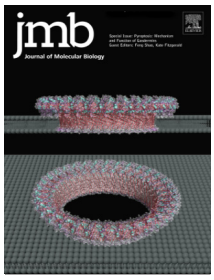
Review Article



Approaching Neutrophil Pyroptosis

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Abstract

All cells must die at some point, and the dogma is that they do it either silently via apoptosis or via pro-inflammatory, lytic forms of death. Amongst these lytic cell death pathways, pyroptosis is one of the best characterized. Pyroptosis depends on inflammatory caspases which activate members of the gasdermin family of proteins, and it is associated with the release of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18. Pyroptosis is an essential component of innate immunity, it initiates and amplifies inflammation and it removes the replication niche for intracellular pathogens. Most of the literature on pyroptosis focuses on monocytes and macrophages. However, the most abundant phagocytes in humans are neutrophils. This review addresses whether neutrophils undergo pyroptosis and the underlying mechanisms. Furthermore, I discuss how and why neutrophils might be able to resist pyroptosis.

Introduction

Not too long ago the world of cell death was relatively simple. We thought that there were two possibilities for a cell to die: active, programmed apoptosis and passive, uncontrolled necrosis. Apoptosis was required for development and swift, immunologically silent removal of obsolete or unwanted cells. Necrosis on the other hand was the outcome of overwhelming stress and resulted in inflammation.

Nowadays the world of cell death, particularly the world of necrosis, is far more complex.[1] Not only have we identified various necrotic forms of cell death which are far from uncontrolled, we also found that many of these pathways interact on several levels. We learned about necroptosis, ferroptosis, Parthanatos, NETosis, PANoptosis and others.[1,2] Some of them are even connected to apoptosis, the long-thought silent death pathway. One of the first regulated forms of necrotic cell death to be identified, however, is pyroptosis. In fact, pyroptosis had already been known as a form of caspase-1-dependent apoptosis[3] for quite some years before it earned its right as an independent form of cell death and obtained its own name ("Pyro" from the Greek word for fire, "ptosis" as a reminder of apoptosis, which itself means "falling off". Hence, pyroptosis is an inflammation-causing form of a programmed cell death pathway.).[4] A bit more than fifteen years after its name day we now have a relatively good understanding of the molecular players at work during this form of cell death. However, most of the literature on pyroptosis focuses on macrophages and there is evidence that other cell types respond differently to pyroptosis

inducers. One of these cell types is the neutrophil, the most abundant human leukocyte. This review will discuss where neutrophil pyroptosis follows the "macrophage dogma", where it differs from it and where we still require more research.

Pyroptosis

Pyroptosis usually results from the activation of inflammasomes (depending on the definition, there are exceptions, see below). Inflammasomes are multi-protein complexes which assemble upon sensing of a broad variety of signals (both danger-associated molecular patterns [DAMPS] and microbe-associated molecular patterns [MAMPS]). There are several different sensor proteins detecting signs of infection (such as flagellin, components of bacterial type 3 secretion systems, pathogen-derived proteases, or cytoplasmic nucleic acids) or disturbed homeostasis. Detailed discussion of the inflammasome-activating mechanisms is beyond the scope of this review, but I refer readers to other reviews covering this topic.[5–7] Briefly, detection of DAMPs or MAMPs leads to multimerization of sensors and the adapter protein ASC (*PYCARD*), which induces recruitment of inflammatory caspases. Caspases (CASP) are proteases with an active site cysteine, which cleave a variety of substrates after aspartate residues. Inflammasome formation therefore results in the multimerization and activation of inflammatory caspases (CASP1/4/5 in humans and CASP1/11 in mice). CASP1 subsequently processes (and thereby activates) the pro-inflammatory cytokines interleukin (IL)-1β and IL-18. These cytokines are then released from cells among other danger signals. IL-1β is a pleiotropic cytokine with diverse functions, including the induction of fever and vasodilation, but also the recruitment of immune cells to an inflammatory site. Although it is possible that cells release IL-1β while remaining alive,[8–10] in most cases activation of inflammatory caspases results in pyroptotic cell death. Exactly this event, activation of inflammatory caspases, was considered the hallmark of pyroptosis for a long time. Murine macrophages deficient for CASP1 and/or CASP11 survive treatment with inflammasome-activating stimuli and mice deficient for caspase-11 survive even harsh inflammatory stimulations such as lipopolysaccharide (LPS)-induced septic shock.[11,12] Researchers around the world coined the dichotomy of apoptotic caspases driving silent apoptosis and inflammatory caspases driving pyroptosis, the latter with rather fulminant and non-silent outcome. It was clear, however, that we were missing pieces of the puzzle. The most obvious was that there had to be a substrate – or several substrates - of inflammatory caspases during pyroptosis. It was only in 2015 when two independent studies found this substrate and demonstrated that the culprit for killing activated cells is a protein called gasdermin D (GSDMD).[13,14] CASP1 and CASP4/5/11 are all able to cleave and activate GSDMD.[13] The cleavage event releases a pore-forming N-terminus (N-GSDMD) from its inhibitory C-terminus, resulting in multimeric N-GSDMD pores which penetrate the plasma membrane and ultimately lead to cell lysis[15–18] (Figure 1). GSDMD-dependent pore formation causes the inflammatory phenotype observed after pyroptosis, since GSDMD-deficient animals (like CASP1/11 deficient mice) resist LPS-induced septic shock.[14] These animals – or their cells – tolerate activation of inflammatory caspases and accumulate mature IL-1β inside the cell,[13,14] demonstrating the involvement of GSDMD downstream of the proteases. GSDMD itself is part of a larger protein family[19] and other members of this family are also able to mediate cell lysis, hence it is possible to redefine pyroptosis from "inflammatory caspase dependent cell death" to "inflammatory cell death dependent on gasdermin activation".[16] Cleavage and liberation of the pore-forming N-terminus of GSDMD (as well as of other gasdermins) is sufficient to mediate cell lysis as shown by expression of the caspase-generated N-terminal fragment in GSDMD-deficient HEK293T cells.[16]

Is the inflammasome-CASP1/11-GSDMD axis the whole story of pyroptosis? Apparently not, as several lines of evidence suggest. First, other proteases can activate gasdermins, sometimes in the context of inflammasomes, but sometimes also in the absence of inflammasome activation. Therefore, if we define pyroptosis as gasdermin-mediated death, it is not strictly dependent on inflammasome activation (Figure 1). Inflammasome-independent gasdermin activation events include processing by other caspases, such as CASP8,[20] but also granzymes[21,22] and neutrophil proteases[23–26] (see below for more details). Furthermore, the apoptotic caspase CASP3 can cleave and inactivate GSDMD,[27] but activate GSDME to promote secondary necrosis after apoptosis.[28,29] These findings demonstrate that, depending on the protease and the gasdermin, there are both activating and inhibitory cleavage events. Second, membrane repair mechanisms induced by GSDMD activation and pore formation can rescue an activated cell's life, at least to some extent.[30] Other proteins and mechanisms also play a role in the survival of N-GSDMD pores.[9,31,32] Third, there seem to be more proteins involved in cell lysis than we previously anticipated. A very recent addition was the protein NINJ1. An elegant forward genetics screen identified NINJ1 as a key executor of pyroptosis and other lytic forms of cell death[33] (Figure 1). NINJ1, as GSDMD, oligomerizes to form pores. These pores induce cell rupture downstream of GSDMD during pyroptosis, as NINJ1-deficient cells showed the characteristic balloon-like shape of pyroptotic cells, but did not burst.[33] These findings demonstrate that plasma

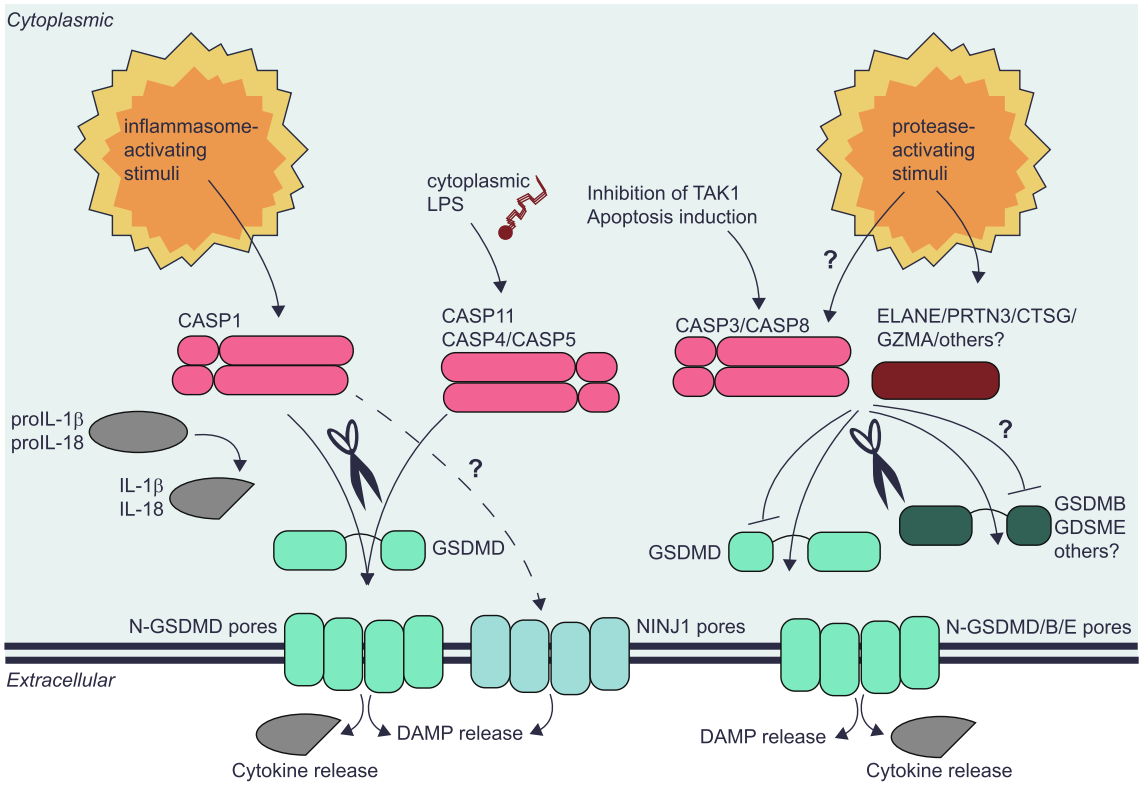

Figure 1. Mechanisms of pyroptosis. Inflammasome-activating stimuli, sensed by inflammasome sensors (not shown), lead to multimerization of sensors and the adaptor protein ASC. This induces activation of CASP1 and downstream processing of the cytokines IL-1β and IL-18 as well as GSDMD (proteolytic processing is indicated by scissors). GSDMD N-termini (N-GSDMD) multimerize to form pores in the plasma membrane. NINJ1 pores also contribute to cell lysis, the mechanism of NINJ1 activation is not known. Detection of cytoplasmic LPS by CASP11 (mouse) or CASP4/CASP5 (human) also activates N-GSDMD and pore formation. Other proteases can cleave GSDMD or other GSDM family members. Depending on the protease and cleavage site this can lead to GSDM activation (for example GSDMB processing by granzymes [GZMA] or GSDMD processing by CASP8) or inhibition (for example GSDMD processing by CASP3). N-GSDM fragments subsequently induce cell lysis and DAMP release in various cell types.

membrane rupture during pyroptosis is not a passive osmotic process, but actively mediated by NINJ1. Therefore, while GSDMD is absolutely required for macrophage pyroptosis, there can be downstream events involved in cell rupture. Fourth, there seem to be mechanisms of posttranslational regulation of gasdermins other than their proteolytic processing. A recent study showed that cells can survive expression of N-GSDMD if they lack a functional Ragulator-Rag-mTORC1 pathway.[34] Absence of this pathway did not affect the plasma membrane localization of N-GSDMD. Rather, disruption of the pathway reduced the levels of mitochondrial ROS. These ROS however were required for N-GSDMD multimerization and pore formation.[34] It seems that the story is not completely written yet and more discoveries are to be made.

How do Neutrophils Die?

Neutrophils are very abundant innate immune cells. They are produced at a continuous, high rate in the bone marrow, from where they enter circulation and patrol the host's body. Neutrophils are very potent microbe hunters, equipped with various efficient antimicrobials, ranging from the production of toxic reactive oxygen species (ROS) to the ability to engulf large amounts of pathogenic microorganisms by phagocytosis or the release of an arsenal of antimicrobials (including antimicrobial peptides and highly active serine proteases) via degranulation. Neutrophils can even use their chromatin to capture extracellular pathogens: when they do so, they undergo a special form of cell death called formation of "Neutrophil extracellular traps" (NETs).[35] NET formation results in the release of neutrophil DNA and proteins (see below). Even though the presence of inflammatory signals can prolong a neutrophil's lifespan the cells are notoriously short-lived with a half-life in circulation of hours to around a day.[36] The combination of their short life and their sheer number (healthy humans have around 1.500 – 8.000 neutrophils per microliter of blood) results in millions of neutrophils dying each day. It is therefore not too difficult to see how important it is that the cell death path-

ways of such a high amount of dying cells are adequately regulated. During homeostatic conditions neutrophils age in circulation and then migrate into various tissues where they undergo apoptosis. Resident macrophage populations and/ or dendritic cells will then swiftly remove apoptotic neutrophils by a phagocytic process called efferocytosis.[37] Apoptosis of neutrophils in the periphery is part of an elaborate signalling loop; phagocytosis of apoptotic neutrophils by macrophages dampens production of the cytokine IL-23 and subsequently of granulocyte-colony stimulating factor (G-CSF). G-CSF is the major cytokine responsible for *de novo* production of neutrophils in the bone marrow. Therefore, as long as there are neutrophils in peripheral tissues, production of new cells in the bone marrow is reduced. Once the amount of tissue neutrophils drops, G-CSF production increases and new cells will be produced and released into the blood stream.[37,38]

Neutrophils do not only undergo apoptosis. Depending on their activation status, the cells also die by necrotic cell death pathways. One particular form of necrotic cell death is the formation of NETs.[35] During NET formation neutrophils release their chromatin, decorated with granule-derived proteins, to the extracellular space. NETs have the capacity to defend the host against infections, since they are able to trap extracellular microbes.[35,39–42] However, at the same time NETs cause damage to host tissue, facilitate thrombosis or favour metastases,[43–45] highlighting their importance in host pathology. As chromatin usually resides inside cells, improperly degraded NETs also serve as a source for autoantigens and both enhanced NET formation as well as reduced NET degradation correlate with autoimmune diseases.[46] Mechanistically, there are two main pathways to NET formation, one depends on ROS production by the protein complex NADPH oxidase, the other proceeds in the absence of NADPH oxidase-derived ROS. It is worth mentioning that several studies also described other forms of NET formation, including the release of NETs in the absence of cell death,[47] but I will focus here on NETs formed during cell death. The dogma is that the pathways leading to NET formation are distinct from other forms of cell death, such as apoptosis, necroptosis or pyroptosis.[39,48,49] However, there is some evidence that NET formation pathways crosstalk to other forms of necrotic cell death (see below for pyroptosis). The focus of this review is neutrophil pyroptosis, therefore I will discuss the mechanisms involved in NET formation relatively briefly to allow to compare them to pyroptotic cell death. For more information about NET formation and the implication of NETs in disease I refer readers to other recent reviews.[50–54]

ROS-dependent NET formation

ROS-dependent NET formation – for example induced by mitogens (such as 12-phorbol 13-myristate acetate [PMA] or concanavalin A), hyphae of the fungal pathogen *Candida albicans* or cholesterol crystals - requires the production of ROS by NADPH oxidase.[39,55,56] Concomitant with ROS production, there is liberation of serine proteases, neutrophil elastase (ELANE), proteinase 3 (PRTN3), and cathepsin G (CTSG) from neutrophil granules. ROS seem to play a direct role in this process, since some species such as H_2O_2 are able to induce the release of ELANE from granules.[57] However, other processes also affect protease release from granules: Cytoplasmic ELANE can activate GSDMD,[23,24,26] and the ELANE-generated N-GSDMD can target and permeabilize granules and thereby induce the release of more ELANE in a feed-forward loop. This feed-forward loop has been proposed in the context of NET formation[23] and was recently confirmed in another setting independent of cell death.[24] Once ELANE leaves the granules, it can also translocate to the nucleus by a poorly understood mechanism. Nuclear ELANE clips histones.[58,59] Although not functionally proven, it is plausible that ELANE-clipped histones allow the chromatin decondensation that we observe during the formation of NETs. ELANE and other neutrophil serine proteases decorate NETs once they are released[35]; however, binding to NETs might interfere with protease activity.[60]

Parallel to ELANE-mediated histone clipping, neutrophils undergoing NET formation also activate components of the cell cycle machinery, in particular the kinase CDK6.[56] It is not clear what the substrates of CDK6 are in this context (also discussed below), but kinase activity is required for NET formation via the ROS-dependent pathway. Concomitant with CDK6 activation the cells also show phosphorylation of nuclear lamins,[56] which could assist breakdown of the nuclear membrane and subsequent chromatin expansion and release. Besides histone cleavage, other enzymes can modify histones during NET formation and might thereby further help chromatin expansion. One example is citrullination (the conversion of an arginine to citrulline) by an enzyme called peptidyl arginine deaminase 4 (PAD4) and citrullinated histones are a marker of NETs, which helps to detect the structures *in vitro* as well as *in vivo*.[61–63] Apoptosis and pyroptosis rely on apoptotic and inflammatory caspases, respectively and soon after the discovery of NETs researchers wondered whether NET formation also depends on caspases. Using broad range caspase inhibitors and rather broadly acting stimuli, several studies concluded that NETs formed via the NADPH oxidase-dependent pathway do not require caspase activity, perhaps due to the very high activity of highly promiscuous serine proteases.[39,48,49] However, recent studies with microbial infection of neutrophils and neutrophils from septic mice and septic patients demonstrated that at least activation of CASP11 can contribute to NET formation,[64,65] as also discussed below. Therefore, there is a crosstalk between NETs and

pyroptosis, depending on the context of neutrophil activation.

Neutrophils produce ROS during their oxidative burst through NADPH oxidase. Patients suffering from x-linked chronic granulomatous disease (CGD) carry mutations in NADPH oxidase subunits and neutrophils isolated from these patients do not form NETs in response to canonical agonists, such as PMA or fungal hyphae.[48,66] These studies genetically confirm the dependence of these inducers on NADPH oxidase-derived ROS. However, it is not entirely clear whether ROS act solely on upstream events of the pathway, such as the liberation of proteases from granules, or whether they also actively take part in killing the cell. Furthermore, the production of ROS through NADPH oxidase is an energy-consuming process. The replenishment of NAPDH occurs through the pentose phosphate pathway and indeed several studies showed that the pentose phosphate pathway is involved in NET formation.[67,68] In addition to these studies, it has recently been suggested that activation of a specific isoform of phosphofructokinase (PFKL) in neutrophils blunts the oxidative burst by favouring glycolysis over the pentose phosphate pathway.[69] Consequently, activation of PFKL reduced NET formation of human neutrophils. However, we still need more studies into the interplay of metabolic pathways during the formation of NETs.

There are other open questions regarding the ROS-dependent pathway of NET formation. One of the most prominent is, how neutrophils lyse to release their nuclear content. It might be that pore-forming proteins, such as gasdermins[23,64] assist the neutrophil in that process. Other studies suggested involvement of mixed lineage kinase domain-like protein (MLKL), which is known to form pores in cells undergoing necroptosis.[70–73] However, the involvement of the necroptotic machinery in NET formation is controversially discussed and might well be context-dependent.[39,74] Many pathogens express toxins with pore-forming, lytic activity and at least some of them can induce the release of NETs.[75–77] Another elegant study addressed the possibility that lysis and plasma membrane rupture during NET formation is a passive process, driven by physical force resulting from chromatin expansion.[78] Summarizing these data, is likely that neutrophil lysis during the formation of NETs does not follow a “one size fits all” scheme, but depends on the stimulus, on the localization of the neutrophil in the host’s body, on its interaction with other cells and probably on other factors.

Yet another open question, tackling the events upstream of lysis, is which signalling pathways are at play during the formation of NETs. Quite some studies showed kinase activation when neutrophils form NETs, including the activation of PKC, Raf-MEK-ERK, JNK, CDK6, PI3K, Src/SYK, IRAK, TBK1, TAK1, and FAK.[56,79–94] The PKC-Raf-MEK-ERK axis results in activating phosphorylation of NADPH oxidase subunits.[81,95] It is therefore a clear example of a kinase activating a substrate with a role in the oxidative burst. However, the involvement of other kinases in the formation of NETs remains less understood. It is unlikely that all the above mentioned kinases are active and required for NET formation during all conditions, particularly when one takes into consideration that the stimuli reached from bacteria and parasites to acids extracted from nut shells. Especially for broadly activating agents (such as PMA) the signal-to-noise ratio might be quite low and we have to interpret results with some caution. Future, systematic studies of kinase activation and kinase substrates during NET formation induced by physiological stimuli will shed light on the signalling events upstream of chromatin expansion and release.

ROS-independent NET formation

In addition to NETs formed upon NADPH oxidase-dependent ROS production, there is a second, much less understood, pathway which proceeds in the absence of NADPH oxidase activity. Typical inducers include calcium ionophores or nigericin.[39] PAD4 activity depends on calcium, hence ionophores are strong activators of PAD4 and histone citrullination serves as a marker for ionophore-induced NETs.[61,62] Mechanistically, we do not understand how exactly ROS-independent NET formation occurs. Whereas ROS-dependent NETs require neutrophil serine proteases, ROS-independent NET formation can progress in the absence of protease activity.[39] At present we do not know whether all NADPH oxidase-independent NET formation events follow the same pathway or whether these are several pathways, some of which might also involve the generation of mitochondrial ROS.[96] It might indeed be that mitochondria and/ or mitochondrial DNA are more important for NADPH oxidase-independent NETs, as a recent study using a neutrophil-like cell line suggested.[97] Therefore, while we have a good marker for these NETs – citrullinated histones – the ROS-independent pathways leading to their release remain rather enigmatic.

Neutrophils and Pyroptosis

Much of the literature on pyroptosis focuses on monocytes and macrophages. Several studies addressed the role of pyroptosis in neutrophils, but altogether these cells are still understudied regarding their ability to undergo pyroptosis. When looking at the few studies directly investigating neutrophil pyroptosis, it is possible to draw three – seemingly disparate - conclusions: 1. Neutrophils activate inflammasomes and release cytokines, but resist pyroptosis, 2. Neutrophils can undergo pyroptosis which also leads to the release of

NETs, 3. Inflammasome stimuli kill neutrophils via NET formation, but independent of inflammasomes and in the absence of cytokine release.

In this section I will first present the evidence for all these conclusions and will then, in the next section, discuss if they can fit together at all. However, there are four points to consider first. 1: Purification method matters. Macrophages and monocytes are much more potent at releasing IL-1β than at least human neutrophils (see Section 'What is the influence of species, timing and priming?'). Therefore, even small contaminations of neutrophil preparations might account for observations of low levels of these cytokines.[98] 2: Readout matters. We know about the sensors and effectors of inflammasomes. There are mouse models deficient for many of these genes and we know of various hallmarks of inflammasome activation (for example ASC speck formation, processing of CASP1/11 and GSDMD). We can – and should - use these hallmarks to carefully control for inflammasome activation and pyroptosis side by side even when we cannot achieve genetic confirmation (i.e. in primary human neutrophils). 3: Species matters. We know that there are substantial differences between neutrophils from different species (in most cases neutrophil studies are performed with either human or murine cells, but there are also studies using zebrafish, rats or other species).[99] Human cells express different effector proteins (for example, high amounts of α-defensins while murine cells do not express α-defensins) and surface markers (the gold standard murine neutrophil marker Ly-6G is absent on human cells), are more abundant in circulation and have a different nuclear morphology than their murine counterparts. Furthermore, mouse neutrophils are usually isolated from bone marrow or the peritoneal cavity whereas human cells are derived from blood.[99] All these model systems are valuable, but results might not be directly transferrable from one to the other. 4: Experimental conditions matter. Although this statement might seem trivial, it is clear that many factors are able to affect the outcome of neutrophil experiments. These include priming,[100] tissue culture media and addition of serum,[78] time point of measurement,[101] purity of cells,[98] age of cells,[102] density of cells,[103] and others. All these factors might affect readouts and the interpretation of results. Underscoring the importance of experimental conditions, studies with broadly activating agents such as PMA or nigericin are likely not directly comparable to more specific and physiological stimuli. Also, the use of bacterial compounds such as LPS might differ from using whole bacteria. One recent example for such a discrepancy is the finding that survival of LPS-induced septic shock in mice depends on macrophage (and to some extent dendritic cell) expression of CASP11. Neutrophil CASP11 and pyroptosis were dispensable. However, they were crucial for defence against the bacterial pathogen *Burkholderia thailandensis.*[104] This elegant study demonstrated *in vivo* how different stimuli reveal different roles for neutrophil pyroptosis in host defence.

Neutrophils activate inflammasomes and release cytokines but resist pyroptosis

Inflammasome activation leads to pyroptotic cell death. Many studies confirmed this dogma in macrophages from various species, but there are reports of other cells and conditions where inflammasome activation allows IL-1β release in the absence of cell death. Some of these were performed in non-professional immune cells such as for example keratinocytes,[8,105] others are reconstitution experiments where inflammasome components were expressed in HEK293T cells.[106] Yet other studies looked at innate immune cells and indeed, under certain conditions immune cells also seem to be able to activate inflammasomes without undergoing cell death. These include human and porcine monocytes,[10] but also dendritic cells and macrophages.[9,32] The mechanisms allowing the cells to resist pyroptosis remain incompletely understood. At least monocytes are able to activate the NLRP3 inflammasome via an alternative pathway, which leads to IL-1β release upon LPS stimulation without requirement for a second activation signal. Also, this pathway does not include CASP1 activation or GSDMD processing..[10,107]

Neutrophils seem to be another exception to the "inflammasome activation leads to pyroptotic cell death" dogma. Although the cells require two signals and therefore do not follow the same alternative activation pathway as monocytes, both NLRC4 and AIM2 inflammasome activation of murine neutrophils led to substantial IL-1β release.[108,109] Interestingly, activation of these inflammasomes resulted in the expected activation of CASP1 and IL-1β, but did not induce neutrophil lysis, a finding that was also reproduced *in vivo* during *Salmonella* infection in mice.[108] Other studies found similar results for activation of the NLRP3 inflammasome.[24,110] Two studies directly looked at GSDMD processing and showed that neutrophils were able to resist N-GSDMD.[24,109] Mature IL-1β localizes into plasma membrane ruffles and this allows its release independent of GSDMD, although this way of release takes longer than through GSDMD pores.[111] This is a possible explanation how neutrophils secrete IL-1 family cytokines. Another possibility is release through an ATG7-dependent process involving autophagosomes.[24] Recent evidence suggests that neutrophils also release IL-1α (which does not require caspase processing for activity) independent of GSDMD via a pathway involving exosomes.[112] Therefore, there is accumulating evidence that neutrophils can release IL-1 family cytokines in the absence of gasdermin pores (or while tolerating gasdermin pores).

GSDMD activation and pore formation induces membrane repair mechanisms in macrophages[30] and it seems likely that such mechanisms are not only at play in macrophages but could also work to protect neutrophils from N-GSDMD pores. Murine neutrophils activated with nigericin to release IL-1β did not seem to activate such membrane repair mechanisms.[24] However, we require more research to understand whether there are situations when neutrophils employ membrane repair mechanisms to resist N-GSDMD pores (Figure 2).

Neutrophils can undergo pyroptosis which also leads to the release of NETs

Whereas classical inflammasome stimuli induce the release of IL-1β from (at least murine) neutrophils without inducing pyroptosis, induction of the non-canonical inflammasome does very efficiently induce neutrophil death.[64] The non-canonical inflammasome detects the presence of cytoplasmic LPS. LPS sensing and activation of the non-canonical inflammasome strictly depends on expression of CASP11 in mice or CASP4/5 in humans.[11,12] Neutrophils express CASP11 and its downstream effector molecule GSDMD, although expression of CASP11 in neutrophils was much lower than in macrophages.[64] Consequently, transfection of LPS into the cytoplasm of murine neutrophils induced pyroptosis.[64] Pyroptosis depended on the expression of CASP11 while neutrophils resisted CASP1 activation, which is less efficient at cleaving GSDMD.[64] Surprisingly though, the dying cells also released their chromatin (Figure 2). A role for CASP11 in NET formation has recently also been shown in murine monosodium urate (MSU)-stimulated neutrophils and in neutrophils from septic mice and patients.[65,113] These findings suggest a crosstalk between pyroptosis and NET formation in neutrophils. During this non-canonical NET formation CASP11 was able to assist in the decondensation of the neutrophil nucleus and GSDMD pores led to neutrophil lysis and NET release.[64]

Inflammasome stimuli kill neutrophils via NET formation in the absence of cytokine release

In addition – or in contrast - to the above-mentioned findings, there are studies describing that inflammasome-activating agonists such as virus infections, nigericin, *Candida albicans*, MSU or cholesterol crystals do actually kill neutrophils via induction of NET formation.[39,55,103,114] These studies do not address if the dying neutrophils produce and release either IL-1β or IL-18 or if they activate inflammatory caspases and gasdermins.[39,55,103,114] However, experiments with caspase inhibitors showed that classical NET formation does not require caspase activity.[23,39,49] Another study demonstrated that stimulation of neutrophils with *Candida albicans* readily induces the production and release of IL-1β. However, the amount of cytokine produced depends on the microbe size.[115] While neutrophils phagocytose yeast particles and induce ROS production on the phagosomal membrane, they cannot phagocytose *Candida* hyphae, which leads to ROS production on the plasma membrane. This localized ROS production determines the amount of IL-1β: phagosomal ROS leaks into the cytoplasm and oxidizes p50, which induces its degradation and curbs cytokine production.[115] Interestingly, the ability of *Candida* to induce NET formation follows the same dichotomy. *Candida* hyphae promote NET release whereas yeasts kill neutrophils in the absence of NET formation.[116] Stimuli that activate inflammasomes in macrophages can therefore probably kill neutrophils via different pathways, some leading to NET formation in the absence of IL-1β/ IL-18 processing and others involving IL-1β activation and release prior to NET formation. It is unclear what the exact molecular nature of these pathways is. Furthermore, in species such as mice, where neutrophils seem to be much more capable of inflammasome activation (see below), it could be that inflammasome and NET formation pathways act in a crosstalk under certain conditions. We need systematic studies comparing macrophage and neutrophil responses of different species side by side to obtain a clearer picture of the mechanisms at play.

Neutrophils and Pyroptosis: An Attempt to Combine Conclusions

The three main conclusions drawn in Section 'Neutrophils and Pyroptosis' are at least partially contradicting each other. In this section I will try to find explanations allowing us to combine these three conclusions into one model or concept. I will also point out where we need more systematic research and actual species or cell type comparisons for final conclusions.

What is the influence of species, timing and priming?

Some studies mentioned in Section 'Neutrophils and Pyroptosis' suggest that stimuli that activate inflammasomes in macrophages will kill neutrophils independent of inflammasomes. Others find that the same activators lead to cytokine release but do not kill neutrophils. This is particularly obvious for NLRP3 activators. Are there explanations for these discrepancies? One could be species differences. Murine neutrophils seem to be much more potent at releasing IL-1β than human cells. Studies with murine cells readily identified the IL-1β in the ng/ml range[108,117] whereas human studies show amounts in the pg/ml range.[118–120] Furthermore, whereas several studies show mRNA expression of human NLRP3,

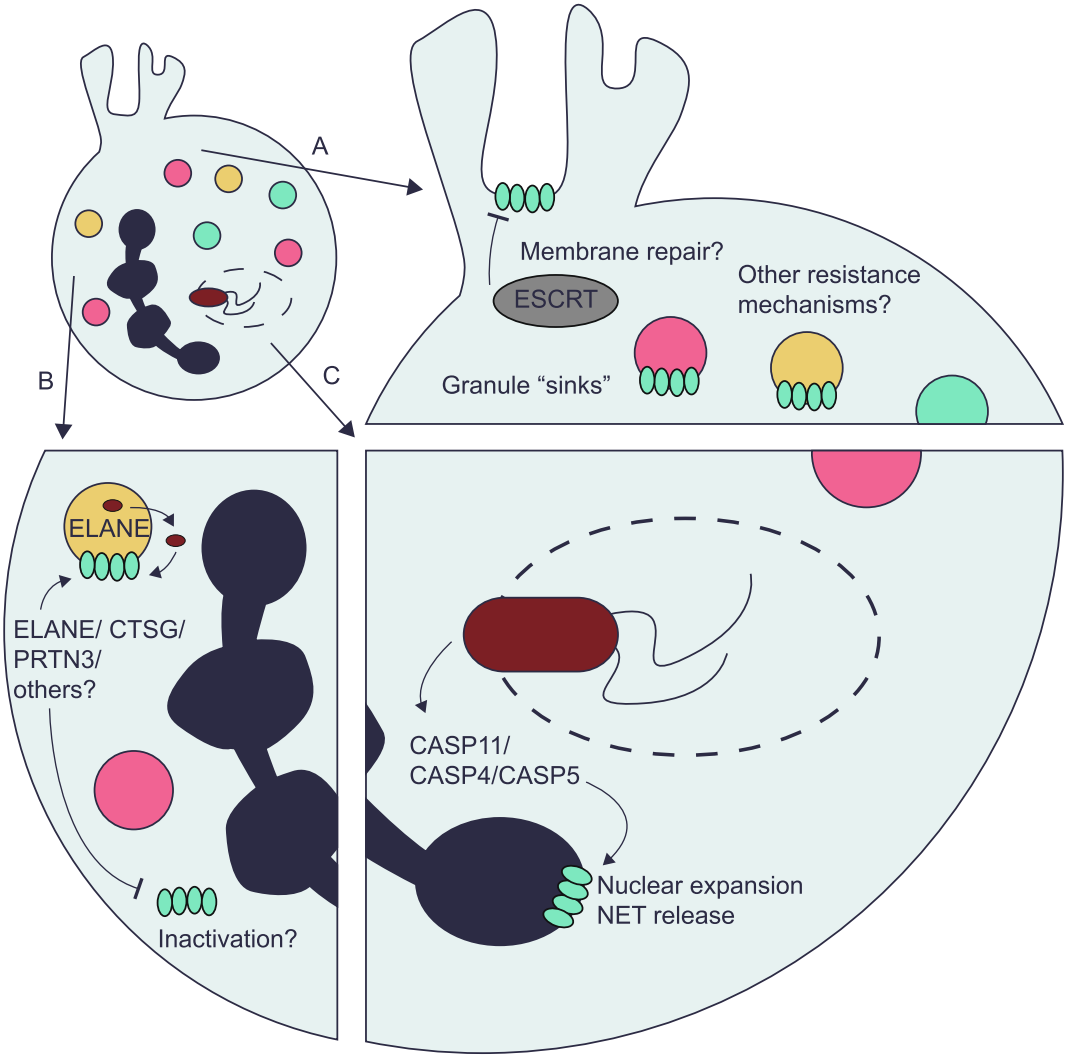

Figure 2. Potential mechanisms of neutrophil pyroptosis and pyroptosis resistance. (A) N-GSDMD (turquoise ovals) plasma membrane pores induce membrane repair mechanisms dependent on the ESCRT complex, which antagonize N-GSDMD pores. It is unclear whether these mechanisms also protect neutrophils. Neutrophil granules can serve as sinks for N-GSDMD pores, thereby protecting the plasma membrane from damage. (B) N-GSDMD pores in primary granules lead to the release of proteases, such as ELANE. ELANE can process and activate GSDMD, thereby leading to more pores. Other proteases, such as PRTN3, might inactivate GSDMD. (C) Detection of cytoplasmic LPS activates CASP11/CASP4/CASP5, which can process and activate GSDMD. This mechanism leads to chromatin decondensation, nuclear expansion and NET release in neutrophils.

they fail to detect NLRP3 protein.[118,119] Consistent with these results, several proteomics studies did not identify NLRP3 protein in human neutrophils while they found protein expression of other inflammasome components such as NLRC4 or ASC.[121–123] It could therefore be that human neutrophils are not well equipped to activate NLRP3 inflammasomes, which would explain why some studies do not identify IL-1β release from these cells. Conceptually, it would make sense for a cell as abundant as a human neutrophil to restrict activation of an inflammasome sensor as promiscuous as NLRP3. Activating mutations of NLRP3 cause autoinflammatory diseases summarized as Cryopyrin-Associated Periodic Syndromes (CAPS).[124,125] Expression of a gain-of-function NLRP3 mutation (L351P) in a neutrophil-specific manner using MRP8-Cre as a driver caused severe CAPS in mice, as a recent study showed.[126] Even though the phenotype of neutrophil-specific expression of NLRP3L351P was not as severe as expression under the pan-myeloid Lys-Cre driver (where mice die at 2 days after birth),[127] neutrophil-specific expression of this activating mutant of NLRP3 still resulted in animals dying within the first week of their life.[126] These results demonstrate the

importance of NLRP3 regulation in mice and they also suggest that neutrophils could be a source of IL-1β in CAPS patients.

There are different mechanisms regulating NLRP3. An exciting finding is that human NLRP3 exists in various splice isoforms and a version lacking exon 5 loses activity.[128] However, at present we can only speculate whether such mechanisms exist in neutrophils and there is no experimental evidence that the cells actively downregulate NLRP3 protein. However, why do some studies conclude that human neutrophils resist activation with NLRP3 agonists such as nigericin[24,110] whereas others show that nigericin treatment leads to NET formation[23,39]? A possible explanation could be the timing. As opposed to macrophage pyroptosis upon nigericin stimulation, which starts at 30–60 minutes, neutrophil nigericin-induced death occurs at later time points starting at 2–2.5 h. Any readout of cytokine release before these time points might therefore catch mostly viable cells and underestimate the killing capacity of nigericin treatment at later time points. This explanation would fit with the findings that neutrophils are better at resisting pyroptosis than macrophages – at early time points one would simply find IL-1β/ IL-18 release in the absence of cell death. At later time points however, neutrophils still die. Other findings could be explained by timing as well. Some of the studies on neutrophil inflammasome activation in the absence of pyroptosis argued that neutrophils are tremendously efficient at killing intracellular pathogens. They are in fact so efficient that subsequent cell death would not help fighting off an infection. Instead, neutrophil resistance to pyroptosis allows sustained IL-1β secretion.[108,109,129] To test this, a study looked at neutrophils derived from NOX2 -/- mice, which lack NAPDH oxidase activity and are therefore less efficient at killing phagocytosed bacteria. Indeed, the authors found that NOX2 -/- neutrophils infected with the bacterial pathogen *Pseudomonas aeruginosa* underwent pyroptosis to a higher extent than wildtype neutrophils.[129] These findings suggest that efficient bacterial killing by neutrophils prevents subsequent induction of pyroptosis. However, there might be confounding factors. Even in the absence of inflammasome activation NAPDH oxidase deficiency prolongs the lifespan of neutrophils and affects their ability to produce cytokines.[130] Furthermore, ROS produced through NADPH oxidase and/ or induced by *Pseudomonas aeruginosa*-derived molecules can block inflammasome activation.[131–133] Therefore, we still need more research to clarify whether reduced bacterial killing rescues the neutrophil's ability to undergo pyroptosis.

One thing timing does not explain is the striking discrepancy of the amounts of IL-1β release found particularly in human neutrophils. This discrepancy could be explained by a requirement for priming. NLRP3 inflammasome activation in macrophages requires a priming signal, provided by stimulation of toll-like receptors (TLRs) to allow transcription of IL-1β and NLRP3. NLRP3 also requires further posttranslational modifications for activity.[5] Priming of neutrophils might require additional signals to TLR stimulation. We know that positive selection of neutrophils during the isolation procedure tends to activate the cells and/or alter their function.[134] Activation or inhibition of neutrophil signalling pathways, occurring during the purification of cells from bone marrow, blood, or peritoneal cavity, might therefore affect how and to what extent the cells respond to TLR agonists and/or inflammasome stimulation.

The gold standard priming agent for macrophages in the context of inflammasome studies is the bacterial cell membrane component LPS. LPS activates TLR4 signalling to drive transcription of proIL-1β and NLRP3 via NF-κB.[135] Human monocytes are able to activate inflammasomes with LPS stimulation alone, without the need for a second stimulus.[10] Interestingly and confusingly, studies investigating cytokine production, inflammasome activation and NET formation in neutrophils came to different conclusions regarding the results of LPS stimulation. Some conclude that LPS enhances neutrophil viability and allows the production of chemokines such as IL-8.[130,136,137] Others use LPS to prime neutrophils for inflammasome activation.[24,64,108,117,118] Yet other studies found LPS to induce cell death via NET formation.[35,138,139] Conceptually one would not expect a priming agent to kill the cell. However, it could be that the NET-inducing ability of LPS depends on the LPS subtype.[138] It could also be that LPS indeed acts mainly as a priming agent and some contaminants present in the tissue culture experiment then indirectly induce NETs in LPS-primed neutrophils. Again, we require more and systematic research to address the requirement of human and murine neutrophils for priming events, both for subsequent NET formation and pyroptosis.

Are neutrophils able to resist GSDMD pore formation?

The dependence of neutrophil death on N-GSDMD pores is a puzzling subject. It is well established that processed GSDMD forms pores in plasma membranes, therefore the findings that N-GSDMD allows ELANE release from granules in the absence of cell death[24] and that neutrophils infected with *Burkholderia thailandensis* resist CASP1-mediated cleavage of GSDMD[109] are surprising. Karmakar *et al.*[24] demonstrated that N-GSDMD targets granule membranes, which exist in abundance in neutrophils. Therefore, granules might serve as a "sink" to capture N-GSDMD and prevent death (also discussed by Kovacs *et al*).[109] The high granule content of neutrophils, both human and murine, might equip them with an addi-

tional property facilitating resistance to GSDMD activation on top of the membrane repair mechanisms[30] and hyperactivation phenotype[9,32] also seen in dendritic cells and macrophages. Interestingly, neutrophil granules are not a static entity, they undergo changes as neutrophils age. Kambara *et al*. described that ELANE release occurred preferentially in aged neutrophils due to enhanced lysosomal membrane permeabilization.[26] Similarly, in conditions of high ROS production, for example upon strong NADPH oxidase activation, granules dissolve[57] and therefore, N-GSDMD might allow further ELANE release, but subsequently – due to reduced amounts of granules - also localize to plasma membranes.[23] On the other hand, ageing neutrophils can spontaneously degranulate and release ELANE to the extracellular space.[140] Thinking about the possibility that granule membranes act as a sink for N-GSDMD, intracellular dissolvement of granules and spontaneous degranulation could be important determinants of N-GSDMD toxicity. Dissolving granules will release ELANE to the cytoplasm where it can activate GSDMD – which then has a higher probability to target the plasma membrane due to fewer granules. Degranulation, however, does not only remove granule membranes but also ELANE itself and should therefore be a protective event. Indeed, the study by Adrover *et al*. demonstrated that degranulated neutrophils are less potent at forming NETs.[140]

One more thing to consider is the membrane specificity of N-GSDMD. We know that N-GSDMD targets eukaryotic plasma membranes from the inside but not from outside.[15] Analysis of GSDMD lipid preferences revealed that the protein binds with high affinity to cardiolipin and the phosphatidylinositol phosphates (PIP) PI(4,5)P2 and PI(4)P.[15] Interestingly, not all gasdermins share this preference,[22] adding another layer of complexity to gasdermin pores. Karmakar *et al*. proposed that N-GSDMD targets neutrophil azurophilic granules and autophagosomes while leaving other granule subtypes more or less intact.[24] This would suggest that different granule subtypes contain different lipids in their membranes. We are currently missing high resolution lipidomics of these subtypes and given that granule subtypes are not necessarily defined entities but rather a continuum[141] such studies might be difficult. However, they would be key to determine whether and how N-GSDMD acts preferentially on particular neutrophil granules. Granules are formed during differentiation and their membranes reflect the plasma membrane composition at the time of granule formation.[141] Therefore, a preference of N-GSDMD for certain granule subtypes would also suggest that developing neutrophils are more susceptible to pyroptosis. However, there is no study addressing if and how neutrophil precursors undergo pyroptosis.

In addition to N-GSDMD membrane preferences, different proteases seem to be able to both activate and inactivate GSDMD in neutrophils, which could affect its pore-forming efficiency. While CASP1/-4 activate GSDMD, apoptotic executioner caspases process GSDMD at position D88 (within the N-terminus) which prevents subsequent pore formation.[27] Neutrophils are short-lived cells with an intrinsic propensity to undergo apoptosis. Therefore, it has been suggested that apoptotic caspase activity in neutrophils might constitutively cleave GSDMD at the D88 position and prevent pyroptosis.[142] However, expression of a non-cleavable GSDMD D88A mutant protein in murine neutrophils did not alter neutrophil lysis in response to *salmonella.*[142] Interestingly, processing and activation of GSDME/DFNA5 by apoptotic caspases contributes significantly to murine neutrophil lysis during Yersinia infection.[143] Macrophage lysis in the same setting was, on the other hand, independent of GSDME.[143] These findings show that the molecular pathways at play during neutrophil and macrophage pyroptosis are not directly comparable. They also show that although neutrophils are – at least to some extent – resistant to N-GSDMD pores, they readily succumb to lysis mediated by other gasdermins. This might be due to the amount of pores being formed, to different membrane preferences of the respective gasdermin N-termini or to other, unknown, mechanisms.

Besides caspases, neutrophils possess highly active serine proteases which can also process gasdermins. Three studies showed that ELANE is able to process and activate GSDMD[23,24,26] whereas another study found no effect of ELANE and concluded that CTSG is most efficient at generating an active N-GSDMD p30 fragment.[25] Interestingly, the authors of the latter study also found PRTN3 to process GSDMD into unstable fragments and they therefore speculate that PRTN3 inhibits N-GSDMD activity.[25] These findings suggest that GSDMD activation in neutrophils depends very much on the amount or availability of different serine proteases. Even though ELANE, PRTN3 and CTSG are all stored in azurophilic granules, it is not a given that they are always present in the same granules in a 1:1:1 stoichiometry. Therefore, imbalances on the protease distribution – for example caused by ageing, degranulation, granule dissolvement or neutrophil heterogeneity – might well affect the outcome of GSDMD processing (Figure 2).

Concluding Remarks

Neutrophils are difficult cells to work with. They are notoriously easy to activate, they are short-lived, and they are not accessible to either genetic manipulation or most cell biological techniques *in vitro*. Despite these obstacles, neutrophil research is going through a revival within recent years. Single-cell technology, systems biology and carefully designed mouse studies have taught us many new things about the unique biology of this

fascinating cell type. Particularly, the developmental program from stem cells to neutrophils and the heterogeneity of mature neutrophils in circulation and tissues were subjects of great interest to researchers.[36,144–146] What we are currently missing are systematic studies investigating the effector functions of neutrophils, including the molecular mechanisms of their death. These effector functions are difficult to study *in vivo* by single cell technology, particularly processes like pyroptosis or NET formation where, by definition, one will lose the dying cells from the analysis. As a further complication, many proteins involved in neutrophil effector functions are transcribed early during maturation and subsequently stored on the protein level in granules.[122,141] We can therefore not expect to be able to assess effector functions by RNA-based studies. However, with regard that neutrophils are by far the most abundant leukocyte in circulation (in humans) and that they are associated with a broad variety of diseases and pathologies, it is crucial that we understand when and how they undergo lytic cell death. What are the conditions that induce robust and reproducible pyroptosis, necroptosis or NET formation? What are the differences between species and between neutrophils from different sources? What are the mechanisms of crosstalk between different forms of cell death? These are only three of many open questions that future research should address. Embracing developments in proteomics and transcriptomics as well as making use of novel tissue culture models of *in vitro* neutrophil differentiation, we are getting to the point where it will be possible to find answers.

CRediT authorship contribution statement

Gabriel Sollberger: Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Visualization, Project administration, Funding acquisition.

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Conflict of interest

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