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Dissecting the role of lysosomal proteases in programmed cell death

Małgorzata Kalinka, MSc, Eng.

Supervisor

Marcin Poręba, PhD, DSc, Prof. of WUST

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Abbreviations

ABPs - activity-based probes
ACD - accidental cell death
AEP - asparaginyl endopeptidase, legumain
AIF - apoptosis-inducing factor
AOMK - acyloxymethyl ketone
Apaf-1 - apoptotic protease activating factor 1
ASC - apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)
ASM - acid sphingomyelinase
ATP - adenosine triphosphate
BAK - BCL-2 antagonist/killer 1
BAX - BCL-2 associated X protein
BCA - Bicinchoninic Acid
Bcl-2 - B cell lymphoma 2 protein family
BH - BCL-2 homology domains
BID - BH3-interacting domain death agonist
BIM - BCL-2-interacting mediator of cell death
BOK - BCL-2 family apoptosis regulator
BMP - bis(monoacylglycero)phosphate
CARDs - caspase recruitment domains
CatB - cathepsin B
CatD - cathepsin D
CatG - cathepsin G
CatL - cathepsin L
CatS - cathepsin S
CatV - cathepsin V
c-FLIP - FLICE-like inhibitory protein (FADD-like IL-1 β -converting enzyme-inhibitory protein)
CFLAR - CASP8 and FADD-like apoptosis regulator (gene encoding c-FLIP)
CytoF - Cytometry by Time-of-Flight or Mass Cytometer (Instrument)
Cy - cyanine
Cys - cysteine
DAMPs - damage-associated molecular patterns
DD - death domains
DED - death effector domains
DIABLO/SMAC - second mitochondria-derived activator of caspases
DISC - death-inducing signaling complex
DPBS - Dulbecco's phosphate-buffered saline
DRP1 - dynamin-related protein 1

ER - endoplasmic reticulum
FADD - Fas-associated protein with death domain
FasL - Fas ligand
FCS - flow cytometry standard
FLICE - FADD-like IL-1 β -converting enzyme
GSDMA - gasdermin A
GSDMB - gasdermin B
GSDMC - gasdermin C
GSDMD - gasdermin D
GSDME / DFNA5 - gasdermin E
GBP1 - guanylate-binding protein 1
GBPs - guanylate-binding proteins
Hsp - heat shock protein
HMGB1 - high mobility group box 1
HyCoSuL - Hybrid Combinatorial Substrate Library
IAPs - inhibitor of apoptosis proteins
ICE - interleukin-1 β -converting enzyme (caspase-1)
ILVs - intraluminal vesicles
IMP – inner membrane peptidase
IQF - internally quenched fluorescence
LAMPs - lysosomal-associated membrane proteins
LDGD - lysosome-dependent cell death
LDH - lactate dehydrogenase
LeuLeu-OMe (LLOMe) - L-leucyl-L-leucyl methyl ester
LIMPs - lysosomal integral membrane proteins
LMP - lysosomal membrane permeabilization
LPS - lipopolysaccharide
LRR - leucine-rich repeat
MCPs - metal-chelating polymers
MOMP - mitochondrial outer membrane permeabilization
MSDH - O-methyl-serine dodecylamide hydrochloride
MSU - monosodium urate crystals
NE - neutrophil elastase
NEK7 - NIMA-related kinase 7
NF- κ B - nuclear factor kappa-light-chain-enhancer of activated B cells
NINJ1 - ninjurin-1
NLRs - nucleotide-binding oligomerization domain-like receptors
NLRP3 - NLR family pyrin domain containing 3
NO - nitric oxide
NOD2 - nucleotide- binding oligomerization domain- containing protein 2
NOXA - phorbol-12-myristate-13-acetate-induced protein 1
NSP4 - Neutrophil Serine Protease 4
NSPs - neutrophil serine proteases
OMM - outer mitochondrial membrane

PAMPs - pathogen-associated molecular patterns
PARP-1 - Poly(ADP-ribose) polymerase-1
PC - phosphatidylcholine
PCD - programmed cell death
PE - phosphatidylethanolamine
PEG - polyethylene glycol
PFD - N-terminal pore-forming domain of gasdermin D
PI - propidium iodide
PJVK - pejvakin
PMA - phorbol 12-myristate 13-acetate
PR3 - proteinase-3
PRRs - pattern recognition receptors
PS - phosphatidylserine
PTMs - post-translational modifications
PUMA - p53 upregulated modulator of apoptosis
PYD - pyrin domain
RCD - regulated cell death
RD - C-terminal repressor domain of gasdermin D
RIPK1 - receptor-interacting protein kinase 1
ROS - reactive oxygen species
SMAC - second mitochondria-derived activator of caspases (see DIABLO)
SMase - sphingomyelinase
SNs - silica nanoparticles
STS - staurosporine
TBID - truncated Bid
TCA - trichloroacetic acid
TLR4 - Toll-like receptor 4
TLRs - Toll- like receptors
TNF - tumor necrosis factor
TNF- α - tumor necrosis factor-alpha
TNF-R - tumor necrosis factor receptor
TRADD - TNFRSF1A-associated via death domain
TRAIL - tumor necrosis factor-related apoptosis-inducing ligand
TUNEL - terminal deoxynucleotidyl transferase dUTP nick end labeling
TOF-ABPs – time-of-flight activity-based probes
UPS – ubiquitin-proteasome system
WR - working reagent

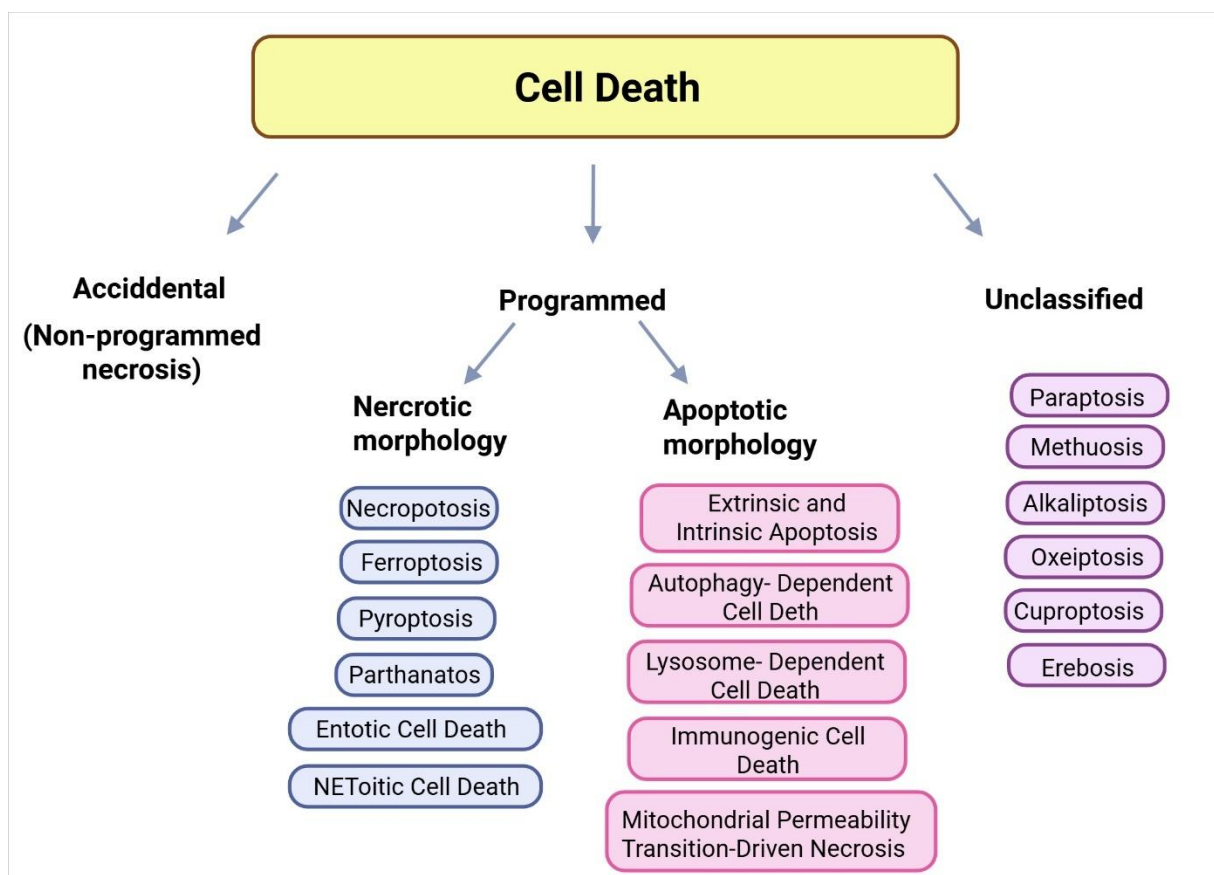
1. INTRODUCTION

1.1. CELL DEATH

Estimated, the subject of a cell death was born in 1970s, but the peak of its growth started in 1990s when the approach to the matter of cell death slightly changed from being an incidental and unregulated process to a crucial and carefully regulated aspect of life with significant medical relevance. First ever major event in the development of this field was discovery of phagocytosis by Ilya Mechnikow, awarded with Nobel prize in 1908 [1]. Followed by 1951 A. Glücksmann's recognition that cell death serves morphogenetic and histogenetic purposes [2] and observation of an apoptotic appearance of nuclei (karyopyknosis) and nuclear fragmentation (karyorrhexis). In the 50s Christian De Duve and his collaborators had recognized the existence of lysosomes [3]. Nearly a decade later, another significant breakthrough revealed that cell death could be delayed using inhibitors of protein or RNA synthesis. It was also discovered that neuronal cell survival depends on extracellular survival factors known as neurotrophins [4, 5]. A breakthrough in cell death research came from Kerr, Wylie, and Currie's ultrastructural study, which identified distinct morphological changes in cells undergoing physiological death [6]. Cells dying from severe stress or injury swell and rupture, a process termed necrosis. Contrary, most cells that die during normal development shrink, exhibit nuclear condensation, preserve membrane integrity, and are rapidly removed by phagocytosis, a process known as apoptosis [7]. Recent research has identified additional types of programmed cell death (PCD), demonstrating that apoptosis is not the sole form of developmental cell death and suggesting that alternative mechanisms may take over when apoptosis is inhibited [8]. Genetic studies in the nematode *C. elegans* provided a major advance in understanding the mechanism of programmed cell death. The discovery of mutations that specifically impact PCD, along with their organization into a genetic pathway, showed that cell death is a programmed developmental outcome, controlled by specific genes that trigger cell suicide [9, 10]. Field of cell death has been gaining attention ever since. Currently it's known that cell death is a naturally occurring process that not only removes damaged, potentially dangerous cells, maintains organismal homeostasis by sustaining cellular equilibrium, compose host defense against infections, addressing diseases such as cancer but also plays role in development. In both vertebrates and invertebrates, it can lead to the elimination of entire regions or organs in certain cases [11, 12].

Cell death can be categorized as either regulated or non-regulated, depending on its reliance on signaling pathways (**Figure 1**). Regulated cell death (RCD) is controlled by specific intracellular signaling mechanisms, whereas non-regulated cell death, accidental cell death (ACD) occurs incidentally due to unforeseen cellular damage by e.g., temperature, high pressure, osmotic stress, extreme pH or shear forces. What explains why ACD is immediate and catastrophic. Worth mentioning that RCD is not limited to multicellular organisms (where there is a need for maintaining organismal homeostasis) but it is as well observed in unicellular

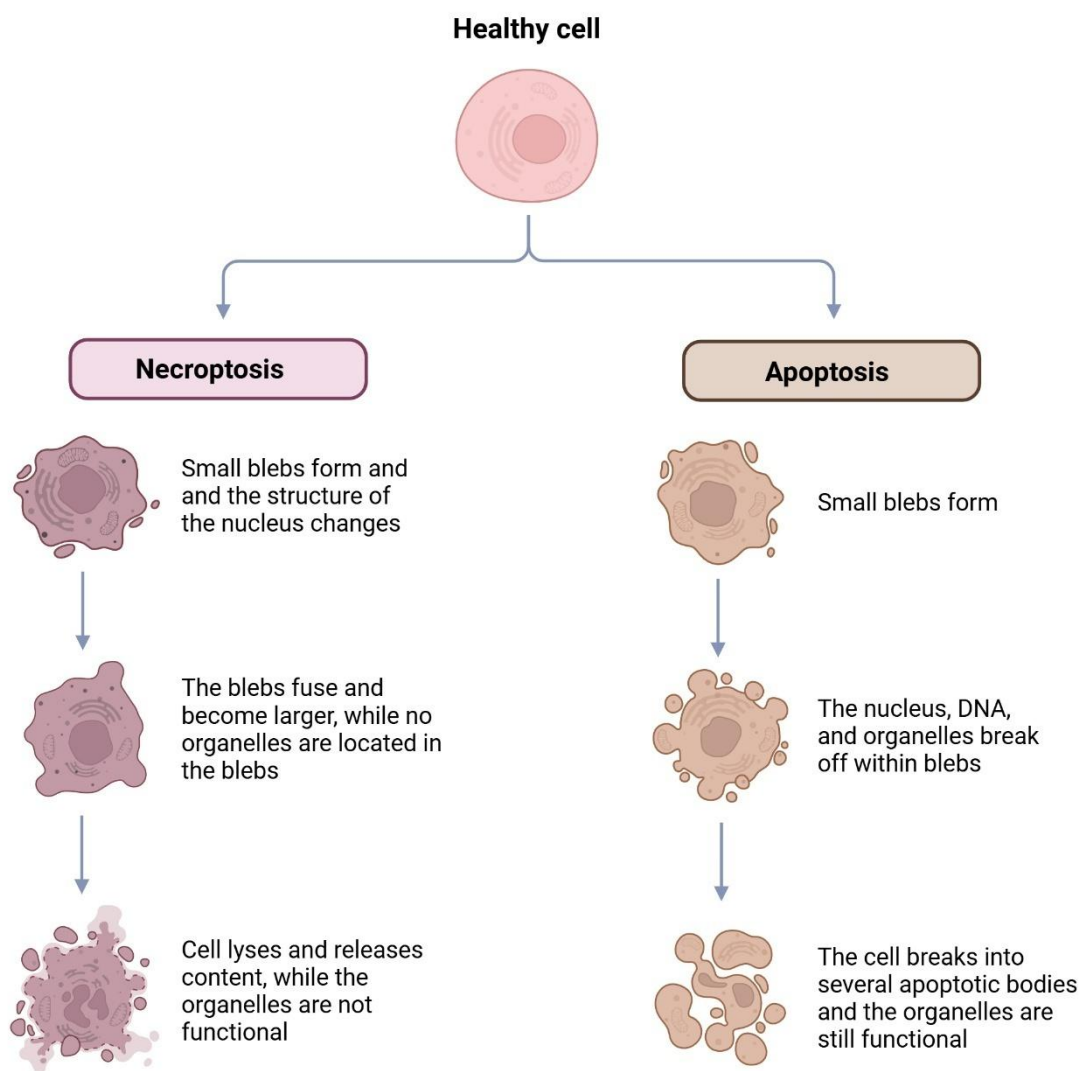
eukaryotes (e.g., *Dictyostelium discoideum*) and in some prokaryotes (e.g., *Escherichia coli*). Moreover, regulated cell death can be further classified into lytic and non-lytic forms, distinguished by their morphological characteristics and underlying molecular mechanisms that define the final cellular outcome [13-15]. Lytic cell death, such as pyroptosis and necroptosis, is driven by the disruption of the plasma membrane through pore formation (**Figure 2**) [16]. This disruption leads to cellular breakdown due to water influx, loss of membrane potential, and swelling, culminating in cell rupture. As a result, intracellular components, such as RNA, DNA, proteins, and lipids, along with materials from any intracellular pathogens, are released into the extracellular environment. In contrast, non-lytic PCD, including apoptosis, involves a carefully orchestrated breakdown of cells into smaller structures called formation of apoptotic bodies. These fragments help contain intracellular components, including DAMPs (damage-associated molecular patterns) and PAMPs (pathogen-associated molecular patterns) when present. Apoptotic cells are primarily recognized and cleared by macrophages, a process thought to limit inflammation by safely removing dying cells [13]. As a result, apoptosis is often referred to as an 'immunologically silent' form of cell death, in contrast to the pro-inflammatory nature of pyroptosis.



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Figure 1. Cell death classification. Schematic representation of major cell death modalities categorized according to their molecular mechanisms and morphological characteristics. The classification follows the 2018 recommendations of the Nomenclature Committee on Cell Death (NCCD) [15].

Numerous internal and external signals are capable of initiating the cascade of programmed cell death process, often in a cell type- and tissue-specific manner [17]. Endogenous factors arise from within the cellular environment and reflect the physiological state of a particular tissue e.g. cellular stress or imbalance, while exogenous triggers, such as toxins, radiation, physical injury, chemical agents or pathological conditions, converge to activate tightly regulated cell death acting more broadly, compromising cellular viability across diverse cell populations. Mostly the combination of cell-damaging agent, specific cell type and critical physiological conditions result in PCD [17-19].



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Figure 2. Morphological differences in lytic vs non-lytic cell death. Schematic representation highlighting the distinct morphological features of necroptosis (left) as a lytic form of cell death and apoptosis (right)-non lytic cell death. During necroptosis, small membrane blebs form and enlarge without containing organelles, eventually leading to cell lysis and the release of intracellular contents, which promotes inflammation. In contrast, apoptosis is characterized by controlled membrane blebbing, chromatin condensation, and fragmentation of the cell into apoptotic bodies that retain functional organelles, ensuring non-lytic and immunologically silent clearance by phagocytes.

Programmed cell death is indispensable for the normal development, and it is tightly regulated process. To date mechanisms of number diverse programmed cell deaths have been studied extensively, uncovering key role proteins in each of them giving a clear idea of those processes and the consequences of dysregulation of those processes. Such dysregulation leads to pathogenesis of various diseases and understanding the mechanism behind cell death enables to identify drug formulation to cure many conditions and some have progressed to clinical trials. Currently it is established that different cell death pathways merge and cell could be dying from number of different pathways at the same time making it more complicated on the one hand but on the other hand that provides more possibilities for establishing its importance in related diseases, searching for potential drug targets and putting forth new therapies. Due to the complexity of those systems and the sensitivity towards multiple stimuli there is a thin line between death and survival and anti-inflammatory and pro-inflammatory character of death. Although the field experienced a lot of crucial and lifechanging discoveries, there are still plenty to do [20].

1.1.1. Apoptosis

The term "apoptosis" was first introduced in 1972 by Kerr, Wyllie, and Currie in a landmark paper to describe a unique form of cell death characterized by specific morphological features [6, 21]. Insights into the mechanisms driving apoptosis in mammalian cells stemmed from studies of programmed cell death during the development of the nematode *C. elegans* [21, 22]. In *C. elegans* formation of 1090 somatic cells takes place during the development, 131 of which undergo apoptosis, also known as "programmed cell death". These specific cells are eliminated at precise stages of development, following a consistent pattern across individual worms, showcasing the exceptional precision and regulation of this process [21]. A variety of external and internal signals can activate apoptosis enabling the removal of excessive cells, aged or infected cells, those that have acquired genetic damage or those exhibiting abnormal development maintaining organism homeostasis. While various signals can trigger apoptosis across different cell types and species, a set of evolutionarily conserved genes governs a unified cell death pathway that is preserved from worms to humans [23, 24]. Progress in the field has identified four key functional categories of molecules that play crucial roles in initiating and regulating apoptosis. These include caspases, adaptor proteins responsible for activating initiator caspases, proteins from the tumor necrosis factor receptor (TNF-R) superfamily, and members of the Bcl-2 (B cell lymphoma 2) protein family [25] (**Table 1**). Each of those proteins are engaged in two main apoptotic pathways: the extrinsic, also called death receptor pathway and the intrinsic pathway, often regarded to as a mitochondrial pathway (**Figure 3**) [15, 21, 26].

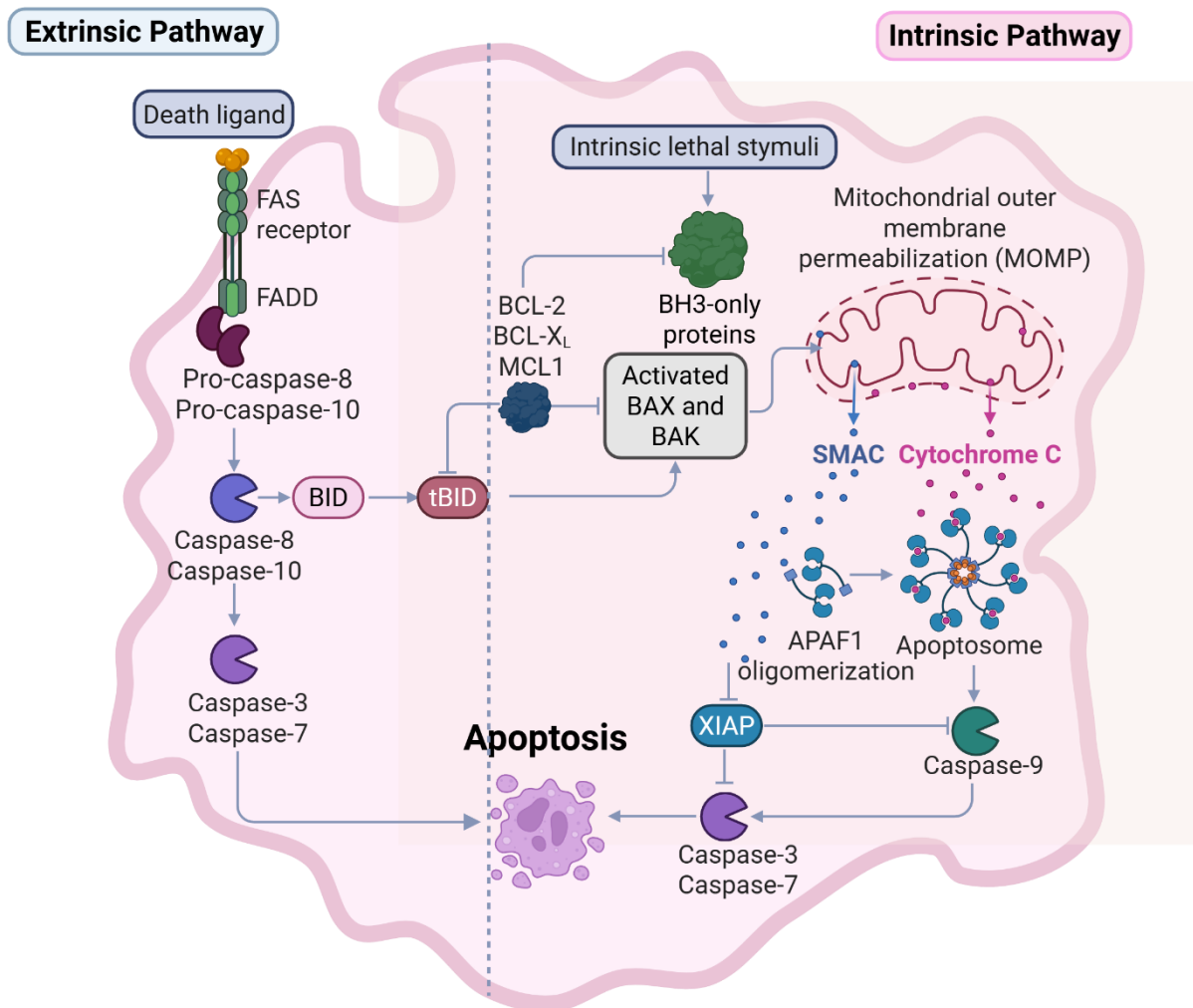
Signals for initiation of extrinsic signaling pathway are based outside of cell and depend on transmembrane receptor-mediated interactions [21]. When extracellular ligands such as tumor necrosis factor- α (TNF- α) or Fas ligand (FasL) bind to their respective death receptors, TNFR and Fas, the extrinsic apoptotic pathway is initiated as part of the cellular response to immune-mediated stress signals [27]. Among the most well-studied ligand-receptor pairs are

FasL/FasR, TNF- α /TNFR1, Apo3L/DR3, and Apo2L/DR4 and DR5 [21, 28-32]. After a ligand binds, cytoplasmic adapter proteins with matching death domains (DD) are recruited to interact with the cognate receptor. The DD is essential for transferring the death signal from the cell surface to intracellular signaling cascades. Binding of the Fas ligand to the Fas receptor leads to the recruitment of the adapter protein FADD (Fas-associated protein with death domain), while the TNF ligand binding to the cognate TNF receptor recruits the adapter protein TRADD (TNFRSF1A associated via death domain), along with FADD and RIPK1 (receptor-interacting protein kinase 1 [13, 21, 33, 34]. After binding to the death receptor, FADD undergoes a conformational change creating a platform that facilitates the recruitment of pro-caspase-8 or c-FLIP (FLICE-like inhibitory protein) to the receptor, thus forming a death-inducing signaling complex (DISC) located on the cytoplasmic tail of dead receptor. The interaction between pro-caspase-8 and the adaptor protein FADD, mediated by homotypic interactions between their death domains and the dimerization of pro-caspase-8 death effector domains, induces the autocatalytic activation of procaspase-8 through spatial proximity [13, 15, 21, 35-37].

Active caspase-8 initiates the downstream apoptotic cascade. Two groups of cells are distinguished based on caspase-3 activation pathway: type I cells mostly lymphoid and type II cells, most cell types. In type I cells, caspase-8 directly cleaves and activates executioner caspases, primarily caspase-3, leading to controlled dismantling of cellular structures. In contrast, type II cells, which compromise most somatic tissue, require mitochondrial amplification of the death signal [38]. In these cells, caspase-8 cleaves the BH3-only protein BID, generating its truncated active form (truncated Bid, tBID), which translocate to the outer mitochondrial membrane (OMM). There tBID promotes BAX (BCL2 Associated X protein) and BAK (BCL2 Antagonist/Killer 1) activation, resulting in MOMP (mitochondrial outer membrane permeabilization). This event enables the release of cytochrome c, DIABLO (second mitochondria-derived activator of caspases or SMAC), and other pro-apoptotic factors that drive apoptosome formation, activation of caspase-9, and full execution of apoptosis via caspases-3, -6, and -7 [13, 39-41]. Subsequently of this activation, execution phase of apoptotic cell death is performed: proteolytic activation of executioner caspases resulting in cell demolition [13, 21]. This tightly regulated process is further modulated by FLICE (FADD-like IL-1 β -converting enzyme or caspase-8)-inhibitory protein (c-FLIP), which competes with procaspase-8 for binding to DISC and can suppress apoptosis in response to cytokines, chemotherapeutics, TNF- α , and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) [37, 42-44]. Additionally, inhibitor of apoptosis protein (IAPs) such as XIAP may interfere with caspase activity downstream, adding an additional checkpoint in the extrinsic pathway [15, 45-47].

The intrinsic apoptosis can be initiated by severe or irreparable DNA damage (e.g., caused by genotoxic drugs, ionizing radiation or UV), growth factor withdrawal, endoplasmic reticulum (ER) stress or microtubular alterations [48-53]. Intrinsic pathway is often called the “mitochondrial pathway” and owes this name to extensive and irreversible mitochondrial outer membrane permeabilization, that represents a pivotal process [54, 55] and tightly regulated event in the intrinsic apoptotic pathway, orchestrated primarily by members of the BCL-2 protein family [48, 56, 57]. These proteins encompass both pro-apoptotic and anti-apoptotic regulators that collectively determine cellular fate in response to apoptotic stimuli. Upon receipt

of such signal, MOMP is executed predominantly by the pro-apoptotic effector BAX and BAK, which oligomerize to form pores in the outer mitochondrial membrane [58, 59]. BOK (BCL2 Family Apoptosis Regulator), another pro-apoptotic BCL-2 family member, also possesses the intrinsic capacity to induce membrane permeabilization. Notably, emerging evidence suggest that BAX, BAK, and BOK may extend their pore-forming activity to other intracellular membranes, highlighting a broader functional repertoire within the cell death machinery [15, 48, 56, 57]. Activation of the pro-apoptotic effectors BAX and BAK during apoptosis is mediated by BH3-only proteins, a subset of the BCL-2 family that functions as sentinels of cellular stress [60, 61]. Members of this protein family are characterized by the presence of conserved BCL-2 homology (BH) domains- designated BH1 through BH4- which mediate their interactions and regulatory functions [62]. While multi-domain members such as BAX, BAK and anti-apoptotic BCL-2 proteins typically contain three or four BH domains (BH1-3 or BH1-4), the BH3-only proteins are defined by the presence of only the BH3 domain, which is critical for their pro-apoptotic activity [15, 63].



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Figure 3. Schematic overview of intrinsic and extrinsic apoptosis pathways. The extrinsic apoptotic pathway is initiated when extracellular death ligands, such as TNF- α or FasL, engage their cognate receptors TNFR or Fas on

the cell surface. This interaction promotes the assembly of the DISC and the activation of initiator caspase-8, ultimately triggering executioner caspases. The intrinsic pathway, in contrast, is activated by internal stressors such as DNA damage, oxidative imbalance, or growth factor deprivation. These stimuli lead to MOMP, release of cytochrome c, and formation of the apoptosome complex with Apaf-1 and caspase-9. Both routes converge at the level of caspase-3/7 activation, resulting in DNA fragmentation, membrane blebbing, and apoptotic body formation, hallmarks of controlled, non-lytic cell death.

These proteins are either transcriptionally upregulated in response to apoptotic signal-such as PUMA (p53 upregulated modulator of apoptosis), BIM (BCL-2-interacting mediator of cell death) and NOXA (phorbol-12-myristate-13-acetate-induced protein 1), or activated through post-translational mechanisms, as is the case with BID (BH3-interacting domain death agonist) [64-68]. Their activation reflects a cellular response to disrupted homeostasis. Upon interaction with BAX and/or BAK, BH3-only proteins initiate conformational rearrangements that facilitate the oligomerization of these effectors. In this context, BAX, which is primarily cytosolic under non-apoptotic conditions, translocate to the mitochondria outer membrane where it inserts and becomes activated. In contrast, BAK is constitutively anchored at the OMM and undergoes activation through structural reconfiguration in situ. This process is self-amplifying, as the release of additional BH3-only proteins promotes further dimer formation, culminating in the assembly of lipidic pores within the mitochondrial outer membrane. These pores alter mitochondrial permeability, serving as a decisive event in the progression of apoptotic cell death [15, 62, 69-74].

Mitochondrial outer membrane permeabilization is tightly regulated by the interplay between pro-apoptotic and anti-apoptotic members of the BCL2 protein family. Antiapoptotic proteins- including BCL-2, BCL-X_L, MCL-1, BCL-W, and BFL-1-share a conserved structure comprising four BH domains and are typically localized at or integrated into the OMM and the ER [48, 56, 57, 75, 76]. Their primary function is to prevent apoptosis by sequestering the pro-apoptotic effectors BAX and BAK, either through direct binding or indirectly via interactions with BH3-only activators (BID, BIM, PUMA), thereby inhibiting BAX/BAK oligomerization and subsequent pore formation [48, 65, 72, 77]. Notably, a subset of BH3-only proteins such as BAD, BMF, and HRK- function as “inactivators”. These proteins do not directly activate BAX or BAK but instead neutralize anti-apoptotic BCL-2 family members, reducing their availability to inhibit BAX/BAK and thus indirectly promoting MOMP [78, 79]. These inactivator proteins, along with other BH3-only members, exhibit distinct binding preferences toward anti-apoptotic targets. For example, BID, BIM, and PUMA are classified as potent activators due to their broad binding capacity across all major anti-apoptotic proteins. In contrast, sensitizers such as BAD preferentially bind BCL-2, BCL-X_L, and BCL-W; NOXA selectively targets MCL-1; and HRK primarily interacts with BCL-X_L [80, 81]. This binding specificity underlies the finely tuned regulation of apoptotic signaling. To maintain this balance, BH3-only proteins are tightly controlled at multiple levels. Their expression and activity are subjected to transcriptional and post-translational regulation, including modulation by transcription factors (such as p53 for PUMA and NOXA), phosphorylation, changes in subcellular localization, and degradation via the ubiquitin-proteasome system. These mechanisms help ensure that BH3-only proteins are activated only under appropriate stress conditions [82-86]. Furthermore, the susceptibility of mitochondria to undergo irreversible outer membrane permeabilization is not solely dictated by

protein interactions; mitochondrial size, morphology, and lipid composition also influence the threshold for MOMP, adding an extra layer of regulation to the intrinsic apoptotic pathway [15, 87-90].

Once MOMP is executed, it facilitates the release of apoptogenic factors from the mitochondrial intermembrane space into the cytosol. Among these are cytochrome c, which under normal conditions functions as an electron carrier in the respiratory chain, and DIABLO/SMAC, which promotes caspase activation by antagonizing inhibitor of apoptosis proteins. The release of these proteins is further enhanced by mitochondrial cristae remodeling, a process governed by the oligomerization and activation of the dynamin-related GTPase OPA1. This remodeling may be preceded or modulated by BAX- and BAK- dependent activation of the mitochondrial protease OMA1, as well as by the fission-promoting activity of DRP1 (dynamin related protein 1). Additionally, [91] nitric oxide (NO) has been shown to induce the release of apoptogenic factors through S-nitrosylation of DRP1 in certain cellular context. In the cytosol cytochrome c binds Apaf-1 (apoptotic protease activating factor 1) and procaspase-9 in deoxyATP-dependent manner (adenosine triphosphate), leading to the assembly of the apoptosome, which catalyzes the activation of caspase-9 and initiates the caspase cascade that drives the execution phase of apoptosis. Studies have shown that the autocatalytic maturation of caspase-9 within the apoptosome proceeds through the formation of caspase-9 homodimers, as well as caspase-9-Apaf-1 heterodimers or multimers. These interactions are mediated by their respective caspase recruitment domains (CARDs), which facilitate the stable assembly and activation of the apoptotic machinery [15, 92-99].

Active caspase-9 initiates the execution phase of apoptosis by proteolytically activating caspase-3 and caspase-7, which mediate the dismantling of cellular components. The pro-apoptotic factor SMAC promotes apoptosis by neutralizing IAPs. For SMAC to exert its IAP-antagonist function, it must undergo proteolytic processing to expose its IAP-binding motif-a step mediated by the inner membrane peptidase (IMP) complex and potentially by the inner mitochondrial membrane protease PARL. Among IAP family members, XIAP is unique in directly inhibiting apoptosis by binding and blocking caspases. In contrast, c-IAP1 (BIRC2) and c-IAP2 (BIRC3) suppress apoptosis indirectly through multiple mechanisms: upregulation of anti-apoptotic regulators like such as c-FLIP, caspase inactivation via their E3 ubiquitin ligase activity, ubiquitination of RIPK1 to promote pro-survival NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells), signaling; and possibly mediating SMAC degradation ale the mitochondria through a BCL-2 dependent process [15, 100-107]. Once activated, executioner caspases orchestrate the biochemical and morphological hallmarks of apoptotic cell death like chromatin condensation, DNA fragmentation, externalization of phosphatidylserine (PS), membrane blebbing and formation of apoptotic bodies. Caspase 3 plays a central role in PS exposure by activating transmembrane phospholipid transporters-scramblases and simultaneously inactivating phospholipid flippases, what results in the translocation of PS to the outer leaflet of the plasma membrane, disrupting membrane asymmetry and promoting recognition by engulfing cells (like phagocytes) to avoid inflammation [108-113].

Apoptosis is traditionally characterized as a non-inflammatory form of programmed cell death, distinguished by the preservation of plasma membrane integrity throughout the execution

phase, in contrast to other forms of cell death in which membrane disruption occurs early. However, it is now well established that, *in vitro*, the terminal phase of apoptosis culminates in efferocytosis, a tightly regulated process in which apoptotic bodies and cellular debris are efficiently cleared by phagocytes, such as macrophages. This clearance is critical in maintaining immune tolerance and preventing secondary inflammation. Notably, both the intrinsic and extrinsic pathways of apoptosis, once considered immunologically silent, are now recognized to have the potential to elicit immune response under certain conditions. Furthermore, in *in vitro* models lacking the phagocytic cells, apoptotic cells that are not promptly cleared undergo secondary necrosis, a process marked by the eventual rupture of the plasma membrane and the release of intracellular contents-morphologically resembling necrotic cell death [15, 24, 114]. Collectively, these findings challenge the long-standing view of apoptosis as an entirely immunologically silent process, highlighting that under specific conditions, it can acquire pro-inflammatory properties and actively contribute to host defense against infection [115-117].

Table 1. Apoptosis key proteins

Protein	Group	Pathway	Role
BAK	Pro-apoptotic effector	Intrinsic	Execution of MOMP by oligomerization and formation of pores in OMM
BAX			
Caspase-9	Initiator caspase		Activation of executioner caspases
HRK	BH3-only sensitizer		Neutralization of anti-apoptotic BCL-2 proteins "sensitize" cells to apoptotic death
BMF			
BAD			
NOXA	BH3-only activator		Direct activation of BAX and BAK
PUMA			
BIM			
BID			
Apaf-1	Apoptosome component		Apoptosome formation and activation initiator caspase-9
Cytochrome c			
BCL-2	Anti-apoptotic		Inhibition of apoptosis by binding and sequestering pro-apoptotic proteins
BCL-W			
MCL-1			
BCL-XL			
BFL-1			

Caspase-3/7	Executioner caspase	Intrinsic and Extrinsic	Degradation of structural and repair proteins, DNA fragmentation, chromatin condensation
Caspase-6	Executioner caspase	Intrinsic and Extrinsic	Nuclear lamin A breakdown and cytoskeletal dismantling
TRADD	Adaptor protein	Extrinsic	Facilitating the connection of activated death receptors and initiator caspases through DISCs assembly
FADD			
FasL	Death ligand		Initiation of extrinsic apoptosis Initiation of extrinsic apoptosis
Fas	Death receptor		
TNFR1			
Caspase-8	Initiator caspase		Activation of executioner caspases
Caspase-10			
c-FLIP	Caspase inhibitor / modulator		Inhibiting apoptosis by competing with caspase-8 for binding to FADD at the DISC

1.1.2. Pyroptosis

The concept of pyroptosis originated in the 1980s, when anthrax toxin was found to induce rapid, lytic death in macrophages. In the years that followed, caspase-1, initially termed interleukin-1 β -converting enzyme (ICE), was identified as a key mediator of IL-1 β maturation. In the early 1990s, Zychlinsky and colleagues described pro-inflammatory cell death in *Shigella flexneri*-infected macrophages, initially misclassified as apoptosis due to shared features like DNA fragmentation and caspase dependence [118]. The term “pyroptosis” was formally introduced in 2001 to define a caspase-1-dependent pro-inflammatory form of programmed cell death that is distinct from the immunologically silent process of apoptosis [119]. It is now well established that pyroptosis is not limited to bacterial infections, but can also be triggered by a variety of infectious and sterile stimuli [119-123]. As implied by the etymology of the term “pyroptosis”, derived from Greek, with “pyro” referring to the fire or fever, this form of cell death is closely associated with inflammation. The subsequent identification of inflammasomes, non-canonical caspase-11 (murine) and caspase-4/5 (human) pathways, and the identification of gasdermin D (GSDMD) as the pore-forming executor protein, together with the recognition of ninjurin-1 (NINJ1) as a key mediator of terminal plasma membrane rupture, established pyroptosis as a caspase-mediated, membrane-disruptive death pathway with potent immunological consequences. The rupture of the plasma leads to the release of DAMPs and the mature form of pro-inflammatory cytokines from pyroptotic cells, which amplify local inflammation and immune cell recruitment. Furthermore, membrane disruption exposes intracellular pathogens to the extracellular environment, enhancing their recognition and clearance by the immune system [119, 124-128].

Like apoptosis, pyroptosis is orchestrated by members of the caspase family of cysteine proteases, specifically the so-called inflammatory caspases. These inflammatory caspases share structural features with apoptotic initiator caspases, including the presence of an N-terminal caspase recruitment domain, allowing them to participate in multiprotein signaling complexes [124, 125]. Although pyroptosis and apoptosis are mechanistically distinct, they share several morphological and biochemical features (**Figure 4**). Both involve caspase activation, DNA fragmentation, and chromatin condensation; however, their membrane dynamics and nuclear integrity differ significantly.

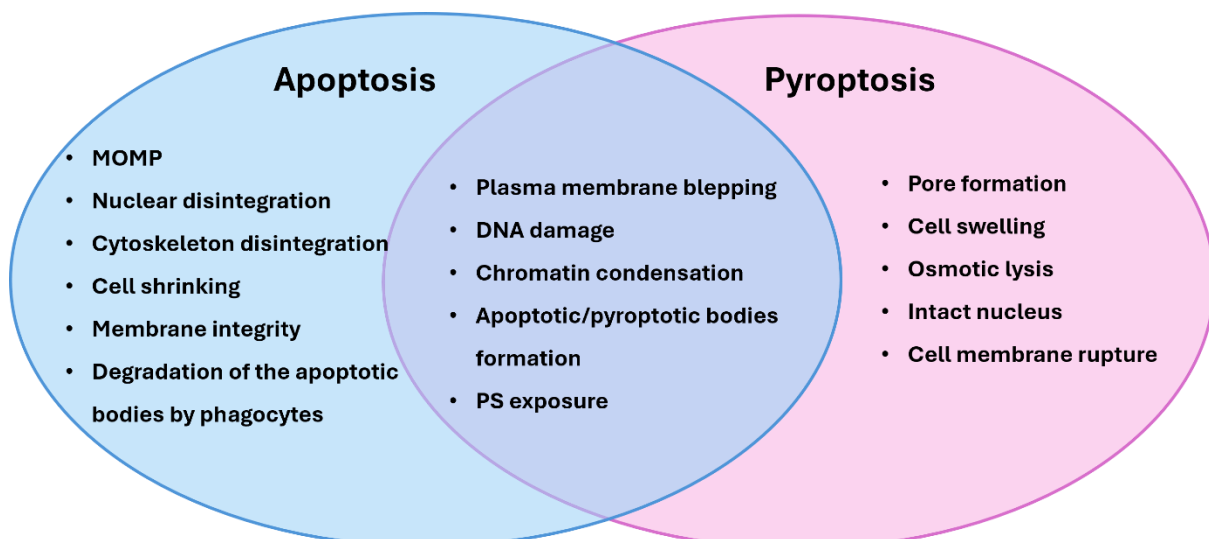


Figure 4. Morphological overlap and divergence between apoptosis and pyroptosis. Both death modes share early nuclear and membrane changes: phosphatidylserine externalization (Annexin-V⁺), DNA damage/TUNEL positivity, chromatin condensation, and membrane blebbing. Small membrane-bound fragments (cellular bodies ~1-5 μ m) can be observed in either context. **Apoptosis:** cells undergo shrinkage, sustained plasma-membrane integrity, and orderly packaging into apoptotic bodies; nuclei typically remain intact until late fragmentation. Consequently, cells are negative for impermeant dyes (e.g. PI) until secondary necrosis. **Pyroptosis:** formation of large plasma-membrane pores leads to rapid cell swelling, osmotic lysis, and loss of membrane integrity, producing pyroptotic bodies rather than classical apoptotic bodies. Cells become PI positive early, reflecting permeabilization. Together, the figure emphasizes that while apoptosis and pyroptosis share several early morphological hallmarks, they diverge at the membrane level: apoptosis remains membrane-contained with shrinkage and apoptotic bodies, whereas pyroptosis is lytic, characterized by pore formation, swelling, dye uptake, and rupture [126].

Pyroptotic cells typically swell and exhibit large bubble-like protrusions on the membrane surface before lysis, whereas apoptotic cells undergo membrane blebbing, a process dependent on caspase-3. Despite superficial similarities, pyroptosis is highly inflammatory and often triggered by microbial infections, toxins, or chemotherapeutic agents, in contrast to apoptosis, which generally proceeds in an immunologically silent manner [6, 126, 129-132]. During pyroptosis, pore formation in the plasma membrane is mediated by the oligomerized N-terminal fragment of gasdermin D, allowing the release of mature cytokines. These pores are approximately 1-2 μ m in diameter and sufficiently large to permit the leakage of caspase-1 (~7.5 nm) and cytokines (~4.5 nm) [133]. The growing osmotic imbalance leads to water influx, cell swelling, and eventual lysis, while the apoptotic cells remain intact during the cell death process. A notable difference between the two processes lies in DNA integrity as well. In apoptosis, DNA fragmentation is ordered and extensive, and nuclear breakdown is evident [134, 135]. In pyroptosis, by contrast, a special form of DNA damage with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) may be observed early, but DNA fragmentation is less intense and more random, with the nucleus generally remaining intact [126, 136-138]. Pyroptosis was initially defined as a caspase-1-dependent process, but accumulating evidence indicates a broader range of caspases can mediate this form of cell death. In humans, caspase-4 and -5 (and caspase-11 in mice) directly sense cytosolic lipopolysaccharide (LPS; a component of the Gram-negative bacterial cell wall) to trigger pyroptosis. Interestingly, caspase-3, classically considered an executioner of apoptosis, can induce pyroptosis via cleavage of gasdermin E, while caspase-9 can contribute indirectly by activating caspase-3 [139-141]. Other caspases traditionally associated with apoptosis also contribute. For instance, caspase-8 may activate pyroptosis through GSDMC cleavage [142] and through GSDMD cleavage as well [143, 144]. Current evidence suggests that caspase-1, -4, -5, and -11 are pyroptosis-specific, whereas caspase-2, -7, and -10 are confined to apoptosis (**Figure 5**) [126]. However, due to the extensive progress made almost every day in the area of cell death, these distinctions may evolve as further mechanistic insights emerge. Moreover, both apoptotic and pyroptotic cells release extracellular vesicles, referred to as apoptotic bodies and pyroptotic bodies, respectively. Both are similar in size (1-5 μ m) [145], but they differ in composition and immunological outcome [126].

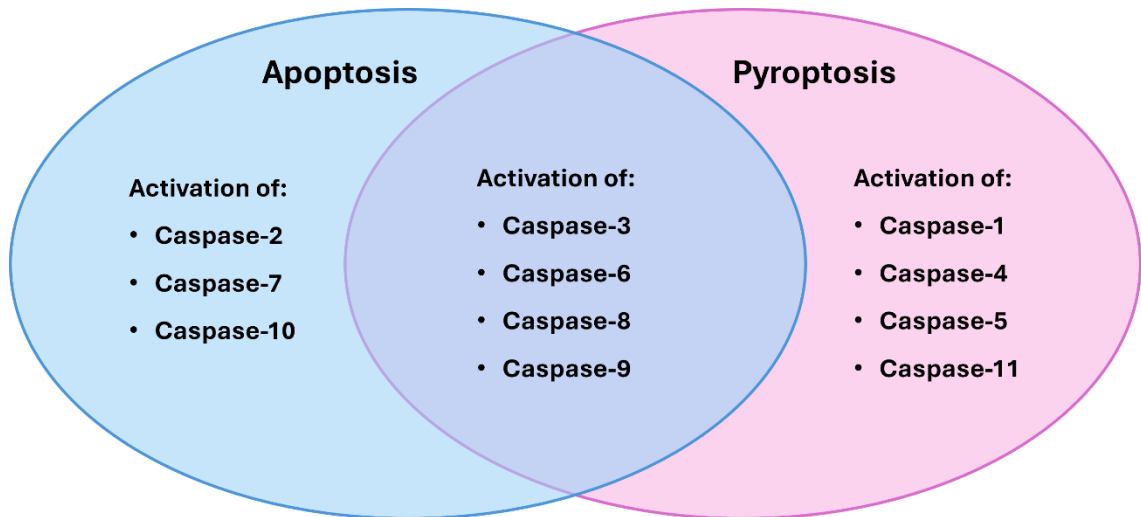


Figure 5. Mapping caspase pathways: separation and overlap between apoptosis and pyroptosis. Apoptotic signaling proceeds through initiator caspases, caspase-8/10 in the extrinsic pathway and caspase-9 in the intrinsic, mitochondrial pathway, that converge on the executioners caspase-3/7 to drive ordered proteolysis (e.g., PARP cleavage) with membranes largely intact. By contrast, pyroptosis is executed by inflammatory caspases: caspase-1 in the canonical pathway and caspase-4/5 in human (caspase-11 in mouse) in the non-canonical pathway, which both process pro-IL-1 β /IL-18 and cleave GSDMD to release a pore-forming N-terminus that precipitates K⁺ efflux, rapid membrane permeabilization, and lytic rupture. These programs intersect at the gasdermins: caspase-3, classically the apoptotic executioner, can cleave GSDME and convert an apoptotic trajectory into secondary pyroptosis where GSDME is expressed; caspase-8 can directly cleave GSDMD to trigger pyroptosis in certain contexts; caspase-9 can feed into this axis indirectly by activating caspase-3, and caspase-6 has been reported to cleave GSDMC. Thus, while apoptosis and pyroptosis are distinguished by their core caspase modules and outcomes (non-lytic versus lytic), context-dependent gasdermin cleavage by apoptotic caspases creates a mechanistic bridge that can pivot cells from classical apoptosis to pore-forming, inflammatory death [126].

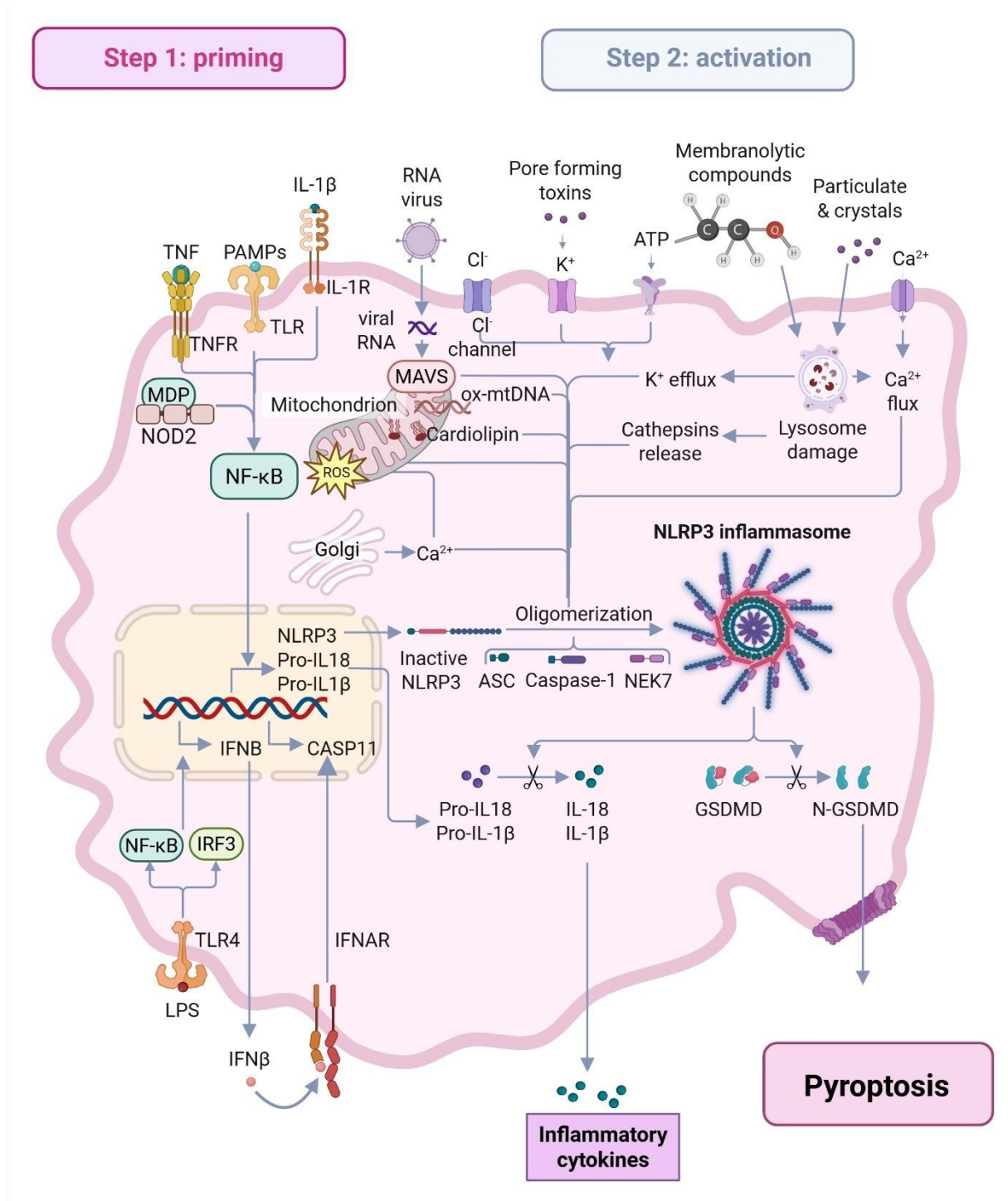
Pyroptosis was formally defined in 2015 as a gasdermin-mediated form of PCD [146]. The gasdermin superfamily includes GSDMA, GSDMB, GSDMC, GSDMD, GSDME (also known as DFNA5), as well as DFNB59 (Pejvakin, or PJVK) in humans, with corresponding homologs in mice [126, 133, 146-148]. Among these, GSDMD and GSDME are the most extensively studied in the context of pyroptosis. All gasdermins, except for Pejvakin, share a conserved architecture composed of an N-terminal pore-forming domain (PFD) and a C-terminal repressor domain (RD) [126, 149-151]. In their inactive state, these domains interact, preventing the pore formation as the proteins are in an autoinhibited conformation [151]. Upon exposure to microbial products, endogenous danger signals, inflammatory caspases (-1, -4, -5, or -11), or granzymes, the full-length 53-kDa GSDMD is cleaved into a 31-kDa N-terminal fragment, a pro-pyroptotic fragment, and a 22-kDa C-terminal repressor fragment. When released, the 31-kDa fragment integrates into the inner leaflet of the cell membrane, oligomerizes, and assembles into pores ranging in diameter from approximately 10 to 33 nm, initiating cell swelling, membrane rupture, and enabling the release of mature cytokines such as IL-1 β and IL-18 [146, 152-154]. The N-terminal fragment also contributes to potassium efflux, further activating NLRP3-dependent caspase-1 inflammasome (NLR family pyrin domain containing 3). Besides acting as the central effector downstream of inflammasome activation, GSDMD's activity is not limited to host cell death, as it plays direct role bactericidal

activity. PFD binds to cardiolipin (a phospholipid abundant in bacterial membranes), enabling pore formation and killing of pathogens such as *Escherichia coli*, *Staphylococcus aureus* and *Bacillus megaterium* [133, 146, 152, 154, 155]. While GSDMD is primarily recognized for mediating pyroptosis, it also participates in autophagic and other non-pyroptotic pathways. Its functional activity- including proteolytic activation, cleavage specificity, and pore formation is finely regulated by disease and microenvironmental factors, ultimately influencing its broader biological and immunological roles [156, 157]. Although several gasdermins have been linked to several human diseases, many aspects of their biological function and regulation remain poorly understood, highlighting the need for continued investigation.

Recognition of GSDMD as being the executor of pyroptosis intensified investigative efforts exploring GSDMD's- most extensively studied member of gasdermin family, structure, expression regulations, molecular activation pathways, involvement in pathology, disease associations and potential therapeutic applications [156]. GSDMD is notably overexpressed in a range of non-cancerous pathological conditions, affecting diverse tissues such as heart, liver, brain, spleen, small intestine and kidneys. It is expressed in both parenchymal (hepatocytes and cardiomyocytes) and immune cells (macrophages, neutrophils and microglia) [156, 158, 159]. In tumors GSDMD expression is shaped by the tumor microenvironment and regulated by both neoplastic and immune cells. Acute inflammatory settings are associated with low GSDMD levels, supporting tumor progression. However, under chronic inflammatory conditions, GSDMD-dependent pyroptosis in immune cells enhances inflammation and promote tumor progression providing insights into dual role of pyroptosis in cancer and inflammatory diseases. Pyroptosis can act both as a tumor suppressor and a facilitator of its progression [160].

Pyroptosis can be initiated through both caspase-1-dependent and caspase-1-independent pathways, referred to as the canonic and non-canonical pathways, respectively. Despite this difference, both routes rely on inflammasome assembly, further triggering caspase activation, GSDMD cleavage and subsequent release of IL-1 β and other modulators (**Figure 6**). Structurally, inflammasomes are cytosolic multiprotein complexes typically composed of a sensor protein, inflammatory caspases and in many cases, an adaptor protein. These platforms are activated in response to a broad array of exogenous and endogenous stimuli, including microbial signals (e.g., bacteria, fungi, parasites, and viruses) and host-derived stress signals (e.g., ion flux, mitochondrial dysfunction, ROS, and metabolic disturbances). The formation of the inflammasome is a tightly regulated process that relies on the coordinated interaction of its key components [126, 154, 161]. In the canonical pyroptosis pathway inflammasome formation is initiated by activation of cytoplasmic pattern recognition receptors (PRRs), which act as inflammasome sensors. These sensors recognize both PAMPs and DAMPs [162, 163] what leads to the induction of type I interferons and pro-inflammatory cytokines release [127]. The activated PRRs oligomerize with adaptor protein ASC and pro-caspase-1 to form a functional inflammasome complex. Canonical inflammasomes are most commonly assembled by sensor such as NLRP1, NLRP3, NLRC4, AIM2, and pyrin. These platforms are composed of three core elements: leucine-rich repeat containing proteins (nucleotide-binding oligomerization domain-like receptors, NLRs), the adapter apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD) (ASC), and pro-caspase-1. NLRs are modular proteins characterized by a leucine-rich repeat (LRR) domain- keeping the protein auto-inhibited until

activation, a central nucleotide-binding oligomerization domain (NACHT), and variable N-terminal domain that mediates protein-protein interactions [164, 165]. Sensors bearing a PYD (pyrin domain) are classified as NLRPs (e.g., NLRP1, NLRP3), whereas those with an N-terminal CARD belong to the NLRC subfamily (e.g., NLRP4) [126, 163, 166]. Owing to its involvement in both innate and adaptive immunity, the NLRP3 inflammasome has emerged as the most thoroughly studied inflammasome complex [167]. Upon danger-signal recognition, NACHT-mediated self-associations brings multiple NLRP3 molecules together. Oligomerized NLRP3 then recruits ASC forming PYD-PYD binding, since ASC contains both PYD and CARD protein interaction domains. Those homotypic PYD-PYD interactions allows helical ASC filament formation, where further number of ASC filaments form a macromolecular ASC speck [168-170]. Once ASC is assembled, it binds caspase-1 via CARD-CARD interactions, facilitating its proximity-driven autocatalytic activation. Caspase-1 in its full-length form contains CARD domain at the N-terminus, followed by a large catalytic domain (p20) in the center and a smaller catalytic domain (p10) at the C-terminus. ASC-bound caspase-1 undergoes autocatalytic cleavage at the linker between its p20 and p10 subunits, producing a complex composed of p33 (containing the CARD and p20) and p10, which remains associated with ASC and retains catalytic activity [171]. Additional cleavage between the CARD and p20 domains releases the p20-p10 heterotetramer from ASC. However, this released complex is unstable within cells, leading to a loss of protease activity [169, 171]. The serine/threonine kinase: NIMA-related kinase 7 (NEK7), is critical for NLRP3 inflammasome function. It selectively binds NLRP3, not interacting with other inflammasome sensors. Upon activation, NEK7 binds and oligomerizes with NLRP3, enabling ASC speck formation and caspase-1 activation, highlighting its sensor-specific regulatory role [172-175].



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Figure 6. Mechanism of pyroptotic cell death. Activation of NLRP3 occurs in two phases. During **priming** (signal 1), detection of cytokines or microbial PAMPs stimulates NF- κ B-dependent transcription, elevating NLRP3 and related inflammasome components together with pro-IL-1 β . The **activation step** (signal 2) can be initiated by many unrelated stimuli, particles and crystals, extracellular ATP, and other PAMPs/DAMPs, that elicit common cellular changes: potassium efflux, calcium fluxes, lysosomal rupture, mitochondrial stress with mtROS, relocalization of cardiolipin to the outer mitochondrial membrane, release of oxidized mitochondrial DNA, and later chloride efflux. RNA viruses may engage NLRP3 via MAVS on mitochondria. Once assembled, the inflammasome activates caspase-1 to mature IL-1 β and IL-18 and to cleave gasdermin D, whose N-terminal fragment forms membrane pores and drives pyroptosis.

As mentioned before, due to its potent pro-inflammatory potential, inflammasome activation requires strict control. This process typically involves two steps. Priming, the first step, increases the expression of key components like NLRP3, caspase-1, and pro-IL-1 β through NF- κ B activation, triggered by recognition of PAMPs and DAMPs through PRRs (e.g. Toll-like receptors, TLRs or nucleotide-binding oligomerization domain-containing protein 2, NOD2) or cytokines (e.g. tumour necrosis factor, TNF and IL-1 β) [176-178]. LPS-driven priming shifts macrophages toward glycolysis, indirectly enhancing IL-1 β production by HIF1 α stabilization [179]. Priming also induces post-translational modifications (PTMs) of NLRP3, keeping NLRP3 inactive but in activation-ready state. These PTMs- such as phosphorylation and ubiquitination, are critical for maintaining inflammasome control [169]. Second step of NLRP3 activation is recognition of activation signal and here NLRP3 inflammasome is unique because it may be activated by a variety of unrelated stimuli, whereas most PRRs have limited specificity for one or few related PAMPs and DAMPs (**Table 2**). After this recognition inflammasome formation is initiated. Collectively, these stimuli are known to induce cellular stress, which is recognized by the NLRP3 inflammasome; however, the exact molecular pathways leading to this recognition and subsequent activation remain incompletely understood. Over the years, multiple mechanisms have been proposed, including mitochondrial perturbations and ROS production, leakage of oxidized mitochondrial DNA, lysosomal damage, cytosolic calcium flux, membrane pore formation and potassium efflux [174, 180]. Among these, potassium depletion has emerged as the most consistent feature across different stimuli. Experimental evidence supports the notion, that reduced intracellular potassium alone is sufficient to initiate NLRP3 activation, suggesting it may serve as a downstream converge point. Still, whether NLRP3 directly senses this ionic change or whether it serves as a proxy for another upstream event remains an open question [169, 174, 181].

Table 2. NLRP3 inflammasome inducers

Adapted from Swanson K. V. et al., (2019), [169].

Activator	Origin	Example
DAMP	Intrinsically generated	cholesterol crystals
		MSU (Monosodium urate crystals)
		calcium oxalate crystals
		calcium pyrophosphate dihydrate crystals
		soluble uric acid
		cathelicidin
		α -synuclein
		amyloid- β
		serum amyloid A
		prion protein,
		biglycan
		hyaluronan
		islet amyloid polypeptide
		hydroxyapatite
		haeme
		oxidized mitochondrial DNA

		membrane attack complex	
		cyclic GMP–AMP	
		lysophosphatidylcholine	
		ceramides	
		oxidized phospholipid 1-palmitoyl-2-arachidonoyl- sn-glycero-3-phosphorylcholine	
		sphingosine	
		ATP	
		Externally sourced	alum
	silica		
	aluminum hydroxide		
	nanoparticles		
	carbon nanotubes		
	chitosan		
	palmitate (also intrinsically generated)		
	UVB		
	resiquimod (R848)		
	imiquimod (R837)/CL097		
	PAMP		Bacterial
		peptidoglycan	
muramyl dipeptide			
trehalose-6,6'-dibehenate			
c- di-GMP–c- di-AMP			
c- di-GMP–c- di-AMP			
RNA–DNA hybrid			
nigericin (<i>Streptomyces hygroscopicus</i>)			
gramicidin (<i>Brevibacillus brevis</i>)			
valinomycin (<i>Streptomyces fulvissimus</i> and <i>Streptomyces tsusimaensis</i>)			
β-haemolysin (<i>Streptococcus sp.</i> ‘group B’)			
α- haemolysin (<i>Staphylococcus aureus</i>)			
M protein (<i>Streptococcus sp.</i> ‘group A’)			
leucocidin (<i>Staphylococcus aureus</i>)			
tetanolysin O (<i>Clostridium tetani</i>)			
pneumolysin (<i>Streptococcus pneumoniae</i>)			
listeriolysin O (<i>Listeria monocytogenes</i>)			
aerolysin (<i>Aeromonas hydrophila</i>)			
streptolysin O (<i>Streptococcus pyogenes</i>)			
enterohaemolysin (<i>Escherichia coli</i> O157:H7)			
haemolysin BL (<i>Bacillus cereus</i>)			
adenylate cyclase toxin (<i>Bordetella pertussis</i>)			
M protein (<i>Streptococcus sp.</i> ‘group A’)			
maitotoxin (<i>Marina spp.</i> dinoflagellates)			
Viral		Double- stranded RNA	
		single- stranded RNA	
Fungal		β- Glucans	
		hyphae	
		mannan	
		zymosan	

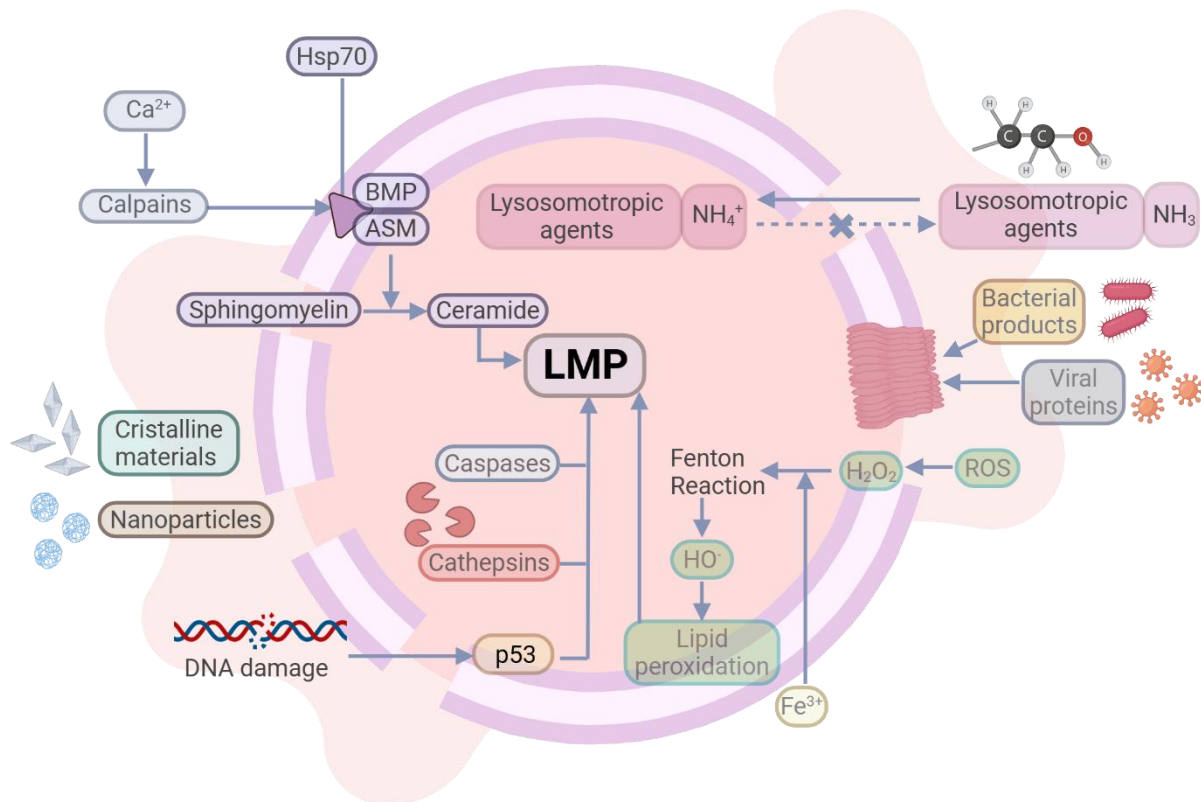
In the non-canonical pyroptosis pathway, activation does not involve classical upstream inflammasome sensors. Instead, human caspase-4 and caspase-5 (and murine caspase-11) function as both sensors and effectors, capable of directly binding cytosolic LPS via their N-terminal CARDs (caspase activation and recruitment domain) [182-184]. In the non-canonical inflammasome pathway, extracellular LPS activates Toll-like receptor 4 (TLR4), initiating a type I interferon response that, together with the complement C3–C3aR axis, upregulates caspase-11 expression [185]. Type I interferons also stimulate the expression of guanylate-binding proteins (GBPs) and the closely related immunity-related GTPase IRGB10. These GTPases are recruited to intracellular bacteria, where they mediate bacterial lysis and facilitate the release of LPS into the cytosol [186, 187]. Upon direct LPS recognition, these caspases undergo oligomerization and autoproteolytic activation [188]. Activated caspase 4/5/11 cleave GSDMD, releasing its N-terminal domain, which oligomerizes and inserts into the plasma membrane forming lytic pores, driving pyroptotic cell death [146]. Unlike caspase-1, caspase 4/5/11 do not process pro-IL-1 β or pro-IL-18 directly. However, GSDMD pore formation induces potassium efflux, which serves as a secondary signal trigger for non-canonical NLRP3 inflammasome activation, leading to caspase-1 activation and the subsequent maturation and release of IL-1 β and IL-18, independent of additional priming signals. Therefore, non-canonical pyroptosis activation mirrors many downstream features of canonical inflammasome responses. Although caspase-4/5/11 were traditionally considered LPS sensors, recent evidence implicated guanylate-binding protein 1 (GBP1) in this process. GBP1 facilitates the detection of intracellular LPS by promoting GBP oligomerization and caspase-4 recruitment, suggesting that LPS sensing in non-canonical inflammasome activation may involve cooperative mechanism upstream of caspase activation [126, 167, 169, 189, 190].

1.1.3. Lysosome-dependent cell death

Eucaryotic cells contain two primary degradative organelles: late endosomes and lysosomes. Both compartments maintain an acidic internal pH and are enriched with hydrolytic enzymes and accessory proteins whose optimal activity depends on this low pH environment. Acidic digestive organelles are highly dynamic, undergoing constant membrane trafficking that results in considerable heterogeneity. A useful distinction defines late endosomes as compartments still receiving enzymes from the trans-Golgi network, while lysosomes represent their mature counterparts. Yet, this classification is imperfect, as most lysosomal digestion occurs in late endosomes, and their properties often overlap due to continuous content exchange [191]. The late endocytic compartments, including lysosomes, play a central role in the degradation of both intracellular and extracellular material internalized via endocytosis, phagocytosis, or autophagy [192]. Through fusion with vesicles such as endosomes, phagosomes and autophagosomes, lysosomes mediate the enzymatic breakdown of a wide array of substrates. Lysosomes also play a broader role in regulating cellular homeostasis, including the processing of pro-proteins, presentation of antigens, breakdown of extracellular matrix components, promotion of angiogenesis, and induction of cell death. To fulfill these diverse roles, lysosomes maintain an acidic lumen and contain a complement of over 60 acid

hydrolases, including proteases, lipases, glycosidases, nucleases, and sulfatases, all enclosed within a single phospholipid bilayer, making them inherently hazardous if membrane integrity is compromised. As first recognized by Christan de Duve, the pioneer of lysosome research, the release of those enzymes into the cytosol poses a considerable risk to cellular integrity [191, 193-195].

Although the lysosomal membrane is generally stable, it can undergo lysosomal membrane permeabilization (LMP) in response to direct damage or cellular stress. LMP includes any damage to the lysosomal membrane that results in leakage of lysosomal contents into the cytoplasm [196] (**Figure 7**). The extent of LMP determines the cellular outcome: while complete rupture can lead to rapid cytosolic acidification, uncontrolled degradation of cellular components, and necrotic cell death, partial and selective release of cathepsins, particularly cathepsin B and D, can activate regulated cell death pathways [197-201]. In such scenarios, cathepsin activity initiates intracellular signaling cascades that culminate in apoptosis-like or lysosome-dependent cell death [196, 202, 203]. In certain contexts, cathepsins released during LMP can activate caspases either directly or through caspase-independent pathways. Moreover, LMP has been implicated in inflammasome activation, leading to pro-inflammatory cytokine release and pyroptotic cell death in macrophages. Notably, pharmacological inhibition of cathepsins has been shown to attenuate LMP-induced cytotoxicity underscoring their pivotal role in this process [197]. Lysosome-mediated cell death is frequently preventable through pharmacological inhibition of cathepsins, with CA-074Me and E64d commonly used to inhibit cathepsin B, and pepstatin A employed to block cathepsin D-dependent pathways [191, 196, 200, 203, 204].



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Figure 7. Converging pathways to lysosomal membrane permeabilization. The diagram centers on a lysosome whose limiting membrane is challenged by multiple, intersecting stresses. Calcium influx activates calpains, diminishing membrane resilience (including via LAMP cleavage) and lowering the threshold for rupture. In parallel, loss of Hsp70-mediated support of the BMP/acid sphingomyelinase (ASM) axis shifts sphingomyelin toward ceramide, a lipid conversion that perturbs bilayer organization. Weakly basic lysosomotropic agents accumulate in the lumen and, together with $\text{NH}_4^+/\text{NH}_3$ -driven neutralization of acidity, impair lysosomal function and repair. Exogenous particulates, including crystalline materials (e.g., silica, MSU, cholesterol crystals) and diverse nanoparticles, are delivered to phagolysosomes, where their physical and chemical interactions further destabilize the membrane. Oxidative routes compound this damage: ROS/ H_2O_2 and Fe^{3+} fuel Fenton chemistry, initiating lipid peroxidation that weakens the bilayer. Once permeabilization begins, protective scaffolds such as LAMPs are compromised, and protein effectors can accelerate leakage: certain viral/bacterial proteins and proapoptotic BCL-2 family members form pore-like lesions, while caspases and escaped cathepsins amplify injury in self-reinforcing loops. Upstream genotoxic stress (via p53) further biases the organelle toward failure. Together, these biochemical, biophysical, and lipid-remodeling inputs converge to breach the lysosomal boundary, culminating in LMP and the cytosolic release of luminal contents that drive downstream cell-death and inflammatory programs.

Lysosomal membrane permeabilization and the resulting release of the lysosomal content into the cytosol initiate a form of regulated cell death termed lysosome dependent cell death (LDCD). This pathway is primarily executed by the lysosomal cathepsin proteases and may exhibit necrotic, apoptotic or intermediate features depending on the degree of the membrane damage and cellular context [205-207]. Lysosome-dependent cell death (LDCD) is marked by the degradation of key lysosomal membrane-associated proteins such as Hsp70, LAMP1, LAMP2, and CD63, leading to compromised membrane integrity. Pharmacological

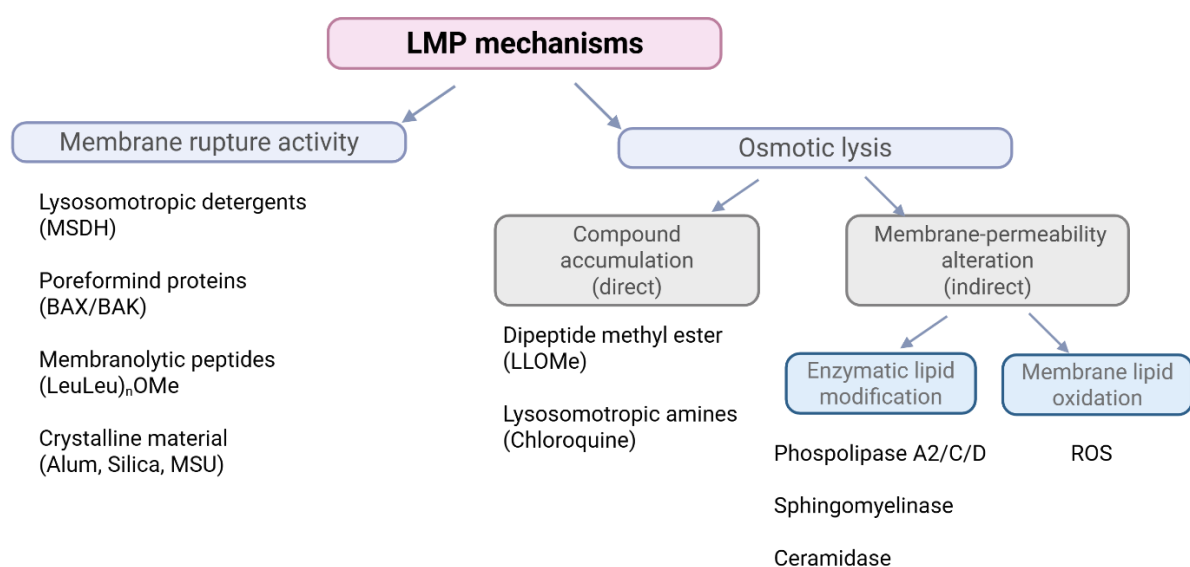
inhibition of cathepsin activity or enhancement of endogenous cathepsin inhibitors has been shown to effectively suppress LDCD. This form of cell death can be initiated by multiple factors, including cysteine proteases, calpains, V-ATPase dysfunction, and reactive oxygen species (ROS) [203]. Among these, ROS-mediated LMP is considered a classical trigger, as lysosomes lack key antioxidant enzymes, making them vulnerable to ROS diffusion and the generation of hydroxyl radicals that promote membrane destabilization. Although initially understudied, LCD has gained increasing attention due to recent advancements in LMP detection methodologies, improved experimental tools for monitoring LMP and the compelling genetic data implicating cathepsins as central effectors of cell death across species [202, 208]. A key mechanism implicated in LDCD involves the formation of pores in the lysosomal membrane, enabling the release of cathepsins into the cytosol. Various venoms and bacterial toxins exploit trigger this process, often resulting in apoptotic cell death [209, 210]. Interestingly, recent findings reveal that not only exogenous agents, but also endogenous proteins with structural similarity to bacterial toxins- such as aerolysin-like proteins, can induce LMP [211]. These proteins are capable of translocating to the lysosomal membrane, where they oligomerize into detergent-stable, high molecular-weight complexes that destabilize lysosome and promote the release of cathepsin B [204].

Beyond direct pore formation, LMP can also be triggered by the accumulation of lysosomotropic agents, small amphiphilic amines capable of diffusing across membranes and accumulating within lysosomes upon protonation inducing membrane destabilization [212] (**Figure 8**). Well-characterized examples include L-leucyl-L-leucyl methyl ester (LeuLeu-OMe, LLOMe), the cationic drug siramesine, known to displace acid sphingomyelinase (ASM) from the membrane, and O-methyl-serine dodecylamide hydrochloride (MSDH) [204, 213-216]. This accumulation is a result of protonation in the acidic lumen and leads to entrapment of lysosomotropic agents within the organelle, where they can disrupt the lipid bilayer, compromise membrane integrity and initiate cell death pathways. Beyond chemical disruption, osmotic imbalance caused by crystalline materials including silica, cholesterol crystals and monosodium urate provoke mechanical stress on the lysosome membrane leading to LMP [217-220].

LMP may also arise under oxidative stress, particularly due to lysosome's high redox-active iron content, which is derived from the degradation of iron-rich macromolecules [221]. Hydrogen peroxide (H_2O_2) can diffuse into lysosomes and engage in iron-catalyzed Fenton reactions, producing highly reactive hydroxyl radicals that damage the membrane and promote leakage of lysosomal content. This damage pathway can be mitigated by iron chelators such as deferoxamine, which has been shown to preserve lysosomal integrity and reduce the susceptibility to oxidative stress causing cell death [204, 222-224].

A key factor in lysosomal resilience is the structural and biochemical composition of the limiting membrane. The inner leaflet of lysosome membrane is shielded by a protective glycocalyx, composed primarily of carbohydrate-rich domains of proteins such as glycosylated lysosomal-associated membrane proteins (LAMPs) and lysosomal integral membrane proteins (LIMPs) [225]. Although the lysosomal membrane has lower content of cholesterol and sphingolipids compared to the plasma membrane, its cholesterol level remains higher than most intracellular organelles [226]. Conversely, intraluminal membranes found within intraluminal

vesicles (ILVs) are enriched with negatively charged lipids such as bis(monoacylglycero)phosphate (BMP), along with phosphatidylinositol and dolichol phosphate [227]. These anionic lipids favor the binding of positively charged hydrolases and activator proteins [228]. Unlike the limiting membrane, ILVs are nearly devoid of cholesterol and sphingomyelin is progressively degraded to ceramide by the acid sphingomyelinase (SMase) [227]. Those alterations also disrupt the stability of lysosomal membrane, maintained by “membrane rafts” formed by cholesterol and sphingomyelin, reducing membrane stability and making the lysosomes more susceptible to oxidative stress damage and membrane permeabilization [226, 229]. Accumulation of ceramide not only perturbs the mechanical integrity of membranes, but can be further metabolized to sphingosine by ceramidase, what promotes membrane rigidity through interactions with lipid acyl chains and has been implicated with promoting LPM [230, 231]. Additionally, sphingosine’s positive charges can neutralize BMP layers, displacing essential lysosomal proteins and impairing membrane function [227]. Notably, the ceramide-sphingosine equilibrium is pH-dependent: at pH 4.5, ceramide predominates, whereas at a more neutral pH (5.5) found in early endosomes, sphingosine levels rise, further influencing membrane properties [191, 232, 233].



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Figure 8. Lysosome membrane permeabilization mechanisms. Adapted from Frederick R. M. et al., (2016).

Molecular chaperones of the heat shock protein (Hsp) family are highly conserved mediators of the cellular stress response. They are rapidly synthesized following exposure to harmful stimuli such as elevated temperature, oxidative stress, hypoxia, inflammatory mediators, or toxic compounds, while the production of most other proteins is simultaneously inhibited [234, 235]. This selective response allows Hsps to preserve protein quality control, sustain homeostasis, and promote cell survival. The Hsp family is divided into subclasses according to molecular size, encompassing small Hsps (Hsp10, Hsp25/27, and Hsp40) as well as Hsp60, Hsp70, Hsp90, and Hsp100 [236]. Notably, Hsp70 represents one of the most highly

inducible members and plays a pivotal role in safeguarding cells from a wide spectrum of environmental and physiological stressors and being an additional regulator of LMP [234-238]. Hsp70 is a multifunctional molecular chaperone that, beyond its canonical roles in protein folding and stress response, exerts a potent stabilizing effect on lysosomal membranes [234, 239, 240]. Localized to the endosomal-lysosomal compartment, Hsp70 interacts directly with the BMP, enhancing the activity of ASM. This activity facilitates sphingomyelin turnover, generating membrane lipid configurations that preserve mechanical integrity and resist destabilization [204, 241]. The protective capacity of Hsp70 is evident in tumor cells, where it effectively blocks lysosomal membrane permeabilization induced by a wide array of stimuli, including cytokines, oxidative stress, γ -irradiation, chemotherapeutic agents, and photodamage. Depletion of Hsp70, in contrast, sensitizes cells to LMP and triggers cathepsin-mediated programmed cell death, which may occur through caspase-dependent or -independent pathways. This anti-LMP mechanism is thought to be a major contributor to the survival advantage conferred by Hsp70 in malignancies [196, 203, 204, 242].

Calpains, a family of calcium-dependent cysteine proteases, can also contribute to LMP by initiating a proteolytic cascade that compromises lysosomal stability [243]. Once activated by elevated intracellular calcium, calpains cleave protective lysosomal proteins such as Hsp70.1 and LAMP2, resulting in membrane destabilization [243, 244]. This process initiates the release of cathepsins into the cytosol, forming a “calpain-cathepsin cascade” that amplifies proteolytic activity and drives cell death [245]. In neurons, this pathway is particularly relevant: calpain activation promotes lysosomal rupture during ischemia, trauma, and neurodegeneration, where necrotic cell death predominates under conditions of ATP depletion. Beyond the nervous system, calpain-dependent LMP also contributes to physiological tissue remodeling, highlighting its role as a versatile regulator of lysosomal stability and cell fate [204, 243, 244, 246].

Lysosome-dependent cell death (LDCD) emerges as a multifaceted process governed by diverse stimuli, including oxidative stress, protease activation, lysosomotropic compounds, and dysregulated calcium signaling. The diversity of its triggers is mirrored by the range of possible outcomes: lysosomal membrane permeabilization may drive caspase-mediated apoptosis, caspase-independent necrosis, or inflammasome-induced pyroptosis, depending on cellular context [195]. This adaptability underscores the role of lysosomes as pivotal decision-makers in determining cell fate. Beyond experimental conditions, LDCD is engaged in physiological processes including developmental tissue regression and immune regulation, while also contributing to pathological settings such as Parkinson’s disease, retinal degeneration, and lysosomal storage disorders. The possibility of modulating LDCD through cathepsin inhibition or lysosomal stabilization highlights its therapeutic relevance, with potential applications both in preventing tissue injury and in promoting the selective elimination of cancer cells [204, 247].

1.1.4. Cell death inducers: NLRP3 activators

The principal classes of NLRP3 inflammasome activators can be grouped into three broad categories: extracellular ATP, K^+ ionophores [248], and crystalline or particulate agents that destabilize lysosomes [249, 250]. Despite their chemical diversity, these stimuli converge on a common downstream event: a reduction in cytosolic K^+ concentration, which is widely recognized as a critical signal for NLRP3 activation [181]. However, the mechanisms by which each class induces K^+ efflux differ markedly. To appreciate these processes, it is useful to first recall the fundamentals of cellular K^+ homeostasis. Under resting conditions, intracellular K^+ levels ($[K^+]_i$, ~140 mM) are much higher than extracellular concentrations ($[K^+]_e$, ~5 mM), a distribution essentially inverted for Na^+ . This gradient is maintained by the electrogenic Na^+/K^+ -ATPase, which pumps three Na^+ ions outward and two K^+ ions inward during each cycle. In mammalian cells, plasma membranes at rest are highly permeable to K^+ relative to Na^+ or Ca^{2+} , thereby sustaining a negative resting membrane potential. Because the plasma membrane functions as an effective electrical insulator, even small ionic shifts can produce significant changes in transmembrane potential [251]. Thus, the sharp drop in $[K^+]_i$ required for NLRP3 activation (estimated at ~20-30%) is expected to be accompanied by compensatory ion movements, either through cation counter-flux or anion co-flux. The diverse activators of NLRP3 exploit distinct routes to achieve this imbalance [252].

Ionophores, literally “ion carriers”, are small, typically lipid-soluble molecules (often of microbial origin) that ferry ions across biological membranes [253]. Among K^+ -targeting ionophores, valinomycin acts as a highly selective K^+ uniporter, whereas nigericin functions as an electroneutral K^+/H^+ exchanger, collapsing the intracellular K^+ pool while driving proton influx [254]. In practice, nigericin has become the workhorse agonist for NLRP3 studies because it produces a rapid, robust fall in cytosolic K^+ and reliably triggers inflammasome activation [255].

Alongside ionophores, a widely used experimental route to lower cytosolic K^+ involves lysosomotropic detergents, small amphiphilic bases that accumulate in acidic compartments and destabilize the lysosomal limiting membrane. Representative agents include imidazole and morpholine, N-dodecylimidazole, O-methyl-serine-dodecyl amide hydrochloride (MSDH), and L-leucyl-leucine methyl ester (LLOMe). By concentrating within lysosomes, these compounds act in a detergent-like manner (and, in the case of LLOMe, via intralysosomal conversion to membranolytic species), leading to lysosomal membrane permeabilization, cathepsin release, and secondary ionic dysregulation that supports the drop in $[K^+]_i$ required for NLRP3 activation. Among this group, LLOMe is the most comprehensively characterized tool compound, and is frequently employed to probe lysosome-dependent pathways upstream of the inflammasome [256].

Another common strategy involves ingested crystals and particulates such as silica, MSU, and alum [250, 257]. Once internalized, these rigid materials accumulate in phagolysosomes, where their physical properties and surface reactivity promote LMP [202]. Subsequent cathepsin escape and oxidative injury at the compromised membrane set off signaling that ultimately reduces cytosolic K^+ and through secondary ion movements and, thereby permitting

NLRP3 activation [181, 219]. The extent of membrane damage shapes the response: limited LMP can favor IL-1 β /IL-18 processing, whereas more severe injury tends to push cells toward pyroptosis [258]. Notably, while cathepsins often act upstream in these pathways, their necessity varies with the stimulus and cell type, highlighting that crystal-driven activation is lysosome-centered yet mechanistically heterogeneous [259].

1.1.4.1. Nigericin

Nigericin (C₄₀H₆₇O₁₁) belongs to the group of polyether antibiotics and is naturally derived from *Streptomyces hygroscopicus* [260]. Structurally, it is a linear molecule composed of multiple oxygen-containing heterocyclic rings interspersed with hydroxyl groups, creating a flexible scaffold capable of coordinating cations (**Figure 9**).

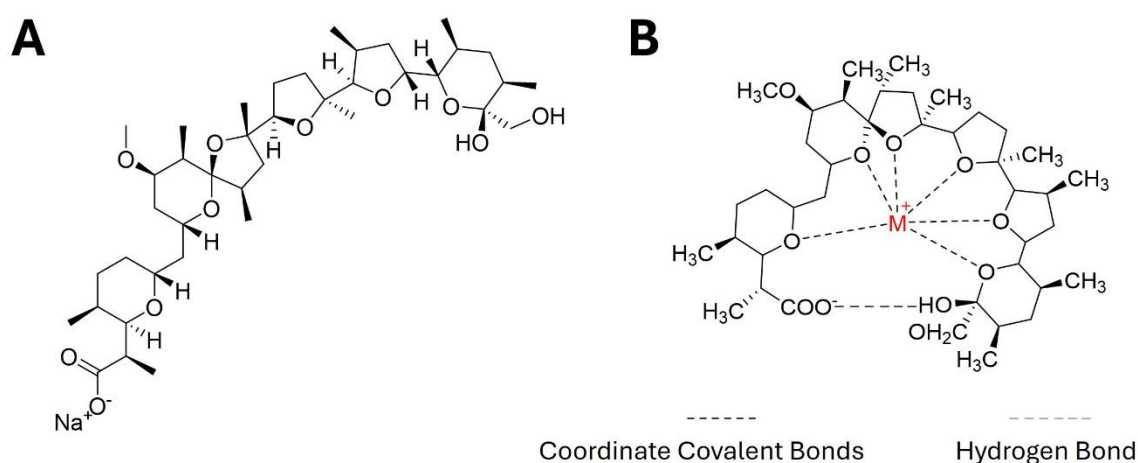
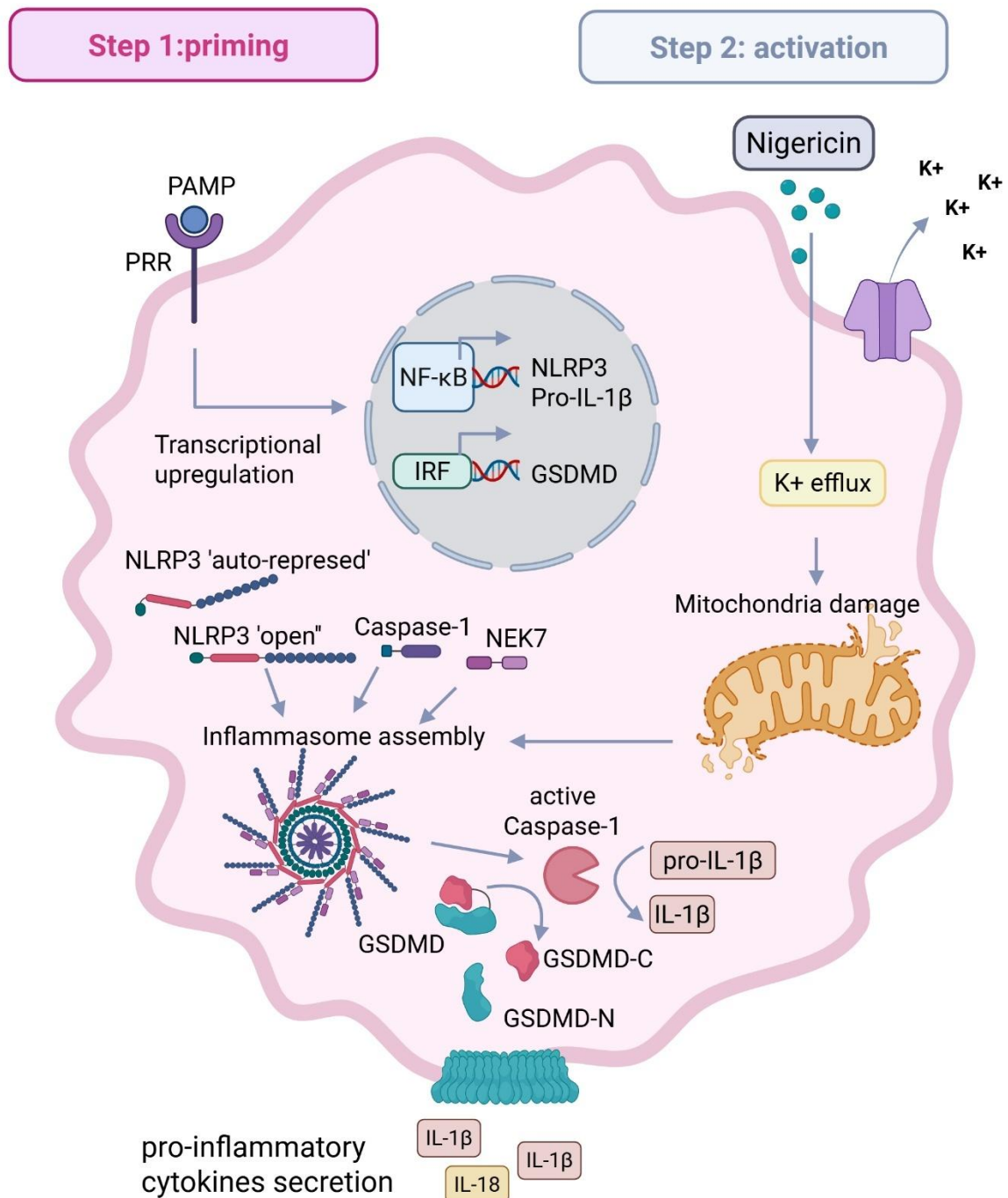


Figure 9. Nigericin chemical structure. **A** Nigericin linear structure **B** Chemical structure of nigericin illustrating intramolecular hydrogen bonds between the carboxylate and hydroxyl moieties, stabilizing a pseudo-cyclic arrangement. M⁺ indicates a coordinated metal ion. The formation of an overall neutral complex enables its passage across lipid membranes.

As an ionophore, nigericin facilitates the translocation of ions across lipid membranes, thereby altering intracellular ionic balance. Such compounds are widely employed as chemical tools to investigate the biological consequences of ion flux and as pharmacological agents for modulating ionic homeostasis *in vivo* [255, 261]. Within lipid bilayers, nigericin undergoes conformational rearrangements that favor interactions between its oxygen-rich interior and metal ions. Through this mechanism, nigericin efficiently catalyzes the electroneutral exchange of potassium (K⁺) for protons (H⁺) across biological membranes [262, 263]. By driving this exchange, nigericin disrupts transmembrane ionic gradients, leading to potassium depletion in the cytosol and an increased influx of protons, which contributes to acidification and loss of pH homeostasis. By disrupting the balance of cytosolic K⁺ and H⁺ concentrations, nigericin profoundly affects cellular physiology. The K⁺ efflux mediated by nigericin is a central event in triggering NLRP3 inflammasome activation (**Figure 10**) [181, 264]. Loss of cytosolic potassium relieves the inhibitory effect on inflammasome assembly, thereby promoting

caspase-1 activation and the subsequent maturation of interleukin-1 β (IL-1 β) and IL-18 [248]. In parallel, the ion imbalance induced by nigericin contributes to mitochondrial dysfunction and ROS generation, further amplifying inflammasome signaling [265]. The culmination of these events is pyroptotic cell death, characterized by gasdermin D-mediated pore formation, plasma membrane rupture, and pro-inflammatory cytokine release. Collectively, these features highlight nigericin not only as a powerful inducer of pyroptosis but also as a valuable model compound for probing the intricate interplay between ion fluxes, inflammasome regulation, and cell fate determination, providing insights into the multifactorial regulation of pyroptosis.



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Figure 10. Two-step mechanism of nigericin-induced NLRP3 activation and pyroptotic cell death in macrophages. Nigericin, a potassium/proton ionophore produced by *Streptomyces hygroscopicus*, activates the NLRP3 inflammasome through a canonical two-step process. During Step 1 (**priming**), engagement of PRRs by PAMPs initiates NF- κ B-mediated transcriptional upregulation of NLRP3, pro-IL-1 β , and pro-IL-18, establishing a responsive intracellular pool of inflammasome components. In Step 2 (**activation**), nigericin promotes a rapid K⁺ efflux, which disturbs ionic equilibrium and induces mitochondrial stress and ROS production, events that facilitate the transition of NLRP3 from its auto-repressed to open conformation. This structural rearrangement enables Nek7 binding and assembly of the active inflammasome complex, culminating in caspase-1 activation, cleavage of pro-IL-1 β , pro-IL-18, and GSDMD, and execution of pyroptotic cell death characterized by membrane pore formation and cytokine release.

1.1.4.2. L-leucyl-L-leucine methyl ester

Leu-Leu-Ome is a lysosomotropic dipeptide ester (**Figure 11**) extensively used as a model compound to study lysosome-dependent cell death. Its cellular uptake occurs mainly via receptor-mediated endocytosis [266, 267], although passive diffusion has also been proposed [268].

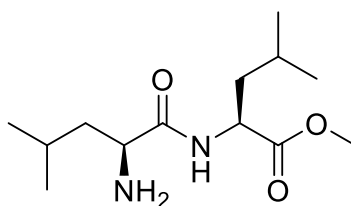
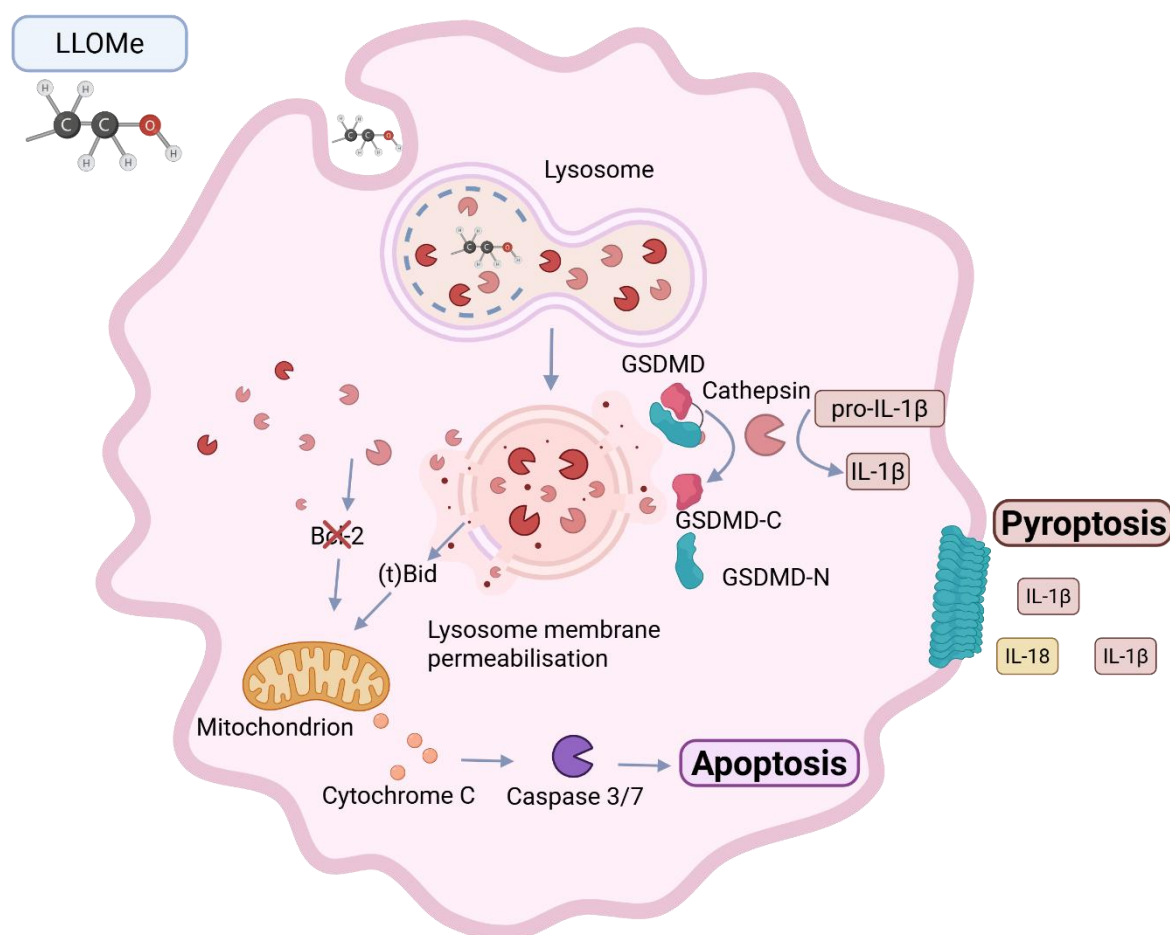


Figure 11. Structure of LLOMe, a lysosomotropic dipeptide methyl ester that causes lysosome membrane permeabilization.

Once internalized, LLOMe selectively accumulates in lysosomes, where the acidic lumen creates a favorable environment for its enzymatic activation. This feature makes LLOMe a reliable experimental tool for probing lysosomal integrity and for dissecting the contribution of lysosomal damage to regulated cell death pathways. Although CatC functions predominately as an exopeptidase in the acidic milieu of intact lysosomes, removing N-terminal dipeptides from peptide and protein substrates. Purified human DPPI efficiently hydrolyzes fluorogenic substrates across an acidic window, with maximal peptidase activity typically observed around pH $\sim$ 4.5-6.5. In contrast, at neutral to mildly alkaline pH the enzyme exhibits transpeptidase activity, enabling polymerization of suitable dipeptide esters or amides rather than simple hydrolysis [266, 269]. In this mode, the enzyme attaches dipeptidyl residues to unprotonated amino groups, which drives the stepwise polymerization of LLOMe into extended oligomers, (Leu-Leu)_n-OMe ($n \geq 3$). These oligomeric products exhibit strong hydrophobic and detergent-like properties, enabling them to integrate into lysosomal membranes and rapidly destabilize them. The acidic environment of the lysosome further enhances this process by both optimizing DPPI activity and stabilizing protonated species with high membranolytic potential [266, 268, 269]. Consequently, lysosomal membrane permeabilization can occur within minutes of exposure [258, 270]. Notably, this mechanism is highly selective: the marked potency of LLOMe reflects cathepsin C-mediated acyl-transfer/polymerization of dipeptide esters within lysosomes, while mono-peptide methyl esters (e.g., Leu-OMe) are far less toxic unless they are

first converted intracellularly to a dipeptide (e.g., Leu-Leu-OMe), which then undergoes DPPI-driven polymerization and promotes LMP [271].

Following LMP, the release of lysosomal proteases into the cytosol initiates multiple cell death pathways (**Figure 12**). On one hand, cathepsins B and L can cleave BID and degrade anti-apoptotic BCL-2 family proteins or XIAP, thereby shifting the intracellular balance toward mitochondrial apoptosis [213, 256, 272, 273]. On the other hand, lysosomal rupture triggered by LLOMe has also been shown to activate the NLRP3 inflammasome, leading to caspase-1 activation, IL-1 β and IL-18 maturation, and pyroptotic cell death [274-277]. Thus, LLOMe functions as a versatile lysosomal destabilizer capable of promoting distinct death modalities depending on cellular context, cathepsin repertoire, and the extent of LMP. Overall, LLOMe serves not only as a mechanistic tool to probe LDCD but also as a paradigm for how enzymatic activation of small molecules within lysosomes can transform them into potent inducers of regulated cell death [258, 266, 268, 270, 273, 278].



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Figure 12. Dual outcomes of L-Leu-L-Leu-OMe after lysosomal damage. LLOMe enters cells predominantly via endocytosis and traffics through the endolysosomal pathway. As a weakly basic lysosomotropic dipeptide methyl ester, it becomes protonated and trapped in acidic lysosomes (ion-trapping), where cathepsin C converts it into membranolytic oligomers. This triggers lysosomal membrane permeabilization and release of cathepsins into the cytosol, from which two outcomes can follow. Apoptotic route (mitochondrial/intrinsic): Cytosolic cathepsins

cleave Bid → tBid and can degrade anti-apoptotic BCL-2 family proteins, promoting mitochondrial outer-membrane permeabilization, cytochrome-c release, and activation of the caspase-9 → caspase-3/-7 cascade. Pyroptotic route (inflammasome-driven in primed myeloid cells): LMP provides signal-2 cues (e.g., cathepsins in cytosol, K⁺ efflux, mtROS-not shown) that activate NLRP3 following prior transcriptional priming. Activated caspase-1 processes pro-IL-1 β /IL-18 and cleaves GSDMD; GSDMD-N forms plasma-membrane pores, leading to pyroptosis and cytokine release, or cathepsins released from lysosomes due to LMP directly cleave GSDMD and/or interleukins leading to pyroptosis as postulated in the study aims and research goals of this dissertation.

1.2. PROTEASES IN CELL DEATH

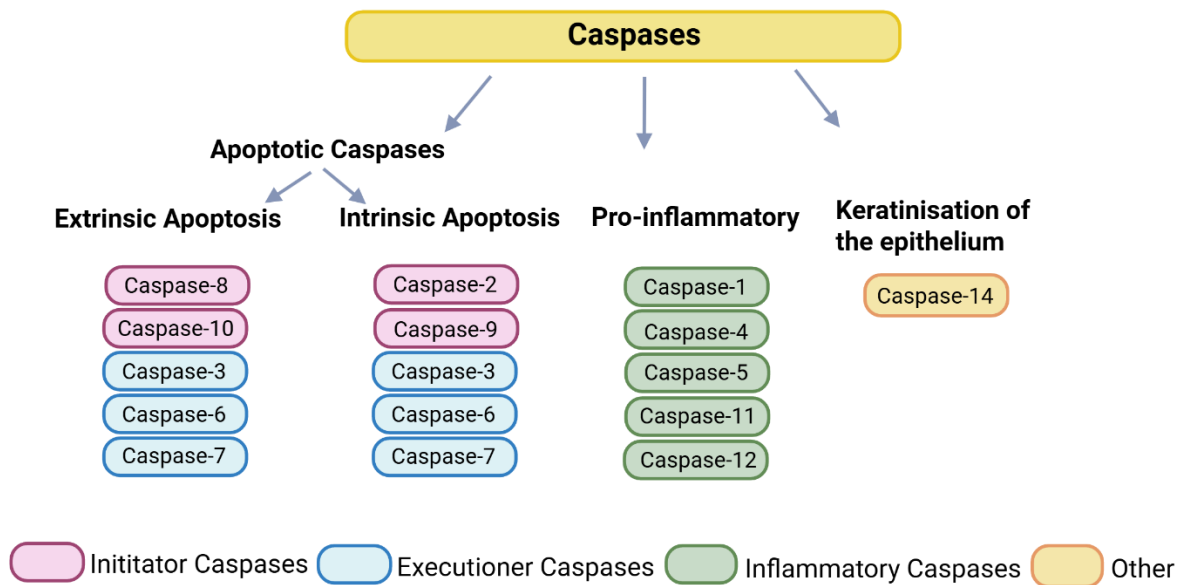
Proteases are central architects of regulated cell death, operating in distinct families and cellular niches. Caspases, cysteine proteases that cleave after aspartate, coordinate apoptosis through initiator modules (caspase-8/10 at death receptors; caspase-9 at the apoptosome) that activate executioners (caspase-3/7) to dismantle key substrates and generate the characteristic apoptotic morphology [279]. Their inflammatory counterparts (caspase-1 and caspase-4/5 in human; -11 in mouse) drive pyroptosis by processing IL-1 β /IL-18 and cleaving gasdermin D to release a pore-forming N-terminus; crosstalk at the gasdermin node allows apoptotic caspases (e.g., caspase-3, caspase-8) to reroute signaling toward lytic death in a context-dependent manner [128, 146]. Within the endo-lysosomal system, cathepsins, primarily cysteine cathepsins (CatB, CatL, CatS) and the aspartyl protease CatD, execute lysosome-dependent cell death once lysosomal membrane permeabilization releases them into the cytosol, where they damage organelles, process pro-death factors, and amplify apoptotic or necrotic outcomes [280]. Building on this protease-centric view, calpains, Ca²⁺-activated cysteine proteases exemplified by calpain-1, remodel cytoskeleton and signaling complexes and can tip fate decisions by cleaving substrates such as BID/BAX [281, 282]. Calpain-driven proteolysis of intermediate filaments (e.g., vimentin) compromises mechanical resilience [283, 284] and, under elevated intracellular Ca²⁺ or limited caspase-1 activity, can support pyroptosis-like lytic outcomes; reports that calpain-1 directly processes GSDMD further couple calcium stress to pore formation [285]. Cytotoxic lymphocytes and natural killer cells deploy granzymes, serine proteases (granzyme A, B, H, K, M), to eliminate infected or malignant cells: granzyme B activates caspase-3 and also cleaves apoptotic substrates directly, whereas granzyme A triggers a morphologically apoptotic yet caspase-independent program involving mitochondrial and nuclear damage; beyond killing, granzymes can modulate immunity by acting on extracellular targets [286, 287]. Regulatory proteolysis further shapes the decision to live or die. The ubiquitin–proteasome system (UPS) marks proteins via the E1/E2/E3 ubiquitin-conjugation cascade for degradation by the 26S proteasome, thereby controlling the abundance of key death regulators. In particular, IAP E3 ligases (e.g., XIAP, c-IAP1/2) harbor BIR domains that bind and inhibit caspases-3/7/9, while their ligase activity promotes ubiquitin-dependent turnover of pro-apoptotic components, restraining executioner protease activity and shaping pathway choice. Besides apoptosis, ubiquitylation modulates many cellular processes, including DNA replication, DNA repair, cell viability, transcription [288, 289]. Another representative of lysosomal cysteine proteases is legumain (asparaginyl endopeptidase, AEP). It is implicated in various pathological processes, including cancer, neurodegeneration, and kidney injury. Recent

studies have highlighted its dual role in regulating cell death. In glioblastoma, AEP overexpression suppresses apoptosis, whereas its silencing increases the number of apoptotic cells. Beyond apoptosis, cytosolic legumain can also promote ferroptosis, a regulated form of cell death driven by iron-dependent lipid peroxidation. Collectively, these findings identify legumain as a multifaceted modulator of cell fate [290-292].

Although multiple protease families contribute to cell-death decisions, this dissertation mainly focuses on caspases and cysteine cathepsins as the principal executioner and lysosomal effector axes, respectively, examining their activation mechanisms, crosstalk, and experimental readouts across apoptosis, pyroptosis and lysosome-dependent cell death.

1.2.1. Caspases

In mammals, proteases, based on the mechanism of catalysis, are divided into five main families: metallo-, serine-, threonine-, aspartic-, and cysteine proteases, depending on the amino acid that defines their catalytic site [293, 294]. Caspases represent a highly conserved family of cysteine proteases that play central roles in apoptosis, inflammation, and other signaling pathways in multicellular organisms. The term caspase, is derived from cysteine-dependent aspartate-specific protease, reflecting two defining biochemical features: they require an aspartate (Asp) residue at the cleavage site for substrate recognition, and they rely on the nucleophilic action of a cysteine (Cys) residue within the active site for catalysis [295, 296]. While many protease families also employ cysteine as a catalytic nucleophile, the strict and exclusive requirement for Asp residues is remarkably uncommon [296-298]. Unlike the proteasome, which promotes wholesale degradation of proteins [299], or calpains, which display broad substrate tolerance [300], caspases engage in highly restricted proteolysis. Typically, cleavage involves only one or a few peptide bonds in a given substrate. This limited proteolysis allows caspases to act as molecular switches, either activating or inactivating key targets, rather than driving nonspecific degradation [301]. Consequently, caspases function less as degradative enzymes and more as precise signaling mediators that reshape cellular physiology [302-306]. Caspases can be broadly grouped according to function, prodomain architecture, and substrate preference [304]. The classical division separates “apoptotic” caspases (caspase-2, -3, -6, -7, -8, -9, and -10) [301, 307] from “pro-inflammatory” caspases (caspase-1, -4, -5, -11, and -12) [308, 309], with caspase-14 remaining a keratinocyte-specific [310, 311], differentiation-linked outlier. However, this binary classification has proven oversimplified. Within the apoptotic group, caspases are further stratified into initiators (apical caspases, including caspase-8, -9, and -10) and executioners (downstream effectors, including caspase-3, -6, and -7) [312] (**Figure 13**).



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Figure 13. Classification of Caspases

Initiator caspases typically harbor long prodomains containing protein-interaction motifs such as CARD of caspase-1, -2, and -9, or DED (death effector domains) of caspase-8 and -10. Their activation mechanism is proximity-induced dimerization, which occurs on oligomeric activation platforms. Examples include the death-inducing signaling complex (DISC) for caspase-8/-10 and the apoptosome for caspase-9 [36, 279, 311]. Unlike initiator caspases, executioner caspases are present as inactive, pre-assembled dimers, and their activation requires cleavage of the linker connecting the large and small catalytic subunits, a reaction generally mediated by upstream initiator caspases [304, 311]. Inflammatory caspases also employ adaptor-mediated dimerization, but their activation platforms are multiprotein inflammasomes, which recruit caspase-1, -4, and -5 via CARD-CARD interactions. These complexes integrate danger and pathogen-associated signals to initiate cytokine maturation (e.g., IL-1 β , IL-18) and pyroptotic cell death [127, 313, 314]. Therefore, unlike many proteases, caspases are not activated by pro-domain removal [311].

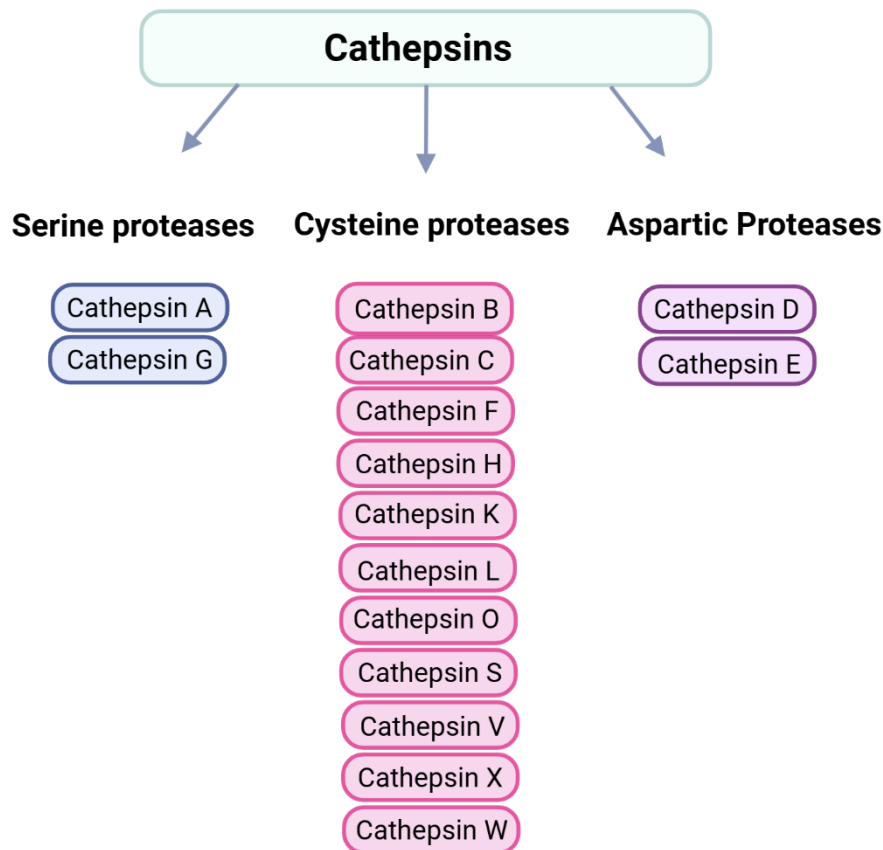
Caspase activation is frequently followed by additional autoproteolytic processing events referred to as maturation. While not essential for the acquisition of catalytic activity, maturation enhances enzyme stability, substrate accessibility, or regulatory interactions. For example, dimerization in the absence of maturation produces a form of active caspase-8 that can promote T-cell activation and proliferation without inducing apoptosis, where active caspase-8 is required [315], therefore highlighting how differential maturation can yield distinct biological outcomes. Because caspase activation is irreversible, cellular systems employ multiple regulatory strategies like caspase inhibition, caspase degradation, and decoy inhibitors, to prevent inappropriate activity. Endogenous inhibitors include viral proteins such as CrmA from the cowpox virus and baculoviral p35, which mimic substrates to nonspecifically block caspase activity [316], as well as the cellular protein XIAP, much more selective mechanism, which directly inhibits caspase-3, -7, and -9 through specialized BIR domains [317]. Additional inhibitory mechanisms involve decoy proteins like FLIP, a pseudo-caspase-8 [318]. Inhibition

through decoy proteins relies on structural similarity to caspase pro-domains, allowing these molecules to compete with functional caspases for adaptor binding sites within activation complexes. Rather than acting as direct enzymatic inhibitors, they essentially serve as 'activation preventers' by blocking the assembly of active complexes. Proteasomal degradation provides another layer of caspase regulation, particularly for the short-lived active forms. IAPs, equipped with RING and ubiquitin-binding domains, likely mediate this process by tagging caspases for degradation [100, 311, 319-321].

These observations emphasize that caspases orchestrate not only apoptosis but also necroptosis suppression, pyroptosis induction, and cross-regulation between death pathways. This crosstalk ensures flexibility of host defense and homeostasis but, when misregulated, drives pathologies such as cancer, autoimmunity, autoinflammation, and neurodegenerative disorders. Although classically associated with apoptosis, caspases have been implicated in a wide spectrum of non-lethal cellular processes. For example, they contribute to neural development, keratinocyte differentiation, immune modulation, and compensatory proliferation following tissue injury [322-324]. In physiological contexts, apoptosis provides a controlled, non-lytic mechanism for tissue sculpting and homeostasis, while inflammatory forms of programmed cell death act as key effectors of innate immunity. Thus, caspases are positioned at the interface of survival, death, and immune defense. These diverse roles suggest that caspases function not only as executioners of cell death but also as versatile modulators of cell fate decisions. The paradoxical capacity of caspases to promote both survival and death highlights the need for precise temporal and spatial regulation of their activity [325-327].

1.2.2. Cathepsins

The term *cathepsin* has been used since 1920 to describe lysosomal proteases, independent of their enzymatic class. This group includes serine proteases (cathepsins A and G), aspartic proteases (cathepsins D and E), and eleven human cysteine proteases (B, C, F, H, K, L, O, S, V, X, and W) [328-330] (**Figure 14**). Cathepsins share common features in localization and biosynthesis, though their expression levels vary. Among lysosomal proteases, cathepsins L, B, and D are the most abundant, reaching concentrations of up to 1 mM within lysosomes [331]. In the human body, serine proteases make up about one-third of all proteases, cysteine proteases account for roughly one-quarter, and aspartic proteases represent around 4% [306, 328, 332]. Cathepsins are most active in the acidic environment of lysosomes, where their proteolytic capacity is optimally tuned to a pH close to 5. Nevertheless, several cathepsins display activity beyond this range, enabling functions outside their classical lysosomal niche [328, 333]. For instance, cathepsin S shows a pH optimum of 6.5 [334, 335], cathepsin D exhibits peak activity at pH 4, but retains detectable activity even at physiological pH 7.4 [336], and cathepsins K and H maintain stable activity at pH 7 [337, 338]. Such flexibility allows cathepsins to exert proteolytic functions not only within lysosomes but also in extracellular or cytoplasmic contexts [306, 339, 340].



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Figure 14. Classification of Cathepsins

Beyond their established role in lysosomal protein recycling, cathepsins have been implicated in a wide spectrum of physiological and pathological processes. They contribute to intracellular homeostasis by degrading proteases and chemokines and play a crucial role in antigen processing during immune responses [328, 341]. Additionally, cathepsins are involved in the maturation of MHC class II complexes, bone remodeling, keratinocyte differentiation, and the regulation of inflammatory mediators. Dysregulated cathepsin activity has been linked to diverse pathologies, including tumor progression and metastasis, rheumatoid arthritis, osteoarthritis, and atherosclerosis and many neurodegenerative processes [306, 341-346]. To prevent uncontrolled proteolysis, cathepsins are controlled through multiple layers of regulation, most prominently by subcellular compartmentalization (lysosomes and other organelles), activation from zymogens, pH dependence, and modulation by both small-molecule inhibitors and endogenous protein inhibitors, often acting in combination to ensure optimal enzyme function [328, 347-349].

Lysosomal cathepsins are produced as prepro-zymogens. As the nascent polypeptide enters the endoplasmic reticulum, the N-terminal signal peptide is removed and the protein becomes N-glycosylated. The remaining propeptide functions as an intramolecular chaperone and inhibitor, ensuring correct folding and preventing premature activity, while M6P tagging in the Golgi directs trafficking via mannose-6-phosphate receptors to endosomes/lysosomes. In the mildly acidic endosomal lumen, cathepsins dissociate from M6PR, and subsequent propeptide removal yields the mature, active enzyme. Depending on the isoform, the mature

cathepsin is found as a single-chain or a two-chain species; in the latter case, the chains are linked by disulfide bonds, with chain status not altering catalytic activity [328, 346, 350-353]. After activation, cathepsin activity is regulated by endogenous protein inhibitors such as cystatins, stefins, thyropins, and serpins, which attach to the enzyme and prevent it from hydrolyzing substrates [347]. For some cathepsins activity might also be regulated through additional mechanisms, such as inactivation through oxidation of the catalytic cysteine residue [331]. Collectively, these processes allow lysosomal cathepsins to support protein turnover and cellular homeostasis while reducing the risk of harmful proteolysis [328, 346].

Besides their roles in protein turnover and cellular homeostasis, cathepsins are key regulators of programmed cell death, including apoptosis and pyroptosis. Cytosolic cathepsins, particularly B, D, and L, have been widely implicated in promoting apoptosis by activating downstream apoptotic proteases. For example, these cathepsins can cleave Bid, producing a truncated form that translocate to mitochondria, triggers cytochrome C release, and subsequently activates caspases, thereby initiating the apoptotic cascade. In parallel, cathepsins facilitate the degradation of anti-apoptotic proteins such as Bcl-2, Bcl-xL, Mcl-1, and XIAP, further tipping the balance toward cell death [273]. In T cells, cathepsin D-mediated apoptosis involves Bax activation and the release of apoptosis-inducing factor (AIF) and cytochrome C, a process that can occur independently of Bid cleavage and directly engage initiator caspase-8 [306, 354-356]. Cathepsins also modulate other forms of programmed cell death. In necroptosis, a regulated necrotic pathway initiated by diverse stimuli, the kinase activity of receptor-interacting serine/threonine kinase 1 is essential. In macrophages, cathepsins B and S can cleave RIPK1, thereby limiting necroptotic signaling [357]. Moreover, cytosolic cathepsins contribute to inflammatory cell death by regulating inflammasome activation. Although they do not directly cleave caspase-1, cathepsins modulate inflammasome assembly and activity. Recent studies indicate that the release of endogenous cathepsin B into the cytosol is required for NLRP3 inflammasome activation, linking lysosomal stress to inflammatory cytokine release [358-361]. Cathepsins S and Z have been shown to compensate for the loss of B, C, and L during LMP-mediated inflammasome activation, suggesting a functional redundancy in this regulatory network, although the exact mechanisms remain unresolved. Also reducing the expression of cathepsins B, C, S, L, and Z led to decreased secretion of IL-1 β , indicating that these lysosomal proteases play a critical upstream role in inflammasome activation and the regulation of pro-inflammatory cytokine release [259, 362, 363]. Cathepsins are increasingly recognized as direct modulators of pyroptotic cell death, a rapidly evolving area of research. Recent studies reveal that cathepsin S cleaves GSDMD, the pore-forming executor of pyroptosis, identifying a mechanism for pyroptotic activation independent of caspase-1 [364]. The same work also demonstrated that cathepsin S processes pro-inflammatory cytokines IL-1 β and IL-18, emphasizing its role in amplifying the inflammatory component of pyroptosis. These findings highlight a novel and expanding dimension of cathepsin function, suggesting that their involvement in cell death extends beyond classical apoptotic and necroptotic pathways. Collectively, this emerging evidence underscores the versatile roles of cathepsins in programmed cell death, acting both upstream of key signaling proteins and through direct cleavage of effector molecules, and points to a developing field with important implications for inflammation and disease [306, 365].

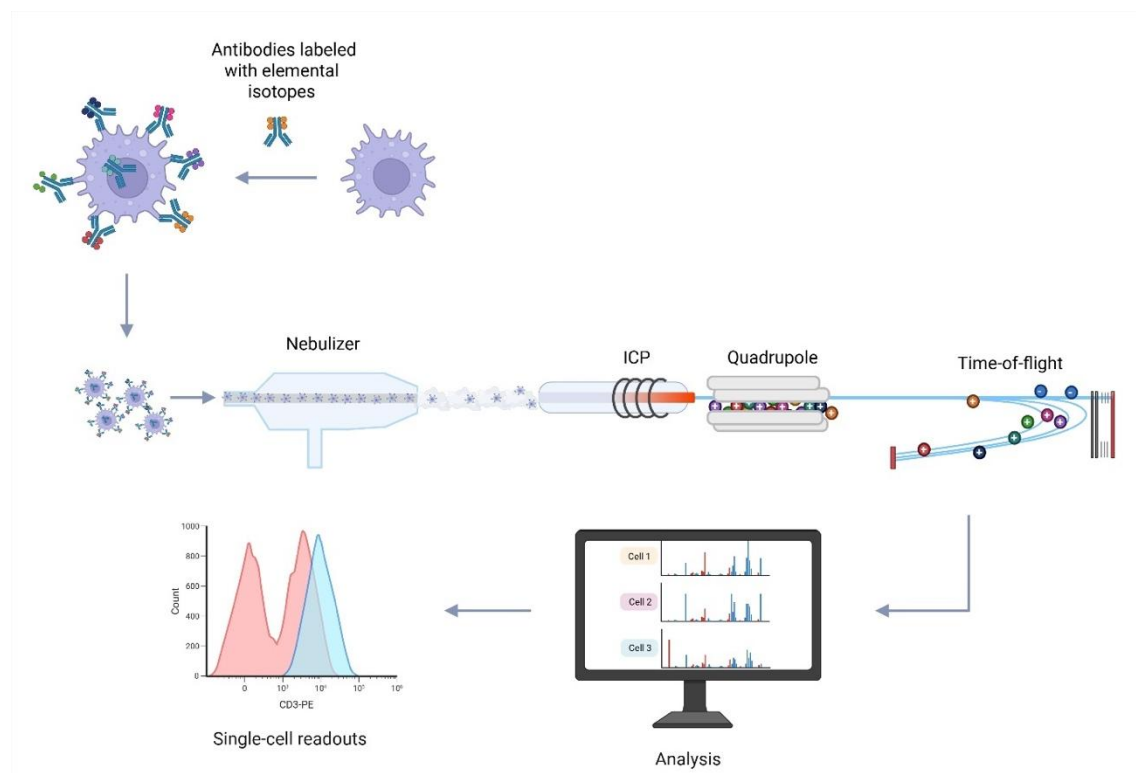
1.3. MASS CYTOMETRY

Mass cytometry, also known as Cytometry by Time-of-Flight (CyTOF), represents an advanced single-cell technology that combines the principles of flow cytometry with the detection power of mass spectrometry [366, 367]. It allows the simultaneous measurement of dozens of cellular parameters at the single-cell level by replacing fluorophores with stable heavy metal isotopes as tags for antibodies [367, 368] or other cellular probes [369]. Unlike conventional flow cytometry, which is restricted by spectral overlap between fluorophores and requires complex compensation matrices, CyTOF can resolve isotopes that differ by as little as 1 atomic mass unit (amu), thereby enabling the analysis of up to 60 parameters per cell with minimal signal interference. The latest generation of instruments detects isotopes within a mass range of 75-209 amu, significantly expanding the palette of available markers. This capability makes mass cytometry one of the most powerful multiparametric approaches for immunophenotyping and functional characterization of cells in complex tissues [366, 370].

Central to CyTOF is the use of antibodies conjugated to metal isotopes. This is typically achieved through covalent attachment to metal-chelating polymers (MCPs), which are water-soluble and can carry up to 30 metal ions. Each antibody is partially reduced to expose sulfhydryl groups, predominantly on the Fc region, that react with a maleimide group on the MCP to form a stable thioether bond [370-372]. On average, four to six MCPs may be attached per antibody, ensuring robust signal without releasing free metals. In addition to lanthanide isotopes, other metals have been adapted for specialized purposes: palladium isotopes (102-110 amu) for cell barcoding [373], rhodium (^{103}Rh) and platinum (^{195}Pt) compounds such as cisplatin for viability discrimination, iodine (127I) for cell cycle tracking [374], iridium ($^{191/193}\text{Ir}$) as DNA intercalators to identify nucleated cells [375], and bismuth (^{209}Bi) as an additional antibody label. Together, these reagents create a versatile toolbox for probing diverse cellular features far beyond surface immunophenotyping [370].

The preparation of samples for mass cytometry (**Figure 15**) involves several critical steps. Cells are typically fixed and permeabilized to preserve morphology and allow detection of both surface and intracellular proteins. Once samples are stained, they are introduced into the CyTOF instrument, where each cell is nebulized into a droplet and subsequently vaporized, atomized, and ionized in an inductively coupled argon plasma [370, 376, 377]. The resulting ion cloud, stripped of light elements such as nitrogen, carbon, phosphorus, and oxygen, contains only the metal isotopes that represent cellular epitopes and functional markers. These ions are separated by their mass-to-charge ratio in the time-of-flight analyzer, with lower-mass isotopes reaching the detector earlier, and the abundance of each isotope corresponds directly to the target molecule's expression level. Each cell generates a transient ion signal that is scanned 20-30 times within $\sim 300\ \mu\text{s}$, and detector outputs are integrated into single-cell events stored in flow cytometry standard (FCS) format [370, 378]. The resulting datasets, which typically encompass millions of single cells characterized by dozens of parameters, demand specialized computational pipelines. This has spurred the development of numerous machine learning-based algorithms for dimensionality reduction, clustering, and visualization, which are now

integral to mass cytometry data analysis [370, 379]. This workflow not only provides single-cell resolution across large populations but also enables detailed interrogation of different biological layers simultaneously. Researchers can measure cell-surface phenotypes alongside intracellular signaling cascades, transcription factor activity, post-translational modifications, proliferation, and cell cycle regulators, all within one experiment [366, 380]. This powerful technology was successfully applied in the complicated area of cell death research [381], where many processes are taking place at the same time, making it still difficult to understand. In the study of cell death, mass cytometry offers significant advantages over classical flow cytometry. While annexin V/propidium iodide staining remains a cornerstone for distinguishing apoptosis from necrosis, CyTOF can capture multiple cell death modalities simultaneously, including apoptotic, necroptotic, pyroptotic, and autophagic signatures. For instance, caspase activation, PARP cleavage, mitochondrial potential loss, DNA fragmentation, and phosphorylation of signaling molecules can all be monitored within a single experiment. This multidimensional profiling allows researchers to dissect how death pathways vary across heterogeneous cell populations—revealing, for example, how immune cells, tumor subclones, or stem/progenitor cells differentially respond to stress or therapy. Another major strength is its capacity to detect rare events, which are often missed in lower-parameter systems. Events such as stem cell apoptosis, pyroptotic bursts in innate immune cells, or drug-induced senescence transitions can be captured and contextualized within the broader cellular landscape. By integrating cell identity markers with functional readouts, mass cytometry provides a systems-level perspective on cell fate decisions, offering insights into how survival and death signals are coordinated in health and disease [376, 377, 381, 382].



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Figure 15. Schematic overview of the CyTOF workflow. Metal-tagged, antibody-stained cells are converted into an aerosol of single-cell droplets using a nebulizer and then atomized and ionized within an inductively coupled

plasma (ICP) torch. The generated ions pass through a quadrupole that excludes cellular debris and non-metallic species, allowing only the heavy metal isotopes to reach the TOF mass spectrometer. The detected isotopic signals correspond to specific antibodies bound to each individual cell, enabling high-dimensional single-cell analysis.

Although the technology has certain drawbacks, such as the inability to recover live cells after analysis and a slightly reduced sensitivity toward very low-abundance targets, its multiplexing power, cost-effectiveness, and depth of information make it a transformative analytical platform. Nonetheless, several limitations must be considered when designing and interpreting CyTOF experiments. Potential biases may arise from panel design, marker selection, and the affinity or specificity of the antibodies used, as these factors directly affect signal accuracy and population resolution. In addition, CyTOF is currently limited to the simultaneous detection of approximately 60 parameters per cell (theoretically up to 120), necessitating focused experimental designs that target selected cell types or signaling pathways [383]. Another important challenge lies in signal variability over time and between instruments, which can complicate multi-site or longitudinal analyses; however, normalization algorithms and the inclusion of anchor samples have been developed to enhance reproducibility and inter-study comparability [383-386]. As with fluorescence-based flow cytometry, certain cellular mediators, particularly small metabolites, remain inaccessible due to the lack of suitable binding probes or fixation-compatible detection methods. Moreover, high-quality affinity reagents with minimal cross-reactivity are essential, although a growing number of validated antibodies and user-friendly probe-design tools [369, 387] have improved accessibility. Importantly, like conventional flow cytometry, mass cytometry can be performed on fixed cells, allowing preservation of biological samples while minimizing artifacts associated with live-cell handling [366, 388].

Mass cytometry has emerged as a transformative tool for advancing the study of regulated cell death. By enabling simultaneous quantification of dozens of molecular markers at the single-cell level, CyTOF provides an unparalleled ability to dissect the complexity of death pathways within heterogeneous cell populations. In oncology, high-dimensional CyTOF profiling has been used to uncover mechanisms of drug resistance and to identify synergistic therapeutic targets through deep characterization of apoptotic signaling networks [381]. In immuno-oncology, CyTOF has elucidated how pyroptosis-driven cell death reshapes the tumor microenvironment, revealing coordinated activation of antigen-presenting and cytotoxic immune cells that contribute to effective antitumor responses [389]. Similarly, in developmental biology, CyTOF has been instrumental in distinguishing the complementary roles of extrinsic apoptosis and necroptosis in sculpting the developing brain, offering new perspectives on how perturbations in cell death programs may lead to neurodevelopmental disorders [390]. Together, these studies highlight the broad applicability and analytical power of mass cytometry in cell death research. Its ability to integrate molecular signaling, cell identity, and functional states at single-cell resolution makes it an essential platform for uncovering context-dependent mechanisms, identifying biomarkers, and guiding the design of targeted therapies. As computational and analytical tools continue to evolve, CyTOF stands out as a cornerstone technology for decoding the cellular heterogeneity and dynamic regulation underlying life-death decisions in health and disease.

2. RESEARCH OBJECTIVES

Pyroptosis is a lytic and proinflammatory form of regulated cell death that plays a central role in host defense but, when dysregulated, contributes to chronic inflammation and tissue pathology. Its canonical execution involves inflammasome assembly, caspase-1 activation, cleavage of gasdermin D, and release of cytokines such as IL-1 β and IL-18 [146, 174]. Despite this well-established caspase-dependent framework, there is mounting evidence that additional proteases, including lysosomal cathepsins, shape the dynamics of pyroptosis by modulating inflammasome activation, cytokine maturation, and execution phases of cell death [250]. However, the mechanisms by which individual cathepsins exert their effects remain poorly defined, largely because of the overlapping substrate specificity and functional redundancy within this protease family.

Cysteine cathepsins reside within lysosomes, maintaining protein homeostasis. When lysosomal membrane permeabilization occurs, these proteases may be released into the cytosol, impacting diverse cell death pathways such as apoptosis and necroptosis [233, 391-393]. Previous studies have suggested that cathepsin B, among others, might act upstream of the NLRP3 inflammasome; however, most of these conclusions stem from experiments using nonspecific inhibitors, leaving ambiguity about true cathepsin-specific functions [219, 250]. This has left a critical gap in understanding whether specific cathepsins truly drive pyroptotic signaling, or whether the observed effects reflect off-target consequences of lysosomal disruption.

The intricate proteolytic landscape associated with regulated cell death led us to hypothesize that pyroptosis and lysosome-dependent cell death intersect at shared molecular checkpoints. We propose that extensive cross-talk occurs among caspases, cathepsins, and calpains, and that this interplay is a key determinant of inflammatory outcomes. Supporting this view, microparticles have been reported to induce cell death even in the absence of caspase-1 and related inflammatory caspases, indicating that alternative protease systems can substitute for canonical executors. Based on these observations, we postulate that cytosolically released cathepsins may directly process GSDMD, thereby contributing to pore formation and the execution of inflammatory cell death independently or in cooperation with caspases.

The overarching goal of this dissertation is to determine the contribution of cysteine cathepsins to pyroptotic cell death and to establish innovative strategies for dissecting their functions. To achieve this, we combined classical cell biology approaches with new generations of chemical biology tools and high-dimensional single-cell technologies. Our unique experimental design centers on the application of tailor-made inhibitors and activity-based probes created using the HyCoSuL (Hybrid Combinatorial Substrate Library) approach, which incorporates natural and non-natural amino acids to generate highly selective substrates, inhibitors and activity-based probes (ABPs) for individual cathepsins [394-396]. These tools overcome one of the most significant, yet unmet challenges in cathepsin research, which is the strong overlap in substrate recognition motifs, allowing functional dissection of specific enzymes in living cells.

Specific Aim 1: Cathepsins as direct mediators of pyroptotic substrate processing

A critical question is whether cathepsins directly cleave pyroptotic substrates, including GSDMD and pro-IL-1 β . Preliminary work suggests that cathepsins may generate active cytokine fragments or modulate gasdermin processing in parallel to caspases. We systematically tested the processing capacity of individual cathepsins using purified proteins, cell-based assays. This defined the potential of cathepsins to directly shape cytokine secretion and pore formation.

Specific Aim 2: Cathepsins in canonical pyroptosis and lysosome dependent cell death

Using selective chemical inhibitors, in infection- and sterile inflammation- based cell models to evaluate whether targeting cysteine cathepsins can alter the magnitude of pyroptotic death and cytokine release. This highlighted the broader biological relevance of cathepsin regulation of pyroptosis and demonstrated the utility of custom probes and inhibitors as research tools for dissecting inflammatory cell death.

Specific Aim 3: Application of selective chemical tools in cell death study

The project leverages a unique set of custom-made cathepsin inhibitors and activity-based probes developed with the HyCoSuL methodology. By screening natural and non-natural amino acid libraries, this strategy produces molecules of exceptional specificity for individual cathepsins, even among enzymes with nearly identical substrate motifs. The strength of this approach lies not only in its biochemical precision but also in its adaptability to cell-based studies, where overlapping proteolytic activity has historically obscured mechanistic insights. This project represents one of the first applications of such chemical tools in live-cell models of pyroptosis, thereby setting a methodological precedent for future protease research.

Specific Aim 4: Systems-level profiling of pyroptotic death using mass cytometry

To integrate protease activity with broader cell death signaling, we employed mass cytometry (CyTOF). This technique allows simultaneous quantification of more than 40 parameters at the single-cell level, making it ideally suited to capture the complexity of pyroptotic responses. **A custom-designed antibody panel**, tailored to detect inflammasome components, cytokine intermediates, markers of mitochondrial integrity, and regulators of lysosomal function, will be used alongside our chemical probes. This combined approach will generate high-dimensional maps of pyroptotic signaling networks, linking cathepsin activity to downstream functional outcomes. Importantly, mass cytometry provides a unique opportunity to capture early events in pyroptotic execution, a feature that may be lost in bulk biochemical assays.

Expected significance:

By uniting precise chemical biology tools with advanced cytometry platforms, this project aims to clarify the underexplored role of cysteine cathepsins in pyroptosis. Our findings will not only expand fundamental knowledge of lysosome-dependent regulation of inflammatory cell death but also demonstrate the power of HyCoSuL-derived inhibitors, activity-based probes, and custom cytometry panels as cutting-edge research tools. Beyond immediate insights into cathepsin biology, the study will establish a methodological framework that can be applied to other protease families and cell death pathways. Ultimately, this work may inform the development of therapeutic strategies targeting cathepsin activity in pathological inflammation, infection, and degenerative diseases.

3. EXPERIMENTAL SECTION

3.1. METHODS

3.1.1. Cell culture methods and Western blotting procedures

Western blotting. The human monocytic cell line THP-1 (TIB-202, ATCC) was maintained under standard culture conditions and differentiated into macrophage-like (M0) cells by treatment with 40 ng/mL phorbol 12-myristate 13-acetate for 16-20 hours, followed by a resting period of 10–12 hours in complete RPMI medium. Subsequently, the medium was replaced with Opti-MEM, and cells were primed with 1 μ g/mL lipopolysaccharide for 10–12 hours. Where indicated, cells were preincubated with 25 μ M of the respective inhibitors for 2 hours prior to stimulation with specific cell death inducers: Nigericin (3 μ M or 25 μ M), LLOMe (500 μ M), MSU (100 μ g/mL), nano-SiO₂ (50 μ g/mL), or Alum (50 μ g/mL) for the time points indicated. Following stimulation, supernatants were collected for protein precipitation using the trichloroacetic acid method and cells were detached from plate using TrypLE™, centrifuged at 350 $\times$ g for 5 minutes. Cell pellets were lysed in 67 μ L of RIPA buffer mixed with 33 μ L of 3 $\times$ SDS/DTT loading buffer, followed by vortexing and boiling for 5 minutes. Equal volumes of cell lysates and precipitated proteins from supernatants were resolved by SDS-PAGE using 4–12% Bis-Tris 15-well gels (20 minutes at 200 V). Proteins were transferred to nitrocellulose membranes (60 minutes at 10 V) and blocked with 5% BSA in TBS-T for 1 hour at room temperature. Membranes were incubated with primary antibodies (Cell Signaling Technology) overnight at 4°C, followed by fluorescently labeled secondary antibodies (anti-rabbit Alexa Fluor 800). Signal detection was performed using the Azure Sapphire imaging system (800 nm laser). Signal intensities were analyzed using Image Studio Lite software.

Analysis of cell death signatures in THP-1 cells. Low-passage THP-1 cells were differentiated with 40 nM PMA for 16-20 hours. Following differentiation, the medium was replaced with fresh complete RPMI and cells were rested for 24 hours. After the resting period, the medium was exchanged for Opti-MEM, and where indicated, cells were primed with 1 μ g/mL lipopolysaccharide (LPS). After 2 hours of priming, 25 μ M of the respective inhibitors was added for an additional 2 hours. Following a total of 4 hours of priming and preincubation, cells were stimulated with cell death inducers, Nigericin (3 μ M, 25 μ M) or LLOMe (500 μ M), in Opti-MEM for the indicated time points. After stimulation, cell culture supernatants were collected into pre-chilled tubes containing 10 $\times$ protease and phosphatase inhibitor cocktail and centrifuged at 10,000 $\times$ g for 10 minutes at 4 °C. On the following day, TCA precipitation was performed on the supernatants. Meanwhile, cells were kept on ice, gently washed with DPBS, and lysed in RIPA buffer supplemented with DNase I and protease/phosphatase inhibitors. After 30 minutes, lysates were collected, sonicated, and quantified using a BCA protein assay. Samples were then stored at -80 °C. Equal amounts of protein were mixed with 5 $\times$ SDS loading buffer and subjected to SDS-PAGE using 4-12% Bis-Tris 15-well gels (200 V, 20 minutes). Proteins were transferred onto nitrocellulose membranes (10 V, 60 minutes) and blocked in 5%

BSA in TBS-T for 1 hour at room temperature. Membranes were incubated with primary antibodies (Cell Signaling Technology) overnight at 4 °C, followed by fluorescently labeled secondary antibodies (anti-rabbit Alexa Fluor 800). Signal detection was performed using the Azure Sapphire imaging system (800 nm laser), and band intensities were quantified with Image Studio Lite software.

Protein precipitation by trichloroacetic acid (TCA). Cell culture supernatants were subjected to protein precipitation using trichloroacetic acid. The volume of each supernatant was measured, and one-half of this volume of ice-cold 30% (w/v) TCA was added to each sample. Tubes were vortexed briefly to ensure complete mixing and incubated on ice for 30 minutes to allow protein precipitation. Following incubation, samples were centrifuged at maximum speed ($\sim 20,000 \times g$) for 30 minutes at 4 °C. The supernatants were carefully discarded, leaving the protein pellet intact. To further purify the precipitate, 100 μ L of ice-cold 10% (w/v) TCA was added to each tube, gently mixed by pipetting, and centrifuged again at maximum speed for 10 minutes at 4 °C. The supernatants were discarded, and pellets were washed with 150 μ L of ice-cold acetone. Samples were gently mixed by pipetting and centrifuged at maximum speed for 5 minutes at 4 °C. Finally, the resulting protein pellets were resuspended in 1 $\times$ SDS sample buffer containing DTT and stored at -20 °C or processed immediately for SDS-PAGE and immunoblot analysis.

Bicinchoninic acid (BCA) assay. A bovine serum albumin stock solution (2 mg/mL) was serially diluted in assay diluent to prepare a standard curve ranging from 0 to 1,000 μ g/mL (final concentrations: 0, 25, 125, 250, 500, and 1,000 μ g/mL). Standards were prepared fresh for each assay. Immediately prior to use, the BCA working reagent (WR) was prepared by mixing 50 parts of BCA solution with 1 part of 4% cupric sulfate solution. The WR was prepared in sufficient quantity to provide 1.0 mL per assay tube. Protein samples and BSA standards (50 μ L each) were pipetted into labeled 12 $\times$ 75 mm polypropylene test tubes in duplicate. To each tube, 1.0 mL of freshly prepared WR was added and mixed by gentle vortexing. Tubes were incubated at 37 °C for 30 minutes. Following incubation, samples were cooled to room temperature prior to measurement. Absorbance was recorded at 562 nm using NanoDrop (Eppendorf, BioPhotometer D301, 1 cm path length cuvettes). Blank-corrected absorbance values were obtained by subtracting the absorbance of the zero standard from all other readings. A standard calibration curve was generated by plotting corrected absorbance versus BSA concentration. Protein concentrations of experimental samples were interpolated from the linear portion of the standard curve and adjusted for dilution factors.

3.1.2. Experiments with recombinant proteins

Analysis of pro-IL-1 β and pro-IL-18 cleavage by recombinant cathepsins. Recombinant human cathepsins B, L, S, and V were diluted in the acidic assay buffer at pH 5.5 (100 mM sodium acetate, 10 mM sodium chloride, 10 mM DTT) and added to the substrate pro-IL-1 β or pro-IL-18 at final enzyme concentrations of 1, 5, 25 or 125 nM. Control conditions included: pro-IL-1 β alone in pH 5.5 buffer, pro-IL-18 alone in pH 5.5 buffer and tested

cathepsin alone in pH 5.5 buffer. All reactions were incubated at 37 °C for 30 minutes. Immediately following incubation, 3× SDS loading buffer was added to each sample, and the mixtures were boiled for 5 minutes at 95 °C. Equal volumes of each reaction were separated by SDS-PAGE (200 V, 20 minutes), and gels were stained with InstantBlue® Coomassie Protein Stain for 1 hour following three washes with dH₂O or subjected to transfer onto nitrocellulose membranes (10 V, 60 minutes) and blocked in 5% BSA in TBS-T for 1 hour at room temperature. Membranes were incubated with primary antibodies (Cell Signaling Technology) overnight at 4 °C, followed by fluorescently labeled secondary antibodies (anti-rabbit Alexa Fluor 800). Protein bands were visualized using the Azure Sapphire Imaging System (Coomassie channel or AlexaFluor 800). Signal intensities were analyzed using Image Studio Lite software.

Analysis of pro-IL-1 β cleavage by recombinant cathepsins. Recombinant human cathepsins B, L, S, and V were diluted in the appropriate assay buffer at either acidic pH 5.5 (100 mM sodium acetate, 10 mM sodium chloride, 10 mM DTT) or neutral pH 7.2 (100 mM HEPES, 10 mM sodium chloride, 10 mM DTT) and added to 500 nM of the substrate- pro-IL-1 β at final enzyme concentrations of 100, 250, or 500 nM. Control conditions included: heat-inactivated cathepsin (500 nM) with pro-IL-1 β at pH 5.5, pro-IL-1 β alone in pH 5.5 buffer, and pro-IL-1 β incubated with caspase-1 (1 μ L; Enzo) in caspase buffer at pH 7.2 (10% w/v sucrose, 20 mM Pipes, 10 mM NaCl, 1 mM EDTA, and 10 mM DTT). All reactions were incubated at 37 °C for 60 minutes. Immediately following incubation, 5× SDS loading buffer was added to each sample, and the mixtures were boiled for 5 minutes at 95 °C. Equal volumes of each reaction were separated by SDS-PAGE (200 V, 20 minutes), and gels were stained with InstantBlue® Coomassie Protein Stain for 1 hour. Gels were then washed three times with dH₂O, and protein bands were visualized using the Azure Sapphire Imaging System (Coomassie channel). Signal intensities were analyzed using Image Studio Lite software.

Analysis of hGSDMD processing in lower concentrations of recombinant cathepsins. Recombinant human cathepsins B, L, S, and V were diluted in the appropriate assay buffer at either acidic pH 5.5 (100 mM sodium acetate, 10 mM sodium chloride, 10 mM DTT) and added to 1.2 μ g of the substrate GSDMD at final enzyme concentrations of 1, 5, 25 or 125 nM. Control conditions included GSDMD alone in pH 5.5 buffer and tested cathepsin alone in pH 5.5 buffer. All reactions were incubated at 37 °C for 30 minutes. Immediately following incubation 3× SDS loading buffer was added to each sample, and the mixtures were boiled for 5 minutes at 95 °C. Equal volumes of each reaction were separated by SDS-PAGE (200 V, 20 minutes), and gels were stained with InstantBlue® Coomassie Protein Stain for 1 hour following three washes with dH₂O or subjected to transfer onto nitrocellulose membranes (10 V, 60 minutes) and blocked in 5% BSA in TBS-T for 1 hour at room temperature. Membranes were incubated with primary antibodies (Cell Signaling Technology) overnight at 4 °C, followed by fluorescently labeled secondary antibodies (anti-rabbit Alexa Fluor 800). Protein bands were visualized using the Azure Sapphire Imaging System (Coomassie channel or AlexaFluor 800). Signal intensities were analyzed using Image Studio Lite software.

Analysis of hGSDMD processing by cathepsins in time dependent manner at acidic pH. Recombinant human cathepsins B, L, S, and V were diluted in the appropriate assay buffer at acidic pH 5.5 (100 mM sodium acetate, 10 mM sodium chloride, 10 mM DTT) and added to 1,2 µg of the substrate GSDMD at final enzyme concentrations of 1, 5, 25 or 125 nM. Control conditions included GSDMD alone in pH 5.5 buffer and tested cathepsin alone in pH 5.5 buffer. All reactions were incubated at 37 °C for marked timepoints of 1, 5, 15, 30, 60 minutes. Immediately following incubation 3× SDS loading buffer was added to each sample, and the mixtures were boiled for 5 minutes at 95 °C. Equal volumes of each reaction were separated by SDS-PAGE (200 V, 20 minutes), and gels were stained with InstantBlue® Coomassie Protein Stain for 1 hour following three washes with dH₂O or subjected to transfer onto nitrocellulose membranes (10 V, 60 minutes) and blocked in 5% BSA in TBS-T for 1 hour at room temperature. Membranes were incubated with primary antibodies (Cell Signaling Technology) overnight at 4 °C, followed by fluorescently labeled secondary antibodies (anti-rabbit Alexa Fluor 800). Protein bands were visualized using the Azure Sapphire Imaging System (Coomassie channel or AlexaFluor 800). Signal intensities were analyzed using Image Studio Lite software.

Analysis of hGSDMD processing by cathepsins at neutral pH. Recombinant human cathepsins B, L, S, and V were diluted in the appropriate assay buffer at neutral pH 7.5 (100 mM HEPES, 10 mM sodium chloride, 10 mM DTT) and added to 1,2 µg of the substrate GSDMD at final enzyme concentrations of 1, 5, 25 or 125 nM. Control conditions included GSDMD alone in pH 7.5 buffer and tested cathepsin alone in pH 7.5 buffer. All reactions were incubated at 37 °C either for 30 minutes or marked timepoints of 1, 5, 15, 30, 60 minutes. Immediately following incubation 3× SDS loading buffer was added to each sample, and the mixtures were boiled for 5 minutes at 95 °C. Equal volumes of each reaction were separated by SDS-PAGE (200 V, 20 minutes), and gels were stained with InstantBlue® Coomassie Protein Stain for 1 hour following three washes with dH₂O or subjected to transfer onto nitrocellulose membranes (10 V, 60 minutes) and blocked in 5% BSA in TBS-T for 1 hour at room temperature. Membranes were incubated with primary antibodies (Cell Signaling Technology) overnight at 4 °C, followed by fluorescently labeled secondary antibodies (anti-rabbit Alexa Fluor 800). Protein bands were visualized using the Azure Sapphire Imaging System (Coomassie channel or AlexaFluor 800). Signal intensities were analyzed using Image Studio Lite software.

Analysis of hGSDMD processing in high concentrations of recombinant cathepsins. Recombinant human cathepsins B, L, S, and V were diluted in the appropriate assay buffer at either acidic pH 5.5 or neutral pH 7.2 and added to 500 nM of the substrate GSDMD at final enzyme concentrations of 100, 250, or 500 nM. Control conditions included: heat-inactivated cathepsin (500 nM) with GSDMD, GSDMD alone in pH 5.5 buffer, and GSDMD incubated with 500 nM of caspase-4 in caspase buffer at pH 7.2. All reactions were incubated at 37 °C for 60 minutes. Immediately following incubation 5× SDS loading buffer was added to each sample, and the mixtures were boiled for 5 minutes at 95 °C. Equal volumes of each reaction were separated by SDS-PAGE (200 V, 20 minutes), and gels were stained with InstantBlue® Coomassie Protein Stain for 1 hour. Gels were then washed three times with

dH₂O, and protein bands were visualized using the Azure Sapphire Imaging System (Coomassie channel). Signal intensities were analyzed using Image Studio Lite software.

Analysis of processing GSDMD in cell lysates. Unstimulated THP-1 and HEK-293T-GSDMD cells were maintained under standard culture conditions, harvested, counted, pelleted, and washed twice with ice-cold DPBS. After decanting the buffer, cell pellets were resuspended in fresh, ice-cold DPBS and lysed by brief sonication on ice. Lysates were vortex-mixed and kept on ice. For each reaction, an equal amount of lysate was combined with recombinant cathepsin B or cathepsin L diluted in the appropriate assay buffer, and mixtures were incubated at 37 °C for 30 min. Reactions were terminated by adding 3X SDS sample buffer to a final 1× concentration, followed by heating at 95 °C for 5 min. Equal volumes of each reaction were resolved by SDS-PAGE on 4-12% Bis-Tris 15-well gels (200 V, 20 min). Proteins were transferred to nitrocellulose membranes (10 V, 60 min) and membranes were blocked in 5% (BSA in TBS-T for 1 h at room temperature. Blots were incubated with primary antibodies (Cell Signaling Technology) overnight at 4 °C, washed with TBS-T, and then incubated with fluorescent secondary antibodies (goat anti-rabbit Alexa Fluor 800). Fluorescent signals were acquired on an Azure Sapphire imaging system (800 nm channel), and band intensities were quantified using Image Studio Lite.

3.1.3. Mass Spectrometry analysis of GSDMD fragments

Identification of cathepsin S-mediated GSDMD cleavage. To identify the cathepsin S cleavage site within the GSDMD protein, recombinant human SUMO-GSDMD-His-tag (2,4 µg) was incubated with 125 µM of purified human cathepsins at pH as specified in the figure legend, in assay buffer for 2 hours at 37°C, in a total reaction volume of 30 µL. Following incubation, reactions were stopped by the addition of 15 µL of 3X SDS/DTT loading buffer, boiled for 5 minutes, and subjected to SDS-PAGE. Protein bands corresponding to the intact SUMO-GSDMD-His-tag (control) and cathepsin S-mediated cleavage fragments were excised and submitted for mass spectrometry analysis (performed by the Mass Spectrometry Laboratory, IBB PAS; <https://mslab-ibb.pl>). Samples were subjected to disulfide bond reduction and alkylation, followed by trypsin digestion. The resulting peptides were separated by liquid chromatography and analyzed by tandem mass spectrometry (LC-MS/MS). Data acquisition included measurement of peptide masses and fragmentation patterns. Search parameters: protease - trypsin; fixed modifications – carbamidomethyl (C); variable modifications – oxidation (M); mass values – monoisotopic; peptide mass tolerance +/- 6.69 ppm, fragment mass tolerance +/- 0.01 Da, instrument type HCD. The database search was performed using Mascot (Matrix Science) against the UniProt database. Identified peptides were mapped to the GSDMD sequence to determine cleavage sites. Sequence coverage (%) was calculated for each sample. The instrumentation used was partially funded by the Centre for Preclinical Research and Technology (CePT), co-financed by the European Regional Development Fund and the Innovative Economy Operational Programme under the National Cohesion Strategy of Poland.

3.1.4. Kinetic analysis of GSDMD-based IQF substrates

Labeling of cathepsin activity in THP-1 cells using activity-based probes. Low-passage THP-1 cells were differentiated as described in the *Cell Culture* and *Western Blotting* sections. Cells were preincubated with different concentrations of inhibitors, 1 μ M, 5 μ M and 25 μ M for 2 hours followed with incubation with activity-based CatB-27 probe (250 nM) for 4 hours. After treatment, cells were washed with DPBS, detached using TrypLE, and centrifuged at $1,000 \times g$ for 10 minutes. Pellets were resuspended in 100 μ L of 1X SDS/DTT loading buffer, boiled at 95 $^{\circ}$ C for 5 minutes, cooled to room temperature, and briefly sonicated. Equal volumes of each sample were separated by SDS–PAGE using 4–12% Bis-Tris gels (150 V, 45 minutes) and transferred to 0.2 μ m nitrocellulose membranes (10 V, 60 minutes). Membranes were blocked with 5% BSA in TBS-T for 1 hour at room temperature. Blots were incubated with primary antibody (R&D Systems) overnight at 4 $^{\circ}$ C, washed with TBS-T, and then incubated with fluorescent secondary antibodies (Donkey anti-Goat Alexa Fluor Plus 800), scanned both at 700 nm (Cy5 channel) and 800nm. Analysis was done using Image Studio Lite software.

Application of cathepsin inhibitors to study cell death mechanisms by immunoblotting. THP-1 cells were differentiated as described in the *Cell Culture Methods* and *Western Blotting Procedures* sections. Cells were preincubated with 25 μ M of the respective inhibitors for 2 hours, followed by treatment with activity-based CatB-27 probe (250 nM) for 4 hours. After stimulation, cells were centrifuged at $400 \times g$ for 5 minutes. Cell pellets were lysed in 67 μ L of RIPA buffer supplemented with 33 μ L of 3 $\times$ SDS/DTT loading buffer, vortexed, and boiled for 5 minutes. Equal volumes of cell lysates were separated by SDS–PAGE using 4–12% Bis-Tris 15-well gels (200 V, 20 minutes). Proteins were transferred to 0.2 μ m nitrocellulose membranes (10 V, 60 minutes) and blocked with 5% BSA in TBS-T for 1 hour at room temperature. Membranes were incubated with primary antibodies (R&D Systems) overnight at 4 $^{\circ}$ C, followed by fluorescently labeled secondary antibody (Donkey anti-Goat Alexa Fluor Plus 800). Detection was performed using the Azure Sapphire imaging system (800 nm and 700 nm laser), and GSDMD signal intensity was quantified using Image Studio Lite software.

3.1.5. Live-cell imaging

Real-time analysis of cell death mechanisms in THP-1 cells using IncuCyte live-cell imaging. Live-cell imaging was performed using the IncuCyte S3 Live-Cell Analysis System (Sartorius) to monitor cell death kinetics in real time. Low-passage THP-1 cells were seeded in a 96-well plate and differentiated with PMA as described in the *Cell Culture Methods* and *Western Blotting Procedures* sections. Where indicated, cells were preincubated with 25 μ M of the respective inhibitor for 2 hours, followed by stimulation with cell death inducers: either Nigericin (3 μ M or 25 μ M) or LLOMe (500 μ M), in the presence of a fluorescent cell death marker, either PI or CellEvent™ Caspase-3/7 Green Detection Reagent. Immediately after treatment, plates were transferred to the IncuCyte system for time-lapse imaging. Scanning was performed using the standard scan type with phase contrast and either green (300 ms acquisition time) or red (400 ms acquisition time) fluorescence channels, using a 10X objective. The Cell-

by-Cell analysis mode was disabled. At the end of the imaging period, Nuclear-ID DNA stain was added to the wells, and a final scan was performed to quantify total cell numbers.

3.1.6. Cell Viability and Cytotoxicity Assessment

MTS cell viability assay and LDH release assay. PMA-differentiated THP-1 cells, following a resting period and LPS priming, were preincubated with 25 μ M of the indicated inhibitors for 2 hours, as detailed in the *Cell Culture and Western Blotting* sections. Subsequently, cells were exposed to cell death inducers for 8 hours, during which live-cell imaging was performed using PI to monitor membrane integrity. Upon completion of the incubation, cells were stained with Nuclear-ID for enumeration, then centrifuged at $150 \times g$ for 1 minute. A 50 μ L aliquot of the supernatant was transferred to a fresh 96-well plate for LDH release assay, while the remaining cells were immediately subjected to an MTS assay, following the manufacturer's instructions. Absorbance signals were measured at 490nm in a FilterMax F5 (Molecular Devices) plate reader. MTS absorbance values were normalized to untreated cells. LDH release was normalized to Triton X-100-treated cells.

3.1.7. Mass cytometry

Conjugation of antibodies with metal isotopes. Antibodies were metal-tagged (**Table 3**) using Maxpar X8 or Maxpar MCP9 Antibody Labeling Kits (Standard BioTools, Canada), designed for coupling lanthanides or cadmium, respectively. Each kit provides the necessary buffers (R, L, C, W), chelating polymers, and corresponding metal reagents. For lanthanide conjugation, 100 μ g of MaxPar X8 polymer was incubated with a 2.5 mM lanthanide solution in L buffer for 1 h to allow metal loading. In parallel, antibodies were exchanged into R buffer using 50 kDa Amicon centrifugal filters (Merck, 505096) and subsequently reduced with 4 mM TCEP (Sigma-Aldrich, 51805-45-9) for 30 min at 37 °C. The metal-loaded polymer was then rinsed using 3 kDa Amicon filters (Merck, UFC500396) with L and C buffers before being combined with the reduced antibody and incubated for 1.5 h at 37 °C to enable covalent attachment. After conjugation, the antibodies were washed with W buffer and centrifuged at $12\,000 \times g$ for 8 min. Final purification was performed by two consecutive elutions with 50 μ L W buffer, and the eluates were transferred to fresh tubes. Protein concentration was determined using a NanoDrop spectrophotometer (Eppendorf, BioPhotometer D301), adjusted to 0.5 mg/ml with antibody stabilizer (Sigma-Aldrich, 55514), and stored at 4 °C in sealed containers.

Table 3. Conjugated antibody and probe panel for mass cytometry

Metal tag	Antibody/TOF-probe
^{141}Pr	WEHD-AOMK (<i>probe</i>)
^{141}Pr	anti-HMGB1
^{142}Ce	anti-cl.IL-1 β
^{147}Sm	anti-FLIP

¹⁵² Sm	anti-cl.caspase-7
¹⁵⁴ Sm	anti-caspase-5
¹⁵⁵ Gd	anti-cl.GSDMD
¹⁵⁹ Tb	IETD-AOMK (<i>probe</i>)
¹⁶⁰ Gd	anti-GSDMD
¹⁶¹ Dy	anti-IL-18
¹⁶² Dy	anti-NINJ1
¹⁶³ Dy	anti-LDH
¹⁶⁴ Dy	anti-TNF- α
¹⁶⁵ Ho	LEHD-AOMK (<i>probe</i>)
¹⁶⁶ Er	anti-cl.Caspase-3
¹⁶⁸ Er	anti-NLRP3
¹⁶⁹ Tm	anti-IL-1 β
¹⁷⁰ Er	anti-caspase-1
¹⁷¹ Yb	anti-caspase-4
¹⁷² Yb	anti-PARP
¹⁷³ Yb	anti-caspase-8
¹⁷⁵ Lu	DEVD-AOMK (<i>probe</i>)
¹⁷⁶ Yb	anti-GSDME

CyTOF data acquisition. Mass cytometry measurements were performed on a CyTOF instrument (Standard BioTools, Canada). Prior to acquisition, cells were washed twice in Cell Staining Buffer (CSB; Standard BioTools, 201068) followed by two washes in Cell Acquisition Solution (CAS; Standard BioTools, 201240). The final cell suspension was prepared in EQ Four Element Calibration Beads (Standard BioTools, 201078) diluted 1:9 in CAS, passed through a 40 μ m cell strainer (SPL Life Sciences, S93040), and transferred into acquisition tubes. Samples were collected at a rate of approximately 500 events per second. The cytometer was tuned daily using Tuning Solution (Standard BioTools, 201072), and instrument performance was confirmed using EQ beads. A full deep-cleaning cycle was carried out every 40 hours of operation. All acquired files were exported in FCS format for downstream analysis.

Normalisation. Cell-bead doublets can be removed by gating out events that are positive for all five bead-associated metal isotopes, without relying on low DNA intensity. More broadly, any bead-derived events can be excluded by eliminating those that fall within the defined region of the characteristic five-element bead cluster. Polystyrene beads remain distinguishable from true cellular events even when some bead metals are included in the antibody panel, provided that the bead signature remains unique. Importantly, no cell should display signal in all five bead channels; partial overlap in four or fewer channels is acceptable.

Bead–bead and bead–cell aggregates can be efficiently removed using the distinctive five-metal profile. For complete separation, one of the bead metals may be dedicated exclusively to bead identification by omitting it from antibody labeling, ensuring clear detection and gating out of bead events [384].

Data analysis. Mass cytometry data were processed in Cytobank using a manual bivariate gating workflow, to isolate high-quality single-cell events from THP-1 cultures induced to cell-death. In cytometry, gating refers to the sequential graphical selection of event populations on two-parameter plots to exclude non-cellular signals and artifacts such as beads, debris, doublets, aggregates, and damaged cells [397]. A comprehensive manual cleanup procedure was applied prior to biological analysis to ensure that only intact, nucleated single cells were subjected to downstream quantification [379, 398]. Approach to Bivariate Analysis of Data Acquired Using the Maxpar Direct Immune Profiling Assay. Technical Note 400248B1 Rev01, Fluidigm, was used as a guide to gating protocol. Cleanup parameters were monitored over time to spot transient issues (clogs, detector drift, debris). Gates were adjusted per sample. First, all events were reviewed to assess run quality and define the parent population. Next, bead events were removed by excluding high ^{140}Ce signal versus time. Signal-quality cleanup followed using vendor Gaussian metrics (Residual, Center, Offset, Width), with outliers (typically aggregates or unstable signals) discarded. Then, DNA1 and DNA2 were each checked versus time to remove debris or dying cells with unstable DNA signals. Finally, nucleated single cells were selected from the stable DNA-positive population to serve as the parent for downstream analyses. The resulting clean, DNA-positive singlet population served as the parent population for all subsequent marker quantification. For each marker or activity-based probe, single-parameter histograms were constructed, with the x-axis reflecting intensity and the y-axis reporting event counts. Overlaying histograms across conditions enabled visualization of shifts in marker abundance, heterogeneity changes and differential population frequencies.

3.2. REAGENTS, BUFFERS, KITS

Recombinant proteins and chemical tools

- Cysteine cathepsins B, L, K, V, and S were provided by Prof Boris Turk, Jozef Stefan Institute, Jamova 39, SL-1000, Ljubljana, Slovenia
- GSDMD, interleukins and caspases were provided by Dr. Katarzyna Grobocz, Scott Snipas and Prof. Guy Salvesen, Sanford Burnham Prebys, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA
- Custom inhibitors, ABPs and TOF-probes synthesis and kinetic study was performed by Julia Nguyen and Vanessa Pippa in the Department of Chemical Biology and Bioimaging, Faculty of Chemistry at WUST

Cell culture media & buffers

- RPMI-1640 medium - Sigma-Aldrich (Merck), R8758
- Opti-MEM™ Reduced Serum Medium - Gibco, 31985-070 (*alt. 11058-021*)
- Dulbecco's Phosphate-Buffered Saline (DPBS), without Ca²⁺/Mg²⁺ - Gibco, 14190-144
- TrypLE™ Express Enzyme (1X), phenol red – Gibco, 12605010

Supplements & antibiotics

- L-Glutamine (200 mM) - Gibco, 25030-081
- Penicillin–Streptomycin (10,000 U/mL; 10,000 µg/mL) - Gibco, 15140-122
- Bovine Serum Albumin (BSA), Fraction V - Sigma-Aldrich (Merck), A7906

Inflammasome/cell-death stimuli & particulates

- Nigericin, sodium salt - Sigma-Aldrich (Merck), N7143 (*alt. Apollo Scientific, BIN0112*)
- L-Leucyl-L-leucine methyl ester (LLOMe), hydrobromide - Sigma-Aldrich (Merck), L7393
- Silicon dioxide nanoparticles (nano-SiO₂) - InvivoGen, tlrl-sio-2
- Aluminum hydroxide (alum), ready-to-use suspension - InvivoGen, tlrl-aloh
- Monosodium urate (MSU) crystals - InvivoGen, tlrl-msu
- Lipopolysaccharide (LPS) from *E. coli* O111:B4 - Sigma-Aldrich (Merck), L3024
- Phorbol 12-myristate 13-acetate (PMA) - Sigma-Aldrich (Merck), P8139
- Staurosporine - AmBeed, A256460

Viability/cytotoxicity & caspase assays

- CellTiter 96® AQueous One Solution (MTS) Cell Proliferation Assay - Promega, G3581
- CytoTox 96® Non-Radioactive Cytotoxicity (LDH) Assay - Promega, G1782
- CellEvent™ Caspase-3/7 Green Detection Reagent - Invitrogen, C10432

Nucleic acids, enzymes & inhibitors

- DNase I - EURx, E1345-02
- Halt™ Protease Inhibitor Cocktail (100×) - Thermo Fisher Scientific, 78430
- Caspase-1 (human), (recombinant, active) – Enzo, ALX-201-056

Small-molecule inhibitors

- Z-VAD-FMK - AbMole BioScience, M3143
- Emricasan - AbMole BioScience, M5612
- VX-765 - AbMole BioScience, M3532
- CA074-Me - AbMole BioScience, M6236
- Calpeptin - AbMole BioScience, M5167
- E64d - AbMole BioScience, M2376
- CA074 - AbMole BioScience, M6237
- E64 - MedChemExpress, HY-15282
- MCC950 - MedChemExpress, HY-12815A

Dyes, stains & indicators

- Propidium iodide (PI) - Thermo Fisher Scientific, P3566
- NUCLEAR-ID® Red DNA stain - Enzo Life Sciences, ENZ-52406
- Trypan Blue stain (0.4%) - Invitrogen (Thermo Fisher Scientific), T10282
- InstantBlue® Coomassie protein stain - Abcam, ab119211

Protein assays, standards & consumables

- SeeBlue™ Plus2 Pre-Stained Protein Standard - Invitrogen, LC5925
- Bolt™ Bis-Tris Plus Mini Protein Gels, 4-12%, 1.0 mm, WedgeWell™ format - Invitrogen™- NW04125BOX
- BCA Protein Assay Kit – Novagen - TB380
- Water, molecular biology grade - Sigma-Aldrich (Merck), W4502

Antibodies used in Western blotting

- anti-IL-1 β (rabbit) - Cell Signaling Technology, 12703; Primary
- anti-cleaved IL-1 β (rabbit) - Cell Signaling Technology, 83186; Primary
- anti-caspase-1 (rabbit) - Cell Signaling Technology, 3866; Primary
- anti-gasdermin D (rabbit) - Cell Signaling Technology, 97558; Primary
- anti-caspase-3 (rabbit) - Cell Signaling Technology, 9662; Primary
- anti-PARP (rabbit) - Cell Signaling Technology, 9542; Primary

- anti-SUMO-1 (rabbit) - Cell Signaling Technology, 4930; Primary
- anti-IL-18 (rabbit) - Cell Signaling Technology, 54943; Primary
- anti- β -actin (mouse) - Santa Cruz Biotechnology, sc-47778; Primary
- anti-cathepsin B (goat) - R&D Systems, AF953; Primary
- Goat anti-rabbit IgG (goat) - Thermo Fisher Scientific, A32735; Secondary
- Donkey anti-goat IgG (donkey) - Thermo Fisher Scientific, A32930; Secondary

Antibodies used in mass cytometry (CyTOF)

- anti-HMGB1 (mouse) - R&D Systems, MAB1690
- anti-GSDMD (rabbit) - Novus Biologicals, NBP2-80427
- anti-IL-1 β (mouse) - R&D Systems, MAB201
- anti-cleaved IL-1 β (rabbit) - Thermo Fisher Scientific, PA5-105048
- anti-FLIP (mouse) - Novus Biologicals, NBP2-37359
- anti-cleaved caspase-7 (rabbit) - Cell Signaling Technology, 8438
- anti-caspase-5 (rabbit) - Thermo Fisher Scientific, MA5-32356
- anti-cleaved GSDMD (rabbit) - Abcam, ab215203
- anti-IL-18 (mouse) - Novus Biologicals, MAB2548
- anti-NINJ1 (mouse) - Novus Biologicals, MAB51051
- anti-LDH (rabbit) - Novus Biologicals, NBP2-67483
- anti-TNF- α (mouse) - R&D Systems, MAB2101
- anti-cleaved caspase-3 (rabbit) - Cell Signaling Technology, 94530
- anti-NLRP3 (rat) - R&D Systems, MAB7578
- anti-caspase-1 (rabbit) - Novus Biologicals, NBP2-67487
- anti-cleaved PARP (mouse) - Novus Biologicals, NBP2-27335
- anti-PARP (rabbit) - Novus Biologicals, NBP2-67482
- anti-caspase-8 (mouse) - Novus Biologicals, NB100-56527
- anti-GSDME (rabbit) - Novus Biologicals, NBP2-80426
- anti-caspase-3 (mouse) - Novus Biologicals, NB100-56708

4. RESULTS

Several experiments in this PhD thesis were conducted with the assistance of researchers from the Department of Chemical Biology and Bioimaging, Faculty of Chemistry at WUST. Long IQF peptides were synthesized on the Liberty Blue system with the help of Abdulla Al Mamun, MSc. Custom inhibitors, probes and TOF-probes synthesis and kinetic study was performed by Julia Nguyen and Vanessa Pippa. Antibody panel design was performed by Katarzyna Groborz, PhD. Antibody panel conjugation and mass cytometry studies were conducted with the help of Natalia Cwilichowska, MSc. The cleavage sites analysis by LC-MS was performed by Marcin Poręba, PhD, DSc. Thank you, all!

4.1. THE SIGNATURE OF CELL DEATH IN THP-1 CELLS UPON VARIOUS STIMULI

Cell death is a tightly regulated and multifactorial process essential for development, immune defense, and tissue homeostasis. Rather than occurring as a uniform event, it proceeds through distinct genetically encoded programs such as apoptosis, pyroptosis, and necroptosis. Each pathway is executed by specialized molecular machinery and yields characteristic, yet often interconnected, cellular outcomes. Their execution involves networks of proteases, pore-forming proteins, metabolic regulators, and signaling intermediates, which generate overlapping but distinguishable biochemical signatures. Owing to this complexity, detailed molecular characterization is required to discriminate among different forms of cell death. A widely used strategy relies on detecting proteolytic processing events in key regulatory proteins.

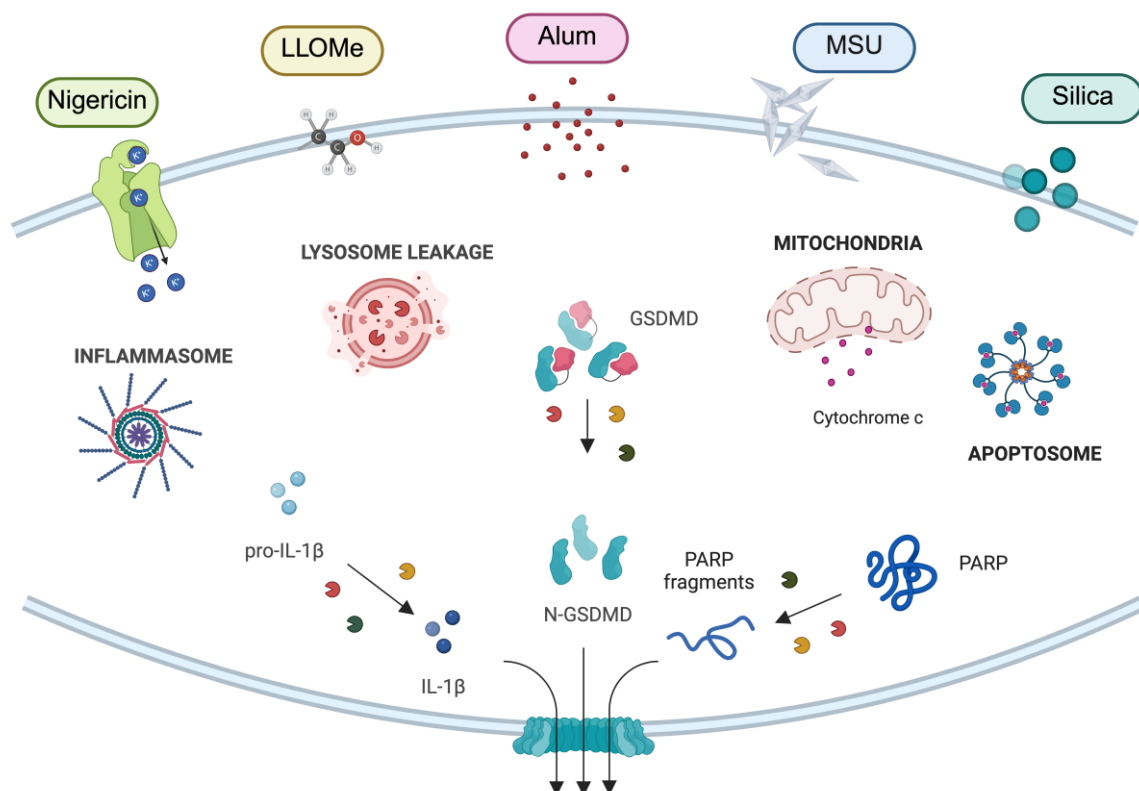


Figure 16. Inflammasome activation and cell death response patterns to various particulate and chemical stimuli. Overview of the experimental setup. PMA-differentiated THP-1 cells ($\pm$ LPS priming) were stimulated with nigericin, LLOMe, alum, MSU, silica, or Val-boroPro to induce cell death. The cleavage signatures of three key proteins, PARP (apoptosis marker), GSDMD, and pro-IL-1 β (pyroptosis markers), were analyzed in culture media.

To investigate the activation patterns of caspase-dependent cell death pathways and inflammasome engagement, PMA-differentiated THP-1 cells (M0 macrophages) were stimulated with a panel of canonical and non-canonical inflammasome activators, including LLOMe, nigericin, alum ($\text{Al}(\text{OH})_3$), silica (SiO_2) and monosodium urate crystals (MSU), in the presence or absence of LPS priming (**Figure 16**). Cleavage patterns of three cell death-related proteins: PARP, GSDMD, and pro-IL-1 β were analyzed. Poly(ADP-ribose) polymerase-1 is a 113-kDa nuclear enzyme that detects DNA strand breaks and catalyzes poly(ADP-ribosylation) to promote DNA repair. Beyond DNA repair, PARP influences transcription, inflammation and neuronal signaling, linking it to many physiological processes [399, 400]. Cleavage of PARP generates specific fragment signatures that serve as biomarkers of apoptotic, necrotic or other death programs. During apoptosis caspases-3/-7 cleave PARP at a canonical DEVD site to produce two hallmark fragments. It was demonstrated that apoptotic cells contain an ~89-kDa C-terminal fragment and a ~24-kDa N-terminal fragment of PARP. The 24-kDa piece comprises the DNA-binding zinc-finger domain and tends to remain bound at DNA breaks, whereas the 89-kDa fragment - which carries the auto-modification and catalytic domains with attached PAR - dissociates from chromatin. This proteolysis inactivates PARP's enzymatic function and is a classic marker of caspase-dependent apoptosis [401]. By contrast, necrotic or harsh stress conditions produce a different PARP cleavage pattern. In necrosis, a dominant ~50-

55-kDa PARP fragment is observed. This zVAD-resistant cleavage is attributable to lysosomal proteases released during necrosis [402]. *In vitro*, lysosomal extracts from necrotic cells, and purified cathepsins B, D and G, cleave PARP into fragments matching those seen in necrotic models. Notably, cathepsin B (and D) cleavage generates ~55-kDa and ~42-kDa C-terminal fragments that retain PARP catalytic activity, along with larger (~62-74 kDa) N-terminal fragments that lack enzymatic function. Importantly, cathepsin cleavage occurs at Leu⁵²⁵ (not an Asp), so that the resulting 55-kDa C-terminal fragment contains the intact catalytic domain, whereas the 62-kDa N-terminal piece is inactive. Thus, caspases and cathepsins yield distinct PARP fragments with different activities. Other proteases also give characteristic patterns: calpains (calcium-activated cysteine proteases) cleave PARP to a ~40-kDa N-terminal fragment, and cytotoxic granzymes cleave at alternative sites (e.g. granzyme A at Lys⁴⁹⁸) to produce ~55-kDa fragments [401]. These distinct cleavage signatures of caspases, cathepsins and other proteases help identify the molecular executioners active in different cell death programs.

Inflammasome activation was long known to involve caspase-1, but the identification of GSDMD established its role as the direct effector of pyroptosis [146, 403]. In seminal studies, caspase-1 and the noncanonical caspases (murine caspase-11 and human caspases-4/5) were shown to cleave GSDMD within its interdomain linker, releasing the ~30 kDa N-terminal pore-forming fragment [146, 404]. The canonical cleavage site lies at Asp²⁷⁵; the motif ²⁷²FLTD²⁷⁵↓G²⁷⁶ (murine ²⁷³LLSD²⁷⁶↓G²⁷⁷); separating the autoinhibitory C-terminal domain. Once freed, the N-terminal fragment oligomerizes and inserts into membranes to assemble large (~18 nm) pores, driving cell lysis and the release of IL-1 family cytokines [405]. Thus GSDMD cleavage is both necessary and sufficient for inflammatory-cell pyroptosis. Beyond inflammatory caspases, apoptotic caspases also process GSDMD under specific conditions. Notably, caspase-8 can directly cleave GSDMD to trigger pyroptosis; for example during *Yersinia* infection or upon TNF/TAK1 blockade [143]. In contrast, caspase-3/7 modify GSDMD in ways that suppress pore formation. Caspase-3/7 cleave GSDMD at Asp⁸⁷, yielding an N-terminal fragment that lacks pore-forming activity [406]. Moreover, during infections that activate caspase-11, co-activation of caspase-3 can cleave the already-active N-terminal GSDMD fragment a second time, producing an inactive ~23 kDa product (p23) and halting pyroptosis [407]. Thus, apoptotic proteases can either switch pyroptosis into apoptosis or actively disable the pore-forming fragment. In neutrophils, non-caspase proteases govern GSDMD activation. For example, neutrophil elastase is released into the cytosol of aging neutrophils and cleaves GSDMD upstream of the canonical site. This elastase-mediated cut produces a fully active N-terminal fragment that oligomerizes and drives neutrophil lytic death (and NETosis) as efficiently as caspase-derived GSDMD^{NT} [408]. Similarly, the neutrophil serine protease cathepsin G cleaves GSDMD at Leu²⁷⁴, just two residues N-terminal to Asp²⁷⁶ (the caspase-1 site), yielding the same ~30 kDa pore-forming fragment [409]. These cleavages by neutrophil proteases underscore that GSDMD can be activated in a caspase-independent manner in certain cell types, contributing to host defense (or inflammation) via pore formation. More recently, it was demonstrated that GSDMD can also be cleaved by calpain-1, a calcium-activated cysteine protease [285]. This cleavage likely occurs at a putative ²³⁷QRTF↓Q²⁴¹ site, located near the canonical caspase-1 cleavage site, potentially supporting the induction of

pyroptosis. In the absence of caspase-1 and under conditions of elevated intracellular calcium, calpain-1 may act as a compensatory protease, enabling pyroptotic-like cell death. These findings identify calpain-1 as a putative noncanonical activator of GSDMD, linking calcium stress to inflammatory cell death through direct GSDMD processing. Another example is cathepsin S. Under conditions of lysosomal disruption, such as reactive oxygen stress induced by antibody, dye conjugates in tumor cells, cathepsin S is released into the cytosol and cleaves GSDMD, as well as the pro-forms of IL-1 β and IL-18, thereby inducing pyroptosis independently of caspase-1 [410]. This work sheds new light on the role of lysosomal cathepsins in supporting the pyroptotic process, revealing alternative proteolytic routes to inflammation beyond classical inflammasome signaling. Since its discovery, caspase-1 has been recognized as the primary protease responsible for GSDMD cleavage and remains the central regulator of pyroptotic cell death. However, accumulating evidence has shown that several other proteases can also directly cleave GSDMD. Some of these, such as calpain-1 and cathepsin S, provide alternative, noncanonical mechanisms to activate pyroptosis and inflammatory signaling, particularly under conditions where caspase-1 is absent or inactive. Others, including caspase-3 and certain apoptotic proteases, cleave GSDMD at distinct sites to inactivate its pore-forming activity, thereby acting as regulatory checkpoints to suppress or fine-tune pyroptosis. Together, these findings highlight the complexity of GSDMD regulation and underscore the existence of multiple proteolytic routes that either promote or restrain pyroptotic cell death depending on the cellular context.

Nigericin stimulation triggered dual activation of caspase-1 and caspase-3, as demonstrated by the appearance of both GSDMD p31 and p45 fragments (**Figure 17**). This response occurred regardless of LPS priming, although IL-1 β maturation was strongly enhanced in LPS-primed cells. What is more, PARP was processed into ~89 kDa and a ~24 kDa fragments, most likely by executioner caspases. These results confirmed that nigericin alone can activate inflammasome signaling, but transcriptional priming is required for effective cytokine output. In contrast, stimulation with LLOMe alone led primarily to caspase-3 activation, as evidenced by the generation of the GSDMD p45 fragment in the absence of mature IL-1 β production (**Figure 17**). Notably, PARP cleavage into p55 and p42 fragments, consistent with lysosomal stress, was also detected under these conditions indicating the release of active cysteine cathepsins into the cytosol. Upon LPS priming, concurrent activation of both caspase-1 and caspase-3 was observed, indicated by the presence of both GSDMD p31 and p45 fragments and robust IL-1 β maturation. Thanks to the enhanced transcription of inflammasome-related genes induced by LPS priming, a redirection towards pyroptotic cell death was observed. This might indicate that some of cathepsins role in this setting might be obscured. In contrast, alum stimulation resulted in weak but selective caspase-1 activation (GSDMD p31) and IL-1 β maturation, with no evidence of caspase-3 activity (absence of GSDMD p45 and PARP p89) (**Figure 17**). Aluminum salts (alum) are widely used vaccine adjuvants, yet their precise mechanisms of action in vivo remain incompletely understood [411]. Alum has been shown to activate the NLRP3 inflammasome, primarily through lysosomal destabilization [250]. It is proposed that many adjuvants, including alum, engage damage-associated molecular patterns and cell death pathways to promote robust immune responses [412]. Specifically, alum-mediated NLRP3 activation drives the caspase-1-dependent cleavage of pro-IL-1 β and pro-IL-

18, generating their biologically active forms. Mechanistically, the phagocytosis of alum, MSU, or silica crystals induces lysosomal destabilization and rupture, which in turn triggers NLRP3 inflammasome activation [250, 413, 414], what is in line with obtained data. Interestingly, distinct non-canonical PARP cleavage products (~50 kDa and 30-35 kDa) were detected, likely produced by cathepsins released upon lysosomal disruption, indicating a caspase-independent component of cell death. A similar pattern was observed following SiO₂ stimulation, which also led to caspase-1-dependent GSDMD p31 cleavage and IL-1 β production (with LPS priming), but no activation of caspase-3 (**Figure 17**). Silica nanoparticles (SNs) are widely employed in industrial, environmental, and biomedical applications; however, their ability to persist and accumulate within organisms raises serious safety concerns [415].

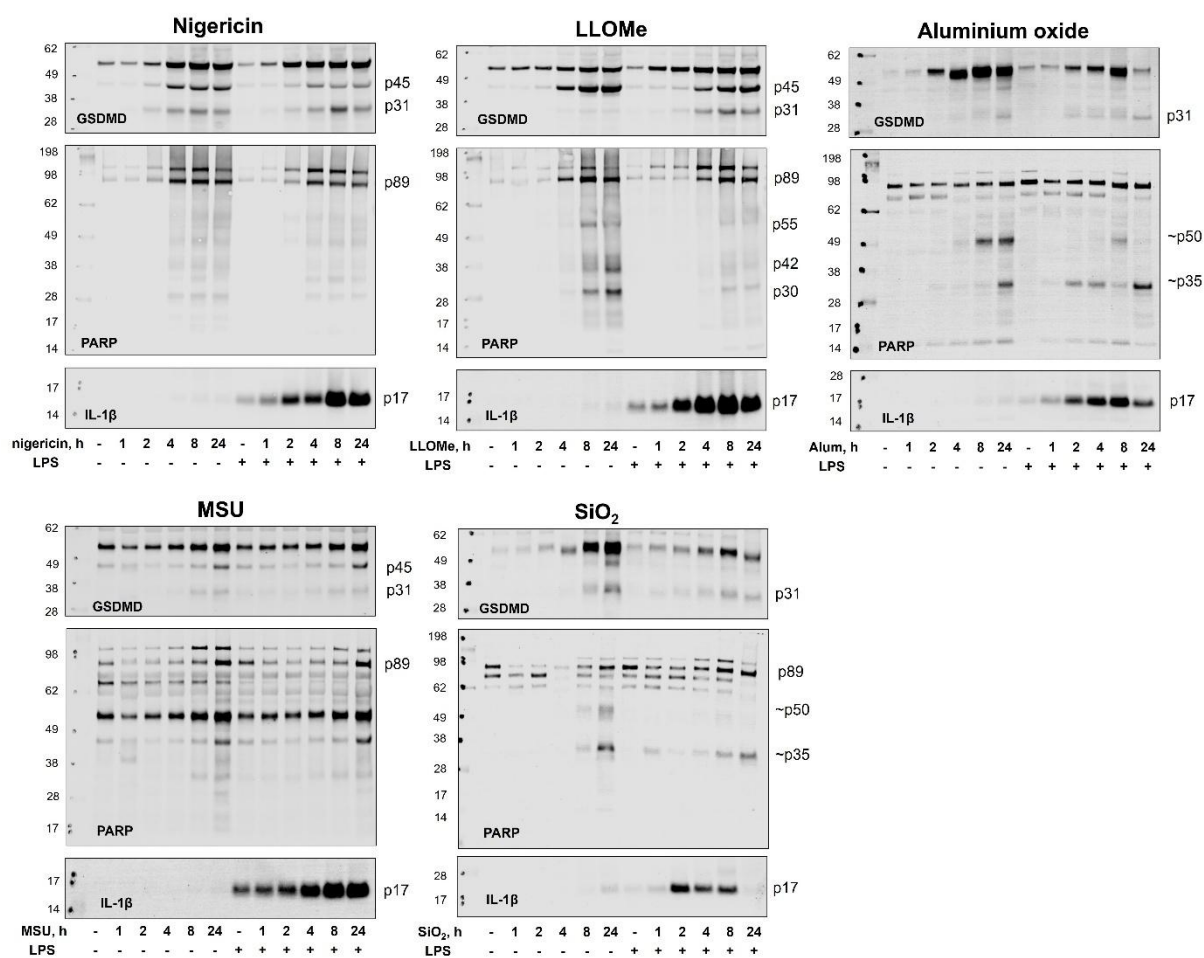


Figure 17. Cell death signatures in response to various particulate and chemical stimuli. Western blot analysis of culture supernatants showing cleavage patterns of three key cell death-related proteins were assessed: PARP (apoptotic marker), GSDMD (pyroptotic pore-forming effector), and pro-IL-1 β (inflammasome-dependent cytokine precursor), following stimulation with NLRP3 activators, applied with or without prior LPS priming, in PMA-differentiated wild-type THP-1 cells.

Both chronic low-dose exposure and acute high-dose exposure to SNs have been shown to cause systemic toxicity and organ damage [416, 417]. Crystalline silica is one of the best-characterized particulate activators of the NLRP3 inflammasome. Upon cellular uptake, it cause

lysosomal destabilization and release of cathepsins, and NLRP3 activation, promoting the cleavage of pro-caspase-1 into its active form and driving the maturation and secretion of the pro-inflammatory cytokines IL-1 β and IL-18 [418]. The role of CatB in contributing to activate NLRP3 was tested by numerous groups and the existing research remains inconclusive with evidence for cathepsin involvement [218, 419, 420] and evidence against cathepsin involvement [360, 421-423]. As with alum, silica/SiO₂ induced non-canonical PARP cleavage patterns, consistent with lysosomal protease activity. Interestingly, in both cases, a non-canonical GSDMD fragment of approximately 50 kDa was observed, suggesting a trimming event rather than cleavage within the interdomain linker of the protein. Next, MSU crystals elicited a strong caspase-3-dominant response, as evidenced by prominent GSDMD p45 and PARP p89 cleavage, with only modest caspase-1 activation (GSDMD p31) (**Figure 17**). MSU crystals, among the most common particulate activators of the second signal, promote NLRP3 inflammasome formation through NLRP3-ASC binding and pro-caspase-1 recruitment. Their uptake by macrophages induces lysosomal damage, reactive oxygen species generation, and potassium efflux, all of which converge to initiate NLRP3 oligomerization and downstream activation [424]. Even though it is the main observed mechanism of action, literature describes also signs of apoptosis caused by MSU treatment [425]. Some studies suggest that oxidative stress-induced mitochondrial apoptosis may facilitate NLRP3 inflammasome activation [426]. IL-1 β maturation was observed only upon LPS priming. This contrasts with SiO₂, despite both being particulate stimuli known to damage lysosomes, suggesting that MSU preferentially triggers apoptotic caspase pathways, whereas SiO₂ favors classical inflammasome-driven pyroptosis.

To conclude, pyroptotic cell death can be triggered by the ionophore nigericin, which activates downstream effector proteins such as PARP, GSDMD, and pro-IL-1 β , in a manner characteristic of caspase activity. During this process, GSDMD is cleaved by caspase-1 into its p30 fragment, which oligomerizes to form membrane pores, marking the execution phase of pyroptosis. Alternatively, GSDMD can be cleaved into a p45 fragment by caspase-3, reflecting engagement of the apoptotic pathway. Concomitant detection of PARP cleavage products provides further evidence for the type of cell death taking place: the appearance of the p89 PARP fragment is a hallmark of caspase-3 activation and apoptotic initiation. Collectively, these findings indicate that nigericin is capable of triggering both apoptosis and pyroptosis in parallel. When cells are exposed to lysosomotropic agents such as LLnMe, or to particulate danger signals including alum [Al(OH)₃], silica (SiO₂), and monosodium urate (MSU) crystals, the proteolytic pattern broadens. In addition to apoptotic PARP fragments (p89), additional cleavage products emerge that are consistent with lysosomal cathepsin activity, suggesting crosstalk between lysosomal damage pathways and caspase signaling. Interestingly, LPS priming alters this balance: under LPS stimulation, these alternative cleavage events are suppressed, and the characteristic caspase cleavage pattern dominates. In the absence of LPS, however, sterile inflammatory stimuli favor caspase-3 activation, as evidenced by the appearance of the GSDMD p45 fragment and the absence of mature IL-1 β . Conversely, LPS priming ensures robust processing of pro-IL-1 β regardless of the upstream trigger, reinforcing the importance of priming in directing inflammasome-driven cell death outcomes.

4.2. CELL DEATH MACHINERY IN THP-1 CELLS LACKING CASPASE-1 OR GSDMD

Following the identification of stimulus-specific cleavage patterns, the contribution of caspase-1 and GSDMD to the execution of cell death was investigated. To this end, THP-1 cells deficient in caspase-1 or GSDMD were employed. To test whether LMP triggers pyroptosis, differentiated cells were stimulated with LLOMe while monitoring propidium iodide (PI) uptake, a common assay for membrane integrity. PI is excluded from viable cells but penetrates cells with compromised membranes, where it intercalates with DNA and emits red fluorescence, thus enabling quantitative detection of dead cells. Using this approach, LLOMe induced cell death was observed, however, this process was independent of caspase-1 and GSDMD, suggesting a non-canonical mechanism of death, potentially linked to lysosomal cathepsin activity. To compare this with a canonical inflammasome stimulus, cells were treated with 3 μ M nigericin, a well-established NLRP3 activator. In contrast to LLOMe, nigericin-induced cell death was markedly delayed and reduced in caspase-1 or GSDMD-deficient cells, confirming the reliance of this pathway on canonical inflammasome activation and pyroptosis (Figure 18).

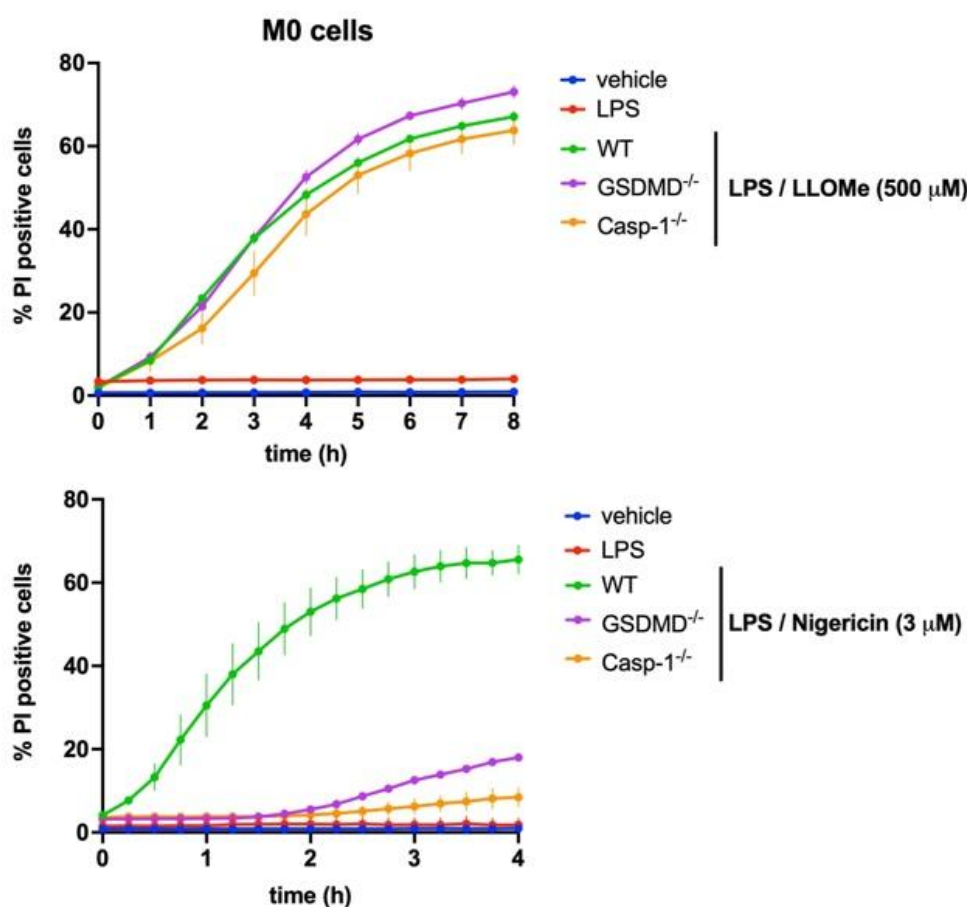


Figure 18. Real-time Incucyte analysis of cell death dynamics in PMA differentiated WT, caspase-1-deficient, and GSDMD-deficient cells exposed to LPS/LLOMe or LPS/nigericin.

Western blotting further substantiated these findings. In WT THP-1 cells, nigericin stimulation produced cleavage patterns characteristic of caspase-1-mediated pyroptosis, evidenced by the appearance of the GSDMD p31 fragment and robust release of mature IL-1 β (**Figure 17**). In contrast, in GSDMD knockout (GSDMD KO) and caspase-1 knockout (Casp-1 KO) cells, this lytic death phenotype was absent (**Figure 19**); instead, caspase-3 activation was detected, as indicated by the GSDMD p45 fragment and a non-canonical PARP cleavage pattern (**Figure 19**). These observations suggest a switch from pyroptotic to apoptotic signaling when the canonical inflammasome effectors are absent. Interestingly, pro-IL-1 β was still cleaved and released into the supernatant even in the absence of caspase-1 (**Figure 19**), implying that alternative proteases may contribute to IL-1 β maturation under certain conditions. Moreover, nigericin treatment of GSDMD KO cells resulted in both caspase-1 and caspase-3 activation, leading to detectable IL-1 β maturation and release, along with a more canonical PARP cleavage pattern (**Figure 19**). This indicates that even without GSDMD-mediated pore formation, compensatory apoptotic pathways remain engaged. LLome treatment produced a comparable pattern of responses (**Figure 20**). In caspase-1-deficient THP-1 cells, caspase-3 was activated, as reflected by PARP cleavage and the generation of the inhibitory p45 fragment of GSDMD (**Figure 20**). By contrast, the characteristic cathepsin-driven non-canonical PARP cleavage was less evident, and the efficiency of IL-1 β processing and secretion was strongly diminished. In cells lacking GSDMD, cytokine release still occurred (**Figure 20**), though at lower levels than in WT controls (**Figure 17**), indicating that IL-1 β can be liberated even in the absence of canonical GSDMD pore formation. Overall, these data show that PMA-differentiated THP-1 cells are not strictly reliant on caspase-1 or GSDMD to undergo lytic demise. Instead, lysosomal membrane permeabilization and subsequent cathepsin activity appear to provide an alternative route of cell death.

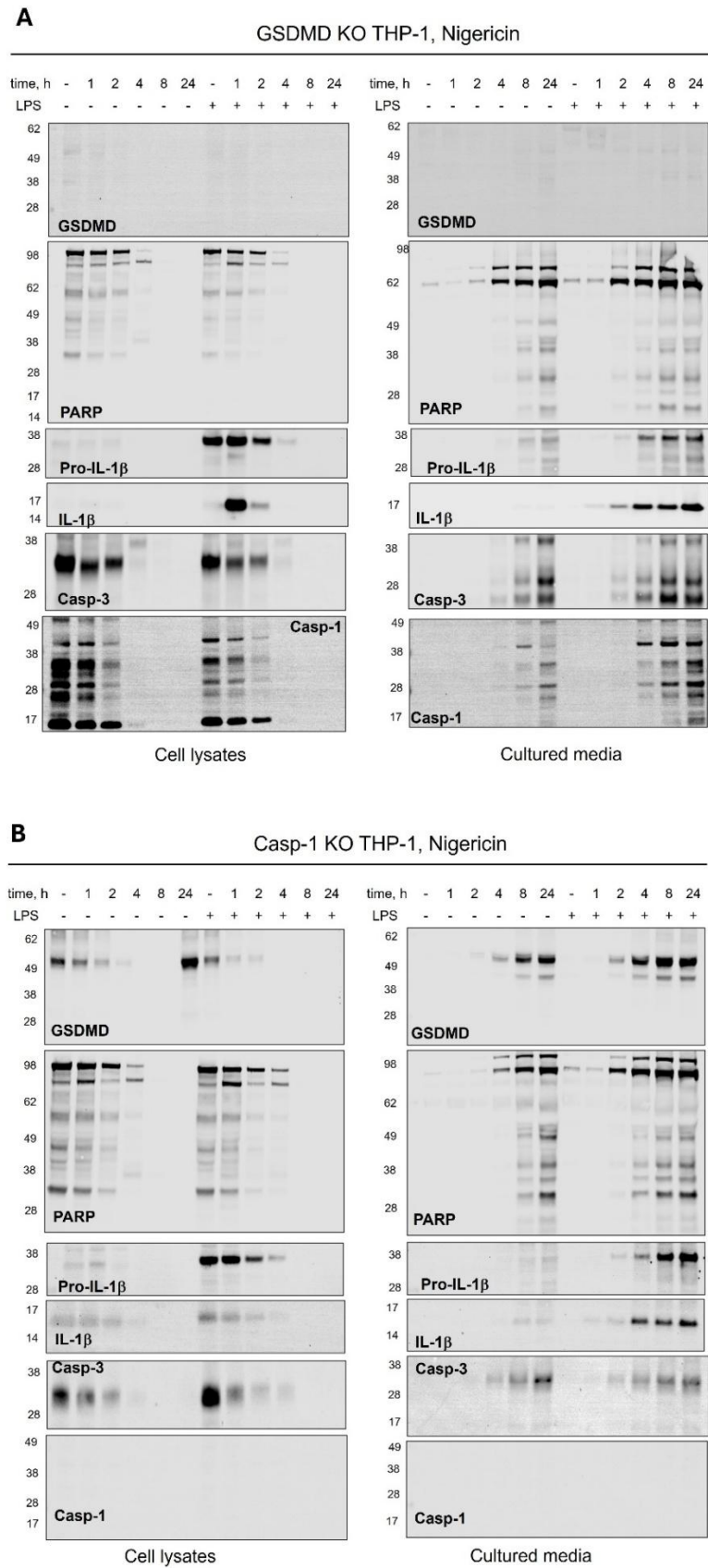


Figure 19. Nigericin induced cell death in Casp-1 and GSDMD knockout cells. Western blot analysis showing the processing patterns of cell-death related proteins in response to nigericin (25μM) in **A** GSDMD KO and **B** caspase-1 KO M0 macrophages over time.

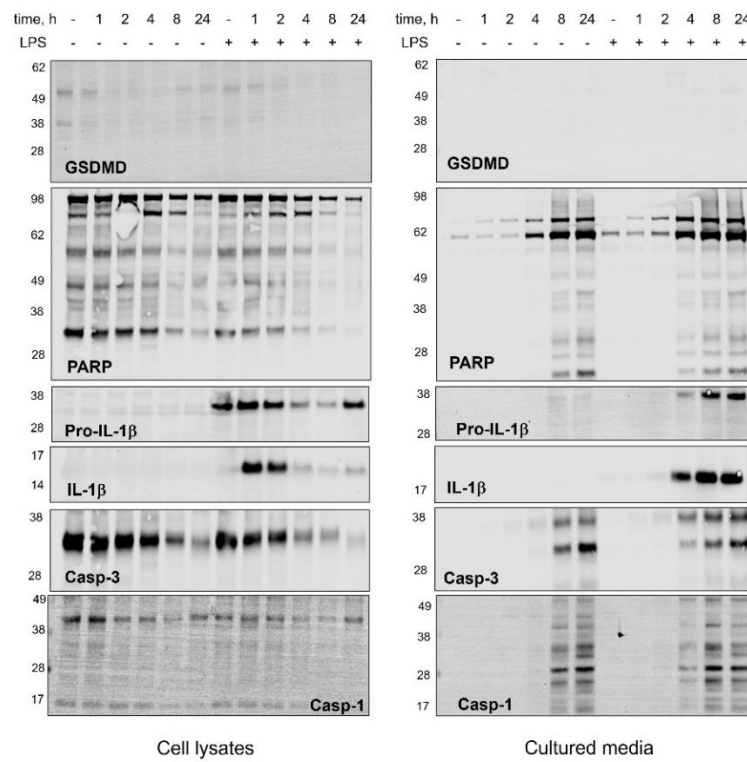
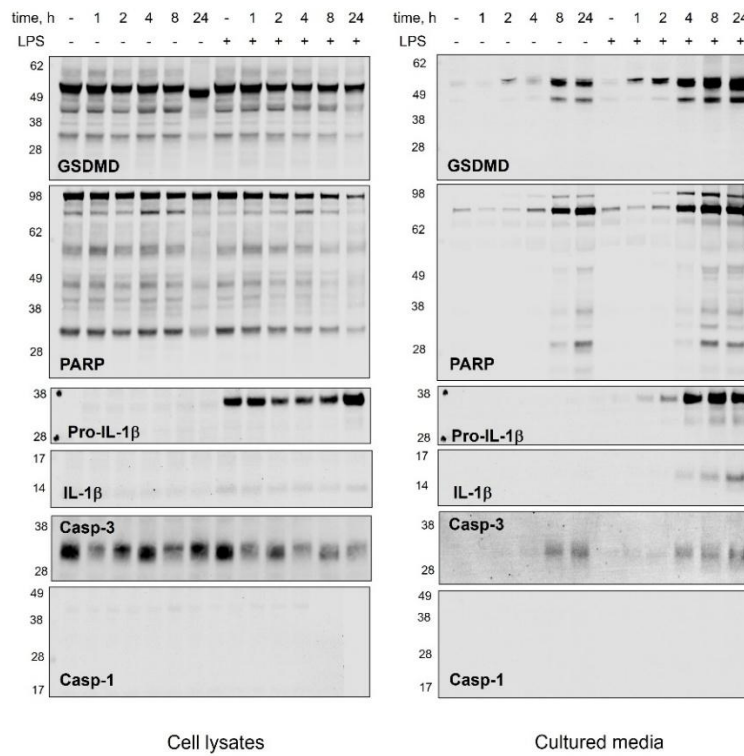
A**GSDMD KO THP-1, LLOMe****B****Casp-1 KO THP-1, LLOMe**

Figure 20. LLOMe stimulated cell death in Casp-1 and GSDMD knockout cells. Western blot analysis showing the processing patterns of cell-death related proteins in response to LLOMe (500 μ M) in **A** GSDMD KO and **B** caspase-1 KO M0 macrophages over time.

4.3. COMPARISON OF EXECUTION OF CELL DEATH RESULTING FROM CANONICAL PYROPTOSIS AND STERILE INFLAMMATION FACTOR

To gain deeper insight into the proteolytic events associated with distinct cell death modalities, we analyzed proteins linked to pyroptosis (Casp-1, GSDMD and IL-1b) and apoptosis (PARP and Casp-3) in both cell lysates and corresponding supernatants. Following stimulation with LLOMe, GSDMD was cleaved into its p45 fragment, a pattern typically attributed to caspase-3 activity, accompanied by a faint signal corresponding to the pyroptotic p30 fragment. This suggests that under sterile, lysosomal stress conditions, caspase-3 exerts dominant control over GSDMD processing. Notably, upon LPS priming, the generation of the p30 fragment was markedly enhanced, indicating that priming not only licenses inflammasome activity but also shifts the proteolytic balance toward GSDMD cleavage that promotes inflammation. Under these conditions, the release of the p20 subunit of caspase-1 into the extracellular supernatant was also detected (**Figure 21**).

Stimulation with 25 μ M nigericin, a canonical NLRP3 agonist, produced robust p30 formation regardless of prior LPS priming. However, the release of caspase-1 was relatively weak and only became more pronounced at longer incubation times. This delayed appearance of caspase-1 fragments suggests that nigericin may also engage secondary proteolytic mechanisms that contribute to GSDMD processing, highlighting the complexity of the pathway (**Figure 21**). This effect was particularly evident when cells were exposed to different concentrations of nigericin (3 μ M vs. 25 μ M) (**Figure 22**). At the lower concentration, cell death progressed more gradually, whereas the higher dose triggered a rapid and irreversible loss of viability, suggesting an immediate and overwhelming activation of lytic pathways. Such high-dose stimulation may bypass tightly regulated inflammasome signaling and instead engage additional, alternative proteolytic mechanisms that accelerate membrane rupture and cell demise.

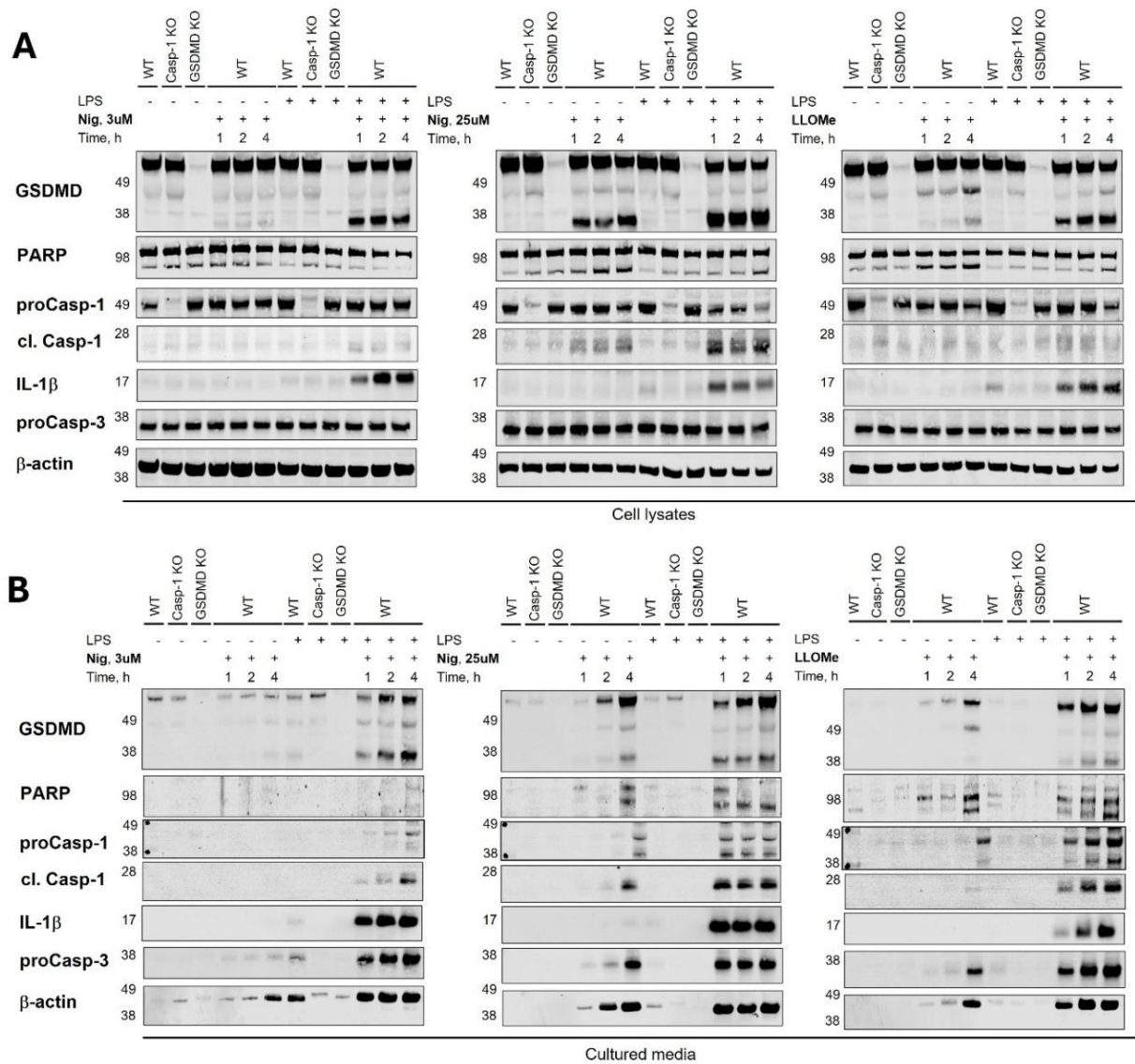


Figure 21. Western blot analysis of proteolytic signatures associated with LLOMe- and nigericin-induced cell death. PMA-differentiated THP-1 cells were either primed or not with LPS, followed by treatment with cell death inducer nigericin (3 μ M or 25 μ M) or LLOMe (500 μ M). At the indicated time points, both cells and culture supernatants were collected and analyzed by Western blotting. A cell lysates B proteins precipitated from culture medium

Across all three models tested, full-length caspase-3 was consistently detected in the supernatant of LPS-primed cells, suggesting that priming not only modulates inflammasome activation but also alters apoptotic signaling. The extracellular release of intact caspase-3, which is typically a cytosolic enzyme, indicates extensive plasma membrane rupture characteristic of lytic forms of cell death. Taken together, these findings demonstrate that LPS priming functions as a critical checkpoint, redirecting cell fate away from a predominantly apoptotic signature toward a pro-inflammatory outcome characterized by caspase-1 activation, GSDMD p30 processing, and robust release of cytosolic proteins, including apoptotic caspase zymogens.

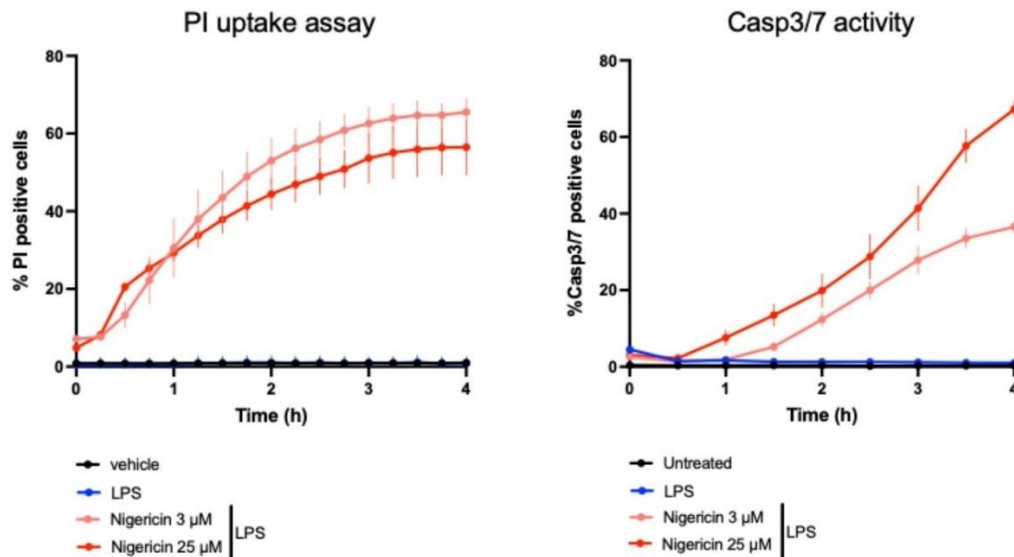


Figure 22. Comparison of cell death kinetics prompted by low and high concentration of nigericin. WT PMA differentiated THP-1 cells were treated with 3 μ M nigericin and 25 μ M nigericin ($\pm$ LPS priming) over time. Cell death was monitored by PI uptake assay and caspase3/7 activity assay.

4.4. CYSTEINE CATHEPSINS DIRECTLY CLEAVE GSDMD AND PRO-IL-1 β

The classical view of cathepsins as proteases restricted to bulk protein turnover within lysosomes has been significantly revised in recent years. It is now well established that these enzymes are not redundant players but rather active regulators of multiple cell death programs. Cathepsins participate in the control of inflammasome activation, particularly in the NLRP3–caspase-1-GSDMD axis, where they act upstream of caspase activation. Among them, cathepsin B (CatB) has been shown to physically interact with NLRP3 and is required for efficient inflammasome assembly and IL-1 β maturation in macrophages [358, 427]. Beyond this canonical pathway, several cathepsins have been implicated in particle-induced cytotoxicity and IL-1 β release independently of inflammasome sensors or caspase-1, suggesting alternative, inflammasome-independent routes of cytokine regulation [259]. Extracellular cathepsin S (CatS) has also been proposed as a sensitive biomarker of lysosomal destabilization, as its release strongly correlates with particulate exposure and subsequent caspase-1 activation [428]. Importantly, evidence of direct substrate cleavage has begun to emerge. While neutrophil serine proteases (NSPs) such as neutrophil elastase (NE), proteinase-3 (PR3), cathepsin G (CatG), and Neutrophil Serine Protease 4 (NSP4) were long recognized for their dual roles in antimicrobial defense and regulation of inflammation, CatG was the first protease demonstrated to directly process GSDMD into its pore-forming fragment in neutrophils and monocytes [409]. This finding expanded the concept that proteolytic enzymes beyond caspases can directly trigger pyroptosis. NSPs, localized in specialized secretory lysosomes, not only degrade microbial proteins but also modulate the inflammatory response through selective cleavage of cytokines, chemokines, opsonins, and receptors. Whether additional cathepsins can directly process GSDMD or cytokine precursors has remained an open question. Recent studies by Chen

et al. provided the first evidence that CatS can cleave not only GSDMD but also pro-IL-1 β , leading to its activation independently of caspase-1 [364]. Intriguingly, CatS was also reported to process pro-IL-18, pointing to a broader role in cytokine maturation. These findings challenge the previous assumption that cathepsins only indirectly modulate cytokine release through inflammasome regulation (as shown for CatB), and instead suggest that cathepsins may act as direct executioners in pyroptotic signaling. However, to date, direct cleavage of pro-inflammatory proteins by other cathepsins, including CatB, CatL, or CatV, has not been demonstrated, leaving an important gap in our understanding of how lysosomal proteases shape inflammatory cell death. To address this knowledge gap, recombinant human cathepsins B, L, V, and S were incubated with recombinant human pro-IL-1 β and pro-IL-18 at pH 5.5. Proteolysis was assessed using two complementary approaches: Western blotting with anti-IL-1 β and anti-IL-18 antibodies to detect specific cleavage products (**Figure 23**), and SDS-PAGE followed by InstantBlue® Coomassie staining to visualize the complete spectrum of protein fragments and exclude the possibility of missing products. Cathepsin B generated only a single fragment with a molecular mass slightly lower than that of the pro-IL-1 β precursor, indicating that under these conditions CatB did not promote cytokine activation (**Figure 23**). In contrast, cathepsin L exhibited strong proteolytic activity toward both substrates, but the pattern suggested extensive degradation rather than specific maturation, as only a fraction of pro-IL-1 β was converted into the ~17 kDa active form. Cathepsins S and V progressively cleaved pro-IL-18 in a concentration-dependent manner, while pro-IL-1 β appeared to undergo non-specific degradation at the same conditions. Overall, these findings indicate that cathepsins are capable of processing pro-inflammatory cytokines, but the outcome is context-dependent, with substrate activation or degradation influenced by enzyme concentration and specificity.

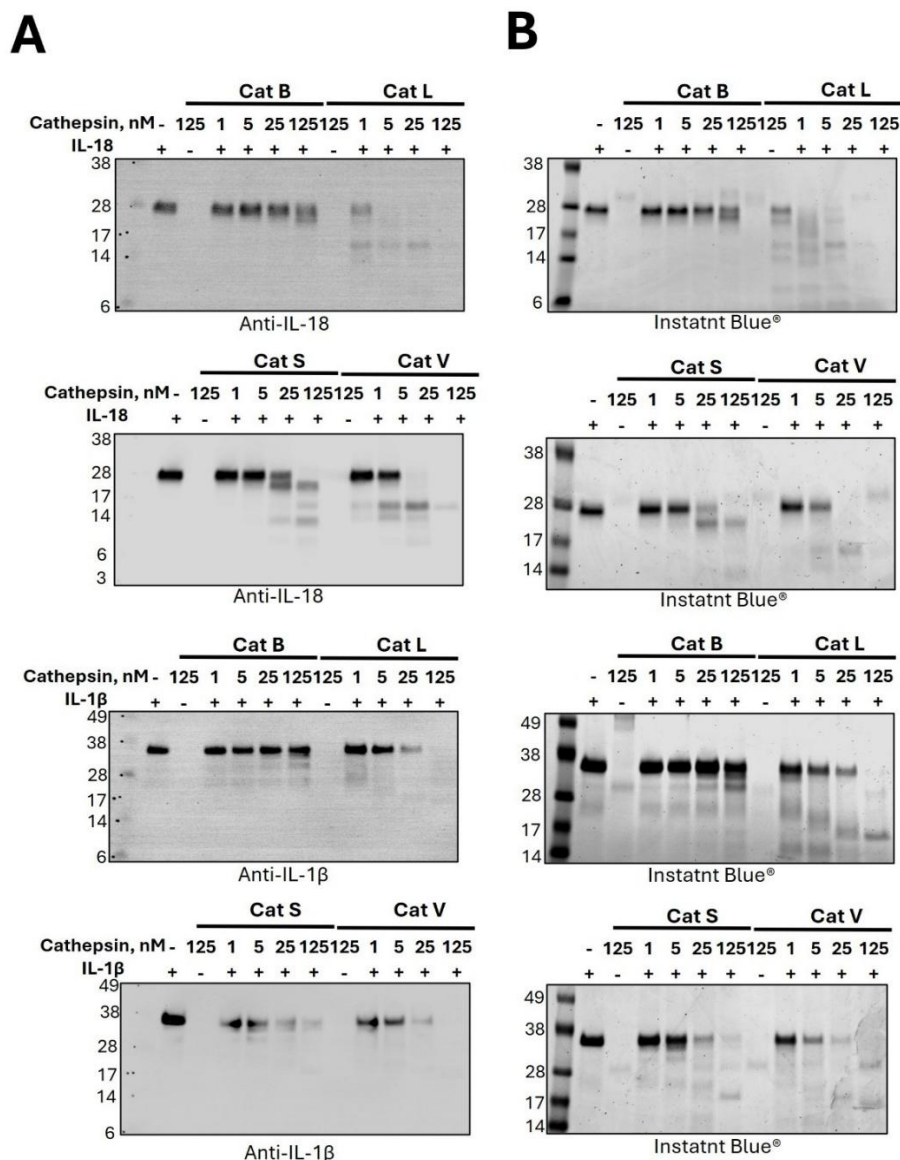


Figure 23. Cathepsins process pro-IL-1 β and IL-18 at pH 5.5. **A** Western blot analysis of pro-IL-1 β and -18 processing by human cysteine cathepsins B, L, S and V at pH 5.5 in concentration dependent manner. **A** Cleavage products were visualized using an anti-IL-1 β and anti-IL-18 antibody depending on interleukin used. **B** SDS-PAGE analysis under the same pH conditions and concentration gradient. For visualization of substrate processing InstantBlue® Coomassie Protein Stain was used.

To investigate this further, recombinant human cathepsins B, L, V, and S were incubated with recombinant human pro-IL-1 β at pH 5.5 and pH 7.2 (**Figure 24**). Recombinant caspase-1 was included as a positive control for cytokine maturation. Cathepsin B displayed a gradual, concentration-dependent cleavage of pro-IL-1 β at pH 5.5, although this activity was markedly diminished at neutral pH. In contrast, the remaining cathepsins exhibited stronger proteolytic capacity. Cathepsin S efficiently processed pro-IL-1 β under both conditions; however, the generation of mature IL-1 β was more evident at pH 5.5, while at pH 7.2 the substrate was primarily subjected to non-specific degradation. Cathepsin L and its closely related homolog cathepsin V (also referred to as cathepsin L2) produced cleavage fragments comparable in size

to those generated by caspase-1, with cathepsin V demonstrating greater efficiency under both pH conditions.

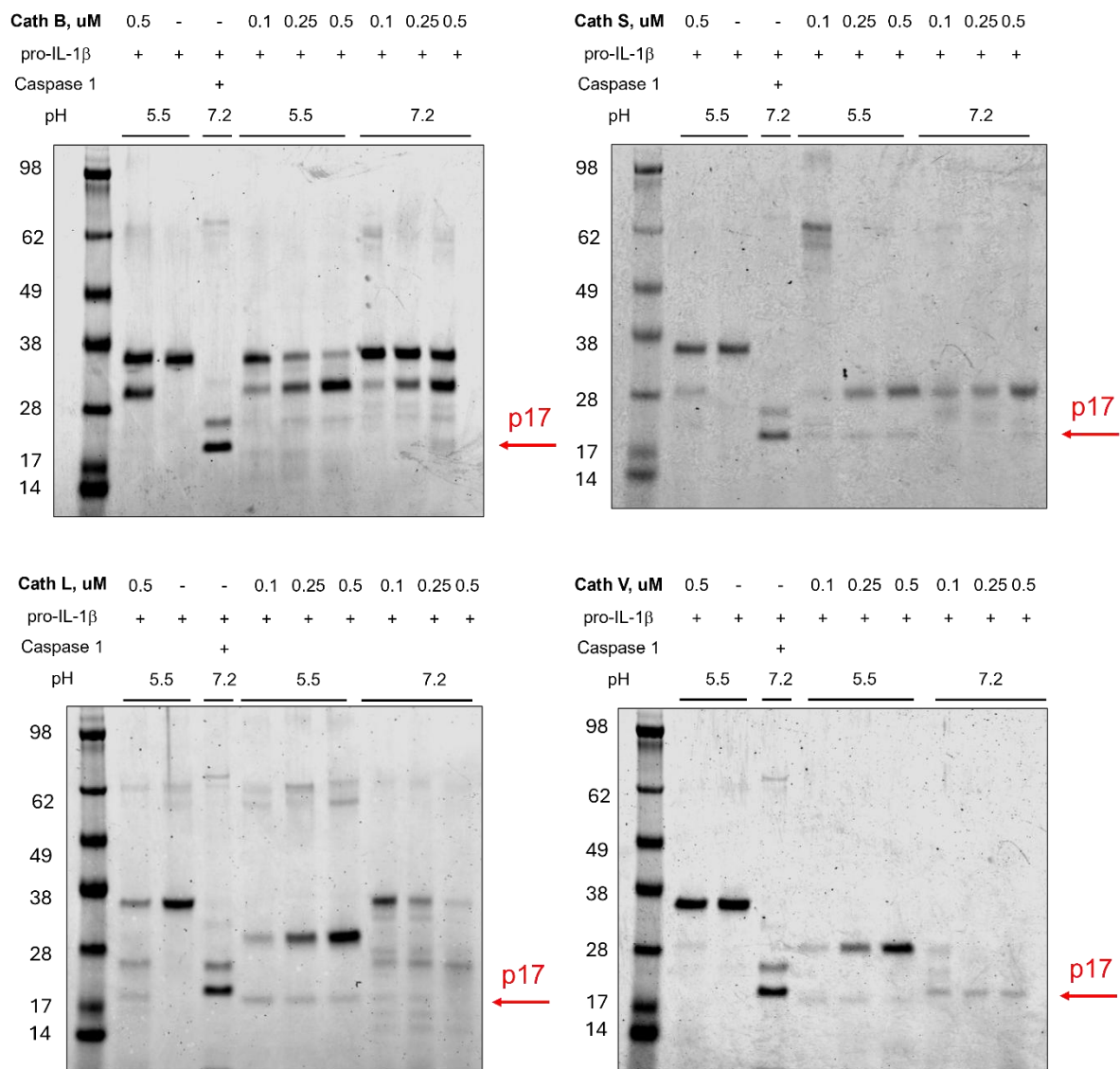


Figure 24. Cysteine cathepsins cleave pro-IL-1 β under both acidic and neutral pH conditions. Western blot analysis of pro-IL-1 β cleavage by human cathepsins B, L, S, and V at pH 5.5 and 7.2 in a concentration dependent manner. Recombinant human caspase-1 was used as a positive control. Cleavage products were visualized using an anti-IL-1 β antibody. The red arrow labeled “p17” indicates a mature IL-1 β fragment corresponding in size to that generated by caspase-1.

As a next step, the ability of cathepsins to process GSDMD was assessed. Recombinant human cathepsins B, L, V, and S were incubated with recombinant human SUMO-GSDMD at pH 5.5, and the resulting products were analyzed by Western blotting using either anti-GSDMD (Figure 25) or anti-SUMO antibodies. To capture all potential cleavage products, SDS-PAGE followed by InstantBlue® Coomassie staining was also performed. Cathepsin B cleaved SUMO-GSDMD only at the highest concentration tested, whereas cathepsin L was active at lower concentrations but caused extensive, non-specific degradation of GSDMD at higher

levels (**Figure 25**). Cathepsins S and V predominantly mediated non-specific degradation of GSDMD under these conditions. Similarly, in low concentration tested in time Cathepsin V, B, L and S showed degrading activity (**Figure 26**) since no cleavage products were visible in time tested. Interestingly, when cathepsins B and S were tested at neutral pH (7.5), both enzymes exhibited concentration-dependent proteolytic activity (**Figure 27**). Cathepsin S appeared more active under these conditions than cathepsin B, generating specific cleavage products at lower concentrations, while at higher concentrations the processing shifted toward more extensive degradation. In contrast, cathepsin B displayed only weak, specific cleavage activity, detectable primarily at the highest enzyme concentration tested. These findings suggest that cathepsin S retains a broader functional activity window at near-physiological pH compared to cathepsin B, which is more restricted in its neutral pH activity. This difference may reflect their distinct physiological adaptations, with cathepsin S being well-documented for its stability and activity in less acidic environments, particularly in antigen-presenting cells, whereas cathepsin B functions optimally in acidic compartments [429-432]. The observed concentration-dependent switch from specific processing to degradation further implies that substrate fate may be tightly influenced by local protease abundance and microenvironmental pH. The processing of untagged GSDMD by recombinant cathepsins was next assessed at both acidic and near-neutral pH, using higher enzyme concentrations to enhance detection of cleavage events (**Figure 28**). Caspase-4 served as a positive control and generated the canonical p31 pyroptotic fragment, confirming assay validity. Cathepsin B cleaved GSDMD to yield a distinct p40 fragment, with cleavage efficiency enhanced under acidic conditions, consistent with its lysosomal activity profile. In contrast, cathepsins L and V cleaved GSDMD at both pH conditions, with only a modest reduction in activity at neutral pH. However, the resulting banding patterns reflected progressive degradation rather than the generation of discrete pyroptotic fragments. Cathepsin S exhibited robust proteolytic activity toward GSDMD, producing widespread cleavage as well as fragments of approximately 35 kDa under both acidic and near-neutral conditions, suggesting that it may contribute to GSDMD activation. These results suggest that cathepsin B retains limited substrate specificity toward GSDMD, whereas cathepsins L and V primarily promote non-specific degradation. This functional divergence may reflect differences in physiological adaptation, with cathepsin B contributing to regulated GSDMD processing under acidic conditions, while cathepsins L and V favor substrate turnover that could restrict GSDMD-mediated pyroptosis. Following the analysis of GSDMD cleavage using recombinant proteins, processing of endogenous GSDMD was examined in cell lysates (**Figure 29**) to determine whether the observed proteolytic patterns could be recapitulated in a cellular context. Two cell lines were selected: HEK-293T cells engineered to overexpress GSDMD, providing a robust system for detecting cleavage products, and wild-type THP-1 monocytes, representing a physiologically relevant immune cell model. In lysates from both cell lines, GSDMD was readily detectable, and protein levels were sufficient to evaluate potential proteolytic processing, enabling direct comparison with the recombinant protein data.

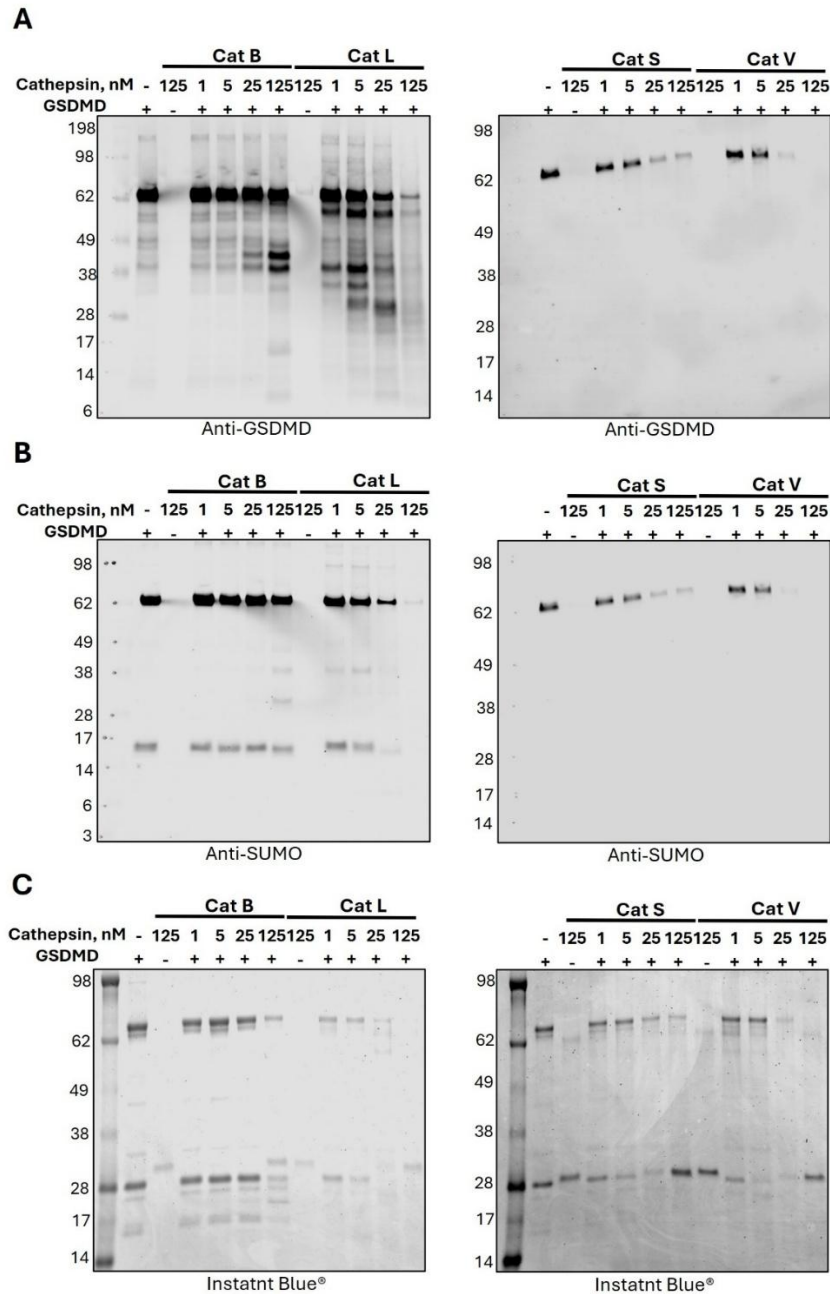


Figure 25. Western blot analysis of GSDMD processing by human cysteine cathepsins B, L, S and V at pH 5.5 in concentration dependent manner. A Cleavage products were visualized using an anti-GSDMD antibody. B Cleavage products were visualized using an anti-SUMO antibody. C. SDS-PAGE analysis under the same pH conditions and concentration gradient. For visualization of GSDMD processing InstantBlue® Coomassie Protein Stain was used.

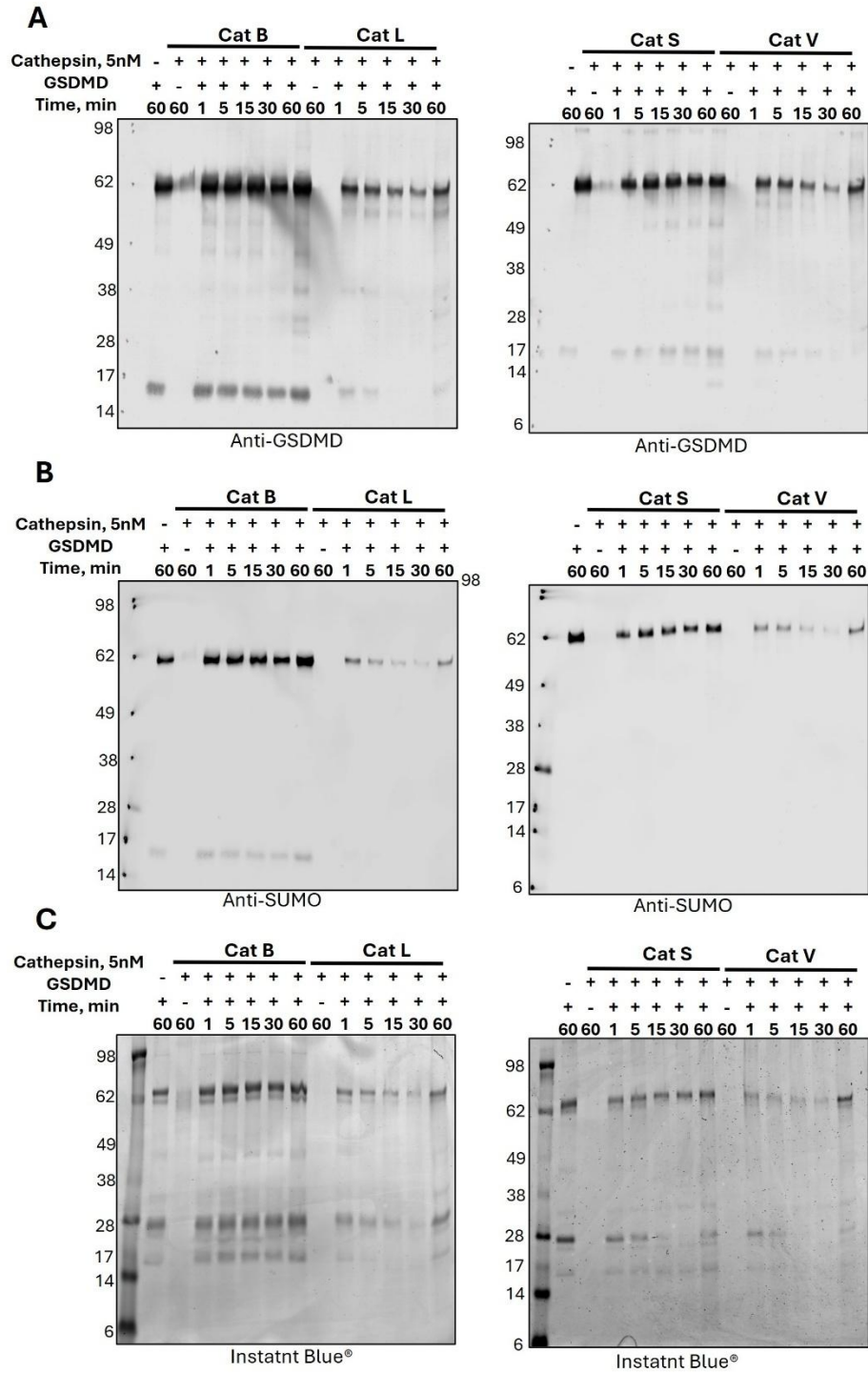


Figure 26. Western blot analysis of GSDMD processing by human cysteine cathepsins B, L, S and V at pH 5.5 in time dependent manner. **A** Cleavage products were visualized using an anti-GSDMD antibody. **B** Cleavage products were visualized using an anti-SUMO antibody. **C** SDS-PAGE analysis under the same pH conditions. For visualization of GSDMD processing InstantBlue® Coomassie Protein Stain was used.

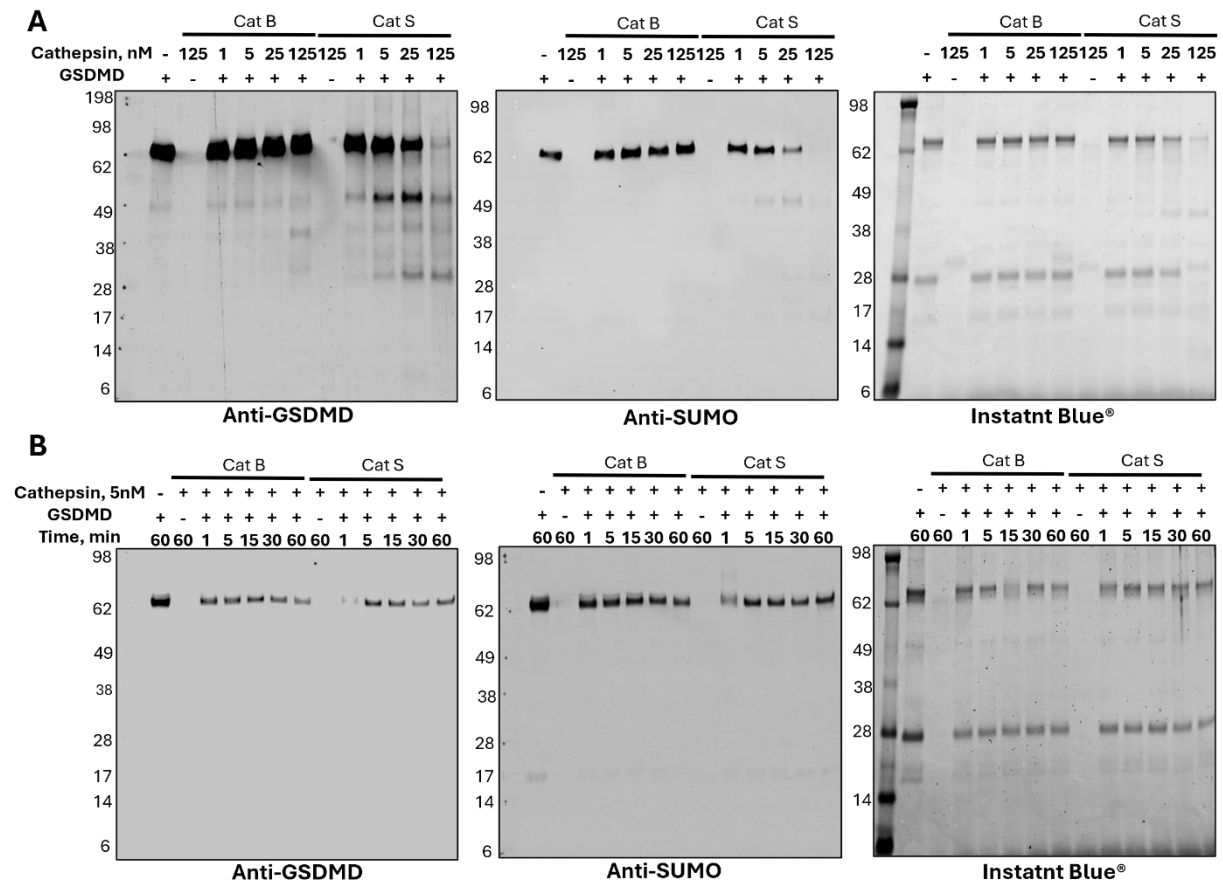
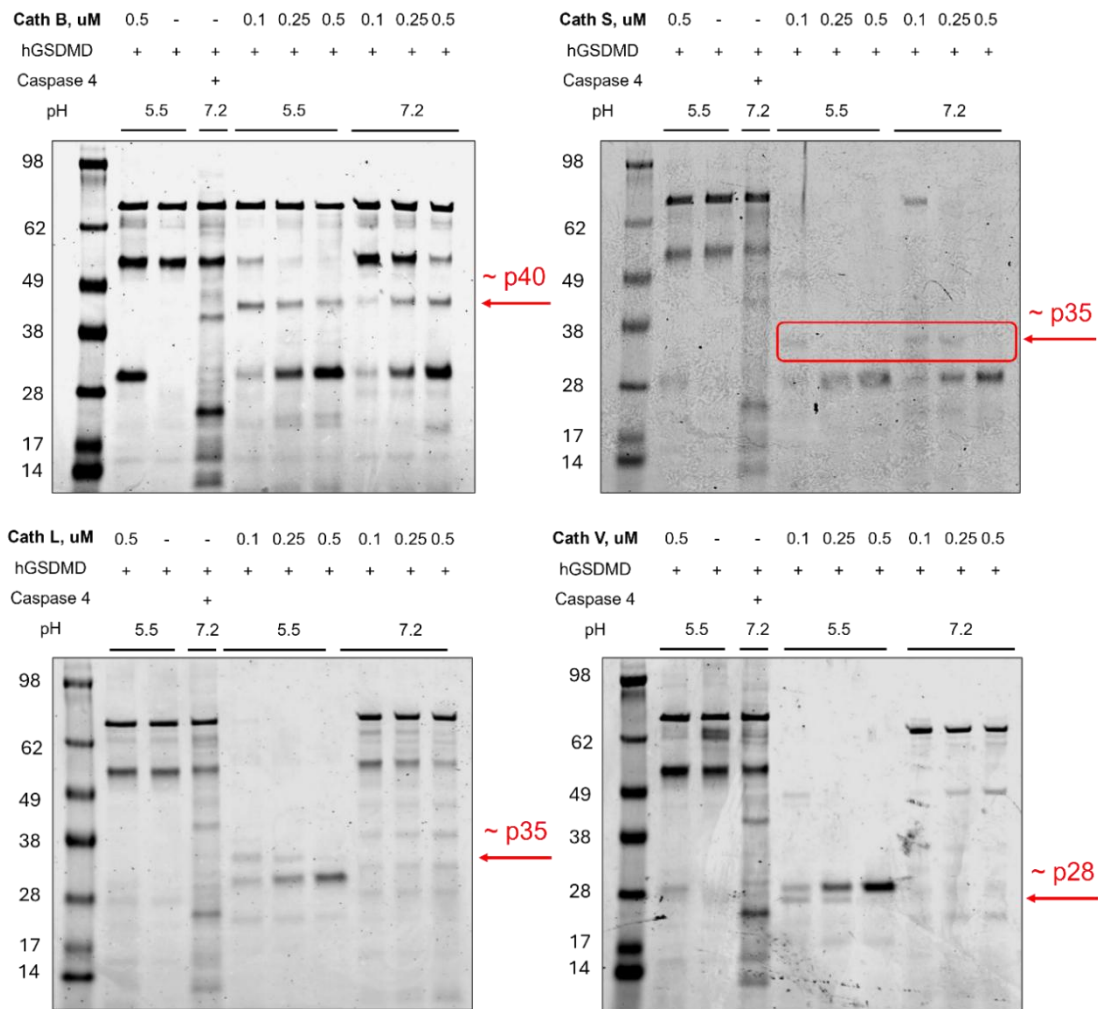


Figure 27. Cysteine cathepsins are active in neutral pH. Western blot analysis of GSDMD processing by human cysteine cathepsins B, and S at pH 7.5 in A concentration dependent manner and B time dependent manner. Cleavage products were visualized using an anti-GSDMD and anti-SUMO antibodies. SDS-PAGE analysis under the same pH conditions and concentration gradient was performed with InstantBlue® Coomassie Protein Staining.



4.5. IDENTIFICATION OF GSDMD CLEAVAGE SITES TARGETED BY CATHEPSINS

Cathepsin S was selected for detailed cleavage site mapping because it consistently showed efficient processing of GSDMD under near-neutral pH conditions, in contrast to cathepsin B, which displayed only limited activity. This was further confirmed by western blot analysis (**Figure 30**) during sample preparation for mass spectrometry, where cathepsin S generated distinct cleavage patterns. Incubation of SUMO-GSDMD with cathepsin S produced three prominent cleavage fragments that were readily visible by InstantBlue® protein staining, allowing precise excision and subsequent LC-MS/MS analysis. These observations indicated that cathepsin S was the most effective of the tested cathepsins in cleaving GSDMD under the examined conditions. To determine the cleavage specificity of cathepsin S, SUMO-GSDMD was incubated with the enzyme and the resulting fragments were analyzed by LC-MS/MS (**Figure 30**). This analysis identified cleavage sites located in close proximity to the canonical caspase-1 site ($^{272}\text{FLTD}\downarrow\text{G}^{276}$), suggesting that cathepsin S targets a similar region of GSDMD.

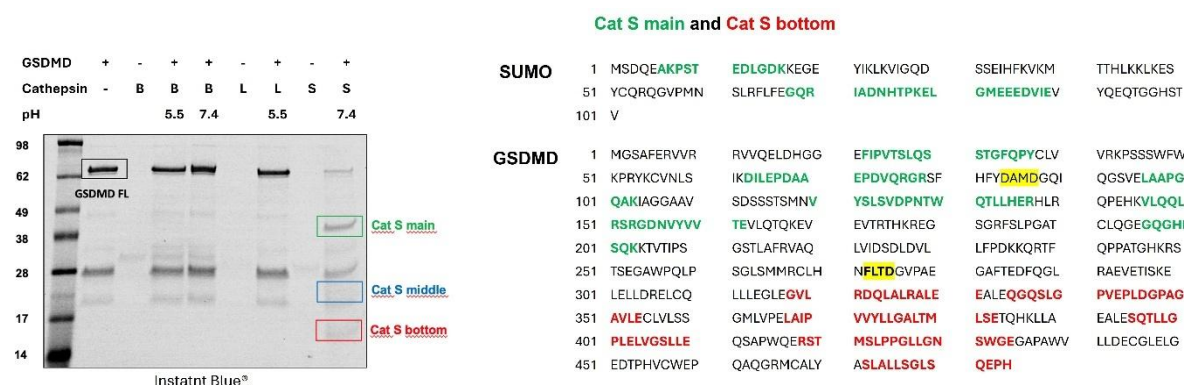


Figure 30. LC-MS/MS analysis of cathepsin S-mediated SUMO-GSDMD. Peptides identified in the gel band corresponding to the primary cleavage product ("Cat S main") are highlighted in green on the GSDMD sequence, while peptides from a lower molecular weight band ("Cat S bottom") are shown in red.

To further validate these observations, a panel of internally quenched fluorescent (IQF) peptide substrates was employed. These substrates consisted of seven amino acids derived from GSDMD, flanked by a fluorophore (ACC) and a quencher (DNP) (**Figure 31**). Given the broad S1 pocket tolerance of cathepsins, a library of 47 IQF substrates containing diverse P1 residues, including Arg, Phe, Trp, and Leu, was synthesized. This approach, previously applied to confirm GSDMD cleavage by caspases and calpain-1[285] was used to determine cleavage sites of cathepsin S, V, B and L (**Figure 32**). Analysis revealed that cathepsin S preferentially cleaved peptides $^{232}\text{VLLF}/\text{PDK}^{238}$ and $^{240}\text{QRTF}/\text{QPP}^{246}$. Both motifs are situated near the caspase-1 cleavage site at $^{272}\text{FLTD}/\text{GVP}^{278}$. Comparative assays showed that cathepsin V displayed highest activity toward $^{272}\text{LHNF}/\text{LTD}^{278}$, while cathepsins B and L preferentially cleaved sites closer to the N-terminus of GSDMD (**Figure 32**). To complement these assays, a set of 24-mer IQF peptides spanning the full GSDMD sequence was used for kinetic analyses (**Figure 33**). These experiments confirmed that cathepsin S most efficiently recognized the peptide encompassing residues $^{229}\text{VLLF}\dots\text{STS}^{252}$, which is consistent with both the LC-MS/MS mapping and the short-peptide substrate results. Together, these data demonstrate that cathepsin

S preferentially cleaves within the central region of GSDMD near the caspase-1 processing site, thereby supporting the idea that it may compete with or modulate caspase-mediated activation of GSDMD.

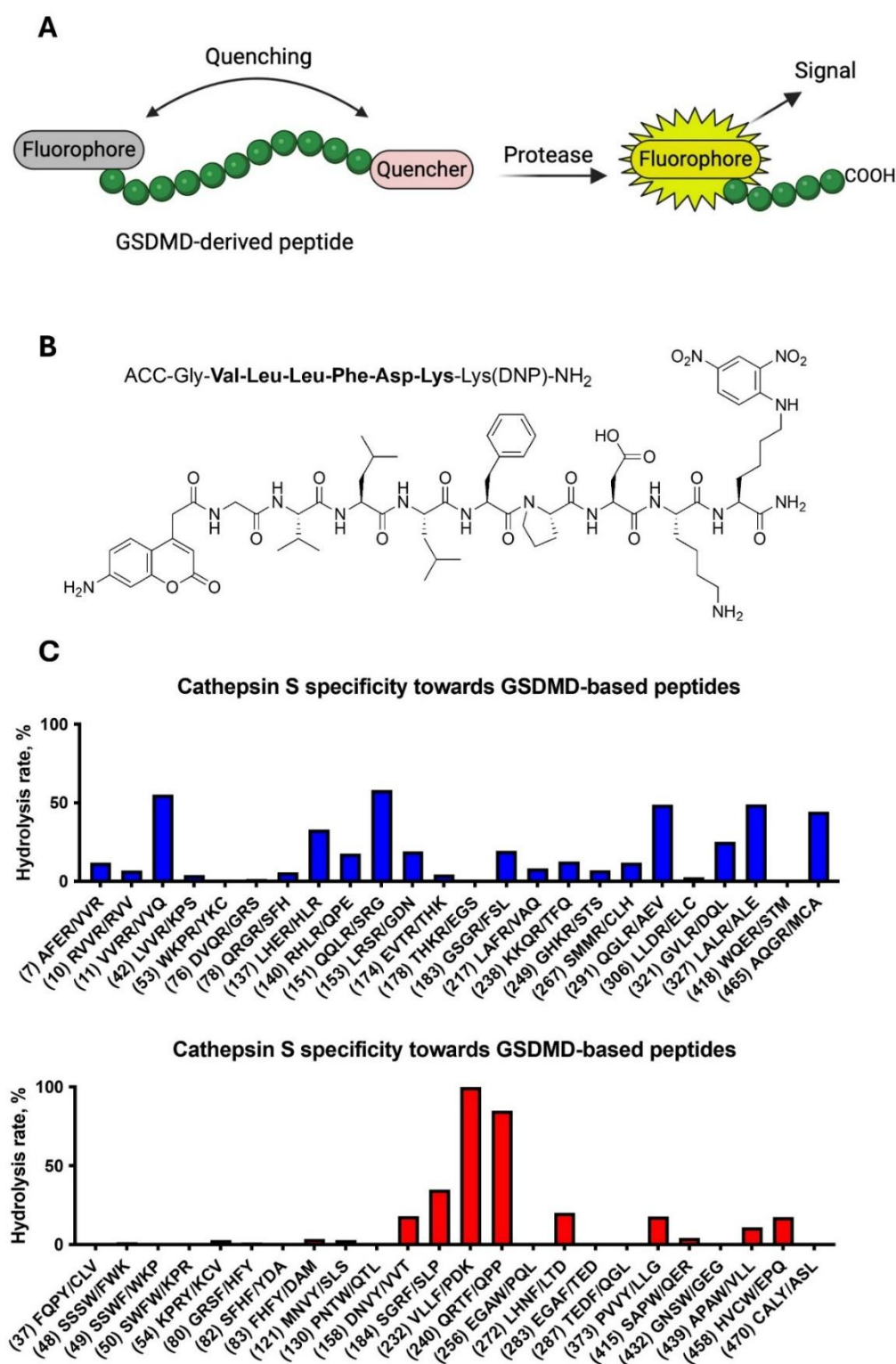


Figure 31. Internally quenched fluorescent (IQF) substrates as a tool for cleavage site investigation. **A** Schematic representation of internally quenched fluorescent (IQF) substrates containing GSDMD-derived peptides. **B** the corresponding peptide structures. **C** Summary of the cleavage preferences of cathepsin S toward selected GSDMD-derived IQF substrates, highlighting optimal P1 residues: Arg (blue), and Trp/Tyr/Phe (red).

substrates processing at pH 7.4

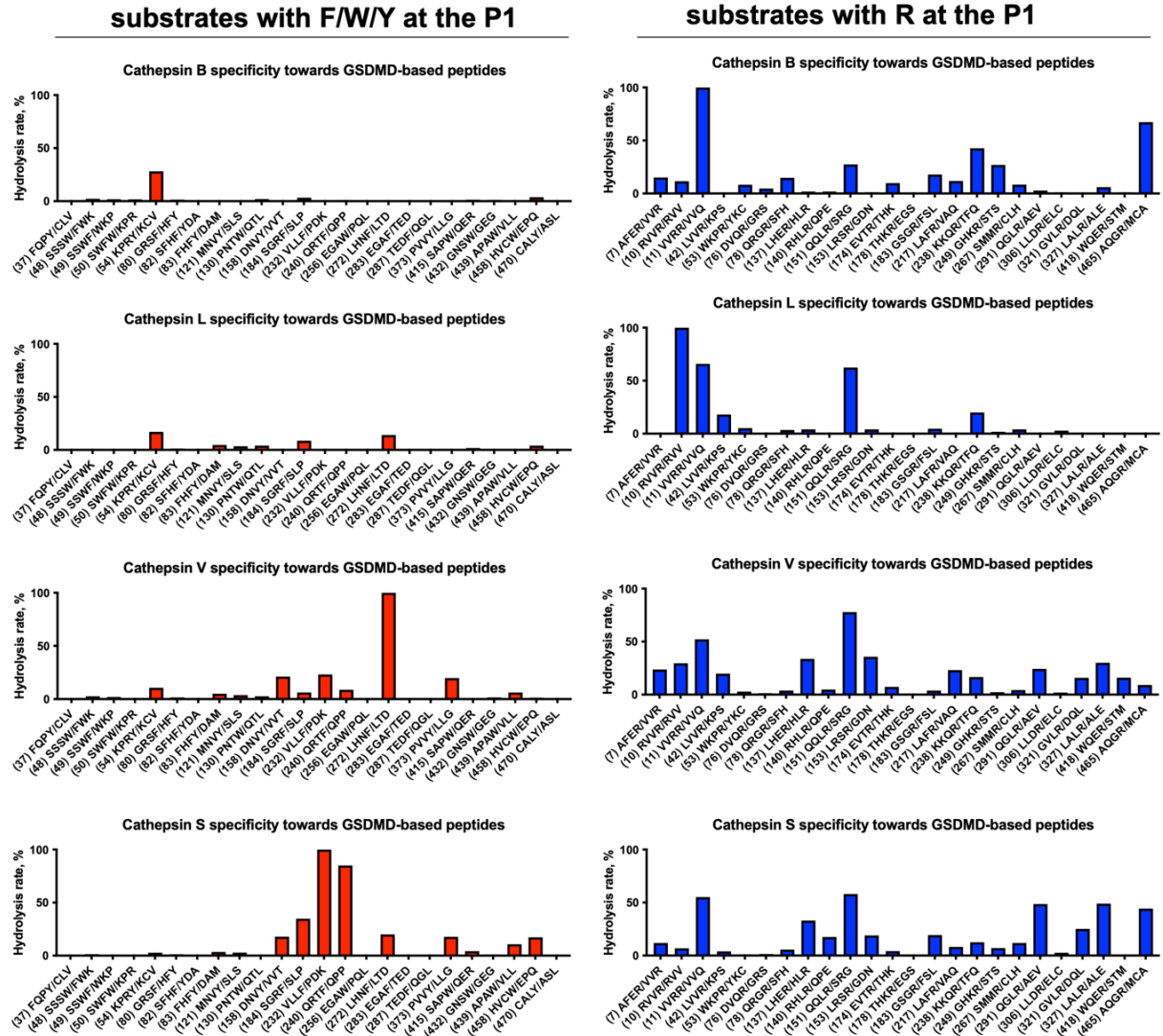


Figure 32. Kinetics analysis of cysteine cathepsins preferences towards GSDMD-based peptide IQF substrates containing diverse P1 residues. Substrates with Phe, Tyr or Trp are presented on the red-bar charts, while substrates with Arg are presented on the blue-bar charts.

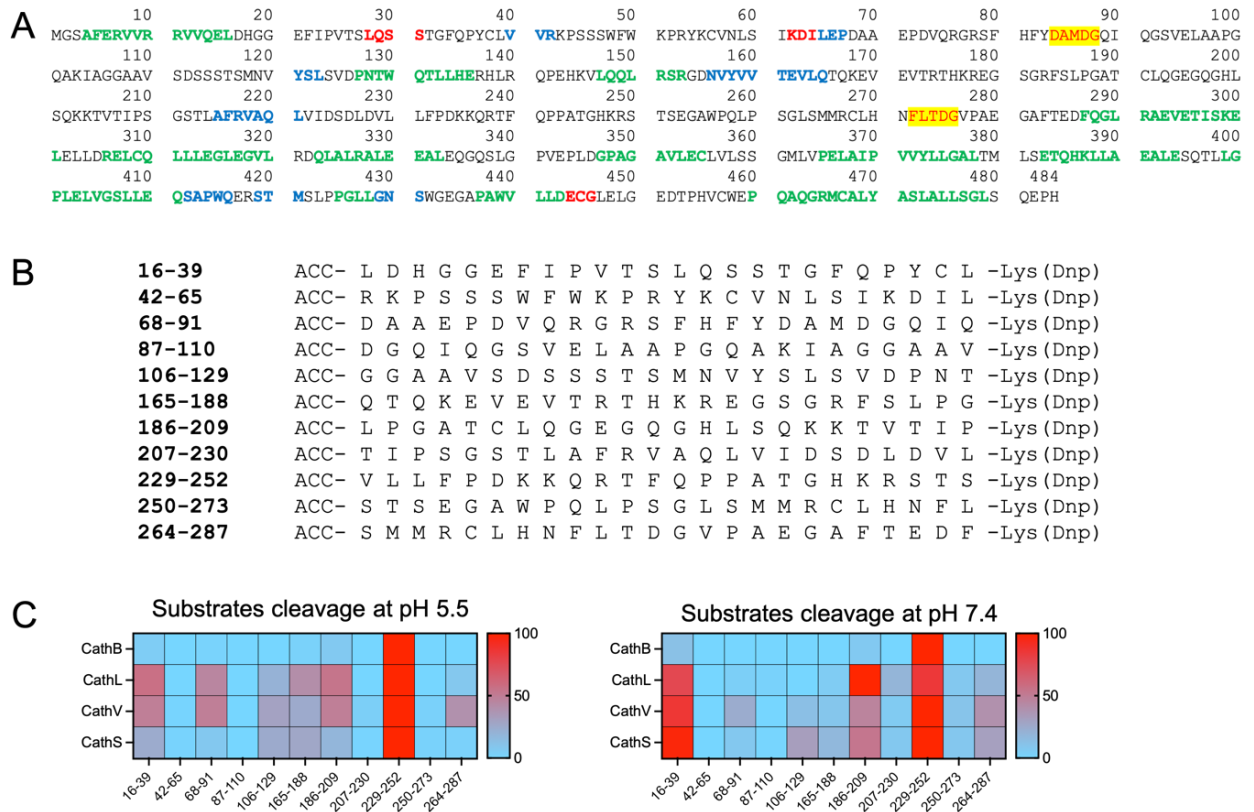


Figure 33. Kinetics analysis of a set of 24-mer IQF peptides spanning the full GSDMD sequence. **A** The sequence of the GSDMD protein is presented in single-letter amino acid code. Alpha-helices are shown in green, beta-sheets in blue, and beta-turns in red. Unstructured peptide motifs are indicated in black. Caspase-1 (FLTD/G) and caspase-3 (DAMD/G) cleavage sites are highlighted with red font and yellow background. **B** Sequence of 24-mer IQF peptides spanning the full GSDMD protein. Only peptide fragments from unstructured regions of GSDMD were selected, since sequences within alpha-helices or beta-sheets are much less prone to proteolytic hydrolysis. **C** Catalytic preferences of cathepsin B, L, V, and S toward long GSDMD fluorescent surrogates tested in acidic (left) and neutral (right) pH. Data is presented as heat-maps based on the cleavage rate of the substrate (RFU/s; relative fluorescence unit per second). The cleavage site in each substrate was confirmed by LC-MS analysis.

Collectively, the results indicate that cysteine cathepsins are capable of directly cleaving pro-IL-1 β , pro-IL-18, and GSDMD. At lower enzyme concentrations, these proteases, particularly cathepsin S, carry out restricted, site-specific proteolysis that generates defined and potentially functional cleavage products. By contrast, at higher concentrations, cleavage becomes less specific, resulting in progressive substrate degradation. Among the tested proteases, cathepsin S consistently displayed the most selective activity and thus emerges as the enzyme with the highest potential to regulate the maturation of pyroptotic mediators in a controlled fashion. These observations prompt the important question of whether cysteine cathepsins, once released from lysosomes into the cytosol, retain the ability to perform limited proteolysis under physiological or pathological conditions. Evidence from previous studies strongly supports this possibility. For example, cathepsin B has been shown to cleave the pro-apoptotic protein Bid at Arg65, generating tBid, which subsequently promotes mitochondrial outer membrane permeabilization and apoptosis [272]. Cathepsins L and S also process Bid at distinct arginine residues, reinforcing the concept that restricted and site-specific proteolysis is

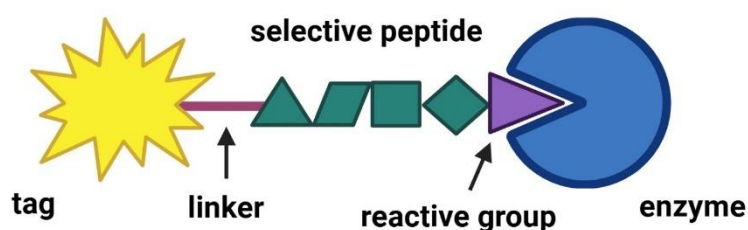
a common feature of this protease family [272]. In addition to Bid, cathepsins B and L cleave anti-apoptotic Bcl-2 family proteins, including Bcl-2, Bcl-xL, and Mcl-1, with well-defined cleavage fragments identified for Bcl-2 and Bcl-xL [273]. Similarly, cathepsin S has been reported to cleave cytosolic BAX, producing truncated forms that enhance apoptotic signaling [433]. In non-apoptotic contexts, cathepsin L has been shown to selectively cleave cytoskeletal regulators such as dynamin [434] and synaptopodin [435] at conserved motifs in kidney podocytes, leading to cytoskeletal remodeling and functional impairment. Beyond apoptosis, cytosolic cathepsins have also been implicated in the regulation of inflammatory pathways. For example, cathepsin C activates pro-granzymes in cytotoxic lymphocytes [436], while cathepsins S and L contribute to antigen processing for MHC class II presentation [365, 437], reflecting their adaptability to different cellular compartments. Moreover, accumulating evidence indicates that lysosomal membrane permeabilization during inflammasome activation can release cathepsins into the cytosol, where they act as upstream regulators of pyroptotic and necroptotic pathways [363, 438]. Taken together, these examples illustrate that cathepsins, although historically classified as degradative enzymes restricted to the lysosome, can function in the cytosol as selective proteases capable of executing precise cleavage events with significant signaling consequences. The ability of cathepsins to act either as site-specific processing enzymes or as broad degradative proteases likely depends on local concentration, substrate accessibility, and pH microenvironment, thereby providing a mechanistic explanation for the dual activities observed in this study.

4.6. DISSECTING ACTIVE CATHEPSIN B USING AN ACTIVITY-BASED PROBE

Lysosomal cysteine cathepsins have attracted growing attention in recent years, as it has become increasingly clear that their proteolytic activity is not strictly confined to the acidic lysosomal lumen. Instead, these enzymes can retain activity and exert functions in neutral or mildly acidic environments, including the cytosol. Despite this, the field has long lacked highly selective inhibitors and activity-based probes (ABPs), and thus the role of cathepsins in regulated cell death pathways has been studied largely using broad-spectrum inhibitors and first-generation probes. Compounds such as E64d, CA-074Me, and K777 are widely used to suppress cathepsin activity and to assess their involvement in inflammasome activation, lysosome-dependent necrosis, and apoptosis. For example, E64d was reported to block particle-induced IL-1 β release and macrophage cell death, linking cathepsin activity to NLRP3 inflammasome regulation. Likewise, CA-074Me has been used extensively in models of LLOMe-induced lysosomal destabilization and pyroptosis, although its originally proposed selectivity for cathepsin B has since been challenged due to significant cross-reactivity with other cysteine proteases. K777, a potent irreversible pan-cathepsin inhibitor, has shown protective effects in vivo by reducing IL-1 β secretion and attenuating inflammatory pathology. More recently, ABPs such as BMV109 have enabled real-time labeling of active cathepsins in live cells, thereby confirming their cytosolic activity during cell death. Nevertheless, the use of broad-spectrum inhibitors inevitably obscures the distinct, non-redundant contributions of

individual cathepsins, given their partially overlapping but unique substrate preferences and cellular functions. To overcome this limitation, the combined application of selective inhibitors with ABPs has been introduced, allowing more precise dissection of cathepsin-specific functions across different modes of cell death.

Activity-based proteomics has emerged as a powerful approach for studying enzyme function directly within complex biological samples. Instead of measuring protein abundance, this strategy focuses on detecting the active fraction of enzymes by using ABPs, small molecules designed to covalently label catalytically competent proteins. Because these probes attach only to enzymes in their active conformation, they allow precise monitoring of enzymatic regulation in cell lysates, intact cells, and even whole organisms. Despite their diversity, ABPs generally share a modular design composed of three functional elements: a reactive group, a linker, and a reporter tag (**Figure 34**). The reactive group, often referred to as the *warhead*, is responsible for forming a stable covalent bond with the catalytic nucleophile present in the enzyme's active site. For proteases such as cysteine, serine, and threonine proteases, the warhead typically contains an electrophilic moiety capable of selectively reacting with the corresponding catalytic residue. Importantly, the electrophile must be sufficiently reactive toward the target nucleophile while remaining inert toward non-specific nucleophiles throughout the proteome. Cysteine proteases are typically targeted using an acyloxymethyl ketone (AOMK) reactive group. The linker region serves primarily to distance the warhead from the tag, improving accessibility to the active site and minimizing steric clashes. Linkers can range from simple alkyl chains or short PEG (polyethylene glycol) spacers to more sophisticated peptide sequences that enhance probe selectivity by mimicking natural substrate preferences. The reporter tag provides a means to visualize or isolate labeled proteins. Early ABPs frequently incorporated radioisotopes, but the introduction of fluorescent tags significantly expanded the versatility of these tools. Fluorophores such as cyanine (Cy) dyes offer high photostability, strong absorption, narrow absorption peaks, and good cell permeability, making them ideal for imaging applications and in-gel detection. These advances enabled the creation of fluorescent probes ideally suited for monitoring the diverse family of cysteine proteases, ranging from caspases to lysosomal cathepsins, legumain, and calcium-dependent calpains. The ability to visualize the active forms of these enzymes has substantially enhanced our understanding of their contributions to cell death pathways, immune signaling, and proteostasis. Overall, the modular architecture and high selectivity of ABPs make them exceptionally powerful tools for interrogating cysteine protease activity and regulation directly within biologically relevant environments [439].

A**Activity based probe****B**

Cy5-PEG(4)-Cha-Leu-hSer(Bzl)-Arg-AOMK

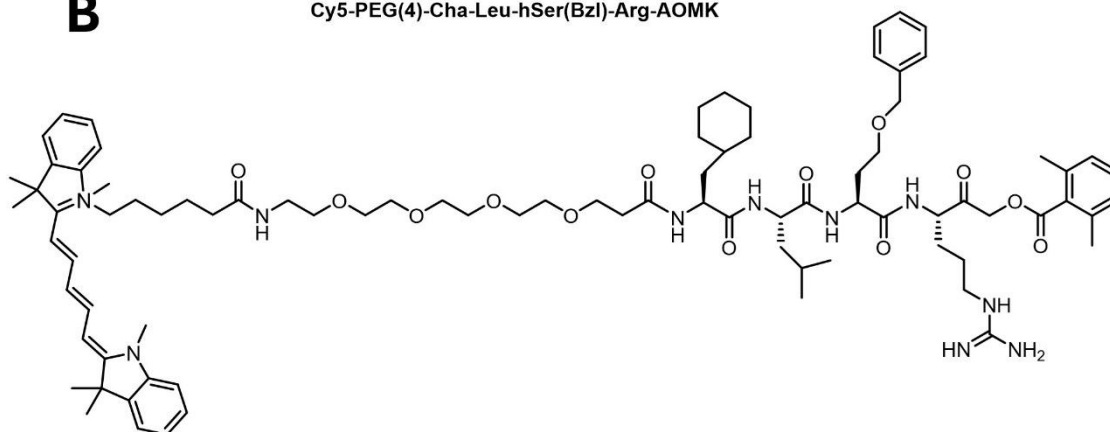


Figure 34. Structural outline of the activity based probe. A Modular layout of ABP (Created in <https://BioRender.com>). B The structure of Cy5-labeled activity-based CatB-27 probe with the sequence Cy5-PEG(4)-Cha-Leu-hSer(Bzl)-Arg-AOMK.

To optimize inhibitor use in our experiments with THP-1 cells, three concentrations of custom cathepsin B inhibitors, CatB-23 and CatB-24, were compared against commonly used inhibitors CA-074Me and E64d (**Figure 35**). Using a CatB-27 probe, we confirmed that all tested inhibitors effectively suppressed cathepsin B activity at 25 μ M. Commercial inhibitors displayed efficacy at lower concentrations, but with reduced selectivity compared to the custom compounds. Importantly, ABPs provided a critical advantage: while anti-cathepsin B antibodies detect both the inactive proenzyme and the mature enzyme, ABPs recognize only the catalytically active form. This highlights the necessity of integrating ABPs into cell-based studies, as they allow direct and selective monitoring of enzyme activity, rather than indirect detection of total protein levels. Cysteine cathepsins are synthesized as preproenzymes containing a signal peptide that directs them into the endoplasmic reticulum, followed by a propeptide domain that ensures correct folding and lysosomal targeting. Activation requires proteolytic removal of this propeptide, either by autocatalytic cleavage or through the action of other proteases [328, 363]. Since only the mature, active form contributes to cell death

pathways, the use of ABPs provides an indispensable tool to distinguish between inactive precursors and functional proteases in living cells.

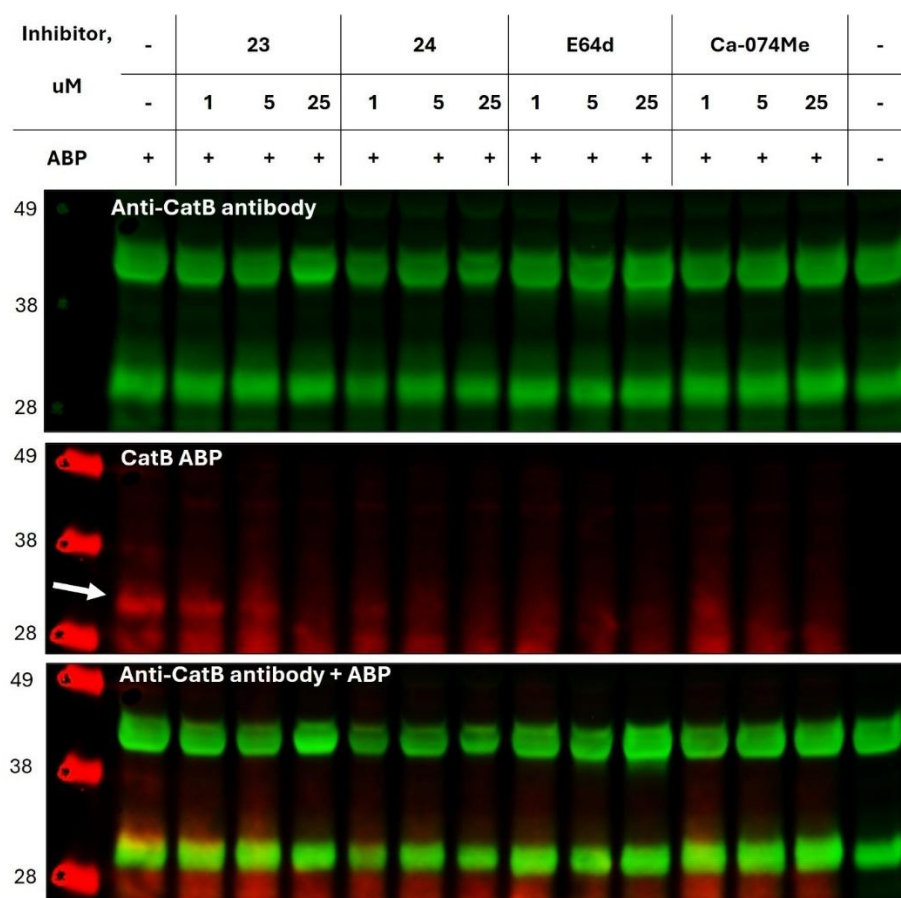


Figure 35. Selective ABP for dissecting cathepsin B in THP-1 cells. Western blot analysis of PMA-differentiated WT THP-1 cells incubated with CatB-24 and CatB-24 inhibitors, and CatB-27 probe. Following incubation, samples were resolved by SDS-PAGE and analyzed by Western blotting. Fluorescence was scanned simultaneously at two wavelengths: 680 nm detecting the secondary antibody fluorophore for anti-cathepsin B and 800 nm detecting the Cy5 fluorophore of the ABP. The red band (white arrow) represents CatB-27 labeling of active cathepsin B (~29 kDa, single-chain form), while the green bands correspond to antibody detection of cathepsin B species, including the proenzyme (~43 kDa) and single-chain (~29 kDa) forms.

4.7. SELECTIVE CHEMICAL TOOLS TOWARDS CYSTEINE PROTEASES

Table 4. Structures of custom inhibitors used

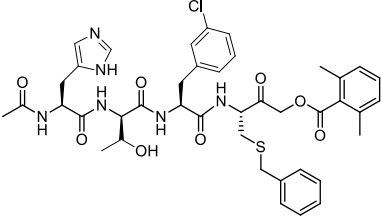
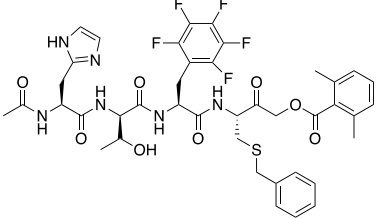
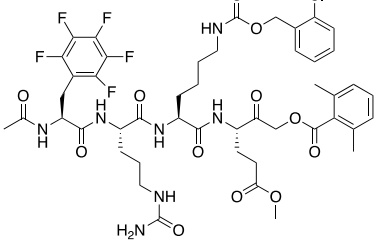
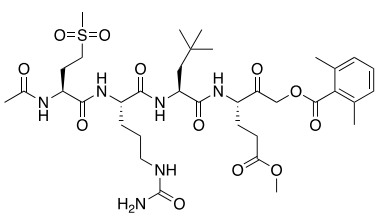
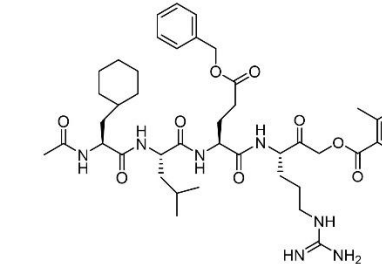
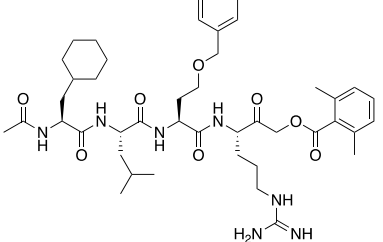
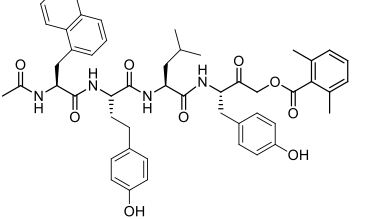
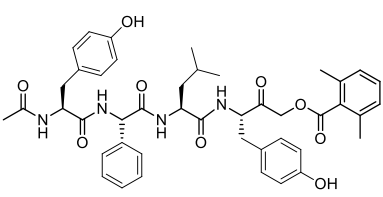
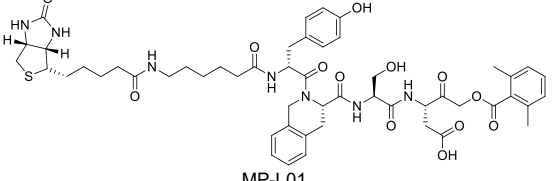
 CatL-19 Ac-His-DThr-Phe(3Cl)-Cys(Bzl)-AOMK	 CatL-20 Ac-His-DThr-Phe(F₃)-Cys(Bzl)-AOMK
 CatS-21 Ac-Phe(F₃)-Cit-Lys(2ClZ)-Glu(Me)-AOMK	 CatS-22 Ac-Met(O₂)-Cit-NptGly-Glu(Me)-AOMK
 CatB-23 Ac-Cha-Leu-Glu(Bzl)-Arg-AOMK	 CatB-24 Ac-Cha-Leu-hSer(Bzl)-Arg-AOMK
 Calp-53 Ac-1Nal-hTyr-Leu-Tyr-AOMK	 Calp-54 Ac-Tyr-Phg-Leu-Tyr-AOMK
 MP-L01 biotin-6-ahx-DTyr-Tic-Ser-Asp-AOMK	

Table 5. Structures of commercially available inhibitors used

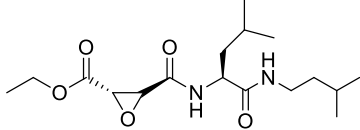
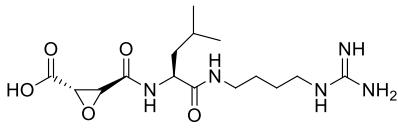
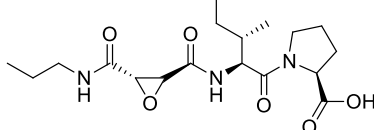
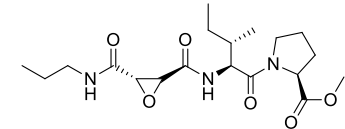
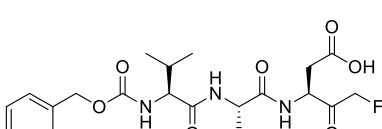
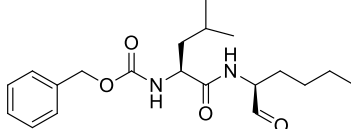
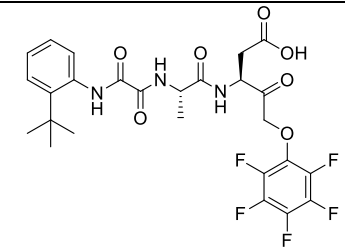
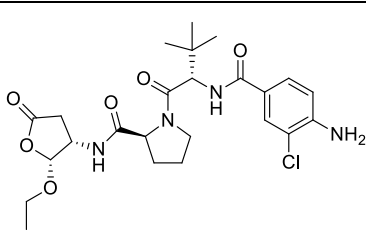
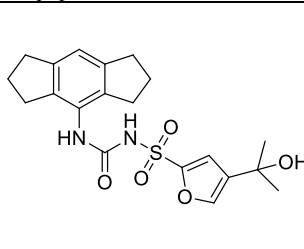
 E64d	 E64	 CA-074
 CA-074Me	 Z-VAD-FMK	 Calpeptin
 Emricasan	 VX-765	 MCC950

Table 6. Kinetic parameters of used inhibitors

	$k_{cat}/[I], M^{-1}s^{-1}$				
Compound	Cathepsin L	Cathepsin S	Cathepsin B	Calpain-1	Legumain
CatL-19	367,00	772	11,600	n.d.	n.d.
CatL-20	7,000	81	213	n.d.	n.d.
CatS-21	867	468,300	19,400	n.d.	n.d.
CatS-22	792	363,000	8,497	n.d.	n.d.
CatB-23	1,500	1,560	337,000	n.d.	n.d.
CatB-24	2,100	2,090	1,298,000	n.d.	n.d.
Calp-53	93,200	13,200	6,460	589,000	n.d.
Calp-54	179,800	82,300	8,640	703,000	n.d.
MP-L01	n.d.	n.d.	n.d.	n.d.	34,000
Calpeptin	2,554,000	62,800	34,500	514,000	n.d.
XV-765	1,100	3,700	3,770	<100	n.d.
Emricasan	1,200	3,060	3,500	<100	n.d.
CA-074	1,300	2,130	55,200	<100	n.d.
E64	32,900	156,000	31,400	42,300	n.d.
Z-VAD-FMK	5,400	23,500	3,780	13,600	n.d.

4.8. TOXICITY TEST OF PROTEASE INHIBITORS

Before initiating the inhibitor-based cell death assays, preliminary toxicity tests were conducted to determine whether the compounds themselves could induce cytotoxicity independent of stimulation. To achieve this, real-time monitoring of plasma membrane integrity was performed using propidium iodide uptake as a readout of cell death. PMA-differentiated WT THP-1 cells, caspase-1 knockout (Casp-1 KO, Casp-1^{-/-}), and GSDMD knockout (GSDMD KO, GSDMD^{-/-}) lines were analyzed over a 24-hour period following inhibitor treatment.

Both commercially available inhibitors and custom-synthesized inhibitors were tested. For the majority of compounds, no significant cytotoxicity was observed within the 24-hour timeframe. However, some specific inhibitors exhibited measurable toxicity: the commercially available Caspase-1 inhibitor caused a pronounced loss of viability after ~12 hours of incubation across all three cell lines. The pan-caspase inhibitor Emricasan displayed mild, time-dependent cytotoxicity, which became detectable in WT cells as early as 4 hours. Similarly, the calpain inhibitor Calpeptin showed low-level toxicity after approximately 12 hours in all tested conditions. By contrast, custom cathepsin L (CatL-19, CatL-20) inhibitors exhibited only slight cytotoxic effects (**Figure 36**). Interestingly, these custom-designed inhibitors consistently demonstrated lower toxicity compared to their commercially available counterparts, suggesting a potentially improved therapeutic index. Specifically, compounds Calp-53 and Calp-54 were less toxic than Calpeptin, while compounds CatS-21, CatS-22, CatB-23 and CatB-24 showed reduced cytotoxicity compared to the widely used lysosomal protease inhibitors CA-074Me and E-64d (**Figure 37**). This comparative analysis highlights that structure-guided custom inhibitors may retain biological activity while minimizing off-target cytotoxicity, an important consideration for downstream mechanistic studies.

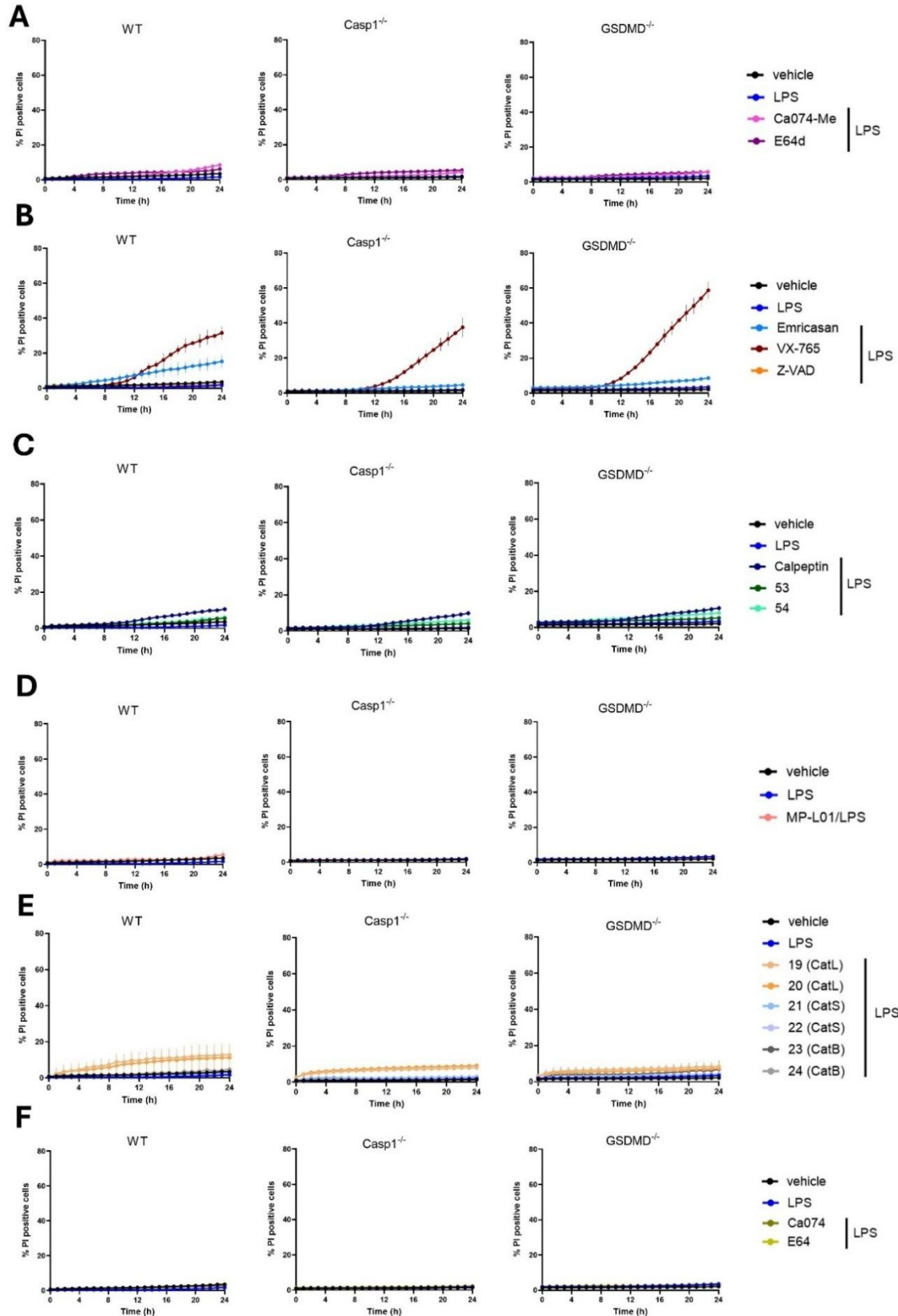


Figure 36. Kinetic assessment of inhibitor toxicity using Incucyte live-cell imaging. Incucyte-based kinetic analysis of cell death in wild-type (WT), caspase-1^{-/-}, and GSDMD^{-/-} M0 THP-1 cells treated with 25 μ M of inhibitors. **A** Commercially available cathepsin inhibitors. **B** Commercially available caspase inhibitors. **C** Commercially available and custom calpain inhibitors. **D** Custom legumain inhibitor. **E** Custom cathepsin inhibitors.

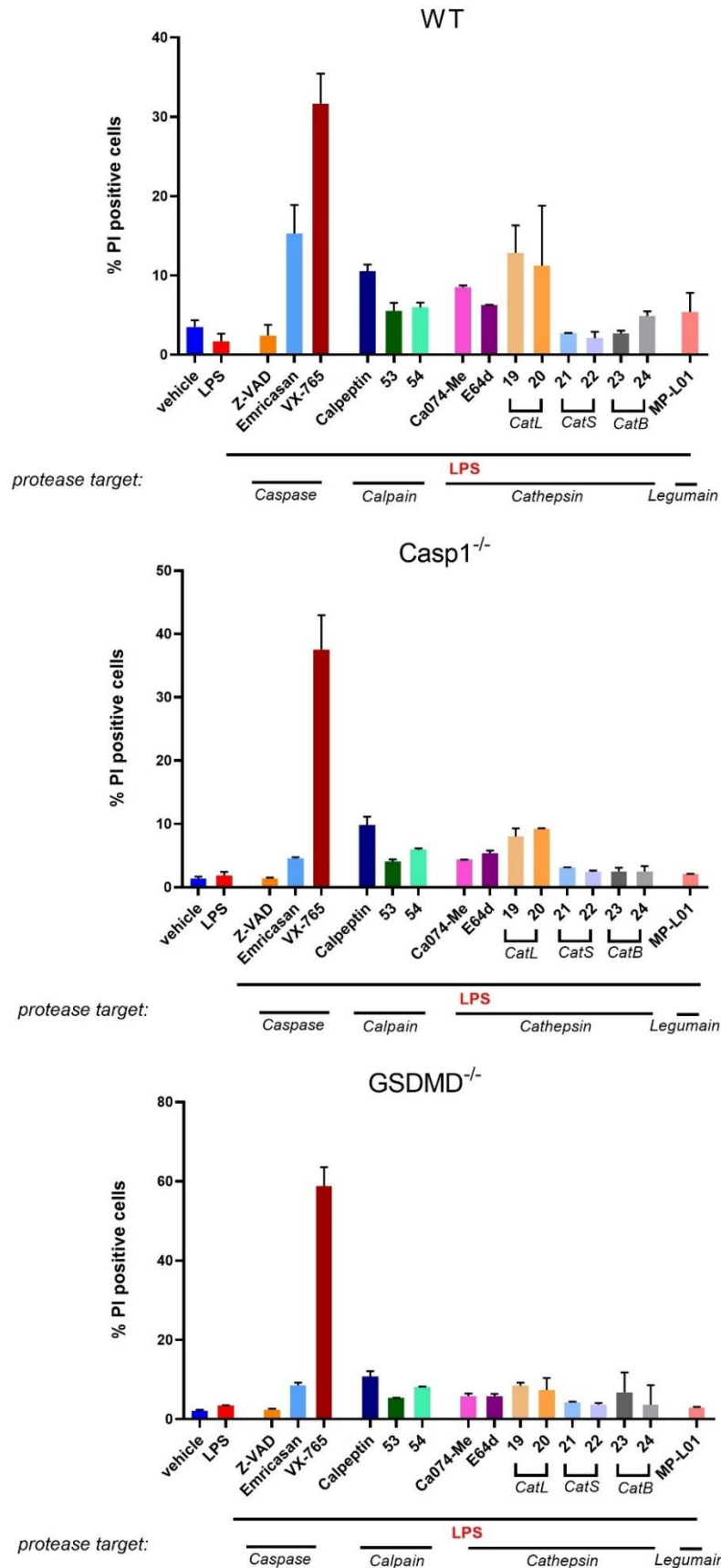


Figure 37. Assessment of inhibitor cytotoxicity by PI uptake after 24 h. PMA-differentiated, LPS-primed wild-type, GSDMD KO, and caspase-1 KO THP-1 cells were incubated for 24 h with 25 μ M of the indicated inhibitors. PI uptake was measured to evaluate potential compound-induced cytotoxicity under resting conditions.

4.9. DISSECTION CATHEPSINS ROLE IN CELL DEATH BY LIVE-CELL IMAGING

Since the newly developed inhibitors did not exhibit cytotoxic effects, their ability to penetrate intact cells was next assessed. Cellular uptake typically occurs either through passive diffusion across the lipid bilayer or, less commonly, under conditions of compromised plasma membrane integrity. To evaluate whether the custom cathepsin B inhibitors could access the intracellular compartment under non-lethal conditions, activity assays were performed in wild-type, unstimulated THP-1 cells using a CatB-27 probe. These compounds were tested in parallel with the widely used reference inhibitors CA-074 and E64, which are not cell-permeable, as well as their membrane-permeable prodrug derivatives CA-074Me and E64d. For passive diffusion to occur, compounds must satisfy several physicochemical requirements, including a balance of hydrophobicity (overly polar molecules display poor permeability, while excessively hydrophobic compounds risk insolubility or membrane trapping), neutral charge (uncharged molecules traverse the membrane more efficiently than ionized forms), relatively small size (molecules <500 Da generally cross more readily), and a limited number of hydrogen bond donors/acceptors (fewer hydrogen-bonding groups increase permeability) [440]. CA-074 is poorly permeable due to the presence of a negatively charged carboxyl group, restricting its application to cell-free assays, lysates, or extracellular inhibition of cathepsin B [441, 442]. In contrast, its methyl ester prodrug, CA-074Me, masks this negative charge, facilitating uptake into live cells where it is hydrolyzed by esterases to release active CA-074. Similarly, E64 is an irreversible broad-spectrum cysteine protease inhibitor that lacks cell permeability due to its charged structure, whereas E64d crosses the membrane efficiently and is subsequently hydrolyzed by intracellular esterases to active E64c, a potent irreversible and nonpermeable synthetic analog of E64 [443-446].

As expected, neither E64 nor CA-074 inhibited cathepsin B activity (**Figure 38**) in intact THP-1 cells, consistent with their inability to cross the plasma membrane. Likewise, the pan-caspase inhibitor emricasan did not affect cathepsin B activity, serving as a negative control. In contrast, all cell-permeable inhibitors tested, including CA-074Me, E64d, and the custom cathepsin B inhibitors CatB-23 and CatB-24, efficiently suppressed cathepsin B activity. Importantly, ABP labeling detected only the active form of cathepsin B as a single band, in contrast to conventional antibody-based detection, which recognizes both the inactive proenzyme and the mature protease. Similarly, live-cell imaging (**Figure 39**) demonstrated that CA-074 and E64 lacked protective activity against nigericin-induced cell death, whereas their prodrug derivatives, CA-074Me and E64d, effectively protected PMA-differentiated wild-type THP-1 cells, as indicated by reduced PI uptake. These findings confirm that both CatB-23 and CatB-24 are cell-permeable, functionally active inhibitors and further highlight the advantage of ABPs in specifically monitoring the active protease pool.

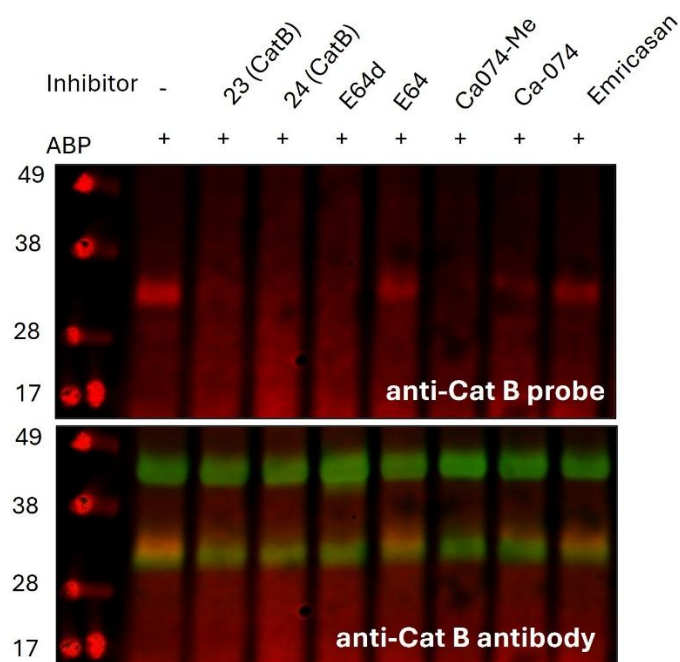


Figure 38. Dissecting cathepsin activity in THP-1 cells using custom CatB-27 probe. M0 WT macrophages were preincubated with 25 μ M of each inhibitor, followed with CatB-27 probe incubation. After incubation, samples were resolved by SDS-PAGE and analyzed by Western blotting. Fluorescence was scanned simultaneously at two wavelengths: 680 nm detecting the secondary antibody fluorophore for anti-cathepsin B and 800 nm detecting the Cy5 fluorophore of the ABP. The red band represents CatB-27 labeling of active cathepsin B (~29 kDa, single-chain form), while the green bands correspond to antibody detection of cathepsin B species, including the proenzyme (~43 kDa) and single-chain (~29 kDa) forms.

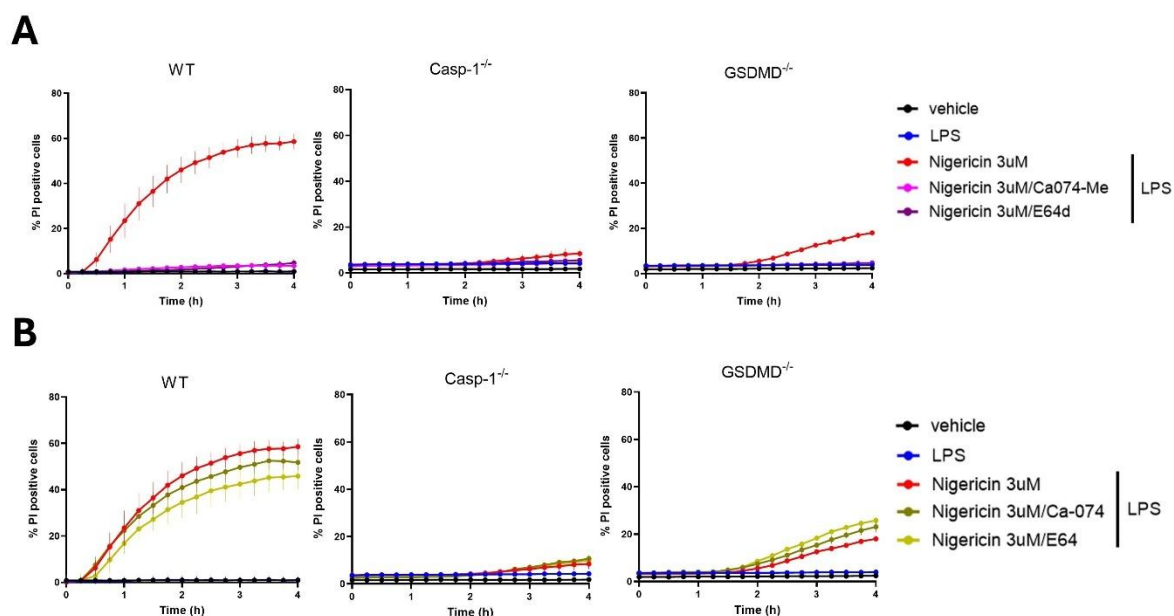


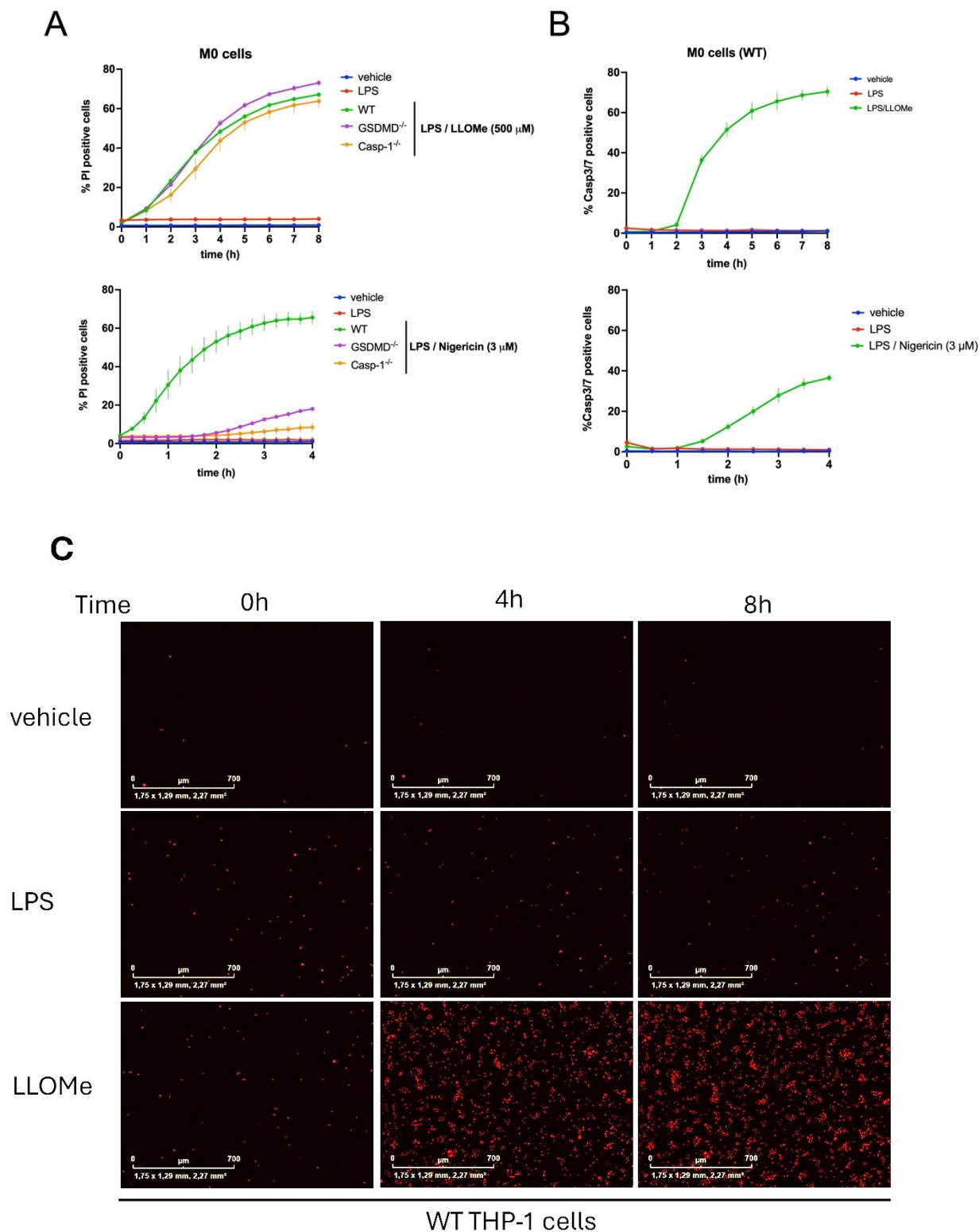
Figure 39. Differential effects of cell-permeable and non-cell-permeable inhibitors on the kinetics of nigericin-induced death in PMA-differentiated, LPS-primed THP-1 cells. **A** Time course of propidium iodide uptake in THP-1 cells treated with nigericin (3 μ M), following preincubation of **A** cell-permeable and **B** non-cell-permeable inhibitors (25 μ M).

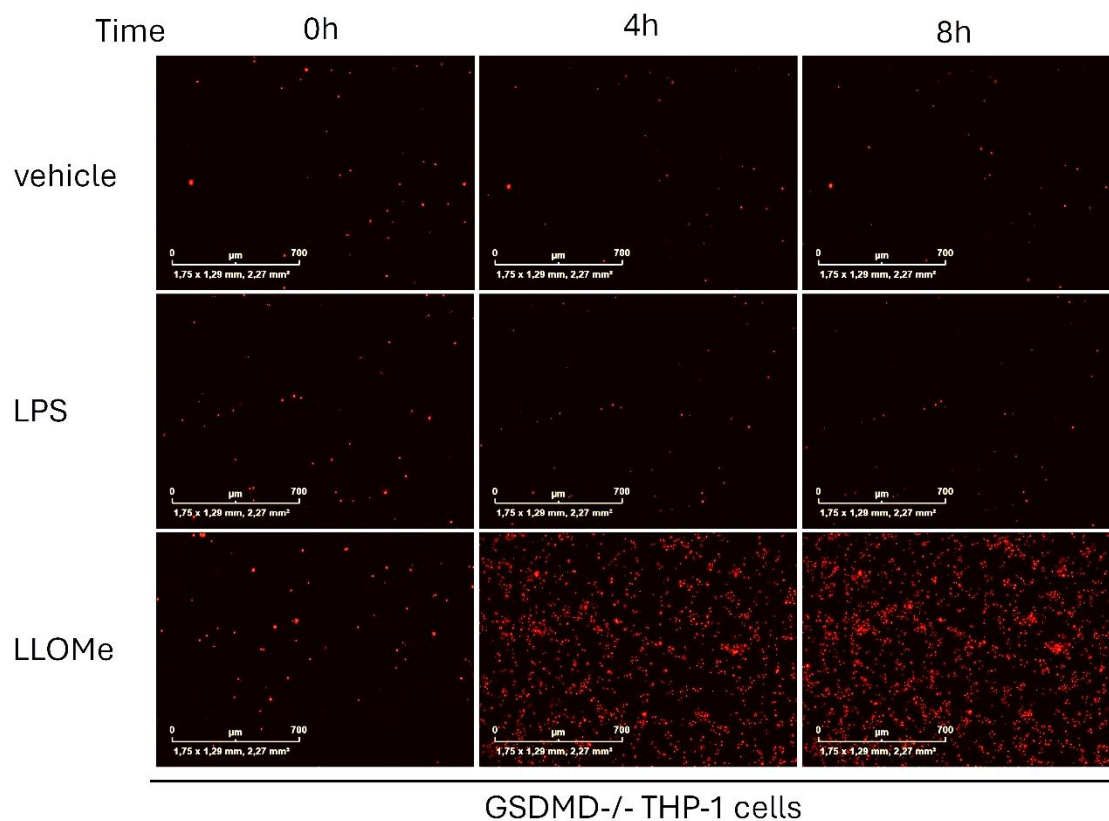
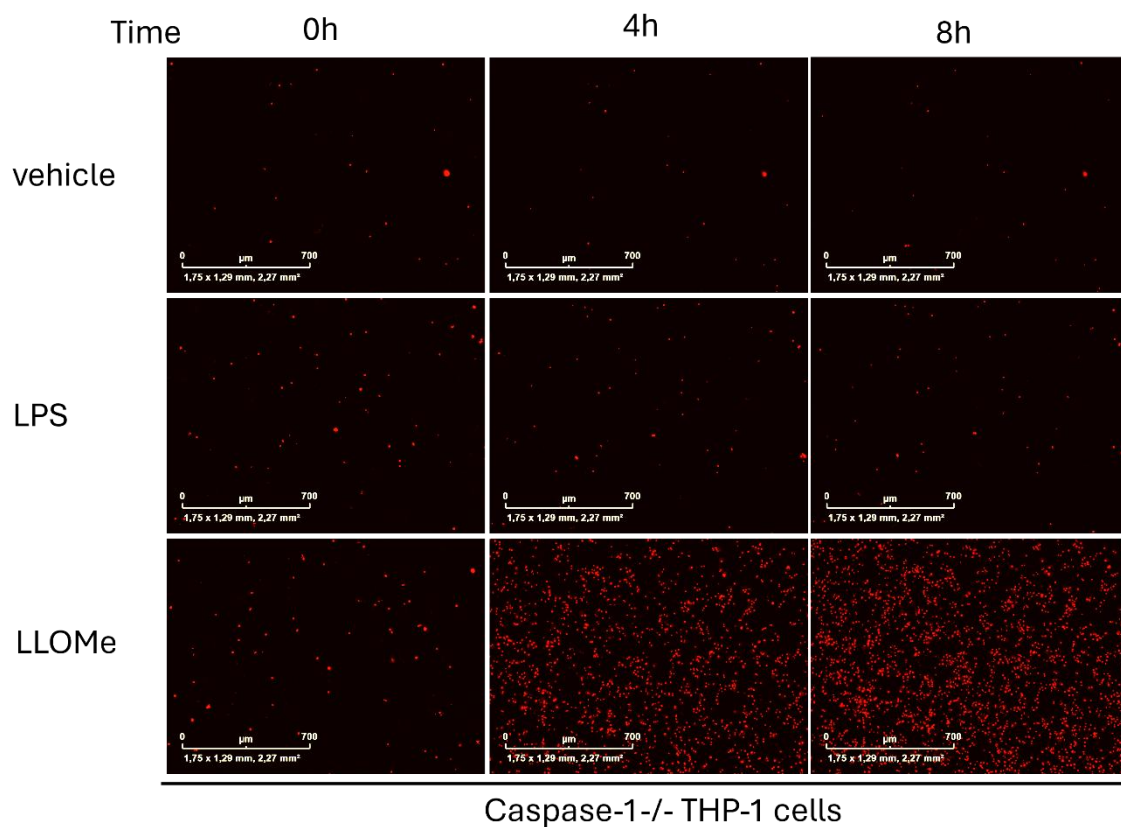
4.10. ANALYSIS OF CATHEPSINS ROLE IN CELL DEATH USING SELECTIVE CHEMICAL TOOLS

Although endpoint assays such as Western blotting provided valuable information about progression of cell death, they offered only a static snapshot of what is inherently a highly dynamic process. Cell death evolves over time, and similar terminal phenotypes can result from distinct pathways operating at different rates or with different temporal hierarchies [447, 448]. This is particularly relevant in the context of lysosomal disruption, where the release of cathepsins into the cytosol can initiate or modulate downstream death pathways [259, 272, 273, 428]. Certain cathepsins operate upstream of caspases, facilitating their activation or contributing to inflammasome assembly. Once activated, caspases-1 and -3 orchestrate distinct cell death programs but may also compete for common substrates, such as GSDMD, particularly in macrophages, where the balance between lytic and non-lytic outcomes is tightly regulated [358, 359, 406]. Importantly, it is not only the presence of these proteases but also the timing of their activation that determines cell fate. Early activation of inflammatory caspases tends to promote pyroptosis, whereas delayed or concurrent activation of apoptotic caspases may suppress pyroptosis or redirect the cell toward apoptosis [406]. To address these temporal aspects and to investigate how specific protease inhibitors influence the kinetics of cell death, we employed live-cell imaging. This real-time approach enabled continuous monitoring of plasma membrane integrity and allowed the onset, progression, and extent of cell death to be followed under different treatment conditions. By moving beyond static endpoints, this strategy provided a more comprehensive understanding of how the trajectory of cell death is shaped by cathepsins, caspases, and their pharmacological inhibition.

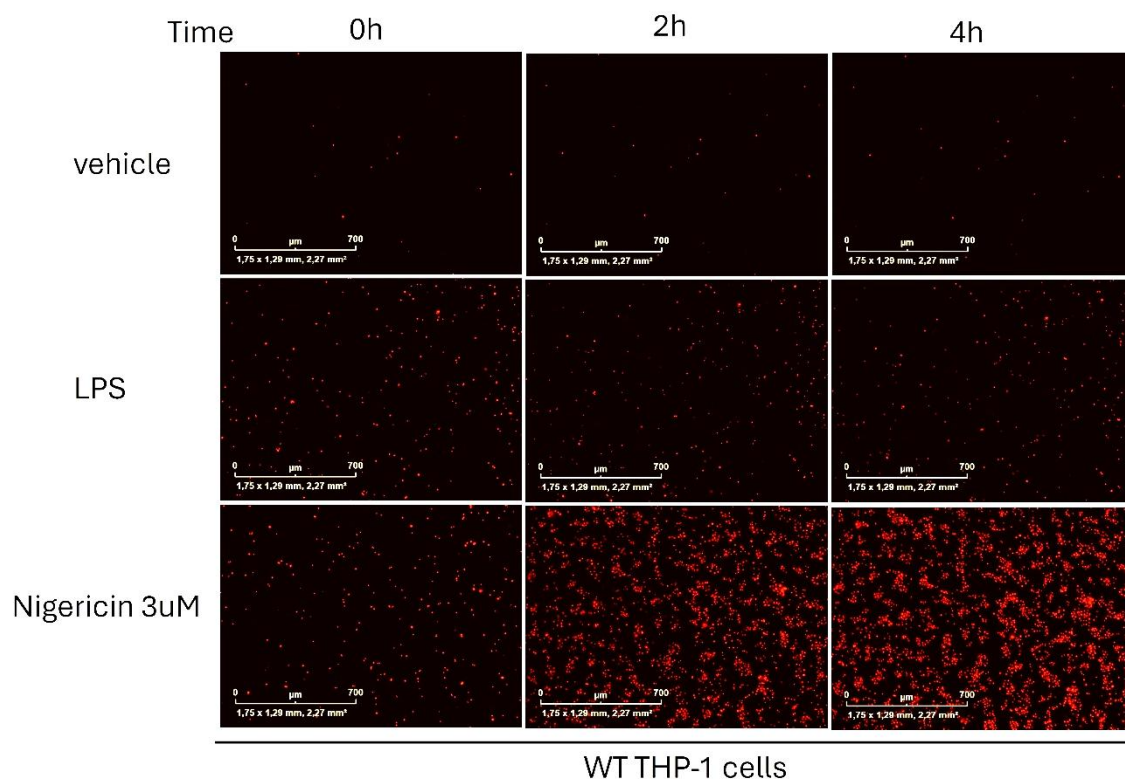
Prior to kinetic analyses of the inhibitor panel, validation experiments confirmed that both LLOMe and nigericin induced a strong, time-dependent response in PMA-differentiated, LPS-primed THP-1 cells, characterized by caspase-3/7 activation (apoptosis) and PI uptake (lytic cell death) (**Figure 40**). In contrast, etoposide and staurosporine (STS) did not trigger significant cell death within the 4-hour observation period (**Figure 41**). Staurosporine and etoposide were included as canonical apoptotic comparators: staurosporine, a broad-spectrum protein kinase inhibitor that classically triggers intrinsic apoptosis via mitochondrial outer membrane permeabilization, cytochrome c release, and caspase-3/7 activation; and etoposide, a topoisomerase II inhibitor that induces p53-dependent mitochondrial apoptosis through DNA double-strand breaks [449-451]. However, under the conditions used and within the 4-hour imaging window, neither agent produced a measurable increase in caspase-3/7 activity or PI uptake, precluding their use as positive apoptotic controls in this dataset. A titration of etoposide over 24 hours confirmed that PI uptake was detectable only after approximately 5 hours of incubation (**Figure 42**), even at higher concentrations, indicating that this agent is not suitable as a control in the 4-hour model of nigericin-induced, inflammasome-dependent cell death. Consequently, they served as low-response/negative controls for early kinetics, while nigericin and LLOMe-both of which elicited rapid, time-dependent responses-were used to resolve inhibitor effects on the trajectory of cell death. This outcome is consistent with reports that staurosporine- or etoposide-induced apoptosis in THP-1-derived macrophage-like cells can

have a slower onset and may require higher doses and/or longer exposure ($\geq 12\text{--}24\text{ h}$) to yield robust terminal readouts [452-454].

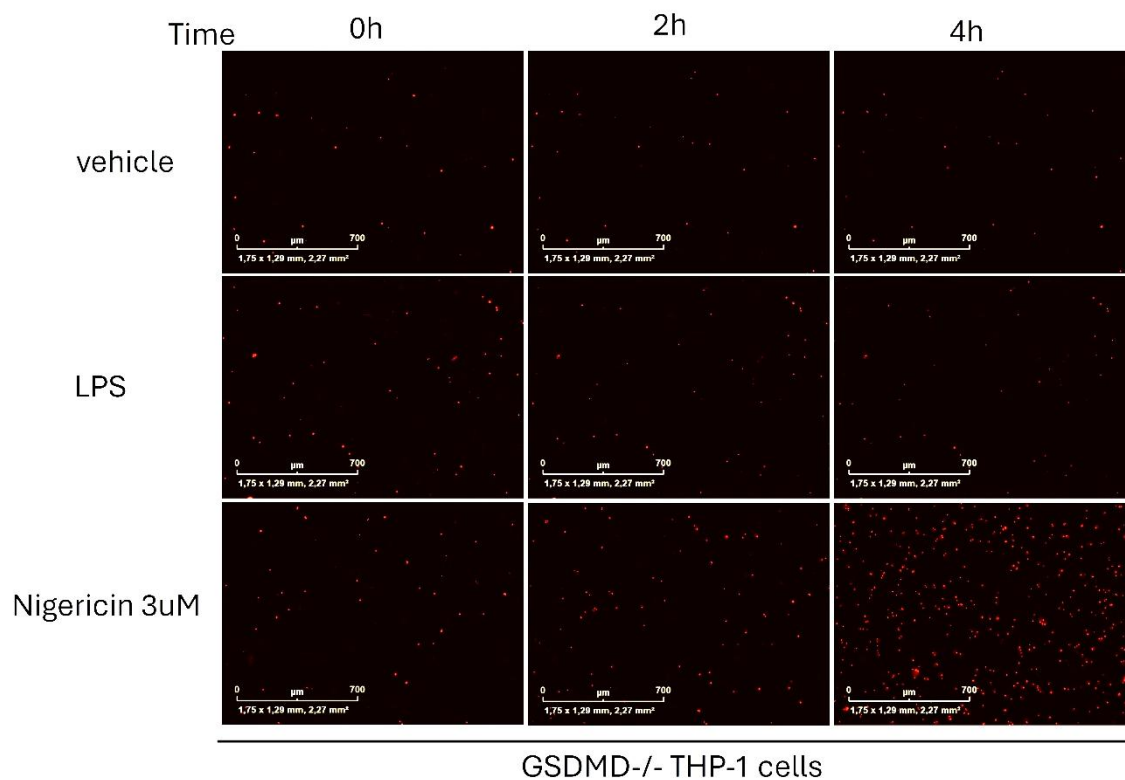


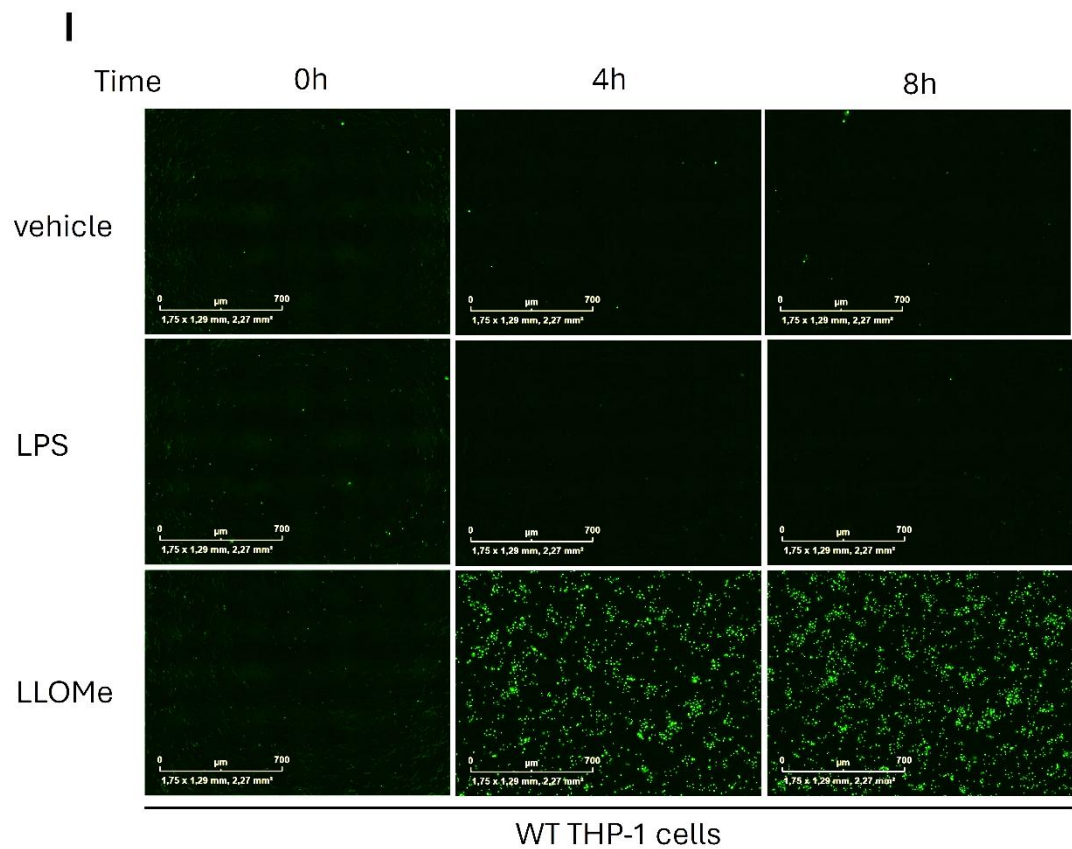
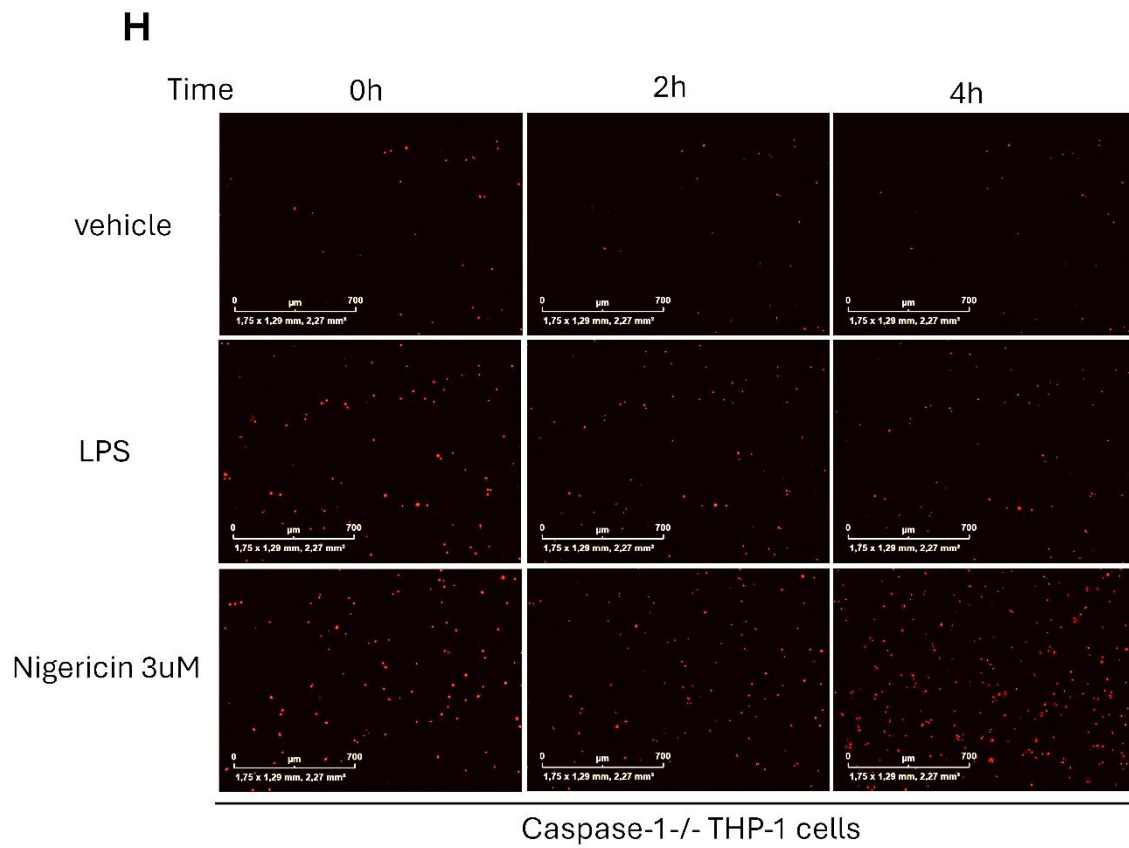
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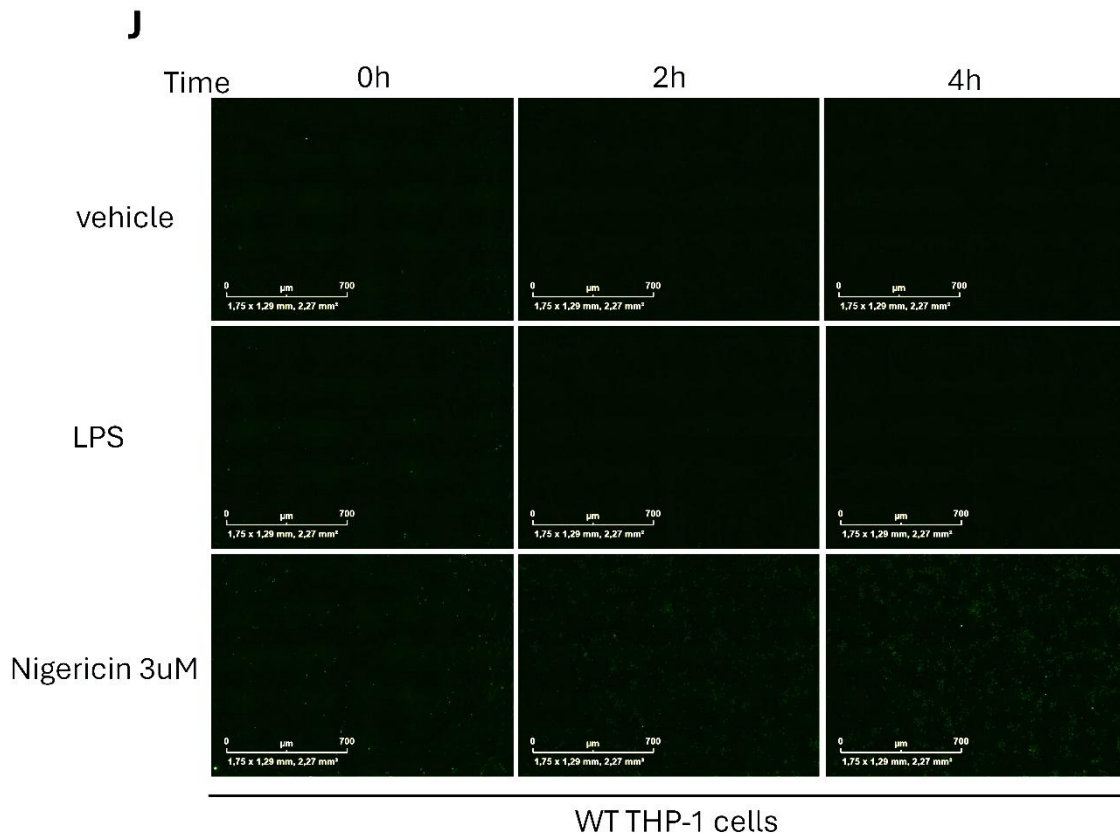


Figure 40. Incucyte®-based kinetic analysis of cell death in PMA differentiated wild-type (WT), caspase-1^{-/-}, and GSDMD^{-/-} cells following stimulation with LPS/LLoMe or LPS/nigericin. A PI uptake marks lytic cell death. B caspase-3/7 activation as a sign of apoptosis. Representative Incucyte® fluorescence images showing PI-positive nuclei following exposure to LLoMe in C WT THP-1 macrophages D GSDMD^{-/-} THP-1 macrophages E Caspase-1^{-/-} THP-1 macrophages. Representative fluorescence micrographs acquired on the Incucyte® system PI uptake after nigericin treatment in F WT THP-1 macrophages G GSDMD^{-/-} THP-1 macrophages H Caspase-1^{-/-} THP-1 macrophages. Representative fluorescence micrograph acquired on the Incucyte® system showing caspase-3/7-positive nuclei following I LLoMe exposure J Nigericin exposure. Each well shown was treated with vehicle control (first row), LPS (second row) and LPS/cell death inducer (third row).

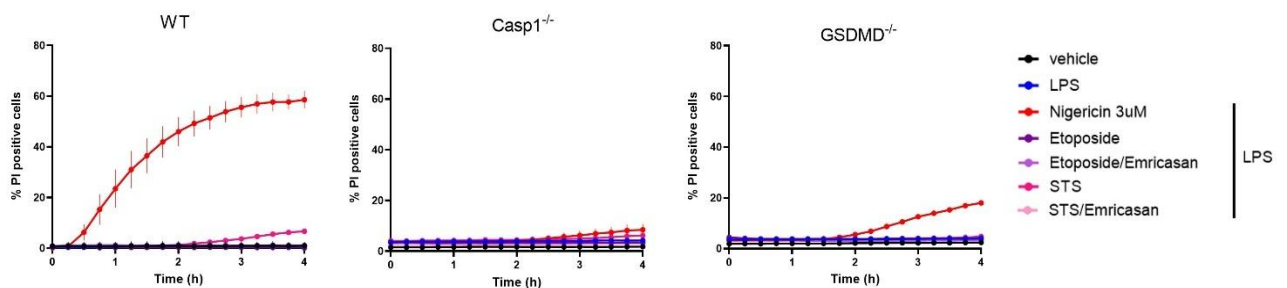


Figure 41. Kinetic study of nigericin induced inflammasome-dependent PI uptake and apoptosis inducers Etoposide (25 μ M) and Staurosporine (1 μ M).

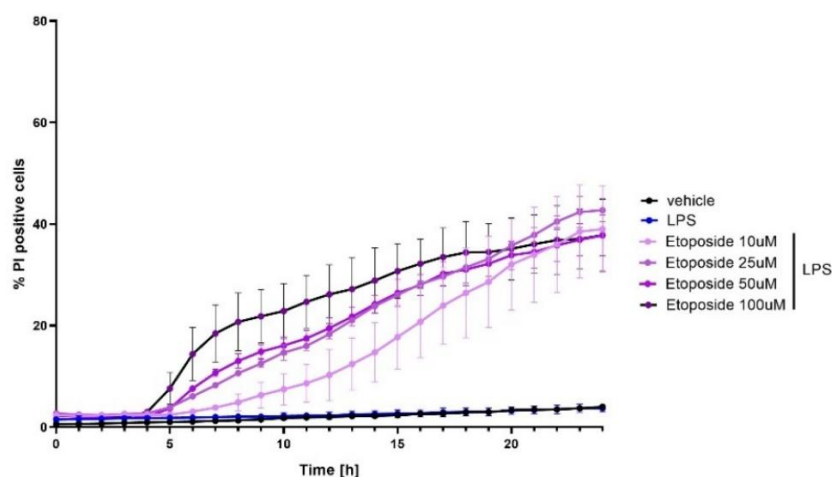


Figure 42. Etoposide titration in LPS-primed M0 macrophages. M0 macrophages primed with LPS were treated with increasing concentrations of etoposide and monitored for 24 h. Cell death was assessed by PI uptake. Even at higher concentrations, detectable membrane permeabilization was only observed after 4-5 h of treatment, indicating that etoposide does not provide an appropriate short-term apoptotic control for comparison with rapid pyroptotic stimuli.

Lipopolysaccharide alone is sufficient to induce lysosomal dysfunction and stress responses [455-457]. Kinetic analyses in PMA-differentiated THP-1 cells indicate that further LLOMe treatment promotes a predominantly inflammatory outcome. LLOMe induces LMP, resulting in the release of lysosomal proteases such as cathepsins into the cytosol. This event has been consistently linked to NLRP3 inflammasome activation. Evidence from cathepsin B-deficient macrophages demonstrates that following NLRP3 stimulation, both IL-1 β production and caspase-1 activation are markedly impaired, reflecting defective inflammasome assembly [358, 359, 458, 459]. In contrast, lactate dehydrogenase (LDH) release, used as a marker of plasma membrane integrity, shows only partial or negligible reduction in cathepsin B-deficient cells, with the extent of the effect depending on the inflammasome stimulus and cellular context [358]. These findings support the view that cathepsin B is indispensable for efficient cytokine maturation and inflammasome signaling, but less critical for the execution of lytic cell death, which can proceed through parallel or redundant pathways. Other cathepsins, including cathepsins L and S, as well as non-lysosomal proteases, have been proposed as potential contributors to maintaining lytic cell death under cathepsin B-deficient conditions, underscoring the functional redundancy of protease networks in inflammasome-driven responses [259, 362, 363]. Building on the initial observations, the contribution of lysosomal proteases to LLOMe-induced cell death was examined using both pharmacological inhibitors and genetic knockout models. Treatment of macrophages with the broad-spectrum cysteine protease inhibitor E64d, or with CA-074Me, a compound more selective toward cathepsin B, conferred almost complete protection against LLOMe-mediated cell death, as measured by plasma membrane integrity assays (**Figure 43**). In contrast, inhibition of caspases did not appreciably alter the cell death outcome; only Emricasan produced a modest delay in the kinetics of cell demise. These findings indicate that classical caspase-dependent apoptosis is not the predominant pathway engaged under these conditions. The role of calpains, which can become activated following LMP through calcium release, was also assessed. Inhibition with calpeptin, a pan-calpain blocker, not only delayed the kinetics of cell death but also preserved

viability in a subset of cells (~20%), as reflected by reduced propidium iodide uptake. The kinetics of PI uptake in LLOMe-treated cells were consistent with LDH release and MTS assay results (**Figure 43**). This partial protection suggests that calpains may act in parallel with cathepsins to amplify the execution phase of LLOMe-induced cell death.

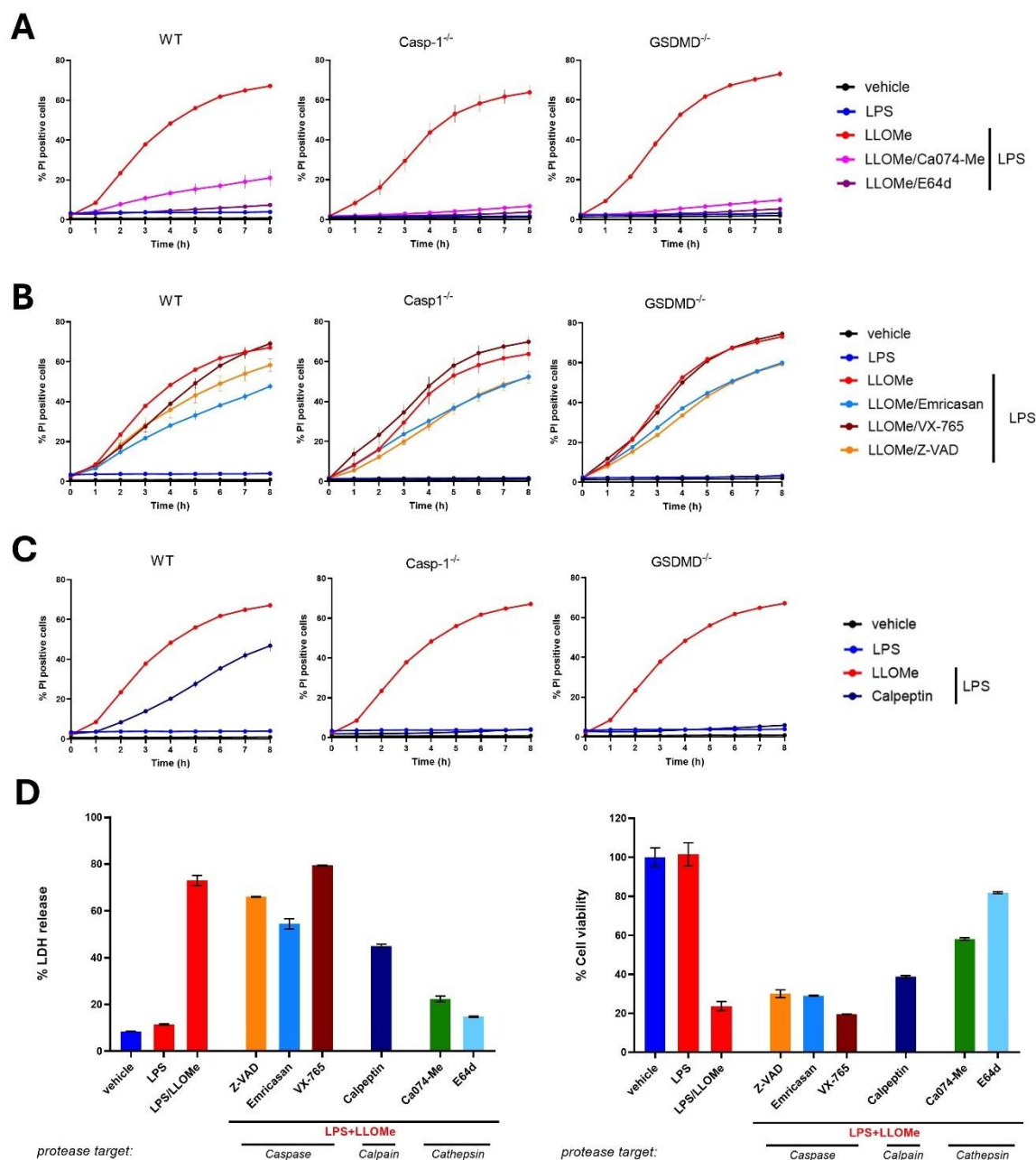


Figure 43. Determinants of LLOMe-induced membrane damage. Kinetics of LPS/LLOMe-induced cell membrane permeability (measured by PI uptake in Incucyte assay) for WT, Casp^{-/-} and GSDMD^{-/-} cell in the presence of **A** pan-cathepsin inhibitors **B** pan-caspase inhibitors **C** pan-calpain inhibitor. **D** Dead cells presented as % LDH release relative to Triton lysis after 8h of LLOMe treatment (left panel) and metabolic activity (MTS) as a % Cell viability after 8h of LLOMe treatment (right panel).

To further dissect the mechanisms of LLOMe-induced cell death, selective cathepsin inhibitors were employed: compounds CatL-19 and CatL-20 targeting cathepsin L, CatS-21 and CatS-22 targeting cathepsin S, and CatB-23 and CatB-24 targeting cathepsin B, along with the

recently developed calpain-1 inhibitors Calp-53 and Calp-54 (named NHI-17 and NHI-18 respectively) [285]. In caspase-1- or GSDMD-deficient backgrounds, treatment with calpeptin, Calp-53 or Calp-54 conferred near-complete protection from LLOMe-induced death, implicating calpain activity as a critical driver of an alternative death pathway that becomes apparent only in the absence of canonical pyroptosis effectors. This protective effect was not observed in wild-type cells, where the caspase-1-GSDMD axis predominates and likely masks the contribution of calpain-dependent mechanisms (**Figure 44**). Application of the highly selective cathepsin inhibitors in caspase-1- and GSDMD-deficient cells revealed that blockade of cathepsin B alone was sufficient to completely rescue cells from death, whereas cathepsin S inhibition yielded partial protection and cathepsin L inhibition exerted little effect after 8h of LLOMe treatment (**Figure 45**). These observations argue against complete functional redundancy among lysosomal proteases in this model, instead highlighting a dominant, non-redundant role for cathepsin B, with cathepsin S acting as a secondary contributor. Cathepsin L, by contrast, appears largely dispensable in the context of LLOMe-induced lysosomal cell death. Together, these findings suggest that when the pyroptotic machinery is disabled, a cathepsin B- and calpain-dependent pathway emerges as the principal executor of LLOMe-induced cytotoxicity, underscoring the hierarchical interplay between lysosomal proteases, calpains, and canonical inflammasome components.

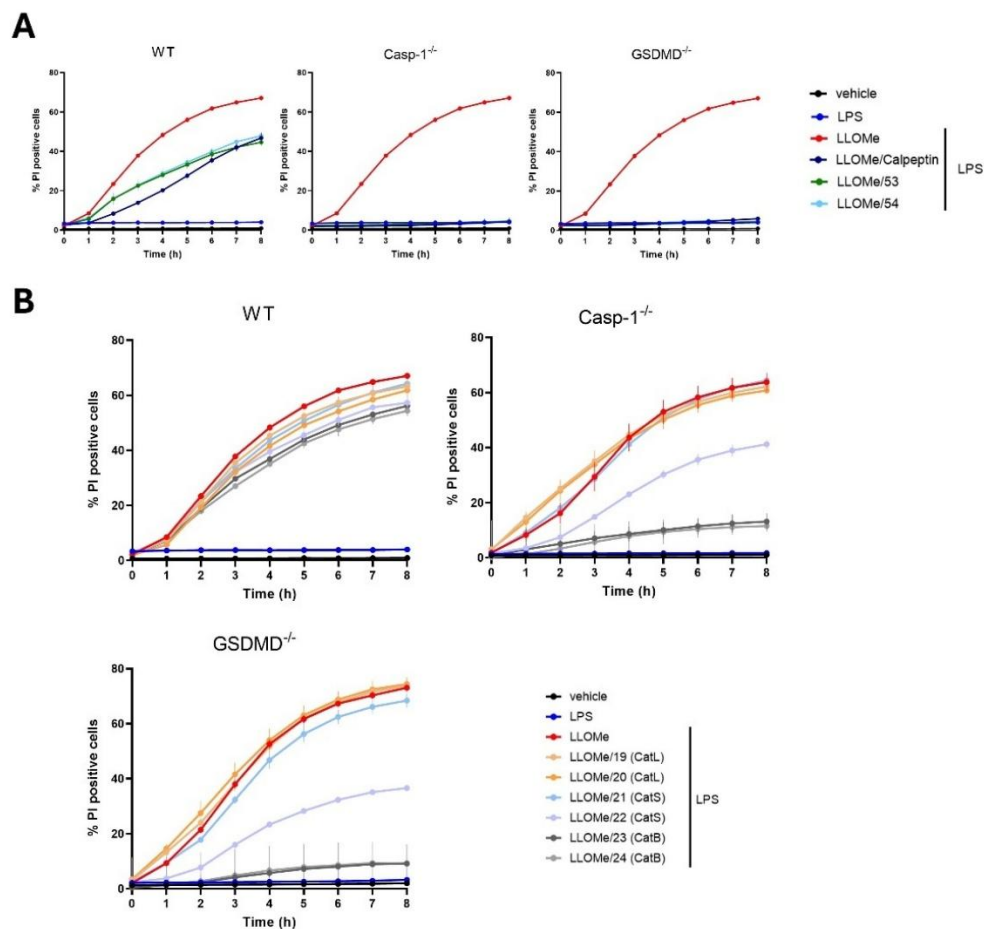


Figure 44. Kinetics of LPS/LLOMe-induced cell membrane permeability (measured by PI uptake in WT, Casp-1^{-/-} and GSDMD^{-/-} cells A pre-incubated with custom calpain-1 inhibitors (commercial Calpeptin inhibitor included for comparison) or B custom cathepsin inhibitors.

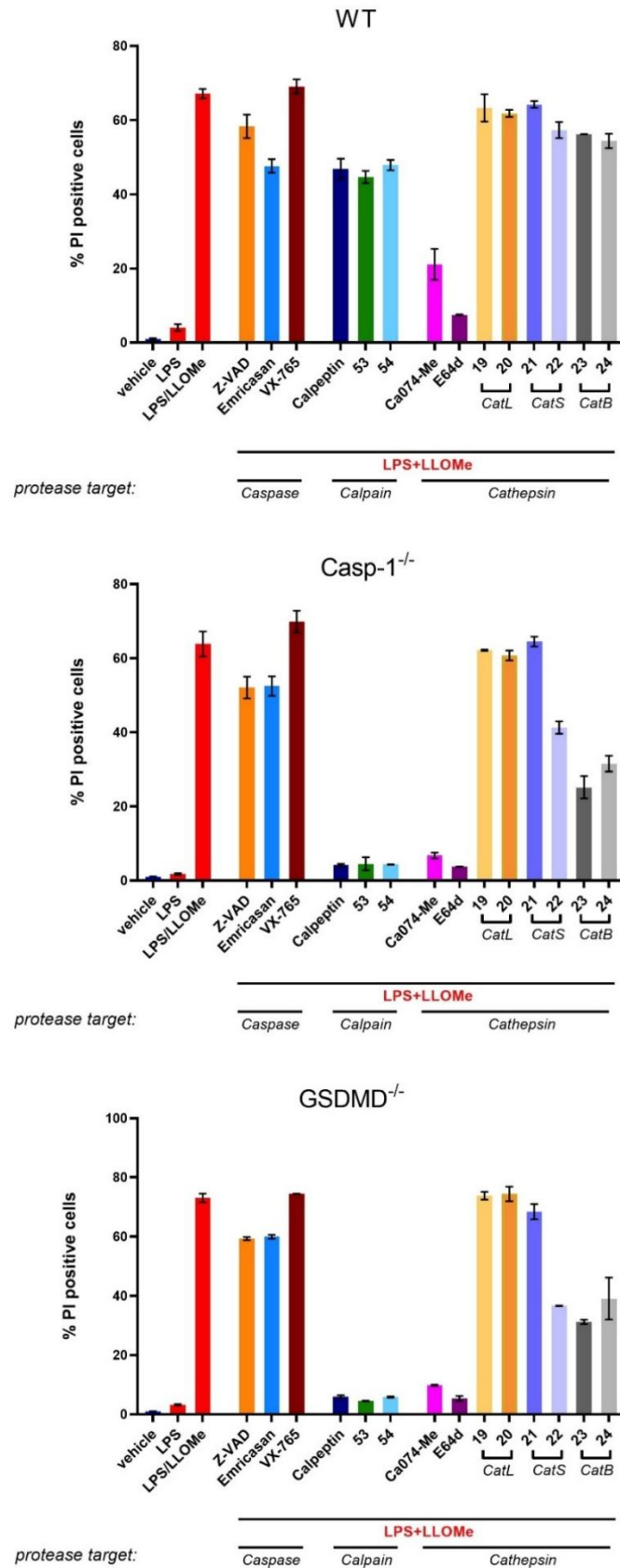


Figure 45. Quantification of PI uptake following LPS/LLOMe treatment in wild-type (WT), caspase-1 knockout (Casp1^{-/-}), and GSDMD knockout (GSDMD^{-/-}) macrophages. Bar graphs represent the effects of broad-spectrum and selective inhibitors (including cathepsin B, cathepsin L, and calpain-1 inhibitors) on membrane damage across genotypes after 8h LLOMe treatment.

To further dissect the mechanisms underlying LLOMe-induced cell death, caspase-3/7 activity was assessed using a DEVD-ase assay in the presence of various inhibitor regimes. As expected, broad-spectrum caspase inhibitors zVAD-fmk and emricasan completely abolished DEVD-ase activity (**Figure 46, 47, 48**), whereas the caspase-1-selective inhibitor VX-765 had no effect. Notably, inhibition of calpains with calpeptin or calpain-1-selective compounds [285], as well as blockade of cathepsins with E64d, CA-074Me, or the cathepsin B-specific inhibitors CatB-23 and CatB-24, markedly reduced LLOMe-induced DEVD-ase activity in M0 macrophages after 8 hours of treatment (**Figure 48**). These findings indicate that cathepsin B and calpain, but not cathepsin L, are critical mediators of executioner caspase activation in this context. Cathepsin S inhibition produced only a minor reduction, further supporting its auxiliary role (**Figure 48**). To evaluate whether this process was NLRP3-dependent, the selective NLRP3 inhibitor MCC950 was applied in the kinetics study. MCC950 delayed caspase-3 activation by approximately one hour, suggesting that LLOMe-induced caspase-3 activation is only partially dependent on NLRP3 signaling. This aligns with the dual consequences of LMP: cathepsin release can activate NLRP3, thereby accelerating inflammasome-driven caspase activation, but LMP can also initiate apoptosis directly through Bid cleavage, mitochondrial permeabilization, and caspase-3 activation. Thus, NLRP3 contributes to the kinetics of apoptotic signaling without being absolutely required. Given that cathepsin B and calpain inhibition prevented caspase-3 activation, the contribution of additional lysosomal proteases was also examined. Remarkably, a custom legumain inhibitor MP-L01 almost completely abrogated caspase-3 activity. Although the role of legumain in lysosome-dependent cell death is less established than that of cathepsins, growing evidence implicates this protease in lysosomal stress responses, regulation of autophagy, and ferroptosis through GPX4 degradation. Our findings suggest that legumain may serve as an unrecognized upstream modulator of executioner caspase activation in lysosomal cell death, extending its functional relevance beyond antigen processing and extracellular matrix remodeling [292, 460].

Taken together, these results indicate that, contrary to models emphasizing complete redundancy among cathepsins, the execution of LLOMe-induced lytic cell death in macrophages is primarily dependent on cathepsin B and calpain, particularly in settings where pyroptotic machinery is compromised. The data further uncover a previously underappreciated role for legumain in linking lysosomal disruption to apoptotic execution. Collectively, these findings highlight the value of combining highly selective pharmacological and genetic tools to resolve the complex interplay of protease networks that orchestrate inflammatory cell death.

A

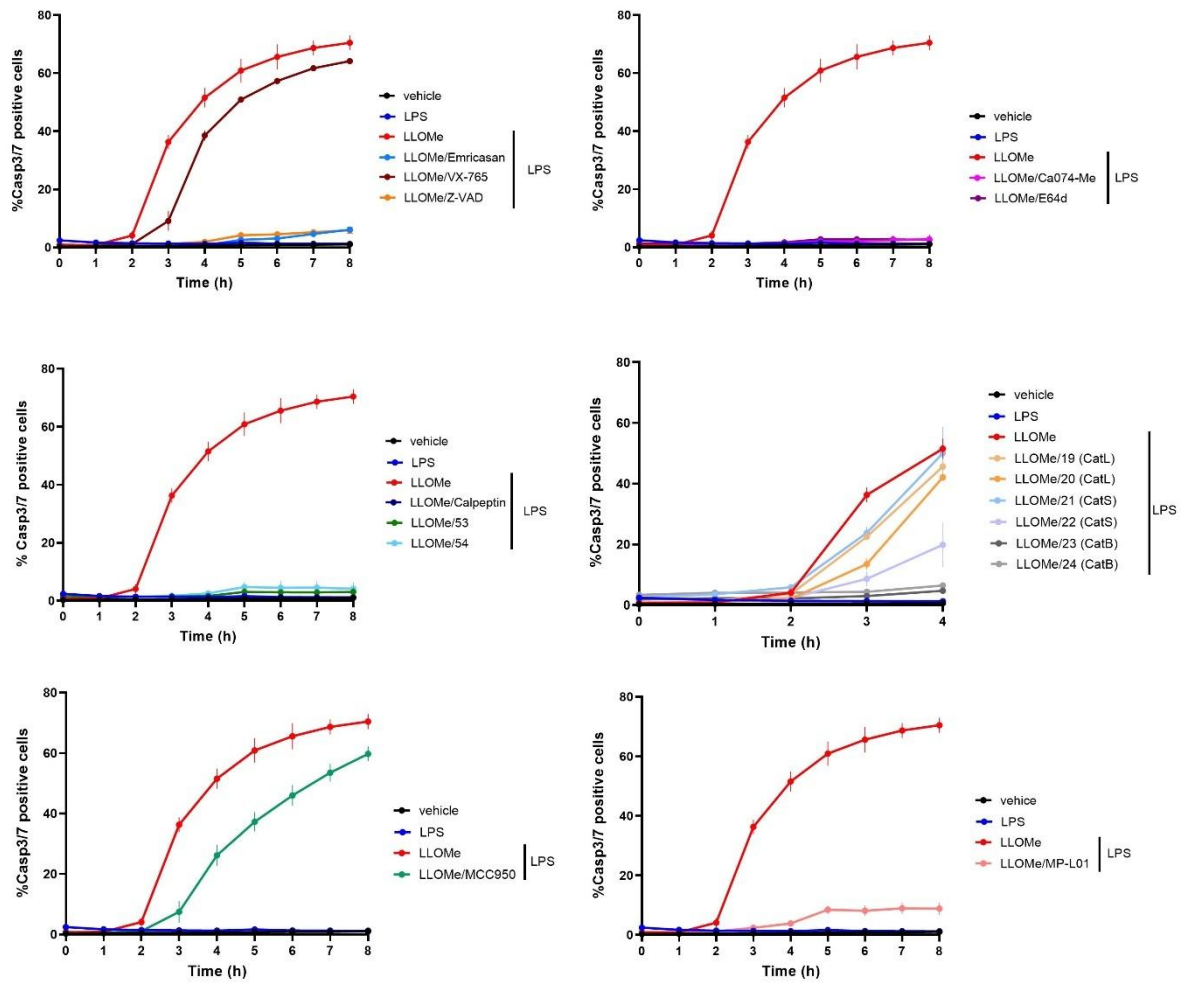


Figure 46. Caspase-3/7 activation after LLOMe treatment. A Time-course analysis of and DEVDase activity in LPS-primed WT M0 THP-1 cells treated with LLOMe, in the presence of inhibitor panel.

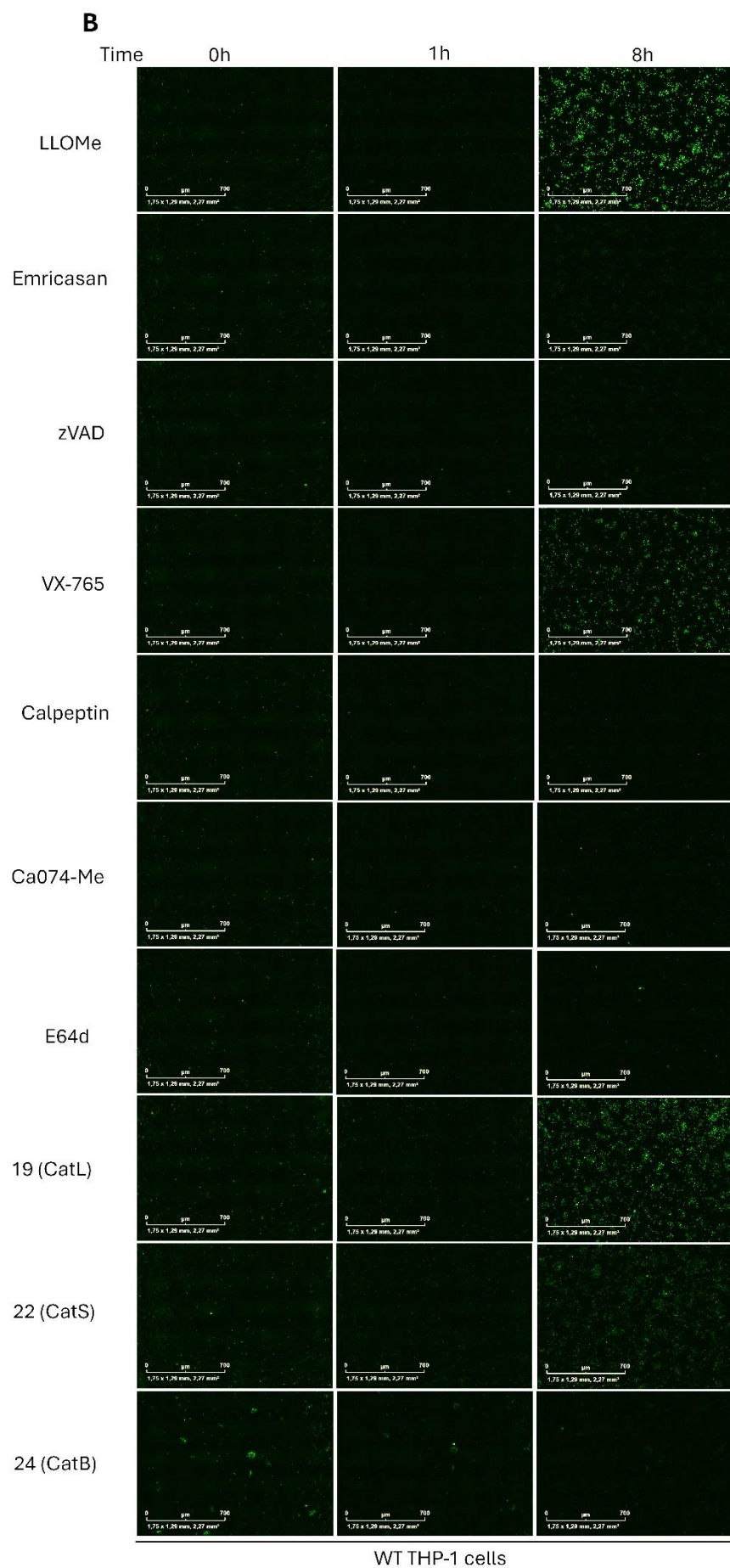


Figure 47. live-cell fluorescence imaging of control and LLOMe treated cells. Representative fluorescence micrograph acquired on the Incucyte® system showing caspase-3/7-positive nuclei (green) in PMA differentiated WT THP-1 cells following LLOMe exposure. Each well shown was treated with LPS/LLOMe (first row) or LPS/25 μ M inhibitor/LLOMe (following rows).

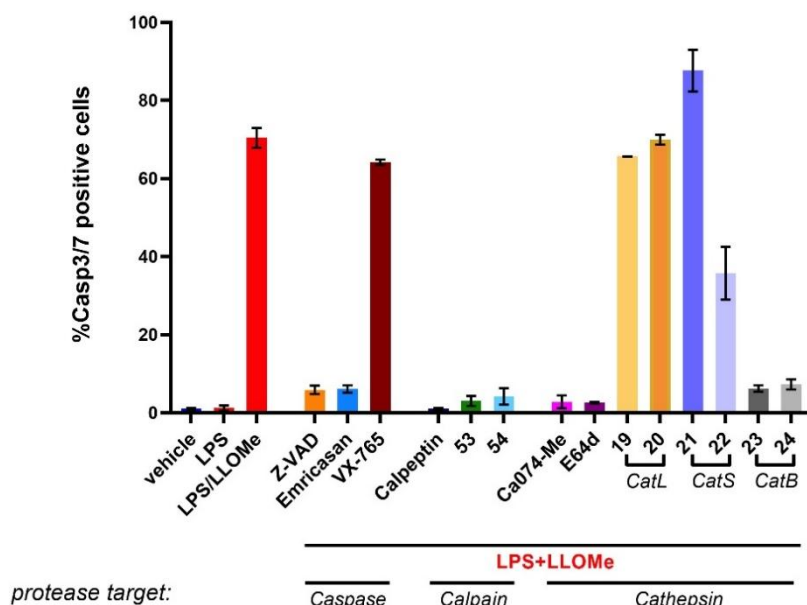


Figure 48. Caspase-3/7 activity assay in WT M0 macrophages treated with LPS/LLOMe, showing the impact of pan-caspase, pan-cathepsin, calpain, and selective protease inhibitors after 8h of LLOMe treatment.

To investigate whether activation of the NLRP3 inflammasome, which drives canonical pyroptosis, requires lysosomal protease activity, M0 WT, Casp^{-/-} and GSDMD^{-/-} macrophages were primed with LPS and subsequently treated with nigericin, a well-established NLRP3 agonist. Plasma membrane integrity was assessed by PI uptake, and executioner caspase activity was monitored using a DEVD-ase assay. At low concentrations (3 μ M), nigericin-induced cell death was substantially reduced by the broad-spectrum cysteine protease inhibitors CA-074Me and E64d (**Figure 49**). This indicates that, similar to LLOMe treatment, multiple parallel cell death pathways are engaged. However, the two stimuli diverge in their dependence on the inflammasome: whereas LLOMe-induced death proceeds independently of caspase-1 and GSDMD, nigericin-induced death is abrogated in cells lacking these effectors (**Figure 43**).

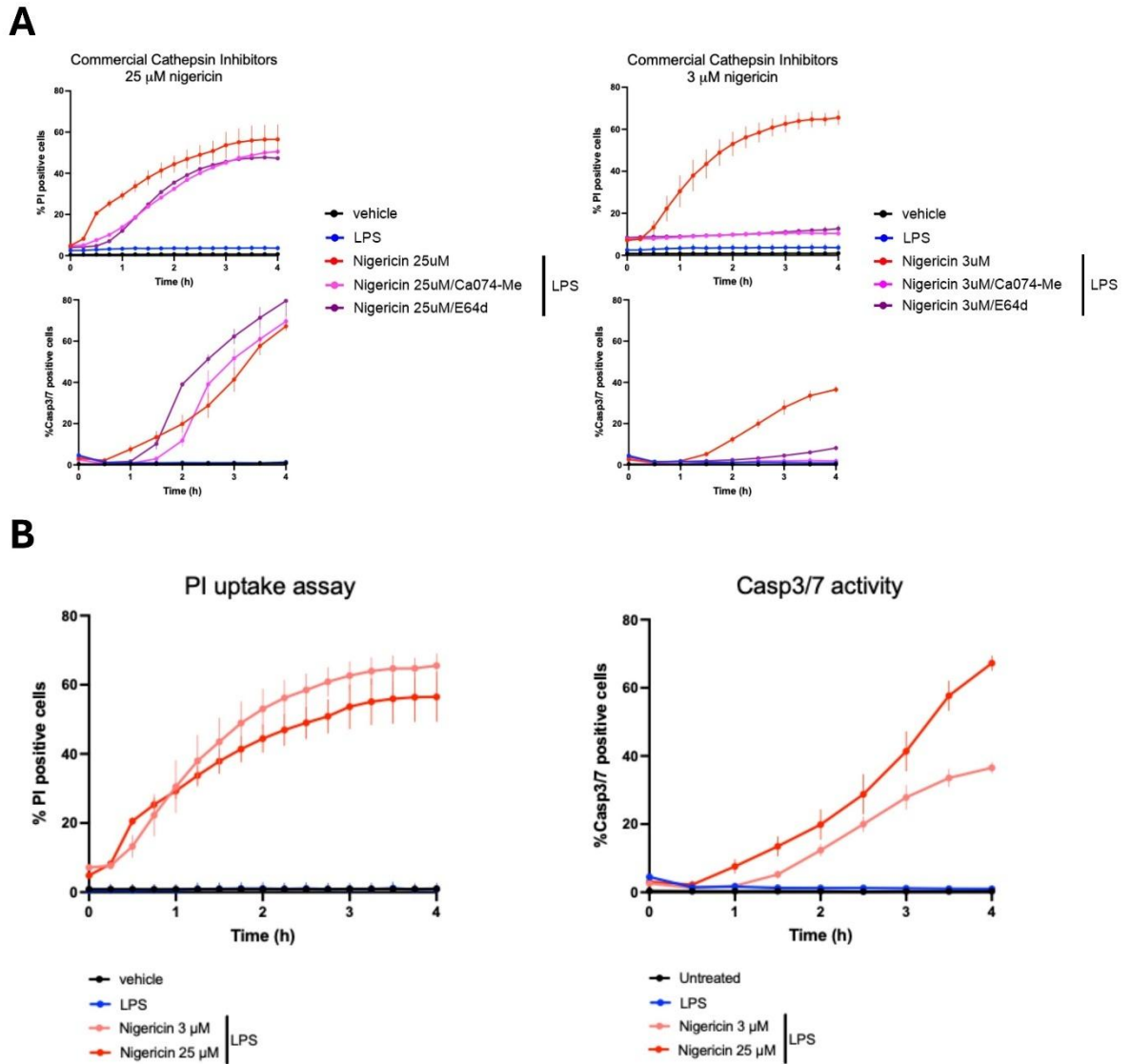


Figure 49. Cathepsin inhibition protects against moderate but not high-dose nigericin. A. Time-course analysis of PI uptake (top) and DEVDase activity (bottom) in LPS-primed M0 THP-1 cells treated with nigericin at either 25 μ M (left) or 3 μ M (right), in the presence of cathepsin inhibitors CA-074Me or E64d. B. PI uptake assay and DEVDase activity in PAM differentiated cells treated with LPS alone or with nigericin (3 or 25 μ M).

Even though elevated nigericin concentrations (25 μ M) produced comparable levels of PI uptake in treated cells (**Figure 49**) and slightly more robust Caspase-3/7 activation, this high-dose response was insensitive to cathepsin inhibition (**Figure 49**), suggesting that elevated nigericin bypasses lysosomal inputs and induces nonspecific effects. Both E64d and CA-074-Me effectively protected WT cells from lytic cell death when lower concentrations of nigericin were applied (**Figure 50, 51**). Emricasan, a pan-caspase inhibitor, also fully preserved cell viability, with no detectable PI signal throughout the 4-hour assay. In contrast, the remaining inhibitors showed minimal to no ability to maintain membrane integrity. The use of selective inhibitors further demonstrated that no single lysosomal protease was indispensable at 3 μ M nigericin (**Figure 51, 52**); instead, their combined activity appears to be required to amplify

inflammasome signaling and promote IL-1 β maturation and release. This observation aligns with the notion that low-dose nigericin does not cause complete lysosomal rupture but instead elicits sublethal lysosomal stress, permitting controlled protease leakage that enhances NLRP3 activation and inflammatory signaling. Under these conditions, pharmacological inhibition or genetic ablation of proteases may allow cells to rebalance ionic fluxes and repair subthreshold membrane damage, thereby limiting pyroptotic progression.

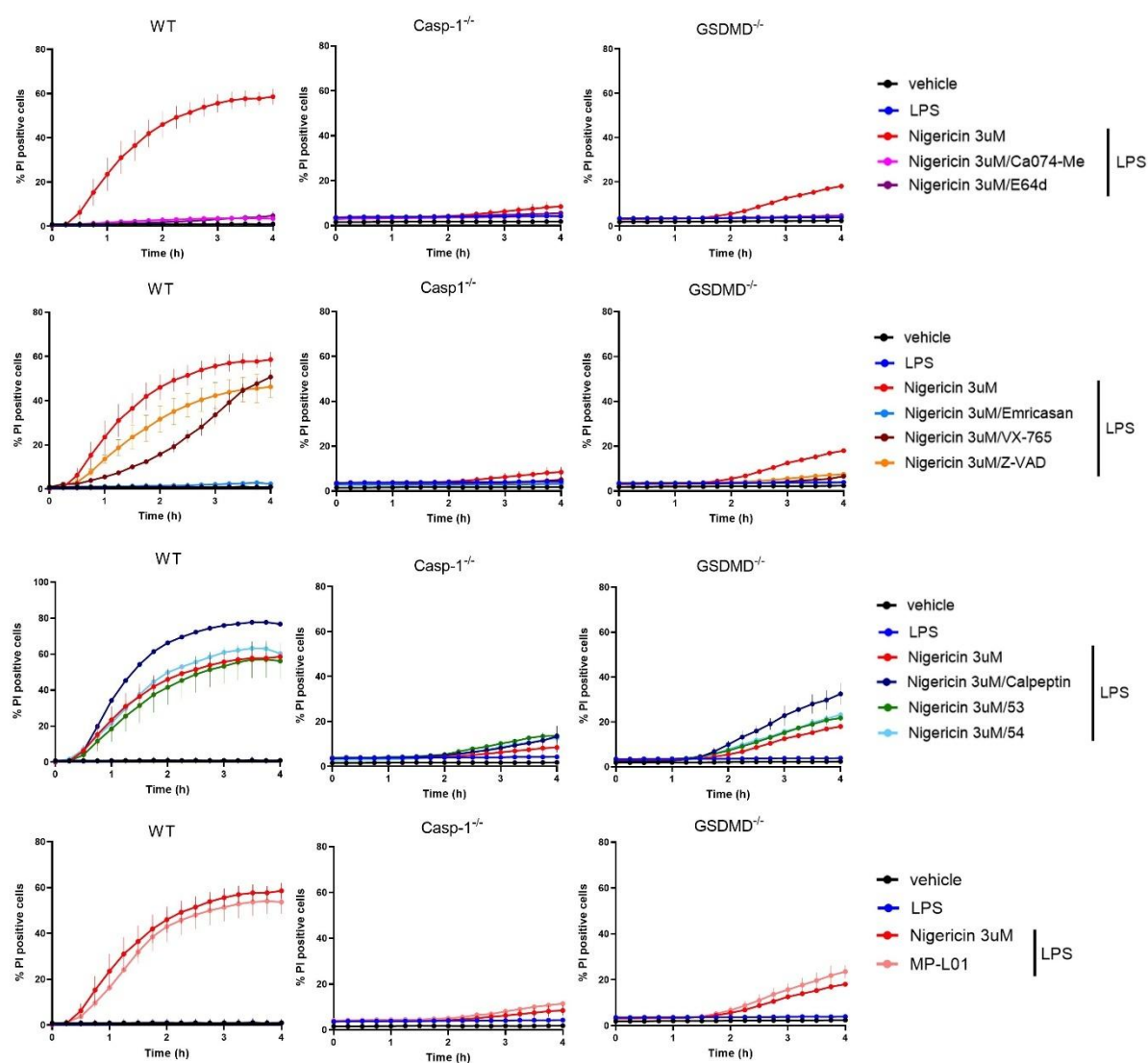


Figure 50. Determinants of nigericin-induced membrane damage. Kinetics of LPS/nigericin-induced cell membrane permeability (measured by PI uptake in Incucyte assay) for WT, Casp^{-/-} and GSDMD^{-/-} cells in the presence of pan-cathepsin inhibitors (CA-074-Me, E64d), pan-caspase inhibitors (emricasan, VX-765, zVAD-fmk), pan-calpain inhibitor (calpeptin), legumain inhibitor (MP-L01).

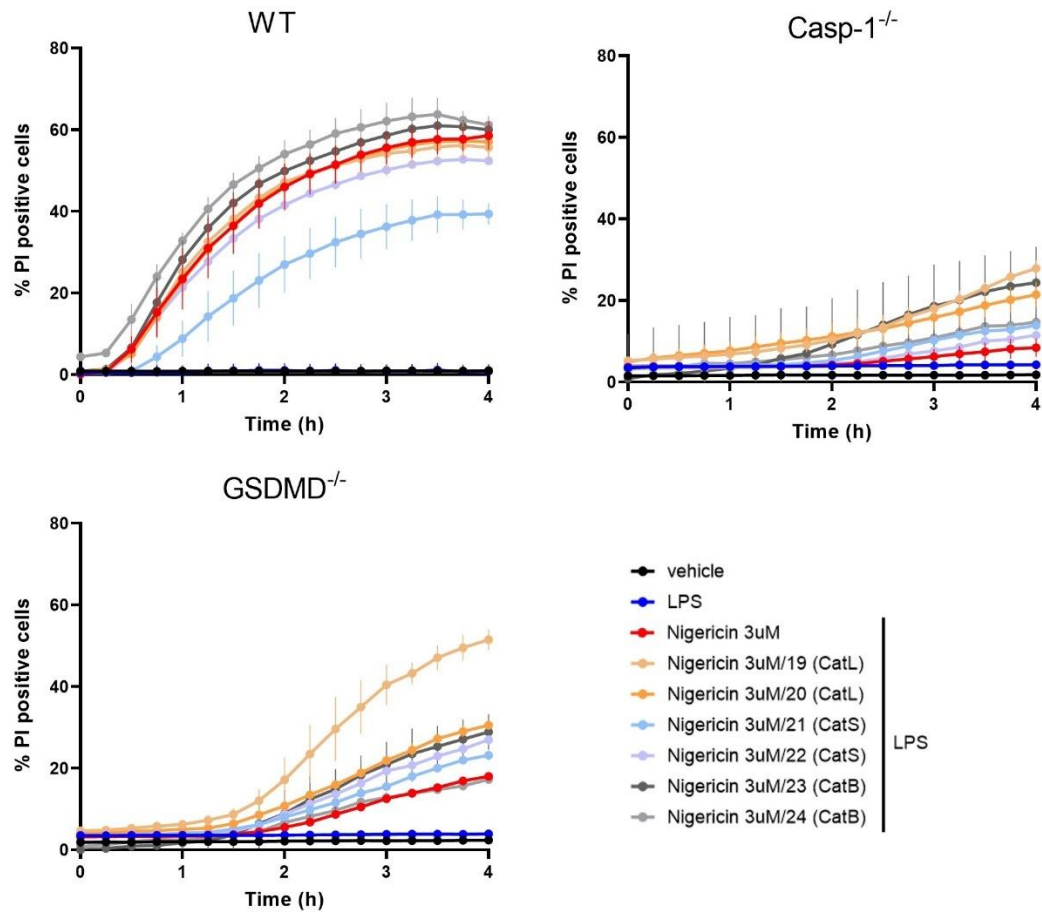


Figure 51. Determinants of nigericin-induced membrane damage. Kinetics of LPS/nigericin (3 μ M)-induced cell membrane permeability (measured by PI uptake in WT, Casp-1^{-/-} and GSDMD^{-/-} cells pre-incubated with custom cathepsin L, cathepsin S and cathepsin B inhibitors.

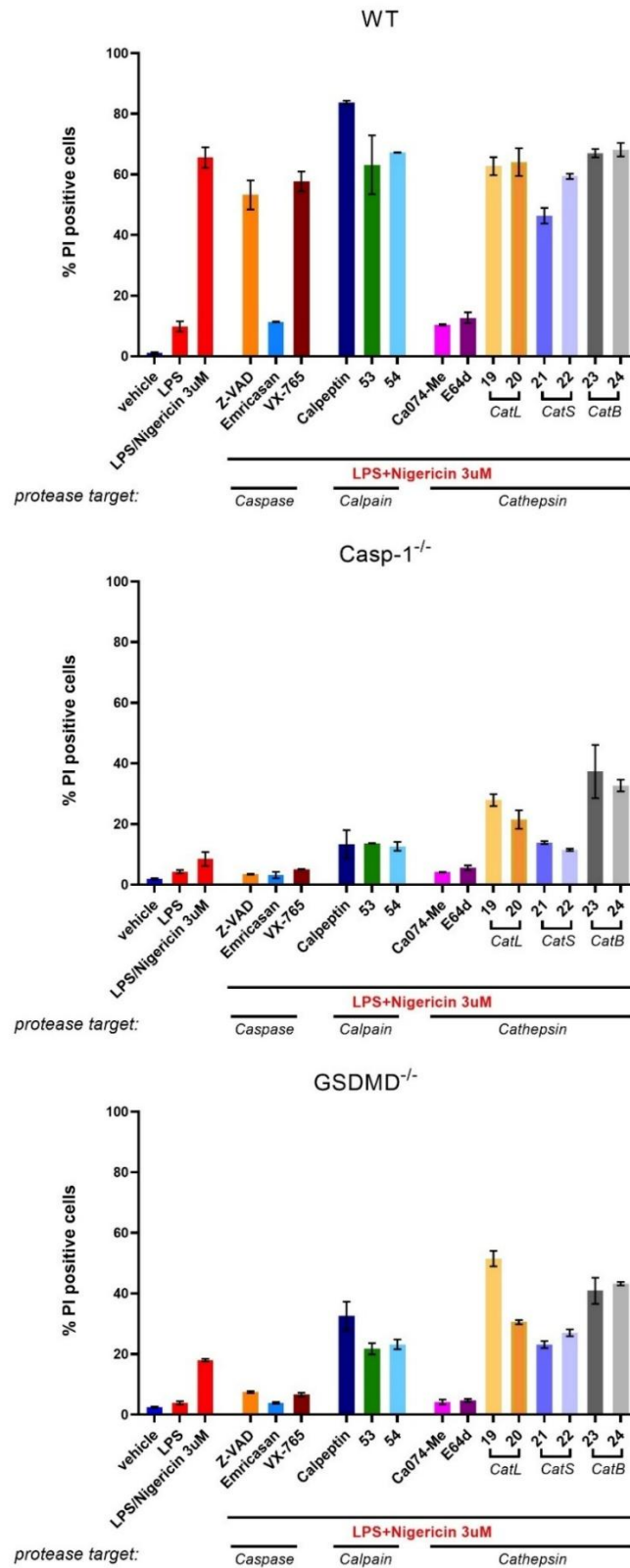


Figure 52. Quantification of PI uptake following LPS/nigericin (3 μ M) treatment in wild-type (WT), caspase-1 knockout (Casp1 KO), and GSDMD knockout (GSDMD KO) macrophages. Bar graphs represent the effects of broad-spectrum and selective inhibitors (including cathepsin B, cathepsin L, calpain-1 and legumain inhibitors) on membrane damage across genotypes after 8h of nigericin (3 μ M)treatment.

DEVD-ase assays further revealed that apoptotic caspase activity was strongly reduced by E64d and CA-074Me but remained unaffected by the caspase-1-selective inhibitor VX-765 (**Figure 53**). This finding demonstrates that apoptotic caspase activation lies downstream of lysosomal protease activity rather than directly downstream of canonical NLRP3 signaling. At higher nigericin concentrations, however, these distinctions could not be resolved: caspase activity persisted regardless of cathepsin inhibition, reflecting the overwhelming cellular damage imposed by excessive ionic dysregulation (**Figure 54**). Such doses, frequently used in the literature, may therefore mask protease-dependent signaling events and obscure physiological mechanisms.

Together, these findings support a model in which cathepsins function as conditional amplifiers of NLRP3 responses under moderate nigericin stress, facilitating both pyroptotic execution and crosstalk with apoptotic caspases. In contrast, supra-physiological nigericin exposure induces catastrophic ionic collapse and organelle failure, overriding protease-regulated checkpoints and committing cells to irreversible lytic death.

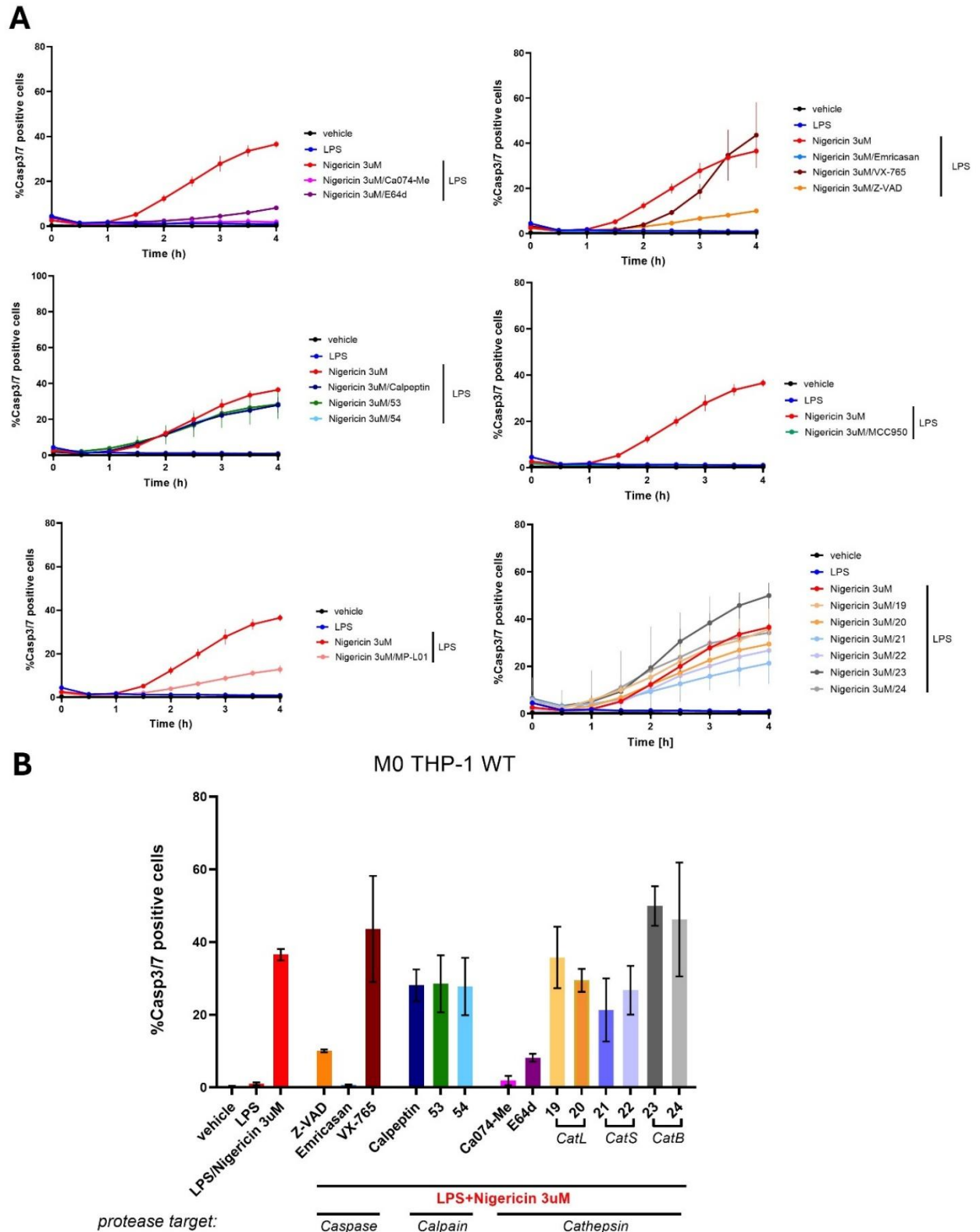


Figure 53. Kinetics of LPS/nigericin (3uM)-induced caspase-3/7 activation in WT M0 macrophages (measured by Caspase3/7 activity Incucyte® assay) in the presence of pan-cathepsin, pan-caspase, calpain inhibitors, selective NLRP3 inhibitor or custom inhibitors towards cysteine cathepsins and legumain.

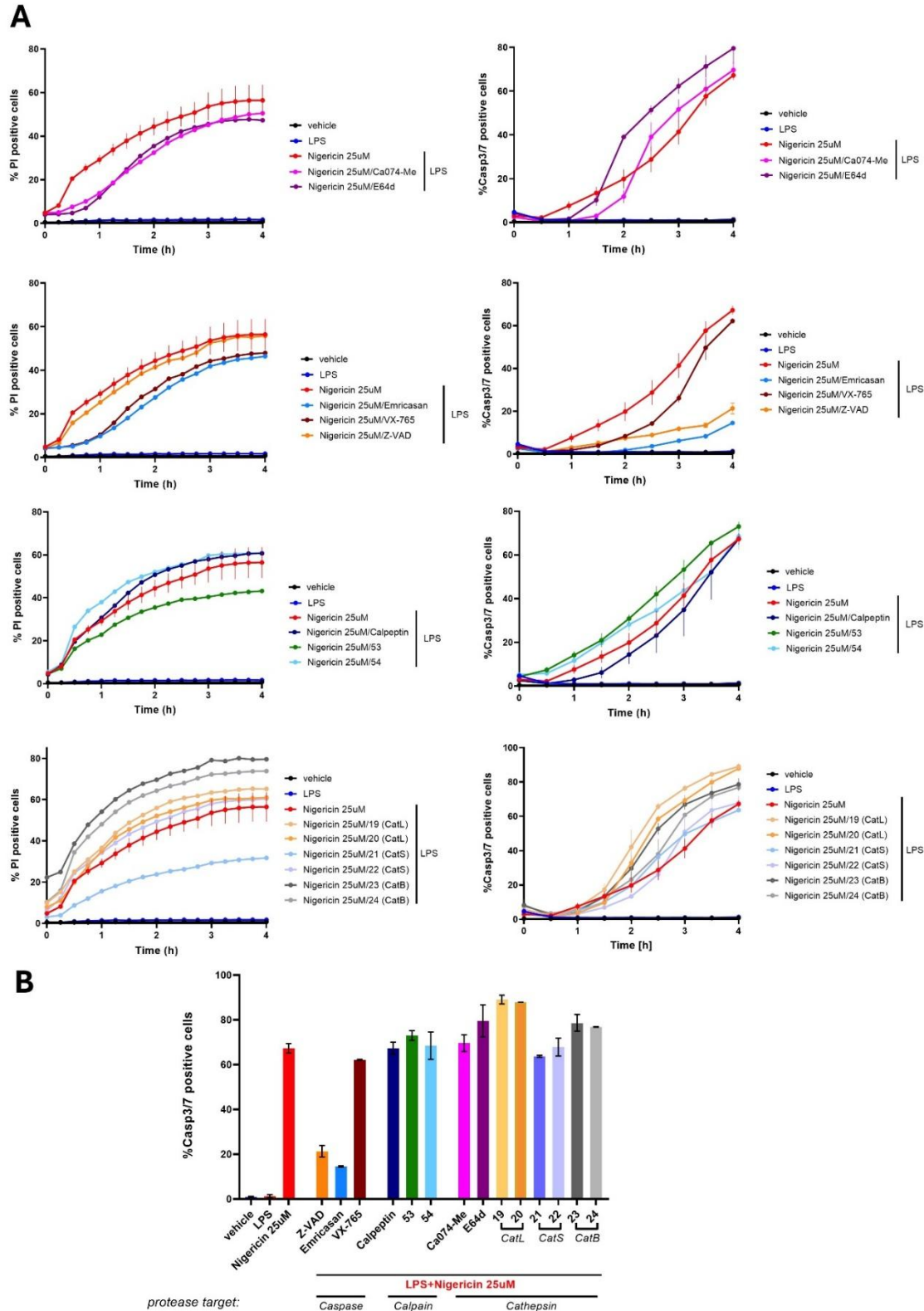
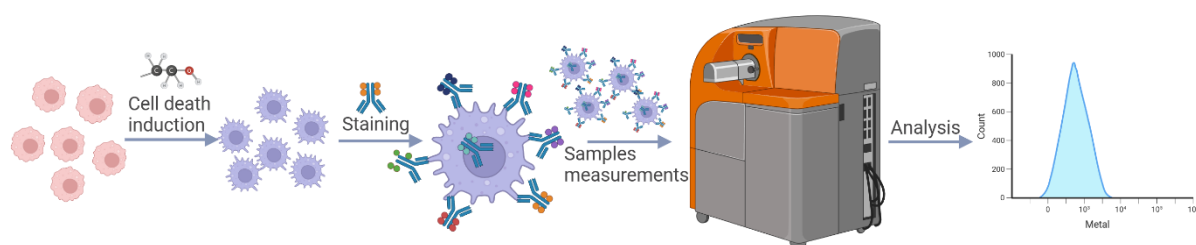


Figure 54. Determinants of nigericin (25 μ M)-induced membrane damage and caspase-3/7 activation in WT macrophages by inhibitor profile. **A** Kinetics of LPS/nigericin-induced cell membrane permeability (measured by PI uptake in Incucyte assay) and caspase-3/7 activation (measured by Caspase3/7 activity Incucyte assay) in the presence of pan-cathepsin, pan-caspase, pan-calpain or custom inhibitors. **B** Caspase-3/7 activity assay in WT M0 macrophages treated with LPS/nigericin, showing the impact of pan-caspase, pan-cathepsin, calpain, and selective protease inhibitors after 4 hours of nigericin treatment.

4.11. MASS CYTOMETRY ANALYSIS OF EARLY CELL DEATH SIGNATURES

To gain deeper insight into the complex processes of cell death, which involve numerous proteins acting at distinct stages, additional analyses were performed beyond endpoint assays such as Western blotting and kinetic studies using Incucyte live-cell imaging. To more precisely interrogate the early events in these pathways, mass cytometry was employed, a powerful single-cell technology capable of simultaneously detecting up to 60 markers and thereby capturing multiple death-associated events in parallel (**Figure 55**). PMA-differentiated wild-type THP-1 cells (M0 macrophages) were stimulated with either LLOMe or nigericin and stained with pyroptosis-associated markers, including antibodies against GSDMD, pro-IL-1 β , and high mobility group box 1 (HMGB1), to compare the responses induced by the two stimuli.



Created in <https://BioRender.com>

Figure 49. Mass cytometry experiment setup

HMGB1, a chromatin-associated protein that normally stabilizes DNA architecture, regulates transcription, and supports DNA repair, is released upon loss of plasma membrane integrity. Once extracellular, it functions as a prototypical damage-associated molecular pattern, promoting inflammatory cytokine production and recruiting immune cells [461-463]. Thus, monitoring HMGB1 release serves as a sensitive readout of terminal lytic death and immunogenic signaling. To interpret these changes at the single-cell level, histogram displays illustrate how a given marker is distributed across the analyzed population. In such plots, the x-axis reflects the detected signal intensity, whereas the y-axis represents the number of events at each intensity value. Shifts in peak position indicate alterations in marker abundance, while differences in peak width or height reveal population heterogeneity and the proportion of cells exhibiting specific expression levels. Extended exposure to either LLOMe or nigericin resulted in nearly complete release of HMGB1, accompanied by complete release of GSDMD and pro-IL-1 β (**Figure 56**). By contrast, shorter treatments with both agents produced only partial release of these markers. This indicates that both stimuli rapidly drive terminal lytic programs, but that the dynamics of marker release depend on treatment duration. Accordingly, to resolve the early regulatory events preceding full membrane rupture, shorter incubation times are required.

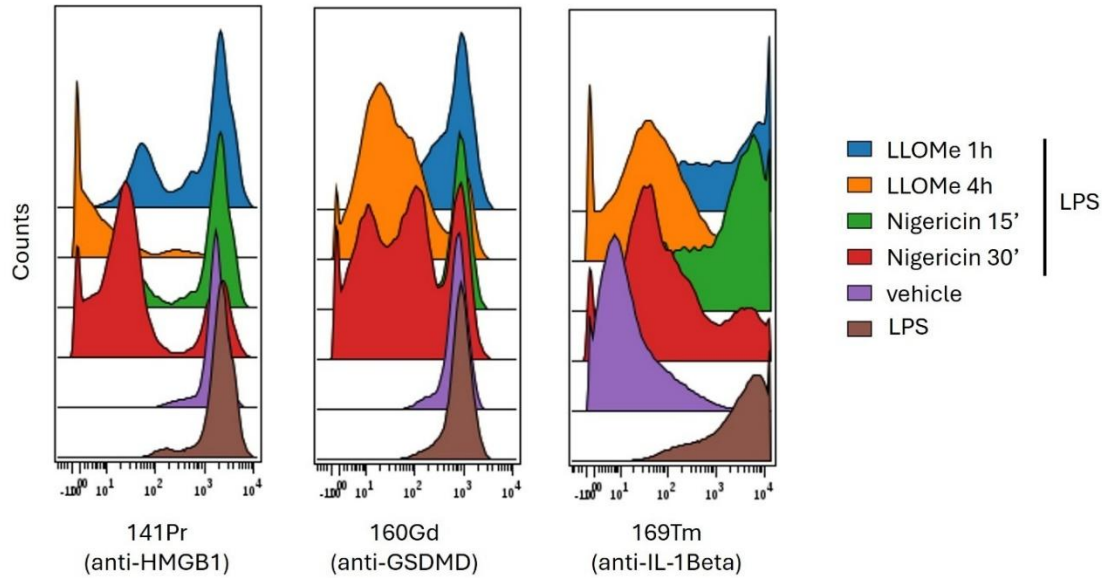


Figure 50. Histogram representation of pyroptosis-associated markers by mass cytometry. PMA-differentiated wild-type THP-1 cells were stimulated with LPS followed by either nigericin (25 μ M) or LLOMe (500 μ M) for the indicated times. Cells were stained with antibodies against GSDMD, IL-1 β , and HMGB1 to monitor pyroptosis-associated protein dynamics.

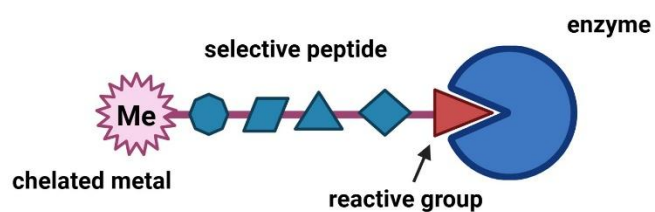
To further dissect the mechanisms underlying LLOMe- and nigericin-induced cell death, PMA-differentiated macrophages were analyzed using a custom-designed mass cytometry panel tailored to capture multiple regulated death pathways in parallel. The panel integrated antibody-based markers for pyroptotic signaling, apoptotic execution, and cross-pathway regulators, along with custom time-of-flight activity-based probes (TOF-ABPs) to directly monitor caspase activity at single-cell resolution. TOF-probe technology was originally developed in the Poreba laboratory and is now an established approach, enabling covalent labeling of active proteases in complex biological systems with single-cell resolution [285, 369]. The pyroptosis arm of the panel included markers for inflammasome-dependent cytokines (cleaved IL-1 β , total IL-1 β , and IL-18), cleaved and total GSDMD (the pore-forming effector of pyroptosis), the NLRP3 sensor, caspase-1 (canonical inflammasome), caspase-4 and caspase-5 (noncanonical inflammasome effectors in humans), and NINJ1, a mediator of plasma membrane rupture downstream of gasdermin pores. Apoptotic pathways were monitored through cleaved caspase-3 and cleaved caspase-7 (executioner caspases), caspase-8 (extrinsic initiator), PARP (a substrate of caspase-3 and hallmark of apoptosis), and FLIP, a caspase-8 regulator and critical checkpoint balancing apoptosis and necroptosis. Cross-talk regulators were incorporated to capture pathway interplay, including TNF- α , a death receptor ligand that triggers both apoptotic and necroptotic signaling, and FLIP, which modulates the caspase-8/RIPK1 node controlling the apoptosis–necroptosis switch. General lytic markers included LDH, a measure of plasma membrane disruption, and GSDME (DFNA5), which can convert apoptotic signaling into a secondary pyroptosis-like phenotype following caspase-3 cleavage. Finally, enzyme-specific TOF-ABPs were included to provide direct readouts of caspase activity: WEHD as a caspase-1 reporter, IETD for caspase-8, DEVD for caspase-3/7, and LEHD for caspase-9 (**Table 7**). Unlike conventional antibodies, which detect the entire pool of

available protein regardless of its activation state, TOF-ABPs covalently label only the enzymatically active forms of their targets. This selectivity enables discrimination between inactive zymogens, processed but inactive intermediates, and catalytically active caspases. The integration of antibody-based markers with TOF-ABPs therefore provides both structural and functional resolution, allowing the dynamics of inflammasome engagement, apoptotic execution, and cross-pathway compensation to be captured within a single experiment.

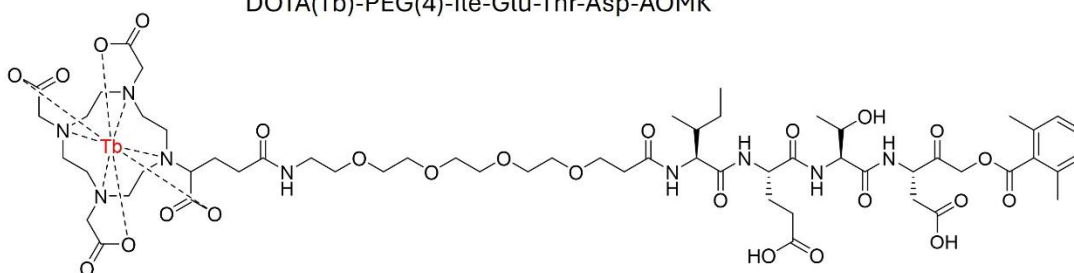
Table 7. The kinetic parameters ($k_{\text{obs}}/[\text{I}]$, $\text{M}^{-1} \text{s}^{-1}$) of TOF-probes toward caspase-1 and caspase-3

$k_{\text{obs}}/[\text{I}]$, $\text{M}^{-1} \text{s}^{-1}$	DOTA(^{159}Tb)-PEG-IETD-AOMK	DOTA(^{165}Ho)-PEG-LEHD-AOMK	DOTA(^{175}Lu)-PEG-DEVD-AOMK	DOTA(^{141}Pr)-PEG-WEHD-AOMK
Caspase-1	65,750	54,000	35,400	57,000
Caspase-3	51,500	11,000	1,634,000	138,000

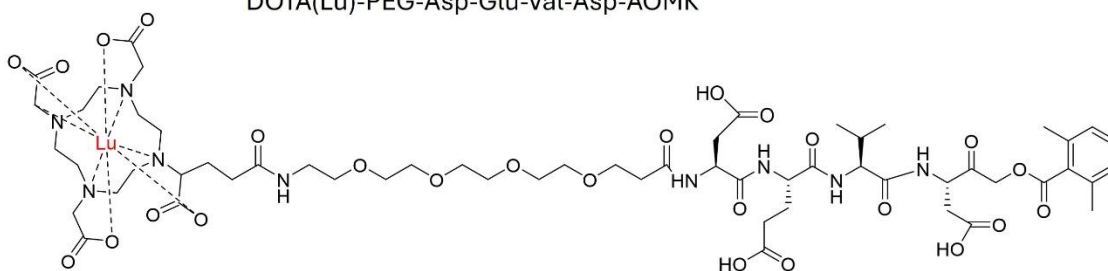
PMA-differentiated WT THP-1 cells, either primed or unprimed with LPS, were exposed to the canonical NLRP3 activator nigericin or to the lysosomotropic agent LLome for short incubation times in order to capture early events that dictate cell fate. In this experiment, HMGB1 was not included, as earlier analyses had already demonstrated that nigericin treatment leads to terminal lytic death with associated immunogenic DAMP release. Instead, an enzyme-specific TOF-probes for caspases (**Figure 57**) were employed to provide a more direct readout of the central protease driving pyroptotic signaling. As anticipated, nigericin stimulation induced a robust pyroptotic profile. Caspase-1 activation was clearly detectable and accompanied by processing and release of IL-1 β and IL-18, as well as cleavage of GSDMD, confirming the canonical inflammasome pathway (**Figure 58**). Some markers appeared difficult to capture, most likely due to their transient activation or loss through GSDMD pores during rapid membrane permeabilization. Nevertheless, increased signals for NINJ1, a downstream mediator of plasma membrane rupture, and TNF- α supported the presence of a pro-inflammatory signaling cascade. Collectively, these results confirm that nigericin triggers a rapid and robust form of inflammasome-dependent pyroptosis with minimal contribution from apoptotic caspases. LLome induced cell death revealed a distinct, apoptosis-like profile. LLome activated executioner caspases such as cl-caspase-3 and cl-caspase-7. In contrast to nigericin, minimal caspase-1 activation suggests caspase-1 engagement, but likely secondary, IL-1 β /IL-18 processing, and GSDMD cleavage and release were present but at reduced levels, suggesting the engagement of cathepsins or alternative pathways. Intracellular LDH staining showed a pronounced decrease, consistent with extensive LDH efflux and advanced membrane damage for both tested inducers, with nigericin inducing stronger leak at the early time point, indicating earlier-stage lysis. Overall, this indicates lysosomal proteases can also drive IL-1 β maturation, albeit less efficiently than ionic flux-driven inflammasome activation and proves previously observed shift from immunologically silent apoptotic mechanism towards pro-inflammatory mechanisms upon LPS treatment.

A**TOF-probe****B**

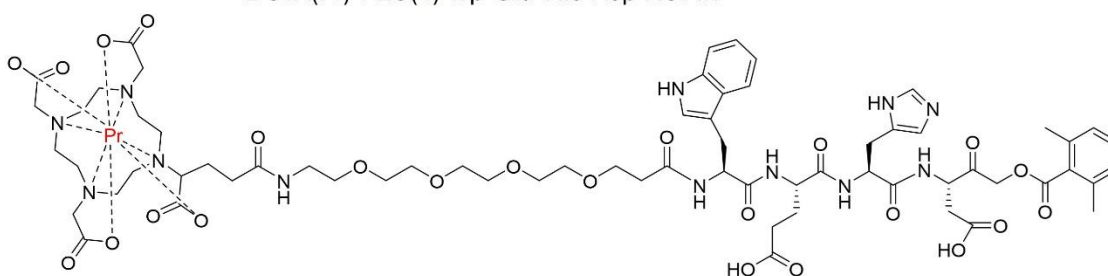
DOTA(Tb)-PEG(4)-Ile-Glu-Thr-Asp-AOMK



DOTA(Lu)-PEG-Asp-Glu-Val-Asp-AOMK



DOTA(Pr)-PEG(4)-Trp-Glu-His-Asp-AOMK



DOTA(Ho)-PEG(4)-Leu-Glu-His-Asp-AOMK

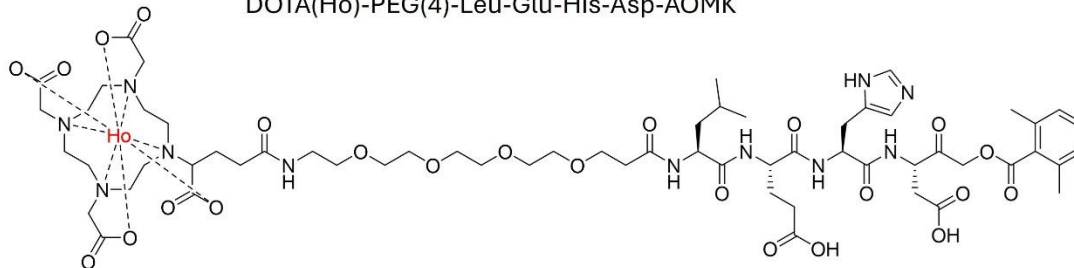


Figure 51. Structural outline of the TOF-probes. **A** Schematic design of TOF probe. Created in <https://BioRender.com>. **B** Structures of custom TOF-probes specific to casape-8 (IETD, ^{159}Tb -tagged), caspase 3 (DEVD, ^{175}Lu -tagged), caspase-1 (WEHD, ^{141}Pr -tagged) and caspase-9 (LEHD, ^{165}Ho -tagged).

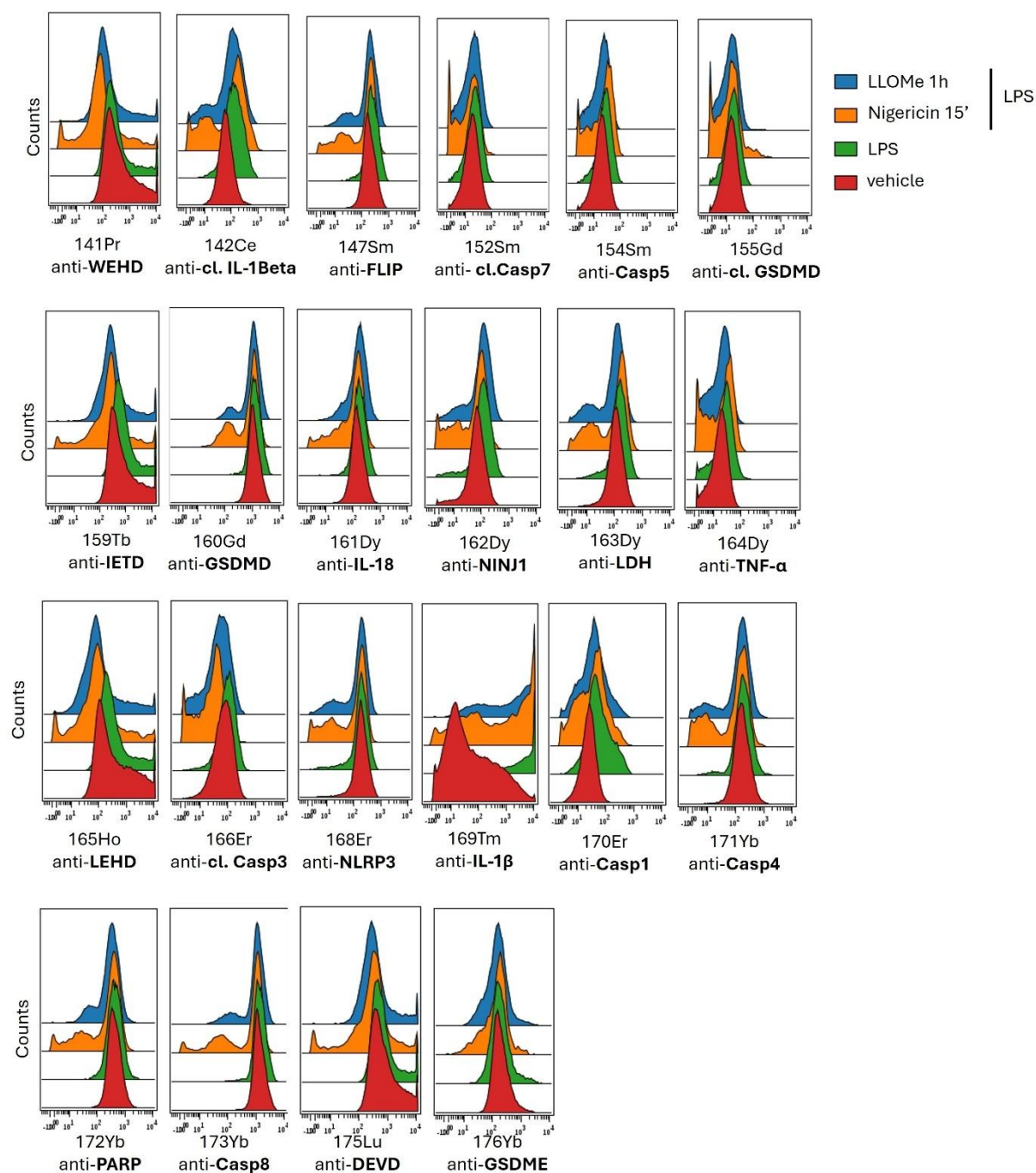


Figure 52. Histogram representation of early pyroptotic events detected by a custom mass cytometry panel.

A panel combining antibodies against cell death-related proteins with TOF-probes targeting caspase activities was applied to monitor early signaling events. LPS-primed M0 macrophages were treated with LLOMe or nigericin for short incubation periods to capture the initial stages of cell death. Following stimulation, cells were stained with the custom panel and analyzed by mass cytometry.

5. DISCUSSION

Cell death represents a universal process essential for maintaining tissue equilibrium, removing cells that are damaged or potentially harmful, supporting immune defense, and guiding organismal development. Its regulation is equally important in pathology, where impaired or excessive cell death contributes to conditions such as cancer, chronic inflammation, and neurodegenerative disorders.

Cell elimination can proceed in fundamentally different ways. When cells die as a result of overwhelming external insults, such as heat shock, mechanical injury, or toxic exposure, the outcome is referred to as accidental cell death. This is an uncontrolled, passive event that lacks regulatory input and often results in catastrophic structural failure. In contrast, programmed cell death follows genetically encoded pathways that operate under physiological conditions. These mechanisms rely on specialized molecular machineries and can be experimentally modified, either slowed or accelerated, through drugs or genetic interventions. Because of its controlled nature, PCD is indispensable for development, immune system function, and tissue repair, and disturbances in these processes are a driving factor in many diseases.

Although each form of PCD displays distinct molecular features, overlaps and pathway convergence are common, sometimes blurring their classification. Classical necrosis provides the best example of unregulated demise, characterized by cell swelling, organelle breakdown, membrane rupture, and the uncontrolled leakage of intracellular contents, typically causing inflammation. In recent years, however, additional forms of regulated cell death, such as necroptosis and pyroptosis, exhibit morphological similarities to necrosis, including plasma membrane rupture and pro-inflammatory outcomes. Importantly, unlike accidental necrosis, these processes are orchestrated by defined signaling pathways and can be altered through inhibitors, activators, or modulation of protein levels [464]. From a morphological perspective, it is often straightforward to distinguish between non-lytic modalities of PCD, such as apoptosis, where the cell disassembles without plasma membrane rupture and thus remains immunologically silent, and lytic forms of PCD, including pyroptosis, necroptosis, and ferroptosis, which involve membrane rupture and release of cellular components that provoke inflammation. This distinction between lytic and non-lytic mechanisms is critical for understanding how cell death contributes to either tissue homeostasis or disease progression.

Comparative profiling of cell death induced by diverse stimuli demonstrates that pyroptosis is not a single, uniform pathway but instead represents a spectrum of mechanistic variants shaped by the initiating trigger and the cellular context. Agents such as nigericin, LLOMe, alum, silica particles and monosodium urate crystals, each elicited distinct proteolytic signatures, involving caspase-1, caspase-3, and various lysosomal cathepsins to different extents. These observations indicate that the downstream trajectory of cell death is dictated not only by the core execution machinery but also by the upstream stressor, which defines whether lysosomal disruption, ionic fluxes, or metabolic stress dominate the process.

This finding aligns with the concept that regulated cell death modalities exist on a continuum, often sharing overlapping features, rather than functioning as strictly discrete

entities. Within this continuum, lysosomal cysteine cathepsins act as context-dependent modulators that intersect with the canonical inflammasome-GSDMD axis, thereby influencing both the efficiency of cytokine maturation and the inflammatory outcome of cell death. The present analysis extends existing models by integrating biochemical readouts with single-cell proteomic profiling, further underscoring the importance of lysosomal proteases in shaping inflammatory demise. It is notable that several NLRP3 activators not only engage canonical inflammasome signaling but also induce lysosomal membrane permeabilization, resulting in protease release into the cytosol. Numerous studies have implicated cathepsins in this process, with cathepsin B most frequently highlighted. To dissect these contributions, genetically modified cell systems, recombinant protein assays, and selective protease inhibitors have been employed. For example, the pan-cathepsin inhibitor K777 suppresses IL-1 β release in response to silica, alum, and cholesterol crystals [363, 465], confirming a catalytic role for lysosomal proteases. However, the interpretation of such findings is complicated by the widespread use of CA-074Me. Although frequently described as cathepsin B-selective, CA-074Me is in fact a methyl ester derivative of the broad-spectrum inhibitor E64 and displays significant cross-reactivity. Thus, results obtained with CA-074Me cannot exclude contributions from other cathepsins, and off-target effects must be considered when assessing its impact on inflammasome activation [363]. Evidence increasingly supports the involvement of multiple cathepsins. CA-074Me reduces NLRP3, ASC, and IL-1 β protein levels by immunofluorescence and western blotting, consistent with a role for cysteine protease activity in inflammasome regulation, yet only cathepsins B and L consistently affect the secretion of mature IL-1 β . In a comprehensive study, Orłowski and colleagues showed that particulate matter and LLOMe trigger inflammasome activation through a combination of cathepsins: knockout of cathepsins B, C, and S, combined with siRNA-mediated knockdown of cathepsins L and Z, produced a partial but significant suppression of IL-1 β secretion and reduced IL-1 β transcript levels in peritoneal macrophages [362, 363]. Taken together, these findings indicate that cathepsins act in a cooperative yet non-redundant manner during NLRP3 inflammasome activation. However, the extent to which each individual cathepsin contributes, and whether these contributions vary by stimulus and cellular context, remains unresolved. This uncertainty highlights the need for highly selective, custom-designed inhibitors as essential tools to accurately define the enzyme-specific roles of lysosomal proteases in regulated cell death.

Cathepsins are lysosomal proteases with optimal activity at acidic pH. However, accumulating evidence indicates that certain cathepsins can retain catalytic activity at near-neutral conditions [466], implying that once released into the cytosol they may remain enzymatically competent to cleave intracellular substrates. This cytosolic activity has been documented for multiple cathepsins and provides a mechanistic basis for their contribution to regulated cell death pathways. Both cathepsins and calpains have previously been implicated in apoptosis, where they promote cell demise by degrading anti-apoptotic regulators [273]. To investigate whether these proteases also target inflammatory cell death effectors, human GSDMD, pro-IL-1 β , and pro-IL-18 were subjected to *in vitro* cleavage assays. Multiple cathepsins were able to directly process these substrates; notably, cathepsin S efficiently cleaved the GSDMD interdomain linker near the canonical caspase-1 site, as well as pro-IL-1 β and pro-IL-18, yielding fragments similar to those generated by caspase-1. Importantly, a

concentration-dependent duality was observed: at low concentrations, cathepsins carried out limited, site-specific proteolysis reminiscent of caspase activity, whereas at higher concentrations they reverted to their characteristic bulk degradative behavior. This switch underscores the context-dependency of cathepsin function and raises questions regarding how closely *in vitro* cleavage reflects physiological activity. Observed divergence suggests functional specialization among cathepsins: cathepsin S may contribute to alternative, context-dependent processing of GSDMD, whereas cathepsins B, L, and V likely function in substrate degradation, potentially restricting GSDMD availability for pyroptotic pore formation.

A major interpretive challenge lies in reconciling the robust substrate cleavage observed in biochemical assays with the relatively modest effects of selective cathepsin inhibitors in cellular systems. Several factors likely contribute. First, the extensive functional redundancy among cathepsins may obscure the role of individual enzymes, such that blocking one protease has little effect when others remain active. Second, the temporal dynamics of lysosomal membrane permeabilization may create transient bursts of cathepsin activity: although enzymes such as cathepsin L rapidly lose activity at neutral pH, they remain catalytically competent long enough to cleave substrates immediately after their release. The high local concentrations reached at the moment of lysosomal rupture may therefore be sufficient to drive critical proteolytic events despite rapid inactivation in bulk cytosol. Our data indicate that, when released into the cytoplasm, cathepsins, especially cathepsin S, are biochemically capable of generating the pore-forming N-terminus of GSDMD and of maturing IL-1 family cytokines independently of caspase-1. Supporting this, Chen et al. recently demonstrated in a tumor model that cathepsin S released during near-infrared phototherapy cleaves GSDMD and initiates bona fide pyroptosis [364]. At the same time, the physiological relevance of this route in immune cells remains uncertain, as canonical inflammasome activators predominantly rely on caspase-1/11 to process GSDMD. For instance, Brojatsch and colleagues reported that deletion of cathepsins, including cathepsin S, did not impair pyroptotic death induced by LPS+ATP or nigericin in macrophages [467]. Together, these findings suggest that cathepsin-mediated cleavage of pyroptotic substrates may act as an auxiliary mechanism in specific contexts, but that caspase-1 remains the principal driver of pyroptosis in innate immune responses.

From an immunological perspective, the ability of cathepsins to process pro-IL-1 β and pro-IL-18 carries important implications for inflammasome biology. Although caspase-1 is recognized as the canonical protease responsible for cytokine maturation, lysosomal proteases may provide an alternative or complementary route for generating active cytokines, particularly under conditions of lysosomal destabilization or impaired inflammasome function. Both the present results and prior studies indicate that cysteine cathepsins act as supportive or parallel effectors within this framework. Cathepsin activity can enhance the inflammatory output of pyroptosis, as these proteases are capable of directly processing pro-IL-1 β and pro-IL-18, and they cooperate with GSDMD-mediated pore formation to maximize cytokine release. For example, Blanco et al. demonstrated that NAIP/NLRC4 inflammasome-driven IL-1 β secretion from macrophages is completely abrogated only when both GSDMD and cathepsins are simultaneously inactivated [468]. This finding underscores the non-redundant contributions of GSDMD pores and lysosomal proteases in ensuring robust cytokine export. Similarly, studies by Orłowski et al. and Hughes et al. confirmed that multiple cathepsins contribute to the priming

and processing of pro-IL-1 β during inflammasome activation [259, 362, 428]. Thus, although cathepsins are not typically required for the initiation of pyroptotic membrane rupture, they significantly amplify inflammation by facilitating cytokine maturation and release. These observations support the concept that inflammasome signaling is not strictly linear but rather modulated by auxiliary proteolytic systems. In this context, cathepsins may act as regulatory nodes that influence the balance between cytokine activation, pyroptotic lysis, and substrate degradation, thereby fine-tuning the magnitude and duration of inflammatory cell death.

Cathepsin B has previously been implicated in NLRP3-dependent pyroptosis induced by the bacterial ionophore nigericin. To further investigate the role of lysosomal proteases in lytic cell death, nigericin was applied as a canonical inducer of the NLRP3-caspase-1-GSDMD axis, whereas LLOMe served as a lysosomotropic agent to study the contribution of cathepsin release. Kinetic analyses of cell death processes demonstrated that the response to nigericin is highly concentration dependent. At lower concentrations, nigericin engaged the classical inflammasome pathway, as indicated by experiments in genetically modified cell lines, where caspase-1-deficient and GSDMD-deficient cells exhibited only residual cell death that could be abolished by combined application of pan-caspase and pan-cathepsin inhibitors. In contrast, exposure to higher concentrations (25 μ M) produced a catastrophic form of lysis that was not strictly dependent on the inflammasome machinery, reflecting an osmotic stress-driven mechanism. Under low-dose conditions, however, partial inhibition by pan-cathepsin and pan-caspase inhibitors suggested that lysosomal destabilization contributes to nigericin-induced pyroptosis by amplifying canonical signaling.

LLOMe-dependent cell death followed a mechanistically distinct trajectory. Lysosomal rupture caused by LLOMe released cathepsins into the cytosol, where they promoted caspase-3 activation and induced a hybrid necrotic/apoptotic-like death program. This cathepsin-caspase axis produced cleavage signatures, including PARP-1 degradation, that diverged from classical apoptosis or pyroptosis and proceeded with slower kinetics than GSDMD pore formation. In this setting, direct GSDMD cleavage by cathepsins was not observed; rather, cathepsin-mediated Bid processing and caspase-3 activation dominated. Consistent with prior studies, apoptotic caspases (caspase-3/7) also functioned as negative regulators of pyroptosis by cleaving GSDMD at Asp87, thereby preventing pore formation. Notably, LPS priming shifted the outcome of LLOMe treatment from a predominantly apoptotic signature toward a pro-inflammatory profile characterized by GSDMD p30 processing and release of mature cytokines such as IL-1 β .

These observations were enabled by the application of a unique panel of selective cathepsin inhibitors targeting cathepsins B, L, and S, which allowed the dissection of individual protease contributions under both canonical and lysosome-disruptive conditions. Although pan-cathepsin inhibitors such as E64d consistently provided stronger protection than single inhibitors, highlighting functional redundancy, the selective blockade of individual cathepsins revealed non-overlapping roles in specific contexts. Kinetic profiling demonstrated that LLOMe-induced death was predominantly cathepsin dependent, whereas nigericin-induced death was governed by the classical caspase-1/GSDMD pathway, with cathepsins acting primarily as auxiliary amplifiers.

To further define the sequence of protease activation, DEVDase activity was monitored as a readout of executioner caspase function. Because cathepsins do not cleave DEVD substrates at physiological pH, all DEVDase activity reflects caspase-3/7 activity. LLOMe treatment induced robust DEVDase activity, which was inhibited by calpeptin, legumain inhibitors, and pan-cathepsin inhibitors, indicating that these enzymes cooperate to promote executioner caspase activation. Among lysosomal proteases, cathepsin B was identified as the principal driver of this activity, in synergy with calpains. In contrast, low-dose nigericin also induced DEVDase activity, which was insensitive to the caspase-1 inhibitor VX-765 but blocked by pan-caspase inhibition, suggesting that lysosomal stress can act upstream of apoptotic caspase activation during inflammasome signaling. At high nigericin concentrations, however, caspase-3/7 activity was only modestly affected by cathepsin inhibition, indicating that alternative stress pathways, consistent with osmotic collapse, contribute to caspase activation during catastrophic cell death.

Activity-based probes enabled the direct detection of catalytically active cathepsin B in THP-1 macrophages. Importantly, ABPs provided a critical advantage: while anti-cathepsin B antibodies detect both the inactive proenzyme and the mature enzyme, ABPs recognize only the catalytically active form. This highlights the necessity of integrating ABPs into cell-based studies, as they allow direct and selective monitoring of enzyme activity, rather than indirect detection of total protein levels. Cysteine cathepsins are synthesized as preproenzymes containing a signal peptide that directs them into the endoplasmic reticulum, followed by a propeptide domain that ensures correct folding and lysosomal targeting. Activation requires proteolytic removal of this propeptide, either by autocatalytic cleavage or through the action of other proteases [328, 363]. Since only the mature, active form contributes to cell death pathways, the use of ABPs provides an indispensable tool to distinguish between inactive precursors and functional proteases in living cells. ABPs were applied to validate the selectivity and efficacy of the custom-designed cathepsin B inhibitors. Importantly, ABPs covalently bind only to the catalytically active form of an enzyme, thereby providing a functional readout that cannot be achieved by antibody-based detection, which recognizes both the inactive proenzyme and the mature protein. In the case of cysteine cathepsins, this distinction is critical, as they are synthesized as preproenzymes containing a signal peptide and an N-terminal propeptide that must be proteolytically removed before full activity is achieved. Using a CatB-27 probe, it was confirmed that the probe produced a single band corresponding to the active enzyme, in contrast to antibodies that detect both the pro-form and the mature protease. This approach provided direct evidence that the custom inhibitors (CatB-23 and CatB-24) effectively suppressed cathepsin B activity within cells. These findings underscore the value of incorporating ABPs into validation workflows, as they enable precise discrimination between inactive and active protease pools and allow inhibitor efficacy to be evaluated at the functional level. Beyond validation, cathepsin-targeted ABPs hold strong potential as investigative tools in cell death research. Because lysosomal proteases are released into the cytosol following lysosomal membrane permeabilization, ABPs could be used to track the timing, localization, and magnitude of cathepsin activation during distinct modes of regulated cell death, including apoptosis, pyroptosis, and necroptosis.

To complement the biochemical and kinetic studies, PMA-differentiated macrophages were analyzed by mass cytometry using a custom-designed panel that combined antibody-based markers of pyroptotic and apoptotic signaling with enzyme-specific TOF activity-based probes. This multiplex approach enabled simultaneous monitoring of inflammasome engagement, caspase activation, and terminal lytic events at the single-cell level. Stimulation with nigericin produced a robust pyroptotic signature. Caspase-1 activation, detected by WEHD-based TOF-ABP, was accompanied by cleavage of GSDMD and maturation of IL-1 β and IL-18. These events correlated with elevated NINJ1 and TNF- α levels, indicating downstream amplification of inflammatory signaling and plasma membrane rupture. Apoptotic markers such as cleaved caspase-3/7 and PARP were minimally detected, consistent with the conclusion that pyroptosis dominates in this setting. By contrast, LLOMe treatment revealed a distinct proteolytic landscape. LLOMe stimulation resulted in strong activation of executioner caspases, as evidenced by cleavage of caspase-3 and caspase-7. In contrast to nigericin, only minimal caspase-1 activation was observed, indicating that inflammasome engagement occurred but played a secondary role. Processing of IL-1 β and IL-18, as well as cleavage and release of GSDMD, were detectable but markedly reduced compared with nigericin, suggesting a greater reliance on cathepsin activity or alternative proteolytic pathways. Analysis of intracellular LDH revealed a substantial reduction in signal, consistent with LDH efflux and advanced membrane permeabilization in response to both stimuli. Notably, nigericin induced a more pronounced LDH decrease at earlier time points, reflecting rapid onset of membrane rupture and earlier lytic events compared with LLOMe. The inclusion of enzyme-specific TOF-ABPs provided functional resolution beyond conventional antibody staining. Whereas antibodies recognized total protein pools, TOF-ABPs selectively marked the active forms of caspases (caspase-1, -3/7, -8, -9), enabling kinetic mapping of protease activation.

Taken together, the findings indicate that cysteine cathepsins are capable of intersecting with pyroptotic signaling, yet under physiological conditions the canonical caspase-1-GSDMD pathway remains the principal executor of membrane rupture. Our kinetic studies with selective inhibitors identified cathepsin B as the dominant lysosomal protease driving necrotic-like lysis, underscoring its non-redundant contribution to cell death downstream of lysosomal membrane permeabilization. While cathepsin S and other family members can cleave pyroptotic substrates *in vitro*, their roles in intact cells appear more context-dependent, emerging primarily under conditions of severe lysosomal stress or atypical triggers such as photodynamic ROS. These observations reinforce the concept that inflammasome signaling is not a linear cascade but a network that can be amplified or modified by auxiliary proteolytic systems. Cathepsins contribute most consistently by facilitating IL-1 β and IL-18 processing and, in some contexts, by providing alternative routes to lytic death when caspase-1-mediated mechanisms are compromised. The fact that cathepsins can substitute for or augment inflammasome activity suggests an evolutionary safeguard, ensuring that infected or stressed cells can still undergo lytic demise and release immunostimulatory signals even when pathogens inhibit canonical pyroptosis.

From a translational perspective, these findings emphasize the importance of cellular context in therapeutic targeting. For pathologies associated with lysosomal disruption, cathepsin inhibitors may offer more effective intervention than inflammasome blockade.

Conversely, inflammasome-driven diseases may remain best addressed by caspase-1-directed approaches. Given the partial redundancy within the cathepsin family, the next generation of therapeutic strategies will likely require selective yet combinatorial inhibition to achieve robust efficacy without broad off-target effects.

Overall, the data support a revised model in which pyroptosis is not a single, rigid pathway but a convergent outcome shaped by multiple proteolytic routes, with cathepsins acting as modulators that can bias the balance between apoptosis, necrosis-like rupture, and inflammasome-driven pyroptosis. This mechanistic flexibility likely reflects evolutionary pressure to maintain resilient host defense across diverse cellular environments. Future studies should address the extent to which cathepsins contribute to pyroptotic signaling *in vivo*, particularly in diseases characterized by lysosomal instability, chronic inflammation, or defective caspase activity.

6. CONCLUSIONS

The role of lysosomal proteases in regulated cell death and their points of intersection with inflammatory execution pathways was investigated. The working premise, that pyroptosis and lysosome-dependent cell death converge at shared proteolytic checkpoints, was tested using selective chemical tools and single-cell readouts. The findings support this premise and are summarized below in relation to the study aims.

Aim 1: Cathepsins as direct mediators of pyroptotic substrate processing

Cathepsin S was shown to cleave the GSDMD interdomain linker near the canonical caspase-1 site and to process pro-IL-1 β and pro-IL-18, producing fragments comparable to caspase-1 products. A concentration-dependent duality was observed: at low concentrations, cathepsins executed limited, site-specific proteolysis consistent with caspase-like editing, whereas higher concentrations produced bulk, degradative proteolysis. These results indicate that, once present in the cytosol, cathepsins, particularly cathepsin S, are biochemically capable of generating pore-forming GSDMD-N and maturing IL-1 family cytokines independently of caspase-1.

Aim 2: Cathepsins in canonical pyroptosis and lysosome-dependent cell death

Obtained results indicate that, contrary to models emphasizing complete redundancy among cathepsins, the execution of LLOMe-induced lytic cell death in macrophages is primarily dependent on cathepsin B and calpain, particularly in settings where pyroptotic machinery is compromised. The data further uncover a previously underappreciated role for legumain in linking lysosomal disruption to apoptotic execution.

Aim 3: Application of selective chemical tools in cell-death studies

Tailor-made inhibitors and activity-based probes derived from HyCoSuL chemistry were applied to delineate individual cathepsin functions. Selective inhibition of cathepsins B, L, and S revealed non-overlapping roles dependent on stimulus and cellular context. Kinetic profiling established that LLOMe-induced death is predominantly cathepsin-dependent. ABPs conferred a functional advantage over antibodies by labeling only catalytically active enzymes; active cathepsin B was visualized in THP-1 macrophages, and custom inhibitors CatB-23 and CatB-24 were verified to suppress cathepsin B in THP-1 cells.

Aim 4: Systems-level profiling of pyroptotic death using mass cytometry

A CyTOF panel combining classical antibody markers of apoptosis/pyroptosis with enzyme-specific TOF-ABPs enabled functional, single-cell resolution beyond antibody staining alone. Active caspases (-1, -3/7, -8, -9) were tracked kinetically, allowing insides of proteolytic events and identification of pathway intersection points.

The results position cathepsins, especially cathepsin B, as the dominant lysosomal protease driving necrotic-like lysis. While not primary initiators of GSDMD-mediated rupture, cathepsins potentiate and shape inflammatory death by editing pyroptotic substrates (GSDMD, pro-IL-1 β , pro-IL-18) under permissive concentrations and by acting in concert with GSDMD pores to enhance cytokine release. In consequence, cathepsin activity possibly fine-tunes the magnitude and duration of inflammatory cell death following LMP.

7. REFERENCES

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8. PUBLICATIONS

1. **Kalinka M**, Nguyen J, Łęcka M, Pippa M, Turk B, Salvesen GS, Grobocz K, Poręba M. Non-redundant roles of cathepsins in promoting necrosis of human macrophages. 2025, *under review*.
2. Ćwilichowska-Puślecka N, Makowiecka A. M, **Kalinka M**, Grobocz K. M, Puślecki T, Drąg M, Simon K, Dąbrowska K, Pazgan-Simon M, Poręba M. Ł, Understanding the long-term interplay of SARS-CoV-2 immune and inflammatory responses with proteases in COVID-19 recovery: a longitudinal study. Frontiers in Immunology. 2025, vol. 16, s. 1-19 DOI: 10.1016/j.chembiol.2025.03.002

3. Horbach N, **Kalinka M**, Ćwilichowska-Puślecka N, Al Mamun A, Mikołajczyk-Martinez A, Turk B, Snipas S, Kasperkiewicz P., Groborz K. M, Poręba M, Visualization of calpain-1 activation during cell death and its role in GSDMD cleavage using chemical probes. *Cell Chemical Biology*. 2025, vol. 32, nr 4, s. 603-619 DOI: 10.1016/j.chembiol.2025.03.002
 4. Groborz K. M, **Kalinka M**, Grzyska J, Kołt S, Snipas S. J, Poręba M, Selective chemical reagents to investigate the role of caspase 6 in apoptosis in acute leukemia T cells. *Chemical Science*. 2023, vol. 14, nr 9, s. 2289-2302 DOI: 10.1039/d2sc05827h
 5. Pohl P, **Kalinka M**, Pieprz M, Development of a very simple and fast analytical methodology for FAAS/FAES measurements of Ca, K, Mg and Na in red beetroot juices along with chemical fractionation of Ca and Mg by solid phase extraction. *Microchemical Journal*. 2019, vol. 147, s. 538-544 DOI: 10.1016/j.microc.2019.03.075
- In addition to the publications listed above, there are two more manuscripts currently in preparations. The data for these manuscripts are included in this PhD thesis.
6. **Kalinka M**, *et al.* Running title: “Cysteine cathepsins cleave GSDMD upon translocation to the cytosol to support pyroptosis”
 7. **Kalinka M**, *et al.* Running title: “Metal-tagged chemical probes for caspases reveal the kinetics of the pyroptotic machinery in macrophages”

9. CONFERENCES

1. **Malgorzata Kalinka**, Stefan Gerhardy, Scott Snipas, Guy Salvesen, Daniel Kirchhofer, Marcin Poręba, Paulina Kasperkiewicz , Alternative pathways of GSDMD processing, 12th General Meeting of the International Proteolysis Society, Singapore, 23-29 June 2023 (flash talk and poster)
2. **Malgorzata Kalinka**, Betsaida Bibo-Verdugo, Boris Turk, Guy Salvesen, and Marcin Poręba, Dissecting the proteolytic crosstalk between caspases and cysteine cathepsins in cell death, Gordon Research Conference on Cell Death, Les Diablerets Conference Center in Les Diablerets, Vaud (fr), Switzerland, 15-20 May 2022, poster
3. **Malgorzata Kalinka**, Katarzyna Groborz, Scott Snipas, Guy Salvesen, Marcin Drag, Marcin Poręba, Caspase selective reagents for diagnosing pyroptotic mechanisms, Gordon Research Seminar on Cell Death, Les Diablerets Conference Center in Les Diablerets, Vaud (fr), Switzerland ,14-15 May 2022, poster
4. **Malgorzata Kalinka**, Katarzyna Groborz, Marcin Poręba, Caspase selective reagents for diagnosing pyroptotic mechanisms, Precision medicine for inflammatory diseases, 9th

Bergen Research School of Inflammation, Myrkdalen, Norway, 16-18 March 2022, oral presentation

5. **Małgorzata Kalinka**, Katarzyna Groborz, Justyna Grzyska, Sonia Kołt, Scott Snipas, Marcin Poręba, Selective chemical reagents to investigate the role of caspase 6 in apoptosis in acute leukemia T cells, 39th Winter School on Proteinases and Inhibitors (online), Tiers, Italy, 16-18 February 2022, oral presentation

6. **Małgorzata Kalinka**, Katarzyna Groborz, Marcin Drąg, Marcin Poręba, Badanie mechanizmów śmierci komórki za pomocą cytometrii masowej, 63. Zjazd Naukowy Polskiego Towarzystwa Chemicznego, Łódź, 13-17 września 2021, poster

10. RESEARCH PROJECTS

1. Grant Project 'Fix Neutropenia (FIXNET): focusing on neutrophil proteases defects which serve as novel diagnostic and therapeutic option' - Contract number POIR.04.04.00-00-1603/18, TEAM NET program, a grant from the Foundation for Polish Science financed by the European Regional Development Fund as part of the Smart Growth Operational Programme (PO IR) 2014-2020 (PO IR), Priority IV, Increasing the scientific research potential

2. Grant Project 'Novel mechanisms of PAD activity regulation. Substrate specificity and activation of peptidyl arginine deiminase in the context of rheumatoid arthritis'- GRIEG, National Science Centre, Poland, 2019/34/H/NZ1/00674

3. Grant Project' Multiparametric analysis of blood from COVID-19 survivors using mass cytometry to study the dynamics of changes in the immune system and identify optimal plasma donors'- National Centre for Research and Development, Poland

4. Grant Project 'Dissecting the mechanisms of proteolytic cross-talk underlying pyroptosis, a programmed inflammatory cell death' – OPUS, National Science Centre, Poland, 2018/29/B/NZ1/02249

11. AWARDS

IPS TRAVEL AWARD - Bestowed in recognition of outstanding research presented at the 12th General Meeting of the International Proteolysis Society, held in Singapore, June 23 to June 29, 2023