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Inflammasome activation in human and mouse macrophages engulfing autophagic dying cells

by

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Abbreviations

AIM2	-absent in melanoma 2
AMP	-adenosine monophosphate
ATP	-adenosine triphosphate
ACAMPs	-apoptotic cell associated molecular patterns
Atg	-autophagy related genes
AVs	-autophagic vacuoles
Beclin 1	-Bcl-2-interacting protein-1
BMDMs	-bone marrow derived macrophages
Ca^{+2}	-calcium
CBX	-carbenoxolone
CFDA-SE	-carboxyfluorescein diacetate N-succinimidyl ester
CARD	-caspase activation and recruitment domain
CBA	-cytokine bead array
CQ	-chloroquine
PtdIns3K	-class III phosphotydylinositol 3-kinase
CM	-conditioned medium
CytD	-cytochalasin D
DAMPs	-damage-associated molecular patterns
ELISA	-enzyme-linked immunosorbent assay
DCC	-estrogen depleted charcoal-stripped-fetal calf serum (FCS)
FCS	-fetal calf serum
ICAM3	-Intercellular adhesion molecule 3
IL-	-Interleukin
KD	-knock down
KO	-knock out
LPS	-lipopolysaccaride
LC3	-microtubule-associated protein light chain 3
MDC	-monodancylcadaverine
NLRP3	-Nod-like receptor 3
NCCD	-Nomenclature Committee on Cell Death
PAMPs	-pathogen-associated molecular patterns
PBMCs	-peripheral blood mononuclear cells
PMA	-phorbol 12-myristate 13-acetate
PS	-phosphatidylserine
KCl	-potassium chloride
ROS	-reactive oxygen species
TLRs	-Toll-like receptors
WT	-wild type
3-MA	-3-methyladenine

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1. Introduction

It has been known that one of the natural functions of immune system is to find and eradicate the aberrant (neoplastic and dysplastic) cells in tissues. This immune surveillance can be impaired due to the unpredictable immune escape strategies of cancer cells and diversity of cancer and immune system interactions. Immune-system and cell death based combination therapies are being used in the treatment of many diseases such as cancers, autoimmune diseases and others. For instance, apoptotic cell death induction by chemotherapy in cancer is being applied to patients even though it has many weak points, such as the fact that apoptotic cells are usually ignored by the immune system since they are immunologically silent and even suppress inflammation.

In 1909, Paul Ehrlich showed that immune system can suppress the growth of carcinomas. After him, other scientists also concluded the similar phenomenon. However, in some cases immune system may promote the growth of cancer cells with reduced immunogenicity. Cancer cells can also escape from immune recognition and destruction due to their immune sculpturing and immune editing capacities. Inducing an immunogenic cell death can promote an efficient clearance of cancerous cells before they become aggressive and lethal. An anticancer immune response to dying cells can be beneficial for the eradication of the diseases.

There was a gap in the literature that how autophagic dying cells are cleared from tissues, particularly from the population of tumor cells, and what is the innate immune response against them. Our experiments were started with answering this question and when we used dying cells with autophagic features it was observed that they have the capacity to start a pro-inflammatory response in macrophages which can engulf them. We have demonstsrated that these autophagic cells could evoke effective innate immune response by inflammasome activation as an early response and clarified the mechanism what is responsible for the activation of the inflammasome by autophagic dying cells.

2. Theoretical Background

2.1 Cell death

In our body, billions of cells (such as aged cells, damaged cells) die daily during homeostasis and immune regulation and are replaced by new ones [1]. Cell death is also important in many diseases such as cancer and auto-immune disorders and cell death type should be defined well due to its potential contribution to treatment of these diseases. Since mid-1960s, cell death subroutines have been mostly classified based on morphological features [2]. Morphological appearance of cells can give an idea about cell death type such as apoptotic, necrotic, autophagic or associated with mitosis [3]. Cell death modalities can be classified also by considering enzymological criteria, functional aspects and immunological characteristics [4]. Particular cell death may be named according to the involvement of particular enzymes such as proteases (caspases, calpains, cathepsins) or transglutaminases [3,4,5]. Metabolic content such as intracellular energy stores, oxygen, adenosine triphosphate (ATP) or glucose levels may play a role in predicting the cell death type [3]. It is accepted as more convenient to determine the cell death type according to involvement of distinct organelles (such as mitochondria or lysosomes) [3]. Lately, it is believed that novel systematic cell death classification is more appropriate to be based on measurable biochemical features [2]. Cell death also can be either immunogenic or non-immunogenic according to their capacity to induce inflammatory pathways [3,4,5].

2.1.1 Apoptosis

Apoptotic cell death is a programmed and controlled breakdown of the cell into apoptotic bodies in development (organ and tissue remodeling), homeostasis (for instance; in post-ovulation and post-lactational mammary gland regression in elimination of already activated immune cells to stop the immune response) [6]. Apoptotic cells have morphological features such as are rounding-up, appearance of pseudopodes, reducing of cellular and nuclear volume

(pyknosis), nuclear fragmentation (karyorrhexis), cytoplasmic organelle modification, plasma membrane blebbing [3,4,5,6]. There are two main evolutionarily conserved protein families which play crucial roles in apoptosis; caspases and Bcl-2 family members [6]. Caspases are cysteine proteases with a central role in apoptosis [7]. Substrates for caspases can be downstream caspases, apoptosis inhibitors or proteins which can cause cell disassembly by cleavage of nuclear lamina, cytoskeleton regulators, adhesion kinases and gelosin [7]. Bcl-2 family members share Bcl-2 homology (BH) domains and can be classified into two groups as pro-apoptotic proteins (apoptogens) and anti-apoptotic proteins [7].

Apoptosis is composed of different biochemical routes such as extrinsic [5] and intrinsic pathways which are triggered by distinct inducers [4]. Apoptotic cell death can occur via extrinsic activation of the death receptors such as TNFR family such as tumor necrosis factor receptor (TNFR), Fas (CD95), TNF related apoptosis inducing ligand (TRAIL) receptor) [6]. FasL (CD95 L), tumour necrosis factor-α (TNF-α), TRAIL-R, also known as APO2 L) or TNF ligand superfamily member 10 (TNFSF10) are the ligands for these death receptors [7]. For instance, FasL and Fas receptor interaction can initiate the recruitment of adaptor proteins such as Fas-associated via death domain (FADD) and further recruitment of initiator caspases such as caspase8/10 which forms death-inducing signaling complex (DISC) [7]. Upon autoproteolytic activity within the complex, caspases can either activate the downstream, major effector caspase 3 leading to apoptosis or caspase 8 mediating the cleavage of the pro-apoptotic Bcl-2 family member Bid which subsequently releases mitochondrial pro-apoptotic factors and links the extrinsic to the intrinsic pathway of apoptosis [7].

Stimuli such as loss of survival/trophic factors, toxins, radiation, hypoxia, oxidative stress, ischaemia-reperfusion and DNA damage can initiate the intrinsic apoptotic pathway which involves central death machinery located at the mitochondria [6,7]. Upon

apoptotic stimuli, mitochondrial outer membrane permeabilization (MOMP) is promoted via Bax/Bak complex and cytochrome-c is released from the mitochondrial inter-membrane space [7]. Effector proteins, Bax, Bak, Bim and Bid interfere with anti-apoptotic family members such as Bcl-2, B-cell lymphoma extra long (Bcl-xL), Mcl-1 which has potential to prevent MOMP [7]. Puma, Noxa and Bad proteins also can sensitize cells for cell death by being antagonist for the anti-apoptotic proteins [7]. Released cytochrome-c can bind to Apaf-1 and a caspase 3 activating apoptosome complex can be formed by recruiting pro-caspase-9 and deoxyribonucleotide adenosine triphosphate (dATP) [7]. Other mitochondrial apoptogens such as Smac/DIABLO and HtrA2/Omi cause inhibition of X-linked inhibitor of apoptosis (XIAP) and lead to apoptotic cell death.

Lately, extrinsic and intrinsic apoptotic cell death types are also classified into subroutines such as extrinsic apoptosis by death receptors or dependence receptors and caspase dependent or independent intrinsic apoptosis [2].

2.1.2 Autophagic cell death

2.1.2.1 Autophagy

Autophagy is a tightly regulated and conserved pathway in all eukaryotes as a stress-induced catabolic process [8]. Autophagy can be classified as chaperon mediated autophagy (CMA), microautophagy and macroautophagy [8]. In CMA, binding of proteins to receptors on lysosomes further leads to translocation of the unfolded protein into lysosomes directly [8]. In microautophagy, cytoplasmic materials translocates into lysosomes by invagination or septation of the lysosomal membrane [8]. In macroautophagy, double-membraned vesicles sequester targets such as organelles, proteins or portions of the cytoplasm and deliver them to the lysosome to be digested [9]. Eukaryotic cells should adapt to external stress conditions created by inducers such as pH, ion concentrations, ultraviolet light, oxygen tension, microbial pathogens,

temperature, hormones and cytokines [10]. These stressors lead to rapid metabolic changes which induce diverse stress response pathways [10]. Macroautophagy (will be called as autophagy throughout the thesis) is one of the stress-induced adaptation and damage control mechanisms [10]. Autophagy can be induced by nutrient (such as glucose, aminoacids) and growth factor (such as those serum contains) deprivation, ER stress, pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), immune signals, hypoxia, redox stress and p53, mitochondrial damage [10]. Numerous upstream signaling pathways regulate macroautophagy such as growth factor signaling, PI3 kinase/Akt, MAP kinase, adenosine monophosphate (AMP) dependent protein kinase, small GTPases, trimeric G proteins, inositol triphosphates and calcium (Ca^{+2}) signaling [11].

In early 1990s, autophagy related genes (Atg) in yeast were characterized and in recent years many mammalian homologues of these genes have been identified [12]. Under nutrient-rich conditions, active mammalian target of rapamycin (mTOR) Ser/Thr kinase (mTORC1) associates with ULK (unc-51-like kinase) complex which is composed of ULK1 (or ULK2), Atg13, FAK-family interacting protein of 200 kDa (FIP200) and Atg101 [8,10,11]. Active mTORC1 phosphorylates ULK1 (or ULK2) and hyperphosphorylates Atg13 which inhibits the kinase activity of ULK1 (or ULK2) and blocks autophagy [8,10,11]. Under stress conditions, autophagy is initiated by activation of ULK complex via inactivation and dissociation of mTORC1 from the complex [8,10,11]. Stress conditions lead to changes in phosphorylation dynamics in ULK complex which further start to autophagy induction [8,10,11]**.** Autophagy continues with the formation of double membraned vesicles (autophagosomes) and sequestration of organelles, proteins or portions of cytoplasm [10]. Vesicle nucleation pathway starts with the recruitment of Atg proteins to the phagophore assembly site (PAS in yeast)-like region where Bcl-2-interacting protein-1 (Beclin1)/class III phosphotydylinositol 3-kinase complex (PtdIns3K)/Vps34 activation is essential [8]. This complex contains other proteins such as UVRAG and Ambra1 which regulate the kinase activity to control

autophagy induction [11]. Vesicle expansion and completion occur via microtubule-associated protein light chain 3 (LC3) and Atg12 ubiquitin-like (Ubl) conjugation systems. Atg7 acts as E1 enzyme and results in the conjugation of phosphotydilethanolamine (PE) and Atg5 respectively to LC3 and Atg12 [11]. Upon conjugation reactions, nascent LC3 becomes mature and resides on vesicle membrane as LC3-phosphatidylethanolamine (PE) (LC3-II) which is further cleaved from PE on the autophagosome membrane after vesicle completion [8]. Meanwhile, Atg5 dissociates from LC3-II which resides on autophagosome membrane [11]. Autophagosome-lysosome fusion requires Rab7 GTPases and lysosome-associated membrane protein (LAMP2) [11]. Sequestered content of autophagosomes are degraded with hydrolitic enzymes due to the autophagosome fusion with lysosomes (autophagolysosomes) [10]. Finally, resulting products are released from lysosomes to cytosol via permeases and used for cell survival [8]. The steps of autophagic machinery can be seen in Figure 1.

Autophagy can be blocked by inhibiting the phosphoinositide 3-kinase (PI3K) (with 3-MA, LY294002, wortmannin), autophagosome-lysosome fusion (with bafilomycin A1, NH_4Cl, chloroquine, protease inhibitors) or microtubule depolimerization thus autophagolysosome formation (with vinblastin, nocodazole) [13,14] (Figure 1).

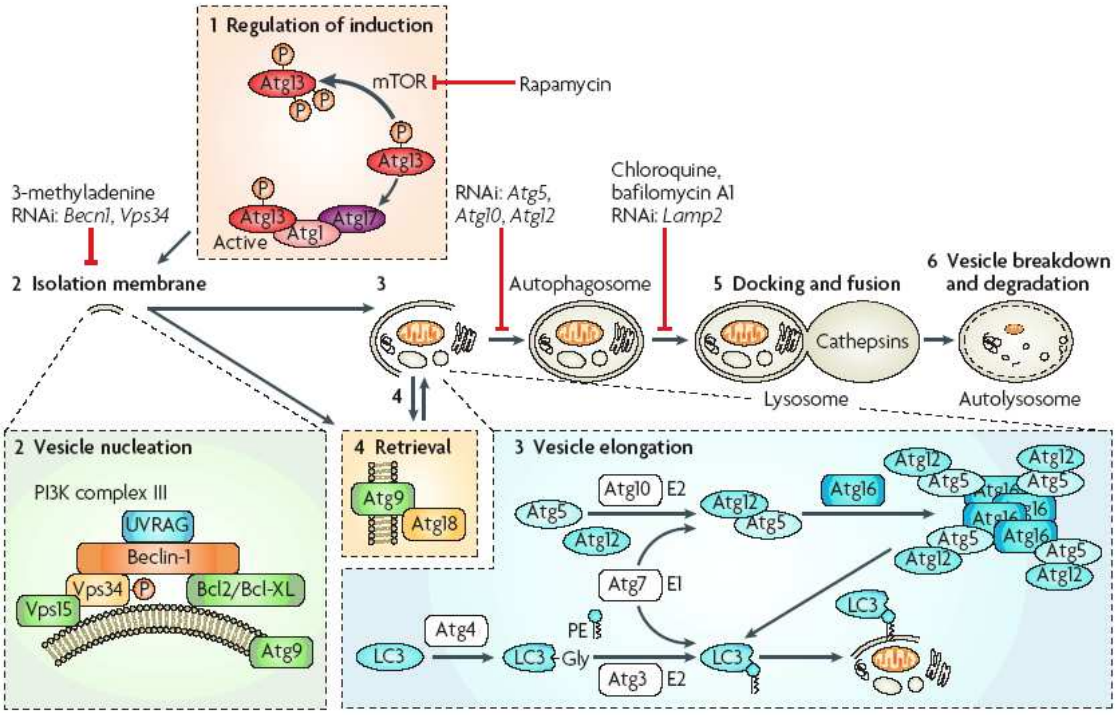


Figure 1: Autophagic machinery and its inhibitors. Upon induction, autophagy starts with the nucleation of the isolation membrane and continues with the engulfment of cytoplasmic material in which autophagy related proteins (Atg proteins) have roles. These proteins can be recycled during or after autophagy. Next step is the fusion of autophagosomes with lysosomes to create autophagolysosomes. The luminal content in autophagic vacuoles (AVs) is degraded by lysosomal enzymes within acidic compartment. Pharmacological inhibitors and small interfering RNAs that can capable of inhibiting the particular steps of autophagy are shown in the figure with red blocking arrows.

Figure adapted from [15]

In order to observe how autophagy is affected by an inducer, LC3-II level comparison is widely used since it is closely related with the number of autophagosomes. Since LC3-II itself is also degraded by autophagy, LC3 turnover should be monitored by measuring the autophagic flux [16]. Autophagic flux is the dynamic process of autophagosome synthesis, autophagosome delivery to lysosomes and degradation of substrates in the lysosomes. Comparing LC3-II levels in the absence or presense of lysosomal inhibitors,

which inhibit the acidification in lysosomes therefore prevent the autophagolysosome fusion, it can be observed how autophagy is changed upon stimulation and it can be clarified whether at its increased level there is blockage in the degradation process (when no change occurs after the inhibitor) or a flux of autophagy when LC3-II is further elevated [16]**.**

Cells primarily use the basal level of autophagy to eliminate the harmful components through catabolic pathway and recycle them to maintain nutrient and energy for survival [10,17]. Autophagy leads to the generation of ATP under stressed conditions, degradation of the unfolded proteins, removal of degraded organelles and genomic stability [17]. Autophagy is also involved in development, senescence, lifespan extension, immunity, defense and human pathologies such as cancer, heart and liver diseases and gastrointestinal disorders [8]. On the other hand, autophagy plays a role in innate immunity by eliminating the intracellular microbes, delivering the cytosolic microbial products to pattern recognition receptors (PRRs) and in adaptive immunity as well by presenting the cytosolic content by MHC Class II molecules [18] [19]. In tumors, autophagy is bearing cytoprotective roles under nutrient and oxygen limited conditions as well as it protects tumor cells from drug-induced apoptosis. When autophagy is inhibited it has been shown that selective form of autophagy called chaperon-associated autophagy (CMA) increases and protects cells against reactive oxygen species (ROS) and ultraviolet (UV) but not Fas/TNF-α induced apoptosis. Apoptosis-deficient tumor cells become necrosis-sensitive when autophagy is inhibited [20].

2.1.2.2 Cell death related to autophagy

Stress conditions trigger numerous pathways in cells such as regulation of nutrient uptake, metabolism, cell cycle and growth control, survival as well as cellular death programs [10]. Excessive bulk self-destruction and selectively targeting key cell survival elements by autophagy can result in autophagic cell death [8,17]. Autophagic cell death is hard to be defined due to the mixed phenotypes of dying cells in a given cell population

[20]. Individual cells can be in different stages during dying process and apoptotic cell population may have a biochemical and functional heterogeneity [4,5]. Therefore, it is important to clarify how autophagy contributes to cell death [20]. Autophagy can contribute to the upstream of apoptosis, it can happen parallel to apoptosis or can assist in eliminating the apoptotic corpse in the final stage of apoptosis [20]. CD4+ T cells infected with human immunodeficiency virus (HIV) and human cancer cells in which nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway is inhibited can die with apoptosis by TNF-α in which autophagy occurs as the upstream process of the cell death [20,21,22]. On the other hand, p53 induced cell death requires both autophagy and other pro-death signals such as Bax, BH3-only proteins, mitochondrial depolarization [20,23]. In this process, lysosomal trans-membrane protein damaged-regulated autophagy modulator (DRAM) can regulate autophagy and autophagy and apoptosis can converge [20,23]. Autophagy can have role in the translocation of 'eat me signal' phosphatidylserine (PS) to the outher leaflet of dying embryonic bodies [20,24]. It has been also shown that ATP levels for PS exposure in dying cells are maintained by autophagy [20,24]. Besides, autophagy can contribute to secretion of 'come and get me' signal lysophosphatidic acid from dying cells which initiating apoptotic cell clearance in embryonic development and morphogenesis [20,24].

Some signaling pathways and central components of apoptosis and autophagy can regulate both pathways which show that there is a crosstalk between these two processes. It has been shown that a known apoptosis inhibitor PI3 kinase/Akt can simultaneously inhibit autophagy [11,25]. On the other hand, a key autophagy protein Beclin 1 has been shown to interact with key apoptosis regulator protein Bcl-2 family members such as anti-apoptotic Bcl-xL and both autophagy and apoptosis are inhibited due to this interaction [11,26]. It has been also shown that Bcl-2 protein can block Ca^{+2} release from ER and released Ca^{+2} may activate Ca^{+2}/calmodulin-dependent protein kinase-β and AMP-activated protein kinase, which further leads to the inhibition of mTOR to activate autophagy [11,27]. Extrinsic apoptotic receptor pathway can also control autophagy; for

instance, FADD and calpains can interact with Atg5 where Atg5 can regulate extrinsic apoptotic pathway [11,28,29]. Apoptotic cell death is also induced in Ba/F3 cells by the cleavage of the autophagy protein, beclin-1, which causes release of cytochrome-c from mitochondria during IL-3 depletion [30]. It has been shown [30] that at the onset of apoptosis, about 6 h after IL-3 withdrawal, it is not the high autophagy rate that kills the cells. Apoptotic cell death and contribution of autophagy in Ba/F3 cells will be discussed more clearly in discussion part since these cells were used in studies of the thesis.

The term of 'autophagic cell death' should be used under caution and biochemical and functional features should be taken into considerations before calling a type of cell death mediated by autophagy [2]. Autophagy inhibition by chemicals (e.g. targeting particular proteins) and/or genetic manuplations (knocking out/down related genes) can serve as a control mechanism whether or not cell death is dependent on autophagy [2]. Autophagy can trigger cell death independently of apoptosis in case of excessive starvation induced cell death in the involution of *Drosophila melanogaster* salivary glands which is the unique *in vivo* study showing cell death is induced by autophagy [20,31]. Degraded ROS by catalase enzyme may also induce autophagic cell death [32]. There are other studies which show that when the apoptotic machinery is defected or caspases are inhibited, autophagy is required for cell death mechanism [33,34]. For instance, autophagic cell death can be induced in MCF-7 cells, which are caspase-3 haplo-insufficient, through autophagy with estrogen depleted charcoal-stripped-fetal calf serum (FCS) (DCC) and concentration dependent anti-estrogen tamoxifen treatment [35,36]. Autophagy and cell death in MCF-7 cells will be discussed more in details since these cells were used in studies mentioned in the thesis.

2.1.3 Anoikis

Cells start to die not only in the absence of hormones or growth factors but they also start to kill themselves as a result of detachment from extracellular matrix (ECM) proteins [37,38,39]. The fate of these cells is to die via anoikis which is generally an apoptotic

process [40]. Anoikis is a physiologically relevant cell death process since correct adhesion of cells is essential to prevent the re-attachment of cells into an improper location and their dysplastic growth [41]. Anoikis generally triggers, not always, the intrinsic apoptotic pathway [2]. When the tumor cells which are apoptosis defective are under metabolic stress, autophagy can lead to survival first and then cells can die eventually with excess amount of autophagy [42]. Detachment of cells from surface (anoikis) has recently been associated with autophagy in caspase-3 haplo-insufficient MCF-7 cells since cell death could be prevented by autophagy inhibition the conclusion was drawn that autophagy was directly related to this cell death type [35,36].

2.1.4 Necrosis

Necrosis has been considered as an uncontrolled form of cell death without showing the features of apoptosis and autophagy [4,6]. It can accidentally happen as a result of severe physical damage (such as hyperthermia or detergent-induces cytolysis) [6]. Recently, it was observed that certain conditions can start programmed necrosis with a strictly regulated signaling events which further cause plasma membrane rupture [6]. TNF activation of the death receptor, TNFR1, may lead to necrosis in which serine/threonine kinases receptor interacting protein 1 and 2 (RIP1-RIP3) pro-necrotic complex formation and kinase activity of these enzymes are the crucial initiator for receptor mediated necrosis, recently called necroptosis [6]. PRRs activation and excessive DNA damage can also induce necroptosis via RIP1 kinase activity [6]. Upon RIP1 kinase activity, induced activation of necrotic mediators (such as ROS, ceramide, cathepsins, calpains, nitric oxide (NO), Ca^{+2} and phospholipases) result in programmed necrosis [6]. Necroptotis, accidentally necrotic and secondary necrotic cells share similar morphological features whereas they are different modes of cell deaths [43]. Cytoplasmic swelling (oncosis), rupture of plasma membrane, cytoplasmic organelle swelling and moderate chromatin condensation are the typical morphological features of these type of dying cells [4].

Necrosis is generally observed with apoptosis as a back-up cell death mechanism, for instance when caspases are inactive for some reasons [6]. It can substitute for apoptosis during embryogenesis to eliminate the unwanted cells, it is also involved in ovulation, in cellular turnover in the small and large intestine, in reducing the T cell number after an immune response and in pathological conditions such as organ injuries, neurodegenerative disorders, stroke, myocardial infarction [6].

2.2 Interactions between dying cells with innate immune cells

Different types of dying cells including apoptotic ones are removed from tissues to prevent immune reactions and maintain tissue homeostasis [35,44,45,46]. These dying cells should be cleared efficiently by either non-professional (neighbouring cells such as epithelial or fibroblastic cells) or professional phagocytes (such as macrophages or immature dendritic cells) [1]. Under normal conditions, basal clearance capacity is high in tissues and it can be defective due to the imbalance between numbers of dying cells, number of phagocytes and low efficiency of phagocytes uptake mechanism [1]. Inability to recognize and remove dead cells could lead to diseases such as autoimmune disorders, cystic fibrosis and asthma [47]. Additionally, phagocytosis contributes to cell growth and wound healing by releasing factors to orchestrate the regeneration and angiogenesis at the site of injury [48].

Complex molecular patterns and dynamic interactions between dying cells and engulfing cells are often called as ‘third synapse” [49]. During the different stages of cell death there are molecules exposed on dying cells (such as scavenger receptors, PS receptors, thrombospondin receptor, integrins, complement receptors) which bind to appropriate receptors on phagocytes (such as intercellular adhesion molecule 3 (ICAM3), Annexin 1, CD47, apoptotic cell associated molecular patterns (ACAMPs), PS, calreticulin, MER). There are also bridging molecules (such as thrombospondin 1 (TSP-1), C1q, collectins, milk fat globule-EGF-factor 8 (MFG-E8)) which interplay between phagocytes and dying cells [49,50].

In case of apoptotic cell clearance, engulfment process is composed of four major steps: (1) 'find-me' signal release from apoptotic cells to attract phagocytes to the site in tissue, (2) 'eat-me' signal exposure on apoptotic cells to promote the recognition of the dying cell and phagocyte, (3) ingestion of the engulfed cell corpse which goes through phagosome formation and degredation process, (4) anti-inflammatory cytokine release from phagocytes which has already recognized and engulfed the apoptotic cells [1]. Apoptotic cells phagocytosed by either professional or non-professional phagocytes before they loose their membrane integrity are thought to be immunologically silent and even suppress inflammation [6,49]. The anti-inflammatory features of apoptotic cells result from exposure of cell surface ACAMPs; for instance, phosphatidylserine was recognized as the first such molecule [51,52].

It would be an oversimplification to state that apoptotic cells are non-inflammatory, non-immunogenic, tolerogenic and even immunesuppressive [3]. During the last couple of years it has become clear that under certain conditions apoptotic cells can also be immunogenic due to the exposure/release of DAMPs [53,54,55,56,57]. For instance, gemcitabine [53] and local γ irradiation [57] induced apoptosis in cells which can elicit antitumor immune response by priming CD8 T cells. Doxorubicin treatment of cancer cells can initiate immunogenic cell death *in vivo*, *ex vivo* and *in vitro* [56]. In contrast to other cytotoxic agents including etoposide and mitomycin C, it was shown that anthracyclin type antibiotics (DNA damaging agents) (e.g. doxorubicin) can cause immunogenic cell death in tumor cells [3,54]. Anthracyclins can be internalized into cells via either passive [58] or selective transport [59]. Doxorubicin-proteasome complex translocates to nucleus and after dissociation of doxorubicin from proteasome, it binds to DNA due to its high affinity and cell death occurs [59]. After treatment cells with anthracyclins, protease activity can be inhibited due to binding of anthracyclins to proteasomes and this proteasome inhibition can activate autophagy for instance in human prostate cancers as a survival mechanism [60] and it may also lead to activation of caspase 8 in an autophagy dependent manner [61]. High doses of doxorubicin can induce

both autophagy and PARP-1 activity and it was concluded that autophagy plays a cytoprotective role against DNA damage via PARP-1 activation [62]. It was shown that DNA damage induced by anti-cancer reagents can induce p53-dependent genes which can also lead to autophagy induction [63]. It is not clear yet how necrotic cells are cleared by macrophages and it was suggested that some macrophage receptors involved in uptake mechanism of apoptotic cells can also involve in necrotic cell clearance [64]. Recent studies have indicated that the internalization of necrotic cells is initiated via macrophage-necrotic cell interaction and this internalization can be preceded by macropinocytic mechanisms [64]. Necrotic cells are believed to be pro-inflammatory after they are recognized and internalized (macropinocytosis) and they release DAMPs [6]. Due to the breakdown of the plasma membrane in necrotic cells, the cytoplasmic contents, including lysosomal enzymes, are released into the extracellular fluid and therefore necrotic cell death initiates an extensive inflammatory response [6]. On the other hand, Galluzzi et al suggest that necrotic cell death is not always pro-inflammatory. It has been shown that when cell lysates or necrotic cells die by freeze-thawing or hypotonic shock and then injected into mice subcutenously, they do not evoke an immunogenic response [3].

2.2.1 Macrophages

Circulating peripheral-blood monocytes (PBMCs) develop from myeloid progenitor cells in bone marrow and migrate into tissues in the steady state or in response to inflammation [65]. They replenish the long-lived tissue macrophages of bone (osteoclasts), alveoli, central nervous system (microglial cells), connective tissue (histiocytes), gastrointestinal track, liver (Kupffer cells), spleen and peritoneum [65]. About 100 years ago, Ilya Mechnikov was awarded with a 1908 Nobel Prize for his discovery of the macrophage functions which gave him the possibility to explain how phagocytes influence development, ensure homeostatis and protect the host from infection through the process ‘innate immunity’ [66]. Macrophages also contribute to the removal of cellular debris generated during tissue remodeling and diseases [65]. Upon clearance the cellular debris,

macrophages release immune mediators depends on how they are stimulated. Phagocytosis receptors stimulate macrophages and induce either induction of pro-inflammatory cytokine gene transcription or production of anti-inflammatory signals [65].

2.3 Initiation of inflammation through innate pattern recognition receptors (PRRs)

Under normal conditions, the immune system can distinguish foreign materials, pathogens (non-self), healthy viable cells (self) and dying cells (altered-self) in order not to stimulate an immunogenic response to self and prevent the possible damage to neighbouring tissues [48]. Innate immune system has roles to detect microbes, coordinate symbiotic responses, mount immune defences against pathogens and signals from dying cells [67]. A "danger theory" proposed by Matzinger states that the immune system can discriminate not only self from non-self but also DAMPs from innocuous ones [68]. DAMPs can be secreted, released and/or exposed on the outer leaflet of the plasma membrane and can provide several kinds of signals: 'find-me' (chemotactic), 'eat-me' (phagocytic), and 'activation' (immune stimulatory) factors [69]. For instance; DAMPs are recognized by membrane-bound or cytoplasmic PRRs, which include Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs) and purinergic receptors [70,71,72].

2.3.1 Toll-like receptors (TLRs)

The discovery of TLRs located on cell membrane and subsequent identifications of cytoplasmic receptors provided the insights into how innate immune cells distinguish self from non-self or altered-self [73]. TLRs are type I membrane glycoproteins which contains an extracellular leucine-rich repeat (LRR) domain, a transmembrane domain and a cytoplasmic Toll/IL (interleukin)-1 receptor (TIR) domain [74]. There are 9 TLRs which is conserved both in humans and mice [74]. TLR1, 2, 4, 5 and 6 are expressed on the cell surface and recognize PAMPs from bacteria, fungi or protozoa [74]. TLR3, 7, 8 and 9 are expressed within endocytic compartments in cytosol and recognize nucleic

acids from bacteria and viruses [74]. TLRs are expressed on immune cells (such as macrophages, dendritic cells, B cells) and on non-immune cells (such as epithelial cells which lie at potential sites of entry, endothelial cells, smooth muscle cells) [75]. TLR signaling is tightly controlled by various negative regulators such as extracellular decoy receptors, intracellular inhibitors [75].

2.3.2 NOD-like receptors (NLRs) and NALP-3 inflammasomes

NOD-like receptors are located in the cytoplasm of cells which have 23 members in humans and almost 34 members in the mice [74]. NLRs comprise three domains: the C-terminal domain contains LRRs which is a sensing module, the N-terminal (apoptosis-associated speck-like protein containing) caspase activation and recruitment (CARD) or pyrin (PYD) domain and an intermediate one consisting of nucleotide-binding and oligomerization (NACHT) domain which mediates NLR oligomerization [74]. NLRs are grouped as NLRA, NLRB, NLRC, NLRP and NLRX (a type of NCLRC) regarding their N terminal effector moldule [76]. NLRA is the only NLR with acidic transactivation domain and other NLRs have protein-protein intraction module on their N terminus [76]. NRLB has BIR, NLRC has CARD and NLRP has PYD domains which sense the ligands [76].

NLRs such as NLRP1 (responds to anthrax lethal toxin [77]), IPAF also known as IPAF (responds to bacterial flagellin [77]), NLRP3 (responds to endogenous danger signals and PAMPs [77], NLRP6 (responds to gut microbial ecology) [78]; RLRs (RIG-I-like receptors which respond to antiviral components) and the cytosolic hemopoietic IFN-inducible nuclear protein 200aa (HIN200) family member absent in melanoma 2 (AIM2) (responds to dsDNA [79]) are capable of forming complexes called inflammasomes [80]. These NLRs trigger IL-1β and IL-18 secretion through inflammasome formation, in addition to the possibility of inducing caspase-1 dependent cell death (pyroptosis), transcription of certain cytokines and chemokinses via MAPK and NF-κB pathways, autophagy and type-1 INF signaling [76]. For instance, NALP-3 inflammasome

complexes can be formed in the cytosol of granulocytes, monocytes, macrophages, dendritic cells, T and B cells, epithelial cells and osteoblasts [81]. NALP-3 inflammasome is composed of multiprotein-complexes which promote the proteolytic activation of inactive form of caspase-1 (pro-caspase-1) which further cleaves the zymogen form of IL-1 family cytokines [74]. Released IL-1β triggers local effects (leukocyte infiltration and lymphocyte activation) and distant effects (fever, acute phase protein induction) by binding to IL-1 receptors (IL1Rs) and IL1R AcP (accessory protein) on immune cells [76]. Upon IL1R activation, MAPK and NF-κB pathways can be induced and further second wave of inflammatory cytokine and chemokine secretion may activate adaptive immune response [76].

2.4 Importance of IL-1β in innate immunity

IL-1β is a multifunctional and pivotal inflammatory cytokine which is known as an endogenous pyrogen due to its capacity to induce fever in animal models [73]. Excess amount of IL-1β lead to local and systemic inflammation [73]. It is the key mediator as an endogenous pyrogen for the body response against infection which evoke fever, hypertension and mediate the production of other pro-inflammatory cytokines and adhesion molecules [67,75]. It is synthesized primarily by macrophages and monocytes [82]. It has a capacity to affect almost all cell types either alone or along with other cytokines [83]. IL-1β is known as lymphocyte-activating factor since it can stimulate T-cell proliferation [73]. For instance, Zitvogel et al have summarized that IL-1β and IL-23 can induce the secretion of IL-17 from γδT cells and stimulates the polarization of CD8+ αβT cells to secrete INF-γ [84]. It can mediate repair responses such as angiogenesis and neutrophil influx to remove the cellular debris [73,85]. The investigation of IL-1β has increased the understanding of the pathologies such as metabolic diseases, autoinflammatory disorders, NALP-3-associated diseases [83]. Its direct roles in fungal, bacterial and viral infections have also been demonstrated [73].

2.4.1 Interplay between TLRs and NALP-3 inflammasome in IL1-β production and maturation

IL-1β production plays a pivotal role in inflammation and during recruitment of neutrophils into tissues [86]. A transcriptional and post-transcriptional regulations [82] have been proposed to explain the tightly controlled production of IL-1β. First, it is transcribed and accumulates in response to signaling through the TLRs which usually activate the transcription factor known as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [87]. Upon induction of IL-1β gene, 35 kDA inactive pro-IL-1β cytokine is expressed [82]. Ultrapure lipopolysaccaride (LPS) can activate TLR4 pathway via myeloid differentiation primary response gene (88) (Myd88)-dependent pathway which further leads to activation of NF-κB pathway for the induction of inflammatory cytokines [74,88]. A secondary stimulus (such as microorganisms, endogenous danger signals, environmental irritants) induces the activation of NALP-3 inflammasomes [89]. There are three main steps for NALP-3 activation: (1) detection of the stress by a sensor; (2) subsequent oligomerization of the sensor and recruitment of effector proteins; (3) activation of effector proteins and cellular responses [85,90]. Under normal conditions, pyrin-domain containing 3 (NALP-3 also known as cryopyrin, NLRP3, CIAS1, PYPAF1 and CLR1.1 [91]) is auto-repressed due to the interaction of NACHT and LRR domains [85,90]. Upon stress detection, interaction of PYD and CARD domains of ASC complex mediate the assembly of the NALP-3 inflammasome complex [85,90]. The CARD domain of ASC recruits pro-caspase-1 which is cleaved into mature caspase-1 and further cleaves pro-IL-18 and pro-IL-1β cytokines [85,90]. Mature cytokines such as IL-18 and IL-1β are released by an unconventional secretion pathway from the cells [85,92]. NALP-3 inflammasomes have immune-modulatory functions and their activity is related to many diseases generally called cryopyrin-associated periodic syndrome (CAPS) in which IL-1β production is dysregulated due to NALP-3 mutations [89,93,94].

2.5 Upstream mechanisms of NALP-3 inflammasome activation

Most of the studies have been done to characterize downstream inflammasome signaling but how inflammasomes sense the particular inducer and initiate the secretion of IL-1β from macrophages has not been clarified in detail [95]. Direct interaction between NALP-3 and its activators has been shown only in a limited number of cases. Bacterial muramyl dipeptide (MDP) and bacterial cell wall component peptidoglycans interact directly with the LRR part of NALP1 and NALP-3, respectively [88]. It is believed that pore forming toxins such as maitotoxin and nigericin directly decrease cellular K^+ by perforating plasma membrane and may mediate the exchange of other cations (H^+, Na^+ and Ca^{+2}) which can induce inflammasome activation [76]. However, NALP-3 inflammasome activation pathways have not been defined for most PAMPs and DAMPs and it seems improbable that the different activators are specifically sensed by the inflammasome. Some mechanisms have been shown to trigger NALP-3 inflammasome activation [91]. It is widely believed that NALP-3 activation can require the generation/activation of a secondary messengers including potassium (K^+) efflux from cytosol due to the opening of non-selective cation channel of the purinergic P_2X_7 receptors via ATP, the generation of ROS, contribution of pannexin-1 channels and lysosomal destabilization [81] (Figure 2).

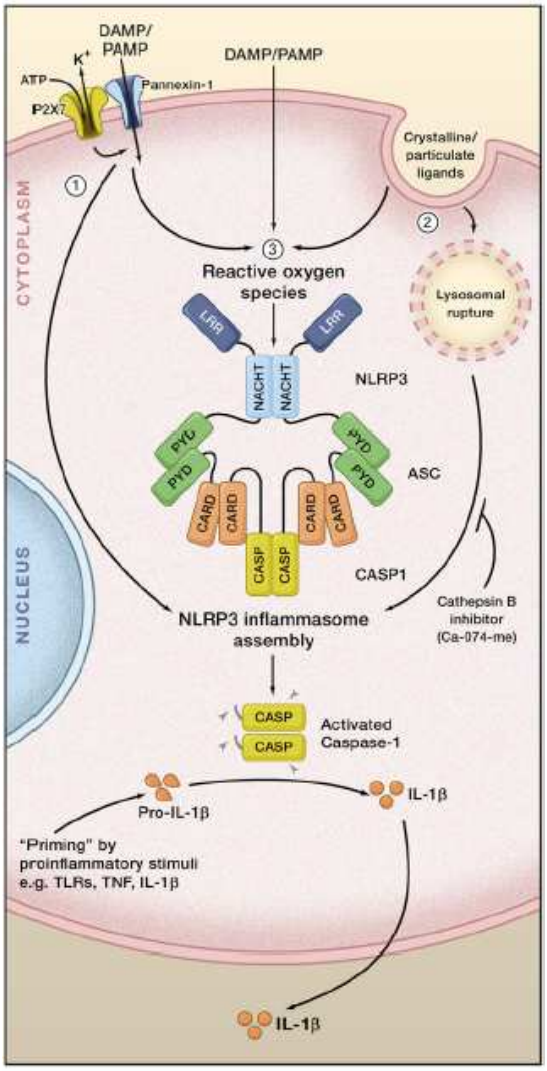


Figure 2: NALP-3 inflammasome activation. NALP-3 inflammasome can be induced via three proposed ways: (1) ATP (NLRP3 agonist) activates P_2X_7 purinergic receptors and triggers pannexin-1 hemichannel based pores which allow the entry of extracellular Nod-like receptor protein 3 (NLRP3) agonists to enter to the cytosol and further to engage with NALP-3 inflammasome directly. (2) Crystalline structured ligands can be engulfed and they can lead to lysosomal rupture due to their physical features such as their size. Upon lysosomal leakage of enzymes; for instance cathepsin B, NALP-3 inflammasome can be activated. (3) It is believed that danger associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs), crystalline structured ligands, ATP trigger reactive oxygen species (ROS) production and it leads to NALP-3 inflammasome activation.

Figure adapted from [95]

2.5.1 P_2X_7 receptor activation by extracellular ATP as one of the DAMPs (danger associated molecular patterns)

ATP is one of the most known danger signal among DAMPs which leads to NALP-3 inflammasome activation [96]. Studies have been done by using NALP-3 deficient mice clearly show that extracellular ATP can act as a danger signal to activate NALP-3 inflammasome via pro-caspase-1 cleavage and further IL-1β maturation [97,98,99].

P2 receptors have phagocytic and chemotactic properties and the $P2X_{1-7}$ receptors are expressed on macrophages and their function in immune responses remain unclarified [64]. They are ligand gated cation channels gated by high concentrations of extracellular ATP which are present at sites of inflammation and injury [100]. Purinergic P_2X_7 receptors are expressed on macrophages and other immune cells [100]. Homeostatic K^+ concentration is ~140-150 mM and it has been shown that exogenous ATP released from cells during inflammation acts on purinergic receptor P_2X_7 on macrophages which can lead to further K^+ efflux from cytosol [90,101,102,103]. Low intracellular K^+ concentration in cytosol (less than 70mM) has been shown as a common trigger of NALP-3 inflammasome activation with ASC domain interaction with pro-caspase-1 and cleavage of pro-IL1-β [90,101,102,103]. On the other hand, it is believed that frustrated phagocytosis of MSU (monosodium urate monohydrate) crystals might lower the K^+ levels in cytosol not via K^+ efflux but via passive water influx through aquaporins and the intracellular osmolarity is compensated by Na^+ release [104].

2.5.2 Contribution of pannexin-1 channels to inflammasome activation

Pannexin-1 channels show homology to gap junction-forming invertebrate innexins [105]. Recently, it has been shown that pannexin-1 channels in brain may conduct small molecules up to ~1kDa such as ions, ATP, inositol triphosphate and amino acids [105]. Pannexin-1 channels can mediate ATP release from astrocytes and arachiodonic acid and its metabolites from red blood cells [105]. It has been also shown that LPS-treated peritoneal macrophages could release IL-1β through the action of ATP in a pannexin-1

channel dependent way [106]. Pore formation has been shown to occur in NALP-3 activation upon stimuli such as bacterial pore-forming toxins as well as ATP via pannexin-1 channels [91]. Opening of pannexin-1 channels via ATP has been implicated in activation of the inflammasome pathway resulting in uptake of bacterial components into cytosol where they are recognized by NALP-3 inflamamsome [107].

2.5.3 ROS production leads to NALP-3 inflammasome activation

Reactive oxygen species such as singlet oxygen, hydroxyl radicals, superoxide, and hydrogen peroxides are highly reactive molecules containing unpaired electrons [32]. They are continuously produced as a byproduct of the mitochondrial respiratory chain in healthy cells at a tolerable level [32]. ROS can damage cell structures due to its capability to oxidize lipids, proteins, and DNA [32]. Redox system contains antioxidants such as superoxide dismutase-1 (SOD1), thioredoxin (TRX) which balance the ROS production and its degradation for cell survival [32]. Due to imbalance, ROS accumulation can initiate cell damage [32]. It was observed that NALP-3 stimuli can not process IL-1β when ROS activity is inhibited chemically [76]. NALP-3 activators such as ATP, asbestos, silica have been shown to trigger excess amount of ROS production which further can lead to K+ efflux [85]. It has not yet been clarified the interplay between ROS production and K^+ efflux but it may be possible K^+ efflux may induce ROS production or vice versa [85]. It has also been shown that ATP treatment of primed macrophages lead to ROS production which stimulates PI3K pathway, subsequent Akt and extracellular signal regulated kinase1/2 (ERK1/2) activation [108]. Besides, ROS production is required for the 'priming' signal and upregulation of NLRP3 expression was shown to be blocked by ROS inhibition but not for inflammasome activation [76,109]. Upon activation of NF-κB and MAPK pathways, it was indicated that ROS promotes the upregulation of NLRP3 and the production of pro-inflammatory cytokines such as pro-IL1-β, IL-6 and TNF [76,109].

2.5.4 Lysosomal rupture and deregulated ion concentrations of Golgi lead to NALP-3 inflammasome activation

Cathepsins are the best known pH-dependent pro-enzymes among lysosomal proteases which are for degradation of lysosomal contents via lysosomal acidification [110]. It has been shown that alum, silica and amyloid-β can trigger the formation and activation of NALP-3 inflammasome in a lysosomal damage dependent manner [91,110,111]. In this model, these crystalline molecules cause lysosomal rupture due to their size upon being phagocytosed and released cathepsin B enzyme lead to NALP-3 inflammasome activation [110,111]. It has not been shown yet whether or not lysosomal rupture model provide any explanation for NALP-3 activation by non-crystalline molecules [91]. On the other hand, M2 protein (Ph-gated H^+ channel) transports H^+ ions from Golgi lumen to cytosol to neutralize the pH of transgolgi pathway and acidify the cytosol [76,112]. Upon influenza A virus infection, deregulated ion concentration in Golgi can activate plasma membrane channels which may induce K+ efflux and subsequent NLRP3 activation [76,112].

3. Aims of the study

- To show the relationship between cell death and increased autophagy in a mouse pro-B cell lymphoma after IL-3 depletion;
- To observe How autophagic dying cells influence the inflammatory response of macrophage;
- To learn the phagocytic and inflammatory response of different types of macrophages triggered by autophagic dying cells;
- To investigate whether or not the NALP-3 inflammasome is involved in the pro-inflammatory response of macrophages to engulfed autophagic dying cells;
- To clarify the upstream mechanisms of inflammasome activation in macrophages triggered by autophagic dying cells

4. Materials and Methods

4.1 Cell culture and treatments

Bone marrow derived IL-3 dependent pro-B cells (Ba/F3) were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma), 10% conditioned medium (CM) from WEHI-3B cells (a source of murine IL-3), 300mg/L L-glutamine (Sigma), 100U/ml penicilline/0.1 mg/ml streptomycin (Sigma), 400µM sodium pyruvate (Sigma), 50µM β-mercapto-ethanol (Sigma).

Human breast adenocarcinoma cells (MCF-7) were grown as a monolayer in DMEM supplemented with 10% FCS (Sigma), L-glutamine (300mg/L) (Sigma) and penicillin/streptomycin antibiotics (Sigma). Cells were detached from the substrate using trypsin/EDTA (0.05:0.02%) (Sigma).
Human acute monocytic leukaemia cells (THP-1) were cultured in RPMI 1640 (Sigma) medium supplemented with 10% FCS, 5% L-glutamine, 5% penicillin/streptomycin. All cell lines were incubated in an atmosphere with 5% CO_2 at 37°C and they were *Mycoplasma* free.

4.2 Cell death induction in MCF-7 and Ba/F3 cells

MCF-7 cells were plated in plastic tissue culture flasks at a density of $7.5x10^3/cm^2$ and the culture medium was replaced by DMEM containing 3% charcoalstripped-FCS (DCC) (Biochrom AG) for 7 days. Then, cells were treated with TAM; for treatment, freshly prepared dilutions of tamoxifen (TAM) in DMSO/ethanol (1:1;v:v) (Sigma) were added directly to the medium to obtain autophagic dying MCF-7 cells. Controls were treated with DMSO/ethanol. For the induction of anoikic dying MCF-7 cells, cells were plated on poly-HEMA covered dishes over a 6-day period in 10% FCS. Apoptotic MCF-7 cells treated with doxorubicin (1200ng/ml) for 72 hrs were used for phagocytosis. Apyrase (2.5units/mL) pre- and co-treatment also was carried on the macrophages engulfing these cells. Lentiviral shRNA gene knock-down system (Mission shRNAi/Sigma) was applied for the downregulation of calreticulin expression in MCF-7 cells. Its efficiency was

confirmed by immunobloting using rabbit polyclonal anti-calreticulin antibody (Thermo Scientific). Autophagic dying Ba/F3 cells were obtained by IL-3 depletion for 6 hr. In some experiments IL-3 depleted and non-depleted cells were treated with chloroquine diphosphate (CQ-25μM) (Fluka, Buchs SG**,** Switzerland**)**. Apoptotic cell death was induced by adding 10 μM Doxorubicin (Sigma) for 16 hrs as it was described by Wirawan E., et al.,2010 [30]. Different concentrations of doxorubicin were used to optimize the apoptotic cell death conditions in Ba/F3 cells which don't lead to autophagic activity. Necrotic cells were prepared by freezing and thawing. After cells were thawed, they were washed with PBS and used in the experiments. Percent of positive autophagic dying Ba/F3 cells for Annexin-V-fluorescein-5-isothiocyanate (FITC)/PI was determined by the Annexin-V fluorescein isothio-cyanate Apoptosis Detection Kit (MBL, Budapest, Hungary) on a FACSCalibur flow cytometer (BD FACSCalibur™ flow cytometer, Franklin Lakes, USA). Autophagosome formation was visualized under fluorescent microscopy (Axiovert-150 Zeiss, Budapest, Hungary) by staining autophagic dying Ba/F3 cells with monodancylcadaverine (MDC) (Sigma) (50μM,1 hr) and acridine orange (Sigma) (1μM, 20 min). The inhibition of autophagy with 10 mM 3-methyladenine (3-MA) (Sigma) and necroptosis with 30μM necrostatin (Sigma) were investigated in both treated Ba/F3 and MCF-7 cells.

4.3 Macrophage preparation

4.3.1 Human macrophages

Human monocytes were isolated from "buffy coats" of healthy blood donors on Ficoll-PaqueTM Plus (GE Healthcare) gradient and a magnetic separation using CD14 human microbeads (Miltenyi Biotec). Human macrophages were obtained through a five-day differentiation process using 5ng/mL macrophage colony stimulating factor (MCSF) (PeproTech).

Oligonucleotides for NALP-3 short hairpin RNA (shRNA) were ordered from Integrated DNA Technologies and the following shRNA sequence was cloned into a pLKO.1 vector

(Addgene, Addgene plasmid): 5'-CAG GTT TGA CTA TCT GTT CTA-3'. Packaging and purification of the lentivirus were performed according to standard procedures. For transduction, lentiviruses were added to $2x10^6$ cell THP-1 cells/2ml on 6-well plate in the presence of 10mg/ml polybrene (Sigma, AL118), and the plates were spun at 1,500g for 2 hrs. After an overnight incubation, medium was replaced and cells were grown for 48 hrs, when they were treated with phorbol 12-myristate 13-acetate (PMA) (Sigma) for differentiation. To control the successful NALP-3 knock down (KD), cells were treated with LPS for 24 hrs and with ATP for 1 hr then NALP-3 was measured from the lysate of the cells using qPCR. All cell lines were incubated in an atmosphere with 5% CO_2 at 37°C.

4.3.2 Mouse macrophages

C57BL/6 mice, 6–9 weeks old, were used in all experiments unless otherwise specified. Animals were maintained in the pathogen-free animal facility of University of Debrecen (Debrecen, Hungary) and at the Department for Molecular Biomedical Research of Ghent University-VIB by 'Etische Commissie Proefdieren VIB Site, Universiteit Gent, Universiteit Gent' under the guidelines and ethically approved protocols. Peritoneal macrophages were obtained by peritoneal lavage from mice that were either injected with 2 ml of 4% thioglycollate or non-injected. Thioglycollate-elicited peritoneal macrophages were collected from the peritoneal cavity of mice three days after injection. For experiments with knockout mice *NALP-3* (C57BL/6 background) or *Caspase-1* (6x back crossed to C57Bl/6) and WT mice of appropriate background were used as controls, and they were bred under the same animal house conditions as the others. Macrophages from some mice were pooled and cells were collected by centrifugation and plated in 96-well plates (Corning, Lowell, MA) at $3x10^5$ cells per well in RPMI-1640 medium (Sigma) supplemented in 10% heat-inactivated FCS, 300 mg/L L-glutamine (Sigma), 100U/ml penicilline/0.1mg/ml streptomycin (Sigma), 1mM sodium pyruvate (Sigma). Macrophages were used for co-incubation experiments on the third day after collection from the peritoneal cavity. Each day, unattached cells were removed by refreshing the medium. Bone marrow derived macrophages (BMDMs) were differentiated from femoral

bone marrow cells with 10% L929 conditioned medium in RPMI medium (Sigma) Every other day, the medium was replaced with the fresh one. On the sixth day, cells were collected with enzyme free cell dissociation buffer (Gibco, Budapest, Hungary) and plated in a 96-well plate. They were used for the co-incubation assay on the third day. All cell lines were incubated in an atmosphere with 5% CO_2 at 37°C.

4.4 Phagocytosis assay

Thioglycollate-elicited peritoneal macrophages, resident macrophages, BMDMs and human macrophages were collected and plated as described above. Mouse macrophages were primed with ultra-pure *E. coli* LPS (Invivogen, Toulouse, France) (0.05ng/ml for resident macrophages, 500ng/ml for thioglycollate-elicited macrophages, 100ng/ml for BMDMs) 4 h before starting the phagocytosis assay. The ratio of phagocytes ($3x10^5$/well) and cells to be engulfed ($1.5x10^6$/well) was set at 1:5. Dying cells were fed to engulfing cells when in their culture autophagy peaked: at day 4 for autophagic dying MCF-7 cells, day 6 for anoikic-autophagic MCF-7 cells; at 6 hrs for autophagic dying Ba/F3 cells. Living, apoptotic and necrotic cells were fed to macrophages as well. Dying/living cells were added to the phagocytes and kept together for 2 hrs with mouse macrophages and 1 hr and additional 17 hrs with human macrophages. After the phagocytosis assay and upon removal of non-engulfed dying cells, we trypsinized macrophages at 37 ^{0}C for 15 min to prevent the cell-to-cell attachment and quantified only the phagocytosis capacity of macrophages. Inhibition of phagocytosis was carried out by pre-treating the macrophages with cytochalasin D (CytD) (Sigma) (15μM) for 45 min at 37^{o}C and throughout the assay. Autophagic dying cells were stained with the viable stains 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA-SE), 15μM, overnight (Invitrogene) and macrophages were labeled with Cell TrackerTM Orange, CMTMR, 3.75μM, overnight (Invitrogene). Upon co-incubation, fluorescence was measured on a BD FACSCalibur flow cytometer, and the percentage of both human and mouse macrophages positive for both CMTMR and 5(6)-CFDA-SE was determined.

4.5 Chemicals used in phagocytosis experiments for determining the upstream mechanisms of inflammasome activation

Studies on the role of P_2X_7R activation was carried with the ATP hydrolyzing apyrase (Sigma) (2.5units/ml) and the P_2X_7 receptor antagonist KN-62 (Sigma) (1μM) (apyrase and KN-62 treatments were done 45 min before and throughout the assay of both human and mouse macrophages). Studies on the role of K^+ efflux were carried out by using medium containing 130mM potassium chloride (KCl) (Sigma) during co-incubation of both human and mouse macrophages. Studies on how the specific caspase-1 inhibitor Z-YVAD-FMK (BioVision Int, Brussels, Belgium) (50μM) affects IL-1β production were carried out by applying it 45 min before and throughout the assay of both human and mouse macrophages. IL-1 receptor antagonist anakinra (0.1mg/10^6 human macrophages) was used 30 minutes prior and throughout the phagocytic assay. The role of the pannexin-1 channel in NALP-3 activation was checked by using carbenoxolone disodium salt (CBX) (Sigma) (5μM) 45 min before and throughout the phagocytosis assay of mouse macrophages. Autophagic dying Ba/F3 cells were also treated with CBX (5μM). Addition of ATP (5mM) (Sigma) (for mouse macrophages) and UA crystals (100μg/well) (for human macrophages) was used as a positive control.

4.6 Western blotting

Anti-IL-1β polyclonal antibody and anti-LC3 polyclonal antibody were purchased from NovusBiologicals, Cambridge, England. Human anti-cleaved IL-1β and human anti-caspase-1 polyclonal antibodies were purchased from Cell signaling. Anti-actin polyclonal antibody and rabbit anti-rat peroxidase-conjugated secondary antibody were purchased from Sigma. Caspase-3 antibody was from BD Pharmingen, Budapest, Hungary. Protein concentrates (up to 40 times) of human macrophage cell supernatants were prepared by Microcon Centrifugal Filter Devices purchased from (Millipore) and separately. Equal amounts of proteins (17.5μg) obtained from Ba/F3 cell lysates and from concentrated supernatants from human macrophages were loaded on the gel and separated on a NuPAGE

15% Bis-Tris polyacrylamide gel (Invitrogen, Merelbeke, Belgium) and transferred to an Immobilon-P membrane (Millipore, Budapest, Hungary; pore size 0.45µm). Membranes were blocked in Tris buffered saline containing 0.05% Tween-20 (TBS-T) and 5% non-fat dry milk (BioRad, Budapest, Hungary) for 1 hr. After blocking, membranes were probed overnight at 4°C with rabbit anti-IL-1β polyclonal antibody (2µg/ml) (NovusBiologicals), anti-actin polyclonal antibody (0.8µg/ml) (Sigma), pro- and mature caspase-3 antibody (1µg/ml) and anti-LC3 polyclonal antibody (2µg/ml) were followed by incubation for 1 h with a rabbit anti-rat peroxidase-conjugated secondary antibody (Sigma) for 1 h at room temperature. Peroxidase activity was detected with SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate (Pierce, Rockford, IL) using a Lumi-Imager (Roche Diagnostics, Mannheim, Germany). Fermentas pre-stained protein ladder was used as protein marker in each blot. Blots in Figure 1B, 1D and 2A, we have not de-stripped the membrane before detecting the actin protein and we have developed the membrane with anti-actin antibody after the washing steps upon developing it for anti-LC3. In Figure 2B and 4A, membranes were de-stripped (15-20 min), washed 3-4 X for 10 min with TBS-T and then re-blocked with 5% non-fat dry milk in TBS-T solution for 1h at room temperature. Then membranes were probed overnight with anti-actin first antibody, then the anti-rabbit secondary antibody for 1 h at room temperature. Detection of the peroxidase activity was done same as explained above. Stripping solution contains 2% SDS, 100 mM beta-mercaptoethanol and 50 mM TRIS, pH 6.8. The ratio of the integrated density of LC3-II to actin was quantified by using Image J (NIH Bethesda).

4.7 Immunocytochemistry

Autophagosome formation was visualized under fluorescent microscopy (Axiovert-150 Zeiss, Budapest, Hungary) by staining of the autophagic cells with and acridine orange (Sigma) (1µM, 20 min). For staining with LC-3 antibody and visualization of autophagosomes living and IL-3 depleted Ba/F3 cells were cytospined and cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min. Blocking was done with 5% BSA (bovine serum albumin) in 0.1% Triton-X-PBS solution for 1 hr. They were then

incubated with anti-LC3 polyclonal antibody (5μg/ml) at room temperature for 2 hrs. Anti-LC3 polyclonal antibody was purchased from Novus Biologicals, Cambridge, England. Secondary antibody was Cy3-labeled goat anti-rabbit (Sigma) and used for 1 hr. Nuclei were labeled with DAPI (0.5μg/ml) (Sigma) and viewed with a fluorescent microscope (Axiovert-150 Zeiss, Budapest, Hungary). Washings were done for 3X5 min with 0.1% Triton-X in PBS.

4.8 Cytokine and ATP quantification

Ultra-pure LPS primed macrophages were co-incubated with appropriate target cells and after the 2 h co-incubation period, supernatants were collected and IL-1β was measured by using enzyme-linked immunosorbent assay (ELISA) (R&D DuoSet, Budapest, Hungary). In experiments where CASPASE-1/NALP-3 knock out mice macrophages were used, immunoreactive levels of IL-1β were measured in CM by using a Milliplex mouse cytokine kit (MPXMCYTO-70K-01, Merck Millipore, Overijse, Belgium) according to the manufacturers' instructions and analyzed on a Bio-Plex 200 (Bio-Rad, Nazareth Eke, Belgium).

Human and mouse macrophages were stimulated or not with 0.5μg/mL crude LPS for 30 min prior to assay and then incubated with autophagic dying cells for 1 hr. After non-ingested dying cells were removed, macrophages were incubated in fresh media without serum for additional 17 hrs or 6 hrs, respectively. The supernatants from crude LPS treated human and mouse macrophages were collected and analyzed for the presence of IL-8, IL-6, IL-1β, TNF-α using the Human Inflammation BD Cytometric Bead Array (CBA) BD Biosciences) kit and only for IL-6 using an ELISA (R&D) kit. Concentration of ATP was measured in supernatants by using ATPliteTM Luminescence Assay System (Perkin Elmer, Budapest, Hungary) according to the manufacturer’s instructions and the light production was measured on a VICTOR2TM (Perkin Elmer) reader.

4.9 Intra-peritoneal injection of autophagic dying Ba/F3 cells and phenotyping of peritoneal exudates cells

Autophagy, apoptosis and necrosis in Ba/F3 cells was induced as described above. Autophagic dying, apoptotic, necrotic and live cells were harvested by centrifugation, washed three times with sterile D-PBS (Invitrogen) and resuspended in D-PBS at a density of $40x10^6$ cells/ml. Syngenic for Ba/F3 cells Balb/c mice (8–10 weeks old, Janvier, Bio Services BV, The Netherlands, n=4-5 mice per group) were intraperitoneally injected with $10x10^6$ cells/mouse in 0.250 ml of D-PBS. Equal volumes of D-PBS were injected as negative controls. Sixteen hrs after injection, animals were euthanized by CO_2 exposure, and peritoneal exudate cells (PECs) were isolated by peritoneal lavage. The red blood cells were lysed with ACK cell lysis buffer (Lonza Walkersville, Basel, Switzerland). The number of PECs was counted in a hematocytometer using trypan blue and phenotyped by flow cytometry. All experimental procedures were approved by the local Ethics Committee of Ghent University–VIB.

PECs (5×10^5) were incubated with rat anti-mouse antibody 2.4G2 (BD Pharmingen, Erembodegem, Belgium) for 30 min at 4°C to block FcγRIIB/III receptors. Since apoptotic cells were treated with doxorubicin, which has a broad range of auto-fluorescence, we divided each sample and used two different stainings in order to identify monocytes, macrophages, eosinophils and neutrophils. In order to quantify monocytes/macrophages/eosinophils, the PECs were stained with anti-mouse antibodies F4/80-APC (clone BM8, eBioscience) and CD11b-APC-Cy7 (clone M1/70, BD Pharmingen). To identify neutrophils, the PECs were stained with anti-mouse antibodies Ly-6G-APC (clone 1A8, BD Pharmingen) and CD11b-APC-Cy7 (clone M1/70, BD Pharmingen). All the stainings were done for 30 min at 4°C in PBS. Just before flow cytometry analysis on BD LSR-II (BD Biosciences), 1.25nM of Sytox Blue dead cell stain was added (Invitrogen) to exclude dead cells from the measurements. Data were acquired and analyzed by BD FACSDiva software (BD Biosciences). The following cell populations were discriminated: macrophages ($F4/80^{high}$ $CD11b^{high}$), monocytes

(F4/80^{medium} CD11b^{high}), eosinophils (F4/80^{medium} CD11b^{medium}) and neutrophils (CD11b^{+} Ly6G^{high}). In order to determine number of cells in each specific cell population, the total cell numbers of PECs were multiplied by the percentage of specific cell population mentioned above.

4.10 Statistical analysis

Results are expressed as mean ± SEM for the number of assays indicated. When results were obtained from experiments in which mouse macrophages were used, for multiple comparisons of groups statistical significance was evaluated by one-way ANOVA followed by Tukey post-hoc test and for comparing of two groups non-bias two-tailed unpaired student t test was used. Statistical significance is indicated by stars shown in graphs. When results obtained from experiments in which human macrophages were used, statistical significance (defined as $p<0.05$) was evaluated by the unpaired student t test. When it is additional information used for statistical analysis, it is written in figure legends. (*$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$,****$p\leq0.0001$)

5. Results

5.1 Autophagy and cell death in Ba/F3 cells

5.1.1 IL-3 deprivation leads to autophagy and apoptosis in Ba/F3 cells

Withdrawal of growth factors triggers both autophagy and apoptosis in Ba/F3 cells [30]. By using anti-LC3 immunostaining and acridine orange staining, we observed that 6 h of IL-3 depletion increased the numbers of autophagolysosomes in Ba/F3 cells (Figure 3A). Western blot analysis showed increased level of LC3-II (a molecular marker of autophagosome formation) relative to controls (Figure 3B). We wanted to determine whether or not IL-3 withdrawal leads to upregulation of autophagy (increased autophagic flux) or a blockage of the autophagic flux (degradation block) with consequent accummulation of autophagic vesicles. For this reason, we treated the cells with the lysosomal inhibitor, chloroquine (CQ), which prevents fusion of autophagosomes with lysosomes [16,113]. CQ treatment led to highly elevated LC3-II protein content in IL-3 depleted Ba/F3 cells, demonstrating that withdrawal of the growth factor resulted in increased autophagic flux but not blockade of autophagic flux (Figure 3B). In the presence of IL-3, CQ treatment also led to high LC3-II content indicating ongoing autophagic flux. It can not be excluded that since the cell suspensions are heterogeneous and the method is not sensitive enough there might be cells in which LC3-II degradation was blocked.

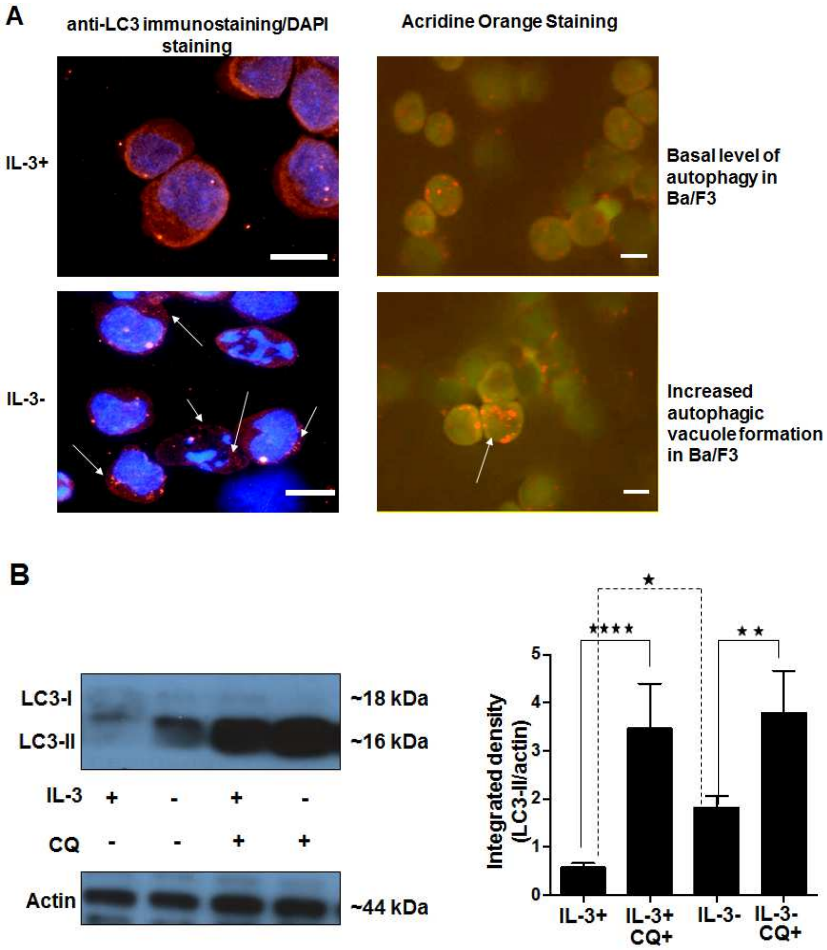


Figure 3: IL-3 depletion increases autophagy in Ba/F3 cells Ba/F3 cells were kept without IL-3 for 6 hrs. **(A)** Both living and dying autophagic Ba/F3 cells were stained with anti-LC3 antibody or acridine orange stain to demonstrate increased autophagosome formation. Arrows represent the increased autophagy with IL-3 depletion. Scale bars are 10µm. **(B)** Proteins in western blots of samples from dying autophagic cells were detected with anti-LC3 antibody. Chloroquine (CQ) was used as lysosomal inhibitor. The right panel presents the quantification of the western blot. Anti-actin polyclonal antibody was used to show that equal amounts of proteins were loaded in western blots. Data represent the mean ± SEM of 13, 19, 5 and 6 independent experiments for IL-3+, IL-3-, IL-3+CQ+ and IL-3-CQ+, respectively. (*p<0.05, **p<0.01, ****p<0.0001)

Upon IL-3 depletion, ~20% of the cells were positive for phosphatidylserine $(PS)^+$ and negative for propidium iodide $(PI)^-$, and ~4% of them were PS^+/PI^+ (Figure 4). When Ba/F3 cells were treated with CQ in the presence of IL-3, accumulation of autophagosomes did not lead to significant increase of cell death, but the combined effect of IL-3 depletion and the addition of the lysosomal inhibitor induced more cell death than IL-3 alone (Figure 4). We also observed that IL-3 depletion led to apoptosis in Ba/F3 cells in accordance with our findings showing caspase-3 processing in cells (Figure 4).

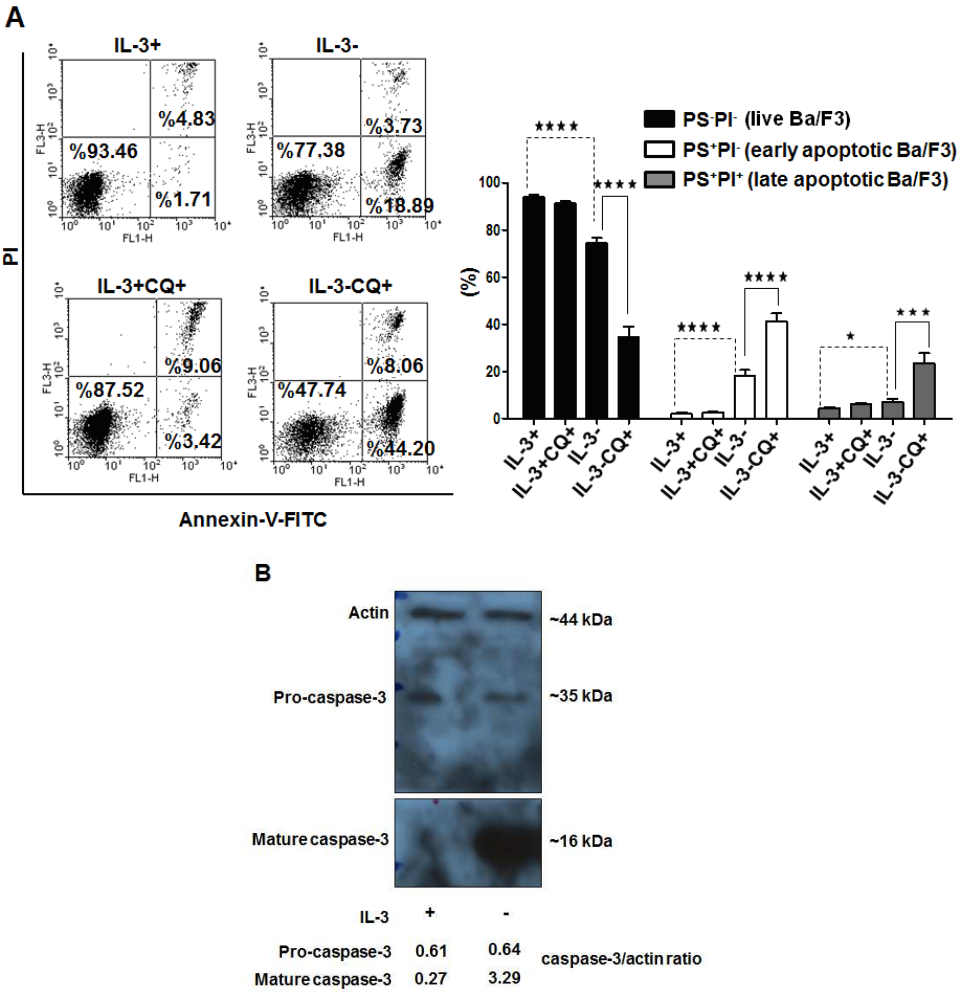


Figure 4: Apoptosis is induced in autophagic Ba/F3 cells by IL-3 depletion. Ba/F3 cells were kept with or without IL-3 for 6 hrs. **(A)** Cell death was quantified by flow cytometric analysis of dying autophagic cells by using Annexin-V-FITC/PI staining. Data represent the mean ± SEM of 7, 10, 3 and 9 independent experiments for IL-3+, IL-3-, IL-3+CQ+, IL-3-CQ+, respectively. PS: Phosphatidylserine, PI: Propidium iodide **(B)** Proteins obtained from dying autophagic cells were detected with caspase-3 antibody. Anti-actin polyclonal antibody was used to show that equal amounts of proteins were loaded in western blots. For simplicity, parts from the same western blots are shown separately in parts B and D. (*p<0.05, ***p<0.001, ****p<0.0001)

5.1.2 Apoptosis and necrosis can be induced in Ba/F3 cells without increased autophagy

Doxorubicin -commonly used in cancer chemotherapy- was used as the apoptotic cell death inducer in Ba/F3 cells [114]. More and more cell death was induced without increasing autophagy at increasing doxorubicin concentrations. With the highest doxorubicin concentration (10μM), cells did not show autophagic activity and most of the cells died by secondary necrosis (Figure 5A). In order to obtain necrotic cells, Ba/F3 cells were frozen at -20^0C and thawed at room temperature. By this procedure, autophagic activity was not elevated and almost 100 % of cells died by necrosis (Figure 5B).

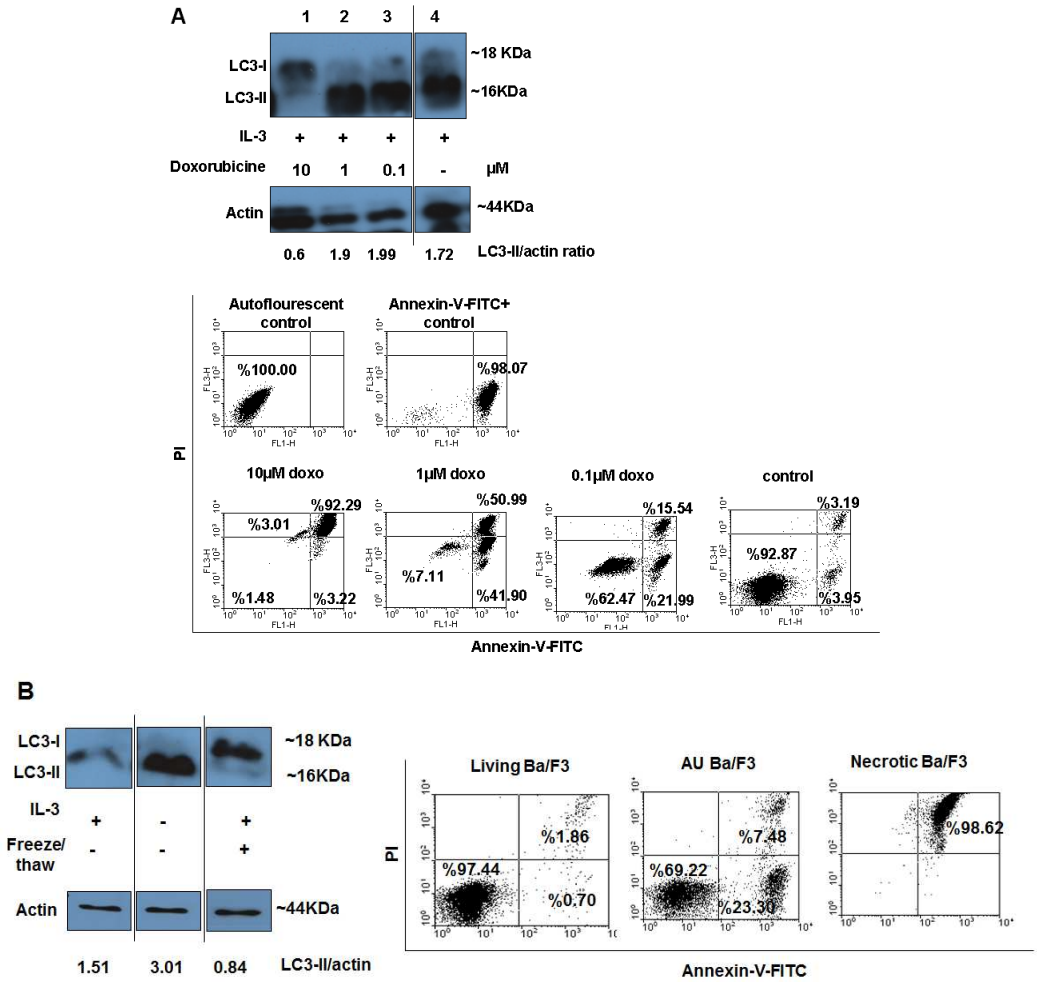


Figure 5: Apoptotic and necrotic Ba/F3 cells do not show autophagic activity (A) (A) Ba/F3 cells were treated with different doses of doxorubicin (0.1, 1, 10µM) in the presence of IL-3 for 16 hrs. Immunoblotting with anti-LC3 antibody was done to detect LC3 protein in cells. Ba/F3 cells which were not treated with doxorubicin in IL-3 containing medium were used as control for the experiment. **(B)** Necrosis in Ba/F3 cells was induced by freeze-thaw. Anti-actin polyclonal antibody was used to show that equal amount of proteins were loaded. For simplicity, parts from the same western blots are shown separately. Cell death was checked by flow cytometric analysis of dying cells by using Annexin-V-FITC/PI staining. Relevant controls (autoflourescent and Annexin-V-FITC positive cells) are also included in the figure.

5.2 Autophagic and dying cells induce inflammasome activation in macrophages

5.2.1 IL-1β release from macrophages engulfing autophagic dying cells

As we have described previously [36], MCF-7 cells die through autophagy with estrogen hormone depletion and tamoxifen treatment and die with autophagy when they were detached from the surface (anoikis). In order to show the response of human monocyte derived macrophages to autophagic dying MCF-7 cells, we conducted co-incubation experiments. It was shown that macrophages released IL1-β cytokine only while engulfing autophagic dying MCF-7 cells but not anoikic-autophagic MCF-7, live and apoptotic ones (Figure 6A). In order to observe the response of mouse resident peritoneal macrophages, they were co-incubated with autophagic dying Ba/F3 cells. Different from human macrophages, mouse macrophages had to be primed with ultra pure LPS. Increased secretion of mature IL-1β was detected during co-incubation with autophagic dying Ba/F3 cells but not with living, apoptotic or necrotic ones (Figure 6B). IL-1β was released in higher amount when CQ treated autophagic dying Ba/F3 cells were co-incubated with the macrophages. Primed thioglycollate elicited peritoneal macrophages and BMDMs also released significantly higher amount of IL-1β after co-incubation with autophagic dying Ba/F3 cells as compared to controls (Figure 6C and D). We measured the uptake capacity of mouse resident peritoneal macrophages. Macrophages engulfed 30% of autophagic dying cells and up to 43% of CQ-treated autophagic dying cells, in contrast to 12% of living cells and up to 27% of CQ-treated living cells during 2 hrs of co-incubation (data not shown).

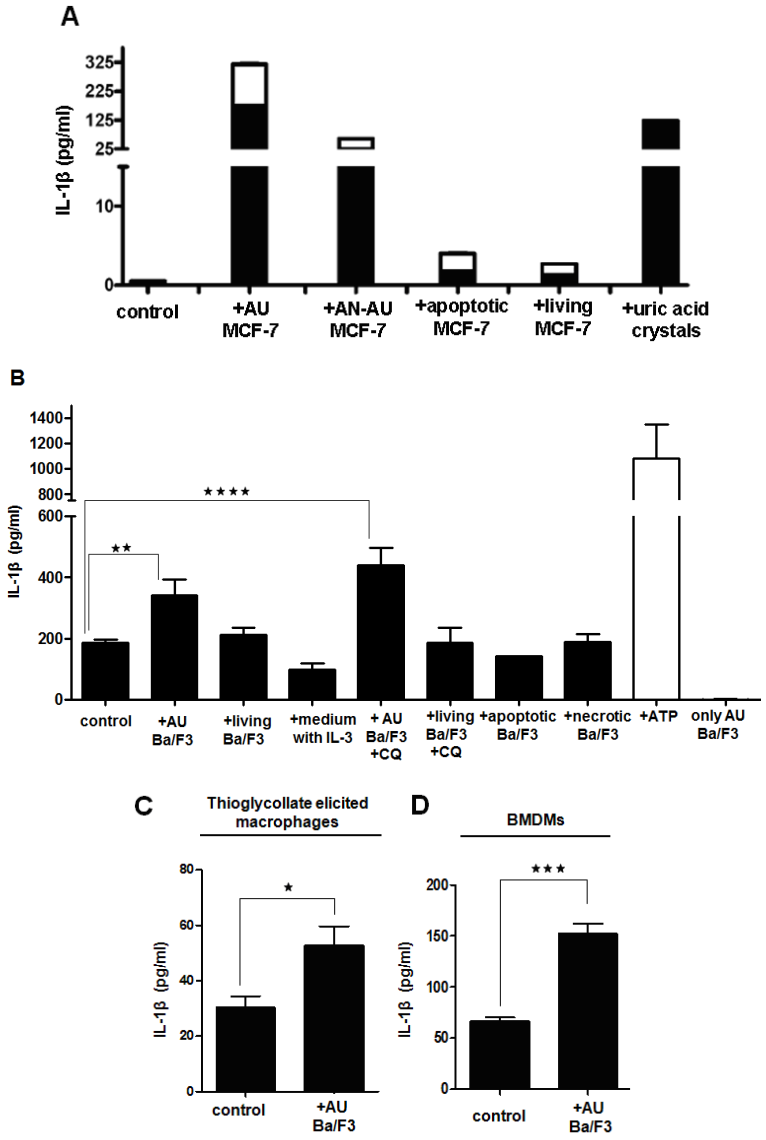


Figure 6: IL-1β release from macrophages engulfing autophagic and dying cells (A) IL-1β upon co-incubation with autophagic dying (AU MCF-7), anoikic-autophagic (AN-AU MCF-7), apoptotic or living MCF-7 cells was measured. Black bars represent 1 hr co-incubation and white bars represent the 17 hrs period after removal of the dying cells. Treatment with 100μg uric acid crystals as a positive control was carried out for 8 hrs. **(B)** IL-3-depleted cells, live cells, apoptotic cells (treated with 10 μM doxorubicin), and necrotic Ba/F3 cells were co-incubated with primed resident macrophages. As a control condition, IL-1β was quantified from supernatant of only dying autophagic Ba/F3 cells in order to analyze if they secrete IL-1β by themselves. ATP, which is a stimulus for the inflammasome activation, was used as a positive control.

Repeated measures one-way ANOVA followed by Tukey post hoc test was used for statistical analysis for part B. **(C, D)** Primed thioglycollate-elicited macrophages and bone marrow derived macrophages (BMDM) were co-incubated with IL-3-depleted dying autophagic cells. Primed macrophages (control) but not co-incubated with any type of Ba/F3 cells. Data represent the mean ± SEM of three independent experiments in parts A, C and D and four independent experiments in part B; all experiments were performed in triplicates. (*p<0.05, **p<0.01, ****p<0.0001)

5.2.2 Autophagy, but not necroptosis is a requisite for inflammasome activation leading to pro-inflammatory response

In order to analyze whether autophagy in the target cells is required for inflammasome activation in human monocyte derived macrophages, it was inhibited by the type III (phosphoinositide 3-kinase) PI3K inhibitor, 3-methyladenine (3-MA) [115]. Upon autophagy inhibition in dying MCF-7 cells, release of IL-1β was inhibited. We next checked the involvement of necroptotic cell death pathway and calreticulin exposure in inflammasome activation. Necrostatin, a necroptosis inhibitor treated autophagic dying MCF-7 cells did not affect the IL-1β release from macrophages (Figure 7A). Similarly, the pro-inflammatory response of mouse resident peritoneal macrophages was decreased when autophagy was inhibited in dying Ba/F3 cells. Necrostatin was ineffective in preventing the IL-1β release from resident macrophages exposed to autophagic dying cells (Figure 7B).

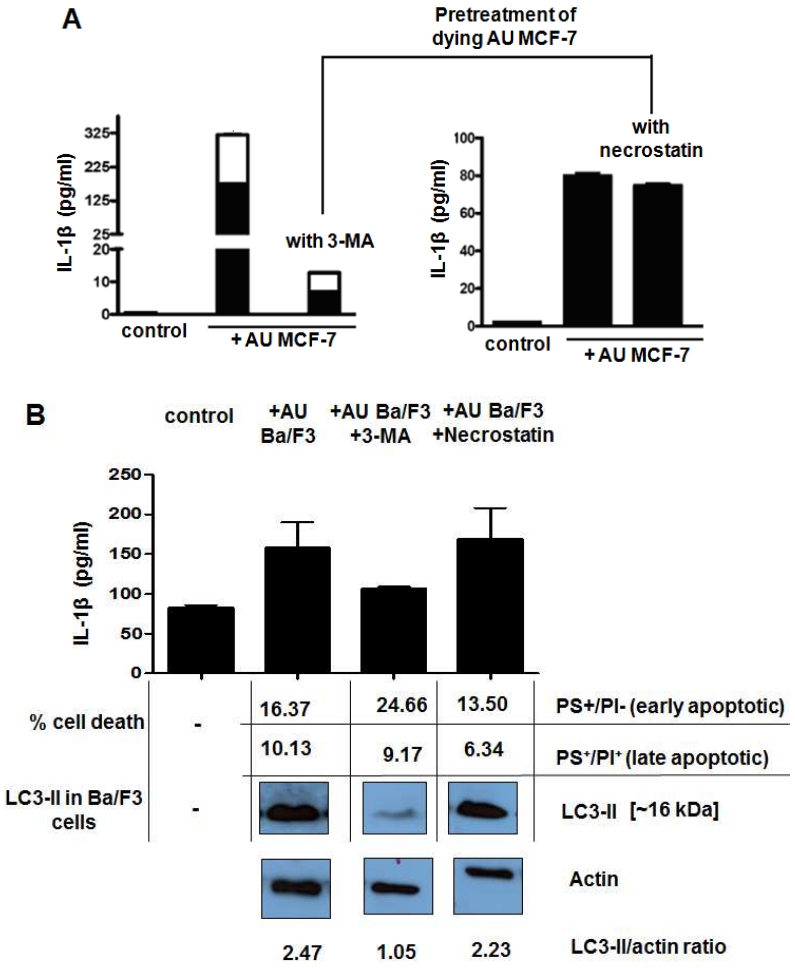
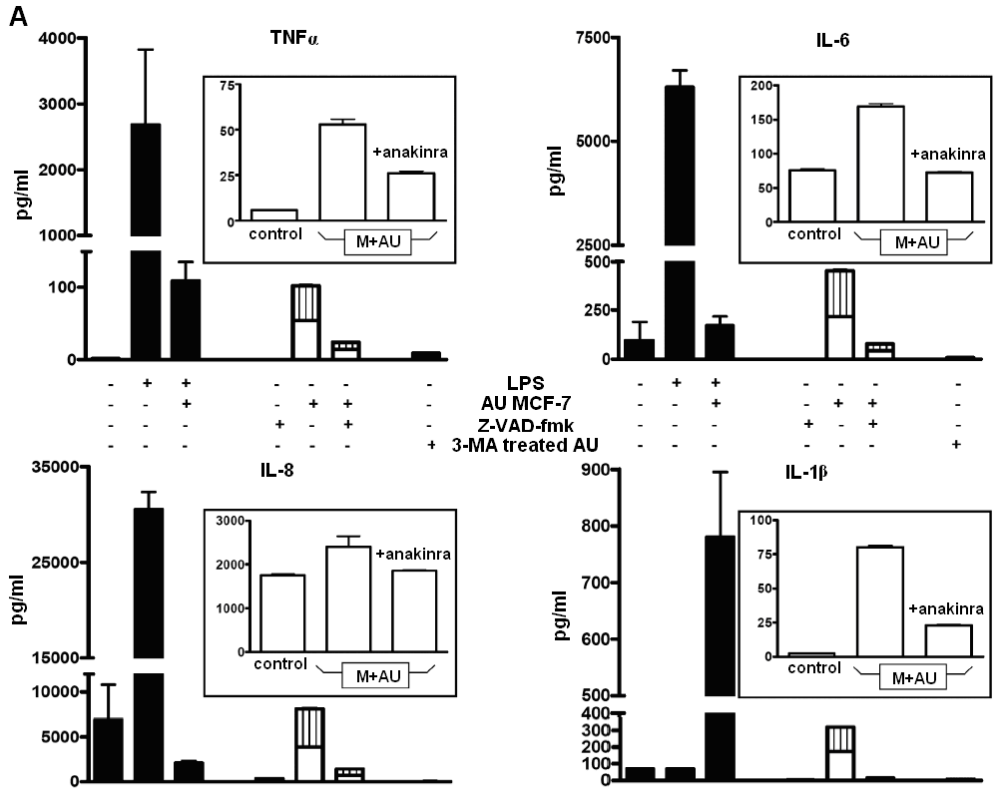


Figure 7: Autophagy but not necroptosis contributes to inflammasome activation in macrophages (A) Human and **(B)** mouse macrophages were co-incubated with autophagic and dying cells and 3-Methyladenine (3-MA) and necrostatin-1 pretreated autophagic dying MCF-7 cells (AU MCF-7) and autophagic dying Ba/F3 cells (AU Ba/F3), respectively. Cell death was checked by flow cytometric analysis of autophagic dying cells. For simplicity, parts of the same western blot are shown separately. Data represent the mean ± SEM of three (for part A) and two (for part B) independent experiments; all experiments were performed in triplicates.

5.2.3 Inhibition of LPS-induced cytokines from macrophages with dying cells

Crude LPS treated human monocyte derived macrophages secreted large amount of pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α. We observed that autophagic dying MCF-7 cells inhibited the release of crude LPS induced pro-inflammatory cytokines from human macrophages while they promoted IL-1β secretion upon crude LPS treatment. Pro-inflammatory cytokine amounts were diminished by inhibition of autophagy in dying MCF-7 cells, prevention of caspase-1 pathway in macrophages and blocking of IL-1β receptor with anakinra on macrophages (Figure 8A). We also observed that IL-1β secretion was higher when autopghagic dying MCF-7 cells were fed to macrophages after LPS treatment (Figure 8A). Similarly, although autophagic dying Ba/F3 cells could induce a pro-inflammatory response in macrophages triggering IL-1β secretion, they could still inhibit the LPS-induced pro-inflammatory response as measured by IL-6 release (Figure 8B).



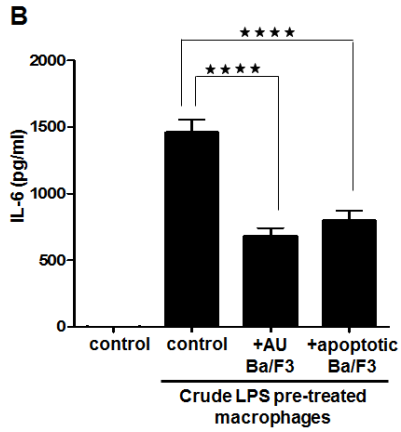
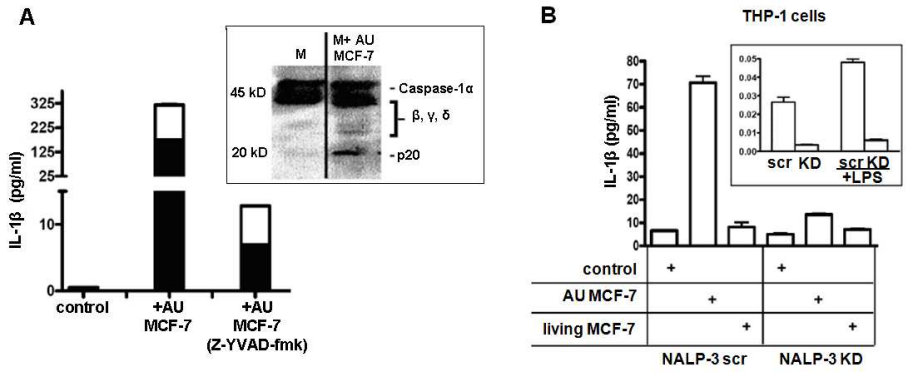


Figure 8: Autophagic and dying cells inhibit the LPS induced pro-inflammatory cytokine production (A) Release of TNF-α, IL-6, IL-8 and IL-1β by human macrophages co-incubated with autophagic dying MCF-7 cells (AU MCF-7) were pre-treated with or without crude lipopolysaccaride (LPS) or Z-YVAD-fmk (caspase-1 inhibitor) or anakinra (IL-1 receptor antagonist). Black bars show the amount of released cytokines after either LPS treatment alone or in phagocytic assays lasting 1 hr either with or without lipopolysaccaride (LPS) treatment. White and striped bars represent 1 and a consequent 17 hrs release of cytokines in the absence of dying cells, respectively. The inserts show the effect of the anakinra on the secretion of pro-inflammatory cytokines. **(B)** IL-6 cytokine release was checked from crude LPS pre-treated elicited peritoneal macrophages co-incubated with IL-3-depleted autophagic dying Ba/F3 cells (AU Ba/F3) or with doxorubicin treated apoptotic cells. In part A, data represent the mean ± SD for main graphs and ± SEM for the inserts which are mean values of three independent experiments; all experiments were performed in triplicates. For part B, data was pooled from one experiment performed in five replicates. (*$p<0.05$, ****$p<0.0001$)

5.3 Uptake of autophagic and dying cells leads to NALP-3 and caspase-1 mediated IL-1β release from macrophages

To check whether caspase-1 activation is responsible for the inflammasome activation in human monocyte derived macrophages, specific caspase-1 inhibitor (Z-YVAD-fmk) was used to treat macrophages during co-incubation with autophagic dying MCF-7 cells. IL1-β release was significantly decreased when caspase-1 was inhibited (Figure 9A). We also

showed the cleavage of pro-caspase-1 happened when macrophages co-incubated with autophagic dying MCF-7 cells (Figure 9A). THP-1 cells (human acute monocytic leukemia cell line) were differentiated into macrophages and NALP-3 gene was knocked down. According to co-incubation results of human monocyte derived macrophages and autophagic dying MCF-7 cells, NALP-3 knocked down macrophages did not respond to autophagic dying MCF-7 cells (Figure 9B). Co-incubation of caspase-1 knock out (KO) mouse elicited peritoneal macrophages with autophagic dying Ba/F3 cells showed that IL-1β release was significantly less from caspase-1 deficient macrophages than from WT ones (Figure 9C-left panel). These data were further confirmed by using a caspase-1 specific inhibitor (Z-YVAD-fmk) which also reduced IL-1β secretion (Figure 9C-right panel). Next, we decided to clarify which inflammasome is activated in macrophages by dying cells which carry autophagic features. Furthermore, macrophages isolated from NALP3 knockout mice had decreased response to dying autophagic cells, indicating that the NALP3 inflammasome can be the mediator of caspase-1 activation and IL-1β secretion (Figure 9D). We did not observe any difference in the phagocytic capacity of each macrophage type engulfing living and autophagic dying Ba/F3 cells (Figure 9E).



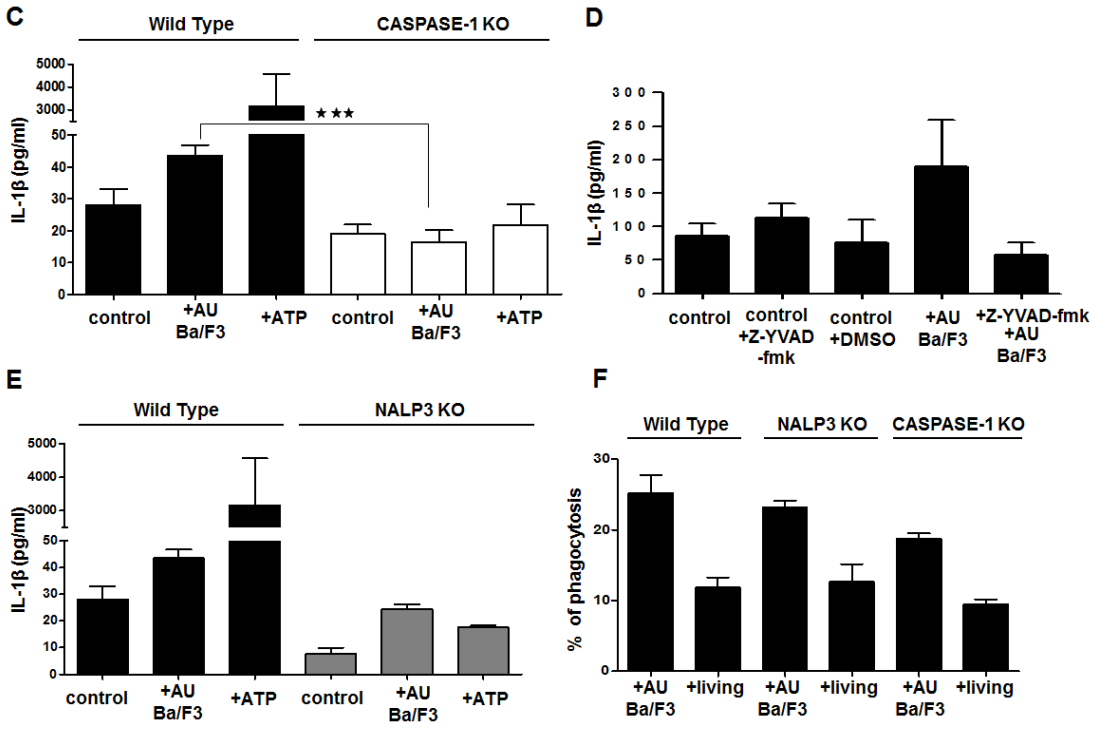


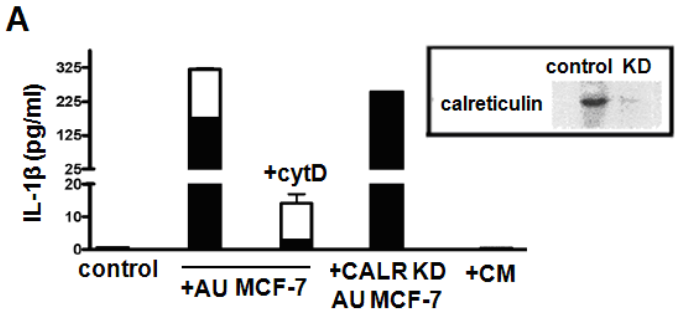
Figure 9: Caspase-1 is required for NALP-3 inflammasome activation with autophagic and dying cells (A) Activation of caspase-1 was inhibited by specific inhibitor (Z-YVAD-fmk). Black bars represent 1hr co-incubation and white bars represent the 17 hrs period after removal of the dying cells. Western blotting shows the caspase-1 activation during the engulfment of autophagic dying MCF-7 cells (AU MCF-7). **(B)** IL-1β release from phorbol 12-myristate 13-acetate (PMA)-treated THP-1 cells engulfing autophagic dying MCF-7 cells were measured. The insert shows the relative expression of NALP-3/cyclophilin and therefore the effectiveness of NALP-3 silencing (knock down (KD)) in THP-1 cells treated with NALP-3 scramble and silencing constructs with or without lipopolysaccaride (LPS) induction. Primed thioglycollate-elicited macrophages from wild type and from CASPASE-1 **(C)** or NALP3 **(E)** knockout mice were co-incubated with IL-3-depleted cells. ATP was used as a positive control. **(D)** Resident macrophages treated with Z-YVAD-fmk (specific caspase-1 inhibitor) were co-incubated with IL-3-depleted dying cells. **(F)** Wild type and NALP3 or CASPASE-1 deficient macrophages were co-incubated with IL-3 depleted dying autophagic (AU) and living Ba/F3 cells and phagocytosis was measured by flow cytometry. Control cells are primed macrophages but not incubated with any type of Ba/F3 cells.

Data represent the mean ± SEM of two independent experiments for part C, three independent experiments for part A, B and D, one experiment for part E, and two independent experiments for part F; all experiments were performed in triplicates (***p<0.001)

5.4 Mechanisms behind NALP-3 inflammasome activation with autophagic and dying cells

5.4.1 Uptake of autophagic and dying cells leads to inflammasome activation

In order to clarify whether or not phagocytosis of the autophagic dying MCF-7 cells by human monocyte derived macrophages was needed for the inflammasome activation, we prevented the engulfment by CytD, an inhibitor of phagocytosis [116] and it led to significant inhibition of IL-1β secretion from macrophages (Figure 10A). On the other hand, CM of autophagic dying MCF-7 cells did not induce inflammasome activation (Figure 10A). Calreticulin knocked down of autophagic dying MCF-7 cells did not affect the IL-1β release from macrophages. We also pre-treated the mouse resident peritoneal macrophages with cytD which reduced the phagocytosis and IL-1β release from macrophages co-incubated with autophagic dying Ba/F3 cells (Figure 10B). Co-incubation of CM obtained from cultures of 6 hrs IL-3 depleted Ba/F3 cells with primed macrophages did not result in IL-1β release (Figure 10B-right panel).



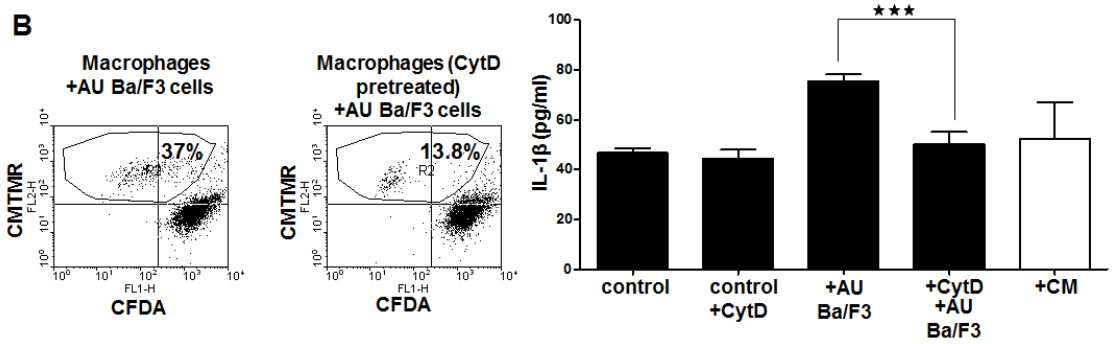


Figure 10: Phagocytosis is needed for inflammasome activation in macrophages engulfing autophagic and dying cells (A) IL-1β secretion from human monocyte derived macrophages engulfing autophagic dying cells (AU MCF-7) was measured. Phagocytosis was inhibited via cytochalasin D (CytD) treatment of macrophages. Conditioned medium (CM) (diluted 50% with serum-free culture medium) was collected from cultures of autophagic dying MCF-7 cells at the time when they were prepared for phagocytosis. In calreticulin (CALR) knock down (KD) MCF-7 cells (western blot insert shows effectiveness of calreticulin silencing) autophagy was induced the same way as in wild type MCF-7 cells. Black bars represent 1 hr co-incubation and white bars represent the 17 hrs period after removal of the dying cells. **(B)** CMTMR-stained macrophages were treated with cytochalasin D (cytD) to inhibit phagocytosis, and cytD treated/non-treated macrophages were co-incubated with CFDA-stained dying autophagic Ba/F3 cells. R2 is the region which shows both the upper left quadrant (macrophages which do not engulf dying cells) and upper right quadrant (macrophages which engulf dying cells). The cells out of R2 region is also shown in the figure. The macrophages were also co-incubated with the non-diluted conditioned medium (CM) from dying autophagic cells. Control cells are macrophages, which were primed but not co-incubated with any type of Ba/F3 cells. Data represent the mean ± SEM of three (for part A), two (for part B) independent experiments; all experiments were performed in triplicates. (***$p<0.001$)

5.4.2 K^+ efflux takes place from macrophages engulfing autophagic dying cells triggering inflammasome activation

Our next goal was to delineate the NALP-3 inflammasome activation pathway triggered by engulfed autophagic dying MCF-7 cells. We first wanted to see whether K^+ efflux, a general inducer of NALP-3 inflammasome activation is required for the autophagic dying

MCF-7 cells to induce release of IL-1β from human monocyte derived macrophages (Figure 11A). Blocking of K^+ efflux from macrophages during phagocytosis of autophagic dying MCF-7 cells inhibited the IL-1β release (Figure 11A). Blocking efflux of K^+ also led to the decrease of IL-1β release from both resident peritoneal (Figure 11B) and thioglycollate-elicited peritoneal macrophages engulfing autophagic dying Ba/F3 cells (Figure 11D).

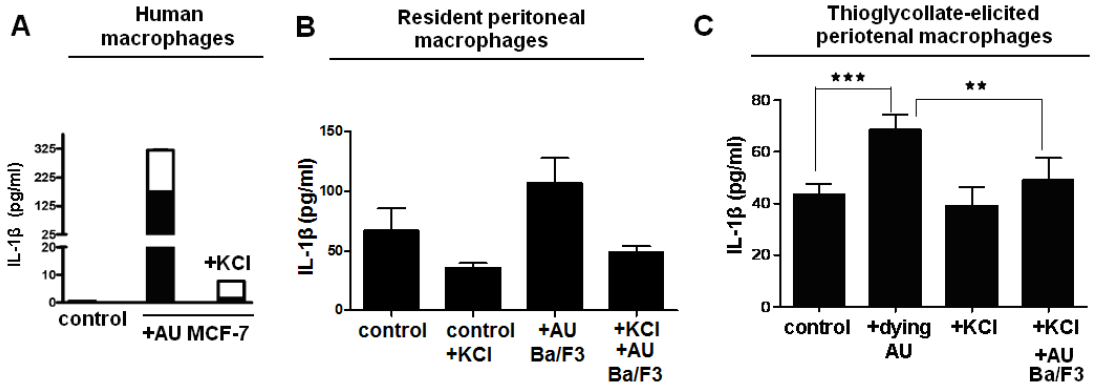
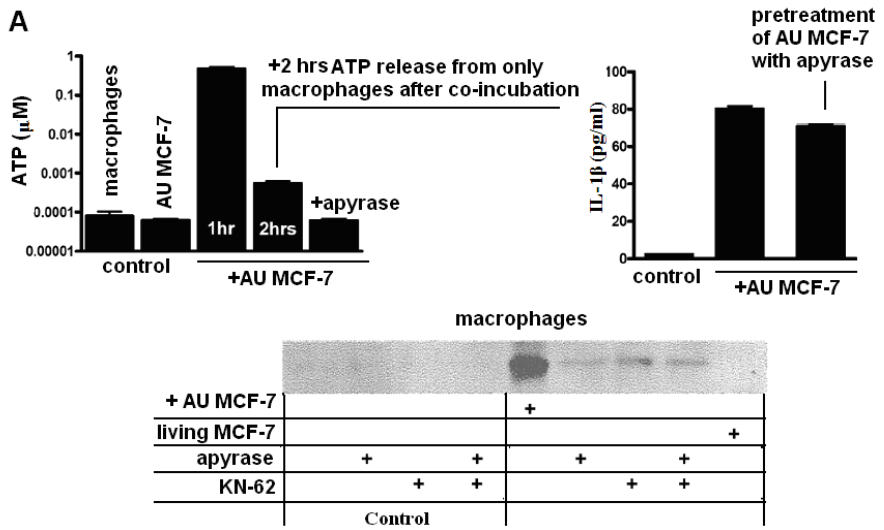


Figure 11: K^+ efflux leads to inflammasome activation in macrophages engulfing autophagic dying cells (A) Potassium chloride (KCl) (130 mM) containing medium was used and IL-1β secretion from human monocyte derived macrophages engulfing autophagic dying MCF-7 cells (AU MCF-7) was measured. **(B,C)** Primed resident and thioglycollate-elicited peritoneal macrophages were co-incubated with IL-3 depleted autophagic dying Ba/F3 cells (AU Ba/F3) in the presence of potassium chloride (KCl). Data represent the mean ± SEM of three independent experiments in all parts; all experiments were performed in triplicates. (***p<0.001, **p<0.01)

5.4.3 ATP is released from macrophages or dying cells during their co-incubation leading to P_2X_7 receptor activation

Next, we aimed to test the possible contribution of ATP to inflammasome activation in macrophages during engulfment of autophagic dying cells. Apyrase treatment inhibited the secretion of IL-1β during co-incubation with autophagic dying MCF-7 cells (Figure 12A, upper left panel). Moreover, macrophages continued to release ATP after washing away the dying cells and further incubated them with fresh medium that shows that ATP

was released from human monocyte derived macrophages but not from dying cells. Consistently, apyrase treatment of autophagic MCF-7 cells secreted same amount of IL-1β during phagocytosis with human macrophages (Figure 12A-upper right panel). The hydrolysis of secreted ATP by apyrase during phagocytosis led to the decrease of IL-1β release from mouse macrophages as well (Figure 12B- left panel). A significant amount of ATP (in the 400-500 nM range) was detected in the culture medium obtained after the co-incubation of mouse resident peritoneal macrophages and autophagic dying Ba/F3 cells in the absence of serum (Figure 12B-left panel). We have further blocked the purinergic receptors by using 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), the activation of the purinergic receptor P_2X_7 by released ATP is essential for inflammasome activation in both human and mouse macrophages engulfing autophagic dying cells (Figure 12A-bottom panel and 12B-right panel).



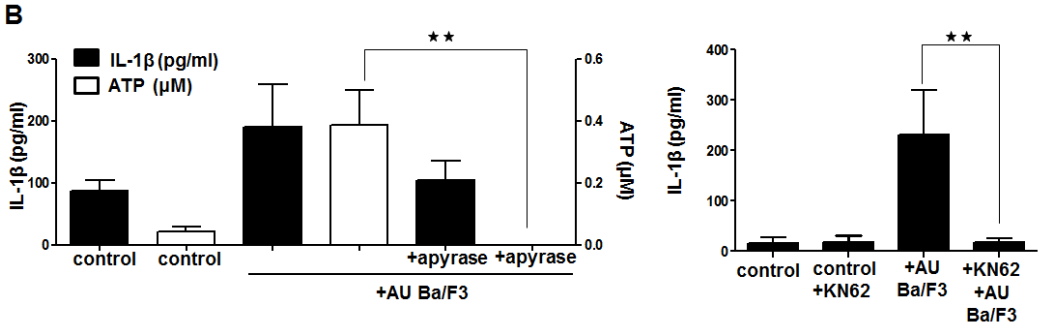


Figure 12: Secreted ATP coupled to P_2X_7 purinergic receptor stimulation activating inflammasome in macrophages (A-upper right and left panel) ATP and IL-1β release from human macrophages and THP-1 cells engulfing autophagic dying MCF-7 cells (AU MCF-7) were measured. (A-bottom panel) Release of IL-1β is shown on western blot of concentrated serum free culture fluid from human macrophages engulfing either living or autophagic dying MCF-7 cells. The effects of apyrase and P_2X_7R inhibitor KN-62 on this process are shown in figure. **(B)** Primed resident macrophages were treated with ATP diphosphohydrolase (apyrase), the purinergic receptor inhibitor (KN-62) and co-incubated with autophagic dying Ba/F3 cells (AU Ba/F3). ATP concentrations were measured from co-culture media of macrophages and the culture medium which was collected from Ba/F3 cells during 6 hrs of IL-3 depletion (IL-3 depleted and serum containing medium). Data represent the mean ± SEM of three (for part A), two (for part B-left panel) and four (for part B-right panel) independent experiments; all experiments were performed in triplicates. (** $p<0.01$)

5.4.4 Contribution of pannexin-1 channels to inflammasome activation

In order to analyze the contribution of pannexin-1 channel in inflammasome activation we used CBX, a specific pannexin-1 channel inhibitor [100] to block its activity during co-incubation of autophagic dying Ba/F3 cells and macrophages. We found that CBX treatment inhibited IL-1β release from either resident macrophages or autophagic dying Ba/F3 cells (Figure 13A). Furthermore, the pannexin-1 channel inhibitor also blocked ATP secretion showing that ATP was released through these channels (Figure 13A). In order to determine the source of ATP release we measured ATP in the CM from autophagic dying Ba/F3 cells cultured alone (Figure 13B). ATP release in the μM range from autophagic dying Ba/F3 cells

could be inhibited by CBX (Figure 13B). We further measured ATP and IL-1β from the medium of macrophages alone upon the removal of autophagic dying Ba/F3 cells during additional 2 hrs (Figure 13C).

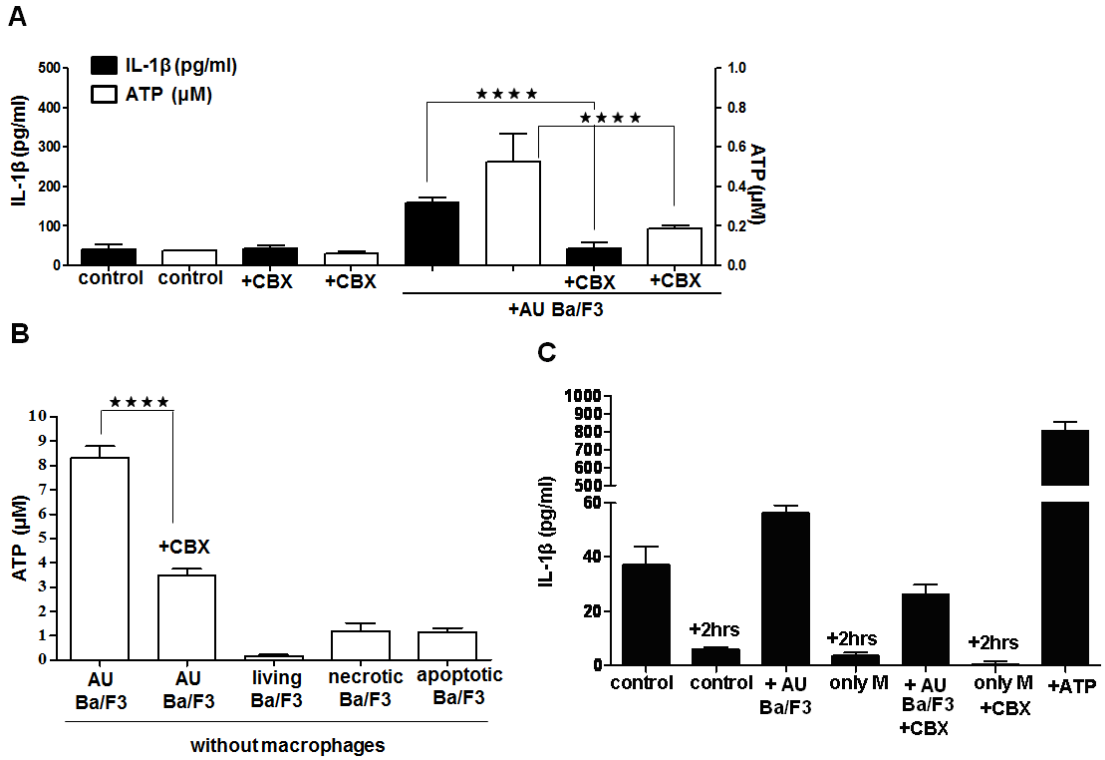


Figure 13: ATP is released through pannexin-1 channel from autophagic dying Ba/F3 cells and contributes to inflammasome activation in mouse macrophages Primed resident macrophages were treated with pannexin-1 channel inhibitor (CBX) and were co-incubated with autophagic dying Ba/F3 cells (AU Ba/F3). **(A,B)** ATP concentrations were measured from co-culture media of macrophages and the conditioned medium (CM) which was collected from Ba/F3 cells during 6 hrs IL-3 depletion (IL-3 depleted and serum containing medium) and CBX treated/non-treated autophagic dying Ba/F3 cells (AU Ba/F3) (in serum free medium without macrophages). **(C)** Macrophages were incubated alone further 2 hrs in fresh medium and IL-1β and ATP were measured from the supernatant. Data represent the mean ± SEM of four (for part A), three (for part B) independent experiments; all experiments were performed in triplicates. (★★★★p<0.0001)

5.5 Inflammatory response in peritoneal cavity of mice exposed to autophagic dying Ba/F3 cells

In our attempt to support our *in vitro* results showing that dying autophagic Ba/F3 cells are pro-inflammatory, we injected dying autophagic cells into the peritoneum of mice. We observed influx of neutrophils into the peritoneal cavity, indicating that the dying autophagic cells induced an acute inflammatory response *in vivo* as well (Figure 14). Living, necrotic and apoptotic Ba/F3 cells (10 μM doxorubicin) were also injected i.p. Apoptotic cells recruited neutrophils, monocytes and eosinophils, but they led to the decrease of macrophages resident in the peritoneum (Figure 14). Living, autophagic and necrotic cells also diminished the number of macrophages. Necrotic and living cells could not induce neutrophil influx. Only necrotic and apoptotic cells recruited eosinophils into the peritoneal cavity.

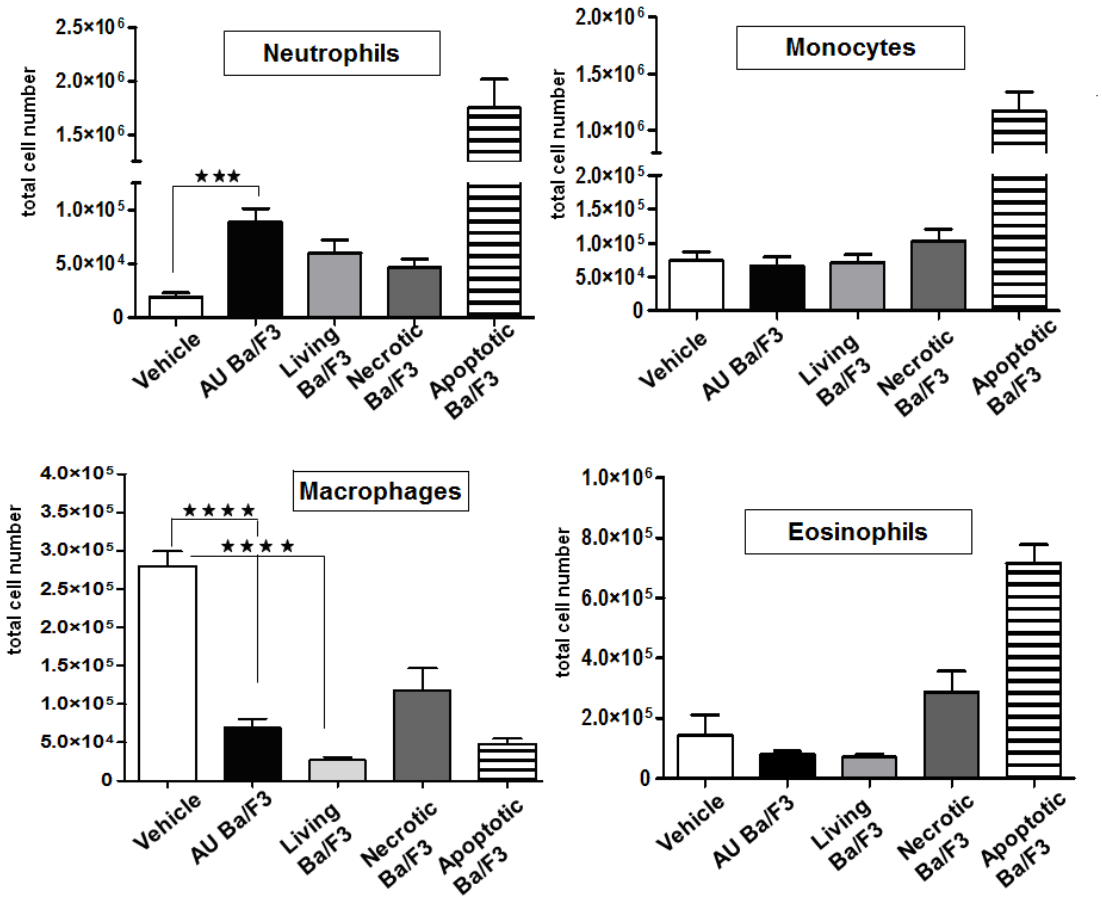


Figure 14: Intraperitoneal injection of dying cells induces a sterile inflammatory response (neutrophil influx) Dying autophagic, apoptotic or necrotic cells and living cells were intraperitoneally injected into wild type BALB/c mice. Equal volumes of D-PBS were injected in mice as negative controls. Peritoneal exudate cells (PECs) were collected 16 hrs later and monocytes, macrophages, eosinophils and neutrophils were stained with anti-mouse antibodies F4/80-APC with CD11b-APC-Cy7 and Ly-6G-APC with CD11b-APC-Cy7 and analyzed on BD LSR-II. Graphs represent the number of macrophages ($F4/80^{high}$ $CD11b^{high}$), monocytes ($F4/80^{medium}$ $CD11b^{high}$), eosinophils ($F4/80^{medium}$ $CD11b^{medium}$) and neutrophils ($CD11b^{+}$ $Ly6G^{high}$) in PECs after injection of AU, necrotic, living or apoptotic Ba/F3 cells. Upon sterile-PBS injection, cells which were collected from peritoneal cavity were named as 'vehicle' in graphs. (***p<0.001, ****p<0.0001)

6. Discussion

6.1 Autophagy contributes to different types of cell death mechanisms in MCF-7 and Ba/F3 cells

Autophagy can contribute to cell death according to cell type, inducer and treatment [117]. Autophagy can lead to cell death (cell death through or with autophagy) or preceed apoptosis. In estrogen-dependent human breast adenocarcinoma cell line (MCF-7), cell death can be induced through autophagy with estrogen depleted charcoal-stripped-fetal calf serum (FCS) (DCC) and concentration dependent anti-estrogen tamoxifen treatment [35,36]. Tamoxifen increases the ceramide levels in cells and eliminates the inhibitory effect of class-I PI3K pathway and up-regulation of Beclin 1 [118]. Upon estrogen depletion and anti-estrogen treatment, majority of dying MCF-7 cells contained autophagic vacuoles (AVs) as an early and predominant feature of cell death whereas a minority of cells showed apoptotic characteristics [35]. High amount of AVs, pyknotic type of chromatin condensation and numerous AVs containing chromatins have been observed during cell death induction [35,36]. The organelles required for protein synthesis such as polyribosomes, ER, Golgi were disappeared and only few number of intact mitochondria were observed in a very close proximity to AVs and nuclear membrane [35] (Figure 15).

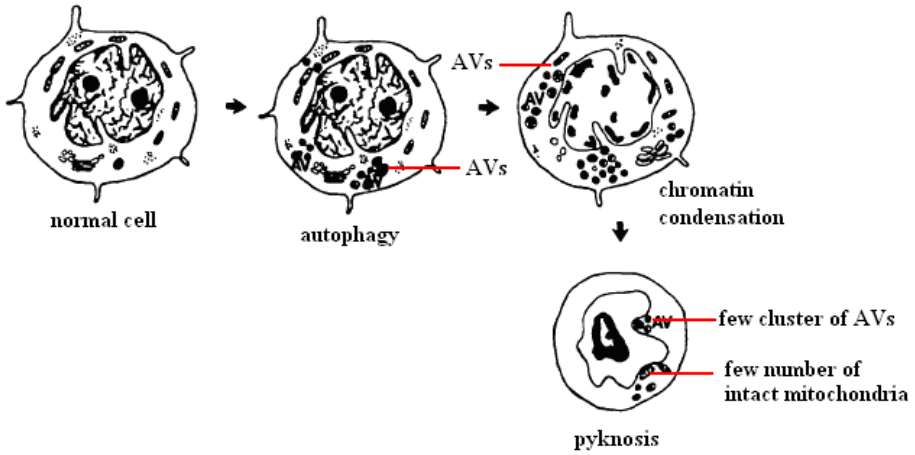


Figure 15: Proposed stages of death in MCF-7 cells upon hormone depletion and tamoxifen treatment Upon tamoxifen treatment, autophagic vacuoles (AVs) are formed which is followed by heterochromatin condensation and detachment from nuclear envelope with few polyribosomes. At the stage of pyknosis, condensed chromatin in the center of still intact nuclear envelope is observed.

Figure adapted from [35].

When the tumor cells which are apoptosis defective are under metabolic stress, autophagy can lead to survival first and then cell can die eventually with excess amount of autophagy [42]. MCF-7 cells are caspase-3 deficient and it is a good model of tumor cell line to induce autophagic cell death by prolonged metabolic stress conditions [35]. Another type of cell death can be associated with autophagy in MCF-7 cells due to the detachment of cells from surface (anoikis). In both autophagic dying MCF-7 cells, the autophagy induction and cell death can be prevented by autophagy inhibition with 3-MA shows that autophagy is directly related to these cell death types [35,36].

We observed increased autophagy in dying murine bone-marrow derived pro-B-cell line (Ba/F3 cells) after IL-3 depletion by anti-LC3 antibody and acridine orange staining as

well as by increased level of LC3-II analysis. These data are in line with previously published results obtained with Ba/F3 cells, which showed that IL-3 dependent murine Ba/F3 pro-B cells respond to growth factor withdrawal by inducing autophagy as a survival mechanism [30]. We have also observed that cells are dying with IL-3 withdrawal. Wirawan et al have already shown that autophagy preceeds apoptosis in IL-3 depleted Ba/F3 cells and there is a crosstalk between apoptosis and autophagy due to Beclin 1 [119] and PI3KC3 cleavage by caspases which implies that Beclin 1 has a pro-apoptotic function [30,120]. Beclin 1 forms a platform with particular proteins and assembles the PI3KC3 complex in order to initiate the autophagosome formation [120]. Upon IL-3 depletion, Beclin 1 protein is cleaved caspase-dependently and Beclin-1C fragments induce the release of cytochrome-c and HtrA2/Omi from mitochondria and apoptosis occurs whereas autophagy stops [30] (Figure 16). Our results indicated that during IL-3 depletion, Ba/F3 cells died more upon inhibition of autophagy with 3-MA which supports the finding that autophagy is for survival in Ba/F3 cells during IL-3 depletion. On the other hand, autophagy related protein Beclin 1 is needed to be cleaved and its cleaved fragment is essential to induce mitochondria dependent apoptotic cell death. It has not yet been shown which caspases can cleave Beclin 1 and how they are involved in this process. Besides, it was also observed that IL-3 withdrawal leads Ba/F3 cells to stay in G0/G1 phase [121] and the most apoptosis-resistant Ba/F3 cells can use autophagy-derived nutrients under growth factor depletion. However, autophagic activity can be a sensitizer for these cells and they become more prone to die by apoptosis [122].

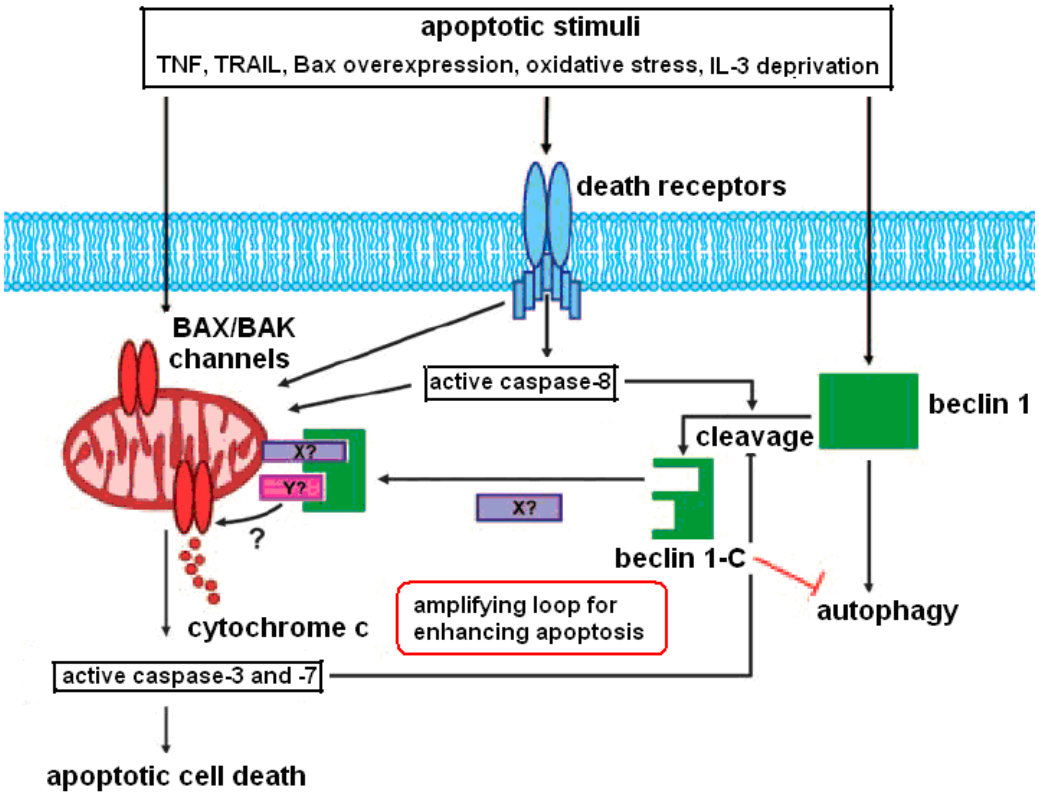


Figure 16: Beclin 1 links autophagy to apoptosis Stimuli such as IL-3 depletion, nutrient deprivation, oxidative stress can induce death receptor- and mitochondria- mediated apoptosis which may also provoke autophagy. Beclin 1 can be cleavaged by caspases into two fragments called Beclin-1C and Beclin-1N. Upon cleavage, beclin 1 becomes incapable of inducing autophagy. One of the fragment of beclin 1 (Beclin-1C) translocates to the mitochondria and leads to cytochrome-c release which further activates caspases for apoptotic cell death.

Figure adapted from [120]

Recently, it has been shown that IL-3 depletion in Ba/F3 cells causes the RIP1 cleavage and the released HtrA2/Omi from mitochondria has roles in caspase-independent cell death by cleaving RIP1 [123]. Additionally, Beclin1 also interacts with the anti-apoptotic proteins Bcl-2 and Bcl-x_L (B-cell lymphoma extra long) which may imply that Beclin 1 has roles in the crosstalk between autophagy and apoptosis [124,125].

Discussion

It was recently shown that accumulation of AVs in glioblastoma cell lines following treatment with a lysosome inhibitor [126] results in a sustained imbalance, in which the rate of autophagic vacuole formation exceeds the rate of autophagic vacuole degradation and promotes development of autophagic stress predisposing to neuronal and glial cells death [127]. Increased autophagy after IL-3 depletion was demonstrated in dying Ba/F3 cells by acridine orange staining and punctuate pattern which represents autophagosomes detected with anti-LC3 antibody, as well as by the increased level of LC3-II. These data are in line with previously published results obtained with Ba/F3 cells, which showed that IL-3 dependent murine Ba/F3 pro-B cells respond to growth factor withdrawal by inducing autophagy as a survival mechanism [30]. Moreover, it has been shown that increased autophagic activity sensitizes Ba/F3 cells for apoptosis through caspase-dependent generation of beclin-1 cleavage fragments and degradation of type III PI3K [30]. IL-3 withdrawal leads Ba/F3 cells to stay in G0/G1 phase [121] and the most apoptosis-resistant Ba/F3 cells can use autophagy-derived nutrients under growth factor depletion. However, autophagic activity can be a sensitizer for these cells and they become more prone to die by apoptosis [122]. We wanted to clarify that IL-3 depletion leads to upregulation of autophagic flux or blockage of autophagic flux (degradation block). LC3-II itself is degraded during the autophagic cycle and increased amount of LC3-II protein at a certain condition and time can not represent by itself the true dynamics of autophagy. When the lysosome inhibitor is added to a cell with increased autophagy and LC3-II level the latter is not changed if the higher number of autophagic vesicles is due to blocking of the autophagic cycle but inhibited in case of increased autophagic flux [16,113]. We have treated our IL-3 depleted/non-depleted Ba/F3 cells with lysosomal inhibitor (CQ) and found that blocking the fusion of autophagosomes with lysosomes by CQ treatment led to accumulation of LC3-II, which further proved that the increased autophagy after IL-3 withdrawal was due to up-regulation of autophagosome formation (autophagic flux) and not to lysosomal blockage. If the IL-3 withdrawal would have resulted in blocking of autophagosome degradation, we would

not have seen any difference in the increased LC3-II levels with CQ treatment. We have also shown that blockage of the lysosomal pathway by CQ treatment increases the percentage of cell death when the cells are depleted of IL-3. When we added CQ to living cells (IL-3+), LC3-II accumulated due to blocking of the basal autophagic activity, but there was no increase in cell death.

6.2 Dying cells with autophagic features are pro-inflammatory and induce inflammasome pathway in macrophages while can inhibit LPS-induced pro-inflammatory cytokine response

Autophagic dying MCF-7 and Ba/F3 cells can both lead to pro-inflammatory cytokine response in human and mouse macrophages via inflammasome activation, respectively. We have shown that IL-1β release from both human and mouse macrophages co-incubated with autophagic dying cells is caspase-1 dependent by using specific caspase-1 inhibition and caspase-1 deficient macrophages. We then checked whether or not the NALP-3 inflammasome plays a role in this pro-inflammatory response. ATP-mediated IL1-β release from NALP-3 knocked down human THP-1 cells or NALP-3 deficient mouse macrophages was almost completely prevented. When we co-incubated NALP-3 deficient mouse macrophages with autophagic dying Ba/F3 cells, the released IL-1β was significantly less than control macrophages but a lower level of inflammasome activation was still observed. Even the known NALP-3 inducer ATP mediated IL1-β release [92] from NALP-3 deficient macrophages was not completely prevented either. Ultra-pure LPS priming of macrophages also led to weaker inflammasome activation in the knockout macrophages. We do not claim that only NALP-3 inflammasome can be activated in macrophages while engulfing dying autophagic cells. We cannot exclude the possibility that inflammasome complexes other than NALP-3 might also be activated by engulfed autophagic dying cells, especially in mice developing in and compensating for the absence of NALP-3. It was recently shown that there is cooperation between NLRP3 and NLRC4 inflammasomes *in vivo* during *S. typhimurium* infection, and that deficiency

of either NLRP3 or NLRC4 does not change the bacterial infection in the mice [128,129]. On the other hand, caspase-1 activity in NLRP3 deficient obese mice decreases but is not abolished in the presence of inflammasome inducers [128,130], suggesting that inflammasome "priming" [76] plays a role at least in the pathophysiology of obesity. Additionally, it is also known that components of the NLRP1 and NLRC4 inflammasomes are constitutively expressed in cells, whereas NLRP3 transcription is triggered by bacterial components through the TLR4 pathway, a process that is also called "priming" [76].

Though 3-MA is a non-specific chemical inhibitor for autophagy we can see the decrease in IL-1β release from both human and mouse macrophages which is co-incubated with autophagy inhibited dying cells. These data indicated that only cells dying with autophagic features due to growth factor depletion result in inflammasome activation in macrophages. Both autophagic dying MCF-7 and Ba/F3 cells are needed to be internalized by macrophages to induce inflammasome pathway. Unlike human macrophages, mouse macrophages had to be primed with ultra pure LPS in order to induce and accumulate the pro-IL-1β which was further cleaved by NALP-3 inflammasome.

Classical apoptotic cells have a strong inhibitory effect on the TLR-mediated, NF-κB dependent inflammatory response of macrophages [51]. This well known anti-inflammatory effect of apoptotic cells can be mediated by cell surface interactions and it may not even require phagocytosis of dying cells [51]. Tamoxifen treatment and estrogen depletion lead to MCF-7 cells to die through autophagy but not much apoptosis. We have showed that autophagic dying Ba/F3 cells are both caspase-3 and LC3-II positive whereas apoptotic Ba/F3 cells died without up-regulating autophagy. We have observed that autophagic dying MCF-7 and Ba/F3 cells can both initiate pro-inflammatory cytokine response in macrophages via inflammasome dependent manner and also inhibit the LPS induced and NF-κB dependent pro-inflammatory as apoptotic cells. Anti-inflammatory features of autophagic dying cells, similarly to apoptotic cells in general, are most

probably due to the surface molecules on cells which can down-regulate the NF-κB dependent transcription. For instance, PS exposure is a well known characteristic of apoptotic cells to be anti-inflammatory and these autophagic dying MCF-7 and Ba/F3 cells expose PS upon autophagy and cell death induction. On the other hand, unlike apoptotic cells, autophagic dying MCF-7 cells alone provoked IL-6 and TNF-α pro-inflammatory cytokine release even though they were anti-inflammatory when added with crude LPS. Most probably, IL-1β released upon inflammasome activation acted in a paracrine or autocrine way resulting in the production of IL-6 and TNF-α. To prove this possibility, we used IL-1β receptor antagonist anakinra and we could prevent the secretion of IL-6 and TNF-α.

Next, we checked whether or not autophagic dying Ba/F3 cells with autophagic stress due to autophagosome accumulation can also lead to an inflammatory response in macrophages. Autophagosome accumulation by itself, induced by CQ treatment in the presence of IL-3, did not induce cell death and was not sufficient to cause caspase-1 activation when these cells were engulfed. Even increased autophagy in dying cells during surface detachment (anoikis) in MCF-7 cells did not induce inflammasome activation. Therefore, it appears that cell death of target cells has to be initiated by autophagy or at least autophagy has to sensitize cells for apoptosis to create the molecular pattern needed for inflammasome activation following phagocytosis of these cells. This conclusion is supported by the finding that a combination of IL-3 depletion and lysosomal inhibitor treatment promotes higher rates of cell death, leads to more efficient engulfment of dying cells and stronger induction of the inflammasome activating pathway, together with the release of more IL-1β from the engulfing macrophages. Our results are valuable since there are phase I/II trials of lysosomal inhibitors in combination with an autophagy and they are currently being developed in several malignancies including breast cancer and lymphoma [131].

Apoptotic MCF-7 or Ba/F3 cells could not induce inflammasome activation in human and mouse macrophages during their engulfment, respectively. According to our results, higher concentration of doxorubicin treated apoptotic Ba/F3 cells did not exert increased autophagic activity as well as did not lead to inflammasome activation in macrophages. However, we have observed elevated autophagic activity with lower concentrations of doxorubicin treatment in Ba/F3 cells. It was observed that autophagy can be induced as a survival mechanism in 3T3 fibroblast cells during DNA damage due to doxorubicin treatment [62]. Recently, it has also been demonstrated that doxorubicin-induced immunogenic apoptotic cells can be recognized and responded to by the TLR-2/TLR-9-Myeloid differentiation primary response gene (88) (Myd88) signaling pathway; this finding provides an alternative explanation for their pro-inflammatory effect [55]. On the other hand, necrotic Ba/F3 cells which necrosis was induced by freeze/thaw method could also not induce inflammasome activation in macrophages. Our results are in line with studies which have reported that necrotic neutrophils via freeze/thaw method can release powerful peptides called as α-defensins which have an anti-inflammatory effects on human macrophages and protect the mice from peritonitis whereas they still have anti-microbial activity [132]. Besides, in our experiments, necrostatin treatment of autophagic dying MCF-7 and Ba/F3 cells did not prevent inflammasome activation during engulfment. This shows that necroptosis was not involved in this pro-inflammatory cell death process.

6.3 Exogeneous ATP released from either macrophages or dying cells is required for purinergic receptor activation for inflammasome activation in macrophages

Several mechanisms that are not mutually exclusive have been proposed to explain how NALP-3 inflammasomes are activated. One of the general mechanisms of inflammasome activation involves extracellular ATP, which generates an activation signal via the purinergic P_2X_7 receptors, followed by rapid K^+ efflux from cytosol leading to low intracellular K^+ levels [85]. Physiologic concentrations of intracellular K^+ can prevent inflammasome assembly and for instance monosodium urate crystals can lead to

inflammasome activation through K^+ efflux from macrophages. Based on these observations, it has been proposed that a lowered K^+ concentration in the cell is a common trigger of inflammasome activation [90]. Here, we have demonstrated that this also takes place when autophagic dying MCF-7 and Ba/F3 cells are taken up by human and mouse macrophages, respectively. In our experiments, inflammasome activation by these autophagic dying cells could be decreased by incubating macrophages with dying cells in a medium containing high concentration of K^+, which prevents K^+ efflux. Inhibition of K^+ efflux also decreased the basal levels of IL-1β released from ultra-pure LPS pre-treated mouse macrophages. This raises the possibility that during recognition and engulfment of autophagic dying cells by macrophages, ATP released in the extracellular space and initiates the above described sequence of events. ATP is a known non-microbial NALP-3 agonist, and different PAMPs and DAMPs have been shown to lead to ATP release from monocytes followed by autocrine stimulation of purinergic receptors such as P_2X_7 [133]. Hydrolyzing ATP by apyrase or blocking the P_2X_7 receptor by a specific antagonist during phagocytosis of both autophagic dying MCF-7 and Ba/F3 cells cells reduced IL-1β secretion. Indeed, we found that a substantial amount of ATP was released during co-incubation of the dying MCF-7 and Ba/F3 cells with macrophages.

During co-incubation of human macrophages and autophagic dying MCF-7 cells, ATP was released from macrophages and it was shown by measuring the higher amount of ATP during the additional 2 hrs incubation of macrophages alone compare to control macrophages after washing away the dying cells. Autophagic dying MCF-7 cells did not release ATP when they were incubated alone. Different from human data, autophagic dying Ba/F3 cells but not mouse macrophages secreted ATP during co-incubation. Moreover, during the additional 2 hrs incubation of macrophages after removal of autophagic dying Ba/F3 cells, we did not quantify any ATP. When we incubated autophagic dying Ba/F3 cells with the absence of IL-3 but in the presence of serum, we could not detect ATP in the conditioned media. However, high amount of ATP was

secreted from the dying cells while they were incubated alone in the absence of macrophages and serum. After 2 hrs of incubation, we have observed that 8–9 μM ATP is released from autophagic dying Ba/F3 cells when they are incubated alone, but the concentration of released ATP is only 0.4–0.6 μM during the co-incubation of ultra-pure LPS primed macrophages with autophagic dying Ba/F3 cells. It is again possible that ecto-ATPases were present in the medium during the co-incubation, which would diminish the amount of ATP released from autophagic dying cells. When we checked whether living, necrotic and apoptotic Ba/F3 cells release ATP, we observed that living cells did not release ATP whereas necrotic and apoptotic cells secreted about 1 μM ATP when they were not co-incubated with macrophages. Since necrotic and apoptotic cells could not up-regulate IL-1β release from macrophages, we assume that this small amount of ATP is also neutralized by ecto-ATPases, preventing activation of the purinergic receptors. Ecto-ATPases (extracellular (E)-ATPases or ecto-apyrase) are a group of ectonuclesidases family members which are expressed on plasma membrane and externally oriented active sites oriented by magnesium and calcium [134,135]. These enzymes can change the concentration of nucleotides which further modulate P2-receptor mediated signaling for instance in nervous system in a dose dependent manner [134].

It has been shown that secretory organelles in cells may store high amount of ATP which may be released and act as a danger signal [67]. Upon physical or physiological stress, adrenal medullary chromaffin granules contain around 100mM, platelet-dense granules contain around 500mM ATP which is very high according to cytosolic ATP concentrations [67]. We have shown that extracellular ATP engages with purinergic receptors, P_2X_7, on both human and mouse macrophages in order to initiate inflammasome activation with autophagic dying cells. Our observations are in line with other studies which showed that ATP released from dying tumor cells acts on P_2X_7 purinergic receptors of dendritic cells, which can induce inflammasome activation and further IL-1β secretion [136]. It was also shown that certain types of necrotic cells can also release ATP and activate the NALP-3 inflammasome in engulfing macrophages

[137]. ATP interaction with P_2X_7 receptors can also have effects on cell death, cell fusion, proliferation and bone formation different than its roles in immune system and inflammation [64]. For instance, stimulation of P_2X_7 receptors can lead PS to flip from the inner leaflet to the outher leaflet on lymphocytes [64].

The pannexin-1 channel was identified as a plasma membrane channel mediating the regulated release of ATP and uridine triphosphate (UTP) (both are "find me" signals for phagocytes) from apoptotic cells as a consequence of its caspase-3 dependent activation [138]. Blocking pannexin-1 channels during co-incubation of mouse macrophages with autophagic dying Ba/F3 cells led to inhibition of ATP release as well as inflammasome activation, which indicates that this channel was involved in the ATP secretion. Using short hairpin (sh)RNA to silence pannexin-1 channels in neurons and astrocytes, it was also demonstrated that pannexin-1 channels are needed for inflammasome activation [139]. Inhibition of the pannexin-1 channel in J774 macrophages shows that the pannexin-1 pathway is essential for caspase-1 activation and mature IL-1β release [100,107,140]. On the other hand, a recent observation shows that in macrophages of pannexin-1 deficient mice, most of the known inflammasome activators can elicit caspase-1 activation, IL-1β maturation and secretion [105], indicating that pannexin-1 is dispensable for the assembly of caspase-1 mediated complexes. However, these mice are deficient in ATP release from cells, including apoptotic cells, and this deficiency is in line with several studies on various cell types showing that this channel is a candidate for ATP release [141].

Based on these data, we believe that ATP is released from autophagic dying Ba/F3 cells through pannexin-1 channels and triggers inflammasome activation in the macrophages engulfing the autophagic dying Ba/F3 cells via P_2X_7 activation pathway. We also found that Ba/F3 cells had to be internalized by macrophages to induce inflammasome activation and IL-1β release. Lack of IL-1β in the medium of the autophagic dying Ba/F3 cells excluded its production by them. On the other hand, CM of autophagic dying MCF-7

and Ba/F3 cells did not contain any inducer for inflammasome meaning that no inducer of IL-1β activation released from autophagic cells dying in the presence of serum. (Figure 17)

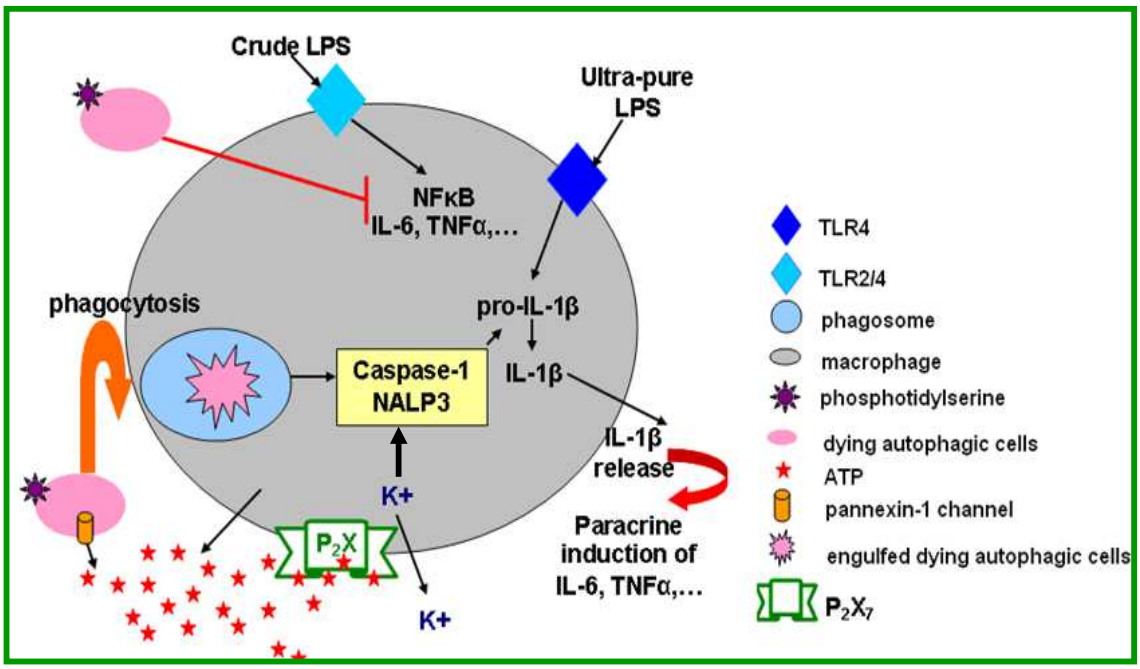


Figure 17: Proposed model of inflammasome activation with autophagic dying cells In our proposed model, similarities and differences in human and mouse macrophages engulfing dying cells carry autophagic features lead to NALP-3 inflammasome activation. Upon, phagocytosis, process begins with P_2X_7 purinergic receptor activation with exogenous ATP. ATP is released either from macrophages or autophagic dying cells depend. K^+ efflux from cytosol leads to NALP-3 inflammasome activation and subsequent IL-1β maturation and secretion.

6.4 Autophagic dying cells display acute inflammatory features by recruiting high amount of neutrophils *in vivo*

Our *in vivo* results show that neutrophil influx (a sign of an acute inflammatory response) was triggered by injection of autophagic dying Ba/F3 cells in the peritoneal cavity of mice. It was also observed that both viable and necrotic Ba/F3 cells could recruit neutrophils into the peritoneum, though to a lesser extent than autophagic dying ones. It is very likely that injected "viable" cells start to die with increased autophagy due to the lack of IL-3 cytokine in the peritoneal cavity. Necrotic cells were frozen-thawed only once and residual intact cells or cellular parts could have been destroyed in the peritoneal cavity. Release of the contents of these newly destroyed cells could recruit neutrophils and eosinophils to the peritoneal cavity. Of note, doxorubicin-killed apoptotic Ba/F3 cells were the most potent inducers of neutrophil attraction in the peritoneum. This is understandable because doxorubicin-treated apoptotic cells have been shown to be the most potent inducers of acute inflammation *in vivo* in several models. Injection of doxorubicin into the peritoneal cavity of mice triggers a rapid neutrophil influx that is associated with the apoptosis of monocytes/macrophages [55]. Therefore, when doxorubicin-killed cells are injected into the peritoneum, doxorubicin itself leaking from the apoptotic cells might induce the death of peritoneal cells. This effect can lead to the recruitment of immune cells to the peritoneum (e.g. neutrophils) as shown by Krysko et al. [55]. Another study has shown that an immunogenic form of apoptosis was induced by mitoxantrone, another prototype of anthracyclines [142,143]. Stimulation of cancer cells with mitoxantrone results in the recruitment of dendritic cells and T lymphocytes to the site of the tumor bed *in vivo* [144]. It has also been shown that this property of dying cancer cells depends on their autophagic features, but this is not relevant for our data because we have shown that apoptotic Ba/F3 cells treated with 10 μM doxorubicin do not exhibit autophagic features. Therefore, it is likely that cells (autophagic dying cells or apoptotic or necrotic cells) that we injected contained danger signals independent of their

dying and autophagic status and that these signals could induce an acute inflammatory response.

Immunogenic cell death in tumor cells lead to immune responses with certain features such as early extracellular secreted ATP, cell surface exposure of calreticulin and/or heat shock proteins (such as HSP70, HSP90) and late release of a pro-inflammatory factor high mobility group box 1 (HMGB1) [145,146]. ATP can be released from damaged cells, dying cells, endothelial or epithelial cells via nonlytic but mechanical stresses such as shear stress, compression, hydrostatic pressure, changes and hypotonic shock [67]. It has been shown that wide range of chemotherapeutics can induce ATP release from tumor cells which is the endogenous inducer with a highest affinity for P_2X_7 receptors [147]. Mitoxantrone (MTX) is injected into an intratumoral area in mice and autophagy competent cancers recruit dendritic cells and T lymphocytes into the tumor [144]. Treatment of cancer cells with anticancer chemicals (such as oxiplatin and mitoxantrone) leads cancer cells to die in an immunogenic manner [144]. It has been shown that dying cells are autophagic and induce an immunogenic response *in vivo* by recruiting dendritic cells and T cells into the tumor by releasing ATP into the extracellular fluid [144]. The authors also indicated that the immunogenicity of dying cells depends on autophagy mediated release of ATP [144]. Our results showed that ATP released from autophagic dying Ba/F3 cells and phagocytosis of autophagic dying cells play a role in inflammasome activation in macrophages. However, human macrophages release ATP during engulfing autophagic dying MCF-7 cells which can imply that there may be other types of dangerous molecules originated from autophagic dying MCF-7 cells which can make them immunogenic. On the other hand, it has been shown that doxorubicin induces immunogenic cell death in cancer cells through the calreticulin exposure pathway as well as inflammasome activation in the phagocytic cells [148]. However, knocking down calreticulin in autophagic dying MCF-7 cells did not prevent inflammasome activation in macrophages taking up these cells.

Autophagy deficient tumor cells failed to induce T and dendritic cell dependent immune response *in vivo* due to the inhibition in releasing ATP from dying cells [144]. Furthermore, such an immunogenic response can also be elicited when autophagy and cell death is induced by cytokine depletion. When we treated Ba/F3 cells with doxorubicin, which is also a known immunogenic anticancer drug, less ATP was released than from autophagic dying cells, and this smaller amount was not sufficient to induce inflammasome activation *in vitro*. This raises the possibility that regulation of the intensity of autophagy and thereby ATP release in dying tumor cells might be important for achieving an effective immunogenic response in the host, like the effect of increasing ATP levels in the tumor environment by inhibiting ecto-ATPases [144]. Kroemer et al have shown that when the tumor is autophagy deficient and tumor itself is treated by chemically by inhibitor of ecto-ATPases, increased intracellular ATP concentrations re-establish the anti-tumoral T and dendritic cell dependent immune response *in vivo* through purinergic receptor dependent way [144].

6.5 Immunogenic autophagic cell death induction can be a useful way for cancer and inflammatory disease treatment through inflammasome activation

Basic research studies on cancer treatment may serve for clinical research which can be useful for treatment of patients. It is important to determine which chemotherapies have the capacity to induce immunogenic cell death to eradicate tumors. For instance, recently it has been shown that dying autophagic tumor cells release ATP and it activates P_2X_7 receptors on DCs which lead to NALP3 inflammasome activation in DCs [136]. The group has also observed that IFN-γ–producing CD8+ T cells cannot be primed by dying tumor cells in the absence of functional IL-1 receptor or in NLRP3 and Caspase-1 deficient mice [136]. It was also mentioned that under treatment with anthracyclines, breast cancer patients with loss-of-function allele of P_2X_7R develop metastatic disease more rapidly than ones who have normal alleles [136]. Our studies also contribute to

basic research literature by providing the upstream inflammasome activation mechanisms which is triggered by tumor cells dying through autophagy cells in different types of mouse and human macrophages. Novel, autophagy targeted therapeutic interventions for cancer and other inflammatory diseases may be designed and tested based these observations.

7. Summary

Phagocytosis of PAMPs, DAMPs and certain dying cells can activate the inflammasome pathway in macrophages. In our study, we show that both human and mouse macrophages display a pro-inflammatory response to autophagic dying MCF-7 and Ba/F3 cells, but not to living, apoptotic, necrotic or necrostatin-1 treated ones. When we investigated this phenomenon, further it was found that caspase-1 was activated and IL-1β was processed and then secreted in a MyD88-independent manner. Neither caspase-1 inhibited nor caspase-1 deficient macrophages could trigger IL-1β release due to the lack of key component for pro-IL-1β cleavage and maturation before its secretion. Next we clarified which inflammasome is activated by autophagic dying cells and found that NALP-3 deficient macrophages displayed reduced IL-1β secretion, which was also observed in macrophages in which the NALP-3 gene was knocked down. Next, we investigated the upstream mechanism of NALP-3 inflammasome activation triggered by autophagic dying cells. Our results show that during phagocytosis of autophagic dying MCF-7 and Ba/F3 cells exogenous ATP is acting through P_2X_7 receptor, initiates K^+ efflux, inflammasome activation and secretion of IL-1β from human and mouse macrophages. Calreticulin exposure on autophagic dying MCF-7 cells do not play role in inflammasome activation. ATP was secreted from human macrophages during co-incubation with autophagic dying MCF-7 cells which did not release ATP. However, autophagic dying Ba/F3 cells were the source the ATP which activated the P_2X_7 receptor and lead to inflammasome activation in mouse macrophages. We further showed that pannexin-1 channel is responsible for ATP secretion from autophagic dying Ba/F3 cells. MCF-7 and Ba/F3 cells dying with involvement of autophagy were capable of preventing crude LPS-induced pro-inflammatory cytokine release but pro-inflammatory cytokines were produced and secreted from human macrophages triggered by autophagic dying cells as a result of the secreted IL-1β. Finally, it was observed that injection of autophagic dying cells intraperitoneally induced an acute inflammatory reaction by recruiting neutrophils and monocytes/macrophages.

8. Perspectives

According to our results, NALP-3 inflammasome assembly, pro-caspase-1 cleavage and further pro-IL-1β maturation and secretion are induced following the engulfment of autophagic dying cells cells by macrophages and subsequent ATP-P_2X_7 purinergic receptor interaction. Human macrophages release ATP during engulfing autophagic dying MCF-7 cells whereas autophagic dying Ba/F3 cells release ATP upon being cleared by mouse macrophages. Since phagocytosis of autophagic dying Ba/F3 cells is also required for inflammasome activation, it should be clarified how the engulfment process and/or the specific components of the autophagic dying cells sensitize macrophages for the ATP-dependent NALP-3 activation. DAMPs can be exposed on or released from autophagic dying cells and trigger inflammasome activation inside macrophages and future studies should focus on identifying the molecular pattern associated with autophagic cells. DNA or caspase-3 exposure on autophagic dying cells, engulfed apoptotic bodies which carry different organelles, factors released from autophagic dying cells, such as HMGB1, can be candidates to induce NALP-3 inflammasome activation in macrophages. It can be valuable to continue the experiments that will show that which soluble factors are released from autophagic dying cells for the recruitment of monocyte/macrophages to the site of inflammation. On the other hand, phagocytic capacity of macrophages can be affected by purinergic receptor activation and can play a role in inflammasome activation. It can also be promising to investigate further whether ROS production and cathepsin B enzyme due to lysosomal rupture have roles in NALP-3 inflammasome activation in macrophages engulfing autophagic dying cells. It can be possible that exogenous ATP activation of purinergic receptor P_2X_7 can lead to ROS production in macrophages. Cross talk between NALP-3 and other inflammasomes can also be investigated for the sake of strengthening the data and learning. Besides, since lysosomal inhibitors are already being used in clinics to treat many diseases such as malaria, cancer, basic research can be further done for clinical studies with the combination of other drugs since combination therapies are thought to be the most effective way to treat diseases.

9. References

9.1 References related to dissertation

1. Ravichandran KS (2010) Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. Journal of Experimental Medicine 207: 1807-1817.
2. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, et al. (2012) Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death and Differentiation 19: 107-120.
3. Galluzzi L, Maiuri MC, Vitale I, Zischka H, Castedo M, et al. (2007) Cell death modalities: classification and pathophysiological implications. Cell Death and Differentiation 14: 1237-1243.
4. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, et al. (2009) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death and Differentiation 16: 3-11.
5. Kroemer G, El-Deiry W, Golstein P, Peter ME, Vaux D, et al. (2005) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. Cell Death and Differentiation 12: 1463-1467.
6. Duprez L, Wirawan E, Vanden Berghe T, Vandenabeele P (2009) Major cell death pathways at a glance. Microbes and Infection 11: 1050-1062.
7. Movassagh M, Foo RSY (2008) Simplified apoptotic cascades. Heart Failure Reviews 13: 111-119.
8. Chen YQ, Klionsky DJ (2011) The regulation of autophagy - unanswered questions. Journal of Cell Science 124: 161-170.
9. He CC, Klionsky DJ (2009) Regulation Mechanisms and Signaling Pathways of Autophagy. Annual Review of Genetics. pp. 67-93.
10. Kroemer G, Marino G, Levine B (2010) Autophagy and the Integrated Stress Response. Molecular Cell 40: 280-293.
11. Thorburn A (2008) Apoptosis and autophagy: regulatory connections between two supposedly different processes. Apoptosis 13: 1-9.
12. Tanida I, Ueno T, Kominami E (2004) LC3 conjugation system in mammalian autophagy. International Journal of Biochemistry & Cell Biology 36: 2503-2518.
13. Mizushima N (2004) Methods for monitoring autophagy. International Journal of Biochemistry & Cell Biology 36: 2491-2502.
14. Munafo DB, Colombo MI (2001) A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. Journal of Cell Science 114: 3619-3629.
15. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G (2007) Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nature Reviews Molecular Cell Biology 8: 741-752.
16. Mizushima N, Yoshimori T (2007) How to interpret LC3 immunoblotting. Autophagy 3: 542-545.

17. Levine B, Kroemer G (2008) Autophagy in the pathogenesis of disease. Cell 132: 27-42.
18. Delgado M, Singh S, De Haro S, Master S, Ponpuak M, et al. (2009) Autophagy and pattern recognition receptors in innate immunity. Immunological Reviews 227: 189-202.
19. Menendez-Benito V, Neefjes J (2007) Autophagy in MHC class II presentation: Sampling from within. Immunity 26: 1-3.
20. Scarlatti F, Granata R, Meijer AJ, Codogno P (2009) Does autophagy have a license to kill mammalian cells? Cell Death and Differentiation 16: 12-20.
21. Espert L, Denizot M, Grimaldi M, Robert-Hebmann V, Gay B, et al. (2006) Autophagy is involved in T cell death after binding of HIV-1 envelope proteins to CXCR4. Journal of Clinical Investigation 116: 2161-2172.
22. Djavaheri-Mergny M, Amelotti M, Mathieu J, Besancon F, Bauvy C, et al. (2006) NF-kappa B activation represses tumor necrosis factor-alpha-induced autophagy. Journal of Biological Chemistry 281: 30373-30382.
23. Crighton D, O'Prey J, Bell HS, Ryan KM (2007) p73 regulates DRAM-independent autophagy that does not contribute to programmed cell death. Cell Death and Differentiation 14: 1071-1079.
24. Qu XP, Zou ZJ, Sun QH, Luby-Phelps K, Cheng PF, et al. (2007) Autophagy gene-dependent clearance of apoptotic cells during embryonic development. Cell 128: 931-946.
25. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, et al. (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 402: 672-676.
26. Pattingre S, Tassa A, Qu XP, Garuti R, Liang XH, et al. (2005) Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122: 927-939.
27. Hoyer-Hansen M, Bastholm L, Szyniarowski P, Campanella M, Szabadkai G, et al. (2007) Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. Molecular Cell 25: 193-205.
28. Pyo JO, Jang MH, Kwon YK, Lee HJ, Jun JIL, et al. (2005) Essential roles of Atg5 and FADD in autophagic cell death - Dissection of autophagic cell death into vacuole formation and cell death. Journal of Biological Chemistry 280: 20722-20729.
29. Yousefi S, Perozzo R, Schmid I, Ziemiecki A, Schaffner T, et al. (2006) Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nature Cell Biology 8: 1124-U1146.
30. Wirawan E, Vande Walle L, Kersse K, Cornelis S, Claerhout S, et al. (2010) Caspase-mediated cleavage of Beclin-1 inactivates Beclin-1-induced autophagy and enhances apoptosis by promoting the release of proapoptotic factors from mitochondria. Cell Death & Disease 1.
31. Berry DL, Baehrecke EH (2007) Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila. Cell 131: 1137-1148.
32. Yu L, Wan FY, Dutta S, Welsh S, Liu ZH, et al. (2006) Autophagic programmed cell death by selective catalase degradation. Proceedings of the National Academy of Sciences of the United States of America 103: 4952-4957.

References

33. Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, et al. (2004) Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. Nature Cell Biology 6: 1221-1228.
34. Yu L, Alva A, Su H, Dutt P, Freundt E, et al. (2004) Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. Science 304: 1500-1502.
35. Bursch W, Ellinger A, Kienzl H, Torok L, Pandey S, et al. (1996) Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: The role of autophagy. Carcinogenesis 17: 1595-1607.
36. Petrovski G, Zahuczky G, Katona K, Vereb G, Martinet W, et al. (2007) Clearance of dying autophagic cells of different origin by professional and non-professional phagocytes. Cell Death and Differentiation 14: 1117-1128.
37. Meredith JE, Fazeli B, Schwartz MA (1993) The extracellular-matrix as a cell-survival factor Molecular Biology of the Cell 4: 953-961.
38. Raff MC (1992) Social controls on cell-survival and cell-death. Nature 356: 397-400.
39. Gilmore AP (2005) Anoikis. Cell Death and Differentiation 12: 1473-1477.
40. Frisch SM, Francis H (1994) Disruption of epithelial cell-matrix interactions induces apoptosis. Journal of Cell Biology 124: 619-626.
41. Chiarugi P, Giannoni E (2008) Anoikis: A necessary death program for anchorage-dependent cells. Biochemical Pharmacology 76: 1352-1364.
42. Mathew R, Karantza-Wadsworth V, White E (2007) Role of autophagy in cancer. Nature Reviews Cancer 7: 961-967.
43. Vanden Berghe T, Vanlangenakker N, Parthoens E, Deckers W, Devos M, et al. (2010) Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features. Cell Death and Differentiation 17: 922-930.
44. Krysko DV, Vanden Berghe T, D'Herde K, Vandenabeele P (2008) Apoptosis and necrosis: Detection, discrimination and phagocytosis. Methods 44: 205-221.
45. Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nature Reviews Molecular Cell Biology 11: 700-714.
46. Gozuacik D, Bialik S, Raveh T, Mitou G, Shohat G, et al. (2008) DAP-kinase is a mediator of endoplasmic reticulum stress-induced caspase activation and autophagic cell death. Cell Death and Differentiation 15: 1875-1886.
47. Krysko O, Vandenabeele P, Krysko DV, Bachert C (2010) Impairment of phagocytosis of apoptotic cells and its role in chronic airway diseases. Apoptosis 15: 1137-1146.
48. Poon IKH, Hulett MD, Parish CR (2010) Molecular mechanisms of late apoptotic/necrotic cell clearance. Cell Death and Differentiation 17: 381-397.
49. Majai G, Petrovski G, Fesus L (2006) Inflammation and the apopto-phagocytic system. Immunology Letters 104: 94-101.
50. Devitt A, Marshall LJ (2011) The innate immune system and the clearance of apoptotic cells. Journal of Leukocyte Biology 90: 447-457.

References

51. Cvetanovic M, Mitchell JE, Patel V, Avner BS, Su Y, et al. (2006) Specific recognition of apoptotic cells reveals a ubiquitous and unconventional innate immunity. Journal of Biological Chemistry 281: 20055-20067.
52. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, et al. (1998) Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. Journal of Clinical Investigation 101: 890-898.
53. Nowak AK, Lake RA, Marzo AL, Scott B, Heath WR, et al. (2003) Induction of tumor cell apoptosis in vivo increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. Journal of Immunology 170: 4905-4913.
54. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, et al. (2007) Calreticulin exposure dictates the immunogenicity of cancer cell death. Nature Medicine 13: 54-61.
55. Krysko DV, Kaczmarek A, Krysko O, Heyndrickx L, Woznicki J, et al. (2011) TLR-2 and TLR-9 are sensors of apoptosis in a mouse model of doxorubicin-induced acute inflammation. Cell Death and Differentiation 18: 1316-1325.
56. Casares N, Pequignot MO, Tesniere A, Ghiringhelli F, Roux S, et al. (2005) Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. Journal of Experimental Medicine 202: 1691-1701.
57. Lugade AA, Moran JP, Gerber SA, Rose RC, Frelinger JG, et al. (2005) Local radiation therapy of B16 melanoma tumors increases the generation of tumor antigen-specific effector cells that traffic to the tumor. Journal of Immunology 174: 7516-7523.
58. Skovsgaard T, Nissen NI (1982) Membrane-transport of anthracyclines Pharmacology & Therapeutics 18: 293-311.
59. Kiyomiya K, Matsuo S, Kurebe M (2001) Mechanism of specific nuclear transport of adriamycin: The mode of nuclear translocation of adriamycin-proteasome complex. Cancer Research 61: 2467-2471.
60. Zhu K, Dunner K, McConkey DJ (2010) Proteasome inhibitors activate autophagy as a cytoprotective response in human prostate cancer cells. Oncogene 29: 451-462.
61. Laussmann MA, Passante E, Dussmann H, Rauen JA, Wurstle ML, et al. (2011) Proteasome inhibition can induce an autophagy-dependent apical activation of caspase-8. Cell Death and Differentiation 18: 1584-1597.
62. Munoz-Gomez JA, Rodriguez-Vargas JM, Quiles-Perez R, Aguilar-Quesada R, Martin-Oliva D, et al. (2009) PARP-1 is involved in autophagy induced by DNA damage. Autophagy 5: 61-74.
63. Kepp O, Tesniere A, Schlemmer F, Michaud M, Senovilla L, et al. (2009) Immunogenic cell death modalities and their impact on cancer treatment. Apoptosis 14: 364-375.
64. Marques-da-Silva C, Burnstock G, Ojcius DM, Coutinho-Silva R (2010) Purinergic receptor agonists modulate phagocytosis and clearance of apoptotic cells in macrophages. Immunobiology 216: 1-11.

65. Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. Nature Reviews Immunology 8: 958-969.
66. Gordon S (2008) Metchnikoff's legacy: macrophage functions in immunity. Immunology 125: 3-3.
67. Martinon F (2008) Detection of immune danger signals by NALP3. Journal of Leukocyte Biology 83: 507-511.
68. Matzinger P (1994) Tolerance, danger, and the extended family Annual Review of Immunology 12: 991-1045.
69. Grimsley C, Ravichandran KS (2003) Cues for apoptotic cell engulfment: eat-me, don't eat-med and come-get-me signals. Trends in Cell Biology 13: 648-656.
70. Garg AD, Nowis D, Golab J, Vandenabeele P, Krysko DV, et al. (2010) Immunogenic cell death, DAMPs and anticancer therapeutics: An emerging amalgamation. Biochimica Et Biophysica Acta-Reviews on Cancer 1805: 53-71.
71. Krysko DV, Agostinis P, Krysko O, Garg AD, Bachert C, et al. (2011) Emerging role of damage-associated molecular patterns derived from mitochondria in inflammation. Trends in Immunology 32: 157-164.
72. Kawai T, Akira S (2007) TLR signaling. Seminars in Immunology 19: 24-32.
73. Guarda G, So A (2010) Regulation of inflammasome activity. Immunology 130: 329-336.
74. Kumar H, Kawai T, Akira S (2011) Pathogen Recognition by the Innate Immune System. International Reviews of Immunology 30: 16-34.
75. Becker CE, O'Neill LAJ (2007) Inflammasomes in inflammatory disorders: The role of TLRs and their interactions with NLRs. Seminars in Immunopathology 29: 239-248.
76. Kersse K, Bertrand MJM, Lamkanfi M, Vandenabeele P (2011) NOD-like receptors and the innate immune system: Coping with danger, damage and death. Cytokine & Growth Factor Reviews 22: 257-276.
77. Lamkanfi M, Kanneganti TD, Franchi L, Nunez G (2007) Caspase-1 inflammasomes in infection and inflammation. Journal of Leukocyte Biology 82: 220-225.
78. Elinav E, Strowig T, Kau AL, Henao-Mejia J, Thaiss CA, et al. (2011) NLRP6 Inflammasome Regulates Colonic Microbial Ecology and Risk for Colitis. Cell 145: 745-757.
79. Zitvogel L, Kepp O, Kroemer G (2010) Decoding Cell Death Signals in Inflammation and Immunity. Cell 140: 798-804.
80. Leemans JC, Cassel SL, Sutterwala FS (2011) Sensing damage by the NLRP3 inflammasome. Immunological Reviews 243: 152-162.
81. Lamkanfi M, Kanneganti TD (2010) Nlrp3: An immune sensor of cellular stress and infection. International Journal of Biochemistry & Cell Biology 42: 792-795.
82. Pope RM, Tschopp J (2007) The role of interleukin-1 and the inflammasome in gout - Implications for therapy. Arthritis and Rheumatism 56: 3183-3188.
83. Church LD, Cook GP, McDermott MF (2008) Primer: inflammasomes and interleukin 1 beta in inflammatory disorders. Nature Clinical Practice Rheumatology 4: 34-42.

84. Zitvogel L, Kepp O, Galluzzi L, Kroemer G (2012) Inflammasomes in carcinogenesis and anticancer immune responses. Nat Immunol 13: 343-351.
85. Tschopp J, Schroder K (2010) NLRP3 inflammasome activation: the convergence of multiple signalling pathways on ROS production? Nature Reviews Immunology 10: 210-215.
86. Netea MG, Simon A, van de Veerdonk F, Kullberg BJ, Van der Meer JWM, et al. (2010) IL-1 beta Processing in Host Defense: Beyond the Inflammasomes. Plos Pathogens 6.
87. Kawai T, Akira S (2007) Signaling to NF-kappaB by Toll-like receptors. Trends Mol Med 13: 460-469.
88. Martinon F, Agostini L, Meylan E, Tschopp J (2004) Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome. Current Biology 14: 1929-1934.
89. Schroder K, Zhou RB, Tschopp J (2010) The NLRP3 Inflammasome: A Sensor for Metabolic Danger? Science 327: 296-300.
90. Petrilli V, Papin S, Dostert C, Mayor A, Martinon F, et al. (2007) Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. Cell Death and Differentiation 14: 1583-1589.
91. Pedra JHF, Cassel SL, Sutterwala FS (2009) Sensing pathogens and danger signals by the inflammasome. Current Opinion in Immunology 21: 10-16.
92. Petrilli V, Dostert C, Muruve DA, Tschopp J (2007) The inflammasome: a danger sensing complex triggering innate immunity. Current Opinion in Immunology 19: 615-622.
93. Bryant C, Fitzgerald KA (2009) Molecular mechanisms involved in inflammasome activation. Trends in Cell Biology 19: 455-464.
94. Lamkanfi M, Walle LV, Kanneganti TD (2011) Deregulated inflammasome signaling in disease. Immunological Reviews 243: 163-173.
95. Schroder K, Tschopp J (2010) The Inflammasomes. Cell 140: 821-832.
96. Dostert C, Guarda G, Romero JF, Menu P, Gross O, et al. (2009) Malarial Hemozoin Is a Nalp3 Inflammasome Activating Danger Signal. Plos One 4.
97. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature 440: 237-241.
98. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, et al. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. Nature 440: 228-232.
99. Sutterwala FS, Ogura Y, Szczepanik M, Lara-Tejero M, Lichtenberger GS, et al. (2006) Critical role for NALP3/CIAS1/cryopyrin in innate and adaptive immunity through its regulation of caspase-1. Immunity 24: 317-327.
100. Pelegrin P, Surprenant A (2006) Pannexin-1 mediates large pore formation and interleukin-1 beta release by the ATP-gated P2X(7) receptor. Embo Journal 25: 5071-5082.
101. Perregaux D, Gabel CA (1994) Interleukin-1-beta maturation and release in response to ATP and nigericin- evidence that potassium-depletion mediated by these agents is a necessary and common feature of their activity

Journal of Biological Chemistry 269: 15195-15203.
102. Kahlenberg JM, Dubyak GR (2004) Mechanisms of caspase-1 activation by P2X(7) receptor-mediated K+ release. American Journal of Physiology-Cell Physiology 286: C1100-C1108.
103. Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, et al. (2006) The P2X(7) receptor: A key player in IL-1 processing and release. Journal of Immunology 176: 3877-3883.
104. Schorn C, Frey B, Lauber K, Janko C, Strysio M, et al. (2011) Sodium Overload and Water Influx Activate the NALP3 Inflammasome. Journal of Biological Chemistry 286: 35-41.
105. Qu Y, Misaghi S, Newton K, Gilmour LL, Louie S, et al. (2011) Pannexin-1 Is Required for ATP Release during Apoptosis but Not for Inflammasome Activation. Journal of Immunology 186: 6553-6561.
106. Pelegrin P, Barroso-Gutierrez C, Surprenant A (2008) P2X(7) receptor differentially couples to distinct release pathways for IL-1 beta in mouse macrophage. Journal of Immunology 180: 7147-7157.
107. Kanneganti TD, Lamkanfi M, Kim YG, Chen G, Park JH, et al. (2007) Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. Immunity 26: 433-443.
108. Cruz CM, Rinna A, Forman HJ, Ventura ALM, Persechini PM, et al. (2007) ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. Journal of Biological Chemistry 282: 2871-2879.
109. Bauernfeind F, Bartok E, Rieger A, Franchi L, Nunez G, et al. (2011) Cutting Edge: Reactive Oxygen Species Inhibitors Block Priming, but Not Activation, of the NLRP3 Inflammasome. Journal of Immunology 187: 613-617.
110. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, et al. (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nature Immunology 9: 847-856.
111. Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, et al. (2008) The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nature Immunology 9: 857-865.
112. Ichinohe T, Pang IK, Iwasaki A (2010) Influenza virus activates inflammasomes via its intracellular M2 ion channel. Nature Immunology 11: 404-U461.
113. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, et al. (2008) Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 4: 151-175.
114. Wang SW, Konorev EA, Kotamraju S, Joseph J, Kalivendi S, et al. (2004) Doxorubicin induces apoptosis in normal and tumor cells via distinctly different mechanisms - Intermediacy of H2O2- and p53-dependent pathways. Journal of Biological Chemistry 279: 25535-25543.

115. Caro LHP, Plomp P, Wolvetang EJ, Kerkhof C, Meijer AJ (1988) 3-methyladenine, an inhibitor of autophagy, has multiple effects on metabolism European Journal of Biochemistry 175: 325-329.
116. Elliott JA, Winn WC (1986) Treatment of alveolar macrophages with cytochalasin-D inhibits uptake and subsequent growth of legiobella-pneumophila. Infection and Immunity 51: 31-36.
117. Gozuacik D, Kimchi A (2007) Autophagy and cell death. Current Topics in Developmental Biology, Vol 78. pp. 217-245.
118. Scarlatti F, Bauvy C, Ventruti A, Sala G, Cluzeaud F, et al. (2004) Ceramide-mediated macroautophagy involves inhibition of protein kinase B and up-regulation of beclin 1. Journal of Biological Chemistry 279: 18384-18391.
119. Liang XH, Kleeman LK, Jiang HH, Gordon G, Goldman JE, et al. (1998) Protection against fatal Sindbis virus encephalitis by Beclin, a novel Bcl-2-interacting protein. Journal of Virology 72: 8586-8596.
120. Djavaheri-Mergny M, Maiuri MC, Kroemer G (2010) Cross talk between apoptosis and autophagy by caspase-mediated cleavage of Beclin 1. Oncogene 29: 1717-1719.
121. Prietzsch H, Brock J, Kleine HD, Liebe S, Jaster R (2002) Interferon-alpha inhibits cell cycle progression by Ba/F3 cells through the antagonisation of interleukin-3 effects on key regulators of G(1)/S transition. Cellular Signalling 14: 751-759.
122. Altman BJ, Wofford JA, Zhao YX, Coloff JL, Ferguson EC, et al. (2009) Autophagy Provides Nutrients but Can Lead to Chop-dependent Induction of Bim to Sensitize Growth Factor-deprived Cells to Apoptosis. Molecular Biology of the Cell 20: 1180-1191.
123. Vande Walle L, Wirawan E, Lamkanfi M, Festjens N, Verspurten J, et al. (2010) The mitochondrial serine protease HtrA2/Omi cleaves RIP1 during apoptosis of Ba/F3 cells induced by growth factor withdrawal. Cell Research 20: 421-433.
124. Maiuri MC, Le Toumelin G, Criollo A, Rain JC, Gautier F, et al. (2007) Functional and physical interaction between Bcl-X-L and a BH3-like domain in Beclin-1. Embo Journal 26: 2527-2539.
125. Oberstein A, Jeffrey PD, Shi YG (2007) Crystal structure of the Bcl-X-L-beclin 1 peptide complex - Beclin 1 is a novel BH3-only protein. Journal of Biological Chemistry 282: 13123-13132.
126. Geng Y, Kohli L, Klocke BJ, Roth KA (2010) Chloroquine-induced autophagic vacuole accumulation and cell death in glioma cells is p53 independent. Neuro-Oncology 12: 473-481.
127. Chu CT (2006) Autophagic stress in neuronal injury and disease. J Neuropathol Exp Neurol 65: 423-432.
128. Strowig T, Henao-Mejia J, Elinav E, Flavell R (2012) Inflammasomes in health and disease. Nature 481: 278-286.
129. Broz P, Newton K, Lamkanfi M, Mariathasan S, Dixit VM, et al. (2010) Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella. Journal of Experimental Medicine 207: 1745-1755.

130. Stienstra R, Joosten LAB, Koenen T, van Tits B, van Diepen JA, et al. (2010) The Inflammasome-Mediated Caspase-1 Activation Controls Adipocyte Differentiation and Insulin Sensitivity. Cell Metabolism 12: 593-605.
131. Amaravadi RK, Thompson CB (2007) The roles of therapy-induced autophagy and necrosis in cancer treatment. Clinical Cancer Research 13: 7271-7279.
132. Miles K, Clarke DJ, Lu WY, Sibinska Z, Beaumont PE, et al. (2009) Dying and Necrotic Neutrophils Are Anti-Inflammatory Secondary to the Release of alpha-Defensins. Journal of Immunology 183: 2122-2132.
133. Piccini A, Carta S, Tassi S, Lasiglie D, Fossati G, et al. (2008) ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1 beta and IL-18 secretion in an autocrine way. Proceedings of the National Academy of Sciences of the United States of America 105: 8067-8072.
134. Wang TF, Guidotti G (1998) Widespread expression of ecto-apyrase (CD39) in the central nervous system. Brain Research 790: 318-322.
135. Klodos I, Fedosova NU, Plesner L (1995) Influence of intramembrane electric charge on Na,K ATPase. Journal of Biological Chemistry 270: 4244-4254.
136. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma YT, et al. (2009) Activation of the NLRP3 inflammasome in dendritic cells induces IL-1 beta-dependent adaptive immunity against tumors. Nature Medicine 15: 1170-U1199.
137. McDonald B (2010) Intravascular danger signals guide neutrophils to sites of sterile inflammation (October, pg 362, 2010). Science 331: 1517-1517.
138. Chekeni FB, Elliott MR, Sandilos JK, Walk SF, Kinchen JM, et al. (2010) Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. Nature 467: 863-U136.
139. Silverman WR, Vaccari J, Locovei S, Qiu F, Carlsson SK, et al. (2009) The Pannexin 1 Channel Activates the Inflammasome in Neurons and Astrocytes. Journal of Biological Chemistry 284: 18143-18151.
140. Pelegrin P, Surprenant A (2007) Pannexin-1 couples to maitotoxin- and nigericin-induced interleukin-1 beta release through a dye uptake-independent pathway. Journal of Biological Chemistry 282: 2386-2394.
141. D'Hondt C, Ponsaerts R, De Smedt H, Vinken M, De Vuyst E, et al. (2011) Pannexin channels in ATP release and beyond: An unexpected rendezvous at the endoplasmic reticulum. Cellular Signalling 23: 305-316.
142. Garg AD, Krysko DV, Verfaillie T, Kaczmarek A, Ferreira GB, et al. (2012) A novel pathway combining calreticulin exposure and ATP secretion in immunogenic cancer cell death. EMBO J 31: 1062-1079.
143. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, et al. (2006) Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med 13: 54-61.
144. Michaud M, Martins I, Sukkurwala AQ, Adjemian S, Ma YT, et al. (2011) Autophagy-Dependent Anticancer Immune Responses Induced by Chemotherapeutic Agents in Mice. Science 334: 1573-1577.
145. Green DR, Ferguson T, Zitvogel L, Kroemer G (2009) Immunogenic and tolerogenic cell death. Nature Reviews Immunology 9: 353-363.

146. Tesniere A, Apetoh L, Ghiringhelli F, Joza N, Panaretakis T, et al. (2008) Immunogenic cancer cell death: a key-lock paradigm. Current Opinion in Immunology 20: 504-511.
147. Martins I, Tesniere A, Kepp O, Michaud M, Schlemmer F, et al. (2009) Chemotherapy induces ATP release from tumor cells. Cell Cycle 8: 3723-3728.
148. Zitvogel L, Kepp O, Senovilla L, Menger L, Chaput N, et al. (2010) Immunogenic Tumor Cell Death for Optimal Anticancer Therapy: The Calreticulin Exposure Pathway. Clinical Cancer Research 16: 3100-3104.

9.2 Publication list prepared by the Kenézy Life Science Library

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List of publications related to the dissertation

1. **Ayna, G.**, Krysko, D.V., Kaczmarek, A., Petrovski, G., Vandenabeele, P., Fésüs, L.: ATP release from dying autophagic cells and their phagocytosis are crucial for inflammasome activation in macrophages.
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2. Petrovski, G., **Ayna, G.**, Majai, G., Hodrea, J., Benkő, S., Mádi, A., Fésüs, L.: Phagocytosis of cells dying through autophagy induces inflammasome activation and IL-1alfa release in human macrophages.
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