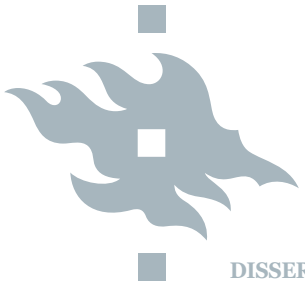

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Immunomodulatory Effects of Engineered Nanomaterials in Healthy and Diseased Lungs and Skin

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Department of Bacteriology and Immunology
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IMMUNOMODULATORY EFFECTS OF ENGINEERED NANOMATERIALS IN HEALTHY AND DISEASED LUNGS AND SKIN

Marit Ilves

ACADEMIC DISSERTATION

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ABSTRACT

Nanotechnology is exerting a huge impact on various sectors of everyday life as it has a tremendous potential for revolutionizing a long list of consumer products and industrial applications. The key to success in the nanotechnology field lies in the fact that materials at the nanoscale possess novel and enhanced properties such as greater strength and improved conductivity when compared with their bulk-sized equivalents.

The most probable occupational and consumer routes of exposure to engineered nanomaterials (ENM) are via the respiratory tract and skin. Due to their small size, ENM are able to bypass physical and chemical barriers in the human body and come into contact with the immune system which is capable of recognizing foreign structures including ENM. However, the downscaling of the materials also increases their chemical reactivity, which in combination with the small size and other physicochemical properties, means that ENM could influence our immune system exerting possibly beneficial but also adverse effects on our health. The aim of this thesis was to investigate modulatory effects and physiological outcomes of ENM on a healthy and a compromised immune system in the lungs and skin.

The main findings of the thesis were that rigid, rod-like but not long and tangled carbon nanotubes (CNT) were able to induce a condition similar to allergic airway inflammation via activation of innate immunity. Although nanofibrillated celluloses triggered acute pulmonary inflammation, their effects subsided within one month and regardless of the material's biopersistence, their health outcomes differed significantly from the long-term pathologies of rigid, rod-like CNT. Uncoated and functionalized CuO nanomaterials demonstrated an ability to worsen allergic asthma by eliciting pulmonary neutrophilia, however it was found that surface PEGylation significantly suppressed the inflammatory potential of the pristine CuO ENM; this effect was especially evident at the transcriptional level. Topical exposure to nano-sized ZnO in a murine model of atopic dermatitis revealed that the particles were able to pass through mechanically injured allergic skin. This penetration of the material resulted in a local inhibition of pro-inflammatory and allergic reactions and a systemic exacerbation of IgE antibody production.

This work provides knowledge of pulmonary and dermal effects of ENM. The results of this thesis demonstrate that ENM with different physicochemical characteristics possess an ability to modulate our immune system. These observations emphasize the diversity and complexity of the materials as well as highlighting their impacts on the immune system and the resulting consequences on health. These data contribute to the safety assessment of ENM as well as information that can be useful in nanomedicine.

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Helsinki, November 2018

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications that are referred to in the text by their Roman numbers:

I Rydman EM*, **Ilves M***, Koivisto AJ, Kinaret PA, Fortino V, Savinko TS, Lehto MT, Pulkkinen V, Vippola M, Hämeri KJ, Matikainen S, Wolff H, Savolainen KM, Greco D, Alenius H. Inhalation of rod-like carbon nanotubes causes unconventional allergic airway inflammation. *Part Fibre Toxicol.* 2014 Oct 16;11:48.

II **Ilves M***, Vilske S*, Aimonen K, Lindberg HK, Pesonen S, Wedin I, Nuopponen M, Vanhala E, Højgaard C, Winther JR, Willemoës M, Vogel U, Wolff H, Norppa H, Savolainen K, Alenius H. Nanofibrillated cellulose causes acute pulmonary inflammation that subsides within a month. *Nanotoxicology*. 2018 May 30:1-18.

III **Ilves M**, Kinaret PA, Ndika J, Karisola P, Marwah V, Fortino V, Fedutik Y, Correia M, Ehrlich N, Löschner K, Wolff H, Savolainen K, Greco D, Alenius H. Surface PEGylation suppresses CuO-induced pulmonary effects in asthmatic airways. Submitted to *Part Fibre Toxicol.*

IV **Ilves M**, Palomäki J, Vippola M, Lehto M, Savolainen K, Savinko T, Alenius H. Topically applied ZnO nanoparticles suppress allergen induced skin inflammation but induce vigorous IgE production in the atopic dermatitis mouse model. *Part Fibre Toxicol.* 2014 Aug 14;11:38.

* Equal contribution

 The thesis contains also supplementary material of the original publications that is not included in this book. Supplementary information is accessible via www.particleandfibretoxicology.com (I, IV) and www.tandfonline.com (II).

AUTHOR'S CONTRIBUTION TO THE PUBLICATIONS

I MI participated in collecting and processing samples of the performed experiments. MI carried out the quantitative assessment of histological samples (activation of mucin-producing goblet cells), prepared samples for mRNA expression analysis and measured the levels of relevant cytokines and chemokines by real-time polymerase chain reaction (PCR) assays. MI designed the microarray experiment and was involved in the interpretation of transcriptomics data. MI participated in interpreting results of the study and writing the manuscript.

II MI took part in the design of the study, supervised the experimental work, contributed to the cytological and histological analysis and participated in interpretation of the results. MI wrote the manuscript.

III MI participated in designing the study. MI performed the experiments and was involved in sample collection and processing. MI performed the sample analyses and participated in the qualitative assessment of the histological changes. MI prepared the samples, designed and conducted the microarray experiment and was involved in analyzing the preprocessed data. MI took part in interpreting the results of the study, and wrote the manuscript.

IV MI participated in designing the study. MI performed the animal experiments and was involved in sample collection and processing. MI took part of restimulations of lymph node cells *in vitro*. MI performed hyperspectral imaging analysis, carried out mRNA extraction of skin samples and performed PCR analyses. MI was involved in interpreting the results and writing the manuscript.

ABBREVIATIONS

AAI	allergic airway inflammation
AAM	alternatively activated macrophages
AD	atopic dermatitis
AHR	airway hyperresponsiveness
Alum	aluminum/magnesium hydroxide
AP	activator protein
APC	antigen-presenting cells
BAL	bronchoalveolar lavage fluid
BNC	bacterial nanocellulose
bZnO	bulk-sized zinc oxide
CBN	carbon-based nanomaterials
CCL	C-C motif chemokine ligand
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CNC	cellulose nanocrystals
CNT	carbon nanotube(s)
cRNA	complementary ribonucleic acid
CuO COOH	carboxylated copper oxide
CuO	core copper oxide
CuO NH3	methylaminated copper oxide
CuO PEG	PEGylated copper oxide
DAMP	damage-associated molecular patterns
DEGs	differentially expressed genes
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DWCNT	double-walled carbon nanotube(s)
ELISA	enzyme-linked immunosorbent assay
ENM	engineered nanomaterial(s)
FBGC	foreign-body giant cells
GI	gastrointestinal
H&E	hematoxylin and eosin
HPF	high power field
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cells
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MCP	monocyte chemoattractant protein
MeO	metal oxide(s)

MGG	May Grünwald-Giemsa
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MWCNT	multi-walled carbon nanotube(s)
NAMP	nanomaterial-associated molecular patterns
NETs	neutrophil extracellular traps
NF	nuclear factor
NFC	nanofibrillated cellulose(s)
NK	natural killer
nZnO	nano-sized zinc oxide
OCT	optimum cutting temperature compound
OEL	occupational exposure limits
OPA	oropharyngeal aspiration
OVA	ovalbumin
OVA/SEB	mixture of ovalbumin and staphylococcal enterotoxin B
PAMP	pathogen-associated molecular patterns
PAS	Periodic acid-schiff
PBS	phosphate buffered saline
PCR	real-time polymerase chain reaction
PEG	polyethylene glycol
Penh	enhanced pause
PMA	phorbol-12-myristate-13-acetate
PMN	polymorphonuclear leukocytes
PPR	pattern-recognition receptors
PSR	picrosirius red
rCNT	rigid, rod-shaped multi-walled carbon nanotubes
RNA	ribonucleic acid
ROS	reactive oxygen species
SEB	staphylococcal enterotoxin B
SWCNT	single-walled carbon nanotube(s)
TB	toluidine blue
tCNT	long, tangled multi-walled carbon nanotubes
TEM	transmission electron microscopy
TGF	transforming growth factor
Th	T helper
THP-1	human monocytic leukemia cell line
TLR	toll-like receptors
TNF	tumor necrosis factor α
Tregs	regulatory T cells
TSLP	thymic stromal lymphopoietin
UV	ultraviolet

1 INTRODUCTION

In 1986, K. Eric Drexler, an American engineer, published a book entitled "*Engines of Creation: The Coming Era of Nanotechnology*" in which he described machines invisible to our eye but being able to renew the human vascular system (Drexler, 1986). This idea sounded like science fiction to many at the time. However, fast forward to 2018, times have changed and so have the opinions of such visions.

Today, the world is in thrall to nanotechnology. It holds an enormous potential for developing and improving the design and manufacture of many consumer and industrial products. Nanotechnology has already exerted a major impact on many sectors of life ranging from electronics, food, textile and cosmetics to construction, sports, military and medicine. Many nanotechnology-based products are already on the market - for example the technology is exploited in carbon-fibre-containing sport ware, sunscreens based on inorganic UV filters, silver-integrated clothes and SiO2-included eye shadow (Danish Ecological Council and Danish Consumer Council, 2013).

In essence, nanotechnology is a field of material manipulation at the atomic scale or in other words – at the nanoscale. The development of these kinds of materials is limited only by the creativity of the human mind, thus, anything that can be imagined, has a potential in nanotechnology. Physicochemically, manufactured nano-sized materials known as engineered nanomaterials (ENM) can be based on all of the elements around us, they can be made in different sizes, shapes and forms and endowed with additional molecular compounds either around or inside the particles. The motivation for creating ENM lies in the fact that particles at the nanoscale have significantly altered, and often improved, properties compared with their larger equivalents.

The main routes of occupational and consumer exposure to ENM are via inhalation and dermal contact. Due to the small nature of primary ENM particles, they might bypass the biological barriers and avoid the clearance mechanisms of human body. Consequently, they encounter the immune system which is designed to recognize the cells that compose our bodies and to combat any new structures with which we come into contact and this includes not only invading bacteria and viruses but also foreign matter such as ENM. However, the downscaling of materials increases their chemical reactivity which when combined with their small size and other properties, confer on the ENM the potential to interact with the immune system and influence its functions. The contact may cause beneficial or detrimental effects on human health under normal but even more likely under compromised physical condition.

The aim of this thesis was to investigate the immunomodulatory effects of ENM under both physiologically healthy and impaired conditions. Firstly, the pulmonary effects of whole-body inhalation exposure to differently shaped

fibrous carbon nanotubes (CNT) were investigated in healthy mice. Secondly, the effects of exposure to nanofibrillated cellulose (NFC) were examined also in the lungs of healthy mice with the particles administered via a different respiratory exposure method called oropharyngeal aspiration (OPA). Thirdly, the ability of uncoated and coated CuO nanomaterials to modulate allergic airway inflammation (AAI) was explored in a murine model of asthma. Lastly, the penetration of nano-sized ZnO (nZnO) through injured and allergic skin, and its subsequent immunomodulatory effects were studied in a mouse model of atopic dermatitis (AD).

2 REVIEW OF THE LITERATURE

2.1 IMMUNE SYSTEM

Humans possess an inbuilt system formed by a variety of cells and molecules that protect us from infectious agents and harmful substances – it is called the immune system. The cells of the immune system lodge in the lymph nodes, reside in different tissues and circulate in the bloodstream. This broad distribution of immune cells throughout the body assures the rapid detection and a vigourous response when pathogens or foreign matter gain access to our bodies.

The immune system is divided into innate immunity, which we are born with and adaptive immunity which develops during our lifetime. Innate immunity responses are nonspecific and rapid, taking place within minutes or the first hours upon encountering a threat, whereas adaptive immunity reactions are specific to a pathogen; they occur days later resulting in the generation of a long-lasting protective immunological memory.

Immune reactions, both innate and adaptive, are mediated by white blood cells, also known as leukocytes. There are many different types of leukocytes and each has its own role to play in the immune response. In addition to killing microorganisms, immune cells also communicate with each other and activate nearby cells or other cell types, therefore their functionality is highly dependent on one another (Murphy *et al.*, 2009).

2.1.1 INNATE IMMUNITY

Innate immunity provides us with a frontline defense barrier against the microorganisms and foreign matter that we encounter on a daily basis. Innate immunity cells reside in the tissues and circulate in the bloodstream to increase the probability of pathogen detection. Cell types that belong to the innate immunity are monocytes and macrophages, dendritic cells, polymorphonuclear leukocytes (PMN), mast cells and innate lymphoid cells (ILC) (Murphy *et al.*, 2009, Artis and Spits, 2015).

Macrophages are resident cells that are found in almost all tissues. They mature from monocytes which circulate in blood and migrate into tissues where they differentiate. Macrophages are professional phagocytes – their role is to recognize, engulf and neutralize microorganisms that have passed through the physical and chemical barriers of our body. Recognition is mediated via cell-surface receptors that can differentiate between the surface molecules of infectious agents from those of the host. Once a pathogen binds to a macrophage receptor, an uptake process, called phagocytosis, occurs. Essentially, it means that the surface-bound invader becomes surrounded by a cell membrane and is then engulfed in an acidic vesicle called the phagosome

where the pathogen dies. Macrophages also store lysosomal granules that consist of enzymes, proteins and peptides used for targeting microbes. Another important function of macrophages is their secretion of signaling molecules called cytokines and chemokines, and other mediators upon their activation by pathogens. This triggers inflammation in the tissue and attracts other types of immune cells to the infected site.

Macrophages, and especially dendritic cells, link innate and adaptive immunity. After the interaction with a pathogen, these cells intracellularly bind fragments from the digested pathogen called antigens to major histocompatibility complex (MHC) molecules which are then displayed on the cell surface. These cells migrate from the infected site to nearby lymph nodes and present the antigens to T cells, a type of adaptive immunity cell. Thus, macrophages and dendritic cells are also called antigen-presenting cells (APC).

PMN are divided into three sub-types – neutrophils, eosinophils and basophils. Since their cytoplasm contains granules, they are also called granulocytes. PMN are short-lived cells that under normal conditions, circulate in blood, however, they are rapidly recruited into tissue to reinforce the actions of the macrophages during times of inflammation (Murphy *et al.*, 2009).

Neutrophils are the most abundant white blood cells; they are so-called professional killers of pathogens. During an infection, they are the first cells recruited to the inflammatory site (Murphy *et al.*, 2009). Neutrophils are able to kill pathogens irrespective of whether the latter are inside and outside the cell. Similar to macrophages, they have an ability to phagocytize foreign matter and neutralize it with the assistance of their granular content. After activation, neutrophils can also become degranulated and form neutrophil extracellular traps (NETs). NETs consist of decondensed chromatin whose purpose is to concentrate the released granule content and thereafter to capture the pathogen into a "net" where it will be killed by anti-microbial components of the granules. Neutrophils either die during the NETosis or become anuclear, in that case they maintain a preserved phagocytic ability (Papayannopoulos, 2018).

Eosinophils fulfill important roles in allergic diseases as well as in combatting helminth, viral and bacterial infections. Historically, eosinophils have been considered as effector cells in providing protection against parasites. In such cases, eosinophils have been observed to migrate and aggregate around parasitic helminths and degranulate to damage the targets (Rothenberg and Hogan, 2006). These cells, however, contribute also to the initiation and propagation of inflammatory responses through the release of cytokines; they can also regulate adaptive immunity by acting as APC to T cells (Rothenberg and Hogan, 2006, Kvarnhammar and Cardell, 2012).

The functions of basophils remain unclear but it is known that in response to cytokines from other cell types, antibody IgE, and antigens, they produce an array of bioactive molecules including cytokines, chemokines and histamine

that contribute to promoting and regulating immune reactions. Similarly to eosinophils, basophils play a role in allergic inflammation and in combatting parasitic infections (Yamanishi and Karasuyama, 2016, Kubo, 2018).

Mature mast cells reside in tissues; they are distributed under mucous membranes, in the skin epithelium as well as along blood vessels. Like eosinophils and basophils, these cells carry high affinity surface receptors, FcεRI, that bind IgE antibodies. Hence, a classical example of the involvement of eosinophils in inflammatory reactions in conjunction with their circulating equivalent, basophils, is the induction of type I hypersensitivity response through FcεRI activation in response to allergens. Contact with the allergen triggers degranulation and the release of pre-formed and newly formed mediators that cause the features typical of allergic responses (Cruvinel *et al.*, 2010).

Innate lymphoid cells (ILC) can be found at the body's barrier surfaces, such as the skin, lung and intestine. These cells are divided into several subsets, including classical natural killer (NK) cells as well as recently discovered non-cytotoxic ILC. NK cells do not carry antigen receptors on their surface and thus, they do not possess antigen specificity (Artis and Spits, 2015). Instead, when activated, NK cells bind to infected cells and release their granules onto the surface of the target cell. The effector proteins of the granules then penetrate through the membrane of the infected cell and induce programmed cell death, i.e. apoptosis. NK cells keep viral infections under control while antigen-specific cytotoxic T cells of the adaptive immunity are being generated (Murphy *et al.*, 2009). Non-cytotoxic ILC respond to cytokine and microbial signals by producing a variety of pro-inflammatory and immunoregulatory cytokines to trigger host-protective effector functions (Artis and Spits, 2015, Sonnenberg and Artis, 2015, Klose and Artis, 2016).

2.1.2 ADAPTIVE IMMUNITY

In the case when a pathogenic microorganism surpasses the protective limit of innate immunity, then the mechanisms of adaptive immune response will be activated. Adaptive immune reactions are the responsibility of lymphocytes that are divided into T and B cells. Following their maturation, these cells circulate in the bloodstream or are located in lymphoid organs such as lymph nodes where they await activation by innate immunity cells.

Naïve T lymphocytes are subcategorized into CD8 and CD4 T cells. In order to become activated, naïve T cells need three signals: MHC molecule:antigen complex binding of APC onto T cell receptor, the presence of APC CD80 (B7.1) or CD 86 (B7.2) co-stimulatory molecule that becomes attached to the CD28 receptor on T cells and cytokines provided by the APC which play a role in determining into which subtype of T cells these naïve cells will differentiate. After activation, T cells themselves produce IL-2 cytokine which acts as a growth factor, supporting T cell division and clonal expansion. Once naïve T

cells are activated, they proliferate and differentiate into effector T cells and subsequently they migrate to the inflammatory site.

Antigen-bound MHC I complex differentiates CD8 cells into cytotoxic CD8 T cells that kill virus-infected cells, whereas the MHC II complex differentiates CD4 cells into several functional subclasses. The main subtypes of these are T helper (Th)1, Th2, Th17 cells and regulatory T cells (Tregs).

Th1 cells control viral infections and enhance the microbial activity of macrophages in killing intracellular bacteria. Th1 cells differentiate in an environment of IFN-γ and IL-12 cytokines and are characterized by their ability to produce IFN-γ for clonal expansion. Th1 cells also induce antibody production (IgG) in B cells. Th2 cells also induce class switching of B cells, especially causing these cells to produce IgE that plays an important part in parasitic infections and allergic responses. Th2 cells produce IL-4, IL-5, IL-13 and IL-10. In the acute phases of the inflammation, both Th1 and Th2 subtypes might participate in order to guarantee an effective response, however, should the condition become chronic, then typically either the Th1 or Th2 subtype becomes dominant (Murphy *et al.*, 2009).

Th17 cells differentiate in an IL-6 and TGF-β environment and produce subset-specific IL-17 cytokines. These cells are stimulated in the early phases of adaptive immunity response to extracellular microbes. Th17 cells induce several cell types to produce pro-inflammatory cyotokines (TNF, IL-1β, IL-6) and chemokines (CXCL1, CXCL8, CXCL10). One of the important functions of Th17 cells is to attract neutrophils to the site of inflammation (Korn *et al.*, 2009).

Tregs become committed from CD4 T cells either when they are still in the thymus, i.e. natural Tregs, or in the periphery, i.e. adaptive or inducible Tregs. These cells produce IL-10 and TGF-β which target T cells directly or indirectly to mainly inhibit their reactions (Murphy *et al.*, 2009, Schmitt and Williams, 2013).

Naïve B lymphocytes can be activated either T cell-independently or T cell-dependently. B cells can be stimulated and their division triggered by non-protein bacterial antigens such as lipopolysaccharide (LPS) alone. More commonly, activation by the majority of antigens requires the help of an effector Th cell. T cell-dependent stimulation takes place in the presence of three signals: binding of the antigen to B cell receptor – surface immunoglobulin, co-stimulatory binding between Th cell ligand CD40L and its B cell receptor CD40, and cytokine release from the T cell (Murphy *et al.*, 2009).

Activated B cells initially differentiate into plasmablasts and then further either into extrafollicular short-lived plasma cells or germinal centre-dependent long-lived plasma cells that contribute to the serological memory. Plasma cells secrete antibodies whose form is determined by T cell-derived cytokines in a process known as class-switching, but these cells no longer possess an antigen-presentation ability. Some of the B cells differentiate into long-lived memory B cells that upon re-encountering the same antigen can

quickly differentiate into plasmablasts and generate class-switched antibodies (Kurosaki *et al.*, 2015).

Antibodies undertake three tasks in immunity. First, they can neutralize pathogens or their toxins by binding onto their surface molecules which the pathogens use for gaining access to cells. Second, they can assist in the uptake and phagocytosis of extracellularly multiplying bacteria in a process called opsonization in which they bind to the antigen-bearing pathogen and are thereafter recognized by Fc receptors on phagocytes. Third, antibodies which have become attached to a pathogen can activate the complement system – a plasma protein cascade of innate immunity, via a classical pathway which results in binding of complement components that in turn are recognized by complement receptors on phagocytes. While IgM, IgG and IgA antibodies can fulfill these functions, they do not sensitize mast cells like IgE. The latter, when bound to mast cells, triggers the secretion of chemicals that cause vomiting, coughing and sneezing – reactions intended to achieve pathogen removal. In addition, IgE mediates allergic responses.

B cells possess the ability to behave also as APC. Their surface-bound antibodies can recognize the antigen which is taken up into the cell, processed and then returned to the cell surface on a MHC II receptor. An antigen which is presented on the surface of a B cell can bind only to antigen-specific Th cells that have differentiated in response to the same stimulus. Upon linked recognition, T cells produce cell-bound and release effector molecules that support B-cell proliferation and immunoglobulin class switching (Murphy *et al.*, 2009).

Successful adaptive immunity response results in the immunological memory. During the primary immune response, some of the T cells differentiate similarly to B cells into long-living memory cells. T and B memory cells ensure that the body has a heightened ability to mount a secondary immune response should re-exposure to the same pathogen occur (Murphy *et al.*, 2009).

2.2 ALLERGIC DISEASES

The occurrence of allergic diseases has insidiously increased in the developed countries over the past 50 years. In addition, the prevalence of the diseases is growing also in developing countries. Furthermore, up to 40% of the global population is sensitized to foreign proteins in the environment and are at an increased risk of developing an allergic disease. Today, allergy has become a major healthcare problem (Pawankar *et al.*, 2011).

2.2.1 ASTHMA

Asthma is a chronic inflammatory disease of the conducting airways that affects over 300 million people all around the world. The typical features of

the disease include bronchial hyper-reactivity, mucus overproduction, remodeling of airway walls and narrowing of the airways. These changes are responsible for the clinical symptoms of asthmatics such as wheezing, shortness of breath and chest tightness (Lambrecht and Hammad, 2015). Asthma has several phenotypes and it can be triggered by several environmental agents and susceptibility genes (Kim *et al.*, 2010). The most general types of asthma are intrinsic (non-allergic) and extrinsic (allergic). Non-allergic asthma is more commonly diagnosed in adults and can be induced by exercise, respiratory infections, air pollution or cold air whereas allergic asthma might be initiated early in life and is triggered by an allergen like house dust mite, pet dander or pollen (Kim *et al.*, 2010, Deckers *et al.*, 2017).

Allergic asthma is the most common form of asthma; it develops with time, after re-exposure to the same allergen (Schatz and Rosenwasser, 2014). The first contact with an allergen - sensitization, results in the production of an antigen-specific IgE. Upon re-encountering the same allergen, a full-blown allergic response takes place (Galli and Tsai, 2012). Within the first minutes, the antigen binds to specific IgE antibodies that attach onto the FcεRI receptor on mast cells and basophils to initiate early-phase reactions. The pre-formed and newly synthesized cellular mediators including histamine, lipid-derived compounds, chemokines, cytokines, will then be released and these cause several symptoms e.g. increased vascular permeability, cell migration, mucus production and bronchoconstriction (Galli *et al.*, 2008, Cruvinel *et al.*, 2010). Late-phase reactions develop 2-6 h later and culminate 6-9 h after allergen exposure. They include activation and migration of CD4+ Th2 cells, eosinophils and other leukocytes with continuous mediator release from local cells (Galli *et al.*, 2008). Chemokine and cytokine environment of allergic asthma is replete with powerful mediators e.g. TNF, IL-1β, IL-5, IL-4, IL-13, IL-10, IL-33, TGF-β, CCL2, CCL7, CCL11 and CCL24 (Lukacs, 2001, Galli *et al.*, 2008, Deckers *et al.*, 2017).

2.2.2 ATOPIC DERMATITIS

Atopic dermatitis (AD) is a chronic, highly pruritic inflammatory disease of the skin that usually starts at an early age (Carmi-Levy et al., 2011). It affects up to 30% of children and up to 10% of adults. The occurrence of AD is increasing among kids and its prevalence has been found highest in Northern Europe. AD is more commonly diagnosed in children who live in urban areas as compared with those from the countryside (Bieber, 2010). Furthermore, it has been estimated that up to 80% of children with AD develop asthma or allergic rhinitis later in life in a process that has become known as the atopic march (Beck and Leung, 2000).

Both genetic and environmental factors play a role in the development of AD. It has been found that loss-of-function mutations in the FLG gene are a major risk factor in the development of AD (Fallon *et al.*, 2009). The gene

encodes profilaggrin/filaggrin production – a protein in keratinocytes that is responsible for keratin filament aggregation and the proper formation of the epidermis layer (McGrath *et al.*, 2008, Fallon *et al.*, 2009). The disturbed skin barrier, however, experiences increased transepidermal water loss and enhanced allergen penetration (Irvine *et al.*, 2011, Watson and Kapur, 2011).

The onset of AD is linked to the production of TSLP by keratinocytes encountering an allergen which leads to the generation of Th2 type milieu in acute skin lesions. However, a class switch from Th2 towards Th1 takes place during the course of the disease and thus, older skin lesions are characterized predominantly by a Th1 environment (He *et al.*, 2008, Werfel, 2009).

The skin of AD patients is richly colonized with *Staphylococcus aureus*. This happens due to the Th2 type reactions in the acute phases of AD which cause a suppression of the production of anti-microbial peptides. However, *S. aureus* produces enterotoxins that act as superantigens – they bind to MHC and TCR molecules non-specifically, causing an uncontrollable activation of T cells and overproduction of cytokines that results in the aggravation of AD (Morishita *et al.*, 1999, Bieber, 2010, Macias *et al.*, 2011).

2.3 NANOTECHNOLOGY AND ENM

The nano era is considered to have begun in 1959 when an American physicist, Richard Feynman gave a lecture entitled "There is plenty of room at the bottom" in which he introduced the idea of manipulating materials at the nanoscale and making molecular machines with atomic precision (Feynman, 1959). Years later, in 1974, a Japanese professor, Norio Taniguchi, formulated and defined the term nanotechnology. He said it "consists of the processing of, separation, consolidation, and deformation of materials by one atom or by one molecule" (Taniguchi, 1974).

The materials to which Feynman and Taniguchi referred are today called ENM; according to the definition provided by the European Commission, these have one or more external dimension in the size range 1 nm - 100 nm (The European Commission, 2011). One nanometer is one thousandth of a micrometer and only one billionth of a meter – ENM are so small that they are invisible to the human eye. It is also noteworthy that the materials are smaller than the cells of our body which confers on them the possibility to interact with our immune system. It may be easier to appreciate the size of ENM by comparing the primary particles of the materials with other common objects in Figure 1.

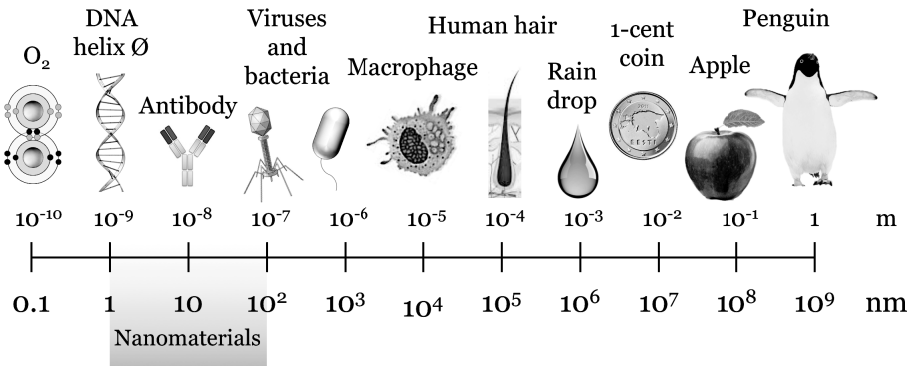

Figure 1 Sizes of different objects (adapted from National Cancer Institute, 2017).

The design of ENM holds seemingly endless possibilities. The materials can be made of basic elements (C, Au, Ag), metal oxides (TiO2, ZnO, SiO), biomolecules (proteins, lipids, DNA) and biomaterials (clay, cellulose). They can be solid or hollow and shaped as spheres, cubes, rods, sheets and fibers. Such core materials can then be coated with functional groups or filled with other nanomaterials or chemical compounds. This needs to be however considered as an incomplete list of examples of first (passive) and second (active) generation ENM. Future generations, i.e. third and fourth generation ENM, include self-assembling ENM and nano-robots, respectively – a level that the nanotechnology field has still to reach (Vogel *et al.*, 2014). However, because so many different ENM have been developed already, it is now apparent that no unified classification of nanomaterials can be devised. More commonly, the materials are categorized based on a specific property of interest, for example those described above.

Another reason why nanomaterials are fascinating and unique, is due to the fact that the size reduction of bulk materials to the nanoscale alters or improves their physicochemical characteristics such as strength and conductivity. For example, at a size of 20 nm, gold turns red, platinum appears yellowish gray and silver has a black color (Khan *et al.*, 2017). In addition, the smaller size increases the material surface area per mass and thus, ENM are chemically more reactive than their bulk-sized counterparts (Krug and Wick, 2011).

The unique properties of ENM have attracted interest from several industrial sectors because they offer numerous possibilities for the development of improved or completely new and innovative products. ENM cover an extremely wide range of application areas such as textiles, renewable energy, electronics, biomedical and cancer treatment, personal care, pharmaceuticals, surface coatings, plastics, paper, health care, food and agriculture, and environmental protection (Tsuzuki, 2009). Today, over 3000 nanomaterial-containing products have reached the European market (Danish Ecological Council and Danish Consumer Council, 2013) and the

European Commission estimated that the global market value of nanotechnology-incorporating products will reach EUR 2 trillion and create 6 million jobs by 2020 (The European Commission, 2013). After years of basic and applied research, the technological and economic growth in this field is now bearing fruit and ENM-containing products are part of our daily lives.

2.3.1 CARBON NANOMATERIALS

Carbon-based nanomaterials (CBN) are one of the most diverse types of nanomaterials because they can be produced in a variety of shapes and forms. This category includes graphene (a layer of carbon atoms arranged hexagonally), graphite (multilayered graphene), fullerenes (empty spheres), nanodiamonds (crystalline carbon allotrope), carbon fibers, nano-onions (fullerenes packed inside one another) and CNT. CNT are further divided into single-walled, double-walled and multi-walled CNT (SWCNT, DWCNT and MWCNT, respectively). A SWCNT is essentially a seamless rolled-up graphene sheet whereas MWCNT consist of several SWCNT cylinders inside each other.

CBN possess many valuable properties. The materials are lightweight with good thermal conductivities and depending on the carbon structure, can behave as conductors, semi-conductors or insulators. Diamond is the hardest material known whereas CNT have been found to be the strongest synthesized material to date (Coville *et al.*, 2011).

Due to the remarkable properties of CNT including high electrical conductivity, tensile strength, elasticity, thermal conductivity and high aspect ratio, potential applications of these ENM are found in field emission lamps, field emission flat panel displays, electronics, gas sensors, composite materials, catalysts, pharmaceutics, bioengineering, medicine and medical devices (Coville *et al.*, 2011, Guo *et al.*, 2012). One well-known example of a CNT-containing product on the market today is light-weight sport equipment, such as bicycle frames, tennis rackets and hockey sticks (Project on Emerging Nanotechnologies, 2005).

2.3.2 METAL OXIDES

Metal oxides (MeO) are a wide nanomaterial group and among the most commonly used ENM. This category includes CuO, ZnO, TiO_2, NiO, Fe_2O_3, Fe_3O_4, CeO_2, Al_2O_3 as well as many others. MeO ENM are produced on a large scale for many household and industrial applications – the materials are used in optical and recording devices, personal care products, water purification and as catalysts. MeO ENM have different electrical properties than metals, semiconductors and insulators, thus they are utilized in sensors, magnets, superconductors, lightning applications and electronics (He *et al.*, 2015, Seabra and Durán, 2015, Parham *et al.*, 2016).

CuO ENM have been specifically manufactured as catalysts, supercapacitors, inks, biocides, coating in food packaging, solar cells, magnetic storage media, antimicrobial textiles, pigments, gas sensors, electrodes in lithium-ion batteries and solar energy conversion (Ahamed *et al.*, 2015, Grigore *et al.*, 2016, Park *et al.*, 2016).

Among their other excellent properties, ZnO ENM have superior UV filtering properties as compared to their bulk-sized substitutes. Thus they are utilized in sun lotions but have also potential uses in bio- and gas sensors, solar cells, catalysts, displays, pigments, and coatings, electronic devices, cosmetics, biomedical imaging as well as potentially in drug delivery and as antimicrobial agents (Zhang *et al.*, 2013).

2.3.3 NANOCELLULOSES

Nanocelluloses are biomaterials that are made of cellulose – the structural compound present in plant cell walls and the most abundant organic polymer in the environment. Nanocellulose materials have become popular due to their renewable and sustainable nature, they are considered a promising solution for replacing fossil-fuel-based products such as plastics (Nair *et al.*, 2014).

Based on the production method of cellulose nanomaterials, they can be categorized into three subgroups. NFC, also called cellulose nanofibrils or microfibrillated cellulose, consist of several micrometer long threads of individualized cellulose fibers. Cellulose nanocrystals (CNC), also called cellulose nanowhiskers, are rigid rods of crystalline cellulose. Although CNC and NFC have a similar diameter, they differ in length. CNC are several hundreds of nanometers long and hence they are shorter than NFC. This is a result of different production methods; NFC are prepared by a mechanical treatment whereas CNC are obtained by acid hydrolysis. The third type of nanocellulose, bacterial nanocellulose (BNC), as the name suggests, is synthesized by bacteria. BNC consists of pure microfibrils that can also be hydrolyzed by acids into bacterial nanocrystals similar to CNC (Börjesson and Westman, 2015). However, large-scale BNC production is rather limited, making NFC and CNC more appealing to manufacturers (Lin and Dufresne, 2014).

Nanocelluloses can be used for example in nanocomposites, paper making, films, tissue engineering, pharmaceutical and food packaging, electronic storage devices and cell culturing as growth matrix. Furthermore, nanocellulose materials have been envisioned to be able to substitute for asbestos (Nair *et al.*, 2014, Park *et al.*, 2018).

2.4 EXPOSURE TO ENM

Throughout evolution, humans have been exposed to natural nano-sized particles since these may be formed during volcanic eruptions or forest fires.

However, human exposure to nanoparticles has increased significantly with global industrialization. Subsequently, we have been exposed to unintentionally generated particles, such as emissions from power plants and diesel exhaust and metal fumes. With the emergence of the nanotechnology field, we are now experiencing also exposure to intentionally made nanoparticles – ENM (Oberdörster et al., 2005, Kendall and Holgate, 2012).

Exposure to an ENM can take place during all of its life-cycle stages. These stages include nanoparticle manufacturing, formulation of nanomaterials and products, their industrial use, consumer use of the products, service period of the products and their waste life phase (Vogel *et al.*, 2014). During the development of these materials, their amounts are small and exposure levels are thought to be low, except if there is an accidental spillage. However, during the ENM commercialization phase and incorporation into products, larger material quantities are being handled and the risk of exposure is much higher, especially during pouring, packaging, cleaning and transportation processes. Once ENM-containing products reach the market, consumers can be exposed via intended usage or unintentionally in case of accidents, misuse or ENM release due to product deterioration. When they are discarded, nanoproducts might undergo physical or thermal treatments in recycling centers (Seaton *et al.*, 2010)(Seaton et al 2010; Fadeel book, chapter 2). In addition, ENM can enter the environment, for example from industrial effluent or through the use of nanoproducts, and human beings may be exposed indirectly via air or water (Oberdöster et al 2005; Kendall et al 2012). For every ENM, several exposure scenarios exist in which their exposure levels vary and their physicochemical nature changes (Fadeel *et al.*, 2012, Vogel *et al.*, 2014).

ENM levels are generally considered to be highest in occupational environments and hence, workplaces represent the greatest possibility for human exposure (Savolainen *et al.*, 2010). However, due to the limited data in this area, in legal terms, an "overexposure" at a workplace cannot occur today because no occupational exposure limits for individual ENM have been devised. Thus, the aim of exposure assessment is to pinpoint ENM release sources, develop measures for eliminating or limiting the release and monitor their effectiveness (Fadeel *et al.*, 2012).

Humans can be exposed to ENM via the respiratory tract (inhalation), gastrointestinal (GI) tract (ingestion), skin, or systemically by injection (Oberdörster *et al.*, 2005). With respect to these entry routes, inhalation is considered as the key exposure route in occupational and consumer scenarios and thus, the majority of the research investigating the health effects of ENM in mammalian systems *in vivo* has been performed with a focus on respiratory exposures (Oberdörster *et al.*, 2005, Kendall and Holgate, 2012). Dermal exposure can occur in workplaces as well as with the use of nanoproducts such as sunscreens. Although, it is generally considered that healthy skin is an effective barrier preventing ENM entry, there is evidence that dermal penetration can lead to systemic exposure (Gulson *et al.*, 2010). The ingestion of ENM may happen via food, water, drugs or cosmetic products. The GI tract,

like the skin, is thought to be of minor significance in terms of ENM exposure because the intestinal epithelium is intended to offer protection against the absorbance of the ENM and the GI tract is able to manage the removal of particles well. Intravenous administration happens intentionally in the case of diagnostic (bioimaging) or therapeutic (drug delivery) purposes (Krug and Wick, 2011). Each entry route has its own defense and clearance mechanisms intended to protect our body from foreign matter. However, these processes may not be effective or may be bypassed, since due to their small size, ENM have an ability to translocate to other areas in our body (Oberdörster *et al.*, 2005). Human exposure routes and biokinetics of the ENM are summarized in Figure 2.

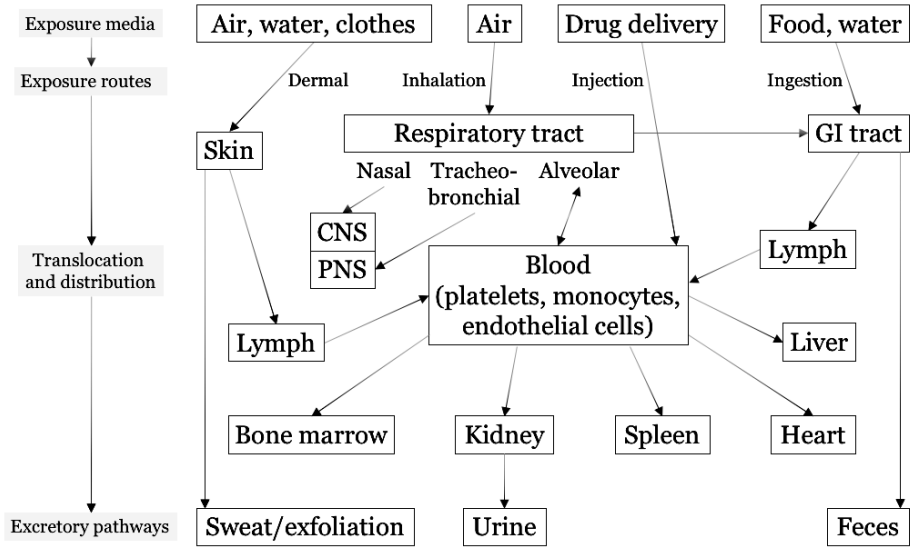

Figure 2 Human exposure routes and biokinetics of the ENM (adapted from Oberdörster *et al.*, 2005). CNS, central nervous system; PNS, peripheral nervous system; GI tract, gastrointestinal tract.

2.5 ENM BEHAVIOR AND EFFECTS IN BIOLOGICAL ENVIRONMENTS

Although nanotechnology-based innovations significantly improve the quality of our lives, ENM with beneficial and novel properties might pose a potential occupational and public health threat should they gain access to our body. To ensure that the materials and devices are safe to use, it is necessary to understand how ENM can affect our health.

The biological effects of ENM are being studied in the fields of nanotoxicology and nanomedicine. The first discipline deals with the adverse health effects of ENM whereas the second represents the positive side of the coin – nanomedicine focuses on developing ENM-based medical applications that aim to improve our health. These two fields provide useful information for each other and are often overlapping because they both investigate ENM interactions with biomolecules, cells and tissues and health outcomes of the triggered biological responses.

2.5.1 NANO-BIO INTERFACE

After an ENM entry into our body, a nano-bio interface is created in which the nanoparticles come into contact with tissue-specific liquids such as a pulmonary surfactant in the lungs or in the circulation, as well as cells. In the contact area between the ENM and such liquid, the ENM surface reacts with components in the surrounding fluid and the particles become coated with biomolecules like proteins, lipids or DNA. This cover is called a biocorona and its composition, i.e. which macromolecules are adsorbed onto the particle, is dependent on the physicochemical characteristics of ENM such as size, shape, surface area and charge, roughness and porosity, functional groups, hydrophobicity or hydrophilicity. In addition, the formation of the corona depends on which components are present in the fluid that surrounds the ENM (Nel *et al.*, 2009, Kendall and Holgate, 2012). Thus, the protein corona can be considered as a fingerprint of an ENM in a certain environment (Fadeel *et al.*, 2012).

The biocorona is described to have two layers. In the “hard” corona, the bound biomolecules interact directly and tightly with the ENM surface and can remain on the particle for a long time whereas the macromolecules on the outer corona layer – “soft” shell – are exchanged dynamically with those in the surrounding fluid (Vilanova *et al.*, 2016).

The formation of the corona causes physical changes in ENM. In addition to covering its initial surface, the presence of the biocorona alters the adhesive properties of the particles which among other factors, affects their agglomeration (Kendall and Holgate, 2012). Furthermore, protein adsorption onto ENM might result in conformational changes in the attached proteins which could subsequently lead to the activation or suppression of their biological function (Farrera and Fadeel, 2015). Nevertheless, the biocorona determines the ENM’s bioactivity and thus, it has a significant effect on toxicological and immunological behavior of the particles. In more specific terms, the composition and presentation of biomolecules in the outer layer of the corona are relevant factors as this is the material detected by the immune cells and it dictates the interactions and uptake between the particles and cells (Fadeel *et al.*, 2012).

2.5.2 DEPOSITION AND CLEARANCE IN THE RESPIRATORY SYSTEM

The respiratory system is divided functionally into the conducting and respiratory zones. The first zone comprises the structures that allow air to flow in and out of the lungs. Inhaled air travels at first through the nose, then passes through the pharynx, that is divided into naso-, oro- and laryngopharynx, larynx, trachea, primary bronchi finally arriving in the lungs. Ultimately, the air is distributed to the terminal bronchioles – the most distant part of the conducting zone, and passes to the alveolar ducts which open into a cluster alveoli where gas exchange takes place.

Inhaled particles deposit fractionally in nasopharyngeal, tracheobronchial and alveolar regions. The predominant deposition mechanism of nano-sized particles is diffusion due to random particle displacement upon collision with air molecules whereas other mechanisms such as inertial impaction, gravitational settling and interception are relevant for larger particles. The diffusion of a particle in the respiratory tract is inversely proportional to its diameter, and greater in areas where the airways are narrower and where the residence time is long. For example, it has been known that 90% of 1-nm particles become deposited in the nasopharyngeal zone, 10% in the tracheobronchial region and none in alveolar region. Five-nm particles deposit equally in all three compartments, whereas 20-nm particles have the highest deposition i.e. ~50% in alveolar region and 15 % in other regions (Oberdörster *et al.*, 2005). In the respiratory system, there are several clearance mechanisms involved in the removal of these particles.

The airways are covered with epithelia that consists of goblet and ciliated cells. When ENM enter the airways, goblet cells become activated and produce mucins – proteins that dissolve in water and form mucus. Mucus, in turn, is intended to trap the inhaled material. Once bound, the foreign matter is cleared from the airways towards the mouth by ciliated cells that move in rhythmical, same-directional movements. Once the mucus-bound particles reach the mouth, they can be swallowed and removed by the GI tract. This clearance mechanism of the lungs is called the mucociliary escalator. However, the mucociliary escalator becomes less effective peripherally and it is considerably slower in the terminal bronchioles. When foreign matter enters the alveoli, the main mechanism of their clearance involves the alveolar macrophages that are experts in engulfing all foreign matter (Fadeel *et al.*, 2012). In addition, the dissolution of ENM in lung fluids is an important aspect of pulmonary clearance (Shinohara *et al.*, 2017).

Very small particles and fibers that have an ability to reach the subpleural alveoli, can be removed to the pleural space and cleared by the outflow through 3-10-μm pores of outer pleura – stomata, to the mediastinal lymph nodes. In contrast, long fibers that cannot pass through stomata will be trapped at the pleura which leads to a prolonged interaction with mesothelial cells and possibly to inflammatory response (Murphy *et al.*, 2011). The knowledge of such exceptional behavior of fibers was gained when extensive use and exposure to asbestos in the 20th century resulted in adverse pulmonary

effects. Consequently, toxicologists established the fiber pathogenicity paradigm which defines a hazardous fiber as one that is thinner than 3 μm, longer than ~15 μm, and biopersistent in the lung tissue and pleura (Murphy *et al.*, 2011). The paradigm is now being applied also in the health and safety research of ENM.

2.5.3 SKIN BARRIER

Skin is an important organ of our body – it is a buffer between internal tissues and the surrounding environment, providing protection from pathogens and hazardous substances. The skin is composed of stratified epidermis and underlying dermis.

The epidermis, which is the skin's external thick avascular layer, consists of keratinocytes, melanocytes, Merkel cells and skin-specific dendritic cells called Langerhans' cells. It divides further into *stratum basale* which is the innermost layer, *stratum spinosum*, *stratum granulosum* and *stratum corneum*. The epidermis is mainly maintained by basal keratinocyte stem cells of *stratum basale* that differentiate and gradually move outwards. In the most superficial layer of the epidermis – *stratum corneum*, the cells are cornified and densely packed to protect the viable cell layers. Due to the constant exposure to external environment – microbes, chemicals, particulate matter including ENM, cornified cells provide efficient protection by continuously shedding from the surface to clean and renew the skin (McGrath *et al.*, 2008).

The dermis consists of connective tissue – a net of fibroblast-derived elastin and collagen fibers, in which other cell types such as mast cells, macrophages and dendritic cells reside. Dermis contains also hair follicles, sweat and sebaceous glands, blood and lymph vessels, and nerves. All of these structures have functional roles; for example, blood and lymph vessels provide the immune system's support in case tissue-resident cells recognize a threat, and sebaceous glands secrete sebum which possesses anti-microbial properties (Drake *et al.*, 2008, McGrath *et al.*, 2008). Sebum flow may be effective also in the removal of ENM that have become trapped into open hair follicules (Nohynek *et al.*, 2007).

2.5.4 CELLULAR UPTAKE OF ENM

The interaction between the particle and the cell starts with membrane contact, adhesion and uptake. Depending on the size and surface treatment, particles can be taken up via all mammalian internalization pathways. Small particles (50-150-nm ENM) are ingested by different endocytotic pathways whereas large, >0.5-μm ENM are engulfed by macropinocytosis or phagocytosis. Very small, <1-nm particles can possibly be translocated into the cells by diffusion through the plasma membrane (Dobrovolskaia and McNeil, 2007, Krug and Wick, 2011, Zhang *et al.*, 2015). However, ENM adherence and engulfment are affected by more features than simply size. The processes are

also dependent on particle shape, surface charge, roughness, agglomeration and biocorona composition. Furthermore, the interacting cell type, its surface features and the differentiation stage determine the attachment and uptake pathway (Nel *et al.*, 2009, Doshi and Mitragotri, 2010, Lunov *et al.*, 2011).

Most ingestion pathways are processes that require specific and nonspecific attractive forces for promoting uptake. This knowledge is used in nanomedicine when designing drugs for targeted delivery. For example, by adding specific ligands onto the particle's surface which bind to complementary compounds or receptors on cell membrane, receptor-mediated endocytosis could be targeted. The surface charge is an example of a nonspecific attractive force that influences cellular contact – cationic functional groups on ENM attach more strongly to the phospholipid layer of the cell than their anionic equivalents (Nel *et al.*, 2009). However, these attractive forces can be diminished by the surface attachment of polymers that create a steric barrier (Kendall and Holgate, 2012). This approach is relevant for designing safe ENM with decreased uptake (passivation) or drug carriers that need to avoid engulfment before reaching their target (Nel *et al.*, 2009).

The most described ENM uptake mechanism is phagocytosis. When a particle is phagocytized, it becomes surrounded by the cell membrane and is internalized in a membrane-enclosed vesicle known as the phagosome. Once closed, the internal of the phagosome's environment becomes acidic and degrades the particle. Phagocytic cells carry also membrane-enclosed granules – lysosomes, that contain enzymes which help with the neutralization process by fusing and releasing their contents into the phagosome and generating a phagolysosome (Murphy *et al.*, 2009).

2.5.5 EARLY EVENTS LEADING TO TOXICITY

When the front line defense cells of our body such as epithelial cells and macrophages in lung and skin, fail to degrade and remove ENM, these particles cause cellular stress. This is considered a key event that determines whether an ENM could trigger inflammation and thus, it is necessary to understand the ways in which nanoparticles can induce cellular stress. Various such crucial early events have been identified for ENM.

The generation of oxygen based radicals – reactive oxygen species (ROS) – after cellular exposure to ENM is considered a major contributor to ENM toxicity (Fadeel *et al.*, 2012). Nanoparticles can produce ROS directly due to their chemical reactivity or indirectly by affecting cellular redox reactions. As most of the ROS are produced in the cell during electron transport of mitochondrial respiration, it is one of the main sources for ENM-induced ROS. In addition, phagocytic cells can produce ROS as a defense mechanism against pathogens and pollutants which is called an oxidative burst. Excessive ROS generation when overwhelming a cell's antioxidant capabilities, results in oxidative stress that can lead to adverse effects such as inflammation, cell death, genotoxicity and carcinogenesis (Manke *et al.*, 2013).

The optimal particle size for phagocytosis by 20-μm alveolar macrophages is 3–6 μm (Krombach *et al.*, 1997, Frohlich, 2015). However, it has been shown that when phagocytes take up long and rigid CNT, they are unable form a phagolysosome which leads to incomplete ingestion known as frustrated phagocytosis (Poland *et al.*, 2008). Consequently, due to the stiff nature of CNT, the fibers rupture the phagolysosome which leads to the release of the lysosomal content into the cytoplasm and consequently, the production of pro-inflammatory cytokines (Schroder and Tschopp, 2010, Palomaki *et al.*, 2011).

Lysosomal destabilization can be caused by ENM with a high positive zeta potential, i.e. the charge that develops at the interface of the particle surface and the surrounding medium. In the biological environment, the biocorona neutralizes their charge but inside lysosomes, due to the acidic conditions and protease/lipase activity, the corona might be removed, revealing the positively charged surface that can interact with the lysosomal membrane, resulting in its rupture and leakage of its contents (Nel *et al.*, 2009, Fadeel *et al.*, 2012).

Nanoparticles generally dissolve poorly at pH 7 but some ENM like ZnO and CuO are acid soluble and thus they dissolve in the lysosomal acidic milieu of the cells. The dissolution of the particle inside the lysosome leads to high local ion concentration, lysosomal destabilization and breakage (Nel *et al.*, 2009, Fadeel *et al.*, 2012). The phenomenon that involves a successful uptake of an ENM in a particle form and its subequent lysosomal dissolution, is defined as the Trojan horse effect (Krug and Wick, 2011).

Lysosomal rupture has been described to result in ROS generation, cell death and activation of the NLRP3 inflammasome complex that plays a role in the maturation and secretion of pro-inflammatory cytokines, such as IL-1β (Schroder and Tschopp, 2010, Stern *et al.*, 2012).

2.5.6 CELL DEATH

Cellular injury may be followed by recovery or the death of the cell. Cell death occurs when there is an irreversible impairment of vital cellular functions that culminate in the loss of cell membrane integrity (Galluzzi et al., 2018). The three main forms of cell death are apoptosis, autophagic cell death and necrosis (Andon and Fadeel, 2013).

Apoptosis is a programmed cell death which happens by chromatin condensation, cell membrane blebbing and finally disintegration into membrane-enclosed parts called apoptotic bodies that are cleaned by nearby phagocytes. Intrinsic apoptosis can be initiated by DNA damage and oxidative stress whereas extrinsic apoptosis is activated by extracellular stress alarmins that are detected by transmembrane death receptors (Fadeel and Orrenius, 2005). There are studies demonstrating that MWCNT, TiO2 and nickel ferrite ($NiFe_2O_4$) nanoparticles can trigger oxidative-stress mediated apoptotic cell death (Bottini *et al.*, 2006, Ahamed *et al.*, 2011, Meena *et al.*, 2012, Ramkumar *et al.*, 2012, Srivastava *et al.*, 2013).

Autophagy is a homeostatic function and survival mechanism if nutrient deficiency occurs that removes malfunctional and injured organelles and recycles them for reuse through their transport to the lysosome. However, uncontrolled autophagy or its inhibition may lead to cell death (Andon and Fadeel, 2013). Cationic dendrimers and carboxylates SWCNT have been shown to induce autophagic cell death with subsequent lung injury in mice (Li *et al.*, 2009, Liu *et al.*, 2011).

Necrosis has been long considered as a non-programmed, premature death that occurs in response to chemical and physical conditions that induce such severe cellular damage that is beyond reasonable to repair. There are accumulating data suggesting that necrosis can be also regulated. In such a case, the process is induced by different stimuli like extracellular TNF and most usually activated when the apoptotic signaling pathway has been inhibited. Cells dying due to any form of necrosis release their intracellular content including pro-inflammatory cytokines into their surroundings, which in turn, initiates inflammation (Feoktistova and Leverkus, 2015).

Theoretically all ENM could cause one or another form of cell death – their cytotoxic potential does not depend only on their physicochemical properties but also on the exposure conditions such as dose and time (De Stefano *et al.*, 2012).

2.5.7 IMMUNOMODULATORY EFFECTS OF ENM

ENM have an ability to manipulate our immune system either by suppression or stimulation. Immunosuppression is essentially a down-regulation of immune reactions. The kind of inhibition caused by ENM has therapeutic prospects in the treatment of inflammatory disorders (Dobrovolskaia and McNeil, 2007). For example, inhaled CNT have been shown to cause systemic immunosuppression via the up-regulation of pulmonary TGF-β transcription which activates the cyclooxygenase pathway in splenocytes, resulting in the production and secretion of prostaglandin and IL-10 that in turn cause impairment of T-cell function and consequently inhibit the systemic immune response (Mitchell *et al.*, 2009). Lipid nanoparticles have been found to bind to cell adhesion molecules called selectins on endothelial cells that results in suppressed transmigration of immune cells into the tissue and ultimately reduced local inflammation (John *et al.*, 2003, Dianzani *et al.*, 2006). In contrast, immunosuppression can be also undesirable. It has been hypothesized that ENM-loaded macrophages might lose their immunological fitness and not be fully functional – they may have a decreased phagocytic ability and be less able to process pathogens during an infection (Fadeel *et al.*, 2012). Such immunosuppressive effects of ENM have, however, not been extensively studied. The majority of the immunomodulatory studies focus on the activation of the immune system and the development of inflammation.

Inflammation is an immediate response of the immune system to cellular or tissue damage by foreign invaders including ENM. The short-term response

– acute inflammation – involves leukocyte recruitment, neutralization and removal of the hazardous agent and tissue repair which usually results in a healing response. In contrast, a protracted, impaired and underperforming response leads to chronic inflammation which includes a continuous on-going inflammation, tissue damage and attempts to repair it (Weiss, 2008). Such prolonged inflammation has been linked to chronic health conditions, including allergy, cardiovascular diseases, cancer, and others (Chen *et al.*, 2018).

2.5.7.1 Inflammation and ENM-induced effects upon respiratory exposure

The initiation of inflammation generally begins with the activation of immune cells like macrophages and epithelial cells on body surfaces. Macrophages, but also monocytes, dendritic cells, neutrophils as well as adaptive immunity T and B cells, are equipped with pattern-recognition receptors (PRR) such as membrane-bound Toll-like receptors (TLR) that scan and detect microbial motifs called pathogen-associated molecular patterns (PAMP) or damage-associated molecular patterns (DAMP) (Murphy *et al.*, 2009). It has been shown that immune cells can recognize also ENM and thus, such immune system activating particles have been defined as nanomaterial-associated molecular patterns (NAMP) (Pallardy *et al.*, 2017). The outcome of recognition by PRR depends on the responding cell type and the molecular pattern. However, most PRR induce the activation of transcription factors like NF-kB and AP-1 that are responsible for encoding cytokines (TNF and IL-1β), chemokines, adhesion molecules and regulators which elicit leukocyte activation and migration (Schroder and Tschopp, 2010). To date, long and rigid MWCNT, SiO2, TiO_2, Ag and several other ENM have been shown to possess an ability to trigger the assembly of the NLRP3 inflammasome that leads to cleavage and maturation of pro-IL-1β and secretion of its biologically active form – IL-1β (Yazdi *et al.*, 2010, Palomaki *et al.*, 2011, Sun *et al.*, 2013). Inflammasome activation is modulated by ROS production, cathepsin B activity and P2X7 receptor (Palomaki *et al.*, 2011).

Leukocyte recruitment is a controlled process consisting of several well-defined steps. The process starts with leukocyte capture onto the endothelial wall, rolling and activation of the cell, its slowing down, arrest, strengthening of adhesion and spreading of the cell, intravascular crawling and transmigration through the endothelial cell membrane. The rolling of the leukocytes is facilitated by the expression of endothelial adhesion molecules – selectins and activated by chemokines derived from tissue macrophages or mast cells. Once the rolling of the cell has slowed down, it adheres firmly to the endothelium, a process which is mediated by leukocyte integrins and endothelial adhesion molecules like ICAM-1, and it transmigrates to the inflammatory site in the tissue (Ley *et al.*, 2007). It is known that nanoparticles are able cross the biobarriers and gain access to the bloodstream

where they interact with the circulatory immune cells (Oberdörster *et al.*, 2005). Carboxyl-coated quantum dots have been shown to enhance leukocyte migration *in vivo*; this process occurs via increased uptake by perivascular macrophages, subsequent activation of mast cells and release of pro-inflammatory cytokines which trigger endothelial activation and eventually elicit leukocyte migration (Rehberg *et al.*, 2010). At the beginning of inflammation, particles that influence leukocyte migration can exert a substantial effect on the efficiency of the immune system.

The nature of ENM-triggered acute inflammation is mainly thought to be neutrophilic. For example, the influx of these cells has been observed in response to SWCNT, DWCNT, MWCNT and TiO2 (Shvedova *et al.*, 2005, Ma-Hock *et al.*, 2009, Aiso *et al.*, 2010, Park *et al.*, 2011, Tian *et al.*, 2013, Sager *et al.*, 2014). Being phagocytes, neutrophils aid macrophages at the site of inflammation. It has been demonstrated that oxidized SWCNT are captured into NETs of phorbol-12-myristate-13-acetate (PMA)-activated human neutrophils and moreover, in purified NETs, the CNT undergo biodegradation through a myeloperoxidase (MPO)-dependent mechanism (Farrera *et al.*, 2014). MPO-mediated clearance of SWCNT has been detected also *in vivo* (Shvedova *et al.*, 2012). However, many ENM have been described as being biopersistent, causing sub-acute and chronic pulmonary effects. Early pulmonary neutrophilia was followed one week after exposure to SWCNT with the production of pro-fibrotic TGF-β, alveolar thickening and diffuse interstitial fibrosis as well as the formation of granulomas – very localized inflammatory sites in the form of immune cell accumulation (Shvedova *et al.*, 2005). Enhanced immune cell migration, early lung fibrosis and subchronic tissue damage were evident in lungs also 4 weeks post-exposure to CNT and ZnO ENM (Cho *et al.*, 2011, Park *et al.*, 2011) and persistent macrophage influx with the presence of MWCNT has been demonstrated even as long as 3 months after their administration (Aiso *et al.*, 2010).

ENM-induced inflammation may also trigger secondary genotoxicity through ROS generation and subsequent DNA damage (Fadeel *et al.*, 2012). DNA damage, caused either directly or indirectly, may promote tumorigenesis. Thus, ENM, especially fibrous ENM that share similar characteristics with asbestos, are feared to have a carcinogenic potential as well as an ability to induce other pathological effects that are known to be caused by these mineral fibers (Fadeel *et al.*, 2012, Pietroiusti, 2012, Doak and Dusinska, 2017).

ENM-triggered effects in subjects with asthma have been described in response to different types of materials. The comparison of these effects reveals that the particles do influence common allergic signs, however, modulation of the disease varies from one ENM type to another. There is evidence that SWCNT can aggravate AAI by increasing Th2 type cytokine and chemokine production and mucus production. In addition, the particles showed adjuvant activity for allergen-specific IgG1 and IgE antibodies (Inoue *et al.*, 2010). Similarly MWCNT, nickel and silica nanoparticles have also been observed to exacerbate AAI (Inoue *et al.*, 2009, Mizutani *et al.*, 2012,

Brandenberger *et al.*, 2013, Glista-Baker *et al.*, 2014, Ronzani *et al.*, 2014). In contrast, TiO2 has been reported to suppress local allergic symptoms (Rossi *et al.*, 2010). Graphene oxide also diminished certain allergic features like eosinophil influx, Th2 type cytokine and antibody levels, but aggravated airway hyperresponsiveness (Shurin *et al.*, 2014).

2.5.7.2 ENM-induced effects upon dermal exposure

Dermal exposure to ENM has generally been considered as insignificant due to the belief that healthy skin is an effective barrier preventing ENM entry. ZnO and TiO2 have been the most extensively studied materials in this area since these compounds are commonly used in cosmetic and personal care products such as sunscreens. Most studies that have investigated the transdermal penetration of ENM either *in vitro* or *in vivo*, have found that the particles are not able pass through the intact skin (Adachi *et al.*, 2010, Sadrieh *et al.*, 2010, Senzui *et al.*, 2010, Lin *et al.*, 2011, Monteiro-Riviere *et al.*, 2011, Holmes *et al.*, 2016). However, there are some controversies around this topic as there are studies demonstrating opposite findings (Wu *et al.*, 2009, Gulson *et al.*, 2010). One of the most intriguing observations was noted in human volunteers, when after repeated application of an isotope-labelled ZnO-containing sunscreen, small amounts of zinc were detected in blood and urine (Gulson *et al.*, 2010). Information about ENM penetration into irritated, injured or diseased skin is very limited and only a few studies exist in this area (Senzui *et al.*, 2010, Lin *et al.*, 2011, Monteiro-Riviere *et al.*, 2011). No information exists on whether the materials, even if able to penetrate through the skin, can cause immunomodulatory effects but hypothetically, ENM-biomolecule interactions, uptake by tissue macrophages and the signals derived from the cells after their activation may evoke these kinds of effects.

3 AIMS OF THE STUDY

Evidence exists that ENM, depending on their characteristics and exposure conditions, have the potential to modulate the immune system. Although some mechanisms of toxicity have been elucidated *in vitro*, there is not enough *in vivo* information about the pathomechanisms and subsequent consequences caused by different ENM types. In addition, most of the nanotoxicology research has been focused on investigating the adverse health effects in the respiratory tract; in contrast, other exposure routes, such as dermal contact, have received little attention. Furthermore, the effects of ENM have been explored much more extensively under normal health conditions but little is known about their influence on vulnerable populations such as subjects with allergic asthma or AD. Furthermore, there is a paucity of data about how certain specific physicochemical properties of ENM such as shape, size and surface alterations, can affect either functional or impaired immunity. The main aim of this thesis was to examine the modulatory effects of various ENM on a healthy and on a compromised immune system in the lungs and skin.

The specific aims of this thesis were:

1. To investigate whether inhalation exposure to rigid, rod-like MWCNT (rCNT) or long and tangled MWCNT (tCNT) can induce asthma-like pathologies in healthy mice.

2. To explore the immunomodulatory properties of different NFC materials upon respiratory exposure, and to compare their effects to those of bulk-sized cellulose fibrils and rCNT.

3. To study how uncoated CuO affects allergic lung inflammation as well as exploring whether different surface functionalizations influence its bioreactivity.

4. To investigate the effects of topically applied nZnO in a mouse model of AD and to compare these outcomes to those induced by bulk-sized ZnO (bZnO).

4 MATERIALS AND METHODS

4.1 NANOMATERIALS

To reach the aims of this thesis, CNT, NFC, CuO and ZnO nanomaterials were used. The primary particle size, description and origin of these ENM are presented in table 1. More details about their physicochemical properties are provided in original publications I-IV.

Table 1 *Nanomaterials used in this thesis. MWCNT, multi-walled carbon nanotubes; NFC, nanofibrillated cellulose; PEG, polyethylene glycol.*

Acronym	Description	Supplier (product code)	Primary particle size	Form	Reference
tCNT	Tangled MWCNT	Cheap Tubes Inc. (MWNTs 8-15 nm OD)	OD 8-15 nm Length 10-50 µm	Powder	I
rCNT	Rigid, rod-like MWCNT	Mitsui & Co., Ltd. (XNRI MWNT-7)	OD >50 nm Length ~13 µm	Powder	I,II
Material 1	Bulk-sized cellulose fibers	Stora Enso Oyi/ UPM Kymmene Oyi	Bulk-sized	Gel	II
Material 2	Non-modified NFC	Stora Enso Oyi/ UPM Kymmene Oyi	Width 2-15 nm Length 2-20 µm	Gel	II
Material 3	Carboxy-methylated NFC	Stora Enso Oyi/ UPM Kymmene Oyi	Width 3-10 nm Length 2-50 µm	Gel	II
Material 4	Carboxylated NFC	Stora Enso Oyi/ UPM Kymmene Oyi	Width 4-10 nm Length 0.5-10 µm	Gel	II
Material 5	Non-modified NFC	Stora Enso Oyi/ UPM Kymmene Oyi	Width 7-20 nm Length 2-20 µm	Gel	II
CuO	Pristine CuO	PlasmaChem GmbH	10-20 nm	Powder	III
CuO COOH	Carboxylated CuO	PlasmaChem GmbH	10-20 nm	Powder	III
CuO NH3	Methyl-aminated CuO	PlasmaChem GmbH	10-20 nm	Powder	III
CuO PEG	PEG-coated CuO	PlasmaChem GmbH	10-20 nm	Water dispersion	III
bZnO	Bulk—sized ZnO	Camden-Grey Essential Oils, Inc.	240 nm	Powder	IV
nZnO	Nano-sized ZnO	Nanostructured & Amorphous Materials, Inc. (5810MR)	20 nm	Powder	IV

4.1.1 CHARACTERIZATION OF THE ENM

The morphology and size of the materials were characterized by scanning electron microscopy, transmission electron microscopy (TEM), laser diffraction and/or atomic force microscopy (I-IV). The composition of

MWCNT and ZnO materials was analyzed in an X-ray energy dispersive spectroscope (EDS ThermoNoran Vantage, Thermo Scientific, Breda, The Netherlands) attached to Jeol JEM 2010 TEM (I, IV). The endotoxin content of MWCNT and cellulose materials was measured by Limulus Amebocyte Lysate assays (I, II). The presence of yeast and mold was assessed in cellulose samples by a MYSK10025 test kit (Merck Millipore), zeta potential was measured by dynamic light scattering (Malvern Zetasizer Nano) and metal traces analysed by inductively coupled plasma mass spectrometry (II). The surface area of CuO materials was determined by nitrogen adsorption/desorption on a Belsorp Mini II instrument (BEL, Japan). The specific surface area of CuO nanoparticles was obtained from nitrogen adsorption isotherms, according to the Brunauer–Emmett–Teller equation (III).

4.1.2 AEROSOL GENERATION AND CHARACTERIZATION

The MWCNT aerosol was generated with a fluidized bed aerosol generator (TSI Model 3400A) for which the materials were used without any pre-treatment. In the generator, CNT agglomerates were mechanically broken by bronze pellets in a continuous air flow. Aerosol concentrations were measured with an optical particle sizer (Grimm Dust Monitor 1.109) and the results of the measurements are presented in Figure 7 of original publication I. Gravimetric samples were collected onto nitrocellulose filter (Millipore) and weighed on an analytical balance. More detailed description of aerosol generation, characterization data and a scheme of the inhalation setup is presented in I.

4.1.3 DISPERSION PREPARATION

4.1.3.1 In vitro

Cellulose materials were provided as gels. Stock suspensions (1 mg/ml) and further dispersions (1, 10 and 100 μg/ml) of the materials were prepared in supplemented RPMI 1640 medium (Invitrogen). All dispersions were mixed on a vortex mixer and thereafter on a shaker for 10 min. Prior to adding the dispersions to the cells, the dilutions were quick-vortexed. More details about the dispersion preparation can be found in II.

4.1.3.2 In vivo

Stock dispersion (4 mg/ml) and further dilutions (200 μg/ml and 800 μg/ml) of cellulose materials were prepared in Dulbecco's phosphate buffered saline (DPBS; Gibco, Life Technologies, Carlsbad, CA) and mixed for 10 minutes (II).

rCNT was suspended in DPBS supplemented with 0.6 mg/ml bovine serum albumin (Sigma-Aldrich, St. Luis, MO) to support deagglomeration. The stock dispersion (1 mg/ml) and further dilutions (200 μg/ml and 800 μg/ml) were sonicated in a water-bath for 20 min at 30 °C (II).

Core CuO (CuO) and its carboxylated (CuO COOH) and methylaminated (CuO NH3) derivates were obtained as powders. Stock suspensions (1 mg/ml) were prepared in endotoxin-free water and sonicated under the conditions described in III with a probe sonifier. Polyethylene glycol (PEG)-coated (CuO PEG) was a 6.5 % aqueous suspension which was diluted 10x in endotoxin-free water to obtain a stock dispersion of 6.5 mg/ml and thereafter sonicated for 2 minutes. Further dilutions (50, 200 and 800 μg/ml) of the materials were prepared in DPBS with or without ovalbumin (OVA; Sigma-Aldrich).

ZnO suspensions were prepared in phosphate buffered saline (PBS) with water-bath sonication for 20 minutes at 30°C (IV). Before application to the patches, the suspensions were diluted to 16.67 mg/ml in vehicle (PBS) or in a mixture of OVA and staphylococcal enterotoxin B (SEB; Sigma-Aldrich) in PBS.

All dispersions were used immediately after preparation and vortexed prior to the administration.

4.2 CELLS

Human monocytic leukemia cell line (THP-1) was maintained in supplemented RPMI 1640 culture medium (Invitrogen) at 37 °C in 5 % CO_2. Cells were regenerated once a week and culture medium was changed twice a week.

In the stimulations, THP-1 cells were seeded onto cell culture plates and differentiated into macrophages with 50 nM PMA (Sigma-Aldrich) for 48 h. A more detailed protocol of cell culturing is described in II.

4.3 ANIMALS

Female C57BL/6 (I-II), BALB/c (I, III-IV) and *Kit*$^{W\text{-}sh}$/HNihrJaeBsmJ (I) mice (7-8 weeks old) were purchased from Scanbur A/S (Karlslunde, Denmark) and quarantined for one week before the start of the experiments. Housing conditions of the mice are described in detail in publications I-IV. The experiments were performed by conforming with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg March 18, 1986, adopted in Finland May 31, 1990), and were approved by the State Provincial Office of Southern Finland.

4.4 EXPOSURES

4.4.1 ENM EXPOSURE *IN VITRO* (II)

After the 48-h differentiation of THP-1 cells, the medium was replaced with 1 ml of a particulate material dispersion at concentrations of 1, 10 and 100 μg/ml (0.1, 1 and 10.4 μg/cm2 respectively). LPS (Sigma-Aldrich) was included as a positive control at a concentration of 100 ng/ml and cell culture medium was used as a negative control. Exposed cells were incubated for 3, 6 or 24 h. At least three stimulations were performed in each experimental group.

4.4.2 WHOLE-BODY INHALATION (I)

Mice were exposed in a whole-body inhalation chamber to rCNT or tCNT aerosol for 4 h/day once or on four consecutive days. Aerosol mass concentrations used for individual experiments were within a range of 6.2-8.2 mg/m^3 for rCNT and 17.5-18.5 mg/m^3 for tCNT. During the experiments, untreated control mice were housed in the same room with CNT-exposed animals. All mice were sacrificed using an overdose of inhaled isoflurane either immediately or 24 h after the exposure(s).

4.4.3 OPA OF CELLULOSE MATERIALS (II)

Mice were exposed to 10 or 40 μg/mouse of cellulose materials (corresponding to 200 or 800 μg/ml dispersions) by single OPA. Briefly, 50 μl of material suspension was delivered to the oropharynx of anesthetized mice under visual control. Control mice received 50 μl of DPBS. All mice were sacrificed using an overdose of inhaled isoflurane 24 h or 28 d after the OPA.

4.4.4 ADMINISTRATION OF CuO ENM IN A MURINE MODEL OF ASTHMA (III)

During the sensitization period, mice received intraperitoneally a mixture of 50 μg of OVA and 2 mg of aluminum/magnesium hydroxide (Alum; Imject® Alum, Pierce Biotechnology, Rockford, IL) in 100 μl of DPBS on day 1 and 10. After 10 days, mice were exposed oropharyngeally to 50 μl of DPBS or 50 μg of OVA in 50 μl of DPBS with or without CuO materials under isoflurane anaesthesia (Univentor 400 Anaesthesia Unit, Abbott Laboratories) on four consecutive days. CuO materials were tested at three doses, 2.5, 10 and 40 μg/mouse/administration. Mice were sacrificed with an overdose of inhaled isoflurane 24 h after the last administration.

4.4.5 ZnO ADMINISTRATION IN A MURINE MODEL OF AD (IV)

During the first sensitization period, on days 1 and 4, anesthetized mice were shaved and tape-stripped to induce a mechanical skin injury, and epicutaneously treated with 100 µl of vehicle (PBS) or a combination of 100 µg of OVA and 2.5 µg of SEB (OVA/SEB; Sigma-Aldrich) that were added onto a gauze patch and secured onto the animals' backs with an adhesive tape. After a recovery period, on days 23, 26 and 29, the mice were shaved and tape-stripped again, and exposed to PBS or OVA/SEB mixture with or without 16.67 mg of bZnO or nZnO per patch. Mice were sacrificed by isoflurane overdose 24 h after the last sensitization.

4.5 AIRWAY HYPERRESPONSIVENESS (I)

Airway hyperresponsiveness (AHR) of BALB/c mice was measured on day 5 using a single chamber, whole-body plethysmograph system (Buxco). Lung reactivity was expressed as enhanced pause (Penh) values. After the measurement, mice were sacrificed and samples were collected for analyses.

4.6 SAMPLE COLLECTION

4.6.1 *IN VITRO* (II)

Cell culture supernatants were collected for cytotoxicity assessment and protein secretion measurements. Attached cells were washed with 1 ml of DPBS and lysed with 500 µl of Trisure (Bioline Reagents Ltd., UK). The lysates were for total RNA isolation and PCR assays.

4.6.2 IN VIVO

The tracheas of mice exposed via respiratory tract (I-III) were cannulated with a blunt syringe and the lungs were lavaged with 800 µl of DPBS. The thoraxes were then opened, and part of the left pulmonary lobe was removed, quick-frozen (I) or kept in RNAlater RNA stabilization reagent (Ambion®, Life Technologies Carlsbad, CA; II-III), and stored for RNA isolation. Another part was immersed in Tissue-Tek optimum cutting temperature compound (OCT; Sakura Finetek, Alphen aan Den Rijn, The Netherlands), quick-frozen and kept at -70 °C for immunohistochemical stainings (II-III). The rest of the lung tissue was formalin-fixed for histological assessment (I-III).

After topical exposures, blood samples from vena cava were collected for antibody analysis. Skin biopsies from treated skin areas were stored for RNA isolation, were formalin-fixed or OCT-immersed and quick-frozen for histological evaluations. Skin draining lymph nodes were collected for cell re-stimulations with OVA or SEB.

4.7 STIMULATION OF LYMPH NODE CELLS (IV)

Collected lymph nodes were ground, the obtained cell suspensions from two mice were pooled and cultured in supplemented RPMI 1640 medium at a density of 3×10^6 cells/well in 24-well plates in the presence of OVA (50 μg/ml) or SEB (1 μg/ml). The cell culture medium was collected 48 h post-stimulation for protein measurement of IL-13 and IFN-γ.

4.8 SAMPLE ANALYSES

4.8.1 CELL DEATH (II)

Cytotoxicity was assessed by the lactate dehydrogenase (LDH) release assay (Cytotoxicity Detection Kit^{PLUS}, Roche). 100 μl of the sample was mixed with 100 μl of the reaction mixture and thereafter the instructions provided by the manufacturer were followed. Optical densities were read at 490 nm in a Victor^3 TM Multilabel counter (Perkin Elmer) and the background values at 620 nm with a Multiscan MS plate reader (Labsystems). A more detailed protocol is described in II.

4.8.2 mRNA EXPRESSION OF CYTOKINES/CHEMOKINES

RNA was extracted from cell lysates, homogenized lung and skin samples by the phenol-chloroform method described in detail in the original publications I-IV. The quantity and purity of isolated RNA was determined by NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific Inc., Wilmington, NC, USA). cDNA was synthesized from 500 ng of total RNA using MultiScribe Reverse Transcriptase and random primers (The High-Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. PCR assays were carried out with Relative Quantification TaqMan 7500 Fast System (7500 Fast Real-Time PCR system, Applied Biosystems) using 1 μl of cDNA sample, pre-developed primers and probes, and TaqMan universal PCR master mix according to the instructions from Applied Biosystems. Ribosomal 18S was used as an endogenous control. The details of cDNA synthesis and PCR analyses are described further in publications I-IV.

4.8.3 CYTOKINE SECRETION AND ANTIBODY ANALYSES

Supernatants of PMA-differentiated THP-1 cells exposed to cellulose materials were analysed with human IL-1β and TNF-α ELISA kits (eBioscience, Inc., San Diego, CA) and supernatants of re-stimulated lymph node cells were analysed

using mouse IL-13 and IFN-γ ELISA (eBioScience, San Diego, CA). All assays were performed according to the instructions given by the manufacturers.

Total antibody levels were assayed by sandwich ELISA and specific-antibody levels by an indirect ELISA method. Antibodies were obtained from BD Biosciences (San Jose, CA) and peroxidase substrate reagents from Kirkegaad & Perry Laboratories (Gaithersburg, MD). BD Pharmingen instructions were followed when performing the ELISA assays.

More details on the used reagents and protocols are presented in original publications II and IV.

4.8.4 CYTOLOGICAL ASSESSMENT

Cells of the bronchoalveolar lavage fluid (BAL) were cytocentrifuged onto slides, stained with May Grünwald-Giemsa (MGG) and counted under light microscopy (Leica DM 4000B; Leica).

4.8.5 HISTOLOGICAL ASSESSMENT

Formalin-fixed lung or skin tissue was embedded in paraffin, cut into 4-µm sections, affixed on slides, and stained with hematoxylin and eosin (H&E; I-III), Periodic acid-schiff (PAS; I-III), picrosirius red (PSR; I-II) or toluidine blue (TB; IV) solutions.

Recruitment of inflammatory cells into the lungs (I-III) or skin (IV) and an assessment of the morphological alterations of the tissue was conducted after H&E staining. PSR-stained lung sections were used to evaluate the formation of fibrosis and the distribution of CNT (I), or cellular activity around cellulose materials (II). The number of activated mucin-producing goblet cells was determined after PAS staining (I-III). TB-stained skin tissue sections were used for counting mast cells (IV).

4.8.6 IMMUNOHISTOCHEMISTRY

Four-µm frozen sections of OCT-immersed lung or skin tissue samples were fixed and stained for counting CD3+, CD4+, CD8+ and F4/80+ cells. The numbers of positively stained cells were counted under a light microscope at 400x magnification as an average of a certain number of randomly selected areas per high power field (HPF). More details are found in publications I-IV.

4.8.7 BIODURABILITY ASSESSMENT OF CELLULOSE MATERIALS (II)

Sections of paraffin-embedded lung tissue were stained with HRP-EXG:CBM as described earlier (Knudsen *et al.*, 2015). The presence of cellulose materials was quantitatively assessed by counting the number of agglomerates and by

measuring their total area. Results are presented as an average from three HPF/slide.

4.8.8 HYPERSPECTRAL IMAGING (IV)

Translocation of ZnO particles in the skin was analysed by CytoViva hyperspectral imaging microscopy system (CytoViva, Inc, Auburn, AL). The images were captured, processed and analysed with CytoViva ENVI 4.8 software. Sonicated 100 μg/ml ZnO dispersions in PBS and unstained 4-μm skin sections were used for creating spectral libraries of the materials and capturing hyperspectral images of the tissue, respectively. The images were processed and spectral libraries of both ZnO materials were filtered with spectra collected from skin samples of control mice not treated with the materials to avoid false positive matches. The Spectral Angle Mapper Classification feature was used to find the locations of pixels matching the spectra of bZnO or nZnO in the images of ZnO-treated skin.

4.8.9 GENOME-WIDE TRANSCRIPTOME ANALYSIS (I, III)

Total RNA was isolated from lung tissue samples and its quality was verified. A pool of two RNA samples was used to synthesize cRNA with the T7 RNA polymerase amplification method (Low Input Quick Amp Labeling Kit, Agilent Technologies). cRNAs were labeled with Cy3 and Cy5 dyes (Agilent Technologies) and thereafter purified using RNeasy Mini spin columns (Qiagen, GmbH, Hilden, Germany). 300 ng of a Cy3-labeled sample and a Cy5-labeled sample were combined (total 600 ng), fragmented and hybridized to the Agilent 2-color 60-mer oligo arrays for 17 h at 65 0C. The slides were washed and scanned with Agilent Microarray Scanner G2505C (Agilent Technologies). Raw intensity values were obtained with the Feature Extraction software, version 11.0.1.1 (Agilent Technologies).

The microarray data have been deposited in NCBI Gene Expression Omnibus (GEO) database (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE50176 (I) and TBA (III).

4.9 DATA ANALYSIS

4.9.1 ANALYSIS OF MICROARRAY DATA

Pre-processing of the microarray data is described in detail in publication I and III. Briefly, the median foreground intensities were analyzed in the R software (R Core Team, 2012) with the BioConductor package limma (Smyth, 2005). Log2 transformation and quantile normalization were performed. Batch effects were removed using the ComBat method implemented in the sva

package (Johnson *et al.*, 2007, Leek *et al.*, 2013). The values of the probes recognizing the same genes were further averaged into the final expression matrix. Differentially expressed genes (DEGs) were identified by using linear models and empirical Bayes pairwise comparison (post hoc adjusted $P < 0.05$ and linear fold change (FC) > |1.5|)(Smyth, 2004). After Benjamini and Hochberg post hoc correction (Benjamini and Hochberg, 1995), the resulting gene sets were considered to be significant and were further studied by using analysis tools further described in publications I and III.

4.9.2 STATISTICS AND GRAPHICS OF ENM PENETRATION, BIODURABILITY AND IMMUNOLOGICAL ENDPOINTS

Statistical analysis of the data was performed and graphs constructed with GraphPad Prism Software (GraphPad Software Inc., San Diego, CA, USA) unless stated otherwise. Analysis of variance was carried out followed by post hoc tests for the pairwise comparisons of interest. A P-value of <0.05 was considered to be statistically significant. The correlation between microarray and PCR results in publication III was calculated with Pearson's correlation test. The details of statistical analyses are described in original publications I-IV.

5 RESULTS

5.1 INHALED rCNT ELICIT ATYPICAL AAI IN HEALTHY LUNGS (I)

The acute pulmonary effects of CNT were examined by exposing mice to aerosolized rCNT and tCNT for four hours on four consecutive days. After the 4-day exposure, rCNT but not tCNT caused a decrease in macrophage numbers and induced a migration of eosinophils into the airways and lung tissue (I, Figure 1a-b, 1c-e). The cytological and histological assessment revealed also rCNT-derived frustrated phagocytosis and the formation of foreign-body giant cells (FBGC; I, Figure 1f-g). Furthermore, it was found that rCNT inhalation induced AHR to inhaled methacholine (I, Figure 2a), activation of mucin-producing goblet cells (I, Figure 2b-e), up-regulation of Th2 cytokines (I, Figure 2f) and eosinophil-attracting chemokines (I, Figure 2h). These results are evidence that inhalation of rCNT can evoke a condition similar to AAI.

In an attempt to understand the role of mast cells in the rCNT-induced condition, mice deficient in that cell type were used. The diminished presence of mast cells affected eosinophil influx (I, Figure 3a) and mRNA expression of Th2 type Il-13 (I, Figure 3c) but it did not influence goblet cell activation (I, Figure 3b) or the transcription of eosinophil-chemoattractants (I, Additional file 2b). These results indicate that mast cells partially regulate the elicitation of rCNT-triggered allergic-like airway inflammation.

CNT-induced early regulatory events were investigated by exposing mice to aerosolized materials once for 4 h with the animals being sacrificed immediately after the end of the exposure or after 24 h. Genome-wide transcriptomics analysis of the lung tissue revealed that the tCNT treatment had a relatively mild effect on the gene expression at 4 h (I, Figure 4a) – innate immunity pathways were not activated or were down-regulated as compared with untreated controls (I, Figure 4c). In contrast, rCNT exposure had activated the pathways essential for innate immunity (I, Figure 4c). At 24 h after the exposure, the effects of tCNT were more pronounced although similar in terms of the size and range of variation to those induced by rCNT. However, there were differences in the gene expression patterns altered by rCNT and tCNT (Additional file 4); this was seen also at the pathway level in the direct comparison of the transcriptomes of mice treated with rCNT and tCNT (Additional file 5). These data reveal that inhaled rCNT rapidly activate the innate immune system.

The involvement of macrophages and mast cells was examined in an attempt to clarify these early rCNT-triggered events. It was found that alveolar macrophages express Il-1β and Tnf, but also Il-33 and Ccl17 along with other cell types in the lung tissue (I, Figure 5a-b). Mast cells were found to express

Il-13 and Il-4 (I, Figure 5b). This information demonstrates that macrophages and mast cells are important cells in the initiation of rCNT-triggered allergy-like responses.

5.2 NFC INDUCES ACUTE PULMONARY INFLAMMATION IN HEALTHY LUNGS THAT DIMINISHES AFTER 28 DAYS (II)

In vitro testing was carried out to examine whether NFC possess immunomodulatory properties. In these experiments, PMA-differentiated THP-1 cells were stimulated with cellulose materials at three concentrations for 3, 6 and 24 h. One NFC – non-functionalized material 2, but not the other nanocellulose materials, exerted significantly increasing cytotoxicity (II, Figure 2), up-regulated mRNA expression and protein secretion of pro-inflammatory IL-1β (II, Figure 3) and TNF (II, Supplementary figure 1). These data suggest that different types of NFC do not commonly activate immune reactions.

In order to characterize the acute NFC-induced pulmonary effects, mice were exposed to 10 and 40 μg of the materials via OPA and sacrificed after 24 h. Bulk-sized cellulose was tested along with NFC to reveal the size-dependent effects and rCNT was included as an inflammagenic nano-sized control material. All of the cellulose materials and rCNT caused neutrophilic influx into BAL (II, Figure 4a) and like rCNT, materials 1, 2 and 5 induced the migration of eosinophils (II, Figure 4b). Aggregated immune cells were clearly seen clustering around rCNT also in lung tissue (II, Figure 5b) whereas no notable activity of the cells was detected in response to material 1 – i.e. the bulk-sized cellulose (II, Figure 5c). The histopathological results of NFC generally supported BAL cell counts. The presence of neutrophils was detected in the lung tissue after exposure to all NFC except material 3, whereas eosinophils were observed only in case of non-functionalized NFC – materials 2 and 5 (II, Figure 5d-g). All of the materials up-regulated pro-inflammatory IL-6 expression (II, Figure 6b); furthermore treatment with rCNT, materials 2 and 5 additionally enhanced IL-1β, TNF and IL-13 transcription (II, Figure 6a, 6c-d). These data demonstrate that NFC trigger innate immunity reactions and evoke acute inflammation in the lungs.

To investigate whether there would be a resolution or a prolongation of NFC-derived inflammatory effects, mice were oropharyngeally treated with 10 and 40 μg of the materials and sacrificed 28 days post-exposure. In response to rCNT, there was a decrease in the number of macrophages and an increase in the numbers of neutrophils and lymphocytes in BAL, whereas in response to NFC materials, no changes in macrophage numbers were detected nor was there any notable influx of inflammatory cells (data not shown). However, the formation of multinucleated cells was seen in BAL collected after rCNT treatment and FBGC were observed also in mice exposed to materials 2 and 5

(II, Figure 7a-c). NFC agglomerates were surrounded in lung tissue with macrophages and had been infiltrated by these cells (II, Figure 7, Supplementary figure 4); this phenomenon was especially visible for materials 2 and 5 (II, Figure 7e-f, 7h-i). Occasional neutrophils, eosinophils and acidophilic (eosinophilic) macrophages were present in the tissue of mice treated with materials 2 and 5 (II, Figure 7). In contrast, rCNT triggered the formation of foreign-body granulomas and acidophilic macrophages (II, Figure 7d, 7g). These results indicate that the NFC-induced acute inflammation had subsided within 28 days.

To clarify whether the cellulose materials had degraded during 28 days, lung tissue sections collected after 24 h were stained with a cellulose-specific stain and compared to those taken at 28 days after the exposure. None of the tested materials showed a lower number of agglomerates in the lungs or exhibited reduced total area of the material after 28 days compared with 24 h post-exposure (II, Figure 8a-b, Supplementary figure 5a-b). However, the number and total area of the agglomerates varied between the materials, for example materials 2 and 5 were present in the lungs in a higher number of agglomerates as compared with the other materials (II, Figure 8a, Supplementary figure 5a). These data reveal that cellulose materials have poor dissolving properties in the biological environment, and are not degraded by immune cells.

5.3 SURFACE PEGylation SUPPRESSES CuO-INDUCED PULMONARY EFFECTS IN ASTHMATIC AIRWAYS (III)

We characterized how CuO nanomaterials influence a pre-existing lung disease by repeatedly exposing mice to 2.5, 10 and 40 μg of core CuO and its surface carboxylated, methylaminated and PEGylated versions via OPA in a mouse model of asthma (III, Figure S1). Irrespective of the pulmonary condition, all CuO materials evoked a significant neutrophilia in the lungs as the cells were clearly detected in BAL (III, Figure 1A) and tissue (III, Figure 2). Histological findings included the presence of nuclear dust in response to all of the materials (Figure 2C-D). Eosinophil population and goblet cell activation were not notably influenced in OVA-challenged mice whereas the numbers of macrophages and lymphocytes were mostly increased in a dose-dependent manner (III, Figure 1A-B). The influx of CD4+ T cells was increased at 10 μg/mouse after exposure to the materials except to CuO COOH. CuO ENM slightly up-regulated the mRNA expression of pro-inflammatory TNF and pro-allergic IL-33 in PBS- and OVA-challenged mice, and mainly suppressed the production of IL-13 in OVA-challenged mice (III, Figure 1C). These findings indicate that inhaled CuO ENM elicit neutrophilia regardless of the pulmonary condition but the materials do not notably affect the common signs of allergic asthma at the cellular level.

A more in-depth, genome-wide gene expression analysis was performed in an attempt to explore the consequences of exposure to CuO materials. Hierarchical clustering of the most differentially expressed genes and numbers of DEGs in experimental groups suggested that CuO PEG had a lower potential to cause changes at the transcriptional level than the other tested materials (III, Figure 3A-B). ENM-specific experimental groups of PBS-challenged or OVA-challenged mice were then combined and their transcriptomes compared against their respective controls as a way of investigating the general effects of every material. As a result, CuO PEG did not differ from controls and produced no DEGs. DEGs specific to CuO, CuO COOH or CuO NH_3 in PBS- challenged mice predicted suppressed Cell Cycle at G2/M DNA Damage Checkpoint Regulation and Antioxidant Action of Vitamin C pathway, and activation of several innate immunity and pro-inflammatory pathways including Aryl Hydrocarbon Receptor Signaling (least activated by core CuO) and Dendritic Cell Maturation pathways (least activated by CuO NH_3) (III, Figure 4B). Comparison of the biological processes enriched by the DEGs revealed 17 common processes that were related to cytokine/chemokine signaling, cell migration and division (III, Figure 4D). No (in)activated canonical pathways were identified from the ENM-specific DEGs in OVA-challenged mice, however, neutrophil degranulation was a commonly enriched biological process associated with CuO, CuO COOH and CuO NH_3 exposure (III, Figure S4). These findings show that regardless of the differing transcriptomes, CuO, CuO COOH and CuO NH_3 do exhibit similarities at the pathway level.

We explored how surface modifications influence core CuO-derived effects, by comparing every dose of each modified material against the same administered dose of the core CuO. Enrichment analysis revealed that DEGs of CuO COOH and CuO NH_3 groups resulted in zero or few significantly enriched biological processes whereas a large number of biological processes were enriched by the set of DEGs in the treatment groups of CuO PEG (III, Figure 5A). When up- and down-regulated genes of the CuO PEG groups were investigated separately, it was found that the surface PEGylation had suppressed the inflammatory processes and pathways induced by core CuO in both PBS-challenged (III, Figure S5-6, 5B) and OVA-challenged mice (III, Figure S7, 5C).

The effects of CuO materials to modulate AAI were compared in the DEGs of ENM-exposed PBS-challenged mice with those of OVA-challenged mice. Hierarchical clustering of canonical pathways revealed that most of the pathways of AAI mice exposed to lower doses of CuO materials, were unenriched or displayed an activation score of zero. However, in other groups, exposure to CuO materials affected several T cell regulation pathways, suppressed cell cycle in G2/M DNA damage checkpoint and activated other cell cycle-related pathways. The ENM activated many inflammatory pathways whose activation was mostly stronger in PBS-challenged mice than in AAI mice (III, Figure 6A).

It was possible that the differences in the modulatory reactions elicited by exposure to CuO materials could be dependent on the condition of the lung. Therefore, activation scores of the predicted canonical pathways were compared in PBS- and OVA-challenged groups and it was found that a large number of the pathways influenced by CuO, CuO COOH or CuO NH3 exposure had a similar activation direction (III, Figure 6B-C, Figure S8B-C). The DEGs of OVA-challenged mice exposed to CuO PEG did not result in predicted (in)activation of any pathways but several pathways had been activated in the respective PBS-challenged mice (III, Figure S8C). These results suggest that there were no notable differences in the PBS- and OVA-challenged mice in the activation of those pathways affected by CuO exposures.

5.4 ZnO NANOPARTICLES PENETRATE THROUGH THE SKIN, SUPPRESS ALLERGEN-INDUCED CUTANEOUS INFLAMMATION AND TRIGGER SYSTEMIC IgE PRODUCTION (IV)

It was decided to study whether nZnO would be able to penetrate skin with a reduced barrier function. Therefore, a murine model of AD (IV, Additional file 2) was used in which a standardized skin injury was generated by tape stripping. bZnO was tested as a size control. An examination of skin sections from the exposed area revealed agglomerates of both materials on the surface of the skin. The presence of nZnO particles was observed in epidermal and dermal skin layers of both PBS-treated and OVA/SEB-challenged mice but particle accumulation was clearly greater in the lesional skin of AD mice, especially in epidermis (IV, Figure 1). In contrast, bZnO was found only on the skin surface and there was no particle penetration into the skin. These data show that nano-sized particles can pass through injured skin and furthermore, the degree of penetration is considerably greater in allergic skin.

Possible changes in features common to the skin condition were examined in order to characterize the local effects of ZnO particles on AD. It was found that nZnO significantly reduced epidermal and dermal skin thickness as well as the total number of inflammatory cells, neutrophils and eosinophils in OVA/SEB-challenged mice (IV, Figure 2A-D). In addition, the numbers of CD3+, DC4+ and CD8+ T cells were reduced after nZnO treatment in AD-like skin (IV, Figure 3A-C). bZnO had a less pronounced effect on these end points except for the eosinophils; the population of these cells increased in response to bZnO. Furthermore, mRNA expression of the pro-inflammatory compounds, IL-1β, IL-6 and TNF, the anti-inflammatory agent, IL-10, the pro-allergic mediators, IL-33, IL-4 and IL-13 and the Th1-type IFN-γ cytokines was down-regulated in OVA/SEB-challenged skin (IV, Figure 4). The production of pro-inflammatory but not of the other cytokines was inhibited also in response to bZnO. A reduction in IL-1β and TNF expression was observed also in PBS-treated mice after ZnO administration. These findings suggest that

local allergen-induced skin inflammation is efficiently suppressed by nZnO whereas bZnO inhibits the inflammation partly by down-regulating pro-inflammatory but not the Th2 type reactions.

Cells from skin draining lymph nodes were collected and treated with OVA in an attempt to determine how ZnO particles could influence allergen-induced systemic effects. A slight, non-significantly reduced secretion of IL-13 as well as IFN-γ was measured upon OVA stimulation in lymphocytes from OVA/SEB-challenged mice treated with nZnO (IV, Figure 5). These results indicate that cutaneous nZnO administration had suppressed systemic T cell reactivity as was evident after their re-exposure to the allergen.

Systemic effects were also evaluated by measuring total and allergen-specific antibody production in serum. ZnO materials were found to elevate the production of total and OVA-specific IgE in OVA/SEB-challenged mice which was greater for nZnO than for bZnO (IV, Figure 6A-B). In contrast, the secretion of OVA-specific IgG1 levels was inhibited significantly by nZnO (IV, Figure 6C). These data reveal that topical application of ZnO particles on an AD-like skin exacerbates the systemic production of the Th2 type IgE antibody.

6 DISCUSSION

Nanotechnology is a fascinating field focusing on the development of various ENM and their incorporation into products that are aimed at enriching and improving our lives. As the production of ENM has increased, our contacts with these materials have also become more common. The most relevant exposure routes of ENM in occupational and consumer scenarios are inhalation and via the skin. Due to the altered and generally improved properties of ENM compared with their larger-sized counterparts, these materials may pose a risk to our health. Although our body has protective mechanisms against foreign invaders, because of their small size, ENM might bypass these systems and come into contact with our immune cells. The role of these cells is to remove the foreign particles, however, the unique properties of ENM confer on them an ability to manipulate the immune system and in some instances, to trigger adverse health effects.

To date, extensive progress has been made in understanding the mechanisms behind ENM toxicity but many knowledge gaps still exist in the field. In this thesis, the pro-allergic potential of inhaled rCNT and tCNT was investigated in healthy mice. In addition, as limited information exists about pulmonary effects of fibrous nanocellulose materials, the ability of NFC to elicit immunomodulatory reactions was studied *in vitro* as well as *in vivo* one day after exposure with the effects being followed up at day 28.

Due to the reduced functionality of the immune system in patients suffering from chronic diseases, exposure to ENM might have stronger adverse impacts on the health of these vulnerable people. Thus, the pulmonary effects of pristine and surface-modified CuO ENM were explored in a murine model of asthma to clarify how the materials and their surface functionalization modulate the symptoms. In addition, dermal effects of nZnO were studied in a mouse model of AD to test the hypothesis that a reduced integrity of the skin barrier would allow particle penetration and furthermore, once penetrated, the ENM would exhibit immunomodulatory reactions.

6.1 CNT EFFECTS DEPEND ON PARTICLE FORM (I)

MWCNT are long, thin and fiber-like structures with a nano-sized diameter. In addition, the CNT are resistant to an acidic environment and high temperatures (Donaldson *et al.*, 2006, Aschberger *et al.*, 2010). Due to their shape and persistency, MWCNT have been compared to asbestos fibers and it is feared that they might cause similar pathologies. According to the classical fiber paradigm, structures longer than ~15 um and thinner than 3 um cause frustrated phagocytosis which leads to toxicity (Poland *et al.*, 2008, Donaldson *et al.*, 2013).

CNT exist in different forms – in addition to varying lengths, the fibers may also be straight and rigid, or tangled. In particular, there is evidence that when administered via intraperitoneal injection, long and rigid MWCNT can induce inflammation and the formation of granulomas in the mesothelial lining of mice (Poland *et al.*, 2008). In publication I, long, rigid and long, tangled MWCNT were administered to mice by aerosol inhalation to test the shape-dependent toxicity hypothesis in a set-up that mimicked a real-life exposure scenario. Both cellular and histological features as well as cytokine expression in lungs were significantly altered after rCNT but not after tCNT exposure. At the transcriptional level, rCNT up-regulated several inflammatory pathways whereas in response to tCNT, some of these pathways were inactivated or even suppressed.

The inability of tCNT to trigger inflammation could be explained by successful phagocytosis of the particles. Due to their bundle-like shape, macrophages can enclose the phagolysosome containing the particles and clear the material from the extracellular space (Donaldson et al 2010). Instead, rCNT cause frustrated phagocytosis, cellular damage and ROS-mediated initiation of inflammation as shown *in vitro* (Palomäki et al. 2010). These data highlight that the form of ENM fibers exerts a substantial impact on their biological activity.

6.2 rCNT CAUSE Th2 TYPE REACTIONS AND A CONDITION SIMILAR TO AAI IN HEALTHY SUBJECTS (I)

Allergic asthma is a chronic airway disease involving airway hyperreactivity and excessive mucus production. The cellular and molecular mediators of asthma include mast cells, eosinophils, Th2 type lymphocytes and cytokines (Galli *et al.*, 2008). CNT have been reported to possess adjuvant properties as exposure to the fibers has been claimed to exacerbate AAI (Inoue *et al.*, 2009, Nygaard *et al.*, 2009, Nygaard *et al.*, 2013, Ronzani *et al.*, 2014). The objective in publication I was to characterize the pulmonary effects of MWCNT in healthy mice without a pre-existing allergen-induced lung disease. It was found that inhaled rCNT triggered characteristics similar to AAI in the murine lungs i.e. the pulmonary milieu induced by the material exhibited eosinophilia, AHR to inhaled methacholine, mucus production, the presence of Th2 type cytokines and eosinophil-specific chemokines. Furthermore, it was observed that mast cells partially regulated the allergy-like inflammatory responses.

Inhalation of aerosolized rCNT caused a reduction in the macrophage population. Furthermore, macrophages undergoing frustrated phagocytosis and the formation of FBGC were observed as described earlier (Poland *et al.*, 2008). FBGC are suggested to be a result of macrophage fusion that takes place due to frustrated phagocytosis. This kind of fusion could be considered as a combined phagocytic force being exerted by individually ineffective

macrophages. It can be also thought of as an isolation mechanism to protect host tissues from continuing injury (McNally and Anderson, 2011). However, multinucleation of cells is recognized as a pathological feature in various diseases such as granulomatous diseases (Helming and Gordon, 2009).

The fusion of phagocytes has been linked to alternatively activated macrophages (AAM), also known as M2 cells, that merge in the presence of IL-4 and IL-13 (Helming and Gordon, 2007, Martinez *et al.*, 2009). Macrophage polarization towards the M2 type can be induced by IL-13 and IL-33. M2 macrophages themselves express IL-13 and several cytokines including CCL17 and CCL24. These signaling molecules mediate both Th2 cell and eosinophil migration – thus, AAM have been suggested to play a role in allergic inflammation (Jiang and Zhu, 2016). The rCNT used in publication I have been shown to change macrophage polarization dynamically during the course of inflammation – the M1 population has been demonstrated to be most evident on day one post-exposure whereas M2 polarization peaks on day 3 and remains elevated until day 7 (Dong and Ma, 2018). rCNT-induced M2 polarization could be a link between frustrated phagocytosis and FBGC formation observed in publication I.

The exploration of early rCNT-induced inflammation by transcriptome analysis revealed a rapid activation of innate immunity pathways which lead to the investigation on how macrophages and mast cells contribute to the development of the allergic consequences. Consequently, it was demonstrated that macrophages solely produce IL-1β, TNF and partially IL-33 and CCL17 whereas mast cells are responsible for providing IL-4 and the majority of IL-13. The data also revealed that some part of IL-33, IL-13 and CCL7 is derived from other cells types in the lung tissue. For example, there is evidence that MWCNT-induced pulmonary effects are regulated by IL-33, likely originating from epithelial cells, followed by the subsequent production of IL-13 by the IL-33-activated ILC2 cells (Beamer *et al.*, 2013).

The development of classical asthma usually involves a sensitization phase during which the first contact with a protein allergen takes place. This leads to the differentiation of allergen-specific Th2 cells and the production of IgE antibodies specific for the allergen (Galli *et al.*, 2008). In the case of rCNT-triggered inflammation which was evident within 5 days, the traditional type of sensitization could not have developed because the time frame was too short and there was also the absence of a conventional allergen. Instead, inhaled rCNT caused an unconventional AAI that took place via innate immunity reactions.

6.3 NFC MATERIALS INDUCE ACUTE PULMONARY INFLAMMATION THAT SUBSIDES WITHIN A MONTH (II)

NFC, a subgroup of nanocelluloses, is characteristically a high aspect ratio nanomaterial with great mechanical strength and good film-forming properties. NFC could be used by the paper and food industry for improving paper quality and for producing bio-based packaging material with better oxygen barrier properties (Nair *et al.*, 2014).

The ENM shape and bioreactivity relationship paradigm for long and thin fibers has gained more support as a high aspect ratio is considered to be one of the major features associated with adverse health outcomes (Fadeel *et al.*, 2012). Thus, NFC might potentially affect the immune system, however, to date, very few studies have focused on investigating the effects of NFC *in vitro* and *in vivo* (Kollar *et al.*, 2011, Pereira *et al.*, 2013, Catalan *et al.*, 2017, Lopes *et al.*, 2017, Park *et al.*, 2018). Pulmonary effects of NFC have been explored 1 and 14 days post-administration but nothing is known about their consequences beyond 2 weeks (Catalan *et al.*, 2017, Park *et al.*, 2018).

An earlier study reported that NFC caused macrophage migration into the airways and DNA damage in lung tissue at doses of 10 and 40 μg/mouse which are the same doses as used in publication II. The authors speculated that the DNA damage could have been a result of inflammation-derived ROS that had been generated after NFC phagocytosis and may have contributed to the recruitment of other inflammatory cells – eosinophils and neutrophils which were seen at higher doses (Catalan *et al.*, 2017). Similarly, exposure to the NFC materials used in publication II resulted after 1 day in acute inflammation which was characterized by neutrophil influx with increased IL-6 expression, however, it took place already at doses 10 and 40 μg/mouse. Furthermore, non-modified NFC (materials 2 and 5) additionally recruited eosinophils into the lungs and up-regulated the transcriptions of IL-1β, TNF and IL-13. These data indicate that NFC have an ability to initiate innate immunity reactions and to trigger acute inflammation in the lungs.

To date, the only study focusing on sub-acute effects of NFC found that 14 days after their exposure, the NFC-triggered local pulmonary environment exhibited a Th1-type phenotype. There was evidence that NFC had elevated the number of polymorphonuclear cells in BAL at a dose of 40 μg/mouse but not those of mononuclear phagocytes or lymphocytes. The cytokine milieu contained up-regulated pro-inflammatory IL-1β, pro-Th1 type IL-12p40, neutrophil-attracting KC and MIP-1α, and monocyte-attracting MCP-1 (CCL2) (Park *et al.*, 2018). At 28 days, none of the NFC caused notable changes in the influx of inflammatory cells into the airways (II). FBGC were found in low numbers in the BAL of mice exposed to non-modified NFC and few acidophilic macrophages were detected in the tissues of these mice with up-regulated IL-13 levels. These kinds of features have been described previously as residues of Th2 type reactions (Rydman *et al.*, 2015).

In summary, cytological, histological and cytokine expression data indicate that non-functionalized NFCs have a higher inflammatory potential than NFC with anionic groups. However, the overall results suggest that NFC have low or no long-term toxicity as the acute inflammatory reactions had subsided in 28 days.

6.4 *IN VITRO* AND *IN VIVO* COMPARISON (II)

With regard to respiratory tract exposure, primary and cell line macrophages are often used *in vitro* for mimicking early innate immunity reactions in alveolar macrophages upon contact with ENM. These models can provide insights into whether the materials could pose a health risk *in vivo*. In publication II, NFC effects were screened *in vitro* in PMA-differentiated THP-1 cells that resemble macrophages and *in vivo* in C57BL/6 mice. The *in vitro* model demonstrated that one of the non-modified NFC (material 2) triggered pro-inflammatory IL-1β and TNF production whereas the *in vivo* model revealed IL-1β, TNF and IL-13 expression in response to both non-modified NFCs (materials 2 and 5), and commonly up-regulated transcription of inflammation-promoting cytokine IL-6 with neutrophil recruitment by all tested NFC materials (materials 2-5). These results indicate that the models are not fully in concordance as the *in vivo* model suggested that more materials possess an inflammatory potential than the *in vitro* model.

Hypotheses of ENM-triggered effects in macrophage *in vitro* models are based on an assumption that the particles reach the alveolar area of the lungs. However, ENM are known to deposit fractionally in different areas of respiratory tract which to a certain extent, results in their interaction with epithelial cells (Oberdörster *et al.*, 2005). This applied also to the NFC because the presence of their agglomerates was evident in the airways as well as in the alveolar space (II, Figure 8). Thus, the sensitivity difference between the *in vitro* and *in vivo* model is likely derived from recognition of ENM by more cell types in whole lungs as compared with macrophages which are the only cells present in the *in vitro* model.

Concurrently with macrophages, the early inflammatory reactions can be initiated by epithelial cells and mast cells. The cytokine profile of epithelial cells includes the production of IL-1β, TNF and IL-6 (Mills *et al.*, 1999), whereas mast cells can produce IL-13 during innate immunity response as demonstrated in the response to rCNT in publication I. In addition, it has been found that the ENM-induced damage to the barrier integrity of epithelial cells triggers IL-33 production which leads to ILC2-derived IL-13 secretion (Katwa *et al.*, 2012, Beamer *et al.*, 2013).

Altogether, when taking the behavior of NFC in the lungs *in vivo* into account, the material testing performed on macrophages *in vitro* resulted in a partial characterization of the immunomodulatory potential of the materials. With regard to the cells present at the air-bio interface, assessing the ENM

deposition by mathematical tools (Miller *et al.*, 2016) or based on published data, could indicate which cell types would provide the most accurate information for predicting *in vivo* toxicity. If deposition in more than one compartment takes place, co-culture models could be used to simulate the initial reactions more precisely.

6.5 SIMILARITIES AND DIFFERENCES BETWEEN EFFECTS OF CNT AND NFC (II)

Respiratory exposure to rCNT via OPA has been compared with asbestos earlier in a similar set-up as used in publication II (Rydman *et al.*, 2015). It was shown that rCNT induced a stronger acute inflammation than asbestos, thus, it was used as a positive ENM control in publication II.

rCNT-triggered effects have been shown to induce acute neutrophilia that changes into a Th2 type inflammation by day 7 with traces of this still being seen at 28 days (Rydman *et al.*, 2015). One day post-administration, rCNT elicited neutrophil influx also in publication II which was accompanied by the presence of eosinophils in BAL and lung tissue, as well as up-regulation of the expression of IL-1β, IL-6, TNF and IL-13. After 28 days, FBGC were found in the BAL of rCNT-exposed mice, the lung tissue exhibited elevated levels of T cell subtypes, acidophilic macrophages and foreign-body granuloma formation around the fibers. In addition, up-regulated transcription of TNF, IL-13, and TGF-β was observed.

Non-modified (materials 2 and 5) but not anionic NFC (materials 3 and 4) triggered Th2 type pulmonary reactions, including the mild recruitment of eosinophils in the early phase with IL-13 transcription at 1 and 28 days after the exposure, with a low number of FBGC in BAL and acidophilic macrophages in lung tissue. Charcot-Layden crystals within acidophilic macrophages are derived from eosinophils and have been linked to eosinophilic diseases (Janin *et al.*, 1993, Lao *et al.*, 1998, Guo *et al.*, 2000). In the study of Rydman et al, rCNT-induced eosinophilia was shown to peak at day 7 (Rydman *et al.*, 2015) and acidophilic macrophages began to appear in the tissue on day 14. After exposure to non-modified NFC (materials 2 and 5), minor indications of Th2 type features were evident at days one and 28 days along with the presence of a low number of acidophilic macrophages. Thus, these materials were proved to have pro-allergic properties like rCNT, however at a significantly lower magnitude as no chronic inflammation was observed on day 28.

The finding that all NFC (materials 2-5) were still present after 28 days in comparable amounts as one day after the exposure, demonstrated that the materials are poorly biodegradable and dissolvable. It is known that humans lack cellulolytic enzymes and thus are not able to degrade cellulose. However, the finding that NFC do not cause chronic inflammation is unusual since biopersistent materials are generally expected to cause long-term adverse effects, for example rCNT-induced foreign-body granulomas. Histologically,

only adhering macrophages around the bundles of NFC (materials 2-5) were seen and in the case of non-modified NFC (materials 2 and 5), few FBGC were detected in BAL (II). Early studies on the effects of bulk-sized cellulose have concluded that the material is generally biocompatible, eliciting only minor, if any, foreign body responses (Miyamoto *et al.*, 1989, Martson *et al.*, 1999). One could speculate that NFC is handled by macrophages similarly as has been postulated for tangled CNT (Donaldson *et al.*, 2010). NFC has low rigidity due to their changing crystalline and amorphous parts (Tomic *et al.*, 2016) and appears in lung tissue mostly in the form of gel-like agglomerates that are larger than one phagocyte. Therefore many macrophages have to adhere to the surface of NFC agglomerates to isolate them from the tissue without the need for promoting inflammation.

6.6 CuO MATERIALS CAUSE NEUTROPHILIA IN HEALTHY AND ALLERGEN-CHALLENGED LUNGS (III)

Extensive information already exists about the toxicity of CuO ENM. CuO has been shown to induce cytotoxicity in different cell types, which is often categorized as apoptosis, occurring with genotoxic effects and ROS generation (Wang *et al.*, 2012, Siddiqui *et al.*, 2013, Karlsson *et al.*, 2014, Semisch *et al.*, 2014, Thit *et al.*, 2015). CuO ENM toxicity has been described to lie in the lysosomal dissolution of the particles and formation of Cu2+ ions, a mechanism defined as the Trojan horse effect (Studer *et al.*, 2010).

The effects of core CuO and its surface carboxylated, methylaminated and PEGylated derivates were explored in a murine model of asthma in publication III. Regardless of the pulmonary status, all CuO nanomaterials caused neutrophilia in the BAL and lung tissue with the presence of nuclear dust in the latter and a mild up-regulated expression of TNF and IL-33. In mice with AAI, the materials did not cause any notable changes in eosinophil numbers and mucin-producing goblet cell activation whereas macrophage and lymphocyte populations had increased and the transcription of IL-13 was mainly suppressed. Furthermore, CD4+ T cell levels were elevated at 10 μg/mouse in response to all of the CuO ENM other than CuO COOH. These data indicate that the materials do not modulate AAI, however, ENM worsen it via the induction of innate immunity reactions. CuO has been found to aggravate asthma in a previous study in which signs of AAI were also influenced – cellular influx into the lungs, AHR, IgE and mucus production were increased along with CuO-triggered ROS generation (Park *et al.*, 2016).

When the bioreactivity of CuO ENM was compared generally at the transcriptome level, CuO, CuO COOH or CuO NH3 in healthy lungs were predicted to suppress Cell Cycle at G2/M DNA Damage Checkpoint Regulation and Antioxidant Action of Vitamin C pathway. CuO materials are known to produce ROS that cause mitochondrial damage via activation of the

mitochondrial-mediated apoptosis pathway, stimulation of p53 and caspase 3, which results in apoptotic death (Siddiqui *et al.*, 2013, Chibber and Shanker, 2017). MeO ENM have also been reported to suppress the cell cycle at G2/M DNA Damage Checkpoint which would lead to cell death (Gao *et al.*, 2016, Yuan *et al.*, 2016). Moreover, suppression of Antioxidant Action of Vitamin C may further promote CuO-induced ROS reactions in the cell. In addition, the materials activated several innate immunity and pro-inflammatory pathways including Aryl Hydrocarbon Receptor Signaling and Dendritic Cell Maturation pathways that have been associated with Th17- and IL-17A-dependent immunity and pulmonary neutrophilia in response to particulate matter (Jaligama *et al.*, 2018). Furthermore, all three materials commonly enriched cytokines/chemokine biological processes that are related to neutrophil trafficking, supporting the cytological and histological findings (III). Although no (in)activated pathways were identified in mice with AAI, neutrophil degranulation stood out as a commonly enriched biological process for these three materials. In contrast, the transcriptomes of CuO PEG groups did not differ significantly from control groups.

Comparison of pathway (in)activation after exposure to CuO nanomaterials between healthy mice and mice with AAI revealed that many pathways in the AAI mice exposed to lower doses of CuO ENM, were unenriched or without an activation score. In other groups, exposure to CuO nanomaterials affected several T cell regulation pathways, suppressed cell cycle in G2/M DNA damage checkpoint, and activated other cell cycle-related pathways as well as many inflammatory pathways. Pathway activation was mostly stronger in PBS-challenged mice than in AAI mice which is most likely due to pre-existing inflammation in the control mice with AAI.

Altogether, these findings show that inhaled CuO ENM elicited innate immunity reactions irrespectively of the pulmonary condition without notably affecting the common signs of allergic asthma at the cellular level. Furthermore, regardless of differing transcriptomes, CuO, CuO COOH and CuO NH3 displayed similarities at the pathway level whereas CuO PEG resembled the controls. These data indicate that PEGylated CuO has a lower potential to trigger adverse effects.

6.7 SURFACE PEGylation SUPPRESSES THE EFFECTS OF PRISTINE CuO (III)

It has been postulated that the surface charge of the ENM affects their uptake and thereby also their inflammatory potential (Nel *et al.*, 2009). In publication III, CuO, CuO COOH, CuO NH3 and CuO PEG were tested to determine whether and how they influence the effects of the core material. CuO COOH represented a negatively-charged, CuO NH3 a positively-charged and CuO PEG a neutral ENM. Based on the cytological analysis of BAL samples, CuO NH3 induced stronger neutrophilia than other materials whereas the

neutrophil influx triggered by CuO COOH was lower as compared with that evoked by CuO. A positive surface charge has been shown to enhance ENM uptake and cationic ENM exhibit a higher engulfment rate than anionic particles (Kenzaoui *et al.*, 2012) which results in higher cytotoxicity than that triggered by anionic or unmodified particles (Ruenraroengsak and Tetley, 2015). The increased uptake of cationic particles is attributable to the negatively-charged nature of the cell plasma membrane that interacts with positive functional groups of cationic ENM (Panariti *et al.*, 2012).

To explore in greater depth the consequences of exposure to CuO materials, a genome-wide gene expression analysis was performed and it was found that CuO PEG had a considerably lower ability than the other CuO materials to induce immunomodulatory changes at the transcriptional level. To unravel how the responses of the materials differ from CuO, transcriptomes of CuO PEG groups were compared against the same administered dose of core CuO. It was observed that large numbers of biological processes were enriched in the DEG sets. Up- and down-regulated genes of CuO PEG groups were then investigated separately which revealed that surface PEGylation suppressed inflammatory processes and pathways induced by core CuO in both healthy mice and mice with AAI. It has been described earlier that the polymer coating can diminish the attractive forces of the pristine material which promote contact with the cell membrane. The PEG coating creates a micelle around the core particle that diminishes the engulfment by phagocytes due to steric hindrance. Thus, PEGylation can be a promising approach in drug delivery for prolonging the circulation time of a medication (Nel *et al.*, 2009, Kendall and Holgate, 2012). However, such prevented uptake by phagocytes could explain the attenuated immune response that was seen in response to PEGylated CuO in publication III.

In summary, it was found that surface PEGylation inhibited the inflammatory reactions caused by the more bioactive core ENM. Based on the previous knowledge, this phenomenon occurred possibly due to hindered uptake of PEGylated particles by phagocytes.

6.8 ZnO NANOPARTICLES BUT NOT THEIR BULK-SIZED COUNTERPARTS PENETRATE MURINE AD-LIKE SKIN (IV)

MeO such as ZnO and TiO2 have been used as UV filters in sunscreens for decades. With the emergence of nanotechnology, these materials acquired superior UV blocking properties when their size was reduced to the nanoscale. Furthermore, the appearance of sunscreens improved as the nano-sized MeO-containing products left a transparent film on the skin as compared with the visible white layer of the older generation sunscreens (Newman *et al.*, 2009). Today, these kinds of modern sunblockers are on the market and more and

more people are being dermally exposed to these nano-sized inorganic nanomaterials.

Exposure of a healthy and intact skin to ENM is considered safe as *stratum corneum* provides an effective barrier against percutaneous absorption of the particles (Krug and Wick, 2011). Theoretically, skin could be penetrated by nanoparticles via paracellular (intercellular), transcellular (through the cells) and transappendageal route that involves entering via hair follicules, sweat pores or sebaceous glands (Smijs and Pavel, 2011). ENM dermal penetration has been studied on various skin models *in vitro* as well as *in vivo* but the results have been controversial (Wu *et al.*, 2009, Adachi *et al.*, 2010, Gulson *et al.*, 2010, Sadrieh *et al.*, 2010, Senzui *et al.*, 2010, Lin *et al.*, 2011, Monteiro-Riviere *et al.*, 2011, Holmes *et al.*, 2016). The discrepancies in the findings may be caused by several factors. First, these experiments have been carried out on murine, porcine or human skin whose thicknesses vary and thus, also penetration can be affected. Even in humans, skin thickness varies in different areas of the body, and depends on age, gender, skin type and its condition (Smijs and Pavel, 2011). Second, *in vitro* models lack the natural skin mobility and due to the absence of flexing, the transdermal transport may be reduced. Third, the physicochemical properties of the ENM, their doses, formulation and exposure schedules vary.

Dermal exposure of UV-injured or diseased skin has been less extensively examined and there is no clear consensus in the data (Senzui *et al.*, 2010, Lin *et al.*, 2011, Monteiro-Riviere *et al.*, 2011, Kim *et al.*, 2016, Pal *et al.*, 2016). In publication IV, the penetration of nano-sized and bulk-sized ZnO was investigated *in vivo* on mechanically injured skin with or without allergen/superantigen-induced AD. While bZnO was observed only on the skin surface, nZnO particles were found in the epidermis and dermis of both PBS-treated and OVA/SEB-challenged mice. Furthermore, particle accumulation was greater in lesional skin of AD mice, especially in the epidermal layers. These data demonstrate that nano-sized ENM are able to enter injured skin, especially allergic skin where their penetration was more extensive.

6.9 nZnO CAUSES SUPPRESSIVE LOCAL BUT AGGRAVATED SYSTEMIC EFFECTS ON AD (IV)

The nanotoxicology field in terms of skin exposure to ENM is currently focused on strategizing the testing of dermal absorption (Gulson *et al.*, 2015). Although it is generally accepted that ENM may pose a health hazard when the skin barrier is diminished (Oberdörster *et al.*, 2005, Lademann *et al.*, 2011), there is little information of the biological effects of ENM on injured skin.

AD is a chronic inflammatory skin disorder characterized by intensely itching eczematous lesions that consequently significantly impact on the patient's life quality. AD is a Th2-polarized disease with elevated serum IgE

levels. Cell constituents in the dermis of acute skin lesions include increased CD4+ and CD8+ T cells, Langerhans cells and eosinophils (Tokura, 2010).

In publication IV, the immunomodulatory effects of nZnO were investigated in a murine model of atopic dermatitis. It was found that nZnO significantly reduced the thickness of skin layers, and decreased the numbers of inflammatory cells including neutrophils, eosinophils and T cell subtypes, as well as inhibiting the expression of several cytokines such as IL-1β, IL-6, TNF, IL-10, IL-33, IL-4, IL-13 and IFN-γ, in AD-like mice. In contrast, bZnO inhibited pro-inflammatory but not Th2 type reactions and thus only partially reduced the allergic skin inflammation. Systemically, nZnO administration suppressed T cell reactivity as after re-exposure of lymph node cells to OVA, there was mildly reduced Th1 and Th2 type cytokine secretion. However, the material exacerbated the systemic production of the Th2 type IgE antibody (IV). The local suppressive effects of topically applied nZnO contrast with the exacerbated reactions observed after intradermal administration of TiO2 nanoparticles in allergic skin, whereas both materials commonly had systemic adjuvant activity (Yanagisawa *et al.*, 2009). In summary, these data show that topically applied MeO materials have an ability to trigger immunomodulatory effects when penetrating through murine skin.

6.10 CHALLENGES AND METHODOLOGICAL CONSIDERATIONS OF ASSESSING THE HEALTH EFFECTS OF ENM

One of the first steps to be taken when designing a nanotoxicological study is the selection of which ENM will be tested. Due to a large number of existing nanomaterials, it has been recommended that the choice should be strategized and rationalized (Nel *et al.*, 2006). In this thesis, CNT and MeO ENM were given a preference due to their high global production volumes and wide use in commercial products whereas NFC were selected because of the Finnish paper and pulp industry's high interest in their generation. In addition, a nanotoxicological study should take into account real-life exposure scenarios of the chosen ENM as these will determine which route is relevant for testing. In terms of exposure in the occupational environment, the most likely route of entry would be the respiratory system whereas dermal contact is more relevant for materials used for example in skin care products. These aspects together with specific scientific questions form the basis for the selection of experimental model, doses, administration technique, exposure duration, time and end points and analysis methods. Here, however, nanotoxicologists face several fundamental and methodological difficulties.

The choice between *in vitro* and *in vivo* models is dependent on the hypothesis of the study. *In vitro* models can be used as a screening tool or for providing mechanistic insights into the toxicity of the ENM being evaluated. Single cell type models reveal responses specific to that cell type but they

cannot reveal intercellular effects such as the cross-talk between inflammatory cells. Co-culture models have been developed in attempts to overcome this limitation; in these systems, usually two cell types are included. *In vivo* models provide comprehensive as well as mechanistic information about local and systemic effects as well as data about the biodistribution of the particles. Furthermore, *in vitro* models are generally restricted in time, thus they are suitable for investigating for example early effects of ENM interaction with the cells, whereas *in vivo* models can be utilized also for long-term studies to examine chronic effects and the biopersistence of ENM. It has been recommended that when investigating mechanisms behind the ENM-triggered effects, the optimal approach is to perform *in vitro* tests integrated with *in vivo* models (Zhao and Liu, 2012). In this thesis, *in vivo* models were chosen because of the complexity of the hypotheses. However, differentiated THP-1 cells were used to supplement the *in vivo* data in publication II.

One of the most challenging aspects in designing experiments that focus on investigating health effects of ENM is choosing the dose – the amount of material determining toxicity. Dose is a result of material concentration and exposure duration, for example, regarding respiratory exposure, it is recommended that the administered ENM amounts should reflect real-life occupational exposure levels (Seaton *et al.*, 2010). However, due to the paucity of experimental data in this area as well as the fact that there are no established regulatory occupational exposure limits (OEL), the decision is complicated. In addition, technical aspects inherent in the experimental system may further challenge the dose selection.

The administered doses in publication I were not precisely known due to the use of a whole-body inhalation method but the aerosol concentrations were an estimation of workplace exposure levels. At the time the experiments were being performed, airborne CNT levels were reported to be around ~1 mg/m^3 (Dahm *et al.*, 2012, Erdely *et al.*, 2013). In the light of this information, the rCNT and tCNT concentrations used in publication I may seem excessive, however, one can justify their use when considering post-production processes during which the exposure levels are likely to be higher.

Since the immunomodulatory potential of NFC was compared with rCNT in publication II, the earlier dose estimations made for CNT exposure were used when designing the study (Porter *et al.*, 2010). Similar nanocellulose doses were however tested also by Shvedova et al who applied OSHA's 8-h time-weighted average permissible exposure limit for the respirable fraction of cellulose (5 mg/m^3) when estimating the ENM amount (Shatkin and Oberdorster, 2016, Shvedova *et al.*, 2016, Park *et al.*, 2018). These calculations can be indirectly applied also to the NFC doses in publication II. As a result, the lower dose of 10 µg/mouse approximates to a reasonable human occupational exposure whereas the higher dose of 40 µg/mouse should be considered as experimental e.g. necessary for determining the relationship between dose and response.

In publication III, the daily administered and cumulative CuO doses were adjusted to those used earlier for CNT, and NFC exposures (Rydman *et al.*, 2015). The rationale for such an approach was to enable an interstudy comparison. Although several other experimental factors vary between these studies, an approximation can be made in terms of the potential of these ENM to induce immunomodulatory responses.

With regard to the dermal exposure, the concentrations of bulk-sized ZnO and TiO2 that are allowed to be used in sunscreen products were used as a basis for choosing the dose (U.S. Food and Drug Administration, 1999). At the time when the study was performed, nano-sized ZnO was not yet allowed to be incorporated into the sun lotions sold in the EU. However, in 2016, the European Commission approved their use in sunscreens at the same concentration as its bulk-sized counterpart – a maximum of 25% (The European Commission, 2016).

The decision on exposure duration can support dose selection in toxicity testing. When considering the fact that workers with industrial diseases have been generally exposed to high concentrations of materials over several years, this could be translated into a repeated exposure *in vivo*, preferably by inhalation of ENM aerosol (Seaton *et al.*, 2010). This kind of approach was used in publication I where mice were exposed for 4 h/day on 4 consecutive days in a whole-body inhalation system. The chosen schedule does not reflect years-long exposure but one may compare it to an inhalation exposure lasting for one working week. However, inhalation experiments have their limitations – they require large amounts of ENM and are time-consuming and labour-demanding. As alternatives, intratracheal instillation and oropharyngeal aspiration techniques have been used; in these approaches, the dose is well-defined and the experiments are less time-consuming and not as labor-intensive. Since oropharyngeal aspiration is less invasive than intratracheal instillation, it was chosen to be used in publications II and III. It has been debated that the bolus dose administered during the aspiration does not apply to the real-life scenario as it has been introduced all at once and therefore cannot reflect exposures occurring over days (Shatkin and Oberdorster, 2016). Thus, in publication III, repeated exposure by oropharyngeal aspiration was used. In fact, a recent study demonstrated that this aspiration technique results in a similar ENM-derived pulmonary inflammation as the inhalation method and can be used as a valid substitute for the latter (Kinaret *et al.*, 2017). The rationale for the dermal exposure schedule was derived from a study performed on human volunteers where repeated application of the materials was chosen to mimic the real-life use of sunscreens (Gulson *et al.*, 2010). In addition to exposure length, also the total duration of an experiment has a relevant role in a study. In this thesis, different time points were selected to cover acute as well as long-term effects of ENM.

A thorough physicochemical characterization of the ENM and their behavior in exposure including size distribution, dissolution and protein binding has been emphasized as one of the key elements of toxicity screening

(Nel *et al.*, 2006, Savolainen *et al.*, 2010, Landsiedel *et al.*, 2012). Various characterization methods were used in this thesis to meet these requirements, however, some shortcomings, such as size distribution and dissolution rate of the ENM in exposure media, protein adhesion, stability of surface modifications and cellular uptake, still remained in one or another publication. Agglomeration, dissolution and protein adhesion onto the particles have been recognized to influence the cellular uptake and alter the particles' distribution in the tissue (Landsiedel *et al.*, 2012). To minimize these processes taking place in ENM suspensions before the exposures, well-established dispersion protocols were applied and the suspensions were administered shortly after their preparation.

Since the ENM distribution influences the responses of the immune system as well as the cellular and histopathological changes, the detection of the material after the exposure provides valuable information for sample analysis and interpretation of the results. However, this task can be technically challenging due to the physicochemical characteristics of the ENM. In this thesis, different approaches were used for locating the tested ENM. CNT materials that are characteristically black, were easy to detect in stained histological samples, especially in such with a light background color. Thus, PSR staining proved to be the best approach for observing CNT localization in the lungs (I). In contrast, histological stainings dyed the NFC materials and thus, they were not as conveniently observed as CNT (II). Therefore, a cellulose specific staining was used to enhance their presence in the tissue. ZnO and CuO materials were undetectable in stained tissue sections (III, IV). In publication IV, a novel microscopy method - hyperspectral imaging technology, was used for detecting ZnO particles in unstained skin sections. Even though the distribution of CuO materials was not determined in publication III, an indirect evaluation could have been made. For example, due to the nature of certain localized histological features, i.e. neutrophil influx and nuclear dust, it could be assumed that CuO particles had deposited in these areas. However, to confirm this uncertainty, hyperspectral imaging or TEM analysis could be performed.

The selection of end points to be analysed is an important part of toxicological testing. In this thesis, a variety of end points was chosen to provide a comprehensive overview of the ENM-derived immunomodulatory effects. Different traditional and novel molecular, cellular and histological analytical methods, which were complementary to each other, were carried out.

Taken together, the success of a nanotoxicological study is challenging due to various factors. To overcome the current uncertainties and even unknowns in the field, rationalized compromises will need to be made when planning and carrying out future toxicological studies of ENM until more knowledge is gained.

6.11 SIGNIFICANCE OF THE FINDINGS FOR OCCUPATIONAL AND PUBLIC HEALTH

Risk assessment is a process consisting of identifying hazards and risk factors that may cause harm, evaluating the risk linked with the hazard, and choosing appropriate ways to eliminate the hazard or control the risk (Canadian Centre for Occupational Health and Safety, 2018). Risk is the probability that a person may suffer detrimental health effects if exposed to a hazard. Thus, the risk level is affected by the hazard and the exposure to the hazard (Health and Safety Authority, 2018). Since no specific OEL have been established in the EU for ENM, it is difficult to evaluate the magnitude of their exposure levels. Due to no regulatory OEL and also limited data on actual occupational exposure levels, mouse to human extrapolation is challenging. Nevertheless, speculative conclusions can be drawn and suggestions made for ensuring the safety of workers handling ENM and consumers who use nanoproducts i.e. preventing potential adverse effects until regulations for these materials have been established.

In publication I, inhalation of aerosolized rCNT was found to cause a rapid innate immunity-mediated allergic airway inflammation in healthy lungs. As tCNT did not trigger a similar outcome, these findings highlight the importance of taking into account the form of fibrous ENM when assessing their detrimental pulmonary effects. Furthermore, foreign-body granuloma formation accompanied by on-going immune reactions were still evident 28 days after rCNT administration in publication II. These data suggest that workers who handle ENM powders with similar characteristics as rCNT might be at risk for developing symptoms of asthma and long-term adverse pulmonary effects if precautions are not taken to avoid continual exposure.

Even though OEL have so far not been set for ENM, the EU legislation on worker protection applies also to nanomaterials (The Council of the European Communities, 1989). The general principles of the Directive include avoiding, evaluating and combating risks by eliminating or minimizing them, or replacing with less hazardous materials. In the case of potential exposure to long and rigid CNT, regular indoor air measurements could be carried out in the occupational environment to monitor, evaluate and control the release of the ENM. In addition, workers' safety could be ensured by providing appropriate personal protective equipment along with regular occupational health checks.

rCNT specifically, also known as MWCNT-7, are now classified as a possibly carcinogenic substance to humans (Group 2B) by the International Agency for Research on Cancer (IARC, 2014). Thus, recommendations of the International Labour Organisation for avoiding occupational cancer may be also taken into consideration when preventing and controlling occupational risks of ENM with similar characteristics to rCNT (International Labour Organization, 1974, International Labour Organisation, 1976).

In contrast to rCNT, airway exposure to non-modified and surface-functionalized NFC demonstrated that the materials had an ability to induce acute, predominantly neutrophilic, lung inflammation, however, there was no evidence of on-going chronic inflammation at 28 days after the exposure even though the materials were still present in the lungs. In the context of occupational respiratory exposure, these findings suggest that NFC do not pose severe health risks and thus, could be ranked as a material with low toxicity. In the case when a workplace risk assessment reveals that exposure to NFC is a possibility, then measures can be taken to minimize the exposure and thereby lower the risk.

Due to the biocompatible characteristics of NFC, one can predict that these materials may have great potential in biomedical applications. For example, it has been earlier suggested that NFC could be used as a substitute implant material for example replacing blood vessels, nucleus pulposus – gelatinous filling of intervertebral disks of the spine, or soft tissue (Lin and Dufresne, 2014).

When identifying vulnerable groups in the workplace, several factors need to be considered such as workers' ages, workers with high levels of exposure due to the nature of the work, specific conditions in the workplace as well as workers with pre-existing health conditions. In publication III, uncoated and modified CuO ENM were tested in compromised lungs and it was found that the materials worsened AAI by adding another inflammatory feature – pulmonary neutrophilia – to the existing condition. As these materials affected also sensitized mice without AAI, the data indicate that both healthy and asthmatic people may suffer detrimental health effects if they are exposed to CuO nanomaterials. However, the exposure may affect asthmatics more severely because of their impaired immune system. Thus, effective preventive measures at workplace should be undertaken to avoid exposure to these materials.

The findings of publication III revealed that surface PEGylation inhibited the inflammatory reactions evoked by the core material. These data suggest that use of PEGylated CuO instead core CuO could be a solution to reduce its risk level in occupational setting.

Sunscreens containing nano-sized TiO2 are on the European market today, and in 2016, the European Commission also approved the use of ZnO nanomaterials as a UV filter in sun lotions (excluding spray products due to the respiratory hazard) at a maximum concentration of 25% (Scientific Committee on Consumer Safety, 2012, The European Commission, 2016). However, the data on dermal penetration and biological effects of MeO nanomaterials are limited and inconclusive in healthy as well as in damaged skin. In publication IV, topically applied nZnO but not bZnO penetrated into injured and allergic skin, suppressed allergic reactions locally but exhibited systemic adjuvant activity. These data indicate that individuals with AD may experience a symptom-relieving effect when using nZnO-containing sunscreens but caution is needed because of the possible exacerbation of IgE-

antibody release. However, due to the elevated risk for skin cancers without UV-protection, the use of sun lotions is important, but individuals with a diminished skin barrier may consider using alternative products, for example sunscreens based on organic instead of inorganic UV filters.

6.12 FUTURE PROSPECTS

Still today, a large part of the health risk evaluation of ENM is being conducted *in vivo* on a material by material basis. However, due to the lack of harmonized protocols, a variety of experimental protocols exist which not only make it challenging to draw conclusions for a specific ENM but also complicate any comparison between different nanomaterials in published studies. Furthermore, the number of new and more complex nanomaterials is raising rapidly along with their increasing use. *In vivo* toxicity testing of all ENM is not feasible as animal experiments are time-consuming and expensive. Thus, there is a growing need to strategize the hazard assessment of ENM.

It has been suggested that due to the extensive variations in the physicochemical properties of ENM, the safety evaluation should focus on associating material characteristics with the pathways of toxicity to which they contribute. However, this task cannot be fulfilled by descriptive animal experiments whose capacity in terms of the number of tested materials is limited. To overcome this issue, the field needs to devise alternative test systems that would allow the screening of many ENM at different concentrations and under various exposure conditions – there is a need for robust assays, such as high-content analysis or high-throughput screening technology, in which the main results are obtained at the cellular and molecular level.

Alternatives for *in vivo* testing may include mono-, 3D-, co-culture, air-liquid interface *in vitro* or artificial tissue models which are based on relevant end points and which reflect *in vivo* consequences. These models, in combination with omics technologies and computational bioinformatics approaches, can provide a large amount of information helping to clarify how ENM interact with biological systems. These approaches should reveal causal associations between ENM characteristics, production of biomolecules and pathways of toxicity (Clark *et al.*, 2011, Nel, 2013, Fadeel *et al.*, 2018). In other words, linking of the physicochemical characteristics of ENM to biological outcomes would make it possible to establish structure–activity relationships which could then be applied as a platform for ranking and predicting the toxicity of ENM. To some extent, animal testing would still be needed for addressing pathophysiological effects or validating *in vitro* tests, but it would be exploited to a significantly lesser extent.

The future of nanotoxicology research is envisioned to lie in predictive toxicology in which alternative experimental and computational methods will be developed in tandem. Such approach would reduce animal testing and

create a scientific foundation for regulating the use of ENM as well as leading to the 'safe-by-design' development of new materials and nanotechnology-based innovations.

7 CONCLUSIONS

The novelty and innovation of ENM can only be fully exploited when it is recognized that their use does not pose a threat to our health or the environment. Therefore it is essential to perform a hazard assessment of these materials in order to ensure their safe use. Impressive progress in understanding the mechanisms behind ENM-induced health effects has been made in recent years, however, with the continuing development and increased use of ENM, new questions arise about both exposure scenarios and the conditions under which the materials are being used and the ways in which contact can occur. The overall aim this thesis was to examine the modulatory effects of different ENM under physiologically normal and conditions when the immune system was impaired.

The main findings of this thesis included rCNT-triggered unconventional allergic airway inflammation, and NFC-induced acute neutrophilic pulmonary inflammation which subsided after a month even though the foreign material was still present in the tissue. In the compromised immune system, CuO as well as its modified derivates caused neutrophilia and thereby worsened allergic airway inflammation. However, the data also revealed that surface PEGylation inhibited the effects of uncoated CuO. Dermal exposure to nZnO indicated that the particles could penetrate into injured and allergic skin. Furthermore, it was observed that nZnO suppressed local inflammatory reactions but systemically it had an adjuvant activity.

This work provides insights into the pulmonary and dermal effects of ENM. The observations of this thesis reveal that different types of ENM in their various forms and shapes and surface chemistries have an ability to elicit, aggravate or suppress the reactions of our immune system. These findings emphasize the diversity and complexity of ENM as well as their complicated interactions with the immune system and the consequences on health. These data contribute to the hazard assessment of nanomaterials, furthermore this information can also be expoited when devising novel applications in nanomedicine.

REFERENCES

Adachi, K., Yamada, N., Yamamoto, K., Yoshida, Y. & Yamamoto, O., 2010. In vivo effect of industrial titanium dioxide nanoparticles experimentally exposed to hairless rat skin. *Nanotoxicology,* 4**,** 296-306.

Ahamed, M., Akhtar, M.J., Alhadlaq, H.A. & Alrokayan, S.A., 2015. Assessment of the lung toxicity of copper oxide nanoparticles: current status. *Nanomedicine (Lond),* 10**,** 2365-77.

Ahamed, M., Akhtar, M.J., Siddiqui, M.A., Ahmad, J., Musarrat, J., Al-Khedhairy, A.A., Alsalhi, M.S. & Alrokayan, S.A., 2011. Oxidative stress mediated apoptosis induced by nickel ferrite nanoparticles in cultured A549 cells. *Toxicology,* 283**,** 101-8.

Aiso, S., Yamazaki, K., Umeda, Y., Asakura, M., Kasai, T., Takaya, M., Toya, T., Koda, S., Nagano, K., Arito, H. & Fukushima, S., 2010. Pulmonary toxicity of intratracheally instilled multiwall carbon nanotubes in male Fischer 344 rats. *Ind Health,* 48**,** 783-95.

Andon, F.T. & Fadeel, B., 2013. Programmed cell death: molecular mechanisms and implications for safety assessment of nanomaterials. *Acc Chem Res,* 46**,** 733-42.

Artis, D. & Spits, H., 2015. The biology of innate lymphoid cells. *Nature,* 517**,** 293-301.

Aschberger, K., Johnston, H.J., Stone, V., Aitken, R.J., Hankin, S.M., Peters, S.A., Tran, C.L. & Christensen, F.M., 2010. Review of carbon nanotubes toxicity and exposure--appraisal of human health risk assessment based on open literature. *Crit Rev Toxicol,* 40**,** 759-90.

Beamer, C.A., Girtsman, T.A., Seaver, B.P., Finsaas, K.J., Migliaccio, C.T., Perry, V.K., Rottman, J.B., Smith, D.E. & Holian, A., 2013. IL-33 mediates multi-walled carbon nanotube (MWCNT)-induced airway hyper-reactivity via the mobilization of innate helper cells in the lung. *Nanotoxicology,* 7**,** 1070-81.

Beck, L.A. & Leung, D.Y., 2000. Allergen sensitization through the skin induces systemic allergic responses. *J Allergy Clin Immunol,* 106**,** S258-63.

Benjamini, Y. & Hochberg, Y., 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J R Statist Soc B,* 57**,** 289-300.

Bieber, T., 2010. Atopic dermatitis. *Ann Dermatol,* 22**,** 125-37.

Börjesson, M. & Westman, G., 2015. Crystalline Nanocellulose — Preparation, Modification, and Properties In Poletto M. (Ed.), Cellulose. IntechOpen.

Bottini, M., Bruckner, S., Nika, K., Bottini, N., Bellucci, S., Magrini, A., Bergamaschi, A. & Mustelin, T., 2006. Multi-walled carbon nanotubes induce T lymphocyte apoptosis. *Toxicol Lett,* 160**,** 121-6.

Brandenberger, C., Rowley, N.L., Jackson-Humbles, D.N., Zhang, Q., Bramble, L.A., Lewandowski, R.P., Wagner, J.G., Chen, W., Kaplan, B.L., Kaminski, N.E., Baker, G.L., Worden, R.M. & Harkema, J.R., 2013. Engineered silica nanoparticles act as adjuvants to enhance allergic airway disease in mice. *Part Fibre Toxicol,* 10**,** 26.

Canadian Centre for Occupational Health and Safety, 2018. *Risk Assessment* [online]. https://www.ccohs.ca/oshanswers/hsprograms/risk_assessment.html [Access Date: 30.08.2018]

Carmi-Levy, I., Homey, B. & Soumelis, V., 2011. A modular view of cytokine networks in atopic dermatitis. *Clin Rev Allergy Immunol,* 41**,** 245-53.

Catalan, J., Rydman, E., Aimonen, K., Hannukainen, K.S., Suhonen, S., Vanhala, E., Moreno, C., Meyer, V., Perez, D.D., Sneck, A., Forsstrom, U.,

Hojgaard, C., Willemoes, M., Winther, J.R., Vogel, U., Wolff, H., Alenius, H., Savolainen, K.M. & Norppa, H., 2017. Genotoxic and inflammatory effects of nanofibrillated cellulose in murine lungs. *Mutagenesis,* 32**,** 23-31.
Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X. & Zhao, L., 2018. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget,* 9**,** 7204-7218.
Chibber, S. & Shanker, R., 2017. Can CuO nanoparticles lead to epigenetic regulation of antioxidant enzyme system? *J Appl Toxicol,* 37**,** 84-91.
Cho, W.S., Duffin, R., Howie, S.E., Scotton, C.J., Wallace, W.A., Macnee, W., Bradley, M., Megson, I.L. & Donaldson, K., 2011. Progressive severe lung injury by zinc oxide nanoparticles; the role of Zn2+ dissolution inside lysosomes. *Part Fibre Toxicol,* 8**,** 27.
Clark, K.A., White, R.H. & Silbergeld, E.K., 2011. Predictive models for nanotoxicology: current challenges and future opportunities. *Regul Toxicol Pharmacol,* 59**,** 361-3.
Coville, N.J., Mhlanga, S.D., Nxumalo, E.N. & Shaikjee, A., 2011. A review of shaped carbon nanomaterials. *South African Journal of Science,* 107.
Cruvinel, W.D.M., Mesquita Júnior, D., Araújo, J.a.P., Catelan, T.T.T., Souza, A.W.S.D., Silva, N.P.D. & Andrade, L.E.C., 2010. Immune system: Part I. Fundamentals of innate immunity with emphasis on molecular and cellular mechanisms of inflammatory response. *Revista Brasileira de Reumatologia,* 50**,** 434-447.
Dahm, M.M., Evans, D.E., Schubauer-Berigan, M.K., Birch, M.E. & Fernback, J.E., 2012. Occupational exposure assessment in carbon nanotube and nanofiber primary and secondary manufacturers. *Ann Occup Hyg,* 56**,** 542-56.
Danish Ecological Council and Danish Consumer Council, 2013. *The Nanodatabase* [online]. http://nanodb.dk [Access Date: 30.08.2018]
De Stefano, D., Carnuccio, R. & Maiuri, M.C., 2012. Nanomaterials toxicity and cell death modalities. *J Drug Deliv,* 2012**,** 167896.
Deckers, J., De Bosscher, K., Lambrecht, B.N. & Hammad, H., 2017. Interplay between barrier epithelial cells and dendritic cells in allergic sensitization through the lung and the skin. *Immunol Rev,* 278**,** 131-144.
Dianzani, C., Cavalli, R., Zara, G.P., Gallicchio, M., Lombardi, G., Gasco, M.R., Panzanelli, P. & Fantozzi, R., 2006. Cholesteryl butyrate solid lipid nanoparticles inhibit adhesion of human neutrophils to endothelial cells. *Br J Pharmacol,* 148**,** 648-56.
Doak, S.H. & Dusinska, M., 2017. NanoGenotoxicology: present and the future. *Mutagenesis,* 32**,** 1-4.
Dobrovolskaia, M.A. & Mcneil, S.E., 2007. Immunological properties of engineered nanomaterials. *Nat Nanotechnol,* 2**,** 469-78.
Donaldson, K., Aitken, R., Tran, L., Stone, V., Duffin, R., Forrest, G. & Alexander, A., 2006. Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety. *Toxicol Sci,* 92**,** 5-22.
Donaldson, K., Murphy, F.A., Duffin, R. & Poland, C.A., 2010. Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. *Part Fibre Toxicol,* 7**,** 5.
Donaldson, K., Poland, C.A., Murphy, F.A., Macfarlane, M., Chernova, T. & Schinwald, A., 2013. Pulmonary toxicity of carbon nanotubes and asbestos - similarities and differences. *Adv Drug Deliv Rev,* 65**,** 2078-86.
Dong, J. & Ma, Q., 2018. Macrophage polarization and activation at the interface of multi-walled carbon nanotube-induced pulmonary inflammation and fibrosis. *Nanotoxicology,* 12**,** 153-168.

Doshi, N. & Mitragotri, S., 2010. Macrophages recognize size and shape of their targets. *PLoS One,* 5**,** e10051.
Drake, D.R., Brogden, K.A., Dawson, D.V. & Wertz, P.W., 2008. Thematic review series: skin lipids. Antimicrobial lipids at the skin surface. *J Lipid Res,* 49**,** 4-11.
Drexler, K.E., 1986. *Engines of Creation: The Coming Era of Nanotechnology* New York: Anchor Books.
Edgar, R., Domrachev, M. & Lash, A.E., 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res,* 30**,** 207-10.
Erdely, A., Dahm, M., Chen, B.T., Zeidler-Erdely, P.C., Fernback, J.E., Birch, M.E., Evans, D.E., Kashon, M.L., Deddens, J.A., Hulderman, T., Bilgesu, S.A., Battelli, L., Schwegler-Berry, D., Leonard, H.D., Mckinney, W., Frazer, D.G., Antonini, J.M., Porter, D.W., Castranova, V. & Schubauer-Berigan, M.K., 2013. Carbon nanotube dosimetry: from workplace exposure assessment to inhalation toxicology. *Part Fibre Toxicol,* 10**,** 53.
Fadeel, B., Farcal, L., Hardy, B., Vazquez-Campos, S., Hristozov, D., Marcomini, A., Lynch, I., Valsami-Jones, E., Alenius, H. & Savolainen, K., 2018. Advanced tools for the safety assessment of nanomaterials. *Nat Nanotechnol,* 13**,** 537-543.
Fadeel, B. & Orrenius, S., 2005. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. *J Intern Med,* 258**,** 479-517.
Fadeel, B., Pietroiusti, A. & Shvedova, A., 2012. *Adverse Effects of Engineered Nanomaterials: Exposure, Toxicology, and Impact on Human Health,* 1st ed.: Academic Press.
Fallon, P.G., Sasaki, T., Sandilands, A., Campbell, L.E., Saunders, S.P., Mangan, N.E., Callanan, J.J., Kawasaki, H., Shiohama, A., Kubo, A., Sundberg, J.P., Presland, R.B., Fleckman, P., Shimizu, N., Kudoh, J., Irvine, A.D., Amagai, M. & Mclean, W.H., 2009. A homozygous frameshift mutation in the mouse Flg gene facilitates enhanced percutaneous allergen priming. *Nat Genet,* 41**,** 602-8.
Farrera, C., Bhattacharya, K., Lazzaretto, B., Andon, F.T., Hultenby, K., Kotchey, G.P., Star, A. & Fadeel, B., 2014. Extracellular entrapment and degradation of single-walled carbon nanotubes. *Nanoscale,* 6**,** 6974-83.
Farrera, C. & Fadeel, B., 2015. It takes two to tango: Understanding the interactions between engineered nanomaterials and the immune system. *Eur J Pharm Biopharm,* 95**,** 3-12.
Feoktistova, M. & Leverkus, M., 2015. Programmed necrosis and necroptosis signalling. *FEBS J,* 282**,** 19-31.
Feynman, R.P., 1959. *Plenty of Room at the Bottom* [online]. http://web.pa.msu.edu/people/yang/RFeynman_plentySpace.pdf [Access Date: 30.08.2018]
Frohlich, E., 2015. Value of phagocyte function screening for immunotoxicity of nanoparticles in vivo. *Int J Nanomedicine,* 10**,** 3761-78.
Galli, S.J. & Tsai, M., 2012. IgE and mast cells in allergic disease. *Nat Med,* 18**,** 693-704.
Galli, S.J., Tsai, M. & Piliponsky, A.M., 2008. The development of allergic inflammation. *Nature,* 454**,** 445-54.
Galluzzi, L., Vitale, I., Aaronson, S.A., Abrams, J.M., Adam, D., Agostinis, P., Alnemri, E.S., Altucci, L., Amelio, I., Andrews, D.W., Annicchiarico-Petruzzelli, M., Antonov, A.V., Arama, E., Baehrecke, E.H., Barlev, N.A., Bazan, N.G., Bernassola, F., Bertrand, M.J.M., Bianchi, K., Blagosklonny, M.V., Blomgren, K., Borner, C., Boya, P., Brenner, C., Campanella, M., Candi, E., Carmona-Gutierrez, D., Cecconi, F., Chan, F.K., Chandel, N.S., Cheng, E.H., Chipuk, J.E., Cidlowski, J.A., Ciechanover, A., Cohen, G.M.,

Conrad, M., Cubillos-Ruiz, J.R., Czabotar, P.E., D'angiolella, V., Dawson, T.M., Dawson, V.L., De Laurenzi, V., De Maria, R., Debatin, K.M., Deberardinis, R.J., Deshmukh, M., Di Daniele, N., Di Virgilio, F., Dixit, V.M., Dixon, S.J., Duckett, C.S., Dynlacht, B.D., El-Deiry, W.S., Elrod, J.W., Fimia, G.M., Fulda, S., Garcia-Saez, A.J., Garg, A.D., Garrido, C., Gavathiotis, E., Golstein, P., Gottlieb, E., Green, D.R., Greene, L.A., Gronemeyer, H., Gross, A., Hajnoczky, G., Hardwick, J.M., Harris, I.S., Hengartner, M.O., Hetz, C., Ichijo, H., Jaattela, M., Joseph, B., Jost, P.J., Juin, P.P., Kaiser, W.J., Karin, M., Kaufmann, T., Kepp, O., Kimchi, A., Kitsis, R.N., Klionsky, D.J., Knight, R.A., Kumar, S., Lee, S.W., Lemasters, J.J., Levine, B., Linkermann, A., Lipton, S.A., Lockshin, R.A., Lopez-Otin, C., Lowe, S.W., Luedde, T., Lugli, E., Macfarlane, M., Madeo, F., Malewicz, M., Malorni, W., Manic, G., et al., 2018. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ,* 25**,** 486-541.

Gao, F., Ma, N., Zhou, H., Wang, Q., Zhang, H., Wang, P., Hou, H., Wen, H. & Li, L., 2016. Zinc oxide nanoparticles-induced epigenetic change and G2/M arrest are associated with apoptosis in human epidermal keratinocytes. *Int J Nanomedicine,* 11**,** 3859-74.

Glista-Baker, E.E., Taylor, A.J., Sayers, B.C., Thompson, E.A. & Bonner, J.C., 2014. Nickel nanoparticles cause exaggerated lung and airway remodeling in mice lacking the T-box transcription factor, TBX21 (T-bet). *Part Fibre Toxicol,* 11**,** 7.

Grigore, M.E., Biscu, E.R., Holban, A.M., Gestal, M.C. & Grumezescu, A.M., 2016. Methods of Synthesis, Properties and Biomedical Applications of CuO Nanoparticles. *Pharmaceuticals (Basel),* 9.

Gulson, B., Mccall, M., Korsch, M., Gomez, L., Casey, P., Oytam, Y., Taylor, A., Mcculloch, M., Trotter, J., Kinsley, L. & Greenoak, G., 2010. Small amounts of zinc from zinc oxide particles in sunscreens applied outdoors are absorbed through human skin. *Toxicol Sci,* 118**,** 140-9.

Gulson, B., Mccall, M.J., Bowman, D.M. & Pinheiro, T., 2015. A review of critical factors for assessing the dermal absorption of metal oxide nanoparticles from sunscreens applied to humans, and a research strategy to address current deficiencies. *Arch Toxicol,* 89**,** 1909-30.

Guo, L., Johnson, R.S. & Schuh, J.C., 2000. Biochemical characterization of endogenously formed eosinophilic crystals in the lungs of mice. *J Biol Chem,* 275**,** 8032-7.

Guo, N.L., Wan, Y.W., Denvir, J., Porter, D.W., Pacurari, M., Wolfarth, M.G., Castranova, V. & Qian, Y., 2012. Multiwalled carbon nanotube-induced gene signatures in the mouse lung: potential predictive value for human lung cancer risk and prognosis. *J Toxicol Environ Health A,* 75**,** 1129-53.

He, R., Oyoshi, M.K., Garibyan, L., Kumar, L., Ziegler, S.F. & Geha, R.S., 2008. TSLP acts on infiltrating effector T cells to drive allergic skin inflammation. *Proc Natl Acad Sci U S A,* 105**,** 11875-80.

He, X., Aker, W.G., Fu, P.P. & Hwang, H.-M., 2015. Toxicity of engineered metal oxide nanomaterials mediated by nano–bio–eco–interactions: a review and perspective. *Environmental Science: Nano,* 2**,** 564-582.

Health and Safety Authority, 2018. *Hazard and Risk* [online]. https://www.hsa.ie/eng/Topics/Hazards/ [Access Date: 30.08.2018]

Helming, L. & Gordon, S., 2007. Macrophage fusion induced by IL-4 alternative activation is a multistage process involving multiple target molecules. *Eur J Immunol,* 37**,** 33-42.

Helming, L. & Gordon, S., 2009. Molecular mediators of macrophage fusion. *Trends Cell Biol,* 19**,** 514-22.

Holmes, A.M., Song, Z., Moghimi, H.R. & Roberts, M.S., 2016. Relative Penetration of Zinc Oxide and Zinc Ions into Human Skin after Application of Different Zinc Oxide Formulations. *ACS Nano,* 10**,** 1810-9.
Iarc, 2014. Volume 111 of the IARC Monographs.
Inoue, K., Koike, E., Yanagisawa, R., Hirano, S., Nishikawa, M. & Takano, H., 2009. Effects of multi-walled carbon nanotubes on a murine allergic airway inflammation model. *Toxicol Appl Pharmacol,* 237**,** 306-16.
Inoue, K., Yanagisawa, R., Koike, E., Nishikawa, M. & Takano, H., 2010. Repeated pulmonary exposure to single-walled carbon nanotubes exacerbates allergic inflammation of the airway: Possible role of oxidative stress. *Free Radic Biol Med,* 48**,** 924-34.
International Labour Organisation, 1976. C139 - Occupational Cancer Convention, 1974 (No. 139).
International Labour Organization, 1974. R147 - Occupational Cancer Recommendation, 1974 (No. 147).
Irvine, A.D., Mclean, W.H. & Leung, D.Y., 2011. Filaggrin mutations associated with skin and allergic diseases. *N Engl J Med,* 365**,** 1315-27.
Jaligama, S., Patel, V.S., Wang, P., Sallam, A., Harding, J., Kelley, M., Mancuso, S.R., Dugas, T.R. & Cormier, S.A., 2018. Radical containing combustion derived particulate matter enhance pulmonary Th17 inflammation via the aryl hydrocarbon receptor. *Part Fibre Toxicol,* 15**,** 20.
Janin, A., Torpier, G., Courtin, P., Capron, M., Prin, L., Tonnel, A.B., Hatron, P.Y. & Gosselin, B., 1993. Segregation of eosinophil proteins in alveolar macrophage compartments in chronic eosinophilic pneumonia. *Thorax,* 48**,** 57-62.
Jiang, Z. & Zhu, L., 2016. Update on the role of alternatively activated macrophages in asthma. *J Asthma Allergy,* 9**,** 101-7.
John, A.E., Lukacs, N.W., Berlin, A.A., Palecanda, A., Bargatze, R.F., Stoolman, L.M. & Nagy, J.O., 2003. Discovery of a potent nanoparticle P-selectin antagonist with anti-inflammatory effects in allergic airway disease. *FASEB J,* 17**,** 2296-8.
Johnson, W.E., Li, C. & Rabinovic, A., 2007. Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics (Oxford, England),* 8**,** 118-127.
Karlsson, H.L., Gliga, A.R., Calleja, F.M., Goncalves, C.S., Wallinder, I.O., Vrieling, H., Fadeel, B. & Hendriks, G., 2014. Mechanism-based genotoxicity screening of metal oxide nanoparticles using the ToxTracker panel of reporter cell lines. *Part Fibre Toxicol,* 11**,** 41.
Katwa, P., Wang, X., Urankar, R.N., Podila, R., Hilderbrand, S.C., Fick, R.B., Rao, A.M., Ke, P.C., Wingard, C.J. & Brown, J.M., 2012. A carbon nanotube toxicity paradigm driven by mast cells and the IL-(3)(3)/ST(2) axis. *Small,* 8**,** 2904-12.
Kendall, M. & Holgate, S., 2012. Health impact and toxicological effects of nanomaterials in the lung. *Respirology,* 17**,** 743-58.
Kenzaoui, B.H., Vila, M.R., Miquel, J.M., Cengelli, F. & Juillerat-Jeanneret, L., 2012. Evaluation of uptake and transport of cationic and anionic ultrasmall iron oxide nanoparticles by human colon cells. *Int J Nanomedicine,* 7**,** 1275-86.
Khan, I., Saeed, K. & Khan, I., 2017. Nanoparticles: Properties, applications and toxicities. *Arabian Journal of Chemistry*.
Kim, H.Y., Dekruyff, R.H. & Umetsu, D.T., 2010. The many paths to asthma: phenotype shaped by innate and adaptive immunity. *Nat Immunol,* 11**,** 577-84.
Kim, S.-H., Heo, Y., Choi, S.-J., Kim, Y.-J., Kim, M.-S., Kim, H., Jo, E., Song, C.-W. & Lee, K., 2016. Safety evaluation of zinc oxide nanoparticles in terms

of acute dermal toxicity, dermal irritation and corrosion, and skin sensitization. *Molecular & Cellular Toxicology,* 12**,** 93-99.
Kinaret, P., Ilves, M., Fortino, V., Rydman, E., Karisola, P., Lahde, A., Koivisto, J., Jokiniemi, J., Wolff, H., Savolainen, K., Greco, D. & Alenius, H., 2017. Inhalation and Oropharyngeal Aspiration Exposure to Rod-Like Carbon Nanotubes Induce Similar Airway Inflammation and Biological Responses in Mouse Lungs. *ACS Nano,* 11**,** 291-303.
Klose, C.S. & Artis, D., 2016. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat Immunol,* 17**,** 765-74.
Knudsen, K.B., Kofoed, C., Espersen, R., Hojgaard, C., Winther, J.R., Willemoes, M., Wedin, I., Nuopponen, M., Vilske, S., Aimonen, K., Weydahl, I.E., Alenius, H., Norppa, H., Wolff, H., Wallin, H. & Vogel, U., 2015. Visualization of Nanofibrillar Cellulose in Biological Tissues Using a Biotinylated Carbohydrate Binding Module of beta-1,4-Glycanase. *Chem Res Toxicol,* 28**,** 1627-35.
Kollar, P., Zavalova, V., Hosek, J., Havelka, P., Sopuch, T., Karpisek, M., Tretinova, D. & Suchy, P., Jr., 2011. Cytotoxicity and effects on inflammatory response of modified types of cellulose in macrophage-like THP-1 cells. *Int Immunopharmacol,* 11**,** 997-1001.
Korn, T., Bettelli, E., Oukka, M. & Kuchroo, V.K., 2009. IL-17 and Th17 Cells. *Annu Rev Immunol,* 27**,** 485-517.
Krombach, F., Munzing, S., Allmeling, A.M., Gerlach, J.T., Behr, J. & Dorger, M., 1997. Cell size of alveolar macrophages: an interspecies comparison. *Environ Health Perspect,* 105 Suppl 5**,** 1261-3.
Krug, H.F. & Wick, P., 2011. Nanotoxicology: an interdisciplinary challenge. *Angew Chem Int Ed Engl,* 50**,** 1260-78.
Kubo, M., 2018. Mast cells and basophils in allergic inflammation. *Curr Opin Immunol,* 54**,** 74-79.
Kurosaki, T., Kometani, K. & Ise, W., 2015. Memory B cells. *Nat Rev Immunol,* 15**,** 149-59.
Kvarnhammar, A.M. & Cardell, L.O., 2012. Pattern-recognition receptors in human eosinophils. *Immunology,* 136**,** 11-20.
Lademann, J., Richter, H., Schanzer, S., Knorr, F., Meinke, M., Sterry, W. & Patzelt, A., 2011. Penetration and storage of particles in human skin: perspectives and safety aspects. *Eur J Pharm Biopharm,* 77**,** 465-8.
Lambrecht, B.N. & Hammad, H., 2015. The immunology of asthma. *Nat Immunol,* 16**,** 45-56.
Landsiedel, R., Fabian, E., Ma-Hock, L., Van Ravenzwaay, B., Wohlleben, W., Wiench, K. & Oesch, F., 2012. Toxico-/biokinetics of nanomaterials. *Arch Toxicol,* 86**,** 1021-60.
Lao, L.M., Kumakiri, M., Nakagawa, K., Ishida, H., Ishiguro, K., Yanagihara, M. & Ueda, K., 1998. The ultrastructural findings of Charcot-Leyden crystals in stroma of mastocytoma. *J Dermatol Sci,* 17**,** 198-204.
Leek, J.T., Johnson, E., Parker, H.S., Jaffe, A.E. & Storey, J.D., 2013. Sva: Surrogate Variable Analysis 3.6.0.
Ley, K., Laudanna, C., Cybulsky, M.I. & Nourshargh, S., 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol,* 7**,** 678-89.
Li, C., Liu, H., Sun, Y., Wang, H., Guo, F., Rao, S., Deng, J., Zhang, Y., Miao, Y., Guo, C., Meng, J., Chen, X., Li, L., Li, D., Xu, H., Wang, H., Li, B. & Jiang, C., 2009. PAMAM nanoparticles promote acute lung injury by inducing autophagic cell death through the Akt-TSC2-mTOR signaling pathway. *J Mol Cell Biol,* 1**,** 37-45.
Lin, L.L., Grice, J.E., Butler, M.K., Zvyagin, A.V., Becker, W., Robertson, T.A., Soyer, H.P., Roberts, M.S. & Prow, T.W., 2011. Time-correlated single photon counting for simultaneous monitoring of zinc oxide nanoparticles

and NAD(P)H in intact and barrier-disrupted volunteer skin. *Pharm Res,* 28, 2920-30.
Lin, N. & Dufresne, A., 2014. Nanocellulose in biomedicine: Current status and future prospect. *European Polymer Journal,* 59, 302-325.
Liu, H.L., Zhang, Y.L., Yang, N., Zhang, Y.X., Liu, X.Q., Li, C.G., Zhao, Y., Wang, Y.G., Zhang, G.G., Yang, P., Guo, F., Sun, Y. & Jiang, C.Y., 2011. A functionalized single-walled carbon nanotube-induced autophagic cell death in human lung cells through Akt-TSC2-mTOR signaling. *Cell Death Dis,* 2, e159.
Lopes, V.R., Sanchez-Martinez, C., Stromme, M. & Ferraz, N., 2017. In vitro biological responses to nanofibrillated cellulose by human dermal, lung and immune cells: surface chemistry aspect. *Part Fibre Toxicol,* 14, 1.
Lukacs, N.W., 2001. Role of chemokines in the pathogenesis of asthma. *Nat Rev Immunol,* 1, 108-16.
Lunov, O., Syrovets, T., Loos, C., Beil, J., Delacher, M., Tron, K., Nienhaus, G.U., Musyanovych, A., Mailander, V., Landfester, K. & Simmet, T., 2011. Differential uptake of functionalized polystyrene nanoparticles by human macrophages and a monocytic cell line. *ACS Nano,* 5, 1657-69.
Ma-Hock, L., Burkhardt, S., Strauss, V., Gamer, A.O., Wiench, K., Van Ravenzwaay, B. & Landsiedel, R., 2009. Development of a short-term inhalation test in the rat using nano-titanium dioxide as a model substance. *Inhal Toxicol,* 21, 102-18.
Macias, E.S., Pereira, F.A., Rietkerk, W. & Safai, B., 2011. Superantigens in dermatology. *J Am Acad Dermatol,* 64, 455-72; quiz 473-4.
Manke, A., Wang, L. & Rojanasakul, Y., 2013. Mechanisms of nanoparticle-induced oxidative stress and toxicity. *Biomed Res Int,* 2013, 942916.
Martinez, F.O., Helming, L. & Gordon, S., 2009. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol,* 27, 451-83.
Martson, M., Viljanto, J., Hurme, T., Laippala, P. & Saukko, P., 1999. Is cellulose sponge degradable or stable as implantation material? An in vivo subcutaneous study in the rat. *Biomaterials,* 20, 1989-95.
Mcgrath, J.A., Eady, R.a.J. & Pope, F.M., 2008. Anatomy and organization of human skin In Griffiths, C. (Ed.), Rook's Textbook of Dermatology, 7th Edition. Wiley-Blackwell.
Mcnally, A.K. & Anderson, J.M., 2011. Macrophage fusion and multinucleated giant cells of inflammation. *Adv Exp Med Biol,* 713, 97-111.
Meena, R., Rani, M., Pal, R. & Rajamani, P., 2012. Nano-TiO2-induced apoptosis by oxidative stress-mediated DNA damage and activation of p53 in human embryonic kidney cells. *Appl Biochem Biotechnol,* 167, 791-808.
Miller, F.J., Asgharian, B., Schroeter, J.D. & Price, O., 2016. Improvements and additions to the Multiple Path Particle Dosimetry model. *Journal of Aerosol Science,* 99, 14-26.
Mills, P.R., Davies, R.J. & Devalia, J.L., 1999. Airway epithelial cells, cytokines, and pollutants. *Am J Respir Crit Care Med,* 160, S38-43.
Mitchell, L.A., Lauer, F.T., Burchiel, S.W. & Mcdonald, J.D., 2009. Mechanisms for how inhaled multiwalled carbon nanotubes suppress systemic immune function in mice. *Nat Nanotechnol,* 4, 451-6.
Miyamoto, T., Takahashi, S., Ito, H., Inagaki, H. & Noishiki, Y., 1989. Tissue biocompatibility of cellulose and its derivatives. *J Biomed Mater Res,* 23, 125-33.
Mizutani, N., Nabe, T. & Yoshino, S., 2012. Exposure to multiwalled carbon nanotubes and allergen promotes early- and late-phase increases in airway resistance in mice. *Biol Pharm Bull,* 35, 2133-40.
Monteiro-Riviere, N.A., Wiench, K., Landsiedel, R., Schulte, S., Inman, A.O. & Riviere, J.E., 2011. Safety evaluation of sunscreen formulations containing

titanium dioxide and zinc oxide nanoparticles in UVB sunburned skin: an in vitro and in vivo study. *Toxicol Sci,* 123**,** 264-80.
Morishita, Y., Tada, J., Sato, A., Toi, Y., Kanzaki, H., Akiyama, H. & Arata, J., 1999. Possible influences of Staphylococcus aureus on atopic dermatitis--the colonizing features and the effects of staphylococcal enterotoxins. *Clin Exp Allergy,* 29**,** 1110-7.
Murphy, F.A., Poland, C.A., Duffin, R., Al-Jamal, K.T., Ali-Boucetta, H., Nunes, A., Byrne, F., Prina-Mello, A., Volkov, Y., Li, S., Mather, S.J., Bianco, A., Prato, M., Macnee, W., Wallace, W.A., Kostarelos, K. & Donaldson, K., 2011. Length-dependent retention of carbon nanotubes in the pleural space of mice initiates sustained inflammation and progressive fibrosis on the parietal pleura. *Am J Pathol,* 178**,** 2587-600.
Murphy, K., Travers, P. & Walport, M., 2009. *Janeway's Immunobiology,* 7th ed. New York: Garland Science.
Nair, S.S., Zhu, J.Y., Deng, Y. & Ragauskas, A.J., 2014. High performance green barriers based on nanocellulose. *Sustainable Chemical Processes,* 2.
National Cancer Institute, U., 2017. *Cancer and Nanotechnology* [online]. https://www.cancer.gov/sites/nano/cancer-nanotechnology [Access Date: 30.08.2018]
Nel, A., Xia, T., Madler, L. & Li, N., 2006. Toxic potential of materials at the nanolevel. *Science,* 311**,** 622-7.
Nel, A.E., 2013. Implementation of alternative test strategies for the safety assessment of engineered nanomaterials. *J Intern Med,* 274**,** 561-77.
Nel, A.E., Madler, L., Velegol, D., Xia, T., Hoek, E.M., Somasundaran, P., Klaessig, F., Castranova, V. & Thompson, M., 2009. Understanding biophysicochemical interactions at the nano-bio interface. *Nat Mater,* 8**,** 543-57.
Newman, M.D., Stotland, M. & Ellis, J.I., 2009. The safety of nanosized particles in titanium dioxide- and zinc oxide-based sunscreens. *J Am Acad Dermatol,* 61**,** 685-92.
Nohynek, G.J., Lademann, J., Ribaud, C. & Roberts, M.S., 2007. Grey goo on the skin? Nanotechnology, cosmetic and sunscreen safety. *Crit Rev Toxicol,* 37**,** 251-77.
Nygaard, U.C., Hansen, J.S., Samuelsen, M., Alberg, T., Marioara, C.D. & Lovik, M., 2009. Single-walled and multi-walled carbon nanotubes promote allergic immune responses in mice. *Toxicol Sci,* 109**,** 113-23.
Nygaard, U.C., Samuelsen, M., Marioara, C.D. & Lovik, M., 2013. Carbon nanofibers have IgE adjuvant capacity but are less potent than nanotubes in promoting allergic airway responses. *Biomed Res Int,* 2013**,** 476010.
Oberdörster, G., Oberdörster, E. & Oberdörster, J., 2005. Nanotoxicology: An Emerging Discipline Evolving from Studies of Ultrafine Particles. *Environmental Health Perspectives,* 113**,** 823-839.
Pal, A., Alam, S., Chauhan, L.K.S., Saxena, P.N., Kumar, M., Ansari, G.N., Singh, D. & Ansari, K.M., 2016. UVB exposure enhanced the dermal penetration of zinc oxide nanoparticles and induced inflammatory responses through oxidative stress mediated by MAPKs and NF-kappaB signaling in SKH-1 hairless mouse skin. *Toxicol Res (Camb),* 5**,** 1066-1077.
Pallardy, M.J., Turbica, I. & Biola-Vidamment, A., 2017. Why the Immune System Should Be Concerned by Nanomaterials? *Front Immunol,* 8**,** 544.
Palomaki, J., Valimaki, E., Sund, J., Vippola, M., Clausen, P.A., Jensen, K.A., Savolainen, K., Matikainen, S. & Alenius, H., 2011. Long, needle-like carbon nanotubes and asbestos activate the NLRP3 inflammasome through a similar mechanism. *ACS Nano,* 5**,** 6861-70.
Panariti, A., Miserocchi, G. & Rivolta, I., 2012. The effect of nanoparticle uptake on cellular behavior: disrupting or enabling functions? *Nanotechnol Sci Appl,* 5**,** 87-100.

Papayannopoulos, V., 2018. Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol,* 18**,** 134-147.
Parham, S., Wicaksono, D.H.B., Bagherbaigi, S., Lee, S.L. & Nur, H., 2016. Antimicrobial Treatment of Different Metal Oxide Nanoparticles: A Critical Review. *Journal of the Chinese Chemical Society,* 63**,** 385-393.
Park, E.J., Khaliullin, T.O., Shurin, M.R., Kisin, E.R., Yanamala, N., Fadeel, B., Chang, J. & Shvedova, A.A., 2018. Fibrous nanocellulose, crystalline nanocellulose, carbon nanotubes, and crocidolite asbestos elicit disparate immune responses upon pharyngeal aspiration in mice. *J Immunotoxicol,* 15**,** 12-23.
Park, E.J., Roh, J., Kim, S.N., Kang, M.S., Han, Y.A., Kim, Y., Hong, J.T. & Choi, K., 2011. A single intratracheal instillation of single-walled carbon nanotubes induced early lung fibrosis and subchronic tissue damage in mice. *Arch Toxicol,* 85**,** 1121-31.
Park, J.W., Lee, I.C., Shin, N.R., Jeon, C.M., Kwon, O.K., Ko, J.W., Kim, J.C., Oh, S.R., Shin, I.S. & Ahn, K.S., 2016. Copper oxide nanoparticles aggravate airway inflammation and mucus production in asthmatic mice via MAPK signaling. *Nanotoxicology,* 10**,** 445-52.
Pawankar, R., Canonica, G.W., Holgate, S.T. & Lockey, R.F., 2011. *World Allergy Organization (WAO) White Book on Allergy* [online]. http://www.worldallergy.org/UserFiles/file/WAO-White-Book-on-Allergy_web.pdf [Access Date: 30.08.2018]
Pereira, M.M., Raposo, N.R., Brayner, R., Teixeira, E.M., Oliveira, V., Quintao, C.C., Camargo, L.S., Mattoso, L.H. & Brandao, H.M., 2013. Cytotoxicity and expression of genes involved in the cellular stress response and apoptosis in mammalian fibroblast exposed to cotton cellulose nanofibers. *Nanotechnology,* 24**,** 075103.
Pietroiusti, A., 2012. Health implications of engineered nanomaterials. *Nanoscale,* 4**,** 1231-47.
Poland, C.A., Duffin, R., Kinloch, I., Maynard, A., Wallace, W.A., Seaton, A., Stone, V., Brown, S., Macnee, W. & Donaldson, K., 2008. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. *Nat Nanotechnol,* 3**,** 423-8.
Porter, D.W., Hubbs, A.F., Mercer, R.R., Wu, N., Wolfarth, M.G., Sriram, K., Leonard, S., Battelli, L., Schwegler-Berry, D., Friend, S., Andrew, M., Chen, B.T., Tsuruoka, S., Endo, M. & Castranova, V., 2010. Mouse pulmonary dose- and time course-responses induced by exposure to multi-walled carbon nanotubes. *Toxicology,* 269**,** 136-47.
Project on Emerging Nanotechnologies, 2005. [online]. http://www.nanotechproject.org [Access Date: 30.08.2018]
R Core Team, 2012. R: A Language and Environment for Statistical Computing.
Ramkumar, K.M., Manjula, C., Gnanakumar, G., Kanjwal, M.A., Sekar, T.V., Paulmurugan, R. & Rajaguru, P., 2012. Oxidative stress-mediated cytotoxicity and apoptosis induction by TiO2 nanofibers in HeLa cells. *Eur J Pharm Biopharm,* 81**,** 324-33.
Rehberg, M., Praetner, M., Leite, C.F., Reichel, C.A., Bihari, P., Mildner, K., Duhr, S., Zeuschner, D. & Krombach, F., 2010. Quantum dots modulate leukocyte adhesion and transmigration depending on their surface modification. *Nano Lett,* 10**,** 3656-64.
Ronzani, C., Casset, A. & Pons, F., 2014. Exposure to multi-walled carbon nanotubes results in aggravation of airway inflammation and remodeling and in increased production of epithelium-derived innate cytokines in a mouse model of asthma. *Arch Toxicol,* 88**,** 489-99.
Rossi, E.M., Pylkkanen, L., Koivisto, A.J., Nykasenoja, H., Wolff, H., Savolainen, K. & Alenius, H., 2010. Inhalation exposure to nanosized and

fine TiO2 particles inhibits features of allergic asthma in a murine model. *Part Fibre Toxicol,* 7**,** 35.
Rothenberg, M.E. & Hogan, S.P., 2006. The eosinophil. *Annu Rev Immunol,* 24**,** 147-74.
Ruenraroengsak, P. & Tetley, T.D., 2015. Differential bioreactivity of neutral, cationic and anionic polystyrene nanoparticles with cells from the human alveolar compartment: robust response of alveolar type 1 epithelial cells. *Part Fibre Toxicol,* 12**,** 19.
Rydman, E.M., Ilves, M., Vanhala, E., Vippola, M., Lehto, M., Kinaret, P.A., Pylkkanen, L., Happo, M., Hirvonen, M.R., Greco, D., Savolainen, K., Wolff, H. & Alenius, H., 2015. A Single Aspiration of Rod-like Carbon Nanotubes Induces Asbestos-like Pulmonary Inflammation Mediated in Part by the IL-1 Receptor. *Toxicol Sci,* 147**,** 140-55.
Sadrieh, N., Wokovich, A.M., Gopee, N.V., Zheng, J., Haines, D., Parmiter, D., Siitonen, P.H., Cozart, C.R., Patri, A.K., Mcneil, S.E., Howard, P.C., Doub, W.H. & Buhse, L.F., 2010. Lack of significant dermal penetration of titanium dioxide from sunscreen formulations containing nano- and submicron-size TiO2 particles. *Toxicol Sci,* 115**,** 156-66.
Sager, T.M., Wolfarth, M.W., Andrew, M., Hubbs, A., Friend, S., Chen, T.H., Porter, D.W., Wu, N., Yang, F., Hamilton, R.F. & Holian, A., 2014. Effect of multi-walled carbon nanotube surface modification on bioactivity in the C57BL/6 mouse model. *Nanotoxicology,* 8**,** 317-27.
Savolainen, K., Alenius, H., Norppa, H., Pylkkanen, L., Tuomi, T. & Kasper, G., 2010. Risk assessment of engineered nanomaterials and nanotechnologies--a review. *Toxicology,* 269**,** 92-104.
Schatz, M. & Rosenwasser, L., 2014. The allergic asthma phenotype. *J Allergy Clin Immunol Pract,* 2**,** 645-8; quiz 649.
Schmitt, E.G. & Williams, C.B., 2013. Generation and function of induced regulatory T cells. *Front Immunol,* 4**,** 152.
Schroder, K. & Tschopp, J., 2010. The inflammasomes. *Cell,* 140**,** 821-32.
Scientific Committee on Consumer Safety, 2012. SCCS/1489/2012: Opinion on Zinc oxide (nano form).
Seabra, A. & Durán, N., 2015. Nanotoxicology of Metal Oxide Nanoparticles. *Metals,* 5**,** 934-975.
Seaton, A., Tran, L., Aitken, R. & Donaldson, K., 2010. Nanoparticles, human health hazard and regulation. *J R Soc Interface,* 7 Suppl 1**,** S119-29.
Semisch, A., Ohle, J., Witt, B. & Hartwig, A., 2014. Cytotoxicity and genotoxicity of nano - and microparticulate copper oxide: role of solubility and intracellular bioavailability. *Part Fibre Toxicol,* 11**,** 10.
Senzui, M., Tamura, T., Miura, K., Ikarashi, Y., Watanabe, Y. & Fujii, M., 2010. Study on penetration of titanium dioxide (TiO(2)) nanoparticles into intact and damaged skin in vitro. *J Toxicol Sci,* 35**,** 107-13.
Shatkin, J.A. & Oberdorster, G., 2016. Comment on Shvedova et al. (2016), "gender differences in murine pulmonary responses elicited by cellulose nanocrystals". *Part Fibre Toxicol,* 13**,** 59.
Shinohara, N., Zhang, G., Oshima, Y., Kobayashi, T., Imatanaka, N., Nakai, M., Sasaki, T., Kawaguchi, K. & Gamo, M., 2017. Kinetics and dissolution of intratracheally administered nickel oxide nanomaterials in rats. *Part Fibre Toxicol,* 14**,** 48.
Shurin, M.R., Yanamala, N., Kisin, E.R., Tkach, A.V., Shurin, G.V., Murray, A.R., Leonard, H.D., Reynolds, J.S., Gutkin, D.W., Star, A., Fadeel, B., Savolainen, K., Kagan, V.E. & Shvedova, A.A., 2014. Graphene oxide attenuates Th2-type immune responses, but augments airway remodeling and hyperresponsiveness in a murine model of asthma. *ACS Nano,* 8**,** 5585-99.

Shvedova, A.A., Kapralov, A.A., Feng, W.H., Kisin, E.R., Murray, A.R., Mercer, R.R., St Croix, C.M., Lang, M.A., Watkins, S.C., Konduru, N.V., Allen, B.L., Conroy, J., Kotchey, G.P., Mohamed, B.M., Meade, A.D., Volkov, Y., Star, A., Fadeel, B. & Kagan, V.E., 2012. Impaired clearance and enhanced pulmonary inflammatory/fibrotic response to carbon nanotubes in myeloperoxidase-deficient mice. *PLoS One,* 7**,** e30923.

Shvedova, A.A., Kisin, E.R., Mercer, R., Murray, A.R., Johnson, V.J., Potapovich, A.I., Tyurina, Y.Y., Gorelik, O., Arepalli, S., Schwegler-Berry, D., Hubbs, A.F., Antonini, J., Evans, D.E., Ku, B.K., Ramsey, D., Maynard, A., Kagan, V.E., Castranova, V. & Baron, P., 2005. Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. *Am J Physiol Lung Cell Mol Physiol,* 289**,** L698-708.

Shvedova, A.A., Kisin, E.R., Yanamala, N., Farcas, M.T., Menas, A.L., Williams, A., Fournier, P.M., Reynolds, J.S., Gutkin, D.W., Star, A., Reiner, R.S., Halappanavar, S. & Kagan, V.E., 2016. Gender differences in murine pulmonary responses elicited by cellulose nanocrystals. *Part Fibre Toxicol,* 13**,** 28.

Siddiqui, M.A., Alhadlaq, H.A., Ahmad, J., Al-Khedhairy, A.A., Musarrat, J. & Ahamed, M., 2013. Copper oxide nanoparticles induced mitochondria mediated apoptosis in human hepatocarcinoma cells. *PLoS One,* 8**,** e69534.

Smijs, T.G. & Pavel, S., 2011. Titanium dioxide and zinc oxide nanoparticles in sunscreens: focus on their safety and effectiveness. *Nanotechnol Sci Appl,* 4**,** 95-112.

Smyth, G.K., 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Statistical applications in genetics and molecular biology* 3.

Smyth, G.K., 2005. Limma: Linear models for microarray data.

Sonnenberg, G.F. & Artis, D., 2015. Innate lymphoid cells in the initiation, regulation and resolution of inflammation. *Nat Med,* 21**,** 698-708.

Srivastava, R.K., Rahman, Q., Kashyap, M.P., Singh, A.K., Jain, G., Jahan, S., Lohani, M., Lantow, M. & Pant, A.B., 2013. Nano-titanium dioxide induces genotoxicity and apoptosis in human lung cancer cell line, A549. *Hum Exp Toxicol,* 32**,** 153-66.

Stern, S.T., Adiseshaiah, P.P. & Crist, R.M., 2012. Autophagy and lysosomal dysfunction as emerging mechanisms of nanomaterial toxicity. *Part Fibre Toxicol,* 9**,** 20.

Studer, A.M., Limbach, L.K., Van Duc, L., Krumeich, F., Athanassiou, E.K., Gerber, L.C., Moch, H. & Stark, W.J., 2010. Nanoparticle cytotoxicity depends on intracellular solubility: comparison of stabilized copper metal and degradable copper oxide nanoparticles. *Toxicol Lett,* 197**,** 169-74.

Sun, B., Wang, X., Ji, Z., Li, R. & Xia, T., 2013. NLRP3 inflammasome activation induced by engineered nanomaterials. *Small,* 9**,** 1595-607.

Taniguchi, N., Year. On the Basic Concept of Nanotechnologyed.^eds. *Proceedings of the International Conference on Production Engineering*, Tokyo, 18-23.

The Council of the European Communities, 1989. Council Directive on the introduction of measures to encourage improvements in the safety and health of workers at work (89/391/EEC).

The European Commission, 2011. *Definition of a nanomaterial* [online]. http://ec.europa.eu/environment/chemicals/nanotech/faq/definition_en.htm [Access Date: 30.08.2018]

The European Commission, 2013. *H2020-EU.2.1.2. - INDUSTRIAL LEADERSHIP - Leadership in enabling and industrial technologies – Nanotechnologies* [online]. https://cordis.europa.eu/programme/rcn/664161_en.html [Access Date: 30.08.2018]

The European Commission, 2016. Commission Regulation (EU) 2016/621 of 21 April 2016 amending Annex VI to Regulation (EC) No 1223/2009 of the European Parliament and of the Council on cosmetic products.
Thit, A., Selck, H. & Bjerregaard, H.F., 2015. Toxic mechanisms of copper oxide nanoparticles in epithelial kidney cells. *Toxicol In Vitro,* 29**,** 1053-9.
Tian, F., Habel, N.C., Yin, R., Hirn, S., Banerjee, A., Ercal, N., Takenaka, S., Estrada, G., Kostarelos, K., Kreyling, W. & Stoeger, T., 2013. Pulmonary DWCNT exposure causes sustained local and low-level systemic inflammatory changes in mice. *Eur J Pharm Biopharm,* 84**,** 412-20.
Tokura, Y., 2010. Extrinsic and intrinsic types of atopic dermatitis. *J Dermatol Sci,* 58**,** 1-7.
Tomic, S., Kokol, V., Mihajlovic, D., Mircic, A. & Colic, M., 2016. Native cellulose nanofibrills induce immune tolerance in vitro by acting on dendritic cells. *Sci Rep,* 6**,** 31618.
Tsuzuki, T., 2009. Commercial scale production of inorganic nanoparticles. *International Journal of Nanotechnology,* 6.
U.S. Food and Drug Administration, 1999. CFR - Code of Federal Regulations Title 21.
Vilanova, O., Mittag, J.J., Kelly, P.M., Milani, S., Dawson, K.A., Radler, J.O. & Franzese, G., 2016. Understanding the Kinetics of Protein-Nanoparticle Corona Formation. *ACS Nano,* 10**,** 10842-10850.
Vogel, U., Savolainen, K., Wu, Q., Van Tongeren, M., Brouwer, D. & Berges, M., 2014. *Handbook of Nanosafety: Measurement, Exposure and Toxicology,* 1st ed.: Academic Press.
Wang, Z., Li, N., Zhao, J., White, J.C., Qu, P. & Xing, B., 2012. CuO nanoparticle interaction with human epithelial cells: cellular uptake, location, export, and genotoxicity. *Chem Res Toxicol,* 25**,** 1512-21.
Watson, W. & Kapur, S., 2011. Atopic dermatitis. *Allergy Asthma Clin Immunol,* 7**,** S4.
Weiss, U., 2008. Inflammation. *Nature,* 454**,** 427.
Werfel, T., 2009. The role of leukocytes, keratinocytes, and allergen-specific IgE in the development of atopic dermatitis. *J Invest Dermatol,* 129**,** 1878-91.
Wu, J., Liu, W., Xue, C., Zhou, S., Lan, F., Bi, L., Xu, H., Yang, X. & Zeng, F.D., 2009. Toxicity and penetration of TiO2 nanoparticles in hairless mice and porcine skin after subchronic dermal exposure. *Toxicol Lett,* 191**,** 1-8.
Yamanishi, Y. & Karasuyama, H., 2016. Basophils and mast cells in immunity and inflammation. *Semin Immunopathol,* 38**,** 535-7.
Yanagisawa, R., Takano, H., Inoue, K., Koike, E., Kamachi, T., Sadakane, K. & Ichinose, T., 2009. Titanium dioxide nanoparticles aggravate atopic dermatitis-like skin lesions in NC/Nga mice. *Exp Biol Med (Maywood),* 234**,** 314-22.
Yazdi, A.S., Guarda, G., Riteau, N., Drexler, S.K., Tardivel, A., Couillin, I. & Tschopp, J., 2010. Nanoparticles activate the NLR pyrin domain containing 3 (Nlrp3) inflammasome and cause pulmonary inflammation through release of IL-1alpha and IL-1beta. *Proc Natl Acad Sci U S A,* 107**,** 19449-54.
Yuan, R., Xu, H., Liu, X., Tian, Y., Li, C., Chen, X., Su, S., Perelshtein, I., Gedanken, A. & Lin, X., 2016. Zinc-Doped Copper Oxide Nanocomposites Inhibit the Growth of Human Cancer Cells through Reactive Oxygen Species-Mediated NF-kappaB Activations. *ACS Appl Mater Interfaces,* 8**,** 31806-31812.
Zhang, S., Gao, H. & Bao, G., 2015. Physical Principles of Nanoparticle Cellular Endocytosis. *ACS Nano,* 9**,** 8655-71.
Zhang, Y., Nayak, T.R., Hong, H. & Cai, W., 2013. Biomedical applications of zinc oxide nanomaterials. *Curr Mol Med,* 13**,** 1633-45.

Zhao, X. & Liu, R., 2012. Recent progress and perspectives on the toxicity of carbon nanotubes at organism, organ, cell, and biomacromolecule levels. *Environ Int,* 40, 244-255.