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MOLECULAR MECHANISMS OF CELL DEATH AND CELL CLEARANCE

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Molecular Mechanisms of Cell Death and Cell Clearance

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As long as you are not aware of the continual law of Die and
Be Again, you are merely a vague guest on a dark Earth.

Johann Wolfgang von Goethe

Learn all there is to learn, and then choose your own path.

Georg Friedrich Händel

To my parents

for their constant support.

ABSTRACT

Programmed cell death (PCD) is a naturally occurring event in multicellular organisms. It is part of the development as well as ensuring tissue homeostasis and cellular turnover. The cell death process and the removal of the dying cells has to be tightly regulated since dysregulated cell death and cell clearance pathways are correlated with various pathological conditions. Notwithstanding the critical importance of individual cell death modes and of cell clearance for the well-being of the organism, our knowledge regarding the underlying mechanisms as well as its consideration in various toxicological or medical settings is still rather limited. The overall aim of this thesis is to shed light on different aspects regarding the mechanisms of programmed cell death and cell clearance. Emphasis was put on the exposure of phosphatidylserine (PS) – a well studied 'eat-me' signal that is known to facilitate recognition and engulfment of dying cells by phagocytes and is known to be an evolutionarily conserved signal. Moreover, we combined a nanotoxicological study with the elucidation of the underlying cell death pathways.

Paper I addresses the effect of different single point mutations on the function of the *Caenorhabditis elegans* aminophospholipid translocase TAT-1 – a protein that was shown to prevent the externalization of PS in the membranes and that is important for endocytic transport. This *in vivo* study shows for the first time that two conserved motifs of TAT-1 – located in the transmembrane domain four and in the following intracellular domain, respectively – are critical for proper protein function. In paper II, apoptosis, necroptosis or ferroptosis was induced in Jurkat cells and cell death was further characterized regarding morphological and biochemical properties. Cell clearance by primary human macrophages was investigated. Interestingly, all three forms of PCD express PS – even though this was previously thought to be a signal specific for apoptotic cells. Apoptotic cells were phagocytosed more efficiently compared to cells undergoing other cell death modes. In paper III, we studied the effect of size and surface functionalization on the toxic properties of gold nanoparticles using multi-omics studies in combination with validation experiments. We found that only the cationic gold nanoparticles caused toxicity and mitochondrial dysfunction. These particles triggered apoptosis, but at high doses cells died by necrosis. The cationic particles also caused *in vivo* lethality in *C. elegans* while carboxylated particles were non-toxic.

Taken together, the 'eat-me' signal PS and the mechanisms leading to its exposure are of central importance for clearance of dying cells. With this work we elucidate the relevance of conserved regions in P4-type ATPases for its PS transport function. Moreover, we highlight that PS externalization is not unique for apoptotic cells and that macrophages differ in the recognition and uptake of different forms of PCD. Finally, we point out the importance to study the mechanisms of cell death in a toxicological setting using multi-omics approaches combined with validation experiments both *in vitro* and *in vivo*.

LIST OF PUBLICATIONS

I.   Chen YZ*, <u>Klöditz K*</u>, Lee ES, Nguyen DP, Yuan Q, Johnson J, Lee-Yow Y, Hall A, Mitani S, Xia NS, Fadeel B, Xue D; Structure and function analysis of the *C. elegans* aminophospholipid translocase TAT-1; *J Cell Sci.*; 2019; 132(5).

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II.   <u>Klöditz K</u>, Fadeel B; Three cell deaths and a funeral: macrophage clearance of cells undergoing distinct modes of cell death; *Cell Death Discov.*; 2019; 5:65.

III.   Gallud A, <u>Klöditz K</u>, Ytterberg J, Östberg N, Katayama S, Skoog T, Gogvadze V, Chen YZ, Xue D, Moya S, Ruiz J, Astruc D, Zubarev R, Kere J, Fadeel B; Cationic gold nanoparticles elicit mitochondrial dysfunction: a multi-omics study; *Sci Rep.*; 2019; 9(1):4366

APPENDIX

I.   <u>Klöditz K</u>, Chen YZ, Xue D, Fadeel B; Programmed cell clearance: From nematodes to humans; *Biochem Biophys Res Commun.*; 2017; 482(3):491-497.

ADDITIONAL PUBLICATION

I.   Balasubramanian K, Maeda A, Lee JS, Mohammadyani D, Dar HH, Jiang JF, St Croix CM, Watkins S, Tyurin VA, Tyurina YY, <u>Klöditz K</u>, Polimova A, Kapralova VI, Xiong Z, Ray P, Klein-Seetharaman J, Mallampalli RK, Bayir H, Fadeel B, Kagan VE; Dichotomous roles for externalized cardiolipin in extracellular signaling: Promotion of phagocytosis and attenuation of innate immunity; *Sci Signal.*; 2015; 8(395):ra95

CONTENTS

ABBREVIATIONS

AIF	Apoptosis inducing factor
APAF1	Apoptotic peptidase activating factor 1
ATPase	Adenosine triphosphatase
ATP11C	phospholipid-transporting ATPase IG
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
BCL2	B-cell lymphoma 2
ccRCC	Clear cell renal cell carcinoma
CED	Cell death abnormal
CL	Cardiolipin
DAMPs	Damage-associated molecular patterns
DED	Death effector domain
DEG	Differentially expressed genes
DIC	differential interference contrast
DISC	Death-inducing signaling complex
DLS	Dynamic light scattering
EGL-1	Egg-lying defective
ER	Endoplasmatic reticulum
FADD	Fas-associated death domain protein
Fer-1	Ferrostatin-1
Gas6	Growth arrest-specific 6
GPX4	Glutathione peroxidase 4
GSH	Glutathione (reduced)
HMDMs	Human monocyte derived macrophages
IAPs	Inhibitor of apoptosis proteins
IL	Interleukin
IPA	Ingenuity Pathway Analysis
LC3	Microtubule-associated proteins 1A/1B light chain 3B
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LMP	Lysosomal membrane permeabilization
LPS	Lipopolysaccharides
M-CSF	Macrophage colony-stimulating factor
MFG-E8	Milk fat globule-EGF factor 8 protein
MLKL	Mixed lineage kinase domain-like pseudokinase
MOMP	Mitochondrial outer membrane permeabilization
NCCD	Nomenclature Committee on Cell Death

Nec-1	Necrostatin-1
NP	Nanoparticle
PC	Phosphatidylcholine
PCD	Programmed cell death
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PI	Propidium iodide
PS	Phosphatidylserine
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
RIPK3	Receptor-interacting serine/threonine-protein kinase 3
ROS	Reactive oxygen species
RSL3	RAS-selective lethal
SLE	Systemic lupus erythematosus
SM	Sphingomyelin
Smac	Second mitochondria-derived activator of caspases
STRT RNA-seq	Single cell tagged reverse transcription RNA sequencing
TAT-1	Transbilayer amphipath transporter 1
TEM	Transmission electron microscopy
TIM4	T cell immunoglobulin mucin receptor 4
TMEM16F	Transmembrane protein 16F
TNFα	Tumor necrosis factor α
ToF-SIMS	Time-of-Flight secondary ion mass spectrometry
TRADD	TNFR1-associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand
TTR-52	Transthyretin 52
WAH-1	Worm AIF homolog
Xkr-8	Xk-related protein 8

1 INTRODUCTION

1.1 CELL DEATH IN HEALTH AND DISEASE

1.1.1 Embryonic development and tissue homeostasis

Cells are the fundamental units of life. In a multicellular organism cells provide the basic structure but moreover work together in a complex network to ensure and maintain proper function of the organism. An adult human body is suggested to consist of approximately 75 to 100 trillion cells and several hundreds of different cell types that form tissues and organs. However, the well-being of the organism requires not only cell proliferation – which ensures replacement and turnover of cells – but also removal of old or unwanted cells. Notably, constant cell division and cell death ensures correct development of the organism as well as homeostasis and tissue renewal and this process is tightly regulated and balanced under physiological conditions. The importance of cell death and its tightly controlled occurrence is summarized by the following quote from the Nobel laureate Robert Horvitz:

> "The number of cells in our bodies is defined by an equilibrium of opposing
> forces: mitosis adds cells, while programmed cell death removes them. Just
> as too much cell division can lead to a pathological increase in cell number,
> so can too little cell death." (Horvitz, 2003).

Therefore, a balance between proliferation and cell death is critical and ensures a constant number of cells in the organism. It is estimated that at least 50 to 70 billion cells die every day through programmed cell death (PCD) in an adult human body. Cell death might be part of the tissue homeostasis or be caused by environmental factors such as infections, exposure to toxins or stress. Moreover, it ensures that old or damaged cells or cells that are no longer required are removed and the quick and efficient removal of these cells is important to prevent them from causing further harm such as tissue damage or inflammation (Ravichandran, 2010). The life time of different cell types varies and ranges from few days up to several decades. As an example, T and B cells are eliminated as part of a quality-control process during their development (Jacobson et al., 1997). Moreover, immune cells that have successfully contained a pathogen infection need to be removed in order to prevent autoreactivity. In contrast, several neurons of the cerebral cortex as well as memory T cells are reported to live several decades. Cell death during embryogenesis is critical for proper development of the organism as it for example ensures limb development, proper shaping of the individual organs or the removal of excess or inappropriately connected neurons (Jacobson et al., 1997).

It is essential that there is not only a balance of the ratio between proliferation and cell death but also between cell death and cell clearance; if phagocytes fail to clear dying cells *in vivo*, this would result in secondary necrosis and tissue damage. The link and tight regulation of proliferation, cell death and cell clearance is essential for the well-being of the organism. Therefore, cell clearance defines the "meaning" of cell death (Savill and Fadok, 2000). Under physiological conditions, cell clearance is highly efficient and apoptotic cells are rarely found even in tissues with high cellular turnover such as the thymus or the bone marrow. Efficient cell clearance as well as its tight regulation – a process referred to as "programmed cell clearance" – is of physiological and pathological importance as it ensures proper function and development of the organism (Witasp et al., 2008). Dysregulated cell death as well as insufficient cell clearance could cause inflammation and tissue damage.

"Programmed cell death" was for many years used as a synonym for "apoptotic cell death" while necrosis was used as a term describing accidental cell death. More recent research revealed the existence of other forms of PCD that are similarly important for development and homeostasis and are associated with various physiological and pathological settings.

1.1.2 Dysregulated cell death and disease progression

Tightly regulated cell death as well as efficient programmed cell clearance is essential for the well-being of the organism and a dysregulation in this process can lead to severe pathological conditions and disease progression. Increased cell death or inefficient cell clearance would result in the accumulation of dying cells leading to persisting inflammation and autoimmunity. Conversely, decreased cell death can cause damaged or transformed cells to remain present in the organism even though these cells should be removed. The following paragraphs describe selected pathologies and the role of cell death or cell clearance in these diseases.

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disorder characterized by the production of autoantibodies – mainly antibodies against phospholipids and cardiolipin as well as nuclear components – which direct the immune system against itself thus causing it to attack healthy tissues. The persistence of apoptotic cells as a result of increased cell death together with deficient cell clearance was reported (Baumann et al., 2002; Shao and Cohen, 2011). If apoptotic cells are not cleared, they undergo secondary necrosis and the exposure of intracellular components to the extracellular environment subsequently causes an increased inflammatory response (Mistry and Kaplan, 2017). Moreover, the exposure of autoantigens would further stimulate the production of autoantibodies.

Type 1 diabetes is an autoimmune disease where the immune system attacks and destroys the β-cells in the pancreas leading to diminished insulin production and inflammation (Bending et al., 2012; Clark et al., 2017). Moreover, type 1 diabetes is reported to be a T cell-mediated

autoimmune disease caused by accumulation of autoreactive CD4^{+} T helper cells and CD8^{+} T cells (Bluestone et al., 2010; Lieberman and DiLorenzo, 2003). The detection of autoantibodies – for example directed against cytoplasmic components of islet cells, insulin or glutamic acid decarboxylase – was reported in patients with type 1 diabetes (Pihoker et al., 2005; Schmidt et al., 2005; Taplin and Barker, 2008).

Atherosclerosis is caused by the accumulation of oxidized lipids (mainly low-density lipoprotein (LDL)) in atherosclerotic lesions – also referred to as plaques – in the vascular wall. Plaque formation is associated with damage of the endothelium and causes inflammation and the recruitment of immune cells. Macrophages ingesting oxidized LDL develop into "foam cells" and die, which further propagates inflammation. Moreover, atherosclerosis is associated with an increase in apoptotic cell death of vascular smooth muscle cells, endothelial cells and macrophages (Littlewood and Bennett, 2003; Rayner, 2017). Additionally, a defective apoptotic cell clearance mechanism and the subsequent occurrence of secondary necrosis causes inflammation, expansion of plaques and leads to further complications such as cardiovascular diseases and stroke (Kojima et al., 2017; Van Vré et al., 2012).

Multiple neurodegenerative disorders – ranging from Alzheimer's disease, Parkinson's disease to Huntington's disease – share a common feature: the loss of specific subsets of neurons (Gorman, 2008). Moreover, the accumulation of certain proteins – e.g. misfolded β-amyloid peptide or mutated huntingtin – was reported. Together, this would directly lead to the manifestation of the symptoms such as cognitive dysfunction and behavioral abnormalities. The mechanism of cell death in these pathologies is still under debate but might be of non-apoptotic nature (Jellinger, 2001; Jellinger and Stadelmann, 2000; Zhang et al., 2017). Understanding the underlying cell death mechanisms of various neurological disorders is essential in order to develop novel, effective treatment strategies which are currently missing (Golde, 2009).

Finally, impaired cell death mechanisms are associated with the onset and progression of tumor development. Resistance to apoptosis is a hallmark of cancer as various forms of cancers suppress the apoptotic pathway and promote survival and proliferation (Hanahan and Weinberg, 2011). Suppression of the cell death program therefore leads to excessive, uncontrolled proliferation of the cells. Recent reports suggest that the increased metabolic activity leads to elevated ROS (reactive oxygen species) production and thus a higher demand of these cells to counterbalance oxidative stress. Therefore, cancer cells that actively suppress apoptosis might be more susceptible to undergo ferroptotic cell death, a form of PCD that targets the ROS-scavenging system (Irani et al., 1997; Szatrowski and Nathan, 1991; Trachootham et al., 2006; Yang et al., 2014). Importantly, addressing alternative cell death pathways in cancer treatment may help to overcome drug resistance, sensitize cancer cells to chemotherapeutic agents and reduce negative side effects (Conrad et al., 2016; Viswanathan et al., 2017; Yu et al., 2015). Various different cancer types – especially clear cell renal cell carcinomas (ccRCC), diffuse large B-cell lymphomas (DLBCL) and triple-negative breast cancer cells – were reported to be sensitive

to ferroptosis induction (Lu et al., 2017) as they are highly dependent on the oxidative defense system (Miess et al., 2018; Timmerman et al., 2013; Yang et al., 2014). However, it is important that anti-cancer therapy does not only aim to induce cell death of the tumor but also to consider the type of cell death as well as its immunologic consequences (Garg and Agostinis, 2017).

Taken together, an imbalance of cell death or cell clearance mechanisms is associated with the pathology of many different diseases and has severe consequences for the well-being of the organism. While most cell death research has focused on cell death induction or cell death inhibition, the cell clearance mechanism – especially with relation to different diseases – have not been studied as extensively. Future studies in this field would be of critical importance and could lead to the development of new treatment strategies. The consideration of cell clearance mechanisms in the treatment of various diseases can be beneficial for the patients.

1.1.3   Relevance of cell death and cell clearance research in toxicology

Throughout our life, we are exposed to different chemicals that can enter our body for example through contact with the skin as well as through ingestion or inhalation. Toxicology aims to study the mode of action and the effect of chemicals or substances on the organism as well as to predict a potential risk and is therefore a field closely linked to cell death research. The huge and constantly increasing number of individual substances and mixtures to which we are exposed sets a high demand for the evaluation of potential adverse effects. In general, the consequences of exposure to high doses for a short time (acute exposure) are opposed to the effect of exposure to low doses over a prolonged time (chronic exposure) and these two cases are not necessarily correlated but can show distinct characteristics and effects. In addition to a potential time dependent effect, a dose-response relationship was observed for different toxic reagents (Klaassen et al., 2019).

The amount of potential toxins to which we are exposed is steadily increasing and ranges from heavy metals in the drinking water to additives in cosmetics, fumes produced from industry or vehicles up to engineered materials. It is hypothesized that the different toxins or materials can interfere with the regulation of the cell death program in multiple ways either through preventing cell death thus being potentially carcinogenic or by causing excessive cell death. Additionally, it is possible that these chemicals interfere with immune cells thus preventing efficient cell clearance or causing inflammation. It is therefore possible to suggest that there is a direct link between the exposure to certain substances, cell death and the onset and progression of various pathologies. Exposure to toxins may not cause an immediate effect but chronic exposure to low doses can nonetheless cause subtle changes and have long-term consequences (Nordberg et al., 2014). Biochemically, different substances have been shown to alter intracellular Ca^{2+} homeostasis and to cause elevated production of ROS. Possible ways to do so include release of ions or direct interaction with proteins or other biological structures. Both, the

intracellular Ca^{2+} level as well as the redox state of a cell are central regulators of various cellular processes and their alteration can directly affect the fate of the cell (Orrenius et al., 2011). Increased oxidative stress may alter protein expression, cause DNA damage and lead to cell death and inflammation (Nel et al., 2006). Moreover, drug induced genotoxicity can influence gene expression or cause mutagenesis.

Nanotoxicology is a more recently expanding field of toxicology that specifically addresses the effect of nanomaterials on the organism and environment. Of note, not all substances are toxic and various nanoparticles are under development for medical applications. Different clinical trials are ongoing where nanoparticles can be a useful tool to image and detect specific structures or are used for diagnostics, drug delivery or treatment (Nasimi and Haidari, 2013). As an example, nanoparticles (NPs) that specifically recognize cancer cells but not healthy cells can be loaded with an anti-cancer drug and thus be used as a cancer treatment. Importantly, nanomaterials can induce autophagy, apoptosis or necrosis in a dose-dependent manner. While autophagy might be an attempt to handle and counterbalance cellular stress, apoptosis can be induced by persistence of the insult. Moreover, necrosis might occur as a result to high concentrations of a toxicant (Orrenius et al., 2013; Orrenius et al., 2011).

A correlation was reported between NP exposure and lung inflammation and some NPs may initiate and support progression of tumor development (Inoue et al., 2006; Stueckle et al., 2017). In depth knowledge regarding the affected pathways and the underlying cell death mode will be of critical importance for the prediction of a possible risk and the consequences that are associated with exposure to the particles as well as for the potential development of novel treatment strategies (Andón and Fadeel, 2013). This is not always an easy task. We are exposed to a great variety of particles and it might therefore be difficult to study the effect of one single substance in a mixture of particles. Nonetheless, the increasing amount of particles in our everyday life demands the development of high-throughput methods to characterize the NPs and to investigate potential adverse effects. Moreover, their use in medical applications requires broad knowledge regarding the effect of the particle on the organism and this then ensures minimal risk and side effects.

While there is a clear link between cell death and toxicology and increasing attempts are made in order to understand the underlying mechanisms (Orrenius et al., 2011), the role of cell clearance has not yet found equal attention in the field of toxicology. However, proper cell clearance as well as the subsequent immune response are similarly important and essentially determine the consequences of the exposure on the organismal level. Various *in vitro*, *in vivo* and *in silico* models are used to address the increasing demand for proper characterization of materials and of potential adverse effects as well as for detailed understanding of the molecular basis of these alterations upon exposure to chemicals and NPs (Fadeel et al., 2017).

1.2   PROGRAMMED CELL DEATH

1.2.1   Historical background

The occurrence of cell death was observed already in the eighteenth and nineteenth century (Clarke and Clarke, 1996; Majno and Joris, 1995). A breakthrough and basis for later cell death research was the discovery of Schwann and Schleiden in the middle of the nineteenth century that organisms are built of cells (Schleiden et al., 1849; Schwann and Hünseler, 1910). Thereafter, several researchers – led by German scientists – described mainly morphological changes during embryogenesis and metamorphosis but do not refer to these observations as cell death (Vogt, 1842) or seem to consider cell death as an especially important or even regulated process and quickly lost interest in it (Weismann, 1864; Weismann, 1866). During that time, it was simply noted that cells "disappear" or "are resorbed" during development. In 1858 Virchow described two forms of cell death – necrobiosis (today referred to as apoptosis) and necrosis – in one of his lectures (Virchow and Chance, 1860). Another milestone was the discovery of phagocytes by Mechnikov (1883), thus for the first time providing evidence not only of cell death but cell clearance mechanisms. In the following years, cell death was observed in various tissues and animals suggesting the general occurrence of this phenomenon (Mayer, 1886; Mayer, 1887; Barfurth, 1887; Goette, 1875).

In 1885 Flemming described morphological characteristics of cells undergoing naturally occurring cell death. He termed these features chromatolysis (Flemming, 1885). Interest in cell death research declined and only few researchers – including Kallius, Glücksmann and colleagues – showed substantial interest in this field (Glücksmann, 1951). Through their work it became more and more evident that cell death was an essential part of the embryonic development and was observed in all species. However, the underlying biochemical signals that regulate cell death as well as the disposal of dying cells remained to be discovered (Saunders, 1966).

The name apoptosis was first proposed by Kerr and colleagues who in detail reported morphological changes of apoptotic cells such as nuclear and cytoplasmic condensation or the formation of apoptotic bodies (Kerr et al., 1972). They further suggest that apoptosis is an active process and a genetically regulated phenomenon that occurs both during embryonic development as well as in the adult organism. They state, that apoptosis is a physiological cell death involved in tissue turnover in the healthy adult organism and that is also linked to various physiological and pathological conditions. Moreover, the uptake of apoptotic cells by phagocytes together with their lysosomal degradation was reported (Kerr et al., 1972).

Importantly, in the 1960s Brenner and colleagues introduced the nematode *C. elegans* as a model organism, which in the following years developed as an important tool in cell death research. This worm consists of 1090 somatic cells out of which 131 cells are eliminated during

the development (Kimble and Hirsh, 1979; Sulston and Horvitz, 1977; Sulston et al., 1983). The observed cell death was found to occur in a highly regulated manner meaning that a specific cell always died in the same spot and at the same time during the development of the organism. In the following years detailed lineage information was achieved and the occurrence of cell division and cell death during development was mapped. This was possible via microscopy of the transparent nematode. The occurrence of cell death was observed though changes in cellular morphology as apoptotic cells show a refractile, raised-button-like appearance. Moreover, screening of mutant strains was performed in order to identify main regulators of the cell death program and this led to the discovery of several genes, including the cell death inducers *ced-3* and *ced-4* (ced stands for cell death abnormal) and the cell death suppressor *ced-9* (Ellis and Horvitz, 1986; Xue and Horvitz, 1997; Xue et al., 1996; Yuan and Horvitz, 1990). Additionally, genes that were required for cell clearance were identified (Ellis et al., 1991). The identification of mammalian homologs then allowed to suggest that the underlying cell death program is evolutionarily conserved (Horvitz, 2003). The term "programmed cell death" was first introduced by Lockshin in his PhD thesis on "Programmed Cell Death in an Insect" in 1963.

Since 2005 the Nomenclature Committee on Cell Death (NCCD) regularly publishes recommendations for definitions of individual cell death modes (Galluzzi et al., 2018; Galluzzi et al., 2012; Kroemer et al., 2005; Kroemer et al., 2009). While cell death was previously defined by morphological features and molecular characterization was missing or unknown (Kroemer et al., 2005), throughout the years the committee changed the view. As the molecular details of different cell death subroutines were discovered, emphasis is put on identifying measurable biochemical properties. In the latest issue, the committee distinguishes between accidental and regulated cell death (Galluzzi et al., 2018). In contrast to accidental cell death, it was stated that regulated cell death can be inhibited through genetic or pharmaceutical means. Moreover, the occurrence of essential and accessory aspects of cell death was described thus further highlighting the point of irreversible manifestation of a cell death. The NCCD defines a reversible initiator phase that aims for repair and adaptation to stress situations. This is followed by an irreversible execution phase, and a propagation phase (Galluzzi et al., 2018). While the recommendations from the NCCD steadily develop and become more and more sophisticated, it might appear confusing that the point of view changes quite drastically from one issue to the other. Of note, the cell clearance mechanisms and immunological consequences are not considered in the recommendations for the definition of a cell death.
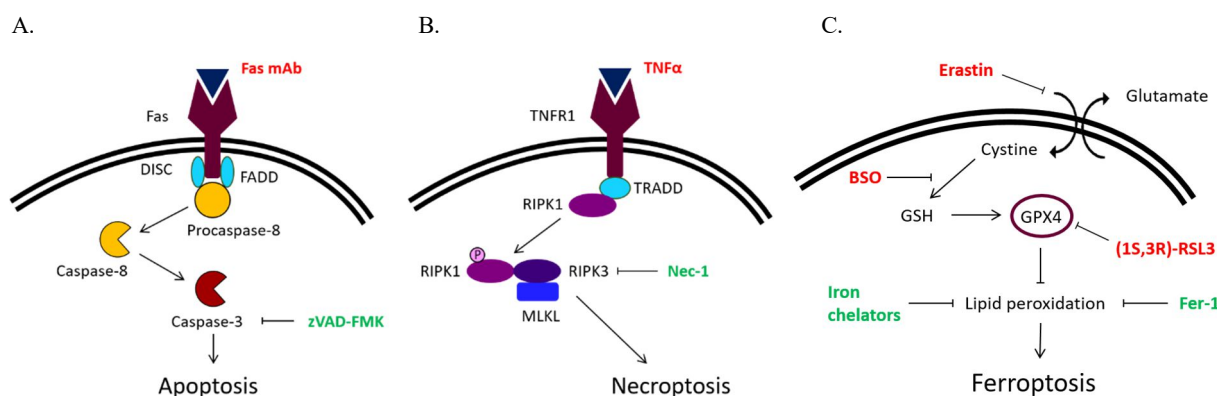
1.2.2 Programmed cell death

Fig. 1: Schematic figure of three cell death modes. Illustration of the pathways leading to (A) death receptor-induced apoptosis, (B) necroptosis or (C) ferroptosis. The cell death inducers are indicated in red, while the cell death specific inhibitors are shown in green. The black double line symbolizes the plasma membrane. Modified from Klöditz and Fadeel, 2019.

1.2.2.1 Apoptosis

The term apoptosis as a form of genetically regulated and programmed cell death was first introduced by Kerr, Wyllie and Currie in 1972 (Kerr et al., 1972) followed by the identification of central cell death genes in the following decades. For that, studies of the nematode *C. elegans* were especially valuable as the key mediators of the apoptotic pathway were first identified in that organism (Horvitz, 2003). Apoptosis occurs as part of the embryonic development and is further critical for tissue homeostasis. Apoptotic cells are characterized by morphological changes such as cell shrinkage, chromatin condensation and large scale DNA fragmentation (~50 kbp), as well as blebbing of the plasma membrane and the formation of "apoptotic bodies" (Kerr et al., 1972). Importantly, the membrane integrity remains intact thus preventing the exposure of intracellular components to the extracellular environment which would cause tissue damage and inflammation. Apoptosis is therefore considered an immunologically silent process meaning it allows sustained cellular turnover in the absence of the activation of the immune system. However, if phagocytes fail to remove apoptotic cells from the system, the apoptotic cell will eventually undergo secondary necrosis thus causing inflammation (Savill and Fadok, 2000). The externalization of phosphatidylserine (PS) – a phospholipid of the cell membrane that under homeostatic conditions is restricted to the cytosolic leaflet – has for a long time been discussed as a signal of apoptotic cells but was more recently shown to also occur in other forms of cell death (Brouckaert et al., 2004; Gong et al., 2017; Li et al., 2015; Zargarian et al., 2017).

Two main apoptotic pathways are distinguished in the mammalian system: the intrinsic (mitochondria-mediated) and the extrinsic (death receptor mediated) pathway (Fadeel and Orrenius, 2005; Scaffidi et al., 1998). The former one is activated by intracellular stress-induced

stimuli such as DNA damage, oxidative stress, ER stress, hypoxia, accumulation of unfolded proteins or growth factor withdrawal as well as developmental signals (Brenner and Mak, 2009). Mitochondria play a central role in this pathway as they are not only required for energy production and cell survival but also tightly regulate cell death. BH3-only proteins belong to the BCL2 protein family. They are shown to trigger apoptosis through activation of Bax and Bak as well as by suppression of anti-apoptotic proteins (Shamas-Din et al., 2011). Oligomerization of Bax and Bak at the mitochondrial outer membrane leads to the formation of pores thus causing mitochondrial outer membrane permeabilization (MOMP) as well as a drop in the mitochondrial membrane potential and allowing mitochondrial intermembrane proteins to translocate to the cytosol (Gogvadze et al., 2001; Jacotot et al., 1999; Lomonosova and Chinnadurai, 2008). Examples for such pro-apoptotic proteins are the apoptosis inducing factor (AIF), cytochrome c, DIABLO/ Smac and EndoG. AIF has an essential role in the regulation of cell survival by facilitating the assembly of mitochondrial electron transport chain complexes I, III and IV. Following apoptosis induction, AIF is cleaved by calpains (Norberg et al., 2008) and released from the mitochondria where it is stabilized by interaction with Scythe (Desmots et al., 2008). AIF was shown to trigger DNA fragmentation in the nucleus as well as scramblase activation and PS exposure at the plasma membrane (Daugas et al., 2000; Preta and Fadeel, 2012). Moreover, following MOMP and translocation of mitochondrial proteins, a cytosolic complex – referred to as the apoptosome – forms and was shown to consist of cytochrome c, APAF1, dATP and the initiator caspase procaspase-9. The dimerization of procaspase-9 leads to autocatalytic cleavage and activation of caspase-9 (Acehan et al., 2002; Fadeel et al., 2008; Yuan and Akey, 2013). DIABLO/ Smac is known as an antagonist of inhibitor of apoptosis proteins (IAPs) and by that expresses pro-apoptotic properties. The release of Smac from the mitochondria abolishes the inhibitory effect of the IAPs thus allowing caspase activation and cell death. Together, the intrinsic apoptotic pathway illustrates the importance of mitochondria as regulators of cell death.

The extrinsic apoptotic pathway is activated through binding of a death ligand to a death receptor of the tumor necrosis factor (TNF) family expressed on the cell surface (Fig. 1A). Binding of the Fas-ligand (CD95), TNFα or the TNF-related apoptosis-inducing ligand (TRAIL) to the specific receptor Fas, to TNFR1 or to TRAIL-R1/2 respectively causes oligomerization of the receptor which allows the intracellular assembly of the death-inducing signaling complex (DISC) (Peter and Krammer, 1998). In this multi-protein complex, procaspase-8 binds to the cytoplasmic tail of the death receptor via the adaptor protein FADD (Fas-associated death domain protein) or TRADD (TNFR1-associated death domain protein) leading to autocatalytic cleavage and activation of caspase-8 (Medema et al., 1997).

Both the intrinsic and extrinsic apoptotic pathway result in the cleavage and activation of executioner caspases such as caspase-3, caspase-6 and caspase-7. These caspases cleave numerous substrates – including structural proteins and negative regulators of apoptosis –

subsequently leading to cell death (Boatright and Salvesen, 2003; Muzio, 1998; Riedl and Shi, 2004). Additionally, cleavage of Bid by caspase-8 causes MOMP and thus links the extrinsic to the intrinsic apoptotic pathway and causes an amplification of the signal (Li et al., 1998; Schug et al., 2011). The presence of zVAD-FMK inhibits caspase activity and thus blocks apoptosis.

NPs that are taken up via the endo-lysosomal pathway and consequently accumulate in lysosomes have the potential to destabilize lysosomal membranes and to cause lysosomal membrane permeabilization (LMP). Hence, lysosomal proteins such as cathepsins can leak into the cytosol, cause mitochondrial outer membrane permeabilization and trigger mitochondria-mediated apoptosis (Aits and Jäättelä, 2013; Johansson et al., 2010; Repnik et al., 2012).

1.2.2.2 Necroptosis

Necroptosis is described as a form of regulated necrosis since this form of PCD displays morphological characteristics of necrosis but – similarly to apoptotic cell death – the underlying cell death mechanism is genetically regulated (Christofferson and Yuan, 2010; Degterev et al., 2008; Degterev et al., 2005; Vanden Berghe et al., 2014). Necroptosis involves receptor-interacting serine/threonine-protein kinase 1 and 3 (RIPK1 and RIPK3) as well as the mixed lineage kinase domain-like pseudokinase (MLKL) as central cell death regulators and is blocked by necrostatin-1 – a RIPK1 inhibitor (Cho et al., 2009; Degterev et al., 2008; He et al., 2009; Sun et al., 2012; Vandenabeele et al., 2010a; Zhang et al., 2009)(Fig. 1B). Similarly to the extrinsic apoptotic cell death pathway, binding of a death ligand to the death receptor causes its oligomerization. In the absence of caspase-8 activation, RIPK1 binds intracellularly to the death receptor – via the FADD or TRADD adaptor molecule – where it is autophosphorylated and activated (Lee et al., 2012). Active RPIK1 then forms a complex with RIPK3 in which RIPK3 becomes phosphorylated and the resulting active RIPK3 further phosphorylates MLKL (Murphy et al., 2013; Zhao et al., 2012). Phosphorylated MLKL was shown to translocate to the plasma membrane where it forms pores and thus results in loss of the membrane integrity (Cai et al., 2014; Dondelinger et al., 2014; Hildebrand et al., 2014; Murphy et al., 2013; Zhao et al., 2012). RIPK1 can interact with both caspase-8 and RIPK3 and thus regulates both apoptosis and necroptosis (Estornes et al., 2014; Meng et al., 2018; Newton, 2015). Together, RIPK1 initiates the necroptotic cell death pathway through activation of RIPK3, while RIPK3-dependent phosphorylation of MLKL executes necroptosis. The subsequent release of intracellular components to the extracellular environment – referred to as DAMPs (damage-associated molecular patterns) – causes inflammation (Kaczmarek et al., 2013). Morphologically, necroptosis is characterized by cellular and organellar swelling, as well as plasma membrane rupture.

Cells infected by viruses can trigger necroptotic cell death and thus contribute to the host defense against pathogens (Cho et al., 2009). Moreover, necroptosis may be associated with

various pathologies ranging from multiple sclerosis to atherosclerosis, ischemic brain injury and neurodegenerative diseases (Degterev et al., 2005; Linkermann and Green, 2014; Ofengeim et al., 2015; Vandenabeele et al., 2010b).

1.2.2.3 Ferroptosis

Ferroptosis describes a recently discovered non-apoptotic, iron-dependent, oxidative form of PCD (Dixon et al., 2012) that was mainly described as a potential way to induce death in several different forms of cancer. Ferroptosis was described to be morphologically, biochemically and genetically distinct from apoptotic, necroptotic or autophagic cell death (Dixon et al., 2012). Mice deficient in the major ferroptosis regulator glutathione peroxidase 4 (GPX4) fail to develop beyond gastrulation stage indicating that a functional oxidative defense mechanism and ferroptosis inhibition are required for proper embryonic development (Brutsch et al., 2015; Conrad et al., 2018; Yant et al., 2003). Tumor cells that have been reported to be susceptible for ferroptosis induction include clear cell renal cell carcinoma (ccRCC), diffuse large B-cell lymphomas (DLBCL) and triple-negative breast cancer cells (Lu et al., 2017; Miess et al., 2018; Timmerman et al., 2013; Yang et al., 2014).

In viable cells, the glutathione peroxidase 4 (GPX4) is catalyzing the reduction of phospholipid hydroperoxides thus counterbalancing ROS production and protecting cells from excessive lipid peroxidation (Thomas et al., 1990). Inhibition of the GPX4 activity therefore causes lethal accumulation of lipid peroxides (Yang et al., 2014)(Fig. 1C). Oxidized phosphatidylethanolamine (PE) species were found to be a marker of ferroptotic cell death (Kagan et al., 2017). Ferroptosis can be induced by depletion of cellular glutathione (GSH) level – the major cellular antioxidant defense system. GSH is a substrate of GPX4 and low cellular GSH level would therefore diminish the GPX4 function. Inhibition of the cystine/glutamate antiporter system x_c^- (type I ferroptosis) by compounds such as artemisinin or erastin results in reduced cellular uptake of cystine and subsequently diminished GSH synthesis (Dixon et al., 2012; Yagoda et al., 2007). Additionally, high extracellular glutamate level (that are inactivating the system x_c^- antiporter and inhibiting cystine uptake) or inhibition of the GSH synthesizing enzyme glutamate cysteine ligase with buthionine-(*S*,*R*)-sulfoximine (BSO) would also result in depletion of cellular GSH level and subsequent ferroptotic cell death. Alternatively, direct inhibition of GPX4 – referred to as type II ferroptosis – for example through the GPX4 inhibitor RAS-selective lethal 3 ((1S, 3R)-RSL3) leads to increased lipid peroxidation and ferroptosis without altering cellular GSH level (Yang et al., 2014; Yang and Stockwell, 2008). Ferroptosis can be blocked by antioxidants or ROS scavengers such as vitamin E/ Trolox, liproxstatin-1 or ferrostatin-1 (Fer-1) or by iron chelators such as Deferoxamine (DFO) or ciclopirox olamine (CPX)(Yang et al., 2014; Yang and Stockwell, 2008). Morphologically, ferroptotic cells were reported to contain altered mitochondrial structures which appeared smaller in size (Dixon et al., 2012; Yagoda et al., 2007).

Resistance towards apoptotic cell death is a hallmark of cancer cells (Hanahan and Weinberg, 2011). The discovery that cancer cells are especially susceptible to ferroptotic cell death could lead to novel treatment strategies. Combined ferroptosis induction and conventional chemotherapy could overcome drug resistance and allow administration of lower doses of the anti-cancer drug thus potentially reducing negative side effects (Yu et al., 2015). Additionally, glutamate-induced neurotoxicity could be blocked by ferroptosis inhibitors thus leading to novel treatment strategies for patients with neurodegenerative disorders such as Huntington's disease or Alzheimer's disease (Danysz and Parsons, 2012; Seiler et al., 2008; Skouta et al., 2014; Wang and Reddy, 2017). Moreover, ferroptosis may also play a role in acute kidney failure (Linkermann et al., 2014) as well as ischemia/reperfusion-induced liver injury (Friedmann Angeli et al., 2014) and ischemia/reperfusion-induced heart injury (Gao et al., 2015).

1.2.2.4 Necrosis

In contrast to the previously described forms of PCD necrosis is described as a passive, accidental form of cell death caused by injury or other external stimuli such as toxins or extreme temperature (Wyllie et al., 1980). Additionally, necrosis has been reported to be associated with different pathological settings such as infarction, mechanical trauma or ischemia. Necrosis is characterized by swelling, loss of membrane integrity and diminished ATP level (Eguchi et al., 1997; Zong and Thompson, 2006). It is further described to release cellular components (DAMPs) to the extracellular environment, thus causing inflammation (Green, 2011; Green and Llambi, 2015; Nikoletopoulou et al., 2013). Importantly, necrosis is a non-regulated process and can therefore not be inhibited by genetic or pharmaceutical means.

Numerous toxicological studies reported necrotic cell death upon exposure to various substances. However, these results have to be regarded with care since a majority of these studies fail to distinguish between individual cell death modes and do not include biochemical characterization of the underlying cell death mechanism.

1.2.3 Autophagy: cell survival

Autophagy is in the first instance a survival mechanism of cells encountering a stress signal (Kroemer et al., 2010; Viry et al., 2014). This process can be initiated from damaged or dysfunctional organelles or deprivation of nutrients or growth factors. The cell developed mechanisms to adapt to this stress in order to survive and maintain the cellular function by recycling cellular components. Autophagy therefore is an adaptive metabolic response to stress that acts to intracellularly remove misfolded proteins, damaged organelles or other macromolecules through lysosomal degradation. Moreover, autophagy is activated as a mechanism for intracellular pathogen clearance (Colombo, 2005; McEwan, 2017).

Three distinct forms of autophagy are known, namely macroautophagy, microautophagy, and chaperone-mediated autophagy (Boya et al., 2013; Parzych and Klionsky, 2014). The latter one describes a form of autophagy where proteins bearing a KFERQ-sequence are shuttled to the lysosome through a process involving HSPA8 (heat shock protein family A [Hsp70] member 8) and LAMP2A (lysosomal-associated membrane protein 2A) (Cuervo and Wong, 2014). Microautophagy is a process of lysosomal membrane invagination that leads to uptake of cytosolic components and their subsequent degradation (Mijaljica et al., 2011). Macroautophagy is characterized by formation of autophagosomes – intracellular vacuoles that are surrounded by a double membrane – around aggregated proteins, damaged organelles or pathogens. The autophagosome then fuses with the lysosome thus forming the autolysosome and leading to degradation of the internal components.

More than 40 highly conserved autophagy-related genes (ATG) have been identified and function for example in the assembly of the autophagosomal membrane. Autophagy is negatively regulated by mTOR (mechanistic target of rapamycin)(Rabanal-Ruiz et al., 2017). This protein acts as a cellular nutrient sensor. Under nutrient-rich conditions, mTOR promotes synthesis of various biomolecules and suppresses autophagy. In case of nutrient deprivation or hypoxia, AMPK inactivates mTOR thus allowing the autophagy program to proceed. Moreover, compounds such as rapamycin inhibit mTOR thus triggering autophagy.

If the cell experiences persistent stress and is not able to counterbalance it through autophagy, the cell will ultimately die through a process termed autophagic cell death. Exposure to NPs can cause oxidative stress and induce autophagy (Li et al., 2010; Li and Ju, 2018; Zabirnyk et al., 2007; Zhou et al., 2018). It is possible to suggest that NPs can cause cellular stress and that the cell activates the autophagic program to cope up with this stress and to intracellularly degrade internalized NPs as well as damaged organelles. Upon prolonged exposure, the autophagic defense mechanism might be exhausted and the cell eventually dies. Of note, inhibition of autophagy can lead to elevated cell death since the cellular cytoprotective mechanism is blocked.

1.3   PROGRAMMED CELL CLEARANCE

"Programmed cell clearance" is a term describing the evolutionarily conserved process of recognition and engulfment of dying cells by phagocytes and the regulation of an appropriate immune response (Witasp et al., 2008)(Fig. 2). As the name indicates, the underlying mechanisms are genetically regulated. Cell death research has to a large extent been focusing on cell death induction/ inhibition and the intracellular pathways associated with a specific form of cell death. However, much less attention has been paid to the recognition and subsequent engulfment and removal of apoptotic cells by phagocytes. Our knowledge regarding how phagocytes recognize and engulf different forms of dying cells is still incomplete and the identity

of involved signals and underlying mechanisms deserves more attention. Moreover, the adaptation of an appropriate immune response following engulfment as well as its correlation to numerous pathological settings remains elusive.
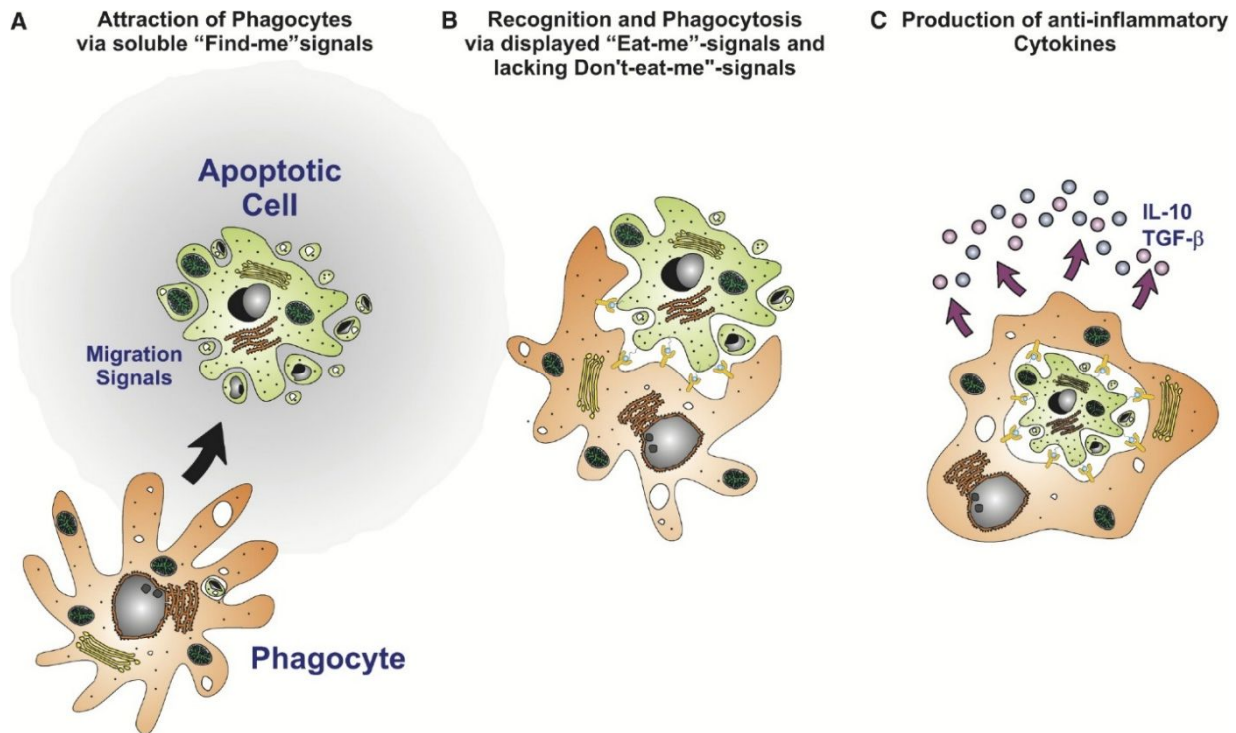

Fig. 2: Schematic figure of the three phases of apoptotic cell clearance. (A) The apoptotic cell attracts the phagocyte by releasing soluble 'find-me' signals. (B) Recognition and engulfment of the apoptotic cell is mediated through ligand-receptor interaction and binding of 'eat-me' signals on the apoptotic cell surface. (C) The last step of cell clearance includes the degradation of the target cell and the secretion of cytokines. Apoptotic cell clearance is an immunologically silent process characterized by the production of anti-inflammatory cytokines, such as IL-10 and TGF-β, by the engulfing cell. Source: Lauber et al., 2004.

1.3.1 Macrophages in tissue homeostasis

Removal of dying cells is facilitated by phagocytes which are present in many species and appear early through evolution. Phagocytes are not only critical for the host defense against pathogens but are further required to remove dying or damaged cells or cells that are no longer required. This process is conserved, highly efficient and tightly regulated in order to ensure well-being as well as proper function and development of the organism. In order to maintain homeostasis, phagocytic cells have to distinguish between viable cells and dying cells. It is of central importance that not only the ratio between proliferation and cell death is balanced, but also that dying cells are efficiently removed. Therefore, cell clearance defines the "meaning" of cell death (Savill and Fadok, 2000) and an imbalance is associated with various pathologies and disease progression. In higher organisms, various types of professional and non-professional phagocytes exist and together ensure rapid removal of dying cells. Examples

of professional phagocytes include macrophages, neutrophils and dendritic cells. This chapter will focus on macrophages – a type of phagocytic cells that was first described by Mechnikov and for whose discovery he received the Nobel Prize in Physiology or Medicine (Mechnikov, 1883). In addition to monocyte-derived macrophages, several tissue specific types of macrophages exist such as Kupffer cells (liver), alveolar macrophages (lung), Hofbauer cells (placenta) and microglia (central nervous system). Macrophages are further distinguished between M1 (classically activated) and M2 (alternatively activated) macrophages (Gordon, 2003). The former type is implicated in host defense against pathogens and elicits a pro-inflammatory phenotype, while the latter one is suggested to be anti-inflammatory as it is involved in wound healing and tissue repair (Mantovani et al., 2013). M2 macrophages represent the predominant phenotype of resident tissue macrophages and are engaged in the recognition and removal of dying cells.

Despite their role in engulfing pathogens and dying cells, macrophages are actively regulating the immune system. Secretion of pro-inflammatory cytokines as a response to bacterial, viral or fungal infection would therefore activate the immune response and recruit additional immune cells. Encountering of dying cells can stimulate either a pro- or an anti-inflammatory response and is dependent on the type of cell death. Apoptotic cells are believed to induce an anti-inflammatory response upon recognition and phagocytosis by macrophages (Hoffmann et al., 2005; Huynh et al., 2002; Savill et al., 2002). Macrophages that have engulfed apoptotic cells actively suppress the secretion of pro-inflammatory cytokines and induced the secretion of anti-inflammatory cytokines (Fadok et al., 1998). In this way, apoptotic cells contribute to the resolution of inflammation and stimulate proliferation of neighboring cells. In contrast, lytic cell death modes such as necrotic or necroptotic cell death expose intracellular material to the extracellular environment thus triggering release of pro-inflammatory cytokines. Conclusively, macrophages regulate an adequate response facilitating either tolerance and homeostasis or the activation of the immune system and inflammation. Of note, the underlying mechanisms by which macrophages recognize and engulf different forms of dying cells may differ and involve various signals that can be both general signals or those that are specific for the encountered cell death mode or the cell type. Both macrophages and dying cells can employ different subsets of ligands or receptors which together ensure efficient clearance (Fond and Ravichandran, 2016; Savill et al., 2002). Our understanding regarding programmed cell clearance – especially of more recently discovered non-apoptotic forms of PCD – is still limited. This involves both the underlying mechanism as well as involved signals or the regulation of the immune response. Phagocytosis efficiency of macrophages engulfing three distinct modes of PCD was studied in paper II.

The efficiency of the cell clearance program *in vivo* is further illustrated by the fact that dying cells are rarely seen in tissues of healthy organisms, including those that show a high cellular turnover. Of note, macrophages are able to engulf more than one target and are specialized in

the recognition of both pathogens and dying cells. Dying cells are taken up through the endolysosomal pathway and digested intracellularly. Importantly, macrophages have developed mechanisms to endure and cope with excess levels of nuclear, protein or lipid content (Han and Ravichandran, 2011). Interestingly, macrophages were shown to differentially regulate their metabolism upon engulfment of apoptotic cells as they promote aerobic glycolysis and downregulate oxidative phosphorylation (Morioka et al., 2018).

1.3.2 'Find-me' and 'eat-me' signals

In a first step of the phagocytic program the dying cell secrets signals to attract phagocytes and promote their migration towards the dying cell based on a chemotactic gradient (Arandjelovic and Ravichandran, 2015; Lauber et al., 2004; Ravichandran and Lorenz, 2007). These signals are referred to as 'find-me' signals. Lysophosphatidylcholine (LPC) or sphingosine-1-phosphate (S1P) have been described as lipid mediators in this first phase of phagocytosis (Gude et al., 2008; Lauber et al., 2003; Truman et al., 2008). However, their role *in vivo* is still questionable and needs to be confirmed since both lipids are present in the circulation and may therefore only act as local signals rather than supporting phagocytic migration from regions more distant to the place of cell death. Additionally, proteins such as fractalkine or nucleotides such as ATP or UTP have been reported to be released from apoptotic cells in a caspase-dependent manner (Elliott et al., 2009; Medina and Ravichandran, 2016). More research is needed in order to decipher the range of individual signals *in vivo* and how macrophages – but not other immune cells – are recruited. Much less in known regarding the identity of signals released from non-apoptotic cells and how these are released in a potentially caspase-independent way. It should be noted that several lytic cell death forms such as necroptotic or ferroptotic cell death are associated with the release of the previously described 'find-me' signals to a much higher concentration compared to apoptotic cells which are suggested to only release 2% of their cellular ATP (Ravichandran, 2010).

Once recruited, the phagocytic cell recognizes and binds to the dying cell. It is critical in terms of immunology and the prevention of inflammation that phagocytes are able to distinguish between healthy cells and potentially harmful dying cells. This process is facilitated via different 'eat-me' signals exposed on the surface of the dying cells but not of viable cells. Today, several different 'eat-me' signals as well as corresponding receptors are known to enable recognition either through direct interaction or indirectly via bridging molecules (Lauber et al., 2004). It is important to note that biological membranes consist of a lipid bilayer and that the lipids are distributed asymmetrically between the two leaflets resulting in a different composition of each of these two leaflets. While phospholipids such as phosphatidylserine (PS), phosphatidylinositol (PI) or phosphatidylethanolamine (PE) are predominantly localized in the cytosolic leaflet of the membrane of viable cells, the outer leaflet mainly consists of phosphatidylcholine (PC) and

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sphingomyelin (SM) (Balasubramanian and Schroit, 2003; Fadeel and Xue, 2009). The asymmetric distribution of the lipids in the membrane is essential for various cellular processes and cells invest a considerable amount of energy to maintain lipid asymmetry at the plasma membrane (Balasubramanian and Schroit, 2003). The exposure of PS to the cell surface has long been described to be a hallmark of apoptotic cells and numerous studies use detection of exposed PS as a signal to distinguish between apoptotic (dying) and viable cells. PS externalization facilitates recognition by phagocytes and is probably the most studied 'eat-me' signal until now (Fadok et al., 1992; Verhoven et al., 1999). However, it has to be noted that more recent studies describe other forms of PCD that show PS externalization suggesting that PS exposure alone cannot be used as a marker of apoptotic cells but may rather be a general feature of dying cells (Gong et al., 2017; Zargarian et al., 2017). It is more likely that several different signals act together in order to facilitate recognition of the dying cell and these signals may differ between different cell types and distinct forms of PCD. While some studies state that PS exposure is sufficient to induce phagocytosis – even of viable cells (Fadok et al., 2001; Segawa et al., 2014) – other studies found that viable PS-expressing cells are not recognized by phagocytes (Birge et al., 2016; Segawa et al., 2011). These discrepancies may result from different cellular models or may be based on the fact that the expression of additional 'eat-me' signals or the absence of 'don't-eat-me' signals is required for phagocytosis of these PS expressing cells. Importantly, PS-containing liposomes are readily engulfed by macrophages and prevention of PS exposure or masking of externalized PS reduces phagocytosis efficiency (Asano et al., 2004; Krahling et al., 1999). These results therefore underline the importance of PS as a signal that facilitates both recognition and engulfment. The occurrence of PS exposure in three different forms of PCD was studied in paper II. Following the discovery of PS as an 'eat-me' signal, several PS-binding receptors and bridging molecules have been identified that support recognition and uptake of apoptotic cells. Among others, MFG-E8 (Hanayama et al., 2004; Hu et al., 2009; Witasp et al., 2007), protein S, Gas6 (Stitt et al., 1995) or annexin I (Dalli et al., 2012) have been described as such PS binding proteins. Moreover, TIM4 (T cell immunoglobulin mucin receptor 4), stabilin-2, integrin receptors and scavenger receptors were suggested to act as phagocytic receptors that recognize different motifs of the externalized PS (Bratton and Henson, 2008; Lauber et al., 2004; Miyanishi et al., 2007; Park et al., 2008; Poon et al., 2014; Rodriguez-Manzanet et al., 2010). Other reported 'eat-me' signals include oxidized PS (PSox) or cardiolipin, which were shown to act as even more efficient signals compared to PS (Balasubramanian et al., 2015; Greenberg et al., 2006; Hazen, 2008; Kagan et al., 2002).

In addition to the exposure of 'eat-me' signals, dying cells are characterized by the absence of 'don't-eat-me' signals which negatively regulate phagocytosis. Examples for such molecules are CD31, CD47 and CD61 (Brown et al., 2002; Schürch et al., 2017).

Conclusively, dying cells actively recruit phagocytes towards the place of cell death, advertise their status through secretion or cell surface externalization of specific signals to allow

discrimination, recognition and uptake, and regulate the immune response (Elliott 2009). Additionally, as different types of dying cells (both different cell types and different forms of PCD) may expose different sets of 'eat-me' signals, so may macrophages show different expression pattern regarding receptors. The tight regulation and synergistic effect of multiple signals, however, ensures rapid and efficient removal of dying cells *in vivo*.

1.3.3 Scramblases and translocases

As mentioned above, lipids are asymmetrically distributed across the plasma membrane and cells invest a considerable amount of energy to maintain this phospholipid asymmetry (Balasubramanian and Schroit, 2003). Under homeostatic conditions, transmembrane proteins known as aminophospholipid translocases (ATPases or flippases) transport lipids such as PS or PE from the extracellular leaflet of the membrane to the cytosolic leaflet in an energy dependent mechanism, thus causing the enrichment of phospholipids on the intracellular side of the plasma membrane (Lopez-Marques et al., 2014). This protein family is highly conserved with respect to its structure and function and shows an overall sequence homology (Lopez-Marques et al., 2015; Puts and Holthuis, 2009; Takatsu et al., 2014). One of the flippases suggested to prevent PS exposure at the plasma membrane is ATP11C – a member of the P4-type adenosine triphosphatase (ATPase) family (Segawa et al., 2014). Irrespective of the well-established function of the protein in phospholipid transport it was suggested that additional factors are involved in maintaining PS asymmetry since knockout of ATP11C did not affect phospholipid asymmetry at the plasma membrane (Yabas et al., 2011). Upon apoptosis induction, phospholipid translocases become inactivated by caspase cleavage and this was shown to lead to PS exposure at the cell surface (Segawa 2014). Moreover, aminophospholipid translocases interact with cell division cycle protein 50 (CDC50) – a functional subunit and chaperone of ATPases – which is required for protein function, substrate specificity and transport of the ligand (Paulusma et al., 2008). However, the exact mechanism of phospholipid transport by ATPases as well as information about its regulation, the substrate specificity of individual ATPases or the exact protein structure remain elusive. One major question addresses the mechanism of phospholipid transport and how these large substrates are transported across the membrane. The phospholipid membrane itself represents a substantial energy barrier where the polar head group of the lipid substrate has to travel through the non-polar/ hydrophobic region of the phospholipid bilayer. Further structural studies are required to address these questions.

A second group of phospholipid transporting proteins are scramblases which facilitate nonspecific, bidirectional transport across the lipid bilayer. These transporters are inactive during homeostasis but become activated upon certain stimuli such as increased intracellular calcium level or the induction of apoptosis. Two examples of such transporters are the transmembrane protein 16F (TMEM16F) and the Xk-related protein 8 (Xkr8)(Suzuki et al., 2013;

Suzuki et al., 2010). While calcium-induced PS exposure is considered a temporary and reversible process that for example occurs in activated cells, caspase-dependent activation of scramblases is irreversible (Malvezzi et al., 2013; Martin et al., 1996).

In *C. elegans*, the transbilayer amphipath transporter 1 (TAT-1) is the only translocase of its protein family that has been shown to maintain PS asymmetry at the plasma membrane. Loss of TAT-1 function disrupts the asymmetric distribution of phospholipids and leads to random loss of healthy cells through PSR-1 and CED-1 dependent phagocytosis (Darland-Ransom et al., 2008). Interestingly, disruption of TAT-1 function also causes defects in lysosomal biogenesis and endocytic sorting, implying that TAT-1 function is not restricted to the plasma membrane but is also required for phospholipid translocation in intracellular membranes (Ruaud et al., 2009). In paper I we investigate the importance of specific residues for the proper function of the aminophospholipid translocase TAT-1 in the nematode *C. elegans*.

Taken together, apoptotic PS exposure is facilitated by inactivation of phospholipid translocases and concomitant activation of scramblases and several proteins are likely to act together in this process to disrupt phospholipid asymmetry. This further illustrates the importance of the plasma membrane as a signaling platform for recognition of dying cells.

1.4 CONSERVATION OF PATHWAYS

1.4.1 Model systems to study cell death

Since the discovery that cell death occurs as a regulated process, several organisms have been used to study PCD. In the early 1960s the 1 mm sized nematode *C. elegans* was proposed as a model organism to study cell death *in vivo*. Using *C. elegans* as a model organism has many advantages ranging from its short reproductive life cycle, to its comparably simple maintenance and the fact that it is transparent and therefore allows to examine the organismal development and morphological changes of all the cells and organs using microscopy techniques. *C. elegans* allows to study cell death mechanisms in various mutants based on counting of dying cells since elevated or reduced level of cell death can easily be scored. The *C. elegans* genome has been completely sequenced (Consortium, 1998) and many of the central cell death regulators have mammalian homologs. Most of the genes and molecular pathways that govern organismal development are shared between the nematode and mammalian system (it is estimated that 60-80% of the genes have a human counterpart). *C. elegans* is highly susceptible to mutagenesis allowing the relatively simple generation of new strains by mutating, silencing or overexpressing different genes of interest. Additionally, the fate of each cell during the development was described in detail meaning that it is know at what time point and in which location each individual cell dies (Sulston et al., 1983). This further highlights the genetically programmed

feature of the underlying cell death mechanisms and its tight regulation. During the development 131 of the 1090 somatic cells are eliminated through cell death (Kimble and Hirsh, 1979; Sulston and Horvitz, 1977; Sulston et al., 1983). Of note, it was shown to be helpful to study cell death mechanism in animals with a CED-1 background (*ced-1(lf)*) since these engulfment mutants show prolonged occurrence of dying cells thus allowing their detection and analysis. This is important since under homeostatic conditions, cell death is rarely observed *in vivo* as cell clearance occurs fast and efficient. Moreover, the nematode is also used for toxicological studies. In paper III we performed cytotoxicity screening and analyzed different mutant strains in order to elucidate the cell death pathway that is triggered by exposure to cationic Au NPs. Together, this makes the nematode a useful tool to study cell death. However, it is important to keep in mind that the nematode is a comparably simple organism that does not contain any circulatory system or specialized immune cells. Dying cell clearance is therefore achieved in *C. elegans* by neighboring phagocytic cells.

Additionally, several mouse model exist to study cell death (Ranger et al., 2001). Importantly, knock out of major cell death related genes such as FADD, caspase-9, caspase-8, cytochrome c, APAF1 or caspase-3 are perinatal lethal. Based on these findings, flox mutants or tissue specific knock out mice were generated and are studied extensively. Moreover, the GPX4 KO mice is lethal at day 7.5 dpc and therefore indicates the importance of the suppression of the ferroptotic cell death mechanism by GPX4 for proper embryonic development (Yant et al., 2003). Mice deficient in the expression of the bridging molecule milk fat globule-EGF factor 8 protein (MFG-E8) develop SLE-type autoimmune disease (Peng and Elkon, 2011).

1.4.2 Conservation of cell death pathways

The nematode *C. elegans* was introduced as a model organism by Brenner and colleagues and has ever since then – together with the fruit fly *Drosophila melanogaster* or different mouse models – been a useful tool in cell death research. Valuable insight into the cell death mechanisms and involved proteins is possible based on the fact that the mitochondria-mediated apoptotic pathway is evolutionarily conserved and remarkably similar between the individual organisms (Fig. 3). Homologous proteins have been identified for the central cell death proteins.

The EGL-1 protein is the corresponding protein of the mammalian BH3-only protein, which is known to induce apoptosis by causing mitochondrial outer membrane permeabilization (MOMP) (Conradt and Horvitz, 1998). EGL-1 directly binds to CED-9 (homolog of the mammalian cell-death inhibitor Bcl-2) and thereby abolishes the inhibitory effect of CED-9 (Conradt and Horvitz, 1998). The mammalian Bcl-2 protein family includes both death antagonists (e.g. Bcl-2, Bcl-X$_L$ and Bcl-w) and death agonists (e.g. Bax, Bak and Bok). As a result of EGL-1 binding to CED-9, the cell death activator CED-4 (a protein similar to the mammalian APAF1) is released from the CED-9/CED-4 protein complex and activates the executioner caspase CED-3, thus leading to cell

death (Lettre and Hengartner, 2006; Yan et al., 2005). In the mammalian system, MOMP causes release of pro-apoptotic proteins from the mitochondrial intermembrane space (Jacotot et al., 1999; Lomonosova and Chinnadurai, 2008). APAF1 forms a cytosolic complex with cytochrome c and procaspase-9 referred to as the apoptosome (Fadeel et al., 2008). In this complex, caspase-9 becomes activated and further activates the main executioner caspases such as caspase-3, caspase-6 or caspase-7. The inhibitor of apoptosis (IAP) protein family suppresses caspase activity, whereas DIABLO/ Smac antagonizes the functions of IAPs, thus promoting caspase activation. Together, the conservation of this pathway also indicates the importance of mitochondria as regulators of cell death. While a death-receptor mediated apoptotic cell death pathway exists in mammals, a similar pathway was not found in the nematode model.
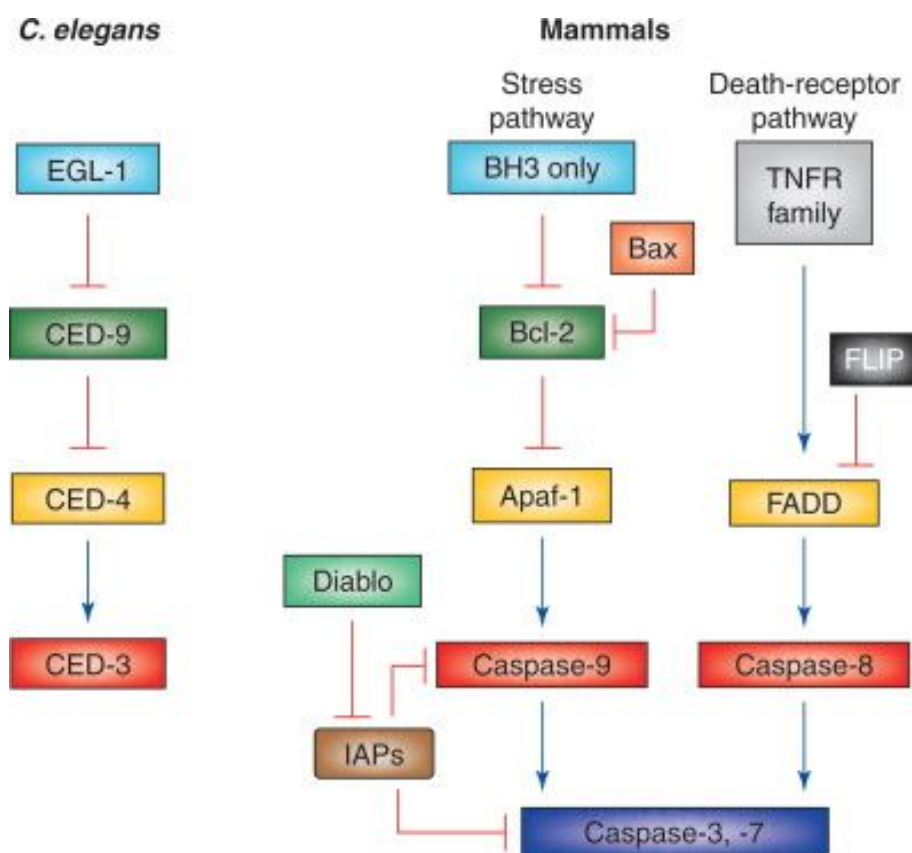
Fig. 3: Conserved components of the cell death pathway in nematodes and mammals. The apoptotic pathways in *Caenorhabditis elegans* (left) and in mammals (right) are shown. In the nematode the inhibitory effect of CED-9 is neutralized through binding of EGL-1 to CED-9 thereby allowing CED-4 to activate the executioner caspase CED-3. In the mammalian system the mitochondria-mediated pathway leads to MOMP and to the activation of initiator caspase-9 while the extrinsic (death receptor-mediated) pathway is triggered though oligomerization of a death receptor at the plasma membrane and leads to the activation of initiator caspase-8. The stress and death receptor pathways are largely independent but may be linked via activation of the BH3-only protein Bid. Both pathways lead to activation of the executioner caspase-3. Identical colors represent homologs. Modified from: Kumar and Cakouros 2004.

Thus far there is no evidence for the existence of RIP-like kinases in lower organisms such as *Drosophila melanogaster* or *C. elegans* suggesting that in these organisms there is no necroptotic-like cell death similar to the one observed in mammals (Chan et al., 2015). Additionally, there have been no reports regarding ferroptotic cell death in the nematode. However, reports of non-apoptotic cell death exist in *C. elegans* (Blum et al., 2012; Kutscher and Shaham, 2017; Malin et al., 2016). More recent studies suggest the establishment of a ferroptosis model in *Drosophila* or report a form of iron-dependent cell death in the fruit fly (Edenharter et al., 2017; Wang et al., 2016). Further studies are required in order to evaluate the physiological relevance of these observations and potentially shed more light on the underlying mechanisms and involved proteins and signals. In paper III, three *C. elegans* mutant strains were analyzed in order to elucidate the underlying cell death mode that was caused by cationic Au NPs. The ced-3(n2433) mutant blocked apoptosis, the clp-1(tm690) mutation inhibits the necrosis pathway, and the lgg-1(bp500) mutation prevents autophagy.

1.4.3 Conservation of cell clearance mechanisms

Even though there are no specialized phagocytes in *C. elegans* the mechanisms leading to engulfment of dying cells are similar to the ones observed in the mammalian system. In the nematode, the neighboring cell acts as a phagocytic cell and is engulfing the dying cell. As mentioned above, PS externalization is recognized as an 'eat-me' signal of central importance for efficient phagocytosis. Both the occurrence of PS as an 'eat-me' signal on the apoptotic cell surface as well as the mechanisms that lead to its exposure are evolutionarily conserved (van den Eijnde et al., 1998; Venegas and Zhou, 2007). The *C. elegans* worm AIF homolog (WAH-1) is a mitochondrial protein that is released from the mitochondria upon apoptosis induction and that subsequently triggers scramblase (SCRM-1) activation and PS exposure (Wang et al., 2007). A similar mechanism was reported in the mammalian system (Preta and Fadeel, 2012). Thus, AIF is regulating caspase-independent apoptosis. Moreover, while CED-8 was shown to be involved in PS exposure in the nematode, its homolog Xkr8 facilitates PS externalization in mammals (Chen et al., 2013; Suzuki et al., 2013).

Several PS receptors exist in *C. elegans* that facilitate recognition of externalized PS on dying cells, indicating that the recognition mechanism is evolutionarily conserved as well. Among these receptors are the phosphatidylserine receptor PSR-1 as well as CED-1 in the nematode or TIM4 and CD36 in the mammalian system (Ellis et al., 1991; Greenberg et al., 2006; Mapes et al., 2012; Miyanishi et al., 2007; Wang et al., 2003; Yang et al., 2015).

Moreover, bridging molecules exist throughout evolution and facilitate recognition of dying cells. The mammalian protein MFG-E8 binds both PS exposed on the apoptotic cell as well as the $\alpha_v\beta_3$ integrin receptor at the phagocytic cell (Hanayama et al., 2002; Witasp et al., 2007). TTR-52 was

reported to be a bridging molecule in the nematode and to bind exposed PS as well as CED-1 (Mapes et al., 2012; Wang et al., 2010).

Macrophages were shown to constitutively express PS on the cell surface which is required for efficient engulfment of apoptotic cells (Schlegel et al., 2000). The exact mechanism and regulation of phagocytic PS exposure remains elusive. Similarly, phagocytic cells in *C. elegans* also express PS on the cell surface and this process involves the ATP-binding cassette (ABC) transporter CED-7, the bridging molecule TTR-52 and the PS receptor CED-1 (Mapes et al., 2012).

1.5   APPLIED CELL DEATH RESEARCH: NANOTOXICOLOGY

1.5.1   Engineered nanomaterials

Nanomaterials per definition are natural, incidental or manufactured materials with a dimension in the nanometer range (1 nm – 100 nm)(EU commission, 2011) which equals the size of biological molecules and structures (Hubbs et al., 2013; Maynard et al., 2011; Shvedova et al., 2010). Although sharing the same characteristic of being small, NPs can greatly differ in their composition and physicochemical properties. Most obviously, NPs of different core elements exist as they can for example compose of polymeric, metal or carbon based core materials. Moreover, it is possible that these core elements are further modified for example by oxidation.

Humans have always been exposed to different kinds of NPs – ranging from uptake of various metal particles in drinking water to the inhalation of campfire combustion or volcanic gases – and adapted the immune system to recognize, remove and correspond to a wide variety of such particles (Buzea et al., 2007; Oberdörster et al., 2007). As an effect of industrialization, the quantity and variety of particles we are exposed to has greatly increased (Oberdörster et al., 2005). The amounts and diversity of different particles that are produced and used in various areas – including medical and diagnostic applications, or the exhaustion from vehicles or industry – is rapidly expanding (Johnston et al., 2013). While the development of nanotechnology may be of great benefit for the society, there is also increasing concern regarding a potential risk correlated with exposure to these particles. Therefore, the necessity arises to carefully characterize the individual materials and to evaluate an associated potential risk for the organism or the environment – which is the main intention of the field of nanotoxicology (Costa and Fadeel, 2016).

1.5.2 Nanotoxicology: mechanisms of toxicity

While some particles are considered to be non-toxic, others may alter cellular functions or lead to inflammation and cell death. Toxicity is determined by multiple characteristics of the particle and is not only dependent on the core element or the dose, frequency and the time of exposure (Fig. 4)(Nel et al., 2013). While particles can differ in their primary core size, they can also appear in various shapes and dimensions – e.g. fibers, tangled particles, spheres, cubes, nanotubes, sheets or nanowires (Kinnear et al., 2017). In general, small particles are considered more toxic than bigger particles. As a consequence of the remarkably small size, the surface area to volume ratio of NPs is extremely high and this greatly affects the properties of the material and allows for numerous interactions with other molecules (Auffan et al., 2009). Moreover, small NPs (<30 nm) can enter the nucleus via passive diffusion through the nuclear pore (Ahlinder et al., 2013; Jang et al., 2013) and can then directly interact with the DNA. Au NPs of 1.4 nm size were shown to cause toxicity by intercalating with the major groove of the DNA (Pan et al., 2007). Of note, various sizes of particles can allow or prevent the particles to cross specific barriers such as the placenta, the blood-brain barrier or the plasma membrane. On the other side, the shape of the NP can also differentially influence toxicity (Kinnear et al., 2017). According to the fibre pathogenicity paradigm, particles that are long, straight, thin and biopersistent have a high potential of being toxic (Davis et al., 1986; Donaldson et al., 2010). It was stated that fibers that are longer than 15-20 nm are potentially toxic since phagocytes are not able to stretch to an extent that would be required for engulfment of these particles. Moreover, the surface properties determine the solubility or the likelihood of the particles to form aggregates. Surface modifications can define the surface chemistry, redox status, charge, solubility or the photoactivation potential of the particle and facilitate or prevent interactions with the environment. Moreover, release of ions can alter intracellular Ca^{2+} level, cause elevated ROS production and lead to cell death (Orrenius et al., 2011).

In biological systems, NPs often interact with proteins and other biomolecules, which are absorbed and form a coating around the particle. This defines the "biological identity" of the particle – also referred to as the bio-corona (Monopoli et al., 2012; Monopoli et al., 2013). Formation of the bio-corona is associated with reduced potential of the NPs to aggregate. Of note, while many toxicological studies are done in cell culture medium in the presence of FBS, there are also studies that invest bio-corona formation in medium containing human plasma. Especially when investigating the effect of a particle that is intended for use in medical applications, this approach appears to be more relevant. Interactions of individual components of the bio-corona occur mainly through hydrophobic or electrostatic interactions, are rather dynamic structures and depend on the composition of the surrounding physiological fluid (Docter et al., 2015; Westmeier et al., 2016). Distinction is made between the primary particle size and the hydrodynamic diameter which depends on the liquid system the particle is in contact with. Formation of the bio-corona can cause a masking effect of the particle but can also result

in deprivation of proteins or other factors that are then no longer available for the cell. Additionally, the properties of the NP can be influenced by introducing different surface functionalization (Fröhlich, 2012). Generally, cationic particles are considered more toxic as they have the potential to disrupt the plasma membrane as well as cause lysosomal rupture, thus causing toxicity. Positively charged particles are suggested to interact with negatively charged phospholipids and membrane proteins, thus causing loss of membrane integrity (Nel et al., 2009). The effect of different sizes and surface functionalization on the toxicity of Au NPs was investigated in paper III.
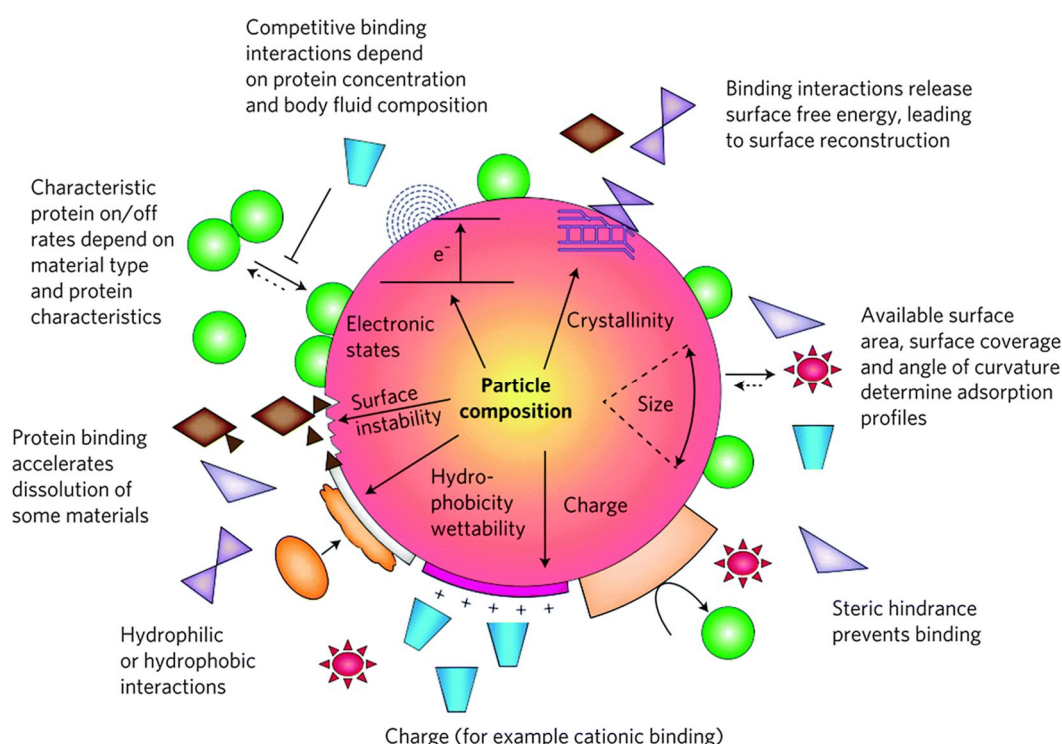
Fig 4: Toxicity of nanoparticles is determined by various physicochemical characteristics. NPs bear unique properties that together affect cytotoxicity. Except from the nature of the core element of the particle and its shape, numerous additional characteristics can influence how these particles interact with the environment. The schematic figure summarizes the key features. Source: Nel et al., 2009.

Interestingly, effects of a certain NP observed at high doses (acute exposure) cannot directly be translated to effects caused by the same particle at low doses over an extended time (chronic exposure). While high doses of single walled carbon nanotubes were shown to induce apoptosis, long-term exposure to low concentrations of the particles caused a transformation of the cells which then became apoptosis-resistant (Naserzadeh et al., 2018; Pongrakhananon et al., 2015). However, the majority of toxicological studies focuses on acute exposure to often unrealistically high doses while long-term studies are rather rare (Krug and Wick, 2011). Of note, it is further important to confirm cellular uptake or interaction of the particle with the cell. If uptake is prevented through a barrier such as the mucus or by means of cilia movement, the potential

effect of the particles on the cell could be diminished. Moreover, limited solubility of the particles can also prevent them from interactions with the cells. Uptake can either occur through diffusion of the particles across the membrane or via pinocytosis or phagocytosis (Krug and Wick, 2011; Kuhn et al., 2014). Depending on the nanomaterial, cells might be able to degrade the particle (Mukherjee et al., 2018). Dissolution of the particles can result in the release of ions – either extracellularly or intracellularly – which can cause ROS production and lead to cytotoxicity (Limbach et al., 2007). Another challenge in nanotoxicology is, that the exact composition of particles or mixtures is not always known and therefore detailed characterization of the material might be incomplete. One example for that is the study of the effect of air pollution on our health. Moreover, production of particles may not always result in pure products but may contain various impurities such as other metals or contain bacterial contaminations. This needs to be considered when performing a nanotoxicological study, especially when investigating the effect of a particle on immune cells. Contamination with endotoxin – a component of bacterial membranes – is a major concern in the field of nanotoxicology (Li and Boraschi, 2016). Excluding the presence of a bacterial contamination of the investigated nanomaterial would provide evidence that the observed effect is indeed induced by the NP and is not a result of the endotoxin (Li et al., 2017a; Li et al., 2017b). Today, the best way to obtain reliable results is the synthesis of the material under endotoxin-free conditions. Several techniques were reported that aim to remove endotoxin contamination – including autoclaving, filtration or chemical treatment – but there is a risk that these procedures could alter the physicochemical properties of the particle and therefore, subsequent detailed characterization of the particle is required (Vetten et al., 2014).

The detailed characterization of the NPs is a first step in the understanding of how certain materials interact with biological systems. These information then have to be expanded in such a way as the consequences of that material on biological samples need to be studied. This includes morphological alterations of the cell as well as changes in gene or protein expression or other molecular characteristics and mechanistic studies (Fadeel et al., 2015).

1.5.3 Gold nanoparticles: from toxicology to medicine

Due to its unique optical and surface plasmon resonance properties one of the main applications for gold nanoparticles (Au NPs) is their use in nanomedicine for treatment purposes as well as for imaging and diagnostics (Boisselier and Astruc, 2009; Singh et al., 2018). An advantage is that the synthesis of Au NPs of various sizes and shapes is rather simple (Dykman and Khlebtsov, 2011) and further alterations with respect to surface functionalization can be introduced. Au NPs are used as a tool for sensitive detection and diagnostics, as targeted drug delivery system, in photothermal therapy or in various microscopy analyzes including TEM (Dykman and Khlebtsov, 2011; Wu et al., 2011a). Moreover, Au NPs were used in various therapeutic purposes for

example to treat rheumatoid arthritis (Lee et al., 2013). In connection with cancer treatment, Au NPs have been suggested for use in photothermal therapy or as a drug delivery system either loaded with a chemotherapeutic drug or with specific siRNAs (Cuenca et al., 2006; LaRocque et al., 2009; Paciotti et al., 2004; Wu et al., 2011a). Even though Au NPs are not yet approved for medical applications by the FDA (Food and Drug Administration), several clinical trials with various Au NPs are ongoing. The promising results from clinical trials using Au NPs further demand to carefully study these particles and investigate a potential health risk. This was the motivation to study different Au NPs as reported in paper III. Based on its inert nature, gold was considered to be biocompatible and non-toxic (Connor et al., 2005; Murphy et al., 2008; Shukla et al., 2005) but conflicting reports exist and other groups report cytotoxic effects of Au NPs (Goodman et al., 2004; Li et al., 2008; Pan et al., 2007). Multiple studies that report Au NP-induced toxicity claim that the observed effect is mainly attributed to be an effect of size or specific surface functionalization. Small particles where generally more toxic compared to bigger particles (Pan et al., 2007; Tsai et al., 2011). Schaeublin and colleagues investigated the effect of different surface charges and state that positively or negatively charged particles induce apoptosis while neutral particles induce necrosis (Schaeublin et al., 2011). Others suggest that cationic Au NPs were toxic while negatively charged particles displayed no toxicity (Goodman et al., 2004). Various Au NPs were reported to induce apoptosis, necrosis or autophagic cell death respectively (Ma et al., 2011; Pan et al., 2007; Schaeublin et al., 2011; Wu et al., 2011b). While these discrepancies may be based on different cellular model or different kinds of particles (varying in size, shape or surface functionalization), not all of the studies manage to characterize the underlying cell death mode on a molecular or biochemical level. The simple distinction between apoptotic and necrotic cells based on annexin V/ PI staining is not sufficient and needs to be complemented with more sophisticated and up-to-date methods. In order to understand how specific Au NPs interact with the biological system and elucidate the mode of action, cell death specific markers should be investigated. Moreover, gene or protein expression level could help to decipher the exact mechanism and effect of Au NPs.

1.5.4   Nanotoxicology: alternative methods

The increased development of engineered nanoparticles and their use in different settings and applications requires in depth knowledge regarding potential adverse effects of these materials on the organism as well as the environment. The evaluation of a potential cytotoxic effect and the understanding of the underlying mechanisms will help develop detailed risk assessments and guidelines for safe handling of the NPs. To this end, various models are commonly used for toxicological studies ranging from cell lines or primary cells to co-culture or *in vivo* models. Recently, more advanced 2D or 3D models were developed and are applied for toxicological studies (Evans et al., 2017). These models simulate a rather complex *in vivo* situation and at the same time follow the 3R principle (Replacement, Reduction and Refinement) of reducing animal

research. Traditional toxicological approaches mainly focused on the characterization of the particles and used basic viability assays to distinguish between viable and dead cells but failed to include further analysis and characterization of affected molecules and pathways. This approach is not only overly simplified but is also missing out on important information regarding the mode of action or the induced form of cell death. Moreover, it is not sufficient to just describe the occurrence of cell death or the presence of a certain morphological feature but to validate such observations with biochemical characteristics that are specific for a certain type of cell death. NPs can differently interact with biomolecules, thus altering cellular functions. In recent years it became therefore more and more evident that nanotoxicological studies should also address the question of how the particles affect the cell or organism, which genes, proteins or metabolites are altered or which form of cell death is induced upon exposure to the NPs (Andón and Fadeel, 2013). Importantly, the underlying cell death mode may vary between different cell types but also be a dose- and time-dependent effect of the exposure to individual NPs.

Toxicological studies of NPs *in vivo* have been performed using model organisms such as the nematode *C. elegans* or the zebrafish model (Nel, 2013). Both organisms are well characterized with respect to their development, replicate quickly and are relatively simple to maintain. These studies provide valuable insight into the mechanism of action of the NP on an organismal level, can be verified and translated to the situation in the mammalian system and help to predict adverse outcomes and potential risks. Importantly, such model organisms allow to study toxicity by high-content screening (Nel et al., 2013). Both the effect on early developmental stages as well as long-term effects associated with prolonged, chronic exposure are tested in these model organisms.

1.5.5 Emerging systems biology approaches

The rapidly increasing production and exposure to nanomaterials demands the evaluation of potential risks and adverse effects on health and environment. This does not only include high quality testing, but also requires standardized, reliable, fast and high throughput analysis as well as economically and ethically acceptable models (Nel et al., 2013). Moreover, the question regarding mechanistic understanding arises. Emerging systems biology approaches are a useful tool to gain comprehensive understanding of the mechanisms and consequences of NP exposure. In contrast to conventional methods, omics analysis allows the measurement and quantification of thousands of end-points – such as proteins (proteomics) or mRNA molecules (transcriptomics) – in high throughput. Moreover, the combination of omics analysis and bioinformatics helps to unravel the underlying, potentially novel pathways and structure-activity relationships, to quantify molecular changes, as well as to understand the consequences of particle exposure and thus to predict a potential risk (Fadeel et al., 2018). Systems biology allows

the identification and quantification of differentially expressed genes, proteins or metabolites and through computational analysis can elucidate functional networks and pathways.

Cytotoxicity assessments are often used as an initial experiment to narrow down an appropriate dose and time point for additional analysis. This is mainly achieved by assays investigating loss of plasma membrane potential, mitochondrial activity or changes of the cellular morphology (Andón and Fadeel, 2013). Proteomics addresses to quantify the expression of proteins but can also be used to identify protein-protein interactions or post-translational modifications. Through genomics it is possible to study histone modifications, DNA damage or DNA methylation pattern but genomics are rather rarely used in toxicology. Transcriptomics allows to quantify mRNA level on a genome wide level (Wang et al., 2009). To this end, two methods have been developed, namely hybridization or microarray techniques and RNA sequencing (RNAseq).

Once the altered end-points have been identified, bioinformatics tools are used in order to pinpoint differentially regulated pathways. Finally, Ingenuity Pathway Analysis (IPA) and Gene Set Enrichment Analysis (GSEA) predict a model of the underlying toxicological pathway as well as identify upstream regulators. Cluster analysis and the generation of heat-maps can help to visualize the observed data. Together, this analysis can then put the results into a biological context.

Ideally, a comprehensive systems toxicology approach aims to characterize the particle with respect to physicochemical properties in detail, includes multiple omics analysis and complements these results with validation experiments both *in vitro* and *in vivo*. Additionally, studies of the final outcome of NP exposure contain valuable information regarding the immune response. This would lead to a global and detailed understanding of the effect of NP exposure on the biological system or environment. Together, these results would not only allow mechanistic insight but also allow prediction of a potential risk associated with NP exposure. Systems toxicology may therefore lead to the development of predictive models and thus support risk assessment (Sturla et al., 2014).

2   AIMS OF THE STUDY

In recent years, cell death research mainly focused on intracellular pathways that could either inhibit or induce cell death. Much less attention has been payed to the question of dying cell recognition, the signaling molecules involved in this process as well as the immunological consequences. Different forms of cell death are triggered by a variety of factors ranging from disease-driving mutations to infections or the exposure to chemicals or toxins. This could lead to an imbalance between proliferation and cell death or between cell death and cell clearance with severe pathological consequences. The overall aim of this study is to increase our knowledge regarding the processes and mechanisms involved in programmed cell death and cell clearance.

The specific aims are:

- To gain insight into the structure and functions of the *C. elegans* aminophospholipid translocase TAT-1 and to identify residues or domains that are critical for PS externalization
- To induce and characterize three distinct forms of programmed cell death using the same cell model, to investigate the phagocytic engulfment of the different cell deaths and whether PS is exposed
- To study the role of size and surface modification for toxicity of gold nanoparticles – both *in vitro* and *in vivo* – by applying conventional toxicity assays and multi-omics approaches

3 METHODS

The following chapter describes and critically evaluates the models and methods used in the different projects. Additional information can be found in the individual publications.

3.1 CELL MODELS

3.1.1 Primary human macrophages

Primary human cells are often used as a cellular model since these cells are non-transformed and therefore do better represent the *in vivo* situation of the respective cell type. The disadvantage is that isolation and potential subsequent differentiation techniques are required which can be time consuming. Primary cells usually do not proliferate to the same extent as cancerous cell lines and have a limited life span which is why primary cells often cannot be kept in culture over a longer period of time. Moreover, primary cells isolated from different donors display a certain divergence representing biological variation while cell lines are usually generated from single clones and do not show these alterations.

Human monocyte derived macrophages (HMDMs) were obtained from peripheral blood mononuclear cells, which were isolated from buffy coats of healthy blood donors by using a Lymphoprep density gradient. All samples were completely anonymized prior to handling in the laboratory. CD14-positive mononuclear cells were then isolated via CD14 magnetic MicroBeads and by using a LS column and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 ng/ml recombinant human M-CSF (macrophage colony-stimulating factor) for four days. Stimulation of monocytes with M-CSF results in their differentiation into so called M2 macrophages. These macrophages show an anti-inflammatory phenotype and are reported to perform cell clearance under homeostatic conditions. Of note, these primary human macrophages are more efficient in the phagocytosis of dying cells compared to THP-1 macrophage-like cells or RAW 264.7 murine macrophages.

3.1.2 Mouse and human cell lines

The human T lymphocytic Jurkat cell line (Jurkat E6.1) was used as target cells to study cell death. This cell line was obtained from peripheral blood of a 14 year-old boy with acute T cell lymphoma at first relapse in 1976. Jurkat cells have been widely used as a suitable model to study cell death and immunological responses. Both Jurkat cells and macrophages represent cell models that are present in the blood circulation and therefore imitate a situation that can possibly occur *in vivo*. Jurkat cells were shown to be susceptible for Fas antibody-induced apoptosis and we successfully

established Jurkat cells as a model to study (1S,3R)-RSL3-induced ferroptosis. FADD-DN (FADD dominant negative) Jurkat cells were used as a model to study necroptotic cell death. These cells lack the death effector domain (DED) of the FADD protein, thus fail to activate caspase-8 and therefore do not undergo death receptor-mediated apoptosis (Lee et al., 2012). Instead, upon TNFα ligation to the death receptor FADD is suggested to bind to RIPK1 and RIPK3 in a so-called necroptosome complex and to trigger necroptosis (Vandenabeele et al., 2010a). Jurkat cells represent a relevant model for all three cell death modalities. It was shown that Jurkat cells can undergo apoptotic cell death induced by various intrinsic or external stimuli (further described in the introduction of the present thesis). Necroptosis was reported to ensure the removal of lymphocytic cells that are no longer required and therefore prevents the accumulation of autoreactive lymphocytes (Lu et al., 2014). Both ferroptotic and necroptotic cell death were suggested to play a role in lymphocytes for host defense during viral infection (Lenardo et al., 2002; Matsushita et al., 2015).

The acute monocytic leukemia cell line THP-1 was chosen as a model to study the effect of Au NPs on immune cells. Au NPs are suggested for use in medical applications as drug delivery systems, for photothermal therapy or for diagnostics and imaging. When circulating in the blood stream these particles would be in direct contact to immune cells such as monocytes/ macrophages and the monocytic THP-1 cells therefore represent a valid model for this *in vivo* situation.

The murine macrophage cell line RAW Difluo RFP::GFP::LC3 is a reporter cell line which allows to study occurrence of autophagy as well as to monitor autophagic flux. These cells express the LC3 protein (microtubule-associated protein 1A/1B-light chain 3) – a central component of the autophagic machinery – which is fused to the two fluorophores RFP and GFP. Upon autophagy induction, LC3 is recruited to the autophagosomal membranes. Subsequently, the formation of autophagosomes can be followed in the reporter cell line based on the appearance of punctate structures via confocal microscopy. Here, both the RFP and the GFP signal can be detected and indicate early stages of the process. Fusion of the autophagosomes with the lysosomes leads to formation of the autolysosome and acidification of the internal environment. Under these acidic conditions, the GFP signal is quenched and only the RFP signal can be detected.

Maintenance of the cells was performed by keeping the cell number between $0.2\text{-}1.5 \times 10^6$ viable cells/ml or by splitting the cells once they reached a confluency of 80-90% and by not exceeding passage number thirty. This ensures that the cells have sufficient amounts of nutrients, don't show altered behavior due to a too high cell number and that cells are maintained while being in the logarithmic growth phase. Moreover, cells were regularly checked for mycoplasma contamination – a rather common bacterial contamination of cell cultures that can deprive nutrients and alter DNA expression or protein synthesis of the affected cells.

3.2 CAENORHABDITIS ELEGANS

3.2.1 Maintenance

The N2 Bristol strain was used as a wild-type strain and the *tat-1*(t*m3117*) deletion mutant served as a positive control to study the function of TAT-1. The individual *tat-1* mutant strains were identified through the *C. elegans* million mutation project and all mutations were confirmed by sequencing. The PS sensor strain *smIs434* which secretes the GFP-Lactadherin fusion protein (GFP::Lact) to the extracellular environment was previously described (Mapes et al., 2012). The strains were cultured and maintained at 20°C or 25°C on nematode growth medium plates that were inoculated with *Escherichia coli* OP50. For a better understanding regarding the basic structure and anatomy of the nematode, a differential interference contrast (DIC) image of the hermaphrodite worm (A) as well as a schematic figure (B) are shown in figure 5. Some of the main organs or body parts are labeled.

Six of the sixteen *tat-1* mutants (P335L, T392I, V490M, G526E, E561K and E1116K) were back-crossed with the wild-type strain in order to exclude the possibility that the observed phenotype is caused by mutations other than the *tat-1* mutation. Additionally, rescue experiments were performed in order to prove that expression of the wild-type TAT-1a rescues the observed phenotype. To this end, the pPD49.83 vector containing the TAT-1a cDNA was injected into the respective *smIs434* strains and ectopic PS exposure in somatic cells as well as the gut phenotype were investigated as described in paper I.

In order to decipher the cell death mode induced by exposure to Au NPs we performed toxicity assays using strains that were deficient in specific cell death pathways. The *ced-3(n2433)* strain is deficient in apoptosis, the *clp-1(tm690)* strain prevents necroptosis, and the *lgg-1(bp500)* strain is an autophagy mutant strain.

As mentioned in the introduction, *C. elegans* is a valuable model to study cell death since many of the involved proteins have homologous proteins in mammals and the underlying pathways are conserved. However, one has to keep in mind that the nematode represents a relatively simple organism that is for example lacking specialized immune cells.

3.2.2 Gonad dissection and annexin V staining

Gonad cells of one-day-old adult hermaphrodite worms of the different *tat-1* strains were stained *ex vivo* with annexin V. To that end, the worms were placed into a depression slide, paralyzed using levamisole and then cut at the head region at the level of the pharynx using two syringes which are placed across of each other and moved in a scissors motion (the location is indicated in Fig. 5A through a dashed red line). This procedure then results in the extrusion of the gonads. Figure 5C represents an image of the exposed gonads which are highlighted by the

yellow dashed line. The main organs or body parts are labeled. The gonads need to be handled carefully since they are very sensitive to mechanical impact as well as dehydration. Therefore, any form of disturbance or mechanical stress to the sample was kept at a minimum and all samples were kept in gonad staining buffer throughout the whole analysis. Following the annexin V and Hoechst 33342 staining – to stain externalized PS and nuclei respectively – and some washing steps, the worms were placed on 1% agarose pads, carefully covered with a cover slide and visualized with the help of an epifluorescence microscope.
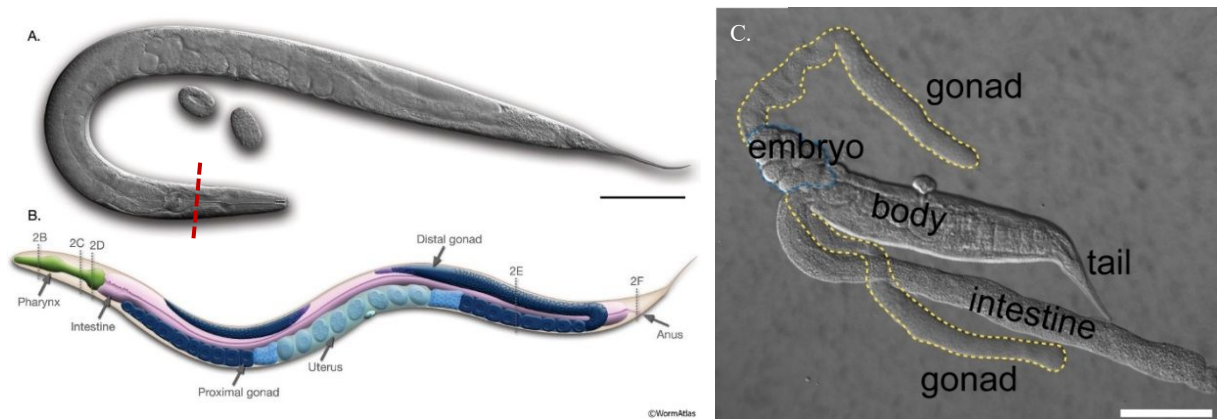

Fig. 5: Anatomy and gonad dissection of adult hermaphrodite nematodes. (A) Anatomy of *C. elegans*. DIC image of an adult hermaphrodite worm together with two embryos is shown. The dotted red line indicates the position where the worm was cut for gonad dissection. A schematic figure of the anatomical structure is shown below (B). The scale bar represents 0.1 mm. Source: http://www.wormatlas.org/hermaphrodite/introduction/mainframe.htm (C) Example of exposed gonads after gonad dissection. The individual organs or body parts are labeled. Source: Yoon et al., 2016.

3.2.3   Intestine vacuolization phenotype assay

The vesicular gut phenotype of the individual *tat-1* strains was investigated by using worms at larval stage 4 (L4). The fact that *C. elegans* is transparent allows examination of the gut phenotype by DIC microscopy. An intestinal defect is visible through accumulation of large vesicles in intestinal cells. Even though the quantification of the vacuolization might be quite subjective, the analysis of several nematodes per strain (a minimum of 20 worms were scored) reduces a potential bias or variation. Moreover, the analysis was performed as a blind test where information of the strain that was analyzed were kept unknown until the end of the experiment thus preventing further risk of prejudice.

3.2.4 PS staining of somatic cells in embryos

The exposure of PS on the surface of somatic cells of the individual *tat-1* strains was investigated by using the PS sensor strain *smIs434* (P$_{hsp}$sGFP::Lact^{C1C2}), which secretes the GFP::Lact fusion protein to the extracellular environment. Lactadherin is a PS-binding protein and therefore labels all cells that expose PS at the plasma membrane. The binding to PS is facilitated via the C1C2 domain of the Lactadherin protein. Embryos at the pre-comma or comma stage were used to study PS externalization of somatic cells and show labeling of the plasma membrane of all cells in case of a defect in PS translocation and diffuse or no staining in case of functional PS translocase activity. In order to induce the expression of the sGFP::Lact protein which is controlled by the heat shock promoter *hsp-16*, the embryos were incubated at 33°C for 45 min and then recovered at room temperature for 2h. GFP::Lact staining of the individual strains was then investigated by fluorescence microscopy. Importantly, heat shock-induced GFP::Lact expression did not alter the function of the TAT-1 protein in facilitating PS transport at the plasma membrane for all the analyzed strains including the temperature sensitive *tat-1*(E1116K) mutant.

3.2.5 TAT-1a constructs and rescue experiments

In order to perform rescue experiments, a construct containing the full length TAT-1a cDNA was used. To that end, total RNA was isolated from N2 animals and the cDNA amplified via PCR. The amplified TAT-1a was subcloned into the pPD49.83 vector and the plasmid was injected into the individual *smIs434* strains together with a second vector which contains a mCherry sequence and was therefore used as an injection marker. Investigation of PS externalization in somatic cells as well as the occurrence of a vacuolar gut phenotype was performed as described before. Since expression of the TAT-1a was under the control of the heat shock promoter, the worms were incubated at 33°C for 45min to induce expression and then kept at room temperature for recovery. Expression of the wild type TAT-1 completely rescued the phenotype in all the mutants analyzed.

3.2.6 *In vivo* viability assay

In order to investigate the potential toxic effect of different Au NPs *in vivo*, toxicity was investigated using the nematode *C. elegans* as a model. To that end, the particles were first mixed with the *E. coli* strain OP50 – which acts as the food source for the nematodes – and sonicated for 5min to ensure homogenous dispersion. Worms at larval stage 4 (L4) were used for analysis and were incubated in liquid culture in M9 buffer in the presence of the indicated concentrations of Au NPs for 24h. Stirring at 100rpm was introduced in order to prevent sedimentation. The survival rate was scored. Mutant strains defective in apoptosis, necrosis or

autophagy were used to decipher the underlying cell death mode. In order to gain robust data, three experiments with 25 animals for each condition were analyzed.

3.3 NANOPARTICLES

3.3.1 Synthesis and characterization

Synthesis of 5 nm and 20 nm Au NPs was performed through reduction of the Au^{3+} to Au^0 with Sodium borohydride ($NaBH_4$) or Sodium citrate ($Na_3C_6H_5O_7$), respectively. The resulting Au NPs contained thiol groups which were attached to the Au core through self-assembly and allowed modification with the individual surface functionalization. The synthesis was done at the University of Bordeaux.

A thorough characterization of the nanomaterial is a central part of nanotoxicological studies. It is important to note that characterization should occur in the presence of a relevant physiological medium as the presence or absence of serum proteins can greatly influence the characteristics of the particle. In the presence of proteins and other biomolecules, a bio-corona forms around the particle and this defines the biological identity of the particle. Moreover, serum proteins can affect the probability of the NP to form aggregates (Walkey and Chan, 2012).

The primary particle size was investigated by transmission electron microscopy (TEM) while the hydrodynamic range was determined by dynamic light scattering (DLS). TEM analysis of the particles further confirmed a spherical shape of the particles. The DLS measurement quantifies the surface charge of a particle – indicated by the zeta potential – and can further be used to evaluate the tendency of the particles to form aggregates or to sediment. These characteristics are highly dependent on the dispersion medium. As expected, we found that for NPs dispersed in water the NR_3^+ Au NPs were positively charged while the COO^- Au NPs had a negative charge and the PEGylated particles were less negatively charged. In cell culture medium containing FBS we observed negative values for the zeta potential of all particles, which is believed to result from the formation of a bio-corona. DLS measures the intensity of the scattered light which is proportional to the diameter of the particle (Fissan et al., 2014). DLS can only be used for spherical particles. Additionally, monodispersed samples are preferred to poly-dispersed particles which contain a big variation of particles with different sizes. A mixture of small and large particles in the sample may therefore not give reliable data (Tomaszewska et al., 2013). UV-Vis spectroscopy was used in order to detect the plasmon band characteristic for Au NPs which is dependent on the size and the shape of the nanoparticle.

3.3.2 Endotoxin assessment

Endotoxin is a form of contamination originating from Gram-negative bacteria. In order to obtain reliable results regarding the effect of a specific nanomaterial, it is important to exclude such a contamination. A widely used assay to detect endotoxin content is the chromogenic-based limulus amebocyte lysate (LAL) assay. We found that Au NPs did interfere with the LAL assay as all samples analyzed – including the negative control, where polymyxin B was added in order to neutralize the potential endotoxin content – resulted in the generation of similar absorbance values at 405 nm. We therefore used an alternative test method – the TNFα expression test (TET) – which is based on the detection of TNFα secretion of primary human macrophages in response to endotoxin (Mukherjee et al., 2016). The TET assay proved that all the tested Au NPs were endotoxin free.

3.4 MICROSCOPY BASED METHODS

3.4.1 Confocal microscopy

The RAW-Difluo mLC3 autophagy reporter cell line was used to study the Au NP-induced activation of the autophagy pathway. This reporter cell line allows to monitor the autophagic flux since it expresses the RFP::GFP::LC3 fusion protein. LC3 is a major component of the autophagosome. Under homeostatic conditions, a rather diffuse green and red cytosolic signal can be detected. Upon autophagy induction, the autophagosome forms and recruitment of LC3 can be followed by formation of puncta structures. Moreover, labeling of the protein with both RFP and GFP allows to distinguish between early stages of autophagy where the autophagosome is formed and both signals can be detected and late stages where the autophagosome has fused with the lysosome and the acidic environment quenches the green fluorescent signal. Therefore, the percentage of RFP-GFP-positive cells and RFP single positive cells can be used as a marker for the autophagic flux. Detection of the RFP and GFP signal was performed by confocal microscopy and the puncta structures of the autophagosome/ autolysosome could be clearly detected. In contrast, fluorescence microscopy or measurement of the fluorescent signal using a plate reader were shown to not be sensitive enough to assess the occurrence and progression of autophagy.

Moreover, the mitochondrial structure and morphology of THP-1 monocytes exposed to the cationic Au NPs was investigated by confocal microscopy after staining of the cells with MitoTracker Red CMXRos. This fluorescent dye accumulates in mitochondria of viable cells and is dependent on the mitochondrial membrane potential. Therefore, MitoTracker-staining allows to study mitochondrial structures and morphology. Mdivi-1 prevents mitochondrial fission through inhibition of Drp1 (dynamin-related protein 1) and was therefore used as a positive control for an elongated mitochondrial network.

3.4.2 Transmission electron microscopy

Transmission electron microscopy (TEM) was performed in two different studies. In the project on distinct cell death modes (paper II), TEM images were used in order to study subcellular changes and morphological characteristics associated with the three different cell deaths. Additionally, TEM was a useful tool to confirm uptake of the dying cells by macrophages and to study the morphology of the dying cells inside of the macrophage. In the Au NP project (paper III), TEM images were used in order to characterize the individual particles regarding primary size and aggregation properties as well as to confirm cellular uptake and subcellular localization of the particles.

This method only captures a limited number of cells or particles – we took approximately 20 to 25 images per condition in each of the analyzed settings – which are subsequently considered to be representative for a larger population of cells. This small sample size cannot be used for quantitative analysis. Moreover, there is a risk for bias and due to the limited amount of analyzed images a chance to miss specific features. However, TEM is very useful since it provides valuable and very detailed information regarding subcellular structures and characteristics. In terms of NP characterization, TEM is useful in order to determine the primary particle size and the shape of the particle.

Prior to the analysis, cells were fixed in 2.5% glutaraldehyde, post-fixed in 2% osmium tetroxide and then step-wise dehydrated in ethanol and acetone. The cells were then embedded in LX-112 and ultrathin sections (50-60 nm) were prepared. After contrasting with uranyl acetate and lead citrate the samples were analyzed using a Hitachi HT7700 120kV transmission electron microscope or a FEI Tecnai 12 Spirit BioTWIN transmission electron microscope in combination with a 2kx2k Veleta OSiS CCD camera.

3.5   CELLULAR AND BIOCHEMICAL ASSAYS

3.5.1   Induction of cell death

Jurkat E6.1 cells were used to study death receptor-induced apoptosis or RSL3-induced ferroptosis, while FADD-DN Jurkat cells were applied as a model for necroptotic cell death triggered by TNFα. The individual cell death pathways are illustrated in figure 1. Binding of a death ligand to the death receptor allows assembly of the DISC complex and activation of caspase-8. Active caspase-8 cleaves further substrates including the executioner caspase-3 thus causing apoptotic cell death. FADD-DN cells lack the death effector domain of the FADD protein. Upon death receptor oligomerization, these cells fail to activate caspase-8 and do not undergo apoptosis. Instead, activation of RIPK1 and RIPK3 leads to MLKL phosphorylation and necroptosis. We were not able to induce ferroptosis in Jurkat cells through Erastin. However, inhibition of GPX4 by RSL3 resulted in lipid peroxidation and ferroptotic cell death. Of note,

previous studies reported that only one RSL3 isoform – the (1S, 3R)-RSL3 – was able to trigger cell death. We found that the cell density was of critical importance for the induction of ferroptosis since a high cell density prevented cell death.

Importantly, cell death as well as cell death specific markers could be blocked by addition of individual cell death inhibitors. Apoptosis was blocked by the caspase-inhibitor zVAD-FMK, necroptosis was inhibited through the allosteric RIPK1 inhibitor necrostatin-1 (Nec-1) and ferroptosis was prevented by addition of the lipid antioxidant ferrostatin-1 (Fer-1).

3.5.2 Cell death/ cell viability assays

3.5.2.1 alamarBlue assay

The alamarBlue cell viability reagent was used to investigate cytotoxicity caused by Au NPs. This assay allows the fluorescence based assessment and quantification of the cell viability and was shown to not interfere with the Au NPs. To this end, resazurin – the active compound of the reagent – was added to the individual samples and incubated with the cells for 4 h. Resazurin is a non-toxic, cell-permeable and non-fluorescent blue reagent. Upon entering the cell it faces the natural reducing environment of viable cells and becomes irreversibly reduced to resorufin thus resulting in the formation of a red, fluorescent product. The resulting fluorescent signal was measured using an excitation at 540 nm and an emission at 590 nm and the fluorescence intensity is direct proportional to the metabolic activity of the cells. The assay can be used for both adherent and suspension cells. In order to compare different samples with each other, it is important that the same cell number is analyzed for each sample. Alternatively to the detection of the fluorescence signal, it is also possible to measure absorbance at 570 nm and 600 nm. However, due to the fact that the oxidized reagent is non-fluorescent and therefore does not interfere with the fluorescence signal, quantification based on the fluorescence signal is more sensitive.

3.5.2.2 LDH release assay

In order to monitor cell death progression, leakage of the cytosolic protein lactate dehydrogenase (LDH) into the extracellular environment was measured after different points of cell death induction by using the Pierce LDH Cytotoxicity Assay Kit. In this assay, LDH first catalyzes the formation of pyruvate from lactate, a reaction which also requires the reduction of NAD^+ to NADH. In a second step, diaphorase uses the NADH to catalyze the reduction of tetrazolium salt to a formazan product. The amount of formazan is therefore directly proportional to the amount of LDH that is present in the sample. Quantification is performed by measurement of the absorbance at 490 nm. In contrast to the previously described

measurement of cell viability by alamarBlue, the quantification of LDH release is a measurement of plasma membrane rupture. We found, that apoptotic cells show lower level of LDH release, even at late time points and hypothesize that apoptotic cells form apoptotic bodies and maintain the plasma membrane integrity even at extended time points. Thus, the amount of plasma membrane rupture and subsequent LDH release is lower in apoptotic cells compared to necroptotic or ferroptotic cells. We conclude that cell death does not always result in rupture of the cell membrane but do not exclude the possibility that membrane rupture occurs at later time points. For comparison, the same amount of cells was analyzed for each sample.

3.5.2.3   ATP measurement

The CellTiter-Glo 2.0 Assay is a luminescence based assay to evaluate cell viability based on measurement of ATP level. Addition of the CellTiter-Glo 2.0 Reagent to the individual samples causes cell lysis. Subsequent measurement of ATP level is based on the activity of the enzyme Ultra-Glo Luciferase which requires ATP in order to catalyze the conversion of Luciferin to Oxyluciferin. Formation of the product can be followed through measurement of the resulting luminescent signal using a plate reader and the detected signal is directly proportional to the amount of ATP in the individual sample. Seeding the same amount of cells allows to directly compare ATP level of individual samples of the same experiment.

3.5.2.4   Caspase-3-like activity assay

The measurement of caspase-3-like activity is based on the cleavage of the DEVD-AMC substrate. Active caspase-3 specifically recognizes the DEVD sequence (Nicholson et al., 1995) and cleavage of the substrate is measured via detection of the resulting fluorescence signal over time. Addition of the reaction buffer would result in lysis of the cells. A time-dependent increase in the fluorescent signal indicates caspase-3-like activity and quantification is possible since the detected signal is directly proportional to the amount of active caspase-3 in the sample. In this assay, it is important to capture the optimal time point since caspases may not be active at very early time points. Conversely, at late time points – when the plasma membrane integrity is compromised or cellular ATP level are depleted – caspase activity may not be detectable. Moreover, in order to compare different samples with each other, it is necessary to analyze the same number of cells in each sample. Finally, even though DEVD may be the main recognition sequence of caspase-3, other caspases such as caspase-7 can also recognize and cleave the DEVD substrate – however, with lower substrate specificity.

3.5.3 Flow cytometry

Flow cytometry was performed in order to study specific markers, mitochondrial function or DNA content by using a BD LSRFortessa or a BD Accuri C6 flow cytometer. Prior to analysis, cells were stained with fluorescent dyes or fluorescent labeled antibodies. On the basis of the detection of individual events, this methods then allows to quantify the expression of specific cellular markers as well as the DNA content, the cellular level of ROS or the mitochondrial membrane potential in a cell population. For each sample 10 000 events were recorded. Extracellular NPs are also detected by flow cytometry as individual events but introducing washing steps prior to the analysis and gating on the cell population in the FSC/SSC plot can exclude or reduce the detection of the particles.

Upon cell death induction, a shift of the cell population can be observed in the FSC/SSC plot. Gating on the healthy cell population in the FSC/SSC plot can therefore be used for quantification of cell death.

PS exposure was investigated by staining the cells with annexin V-FITC or a FITC-labeled PS antibody. Annexin V is a PS-binding protein but also recognizes other phospholipids (e.g. PE or CL). Binding of annexin V to PS occurs in a Ca^{2+} dependent manner. Staining with annexin V was performed for 30min in the dark before propidium iodide (PI) was added and the samples were analyzed through flow cytometry. PI is a cell impermeable dye that only enters cells with ruptured plasma membrane but not healthy cells. In cells with diminished membrane integrity, PI can enter the cell and intercalate with the DNA. Annexin V^+/PI$^-$ cells are considered as apoptotic cells, while double positive cells are referred to as (secondary) necrotic cells. However, PS exposure was observed in non-apoptotic settings. Analysis was performed on non-fixed cells since fixation could lead to permeabilization of the plasma membrane. In case of staining with the PS antibody, a matching isotope control antibody was used in order to confirm specificity.

Cell cycle analysis was performed by staining fixed and permeabilized cells with PI. RNA was degraded by adding RNaseA to prevent interference with the staining. The DNA content was investigated by flow cytometry and the sub-G1 population in the histograms was quantified and used as a measurement for apoptosis.

The expression of the 'don't-eat-me' signal CD31 was studied via antibody staining. A matching isotope control antibody was used to confirm specificity of the CD31-FITC antibody.

The mitochondrial membrane potential was investigated through TMRE (tetramethylrhodamine ethyl ester) staining. This dye enters the cell and accumulates in mitochondria of healthy cells. Active mitochondrial membrane potential is required for that, which is why only viable cells show TMRE staining of the mitochondria while diminished mitochondrial potential – as found in dying/dead cells – would result in a reduced staining. Therefore, TMRE staining can be used to quantify mitochondrial membrane potential.

Phospholipid peroxidation as a marker of ferroptotic cells was investigated through staining with BODIPY 581/591. The dye was added to the cells where it intercalates with the membrane and is susceptible for oxidation. Staining was performed for 30min. Oxidation of BODIPY 581/591 can be measured through a detection of the reduced red fluorescence signal and increase in the green fluorescence signal. The cells were washed with PBS and then analyzed by flow cytometry.

Mitochondrial ROS production was measured by staining with the MitoSOX Red Mitochondrial Superoxide Indicator for 30min. Oxidation of the dye through contact with mitochondrial superoxide results in a red fluorescent signal. The dye is specific to detect mitochondrial superoxide production and not other ROS or RNS (reactive nitrogen species) species. The cells were washed three time before analysis by flow cytometry.

3.5.4   Western blot

Western blot analysis was used to investigate the expression and quantification of different proteins. To that end, lysates were prepared from whole worms of the different *tat-1* mutant strains or from cell pellets using RIPA buffer. In order to prevent protein degradation protease inhibitors as well as phosphatase inhibitors were freshly added. The RIPA buffer has a relatively short shelf life and it is therefore critical to use fresh RIPA buffer. Lysis was performed over night at 4°C and cell debris as well as the DNA content of the samples were removed by centrifugation for 10min at 13000 rpm.

Subsequently, 4-12% gradient gels were used to separate the proteins via electrophoresis according to their molecular mass. For comparison of the individual samples, the same amount of total protein – previously determined by BCA assay – was loaded to each well of the gel. Incubating the samples at 95°C for 5min promotes denaturation of the proteins. Following the electrophoretic separation, the proteins were transferred to a PVDF membrane. Quantification of the individual proteins was achieved through binding of specific primary antibodies followed by detection via fluorescent labeled secondary antibodies and scanning of the membrane using a Licor Odyssey Scanner. Western blot analysis in the TAT-1 project was performed through separation on polyacrylamide gels. The primary antibody is described below. The secondary antibody was coupled to HRP (horseradish peroxidase) and addition of a HRP substrate subsequently allowed detection through a photographic film.

In general, monoclonal antibodies show a higher specificity compared to polyclonal antibodies since they only recognize one epitope. In contrast to the native-PAGE, electrophoretic separation of proteins in the SDS-PAGE occurs under denaturing conditions. It is therefore critical that the chosen primary antibody is able to detect the specific epitope in the denatured protein. Polyclonal antibodies have the advantage that they detect several epitopes of the same protein and are therefore less dependent on one specific epitope. Therefore, polyclonal antibodies may

give more sensitive results but also bear the risk of more unspecific binding compared to monoclonal antibodies. The actual detection of the protein of interest occurs via a secondary antibody that is coupled to a fluorescent dye and that recognizes the constant domain of the primary antibody. The choice of the secondary antibody is therefore dependent on the species in which the primary antibody was produced. Several secondary antibodies can bind to one single primary antibody thus resulting in an amplification of the signal.

Western blot analysis is a semi-quantitative method that allows not only detection but also quantification of the expression of the specific protein – especially with respect to a suitable housekeeping gene or loading control – based on the intensity of the band. Examples of such housekeeping genes are GAPDH or α-tubulin.

Antibodies that specifically detect the nematode TAT-1 protein are not commercially available. Therefore, monoclonal antibodies were raised in mice through injection of recombinant TAT-1 (amino acids 369-734). The isolated antibody therefore detects the C-terminus of the protein. Screening for monoclonal antibodies and injection of selected hybridoma cultures in mice allowed subsequent amplification and purification of the monoclonal antibodies. Results from the TAT-1 7G1 antibody are shown in paper I. These results were evaluated with a second antibody (TAT-1 1H5) to confirm that reduced detection of the protein is a result of the TAT-1 mutation and not based on an altered epitope recognition site (data not shown).

3.5.5   Mitochondrial respiration

The oxygen consumption of THP-1 monocytes exposed to Au NPs was investigated with the help of an Clark-type oxygen electrode (Hansatech Instruments, Norfolk, UK). Untreated cells were used as a negative control and STS treated cells as a positive control. Following the exposure, cells were collected, resuspended in fresh cell culture medium and introduced to the respiratory chamber of the Oxygraph instrument. The chamber was closed and moderate stirring was introduced to the sample to ensure homogenous distribution. After the signal has stabilized, basal respiration was monitored for approximately 3min. The maximal activity of the respiratory chain was measured after addition of the mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP). The mitochondrial respiration was quantified based on the slope of the curve and normalized to the number of cells of each sample. This method therefore allows sensitive quantification of the oxygen consumption rate.

3.5.6   Phagocytosis assays

Human monocyte derived macrophages were obtained from buffy coats of healthy blood donors after differentiation with M-CSF for four days. Additionally, apoptotic, necroptotic or ferroptotic

cell death was triggered in wild-type Jurkat cells or FADD-DN Jurkat cells, respectively, for different time points (for more details refer to the corresponding sections on the isolation of macrophages or on cell death induction). In order to identify the target cells in the co-culture with macrophages, Jurkat cells were labeled with TAMRA (Witasp et al., 2007) – an amine-reactive dye that non-specifically binds to proteins. The Jurkat cells were then collected and resuspended in fresh cell culture medium. The cell density of the dying Jurkat cells was determined through cell counting and the same amount of target cells was then added to each sample of macrophages thus keeping the ratio between macrophages and target cells the same for all analyzed samples. This then allows to directly compare uptake of the different cell death modes by the macrophages. To this end, co-culture was performed for 1h and non-engulfed Jurkat cells were removed through extensive washing. The cells were fixed in 4% formaldehyde in PBS, nuclei stained with Hoechst 33342 and phagocytosis efficiency investigated through fluorescence microscopy. Images from at least six optical fields were taken and analyzed per sample. The phagocytosis efficiency was quantified and is defined as the percentage of macrophages, that have engulfed at least one Jurkat cell (as observed by TAMRA staining).

3.6   MULTI – OMICS ANALYSIS

3.6.1   RNA sequencing

RNA sequencing is a method to identify and quantify mRNA molecules. It provides robust insight into the expression of individual transcripts with low background noise and high dynamic range. In order to prepare samples for RNA sequencing, RNA was extracted from THP-1 cells that were exposed to low (EC_{10}) doses of the Au NPs for a short time. These sub-cytotoxic doses allowed to capture early events and detect changes on the transcript level before excessive cell death occurred. All samples were prepared in triplicates. The quality of the extracted RNA was found to be suitable for RNA sequencing and was confirmed for all samples via Nanodrop, Qbit fluorometer and Bioanalyzer. RNA sequencing allows the quantification of changes on the transcript level in response to a certain treatment and to discriminate between individual variants. In paper III we used single cell tagged reverse transcription (STRT) RNA sequencing (Islam et al., 2011). This method allows full-length coverage of the transcript, is highly sensitive and allows multiplex analysis. It is based on the creation of a cDNA library, followed by amplification and sequencing. The individual transcripts were identified with the help of computational alignment to the human genome and the expression level was quantified based on the number of reads. Data analysis was performed as described below. Significant changes in gene expression were mainly detected for samples exposed to the cationic particles. The most significantly affected pathways include mitochondrial dysfunction, oxidative phosphorylation and protein ubiquitination.

3.6.2 Mass spectrometry

THP-1 cells were seeded and exposed to the EC_{50} doses of Au NPs for 24h. Compared to the samples for RNA sequencing, the prolonged exposure time and increased concentration of the particles assured that changes on protein level could be detected. All samples were prepared in triplicates. Following exposure and washing of the cells, proteins were extracted, digested and analyzed via mass spectrometry. The data from this analysis were searched against the human complete proteome database (Uniprot) and further processed via statistical analysis. In order to compare individual samples, Student's t-test was applied. Significant changes were mainly found for THP-1 cells exposed to the cationic Au NPs. The correlation between RNA and protein level is suggested to be about 40% (de Sousa Abreu et al., 2009; Vogel and Marcotte, 2012). Based on these findings, it is important to not only study the transcriptome but also the effect of the exposure on the protein expression. Similar to the results from RNA sequencing, the cationic Au NPs were shown to induce significant alterations in protein expression. Mitochondrial dysfunction, oxidative phosphorylation and protein ubiquitination were among the most affected pathways both on transcript and protein level.

3.6.3 Bioinformatics analysis

Omics analysis results in the generation of immense amounts of data that have to be further processed in order to determine relevant changes in the expression level as well as to put these results into a biological context. Bioinformatics analysis was performed in paper III on data from RNA sequencing as well as mass spectrometry. Hence, samples from untreated THP-1 cells and THP-1 cells exposed to different Au NPs were compared with each other. Untreated cells were used as a negative control. The human complete proteome database (Uniprot) was used as a reference. For the data from RNA sequencing, differentially expressed genes (DEGs) were identified. Gene ontology (GO) enrichment analysis and pathway analysis were performed in order to investigate the biological relevance of the data. For both the transcriptomics and proteomics analysis hierarchical cluster analysis was performed in order to identify individual clusters. We also performed ingenuity pathway analysis (IPA) in order to elucidate the downstream pathways that were significantly altered in response to NP exposure.

This data analysis provides a collection of significantly up-or downregulated genes, proteins or pathways and is considered as an unbiased way of data analysis. These data can further build the basis to develop a new hypothesis and were used as a starting point for further validation experiments.

In paper I the tree bioinformatics programs PROVEAN, SIFT and PolyPhen-2 were used in order to predict the effect of individual *tat-1* mutations on the protein function. Most of the mutations were predicted to be deleterious by at least one of the programs.

3.7 STATISTICS

At least three independent experiments were performed for each assay and for each condition technical duplicates or triplicates were analyzed. Graphs represent average values and error bars show standard deviation (S.D.). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. This statistical method allows to compare individual groups of samples with each other. Statistical significance was considered for $p < 0.05$.

4 RESULTS

4.1 PAPER I

The basic cell death mechanisms as well as the main cell death signals are evolutionarily conserved. As an example, the exposure of PS as an 'eat-me' signal as well as the mechanisms that lead to its externalization are highly similar in nematodes and the mammalian system.

The *C. elegans* aminophospholipid translocase TAT-1 is a transmembrane protein that is critical for endocytic sorting as well as for maintaining PS asymmetry at the plasma membrane (Chen et al., 2010; Darland-Ransom et al., 2008; Ruaud et al., 2009). Loss of the TAT-1 protein function leads to accumulation of vesicles in the intestine as well as exposure of PS at the plasma membrane of all cells. Despite the importance of this protein for multiple biological processes, information regarding the structure, regarding specific residues and domains that are critical for the proper protein function or regarding the mechanism of action are greatly missing. In this project, we studied the effect of 16 different single point mutations in the *tat-1* gene – identified by the *C. elegans* million mutation project (Thompson et al., 2013) – on the functions and stability of the protein. These mutations are distributed across the entire protein and result in amino acid changes with opposite physicochemical properties, which are often predicted to be deleterious.

For each of the strains we investigated the occurrence of a vacuolar gut phenotype – a marker of defective endocytic sorting. Moreover, we studied exposure of PS at the cell surface of gonad cells and somatic cells as well as protein expression and stability.

Surprisingly, most of the mutant strains investigated did not show any phenotype different from the one observed in wild type worms, suggesting that the TAT-1 protein is remarkably resilient towards local amino acid substitutions even though these may result in substitutions by amino acids with opposite characteristics or most likely induce conformational changes. However, two mutations that disrupt either a conserved PISL motif – shown to be important for substrate specificity – or a highly conserved DKTGT motif – critical for phosphorylation of the protein during the catalytic cycle (Andersen et al., 2016; Okamura et al., 2003; Roland and Graham, 2016; Vestergaard et al., 2014) – did disrupt both functions of TAT-1. Interestingly, while the mutation of the PISL motif does not affect protein expression and stability, mutation of the DKTGT motif resulted in greatly reduced protein level of the TAT-1 protein. Therefore, mutations of the DKTGT motif are likely to destabilize the protein thus diminishing its function. Another mutation that – similar to the mutation of the DKTGT motif – affects a residue located in the intracellular phosphorylation and nucleotide-binding domain between transmembrane domain four and five resulted in a weak vesicular gut phenotype. No ectopic PS exposure was observed in this mutant. Moreover, we identified a temperature sensitive mutant (E1116K) – bearing an amino acid

substitution in the C-terminal region of the TAT-1 protein – which expressed a vesicular gut phenotype at increased temperature. We did not detect externalization of PS at the cell surface of this mutant strain – even at increased growth temperature. Interestingly, five additional mutants also bearing amino acid changes in the C-terminus did not alter the TAT-1 functions.

In order to confirm that the observed phenotype is caused by loss of the TAT-1 function, we performed rescue experiments. We show that expression of wild-type TAT-1a fully rescues the vacuolar gut phenotype and PS externalization defect observed in the individual mutant strains that are described above.

With this project, we gained further insight into the mechanism of phospholipid translocases and provide *in vivo* evidence for the importance of two conserved motifs of this protein family for the protein functions.

4.2 PAPER II

Ever since the discovery of several distinct forms of PCD and their involvement in different physiological and pathological settings the question regarding the final outcome of a specific cell death mode and its relevance for the organism became more and more evident. On an organismal level, the underlying cell death program may be less important than the consequence of this cell death mode. However, while cell death research mainly focused on elucidating the mechanism of the specific cell death and on inducing or blocking it, much less focus was put on the cell clearance mechanisms and immunological responses associated with different cell deaths. Even more so, while different studies addressing cell clearance mainly focus on apoptotic cell clearance, studies on the recognition and engulfment of necroptotic or ferroptotic cells as well as involved molecules are greatly missing.

In this project, we first established a cellular model that allows the induction of three different forms of PCD – apoptosis, necroptosis and ferroptosis – using the same cell line. Jurkat cells were used to induce apoptosis (Fas mAb), necroptosis (TNFα) and ferroptosis (RSL3) respectively. The occurrence of cell death was confirmed and monitored by measurement of LDH release into the supernatant and could be blocked by cell death specific inhibitors. Distinct morphological changes associated with each cell death were investigated by TEM imaging and gave detailed insight into the subcellular alterations of apoptotic, necroptotic or ferroptotic cell death. This includes chromatin condensation in case of apoptotic cells, organelle swelling and mitochondrial membrane rupture in necroptotic cells or diminished mitochondrial cristae structure and altered mitochondrial morphology in ferroptotic cells. Additionally, cell death specific biochemical markers were investigated. Apoptotic cells were characterized by the occurrence of caspase-3-like activity, necroptotic cells displayed phosphorylation of MLKL and ferroptotic cells show time dependent increase of lipid peroxidation. These features could be blocked by cell death specific inhibitors.

Importantly, our study confirms for the first time that PS is exposed to the cell surface in all three forms of cell death. PS exposure has long been described as an 'eat-me' signal specific for apoptotic cell death and is still today often used as a marker of apoptotic cell death. We now show that PS externalization occurs in other forms of cell death, which was proven by annexin V staining as well as staining with a specific PS antibody. PS exposure occurred prior to the rupture of the plasma membrane and could be blocked by the individual cell death specific inhibitors. We therefore provide evidence that PS exposure is not unique for apoptotic cell death and should not be used as a specific marker of apoptosis. Our results let us suggest that PS exposure could have a role in facilitating phagocytosis not only of apoptotic cells but also of necroptotic and ferroptotic cells. However, the underlying mechanisms leading to the externalization of PS in the individual cell death modes remain to be studied.

We next investigated phagocytosis efficiency of the three cell deaths and confirm that primary human macrophages are able to engulf apoptotic, necroptotic as well as ferroptotic Jurkat cells. We show that induction of apoptosis, necroptosis and ferroptosis resulted in a comparable degree of cell death at 24h. Moreover, the ratio of macrophage to target cell was kept the same for all samples, therefore allowing the comparison of the engulfment efficiency of the individual cell death modes. Interestingly, we show that the macrophages preferably engulf apoptotic cells and that the majority of macrophages internalized several apoptotic target cells as confirmed by TEM analysis. In contrast, phagocytosis of more than one necroptotic or ferroptotic target cells per macrophage was observed less frequently. Furthermore, our analysis suggests that necroptotic and ferroptotic cells are internalized as cell fragments rather than whole cells. The number of macrophages that engulfed at least one ferroptotic cell was significantly reduced compared to apoptotic or necroptotic cells. We also show that early time points of all three cell deaths are less efficiently engulfed compared to late time points.

These results could have an impact on the way different cell death related diseases are treated and potentially open the way to new treatment strategies. However, more studies are required in order to understand the mechanisms and consequences of cell clearance of different forms of PCD as well as its correlation to different pathological settings.

4.3 PAPER III

Based on the unique optical and physicochemical properties, gold nanoparticles (Au NPs) are widely used in various biomedical applications as well as for imaging purposes or diagnostics. Intriguingly, there are discrepancies regarding the biocompatibility or toxicity of different Au NPs. While some studies found that Au NPs are non-toxic, others observed the induction of different forms of cell death. In a comprehensive study, we here investigate the effect of Au NPs of two different sizes and three different surface functionalizations by applying multi-omics analysis as well as by complementing these results with validation experiments both *in vitro* and *in vivo*.

The synthesis of the particles was performed by catalyzing the growth of the NP core and simultaneously introducing the individual surface modifications through binding to a thiol monolayer. Conventional nanotoxicology methods were used for initial characterization of the individual particles. The size distribution as well as physicochemical characteristics were investigated by TEM, as well as DLS and UV-Vis spectroscopy. The Au NPs were found to be free of endotoxin contamination. Furthermore, cytotoxicity of the NPs was investigated both *in vitro* and *in vivo*. We show that the cationic (NR_3^+-functionalized) Au NPs were toxic, while anionic (COO^--functionalized) or PEGylated Au NPs were non-toxic. Additionally, the bigger (20 nm) particles were more toxic than the small (5 nm) particles. Analyzing the dose-dependent cytotoxicity further enabled to select appropriate concentrations and time points for further experiments. Moreover, uptake of the Au NPs was confirmed through TEM imaging of exposed THP-1 monocytes. This high resolution analysis further allowed to investigate the intracellular localization of the particles as well as subcellular morphological changes. Importantly, the cationic Au NPs were found to localize in mitochondria.

Following the initial characterization of the individual particles, transcriptomics and proteomics analysis was performed. Sub-cytotoxic doses and early time points were selected for transcriptomics analysis in order to capture early events that occur prior to cell death. Following RNA extraction, single-cell tagged reverse transcription sequencing (STRT-seq) was performed. Proteomics analysis was applied on cells exposed to the Au NPs at EC_{50} doses for 24h thus allowing to detect changes on protein level. Proteins were extracted, digested and analyzed via mass spectrometry. Hierarchical clustering as well as pathway analysis were carried out on the transcripts or proteins which were suggested to be significantly altered by RNA-sequencing or mass spectrometry, respectively. Importantly, we show that on both the transcript and the protein level, the pathways which were most affected by exposure to the cationic Au NPs were related to mitochondrial dysfunction, oxidative phosphorylation and protein ubiquitination. With respect to oxidative phosphorylation, we found that a pronounced majority of genes related to the subunits of the mitochondrial electron transport chain were downregulated in cells exposed to the cationic Au NPs.

In validation experiments specifically addressing mitochondrial function, we found that the NR_3^+-Au NPs inhibit mitochondrial respiration. Moreover, we show reduced mitochondrial membrane potential and increased mitochondrial superoxide production in response to the cationic Au NPs. When investigating the mitochondrial morphology we found that cells exposed to the cationic Au NPs elicit elongated and swollen mitochondria with diminished cristae structures.

Investigating the underlying cell death mode triggered by the cationic particles, we found evidence for involvement of autophagy related pathways as well as induction of apoptotic cell death. We observed formation of autophagosomes using the RAW-Difluo mLC3 reporter cell line and show that inhibition of autophagy resulted in increased cell death. These results suggest that activation of the autophagy program is a survival mechanism of the cells. Ultimately, cells exposed to the cationic Au NPs undergo apoptosis, which is further supported by the detection of caspase 3-like activity and the observation that caspase inhibition by zVAD-FMK prevents cell death. Of note, prolonged exposure to the cationic particles or high concentrations would cause depletion of cellular ATP level and lead to necrotic cell death. These results were confirmed in the nematode *C. elegans*.

Together, we found that toxicity of Au NPs is dependent on the size and the surface functionalization of the particle and provide evidence for a mitochondria-related pathway of toxicity induced by cationic Au NPs. These results are of relevance for various medical applications where Au NPs are suggested for use in treatment or detection and may thus lead to the production of novel materials and treatment strategies.

5 DISCUSSION

5.1 PHOSPHATIDYLSERINE – A KEY SIGNAL OF DYING CELLS

Cell death is a central feature in multicellular organisms ensuring proper development, cellular turnover and homeostasis. The main pathways, as well as the involved signals are evolutionarily conserved and tightly regulated. Among these, PS exposure on the surface of dying cells is the most extensively studied 'eat-me' signal. While PS is restricted to the cytosolic leaflet of the plasma membrane under homeostatic conditions, its externalization to the extracellular leaflet of the plasma membrane of dying cells facilitates recognition and engulfment by phagocytes. The presence of 'eat-me' signals and the concurrent absence of 'don't-eat-me' signals on dying cells allows their discrimination from viable cells and thus enables engulfment. Interestingly, PS exposure on macrophages is required to facilitate efficient cell clearance (Callahan et al., 2000).

PS exposure was shown to occur in various species ranging from the nematode *C. elegans* to mammalian organisms and was initially reported to be characteristic for apoptotic cells. Even today, PS exposure is often referred to as a marker for apoptotic cells. However, it becomes more and more evident that various non-apoptotic forms of PCD display PS on their surface and therefore are likely to facilitate PS-dependent recognition as well. Necroptotic cells were shown to release PS-positive necroptotic 'bubbles' and PS externalization was reported to occur prior to the loss of plasma membrane integrity of these cells (Gong et al., 2017; Zargarian et al., 2017). There are controversial reports regarding the occurrence of PS exposure in ferroptotic cells and these discrepancies may be the result of different ferroptosis inducers or cell models (Seiler et al., 2008). However, in our study on RSL3-induced ferroptotic Jurkat cells we clearly show PS externalization, which occurs before plasma membrane rupture and which was confirmed both by annexin V-staining and by staining with a specific PS antibody. Similarly, we confirm apoptotic and necroptotic PS exposure in our model. We therefore conclude, that PS exposure is a rather general feature of dying cells and not specific for a certain cell death mode.

When analyzing the mode of cell death in a certain setting, it is therefore important to investigate cell death specific markers instead of drawing a conclusion that is based only on the externalization of PS. Addressing the activity of specific proteases as well as biochemical characteristics or the effect of cell death inhibitors may help to correctly identify the underlying form of cell death.

Moreover, PS exposure might not be the only 'eat-me' signal but there are likely to be additional signals involved in facilitating recognition of dying cells. While some of these features might be a general characteristic of dying cells, others could be more specific for one form of cell death. Together, it might be possible to suggest that the occurrence or absence of multiple signals is required to facilitate efficient phagocytosis and that these signals work together in a synergistic

network. There is more and more evidence that PS exposure is neither the only 'eat-me' signal, nor a specific marker of a certain cell death mode. The combination of different signals may vary between different cell types as well as between different cell death modes. It is not clear – and probably rather unlikely – if there is a single molecule that is unique for a certain cell death mode or if there is a combination of multiple signals which allows discrimination. There might be an overlap between the occurrence of such signals in different cell death modes. Evolutionarily, it is reasonable to suggest that different cell death modes express similar characteristics – which allow discrimination between living and dying cells by phagocytes – rather than expressing a single, cell death specific signal – which would require more specialized individual recognition mechanisms. PS exposure might be just one of these characteristics of dying cells – and probably a rather general one – instead of being specific for only one type of cell death. Nonetheless, the occurrence of distinct immune responses associated with a specific form of cell death suggest that more cell death specific characteristics exist. Of note, we found that dying cells at late time points of cell death induction were more efficiently engulfed compared to early stages. This further suggests that additional molecules might be involved in facilitating the recognition of late cell death stages. Moreover, different types of macrophages may express distinct sets of receptors and the cooperation of specific ligands and receptors subsequently ensures rapid phagocytosis. The high efficiency of the phagocytosis program *in vivo* is further illustrated by the fact that in a healthy organism the occurrence of dying cells is rarely observed. Importantly, the mechanism by which phagocytes are taking up different modes of dying cells may differ between the forms of cell death (Cocco and Ucker, 2001; Krysko et al., 2006). However, only few studies have addressed this question thus far. More research is required in order to identify such molecules and mechanisms and this could potentially lead to the discovery of specific cell death markers. Model organisms such as the nematode *C. elegans* are a useful tool to study such mechanisms. The conservation of the underlying pathways and the high degree of homology allows to translate the findings to its counterparts in higher (mammalian) organisms.

While PS exposure is a conserved 'eat-me' signal, the mechanism that leads to its exposure – at least during apoptosis – are evolutionarily conserved as well. Apoptosis induction leads to inactivation of phospholipid translocases as well as activation of scramblases and this causes PS externalization (Bratton et al., 1997). Although the general function of these transmembrane proteins is known, our understanding regarding the exact transport mechanism is still limited. Translocases show structural similarity to ion transporters but in contrast, the lipid substrate is several magnitudes bigger than ions. More research is needed to elucidate the exact structure of these proteins, the transport mechanism and substrate specificity as well as critical residues in this process. Moreover, the mechanisms that lead to PS externalization in non-apoptotic forms of cell death may be distinct from the apoptotic ones. While apoptotic PS exposure occurs in a caspase-dependent manner (Vanags et al., 1996), necroptotic or ferroptotic PS externalization is likely to happen in the absence of caspase activity. The involved transporters that facilitate non-apoptotic PS exposure as well as their mechanism of action and their regulation remain to be

identified. It is possible to suggest that alterations of the phosphorylation status of the transporter can activate or inactivate the protein. Additionally, our knowledge about the mechanism and regulation of temporary PS exposure in viable (activated) cells is still limited.

The *C. elegans* aminophospholipid translocase TAT-1 was shown to facilitate PS transport in the nematode (Darland-Ransom et al., 2008). Of note, attempts to purify TAT-1 and to express this membrane protein *in vitro* failed. Therefore, further biochemical analysis addressing phospholipid flippase activity could not yet be performed. Moreover, the crystal structure of the protein was not yet obtained. More research is also needed in order to understand the regulation of these transporters, the exact transport mechanism as well as potential interaction with additional factors. In paper I we performed mutational analysis in order to study the structure-function relationship of the TAT-1 protein. Importantly, our study shows that two conserved motifs are critical for the protein function of the aminophospholipid translocase. Attempts like this are useful to elucidate residues or domains that are required for the protein function *in vivo*.

One of the methods with which we and others commonly detect externalized PS at the cell surface is the staining with annexin V – a PS binding protein. However, annexin V is not specific for PS but also recognizes other lipids such as PE or CL (Balasubramanian et al., 2015). In order to further confirm the exposure of PS at the cell surface, alternative methods are required. This could include ToF-SIMS (Time-of-Flight secondary ion mass spectrometry) or staining with specific antibodies. While the former method allows the identification of individual lipid species, the latter one displays a rather simple approach to identify individual lipids but requires antibodies that are able to detect a specific epitope. It is possible that certain subspecies of a lipid – such as its oxidized forms (Kagan et al., 2002) – are not recognized by the antibody or that antibodies against individual lipid species are not commercially available.

In addition to the question if PS is a signal that is unique for apoptotic cells, it has been discussed if PS exposure alone is sufficient to induce phagocytosis. Studies using PS-containing liposomes show that phagocytes readily recognize and engulf these liposomes. Additionally, prevention of PS exposure or masking of exposed PS by PS-binding molecules results in reduced engulfment (Asano et al., 2004). Raji cells were shown to undergo apoptosis in the absence of PS exposure – later shown to be caused by strongly reduced *Xkr8* mRNA expression and therefore lack of the phospholipid scramblase activity in these cells – and as a consequence are less efficiently phagocytosed (Fadeel et al., 1999; Kagan et al., 2002; Suzuki et al., 2013). These findings suggest that PS is required for efficient recognition and cell clearance. In contrast, pre-apoptotic cells that did not yet expose PS were readily engulfed by macrophages (Zhang et al., 2008). Moreover, artificial enrichment of PS on the surface of viable cells did not facilitate their engulfment. It is therefore likely that PS is one out of several 'eat-me' signals which act together in a synergistic network in order to ensure efficient cell clearance. Notably, different cell types may express different sets of these molecules. Additionally, the occurrence of various 'don't-eat-me' signals

on viable cells prevents their uptake by phagocytes (Brown et al., 2002). Together, PS exposure might be required but not sufficient for the recognition and engulfment of dying cells and additional signals are likely to exist.

5.2 IMPORTANCE OF THE UNDERLYING CELL DEATH MODE

On an organismal level, it is not only important that the cell death program is balanced but also that dying cells are efficiently removed before causing any harm. Today, several distinct cell death modes are known. One issue in cell death related fields is that cell death is often reported in an overly simplified way. While a great number of toxicological studies only distinguishes between viable and dead cells, other studies further discriminate between programmed (apoptotic) or accidental (necrotic) cell death. The distinction between apoptotic and necrotic cells is often based on staining of the cells with annexin V and propidium iodide (PI) in order to detect PS externalization and plasma membrane rupture. As mentioned above, PS exposure is not an apoptosis specific feature and therefore the characterization of the cell death mode solely based on annexin V-positivity is not appropriate. Moreover, "programmed cell death" is often used synonymously for "apoptotic cell death" even though non-apoptotic forms of programmed cell death have been identified. This simplified nomenclature dates back to a time where naturally occurring cell death was first described and where apoptosis was the only known form of PCD. Apoptosis was then described to be morphologically and mechanistically distinct from necrotic cell death and most importantly to be genetically regulated. Still today, many studies only distinguish between these two forms of cell death. This classification, however, needs to be updated. Moreover, it is not sufficient to only describe morphological features of cell death or the pure occurrence of cell death, but it is essential to address molecular and biochemical characteristics of a certain type of cell death. This would not only result in the disclosure of the underlying mechanisms but can also lead to a better understanding regarding the outcome of the observed cell death on an organismal level (Kumar et al., 2017). It is necessary to understand the immunological consequences of the cell death mode – e.g. does it cause a pro-inflammatory or an anti-inflammatory response – as well as to identify the underlying pathways and involved signals. While an inflammatory response could cause tissue damage, an anti-inflammatory response would support tissue renewal and tolerance. Moreover, addressing distinct cell death pathways and considering cell clearance mechanisms in the treatment of various pathologies that are linked to a dysregulated cell death/ cell clearance program could be of great benefit for the patient as it could reduce negative side effects and could help to generally improve the quality of life. Future studies in various cell death related fields – such as toxicology, pharmacy or medicine – should therefore aim to more carefully identify the underlying cell death mechanisms by addressing several markers and characteristics as well as to consider subsequent cell clearance and immunological consequences.

As an example, resistance of cancer cells towards apoptotic cell death was described to be one of the hallmarks of cancer (Hanahan and Weinberg, 2011). Moreover, cancer cells are dependent on an efficient antioxidant defense mechanism to counterbalance excessive ROS production that occurs due to increased metabolic activity. These cells could therefore be more sensitive for ferroptosis induction (Dixon et al., 2012). The discovery of ferroptosis and its potential in specifically killing tumor cells gives raise to novel treatment strategies that are of special interest for the treatment of drug resistant tumors (Yu et al., 2015). However, we now report that engulfment of ferroptotic cells by monocyte derived macrophages is less efficient compared to necroptotic or apoptotic cells. While our study only focused on one type of primary human macrophages, this does not rule out the possibility that other phagocytic cells can recognize and engulf ferroptotic cells with higher efficiency. Nonetheless, we aim to raise awareness that it is critical to not only consider the cell death mechanism, but also the consequences of the cell death since persistent cell death is likely to cause inflammation and tissue damage. Importantly, cell death *in vivo* does not stop with the dying cell but also includes the fate of that dying cell and the corresponding immune response.

Ferroptosis was first described as a cell death mode that is specific for RAS mutated tumor cells (Dixon et al., 2012). However, later studies successfully induced ferroptosis in cells expressing wild-type RAS – which also includes the Jurkat cells used in our study. Of note, in preceding experiments we failed to induce ferroptosis in Jurkat cells by addition of Erastin or a combination of Erastin and a Smac peptide. It might be possible that certain cells are sensitive for induction of type II ferroptosis (triggered by inhibition of GPX4) but not for type I ferroptosis (caused by depletion of cellular GSH level). Similarly, Yu and colleagues found that Jurkat cells were resistant to Erastin-induced ferroptosis and further conclude that resistance towards Erastin is not correlated with RAS mutation (Yu et al., 2015). Another study leads to the suggestion that the resistance of Jurkat cells towards Erastin-treatment is caused by reduced expression of the cystine/glutamate antiporter system x_c^- in these cells (Kakazu et al., 2011). Nonetheless, studies from the Fulda group reported to successfully induce ferroptosis in Jurkat cells using Erastin, BSO or RSL3 either alone or in combination with Smac mimetics (Dächert et al., 2016; Haß et al., 2016; Schoeneberger et al., 2015). Importantly, the cell density is critical for the induction of ferroptosis and a high cell density results in reduced susceptibility of the cells to undergo ferroptotic cell death. This observation may further explain the discrepancies between the individual studies.

Together, it is of critical importance that individual cell death pathways are characterized based on specific biochemical properties. The mechanisms and signals involved in cell clearance have not yet received much attention – both in medical research as well as in toxicology. A detailed understanding of these pathways will be beneficial for the various cell death related fields. It will not only provide molecular insight regarding the outcome of a certain treatment or exposure, but can lead to the development of novel treatment strategies for various cell death related diseases, reduce negative side effects and can increase the quality of life of the patient.

5.3 NANOTOXICOLOGY AND CELL DEATH

While early nanotoxicological studies mainly focused on the characterization of the NPs and only report the occurrence or absence of cell death, there is now an increasing demand for more detailed analysis and fundamental understanding of the underlying mechanisms and consequences for the organism and environment. NPs can differentially interact with the biological system and alter its function. In that, NPs can either directly interact with biomolecules such as proteins or DNA, or affect the cellular redox state (Stark, 2011). Increased ROS level might act as signaling molecules and can either be the cause or the consequence of cell death. Systems biology approaches represent a valuable tool for high-throughput analysis and can lead to a comprehensive description of particle interaction with the biological system. Of note, this does not diminish the importance for thorough characterization of the particles. Understanding the physicochemical properties of the particle is of central importance but needs to be expanded. Through systems biology it is not only possible to identify novel pathways and interactions as well as biomarkers that are correlated with the exposure to a certain material, but it can also be used to predict a potential adverse effect. Systems biology therefore enables detailed understanding of interactions and its consequences and this can further be used to perform a risk assessment (Fadeel et al., 2018). Moreover, it will be important to consider the immunological consequences of a certain cell death. Investigation of the cell clearance mechanisms as well as of a pro- or an anti-inflammatory response will lead to a broad understanding of the effects of a certain particle on the organism. This is of special importance in medical applications where NPs could be useful tools for various purposes (Pelaz et al., 2017). NPs that are for example used for diagnostics or imaging purposes should be non-toxic and biodegradable while those used in cancer therapy or as a drug carrier should fulfill the designated purpose with high specificity without altering or affecting healthy tissue or causing excessive inflammation. Therefore, evaluation of a potential toxic effect should start by careful characterization of the material, investigate its interaction with the biological system in detail and also consider the outcome – meaning the underlying cell death mode and immunological consequences – for the organism. Only such comprehensive understanding will allow a robust prediction of a potential risk. This knowledge can then be used for reasonable regulations regarding a safe handling of the particles but could also lead to the production of new materials following the safe-by-design paradigm (Cobaleda-Siles et al., 2017; Morose, 2010).

The nematode *C. elegans* has been an extensively studied model organism in cell death research. Many key regulators and mechanisms facilitating cell death are conserved and homologous proteins and pathway similarities are found in the mammalian system. More recently, *C. elegans* has been used in nanotoxicology as a tool to study the effect of NPs on the organismal level (Gonzalez-Moragas et al., 2015). The availability of various mutant strains makes the nematode a valuable model for validation experiments to elucidate the mechanism of action.

In our study of six different Au NPs we investigated the effect of size and surface functionalization on the toxicity of the particles. We found that only the ammonium-functionalized particles were toxic, while carboxylated or PEGylated Au NPs were non-toxic. Moreover, the larger particles were more toxic than the smaller ones. Further investigation of the downstream mechanism – both through multi-omics analysis as well as numerous validation experiments – demonstrates that the cationic Au NPs cause cytotoxicity though induction of mitochondrial dysfunction. These results are critical for the safe design and application of Au NPs in various biomedical settings.

6 CONCLUSIONS

Cell death and cell clearance mechanisms are central elements in the development and homeostasis of multicellular organisms. Dysregulation of these pathways is associated with various pathologies. However, our knowledge about involved signals and the underlying mechanisms is still limited. While cell death research has to a great extent focused on apoptotic cell death, other – more recently discovered – forms of PCD have received less attention. Additionally, much less attention was put on mechanisms of programmed cell which regulates recognition and engulfment of the dying cell and adapts an appropriate immune response. Importantly, the efficiency with which macrophages engulf distinct modes of cell death can vary and therefor directly influence the consequences of the cell death. Consideration of individual cell death modes as well as the final outcome for the organism will have a great impact in cell death related fields such as medicine or toxicology. Detailed knowledge of these aspects can lead to the development of novel treatment strategies or help to better understand the consequences of a certain form of cell death.

Of note, while various (toxicological) studies only report the occurrence or absence of cell death or discriminate between apoptotic (programmed) and necrotic (accidental) cell death, these studies fail to address biochemical characteristics of the observed cell death mode. The expression "programmed cell death" as a synonym for "apoptosis" is overly simplified and outdated since several non-apoptotic forms of PCD were identified. The consideration of alternative cell death pathways in various cell death related areas as well as their proper identification through biochemical and molecular characteristics may result in a better understanding of these pathways and could lead to the development of novel treatment strategies for diseases with dysregulated cell death program.

One extensively studied signal of apoptotic cells is the exposure of phosphatidylserine (PS). While PS externalization was previously suggested to be a marker of apoptotic cells, it becomes now evident that other forms of PCD adopt the same signal. In paper II we found that PS is exposed in apoptotic, necroptotic and ferroptotic cells thus suggesting that it is not a feature that is unique for apoptotic cells. The mechanisms and regulation of PS exposure in non-apoptotic settings remain to be studied. Transmembrane proteins that were suggested to be involved in regulating PS distribution are P4-type ATPases. The *C. elegans* protein TAT-1 belongs to this family and it was shown that disruption of the protein function diminishes asymmetric phospholipid distribution (Darland-Ransom et al., 2008). In paper I we performed mutational analysis in order to elucidate the structure-function relationship of this protein. We thus identified two domains that are critical for the function of the protein *in vivo*. This study helps to identify important residues and helps to decipher the mechanism of phospholipid transport. The

structure and function of ATPases are highly conserved and it is therefore possible to suggest that similar mechanisms occur in the mammalian system.

The nematode *C. elegans* has been a valuable model organism for cell death research. Many of the major cell death regulators and pathways have first been identified in the worm and were later found to have equivalent mechanisms and counterparts in the mammalian system. Even though the mammalian system is more complex, there is still a remarkable homology between the species. The discovery that the basic mechanisms are evolutionarily conserved followed by the identification of the main cell death regulators led to a quick expansion of the cell death field which rapidly developed "from worm to clinic" within few decades. Nonetheless, additional non-apoptotic cell death modes have recently been identified and are relevant in various disease settings or other pathologies. Consideration of these cell death modes is important not only in the medical field but also in toxicology. Individual forms of cell death are not only associated with various diseases but can also be triggered by exposure to particles or chemicals. The exposure to NPs can cause cell death and detailed knowledge on the underlying mechanism, the form of cell death and the potential adverse effects for the organism is critical in order to predict an associated risk. The increased occurrence of NPs in our everyday life as well as the occupational or accidental exposure to these substances demands thorough characterization and investigation of its effects. Moreover, the utilization of nanomaterials in various medical applications requires safe handling and know-how of the mode of action. Emerging systems biology approaches as well as validation experiments both *in vitro* and *in vivo* are ways to achieve this and the nematode model can provide a valuable model also in toxicology. In paper III we performed comprehensive analysis of the effect of size and surface functionalization of Au NPs both *in vitro* and *in vivo*. We applied multi-omics analyses and performed various validation experiments in order to elucidate the downstream mechanism. Au NPs are promising tools for biomedical applications and detailed knowledge on its potential toxicity will help to design novel materials that can be uses in treatment or diagnostics without causing adverse effects.

To summarize, the distinct cell death and cell clearance mechanisms require more attention and proper characterization based on specific biochemical features. Our knowledge regarding the mode of action, involved signals or the final outcome of a certain cell death are still limited. The consideration of individual cell death modes and their consequences in toxicology, medicine or other related fields would be of great benefit. For that, model organisms such as the nematode *C. elegans* are valuable tools to study individual proteins or underlying mechanisms *in vivo*. Moreover, systems biology approaches are tools to elucidate underlying mechanisms in high throughput as well as to put these results into a biological context. Detailed knowledge of potential adverse effects is the basis of reliable risk assessment.

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