Investigation Of Nanoparticles Induced Cell Responses In The Presence Of Innate Immune Factors

Basudev Paudyal

Doctor of Philosophy

April 2018

Declaration

I hereby declare that this thesis has been submitted exclusively for the degree of Doctor of Philosophy at Kingston University London and has not formed the foundation for any other award at any university or tertiary institute.

This thesis contains are my original research and any contribution to this work by other individuals has been fully acknowledged. Where previously published work of others has been consulted or quoted, the authors of the work have been given full acknowledgment via referencing

Basudev Paudyal

Abstract

Nanoparticles (NPs) are progressively being investigated for use in biomedical applications, including biological agents delivery like gene delivery, drug and protein delivery. Activation of complement pathways and interactions with immune recognition subcomponents can modulate the clearance of the NPs and subsequent inflammatory response. Such modulation could affect the intended translational applications either in the development as tissue-specific drug delivery platform or in the treatment of pulmonary diseases such as tuberculosis and lung cancer thus, poses challenges to develop them for *in vivo* applications. Here, we set out to study the interaction between innate immune components such as properdin, a small fragment of properdin, TSR4+5, a key lung pattern recognition molecule, surfactant protein D (SP-D), and carbon nanotubes (CNTs), and potential downstream effects on the immune response *via* macrophages. We report, that human properdin, an up-regulator of the complement alternative pathway and stabilizer of C3 convertase can opsonize CNTs via its thrombospondin type I repeat (TSR) 4 and 5. Uptake of properdin bound CNTs was enhanced by a macrophage cell line, THP-1, surging a robust pro-inflammatory immune response. In addition, recombinant TSR4+5 on CNTs, inhibited complement consumption, suggesting that TSR4+5, can be potentially used as a complement inhibitor in a number of pathological circumstances arising due to unintentional complement activation. Similarly, a recombinant fragment of human SP-D (rfhSP-D) bound to CNTs *via* its C-type lectin domain and augmented phagocytosis by THP-1 monocytic cell lines, together with an increased pro-inflammatory response. Furthermore, rfhSP-D opsozined CNTs continued to activate complement pathway via the classical pathway. Complement deposition on the rfhSP-D opsonised CNTs led to dampening of the pro-inflammatory immune response. Furthermore, like CNTs, Iron oxide nanoparticles are also recognized by complement pathway, but mainly by alternative complement pathway. Complement deposition enhanced their uptake by activated THP-1 macrophages and dampened the pro-inflammatory responses. These studies emphasise the significance of understanding the interaction between innate immune humoral factors including complement in developing nanoparticle-based drug delivery strategies.

Acknowledgement

First, I am grateful to **Dr Kishore**, **Dr Stenbeck** for giving me an opportunity to do a PhD at Brunel University and thank you to **Dr Jones** for accepting me as her PhD student at Kingston University. I would not be a PhD student without the acceptance from both universities. I would like to thank **Dr Stenbeck**, for her support, guidance, and encouragement during the course of study. The door to **Dr Stenbeck** was always open whenever I ran into a trouble spot or had a question or queries.

Thank you to **Dr Kishore** for his diligence and constructive criticism during the research and while writing the thesis. He consistently encouraged and steered me in the right direction whenever she thought I needed it. I also greatly appreciate his views for details. He continuously encouraged me with comments, criticism or with stories, jokes, sweets, cakes and lovely meal.

I would also like to thank **Dr Jones** for her support in finalising reports and thesis. I am gratefully indebted to her for very valuable support both academically and non-academically when in need.

I would also like to acknowledge **Dr Natasha Hill** for her encouragement in pursuing PhD while doing a master degree with her.

I am very grateful to have them as my PhD supervisors and without their passionate participation and input, PhD would not have been possible to complete.

I would also like to thank **Prof. Bob Sim**, Oxford University and **Dr Pondman** for igniting interest inside me on nanoparticles. I am very grateful to be publishing several papers with you. I would also like to thank **Dr Emmanuel Flahaut**, Toulouse University for providing various forms of nanotubes for this PhD work. I would also like to acknowledge all the staff of ETC, Brunel University, particularly **Dr Ashley Howkins** for giving me his valuable time and assisting me in preparation for TEM imaging. I would also like to acknowledge to **Dr Tsolaki** in providing training and support in qPCR, **Dr Pathan** in being always available in drawing blood and macrophages separation for the study.

I would like to acknowledge my entire friend, **Suliman**, **Arshad**, **Anu** for their company in office and in the lab. I would like to thank **Lubna Kouser** for her support and being a very strong and productive lab buddy and friend. Thank you to **Iesha** for being there when I need any advice and accompany in Kingston events.

Finally, I must express my very profound gratitude to my parents and to my lovely wife, **Niru**, for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. I am grateful to have a little sweet daughter, **Nisu**, who continuously deterred me from my work but thoroughly enjoyed her company. This accomplishment would not have been possible without them. Thank you.

Author

Basudev Paudyal

Table of Contents

Decleration......i

Abstract…......ii

Acknowledgements......iii

Table of Contents......v

List of Abbreviations......x

List of Figures......xv

List of Tables......xviii

1 Chapter I...... 1

Review of literature: Complement activation by nanoparticles and implications

1.1 Nanomedicine...... 2

1.2 Nanoparticles...... 3

1.2.1 Characteristics of NPs...... 5

1.2.2 Magnetic NPs...... 5

1.2.3 Carbon NPs...... 7

1.3 Delivery of nanoparticle: tumour-targeting mechanism...... 9

1.3.1 Passive targeting...... 10

1.3.2 Active targeting...... 12

1.4 Nanoparticle clearance...... 14

1.5 Immune system...... 16

1.5.1 Innate immunity...... 17

1.5.2 Adaptive immunity...... 21

1.6 NPs interaction with innate immune components...... 24

1.6.1 Human plasma proteins...... 24

1.6.2 Neutrophils...... 25

1.6.3 Basophil...... 26

1.6.4 Eosinophils ... *27*

1.6.5 Mast cells ... *28*

1.6.6 Natural Killer (NK) cells ... *29*

1.6.7 Complement pathway ... *29*

1.7 Intracellular distribution ... 41

1.8 Cytokine, chemokine, inflammation and immune response ... 43

1.8.1 Minimizing the pro-inflammatory nature of NPs ... *49*

1.9 Lung innate immunity and pulmonary surfactant proteins ... 50

1.9.1 Functions of SP-A and SP-D ... *51*

1.9.2 Ligands and receptors for SP-A and SP-D ... *57*

1.9.3 Pulmonary toxicity and immune reactivity due to NPs ... *59*

1.10 Genomic toxicity associated with NPs ... 61

1.11 Thesis scope ... 65

2 Chapter II ... 69

General Materials and Methods

2.1 Preparation of NPs (CNTs and IONP) ... 70

2.2 Transmission electron microscopy contrast staining ... 70

2.2.1 Preparation of uranyl acetate and lead citrate ... *70*

2.2.2 Staining for TEM ... *71*

2.3 Biotinylation of CNTs ... 71

2.4 Coating of CNTs with Proteins. ... 72

2.5 Complement activation and consumption assay ... 72

2.5.1 Classical pathway ... *72*

2.5.2 Alternative pathway ... *73*

2.5.3 Activity of bound properdin in complement consumption ... *74*

2.6 SDS (Sodium dodecyl sulphate) and sample preparation ... 74

2.7 Western blot ... 75

2.8 Fluorescence Microscopy 76
2.8.1 Cell culture and differentiation of monocytes 76
2.8.2 Microscopy of uptake of CNTs 76
2.8.3 Co-localization of IONPs with dextran (a marker for fluid phase endocytosis). 77
2.8.4 Co-localization of IONPs with lysotracker 77
2.8.5 NF-kB nuclear translocation 78
2.9 Determination of cytotoxicity of NPs 78
2.10 Quantification of uptake of CNTs 79
2.11 Measurement of THP-1 cell cytokine and transcription factor mRNA expression using quantitative RT-PCR 80
2.12 Multiplex cytokine array analysis 81
2.12.1 Preparation of reagents and sample for immunoassay 83
2.12.2 Multiplex procedures 83
2.13 Statistical analysis 84

3 Chapter III 85

Interaction between human properdin and carbon nanotubes

3.1 Abstract 86
3.2 Introduction 87
3.3 Results 90
3.3.1 Properdin and TSR4+5 bind CNTs. 90
3.3.2 MBP-TSR4+5 coated on CNTs inhibited complement consumption via the alternative pathway 92
3.3.3 Properdin, but not TSR4+5, enhanced CNT uptake by THP-1 cells 94
3.3.4 Pro-inflammatory cytokines are upregulated by both properdin and TSR4+5 CNTs 97
3.3.5 Properdin or TSR4+5 CNTs up-regulate pro-inflammatory cytokines/chemokines and other soluble factors in THP-1 cells 99
3.3.6 Properdin or TSR4+5 -CNTs induced nuclear translocation of NF-κB 104

3.4 Discussion 106

4 Chapter IV 111

Interaction between Surfactant Protein D and carbon nanotubes

4.1 Abstract 112

4.2 Introduction 113

4.3 Results 116

4.3.1 SDS PAGE and TEM analysis showing rfhSP-D bound to CNTs 116

4.3.2 Coating of CNTs with rfhSP-D enhances complement consumption 117

4.3.3 rfhSP-D enhances uptake of CNTs by THP-1 macrophage cell lines 118

4.3.4 Cytokine and transcription factor mRNA expression THP-1 treated with rfhSP-D and complement bound CNTs 121

4.3.5 Multiplex array analysis revealed dramatic down-regulation of pro-inflammatory cytokines/chemokines by THP-1 cells when challenged with complement deposited rfhSP-D coated CNTs. 123

4.3.6 NF-κB nuclear translocation in response to rfhSP-D coated CNTs can be halted by complement deposition 127

4.4 Discussion 129

5 Chapter V 135

Immune Interaction with Iron Oxide Nanoparticles

5.1 Abstract 136

5.2 Introduction 137

5.3 Results 140

5.3.1 CNTs and Iron oxides NPs activates the complement pathway 140

5.3.2 Cytotoxicity of IONPs 141

5.3.3 Differential uptake of IONPs by THP-1 and U937. 142

5.3.4 Opsonisation enhanced IONPs' endocytosis but does not modulate intracellular fate 143

5.3.5 Complement deposition on IONPs modulates cytokine expression at transcriptional level. 146

5.3.6 Serum deposition on IONPs modulates the expression of cytokines, chemokines and other soluble factors. 148

5.4 Discussion 152

6 Chapter VI 159

General Conclusion and Future Perspective

7 References 165

8 List of publications 234

List of Abbreviations

4-HHE: 4-hydroxy-(2E)-hexanal

AAV: Antibody-associated vasculitis

APC: Antigen presenting cells

ATCC: American Type Culture Collection

ATP: Adenosine Triphosphate

BALF: Bronchoalveolar lavage fluid

BCR: B-cell receptor

CARPA: complement-activation related pseudoallergy

CCL:chemokine (C-C motif) ligand

CD: Cluster of differentiation

CIE: Clathrin-independent endocytosis

CLR: C-type lectin receptor

CMC: Carboxymethyl cellulose

CME: Clathrin-mediated endocytosis

CNTs: Carbon nanotubes

COPD: Chronic obstructive pulmonary disease

CR: Complement receptors

CRD: Carbohydrate recognition domain

CSC: cancer stem cells

Ct: cycle threshold

CTL: Cytotoxic T cell

CXCL:chemokine (C-X-C motif) ligand

DAMP: Damage-associated molecular patterns

DC: Dendritic cells

DOX: Doxorubicin

DPPC: Dipalmitoylphosphatidylcholine

DWNTs: Double-walled nanotubes

EDTA: Ethylenediaminetetraacetic Acid

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptors

EGTA: ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

EPO: Eosinophil peroxidase

EPR: Enhanced permeability and retention effect

ESF: European science foundation

FGF: Fibroblast growth factor

FLT-3L: FMS-like tyrosine kinase 3 ligand

GM-CSF: Granulocyte macrophages-colony-stimulating factor

GO: Graphene oxide

gp120: Glycoprotein 120

GRO: Growth related oncogen

HER: Human epidermal receptor

IFN: Interferon

IL: Interleukin

ILC: Innate lymphoid Cell

IONP: Iron oxide nanoparticles

IP: Interferon induced protein

IP3: Inosito; 1,4,5-triphosphate

LPS: Lipopolysaccharides

MAC: Membrane attack complex

MAPK: Mitogen activated protein kinase

MASP: membrane-associated serine proteases

MBL: mannose-binding lectin

MCP: Monocyte chemotactic protein

MDA: Malondialdehyde

MDC:Macrophage-derived chemokine

MFI: Mean fluorescent intensity

MHC: Major histocompatibility complex

MIG:Monokine induced by gamma interferon

MIP:Macrophage Inflammatory Proteins

MMP: matrix metalloproteinase

MN: Micronucleus

MPO: Myeloperoxidase

MR: Mannan receptor

MRI: Magnetic resonance imaging

MTT: 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide

MWNTs: Multi-walled nanotubes

NADPH:Nicotinamide adenine dinucleotide phosphate

NAMP: Nanoparticle-associated molecular patterns

ND: Nanodiamonds

NET: Neutrophil extracellular traps

NF-κB: Nuclear factor *kappa* B

NK: Natural killer cells

NLR: Nucleotide-binding oligomerization domain (NOD) like receptor

NLRP3: Nod-like receptor protein 3

NPs: Nanoparticles

OPD: o-Phenylenediamine dihydrochloride

PAMP: Pathogen-associated molecular patterns

PARP: Poly(ADP-ribose) polymerase

PBMC: Peripheral blood monocytes

PBS: Phosphate buffer saline

PCL: Polycaprolactone

PCR: Polymerase chain reaction

PDGF: Platelets derived growth factor

PE: Phycoerythrin

PEG: Polyethylene glycol

PEO: Polyethylene oxide

PLC: Phospholipid C

PLGA: Polyactic-co-glycolic acid

PMA:Phorbol myristate acetate

PRR: Pattern recognition receptors

PS: Polystyrene

rGO: reduced Graphene Oxides

ROS: Reactive oxygen species

SAEC: small airway epithelial cells

sCD40L: soluble CD40 ligand

SIRPα:Signal regulatory protein α

SP-A: Surfactant protein-A

SP-D: Surfactant protein –D

SPION: Superparamagnetic iron oxide nanoparticle

SR: Scavenger receptor

SWNTs: Single-walled nanotubes

TCR: T-Cell receptor

TEM: Transmission electron microscopy

TGF: Transforming growth factor

TLR: Toll-like receptors

TNF: Tumour necrosis factor

TSR: Thrombospondin type I repeat

VCAM: Vascular cell adhesion molecule

VDJ: Variable, diversity, joining

VEGF: vascular endothelial growth factor

List of figures

Chapter I

Figure 1.1-1 Examples of commonly used NPs in biomedical applications. 4

Figure 1-1-2 Structure of starch functionalized magnetic nanoparticle used in this study. 6

Figure 1-1-3 Sketch of carbon-based nanoparticles used in biomedicine. 9

Figure 1-1-4 Nanoparticle drug delivery methods. 13

Figure 1-5 Nanoparticles interaction with the immune system 17

Figure 1-6 The three complement pathways: Classical, Lectin and Alternate pathways with different recognition strategies. 30

Figure 1-7 Pathways of entry of NPs into the cells. 41

Figure 1-8: Multimeric structure of SP-A and SP-D. 52

Chapter II

Figure 2-1: The schematic representation of the principle of the Milliplex assay. 82

Chapter III

Figure 3-1: Sketch of trimeric properdin (Left) and its TSR modules (Right). 89

Figure 3-2: Binding of recombinant full-length properdin and MBP-TSR4+5 to CNTs91

Figure 3-3: Complement alternative pathway activation by properdin and TSR 4+5 coated CNTs. 93

Figure 3-4: Uptake of biotin-CNTs coated with properdin and TSR 4+5 by differentiated THP-1 96

Figure 3-5: Cytokine gene expression profile of THP-1 cells treated with properdin or TSR 4+5 coated or uncoated CNTs. 98

Figure 3-6: Multiplex array analysis of supernatants of THP-1 cells following treatment with properdin and TSR 4+5 or uncoated CNTs. *100*

Figure 3-7: Multiplex array analysis of supernatants of THP-1 cells following treatment with properdin and TSR 4+5 or uncoated CNTs. *101*

Figure 3-8: Multiplex array analysis of supernatants of THP-1 cells following treatment with properdin and TSR 4+5 or uncoated CNTs. *102*

Figure 3-9: Multiplex array analysis of supernatants of THP-1 cells following treatment with properdin and TSR 4+5 or uncoated CNTs. *103*

Figure 3-10: Properdin-CNTs and TSR4+5-CNTs induce nuclear translocation of NF-κB in THP-1 cells. *105*

Chapter IV

Figure 4-1: Binding of rfhSP-D to CNTs. *116*

Figure 4-2: Complement consumption by rfhSP-D coated CNTs. *117*

Figure 4-3: Differential uptake of CNTs by macrophage (THP-1) after coating with rfhSP-D, and with or without serum treatment (i.e. complement deposition). *119*

Figure 4-4: Differential uptake of CNTs by macrophage (THP-1) after coating with rfhSP-D, and with or without serum treatment (i.e. complement deposition). *120*

Figure 4-5: Cytokine gene expression of differentiated THP-1 cells challenged with rfhSP-D coated CNT with or without serum deposition. *122*

Figure 4-6: Cytokines analysis of supernatants of THP-1 cells treated with rfhSP-D bound CNT with or without serum. *124*

Figure 4-7: Growth factors, ligand and receptor analysis of supernatants of THP-1 cells treated with rfhSP-D bound CNT with or without serum. *125*

Figure 4-8: Chemokine analysis of supernatants of THP-1 cells treated with rfhSP-D bound CNT with or without serum. *126*

Figure 4-9: Effect of rfhSP-D and /or complement deposition on CNT on nuclear translocation of NF-kB..128

Chaptter V

Figure 5-1 Complement activation via a classical and alternative pathway................140

Figure 5-2: MTT assay to determine cytotoxicity of different NPs...............................141

Figure 5-3: Differential uptake of IONPs by human monocytic cell lines THP-1 (A) and U937 (B)...142

Figure 5-4: Co-localization of IONPs with fluorescent dextran with MW20,000 (a marker for fluid phase endocytosis)..144

Figure 5-5: Co-localization of IONPs with lysotracker 145

Figure 5-6: Cytokine gene expression profile of THP-1 cells in response to serum treated or untreated IONPs..147

Figure 5-7: Cytokines analysis of supernatants of THP-1 cells following treatment with Iron oxides particles (with and without serum) 149

Figure 5-8: Multiplex cytokine array analysis of supernatants of THP-1 cells following treatment with Iron oxides particles (with and without serum)...................................150

Figure 5-9: Multiplex cytokine array analysis of supernatants of THP-1 cells following treatment with Iron oxides particles (with and without serum)...................................151

List of Tables

Table 1-1: Preclinical staged carbon-based NPsfor drug delivery in cancer 10

Table 1-2: Passively tumour-targeted NPs in cancer therapy 11

Table 1-3 List of cytokine released by macrophages in response to various CNTs and IONP 45

Table 1-4: List of chemokines released by macrophages in response to various CNTs and IONP 46

Table 1-5 List of growth factors, ligand and receptors released by macrophages in response to CNTs and IONP 47

Table 1-6: The diverse function of SP-D, SP-A, and a recombinant fragment of human SP-D (rfhSP-D)] 53

Table 1-7: Ligands and receptors of SP-A and SP-D 58

Table 2-1: Components and volume for preparing 12% and 15% SDS-PAGE 75

Table 2-2: Terminal primers used for qPCR analysis 81

1 Chapter I

Review of literature: Complement activation by nanoparticles and implications

1.1 Nanomedicine

Delivering biological or therapeutic agents to the desired site with precision is a major problem in the treatment of many diseases. Problems such as limited effectiveness and lack of specificity with rapid clearance and undesired side effects have rendered the conventional use of drugs ineffective. Thus, strategies should be implemented to device a controlled drug delivery system to overcome these limitations. Such delivery systems not only provide protection against rapid drug degradation or clearance from the system but also lower the doses of drug required by enhancing drug concentration in targeted organ or tissues. Targeting cells or specific tissues by means of designed carriers for drugs or any other bioactive compounds is more a fundamental and successful approach that forms the basis of nanotechnology. This concept dates back to 1900s when Paul Ehrlich postulated the term "Magic Bullet" in which the drug is precisely targeted to the site of interest at the right time (Ehrlich and Sachs, 1902). Nanoparticles have a great potential to be used as drug carriers; in fact, nanotechnology is not far away from the model termed by Paul Ehrlich. It is aimed at enhancing release, reducing toxicity, enhancing solubility and bioavailability of drugs. In 2005, European science foundation (ESF) has embrace disciplines such as analytical tools, nano-imaging, nanomaterials and nano-devices, novel drug delivery system, clinical regulatory and toxicological issues to improve human health.

The use of nanotechnology in various sectors of therapeutics has revolutionized the field of medicine by designing biomedical tools for therapeutics and diagnosis. Designed nanostructures have possibilities to offer better pharmacokinetic properties, controlled and sustained release targeting a specific part of the body. Such targeted delivery system can decrease drug resistance by reducing dose needed (Lee *et al.*, 2006; Dutta and Jain, 2007), decrease toxicity by increasing the stability of drug and

formulation(Brewer, Coleman and Lowman, 2011), enhance solubility and bioavailability (Farokhzad and Langer, 2006). Such advantages have led to the revolutionisation of the drug development process and have changed the landscape of the pharmaceutical industry.

1.2 Nanoparticles

Nanoparticles (NPs) are ultrafine-engineered materials having the dimension in the order of 100th of nm or less. They are engineered in different sizes, shapes, composition based on requirement or functionality (Figure 1.1-1). NPs used in biomedicine can be divided into different categories: hard and solid particles; soft particles and others (Bhatia, 2016). Hard and solid particles include metal and ceramic NPs such as gold particles, iron oxides, and carbon nanotubes. They have a defined core size though overall size and shape may vary based on functionalization. The second categories, soft particles, typically include polymers like micelles, vesicles, liposomes, core-shell structure, polymer-drug conjugates, and gels. Their shape and size heavily vary based on environmental factors like pH, ionic strength, and temperature. In a third category, others, antibody-drug conjugates, albumin particles and composite NPs have been considered as (Bhatia, 2016).

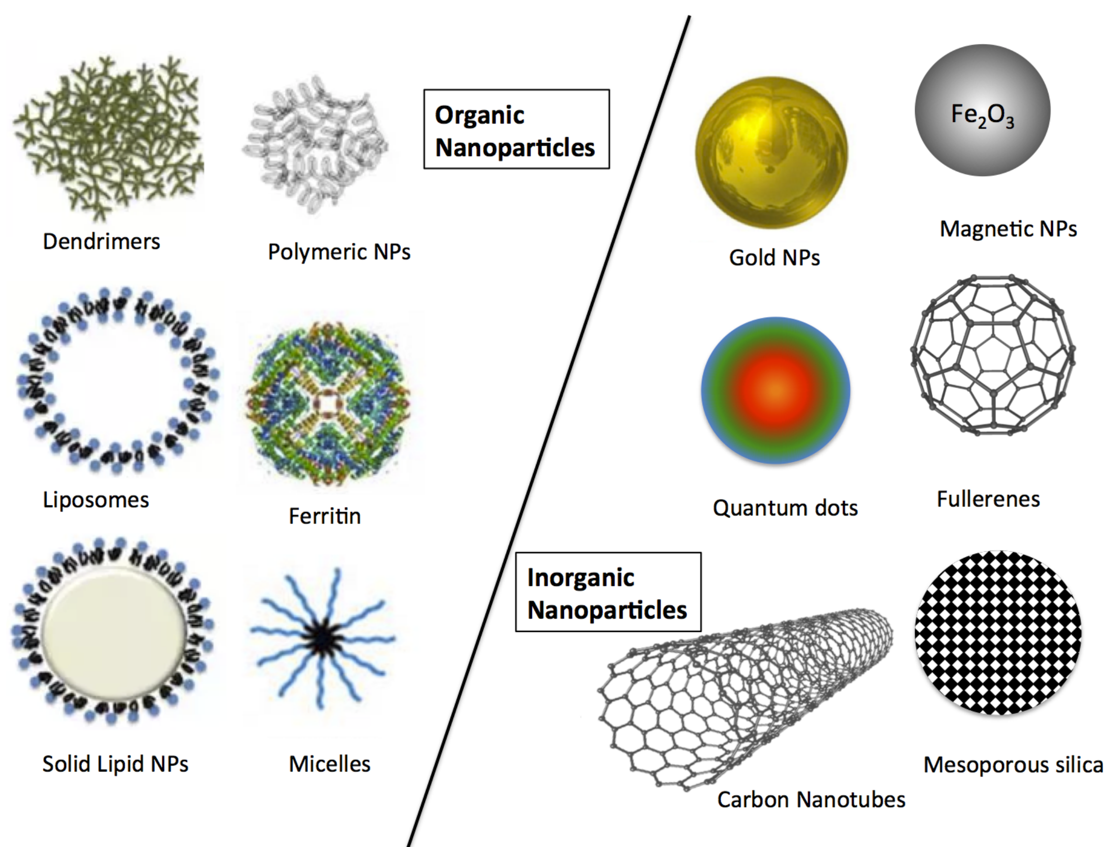


Figure 1.1-1 Examples of commonly used NPs in biomedical applications.

Organic NPs includes Dendrimers, polymers, liposomes, ferritin, micelles etc. Inorganic NPs included metallic NPs like iron oxides, gold, quantum, silica etc. and carbon-basedNPs like carbon nanotubes, fullerenes etc.

Furthermore, based on the chemical composition of nanoparticle core, NPs can be divided into organic and inorganic NPs (Xing *et al.*, 2014). Organic NPs consist of Dendrimers (Abbasi *et al.*, 2014), micelles (La *et al.*, 1996), liposomes (Akbarzadeh *et al.*, 2013), ferritin (Drysdale, 1976), hybrid and compact polymeric NPs (Lipid polymers, lipid-PEG shells) (Wang *et al.*, 2017b) etc. whereas gold NPs (Liang, Wang and Liu, 2009), quantum dots (Juzenas *et al.*, 2008), silica (Liberman *et al.*, 2014), iron oxides NPs (Woo *et al.*, 2004), carbon based NPs (fullerenes, carbon NPs, graphene) (Cha *et al.*, 2013) etc. are considered as inorganic NPs.

1.2.1 Characteristics of NPs

NPs surface to volume ratio increases with decreasing their size and for the small particle size; their properties are no longer dominated by the bulk of the materials. Thus, NPs properties behave differently than their larger counterparts in terms of their size, diameter and charge, which influence the in vivo behaviour of NPs and their physical stability in the biological environment. Features like in vivo performance, drug release profile and stability are affected by the surface charge of the NPs.

Various other properties are considered in the design of NPs for biomedical use. Properties like Carrier-drug interaction, charge determination, chemical analysis of surface, drug stability, NPs dispersion stability, particle size and distribution, release profile, surface hydrophobicity is highly considered (Rodzinski *et al.*, 2016). These factors also influence rapid clearance from the system during systemic delivery (Alexis *et al.*, 2008)

1.2.2 Magnetic NPs

Magnetic NPs or iron oxide NPs (IONP) are developed with a view to targeting specific sites of the body using an external magnetic field (Child *et al.*, 2011). Metallic magnetic particles are composed of magnetic elements such as cobalt, nickel, iron. Magnetite, maghemite, cobalt ferrite and chromium dioxide, oxide form of metals, are used in biomedicine (Bao *et al.*, 2016). Magnetic NPs on their own are poorly biocompatible, non-biodegradable and chemically unstable in a physiological environment. They need to be functionalized to attain colloidal stability, longer circulation life and lower toxicity (Fratila *et al.*, 2014). Due to the large surface area to volume ratio, NPs tend to aggregate. Functionalization prevents from undesirable aggregation by reducing surface energyand provides uniformity in the solution. Functionalization can be achieved by ligand addition or ligand exchange. Ligand addition involves adsorption of polymers

such as dextran(Easo and Mohanan, 2013), chitosan (Kostevsek *et al.*, 2016), polyethylene glycol (PEG) (Lak *et al.*, 2013), polyvinylpyrrolidone (PVP) (Merrifield *et al.*, 2013) etc to the surface by electrostatic or hydrogen bonding. In ligand exchange, the native surface is replaced with functional groups such as amines, carboxylic acids, thiols etc. Such functional groups assist in covalently linking of proteins, enzymes, receptors, oligonucleotides, and peptides for specific cell target (Lin *et al.*, 2008). However, the choice of the coating is reliant on the intended biomedical application. Figure 1-1-2 shows the sketch and transmission electron microscopy (TEM) figure of starched functionalized magnetite (Iron oxide NPs) used in this study.

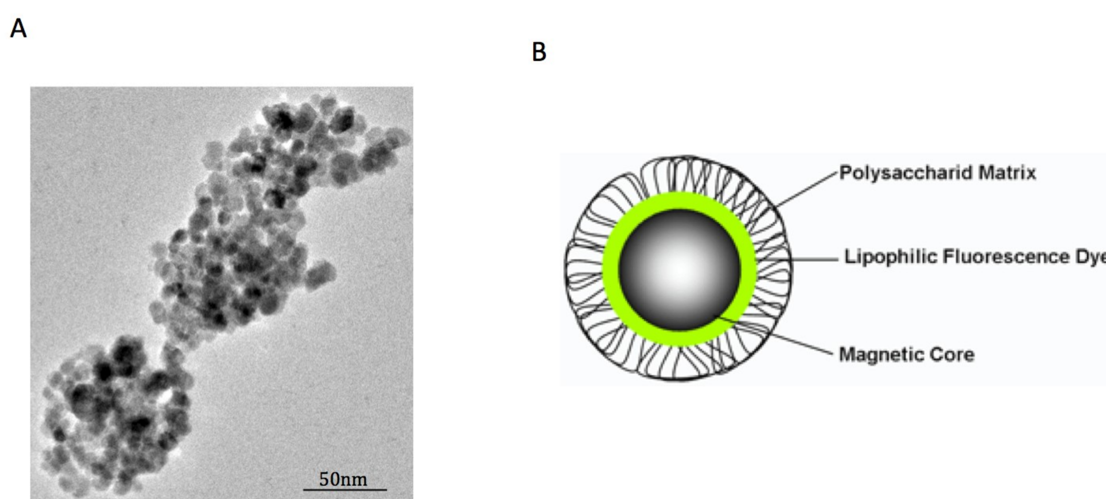


Figure 1-1-2 Structure of starch functionalized magnetic nanoparticle used in this study.

A) TEM image of magnetic NPs. An aqueous suspension of IONPs was adsorbed on carbon grid and micrographs were recorded using a JEOL 2100 FEG-TEM operating at 80Kv, and the images were processed using Gatan microscopy suite software (Gatan, Inc). **B)** Sketch of starch functionalized iron oxide nanoparticle. Iron oxide core is covalently functionalized with lipophilic fluorescence dye and polysaccharide matrix, starch for uniform suspension in aqueous solution.

Magnetic NPs have been widely used as contrast agent (Wei *et al.*, 2017) or iron supplement (Balakrishnan *et al.*, 2009). Some of the approved magnetic NPs are Feridex, Peraheme, Resovist, Ferristene, Ferumoxsil, ferumoxytol (Schutz *et al.*, 2013). However, Feridex has been discontinued due to complement activation and

hypersensitivity (Banda *et al.*, 2014). IONPs have also been studied as potential cancer therapy inducing hyperthermia (Lee *et al.*, 2011). Magnetic particles (magnetite cationic liposomes) are injected into a tissue and exposed to external magnetic field allowing heat generation up to the range of 42-45^{0}C, thus irreversibly damaging the cancer tissues by causing necrosis (Tanaka *et al.*, 2005).

1.2.3 Carbon NPs

Carbon nanotubes (CNTs) are tubular structures of carbon, first reported in 1991 by Iijima (Iijima, 1991). Each carbon atom is connected to another 3 carbon atom in hexagonal networks or honeycomb attics arranged in a graphite sheet rolled up into a cylinder (Figure 1-1-3). One or both ends are closed by a buckyball. Based on the rolling orientation of graphene sheet, CNTs are classified as an armchair, zigzag and chiral nanotubes. Depending on the number of concentric carbon tubes, they can be divided into single-walled nanotubes (SWNTs) (Iijima and Ichihashi, 1993), double-walled (DWNTs) or multi-walled nanotubes (MWNTs) (Iijima, 1991). DWNTs have been studied in this work. Apart from carbon nanotubes, other allotropic carbon NPs have been described including graphene (graphene oxide and reduced graphene oxides) (Novoselov *et al.*, 2004), nanodiamonds (Kurbatov *et al.*, 2010), fullerene (Kroto *et al.*, 1985; Krätschmer *et al.*, 1990) and nano-onions(Ugarte, 1992). Graphene isthe 2D structure of a thin layer of trigonally bonded sp^2carbon atoms together in a hexagonal honeycomb lattice (Figure 1-1-3). Oxidised and reduced version of graphene, graphene oxides (GO) and reduced graphene oxides (rGO) consists of partly tetrahedrally bonded sp^3 carbon atoms (Schniepp *et al.*, 2006). Unlike graphene, nanodiamonds (ND) are crystal in structure with 2-8 nanometers in diameter consisting of two closely packed interchangeable graphite (sp^2) and diamond (sp^3) bonds (Kurbatov *et al.*, 2010) (Figure 1-1-3). An interchangeable bond provides ND with a very special property of flexibility.

Similarly, fullerenes are 3D in structure, stable and smallest known nanostructure consisting 20(C_{20}) or 60 (C_{60}) or 70 (C_{70}) sp^2 carbon atoms arranged in hexagons and pentagons to form a spherical structure (Krätschmer *et al.*, 1990) (Figure 1-1-3). Concentric fullerenes of different carbon atoms arranged in a multilayer resembling that of an onion like shape are called carbon nano-onions (Figure 1-1-3).They are often called as multi-layered fullerenes (Ugarte, 1992). Like in multi-walled CNTs, each concentric closed layers are held together by van der Waals force. Carbon nano-onions can be of several layered, however, the stable configuration exists with inner layered fullerenes with carbon 60 (C_{60}) followed by fullerenes with carbon 240 (C_{240}), 540 (C_{540}) and 960 following the sequence: (C_{960}) (C_{60} @ C_{240} @ C_{540} @ C_{960}…..C_{60n}2) (n=1, 2, 3, 4, …..n) (Yoshida and Ōsawa, 1993).

The characteristics of CNTs are their unique size, geometry, atomic structure, ultra-lightweight, high electrical conductivity, high surface area to volume ratio, high mechanical strength, metallic or semi-metallic behaviour, collectively making them versatile carriers for biological active ingredients like drugs and genetic material. Despite showing promising results *in vitro* and *in vivo* in biomedical use like cell specificity, gene delivery, drug efficacy, gene silencing, and diagnosis, to date, none of the carbon-based drug delivery has been approved. Some of the preclinical phase carbon nanotubes based cancer drug conjugates are listed in Table 1-1

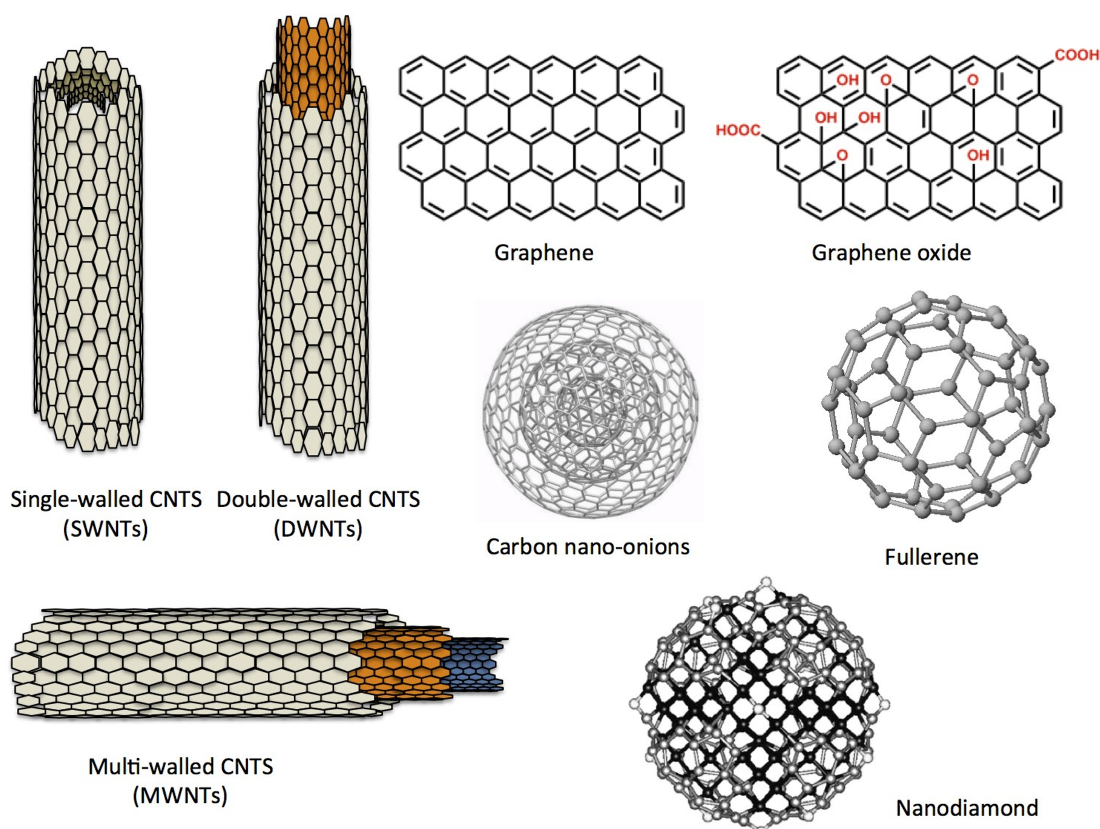


Figure 1-1-3 Sketch of carbon-based nanoparticles used in biomedicine.

Carbon-based nanoparticles cover a broad range of shape and size. Carbon nanotubes are hallowed tubes like structure with (DWNT and MWNT) or without (SWNT) concentric tubes. Fullerenes are caged-like fused-ring structure. Several concentric fullerenes give a structure of carbon nano-onions. Nanodiamonds are crystal structure fused with graphite and diamond bonds.

1.3 Delivery of nanoparticle: tumour-targeting mechanism

Use of NPs in drug delivery of cancer therapeutics is continuously growing. Furthermore, anti-cancer drugs are usually toxic to the human body and making them into systemic administrations is often associated with serious side effects. Nanoparticle-based anti-cancer drug delivery has become particularly attractive. Ideally, drug-loaded NPs should reach the targeted part without affecting the tissues and organ en route, minimal loss of their volume in blood circulation, and release of the drug after reaching the target. After releasing the drug, NPs should make their way to elimination from the

body. Targeting in tumour microenvironment can be achieved through passive or non-specific strategy and active or specific strategy

Table 1-1:Preclinical staged carbon-based NPs for drug delivery in cancer

Agent delivered	Status	Details of study	References
Cisplatin	Preclinical	Cisplatin loaded carbon nanohorn enhanced efficacy in murine lung cancer in vivo	(Ajima *et al.*, 2008)
Paclitaxel	Preclinical	Paclitaxel-loaded CNTs enhanced circulation life, tumour killing in 4T1 murine breast cancer	(Liu *et al.*, 2008)
Doxorubicin	Preclinical	Enhanced tumour cytotoxicity, blood circulation half-life and increase survival rate in chemo-resistant breast and liver cancer in mouse	(Chow *et al.*, 2011)
Epirubicin	Preclinical	Tumour regression in a mouse model of human triple negative breast cancer targeting EGFR.	(Moore *et al.*, 2013)
SiRNA	Preclinical	siRNA delivery using MWNTs in mice inhibited tumour growth.	(Podesta *et al.*, 2009)
Melittin peptide	Preclinical	Perfluorocarbon NPs enhanced therapeutic efficacy against human breast and skin cancer mouse xenograft tumour	(Soman *et al.*, 2009)
Radionuclide	Preclinical	Radio metal ion conjugated effectively targeted in vivo in murine xenograft lymphoma model	(McDevitt *et al.*, 2007)

1.3.1 Passive targeting

A tumour needs a continuous supply of nutrients and oxygen to grow and spread. For that purpose, the development of new blood vessels is inevitable. Development of new blood vessels is called angiogenesis and are often very rapid, vigorous and irregular (Folkman, 1971b), resulting in impaired and leaky blood vessels in a tumour where drugs or nanoparticles leak into tumour tissue through permeable vessels and retain in the tumour microenvironment. Such phenomenon is known as enhanced permeability and retention effect (EPR) first described by Maeda and Matsumura (Matsumura and Maeda, 1986; Maeda and Matsumura, 1989). EPR is now becoming the gold standard in

the passive targeting of cancer. Most of the NPs for drug delivery (nanocarriers) use the EPR effect to deliver the drug (Figure 1-1-4). It has been reported that within 1-2 days, 10-50 fold higher concentrations of drug-loaded NPs can be seen in tumour sites than in normal tissues (Iyer *et al.*, 2006). However, the concentration of NPs in tumour site depends upon the size of NPs. To this end, ideal nanocarrier size is suggested to be between 10 and 100nm for efficient extravasation in the leaky vasculature and to avoid filtration by kidney glomerular wall-sized 4-6nm. The charge of the particles should be as neutral as possible and must be hidden from the reticulo-endothelial system to avoid any opsonization and phagocytosis (Gullotti and Yeo, 2009). Apart from the surface charge, nanocarrier should be hydrophilic to escape possible macrophage engulfment. Hydrophilic functionalization can be achieved by coating the surface with a hydrophilic polymer like polyethylene glycol (PEG) and polyethylene oxide (PEO). PEG and PEO prevent opsonisation (Jeon *et al.*, 1991; Peracchia *et al.*, 1999) and thus providing longer blood circulation life (Kwon *et al.*, 1994). Passive targeting is restricted to use only when the vessels are permeable unlike a hypovascular tumour such as (Unezaki *et al.*, 1996) prostate and pancreatic cancer (Unezaki *et al.*, 1996; Maeda, Bharate and Daruwalla, 2009).

Table 1-2: Passively tumour-targeted NPs in cancer therapy

Drug	Name	Target	References
Albumin-Paclitaxel	Abraxane®	Metastatic breast cancer	(Abraxane, 2017)
Doxorubicin paclitaxel	Livatag®	Hepatocarcinoma	(Trochon-joseph *et al.*, 2016)
Liposomes-doxorubicin	Doxil®	Ovarian Metastatic breast cancer Kaposi sarcoma	(Janssen, 2015)
Liposomes-doxorubicin	Myocet®	Breast cancer	(Harris *et al.*, 2002)
Liposomes-Daunorubicin	DaunoXome®	Kaposi sarcoma	(Rosenthal *et al.*, 2002)
Liposomes-Vincristine	Onco-TCS®	Non-Hodgkin lymphoma	(INEX, 2004)

1.3.2 Active targeting

In active targeting, antibodies, peptides, and ligands are coupled to the nanocarrier to act as receptors to the target site (Figure 1-1-4). Use of magnetic NPs is considered as active targeting because the external magnetic field is created to guide them to the site of interest. Such approach is revolutionizing the use of nanomedicine in cancer treatment. Though it is highly selective, versatile and less likely to cause side effects, it has to overcome some barriers before interacting with receptors on tumour and release the conjugated drug. As the main principle behind active targeting is to facilitate receptor-ligand interactions, unavailability of receptor on cells or saturation of receptors by endogenous ligand should be overcome for successful delivery of drug to the target site. Drug conjugated nanoparticles should not be immunogenic because ligand for receptor conjugated on nanoparticles could activate the immune system causing the rapid clearance of by macrophages (Tolcher *et al.*, 1999). In some cases, receptors may not be expressed on the surface rather they might reside inside the cells or are internalized making them very hard to reach. So there should be an abundant and homogenous expression of receptors on the target cells or a tumour(Capone, Papsidero and Chu, 1984). Receptors could be already bound with ligand resulting in difficulties of binding of NPs.

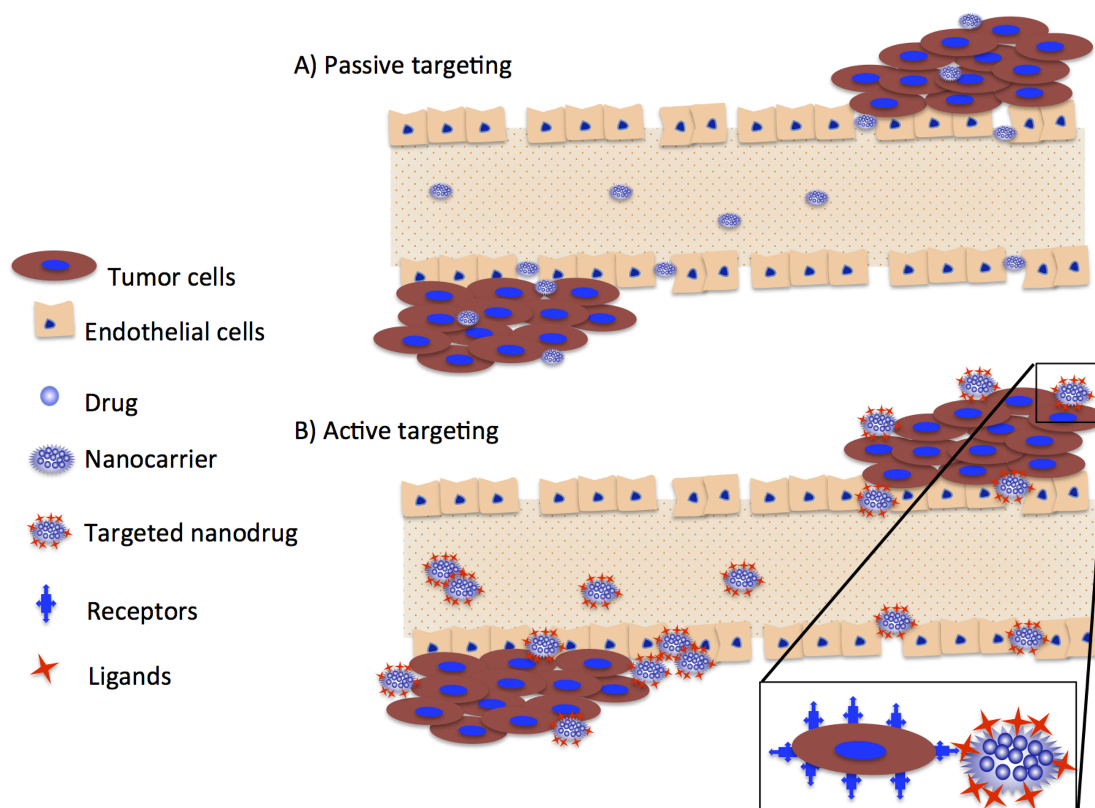


Figure 1-1-4 Nanoparticle drug delivery methods.

Nanocarriers reach tumour site by using either passive targeting or active targeting. A) Passively targeted delivery. Nanocarriers reach target sites by extravasation via leaky vessels. Nanocarriers are accumulated in the extracellular environment, not within tumour cells. B) Actively targeted delivery: Antibody decorated on NPs is targeted against tumour cells. NPs extravagate through leaky blood vessels and accumulate on tumour cell membranes and enter the cells. (Danhier, Feron and Preat, 2010)

In the active targeting approach, tumours are targeted via two methods; targeting the cancer cell and targeting the tumoural endothelium. Cancer cells over express numerous receptors on their surface. The aim of tumour cancer cells based target strategy is to improve the cellular uptake of the nanocarriers upon binding of the ligand on the NPs to a receptor on the cell surface (Kirpotin *et al.*, 2006). Internalised NPs release the drug inside the cells thus causing cell death. Such approach is highly efficient in case of delivery of DNA, RNA, siRNA or protein. The popular receptors targeted strategy includes transferrin receptor (Daniels *et al.*, 2006; Dixit *et al.*, 2015), folate receptor (Muralidharan *et al.*, 2016), glycoproteins (Punfa *et al.*, 2014; Iangcharoen *et al.*, 2011), epidermal growth factor receptors (EGFR) (Acharya,

Dilnawaz and Sahoo, 2009) and human epidermal receptor-2 (HER-2) (Yao *et al.*, 2016). These receptors are expressed by up to 100 fold compared to expression in normal cells making them available for interaction with the conjugated ligand on NPs.

Tumours are highly vascularized. Targeting the tumoural endothelium and the associated marker looks promising by causing blockage in oxygen and nutrient supply. In fact, the idea of killing cancer cells by cutting off their nutrient supply dates back to 1971 (Folkman, 1971a). The main targets of the tumoural endothelium includes vascular endothelial growth factor (VEGF) (Shi *et al.*, 2015), $\alpha_v\beta_3$ integrin (Desgrosellier and Cheresh, 2010), vascular cell adhesion molecule-1 (VCAM-1) (Gosk *et al.*, 2008) and matrix metalloproteinase (MMPs) (Hatakeyama *et al.*, 2007)

Passive target based nanocarriers have already been approved and active target strategy looks more specific and versatile. However, as of now only a median of 0.7% administered are found to be localized within a solid tumour (Wilhelm *et al.*, 2016). In this scenario, to increase the efficiency, we need to increase the amount of administered dose. As more than 99% of administered NPs end up residing in the off-target site, high nanoparticle doses may increase the risk of toxicity. This brings several questions to light regarding the fate of administration and other players in nanoparticles-based drug delivery system, which could influence their efficacy.

1.4 Nanoparticle clearance

Immune system strongly influences the effectiveness of intravenously administered NPs. Innate immune components recognize, sequester, degrade and eliminate foreign particles like viruses, fungi, parasites, and bacteria. The innate immune system is the first line of defence and is non-specificwhereasthe adaptive immune system is more specific and comprises of B cells and T cells. Both systems work in efficient removals of foreign particles in circulation. In brief, recognition of foreign particles starts with

opsonisation of foreign particles by complement components and other abundantly available proteins like albumin, antibodies and collectins. Opsonisation helps in recognition of particles, which results in activation of macrophages, dendritic cells, and leukocytes. Phagocytosis occurs by professional phagocytic cells like macrophages, neutrophils. Fragments of the foreign particle are presented on the cell surface by antigen presenting cells (APC) such as dendritic cells, macrophages, B-lymphocytes for recognition by T-lymphocytes. Recognition of antigen presented on the APC results in activation of the acquired immune system. Activation of B-cells of the acquired immune system produces antibodies against those foreign particles further assisting in phagocytosis and degradation of particles. During the activation of both immune systems, several cytokines and chemokines are also produced. These small signalling molecules regulate the process to give a balanced immune response (Lanzavecchia, 1985; Clark and Kupper, 2005).

Apart from the immune system, mononuclear phagocytic cells in bone marrow, skin, lymph nodes, liver, and spleen sequester administered NPs. Most of the systemic NPs are sequestered by liver and spleen where degradation occurs for final elimination. Liver includes kupffer cells expressing scavenger receptors, which recognizes the opsonins on the NPs and trap them (Gustafson *et al.*, 2015). Similarly, macrophages residing in the white and red compartment of spleen known as splenic macrophages, engulf NPs and eliminate them (Bronte and Pittet, 2013).

Another system competing with tumours for circulating NPs is the renal system (Liu *et al.*, 2013). The epithelial lining of kidney glomeruli contains filtration slits of 4-6nm in width (Venkatachalam and Rennke, 1978). Any NPs with a cut-off size smaller than filter slit can pass through the epithelial lining and get collected into the proximal

convoluted tubule. Some NPs are endocytosed by the cells whereas the rest is passed to the bladder and eliminated (Nair *et al.*, 2015).

1.5 Immune system

Nanoparticles interact with the body either by accidental exposure such as industrial or environmental or deliberate exposure such as therapeutics through vaccination or drug delivery. Nanoparticles may enter the body through the various route of entry like inhalation, ingestion, systemic injection or direct contact. Thus, the interaction of nanoparticles with either blood or tissue or lung is unavoidable. Therefore, it is essential to study and understand the interactions between nanoparticles and all immune components. Here, the interaction of nanoparticles, mainly carbon nanotubes and magnetic nanoparticles, with the innate immune system has been studied.

The two wings of the immune system, innate and adaptive immune system (Figure 1-5), comprised of highly specialized immune cells, proteins and various other components which are very effective in recognition and clearance of foreign or altered self-agents as rapidly and safely as possible. Both systems get activated when foreign materials invade the body and undesirable activation of the immune response may lead to harmful consequences and may affect the fate of drug-laden NPs.

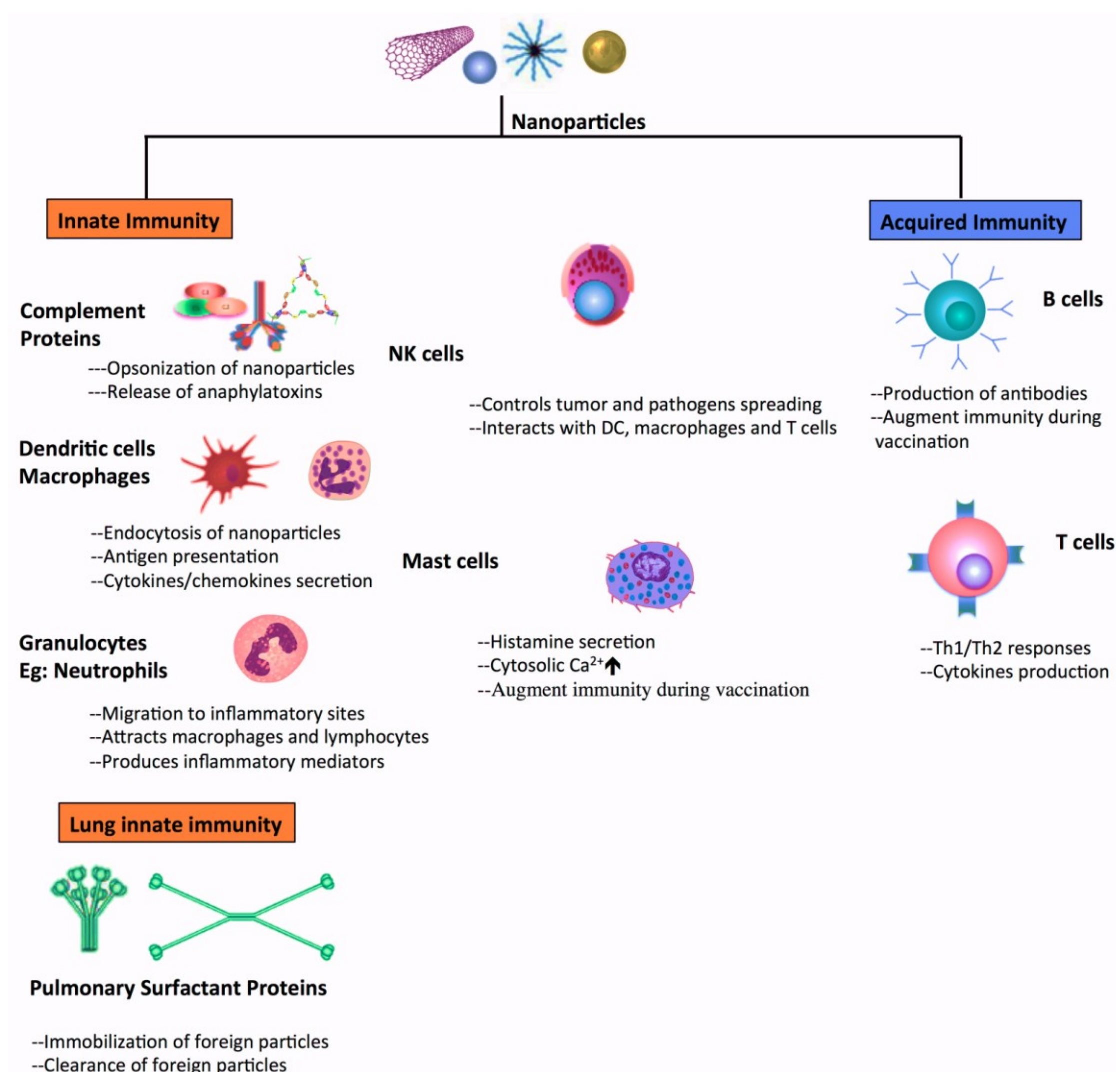


Figure 1-5 Nanoparticles interaction with the immune system

The immune system can be divided into two interacting parts, the innate immunity and adaptive immunity. Innate immunity is fast with broader specificity and comprises of various immune cells like a dendritic cell, macrophages, neutrophils, mast cells, NK-cells and soluble components like complement components. Adaptive immunity is rather slow but very specific and comprises of T-cells and B cells and antibodies. (Adapted from (Luo, Chang and Lin, 2015)

1.5.1 Innate immunity

Innate immunity is a broad-specific, rapid and first line defence system, which relies on pattern recognition receptors (PRRs) for the recognition of molecular pattern associated with pathogens known as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Janeway and Medzhitov, 2002; Matzinger, 1994). The main components of innate immune systems include physical

epithelial barriers (skin, tracts and secretions), antigen presenting cells such as dendritic cells; macrophages, monocytes, neutrophils, basophils, mast cells, eosinophils, natural killer (NK) cells and complement components. Phagocytes such as macrophages, monocytes, neutrophils and epithelial cells express PRRs on their surfaces (membrane-bound) or in the cytoplasm (Mogensen, 2009). These receptors are a key element in sensing and the clearance of altered self or non-self materials (eg. Nanoparticles and microbes) and trigger downstream effector function to eliminate the danger. These receptors include pattern classical pattern recognition receptors such as toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) (Brubaker *et al.*, 2015). PRRs bind to PAMPs such as microbial nucleic acids, lipoproteins, membrane components [peptidoglycans, Lipopolysaccharides (LPS)] and DAMPs such as nucleic acids, heparin sulphate, uric acid, ATP and intracellular organelles; mitochondria. Binding of PRR to the ligands leads to activation of phagocytes and transmit the signals to release signalling molecules such as cytokines, chemokines, growth factors etc. Recently, molecular docking studies showed that TLR4 directly interacts and binds with both CNTs, pristine and carboxylated CNTs with its side-walls and tip region and the binding mode is hydrophobic for pristine and electrostatics interactions for carboxylated CNTs (Mukherjee *et al.*, 2018a). Furthermore, it has also been shown that TLR2/4 sensed SWNTs resulting in MyD88 dependent activation of NF-kB leading to secretion of CCL5 in human macrophages. This finding shows that nanoparticle have a unique molecular pattern, termed as nanoparticle-associated molecular patterns (NAMPs), can be recognized by PRR on the surface of phagocytic cells (Fadeel, 2012). The activation of PRR stimulated immune cells to phagocytes, direct killing of pathogens, migration of cell to inflammatory sites,

the release of inflammatory cytokines and anti-microbial peptides (Takeda, Kaisho and Akira, 2003). Professional phagocytes when stimulated by PRR, phagocytes via either receptor-mediated endocytosis process such as involving complement receptors (CR), scavenger receptors (SR) (Section 1.6.7.3) or non-receptor mediated phagocytosis (Figure 1-7). Internalised foreign agents fuses with lysosomes containing degrading enzymes such as proteases, nucleases, hydrolytic enzymes, lipases and break into certain small products in low pH (3.8-5.0) condition (Turk *et al.*, 2002). Biodegradability of carbon nanotubes is not fully understood despite tremendous applications but studies carried out intracellular biodegradation show similar mechanism involving reactive oxygen-mediated degradation. Studies have shown that functionalized MWCNTs and iron oxide hybrids with CNTs are degraded by macrophages via Nrf2 signalling pathway by producing reactive oxygen species such as O_2*, H_2O_2, and OH* (Elgrabli *et al.*, 2017). CNTs length and functionalization (oxidisation) also influence in their biodegradation inside macrophages where short pristine CNTs are more prone to degradation than long CNTs (pristine or functionalized) compared to short functionalized. Degradation is intracellular pH-dependent (Landry *et al.*, 2016) and mediated by respiratory burst initiated by activation of NOX (NADPH Oxidase) (Hou *et al.*, 2016). Some reports have demonstrated that animal peroxidases such as myeloperoxidase (MPO) (Shvedova *et al.*, 2012), plant peroxidise (horseradish peroxidase) (Allen *et al.*, 2009) and eosinophil peroxidase (EPO) (Andon *et al.*, 2013) can degrade carbon-based nanoparticles. Antigen-presenting cells (APC) such as dendritic cells, monocytes/macrophages, B cells process these digested and fragmented foreign agents and presents on the cell surface via major histocompatibility complex class II (MHC II) to be recognized by Helper T lymphocytes ($CD4^+$ T cells). Cytotoxic T lymphocytes ($CD8^+$ T cells) recognizes the

processed antigen only via MHC I which is present in all nucleated cells and platelets except red blood cells (Gruen and Weissman, 1997). Neutrophils though are professional phagocytes, do not express MHC II and directly play role in $CD4^+$T cells activation. However, upon activation by GM-CSF, IFN-γ, neutrophil express surface MHC-II and costimulatory molecules but does not enable them to function as antigen-presenting cells to $CD4^+$ T cells (Gosselin *et al.*, 1993). Recently, neutrophils stimulated with cytomegalovirus pp65 and influenza hemagglutinin was able to express MHC-II molecules and co-stimulatory molecules activate memory $CD4^+$ T cells (Vono *et al.*, 2017) The concept of antigen presentation to either $CD4^+$T cells or $CD8^+$ T cells have been exploited in nanoparticles based antigen delivery for vaccination. Carbon-polymer composite conjugated with T cells growth factor IL-2, MHC class I and co-stimulatory ligand for $CD8^+$ T cells (anti-CD28) acted as APC and showed greater expansion of human $CD8^+$ T cells and expanded lymphocytes significantly delaying tumour growth in murine melanoma model (Fadel *et al.*, 2014). Similarly, nanoparticles, (polylactic-co-glycolic acid) (PLGA), loaded with a tumour associated antigen were efficiently internalised by DCs and enhanced the presentation of murine MHC -I and MHC II thereby, enhancing the production of IFN-γ by CD8 T cells and proliferation of CD4 T cells respectively (Ma *et al.*, 2011), thus providing a promising strategy for tumour immunotherapy. Antigen presentation by APCs, stimulation of T lymphocytes (helper T cells and cytotoxic T cells) and subsequent release of small signalling molecules like cytokines, chemokines and growth factors leads to adaptive immunity where APCs especially DC acts as a bridge between innate immunity and adaptive immunity due to their ability to activate naïve T cells by processing and presenting antigen via both MHC I and MHC II molecules to both CD8 and CD4 T cells (Rescigno *et al.*, 1998). DCs develop in bone marrow and mature upon exposure to PAMP (eg. LPS),

inflammatory cytokines (eg. IL-2, IL-7, IL-13, hepatocyte growth factor) and cell surface receptors (eg.CD40) and viral products (eg. Double-stranded RNA) and up-regulates co-stimulatory molecules, B7 (CD80/CD86) (de Saint-Vis *et al.*, 1998).

1.5.2 Adaptive immunity

The adaptive immunity is mediated by two cellular elements, T and B-lymphocytes. Both B and T cells can recognize any antigen due to this ability to create novel and unique antigen receptors by the process known as VDJ (variable, diversity, joining) recombination (Grawunder, West and Lieber, 1998), unlike cells in an innate immune system where cells have fixed inherited receptors. B cells bind to antigen via B-cell receptors (BCR) also known as membrane-bound immunoglobulin. Binding of antigen via BCR results in secretion of antibodies specific to bound antigen by differentiated B cells, the plasma cells (Treanor, 2012). T cell receptors (TCR), on the other hand, differ from BCR in several ways. BCR can be secreted as immunoglobulin unlike TCR, which is always cell surface bound. BCR binds to the native tertiary structure of antigen where TCR binds to the primary structure of antigen only presented via MHC II and I molecules (Griesser and Mak, 1994). Crosstalk between B cells and T cells is prominent in the adaptive immune system. $CD4^+$ T cells stimulate naïve B cells to proliferate and differentiate to memory cells and plasma cells by recognizing antigen presentation by B cells via MHC II molecules to T cells and secretion of a cytokine such as IL-4, IL-5 and IL-6 (Flynn *et al.*, 1998). T cells can be functionally divided into $CD4^+$ helper T cells and $CD8^+$ T cells (cytotoxic T cells, Tc cells, CTL). T cells are developed in bone marrow and maturation takes place in the thymus. Activation of CTL and $CD4^+$ T cells and differentiation into effector cell takes place by a combination of signal 1 and signal 2. TCR binding to MHC I (for CTL) and MHC II (for CD4 T cells) generate signal 1. Signal 2 is provided by costimulatory proteins, B7 proteins recognize the co-receptor

protein CD28 on the surface of T cells. Thus activated T cells produce IL-2 and binding of IL-2 on their receptors induces differentiation and proliferation of T cells. Activated CTL provides cell-mediated immunity by recognizing foreign antigen expressed on the cell surface, virally infected cells and even protecting against spontaneous malignant tumours (Castelli *et al.*, 2000). Activation of CTL is tightly regulated and inhibited by the inhibitory receptor, CTLA-4 regulating self-tolerance and T cell activation (Lohr *et al.*, 2003). Signal 1 and signal 2 on $CD4^{+}$T helper cells to polarize them into T_H1, T_H2 and Treg (T_H-regulatory) cells. Polarization is mainly dependent upon cytokine signal from DCs. Th1-polarising factor (IL-12. IL-27), T_H2 polarizing factor (CCL2) and Treg-polarizing factor (IL-10, TGB-β) from DCs polarizes CD4+T helper cells into T_H1, T_H2 and Treg cells respectively. T_H1 cells secrete Th1 cytokines (IFN-γ, TNF-β) and stimulate Tc cells inducing cell-mediated immunity. Th2 T cells secrete Th2 cytokines (IL-4. IL-5 and IL-13) and stimulate B cells. Treg cells secrete IL-19 and TGF-β and suppress the response of other T cells (Bilsborough *et al.*, 2003). Thus, T cells polarization helps to tune and regulate the adaptive immune response directly though, the signal comes from innate immune system intermediate cells, APCs.

1.5.2.1 Interaction of NPs with T and B cells

B and T lymphocytes, as a model for circulating cells, have been used to analyse the toxicity and the stimulatory effect of functionalized and non-functionalized CNTs. Amphotericin B, an antifungal drug, conjugated CNTs have shown less cytotoxicity to T cells than equal concentration of amphotericin in soluble form (Jurkat cell line) and result showed that amphotericin B conjugated CNTs can provide an antifungal activity better than amphotericin B alone (Benincasa *et al.*, 2011). Functionalized CNTs (oxidized-CNTs) have been studied as an immunotherapy by stimulating T cells. Anti-CD3 (T-cell stimulating antibody) and anti-CD28 (Co-stimulatory ligand) conjugated

with SWNTs revealed better T cells stimulation than similar concentration of soluble anti-CD3 and anti-CD28 in mouse splenocytes (Fadel *et al.*, 2008) and considerably more stimulation was observed in functionalized CNTs than their non-functionalized counterparts (Fadel *et al.*, 2010). Furthermore, a genotoxicity study showed that functionalized (amidation) CNTs did not affect in T cells proliferation contrast to non-functionalized CNTs (Cveticanin *et al.*, 2010). Functionalized (Oxidised and ammonium functionalized) MWNTs showed no cytotoxicity to peripheral blood monocytes (PBMCs) derived from healthy donors and none of these MWNTs induced T cells stimulation and expansion (Cveticanin *et al.*, 2010) contrast to earlier study which reported that oxidised MWNTs induces apoptosis of Jurkat T cells (Bottini *et al.*, 2006). MWNTs and SWNTs exposure to B cells have shown significant cytotoxicity and SWNTs were more severe cytotoxic than MWNTs as SWNTs caused cell membrane penetration as revealed by TEM images and further revealed that lymphocytes are more sensitive to CNTs than skin (HaCat) and lung (MSTO-211H) cell lines (Hu *et al.*, 2010). However, cytotoxicity was not observed in lymphocytes co-incubated with breast cancer cells line (MCF-7) with carboxylate MWNTs rather cytotoxicity was observed in MCF-7 cells and was T lymphocytes mediated by up regulating NF-κB expression and subsequent IFN-γ and TNF-α secretion (Sun *et al.*, 2011). One of the properties of CNTs, needling effect, has been exploited in the delivery of short interfering RNA (siRNA) inside $CD4^{+}$T cells to silent HIV-specific cell-surface receptor (CD4) and co-receptors (CXCR4/CCR5). PEGylated NPs were shown to interact with splenic B cells to secret anti-PEG-IgM in T cell-independent manner (Ichihara *et al.*, 2010; Ishida *et al.*, 2007). This raise an alert in the safe use of functionalized nanoparticles as functionalization have been considered less immunogenic and safe to immune cells due to less-toxic, more biodegradable and bio-

tolerable (Bourdiol *et al.*, 2013). A recent study showed that B cells actively uptake CMC functionalized CNTs (and augments pro-inflammatory cytokine secretion (TNF-α, IL-1β). Down-regulation of proinflammatory cytokines and upregulation of anti-inflammatory cytokines, IL-10 was observed by complement deposition on them (Pondman *et al.*, 2016). Similar, immune modulation was observed in T cells (Jurkat cells) however; uptake was much lesser than that of B cells (Pondman *et al.*, 2016).

1.6 NPs interaction with innate immune components

1.6.1 Human plasma proteins

Blood plasma contains 1,000 different proteins in various concentrations. Therefore, intravenously administrated NPs see competition between various biological molecules to adsorb on its surface. At first, smaller molecular weight and abundant protein (albumin and fibrinogen) get adsorbed and other proteins may later displace these proteins with a higher affinity towards the particle surface. This effect is known as Vroman'effect (Vroman, 1962). The layers of bound or adsorbed proteins around the particles are known as protein corona. Protein adsorbed with high-affinity form a "hard corona" where as proteins with low-affinity form the "soft corona". The bound molecule forms a corona that alters the size and interfacial composition of nanoparticles like effective size, surface charge, physicochemical properties. This gives a new identity to the nanoparticles, which is seen by immune cells differently as surface proteins or lipids provide different recognition patterns, alter the interaction with the immune components and modulate the immune response (Maiorano *et al.*, 2010). Corona, however, does not completely mask the native nanomaterial or functional group suggesting that the nanoparticles surface is exposed enough for receptor recognition by immune cells (Simberg *et al.*, 2009).

In a study on dextran functionalized magnetic particles, the formation of protein corona did not significantly modulate the nanoparticles circulation lifetime. In another study, adsorption of more serum proteins have been observed in negative and neutral surface charged super paramagnetic iron oxide nanoparticles (SPION) coated with polyvinyl alcohol polymer than dextran coated SPION (Sakulkhu *et al.*, 2014). Similarly, more protein adsorption was observed in negatively charged oxidized CNTs (Shannahan *et al.*, 2013; Rybak-Smith *et al.*, 2011). However, the adsorption of some abundant proteins such as serum albumin and low abundance proteins such as serotransferrin, α-fetoprotein and kininogen-1 are found to be non-specific to nanomaterial or the surface charge. Simberg *et al* have demonstrated the existence of IONP core binding proteins such as a histidine-proline rich glycoprotein, kininogen-1 and dextran binding protein such as mannose-binding lectins A and C (Simberg *et al.*, 2009). Serum albumin was found to be the most abundant protein on CNTs unlike on metallic nanoparticles (Deng *et al.*, 2009). Irrespective of functionalisation, CNTs were observed to bind serum albumin, apolipoprotein types, titin, keratin, α-S1-casein, α2-HS-glycoprotein and α1-antiproteinase (Shannahan *et al.*, 2013). Thus, protein corona is very multifaceted and different coronas exist for all nanoparticles of different size, surface charge, and coating. Thus, the composition of the proteincorona is exclusive to the nanomaterial and varies in many parameters.

1.6.2 Neutrophils

Neutrophils play a key role in the defence during acute inflammation via several strategies such as actively engulfment and degradation, degranulation and secretion of myeloperoxidase (MPO) and neutrophil extracellular traps (NETs) for the non-phagocytic killing of microbes (Papayannopoulos and Zychlinsky, 2009). They migrate first to an inflammatory site and produce inflammatory mediators including chemokines

such as IL-8, GROα, MIG, IP-10, I-TAC, MCP-1, MIP-1α, MIP-1β to attract other immune cells such as DCs, monocytes, NK cells, T-lymphocytes (Scapini *et al.*, 2000). Nanoparticles, gold and graphene oxides (GO), were found to trigger NETs release in primary human neutrophils (Bartneck *et al.*, 2010; Mukherjee, Bottini and Fadeel, 2017). More NETs release was observed in response to micrometre-sized GO and also induced disruption of lipid rafts in neutrophils (Mukherjee, Bottini and Fadeel, 2017). Recently, GO was found to undergo degradation by MPO and degraded GO was non-toxic to human lung cells (Mukherjee *et al.*, 2018b). MPO mediated intracellular degradation of carbon nanotubes by neutrophils have been reported. Like GO, biodegraded CNTs showed no pulmonary inflammation when aspirated into the mice lungs (Kagan *et al.*, 2010). In an inflammatory condition such as acute lung injury and ischemic tissue injury, where neutrophils are adhered on to the lining of vascular endothelium, piceatannol-loaded albumin nanoparticles have prevented vascular inflammation by internalization of drug-loaded nanoparticle and signalling to β2 integrin to detach, thereby detaching and dampening pro-inflammatory function (Wang *et al.*, 2014). Such therapeutic approaches are promising in the treatment of inflammatory diseases caused by neutrophil sequestration and unwanted activation.

1.6.3 Basophil

Basophil and eosinophil are bone marrow-derived cells that contribute to the inflammation associated with allergic diseases, asthma and helminth infections. They share the common phenotypic features in terms of their involvement in adhesion and migration via beta 1 and 2 integrins and stimulation and differentiation by common cytokines such as IL-3, GM-CSF and IL-5 (Valent *et al.*, 1989). Interaction of these cells with nanoparticles has not been studied efficiently despite the wide range of nanomaterials proposed in the potential development of nanomedicine. Using the blood

of healthy and allergic volunteers, SWNTs triggered significant basophil activation and development of allergic reaction observed by cell-surface CD63 expression (Kiss-Toth Dojcsak *et al.*, 2014). However, another carbon-based nanoparticle, fullerenes, showed anti-allergic properties *in vitro* and *in vivo* (Ryan *et al.*, 2007). Fullerenes showed no cytotoxicity to human basophils and dampened the release of basophil mediators such as histamine and proteoglycans. Furthermore, it inhibited basophil allergic responses by reducing antigen-induced cytoplasmic ROS levels and anaphylaxis in IgE- sensitized mice. Complement activation by fullerenes has not been reported. However, the presence of anti-IgGisotype-specific to fullerene in mouse indicates them as complement activating nanoparticles (Chen *et al.*, 1998). Complement activation and subsequent release of anaphylatoxins such as C3a and C5a would potentially stimulate basophils through their C3aR and C5aR receptors. Thus, further study is necessary to understand the allergic properties of NPs.

1.6.4 Eosinophils

Studies have shown that SWCNTs and MWCNTs promote the stimulation and infiltration of eosinophils in the lung, bronchoalveolar fluid and blood and promoting allergic immune responses in mice (Erdely *et al.*, 2011). Like neutrophils, eosinophil express peroxidase known as eosinophil peroxidase (EPO) is mainly responsible for destroying invading parasites including helminths. Reports suggest that eosinophil triggers the release of EPO in response to CNTs in mice lungs (Girtsman *et al.*, 2014) and EPO in vitro and ex vivo by activated eosinophils has shown to degrade oxidised SWNTs (Andon *et al.*, 2013). Other than CNTs, metallic NPs like nickel oxide has been shown to recruit eosinophils and neutrophil and involves the release of chemokines, eotaxin (Lee *et al.*, 2016).

1.6.5 Mast cells

Mast cells are non-phagocytic cells and play a vital role in allergic reactions, like basophils and eosinophils. They release inflammatory mediators such as histamine, proteases, cytokines/chemokines (IL-4, IL-13, and TNF-α), lipid mediators (leukotrienes, prostaglandins) (Borish and Joseph, 1992). During an allergic reaction, B-cells release IgE and IgE binds to mast cells surface via FcεR1 receptors. Binding of IgE triggers mast cells to degranulation. Degranulation is also induced by external toxins and endogenous cationic proteins secreted by eosinophils and neutrophils (Hogan and Schwartz, 1997). A recent study demonstrates that differentially functionalized CNTs can inhibit the cell viability, degranulation and histamine release in mast cell line model (Ede *et al.*, 2018). Pristine CNTs not only significantly reduce the cell viability but also inhibited IgE-FCεR1 mediated degranulation compared to carboxylated CNTs. Furthermore; SWNTs exposure resulted in a concomitant reduction in MAPK/ERK activation (Ede *et al.*, 2018). This indicates that pristine SWNTs can interact with IgE receptors on the mast cell surface preventing the binding of IgE. Suppression of mast cell pro-inflammatory degranulation function by SWNTs has previously been reported by Umemoto *et al.* (Umemoto *et al.*, 2014). SWNTs induce membrane rearrangement and subsequent suppression of receptor-mediated signalling pathway involving FCεR1 receptor. Similar effects were also observed with fullerenes in a mouse model (Ryan *et al.*, 2007). These reports suggested that carbon-based NPs area negative regulator of allergic mediator in mast cells. Similar effects were observed with different kind of NPs, polymeric NPs (PLGA NPs), in a mouse model in which NPs administration suppressed the antigen-induced systemic allergic response and inhibited histamine release (Tahara *et al.*, 2012). However, in contrast to these reports, Chen et al, demonstrated that metallic NPs (TiO_2) increase histamine release in rat mast cells.

These conflicting results indicate that systemic circulation of NPs may suppress or promote degranulation depending upon nanomaterials.

1.6.6 Natural Killer (NK) cells

NK cells are lymphocytes of innate immune system sharing the common lymphoid progenitor with B and T cells. They are associated with recognition and killing of virally infected cells and neoplastic cells via secretion of membrane-disrupting protein, perforin, granzymes and initiation of caspase-dependent apoptosis involving association of death receptors (Fas/CD95) on the target cells with ligands such as FasL and TRIAL on NK cells (Zamai *et al.*, 1998). NPs have been shown to affect NK cells activity and stimulation. NK function has been significantly suppressed in mice upon inhalation of MWNTs as shown by the loss of target cell lysis function (Mitchell *et al.*, 2007) However, human NK cells have demonstrated an increase in their activity and stimulated upon exposure (Delogu *et al.*, 2012). Recently, Loss of NK cells activity to kill target cell upon exposure to SWNTS and functionalized SWNTs have also been reported (Alam and Saxena, 2017). Alam and Saxena extensively studied two types of CNTs, pristine SWNTs and functionalized SWNTs. They reported that functionalized SWNTs induced inhibition of cellular proliferation, increased apoptosis, decreased degranulation, lowered ligand-death receptor interaction and lowered inflammatory cytokine (IFN-γ, TNF-α) release in NK cells. These results suggest that CNTs possibly have a negative effect on NK cells normal functions.

1.6.7 Complement pathway

A key component of the immune system that plays an important role in seeing and sensing foreign particles, their handling and eventually elimination through a series of the enzymatic reactions is the complement system. Complement recognizes foreign invades primarily through pattern recognition. It consists of at least 30 soluble and

membrane-bound proteins working in conjunction to maintain host defence, balanced inflammatory response, homeostatic and immune reactions (Ricklin *et al.*, 2010). There are three established pathways of complement activation: classical pathway, lectin pathway and alternative pathway (Figure 1-6). Pathways are initiated by the binding of initial complement components; Antibody and C1q in classical pathway, mannose-binding lectin and ficolin in lectin pathway and C3b and properdin in alternative pathway.

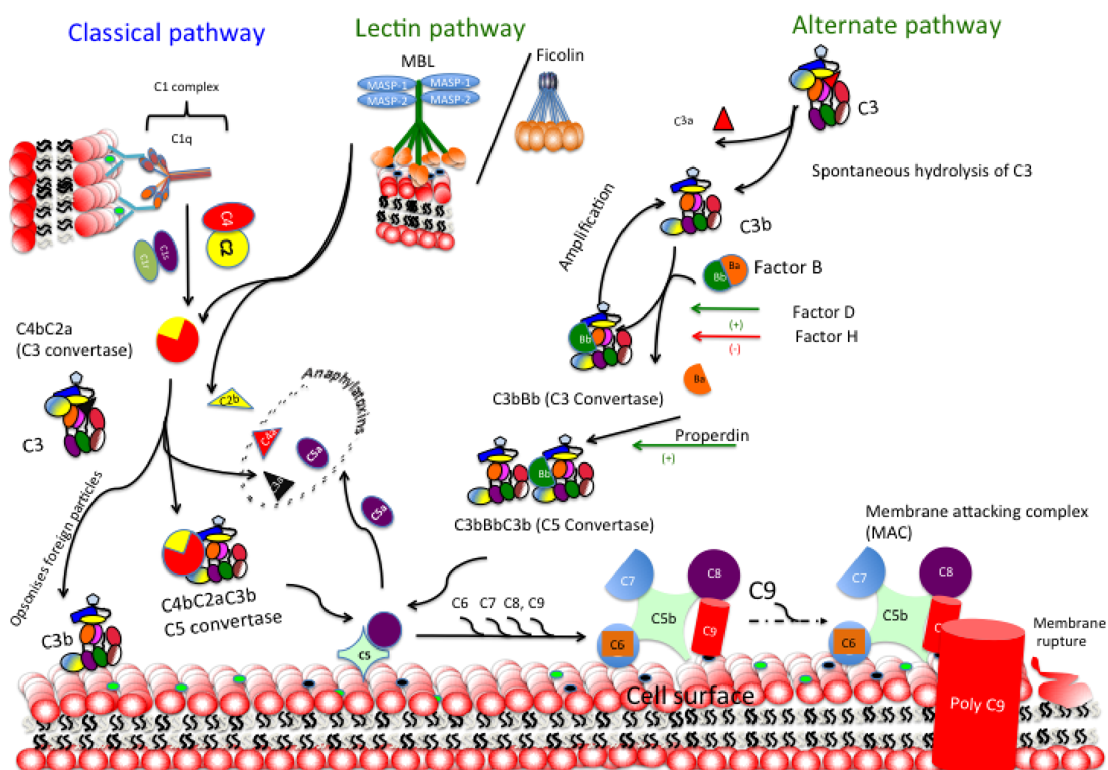


Figure 1-6 The three complement pathways: Classical, Lectin and Alternate pathways with different recognition strategies.

The classical pathway initiates by recognition and binding of antigen-antibodycomplexes via C1q, lectin pathway with the binding of mannose or carbohydrates moieties with MBL. Classical and lectin pathway converge in common C3 convertase (C4b2a). The alternative pathway is initiated with spontaneous hydrolysis of C3 to C3b. All pathways converge at the formation of C5 convertase and results in the formation of membrane attack complex (MAC) leading to cell lysis. Potent anaphylatoxins; C4a, C3a and C5a are generated which enhance the inflammation.

C1q, a charge pattern recognition protein consists of 18 homologous polypeptide chains 6 each of A, B and C domain. Each of the chains consists of a short N-terminal region, the collagen-like domain at the middle followed by C-terminal globular head domain (Reid, Bentley and Wood, 1984; Sellar, Blake and Reid, 1991). The globular head domain recognizes the PAMPs and binds to charge clusters or hydrophobic patches on the targets (Kishore *et al.*, 2003; Kishore *et al.*, 2004). Binding of C1q to its targets results in activation of C1r and C1s of C1 complex, which in turn cleaves C4 and C2 forming C3 convertase (C4bC2a). In lectin pathway, MBL binds to mannose or carbohydrate moieties activating membrane-associated serine proteases (MASPs), which cleave C4 and C2 forming common C3 convertase (C4bC2a). C3 convertase cleaves C3 into C3b and C3a. C3b opsonizes the targets and binds to complement receptors expressed on macrophages for phagocytosis. C3b also binds to C3b convertase forming C5 convertase (C4bC2aC3b) and cleaves C5 into C5a and C5b. C5b forms a complex with C6, C7, C8 and C9 forming membrane attack complex (MAC). MAC disrupts the lipid bilayer of cells (Ricklin *et al.*, 2010).

The alternative pathway involves the slow hydrolysis of inactive C3 into C3 (H_2O), altering its conformation and exposing new binding sites facilitating binding of factor B. Factor D cleaves factor B and forms C3 convertase (C3bBb). Properdin (factor P) stabilizes C3bBb to C3bBbP (Fearon and Austen, 1975). C3bBbP in turns cleaves C3 leading to amplification of C3b formation and C5 convertase (C3bBbC3b). C3 convertase is stabilized by properdin while Factor H acts as an antagonist. Factor H inactivates C3b to form inactive iC3b. C5 convertase cleaves C5 forming C5b, which combines with C6, C7, C8 and C9 to form membrane attack complex (MAC) leading to cell lysis.

1.6.7.1 Complement activation by nanoparticles

One of the liposomal doxorubicin formulated nanomedicines, DOXIL highlight the importance of complement activation. DOXIL is infused at a very low rate to avoid complement activation and subsequent hypersensitivity. Even administration at a very slow rate induces serious cardiopulmonary distress with anaphylactic shock in 10% of patients (Szebeni *et al.*, 2002). Another example of nanomedicine, which was approved for contrast agent and later discontinued, is Feridex due to alternative pathway activation (Szebeni *et al.*, 2011). In addition to these two drugs, a diverse range of nanomaterials including Poly (lactic-co-glycolic acid (PLGA) nanoparticles (Fornaguera *et al.*, 2015) and CNTs activate complement via either alternative or classical pathway (Pondman *et al.*, 2014). C1q has been shown to crystallize on pristine and functionalized CNTs in a confirmation that does not promote classical pathway (Ling *et al.*, 2011). However, another study (Pondman *et al.*, 2015) reported a high-affinity direct binding of C1q to CNTs involving hydrophobic interactions that led to complement activation. The ways other complement components assemble on nanoparticle surface have still remained unclear. Recently, it has been shown that non-specifically adsorbed serum and plasma protein on the dextran functionalized IONP forms a covalent link with C3 thus initiating alternative pathway rather than conventional direct binding of C3 to dextran shell of IONP via thioester bond (Chen *et al.*, 2017). Such surface bound proteins accelerate the assembly of the complement components in complement activation thus poses a challenge in the reduction of complement consumption by nanomedicine.

1.6.7.2 Strategy to dampen complement activation

C4a, C3a and C5a generated during complement activation act as anaphylatoxins. These peptides cause the release of histamine, enhance vascular permeability and smooth

muscle contraction (van Lookeren Campagne, Wiesmann and Brown, 2007) eventually causing a host complement-activation related pseudoallergy (CARPA) (Szebeni *et al.*, 2012). Liberated C5a results in recruitment of regulatory T cells, immunosuppressive monocytes into malignant tumours and thus affecting the prognosis of cancer (Markiewski *et al.*, 2008; Moghimi and Farhangrazi, 2014).

Complement activation, therefore, is a thoughtful concern for the successful development of intravenous nano-based drug delivery. In order to dampen the inevitable complement activation on NPs surface, modified sugar alcohols of surface polysaccharides, dextran with alkylating, acetylating, or crosslinking agents have been used but these modifications have failed to inhibit complement activation and C3 opsonisation (Wang *et al.*, 2016). In separate studies, Kishore and colleagues extensively investigated the complement deactivation using recombinant individual globular head modules of C1q molecules and factor H (Pondman *et al.*, 2014; Pondman *et al.*, 2015). The recombinant globular head module of C1q (ghA, ghB and ghC) and factor H dampened the complement activation when adsorbed on CNTs. It has also been shown that individual globular heads bind to CNTs via hydrophobic interaction through the globular head, ligand recognition domain of C1q. Pre-coating with globular heads inhibited the direct interaction of C1q with CNTs possibly by occupying same binding pattern on the surface and thus inhibiting stable binding of C1q resulting in inhibition of classical pathway activation (Pondman *et al.*, 2016). Adsorption of Factor H on graphene oxide and reduced graphene oxide has also been shown to inhibit complement activation to naturally occurring levels (Belling *et al.*, 2016). Surface adsorption of soluble complement regulators such as globular heads of C1q and factor H have offered a novel way of controlling complement pathway activation and also down-regulation of

inflammation caused by nanoparticles, important factors in the design of immune-safe nanomedicine.

Besides complement components, polymers like PEG and its derivatives have also been extensively studied in inhibition of complement activation. It has been considered to provide a shielding or masking of the surface of the nanomaterial from complement recognition (Moghimi and Szebeni, 2003). On the contrary, recent biochemical and immunological studies have shown that such polymeric derivatives also trigger complement, in spite of their protective PEG armour (Hamad *et al.*, 2008). Mechanisms of PEG-mediated complement activation are still a matter of debate. Various factors such as PEG chain length, surface density and molecular weight may affect the degree of complement activation. However, some studies have suggested that the immune system can produce PEG-specific antibodies after intravenous administration of PEG-liposomes (Ishida *et al.*, 2007), which further give an insight into one of the various mechanisms of PEG-mediated complement activation (Taguchi *et al.*, 2009).

1.6.7.3 Complement/receptor-mediated endocytosis of NPs

Complement activation and opsonisation of NPs with C3b follows the interactions with complement receptors: CR1, CR2, CR3, CR4 (Zipfel and Skerka, 2009). C3b upon degradation by inhibitory factor I is converted into iC3b. iC3b, though, retains the capacity to act as an opsonins, it no longer acts as C3b in the amplification of complement alternative pathway. Further cleavage of iC3b by factor I results in the formation of C3d. C3b, iC3b and C3d have an affinity toward different complement receptors present on the membrane of host immune cells and may signal for different functions. CR1 binds to C3b, iC3b and C4b and mediates the clearance of immune complex and enhances phagocytosis (Medof *et al.*, 1982). CR1 is abundant

onerythrocytes (Nielsen *et al.*, 1997) and follicular dendritic cells (Reynes *et al.*, 1985). iC3b has a lower affinity toward CR1 but higher affinity towards CR3 and CR4, commonly found on professional phagocytic cells (Helmy *et al.*, 2006). Thus, C3b decorated particles bound to erythrocytes via CR1 are transferred into the liver for clearance. CR2 is abundantly present on B cells. Both CR1 and CR2 act as co-receptors for surface-bound immunoglobulin and regulate B cell differentiation and maturation (Zipfel, 2009). CR2 on B cells binds to C3d and instructs them to respond to C3d-coupled foreign antigens thus linking innate and adaptive immune system.

Complement activating nanoparticles are readily endocytosed and cleared by macrophages (U937) and B cells (Raji) *in vitro* (Pondman *et al.*, 2017b; Pondman *et al.*, 2015; Pondman *et al.*, 2014). B cells are known to present the highest number of CR2 and CR1 complement receptors on their surface. Macrophages, also express abundant CR1, CR3 and CR4 receptors. Thus, rapid endocytosis of complement deposited nanoparticles by macrophages and B cells is an indicator of the involvement of complement receptors. Interestingly, no difference was observed in the uptake of complement deposited and untreated CNTs by Jurkat cells (T-cell line), which are known to lack complement receptors. These evidences further advocate the role of complement receptors in endocytosis and clearance of complement activating nanoparticles. Pre-coating of CNTs with recombinant globular heads of human C1q and factor H increased and decreased phagocytosis respectively by macrophages (Pondman *et al.*, 2015).

Besides, CR, macrophages and dendritic cells are equipped with other receptors like Toll-like receptors (TLR), scavenger receptors (SR), integrins, lectin-like receptors and Fc receptors to recognize, bind and phagocyte foreign particles. Antigen-presenting cells like monocytes/macrophages, dendritic and B cells bind and either phagocyte

(macrophages and dendritic cells) or receptor-mediated endocytose (B cells) foreign agents and process them into peptide fragments (epitope) and then display them on their plasma membrane through class II and class II MHC molecules (Kambayashi and Laufer, 2014) for T lymphocyte recognition through TCR (T cell receptor). Thus, bridging innate and acquired immunity. Other phagocytic receptors such as scavenger (SR) and mannose receptors are also involved in clearance of nanoparticles in a complement-independent manner. Non-opsonized NPs were shown to be taken up via sugar receptor-mediated processes involving mannose receptors (Artursson, Johansson and Sjoholm, 1988). Furthermore, it has been shown that complement receptor-mediated phagocytosis overrides the sugar receptor-mediated process in the case of phagocytosis of the particles that have a tendency to adsorbed significant amounts of serum proteins. Involvement of SR-A mediated endocytosis has also been reported in macrophage uptake of SPION, ferumoxides and ferumoxtran-10 contrast agents (Raynal *et al.*, 2004). Involvement of TLRs in phagocytosis is still not clear. However, TLR 4 has been shown to enhance the phagocytosis of titanium dioxide nanoparticles in TLR-transfected human epithelial cell lines independent of its co-receptor, CD14 (NCI-H292) (Mano, Kanehira and Taniguchi, 2013). However, the mechanism of phagocytosis is not well defined but it has been shown that titanium dioxide stimulates TLR4 expression and increases the phagocytic activity and p38 MAPK signalling pathway in sea urchin immune cells (Pinsino *et al.*, 2015). Furthermore, TLR4 stimulants, such as alginates, are required to enhance the uptake of gold nanoparticles by murine macrophages (Bi *et al.*, 2017). Aliginate was shown to upregulate TLR4 expression and the activation of Akt/NF-kB and p38 MAPK signalling pathways. These findings show that TLR4 may not bind to nanoparticles directly but stimulation of

TLR4 enhances uptake through other routes for example by upregulating SR expression (Bi *et al.*, 2017)

1.6.7.4 Non-complement dependent endocytosis of NPs

The latest information on cellular uptake of nanoparticles suggests the involvement of phagocytosis, pinocytosis, caveolae-dependent and clathrin-mediated endocytosis in their uptake (Figure 1-7). These pathways are highly dependent on a particle's physicochemical properties, experimental conditions and the cell type (phagocytic or non-phagocytic cells) (Kettler *et al.*, 2014). Endocytosis processes are energy dependent and highly conserved in eukaryotes. Foreign objects greater than 750 nm are engulfed and transported into the cells via phagocytosis. Smaller particles of size around 100 nm are mostly internalized by pinocytosis or macropinocytosis. Clathrin-mediated endocytosis or receptor-mediated endocytosis is probably the best-characterized mechanisms for nanoparticle internalization in which particles are deposited in small endocytic vesicles which later fuse with early endosomes (Zhao *et al.*, 2011). Organic molecule coated particles take a different route of internalisation compared to their uncoated counterpart. Pristine gold nanoparticles are endocytosed via macropinocytosis and clathrin-mediated endocytosis whereas PEG-coated gold nanoparticles mostly enter cells via clathrin-mediated endocytosis, not by macropinocytosis (Brandenberger *et al.*, 2010). However, there was no difference in their intracellular distribution after endocytosis. Similar intracellular localization patterns were observed with both uncoated liposomes and hyaluronic acid coated liposomes though they entered via two different pathways; fusion with the plasma membrane and receptor-mediated internalization (Nazarenus *et al.*, 2014).

Moreover, a combination of three pathways has been reported in the uptake of CNTs; clathrin-mediated, caveolae mediate and macropinocytosis (Maruyama *et al.*,

2015). Furthermore, very recently Cui et al also suggested the involvement of multiple pathways simultaneously but in an orderly fashion; with macropinocytosis being the preferred entry pathway then caveolae-mediated and clathrin-dependent endocytosis as a rare pathway in CNT uptake (Cui *et al.*, 2017). Long CNTs (630±171nm) have been shown to be internalized by direct penetration or needling through plasma membrane (Cui *et al.*, 2014). After NPs uptake by macrophages, NPs could localise in the cytoplasm or within the endo-lysosomal system (Kang *et al.*, 2008; Cui *et al.*, 2014; Osman *et al.*, 2012). Nuclear localisation of NPs is very rare but in some cases, endosomal escape and nuclear translocation have been observed (Mu, Broughton and Yan, 2009). Some studies have suggested that silica NPs can accumulate in lysosomes (Lesniak *et al.*, 2012) and SWCNTs can reside in intracellular vacuoles in Hela cells (Yehia *et al.*, 2007). This differential localisation of NPs is dependent on ligand coating (Gao *et al.*, 2013). The uptake of IL-13 peptide linked PEG-PCL NPs was higher and the endocytosed particles were distributed throughout the cell compared to PEG-PCL NPs and PEG-PCL NPs which co-localised with endosomes (Gao *et al.*, 2013).

Phagocytosis

Phagocytosis is a common endocytic process widely observed in cells types such as epithelial cells, professional immune cells (monocytes, neutrophils, macrophages, basophils, eosinophil, mast cells, NK cells) and fibroblasts (Aderem and Underhill, 1999). Foreign particles, cells and infectious microorganisms are engulfed and disabled by the cells' plasma membrane protrusions as a response to innate and adaptive immunity. The process of phagocytosis occurs through the interaction of cell surface receptors with foreign agents. Receptors like Fc receptor family for IgG (FcYRI, FcYRIIA, FcYRIIB2, FcεRI. FcαRI) and complement receptors (CR1, CR3 and CR4) and α5β1 integrin (Underhill and Goodridge, 2012) stimulate uptake. Large particles (>250nm) are endocytosed through this process and form phagosomes. The phagosome matures by fusion with lysosomes and engulfed agents are degraded by hydrolytic enzymes in the low pH environment of the phagolysosome (Figure 1-7), Degradation of the foreign agents leads to detection of epitopes inside the cells, which stimulates signalling to the nucleus to trigger an inflammatory response and presentation of peptides derived from the foreign agent to other immune cells via MHC class II.

Macropinocytosis

It is an actin-driven endocytic process in which solute molecules; nutrients and antigens are internalized forming vesicles called macropinosomes. It is a route of uptake of viruses, bacteria and apoptotic cells and in dendritic cells material taken up by macropinocytosis is processed and subsequently presented by MHC II. After formation, macropinosomes undergo a maturation process during which they shrink and the content is concentrated. Macropinosomes ultimately fuse with the endo-lysosome system where loading of MHCII molecules takes place for the subsequent presentation of the antigen to T cells (Racoosin and Swanson, 1993)(Figure 1-7).

Clathrin-mediated endocytosis (CME)

CME is a complex endocytosis process in eukaryotic cells in which cargo is packed in vesicles containing a clathrin coat. The vesicle forms with the participation of a series of protein modules (Complexes) starting with the induction of curvature in the membrane and addition of adaptor protein complex (AP2) forming a spherical clathrin-coated pit (Figure 1-7)(McMahon and Boucrot, 2011). Once the vesicles are

formed, the GTPase dynamin is assembled around the neck of the vesicle and initiates its release from the plasma membrane. Released vesicles lose their clathrin coat by the action of auxilin and heat shock protein 70 (HSC70) (McMahon and Boucrot, 2011). The vesicles are then trafficked to endosomes and where their content is sorted to either recycle back to the plasma membrane or to be digested in the lysosome. CME is also called receptor-mediated endocytosis.

Clathrin-independent endocytosis (CIE)

Unlike CME, CIE does not require coat proteins for vesicle formation and invagination. CIE pathway uses actin and actin-associated small GTPases like Arf-6, RhoA and Cdc42 for vesicle formation (Ferreira and Boucrot, 2017). The formed vesicles are delivered to the early endosomes and later to late endosomes (Figure 1-7) which subsequently fuse with lysosomes for degradation. Cargo may be also trafficked to the trans-Golgi network or recycled to plasma membrane similar to what is observed with CME (Grant and Donaldson, 2009)

Caveolae

Caveolae are small (60-80nm) plasma membrane invaginations abundantly observed in fibroblasts, adipocytes, endothelial cells but absent in neurons and leukocytes. Caveolae are coated with caveolin proteins (CAV1, CAV2 and CAV3). Cavins (Cavin 1-4) are associated with caveolins to regulate the formation of caveolae. These proteins also regulate the signal transduction and govern the fate of caveolae (Parton and del Pozo, 2013). The endocytosed cargo is localized into neutral pH vesicles called caveosomes (Figure 1-7). Due to the physiological pH inside caveosomes, their content is not degraded hydrolytic enzyme of lysosomes. Caveosomes may later deliver their cargo to the Golgi apparatus and endoplasmic reticulum. Negative surface charged particles have been found to be endocytosed predominantly via caveolae (Sahay, Alakhova and Kabanov, 2010).

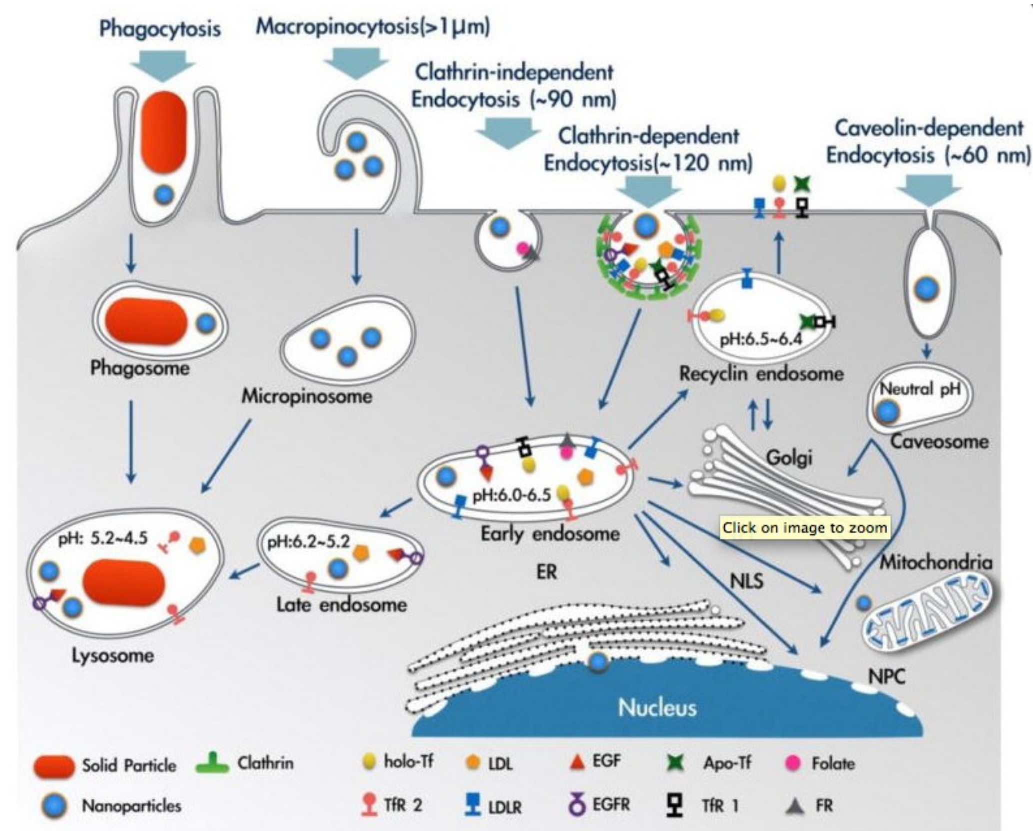


Figure 1-7 Pathways of entry of NPs into the cells.

Phagocytosis engulfs large particles using surface receptors and plasma membrane. Macropinocytosis mainly involves internalization of extracellular fluids. Clathrin-dependent endocytosis involves the organization of clathrin and adaptor proteins on the plasma membrane to form a nascent vesicle for internalization. Caveolin-dependent endocytosis involves the assembly of caveolin coated and forms vesicle for internalization. Clathrin-independent endocytosis does not require coat proteins for vesicle formation and internalization but actin and associated proteins help to form the membrane into a vesicle. Except for caveolin-dependent endocytosis, all other endocytosis eventually leads to fusion with a lysosome. Internalized cargo may escape from early endosome and reside in other subcellular compartment like Golgi, mitochondria, endoplasmic reticulum (ER) and nucleus. Image is taken from (Yameen *et al.*, 2014)

1.7 Intracellular distribution

The therapeutic potential of the nano drug is highly dependent upon the endocytosis process and subsequent intracellular distribution, thus it is important to understand the physicochemical properties of NPs so that necessary modulation can be carried out to lessen the effect of any digestive process after cellular uptake and determine their intracellular fate.

In general, the intracellular localisation and distribution of the NPs are strongly guided by the route of entry. Clathrin-mediated endocytosis pathway occurs upon specific interaction of ligand and receptor on the extracellular surface. Particles are internalized and are generally delivered to endosomes. These endosomes later fuse with acidic lysosomes containing lysosomal enzymes resulting in degradation of the bioactive molecules carried by the NPs (Watson, Jones and Stephens, 2005). Thus, it is warranted to programme the NPs to be internalised through an alternative route like caveolae. This pathway is non-specific and is devoid of both acidic and digestive degradation. Caveolae leads to the formation of a large endocytic compartment formed from the plasma membrane and do not fuse with lysosome thus avoiding lysosomal degradation of the nano-conjugates (Parton and del Pozo, 2013). This results in not only prevention of degradation but also provides an opportunity to distribute to other cellular organelles. The endocytosed NPs can be delivered to the Golgi complex, endoplasmic reticulum or even exocytosed. In this way, drugs targeting ER and Golgi complex can be programmed to be delivered via caveolae-mediated endocytosis and macropinocytosis mediated uptake (Sneh-Edri, Likhtenshtein and Stepensky, 2011). Very recently, such strategy has been employed to avoid lysosomal degradation by surface modification. PEG-modified zirconium terephthalate porous metal-organic frameworks (UiO-66) has been tuned to escape lysosomal degradation by enhancing caveolae-mediated uptake (Abanades Lazaro *et al.*, 2017). Furthermore, surface modification of nanoparticles diverts or enhances quick escape from nanoparticles from lysosomal fusion and allows reaching target organelles. Such modification has been very successful in the delivery of doxorubicin to nucleus and mitochondria (Li *et al.*, 2017b; Zeng, Morgenstern and Nystrom, 2014; Qu, Ma and Zhao, 2015). Fusion with lysosome is sometimes favourable when NPs are designed for efficient drug release in

tumour cells where decreased pH is commonly observed in solid tumours. Fusion with lysosomes and release of the drug has three stages; minimum leakage of the drug in the blood circulation en route to the target site, a burst release at low pH generally at lysosomal fusion inside tumour cells and a slow and steady release of the drug. In order to achieve lysosomal fusion and drug release, NPs are modified to take the receptor-mediated endocytosis route, which results in delivery to the endosome and later fusion with lysosomes. Functionalized carbon nanohorns were formulated with pH-sensitive phospholipids and polyethylene glycol and loaded with paclitaxel. Paclitaxel was released steadily over 30 days with a cumulative release of 90% of the loaded drug in breast cancer cells (Huang *et al.*, 2013). Similarly, Poly (ethylene glycol)-poly(aspartic acid) [PEG-p(Asp)] micelles conjugated with doxorubicin (DOX) via a pH-sensitive hydrazine bond, were tuned to release the drug in the acidic (pH- 4-6) environment of endosomes and lysosomes controlling the systemic, local and cellular distribution of the drug (Ponta and Bae, 2010).

1.8 Cytokine, chemokine, inflammation and immune response

NPs have been shown to interact with serum plasma, complement system, and antibodies. Further understanding the profile of released cytokines and chemokines by immune cells can unveil the nature of nanoparticle interactions with the immune system. In fact, cytokine profiles can be used as a biomarker of immunostimulation by nanomaterials. High levels of pro-inflammatory cytokines such as TNF-α and IL-1 β are usually associated with toxicity, low therapeutic outcome and adverse reactions. Hence, analysing and studying these signalling proteins would help to predict the immunotoxicity possessed by NPs.

Due to their fibrous morphology and high aspect ratio (length many times than diameter), CNTs are similar to asbestos; a mineral fibre have shown to cause pleural mesothelioma (Jaurand, Renier and Daubriac, 2009). Recent studies have shown that carbon-based NPs are pro-inflammatory (Tan *et al.*, 2014; van Berlo *et al.*, 2014). The interaction between MWNTs and murine macrophages leads to secretion of macrophage-induced protein (MIP)-1α and MIP-2 that recruits naïve macrophages, and produces angiogenesis factors, Matrix metalloproteinase (MMP)-9 and vascular endothelial growth factor (VEGF) (Meng *et al.*, 2015). The size of the CNTs plays a vital role in the nature of inflammatory response: long MWNTs induce significant release of IL-1β, TNF-α, IL-6 and IL-8 in breast cancer cell line (MCF-7) (Murphy *et al.*, 2012) and murine macrophage cell line (RAW) (Liu *et al.*, 2012). A recent study has demonstrated that carboxylic MWNTs on their own act as chemoattractant and activate the PLC/IP3 (Phospholipid C/ Inositol 1,4,5-triphosphate) signalling pathway and stimulate the migration of RAW264.7 macrophages (Li *et al.*, 2017a). A list of secreted small soluble proteins in response to CNTs and IONP administration studied in this thesis are presented in Table 1-3, Table 1-4 and Table 1-5.

Table 1-3 List of cytokine released by macrophages in response to various CNTs and IONP

Types	**Main source**	**Target cells**	**Major functions**	References
IL-1β **IL-1α**	Macrophages, B cells, DCs	B cells, NK cells, T-cells	Pyrogenic, pro-inflammatory, BM cell proliferation	(Dinarello, 1985)
TNF- α	Macrophages	Macrophages	Pro-inflammatory, phagocyte cell activation, endotoxin shock	(Pfeffer, 2003)
TNF-β	Monocytes, T-cells	Tumour cells, phagocytes	Oncostatic, cachexia, pro-inflammatory, tumour cytotoxicity	(Sugarman *et al.*, 1985) (Pfeffer, 2003)
IL-6	Th cells, macrophages	Activated B-cells	Production and release of acute phase proteins	(Tanaka, Narazaki and Kishimoto, 2014)
IL-9	T cells, macrophages	T cells	Growth and proliferation	(Schmitt and Bopp, 2012)
IL-10	T cells, macrophages	B cells, macrophages	Anti-inflammatory	(Iyer and Cheng, 2012)
IL-12 (p70) **IL-12 (p40)**	T cells and macrophages	NK cells	Activates NK cells by stimulating interferon γ.	(Iyer and Cheng, 2012; Ma *et al.*, 2015)
IL-13	Th cells, macrophages, basophils	Basophils	Anti-parasitic agent, stimulate an isotype switch to IgE	(Hershey, 2003)
IL-15	Macrophage	NK cells and Tc cells	Stimulation of NK and Tc cells	(William E. Carson *et al.*, 1994)
IL-17A	Th cells,	Neutrophils	Pro-inflammatory signalling	(Gaffen, 2008)
IFN- α	Leukocytes, T cells	Various	Anti-viral	(Kamijo *et al.*, 1994; Samuel, 2001)
IFN- γ	Th1 and NK cells	Macrophage	Macrophage activation, increase MHC expression	(Kamijo *et al.*, 1994)

Table 1-4: List of chemokines released by macrophages in response to various CNTs and IONP

Types	Main source	Target cells	Major functions	References
MCP-1	Endothelial, monocytes, fibroblast,	Monocytes, NK cells, T lymphocytes	Regulate migration and infiltration of monocytes	(Deshmane *et al.*, 2009)
MCP-3	Macrophages, tumour cells	Macrophage, neutrophils	Chemo attracts monocytes and neutrophils and regulates macrophage function	(Dahinden *et al.*, 1994; Xu *et al.*, 1995)
MDC	Macrophage	DC, NK cells and T cell subsets	Polarizes Th2 and Tc2 cells.	(Godiska *et al.*, 1997)
Eotaxin	Eosinophil, macrophages, T cells	Basophils,	Induce histamine-release from basophils, respiratory burst and leukotriene-release	(Matthews *et al.*, 1998)
Fractalkine	Microglial, endothelial cells	T cells and monocytes	Chemo attractants to T cells and monocytes. Promotes adhesion of leukocytes to endothelial cells	(Owlasiuk *et al.*, 2009)
GRO	Macrophages, Neutrophils Epithelial cell	Neutrophil	Angiogenesis, inflammation, wound healing	(Ahuja and Murphy, 1996)
IL-8	Macrophages	Neutrophils	Chemo taxis	(Bickel, 1993)
IP-10	Monocytes, fibroblast, endothelial cells	T cells, NK cells, DCs	Anti-cancer activity, attract T cells NK cells and dendritic cells	(Dufour *et al.*, 2002)
MIP-1α	Macrophages, DC, lymphocytes	Granulocyte, Endothelial cells	Inhibit proliferation of hematopoietic stem cells, activate granulocytes.	(Dorner *et al.*, 2002)
MIP-1β	Macrophages, DC, lymphocytes	NK cells, monocytes	Chemo attractant for NK cells, monocytes.	(Dorner *et al.*, 2002)

Table 1-5 List of growth factors, ligand and receptors released by macrophages in response to CNTs and IONP

Types	Main source	Target cells	Major functions	References
EGF	Macrophages, fibroblast and other	M +	Cellular proliferation, differentiation and survival	(Todderud and Carpenter, 1989)
G-CSF	Macrophages, endothelial cells, fibroblast	Stem cells	Granulocyte production	(Bendall and Bradstock, 2014)
GM-CSF	Macrophages, endothelial cells, T cells	Stem cells	Differentiation and growth of stem cells and monocytes progenitors. Development of osteoclasts.	(Shi *et al.*, 2006)
VEGF	Macrophages, endothelial cells, fibroblast	Stem cells, endothelial cells	Stimulation of endothelial cells. Angiogenesis	(Ferrara *et al.*, 1992)
FGF-2	Fibroblast, macrophages	Fibroblast	Angiogenesis wound healing, embryonic development.	(Yun *et al.*, 2010)
IL-2	T cells	Activated T and B cells	Proliferation and activation, Development of T regulatory cells	(Malek, 2003)
IL-3	T cells, NK cells	Stem cells	Hematopoietic precursor proliferation and differentiation	(Lindemann and Mertelsmann, 1993)
IL-4	T_h cells	B cells, T cells, Macrophages	The proliferation of B and Tc cells, IgG and IgE production	(Brown and Hural, 1997)
IL-5	Th cells	B cells, Eosinophil	IgA and IgM production, maturation and proliferation	(Greenfeder *et al.*, 2001)
IL-7	BM stromal cells	Stem cells, T cells	B and T cells growth factor	(ElKassar and Gress, 2010)
TGF- α	Macrophages, keratinocytes, astrocytes	Various	Wound healing, cellular proliferation	(Snedeker, Brown and DiAugustine, 1991)

FLT-3L	Lymphocytes, macrophages	Lymphocytes	Activate hematopoietic progenitors	(Harada *et al.*, 2007)
IL-1RA	Macrophages, epithelial cells, adipocytes	Various	Inhibitor of the pro-inflammatory effect of IL-1β and IL-1α	(Arend and Gabay, 2000)
sCD40L	Macrophages, endothelial cells, muscle cells	Platelet	Platelet activation, the release of inflammatory mediators, increase the activity of MMP and coagulation cascade.	(Aoui *et al.*, 2014; Bou Khzam *et al.*, 2013)

1.8.1 Minimizing the pro-inflammatory nature of NPs

Strategies to lower the inflammatory properties of CNTs have been implemented by using biocompatible polymers. Use of medical grade polycarbonate urethane in the functionalization of MWNTs has been shown to significantly reduce TNF-α and IL-1β production by mouse macrophages. Furthermore, the release of nitric oxide was also decreased in mouse macrophages (Khang, 2015). Similarly, Pondman *et al* have shown the reduction in expression of pro-inflammatory cytokines and increased expression of anti-inflammatory cytokines (IL-10 and TGF-β) in monocytes and macrophages upon exposure to complement deposited CNTs (Pondman *et al.*, 2014; Pondman *et al.*, 2016). This suggests that under physiological conditions, the threat posed by complement-activating NPs like CNTs and SPION could be lower than as observed in vitro and complement deposition might signal the cells to remove the NPs swiftly. Though complement deposited CNTs lower pro-inflammatory cytokine release but there are no marked decrease in chemokine secretion (Pondman *et al.*, 2016) Members of IL-1 and TNF receptor families and TLR receptors are thought to be involved in inflammation signalling (Dinarello, 2009; Aggarwal *et al.*, 2002; Drexler and Foxwell, 2010). Signal transduction to nuclear factor-kappa B (NF-kB), a regulator of expression of pro-inflammatory genes, through these receptors leads to transcription of pro-inflammatory cytokines (Ghosh and Karin, 2002). Some NPs induce nod-like receptor protein 3 (NLRP3) inflammasome resulting in secretion of pro-inflammatory cytokines, IL-1β. DWNTs enhanced the secretion of IL-1β from monocytes via NLRP3 activation pathway (Meunier *et al.*, 2012). Interestingly, complement-activating NPs could induce the release of various cytokines via complement component receptors, C3aR and C5aR (Wolf-Grosse *et al.*, 2017). Anaphylatoxins (C5a and C3a) generated during complement activation of IONP

trigger C5aR and C3aR to release IL-1β, TNF- α, IL-8, MCP-1 and MIP-1β. Blockage of these receptors resulted in a reduction of cytokine release suggesting a complement-dependent manner of cytokine secretion (Wolf-Grosse *et al.*, 2017). Intravenously administered NPs have the ability to polarize the balance of Th_1 and Th_2 cytokines. Metallofullerenes have been reported to show specific immunomodulatory effects on T cells and macrophages and polarized the cytokine balance toward Th_1 cytokines (IL-2, IFN-γ, TNF- α) by decreasing the production of Th2 cytokines (IL-4, IL-5 and IL-6) (Liu *et al.*, 2009)in a mice serum by differentiating T cell into Th1 cells in contrast to SWNTs and MWNTs (Wang *et al.*, 2013).

1.9 Lung innate immunity and pulmonary surfactant proteins

The lung innate immune system comprises soluble proteins, leukocytes and the large epithelial lining the alveolar surface. Inhaled foreign agents are trapped by the mucociliary surface in the airways passage and propelled upward by the force of the mucociliary system. Some particles may find them on the alveolar surface where they interact with soluble components in alveolar fluids that opsonize the particle and alveolar macrophages clear them. Lung innate immune defence is governed by these soluble components such as IgG, complement, surfactant and surfactant-associated hydrophilic proteins (SP-B and SP-C).

Pulmonary surfactant is a multi-layered lipoprotein constituted of up to 10% proteins and 90% lipid (Goerke, 1998). These lipoproteins line the alveolar epithelial surface and perform two important functions in the lung; defence against microbial infection and reduction of alveolar surface tension. Four surfactant proteins (SPs); SP-A (5.3%), SP-B (0.7%), SP-C (0.4%) and SP-D (0.6%) are found associated with surfactant lipids (90%) (Kishore *et al.*, 2002; Weaver and Whitsett,

1991). SP-B and SP-C are small hydrophobic proteins having the main role in reducing surface tension, phospholipid packaging and adsorption and organization of air-liquid interface (Ding *et al.*, 2001; Tchoreloff *et al.*, 1991). SP-A and SP-D are large collagenous and hydrophilic proteins. They have an important role in surfactant homeostasis, a defence mechanism against pathogens and allergens (Kishore *et al.*, 2002; Kishore *et al.*, 2006). SP-A and SP-D have a multimeric structure similar to C1q and MBL and belong to a family of mammalian C-type lectins, called collectins. The primary structural organization consists of a cysteine-containing N-terminal region, a triple-helical collagen region composed of repeating Gly-X-Y triplets, an α-helical coiled-coil neck region, and a globular structure at the C-terminal comprising a C-type lectin or CRD (carbohydrate recognition domain) (Figure 1-8) (Kishore *et al.*, 2006; Crouch *et al.*, 1994). CRD region binds to invading pathogens, carbohydrate, phospholipid, etc. in a Ca^{2+}dependent manner. SP-A has a hexameric structure consisting of 6 structural subunits of 105 kDa each to form a molecule of 630 kDa (King, Simon and Horowitz, 1989). Each polypeptide is held together by disulphide bonds located in the N-terminal halves of the chains forming a similar structure to C1q (Figure 1-8). SP-D is composed of oligomers of 130 kDa each subunit comprising of three identical polypeptide chains of 43 kDa, assembled into a 520-kDa tetrameric structure. Presence of trimeric, dimeric and monomeric SP-D is also reported. SP-D might undergo further oligomerization to form multimeric SP-D of up to 96 CRDs (Crouch *et al.*, 1994).

1.9.1 Functions of SP-A and SP-D

SP-A and SP-D bind to carbohydrate moiety present on various self and non-self ligands via CRD region in a calcium-dependent manner (Ogasawara, Kuroki and Akino, 1992; Ogasawara *et al.*, 1994). Gardai *et al* have shown that both SP-A and

SP-D binds to LPS and apoptotic cells via CRD regions and interacts with macrophages via collagen region thereby inducing phagocytosis, pro-inflammatory cytokine production and enhancement of adaptive immune response (Gardai *et al.*, 2003). Both SP-A and SP-D are very versatile proteins and play an important role in dampening inflammation and allergic reactions, binding to various fungi and opportunistic fungal pathogens and enhancing phagocytosis. SP-A and SP-D are also found in the non-pulmonary region such as in gastric tract, urinary tract, and female reproductive tract and in amniotic fluid and highly expressed during labour. They play a role in non-antibody-mediated innate immune responses such as modulation of host defence and inflammation. Some of their functions are summarized in Table **1-6**.

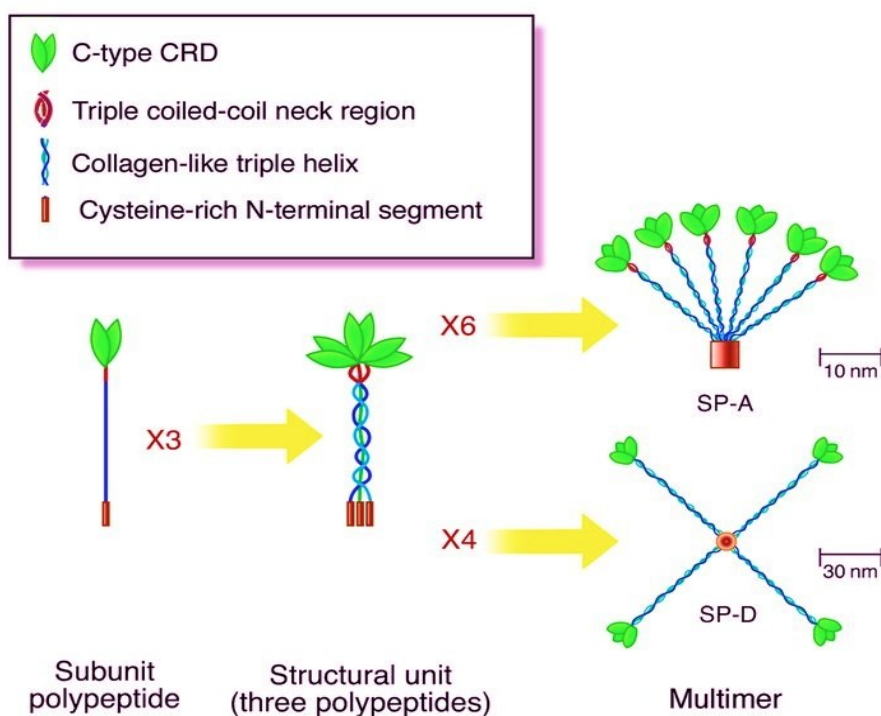


Figure 1-8: Multimeric structure of SP-A and SP-D.

SP-A and SP-D are assembled from three-polypeptide subunit. Six and four structural unit assembles at cysteine-rich N-terminal to form SP-A and SP-D respectively. SP-D and SP-A have N-terminal triple-helical collagen region, followed by a trimerizing alpha-helical coiled neck region and C-terminal homotrimeric carbohydrate recognition domain (CRD). (Image is taken from (Jakel *et al.*, 2013)

Table 1-6: The diverse function of SP-D, SP-A, and a recombinant fragment of human SP-D (rfhSP-D)]

	Functions	SP	Reference:
Lung homoeostasis Properties	Control the secretion and uptake of phosphatidylcholine to regulates its own secretion by type II cells	SP-A	(Dobbs *et al.*, 1987; Rice *et al.*, 1987)
	Protect surface activity properties of surfactant from functional inhibitors such as fibrinogen and albumin.	SP-A	(Cockshutt, Weitz and Possmayer, 1990)
	Inhibition of lung phospholipase A2 that promote inflammation during lung disease.	SP-A SP-D	(Fisher, Dodia and Chander, 1994)
	Formation of tubular myelin such as tubular myelin	SP-A	(Suzuki, Fujita and Kogishi, 1989)
	Lung remodelling in idiopathic pulmonary fibrosis by regulating macrophages, fibrocytes, cytokine expression	SP-D	(Aono *et al.*, 2012)
	Regulates surfactant phospholipid homeostasis and maintain type II cells morphology.	SP-D	(Korfhagen *et al.*, 1998; Botas *et al.*, 1998)
Anti-viral properties	Influenza A virus inactivation by inhibiting hemagglutination and promote viral precipitation and aggregation	SP-A SP-D	(Hartshorn *et al.*, 1997; Hartshorn *et al.*, 2010)
	Defence against respiratory syncytial virus and adenovirus	SP-A SP-D	(Hartshorn *et al.*, 2010)
	Enhancement of phagocytosis by macrophages and superoxidative burst by neutrophils	SP-A SP-D	(Hartshorn *et al.*, 1994; Hartshorn *et al.*, 1996)
	Binds to glycosylated HIV envelope protein, gp120 of HIV and inhibit its binding to CD4 T cells	SP-A SP-D	(Madsen *et al.*, 2013; Gaiha *et al.*, 2008)
Anti-bacterial properties	Antibody-independent pathogen recognition and clearance of *H.pylori* interacting with O-antigen causing immobilization and aggregation	SP-D	(Khamri *et al.*, 2005)
	Agglutinates *E.coli, Salmonella, Pseudomonas aeruginosa, Klebsiella pneumoniae* by interacting with lipopolysaccharides and phospholipids.	SP-D	(Kuan, Rust and Crouch, 1992; Kishore *et al.*, 1996)

	Binding to Mycobacterium and increases fusion of phagosomes with lysosomes.	SP-D SP-A	(Ferguson *et al.*, 2006; Ferguson*et al.*, 1999; Lemos, McKinney and Rhee, 2011)
	Aggregate and opsonizes H. influenza type A	SP-A	(McNeely and Coonrod, 1994)
	Modulate reactive oxygen and nitrogen intermediates in the killing of *Mycobacterium* spp, *Mycoplasma pulmonis*	SP-A SP-D	(Weikert *et al.*, 2000; Hickman-Davis *et al.*, 1999)
	Protection in ocular *S. aureus* infection	SP-D	(Zhang *et al.*, 2015)
	Binds to flagella of *Pseudomonas aeruginosa* enhancing phagocytosis but induction of IL-1β through inflammasome pathway	SP-A	(Ketko *et al.*, 2013)
Anti-fungal properties	Growth inhibition of *Histoplasma capsulatum* by increasing the permeability of the cell membrane	SP-A SP-D	(McCormack *et al.*, 2003)
	Agglutination of *C. neoformans* and *A. fumigatus*	SP-D SP-A	(Schelenz *et al.*, 1995)(Madan *et al.*, 1997)
	Reduction in the growth of fungal hyphae of *Aspergillus fumigatus* in murine lungs.	SP-D	(Singh *et al.*, 2009)
	Binds to glycoproteins secreted by *Pneumocystis jirovecii and S. cerevisiae*	SP-D SP-A	(Zimmerman *et al.*, 1992; Allen, Voelker and Mason, 2001)
	Enhance the phagocytosis of *C.neoformans* by murine alveolar macrophages	SP-D SP-A	(Geunes-Boyer *et al.*, 2009)
	Binds to β-glucan on the surface of B. dermatitidis blocking the access to receptors on alveolar macrophages to stimulate TNF-α production.	SP-D	(Lekkala *et al.*, 2006)
Allergens and Hypersensitiv ity	Reduces allergic responses in mice sensitized to house dust mite allergens.	rfhSP-D	(Strong *et al.*, 2003)
	Suppresses basophil activation and Th2 cytokine production and IgE synthesis in response to grass pollen.	rfhSP-D	(Qaseem *et al.*, 2017)
	Binds to pollen grains, *Aspergillus fumigatus* allergen, *Afu* and inhibit specific IgE bindings to allergens	SP-A SP-D	(Madan *et al.*, 1997b; Malhotra *et al.*, 1993; Kishore *et al.*, 2002)

	Inhibition of the differentiation of Th2 cells in the nasal mucosa, reduced the level of Th2 cytokines, inhibition of increase of specific IgE production in Allergic rhinitis	SP-A	(Ren *et al.*, 2013)
	Increase in selective induction of phagocytosis of allergic eosinophil in inflammation	rfhSP-D	(Mahajan *et al.*, 2008)
	Enhance binding, aggregation and phagocytosis of starch granules containing grass pollen allergens via alveolar macrophages.	Dodecameric SP-D	(Erpenbeck *et al.*, 2005)
Modulation of apoptosis and necrotic cells	Binds genomic DNA, apoptotic cells and enhance their clearance	SP-D SP-A	(Palaniyar *et al.*, 2003; Vandivier *et al.*, 2002)
	Attenuates sepsis-induced acute pancreatic injury through modulating apoptosis and NF-kB mediated inflammation	SP-D	(Liu *et al.*, 2015)
	Induction of apoptosis in pancreatic cancer cell lines via Fas-mediated pathway	rfhSP-D	(Kaur et al., 2018)Frontiers in immunology, in press
Pulmonary inflammation and signalling	Downregulation of IL-13 and IL-5 responsible for eosinophilia and lung tissue damage.	SP-A	(Erpenbeck *et al.*, 2006)
	Binds to calreticulin/CD91 receptor complex and initiates a pro-inflammatory response through NF-kB activation	S.nitrosylated SP-D	(Guo *et al.*, 2008)
	Chemotaxis: CRD region of SP-D and collagen region of SP-A being chemotactic	SP-D SP-A	(Madan *et al.*, 1997a)
	Enhancement of TNF-α, IL-1α, IL-1β, IL-6 and IFN-γ production by human PBMCs	SP-D SP-A	(Wright, 2005)
Non-pulmonary function in the Female	Innate immune protection against sexually transmitted infection caused by Chlamydia	SP-A SP-D	(Oberley *et al.*, 2004)
	Protects amnion and amniotic cavity from inflammation during pregnancy and preventing preterm birth	SP-A	(Lee *et al.*, 2010)

	Regulation of onset of labour by inhibiting TNF-α production in response to prostaglandin $F_{2\alpha}$ released by intrauterine tissue macrophages	SP-D SP-A	(Alcorn and Wright, 2004; Norwitz, Lopez Bernal and Starkey, 1992)
	Suppress preterm delivery and inflammation via TLR2 signalling	SP-A	(Agrawal *et al.*, 2013)
	Role in intrauterine tissue remodelling during parturition and controls inflammation during pregnancy	SP-D	(Nadesalingam *et al.*, 2003; Groeneveld *et al.*, 2005)
	Reduces bacterial (*E.coli*) adherence to the human bladder and inhibit the growth	SP-A SP-D	(Hu *et al.*, 2016)
	Control inflammatory bowel disease, Crohn's disease and necrotising enterocolitis	SP-A SP-D	(Saka *et al.*, 2016; Quintanilla *et al.*, 2015)
	Upregulation during inflammation of salivary gland and periodontal diseases	SP-A SP-D	(Schicht *et al.*, 2015)

1.9.2 Ligands and receptors for SP-A and SP-D

The wide range of functions of SP-A and SP-D is due to their ability to bind and interact with various molecules and receptors expressed on micro-organisms, viruses, pathogens, self and non-self agents, apoptotic and necrotic cells via their collagens or CRD region. They recognize bacteria, fungi and viruses by binding to surface carbohydrates such as mannose, fucose, glucose, galactose, N-acetylglucosamine residues mainly through the CRDs region in a calcium-dependent manner (Persson, Chang and Crouch, 1990; Childs *et al.*, 1992). However, LPS and phospholipids require alpha-helical neck region of SP-D to facilitate CRD region to bind them. SP-A binds to lipid derivatives such as dipalmitoylphosphatidylcholine (DPPC) (Kuroki and Akino, 1991) glycolipids (Kuroki *et al.*, 1992) phosphatidylserine (Jakel, Reid and Clark, 2010) via CRD region in sugar moiety independent manner. Furthermore, both, SP-D and SP-A have been shown to bind nucleic acid (DNA and RNA), predominately by SP-D, for the clearance of apoptotic and necrotic cells via their CRDs as well as collagen-like regions (Palaniyar *et al.*, 2003; Palaniyar *et al.*, 2004). SP-D and SP-A interact with cell-surface receptors such as SPR-210, CD14, calreticulin-CD91 complex, SIRPα, TLR4, MD-2 and CR3, and binds to soluble molecules (ligands) (Gp 340, MPO, C1q, Ig, defensins, decorin) acting as receptors. The mechanism of bindings and their functions are summarized in Table 1-7

Table 1-7:Ligands and receptors of SP-A and SP-D

	Protein	**Cells expressing**	Protein and domain	Mechanism/ functions	Reference
Ligands	Glycoprotein (Gp) 340	Soluble proteins/opsonin	SP-A / SP-D CRD region	Ca^{2+} dependent protein-protein interaction	(Holmskov *et al.*, 1997)
	Myeloperoxidase (MPO)	Neutrophils	SP-A / SP-D CRD region	Ca^{2+} independent binding to late apoptotic neutrophils expressing MPO	(Jakel *et al.*, 2010)
	C1q	Soluble proteins	SP-A / SP-D CRD region	Ca^{2+} dependent	(Watford *et al.*, 2002; Oosting and Wright, 1994)
	Igs (IgG, IgM, IgE, secretory IgA)	Soluble opsonins	SP-A rfhSP-D, CRD region	Ca^{2+} dependent binding to Fab and Fc domain of Ig.	(Oosting and Wright, 1994; Qaseem *et al.*, 2017; Lin and Wright, 2006)
	Defensins	Neutrophils	SP-D/Neck and or CRD region	Ca^{2+} dependent binding to Neck /CRD region of SP-D	(Hartshorn *et al.*, 2006)
	Decorin	Endothelial cells, tumour cells	SP-D CRD region	Ca^{2+} dependent binding to a sulfated moiety of decorin	(Nadesalingam *et al.*, 2003)
Receptor	SPR-210/ mysosin 18A	Bone marrow-derived macrophages, Alveolar macrophages type II cells	SP-A Collagen region	Phagocytosis, inhibition of T-cell proliferation,	(Chroneos *et al.*, 1996; Yang *et al.*, 2005)
	CD14	Alveolar macrophages	SP-A(Neck region) SP-D(CRD)	Modulation of CD14-LPs interaction and release of pro-inflammatory cytokines	(Sano *et al.*, 2000; Sano *et al.*, 1999)
	Calreticulin-CD91 complex	Ubiquitous	SP-A/SP-D(Collagen region)	Phagocytosisandproduction of pro-inflammatory cytokines	(Vandivier *et al.*, 2002)
	Signal-inhibitory regulatory protein α(SIRP α)	Myeloid cells, neurons, B-cells, APCs, Endothelial cells	SP-A / SP-D(Collagenous region)	Inhibition of pro-inflammatory cytokine production	(Gardai *et al.*, 2003)
	TLR2, TLR4 MD-2	Macrophages, B-cells, T-cells, Endothelial cells	SP-A, SP-D(CRD)	Modulation of cellular response through TLRs	(Nie *et al.*, 2008; Yamada *et al.*, 2006)
	CR3(CD11b, CD18)	Alveolar macrophage,macrophage, NK and T cells	SP-A(CRD)	Enhance CR3 mediated phagocytosis	(Gil, McCormack and Levine, 2009)

1.9.3 Pulmonary toxicity and immune reactivity due to NPs

Animal models demonstrated that SP-D expression is upregulated in thelung in response to carbon nanotubes exposure (Clement *et al.*, 2008; Kang *et al.*, 2010). Inhaled or instilled, the NPs are expected to come in contact with innate immune molecules in the lungs such as SP-A and SP-D. In this study, we have examined the interaction between SP-D and CNTs and possible downstream effects on the immune response via macrophages response (Chapter 4).

Due to the potential use in the area of electronics, automobiles, constructions, aerospace, and medicine, occupational exposure to carbon NPs is considerably higher. Inhaled NPs deposit in the lung and interact with lung surfactant components before cellular interactions (Lundqvist *et al.*, 2008). Extracellular molecules such as fibrinogen, fluid can modulate NPs properties, reduce surface charge as measured by zeta potential and promote particle agglomeration (Kendall, Ding and Kendall, 2011). Once inside the airways, NPs adsorb onto the mucosal surface and pulmonary surfactant lining of the lower airways. Similar to the formation of plasma corona in blood, pulmonary biological molecules form a corona, which influences the clearance, bioavailability and toxicity of the NPs (Kendall and Holgate, 2012). Human exposure to MWNTs at manufacturing sites and its consequences on human health was first examined by Lee et al (Lee *et al.*, 2015) and shown to have higher levels of oxidative stress markers such as malondialdehyde (MDA), 4-hydroxy-(2E)-hexanal (4-HHE) and n-hexanal. Fibrogenic and tumorigenic potential of CNTs have also been reported (Voronkova *et al.*, 2017; Luanpitpong *et al.*, 2014b). However, the extent of damage to the lung is partly determined by physical properties like diameter and length and partly by fibrous or straight nature of CNTs (Luanpitpong *et al.*, 2014b). Mercer et al showed

the progressive presence of collagen deposition in the mouse lung over a period of 11 months post-exposure to long MWNTs (Mercer *et al.*, 2011)

Granuloma formation and fibrosis after intratracheal instillation of SWNTs in mice have also been reported (Lam *et al.*, 2004; Ryman-Rasmussen *et al.*, 2009). Lung exposure to MWNTs has been shown to stimulate inflammatory cytokine and chemokine secretion, production of fibrogenic growth factors such as TGF-β and PDGF that can promote the transformation of lung fibroblasts into myofibroblasts; a potential mechanism in human lung fibrosis (He *et al.*, 2011). Similarly, an increased cellular influx in BALF (Bronchoalveolar lavage fluid), altered expression of genes involved in cell cycle and microtubule assembly and interstitial pneumonia were observed after exposure to small (0.8 ± 0.1µm in length) and long CNTs (4 ± 0.4 µm in length). Only long CNTs caused early inflammation by infiltration of neutrophils, eosinophils and macrophages, DNA strand break and induction of hepatic fibrosis as indicated by expression of fibrosis-associated genes such as matrix metallopeptidases (MMP) 12, Mmp12 in mouse lungs (Poulsen *et al.*, 2015). Recently, Kinaret et al have demonstrated that inhalation and low-dose aspiration of long rod-like CNTs (~13µm in length) enhanced accumulation of neutrophils in the airways and giant cell formation in a mouse model (Kinaret *et al.*, 2017). Such size dependent variability in the in vivo results can be attributed to the variations in CNT types; multiwalled, double walled, single-walled; shape and size, metal contamination, aggregation, route of administration and surface coating (Madani, Mandel and Seifalian, 2013). A link between inhalation of IONP and COPD (chronic obstructive pulmonary disease) and atherosclerosis has been suggested due to increase in iron oxide-mediated oxidation of α1-antitrypsin and low-density lipoprotein in an alveolar-capillary co-culture model (Sun *et al.*, 2016). Rats exposed to magnetite dust particles showed increased neutrophils in BALF and

eosinophilic globules in lower and upper respiratory tract, concomitant with translocation of particles into local lymph nodes (Pauluhn, 2012).

1.10 Genomic toxicity associated with NPs

SWNTs have been shown to cause alveolus haemorrhage, infiltration of alveolar macrophages and neutrophils in a mouse model after intra tracheal instillation, without leading to tumour formation (Naya *et al.*, 2012). However, chronic exposure of the non-tumorigenic human lung epithelial cell to SWNTs induced the appearance of cancer stem cell (CSC)-like properties as evident by over expression of stem cell markers such as SOS2, SOX17 and repression of E-cadherin (Luanpitpong *et al.*, 2014a). Furthermore, tumour formation has been observed when these CSC-like cells were implanted subcutaneously into mice (Luanpitpong *et al.*, 2014a). It has also been shown that induction of tumours in mice was due to overexpression of tumour promoter caveolin-1 (Cav-1) and p53 dysregulation in lung stem-cell-like cells (Luanpitpong *et al.*, 2014c). Similar effects of SWNTs, lesser than MWNTs and carboxy functionalized MWNTs, were observed in primary human lung fibroblasts upon exposure to SWNTs which induced appearance of cancer-associated fibroblast (CAF) cells as indicated by overexpression of α-smooth muscle actin (α-SMA), podoplanin and increased secretion of TGF-β (Luanpitpong *et al.*, 2016). Like CSC-like cells, CAF cells also induced lung carcinoma in mice subcutaneously injected with CAF cells. Furthermore, long-term exposure of mice (1yr post-exposure) to SWNTs (40 μg/mouse) and carbon nanofibres (40 μg/mouse), regardless of the mode of administration (pharyngeal aspiration or inhalation), induced oncogenic K-ras mutation in the lung (Shvedova *et al.*, 2014). This potentially suggests the tumourigenic activity of CNTs. Exposure to MWNTs has been shown to induce genetic aberration and can induce tumours if

humans are co-exposed to mutagens like cigarette smoke and particles present in diesel fumes such as radon (radioactive noble gas), naphthalene (polycyclic aromatic hydrocarbon) and anthracene (polycyclic aromatic hydrocarbon) acting as initiators of tumour formation (Sargent *et al.*, 2014). The presence of iron contamination in CNTs has been related to ROS generation, which could account for the observed genotoxicities. CNTs have been shown to physically interact with DNA in G-C rich regions (Kato *et al.*, 2013; van Berlo *et al.*, 2012). Furthermore, CNTs, irrespective of their physical properties, induce DNA strand breaks, the formation of gammaH2Ax foci (a marker of double DNA strand breakage) and activation of poly(ADP-ribose)polymerase (PARP) enzyme for DNA repair in lung epithelial cells (van Berlo *et al.*, 2012). CNTs have also been linked to interfering with cell division by inducing aneuploidy, formation of three spindle poles, centrosome and microtubules fragmentation and cell division arresting in G1/S and G2/M phase in human small airway epithelial cells (SAEC) (Siegrist *et al.*, 2014). Further to their toxicities to humans and other animals, CNTs have been shown to physically interact with aquatic microalgae causing oxidative stress, agglomeration, and inhibition of photosynthesis (reviewed in (Jafar, Alshatti and Ahmad, 2016).

IONPs, on the other hand, are generally considered safe due to their biocompatibility and have shown no severe side effects and toxicity in vivo, therefore, a few SPIONs are already FDA approved for use in the clinical settings as MRI contrast agents and also as iron supplement, others are still undergoing clinical trials (Jin *et al.*, 2014). Despite the FDA approval and their usefulness, there are continuous controversies on their toxicity. Oxidative stress caused by ROS generation by metallic NPs are well documented in various studies (Abdal Dayem *et al.*, 2017; Fu *et al.*, 2014; Qiu *et al.*, 2016). Buzea et al have demonstrated that ROS generation and oxidative

stress development would lead to an inflammatory response (Buzea, Pacheco and Robbie, 2007). One of the proposed mechanisms of inflammatory signalling and response is via modulating intracellular calcium concentration (Moller *et al.*, 2010). Dextrose functionalized SPION are found to cause DNA strand break as evident by comet assay in a dose-dependent manner. Furthermore, genotoxicity in human hepatoma (HepG2) cells line was also evaluated by observing micronucleus formation, which was due to reactive oxygen species (ROS) generation (Seo *et al.*, 2017). High ROS levels have been linked to lipid peroxidation, protein oxidation, DNA aberration and mitochondrial damage. These disruptions can lead to up-regulation of calcium-dependent signalling mechanisms resulting in an imbalance of homeostasis and ultimately in cell apoptosis and death (Stroh *et al.*, 2004). ROS production as a key mediator of cytotoxicity has been further proven by depletion of glutathione and inactivation of several antioxidant enzymes as shown in Chinese hamster lung cells upon exposure to IONPs (Zhang *et al.*, 2012). IONPs, upon endocytosis and metabolization, release free iron and thus modulate the intracellular iron concentration. Iron, due to its ability to switch between ferrous (Fe^{2+}) and ferric (Fe^{3+}), is essential for life. This redox reaction is critical to DNA synthesis, oxygen transport, cytochrome P450 function and oxidative phosphorylation (Shander, Cappellini and Goodnough, 2009). Imbalances in intracellular iron concentration, which is tightly regulated, would be extremely toxic. Increases in intracellular iron concentration alter the expression of transferrin receptor and cellular proliferation. Furthermore, vital organs with highly active mitochondria are likely to be susceptible to toxicity from a high iron concentration environment (Huang *et al.*, 2009).

There are conflicting and inconsistent results regarding genotoxicity of IONPs in various cells regardless of IONP dose and functionality. Micronucleus (MN) tests

and comet assays show genotoxicity in MCL5 lymphoblastoid cells (Singh *et al.*, 2012), A549 alveolar adenocarcinoma cells (Konczol *et al.*, 2011), murine fibroblast cells (L-929) (Hong *et al.*, 2011) and human lung fibroblasts (IMR-90). Similarly, DNA adduct formation was observed in human BEAS-2B bronchial epithelial cells but no DNA breakage (Bhattacharya *et al.*, 2009). Contradictory to these reports, Karlsson et al found no DNA damage in A549 cells upon exposure to IONPs (Fe_2O_3), although Fe_3O_4 produces considerably higher oxidative DNA damage (Karlsson *et al.*, 2009). Similarly, no increase in micronucleus formation and DNA damage as revealed by comet assay in human lymphoblast cells (Singh *et al.*, 2012) and Syrian hamster embryo cells (Guichard *et al.*, 2012) treated with magnetite NPs regardless of functionalization. Mutagenic potentials of IONP have also been investigated which turned out to be negative in different strains of *S. Typhimurium* treated with AMI-25 ION and ferumoxtran-10, an FDA approved contrast agent (Weissleder *et al.*, 1989; Bourrinet *et al.*, 2006). In vivo genotoxicity of IONPs has been studied in mice exposed to Fe_3O_4, which developed DNA-protein crosslinks and oxidative DNA damage in hepatic and renal tissues (Ma *et al.*, 2012). In contrast, orally administration of Fe_2O_3 to wistar rats indicated that IONPs were accumulating in the liver, kidney, spleen and bone barrow upon crossing intestinal barrier but not inducing genotoxicity as evaluated by the comet assay in leucocytes and bone marrow cells (Singh *et al.*, 2013). Intraperitoneal exposure to IONPs increased MN frequency in bone marrow cells of mice (Freitas *et al.*, 2002), in contrast to reports from Estevanato et al (Estevanato *et al.*, 2011) and Bourinet et al (Bourrinet *et al.*, 2006).

These studies show that IONPs induce ROS and have the potential to induce DNA breaks due to oxidative damage to DNA. However, the ability to cause these complications is hugely based on shape, size and functionality of NPs used.

Nevertheless, a large percentage of studies are also inconclusive due to inconsistency. More studies are needed to pinpoint the underlying DNA damage induced by these NPs.

1.11 Thesis scope

The successful translation of nanomedicine from concept through pre-clinical testing to therapeutic use in the clinical setting is very challenging. Despite the tremendous medical need for anti-cancer therapeutic and numerous NPs that have been developed to meet the requirement, only a few have been approved for use. The interactions between NPs and the immune system is one of the key players in creating such challenges which is evident by the withdrawal of approved MRI contrast agents, Feridex, Sinerem, Combidex from clinical use on the ground of complement-related side effects and hypersensitivity in patients (Banda *et al.*, 2014). In recent years, our understanding of the interaction of NPs with the immune system has significantly improved, but several questions still remain unanswered.

Before the biological effects of radiation were known to the scientist, many physicians and corporations had marketed radioactive substances like radium as patented medicine, Radithor or Radium water and advertised as ‘cure for the living dead’. However, later on, risk and safety of these radioactive substances were fully characterized and they were banned from use as tonic water. Likewise, we do not want the history to repeat itself due to the poor characterization of NPs and their use in biomedicine. So, a detailed understanding of risks and safety of nanoparticles is needed.

This thesis combines the development of new NPs with the careful characterization of the response of the immune system towards CNTs and IONPs or magnetite NPs. In the study of the immune system, the focus lays on the innate immune system and the way the response of the complement system influences further immune response.

The thesis includes 6 chapters.

Chapter 2: It includes all the methods and methodologies.

Chapter 3: It includes an in-depth study of a complement protein, properdin and its recombinant small fragment, TSR4+5. Properdin, a C3 convertase stabilizer in alternative pathway also acts as PRR and bound properdin on CNTs modulates the immune response. TSR4+5 on the other hand also adsorb onto NPs and do not allow complement pathway activation, unlike properdin and uncoated NPs. TSR4+5: CNT is able to inhibit the alternative pathway, which may have potential implications on therapeutic drug delivery. On the other hand, we have demonstrated that adsorbed properdin on CNTs still retains its bioactivity and can stabilize C3 and C5 components.

Chapter 4: It includes pulmonary lung surfactant protein D (SP-D) and its bindings to NPs and immune modulation. We found that CNTs coated with a recombinant fragment of SP-D (rhSP-D) were cleared by macrophages very rapidly and enhanced the production of pro-inflammatory cytokines. However, adding complement components on SP-D coated CNTs reduced both phagocytosis and enhanced anti-inflammatory cytokines release. We have demonstrated that complement deposition can be vital in designing NPs that reduce immune modulation.

Chapter 5: Here, IONPs immune response has been studied particularly; activation of the complement system as part of the innate immune response. Analysis of cytokine released by macrophages after complement activation and phagocytosis will be included. In brief, intracellular localization of IONPs will be discussed. Activation of the complement system is generally considered to have an adverse effect. However, we show that complement activation by both NPs could have a positive effect on their uptake by phagocytes and subsequent immune response by these cells.

Chapter 6: It provides the general conclusion and perspectives of the results presented in this thesis.

2 Chapter II

General Materials and Methods

2.1 Preparation of NPs (CNTs and IONPs)

Purified catalytic vapour deposition DWNTs were provided courtesy of Dr E. Flahaut (CIRIMAT, Toulouse University, France). Preparation and purification of these CNTs were described earlier (Bortolamiol *et al.*, 2014; Flahaut *et al.*, 2003). Dispersion of these pristine DWNTs was done using carboxymethyl Cellulose (CMC) (Sigma; CN: 2101). 1mg of CNTs was added to 2% w/v CMC solution prepared in PBS. The solution was ultrasonicated for 2 min. After sonication, the samples were centrifuged at 8000g for 5 min to remove aggregates. The supernatants were washed by vacuum filtering using a 0.2 μm polycarbonate filter (Whatman) with PBS. Functionalized CNTs were re-suspended in PBS-EDTA. Magnetic IONP (nano-screenMAG/R) were purchased from Chemicell. These particles consist of a magnetite core first covered by a lipophilic fluorescence dye and second a layer enveloped with a hydrophilic polysaccharide (starch).

2.2 Transmission electron microscopy contrast staining

Uranyl acetate and lead citrate stain were prepared to enhance the contrast of organic material within the TEM.

2.2.1 Preparation of uranyl acetate and lead citrate

Uranyl acetate was dissolved in distilled water until the solution is saturated. Saturated solution was filtered through a 0.45μm filter and stored in an opaque sealed container for up to 1 week. Distilled water was boiled to get rid of dissolved carbon dioxide and allowed to cool. 33.1g of lead nitrate, 37.7g of tri-sodium and 4g of sodium hydroxide was dissolved in 100ml of cool boiled water and labelled as solution A, solution B and solution C. 3ml of solution A and 2ml of solution B was mixed and 16ml of boiled distilled water was added. The mixture was stirred and left for 30min. 4ml of solution C

was added to the mixture of solution A and solution B to dissolve the precipitate. Thus, prepared lead citrate was filtered through a 0.45um filter and stored up to 1 week.

2.2.2 Staining for TEM

2 µg of well-dispersed CNTs and protein-coated CNTs were adsorbed onto carbon-coated grids. The grid was placed on to parafilm and placed on Petri dishes with potassium hydroxide on it. 20µl of uranyl acetate was placed onto the grid and incubated for 60 min. After incubation, grids were rinsed in distilled water. 20µl of lead citrate was added to the grid in close proximity to the potassium hydroxide pellets to absorb any carbon dioxide. Grids were incubated for 5 min, rinsed in distilled water and allowed to dry. Dried grids are ready for TEM inspection. Micrographs were recorded using a JEOL 2100 FEG-TEM operating at 80Kv, and the images were processed using Gatan microscopy suite software (Gatan, Inc). Surface visualization of CNTs was performed using a Zeiss Supra 35vP scanning electron microscope operating at 5 keV.

2.3 Biotinylation of CNTs

CNTs were biotinylated as follows: 1 mg of CNTs was suspended in 1 mL 0.1 M MES buffer (2-(*N*-morpholino) ethanesulfonic acid, pH 5.0) (Sigma; CN:1.06126). 1 mg of pentylamine biotin (Thermo Fisher Scientific; CN:21345) and 4 µg EDC [1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide] (Sigma) were added in the suspension and mixed for 2 h at room temperature and the reaction was stopped by addition of 0.1 M ethanolamine (Sigma; CN:E9508). The resulting biotin-CNTs were centrifuged at 10,000 g for 10 min in order to remove remaining reactants and MES. Finally, labelled CNTs were resuspended by sonication using water bath sonicator (Transsonic t460 water bath sonicator, 85 Watt, 35 kHz), for 5-10 min. The efficiency of biotinylation was determined by incubating 10 µg of biotinylated CNTs with Alexa Fluor 488-

conjugated streptavidin(1:1000) (Thermofisher Scientific; CN:S11223) for 1 h and then washed extensively via centrifugation at 17,000 g for 10 min three times and re-dispersed. Dispersed biotinylated CNTs were observed under a Leica microscope and images were taken using manufacturer supplied software, Leica application software.

2.4 Coating of CNTs with Proteins.

Recombinant full-length human properdin was expressed in human embryonic kidney (HEK) cells. Purification of properdin was carried out as explained in Kouser *et al.* (Kouser *et al.*, 2018). MBP fused TSR4+5 and MBP was expressed on *E.coli* BL21 as explained in Kouser *et al.*(Kouser *et al.*, 2016). Purified properdin and LPS free TSR 4+5 and MBP was kindly provided by Dr Lubna Kouser (Brunel University) to conduct this study. rfhSP-D was expressed in *E.coli* (λDE3) pLysS and purified as described in Pondman *et al.*(Pondman et al., 2017). Purified LPS free rfhSP-D was kindly provided by Dr Anuvinder Kaur (Brunel University) for the study. Purified properdin, TSR4+5, MBP or BSA and rhfSP-D were incubated in a w/w ratio of 2:1 with 100μg of CNTs in the affinity buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 5mM $CaCl_2$), overnight at 4°C. Excess protein was removed by repeated centrifugation and washing at 17000 g for 10 min to wash away any unbound proteins trapped within the CNTs. CNTs were re-dispersed in affinity buffer between centrifugations.

2.5 Complement activation and consumption assay

2.5.1 Classical pathway

To examine complement consumption in human serum, CNTs and various protein coated 10 μg CNTs, 10 μg of IONP and 10 μg of zymosan (Sigma; CN:Z4250) as positive control were incubated in a ratio 1:1 diluted normal human serum in Dextrose

veronal buffer with Mg^{2+} and Ca^{2+} (DGVB $^{++}$) [2.5 mM sodium barbital (Sigma; CN:B0500), 71 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.5% w/v glucose, 0.1% w/v gelatin (Sigma; CN:, pH 7.4) for 1 hr at 37^0C following the standard protocol for complement consumption assay(Whaley and North, 1997). After incubation, NPs were centrifuged at maximum speed in a microcentrifuge for 10 min and the supernatant was collected. The capacity to lyse antibody-sensitized sheep erythrocytes (EA) (TCS, Buckingham) was tested. Sheep erythrocytes were washed twice for 10 min with PBS-EDTA until the supernatant was cleared and resuspended in $DGVB^{++}$ buffer. Cells were adjusted to 10^9/ml by measuring the absorbance at 540 nm. Absorbance 0.7 at 541nm will give approximately 10^9/ml cells. 10µl of haemolysin (Sigma; CN:S1389) was added to 1ml of cells and incubated for 15 min at 37^0C followed by 15 min on ice. Cells were washed with PBS and suspended in $DGVB^{++}$ to 10^8 cells/ml. The serum was serially diluted 2-fold (1/10-1/5120) in $DGVB^{++}$) in 96 well plate. Then, 100 µl of EA cells at 10^8cells/ml were added to each dilution and incubated for 1 hr at 37^0C. Total haemolysis (100%) was measured by lysing EA with undiluted normal human serum.

2.5.2 Alternative pathway

For alternative pathway, CNTs and various protein coated 10 µg CNTs, 10 µg of IONP and 10 µg of zymosan (Sigma; CN:Z4250) as positive control were incubated in a ratio 1:1 diluted normal human serum in DGVB-MG-EGTA buffer ((2.5 mM sodium barbital, 71 mM NaCl, 7 mM $MgCl_2$, 10 mM EGTA, 2.5% w/v glucose, 0.1% gelatin, pH 7.4) for 1h at 37^0C following the standard protocol (Whaley and North, 1997). This buffer prevents the activation of classical and lectin pathway because of lack of Ca^{2+} in the buffer. After incubation, NPs were centrifuged at maximum speed in a micro centrifuge for 10 min and the supernatant was collected. The capacity to lyse rabbit erythrocytes (TCS, Buckingham) was tested. Rabbit erythrocytes were washed by

repeated centrifugation for 10min, 700g in PBS + 5mM EDTA, pH 7.4 until the supernatant was clear. The cells concentration was adjusted to $1X10^9$/ml in DGVB-MG-EGTA. The serum samples were serially diluted 2-fold (1/5-1/320) in DGVB-MG-EGTA and 100µl of rabbit erythrocytes at 10^9cells/ml concentration was added to each dilution and incubated for 1 hr at 37^0C. After incubation, cells were pelleted by spinning (700g, 10min) and released haemoglobin was measured at 541nm in the supernatant.

2.5.3 Activity of bound properdin in complement consumption

To check whether the CNTs with bound properdin retained capacity to activate the complement alternative pathway, we used properdin deficient serum obtained from the properdin gene-deficient mice kindly gifted by Dr Stover, Leicester University. Properdin-coated CNTs (properdin-CNT), TSR-coated CNTs (TSR4+5-CNT) and CNT alone were incubated with properdin deficient serum (1/2 dilution) in DGVB-Mg-EGTA buffer and incubated for 1 h at 37^oC. After incubation, CNTs were removed by centrifugation at 17000 g for 10 min, and serum was collected. To each of the collected sera, purified properdin (1 µg/ml) was added and the reconstituted sera were tested for the lysis of rabbit erythrocytes. Rabbit erythrocytes were prepared as described above. After incubation, cells were pelleted by spinning (700g, 10min) and released haemoglobin was measured at 541 nm in the supernatant.

2.6 SDS (Sodium dodecyl sulphate) and sample preparation

SDS-PAGE has carried out bye the method of Laemmli (Laemmli, 1970) to detect purified proteins, rhSP-D, TSR4&5 and properdin or adsorbed protein in CNTs. Sample was denatured in 2 X treatment buffer (50 mM Tris pH 6.8, 2% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) and heated at 100^0C for 7 min prior to running on a 12% SDS-PAGE. SDS gel was prepared using the ingredient as shown in

Table 3.1. 20ul of the sample was loaded on the gel and ran for 90 min at 120V for analysis of the expressed protein in the samples. After the completion of SDS-PAGE, the gel was either transferred to nitrocellulose membrane (Thermofisher, CN:LC2006) using western blot method or stained using staining solution ((1 g of brilliant blue, 50% v/v Methanol, 10% v/v acetic acid, 40 ml d.H_2O), and left overnight on a rotary shaker. The gel was then de-stained using a de-staining solution (40% v/v methanol, 10% v/v acetic acid) for 1 h on a rotary shaker or until the protein bands were visible.

Table 2-1: Components and volume for preparing 12% and 15% SDS-PAGE

Resolving gel	12%	15%	Stacking gel	
Components	Volume (ml)		Components	Volume (ml)
dH_2O	3.3	2.3	dH_2O	3.4
30% Bis-Acrylamide	4	5	30% Bis Acrylamide	0.83
1.5M Tris-HCl, pH8.8	2.5	2.5	1.0M Tris-HCl, pH6.8	0.63
10% SDS	0.1	0.1	10% SDS	0.05
10% APS (Ammonium Persulfate)	0.1	0.1	10% APS	0.05
TEMED (Tetramethylethylenediamine)	0.015	0.015	TEMED	0.014

2.7 Western blot

Initially, an SDS-PAGE is prepared to separate protein based on their molecular weight as mentioned above. The protein bands were transferred on to a nitrocellulose membrane in transfer buffer (25 mM Tris, 192 mM Glycine, 20% v/v methanol, pH 8.3) at 320 mA for 2 h. The membrane was then blocked with 5% semi-skimmed milk powder (Tesco, UK) in PBS, pH 7.4 overnight at 4°C and washed with 20ml 1X PBS. Rabbit anti-human properdin (0.92 mg IgG/ml) polyclonal antibodies (donated by Prof. R.B Sim, Oxford University) were diluted 1:500 in PBS and incubated with the membrane for 2 h at room temperature. The membrane was washed three times, 10 min each, with PBS + 0.05% Tween 20 (PBST). Protein A-Horseradish peroxidase (PA-

HRP; 1:1000) (Thermo Scientific, CN:21041) in PBS was added and left at room temperature for 1 h. The blot was washed again with PBST three times and the colour was developed using 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich, CN:8001)).

2.8 Fluorescence Microscopy

2.8.1 Cell culture and differentiation of monocytes

THP-1 cells(ATCC TIB-202), Human embryonic kidney (ATCC 87258) and U937 (ATCC CRL-1593.2) were maintained in complete RPMI 1640 (Sigma, CN: R6504) containing 10% v/v Fetal Bovine Serum (FBS) (Thermofisher, CN:11573397), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Thermofisher, CN:15140122) and 1 mM sodium pyruvate (Thermofisher, CN:11360039). Differentiation of monocytes, THP-1 and U937, into macrophages was carried out with 100 nM Phorbol myristate acetate (PMA) (Sigma, CN:P8139) for 24 h. Differentiated cells were washed three times with PBS to remove excess PMA and then rested for another 24 h in complete RPMI media prior to the experiment.

2.8.2 Microscopy of uptake of CNTs

Uptake of biotinylated CNTs, coated with various proteins or uncoated was examined using differentiated THP-1 macrophages. For fluorescence microscopy, 1 x 10^5 THP-1 cells were plated and differentiated with PMA on 13 mm coverslips in complete RPMI 1640 medium. Differentiated cells were exposed to 4 µg/ml biotinylated CNT, proteins coated or uncoated in 500 µl of serum-free RPMI medium for 2 h. After washing the cells with PBS, cells were fixed with 4% paraformaldehyde (PFA) (Sigma, CN:158127) for 10 min. Coverslips were washed, mounted on mounting slide and processed for fluorescence microscopy. The cells on coverslips were permeabilized using a permeabilizing buffer (20 mM HEPES pH 7.4, 300 mM sucrose, 50 mM sodium

chloride, 3 mM $MgCl_2$, 0.5% Triton X-100) for 5 min on ice and then stained for 30 min with Hoechst 33342 (1:10,000) (Thermofisher, H3570), Alexa-Fluor546-conjugated wheat germ agglutinin (1:250) (ThermoFisher, W11261) and Alexa fluor 488-conjugated streptavidin (1:500) (Thermo Scientific, CN:S11223) to reveal biotinylated CNTs. Cells were washed, mounted on the glass mounting slide using Citifluor anti-fade (Citifluor, UK), and viewed under a Nikon Eclipse TE2000-S confocal microscope with 62 X oil lens.

2.8.3 Co-localization of IONPs with dextran (a marker for fluid phase endocytosis).

$5x10^4$THP-1 cells were seeded in 24 well plates with 13mm coverslips. Cells were differentiated with 100 nM of PMA for 24 h and left to rest for another 24 h. Cells were treated with 10ug/ml of complement deposited and undeposited IONPs together with 100ug/ml of FITC labelled dextran particles (M. wt: 21,200) (Sigma, CN:FD20) for 2 h and a further 18h after washing (pulse chase). After incubation, cells were washed with PBS three times and fixed with 4% PFA, mounted and observed under a Leica fluorescent microscope using LAS software.

2.8.4 Co-localization of IONPs with lysotracker

THP-1 cells were differentiated and maintained on coverslips as described above. After incubation and resting for 24h, cells were treated with 10ug/ml of serum coated and uncoated IONPs for 2h, 4h, 6h and 24 h continuous exposure as well as for 2h with subsequent 18h chase. 15 min prior to the observation, 100 nM of lysotracker green (Cell Signalling, CN:8783S) was added to the cells and incubated. Cells were washed three times with warm PBS, mounted using imaging medium (140mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1.0mM $MgCl_2$, 20mM HEPES, pH=7.4)on mounting slides and observed under a microscope.

2.8.5 NF-kB nuclear translocation

To observe nuclear translocation of NF-κB, $1x10^5$THP-1 cells were differentiated with 100 nM PMA, as described above, in 13mm of glass coverslip in 24 well plate and incubated with 4 µg/ml of rfhSP-D bound CNTs (rhSP-D + CNT), rfhSP-D bound and complement deposited CNTs (rfhSP-D + serum + CNT), complement deposited CNTs (serum + CNTs), properdin-CNTs, TSR4+5-CNTs or CNTs alone in 500 µl of serum-free medium for 2 h. Cells were fixed and permeabilized using permeabilization buffer for 5 min on ice. The cells were probed with rabbit anti-NF-κB p65 polyclonal antibodies (1:250) (Santa Cruz Biotech, CN:sc8008), followed by Alexa Fluor 488-goat anti-rabbit antibody (1:500) (Abcam, CN:ab150077), and viewed under a leica fluorescent microscope. Images were processed using LAS software.

2.9 Determination of cytotoxicity of NPs

Cytotoxicity assay was performed to determine the cytotoxicity of NPs. $1x10^4$of THP-1 cells per well were differentiated in 96-well cell culture plate and left overnight with PMA. Cells were washed and fresh RPMI medium was added and cells were rested for another 24h. 10 µg/ml of IONPs and 5 µg/ml of CNTs and their respective complement deposited version were added to each well in serum free medium and incubated for 24h. 0.05% saponin (Sigma, CN:47036) was used as positive control and cell only well was used as negative control. After incubation, medium was aspirated and replaced with 100 µl of fresh culture medium. 12 mM of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, CN:M5655) was prepared in sterile PBS. 10 µl of 12 mM freshly made MTT solution was added to each well and incubated at 37^0C for 4h. After incubation, the media was discarded gently and 50ul of DMSO (Dimethly sulfoxide) (Sigma. CN: W387520) was added to each well to solubilize the formazan crystals and mixed thoroughly shaking on a shaker for 10min. The absorbance of

formazan was read at 540 nm using a BioRad plate reader. Number of viable cells were expressed in percentage with respect to negative control (cells only) using the following formula:% of viable cells =(Mean absorbance of sample/Mean absorbance of negative control)x100%.

2.10 Quantification of uptake of CNTs

For quantification, 5 x 10^5 THP-1 cells were plated in 12 well plates and differentiated with PMA for 24h and rested for 24 in complete RPMI media. Cells were washed with PBS and challenged with 4 µg/ml biotinylated CNTs coated with properdin (properdin-CNTs), MBP-TSR4+5 (TSR4+5-CNT), or biotin-CNTs, SP-D or serum deposited SP-D in 500 µl of serum-free RPMI medium for 2 h. Cells were washed three times with PBS and lysed with lysis buffer (10 mM HEPES, 20 mM NaCl, 0.5 mM EDTA, 1% v/v Triton X 100). An ELISA type assay was employed to quantify the number of CNTs taken up by THP-1 cells (Pondman *et al.*, 2016). Microtitre wells (NUNC, polysorbate) were coated with 100 µl Avidin (Sigma, CN:9275,) at 50 µg/ml in 0.1 M carbonate-bicarbonate buffer, pH 9 (Sigma, CN: 08058) for 1 h at RT, followed by blocking with 0.05% of Bovine serum albumin (BSA) for 1 h at RT. 50 µl of a solution or cell lysate containing biotin- CNTs and 50 µl of 0.05% BSA were added in each well and incubated for 1 h at RT. The plate was washed with PBS to remove unbound CNTs and then incubated with 1:2000 dilution of Streptavidin-HRP (Invitrogen, CN:434323) for 1 h at RT. Following washing again, O-Phenylenediamine dihydrochloride (OPD) (Sigma, CN:P9187) was used as a substrate for the HRP and the yellow 2, 3-Diaminophenazine product was read at 450 nm using a BioRad plate reader.

2.11 Measurement of THP-1 cell cytokine and transcription factor mRNA expression using quantitative RT-PCR

In a 12 well cell culture plate (Nunc), THP-1 cells (1 x 10^6/well) were differentiated with 100 nM PMA in RPMI 1640 complete medium for 24 h and then rested (without PMA) for 24 h. Cells were incubated with 5 µg/ml of various protein coated or CNT alone and 10 µg/ml of IONP and various protein coated IONP to wells in serum-free RPMI 1640 medium and incubated for 30, 60, 120 or 360 min. Cells at each time point were washed with PBS and lysed within the wells using lysis buffer from GenElute Mammalian Total RNA Purification Kit (Sigma, CN:RTN350). Total RNA was extracted from the lysate using the GenElute Mammalian Total RNA Purification Kit (Sigma). To inactivate both DNase I and RNase, samples were heated at 70°C for 10 min and cooled on ice. The concentration and purity of extracted RNA were determined using a NanoDrop 2000/2000c spectrophotometer (Thermo-Fisher Scientific). The cDNA was synthesized using High Capacity RNA to cDNA Kit (Applied Biosystems, CN:4387406) following manufacturer protocol using 2 µg of RNA. Primers (Table 2-2) were used and designed using Primer-BLAST(http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The qPCR reaction mixture was prepared following manufacturer protocol containing 5µl Power SYBR Green MasterMix (Applied Biosystems, CN:4367659) which contains SYBR green dye, DNA polymerase, dNTPs, passive reference and optimized buffer components, 75 nM of forward primer, 75 nM of reverse primers and 500 ng template cDNA.. PCR was carried out in a Step One Plus Real-Time PCR System (Applied Biosystems). The initial steps involved 2 min at 50^0 C and 10 min at 95^0 C. The template was amplified for 40 cycles, each cycle consists of 15 s at 95^0C and 1 min at 60^0C.Human 18S rRNA target was used as an endogenous control and data were analysed using manufacturer software, the Step One software v2.3. Ct (cycle

threshold) values for each cytokine target gene were extracted and relative expression of each target gene was calculated using the equation: RQ = $2^{-\Delta\Delta Ct}$ for each cytokine target gene and log10 of relative expression (RQ) was plotted, and comparing relative expression with that of the 18S rRNA constitutive gene product.

Table 2-2: Terminal primers used for qPCR analysis

Targets	Forward primer	Reverse primer
18S	ATGGCCGTTCTTAGTTGGTG	CGCTGAGCCAGTCAGTGTAG
IL-1β	GGACAAGCTGAGGAAGATGC	TCGTTATCCCATGTGTCGAA
IL-6	GAAAGCAGCAAAGAGGCACT	TTTCACCAGGCAAGTCTCCT
IL-10	TTACCTGGAGGAGGTGATGC	GGCCTTGCTCTTGTTTTCAC
IL-12	AACTTGCAGCTGAAGCCATT	GACCTGAACGCAGAATGTCA
TGF-β	GTACCTGAACCCGTGTTGCT	GTATCGCCAGGAATTGTTGC
TNF-α	AGCCCATGTTGTAGCAAACC	TGAGGTACAGGCCCTCTGAT
NF-κB	GTATTTCAACCACAGATGGCACT	AACCTTTGCTGGTCCCACAT

2.12 Multiplex cytokine array analysis

Supernatant from THP-1 cells, incubated with various protein coated or uncoated CNTs for 24 h and 48 h, were collected to measure Secreted cytokines [interleukin (IL)-6, IL-10, IL-12p40, IL-12p70, IL-1α, IL-1β, TNF-α, IL-15, IL-17A, IL-9, TNF-β, IFN-α2], chemokines [monocyte chemo attractant protein (MCP)-3, interferon gamma-induced protein (IP)-10, eotaxin, growth regulated oncogene (GRO), IL-8, MCP-1, macrophage-derived chemokine (MDC), fractalkine, macrophage inflammatory protein (MIP)-1α, MIP-1β, growth factors [vascular endothelial growth factor (VEGF), IL-9, fibroblast growth factor (FGF)-2, IL-2, epidermal growth factor (EGF), granulocyte macrophage (GM)-CSF, IL-3, granulocyte colony stimulating factor (G-CSF), IL-7], and ligand and receptors [FMA like tyrosine kinase 3 ligand (FLT-3L), sCD40L, interleukin 1 receptor antagonist (IL-1RA)].

The analytes were measured using MagPix Milliplex kit (EMD Millipore) following the manufacturer's protocol. MagPix Milliplex kit consists of quality controls (QCs) for quality assay performance, standard (calibrator) to a reference lot to ensure lot-to-lot consistency, optimized assay buffer to mimic native analyte environment, and a cocktail of detection antibody for each analyte bound on magnetic beads. MILLIPLEX map is based on the Luminex xMAP technology capable of performing a variety of immunoassays on the surface of fluorescent-coded magnetic beads known as MagPlex-C microspheres. Magnetic beads are 6.45µm in size and each is coated with antibodies against analytes. These antibodies bind to analytes like TNF-α, IL-1β etc. Detection antibody conjugated with biotin binds to these captured analytes and was detected using streptavidin conjugated with phycoerythrin (PE) (Figure 2-1).

;

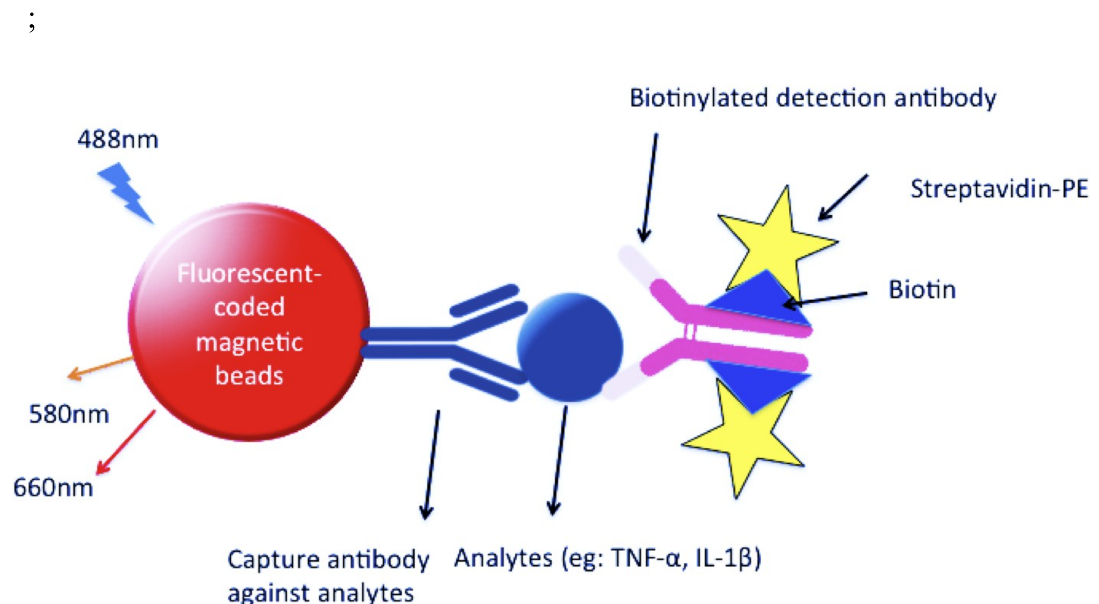


Figure 2-1: The schematic representation of the principle of the Milliplex assay.

Fluorescent beads coupled with capture antibody are used to capture analyte target. The analyteis detected using biotinylated detection antibody. Detection antibodies are detected using streptavidin conjugated with phycoerythrin. The assay is carried out in 96-assay well plate and read using Luminex Magpix instrument and data is analysed using Milliplex analyst software.

2.12.1 Preparation of reagents and sample for immunoassay

Each individual vial of beads was sonicated for 30 seconds and vortexed for 1 minute. 70 µl of each bead were mixed with bead diluent and made the final volume up to 3.5ml. Diluted beads were mixed welled and stored at 2-8^{0}C for up to a month. Manufacture provided 10X wash buffer was diluted to a 1X working buffer in deionized water. THP-1 supernatant of various time points (24h and 48h) from each experiment were stored at -80^{0}C. The supernatantwas thawed and centrifuged to remove debris. Prior to the use of the assay, all reagents were warm to room temperature.

2.12.2 Multiplex procedures

The assay plate was washed with 200 µl of wash buffer by shaking for 10 min. Wash buffer was discarded from all wells by inverting the plate and tapping it onto absorbent several times. 25 µl of standard, or control or test supernatant was added. 25 µl of assay buffer provided by the manufacturer was added to each sample well. 25 µl of matrix solution was added to the background, standard and control well. Culture medium (RPMI 1640) was used as matrix solution. 25 µl of a diluted mixed cocktail of magnetic beads were added to each well. The plate was sealed with a plate sealer and incubated for overnight at 4^{0}C. The plate was washed 2 times with 200 µl of wash buffer using handheld magnet plate washer. 25 µl of detection antibodies were added to each well, sealed and incubated with agitation on a plate shaker for 1h at room temperature. 25 µl of streptavidin-PE was added to each well-containing detection antibodies. The plate was sealed and incubated again with agitation on a plate shaker for 30 min at room temperature. Contents on the plate were washed 2 times with 200 µl of wash buffer using handheld magnetic plate washer. 150 µl of sheath fluid (manufacturer provided) was added to each well to serve as the delivery medium to transport the sample to the

instrument's optics. The beads were resuspended by shaking on the plate shaker. The plate was read using Luminex Magpix instrument and results were obtained using Milliplex analyst software. Medium fluorescent intensity (MFI) data was exported and presented using GraphPad Prism 7.0 software.

2.13 Statistical analysis

Statistical analysis was conducted using GraphPad Prism version 7.0 (GraphPad software). An unpaired to two-sided*t*-test and 2 –way ANOVA test was used on the data for analysing any significant difference between test samples. *P* values were computed and graphs were compiled and analysed using GraphPad Prism version 7.0.

3 Chapter III

Interaction between human properdin and carbon nanotubes

3.1 Abstract

Development of NPs as tissue-specific drug delivery platform can be significantly influenced by complement components due to their inherent pro-inflammatory and tumorigenic consequences. Activation of complement pathways and interactions with recognition subcomponents can modulate the clearance of the NPs and subsequent inflammatory response. Such modulation could affect the intended translational applications. Here, we report, that human properdin, an up-regulator of the complement alternative pathway and stabilizer of C3 convertase, can opsonize CNTs via its thrombospondin type I repeat (TSR) 4 and 5. Binding of properdin and TSR4+5 is likely via charge pattern/polarity recognition on the surface of NPs. Uptake of properdin bound CNTs was enhanced by a macrophage cell line, THP-1, surging a robust pro-inflammatory immune response, as evident by qRT-PCR, multiplex cytokine array, and NF-κB nuclear translocation tests. Thus, properdin interaction with NPs is of considerable importance as immune cells can locally synthesize properdin in an inflammatory microenvironment. In addition, recombinant TSR4+5 on CNTs, inhibited complement consumption, suggesting that TSR4+5, can be potentially used as a complement inhibitor in a number of pathological circumstances arising due to unintentional complement activation. Furthermore, properdin coated NPs are still functionally active and can regulate alternative pathway activation. Properdin-CNTs can be used as a platform for drug delivery where properdin deficiency causes susceptibility to a range of infections in the condition like a rare X-linked properdin deficiency disease in human.

3.2 Introduction

Properdin, a highly positively charged molecule at neutral pH (Smith *et al.*, 1984). The human properdin monomer has seven TSRs denoted as TSR0-TSR6. Properdin consists of identical monomers of 53 kDa joined with each other in a head-to-tail manner (Nolan *et al.*, 1992; Discipio, 1982) to form cyclic polymers (dimers, trimers, and tetramers) in a ratio of 26:54:20 (Smith *et al.*, 1984) (Figure 3-1). A recent electronic microscopic study has shown that vertexes of oligomeric properdin are formed by four TSR from two neighbouring monomers (Alcorlo *et al.*, 2013). Each TSR is 60 amino acids long and has sequence similarities with circumsporozoite protein of malaria parasite thrombospondin (adhesive glycoprotein) (Dame *et al.*, 1984,Lawler and Hynes, 1986) and a membrane-attack component of component C9 (DiScipio *et al.*, 1988). TSR4 and TSR5 module in properdin helps in the stabilization of C3 convertase by binding to C3b (Higgins *et al.*, 1995; Perdikoulis, Kishore and Reid, 2001; Kouser *et al.*, 2016).

The concentration of properdin is 25 μg/ml in plasma (Pillemer *et al.*, 1954). However, in some condition such as anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV), properdin level is lowered (Gou *et al.*, 2013). It can be locally secreted by variety of stimulated cells, such as endothelial cells (Bongrazio, Pries and Zakrzewicz, 2003), neutrophils (Wirthmueller *et al.*, 1997), dendritic cells (Dixon *et al.*, 2017), peripheral blood monocytes (Schwaeble *et al.*, 1994), and both $CD4^+$ and $CD8^+$ T cells (Schwaeble *et al.*, 1993). Local production of properdin is crucial for immune cells such as DCs, macrophages and T cell response. DCs treated with properdin-siRNA shown a reduction in proliferation of allogenic T cells and the effect were more prominent in combined with IFN-γ stimulation (Dixon *et al.*, 2017). Furthermore, macrophages in properdin-deficient mice have associated with the tumour-promoting activity by increasing production in the M2 phenotype (arginase-1, MCP-1 and IL-10)

and reducing M1 phenotype (IL-1β) thereby, enhancing tumour environment(Al-Rayahi, Browning and Stover, 2017).

The structural organization of properdin (multi-subunit, multiple potential binding domains, potential multivalency) appears to suggest a possible role as a soluble PRR as evident by various published reports. It has been reported to bind to several self and non-self-target ligands such as zymosan (Harboe *et al.*, 2012) inducing-antibody-independent complement activation (Spitzer *et al.*, 2007), rabbit erythrocytes, *Neisseria gonorrhoeae* (Spitzer *et al.*, 2007), apoptotic CD4^{+} T cells facilitated by the glycosaminoglycan chains of surface proteoglycans (Kemper *et al.*, 2008), human proximal tubular epithelial cells (Gaarkeuken *et al.*, 2008), and cartilage oligomeric matrix protein (Happonen *et al.*, 2010). Properdin directly interacts with target ligands such as heparin (Yu *et al.*, 2005), heparan sulphate (Zaferani *et al.*, 2012; Kemper *et al.*, 2008), dextran sulfate, fucoidan (Holt, Pangburn and Ginsburg, 1990), and chondroitin sulfate (Kemper *et al.*, 2008). Properdin also binds to activated platelets via surface GAGs (Saggu *et al.*, 2013). Properdin, secreted by DCs and tolerogenic DCs can bind DNA on late apoptotic and necrotic cells (Ferreira, Cortes and Pangburn, 2010) in a C3b-independent manner (Xu *et al.*, 2008) and also bacterial LPS of *E. coli* (Kimura *et al.*, 2008). Furthermore, properdin can also bind to natural cell activating receptor (NKp46) on NK cells and innate lymphoid cell (ILC) 1 and ILC3, natural cell activating receptor required for the fight against *Neisseria meningitidis* infections. Properdin's direct interaction with such large number of cell surface molecules may indicate its selective pattern recognition properties and thus, can be hypothesized that it can also bind to a wide range of nanotherapeutics.

Here, we showed that properdin binds to CNTs as a pattern recognition receptor via at least TSR4+5 domains, and enhances their uptake by macrophages. Properdin

bound CNTs promoted a robust pro-inflammatory immune response. TSR4+5, when coated on CNTs, inhibited complement alternative pathway activation, reduced macrophage sequestration but induced a robust pro-inflammatory immune response.

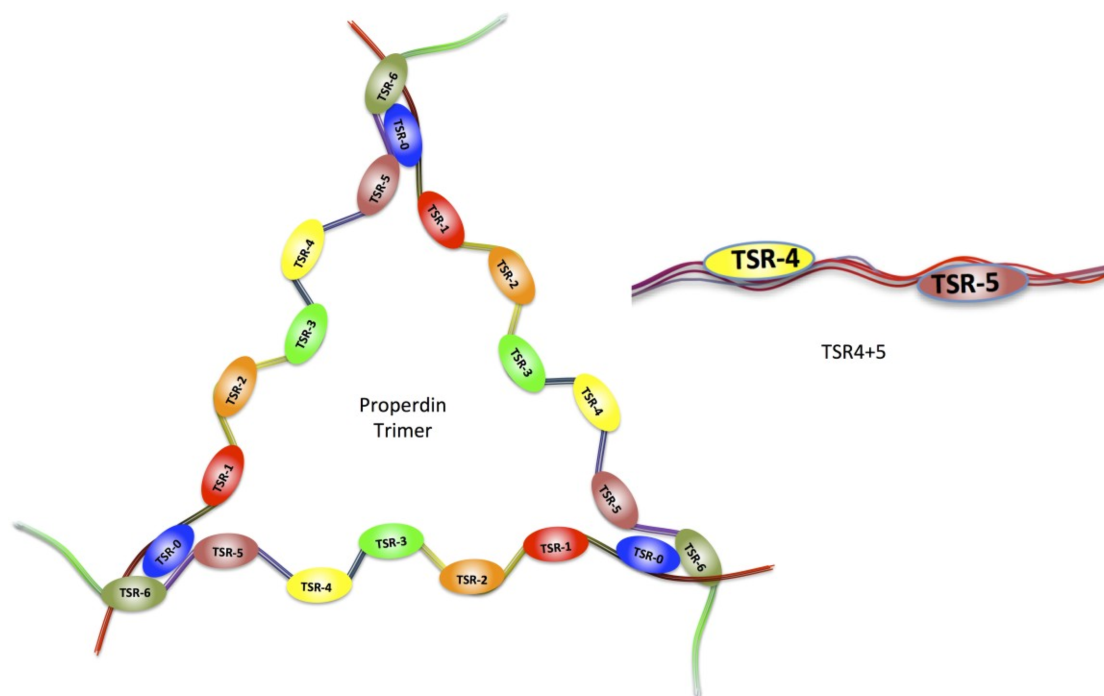


Figure 3-1: Sketch of trimeric properdin (Left) and its TSR modules (Right).

Schematic representation of the modular organization of human properdin including thrombospondin type 1 repeats (TSRs) domain. In serum, properdin is found in monomers, dimers or cyclic trimer or tetramers joined together with head to the tail orientation of monomers made up of seven TSRs domain. N-terminal, TSR0 is connected with C-terminal TSR6 thus forming a cyclic structure. TSR4 and TSR5 domain, main C3b binding domain, are expressed in *E.coli.*

3.3 Results

3.3.1 Properdin and TSR4+5 bind CNTs.

Well-dispersed CNTs were incubated overnight with purified recombinant full-length properdin as well as TSR4+5 modules. After incubation, CNTs were washed with PBS extensively via centrifugation. Western blot analysis showed that properdin and MBP-TSR4+5 bound CNTs, in their denatured formed, and appeared in the 12% SDS-PAGE at their expected molecular weight at ~ 55kDa. However, CNTs remained in the loading wells (Figure 3-2). This suggested that properdin and TSR4+5 can bind to CNTs and binding to CNTs surface is likely to be through charge pattern/polarity recognition on the surface.

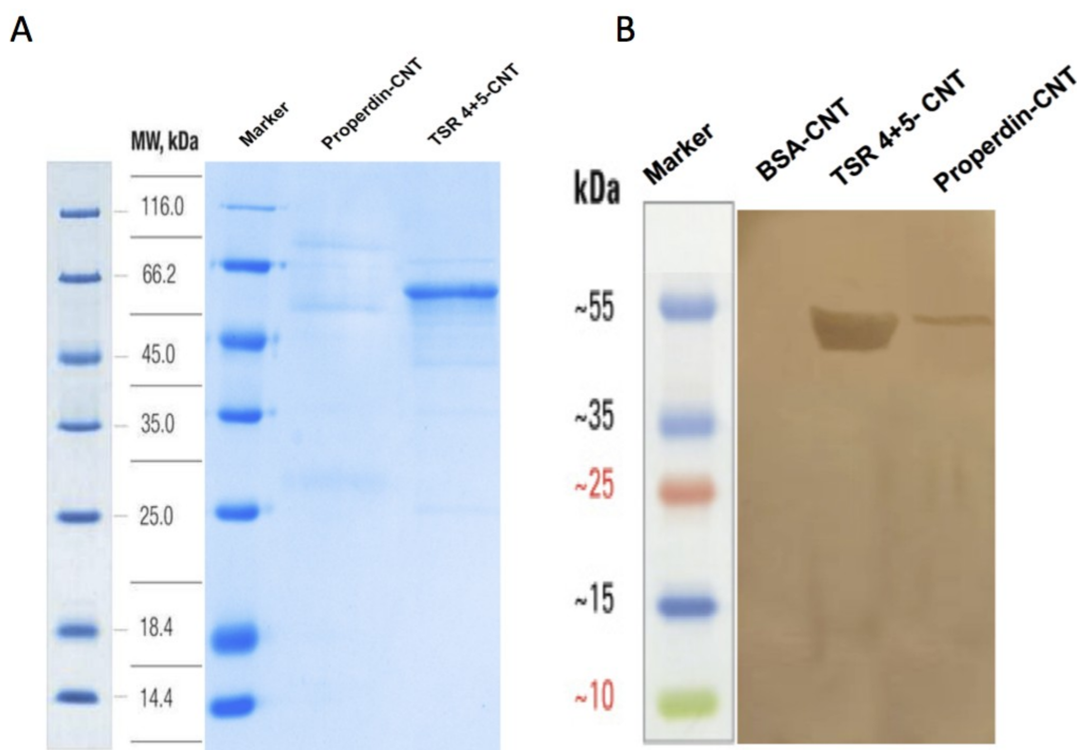


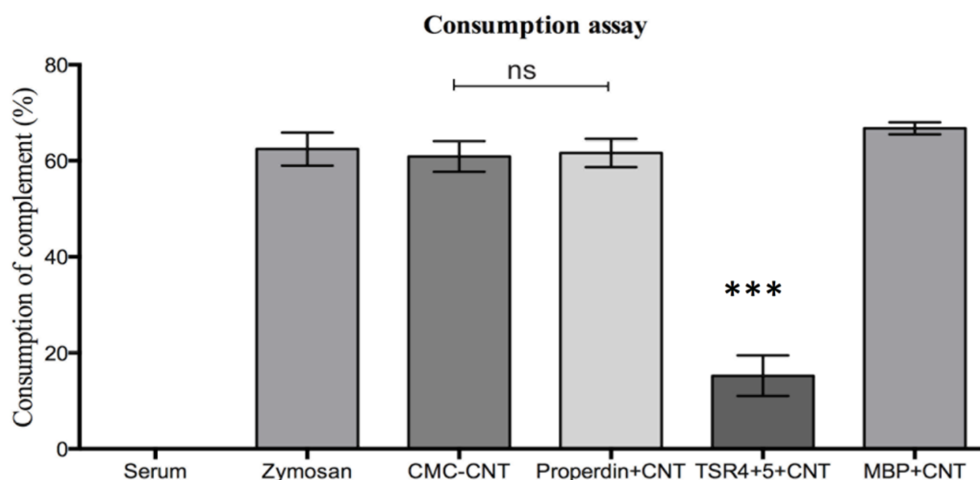
Figure 3-2: Binding of recombinant full-length properdin and MBP-TSR4+5 to CNTs

(A) SDS-PAGE: CNTs were incubated with recombinant human properdin, or MBP-TSR4+5 (kindly provided by Dr Lubna Kouser) and BSA overnight in the affinity buffer. CNTs were washed extensively via centrifugation and ran on an SDS-PAGE (12%) under reducing conditions. Properdin and MBP-TSR4+5 migrated as a single band at ~55 kDa and ~53 kDa respectively. **(B) Western blot of properdin and TSR4+5 binding to CNTS**. Separated proteins were blotted onto nitrocellulose membrane for 2 h at 320 mA. The blot was blocked with semi skimmed milk and probed with anti-human properdin (polyclonal) antibodies, followed by Protein A-HRP conjugate and developed using DAB.

3.3.2 MBP-TSR4+5 coated on CNTs inhibited complement consumption via the alternative pathway

Properdin-coated and MBP-TSR4+5 coated CNTs were examined for their ability to activate the complement alternative pathway compared with uncoated CNTs, zymosan (positive control) and serum (negative control). Properdin coated CNTs did not interfere with alternative pathway activation; showing the same extent of complement activation as CNTs alone and MBP-CNTs. However, TSR4+5-bound CNTs showed ~60% less complement consumption. This suggests that the CNT-surface bound TSR4+5 acted as an inhibitor of the complement alternative pathway. TSR4+5 have previously been shown inhibit complement alternative pathway in solution (Kouser *et al.*, 2016). These results suggest that binding of properdin to CNTs is via TSR4+5 domain and that pre-coated TSR4+5 inhibited the binding of serum properdin, and thereby weakening the alternative pathway activation. Properdin-coated CNTs also consumed (activated) the alternative pathway (Figure 3-3A), in properdin-deficient serum. Complement consumption was upregulated by ~60% compared to uncoated CNTs (Figure 3-3B). Complement activation by TSR4+5-coated CNTs was not significant compared to uncoated CNTs. This suggested that the properdin activity was not inhibited upon binding to CNTs.

A)



B)

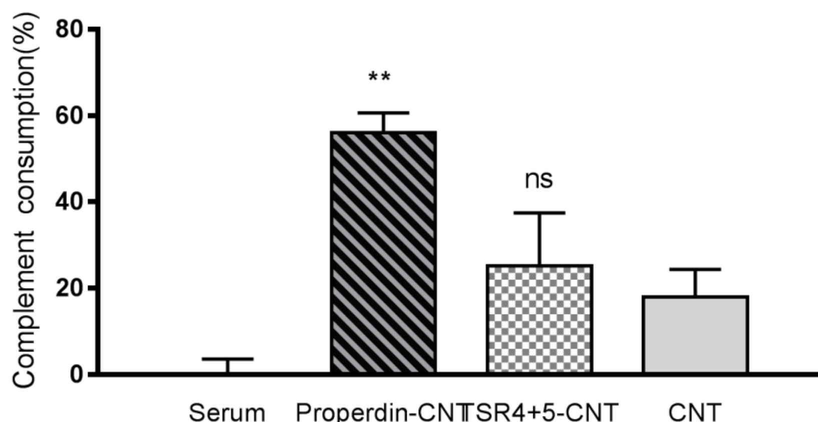


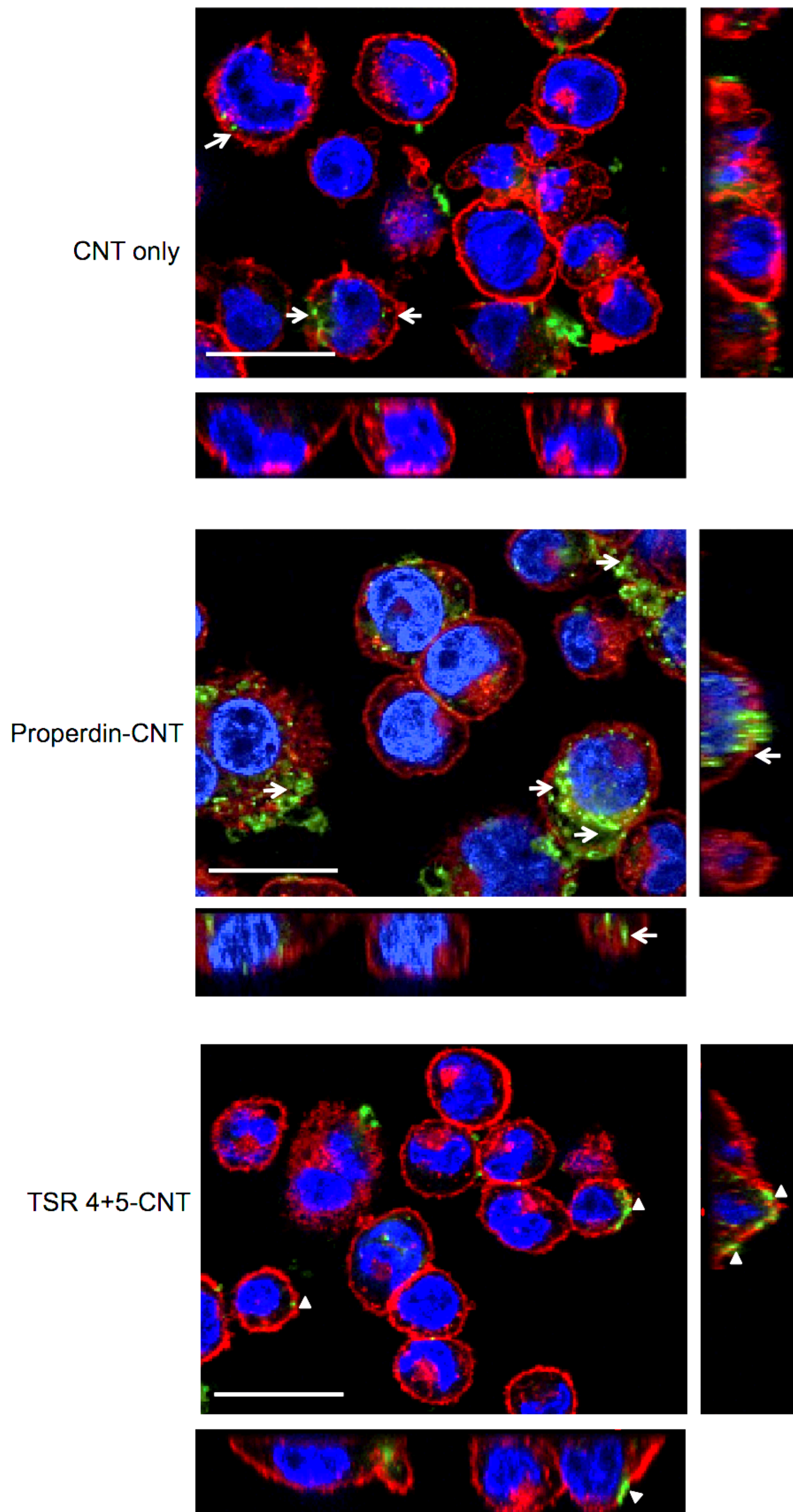
Figure 3-3: Complement alternative pathway activation by properdin and TSR 4+5 coated CNTs.

A) Properdin, TSR4+5, or MBP coated CNTs were incubated with human serum in 1:5 dilution with alternative pathway buffer (DGVB-Mg-EGTA buffer) for 1 h shaking at 37°C. CNTs were spun down and serum was collected for complement consumption assay. Zymosan and serum only were used as positive and negative control respectively. **(B)** Protein-coated CNTs were incubated with mouse properdin deficient serum diluted in 1:2 with DGVB ¯Mg-EGTA buffer for 1h at 37°C. The samples were centrifuged and properdin deficient serum supernatant was collected. Collected serum was reconstituted by adding purified recombinant properdin to give a final concentration of 1µg/ml. Serum was then assayed for complement consumption. Percentage complement consumption was calculated using $(C - \mathrm{Ci})/C \times 100\%$, where C represents the % hemolysis of the negative control, and Ci is the % hemolysis with the test samples; properdin or TSR4&5 coated CNTs or uncoated CNTs or zymosan-treated sampleThe experiments were repeated 3 times; error bars represent ± standard deviation. Ns: not significant (p>0.05)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$) (a 2-tailed, unpaired t-test between complement consumption of properdin coated CNTs and TSR4+5 coated CNTs with CNTs only)

3.3.3 Properdin, but not TSR4+5, enhanced CNT uptake by THP-1 cells

PMA differentiated THP-1 macrophages were challenged with properdin and TSR4+5 bound CNTs for analysis of their uptake. ELISA results showed that full-length recombinant properdin enhanced the uptake of CNTs more than TSR4+5 bound CNTs compared to uncoated at 2 h in a complement-independent manner (Figure 3-4B). Previous studies reported that pre-coating with C1q enhanced phagocytosis of CNTs by U937 monocytes and human monocytes, whereas, factor H, a negative regulator of the complement system, inhibited the uptake of CNTs and CNTs were observed adhered on the surface of the plasma membrane (Pondman *et al.*, 2015). Here, confocal microscopic images showed that properdin-CNTs were significantly taken up more by THP-1 cells, as compared to CNT alone and TSR4+5-CNT (Figure 3-4). CNTs (green) were observed within the cell membrane boundary as revealed by staining with Alexa Fluor 546 conjugated wheat germ agglutinin (red) for plasma membrane and the nucleus (Hoechst, blue) (Figure 3-4). The orthogonal confocal sections (right panel) revealed Properdin-CNT within the cell; however, very few CNTs could be seen intracellular in the case of TSR4+5-CNT (Figure 3-4A). This suggests that full-length properdin molecule is required for this function as 'opsonin'. This also indicates that macrophage may interact with other TSRs domains, not limited to TSR4+5 only.

A)



B)

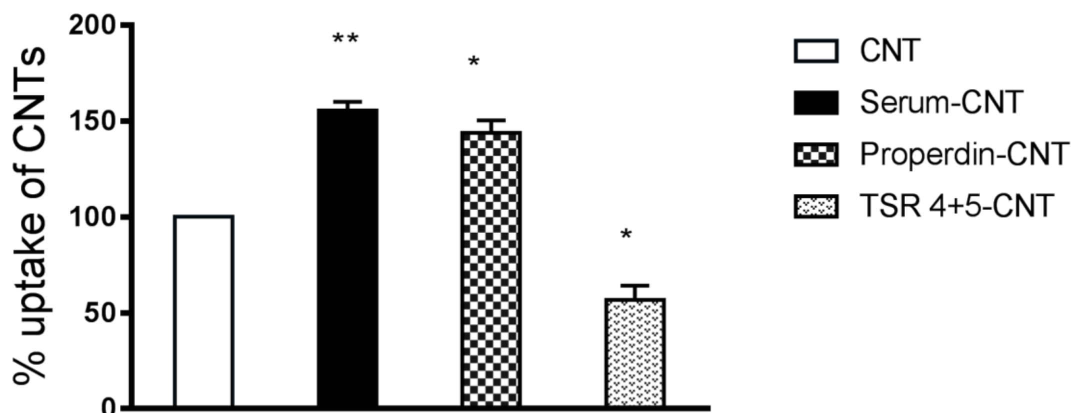


Figure 3-4: Uptake of biotin-CNTs coated with properdin and TSR 4+5 by differentiated THP-1

A) To observe internalization of CNTs, PMA-differentiated THP-1 cells were treated with properdin or MBP-TSR4+5 coated biotinylated CNTs and uncoated biotinylated CNTs for 2 h. Cells were washed, fixed, permeabilised and stained with Alexafluor-488 labelled Streptavidin (green) to reveal internalized biotin-CNTs. Orthogonal views of the confocal images demonstrate internal localisation of CNTs (green), plasma membrane in red, scale bar 20µm, arrow heads point to the CNTs adhering to the plasma membrane**B)** To quantify the amount of uptake of protein-coated or uncoated CNTs, THP-1 cells were incubated with 4µg/ml of protein-coated biotin CNTs or uncoated CNTs for 2 h. The cells were lysed and the amount of CNTs was quantified by an ELISA type assay. All experiments were done in triplicate; error bars represent mean± SDNs: not significant (p>0.05)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$)(An unpaired t-test, 2-tailed between the uptake of properdin coated CNTs and TSR4+5 coated CNTs with CNTs only)

3.3.4 Pro-inflammatory cytokines are upregulated by both properdin and TSR4+5 CNTs

Properdin-CNT and TSR4+5:CNTs significantly upregulated transcriptional expression of TNF-α, IL-1β, IL-6 and IL-12 in THP-1 cells (Figure 3-5). In contrast, IL-10 and TGF-β were initially up-regulated at 30 min, but decreased by 6 h, suggesting that the anti-inflammatory response was very transient and dampened later. Consistent with the up-regulation of TNF-α, properdin and TSR4+5 coated CNTs upregulated the expression of NF-κB at 6h incubation. However, the NLRP3 expression was not significant in all cases, suggesting that none of the CNTs activated NLRP3 inflammasome formation.

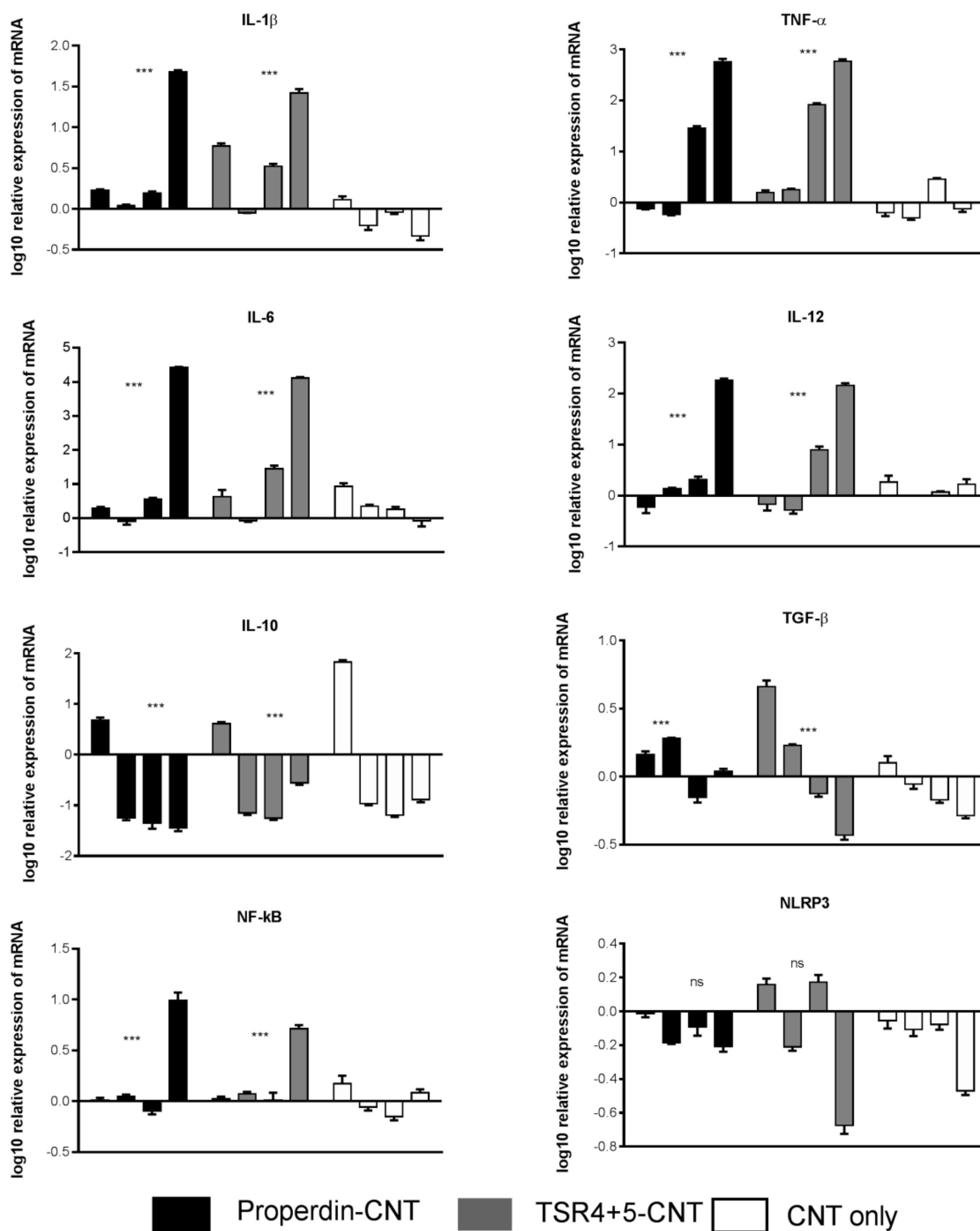


Figure 3-5: Cytokine gene expression profile of THP-1 cells treated with properdin or TSR 4+5 coated or uncoated CNTs.

mRNA expression of pro- and anti-inflammatory target genes were measured. THP-1 cells were incubated with coated and uncoated CNTs for 30 min, 1h, 2h and 6h (X-axis). The data were normalized using 18S rRNA gene expression as an endogenous control. Error bars represent ± S.D. Ns: not significant (p>0.05)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$)(A 2-way ANOVA test between properdin or TSR4+5 coated CNTs with uncoated CNTs.

3.3.5 Properdin or TSR4+5 CNTs up-regulate pro-inflammatory cytokines/chemokines and other soluble factors in THP-1 cells

Multiplex array analysis on supernatants collected at 24 h and 48 h time points from the phagocytosis assay showed a dramatic increase in the secretion of pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α, IL-6, IL12p40, IL12p70, IL-13, IL-15, IL-9) (Figure 3-6) for both properdin-CNTs and TSR4+5-CNTs. There was an enhancement in chemokinereleases, such as MCP-3, MDC, Eotaxin, Fractalkine, GRO, IL-8, IP-10, MCP-1, MIP-1α (Figure 3-7) by properdin and TSR 4+5-CNTs. A number of anti-inflammatory cytokines, chemokines, growth factors and immune ligands were also differentially regulated by exposure to protein-coated CNTs. This suggests that properdin and its small fragment, TSR4+5 are very pro-inflammatory in nature when bound to NPs. Furthermore, properdin and TSR 4+5 have been shown to both enhance the chemoattractant function of CNTs. Moreover, CNTs themselves act as a chemoattractants and stimulate macrophages migration by activating the PLC/IP3/CRAC channel signalling pathway (Li *et al.*, 2017a).

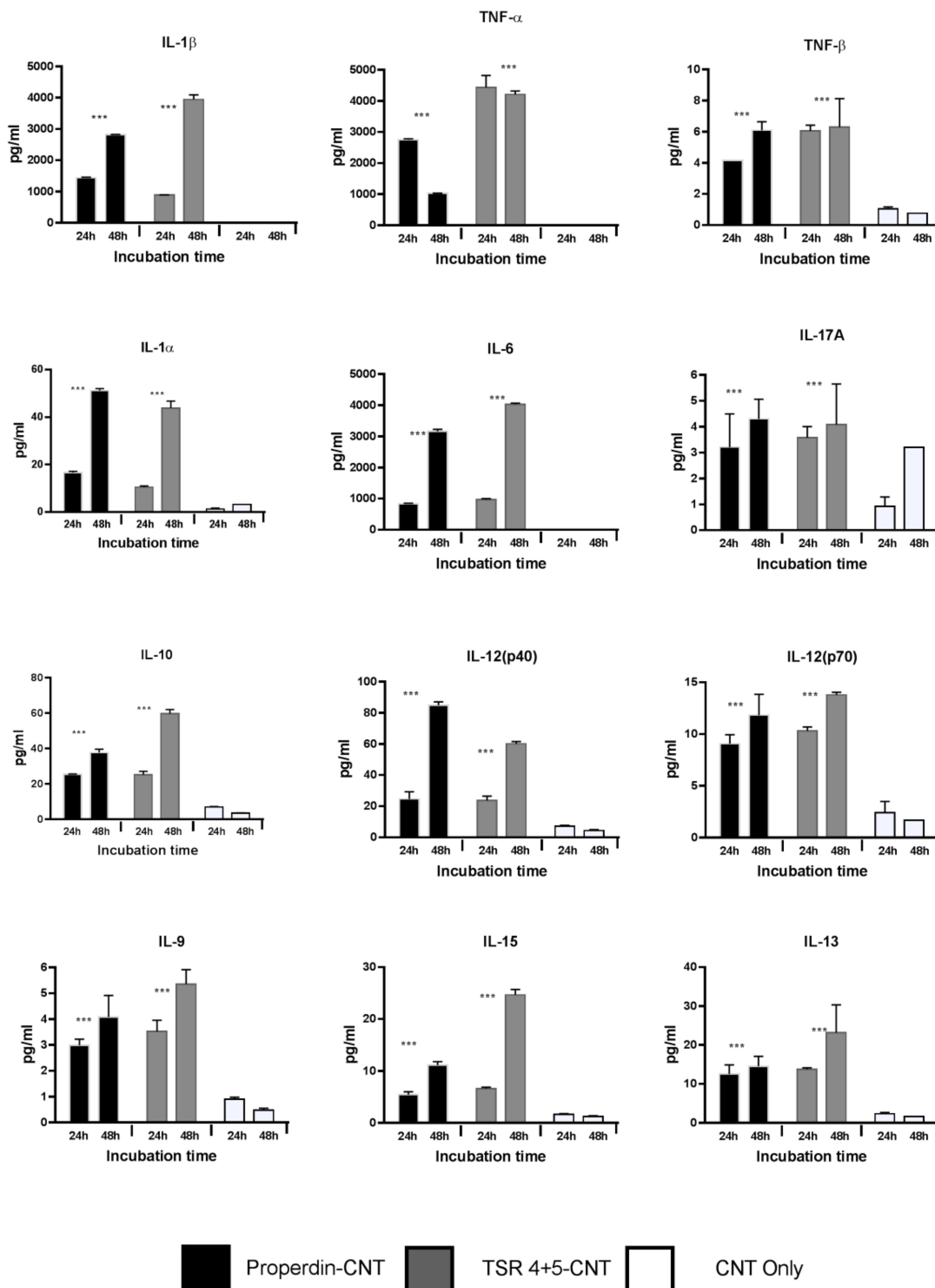


Figure 3-6: Multiplex array analysis of supernatants of THP-1 cells following treatment with properdin and TSR 4+5 or uncoated CNTs.

Protein-coated CNTs (properdin and TSR4+5) were incubated with THP-1 cells for 30 min, 1h, 2h, 6h, 12h, 24h and 48h. Supernatant from 24h and 48h time points (X-axis) were used for the measurement of the levels of secreted **cytokines (**IL-1α, IL-1β, TNF-α, IL-6, IL-10, IL12p40, IL12p70, TNF-β, IL-15, IL-17A, IL-9, IL-13)) by using a MagPix Milliplex kit. Error bars represent ± standard deviation Ns: not significant (p>0.05)*$p <$ 0.05, *$p <$ 0.01, and ***$p <$ 0.001 (2-way ANOVA test between properdin or TSR4+5 coated CNTs with uncoated CNTs).

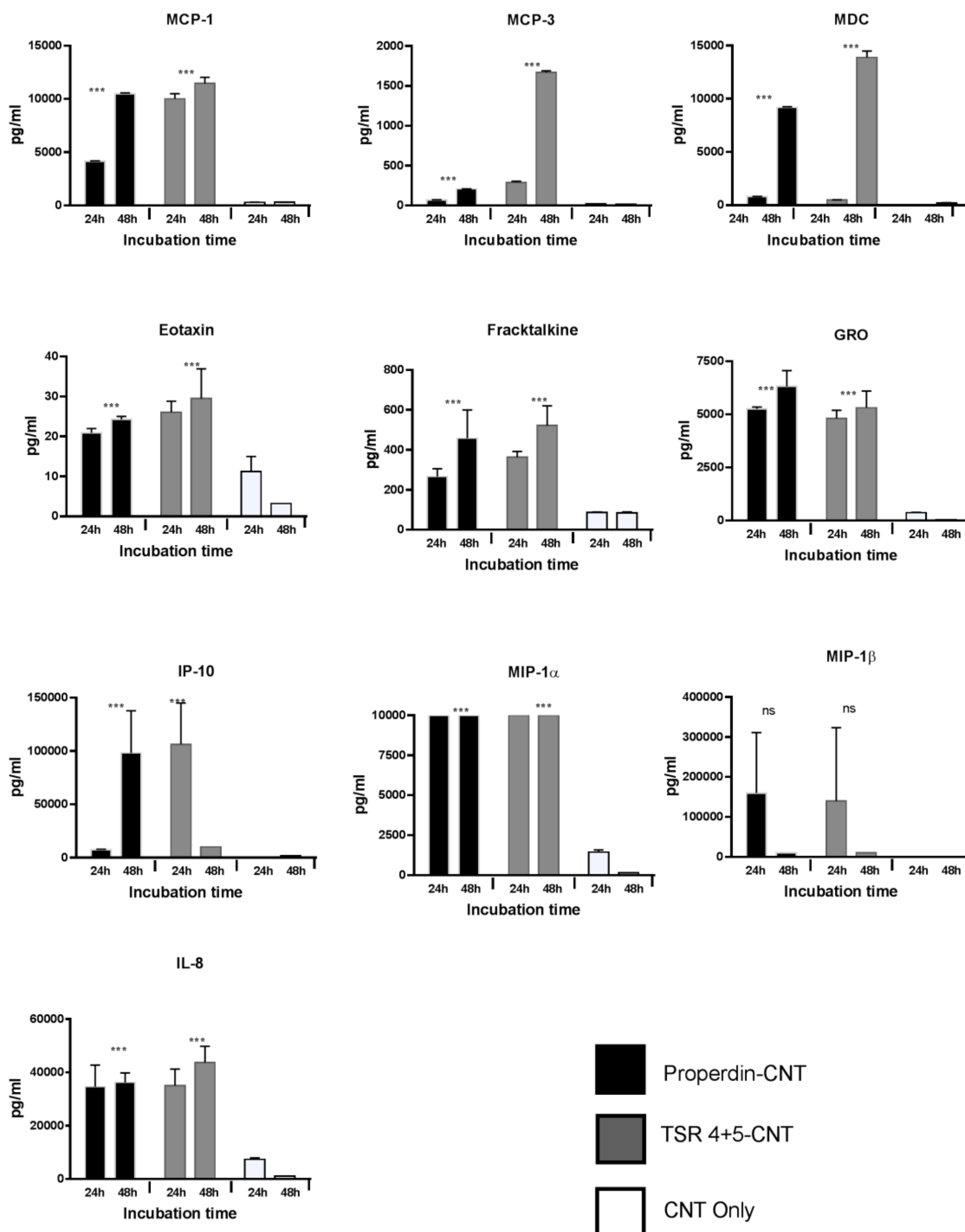


Figure 3-7: Multiplex array analysis of supernatants of THP-1 cells following treatment with properdin and TSR 4+5 or uncoated CNTs.

Protein-coated CNTs (properdin and TSR4+5) were incubated with THP-1 cells for 30 min, 1h, 2h, 6h, 12h, 24h and 48h. Supernatant from 24h and 48h time points (X-axis) were used for the measurement of the levels of secreted **chemokines** (MCP-3, MDC, Eotaxin, MIP-1α, MIP-1β, Fractalkine, GRO, IL-8, IP-10, MCP-1) by using a MagPix Milliplex kit. Error bars represent ± standard deviation Ns: not significant ($p>0.05$)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$ (2-way ANOVA test between properdin or TSR4+5 coated CNTs with uncoatedCNTs).

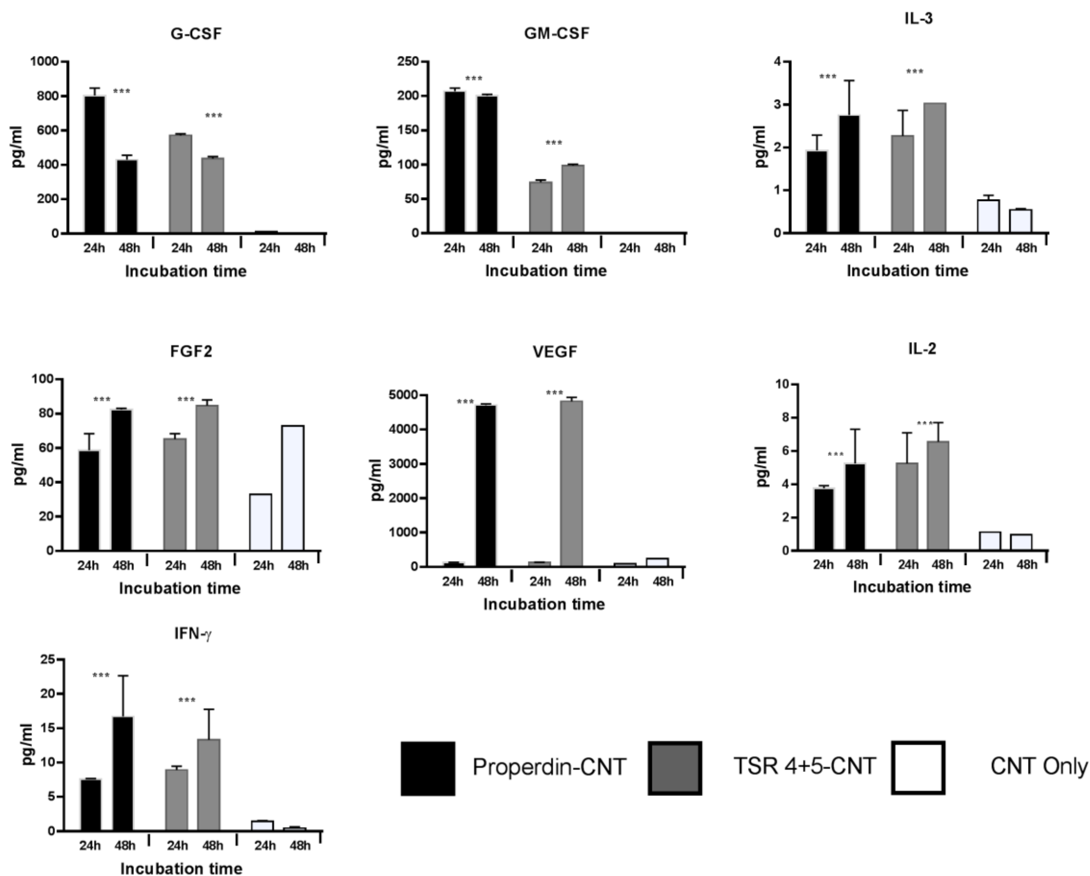


Figure 3-8: Multiplex array analysis of supernatants of THP-1 cells following treatment with properdin and TSR 4+5 or uncoated CNTs.

Protein-coated CNTs (properdin and TSR4+5) were incubated with THP-1 cells for 30 min, 1h, 2h, 6h, 12h, 24h and 48h. Supernatant from 24h and 48h time points (X-axis) were used for the measurement of the levels of secreted **growth factors** (IL-2, FGF-2, G-CSF, GM-CSF, IL-3, VEGF) by using a MagPix Milliplex kit. Error bars represent ± standard deviation Ns: not significant (p>0.05)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$(2-way ANOVA test with column mean effect was used in between properdin or TSR4+5 coated CNTs with uncoated CNTs).

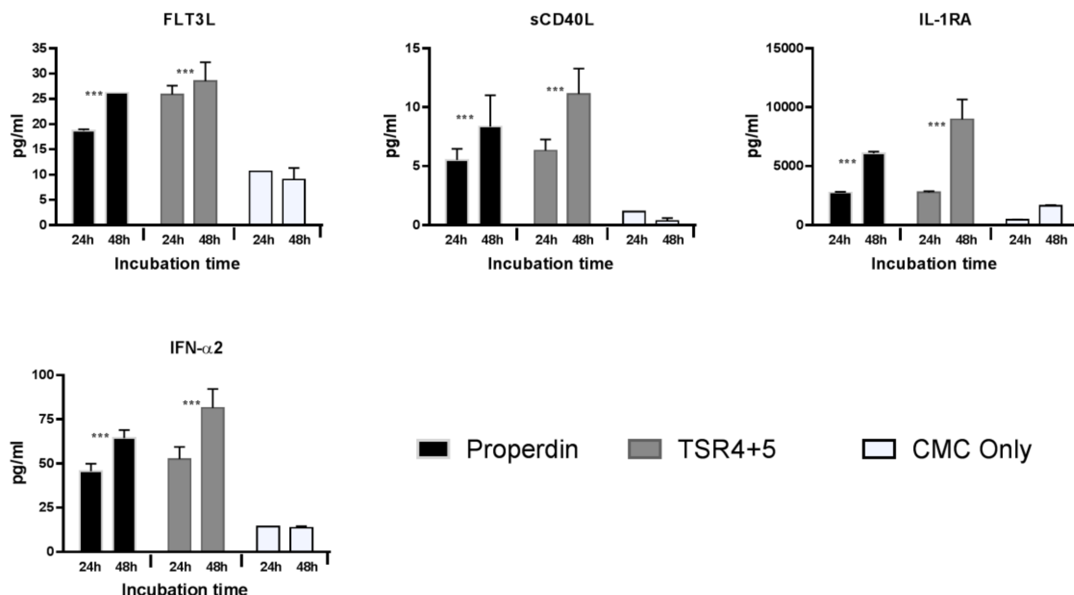


Figure 3-9: Multiplex array analysis of supernatants of THP-1 cells following treatment with properdin and TSR 4+5 or uncoated CNTs.

Protein-coated CNTs (properdin and TSR4+5) were incubated with THP-1 cells for 30 min, 1h, 2h, 6h, 12h, 24h and 48h. Supernatant from 24h and 48h time points (X-axis) were used for the measurement of the levels of **related ligands and receptors** (IFN-γ, FLT-3L, IL-1RA, sCD40L) by using a MagPix Milliplex kit. Error bars represent ± standard deviation Ns: not significant (p>0.05)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$(2-way ANOVA test between properdin or TSR4+5 coated CNTsand uncoated CNTs.)

3.3.6 Properdin or TSR4+5 -CNTs induced nuclear translocation of NF-κB

Fluorescence microscopy images revealed the translocation of NF-κB into the nucleus following challenge with properdin-CNTs or TSR4+5-CNTs. The transcription factor, NF-κB, has a main role in the downstream signalling pathway of many pro-inflammatory cytokines that are activated when cells are exposed to external stimuli. THP-1 cells were incubated with properdin-CNTs or TSR4+5-CNTs for 2 h and subsequently stained with an antibody against the p65 subunit of NF-κB (green) (Figure 3-10). The merged image shows induction of translocation of NF-κB to the nucleus (blue), which is significantly enhanced by properdin-CNTs and TSR4+5-CNTs, compared to CNTs alone. Properdin & TSR4+5 coated CNTs also induced an up-regulation of NF-κB mRNA levels at 6 h by THP-1 cells (Figure 3-5). This is consistent with the nuclear localization of NF-κB (Figure 3-10). This reflects on the up-regulation of the pro-inflammatory response by TNF-α, IL-1β, IL-2 and IL-6 (Figure 3-5). Upregulation of pro-inflammatory cytokines (TNF-α, IL-1β) and nuclear translocation of NF-κB suggests that properdin-CNTs and TSR4+5-CNTs might have triggered TLRs, especially TLR4 via the MyD88-dependent pathway, on the macrophages to initiate transcription of pro-inflammatory cytokines (Yamamoto *et al.*, 2004).

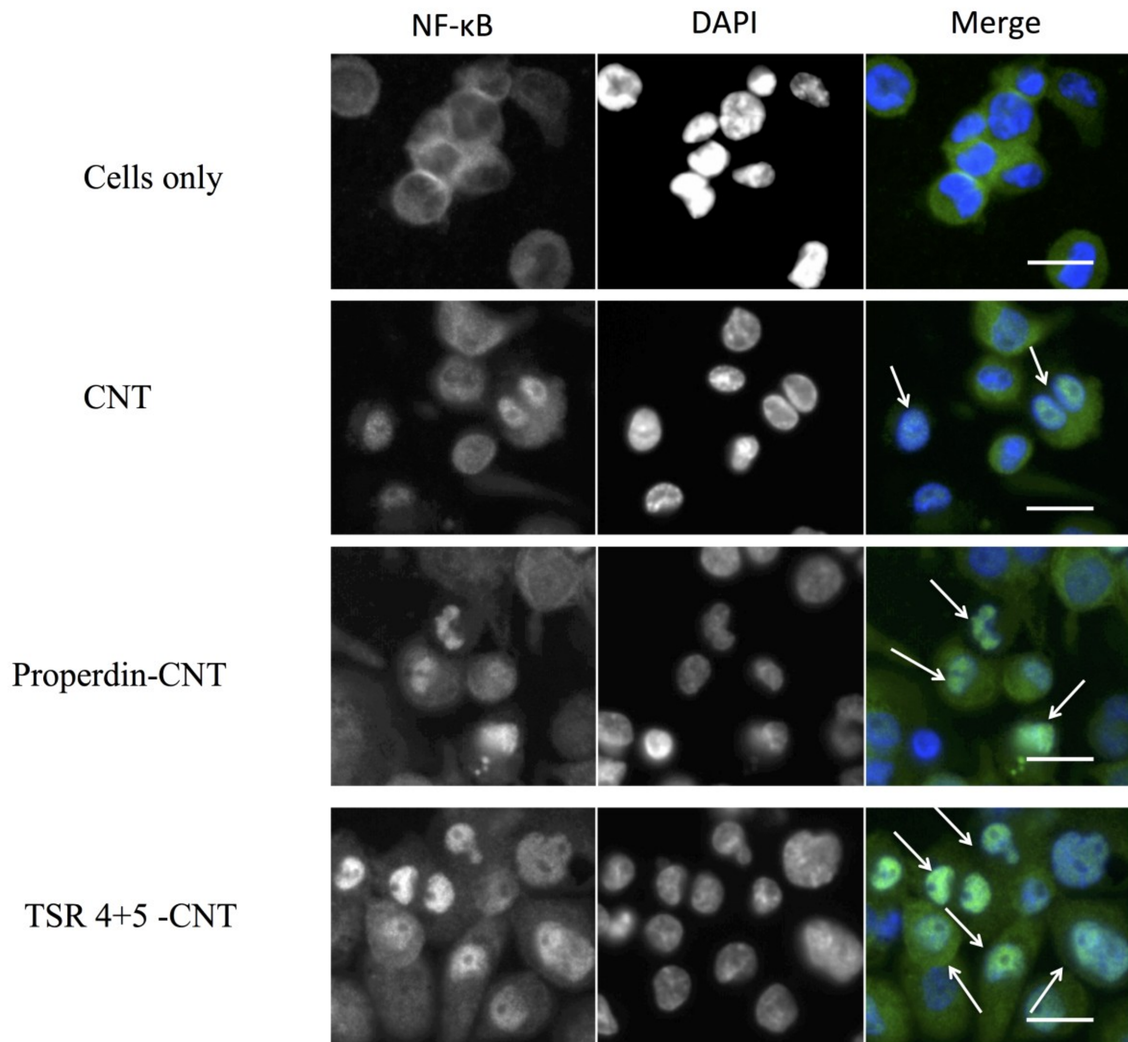


Figure 3-10: Properdin-CNTs and TSR4+5-CNTs induce nuclear translocation of NF-κB in THP-1 cells.

Differentiated THP-1 cells were incubated with properdin-coated or TSR4+5-coated CNTs for 2 h. Cells were washed and fixed using 4% PFA, permeabilized, blocked with 5% FBS and incubated with rabbit anti-NF-κB p65 antibodies, followed by secondary Alexa Fluor 488-goat anti-rabbit antibody (Green). The nucleus was stained with Hoechst 33342 (Blue). Arrowhead in merged images shows the translocation of NF-κB from the cytoplasm to nucleus. Scale bar 20 μm.

3.4 Discussion

Previously, we have shown that complement components like C1q and Factor-H (F-H) can absorb on CNTs and modulate the uptake of opsonized CNTs in which C1q enhanced the uptake of CNTs in PBMCs in contrast to F-H (Pondman *et al.*, 2015; Pondman *et al.*, 2014). Various CNTs, pristine, Ox-CNTs, CMC-CNTs, appeared to provide molecular charge pattern recognition for C1q to bind and initiate classical pathway activation. C1q appeared to bind CNTs via its globular head (gC1q), as revealed by binding using individual globular head domains, enhanced uptake of CNTs by human monocytic cell lines (U937) and up-regulated the pro-inflammatory immune response (Pondman *et al.*, 2015). Although complement factor H, a negative regulator of the alternative pathway, also bound to CNT derivatives, it did not enhance uptake but exerted an anti-inflammatory response. Bound recombinant forms of globular head modules of C1q (ghA, ghB and ghC) on CNTs inhibited classical pathway activation but augmented the uptake of CNTs leading to a significant dampening of pro-inflammatory response (Pondman *et al.*, 2014). Thus, pre-coated NPs with recombinant globular heads domain have been proposed to be a good strategy to avoid complement activation and the subsequent release of anaphylatoxins (C3a, C5a, C2b) there by suppressing pro-inflammatory cytokine/chemokine responses (Pondman *et al.*, 2015). Here, we have examined whether properdin can interact with NPs, thus potentially acting as a pattern recognition innate immune molecule, similar to C1q, globular heads modules and factor H, and modulate CNT handling by macrophages.

CNTs are hydrophobic in nature but are made biocompatible by coating with polymers such as Carboxymethyl cellulose (CMC), which gives negative polarity in solution, making the particles water soluble, less -toxic, and more biodegradable and bio-

tolerable (Bourdiol *et al.*, 2013). Properdin is a highly positive charged molecule at neutral pH (Smith *et al.*, 1984). Many cell surface molecules identified so far that interact directly with properdin are negatively charged. Thus, the direct and stable binding of properdin and TSR 4+5 proved to be a charge/polarity interaction in the way to retain its biological activity as evidenced by activation of complement alternative pathway (Figure 3-3). Contrasting to properdin binding and retaining its biological activity when bound to CNTs, C1q has been shown to adsorb on CNTs upon binding, to crystallize and lose its biological activity (Ling *et al.*, 2011). This report is in contrast to recent findings from Pondman et al where they have shown that adsorbed C1q does activate complement classical pathway and enhance the uptake of CNTs and modulate the immune response. Another complement component, factor-H.can also binds to CNTs (Pondman *et al.*, 2015).graphene oxide and reduced graphene which protects the NPs against complement activation (a "stealth effect") (Belling *et al.*, 2016). Here, we have shown that one of the key players in the alternative pathway, properdin does act as soluble pattern recognition receptor and bind to CNTs. Several studies have demonstrated the role of properdin as a PRR. A recent study by Dixon et al., 2017 has shown that properdin produced by DCs and tolerogenic DCs binds to necrotic cells. This finding is consistent with previous observations that properdin interacts with apoptotic and necrotic cells (Kemper *et al.*, 2008; Xu *et al.*, 2008) in a complement-independent manner due to its local synthesis. Furthermore, properdin is also involved in the interaction of DC and T cells (Dixon *et al.*, 2017). Properdin also binds to several pathogen surfaces such as Neisseria gonorrhoeae, Salmonella typhimurium lipopolysaccharide (LPS), *Neisseria meningitidis* lipooligosaccharide (LOS), and Chlamydia pneumonia, Zymosan, E-coli strains, live human leukemia T cell lines, as well as rabbit erythrocytes as mentioned by several studies described in the introduction

section. Properdin's direct interaction with cell surface moieties may further confirms itas an independent recognition molecule. Such locally released properdin has a potential to bind to therapeutic nano drugs and can influence their pharmacokinetics and biodistribution.

In contrast to properdin, TSR4+5 bound CNTs dampened alternative pathway activation in vitro similar to the globular heads modules of C1q (Pondman *et al.*, 2014). The recombinant TSR4+5 has recently been shown to compete with properdin binding to C3b (and other ligands), and to inhibit the alternative pathway in solution (Kouser *et al.*, 2016). Here, TSR4+5 bound CNTs inhibited alternative pathway activation on the surface of the bound NPs. Coating complement inhibitors onto therapeutic NPs like CNTs, IONPs, gold NPs, offers a simple approach to regulate immune response triggered by different NPs and improve their presence and persistence in the blood. Inhibition of complement alternative pathway has been previously reported to be advantageous in pathological conditions using antibody against other regulatory complement components such as mAb 1379, (anti-factor B antibody) against anti-phospholipid antibody-induced complement activation (Thurman *et al.*, 2005), eculizumab (Anti-complement C5 antibody) against paroxysmal nocturnal hemoglobinuria (Wong and Kavanagh, 2015).

We next assessed the uptake of properdin-CNT and TSR4+5-CNT by THP-1 macrophages (Figure 3-4). Properdin was able to enhance the phagocytosis of CNTs considerably more than TSR4+5. Low uptake of TSR4+5-CNTs indicates the requirement of additional TSR modules or multimers of properdin to engage with receptors on the cell surface. Properdin proved to act as an opsonin for CNTs without involving complement recruitment and activation, and enhance their uptake and clearance by a macrophage cell line. However, it is possible that THP-1 cells could

synthesise a sufficient quantity of complement proteins (eg C3) to contribute to this apparent opsonisation as THP-1 harbour properdin RNA and traces of C3 RNA as reported by The World Protein Atlas (http://www.proteinatlas.org). However, Takizawa et al (1996) argued that addition of serum as a complement source was required to observe phagocytosis of apoptotic cells by activated THP-1 cells (Takizawa, Tsuji and Nagasawa, 1996).

Although uptake of CNTs was exclusive to properdin, TSR4+5 and properdin were able to trigger the pro-inflammatory cytokine response. This suggests that though additional receptors were needed for phagocytic uptake, cell stimulation and cytokine/chemokine release did not require more modules. CNTs on their own were not able to produce a significant signal for tested cytokines compared to protein coated. However, expression of IL-10 was up-regulated at an early time point (30min) dampening the activation of macrophages which may explain the lower uptake of uncoated CNTs. Expression of pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6, was dramatically up-regulated at 6 h, suggesting that both properdin and TSR4+5 coated CNTs can alter the immune response. Further analysis was carried out to measure the secreted cytokine levels at late time points (24 h and 48 h) following THP-1-CNT interaction, using multiplex array analysis (Figure 3-9). A dramatic up-regulation of pro-inflammatory cytokine response was observed consistent with the early mRNA response for TNF-α, IL-1β, and IL-6. These cytokines are potent inducers of GM-CSF (Chung, Kim and Ma, 2006) as a result GM-CSF was dramatically up-regulated by both protein coated CNTs. Upregulation of IL-1α and IL-1β (Figure 3-9) indicates the activation of TLR-Myd88 mediated pro-inflammatory pathway resulting NF-κB nuclear translocation (Figure 3-10). Additionally, IL-8, a chemoattractant for neutrophils was also upregulated, which in vivo may induce local production of

properdin by neutrophils and enhance activation of the alternative pathway (Wirthmueller *et al.*, 1997). Complement proteins have a significant contribution to the tumour microenvironment and have become a focus of intense research. An immunosuppressed microenvironment facilitates tumour cells growth. To this effect, conditioned medium from melanoma B16F10 tumour cells has been shown to prime M1 macrophages, characterised by upregulation of iNOS, IL-1β, TNF-α, IL-12, IL-23, CXCL-9 and CXCL-10 (Al-Rayahi, Browning and Stover, 2017). Thus, locally synthesized properdin could potentially modulate the tumour microenvironment by binding to CNTs if these were used for drug delivery.

From a therapeutic point of view, properdin-CNTs not only allowed alternative pathway activation but also retained its biological activity and ability to activate the alternative pathway in properdin deficient conditions. These results pave the way for its development as a prophylactic and therapeutic agent for treatment in the rare condition of X-linked properdin deficiency. Properdin-CNTs used as a platform for drug delivery may mediate a protective role in this disease. These results are of pathophysiological significance since properdin deficiency has been linked with a range of bacterial infections especially Neisseria (Soderstrom *et al.*, 1991). The properdin gene-deficient mice are susceptible to a number of bacterial infections (Stover *et al.*, 2008). Furthermore, an inhibitor of the alternative pathway, TSR4+5 delayed endocytosis, which has potential implications for therapeutic drug delivery in alternative pathway related diseases. Though we observed a significant upregulation of pro-inflammatory cytokines and chemokine release upon exposure to properdin and TSR4+5 coated CNTs, this can be successfully reduced by C3b deposition on the CNTs as reported by Pondman et al. where they downregulated pro-inflammatory nature of SP-D coated CNTs by complement deposition (Pondman *et al.*, 2017a).

4 Chapter IV

Interaction between Surfactant Protein D and carbon nanotubes

4.1 Abstract

Carbon nanotubes (CNTs) are progressively being investigated for use in biomedical applications, including biological agents delivery like gene delivery, drug and protein delivery. One of the most promising applications under assessment is in treating pulmonary diseases such as tuberculosis and lung cancer. Besides therapeutic NPs, exposure to airborne NPs is not a new phenomenon. Once inhaled either intentional or accidental, the NPs are likely to be recognized by innate immune molecules in the lungs such as hydrophilic pulmonary surfactant proteins with the ability to bind or opsonise foreign particles. Here, we set out to study the interaction between surfactant protein D (SP-D), a key lung pattern recognition molecule and NPs, CNTs, and potential downstream effects on the immune response *via* macrophages. We observed that a recombinant form of human SP-D (rfhSP-D) bound to carboxymethyl cellulose (CMC) coated CNTs *via* its C-type lectin domain and augmented phagocytosis by THP-1 macrophages/monocytic cell lines, together with an increased pro-inflammatory response. This suggests that immobilization of SP-D by CNTs in the lungs can initiate an unfavourable immune response. Furthermore, rfhSP-D opsozined CNTs continued to activate complement pathway via the classical pathway, suggesting that SP-D and C1q, an initiator of classical pathway do not interfere in the complement activation and SP-D and C1q have distinct pattern recognition sites on the CNTs. Complement deposition on the rfhSP-D opsonised CNTs led to dampening of the pro-inflammatory immune response by THP-1 macrophages, as evident from qPCR, cytokine array and NF-κB nuclear translocation examination. This study emphasises the significance of understanding the interaction between innate immune humoral factors including complement in developing nanoparticle-based drug delivery strategies.

4.2 Introduction

The assessments of how the innate immune system is able to recognize and process these NPs, with the goal of extrapolating this knowledge for the development of a new generation of nanotherapeutics has already been carried out in previous chapters. One of the most promising applications under assessment is in treating pulmonary diseases such as tuberculosis and lung cancer. Drug delivery to the lung through aerosol inhalation is widely used for the treatment of several diseases such as cystic fibrosis, asthma, COPD and more recently for the treatment of lung cancer (Hershey *et al.*, 1999). But still, the nano-based drug delivery has not reached to the acceptable stage and studies have been regularly conducted to examine the challenges in delivering for the treatment of pulmonary diseases like tuberculosis (Bonner, 2011; Choudhary and Kusum Devi, 2015). Therefore, it is vital to understand the way therapeutic NPs interacts with the pulmonary innate immune system and their mechanism and consequences.

Recent studies in animal model demonstrated that SP-D expression is upregulated in the lung in response to ambient particulate matter from air pollution (Clement *et al.*, 2008; Kang *et al.*, 2010). Accidentally inhaled or therapeutically administered NPs both would be recognized and processed by innate immune molecules in the lungs such as pulmonary surfactant proteins. Pulmonary surfactant is a multi-layered lipoprotein constituted up to 10% proteins and 90% lipid. (Goerke, 1998). These lipoproteins line the alveolar epithelial surface and provide two important functions to the lung; defence to the microbial infection and reducing alveolar surface tension. Both SP-A and SP-D opsonise pathogens and foreign particles and interact with the alveolar macrophages *via* putative receptors. Opsonisation assists in their uptake and downstream immune response (Kishore *et al.*, 2006), suggesting that they can have a critical role in the processing of inhaled NPs.

Homotrimeric C-type lectin domain or carbohydrate recognition domain (CRD) facilitates the binding of SP-D and SP-A with Oxidized (Ox) double-walled CNTs (DWNTs), as shown by transmission electron microscopy (Rybak-Smith *et al.*, 2011; Salvador-Morales *et al.*, 2007). Bound SP-A on CNTs enhanced their uptake by alveolar macrophages, without any significant inflammatory response evident by no marked rise of nitric oxide levels (Salvador-Morales *et al.*, 2013). Similarly, the formation of lung surfactant protein corona on NPs consisting of SP-A and SP-D enhances the uptake of SWNTs by RAW murine macrophages (Kapralov *et al.*, 2012). Furthermore, pulmonary surfactant proteins have shown to stabilize the suspensions of CNTs, however, cluster of CNTs was observed in monocytes derived-macrophages (Gasser *et al.*, 2012). This could be due to enhanced uptake of CNTs, accumulation and prolonged holding of CNTs inside the macrophages. Enhanced uptake of CNTs has also been reported upon coating with SP-A as well as phosphatidylserine (Konduru *et al.*, 2009; Stringer and Kobzik, 1996). As surfactants proteins are available in very low amount, immobilization of these proteins on NPs can potentially cause significant depletion of proteins, which can have a detrimental effect on the lung innate immune defence (Salvador-Morales *et al.*, 2007). In contrast, bound SP-A or SP-D on unmodified polystyrene (U-PS) and amino-modified polystyrene (A-PS) NPs were found to neutralise influenza A viral cellular infection *in vitro* (McKenzie *et al.*, 2015a). Recently, Kendall et al group found that SP-D adsorbs on the various NPs (polystyrene, carbon black and silica NPs) and enhance their uptake by alveolar macrophages and mouse dendritic cells (Kendall *et al.*, 2013). In a mouse model, alveolar macrophages, SP-A was found to inhibit agglomeration and uptake of A-PS, whilst it promoted uptake of U-PS (McKenzie *et al.*, 2015b).

The mechanistic pulmonary toxicity of NPs still remains unexplained but surface area, insolubility of NPs, particle sizes are significant factors (Kendall, Brown and Trought, 2004). Inhaled NPs deposits in the lungs and interacts with lung surfactant components before involving cellular interactions (Lundqvist *et al.*, 2008). Extracellular molecules modulate the NPs properties, reduce surface charge as measured by zeta potential and promote particle agglomeration (Kendall, Ding and Kendall, 2011). Once inside the airways, NPs adsorb onto the mucosal surface and pulmonary surfactant lining of the lower airways. Similar to the formation of plasma corona in blood, pulmonary biological molecules form a corona, which influences the clearance, bioavailability and toxicity of the NPs (Kendall and Holgate, 2012).

Here, we have set out to investigate the interaction of SP-D with CNT and their subsequent effect on complement activation, phagocytosis and immune response by macrophage cell lines.

4.3 Results

4.3.1 SDS PAGE and TEM analysis showing rfhSP-D bound to CNTs

CNTs were incubated with rfhSP-D for overnight at 4^0C shaking. CNTs were washed extensively and redispersed to wash away any trapped protein. Uniformly suspended CNTs ran in 12% SDS-PAGE to analyse the binding of rfhSP-D on CNTs. TEM micrograph further also revealed that rfhSP-D was bounded on to the CNTs. Figure 4-1 shows the rfhSP-D were bound to CNTs NPs stably.

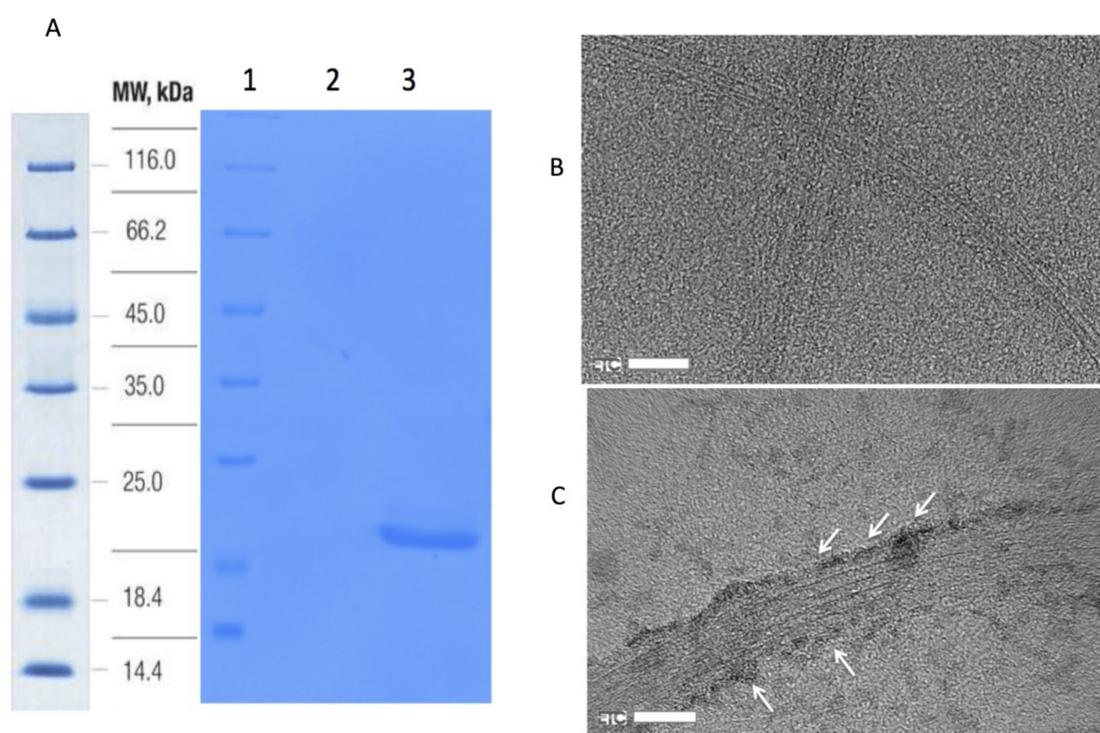


Figure 4-1: Binding of rfhSP-D to CNTs.

A) rfhSP-D bound to CNTs. CNTs were incubated with rfhSP-D in 1 : 2 weight ratio for overnight and repeatedly shaking. CNTs were washed extensively *via* centrifugation to remove unbound or trapped SP-D. Lane 1: molecular weight marker; lane 2: CNTs only, lane 3: rfhSP-D coated CNTs. CNTs incubated with rfhSP-D was adsorbed on carbon grid and counterstained with uranyl acetate and lead citrate and micrographs were recorded using a JEOL 2100 FEG-TEM operating at 80Kv, and the images were processed using gatan microscopy suite software (Gatan, Inc). **B)** CNTS only **C)** CNTs coated with rfhSP-D as shown by the arrow.

4.3.2 Coating of CNTs with rfhSP-D enhances complement consumption

It has been established that CNTs can activate complement classical pathway. Here, we set out to investigate whether CNT coated with rfhSP-D can activate complement pathway and what extent it does. rfhSP-D coated CNTs showed a very significant enhancement (≈2 fold) in complement consumption compared to uncoated CNTs (Figure 4-2). Complement activation, therefore, appears to be facilitated by rfhSP-D coating on CNTs, suggesting that rfhSP-D and C1q do not compete for binding to the same sites on the CNTs, *i.e.* rfhSP-D does not inhibit C1q binding, an initiator of the classical pathway. Bound rfhSP-D could provide more site for the covalent binding of C3b or C4b, thus enhancing formation of the convertase enzymes, C3bBb or C4b2a., which would increase consumption (activation) of complement as evident by earlier report stating that coating of DWNT with other protein, e.g. BSA and fibrinogen, can also enhance complement consumption (Pondman *et al.*, 2014).

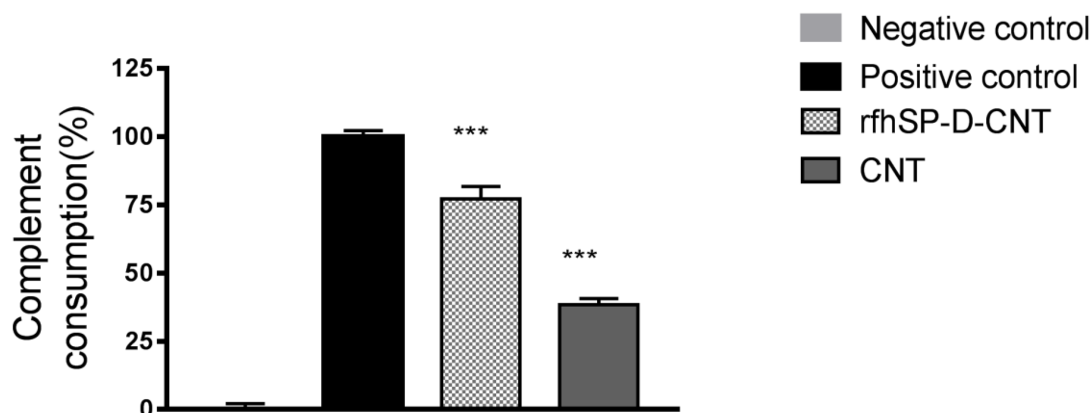


Figure 4-2: Complement consumption by rfhSP-D coated CNTs.

rfhSP-D coated CNTs and CNTs alone were incubated with human serum (1/10 diluted in DGVB for a classical pathway for 1h at 37^0C. Samples were spun down and serum was collected for complement inhibition assay. Zymosan was used as positive control and serum only was used as negative control. Percentage complement consumption was calculated using $(C - \mathrm{Ci})/C \times 100\%$, where C represents the % hemolysis of the negative control, and Ci is the % hemolysis with the rhSP-D coated or uncoated CNTs or zymosan-treated sample. Experiments were conducted in triplicate. Multiple T-test was performed to determine significant differences between each uncoated and coated CNTs. All error bars represent ± standard error of the mean, Ns: not significant (p>0.05)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$)

4.3.3 rfhSP-D enhances uptake of CNTs by THP-1 macrophage cell lines

Like serum treated CNTs (serum+CNTs), rfhSP-D enhanced the uptake of CNTs by differentiated THP-1 cells as revealed by confocal microscopy (Figure 4-3). Orthogonal section of the THP-1 cells shows the endocytosed CNTs inside the cells within plasma membrane boundary (Figure **4-4**A). Interestingly, at both, early (2 hours) and late (4 hours) time points complement deposition on rfhSP-D coated CNTs (SP-D+serum-CNTs) reduced uptake compared to rfhSP-D coating alone or serum treatment alone. Quantitative analysis of uptake of CNTs shows that binding of rfhSP-D increased cellular uptake by about 40% (Figure 4-4B), similar to previously shown increase in uptake of CNTs coated with serum and C1q (Pondman *et al.*, 2015; Pondman *et al.*, 2016). In contrast, uptake of rfhSP-D coated and complement deposited CNTs were inhibited by three-time compared to rfhSP-D coated CNTs The reduction in uptake of rfhSP-D deposited CNTs with additional complement deposition could be due to steric hindrance or competition for a receptor.

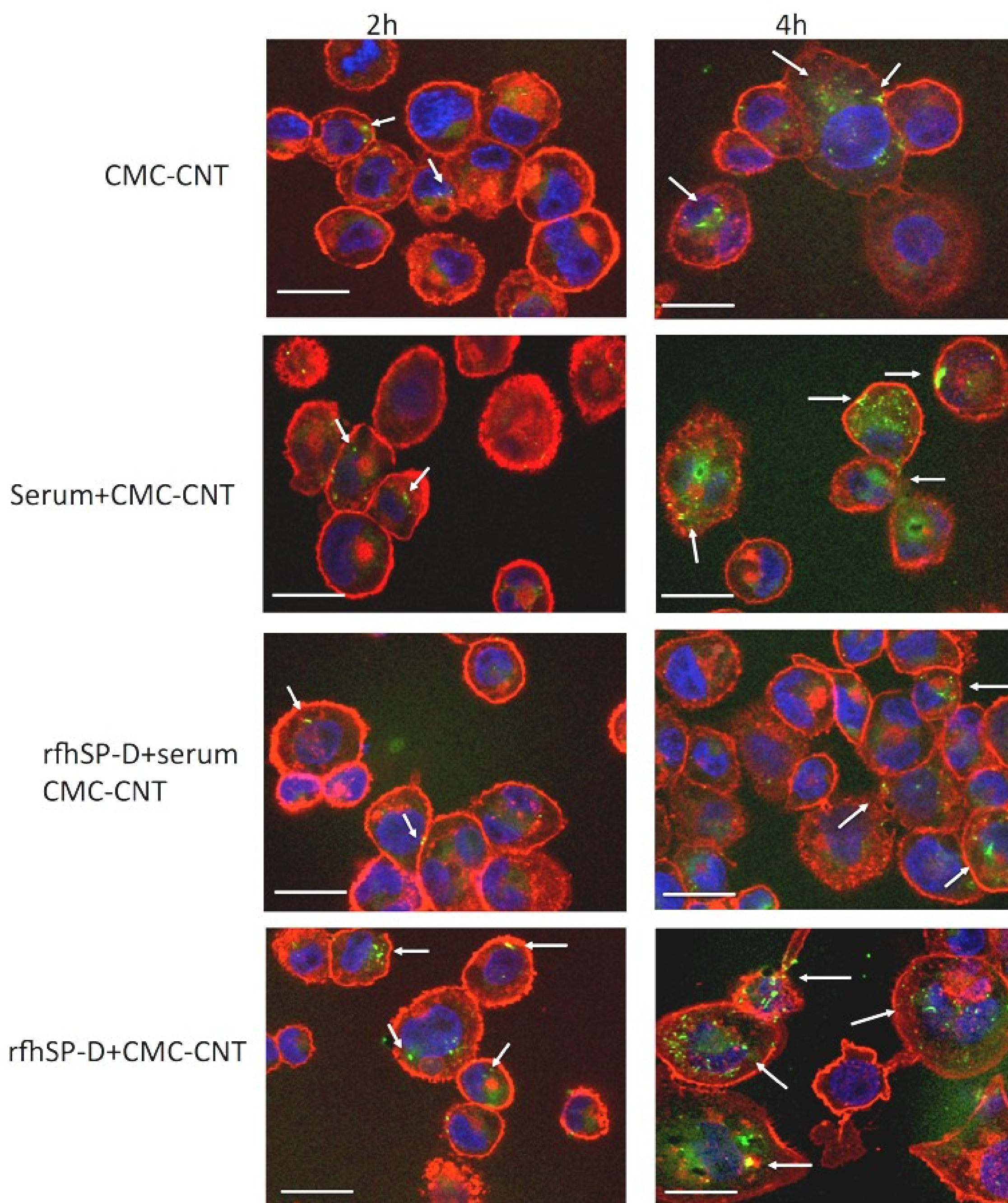
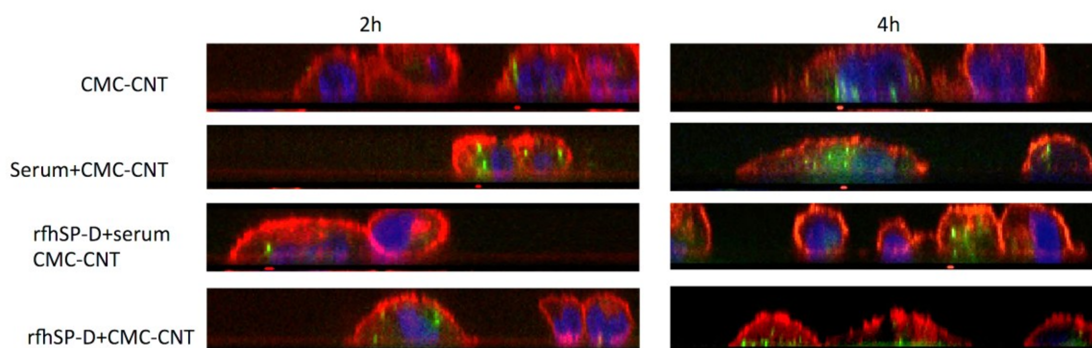


Figure 4-3: Differential uptake of CNTs by macrophage (THP-1) after coating with rfhSP-D, and with or without serum treatment (i.e. complement deposition).

THP-1 cells on a 13mm glass coverslip were exposed to rfhSP-D bound biotinylated CNTs (rfhSP-D+CNT), rfhSP-D deposited biotinylated CNT with complement deposition (rfhSP-D+serum+CNT), complement deposited biotinylated CNTs (serum+CNTs), or biotinylated CNTs alone for 2 h and 4 h. After incubation, cells were washed, fixed, permeabilised. Alexafluor-488 labelled streptavidin and alexafluor 546-conjugated wheat germ agglutinin were to stain internalized biotinylated CNTs and plasma membrane (red) respectively. The nucleus was stained with Hoechst 33342 (Blue). Prepared slides were viewed under Nikon confocal microscope; scale bar 20µm. Arrows highlight internalised CNTs. **(A)** Confocal microscopy images showing uptake of coated or uncoated CNTs

A)



B)

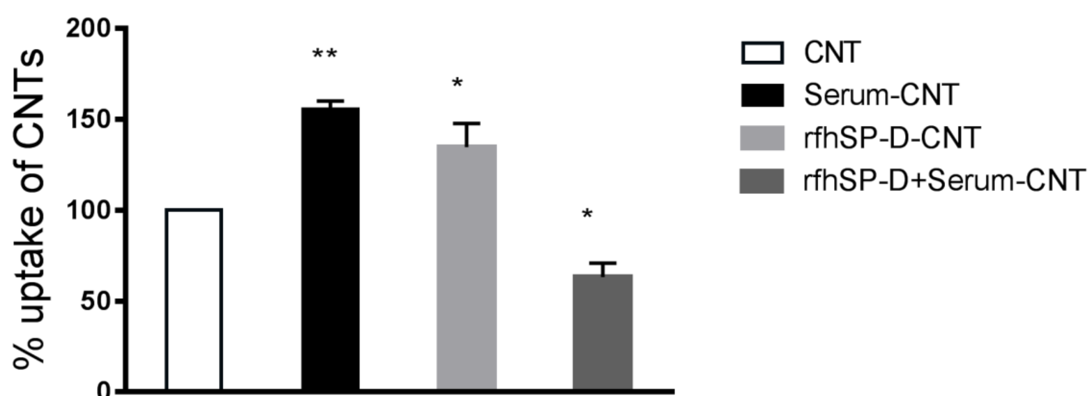


Figure 4-4: Differential uptake of CNTs by macrophage (THP-1) after coating with rfhSP-D, and with or without serum treatment (i.e. complement deposition).

THP-1 cells on 13mm glass coverslip were exposed to rfhSP-D bound biotinylated CNTs (rfhSP-D+CNT), rfhSP-D deposited biotinylated CNT with complement deposition (rfhSP-D+serum+CNT), complement deposited biotinylated CNTs (serum+CNTs), or biotinylated CNTs alone for 2 h and 4 h. After incubation, cells were washed, fixed, permeabilised. Alexafluor-488 labelledStreptavidin and alexafluor 546-conjugated wheat germ agglutinin was to stain internalized biotinylated CNTs and plasma membrane (red) respectively. The nucleus was stained with Hoechst 33342 (Blue). Prepared slides were viewed under Nikon confocal microscope; scale bar 20µm. Arrows highlight internalised CNTs.**(A)** Orthogonal views of the confocal images demonstrate internal localisation of CNTs (green), plasma membrane in red, scale bar 20µm. **(B)** For quantification of internalized CNTs, differentiated THP-1 cells were incubated with 20µg of various versions of CNTs for 2h. Following extensive washing in PBS, the cells were lysed after extensive washing and the amount of internalised CNTs was quantified by an ELISA assay. All experiments were carried out in triplicate; error bars represent ± SEM, Ns: not significant (p>0.05)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$) (A 2-tailed, unpaired t-test between uptake of CNTs between rfhSP-D

coated CNTs and complement deposited rfhSP-D coated CNTs (rfhSP-D+serum CNT) with CNTs only)

4.3.4 Cytokine and transcription factor mRNA expression THP-1 treated with rfhSP-D and complement bound CNTs

Cytokine and transcription factor gene expression profiles of THP-1 cells were measured at six time-points up to 6 h, following treatment with various CNTs, with and without rfhSP-D and serum coatings. In THP-1 cells, mRNA levels for the pro-inflammatory cytokines (IL-1β, TNF-α) were up-regulated by rfhSP-D CNTs, when compared to CNTs (Figure 4-5) but significantly downregulated by complement deposited rfhSP-D coated CNTs. However, IL-12 mRNA level showed an opposite trend. For anti-inflammatory cytokines, unlike IL-10, complement-deposited and rfhSP-D coated CNTs slightly upregulated the expression of TGF-β. NLRP3 was upregulated however, NF-κB mRNA level was downregulated by complement deposition on the rfhSP-D coated CNTs. Thus, rfhSP-D coated CNTs are pro-inflammatory but the pro-inflammatory effect was dampened by complement deposition on CNTs.

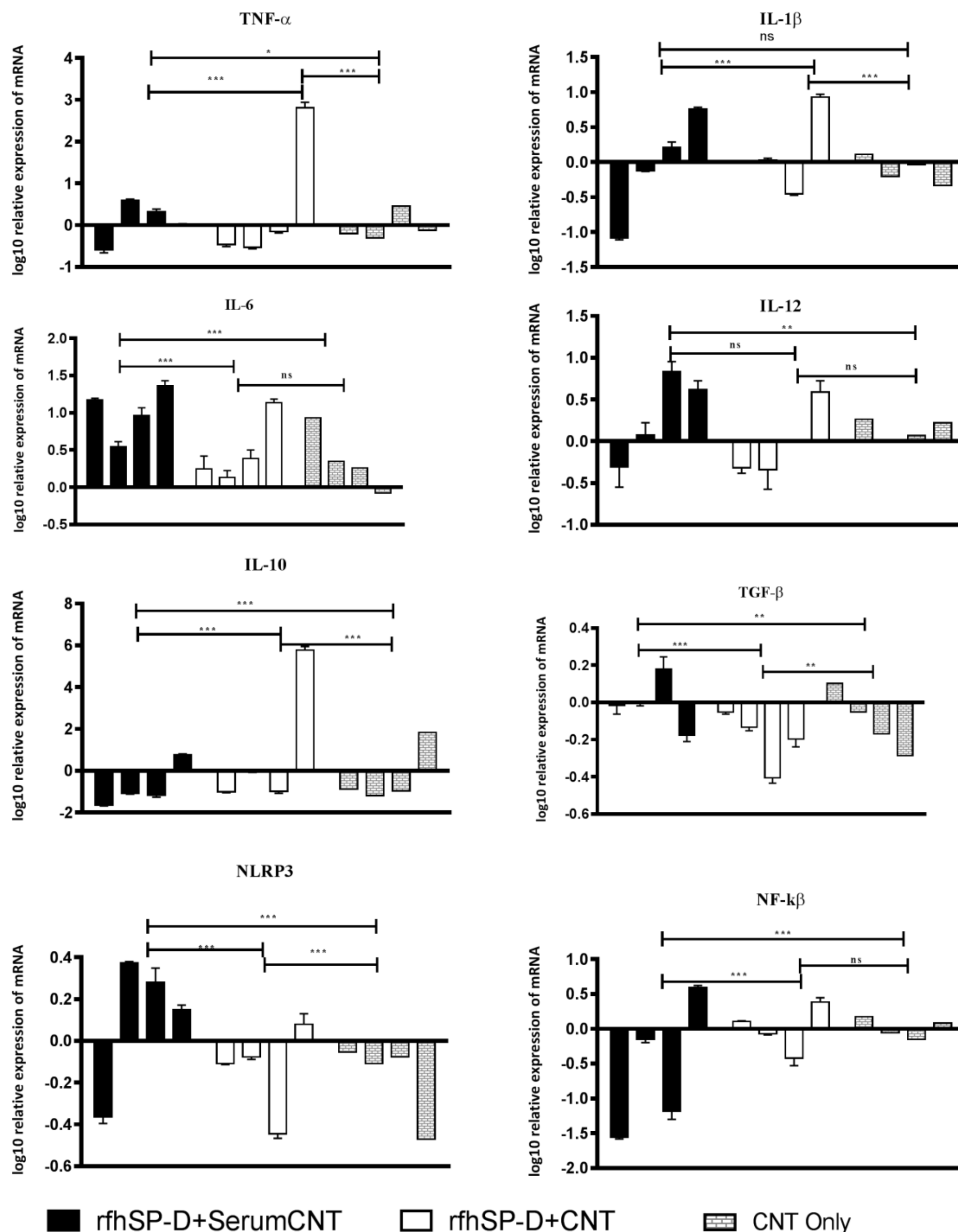


Figure 4-5: Cytokine gene expression of differentiated THP-1 cells challenged with rfhSP-D coated CNT with or without serum deposition.

For the measurement of mRNA expression of pro- and anti-inflammatory target genes, PMA differentiated THP-1 cells were exposed with each CNTs 30 min, 1h, 2h and 6h (X-axis). Error bars represent ±standard deviation. Ns: not significant (p>0.05)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$) (A 2-way ANOVA test to determine significant differences in expression among various version of CNTs; uncoated (CNT only), serum-rfhSP-D coated (rfhSP-D +serum CNT) and rfhSP-D coated CNTs (rfhSP-D+CNT).

4.3.5 Multiplex array analysis revealed dramatic down-regulation of pro-inflammatory cytokines/chemokines by THP-1 cells when challenged with complement deposited rfhSP-D coated CNTs.

mRNA expression suggests the pro-inflammatory nature of rfhSP-D bound on CNTs. However, mRNA expression at early time points does not always give all the clues about immune response over an extended period, cytokine array analysis was performed on the supernatants obtained at 24h and 48 h after treatment of THP-1 and with CNTs. Significant secretion amount of IL-1α, IL-1β, TNF-α, IL-12p40 and IL-12p70, indicates the pro-inflammatory properties of rfhSP-D bound CNTs (Figure 4-6). A similar profile of chemoattractant was seen in IL-8, MDC, IP10, MCP-3, MCP-1,MIP-1α and MIP-1β indicating inflammatory potential of rfhSP-D(Figure 4-8). There was also an increase in G-CSF levels (Figure 4-7), in CNTs coated with rfhSP-D, suggesting a dampening of fibrotic responses consistent with an increase in the inflammatory response (Romero *et al.*, 2015). rfhSP-D coated CNTs downregulated the level of IL-10 levels.

Though, rfhSP-D-coated CNTs produced an increased pro-inflammatory response in THP-1 cells upon challenged, this effect was prominently reduced by complement deposition. We, observed a dramatic downregulation of IL-1β, TNF-α, IL-1α, IL-6, IL-8, MCP-1, MCP-3, MDC, GRO, IP-10, MIP-1β, C-CSF, GM-CSF and VEGF when challenged with complement deposition on rfhSP-D coated CNTs (Figure 4-6,Figure 4-7,Figure 4-8). The levels of TNF-β, IL-17A, IL-12, IL-9 and IL-15 were also significantly downregulated (Figure 4-6). Targets like IL-10, IL-12p40, eotaxin, IFN-α2, MIP-1α, fractalkine, EGF, FGF-2, IL-3, IL-7, FLT3L and soluble CD40L were not significantly altered. qPCR analysis and multiplex analysis, both demonstrate that complement deposition on rfhSP-D coated CNTs, modulates the immune response and polarize to anti-inflammation.

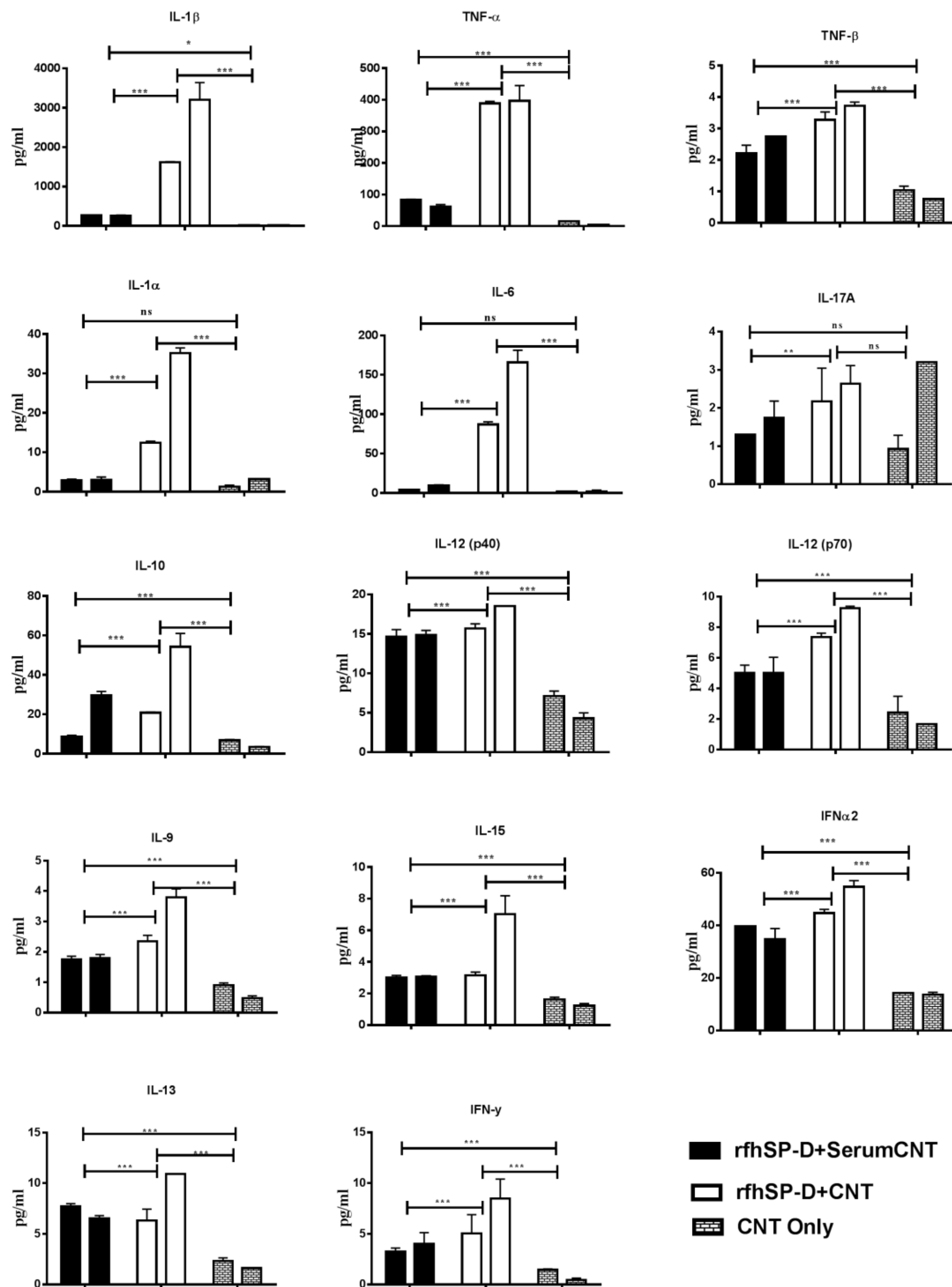


Figure 4-6: Cytokines analysis of supernatants of THP-1 cells treated with rfhSP-D bound CNT with or without serum.

rfhSP-D bound CNTs (rfhSP-D+CNT), rfhSP-D and complement deposited CNTs (rfhSP-D +serum CNT) and CNT only were incubated with differentiated THP-1 cells for various time points (30 min, 1 h, 2 h, 6 h, 12 h, 24 h and 48 h). Supernatants from time points; 24 h and 48 h (X-axis) were taken for measurement of different **cytokines** (IL-6, IL-10, IL-12p40, IL-12p70, IL-1α, IL-1β, TNF-α, IL-13, IL-15, IL-17A, IL-9, TNF-β, IFN-α2, IFN-γ),by using a MagPix Milliplex kit (EMD Millipore). Error bars represent ±standard deviation. Ns: not significant (p>0.05)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$) (A 2-way ANOVA test to determine significant differences in expression among various version of CNTs.

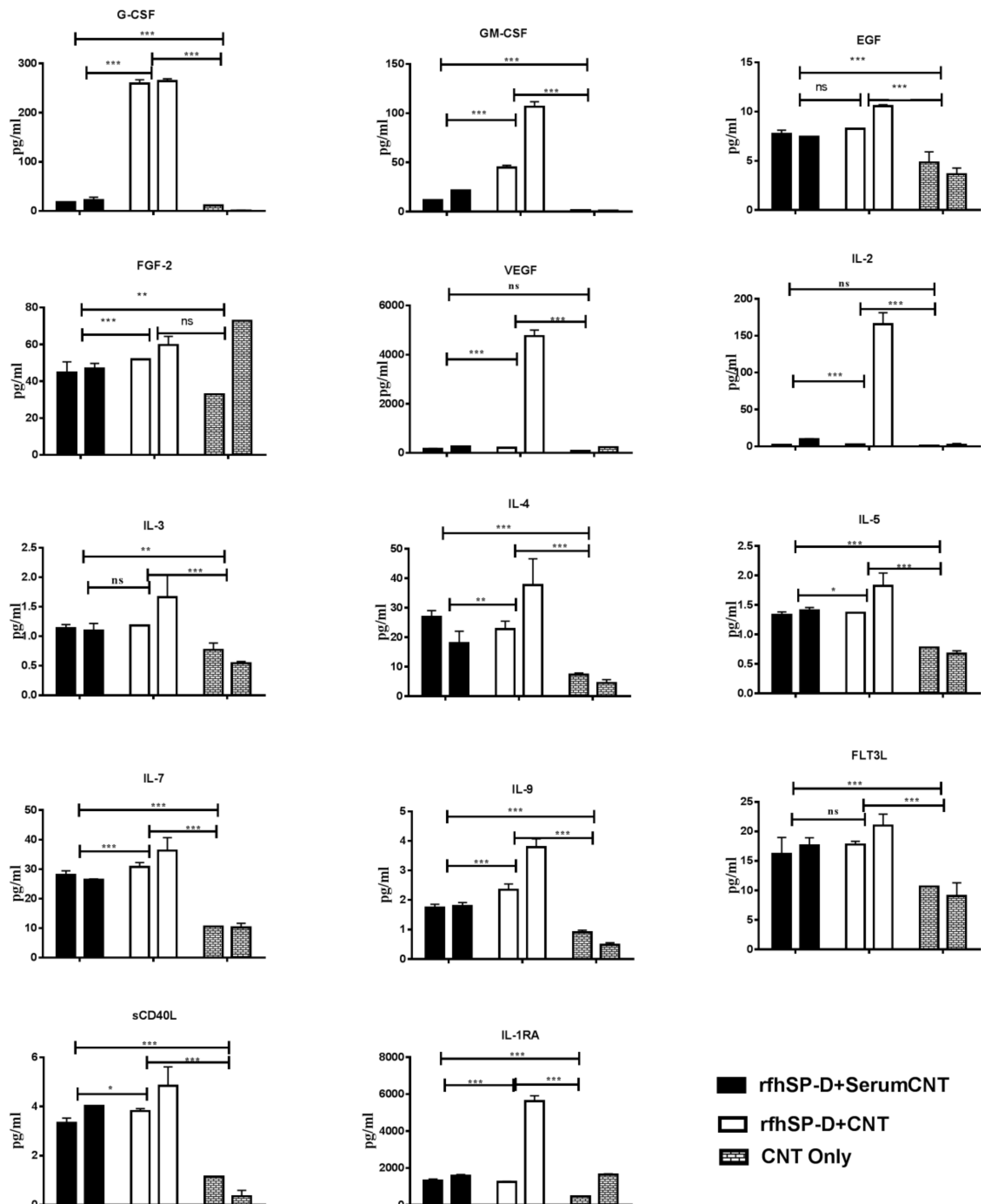


Figure 4-7: Growth factors, ligand and receptor analysis of supernatants of THP-1 cells treated with rfhSP-D bound CNT with or without serum.

rfhSP-D bound CNTs (rfhSP-D+CNT), rfhSP-D and complement deposited CNTs (rfhSP-D +serum CNT) and CNT only were incubated with differentiated THP-1 cells for various time points (30 min, 1 h, 2 h, 6 h, 12 h, 24 h and 48 h). Supernatants from time points; 24 h and 48 h (X-axis) were taken for measurement of **growth factors, ligands and receptors** (IL-2, EGF, FGF-2, G-CSF, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-7 VEGF, (FLT-3L, IL-1RA and sCD40L),by using a MagPix Milliplex kit (EMD Millipore). Error bars represent ±standard deviation. Ns: not significant (p>0.05)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$) (A 2-way ANOVA test to determine significant differences in expression between various version of CNTs)

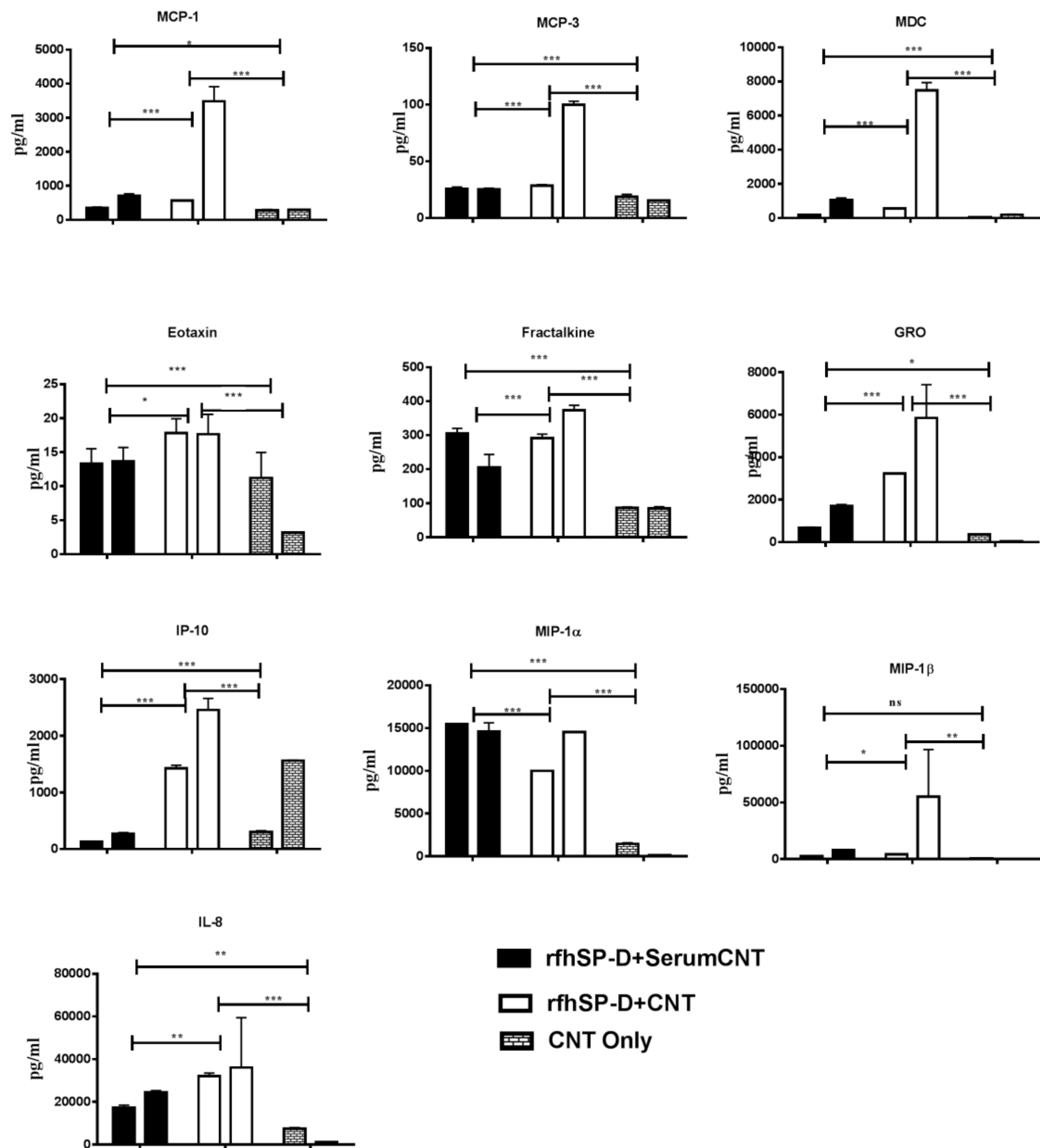


Figure 4-8: Chemokine analysis of supernatants of THP-1 cells treated with rfhSP-D bound CNT with or without serum.

rfhSP-D bound CNTs (rfhSP-D+CNT), rfhSP-D and complement deposited CNTs (rfhSP-D +serum CNT) and CNT only were incubated with differentiated THP-1 cells for various time points (30 min, 1 h, 2 h, 6 h, 12 h, 24 h and 48 h). Supernatants from time points; 24 h and 48 h (X-axis) were taken for measurement of **chemokines** (MCP-3, fractalkine, MDC, IL-8, IP-10, eotaxin, GRO, MCP-1, MIP-1α and MIP-1β),by using a MagPix Milliplex kit (EMD Millipore). Error bars represent ±standard deviation. Ns: not significant ($p>0.05$)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$) (A 2-way ANOVA test to determine significant differences in expression between various version of CNT.

4.3.6 NF-κB nuclear translocation in response to rfhSP-D coated CNTs can be halted by complement deposition

A transcription factor, NF-κB, is a central regulator of transcription expression of various pro-inflammatory cytokines induced in response to external stimuli. THP-1 cells were used to observe nuclear translocation of NF-κB after CNT treatment was observed in THP-1 cells using fluorescent staining techniques. Figure 4-9 shows fluorescence in cells exposed to CNTs for 2 h. As shown in the figure, CNT exposure stimulated NF-κB nuclear translocation compared to the control (cells only). rfhSP-D coated CNTs induced prominent nuclear translocation unlike complement deposited, rfhSP-D-coated CMC-CNTs. This suggests that serum deposition on rfhSP-D decreases or negates the pro-inflammatory potential of rfhSP-D coated CNTs. This effect is further marked by the downregulation of TNF-α and IL-1β mRNA levels (Figure 4-5) and the secretion of pro-inflammatory cytokines (TNF-α, IL-1β, IL-2 and IL-6), and chemokines (IL-8, MCP-1, IP-10) (Figure 4-6, Figure 4-8)

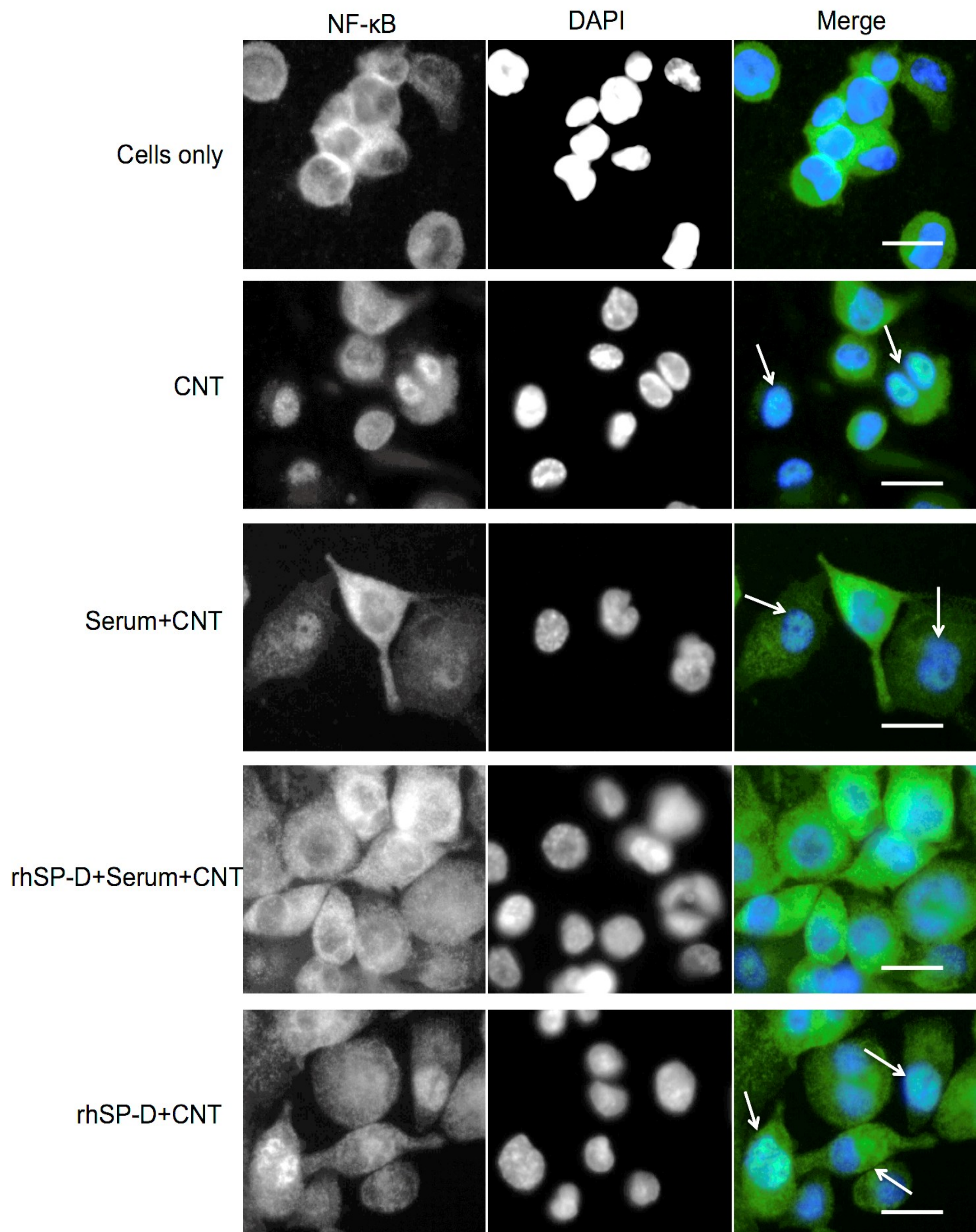


Figure 4-9: Effect of rfhSP-D and /or complement deposition on CNT on nuclear translocation of NF-κB.

Differentiated THP-1 cells on 13mm glass coverslips were treated with rfhSP-D bound CNTs (rfhSP-D + CNT), rfhSP-D bound CNT with serum deposition (rfhSP-D + serum + CNT), complement deposited CNTs (serum + CNTs), or CNT only for 2 h. After incubation, cells were washed, fixed and permeabilised, probed with rabbit anti-NF-κB p65 antibodies and revealed using alexa Fluor 488-goat anti-rabbit antibody (green). To reveal nucleus, Hoechst 33342 (blue) were used. Scale bar 20 μm. Arrows highlight nuclear NF-κB in the merged images.

4.4 Discussion

Carbon nanotubes (CNTs) have exceptional physical properties that make them attractive and amenable for their exciting use as drug delivery in the lungs (Li *et al.*, 2007; Bianco, Kostarelos and Prato, 2005; Bonner, 2011; Choudhary and Kusum Devi, 2015) Thus, it is of great importance to understand the biological interactions these CNTs govern in the lungs, especially with innate immune components, which are likely to be the first host defence system to recognize and process these NPs (Pondman *et al.*, 2015; Pondman, Sim and Kishore, 2015). Inevitably, use of CNTs into the lungs for a therapeutic purpose or even accidental exposure of various other nanomaterials will bring them into contact with pulmonary surfactant, including hydrophilic surfactant proteins, SP-A and SP-D, which are Ca^{2+}-dependent pattern recognition innate immune molecules (Kishore *et al.*, 2006).

Of the two hydrophilic surfactant proteins, SP-D has been shown to mount a strong pro-inflammatory response when engaging with a diverse of pathogens (Kishore *et al.*, 2005; Nayak *et al.*, 2012). The primary structural organization consist of a cysteine-containing N-terminal, a triple-helical collagen region composed of repeating Gly-X-Y triplets, an α-helicalcoiled- neck region and a globular structure at the C-terminal comprising a C-type lectin or CRD (carbohydrate recognition domain). The C-terminal homotrimeric CRD region being the principal recognition and ligand-binding domain of SP-D that binds carbohydrate and/or charge patterns on foreign moieties like carbohydrate on pathogens, allergens, receptors/binding proteins, and apoptotic cells (Silveyra and Floros, 2012). A modified SP-D, with three CRDs, a neck and eight Gly-Xaa-Yaa collagen region repeats was used in this study. Xaa and Yaa could be any amino acid in the collagen region. CRD region acts as a pattern recognizing receptor and binds the ligands causing opsonic effects, enhanced phagocytosis and clearance

mechanisms via superoxidative burst and mostly pro-inflammatory immune response by phagocytic cells (Kishore *et al.*, 2002). SP-D knock-out studies have revealed that SP-D can modulate adaptive immune responses further downstream from its effector functions that can include helper T cell polarisation (Madan *et al.*, 2001)as demonstrated in *in vivo* models of infection and allergy (Madan *et al.*, 2001; Madan *et al.*, 2005; Qaseem *et al.*, 2013) It also caused apoptosis induction in immune-activated cells (Pandit *et al.*, 2016; Mahajan *et al.*, 2013). This shows that there is some sort of interaction between SP-D and NPs and such interaction can have a profound and wide-ranging impact on the lung immune, innate and adaptive, mechanisms.

Both SP-A and SP-D can be adsorbed and immobilize with NPs and modulate their phagocytosis. Schulze et al have shown that metallic oxide NPs like IONP, gold nanoparticle is able to sequester SP-A purified from bronchoalveolar lavage (Schulze *et al.*, 2011). SP-A prefers hydrophobic coating whilst SP-D prefers hydrophilic coating on NPs and binding of these proteins on NPs enhance the uptake of NPs by alveolar macrophages (Ruge *et al.*, 2012; Ruge *et al.*, 2011). Enhanced uptake of NPs upon coating of these surfactant proteins has further proved by demonstrating the decreased uptake of NPs in alveolar macrophages and lung dendritic cells isolated from SP-D knockout mice when compared to wild-type mice (Kendall *et al.*, 2013).

In this study, we set out to examine the effect of rfhSP-D binding to the surface of CNTs on their uptake by macrophage cell lines. Haniu *et al.* observed that SP-A coated particles are endocytosed via receptor-mediated and reported that CR3 recognizes, binds and internalise (Haniu *et al.*, 2013). Likewise, here, internalisation of rfhSP-D coated CNTs could also be via CR3 mediated. Moreover, THP-1 cells constitutively express complement receptor 3 (CR3) and their surface expression is further upregulated by PMA treatment (Plescia and Altieri, 1996). The hemolytic assay

was conducted to test whether rfhSP-D coated CNTs continue to activate the complement classical pathway like CNTs alone, exploring the possibility of independent and multiple interactions of key innate immune molecules with CNTs. It has been already established that serum treatment (i.e. complement deposition) on CNTs enhances their uptake by macrophages and B cells (Pondman *et al.*, 2016) and human bronchial epithelial cells (Haniu *et al.*, 2013).

Components deposition on CNTs enhances their uptake by complement receptors on macrophages, whilst down-regulating the pro-inflammatory cytokine response as evident by the up-regulation of potent anti-inflammatory cytokine, IL-10. Pondman et al have previously reported that C1q binds to CNTs via its ligand recognizing globular (gC1q) domain, as evident by the use of recombinant globular head modules (ghA, ghB and ghC) (Pondman *et al.*, 2015; Pondman *et al.*, 2016) and binding of C1q and globular head domain to CNTs is likely though recognition of charge patterns or polarisation (Pondman *et al.*, 2015; Kishore and Reid, 2000; Pondman *et al.*, 2016; Ghai *et al.*, 2007). Being a similar structure to C1q, SP-D is mainly a carbohydrate or a charge pattern recognition molecule, which perhaps recognize vicinal diol groups as does its homologue Mannose-Binding Lectin (Salvador-Morales *et al.*, 2007). Thus, in addition to observation of rfhSP-D binding to CNTs, we also investigated the effect of rfhSP-D bound CNTs in the modulation of complement activation. Intriguingly, we observed the 2-fold enhancement in complement activation by rfhSP-D CMC-CNTs. These observations advocate the presence of differential recognition sites on CNTs by SP-D and C1q and thus do not inhibit binding of C1q to CNTs. Enhanced complement activation by rfhSP-D bound CNTs is most likely caused by the formation of additional binding sites for C4b and C3b and facilitating their binding by rfhSP-D. In contrast to the effect of rfhSP-D on

CNTs, the recombinant globular head of C1q (ghA, ghB and ghC), bound to CNTs, have been shown to diminish classical pathway activation (Pondman *et al.*, 2015; Pondman *et al.*, 2016) as they compete with native C1q for binding to the CNTs and activation of complement pathway.

Downstream cytokine response from macrophage cell lines treated with CNTs with and without rfhSP-D coating showed modulation of cytokines release. rfhSP-D bound CNTs up-regulated the pro-inflammatory response in differentiated THP-1 macrophages. Upregulation is more likely through the interaction of rfhSP-D bound CNTs with Toll-like receptor 4 (TLR4), and resulting in initiation of the IL-1β pathway and NLRP3 inflammasome activation (Meunier *et al.*, 2012). The up-regulation of IL-12 may indicate the polarisation of Th1 cell (Athie-Morales *et al.*, 2004), signifying the switches from Th2 to a th1 response during interaction with CNTs. These findings are consistent with previously reported by Pondman et al (Pondman *et al.*, 2016). The rfhSP-D coating on both types of CNTs was also found to up-regulate G-CSF and IL-8, which are important for the mobilisation of neutrophils and T cells, respectively. IL-8, as a crucial neutrophil chemoattractant, has been shown to be involved in acute lung injury (Allen and Kurdowska, 2014), which has been reduced in secretion by complement deposition. Nuclear translocation of NF-κB by NPs has been previously demonstrated in human kidney cells (Pujalte *et al.*, 2011). Activation of NF-κB and subsequent release of pro-inflammatory signalling molecules such as TNF-α, IL-1β, IL-8 and IL-6 by metallic NPs (Monteiller *et al.*, 2007) and single-walled CNTs (Pacurari *et al.*, 2008) has been previously observed in *in vitro* and in *in vivo*. This further indicates the potential of rfhSP-D to produce an inflammatory response in combination with CNTs.

IL-1α and IL-1β are potent inflammatory cytokines, which can additionally induce endothelial cells to produce infiltration-causing MCP-1 release (Sica *et al.*, 1990). Furthermore, IL-1 also induces IFN- γ production by Th1 and NK cells via IL-12 secretion by antigen presenting cells (Billiau, 1996). In addition, IL-1 induces metallomatrix proteins (MMP) production by lung fibroblasts resulting extracellular matrix degradation. Thus, suppression of these cytokines, whose generation involves lung fibrosis, inflammasome and TLR4, is favourable to maintain pulmonary homeostasis. IL-1 also upregulates the pyrogenic and acute phase protein TNF-α and TLR4 mediated interaction involving SP-D and CNT (Yamazoe *et al.*, 2008). Upregulation of IL-13 and eotaxin by rfhSP-D bound CNTs strongly suggests the likeliness of idiopathic lung fibrosis, pulmonary inflammation and asthma (Passalacqua *et al.*, 2017). One crucial up-regulation that we observed in response to CNTs is VEGF. VEGF is known to increase oedema (vascular permeability) involving Th2 dependent sensitisation and pulmonary inflammation and asthma (Lee *et al.*, 2004). It has been established that CNT-bound rfhSP-D differs with the soluble rfhSP-D in murine models (Madan *et al.*, 2001); however, this effect can be overcome by complement deposition on them.

Here we observed that functionalized CNTs act as a charge pattern recognition target, for SP-D and C1q simultaneously but the binding sites are not overlapping. Binding of several other proteins and subsequent modulation of complement activation has been previously reported. This includes efficient binding of human serum albumin (HSA), fibrinogen (Salvador-Morales, Green and Sim, 2007), human C1q, bovine serum albumin (BSA) and recombinant fragments of C1q (Pondman *et al.*, 2014; Pondman *et al.*, 2015) and SP-A (Salvador-Morales *et al.*, 2008; Salvador-Morales *et*

al., 2013). Furthermore, a coating of CNTs with other proteins like BSA and fibrinogen enhance complement activation (Pondman *et al.*, 2014).

SP-D opsonisation leads to enhanced uptake of CNTs by macrophages, similar to complement deposition. However, we observed a restriction of entry of the CNTs, when both opsonins (SP-D and complement) were bound together and also suppressed pro-inflammatory response by rfhSP-D bound CNTs. These findings make a good argument for coating of CNTs with complement components for therapeutic applications.

As some of the properties of CNTs resembles with asbestos, for the use of therapeutic, a careful consideration is a warrant due to their interaction with SP-D and subsequent potential harmful inflammation (Cassel *et al.*, 2008; Dostert *et al.*, 2008). Furthermore, it has been demonstrated that SP-D gets completely sequestered from suspension by binding to carbon black particles (Kendall, Brown and Trought, 2004). Such sequestration of SP-D intensified *Streptococcus pneumonia* infection in rats exposed to airborne particles (Zelikoff *et al.*, 2003) It has also been reported that immobilization of SP-D and SP-A by NPs was shown to modulate the function of these proteins to combat and neutralize influenza A infection *in vitro* (McKenzie *et al.*, 2015a). Sequestration of these immune molecules by NPs make host prone to viral and bacterial infection. The SP-D bindings should thus be considered separately for every modification (also for therapeutics bound onto the surface coating).

5 Chapter V

Immune Interaction with Iron Oxide Nanoparticles

5.1 Abstract

NPs have promised a range of diagnostic and therapeutic applications in biomedicine. They are attractive drug delivery vehicles for targeted organs or tissues. However, their interaction with the immune system is very intriguing and poses challenges to develop themfor i*n vivo* applications. Here, we report that iron oxide particles (IONP) are recognized by complement pathway, mainly by alternative complement pathway. Complement deposition enhanced their uptake by activated THP-1 macrophages. Furthermore, secreted cytokines, chemokines and growth factors analyses showed complement-dependent downregulation of pro-inflammatory cytokines, TNF-α, IL-1β, IL-6, IL-12, although the intracellular fate of IONP is independent of complement deposition. We propose that complement activation by the iron particles may cause immune cells activation and infiltration due to the release of cytokines and chemokines and growth factors.

5.2 Introduction

IONPs have been widely used as contrast agents for magnetic resonance imaging (Daldrup-Link *et al.*, 2011). They have also been used as intravenous iron preparations, iron dextran, iron sucrose, iron gluconate, ferric carboxymaltose, iron isomaltoside 1000, and ferumoxytol, to treat iron deficiency and as an iron replacement (Singh *et al.*, 2008; Auerbach and Ballard, 2010). Recently, ferumoxytol, one of the Food and Drug Administration (FDA)-approved iron supplements, has shown an intrinsic therapeutic effect on early breast cancer and metastatic lung cancer by increasing caspase-3 activity in macrophages. Furthermore, in vivo, it significantly inhibited proliferation of subcutaneous adenocarcinoma in mice and prevented the development of liver metastasis accompanied by the pro-inflammatory immune response with M1 macrophage polarization in the tumour microenvironment (Zanganeh *et al.*, 2016).

It is inevitable that intravenously delivered NPs will have direct access to blood and hence the immune system. NPs injected into the bloodstream encounter the immune system and can cause an inflammatory response. The immune system attempts to clear them, as they are foreign materials. Therefore, the immune response to NPs must be considered prior to the development of *in vivo* applications. NPs in the blood may undergo immune-mediated clearance via phagocytosis or damage the immune system via immunotoxicity or there could be no interference with immune responses due to immunocompatibility (Boraschi, Costantino and Italiani, 2012). Immune components, especially macrophages and complement proteins, actively participate in clearance of foreign particles. Macrophages and dendritic cells express receptors like Toll-like receptors (TLRs), scavenger receptors, complement receptors, integrins, lectin-like receptors and Fc receptors. These receptors recognize and bind foreign particles and

help in phagocytic clearance, ultimately influencing the fate of NPs (Silva *et al.*, 2016; Yameen *et al.*, 2014).

Surface coatings of NPs interact with biological systems first and provide a physical barrier between the NP core and the immune system. It has been shown that surface properties of NPs like the presence of the functional group, the net charge of surfactants and overall size are critical for their cellular uptake (Mahmoudi *et al.*, 2011), bio-distribution and toxicity (Albanese, Tang and Chan, 2012; Salatin, Maleki Dizaj and Yari Khosroushahi, 2015). Besides NP surfaces, size and charges, non/specific absorption of serum proteins also critically affect NP–immune cell interactions. Contact of NPs with a biological system like blood, cells and tissues, forms a thin layer of soluble proteins called 'protein corona' (Pelaz *et al.*, 2015; Vroman, 1962). The formation of protein corona reconfigures the native structure of NPs and, subsequently, affects their internalization (Pondman *et al.*, 2014) and has an impact on cell response eg. Cytotoxicity, immunotoxicity (Monopoli *et al.*, 2011) and intracellular localization (Lesniak *et al.*, 2012). On the other hand, there is a continuous and rapid turnover of adsorbed proteins based on their binding affinity. Adsorbed proteins could alter their conformation, leading to activation of particular cell signalling pathways (Deng *et al.*, 2011) or facilitate the absorption of another protein forming protein-protein interactions (Vroman, 1962). Such protein-NP interaction can be predominately seen in one of the components of the innate immune system, specifically the complement pathway, which is initiated by the binding of the antibody complex by C1q (classical pathway) or C3b deposition (alternative pathway) on NPs or recognition by pattern recognition molecules such as MBL (Lectin pathway) (Merle *et al.*, 2015). Initial binding leads to sequential adsorption of other small complement proteins eventually forming membrane attack complex (MAC) (Serna *et al.*, 2016). Thus, activation of the complement system can

influence therapeutic activity and effectiveness of NPs, as it can, in principle, cause NPs to adhere to immune cells (Dobrovolskaia *et al.*, 2008). Further understanding of the interactions between NPs and human monocyte cell lines will be beneficial for predicting NP-induced inflammatory responses and provide valuable information for NP design to alleviate inflammation in biological systems facilitating strategic and specific in vivo delivery.

Previously, we have reported that pristine CNTs, CMC-functionalized CNTs, Oxidized CNTs activate the complement pathway prerdominantly via classical pathway (Pondman *et al.*, 2014). Complement deposited CNTs modulated the immune response by downregulating pro-inflammatory cytokines and upregulating anti-inflammatory cytokines in human monocytic cells (U937), B cells (Raji) and T (Jurkat) cells (Pondman *et al.*, 2016). Furthermore, complement deposition on CNTs enhanced their uptake by U937 (Pondman *et al.*, 2016; Pondman *et al.*, 2014; Pondman *et al.*, 2015). Here, we have aimed to analyse whether complement deposition, modulation of immune response and recognition by the immune cell is consistently observed with other differently functionalized NPs. We have also aimed to analyse the effect of complement deposition on nanoparticles' intracellular fate. Here, the surface effects of IONPs on cellular uptake and their ability to induce immune responses were studied in human monocyte cell line, THP-1. We found that starch coated IONPs activate the complement system predominantly via alternative pathway unlike carboxymethyl cellulose coated carbon nanotubes (CMC-CNTs) (Pondman *et al.*, 2014). Complement opsonisation enhanced the uptake of IONPs by THP-1 cells, leading to modulation of pro-inflammatory and anti-inflammatory cytokine expression as evident by qPCR and multiplex assays. Intracellular localization of serum coated (complement deposited) and

uncoated IONPs showed no distinct differences. Both, complement deposited and uncoated were shown residing in lysosomes.

5.3 Results

5.3.1 CNTs and Iron oxides NPs activates the complement pathway

Both functionalized and pristine IONPs were assessed for their ability to activate the complement pathway. Functionalized IONPs predominantly activated alternative pathway (Figure 5-1). The activation was 5 times more than that of the classical pathway. Pristine IONPs activated both complement pathways equally but for the alternative pathway to a lesser extent than functionalized IONPs. This showed that starch functionalized IONPs activate the alternative pathway more readily.

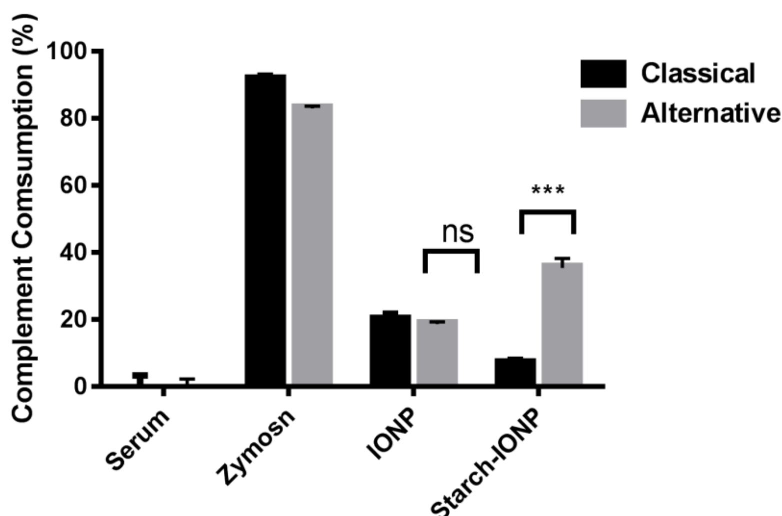


Figure 5-1 Complement activation via a classical and alternative pathway.

Starch functionalized IONPs and IONPs were incubated with human serum (1/10 diluted in DGVB for classical pathway and 1/5 diluted in DGVB-Mg-EGTA buffer) for 1h at 37^{0}C. Samples were spun down and serum was collected for complement inhibition assay. Zymosan was used as positive control and serum only was used as negative control. Percentage complement consumption was calculated separately for each pathway analysis and results were merged in one figure. Percentage complement consumption was calculated using $(C - \mathrm{Ci})/C \times 100\%$, where C represents the % hemolysis of the negative control, and Ci is the % hemolysis with the IONPs or zymosan-treated sample. Multiple T-test was performed to determine significant differences in complement activation between the two types of consumption pathway. All experiments were carried out in triplicate. All error bars represent ± standard deviation,; ns: not significant p>0.05, *p<0.05, **p<0.01, ***p<0.001.

5.3.2 Cytotoxicity of IONPs

THP-1 cells were tested for potential cytotoxicity of IONPs before analysing for cytokine expression and various signalling molecule levels using multiplex. 10 µg /ml of IONPs and equal concentrations of complement deposited IONPs (IONP+serum) were incubated with differentiated THP-1 for 24 h. None of the NPs at given concentration possessed any cytotoxic effect on THP-1 macrophages as shown in figure (Figure 5-2). Thus, based on the cytotoxic result, the concentration of 10ug/ml of IONPs was used for other analysis such as endocytosis assay, cytokine mRNA expression and multiplex cytokine/chemokine assays.

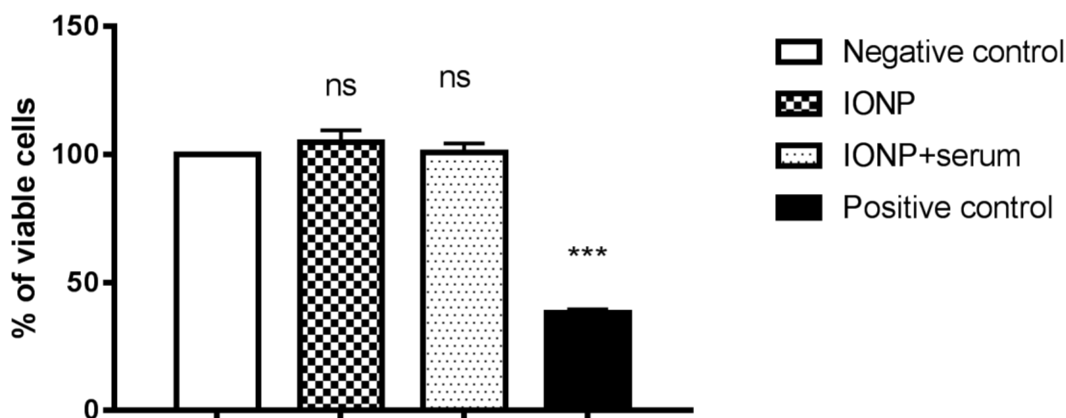


Figure 5-2: MTT assay to determine cytotoxicity of different NPs.

Differentiated THP-1 cells were incubated with 10ug/ml of serum treated or untreated IONPs for 24 h in serum-free medium. Percentage of viable cells was determined by MTT assay. 0.05% saponin was used as positive control. The experiment was carried out in triplicate. All error bars represent ± SD, Ns: not significant ($p>0.05$)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$) (An unpaired t-test with Welch's correction was carried out to test the difference in cytotoxicity between the negative control and NPs)

5.3.3 Differential uptake of IONPs by THP-1 and U937.

To determine the rate of NPs' internalization, serum treated and untreated IONPs were incubated with U937 and THP-1 cells lines for 15 min and 2 hrs (Figure 5-3). Internalization of serum treated IONPs were high in both U937 and THP-1 cell lines. Serum treatment enhances uptake of NPs but the amount of particle internalization is highly dependent upon cell type. Furthermore, at the earliest time point (15 min), internalization of particles was independent of serum treatment in U937. Internalization of serum treated particles was very prominent in THP-1 cells. At later time points (2 hrs), IONPs accumulated in the perinuclear region.

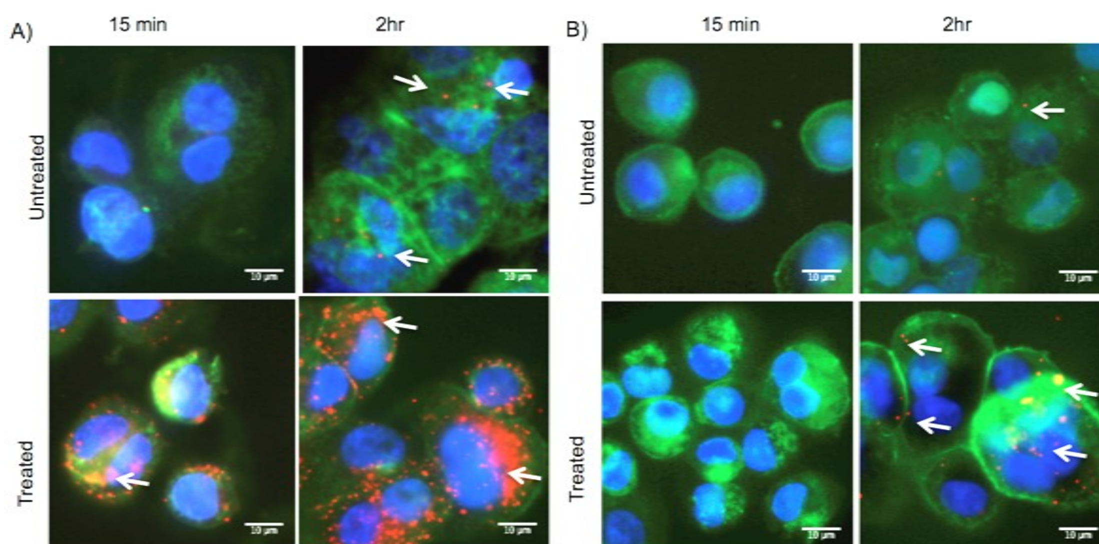


Figure 5-3: Differential uptake of IONPs by human monocytic cell lines THP-1 (A) and U937 (B)

10 µg/ml of IONPs, serum treated or untreated (Red) was added to THP-1 (A) and U937 (B) cells and incubated for 15min and 2 hrs. Cells were washed, fixed and stained with AlexaFluor546-labeled WGA (Green) to reveal plasma membrane and Hoechst 33342 to stain nucleus (Blue). Scale bars, 10µm.

5.3.4 Opsonisation enhanced IONPs' endocytosis but does not modulate intracellular fate

The efficiency of phagocytosis of IONPs by THP-1 cells is enhanced by the presence of serum proteins on their surface as shown in Figure 5-4 and Figure 5-5. Fluorescent microscopy shows that IONPs were readily endocytosed at 2h incubation when serum deposited but less so when left uncoated. After longer incubation times this difference was lost. This suggests that presence of serum protein on the surface makes the particles more visible to macrophages possibly mimicking opsonized bacteria or other pathogens. In contrast, uncoated iron particle devoid of complement fragments may take another route of uptake.

Figure 5-4 shows that internalized IONPs co-localize with dextran particles that accumulate in cellular lysosomes. Co-localization is very prominent in serum deposited particles. However, after longer incubation, co-localization with dextran is observed independent of serum deposition.

Similarly, serum treated IONPs colocalized with Lysotracker, which accumulates in the acidic environment of late endosomes and lysosomes, in early time points (6h) but upon longer incubation (18h and 24h continuous exposure), uncoated particles were also seen to co-localise with lysotracker.

It shows that serum deposition is not modulating the intracellular fate of the particles but that it enhances uptake and may also contribute to the swift movement of IONPs within the different vesicles involved in the endocytic pathway.

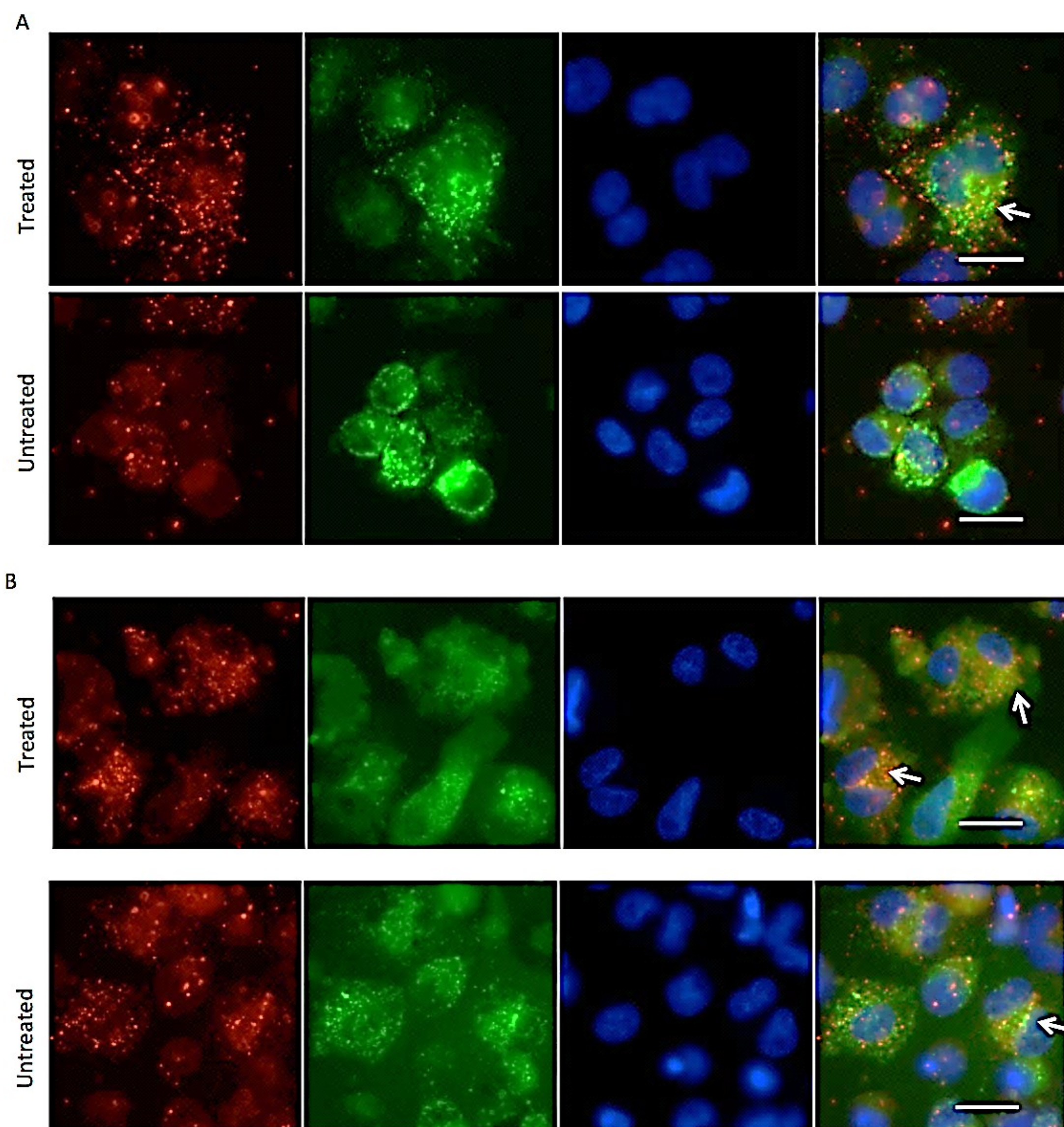


Figure 5-4: Co-localization of IONPs with fluorescent dextran with MW20,000Da marker for fluid phase endocytosis)

Serum coated and uncoated iron oxides particles (Red) and dextran (green) were co-incubated with differentiated THP-1 cells for 2h **(A)** and 2h followed by18h of the chase in normal media (pulse-chase)**(B).** Cells were washed with PBS, fixed with 4% PFA, mounted and observed under a microscope. Green: dextran, Red: IONPs, Blue: Nucleus. Scale 20 µm.

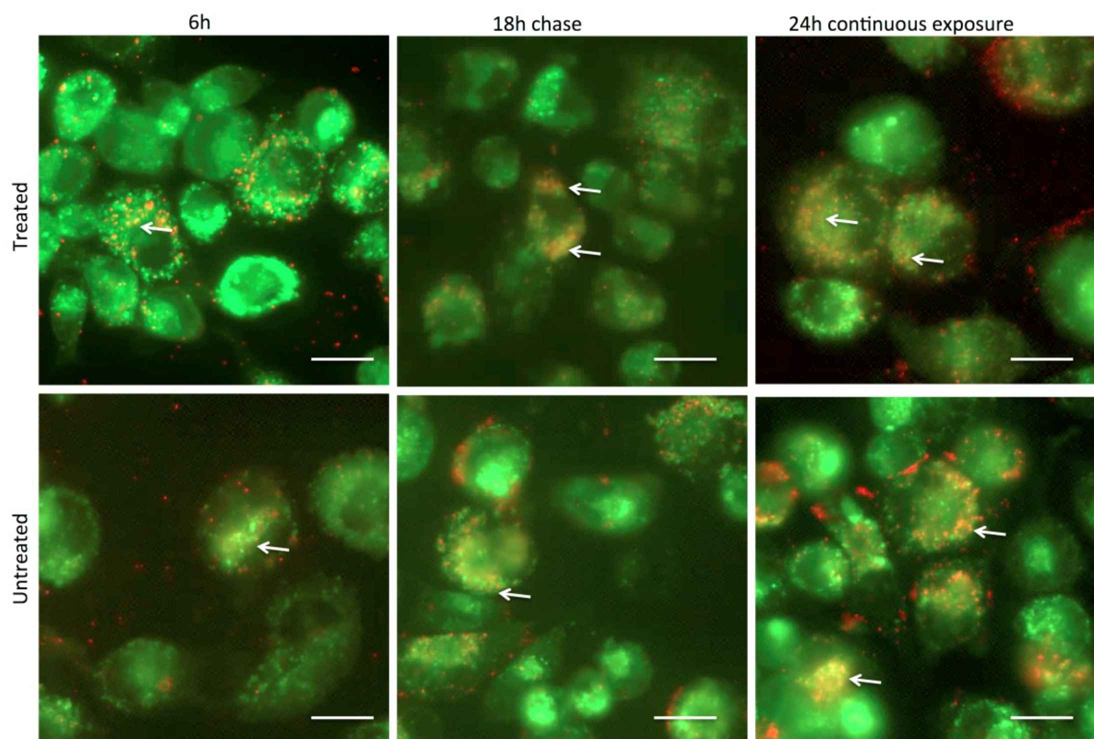


Figure 5-5: Co-localization of IONPs with lysotracker

Serum coated and uncoated iron oxides particles (Red) were incubated with differentiated THP-1 cells for 6h, 2h followed by18h of the chase in normal media (pulse-chase) and 24h continuous exposure. Prior to observation, lysotracker was added to the cells for 15 min, the cells were then washed and mounted on the slide. Green: lysotracker, Red: IONPs Scale 20μm

5.3.5 Complement deposition on IONPs modulates cytokine expression at transcriptional level.

Though, both IONP activated the complement pathway, here, we used starched functionalized IONPs to study the immune response they modulate in macrophages. Cytokine profiles of THP-1 cells were measured at four time points up to 6h (30min, 1h, 2h and 6h) via qPCR following treatment with iron oxides particles with and without serum deposition (Figure 5-6). mRNA levels of pro-inflammatory cytokine TNF-α were significantly downregulated by serum deposited IONPs compared to untreated IONPs in a time-dependent manner. No difference was seen in the case of IL-1β at transcription level in THP-1 cells exposed to IONPs. However, IL-12 was downregulated by both serum treated and untreated IONPs. For anti-inflammatory cytokines, IL-10 was slightly downregulated by untreated IONPs. Similarly, TGF-β was downregulated by serum deposited IONPs. No significant changes were observed for NLRP3, suggesting that NLRP3 inflammasome activation was limited. Overall, serum deposited IONPs induce an anti-inflammatory response in THP-1 cells.

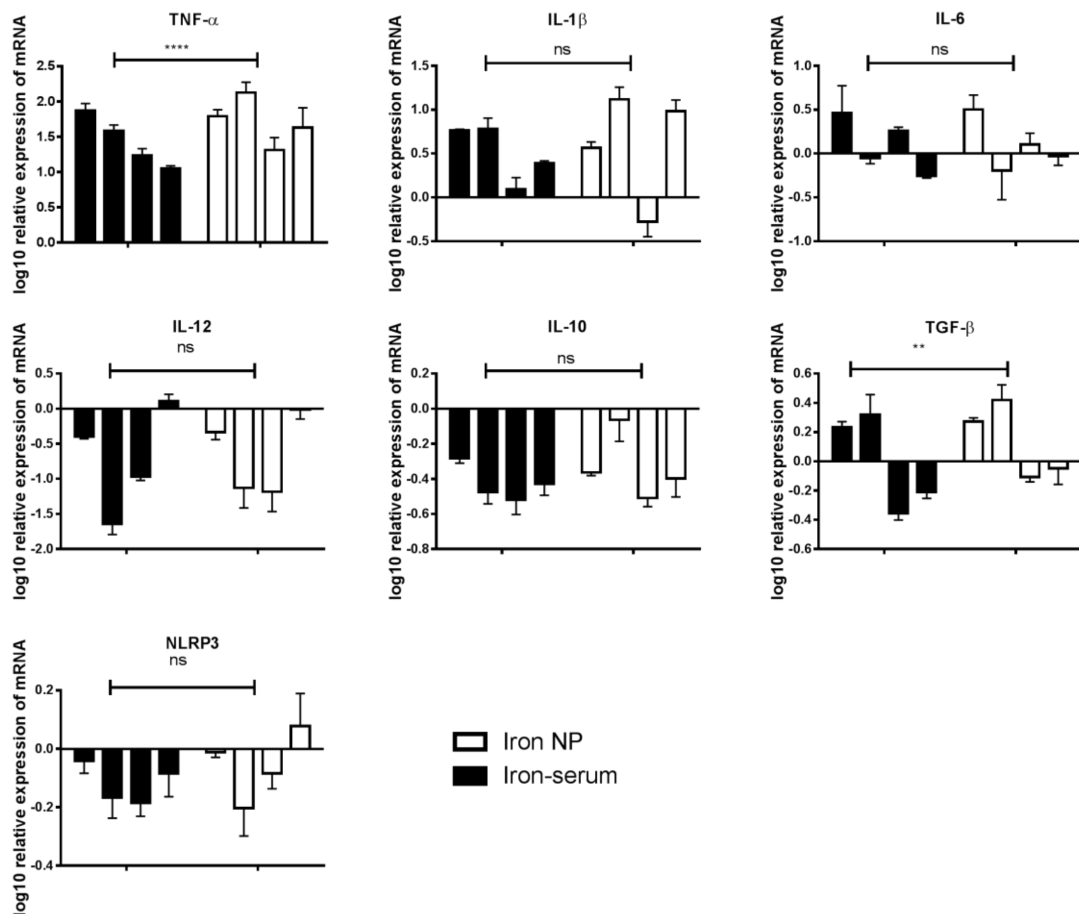


Figure 5-6: Cytokine gene expression profile of THP-1 cells in response to serum treated or untreated IONPs

For the measurement of mRNA expression of pro- and anti-inflammatory target genes, PMA differentiated THP-1 cells were exposed to serum treated or untreated IONPs for 30 min, 1h, 2h and 6h (X-axis). All error bars represent ± standard deviation, Ns: not significant (p>0.05)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$) (2-way ANOVA test was performed between each uncoated and coated IONPs to determine the significance difference in the level of expression)

5.3.6 Serum deposition on IONPs modulates the expression of cytokines, chemokines and other soluble factors.

Increased pro-inflammatory responses were observed in THP-1 cells when challenged with IONPs. Levels of chemokine secretion were also significantly high compared to cells only control (Figure 5-9). However, this effect was considerably downregulatedin samples treated with serum deposited IONPs, as indicated by IL-1β, IL-6, TNF-α, IL-12 (p40). A similar trend was observed for the chemoattractant array in MCP-1, MDC, MCP-3, GRO, MIP-1α and IL-8 by serum deposited IONPs. However, MIP-1β and IP-10 were significantly higher in the serum deposited IONP samples. IFNα2 and IFN-γ were upregulated by serum deposited IONPs as a result, Interferon gamma-induced protein 10 (IP-10) was significantly upregulated after 48h but not after 24 h. There was also significant downregulation of some growth factors; like GM-CSF, VEGF and TGF-α. However, EGF and IL-2 showed significant upregulation. Other targets in the analyte arrays such as FGF-2, FLT-3L, IL-15, IL-12 (p70), IL-17A and were not altered upon IONPs exposure.

A)

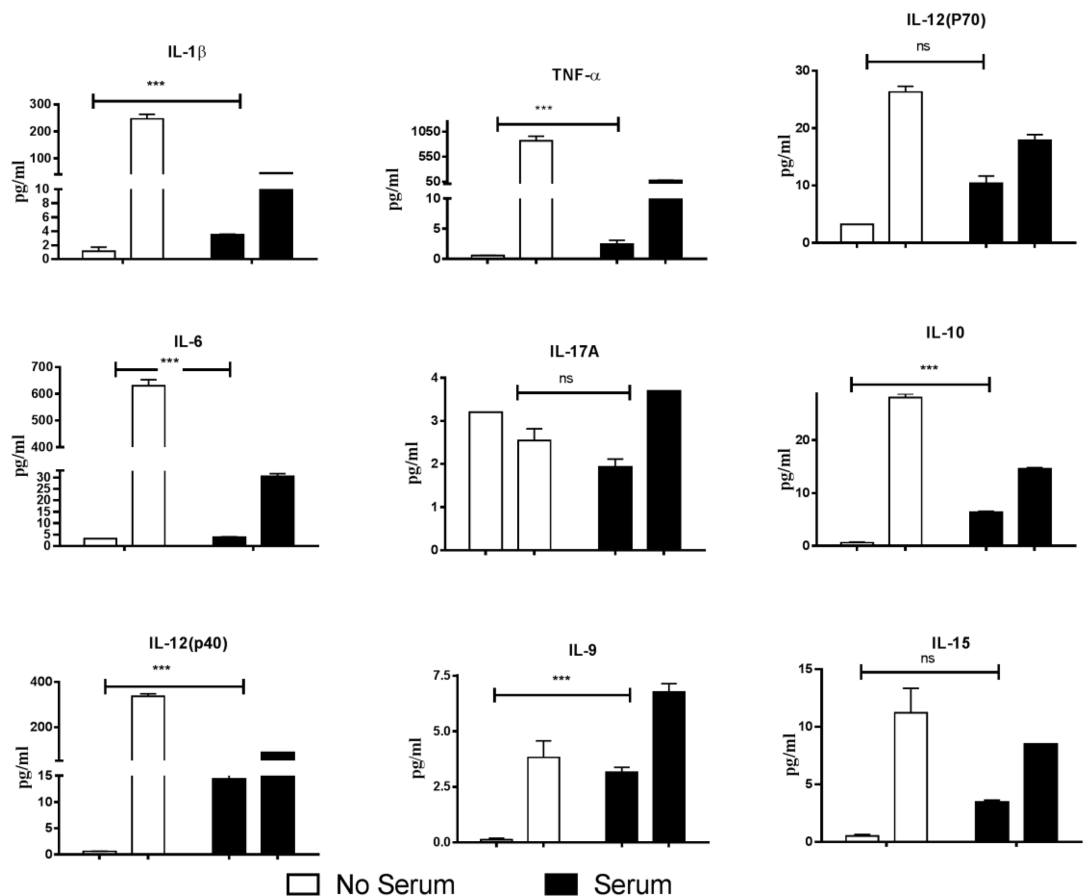


Figure 5-7: Cytokines analysis of supernatants of THP-1 cells following treatment with Iron oxides particles (with and without serum)

THP-1 cells were incubated 30 min, 1h, 2h, 6h, 12h, 24h and 48h with serum treated and untreated IONPs. Supernatants from 24h and 48 h (X-axis) incubations were used for the measurement of the levels of **cytokines released** (IL-6, IL-10, IL12p40, IL12p70, IL-1β, TNF-α, IL-15, IL-17A, IL-9) by using a MagPix Milliplex kit (EMD Millipore). All error bars represent ± standard deviation, Ns: not significant (p>0.05)*p < 0.05, *p < 0.01, and ***p < 0.001) (2-way ANOVA test was performed between each uncoated and coated IONPs to determine the significance difference in the level of expression)

B)

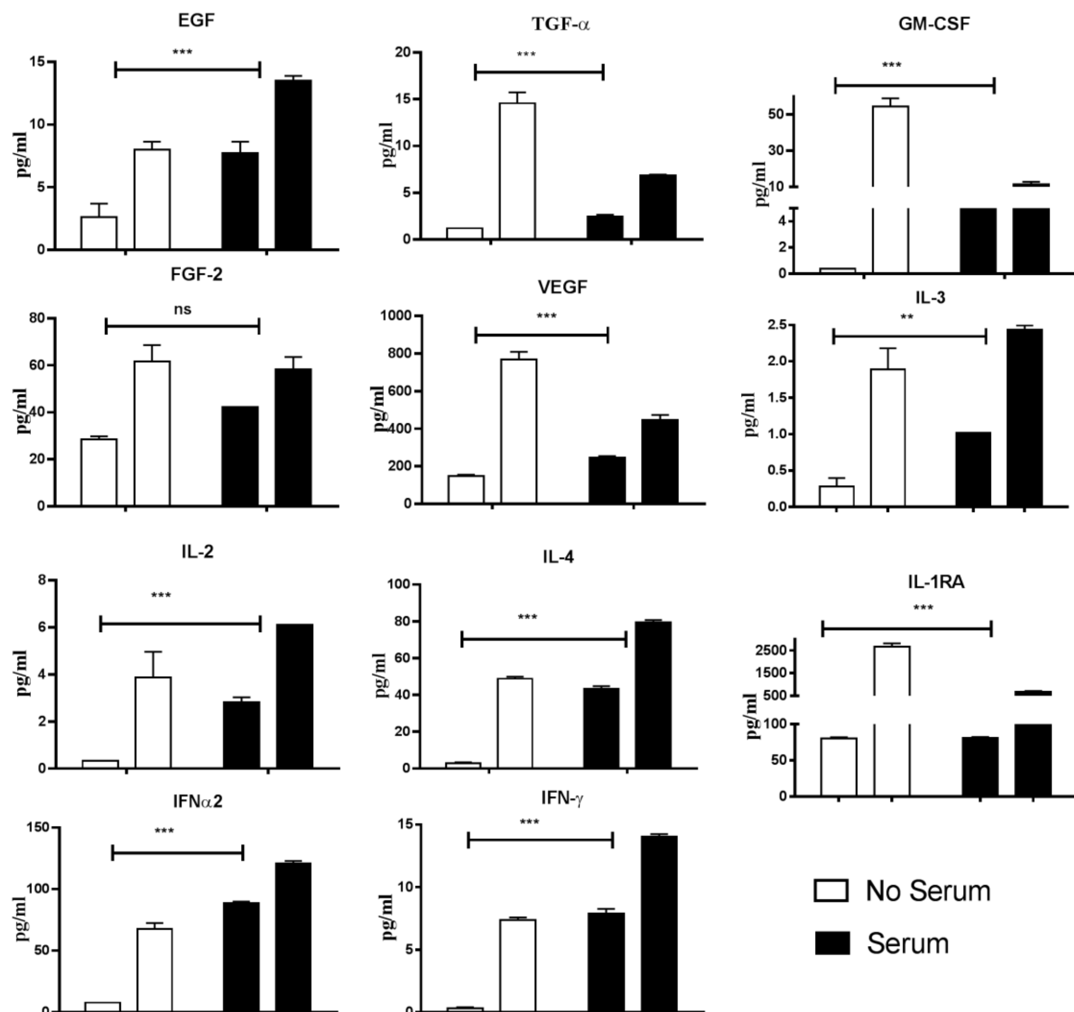


Figure 5-8: Multiplex cytokine array analysis of supernatants of THP-1 cells following treatment with Iron oxides particles (with and without serum)

THP-1 cells were incubated 30 min, 1h, 2h, 6h, 12h, 24h and 48h with serum treated and untreated IONPs. Supernatants from 24h and 48 h (X-axis) incubations were used for the measurement of **growth factors** (IL-2, EGF, FGF-2, TGF- α, GM-CSF, IL-3, IL-4, VEGF) and **related ligands and receptors** (IFNα2, IFN-γ, IL-1RA), by using a MagPix Milliplex kit (EMD Millipore). All error bars represent ± standard deviation, Ns: not significant (p>0.05)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$) (2-way ANOVA test was performed between each uncoated and coated IONPs to determine the significance difference in the level of expression)

C)

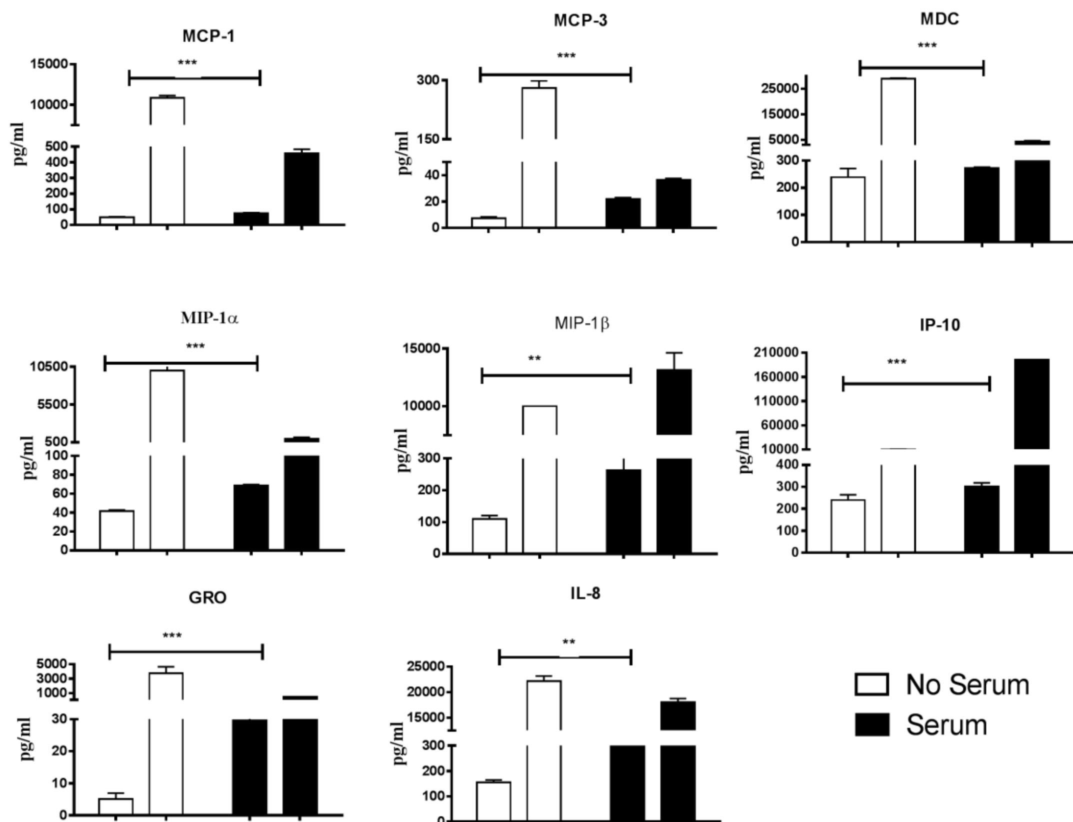


Figure 5-9: Multiplex cytokine array analysis of supernatants of THP-1 cells following treatment with Iron oxides particles (with and without serum)

THP-1 cells were incubated 30 min, 1h, 2h, 6h, 12h, 24h and 48h with serum treated and untreated IONPs. Supernatants from 24h and 48 h (X-axis) incubations were used for the measurement of **chemokines** (MCP-3, MDC, GRO, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β), by using a MagPix Milliplex kit (EMD Millipore). All error bars represent ± standard deviation, Ns: not significant (p>0.05)*$p < 0.05$, *$p < 0.01$, and ***$p < 0.001$) (2-way ANOVA test was performed between each uncoated and coated IONPs to determine the significance difference in the level of expression)

5.4 Discussion

Understanding of the interactions of nanomaterials with host immune system is instrumental to illustrate the biological and medical effects of these materials. Here, we have attempted to elucidate the activation of complement pathway, a crucial part of the innate immune system and subsequent immune modulation by complement activating NPs. Previously, we have established that CNTs activate the complement pathway mainly via a classical pathway in human sera (Pondman *et al.*, 2014). Here, we have reported that, unlike CNTs, starch-IONPs activate complement pathway predominantly via the alternative pathway. However, pristine IONPs showed no difference between classical and alternative pathway activation but still, considerable activation of classical pathway took place compared to starch-IONPs. This result highlights that starch functionalization can be a key activator of the alternative pathway. The starch surface is likely to induce the formation of fluid-phase AP convertase, C3 (H_20) Bb, the initial deposition of C3b on the foreign surface (Bexborn *et al.*, 2008). Deposition of C3b component induces the activation of alternative pathway cascade. It has also been reported that polymer like polystyrene can adsorbed C3 component to its surface causing conformational changes that turn C3 into a C3B-like component binding factor B forming a functional C3Bb convertase (Andersson *et al.*, 2002; Nilsson *et al.*, 1993). Alternative pathway activation has also been studied on other polymeric biomaterial surfaces like silicon and polypropylene (Gobel *et al.*, 1987). Furthermore, the hydroxyl group of starch ionizes in aqueous condition behaving as a nucleophilic group, which can not only bind to nascent C3b by attacking the thioester but also allows C3 to adsorbed through electrostatic or hydrophobic interactions (Andersson *et al.*, 2002). Such duel effects might have contributed to the observed higher activation of alternative pathway than in case of non-functionalized IONPs. Besides, binding C3 and C3b

components on the surface, glucose and dextrose derived polysaccharides also bind to MBL (Mannose-binding lectins) and MBL –associated serine proteases like MASP-1 and MASP-2 (Simberg *et al.*, 2009) suggesting the activation of lectin pathway (Fujita, Matsushita and Endo, 2004), while negatively charge part of iron core binds to histidine-rich glycoprotein and kininogen (Simberg *et al.*, 2009) thus playing a significant role in immunomodulation. As complement system is regarded as central in the sensing of foreign particles, their processing and elimination, activation of such cascade and the release of extremely potent anaphylatoxins like C3a and C5a may bring serious issues regarding IONPs' safety and efficacy (Peng *et al.*, 2009). Liberated C5a recruits regulatory T cells, immunosuppressive monocytes into malignant tumours seriously affecting the prognosis of cancer (Markiewski *et al.*, 2008; Moghimi and Farhangrazi, 2014). Complement activation, therefore, is of concern for the successful development of intravenous nano-based drug delivery. In order to dampen the inevitable complement activation, Moghimi and colleagues have modified sugar alcohols of surface polysaccharides in dextran with alkylating, acylating, or crosslinking agents but unfortunately did not overcome complement activation and C3 opsonization (Wang *et al.*, 2016). Recently, our group has extensively investigated inhibition of complement activation using recombinant globular head modules of the human C1q molecule as well as factor H (Pondman *et al.*, 2015). Surface adsorption of these soluble complement regulators offered a novel way of controlling complement pathway activation and also down-regulation of inflammation caused by NPs and design of immune-safe nanomedicine.

Another part of the innate immune system, macrophages, was used to study the response to NPs. As we have already established that NPs do activate complement pathway and upon activation, NPsopsonize with C3b molecules and other plasma

proteins. Here we tested fixed concentration of IONPs (10ug/ml) for potential cytotoxicity. Based on MTT assay, none of the concentration was cytotoxic to the cells until 24h. These non-toxic concentrations of IONPs were used for all other assays. IONPs have been shown to have the cytotoxic potential to mammalian cells in higher concentration than 10μg/ml and cytotoxicity has been shown to be mainly due to high ROS generation, oxidative stress and induction of apoptosis (Naqvi *et al.*, 2010). Oxidative stress-mediated cytotoxicity has also been reported recently using *in vivo* test in Artemia salina cysts and three stages of larvae (Wang *et al.*, 2017a). IONPs were highly pro-inflammatory as shown by qPCR and multiplex results. We have studied the cytokines, chemokines, growth factors and other ligand secretion by macrophage-like cell lines, in response to opsonized IONPs. We observed very early transcriptional up-regulation of pro-inflammatory cytokines, TNF-α and IL-1β. Serum treated IONPs significantly downregulated the expression of TNF-α in contrast to results observed by Brown et al. (Brown *et al.*, 2014) where they reported that IONPs incubated with culture media with serum enhanced cytokine response in mouse macrophage cell line (J774A.1). High levels of cytokine production in both healthy and patient's donor have been reported upon iron sucrose and iron dextrose administration (Pai *et al.*, 2011; Fell *et al.*, 2014). Both types of iron were pro-inflammatory and activated ROS generation. The release of reactive oxygen metabolites has previously been observed in starch coated IONPs via interaction of abundantly adsorbed immunoglobulin on IONPs with Fc receptors on macrophages (Artursson *et al.*, 1987). We observed a dramatic dampening of pro-inflammatory cytokines, IL-1β, IL-6, TNF-α, IL-12 (p40), and IL-12(P70), and chemokines MCP-1, MDC, MCP-3, GRO, MIP-1α by serum deposited IONPs. Dampening of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines has previously been reported in human macrophages in

response to serum deposited CNTs (Pondman *et al.*, 2014; Pondman *et al.*, 2016). Though significant differences were not observed at the transcriptional level of these cytokines except for TNF-α, measurement of secreted cytokines and chemokines showed a shift of the pro-inflammatory response toward an anti-inflammatory environment by serum deposited IONPs.

The production of IL-12 may indicate Th1 cell polarisation switching Th2 to Th1 response during interaction with IONPs. IL-1α and IL-1 β are prototypical inflammatory cytokines mostly induced by TLR signalling pathways (Chen, Kanehira and Taniguchi, 2013; Verhoef *et al.*, 2017). Production of IL-1β induces Th1 type cytokine, IFN-γ (Cardozo *et al.*, 2003) which in turn stimulates the production of IFN- γ induced protein 10 (IP 10). Here, we also observed an increased level of IL-6 and TNF-α, though downregulated by serum deposited IONPs, which can otherwise activate inflammatory cells, induce vascular permeability causing swelling, redness and fever (Elsabahy and Wooley, 2013). Secretion of Th2-type cytokines like IL-4, IL-6 and IL-10 indicate the activation of humoral immunity and IL-2, IFN- γ and TNF-β indicates the activation of cell-mediated immunity (Osugi *et al.*, 1997; Rajananthanan *et al.*, 1999). IL-8, as a crucial chemoattractant can be associated with induction of chemotaxis in target cells, attracting neutrophils to the site of infection and promote angiogenesis (Baggiolini and Clark-Lewis, 1992).

Another aspect of the innate immune system is a clearance of foreign particles via several endocytic pathways. Here, we studied how serum opsonized IONPs are cleared by macrophages and their intracellular fate after uptake. Complement deposition modulated the rate of uptake of IONPs by macrophages. Professional phagocytic cells deploy mostly receptor-mediated endocytosis like mannose receptor (MR), complement receptor (CR), Fcγ receptor (FcγR) and several scavenger receptors (SR). Mannose,

complement proteins and immunoglobulins are opsonins targeting the MR, CR and FcγR routes respectively (Aderem and Underhill, 1999). Internalization of starch-IONPs was enhanced by complement deposition. Serum treated NPs were not only endocytosed quickly and readily but also held for the considerably longer period than uncoated NPs (Pondman *et al.*, 2014). The higher level of uptake of IONPs in the presence of serum suggests the involvement of complement receptor and receptor-mediated internalization. Involvement of CR receptors in endocytosis of starch and mannan NPs has been reported earlier (Artursson, Johansson and Sjoholm, 1988). THP-1 cells express high levels of CR3 on their surface. These receptors, alone or in combination, may be involved in their uptake. High levels of particle internalization by THP-1 could be due to extra complement receptor such as FCγRI and FCγRII receptors present on the surface of THP-1. These receptors are constitutively expressed three to four-fold more on THP-1 than U937 cells (Fleit and Kobasiuk, 1991). However, non-opsonized IONPs could be taken up via a sugarr eceptor-mediated process involving MR (Artursson, Johansson and Sjoholm, 1988). It can now be suggested that complement receptor-mediated phagocytosis could override the sugar receptor-mediated (Mannon receptor) process in the case of phagocytosis of the particles that have a tendency to adsorbed significant amounts of serum protein. Involvement of SR-A mediated endocytosis has also been reported in macrophage uptake of non-opsonized superparamagnetic iron particles, ferumoxides and ferumoxtran-10, contrast agents (Raynal *et al.*, 2004).

Moreover, size of particles and surface coating highly influence their method of uptake. The known endocytic processes that cells employ are mainly phagocytosis, pinocytosis, Caveolae dependent or Clathrin-mediated endocytosis. These processes are energy dependent. Particles greater than 750nm are engulfed and transported into the cells via

phagosomes. Smaller particles of size around 100nm are mostly internalized by pinocytosis or macropinocytosis. Clathrin-mediated endocytosis or receptor-mediated endocytosis is probably the primary characterized mechanisms for NPs internalization in which particles are deposited in small endocytic vesicles which later fuse with early endosomes (Zhao *et al.*, 2011). Organic molecules coated particles are endocytosed through different processes compared to their uncoated counterpart. Pristine gold NPs are endocytosed via macropinocytosis and clathrin-mediated endocytosis whereas PEG-coated gold NPs mostly enter cells via clathrin-mediatedendocytosis, not by macropinocytosis (Brandenberger *et al.*, 2010). Therefore, the mechanism of endocytosis of NPs cannot be generalized due to the fact that surface modification of particles, size of particles and cell type govern the uptake process.

Though complement deposition and surface modification influenced the route of endocytosis it did not affect the intracellular localisation of IONPs, suggesting the fate of NPs is independent of opsonisation. Studies have shown conflicting results concerning localization of NPs. Here we found that non-opsonized IONPs of size 100 nm were found within a lysosomal compartment at early time points (6h). However, at late time points (18h and 24h), both particles appeared to be localized within lysosomes. Some studies have shown that similar sized IONPs accumulated in endosomes by 24 h in human embryonic kidney cells (HEK) cells (Osman *et al.*, 2012) and mouse macrophages (Raw 264.7) (Billotey *et al.*, 2003). This suggests that different cell types employ their own preferred methods of endocytosis.

This study has highlighted the ability of IONPs to bind complement proteins and to activate the complement pathway. The complement activation leads to altered uptake and cytokine expression by macrophages. Understanding of nanomaterials properties and their interaction with the immune system is a prerequisite for the design and

engineering of nanomedicine for safer use. Many pathogens have deployed innovative ways to mask themselves from immune recognition and clearance. Translation of such strategies could be pivotal in designing complement safe and phagocyte resistant drug carriers. Furthermore, complement activation is not always detrimental as evident by inhibition of pro-inflammatory cytokines and chemokines.

6 Chapter VI

General Conclusion and Future Perspective

Results presented here demonstrate the immune response to nanoparticle exposure, in terms of complement activation, endocytosis and cytokine expression and secretion. Knowing the mechanism of immune response to any NPs designed for therapeutic use is vital to overcoming the barriers presented by the body's defence systems, which rapidly eliminate the particles from systemic circulation. Thus, one of many obstacles in nanomedicine is programming the nanoparticles to maximize the amount that reaches target the location thereby minimizing side effects. Undesired immune system activation can lead to fatal acute and chronic inflammatory reactions. Therefore, understanding the interaction between nanoparticles and the immune system is of outmost importance.

Previously, analysis of nanoparticle uptake and immune response in presence and absence of complement have been investigated extensively in monocytes (U937), B cells (Raji cells) and T-cells (Jurkat cell) (Pondman *et al.*, 2016; Pondman *et al.*, 2014; Pondman *et al.*, 2015). CNTs were shown to modulate expression and release of cytokines. Cytokine array studies in these cells lines have given a wide range of information on how individual cells respond to CNTs with or without complement deposition. We also investigated how classical complement components, C1q, individual globular head of C1q (ghA, ghB, ghC) and f-H, modulate immune responses upon depositing them on various CNTs. We demonstrated that f-H not only reduced complement pathway activation but also inhibited CNT uptake by macrophages (Pondman *et al.*, 2015). Furthermore, C1q enhanced classical complement activation and also enhanced uptake of CNTs whereas recombinant globular heads inhibited classical complement activation but still enhanced the uptake of CNTs. This shows that each complement component recognizes different recognition pattern on CNTs and modulates immune response differently. In this thesis, we aimed to understand how

other soluble immune factors would modulate the immune response. With a view to understanding their immune modulatory effect, here, we have used recombinant full-length properdin, the small fragment of properdin, recombinant TSR4+5 module, as well as a recombinant human fragment (rfhSP-D) and adsorbed them on CNTs. Their effect on complement activation, uptake by macrophages and inflammatory response in macrophages was then analysed.

In Chapter 3, we reported that CNTs can bind to pulmonary surfactant protein SP-D and activate complement via the classical pathway. Complement deposition is more prominent in SP-D bound CNTs than CNTs alone. This shows that SP-D binds to CNTs in the orientation that activates the classical pathway. In addition, we report that SP-D deposition enhances the uptake of CNTs by macrophages. Most surprisingly, we show that complement deposition on SP-D coated CNT surface reduces the uptake of CNTs. Furthermore, complement deposited SP-D coated CNTs dampen pro-inflammatory cytokine production significantly compared to SP-D coated CNTs. These findings give an insight into the ability of complement to suppress pro-inflammatory responses elicited by rfhSP-D bound CNTs, reducing their quick clearance by immune cells. This supports the hypothesis that coating of CNTs with small fragments of recombinant complement components provides a promising avenue for therapeutic applications.

In chapter 4, we report for the first time, that properdin, a complement up-regulator, which stabilizes alternative pathway C3-convertases, can bind on CNTs as a pattern recognition receptor and induce a massive pro-inflammatory response. Furthermore, biological activities of CNT bound properdin are not affected as evident by activation of the alternative pathway in properdin-deficient serum. These findings highlight the use of CNT bound properdin in properdin deficient disease like X-linked

properdin deficiency disease. We also report that a recombinant small fragment of human properdin, TSR4+5, when coated on CNTs, acts as a potent inhibitor of alternative pathway activation and interestingly, reduces endocytosis of TSR4+5 coated CNTs. These findings address two features needed for an ideal nanocarrier, low immunogenicity with stealth properties and slow elimination by the immune system.

In chapter 5, we tested the interaction of IONPs with complement and their interaction with immune cells. It is evident from our study that all NPstested are viewed by complement recognition subcomponents as a non-self-pattern, which leads to theirsafesequestrationanda dampening of the pro-inflammatory cytokine, chemokines and growth factors release. Understanding how complement and immune cells engage with IONPs is likely to be crucial in the future design of nanotherapeutics.

In this thesis, immune responses to NPs were studied in the macrophage-like cell line (THP-1), which has given an insight into how the immune system responds challenges with CNTs and IONPs at the single cell level. However, as the immune system is a complex network of various components and works in constant communication among these, studies in cell lines do not always reflect the actual polarization of the immune response. To overcome this drawback, an ex vivo model can be implemented to study NPsinteraction with the immune system. An ex vivo lepirudin-based human whole blood model, as first described by Mollnes *et al*, was adapted to assess the inflammatory effects of IONPs in a complex biological environment (Mollnes *et al.*, 2002). The ex vivo whole blood model is presently one of the systems to closest mimic an in vivo situation. This model uses lepirudin as an anti-coagulant as it specifically inhibits thrombin without affecting the complement system, unlike heparin. The ex vivo human whole blood model would be a highly suitable model to study the interaction between NPs and the immune system. This system has advantages over the use of monoculture

model, although it lacks the complexity of a whole organism (Joris *et al.*, 2013), importantly, it allows for intercellular communication and crosstalk with the protein cascades in the blood.

Another important factor to be considering in nanomedicine is the overall surface charge and size of the nanoparticle. Adsorption of proteins on the nanoparticle is aided by several forces such as Van der Waals force, hydrogen bonds, charge/polarity interaction, hydrophobic/hydrophilic interaction etc. The overall surface charge of NPs depends upon the combination of NPs charge and adsorbed proteins. The surface charge of NPs heavily affects NP diffusion, sedimentation and aggregation determining the fate of NPs in colloidal solution, a concept is known as pharmacokinetics (Teeguarden *et al.*, 2007). Here, the complement components, properdin, TSR4+5 module and rfhSP-D, may have altered the surface charge and overall size of the NPs, moulding them to behave differently in terms of their uptake and immunomodulation. So, the study of overall surface charge and size is necessary in this case. Zeta potential analysis can be carried out to measure the surface charge of NPs and their protein-coated version in solutions. Moreover, charge modifications of NPs prolongthe half-life of a drug in systemic circulation,enhancing the possibility of their interaction with target cells.

Recent reports demonstrate that rfhSP-D induces apoptosis in prostate cancer cell lines (PC3) and primary prostate cancer cells via mitochondrial intrinsic pathway and P53 mediated apoptosis (Thakur *et al.*, 2017) and in pancreatic cancer cells via Fas-mediated pathway (Kaur *et al.,* 2018). Similarly, the CRD region of rfhSP-D has shown to induce apoptosis in eosinophil leukemic cell line (Mahajan *et al.*, 2013). This indicates that rfhSP-D could potentially be developed as an anti-cancer therapeutic. To study the anti-cancer effect of rfhSP-D-CNTs, rfhSP-D can be linked to CNTs via its collagen region to functionalised CNTs with a linker such as EDC and

BS3 [(Bis (sulfocyccinimidyl) suberate] thus freeing the CRD region to actively participate in recognition of different moieties on cancer cells. Covalently linking rfhSP-D to CNTs would be an interesting idea to analyse whether CNT bound rfhSP-D would have a more inhibitory effect on cancer cells than rfhSP-D alone and could potentially be tested in vivo.

7 References

Abanades Lazaro, I., Haddad, S., Sacca, S., Orellana-Tavra, C., Fairen-Jimenez, D. and Forgan, R. S. (2017) 'Selective Surface PEGylation of UiO-66 Nanoparticles for Enhanced Stability, Cell Uptake, and pH-Responsive Drug Delivery', *Chem,* 2 (4), pp. 561-578.

Abbasi, E., Aval, S. F., Akbarzadeh, A., Milani, M., Nasrabadi, H. T., Joo, S. W., Hanifehpour, Y., Nejati-Koshki, K. and Pashaei-Asl, R. (2014) 'Dendrimers: synthesis, applications, and properties', *Nanoscale Research Letters,* 9 (1), pp. 247-247.

Abdal Dayem, A., Hossain, M. K., Lee, S. B., Kim, K., Saha, S. K., Yang, G. M., Choi, H. Y. and Cho, S. G. (2017) 'The Role of Reactive Oxygen Species (ROS) in the Biological Activities of Metallic Nanoparticles', *International Journal of Molecular Sciences,* 18 (1), pp. 10.3390/ijms18010120.

Abraxane (2017) *Abraxane for injectable suspension (Paclitaxel protein-bound particles for injectable suspension) (albumin-bound).*Available at: http://www.abraxane.com/mbc/abraxane-and-advanced-breast-cancer/ (Accessed: 02/03).

Acharya, S., Dilnawaz, F. and Sahoo, S. K. (2009) 'Targeted epidermal growth factor receptor nanoparticle bioconjugates for breast cancer therapy', *Biomaterials,* 30 (29), pp. 5737-5750.

Aderem, A. and Underhill, D. M. (1999) 'Mechanisms of phagocytosis in macrophages', *Annual Review of Immunology,* 17 pp. 593-623.

Aggarwal, B. B., Shishodia, S., Ashikawa, K. and Bharti, A. C. (2002) 'The role of TNF and its family members in inflammation and cancer: lessons from gene deletion', *Current Drug Targets.Inflammation and Allergy,* 1 (4), pp. 327-341.

Agrawal, V., Smart, K., Jilling, T. and Hirsch, E. (2013) 'Surfactant protein (SP)-A suppresses preterm delivery and inflammation via TLR2', *PloS One,* 8 (5), pp. e63990.

Ahuja, S. K. and Murphy, P. M. (1996) 'The CXC Chemokines Growth-regulated Oncogene (GRO) α, GROβ, GROγ, Neutrophil-activating Peptide-2, and Epithelial Cell-derived Neutrophil-activating Peptide-78 Are Potent Agonists for the Type B, but Not the Type A, Human Interleukin-8 Receptor', *Journal of Biological Chemistry,* 271 (34), pp. 20545-20550.

Ajima, K., Murakami, T., Mizoguchi, Y., Tsuchida, K., Ichihashi, T., Iijima, S. and Yudasaka, M. (2008) 'Enhancement of in vivo anticancer effects of cisplatin by incorporation inside single-wall carbon nanohorns', *ACS Nano,* 2 (10), pp. 2057-2064.

Akbarzadeh, A., Rezaei-Sadabady, R., Davaran, S., Joo, S. W., Zarghami, N., Hanifehpour, Y., Samiei, M., Kouhi, M. and Nejati-Koshki, K. (2013) 'Liposome: classification, preparation, and applications', *Nanoscale Research Letters,* 8 (1), pp. 102-276X-8-102.

Alam, A. and Saxena, R. K.(2017) 'Natural Killer Cells Interaction with Carbon Nanoparticles', in Aribi, M. (ed.) *Natural Killer Cells.* Rijeka: InTech, .

Albanese, A., Tang, P. S. and Chan, W. C. (2012) 'The effect of nanoparticle size, shape, and surface chemistry on biological systems', *Annual Review of Biomedical Engineering,* 14 pp. 1-16.

Alcorlo, M., Tortajada, A., Rodriguez de Cordoba, S. and Llorca, O. (2013) 'Structural basis for the stabilization of the complement alternative pathway C3 convertase by properdin', *Proceedings of the National Academy of Sciences of the United States of America,* 110 (33), pp. 13504-13509.

Alcorn, J. F. and Wright, J. R. (2004) 'Surfactant protein A inhibits alveolar macrophage cytokine production by CD14-independent pathway', *American Journal of Physiology.Lung Cellular and Molecular Physiology,* 286 (1), pp. L129-36.

Alexis, F., Pridgen, E., Molnar, L. K. and Farokhzad, O. C. (2008) 'Factors Affecting the Clearance and Biodistribution of Polymeric Nanoparticles', *Molecular Pharmaceutics,* 5 (4), pp. 505-515.

Allen, B. L., Kotchey, G. P., Chen, Y., Yanamala, N. V., Klein-Seetharaman, J., Kagan, V. E. and Star, A. (2009) 'Mechanistic investigations of horseradish peroxidase-catalyzed degradation of single-walled carbon nanotubes', *Journal of the American Chemical Society,* 131 (47), pp. 17194-17205.

Allen, M. J., Voelker, D. R. and Mason, R. J. (2001) 'Interactions of surfactant proteins A and D with Saccharomyces cerevisiae and Aspergillus fumigatus', *Infection and Immunity,* 69 (4), pp. 2037-2044.

Allen, T. C. and Kurdowska, A. (2014) 'Interleukin 8 and acute lung injury', *Archives of Pathology & Laboratory Medicine,* 138 (2), pp. 266-269.

Al-Rayahi, I. A., Browning, M. J. and Stover, C. (2017) 'Tumour cell conditioned medium reveals greater M2 skewing of macrophages in the absence of properdin', *Immunity, Inflammation and Disease,* 5 (1), pp. 68-77.

Andersson, J., Ekdahl, K. N., Larsson, R., Nilsson, U. R. and Nilsson, B. (2002) 'C3 adsorbed to a polymer surface can form an initiating alternative pathway convertase', *Journal of Immunology (Baltimore, Md.: 1950),* 168 (11), pp. 5786-5791.

Andon, F. T., Kapralov, A. A., Yanamala, N., Feng, W., Baygan, A., Chambers, B. J., Hultenby, K., Ye, F., Toprak, M. S., Brandner, B. D., Fornara, A., Klein-Seetharaman, J., Kotchey, G. P., Star, A., Shvedova, A. A., Fadeel, B. and Kagan, V. E. (2013) 'Biodegradation of single-walled carbon nanotubes by eosinophil peroxidase', *Small (Weinheim an Der Bergstrasse, Germany),* 9 (16), pp. 2721-9, 2720.

Aono, Y., Ledford, J. G., Mukherjee, S., Ogawa, H., Nishioka, Y., Sone, S., Beers, M. F., Noble, P. W. and Wright, J. R. (2012) 'Surfactant protein-D regulates effector cell function and fibrotic lung remodeling in response to bleomycin injury', *American Journal of Respiratory and Critical Care Medicine,* 185 (5), pp. 525-536.

Aoui, C., Prigent, A., Sut, C., Tariket, S., Hamzeh-Cognasse, H., Pozzetto, B., Richard, Y., Cognasse, F., Laradi, S. and Garraud, O. (2014) 'The Signaling Role of CD40 Ligand in Platelet Biology and in Platelet Component Transfusion', *International Journal of Molecular Sciences,* 15 (12), pp. 22342-22364.

Arend, W. P. and Gabay, C. (2000) 'Physiologic role of interleukin-1 receptor antagonist', *Arthritis Research,* 2 (4), pp. 245-248.

Artursson, P., Arro, E., Edman, P., Ericsson, J. L. and Sjoholm, I. (1987) 'Biodegradable microspheres. V: Stimulation of macrophages with microparticles made of various polysaccharides', *Journal of Pharmaceutical Sciences,* 76 (2), pp. 127-133.

Artursson, P., Johansson, D. and Sjoholm, I. (1988) 'Receptor-mediated uptake of starch and mannan microparticles by macrophages: relative contribution of receptors for complement, immunoglobulins and carbohydrates', *Biomaterials,* 9 (3), pp. 241-246.

Athie-Morales, V., Smits, H. H., Cantrell, D. A. and Hilkens, C. M. (2004) 'Sustained IL-12 signaling is required for Th1 development', *Journal of Immunology (Baltimore, Md.: 1950),* 172 (1), pp. 61-69.

Auerbach, M. and Ballard, H. (2010) 'Clinical use of intravenous iron: administration, efficacy, and safety', *Hematology.American Society of Hematology.Education Program,* 2010 pp. 338-347.

Baggiolini, M. and Clark-Lewis, I. (1992) 'Interleukin-8, a chemotactic and inflammatory cytokine', *FEBS Letters,* 307 (1), pp. 97-101.

Balakrishnan, V. S., Rao, M., Kausz, A. T., Brenner, L., Pereira, B. J., Frigo, T. B. and Lewis, J. M. (2009) 'Physicochemical properties of ferumoxytol, a new intravenous iron preparation', *European Journal of Clinical Investigation,* 39 (6), pp. 489-496.

Banda, N. K., Mehta, G., Chao, Y., Wang, G., Inturi, S., Fossati-Jimack, L., Botto, M., Wu, L., Moghimi, S. M. and Simberg, D. (2014) 'Mechanisms of complement activation by dextran-coated superparamagnetic iron oxide (SPIO) nanoworms in mouse versus human serum', *Particle and Fibre Toxicology,* 11 pp. 64-014-0064-2.

Bao, Y., Wen, T., Samia, A. C., Khandhar, A. and Krishnan, K. M. (2016) 'Magnetic Nanoparticles: Material Engineering and Emerging Applications in Lithography and Biomedicine', *Journal of Materials Science,* 51 (1), pp. 513-553.

Bartneck, M., Keul, H. A., Zwadlo-Klarwasser, G. and Groll, J. (2010) 'Phagocytosis independent extracellular nanoparticle clearance by human immune cells', *Nano Letters,* 10 (1), pp. 59-63.

Belling, J. N., Jackman, J. A., Yorulmaz Avsar, S., Park, J. H., Wang, Y., Potroz, M. G., Ferhan, A. R., Weiss, P. S. and Cho, N. J. (2016) 'Stealth Immune Properties of Graphene Oxide Enabled by Surface-Bound Complement Factor H', *ACS Nano,* 10 (11), pp. 10161-10172.

Bendall, L. J. and Bradstock, K. F. (2014) 'G-CSF: From granulopoietic stimulant to bone marrow stem cell mobilizing agent', *Cytokine & Growth Factor Reviews,* 25 (4), pp. 355-367.

Benincasa, M., Pacor, S., Wu, W., Prato, M., Bianco, A. and Gennaro, R. (2011) 'Antifungal activity of amphotericin B conjugated to carbon nanotubes', *ACS Nano,* 5 (1), pp. 199-208.

Bexborn, F., Andersson, P. O., Chen, H., Nilsson, B. and Ekdahl, K. N. (2008) 'The tick-over theory revisited: formation and regulation of the soluble alternative complement C3 convertase (C3(H2O)Bb)', *Molecular Immunology,* 45 (8), pp. 2370-2379.

Bhatia, S. (2016) 'Nanoparticles Types, Classification, Characterization, Fabrication Methods and Drug Delivery Applications', in Bhatia, S. (ed.) *Natural Polymer Drug Delivery Systems: Nanoparticles, Plants, and Algae.* Cham: Springer International Publishing, pp. 33-93.

Bhattacharya, K., Davoren, M., Boertz, J., Schins, R. P., Hoffmann, E. and Dopp, E. (2009) 'Titanium dioxide nanoparticles induce oxidative stress and DNA-adduct formation but not DNA-breakage in human lung cells', *Particle and Fibre Toxicology,* 6 pp. 17-8977-6-17.

Bi, D., Zhou, R., Cai, N., Lai, Q., Han, Q., Peng, Y., Jiang, Z., Tang, Z., Lu, J., Bao, W., Xu, H. and Xu, X. (2017) 'Alginate enhances Toll-like receptor 4-mediated phagocytosis by murine RAW264.7 macrophages', *International Journal of Biological Macromolecules,* 105 (Pt 2), pp. 1446-1454.

Bianco, A., Kostarelos, K. and Prato, M. (2005) 'Applications of carbon nanotubes in drug delivery', *Current Opinion in Chemical Biology,* 9 (6), pp. 674-679.

Bickel, M. (1993) 'The role of interleukin-8 in inflammation and mechanisms of regulation', *Journal of Periodontology,* 64 (5 Suppl), pp. 456â€”460.

Billiau, A. (1996) 'Interferon-gamma: biology and role in pathogenesis', *Advances in Immunology,* 62 pp. 61-130.

Billotey, C., Wilhelm, C., Devaud, M., Bacri, J. C., Bittoun, J. and Gazeau, F. (2003) 'Cell internalization of anionic maghemite nanoparticles: quantitative effect on magnetic resonance imaging', *Magnetic Resonance in Medicine,* 49 (4), pp. 646-654.

Bilsborough, J., George, T. C., Norment, A. and Viney, J. L. (2003) 'Mucosal CD8α+DC, with a plasmacytoid phenotype, induce differentiation and support function of T cells with regulatory properties.',*Immunology,* 108 (4), pp. 481-492.

Bongrazio, M., Pries, A. R. and Zakrzewicz, A. (2003) 'The endothelium as physiological source of properdin: role of wall shear stress', *Molecular Immunology,* 39 (11), pp. 669-675.

Bonner, J. C. (2011) 'Carbon nanotubes as delivery systems for respiratory disease: do the dangers outweigh the potential benefits?',*Expert Review of Respiratory Medicine,* 5 (6), pp. 779-787.

Boraschi, D., Costantino, L. and Italiani, P. (2012) 'Interaction of nanoparticles with immunocompetent cells: nanosafety considerations', *Nanomedicine (London, England),* 7 (1), pp. 121-131.

Borish, L. and Joseph, B. Z. (1992) 'Inflammation and the allergic response', *The Medical Clinics of North America,* 76 (4), pp. 765-787.

Bortolamiol, T., Lukanov, P., Galibert, A., Soula, B., Lonchambon, P., Datas, L. and Flahaut, E. (2014) 'Double-walled carbon nanotubes: Quantitative purification assessment, balance between purification and degradation and solution filling as an evidence of opening', *Carbon,* 78 pp. 79-90.

Botas, C., Poulain, F., Akiyama, J., Brown, C., Allen, L., Goerke, J., Clements, J., Carlson, E., Gillespie, A. M., Epstein, C. and Hawgood, S. (1998) 'Altered surfactant homeostasis and alveolar type II cell morphology in mice lacking surfactant protein D', *Proceedings of the National Academy of Sciences of the United States of America,* 95 (20), pp. 11869-11874.

Bottini, M., Bruckner, S., Nika, K., Bottini, N., Bellucci, S., Magrini, A., Bergamaschi, A. and Mustelin, T. (2006) 'Multi-walled carbon nanotubes induce T lymphocyte apoptosis', *Toxicology Letters,* 160 (2), pp. 121-126.

Bou Khzam, L., Boulahya, R., Abou-Saleh, H., Hachem, A., Zaid, Y. and Merhi, Y. (2013) 'Soluble CD40 Ligand Stimulates the Pro-Angiogenic Function of Peripheral Blood Angiogenic Outgrowth Cells via Increased Release of Matrix Metalloproteinase-9', *PLoS ONE,* 8 (12), pp. e84289. doi:10.1371/journal.pone.0084289.

Bourdiol, F., Mouchet, F., Perrault, A., Fourquaux, I., Datas, L., Gancet, C., Boutonnet, J., Pinelli, E., Gauthier, L. and Flahaut, E. (2013) 'Biocompatible polymer-assisted dispersion of multi walled carbon nanotubes in water, application to the investigation of their ecotoxicity using Xenopus laevis amphibian larvae', *Carbon,* 54 pp. 175-191.

Bourrinet, P., Bengele, H. H., Bonnemain, B., Dencausse, A., Idee, J. M., Jacobs, P. M. and Lewis, J. M. (2006) 'Preclinical safety and pharmacokinetic profile of ferumoxtran-10, an ultrasmall superparamagnetic iron oxide magnetic resonance contrast agent', *Investigative Radiology,* 41 (3), pp. 313-324.

Brandenberger, C., Muhlfeld, C., Ali, Z., Lenz, A. G., Schmid, O., Parak, W. J., Gehr, P. and Rothen-Rutishauser, B. (2010) 'Quantitative evaluation of cellular uptake and trafficking of plain and polyethylene glycol-coated gold nanoparticles', *Small (Weinheim an Der Bergstrasse, Germany),* 6 (15), pp. 1669-1678.

Brewer, E., Coleman, J. and Lowman, A. (2011) 'Emerging Technologies of Polymeric Nanoparticles in Cancer Drug Delivery', *Journal of Nanomaterials,* 2011 .

Bronte, V. and Pittet, M. J. (2013) 'The spleen in local and systemic regulation of immunity', *Immunity,* 39 (5), pp. 806-818.

Brown, D. M., Johnston, H., Gubbins, E. and Stone, V. (2014) 'Serum enhanced cytokine responses of macrophages to silica and iron oxide particles and nanomaterials: a comparison of serum to lung lining fluid and albumin dispersions', *Journal of Applied Toxicology : JAT,* 34 (11), pp. 1177-1187.

Brown, M. A. and Hural, J. (1997) 'Functions of IL-4 and Control of Its Expression', *Critical Reviews™ in Immunology,* 17 (1), pp. 1-32.

Brubaker, S. W., Bonham, K. S., Zanoni, I. and Kagan, J. C. (2015) 'Innate Immune Pattern Recognition: A Cell Biological Perspective', *Annual Review of Immunology,* 33 pp. 257-290.

Buzea, C., Pacheco, I. I. and Robbie, K. (2007) 'Nanomaterials and nanoparticles: Sources and toxicity', *Biointerphases,* 2 (4), pp. MR17-MR71.

Capone, P. M., Papsidero, L. D. and Chu, T. M. (1984) 'Relationship between antigen density and immunotherapeutic response elicited by monoclonal antibodies against solid tumors', *Journal of the National Cancer Institute,* 72 (3), pp. 673-677.

Cardozo, A. K., Proost, P., Gysemans, C., Chen, M. C., Mathieu, C. and Eizirik, D. L. (2003) 'IL-1beta and IFN-gamma induce the expression of diverse chemokines and IL-15 in human and rat pancreatic islet cells, and in islets from pre-diabetic NOD mice', *Diabetologia,* 46 (2), pp. 255-266.

Cassel, S. L., Eisenbarth, S. C., Iyer, S. S., Sadler, J. J., Colegio, O. R., Tephly, L. A., Carter, A. B., Rothman, P. B., Flavell, R. A. and Sutterwala, F. S. (2008) 'The Nalp3 inflammasome is essential for the development of silicosis', *Proceedings of the National Academy of Sciences of the United States of America,* 105 (26), pp. 9035-9040.

Castelli, C., Rivoltini, L., Andreola, G., Carrabba, M., Renkvist, N. and Parmiani, G. (2000) 'T-cell recognition of melanoma-associated antigens', *Journal of Cellular Physiology,* 182 (3), pp. 323-331.

Cha, C., Shin, S. R., Annabi, N., Dokmeci, M. R. and Khademhosseini, A. (2013) 'Carbon-Based Nanomaterials: Multi-Functional Materials for Biomedical Engineering', *ACS Nano,* 7 (4), pp. 2891-2897.

Chen, B. X., Wilson, S. R., Das, M., Coughlin, D. J. and Erlanger, B. F. (1998) 'Antigenicity of fullerenes: antibodies specific for fullerenes and their characteristics', *Proceedings of the National Academy of Sciences of the United States of America,* 95 (18), pp. 10809-10813.

Chen, F., Wang, G., Griffin, J. I., Brenneman, B., Banda, N. K., Holers, V. M., Backos, D. S., Wu, L., Moghimi, S. M. and Simberg, D. (2017) 'Complement proteins bind to nanoparticle protein corona and undergo dynamic exchange in vivo', *Nature Nanotechnology,* 12 (4), pp. 387-393.

Chen, P., Kanehira, K. and Taniguchi, A. (2013) 'Role of toll-like receptors 3, 4 and 7 in cellular uptake and response to titanium dioxide nanoparticles', *Science and Technology of Advanced Materials,* 14 (1), pp. 015008.

Child, H. W., del Pino, P. A., Fuente, D. L., Hursthouse, A. S., Stirling, D., Mullen, M., McPhee, G. M., Nixon, C., Jayawarna, V. and Berry, C. C. (2011) 'Working Together: The Combined Application of a Magnetic Field and Penetratin for the Delivery of Magnetic Nanoparticles to Cells in 3D', *ACS Nano,* 5 (10), pp. 7910-7919.

Childs, R. A., Wright, J. R., Ross, G. F., Yuen, C. T., Lawson, A. M., Chai, W., Drickamer, K. and Feizi, T. (1992) 'Specificity of lung surfactant protein SP-A for both the carbohydrate and the lipid moieties of certain neutral glycolipids', *The Journal of Biological Chemistry,* 267 (14), pp. 9972-9979.

Choudhary, S. and Kusum Devi, V. (2015) 'Potential of nanotechnology as a delivery platform against tuberculosis: current research review', *Journal of Controlled Release : Official Journal of the Controlled Release Society,* 202 pp. 65-75.

Chow, E. K., Zhang, X. Q., Chen, M., Lam, R., Robinson, E., Huang, H., Schaffer, D., Osawa, E., Goga, A. and Ho, D. (2011) 'Nanodiamond therapeutic delivery agents mediate enhanced chemoresistant tumor treatment', *Science Translational Medicine,* 3 (73), pp. 73ra21.

Chroneos, Z. C., Abdolrasulnia, R., Whitsett, J. A., Rice, W. R. and Shepherd, V. L. (1996) 'Purification of a cell-surface receptor for surfactant protein A', *The Journal of Biological Chemistry,* 271 (27), pp. 16375-16383.

Chung, E. Y., Kim, S. J. and Ma, X. J. (2006) 'Regulation of cytokine production during phagocytosis of apoptotic cells', *Cell Research,* 16 (2), pp. 154-161.

Clark, R. and Kupper, T. (2005) 'Old meets new: the interaction between innate and adaptive immunity', *The Journal of Investigative Dermatology,* 125 (4), pp. 629-637.

Clement, C. G., Evans, S. E., Evans, C. M., Hawke, D., Kobayashi, R., Reynolds, P. R., Moghaddam, S. J., Scott, B. L., Melicoff, E., Adachi, R., Dickey, B. F. and Tuvim, M. J. (2008) 'Stimulation of lung innate immunity protects against lethal pneumococcal pneumonia in mice', *American Journal of Respiratory and Critical Care Medicine,* 177 (12), pp. 1322-1330.

Cockshutt, A. M., Weitz, J. and Possmayer, F. (1990) 'Pulmonary surfactant-associated protein A enhances the surface activity of lipid extract surfactant and reverses inhibition by blood proteins in vitro', *Biochemistry,* 29 (36), pp. 8424-8429.

Crouch, E., Persson, A., Chang, D. and Heuser, J. (1994) 'Molecular structure of pulmonary surfactant protein D (SP-D)', *The Journal of Biological Chemistry,* 269 (25), pp. 17311-17319.

Cui, H., Wu, W., Okuhira, K., Miyazawa, K., Hattori, T., Sai, K., Naito, M., Suzuki, K., Nishimura, T., Sakamoto, Y., Ogata, A., Maeno, T., Inomata, A., Nakae, D., Hirose, A. and Nishimaki-Mogami, T. (2014) 'High-temperature calcined fullerene nanowhiskers as well as long needle-like multi-wall carbon nanotubes have abilities to induce NLRP3-mediated IL-1β secretion', *Biochemical and Biophysical Research Communications,* 452 (3), pp. 593-599.

Cui, X., Wan, B., Yang, Y., Ren, X. and Guo, L. H. (2017) 'Length effects on the dynamic process of cellular uptake and exocytosis of single-walled carbon nanotubes in murine macrophage cells', *Scientific Reports,* 7 (1), pp. 1518-017-01746-9.

Cveticanin, J., Joksic, G., Leskovac, A., Petrovic, S., Sobot, A. V. and Neskovic, O. (2010) 'Using carbon nanotubes to induce micronuclei and double strand breaks of the DNA in human cells', *Nanotechnology,* 21 (1), pp. 015102-4484/21/1/015102. Epub 2009 Nov 30.

Dahinden, C. A., Geiser, T., Brunner, S. T., Tscharner, V. V., Caput, S. D., Ferrara, P., Minty, A. and Baggiolini, M. (1994) 'Monocyte chemotactic protein 3 is a most effective basophil- and eosinophil-activating chemokine', *The Journal of Experimental Medicine,* 179 (2), pp. 751-756.

Daldrup-Link, H. E., Golovko, D., Ruffell, B., Denardo, D. G., Castaneda, R., Ansari, C., Rao, J., Tikhomirov, G. A., Wendland, M. F., Corot, C. and Coussens, L. M. (2011) 'MRI of tumor-associated macrophages with clinically applicable iron oxide nanoparticles', *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research,* 17 (17), pp. 5695-5704.

Dame, J. B., Williams, J. L., McCutchan, T. F., Weber, J. L., Wirtz, R. A., Hockmeyer, W. T., Maloy, W. L., Haynes, J. D., Schneider, I. and Roberts, D. (1984) 'Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite Plasmodium falciparum', *Science (New York, N.Y.),* 225 (4662), pp. 593-599.

Danhier, F., Feron, O. and Preat, V. (2010) 'To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery', *Journal of Controlled Release : Official Journal of the Controlled Release Society,* 148 (2), pp. 135-146.

Daniels, T. R., Delgado, T., Helguera, G. and Penichet, M. L. (2006) 'The transferrin receptor part II: targeted delivery of therapeutic agents into cancer cells', *Clinical Immunology (Orlando, Fla.),* 121 (2), pp. 159-176.

de Saint-Vis, B., Fugier-Vivier, I., Massacrier, C., Gaillard, C., Vanbervliet, B., Ait-Yahia, S., Banchereau, J., Liu, Y. J., Lebecque, S. and Caux, C. (1998) 'The cytokine profile expressed by human dendritic cells is dependent on cell subtype and mode of activation', *Journal of Immunology (Baltimore, Md.: 1950),* 160 (4), pp. 1666-1676.

Delogu, L. G., Venturelli, E., Manetti, R., Pinna, G. A., Carru, C., Madeddu, R., Murgia, L., Sgarrella, F., Dumortier, H. and Bianco, A. (2012) 'Ex vivo impact of functionalized carbon nanotubes on human immune cells', *Nanomedicine (London, England),* 7 (2), pp. 231-243.

Deng, Z. J., Liang, M., Monteiro, M., Toth, I. and Minchin, R. F. (2011) 'Nanoparticle-induced unfolding of fibrinogen promotes Mac-1 receptor activation and inflammation', *Nature Nanotechnology,* 6 (1), pp. 39-44.

Deng, Z. J., Mortimer, G., Schiller, T., Musumeci, A., Martin, D. and Minchin, R. F. (2009) 'Differential plasma protein binding to metal oxide nanoparticles', *Nanotechnology,* 20 (45), pp. 455101-4484/20/45/455101.Epub 2009 Oct 13.

Desgrosellier, J. S. and Cheresh, D. A. (2010) 'Integrins in cancer: biological implications and therapeutic opportunities', *Nature Reviews.Cancer,* 10 (1), pp. 9-22.

Deshmane, S. L., Kremlev, S., Amini, S. and Sawaya, B. E. (2009) 'Monocyte chemoattractant protein-1 (MCP-1): an overview', *Journal of Interferon & Cytokine Research : The Official Journal of the International Society for Interferon and Cytokine Research,* 29 (6), pp. 313-326.

Dinarello, C. A. (2009) 'Immunological and inflammatory functions of the interleukin-1 family', *Annual Review of Immunology,* 27 pp. 519-550.

Dinarello, C. A. (1985) 'An update on human interleukin-1: From molecular biology to clinical relevance', *Journal of Clinical Immunology,* 5 (5), pp. 287-297.

Ding, J., Takamoto, D. Y., von Nahmen, A., Lipp, M. M., Lee, K. Y., Waring, A. J. and Zasadzinski, J. A. (2001) 'Effects of lung surfactant proteins, SP-B and SP-C, and palmitic acid on monolayer stability', *Biophysical Journal,* 80 (5), pp. 2262-2272.

DiScipio, R. G., Chakravarti, D. N., Muller-Eberhard, H. J. and Fey, G. H. (1988) 'The structure of human complement component C7 and the C5b-7 complex', *The Journal of Biological Chemistry,* 263 (1), pp. 549-560.

Discipio, R. G. (1982) 'Properdin is a trimer', *Molecular Immunology,* 19 (4), pp. 631-635.

Dixit, S., Novak, T., Miller, K., Zhu, Y., Kenney, M. E. and Broome, A. M. (2015) 'Transferrin receptor-targeted theranostic gold nanoparticles for photosensitizer delivery in brain tumors', *Nanoscale,* 7 (5), pp. 1782-1790.

Dixon, K. O., O'Flynn, J., Klar-Mohamad, N., Daha, M. R. and van Kooten, C. (2017) 'Properdin and factor H production by human dendritic cells modulates their T-cell stimulatory capacity and is regulated by IFN-gamma', *European Journal of Immunology,* 47 (3), pp. 470-480.

Dobbs, L. G., Wright, J. R., Hawgood, S., Gonzalez, R., Venstrom, K. and Nellenbogen, J. (1987) 'Pulmonary surfactant and its components inhibit secretion of phosphatidylcholine from cultured rat alveolar type II cells', *Proceedings of the National Academy of Sciences of the United States of America,* 84 (4), pp. 1010-1014.

Dobrovolskaia, M. A., Aggarwal, P., Hall, J. B. and McNeil, S. E. (2008) 'Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle biodistribution', *Molecular Pharmaceutics,* 5 (4), pp. 487-495.

Dorner, B. G., Scheffold, A., Rolph, M. S., Hüser, M. B., Kaufmann, S. H. E., Radbruch, A., Flesch, I. E. A. and Kroczek, R. A. (2002) 'MIP-1α MIP-1β RANTES, and ATAC/lymphotactin function together with IFN- γ type 1 cytokines', *Proceedings of the National Academy of Sciences,* 99 (9), pp. 6181-6186.

Dostert, C., Petrilli, V., Van Bruggen, R., Steele, C., Mossman, B. T. and Tschopp, J. (2008) 'Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica', *Science (New York, N.Y.),* 320 (5876), pp. 674-677.

Drexler, S. K. and Foxwell, B. M. (2010) 'The role of toll-like receptors in chronic inflammation', *The International Journal of Biochemistry & Cell Biology,* 42 (4), pp. 506-518.

Drysdale, J. W. (1976) 'Ferritin phenotypes: structure and metabolism', *Ciba Foundation Symposium,* (51) (51), pp. 41-67.

Dufour, J. H., Dziejman, M., Liu, M. T., Leung, J. H., Lane, T. E. and Luster, A. D. (2002) 'IFN-γ-Inducible Protein 10 (IP-10; CXCL10)-Deficient Mice Reveal a Role for IP-10 in Effector T Cell Generation and Trafficking', *The Journal of Immunology,* 168 (7), pp. 3195-3204.

Dutta, T. and Jain, N. K. (2007) 'Targeting potential and anti-HIV activity of lamivudine loaded mannosylated poly (propyleneimine) dendrimer', *Biochimica Et Biophysica Acta,* 1770 (4), pp. 681-686.

Easo, S. L. and Mohanan, P. V. (2013) 'Dextran stabilized iron oxide nanoparticles: synthesis, characterization and in vitro studies', *Carbohydrate Polymers,* 92 (1), pp. 726-732.

Ede, J. D., Ortega, V. A., Boyle, D., Felix, L., Stafford, J. L., Martinez-Rubi, Y., Simard, B. and Goss, G. G. (2018) 'Carbon nanotubes diminish IgE-mediated degranulation in the rat basophilic leukemia (RBL)-2H3 cell line', *NanoImpact,* 9 pp. 31.

Ehrlich, P. and Sachs, H. (1902) 'Ueber den Mechanismus der Antiamboceptorwirkung.',*Berliner Klinische Wochenschrift,* 39 (21), pp. 492-558.

Elgrabli, D., Dachraoui, W., Marmier, H., Menard-Moyon, C., Begin, D., Begin-Colin, S., Bianco, A., Alloyeau, D. and Gazeau, F. (2017) 'Intracellular degradation of functionalized carbon nanotube/iron oxide hybrids is modulated by iron via Nrf2 pathway', *Scientific Reports,* 7 pp. 40997.

ElKassar, N. and Gress, R. E. (2010) 'An Overview of IL-7 Biology and Its Use in Immunotherapy', *Journal of Immunotoxicology,* 7 (1), pp. 1-7.

Elsabahy, M. and Wooley, K. L. (2013) 'Cytokines as biomarkers of nanoparticle immunotoxicity', *Chemical Society Reviews,* 42 (12), pp. 5552-5576.

Erdely, A., Liston, A., Salmen-Muniz, R., Hulderman, T., Young, S. H., Zeidler-Erdely, P. C., Castranova, V. and Simeonova, P. P. (2011) 'Identification of systemic markers from a pulmonary carbon nanotube exposure', *Journal of Occupational and Environmental Medicine,* 53 (6 Suppl), pp. S80-6.

Erpenbeck, V. J., Malherbe, D. C., Sommer, S., Schmiedl, A., Steinhilber, W., Ghio, A. J., Krug, N., Wright, J. R. and Hohlfeld, J. M. (2005) 'Surfactant protein D increases phagocytosis and aggregation of pollen-allergen starch granules', *American Journal of Physiology.Lung Cellular and Molecular Physiology,* 288 (4), pp. L692-8.

Erpenbeck, V. J., Ziegert, M., Cavalet-Blanco, D., Martin, C., Baelder, R., Glaab, T., Braun, A., Steinhilber, W., Luettig, B., Uhlig, S., Hoymann, H. G., Krug, N. and Hohlfeld, J. M. (2006) 'Surfactant protein D inhibits early airway response in Aspergillus fumigatus-sensitized mice', *Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology,* 36 (7), pp. 930-940.

Estevanato, L., Cintra, D., Baldini, N., Portilho, F., Barbosa, L., Martins, O., Lacava, B., Miranda-Vilela, A. L., Tedesco, A. C., Bao, S., Morais, P. C. and Lacava, Z. G. (2011) 'Preliminary biocompatibility investigation of magnetic albumin nanosphere designed as a potential versatile drug delivery system', *International Journal of Nanomedicine,* 6 pp. 1709-1717.

Fadeel, B. (2012) 'Clear and present danger? Engineered nanoparticles and the immune system', *Swiss Medical Weekly,* 142 pp. w13609.

Fadel, T. R., Look, M., Staffier, P. A., Haller, G. L., Pfefferle, L. D. and Fahmy, T. M. (2010) 'Clustering of stimuli on single-walled carbon nanotube bundles enhances cellular activation', *Langmuir : The ACS Journal of Surfaces and Colloids,* 26 (8), pp. 5645-5654.

Fadel, T. R., Sharp, F. A., Vudattu, N., Ragheb, R., Garyu, J., Kim, D., Hong, E., Li, N., Haller, G. L., Pfefferle, L. D., Justesen, S., Herold, K. C. and Fahmy, T. M. (2014) 'A carbon nanotube-polymer composite for T-cell therapy', *Nature Nanotechnology,* 9 (8), pp. 639-647.

Fadel, T. R., Steenblock, E. R., Stern, E., Li, N., Wang, X., Haller, G. L., Pfefferle, L. D. and Fahmy, T. M. (2008) 'Enhanced cellular activation with single walled carbon nanotube bundles presenting antibody stimuli', *Nano Letters,* 8 (7), pp. 2070-2076.

Farokhzad, O. C. and Langer, R. (2006) 'Nanomedicine: developing smarter therapeutic and diagnostic modalities', *Advanced Drug Delivery Reviews,* 58 (14), pp. 1456-1459.

Fearon, D. T. and Austen, K. F. (1975) 'Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase', *The Journal of Experimental Medicine,* 142 (4), pp. 856-863.

Fell, L. H., Zawada, A. M., Rogacev, K. S., Seiler, S., Fliser, D. and Heine, G. H. (2014) 'Distinct immunologic effects of different intravenous iron preparations on monocytes', *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association,* 29 (4), pp. 809-822.

Ferguson, J. S., Martin, J. L., Azad, A. K., McCarthy, T. R., Kang, P. B., Voelker, D. R., Crouch, E. C. and Schlesinger, L. S. (2006) 'Surfactant protein D increases fusion of Mycobacterium tuberculosis-containing phagosomes with lysosomes in human macrophages', *Infection and Immunity,* 74 (12), pp. 7005-7009.

Ferguson, J. S., Voelker, D. R., McCormack, F. X. and Schlesinger, L. S. (1999) 'Surfactant protein D binds to Mycobacterium tuberculosis bacilli and lipoarabinomannan via carbohydrate-lectin interactions resulting in reduced phagocytosis of the bacteria by macrophages', *Journal of Immunology (Baltimore, Md.: 1950),* 163 (1), pp. 312-321.

Ferrara, N., Houck, K., Jakeman, L. and Leung, D. W. (1992) 'Molecular and Biological Properties of the Vascular Endothelial Growth Factor Family of Proteins', *Endocrine Reviews,* 13 (1), pp. 18-32.

Ferreira, A. P. A. and Boucrot, E. (2017) 'Mechanisms of Carrier Formation during Clathrin-Independent Endocytosis', *Trends in Cell Biology,* 28 (3), pp. 188-200.

Ferreira, V. P., Cortes, C. and Pangburn, M. K. (2010) 'Native polymeric forms of properdin selectively bind to targets and promote activation of the alternative pathway of complement', *Immunobiology,* 215 (11), pp. 932-940.

Fisher, A. B., Dodia, C. and Chander, A. (1994) 'Inhibition of lung calcium-independent phospholipase A2 by surfactant protein A', *The American Journal of Physiology,* 267 (3 Pt 1), pp. L335-41.

Flahaut, E., Bacsa, R., Peigney, A. and Laurent, C. (2003) 'Gram-scale CCVD synthesis of double-walled carbon nanotubes', *Chemical Communications (Cambridge, England),* (12) (12), pp. 1442-1443.

Fleit, H. B. and Kobasiuk, C. D. (1991) 'The human monocyte-like cell line THP-1 expresses Fc gamma RI and Fc gamma RII', *Journal of Leukocyte Biology,* 49 (6), pp. 556-565.

Flynn, S., Toellner, K. M., Raykundalia, C., Goodall, M. and Lane, P. (1998) 'CD4 T Cell Cytokine Differentiation: The B Cell Activation Molecule, OX40 Ligand, Instructs CD4 T Cells to Express Interleukin 4 and Upregulates Expression of the Chemokine Receptor, Blr-1', *The Journal of Experimental Medicine,* 188 (2), pp. 297-304.

Folkman, J. (1971a) 'Transplacental carcinogenesis by stilbestrol', *The New England Journal of Medicine,* 285 (7), pp. 404-405.

Folkman, J. (1971b) 'Tumor Angiogenesis: Therapeutic Implications', *The New England Journal of Medicine,* 285 (21), pp. 1182-1186.

Fornaguera, C., Caldero, G., Mitjans, M., Vinardell, M. P., Solans, C. and Vauthier, C. (2015) 'Interactions of PLGA nanoparticles with blood components: protein adsorption, coagulation, activation of the complement system and hemolysis studies', *Nanoscale,* 7 (14), pp. 6045-6058.

Fratila, R. M., Mitchell, S. G., del Pino, P., Grazu, V. and de la Fuente, J. M. (2014) 'Strategies for the biofunctionalization of gold and iron oxide nanoparticles', *Langmuir : The ACS Journal of Surfaces and Colloids,* 30 (50), pp. 15057-15071.

Freitas, M. L. L., Silva, L. P., Azevedo, R. B., Garcia, V. A. P., Lacava, L. M., GrisÃ³lia, C. K., Lucci, C. M., Morais, P. C., Silva, M. F. D., Buske, N., Curi, R. and Lacava, Z. G. M. (2002) 'A double-coated magnetite-based magnetic fluid evaluation by cytometry and genetic tests', *Journal of Magnetism and Magnetic Materials,* 252 (Supplement C), pp. 396.

Fu, P. P., Xia, Q., Hwang, H., Ray, P. C. and Yu, H. (2014) *Mechanisms of nanotoxicity: Generation of reactive oxygen species.* Nanomaterials - Toxicology and Medical Applications.

Fujita, T., Matsushita, M. and Endo, Y. (2004) 'The lectin-complement pathway--its role in innate immunity and evolution', *Immunological Reviews,* 198 pp. 185-202.

Gaarkeuken, H., Siezenga, M. A., Zuidwijk, K., van Kooten, C., Rabelink, T. J., Daha, M. R. and Berger, S. P. (2008) 'Complement activation by tubular cells is mediated by properdin binding', *American Journal of Physiology.Renal Physiology,* 295 (5), pp. F1397-403.

Gaffen, S. L. (2008) 'An overview of IL-17 function and signaling', *Cytokine,* 43 (3), pp. 402-407.

Gaiha, G. D., Dong, T., Palaniyar, N., Mitchell, D. A., Reid, K. B. and Clark, H. W. (2008) 'Surfactant protein A binds to HIV and inhibits direct infection of CD4+ cells, but enhances dendritic cell-mediated viral transfer', *Journal of Immunology (Baltimore, Md.: 1950),* 181 (1), pp. 601-609.

Gao, H., Yang, Z., Zhang, S., Cao, S., Shen, S., Pang, Z. and Jiang, X. (2013) 'Ligand modified nanoparticles increases cell uptake, alters endocytosis and elevates glioma distribution and internalization', *Scientific Reports,* 3 pp. 2534.

Gardai, S. J., Xiao, Y. Q., Dickinson, M., Nick, J. A., Voelker, D. R., Greene, K. E. and Henson, P. M. (2003) 'By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation', *Cell,* 115 (1), pp. 13-23.

Gasser, M., Wick, P., Clift, M. J., Blank, F., Diener, L., Yan, B., Gehr, P., Krug, H. F. and Rothen-Rutishauser, B. (2012) 'Pulmonary surfactant coating of multi-walled carbon nanotubes (MWCNTs) influences their oxidative and pro-inflammatory potential in vitro', *Particle and Fibre Toxicology,* 9 pp. 17-8977-9-17.

Geunes-Boyer, S., Oliver, T. N., Janbon, G., Lodge, J. K., Heitman, J., Perfect, J. R. and Wright, J. R. (2009) 'Surfactant protein D increases phagocytosis of hypocapsular Cryptococcus neoformans by murine macrophages and enhances fungal survival', *Infection and Immunity,* 77 (7), pp. 2783-2794.

Ghai, R., Waters, P., Roumenina, L. T., Gadjeva, M., Kojouharova, M. S., Reid, K. B., Sim, R. B. and Kishore, U. (2007) 'C1q and its growing family', *Immunobiology,* 212 (4-5), pp. 253-266.

Ghosh, S. and Karin, M. (2002) 'Missing pieces in the NF-kappaB puzzle', *Cell,* 109 Suppl pp. S81-96.

Gil, M., McCormack, F. X. and Levine, A. M. (2009) 'Surfactant protein A modulates cell surface expression of CR3 on alveolar macrophages and enhances CR3-mediated phagocytosis', *The Journal of Biological Chemistry,* 284 (12), pp. 7495-7504.

Girtsman, T. A., Beamer, C. A., Wu, N., Buford, M. and Holian, A. (2014) 'IL-1R signalling is critical for regulation of multi-walled carbon nanotubes-induced acute lung inflammation in C57Bl/6 mice', *Nanotoxicology,* 8 (1), pp. 17-27.

Gobel, R. J., Janatova, J., Googe, J. M. and Apple, D. J. (1987) 'Activation of complement in human serum by some synthetic polymers used for intraocular lenses', *Biomaterials,* 8 (4), pp. 285-288.

Godiska, R., Chantry, D., Raport, C. J., Sozzani, S., Allavena, P., Leviten, D., Mantovani, A. and Gray, P. W. (1997) 'Human Macrophageâ€“derived Chemokine (MDC), a Novel Chemoattractant for Monocytes, Monocyte-derived Dendritic Cells, and Natural Killer Cells', *The Journal of Experimental Medicine,* 185 (9), pp. 1595-1604.

Goerke, J. (1998) 'Pulmonary surfactant: functions and molecular composition', *Biochimica Et Biophysica Acta,* 1408 (2-3), pp. 79-89.

Gosk, S., Moos, T., Gottstein, C. and Bendas, G. (2008) 'VCAM-1 directed immunoliposomes selectively target tumor vasculature in vivo', *Biochimica Et Biophysica Acta,* 1778 (4), pp. 854-863.

Gosselin, E. J., Wardwell, K., Rigby, W. F. and Guyre, P. M. (1993) 'Induction of MHC class II on human polymorphonuclear neutrophils by granulocyte/macrophage colony-stimulating factor, IFN-gamma, and IL-3', *Journal of Immunology (Baltimore, Md.: 1950),* 151 (3), pp. 1482-1490.

Gou, S. J., Yuan, J., Chen, M., Yu, F. and Zhao, M. H. (2013) 'Circulating complement activation in patients with anti-neutrophil cytoplasmic antibody-associated vasculitis', *Kidney International,* 83 (1), pp. 129-137.

Grant, B. D. and Donaldson, J. G. (2009) 'Pathways and mechanisms of endocytic recycling', *Nature Reviews.Molecular Cell Biology,* 10 (9), pp. 597-608.

Grawunder, U., West, R. B. and Lieber, M. R. (1998) 'Antigen receptor gene rearrangement', *Current Opinion in Immunology,* 10 (2), pp. 172.

Greenfeder, S., Umland, S. P., Cuss, F. M., Chapman, R. W. and Egan, R. W. (2001) 'Th2 cytokines and asthma-The role of interleukin-5 in allergic eosinophilic disease', *Respiratory Research,* 2 (2), pp. 71-79.

Griesser, H. and Mak, T. W. (1994) 'The T-cell receptor--structure, function, and clinical application', *Hematologic Pathology,* 8 (1-2), pp. 1-23.

Groeneveld, T. W., Oroszlan, M., Owens, R. T., Faber-Krol, M. C., Bakker, A. C., Arlaud, G. J., McQuillan, D. J., Kishore, U., Daha, M. R. and Roos, A. (2005) 'Interactions of the extracellular matrix proteoglycans decorin and biglycan with C1q and collectins', *Journal of Immunology (Baltimore, Md.: 1950),* 175 (7), pp. 4715-4723.

Gruen, J. R. and Weissman, S. M. (1997) 'Evolving views of the major histocompatibility complex', *Blood,* 90 (11), pp. 4252-4265.

Guichard, Y., Schmit, J., Darne, C., Gate, L., Goutet, M., Rousset, D., Rastoix, O., Wrobel, R., Witschger, O., Martin, A., Fierro, V. and Binet, S. (2012) 'Cytotoxicity and genotoxicity of nanosized and microsized titanium dioxide and iron oxide particles in Syrian hamster embryo cells', *The Annals of Occupational Hygiene,* 56 (5), pp. 631-644.

Gullotti, E. and Yeo, Y. (2009) 'Extracellularly activated nanocarriers: a new paradigm of tumor targeted drug delivery', *Molecular Pharmaceutics,* 6 (4), pp. 1041-1051.

Guo, C. J., Atochina-Vasserman, E. N., Abramova, E., Foley, J. P., Zaman, A., Crouch, E., Beers, M. F., Savani, R. C. and Gow, A. J. (2008) 'S-nitrosylation of surfactant protein-D controls inflammatory function', *PLoS Biology,* 6 (11), pp. e266.

Gustafson, H. H., Holt-Casper, D., Grainger, D. W. and Ghandehari, H. (2015) 'Nanoparticle Uptake: The Phagocyte Problem', *Nano Today,* 10 (4), pp. 487-510.

Hamad, I., Christy Hunter, A., Rutt, K. J., Liu, Z., Dai, H. and Moein Moghimi, S. (2008) 'Complement activation by PEGylated single-walled carbon nanotubes is independent of C1q and alternative pathway turnover', *Molecular Immunology,* 45 (14), pp. 3797-3803.

Haniu, H., Saito, N., Matsuda, Y., Tsukahara, T., Maruyama, K., Usui, Y., Aoki, K., Takanashi, S., Kobayashi, S., Nomura, H., Okamoto, M., Shimizu, M. and Kato, H. (2013) 'Culture medium type affects endocytosis of multi-walled carbon nanotubes in BEAS-2B cells and subsequent biological response', *Toxicology in Vitro : An International Journal Published in Association with BIBRA,* 27 (6), pp. 1679-1685.

Happonen, K. E., Saxne, T., Aspberg, A., Morgelin, M., Heinegard, D. and Blom, A. M. (2010) 'Regulation of complement by cartilage oligomeric matrix protein allows for a novel molecular diagnostic principle in rheumatoid arthritis', *Arthritis and Rheumatism,* 62 (12), pp. 3574-3583.

Harada, S., Kimura, T., Fujiki, H., Nakagawa, H., Ueda, Y., Itoh, T., Yamagishi, H. and Sonoda, Y. (2007) 'Flt3 ligand promotes myeloid dendritic cell differentiation of human hematopoietic progenitor cells: possible application for cancer immunotherapy', *International Journal of Oncology,* 30 (6), pp. 1461-1468.

Harboe, M., Garred, P., Lindstad, J. K., Pharo, A., Muller, F., Stahl, G. L., Lambris, J. D. and Mollnes, T. E. (2012) 'The role of properdin in zymosan- and Escherichia coli-induced complement activation', *Journal of Immunology (Baltimore, Md.: 1950),* 189 (5), pp. 2606-2613.

Harris, L., Batist, G., Belt, R., Rovira, D., Navari, R., Azarnia, N., Welles, L., Winer, E. and TLC D-99 Study Group (2002) 'Liposome-encapsulated doxorubicin compared with conventional doxorubicin in a randomized multicenter trial as first-line therapy of metastatic breast carcinoma', *Cancer,* 94 (1), pp. 25-36.

Hartshorn, K. L., Crouch, E. C., White, M. R., Eggleton, P., Tauber, A. I., Chang, D. and Sastry, K. (1994) 'Evidence for a protective role of pulmonary surfactant protein D (SP-D) against influenza A viruses', *The Journal of Clinical Investigation,* 94 (1), pp. 311-319.

Hartshorn, K. L., Reid, K. B., White, M. R., Jensenius, J. C., Morris, S. M., Tauber, A. I. and Crouch, E. (1996) 'Neutrophil deactivation by influenza A viruses: mechanisms of protection after viral opsonization with collectins and hemagglutination-inhibiting antibodies', *Blood,* 87 (8), pp. 3450-3461.

Hartshorn, K. L., White, M. R., Shepherd, V., Reid, K., Jensenius, J. C. and Crouch, E. C. (1997) 'Mechanisms of anti-influenza activity of surfactant proteins A and D: comparison with serum collectins', *The American Journal of Physiology,* 273 (6 Pt 1), pp. L1156-66.

Hartshorn, K. L., White, M. R., Smith, K., Sorensen, G., Kuroki, Y., Holmskov, U., Head, J. and Crouch, E. C. (2010) 'Increasing antiviral activity of surfactant protein d trimers by introducing residues from bovine serum collectins: dissociation of mannan-binding and antiviral activity', *Scandinavian Journal of Immunology,* 72 (1), pp. 22-30.

Hartshorn, K. L., White, M. R., Tecle, T., Holmskov, U. and Crouch, E. C. (2006) 'Innate Defense against Influenza A Virus: Activity of Human Neutrophil Defensins and Interactions of Defensins with Surfactant Protein D', *The Journal of Immunology,* 176 (11), pp. 6962-6972.

Hatakeyama, H., Akita, H., Ishida, E., Hashimoto, K., Kobayashi, H., Aoki, T., Yasuda, J., Obata, K., Kikuchi, H., Ishida, T., Kiwada, H. and Harashima, H. (2007) 'Tumor targeting of doxorubicin by anti-MT1-MMP antibody-modified PEG liposomes', *International Journal of Pharmaceutics,* 342 (1-2), pp. 194-200.

He, X., Young, S. H., Schwegler-Berry, D., Chisholm, W. P., Fernback, J. E. and Ma, Q. (2011) 'Multiwalled carbon nanotubes induce a fibrogenic response by stimulating reactive oxygen species production, activating NF-kappaB signaling, and promoting fibroblast-to-myofibroblast transformation', *Chemical Research in Toxicology,* 24 (12), pp. 2237-2248.

Helmy, K. Y., Katschke, K. J.,Jr, Gorgani, N. N., Kljavin, N. M., Elliott, J. M., Diehl, L., Scales, S. J., Ghilardi, N. and van Lookeren Campagne, M. (2006) 'CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens', *Cell,* 124 (5), pp. 915-927.

Hershey, A. E., Kurzman, I. D., Forrest, L. J., Bohling, C. A., Stonerook, M., Placke, M. E., Imondi, A. R. and Vail, D. M. (1999) 'Inhalation chemotherapy for macroscopic primary or metastatic lung tumors: proof of principle using dogs with spontaneously occurring tumors as a model', *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research,* 5 (9), pp. 2653-2659.

Hershey, G. K. (2003) 'IL-13 receptors and signaling pathways: an evolving web', *The Journal of Allergy and Clinical Immunology,* 111 (4), pp. 677-90; quiz 691.

Hickman-Davis, J., Gibbs-Erwin, J., Lindsey, J. R. and Matalon, S. (1999) 'Surfactant protein A mediates mycoplasmacidal activity of alveolar macrophages by production of peroxynitrite', *Proceedings of the National Academy of Sciences of the United States of America,* 96 (9), pp. 4953-4958.

Higgins, J. M., Wiedemann, H., Timpl, R. and Reid, K. B. (1995) 'Characterization of mutant forms of recombinant human properdin lacking single thrombospondin type I repeats. Identification of modules important for function', *Journal of Immunology (Baltimore, Md.: 1950),* 155 (12), pp. 5777-5785.

Hogan, A. D. and Schwartz, L. B. (1997) 'Markers of mast cell degranulation', *Methods (San Diego, Calif.),* 13 (1), pp. 43-52.

Holmskov, U., Lawson, P., Teisner, B., Tornoe, I., Willis, A. C., Morgan, C., Koch, C. and Reid, K. B. (1997) 'Isolation and characterization of a new member of the scavenger receptor superfamily, glycoprotein-340 (gp-340), as a lung surfactant protein-D binding molecule', *The Journal of Biological Chemistry,* 272 (21), pp. 13743-13749.

Holt, G. D., Pangburn, M. K. and Ginsburg, V. (1990) 'Properdin binds to sulfatide [Gal(3-SO4)beta 1-1 Cer] and has a sequence homology with other proteins that bind sulfated glycoconjugates', *The Journal of Biological Chemistry,* 265 (5), pp. 2852-2855.

Hong, S. C., Lee, J. H., Lee, J., Kim, H. Y., Park, J. Y., Cho, J., Lee, J. and Han, D. W. (2011) 'Subtle cytotoxicity and genotoxicity differences in superparamagnetic iron oxide nanoparticles coated with various functional groups', *International Journal of Nanomedicine,* 6 pp. 3219-3231.

Hou, J., Wan, B., Yang, Y., Ren, X. M., Guo, L. H. and Liu, J. F. (2016) 'Biodegradation of Single-Walled Carbon Nanotubes in Macrophages through Respiratory Burst Modulation', *International Journal of Molecular Sciences,* 17 (3), pp. 409.

Hu, F., Ding, G., Zhang, Z., Gatto, L. A., Hawgood, S., Poulain, F. R., Cooney, R. N. and Wang, G. (2016) 'Innate immunity of surfactant proteins A and D in urinary tract infection with uropathogenic Escherichia coli', *Innate Immunity,* 22 (1), pp. 9-20.

Hu, X., Cook, S., Wang, P., Hwang, H., Liu, X. and Williams, Q. L. (2010) 'In vitro evaluation of cytotoxicity of engineered carbon nanotubes in selected human cell lines', *Science of the Total Environment,* 408 (8), pp. 1812.

Huang, D. M., Hsiao, J. K., Chen, Y. C., Chien, L. Y., Yao, M., Chen, Y. K., Ko, B. S., Hsu, S. C., Tai, L. A., Cheng, H. Y., Wang, S. W., Yang, C. S. and Chen, Y. C. (2009) 'The promotion of human mesenchymal stem cell proliferation by superparamagnetic iron oxide nanoparticles', *Biomaterials,* 30 (22), pp. 3645-3651.

Huang, W., Zhang, J., Dorn, H. C. and Zhang, C. (2013) 'Assembly of bio-nanoparticles for double controlled drug release', *PloS One,* 8 (9), pp. e74679.

Iangcharoen, P., Punfa, W., Yodkeeree, S., Kasinrerk, W., Ampasavate, C., Anuchapreeda, S. and Limtrakul, P. (2011) 'Anti-P-glycoprotein conjugated nanoparticles for targeting drug delivery in cancer treatment', *Archives of Pharmacal Research,* 34 (10), pp. 1679-1689.

Ichihara, M., Shimizu, T., Imoto, A., Hashiguchi, Y., Uehara, Y., Ishida, T. and Kiwada, H. (2010) 'Anti-PEG IgM Response against PEGylated Liposomes in Mice and Rats', *Pharmaceutics,* 3 (1), pp. 1-11.

Iijima, S. (1991) 'Helical microtubules of graphitic carbon', *Nature,* 354 pp. 56-58.

Iijima, S. and Ichihashi, T. (1993) 'Single-shell carbon nanotubes of 1-nm diameter', *Nature,* 363 pp. 603.

INEX. (2004) 'Vincristine liposomal--INEX: lipid-encapsulated vincristine, onco TCS, transmembrane carrier system--vincristine, vincacine, vincristine sulfate liposomes for injection, VSLI', *Drugs in R&D,* 5 (2), pp. 119-123.

Ishida, T., Wang, X., Shimizu, T., Nawata, K. and Kiwada, H. (2007) 'PEGylated liposomes elicit an anti-PEG IgM response in a T cell-independent manner', *Journal of Controlled Release,* 122 (3), pp. 349-355.

Iyer, A. K., Khaled, G., Fang, J. and Maeda, H. (2006) 'Exploiting the enhanced permeability and retention effect for tumor targeting', *Drug Discovery Today,* 11 (17-18), pp. 812-818.

Iyer, S. S. and Cheng, G. (2012) 'Role of Interleukin 10 Transcriptional Regulation in Inflammation and Autoimmune Disease', *Critical Reviews in Immunology,* 32 (1), pp. 23-63.

Jafar, A., Alshatti, Y. and Ahmad, A. (2016) 'Carbon nanotube toxicity: The smallest biggest debate in medical care', *Cogent Medicine,* 3 (1), pp. 1217970.

Jakel, A., Clark, H., Reid, K. B. and Sim, R. B. (2010) 'Surface-bound myeloperoxidase is a ligand for recognition of late apoptotic neutrophils by human lung surfactant proteins A and D', *Protein & Cell,* 1 (6), pp. 563-572.

Jakel, A., Qaseem, A. S., Kishore, U. and Sim, R. B. (2013) 'Ligands and receptors of lung surfactant proteins SP-A and SP-D', *Frontiers in Bioscience (Landmark Edition),* 18 pp. 1129-1140.

Jakel, A., Reid, K. B. and Clark, H. (2010) 'Surfactant protein A (SP-A) binds to phosphatidylserine and competes with annexin V binding on late apoptotic cells', *Protein & Cell,* 1 (2), pp. 188-197.

Janeway, C. A.,Jr and Medzhitov, R. (2002) 'Innate immune recognition', *Annual Review of Immunology,* 20 pp. 197-216.

Janssen (2015) *About DOXIL.DOXIL.com.*Available at: https://www.doxil.com/ovarian-cancer/about-doxil .

Jaurand, M. C., Renier, A. and Daubriac, J. (2009) 'Mesothelioma: Do asbestos and carbon nanotubes pose the same health risk?',*Particle and Fibre Toxicology,* 6 pp. 16-8977-6-16.

Jeon, S. I., Lee, J. H., Andrade, J. D. and De Gennes, P. G. (1991) 'Proteinâ€"surface interactions in the presence of polyethylene oxide: I. Simplified theory', *Journal of Colloid and Interface Science,* 142 (1), pp. 149-158.

Jin, R., Lin, B., Li, D. and Ai, H. (2014) 'Superparamagnetic iron oxide nanoparticles for MR imaging and therapy: design considerations and clinical applications', *Current Opinion in Pharmacology,* 18 pp. 18-27.

Joris, F., Manshian, B. B., Peynshaert, K., De Smedt, S. C., Braeckmans, K. and Soenen, S. J. (2013) 'Assessing nanoparticle toxicity in cell-based assays: influence of cell culture parameters and optimized models for bridging the in vitro-in vivo gap', *Chemical Society Reviews,* 42 (21), pp. 8339-8359.

Juzenas, P., Chen, W., Sun, Y. P., Coelho, M. A., Generalov, R., Generalova, N. and Christensen, I. L. (2008) 'Quantum dots and nanoparticles for photodynamic and radiation therapies of cancer', *Advanced Drug Delivery Reviews,* 60 (15), pp. 1600-1614.

Kagan, V. E., Konduru, N. V., Feng, W., Allen, B. L., Conroy, J., Volkov, Y., Vlasova, I. I., Belikova, N. A., Yanamala, N., Kapralov, A., Tyurina, Y. Y., Shi, J., Kisin, E. R., Murray, A. R., Franks, J., Stolz, D., Gou, P., Klein-Seetharaman, J., Fadeel, B., Star, A. and Shvedova, A. A. (2010) 'Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation', *Nature Nanotechnology,* 5 (5), pp. 354-359.

Kambayashi, T. and Laufer, T. M. (2014) 'Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell?',*Nature Reviews.Immunology,* 14 (11), pp. 719-730.

Kamijo, R., Shapiro, D., Gerecitano, J., Le, J., Bosland, M. and Vilcek, J. (1994) 'Biological functions of IFN-gamma and IFN-alpha/beta: lessons from studies in gene

knockout mice', *[Hokkaido Igaku Zasshi] the Hokkaido Journal of Medical Science,* 69 (6), pp. 1332-1338.

Kang, B., Yu, D. C., Chang, S. Q., Chen, D., Dai, Y. D. and Ding, Y. (2008) 'Intracellular uptake, trafficking and subcellular distribution of folate conjugated single walled carbon nanotubes within living cells', *Nanotechnology,* 19 (37), pp. 375103-4484/19/37/375103. Epub 2008 Aug 1.

Kang, X., Li, N., Wang, M., Boontheung, P., Sioutas, C., Harkema, J. R., Bramble, L. A., Nel, A. E. and Loo, J. A. (2010) 'Adjuvant effects of ambient particulate matter monitored by proteomics of bronchoalveolar lavage fluid', *Proteomics,* 10 (3), pp. 520-531.

Kapralov, A. A., Feng, W. H., Amoscato, A. A., Yanamala, N., Balasubramanian, K., Winnica, D. E., Kisin, E. R., Kotchey, G. P., Gou, P., Sparvero, L. J., Ray, P., Mallampalli, R. K., Klein-Seetharaman, J., Fadeel, B., Star, A., Shvedova, A. A. and Kagan, V. E. (2012) 'Adsorption of surfactant lipids by single-walled carbon nanotubes in mouse lung upon pharyngeal aspiration', *ACS Nano,* 6 (5), pp. 4147-4156.

Karlsson, H. L., Gustafsson, J., Cronholm, P. and Moller, L. (2009) 'Size-dependent toxicity of metal oxide particles--a comparison between nano- and micrometer size', *Toxicology Letters,* 188 (2), pp. 112-118.

Kato, T., Totsuka, Y., Ishino, K., Matsumoto, Y., Tada, Y., Nakae, D., Goto, S., Masuda, S., Ogo, S., Kawanishi, M., Yagi, T., Matsuda, T., Watanabe, M. and Wakabayashi, K. (2013) 'Genotoxicity of multi-walled carbon nanotubes in both in vitro and in vivo assay systems', *Nanotoxicology,* 7 (4), pp. 452-461.

Kaur, A., Riaz, M.S., Murugaish, V., Varghese, P.M., Singh, S.K.and Kishore, U.(2018) 'A recombinant fragment of Human Surfactant Protein D induces apoptosis in pancreatic cancer cell lines via Fas-mediated pathway', *Frontiers in immunology*, in press

Kemper, C., Mitchell, L. M., Zhang, L. and Hourcade, D. E. (2008) 'The complement protein properdin binds apoptotic T cells and promotes complement activation and

phagocytosis', *Proceedings of the National Academy of Sciences of the United States of America,* 105 (26), pp. 9023-9028.

Kendall, M., Brown, L. and Trought, K. (2004) 'Molecular adsorption at particle surfaces: a PM toxicity mediation mechanism', *Inhalation Toxicology,* 16 Suppl 1 pp. 99-105.

Kendall, M., Ding, P. and Kendall, K. (2011) 'Particle and nanoparticle interactions with fibrinogen: the importance of aggregation in nanotoxicology', *Nanotoxicology,* 5 (1), pp. 55-65.

Kendall, M., Ding, P., Mackay, R. M., Deb, R., McKenzie, Z., Kendall, K., Madsen, J. and Clark, H. (2013) 'Surfactant protein D (SP-D) alters cellular uptake of particles and nanoparticles', *Nanotoxicology,* 7 (5), pp. 963-973.

Kendall, M. and Holgate, S. (2012) 'Health impact and toxicological effects of nanomaterials in the lung', *Respirology (Carlton, Vic.),* 17 (5), pp. 743-758.

Ketko, A. K., Lin, C., Moore, B. B. and LeVine, A. M. (2013) 'Surfactant protein A binds flagellin enhancing phagocytosis and IL-1beta production', *PloS One,* 8 (12), pp. e82680.

Kettler, K., Veltman, K., van de Meent, D., van Wezel, A. and Hendriks, A. J. (2014) 'Cellular uptake of nanoparticles as determined by particle properties, experimental conditions, and cell type', *Environmental Toxicology and Chemistry,* 33 (3), pp. 481-492.

Khamri, W., Moran, A. P., Worku, M. L., Karim, Q. N., Walker, M. M., Annuk, H., Ferris, J. A., Appelmelk, B. J., Eggleton, P., Reid, K. B. and Thursz, M. R. (2005) 'Variations in Helicobacter pylori lipopolysaccharide to evade the innate immune component surfactant protein D', *Infection and Immunity,* 73 (11), pp. 7677-7686.

Khang, D. (2015) 'Real time macrophage migration analysis and associated pro-inflammatory cytokine release on transparent carbon nanotube/polymer composite nano-film', *Nanotechnology,* 26 (32), pp. 325101-4484/26/32/325101.Epub 2015 Jul 23.

Kimura, Y., Miwa, T., Zhou, L. and Song, W. C. (2008) 'Activator-specific requirement of properdin in the initiation and amplification of the alternative pathway complement', *Blood,* 111 (2), pp. 732-740.

Kinaret, P., Ilves, M., Fortino, V., Rydman, E., Karisola, P., Lahde, A., Koivisto, J., Jokiniemi, J., Wolff, H., Savolainen, K., Greco, D. and 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 (1), pp. 291-303.

King, R. J., Simon, D. and Horowitz, P. M. (1989) 'Aspects of secondary and quaternary structure of surfactant protein A from canine lung', *Biochimica Et Biophysica Acta (BBA) - Lipids and Lipid Metabolism,* 1001 (3), pp. 294-301.

Kirpotin, D. B., Drummond, D. C., Shao, Y., Shalaby, M. R., Hong, K., Nielsen, U. B., Marks, J. D., Benz, C. C. and Park, J. W. (2006) 'Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models', *Cancer Research,* 66 (13), pp. 6732-6740.

Kishore, U., Bernal, A. L., Kamran, M. F., Saxena, S., Singh, M., Sarma, P. U., Madan, T. and Chakraborty, T. (2005) 'Surfactant proteins SP-A and SP-D in human health and disease', *Archivum Immunologiae Et Therapiae Experimentalis,* 53 (5), pp. 399-417.

Kishore, U., Ghai, R., Greenhough, T. J., Shrive, A. K., Bonifati, D. M., Gadjeva, M. G., Waters, P., Kojouharova, M. S., Chakraborty, T. and Agrawal, A. (2004) 'Structural and functional anatomy of the globular domain of complement protein C1q', *Immunol Lett,* 95 (2), pp. 113-128.

Kishore, U., Greenhough, T. J., Waters, P., Shrive, A. K., Ghai, R., Kamran, M. F., Bernal, A. L., Reid, K. B., Madan, T. and Chakraborty, T. (2006) 'Surfactant proteins SP-A and SP-D: structure, function and receptors', *Molecular Immunology,* 43 (9), pp. 1293-1315.

Kishore, U., Gupta, S. K., Perdikoulis, M. V., Kojouharova, M. S., Urban, B. C. and Reid, K. B. (2003) 'Modular organization of the carboxyl-terminal, globular head region

of human C1q A, B, and C chains', *Journal of Immunology (Baltimore, Md.: 1950),* 171 (2), pp. 812-820.

Kishore, U., Madan, T., Sarma, P. U., Singh, M., Urban, B. C. and Reid, K. B. (2002) 'Protective roles of pulmonary surfactant proteins, SP-A and SP-D, against lung allergy and infection caused by Aspergillus fumigatus', *Immunobiology,* 205 (4-5), pp. 610-618.

Kishore, U. and Reid, K. B. (2000) 'C1q: structure, function, and receptors', *Immunopharmacology,* 49 (1-2), pp. 159-170.

Kishore, U., Wang, J. Y., Hoppe, H. J. and Reid, K. B. (1996) 'The alpha-helical neck region of human lung surfactant protein D is essential for the binding of the carbohydrate recognition domains to lipopolysaccharides and phospholipids', *Biochemical Journal,* 318 pp. 505-511.

Kiss-Toth Dojcsak, E., Ferenczi, Z., Szalai Juhasz, A., Kiss-Toth, E., Racz, O. and Fodor, B. (2014) 'Flow cytometric analysis of the basophil cell activating impact of potential drug delivery nanoparticle-candidate', *Acta Microbiologica Et Immunologica Hungarica,* 61 (1), pp. 49-60.

Konczol, M., Ebeling, S., Goldenberg, E., Treude, F., Gminski, R., Giere, R., Grobety, B., Rothen-Rutishauser, B., Merfort, I. and Mersch-Sundermann, V. (2011) 'Cytotoxicity and genotoxicity of size-fractionated iron oxide (magnetite) in A549 human lung epithelial cells: role of ROS, JNK, and NF-kappaB', *Chemical Research in Toxicology,* 24 (9), pp. 1460-1475.

Konduru, N. V., Tyurina, Y. Y., Feng, W., Basova, L. V., Belikova, N. A., Bayir, H., Clark, K., Rubin, M., Stolz, D., Vallhov, H., Scheynius, A., Witasp, E., Fadeel, B., Kichambare, P. D., Star, A., Kisin, E. R., Murray, A. R., Shvedova, A. A. and Kagan, V. E. (2009) 'Phosphatidylserine targets single-walled carbon nanotubes to professional phagocytes in vitro and in vivo', *PloS One,* 4 (2), pp. e4398.

Korfhagen, T. R., Sheftelyevich, V., Burhans, M. S., Bruno, M. D., Ross, G. F., Wert, S. E., Stahlman, M. T., Jobe, A. H., Ikegami, M., Whitsett, J. A. and Fisher, J. H. (1998) 'Surfactant protein-D regulates surfactant phospholipid homeostasis in vivo', *The Journal of Biological Chemistry,* 273 (43), pp. 28438-28443.

Kostevsek, N., Locatelli, E., Garrovo, C., Arena, F., Monaco, I., Nikolov, I. P., Sturm, S., Zuzek Rozman, K., Lorusso, V., Giustetto, P., Bardini, P., Biffi, S. and Comes Franchini, M. (2016) 'The one-step synthesis and surface functionalization of dumbbell-like gold-iron oxide nanoparticles: a chitosan-based nanotheranostic system', *Chemical Communications (Cambridge, England),* 52 (2), pp. 378-381.

Kouser, L., Abdul-Aziz, M., Tsolaki, A. G., Singhal, D., Schwaeble, W. J., Urban, B. C., Khan, H. A., Sim, R. B. and Kishore, U. (2016) 'A recombinant two-module form of human properdin is an inhibitor of the complement alternative pathway', *Molecular Immunology,* 73 pp. 76-87.

Kouser, L., Paudyal, B., Kaur, A., Stenbeck, G., Jones, L. A., Abozaid, S. M., Stover, C. M., Flahaut, E., Sim, R. B. and Kishore, U. (2018) 'Human Properdin Opsonizes Nanoparticles and Triggers a Potent Pro-inflammatory Response by Macrophages without Involving Complement Activation', *Frontiers in Immunology,* 9 pp. 131.

Krätschmer, W., Lamb, L. D., Fostiropoulos, K. and Huffman, D. R. (1990) 'Solid C60: a new form of carbon', *Nature,* 347 pp. 354.

Kroto, H. W., Heath, J. R., O'Brien, S. C., Curl, R. F. and Smalley, R. E. (1985) 'C60: Buckminsterfullerene', *Nature,* 318 pp. 162.

Kuan, S. F., Rust, K. and Crouch, E. (1992) 'Interactions of surfactant protein D with bacterial lipopolysaccharides. Surfactant protein D is an Escherichia coli-binding protein in bronchoalveolar lavage', *The Journal of Clinical Investigation,* 90 (1), pp. 97-106.

Kurbatov, A. V., Mayewski, P. A., Steffensen, J. P., West, A., Kennett, D. J., Kennett, J. P., Bunch, T. E., Handley, M., Introne, D. S., Que Hee, S. S., Mercer, C., Sellers, M., Shen, F., Sneed, S. B., Weaver, J. C., Wittke, J. H., Stafford, T. W., Donovan, J. J., Xie, S., Razink, J. J., Stich, A., Kinzie, C. R. and Wolbach, W. S. (2010) 'Discovery of a nanodiamond-rich layer in the Greenland ice sheet', *Journal of Glaciology,* 56 (199), pp. 747-757.

Kuroki, Y. and Akino, T. (1991) 'Pulmonary surfactant protein A (SP-A) specifically binds dipalmitoylphosphatidylcholine', *The Journal of Biological Chemistry,* 266 (5), pp. 3068-3073.

Kuroki, Y., Gasa, S., Ogasawara, Y., Makita, A. and Akino, T. (1992) 'Binding of pulmonary surfactant protein A to galactosylceramide and asialo-GM2', *Archives of Biochemistry and Biophysics,* 299 (2), pp. 261-267.

Kwon, G., Suwa, S., Yokoyama, M., Okano, T., Sakurai, Y. and Kataoka, K. (1994) 'Enhanced tumor accumulation and prolonged circulation times of micelle-forming poly (ethylene oxide-aspartate) block copolymer-adriamycin conjugates', *Journal of Controlled Release,* 29 (1), pp. 17-23.

La, S. B., Kataoka, K., Okano, T. and Sakurai, Y. (1996) *Development of Polymeric Micelles for Drug Delivery of Indomethacin.* Tokyo: Springer Japan.

Laemmli, U. K. (1970) 'Cleavage of structural proteins during the assembly of the head of bacteriophage T4', *Nature,* 227 (5259), pp. 680-685.

Lak, A., Dieckhoff, J., Ludwig, F., Scholtyssek, J. M., Goldmann, O., Lunsdorf, H., Eberbeck, D., Kornowski, A., Kraken, M., Litterst, F. J., Fiege, K., Mischnick, P. and Schilling, M. (2013) 'Highly stable monodisperse PEGylated iron oxide nanoparticle aqueous suspensions: a nontoxic tracer for homogeneous magnetic bioassays', *Nanoscale,* 5 (23), pp. 11447-11455.

Lam, C. W., James, J. T., McCluskey, R. and Hunter, R. L. (2004) 'Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation', *Toxicological Sciences : An Official Journal of the Society of Toxicology,* 77 (1), pp. 126-134.

Landry, M., Pinault, M., Tchankouo, S., Charon, E., Ridoux, A., Boczkowski, J., Mayne-L'Hermite, M. and Lanone, S. (2016) 'Early signs of multi-walled carbon nanotbues degradation in macrophages, via an intracellular pH-dependent biological mechanism; importance of length and functionalization', *Particle and Fibre Toxicology,* 13 (1), pp. 61-016-0175-z.

Lanzavecchia, A. (1985) 'Antigen-specific interaction between T and B cells', *Nature,* 314 (6011), pp. 537-539.

Lawler, J. and Hynes, R. O. (1986) 'The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins', *The Journal of Cell Biology,* 103 (5), pp. 1635-1648.

Lee, C. C., Gillies, E. R., Fox, M. E., Guillaudeu, S. J., Frechet, J. M., Dy, E. E. and Szoka, F. C. (2006) 'A single dose of doxorubicin-functionalized bow-tie dendrimer cures mice bearing C-26 colon carcinomas', *Proceedings of the National Academy of Sciences of the United States of America,* 103 (45), pp. 16649-16654.

Lee, C. G., Link, H., Baluk, P., Homer, R. J., Chapoval, S., Bhandari, V., Kang, M. J., Cohn, L., Kim, Y. K., McDonald, D. M. and Elias, J. A. (2004) 'Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung', *Nature Medicine,* 10 (10), pp. 1095-1103.

Lee, D. C., Romero, R., Kim, C. J., Chaiworapongsa, T., Tarca, A. L., Lee, J., Suh, Y. L., Mazaki-Tovi, S., Vaisbuch, E., Mittal, P., Draghici, S., Erez, O., Kusanovic, J. P., Hassan, S. S. and Kim, J. S. (2010) 'Surfactant protein-A as an anti-inflammatory component in the amnion: implications for human pregnancy', *Journal of Immunology (Baltimore, Md.: 1950),* 184 (11), pp. 6479-6491.

Lee, J. H., Jang, J. T., Choi, J. S., Moon, S. H., Noh, S. H., Kim, J. W., Kim, J. G., Kim, I. S., Park, K. I. and Cheon, J. (2011) 'Exchange-coupled magnetic nanoparticles for efficient heat induction', *Nature Nanotechnology,* 6 (7), pp. 418-422.

Lee, J. S., Choi, Y. C., Shin, J. H., Lee, J. H., Lee, Y., Park, S. Y., Baek, J. E., Park, J. D., Ahn, K. and Yu, I. J. (2015) 'Health surveillance study of workers who manufacture multi-walled carbon nanotubes', *Nanotoxicology,* 9 (6), pp. 802-811.

Lee, S., Hwang, S. H., Jeong, J., Han, Y., Kim, S. H., Lee, D. K., Lee, H. S., Chung, S. T., Jeong, J., Roh, C., Huh, Y. S. and Cho, W. S. (2016) 'Nickel oxide nanoparticles can recruit eosinophils in the lungs of rats by the direct release of intracellular eotaxin', *Particle and Fibre Toxicology,* 13 (1), pp. 30-016-0142-8.

Lekkala, M., LeVine, A. M., Linke, M. J., Crouch, E. C., Linders, B., Brummer, E. and Stevens, D. A. (2006) 'Effect of lung surfactant collectins on bronchoalveolar macrophage interaction with Blastomyces dermatitidis: inhibition of tumor necrosis factor alpha production by surfactant protein D', *Infection and Immunity,* 74 (8), pp. 4549-4556.

Lemos, M. P., McKinney, J. and Rhee, K. Y. (2011) 'Dispensability of surfactant proteins A and D in immune control of Mycobacterium tuberculosis infection following aerosol challenge of mice', *Infection and Immunity,* 79 (3), pp. 1077-1085.

Lesniak, A., Fenaroli, F., Monopoli, M. P., Ãberg, C., Dawson, K. A. and Salvati, A. (2012) 'Effects of the Presence or Absence of a Protein Corona on Silica Nanoparticle Uptake and Impact on Cells', *ACS Nano,* 6 (7), pp. 5845-5857.

Li, H., Tan, X. Q., Yan, L., Zeng, B., Meng, J., Xu, H. Y. and Cao, J. M. (2017a) 'Multi-walled carbon nanotubes act as a chemokine and recruit macrophages by activating the PLC/IP3/CRAC channel signaling pathway', *Scientific Reports,* 7 (1), pp. 226-017-00386-3.

Li, J. G., Li, W. X., Xu, J. Y., Cai, X. Q., Liu, R. L., Li, Y. J., Zhao, Q. F. and Li, Q. N. (2007) 'Comparative study of pathological lesions induced by multiwalled carbon nanotubes in lungs of mice by intratracheal instillation and inhalation', *Environmental Toxicology,* 22 (4), pp. 415-421.

Li, W. Q., Wang, Z., Hao, S., He, H., Wan, Y., Zhu, C., Sun, L. P., Cheng, G. and Zheng, S. Y. (2017b) 'Mitochondria-Targeting Polydopamine Nanoparticles To Deliver Doxorubicin for Overcoming Drug Resistance', *ACS Applied Materials & Interfaces,* 9 (20), pp. 16793-16802.

Liang, X., Wang, Z. J. and Liu, C. J. (2009) 'Size-controlled synthesis of colloidal gold nanoparticles at room temperature under the influence of glow discharge', *Nanoscale Research Letters,* 5 (1), pp. 124-129.

Liberman, A., Mendez, N., Trogler, W. C. and Kummel, A. C. (2014) 'Synthesis and surface functionalization of silica nanoparticles for nanomedicine', *Surface Science Reports,* 69 (2-3), pp. 132-158.

Lin, M. M., Kim, D. K., El Haj, A. J. and Dobson, J. (2008) 'Development of superparamagnetic iron oxide nanoparticles (SPIONS) for translation to clinical applications', *IEEE Transactions on Nanobioscience,* 7 (4), pp. 298-305.

Lin, P. M. and Wright, J. R. (2006) 'Surfactant protein A binds to IgG and enhances phagocytosis of IgG-opsonized erythrocytes

', *Am J Physiol Lung Cell Mol Physiol,* 291 pp. L1199-206.

Lindemann, A. and Mertelsmann, R. (1993) 'Interleukin-3: Structure and Function', *Cancer Investigation,* 11 (5), pp. 609-623.

Ling, W. L., Biro, A., Bally, I., Tacnet, P., Deniaud, A., Doris, E., Frachet, P., Schoehn, G., Pebay-Peyroula, E. and Arlaud, G. J. (2011) 'Proteins of the innate immune system crystallize on carbon nanotubes but are not activated', *ACS Nano,* 5 (2), pp. 730-737.

Liu, D., Wang, L., Wang, Z. and Cuschieri, A. (2012) 'Different cellular response mechanisms contribute to the length-dependent cytotoxicity of multi-walled carbon nanotubes', *Nanoscale Research Letters,* 7 (1), pp. 361-276X-7-361.

Liu, J., Yu, M., Zhou, C., Yang, S., Ning, X. and Zheng, J. (2013) 'Passive tumor targeting of renal-clearable luminescent gold nanoparticles: long tumor retention and fast normal tissue clearance', *Journal of the American Chemical Society,* 135 (13), pp. 4978-4981.

Liu, Y., Jiao, F., Qiu, Y., Li, W., Lao, F., Zhou, G., Sun, B., Xing, G., Dong, J., Zhao, Y., Chai, Z. and Chen, C. (2009) 'The effect of Gd@C82(OH)22 nanoparticles on the release of Th1/Th2 cytokines and induction of TNF-alpha mediated cellular immunity', *Biomaterials,* 30 (23-24), pp. 3934-3945.

Liu, Z., Chen, K., Davis, C., Sherlock, S., Cao, Q., Chen, X. and Dai, H. (2008) 'Drug delivery with carbon nanotubes for in vivo cancer treatment', *Cancer Research,* 68 (16), pp. 6652-6660.

Liu, Z., Shi, Q., Liu, J., Abdel-Razek, O., Xu, Y., Cooney, R. N. and Wang, G. (2015) 'Innate Immune Molecule Surfactant Protein D Attenuates Sepsis-induced Acute

Pancreatic Injury through Modulating Apoptosis and NF-ÎºB-mediated Inflammation', 5 pp. 17798.

Lohr, J., Knoechel, B., Jiang, S., Sharpe, A. H. and Abbas, A. K. (2003) 'The inhibitory function of B7 costimulators in T cell responses to foreign and self-antigens', *Nature Immunology,* 4 (7), pp. 664-669.

Luanpitpong, S., Wang, L., Castranova, V., Dinu, C. Z., Issaragrisil, S., Chen, Y. C. and Rojanasakul, Y. (2016) 'Induction of cancer-associated fibroblast-like cells by carbon nanotubes dictates its tumorigenicity', *Scientific Reports,* 6 pp. 39558.

Luanpitpong, S., Wang, L., Castranova, V. and Rojanasakul, Y. (2014a) 'Induction of stem-like cells with malignant properties by chronic exposure of human lung epithelial cells to single-walled carbon nanotubes', *Particle and Fibre Toxicology,* 11 pp. 22-8977-11-22.

Luanpitpong, S., Wang, L., Manke, A., Martin, K. H., Ammer, A. G., Castranova, V., Yang, Y. and Rojansakul, Y. (2014b) 'Induction of stemlike cells with fibrogenic properties by carbon nanotubes and its role in fibrogenesis', *Nano Letters,* 14 (6), pp. 3110-3116.

Luanpitpong, S., Wang, L., Stueckle, T. A., Tse, W., Chen, Y. C. and Rojanasakul, Y. (2014c) 'Caveolin-1 regulates lung cancer stem-like cell induction and p53 inactivation in carbon nanotube-driven tumorigenesis', *Oncotarget,* 5 (11), pp. 3541-3554.

Lundqvist, M., Stigler, J., Elia, G., Lynch, I., Cedervall, T. and Dawson, K. A. (2008) 'Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts', *Proceedings of the National Academy of Sciences of the United States of America,* 105 (38), pp. 14265-14270.

Luo, Y. H., Chang, L. W. and Lin, P. (2015) 'Metal-Based Nanoparticles and the Immune System: Activation, Inflammation, and Potential Applications', *BioMed Research International,* 2015 pp. 143720.

Ma, P., Luo, Q., Chen, J., Gan, Y., Du, J., Ding, S., Xi, Z. and Yang, X. (2012) 'Intraperitoneal injection of magnetic Fe(3)O(4)-nanoparticle induces hepatic and renal

tissue injury via oxidative stress in mice', *International Journal of Nanomedicine,* 7 pp. 4809-4818.

Ma, W., Smith, T., Bogin, V., Zhang, Y., Ozkan, C., Ozkan, M., Hayden, M., Schroter, S., Carrier, E., Messmer, D., Kumar, V. and Minev, B. (2011) 'Enhanced presentation of MHC class Ia, Ib and class II-restricted peptides encapsulated in biodegradable nanoparticles: a promising strategy for tumor immunotherapy', *Journal of Translational Medicine,* 9 pp. 34-5876-9-34.

Ma, X., Yan, W., Zheng, H., Du, Q., Zhang, L., Ban, Y., Li, N. and Wei, F. (2015) 'Regulation of IL-10 and IL-12 production and function in macrophages and dendritic cells', *F1000Research,* 4 pp. 10.12688/f1000research.7010.1.

Madan, T., Eggleton, P., Kishore, U., Strong, P., Aggrawal, S. S., Sarma, P. U. and Reid, K. B. (1997a) 'Binding of pulmonary surfactant proteins A and D to Aspergillus fumigatus conidia enhances phagocytosis and killing by human neutrophils and alveolar macrophages', *Infection and Immunity,* 65 (8), pp. 3171-3179.

Madan, T., Kishore, U., Shah, A., Eggleton, P., Strong, P., Wang, J. Y., Aggrawal, S. S., Sarma, P. U. and Reid, K. B. (1997b) 'Lung surfactant proteins A and D can inhibit specific IgE binding to the allergens of Aspergillus fumigatus and block allergen-induced histamine release from human basophils', *Clinical and Experimental Immunology,* 110 (2), pp. 241-249.

Madan, T., Kishore, U., Singh, M., Strong, P., Clark, H., Hussain, E. M., Reid, K. B. and Sarma, P. U. (2001) 'Surfactant proteins A and D protect mice against pulmonary hypersensitivity induced by Aspergillus fumigatus antigens and allergens', *The Journal of Clinical Investigation,* 107 (4), pp. 467-475.

Madan, T., Reid, K. B., Singh, M., Sarma, P. U. and Kishore, U. (2005) 'Susceptibility of mice genetically deficient in the surfactant protein (SP)-A or SP-D gene to pulmonary hypersensitivity induced by antigens and allergens of Aspergillus fumigatus', *Journal of Immunology (Baltimore, Md.: 1950),* 174 (11), pp. 6943-6954.

Madani, S. Y., Mandel, A. and Seifalian, A. M. (2013) 'A concise review of carbon nanotube's toxicology', *Nano Reviews,* 4 pp. 10.3402/nano.v4i0.21521.

Madsen, J., Gaiha, G. D., Palaniyar, N., Dong, T., Mitchell, D. A. and Clark, H. W. (2013) 'Surfactant Protein D modulates HIV infection of both T-cells and dendritic cells', *PloS One,* 8 (3), pp. e59047.

Maeda, H., Bharate, G. Y. and Daruwalla, J. (2009) 'Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect', *European Journal of Pharmaceutics and Biopharmaceutics : Official Journal of Arbeitsgemeinschaft Fur Pharmazeutische Verfahrenstechnik E.V,* 71 (3), pp. 409-419.

Maeda, H. and Matsumura, Y. (1989) 'Tumoritropic and lymphotropic principles of macromolecular drugs', *Critical Reviews in Therapeutic Drug Carrier Systems,* 6 (3), pp. 193-210.

Mahajan, L., Madan, T., Kamal, N., Singh, V. K., Sim, R. B., Telang, S. D., Ramchand, C. N., Waters, P., Kishore, U. and Sarma, P. U. (2008) 'Recombinant surfactant protein-D selectively increases apoptosis in eosinophils of allergic asthmatics and enhances uptake of apoptotic eosinophils by macrophages', *International Immunology,* 20 (8), pp. 993-1007.

Mahajan, L., Pandit, H., Madan, T., Gautam, P., Yadav, A. K., Warke, H., Sundaram, C. S., Sirdeshmukh, R., Sarma, P. U., Kishore, U. and Surolia, A. (2013) 'Human surfactant protein D alters oxidative stress and HMGA1 expression to induce p53 apoptotic pathway in eosinophil leukemic cell line', *PloS One,* 8 (12), pp. e85046.

Mahmoudi, M., Laurent, S., Shokrgozar, M. A. and Hosseinkhani, M. (2011) 'Toxicity evaluations of superparamagnetic iron oxide nanoparticles: cell "vision" versus physicochemical properties of nanoparticles', *ACS Nano,* 5 (9), pp. 7263-7276.

Maiorano, G., Sabella, S., Sorce, B., Brunetti, V., Malvindi, M. A., Cingolani, R. and Pompa, P. P. (2010) 'Effects of cell culture media on the dynamic formation of protein-nanoparticle complexes and influence on the cellular response', *ACS Nano,* 4 (12), pp. 7481-7491.

Malek, T. R. (2003) 'The main function of IL-2 is to promote the development of T regulatory cells', *Journal of Leukocyte Biology,* 74 (6), pp. 961-965.

Malhotra, R., Haurum, J., Thiel, S., Jensenius, J. C. and Sim, R. B. (1993) 'Pollen grains bind to lung alveolar type II cells (A549) via lung surfactant protein A (SP-A)', *Bioscience Reports,* 13 (2), pp. 79-90.

Mano, S. S., Kanehira, K. and Taniguchi, A. (2013) 'Comparison of cellular uptake and inflammatory response via toll-like receptor 4 to lipopolysaccharide and titanium dioxide nanoparticles', *International Journal of Molecular Sciences,* 14 (7), pp. 13154-13170.

Markiewski, M. M., DeAngelis, R. A., Benencia, F., Ricklin-Lichtsteiner, S. K., Koutoulaki, A., Gerard, C., Coukos, G. and Lambris, J. D. (2008) 'Modulation of the antitumor immune response by complement', *Nature Immunology,* 9 (11), pp. 1225-1235.

Maruyama, K., Haniu, H., Saito, N., Matsuda, Y., Tsukahara, T., Kobayashi, S., Tanaka, M., Aoki, K., Takanashi, S., Okamoto, M. and Kato, H. (2015) 'Endocytosis of Multiwalled Carbon Nanotubes in Bronchial Epithelial and Mesothelial Cells', *BioMed Research International,* 2015 pp. 793186.

Matsumura, Y. and Maeda, H. (1986) 'A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs', *Cancer Research,* 46 (12 Pt 1), pp. 6387-6392.

Matthews, A. N., Friend, D. S., Zimmermann, N., Sarafi, M. N., Luster, A. D., Pearlman, E., Wert, S. E. and Rothenberg, M. E. (1998) 'Eotaxin is required for the baseline level of tissue eosinophils', *Proceedings of the National Academy of Sciences,* 95 (11), pp. 6273-6278.

Matzinger, P. (1994) 'Tolerance, danger, and the extended family', *Annual Review of Immunology,* 12 pp. 991-1045.

McCormack, F. X., Gibbons, R., Ward, S. R., Kuzmenko, A., Wu, H. and Deepe, G. S.,Jr (2003) 'Macrophage-independent fungicidal action of the pulmonary collectins', *The Journal of Biological Chemistry,* 278 (38), pp. 36250-36256.

McDevitt, M. R., Chattopadhyay, D., Kappel, B. J., Jaggi, J. S., Schiffman, S. R., Antczak, C., Njardarson, J. T., Brentjens, R. and Scheinberg, D. A. (2007) 'Tumor targeting with antibody-functionalized, radiolabeled carbon nanotubes', *Journal of Nuclear Medicine : Official Publication, Society of Nuclear Medicine,* 48 (7), pp. 1180-1189.

McKenzie, Z., Kendall, M., Mackay, R. M., Tetley, T. D., Morgan, C., Griffiths, M., Clark, H. W. and Madsen, J. (2015a) 'Nanoparticles modulate surfactant protein A and D mediated protection against influenza A infection in vitro', *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences,* 370 (1661), pp. 20140049.

McKenzie, Z., Kendall, M., Mackay, R. M., Whitwell, H., Elgy, C., Ding, P., Mahajan, S., Morgan, C., Griffiths, M., Clark, H. and Madsen, J. (2015b) 'Surfactant protein A (SP-A) inhibits agglomeration and macrophage uptake of toxic amine modified nanoparticles', *Nanotoxicology,* 9 (8), pp. 952-962.

McMahon, H. T. and Boucrot, E. (2011) 'Molecular mechanism and physiological functions of clathrin-mediated endocytosis', *Nature Reviews.Molecular Cell Biology,* 12 (8), pp. 517-533.

McNeely, T. B. and Coonrod, J. D. (1994) 'Aggregation and opsonization of type A but not type B Hemophilus influenzae by surfactant protein A', *American Journal of Respiratory Cell and Molecular Biology,* 11 (1), pp. 114-122.

Medof, M. E., Iida, K., Mold, C. and Nussenzweig, V. (1982) 'Unique role of the complement receptor CR1 in the degradation of C3b associated with immune complexes', *The Journal of Experimental Medicine,* 156 (6), pp. 1739-1754.

Meng, J., Li, X., Wang, C., Guo, H., Liu, J. and Xu, H. (2015) 'Carbon Nanotubes Activate Macrophages into a M1/M2 Mixed Status: Recruiting Naïve macrophages and Supporting Angiogenesis', *ACS Applied Materials \& Interfaces,* 7 (5), pp. 3180-3188.

Mercer, R. R., Hubbs, A. F., Scabilloni, J. F., Wang, L., Battelli, L. A., Friend, S., Castranova, V. and Porter, D. W. (2011) 'Pulmonary fibrotic response to aspiration of multi-walled carbon nanotubes', *Particle and Fibre Toxicology,* 8 pp. 21-8977-8-21.

Merle, N. S., Church, S. E., Fremeaux-Bacchi, V. and Roumenina, L. T. (2015) 'Complement System Part I - Molecular Mechanisms of Activation and Regulation', *Frontiers in Immunology,* 6 pp. 262.

Merrifield, R. C., Wang, Z. W., Palmer, R. E. and Lead, J. R. (2013) 'Synthesis and characterization of polyvinylpyrrolidone coated cerium oxide nanoparticles', *Environmental Science & Technology,* 47 (21), pp. 12426-12433.

Meunier, E., Coste, A., Olagnier, D., Authier, H., Lefevre, L., Dardenne, C., Bernad, J., Beraud, M., Flahaut, E. and Pipy, B. (2012) 'Double-walled carbon nanotubes trigger IL-1beta release in human monocytes through Nlrp3 inflammasome activation', *Nanomedicine : Nanotechnology, Biology, and Medicine,* 8 (6), pp. 987-995.

Mitchell, L. A., Gao, J., Wal, R. V., Gigliotti, A., Burchiel, S. W. and McDonald, J. D. (2007) 'Pulmonary and systemic immune response to inhaled multiwalled carbon nanotubes', *Toxicological Sciences : An Official Journal of the Society of Toxicology,* 100 (1), pp. 203-214.

Mogensen, T. H. (2009) 'Pathogen recognition and inflammatory signaling in innate immune defenses', *Clinical Microbiology Reviews,* 22 (2), pp. 240-73, Table of Contents.

Moghimi, S. M. and Farhangrazi, Z. S. (2014) 'Just so stories: the random acts of anti-cancer nanomedicine performance', *Nanomedicine : Nanotechnology, Biology, and Medicine,* 10 (8), pp. 1661-1666.

Moghimi, S. M. and Szebeni, J. (2003) 'Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties', *Progress in Lipid Research,* 42 (6), pp. 463-478.

Moller, P., Jacobsen, N. R., Folkmann, J. K., Danielsen, P. H., Mikkelsen, L., Hemmingsen, J. G., Vesterdal, L. K., Forchhammer, L., Wallin, H. and Loft, S. (2010) 'Role of oxidative damage in toxicity of particulates', *Free Radical Research,* 44 (1), pp. 1-46.

Mollnes, T. E., Brekke, O. L., Fung, M., Fure, H., Christiansen, D., Bergseth, G., Videm, V., Lappegard, K. T., Kohl, J. and Lambris, J. D. (2002) 'Essential role of the C5a receptor in E coli-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation', *Blood,* 100 (5), pp. 1869-1877.

Monopoli, M. P., Walczyk, D., Campbell, A., Elia, G., Lynch, I., Bombelli, F. B. and Dawson, K. A. (2011) 'Physical-chemical aspects of protein corona: relevance to in vitro and in vivo biological impacts of nanoparticles', *Journal of the American Chemical Society,* 133 (8), pp. 2525-2534.

Monteiller, C., Tran, L., MacNee, W., Faux, S., Jones, A., Miller, B. and Donaldson, K. (2007) 'The pro-inflammatory effects of low-toxicity low-solubility particles, nanoparticles and fine particles, on epithelial cells in vitro: the role of surface area', *Occupational and Environmental Medicine,* 64 (9), pp. 609-615.

Moore, L. K., Chow, E. K., Osawa, E., Bishop, J. M. and Ho, D. (2013) 'Diamond-lipid hybrids enhance chemotherapeutic tolerance and mediate tumor regression', *Advanced Materials (Deerfield Beach, Fla.),* 25 (26), pp. 3532-3541.

Mu, Q., Broughton, D. L. and Yan, B. (2009) 'Endosomal leakage and nuclear translocation of multiwalled carbon nanotubes: developing a model for cell uptake', *Nano Letters,* 9 (12), pp. 4370-4375.

Mukherjee, S. P., Bondarenko, O., Kohonen, P., Andon, F. T., Brzicova, T., Gessner, I., Mathur, S., Bottini, M., Calligari, P., Stella, L., Kisin, E., Shvedova, A., Autio, R., Salminen-Mankonen, H., Lahesmaa, R. and Fadeel, B. (2018a) 'Macrophage sensing of single-walled carbon nanotubes via Toll-like receptors', *Scientific Reports,* 8 (1), pp. 1115-018-19521-9.

Mukherjee, S. P., Bottini, M. and Fadeel, B. (2017) 'Graphene and the Immune System: A Romance of Many Dimensions', *Frontiers in Immunology,* 8 pp. 673.

Mukherjee, S. P., Gliga, A. R., Lazzaretto, B., Brandner, B., Fielden, M., Vogt, C., Newman, L., Rodrigues, A. F., Shao, W., Fournier, P. M., Toprak, M. S., Star, A., Kostarelos, K., Bhattacharya, K. and Fadeel, B. (2018b) 'Graphene oxide is degraded by

neutrophils and the degradation products are non-genotoxic', *Nanoscale,* 10 (3), pp. 1180-1188.

Muralidharan, R., Babu, A., Amreddy, N., Basalingappa, K., Mehta, M., Chen, A., Zhao, Y. D., Kompella, U. B., Munshi, A. and Ramesh, R. (2016) 'Folate receptor-targeted nanoparticle delivery of HuR-RNAi suppresses lung cancer cell proliferation and migration', *Journal of Nanobiotechnology,* 14 (1), pp. 47-016-0201-1.

Murphy, F. A., Schinwald, A., Poland, C. A. and Donaldson, K. (2012) 'The mechanism of pleural inflammation by long carbon nanotubes: interaction of long fibres with macrophages stimulates them to amplify pro-inflammatory responses in mesothelial cells', *Particle and Fibre Toxicology,* 9 pp. 8-8977-9-8.

Nadesalingam, J., Bernal, A. L., Dodds, A. W., Willis, A. C., Mahoney, D. J., Day, A. J., Reid, K. B. and Palaniyar, N. (2003) 'Identification and characterization of a novel interaction between pulmonary surfactant protein D and decorin', *The Journal of Biological Chemistry,* 278 (28), pp. 25678-25687.

Nair, A. V., Keliher, E. J., Core, A. B., Brown, D. and Weissleder, R. (2015) 'Characterizing the interactions of organic nanoparticles with renal epithelial cells in vivo', *ACS Nano,* 9 (4), pp. 3641-3653.

Naqvi, S., Samim, M., Abdin, M., Ahmed, F. J., Maitra, A., Prashant, C. and Dinda, A. K. (2010) 'Concentration-dependent toxicity of iron oxide nanoparticles mediated by increased oxidative stress', *International Journal of Nanomedicine,* 5 pp. 983-989.

Naya, M., Kobayashi, N., Endoh, S., Maru, J., Honda, K., Ema, M., Tanaka, J., Fukumuro, M., Hasegawa, K., Nakajima, M., Hayashi, M. and Nakanishi, J. (2012) 'In vivo genotoxicity study of single-wall carbon nanotubes using comet assay following intratracheal instillation in rats', *Regulatory Toxicology and Pharmacology : RTP,* 64 (1), pp. 124-129.

Nayak, A., Dodagatta-Marri, E., Tsolaki, A. G. and Kishore, U. (2012) 'An Insight into the Diverse Roles of Surfactant Proteins, SP-A and SP-D in Innate and Adaptive Immunity', *Frontiers in Immunology,* 3 pp. 131.

Nazarenus, M., Zhang, Q., Soliman, M. G., Del Pino, P., Pelaz, B., Carregal-Romero, S., Rejman, J., Rothen-Rutishauser, B., Clift, M. J., Zellner, R., Nienhaus, G. U., Delehanty, J. B., Medintz, I. L. and Parak, W. J. (2014) 'In vitro interaction of colloidal nanoparticles with mammalian cells: What have we learned thus far?',*Beilstein Journal of Nanotechnology,* 5 pp. 1477-1490.

Nie, X., Nishitani, C., Yamazoe, M., Ariki, S., Takahashi, M., Shimizu, T., Mitsuzawa, H., Sawada, K., Smith, K., Crouch, E., Nagae, H., Takahashi, H. and Kuroki, Y. (2008) 'Pulmonary surfactant protein D binds MD-2 through the carbohydrate recognition domain', *Biochemistry,* 47 (48), pp. 12878-12885.

Nielsen, C. H., Matthiesen, S. H., Lyng, I. and Leslie, R. G. (1997) 'The role of complement receptor type 1 (CR1, CD35) in determining the cellular distribution of opsonized immune complexes between whole blood cells: kinetic analysis of the buffering capacity of erythrocytes', *Immunology,* 90 (1), pp. 129-137.

Nilsson, U. R., Storm, K. E., Elwing, H. and Nilsson, B. (1993) 'Conformational epitopes of C3 reflecting its mode of binding to an artificial polymer surface', *Molecular Immunology,* 30 (3), pp. 211-219.

Nolan, K. F., Kaluz, S., Higgins, J. M., Goundis, D. and Reid, K. B. (1992) 'Characterization of the human properdin gene', *The Biochemical Journal,* 287 (Pt 1) (Pt 1), pp. 291-297.

Norwitz, E. R., Lopez Bernal, A. and Starkey, P. M. (1992) 'Tumor necrosis factor-alpha selectively stimulates prostaglandin F2 alpha production by macrophages in human term decidua', *American Journal of Obstetrics and Gynecology,* 167 (3), pp. 815-820.

Novoselov, K. S., Geim, A. K., Morozov, S. V., Jiang, D., Zhang, Y., Dubonos, S. V., Grigorieva, I. V. and Firsov, A. A. (2004) 'Electric field effect in atomically thin carbon films', *Science (New York, N.Y.),* 306 (5696), pp. 666-669.

Oberley, R. E., Goss, K. L., Ault, K. A., Crouch, E. C. and Snyder, J. M. (2004) 'Surfactant protein D is present in the human female reproductive tract and inhibits

Chlamydia trachomatis infection', *Molecular Human Reproduction,* 10 (12), pp. 861-870.

Ogasawara, Y., Kuroki, Y. and Akino, T. (1992) 'Pulmonary surfactant protein D specifically binds to phosphatidylinositol', *The Journal of Biological Chemistry,* 267 (29), pp. 21244-21249.

Ogasawara, Y., McCormack, F. X., Mason, R. J. and Voelker, D. R. (1994) 'Chimeras of surfactant proteins A and D identify the carbohydrate recognition domains as essential for phospholipid interaction', *The Journal of Biological Chemistry,* 269 (47), pp. 29785-29792.

Oosting, R. S. and Wright, J. R. (1994) 'Characterization of the surfactant protein A receptor: cell and ligand specificity', *The American Journal of Physiology,* 267 (2 Pt 1), pp. L165-72.

Osman, O., Zanini, L. F., Frenea-Robin, M., Dumas-Bouchiat, F., Dempsey, N. M., Reyne, G., Buret, F. and Haddour, N. (2012) 'Monitoring the endocytosis of magnetic nanoparticles by cells using permanent micro-flux sources', *Biomedical Microdevices,* 14 (5), pp. 947-954.

Osugi, Y., Hara, J., Tagawa, S., Takai, K., Hosoi, G., Matsuda, Y., Ohta, H., Fujisaki, H., Kobayashi, M., Sakata, N., Kawa-Ha, K., Okada, S. and Tawa, A. (1997) 'Cytokine production regulating Th1 and Th2 cytokines in hemophagocytic lymphohistiocytosis', *Blood,* 89 (11), pp. 4100-4103.

Owlasiuk, P., Zajkowska, J. M., Pietruczuk, M., Pancewicz, S. A. and Hermanowska-Szpakowicz, T. (2009) 'Fractalkine--structure, functions and biological activity', *Polski Merkuriusz Lekarski : Organ Polskiego Towarzystwa Lekarskiego,* 26 (153), pp. 253-257.

Pacurari, M., Yin, X. J., Zhao, J., Ding, M., Leonard, S. S., Schwegler-Berry, D., Ducatman, B. S., Sbarra, D., Hoover, M. D., Castranova, V. and Vallyathan, V. (2008) 'Raw single-wall carbon nanotubes induce oxidative stress and activate MAPKs, AP-1, NF-kappaB, and Akt in normal and malignant human mesothelial cells', *Environmental Health Perspectives,* 116 (9), pp. 1211-1217.

Pai, A. B., Conner, T., McQuade, C. R., Olp, J. and Hicks, P. (2011) 'Non-transferrin bound iron, cytokine activation and intracellular reactive oxygen species generation in hemodialysis patients receiving intravenous iron dextran or iron sucrose', *Biometals : An International Journal on the Role of Metal Ions in Biology, Biochemistry, and Medicine,* 24 (4), pp. 603-613.

Palaniyar, N., Clark, H., Nadesalingam, J., Hawgood, S. and Reid, K. B. (2003) 'Surfactant protein D binds genomic DNA and apoptotic cells, and enhances their clearance, in vivo', *Annals of the New York Academy of Sciences,* 1010 pp. 471-475.

Palaniyar, N., Nadesalingam, J., Clark, H., Shih, M. J., Dodds, A. W. and Reid, K. B. (2004) 'Nucleic acid is a novel ligand for innate, immune pattern recognition collectins surfactant proteins A and D and mannose-binding lectin', *The Journal of Biological Chemistry,* 279 (31), pp. 32728-32736.

Pandit, H., Thakur, G., Koippallil Gopalakrishnan, A. R., Dodagatta-Marri, E., Patil, A., Kishore, U. and Madan, T. (2016) 'Surfactant protein D induces immune quiescence and apoptosis of mitogen-activated peripheral blood mononuclear cells', *Immunobiology,* 221 (2), pp. 310-322.

Papayannopoulos, V. and Zychlinsky, A. (2009) 'NETs: a new strategy for using old weapons', *Trends in Immunology,* 30 (11), pp. 513-521.

Parton, R. G. and del Pozo, M. A. (2013) 'Caveolae as plasma membrane sensors, protectors and organizers', *Nature Reviews.Molecular Cell Biology,* 14 (2), pp. 98-112.

Passalacqua, G., Mincarini, M., Colombo, D., Troisi, G., Ferrari, M., Bagnasco, D., Balbi, F., Riccio, A. and Canonica, G. W. (2017) 'IL-13 and idiopathic pulmonary fibrosis: Possible links and new therapeutic strategies', *Pulmonary Pharmacology & Therapeutics,* 45 pp. 95-100.

Pauluhn, J. (2012) 'Subchronic inhalation toxicity of iron oxide (magnetite, Fe(3) O(4)) in rats: pulmonary toxicity is determined by the particle kinetics typical of poorly soluble particles', *Journal of Applied Toxicology : JAT,* 32 (7), pp. 488-504.

Pelaz, B., del Pino, P., Maffre, P., Hartmann, R., Gallego, M., Rivera-Fernandez, S., de la Fuente, J. M., Nienhaus, G. U. and Parak, W. J. (2015) 'Surface Functionalization of Nanoparticles with Polyethylene Glycol: Effects on Protein Adsorption and Cellular Uptake', *ACS Nano,* 9 (7), pp. 6996-7008.

Peng, Q., Li, K., Sacks, S. H. and Zhou, W. (2009) 'The role of anaphylatoxins C3a and C5a in regulating innate and adaptive immune responses', *Inflammation & Allergy Drug Targets,* 8 (3), pp. 236-246.

Peracchia, M. T., Harnisch, S., Pinto-Alphandary, H., Gulik, A., Dedieu, J. C., Desmaele, D., d'Angelo, J., Muller, R. H. and Couvreur, P. (1999) 'Visualization of in vitro protein-rejecting properties of PEGylated stealth polycyanoacrylate nanoparticles', *Biomaterials,* 20 (14), pp. 1269-1275.

Perdikoulis, M. V., Kishore, U. and Reid, K. B. (2001) 'Expression and characterisation of the thrombospondin type I repeats of human properdin', *Biochimica Et Biophysica Acta,* 1548 (2), pp. 265-277.

Persson, A., Chang, D. and Crouch, E. (1990) 'Surfactant protein D is a divalent cation-dependent carbohydrate-binding protein', *The Journal of Biological Chemistry,* 265 (10), pp. 5755-5760.

Pfeffer, K. (2003) 'Biological functions of tumor necrosis factor cytokines and their receptors', *Cytokine & Growth Factor Reviews,* 14 (3-4), pp. 185-191.

Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W. and Wardlaw, A. C. (1954) 'The properdin system and immunity. I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena', *Science (New York, N.Y.),* 120 (3112), pp. 279-285.

Pinsino, A., Russo, R., Bonaventura, R., Brunelli, A., Marcomini, A. and Matranga, V. (2015) 'Titanium dioxide nanoparticles stimulate sea urchin immune cell phagocytic activity involving TLR/p38 MAPK-mediated signalling pathway', *Scientific Reports,* 5 pp. 14492.

Plescia, J. and Altieri, D. C. (1996) 'Activation of Mac-1 (CD11b/CD18)-bound factor X by released cathepsin G defines an alternative pathway of leucocyte initiation of coagulation', *The Biochemical Journal,* 319 (Pt 3) (Pt 3), pp. 873-879.

Podesta, J. E., Al-Jamal, K. T., Herrero, M. A., Tian, B., Ali-Boucetta, H., Hegde, V., Bianco, A., Prato, M. and Kostarelos, K. (2009) 'Antitumor activity and prolonged survival by carbon-nanotube-mediated therapeutic siRNA silencing in a human lung xenograft model', *Small (Weinheim an Der Bergstrasse, Germany),* 5 (10), pp. 1176-1185.

Pondman, K. M., Paudyal, B., Sim, R. B., Kaur, A., Kouser, L., Tsolaki, A. G., Jones, A. L., Khan, S.,C., Khan, A. H., Ten Haken, B., Stenbeck, G. and Kishore, U. (2017a) 'Pulmonary surfactant protein SP-D opsonises carbon nanotubes and augments their phagocytosis and subsequent pro-inflammatory immune response', *Nanoscale,* 9 (3), pp. 1097-1109.

Pondman, K. M., Pednekar, L., Paudyal, B., Tsolaki, A. G., Kouser, L., Khan, H. A., Shamji, M. H., Ten Haken, B., Stenbeck, G., Sim, R. B. and Kishore, U. (2015) 'Innate immune humoral factors, C1q and factor H, with differential pattern recognition properties, alter macrophage response to carbon nanotubes', *Nanomedicine : Nanotechnology, Biology, and Medicine,* 11 (8), pp. 2109-2118.

Pondman, K. M., Salvador-Morales, C., Paudyal, B., Sim, R. B. and Kishore, U. (2017b) 'Interactions of the innate immune system with carbon nanotubes', *Nanoscale Horizons,* 2 pp. 174-186.

Pondman, K. M., Sobik, M., Nayak, A., Tsolaki, A. G., Jakel, A., Flahaut, E., Hampel, S., Ten Haken, B., Sim, R. B. and Kishore, U. (2014) 'Complement activation by carbon nanotubes and its influence on the phagocytosis and cytokine response by macrophages', *Nanomedicine : Nanotechnology, Biology, and Medicine,* 10 (6), pp. 1287-1299.

Pondman, K. M., Tsolaki, A. G., Paudyal, B., Shamji, M. H., Switzer, A., Pathan, A. A., Abozaid, S. M., Ten Haken, B., Stenbeck, G., Sim, R. B. and Kishore, U. (2016) 'Complement Deposition on Nanoparticles Can Modulate Immune Responses by

Macrophage, B and T Cells', *Journal of Biomedical Nanotechnology,* 12 (1), pp. 197-216.

Pondman, K. M., Sim, R. B. and Kishore, U.(2015) 'Interaction of the Immune System with Nanoparticles', in Bhushan, B. (ed.) *Encyclopedia of Nanotechnology.* Dordrecht: Springer Netherlands, pp. 1-8.

Ponta, A. and Bae, Y. (2010) 'PEG-poly(amino acid) block copolymer micelles for tunable drug release', *Pharmaceutical Research,* 27 (11), pp. 2330-2342.

Poulsen, S. S., Saber, A. T., Williams, A., Andersen, O., Kobler, C., Atluri, R., Pozzebon, M. E., Mucelli, S. P., Simion, M., Rickerby, D., Mortensen, A., Jackson, P., Kyjovska, Z. O., Molhave, K., Jacobsen, N. R., Jensen, K. A., Yauk, C. L., Wallin, H., Halappanavar, S. and Vogel, U. (2015) 'MWCNTs of different physicochemical properties cause similar inflammatory responses, but differences in transcriptional and histological markers of fibrosis in mouse lungs', *Toxicology and Applied Pharmacology,* 284 (1), pp. 16-32.

Pujalte, I., Passagne, I., Brouillaud, B., Treguer, M., Durand, E., Ohayon-Courtes, C. and L'Azou, B. (2011) 'Cytotoxicity and oxidative stress induced by different metallic nanoparticles on human kidney cells', *Particle and Fibre Toxicology,* 8 pp. 10-8977-8-10.

Punfa, W., Suzuki, S., Pitchakarn, P., Yodkeeree, S., Naiki, T., Takahashi, S. and Limtrakul, P. (2014) 'Curcumin-loaded PLGA nanoparticles conjugated with anti- P-glycoprotein antibody to overcome multidrug resistance', *Asian Pacific Journal of Cancer Prevention : APJCP,* 15 (21), pp. 9249-9258.

Qaseem, A. S., Singh, I., Pathan, A. A., Layhadi, J. A., Parkin, R., Alexandra, F., Durham, S. R., Kishore, U. and Shamji, M. H. (2017) 'A Recombinant Fragment of Human Surfactant Protein D Suppresses Basophil Activation and T-Helper Type 2 and B-Cell Responses in Grass Pollen-induced Allergic Inflammation', *American Journal of Respiratory and Critical Care Medicine,* 196 (12), pp. 1526-1534.

Qaseem, A. S., Sonar, S., Mahajan, L., Madan, T., Sorensen, G. L., Shamji, M. H. and Kishore, U. (2013) 'Linking surfactant protein SP-D and IL-13: implications in asthma and allergy', *Molecular Immunology,* 54 (1), pp. 98-107.

Qiu, T. A., Gallagher, M. J., Hudson-Smith, N. V., Wu, J., Krause, M. O. P., Fortner, J. D. and Haynes, C. L. (2016) 'Research highlights: unveiling the mechanisms underlying nanoparticle-induced ROS generation and oxidative stress', *Environ.Sci.: Nano,* 3 (5), pp. 940-945.

Qu, Q., Ma, X. and Zhao, Y. (2015) 'Targeted delivery of doxorubicin to mitochondria using mesoporous silica nanoparticle nanocarriers', *Nanoscale,* 7 (40), pp. 16677-16686.

Quintanilla, H. D., Liu, Y., Fatheree, N. Y., Atkins, C. L., Hashmi, S. S., Floros, J., McCormack, F. X., Rhoads, J. M. and Alcorn, J. L. (2015) 'Oral administration of surfactant protein-a reduces pathology in an experimental model of necrotizing enterocolitis', *Journal of Pediatric Gastroenterology and Nutrition,* 60 (5), pp. 613-620.

Racoosin, E. L. and Swanson, J. A. (1993) 'Macropinosome maturation and fusion with tubular lysosomes in macrophages', *The Journal of Cell Biology,* 121 (5), pp. 1011-1020.

Rajananthanan, P., Attard, G. S., Sheikh, N. A. and Morrow, W. J. (1999) 'Novel aggregate structure adjuvants modulate lymphocyte proliferation and Th1 and Th2 cytokine profiles in ovalbumin immunized mice', *Vaccine,* 18 (1-2), pp. 140-152.

Raynal, I., Prigent, P., Peyramaure, S., Najid, A., Rebuzzi, C. and Corot, C. (2004) 'Macrophage endocytosis of superparamagnetic iron oxide nanoparticles: mechanisms and comparison of ferumoxides and ferumoxtran-10', *Investigative Radiology,* 39 (1), pp. 56-63.

Reid, K. B., Bentley, D. R. and Wood, K. J. (1984) 'Cloning and characterization of the complementary DNA for the B chain of normal human serum C1q', *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences,* 306 (1129), pp. 345-354.

Ren, J., Deng, Y., Xiao, B., Wang, G. and Tao, Z. (2013) 'Protective effects of exogenous surfactant protein A in allergic rhinitis: a mouse model', *The Annals of Otology, Rhinology, and Laryngology,* 122 (4), pp. 240-246.

Rescigno, M., Citterio, S., ThÃ¨ry, C., Rittig, M., Medaglini, D., Pozzi, G., Amigorena, S. and Ricciardi-Castagnoli, P. (1998) 'Bacteria-induced neo-biosynthesis, stabilization, and surface expression of functional class I molecules in mouse dendritic cells', *Proc Natl Acad Sci USA,* 95 (9), pp. 5229.

Reynes, M., Aubert, J. P., Cohen, J. H., Audouin, J., Tricottet, V., Diebold, J. and Kazatchkine, M. D. (1985) 'Human follicular dendritic cells express CR1, CR2, and CR3 complement receptor antigens', *Journal of Immunology (Baltimore, Md.: 1950),* 135 (4), pp. 2687-2694.

Rice, W. R., Ross, G. F., Singleton, F. M., Dingle, S. and Whitsett, J. A. (1987) 'Surfactant-associated protein inhibits phospholipid secretion from type II cells', *Journal of Applied Physiology (Bethesda, Md.: 1985),* 63 (2), pp. 692-698.

Ricklin, D., Hajishengallis, G., Yang, K. and Lambris, J. D. (2010) 'Complement - a key system for immune surveillance and homeostasis', *Nature Immunology,* 11 (9), pp. 785-797.

Rodzinski, A., Guduru, R., Liang, P., Hadjikhani, A., Stewart, T., Stimphil, E., Runowicz, C., Cote, R., Altman, N., Datar, R. and Khizroev, S. (2016) 'Targeted and controlled anticancer drug delivery and release with magnetoelectric nanoparticles', *Scientific Reports,* 6 pp. 20867.

Romero, F., Shah, D., Duong, M., Penn, R. B., Fessler, M. B., Madenspacher, J., Stafstrom, W., Kavuru, M., Lu, B., Kallen, C. B., Walsh, K. and Summer, R. (2015) 'A pneumocyte-macrophage paracrine lipid axis drives the lung toward fibrosis', *American Journal of Respiratory Cell and Molecular Biology,* 53 (1), pp. 74-86.

Rosenthal, E., Poizot-Martin, I., Saint-Marc, T., Spano, J. P., Cacoub, P. and DNX Study Group (2002) 'Phase IV study of liposomal daunorubicin (DaunoXome) in AIDS-related Kaposi sarcoma', *American Journal of Clinical Oncology,* 25 (1), pp. 57-59.

Ruge, C. A., Kirch, J., Canadas, O., Schneider, M., Perez-Gil, J., Schaefer, U. F., Casals, C. and Lehr, C. M. (2011) 'Uptake of nanoparticles by alveolar macrophages is triggered by surfactant protein A', *Nanomedicine : Nanotechnology, Biology, and Medicine,* 7 (6), pp. 690-693.

Ruge, C. A., Schaefer, U. F., Herrmann, J., Kirch, J., Canadas, O., Echaide, M., Perez-Gil, J., Casals, C., Muller, R. and Lehr, C. M. (2012) 'The interplay of lung surfactant proteins and lipids assimilates the macrophage clearance of nanoparticles', *PloS One,* 7 (7), pp. e40775.

Ryan, J. J., Bateman, H. R., Stover, A., Gomez, G., Norton, S. K., Zhao, W., Schwartz, L. B., Lenk, R. and Kepley, C. L. (2007) 'Fullerene nanomaterials inhibit the allergic response', *Journal of Immunology (Baltimore, Md.: 1950),* 179 (1), pp. 665-672.

Rybak-Smith, M., Pondman, K. M., Flahaut, E., Salvador-Morales, C. and Sim, R. B.(2011) 'Recognition of Carbon Nanotubes by the Human Innate Immune System', in Klingeler, R. &Sim, B. R. (eds.) *Carbon Nanotubes for Biomedical Applications.*Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 183-210.

Ryman-Rasmussen, J. P., Tewksbury, E. W., Moss, O. R., Cesta, M. F., Wong, B. A. and Bonner, J. C. (2009) 'Inhaled multiwalled carbon nanotubes potentiate airway fibrosis in murine allergic asthma', *American Journal of Respiratory Cell and Molecular Biology,* 40 (3), pp. 349-358.

Saggu, G., Cortes, C., Emch, H. N., Ramirez, G., Worth, R. G. and Ferreira, V. P. (2013) 'Identification of a novel mode of complement activation on stimulated platelets mediated by properdin and C3(H2O)', *Journal of Immunology (Baltimore, Md.: 1950),* 190 (12), pp. 6457-6467.

Sahay, G., Alakhova, D. Y. and Kabanov, A. V. (2010) 'Endocytosis of nanomedicines', *Journal of Controlled Release : Official Journal of the Controlled Release Society,* 145 (3), pp. 182-195.

Saka, R., Wakimoto, T., Nishiumi, F., Sasaki, T., Nose, S., Fukuzawa, M., Oue, T., Yanagihara, I. and Okuyama, H. (2016) 'Surfactant protein-D attenuates the

lipopolysaccharide-induced inflammation in human intestinal cells overexpressing toll-like receptor 4', *Pediatric Surgery International,* 32 (1), pp. 59-63.

Sakulkhu, U., Mahmoudi, M., Maurizi, L., Salaklang, J. and Hofmann, H. (2014) 'Protein corona composition of superparamagnetic iron oxide nanoparticles with various physico-chemical properties and coatings', *Scientific Reports,* 4 pp. 5020.

Salatin, S., Maleki Dizaj, S. and Yari Khosroushahi, A. (2015) 'Effect of the surface modification, size, and shape on cellular uptake of nanoparticles', *Cell Biology International,* 39 (8), pp. 881-890.

Salvador-Morales, C., Green, M. L. H. and Sim, R. B.(2007) 'Interaction between carbon nanotubes and biomolecules', in Basiuk, E. V,Basiuk, V.A (ed.) *Chemistry of carbon nanotubes.* American Scientific Publishers, .

Salvador-Morales, C., Townsend, P., Flahaut, E., Vénien-Bryan, C., Vlandas, A., Green, M. L. H. and Sim, R. B. (2007) 'Binding of pulmonary surfactant proteins to carbon nanotubes; potential for damage to lung immune defense mechanisms', *Carbon,* 45 (3), pp. 607-617.

Salvador-Morales, C., Basiuk, E. V., Basiuk, V. A., Green, M. L. and Sim, R. B. (2008) 'Effects of covalent functionalization on the biocompatibility characteristics of multi-walled carbon nanotubes', *Journal of Nanoscience and Nanotechnology,* 8 (5), pp. 2347-2356.

Salvador-Morales, C., Khan, Z., Zamory, J., Tran, V., Cedeno, A., Umanzor-Alvarez, J., Kishore, U. and Sim, R. B. (2013) 'Acid-Treated Multi-Walled Carbon Nanotubes Coated with Lung Surfactant Protein SP-A Do Not Induce a Lung Inflammatory Response', *J. Adv. Microsc. Res,* 8 (2), pp. 93-99.

Samuel, C. E. (2001) 'Antiviral Actions of Interferons', *Clinical Microbiology Reviews,* 14 (4), pp. 778-809.

Sano, H., Chiba, H., Iwaki, D., Sohma, H., Voelker, D. R. and Kuroki, Y. (2000) 'Surfactant proteins A and D bind CD14 by different mechanisms', *The Journal of Biological Chemistry,* 275 (29), pp. 22442-22451.

Sano, H., Sohma, H., Muta, T., Nomura, S., Voelker, D. R. and Kuroki, Y. (1999) 'Pulmonary surfactant protein A modulates the cellular response to smooth and rough lipopolysaccharides by interaction with CD14', *Journal of Immunology (Baltimore, Md.: 1950),* 163 (1), pp. 387-395.

Sargent, L. M., Porter, D. W., Staska, L. M., Hubbs, A. F., Lowry, D. T., Battelli, L., Siegrist, K. J., Kashon, M. L., Mercer, R. R., Bauer, A. K., Chen, B. T., Salisbury, J. L., Frazer, D., McKinney, W., Andrew, M., Tsuruoka, S., Endo, M., Fluharty, K. L., Castranova, V. and Reynolds, S. H. (2014) 'Promotion of lung adenocarcinoma following inhalation exposure to multi-walled carbon nanotubes', *Particle and Fibre Toxicology,* 11 pp. 3-8977-11-3.

Scapini, P., Lapinet-Vera, J. A., Gasperini, S., Calzetti, F., Bazzoni, F. and Cassatella, M. A. (2000) 'The neutrophil as a cellular source of chemokines', *Immunological Reviews,* 177 pp. 195-203.

Schelenz, S., Malhotra, R., Sim, R. B., Holmskov, U. and Bancroft, G. J. (1995) 'Binding of host collectins to the pathogenic yeast Cryptococcus neoformans: human surfactant protein D acts as an agglutinin for acapsular yeast cells', *Infection and Immunity,* 63 (9), pp. 3360-3366.

Schicht, M., Stengl, C., Sel, S., Heinemann, F., Gotz, W., Petschelt, A., Pelka, M., Scholz, M., Rausch, F., Paulsen, F. and Brauer, L. (2015) 'The distribution of human surfactant proteins within the oral cavity and their role during infectious diseases of the gingiva', *Annals of Anatomy = Anatomischer Anzeiger : Official Organ of the Anatomische Gesellschaft,* 199 pp. 92-97.

Schmitt, E. and Bopp, T. (2012) 'Amazing IL-9: revealing a new function for an â€œoldâ€• cytokine', *The Journal of Clinical Investigation,* 122 (11), pp. 3857-3859.

Schniepp, H. C., Li, J. L., McAllister, M. J., Sai, H., Herrera-Alonso, M., Adamson, D. H., Prud'homme, R. K., Car, R., Saville, D. A. and Aksay, I. A. (2006) 'Functionalized single graphene sheets derived from splitting graphite oxide', *The Journal of Physical Chemistry.B,* 110 (17), pp. 8535-8539.

Schulze, C., Schaefer, U. F., Ruge, C. A., Wohlleben, W. and Lehr, C. M. (2011) 'Interaction of metal oxide nanoparticles with lung surfactant protein A', *European Journal of Pharmaceutics and Biopharmaceutics : Official Journal of Arbeitsgemeinschaft Fur Pharmazeutische Verfahrenstechnik E.V,* 77 (3), pp. 376-383.

Schutz, C. A., Juillerat-Jeanneret, L., Mueller, H., Lynch, I., Riediker, M. and NanoImpactNet Consortium (2013) 'Therapeutic nanoparticles in clinics and under clinical evaluation', *Nanomedicine (London, England),* 8 (3), pp. 449-467.

Schwaeble, W., Dippold, W. G., Schafer, M. K., Pohla, H., Jonas, D., Luttig, B., Weihe, E., Huemer, H. P., Dierich, M. P. and Reid, K. B. (1993) 'Properdin, a positive regulator of complement activation, is expressed in human T cell lines and peripheral blood T cells', *Journal of Immunology (Baltimore, Md.: 1950),* 151 (5), pp. 2521-2528.

Schwaeble, W., Huemer, H. P., Most, J., Dierich, M. P., Strobel, M., Claus, C., Reid, K. B. and Ziegler-Heitbrock, H. W. (1994) 'Expression of properdin in human monocytes', *European Journal of Biochemistry,* 219 (3), pp. 759-764.

Sellar, G. C., Blake, D. J. and Reid, K. B. (1991) 'Characterization and organization of the genes encoding the A-, B- and C-chains of human complement subcomponent C1q. The complete derived amino acid sequence of human C1q', *Biochemical Journal,* 274 (Pt 2), pp. 481-490.

Seo, D. Y., Jin, M., Ryu, J. and Kim, Y. (2017) 'Investigation of the genetic toxicity by dextran-coated superparamagnetic iron oxide nanoparticles (SPION) in HepG2 cells using the comet assay and cytokinesis-block micronucleus assay', *Toxicology and Environmental Health Sciences,* 9 (1), pp. 23-29.

Serna, M., Giles, J. L., Morgan, B. P. and Bubeck, D. (2016) 'Structural basis of complement membrane attack complex formation', *Nature Communications,* 7 pp. 10587.

Shander, A., Cappellini, M. D. and Goodnough, L. T. (2009) 'Iron overload and toxicity: the hidden risk of multiple blood transfusions', *Vox Sanguinis,* 97 (3), pp. 185-197.

Shannahan, J. H., Brown, J. M., Chen, R., Ke, P. C., Lai, X., Mitra, S. and Witzmann, F. A. (2013) 'Comparison of nanotube-protein corona composition in cell culture media', *Small (Weinheim an Der Bergstrasse, Germany),* 9 (12), pp. 2171-2181.

Shi, Y., Liu, C. H., Roberts, A. I., Das, J., Xu, G., Ren, G., Zhang, Y., Zhang, L., Yuan, Z. R., Tan, H. S., Das, G. and Devadas, S. (2006) 'Granulocyte-macrophage colony-stimulating factor (GM-CSF) and T-cell responses: what we do and don't know', *Cell Research,* 16 (2), pp. 126-133.

Shi, Y., Zhou, M., Zhang, J. and Lu, W. (2015) 'Preparation and cellular targeting study of VEGF-conjugated PLGA nanoparticles', *Journal of Microencapsulation,* 32 (7), pp. 699-704.

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. and Kagan, V. E. (2012) 'Impaired clearance and enhanced pulmonary inflammatory/fibrotic response to carbon nanotubes in myeloperoxidase-deficient mice', *PloS One,* 7 (3), pp. e30923.

Shvedova, A. A., Yanamala, N., Kisin, E. R., Tkach, A. V., Murray, A. R., Hubbs, A., Chirila, M. M., Keohavong, P., Sycheva, L. P., Kagan, V. E. and Castranova, V. (2014) 'Long-term effects of carbon containing engineered nanomaterials and asbestos in the lung: one year postexposure comparisons', *American Journal of Physiology.Lung Cellular and Molecular Physiology,* 306 (2), pp. L170-82.

Sica, A., Wang, J. M., Colotta, F., Dejana, E., Mantovani, A., Oppenheim, J. J., Larsen, C. G., Zachariae, C. O. and Matsushima, K. (1990) 'Monocyte chemotactic and activating factor gene expression induced in endothelial cells by IL-1 and tumor necrosis factor', *Journal of Immunology (Baltimore, Md.: 1950),* 144 (8), pp. 3034-3038.

Siegrist, K. J., Reynolds, S. H., Kashon, M. L., Lowry, D. T., Dong, C., Hubbs, A. F., Young, S. H., Salisbury, J. L., Porter, D. W., Benkovic, S. A., McCawley, M., Keane, M. J., Mastovich, J. T., Bunker, K. L., Cena, L. G., Sparrow, M. C., Sturgeon, J. L.,

Dinu, C. Z. and Sargent, L. M. (2014) 'Genotoxicity of multi-walled carbon nanotubes at occupationally relevant doses', *Particle and Fibre Toxicology,* 11 pp. 6-8977-11-6.

Silva, F., Zambre, A., Campello, M. P., Gano, L., Santos, I., Ferraria, A. M., Ferreira, M. J., Singh, A., Upendran, A., Paulo, A. and Kannan, R. (2016) 'Interrogating the Role of Receptor-Mediated Mechanisms: Biological Fate of Peptide-Functionalized Radiolabeled Gold Nanoparticles in Tumor Mice', *Bioconjugate Chemistry,* 27 (4), pp. 1153-1164.

Silveyra, P. and Floros, J. (2012) 'Genetic variant associations of human SP-A and SP-D with acute and chronic lung injury', *Frontiers in Bioscience (Landmark Edition),* 17 pp. 407-429.

Simberg, D., Park, J. H., Karmali, P. P., Zhang, W. M., Merkulov, S., McCrae, K., Bhatia, S. N., Sailor, M. and Ruoslahti, E. (2009) 'Differential proteomics analysis of the surface heterogeneity of dextran iron oxide nanoparticles and the implications for their in vivo clearance', *Biomaterials,* 30 (23-24), pp. 3926-3933.

Singh, A., Patel, T., Hertel, J., Bernardo, M., Kausz, A. and Brenner, L. (2008) 'Safety of ferumoxytol in patients with anemia and CKD', *American Journal of Kidney Diseases : The Official Journal of the National Kidney Foundation,* 52 (5), pp. 907-915.

Singh, M., Madan, T., Waters, P., Sonar, S., Singh, S. K., Kamran, M. F., Bernal, A. L., Sarma, P. U., Singh, V. K., Crouch, E. C. and Kishore, U. (2009) 'Therapeutic effects of recombinant forms of full-length and truncated human surfactant protein D in a murine model of invasive pulmonary aspergillosis', *Molecular Immunology,* 46 (11-12), pp. 2363-2369.

Singh, N., Jenkins, G. J., Nelson, B. C., Marquis, B. J., Maffeis, T. G., Brown, A. P., Williams, P. M., Wright, C. J. and Doak, S. H. (2012) 'The role of iron redox state in the genotoxicity of ultrafine superparamagnetic iron oxide nanoparticles', *Biomaterials,* 33 (1), pp. 163-170.

Singh, S. P., Rahman, M. F., Murty, U. S., Mahboob, M. and Grover, P. (2013) 'Comparative study of genotoxicity and tissue distribution of nano and micron sized iron

oxide in rats after acute oral treatment', *Toxicology and Applied Pharmacology,* 266 (1), pp. 56-66.

Smith, C. A., Pangburn, M. K., Vogel, C. W. and Muller-Eberhard, H. J. (1984) 'Molecular architecture of human properdin, a positive regulator of the alternative pathway of complement', *The Journal of Biological Chemistry,* 259 (7), pp. 4582-4588.

Snedeker, S. M., Brown, C. F. and DiAugustine, R. P. (1991) 'Expression and functional properties of transforming growth factor alpha and epidermal growth factor during mouse mammary gland ductal morphogenesis', *Proceedings of the National Academy of Sciences of the United States of America,* 88 (1), pp. 276-280.

Sneh-Edri, H., Likhtenshtein, D. and Stepensky, D. (2011) 'Intracellular targeting of PLGA nanoparticles encapsulating antigenic peptide to the endoplasmic reticulum of dendritic cells and its effect on antigen cross-presentation in vitro', *Molecular Pharmaceutics,* 8 (4), pp. 1266-1275.

Soderstrom, C., Braconier, J. H., Sjoholm, A. G. and Thuresson, B. (1991) 'Granulocyte functions and Neisseria meningitidis: influence of properdin-deficient serum', *APMIS : Acta Pathologica, Microbiologica, Et Immunologica Scandinavica,* 99 (11), pp. 965-971.

Soman, N. R., Baldwin, S. L., Hu, G., Marsh, J. N., Lanza, G. M., Heuser, J. E., Arbeit, J. M., Wickline, S. A. and Schlesinger, P. H. (2009) 'Molecularly targeted nanocarriers deliver the cytolytic peptide melittin specifically to tumor cells in mice, reducing tumor growth', *The Journal of Clinical Investigation,* 119 (9), pp. 2830-2842.

Spitzer, D., Mitchell, L. M., Atkinson, J. P. and Hourcade, D. E. (2007) 'Properdin can initiate complement activation by binding specific target surfaces and providing a platform for de novo convertase assembly', *Journal of Immunology (Baltimore, Md.: 1950),* 179 (4), pp. 2600-2608.

Stover, C. M., Luckett, J. C., Echtenacher, B., Dupont, A., Figgitt, S. E., Brown, J., Mannel, D. N. and Schwaeble, W. J. (2008) 'Properdin plays a protective role in polymicrobial septic peritonitis', *Journal of Immunology (Baltimore, Md.: 1950),* 180 (5), pp. 3313-3318.

Stringer, B. and Kobzik, L. (1996) 'Alveolar macrophage uptake of the environmental particulate titanium dioxide: role of surfactant components', *American Journal of Respiratory Cell and Molecular Biology,* 14 (2), pp. 155-160.

Stroh, A., Zimmer, C., Gutzeit, C., Jakstadt, M., Marschinke, F., Jung, T., Pilgrimm, H. and Grune, T. (2004) 'Iron oxide particles for molecular magnetic resonance imaging cause transient oxidative stress in rat macrophages', *Free Radical Biology & Medicine,* 36 (8), pp. 976-984.

Strong, P., Townsend, P., Mackay, R., Reid, K. B. and Clark, H. W. (2003) 'A recombinant fragment of human SP-D reduces allergic responses in mice sensitized to house dust mite allergens', *Clinical and Experimental Immunology,* 134 (2), pp. 181-187.

Sugarman, B. J., Aggarwal, B. B., Hass, P. E., Figari, I. S., Palladino Jr., M. A. and Michael Shepard, H. (1985) 'Recombinant human tumor necrosis factor-a: Effects on proliferation of normal and transformed cells in vitro', *Science,* 230 (4728), pp. 943-945.

Sun, Z., Liu, Z., Meng, J., Meng, J., Duan, J., Xie, S., Lu, X., Zhu, Z., Wang, C., Chen, S., Xu, H. and Yang, X. D. (2011) 'Carbon nanotubes enhance cytotoxicity mediated by human lymphocytes in vitro', *PloS One,* 6 (6), pp. e21073.

Sun, Z., Yang, L., Chen, K. F., Chen, G. W., Peng, Y. P., Chen, J. K., Suo, G., Yu, J., Wang, W. C. and Lin, C. H. (2016) 'Nano zerovalent iron particles induce pulmonary and cardiovascular toxicity in an in vitro human co-culture model', *Nanotoxicology,* 10 (7), pp. 881-890.

Suzuki, Y., Fujita, Y. and Kogishi, K. (1989) 'Reconstitution of tubular myelin from synthetic lipids and proteins associated with pig pulmonary surfactant', *The American Review of Respiratory Disease,* 140 (1), pp. 75-81.

Szebeni, J., Baranyi, L., Savay, S., Milosevits, J., Bunger, R., Laverman, P., Metselaar, J. M., Storm, G., Chanan-Khan, A., Liebes, L., Muggia, F. M., Cohen, R., Barenholz, Y. and Alving, C. R. (2002) 'Role of complement activation in hypersensitivity

reactions to doxil and hynic PEG liposomes: experimental and clinical studies', *Journal of Liposome Research,* 12 (1-2), pp. 165-172.

Szebeni, J., Bedocs, P., Rozsnyay, Z., Weiszhar, Z., Urbanics, R., Rosivall, L., Cohen, R., Garbuzenko, O., Bathori, G., Toth, M., Bunger, R. and Barenholz, Y. (2012) 'Liposome-induced complement activation and related cardiopulmonary distress in pigs: factors promoting reactogenicity of Doxil and AmBisome', *Nanomedicine : Nanotechnology, Biology, and Medicine,* 8 (2), pp. 176-184.

Szebeni, J., Muggia, F., Gabizon, A. and Barenholz, Y. (2011) 'Activation of complement by therapeutic liposomes and other lipid excipient-based therapeutic products: prediction and prevention', *Advanced Drug Delivery Reviews,* 63 (12), pp. 1020-1030.

Taguchi, K., Urata, Y., Anraku, M., Watanabe, H., Kadowaki, D., Sakai, H., Horinouchi, H., Kobayashi, K., Tsuchida, E., Maruyama, T. and Otagiri, M. (2009) 'Hemoglobin vesicles, polyethylene glycol (PEG)ylated liposomes developed as a red blood cell substitute, do not induce the accelerated blood clearance phenomenon in mice', *Drug Metabolism and Disposition: The Biological Fate of Chemicals,* 37 (11), pp. 2197-2203.

Tahara, K., Tadokoro, S., Yamamoto, H., Kawashima, Y. and Hirashima, N. (2012) 'The suppression of IgE-mediated histamine release from mast cells following exocytic exclusion of biodegradable polymeric nanoparticles', *Biomaterials,* 33 (1), pp. 343-351.

Takeda, K., Kaisho, T. and Akira, S. (2003) 'Toll-like receptors', *Annual Review of Immunology,* 21 pp. 335-376.

Takizawa, F., Tsuji, S. and Nagasawa, S. (1996) 'Enhancement of macrophage phagocytosis upon iC3b deposition on apoptotic cells', *FEBS Letters,* 397 (2-3), pp. 269-272.

Tan, X. Q., Cheng, X. L., Zhang, L., Wu, B. W., Liu, Q. H., Meng, J., Xu, H. Y. and Cao, J. M. (2014) 'Multi-walled carbon nanotubes impair Kv4.2/4.3 channel activities, delay membrane repolarization and induce bradyarrhythmias in the rat', *PloS One,* 9 (7), pp. e101545.

Tanaka, K., Ito, A., Kobayashi, T., Kawamura, T., Shimada, S., Matsumoto, K., Saida, T. and Honda, H. (2005) 'Heat immunotherapy using magnetic nanoparticles and dendritic cells for T-lymphoma', *Journal of Bioscience and Bioengineering,* 100 (1), pp. 112-115.

Tanaka, T., Narazaki, M. and Kishimoto, T. (2014) 'IL-6 in Inflammation, Immunity, and Disease', *Cold Spring Harbor Perspectives in Biology,* 6 (10), pp. a016295.

Tchoreloff, P., Gulik, A., Denizot, B., Proust, J. E. and Puisieux, F. (1991) 'A structural study of interfacial phospholipid and lung surfactant layers by transmission electron microscopy after Blodgett sampling: influence of surface pressure and temperature', *Chemistry and Physics of Lipids,* 59 (2), pp. 151-165.

Teeguarden, J. G., Hinderliter, P. M., Orr, G., Thrall, B. D. and Pounds, J. G. (2007) 'Particokinetics in vitro: dosimetry considerations for in vitro nanoparticle toxicity assessments', *Toxicological Sciences : An Official Journal of the Society of Toxicology,* 95 (2), pp. 300-312.

Thakur, G., Prakash, G., Murthy, V., Sable, N., Menon, S., Bakshi, G., Kishore, U. and Madan, T. (2017) 'Involvement of mitochondrial intrinsic pathway in rhSP-D (recombinant human Surfactant Protein D) induced apoptosis of prostate cancer cells', *Can J Biotech,* 1 pp. 172.

Thurman, J. M., Kraus, D. M., Girardi, G., Hourcade, D., Kang, H. J., Royer, P. A., Mitchell, L. M., Giclas, P. C., Salmon, J., Gilkeson, G. and Holers, V. M. (2005) 'A novel inhibitor of the alternative complement pathway prevents antiphospholipid antibody-induced pregnancy loss in mice', *Molecular Immunology,* 42 (1), pp. 87-97.

Todderud, G. and Carpenter, G. (1989) 'Epidermal growth factor: the receptor and its function', *BioFactors (Oxford, England),* 2 (1), pp. 11â€”15.

Tolcher, A. W., Sugarman, S., Gelmon, K. A., Cohen, R., Saleh, M., Isaacs, C., Young, L., Healey, D., Onetto, N. and Slichenmyer, W. (1999) 'Randomized phase II study of BR96-doxorubicin conjugate in patients with metastatic breast cancer', *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology,* 17 (2), pp. 478-484.

Treanor, B. (2012) 'B-cell receptor: from resting state to activate', *Immunology,* 136 (1), pp. 21-27.

Trochon-joseph, V., Lemarchand, C., Hayes, V., Rayah, Y., Labernardière, J. and Dixon, G. (2016) 'Mechanistic study of the relative cytotoxicity of doxorubicin loaded nanoparticle formulation compared to free doxorubicin in hepatocellular carcinoma (HCC) cell lines', *Experimental and Molecular Therapeutics,* 76 (14 Supplement), pp. 2143-2143.

Turk, B., Stoka, V., Rozman-Pungercar, J., Cirman, T., Droga-Mazovec, G., Oresic, K. and Turk, V. (2002) 'Apoptotic pathways: involvement of lysosomal proteases', *Biological Chemistry,* 383 (7-8), pp. 1035-1044.

Ugarte, D. (1992) 'Curling and closure of graphitic networks under electron-beam irradiation', *Nature,* 359 (6397), pp. 707-709.

Umemoto, E. Y., Speck, M., Shimoda, L. M., Kahue, K., Sung, C., Stokes, A. J. and Turner, H. (2014) 'Single-walled carbon nanotube exposure induces membrane rearrangement and suppression of receptor-mediated signalling pathways in model mast cells', *Toxicology Letters,* 229 (1), pp. 198-209.

Underhill, D. M. and Goodridge, H. S. (2012) 'Information processing during phagocytosis', *Nature Reviews.Immunology,* 12 (7), pp. 492-502.

Unezaki, S., Maruyama, K., Hosoda, J., Nagae, I., Koyanagi, Y., Nakata, M., Ishida, O., Iwatsuru, M. and Tsuchiya, S. (1996) *Direct measurement of the extravasation of polyethyleneglycol-coated liposomes into solid tumor tissue by in vivo fluorescence microscopy.*

Valent, P., Schmidt, G., Besemer, J., Mayer, P., Zenke, G., Liehl, E., Hinterberger, W., Lechner, K., Maurer, D. and Bettelheim, P. (1989) 'Interleukin-3 is a differentiation factor for human basophils', *Blood,* 73 (7), pp. 1763-1769.

van Berlo, D., Clift, M. J., Albrecht, C. and Schins, R. P. (2012) 'Carbon nanotubes: an insight into the mechanisms of their potential genotoxicity', *Swiss Medical Weekly,* 142 pp. w13698.

van Berlo, D., Wilhelmi, V., Boots, A. W., Hullmann, M., Kuhlbusch, T. A., Bast, A., Schins, R. P. and Albrecht, C. (2014) 'Apoptotic, inflammatory, and fibrogenic effects of two different types of multi-walled carbon nanotubes in mouse lung', *Archives of Toxicology,* 88 (9), pp. 1725-1737.

van Lookeren Campagne, M., Wiesmann, C. and Brown, E. J. (2007) 'Macrophage complement receptors and pathogen clearance', *Cellular Microbiology,* 9 (9), pp. 2095-2102.

Vandivier, R. W., Ogden, C. A., Fadok, V. A., Hoffmann, P. R., Brown, K. K., Botto, M., Walport, M. J., Fisher, J. H., Henson, P. M. and Greene, K. E. (2002) 'Role of surfactant proteins A, D, and C1q in the clearance of apoptotic cells in vivo and in vitro: calreticulin and CD91 as a common collectin receptor complex', *Journal of Immunology (Baltimore, Md.: 1950),* 169 (7), pp. 3978-3986.

Venkatachalam, M. A. and Rennke, H. G. (1978) 'The structural and molecular basis of glomerular filtration', *Circulation Research,* 43 (3), pp. 337-347.

Verhoef, J. J. F., de Groot, A. M., van Moorsel, M., Ritsema, J., Beztsinna, N., Maas, C. and Schellekens, H. (2017) 'Iron nanomedicines induce Toll-like receptor activation, cytokine production and complement activation', *Biomaterials,* 119 pp. 68-77.

Vono, M., Lin, A., Norrby-Teglund, A., Koup, R. A., Liang, F. and Lore, K. (2017) 'Neutrophils acquire the capacity for antigen presentation to memory CD4(+) T cells in vitro and ex vivo', *Blood,* 129 (14), pp. 1991-2001.

Voronkova, M. A., Luanpitpong, S., Rojanasakul, L. W., Castranova, V., Dinu, C. Z., Riedel, H. and Rojanasakul, Y. (2017) 'SOX9 Regulates Cancer Stem-Like Properties and Metastatic Potential of Single-Walled Carbon Nanotube-Exposed Cells', *Scientific Reports,* 7 (1), pp. 11653-017-12037-8.

Vroman, L. (1962) 'Effect of Adsorbed Proteins on the Wettability of Hydrophilic and Hydrophobic Solids', *Nature,* 196 (4853), pp. 476-477.

Wang, C., Jia, H., Zhu, L., Zhang, H. and Wang, Y. (2017a) 'Toxicity of alpha-Fe2O3 nanoparticles to Artemia salina cysts and three stages of larvae', *The Science of the Total Environment,* 598 pp. 847-855.

Wang, G., Chen, F., Banda, N. K., Holers, V. M., Wu, L., Moghimi, S. M. and Simberg, D. (2016) 'Activation of Human Complement System by Dextran-Coated Iron Oxide Nanoparticles Is Not Affected by Dextran/Fe Ratio, Hydroxyl Modifications, and Crosslinking', *Frontiers in Immunology,* 7 pp. 418.

Wang, T., Xue, J., Hu, Q., Zhou, M., Chang, C. and Luo, Y. (2017b) 'Synthetic surfactant- and cross-linker-free preparation of highly stable lipid-polymer hybrid nanoparticles as potential oral delivery vehicles', *Scientific Reports,* 7 (1), pp. 2750-017-02867-x.

Wang, X., Podila, R., Shannahan, J. H., Rao, A. M. and Brown, J. M. (2013) 'Intravenously delivered graphene nanosheets and multiwalled carbon nanotubes induce site-specific Th2 inflammatory responses via the IL-33/ST2 axis', *International Journal of Nanomedicine,* 8 pp. 1733-1748.

Wang, Z., Li, J., Cho, J. and Malik, A. B. (2014) 'Prevention of vascular inflammation by nanoparticle targeting of adherent neutrophils', *Nature Nanotechnology,* 9 (3), pp. 204-210.

Watford, W. T., Smithers, M. B., Frank, M. M. and Wright, J. R. (2002) 'Surfactant protein A enhances the phagocytosis of C1q-coated particles by alveolar macrophages', *American Journal of Physiology.Lung Cellular and Molecular Physiology,* 283 (5), pp. L1011-22.

Watson, P., Jones, A. T. and Stephens, D. J. (2005) 'Intracellular trafficking pathways and drug delivery: fluorescence imaging of living and fixed cells', *Advanced Drug Delivery Reviews,* 57 (1), pp. 43-61.

Weaver, T. E. and Whitsett, J. A. (1991) 'Function and regulation of expression of pulmonary surfactant-associated proteins', *Biochemical Journal,* 273 pp. 249-264.

Wei, H., Bruns, O. T., Kaul, M. G., Hansen, E. C., Barch, M., Wisniowska, A., Chen, O., Chen, Y., Li, N., Okada, S., Cordero, J. M., Heine, M., Farrar, C. T., Montana, D. M., Adam, G., Ittrich, H., Jasanoff, A., Nielsen, P. and Bawendi, M. G. (2017) 'Exceedingly small iron oxide nanoparticles as positive MRI contrast agents', *Proceedings of the National Academy of Sciences of the United States of America,* 114 (9), pp. 2325-2330.

Weikert, L. F., Lopez, J. P., Abdolrasulnia, R., Chroneos, Z. C. and Shepherd, V. L. (2000) 'Surfactant protein A enhances mycobacterial killing by rat macrophages through a nitric oxide-dependent pathway', *American Journal of Physiology.Lung Cellular and Molecular Physiology,* 279 (2), pp. L216-23.

Weissleder, R., Stark, D. D., Engelstad, B. L., Bacon, B. R., Compton, C. C., White, D. L., Jacobs, P. and Lewis, J. (1989) 'Superparamagnetic iron oxide: pharmacokinetics and toxicity', *AJR.American Journal of Roentgenology,* 152 (1), pp. 167-173.

Whaley, K. and North, J.(1997) 'Haemolytic assays for whole complement activity and individual components', in A.W. Dodds, R. B. S. (ed.) *Complement, A Practical Approach.* UK: IRL Press Oxford, pp. 19-47.

Wilhelm, S., Tavares, A. J., Dai, Q., Ohta, S., Audet, J., Dvorak, H. F. and Chan, W. C. W. (2016) 'Analysis of nanoparticle delivery to tumours', 1 pp. 16014.

William E. Carson, W. E., Giri, J. G., Lindemann, M. J., Linett, M. L., Ahdieh, M., Paxton, R., Anderson, D., Eisenmann, J., Grabstein, K. and Caligiuri, M. A. (1994) 'Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor', *The Journal of Experimental Medicine,* 180 (4), pp. 1395-1403.

Wirthmueller, U., Dewald, B., Thelen, M., Schafer, M. K., Stover, C., Whaley, K., North, J., Eggleton, P., Reid, K. B. and Schwaeble, W. J. (1997) 'Properdin, a positive regulator of complement activation, is released from secondary granules of stimulated peripheral blood neutrophils', *Journal of Immunology (Baltimore, Md.: 1950),* 158 (9), pp. 4444-4451.

Wolf-Grosse, S., Rokstad, A. M., Ali, S., Lambris, J. D., Mollnes, T. E., Nilsen, A. M. and Stenvik, J. (2017) 'Iron oxide nanoparticles induce cytokine secretion in a complement-dependent manner in a human whole blood model', *International Journal of Nanomedicine,* 12 pp. 3927-3940.

Wong, E. K. and Kavanagh, D. (2015) 'Anticomplement C5 therapy with eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome', *Translational Research : The Journal of Laboratory and Clinical Medicine,* 165 (2), pp. 306-320.

Woo, K., Hong, J., Choi, S., Lee, H., Ahn, J., Kim, C. S. and Lee, S. W. (2004) 'Easy Synthesis and Magnetic Properties of Iron Oxide Nanoparticles', *Chemistry of Materials,* 16 (14), pp. 2814-2818.

Wright, J. R. (2005) 'Immunoregulatory functions of surfactant proteins', *Nature Reviews.Immunology,* 5 (1), pp. 58-68.

Xing, Y., Zhao, J., Conti, P. S. and Chen, K. (2014) 'Radiolabeled nanoparticles for multimodality tumor imaging', *Theranostics,* 4 (3), pp. 290-306.

Xu, L. L., McVicar, D. W., Ben-Baruch, A., Kuhns, D. B., Johnston, J., Oppenheim, J. J. and Wang, J. M. (1995) 'Monocyte chemotactic protein-3 (MCP3) interacts with multiple leukocyte receptors: binding and signaling of MCP3 through shared as well as unique receptors on monocytes and neutrophils', *European Journal of Immunology,* 25 (9), pp. 2612-2617.

Xu, W., Berger, S. P., Trouw, L. A., de Boer, H. C., Schlagwein, N., Mutsaers, C., Daha, M. R. and van Kooten, C. (2008) 'Properdin binds to late apoptotic and necrotic cells independently of C3b and regulates alternative pathway complement activation', *Journal of Immunology (Baltimore, Md.: 1950),* 180 (11), pp. 7613-7621.

Yamada, C., Sano, H., Shimizu, T., Mitsuzawa, H., Nishitani, C., Himi, T. and Kuroki, Y. (2006) 'Surfactant Protein A Directly Interacts with TLR4 and MD-2 and Regulates Inflammatory Cellular Response: IMPORTANCE OF SUPRATRIMERIC OLIGOMERIZATION', *Journal of Biological Chemistry,* 281 (31), pp. 21771-21780.

Yamamoto, M., Yamazaki, S., Uematsu, S., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Kuwata, H., Takeuchi, O., Takeshige, K., Saitoh, T., Yamaoka, S., Yamamoto, N., Yamamoto, S., Muta, T., Takeda, K. and Akira, S. (2004) 'Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta', *Nature,* 430 (6996), pp. 218-222.

Yamazoe, M., Nishitani, C., Takahashi, M., Katoh, T., Ariki, S., Shimizu, T., Mitsuzawa, H., Sawada, K., Voelker, D. R., Takahashi, H. and Kuroki, Y. (2008) 'Pulmonary surfactant protein D inhibits lipopolysaccharide (LPS)-induced inflammatory cell responses by altering LPS binding to its receptors', *The Journal of Biological Chemistry,* 283 (51), pp. 35878-35888.

Yameen, B., Choi, W. I., Vilos, C., Swami, A., Shi, J. and Farokhzad, O. C. (2014) 'Insight into nanoparticle cellular uptake and intracellular targeting', *Journal of Controlled Release : Official Journal of the Controlled Release Society,* 190 pp. 485-499.

Yang, C., Szeliga, J., Jordan, J., Faske, S., Sever-Chroneos, Z., Dorsett, B., Christian, R. E., Settlage, R. E., Shabanowitz, J., Hunt, D. F., Whitsett, J. A. and Chroneos, Z. C. (2005) 'Identification of the Surfactant Protein A Receptor 210 as the Unconventional Myosin 18A', *Journal of Biological Chemistry,* 280 (41), pp. 34447-34457.

Yao, V. J., D'Angelo, S., Butler, K. S., Theron, C., Smith, T. L., Marchio, S., Gelovani, J. G., Sidman, R. L., Dobroff, A. S., Brinker, C. J., Bradbury, A. R., Arap, W. and Pasqualini, R. (2016) 'Ligand-targeted theranostic nanomedicines against cancer', *Journal of Controlled Release : Official Journal of the Controlled Release Society,* 240 pp. 267-286.

Yehia, H. N., Draper, R. K., Mikoryak, C., Walker, E. K., Bajaj, P., Musselman, I. H., Daigrepont, M. C., Dieckmann, G. R. and Pantano, P. (2007) 'Single-walled carbon nanotube interactions with HeLa cells', *Journal of Nanobiotechnology,* 5 pp. 8.

Yoshida, M. and Ōsawa, E. (1993) 'Molecular Mechanics Calculations of Giant- and Hyperfullerenes with Eicosahedral Symmetry', *Fullerene Science and Technology,* 1 (1), pp. 55-74.

Yu, H., Munoz, E. M., Edens, R. E. and Linhardt, R. J. (2005) 'Kinetic studies on the interactions of heparin and complement proteins using surface plasmon resonance', *Biochimica Et Biophysica Acta,* 1726 (2), pp. 168-176.

Yun, Y. R., Won, J. E., Jeon, E., Lee, S., Kang, W., Jo, H., Jang, J. H., Shin, U. S. and Kim, H. W. (2010) 'Fibroblast Growth Factors: Biology, Function, and Application for Tissue Regeneration', *Journal of Tissue Engineering,* 2010 pp. 10.4061/2010/218142.

Zaferani, A., Vives, R. R., van der Pol, P., Navis, G. J., Daha, M. R., van Kooten, C., Lortat-Jacob, H., Seelen, M. A. and van den Born, J. (2012) 'Factor h and properdin recognize different epitopes on renal tubular epithelial heparan sulfate', *The Journal of Biological Chemistry,* 287 (37), pp. 31471-31481.

Zamai, L., Ahmad, M., Bennett, I. M., Azzoni, L., Alnemri, E. S. and Perussia, B. (1998) 'Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells', *The Journal of Experimental Medicine,* 188 (12), pp. 2375-2380.

Zanganeh, S., Hutter, G., Spitler, R., Lenkov, O., Mahmoudi, M., Shaw, A., Pajarinen, J. S., Nejadnik, H., Goodman, S., Moseley, M., Coussens, L. M. and Daldrup-Link, H. E. (2016) 'Iron oxide nanoparticles inhibit tumour growth by inducing pro-inflammatory macrophage polarization in tumour tissues', *Nature Nanotechnology,* 11 (11), pp. 986-994.

Zelikoff, J. T., Chen, L. C., Cohen, M. D., Fang, K., Gordon, T., Li, Y., Nadziejko, C. and Schlesinger, R. B. (2003) 'Effects of inhaled ambient particulate matter on pulmonary antimicrobial immune defense', *Inhalation Toxicology,* 15 (2), pp. 131-150.

Zeng, X., Morgenstern, R. and Nystrom, A. M. (2014) 'Nanoparticle-directed sub-cellular localization of doxorubicin and the sensitization breast cancer cells by circumventing GST-mediated drug resistance', *Biomaterials,* 35 (4), pp. 1227-1239.

Zhang, T., Qian, L., Tang, M., Xue, Y., Kong, L., Zhang, S. and Pu, Y. (2012) 'Evaluation on cytotoxicity and genotoxicity of the L-glutamic acid coated iron oxide nanoparticles', *Journal of Nanoscience and Nanotechnology,* 12 (3), pp. 2866-2873.

Zhang, Z., Abdel-Razek, O., Hawgood, S. and Wang, G. (2015) 'Protective Role of Surfactant Protein D in Ocular Staphylococcus aureus Infection', *PloS One,* 10 (9), pp. e0138597.

Zhao, F., Zhao, Y., Liu, Y., Chang, X., Chen, C. and Zhao, Y. (2011) 'Cellular uptake, intracellular trafficking, and cytotoxicity of nanomaterials', *Small (Weinheim an Der Bergstrasse, Germany),* 7 (10), pp. 1322-1337.

Zimmerman, P. E., Voelker, D. R., McCormack, F. X., Paulsrud, J. R. and Martin, W. J.,2nd (1992) '120-kD surface glycoprotein of Pneumocystis carinii is a ligand for surfactant protein A', *The Journal of Clinical Investigation,* 89 (1), pp. 143-149.

Zipfel, P. F. (2009) 'Complement and immune defense: from innate immunity to human diseases', *Immunology Letters,* 126 (1-2), pp. 1-7.

Zipfel, P. F. and Skerka, C. (2009) 'Complement regulators and inhibitory proteins', *Nature Reviews.Immunology,* 9 (10), pp. 729-740.

8 List of publications

Paudyal, B., Kouser, L., Jones, L., Sim, R.B., Kishore, U. Stenbeck, G. (2018) Iron Particle induces cytokine, chemokines and growth factor production and complement activation. **Manuscript in preparation**.

Kouser, L., **Paudyal, B**., Kaur, A., Stenbeck, G., Jones, L. A., Abozaid, S. M., Stover, C. M., Flahaut, E., Sim, R. B. and Kishore, U. (2018) 'Human Properdin Opsonizes Nanoparticles and Triggers a Potent Pro-inflammatory Response by Macrophages without Involving Complement Activation', *Frontiers in Immunology,* 9 pp. 131. (**Joint first authorship)**

Pondman, K. M., **Paudyal, B**., Sim, R. B., Kaur, A., Kouser, L., Tsolaki, A. G., Jones, A. L., Khan, S.,C., Khan, A. H., Ten Haken, B., Stenbeck, G. and Kishore, U. (2017a) 'Pulmonary surfactant protein SP-D opsonises carbon nanotubes and augments their phagocytosis and subsequent pro-inflammatory immune response', *Nanoscale,* 9 (3), pp. 1097-1109. (**Joint first authorship)**

Pondman, K. M., Salvador-Morales, C., **Paudyal, B**., Sim, R. B. and Kishore, U. (2017b) 'Interactions of the innate immune system with carbon nanotubes', *Nanoscale Horizons,* 2 pp. 174-186.

Pondman, K. M., Tsolaki, A. G., **Paudyal, B**., Shamji, M. H., Switzer, A., Pathan, A. A., Abozaid, S. M., Ten Haken, B., Stenbeck, G., Sim, R. B. and Kishore, U. (2016) 'Complement Deposition on Nanoparticles Can Modulate Immune Responses by Macrophage, B and T Cells', *Journal of Biomedical Nanotechnology,* 12 (1), pp. 197-216.

Pondman, K. M., Pednekar, L., **Paudyal, B**., Tsolaki, A. G., Kouser, L., Khan, H. A., Shamji, M. H., Ten Haken, B., Stenbeck, G., Sim, R. B. and Kishore, U. (2015) 'Innate immune humoral factors, C1q and factor H, with differential pattern recognition properties, alter macrophage response to carbon nanotubes', *Nanomedicine : Nanotechnology, Biology, and Medicine,* 11 (8), pp. 2109-2118.