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Unconventional protein secretion by gasdermin pores

Petr Broz

Department of Immunobiology, University of Lausanne, Switzerland

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ABSTRACT

Unconventional protein secretion (UPS) allows the release of specific leaderless proteins independently of the classical endoplasmic reticulum (ER)-Golgi secretory pathway. While it remains one of the least understood mechanisms in cell biology, UPS plays an essential role in immunity as it controls the release of the IL-1 family of cytokines, which coordinate host defense and inflammatory responses. The unconventional secretion of IL-1β and IL-18, the two most prominent members of the IL-1 family, is initiated by inflammasome complexes – cytosolic signaling platforms that are assembled in response to infectious or noxious stimuli. Inflammasomes activate inflammatory caspases that proteolytically mature IL-1β/− 18, but also induce pyroptosis, a lytic form of cell death. Pyroptosis is caused by gasdermin-D (GSDMD), a member of the gasdermin protein family, which is activated by caspase cleavage and forms large β-barrel plasma membrane pores. This pore-forming activity is shared with other family members that are activated during infection or upon treatment with chemotherapy drugs. While the induction of cell death was assumed to be the main function of gasdermin pores, accumulating evidence suggests that they have also non-lytic functions, such as in the release of cytokines and alarmins, or in regulating ion fluxes. This has raised the possibility that gasdermin pores are one of the main mediators of UPS. Here, I summarize and discuss new insights into gasdermin activation and pore formation, how gasdermin pores achieve selective cargo release, and how gasdermin pore formation and ninjurin-1-driven plasma membrane rupture are executed and regulated.

1. Introduction

The innate immune system provides a first line of defense against invading microbes [1]. To detect the presence of these invaders, innate immune cells like macrophages and dendritic cells (DCs) constantly patrol the body and sample their environment for specific signs of tissue damage or infection. These signals, often referred to as pathogen- or danger-associated molecular patterns (PAMPs, DAMPs respectively) comprise microbial molecules such as lipopolysaccharide (LPS), peptidoglycan or flagellin; or endogenous molecules released from dying cells or damaged organelles, such as S100 proteins, nuclear or mitochondrial DNA or heat-shock proteins [2]. Immune cells use membrane bound or cytosolic pattern recognition receptors such as the Toll-like receptors (TLRs) to detect these signals and engage conserved signaling pathways that trigger the production and release of pro-inflammatory cytokines, a class of signaling molecules that are essential for mounting an inflammatory response [3]. Most cytokines (among them TNFα, IL-8, IL-6, and others) are secreted by the classical secretory pathway comprising the endoplasmic reticulum and the Golgi apparatus. A subset of cytokines, namely the 11 members of the IL-1 cytokine family, does not follow this route of secretion and is instead released by unconventional protein secretion (UPS) [4]. IL-1 family members play important roles in coordinating various inflammatory responses, with seven members having potent pro-inflammatory activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ) and four members acting as antagonists or having anti-inflammatory activities (IL-1 receptor antagonist (IL-1Ra), IL-36Ra, IL-37, IL-38) [5]. Unconventional or non-classical secretion of IL-1 family members has puzzled researchers for a long time and over the years many possible mechanisms were proposed to control their release [4]. Here, I discuss the gasdermins [6], a novel class of pore-forming proteins that are best known as executors of pyroptotic cell death, but that have also emerged as important mediators of the unconventional secretion of leaderless cytokines and danger signals.

2. Unconventional protein secretion

Classically secreted proteins carry a defined signal peptide and/or a transmembrane domain that directs the ribosome together with the nascent peptide to the endoplasmic reticulum (ER), where their synthesis is completed together with their translocation into the lumen of

E-mail address: petr.broz@unil.ch.

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ER [7]. From there they reach the Golgi apparatus via COPII-coated vesicles, and eventually traffic to the plasma membrane via secretory vesicles. Research over the last decades has shown that alternative pathways of secretion exist as well, commonly referred to as unconventional protein secretion (UPS) [8,9]. Two main categories of unconventionally secreted proteins exist. The first are proteins with a signal peptide that enter the ER but bypass the Golgi apparatus (Golgi bypass pathway). The second category are leaderless cytosolic proteins that do not have a signal peptide and/or transmembrane domain but still cross the plasma membrane to reach the extracellular space. Among the best studied of these proteins are mammalian growth factors FGF (fibroblast growth factor)– 1 and – 2, annexins, alarmins like HMGB-1 and Hsp90, and the IL-1 family cytokines [9]. How UPS is regulated remains unknown, but often UPS is linked to cell stress in form of heat shock, starvation and autophagy induction, oncotic stress, infections, or treatment with noxious substances. Comparably few substrates have been reported to be released under normal conditions, and even their release is enhanced by stress [9]. Yet, how cellular stress induces UPS is unclear. It might impair the classical secretory pathway, thus driving the need for alternative routes, or activate novel secretory pathways and mechanism that mediate UPS. Over the years, many different mechanisms have been proposed to drive UPS, such as direct pore-mediated translocation across the plasma membrane, ABC transporter based secretion, or the secretion through membrane-bound structures, such as multi-vesicular bodies (MVBs), secretory lysosomes, exosomes, secretory autophagy and amphisomes, CUPs (compartments for unconventional protein secretion) or microvesicles (for recent reviews see [4,8,9]). These release mechanisms have been observed in a variety of cell types and using different stimuli, suggesting that for a given substrate different mechanisms can exist and that these are engaged in a highly context dependent manner. It is also unknown how secreted proteins are directed to these pathways and if these pathways exert any substrate- or cargo-specificity. And finally, it remains unclear if UPS conveys any advantage over the classical secretory pathway. The rate at which secretion occurs might dictate the requirement for UPS. Unconventional protein secretion is known to occur very rapidly and as a reaction to cellular stress, such as shown for the release of IL-1β, which might be a requirement that constitutive co-translation secretion might not meet. Given the important functions that leaderless proteins have in homeostasis and disease [8], new insights into their secretion mechanism are urgently needed.

2.1. *Inflammasomes control the unconventional secretion of IL-1β and IL-18*

The best studied IL-1 family members are the two cytokines IL-1β and IL-18, which have both strong pro-inflammatory activities [5]. IL-1β signals via the IL-1 receptor and can activate immune cells or amplify the pro-inflammatory response by producing cytokines and chemokines to induce fever and attract immune cells. IL-18 is by contrast mainly known for promoting the production of interferon (IFN)-γ by NK and T cells, either independently or in synergy with IL-12, and thus leading to a rapid activation of monocytes and macrophages. Both cytokines are produced as biologically inactive precursors (pro-IL-1β, –18) that are stored in the cytosol and require proteolytic removal of the pro-domain – the so-called 'maturation' step – to exert biological activity. Proteolytic maturation of IL-1β and IL-18, as well as their subsequent release, is controlled by an innate immune signaling pathway known as the 'inflammasome pathway' (Fig. 1) [10,11]. Inflammasomes are signaling complexes that are assembled by certain cytosolic PRRs that acts as

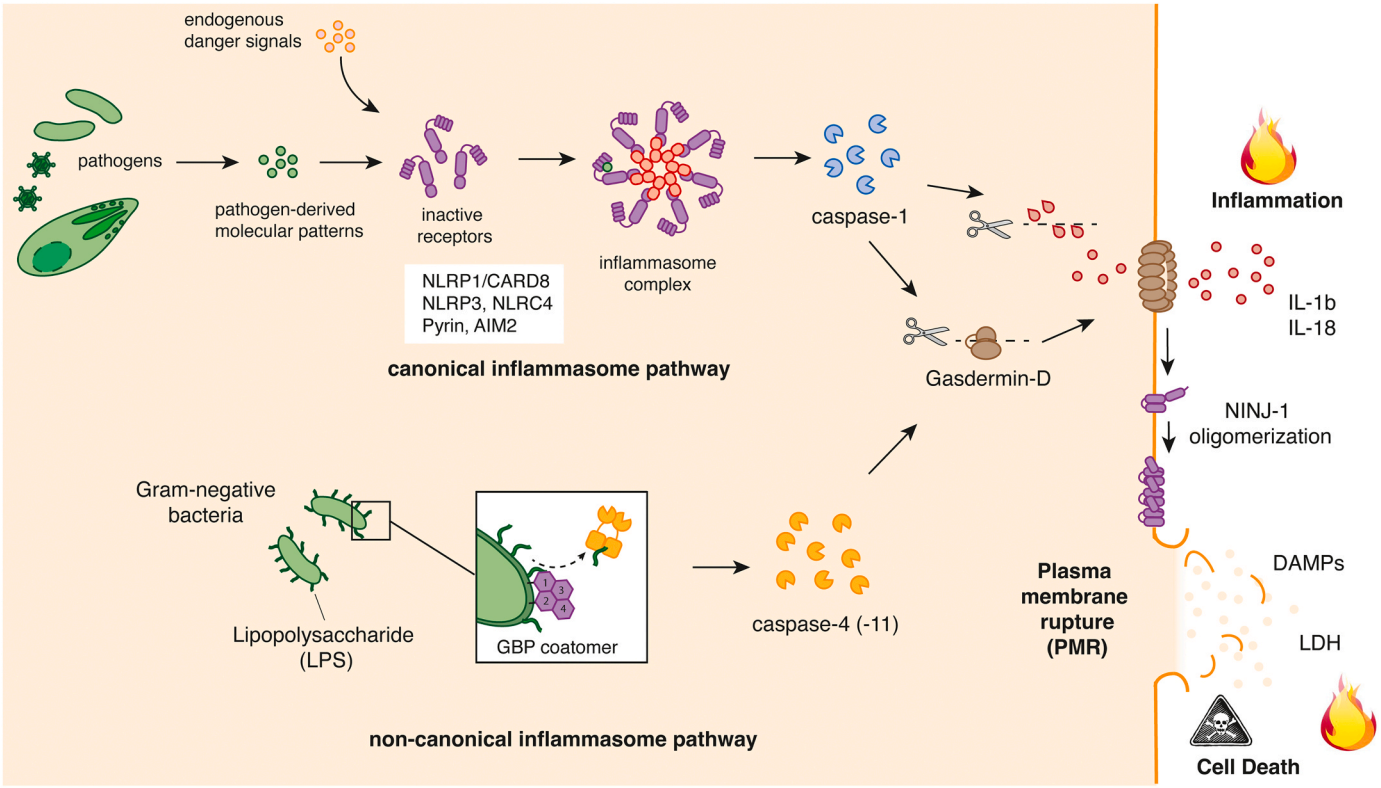

Fig. 1. Inflammasome-induced activation of GSDMD. Detection ofpathogen-derived or endogenous danger signals initiates the assembly ofinflammasome complexes, which act as activation platforms for caspase-1. In thenon-canonical pathway, GBP promote the binding of LPS to caspase-4 (-11),resulting in its activation. Caspase-1 processes the pro-forms of IL-1b and IL-18 to their mature form. Both caspase-1and -4 (-11) process GSDMD, releasing the cytotoxic N-terminal domain thatforms plasma membrane pores. Mature IL-1b/-18 exit through GSDMD pores to driveinflammation. GSDMD pore formation initiates the oligomerization of NINJ1 intoamphipathic filaments by a yet unrecognized signal. NINJ1 filaments rupture theplasma membrane thereby releasing LDH and larger DAMPs.

sensors for pathogen, cellular damage or the disruption of cellular homeostasis. The complexes control the activation of inflammatory caspases, a group of aspartate-specific cysteine dependent proteases, that are expressed as low activity zymogens and require dimerization and auto-processing to unleash their full activity [12]. Inflammasomes can be sub-divided into canonical and non-canonical inflammasomes depending on the caspase they activate [10]. Canonical inflammasomes are the largest group and control the activation of caspase-1 in humans and mice. Among the sensors that can assemble canonical inflammasome complexes are the proteins AIM2, CARD8 and Pyrin, and several members of the NOD-like receptors (NLR) family (NLRP1, NLRP3, NLRP6, NLRC4). Inflammasome forming sensors are modular proteins featuring protein-protein interaction domains for downstream signaling (PYD, CARD), oligomerization domains (such as the NACHT domain found in NLRs) and ligand binding domains. The exact signals and activation mechanisms of these receptors have extensively been reviewed and will not be covered in detail [10,13,14]. A critical step in the activation of these sensors is their oligomerization into signaling platforms, which happens in an ATP-dependent manner as shown for the NACHT domain, or by interacting with the ligand as shown for AIM2 that clusters on double-stranded cytosolic DNA. In either case, oligomerization of the receptors allows a clustering of the protein-protein interaction domains and the recruitment of the signaling adaptor ASC [15], which itself features a PYD and CARD. ASC can then recruit the caspase-1 zymogen via homotypic CARD-CARD interactions and promote its auto-activation. While some receptors can recruit pro-caspase-1 directly, most still rely on ASC since the adaptor oligomerizes into filaments which represents an important signal amplification step [16,17]. These filaments are formed via the ASC^{PYD} and expose multiple free ASC^{CARD} that recruit pro-caspase-1. ASC polymers can often be detected in cells as a single filamentous ASC speck that can be up to 1 μm in diameter [15]. Canonical inflammasomes are complemented by the non-canonical inflammasome pathway that controls the activation of caspase-4/− 5 in human cells and caspase-11 in mice [18]. The mechanistic details of this pathway are less-well understood, but it is known that Lipopolysaccharide (LPS), a component of the Gram-negative bacterial cell wall, binds these caspases and promotes their activation [19–21]. A family of IFN-induced GTPases, so-called guanylate-binding proteins (GBPs), is also required for LPS sensing [22]. The GBPs interact with membrane-bound LPS to assemble coat-like structures on cytosolic bacteria [23,24], which recruit the caspase to LPS containing membrane, thereby promoting its activation.

Soon after the discovery of IL-1β it was recognized that the cytokine is synthesized as 33 kDa precursors but released from cells as 17 kDa mature protein [25]. Moreover, it was apparent that activated immune cells, such as monocytes and THP1 cells, feature an interleukin-1 converting enzyme (ICE), but its identity was unknown until 1992, when Thornberry et al. identified it as caspase-1 [26]. That caspase-1 also cleaves IL-18 was found soon thereafter [27,28]. In the mouse, the ability to cleave IL-1 cytokines is restricted to caspase-1 since caspase-11, the effector of the non-canonical inflammasome, does not efficiently process these cytokines [18]. By contrast, in vitro and cell-based studies showed that human caspase-4 can at least process IL-18 and promotes its release [23,24,29]. Interestingly, over the years inflammatory caspases were also implicated in the release of other IL-1 family members that do not require proteolytic maturation. For example, it was found that monocytes from *Casp1*-deficient mice fail to release IL-1β upon stimulation with LPS [30]. This led the concept that active caspase-1 might be a general regulator of UPS [31], yet how caspase-1 controlled UPS remained unclear (Fig. 2). Necrotic cell lysis emerged as one possible mechanism, since it was found that besides processing IL-1 cytokines active inflammatory caspases induce a lytic form of cell death known as pyroptosis [32].

2.2. Pyroptosis, a lytic form of death induced by gasdermin family members

Pyroptosis was first reported in the late 1990 s as a form of cell death induced in macrophages upon treatment with Anthrax lethal toxin or infection with *Shigella flexneri* [33,34]. Later studies found that this form of cell death required caspase-1 but none of the apoptotic caspases, and that it correlated with the release of mature IL-1β/− 18 [35]. To underline the pro-inflammatory nature of this form of cell death the term pyroptosis was coined, using the Greek roots ‘pyro’ (fever or fire) and ‘ptosis’ (falling off of leaves) [36]. A hallmark of pyroptosis is a marked swelling and enlargement of the cell that eventually results in cell lysis and the release of cytosolic content [37]. Supplementing the cell medium with osmoprotectants such as differentially sized PEGs (polyethylene glycols) or the cytoprotectant glycine were found to block cell lysis [38]. This gave rise to the hypothesis that pyroptosis is a two-step process involving the formation of a membrane pore that then initiates non-specific ion fluxes and osmotic lysis [38]. However, the identity of the pyroptosis-inducing pore remained elusive until 2015, when gasdermin-D (GSDMD), a member of the poorly studied gasdermin protein family, emerged as a substrate of inflammatory caspases and the single executor of inflammasome-associated pyroptosis [39–41].

The gasdermin family comprises 6 members in humans (GSDMA, -B, -C, -D, -E (known as DFNA5) and PJVK (pejvakin, known as DFNB59) and 10 in mice (GSDMA1, -A2, -A3, -C1, C2, -C3, -C4, -D, -E and PVJK) [6]. Their names originate from the observation that murine GSDMA1 is most abundantly expressed in the intestinal tract and the dermis. The function of gasdermins remained unclear for over 15 years, but with time their links to cell death and inflammation started to emerge. For example, gain-of-function mutations in *Gsdma3* were found to cause alopecia with bulge stem cell depletion, hyperkeratosis and inflammation in the mouse [42,43]. Moreover, the C-terminally truncated form of GSDME caused cell cycle arrest and cell death when expressed ectopically in human cells [44]. However, whether gasdermins are components of a programmed cell death pathway and what type of cell death is controlled by these proteins remained unclear.

All gasdermin members, except for DFNB59, share a similar domain architecture consisting of an N-terminal ($GSDMD^{NT}$) and a C-terminal domain ($GSDMD^{CT}$) that are connected by an interdomain linker. Inflammatory caspases cleave GSDMD within the interdomain linker ($_{272}FLTD_{275}$ in humans and $_{273}LLSD_{276}$ in mice) which results in the separation of the two domains [39–41]. $GSDMD^{NT}$ was found to be the cytotoxic part of the protein and when expressed ectopically it is sufficient for pyroptosis induction. The $GSDMD^{CT}$ is non-toxic and serves as an inhibitory domain that keeps $GSDMD^{NT}$ inactive. Interestingly, GSDMD was found to be required for both pyroptosis induction and the release of IL-1β/− 18, indicating that GSDMD or the resulting lysis is responsible the unconventional secretion of IL-1 cytokines [39,40]. The cytotoxic activity of $GSDMD^{NT}$ depends on its ability to associate with and integrate into cellular membranes [45–48]. *In vitro* studies using liposomes showed that this insertion promotes the oligomerization of $GSDMD^{NT}$ into large pores that can permeate membranes (Fig. 1). The GSDMD pores were estimated to have an internal diameter of 20 nm and adopt a β-barrel structure formed by around 27 protomers [45–48]. The pore-forming function is not restricted to GSDMD; the N-terminal domains of other family members (with the exception of DFNB59) were also shown to kill cells upon ectopic expression and to form pores in liposomal membranes [48]. Cell death induced by other $GSDM^{NT}$ resembles GSDMD-induced pyroptosis, thus redefining pyroptosis as a form of cell death initiated by gasdermin pore formation in the plasma membrane [6]. Of note, while the plasma membrane appears to be the main target of gasdermin pores, they have been shown to also permeabilize other membranes or organelles, such as the nucleus [49], mitochondria [50,51] and neutrophil organelles [52].

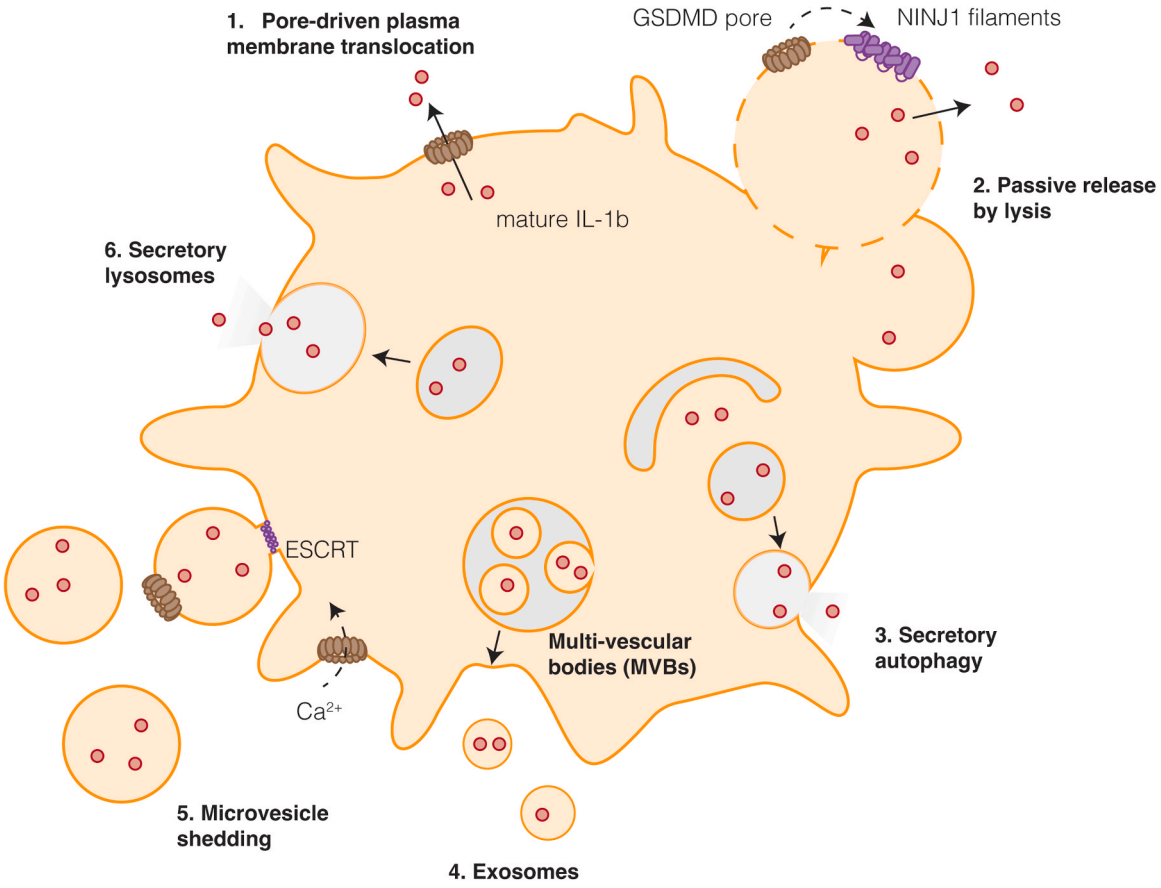

Fig. 2. Mechanisms mediating unconventional secretion of IL-1β Various mechanisms have been proposed to control the unconventional secretion of IL-1β, among them: 1. Mature IL-1β is released by GSDMD pores. Alternatively, other pores or a pore-independent mechanism might account for direct translocation across the plasma membrane. 2. IL-1β is released passively during cell lysis, triggered for example by NINJ1-dependent PMR. 3. Mature IL-1β is engulfed in autophagosomes and released via secretory autophagy. 4. Mature IL-1β is packaged into multivesicular bodies (MVBs) and released within exosomes. 5. Mature IL-1β is packaged into exosomes, that are formed by the ESCRT machinery. ESCRT assembly could be triggered by Calcium that enters the cell via GSDMD pores. 6. Mature IL-1β is taken up into secretory lysosomes and released.

Since their discovery as executors of pyroptosis, the activation mechanism of the gasdermins and their biological functions received a lot of attention (Fig. 3a). GSDMA, which is highly expressed in the skin, has for example been shown to be important for host defense against *Streptococcus pyogenes* infections [53,54]. During infections, human GSDMA or murine GSDMA1 get cleaved by the protease SpeB, an important *S. pyogenes* virulence factor, which results in pyroptosis induction and thus in the death of the infected keratinocytes, which restricts pathogen spread. GSDMB has been found to be cleaved by granzyme A (GrzA) in vitro, and consistently expression of GSDMB in cells resulted in the induction of pyroptosis after the attack by cytotoxic lymphocytes, which enhances immune clearance of tumors in mouse models [55]. GSDMC on the other hand has been found to be a substrate of apoptotic caspases in certain cases and can restrict tumor growth [56,57]. Surprisingly, it has also been found that GSDMD is not only cleaved by inflammatory caspases, but also by a variety of other proteases, thus expanding its function beyond inflammasome signaling. For example, neutrophil elastase processes GSDMD to induce NETosis [58], and caspase-8 (when activated by TNFa complex IIb) can process GSDMD at the same site as caspase-1 to promote host defense or TNF-induced inflammation [59–62]. Finally, GSDME was found to feature a caspase-3 cleavage site in its interdomain linker (D270 in humans and mice), which can be cleaved by apoptotic executioner caspases-3 and − 7 [63–65] or by granzyme B (GrzB) upon delivery by cytotoxic cells [66]. Activation of GSDME reroutes cell fate from apoptosis to pyroptosis and thus to a proinflammatory outcome. Additionally, GSDME has also been proposed to permeabilize the mitochondrial outer membrane to augment apoptosis induction [50]. Switching apoptosis to pyroptosis has been shown to be important in anti-cancer immunity, and consistently it was found that many tumor cells downregulate GSDME expression [67]. It can however also augment anti-bacterial or anti-viral immunity since caspase-3-dependent activation of GSDME in neutrophils or keratinocytes provides protection against infections with *Yersinia pseudotuberculosis* or Vesicular stomatitis virus (VSV) [68,69].

In summary, these findings firmly established the gasdermins as a family of pore-forming proteins and highlighted their physiological function in host defense and immunity. Since gasdermin cleavage was often found to correlate with the induction of pyroptosis, these studies led to the prevailing view that cell lysis is the only effector mechanism by which gasdermins exert their biological functions. However, this view has been recently challenged by several studies that demonstrate that gasdermin pores might have both lytic and non-lytic functions [70].

3. Gasdermin pores as conduits for unconventional protein secretion

Unconventional protein secretion has not only been reported for IL-1 family cytokines, but also for other leaderless cytosolic proteins such as HMGB1, FGF2 and α-synuclein [8,9]. While different mechanisms have been proposed to mediate the release of IL-1β and − 18 [4,71–78] (Fig. 2), the vast majority of studies that examined the role of GSDMD in this process found that deletion of *GSDMD* abrogates the release of these

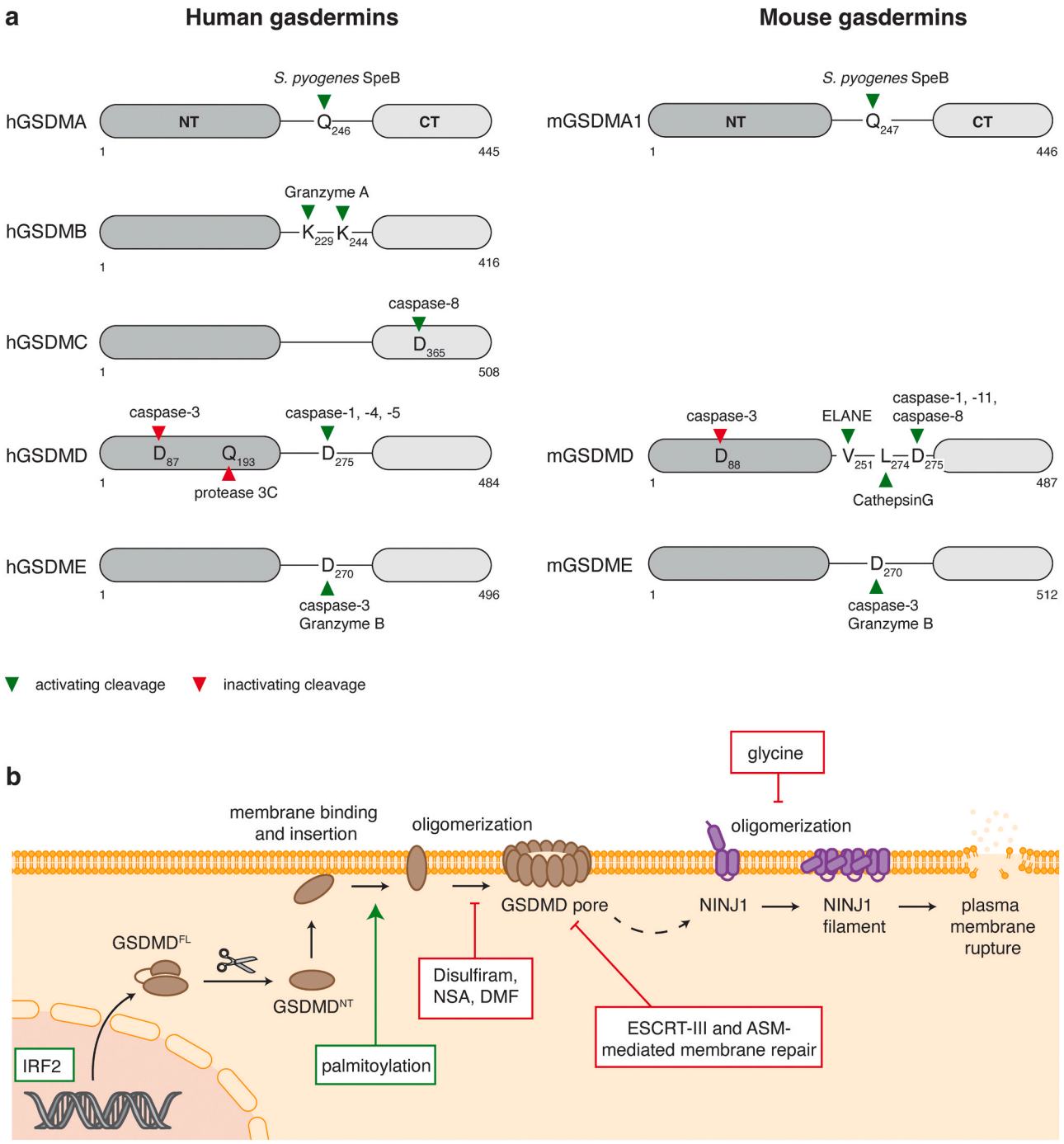

Fig. 3. Activation and regulation of gasdermins. a. Schematic drawing showing the known cleavage sites in human and mouse gasdermins, and the respective protease targeting the site. Activating cleavages are indicated by a green arrowhead, inactivating cleavages by a red arrowhead. b. Regulation of GSDMD-induced pyroptosis. IRF2 promotes the induction of GSDMD expression thus controlling GSDMD protein levels. After processing, palmitoylation at C191 (C192) promotes the interaction of $GSDMD^{NT}$ with membranes and oligomerization into GSDMD pores. GSDMD insertion and oligomerization is blocked by inhibitors acting on C191, thus preventing GSDMD pore formation. Once assembled GSDMD pores can be removed from the plasma membrane by membrane repair mechanisms, specifically ESCRT-III induced formation and shedding of microvesicles, or the internalization of damaged membranes induced by acid sphingomyelinase (ASM). Downstream of GSDMD NINJ1 oligomerizes into filaments that induce PMR (plasma membrane rupture), a step that can be blocked by glycine.

cytokines in both human and mouse cells [39,40,79,80]. This supports a model in which GSDMD pores are either an integral part of UPS of IL-1 cytokines or at least trigger a pathway of UPS. Whether this function of GSDMD pores extents to the release of other proteins released by UPS (such as FGF1/−2) remains to be determined but is rather unlikely since UPS does not always involve caspase-1. Yet, given the broad range of proteases that activate gasdermins and the fact that cellular stress can often leads to protease activation [81], it is well possible that other gasdermins are implicated in UPS independently of caspase-1 and GSDMD. If gasdermin pores are mediators of UPS it raises question of how they promote protein secretion. Do they in all cases promote pyroptosis and cell lysis that releases cytosolic content, do gasdermin pores act as conduits for secretion independently of cell lysis, or could gasdermin pores trigger UPS in an indirect manner?

3.1. GSDMD can promote IL-1 release in absence of lysis

While in most cases inflammasome activation results in IL-1β/− 18 release concomitant with cell lysis (measured by LDH release), several studies have now reported that live cells can release IL-1β as well and that this requires formation of GSDMD pores. Neutrophils for example release IL-1β after canonical inflammasome activation without measurable cell lysis (as measured by LDH release), while under the same conditions, macrophage lysis is detectable [82–84]. Neutrophil IL-1β release is GSDMD dependent, suggesting that GSDMD membrane pores are formed, but they do not induce plasma membrane rupture [80,83]. Consistently, GSDMD-dependent plasma membrane permeabilization can still be detected (measured by SYTOX-Green influx), while PMR measured by LDH release remains low [85]. Lysis-independent IL-1β release in neutrophils appears to be however highly stimulus and context dependent, since non-canonical inflammasome activation or infection with *P. aeruginosa* were reported to induce GSDMD-dependent LDH release from neutrophils [49,86]. It is unclear what determines if neutrophils undergo PMR, but it might depend on how much caspase and GSDMD is activated, the type of caspase that is activated or the engagement of addition, yet unknown factors.

Dendritic cells are another cell type that can release IL-1β in absence of cell death, for example when stimulated with oxidized phospholipids (oxPAPC) [87]. OxPAPC are bound by CD14 on the cell surface, resulting in their internalization into endosomes and translocation across the endosomal membrane by an unknown mechanism [88]. In the cytosol, oxPAPC were proposed to bind caspase-11 and activate NLRP3 which resulted in a so-called 'hyperactivated' state in which cells secrete IL-1β without any detectable release of LDH [87]. Interestingly such 'hyperactivated' cells were even reported to remain alive while forming ASC specks [89]. Hyperactivated cells release IL-1β in a GSDMD-dependent manner, indicating that they form GSDMD pores but fail to undergo PMR and terminal lysis, similarly to neutrophils. Hyperactivated DCs were found to induce superior cross-presentation, migration and long-lasting anti-tumor immunity compared to pyroptotic DCs or DCs stimulated by LPS [90]. Since this first report, other hyperactivating stimuli have been identified. Peptidoglycan (PGN)-derived N-acetylglucosamine (NAG) or infections with an Δ*oatA* mutant of *Staphylococcus aureus* trigger NLRP3 and GSDMD-dependent IL-1β release in absence of pyroptosis in macrophages [91]. Moreover, L18-MDP, a lipidated form of MDP that improves uptake of the molecule, induced a state of hyperactivation in cDC2s [92].

These few reports indicate that under certain conditions IL-1 release and even GSDMD-dependent membrane permeabilization (PI or SYTOX uptake) can be detected without common signs of PMR (LDH release). Yet, many questions about hyperactivation remain unanswered. For example, it is not clear if hyperactivated cells remain alive or if they just die without undergoing PMR, for example because they have low NINJ1 levels, or they fail to efficiently activate and polymerize NINJ1 (see below). On the other hand, it is also possible that hyperactivating stimuli induce inflammasome activation in only a small fraction of cells, which release enough IL-1β to be detected, but do not result in detectable levels of LDH release due to differential sensitivity of these assays. Indeed, the amount of IL-1β detected upon treatment with hyperactivating stimuli is much lower than after treatment with classical inflammasome activators, and titrating down the classical inflammasome activator Nigericin was shown to result in conditions where IL-1β release can still be detected while LDH release cannot [93]. Thus, careful single-cell time-resolved analysis of viability, PMR and IL-1β release will be necessary to better understand the phenomenon of hyperactivation. Nevertheless, together with the observations that osmoprotection by large PEGs or treatment with glycine efficiently blocks GSDMD-dependent cell lysis without abrogating GSDMD-dependent IL-1β release, these studies support the conclusion that GSDMD pores are large enough to serve as conduit for a direct translocation of IL-1β and potentially other cytokines across the plasma membrane.

3.2. Gasdermin pores allow a selective passage of small neutral or positively charged cargo

Since the molecular diameter of mature IL-1β (17 kDa) has been estimated to be around 4.5 nm, the inner diameter GSDMD pores must accommodate for it [48]. Early work by Fink and Cookson estimated to size of the pyroptotic pores to be between 1.1 and 2.4 nm, as PEG2000 but not the smaller PEG200 blocked cell lysis, which would be too small to allow IL-1β release [38]. Later studies using atomic force microscopy or electron microscopy however found that GSDMD pores have an inner diameter of 20–25 nm [45,47,48,94]. The size estimate was further refined by the recent cryo-EM structures of the GSDMA3 and GSDMD pore showing that these pores are β-barrels with an inner diameter of 18 nm or 21.5 nm, respectively [93,95]. Interestingly, pore size is not uniform, as these studies also reveal that GSDMA3 pores can be formed by 26–28 protomers, while the symmetry of GSDMD pores varies from 31 to 34-fold. Gasdermin pores are thus large enough to allow the passage of mature IL-1β, but do they also allow passage of larger cargo, and is there a size and cargo specificity? Experimental data argue that gasdermin pores allow a selective passage of cargo, and that size alone, measure by the molecular diameter, is not the only criterium [83]. The molecular diameter of LDH, which forms a 147-kDa heterotetramer, has for example been estimated to be only 8.7 nm, and still LDH release is only observed if cells lyse. Similarly, other proteins with small size, such as the caspase-1 p10/p20 complex (60 kDa, 6.8 nm molecular diameter) or HMGB-1 (26 kDa, 7.9 nm molecular diameter) are not released from glycine protected cells after inflammasome activation. Even pro-IL-1β, which is only slightly larger than the mature protein, was retained in cells if cell lysis was blocked by glycine [83].

Thus, what other criterium is employed by gasdermin pores to select cargo specifically? Here, the cryo-EM structure of the GSDMD pore provided novel insights into this question [93], as it revealed that the inner channel surface of GSDMD pores contains 4 predominantly acidic patches containing aspartate and glutamate residues. Interestingly, both the IL-1β and IL-18 pro-domain was also found to be mostly acidic, implying that repulsion between the acidic patches in the channel conduit and the pro-domain might repel the IL-1β pre-cursors and thus prevent its passage. Maturation of the cytokine by caspase cleavage would thus removes the acidic domain thereby facilitating the passage of the mature cytokine through the pore. Consistently, it could be shown that mutating the acidic patches in either GSDMD or the IL-1β pro-domain led to enhanced release of pro-IL-1β from glycine protected cells. Interestingly, GSDMA3 pores also feature such a predominantly acidic inner channel and were shown to exercise charge-based retention of pro-IL-1β. Thus, in summary, these findings showed that besides inducing cell death, GSDMD and GSDMA3 pores exercise electrostatic and size-based filtering that selects which cargo can pass through the channel [93]. If other gasdermin pores exhibit the same cargo-selectivity for IL-1β or other IL-1 family members, and if additional means of selecting cargo exist is not yet known. Interestingly, the authors predict that IL-1α which is predominantly acidic (both pro- and mature form) should not be able to pass through the GSDMD pore [93]. However experimental evidence contradicts this prediction, as IL-1α release is blocked by glycine [96] and does not require NINJ1, while being GSDMD dependent [97]. IL-1αhas also been reported to be released via GSDME pores from activated T_H17 cells even in absence of cell lysis, but whether GSDME pores exhibit electrostatic filtering remains to be shown [98]. A recent study suggested that IL-33 is released via GSDMD pores independently of cell lysis after exposure of airway epithelial cells to allergen proteases [99]. The authors however implicated an atypical 40 kDa GSDMD fragment in the release of IL-33 and it is unclear if such a fragment can form pores and if these have the same cargo-specificity as canonical GSDMD pores. IL-33 can also be released by GSDMC pores from intestinal epithelial cells upon helminth infection, where it contributes to type 2 immune activation and anti-helminth immunity. IL-36γ has also been reported to be secreted in a manner requiring

GSDMD and the P2X7R pores [100]. Given these reports, it would thus be interesting to investigate if the surface charge of other IL-1 family members correlates with their release through pores or cell lysis to make predictions about their release mechanism. Besides mediating the release of IL-1 family members, gasdermin pores have been shown to promote the release of galectin-1, which acts as an alarmin, and the non-canonical secretion of interferon beta [101,102].

From these data, we can conclude that passage of certain cargo can happen as soon as first gasdermin pores are formed and that this does not require terminal lysis. It is important to point out that conditions where IL-1 release precedes cell lysis or happens completely without cell lysis are rare and often artificially created by glycine protection. In most cases, IL-1 release is observed concomitant with LDH release, and under these conditions both mechanisms, e.g., pore-mediated and lysis-mediated release will happen. It does also not exclude that GSDMD pores could promote other forms of unconventional IL-1β secretion, such as via exosomes or ectosomes. It is well-known that after inflammasome activation cells shed a large number of vesicles [72], for example as a result of ESCRT-driven plasma membrane repair (see below) [69], which might carry IL-1β and transport it over long distances due to their extraordinary stability.

3.3. Ion fluxes through gasdermin pores regulate cellular signaling

Besides allowing the passage of proteins, GSDMD pores are large enough to permit the non-selective passage of ions. Investigating the earliest events caused by GSDMD pore formation, Chen et al. found that GSDMD pore formation led to potassium efflux, the entry of calcium and sodium ions, and the collapse of the ionic gradient across the plasma membrane [104]. These ion fluxes can have important impact on signaling pathways, even as far as changing cell fate after GSDMD pore formation. Potassium efflux for example is a well-known trigger of NLRP3 activation [105], and several reports suggest that GSDMD pore formation can drive NLRP3 activation. For example, NLRP3 activation following the activation of the non-canonical inflammasome requires the GSDMD and potassium efflux [106], and caspase-8-driven GSDMD pore formation in apoptotic cells has been proposed to trigger activation of NLRP3 by potassium efflux [59]. Potassium efflux has also been shown to have an impact on other cellular processes, in particular innate immune sensing of DNA by the cGAS-STING pathway. Cytosolic DNA is recognized by the 2'3' cyclic guanosinemonophosphate-adenosinmono phasphate (cGAMP) synthase cGAS to produce the second messenger cGAMP, which binds to STING (Stimulator of interferon genes) to activate IRF3-depedent production of type-I-IFNs. Banershee et al. report that activation of the DNA sensor AIM2, which forms an inflammasome and activates caspase-1 in response to DNA transfection or *Francisella novicida* infection, drives GSDMD-dependent K+ efflux which suppressed the enzymatic activity of cGAS thus reducing cGAMP production and subsequent IFN production [107]. Ion influx on the other hand might allow cells to resist GSDMD-dependent cell death by inducing plasma membrane repair mechanism (see below). In summary, the examples illustrate that GSDMD pore formation can influence cellular signaling pathways prior or even independently of lysis in many ways, yet to define if these effects are mere side effects of gasdermin-driven pyroptosis or if cells use transient non-lethal gasdermin pore formation to regulate cytosolic signaling events will require additional work.

3.4. Ninjurin-1 drives plasma membrane rupture and cell lysis downstream of gasdermin pores

Many studies have shown that gasdermin-driven cell lysis can be blocked in cell culture by adding osmoprotectants, glycine or a number of other small molecules to the extracellular medium [38,83,89,108], allowing to study which proteins can be directly released by gasdermin pores. But understanding if direct release of cytokines through pores or subsequent cell lysis is important for host defense or auto-immunity in vivo would require to genetically separate these events.

Pyroptosis was postulated to be a two-step process that starts with the formation of a plasma membrane pore formation (e.g., the gasdermin pore), followed by plasma membrane rupture and cell lysis. This second step was generally assumed to be a passive process, caused by uncontrolled water influx via the pores that leads to increased osmotic pressure and plasma membrane rupture (PMR) (e.g. osmotic lysis) [109]. However, quite unexpectedly, it could recently be shown that PMR is an active process executed by the protein ninjurin-1 (NINJ1, nerve injury induced protein 1) [97]. Using an ENU mutagenesis screen to search for factors modulating inflammasome-induced cell lysis, Kayagaki et *al.* found that deletion of *Ninj1* resulted in a loss of cell lysis (measured by LDH release) in murine BMDMs treated with canonical and non-canonical inflammasome activators. Moreover, *Ninj1*-deficiency abrogated the release of around 780 intracellular proteins, among many DAMPs such as HMGB-1. Surprisingly, GSDMD processing, PI uptake and IL-1β release remained unchanged, indicating that NINJ1 only plays a role downstream of GSDMD processing and pore formation. This phenotype strongly resembles hyperactivated cells, however, *Ninj1*-deficient cell still succumbed to cell death, probably due to extensive GSDMD membrane pore formation. While it was not addressed experimentally by the authors, it is likely that NINJ1 executes the cell lysis downstream of other gasdermin pores which have all been shown to induce efficient LDH release. Notably, the role of NINJ1 was not restricted to pyroptosis alone since *Ninj1*-deficiency abrogated PMR in cell treated with the pore-forming toxins LLO and SLO, and even in cells undergoing secondary necrosis after activation of apoptotic caspases. The role of NINJ1 was though not universal, as it only played a partial role in necroptosis induced PMR. The discovery that NINJ1 is a mediator of PMR downstream of several major pathways of cell death has been a game-changer, as it allowed to test to what extent programmed necrosis contributes to immunity and inflammation. *Ninj1*-deficiency was for example found to contribute to host defense against *Citrobacter rodentium* infections in mice, but not to LPS induced lethality [97]. The importance of NINJ1 as a driver of inflammation as also highlighted by recent studies that showed that therapeutic antibodies that inhibit NINJ1 oligomerization or *Ninj1*-deficiency ameliorated hepatocellular PMR induced with TNF plus D-galactosamine, concanavalin A, Jo2 anti-Fas agonist antibody or ischaemia-reperfusion injury, resulting in lower plasma LDH levels, reduced DAMP release and reduced infiltration of immune cells [110,111]. While these studies provide first insights into the role of NINJ1 in vivo, additional studies will be necessary to further dissect the contribution of DAMP release vs. cytokine production. PMR has for example been proposed to be important in removing the replicative niche of *Salmonella enterica* serovar typhimurium [112] or to trap intracellular bacteria within dead cell corpses known as PITs (pyroptosis-induced traps) [113]. Testing *NINJ1*-deficient cells, will also allow to determine if cell lysis is the main effector mechanism of GSDME pores in anti-tumor immunity [64] or of GSDMA pores in restricting *Streptococcus* infections [53,54], or if in these cases gasdermin pore formation is sufficient. Finally, it will be interesting to determine if NINJ1 expression levels are the driver of hyperactivation in DCs, or if neutrophils for example feature reduced NINJ1 protein levels and therefore do not undergo cell lysis [82]. It is also possible that in these cases NINJ1 activation is modulated, and thus it will be important to understand how NINJ1 is activated and how it ruptures membrane as altering these processes could also have an impact on whether a cell lyses or not.

Recent work by us has shed new light on the molecular mechanism of NINJ1-induced PMR [114]. NINJ1 is a small 16-kDa plasma membrane protein that features an N-terminal extracellular α-helix followed by two transmembrane α-helices. Mutagenesis studies have shown that residues within the N-terminal α-helix are required for NINJ1-dependent cell death, but the exact role of the N-terminal helix in membrane rupture remained unclear. A hallmark of NINJ1 activation is that the protein rapidly clusters to form oligomers that can be distinguished as larger dots by confocal microscopy. Using STORM super-resolution

microscopy, we found that while NINJ1 forms only monomers or smaller assemblies in unstimulated cell, it oligomerizes into structurally diverse assemblies in the membrane of dying cells, in particular large filamentous assemblies with branched morphology. These assemblies extended for up to 2000 nm and were often connected by thin filaments of NINJ1, suggesting that oligomeric NINJ1 adopt a predominantly filamentous conformation. Using cryo-EM we resolved the atomic structure of recombinant filamentous NINJ1, revealing a tightly packed fence-like array of transmembrane α-helices. Two antiparallel helices (α3 and α4), which correspond to the transmembrane helices of the monomer, form the core of the filament. The N-terminal extracellular α-helix is split up into α1 and α2 that are separated by a distinct kink at L56. Helix α-2 adopts a parallel orientation with respect to α3 and α4, while α1 protrudes in nearly 90° angle from the helical bundle and connects to the adjacent protomer via an extensive polymerization interface. Extensive mutagenesis confirmed the structural model in human cells and mouse macrophages, linking filament formation with PMR. The NINJ1 filament features a hydrophilic and a hydrophobic side, and molecular dynamics simulations evidence it can stably cap membrane edges. In summary, these data suggest that during lytic cell death, the extracellular a-helix of NINJ1 inserts into the plasma membrane to polymerize NINJ1 monomers into amphipathic filaments that rupture the plasma membrane. What triggers the insertion of the extracellular helix remains to be determined. It is likely that specific changes occurring upon cell death induction such as membrane lipid composition, membrane tension or ion fluxes might promote the association of the N-terminal helix with the plasma membrane. Interestingly, glycine was found to block NINJ1 oligomerization, but if it prevents insertion of the N-terminal helix or blocks NINJ1 oligomerization by other means remains to be shown [115]. In summary, the discovery of NINJ1 as mediator of PMR allows to specifically investigate role of gasdermin pores as conduits for unconventional proteins secretion, thus accelerating research into non-lytic functions of gasdermin.

4. Regulation of gasdermin pore formation

Given the important function of gasdermin pores in host defense and (auto) inflammation, it is evident that cells need to regulate it activation tightly. The most important regulation is at the level of gasdermin processing, as this is directly involved in the release of the N-terminal pore-forming domain. This form of regulation, which occurs at the level of the controlling signaling pathway and the protease will not be discussed here (for a review see [6116]). Here, we will instead focus on the regulation of gasdermin expression, and on post-translation and post-processing regulation (Fig. 3b).

4.1. Transcriptional regulation of gasdermin expression

The expression level of gasdermin family members determines if a cell undergoes pyroptosis, since enough gasdermin pores need to be formed to overwhelm plasma membrane repair mechanisms and induce pyroptosis. Different gasdermin family members show vastly different expression profiles across tissues and cell types, with GSDMD being expressed in most cell types, while GSDMA expression is restricted to the gut and skin [6]. Moreover, reducing GSDME expression has been shown to be common in many tumor cell lines [64], highlighting that regulating gasdermin expression can be an efficient way to reduce the level of gasdermin-dependent cell death and cytokine release. The transcription factors that control gasdermin expression at steady-state or regulate its expression during disease are only emerging, and studies on GSDMD once again provide the first concrete insights into its regulation. In murine macrophages, GSDMD is constitutively expressed and priming with TLR agonists has only a minor role [40]. By contrast, NFkB signaling appears to be important for inducing GSDMD in adipocytes [117]. Steady-state levels of GSDMD in mouse macrophages are maintained by IRF (IFN-regulated factor)− 2, which binds to site at the transcriptional start site of GSDMD [118]. Deletion of IRF2 results in strongly reduced steady-state levels of GSDMD expression, and reduced levels of cell death and IL-1β release after inflammasome activation. In human monocytes, by contrast, it was found that IRF2 did not regulate GSDMD expression [119], while it is necessary in human EA.hy926 hybrid endothelial cells. Additional work will be necessary to define which pathways maintain and induce GSDMD expression and if these also regulate other gasdermin family members.

4.2. Regulating the pore-forming activity of GSDMD^N

Once cleavage has occurred, GSDMD^{NT} targets organellar membranes and the plasma membrane, where it inserts and oligomerizes into pores. Membrane insertion is favored by negatively charged lipids such as phosphoinositides and cardiolipin, which can be found in the inner leaflet of the plasma membranes or in the membranes of mitochondria, respectively. Recent reports suggest that besides lipid composition other factors are necessary for efficient gasdermin pore formation. Evavold et *al.* for example report that GSDMD pore formation requires ROS production that is dependent on signaling via the Ragulator-Rag-mTORC1 pathway [120]. Following up on these findings, the same authors show that ROS enhance GSDMD pore forming activity through oxidative modification of cysteine 192, although how oxidation of this critical residue promotes pore formation remained unclear [121]. The authors do not expect a role for disulfide bonds, as this is inconsistent with the positioning of C192 in the high-resolution structure of oligomerized GSDMD. Interestingly, another study published simultaneously with the first, also shows that the Rag-Ragulator complex is important for GSDMD pore formation, but only if GSDMD is activated by caspase-8 after TAK1 inhibition, but not downstream of canonical inflammasomes like NLRP3 or NLRP1 [122]. The authors show that during TAK1 inhibition, Rag-Ragulator is required to recruit RIPK1 and caspase-8 to lysosomal membranes which enhances RIPK1 phosphorylation and caspase activation.

Recent pre-prints might provide new insights into why Cys191/Cys192 (human/mouse) plays such a critical role in GSDMD activity [123,124]. The first study reports that both GSDMD^{FL} and GSDMD^{NT} are palmitoylated and that treatment with palmitoylation inhibitors is critical for pyroptosis induction in macrophages [123]. They also find that mutation of Cys192 abrogates palmitoylation as well as pyroptosis induction, and that zinc finger and DHHC motif-containing (ZDHHC) proteins 5 and 9 that transfer palmitate onto the thiol group of cysteine from cytosolic palmitoyl-CoA is required. Finally, they find that a cell-permeable, GSDMD palmitoylation-specific competitive peptide suppresses macrophage pyroptosis and alleviates sepsis. The second study shows similar findings, but in addition reports that palmitoylation is regulated by the redox state of the cell. They find that ROS production that is caused by common inflammasome activators also promotes GSDMD palmitoylation as a licensing modification to facilitate GSDMD pore formation [124]. These studies are the first to provide a potential explanation for the many studies that find a critical role for Cys191 (Cys192) in GSDMD pore formation, explaining also why it is a common target for pharmacological inhibition of GSDMD (see below).

4.3. Membrane repair

Mammalian cells can repair small (<10–20 nm) to medium (<100 nm) sized plasma membrane lesions through various mechanisms. The pathways that are best described at the molecular levels are Acidic Sphingomyelinase (ASM)-dependent endocytosis of plasma membrane pores and ESCRT (endosomal sorting complexes required for transport)-mediated shedding of damaged plasma membrane [125]. Recently, both pathways were implicated in restricting membrane damage caused by GSDMD pores. Rühl et al. for example found that influx of extracellular Calcium via GSDMD pores restricted LDH release in macrophages after inflammasome activation [103]. Calcium influx

has been previously shown to initiate membrane repair programs upon treatment with detergents or pore-forming toxins, among these a membrane repair pathway that relies on a targeted recruitment of ESCRT to membrane lesions. A critical feature of ESCRT is that it is the only machinery known in mammals that is capable of deforming membranes away from the cytosol, thus allowing MVB generation or exosome shedding. For its function in membrane repair, components of ESCRT-0, -I and − 2 subcomplexes are dispensable for this activity (except ALIX and ALG-2 that are probably required to sense Ca^{2+} influx), while ESCRT-III is essential for membrane repair. The authors show that ESCRT-III components are recruited to the plasma membrane upon pyroptosis induction and that this initiates the shedding of exosomes that contain ESCRT-III components as well as GSDMD pores. These findings not only provide the first example of cells actively regulating pyroptosis-induced membrane damage, but also could provide a molecular explanation for the vesicle release that has previously been observed upon inflammasome activation. Moreover, as IL-1β has been proposed to be released in exosomes as well, GSDMD pore formation might indirectly via Calcium influx control this pathway of UPS.

ASM is a sphingomyelin-converting enzyme that is located inside the lysosome and is released by lysosomal exocytosis that is triggered by Calcium influx upon membrane damage [126]. Extracellular ASM hydrolyses sphingomyelin to ceramide on the outer leaflet of the plasma membrane thereby promoting formation of lipid rafts around the site of exocytosis. These lipid rafts promote local membrane invagination which allows for enhanced endocytosis around the site of plasma membrane damage, removing stable transmembrane pores. Recently, Nozaki et *al.* reported that this pathway has also the ability to antagonize GSDMD pore-mediated cell death [127]. Previous reports had shown that active caspase-1 also cleaves the apoptotic effector caspase-7 [128], but the function of this caspase remained unclear as no apoptosis could be observed. In their study, the authors found that GSDMD pore formation was enhanced in *Casp7*-deficient cells, indicating that caspase-7 somehow antagonized GSDMD pore formation. This antagonism was shown to depend on caspase-7-mediated cleavage of ASM in cells activating the NLRC4 inflammasome, in line with previous work that had shown that ASM can be cleaved by caspase-7 to enhance its sphingomyelin-catalyzing activity [129]. In what context, would caspase-1 need to restrict GSDMD pore formation? Interestingly, the authors report that by slowing down GSDMD pore formation, caspase-7 delays pyroptosis thereby mitigates intestinal pathology during *Salmonella* infection. This delay in pyroptosis and PMR allows the tissue to extrude infected enterocytes into the gut lumen, which would have otherwise undergone PMR while still embedded in the epithelium.

In summary, both ESCRT- and ASM-dependent pathways restrict GSDMD pore formation in the plasma membrane, thereby raising the resistance of cells to pyroptosis and delaying the induction of cell lysis. Together, these reports argue that cells actively try to suppress GSDMD-induced cell death by engaging several pathways of membrane repair. It is thus possible that in vivo GSDMD-induced cell lysis might be normally a very late event or even completely avoided. If highly active membrane repair underlies to low level of lysis in neutrophils or the hyperactivation phenotype observed by others remains to be determined.

4.4. Pharmacological inhibition of gasdermins

Current anti-IL-1 therapies target either the cytokine itself or its receptors (Anakinra, Canakinumab and Rilonacept) or the inflammasome complex that controls caspase-1 activation (MCC950). But blocking GSDMD pore formation directly might also be an alternative approach to reduce the release of mature cytokines during inflammasome-driven sterile inflammatory disease, auto-inflammatory disorders or systemic inflammation of cytokine storm during sepsis. Over the last years, several reports have identified compounds that block GSDMD-dependent cell death and cytokine release. A critical target of drugs like di-methyl-fumarate (DMF), disulfiram, necrosulfonamide (NSA) appears to be Cys191 in human GSDMD (Cys 192 in mice) [130–132]. All of these modify this residue, leading to reduced pore formation, potentially by blocking the palmitoylation of this residue. Importantly, these drugs are quite unspecific and have been shown to also block other steps in the inflammasome pathway. DMF, for example, also inhibits the interaction of GSDMD with active caspase-1 [130]. Disulfiram on the other hand has been long known to be a potent inhibitor of caspase activation [133]. But in THP-1 cells stimulated with inflammasome activators it did not seem to reduce caspase activity, ASC speck formation or GSDMD processing, suggesting that it specifically targets the pore-forming activity of GSDMD [131]. Interestingly, the same study also evaluated the effect of DMF and NSA on GSDMD-induced pore formation but found only low activity [131]. Another GSDMD inhibitor that was reported to block GSDMD activation in neutrophils and subsequent NETosis is LDC7559. However, a recent study showed that the inhibition of NETosis by LDC7559 [58] stems from its ability to activate the glycolytic enzyme phosphofructokinase-1 liver type (PFKL) and dampening flux through the pentose phosphate pathway, which inhibits the NOX2-dependent oxidative burst in neutrophils that is required for NETosis [134]. Unfortunately, Cys191 is not conserved in other gasdermins, making it unlikely that the same drugs might also block other family members. Thus, improving our understanding of the critical residues in the region encompassing Cys191 might be needed to guide the development of novel approaches to also inhibit other gasdermin family members.

5. Future perspectives

Since their discovery as pore-forming executors of pyroptotic cell death less then 10 years ago, the gasdermin protein family has been shown to control various other functions, thus transforming them into multifunctional tools for unconventional protein secretion and regulators of cellular signaling. A surprisingly large number of proteins are released by ER-Golgi independent UPS, often triggered by cell stress or disease. As gasdermin family members have emerged as substrates of various proteases that are activated in response to cell stress, it will be important to define if they play a general role in unconventional protein secretion. Besides revealing new functions for gasdermins, this might potentially uncover new insight into the activation mechanism of gasdermins. In addition, it will be interesting to define if gasdermin pores always act as conduits for UPS, or if they indirectly control other release mechanisms. It has been shown that UPS can also involve secretory granules or synaptic vesicle fusion with the plasma membrane, or ESCRT-dependent macrovesicle shedding that is triggered by calcium influx. But if gasdermin pore formation is linked to these pathways remains to be shown. Finally, UPS has also been observed in protists parasites, yeast and drosophila, yet if gasdermins or gasdermin-like protein are involved in these organism remains to be determined.

Another open question is if all gasdermins allow the passage of a similar set of proteins, or if they display differential cargo-specificity. GSDMD and GSDMA3 have both an acidic channel, allowing the passage of predominantly neutral or positively charged cargo, yet nothing is known about other family members. In this context, the recently discovered PMR-executor NINJ1 will play a defining role as it allows to genetically uncouple gasdermin pore formation from cell lysis. Moreover, *Ninj1*-deficient mice should be used to investigate to what extent gasdermin pore-dependent unconventional protein release occurs in vivo and how important it is in driving host defense mechanism, anti-tumor immunity or the development of (auto)-inflammatory diseases, such as in the context of uncontrolled inflammasome activation. While several lines of evidence suggest that gasdermin pores can cause a limited, sub-lytic and even sub-lethal plasma membrane permeabilization, dubbed ‘hyperactivation’, it remains to be determined if this is a common phenomenon, how long such phases persist and how this is regulated. A long-standing issue that needs to be resolved is if cell death-independent IL-1 release is not an artifact caused by differential

sensitivities of commonly used assays to detect cell lysis and IL-1 secretion. Here careful single cell assays measuring plasma membrane rupture, gasdermin pore formation and IL-1 release will need to be performed using classical inflammasome activator as well as hyper-activating stimuli and performed over a variety of cell types (macrophages, DCs, neutrophils). Another point to investigate will be if this is regulated by posttranslational mechanisms or membrane repair that have been shown to regulate gasdermin pore formation, or if regulation happens downstream of pore formation at the level of PMR. It is for example not yet known how NINJ1 expression levels are regulated, how it is activated and if its filament-forming activity is regulated by interactors or post-translational modifications. Finally, it will be important to further develop new approach to inhibit gasdermin pore formation and NINJ1-dependent PMR using small-molecule inhibitors, or neutralizing antibodies and test their efficacy in animal models. In summary, despite all these advances we are only now beginning to understand the multifaceted roles of this novel pore-forming protein family in immunity and beyond, and we expect that future research will provide additional exciting insights into gasdermin regulation and function.

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