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Inflammasome activation in human and mouse macrophages engulfing autophagic dying cells

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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 cells in tissues. Immune-system and cell death based combination therapies are being used in the treatment of many diseases such as cancers, autoimmune diseases. It was showed that immune system can suppress the growth of carcinomas. However, immune system may also promote the growth of cancer cells with reduced immunogenicity. There was a gap in the literature that how autophagic dying cells are cleared from tissues and what is the innate immune response against them. When we used dying cells with autophagic features we have 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.

1.1 Cell death

In our body, billions of cells die daily during homeostasis and immune regulation and they are replaced by new ones. Cell death modalities can be classified also by considering enzymological criteria, functional aspects and immunological characteristics. Cell death type can be also immunogenic or non-immunogenic according to their capacity to induce inflammatory pathways.

1.1.1 Apoptosis

Apoptotic cell death is a programmed and controlled breakdown of the cell into apoptotic bodies. Some of the morphological features of apoptotic cells are rounding-up, nuclear fragmentation, membrane blebbing. There are two main evolutionarily conserved protein families which play crucial roles in apoptosis; caspases and Bcl-2 family members. Apoptosis is composed of two biochemical routes; extrinsic or intrinsic pathways which are triggered by distinct inducers. 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.

1.1.2 Autophagic cell death

1.1.2.1 Autophagy

Cells primarily use the basal level of autophagy to eliminate the harmful components through catabolic pathways and recycle them to maintain nutrient and energy for survival. Autophagy is a tightly regulated and conserved pathway in all eukaryotes as a stress-induced catabolic process. Eukaryotic cells should adapt to external stress conditions. These stressors lead to rapid metabolic changes which induce diverse stress response pathways. Autophagy can be induced by nutrient and growth factor deprivation, ER stress, pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), hypoxia, redox stress, p53 and mitochondrial damage. In autophagy, initially cytoplasmic contents such as organelles or long-lived proteins are enwrapped by double membraned membrane called as autophagosome. Furthermore, autophagosomes fuse with lysosomes which provide hydrolases that can degrade the enwrapped constituents.

1.1.2.2 Cell death related to autophagy

Excessive bulk self-destruction and selectively targeting key cell survival elements by autophagy can result in autophagic cell death. 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. 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. Autophagy can also trigger cell death independently of apoptosis.

1.1.3 Anoikis

Cells can start to kill themselves as a result of detachment from extracellular matrix proteins. Anoikis is a physiological cell death mechanism since correct adhesion of cells is essential to prevent the re-attachment of cells into an improper location and their dysplastic growth. When the apoptosis defective tumor cells which are under metabolic stress, autophagy can lead to survival first and then cells can die eventually with excess amount of autophagy.

1.1.4 Necrosis

Necrosis has been considered as an uncontrolled form of cell death without showing the features of apoptosis and autophagy. It can accidentally happen as a result of severe physical damage. Recently, it was observed that certain conditions can start programmed necrosis with strictly regulated signaling events which further cause plasma membrane rupture.

1.2 Interactions between dying cells with innate immune cells

To prevent immune reactions and maintain tissue homeostasis, dying cells should be cleared efficiently by either non-professional or professional phagocytes. Complex molecular patterns and dynamic interactions between dying cells and engulfing cells are often called as 'third synapse". There are molecules exposed on dying cells (scavenger receptors, PS receptors, thrombospondin receptor) which bind to appropriate receptors on phagocytes (intercellular adhesion molecule 3 (ICAM3), apoptotic cell associated molecular patterns (ACAMPs), PS, calreticulin). 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.

1.2.1 Macrophages

Circulating monocytes develop from myeloid progenitor cells in bone marrow and migrate into tissues in the steady state or in response to inflammation. Macrophages are phagocytes which influence development, ensure homeostatis and protect the host from infection through the process 'innate immunity'. Macrophages also contribute to the removal of cellular debris generated during tissue remodeling and diseases. Upon clearance the cellular debris, macrophages release immune mediators depends on how they are stimulated.

1.3 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.

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. 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’, ‘eat-me’, and ‘activation’ factors.

1.3.1 Toll-like receptors (TLRs)

TLRs can recognize PAMPs from bacteria, fungi or protozoa or nucleic acids from bacteria and viruses. 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).

1.3.2 NOD-like receptors (NLRs) and NALP3 inflammasomes

NOD-like receptors comprise three domains: C-terminal domain contains LRRs, N-terminal caspase activation and recruitment or pyrin domain and an intermediate one consisting of nucleotide-binding and oligomerization domain. NLRs are grouped as NLRA, NLRB, NLRC, NLRP and NLRX regarding their N terminal effector moldule. NALP-3 inflammasome is composed of multiprotein-complexes which promote the proteolytic activation of inactive form of caspase-1. Upon stress detection and the assembly of the NALP3 inflammasome, pro-caspase-1 is recruited which is cleaved into caspase-1 and further cleaves pro-IL-18 and pro-IL-1β cytokines. Mature cytokines such as IL-18 and IL-1β (17 kDA) are released by an unconventional secretion pathway from the cells.

1.4 Importance of IL-1β in innate immunity

IL-1β is a multifunctional inflammatory cytokine which is known as an endogenous pyrogen for the body response against infection which evokes fever, hypertension and mediates the production of other pro-inflammatory cytokines and adhesion molecules. It has a capacity to affect almost all cell types such as T lymphocytes either alone or along with other cytokines. It can mediate repair responses such as angiogenesis and neutrophil influx to remove the cellular debris.

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

IL-1β production is a tightly controlled process playing a pivotal role in inflammation and during recruitment of neutrophils into tissues. First, pro-IL-1β 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) and the activity of the IL-1β promoter.

1.5 Upstream mechanisms of NALP3 inflammasome activation

How inflammasomes sense the particular inducer and lead to secretion of IL-1β from macrophages has not been clarified in detail. It is widely believed that NALP-3 activation can require the 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.

1.5.1 P_2X7 receptor activation by extracellular ATP as one of the DAMPs (danger associated molecular patterns)

ATP is one of the danger signal which leads to NALP-3 inflammasome activation. P_2X_{1-7} receptors are known as ligand gated channels activated by high concentrations of extracellular ATP. 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.

1.5.2 Contribution of pannexin-1 channels to inflammasome activation

Pannexin-1 channels show homology to gap junction-forming invertebrate innexins. It has been shown that pannexin-1 channels may conduct small molecules up to ~1kDa such as ions, ATP, inositol triphosphate and amino acids. 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.

1.5.3 ROS production leads NALP-3 inflammasome activation

Reactive oxygen species such as singlet oxygen, hydroxyl radicals are highly reactive molecules containing unpaired electrons. They are continuously produced as a byproduct of the mitochondrial respiratory chain in healthy cells at a tolerable level. ROS can damage cell structures due to its capability to oxidize lipids, proteins, and DNA. 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. Besides, ROS production is required for the 'priming' signal and upregulation of NLRP3 expression.

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

Cathepsins function for degradation of lysosomal contents via lysosomal acidification. It has been shown that alum, silica and amyloid-β can trigger the activation of NALP-3 inflammasome in a lysosomal damage dependent way. Crystalline molecules cause lysosomal rupture due to their size upon being phagocytosed and released cathepsin B enzyme lead to NALP-3 inflammasome activation. 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.

2. Aims of the studies

- 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

3. Materials and Methods

3.1 Cell culture and treatments

Ba/F3 were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 10% medium from WEHI-3B cells (a source of murine IL-3), 300mg/L L-glutamine (Sigma), 100U/ml penicilline/0.1mg/ml streptomycin, 400µM sodium pyruvate (Sigma), 50µM β-mercapto-ethanol. MCF-7 cells were grown in DMEM supplemented with 10% FCS, L-glutamine (300mg/L) and penicillin/streptomycin antibiotics. Cells were detached from the substrate using trypsin/EDTA (0.05:0.02%). THP-1 were cultured in RPMI 1640 medium supplemented with 10% FCS, 5% L-glutamine, 5% penicillin/streptomycin.

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

MCF-7 cells were plated in the culture medium was replaced by DMEM containing 3% charcoalstripped-FCS (DCC) for 7 days. Then, cells were treated with TAM; for treatment, freshly prepared dilutions of TAM were added to obtain autophagic dying MCF-7 cells. Controls were treated with DMSO/ethanol. For the induction of anoikis, MCF-7 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. Apyrase (2.5units/mL) pre- and co-treatment also was carried on the macrophages engulfing these cells. Lentiviral shRNA gene knock-down system was applied for the downregulation of calreticulin expression in MCF-7 cells. Its efficiency was confirmed by immunobloting using rabbit polyclonal anti-calreticulin antibody. Autophagic dying Ba/F3 cells were obtained by IL-3 depletion for 6 hrs. In some experiments IL-3 depleted and non-depleted cells were treated with chloroquine diphosphate (CQ-25µM). Apoptotic cell death was induced by adding 10 µM doxorubicin for 16 hrs. Necrotic cells were prepared by freezing and thawing. 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 on a FACSCalibur flow cytometer. Autophagosome formation was visualized under fluorescent microscopy by staining autophagic dying Ba/F3 cells with monodancylcadaverine (MDC) (50µM,1hr) and acridine orange (1µM, 20min). The

inhibition of autophagy with 10mM 3-methyladenine (3-MA) and necroptosis with 30μM necrostatin 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 gradient and a magnetic separation using CD14 human microbeads. Human macrophages were obtained through a five-day differentiation process using 5ng/mL macrophage colony stimulating factor (MCSF). Oligonucleotides for NALP-3 short hairpin RNA (shRNA) were used and the following shRNA sequence was cloned into a pLKO.1 vector: 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 THP-1 cells in the presence of 10mg/ml polybrene, 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) for differentiation. To control the successful NALP-3 knock down, 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.

4.3.2 Mouse macrophages

C57BL/6 mice were used in all experiments unless otherwise specified. Animals were maintained in the pathogen-free animal facility of University of Debrecen and at the Department for Molecular Biomedical Research of Ghent University-VIB. Peritoneal macrophages were obtained by peritoneal lavage from mice that were either injected of 4% thioglycollate or non-injected. 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. Macrophages from some mice were pooled and cells were collected by centrifugation and plated in RPMI-1640 medium supplemented in 10% heat-inactivated FCS, 300 mg/L L-glutamine, 100U/ml penicilline/0.1mg/ml streptomycin, 1mM sodium pyruvate. 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 were differentiated from femoral bone marrow cells with 10% L929 conditioned medium in RPMI medium and the medium was replaced with the fresh one. On the sixth day, cells were collected with enzyme free cell dissociation buffer and plated in a 96-well plate. They were used for the co-incubation assay on the third day.

4.4 Phagocytosis assay

Mouse macrophages were primed with ultra-pure *E. coli* LPS (0.05ng/ml for resident macrophages, 500ng/ml for thioglycollate-elicited macrophages, 100ng/ml for BMDMs) 4 hrs before starting the phagocytosis assay. The ratio of phagocytes and cells to be engulfed 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. 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) (0.1μM for mouse, 15μM for human macrophages) for 45 min 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 and macrophages were labeled with Cell TrackerTM Orange, CMTMR, 3.75μM, overnight. 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 (2.5units/ml) and the P_2X_7 receptor antagonist KN-62 (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) during co-incubation of both human and mouse macrophages. Studies on how the specific caspase-1 inhibitor Z-YVAD-FMK (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 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) (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) (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, rabbit anti-rat peroxidase-conjugated secondary antibody and caspase-3 antibody were used. 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 and transferred to an Immobilon-P membrane. Membranes were blocked in Tris buffered saline containing 0.05% Tween-20 and 5% non-fat dry milk for 1 hr. After blocking, membranes were probed overnight at 4°C with rabbit anti-IL-1β polyclonal antibody (2μg/ml), anti-actin polyclonal antibody (0.8μg/ml), pro- and mature caspase-3 antibody (1μg/ml) and anti-LC3 polyclonal antibody (2μg/ml) were followed by incubation for 1 hr with a rabbit anti-rat peroxidase-conjugated secondary antibody for 1 hr at room temperature. Peroxidase activity was detected with SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate using a Lumi-Imager. The ratio of the integrated density of LC3-II to actin was quantified by using Image J.

4.7 Immunocytochemistry

Autophagosome formation was visualized under fluorescent microscopyby staining of the autophagic cells with and acridine orange (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 in PBS for 15 min. Blocking step was done with 5% BSA 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 used. Secondary antibody was Cy3-labeled goat anti-rabbit and used for 1 hr. Nuclei were labeled with DAPI (0.5μg/ml) and viewed with a fluorescent microscope. 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 hrs co-incubation period, supernatants were collected and IL-1β was measured by using enzyme-linked immunosorbent assay. 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 according to the manufacturers' instructions and analyzed on a Bio-Plex 200.

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 kit and only for IL-6 using an ELISA kit. Concentration of ATP was measured in supernatants by using ATPliteTM Luminescence Assay System. The light production was measured on a VICTOR2TM 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 and resuspended in D-PBS at a density of $40x10^6$ cells/ml. Syngenic for Ba/F3 cells Balb/c mice (n=4-5 mice per group) were intraperitoneally injected with $10x10^6$ cells/mouse. 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. The number of PECs was counted in a hematocytometer using trypan blue and phenotyped by flow cytometry.

PECs were incubated with rat anti-mouse antibody 2.4G2 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 and CD11b-APC-Cy7. To identify neutrophils, the PECs were stained with anti-mouse antibodies Ly-6G-APC and CD11b-APC-Cy7. All the stainings were done for 30 min at 4°C in PBS. Just before flow cytometry analysis on BD LSR-II, 1.25nM of Sytox Blue dead cell stain was added to exclude dead cells from the measurements. Data were acquired and analyzed by BD FACSDiva software. 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.

4.11 Statistical analysis

Results are expressed as ± 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. (*$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$,****$p\leq0.0001$)

4. Results

4.1 Autophagy and cell death in Ba/F3 cells

4.1.1 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. We observed that 6 h of IL-3 depletion increased the numbers of autophagolysosomes in Ba/F3 cells. 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. 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. In the presence of IL-3, CQ treatment also led 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.

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

With the highest doxorubicin concentration (10μM), cells did not show autophagic activity and most of the cells died by secondary necrosis without increased autophagic activity.

4.2 Autophagic and dying cells induce inflammasome activation in macrophages whereas they inhibit crude LPS-induced pro-inflammatory cytokines

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

Increased secretion of IL-1β was detected during co-incubation with autophagic dying MCF-7 and Ba/F3 cells but not with living, apoptotic or necrotic ones. IL-1β was released in higher amount when CQ treated autophagic dying Ba/F3 cells were co-incubated with the macrophages. Thioglycollate elicited peritoneal macrophages and bone marrow derived macrophages also released significantly high amount of IL-1β.

4.2.2 Inflammasome activation depends on autophagic components of dying cells and necroptosis is not involved in this pro-inflammatory cell death mechanism

Released IL-1β was inhibited when the autophagy was (by 3-MA) in autophagic dying MCF-7 and Ba/F3cells. Necrostatin, a necroptosis inhibitor treated autophagic dying MCF-7 cells did not affect the IL-1β release from macrophages. Necrostatin was ineffective in preventing the IL-1β release from resident macrophages exposed to autophagic dying cells.

4.2.3 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 LPS induced pro-inflammatory cytokines from human macrophages. 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. We also observed that IL-1β secretion was higher when autophagic dying MCF-7 cells were fed to macrophages after LPS treatment. Similarly, autophagic dying Ba/F3 cells could also inhibit the LPS-induced pro-inflammatory response as measured by IL-6 release.

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

IL1-β release from mactophages was significantly decreased during autophagic dying cells uptake when caspase-1 was inhibited. We also showed the cleavage of pro-caspase-1 happened when macrophages co-incubated with autophagic dying MCF-7 cells. Co-incubation of caspase-1 knock out 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. NALP-3 knocked down THP-1 macrophages did not respond to autophagic dying MCF-7 cells. Furthermore, we wanted to confirm our results with NALP-3 deficient peritoneal macrophages isolated from NALP-3 KO mice and they did not respond to autophagic dying Ba/F3 cells. We did not observe any difference in the phagocytic capacity of each macrophage type engulfing living and autophagic dying Ba/F3 cells.

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

4.4.1 Uptake of autophagic and dying cells leads to inflammasome activation

We prevented the engulfment by cytD, an inhibitor of phagocytosis and it led to significant inhibition of IL-1β secretion from macrophages. We pre-treated the macrophages with cytD which reduced the phagocytosis and IL-1β release from macrophages co-incubated with autophagic dying Ba/F3 and MCF-7 cells. Calreticulin knocked down of autophagic dying MCF-7 cells did not affect the IL-1β release from macrophages. Co-incubation of medium obtained from cultures of autophagic dying MCF-7 and Ba/F3 cells with macrophages did not result in IL-1β release.

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

We wanted to see whether K^+ efflux is required for the autophagic dying cells to induce release of IL-1β from macrophages. Blocking of K^+ efflux from macrophages during phagocytosis of autophagic dying MCF-7 and Ba/F3 cells decreased the IL-1β release.

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

ATP was released from human macrophages but not from dying cells during phagocytosis. Apyrase treatment inhibited the secretion of IL-1β during co-incubation with autophagic dying MCF-7 cells. Moreover, macrophages continued to release ATP after washing away the dying cells and further incubated them with fresh medium cells. We have further blocked the purinergic receptors by using KN-62, the activation of the purinergic receptor P_2X_7 by released ATP is essential for inflammasome activation in both macrophages. We also found that ATP was released from autophagic dying Ba/F3 cells incubated without macrophages in the μM range measured from serum free medium. A significant amount of ATP (in the 400-500 nM range) was also detected in the medium obtained after the co-incubation of peritoneal macrophages and autophagic dying Ba/F3 cells in the absence of serum.

4.4.4 Contribution of pannexin-1 channels to inflammasome activation

We used CBX, a specific pannexin-1 channel inhibitor 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. Furthermore, the pannexin-1 channel inhibitor also blocked ATP secretion showing that ATP was released through these channels. In order to determine the source of ATP release we measured ATP in the medium from autophagic dying Ba/F3 cells cultured alone. ATP release in the μM range from autophagic dying Ba/F3 cells could be inhibited by CBX. ATP and IL-1β from the medium of macrophages alone upon the removal of autophagic dying Ba/F3 cells during additional 2 hrs could not be detected.

4.5 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. Living, necrotic and apoptotic Ba/F3 cells were also injected i.p. Apoptotic cells recruited neutrophils, monocytes and eosinophils, but they led to the decrease of macrophages resident in the peritoneum. 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.

5. Discussion

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

In MCF-7 cells, cell death can be induced through autophagy with estrogen depleted serum and anti-estrogen tamoxifen treatment. Upon treatments, majority of dying MCF-7 cells contained autophagic vacuoles as an early and predominant feature of cell death whereas a minority of cells showed apoptotic characteristics. MCF-7 cells are caspase-3 deficient cells in which autophagic cell death can be induced by prolonged metabolic stress conditions. 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. We have also observed increased autophagy in 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 and cells were 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 and PI3KC3 cleavage by caspases which implies that Beclin 1 has a pro-apoptotic function. 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 the accumulation of autophagic vacuoles in glioblastoma cells following treatment with a lysosome inhibitor results in which the rate of autophagic vacuole formation exceeds the rate of autophagic vacuole degradation and promotes the cells to die due to autophagic stress. In our study, it has also been shown that autophagy induced by IL-3 withdrawal in Ba/F3 cells was due to autophagic flux. According to our results, there is a basal autophagy rate in Ba/F3 cells. 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. On the other hand, IL-3

depletion led to significant elevation of basal level autophagic activity and cell death. 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.

5.2 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 pro-inflammatory cytokine response in human and mouse macrophages via inflammasome activation, respectively. We have shown that IL-1β release from 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 the NALP3 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. Ultra-pure LPS priming of macrophages also led to weaker inflammasome activation in the knockout macrophages. We also 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.

We 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. These data indicated that only cells dying with autophagic features due to growth factor depletion result in inflammasome activation in macrophages. Though 3-MA is a non-specific chemical inhibitor for autophagy, we have shown that autophagic components of the dying MCF-7

and Ba/F3 cells are required for inflammasome activation. Both autophagic dying MCF-7 and Ba/F3 cells 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. We have observed that autophagic dying MCF-7 and Ba/F3 cells can also inhibit the LPS induced and NF-κB dependent pro-inflammatory as apoptotic cells. Anti-inflammatory features of autophagic dying cells 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 autophagic dying MCF-7 cells alone provoked IL-6 and TNF-α pro-inflammatory cytokine release even though they were anti-inflammatory. IL-1β released upon inflammasome activation acted in a paracrine or otocrine way resulting in the production of IL-6 and TNF-α. Besides, 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. 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. Apoptotic MCF-7 or Ba/F3 cells could not induce inflammasome activation in human and mouse macrophages, respectively. 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. 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.

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

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. Physiologic concentrations of intracellular K^+ can prevent inflammasome assembly. It has been proposed that a lowered K^+ concentration is a common trigger of inflammasome activation. 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. 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. 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 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. During the additional 2 hrs incubation of macrophages after removal of autophagic dying Ba/F3 cells, we did not quantify any ATP. 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 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 1μM 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. We have also shown that extracellular ATP engages with purinergic receptors, P_2X_7, on both macrophages 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.

The pannexin-1 channel was identified as a plasma membrane channel mediating the regulated release of ATP and “find me” signals from apoptotic cells as a consequence of its caspase-3 dependent activation. 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. 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.

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

Our *in vivo* results show that neutrophil influx was triggered by injection of autophagic dying Ba/F3 cells in the peritoneal cavity of mice. Viable and necrotic Ba/F3 cells could also 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. Residual intact cells or cellular parts of necrotic cells could have been destroyed in the peritoneal cavity which may lead inflammasome activation. Doxorubicin-killed apoptotic Ba/F3 cells were the most potent inducers of neutrophil attraction in the peritoneum. Doxorubicin-treated apoptotic cells have already 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. Another study has shown that an immunogenic form of apoptosis was induced by mitoxantrone, another prototype of anthracyclines. 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. It has been shown that wide range of chemotherapeutics (such as oxiplatin and mitoxantrone) can induce ATP release from tumor cells which is the endogenous inducer with a highest affinity for P_2X_7 receptors.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. 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. Iit 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. 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. 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 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.

5.5 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. 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. 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. 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.

6. 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 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.

7. Publications

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Register Number: DEENKÉTK/190/2012.
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Subject: Ph.D. List of Publications

Candidate: Gizem Ayna

Neptun ID: LCZSAM

Doctoral School: Doctoral School of Molecular Cell and Immune Biology

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.
   PLoS One. "accepted by publisher", 2012.
   DOI: http://dx.doi.org/10.1371/journal.pone.0040069
   IF:4.411 (2010)

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.
   Autophagy. 7 (3), 321-330, 2011.
   DOI: http://dx.doi.org/10.4161/auto.7.3.14583
   IF:6.643 (2010)

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H-4032 Debrecen, Egyetem tér 1. e-mail: publikaciok@lib.unideb.hu

Oral and Poster Presentations

Oral Presentations

Ayna G. Advanced ICAS/ApopTrain Training Course "Advances in Cell Death Research–from Basic Principles to New Therapeutic Concepts" in Ulm, Germany, July 16-20, 2008

Ayna G. ApopTrain Mid-Term Meeting in Frankfurt, Germany, June 25-26, 2009

Ayna G. 4th Molecular Cell Immune Biology Winter School in Galyateto, Hungary, 11-14 January 2011

Ayna G. 3rd Molecular Cell Immune Biology Winter School in Mariazell in Austria, 7-10 January 2010

Ayna G. 1st and 2nd Molecular Cell Immune Biology Winter School in Slovakia, 9-12 January 2009

Poster Presentations

Gordon Research Conferences, Apoptotic Cell Recognition & Clearance, Death and Damage in Development and Disease, Bates College, Lewiston, ME, USA, July 17-22, 2011

18th Euroconference on Apoptosis 7th Training course on 'Concepts and Methods in Programmed Cell Death' Ghent, Belgium, September 1-4, 2010

35th FEBS congress - Molecules of life, Gothenburg, Sweden, June 26-July 1, 2010

International Cell Death Society (ICDS) Conference on: "Multidisciplinary Approaches in Cell Death Research" Kemer, Antalya, Turkey, 28-31 May 2010

EMBO conference Monte Verita Autophagy: Cell biology, physiology and pathology in Ascona Switzerland, October 18-21, 2009

17th Euroconference on Apoptosis. Destruction, Degredation and Death, Cell death control in cancer and neurodegeneration Pasteur Intitute, Paris, France, September 23-26, 2009

16th Euroconference on Apoptosis and 5th Swiss Apoptosis Meeting & 5th Training course on "Concepts and Methods in Programmed Cell Death" in Bern, Switzerland, September 6-9, 2008

15th Euroconference on Apoptosis and 4th Training course on "Concepts and Methods in Programmed Cell Death" in Protoroz, Slovenia, October 26-31, 2007

8. Keywords

Ba/F3, MCF-7, autophagic cell death, phagocytosis, macrophages, NALP-3, inflammasome activation, IL-1β secretion, ATP, P_2X_7, pannexin-1 channel

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