Cell biology of inflammasome activation

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Abstract

Organelles are critical structures in mediating the assembly and activation of inflammasomes in mammalian cells, resulting in inflammation and cell death. Assembly of inflammasomes can occur at the mitochondria, endoplasmic reticulum, nucleus, trans-Golgi network, or pathogen surface, facilitated by the overarching architecture of the cytoskeleton. NLRP3 and Pyrin inflammasome sensors may form smaller speckles and converge on a single larger speck at the microtubule-organizing center or MTOC. This signaling hub activates multiple mammalian inflammatory and apoptotic caspases, cytokine substrates, the pore-forming protein gasdermin D, and the plasma membrane rupture protein ninjurin-1 or NINJ1, allowing pyroptosis, cellular disintegration, and inflammation to ensue. In this review, we highlight the role of mammalian cell types and organellar architectures in executing inflammasome responses.

An overview of Inflammasome Biology

Inflammasome activation signifies a central pillar of innate immune signaling leading to inflammation and cell death [1]. Inflammasome sensor proteins are triggered by a plethora of pathogens and danger signals. The identity of the inflammasome complex is designated by the inflammasome sensor that is being activated, including AIM2, NLRP1, NLRP3, NLRP6, NLRP9b, Pyrin, and caspase-11 (Figure 1). On activation, most inflammasome sensors recruit an adaptor protein called apoptosis-associated speck-like protein containing a CARD (also known as ASC or PYCARD) and the **cysteine protease** (see Glossary) called caspase-1. The activated inflammasome sensor, ASC and caspase-1 unify to form a functional inflammasome complex, from which caspase-1 drives the proteolytic cleavage of proinflammatory cytokines pro-interleukin (IL)-1β and IL-18, rendering these cytokines biologically active. Caspase-1 also induce the proteolytic cleavage of the pore-forming protein **gasdermin D (GSDMD)** [2, 3], leading to the generation of an active N-terminal domain which oligomerizes to form pores in the plasma membrane [2-8]. These pores provide channels through which IL-1β, and IL-18 are secreted by the cell [2-4, 9, 10]. Further, another protein called **ninjurin-1** or NINJ1 is activated via an as-yet-defined mechanism to induce plasma membrane rupture and pyroptosis [11].

Inflammasome components are expressed in both immune and non-immune cells of humans and mice and at multiple subcellular locations (Figure S1 and Figure S2 [12, 13]). However, it is important to note that the expression of genes encoding inflammasome components does not indicate that the inflammasome is formed or activated in a cell. Mouse bone-marrow-derived macrophages (BMDMs) are the predominant cell type used to study and characterize inflammasome biology, which is, in part, owing to a simple and easy cultivation system that can generate millions of

macrophages expressing inflammasome components from the bone marrow of a single mouse [14]. Inflammasomes are also found in bone-marrow-derived neutrophils, and intestinal epithelial cells from mice, and monocytes, macrophages, airway epithelial cells, peripheral blood mononuclear cell (PBMCs), neutrophils and platelets from humans, as well as $CD4^+$ and $CD8^+$ T cells from humans and rodents (Table 1). However, different cell types and host species assemble different inflammasome complexes (Table 1), and the activation of inflammasomes in these cell types have different biological outcomes and consequences.

The activation mechanisms of inflammasome sensors in response to their corresponding activators, and the biochemical regulation of pyroptosis have been reviewed previously [15-17] and are not the focus of this article. Instead, we provide an overview of inflammasome functionalities in different mammalian cell types and discuss the organellar processes and network in orchestrating the assembly and activation of inflammasome complexes.

Which cell types express inflammasome components and form inflammasome complexes?

Innate immune cells

In mice, the NLRP1a, NLRP1b, NLRP3, NLRP6, NAIP-NLRC4, AIM2, Pyrin and caspase-11-NLRP3 inflammasomes can form in macrophages, with a majority of studies using BMDMs as a model system [14]. Unlike other inflammasome sensors, the activation of NLRP3 inflammasome in BMDMs generally requires a priming signal (also known as Signal 1), such as stimulation with lipopolysaccharide (LPS), an outer membrane component of Gram-negative bacteria, which facilitates the upregulation of

NLRP3 and IL-1β. NLRP3 can then subsequently be activated by an activation signal (also known as Signal 2), such as ATP or nigericin. Following activation of inflammasome sensors in mouse BMDMs, cleavage of caspase-1 and GSDMD, formation of the ASC specks (also known as ASC foci), secretion of IL-1β and IL-18, and induction of pyroptosis can readily be observed [18]. Inflammasome activation can also be seen in bone-marrow-derived dendritic cells (BMDCs). However, BMDCs are often generated using cell culture media supplemented with **granulocyte-macrophage colony-stimulating factor** (GM-CSF) [19], which may affect the proportion of differentiated cells that can respond to inflammasome activators. Indeed, profiling bone marrow–derived cell populations grown in cell culture media containing GM-CSF revealed that, within a heterogeneous cell population, monocyte-derived macrophages rather than BMDCs were responsible for inflammasome activation and IL-1β secretion [19]. Determining why BMDCs are largely unresponsive to inflammasome activators may reveal key inhibitory checkpoints of inflammasomes.

Mouse bone-marrow-derived neutrophils undergo sustained caspase-1 activation and IL-1β release in response to inflammasome activation. However, pyroptosis is induced by LPS but not by other classical inflammasome activators [20-22]. LPS-induced caspase-11 activation leads to cell death and the release of **neutrophil extracellular traps** in mouse bone-marrow-derived neutrophils [21, 22], potentially via GSDMD pores and/or NINJ1-dependent membrane rupture. Why other inflammasome activators do not trigger pyroptosis in mouse bone-marrow-derived neutrophils is not entirely clear. Compared to macrophages, smaller-sized ASC specks are assembled in neutrophils following stimulation with the classical NLRP3 activator nigericin [23], which might lead to a reduction in the recruitment and auto-processing of inflammasome components in mouse neutrophils and impaired pyroptosis. Moreover,

GSDMD pores are predominantly localized to **azurophilic granules** and **autophagosomes** within human and mouse neutrophils following stimulation with nigericin or ATP [24]. The localization of GSDMD pores in these organelles within neutrophils might lead to a reduced availability of pores in the plasma membrane and might explain the observed attenuated pyroptotic response.

Cell-type-specific inflammasome responses in innate immune cells can be further exemplified by the way they sense cytosolic DNA. Detection of cytosolic DNA in mouse and human macrophages requires the DNA sensor AIM2, which triggers an inflammasome response [17]. However, in human monocytes, the DNA sensor cyclic GMP–AMP synthase (cGAS) and its adaptor stimulator of interferon genes (STING) mediate cytosolic DNA recognition, leading to efflux of potassium and activation of the NLRP3 inflammasome [25, 26]. These findings suggest that different cell types may use a different selection of pattern-recognition receptors for sensing the same pathogen-associated molecular pattern (PAMP) or danger-associated molecular pattern (DAMP) to trigger an inflammasome response and can be dependent on whether or not these receptors are expressed (Figure S1). Further investigation into inflammasome formation in different cell types requires more refinement in routine culturing techniques and sustaining large numbers of these cells in the laboratory (See Outstanding questions).

Adaptive immune cells

Certain cells of the adaptive immune system express and assemble inflammasomes (Table 1 and Figure S1), and emerging evidence support the interplay between inflammasomes and adaptive immune responses. B cells express several

inflammasome components (Figure S1), but the ability of inflammasome complexes to be formed within B cells is unknown. Resting $CD4^+$ and $CD8^+$ T cells from mice and humans undergo pyroptosis following treatment with small molecule inhibitors of dipeptidyl peptidases 8 and 9 (DPP8 and 9). The sensors triggering this inflammasome response are mouse NLRP1a or NLRP1b and a newly characterized human inflammasome sensor called CARD8 [27, 28]. Similarly, another study found that human $CD4^+$ T cells undergo pyroptosis following activation of the CARD8 inflammasome, a mechanism potentially used to expel HIV-1 [29]. The revelation of these inflammasomes further broadens the role of innate immune sensing pathways in T cell biology and adaptive immunity.

Inflammasome-independent functions of inflammasome sensors have also been described in T cells [30, 31]. In mouse $CD4^+$ T cells, upregulation of NLRP3, by the signal transducer and activator of transcription 5 and IL-2, promotes the differentiation of T_H2 cells [30], whereas activation of AIM2 in mouse T-regulatory (T_{reg}) cells leads to inhibition of the **AKT–mTOR signaling** pathway, which controls the development of autoimmune diseases, including multiple sclerosis and inflammatory bowel disease [31]. Given that both T and B cells express many other inflammasome components (Figure S1), inflammasomes may have additional bifurcated inflammasome-dependent and inflammasome-independent signaling functions, which could be identified by the use of mouse strains lacking inflammasome components in specialized subsets of T and B cells.

Epithelial cells

The intestinal barrier and the gut microbiota form an intimate relationship to maintain homeostasis in the gastrointestinal tract. Inflammasomes in cells within the gastrointestinal tract, such as NAIP-NLRC4, NLRP6, NLRP9b and AIM2, contribute to the host defense against infections and the development of gastrointestinal inflammation and cancer [14]. Intestinal epithelial cells exhibit unique inflammasome responses to bacteria, viruses, or danger signals. In responses to *Salmonella* Typhimurium infection of intestinal epithelial cells, the NAIP-NLRC4 inflammasome induces the production of eicosanoid **prostaglandin** PGE2, cell death and expulsion of the infected cell from the epithelial layer [32]. Mouse intestinal epithelial cells also undergo caspase-11-mediated pyroptosis in response to LPS derived from *Escherichia coli* and *Salmonella* Typhimurium [33, 34]. Additionally, endotoxemia induced by LPS activates caspase-11, which in turn promotes caspase-8-mediated apoptosis of mouse intestinal epithelial cells [34]. These findings suggests that therapeutic interventions targeting pyroptotic caspases such as caspase-11, and apoptotic caspases such as caspase-8 may potentially block bacterial dissemination and provide benefit against bacterial sepsis. Moreover, the inflammasome sensor NLRP6 controls infection of intestinal bacterium *Citrobacter rodentium*, facilitates mucus granule exocytosis, and have been suggested to prevent **dysbiosis** in mice [35]. The exact cell types involved in this process is not known and can be further identified using mouse strains with a deletion of NLRP6 in more defined intestinal cell types. Indeed, NLRP6 is expressed in mouse intestinal goblet cells and can induce the release of IL-18 in response to bile acid-derived taurine [36].

Other inflammasomes of importance in the intestinal epithelium include NLRP9b and AIM2. In mouse intestinal epithelial cells, NLRP9b serves as an adaptor protein to the

RNA helicase DHX9 which binds to dsRNA, triggering inflammasome responses against RNA virus, rotavirus [37]. Activation of AIM2 in non-hematopoietic cells, which include intestinal epithelial cells, restricts the AKT-mTOR pathway and the development of colitis-associated colorectal cancer in mice [38, 39].

Additional epithelial cell types which execute inflammasome functions are primary human airway epithelial cells and the human keratinocyte cell line HaCaT. Investigations into these cell types revealed an ability of NLRP1 to sense the protease activity of picornaviruses, leading to inflammasome activation, pyroptosis and clearance of the virus [40, 41]. Similarly, in primary human keratinocytes and immortalized human bronchial epithelial cells, NLRP1 was found to bind directly to dsRNA during infection by RNA viruses [42].

Inflammasome biology has also been studied in human and mouse endothelial cells. The activation of human caspases-4 or -5 by LPS can lead to pyroptosis in human endothelial cell lines, such as human umbilical vein endothelial cells [43]. Similarly, activation of mouse caspase-11 by LPS triggers pyroptosis in mouse lung microvascular endothelial cells and during endotoxemia and sepsis [43, 44]. Furthermore, reactive oxygen species generated during hemorrhagic shock can activate the NLRP3 inflammasome in mouse lung endothelial cells, leading to IL-1β release and pyroptosis [45]. Given the mammalian vascular system is lined with endothelial cells, precise therapeutics targeting inflammasome signaling and/or inflammatory caspases, such as caspase-1 or caspase-4, might be beneficial in the treatment of acute lung injury and septic shock.

Although the field of inflammasome is growing rapidly, the existence inflammasomes and/or consequence of inflammasome activation are relatively poorly understood in many cell types, such as B cells, mast cells and microglial cells (Figure S1). Perhaps

specific inflammasome complexes exist in certain cell types to coincide with natural pathogens of those cells. For example, NLRP6 and NLRP9b are found in intestinal epithelial cells because these sensors are specialized in detecting pathogens infecting the intestinal tract, whereas NLRP1 is found in keratinocytes because it is specialized in responding to viruses infecting the skin. Multiple inflammasomes are found in macrophages to enable a rapid response to all types of phagocytosed PAMP and DAMP. A better understanding of cell-type-specific roles of inflammasomes and novel pattern-recognition mechanisms might provide insights into designing precision therapeutics targeting inflammasome activities in human diseases such as infection, autoimmunity, and cancer.

How do organelles facilitate inflammasome activation?

Mitochondria

In addition to its role in generating ATP to sustain cellular functions, mitochondria have an emerging role in inflammasome activation. Earlier studies found that nicotinamide dinucleotide phosphate oxidase (NOX)-dependent reactive oxygen species (ROS) production induced by the phagocytosis of crystalline material leads to activation of the NLRP3 inflammasome in human THP-1 monocytes and mouse BMDMs [46, 47]. However, activation of the NLRP3 inflammasome can occur independent of NOX-mediated ROS production in human PBMCs [48-50]. Indeed, NOX is not the only source of ROS production. Further studies have shown that mitochondria produce ROS in response to cellular stress, which can trigger activation of NLRP3 [51, 52]. Of note is that inhibitors of ROS, such as Diphenyliodonium and N-Acetyl-L-cysteine, can

block transcriptional upregulation of NLRP3 [53], making untangling the role of mitochondrial ROS in the activation of NLRP3 more difficult.

Further evidence emerged connecting mitochondrial components and NLRP3. ATP-induced mitochondrial dysfunction leads to the release of oxidized mitochondrial DNA, which is suspected to bind to and activate the NLRP3 inflammasome [54]. Indeed, increased levels of cytosolic mitochondrial DNA caused by deficiency in autophagy proteins, such as the microtubule-associated protein 1 light chain 3B and the autophagy regulator Beclin 1, can promote NLRP3 inflammasome activation induced by LPS and ATP [51]. Further, genetic aberration leading to the elongation of mitochondria [55] or a failure to remove damaged mitochondria by means of **mitophagy** or autophagy [56] can both lead to overt activation of the NLRP3 inflammasome. It is possible that either scenario leads to an excessive release of oxidized mitochondrial DNA. Indeed, newly synthesized mitochondrial DNA has been reported to associate with and activate NLRP3 [57]. Importantly, the oxidized version of either mitochondrial DNA or nuclear DNA can induce the activation of NLRP3, but not AIM2, in BMDMs [57], suggesting that oxidized host DNA might be a putative ligand of NLRP3, and that modification of host DNA leads to its differential recognition by inflammasome sensors (Figure 2**;** Key Figure).

The rapid response of NLRP3 to oxidized mitochondrial DNA or mitochondrial damage in general might in part be explained by the cellular localization of NLRP3. Once activated in HEK293T cells and BMDMs, NLRP3 can be translocated from the cytoplasm to the mitochondria by associating with lipids on the mitochondrial membrane [52, 58, 59]. Cardiolipin, a lipid localized to the inner membrane of mitochondria, can translocate to the outer membrane in response to LPS stimulation [59, 60] (Figure 2A). Externalized cardiolipin can recruit NLRP3 and caspase-1 to the

mitochondria in response to the NLRP3 activators ATP or silica [59, 60]. The release of DNA from within the mitochondria has been suggested to involve mitochondrial permeability transition pore formation, a process which permeabilizes the inner mitochondrial membrane; however, genetic deletion of cyclophilin D, a component required for mitochondrial permeability transition pore formation, did not impair NLRP3 inflammasome activation in mouse BMDMs [61]. These findings suggest that alternative machinery, such as macropores formed by oligomerization of voltage-dependent anion channel oligomers [62], might mediate mitochondrial DNA release leading to inflammasome activation.

The translocation and activation of NLRP3 may require mitochondrial anti-viral signaling protein (MAVS, also known as CARDIF, IPS1 or VISA) [58, 63] (Figure 2A). MAVS is found on the mitochondrial membrane and the **mitochondria-associated endoplasmic reticulum membrane** (**MAM**) [64]. Further genetic data have shown that BMDMs lacking MAVS have a partial reduction in the secretion of IL-1β only in response to synthetic dsRNA poly(I:C), vesicular stomatitis virus or the bacterium *Escherichia coli* [65], but not in response to nigericin, ATP or silica [65, 66]. Given that MAVS is required to produce type I interferons following cytosolic recognition of RNA molecules, it is likely that the role of MAVS rests with the upregulation, rather than activation, of NLRP3 proteins.

Golgi Apparatus

The Golgi apparatus functions in sorting proteins and lipids from the ER for transportation to the plasma membrane, secretory vesicles, and lysosomes [67], indicating that the Golgi apparatus might receive signals from and/or transmit signals

to inflammasomes. For example, protein kinase D (PKD), which is responsible for cargo transportation on the ***trans*-Golgi network** (**TGN**), can phosphorylate NLRP3 and mediate NLRP3 translocation from MAMs to the Golgi apparatus within BMDMs [68] (Figure 2B). In human epithelial HeLa cells and mouse BMDMs, TGN is dispersed in response to ATP or nigericin [69]. The phospholipids, phosphatidylinositol-4-phosphate (PtdIns4P) on TGN can recruit NLRP3 via ionic binding [69] (Figure 2B). NLRP3 forms smaller puncta on the dispersed TGN and then oligomerizes with ASC [69].

The translocation of NLRP3 to the Golgi apparatus mediated by phospholipids might also be affected by lipid metabolism because this metabolic process controls the abundance of phospholipids on cellular membranes. Indeed, two lipid homeostasis mediators, the sterol regulatory element-binding proteins (SREBPs) and SREBP cleavage-activation protein (SCAP), can mediate NLRP3 translocation from the ER to the Golgi apparatus [70]. In response to LPS stimulation, NLRP3 binds to both SREBP and SCAP, forming a ternary complex which then translocates to the Golgi apparatus [70] (Figure 2B). Absent in this complex are ASC and NIMA-related kinase 7 (NEK7), key components of the NLRP3 inflammasome [71-73], suggesting that additional steps are required to fully assemble the NLRP3 inflammasome, such as migration of the NLRP3-SREBP-SCAP complex to a secondary location for the recruitment of ASC and NEK7 into this complex.

Given that earlier studies have also shown that NLRP3 can directly bind to mitochondria, how mitochondria might contribute to the translocation of NLRP3 to the Golgi apparatus remains unclear. NLRP3 can be translocated to the Golgi apparatus that is adjacent to a mitochondrial cluster in response to mitochondrial damage

induced by nigericin in BMDMs and THP-1 cells [68, 70]. Therefore, mitochondria might provide signals to initiate the translocation of NLRP3 to the Golgi apparatus.

Endoplasmic Reticulum

As the largest organelle in mammalian cells, the endoplasmic reticulum (ER) contributes to protein synthesis and transportation, and by extension, controls inflammasome signaling. Indeed, ER has been found to directly associate with NLRP3 [52, 74]. In BMDMs and THP-1 cells, stably expressed Flag-tagged NLRP3 resides on the ER membrane where it further interacts with ASC following nigericin treatment [52, 74] (Figure 2C). Pharmacological inhibition of 2-aminoethoxydiphenyl borate (2-APB) and BAPTA-AM or knockdown of inositol 1,4,5-trisphosphate (IP_3) receptors, both of which disrupt ER-mitochondria calcium fluxes [75], can reduce NLRP3 inflammasome activation in BMDMs in response to ATP stimulation [61, 76] (Figure 2C). The mechanisms by which ER fine-tunes NLRP3 inflammasome assembly might be difficult to decipher owing to the interconnectedness between the ER and many other organelles, such as the Golgi apparatus.

Cytoskeleton

The cytoskeleton consists of microfilaments, microtubules, and intermediate filaments. Given that inflammasome assembly requires multiple components to converge to a single inflammasome speck, it is conceivable that the cytoskeleton would in some way contribute to this process. Indeed, colchicine, an inhibitor of microtubule assembly, blocks NLRP3 inflammasome activation induced by uric acid crystals in THP-1 cells and Pyrin inflammasome activation by bacterial toxins in BMDMs [77, 78]. A further

study has revealed that acetylated α-tubulin mediates the transportation of mitochondria to ER, leading to the association between mitochondria-bound ASC and ER-bound NLRP3 [74]. However, another study has shown that two inhibitors of microtubule assembly, colchicine and nocodazole, did not inhibit NLRP3 inflammasome activation in mouse BMDMs treated with nigericin [71]. This finding suggests that certain but not all NLRP3 inflammasome activators require the microtubule to initiate inflammasome assembly.

The Pyrin inflammasome is sequestered by the Ras homolog family member (Rho) A, a cytoskeleton-associated Rho guanosine triphosphatase [78]. RhoA is also responsible for cytoskeletal rearrangements which are essential for cell adhesion and migration [79]. The Pyrin inflammasome is activated in response to perturbations of microtubule dynamics, which can be induced by RhoA inhibitors, such as the glucosyltransferase cytotoxin TcdB from the bacterium *Clostridium difficile* [78]. These studies highlight the role of microtubules in organizing the subcellular locations of inflammasome sensors and activity of inflammasomes.

The **microtubule-organizing center** (**MTOC**) is suspected to be the main site where a single inflammasome speck can form and possibly the final destination for certain inflammasomes [80, 81]. AIM2 was reported to form an inflammasome complex at the MTOC in human nasopharyngeal carcinoma cells, mediated by End-binding Protein 1 [82]. However, colocalization of the AIM2 inflammasome and the MTOC in human monocytes or mouse macrophages was not observed [80, 81], suggesting that the cellular positioning of the inflammasome may differ between cell types. In THP-1 cells and BMDMs, NLRP3 can bind to the microtubule-affinity regulating kinase 4 (MARK4) which drives NLRP3 to the MTOC to form an inflammasome speck [80] (Figure 2D). Another transporter of inflammasome sensors is the dynein adaptor histone

deacetylase 6 (HDAC6) [81]. In immortalized BMDMs and THP-1 cells, HDAC6 is required for the translocation of NLRP3 and Pyrin to the MTOC where NLRP3 and Pyrin each forms a single inflammasome complex [81] (Figure 2D). Inhibitors of HDACs, such as suberoylanilide hydroxamic acid and hydroxamic acid-derived compound ITF2357, can also suppress the production of IL-1β in primary human monocytes stimulated with LPS and ATP [83]. In the absence of HDAC6, NLRP3 remained on the TGN [69, 81].

Why only a subset of inflammasomes localize to the MTOC and why this process occurs in certain cell types requires further investigation. Given that actin, a component of the microfilament, can directly bind to NLRP3 and Pyrin [84-86], and that the type III intermediate filaments, vimentin, interact with NLRP3 [87], perhaps the NLRP3 and Pyrin inflammasome complexes require MTOC and the cytoskeleton for maintaining stability for sustained activity.

Nucleus

The nucleus provides a site where two inflammasome sensors, AIM2 and interferon-inducible protein 16 (IFI16), have been shown to activate under certain conditions. To avoid unwarranted activation of either DNA sensors under homeostatic conditions, inhibitory proteins may interfere with these DNA sensors or that the nuclear DNA may be packaged in a way that is inaccessible to these DNA sensors. In primary mouse macrophages exposed to ionizing radiation, AIM2 can sense double strand DNA and forms a complex with ASC in the nucleus [88]. In primary adult human dermal microvascular endothelial cells infected with Kaposi Sarcoma herpesvirus, IFI16 can interact with ASC in the nucleus [89]. Both AIM2 and IFI16 then translocate to the

cytoplasm and form an inflammasome complex in the perinuclear region [88, 89]. Although the mechanisms of the nuclear-to-cytoplasmic translocation of AIM2 and IFI16 remain unclear, it is possible that these inflammasome sensors and ASC may have nuclear localization signals, similar to what has been reported for caspase-1 [90].

Stress granules

Stress granules are aggregates formed by untranslating messenger ribonucleoproteins in response to cellular stress [91]. In contrast to inflammasome activation which can induce cell death, stress granules can promote host cell survival [91]. The stress granule protein, DDX3X, has been reported to bind NLRP3 and drive inflammasome activation [92]. Formation of stress granules induced by the osmotic stress-inducing agent arsenite can sequester DDX3X, and thereby, inhibiting NLRP3 inflammasome formation [92]. It is also possible that stress granules may inhibit NLRP3 inflammasome activation by hindering the translation of NLRP3 inflammasome components such as NEK7 or substrates such as pro-IL-1β.

Ribosomes

Ribosomes maintain cellular translation and are especially important for generating components of inflammasomes. The function of ribosomes can be inhibited by a fungal cell wall component, the polysaccharide galactosaminogalactan of *Aspergillus fumigatus,* and thus causing cellular stress in BMDMs which triggers activation of the NLRP3 inflammasome [93]. Similarly, other translation inhibitors targeting ribosomes, such as anisomycin, puromycin and cycloheximide, can also activate the NLRP3 inflammasome [93]. Conversely, stalled translation of mRNA in ribosomes can lead to

391 the formation of stress granules which can inhibit NLRP3 inflammasome activation
392 [92]. It is possible that ribosomes are in fact a cytosolic sensing hub such that they
393 respond to pathogens or danger signals and dictate activation or inhibition of
394 inflammasomes.

395

396 ## Intracellular pathogens as inflammasome-activating "organelles"

397 Emerging evidence suggests that cytosolic pathogens, once inside the host
398 cytoplasm, can serve as a signaling hub to promote inflammasome activation. Host
399 cells express interferon-inducible proteins, such as guanylate-binding proteins
400 (GBPs), in response to microbial infection [94]. In human epithelial cells infected with
401 the bacterium *Salmonella* Typhimurium or *Shigella flexneri*, GBP1 produced by the
402 host cell can localize to the bacterial surface and bind to LPS [95-97] (Figure 2E). This
403 GBP1-LPS binding mediates the recruitment of several other GBPs and caspase-4,
404 the human ortholog of mouse caspase-11, onto the bacterial surface, ultimately
405 inducing the assembly of an inflammasome complex [95-97] (Figure 2E). These
406 intriguing findings argue that foreign membrane-bound structures, such as cytosolic
407 bacteria, can act as organelles and provide anchoring sites for inflammasome
408 assembly. A similar conceptual ideology could be applied to pathogen-containing
409 vacuoles, outer membrane vesicles produced by certain bacteria such as *Escherichia*
410 *coli*, or viral-induced replication complexes. Some of these structures are targeted and
411 ruptured by GBPs within the host cytoplasm, driving activation of inflammasomes [98-
412 102]. These structures, could therefore, be viewed as organellar platforms for the
413 initiation of inflammasome signaling.

414

Concluding Remarks

Inflammasome components are found in virtually almost all organelles within the mammalian cell, often orchestrating cell-type-specific inflammasome responses. Research studies using cell culture systems and mouse models involving bone marrow chimera and cell-type-specific deletion of inflammasome components have provide unprecedented insights into the role of inflammasomes in myeloid cells, intestinal epithelial cells, and T cells. Although time-consuming and expensive, further investigation into inflammasome biology using mouse strains with a genetic deletion of inflammasome components in more specialized cell subsets, such as those with the highest levels of expression of inflammasome components (Figure S1), would further reveal the cell biological functions of existing and potentially novel inflammasomes. The development and use of cell culture systems which can support the growth or viability of currently unculturable cells would provide alternative approaches to study the biology of mammalian inflammasomes. Indeed, investigations into cell types other than mouse macrophages, such as human T cells, have already unveiled new inflammasome complexes, such as the human CARD8 inflammasome. The use of high throughput screening methods, such as single cell proteomic analysis, may be used profile the kinetics of inflammasome activation in cultured cells or a mixed population of cells derived from tissues and organs.

While the biochemical regulation of inflammasome sensors and pyroptosis, such as post-translation modifications, the identification of ligands and activators, and relevant disease mechanisms remain at the forefront of inflammasome research, organelles are emerging pieces within the inflammasome puzzle (see Outstanding questions). The contributions of organelles in the assembly and activation of newer complexes such as CARD8, NLRP6 and NLRP9b inflammasomes, if any, remain to be

440 determined. These investigations would require the availability of imaging,
441 biochemical and structure-guided techniques and reagents which enable detection of
442 the changes and localization of inflammasome components at different stages of
443 activation and assembly.

444 A role for multiple organelles in the activation of inflammasomes is perhaps not
445 surprising, given that mammalian cells must be able to respond to a large variety of
446 pathogenic, stress and danger signals which may affect more than one organelle. In
447 response, the cell must be able to transduce signals emanating from more than one
448 organelle. Future research investigating the role of organelles would need to take into
449 account the highly intertwined nature of these structures and that it is likely that no
450 single organelle is solely responsible for the assembly and activation of inflammasome
451 complexes. Targeting organelle-specific inflammasome proteins and/or
452 inflammasome-related proteins using small molecule compounds, nanobodies or
453 monoclonal antibodies could provide new information on the cellular positions of
454 inflammasomes, but also offer alternatives for the treatment of human diseases
455 associated with excessive inflammasome activation.

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Conflicts of interest

None to declare.

References

1. Vanaja, S.K. et al. (2015) Mechanisms of inflammasome activation: recent advances and novel insights. Trends Cell Biol. 25, 308-315.
2. Kayagaki, N. et al. (2015) Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. Nature 526, 666-671.
3. Shi, J. et al. (2015) Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature 526, 660-665.
4. He, W.T. et al. (2015) Gasdermin D is an executor of pyroptosis and required for interleukin-1beta secretion. Cell Res. 25, 1285-1298.
5. Liu, X. et al. (2016) Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. Nature 535, 153-158.
6. Ding, J. et al. (2016) Pore-forming activity and structural autoinhibition of the gasdermin family. Nature 535, 111-116.
7. Sborgi, L. et al. (2016) GSDMD membrane pore formation constitutes the mechanism of pyroptotic cell death. EMBO J. 35, 1766-1778.
8. Aglietti, R.A. et al. (2016) GsdmD p30 elicited by caspase-11 during pyroptosis forms pores in membranes. Proc. Natl. Acad. Sci. U. S. A. 113, 7858-7863.
9. Evavold, C.L. et al. (2018) The Pore-Forming Protein Gasdermin D Regulates Interleukin-1 Secretion from Living Macrophages. Immunity 48, 35-44.e6.
10. Heilig, R. et al. (2018) The Gasdermin-D pore acts as a conduit for IL-1beta secretion in mice. Eur. J. Immunol. 48, 584-592.
11. Kayagaki, N. et al. (2021) NINJ1 mediates plasma membrane rupture during lytic cell death. Nature 591, 131-136.
12. Wu, C. et al. (2016) BioGPS: building your own mash-up of gene annotations and expression profiles. Nucleic Acids Res. 44, D313-D316.
13. UniProt, C. (2021) The universal protein knowledgebase in 2021. Nucleic Acids Res. 49, D480-D489.
14. Man, S.M. (2018) Inflammasomes in the gastrointestinal tract: infection, cancer and gut microbiota homeostasis. Nat. Rev. Gastroenterol. Hepatol. 15, 721-737.
15. De Schutter, E. et al. (2021) Punching Holes in Cellular Membranes: Biology and Evolution of Gasdermins. Trends Cell Biol. 31, 500-513.
16. Tweedell, R.E. and Kanneganti, T.D. (2020) Advances in Inflammasome Research: Recent Breakthroughs and Future Hurdles. Trends Mol. Med. 26, 969-971.
17. Xue, Y. et al. (2019) Emerging Activators and Regulators of Inflammasomes and Pyroptosis. Trends Immunol. 40, 1035-1052.
18. Man, S.M. and Kanneganti, T.D. (2015) Regulation of inflammasome activation. Immunol. Rev. 265, 6-21.
19. Erlich, Z. et al. (2019) Macrophages, rather than DCs, are responsible for inflammasome activity in the GM-CSF BMDC model. Nat. Immunol. 20, 397-406.
20. Chen, K.W. et al. (2014) The neutrophil NLRC4 inflammasome selectively promotes IL-1beta maturation without pyroptosis during acute Salmonella challenge. Cell Rep. 8, 570-582.
21. Sollberger, G. et al. (2018) Gasdermin D plays a vital role in the generation of neutrophil extracellular traps. Sci. Immunol. 3, eaar6689.
22. Chen, K.W. et al. (2018) Noncanonical inflammasome signaling elicits gasdermin D-dependent neutrophil extracellular traps. Sci. Immunol. 3, eaar6676.
23. Boucher, D. et al. (2018) Caspase-1 self-cleavage is an intrinsic mechanism to terminate inflammasome activity. J. Exp. Med. 215, 827-840.
24. Karmakar, M. et al. (2020) N-GSDMD trafficking to neutrophil organelles facilitates IL-1beta release independently of plasma membrane pores and pyroptosis. Nat. Commun. 11, 2212.

25. Gaidt, M.M. et al. (2017) The DNA Inflammasome in Human Myeloid Cells Is Initiated by a STING-Cell Death Program Upstream of NLRP3. Cell 171, 1110-1124 e18.
26. Swanson, K.V. et al. (2017) A noncanonical function of cGAMP in inflammasome priming and activation. J. Exp. Med. 214, 3611-3626.
27. Linder, A. et al. (2020) CARD8 inflammasome activation triggers pyroptosis in human T cells. EMBO J. 39, e105071.
28. Johnson, D.C. et al. (2020) DPP8/9 inhibitors activate the CARD8 inflammasome in resting lymphocytes. Cell Death Dis. 11, 628.
29. Wang, Q. et al. (2021) CARD8 is an inflammasome sensor for HIV-1 protease activity. Science 371, eabe1707.
30. Bruchard, M. et al. (2015) The receptor NLRP3 is a transcriptional regulator of TH2 differentiation. Nat. Immunol. 16, 859-870.
31. Chou, W.C. et al. (2021) AIM2 in regulatory T cells restrains autoimmune diseases. Nature 591, 300-305.
32. Rauch, I. et al. (2017) NAIP-NLRC4 Inflammasomes Coordinate Intestinal Epithelial Cell Expulsion with Eicosanoid and IL-18 Release via Activation of Caspase-1 and -8. Immunity 46, 649-659.
33. Knodler, L.A. et al. (2014) Noncanonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses against enteric bacterial pathogens. Cell Host Microbe 16, 249-256.
34. Mandal, P. et al. (2018) Caspase-8 Collaborates with Caspase-11 to Drive Tissue Damage and Execution of Endotoxic Shock. Immunity 49, 42-55.e6.
35. Wlodarska, M. et al. (2014) NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion. Cell 156, 1045-1059.
36. Levy, M. et al. (2015) Microbiota-Modulated Metabolites Shape the Intestinal Microenvironment by Regulating NLRP6 Inflammasome Signaling. Cell 163, 1428-1443.
37. Zhu, S. et al. (2017) Nlrp9b inflammasome restricts rotavirus infection in intestinal epithelial cells. Nature 546, 667-670.
38. Man, S.M. et al. (2015) Critical Role for the DNA Sensor AIM2 in Stem Cell Proliferation and Cancer. Cell 162, 45-58.
39. Wilson, J.E. et al. (2015) Inflammasome-independent role of AIM2 in suppressing colon tumorigenesis via DNA-PK and Akt. Nat. Med. 21, 906-913.
40. Robinson, K.S. et al. (2020) Enteroviral 3C protease activates the human NLRP1 inflammasome in airway epithelia. Science 370, eaay2002.
41. Tsu, B.V. et al. (2021) Diverse viral proteases activate the NLRP1 inflammasome. Elife 10, e60609.
42. Bauernfried, S. et al. (2021) Human NLRP1 is a sensor for double-stranded RNA. Science 371, eabd0811.
43. Cheng, K.T. et al. (2017) Caspase-11-mediated endothelial pyroptosis underlies endotoxemia-induced lung injury. J. Clin. Invest. 127, 4124-4135.
44. Deng, M. et al. (2018) The Endotoxin Delivery Protein HMGB1 Mediates Caspase-11-Dependent Lethality in Sepsis. Immunity 49, 740-753.e7.
45. Xiang, M. et al. (2011) Hemorrhagic shock activation of NLRP3 inflammasome in lung endothelial cells. J. Immunol. 187, 4809-4817.
46. Dostert, C. et al. (2008) Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science 320, 674-677.
47. Cassel, S.L. et al. (2008) The Nalp3 inflammasome is essential for the development of silicosis. Proc. Natl. Acad. Sci. U. S. A. 105, 9035-9040.

592 48. van de Veerdonk, F.L. et al. (2010) Reactive oxygen species-independent activation of the
593 IL-1beta inflammasome in cells from patients with chronic granulomatous disease. Proc. Natl.
594 Acad. Sci. U. S. A. 107, 3030-3033.
595 49. van Bruggen, R. et al. (2010) Human NLRP3 inflammasome activation is Nox1-4
596 independent. Blood 115, 5398-5400.
597 50. Meissner, F. et al. (2010) Inflammasome activation in NADPH oxidase defective
598 mononuclear phagocytes from patients with chronic granulomatous disease. Blood 116, 1570-
599 1573.
600 51. Nakahira, K. et al. (2011) Autophagy proteins regulate innate immune responses by
601 inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat.
602 Immunol. 12, 222-230.
603 52. Zhou, R. et al. (2011) A role for mitochondria in NLRP3 inflammasome activation. Nature
604 469, 221-225.
605 53. Bauernfeind, F. et al. (2011) Cutting edge: reactive oxygen species inhibitors block
606 priming, but not activation, of the NLRP3 inflammasome. J. Immunol. 187, 613-617.
607 54. Shimada, K. et al. (2012) Oxidized mitochondrial DNA activates the NLRP3
608 inflammasome during apoptosis. Immunity 36, 401-414.
609 55. Park, S. et al. (2015) Defective mitochondrial fission augments NLRP3 inflammasome
610 activation. Sci. Rep. 5, 15489.
611 56. Rocha, M. et al. (2020) Mitochondria and T2D: Role of Autophagy, ER Stress, and
612 Inflammasome. Trends Endocrinol. Metab. 31, 725-741.
613 57. Zhong, Z. et al. (2018) New mitochondrial DNA synthesis enables NLRP3 inflammasome
614 activation. Nature 560, 198-203.
615 58. Subramanian, N. et al. (2013) The adaptor MAVS promotes NLRP3 mitochondrial
616 localization and inflammasome activation. Cell 153, 348-361.
617 59. Elliott, E.I. et al. (2018) Cutting Edge: Mitochondrial Assembly of the NLRP3
618 Inflammasome Complex Is Initiated at Priming. J. Immunol. 200, 3047-3052.
619 60. Iyer, S.S. et al. (2013) Mitochondrial cardiolipin is required for Nlrp3 inflammasome
620 activation. Immunity 39, 311-323.
621 61. Lee, G.S. et al. (2012) The calcium-sensing receptor regulates the NLRP3 inflammasome
622 through Ca2+ and cAMP. Nature 492, 123-127.
623 62. Kim, J. et al. (2019) VDAC oligomers form mitochondrial pores to release mtDNA
624 fragments and promote lupus-like disease. Science 366, 1531-1536.
625 63. Park, S. et al. (2013) The mitochondrial antiviral protein MAVS associates with NLRP3
626 and regulates its inflammasome activity. J. Immunol. 191, 4358-4366.
627 64. Horner, S.M. et al. (2011) Mitochondrial-associated endoplasmic reticulum membranes
628 (MAM) form innate immune synapses and are targeted by hepatitis C virus. Proc. Natl. Acad.
629 Sci. U. S. A. 108, 14590-14595.
630 65. Franchi, L. et al. (2014) Cytosolic double-stranded RNA activates the NLRP3
631 inflammasome via MAVS-induced membrane permeabilization and K+ efflux. J. Immunol.
632 193, 4214-4222.
633 66. Allam, R. et al. (2014) Mitochondrial apoptosis is dispensable for NLRP3 inflammasome
634 activation but non-apoptotic caspase-8 is required for inflammasome priming. EMBO Rep. 15,
635 982-990.
636 67. Boncompain, G. and Weigel, A.V. (2018) Transport and sorting in the Golgi complex:
637 multiple mechanisms sort diverse cargo. Curr. Opin. Cell Biol. 50, 94-101.
638 68. Zhang, Z. et al. (2017) Protein kinase D at the Golgi controls NLRP3 inflammasome
639 activation. J. Exp. Med. 214, 2671-2693.
640 69. Chen, J. and Chen, Z.J. (2018) PtdIns4P on dispersed trans-Golgi network mediates NLRP3
641 inflammasome activation. Nature 564, 71-76.

70. Guo, C. et al. (2018) Cholesterol Homeostatic Regulator SCAP-SREBP2 Integrates NLRP3 Inflammasome Activation and Cholesterol Biosynthetic Signaling in Macrophages. Immunity 49, 842-856.e7.
71. He, Y. et al. (2016) NEK7 is an essential mediator of NLRP3 activation downstream of potassium efflux. Nature 530, 354-357.
72. Shi, H. et al. (2016) NLRP3 activation and mitosis are mutually exclusive events coordinated by NEK7, a new inflammasome component. Nat. Immunol. 17, 250-258.
73. Schmid-Burgk, J.L. et al. (2016) A Genome-wide CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Screen Identifies NEK7 as an Essential Component of NLRP3 Inflammasome Activation. J. Biol. Chem. 291, 103-109.
74. Misawa, T. et al. (2013) Microtubule-driven spatial arrangement of mitochondria promotes activation of the NLRP3 inflammasome. Nat. Immunol. 14, 454-460.
75. Loncke, J. et al. (2021) Balancing ER-Mitochondrial Ca(2+) Fluxes in Health and Disease. Trends Cell Biol. (in press)
76. Murakami, T. et al. (2012) Critical role for calcium mobilization in activation of the NLRP3 inflammasome. Proc. Natl. Acad. Sci. U. S. A. 109, 11282-11287.
77. Martinon, F. et al. (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature 440, 237-241.
78. Xu, H. et al. (2014) Innate immune sensing of bacterial modifications of Rho GTPases by the Pyrin inflammasome. Nature 513, 237-41.
79. Hodge, R.G. and Ridley, A.J. (2016) Regulating Rho GTPases and their regulators. Nat Rev Mol. Cell Biol. 17, 496-510.
80. Li, X. et al. (2017) MARK4 regulates NLRP3 positioning and inflammasome activation through a microtubule-dependent mechanism. Nat. Commun. 8, 15986.
81. Magupalli, V.G. et al. (2020) HDAC6 mediates an aggresome-like mechanism for NLRP3 and pyrin inflammasome activation. Science 369, eaas8995.
82. Wang, L.J. et al. (2014) The microtubule-associated protein EB1 links AIM2 inflammasomes with autophagy-dependent secretion. J. Biol. Chem. 289, 29322-29333.
83. Carta, S. et al. (2006) Histone deacetylase inhibitors prevent exocytosis of interleukin-1beta-containing secretory lysosomes: role of microtubules. Blood 108, 1618-1626.
84. Burger, D. et al. (2016) F-actin dampens NLRP3 inflammasome activity via Flightless-I and LRRFIP2. Sci. Rep. 6, 29834.
85. Waite, A.L. et al. (2009) Pyrin and ASC co-localize to cellular sites that are rich in polymerizing actin. Exp. Biol. Med. (Maywood) 234, 40-52.
86. Akkaya-Ulum, Y.Z. et al. (2015) Pyrin-PSTPIP1 colocalises at the leading edge during cell migration. Cell Biol. Int. 39, 1384-1394.
87. dos Santos, G. et al. (2015) Vimentin regulates activation of the NLRP3 inflammasome. Nat. Commun. 6, 6574.
88. Hu, B. et al. (2016) The DNA-sensing AIM2 inflammasome controls radiation-induced cell death and tissue injury. Science 354, 765-768.
89. Kerur, N. et al. (2011) IFI16 acts as a nuclear pathogen sensor to induce the inflammasome in response to Kaposi Sarcoma-associated herpesvirus infection. Cell Host Microbe 9, 363-375.
90. Mao, P.L. et al. (1998) Activation of caspase-1 in the nucleus requires nuclear translocation of pro-caspase-1 mediated by its prodomain. J. Biol. Chem. 273, 23621-23624.
91. Protter, D.S.W. and Parker, R. (2016) Principles and Properties of Stress Granules. Trends Cell Biol. 26, 668-679.
92. Samir, P. et al. (2019) DDX3X acts as a live-or-die checkpoint in stressed cells by regulating NLRP3 inflammasome. Nature 573, 590-594.

691 93. Briard, B. et al. (2020) Galactosaminogalactan activates the inflammasome to provide host
692 protection. Nature 588, 688-692.
693 94. Ngo, C.C. and Man, S.M. (2017) Mechanisms and functions of guanylate-binding proteins
694 and related interferon-inducible GTPases: Roles in intracellular lysis of pathogens. Cell
695 Microbiol. 19, e12791.
696 95. Wandel, M.P. et al. (2020) Guanylate-binding proteins convert cytosolic bacteria into
697 caspase-4 signaling platforms. Nat. Immunol. 21, 880-891.
698 96. Santos, J.C. et al. (2020) Human GBP1 binds LPS to initiate assembly of a caspase-4
699 activating platform on cytosolic bacteria. Nat. Commun. 11, 3276.
700 97. Kutsch, M. et al. (2020) Direct binding of polymeric GBP1 to LPS disrupts bacterial cell
701 envelope functions. EMBO J. 39, e104926.
702 98. Man, S.M. et al. (2015) The transcription factor IRF1 and guanylate-binding proteins target
703 activation of the AIM2 inflammasome by Francisella infection. Nat. Immunol. 16, 467-475.
704 99. Meunier, E. et al. (2015) Guanylate-binding proteins promote activation of the AIM2
705 inflammasome during infection with Francisella novicida. Nat. Immunol. 16, 476-484.
706 100. Meunier, E. et al. (2014) Caspase-11 activation requires lysis of pathogen-containing
707 vacuoles by IFN-induced GTPases. Nature 509, 366-370.
708 101. Finethy, R. et al. (2017) Inflammasome Activation by Bacterial Outer Membrane Vesicles
709 Requires Guanylate Binding Proteins. mBio 8, e01188.
710 102. Man, S.M. et al. (2016) IRGB10 Liberates Bacterial Ligands for Sensing by the AIM2
711 and Caspase-11-NLRP3 Inflammasomes. Cell 167, 382-396.e17.
712 103. Orning, P. et al. (2018) Pathogen blockade of TAK1 triggers caspase-8-dependent
713 cleavage of gasdermin D and cell death. Science 362, 1064-1069.
714 104. Sarhan, J. et al. (2018) Caspase-8 induces cleavage of gasdermin D to elicit pyroptosis
715 during Yersinia infection. Proc. Natl. Acad. Sci. U. S. A. 115, E10888-E10897.
716 105. Chen, K.W. et al. (2019) Extrinsic and intrinsic apoptosis activate pannexin-1 to drive
717 NLRP3 inflammasome assembly. EMBO J. 38, e101638.
718 106. Demarco, B. et al. (2020) Caspase-8-dependent gasdermin D cleavage promotes
719 antimicrobial defense but confers susceptibility to TNF-induced lethality. Sci. Adv. 6,
720 eabc3465.
721 107. Muendlein, H.I. et al. (2020) cFLIPL protects macrophages from LPS-induced pyroptosis
722 via inhibition of complex II formation. Science 367, 1379-1384.
723 108. Kambara, H. et al. (2018) Gasdermin D Exerts Anti-inflammatory Effects by Promoting
724 Neutrophil Death. Cell Rep. 22, 2924-2936.
725 109. Burgener, S.S. et al. (2019) Cathepsin G Inhibition by Serpinb1 and Serpinb6 Prevents
726 Programmed Necrosis in Neutrophils and Monocytes and Reduces GSDMD-Driven
727 Inflammation. Cell Rep. 27, 3646-3656.e5.
728 110. Hou, J. et al. (2020) PD-L1-mediated gasdermin C expression switches apoptosis to
729 pyroptosis in cancer cells and facilitates tumour necrosis. Nat. Cell. Biol. 22, 1264-1275.
730 111. Rogers, C. et al. (2017) Cleavage of DFNA5 by caspase-3 during apoptosis mediates
731 progression to secondary necrotic/pyroptotic cell death. Nat. Commun. 8, 14128.
732 112. Wang, Y. et al. (2017) Chemotherapy drugs induce pyroptosis through caspase-3 cleavage
733 of a gasdermin. Nature 547, 99-103.
734 113. Liu, Y. et al. (2020) Gasdermin E-mediated target cell pyroptosis by CAR T cells triggers
735 cytokine release syndrome. Sci. Immunol. 5, eaax7969.
736 114. Zhang, Z. et al. (2020) Gasdermin E suppresses tumour growth by activating anti-tumour
737 immunity. Nature 579, 415-420.
738 115. Zhou, Z. et al. (2020) Granzyme A from cytotoxic lymphocytes cleaves GSDMB to trigger
739 pyroptosis in target cells. Science 368, eaaz7548.

116. Broz, P. et al. (2020) The gasdermins, a protein family executing cell death and inflammation. Nat. Rev. Immunol. 20, 143-157.
117. Wang, Q. et al. (2020) A bioorthogonal system reveals antitumour immune function of pyroptosis. Nature 579, 421-426.
118. Pizzuto, M. and Pelegrin, P. (2020) Cardiolipin in Immune Signaling and Cell Death. Trends Cell Biol. 30, 892-903.
119. Rogers, C. et al. (2019) Gasdermin pores permeabilize mitochondria to augment caspase-3 activation during apoptosis and inflammasome activation. Nat. Commun. 10, 1689.
120. Ruan, J. et al. (2018) Cryo-EM structure of the gasdermin A3 membrane pore. Nature 557, 62-67.
121. Liu, Z. et al. (2019) Crystal Structures of the Full-Length Murine and Human Gasdermin D Reveal Mechanisms of Autoinhibition, Lipid Binding, and Oligomerization. Immunity 51, 43-49.e4.
122. Kuang, S. et al. (2017) Structure insight of GSDMD reveals the basis of GSDMD autoinhibition in cell pyroptosis. Proc. Natl. Acad. Sci. U. S. A. 114, 10642-10647.
123. Mulvihill, E. et al. (2018) Mechanism of membrane pore formation by human gasdermin-D. EMBO J. 37, e98321.
124. Wang, K. et al. (2020) Structural Mechanism for GSDMD Targeting by Autoprocessed Caspases in Pyroptosis. Cell 180, 941-955.e20.
125. Ruhl, S. et al. (2018) ESCRT-dependent membrane repair negatively regulates pyroptosis downstream of GSDMD activation. Science 362, 956-960.
126. Pandey, A. et al. (2019) Inflammasomes in Colitis and Colorectal Cancer: Mechanism of Action and Therapies. Yale J. Biol. Med. 92, 481-498.
127. Shen, C. et al. (2020) Gasdermins deliver a deadly punch to cancer. Cell Res. 30, 463-464.
128. Russo, A.J. et al. (2021) Intracellular immune sensing promotes inflammation via gasdermin D-driven release of a lectin alarmin. Nat. Immunol. 22, 154-165.
129. Zanoni, I. et al. (2016) An endogenous caspase-11 ligand elicits interleukin-1 release from living dendritic cells. Science 352, 1232-1236.
130. Volchuk, A. et al. (2020) Indirect regulation of HMGB1 release by gasdermin D. Nat. Commun. 11, 4561.
131. Keller, M. et al. (2008) Active caspase-1 is a regulator of unconventional protein secretion. Cell 132, 818-831.

Figure legends

Figure 1. Inflammasome sensors can bind to a specific activator or respond to physiological changes**.** NACHT, LRR and PYD domains-containing protein 3 (NLRP3) is activated by pathogen-associated molecular patterns (PAMPs) and danger-associated molecular pattern molecules (DAMPs). NLRP6 is activated by lipoteichoic acid and microbial metabolites. NLRP9b is activated by the double-stranded RNA (dsRNA) virus Rotavirus. Pyrin is activated in response to toxin-induced modification of Rho GTPases. Absent in melanoma 2 (AIM2) recognizes dsDNA. Caspases (casp) including Caspase-4/5 (Casp-4/5) from humans and Caspase-11 (Casp-11) from mice recognize lipopolysaccharides (LPS), which induce potassium efflux and further activate NLRP3. Neuronal apoptosis inhibitory proteins (NAIPs) recognize bacterial flagellin and certain proteins within the Type III secretion system (T3SS) apparatus, which further activate NLRC4. Caspase recruitment domain–containing protein 8 (CARD8) is activated by HIV protease activity mediated by dimerization of the HIV Gag–Pol polyprotein, and the inhibitor of post-proline cleaving serine proteases called Val-boroPro (VbP). Human NLRP1 (hNLRP1) is activated by certain viral proteases such as the 3C protease of human rhinovirus, dsRNA and VbP. Mouse NLRP1b (mNLRP1b) is activated by certain bacterial proteases such as the lethal factor of *Bacillus anthracis*, the *Shigella flexneri* IpaH7.8 ubiquitin ligase, and VbP.

Figure 2. Key mechanisms of inflammasome activation modulated by cellular organelles**.** (A) Mitochondria contribute to activation of the NLRP3 inflammasome either by providing signals such as reactive oxygen species (ROS) or oxidized mitochondrial DNA (mtDNA) or by recruiting NLRP3 via mitochondrial anti-viral signaling protein (MAVS) or cardiolipin. (B) The Golgi apparatus recruits NLRP3 from

ER or cytoplasm which is mediated by sterol regulatory element-binding proteins (SREBPs), SREBP cleavage-activation protein (SCAP) and protein kinase D (PKD) or phosphatidylinositol-4-phosphate (PtdIns4P) (on trans-Golgi network, TGN) and can promote NLRP3 inflammasome activation. PKD can also phosphorylate NLRP3 and trigger inflammasome activation. (C) The endoplasmic reticulum (ER) is associated with NLRP3 and can promote the oligomerization of NLRP3 with ASC. Moreover, calcium (Ca^{2+}) release mediated by inositol 1,4,5-trisphosphate receptors (IP3R) can also trigger NLRP3 inflammasome activation. (D) The microtubule-organizing center (MTOC) supports inflammasome complex formation initiated by NLRP3 or Pyrin. Translocation of NLRP3 is mediated by microtubule-affinity regulating kinase 4 (MARK4) or histone deacetylase 6 (HDAC6). Translocation of Pyrin is mediated by HDAC6. (E) The membrane of cytosolic bacteria serves as a platform for caspase-4 inflammasome signaling. Formation of this platform is initiated by human guanylate-binding protein 1 (GBP1) followed by the binding of GBP2, GBP3, GBP4 and eventually caspase-4.

Figure S1. Expression of inflammasome sensors and related molecules**.** Data represent log2 transformed guanine cytosine robust multi-array analysis (gcrma) values from the BioGPS database [12]. (A), Expression in humans. (B), Expression in mice. Left panels, Expression of inflammasome sensors. Right panels, Expression of inflammasome-related molecules. AIM2, Absent in melanoma 2; CASP, Caspase; MEFV, Mediterranean fever, also called Pyrin; GSDMD, Gasdermin D; NAIP, Neuronal apoptosis inhibitory protein; NLRC4, Nucleotide-binding domain, leucine-rich repeat-containing protein (NLR) family CARD domain-containing protein 4; NLRP, NACHT, LRR and PYD domains-containing protein. PYCARD, ASC [apoptosis-

associated speck-like protein containing a caspase activation and recruitment domain (CARD)].

Figure S2. Subcellular location of inflammasome sensors and related molecules. Data represent the locations of mature proteins as annotated in the Uniprot database [13]. (A), Expression in humans. (B), Expression in mice. AIM2, Absent in melanoma 2; ASC, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD); NAIP, Neuronal apoptosis inhibitory protein; NLRC4, Nucleotide-binding domain, leucine-rich repeat-containing protein (NLR) family CARD domain-containing protein 4; NLRP, NACHT, LRR and PYD domains-containing protein.

Figure I. Proteolytic cleavage of members of the gasdermin family. The serine protease granzyme A, secreted from lymphocytes and natural killer cells, is able to cleave gasdermin (GSDM) B [115]. Caspase-8 has been reported to cleave GSDMC [110]. Inflammatory caspases, caspase-8 [103-107], neutrophil elastase [24, 108] and cathepsin G [109] can cleave and activate GSDMD. Caspase-3, an executioner of apoptosis, can cleave and activate GSDME, leading to a switch from apoptosis to pyroptosis [111, 112]. In addition, the serine protease granzyme B can cleave GSDME [113, 114]. In this context, cytotoxic T lymphocytes and natural killer cells secret perforin and granzyme B. Perforin generates pores on the cell membrane of the target cancer cell, leading to the entrance of granzyme B. Granzyme B-mediated cleavage of GSDME releases the N-terminal domain of GSDME to induce pyroptosis and death of the cancer cell [113, 114].

Figure II. Cell biology of pyroptosis. Cleavage of gasdermin D (GSDMD) by caspase-1, caspase-8, caspase-11, neutrophil elastase or cathepsin G releases the N-terminal domain of GSDMD, which then form pores on the plasma membrane. Active IL-1β and IL-18 are secreted via these pores. During programmed cell death (including pyroptosis), activated ninjurin1 (NINJ1) undergoes oligomerization, leading to rupture of the plasma membrane at the final stages of pyroptosis. Plasma membrane rupture mediates the release of larger cytosolic proteins, such as lactate dehydrogenase (LDH) and high mobility group box (HMGB) 1. Abbreviations: Casp, Caspase.

Glossary

Aggresomes: Aggregates of misfolded proteins formed in response to proteasomal inhibition or overexpression of aggregation-prone proteins and are located near the nuclear envelope at the microtubule-organizing center.

AKT–mTOR signaling pathway: An intracellular signaling pathway regulating cell cycle leading to cell proliferation.

Apoptosis: A non-inflammatory form of programmed cell death mediated by apoptotic caspases and characterized by membrane blebbing, DNA fragmentation and cell shrinkage.

Autophagosomes: Double-membraned vesicles that contain cellular material to be degraded by autophagy.

Azurophilic granules: Membrane-bound organelles which act as reservoirs for digestive enzymes and antimicrobial proteins including myeloperoxidase, α-defensins, elastase, proteinase-3, and cathepsin G.

Cysteine protease: A family of enzymes with a central role in programmed cell death pathways, including pyroptosis, by processing their substrates at specific aspartate residues.

Dysbiosis: A condition of having imbalances in the microbial communities of the gastrointestinal tract or other parts of the body. Dysbiosis in the intestine has been linked to diseases such as inflammatory bowel disease.

Granulocyte-macrophage colony-stimulating factor (GM-CSF): A hematopoietic growth factor responsible for promoting proliferation and differentiation of stem cells into granulocytes (neutrophils, eosinophils, and basophils), macrophages, and dendritic cells.

884 **High mobility group box (HMGB) 1:** A non-histone nuclear protein released during
885 cell injury or cell death.

886 **Lactate dehydrogenase (LDH):** A hydrogen transfer enzyme found in the cytoplasm
887 of most cells and are released during cell injury or cell death.

888 **Microtubule-organizing center (MTOC):** A cellular structure composed of two
889 orthogonally arranged centrioles and pericentriolar material, which nucleates and
890 anchors microtubules.

891 **Mitochondria-associated endoplasmic reticulum membrane** (**MAM**)**:** A dynamic
892 cellular connection between subdomain of the endoplasmic reticulum and the outer
893 mitochondrial membrane. MAMs are involved in mediating autophagy, Ca^{2+} transport
894 and lipid metabolism.

895 **Mitophagy:** A form of autophagy that selectively degrades damaged mitochondria.

896 **Necroptosis:** A form of non-apoptotic cell death that can be activated by ligands of
897 death receptors and stimuli that induce the expression of death receptor ligands under
898 certain conditions.

899 **Neutrophil extracellular traps:** A web-like chromatin–protein structures released into
900 the extracellular space during neutrophil cell death, which is used to capture microbes,
901 promote coagulation, and activate myeloid cells.

902 **Ninjurin1 (NINJ1):** A homophilic cell adhesion molecule induced by nerve injury.
903 NINJ1 mediates plasma membrane rupture following programmed cell death
904 pathways.

905 **Prostaglandins (PGs)**: A family of fatty acid eicosanoids synthesized from
906 arachidonic acid via cyclooxygenase, an enzyme involved in the synthesis of PGs,

907 prostacyclin, and thromboxane, and the target of anti-inflammatory drugs such as
908 ibuprofen.

909 ***trans*-Golgi network:** A major secretory pathway and sorting station that directs newly
910 synthesized proteins to different subcellular destinations.

911

912

Box 1

The gasdermin family and activation mechanisms

The gasdermin (GSDM) family in humans comprises six members: GSDMA, GSDMB, GSDMC, GSDMD, GSDME (also known as DFNA5) and PJVK (also known as DFNB59) [116]. Mice lack GSDMB but carry three GSDMA isoforms (GSDMA1-3), four GSDMC isoforms (GSDMC1-4), and one of GSDMD, GSDME and PJVK [116]. GSDMA, GSDMB, GSDMC, GSDMD and GSDME, via their N-terminal domain, have been shown to induce pyroptosis [2-4, 6, 110-115, 117]; however, different proteases mediate their proteolytic cleavage (Figure I).

Following inflammasome activation, the inflammatory caspases, caspase-1 and caspase-11 in mice, and caspase-1, caspase-4, and caspase-5 in humans induce the cleavage of GSDMD, releasing the N-terminal domain from its C-terminal autoinhibitory domain [2-4] (Figure I). In addition to inflammatory caspases, other proteases have been reported to cleave GSDMD, suggesting that GSDMD can be used as a substrate and executor of cell death by multiple signaling pathways (Figure I).

The N-terminal domain of GSDMD can directly bind to membrane lipids, including phosphatidylinositol phosphates, phosphatidylserine, and cardiolipin [5, 6]. Phosphatidylinositol phosphates and phosphatidylserine are only present in the leaflet of the plasma membrane facing the cytoplasm of a mammalian cell [5, 6], suggesting GSDMD can only trigger cell death when they are present in the cytoplasm, possibly to prevent unwarranted cell death and tissue damage when they are released into the extracellular milieu [6]. In addition, cardiolipins found in the inner membrane of mitochondria can be translocated to the outer membrane of mitochondria, exposing

937 them to the cytoplasm [118]. This process may trigger the binding between cardiolipin
938 and the N-terminal domain of GSDMD, resulting in permeabilization and disruption of
939 mitochondria and apoptosis [5, 6, 119].

940 Following lipid binding on the plasma membrane, oligomerization of the N-terminal
941 domain of GSDMD is required for pore formation [5, 6, 120, 121]. Structural studies of
942 GSDMA3 elucidated a possibly conserved mechanism of oligomerization and pore
943 formation in the gasdermin family [6, 120]. Based on this mechanistic model, the N-
944 terminal domain of GSDMD forms arc-shaped oligomers and grows into ring-shaped
945 oligomers, punching pores of 10-20 nm in inner diameter on the plasma membrane
946 [5, 6, 120-124]. The formation of GSDMD pores on the plasma membrane does not
947 always lead to pyroptosis. Membrane damage induced by GSDMD pores can be
948 repaired by the endosomal sorting complexes required for transport (also known as
949 ESCRT) machinery [125]. The ESCRT machinery is initiated by influx of calcium via
950 GSDMD pores, followed by membrane budding and release of damaged membrane
951 to prevent cell death [125].

952

953 **Box 2**

954 **Cell biology of pyroptosis and cell fates following pyroptosis**

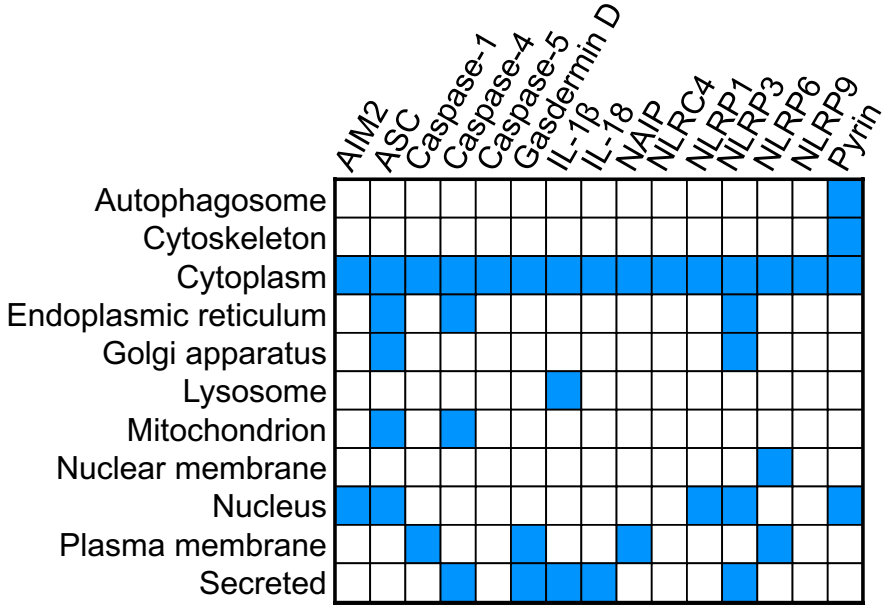
955 Pyroptosis is mediated by pore-forming proteins of the gasdermin family (Box 1) [116].
956 The induction of pyroptosis is characterized by extensive plasma membrane blebbing
957 and swelling, followed by a loss of membrane integrity and membrane rupture,
958 characteristics which have been biochemically investigated [116]. Of the gasdermin
959 family members (Box 1), gasdermin D (GSDMD) is the best characterized. Pyroptosis
960 induced by GSDMD is one of the ways in which compromised mammalian cells, such

961 as cancer cells or cells infected with a pathogen, can be removed from a multicellular
962 organism [17, 126, 127]. Furthermore, pyroptosis leads to the release of inflammatory
963 cytokines and endogenous molecules, such as IL-1β and IL-18 (Figure II). Moreover,
964 GSDMD pores mediate the release of an endogenous β-galactoside-binding lectin
965 called galectin-1, leading to inflammatory responses [128]. In some cases, mouse
966 macrophages and dendritic cells stimulated with certain inflammasome activators,
967 such as oxidized phospholipids, secrete IL-1β [9, 129], and IL-18 [9] without
968 undergoing pyroptosis.

969 The pores formed by members of the gasdermin family are not considered to be the
970 final step of pyroptosis. Instead, NINJ1 has been proposed as a terminal executor of
971 plasma membrane rupture during pyroptosis and other cell death modalities, such as
972 **apoptosis** and **necroptosis** [11]. In mouse macrophages, NINJ1 exists as a dimer or
973 trimer, which further oligomerizes to cause plasma membrane rupture. Endogenous
974 molecules which are too large to be secreted via GSDMD pores, such as **high**
975 **mobility group box (HMGB) 1** and **lactate dehydrogenase (LDH)** [130], are
976 released via plasma membrane rupture (Figure II) [11]. Mouse macrophages lacking
977 NINJ1 are no longer alive but maintain their “swelled up” or “balloon” morphology with
978 a largely intact plasma membrane integrity [11]. The role of NINJ1 is perhaps to
979 amplify inflammation and to break down the pyroptotic cell to smaller pieces for
980 degradation and clearance by other host cells. Proteomic studies confirmed that
981 inflammasome activation is associated with release of numerous proteins, such as the
982 chaperone calnexin, the pro-apoptotic protein Bid, and the endopeptidase MMP14
983 [131], suggesting that pyroptosis, GSDMD, and/or NINJ1 may have broader
984 physiological effects. The mechanisms of how NINJ1 is activated and induce
985 membrane rupture have remained unclear. It is possible that one or more substrates

activated by inflammatory caspases, or ion fluxes mediated by GSDMD or other pores in the plasma membrane, trigger the activation of NINJ1.

(A)


AIM2
ASC
Caspase-1
Caspase-4
Caspase-5
Gasdermin D
IL-1β
IL-18
NAIP
NLRC4
NLRP1
NLRP3
NLRP6
NLRP9
Pyrin
Autophagosome
Cytoskeleton
Cytoplasm
Endoplasmic reticulum
Golgi apparatus
Lysosome
Mitochondrion
Nuclear membrane
Nucleus
Plasma membrane
Secreted

(B)

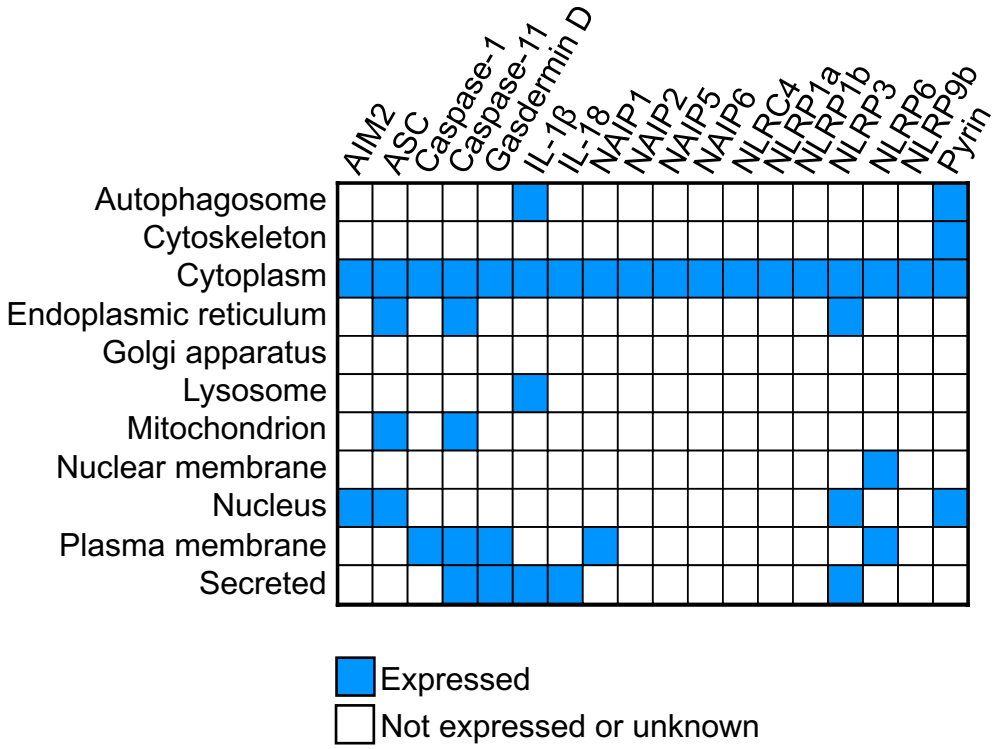

AIM2
ASC
Caspase-1
Caspase-11
Gasdermin D
IL-1β
IL-18
NAIP1
NAIP2
NAIP5
NAIP6
NLRC4
NLRP1a
NLRP1b
NLRP3
NLRP6
NLRP9b
Pyrin
Autophagosome
Cytoskeleton
Cytoplasm
Endoplasmic reticulum
Golgi apparatus
Lysosome
Mitochondrion
Nuclear membrane
Nucleus
Plasma membrane
Secreted
Expressed
Not expressed or unknown

Figure S2

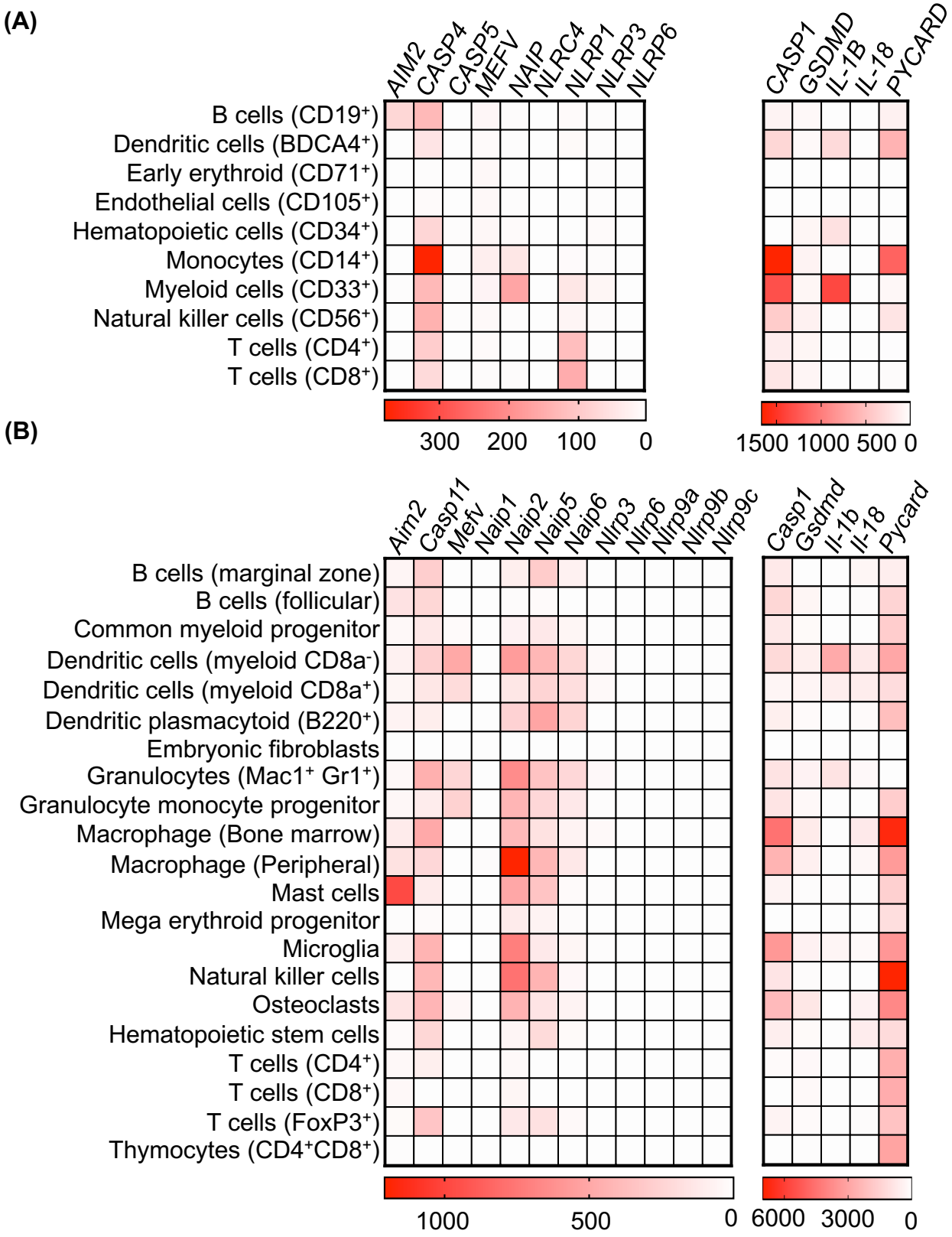

(A)
AIM2
CASP4
CASP5
MEFV
NAIP
NLRC4
NLRP1
NLRP3
NLRP6
CASP1
GSDMD
IL-1B
IL-18
PYCARD
B cells (CD19+)
Dendritic cells (BDCA4+)
Early erythroid (CD71+)
Endothelial cells (CD105+)
Hematopoietic cells (CD34+)
Monocytes (CD14+)
Myeloid cells (CD33+)
Natural killer cells (CD56+)
T cells (CD4+)
T cells (CD8+)
300 200 100 0
1500 1000 500 0
(B)
Aim2
Casp11
Mefv
Naip1
Naip2
Naip5
Naip6
Nlrp3
Nlrp6
Nlrp9a
Nlrp9b
Nlrp9c
Casp1
Gsdmd
Il-1b
Il-18
Pycard
B cells (marginal zone)
B cells (follicular)
Common myeloid progenitor
Dendritic cells (myeloid CD8a-)
Dendritic cells (myeloid CD8a+)
Dendritic plasmacytoid (B220+)
Embryonic fibroblasts
Granulocytes (Mac1+ Gr1+)
Granulocyte monocyte progenitor
Macrophage (Bone marrow)
Macrophage (Peripheral)
Mast cells
Mega erythroid progenitor
Microglia
Natural killer cells
Osteoclasts
Hematopoietic stem cells
T cells (CD4+)
T cells (CD8+)
T cells (FoxP3+)
Thymocytes (CD4+CD8+)
1000 500 0
6000 3000 0

Figure S1

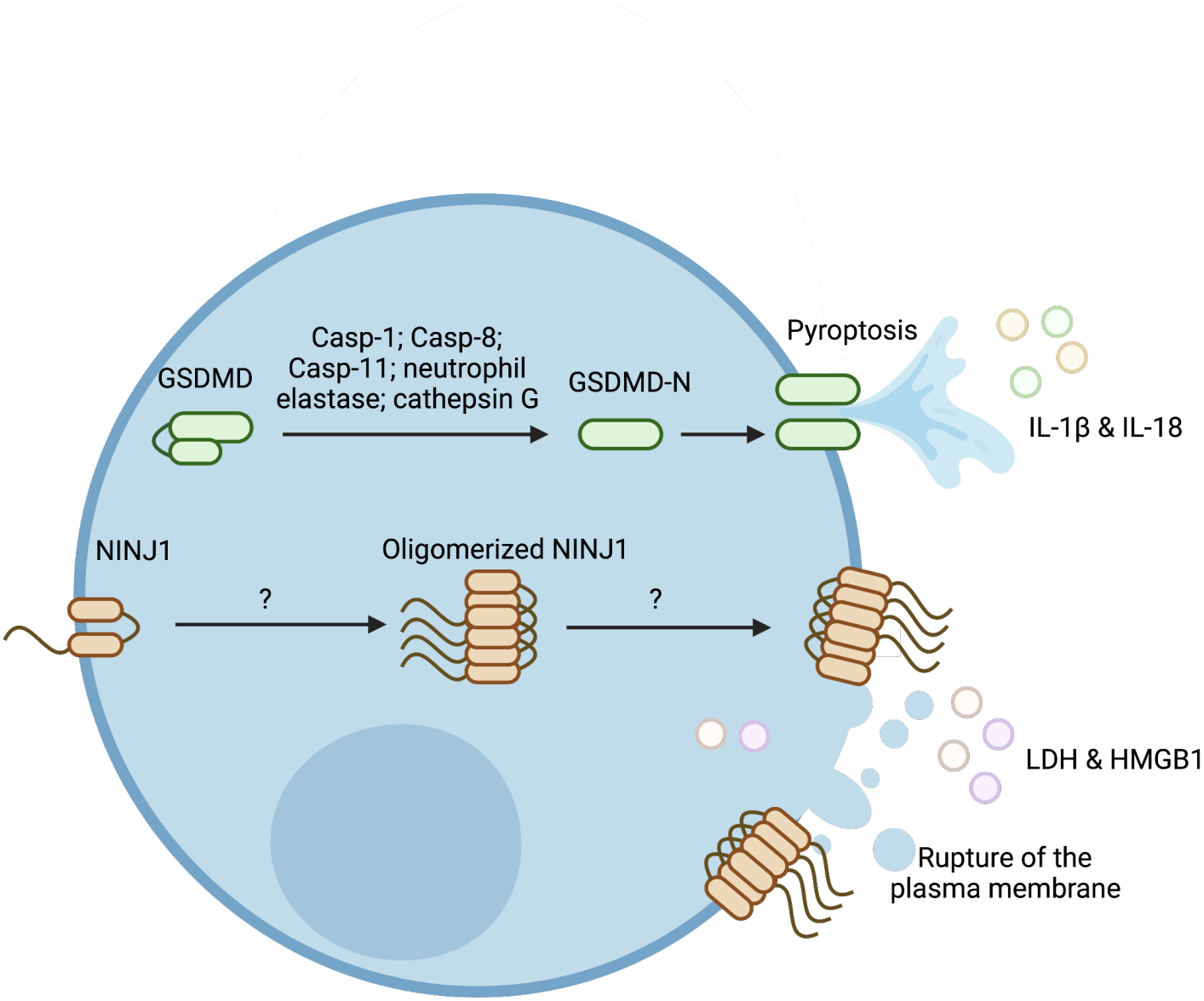

GSDMD
Casp-1; Casp-8;
Casp-11; neutrophil
elastase; cathepsin G
GSDMD-N
Pyroptosis
IL-1β & IL-18
NINJ1
?
Oligomerized NINJ1
?
LDH & HMGB1
Rupture of the
plasma membrane

Figure II

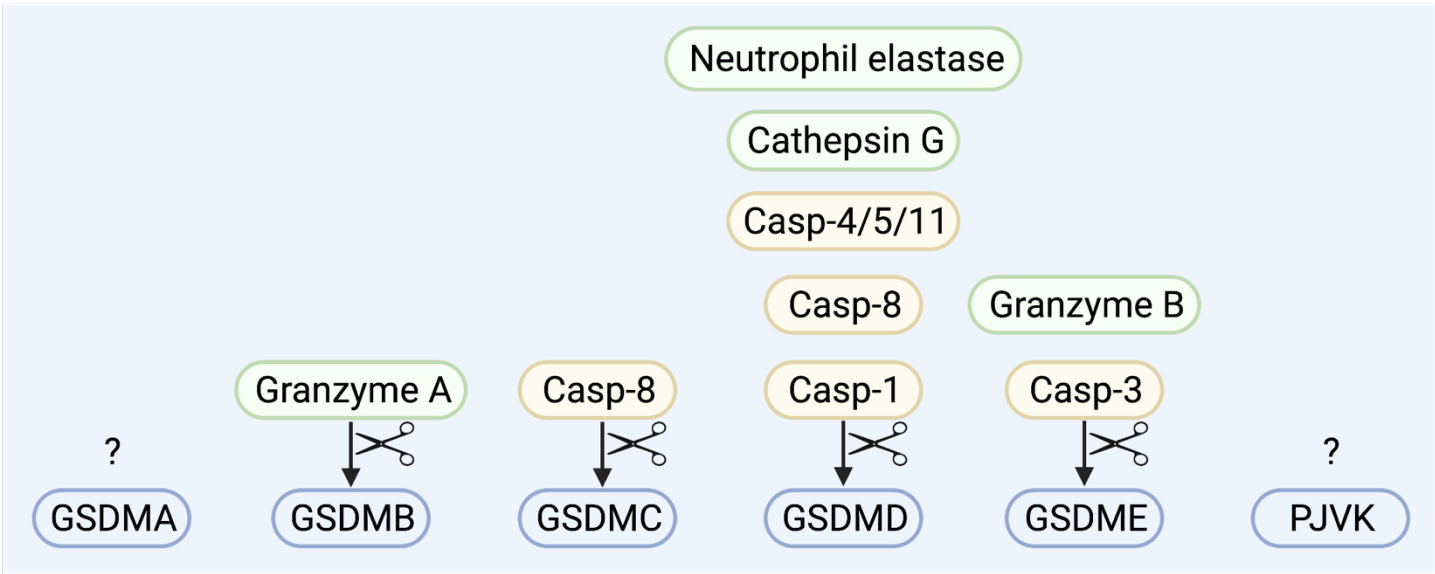

Neutrophil elastase
Cathepsin G
Casp-4/5/11
Casp-8
Granzyme B
Granzyme A
Casp-8
Casp-1
Casp-3
?
?
GSDMA
GSDMB
GSDMC
GSDMD
GSDME
PJVK

Figure I

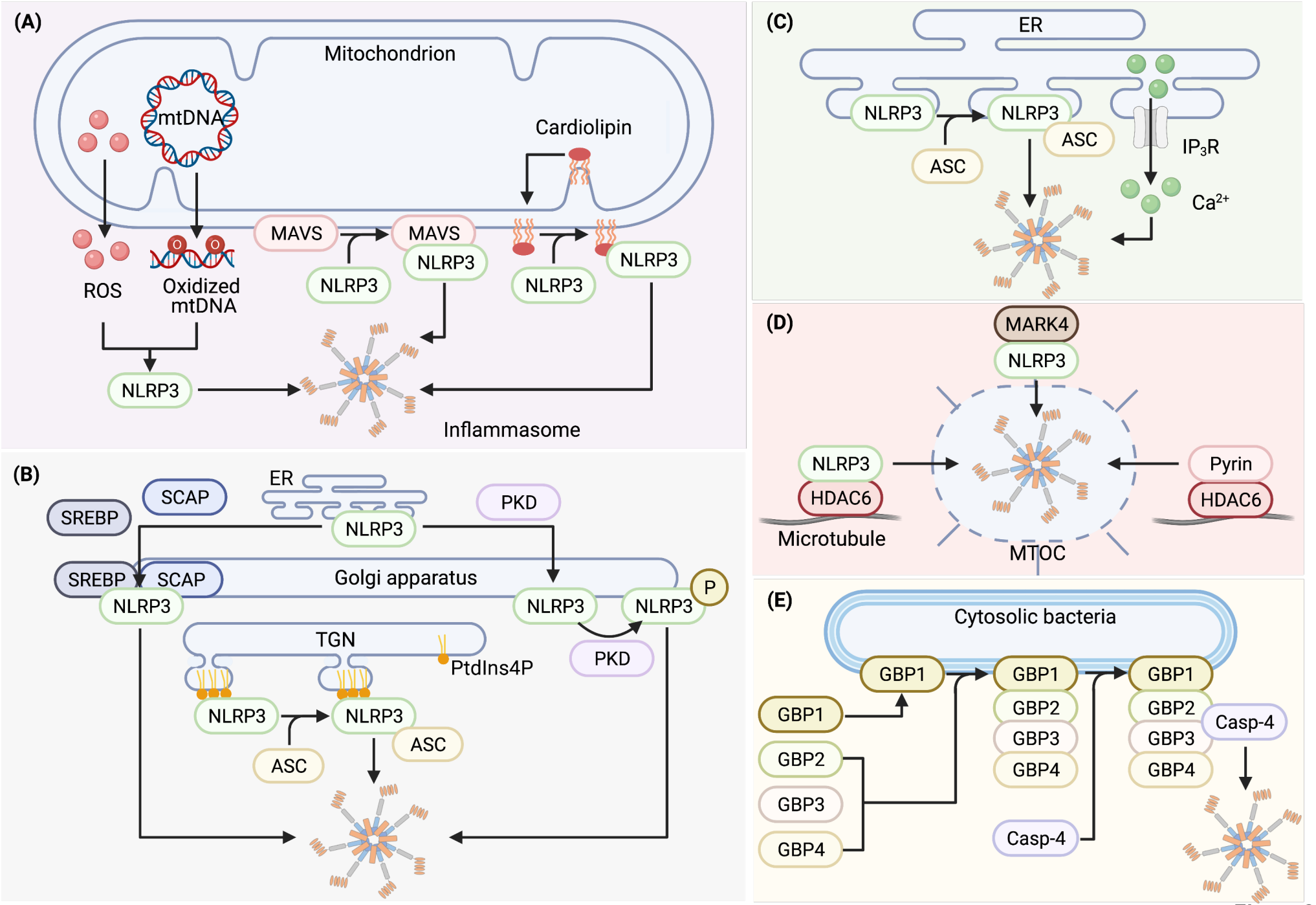

(A)
Mitochondrion
mtDNA
Cardiolipin
ROS
Oxidized mtDNA
MAVS
MAVS
NLRP3
NLRP3
NLRP3
NLRP3
NLRP3
Inflammasome
(B)
ER
SCAP
SREBP
NLRP3
PKD
SREBP
SCAP
Golgi apparatus
NLRP3
NLRP3
NLRP3
P
TGN
PtdIns4P
PKD
NLRP3
NLRP3
ASC
ASC
(C)
ER
NLRP3
NLRP3
ASC
ASC
IP3R
Ca2+
(D)
MARK4
NLRP3
NLRP3
HDAC6
Microtubule
Pyrin
HDAC6
MTOC
(E)
Cytosolic bacteria
GBP1
GBP1
GBP1
GBP1
GBP2
GBP3
GBP4
GBP2
GBP3
GBP4
GBP2
GBP3
GBP4
Casp-4
Casp-4

Figure 2

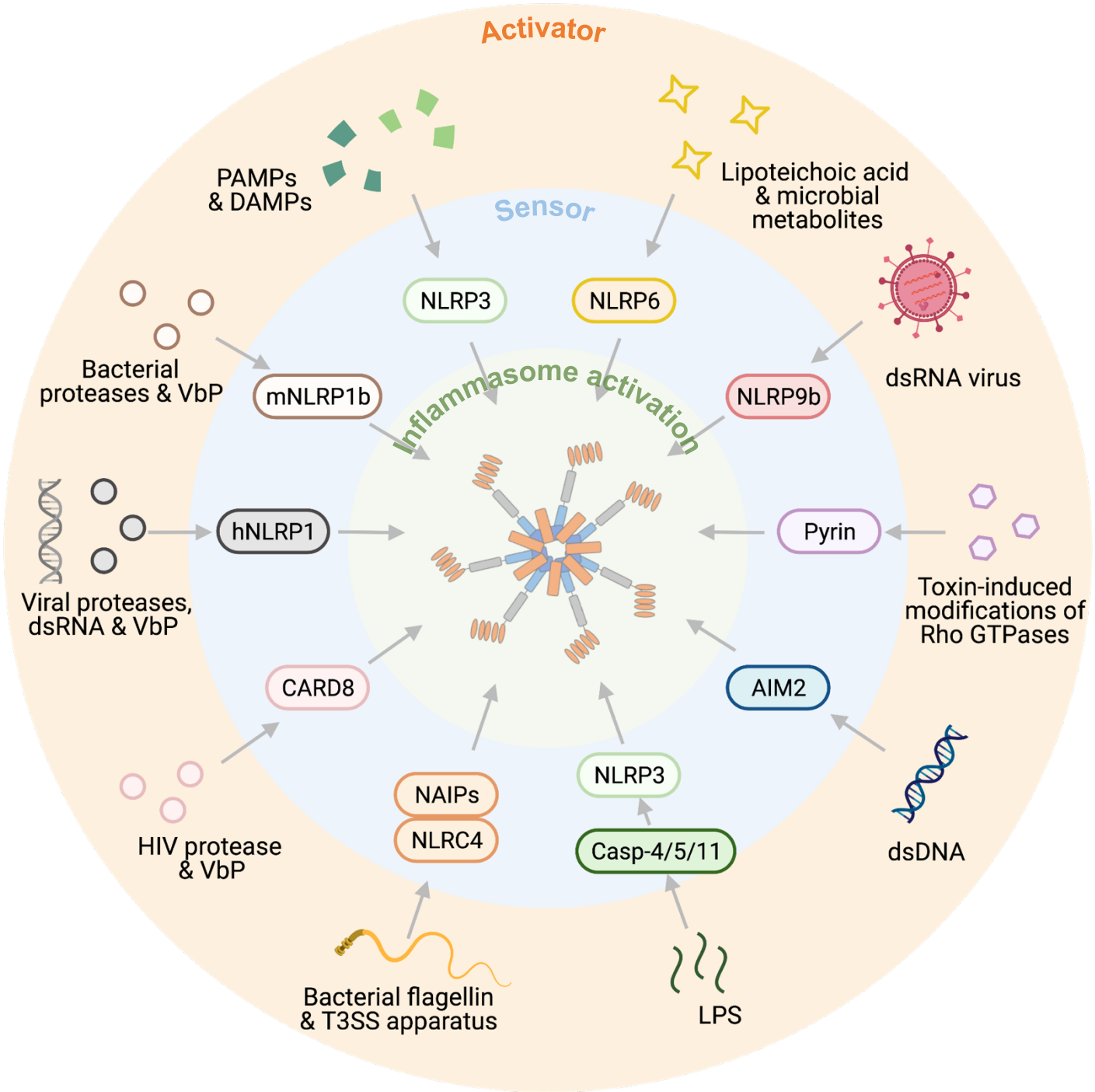

Activator
PAMPs
& DAMPs
Lipoteichoic acid
& microbial
metabolites
Sensor
NLRP3
NLRP6
Bacterial
proteases & VbP
mNLRP1b
Inflammasome activation
NLRP9b
dsRNA virus
hNLRP1
Pyrin
Viral proteases,
dsRNA & VbP
Toxin-induced
modifications of
Rho GTPases
CARD8
AIM2
NAIPs
NLRC4
NLRP3
Casp-4/5/11
HIV protease
& VbP
dsDNA
Bacterial flagellin
& T3SS apparatus
LPS

Figure 1