocol for nanoparticle powders (I)

There were altogether five exposure groups (eight mice per group). The first two groups were exposed once for 2 h and the second two groups were exposed for 2 h on four consecutive days, one of the replica groups was always sacrificed at 4 h and the other 24 h after the last exposure. The fifth group was exposed for 2 h a day on four consecutive days for 4 weeks and sacrificed 24 h after the last exposure. The exposure concentration was $10 \pm 2 \text{ mg/m}^3$ in all tests. This exposure setting was chosen to mimic occupational conditions where workers are exposed to concentrations of around 5 mg/m$^3$ (Boffetta *et al.* 2004).

3.3.2 The asthma model - sensitization and exposure protocol (II)

BALB/c/Sca mice were randomized into two exposure and two control groups (8 mice/group, table 5). Mice were sensitized intraperitoneally with 20 μg of ovalbumin (OVA) in alum (Sigma-Aldrich, St Louis, MO) in 100 μl of phosphate-buffered saline (PBS) on days 1 and 14 of the experiment (figure 9). The control group was given alum in 100 μl of PBS. Exposure groups were exposed three times a week for 2 hours for the duration of the four week experiment, concurrently with the asthma sensitization. The exposure concentration was $10 \pm 2 \text{ mg/m}^3$ in all tests. This concentration was chosen to mimic occupational conditions where workers would be exposed to concentrations of around 5 mg/m$^3$ (Boffetta *et al.* 2004). On days 25-27, all mice were challenged with 1% OVA solution delivered for 20 min via the airways by an ultrasonic nebulizer (DeVilbiss, Glendale Heights, IL). The last two exposures were given on days 25 and 27. Different materials were studied on separate occasions.

*Table 5.* Mice groups used in publication II.

<table>
<thead>
<tr>
<th>Mice groups</th>
<th>Not exposed</th>
<th>nano TiO$_2$ exp.</th>
<th>fine TiO$_2$ exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy (PBS)</td>
<td>8+8 mice</td>
<td>8 mice</td>
<td>8 mice</td>
</tr>
<tr>
<td>Asthmatic (OVA)</td>
<td>8+8 mice</td>
<td>8 mice</td>
<td>8 mice</td>
</tr>
</tbody>
</table>
3.3.2.1 Measurement of airway responsiveness (II, III)

In publication II, the airway responsiveness was measured on day 28 using a single chamber, whole-body plethysmograph system (Buxco, Troy, NY) as described earlier (Hamelmann et al. 1997). Briefly, mice were exposed to increasing concentrations (1, 3, 10, 30 and 100 mg/ml) of metacholine (MCh; Sigma Aldrich) in PBS delivered via an AeroSonic 5000 D ultrasonic nebulizer (DeVilbiss, ITW, Glendale Heights, IL). Before the MCh exposure, the baseline was measured for three minutes. After the baseline measurements, the MCh was nebulized for 1.5 minutes and airway reactivity was assessed for 5 minutes per concentration. Lung reactivity parameters were expressed as Penh (enhanced pause) values. After measurement of lung responsiveness, the mice were sacrificed with an overdose of isoflurane and samples were collected for analysis.

In publication III, the airway responsiveness of BALB/c mice was measured on day 5 using a single chamber, whole-body plethysmograph system (Buxco, Troy, NY, USA) as described above.
3.3.3 *Ex vivo* spleen cell stimulations (II)

The spleens were dissected from the mice onto 6-well plates with PBS. The spleens were then mechanically disrupted to form a paste and filtered to remove larger pieces. The cells were re-suspended in RPMI media, counted under light microscopy and plated at a concentration of one million cells/ml. Cells to be stimulated were plated in RPMI containing 100 µg/ml ovalbumin. All cells were incubated for 48 hours after which the supernatant was collected and stored at -70°C prior to analysis.

3.3.4 Exposure protocol for fibrous materials (III)

Mice were exposed to aerosolized rCNT or tCNT for 4 hours at a time once or on four consecutive days in a whole-body inhalation chamber. The aerosol mass concentrations used for individual experiments were within a range of 6.2-8.2 mg/m³ for rCNT and 17.5-18.5 mg/m³ for tCNT. A greater concentration of control material, (tCNT), was used to confirm that rCNT-induced inflammatory reactions were rCNT-specific and biologically relevant. During the experiments, untreated control mice were housed in the same room with CNT-exposed animals.

3.3.5 Exposure protocol for aspiration (IV)

In single exposures by pharyngeal aspiration, a dose of 10 µg per mouse (50 µl of a 0.2 mg/ml dispersion) was used for short –term time points (4 and 16h) whereas for the 7, 14 and 28-day exposure, two doses, 10 µg and 40 µg (50 µl of a 0.8 µg/ml dispersion), per mouse were used. The doses were selected to mimic occupational conditions (Porter *et al.* 2010).

3.3.5.1 Antagonists (IV)

In the antagonist experiment, intraperitoneal injections were delivered and 16 h later the mice were subjected to the pharyngeal aspiration (table 6). The animals were sacrificed at 4 h after the aspiration exposure. PBS contained 0.6 mg/ml BSA, 10 µg of R/CNT was given per mouse (0.2 mg/ml dispersion) and the mice were treated with antagonists, either anakinra (Kineret; Biovitrum AB, Stockholm, Sweden) or etanercept alone or in combination (Enbrel; Wyeth Pharmaceuticals, Hampshire, UK) 200 µg/mouse both times.
Table 6. Exposure groups of the antagonist experiment.

<table>
<thead>
<tr>
<th>intraperitoneal injection</th>
<th>pharyngeal aspiration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS/BSA</td>
<td>PBS/BSA</td>
</tr>
<tr>
<td>PBS/BSA + etanercept</td>
<td>PBS/BSA + R/CNT</td>
</tr>
<tr>
<td>PBS/BSA + anakinra</td>
<td>PBS/BSA + R/CNT + anakinra</td>
</tr>
<tr>
<td>PBS/BSA + etanercept + anakinra</td>
<td>PBS/BSA + R/CNT + etanercept + anakinra</td>
</tr>
</tbody>
</table>

3.4 *In vitro experiments*

3.4.1 Cell lines (I)

Murine macrophage RAW 264.7 and human pulmonary fibroblast MRC-9 cells were purchased from the American Type Culture Collection (Manassas, VA). RAW 264.7 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) and MRC-9 cells in MEM + GlutaMAX-1 medium (Invitrogen) supplemented with 10% of heat-inactivated fetal bovine serum, 1% penicillin-streptomycin (PEST) antibiotics, L-glutamine, 1× nonessential amino acids, and 1× Na-pyruvate. All cells were cultured at +37°C in 5% CO₂.

3.4.2 Differentiation of macrophages from peripheral blood–derived monocytes (I)

The peripheral blood–derived monocytes were isolated from the buffy coats by Ficoll-Paque gradient centrifugation after which the mononuclear cell layer was harvested. After washing the harvested cells three times with PBS (pH 7.4), the mononuclear cells were allowed to adhere onto six-well plates (10 million cells per well) for 50 min covered in RPMI 1640 medium supplemented with 2mM L-glutamine and 1% PEST at 37°C and an 5% CO₂ atmosphere. The cells were then washed three times with PBS. After the last wash, the adhered cells were given 1 ml serum-free macrophage medium (Invitrogen) supplemented with granulocyte-macrophage colony-stimulating factor (ImmunoTools, GmbH, Germany) 10 ng/ml and 1% PEST. The macrophage medium was changed every second day. Maturated macrophages were used for stimulations on the seventh day after the cell harvesting. All cell stimulations were done using cells obtained from three different blood donors.
The phenotype of these macrophages resembles closely that of human alveolar macrophages (Akagawa et al. 2006).

3.4.3 Exposure protocol (I)

One day before the exposures, all the cells were provided with fresh medium. The cells were exposed with medium containing the desired amount of the nanoparticles, which had been beforehand sonicated for 20 min in Elmasonic S 15 H water bath sonicator (37 kHz, 35 W; Elma GmbH & Co. KG, Singen, Germany) before delivering it to the cells. Lipopolysaccharide (Escherichia coli O111:B4; Sigma-Aldrich) was used as a positive control at a concentration of 100 ng/ml. Tumor necrosis factor-alpha (TNF-α) was from BioSource International (Camarillo, CA), and the concentration used was 10 ng/ml.

3.5 Sample collection and analyses

3.5.1 Collection of in vivo samples and preparation (I, II, III, IV)

The mice were sacrificed using an overdose of isoflurane. Blood was collected from the vena cava (hepatic vein), and the lungs were lavaged with PBS (800 μl for 10 s) via the tracheal tube. The bronchoalveolar lavage (BAL) sample was cytocentrifuged on a slide, and later, the cells were stained using the May Grünwald-Giemsa (MGG) stain and counted under light microscopy. The BAL supernatant was stored at −70°C for cytokine analysis, and the remaining cells were fixed in 1:1 ethanol-DPBS mixture. The blood was kept on ice and later centrifuged. The supernatant was then collected and kept at −70°C for subsequent analysis. The mouse chest was opened and half of the left pulmonary lobe was removed, quick-frozen, and kept at −70°C for later RNA isolation. A slice of the lungs and BAL cells were collected from each mouse, fixed with glutaraldehyde, and then prepared for electron microscopy. A part of the left lobe was embedded in Tissue-Tek oxacalcitriol compound (OCT, Sakura Finetek, Alphen aan den Rijn, Netherlands), quick-frozen and kept at -70°C for later immunohistochemical staining. (IV) The rest of the lungs were formalin fixed, embedded in paraffin, cut, affixed on slides, and stained with hematoxylin and eosin (H&E), Periodic acid-Schiff (PAS) and picrosirius red (PSR, III) solutions.
MATERIALS AND METHODS

3.5.2 Histology

3.5.2.1 Analysis of mucus producing cells (II, III)

The lung tissue was analyzed from formalin-fixed, paraffin-embedded sections. These sections were stained for mucus secreting goblet cells using periodic acid-Schiff (PAS) -stain. The data was analyzed with Leica Image Manager IM50 version 4.0 (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). PAS+ cells were counted as an average of PAS+ cells found in 100 μm (I) or 200 μm (II) of bronchus counted from three bronchioles of similar size per mouse (n = 8 mice per group).

3.5.2.2 Lung morphology (III)

Recruitment of inflammatory cells into the lungs and morphological alterations of the tissue were assessed after H&E staining. Lung sections stained with PSR solution were used to evaluate the formation of fibrosis but also the distribution of CNT within lungs since these black fibers were easily detectable against the light background.

3.5.3 Bronchoalveolar lavage cells (I, II, III, IV)

MGG-stained BAL cells were counted from three high-power fields in a light microscope (Leica DM 4000B; Leica, Wetzlar, Germany).

3.5.4 mRNA analyses

3.5.4.1 RNA isolation from the lung tissues (I, II, III, IV) and cultured cells (I)

Due to the very local mode of action of cytokines and chemokines, they are known to circulate in the organism at very low levels (<10 pg/ml) and used rapidly by the cells. (Bienvenu et al. 2000) Therefore cytokines and chemokines especially from the in vivo studies are often below the detection limit of many protein assays. In an attempt to avoid possible false negative results, it was decided to focus on the key cytokines/chemokines and to assess them via their mRNA expression levels using quantitative RT-qPCR which is a more rapid and sensitive method compared with protein analysis.
The lung samples were homogenized in a FastPrep FP120 (BIO 101; Thermo Savant, Waltham, Mass) machine, and RNA was extracted using the FastRNA Pro Green Kit (Qbiogene/MP Biomedicals, Illkirch, France) according to recommendations of the manufacturer. The quantity and purity of extracted RNA was determined by NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies, Inc., Wilmington, DE). Isolated total RNA was dissolved in diethyl pyrocarbonate (DEPC) water and stored at −70°C.

Before RNA isolation from the cells, the nanoparticles were removed from the solution by centrifuging the samples at 14,000 × g 10 min + 4°C. At this point, the triplicate samples of human macrophages were pooled. Total RNA from cultured cells was isolated by phenol-chloroform method with Eurozol reagent (EuroClone S.p.A-Life Sciences Division, Siziano, Italy) according to the manufacturer’s instructions and precipitated with isopropanol. Total RNA was dissolved in DEPC-treated milli-Q water.

3.5.4.2 Complementary DNA synthesis (I, II, III, IV)

Complementary DNA (cDNA) was synthesized from 1 μg of total RNA in a 25 μl reaction using MultiScribe Reverse Transcriptase and random primers (The High-Capacity cDNA Archive Kit; Applied Biosystems, Foster City, CA) using the manufacturer’s protocol. The synthesis was performed in a 2720 Thermal Cycler (Applied Biosystems, Carlsbad, CA) starting with 25°C for 10 min and continuing with 37°C for 120 min.

3.5.4.3 PCR amplification (I, II, III, IV)

PCR primers and probes were ordered as predeveloped assay reagents from Applied Biosystems. The real-time quantitative PCR was performed in a 96-well optical reaction plate with Relative Quantification 7500 Fast System (7500 Fast Real-Time PCR system; Applied Biosystems) using the manufacturer’s instructions. Amplifications were done in 11 μl reaction volume containing 20 ng cDNA and TaqMan universal PCR master mix and primers provided by Applied Biosystems. Endogenous 18S was used as the housekeeping gene.
MATERIALS AND METHODS

3.5.5 Protein analyses

3.5.5.1 Enzyme-linked immunosorbent assay (I, II, IV)

ELISAs (eBioscience, San Diego, CA) were performed according to the manufacturer’s instructions. An ELISA plate microtiter reader (Multiskan MS, Labsystems, Helsinki, Finland) was used to read the results.

3.5.5.2 Luminex (IV)

The Milliplex Mouse Cytokine/Chemokine Immunoassay was used in the analysis of proteins in BAL fluid supernatants (Millipore Corporation, Billerica, MA) according to the manufacturer’s protocol. Then, 3% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO) in PBS was added at a concentration of 0.5% to samples, controls and standards to ensure sufficient protein amounts for the assay. The assay was performed using Luminex xMAP Technology (Bio-Plex 200 System, BioRad, Hercules, CA).

3.5.6 Measurement of serum antibodies (II)

For the analysis of OVA-specific IgE, ELISA plates were coated with Purified Rat Anti-mouse IgE antibody (BD Biosciences, San Jose, CA) and incubated overnight. On the second day, the plates were blocked with 3% BSA in PBS, serum samples (1:10 and 1:20 dilution) were added to the plate and incubated overnight. Biotinylated OVA was added and streptavidin horseradish peroxidase (Pharmingen 13047E, BD Biosciences) followed by ABTS solution (ABTS Microwell Peroxidase Substrate System, KPL, Gaithersburg, MD). Absorption was read at 405 nm with ELISA plate absorbance reader (Multiskan MS, Labsystems, Helsinki, Finland).

For OVA-specific IgG2a analysis, ELISA plates were coated with ovalbumin (BD Biosciences) and incubated overnight. On the second day, the plates were blocked with 3% BSA in PBS, serum samples (1:60, 1:180, 1:540 and 1:1620 dilution) were added to the plate and incubated overnight. Biotinylated anti-mouse IgG2a (Pharmingen 02012 D, BD Biosciences) was added and streptavidin horseradish peroxidase (BD Biosciences). ABTS solution and peroxidase were mixed 50:50 and added before reading absorption at 405 nm with an ELISA plate absorbance reader.
3.5.7 DNA microarrays (III)

Total RNA samples isolated from lung tissue as described above were quantified by NanoDrop and the quality was verified by Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Independent pools of two RNA samples each (total of 600 ng) were labeled using a T7 RNA polymerase amplification method (Low Input Quick Amp Labeling Kit, Agilent Technologies), according to the instructions of the manufacturer. cRNAs were then labeled with Cy3 and Cy5 dyes (Agilent Technologies) and hybridized to the Agilent 2-color 60-mer oligo arrays (Agilent SurePrint G3 Mouse GE 8x60K). The slides were washed and scanned with Agilent Microarray Scanner G2505C (Agilent Technologies) and the raw intensity values were obtained with the Feature Extraction software, version 11.0.1.1 (Agilent Technologies). Raw data was quality checked according to the Agilent standard procedures. The median foreground intensities were imported into the R software version 3.0.0 (http://cran.r-project.org) (Team 2012) and analyzed with the BioConductor package limma (Smyth 2005). Log2 transformation and quantile normalization was performed on the single channel data separately, similarly to published methods (Kilpinen et al. 2013, Palgi et al. 2012, Procaccini et al. 2012) and according to the proposals by Smyth and Altman (Smyth et al. 2013). No background correction was carried out, as suggested by Zahurak et al. (Zahurak et al. 2007) Subsequently the batch effect derived from the labeling was removed using the ComBat method (Benjamini et al. 1995) implemented in the sva package. (Johnson et al. 2007) The values of the probes recognizing the same NCBI Entrez Gene Ids (Coordinators 2013) were further averaged into the final expression matrix. Differentially expressed genes were identified by using linear models and empirical Bayes pairwise comparisons (post hoc adjusted P<0.01 and linear FC > |1.5|). (Smyth 2004) The resulting gene sets after Benjamini and Hochberg post hoc correction (Benjamini et al. 1995) were considered to be significant and were studied further by the DAVID version 6.7 annotation tool (Huang 2009) with the default parameters.
3.5.8 **Electron microscopy (I, IV)**

Samples were fixed in 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide, dehydrated and embedded in epon (I) or LX-112 (IV) (Ladd Research, Williston, VT, USA). Thin sections were collected on uncoated copper grids, stained with uranyl acetate and lead citrate and then examined with a transmission electron microscope operated at 80 (I) or 100KV (IV) (JEM-1220, Jeol Ltd., Tokyo, Japan) and photographed with a Veleta TEM CCD camera (Olympus Soft Imaging Solutions Gmph, Germany).

3.5.9 **Immunohistochemical staining (IV)**

The frozen OCT-fixed lung tissue samples were cut into 4 μm thick slides, air-dried and fixed with -20C acetone for 10 minutes and then stained with the Autostainer 360 (Thermo Scientific) using primary antibodies CD3 (clone 17A2, BD Pharmingen, San Diego, CA), CD4 (clone RM4-5, BD Pharmingen), CD8 (clone 53-6.7, BD Pharmingen), CD11c (clone HL3, BD Pharmingen) and F4/80 (clone BM8, Acris Antibodies GmbH, Herford, Germany). Positive cells were counted under light microscopy at x400 magnification as an average of at least 5 randomly selected areas of 2 sections.

3.5.10 **Inductively coupled plasma-mass spectrometry (I)**

The titanium concentration of the lung tissues was analyzed from the formalin-fixed lungs by inductively coupled plasma-mass spectrometry (ICP-MS, Thermo X series II, Thermo Electron, Bremen, Germany). The samples were dried to constant weight, the organic material was combusted with acids, and the titanium concentration was measured by ICP-MS using germanium as an internal standard.
3.5.11 Statistical analysis (I, II, III, IV)

The toxicological data were analyzed and the graphs were created with the GraphPadPrism software (GraphPadPrism Software, Inc., San Diego, CA). In all statistical analyses, first analysis of variance was performed using one-way ANOVA (nonparametric Kruskal-Walles test) and when the ANOVA was positive, then appropriate post-hoc tests were performed. The different groups were compared using an unpaired t-test or nonparametric Mann-Whitney $U$-test, and $p$ values less than 0.05 were considered statistically significant. Regression analyses and calculations of probabilities in the analysis of ·OH radical formation were done using the standard equations in MS Office Excel versus 2003 and Minitab 15.0.0 (Minitab Inc., State College, PA).
# 4 RESULTS

**Table 7.** Simplified summary of the *in vivo* results from all the publications of this thesis.

<table>
<thead>
<tr>
<th>Name - product number</th>
<th>Publication</th>
<th>neutrophilia</th>
<th>eosinophilia</th>
<th>mRNA cytokines, chemokines</th>
<th>AHR</th>
<th>PAS</th>
<th>granulomas</th>
<th>crystal formation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>nano TiO$_2$+SiO$_2$ - 637262</strong></td>
<td>I, II</td>
<td>I: +</td>
<td>I: none</td>
<td>I: CXCL1+, TNFα+, CXCL8+, CXCL5+</td>
<td>II: -</td>
<td>II: -</td>
<td>none</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II: +</td>
<td></td>
<td>II: IL-1β-, TNFα-, IL-4-, IL-13-, IL10-, Foxp3+, CCL3-, CXCL5-, CXCL2-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>nano TiO$_2$ - 637254</strong></td>
<td>I</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>n/a</td>
<td>n/a</td>
<td>none</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>nano TiO$_2$ - 4850MR</strong></td>
<td>I</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>n/a</td>
<td>n/a</td>
<td>none</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>nano TiO$_2$-generated</strong></td>
<td>I</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>n/a</td>
<td>n/a</td>
<td>none</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>coarse/ fine TiO$_2$ - 224227</strong></td>
<td>I, II</td>
<td>none</td>
<td>none</td>
<td>II: IL-1β-, TNFα-, IL-4-, IL-13-, IL-10-, Foxp3+, CCL3-, CXCL5-, CXCL2-</td>
<td>II: +</td>
<td>II: -</td>
<td>none</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>nano SiO$_2$-5485HT</strong></td>
<td>I</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>n/a</td>
<td>n/a</td>
<td>none</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>tangled CNT - CheapTube</strong></td>
<td>III, IV</td>
<td>none</td>
<td>7,14d: low +</td>
<td>III: IL-5+, CCL11+, CCL17+</td>
<td>IV: none</td>
<td>IV: 14d: +</td>
<td>IV: 28d: +</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IV: 7d: IL-13+, CCL11+, CCL24+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>rod-like CNT- XNRI MWNT-7</strong></td>
<td>III, IV</td>
<td>III: +</td>
<td>III: +</td>
<td>III: IL-13+, IL-5+, IFN-γ, CCL11+, CCL24+, CCL17+</td>
<td>III: +</td>
<td>IV: 1d: IL-1β+, CXCL1+, CXCL9+</td>
<td>IV: 28d: +</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV: 1,28d: +</td>
<td>IV: 7d: IL-1β+, TNFα+, IL13+, TGF-β+, CCL11+, CCL24+</td>
<td></td>
<td>IV: 7,14, 28d: +</td>
<td>IV: 14, 28d: +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IV: 14,28d:-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>crocidolite asbestos</strong></td>
<td>IV</td>
<td>1d: +</td>
<td>7d: +, 14,28d:-</td>
<td>7d: IL-13+, CCL11+, CCL24+</td>
<td>n/a</td>
<td>7, 14d:+</td>
<td>none</td>
<td>14,28d: +</td>
</tr>
</tbody>
</table>

"+" sign indicates increased expression, whereas "-" stands for decreased expression. "none" means that there was no change in expression and "n/a" that this endpoint was not analyzed. In publication II, the results are from the asthmatic mice group exposed to NPs. In publication III the results are from mice exposed for 4h a day on 4 consecutive days and sacrificed on day 5. In publication IV the results are divided to 1, 7, 14 and 28 days (d) after exposure.
The results summarized in table 7 before, are described in more detail in the following chapters.

4.1 Effects of inhalation exposure to different TiO$_2$ (I)

This original publication revealed that even a material that is thought to be inert and safe, after small modifications, can become harmful. Here, four different nanosized TiO$_2$ (one coated with SiO$_2$, cnTiO$_2$), coarse TiO$_2$ and nanosized SiO$_2$ were compared. Out of these six materials, only the cnTiO$_2$ evoked inflammation in the lungs of the mice after one week’s inhalation exposure. The presence of pulmonary neutrophilia (I, Fig. 2a) was confirmed by the expression of the neutrophil-attracting chemokine CXCL1 (I, Fig. 3b) and the major pro-inflammatory cytokine TNF-$\alpha$ (I, Fig. 3b).

The mRNA results from in vivo studies suggested that macrophages were the main producers of TNF-$\alpha$ and lung cells of neutrophil-attracting chemokines. This was further verified using murine alveolar macrophages (RAW 264.7) and monocyte-derived human macrophages. Both macrophages were identified as a major source of the pro-inflammatory cytokine TNF-alpha (I, Fig. 5a,b and 6a) after exposure to cnTiO$_2$. Human lung fibroblasts (MRC-9) on the other hand were exposed to cytokines secreted by cnTiO$_2$-exposed macrophages, which resulted in a strong expression of CXCL1 and CXCL8.

Analyzing the lung tissues revealed that particles accumulated mainly in the phagosomes of pulmonary macrophages (I, Fig. 4a). In contrast to the other particles, cnTiO$_2$ particles seemed to be free in the cytosol, suggesting that they had broken out of the phagosomes and been released into the cytoplasm (I, Fig 4c).
4.2 Effects of inhalation exposure to nanosized and fine TiO₂ on allergic asthma (II)

This study revealed important results emphasizing the heterogeneity of the human population. Here healthy and asthmatic mice were exposed to nanosized (n) and fine (f) TiO₂ for three times a week for four weeks. A murine asthma model was used to mimic reactions in a more vulnerable part of the population. As seen in the previous study, exposure of healthy mice to nTiO₂ (same as cnTiO₂ in the previous study) resulted in clear pulmonary neutrophilia (II, Fig. 3) and an induction of neutrophil-attracting chemokine CXCL5 (II, Fig. 7b).

Eosinophilia (II, Fig. 3), lymphocytes (II, Fig. 3) and the expression of mucus secreting PAS+ cells (II, Fig. 4), all known features of allergic asthma, were dramatically down-regulated in both TiO₂ exposed mice. All measured cell signaling molecules, including pro-inflammatory (II, Fig. 6a), regulatory (II, Fig. 6c) and Th2-type cytokines (II, Fig. 6b), were up-regulated in the asthmatic mice, but were similar to levels found in healthy mice in both TiO₂ exposed groups. The same was true for all measured chemokines (II, Fig. 7).

Airway hyper-responsiveness, a hallmark of allergic asthma, revealed a difference in the two TiO₂ materials - while nTiO₂ reduced responsiveness (II, Fig. 5a), fTiO₂ showed a modest exacerbation of this asthmatic symptom (II, Fig. 5b). Finally, the results obtained with OVA-stimulated ex vivo spleen cells pointed to a systemic immune suppression after exposure to nTiO₂ (II, Fig. 8) while fTiO₂ reduced levels of OVA-specific IgE (II, Fig. 9a).
4.3 Unconventional allergic-like airway inflammation evoked by inhalation exposure to CNT (III)

The aim of this study was to compare two differently shaped carbon nanotubes, flexible tangled and rigid rod-like CNT. Mice were exposed by inhalation for four hours a day once or on four consecutive days. The focus of this study was on early events with the mice being sacrificed immediately and 24 hours after a single exposure. The four day exposure mimicked an occupational week. Mast cell deficient mice were used to examine the role of mast cells in the inflammation and the transcriptomics analysis was used to explore the early transcriptional responses, which may play an important role in initiating the airway inflammation.

It was found that even a short-term inhalation of the rod-like CNT (rCNT) was able to induce a novel allergic-like inflammation mediated by the innate immunity. This inflammation was characterized by pulmonary eosinophilia (III, Fig. 1b) and relevant chemokines (III, Fig. 2g), mucus hypersecretion (III, Fig. 2b,d), airway hyper-reactivity (III, Fig. 2a) and Th2-type cytokines (III, Fig. 2e). All of the above mentioned responses are considered as hallmarks of allergic asthma.

Mast cells are thought to act as major mediators in immediate allergic reaction. These cells were found to partially regulate the allergic-like inflammation caused by rCNT. Using mast cell deficient Kit\textsuperscript{W\textasciitilde}sh mice and the four day exposure routine, the mast cells were shown to mediate eosinophilia (III, Fig. 3a) and the production of IL-13 (III, Fig. 3c).

Transcriptomics analysis was used to identify early molecular events taking place in the resident cells, which finally result in allergic-like airway inflammation seen after four days of rCNT exposure. Immediately after the 4 hour rCNT exposure, the pathways induced were almost exclusively related to innate immunity (III, Fig. 4c). On the contrary, this phenomenon was not found with tCNT (III, Fig. 4c).
RESULTS

Next, cytokine and chemokine expression were compared in alveolar macrophages, resident lung cells and mast cells using BAL, lung tissue and mast cell deficient mice respectively. It was found that pro-inflammatory cytokine IL-1β and TNF-α were mainly produced by alveolar macrophages (III, Fig. 5a) and IL-13 and IL-4 by mast cells (III, Fig. 5c).
RESULTS

4.4 A Th2-type pulmonary inflammation resulting from aspiration exposure to CNT (IV)

This study utilized a different exposure technique, pharyngeal aspiration, which permits the use of more time points and doses as well as a more precise dosage when compared to inhalation. It is also a safer method to use with hazardous materials. The same two CNT as examined in the previous study were examined but in addition, crocidolite asbestos was included as a control material of interest. Doses of 10 or 40 μg per mouse were administered and the mice were sacrificed at 4 or 16 hours or 7, 14 and 28 days after a single exposure.

At 4 hours after exposure, the mRNA expression of neutrophil attracting chemokine CXCL1 was observed with all materials (IV, Fig. 1c). There was also evidence of pulmonary neutrophilia in all groups and this increased in a time-dependent manner from 4 to 16 hours, with the rod-like CNT (R/CNT) eliciting the fastest and highest reaction (IV, Fig. 1a). R/CNT alone induced the mRNA expression of pro-inflammatory cytokine IL-1β and lymphocyte attractant CXCL9 at the 4 hour point (IV, Fig. 1b, d).

At 7 days after exposure, there was a drastic shift in the influxing cells as the numbers of neutrophils had diminished whereas a strong eosinophilia was present in the R/CNT and asbestos exposed groups (IV, Fig. 9b, c). R/CNT also elicited strongest mRNA expression of IL-1β, TNF, TGF-β, CCL11 and CCL24 and increase in numbers of goblet cells (IV, Supplementary Fig.2c, 3a-d).

At 14 days, it was possible to detect Charcot-Leyden-like crystals in R/CNT and asbestos exposed mice. At this time point, the numbers of eosinophils had significantly reduced (IV, Fig.9). The numbers of mucin-producing goblet-cells had however still increased when compared to day 7 (IV, Supplementary Fig.5).
RESULTS

After 28 days, the strong effect of R/CNT remained as there were still up-regulated mRNA levels of pro-inflammatory cytokines TNF-α and IL-1β (IV, Fig. 2d,e) and clear signs of pulmonary neutrophilia (IV, Fig. 2a). At this time, eosinophilia had disappeared completely (IV, Fig.9). Instead pro-fibrotic TGF-beta levels remained high (IV, Fig. 2f). The effects of asbestos and tangled CNT (T/CNT) had either disappeared, or in the case of TGF-beta, were even down-regulated.

Other changes observed at the later time point included interstitial macrophages around R/CNT fibers and bundles (IV, Fig. 3a-b), granulomas (IV, Fig. 4a), acidophilic macrophages containing Charcot-Leyden-like crystals (IV, Fig. 5a-c), mucus hypersecretion (IV, Fig. 6a) and up-regulated mRNA expression of Th2 type cytokine IL-13 (IV, Fig. 6c). Asbestos and T/CNT also caused some of these changes, but to a much smaller degree.

The role of a major pro-inflammatory player IL-1β was studied together with its close companion TNF-α using antagonists and IL-1-receptor knock-out (KO) mice. At 4 hours, both antagonists, anakinra (IL-1β) and etanercept (TNF-α), given either together and separately abolished mRNA expression of IL-1β and TNF-α as well as abolishing the neutrophilia (IV, Fig. 7a). This was in line with results from KO mice, where the levels of neutrophils and neutrophil attracting chemokines CXCL5, 1 and 2 were significantly down-regulated when compared to wild type (WT) mice (IV, Fig. 7b). At 28 days, the KO mice showed continuous impairment in terms of neutrophilia and the expression of TNF-α mRNA (IV, Fig. 8a,e). Th2-related signals, IL-13 and mucus production, as well as pro-fibrotic TGF-β however remained unaffected (IV, Fig. 8f-g).
5 DISCUSSION

As nanomaterials have become increasingly common and product development on ENM is more intensive than ever, it is crucially important that research should be conducted into the safety of these materials. The tragedy surrounding asbestos, a material responsible for so many deaths following its widespread use, is often highlighted as a warning example of how apparently safe materials can be incredibly dangerous. This time with ENM, we should be wise ahead of time, to avoid another asbestos disaster. As researchers in the nanosafety field have come to realize, the task is not as easy as one would think. The number of different materials is increasing every day and even a small modification can dramatically change how a material reacts with the human body. Ultimately this means that it is extremely hard to draw a demarcation line between a safe and a harmful material. As a result, to be guaranteed safe, each material should be studied on its own or treated as a potentially harmful material. The work conducted to evaluate the safety of ENM in this thesis have definitely proven that even if you think you know how a material will react, you will most likely be surprised.

5.1 TiO$_2$ nanoparticles – harmful, neutral or healing ENM?

As larger particles, TiO$_2$ have long been used as a negative control. However when nanosized particles entered the market, this situation changed. At present, nTiO$_2$ has been rather extensively investigated in different \textit{in vivo} and \textit{in vitro} models. nTiO$_2$ has been demonstrated to evoke both local and systemic symptoms and also to aggravate pre-existing symptoms (Li \textit{et al.} 2013, Zhang \textit{et al.} 2015). In addition to the lungs, other exposure routes have been less extensively studied, but should not be forgotten. nTiO$_2$ are the most commonly used nanoparticle in dermally applicable consumer products (Robertson \textit{et al.} 2010) and present in many orally administered items such as food colorants and nutritional supplements. They are also used as carriers in nanomedicines that may be administered intravenously or subcutaneously. It has been shown that nTiO$_2$ can be absorbed through the lungs and GIT into the systemic circulation and because of this possibility, other organ injuries and inflammatory reactions should be investigated. Continual use may lead to chronic levels of exposure and accumulation in many organs. Most nTiO$_2$ studies have employed doses that are not realistic in occupational settings and
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as such, extrapolation to human exposures can be difficult (Shakeel et al. 2015, Shi et al. 2013).

The results from publications I are in line with common observation of nTiO$_2$ (Li et al. 2013, Sha et al. 2015, Zhang et al. 2015). In publication I, it was concluded that most nTiO$_2$ are rather harmless, when the exposure is to modest doses. At least doses under the two recommended levels (0.3 mg/m$^3$ by NIOSH, (Dankovic et al. 2011) and 1.2 mg/m$^3$ by NEDO, (Morimoto et al. 2010)) can with ease be considered modest. In a recent study by (Spinazzè et al. 2016) worker exposure levels remained at 300 μg/m$^3$ and in another recent study also well below the RELs (Vaquero et al. 2015). If then exposures of 10 mg/m$^3$ result in only modest inflammation, following RELs in occupational settings can well be presumed safe.

In the acute TiO$_2$ exposures of this thesis, the materials used did not provoke any notable reactions in the mouse lungs. Only the SiO$_2$ coated nTiO$_2$ (cnTiO$_2$) caused pulmonary inflammation after four days of exposure causing neutrophilia, high mRNA levels of pro-inflammatory TNF-α and the neutrophil attracting compound, CXCL1. The results after four weeks remained at the levels close to those observed after one week, indicating that the body was able to clear the particles and the inflammation levels remained somewhat stable. If the particles hadn't been cleared at all, they would have been accumulating in the lungs and thus likely been increasing the inflammatory reaction. Results from ICP-MS analyses confirmed that the clearance rate indeed was around 60%. Four weeks of exposure to coarse TiO$_2$ on the contrary did not cause signs of inflammation.
An inflammatory cascade was also proposed for the SiO$_2$ coated TiO$_2$ (figure 10) which started with alveolar macrophages engulfing SiO$_2$ coated TiO$_2$ particles into their phagosomes. After phagosomal entrapment, SiO$_2$ coated TiO$_2$ seemed to emerge into the cytoplasm, possibly causing the release of TNF-$\alpha$ from the macrophages, which in turn caused the release of CXCL1 mRNA from the epithelial cells leading to pulmonary neutrophilia. This was also supported by in vitro data obtained from human and murine macrophages and human lung fibroblasts. Since neither plain SiO$_2$ nor TiO$_2$ caused the observed effects, it is likely that coating TiO$_2$ with SiO$_2$ caused the material to convert from being inert to harmful. In an intratracheal instillation study done in mice, (Saber et al. 2012) also concluded that two coated TiO$_2$ were more harmful than an uncoated one. On the other hand coating can also reduce toxicity as was concluded in an in vitro study by (Tedja et al. 2012). There the surface adsorbed serum components were found to provide some protection from the cytotoxic effect of endocytosed TiO$_2$ nanoparticles.
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It would be interesting to find out what causes SiO$_2$ coated TiO$_2$ to be released from the phagosomes as was seen in publication I. It was speculated in the publication that it had something to do with the material’s Z-potential. Interestingly, in a publication about several different silica nanoparticle exposures on macrophages it was suggested that the IL-1$\beta$ responses induced by the particles in the RAW264.7 macrophages involved activation of the vacuolar H$^+$-ATPase, and induction of cathepsin B release, subsequently leading to phagosomal destabilization (Sandberg et al. 2012). (Palomäki et al. 2010) studied immunological effects of SiO$_2$ coated TiO$_2$ on antigen presenting cells (APC, e.g. RAW264.7) in vitro and found that the material elicited a very slight increase in expression of IL-1$\beta$ and in addition expressions of IL-6, MIP-1$\alpha$ and TNF-$\alpha$. If not through this exact pathway, then possibly through something similar, the SiO$_2$ coated TiO$_2$ causes phagosomes to break.

In publication II, a murine model was used to assess effects of nTiO$_2$ exposure on asthmatic and non-asthmatic mice. The same nTiO$_2$ was used as in publication I (referred to as cnTiO$_2$ in that article). This was compared to a larger TiO$_2$, referred to as fTiO$_2$. The working hypothesis was that there would be exacerbation of the allergic inflammation, but on contrary, most markers of inflammation were reduced due to TiO$_2$ exposure. Known signs of asthma: eosinophilia, lymphocytes, mucus production and relevant mRNA of cytokines IL-1$\beta$, TNF-$\alpha$, IL-4, IL-13 and chemokine CCL3, were all downregulated in nano- and finesized TiO$_2$ exposed groups. The two different sizes of TiO$_2$ differed in the measured AHR, i.e. fTiO$_2$ caused a slight increase whereas nTiO$_2$ followed the suppressive trend. Another difference between these materials was the reduced level of IgE-antibody following fTiO$_2$ exposure, but not nTiO$_2$. It was hypothesized that many particles that cause exacerbation of asthmatic symptoms also contain organic matter and as such, also contain pathogen-associated molecular patterns (PAMP) that activate innate immunity. It is possible that the lack of this PAMP-signal had prevented the symptoms from developing. This would however only explain why the symptoms did not become more severe, but it gives no clear clues as to why it seemed as if the asthma-like symptoms were disappearing. In an article by (Whitehead et al. 2014) it was shown that inhalation of moderate doses of LPS
during sensitization induces regulatory responses that, after multiple allergen exposures, limit the severity and longevity of asthma-like features. In this case a plain PAMP-signal inhibits asthmatic inflammation.

Available research shows that similar to immunostimulation, nanoparticle-mediated suppression and inhibition of immune function exists in some cases and is determined by the nanoparticle's physicochemical properties (Ilinskaya et al. 2014). In case of TiO$_2$ significant immunosuppression *in vivo* has been shown e.g. when systemic administration of nTiO$_2$ inhibited T-cells, B-cells, macrophages and NK cells in one study (Andersson-Willman et al. 2012) and in another study exposure was associated with greater susceptibility to a melanoma challenge (Moon et al. 2011).

While one could speculate that inhaling TiO$_2$ particles would be a cure for asthma, unfortunately this is most likely not the case. One possible explanation is that the sensitization phase is strongly modulated by the concurrent exposure to TiO$_2$ and that the particles cause a competing pro-inflammatory response, which in some unknown manner overwhelms the Th2 response. This could be true for the nTiO$_2$, but as fTiO$_2$ seems to be quite neutral by itself, it does not explain how both materials are able to block most asthmatic responses. This does however leave the option that the modulation is a result of some underlying mechanisms that are activated by both types of TiO$_2$ exposure, but only cause inflammation in the case of the more irritant material, the nTiO$_2$. An interesting study done on OVA-sensitized rats showed that allergic pulmonary inflammation is not up-regulated by inhalation of nanosized TiO$_2$, but on the contrary decreases lung inflammation (Scarino et al. 2012). They speculated that the inhibition might be due to the route of exposure (inhalation) or the timing of the nanoparticle exposure in relation to the induction of the asthmatic lung inflammation. Their single exposure was performed subsequent to the allergen challenge. The OVA-challenges in publication II were given on three concurrent days and TiO$_2$ exposures on first and last of those days. Exposures were however also given many weeks in advance. Results in a mouse model of di-isocyanate-induced asthma show in contrast that low, intrapulmonary doses of nTiO$_2$ can aggravate pulmonary
inflammation and AHR (Hussain *et al.* 2011). Plain silica on the other hand has been shown to exacerbate OVA-induced allergic airway inflammation in mica and to exert adjuvant effects (Han *et al.* 2016).

In conclusion, it seems that nanosized TiO$_2$ are able to cause moderate toxicity which in case of allergic asthma seems to suppress the Th2-type immune reaction. Toxicity is most commonly seen as pulmonary inflammation and according to the literature (Dankovic *et al.* 2011, Shi *et al.* 2013), nanosized TiO$_2$ are generally able to induce greater responses than their larger counterparts. In addition to inflammation, other studies show production of reactive oxygen species (ROS) leading to increased oxidative stress levels, histopathological alterations, genotoxicity, metabolic chance, carcinogenesis and immune disruption. As seen also in the present studies, the extent of the damage depends on material-based characteristics such as crystal structure, size and purity of the TiO$_2$ (Fedora *et al.* 2016, Shakeel *et al.* 2015, Shi *et al.* 2013).
5.2 Rigid long MWCNT – a close cousin of asbestos

In light of growing evidence about the possible toxicity of CNTs and on the other hand their great commercial potential, especially in electronics and medicine, it is not surprising that controversy surrounds these impressive materials. It seems that there is some validity in comparing this material to asbestos, however the heterogeneity of the CNT leaves many questions unanswered.

Publication III demonstrated that the shape and length of a MWCNT could greatly affect its toxicity. In this study, the rigid rod-like material (rCNT) resembling asbestos fibers in terms of shape, and slightly in size, caused a dramatic pulmonary inflammation with many characteristics similar to allergic asthma, while this was not observed with a tangled CNT (tCNT). In agreement with the present studies, it has been shown across the CNT field that the toxicity of CNT is mainly length dependent. There is convincing evidence that short or tightly bundled aggregates with no isolated long fibers are less inflammogenic in fiber-specific assays (Osmond-McLeod et al. 2011). However, short 5μm MWCNT may also be pro-inflammatory if delivered in high bolus doses or as large aggregates (Kolosnjaj-Tabi et al. 2010, Oberdörster et al. 2015). In addition to the length of the fiber, also its metal content/purity, rigidity, reactivity, diameter, surface and crystal structure may play some roles in defining the overall toxicity (Boyles et al. 2015, Wang et al. 2013, Vietti et al. 2016).

Macrophages have a major role in CNT induced toxicity as the key problem seems to be frustrated phagocytosis, leading to the continued production of pro-inflammatory cytokines and reactive oxygen species (ROS) as a result of the phagocytic burst. Macrophages were first-line responders also in the present studies, trying to engulf the foreign material, resulting in incomplete phagocytosis, carbon bridges being formed between macrophages and foreign body giant cells (FBGC) after exposure to rCNT. Macrophages were also shown to express pro-inflammatory cytokines IL-1β and TNF-α. In addition to frustrated phagocytosis, long fibers have been shown to induce


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inflammation should they accumulate in the parietal pleura. This is due to the size of the lymphatic stomata, which are about 8-10 μm, allowing only smaller fibers to pass (Donaldson *et al.* 2010). In addition to macrophages, other strongly responding cells included eosinophils and mucus producing cells that both appeared in great numbers after rCNT exposure.

As foreign body giant cells, eosinophils and mucus production indicated a Th2 type inflammation, other signs of allergic asthma were also sought. The expression of Th2 cytokines, AHR and also eosinophil chemoattractants were studied. In a more detailed search for other allergic mediators, mast cells were examined and it was found that they were indeed participating in the rCNT induced allergic-like airway inflammation, at least by producing IL-4 and IL-13. However, this allergic-like inflammation was unusual due to the short time-span before its appearance. The conventional sensitization phase cannot occur in just one week which indicates that it is the innate immune system which is the driving force behind these inflammatory reactions. Transcriptomic profiling also pointed towards an innate mediated reaction. The airway inflammation witnessed in this study seems to be somewhere between classical asthma and IrIA. Even though the inflammation lacked a sensitization phase and, in terms of its fast response, appeared to be innate related, it still involved Th2 cytokines, mucus production and eosinophilia. Rather than allergens, the rCNT seem to act more like irritants in inducing a rapid and dramatic airway inflammation similar to that seen in allergic asthma. Recently a new non-allergic eosinophilic airway inflammation has been suggested, which describes a way of generating type 2 cytokines and eosinophils without the intervention of the adaptive immune system. This inflammation involves especially ILC2 (innate lymphoid cell, formerly “nuocytes” or “natural helper cells”) that are stimulated by the expression of IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) from activated epithelial cells in the presence of irritants and environmental pollutants (Lambrecht *et al.* 2015). This type of mechanism would also seem a plausible explanation for the fast Th2 type inflammation observed in publication III. The role of ILC2 was examined by measuring markers IL-25 and ROR-α, but there was no evidence of up-regulation in their expression. However many other markers have been identified to associate with ILC2 and it is possible
that it is these agents and not those assayed here which are active in this case (Barlow et al. 2014).

tCNT and rCNT were compared to asbestos in publication IV. In this exposure study done by aspiration, there were signs of acute inflammation dominated by pulmonary neutrophilia that converted to eosinophilia within a week. This was seen with all three materials, but rCNT showed the most dramatic effects. Throughout the study, rCNT showed more dramatic responses than encountered with asbestos. There are also other studies demonstrating that CNT can indeed be more toxic than asbestos (Boyles et al. 2015, Sanchez et al. 2009).

After 28 days, the inflammation was still evident, with mRNA expression of pro-inflammatory cytokines IL-1β and TNF-α and pro-fibrotic TGF-β. The tissues showed signs of granuloma formation and Charcot-Leyden-like crystals in acidophilic macrophages. These changes indicate possible fibrosis at a later time point and also reveal that the materials are not being effectively cleared out from the lungs. As in publication III, also in this study there was evidence of Th2-associated signals such as IL-13 and goblet cell hyperplasia. The finding of Charcot-Leyden-like crystals in article IV is an interesting one that apparently has only once before been linked to CNT (Købler et al. 2015). In that study, the investigators detected crystalline bodies in murine alveolar macrophages, multinucleated cells and lung tissue at 28 days after intratracheal exposure to MWCNT. They concluded that these crystals resembled the structures seen in eosinophilic crystalline pneumonia (ECP) that is especially prevalent in the C57BL/6 mouse strain. According to the literature, the crystals are formed after the breakdown of eosinophils and are
strongly linked to the presence of IL-13 (Zhu et al. 1999). This suggests a possible route map describing the mechanism for fibrosis (figure 11) where initially the responding macrophages express CXCL1 which attracts neutrophils. Macrophages, epithelial cells and possibly even neutrophils (Chen et al. 2014) then secrete IL-13 that targets the eosinophils which then break down, depositing their granule proteins, leading to the formation of Charcot-Leyden-like crystals, acidophilic macrophages and ultimately, possibly to lung fibrosis. The role of IL-13 and crystal formation in fibrosis induced by CNT should be studied in the future.

Figure 11. Inflammatory effects caused by R/CNT exposure.
Several studies have shown that in addition to inflammation and lung damage, many CNT can indeed cause granulomas and interstitial fibrosis. MWCNT have also been shown to be able to migrate to the pleura and diaphragm from the lungs (Oberdörster et al. 2015, Ryman-Rasmussen et al. 2009). Pulmonary fibrosis is induced due to prolonged tissue injury or chronic inflammation. This involves ever-increasing numbers of fibroblasts, their differentiation into myofibroblasts, overproduction of extracellular matrix (ECM) proteins resulting in scarring of the lung tissue and eventually severe respiratory insufficiency (Wynn 2011). The cellular and molecular mechanisms through which this process takes place are not yet established, but pieces of this intriguing puzzle are emerging. For example, macrophages have been shown to act through the inflammasome, ROS, IL-1β and IL-18, platelet-derived growth factor (PDGF) and ERK1/2 pathway or through the NF-κB, TNF-α, TGF-β, Smad/ERK1/2 pathway, ultimately causing collagen production, differentiation, proliferation and transition in fibroblasts and ECM accumulation. Another route mediated through Smad is the transition of lung epithelial cells into fibroblasts called the epithelial-mesenchymal transition (EMT) (Wang et al. 2015, Vietti et al. 2016).

The role of IL-1β was studied in more detail as it has been linked to CNT and fiber induced inflammation in several publications (Hussain et al. 2014, Meunier et al. 2012, Murphy et al. 2012, Palomaki et al. 2011). In both knock-out and antagonist experiments, the lack of bioactive IL-1β was able to prevent acute inflammation. This would seem to indicate that the use of these antagonists could be a potential way to treat acute inflammation caused by CNT exposure. In more chronic conditions, however it appears that Th2 type symptoms are not prevented by the lack of IL-1β. This also suggests a role for IL-1β in neutrophilia, but not in the Th2 type reaction.

All in all, there does appear to be some validity in comparing CNT to asbestos. However as with the TiO₂ materials, the inflammatory potential of different materials varies enormously. As described in the introduction, there are many factors influencing the possible toxicity of CNT, some relating to the nanoscale and others to the fibrous shape. In the case of the materials studied
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in these publications, it seems that at least the shape of the material is a potential factor as well as what kind of agglomerates they produce. The rCNT that proved very toxic in all these experiments, has subsequently been classified as possibly carcinogenic to humans (Group 2B) by the IARC (Grosse et al. 2014). However this is the only CNT- material classified so far which underlines the diversity found throughout this novel class of materials. Although toxic responses across different CNT studies seem qualitatively similar, quantitative differences in bioactivity are more than possible (Oberdörster et al. 2015). There have been some efforts to control toxicity e.g. through functionalization (e.g. added functional groups) and at least doping of MWCNT with nitrogen has been shown to control inflammation (Carrero-Sánchez et al. 2006). Similarly, carboxylation of CNT has been shown to reduce bio-persistence, thus also reducing toxicity (Oberdörster et al. 2015) This toning down of toxicity will be an important field of study in the future.
5.3 Challenges in assessing toxicity of ENM

As already stated in the introduction of this thesis, the choice of material has a major impact on the results as even minor changes in a material can dramatically alter its effects in the body. Some guidelines can be drawn, e.g. it is recommended to examine materials that are widely used, but ultimately the choice is often dictated by chance – research partners, availability etc. A good example of chance was the material referred to as cnTiO$_2$ in the first two publications. This material was purchased as a pure rutile TiO$_2$ from the vendor and only later was found to have been coated with SiO$_2$. This naturally gave the interpretation of results a whole new aspect and also underlined the importance of conducting a thorough characterization of the materials being investigated. It is still however difficult to draw lines to what is relevant and sufficient in terms of characterization and there is a wide range of practices in the different laboratories (Bussy et al. 2012).

As this thesis was focused on obtaining relevant answers especially to problems existing in the occupational world, the time points, doses and routes of exposure were chosen with occupational settings in mind. As inhalation has been proven as the most relevant route of exposure to nanoparticles in the workplace (Krug et al. 2011, Oberdörster et al. 2015), inhalation was a natural choice as an exposure method. Inhalation exposures, however valid and important, are laborious and have many restrictions, not to mention safety issues. This did limit the amount of exposure groups and there had to be emphasis placed on proper experimental design. The protocol was based on the best available knowledge at that point in time and limited knowledge sometimes complicates experimental design. Unfortunately, there is still limited amounts of information about the concentrations found on workplaces due to the novelty of ENM and partly due to the challenge of measuring the amounts of airborne nanomaterials. Since TiO$_2$ has been on the market for a longer time, there were already some studies to give direction at the beginning of this thesis (Boffetta et al. 2004). Some recommended exposure levels were also already available for fine TiO$_2$ particles. The American Conference of Governmental Industrial Hygienists (ACGIH) had assigned TiO$_2$ fine particles a threshold limit value (TLV) of 10mg/m$^3$ and the Occupational Safety &
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Health Administration (OSHA) had set a permissible exposure limit (PEL) of 15mg/m³ (Shi et al. 2013). However publications on measurements from CNT workplaces only started emerging by the time that most of the CNT inhalation exposures had been completed (Dahm et al. 2012, Erdely et al. 2013).

In all inhalation experiments, the amount of airborne material was on average 10 mg/m³. This still seems relevant in occupational TiO₂ settings. Although according to the few studies already mentioned, these CNT doses might seem somewhat excessive, it is possible to justify their adoption. During CNT synthesis, concentrations are most probably in the order of micrograms/m³ or less, but it is likely that exposure concentrations to large CNT granulates may be high (e.g. milligrams/m³) during the post-processing of CNT powders. When interpreting results from high dose studies, researchers sometimes refer to a phenomenon called lung particle overload described by Morrow already in 1988, which can lead to impairment of the alveolar macrophage clearance function. Originally volumetric loading of AM was used to define particle overload and this can still be applied for larger particles, but the particle surface area dose-metric has been proposed as an alternative parameter, especially for ENM (Borm et al. 2015). The different technical processes used to formulate CNT have also been proven to exert dramatic effects on their pulmonary fate and subsequent toxicity (Pauluhn et al. 2014). The durations of daily exposures were chosen based more on technical aspects, e.g. restrictions on machinery, ethical considerations and time required for processing of samples on sacrifice days. Even if the exposure times used in these studies (2 and 4h) are shorter than a normal working day of 8 hours, it is most unlikely that workers would be exposed continuously for such long periods on a daily basis.

Although it is possible to determine the concentrations of airborne particles, it is much more difficult to measure the exact doses received by individual animals. In the TiO₂ studies, a relatively straightforward method existed for measuring the doses of individual mice. With inductively coupled plasma-mass spectrometry (ICP-MS), it was possible to observe that TiO₂ accumulated in the lungs in a clear dose-dependent manner, with ~60%
clearance rate and with no significant differences between different TiO₂ materials. Lung deposition analysis was also estimated mathematically in the article of Koivisto et al. (2011). On the other hand, the evaluation of the actual CNT exposure doses of individual animals after CNT inhalation has proved very challenging for all researchers in the field (Pauluhn et al. 2014). As carbon is naturally abundant in the human body, it is hard to distinguish the CNTs from tissues and as such, it was necessary to utilize stained tissue samples to prove that the material had actually reached its target tissues. Mathematical and other estimations are possible (Koivisto et al. 2011, Koivisto et al. 2012), e.g. based on the size distribution and airborne concentration, it can be assumed that exposure dose of tangled-CNT is likely higher when compared to rigid-CNT. (Pauluhn et al. 2014) have since successfully used an elemental/organic total carbon method to analytically determine the lung burden from digested lungs. However, before it will be possible to make a valid comparison of results from different studies, it would be necessary to determine lung burdens rather than simply measuring exposure concentrations.

Estimating dosage after pharyngeal aspiration exposure poses other problems. With aspiration, it is possible to deliver a relatively constant dose to all exposed animals, but estimating how it corresponds to inhalation doses can be more complicated. According to some studies (Porter et al. 2010, Shvedova 2005) an aspirated dose of 20 μg should be equivalent to 20 days of inhalation exposure for 8 hours a day by humans at a concentration of 5 mg/m³ or an aspirated dose of 10 μg should be equivalent to a deposited dose in the alveolar region of a worker performing light work for 30 days at 400 μg/m³ (Han et al. 2008). The doses used here were 10 and 40 μg per mouse, which according to available research, would seem relatively relevant from the occupational viewpoint. Naturally there is a large difference in the dose rate between inhalation and aspiration, as inhalation exposure happens gradually over a long period of time and aspiration is a single instantaneous bolus. The dose rate likely especially affects acute effects and high single doses can induce responses through mechanisms of homeostasis or particle overload that would not be relevant with low doses (Slikker Jr et al. 2004). More research is needed to clarify the differences between these two methods, but it does seem
that aspiration can be used for qualitative but not quantitative risk assessment. Recent experiments in our lab done by repeated aspirations seem to provide data that is more comparable to inhalations than single doses delivered by aspiration (Kinaret et al. submitted), however repeated exposure requires repeated anesthesia, which can increase stress in the animals and this could obviously alter the outcome of the experiment. Another problem with aspiration is the fact that the particles must be suspended in a vehicle medium in order to be delivered to the lungs. Most nanoparticles agglomerate when placed in a medium, making pretreatment with dispersants necessary. This alters the surface properties of the particles and thus impacts on their biokinetics and how cells perceive the nanoparticles. Exposure through aspiration also usually results in a more uneven distribution of the material (Oberdörster et al. 2015). In publication IV we used PBS containing 0.6 mg/ml bovine serum albumin to disperse the material. This has been proven to be a good way of preventing excess agglomeration (Bihari et al. 2008).

Finally, it is necessary to briefly consider the mouse strains used in this thesis. As in the material selection, chance can somewhat dictate the strains being used. As our lab had been deeply focused on allergy research long before the start of nano-research, the balb/c-strain, which is considered a Th2-type strain, had been the most widely used, most familiar and therefore the natural strain to adopt. The c57bl6-strain is also used in asthma research, but it was chosen here because these experiments were collaborations with genotoxicologists and this was a strain with which they were more familiar. Comparing results even from these two rather similar and basic mouse strains is difficult and only broad lines can be drawn with confidence. For example, it was possible to discern Th2-type players activated throughout the studies as a result of ENM exposure, regardless of mouse strain, but whereas c57bl6 responded strongly with eosinophilia, balb/c mice mainly exhibited neutrophilia. It is known that the c57bl6 mice are more skewed to eosinophilia, which possibly explains this difference, but as (Watanabe et al. 2004) and (Gueders et al. 2009) both point out, there are multiple differences between these mouse strains. It is important to note, however, that as described earlier in the introduction, asthma is a heterogeneous disease and some patients exhibit neutrophil-predominant asthma sub-phenotypes (Bogaert et al. 2011). In addition, a few more specific
strains were used, IL-1R KO and mast cell KO mice, that were both bred from the same background as the WT mouse; these were used to investigate very specific questions. The results obtained from these mice strains were therefore much more straightforward to interpret.

As a summary, it is important to keep in mind the different factors affecting the results. The inhalation and aspiration doses used in this thesis compare quite well and are relevant also in occupational settings. Instruments and techniques used to evaluate occupational exposure are developing rapidly and soon we will likely have more and better tools available. If aspiration proves a reliable substitute to inhalation in assessing pulmonary toxicity, it will make testing materials \textit{in vivo} easier. The results seen are true for each experiment and mouse strain. Putting them in perspective between strains and eventually relative to humans is not an easy endeavor.
6 CONCLUSIONS AND FUTURE PERSPECTIVES

In light of growing use of engineered nanomaterials (ENM) in a wide variety of applications, it has become evident that research on their safety must also be conducted. This thesis aimed at uncovering the possible toxicity of two commonly used nanomaterials, titanium dioxide (TiO$_2$) and carbon nanotubes (CNT), especially in the occupational context. This work focused on the direct effects of repeated nanoparticle exposure on the development of lung inflammation \textit{in vivo}. Whether exposure to nanoparticles modulates development of allergic asthma was also investigated.

When looking at the direct effects following TiO$_2$ or CNT exposure, it can be concluded that they reside on the opposite sides of the spectrum. In general TiO$_2$ nanoparticles do not pose alarming risks to pulmonary health. In this thesis they only caused mild or moderate inflammatory effects, which were characterized by activated macrophages and following neutrophilia. Out of the two different CNT studied in this thesis, one was rather harmless (tCNT) and another with potentially very toxic effects (rCNT). The granulomas, AHR, eosinophilia, mucus producing cells, Charcot-Leyden-like crystals and inflammatory mediators seen in four weeks after a single aspiration exposure exceeded the effects seen with crocidolite asbestos. Inhalation exposure to rCNT revealed a novel allergic-like inflammation mediated by the innate immunity. It seems that rigid fibre-like CNT pose a special threat to pulmonary health and may eventually cause serious fibrosis and impairment of the lungs.

When asthmatic mice were exposed to TiO$_2$, the discoveries were rather surprising. It seemed that exposure to certain nanosized and fine TiO$_2$ caused modulation in the allergic inflammation, resulting in local and systemic reduction of several features of experimental asthma. In contrast with e.g. severe effects of particulate air pollution (Gavett \textit{et al}. 2001, Pandya \textit{et al}. 2002) on allergic asthma, a similar suppressive phenomenon has been seen with e.g. oak dust (Määttä \textit{et al}. 2007) and microbe exposure (Whitehead \textit{et al}. 2014). It would be interesting to pursue this lead and to find out more about
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the mechanisms behind this suppression. The results could open new doors in understanding mechanisms of human allergic asthma.

Considering human exposure to nanosized TiO$_2$ in general, it could be concluded that exposure to large enough quantities of TiO$_2$ to cause serious harm is somewhat unlikely. However considering the very wide use of TiO$_2$ it is crucial to design products and manufacturing processes in ways that do not allow TiO$_2$ to accumulate in humans or the environment. In the case of CNT, significant release and following exposure from consumer products is unlikely, if one excludes manufacturing and processing, tires, recycling and their potential use in textiles. Even in the above mentioned scenarios, the exposure levels will be low (Guseva Canu et al. 2016). Special attention should still be paid to new MWCNT that are designed, produced and used in application. Without thorough toxicity evaluation (e.g. LOAEL, follow-up studies) they should always be treated as potentially hazardous materials.

As both studied materials, TiO$_2$ and MWCNT, are already being introduced into novel areas e.g. in diagnostics, pharmacology and therapeutics, it is good to also keep in mind the numerous advantages of these materials. For example, TiO$_2$ has demonstrated potential as a cancer drug due to its photocatalytic killing effect (Sha et al. 2013). CNT on the other hand, can be used to deliver drugs to specific sites in the organism due to the fact that they are hollow and with functionalization easily targeted to specific sites (Amenta et al. 2015). This exciting development should still always proceed hand-in-hand with toxicological testing.

Due to the rapidly growing numbers of different ENM, vivid toxicity testing cannot be performed on all new materials. Standard in vitro tests to assess basic ENM toxicity will hopefully become available in the near future and they will most likely be enough for pre-evaluated low risk ENM. For example thorough characterisation can give vital clues about the possible toxicity of ENM. The situation at the moment calls for actual exposure studies to be conducted to provide solid evidence of possible exposure scenarios. However,
the analytical methods available have great problems monitoring exposure in real-world conditions (Guseva Canu et al. 2016, Liou et al. 2015).

The main contributions of this thesis could be summarized as providing information on a potential inhibitory mechanism of allergic asthma, helping to unveil mechanisms of a new Th2 type eosinophilic airway inflammation and providing new information on diverse abilities of different ENM to impact the immune system. This information can be further utilized in hazard assessment.
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