From the Institute of Environmental Medicine
Division of Biochemical Toxicology
Karolinska Institutet, Stockholm, Sweden

PROGRAMMED CELL CLEARANCE: MECHANISMS AND CONSEQUENCES OF PHAGOCYTOSIS OF APOPTOTIC CELLS

Erika Witasp

Karolinska Institutet

Stockholm 2009

Cover: TEM-image of a primary human macrophage engulfing an apoptotic neutrophil (photo: Kjell Hultenby).

Published by Karolinska Institutet. Printed by Universitetsservice US-AB.

ISBN 978-91-7409-293-6

Till mina nära och kära

ABSTRACT

Programmed cell clearance involves the engulfment of apoptotic cells by professional phagocytes (macrophages) or neighboring cells. During normal development and tissue regeneration a massive number of cells undergo apoptosis. The subsequent removal of the cell corpses by phagocytes is crucial to prevent disintegration of the cell membrane and leakage of noxious constituents into surrounding tissues. Efficient phagocytosis requires recognition or "eat-me" signals, soluble bridging molecules and phagocytosis receptors. Phosphatidylserine (PS) is externalized on the plasma membrane during apoptosis and is one of the most well-studied recognition signals for phagocytosis. The importance of PS exposure was addressed in the present thesis using different *in vitro* models. We found that plasma membrane blebbing could be dissociated from other features of the apoptotic program. PS-positive cells that failed to display membrane blebbing during apoptosis were observed to escape engulfment by macrophages. However, the PS-binding bridging molecule milk fat globule epidermal growth factor 8 (MFG-E8) increased the efficiency of phagocytosis of non-blebbing apoptotic cells. To further study the importance of PS in the clearance of neutrophils we established a model of macrophage-induced PS exposure. Macrophage-differentiated PLB-985 cells triggered caspase- and NADPH oxidase-independent PS externalization in primary human neutrophils. These neutrophils exhibited similar levels of PS exposure as neutrophils undergoing constitutive apoptosis. However, the phagocytosis of PLB-985-co-cultured neutrophils by human monocyte-derived macrophages (HMDM) was considerably lower, indicating that PS externalization alone is not sufficient for macrophage disposal of neutrophils. The addition of recombinant MFG-E8 restored macrophage engulfment of these cells. Moreover, PLB-985-co-cultured neutrophils displayed significantly lower surface expression and release of annexin I compared to spontaneous apoptotic neutrophils. Phagocytosis of macrophage-co-cultured neutrophils was promoted when annexin I-enriched cell culture medium was added, and this process was blocked by Boc1 (formyl peptide receptor/lipoxin receptor antagonist). A role for annexin I was also found in the engulfment of pre-apoptotic Jurkat cells briefly treated with agonistic anti-Fas antibody or recombinant Fas ligand. These cells secreted annexin I, and were ingested prior to the occurrence of common biomarkers of apoptosis, including PS exposure. Moreover, Boc1 markedly attenuated their engulfment. Similar findings were obtained when using primary human T cells. Furthermore, pre-apoptotic Jurkat cells induced lower macrophage production of TNF-α and higher production of IL-10 in comparison to apoptotic target cells. Finally, the interaction of HMDM and mesoporous silica particles was examined. Efficient and active internalization of mesoporous silica particles of different sizes was observed and appeared to occur through a process of endocytosis. Uptake of mesoporous silica particles did not affect viability of human macrophages, or the function of these cells, including the ingestion of various classes of apoptotic or opsonized target cells. In summary, these studies contribute to our understanding of the important physiological process of programmed cell clearance and may have implications for chronic inflammation and autoimmune disease. Studies of the interaction of nanomaterials with phagocytes are also relevant for the development of these materials for biomedical applications.

LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. Witasp E., Uthaisang W., Elenström-Magnusson C., Hanayama R., Tanaka M., Nagata S., Orrenius S., Fadeel B. (2007) Bridge over troubled water: milk fat epidermal growth factor 8 (MFG-E8) promotes human monocyte-derived macrophage clearance of non-blebbing phosphatidylserine-positive target cells. Cell Death Differ. 14:1063-5 + Supplementary information

II. Jitkaew S., Witasp E., Zhang S., Kagan V., Fadeel B. (2008) Induction of caspase- and reactive oxygen species-independent phosphatidylserine externalization in primary human neutrophils: role in macrophage recognition and engulfment. J. Leukoc. Biol. Dec 23 [Epub ahead of print]

III. Zhang S., Witasp E., Lauwen M., Fadeel B. (2008) Brief cross-linking of Fas/APO-1 (CD95) triggers engulfment of pre-apoptotic target cells. FEBS Lett. 582: 3501-3508

IV. Witasp E., Kupferschmidt N., Bengtsson L., Hultenby K., Garcia-Bennett A., Fadeel B. (2008) Evaluating the toxicity and internalization of mesoporous silica particles of different sizes in human monocyte-derived macrophages. *(submitted for publication)*

Appendix I Witasp E., Kagan V., Fadeel B. (2008) Programmed cell clearance: molecular mechanisms and role in autoimmune disease, chronic inflammation, and anti-cancer immune responses. Curr. Immunol. Rev. 4: 53-69

ADDITIONAL RELEVANT PUBLICATIONS

I. Konduru N., Tyurina Y., Basova L., Feng W.H., Bayir H., Clark K., Rubin M., Stolz D., Vallhov H., Scheynius A., Witasp E., Fadeel B., Kichambare P., Star A., Kisin E., Murray A., Shvedova A., Kagan V. (2008) Phosphatidylserine functionalization targets single-walled carbon nanotubes to professional phagocytes *in vitro* and *in vivo*. PLoS ONE *(in press)*

II. Witasp E., Shvedova A., Kagan V., Fadeel B. (2008) Single-walled carbon nanotubes impair human macrophage engulfment of apoptotic cell corpses. Inhal. Toxicol. *(in press)*

CONTENTS

1 INTRODUCTION 1
1.1 APOPTOSIS 1
1.1.1 Apoptosis: programmed cell death 1
1.1.2 Apoptosis pathways 2
1.1.3 Role of apoptosis in disease 2
1.2 PROGRAMMED CELL CLEARANCE 3
1.2.1 Evolutionarily conserved genes in the engulfment of apoptotic cells 3
1.2.2 Mechanisms of clearance of apoptotic cells 4
1.2.3 Consequences of clearance of apoptotic cells 9
1.3 PROGRAMMED CELL CLEARANCE: ROLE IN DISEASE 10
1.3.1 Chronic inflammatory diseases 10
1.3.2 Role in autoimmune disease 12
1.3.3 Role in cancer treatment 13
1.4 ENGINEERED NANOPARTICLES 14
1.4.1 Size and definitions 14
1.4.2 Specific properties of nanoparticles 15
1.4.3 Engineered nanomaterials in medicine 15
1.5 NANOTOXICOLOGY: AN EMERGING DISCIPLINE 16
1.5.1 Potentially harmful particles 16
1.5.2 Mechanisms of toxicity 18
1.5.3 Cellular uptake of nanoparticles 19
1.5.4 Immunotoxicity of nanoparticles 20
2 AIMS OF THE STUDY 22
3 MATERIALS AND METHODS 23
3.1 CELL CULTURE PROCEDURES 23
3.2 VIABILITY ASSAYS 25
3.3 APOPTOSIS DETECTION 25
3.4 PHAGOCYTOSIS ASSAYS 26
3.5 DETECTION OF CELL SURFACE MARKERS 27
3.6 PROTEIN PRODUCTION AND ANALYSIS 28
3.7 MEASUREMENT OF CYTOKINES 29
3.8 ROS GENERATION 30
3.9 PREPARATION AND CHARACTERIZATION OF PARTICLES 30
3.10 ANALYSIS OF PARTICLE UPTAKE 32
4 RESULTS 34
4.1 PAPER I: Bridge over troubled water: milk fat globule epidermal growth factor 8 promotes human monocyte-derived macrophage clearance of non-blebbing phosphatidylserine-positive target cells 34
4.2 PAPER II: Induction of caspase- and reactive oxygen species-independent phosphatidylserine externalization in primary human neutrophils: role in macrophage recognition and engulfment 35
4.3 PAPER III: Brief cross-linking of Fas/APO-1 (CD95) triggers engulfment of pre-apoptotic target cells 37

4.4 PAPER IV: Evaluating the toxicity and internalization of mesoporous silica particles of different sizes in human monocyte-derived macrophages 38
5 GENERAL DISCUSSION 41
6 CONCLUSIONS 47
7 ACKNOWLEDGEMENTS 49
8 REFERENCES 51
9 APPENDIX I 63

LIST OF ABBREVIATIONS

APAF-1	Apoptotic protease-activating factor-1
ATP	Adenine triphosphate
BAI-1	Brain specific angiogenesis inhibitor 1
Bcl-2	B-cell lymphoma gene 2
Boc1	N-t-Boc-Phe-D-Leu-Phe-D-Leu-Phe
BSA	Bovine serum albumin
Caspase	Cysteine-dependent aspartate-specific protease
CB	Cytochalasin B
CD	Cytochalasin D
CED	Cell death abnormal
CGD	Chronic granulomatous disease
CNT	Carbon nanotubes
CRT	Calreticulin
DEVD-AMC	Asp-glu-val-asp-7-amino-4-methyl-coumarin
DMSO	Dimethyl sulfoxide
DPI	Diphenyliodonium
FADD	Fas-associating protein with death domain
FBS	Fetal bovine serum
FPRL-1	Formyl-peptide receptor-like-1
HMDM	Human monocyte-derived macrophage
HMGB1	High mobility group box 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MFG-E8	Milk fat globule epidermal growth factor 8
MTT	3-(4,5-dimethylthiazolyl-2) -2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PI	Propidium iodide
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear neutrophil
PS	Phosphatidylserine
PSR	PS-receptor
ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus
STS	Staurosporine
SWCNT	Single-walled carbon nanotubes
TAMRA	5(6)-carboxytetramethyl-rhodamine N-hydroxy-succimide ester
Tim4	T-cell immunoglobulin- and mucin-domain-containing molecule
TNF-α	Tumour necrosis factor-α
TRAIL	TNF-associated apoptosis-inducing ligand
zVAD-fmk	Benzyloxycarbonyl-val-ala-asp-fluoromethylketone

1 INTRODUCTION

1.1 APOPTOSIS

1.1.1 Apoptosis: programmed cell death

Apoptosis is a morphologically defined type of cell death first described by Kerr, Wyllie, and Currie (Kerr et al., 1972) visibly distinct from death occurring in pathological conditions. Naturally occurring cell death during development was reported earlier in the literature (Lockshin et al., 2000) and was described as programmed cell death. Later it became evident that programmed cell death not only was initiated at a certain time and at specific sites, but also that it was controlled by a genetic program. Apoptosis is a physiological process of cell death playing a crucial role during development and normal tissue homeostasis (Jacobson et al., 1997). Moreover, apoptosis is also important for a functional immune system in removing undesirable cells (Thompson, 1995). The apoptotic cell undergoes biochemical and morphological changes by a sequential series of strictly controlled events finally resulting in the phagocytosis of the dying cells. A family of cysteine-dependent aspartate proteases termed caspases are to a high degree responsible for the morphological and biochemical changes in apoptosis (Hengartner, 2000). Extensive studies of cell death during embryogenesis in *Caenorhabditis elegans* have revealed conserved pathways of programmed cell death, and resulted in the identification of genes that are regulating cell death (Ellis and Horvitz, 1986; Ellis et al., 1991b). The protein encoded by the ced-3 gene in *C. elegans* is homologous to the mammalian family of caspases (Yuan et al., 1993). Morphologically, apoptosis is characterized by cell shrinkage, membrane blebbing, condensation of the nucleus, DNA cleavage at specific sites and formation of apoptotic bodies. The plasma membrane undergoes biochemical alterations allowing macrophages and other phagocytic cells to recognize and swiftly remove these dead cells. Importantly, this occurs before the plasma membrane integrity is lost. Apoptosis is therefore believed to be non-inflammatory (Savill and Fadok, 2000). In contrast, necrosis is characterized by a structural and functional collapse of the cell causing the release of cytotoxic mediators into the surrounding tissue. Ultimately this leads to an inflammatory response.

1.1.2 Apoptosis pathways

The process of apoptosis is initiated by a mitochondrial insult (intrinsic pathway) or by death receptor ligation (extrinsic pathway) (Danial and Korsmeyer, 2004). Activation of the Fas/APO-1 receptor triggers recruitment of the adapter protein Fas-associating protein with death domain (FADD) and processing of procaspase-8. Downstream events results in the activation of the main effector caspase-3. The intrinsic pathway is distinguished by activation of the mitochondria and involves loss of mitochondrial membrane potential with the concomitant release of pro-apoptotic proteins including cytochrome c, Smac/DIABLO and AIF. In the cytosol, cytochrome c associates with a protein complex termed the apoptosome together with apoptotic protease-activating factor (Apaf-1) and procaspase-9 in an ATP-dependent reaction (Fadeel et al., 2008). This results in processing of procaspase-9 which initiates a downstream cascade of proteolysis and activation of caspase-3 and caspase-7. In some types of cells (type II), the extrinsic and intrinsic pathways are connected via the targeting of caspase-8 to Bid. Truncated Bid translocates to the mitochondria where it promotes cytochrome c release, thereby serving as an amplification loop of the death receptor pathway. The apoptotic program is tightly regulated by proteins of the Bcl-2 family such as the anti-apoptotic Bcl-2, Bcl-XL and the pro-apoptotic Bax, Bak, Bad and Bid (Kim et al., 2006).

1.1.3 Role of apoptosis in disease

Apoptosis is crucial in the ablation of cells that have been produced in excess, that have developed improperly, or that have acquired genetic damage. Sometimes these processes are malfunctioning. Apoptosis is involved in various diseases when the apoptotic process is dysfunctional creating an imbalance between cell proliferation and cell death (Thompson, 1995). Essentially, this means too much or too little apoptosis. Inappropriate triggering of the cell death program leads to excessive loss of cells and degeneration of the tissue which is the principle mechanism for degenerative diseases. Extensive neuronal loss is implicated in neurodegenerative conditions including e.g. Alzheimer's and Parkinson's disease, and amytrophic lateral sclerosis (ALS). Moreover, in autoimmune diabetes T-lymphocytes kill pancreatic insulin-producing β-cells via interaction of Fas and Fas-ligand. Deterioration of the apoptotic machinery, on the other hand, leads to cell accumulation. Apoptosis plays a major role in the progression of cancer where mutations in genes involved in proliferation and the cell cycle causes excessive proliferation and insufficient apoptosis (Fadeel

and Orrenius, 2005). Apoptosis resistance is one of the six hallmarks of cancer described by Hanahan and Weinberg (2000).

In cancer therapy, new understandings of the apoptotic processes have directed the search of new potential drugs. Different approaches to produce novel cancer treatments are being realized, such as gene therapy and molecules that act on specific targets in the apoptotic pathway or regulate genes involved in apoptosis. For example, to overcome the resistance to apoptosis, attempts have been made to restore a functional p53 protein, a tumor suppressor that can upregulate the expression of a number of pro-apoptotic genes. Moreover, death receptor mediated signaling is being explored for cancer treatment; TNF-associated apoptosis-inducing ligand (TRAIL) is for instance tested in clinical and pre-clinical studies (Fadeel and Orrenius, 2005).

1.2 PROGRAMMED CELL CLEARANCE

1.2.1 Evolutionarily conserved genes in the engulfment of apoptotic cells

The execution of apoptosis is genetically programmed, and this also includes the final step: the clearance of the dying cell. Moreover, the process of clearance of apoptotic cells also appears to be conserved through evolution (Reddien and Horvitz, 2004). There are several genes responsible for engulfment described in the nematode *C. elegans*, and mammalian homologues have been identified. Most of the characterized genes so far are expressed in the engulfing cell, and only a few in the dying cell.

Two major pathways for the engulfment process in *C. elegans* have been recognized. The first pathway consists of CED-2, CED-5 and CED-12 (mammalian homologues CrkII, Dock180 and ELMO, respectively). In the second pathway CED-1, CED-6, and CED-7 (mammalian homologues CD91, GULP and ABCA1 respectively) cooperate. The two pathways are functionally linked by the downstream activation of CED-10 (Rac1) GTPase that leads to cytoskeletal reorganization necessary for engulfment (Kinchen et al., 2005). Mutations in only one of these sets of genes have relatively few unengulfed cell corpses in *C. elegans*. In contrast, animals with mutations in both sets of genes have many unengulfed corpses. These observations suggest that the two sets of genes are involved in distinct and partially redundant processes that act in the engulfment of cell corpses (Ellis et al., 1991a). Recent work

has shown that the engulfment pathways encoded by these genes in *C. elegans* are not specific for cells undergoing apoptotic cell death (Chung et al., 2000). Rather, these genes are required for the clearance of both apoptotic and necrotic corpses.

An important difference distinguishing C. *elegans* from mammals is that the nematode lacks an immune system and therefore dead cells are usually removed by neighboring cells (Robertson and Thomson, 1982). In mammalian systems both professional and non-professional phagocytes are crucial in the removal of cell corpses. The fruit fly, *Drosophila melanogaster* have professional phagocytes (haemocytes) as well, and studies have revealed genes that are essential for cell corpse engulfment. The *Drosophila* homologue of CED-1, Draper, has been implicated in phagocytosis of apoptotic cells (Manaka et al., 2004). Recently, a *Drosophila* Junctophilin protein Undertaker (UTA) was found to link Draper-mediated phagocytosis to Ca^{2+} homeostasis, revealing a previously uncharacterized role for the CED1/6/7 pathway (Cuttell et al., 2008). Furthermore, macrophages in the *Drosophila* embryo specifically express Croquemort (related to the mammalian receptor CD36) which is essential for efficient phagocytosis of apoptotic corpses (Franc et al., 1999).

1.2.2 Mechanisms of clearance of apoptotic cells

The mechanisms of programmed cell clearance involve receptor-ligand interactions and include Fc receptor-mediated, complement-dependent and pattern recognition molecular controlled pathways (Appendix I). The great numbers of different recognition signals, co-factors and receptors involved in the phagocytosis process have some redundant functions; however, some recognition systems are cell-specific with non-overlapping function. Furthermore, the recognition signals may play a role in the attachment of the target cell to the macrophage, others are important for the subsequent event of internalization of the cell corpse (Hoffmann et al., 2001). This two-step process of tethering and engulfment may be crucial for the tight regulation of the cell clearance process and protect against the uptake of viable cells. Receptor-ligand interactions may act in a proposed model of an engulfment synapse (Grimsley and Ravichandran, 2003) that would allow signal amplification in the phagocyte of low avidity interactions between ligand and receptor (Gardai et al., 2006). This requires a redistribution of ligands into patches on the plasma membrane of the dying cells and adds another dimension to the regulation of apoptotic cell clearance.

1.2.2.1 Ligands

Various types of recognition molecules or eat me signals including sugars, proteins lipids and nucleic acids have been implicated in the clearance of apoptotic cells by macrophages (Appendix I). Phosphatidylserine (PS) is one of the best studied and well recognized "eat-me" signals that trigger specific recognition and removal by macrophages (Fadok et al., 1992). PS exposure on apoptotic cells is considered to be a general feature of the phagocytosis of apoptotic lymphocytes by macrophages (Krahling et al., 1999). In addition, oxidation of the externalized PS contributes to the efficient recognition and phagocytosis (Arroyo et al., 2002; Kagan et al., 2002). The role of PS as a recognition signal for macrophage engulfment was first observed in erythrocytes with symmetric distribution of membrane phospholipids (McEvoy et al., 1986). Normally, the distribution of phospholipids in the cell plasma membrane is asymmetric, with restricted localization of PS in the inner part of the plasma membrane facing the cytosol. During apoptosis PS is externalized on the outer leaflet of the plasma membrane by a mechanism that is not fully understood; however, the process has been demonstrated to require calcium and ATP, and to be a mitochondria-dependent event. PS externalization and the subsequent recognition and engulfment can be dissociated from other features of the apoptotic program in Fas type I (mitochondria independent) cells (Uthaisang et al., 2003). The present view on PS translocation involves activation of a phospholipid scramblase that enhances bidirectional phospholipid flip-flop across the membrane bilayer and inactivation of an aminophospholipid translocase, which would normally return PS to the inner membrane leaflet (Bratton et al., 1997; Gleiss et al., 2002). ABC1 (ATP-binding cassette transporter), implicated in the normal cell membrane-lipid turnover has also been shown to promote Ca^{2+}-induced exposure of PS at the cell membrane (Hamon et al., 2000). Recent studies have demonstrated PS exposure on the surface of apoptotic cells in *C. elegans* and a role of PS in cell corpse engulfment indicating that PS externalization is a conserved recognition signal in nematodes (Wang et al., 2007; Venegas and Zhou, 2007; Zullig et al., 2007).

The glucocorticoid-inducible protein annexin I is an anti-inflammatory mediator expressed on the surface of apoptotic Jurkat cells and primary T cells, but not on apoptotic thymocytes (Arur et al., 2003; Fan et al., 2004). Annexin I promotes calcium-dependent apoptosis in neutrophils (Solito et al., 2003) and was

demonstrated to be required for efficient uptake of apoptotic cells by non-professional phagocytes (Fan et al., 2004). Apoptotic cells release annexin I and its cleavage products (Maderna et al., 2005; Scannell et al., 2007) promoting non-inflammatory phagocytosis by acting as bridging molecules that link phagocytes and target cells (Fan et al., 2004). Annexin I seems to co-localize with PS, and PS receptor clustering around the dying cells is reduced in cells with silenced annexin I expression. In addition, downregulation of the annexin I homolog nex-1 in *C. elegans* impaired phagocytosis of cell corpses supporting the idea of conservation of this uptake pathway (Arur et al., 2003). Generation of annexin $I^{-/-}$ mice confirmed the functional importance of annexin I in the resolution of inflammation (Hannon et al., 2003) and in the clearance of zymosan and bacteria (Yona et al., 2006).

1.2.2.2 Receptors

A variety of macrophage receptors are involved in the recognition and engulfment of apoptotic cells (Appendix I). At present, more engulfment receptors than recognition signals have been identified. However, while most receptors have been demonstrated to be involved in the recognition of apoptotic cells *in vitro*, a considerable less number has been shown to be important *in vivo* (Henson et al., 2001). The rationale for the great number of phagocyte receptors can to some extent be tissue and cell type specific differences in the engagement of receptors. Further, the phagocytosis process may systematically activate different receptors, some involved in the initial tethering of apoptotic cells and some involved in cytoskeletal rearrangement needed for the internalization (Henson et al., 2001). Distinct receptor ligation may also give rise to either pro- or anti-inflammatory responses as a consequence of engulfment (Stuart and Ezekowitz, 2005). The engulfment receptors include lectin-like receptors, the so-called PS-receptor (PSR), the class A scavenger receptor (SRA), CD36 (a class B scavenger receptor), CD68 (macrosialin, a class D scavenger receptor), the integrin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$, the LPS receptor CD14, and the calreticulin-CD91 complex.

The class B scavenger receptor CD36 have a wide variety of different ligands (Febbraio et al., 2001) and share homology with *Drosophila melanogaster* Crouqemort (Franc et al., 1999). CD36 was first reported to cooperate with thrombospondin and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis by Savill and colleagues (Savill et al., 1992) and also serves a co-factor during PS dependent clearance (Fadok et al., 1998b).

Moreover, studies by Kagan et al (Kagan et al., 2003) showed that anti-CD36 antibodies were unable to inhibit phagocytosis of Jurkat cells enriched with only PS. In contrast, anti-CD36 together with anti-PSR were effective in suppressing phagocytosis of target cells enriched with both PS and oxidized PS indicating that the preferential ligand for CD36 was oxidized PS. Indeed, macrophage recognition of apoptotic cells via CD36 was recently demonstrated to occur via interactions with membrane associated oxidized PS and to a lesser extent with oxidized phosphatidylcholine but not nonoxidized PS molecular species (Greenberg et al., 2006). Importantly, CD36 has an essential role in macrophage clearance of apoptotic cells *in vivo* (Greenberg et al., 2006). In CD36 knock out mice apoptotic cells were accumulated at inflammatory sites indicating compromised ability to remove apoptotic cells.

Divergent reports have discussed the involvement and importance of a candidate receptor for PS (PSR) in apoptotic cell clearance. The first study, published in 2000 by Fadok and colleagues (Fadok et al., 2000), identified a protein mediating specific recognition and phagocytosis of apoptotic cells in a PS dependent manner. The protein was found to be highly homologous to genes in *Caenorhabditis elegans* and *Drosophila melanogaster*. Indeed, a PSR-homolog was found in *C. elegans* upstream of the engulfment genes CED-2, CED-5, CED-10 and CED-12 that was able to bind to PS (Wang et al., 2003). Loss of function mutant revealed a moderate impairment of corpse clearance in the nematode. In contrast, Arur and colleagues (Arur et al., 2003) found no significant accumulation of *C. elegans* pharyngeal corpses when PSR was downregulated.

Using erythrocytes coated with different surface modifications Hoffman (Hoffmann et al., 2001) showed that cell surface exposure of PS was absolutely crucial for engulfment by human macrophages. However, ligation of PS to the PSR was not sufficient to stimulate tethering or ingestion of apoptotic cells by macrophages. The initial attachment to the phagocyte required activation of additional receptors, and subsequently PS-PSR-dependent internalization was enabled. More recent studies have demonstrated a nuclear localization of the PSR and a number of nuclear localization signals were identified in the PSR sequence (Cui et al., 2004). The question of the physiological relevance of PSR in mammals has been approached with PSR-knockout mice by several groups. The common findings from these studies

appear to be abnormal developmental effects and neonatal lethality. Impairment in cell corpse engulfment has however only been demonstrated by some of these studies (Li et al., 2003; Kunisaki et al., 2004). In contrast, Böse and colleagues (Böse et al., 2004) concluded that engulfment of apoptotic cells was normal in PSR knockout mice; however, the cytokine production after stimulation with apoptotic cells or LPS was affected.

Recently, several new candidates for PSR have been reported. An antibody against mouse peritoneal macrophages was found to strongly inhibit the PS-dependent engulfment of apoptotic cells. The identified protein was a type I transmembrane protein called Tim4 (T-cell immunoglobulin- and mucin-domain-containing molecule) and was expressed in Mac1-positive cells in various mouse tissues, including spleen, lymph nodes and fetal liver. The ability of fibroblasts to ingest apoptotic cells was enhanced by the expression of Tim4. Moreover, mice administered with anti-Tim4 antibodies exhibited impaired engulfment of apoptotic cells by thymic macrophages, and development of autoantibodies (Miyanishi et al., 2007). The membrane bound multifunctional receptor stabilin-2 was reported to recognize PS on aged red blood cells and apoptotic cells. Downregulation of stabilin-2 expression in macrophages significantly inhibited phagocytosis, and anti-stabilin-2 monoclonal antibody provoked the release of anti-inflammatory cytokine TGF-β (Li and Baker, 2007). Brain specific angiogenesis inhibitor 1 (BAI1) was identified as a receptor that recognizes apoptotic cells by direct binding to PS. This receptor acts upstream of ELMO and forms trimeric complex with ELMO and Dock180. Functional studies suggest that BAI1 cooperate with ELMO/Dock180/Rac to directly recruit Rac-GEF complex to promote engulfment of apoptotic cells. Blocking the PS-binding domains of BAI1 inhibited engulfment of apoptotic cells by primary murine astrocytes *in vitro* and by peritoneal macrophages *in vivo* (Park et al., 2007).

1.2.2.3 Bridging molecules

Recognition signals can bind either directly to phagocytosis receptors or indirectly via membrane-bound co-factors, or via soluble bridging molecules (Appendix I). There are several known bridging molecules including thrombospondin (TSP1) (Savill et al., 1992), β2 glycoprotein 1 (β2GP1), protein S (Anderson et al., 2003), the growth arrest specific gene product GAS-6 (Wu et al., 2005), complement activation

products, and milk-fat globule epidermal growth factor 8 (MFG-E8) (Hanayama et al., 2002).

MFG-E8 (also known as lactadherin) is a glycoprotein first found associated with mammary epithelial cells. Moreover, MFG-E8 is secreted by certain classes of macrophages (Hanayama et al., 2002) and immature dendritic cells and serves a molecular bridge between PS, preferably its oxidized counterpart (Borisenko et al., 2004), on the apoptotic cell and integrin receptors $\alpha_v\beta_3$ or $\alpha_v\beta_5$ through the RGD motif on the phagocyte. Upon ligation of MFG-E8 to the $\alpha_v\beta_5$ integrin on the phagocyte DOCK180-dependent Rac1 activation is triggered for the phagocytosis of apoptotic cells (Akakura et al., 2004). Developmental endothelial locus-1 (Del-1) is functionally homologous to MFG-E8 and is found in fetal liver and thymic (resident) macrophages. In contrast, MFG-E8 is expressed by thiolglycollate-elicited macrophages in germinal centers. These two opsonins are expressed by different subsets of phagocytes and therefore play a non-redundant role in cell clearance of apoptotic cells (Akakura et al., 2004). Mice deficient in MFG-E8 developed splenomegaly, with the formation of numerous germinal centers, and suffered from glomerulonephritis as a result of autoantibody production demonstrating a critical role of MFG-E8 in removing apoptotic B cells in the germinal centers (Hanayama et al., 2004). Similarly, mice injected with a MFG-E8 mutant carrying a point mutation in RGD motif developed autoantibodies and IgG deposition in the glomeruli (Asano et al., 2004). Moreover, MFG-E8 was upregulated and shown to be critical during the involution of the mammary gland in mice. MFG-E8 deficiency caused the accumulation of a large number of milk-fat globules and apoptotic epithelial cells in the mammary ducts during involution (Hanayama and Nagata, 2005; Nakatani et al., 2006).

1.2.3 Consequences of clearance of apoptotic cells

The rapid *in vivo* clearance of apoptotic cells by macrophages is a crucial physiological process fundamental in tissue homeostasis in several ways. By avoiding intracellular antigens to be released from the dying cells autoimmune responses is prevented. Importantly, programmed clearance also plays an active role in the resolution of inflammation. Neutrophils are key players in the inflammatory response and are recruited and accumulated upon tissue insult. At inflammatory sites, apoptotic neutrophils are recognized and ingested by macrophages thereby limiting the degree

of tissue injury by preventing cell lysis and liberation of harmful cellular constituents (Savill et al., 1989). In addition, phagocytosis of apoptotic neutrophils inhibits pro-inflammatory cytokine production and increases production of anti-inflammatory mediators (Fadok et al., 1998a). Furthermore, engulfment of apoptotic cells can attenuate activation of macrophages by reducing signal transduction pathways (Tassiulas et al., 2007). Convergent studies demonstrate that the regulation of inflammatory responses by the apoptotic cells is exerted directly upon binding to the macrophage, independent of subsequent engulfment (Cvetanovic and Ucker, 2004; Kim et al., 2004; Lucas et al., 2006) Cell-cell contact with apoptotic cells or treatment of macrophages with PS suppresses the pro-inflammatory cytokine production and promotes the release of anti-inflammatory cytokines (Kim et al., 2004). A newly suggested role for macrophages in the active resolution of inflammation was reported by Tyurina and colleges (Tyurina et al., 2007). The study suggested that macrophages can directly participate in apoptotic cell clearance by triggering viable target cells to expose PS. Production of reactive oxygen and nitrogen species by activated macrophages inhibited the aminophospholipid translocase (APLT) by S-nitrosylation/oxidation and induced PS exposure in the target cells with no other apoptotic characteristics. The subsequent recognition and engulfment dampened the inflammatory mechanism.

Phagocytosis of apoptotic cells may result both in life and death. Growth factors such as vascular endothelial growth factor can be produced by nonprofessional phagocytes, such as endothelial or epithelial cells, when engulfing neighboring apoptotic cells (Golpon et al., 2004). In contrast, active promotion of cell death by phagocyte clearance of target cells has also been proposed. Genes involved in cell corpse clearance in *C. elegans* have been shown to cooperate with CED-3 to push early apoptotic cells to definitively proceed into irreversible death. Thus, genes that mediate corpse removal can also function to actively kill cells (Hoeppner et al., 2001; Reddien et al., 2001).

1.3 PROGRAMMED CELL CLEARANCE: ROLE IN DISEASE

1.3.1 Chronic inflammatory diseases

Inflammatory responses are necessary for the defense against invading pathogens; however, these immune responses need to be tightly regulated since persistence of

inflammation can lead to tissue damage and impairment of organ function. Chronic granulomatous disease (CGD) is a rare hereditary chronic inflammatory condition characterized by mutations in different NADPH oxidase subunits in neutrophils and macrophages (Dinauer, 2005). Patients with CGD suffer from recurrent, severe bacterial and fungal infections and frequently have chronic tissue granulomas. Neutrophils from CGD patients are able to undergo normal apoptosis but they are defective for phorbol myristate acetate (PMA)-induced plasma membrane exposure of PS (Fadeel et al., 1998) and have impaired production of the anti-inflammatory mediator prostaglandin D(2) (Brown et al., 2003). Hampton and colleagues (Hampton et al., 2002) injected bacteria into peritoneum of mice lacking a functional NADPH-oxidase and observed an elevated number of neutrophils in the inflammatory exudates, indicating impaired recognition and clearance of the neutrophils. Similarly, treatment of normal neutrophils with a pharmacological inhibitor of the NADPH oxidase blocked PMA-induced PS exposure and these cells escaped engulfment by macrophages *in vitro* (Sanmun et al., submitted manuscript). Moreover, macrophages from CGD patients showed decreased phagocytic capacity of normal PS-positive target cells (Sanmun et al., submitted manuscript). In a study by Brown and colleagues (Brown et al., 2003) TGF-β production was shown to be diminished in CGD-macrophages during phagocytosis of apoptotic debris and invading pathogens. This suggests that programmed cell clearance is dependent on a functional NADPH oxidase not only in the target cell, but also in macrophages. Defective cell clearance could perhaps contribute to the formation of tissue-destructive granulomas and chronic inflammation evidenced in CGD patients.

On the other hand, successful phagocytosis of apoptotic cells was reported to be an important mechanism in the resolution of joint inflammation (van Lent et al., 2001). Injection of apoptotic leukocytes into the knee joint in mice prior to induction of immune complex-mediated arthritis significantly blocked neutrophil infiltration and inhibited the onset of experimental arthritis. As a therapeutic intervention for persistent inflammation it can be envisioned that this concept could be utilized to modulate immune responses. Clearly, the interaction of apoptotic cells and macrophages triggers production of anti-inflammatory cytokines (Fadok et al., 1998a), suppress ROS and RNS production (Serinkan et al., 2005) and limit tissue injury. These effects are largely PS dependent and also living cells coated with PS

and PS enriched liposomes can stimulate anti-inflammatory responses (Fadok et al., 2001).

1.3.2 Role in autoimmune disease

Disruption of programmed cell clearance may trigger undesirable immune responses to self that eventually could lead to systemic autoimmunity. Apoptotic cells proceed into secondary necrosis if they escape engulfment resulting in the release of danger signals directing the immune response to be immunogenic (Savill and Fadok, 2000). Accumulation of dying cells, or failure in the target cell-triggered signaling in the responding phagocyte, plays a role in the development of autoimmunity. This has been shown in several experimental models. Systemic exposure of normal mice to irradiated syngeneic apoptotic thymocytes induced production of anti-nuclear, anticardiolipin and anti-ssDNA antibodies (Mevorach et al., 1998). Moreover, knock out models of C1q, mannose binding lectin, C-reactive protein, serum amyloid P, and pentraxin 3, all involved in acute phase responses and in the clearance of apoptotic cells, displayed induced autoimmune responses (Kravitz and Shoenfeld, 2006). MFG-E8 deficient mice developed splenomegaly, with the formation of numerous germinal centers, and suffered from glomerulonephritis as a result of autoantibody production (Hanayama et al., 2004). Similarly, intravenous injection of a MFG-E8 mutant into mice induced production of autoantibodies, including antiphospholipid antibodies and antinuclear antibodies (Asano et al., 2004).

PS exposed on surface blebs of apoptotic cells has been found to induce the production of antiphospholipid antibodies and contribute to the immunogenicity of systemic lupus erythematosus (SLE) (Casciola-Rosen et al., 1994). SLE is an autoimmune condition associated with defects in apoptotic cell clearance. Accumulation of apoptotic cells in the germinal centers of the lymph nodes is frequently observed in SLE-patients. Moreover, granulocytes and monocytes in whole blood of SLE patients have reduced engulfing capacity and attraction signals for macrophages are reduced (Gaipl et al., 2007). Production of autoantibodies to various immunogenic epitopes direct non-inflammatory engulfment of apoptotic cells to become inflammatory by immune complex formation (Herrmann et al., 1998). Deficiencies in the complement system have been shown to be implicated in the pathogenesis of SLE in multiple ways (Lewis and Botto, 2006). In addition, autoantibodies against scavenger receptors MARCO and SR-A have been found in

sera from SLE patients (Wermeling et al., 2007). In knock out mice, these receptors have been shown to be critical for proper cell clearance in the marginal zone which is necessary for the regulation of B cell tolerance. When deleting MARCO, SR-A or both receptors, mice exhibited lower tolerance threshold and developed higher anti-DNA antibody titres spontaneously and after injection of apoptotic cells.

1.3.3 Role in cancer treatment

Programmed cell clearance of apoptotic cells is predominantly a non-immunogenic process, however in some cases engulfment of apoptotic cells by antigen-presenting dendritic cells may lead to an immunogenic response. This is important in the normal protection against cancer cells by the immune system. Numerous innate and adaptive immune effector cells and molecules participate in the recognition and destruction of cancer cells, a process that is known as cancer immunosurveillance (Zitvogel et al., 2006). The immunogenicity of apoptotic cells appears to be strongly dependent on the antigen presenting cells involved in the processing and presentation of the antigenic material contained in the apoptotic cells, and on the balance among immunomodulatory cytokines. Immunization with dendritic cells, but not macrophages, pulsed with apoptotic cells primes tumor-specific cytotoxic T lymphocytes and confers protection against tumor challenge (Ronchetti et al., 1999).

In the case of chemotherapy treatment of cancer, induction of immunogenic tumor cell death may be advantageous because it would trigger the immune system to contribute by abolishment of chemotherapy-resistant cancer cells. One could speculate that among the vast range of recognition signals, some may play a role as a non-inflammatory mediator, while other signals may be important in signaling for activation of the immune system. Thrombospondin and its heparin-binding domain, released from apoptotic cells, bind to immature dendritic cells and improve engulfment and induce tolerance to the engulfed material prior to the interaction with apoptotic cells (Krispin et al., 2006). In contrast, exposure of calreticulin (CRT) on apoptotic cancer cells has been demonstrated to induce an immunogenic signal in immunocompetent mice (Obeid et al., 2007). CRT by itself is not immunostimulatory, since neither CRT alone nor viable cells coated with CRT trigger this response (Obeid et al., 2007). A key feature of the immunogenic apoptosis seems to be caspase-mediated cell death with CRT exposure (Obeid et al., 2007; Waterhouse

and Pinkoski, 2007) in combination with danger signals e.g. heat shock proteins and high mobility group box 1 (HMGB1) (Tesniere et al., 2008).

There are some studies suggesting that tumor cells have the ability to engulf other tumor cells. In metastatic melanoma this cannibalistic activity seemed to increase the survival of the tumor cells when nutrient supply was scarce (Lugini et al., 2006). In contrast, both in cell lines of human small cell carcinoma of the lung (SCCL) and in fresh tumor biopsies from SCCL patients cells were observed to have internalized neighboring cells, leading to death of the engulfed cells. This cell cannibalism was dependent of unknown serum factor(s) and is suggested to have therapeutic potential (Brouwer et al., 1984). A similar notion is presented by the "buried alive" hypothesis (Fadeel et al., 2004) suggesting that directed phagocytosis of cancer cells in the absence of a death signal could be a novel approach to remove these unwanted cells without the undesired effects observed during conventional treatment.

Cancer cell cannibalism of neighboring cells may result in transfer of genetic material within a solid tumor. The incoming DNA can be re-utilized if the receiving cell does not have an intact DNA-repair system (Bergsmedh et al., 2001). This horizontal gene transfer may result in an altered tumorogenic phenotype that gives growth advantages to the tumor (Bergsmedh et al., 2001).

1.4 ENGINEERED NANOPARTICLES

1.4.1 Size and definitions

The definition of nanoparticles is materials with at least one dimension in the nano-scale, i.e. less than 100 nm (Lewinski et al., 2008). Nanomaterials are found naturally everywhere in various biological contexts from the gecko foot-hair with its hundreds of nano-tips, to viruses, exosomes (endogenously produced nanoparticles), and biomolecules. Humans have been exposed to nanoparticles throughout their evolutionary phases; however, this exposure has been significantly increased in modern life mainly because of the industrial revolution (Oberdörster et al., 2005). One example is ultrafine particles derived from combustion sources such as motor vehicle and industrial emissions. More recently, development of nanoscale technologies further adds to the potential sources of human exposure to nanomaterials (Gwinn and Vallyathan, 2006). The main characteristics of engineered nanomaterials,

quite distinct from ambient nanomaterials or unintentionally generated nanoparticles, are obviously the homogeneity of the material composition and the narrow size distribution (Oberdörster et al., 2005). Nanotechnology is envisioned as a new industrial revolution with major impact of the life of citizens (Nel et al., 2006). The specific physicochemical properties of nanoparticles make them very attractive for commercial and medical development (Nel et al., 2006). Nanoparticles are increasingly used in cosmetics, clothes, electronics, aerospace and computer industry (Nel et al., 2006). There is also a potential use of nanomaterials in various biomedical applications including diagnostics, as active biological nanosensors and in targeted drug delivery (Moghimi et al., 2005).

1.4.2 Specific properties of nanoparticles

Nanomaterials have specific physicochemical properties different to bulk materials of the same composition. The key factor is evidently their small size which is dramatically illustrated when comparing the number of particles and the surface area of nanoparticles versus that of larger particles with a fixed mass. The number of nanoparticles increases exponentially along with the surface area as the size decreases. Furthermore, the ratio of surface to total molecules increases exponentially as particle size is reduced at the nanoscale. This plays a crucial role in determining the reactivity of the material and is an important feature to define the chemical and biological properties of nanoparticles (Oberdörster et al., 2005).

Eight to ten atoms span one nanometer and accordingly, at the nanoscale (below 50 nm), materials acquires some of the properties of molecules resulting in optical, electrical and magnetic behaviors different from those of the same material at a larger scale. Hence, the characteristics of engineered nanomaterials are determined by size, chemical composition, surface structure, solubility, shape and aggregation (Nel et al., 2006).

1.4.3 Engineered nanomaterials in medicine

Various different classes of nanoparticles are intended for use in medicine: liposomes, emulsions, polymers, ceramic nanoparticles, metallic particles, gold shell nanoparticles, carbon nanomaterials and quantum dots (Medina et al., 2007). Carbon nanomaterials are possibly the most renowned products of nanotechnology to date, including fullerenes, nanofibers, nanotubes and a variety of related forms. The 2008

Kavli Prize in Nanoscience was awarded to Dr Sumio Iijima for his contribution to the field of carbon nanotubes (CNT) (Javey, 2008). CNT are rolled-up sheets of graphite and are extremely strong fibers with good semiconducting properties. In addition to the technical applications, CNT also have a potential medical use ranging from sensors for detection of genetic or other molecular abnormalities, to substrates for cell growth in tissue regeneration, and in drug delivery systems (Lacerda et al., 2006).

The utilization of nanomaterials in medicine focuses to a large extent on the improved and targeted delivery of pharmaceutical, therapeutic and diagnostic agents. Through encapsulation of the agent of interest, diffusion controlled release of the active molecule can be achieved. The problem with water insoluble drugs can be solved by coated nanocrystals. These approaches would potentially improve the response and reduce the unwanted side effects. Moreover, the use of nanocarriers in the development of effective vaccines is very promising (Xiang et al., 2006).

Specific targeting of tumor cells for selective killing is highly desirable. Nanocarriers with tumor-specific antibodies or ligands attached are explored for this purpose. *In vivo* studies in mice with radiolabeled single-walled carbon nanotubes (SWCNT) coated with polyethylene-glycol linked to an RGD-peptide revealed highly efficient uptake in tumors positive for integrin receptors (Liu et al., 2007). Another example is to take advantage of the fact that the folate receptor expression is restricted to certain cancers and generally not present in most other tissues (Lu and Low, 2002). Folic acid could therefore be conjugated to nanocarriers for targeted delivery to cancer cells (Sudimack and Lee, 2000). In order to kill the tumor cells, the nanoparticles could either be loaded with cytotoxic drugs, or the properties of heat emission could be utilized (Moghimi et al., 2005). Continuous near-infrared radiation was reported to kill cancer cells *in vitro* because of excessive local heating by SWCNT (Kam et al., 2005).

1.5 NANOTOXICOLOGY: AN EMERGING DISCIPLINE

1.5.1 Potentially harmful particles

The rapid and promising development of nanotechnology will most likely benefit human life in various ways, but may also bring potential risks to health (Maynard et

al., 2006). Therefore an increasing area of research, i.e. nanotoxicology, has developed in parallel with nanorevolution (Donaldson et al., 2004) (Figure 1). To avoid past mistakes as new technologies are developed it is of great importance to take precautionary measures to identify potential risks with nanotechnologies as early as possible (Foss Hansen et al., 2008). As new nanomaterials are moving from the lab to the marketplace the exposure in occupational settings and through intentional administration will increase, and consequently also the environment will be exposed (Colvin, 2003). The problem is that a there is no comprehensive picture of the effects that engineered nanomaterials could have on living organisms and the environment (Maynard et al., 2006). Currently, there are too few studies addressing these questions. Some lessons from toxicological studies of airborne ultrafine particles can be learned (Oberdörster et al., 2005). The comparability of engineered nanoparticles to ultrafine particles suggests that the human health effects are expected to be similar (Gwinn and Vallyathan, 2006). However, as yet, there is no standard or consensus as to how toxicity of nanoparticles should be measured (Maynard and Aitken, 2007).

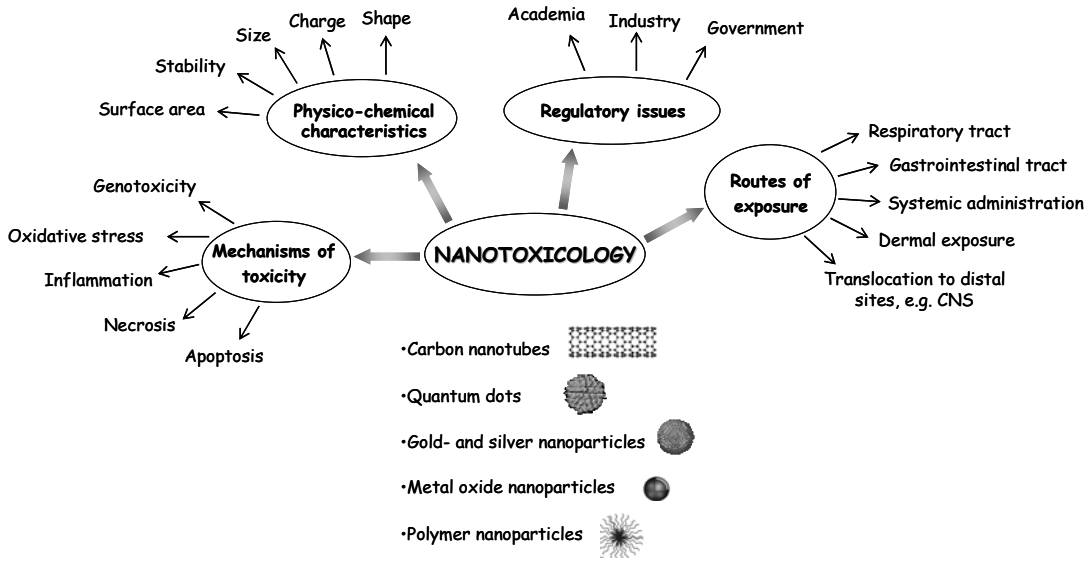

Figure 1. Complexity of nanotoxicology. Adapted from Vega-Villa et al., 2008.

Nanoparticles can enter the body through the skin, lungs or intestinal tract, and could induce adverse effects on various target organs (Fadeel et al., 2007). Moreover, engineered nanomaterials are intentionally administrated for various biomedical applications and could be designed to specifically target cells or tissues. Some nanoparticles readily travel throughout the body, deposit in target organs, penetrate

cell membranes, translocate to mitochondria, and may trigger adverse responses (Nel et al., 2006). Nanomaterials can be on the same scale as elements of living cells, including proteins, nucleic acids, lipids and cellular organelles. Being in the same size range as endogenous cell components opens up for wide possibilities but at the same time this property is also what makes them potentially dangerous (Fadeel et al., 2007). The smaller the particles are, the greater surface area they have per unit mass; and this feature makes nanoparticles very reactive in the cellular environment (Oberdörster et al., 2005).

A great challenge lies ahead to predict effects of nanoparticles in biological systems. The behavior of nanoparticles within the biological microenvironment, and the stability and cellular distribution of nanomaterials varies significantly with their chemical composition, morphology and size (Moghimi et al., 2005). Moreover, particle dosimetry is important to consider when doing systematic investigations of toxicity (Teeguarden et al., 2007). Using mass, number of particles, or surface area as measure of dose will influence the outcome and the conclusions of different studies. The challenge is to identify key features or tests that can be used to predict toxicity making it conceivable for the development of new and safe nanoparticles (Stone and Donaldson, 2006).

1.5.2 Mechanisms of toxicity

Various toxicological aspects of nanoparticles have been described in detail in several reports. However, the exact mechanism of nanoparticle toxicity in the target cell is still not completely clarified. The main interaction of nanoparticles with their biological environment occurs at the cellular level and in addition to the cell membrane, mitochondria and cell nucleus are considered as major cell compartments important for possible nanoparticle-induced toxicity (Unfried et al., 2007).

ROS generation and oxidative stress is currently a well accepted paradigm to explain the toxic effects of nanoparticles (Donaldson et al., 2001; Nel et al., 2006). ROS may be produced directly on the particle surface or indirectly produced by the cell as a response to the particle (Nel et al., 2006). In addition, metal trace impurities in nanomaterials may act as catalysts of oxidative stress (Kagan et al., 2006). If the generated ROS exceed the scavenging capacity of the cell oxidative stress and cellular damage will be generated. Cellular toxicity can be induced by impairment of

mitochondrial respiration. By targeting mitochondria silica particles inhibited the cellular respiration in human cancer cell lines in a time- and dose-dependent manner (Tao et al., 2008). Moreover, ROS function as second messenger in various signaling pathways; consequently, ROS production stimulated by nanoparticles may induce aberrant activation of signaling pathways leading to proliferation, or cell death.

In addition to ROS-related toxicity, other forms of injury, such as protein denaturation, membrane damage, DNA damage, immune reactivity, and the formation of foreign body granulomas may be caused by nanomaterials (Nel et al., 2006). For example, translocation of silica nanoparticles into the nucleus induced formation of intranuclear protein aggregates that inhibited transcription and cell proliferation, but not cell viability, in a panel of different cancer cell lines (Chen and von Mikecz, 2005).

An alternative theory on the mechanisms of toxicity by nanoparticles was proposed by Moss and Wong (2006). Their hypothesis is that the toxicity may be related to obstruction of cellular mechanisms due to particles masking or covering the inner and the outer surface of exposed cells. A study by Oberdörster et al. (Oberdörster et al., 1994) was reevaluated according to this hypothesis. Oberdörster et al. reported that the phagocytosing ability of alveolar macrophages was impaired by TiO_2 nanoparticles and suggested that the toxicity was related to the particle surface area. However, Moss and Wong calculated that the potential of the dose of nanoparticles used in the Oberdörster study was enough to cover each macrophage surface four times, and thus they conclude that the potential of obstruction of cellular processes existed.

1.5.3 Cellular uptake of nanoparticles

At present, the mechanisms of cellular uptake of engineered nanomaterials are not well described. Understanding cellular uptake is, however, important for predicting toxicity. Studies with various nanomaterials have revealed that several uptake mechanisms may be involved and the processes depend both on the cell type and the characteristics of the material. Some reports indicate that internalization of nanoparticles is completely independent of any of the known pinocytosis or endocytosis pathways. For instance, fluorescent polystyrene microspheres (1, 0.2, and 0.078 μm) were internalized through diffusion or adhesive interactions in pulmonary

macrophages and red blood cells *in vitro* (Geiser et al., 2005). In contrast, cationic polystyrene nanospheres (60 nm) were found to be taken up by cell specific pathways in RAW 264.7 macrophages and epithelial BEAS-2B cells respectively (Xia et al., 2008). In RAW macrophages the particles entered a LAMP-1 positive lysosomal compartment and in epithelial BEAS-2B cells the particles were taken up by a caveolae-mediated pathway. Furthermore, Harush-Frenkel and co-workers (Harush-Frenkel et al., 2007) reported that positively charged nanoparticles were internalized via the clathrin-mediated pathway. Internalization of mesoporous silica nanoparticles (110 nm) in human mesenchymal stem cells was demonstrated to be energy-dependent and the uptake was decreased by inhibitors of either phagocytosis or clathrin-mediated endocytosis (Huang et al., 2008). The importance of receptor-mediated uptake of unopsonized particles has been investigated by Arredouani and colleagues (Arredouani et al., 2005) and their findings established a major role of the class A scavenger receptor MARCO in this process. In addition, silica particle uptake and toxicity in alveolar macrophages from C57BL/6 mice was also demonstrated to be mediated by MARCO (Hamilton et al., 2006).

1.5.4 Immunotoxicity of nanoparticles

Studies have shown that nanoparticles have the ability to stimulate and/or suppress immune responses (Dobrovolskaia and McNeil, 2007). Upon systemic administration of engineered nanoparticles immune cells are potential targets and therefore it is highly relevant to study the interaction between these cells and nanomaterials. Nanomaterials can have either immunostimulatory properties, including inflammatory responses, or immunosuppressive properties. The immunological properties of nanomaterials could be desired (e.g. adjuvant effects) or considered as harmful side effects. In the case of undesired immune responses it is crucial to control every step of the production and handling of the material to avoid contamination of endotoxins e.g. lipopolysaccharide (LPS) (Vallhov et al., 2007) that would otherwise activate immune-competent cells.

Macrophages are activated in response to pathogens and an inflammatory response is induced involving the release of cytokines. Macrophages may act in a similar way in response to nanomaterials. *In vivo* studies of intratracheal aspiration of SWCNT in C57BL76 mice demonstrated an unusual inflammatory response with early onset of fibrosis and granulomas, accumulation of inflammatory cells and induced cytokine

production (Shvedova et al., 2005). Moreover, alveolar macrophages in SWCNT-challenged mice exhibited decreased bacterial clearance (Shvedova et al., 2005). Additional *in vitro* studies in human alveolar macrophages have shown that diesel exhaust particles impaired bacterial clearance (Lundborg et al., 2006), and phagocytic capacity in rat alveolar macrophages was decreased by aluminium nanoparticles (Wagner et al., 2007).

In order to make nanomaterials either more non-immunogenic or more recognizable to immune cells, the surface of nanomaterials may be functionalized. Macrophages poorly recognize and internalize pristine carbon nanotubes; however, coating of nanotubes with the phospholipid "eat-me" signal PS results in a directed recognition and efficient uptake of carbon nanotubes (Konduru et al., 2008). Recognition of PS-positive target cells triggers anti-inflammatory responses in macrophages, and accordingly PS-coated SWCNT were capable of suppressing LPS-induced TNF-α. One could therefore envision specific targeting of PS-coated SWCNT to macrophages and the regulation of functions of these cells, which could serve to mitigate toxic responses to these nanomaterials.

2 AIMS OF THE STUDY

The overall aim of the current thesis project is to investigate the mechanism of programmed cell clearance, i.e. the process of macrophage recognition and engulfment of apoptotic cells. The specific aims are to determine:

- the role of MFG-E8 in the phosphatidylserine (PS)-dependent uptake of apoptotic cells,

- the role of macrophage-induced PS exposure and annexin I in the engulfment of neutrophils,

- the role of annexin I in macrophage phagocytosis of pre-apoptotic target cells,

- the uptake of nanoparticles by primary human macrophages, and their effect on programmed cell clearance.

3 MATERIALS AND METHODS

Detailed descriptions of the materials and methods used in our studies can be found in the publications and manuscripts included in this thesis. The following sections provide an overview of methods used.

3.1 CELL CULTURE PROCEDURES (PAPER I-IV)

3.1.1 Macrophage isolation and cell culture

Mononuclear cells were prepared from buffy coats obtained from healthy adult blood donors by density gradient centrifugation using Lymphoprep, as described previously (Kagan et al., 2002) (Paper I-IV). In brief, cells were washed and resuspended at $5 \cdot 10^6$ cells/ml in RPMI-1640 medium and seeded in 24-well cell culture plates. Monocytes were then separated by adhesion to tissue culture plastic for 1 h at 37ºC with a 5% CO_2 atmosphere and non-adherent cells were removed by several washes with PBS. Human monocyte-derived macrophages (HMDM) were cultured for 7-10 days in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin (Paper I). In some studies, HMDM were activated with 50 ng/ml human recombinant macrophage-colony-stimulating factor (M-CSF) and used on day 3-4 (Paper II-IV).

3.1.2 Neutrophil isolation and cell culture

Peripheral blood neutrophils were isolated from buffy coats of healthy adult blood donors by a method of dextran sedimentation and density gradient centrifugation as previously described (Fadeel et al., 1998). Residual erythrocytes were removed by hypotonic lysis. Purified neutrophils were cultured in 12 or 24-well tissue culture plates in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U penicillin/ml and 100 μg streptomycin/ml. To induce spontaneous apoptosis, $2.0 \cdot 10^6$ cells/ml were cultured in 24-well tissue culture plates for 22 h at 37ºC in full medium supplemented as above. Freshly isolated neutrophils or neutrophils maintained at 15ºC for 22 h were used as a non-apoptotic control (Paper II and IV).

3.1.3 T cell isolation and culture

Peripheral blood mononuclear cells were isolated from heparinized blood obtained from healthy adult donors by density gradient centrifugation. After separation, cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were activated for 2 days in the presence of phytohaemagglutinin (PHA) (5 μg/ml) and then cultured for 24 h with recombinant IL-2 (50 IU/ml) prior to Fas/APO-1 cross-linking experiments (Paper III).

3.1.4 Cell line culture and differentiation

3.1.4.1 Jurkat T cells

The T cell leukemia cell line Jurkat was grown in RPMI-1640 medium (Sigma) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in logarithmic growth phase for all experiments (Paper I-III).

3.1.4.2 J774A.1 cells

The murine macrophage-like cell line J774A.1 was cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin sulfate (Paper I).

3.1.4.3 THP.1 cells

The human monocytic leukemia cell line THP.1 was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM Na-pyruvate and 50 μM β-mercaptoethanol. To induce differentiation into macrophage-like cells, $5.0 \cdot 10^5$ THP.1 cells/ml were stimulated with PMA at 150 nM for 3 days (Paper I and II).

3.1.4.4 PLB-985 cells

The X chromosome-linked chronic granulomatous disease (X-CGD) human promyelocytic leukemia PLB-985 cell line lacking $gp91^{phox}$ (Zhen et al., 1993) and the X-CGD cell line re-transfected with $gp91^{phox}$ (X-CGD-$gp91^{phox}$) (Ding et al., 1996) were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml

streptomycin. To induce differentiation into macrophage-like cells, PLB-985 cells were seeded at a density of $5.0 \cdot 10^5$ cells/ml in 24-well tissue culture plates and stimulated using a combination of 30 nM PMA and 200 nM vitamin D_3 for 3 days (Bhatia et al., 1994). Differentiation into macrophage-like cells was determined by assessment of plastic adherence, morphological features, and detection of phenotypic cell surface markers (Paper II).

3.2 VIABILITY ASSAYS (PAPER IV)

3.2.1 Trypan blue exclusion assay

Cell viability was assessed based on vital dye exclusion. In brief, cells were exposed to specified stimuli and harvested at the indicated time-points. Aliquot from the cell suspension was mixed 1:1 with Trypan blue (0.5%) and cells were examined by light microscopy. The percentage of viable and dead cells was calculated (Paper IV).

3.2.2 MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) assay was employed to investigate the metabolic activity in mitochondria as a measure of cell viabilty. Cells were seeded in 96-well plates, and exposed to indicated stimuli for various time-points in complete (10% FBS) or serum-free medium. At the end of treatment period, the medium was removed and cells were incubated for 3 h in 100 µl MTT-solution (0.5 mg/ml). Formazan crystals was dissolved in 50 µl DMSO, and the color intensity was measured in a scanning multi-well spectrophotometer at 590 nm with a reference at 650 nm (Mosmann, 1983) (Paper IV).

3.3 APOPTOSIS DETECTION (PAPER I-III)

3.3.1 PS externalization

Plasma membrane exposure of phosphatidylserine (PS) was quantified as detailed in the annexin V-FITC apoptosis detection kit (and see van Genderen et al., 2006). Cells were co-stained with propidium iodide (125 ng/ml) prior to analysis on a FACScan equipped with a 488 nm argon laser. Ten thousand events were collected for each sample and analyzed using CellQuest software. Low-fluorescence detritus was gated out before analysis (Paper I-III).

3.3.2 Caspase-3-like enzyme activity

Cleavage of the fluorogenic peptide substrate DEVD-AMC, indicative of caspase-3-like enzyme activity, was determined as previously described (Fadeel et al., 1998). Briefly, cell lysates and DEVD-AMC substrate (50 μM) were combined in a standard reaction buffer (100 mM HEPES, 10% sucrose, 5 mM dithiothreitol, and 0.1% CHAPS; pH 7.25) and enzyme-catalyzed release of AMC was measured every 70 s during a 30 min period in a Fluoroscan II plate reader using 355 nm excitation and 460 nm emission wavelengths (Paper I-III).

3.3.3 Determination of nuclear morphology

Nuclear apoptosis was determined as described before (Tyurina et al., 2007). Briefly, cells were harvested and resuspended in 2% paraformaldehyde. Cytospin preparations were stained with 1 μg/ml Hoechst 33342. Cells were scored using a fluorescence microscope (Paper II).

3.3.4 Assessment of hypodiploid DNA content

DNA content was assessed as previously reported (Karpova et al., 2006). In brief, $0.5x10^6$ cells were harvested and resuspended in staining solution containing 50 μg/ml propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate in PBS. The percentage of cells displaying hypodiploid (sub-G1) DNA content was then determined using a FACScan operating with CellQuest software (Paper II).

3.4 PHAGOCYTOSIS ASSAYS (PAPER I-IV)

3.4.1 Phagocytosis of apoptotic/activated target cells

Phagocytosis assays were performed as previously described (Kagan et al., 2002). Briefly, target cells (typically $30{\cdot}10^6$) were labeled with 50 μg/ml of the fluorescent dye TAMRA in serum-free medium for 15 min at 37ºC and then cultured at $2{\cdot}10^6$ cells/ml in the presence or absence of the indicated stimulus. Target cells were then washed in PBS, resuspended in medium and added to macrophages at a ratio of 10:1 in 24-well tissue culture plates. After co-cultivation at 37ºC for 1 h (for THP.1 and PLB-985 macrophages, and M-CSF-stimulated HMDM) or 2 h (for non-activated HMDM), non-ingested cells were washed off with several washes in cold PBS and the remaining cells were fixed in 2% paraformaldehyde for 15 min. Nuclei of both target cells and macrophages were counterstained with Hoechst 33342. Phagocytosis

was evaluated by counting macrophages in phase contrast light and thereafter counting TAMRA-labeled target cells within macrophages under UV illumination using a fluorescence microscope. At least 400 macrophages/experimental condition were counted. Data are reported as % phagocytes positive for uptake of fluorescent target cells, or ratio of phagocytosis of treated versus untreated target cells (for M-CSF-stimulated HMDM) (Paper I-IV).

3.4.2 Fcγ-receptor-dependent phagocytosis assay

Sheep red blood cells (SRBC) were stained with TAMRA and opsonized with anti-SRBC IgG antibodies (1:5000 dilution) (Sigma) in RPMI-1640 complete medium at 37ºC for 30 min. $10 \cdot 10^6$ IgG-coated SRBC were added to each well of M-CSF-stimulated HMDM and co-cultured for 40 min at 37ºC. The unattached target cells were then washed out with PBS and the HMDM were further incubated for 20 min to allow for internalization of attached SRBC. Finally, co-cultures were washed additional times with cold PBS, fixed in 2% PFA and stained with Hoechst 33342. Phagocytosis was evaluated as described above (Paper IV).

3.4.3 Co-cultivation of normal neutrophils and PLB-985 macrophages

PLB-985 WT or X-CGD macrophages were extensively washed before co-cultivation in order to remove residual amounts of the differentiating agents. Neutrophils (pre-chilled or freshly isolated) were then added to PLB-985 cultures in 24-well tissue culture plates at a ratio of 10:1. After co-cultivation at 37ºC, supernatants were collected at the indicated time-points and spun down at 1200 rpm for 5 min to obtain (non-engulfed) neutrophils for further analysis (Paper II).

3.5 DETECTION OF CELL SURFACE MARKERS (PAPER II AND III)

3.5.1 Monocyte/macrophage cell surface markers

Expression of cell surface markers was analyzed by flow cytometry as described previously (Selmeczy et al., 2003). Briefly, adherent PLB-985 or THP-1 cells differentiated as described above were removed by trypsinization and incubated with FITC-conjugated monoclonal anti-human CD11b antibody (IgG1), PE–conjugated monoclonal anti-human CD14 antibody (IgG2a), or isotype-matched control antibodies for 30 min at RT. Cells were then resuspended in 2% paraformaldehyde and analyzed on a FACScan. Ten thousand events were collected for each sample and

data were analyzed using CellQuest software. Low-fluorescence detritus was gated out before analysis (Paper II).

3.5.2 Annexin I surface expression

Cells were harvested and resuspended in antibody-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$; pH 7.4) containing either polyclonal rabbit anti-human annexin I antibodies or negative control rabbit immunoglobulin fraction from serum. Cells were then incubated at room temperature for 30 min, washed with ice-cold antibody binding buffer, and resuspended in antibody-binding buffer containing secondary FITC-conjugated swine anti-rabbit polyclonal antibodies (1:1000). Cells were incubated for 1 h at room temperature and then washed and co-stained with 0.5 μg/ml PI. Samples were analyzed by flow cytometry using a FACScan flow cytometer equipped with a 488-nm argon laser. All PI-positive cells were excluded from further analysis (Paper II and III).

3.5.3 CD31 staining

Cells were resuspended in PBS containing 1% bovine serum albumin (BSA) and incubated with FITC-conjugated monoclonal anti-human CD31 antibody or isotype-matched control antibody for 1 h at RT. CD31 expression was analyzed on a FACScan using the CellQuest software (Paper II).

3.5.4 Neutrophil degranulation

Neutrophils co-cultivated with PLB-985 macrophages were resuspended in PBS containing 1% BSA and incubated for 30 min with FITC-conjugated monoclonal anti-human MPO antibody or isotype-matched control antibody for 30 min at RT. Membrane expression of myeloperoxidae (MPO) was then analyzed using a FACScan. As a positive control for neutrophil degranulation, cells were preincubated with cytochalasin B (CB) (10 μg/ml) for 5 min and then stimulated with fMLP (10 μM) for 15 min (Paper II).

3.6 PROTEIN PRODUCTION AND ANALYSIS (PAPER I-III)

3.6.1 MFG-E8 production

Recombinant MFG-E8 and D89E mutant proteins were produced as described previously (Hanayama et al., 2002). In brief, expression plasmids of FLAG-tagged

MFG-E8 and mutant MFG-E8 (the generous gift of Prof. Shigekazu Nagata, Kyoto University, Kyoto, Japan) were introduced into human 293T cells using the Lipofectamine™ 2000 reagent, and the proteins secreted into the medium were purified using anti-Flag M2 affinity gel. To examine the purity of the proteins, they were subjected to SDS-PAGE and Western blotting using anti-FLAG antibody (Paper I and II).

3.6.2 Western blot analysis

Equal volumes of supernatants from cell cultures, or equal amounts of protein were resolved by electrophoresis on a 12% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The membranes were incubated with blocking buffer containing 0.1% Tween-20 and 5% skimmed milk and probed overnight with primary antibodies. Following incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies, bound antibody was visualized with enhanced chemiluminescence (Paper I-III).

3.7 MEASUREMENT OF CYTOKINES (PAPER II-IV)

3.7.1 Cytokine bead array

Supernatants from co-cultivation of HMDM and target cells were collected and frozen at –70°C until needed for cytokine analysis. TNF-α and IL-10 expression was measured using the Cytometric Bead Array (CBA) to the manufacturer's instructions. Cytometric analysis was carried out using the FACScalibur and data were analyzed using dedicated CBA software (Paper II and III).

3.7.2 ELISpot

ELISpot plates were pre-treated with 70% ethanol and washed with sterile water. Capture anti-cytokine antibodies for TNF-α, IL-1β and IL-10 (15 µg/ml) were added to the plates (100 µl/well). After incubation overnight at +4ºC, unbound antibodies were removed by washing in sterile PBS followed by blocking for 30 min with cell culture medium (RPMI medium supplemented with 10% FBS, 0.5 mM HEPES, 2 mM L-glutamine, 100 U penicillin/ml and 100 µg streptomycin/ml). Five hundred HMDM in 100 µl cell culture medium were added to each well and incubated with indicated stimui. After 18 h the cells were washed out with PBS and the plates were incubated for 2 h at RT with 100 µl/well of biotinylated antibodies to TNF-α, IL-1β

or IL-10 diluted to 1 µg/ml in PBS with 0.5% FBS. Then, the plates were washed in PBS and incubated for 1 h at RT with streptavidin-alkaline phosphatase diluted 1:1000 in PBS with 0.5% FBS. Finally, after a final round of washing, the plates were developed with BCIP/NBT substrate solution at RT until distinct spots emerged (approx. 10 min). The colour development was stopped by repeated washings with tap water. After drying, the spots were counted with an ELISpot reader using AID ELISPOT software (Paper IV).

3.8 ROS GENERATION (PAPER II)

Production of superoxide was assessed by oxidation of dihydroethidium (DHE) to ethidium. Cells were incubated with 5 µM DHE in tissue culture medium for 45 min at 37˚C and then washed, resuspended in PBS and submitted to flow cytometric analysis using a FACScan flow cytometer operating with CellQuest software. Cellular debris and necrotic cells were excluded based on forward and side scatter characteristics.

3.9 PREPARATION AND CHARACTERIZATION OF PARTICLES (PAPER IV)

3.9.1 Material synthesis

Preparation of the anionic-templated mesoporous solid (AMS) materials utilized in our study has been described previously (Atluri et al., 2008; Garcia-Bennett et al., 2005). Briefly, for the synthesis of AMS-6, *N*-Lauroyl-*L*-Alanine is used as surfactant, 3-aminopropyl triethoxysilane (APES) as an anionic co-structure directing agent (CSDA), and tetraethyl orthosilicate (TEOS) as silica source. For the synthesis of AMS-8 *N*-lauroyl-L-glutamic acid (C_{12}GlutA) is used as surfactant. Removal of surfactant was conducted by calcination at 550ºC (1 h in flowing N_2, followed by 6 h in O_2), to give porous AMS-n silicates. Alternatively, the surfactant was removed by solvent extraction in a soxhlet apparatus using ethanol/HCl mixture for 3 days, allowing the surfactant to be extracted while retaining the amine-functionalized silica surface.

3.9.2 Covalent loading of fluorescein isocyanate (FITC)

Covalent loading of FITC was achieved by the reaction of amine-functionalised mesoporous AMS-6 and AMS-8 with the fluorochrome under alkaline conditions in order to produce the imminothioester bond. For the purpose of covalent attachment to FITC, 1 g of extracted mesoporous material was refluxed in 100 ml dry methanol containing the desired amount of fluorochrome. The pH of the solvent was raised to 11 by addition of NaOH pellets and the mixture allowed to reflux for a period of 24 h after which the remaining orange solid was filtered, washed with ample methanol followed by distilled water, and left to dry at RT.

3.9.3 Low angle X-ray powder diffraction (XRD)

Low angle X-ray powder diffraction (XRD patterns were performed on a X`Pert Pro diffractometer using Cu Kα radiation (λ=1.5418 Å) at 45kV and 40mA. The diffraction patterns were recorded between 1^0 and 6^0 2θ using an increment of 0.04^0 2θ and a 60 s step time.

3.9.4 Nitrogen adsorption/desorption isotherms

Nitrogen adsoption/desrption isotherms were measured at liquid nitrogen temperature (-196 ºC) using a Micromeritics ASAP2020 volumetric adsorption analyzer. Before the measurements the calcined silica samples were outgassed under vacuum for 10 h at 120ºC. The BET (Brunauer-Emmett-Teller) equation was used to calculate the surface area (S_{BET}) from adsorption data obtained in the relative pressure (p/p^0) range of 0.05 and 0.3. The total pore volume (V_{tot}) was calculated from the amount of gas adsorbed at $p/p^0 = 0.91$. Pore size distribution curves were derived using the density functional theory (DFT) method assuming a cylindrical pore model (Ravikovitch and Neimark, 2000).

3.9.5 Thermogravimetric analyses (TGA)

TGA were performed on Mettler TGA instrument. The as-synthesized samples were heated from 25 to 900ºC at a heating rate of 1ºC/min on an alumina holder under the flow of air at 20 ml/min.

3.9.6 Transmission electron microscopy (TEM)

TEM of calcined and extracted samples was conducted with a JEOL-3010 microscope, operating at 300 kV (Cs 0.6 mm, resolution 1.7 Å). Images were

recorded using a CCD camera (model Keen View, SIS analysis, size 1024 x 1024, pixel size 23.5 x 23.5 μm) at 30 000-100 000x magnification using low-dose conditions on as-synthesized and calcined samples.

3.9.7 Scanning electron microscopy (SEM)

SEM images were obtained using LEO 1550 operating at 2-3kV with no gold coating.

3.9.8 TEM studies of mesoporous particles dispersed in cell culture medium

To characterize material incubated in biological medium TEM studies were performed as above, except samples were not crushed prior to transfer to the microscope grid. Due to the larger size of the mesoporous particles AMS-8 and STA-11 it was not possible to observe the internal structure of these solids. Hence, only AMS-6 particles could be studied in this way.

3.9.9 Fourier Transform Infrared (FT-IR) spectroscopy

FT-IR was performed on a Perkin Elmer spectrum one FT-IR spectrometer.

3.9.10 Hydrodynamic particle size analysis

Hydrodynamic particle size analysis of calcined, solvent extracted (amine functionalized), and FITC-conjugated AMS-6 particles was performed on an IB90 AT Particle Sizer v5.2. The particles where dispersed in distilled water, phosphate-buffered saline, cell culture medium, or cell culture medium supplemented with 10% fetal bovine serum. All measurements where performed at a particle concentration of 1 mg/ml.

3.10 ANALYSIS OF PARTICLE UPTAKE (PAPER IV)

3.10.1 TEM analysis of macrophages

HMDM were incubated with silica particles for 1 or 6 h and fixed in 2 % glutaraldehyde and 0.5 % paraformaldehyde in 0.1 M sodiumcacodylate buffer containing 0.1 M sucrose and 3 mM $CaCl_2$, pH 7.4 for 30 min. Cells were gently scraped to detach from cell culture plastics, pelleted and fixed for additional 24 h in fixation solution mentioned above. The pellets were then rinsed in 0.15 M sodiumcacodylate buffer containing 3 mM $CaCl_2$, pH 7.4. Post-fixation of pellets was

carried out in 2 % osmium tetroxide in 0.07 M sodiumcacodylate buffer containing 1.5 mM $CaCl_2$, pH 7.4 at 4 °C for 2 h, dehydrated in ethanol followed by acetone and embedded in LX-112. Sections were contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 10 transmission electron microscope at 80 kV. Digital images were taken by a MegaView III digital camera.

3.10.2 Fluorescence microscopy

To study the internalization of mesoporous silica particles HMDM were grown on sterile cover slips in 24-well plates as described above and incubated with FITC-conjugated silica particles for 1 h. HMDM were then fixed in 2 % formaldehyde for 15 min and permeabilized in 0.5% Triton X-100 and 1 % BSA. Staining was carried out using phalloidin-TRITC (1:1000) and Hoechst 33342 (1 μg/ml). Slides were mounted with Vectashield. Florescent images were acquired on a NIKON Eclipse TE-2000 fluorescence microscope equipped with a DS-5M digital camera operating with NIS elements software. To investigate the mechanism of uptake, HMDM were pretreated with various inhibitors for 30 min at 37ºC before addition of FITC-labeled silica particles; alternatively, HMDM were incubated at 4ºC for 30 min, or in the absence of serum (FBS) before and during exposure to particles. The inhibitors used were cytochalasin D (10 μg/ml), 5-(*N*,*N*-dimethyl)amiloride hydrochloride (DMA) (100 μM), or a cocktail of endocytosis inhibitors including nystatin (25 μg/ml), genistein (200 μM), chlorpromazine (6 μg/ml), and brefeldin A (10 μg/ml).

3.10.3 Flow cytometric analysis of particle uptake

The uptake of mesoporous silica particles by HMDM was quantified by flow cytometric detection of FITC-conjugated particles. HMDM were exposed to FITC-labeled AMS-6 or AMS-8 in fresh medium for 1 h. After the indicated time-points cells were washed with PBS, fixed in 2 % formaldehyde and detached from cell culture plastics with a cell scraper. The fluorescence intensity of HMDM was measured with a FACScan flow cytometer equipped with a 488 nm argon laser. Ten thousand events were collected for each sample and data were analyzed using CellQuestPro software. For studies of the mechanism of uptake, HMDM were pre-treated as described above for 30 min prior to exposure to particles, and flow cytometric detection of particle uptake.

4 RESULTS

4.1 PAPER I: BRIDGE OVER TROUBLED WATER: MILK FAT GLOBULE EPIDERMAL GROWTH FACTOR 8 PROMOTES HUMAN MONOCYTE-DERIVED MACROPHAGE CLEARANCE OF NON-BLEBBING PHOSPHATIDYLSERINE-POSITIVE TARGET CELLS

In this study, the putative importance of plasma membrane blebbing for engulfment of dying cells was investigated. In addition, the ability of the bridging molecule MFG-E8 to increase the efficiency of phagocytosis of apoptotic cells was studied. First, we confirmed that agonistic anti-Fas antibody-stimulated Jurkat T cells undergoing apoptosis exhibited membrane blebbing. Furthermore, the redistribution of PS into segregated membrane microdomains or lipid rafts is believed to play an important role in cell clearance. These aggregations of PS can be found on membrane blebs on the surface of apoptotic cells (Casciola-Rosen et al., 1996). To obtain more information on the putative involvement of lipid rafts in PS exposure in Fas-triggered cells, we co-labeled Jurkat cells with annexin V and cholera toxin B (a marker of lipid rafts) and examined the cells under a confocal microscope. Our data suggest that PS molecules are co-localized with lipid rafts in Fas-triggered cells. Pre-treatment of Fas-triggered cells with cytochalasin B prevented blebbing, but did not affect PS externalization or caspase-activation. Similarly, the ROCK I kinase inhibitor, Y-27632, also inhibited blebbing in Fas-triggered cells, in line with previous studies (Coleman et al., 2001; Sebbagh et al., 2001).

To elucidate the role of membrane blebbing in cell clearance, PS-positive target cells were co-cultured with macrophages and phagocytosis was quantified. We observed that non-blebbing Jurkat cells appeared to be less efficiently engulfed by macrophages than blebbing target cells with a similar level of PS externalization. The protein kinase C inhibitor staurosporin (STS), a potent inducer of apoptosis, triggered PS externalization and caspase-activation in treated cells; however, no membrane blebbing was detected. Accordingly, phagocytosis of these cells was also diminished.

MFG-E8 is a soluble PS-binding protein previously shown to facilitate ingestion of PS-positive target cells by macrophages (Hanayama et al., 2002). Therefore, recombinant MFG-E8 was added to co-cultures with non-blebbing cells and

macrophages. When MFG-E8 was present in co-cultures, the number of phagocytosis-positive macrophages increased and the number of engulfed target cells per macrophage was elevated. In co-cultures with blebbing cells, only the phagocytic index, i.e. the number of engulfed target cells per macrophage, was increased.

To conclude, our data show that plasma membrane blebbing and PS externalization can be dissociated during apoptosis. Non-blebbing target cells are less efficiently ingested when compared to their blebbing counterparts; however, MFG-E8 was found to restore cell clearance.

4.2 PAPER II: INDUCTION OF CASPASE- AND REACTIVE OXYGEN SPECIES-INDEPENDENT PHOSPHATIDYLSERINE EXTERNALIZATION IN PRIMARY HUMAN NEUTROPHILS: ROLE IN MACROPHAGE RECOGNITION AND ENGULFMENT

In the second paper, we aimed to study the novel mechanism of macrophage-induced PS externalization in human peripheral blood neutrophils and clearance of these cells by activated human macrophages. Accordingly, we set up an *in vitro* model of macrophage-differentiated PLB-985 cells and primary neutrophils. We observed that macrophage-differentiated PLB-985 cells induced caspase-independent PS externalization in human neutrophils. A similar degree of PS externalization was seen when neutrophils were co-cultured with $gp91^{phox}$-deficient PLB-985 macrophages and when PLB-985 WT macrophages were pre-treated with DPI, a non-specific inhibitor of the NADPH oxidase prior to co-cultivation with neutrophils, thus demonstrating that macrophage-induced PS externalization was NADPH oxidase-independent. In addition, inhibition of inducible nitric oxide synthase (iNOS) also failed to prevent PS externalization. Macrophage-induced PS externalization required cell-to-cell contact and was shown to correlate with neutrophil degranulation. Pre-treatment of neutrophils with the protein kinase C inhibitor STS before co-incubation with PLB-985 macrophages resulted in complete block of the macrophage-induced externalization of PS. Additional studies with various kinase inhibitors revealed that genistein significantly decreased macrophage-induced externalization of PS whereas the selective PKC inhibitors, H-7 and rottlerin were ineffective. This indicates that the activation of PKC is not solely responsible for this phenomenon. Indeed, both

genistein and STS are also well known inhibitors of tyrosine kinases (Geissler et al., 1990).

PS-positive neutrophils activated by PLB-985 macrophages exhibited comparable levels of PS exposure as neutrophils undergoing constitutive apoptosis. However, the degree of engulfment of such PS-positive neutrophils by activated human monocyte-derived macrophages was considerably lower. To determine whether engulfment of apoptotic neutrophils was PS-dependent, the inhibitor of PS-mediated uptake, phospho-L-serine (PLS) was added to co-cultures of apoptotic neutrophils and HMDM and the percentage of phagocytosis-positive macrophages was calculated. Administration of PLS resulted in significant inhibition of phagocytosis, which is in accordance with previous studies (Fadok et al., 1992). Nevertheless, these results indicate that PS externalization alone may not be sufficient for macrophage disposal of neutrophils, and suggest therefore that surface expression and/or release of additional recognition ligand(s) is lacking in this model. Indeed, addition of recombinant MFG-E8, a PS-binding protein, restored engulfment of the macrophage-co-cultured target cells.

Proteins such as annexin I and its peptide derivatives have been shown to facilitate uptake of cells undergoing spontaneous apoptosis (Scannell et al., 2007). Interestingly, we found that neutrophils undergoing spontaneous apoptosis, but not macrophage-co-cultured neutrophils displayed surface expression and release of annexin I, and the addition of Boc1, a formyl peptide receptor/lipoxin receptor antagonist suppressed clearance of apoptotic neutrophils. Conditioned medium from apoptotic neutrophils also promoted the engulfment of macrophage-co-cultured neutrophils, and Boc1 blocked this process.

Taken together, these studies highlight a novel pathway of PS externalization in primary human neutrophils and also provide evidence for an auxiliary function of annexin I in macrophage clearance of neutrophils.

4.3 PAPER III: BRIEF CROSS-LINKING OF FAS/APO-1 (CD95) TRIGGERS ENGULFMENT OF PRE-APOPTOTIC TARGET CELLS

Initially we studied the kinetics of apoptosis triggered by antibody-mediated Fas/APO-1 cross-linking on the surface of Jurkat cells. We observed a time-dependent increase in apoptosis, with caspase activation, cleavage of PARP, and PS externalization starting at approx. 2 h of cross-linking, and at 4 h more than 40% of the cells were PS positive. Fas-triggered cells were subsequently co-cultured with M-CSF stimulated primary human monocyte-derived macrophages. Following 1 h of co-culture, PS-positive apoptotic Jurkat cells stimulated 4 h with anti-Fas antibodies were rapidly engulfed by macrophages. Surprisingly, Jurkat cells briefly treated (15 min) with anti-Fas antibodies were also efficiently engulfed by human macrophages prior to the externalization of PS. The possibility that this effect was limited to anti-Fas antibody-mediated cross-linking was excluded in studies with recombinant Fas-ligand. Time-course studies of Fas ligand-treated cells showed that PS was externalized after 2 h; however, these cells were engulfed after 15 min of treatment.

Hence, pre-apoptotic (PS-negative) and apoptotic (PS-positive) target cells were engulfed to a similar extent by activated HMDM. To verify our findings in another cell type, primary human T cells from peripheral blood were subjected to treatment with agonistic anti-Fas antibodies for various time-points, and PS externalization and phagocytosis by HMDM was monitored. Primary T cells were effectively engulfed upon brief (15 min) cross-linking of Fas/APO-1 and the degree of phagocytosis was comparable after 6 h of cross-linking. PS externalization, however, was not evident at 15 min of treatment and was detected only at prolonged (6 h or longer) treatment with agonistic anti-Fas antibodies.

Macrophage engulfment of pre-apoptotic target cells was found to be largely dependent on serum. In contrast, engulfment of Jurkat cells treated with anti-Fas for 4 h seemed less sensitive to serum deprivation. Moreover, pre-treatment of target cells with the pan-caspase inhibitor prior to co-cultivation with HMDM had no effect on the engulfment of pre-apoptotic target cells whereas the engulfment of apoptotic target cells was significantly reduced. However, addition of zVAD-fmk to the co-culture system partially suppressed macrophage engulfment of pre-apoptotic cells and further decreased the engulfment of apoptotic cells.

In the search for additional factors involved in the engulfment of pre-apoptotic cells, we discovered that these cells secreted annexin I to the same extent as apoptotic cells. Moreover, administration of Boc1 markedly attenuated the engulfment of pre-apoptotic Jurkat cells, and phagocytosis of apoptotic cells was partially inhibited.

Finally, the cytokine release by macrophages when encountering Fas-triggered Jurkat cells was analyzed. Pre-apoptotic Jurkat cells induced lower macrophage production of TNF-α and higher production of IL-10 in comparison to apoptotic target cells. HMDM co-cultured with pre-apoptotic Jurkat cells produced low levels of TNF-α, comparable to macrophages co-cultured with control cells while macrophages incubated with apoptotic target cells produced significantly higher levels of TNF- α. Moreover, IL-10 production was lower for macrophages co-cultured with apoptotic Jurkat cells when compared to macrophages co-cultured with pre-apoptotic or control cells

To conclude, our studies show for the first time that brief cross-linking of Fas/APO-1 is sufficient to target Jurkat cells and primary T cells for engulfment by activated human macrophages. Our results point to a role for annexin I in this process. In addition, we have shown that macrophages engulfing pre-apoptotic cells produced significantly lower levels of the pro-inflammatory cytokine, TNF-α, and higher amounts of IL-10, representative of anti-inflammatory cytokines.

4.4 PAPER IV: EVALUATING THE TOXICITY AND INTERNALIZATION OF MESOPOROUS SILICA PARTICLES OF DIFFERENT SIZES IN HUMAN MONOCYTE-DERIVED MACROPHAGES

These studies were aimed at studying the interactions of mesoporous silica particles with primary human monocyte-derived macrophages, with focus on uptake and potential toxic effects. To this end, mesoporous silica particles AMS-6 (approx. 250 nm) with cubic pore geometries and covalently fluorescein-grafted particles were synthesized through a novel route. First, to address whether particles retain their original size in biological medium, or whether particle agglomeration occurs, we determined the hydrodynamic particle size using dynamic light scattering. The material was confirmed to have intact shape and size after incubation in cell culture

medium and showed increased particle dispersion in cell culture medium supplemented with 10% fetal bovine serum.

Subsequently, we determined the internalization of AMS-6 by HMDM using TEM. Massive internalization of AMS-6 particles was evident already after one hour and particles within membrane-enclosed structures in the cytosol of macrophages pointed to an active uptake mechanism. For comparison, the micron-sized mesoporous silica particles STA-11 were also readily engulfed by HMDM. However, STA-11 particles were not surrounded by visible membrane structures upon internalization. The mechanism of uptake was further investigated utilizing FITC-labeled silica particles. Massive uptake of AMS-6 particles was seen in a majority of HMDM after 1 h, and for comparison, mesoporous AMS-8 particles (approx. 2.5 μm) were also taken up; however, fewer macrophages were positive for uptake of AMS-8. The internalization of the silica particles was found to be energy-dependent, and cytochalasin D, an inhibitor of actin polymerization, blocked uptake of AMS-8 particles and decreased uptake of AMS-6 particles to some extent. Moreover, pre-treatment of HDMD with a cocktail of endocytosis inhibitors decreased uptake of both AMS-6 and AMS-8; this inhibition was more pronounced for the larger particles. The macropinocytosis inhibitor, 5-(*N*,*N*-dimethyl)amiloride hydrochloride (DMA) failed to prevent internalization of silica particles. In addition, the process of internalization of both particles was serum-independent.

Uptake of mesoporous silica particles did not affect viability of human macrophages, as assessed with Trypan blue exclusion assay, after 6 or 24 h of incubation with AMS-6 or STA-11 particles at the dose studied. However, using the MTT assay, the metabolic activity of mitochondria in HMDM was decreased when incubated with AMS-6 for 6 and 24 h, but not after 1 h of incubation with particles. This effect was more pronounced when experiments were performed in the absence of FBS in the culture medium. For comparison, STA-11 silica particles showed less toxicity at the same dose.

Thereafter, the subsequent phagocytosis of target cells was monitored. HMDM were pre-incubated with FITC-conjugated AMS-6 or AMS-8 particles for 1 h and thereafter the capacity of HMDM to engulf various target cells was assessed. These studies show that the ability of HMDM to engulf apoptotic Jurkat cells or neutrophils

was not impaired by the pre-loading of mesoporous particles. Moreover, Fc receptor-mediated phagocytosis of IgG-opsonized sheep red blood cells was also unaffected by pre-treatment of HMDM with AMS-6 particles. Studies with STA-11 and AMS-8-FITC demonstrated similar findings. Furthermore, the cytokine response of HMDM to LPS was not affected by AMS-6 or STA-11, and the particles alone did not induce cytokine release.

In conclusion, the present studies have shown that mesoporous silica particles are avidly taken up by HMDM through an active process of endocytosis. Serum factors were apparently not required for this process. Furthermore, uptake of mesoporous particles was not associated with a decrease in viability of macrophages or in any subsequent impairment of macrophage function, including ingestion of apoptotic cell corpses, and cytokine secretion. The current findings are relevant to the development of mesoporous materials for drug delivery and other biomedical applications.

5 GENERAL DISCUSSION

The continuous turnover of cells demands efficient clearance of cell corpses. Tissue homeostasis is dependent not only on the balance between cell proliferation and apoptosis, but also on the rate of apoptosis versus that of cell clearance (Fadeel and Orrenius, 2005). The removal of dead cells is important to prevent inappropriate detrimental material and intracellular antigens to be released from the cell corpses that may cause inflammatory or autoimmune responses. Apoptotic cells undergo morphological changes that render them appetizing for engulfment by professional phagocytes or neighboring cells and they are subsequently removed in a non-phlogistic manner. Thus, macrophage clearance of apoptotic cells may be viewed as the meaning of cell death (Savill and Fadok, 2000).

The studies presented in the current thesis are aimed at elucidating the mechanisms of programmed cell clearance. For this purpose we have used different model systems to study the role of PS for phagocytosis. It is clear from several studies that PS is involved in apoptotic cell clearance. Moreover, enrichment of the plasma membrane of viable cells with PS target cells for macrophage engulfment (Kagan et al., 2002). The question is if PS is always sufficient for macrophage engulfment? In the first paper we demonstrate that not only the presence of signals on the cell surface of apoptotic cells is necessary for recognition and clearance, but also additional surface alterations may be required. The redistribution of PS into segregated membrane microdomains or lipid rafts is believed to play an important role in cell clearance. These aggregations of PS can be found on membrane blebs on the surface of apoptotic cells. Previous studies have shown that PS externalization in anti-IgM-treated B cells and in neutrophils stimulated with chemotactic peptides may occur in lipid rafts (Dillon et al., 2000; Frasch et al., 2004). Furthermore, recent studies suggest that lipid rafts may be important for the maintenance of PS on the cell surface during doxorubicin-induced apoptosis (Ishii et al., 2005). Indeed, our data suggest that PS molecules are co-localized with lipid rafts in Fas-triggered cells. However, further studies are required to determine the functional importance of lipid raft domains for PS-dependent signaling. It is plausible that other signals accumulated in membrane blebs could play a role in the phagocytosis signaling as well, or that several signals in combination brought together in close proximity in rafts are needed.

Aggregation of signaling molecules could also be important for amplifying the signal and bring an additional level of control for the discrimination between living and dying cells (Appendix I).

MFG-E8 was able to bind to both blebbing and non-blebbing PS-positive apoptotic cells and enhance phagocytosis. MFG-E8 is believed to crosslink PS-molecules and thereby facilitates clearance of apoptotic cells by macrophages (Hanayama et al., 2002). However, we cannot exclude the possibility that the observed increase in phagocytosis is because MFG-E8 triggers downstream signaling pathways in macrophages. In dendritic cells, MFG-E8 binding to integrin $\alpha_v\beta_5$ was linked to the downstream signaling pathway of Crk-Dock180-Rac1 in phagocytosing cells (Akakura et al., 2004). It will be of interest to assess the importance of endogenous MFG-E8 by small interfering RNA techniques targeting MFG-E8 and/or downstream signaling molecules such as DOCK180 in human macrophages, and to determine the functional outcome (e.g. cytokine response) of phagocytosis receptor ligation of PS alone or in conjunction with MFG-E8, using the co-cultivation model described in Paper I.

In the second paper we demonstrate a novel pathway of macrophage-induced PS externalization in neutrophils. The process was caspase-independent and the neutrophils showed minor apoptosis-associated changes, including DNA-fragmentation and nuclear condensation. Several studies have shown that non-apoptotic cells can express PS. For example, PS has a normal physiological role in stimulating the coagulation cascade when expressed on the surface of aged erythrocytes and is also exposed on the plasma membrane of activated platelets and neutrophils (Fadeel et al., 1998). B-cell receptor cross-linking in Bcl-2 overexpressing lymphoma cells (that are protected from apoptosis) rendered the cells annexin V positive (Holder et al., 2006). Moreover, a great number of circulating annexin V binding neutrophils with no other sign of apoptosis can be observed in patients with Barth syndrome (Kuijpers et al., 2004). To control for unspecific annexin V-FITC binding to PLB-985 macrophage co-cultured neutrophils we took additional measures to analyze phospholipid asymmetry/PS exposure in the plasma membrane using two other approaches: merocyanine 540 (MC540) staining, and anti-PS monoclonal antibodies. Our results provide support for the specificity of the annexin V assay as it is applied to the current model.

Furthermore, we observed that macrophage-induced PS externalization in pre-apoptotic neutrophils was NADPH oxidase and iNOS-independent, and based on inhibitor studies, it is likely that tyrosine kinases are involved. In addition, cell-to-cell contact was required for the induced PS externalization. Cell-to-cell contact between neutrophils and certain classes of macrophages with the activation of adhesion molecules has been demonstrated to trigger degranulation of neutrophils with concomitant exposure of PS on the cell surface. For comparison, previous studies have documented degranulation-associated PS externalization in human and murine mast cells (Martin et al., 2000). Indeed, an association between PS externalization and the degranulation marker MPO was observed in the PLB-985 macrophage co-cultured neutrophils.

Despite high levels of PS, neutrophils with PLB-985 macrophage-induced PS externalization was not efficiently engulfed by primary human macrophages. However, MFG-E8 could, in addition to promoting engulfment of PS positive non-blebbing Jurkat cells (Paper I), also facilitate clearance of PS-positive pre-apoptotic neutrophils. Macrophage engulfment of neutrophils undergoing constitutive apoptosis was partially blocked by phospho-L-serine (PLS), and accordingly, additional signals must play a role in the engulfment of neutrophils. We observed that macrophage-induced neutrophils downregulated the expression of the detachment signal, CD31 and therefore failure to downregulate CD31 could not explain the poor engulfment of these cells. Moreover, we found that ingestion of apoptotic neutrophils was partially inhibited by Boc1, a formyl peptide receptor/lipoxin receptor antagonist that is known to block annexin I-dependent uptake of cells (Maderna et al., 2005). For that reason, we compared the surface expression of neutrophils undergoing spontaneous apoptosis and neutrophils co-cultured with PLB-985 macrophages, and we discovered that apoptotic neutrophils expressed annexin I on the surface whereas co-cultured neutrophils failed to do so. In addition, apoptotic neutrophils secreted annexin I into the supernatant, as reported recently by others (Scannell et al., 2007) whereas no annexin I secretion was seen in neutrophils co-cultured with PLB-985 macrophages. However, conditioned medium from apoptotic neutrophils, with high levels of annexin I, was shown to promote uptake of co-cultured neutrophils by primary human macrophages, and this uptake was also suppressed in the presence of Boc1.

Neutrophils contain large amounts of annexin I in the cytosol. Annexin I lacks a signal peptide and therefore cannot be exported through the classical secretory pathway. (Perretti and Flower, 2004). However, annexin I is localized within the gelatinase granules and is externalized from the neutrophils as these granules and their contents is exported on the cell surface by a process of exocytosis. We observed that PS externalization in PLB-985 co-cultured neutrophils was associated with degranulation as indicated by the surface expression of MPO. MPO is primarily an azurophilic granule-protein and therefore the two neutrophil granule proteins may not be externalized or secreted together upon neutrophil co-culture with PLB-985 macrophages.

We initially considered PS to be the main recognition signals for macrophage engulfment of primary neutrophils; however, our findings presented in Paper II prompted us to reevaluate this idea. A detailed analysis of the differences between neutrophils undergoing spontaneous apoptosis and neutrophils subjected to co-culture with PLB-985 macrophages revealed an important role for annexin I, and possibly other signals, in the phagocytosis of these cells. In continuation, it will be of interest to further explore the involvement of additional recognition signals and to clarify the mechanism for macrophage-induced PS externalization.

In the third study we report on a role of annexin I in the phagocytosis of pre-apoptotic (PS-negative) Jurkat T cells. Brief treatment (15 min) of Jurkat cells with agonistic anti-Fas antibodies resulted in release of annexin I. Boc1 significantly blocked the engulfment of pre-apoptotic Jurkat cells and partly the phagocytosis of apoptotic cells. In cells without granular storage of annexin I it is not entirely clear how annexin I is exported from cells (D'Acquisto et al., 2008). Some evidence point to the involvement of an ABCA1 transporter (Chapman et al., 2003). Fas-triggering of Jurkat cells has previously been shown to result in a caspase-dependent translocation of Annexin I from the cytosol to the outer plasma membrane leaflet, where it colocalizes with PS, and act as recognition signal required for efficient clearance of apoptotic cells (Arur et al., 2003). In the model system used in Paper III it is still unclear what triggers the release and cleavage of annexin I. We have not dissected the downstream signaling of Fas/APO-1 upon brief cross-linking with anti-IgM antibodies or the mechanism for generating pre-apoptotic recognition signals. Nonetheless, it is clear that the recognition signal is upstream of caspase 3-like

activation and is externalized/released before PS exposure. Previous studies have revealed that caspase activation in the Jurkat cell line is delayed until approx. 1 h after ligation of Fas/APO-1 (Scaffidi et al., 1998). In contrast, other signaling events occur very soon after cross-linking of Fas/APO-1; reportedly translocation of the p65 component of NFκB to the nucleus occurs at 5 min after cross-linking of Fas/APO-1 (Kang et al., 2006). Future studies to examine the mechanism for the early re-distribution of annexin I upon Fas/APO-1 triggering will be of significant interest.

One might question why the non-blebbing apoptotic Jurkat cells are not engulfed (Paper I) since PS does not appear to be the sole determinant for the engulfment of these cells (Paper III). The reverse question could also be asked: why is not blebbing required for phagocytosis of pre-apoptotic Jurkat cells? Part of the explanation is that different types of macrophages were studied in Paper I and Paper III. In Paper I unstimulated HMDM and the murine cell line J77A.4 have been employed, compared to the M-CSF activated HMDM used in Paper III. Different recognition signals and phagocytosis receptors seem to have distinct roles in different classes of phagocytes. J774A.1 is a murine macrophage cell line resembling non-activated macrophages similarly to human monocyte-derived macrophages cultured for 7-10 days without M-CSF. In contrast, M-CSF activated HMDM resembles activated peritoneal macrophages and phagocytosis by unactivated versus that of activated macrophages involves diverse receptor-systems (Pradhan et al., 1997). Moreover, different mechanism of cell clearance may also be depending on the stage of apoptosis (early or late) of the target cell. Our findings do not exclude the potential involvement of additional recognition signal(s) for macrophage engulfment of pre-apoptotic cells. It is likely that “eat-me” signals operate in a sequential manner with carbohydrate-mediated mechanisms of cell clearance having a role during early stages, and PS-dependent mechanisms coming into play at later stages of apoptosis (Yamanaka et al., 2005). It remains to be investigated whether pre-apoptotic cells express additional signals known to be implicated in phagocytosis.

In the last paper, we studied the interaction between macrophages and mesoporous silica particles. We have investigated whether mesoporous silica AMS-6 particles (250 nm) are taken up by primary human macrophages and whether the interaction with these nanomaterials is deleterious to the cells. We have compared AMS-6 with the larger mesoporous silica particles STA-11 (5 μm) or FITC-conjugated AMS-8

(2.5 µm) to investigate any size-dependent effects. Nanoparticles have been shown to target distinct cell populations according to their size (Manolova et al., 2008). *In vivo* studies demonstrated a translocation of nanoparticles (20-200 nm) to lymph nodes where they targeted lymph node-resident dendritic cells and macrophages. In contrast, the larger particles (500-2000 nm) were mainly found in dendritic cells at the local injection site. We found the uptake of AMS-6 was higher compared to AMS-8, but this might just reflect the fact that per mass dose the number of AMS-6 is greater than AMS-8. Consequently, fewer AMS-8 particles per macrophage are available. Incubation at 4ºC impaired the efficiency of the number of particles internalized in each macrophage; however, the total number of particle-positive macrophages was only decreased for AMS-8. Similar results were found when macrophages were pre-treated with a cocktail of endocytosis inhibitors or cytochalasin D (CD). CD is commonly used to inhibit the process of phagocytosis. However, actin-dependent processes are also considered to be important in clathrin- and caveolae-mediated endocytosis (Kaksonen et al., 2006). The suppression of AMS-6 internalization may thus be explained, in part, by the disruptive effect of CD on endocytosis. In line with our observations, recent studies have shown that the internalization of mesoporous silica nanoparticles by bone marrow-derived human mesenchymal stem cells is decreased by CD (Huang et al., 2008). The notion that several pathways contribute to the internalization of particles may partly be explained by the state of aggregation of the particles with different internalization-mechanisms for larger aggregates compared to single particles. Furthermore, opsonization by serum proteins probably does not play a role in the uptake the mesoporous silica particles. However, we cannot exclude the involvement of receptor-mediated pathways. The class A scavenger receptor MARCO binds unopsonized environmental particles (Arredouani et al., 2005) and is the main receptor mediating crystalline silica uptake and toxicity in murine alveolar macrophages (Hamilton et al., 2006). Future studies should be directed to the assessment of scavenger receptors such as MARCO for uptake of nanoparticles.

6 CONCLUSIONS

In summary, the main results obtained from this thesis suggest the following:

Paper I:

- Membrane blebbing and PS externalization can be dissociated during apoptosis
- Membrane blebbing may be important for aggregation of PS and efficient engulfment of apoptotic cells
- The PS-binding protein MFG-E8 can enhance macrophage uptake of PS-positive non-blebbing target cells

Paper II:

- PLB-985 differentiated macrophages are capable of inducing caspase- and NADPH-oxidase independent PS exposure in primary neutrophils
- Neutrophils with macrophage-induced PS exposure are poorly engulfed by primary human M-CSF stimulated macrophages
- MFG-E8 facilitates clearance of neutrophils with macrophage-induced PS exposure
- Annexin I plays a complementary role for engulfment of primary neutrophils

Paper III:

- Pre-apoptotic Jurkat cells (PS negative) are efficiently engulfed by M-CSF stimulated primary human macrophages
- Annexin I may play a role for engulfment of pre-apoptotic target cells
- Pre-apoptotic Jurkat cells induce lower production of TNF-α and higher production of IL-10 in comparison to apoptotic target cells

Paper IV:

- Mesoporous silica particles are efficiently taken up by HMDM through an active process of endocytosis.
- Serum factors are not required for the internalization of mesoporous silica particles

- Macrophage uptake of mesoporous particles was not associated with a decrease in viability
- Macrophage function, including ingestion of apoptotic cell corpses, and cytokine secretion was not affected by the mesoporous silica particles

Overall, these studies have us informed on the mechanisms of macrophage clearance of target cells and particles.

7 ACKNOWLEDGEMENTS

This work was performed at the Institute of Environmental Medicine, Division of Molecular Toxicology and Division of Biochemical Toxicology. I would like to express my deepest gratitude to all the people that I have been working and interacting with at Karolinska Institutet. In particular I wish to thank the following people:

Associate professor Bengt Fadeel, my main supervisor for accepting me as a PhD student and introducing me to the field of apoptosis, programmed cell clearance and nanotoxicology, and for making this thesis possible. For all your support, for being dedicated and always taking your time. Your devotion to science has been a constant source of inspiration.

Professor Valerian Kagan, my co-supervisor for support and critical input during these years.

Professor Ralf Morgenstern, for always being supportive and for creating a good and relaxed working atmosphere at the Division of Biochemical Toxicology.

All our collaborators and co-authors for their essential contributions. A special thanks to Siriporn Jitkaew, Shouting Zhang, Alfonso Garcia-Bennett and Natalia Kupferschmidt for fruitful collaboration.

The past and present members of the Cell Death group for being great colleagues: Astrid, my stand-in sister in the lab – there is absolutely no way I could have done this without you; Jingwen, for being a great colleague and for sharing nano-work with me; Marjolein, for always being enthusiastic and smiling, Shouting for your optimism; Dao and Amm, for being enjoyable to work with; Sun, for giving me the best start in the group; Alicja, Sandra, Lina, Neus, Linneá, Mei, Daniel, Camilla, Diana, Magnus, Lovisa.

The past and present members of the Division of Biochemical Toxicology and other colleagues at the third floor. A special thanks to: Katarina, for your warmth and care; Åse, for always having a helping hand; Rebecca, for all the encouraging small talks and for letting me occupy your room; Therese, for being a nice roommate; Johan, for sharing your biochemical expertise; Vanina for keeping me company in the cell lab during weekends.

The members of the former Division of Molecular Toxicology. Thank you for sharing your expertise and helping me with various methods and techniques, and for all the nice “fikas”.

Colleagues at the 5th floor. Special thanks to Ullis for all the help, for being a great friend, always a patient listener; Helin and Poppi for all your help and all the great fun we have had at conferences; Bertrand Joseph and Christoffer Tamm for excellent assistance with confocal microscopy.

Ida von Schéele at Retzius, for being the best lunch-date and a great friend, for your support and for guiding me in the jungle of statistical tests.

The members of the NanoNet, for nice discussions and for sharing your knowledge. A special thanks to Helen Vallhov for always having a helping hand, for sharing reagents and protocols, and for being great fun to collaborate with.

Jag skulle också vilja tacka:

Mina underbara vänner för att ni alltid är på min sida, för att ni sätter guldkant på min tillvaro och fyller mig med energi. Jag vill framför allt tacka Astrid, för ditt ovärderliga stöd och för att vi alltid har så roligt ihop, jag har haft en sån himla tur att få träffa och jobba ihop med en fantastisk vän nästan varje dag! Extra varmt tack också till Anna S, sedan dagis har du varit den bästa av vänner och en riktig klippa, en 3B-träff med dig och jag är på topp igen!

Familjen Henriksson, för alla uppmuntrande tillrop och all hjälp!

Min kära familj: mamma, pappa och Birgitta, mina fantastiska systrar Anna, Lina, Sanna och Victoria - go 5sis! Farmor och Calle. Tack för all omtanke och kärlek! Min bästa klon Anna, vad hade jag gjort utan dig och Kennet? Ni har verkligen hjälpt mig med ALLT. Ständig uttryckning dygnet runt vad det än har gällt. Jag hade inte tagit mig igenom den här tiden utan er och jag kan inte tacka er nog. Ni är bäst!

Min älskade Richard, för att du är min bästa supporter i alla kategorier. Du är min lycka varje dag och allt för mig ♥

8 REFERENCES

Akakura, S., Singh, S., Spataro, M., Akakura, R., Kim, J.I., Albert, M.L., Birge, R.B. (2004). The opsonin MFG-E8 is a ligand for the alphavbeta5 integrin and triggers DOCK180-dependent Rac1 activation for the phagocytosis of apoptotic cells. Exp Cell Res *292*, 403-416.

Anderson, H.A., Maylock, C.A., Williams, J.A., Paweletz, C.P., Shu, H., Shacter, E. (2003). Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. Nat Immunol *4*, 87-91.

Arredouani, M.S., Palecanda, A., Koziel, H., Huang, Y.C., Imrich, A., Sulahian, T.H., Ning, Y.Y., Yang, Z., Pikkarainen, T., Sankala, M., *et al.* (2005). MARCO is the major binding receptor for unopsonized particles and bacteria on human alveolar macrophages. J Immunol *175*, 6058-6064.

Arroyo, A., Modriansky, M., Serinkan, F.B., Bello, R.I., Matsura, T., Jiang, J., Tyurin, V.A., Tyurina, Y.Y., Fadeel, B., Kagan, V.E. (2002). NADPH oxidase-dependent oxidation and externalization of phosphatidylserine during apoptosis in Me2SO-differentiated HL-60 cells. Role in phagocytic clearance. J Biol Chem *277*, 49965-49975.

Arur, S., Uche, U.E., Rezaul, K., Fong, M., Scranton, V., Cowan, A.E., Mohler, W., Han, D.K. (2003). Annexin I is an endogenous ligand that mediates apoptotic cell engulfment. Dev Cell *4*, 587-598.

Asano, K., Miwa, M., Miwa, K., Hanayama, R., Nagase, H., Nagata, S., Tanaka, M. (2004). Masking of phosphatidylserine inhibits apoptotic cell engulfment and induces autoantibody production in mice. J Exp Med *200*, 459-467.

Atluri, R., Hedin, N., Garcia-Bennett, A.E. (2008). Hydrothermal Phase Transformation of Bicontinuous Cubic Mesoporous Material AMS-6. Chem Mater *20*, 3857-3866.

Bergsmedh, A., Szeles, A., Henriksson, M., Bratt, A., Folkman, M.J., Spetz, A.L., Holmgren, L. (2001). Horizontal transfer of oncogenes by uptake of apoptotic bodies. Proc Natl Acad Sci U S A *98*, 6407-6411.

Bhatia, M., Kirkland, J.B., Meckling-Gill, K.A. (1994). M-CSF and 1,25 dihydroxy vitamin D3 synergize with 12-O-tetradecanoylphorbol-13-acetate to induce macrophage differentiation in acute promyelocytic leukemia NB4 cells. Leukemia *8*, 1744-1749.

Borisenko, G.G., Iverson, S.L., Ahlberg, S., Kagan, V.E., Fadeel, B. (2004). Milk fat globule epidermal growth factor 8 (MFG-E8) binds to oxidized phosphatidylserine: implications for macrophage clearance of apoptotic cells. Cell Death Differ *11*, 943-945.

Bratton, D.L., Fadok, V.A., Richter, D.A., Kailey, J.M., Guthrie, L.A., Henson, P.M. (1997). Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. J Biol Chem *272*, 26159-26165.

Brouwer, M., de Ley, L., Feltkamp, C.A., Elema, J., Jongsma, A.P. (1984). Serum-dependent "cannibalism" and autodestruction in cultures of human small cell carcinoma of the lung. Cancer Res *44*, 2947-2951.

Brown, J.R., Goldblatt, D., Buddle, J., Morton, L., Thrasher, A.J. (2003). Diminished production of anti-inflammatory mediators during neutrophil apoptosis and macrophage phagocytosis in chronic granulomatous disease (CGD). J Leukoc Biol *73*, 591-599.

Böse, J., Gruber, A.D., Helming, L., Schiebe, S., Wegener, I., Hafner, M., Beales, M., Kontgen, F., Lengeling, A. (2004). The phosphatidylserine receptor has essential functions during embryogenesis but not in apoptotic cell removal. J Biol *3*, 15.

Casciola-Rosen, L., Rosen, A., Petri, M., Schlissel, M. (1996). Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythematosus. Proc Natl Acad Sci U S A *93*, 1624-1629.

Casciola-Rosen, L.A., Anhalt, G., Rosen, A. (1994). Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J Exp Med *179*, 1317-1330.

Chapman, L.P., Epton, M.J., Buckingham, J.C., Morris, J.F., Christian, H.C. (2003). Evidence for a role of the adenosine 5'-triphosphate-binding cassette transporter A1 in the externalization of annexin I from pituitary folliculo-stellate cells. Endocrinology *144*, 1062-1073.

Chen, M., von Mikecz, A. (2005). Formation of nucleoplasmic protein aggregates impairs nuclear function in response to SiO2 nanoparticles. Exp Cell Res *305*, 51-62.

Chung, S., Gumienny, T.L., Hengartner, M.O., Driscoll, M. (2000). A common set of engulfment genes mediates removal of both apoptotic and necrotic cell corpses in C. elegans. Nat Cell Biol *2*, 931-937.

Coleman, M.L., Sahai, E.A., Yeo, M., Bosch, M., Dewar, A., Olson, M.F. (2001). Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat Cell Biol *3*, 339-345.

Colvin, V.L. (2003). The potential environmental impact of engineered nanomaterials. Nat Biotechnol *21*, 1166-1170.

Cui, P., Qin, B., Liu, N., Pan, G., Pei, D. (2004). Nuclear localization of the phosphatidylserine receptor protein via multiple nuclear localization signals. Exp Cell Res *293*, 154-163.

Cuttell, L., Vaughan, A., Silva, E., Escaron, C.J., Lavine, M., Van Goethem, E., Eid, J.P., Quirin, M., Franc, N.C. (2008). Undertaker, a Drosophila Junctophilin, links Draper-mediated phagocytosis and calcium homeostasis. Cell *135*, 524-534.

Cvetanovic, M., Ucker, D.S. (2004). Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled directly to specific recognition. J Immunol *172*, 880-889.

D'Acquisto, F., Perretti, M., Flower, R.J. (2008). Annexin-A1: a pivotal regulator of the innate and adaptive immune systems. Br J Pharmacol *155*, 152-169.

Danial, N.N., Korsmeyer, S.J. (2004). Cell death: critical control points. Cell *116*, 205-219.

Dillon, S.R., Mancini, M., Rosen, A., Schlissel, M.S. (2000). Annexin V binds to viable B cells and colocalizes with a marker of lipid rafts upon B cell receptor activation. J Immunol *164*, 1322-1332.

Dinauer, M.C. (2005). Chronic granulomatous disease and other disorders of phagocyte function. Hematology Am Soc Hematol Educ Program, 89-95.

Ding, C., Kume, A., Bjorgvinsdottir, H., Hawley, R.G., Pech, N., Dinauer, M.C. (1996). High-level reconstitution of respiratory burst activity in a human X-linked chronic granulomatous disease (X-CGD) cell line and correction of murine X-CGD bone marrow cells by retroviral-mediated gene transfer of human gp91phox. Blood *88*, 1834-1840.

Dobrovolskaia, M.A., McNeil, S.E. (2007). Immunological properties of engineered nanomaterials. Nat Nanotechnol *2*, 469-478.

Donaldson, K., Stone, V., Seaton, A., MacNee, W. (2001). Ambient particle inhalation and the cardiovascular system: potential mechanisms. Environ Health Perspect *109*, 523-527.

Donaldson, K., Stone, V., Tran, C.L., Kreyling, W., Borm, P.J. (2004). Nanotoxicology. Occup Environ Med *61*, 727-728.

Ellis, H.M., Horvitz, H.R. (1986). Genetic control of programmed cell death in the nematode C. elegans. Cell *44*, 817-829.

Ellis, R.E., Jacobson, D.M., Horvitz, H.R. (1991a). Genes required for the engulfment of cell corpses during programmed cell death in Caenorhabditis elegans. Genetics *129*, 79-94.

Ellis, R.E., Yuan, J.Y., Horvitz, H.R. (1991b). Mechanisms and functions of cell death. Annu Rev Cell Biol *7*, 663-698.

Fadeel, B., Åhlin, A., Henter, J.I., Orrenius, S., Hampton, M.B. (1998). Involvement of caspases in neutrophil apoptosis: regulation by reactive oxygen species. Blood *92*, 4808-4818.

Fadeel, B., Kagan, V., Krug, H., Shvedova, A., Svartengren, M., Tran, L., Wiklund, L. (2007). There's plenty of room at the forum: Potential risks and safety assessment of engineered nanomaterials. Nanotoxicology *1*, 73-84.

Fadeel, B., Orrenius, S. (2005). Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. J Intern Med *258*, 479-517.

Fadeel, B., Orrenius, S., Pervaiz, S. (2004). Buried alive: a novel approach to cancer treatment. FASEB J *18*, 1-4.

Fadeel, B., Ottosson, A., Pervaiz, S. (2008). Big wheel keeps on turning: apoptosome regulation and its role in chemoresistance. Cell Death Differ *15*, 443-452.

Fadok, V.A., Bratton, D.L., Henson, P.M. (2001). Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. J Clin Invest *108*, 957-962.

Fadok, V.A., Bratton, D.L., Konowal, A., Freed, P.W., Westcott, J.Y., Henson, P.M. (1998a). Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest *101*, 890-898.

Fadok, V.A., Bratton, D.L., Rose, D.M., Pearson, A., Ezekewitz, R.A., Henson, P.M. (2000). A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature *405*, 85-90.

Fadok, V.A., Savill, J.S., Haslett, C., Bratton, D.L., Doherty, D.E., Campbell, P.A., Henson, P.M. (1992). Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. J Immunol *149*, 4029-4035.

Fadok, V.A., Warner, M.L., Bratton, D.L., Henson, P.M. (1998b). CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor ($\alpha_v ß_3$). J Immunol *161*, 6250-6257.

Fan, X., Krahling, S., Smith, D., Williamson, P., Schlegel, R.A. (2004). Macrophage surface expression of annexins I and II in the phagocytosis of apoptotic lymphocytes. Mol Biol Cell *15*, 2863-2872.

Febbraio, M., Hajjar, D.P., Silverstein, R.L. (2001). CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. J Clin Invest *108*, 785-791.

Foss Hansen, S., Maynard, A., Baun, A., Tickner, J.A. (2008). Late lessons from early warnings for nanotechnology. Nat Nanotechnol *3*, 444-447.

Franc, N.C., Heitzler, P., Ezekowitz, R.A., White, K. (1999). Requirement for croquemort in phagocytosis of apoptotic cells in Drosophila. Science *284*, 1991-1994.

Frasch, S.C., Henson, P.M., Nagaosa, K., Fessler, M.B., Borregaard, N., Bratton, D.L. (2004). Phospholipid flip-flop and phospholipid scramblase 1 (PLSCR1) co-localize to uropod rafts in formylated Met-Leu-Phe-stimulated neutrophils. J Biol Chem *279*, 17625-17633.

Gaipl, U.S., Munoz, L.E., Grossmayer, G., Lauber, K., Franz, S., Sarter, K., Voll, R.E., Winkler, T., Kuhn, A., Kalden, J., *et al.* (2007). Clearance deficiency and systemic lupus erythematosus (SLE). J Autoimmun *28*, 114-121.

Garcia-Bennett, A.E., Kupferschmidt, N., Sakamoto, Y., Che, S., Terasaki, O. (2005). Synthesis of mesocage structures by kinetic control of self-assembly in anionic surfactants. Angew Chem Int Ed Engl *44*, 5317-5322.

Gardai, S.J., Bratton, D.L., Ogden, C.A., Henson, P.M. (2006). Recognition ligands on apoptotic cells: a perspective. J Leukoc Biol *79*, 896-903.

Geiser, M., Rothen-Rutishauser, B., Kapp, N., Schurch, S., Kreyling, W., Schulz, H., Semmler, M., Im Hof, V., Heyder, J., Gehr, P. (2005). Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. Environ Health Perspect *113*, 1555-1560.

Geissler, J.F., Traxler, P., Regenass, U., Murray, B.J., Roesel, J.L., Meyer, T., McGlynn, E., Storni, A., Lydon, N.B. (1990). Thiazolidine-diones. Biochemical and biological activity of a novel class of tyrosine protein kinase inhibitors. J Biol Chem *265*, 22255-22261.

Gleiss, B., Gogvadze, V., Orrenius, S., Fadeel, B. (2002). Fas-triggered phosphatidylserine exposure is modulated by intracellular ATP. FEBS Lett *519*, 153-158.

Golpon, H.A., Fadok, V.A., Taraseviciene-Stewart, L., Scerbavicius, R., Sauer, C., Welte, T., Henson, P.M., Voelkel, N.F. (2004). Life after corpse engulfment: phagocytosis of apoptotic cells leads to VEGF secretion and cell growth. FASEB J *18*, 1716-1718.

Greenberg, M.E., Sun, M., Zhang, R., Febbraio, M., Silverstein, R., Hazen, S.L. (2006). Oxidized phosphatidylserine-CD36 interactions play an essential role in macrophage-dependent phagocytosis of apoptotic cells. J Exp Med *203*, 2613-2625.

Grimsley, C., Ravichandran, K.S. (2003). Cues for apoptotic cell engulfment: eat-me, don't eat-me and come-get-me signals. Trends Cell Biol *13*, 648-656.

Gwinn, M.R., Vallyathan, V. (2006). Nanoparticles: health effects--pros and cons. Environ Health Perspect *114*, 1818-1825.

Hamilton, R.F., Jr., Thakur, S.A., Mayfair, J.K., Holian, A. (2006). MARCO mediates silica uptake and toxicity in alveolar macrophages from C57BL/6 mice. J Biol Chem *281*, 34218-34226.

Hamon, Y., Broccardo, C., Chambenoit, O., Luciani, M.F., Toti, F., Chaslin, S., Freyssinet, J.M., Devaux, P.F., McNeish, J., Marguet, D., *et al.* (2000). ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. Nat Cell Biol *2*, 399-406.

Hampton, M.B., Vissers, M.C., Keenan, J.I., Winterbourn, C.C. (2002). Oxidant-mediated phosphatidylserine exposure and macrophage uptake of activated neutrophils: possible impairment in chronic granulomatous disease. J Leukoc Biol *71*, 775-781.

Hanahan, D., Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hanayama, R., Nagata, S. (2005). Impaired involution of mammary glands in the absence of milk fat globule EGF factor 8. Proc Natl Acad Sci U S A *102*, 16886-16891.

Hanayama, R., Tanaka, M., Miwa, K., Nagata, S. (2004). Expression of developmental endothelial locus-1 in a subset of macrophages for engulfment of apoptotic cells. J Immunol *172*, 3876-3882.

Hanayama, R., Tanaka, M., Miwa, K., Shinohara, A., Iwamatsu, A., Nagata, S. (2002). Identification of a factor that links apoptotic cells to phagocytes. Nature *417*, 182-187.

Hannon, R., Croxtall, J.D., Getting, S.J., Roviezzo, F., Yona, S., Paul-Clark, M.J., Gavins, F.N., Perretti, M., Morris, J.F., Buckingham, J.C., *et al.* (2003). Aberrant inflammation and resistance to glucocorticoids in annexin 1-/- mouse. FASEB J *17*, 253-255.

Harush-Frenkel, O., Debotton, N., Benita, S., Altschuler, Y. (2007). Targeting of nanoparticles to the clathrin-mediated endocytic pathway. Biochem Biophys Res Commun *353*, 26-32.

Hengartner, M.O. (2000). The biochemistry of apoptosis. Nature *407*, 770-776.

Henson, P.M., Bratton, D.L., Fadok, V.A. (2001). The phosphatidylserine receptor: a crucial molecular switch? Nat Rev Mol Cell Biol *2*, 627-633.

Herrmann, M., Voll, R.E., Zoller, O.M., Hagenhofer, M., Ponner, B.B., Kalden, J.R. (1998). Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. Arthritis Rheum *41*, 1241-1250.

Hoeppner, D.J., Hengartner, M.O., Schnabel, R. (2001). Engulfment genes cooperate with ced-3 to promote cell death in Caenorhabditis elegans. Nature *412*, 202-206.

Hoffmann, P.R., deCathelineau, A.M., Ogden, C.A., Leverrier, Y., Bratton, D.L., Daleke, D.L., Ridley, A.J., Fadok, V.A., Henson, P.M. (2001). Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. J Cell Biol *155*, 649-659.

Holder, M.J., Barnes, N.M., Gregory, C.D., Gordon, J. (2006). Lymphoma cells protected from apoptosis by dysregulated bcl-2 continue to bind annexin V in response to B-cell receptor engagement: a cautionary tale. Leuk Res *30*, 77-80.

Huang, D.M., Chung, T.H., Hung, Y., Lu, F., Wu, S.H., Mou, C.Y., Yao, M., Chen, Y.C. (2008). Internalization of mesoporous silica nanoparticles induces transient but not sufficient osteogenic signals in human mesenchymal stem cells. Toxicol Appl Pharmacol *231*, 208-215.

Ishii, H., Mori, T., Shiratsuchi, A., Nakai, Y., Shimada, Y., Ohno-Iwashita, Y., Nakanishi, Y. (2005). Distinct localization of lipid rafts and externalized phosphatidylserine at the surface of apoptotic cells. Biochem Biophys Res Commun *327*, 94-99.

Jacobson, M.D., Weil, M., Raff, M.C. (1997). Programmed cell death in animal development. Cell *88*, 347-354.

Javey, A. (2008). The 2008 Kavli prize in nanoscience: Carbon nanotubes. ACS Nano *2*, 1329-1335.

Kagan, V.E., Borisenko, G.G., Serinkan, B.F., Tyurina, Y.Y., Tyurin, V.A., Jiang, J., Liu, S.X., Shvedova, A.A., Fabisiak, J.P., Uthaisang, W., *et al.* (2003). Appetizing rancidity of apoptotic cells for macrophages: oxidation, externalization, and recognition of phosphatidylserine. Am J Physiol Lung Cell Mol Physiol *285*, L1-17.

Kagan, V.E., Gleiss, B., Tyurina, Y.Y., Tyurin, V.A., Elenstrom-Magnusson, C., Liu, S.X., Serinkan, F.B., Arroyo, A., Chandra, J., Orrenius, S., *et al.* (2002). A role for oxidative stress in apoptosis: oxidation and externalization of phosphatidylserine is required for macrophage clearance of cells undergoing Fas-mediated apoptosis. J Immunol *169*, 487-499.

Kagan, V.E., Tyurina, Y.Y., Tyurin, V.A., Konduru, N.V., Potapovich, A.I., Osipov, A.N., Kisin, E.R., Schwegler-Berry, D., Mercer, R., Castranova, V., *et al.* (2006). Direct and indirect effects of single walled carbon nanotubes on RAW 264.7 macrophages: role of iron. Toxicol Lett *165*, 88-100.

Kaksonen, M., Toret, C.P., Drubin, D.G. (2006). Harnessing actin dynamics for clathrin-mediated endocytosis. Nat Rev Mol Cell Biol *7*, 404-414.

Kam, N.W., O'Connell, M., Wisdom, J.A., Dai, H. (2005). Carbon nanotubes as multifunctional biological transporters and near-infrared agents for selective cancer cell destruction. Proc Natl Acad Sci U S A *102*, 11600-11605.

Kang, N.Y., Kang, S.K., Lee, Y.C., Choi, H.J., Lee, Y.S., Cho, S.Y., Kim, Y.S., Ko, J.H., Kim, C.H. (2006). Transcriptional regulation of the human GD3 synthase gene expression in Fas-induced Jurkat T cells: a critical role of transcription factor NF-kappaB in regulated expression. Glycobiology *16*, 375-389.

Karpova, M.B., Schoumans, J., Blennow, E., Ernberg, I., Henter, J.I., Smirnov, A.F., Nordenskjold, M., Fadeel, B. (2006). Combined spectral karyotyping, comparative genomic hybridization, and in vitro apoptyping of a panel of Burkitt's lymphoma-derived B cell lines reveals an unexpected complexity of chromosomal aberrations and a recurrence of specific abnormalities in chemoresistant cell lines. Int J Oncol *28*, 605-617.

Kerr, J.F., Wyllie, A.H., Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*, 239-257.

Kim, R., Emi, M., Tanabe, K. (2006). Cancer immunosuppression and autoimmune disease: beyond immunosuppressive networks for tumour immunity. Immunology *119*, 254-264.

Kim, S., Elkon, K.B., Ma, X. (2004). Transcriptional suppression of interleukin-12 gene expression following phagocytosis of apoptotic cells. Immunity *21*, 643-653.

Kinchen, J.M., Cabello, J., Klingele, D., Wong, K., Feichtinger, R., Schnabel, H., Schnabel, R., Hengartner, M.O. (2005). Two pathways converge at CED-10 to mediate actin rearrangement and corpse removal in C. elegans. Nature *434*, 93-99.

Konduru N., Tyurina Y., Basova L., Feng W.H., Bayir H., Clark K., Rubin M., Stolz D., Vallhov H., Scheynius A., Witasp E., Fadeel B., Kichambare P., Star A., Kisin E., Murray A., Shvedova A., Kagan V. (2008) Phosphatidylserine functionalization targets single-walled carbon nanotubes to professional phagocytes in vitro and in vivo. PLoS ONE (in press)

Krahling, S., Callahan, M.K., Williamson, P., Schlegel, R.A. (1999). Exposure of phosphatidylserine is a general feature in the phagocytosis of apoptotic lymphocytes by macrophages. Cell Death Differ *6*, 183-189.

Kravitz, M.S., Shoenfeld, Y. (2006). Autoimmunity to protective molecules: is it the perpetuum mobile (vicious cycle) of autoimmune rheumatic diseases? Nat Clin Pract Rheumatol *2*, 481-490.

Krispin, A., Bledi, Y., Atallah, M., Trahtemberg, U., Verbovetski, I., Nahari, E., Zelig, O., Linial, M., Mevorach, D. (2006). Apoptotic cell thrombospondin-1 and heparin-binding domain lead to dendritic-cell phagocytic and tolerizing states. Blood *108*, 3580-3589.

Kuijpers, T.W., Maianski, N.A., Tool, A.T., Becker, K., Plecko, B., Valianpour, F., Wanders, R.J., Pereira, R., Van Hove, J., Verhoeven, A.J., *et al.* (2004). Neutrophils in Barth syndrome (BTHS) avidly bind annexin-V in the absence of apoptosis. Blood *103*, 3915-3923.

Kunisaki, Y., Masuko, S., Noda, M., Inayoshi, A., Sanui, T., Harada, M., Sasazuki, T., Fukui, Y. (2004). Defective fetal liver erythropoiesis and T lymphopoiesis in mice lacking the phosphatidylserine receptor. Blood *103*, 3362-3364.

Lacerda, L., Bianco, A., Prato, M., Kostarelos, K. (2006). Carbon nanotubes as nanomedicines: from toxicology to pharmacology. Adv Drug Deliv Rev *58*, 1460-1470.

Lewinski, N., Colvin, V., Drezek, R. (2008). Cytotoxicity of nanoparticles. Small *4*, 26-49.

Lewis, M.J., Botto, M. (2006). Complement deficiencies in humans and animals: links to autoimmunity. Autoimmunity *39*, 367-378.

Li, M.O., Sarkisian, M.R., Mehal, W.Z., Rakic, P., Flavell, R.A. (2003). Phosphatidylserine receptor is required for clearance of apoptotic cells. Science *302*, 1560-1563.

Li, W., Baker, N.E. (2007). Engulfment is required for cell competition. Cell *129*, 1215-1225.

Liu, Z., Cai, W., He, L., Nakayama, N., Chen, K., Sun, X., Chen, X., Dai, H. (2007). In vivo biodistribution and highly efficient tumour targeting of carbon nanotubes in mice. Nat Nanotechnol *2*, 47-52.

Lockshin, R.A., Osborne, B., Zakeri, Z. (2000). Cell death in the third millennium. Cell Death Differ *7*, 2-7.

Lu, Y., Low, P.S. (2002). Folate-mediated delivery of macromolecular anticancer therapeutic agents. Adv Drug Deliv Rev *54*, 675-693.

Lucas, M., Stuart, L.M., Zhang, A., Hodivala-Dilke, K., Febbraio, M., Silverstein, R., Savill, J., Lacy-Hulbert, A. (2006). Requirements for apoptotic cell contact in regulation of macrophage responses. J Immunol *177*, 4047-4054.

Lugini, L., Matarrese, P., Tinari, A., Lozupone, F., Federici, C., Iessi, E., Gentile, M., Luciani, F., Parmiani, G., Rivoltini, L., *et al.* (2006). Cannibalism of live lymphocytes by human metastatic but not primary melanoma cells. Cancer Res *66*, 3629-3638.

Lundborg, M., Dahlen, S.E., Johard, U., Gerde, P., Jarstrand, C., Camner, P., Lastbom, L. (2006). Aggregates of ultrafine particles impair phagocytosis of microorganisms by human alveolar macrophages. Environ Res *100*, 197-204.

Maderna, P., Yona, S., Perretti, M., Godson, C. (2005). Modulation of phagocytosis of apoptotic neutrophils by supernatant from dexamethasone-treated macrophages and annexin-derived peptide Ac(2-26). J Immunol *174*, 3727-3733.

Manaka, J., Kuraishi, T., Shiratsuchi, A., Nakai, Y., Higashida, H., Henson, P., Nakanishi, Y. (2004). Draper-mediated and phosphatidylserine-independent phagocytosis of apoptotic cells by Drosophila hemocytes/macrophages. J Biol Chem *279*, 48466-48476.

Manolova, V., Flace, A., Bauer, M., Schwarz, K., Saudan, P., Bachmann, M.F. (2008). Nanoparticles target distinct dendritic cell populations according to their size. Eur J Immunol *38*, 1404-1413.

Martin, S., Pombo, I., Poncet, P., David, B., Arock, M., Blank, U. (2000). Immunologic stimulation of mast cells leads to the reversible exposure of phosphatidylserine in the absence of apoptosis. Int Arch Allergy Immunol *123*, 249-258.

Maynard, A.D., Aitken, R.J. (2007). Assessing exposure to airborne nanomaterials: Current abilities and future requirements. Nanotoxicology *1*, 26-41.

Maynard, A.D., Aitken, R.J., Butz, T., Colvin, V., Donaldson, K., Oberdörster, G., Philbert, M.A., Ryan, J., Seaton, A., Stone, V., *et al.* (2006). Safe handling of nanotechnology. Nature *444*, 267-269.

McEvoy, L., Williamson, P., Schlegel, R.A. (1986). Membrane phospholipid asymmetry as a determinant of erythrocyte recognition by macrophages. Proc Natl Acad Sci U S A *83*, 3311-3315.

Medina, C., Santos-Martinez, M.J., Radomski, A., Corrigan, O.I., Radomski, M.W. (2007). Nanoparticles: pharmacological and toxicological significance. Br J Pharmacol *150*, 552-558.

Mevorach, D., Zhou, J.L., Song, X., Elkon, K.B. (1998). Systemic exposure to irradiated apoptotic cells induces autoantibody production. J Exp Med *188*, 387-392.

Miyanishi, M., Tada, K., Koike, M., Uchiyama, Y., Kitamura, T., Nagata, S. (2007). Identification of Tim4 as a phosphatidylserine receptor. Nature *450*, 435-439.

Moghimi, S.M., Hunter, A.C., Murray, J.C. (2005). Nanomedicine: current status and future prospects. FASEB J *19*, 311-330.

Mosmann, T. (1983). Rapid colorimetric assay for cell growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods *65*, 55-63.

Moss, O.R., Wong, V.A. (2006). When nanoparticles get in the way: impact of projected area on in vivo and in vitro macrophage function. Inhal Toxicol *18*, 711-716.

Nakatani, H., Aoki, N., Nakagawa, Y., Jin-No, S., Aoyama, K., Oshima, K., Ohira, S., Sato, C., Nadano, D., Matsuda, T. (2006). Weaning-induced expression of a milk-fat globule protein, MFG-E8, in mouse mammary glands, as demonstrated by the analyses of its mRNA, protein and phosphatidylserine-binding activity. Biochem J *395*, 21-30.

Nel, A., Xia, T., Madler, L., Li, N. (2006). Toxic potential of materials at the nanolevel. Science *311*, 622-627.

Obeid, M., Tesniere, A., Ghiringhelli, F., Fimia, G.M., Apetoh, L., Perfettini, J.L., Castedo, M., Mignot, G., Panaretakis, T., Casares, N., *et al.* (2007). Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med *13*, 54-61.

Oberdörster, G., Ferin, J., Lehnert, B.E. (1994). Correlation between particle size, in vivo particle persistence, and lung injury. Environ Health Perspect *10*, 173-179.

Oberdörster, G., Oberdörster, E., Oberdörster, J. (2005). Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect *113*, 823-839.

Park, D., Tosello-Trampont, A.C., Elliott, M.R., Lu, M., Haney, L.B., Ma, Z., Klibanov, A.L., Mandell, J.W., Ravichandran, K.S. (2007). BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. Nature *450*, 430-434.

Perretti, M., Flower, R.J. (2004). Annexin 1 and the biology of the neutrophil. J Leukoc Biol *76*, 25-29.

Pradhan, D., Krahling, S., Williamson, P., Schlegel, R.A. (1997). Multiple systems for recognition of apoptotic lymphocytes by macrophages. Mol Biol Cell *8*, 767-778.

Ravikovitch, P.I., Neimark, A.V. (2000). Relations between structural parameters and adsorption characterization of templated nanoporous materials with cubic symmetry. Langmuir *16*, 2419-2423.

Reddien, P.W., Cameron, S., Horvitz, H.R. (2001). Phagocytosis promotes programmed cell death in C. elegans. Nature *412*, 198-202.

Reddien, P.W., Horvitz, H.R. (2004). The engulfment process of programmed cell death in caenorhabditis elegans. Annu Rev Cell Dev Biol *20*, 193-221.

Robertson, A., Thomson, J. (1982). Morphology of programmed cell death in the ventral nerve cord of *Caenorhabditis elegans* larvae. J Embryol Exp Morphol *67*, 89-100.

Ronchetti, A., Rovere, P., Iezzi, G., Galati, G., Heltai, S., Protti, M.P., Garancini, M.P., Manfredi, A.A., Rugarli, C., Bellone, M. (1999). Immunogenicity of apoptotic cells in vivo: role of antigen load, antigen-presenting cells, and cytokines. J Immunol *163*, 130-136.

Savill, J., Fadok, V. (2000). Corpse clearance defines the meaning of cell death. Nature *407*, 784-788.

Savill, J., Hogg, N., Ren, Y., Haslett, C. (1992). Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. J Clin Invest *90*, 1513-1522.

Savill, J.S., Wyllie, A.H., Henson, J.E., Walport, M.J., Henson, P.M., Haslett, C. (1989). Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. J Clin Invest *83*, 865-875.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H., Peter, M.E. (1998). Two CD95 (APO-1/Fas) signaling pathways. EMBO J *17*, 1675-1687.

Scannell, M., Flanagan, M.B., deStefani, A., Wynne, K.J., Cagney, G., Godson, C., Maderna, P. (2007). Annexin-1 and peptide derivatives are released by apoptotic cells and stimulate phagocytosis of apoptotic neutrophils by macrophages. J Immunol *178*, 4595-4605.

Sebbagh, M., Renvoize, C., Hamelin, J., Riche, N., Bertoglio, J., Breard, J. (2001). Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. Nat Cell Biol *3*, 346-352.

Selmeczy, Z., Szelenyi, J., Nemet, K., Vizi, E.S. (2003). The inducibility of TNF-alpha production is different in the granulocytic and monocytic differentiated forms of wild type and CGD-mutant PLB-985 cells. Immunol Cell Biol *81*, 472-479.

Serinkan, B.F., Gambelli, F., Potapovich, A.I., Babu, H., Di Giuseppe, M., Ortiz, L.A., Fabisiak, J.P., Kagan, V.E. (2005). Apoptotic cells quench reactive oxygen and nitrogen species and modulate TNF-alpha/TGF-beta1 balance in activated macrophages: involvement of phosphatidylserine-dependent and -independent pathways. Cell Death Differ *12*, 1141-1144.

Shvedova, A.A., Kisin, E.R., Mercer, R., Murray, A.R., Johnson, V.J., Potapovich, A.I., Tyurina, Y.Y., Gorelik, O., Arepalli, S., Schwegler-Berry, D.*, et al.* (2005). Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. Am J Physiol Lung Cell Mol Physiol *289*, L698-708.

Solito, E., Kamal, A., Russo-Marie, F., Buckingham, J.C., Marullo, S., Perretti, M. (2003). A novel calcium-dependent proapoptotic effect of annexin 1 on human neutrophils. FASEB J *17*, 1544-1546.

Stone, V., Donaldson, K. (2006). Nanotoxicology: signs of stress. Nat Nanotechnol *1*, 23-24.

Stuart, L.M., Ezekowitz, R.A. (2005). Phagocytosis: elegant complexity. Immunity *22*, 539-550.

Sudimack, J., Lee, R.J. (2000). Targeted drug delivery via the folate receptor. Adv Drug Deliv Rev *41*, 147-162.

Tao, Z., Morrow, M.P., Asefa, T., Sharma, K.K., Duncan, C., Anan, A., Penefsky, H.S., Goodisman, J., Souid, A.K. (2008). Mesoporous silica nanoparticles inhibit cellular respiration. Nano lett *8*, 1517-1526.

Tassiulas, I., Park-Min, K.H., Hu, Y., Kellerman, L., Mevorach, D., Ivashkiv, L.B. (2007). Apoptotic cells inhibit LPS-induced cytokine and chemokine production and IFN responses in macrophages. Hum Immunol *68*, 156-164.

Teeguarden, J.G., Hinderliter, P.M., Orr, G., Thrall, B.D., Pounds, J.G. (2007). Particokinetics in vitro: dosimetry considerations for in vitro nanoparticle toxicity assessments. Toxicol Sci *95*, 300-312.

Tesniere, A., Panaretakis, T., Kepp, O., Apetoh, L., Ghiringhelli, F., Zitvogel, L., Kroemer, G. (2008). Molecular characteristics of immunogenic cancer cell death. Cell Death Differ *15*, 3-12.

Thompson, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. Science *267*, 1456-1462.

Tyurina, Y.Y., Basova, L.V., Konduru, N.V., Tyurin, V.A., Potapovich, A.I., Cai, P., Bayir, H., Stoyanovsky, D., Pitt, B.R., Shvedova, A.A., *et al.* (2007). Nitrosative stress inhibits the aminophospholipid translocase resulting in phosphatidylserine externalization and macrophage engulfment: implications for the resolution of inflammation. J Biol Chem *282*, 8498-8509.

Unfried, K., Albrecht, C., Klotz, L.-O., Von Mikecz, A., Grether-Beck, S., Schins, R.P.F. (2007). Cellular responses to nanoparticles: Target structures and mechanisms. Nanotoxicology *1*, 52-71.

Uthaisang, W., Nutt, L.K., Orrenius, S., Fadeel, B. (2003). Phosphatidylserine exposure in Fas type I cells is mitochondria-dependent. FEBS Lett *545*, 110-114.

Wagner, A.J., Bleckmann, C.A., Murdock, R.C., Schrand, A.M., Schlager, J.J., Hussain, S.M. (2007). Cellular interaction of different forms of aluminum nanoparticles in rat alveolar macrophages. J Phys Chem B *111*, 7353-7359.

Vallhov, H., Gabrielsson, S., Stromme, M., Scheynius, A., Garcia-Bennett, A.E. (2007). Mesoporous silica particles induce size dependent effects on human dendritic cells. Nano lett *7*, 3576-3582.

van Genderen, H., Kenis, H., Lux, P., Ungeth, L., Maassen, C., Deckers, N., Narula, J., Hofstra, L., Reutelingsperger, C. (2006). In vitro measurement of cell death with the annexin A5 affinity assay. Nat Protoc *1*, 363-367.

van Lent, P.L., Licht, R., Dijkman, H., Holthuysen, A.E., Berden, J.H., van den Berg, W.B. (2001). Uptake of apoptotic leukocytes by synovial lining macrophages inhibits immune complex-mediated arthritis. J Leukoc Biol *70*, 708-714.

Wang, X., Wang, J., Gengyo-Ando, K., Gu, L., Sun, C.L., Yang, C., Shi, Y., Kobayashi, T., Shi, Y., Mitani, S., *et al.* (2007). C. elegans mitochondrial factor WAH-1 promotes phosphatidylserine externalization in apoptotic cells through phospholipid scramblase SCRM-1. Nat Cell Biol *9*, 541-549.

Wang, X., Wu, Y.C., Fadok, V.A., Lee, M.C., Gengyo-Ando, K., Cheng, L.C., Ledwich, D., Hsu, P.K., Chen, J.Y., Chou, B.K., *et al.* (2003). Cell corpse engulfment mediated by C. elegans phosphatidylserine receptor through CED-5 and CED-12. Science *302*, 1563-1566.

Waterhouse, N.J., Pinkoski, M.J. (2007). Calreticulin: raising awareness of apoptosis. Apoptosis *12*, 631-634.

Vega-Villa, K.R., Takemoto, J.K., Yanez, J.A., Remsberg, C.M., Forrest, M.L., Davies, N.M. (2008). Clinical toxicities of nanocarrier systems. Adv Drug Deliv Rev *60*, 929-938.

Venegas, V., Zhou, Z. (2007). Two alternative mechanisms that regulate the presentation of apoptotic cell engulfment signal in Caenorhabditis elegans. Mol Biol Cell *18*, 3180-3192.

Wermeling, F., Chen, Y., Pikkarainen, T., Scheynius, A., Winqvist, O., Izui, S., Ravetch, J.V., Tryggvason, K., Karlsson, M.C. (2007). Class A scavenger receptors regulate tolerance against apoptotic cells, and autoantibodies against these receptors are predictive of systemic lupus. J Exp Med, *204*, 2259-2265.

Wu, Y., Singh, S., Georgescu, M.M., Birge, R.B. (2005). A role for Mer tyrosine kinase in $\alpha_v\beta_5$ integrin-mediated phagocytosis of apoptotic cells. J Cell Sci *118*, 539-553.

Xia, T., Kovochich, M., Liong, M., Zink, J.I., Nel, A.E. (2008). Cationic polystyrene nanosphere toxicity depends on cell-specific endocytic and mitochondrial injury pathways. ACS Nano *2*, 85-96.

Xiang, S.D., Scholzen, A., Minigo, G., David, C., Apostolopoulos, V., Mottram, P.L., Plebanski, M. (2006). Pathogen recognition and development of particulate vaccines: does size matter? Methods *40*, 1-9.

Yamanaka, M., Eda, S., Beppu, M. (2005). Carbohydrate chains and phosphatidylserine successively work as signals for apoptotic cell removal. Biochem Biophys Res Commun *328*, 273-280.

Yona, S., Heinsbroek, S.E., Peiser, L., Gordon, S., Perretti, M., Flower, R.J. (2006). Impaired phagocytic mechanism in annexin 1 null macrophages. Br J Pharmacol *148*, 469-477.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M., Horvitz, H.R. (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell *75*, 641-652.

Zhen, L., King, A.A., Xiao, Y., Chanock, S.J., Orkin, S.H., Dinauer, M.C. (1993). Gene targeting of X chromosome-linked chronic granulomatous disease locus in a human myeloid leukemia cell line and rescue by expression of recombinant gp91phox. Proc Natl Acad Sci U S A *90*, 9832-9836.

Zitvogel, L., Tesniere, A., Kroemer, G. (2006). Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol *6*, 715-727.

Zullig, S., Neukomm, L.J., Jovanovic, M., Charette, S.J., Lyssenko, N.N., Halleck, M.S., Reutelingsperger, C.P., Schlegel, R.A., Hengartner, M.O. (2007). Aminophospholipid translocase TAT-1 promotes phosphatidylserine exposure during C. elegans apoptosis. Curr Biol *17*, 994-999.

APPENDIX I