IMMUNOTOXICOLOGICAL EFFECTS
INDUCED BY ENGINEERED
NANOMATERIALS

Jaana Palomäki

ACADEMIC DISSERTATION

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Helsinki 2014
’Nainen tarvitsee elämässään kahta asiaa: huumoria ja punaiset korkokengät. Tohtorintutkinto on hyväksi muttei välttämätön.’

[Riikka Pulkkinen: Totta]
The human body is protected from invading pathogens and foreign material by its immune system. The most common routes for pathogens to enter the human body are via inhalation, digestion or dermal penetration. The first stage of protection is a layer of epithelial cells forming a physical barrier on the body surfaces. This may be accompanied with mucus secretion and special cells, i.e. ciliated cells in the lungs and a biochemical barrier i.e. enzymes. These non-specific barriers are part of the innate immunity: Rapid and non-specific immune system. Innate immunity also possesses specific cells operating in the body surfaces. The most important of these cells are phagocytosing cells, which scavenge foreign material in the body cavities and secreting signalling molecules to further activate other cells of the immune response. Macrophages and dendritic cells are not the only phagocytes but they are also able to present antigens to the cells of the adaptive immunity, and therefore further activate this specific response against pathogens via T and B cell activation. When the immune system functions in its regular manner, it is practically invincible, but if there are any imbalances in the response, different outcomes such as inflammation and allergic diseases may be induced.

Engineered nanomaterials (ENM) are widely used in the industry. Their unique and attractive characteristics are said to improve competitiveness and potential for innovations in the different sectors of industry, and it has been estimated that the market size of nanotechnological innovations will exceed 2 trillion euros in 2015. However, the toxicological hazard and risk assessment of different nanomaterials is far from complete. There is some concern that nanomaterials may cause toxic outcomes, and more research efforts are now being put into the hazard assessment. However, the risk assessment of nanomaterials requires not only information on their hazardous properties but also awareness about the risks of exposure, and therefore cooperation between different expertise areas is required. In addition, the huge amount of differently modified nanomaterials poses a challenge for toxicologists who will have to develop new, quicker and cheaper approaches for hazard and risk assessment to guarantee the human health.

In this thesis, an immunotoxicological approach was used to compare different types of ENM and the effect of size, shape and surface modifications on the toxicity of ENM. The results confirm that the materials possess different hazardous properties and provide evidence of the mechanisms behind fibre-induced inflammation. The studies performed with cell models suggest that different ENM induce different responses in antigen presenting cells. Furthermore, the results show that surface modifications of metal oxide nanoparticles as well as shape of
the carbon nanomaterials drastically influence their toxicity. The mechanistical study performed with carbon nanotubes (CNT) \textit{in vitro} clearly demonstrated that long, rigid carbon nanotubes resemble asbestos fibres in their ability to activate the NLRP3 inflammasome essential for efficient immune response. The proteomics study on the secretomes of the macrophages, which had been exposed with different CNT, and asbestos fibres indicated that different shaped CNT can induce different responses in macrophages, confirming the finding that not all CNT are identical to asbestos fibres. In addition, the effects of either bulk-sized or nano-sized ZnO particles were compared in the mouse atopic dermatitis (AD) skin model. The results clearly demonstrated that ZnO particles were able to suppress local inflammation, however, there was also an induction of systemic antibody (Ab) levels. These outcomes were clearly higher if the mice skin had been treated with nano-ZnO shown also to penetrate into the deeper layers on mouse allergic skin. These results highlight that particle size is an important parameter in determining the toxicological outcome and suggest that the effect may be attributable to the higher active surface area on smaller particles.

If these results are considered in the light of current risk assessment protocols, they emphasize the importance of careful physico-chemical characterisation of ENM if one wishes to compare the results with those in the literature. Furthermore, mechanistic studies revealing the pathways activated in the toxic outcomes \textit{in vitro} should be further validated \textit{in vivo} in order to utilise this information in the future in the more rapid screening of fibre toxicity. Taken together, these results highlight the weaknesses and strengths in the scientific data in the traditional risk assessment approach and these have to be taken into account in the planning of the future studies.


Tässä väittöskirjassa vertailtiin erilaisia nanomateriaaleja ja tutkittiin materiaalien koon, muodon ja pintakäsittelyn vaikutuksia niiden terveydelle haitallisista ominaisuuksien. Tulokset osoittavat, että kemiallisesti erilaisilla nanomateriaalleilla on erilaiset terveydelle haitalliset ominaisuuksut. Lisäksi tulokset vahvistavat, että erilaiset nanomateriaalit aiheuttavat erilaisen vasteen antigeenien esittelevässä soluissa ja että metallioksidien pintakäsittely ja hiilinanoputkien muoto vaikuttavat voimakkaasti

Kun tuloksia analysoitiin nykyisillä riskinarvointitavoilla, oli selvää, että hiilinanoputkien siltamaisuuden ja näiden toksikologisten mekanismien vaikutusten elämänlailla olisi väittämättöntä tulosten vertailtavuuden kannalta. Mekanistinen tutkimus osoitti solunsisäisten reittien aktivoitumisen solumalleissa, ja näiden toksikologisten mekanismien aktivoitumisen oikeaksi eläimintehoksi olisi väittämättöntä, jotta tuloksia voisisiin hyödyntää tulevaisuudessa kuitujen vaarallisuuden nopeassa seurannassa. Väitöskirjan osatoimen tarkastelu kemikaaliriskinarvioinnin näkökulmasta paljastaa tieteellisesti tuotetun tiedon puutteet ja vahvuutet perinteisessä riskinarviointitavassa. Tulokset auttavat myös ottaamaan huomioon tekijöitä, jotka ovat tärkeitä suunniteltaessa ja toteutettaessa uusia toksisuustutkimuksia.
6.1 Nanomaterials and the suspension preparation protocol (I–IV) ..... 41
6.2 Cell models (I-III) ..............................................................................42
6.3 The mouse model of atopic dermatitis (IV).................................42
6.4 Methods ..............................................................................................43
  6.4.1 Cell death analysis (I, III)........................................................43
  6.4.2 Cytokine/Chemokine expression analysis (I, IV) ...................44
  6.4.3 Cytokine/Chemokine secretion and serum antibody analysis (I, II, IV)..........................................................45
  6.4.4 Analysis of cell surface molecules (I) .......................................46
  6.4.5 SiRNA treatment and pharmacological blockade (II) ..........46
  6.4.6 Western blot analysis (II, III) ......................................................46
  6.4.7 Microscopy ..............................................................................47
    6.4.7.1 Light microscopy (I, III) .................................................... 47
    6.4.7.2 Transmission electron microscope (II) ..........................47
    6.4.7.3 Fluorescent microscopy (II)...........................................47
    6.4.7.4 Hyperspectral imaging (IV) ......................................47
  6.4.8 Proteomics methods .....................................................................48
    6.4.8.1 DIGE labeling (III) .................................................... 48
    6.4.8.2 2-dimensional electrophoresis (III) ..........................48
    6.4.8.3 DeCyder analysis (III) ...............................................49
    6.4.8.4 Protein identification (III) ...........................................49
    6.4.8.5 Bioinformatics analysis (III) ...........................................49
  6.4.9 Histology (IV) ..........................................................................49
  6.4.10 Lymph node stimulations (IV) ..................................................50
  6.4.11 Data Analysis (I, II, IV) ............................................................50
7  RESULTS ................................................................................................52
  7.1 Metal oxide nanoparticles (I, IV) ..................................................52
    7.1.1 Major differences in the levels of cytotoxicity of different TiO$_2$ and ZnO nanoparticles (I).................................52
    7.1.2 Surface modification influences the TiO$_2$ NP induced immunotoxicity (I) ..........................................................52
    7.1.3 ZnO nanoparticles activate macrophages and dendritic cells in vitro (I)..........................................................53
7.1.4 Particle size modulates the inflammatory response in vivo (IV)...............................................................................53

7.1.4.1 The local allergic reaction is significantly diminished after skin treatment with nano-ZnO......53

7.1.4.2 The levels of systemic antibodies in serum are increased after skin treatment with nano-ZnO ......54

7.2 Carbon nanomaterials (I, II, III) ........................................................................................55

7.2.1 Short carbon nanotubes are weak inducers of a pro-inflammatory response in mouse in vitro –cell models (I) ................................................................................55

7.2.1.1 Rigid multi-walled carbon nanotubes but no other types of carbon nanomaterials activate profound IL-1 –family cytokine secretion from human primary macrophages (II)..............................55

7.2.2 Rigid multi-walled carbon nanotubes activate theN LRP3 inflammasome in a similar manner as asbestos fibres (II) ....56

7.2.2.1 NLRP3 inflammasome activation ........................................56

7.2.2.2 Formation of reactive oxygen species (ROS) ...............56

7.2.2.3 Leakage of lysosomal contents to the cytoplasm ......56

7.2.2.4 P2X7 receptor activation ............................................56

7.2.2.5 Src and Syk tyrosine kinase activation .....................56

7.2.3 The full proteomic picture of rigid MCNT is more similar to asbestos fibres than it is to tangled MCNT (III)........57

7.2.3.1 Protein clustering reveals similarities in the macrophage response towards rigid MCNT and asbestos....................................................................... 57

7.2.3.2 Rigid MCNT activates signalling cascades leading to cell death .........................................................57

8 DISCUSSION.......................................................................................................................... 59

8.1 Toxicity and inflammatory effects of engineered nanomaterials in vitro (I-III) ...............................................................59

8.2 Mechanisms of immunotoxicity induced by engineered nanomaterials in vitro (I-III)..........................................................61

8.3 Nano-sized ZnO penetrate into the diseased skin and cause both local and systemic effects.................................62

8.4 Usability of the collected data in the hazard assessment ............63

8.5 Future prospects.................................................................................65

9 CONCLUDING REMARKS ...................................................................................................... 67

ACKNOWLEDGEMENTS ........................................................................................................69

REFERENCES ..........................................................................................................................71
ABBREVIATIONS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AD</td>
<td>Atopic dermatitis</td>
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<td>ADI</td>
<td>Accepted daily intake</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>Bcl</td>
<td>B-cell lymphoma</td>
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<tr>
<td>bmDC</td>
<td>Bone-marrow derived dendritic cells</td>
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<tr>
<td>bZnO</td>
<td>bulk-sized ZnO</td>
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<tr>
<td>CB</td>
<td>Carbon black</td>
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<tr>
<td>CNM</td>
<td>Carbon nanomaterials</td>
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<tr>
<td>CNT</td>
<td>Carbon nanotubes</td>
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<tr>
<td>DAMP</td>
<td>Danger-associated molecular pattern</td>
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<td>DC</td>
<td>Dendritic cells</td>
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<td>DCNT</td>
<td>Double-walled carbon nanotubes</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EChA</td>
<td>European Chemicals Agency</td>
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<td>ENM</td>
<td>Engineered nanomaterials</td>
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<tr>
<td>FACS</td>
<td>Flow cytometry</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GHS</td>
<td>Globally Harmonized System of Classification and Labelling of Chemicals</td>
</tr>
<tr>
<td>GI</td>
<td>Gastro-intestinal</td>
</tr>
<tr>
<td>GLP</td>
<td>Good laboratory practice</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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<tr>
<td>HARN</td>
<td>High aspect ratio nanomaterials</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IPCS</td>
<td>International Programme of Chemical Safety</td>
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<tr>
<td>LC</td>
<td>Langerhan’s cells</td>
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<tr>
<td>LDH</td>
<td>Lactase dehydrogenase</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MoA</td>
<td>Mode of action</td>
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<tr>
<td>MOx</td>
<td>Metal oxide</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>MCNT</td>
<td>Multi-walled carbon nanotubes</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>MW</td>
<td>Multi-walled</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NM</td>
<td>Nanomaterials</td>
</tr>
<tr>
<td>NOAEL</td>
<td>No observed adverse effect level</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>nZnO</td>
<td>Nano-sized ZnO</td>
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<tr>
<td>OECD</td>
<td>Organisation for Economic Co-operation and Development</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PRR</td>
<td>Pattern-recognition receptor</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure-activity relationship</td>
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<tr>
<td>RA</td>
<td>Risk assessment</td>
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<tr>
<td>REACH</td>
<td>Registration, Evaluation, Authorisation and Restriction of Chemicals</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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<tr>
<td>SCNT</td>
<td>Single-walled carbon nanotubes</td>
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<tr>
<td>SDS</td>
<td>Safety data sheet</td>
</tr>
<tr>
<td>SEB</td>
<td>Staphylococcal enterotoxin B</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
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<tr>
<td>SVHC</td>
<td>Substances of very high concern</td>
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<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>SW</td>
<td>Single-walled</td>
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<tr>
<td>S. aureus</td>
<td>Staphylococcus aureus</td>
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<tr>
<td>Tc</td>
<td>T cytotoxic</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TDI</td>
<td>Tolerable daily intake</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>TFH</td>
<td>T follicular helper cell</td>
</tr>
<tr>
<td>TG</td>
<td>Test guidelines</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>T reg</td>
<td>T regulator</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WOE</td>
<td>Weight of evidence</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on following publications, which are referred to by Roman numerals in the text.


*Equal contribution
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The immune system protects human beings from foreign agents such as viruses, bacteria and particular matter (Murphy, 2011). Skin, respiratory system and gastrointestinal tract are the main routes for foreign agents to enter the human body, and they are protected by a sophisticated network of functions. The mechanistical barrier, the epithelium, covers body surfaces and prevents entrance of foreign agents into the body. In addition to this property, chemical protection such as mucus which contains e.g. enzymes and proteases in the lungs strengthen the protective barrier in the borders of the body. The first line defence cells of immunity, such as macrophages and dendritic cells functionally protect the body by phagocytosing foreign agents entering the body. These cells act as general scavengers in the borders of the body and aim to destroy and inactivate pathogens, or to activate other types of immune cells by secreting inflammatory mediators and/or antigen presentation. If the immune reaction is successful, the foreign agent will be destroyed but if this fails and immune reaction becomes chronic, then health problems may ensue. Chronic inflammation may cause damage to the surrounding tissue and induce severe pathogenic outcomes, even the development of cancer.

Innate immune system cells operate in the first step of the immune reaction (Stampfli and Anderson, 2009). The response is non-specific and rapid and does not create immunological memory. Innate immunity cells may activate adaptive immune response which in turn is specific, slower but does leave an immunological memory ((Bonilla and Oettgen, 2010; Stampfli and Anderson, 2009). Cytokines and chemokines are secreted by different cell types to strengthen, weaken or to polarise immune response (Joshi and Knecht, 2013). Since it is the professional phagocytes that operate in the innate immunity reactions, T and B cells are mostly responsible of adaptive immune responses. In the successful immune response, there is a complex network of cells all operating simultaneously to ensure that invading foreign agents are neutralized.

Engineered nanomaterials (ENM) are widely used in many industries, and it has been estimated that nanotechnology-based manufacturing companies will employ millions of workers in the following decade (Castranova, 2011; Medina et al., 2007). ENM are defined as particles or materials with at least one dimension between 1–100 nm (EU, 2009). Multiple different types of nanomaterials exist, such as carbon-based nanomaterials (NM), metal and metal oxide nanoparticles (NP) (Elsaesser and Howard, 2012; Shi et al., 2013). Carbon-based nanomaterials, such as carbon black (CB) particles and carbon nanotubes (CNT), may be used in electronics, sports equipment, car tires or inks, to mention a few sectors. Metal
oxide (MO\textsubscript{x}) NP, such as titanium dioxide (TiO\textsubscript{2}), silicon dioxide (SiO\textsubscript{2}) and zinc oxide (ZnO), are used for example in the cosmetics, pharmaceuticals, inks, food products or plastics. It is alarming that despite the wide use of ENM their health effects have remained poorly characterised (Castranova, 2011).

The special characteristics of ENM i.e their microscopic size (<100 nm) influence on their toxicity (Castranova, 2011; Elsaesser and Howard, 2012). Small particle size means a large surface area and increased reactivity, because reactivity of a particle is associated with its surface area and other surface properties. Particle surface functionalization, reactive oxygen species (ROS) generation and ion dissolution have been assumed to influence the ENM toxicity, as well as physical characteristics of ENM such as their shape. In addition, the tendency of ENM to form weakly bound complexes with similar active surface area as single particles (agglomerate) or the tendency to form strongly bound complexes with smaller active surface area than in agglomerates (aggregate) has a high influence on the toxic properties of ENM (Nel et al. 2006). Toxicological studies have shown that some types of CNT can induce local inflammation, fibrosis and even cancer \textit{in vivo} (Poland et al., 2008; Takagi et al., 2008). On the other hand, some types of CNT do not seem to be hazardous to health at least \textit{in vitro} or \textit{in vivo} experiments. The identification of toxic characteristics and the mechanism of ENM toxicity are essential for grouping of ENM into non-hazardous and hazardous materials (Nel et al., 2013).

The traditional risk assessment (RA) requires information on a chemical's hazardous characteristics and data from human exposure in order to characterise and manage risks (WHO/IPCS 2010). Hazard identification and characterization are based on standardized testing protocols performed under good laboratory practice (GLP) in testing laboratories. GLP is referred as a management system aiming to ensure quality, i.e. reliability, reproducibility and consistency, of the studies (OECD, 1998). The non-clinical safety-test pattern is expensive and time consuming as the RA process for one chemical may take years. On the other hand, based on the RA efficient risk management measures can be set and risk for health hazards can be minimized. It has been emphasized that in the case of nanomaterials, the current approach of RA is not applicable to protect people, especially in the industrial processes (Hristozov et al., 2012). In addition, immunotoxicology testing which is commonly utilized as a first step of nanotoxicity assessment is not recognised as a part of non-clinical testing of chemicals. As thousands of differentially modified ENM are used in the manufacturing of products, a more efficient RA method should be developed and the data published in science should be analysed as a part of risk evaluation (Nel, 2013). In addition, the suitability of standardized testing protocols needs to be clarified as there is the possibility for false negative or positive results if ENM interfere with assays (Nel et al., 2013).
The aim of this thesis was to investigate the effects and mechanisms of immune response caused by different ENM. Professional phagocytes, macrophages and dendritic cells, were used as cell models of the innate immunity cells able to activate adaptive immune responses. The activation of production and secretion of cytokines and chemokines was studied in order to compare different ENM, and intracellular cascades regulating pro-inflammatory, e.g. IL-1 family, cytokine secretion were investigated after CNT and asbestos exposure. Proteomics tools were used to characterise the full picture of secreted proteins from macrophages and different CNT were compared with asbestos fibres. Furthermore, the effects of nano- and fine-sized ZnO particles were investigated in a mouse atopic dermatitis disease model. Finally, the usefulness of scientific data in the hazard identification and characterisation in the RA process was discussed.
2 IMMUNITY AND CELL DEATH

The human body is protected from pathogens and invasion of foreign matter by the immune system (Murphy, 2011). The immune system is a complex network divided into two functional parts: innate and adaptive immune responses which both consist of a wide range of specialised cells and signalling molecules. A substance inducing an immune reaction is called an antigen, and the proteins produced by specialised cells against antigens are antibodies. The immune system has to create an effective immune response to protect the body from invading pathogens or foreign materials (Murphy, 2011). Cell death is also tightly associated with inflammation. Necrosis induces cell swelling and the release of cell contents leads to the promotion of inflammation in the surrounding tissue whereas activation of inflammation-associated cell signalling cascades triggers different forms of cell death, e.g. apoptosis or pyroptosis (Galluzzi et al., 2012).

2.1 ACTIVATION OF THE INNATE IMMUNITY

2.1.1 CELLS OF THE INNATE IMMUNITY

Innate immunity is a non-specific system intended to destroy different pathogens or foreign matter invading the body (Murphy, 2011). It does not create an immunological memory but is a rapid and effective system for eliminating invading agents, as it is the first line of defence capable of activating adaptive immunity. Physical and biochemical barriers such as skin and surface of respiratory and gastrointestinal (GI) tract as well as different enzymes in the body are important parts of the innate immunity, but the main participants in the response are the immune cells such as neutrophils, macrophages and dendritic cells (DC). These cells are capable of phagocytosis, and they are the main part of innate immunity. They are professional phagocytes acting in the innate immunity and besides their scavenging role of body cavities, these phagocytes are also capable of producing large amounts of toxic chemicals, degradative enzymes and pro-inflammatory mediators to protect body against pathogens. Neutrophils are present throughout the body and are usually the first type of cells attracted to the site of inflammation. They phagocytose and destroy pathogens in their intracellular vesicles with degradative enzymes. They are incapable of antigen presentation but effectively activate bacteriocidal mechanisms (Murphy, 2011). Macrophages and DC are a link between innate and adaptive immunity as they are capable of priming and activating other immune cells by antigen presentation (Barton, 2008; Stampfli and Anderson, 2009). Therefore
they are called professional antigen presenting cells (APC) and these cells are the most important activators of adaptive immune response. A simplified view of APC on the surface of body cavities is represented in the Figure 2.1.

![Figure 2.1. Antigen presenting cells located and functioning in the epithelial lining. 1. Macrophages in the body cavity; 2. Immature DC recruited from bloodstream; 3. Mature DC migrate to the local lymph nodes.](image)

Monocytes circulate in blood and migrate into the tissues where they mature into macrophages. Macrophages are long living phagocytic cells resident in almost all tissues and body cavities, and they have an important role in both innate and adaptive immunity responses (Gardner, 1984). The most important functions of macrophages are to phagocytose invading micro-organisms and particles as well as dead cells and debris as scavenger cells. In addition, they are also able to orchestrate the immune response by secreting signalling molecules (Murphy, 2011). As an example, the macrophages present in the lungs are called alveolar macrophages. They can recognise and kill pathogens in the early course of an infection to prevent them entering to the circulation. The production of pro-inflammatory mediators, reactive oxygen species (ROS) and proteolytic enzymes strives to eliminate pathogens (Joshi and Knecht, 2013; Stampfli and Anderson, 2009).

Dendritic cells (DC) are present throughout the body, e.g. in lungs, skin, liver and on the surfaces of body cavities. In addition to phagocytosis, they act to prime adaptive immune responses, to induce self-tolerance and to eliminate viral infections (Banchereau and Steinman, 1998). There are different types of DC in the different areas of body surfaces such as in the epidermis and in the gut mucosa (Langerhan’s cells; LC) and lungs (alveolar DC) (Banchereau and Steinman, 1998; Novak and Bieber, 2008; Shaw et al., 2012) where they are very responsive to the invading pathogens and foreign matter as well as to the allergens in chemical agents (Kang and Companas, 2009; Shaw et al., 2012). DC engulf pathogens in tissues, and this leads to processing of pathogen antigens which are then presented onto the DC
surface. This process is called maturation, and during this phase, DCs migrate to the local lymph nodes where the T cells come into the contact with peripheral antigens presented by DC. In addition to engulfment of pathogens, the signals leading to DC maturation may be microbial patterns, danger signals or pro-inflammatory cytokines (Sousa, 2006). Maturated DCs are incapable of phagocytosing but are highly efficient in antigen presentation as well as in presenting co-stimulatory and adhesion molecules on their surface (Vermaelen and Pauwels, 2005). This process is an essential stage in evoking a powerful antigen-specific activation of T cells and B cells as well as in the regulation of their functions and responses, leading to efficient adaptive immune response (Banchereau and Steinman, 1998; Kang and Compans, 2009).

### 2.1.2 NLRP3 INFLAMMASOME COMPLEX

Macrophages and dendritic cells as well as other types of immune cells recognise evolutionarily microbial motifs through their pattern-recognition receptors (PPRs) such as Toll-like receptors (TLR) recognising pathogen-associated molecular patterns (PAMPs) and NOD-like receptors (NLR) recognising PAMPs and danger-associated molecular patterns (DAMPs) (Schroder and Tschopp, 2010). Some PPRs are assembled into high molecular weight platforms, inflammasomes, controlling the maturation and secretion of interleukin (IL) -1 – family cytokines, such as IL-1β and IL-18 (Martinon et al., 2002). IL-1β is an important pro-inflammatory cytokine, which activation is tightly controlled by expression, maturation and secretion (Dinarello, 2010). Two signals are required for the secretion of active IL-1β: at first, a pro-inflammatory signal activates the expression of inactive pro-IL-1β in the cell cytoplasm. Secondly, another signal is required for assembly of inflammasome complex to activate caspase-1 enzyme cleaving pro-IL-1β into its active form which is then secreted (Schroder and Tschopp, 2010).
Figure 2.2. Large intracellular NLRP3 inflammasome complex consists NLRP3 scaffolds, ACS (PYD) adaptor molecule and pro-caspase-1 (pro-CASP-1) protein. Pro-CASP-1 is a complex of cardinal-1 (CARD) and caspase-1 (CASP-1) proteins. When the inflammasome is activated, active CASP-1 is released to cleave the inactive pro-IL-1β protein to active IL-1β protein to be secreted out from the cell. The figure represents one part of the large NLRP-3 inflammasome complex with still unknown number of units forming a full complex.

NLRP3 inflammasome consists NLRP3 scaffold, the ASC adaptor molecule and pro-caspase-1 protein. As NLRP3 inflammasome is activated, pro-caspase-1 is spliced into the active caspase-1 protein then able to cleave pro-IL-1β protein into its mature form (Figure 2.2). NLRP3 inflammasome is activated by multiple signals, such as whole pathogens, some PAMPs and DAMPs as well as environmental irritants such as asbestos fibres (Cassel et al., 2008) and silica particles (Hornung et al., 2008). Multiple mechanisms have been shown to activate the NLPR3 inflammasome such as K⁺ efflux via the P2X₇ ATP gated ion channel, lysosomal damage and release of lysosomal contents into the cytoplasm especially if caused by particulate materials, generation of reactive oxygen species (ROS), activation of specific tyrosine kinases (Src and spleen tyrosine kinase Syk), and autophagy (Cassel et al., 2008; Gross et al., 2009; Hornung et al., 2008; Saitoh et al., 2008). However, it is still not known whether the distinct NLRP3 inflammasome activation cascades are inter-regulated or required independently for the inflammasome assembly.

2.2 ACTIVATION OF THE ADAPTIVE IMMUNITY

2.2.1 CELLS OF THE ADAPTIVE IMMUNITY
Tissues, cells and molecules, which act together to provide specific and therefore effective defence system, form the adaptive immunity. The main characteristics of adaptive immunity are specificity, memory and discrimination between self and non-self. The main effector cells of adaptive immunity are lymphocytes, T and B cells. They
are present in the secondary lymphoid organs, lymph nodes and spleen and their task is to eliminate evading pathogens. T and B cells can be activated by the circulating pathogens themselves through antigen binding on the receptors on the surface of T and B cells. In addition, antigen presenting cells activate and functionalise T and B cells via T and B cell receptors (Bonilla and Oettgen, 2010). These very specific receptors recognise a particular antigen being presented by APC and focus the immune response on it. Immunological memory is also based on the large amount of rearranged receptors on the lymphocyte surface. In order to eliminate invading pathogens successfully, several levels of interactions between innate and adaptive immunity are required. Even although the adaptive immune reactions are more specific towards specific antigens, innate immunity still determines the nature of the adaptive T and B cell response (Kang and Compans, 2009).

T cells mature in the thymus and these are the major effector cells in cell-mediated immunity as T cells are activated by APC to create an adaptive immune response. T cells are classified as T cytotoxic (Tc) cells, T regulator (T reg) cells and T helper (Th) cells according to their distinctive cell surface markers. Tc cells express CD8+ surface marker, and recognise antigens bound onto the major histocompatibility complex I (MHCI) molecule whereas T reg and Th cells express CD4+ on their surface and recognise antigens bound to MHCII molecules. For example cytotoxic T cells destroy virus-infected cells by secreting effector molecules and are activated by APC and, in some cases, by Th cells. T reg cells suppress immune response by secreting TGF-β and IL-10 or direct cell-cell contact with for example Tc cells. On the other hand, Th cells activate macrophages, B cells and neutrophils to strengthen the adaptive immune response (Auriemma et al., 2013; Tangye et al., 2012). Th cells are divided into different subclasses based on the cytokines produced during the active phase. These subtypes are presented in Table 2.1. The differentiation of T cells into different lineages of response type is a complex process depending on multiple factors, such as the dose and type of antigen. Furthermore, T memory cells are generated during the first contact with antigen and these are responsible for creating the immunological memory capable of producing rapid immune response when challenged with the same antibody again. For example, macrophages and B cells are able to activate long-living T memory cells to destroy invading pathogen.
### Table 2.1 Different T helper cell subsets, cytokines produced and their targets.

<table>
<thead>
<tr>
<th>T helper cell (Th) type</th>
<th>Typical cytokine production profile</th>
<th>Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>IFN-γ, TNF-α</td>
<td>Intracellular pathogens</td>
</tr>
<tr>
<td>Th2</td>
<td>IL-4, IL-5, IL-13, IL-31</td>
<td>Extracellular pathogens Ab production by B cells</td>
</tr>
<tr>
<td>Th9</td>
<td>IL-9, IL-10</td>
<td>Recruitment of mast cells</td>
</tr>
<tr>
<td>Th17</td>
<td>IL-6, IL-8, IL-17, IL-21, IL-22,</td>
<td>Innate immunity regulation Neutrophil recruitment</td>
</tr>
<tr>
<td>Th22</td>
<td>IL-22, IL-31</td>
<td>Unknown</td>
</tr>
<tr>
<td>TFH</td>
<td>IL-21</td>
<td>B cell activation</td>
</tr>
</tbody>
</table>

B cells mature in the bone marrow and act in the humoral immunity by producing antibodies that have a role in pathogen elimination when they bind to the pathogens. This leads to their neutralisation by the complement system or their removal by phagocytic cells such as macrophages. The sophisticated communication network between T cells or DC with B cells leads to B cell differentiation into antibody producing plasma cells or memory B cells. These cells provide fast and effective removal of a pathogen after the subsequent appearance of the same pathogen in the body (Bonilla and Oettgen, 2010). Plasma cells produce antibodies (Ab) of different isotypes dependent on the antigen: IgA, IgD, IgE, IgG and IgM are produced in order to eliminate the pathogen. Different isotypes have different functions in the adaptive immune response. IgG is the most potent isotype secreted in humans opsonising pathogens to be ingested by phagocytes whereas IgE has an important function in the pathomechanisms underpinning allergy (Bonilla and Oettgen, 2010; Tangye et al., 2012).

#### 2.2.2 ALLERGIC RESPONSES

Allergic response is a hypersensitivity immune reaction targeted at a substance that would not induce immune effects in a non-sensitized individual (Barnes, 2011). Allergic diseases are divided into types I-IV characterised by their immunoglobulin secretion. Type I allergy is mainly driven by immunoglobulin E (IgE) -dependent mechanisms and several inflammatory cells are involved in the allergic inflammation. Dendritic cells, lymphocytes, mast cells, basophils, eosinophils and neutrophils secrete inflammatory mediators to modify the functions of epithelial cells, fibroblasts or vascular cells, etc. The pattern of secreted inflammatory mediators varies extensively, and it may lead to immediate, delayed or chronic allergy. Other factors, such as the genetic background of individual or environmental factors, can all affect the outcome of an allergic inflammation therefore inducing chronic responses and
creating a complex group of allergic diseases such as allergies, atopic dermatitis and rhinitis. These diseases share some common features but have major differences in clinical outcomes. Mast cells initiate allergic immune responses through an IgE-dependent mechanism, which in turn attracts DC to the site of allergic inflammation. DC recruit T cells to the site of inflammation to secrete cytokines important in the immune response. T reg cells are also present at the site of allergic inflammation where they strive to suppress allergic inflammation (Barnes, 2011; Johansson, 2011). Cytokines then activate eosinophils and B cells to release IgE further activating mast cells. In addition, the histamine, leukotrienes, prostaglandins and cytokines secreted by mast cells worsen allergic reactions, independently of the type of allergic disease (Johansson, 2011).

One example of an allergic disease is atopic dermatitis (AD), which is a chronic and relapsing inflammatory skin disease characterised by the presence on lesions and pruritus as well as eosinophil infiltration in the skin and high levels of IgE in plasma. The prevalence of AD is 15–30% in children and 2–10% in adults, and it has been a growing problem in the industrial countries over last decades suggesting that there may be an even higher disease prevalence in the future (Bieber, 2010; Johansson and Bieber, 2002). The allergic reaction in the skin causes itching, which leads to scratching mechanically damaging epithelial barrier. This worsens the inflammation locally in the skin. Infective bacteria, e.g. Staphylococcus aureus (S. aureus), are common in the skin but they do not cause any pathogenic effects in the undamaged skin (Barnes, 2011). However, in the damaged and diseased skin, these bacteria are able to form colonies due to the decreased production of antimicrobial peptides. S. aureus colonisation leads to the release of its enterotoxins such as the superantigen Staphylococcal enterotoxin B (SEB). This activates B and T cell proliferation and extensive IgE production (Barnes, 2010) subsequently causing to IgE sensitisation. It is known that the alteration of the functions of T reg cells is an important step in the pathogenesis of AD (Bieber, 2010).

2.3 INFLAMMATORY MEDIATORS

Immune cells signal to each other by secreting small proteins, peptides or glycoproteins called cytokines or chemokines or by expressing co-stimulatory molecules on their surface. Cells may also secrete other kinds of mediators, such as lipids, purines or reactive oxygen species (ROS) to activate the inflammatory cascade (Barnes, 2011). In particular epithelial cells, macrophages and T cells secrete mainly cytokines whereas antigen-presenting cells (APC), such as dendritic cells, may activate T cells by expressing co-stimulatory molecules on their cell surface. Multiple different cytokines are secreted after the appearance of the activation stimulus. In general,
inflammatory responses are induced by cytokines binding to specific receptors, which then activate signalling cascade resulting in a cytokine-specific response. This drives immune response by cell recruitment and cell differentiation into the specific subtypes (Auriemma et al., 2013).

Cytokines activating different innate immune responses are most often IL-1–family cytokines, such as IL-1β and IL-18, or IL-6, tumor necrosis factor (TNF) -α or interferon (IFN) -γ. IL-1–family cytokines, IL-6 and TNF-α induce pro-inflammatory reactions against different pathogens such as bacteria, whereas IFN-γ exerts significant antiviral activity. IL-8 is also associated with innate immune responses as it attracts neutrophils to the infection site. These cytokines are produced not only by phagocytes but also by numerous other cell types such as endothelial cells and keratinocytes. TGF-β and IL-10 are the cytokines responsible for anti-inflammatory actions. TGF-β activates T reg cells which then secrete IL-10 suppressing many inflammatory reactions such as the IgE production by mast cells. In addition, the chemokines secreted by immune cells induce chemotaxis of nearby responsive cells such as monocytes and neutrophils to the site of inflammation. All secreted inflammation mediators together form a sophisticated balanced immune response which should successfully eliminate any pathogen (Borish and Steinke, 2003).

Activated APC express co-stimulatory molecules (CD40, CD80, CD86) on their surface mostly through pattern recognition receptor (PRR) -mediated recognition of a pathogen. The presentation of co-stimulatory molecules to naïve T CD4+ cells can promote their differentiation into Th1 cells or Th2 cells (Kang and Compans, 2009). In order to activate T cells efficiently, APC must express T cell co-stimulatory molecules to be bound to T cells. The non-specific interaction between these co-receptors and their ligands strengthens the specific interactions between T cell receptor (TCR) and antigenic peptides associated with major histocompatibility complex (MHC) class I or MHC class II and leads to activation of T cells in the adaptive immune response (Neefjes et al., 2011).

2.4 CELL DEATH

Cell death is one of most primitive host defences against an invading pathogen and it is also essential to maintain cellular homeostasis. Programmed cell death achieves the removal of unnecessary cells during embryogenesis, virus infected cells are recognised and killed by specialised cells to avoid the spread of infection and tumorous cells are intended to be recognised and destroyed. A failure in the cell death processes can lead e.g. to tumour development. Multiple proteins, cascades and recognition mechanisms tightly regulate cell death processes since these are
essential for the normal functions of the organism. Different cell death types can be distinguished based on the morphological criteria in conjunction with the biochemical and functional characteristics. The different forms of cell death have their unique characteristics but also share some common features. In the following sections, some common forms of cell death, necrosis, apoptosis and pyroptosis, will be described briefly (Galluzzi et al., 2012). The successful cell death process requires crosstalk between different cell death subroutines with a high level of complexity, and leads to the phagocytosis of dead cells by professional and non-professional phagocytes (Vandenabeele et al., 2010).

2.4.1 NECROSIS

Necrosis is a pathological process, where a group of cells or even tissues die as a response to some overwhelming trauma (Leist and Jaattela, 2001). In the necrotic process, the cell and its components such as mitochondria swell due to water influx and this leads to the disruption of ion homeostasis. Eventually cell membrane integrity is lost, the cell swells more and breaks down releasing cell components into the surrounding tissue. This typically induces profound inflammation (Vanderabeele et al., 2010). For example, adenosine triphosphate (ATP) released from necrotic cells activates NLRP3 inflammasome in the surrounding cells leading to release of IL-1-family cytokines. Necrosis is considered to be an accidental form of cell death, but recent studies have indicated that it may be regulated by a set of signal transduction pathways and catabolic mechanisms (Galluzzi et al., 2014). This regulated cell death process is commonly called necroptosis, however, there are some aberrations in the nomenclature (Galluzzi et al., 2012). The regulated necrosis has a role in some physiological and pathological settings such as alkylating deoxyribonucleic acid (DNA) damage, and it is characterised with regard to specific signalling molecules.

2.4.2 APOPTOSIS

Apoptosis is a highly controlled process leading to cell death of single cells. It is energy-dependent, and leads to deletion of unwanted cells in the physiological processes and in the pathological conditions it may be an attempt to maintain homeostasis (Kerr et al., 1972). The apoptotic process depends on the anti-apoptotic and pro-apoptotic signals. Inhibition of apoptosis is intended to allow cells time to repair the cellular damage, and multiple stimuli such as growth factors, cell matrix, and some viral proteins have anti-apoptotic functions. Apoptosis may be induced by intracellular stress conditions such as DNA damage, oxidative stress, cytosolic
calcium (Ca\(^{2+}\)) overload, or pathological stimuli such as ionising radiation (Galluzzi et al., 2012). In mammalian cells, apoptosis may be an extrinsic or intrinsic process i.e. the extrinsic process is dependent on so-called death receptors expressed in plasma membrane and caspase signalling whereas the intrinsic process is dependent on mitochondria-controlled signalling and may be either a caspase-dependent or a caspase-independent process (Andon and Fadeel, 2012).

Morphological criteria associated with apoptosis have been used to recognise the process by observation under the light microscope (Galluzzi et al., 2012). The apoptotic cell becomes round-shaped, the cellular volume is reduced (pyknosis), chromatin and nucleus are fragmented (karyorrhexis) and pseudopods are retracted but cytoplasmic organelles including lysosomes remain intact. Cell membrane integrity is maintained and the cell first shrinks and then fragments to form apoptotic bodies which can be phagocytosed and eliminated by phagocytosing cells (Kerr et al., 1972). Multiple proteins, such as B-cell lymphoma (Bcl) -2 protein family, are involved in the regulation of apoptosis. These proteins have important roles in the control of intrinsic apoptosis by controlling the formation of the apoptosome. The apoptosome is a multi-protein complex consisting of cytochrome c, deoxyadenosine triphosphate (dATP), apoptotic protease-activating factor-1 (Apaf-1) and procaspase-9 and it serves as a platform to activate caspases (Andon and Fadeel, 2012). Caspases are enzymes belonging to a family of cysteine-dependent proteases that cleave their substrates in the cytoplasm. They function as initiators or executors of apoptosis and are involved in the different processes of inflammation such as IL-1 -family cytokine maturation (Lamkanfi et al., 2009) as well in the process of pyroptosis described below (Bergsbaken et al., 2009).

2.4.3 PYROPTOSIS

Pyroptotic cells share morphological features with both necrosis and apoptosis but pyroptosis is a biochemically distinct process (Bergsbaken et al., 2009). The biochemical feature of pyroptotic cell death is the activation of caspase-1 in the early phase of the cell death process, and this has been associated with the NLRP3 inflammasome, also called the pyroptosome, activation. As described in chapter 2.1.2, NLRP3 inflammasome regulates the activation and secretion of known pyrogenic cytokines, IL-1 -family cytokines. It is still unclear why caspase-1 activation sometimes leads to cytokine secretion without cell death and sometimes triggers pyroptosis, more research into this process will need to be conducted in the future (Galluzzi et al., 2012). Caspase-1 is not involved in apoptosis or necrosis and in this respect it differs from the apoptotic caspases. In turn, caspase-3 or caspase-8, are not involved in pyroptosis (Bergsbaken et al., 2009).
Pyroptotic cells suffer from rapid plasma-membrane rupture by pore formation in the plasma membrane leading to water influx, cell swelling and a significant increase in cell size and, eventually, to osmotic lysis. The uncontrolled cell lysis leads to release of pro-inflammatory contents into the surrounding tissue causing extensive inflammation. All of the functions and targets of caspase-1 are still not known, and more research is currently being conducted in attempts to clarify the mechanism and features of pyroptosis (Bergsbaken et al., 2009).
The European Union defined nanomaterials in 2011 as ‘a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or agglomerate; where 50% or more of the particles exhibited, one or more external dimensions in the size range of 1–100 nm’ (EU, 2011). Figure 3.1 demonstrates the size relation of nanomaterials to different sized objects such as lung alveoli and salt grain. Engineered nanomaterials (ENM) are widely utilised by different industries and nanotechnology has been claimed to provide possibilities to improve the competitiveness of the products and industrial sectors responding to societal challenges (Medina et al., 2007). In fact, EU has nominated nanotechnology as a key enabling technology of industry (EU, 2009). Many industrial branches use ENM to make their products user-friendlier or to improve their market competitiveness, for example in the electronics, cosmetics and sports industry sectors as well as in pharmaceuticals, diagnostic tools and medical imaging. It has been estimated that millions of workers worldwide operate in the nanofield before 2020 (Castranova, 2011). Therefore, the most common way to become exposed to ENM is in occupational settings, either via inhalation or dermal exposure. The challenge is that there is not sufficient knowledge of risk of the hazard, toxicity or the environmental effects of ENM (Hristozov et al., 2014b; Nel, 2013). As human exposure to ENM is likely to increase, substantially in the future identification and quantifying of potential health hazards of ENM is essential for the human health risk assessment and for setting the appropriate risk management measures to protect people for the health effects induced by exposure to ENM (Hristozov et al., 2012; Kroll et al., 2009).

![Figure 3.1](image.png)

**Figure 3.1.** An illustration describing the size of different objects in comparison to engineered nanomaterials.
3.1 GENERAL PROPERTIES

There are several groups of nanomaterials classified by their physical and chemical characteristics (Elsaesser and Howard, 2012). Metal nanoparticles (NP), metal oxide NP, carbon nanomaterials (CNM) and carriers or vehicles in the drug delivery are examples of different groups of ENM and are described as follows. For example, gold (Au), silver (Ag) and copper (Cu) can be produced as nanosized particles. In the industrial use, metal oxide NP are excellent catalysts and have interesting optical properties depending on particle size and shape. For instance, nano-sized iron oxide (FeO\textsubscript{x}), titanium dioxide (TiO\textsubscript{2}), zinc oxide (ZnO) and copper oxide (CuO) represent metal oxide nanoparticles. These materials are used commonly in biomedical imaging and in cosmetics to improve properties of beauty products and to produce user-friendly sunscreens, and they can also be utilised in drug delivery (Medina et al., 2007; Nath and Banerjee, 2013; Shi et al., 2013). Another main class of nanomaterials are made out of carbon. Different types of CNM such as carbon black (CB), fullerenes, graphene, single-walled (SCNT), double-walled (DCNT) and multi-walled (MCNT) carbon nanotubes have high electrical conductivity and excellent strength. In an ideal carbon nanotube (CNT), a graphene sheet is rolled and welded into a seamless tube (Luo et al., 2013). MCNT has more graphene layers, and is thicker and more rigid whereas one-layered graphene roll forms SCNT (De Volder et al., 2013). CNT are commonly used in many sectors of industry, such as electronics, catalytic processes and in sports equipment such as tennis rackets (Dumortier, 2013).

Several types of ENM have been used as carriers in the drug delivery, utilising liposome as a carrier structure. These lipid vesicles carry drugs to the target cells and release their contents (Lian and Ho, 2001). In addition to pharmaceutical products, liposomes are used in cosmetics, such as in shampoos and skin care products (Hoet et al., 2004). Microemulsions are commonly used to enhance the solubility of insoluble materials and to aid the transport of pharmaceuticals in the body (Spernath and Aserin, 2006). Biodegradable polymers have been developed for use in drug delivery systems and implant materials. Ceramic NM are inorganic systems with porous characteristics and they have been claimed to serve as drug vehicles in cancer therapy but they seem to be non-biodegradable and a major concern is the possible accumulation in the body causing unwanted effects (Medina et al., 2007; Yih and Al-Fandi, 2006). The ability of certain ENM to cross the body’s protective barriers may serve as a tool for therapeutics but also a source of unwanted effects, for example should an ENM cross the placental barrier (Pietroiusti et al., 2013).
3.2 UNIQUE CHARACTERISTICS

Nanomaterials may be potentially more toxic than fine-sized particles of the same materials because of their unique physicochemical properties such as shape, particle size distribution, surface characteristics like coating and charge, solubility and level of aggregation or agglomeration (Elsaesser and Howard, 2012). When size decreases, the specific surface area per mass unit increases and the NP becomes more reactive than its fine-sized particle counterpart (Castranova, 2011). For example, it has been demonstrated that solubility of ZnO particles increases when its size decreases (Reed et al., 2012). The coating of a NP may change its properties drastically, for example from being hydrophilic to hydrophobic, and modify their surface reactivity (Nel et al., 2006; Shi et al., 2013). Although these unique characteristics may be considered as an attractive property of ENM, they also may pose human health risks. Therefore a careful characterisation of material properties should be required for all studies performed with ENM to allow comparison of data and to provide information for risk assessment purposes (Shi et al., 2013). In addition, surface characteristics of ENM can affect on the formation of the protein corona on the material surface, which will be further discussed in 3.5.

3.3 IMMUNE RESPONSE AND TOXICITY

Generally, when foreign materials enter the body they are removed by the normal defence systems in the body such as ciliated cells and mucus secretion in the lungs or engulfed by the phagocytosing cells such as macrophages. This may lead to total cleavage of materials but scavenger cells may also secrete pro-inflammatory signalling molecules to activate other types of immune cells (Gwinn and Vallyathan, 2006). ENM may also evoke the production of reactive oxygen species (ROS) inducing oxidative stress capable of activating innate immune response (Hornung et al., 2008) or induce release of pro-inflammatory cytokines independently of ROS production (Lu and Liu, 2009). In addition, it has been postulated that NM may escape phagocytosis, cross cell membranes and redistribute to other sites of body and lead to systemic effects such as cardiovascular diseases or dissolve to reactive ions after entering the cell, for example in the acidic environment in lysosomes (Reed et al., 2012, Kettiger et al., 2013). However, the ability of ENM to cross membranes may be used in treatment of diseases e.g. in the targeted cancer therapy (Elsaesser and Howard, 2012).

ENM may evoke different types of cell death, such as necrosis or apoptosis (Andon and Fadeel, 2012). Apoptosis may be induced by mitochondrial perturbation
and the release of pro-apoptotic factors. Frustrated apoptosis by macrophages in the case of high aspect ratio nanomaterials (HARN) may lead to release of pro-inflammatory mediators and eventually to the development of fibrosis in the target organ (Gou et al., 2010). Impurities such as residual transition metals, like iron (Fe) or nickel (Ni), from the CNT production process possibly potentiate CNT reactivity with macrophages and epithelial cells (Castranova et al., 2013; Luo et al., 2013). Other types of injuries are also possible after the exposure to ENMs including protein denaturation, membrane or DNA damage, the formation of foreign body granulomas and binding of the proteins to ENM surface leading to cellular malfunctions (Elsaesser and Howard, 2012; Luo et al., 2013; Nel et al., 2006).

To summarise, there is an urgent need for more precise knowledge of mechanisms of ENM toxicity (Clift and Gehr 2011). Mechanistical information can form a basis for developing qualified risk assessment methods for screening of nanomaterial toxicity. *In vitro*–assays are rapid, simple and cheap as compared to *in vivo*–studies but they do not reveal the entire scope of the possible toxicity. In addition, co-cultures of multiple cell types are a valid alternative for *in vitro*–screening of toxicity (Dekali et al., 2012). On the other hand, the possibility of ENM interference with assays has to be studied carefully to avoid relying on false positive or negative results in toxicity testing. One example of known interference occurs with Organisation for Economic Co-operation and Development (OECD) guideline 471, Bacterial reverse mutation assay, this test for genotoxicity (Ames test) and it should be excluded from the ENM genotoxicity test battery (Clift et al 2013). To conclude, one crucial part of the nanomaterial toxicity testing is to develop suitable testing assays and to ensure that the existing methods are suitable for evaluating ENM.

### 3.4 POTENTIAL EXPOSURE ROUTES

The most common exposure routes to ENMs are inhalation, dermal and gastrointestinal exposure. Inhalation exposure to ENMs occurs mostly due to occupational exposure during the production of nanomaterials or nanomaterial-based products. The upper airways (nose) and lower airways (lungs) are exposed to dusts when the individual breathes, and there are several defence mechanisms to keep the mucosal surfaces free from deposited particles. Should ENM be able to escape or alter defence mechanisms, they may induce local toxic effects. The phagocytes which are cells involved in cleaning body cavities, may be targets of exposure to nanomaterials (Geiser and Kreyling, 2010). Oxidative injury, inflammation, fibrosis, cytotoxicity and inflammation mediator release have been reported to be the main effects after inhalation exposure to fibrous asbestos (Nel et al., 2006), and *in vivo*–studies have shown that the high aspect ratio CNT induce inflammation, fibrosis
and mesothelioma (Poland et al., 2008; Ryman-Rasmussen et al., 2009; Takagi et al., 2008). A long-term study in mice also indicated that SCNT could cause more extensive fibrosis in lungs than asbestos fibres (Shvedova et al., 2014). It remains unclear whether ENM are able to cross the air-blood barrier to be translocated to other sites of the body or induce systemic inflammation (Castranova, 2011).

Dermal exposure to ENMs occurs mostly through application of cosmetic products and sunscreens as well as in the occupational settings (Elsaesser and Howard, 2012). Metal oxide nanoparticles TiO$_2$ and ZnO are used in the cosmetics and sunscreens. These nanoparticles are often coated in order to change the surface properties of the NP and to minimise their reactivity. Several studies suggest that NP cannot pass through intact skin because of the unique layered structure of skin. However, if skin is damaged by mechanical injury, ultraviolet (UV) -radiation or allergic inflammation, translocation to deeper layers of skin may occur. Then they may be engulfed by phagocytic cells and to end up in the regional lymph nodes, or travel in the blood circulation to distant organs. Cytotoxicity, inflammatory response, possible accumulation into the skin, metabolism and phototoxicity are all important areas to needing to be evaluated when assessing dermal toxicity (Tsuji et al., 2006).

GI-tract may also be a route for ENM to the body especially when they are being used in the food products, water and pharmaceuticals (Shi et al., 2013). Inhaled ENM may also be swallowed once mucociliary escalator cleared them from the respiratory tract. It remains unclear whether ENM are absorbed from the gut and translocated to systemic circulation and distribute to other sites of the body (Powell et al., 2010). ENM may also induce a local inflammatory response in the GI-tract (Medina et al., 2007). However, in vivo –studies suggest that most ENM in GI-tract are rapidly discarded via faeces.

### 3.5 TARGET ORGANS

If ENM gain access to the circulation, they may exert effects in the blood vessels or end up to other organs (Kettiger et al., 2013). The distribution of ENM to organs depends on the site of translocation into the blood stream. Furthermore, if ENM tend to agglomerate, they may also cause an embolism in the blood circulation which is a serious health concern. ENM can be distributed to all sites of body, such as liver, spleen, kidneys, heart, brains and central nervous system and they may travel through placenta (Pietroiusti et al., 2013). The most important factors that influence ENM targeting of organs are their size, shape and their surface functionalities but also the cells of the translocation site affect on the outcome (Albanese et al., 2013). It has been speculated that spherical particles are taken up more rapidly than fibrous materials, and that the most efficiently uptaken particles will have a diameter of
This knowledge can be utilised in the development of nanotechnology-based drug carriers. In addition, the effects of ENM on platelet activation and thrombus formation as well as the effects of ENM on the microcirculation have been studied in vivo. Investigations into engineered nanomaterials showed that SCNT could interfere with platelet function and cause pro-thrombic effects on microcirculation whereas diesel exhaust and titanium dioxide did not have any effects on the blood vessels (Bihari et al., 2010). The effect of different ENM in blood vessels and their distribution to distant organs has to be studied more extensively in the future.

The positive or negative effects induced by ENM are modified by protein corona formation (Kettiger et al., 2013, Tenzer et al. 2013). The attachment of biomolecules on the material surface is shown to be dependent on multiple factors such as material size, shape, chemical composition and surface charge. Furthermore, the absorption of biomolecules, such as proteins, to their ENM surface depends on the type of biological fluid with which they are surrounded (Tenzer et al., 2013). The ENM route of entry to the body modifies the biomolecular layer in the ENM surface, and this protein corona may be responsible for the true biological identity of ENM. The protein corona may enhance the ENM uptake into the cells, induce their active or passive transport through body barriers and cause interactions with cellular components. This may lead to toxicity in exposure routes themselves or in the translocation sites of ENM (Pietroiusti et al., 2013). Therefore, the material-protein binding studies will need to be included into the hazard assessment of ENM.
4 HEALTH RISK ASSESSMENT

4.1 PRINCIPLE

Risk is a combination of the hazard and the exposure, and therefore risk assessment (RA) is divided into different phases. Different organisations, such as European Chemicals Agency (ECHA) and World Health Organisation (WHO) describe the process in somewhat different ways but the purpose is the same: To ensure chemical safety. Hazard and exposure assessment are essential for the analysis of risk, and risk management is a regulatory process intended to minimise the risk (WHO/IPCS 2010; ECHA 2011, ECHA 2012). RA may lead to the development of guidance values for chemical residuals e.g. in food, to setting of exposure limits for occupational exposure and even restriction of use of a substance. The simplified schematic flow of the RA process used in both substance and product evaluation is represented in the Figure 4.1.

![Figure 4.1. A schematic flow of risk assessment process.](image)

Hazard assessment is the process where a specific chemical hazard is identified and one analyses its potential to cause toxic effects on humans (ECHA 2011). The identity of a chemical is determined by evaluation of existing safety data. When possible, the quantitative structure-activity relationship (QSAR) data is analysed in silico, data from in vitro and in vivo studies is evaluated according to the current RA paradigm. If any epidemiological data is available, it can be taken into account in the hazard and risk assessment at chemical. The in vivo –data forms a basis of hazard identification, however, public pressure to limit unnecessary animal suffering, and this has been taken into account in REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) legislation (1907/2006/
EC) by promotion of development of alternative methods for the hazard assessment. In addition to this, the main aims of REACH Regulation are to ensure the high level of protection of human health and environment and to improve industrial competitiveness and innovation potential by unifying means of hazard assessment in the EU level. REACH requires authorization of chemicals produced more than 1 tonnage, and if a chemical poses a risk for human health or environment, it may not be authorized or it will be added to Annex XIV. Substances in Annex XIV are to be replaced when safer alternatives for the same use are available.

In the Globally Harmonized System of Classification and Labelling of Chemicals (GHS; UNECE 2010) specific criteria on hazard classification as well as guidance on labelling and for safety data sheets (SDS) have been adopted in order to improve hazard communication. GHS also describes the endpoints required to the human health risk assessment, such as acute toxicity, skin corrosion/irritation, carcinogenicity, respiratory sensitization and toxicity to reproduction, and these effects can be further categorised by a chemical’s potency to induce toxicity (WHO/IPCS 2010). Toxic endpoints are studied with specific and validated test guidelines (TG) for chemicals such as TG provided by OECD or with other suitable, justified methods. The quantitative or qualitative description of a chemical’s hazard characteristics is performed with the results obtained from these studies. In addition, the hazard assessment should include the identification and description of mode of action (MoA) for critical steps in toxicity such as clarifying molecular and cellular pathways (ECHA 2012). Table 4.1 summarises the most common abbreviations used in the risk assessment.

The human contact with a potentially hazardous chemical as well as exposure route, length and volume of exposure are evaluated in the exposure assessment (WHO/IPCS 2010). Different scenarios are available to routinely calculate exposure of different groups, like adults or infants via possible routes, such as food, cosmetics or toys based on the chemicals concentration on the items and on the estimated exposure route and length. Certain parameters, for example human body weight, are set to represent the average person, in the exposure assessment calculations. REACH legislation provides guidance by describing exposure scenarios in attempts to improve the control of risk (ECHA 2007). In addition, if there are known biomarkers of exposure, they may be measured from biological samples such as blood and urine and utilised in the exposure assessment calculations. With knowledge of hazard and exposure parameters, the risk can be characterised by comparing calculated human exposure levels to delivered guidance values. Then, risk prevention by personal protection, risk mitigation measures and risk communication can be utilised as a tool of risk management.
Table 4.1. The description of the common abbreviations used in the risk assessment (ECHA, 2013).

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADI</td>
<td>Acceptable Daily Intake</td>
<td>An amount of specific chemical that can be ingested on a daily basis during the lifetime without a health risk, e.g. food additives.</td>
</tr>
<tr>
<td>DNEL</td>
<td>Derived no-effect level</td>
<td>An amount exposure of a specific chemical that above humans should not be exposed. In REACH, DNEL determination is required for chemicals produced 10 tonnes or more per year.</td>
</tr>
<tr>
<td>MoA</td>
<td>Mode of action</td>
<td>Functional of anatomical change in cellular level resulting from the exposure to the chemical.</td>
</tr>
<tr>
<td>NOAEL</td>
<td>No observed adverse effect level</td>
<td>The highest dose of chemical that is not causing any adverse effects in the testing organism.</td>
</tr>
<tr>
<td>OECD TG</td>
<td>OECD test guidelines</td>
<td>A collection of internationally agreed test methods used by government, industry and independent laboratories. OECD TG are used to determine the safety of chemicals and chemical preparations.</td>
</tr>
<tr>
<td>SDS</td>
<td>Safety data sheet</td>
<td>SDS is intended to provide people procedures for handling or working with that chemical in a safe manner. Requirements are escribed in REACH article 31.</td>
</tr>
<tr>
<td>TDI</td>
<td>Tolerable daily intake</td>
<td>An amount of a chemical that can be ingested on a daily basis during the lifetime without a health risk. TDI is used in the case of a specific chemical that is not used intentionally in food, e.g. contaminants.</td>
</tr>
<tr>
<td>WOE</td>
<td>Weight of evidence</td>
<td>WOE is seldom defined. The most common use is a general concept to summarize, synthesize and interpret a body of evidence.</td>
</tr>
</tbody>
</table>

4.2 CHALLENGES WITH NANOMATERIALS

Nanomaterials may fall under different regulations in the EU area, such as REACH (1907/2006/EC), even if ENM are not referred in Regulation 1907/2006. However, as REACH deals with substances in whatever size, shape and chemical composition, ENM are covered by REACH. More specific product type regulations refer ENM such as biocide (528/2012/EU), plant protection products (1107/2009/EC) or pharmaceuticals regulation (726/2004/EC). These legislations strive to safeguard and guarantee the appropriate use of different chemicals, and provide regulation for placing products into the market and use of the products.

The greatest challenge in ENM risk assessment and management is the diversity of materials and the lack of requirements for the material characterization (Krug and Wick, 2011). Since the material characteristics drastically affect their toxic properties such as their capacity to deposit to the different regions of lungs, enter to the systemic circulation or cross biological barriers, a specific guidance for characterization requirements should be provided. For example size, shape, and surface chemistry data should be provided when nanotoxicity results have been published to make it possible to identify which physico-chemical characteristics are associated with
toxicity (Hristozov et al., 2012). In addition, those ENM properties important to the identification of specific nanomaterial rather than to the characterization should be recognized (OECD, 2013). Furthermore, it is important to document the behaviour of the materials in the suspension and their tendency to form agglomerates or aggregates. The OECD has published a ‘Guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials’ to unify material handling protocols to be used in the nanosafety testing. However, it will take time to set up the protocols in the different laboratories (OECD, 2012). As risk is also dependent on the route of exposure, the material-specific exposure routes and doses will need to be identified (Krug and Wick, 2011). In REACH, is is suggested that substances identified by their chemical composition may need to be further identified by additional identifiers, such as characterization data (1907/2006/EC). The ENM separation from bulk-form of the same chemical composition is challenging, even if it is stated in REACH that material ‘sameness’ has to be analyzed by the comparison of physical properties, not by comparing the chemical composition. Nevertheless, traditional approaches to RA using physico-chemical properties as a predictor of toxicity do not seem to be suitable for the RA of ENM, and the utilization of high-throughput screening approaches for the identification of materials characteristics associated with the hazard is in progress (Nel et al., 2013). Also, the adjustments for the information requirements in REACH (1907/2006/EC) are to be performed to assess ENM behavior and effects on individuals, and to develop new, nano-relevant exposure scenarios as well as risk management measures.

The huge number of different forms of ENM being used by different industries poses a challenge for traditional RA requirements (Hristozov et al., 2012). There are pressures to reduce the numbers of animals used in the chemical hazard evaluation, and therefore new protocols for chemical testing in vitro or the evaluation of QSAR in silico are required (1907/2006/EC). In vitro –studies can be utilized in the identification of hazard mechanisms to understand and recognize the pathways activated by the ENM, and to predict the toxicological outcome (Hristozov et al., 2014b). An awareness of MoA associated with physico-chemical properties of material could also help in the grouping of materials which then could be used in the extrapolation to other related compounds in their hazard characterization (Nel et al., 2013). However, animal testing is still and will be an essential part of the hazard evaluation of ENM: Chronic studies are required and in vivo –studies are essential for validation, verification and bridging cell experiments with systemic results and to extrapolate exposure-dose –response (Nel, 2013). Building data bases by sharing data of physico-chemically and toxicologically (in vitro) characterized nanomaterials would enable the use of a systems biology approaches for large data sets, and this is hoped to eventually lead to a better understanding of key elements in nano hazard identification (Hristozov et al., 2014b; Nel et al., 2013).
However, the lack of benchmark or reference materials to different testing protocols in nanotoxicology poses a challenge in the evaluation of the quality of data and to the validation of test methods (Nel et al., 2013; Schrurs and Lison, 2012).

To conclude, the main challenges in the hazard assessment of ENM are the large number of materials which need to be evaluated case-by-case (Hristozov et al., 2012). This is time consuming and expensive and has high requirements since hazard assessment is based on in vivo –testing, and current RA protocols may require modification, regarding both test methods for the identification of hazard and exposure assessment. Different approaches to be used, i.e. ENM grouping, hazard scanning, QSAR evaluation, testing of toxicological potency in vitro or predictive toxicological modelling, have been suggested, however, there are knowledge gaps still to be filled before any of the postulated methods can be applied routinely (Nel et al., 2013). Furthermore, a quantitative weight of evidence (WOE) approach could be utilized to calculate hazard scores from scientific studies (Hristozov et al., 2014b). The problem with the WOE approach is that the more data gaps that exist in the reporting of study, the more expert judgment is required and thus the more uncertain is the final result. This uncertainty has been and is analyzed in large international project funded by 7th framework program of EU, such as in NANoREG (http://www.nanoreg.eu) and MARINA (http://www.marina-fp7.eu). However, good quality data collected from scientific research should also be taken into the account in the regulatory RA of ENM in order to minimize the costs of nanosafety research, and to promote the development of appropriate methods for hazard identification and characterization of ENM (Hristozov et al., 2014a). According to REACH, a scientific evidence of probable serious effects may lead to classification of chemical to the list of substances of very high concern (SVHC) to be replaced by suitable alternatives, and this approach may be utilized with ENM with an alarming scientific data (1907/2006/EC).
5 AIMS OF THE STUDY

The safety of engineered nanomaterials is an inadequately-studied area, and there are concerns about the well-being and health of industrial workers handling ENM as well as consumers using nano-based products. Therefore risk assessment and management of different nanomaterials are important issues. The overall aim of this thesis was to study the toxicity of selected nanomaterials, their ability to induce inflammatory reactions, the mechanisms behind these phenomena and in this way contribute to the development of risk assessment protocols for engineered nanomaterials.

The specific aims of this thesis were to:

• Evaluate the ability of different engineered nanomaterials to activate inflammatory cascade (I, II)

• Investigate the mechanisms of pro-inflammatory reactions induced by carbon nanotubes and compare these with asbestos fibres (A high aspect ratio theory: II, III)

• Compare the penetration and effects of different sized ZnO particles on diminished skin epithelial barrier with or without allergic inflammation (IV)

• Evaluate how current approach for chemical hazard assessment serves the needs of nanomaterial risk assessment (I–IV)
6 SUMMARY OF MATERIALS AND METHODS

The materials and methods used in the study are described in more detail in the original publications I–IV.

6.1 NANOMATERIALS AND THE SUSPENSION PREPARATION PROTOCOL (I–IV)

The research on the thesis was concentrated on metal oxide NP (TiO$_2$ and ZnO) and different CNM. All materials studied and their references are represented in Tables 6.1 and 6.2.

Table 6.1. Metal oxide (MOx) nanoparticles studied, their basic characteristics and references.

<table>
<thead>
<tr>
<th>Material</th>
<th>TiO$_2$</th>
<th>TiO$_2$(SiO$_2$)</th>
<th>ZnO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>30-40 nm</td>
<td>10 x 40 nm</td>
<td>20 nm</td>
</tr>
<tr>
<td>Elements</td>
<td>Ti, O</td>
<td>Ti, O, Si</td>
<td>Zn, O</td>
</tr>
<tr>
<td>Product number</td>
<td>5485HT</td>
<td>637262</td>
<td>5810MR</td>
</tr>
<tr>
<td>Producer</td>
<td>NanoAmor</td>
<td>Sigma-Aldrich</td>
<td>NanoAmor</td>
</tr>
<tr>
<td>References</td>
<td>I</td>
<td>I</td>
<td>I, IV</td>
</tr>
</tbody>
</table>

The protocol for suspension preparation was similar in all studies. First, materials were weighed into glass tubes and a stock solution [1000 μg/ml] was diluted in the presence of 2% fetal bovine serum (FBS). Secondly, the stock solution was water-path sonicated in 30°C for 20 minutes and further diluted to the concentrations used in each study. If multiple concentrations were used in the experiments, serial dilutions of NM were prepared.Study concentrations were then water-bath sonicated at 30°C for 20 minutes before application to the experimental model.
Table 6.2. Carbon nanomaterials studied, their basic characteristics and references.

<table>
<thead>
<tr>
<th>Material</th>
<th>Carbon black (CB)</th>
<th>SCNT</th>
<th>MCNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>14 nm</td>
<td>&lt; 2 nm, 1-5 Qm</td>
<td>10-30 nm, 1-3 Qm</td>
</tr>
<tr>
<td>Composition</td>
<td>C</td>
<td>C, traces of Co</td>
<td>C, traces of Ni</td>
</tr>
<tr>
<td>Trade name / Product number</td>
<td>Printex-90</td>
<td>900-1351</td>
<td>900-1260</td>
</tr>
<tr>
<td>Producer</td>
<td>Evonik Industries</td>
<td>SES Research</td>
<td>SES Research</td>
</tr>
<tr>
<td>References</td>
<td>II</td>
<td>I</td>
<td>I</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Material</th>
<th>sMCNT</th>
<th>tMCNT</th>
<th>rMCNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>5-20 nm, 1-10 Qm</td>
<td>8-15 nm, 10-50 Qm</td>
<td>&gt; 50 nm, 13 Qm</td>
</tr>
<tr>
<td>Composition</td>
<td>C (&gt;99%)</td>
<td>C (&gt;99%)</td>
<td>C (&gt;99%)</td>
</tr>
<tr>
<td>Trade name</td>
<td>Baytubes C 150 HP</td>
<td>MWCNT B-15 OD</td>
<td>Mitsui-7</td>
</tr>
<tr>
<td>Producer</td>
<td>Bayer Material Science</td>
<td>Cheaptubes, Inc.</td>
<td>Mitsui&amp;Co</td>
</tr>
<tr>
<td>References</td>
<td>II</td>
<td>II, III</td>
<td>II, III</td>
</tr>
</tbody>
</table>

6.2 CELL MODELS (I-III)

RAW 264.7 mouse macrophages (TIB71, ATCC, US), mouse bone marrow derived dendritic cells and human primary macrophages were used in the studies. The mouse bone marrow derived cells were isolated from balb/c mice (Scanbur, Sweden). Briefly, bone marrow was flushed out from mice hind legs, cultured in RPMI 1640 media (Invitrogen, UK) with suitable supplements (detailed description in the I) and mouse granulocyte macrophage colony stimulating factor (GM-CSF) for six days before experiments to allow their differentiation into dendritic cells. Human monocyte-derived primary macrophages were differentiated from leukocyte-rich buffy coats from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Finland) by low-speed density gradient centrifugation on Ficoll-Paque Plus (Amersham Biosciences, Sweden). The non-adherent cells were washed away and adherent monocytes were cultured in serum-free macrophage medium (Macrophage-SFM; Invitrogen, UK) supplemented with human GM-CSF (BioSource, USA) for 7 days to allow monocyte differentiation into macrophages which were then exposed to different ENM (described in II and III).

6.3 THE MOUSE MODEL OF ATOPIC DERMATITIS (IV)

The mice were epicutaneously treated with phosphate buffered saline (PBS, vehicle), bulk-sized ZnO particles (bZnO), nano-sized ZnO particles (nZnO), combination of ovalbumin (OVA, 100 μg, Sigma-Aldrich Co, US) and staphylococcal enterotoxin B
(SEB, 2.5 μg, Sigma-Aldrich Co, US), and a combination of OVA, SEB and bZnO or a combination of OVA, SEB and nZnO under isoflurane anesthesia. The exposure mass of ZnO particles was 1.66 mg. The backs of the mice were shaved with an electronic razor and tape-stripped by adhesive tape. Exposing agents (V=100 μl) were added on the sterile gauze (1 cm²) then secured onto the back skin of the mice with transparent adhesive tape twice a week (the first sensitization week). After a two week recovery period, the mice were again tape-stripped (the second sensitization week), and an identical patch was reapplied to the same skin site. Patches were applied twice during the first sensitization week and three times during the second sensitization week. Mice were sacrificed and samples collected 24 h after the last sensitization. The schematic flow of the sensitization and exposure protocol is represented in Figure 6.1.

![Figure 6.1](image)

**Figure 6.1.** Mouse AD model: A Schematic flow of sensitization and exposure protocol. Mouse patching was performed with either PBS or combination of OVA (100 μg/patch) and SEB (2.5 μg/patch). Arrows indicate days of sensitization. Elicitations were performed with PBS, OVA+SEB or NP alone or with combination of OVA+SEB and NP.

### 6.4 METHODS

Only a brief description of methods will be provided. All methods utilized in the study and their original publications are represented in Table 6.7. at the end of this chapter.

#### 6.4.1 CELL DEATH ANALYSIS (I, III)

Three different assays were used to study cell viability: Trypan blue and Lactate dehydrogenase® (LDH) assay for general cytotoxicity and Apoptosis Percentage Apoptosis Assay® for apoptosis studies. Trypan blue stains dead cells whereas live
cells remain unstained, and the percentage of dead cells was calculated under the light microscopy. The LDH assay (Cytotoxicity Detection KitPLUS, Roche, Germany) was performed and results were analysed according to the instructions given by the manufacturer. APOPercentage apoptosis assay (Biocolor Life Sciences Assays, UK) was used according to the manufacturer’s instructions and apoptotic cells were manually calculated on the light microscopy.

6.4.2 CYTOKINE/CHEMOKINE EXPRESSION ANALYSIS (I, IV)

Quantitative real-time reverse transcription-PCR (RT-PCR) analyses were performed with 7500 Fast Real-Time PCR system (Applied Biosystems, US). RNA was extracted using Trisure® Reagent (Bioline, UK) according to reagent instructions. Total RNA concentration and purity were determined by NanoDrop ND-100 Spectophometer (Thermo Scientific, DE) and complementary DNA (cDNA) was synthesized with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The relative expressions of a target gene messenger-RNA (mRNA) were analyzed with PCR primers and probes were obtained from Applied Biosystems as pre-developed reagents (Table 6.4.). The gene expression between different samples was normalized to 18S housekeeping gene.
Table 6.4. Mouse primers and probes used in RT-PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Probe</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL-5</td>
<td>Mm00436451_g1</td>
<td>I</td>
</tr>
<tr>
<td>CXCL-9</td>
<td>Mm00434946_m1</td>
<td>I</td>
</tr>
<tr>
<td>CXCL-10</td>
<td>Mm00445235_m1</td>
<td>I</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Hs00174143_m1</td>
<td>IV</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Mm00434228_m1</td>
<td>I, IV</td>
</tr>
<tr>
<td>IL-4</td>
<td>Hs00174122_m1</td>
<td>IV</td>
</tr>
<tr>
<td>IL-6</td>
<td>Mm00446190_m1</td>
<td>I, IV</td>
</tr>
<tr>
<td>IL-10</td>
<td>Mm00439616_m1</td>
<td>IV</td>
</tr>
<tr>
<td>IL-13</td>
<td>Hs00174379_m1</td>
<td>IV</td>
</tr>
<tr>
<td>IL-33</td>
<td>Hs00369211:m1</td>
<td>IV</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Mm00441258_m1</td>
<td>I</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Mm00443258_m1</td>
<td>I, IV</td>
</tr>
</tbody>
</table>

6.4.3 CYTOKINE/CHEMOKINE SECRETION AND SERUM ANTIBODY ANALYSIS (I, II, IV)

Secretion of cytokines and chemokines to cell culture supernatants (I, II) as well as total antibody levels (IV) in the mouse serum was analyzed with standard sandwich ELISA and serum levels of antigen-specific antibody levels (IV) were analysed with straight ELISA according to the manufacturer’s instructions. All ELISA assays used in the study are summarized in Table 6.5.

Table 6.5. ELISA assays used in the study.

<table>
<thead>
<tr>
<th>Cytokine / Chemokine</th>
<th>Manufacturer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human IL-1α</td>
<td>Diaclone, France</td>
<td>II</td>
</tr>
<tr>
<td>Human IL-1β</td>
<td>Diaclone, France</td>
<td>II</td>
</tr>
<tr>
<td>Human IL-18</td>
<td>Bender MedSystems, Austria</td>
<td>II</td>
</tr>
<tr>
<td>Mouse IL-13</td>
<td>eBioscience, US</td>
<td>IV</td>
</tr>
<tr>
<td>Mouse INF-γ</td>
<td>eBioscience, US</td>
<td>IV</td>
</tr>
<tr>
<td>Mouse MIP-1α</td>
<td>R&amp;D Systems, US</td>
<td>I</td>
</tr>
<tr>
<td>Mouse TNF-α</td>
<td>eBioscience, US</td>
<td>I</td>
</tr>
<tr>
<td>Total / OVA / SEB specific IgE</td>
<td>BD Pharmingen, US</td>
<td>IV</td>
</tr>
<tr>
<td>Total / OVA / SEB specific IgG2a</td>
<td>BD Pharmingen, US</td>
<td>IV</td>
</tr>
</tbody>
</table>
6.4.4 ANALYSIS OF CELL SURFACE MOLECULES (I)

Flow cytometry (FACS Canto II, BD BioSciences, US) was used to study cell surface molecules. ENM exposed cells were removed from cell culturing plates, washed and stained with different fluorescently labelled antibodies (Table 6.6.). After the pre-defined incubation periods, samples were washed, run into the FACS and data was analysed with FlowJo software (TreeStar Inc, US).

Table 6.6. Specification of FACS fluorescently labelled antibodies used in Reference I.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Isotype</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1d</td>
<td>PE</td>
<td>Rat IgG2b</td>
<td>1B1</td>
</tr>
<tr>
<td>CD11c</td>
<td>PerCP_CY5.5</td>
<td>Rat IgG2b</td>
<td>M1/70</td>
</tr>
<tr>
<td>CD40</td>
<td>FITC</td>
<td>Armenian hamster IgM</td>
<td>HM40-3</td>
</tr>
<tr>
<td>CD86</td>
<td>FITC</td>
<td>Rat IgG2a</td>
<td>GL1</td>
</tr>
<tr>
<td>F4/80</td>
<td>PE_Cy7</td>
<td>Rat IgG2a</td>
<td>BM8</td>
</tr>
<tr>
<td>MHCII</td>
<td>PE</td>
<td>Rat IgG2b</td>
<td>M5/114.15.2</td>
</tr>
</tbody>
</table>

6.4.5 SIRNA TREATMENT AND PHARMACOLOGICAL BLOCKADE (II)

Cells were transfected with 200 nM non-targeting control small interfering RNA (siRNA) (AllStars Negative Control siRNA, Qiagen, US) and with 50 nM of four different NLRP3 target siRNAs (Hs_CIAS1_6, Hs_CIAS1_9, Hs_CIAS1_10, Hs_CIAS1_11, Qiagen) or 100 nM of two different P2X7 target siRNAs (Hs_P2RX7_1, Hs_P2RX7_2, Qiagen) using the HiPerFect Transfection Reagent (Qiagen) according to the manufacturer’s instruction one day prior to exposure with ENM or asbestos. After 4 h incubation, cell culture media was changed. Pharmacological blockade was performed with the cell-permeable cathepsin B inhibitor Ca-047-Me (10 μM, Calbiochem, Germany), the cell-permeable reactive oxidative species (ROS) inhibitor N-acetyl-L-cysteine (NAC, 10 mM; Sigma-Aldrich, Germany) and the P2X7 receptor inhibitor AZ11645373 (1μM, Sigma-Aldrich). When Abs or inhibitors were used, they were added to the cell culture plates 1 h prior to stimulation with either the nanomaterials or asbestos.

6.4.6 WESTERN BLOT ANALYSIS (II, III)

Cell culture supernatants were concentrated by Amicon UItra-15 –tubes, (Millipore, US) according to the manufacturer’s instructions. Proteins were separated on SDS-PAGE at 200 V and transferred onto Immobilon-P Transfer Membranes (Millipore) at +4ºC. The unspecific binding was blocked in PBS containing 5% non-fat milk.
and membranes were incubated with appropriate HRP-conjugated secondary Abs (Dako A/S, Denmark). Proteins were then visualized by the Image Quant LAS 4000 mini quantitative imager (GE Healthcare, Fairfield, CT, US). ImageQuant TL (GE Healthcare Biosciences) was used to analyze the intensities of protein bands. The antibodies used in the western blot (WB) analysis were anti-IL-1β (II), anti-cathepsin B (II), anti-ACS (II) and anti-2AAA (III).

6.4.7 MICROSCOPY

6.4.7.1 Light microscopy (I, III)

Light microscopy was used in the cell viability studies as described earlier and in the skin histology analysis described in section 4.4.9.

6.4.7.2 Transmission electron microscope (II)

Exposed cells were washed twice with PBS, fixed with 2.5% glutaraldehyde in phosphate buffer, and removed from the plate by scraping. Then cells were post-fixed in 1% osmium tetroxide, dehydrated and embedded in epon. Thin sections were collected on uncoated copper grids, stained with uranyl acetate and lead citrate and then examined with a transmission electron microscope (TEM) operating at an acceleration voltage of 80 kV (JEM-1220, Jeol Ltd., Japan).

6.4.7.3 Fluorescent microscopy (II)

ENM exposed cells cultured on cover slides were washed twice with PBS and the stained with acridine orange (AO 0.004% in Sorensen buffer, Sigma-Aldrich). The cover slides with AO stained cells were washed and placed upside-down onto the object glass with a drop of Sorensen buffer. Fluorescent microscopy analysis of slides was performed by using a Zeiss Plan Apochromat 40 objective mounted on a Zeiss AxioImager microscope (Carl Zeiss MicroImaging GmbH, Germany) equipped with an ISIS fluorescent imaging system version 5.1 (MetaSystems GmbH, Germany).

6.4.7.4 Hyperspectral imaging (IV)

To illustrate material penetration to the skin, the Hyperspectral Imaging (CytoViva; Aetos Technologies, Inc, AL) illumination and imaging system was used. DAGE-
6.4.8 PROTEOMICS METHODS

6.4.8.1 DIGE labeling (III)

Protein concentrations of the samples were determined with BioRad DC Protein Assay Kit (BioRad, US) and all samples were treated with ReadyPrep 2D Cleanup Kit to remove contaminants (BioRad, US). The samples were then re-suspended in DIGE labelling buffer (7M urea, 2M thiourea, 30mM tris-HCl, 4% CHAPS) to a final concentration of 50 mg / 20 µl. DIGE labelling was performed according to the instructions given by the manufacturer (Amersham CyDye DIGE fluoros (minimal dye) for Ettan DIGE, GE Healthcare Biosciences, US). 75 µg of protein mixture was required, 50 µg for secretome analysis and 25 µg for internal standard.

6.4.8.2 2-dimensional electrophoresis (III)

SERVA IPG Blue strips 3-10 NL (18 cm, SERVA Electrophoresis GmbH, Germany) were rehydrated in lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 0.04% bromophenol blue) supplemented with 3-10 IPG buffer (GE Healthcare Biosciences, US) and DeStreak reagent (GE Healthcare Biosciences, US). A cup loading method was used to introduce the proteins to IPG strips. Each IPG strip was loaded with proteins labeled with Cy5, Cy3 and Cy2. The final volume of sample was adjusted to 120 µl / strip with 2 x lysis buffer supplemented with 2% 3-10 IPG buffer and 2% DTT. Isoelectric focusing (IEF) was performed with Ettan IPGphor IEF system (GE Healthcare Biosciences, US). After IEF, the strips were equilibrated at first for 15 min in the mixture of 6M urea, 30% glycerol, 2% SDS, 50mM tris-HCl, 2% iodoacetamide, 0.04% bromophenol blue, then for another 15 min in the mixture of 6M urea, 30% glycerol, 2% SDS, 50mM tris-HCl, 1% dithiothreitol, 0.04% bromophenol blue. Second dimension, separation of proteins according to their size by polyacrylamide gel electrophoresis (PAGE), was performed using Ettan DALTsix Electrophoresis Unit (GE Healthcare Biosciences, US), with 12.5% reducing gels in Laemmli buffer. After electrophoresis, the gels were scanned with Ettan DIGE Imager (GE Healthcare Biosciences, US).
6.4.8.3 DeCyder analysis (III)

DeCyder 2-D Differential Analysis Software v.7.0 (GE Healthcare Biosciences) was used for the analysis of scanned gel images and for the statistical testing. Spots with \( \geq |1.5| \) fold change in expression compared to control, with p-value \( \leq 0.05 \) were selected for identification by mass spectrometry.

6.4.8.4 Protein identification (III)

Gels were silver-stained and selected spots were picked from the gels. In-gel digestion was performed and the resulting peptides were extracted as previously described (Korolainen et al. 2006). Peptide extracts were dried in a vacuum centrifuge and diluted to 2% HCOOH. Each peptide mixture was analyzed by automated nanoflow capillary LC–MS/MS using a CapLC system (Waters, US) coupled to an electrospray ionization quadrupole time-of-flight mass spectrometer (Q-TOF Global, Waters, US). The obtained mass fragment spectra were searched in Uniprot database against human entries using in-house Mascot v.2.1 (Matrix Science Ltd., UK).

6.4.8.5 Bioinformatics analysis (III)

Hierarchical clustering was performed for Cy2 standardized intensity values exported from DeCyder with R software version 3.0.0. (R Core team, Austria). Differentially secreted proteins were tested by the limma package (Limma, 2013) implemented in the bioconductor (Gentleman et al 2004). Nominal P-value <0.01 were considered significant. Information on gene ontology was gathered from Protein knowledge base of UniProt (www.uniprot.org) and the KEGG pathway analysis and enriched functional domain analysis of identified proteins was performed by DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/, Huang 2009).

6.4.9 HISTOLOGY (IV)

For histology analysis, tissue biopsies were fixed in 10% buffered formalin and embedded in paraffin. 4-μm skin sections were cut and stained with haematoxylin and eosin (H&E) for cell counts and to measure epidermal or dermal thicknesses, and with o-toluidine blue for mast cell counts. For immunohistochemistry, skin specimens were embedded in Tissue-Tek oxacalcitriol compound (Sakura Finetek Europe B.V., The Netherlands), frozen and staining of different subtypes of T-cells (CD3+, CD4+ and CD8+) and macrophages were performed (detailed description...
in IV). The sections were examined under light microscopy (Leica DM 4000B, Germany). Inflammatory cells were counted from 15 high-power fields (HPF) at 1,000 x magnification, mast cells, T-cell subtypes and macrophages at 400 x magnification.

6.4.10 LYMPH NODE STIMULATIONS (IV)

Cells from draining lymph nodes were collected and cultured in RPMI 1640 with suitable supplements in the presence of OVA (50 μg/ml) or SEB (1 μg/ml) for 6 hours (described in detail in IV). IL-13 and INF-γ ELISAs were used according to the manufacturer’s instructions to measure cytokine concentrations from cell culture supernatants (see Table 6.5.).

6.4.11 DATA ANALYSIS (I, II, IV)

Data were analysed using GraphPad Prism 4 Software (GraphPad Software Inc., US). The statistical tests were selected based on the assay; an unpaired (students) t-test or Mann-Whitney U-test was used to compare the differences between the groups. Significance levels p<0.05: *, p<0.01: ** and P<0.001: *** were used. All data are represented as means ± standard error (SD).
<table>
<thead>
<tr>
<th>Method</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Cell culturing</strong></td>
<td></td>
</tr>
<tr>
<td>RAW 264.7 cells</td>
<td>I</td>
</tr>
<tr>
<td>Mouse bone marrow derived DC</td>
<td>I</td>
</tr>
<tr>
<td>Human primary macrophages</td>
<td>II, III</td>
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<tr>
<td>Lymph node restimulation</td>
<td>IV</td>
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<tr>
<td><strong>Cell death assays</strong></td>
<td></td>
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<tr>
<td>Trypan blue</td>
<td>I</td>
</tr>
<tr>
<td>LDH cytotoxicity assay</td>
<td>II, III</td>
</tr>
<tr>
<td>ApoPercentage apoptosis assay</td>
<td>III</td>
</tr>
<tr>
<td><strong>Basic techniques in molecular biology</strong></td>
<td></td>
</tr>
<tr>
<td>Cytokine/Chemokine expression analysis (RT-PCR)</td>
<td>I, IV</td>
</tr>
<tr>
<td>Cytokine/Chemokine secretion analysis (ELISA)</td>
<td>I, II, IV</td>
</tr>
<tr>
<td>Serum antibody analysis (ELISA)</td>
<td>IV</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>I</td>
</tr>
<tr>
<td>Pharmacological Blockade</td>
<td>II</td>
</tr>
<tr>
<td>Small interfering RNA treatment</td>
<td>II</td>
</tr>
<tr>
<td>Western blotting</td>
<td>II, III</td>
</tr>
<tr>
<td><strong>Microscopy</strong></td>
<td></td>
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<tr>
<td>Light microscopy</td>
<td>I, III, IV</td>
</tr>
<tr>
<td>Transmission electron microscopy</td>
<td>II</td>
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<tr>
<td>Fluorescent microscopy</td>
<td>II</td>
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<tr>
<td>Hyperspectral imaging (Cytoviva)</td>
<td>IV</td>
</tr>
<tr>
<td><strong>Proteomics techniques</strong></td>
<td></td>
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<tr>
<td>DIGE labeling</td>
<td>III</td>
</tr>
<tr>
<td>2-DIGE</td>
<td>III</td>
</tr>
<tr>
<td>DeCyder Analysis</td>
<td>III</td>
</tr>
<tr>
<td>Protein identification</td>
<td>III</td>
</tr>
<tr>
<td>Bioinformatics analysis</td>
<td>III</td>
</tr>
<tr>
<td>Histology</td>
<td>IV</td>
</tr>
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7 RESULTS

7.1 METAL OXIDE NANOPARTICLES (I, IV)

7.1.1 MAJOR DIFFERENCES IN THE LEVELS OF CYTOTOXICITY OF DIFFERENT TiO₂ AND ZnO NANOPARTICLES (I)

The cytotoxicity of TiO₂, TiO₂-silica and ZnO NP was studied with mouse macrophage cell line (RAW264.7) and mouse bone marrow derived dendritic cells (bmDC) with the Trypan blue cell staining assay. The cytotoxicity of different TiO₂ NP was studied in 30, 100 and 300 μg/ml with a clear dose-response evident with both cell types. There was no difference in the levels of occurring cytotoxicity in antigen presenting cells in response to the different types of TiO₂ particles. In contrast, ZnO NP were highly cytotoxic to both cell types, and 100 times lower concentrations were used in the experiments. The highest dose (30 μg/ml) was able to induce cell death in >50% of both cell types, in contrast mouse dendritic cells were clearly more sensitive than mouse macrophage cell line cells to the ZnO induced cell death.

7.1.2 SURFACE MODIFICATION INFLUENCES THE TiO₂ NP INDUCED IMMUNOTOXICITY (I)

The ability of TiO₂ and TiO₂-silica NP to induce cytokine and chemokine mRNA transcription in RAW 264.7 cells and bmDC was studied at two time points, 6 and 24 h. In addition, selected cytokines were studied at the protein level from the cell culture supernatants of NP-exposed cells after 24 h. Overall, the results suggest that TiO₂-silica is a stronger activator of pro-inflammatory response than uncoated TiO₂ as it induced a high expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α as well as the neutrophil attracting chemokine MIP-1α in RAW 264.7 cells. Neither uncoated nor coated TiO₂ NP induced the expression of studied cytokines/chemokines in the primary dendritic cells. At the protein level, both TiO₂ NP induced the secretion of TNF-α and MIP-1α from macrophages; however, the TiO₂-silica NP induced profound secretion of both proteins in lower doses. The in vitro-analysis revealed that TiO₂ NP exposed cells had been activated to produce pro-inflammatory mediators in both mRNA and protein level, whereas surface treatment of TiO₂ particles may have had a high effect on their potency to activate cells.

In the flow cytometry analysis of cell surface markers, both TiO₂ NP induced expression of macrophage maturation marker CD11c, antigen presentation markers
MHCII and CD1d and co-stimulation molecules CD86 and CD40 in RAW 264.7 cells after 24 h exposure to TiO$_2$-silica NP being more potent activator of cells. However, after 48 h exposure uncoated TiO$_2$ exposed cells showed greater potency to induce expression of these cell surface molecules. These in vitro –experiments indicate that both uncoated and coated TiO$_2$ NP had been able to activate macrophages to act as the first step in the cascade of pro-inflammatory reaction.

7.1.3 ZnO NANOPARTICLES ACTIVATE MACROPHAGES AND DENDRITIC CELLS IN VITRO (I)

ZnO NP evoked a powerful induction of the expression of the pro-inflammatory cytokines IL-1β and IL-6 as well as neutrophil-attracting chemokine CXCL-9 in RAW 264.7 macrophages. In addition to these inflammation mediators, the expressions of TNF-α, CXCL-5 and CXCL-10 were induced in bmDC suggesting that a pro-inflammatory cascade in both important antigen presenting cell types had been activated by ZnO NP. Indeed, when studying cell surface markers in RAW 264.7 cells, the expression of macrophage maturation marker CD11c, T-cell co-stimulation markers DC86 and CD40 and antigen presentation markers MHCII and CD1d were seen after 24 h of exposure with ZnO NP, evident of the potency of nano-ZnO to activate inflammatory responses and maturate macrophages, potentially leading to decreased capability of phagocytosis.

7.1.4 PARTICLE SIZE MODULATES THE INFLAMMATORY RESPONSE IN VIVO (IV)

7.1.4.1 The local allergic reaction is significantly diminished after skin treatment with nano-ZnO

Patients with AD have an increased skin thickness and there is infiltration of inflammatory cells within skin lesions, and the effect of different sized ZnO particles was studied in this phenomenon. The histological analysis of mice back skin revealed that both bulk-ZnO (bZnO) and nano-ZnO (nZnO) decreased the thickness of skin and the numbers of infiltrating inflammatory cells in OVA+SEB sensitised mice, where the suppression of nZnO in the inflammation was stronger than that achieved with bZnO. On the contrary, macrophage numbers in the skin histological samples were increased in particle-treated groups, suggesting that macrophages had been attracted to scavenge the treatment site to seek out particles. This effect was independent of OVA+SEB sensitisation and stronger in bZnO treated skin. Hyperspectral imaging revealed that both particle types were agglomerated into the
skin surface; bZnO were detected from the epidermis of the damaged or allergic skin whereas nZnO was found not only from epidermis and hair follicles but also from the dermis of the allergic skin.

Since AD patients have local inflammation in the skin lesions, the effect of ZnO particles was studied on the expression of pro-inflammatory, Th1-type, Th-2-type and anti-inflammatory cytokines. The analysis showed that mRNA expression of important pro-inflammatory cytokines IL-1β, IL-6 and TNF-α was induced in the skin. In addition, expression of Th1 type cytokine IFN-γ, Th2 type cytokines IL-4, IL-13 and IL-33 associated with allergy and anti-inflammatory cytokine IL-10 was upregulated in the allergic skin. In contrast, if the AD model of allergic skin was treated with nZnO, the expression levels of all cytokines studied was significantly lower. In addition, the bulk-ZnO showed a similar, but weaker response in the OVA+SEB sensitised mice skin tissue but it failed to suppress Th2-type cytokine expression in the allergic skin. When cells from local lymph nodes were isolated and stimulated with OVA, the production of IL-13 and IFN-γ were strongly increased in the OVA+SEB sensitised mice. The protein levels were suppressed if mice skin was treated with nZnO but not if the skin was treated with bZnO, however, the level of suppression was not statistically significant.

7.1.4.2 The levels of systemic antibodies in serum are increased after skin treatment with nano-ZnO

Patients suffering AD have elevated levels of IgE antibodies in their blood circulation. Therefore, total and OVA or SEB specific IgE and IgG2a levels were measured from mice sera. ZnO treatment drastically induced serum IgE levels, both total and OVA-specific IgE whereas there was no effect in the total of OVA-specific IgG2a levels or in the SEB-specific IgE or IgG2a levels. The induction was more intense if mice were locally treated with nZnO than bZnO. These results suggest that both bulk- and nano-ZnO induce production of systemic Th2-type antibodies, leading to systemic sensitization.
7.2 CARBON NANOMATERIALS (I, II, III)

7.2.1 SHORT CARBON NANOTUBES ARE WEAK INDUCERS OF A PRO-INFLAMMATORY RESPONSE IN MOUSE IN VITRO –CELL MODELS (I)

The cytotoxicity of short single-walled (S) and multi-walled (M) carbon nanotubes was evaluated in mouse RAW 264.7 cells and primary bone-marrow derived dendritic cells. In mouse macrophages, there was no significant cytotoxicity noted with any of the doses (30, 100, 300 μg/ml) tested. In DC, dose-dependent cytotoxicity was observed, however, there was no difference in the short SCNT and MCNT induced response. The expression of different cytokines and chemokines was not induced in DC by the treatment of short CNT, and in macrophage cell line short MCNT but not SCNT induced MIP-1α mRNA expression and protein secretion. However, no other changes were observed. The flow cytometry analysis of RAW 264.7 cells also failed to show any induction in the expression of cell surface molecules by the treatment of cells with short CNT. These materials seem unable to induce pro-inflammatory reactions in the chosen mouse in vitro –cell models.

7.2.1.1 Rigid multi-walled carbon nanotubes but no other types of carbon nanomaterials activate profound IL-1 –family cytokine secretion from human primary macrophages (II)

Short MCNT, long and tangled MCNT as well as long and rigid MCNT were studied for their ability to induce IL-1 –family cytokine secretion in LPS-primed human primary macrophages after 6 h of exposure. Carbon black nanoparticles were used as a material control and crocidolite asbestos as a positive control for the experiments. The IL-1 –family cytokine secretion is a highly controlled process in macrophages, and the expression of pro-IL-1α/β is not constant. Their transcription is known to be activated by Toll-like receptor-4 (TLR4) activation by bacterial lipopolysaccharide (LPS), and therefore LPS treatment of macrophages was used prior to exposure to carbon nanomaterials and asbestos fibres. The secretions of both IL-1α and IL-1β were clearly induced by treatment with long, rigid MCNT and asbestos fibres whereas other CNM studied failed to activate the secretion. The analysis of IL-18 displayed similar phenomena.
7.2.2 RIGID MULTI-WALLED CARBON NANOTUBES ACTIVATE THEN LRP3 INFLAMMASOME IN A SIMILAR MANNER AS ASBESTOS FIBRES (II)

7.2.2.1 NLRP3 inflammasome activation

The NLRP3 inflammasome regulates the secretion of IL-1 –family cytokines. The NLRP3 gene silencing by siRNA treatment clearly achieved a suppression of IL-1β secretion after exposure with long, rigid MCNT and asbestos fibres. In addition, the WB analysis revealed the secretion of NLRP3 inflammasome component ASC to the cell culture supernatant confirming the formation of NLRP3 protein complex.

7.2.2.2 Formation of reactive oxygen species (ROS)

The formation of reactive oxygen species has been speculated to be an important component leading to the inflammation induced by nanomaterials. It has also been shown to be an upstream effector of NLRP3 inflammasome activation. Therefore a pharmacological blockade with two antioxidants, APDC and NAC, was performed. Indeed, macrophage treatment with both antioxidants prior to rigid fibre exposure independently suppressed IL-1β secretion.

7.2.2.3 Leakage of lysosomal contents to the cytoplasm

Cathepsins such as cathepsin B are present in the lysosomal compartment of the cells. The lysosomal damage triggers the leakage of cathepsins to the cytosol then activating upstream the cascades of the NLRP3 inflammasome complex formation. The role of cathepsin B in NLRP3 inflammasome activation after macrophage exposure with rigid MCNT and asbestos fibres was studied with cathepsin B inhibitor (Ca-047-Me) and with WB analysis of cathepsin B protein from the cell culture supernatant. The impaired secretion of IL-1β accompanied with the observation of mature cathepsin B was pointing to a role of lysosomal damage in the inflammation induced by rigid MCNT.

7.2.2.4 P2X7 receptor activation

Extracellular ATP-gating cation channel P2X7 is an upstream signal for NLRP3 inflammasome activation. P2X7 allows cations such as K+, to pass through the cell membrane leading to NLRP3 inflammasome activation. The silencing of the P2X7 gene and pharmacological blockade of P2X7 receptor in macrophages prior to
exposure to long, rigid MCNT and asbestos fibres achieved a significant suppression of IL-1β secretion, demonstrating that P2X7 receptor activation is an important upstream signal for fibre-induced NLRP3 inflammasome activation.

### 7.2.2.5 Src and Syk tyrosine kinase activation

Tyrosine kinases Src and spleen tyrosine kinase (Syk) are believed to act upstream of NLRP3 inflammasome activation. To study whether these pathways are involved in fibre-induced NLRP3 activation, a Src kinase inhibitor (PP2) and a Syk-kinase inhibitor (SYKII) were administered to the macrophages. Resulting dose-dependent inhibition of IL-1β secretion is clear evidence for the involvement of tyrosine kinases in the inflammation induced by rigid MCNT and asbestos.

### 7.2.3 THE FULL PROTEOMIC PICTURE OF RIGID MCNT IS MORE SIMILAR TO ASBESTOS FIBRES THAN IT IS TO TANGLES MCNT (III)

#### 7.2.3.1 Protein clustering reveals similarities in the macrophage response towards rigid MCNT and asbestos

The proteomics tools were used to analyse the full proteomic picture of secretome of macrophages exposed for 6 h with long, tangled MCNT; long, rigid MCNT and asbestos fibres. Hierarchical clustering confirmed these results showing similar profiles in up-regulated and down-regulated proteins in the secretomes of long, rigid MCNT and asbestos fibres whereas long, tangled MCNT showed different response. However, the Venn diagram analysis showed that all of the materials had a unique set of secreted proteins where long, rigid MCNT had the highest set of unique proteins compared to the other treatment groups.

#### 7.2.3.2 Rigid MCNT activates signalling cascades leading to cell death

A bioinformatics analysis of the data collected from macrophages exposed to different fibres displayed a protein profile providing evidence that the cell death pathway had been activated in macrophages exposed to long, tangled MCNT but not with other fibres. Indeed, an analysis of lactate dehydrogenase enzyme from cell culture supernatants showed leakage of the enzyme to the supernatant suggestive of cellular damage after 18 h of exposure with long, rigid MCNT. In order to confirm whether the cells are undergoing necrosis or apoptosis, an apoptosis light microscope staining was performed. This suggested that long, rigid MCNT had induced apoptosis.
in macrophages after 6 and especially after 18 hours of exposure whereas long, tangled MCNT and asbestos fibres were incapable of activating this type of cell death pathway. David enrichment analysis revealed that all fibres had enriched lysosomal pathway suggesting that the particles had been actively ingested to the lysosomal compartment of macrophages, however, only rigid MCNT seem to be able to induce biological responses associated with lysosomal damage such as apoptosis.

Summary of the effects induced by rigid CNT in human primary macrophages is presented in Figure 7.1.

**Figure 7.1.** Rigid MCNT induced P2X<sub>7</sub> receptor activation, lysosomal damage, ROS production, Src and Syk kinase activation and release of cathepsins in the cell cytoplasm leads to NLRP3 inflammasome assembly, activation of caspase-1 and secretion of active IL-1β. Cathepsin release from the lysosomes to the cytoplasm also leads to apoptosis and furthermore, caspase-1 activation is speculated to induce pyroptosis in macrophages evoking inflammation in the surrounding tissue.
Engineered nanomaterials (ENM) possess unique specific characteristics, and depending on their physico-chemical properties, they may induce different responses than their non-nano counterparts in humans (Nel et al., 2013). The most likely exposure routes to ENM are via lungs and skin, where body has effective protection mechanisms to prevent foreign materials from entering to the circulation (Geiser and Kreyling 2010). The most sophisticated protective system is called immunity and it protects body from invading pathogens and foreign materials, however, if the cleavage is not successful, inflammation may become chronic and cause unwanted effects (Janeway’s Immunobiology, 2011). In this thesis, the immunotoxicological effects of metal nanoparticles and carbon nanotubes were studied in the antigen presenting cells (APC), macrophages and dendritic cells, acting as first line of defense in the body surfaces, and the mechanisms behind these effects were then further investigated. In addition, the atopic dermatitis (AD) disease model was utilized to investigate particle penetration and ZnO effects in diseased skin. New insights from the mechanisms of toxicity of ENM as well as from the particle penetration and effects in the damaged, allergic skin were gained from these studies. According to these results, it can be postulated that an immunotoxicological analysis may be able to separate hazardous ENM from non-hazardous ones by their mode of action (MoA) and should be consireded as one of the first steps in the hazard assessment of ENM. As more knowledge is gained from mechanisms of nanotoxicity, it should be possible to develop more efficient risk assessment protocols suitable for ENM and thus to assure the nanosafety of individuals, especially for the manufacturers, importers and the downstream users of products based on nanotechnology.

8.1 TOXICITY AND INFLAMMATORY EFFECTS OF ENGINEERED NANOMATERIALS IN VITRO (I-III)

Engineered nanomaterials with different chemical background have suggested inducing different outcomes in both in vitro and in vivo (Wu et al., 2014). Also, the ENM modifications, such as size, surface coating and shape influence on the hazards posed by to the living organisms (Shi et al., 2013). The TiO₂, ZnO and CNT used in the studies induced different results in the cell models used. In the mouse macrophages ZnO, was the most cytotoxic material (I), most probably due to its ability to release Zn²⁺ ions (Ng et al., 2013). In the phagocytosing cells such as macrophages, the release of this ion may be even higher than in the phosphate
buffers, because after digestion the particles are normally located in the highly acidic (ph < 4) lysosomes possibly inducing the dissolution of particles. This ‘trojan horse’ effect is a well-known pathway of particle toxicity (Studer et al., 2010; Cronholm et al., 2013). BmDC were more susceptible to the Zn-effect than macrophages, perhaps due to their origin as primary cells rather than immortal cell lines. However, ZnO NP showed high cytotoxicity when administered to bmDC even in very low concentrations, emphasizing the toxic nature of ZnO NP as compared to the other types of ENM studied (Hsin et al., 2008). As different CNT were studied (I-III), short SCNT and short MCNT did not seem to induce cell death in mouse antigen presenting cells (APC; I) whereas the long, rigid MCNT induced the highest cytotoxicity in human macrophages at the late 18 h time point (II-III), whereas other types of MCNT were not cytotoxic in human cells. These results suggest that both ENM chemistry as well as its surface modifications influence on their cytotoxicity.

When investigating expression of cytokine mRNA in macrophages and DC, ZnO and TiO$_2$ induced the expression of different cytokines. However, the levels of cytokine TNF-α and chemokine MIP-1α secreted from macrophages were higher if the cells were treated with TiO$_2$ than with ZnO, suggesting that ZnO had induced its effects via cytotoxic pathways whereas TiO$_2$ may induce pro-inflammatory cascades. This speculation is supported by an analysis of co-stimulatory molecule CD86 on the surface of macrophages, as TiO$_2$ was the most potent inducer of the cell surface marker associated with cell maturation. To compare TiO$_2$ NP with different surface modifications, SiO$_2$ coated TiO$_2$ seemed to possess slightly higher potency to incude the mRNA and protein levels of pro-inflammatory cytokines and chemokines than uncoated TiO$_2$ NP, as was also noted in the cell maturation markers on macrophage surface. One can speculate on whether this is a coating effect or whether it is due to the different shapes of the TiO$_2$ particles because the uncoated TiO$_2$ was spherical with specific surface area of 23 m$^2$/g and SiO$_2$-coated TiO$_2$ particles were more needle-like with a more than five times higher specific surface area (132 m$^2$/g). The different effects of these TiO$_2$ particles on the inflammatory cascade have been further confirmed in vivo (Rossi et al., 2010) but the mechanism behind these phenomena remains unclear. In the studies with mouse cells (I), SCNT and MCNT did not seem to activate pro-inflammatory reactions in mouse APC, however, both of the CNT studied were short and therefore resembled the more carbon black particles rather than high aspect ratio nanomaterials (HARN). As investigating HARN (II), more precisely long, rigid MCNT, the secretion of the important pro-inflammatory cytokine IL-1β was determined, suggesting that rigid MCNT is a potent activator of pro-inflammatory cascade in a similar manner than silica crystals and asbestos fibers (Hornung et al., 2008). These results greatly reflect data collected from in vivo –studies, as ridig fibers have shown to be also more harmful if administered by inhalation to mice and rat (Braakhuis et al., 2014).
8.2 MECHANISMS OF IMMUNOTOXICITY INDUCED BY ENGINEERED NANOMATERIALS IN VITRO (I-III)

IL-1-family cytokine secretion is regulated by NLRP3 inflammasome activation. It is known that larger but structurally similar fiber, crocidolite asbestos as well as other crystalline materials such as silica and cholesterol crystals activate the NLRP3 inflammasome by inducing ROS production in macrophages (Duewell et al., 2010; Hornung et al., 2008). In our study, it was clearly seen that NLRP3 inflammasome is activated after exposure of macrophages with long, rigid MCNT via ROS production, lysosomal damage, ATP-gated P2X7 receptor activation and Src and Syk tyrosine kinase activation (II). The importance of these pathways in the cytotoxicity and inflammation of certain types of MCNT has been shown also in different cell types in vitro ((Frohlich, 2013; Singh et al., 2014).). In the investigation of the mechanisms behind these phenomena, rigid MCNT and asbestos were identical, supporting the high aspect ratio (HAR) theory (Donaldson et al., 2011). Carbon nanotubes may differ in their physical identity in many ways: Their length, width, number of carbon layers; impurities and rigidity are some of the numerous factors that may influence their toxic properties (Lanone et al., 2013), and this study clearly showed that a specific fiber-like physical identity is required to activate the pro-inflammatory cascade. According to these results, the shape and rigidity of MCNT seems to be a key characteristic in the induction of toxicity. It has been speculated that impurities such as nickel (Ni) from the manufacturing process of CNT may influence on their toxicity (Sohaebuddin et al., 2010), however, as materials used in these studies were more than 99% pure carbon, the NLRP3 inflammasome activation is most likely not due the residual metals.

Mechanisms of toxicity of MCNT were investigated by using proteomics approach (III) to understand the whole picture of secreted proteins. In these experiments macrophages were exposed to with long, tangled MCNT; long, rigid MCNT and crocidolite asbestos and secretome profiles of macrophages were analyzed and compared. This revealed that the secretome induced by rigid MCNT displayed more common features with asbestos fibers, however, analysis also showed own unique set of proteins for rigid MCNT. They seemed to have a greater ability to induce cell death, and that may be pathway exerting the tissue damage seen in vivo (Poland et al., 2008; Takagi et al., 2008; Donaldson et al., 2011). The activation of cell death cascades may indicate mechanisms of toxicity in the tissues, as cell contents released to the tissue may result in excessive local inflammation (Hussain et al., 2014). However, these results and their importance from a mechanistical view in nanotoxicity will need to be validated in further studies which will concentrate on comparing the different cell death pathways in different cell types, whole tissues or cell co-cultures, and also to evaluate the effects of cell death signalling on neighboring
cells (Vandenabeele et al., 2010; Andon and Fadeel, 2012; Joshi and Knecht, 2013).

The lysosomal damage has been reported as an crucial step of ENM induced inflammation (Frohlich, 2012, II), and it can be speculated that fiber-ingestion by macrophages leads to leakage of lysosomal contents to cytosol, excessive ROS production and either activation of pro-inflammatory cascade by NLRP3 inflammasome complex formation or to the cell death, leading to release of intracellular compartments to the surrounding tissue. On the other hand, the ROS production is commonly speculated to be a main pathway to the nanomaterial toxicity, and it may be also induced in cell membranes or mitochondria independently of lysosomal damage leading also to the non-beneficial outcomes as uncontrolled cell death (Fu et al., 2014). However, more studies are required to confirm the mechanisms of toxicity and especially the sequence of pro-inflammatory events. In the III study, the secretome profile of tangled MCNT resembled more unexposed control cells than fiber-exposed cells further confirming the importance of results obtained from NLRP3 inflammasome study (II) and suggesting that not all CNT are similarly hazardous. These results underline the HAR effect, suggesting that width and length –ratio of the MCNT as well as their rigidity determine their toxicity, as also revealed in a proteomics study with SCNT in vivo (Teeguarden et al., 2011).

The evaluation of the importance of NLRP3 inflammasome in the fiber-induced inflammation has been confirmed in several studies (Meunier et al., 2012; Yang et al., 2013; Girtsman et al., 2014), and the free radical formation essential for the NLRP3 inflammasome activation has been shown to be associated with the most toxic fibers (Nymark et al., 2014). Therefore, it is evident that this pathway is important in the fiber-induced inflammation, however, the importance of P2X7 receptor activation and roles of different tyrosine kinases are to be further investigated in the future.

### 8.3 NANO-SIZED ZnO PENETRATE INTO THE DISEASED SKIN AND CAUSE BOTH LOCAL AND SYSTEMIC EFFECTS

Engineered nanoparticles are widely used as UV-filtering agents in commercial sunscreens. Bulk-sized ZnO particles are also commonly used in anti-inflammatory pastas commonly applied to the damaged skin, such as into the rash caused by nappy usage for babies, as they have antibacterial activity (Fu et al., 2014). It has been suggested that different-sized particles do not penetrate healthy skin but the phenomena in the diseased skin was unknown (Lin et al 2011). In the mouse atopic dermatitis (AD) model (IV) it was clearly shown that nano-ZnO caused higher responses than bulk-ZnO. Nano-ZnO penetrated deeper into the layers of the damaged, allergic skin, suppressed local inflammation more efficiently and
induced extensive production of allergy-associated IgE antibody in the systemic circulation. However, if skin was only damaged, not inflamed, nano-ZnO particles were not able to penetrate to the deeper layers of the skin. Most probably observed effects are mainly attributable to the higher \( \text{Zn}^{2+} \) ion release from the larger particle surface area (Ng et al., 2013), accompanied by the nanoparticle transport into the deeper layers to the skin. In this study, it was clearly demonstrated that size of the particle with same chemical composition is an important parameter when assessing particle-induced toxicity, which is line with current understanding (Krug and Wick, 2011; Shi et al., 2013).

The study indicates that role of allergic inflammation have a great role in the particle penetration to the skin. The local inflammation may be caused by sunburns, which may in turn influence on particle penetration as sunscreens are commonly applied in these situations (Ryser et al., 2014). If observed effects in the AD skin are taken together, cell infiltration in the treated skin demonstrate that allergen induced skin inflammation is significantly reduced especially by the topical exposure to nZnO particles. In contrast, exposure to bZnO increases the infiltration of eosinophils as well as macrophages in AD-like skin lesions. This is further confirmed by analysis of cytokine expression in the skin, as cytokine levels were downregulated when mice were treated with nZnO suggesting that nZnO particles suppress local inflammation whereas bZnO failed to suppress Th2-type cytokine expression. As eosinophils are known effector cells in the Th2 –type immune response (Spencer and Weller, 2010), these results suggest that even there are similarities in the particle effects on treated skin, there are also differences in the suppression of local inflammation, nZnO being the most efficient inhibitor. On the contrary, both sizes of ZnO induced antibody levels on blood stream, suggesting elevation of systemic inflammation, often associated with topical sensitization to chemicals (Warbrick et al., 2002). This phenomenon has to be further studied to understand the full picture of ZnO effects via dermal application. However, as UV-light is known to cause malignant mesothelioma, it is of importance to use UV-filtering agents, such as nano-ZnO. Nevertheless, if skin is damaged and inflamed, the consideration of using protective clothing instead of sunscreens may be necessary.

### 8.4 Usability of the Collected Data in the Hazard Assessment

Nanomaterial hazard assessment is a growing field of science, and a plethora of toxicological studies have been published in the last decade (NAS, 2012). Nevertheless, the suitability of these studies as tools of risk assessment needs to be critically evaluated (Klimisch et al., 1997). In immunotoxicology, the lack of
guidelines, common endpoints, benchmark materials and the variability on cell types used in the in vitro –studies complicate any comparison of results from different studies. In addition, the lack of any consensus in the nanomaterial characterization data required for publishing the results increases uncertainty on the data assessment. The OECD is providing recommendations for the requirements of material characterization for genotoxicity studies (OECD 2014), and these data should be equally required from all toxicity screening studies: Primary particle size distribution, aggregation and agglomeration state, shape and aspect ratio, crystallinity, specific surface area, dispersibility, dustiness (aerosol exposure studies) and ENM solubility, and all has to be analyzed in the test media used. In addition, in order to make the data comparable, common benchmark materials and doses should be set and used to validate the results (Klimisch et al., 1997; Nel et al., 2013). In this thesis, the first study (I) was a traditional screening study for different ENM. The characterization data from ENM was somewhat incomplete, but in fact, it highlights rather well that lack of benchmark doses and study guidelines makes the data comparison challenging. On the other hand, in the NLRP3 inflammasome study (II), both negative and positive material controls were used to improve the data quality, but the third study (III) again lacked a negative benchmark material. As scientific data quality varies a lot, the careful expert judgement of usability of data is required to ensure usability of collected data on hazard assessment (Klimisch et al., 1997; Beronius et al., 2013).

Usually, the term biological identity describes the biomolecules that absorb onto the material surface, which is dependent on the route of entry into the body as well as on the dispersion vehicle such as cell culturing media (Bhattacharya et al., 2013; Tenzer et al., 2013). The biological identity of ENM used in the studies (I-IV) remains uncharacterized. The status of material agglomeration and aggregation remains unclear and it cannot be confirmed whether or not the toxic phenomena were due to the single particles/fibers or –more likely- to the larger mass of bound particles/fibers. In addition, the status of biomolecule absorption on the material surface remains uncharacterized. In a biological system, this depends on route of entry, but in in vitro –studies in presence of serum in cell culturing media may lead to the formation of a protein corona around the single particle modifying its size, reactivity and toxicological properties. It was suggested that protein corona formation may differ extensively between different types of TiO₂ nanoparticles (Sund et al., 2011), and therefore it can be postulated that even if materials may have the same chemical identity, their biological identities may be very different, and therefore they may induce different toxicological outcomes (Tenzer et al., 2013).

In principle, these results suggest that the use of high-aspect ratio nanomaterials, more specifically long, rigid CNT should be avoided due to their high toxicity. According to REACH regulation, if the production of these nanotubes would be
more than 1 tonnage, the scientific data should be taken into account and either not authorize rigid CNT or add it to Annex XIV (substances to be replaced) until other types of CNT have been proved to have same characteristics to be utilized in the industrial processes. In addition to this, the toxicity data from other types of CNT should support the replacement by showing less toxic properties, as suggested in II and III.

8.5 FUTURE PROSPECTS

Mechanistical data collected from in vitro –studies may help to recognize certain pathways, which are essential for toxicological outcome (Nel, 2013). The knowledge of mechanism of action (MoA) is important in the development of guidelines (GL) for toxicity testing, in the material selection and prioritization in the further testing of the hazard and to create faster, cheaper and more transparent hazard screening processes or to justify the prioritization of certain materials in the first step of hazard assessment.

A successful nanomaterial hazard assessment will most probably be based on the development of new tools to achieve an efficient risk characterisation and management (Hristozov et al., 2012). However, the evaluation and utilisation of existing data are also important (Klimisch et al., 1997). As thousands of differently modified materials are being incorporated into the industrial products, occupational safety has to be ensured. Since protection protocols are expensive, it would be beneficial to be able to differentiate hazardous nanomaterials from their non-hazardous counterparts (Nel et al., 2013). The communication between basic scientists and regulators has to improve, as does the transparency of the processes, and the publication bias in i.e. the reluctance of scientific journals to publish negative results has to be solved by creating data banks accepting all data as it has emerged from well-characterised good-quality studies (Hristozov et al., 2014b). These open-access databanks could be used for data mining by experts in bioinformatics and computational sciences perhaps to develop algorithms which would make it possible to recognize appropriate toxicological endpoints and characteristics, and to develop a predictive approach for risk assessment to replace the descriptive approach now being used by the regulatory authorities (Nel et al., 2013).

The chemical risk assessment process is not simple, and it commonly requires expert judgement to set the most suitable risk management measures to minimise the possibility of health risks (Beronius et al., 2013; Hristozov et al., 2014b). Existing expertise has to be used when developing suitable means to assess hazard and exposure for ENM. The suitability of the current practises of classification and labelling of chemicals as nanomaterials should also be discussed and, if needed,
legislation should be updated according to the knowledge of hazard mechanisms in order to ensure nanosafety.

There will need to be collaboration between toxicologists, computational scientists, material chemists, physics experts and occupational hygienists in order to develop functional nanosafety approaches. The online-measurements of the nanomaterial exposure in the industrial workers would describe the exposure and bring new information of the exposure levels in the working places then be further utilized in the development of exposure scenarios in the risk assessment. Extensive characterisation of material behaviour in different test media would help toxicologists to create scenarios for health hazards, eventually to lead to a better awareness of the nanorisk. Before the hazard mechanisms of nanomaterials are understood, it is only possible to adopt efficient protection measures to avoid exposure and treat all ENM as if they were hazardous to human health, even if this approach is more costly and slower, because safety comes first.
Nanosafety research is a rapidly growing field, however, the most suitable assays, relevant endpoints and detailed requirements for hazard assessment are still being debated. The goal of this thesis was to contribute to the assessment of nanohazards by producing mechanistical data: By comparing ENM to other types of nanomaterials or to their surface-modified, larger or different shaped counterparts in different cell models of immunotoxicity or in the mouse skin model of atopic dermatitis.

The results clearly show that each ENM studied has its own unique characteristics, which affects on its toxicity. Different metal oxide nanoparticles, TiO$_2$ and ZnO, induce different responses in mouse macrophages and dendritic cells most probably due to their ability to release free ions from their surface. SiO$_2$-coated TiO$_2$ ENP possessed higher potency to activate mouse macrophages in vitro than non-coated TiO$_2$ indicating that surface modifications influence their toxic properties of ENP. In addition, it was demonstrated that particle size is an important parameter as nano-sized ZnO particles were more potent suppressors of local inflammation in allergic skin in vivo than their bulk-sized counterparts. In addition, nano-ZnO were greater inducers of systemic antibodies than bulk-ZnO suggesting that particle penetration through damaged, allergic skin and the higher active surface area of the smaller particles are relevant in the body’s response to these particles.

The HARN materials have been speculated to induce similar hazardous outcomes as asbestos fibres. In this thesis, it was shown that long, rigid CNT could indeed activate similar pro-inflammatory pathways in human primary macrophages as asbestos fibres. When studying the cellular effects with proteomics tools, the analysis clearly showed that complete photographs of secreted proteins of long, rigid CNT and asbestos fibres resembled each other even although both materials had their unique sets of secreted proteins. More importantly, the other types of CNT studied showed no asbestos-like effects, suggesting that not all CNT are equally hazardous. Short MCNT or long, tangled CNT did not activate the NLRP3 inflammasome nor did the full proteomic picture of long, tangled CNT resemble the secretome of long, rigid CNT. These results highlight the mechanisms behind the toxic outcomes of certain types of CNT underlining the importance of the development of screening assays, which would be suitable for studying these endpoints, especially the NLRP3 inflammasome activation, in the fibre-induced hazard assessment.

The challenges faced by nanosafety research are the numbers of materials to be studied and time and money consuming, animal test -based, risk assessment guidelines in chemical testing. Faster screening protocols will need to be developed, with improved collaboration between the scientific community, and the regulatory
agencies and legislation has to be updated in order to utilise collected data more efficiently. Nonetheless, the quality of data has to be ensured; there must be detailed material characterisation, and the development of an open-access data bank to store high-quality data which will permit computational analysis. In the last decade, nanosafety research has been extensively funded and many publications have appeared. Now the scientific community need to find some way to communicate their results to the general public. In addition, the regulatory authorities need to understand how to improve hazard assessment and characterisation, develop techniques to assess exposure to ENM and help to develop new ways of risk assessment.
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