

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Studying the signal transduction of regulated necrosis

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1. Introduction

1.1. About cell death

The development and homeostasis of multicellular organisms depends on the balance between cell proliferation and cell death. After the initial steps, various executing pathways conclude into irreversible outcomes (which can be a strong caspase activation, the collapse of the mitochondrial membrane potential, or the complete permeabilization of the mitochondrial outer membrane). According to the Nomenclature Committee on Cell Death a cell should be considered dead when any of the following molecular or morphological criteria are met: (1) the cell has lost the integrity of the plasma membrane; (2) the cell including its nucleus has undergone complete fragmentation into discrete bodies; (3) its corpse (or its fragments) have been engulfed by an adjacent cell.

In the life of multi-celled organisms, the death of a cell is not necessarily a tragedy, rather it is often required for maintaining homeostasis. Apoptosis, for example, is a physiological process designed to remove cells that have already performed their function (tissue turnover, embryogenesis), dysfunctional, or pose a threat to other cells in the body (lymphocyte maturation, elimination of autoreactive T lymphocytes). Of the nearly 10^{13} – 10^{14} cells in the human body, approximately 10^{11} – 10^{12} die or are replaced by new cells every day.

Cells that are exposed to extreme physical (pressure, temperature or osmotic stress), chemical (extreme pH), or mechanical effects die in an uncontrollable manner. Their cellular structure is damaged irreparably, thus death itself is unavoidable. In these events the cell suffers the events that are happening as just a passive bystander. In most cases however, the cell death process is executed according to a well-orchestrated program which is fine-tuned by signaling molecules as well as by various control mechanisms and can be influenced (accelerated or delayed) by pharmacological agents or genetic interventions. Such regulated cell death can be initiated independently of the intra- or extracellular environmental factors by the activation of a built-in and predetermined program. These programmed cell death processes are paramount during embryonic development and in normal tissue turnover. On the other hand, regulated cell death can also be induced by stimuli originating from the internal or external microenvironment that trigger downstream signaling processes by activating specific receptors.

In terms of immunological outcome, the cell death can be either pro- or anti-inflammatory, as well as tolerogenic or immunogenic. The dominance of apoptosis over necroptosis under normal conditions ensures the tolerogenic outcome and it prevents inflammation. However, if this dominance cannot be established, then the danger signals

released from the necroptotic cells can activate the adaptive immune system and at the same time these can also activate the cross-presentation of dendritic cells.

1.2. Necroptosis

Over the past couple of decades extensive studies have uncovered novel cell death pathways, which are independent of apoptosis. Among these is necroptosis, a tightly regulated, inflammatory form of cell death. In terms of morphology, it closely resembles necrosis, as it is characterized by cellular swelling, the rupture of the cell membrane, the release of damage-associated molecular patterns (DAMPs) which initiate inflammation. In its mechanism however, it is a classical regulated process, as it is induced by receptor activation, followed by the assembly of a signaling platform that leads to the activation and oligomerization of mixed lineage kinase domain-like proteins (MLKL) which are responsible for forming pores in the cell membranes. According to published results, many stimuli have the ability to induce necroptosis including (but not limited to) cell death ligands, for example: Fas ligand (FasL), tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (Trail), and TNF-related weak inducer of apoptosis (TWEAK), T-cell receptor activation, genotoxic stress, activation of adhesion receptors, and many chemotherapeutics (e.g., mitoxantrone, cisplatin, dasatinib, shikonin, GX15-070). Pathogen-associated molecular patterns are also well-known triggers of necroptosis: dsRNA through activating Toll-like receptor 3 (TLR3), lipopolysaccharide (LPS) - TLR4, pathogenic DNA through activating the cyclic GMP-AMP Synthase- Stimulator of Interferon Genes (cGAS-STING) or the Z-DNA-binding protein 1 (ZBP1, also known as DAI) pathways, as well as retinoic acid inducible gene I (RIG-1) receptors.

During our work, we focused on death receptor (DR) induced necroptosis. These receptors (FasR, TNFR, TrailR) all belong to the tumor necrosis superfamily and they also play roles in cell proliferation and differentiation. They consist of an extracellular ligand-binding, a transmembrane, and an intracellular death domain (DD). The binding of their specific ligand (Fas, TNF, Trail) initiates the receptor oligomerization and activates the signaling cascade. TNF receptor signaling pathway is the best known and the most widely studied among the DRs. The initial intracellular signaling molecules of Fas and Trail receptors are somewhat different from TNFR, but the association of RIPK1-RIPK3 and subsequent steps are the same among all three DRs during necroptosis.

1.3. The TNF signaling pathway

1.3.1. Initiation steps

According to our current knowledge TNFR activation – based on the state, activity and availability of intracellular signaling partners – can lead to the activation of at least four different signaling cascades. These pathways include the NF- κ B pathway (which mediates cell activation), the RIPK1-dependent and independent extrinsic apoptosis (which leads to the elimination of the cell in an anti-inflammatory and tolerogenic manner) and necroptosis (which is inflammatory and immunogenic cell death process).

Binding of TNF induces the oligomerization, thus activation of TNFR, which is prerequisite for the recruitment of several adaptor molecules, among them RIPK1. In this complex RIPK1 will be polyubiquitinated in a multistep process. Polyubiquitinated RIPK1 then can serve as a scaffold for transforming growth factor- β (TGF- β)-activated kinase 1 (TAK1) and the TAK1-binding protein, as well as to the IKK complex, which consists of inhibitor of nuclear factor kappa-b kinase subunit alpha (IKK α), IKK β and NF-kappa-B essential modulator (NEMO). These proteins activate mitogen-activated protein kinases (MAPKs), for example c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase 1 and 2 (ERK1 / 2) and p38 kinases, through phosphorylation and ubiquitination steps. This leads to the activation of the transcription factor activator protein 1 (AP-1) and the NF- κ B pathway (complex I).

After the activation the of NF- κ B cascade, the signal from the TNF receptor silences, the intracellular signaling complexes will disintegrate to its components. This provides an opportunity for the formation of another type of complex, namely the complex IIa, where TRADD associates with the FAS-associated death domain protein (FADD), the long isoform of the FLICE-like inhibitory protein (FLIP_L), and procaspase-8. This complex shifts the signaling towards apoptosis through the homodimerization of procaspase-8 which is followed by its subsequent cleavage and activation. Activated caspase-8 besides initiating the apoptotic program, at the same time it inhibits the activation necroptosis through the cleavage of RIPK1 and RIPK3. In most cases however, the complex IIa-mediated apoptosis is inhibited by the activated NF- κ B pathway through the increased expression of FLIP_L and the ubiquitination of RIPK1. Because of this mechanism, the cells are normally protected against TNF-induced apoptosis.

Deubiquitination of RIPK1 can be observed when the mitochondrial second mitochondria derived activator of caspase (SMAC) binds to cIAP1 and cIAP2 proteins, leading to their autoubiquitination and proteolytic degradation. This effect can also be

induced by the use of IAP antagonists (or SMAC mimetics). An alternative complex, namely complex IIb is then assembled, in which RIPK1 binds FADD and procaspase-8 instead of TRADD, resulting in the activation of RIPK1-dependent apoptosis. The assembled complex IIb is often referred to as the ripoptosome.

In addition, necroptosis may occur, but to our current knowledge, only if RIPK1 is deubiquitinated and caspase-8 activation is inhibited. The signaling platform of necroptosis, namely necrosome can only be formed, under these circumstances.

1.3.2. Necrosome and the downstream steps of necroptosis

Both RIPK1 and RIPK3 kinases, which play a key role in necroptosis, each have a RIP homotypic interaction motif (RHIM) located at their C-terminus. Following necroptotic stimuli, several RIPK1 and RIPK3 proteins bind together through their RHIM domains to form oligomers and assemble into an amyloid-like complex that acts as the structural backbone of the necrosome. In this complex, both RIPK1 and RIPK3 will be phosphorylated (in human proteins, RIPK1 at serine 161 and RIPK3 at serine 277).

MLKL is a pseudokinase consisting of an N-terminal four-helix bundle domain (4HBD) that plays a key role in oligomerization and association with plasma membranes, a C-terminal pseudokinase domain, and a linker region connecting these two. In contrast to RIPK1 and RIPK3, which have kinase activity and which is essential during necroptosis, the MLKL pseudokinase domain functions only as an interaction domain during the MLKL-RIPK3 association. In addition to this, it also plays an important self-regulatory role by binding the 4HBD. This interaction is responsible for enclosing the 4HBD domain in the inner part of the molecule, thus – in the absence of phosphorylation – keeping the MLKL molecule in a resting state. When inactive, MLKL is located in the cytoplasm as a monomer, however, after phosphorylation mediated by RIPK3 (which occurs in human cells on threonine 357 and serine 358), MLKL transforms into an active conformation. As a result, the 4HBD is released from inhibition and shifts to the surface of the molecule, which is now capable of binding the phosphatidylinositol phosphate groups in the various membranes. An important step after the phosphorylation is the oligomerization of MLKL. In mouse cells these proteins typically assemble into trimers while in human cells they form tetramers. MLKL oligomers now in an active state then detach from the necrosome and are responsible for forming pores after embedding into membranes. The resulting water and ions influx through these pores then compromise the osmotic balance of the cell, leading to cellular swelling and the typical morphologic changes of necrosis.

1.4. The role of necroptosis in diseases

In recent years the number of necroptosis associated research is growing exponentially, as more and more scientific evidence suggests that necroptosis plays a significant role in the pathogenesis of many diseases. Both up and down-regulation of necroptosis and misregulation of the apoptosis-necroptosis transition – which modifies the immunological outcome of cell death – contribute to the evolution of degenerative disorders, autoimmune processes, inflammatory diseases or the immune surveillance of tumors.

A significant part of necroptosis research deals with cell death in response to hypoxia, as myocardial infarction, stroke, pulmonary embolism is considered as the leading causes of death nowadays. During surgical procedures, the closure and reopening of a vascular section (ischemia / reperfusion, IR) is a common phenomenon. In these cases, the expression of pro-necroptotic proteins (RIPK1, RIPK3, MLKL) was shown to be increased. It is also known that during the prolonged starvation of cells (glucose and oxygen deprivation) the level of one of the most well-known negative regulator of necroptosis (caspase 8) decreases.

The abnormal overactivation of cell death pathways has been observed in several neurodegenerative diseases. Necroptosis has been shown to contribute to the development of diseases such as Alzheimer's disease, multiple sclerosis (MS) or amyotrophic lateral sclerosis (ALS). Activated forms of RIPK1, RIPK3 and MLKL were detected in the cortical lesions of human MS samples. In ALS patients, multiple biochemical hallmarks of necroptosis including increased levels of RIPK1, RIPK3 and MLKL and elevated pRIPK1 and pMLKL were detected in both microglia and oligodendrocytes.

The dominance of apoptosis over necroptosis provides a tolerogenic and anti-inflammatory result. However, if this dominance is disrupted for some reason, the immunological outcome is altered, which can lead to chronic inflammation typically affecting the digestive system, kidneys, skin, and the respiratory system. Regarding gastrointestinal diseases, increased RIPK3 expression was detected in liver biopsies from patients with alcoholic liver disease, while both RIPK3 and MLKL expression was increased in non-alcoholic fatty liver diseases, as well as elevated MLKL phosphorylation in drug-induced liver injury. Similarly, increased levels of RIPK3 were documented in the terminal ileum of patients with Crohn's disease, and elevated RIPK3 and MLKL levels were observed in inflamed tissues of inflammatory bowel disease (IBD) and allergic colitis patients, whereas the expression of caspase-8 in these tissues was reduced.

Tumor cells are known to avoid cell death processes, as this is one of the prerequisite steps during their formation and is necessary for their survival. To the best of our knowledge, the role of necroptosis in the development and progression of cancer is quite bipartite. On the one hand, in a wide variety of tumor samples, levels of RIPK3 and /or MLKL proteins have been reduced or dysfunctional variants have been expressed. In contrast, several studies report that inflammation caused by necroptosis promotes tumor development and contributes to the formation of the tumor microenvironment. There are also data that tumors are able to induce necroptosis of nearby endothelial cells, which contributes to the spread of tumors and the development of metastases.

Although there is currently no accepted specific therapeutic agent for regulating necroptosis, more than 20 approved and already used drugs for the treatment of other diseases are known that have the potential to regulate (inhibiting or inducing) necroptosis. It would be worth considering and developing protocols on how these agents could be used to treat other diseases as well.

1.5. Pathways leading to the activation of apoptosis

It has been almost five decades since John Foxton Ross Kerr and his colleagues introduced apoptosis as the concept of regulated cell death into scientific life. Although over the years several regulated forms of cell death have been described, apoptosis has been served as a reference point for understanding other cell death pathways ever since. Apoptosis can be activated by the signals emerging from both the extra- and intracellular microenvironment.

The first step in the activation of extrinsic apoptosis is the ligand binding and subsequent oligomerization of cell death receptors (TNFR, TrailR, FasR). This process leads to the assembly of death-inducing signaling complex (DISC) which facilitates the activation of procaspase-8 and 10. Depending on the cell type, the signaling pathway can proceed in two ways: type I cells produce large amounts of active caspase-8, which immediately activate effector caspases (procaspase-3 and -7). However, in type II cells, DISC assembly is not efficient enough, resulting in a delayed or weak procaspase-8 activation, where the few active caspase-8 molecules cleave the BH3 interacting domain death agonist (Bid) proteins. The truncated, pro-apoptotic tBid induces mitochondrial permeabilization through oligomerization of BAK, thus turning on the mitochondrial pathway of apoptosis.

The intrinsic or mitochondrial pathway of apoptosis can be triggered by a wide variety of intracellular stressors, including (but not limited to) growth factor deprivation, cytoskeletal injury, DNA damage, hypoxia and the accumulation of defective, damaged or poorly coiled

proteins. The fate determining step of the pathway is the permeabilization of the outer mitochondrial membrane, which is regulated by the relative balance of the pro- and anti-apoptotic proteins belonging to the Bcl-2 family. Mitochondria contain several proteins that are essential in various cellular functions (e.g. cytochrome c, apoptosis-inducing factor (AIF), endonuclease G, SMAC (also known as DIABLO), high temperature requirement protein A2 (HtrA2 / Omi)), but when released into the cytosol, they act as pro-apoptotic molecules.

BAK and BAX are known as pore-forming proteins which, upon activation (typically homodimerization), are able to permeabilize the outer membrane of the mitochondria, thereby causing the release of pro-apoptotic proteins into the cytosol. Anti-apoptotic Bcl-2 members are known as the main repressors for apoptosis. They may inhibit BAK and BAX directly (forming heterodimers with them), but they may also prevent apoptosis by blocking the activation of several pro-apoptotic Bcl-2 members (also with heterodimerization). This inhibition can be resolved either by increasing the expression or activity of pro-apoptotic members of the Bcl-2 family, or by the post-translational modifications of anti-apoptotic Bcl-2 proteins.

The interactions between the Bcl-2 family members – which determine if the mitochondrial apoptotic pathway is activated or not – always depends on the quality (posttranslational state) and quantity (level of expression) of the members in a given situation.

1.6. The structure and activation of caspase-9

Caspases are cysteine protease enzymes that cleave their substrate along a typically 4 amino acid motif, which typically ends with an aspartic acid. In all cases, this extremely precise and only partial cleavage (limited proteolysis) always occurs along these motifs (typically one site can be found in a given substrate). About the 12 caspases present in the human body 7 are involved in apoptosis. These are termed initiator (2, 8, 9, and 10) and effector (3, 6, and 7) caspases. A common feature of all caspases is that they are synthesized in an inactive (zymogenic) form and they are structurally composed of a pro-domain located at the N-terminus of the protein which is followed by a large and a small subunit. They are activated through the formation of procaspase-procaspase dimers (in which the pro-domain functions as an interaction domain) followed by limited proteolysis (the cleavage of the pro-domain from the molecule).

Caspase-9 is widely known as the initiator caspase of intrinsic apoptosis. This pathway can be triggered by a wide variety of stimuli that result in the BAK and BAX mediated permeabilization of the outer mitochondrial membrane. Following this a number of pro-

apoptotic proteins are released, including (but not limited to) cytochrome c, which forms a heterodimer with apoptotic protease activating factor 1 (Apaf-1). Seven Apaf-1-cytochrome c complexes then bind together to form the wheel-shaped apoptosome which acts as a signaling platform by recruiting several pro-caspase-9 molecules. Although the mitochondrial pathway of apoptosis has long been known, the exact and detailed mechanism of pro-caspase-9 activation has still not been fully elucidated yet. Currently, two theories are known: the “induced conformation model” and the “induced proximity model”. There are countless published results which support either one or the other theory, but currently none of these two models can explain all these results alone. According to the “induced conformational change model,” the binding of pro-caspase-9 to Apaf-1 induce a conformational change in the pro-caspase-9 which is both is essential and sufficient for their activation. But the “induced proximity model” states that, the apoptosome only serves as a platform to bring many pro-caspases-9 proteins in close proximity, thereby allowing their homodimerization and subsequent limited proteolysis.

As the initiator of mitochondrial apoptosis, caspase-9 plays an essential role in the development of apoptosis and maintaining the homeostasis of the human body. This is why the precise regulation and fine-tuning of all the steps leading to pro-caspase-9 activation is of utmost importance.

1.7. The expression of caspase-9 under physiological and pathological conditions

The regulated elimination of cells is a normal and more importantly an essential process during the life of multicellular organisms. Removal of excess, aged, dysfunctional, or endangered cells is largely accomplished through mitochondrial apoptosis. Caspase-9 is expressed in almost every cell in the body. According to published results the alterations in the expression of caspase-9 or genetic mutations lead to various diseases, such as developmental disorders, degenerative processes and cancers.

Based on the experience gathered in transgenic mice, whole-body knockout of caspase-9 – although not with 100% penetrance – leads to perinatal lethality. It has been shown that these embryos suffer from serious brain defects (brain hyperplasia caused by the excess proliferation of cells – mainly neurons – that are unresponsive to apoptotic stimuli).

One of the potential evasion and survival mechanisms of tumor cells is to inhibit the various regulated cell death pathways. Disorders during the activation of caspase-9 or single-nucleotide polymorphisms – leading to the expression of a dysfunctional protein, which contributes to the survival of tumor cells – have been observed in various cancers affecting

the lung, bladder, stomach, colon, rectum or pancreas. Besides the inhibition of stress-induced apoptosis, the overactivation of signal transduction also causes pathological conditions. Huntington's disease is a disorder of the central nervous system caused by progressive degeneration of brain cells. Significantly elevated levels of active caspase-9 and caspase-3 have been detected in end-stage patients, suggesting that excessive neuronal apoptosis contributes to the final stage of the disease.

These results demonstrate that caspase-9 plays an essential role in maintaining the homeostasis in the body.

2. Objectives

It is well-known that DR-mediated apoptosis dominates necroptosis, but the link between necroptosis and intrinsic apoptosis has not been studied in detail. Although several substrates of caspase-8 (RIPK1, RIPK3, CYLD, TRIF) have been described as critical regulators of necroptosis, however, its most well-known substrates, the downstream caspases, have been only marginally studied during necroptosis. Based on published data, the silencing of caspase-3 or -7 did not affected TNF-induced necroptosis, but the role of caspase-9 in necroptosis has not yet been investigated.

We hypothesize that caspase-9 is essential in the signaling pathway of necroptosis and may regulate the transition between mitochondrial apoptosis and necroptosis.

In our research, we used wild-type and caspase-9 deficient cell lines (of human and mouse origins) to investigate that

- Does caspase-9 play a role in signaling of cell death receptor or pattern recognition receptor induced necroptosis?
- Which of the currently known steps of necroptosis (formation of molecular complexes, post-translational modifications) prove to be dependent of caspase-9?
- Which are the interaction partners of caspase-9 during necroptosis?
- What is the function of caspase-9 in necroptosis?
- Does the specific deletion of caspase-9 in acinar cells reduce the severity of cerulein-induced acute pancreatitis?

3. Materials and methods

3.1. Cell lines

In the experiments the following cell lines were used: JA3 Jurkat (human T lymphocyte), JA3-C9 (caspase-9 negative Jurkat), JA3 retr (JA3-C9 retransfected with caspase-9), MEF (mouse embryonic fibroblast), MEF-C9 (caspase-9 negative MEF). Jurkat cells were cultured in RPMI-1640 (Sigma-Aldrich; #R5886) while MEFs were cultured in Dulbecco's Modified Eagle Medium (DMEM) low glucose (Sigma-Aldrich; #D5546) in a humidified atmosphere of 5% CO₂ at 37 °C, respectively. All media were supplemented with 10 % FBS, 2 mM L-glutamine, and 40 mg/l gentamicin.

3.2. Measurement of cell viability

A total of 5×10^5 cells were pretreated with the indicated reagents: 10–50 μ M Z-VAD (ApexBio, #A1902), 1 μ M BV6 (a kind gift from Genentech), 2.5 μ M CCT137690 (ApexBio, #A4132), 20 μ M AR-A014418 (Selleck Chemicals, #S7435), 100 ng/ml recombinant flagellin (Sigma-Aldrich, #SRP8029-10UG), 40 μ M Nec-1 (Abcam, #ab141053), 1 μ M necrosulfonamide (Tocris Bioscience, #5025) or 7.5 μ M GSK'872 (Selleck Chemicals, #S8465) for 1 h and activated with: 30 ng/mL flag-tagged recombinant FasL (Enzo Life Sciences, #ALX-522-001-C010) crosslinked with anti-FLAG M2 antibody (Sigma-Aldrich; #F1804), 50 ng/mL human TNF α (BioLegend, #570104), 40 ng/mL flag tagged recombinant Trail (Enzo Life Sciences, #ALX-522-003-C010) cross-linked with anti-FLAG M2 antibody, 400 μ M H₂O₂ (Sigma-Aldrich, #H1009), 0.74–6.7 μ M ionomycin (Sigma-Aldrich, #I0634), 100 ng/mL LPS (Sigma-Aldrich, #L8643) or infected with HHV-1 (ATCC-VR-1493) at MOI 5. Cell death was determined after 24 h by propidium iodide (PI, Sigma-Aldrich, #P4170) staining (10 μ g/mL) using BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed by FlowJo software (FlowJo X, v10.0.7r2, Tree Star).

3.3. Caspase activity assay

We used Apo-ONE Homogeneous Caspase-3/7 Assay (Promega; #G7792) to evaluate the activity of Caspase-3 and Caspase-7, respectively. Jurkat cells (2×10^4) were pre-treated with 10 μ M Z-VAD, 1 μ M BV6 and 40 μ M necrostatin-1 for 1 hour and activated for 24 hours with 50 ng/ml human TNF α in 96-well black tissue culture plates (SPL Life Sciences; #31296). The reactions to detect Caspase-3/7 activity was performed following the manufacturer's protocol. Fluorescence was measured using Synergy HT Multi-Mode

Microplate Reader configured to detect Caspase-3/7 activity at an excitation wavelength range of 485 ± 20 nm and an emission wavelength range of 528 ± 20 nm.

3.4. Western blotting

Protein extraction was performed by lysing 10^6 cells in 100 μ l 2X Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris HCl, pH 6.8). Proteins were separated by SDS gel electrophoresis using 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories; #1620115). Nonspecific binding was blocked by TBS-Tween with 5% non-fat dry milk. Transfer membranes were immunoblotted with the indicated antibodies: Caspase-9 (MBL Life science; #M054-3), Caspase-8 (Cell Signaling Technology; #9746S), RIPK1 (BD Biosciences; #610459), RIPK3 (Cell Signaling Technology; #13526), GSK3 β (Cell Signaling Technology; #9315), Aurora A (Cell Signaling Technology; #14475), TAK1 (Cell Signaling Technology; #5206S), CYLD (Sigma-Aldrich; #SAB4200060), pRIPK1 (Cell Signaling Technology; #65746), pRIPK3 (Abcam; #ab209384), Phospho-Aurora A (Thr288) (Cell Signaling Technology; #2914), and β -actin (Santa Cruz Biotechnology; #sc-47778) all diluted 1:1000. Anti-rabbit (GE Healthcare; #NA934), anti-mouse (GE Healthcare; #NA931) antibodies or anti-rat (Sigma-Aldrich; #A9037) antibodies conjugated to horseradish peroxidase were used as secondary antibodies in the dilution of 1:5000. For mouse cells the following mouse specific antibodies were used: Caspase-8 (Cell Signaling Technology; #4927S), Phospho-RIP (Ser166) Antibody (Rodent Specific) (Cell Signaling Technology; #31122), Phospho-RIP3 (Thr231/Ser232) (Cell Signaling Technology; #91702).

3.5. Immunoprecipitation

A total of 5×10^7 Jurkat cells were pretreated with 10 μ M Z-VAD and 1 μ M BV6 for 1 hour and activated for the indicated times with 50 ng/ml human TNF α . Cells were lysed using 1% Triton X-100 with protease inhibitor (Sigma-Aldrich; #P8340) and phosphatase inhibitor (Sigma-Aldrich; #P2850) in lysis buffer (30 mM Tris, 140 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 50 mM NaF, 2 mM Na₄P₂O₇, pH 7.6). Protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific; #23227). Equal amounts of total protein were added to protein A/G PLUS-agarose beads (Santa Cruz Biotechnology; #sc-2003) precoated with the indicated antibodies: Caspase-9 (LifeSpan BioSciences; #LS-C148248), RIPK1 (BD Biosciences; #610459), RIPK3 (Cell Signaling Technology;

#13526). The content was gently rotated at 4 °C overnight. The next day, the beads were washed four times with lysis buffer on ice, and then subjected to western blotting analysis. Anti-rabbit light chain (Merck; #MAB201) or anti-mouse light chain (Merck; #AP200P) antibodies conjugated to horseradish peroxidase were used as the secondary antibodies in the dilution of 1:5000.

3.6. Plasmids and constructs

To generate the lentivector expressing RIPK1, pCR3-FLAGhRIP1 (#LMBP 4850) were purchased from CABRI, for RIPK3, hRIP3 GFP wt was a gift from Francis Chan (Addgene plasmid #41387) and for Caspase-9, pET23b-Casp9-His was a gift from Guy Salvesen (Addgene plasmid #11829). RIPK1, RIPK3 and Caspase-9 were first cloned into pENTR4-FLAG provided by Eric Campeau & Paul Kaufman (Addgene plasmid #17423). RIPK1 and RIPK3 were further subcloned into the all-in-one doxycycline-inducible lentiviral destination vector, pCW57.1 (a gift from David Root, Addgene plasmid #41393), while Caspase-9 was subcloned into pLenti CMV Blast DEST (a gift from Eric Campeau & Paul Kaufman, Addgene plasmid #17451) and used for lentiviral transduction of wild-type Jurkat cells (RIPK1 JA3 and RIPK3 JA3), and their Caspase-9 deficient counterparts (RIPK1 JA3-C9 and RIPK3 JA3-C9, JA3-C9 retr). All cloning was checked by restriction analysis and DNA sequencing (capillary sequencing runs were performed by UD-GenoMed Medical Genomic Technologies Ltd.).

3.7. Lentivirus production and transduction

Replication-deficient lentivirus used in the gene transfer experiments were created as previously described. Briefly, 22 µg pCW57.1-RIPK1, pCW57.1-RIPK3 or pLENTI-Casp9 lentiviral backbone vector together with 15 µg pMD2.G and 5 µg psPAX2 were combined together in a 50 ml centrifuge tube. pMD2.G (Addgene #12259) and psPAX2 (Addgene #12260) was a gift from Didier Trono. Calcium phosphate precipitation was performed by adding 1.35 ml distilled H₂O and 150 µl 2.5 M CaCl₂ and 1.5 mL 2x HEPES-buffered saline (280 mM NaCl, 50 mM HEPES, 1.5 mM Na₂HPO₄, pH 7.0) was added dropwise into the centrifuge tube under constant agitation. The solution was incubated at room temperature for 30 minutes and then added dropwise to HEK293FT cells grown in T75 cell culture flask. 16 hours post-transfection the medium was replaced with fresh DMEM high glucose (Sigma-Aldrich; #D5796) supplemented with 10 % FBS, 2 mM L-glutamine. Viral supernatants were collected at 48- and 72-hours post-transfection and passed through a 0.45 µm filter. Target

cells were incubated overnight with lentiviral supernatant in the presence of 4 µg/ml polybrene. Infected cells were selected with 5 µg/ml puromycin or 10 µg/ml blasticidin for 7 days.

3.8. Acute pancreatitis model

The symptoms and severity of cerulein induced acute pancreatitis in wild-type and caspase-9 deficient mice was studied by our collaboration partners: Dr. Petra Pallagi, Dr. Bálint Tél and Dr. József Maléth (First Department of Medicine, University of Szeged, Szeged, Hungary and HAS-USZ Momentum Epithelial Cell Signaling and Secretion Research Group, University of Szeged, Szeged, Hungary).

Mice received 8, hourly i.p. injections of 50 µg/bwkg cerulein (Bachem; #H-3220) and were sacrificed 12 hours after the induction of AP, (n = 6-8, 8 weeks old mice per group). All animals were randomly divided into the control and treated groups, No specific exclusion criteria were applied. Blood and tissue samples were collected after terminal anesthesia immediately. Blood samples were centrifuged at 2500 RCF for 15 min at 4 °C and serums were stored at -20 °C. Pieces of pancreatic tissue were placed into a 4% formaldehyde solution and stored at 4 °C until histology. Serum amylase activity was measured by a colorimetric kit (Diagnosticum; #47462) according to the manufacturer's instruction and absorbance was detected at 405 nm using a FLUOstar OPTIMA (BMG Labtech) microplate reader. For routine histology formaldehyde-fixed pancreas samples were embedded in paraffin, and 4 µm thick sections stained with hematoxylin–eosin. Histological scores for edema, inflammatory cell infiltration and necrosis were evaluated by three independent investigators blinded to the protocol (0–5 points for edema, leukocyte infiltration and % of the total area for necrosis).

3.9. Total protein isolation from whole pancreatic tissue

Protein isolation from the pancreas of treated animals was performed by our collaboration partners: Dr. Petra Pallagi, Dr. Bálint Tél and Dr. József Maléth.

Whole mouse pancreatic tissue was cutted to small pieces with a razor blade and resolved in 1 ml RIPA lysis buffer (Merck Millipore; #20-188) completed with EASYpack Protease Inhibitor Cocktail (Roche; #05892970001). Branson Sonifier SFX150 was used for the tissue homogenization, during which samples were kept on ice. After the tissue disruption, samples were centrifuged at 3500 rpm for 10 minutes on 4 °C. Finally, supernatant was frozen by the „snap-freeze” method. Samples were stored at -80 °C until use.

3.10. Statistical analysis

Two-way ANOVA or one-way ANOVA, followed by *Sidak's multiple comparisons test* was used for multiple comparisons. Results are expressed as mean $\pm$ SD. The results are expressed as mean+SD. All analyses were performed by using GraphPad Prism software, version 6.0. Differences were considered to be statistically significant at $P < 0.05$. Significance is indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$, **** $P < 0.001$.

4. Results

4.1. Caspase-9 deficiency inhibits necroptosis in human Jurkat cells

At the beginning of our research, we induced necroptosis in JA3 (wild-type Jurkat), in their caspase-9-negative subclone (JA3-C9) and also in JA3-C9 cells retransfected with wild-type caspase-9 (JA3-C9 retr). First, we demonstrated that the caspase-9 deficient cells did not express caspase-9, whereas the original wild-type and retransfected cell lines did. We also showed that the absence of caspase-9 did not affect the expression of key necroptotic proteins. Our results showed that RIPK1, RIPK3, MLKL, CYLD, caspase-8, and TAK1 were expressed at the same level in JA3, JA3-C9, and JA3 retr cells.

To prevent the activation of caspase-8 and thus the inhibition of necroptosis, cells were pretreated with a pan-caspase inhibitor (Z-VAD) 1 hour prior to the administration of death ligands. Necroptosis can only be observed when the RIPK1 is not ubiquitinated. Therefore, cells were pretreated with a IAP antagonist (BV6) to promote the degradation of IAP proteins and thus to prevent the ubiquitination of RIPK1. To confirm that the induced cell death is necroptosis, we administered a specific necroptosis inhibitor, necrostatin-1 (Nec1).

According to our results, necroptosis has been induced in wild-type cells, but this was not observed in the absence of caspase-9. To confirm that the observed cell death is strictly caspase-9 dependent, we retransfected the JA3-C9 subclone with wild-type caspase-9 (JA3-C9 retr). Reconstitution of caspase-9 successfully restored the necroptosis sensitivity of the cells. The cell death induced in the JA3 and JA3 retr cells was completely inhibited by Nec1, confirming the cell death is indeed necroptosis. In summary, we provided evidence that caspase-9 has an essential role in DR-mediated necroptosis in human Jurkat cells.

4.2. Investigating the necroptotic role of caspase-9 on MEF cells

Necroptosis has been published to highly depend on the cell type and on the sorts of stimuli. To determine whether the role of caspase-9 in necroptosis is a general requirement or specific to the Jurkat cells, we studied the necroptotic sensitivity of caspase-9-deficient MEF cells (MEF-C9) and their wild-type counterparts. Similarly to our observations in Jurkat cells, TNF and Fas receptor activation induced necroptosis in wild-type, but not in the caspase-9-deficient MEFs. We also checked the expression of the main necroptosis regulatory molecules (RIPK1, RIPK3, MLKL, caspase-8, CYLD, TAK1) and it was comparable in both wild-type and caspase-9-deficient MEF cells.

Our results indicate that caspase-9 plays a general role in necroptosis, as caspase-9 deficiency prevents necroptosis in both human and murine cells under various stimuli.

4.3. Caspase-9 does not play a role in RIPK1 dependent apoptosis

In addition to RIPK1-mediated necroptosis, RIPK1-dependent apoptosis has also been documented, primarily in the presence of SMAC mimetics under conditions that allow caspase activation. JA3 and JA3-C9 were stimulated with TNF and BV6, and the activity of effector caspases was investigated. This treatment led to potent effector caspase activation, confirming that in these samples apoptosis was induced. In accordance with published results, the absence of caspase-9 did not modify the intensity of RIPK1-mediated apoptosis. Our results indicate that in addition to its well-known role in intrinsic apoptosis, caspase-9 is specifically required for necroptosis, whereas it has no role in RIPK1-mediated apoptosis.

4.4. Ionomycin and hydrogen peroxide induced necrosis is independent of caspase-9

Ionomycin is known as calcium ionophore. While in low concentrations (<250 nM) it induces apoptosis, its micromolar concentration leads to classical necrosis due to the generated osmotic shock. To study whether caspase-9 acts on necroptosis only or controls necrosis in general, we treated the cells with ionomycin and observed that the intensity of ionomycin-induced necrosis was comparable in both JA3 and JA3-C9 cells. In addition to ionomycin, treatment with hydrogen peroxide has also been shown to induce both apoptosis and classical necrosis in Jurkat cells. The role of caspase-9 is well-known in stress-induced apoptosis and consequently H₂O₂ induced apoptosis only in caspase-9-positive cells, but in the presence of Z-VAD, H₂O₂ treatment resulted in equally intense necrosis in both JA3 and JA3-C9 cells. Our results indicate that in addition to its well-known role in intrinsic apoptosis, caspase-9 is specifically required for necroptosis, whereas it has no role in necrosis.

4.5. The role of the caspase-9 in Toll-like receptor induced necroptosis

Necroptosis has been observed not only after DR-mediated signals, but also as a result of various other stimuli, among them pattern recognition receptor activation has been investigated the most intensely. Wild-type and caspase-9 negative MEF cells were treated with lipopolysaccharide (LPS) in the presence of Z-VAD. Similarly to the results detected after DR activation, in the absence of caspase-9 significantly fewer dead cells were detected after LPS exposure compared to the wild-type cells. Mouse cells have been published to be sensitive to human herpesvirus 1 (HHV-1 or HSV-1) induced necroptosis. MEF and MEF-C9 cells were infected with HSV-1 in the presence of Z-VAD and it was observed that the lack of caspase-9 protected MEF cells from HSV-1 induced necroptosis.

Therefore, it has been proven that caspase-9 plays an important role not only in DR-mediated necroptosis, but also in pattern recognition receptor induced necroptosis as well.

4.6. TLR5 activation enhances the RIPK1-dependent cell death processes

In the majority of cases the elimination of infected cells is achieved by FasL mediated apoptosis. However, in infected cells TLR mediated pathways can also be activated or may already be active in response to the presence of PAMPs and DAMPs. Although it is a very common phenomenon, the effect of the simultaneous activation of TLRs and DRs on the cell death processes is still not fully clarified yet.

We were wondering whether the activation of TLR5 affects the DR-mediated apoptosis. Therefore, JA3 and its RIPK1 deficient counterparts (JA3-RIPK1) were pre-treated with recombinant flagellin for 1 hour and then activated with FasL for 24 hours. According to our results, both the wild type and JA3-RIPK1 were sensitive to FasL-induced cell death. Flagellin pre-treatment resulted in significantly more intense cell death in the wild type cells compared to the RIPK1 deficient cells upon FasL stimulation. To further confirm the impact of flagellin on other types of death receptor-mediated signaling events, we also activated the cells with TNF or the supernatant of phytohemagglutinine-activated T-cells (T-sup) after flagellin pre-treatment. Our results showed that, in contrast to the wild type cells, flagellin did not increase the intensity of T-sup- and TNF-induced cell death in the RIPK1 deficient cell lines. To determine whether flagellin affects DR-stimulated necroptosis or its effect is restricted exclusively to DR-mediated apoptosis, we treated the JA3 cells with flagellin and co-cultured them with FasL-expressing WSU B-cells in the presence of Z-VAD.

Strikingly, flagellin pre-treatment of Jurkat cells was able to increase Fas-induced necroptosis to an extent similar to that observed in apoptotic conditions. In the presence of Z-VAD, the cell death process could be completely blocked by Nec1 confirming that the observed cell death is necroptosis. These results altogether demonstrate that flagellin pretreatment supports both the RIPK1 dependent apoptotic and necroptotic pathways.

4.7. Identifying the interaction partners of caspase-9 during necroptosis

Considering that DR-induced necroptosis takes place at caspase-compromised conditions, the protease activity of caspase-9 is not required for its role in the signaling. Therefore, we assumed that caspase-9 contributes to necroptosis as an adaptor protein. We checked the molecules recruited to caspase-9, in particular, its association with RIPK1, RIPK3, and MLKL. Although we were unable to detect any interaction between caspase-9

and RIPK1 or MLKL, but we observed a weak and transient association of RIPK3 with caspase-9, which peaked at 3 h after the initiation of necroptosis. Immunoprecipitation with anti-RIPK3 Ab has confirmed RIPK3-caspase-9 association. According to our data, caspase-9 can be considered as a regulator of necroptosis that interacts with RIPK3.

4.8. Investigating the assembly and activation of the necrosome

The signal paths that can be started with a wide variety of stimuli ultimately leads to the assembly of the necrosome. The first step in the formation of the necrosome is the RIPK1/RIPK3 interaction, which is followed by the phosphorylation of these proteins.

To dissect the role of caspase-9 in the molecular background of necroptosis, we compared the association of RIPK1 with RIPK3 in the presence and absence of caspase-9 to analyze the regulated events in the course of the necroptotic process. We detected the RIPK1/RIPK3 interaction by immunoprecipitation 3–6 h following TBZ activation in wild-type Jurkat cells, but we could not observe this association in the absence of caspase-9. To further evaluate how the absence of caspase-9 affects the signaling events, we examined the phosphorylation of the core components of the necrosome. RIPK1 phosphorylation (S166) was strongly elevated in JA3 cells following TBZ activation, but only marginal phosphorylation of RIPK1 was found in JA3-C9 cells. Similarly, more intense RIPK3 phosphorylation (S227) was detected in wild-type, than in JA3-C9 cells 6 h post activation. The failure of RIPK1/RIPK3 interaction in JA3-C9 cells indicates that caspase-9 regulates the upstream events of necroptotic signaling.

4.9. RIPK1 and RIPK3 overexpression restores necroptosis

We overexpressed RIPK1 and RIPK3 assisted by a Tet-On inducible system in both JA3 and JA3-C9 cells to verify the regulation of the necrosome assembly by caspase-9. In non-overexpressed cells, doxycycline pre-treatment had no effect on cell death intensity, non-infected JA3-C9 cells were still proved to be unresponsive to the stimulus. However, on the infected cells, the doxycycline pre-treatment initiated the overexpression of RIPK1 and RIPK3 (confirmed by Western Blotting) and it reconstituted the sensitivity of JA3-C9 cells to necroptosis. These results demonstrate that the downstream parts of the necroptotic pathway still function normally in the absence of caspase-9 in both RIPK1- and RIPK3-overexpressing cells.

4.10. AURKA and caspase-9 interact during necroptosis

We have shown that caspase-9 regulates the initial phase of necroptotic signaling. Caspase-9 interacts weakly with the basic components of the necroptotic signaling pathway; therefore, we hypothesized that it acts primarily on molecules that regulates the necrosome assembly. AURKA has been identified recently as an interaction partner of RIPK3 and RIPK1. We tested the association of caspase-9 with AURKA under necroptotic conditions in Jurkat cells and found that AURKA was associated with caspase-9 in a stimulus-dependent manner. Caspase-9-mediated regulation of AURKA was confirmed by the investigation of AURKA phosphorylation. AURKA phosphorylation on T288 was observed only in JA3 but not in JA3-C9 cells, and this phosphorylation was elevated upon TBZ stimuli indicating that the activity of this kinase is regulated by caspase-9.

In summary, caspase-9 and the AURKA mediated pathways interact in the regulation of necroptosis in a still unidentified way.

4.11. AURKA inhibitor restores the necroptosis sensitivity of caspase-9-deficient cells

AURKA and its downstream target, GSK3 β , have been published to downregulate necroptosis. We tested the TBZ-induced necroptosis in the presence of AURKA inhibitor (CCT137690) or GSK3 β inhibitor (AR-A014418) in JA3 and JA3-C9 cells. Low doses of CCT137690 (<2.5 μ M) or AR-A014418 (<20 μ M) had only minimal effects on the cell survival; however, higher doses of CCT137690 ($\geq$ 2.5 μ M) and AR- A014418 ($\geq$ 20 μ M) elevated the ratio of dead cells. Co-treatment with low doses of either CCT137690 or AR-A014418 with TBZ increased necroptosis, which was effectively blocked by all of the applied necroptosis inhibitors (Nec1, GSK'872, and necrosulfonamide). Importantly, co-treatment of either CCT137690 or AR-A014418 with TBZ restored the necroptosis sensitivity of the caspase-9-deficient cells.

These results indicate that caspase-9 may promote necroptosis against the AURKA - GSK3 β pathway mediated necroptosis inhibition, which control mechanism is hyperactivated in the absence of caspase-9

4.12. Verifying the necroptotic function of caspase-9 in acute pancreatitis

To confirm the role of caspase-9 in necroptosis in vivo, we compared the severity of cerulein-induced experimental acute pancreatitis (AP) in wild-type and pancreas acinus-specific caspase-9 knockout mice.

In this experiment, mice were i.p. injected hourly (eight times) with either physiological saline (control) or 50 µg/bwkg cerulein to induce AP. Overall, in this experimental model we observed a remarkable improvement of pancreatitis morphology in the caspase-9 knockout animals. The control mice had normal pancreatic histology in both groups, whereas cerulein stimulation caused pancreatic damage. Knockout of caspase-9 in pancreatic acinar cells significantly decreased the histological scores and improved serum amylase activity. The extent of interstitial edema and leukocyte infiltration was significantly improved, and the extent of necrosis was completely restored to the control level.

These results highlight the major role of caspase-9 during necroptosis in the development of experimental AP.

4.13. The activation of necroptotic proteins following cerulein treatment

Although it is widely accepted that necroptosis is responsible for the destruction of the pancreas and for the appearance of characteristic clinical symptoms in the cerulein-induced acute pancreatitis model, but we still wanted to confirm, that cerulein treatment activates necroptosis and not other cell death processes. For this we examined the phosphorylation of necrosome components. We detected increased RIPK1 and RIPK3 phosphorylation in pancreatic tissue samples following cerulein treatments in wild-type mice, but not in caspase-9-deficient animals.

These results confirm that cerulein stimulation leads to necroptosis, but only in the presence of caspase-9.

5. Discussion

Cell death is an indispensable process that affects billions of cells each day in the human body. In addition to intensity, the immunological outcome of cell death also has fundamental impact on the evolution of various inflammatory diseases, autoimmune processes, degenerative disorders, and immune surveillance of tumors. What was limited only to two separate processes (apoptosis and necrosis) in the past, is now considered as a dynamically developing scientific field. Thanks to the rapid development of the past 20 years, we now know that the removal of the cells can be potentially achieved through several distinct cell death mechanisms.

There is more and more scientific evidence suggesting, that the highly diverse signaling processes that carry out the different cell death subtypes are inter-connected more strictly than previously thought. In certain circumstances, apoptosis and autophagy are in a synergistic relationship, while in other situations autophagy can only be activated when apoptosis is inhibited. Necroptosis is still considered as a backup mechanism of apoptosis. However, shikonin – which is widely used in Chinese traditional medicine – is capable of inducing necroptosis directly without the need of caspase inhibition. However, the combined treatment of shikonin plus necroptosis inhibitors activates apoptosis, so in these cases apoptosis serves as a backup mechanism for necroptosis. In the previous chapters, I have described that the activation of various receptors can lead to the development of necroptosis, but these receptors are often the starting points of other routes as well. While the activation of the pro-caspase-8 is commonly associated with extrinsic apoptosis, the same protein can also induce inflammasome assembly and thus pyroptosis. Similarly, on mouse bone derived macrophages, LPS treatment leads to cell activation (if IAPs are not inhibited), apoptosis and NLRP3 dependent pyroptosis (in the absence or under the inhibition of IAPs) and necroptosis (when both IAPs and caspase-8 are inhibited). In all four cases, very similar complexes are formed in which common molecules are involved. Caspase-9 has been discovered as the initiator caspase of the mitochondrial apoptotic pathway, but its role in both nonapoptotic cell death processes and in autophagy has also been documented. FasL plus caspase inhibitor or cytotoxic T cell-induced necrotic cell death were also reduced in caspase-9-deficient cells.

In this work, we investigated whether the loss of caspase-9 have an effect on necroptosis or not. We have shown in different cell lines of human and murine origin that necroptosis is blocked in the absence of caspase-9. These results have been confirmed upon stimulation of all DRs, PRRs and after HSV1 infection. In all these experiments, necroptosis was induced under caspase-compromised conditions which indicate that caspase-9 acts as an

adaptor protein in necroptotic signaling. In accordance with this scenario, previous studies have demonstrated that the inhibition of caspase-9 did not modify necroptosis intensity.

The *in vivo* appearance of necroptosis indicates that in addition to caspase-mediated processes, various regulatory mechanisms control necroptosis that are independent of caspase activity. The expression, the interaction partners, the posttranslational modifications, and the localization of necrosome components are tightly regulated to control necroptosis. We demonstrated that the expression levels of RIPK1 and RIPK3 did not depend on caspase-9, but it was required for optimal RIPK1/RIPK3 association. We also identified caspase-9 as a new binding partner of RIPK3. The phosphorylation of RIPK1 and RIPK3 decreased in the absence of caspase-9. It is important to note that although RIPK1 initiates RIPK3 activation during DR -driven necroptosis, it inhibits the activation of RIPK3 in PRR-induced necroptosis. We assume that caspase-9 may act more on RIPK3. This process can consequently control RIPK1 phosphorylation in DR-induced necroptosis because the stability of the necrosome has been reported to regulate the phosphorylation of both RIPK1 and RIPK3. The dimerization of RIPK3 is one of the most critical points of necroptosis induction, and increased expression of RIPK3 can induce its oligomerization and can initiate necroptosis. We have shown that overexpression of RIPK1 and RIPK3 restores the necroptosis sensitivity of caspase-9-deficient cells and can bypass caspase-9-mediated regulation of necroptosis.

Aurora kinases (Aurora A / B / C) are known to play important roles in both mitosis and meiosis. Their excessive activation is reported in many cancers, that points out their potential oncogenic function. Therefore, the aurora kinases serve as a promising target for cancer therapy. Up until now, several Aurora kinase inhibitors have already been discovered, some of them (AT9283, AZD1152, Danusertib, MLN8054) are already being evaluated in clinical trials. It is known that beyond its role during cell division AURKA, interacts with RIPK1 and RIPK3 with its interaction partner (GSK3 β) acts as one of the negative regulators of necroptosis. In our experiments, we examined whether the caspase-9 deficiency affects the AURKA/ GSK3 β pathway or not. We revealed that AURKA associates with caspase-9 upon necroptotic stimulus, but further investigations are needed to determine the exact molecular background of caspase- 9 and AURKA interaction. The phosphorylation of AURKA, was also proved to be caspase-9 dependent during necroptosis. Finally, similarly to the overexpression of RIPK1 and RIPK3, the inhibition of either AURKA or its downstream substrate, GSK3 β , restored necroptosis sensitivity of caspase-9-deficient cells. Altogether, our results indicate that caspase- 9 acts as a newly identified regulator in the finetuning of

necroptotic signaling, and can control the cross-regulation of intrinsic apoptosis and necroptosis.

Certain cell death subtypes have the ability to invoke the inflammatory response of the surrounding cells and tissues. A common feature of such "lithic" cell death processes is the massive release of DAMPs into the surrounding area when the integrity of their cell membrane is lost. DAMPs are both structurally and functionally highly varied molecules and are normally found inside the cells. Under normal conditions, they are indispensable for several cellular processes, but after certain effects (such as physical trauma, irradiation, osmotic stress, toxins, hypoxia, pathogenic infection) they are released into the extracellular space, where these molecules activate the inflammatory response of other cells through pattern recognition receptors.

Cell death can be result, but in some events, it can be the initiator of inflammation also. The most well-known DAMP-associated cell death is necrosis, in which very diverse DAMPs are released due to the complete breakdown of the cell membrane. However, there are certain DAMPs, that are frequently associated to a specific forms of cell deaths (apoptosis, necroptosis, pyroptosis, ferroptosis), thus these cell death subtypes activate inflammation through different pathways. Apoptosis, for example, leads to the release of nuclear proteins, mainly due to chromatin condensation and DNA fragmentation. Antimicrobial peptides and nuclear proteins are typical features of neutrophil extracellular traps (NETs). Characterizing DAMPs can be used to differentiate between various cell deaths. For example, 180 bp DNA fragments are typically released from apoptotic cells, while during necrosis these can be as long as 10 000 bp. During pyroptosis the released high-mobility group protein 1 (HMGB1) in most cases is hyperacetylated, while this is not observed in neither apoptosis or necroptosis.

Activation of the pro-inflammatory signaling pathways is one of the earliest events in the immune response. Acute inflammation is an important defense mechanism which is triggered by immune cells (macrophages, mast cells), due to the activation of their cell surface or intracellular PRRs. The resolution of inflammation is a very important step to restore the normal tissue microenvironment after the elimination of the pathogen. If this step cannot be executed for some reason (for example because the overactivation of positive feedback mechanisms), the acute inflammation becomes chronic, in which the immune response will continue, causing tissue damage and finally lead to diseases associated with chronic inflammation. Chronic inflammation is a common feature of many diseases affecting the internal (such as the gastrointestinal tract associated Crohn's disease or colitis ulcerosa) and external (eczema and psoriasis in the skin) body surface. Inflammation can often be the cause

of several rheumatic diseases such as systemic lupus erythematosus, rheumatoid arthritis, psoriatic arthritis, osteoarthritis, spondylitis ankylopoietics (Bechterew disease) or gout. Therefore, switching from lithic cell death types to other tolerogenic cell death processes would be of paramount therapeutic importance in such diseases.

It is reasonable to assume that the common molecules of different cell death signals regulate the transition for one cell death form to another, and thus, caspase-9 poses an attractive target for pharmacological interventions. We checked the role of caspase-9 in one of the most intensely studied inflammatory disorders, AP. Necroptosis inhibitors, as well as RIPK3 or MLKL deficiency, have been published to block AP, but caspase inhibitors remain ineffective in *in vivo* pancreatitis models. Consistent with the demonstrated role of caspase-9 in *in vitro* experiments, we presented here that acinar cell-specific depletion of caspase-9 protected mice from cerulein-induced AP. Necroptosis is considered as a backup mechanism of apoptosis, because the absence of caspase-8 or caspase inhibition activates necroptosis. We can assume that preventing one type of regulated cell death induces the activation of an alternative cell death pathway to compensate the failure. In accordance with this hypothesis, decreased apoptosis markedly stimulated necrosis in pancreatitis model. Surprisingly, our data indicate that the intensity of necroptosis was reduced or even ceased in the absence of caspase-9 both in *in vitro* and *in vivo* experimental settings. Our results suggest that caspase-9 is also involved in the signaling of two different cell death pathways.

Overactivation of cell death pathways (neurodegenerative diseases) are just as detrimental as their downregulation (bacterial and viral infections, formation of tumors). In a separate group of diseases, the balance of apoptosis-necroptosis shifts strongly towards necroptosis. This leads to events in which the removal of cells is not carried out in a tolerogenic manner, but by one of the lithic cell death types causing inflammation and strong autoimmunity. Therapeutic possibilities may include cell death inhibitors (necrostatins) or various chemotherapeutic agents. The molecular programs that govern switches between the different modes of cell death remain obscure despite the enormous potential of putative therapeutic applications. For this, it would be necessary to explore the common molecules between different cell death pathways and to identify the possible switching mechanisms. We hope that in our work we have been able to contribute to our current understanding of the dialogue between mitochondrial apoptosis and necroptosis and thus will opened up new possibilities in the treatment of chronic inflammatory conditions

6. Summary

Cell death has a fundamental impact on the evolution of degenerative disorders, autoimmune processes, inflammatory diseases, tumor formation and immune surveillance. Over the past couple of decades extensive studies have uncovered novel cell death pathways, which are independent of apoptosis. Among these is necroptosis, a tightly regulated, inflammatory form of cell death. Based on current data, necroptosis serves as a backup mechanism when death receptor-induced apoptosis is inhibited or absent. Several substrates of Caspase-8 have been documented as critical regulators of necroptosis (such as RIPK1, RIPK3 and CYLD), however the necroptotic role its most well-known substrates, the downstream caspases, have been only marginally studied. As the most widely studied initiator caspase, Caspase-9 is a key player in the intrinsic or mitochondrial apoptotic pathway which is involved in many various stimuli, among them hypoxia, radiation and chemotherapeutic agents. Deficiencies in the Caspase-9 activation has profound physiological and pathophysiological outcomes, leading to degenerative diseases, developmental defects and even cancer.

Despite the huge clinical potential of switching between cell death modalities to avoid the unwanted inflammatory outcome of cell death in various inflammatory diseases, the necroptotic role of the proteins involved in mitochondrial apoptosis has not been investigated. Here we demonstrated for the first time that the stimulation of several death and pattern recognition receptors induced necroptosis under caspase-compromised conditions in wild-type, but not in Caspase-9 negative human Jurkat and murine MEF cells. It is reasonable to assume that the common molecules of different cell death signals regulate the transition for one cell death form to another and thus Caspase-9 poses an attractive target for pharmacological interventions. We checked the role of Caspase-9 in one of the most intensely studied inflammatory disorders, acute pancreatitis. According to our results, cerulein-induced pancreatitis was significantly reduced in mice with acinar cell restricted Caspase-9 gene knockout. The signaling pathway of necroptosis is regulated on multiple levels, from the transcription, to the stability and posttranslational modifications of the necrosome components, to the availability of molecular interaction partners and the localization of RIPK1, RIPK3 and MLKL. We demonstrated that the absence of Caspase-9 led to impaired association of RIPK1 and RIPK3 and resulted in decreased phosphorylation of RIP kinases. RIPK3 dimerization is the most critical points of necroptosis induction and increased expression of RIPK3 can induce its oligomerization and can initiate necroptosis. Here we have shown that the overexpression of RIPK1 or RIPK3 restored the necroptosis sensitivity of

Caspase-9 deficient cells and can bypass Caspase-9-mediated regulation of necroptosis. It has been published that AURKA-GSK3 β axis downregulates necrosome formation. Inhibition of either AURKA or its known substrate, GSK3 β restored necroptosis sensitivity of Caspase-9 deficient cells, indicating an interplay between Caspase-9 and AURKA-mediated pathways to regulate necroptosis. Our findings suggest that Caspase-9 acts as a newly identified regulator of necroptosis and thus Caspase-9 provides a promising therapeutic target to manipulate the immunological outcome of cell death.

7. List of publications



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List of publications related to the dissertation

1. **Molnár, T.**, Pallagi, P., Tél, B., Király, R., Csoma, E., Jenei, V., Varga, Z., Gogolák, P., Odile Hueber, A., Máté, Z., Erdélyi, F., Szabó, G., Pettkó-Szandtner, A., Bácsi, A., Virág, L., Maléth, J., Koncz, G.: Caspase-9 acts as a regulator of necroptotic cell death.
FEBS J. [Epub ahead of print], 2021.
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Immunol. Lett. 193, 42-50, 2018.
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IF: 2.552

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3. Varga, Z., Rácz, E., Türk-Mázló, A., Korodi, M., Szabó, A., **Molnár, T.**, Szőör, Á., Veréb, Z., Bácsi, A., Koncz, G.: Cytotoxic activity of human dendritic cells induces RIPK1-dependent cell death.
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4. Varga, Z., **Molnár, T.**, Türk-Mázló, A., Kovács, R., Jenei, V., Kerekes, K., Bácsi, A., Koncz, G.: Differences in the sensitivity of classically and alternatively activated macrophages to TAK1 inhibitor-induced necroptosis.
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Cell Death Dis. 10 (11), 1-21, 2019.
DOI: <http://dx.doi.org/10.1038/s41419-019-2094-z>
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8. Keywords

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