# Interleukin-1$\beta$ Maturation Triggers Its Relocation to the Plasma Membrane for Gasdermin-D-Dependent and -Independent Secretion

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Cell Reports



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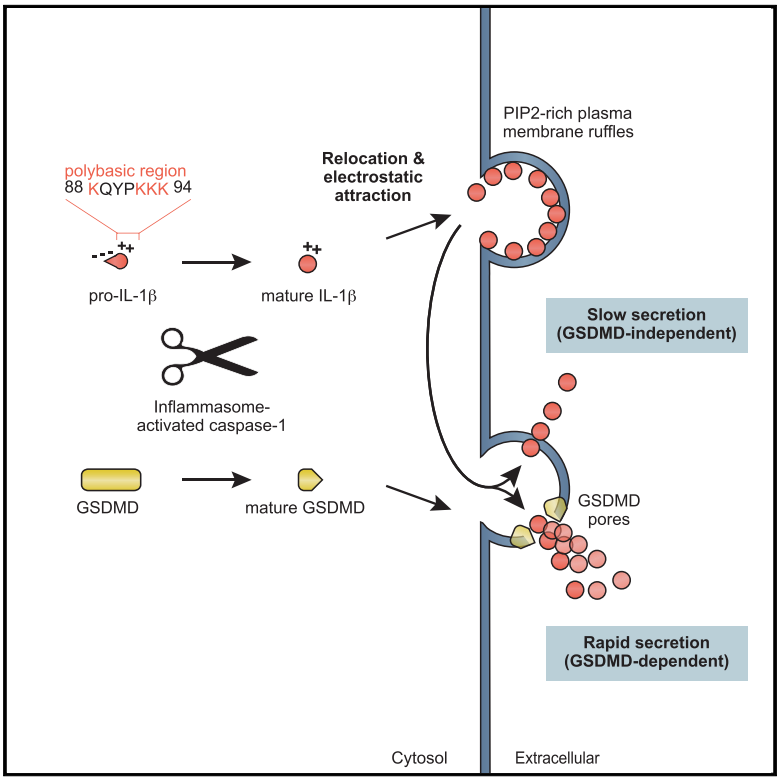
## Graphical Abstract




## Highlights

- IL-1β cleavage by caspase-1 is both necessary and sufficient for IL-1β secretion
- IL-1β maturation enables its relocation from the cytosol to the plasma membrane
- Caspase-1 activity increases the speed of IL-1β secretion, via GSDMD
- PIP2 membrane microdomains support GSDMD-dependent and -independent IL-1β exit

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## In Brief

Interleukin-1β is a potent pro-inflammatory cytokine whose dysregulated production drives a myriad of human diseases. Monteleone et al. uncover the trafficking mechanisms driving the unconventional secretion of mature interleukin-1β in non-pyroptotic and pyroptotic myeloid cells and reveal functions for caspase-1 and GSDMD therein.

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# Interleukin-1β Maturation Triggers Its Relocation to the Plasma Membrane for Gasdermin-D-Dependent and -Independent Secretion

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## SUMMARY

**IL-1β requires processing by caspase-1 to generate the active, pro-inflammatory cytokine. Acute IL-1β secretion from inflammasome-activated macrophages requires caspase-1-dependent GSDMD cleavage, which also induces pyroptosis. Mechanisms of IL-1β secretion by pyroptotic and non-pyroptotic cells, and the precise functions of caspase-1 and GSDMD therein, are unresolved. Here, we show that, while efficient early secretion of endogenous IL-1β from primary non-pyroptotic myeloid cells *in vitro* requires GSDMD, later IL-1β release *in vitro* and *in vivo* proceeds independently of GSDMD. IL-1β maturation is sufficient for slow, caspase-1/GSDMD-independent secretion of ectopic IL-1β from resting, non-pyroptotic macrophages, but the speed of IL-1β release is boosted by inflammasome activation, via caspase-1 and GSDMD. IL-1β cleavage induces IL-1β enrichment at PIP2-enriched plasma membrane ruffles, and this is a prerequisite for IL-1β secretion and is mediated by a polybasic motif within the cytokine. We thus reveal a mechanism in which maturation-induced IL-1β trafficking facilitates its unconventional secretion.**

## INTRODUCTION

Interleukin (IL)-1β induces fever and recruits and activates immune cells, mounting responses that are generally beneficial during infection but that can also drive pathology in a wide variety of human diseases. While most cytokines contain a signal sequence to direct their cellular exit via secretory vesicles, IL-1β has no such sequence and, thus, follows an unconventional pathway for secretion (unconventional protein secretion; UPS). IL-1β is also unusual in that it expressed as a precursor, pro-IL-1β (p-IL-1β), which requires maturation for activity. The inflammasome pathway controls both p-IL-1β cleavage in the cytosol and the unconventional secretion of the mature cytokine (p17, or m-IL-1β) (Monteleone et al., 2015).

Inflammasomes are cytosolic signaling complexes that activate caspase-1 (Schroder and Tschopp, 2010). The most studied inflammasome is nucleated by the danger-sensor protein, NLRP3. Upon sensing signals indicative of infection, injury, or cell stress, NLRP3 self-oligomerizes, recruits the ASC adaptor, and drives ASC polymerization; polymerized ASC then recruits caspase-1, facilitating caspase-1 dimerization and activation (Boucher et al., 2018). Caspase-1 mediates the cleavage-induced activation of IL-1β and enables its secretion (Schroder and Tschopp, 2010). Caspase-1 also cleaves gasdermin D (GSDMD), triggering the formation of GSDMD pores in the plasma membrane (Aglietti et al., 2016; Ding et al., 2016; Liu et al., 2016; Sborgi et al., 2016) to drive a form of osmotic cell lysis, termed pyroptosis (He et al., 2015; Kayagaki et al., 2015; Shi et al., 2015). Cell lysis and m-IL-1β secretion are temporally associated (Brough and Rothwell, 2007; Cullen et al., 2015; Liu et al., 2014; Shirasaki et al., 2014), and, consequently, m-IL-1β is often proposed to be passively released during cell rupture (reviewed in Monteleone et al., 2015). However, m-IL-1β release can be uncoupled from cell rupture by culturing cells in an osmoprotectant (Evavold et al., 2018; Fink and Cookson, 2006; Pelegrin et al., 2008; Verhoef et al., 2004), and pyroptosis is not a universal outcome of caspase-1 activation in all cell types; neutrophils can activate caspase-1 and secrete m-IL-1β without dying in a caspase-1-dependent manner (Chen et al., 2014), and a growing number of studies report m-IL-1β release from non-pyroptotic or, indeed, living cells (Conos et al., 2016; Diamond et al., 2017; Gaidt et al., 2016; Kang et al., 2013; Wolf et al., 2016; Zanoni et al., 2016). Here, we investigate cellular mechanisms of m-IL-1β secretion, including roles for caspase-1 and GSDMD, in pyroptotic and

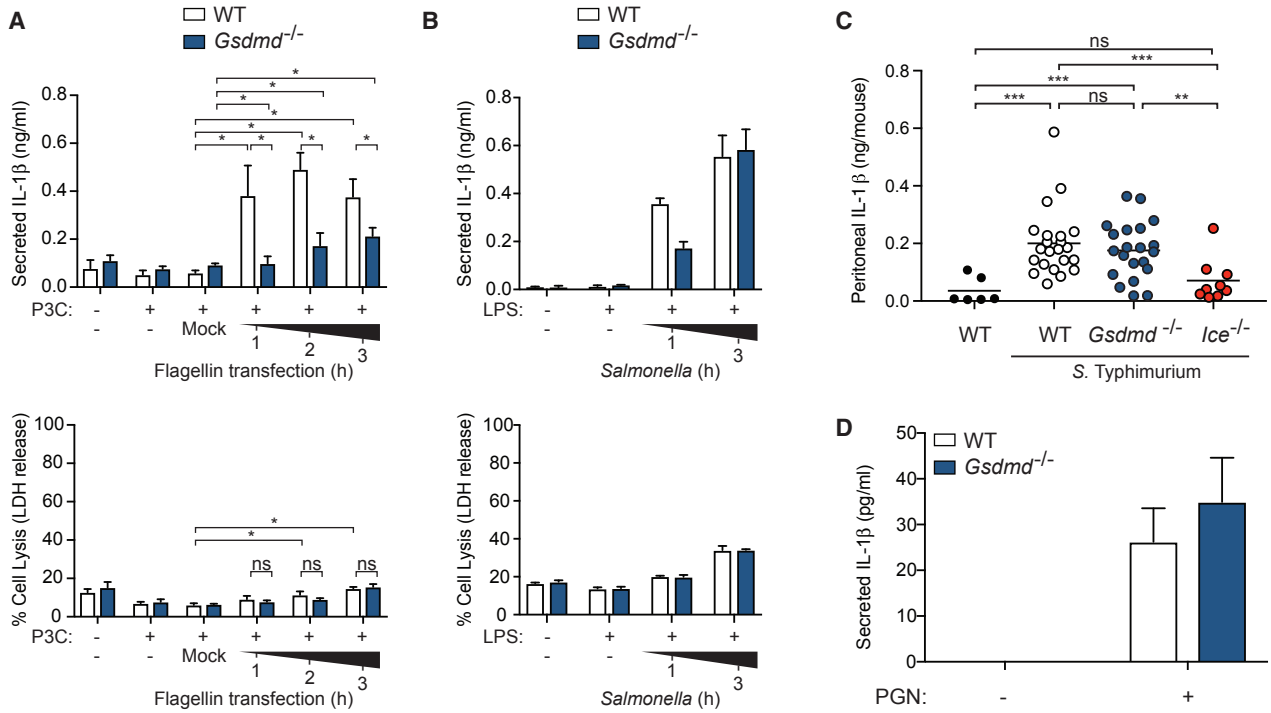



**Figure 1. Rapid IL-1β Secretion Requires GSDMD, while IL-1β Release at Later Time Points Is Partially or Fully GSDMD Independent in Non-pyroptotic Primary Myeloid Cells**

(A and B) Primary neutrophils were primed with Pam3CSK4 (P3C) or LPS for 4 hr before transfection with bacterial flagellin (A) or infection with *Salmonella* Typhimurium (SL1344) (B). Data indicate means + SEM of 4 biological replicates, with significance tested by non-parametric test (Mann-Whitney; *$p < 0.05$ in A), or mean + SD of technical triplicates and are representative of at least 3 biological replicates in (B). ns, not significant.

(C) WT versus *Gsdmd*$^{-/-}$ and *Ice*$^{-/-}$ mice were challenged intraperitoneally (i.p.) with *Salmonella* Typhimurium ($10^6$ CFU at log phase), or WT mice were injected with PBS as controls, and IL-1β in the peritoneal lavage fluid was quantified by ELISA. Each data point represents an individual mouse. **$p \leqslant 0.001$; ***$p \leqslant 0.001$.

(D) Primary WT and *Gsdmd*$^{-/-}$ macrophages were stimulated with 20 μg/mL peptidoglycan (PGN) for 18 hr. IL-1β secretion into the cell culture supernatant was monitored by ELISA. Data indicate means + SEM of 3 biological replicates.

See also Figure S1.

non-pyroptotic myeloid cells. We reveal that IL-1β cleavage by caspase-1 triggers the transport of the mature cytokine to plasma membrane projections for both GSDMD-dependent and -independent release from the cell.

## RESULTS

### Primary Myeloid Cells Release IL-1β through GSDMD-Dependent and -Independent Mechanisms *In Vitro* and *In Vivo*

Early IL-1β release from inflammasome-stimulated macrophages requires GSDMD, which also mediates rapid cell lysis (Kayagaki et al., 2015) (confirmed in Figures S1A–S1C), while GSDMD is dispensable for caspase-1-dependent maturation of IL-1β (Figure S1C) (Boucher et al., 2018). Nonetheless, some cell types, such as neutrophils (Chen et al., 2014; Karmakar et al., 2015) and monocytes (Diamond et al., 2017; Stoffels et al., 2015; Viganò et al., 2015), use an undefined mechanism to produce caspase-1-dependent m-IL-1β without dying by pyroptosis. Thus, we investigated the GSDMD dependency of endogenous IL-1β release in NLRC4 inflammasome-activated primary neutrophils transfected with bacterial flagellin or infected with *Salmonella*. In both cases, early IL-1β release fully or partially required GSDMD, but this was followed by GSDMD-independent release at 3 hr post-challenge (Figures 1A and 1B). Neutrophils are major contributors to IL-1β secretion during murine challenge with *Salmonella* (Chen et al., 2014), and *in vivo* IL-1β secretion at 6 hr post-challenge was similarly independent of GSDMD, while it required caspase-1 (Figure 1C). While neither NLRC4 activator induced GSDMD-dependent neutrophil pyroptosis, GSDMD-independent IL-1β secretion in both cases was associated with low levels of non-pyroptotic cell death. Thus, we cannot rule out the possibility that GSDMD-independent, non-pyroptotic cell death signaling pathways promote IL-1β release under these conditions as they do during necroptosis or ripoptosome signaling (Conos et al., 2017; Gutierrez et al., 2017; Lawlor et al., 2017; Yoon et al., 2017). However, recent reports of IL-1β release from living cells provide a strong argument against an obligatory requirement for cell death and rupture for IL-1β secretion (Conos et al., 2016; Diamond et al., 2017; Gaidt et al., 2016; Wolf et al., 2016; Zanoni et al., 2016). As extended exposure to peptidoglycan triggers NLRP3- and caspase-1-dependent mature IL-1β secretion from living macrophages (Wolf et al., 2016), we speculated that peptidoglycan-stimulated

macrophages may secrete IL-1β in a caspase-1-dependent but GSDMD-independent manner, which was, indeed, the case (Figures 1D and S1D). Thus, there are at least two mechanisms for IL-1β secretion, and the GSDMD-independent secretory pathway may be the sole route for release utilized by myeloid cells in specific contexts.

## IL-1β Maturation Is Necessary and Sufficient for Slow IL-1β Secretion, while Rapid Secretion Requires Caspase-1 and GSDMD

Caspase1 is required for both the maturation and secretion of IL-1β (Kuida et al., 1995), but the relationship between cytokine *processing* and *secretion* is unresolved. IL-1β maturation and secretion may be temporally coupled, because they each independently rely on caspase-1 activity, or because IL-1β processing by caspase-1 is obligatorily required for secretion. To investigate the requirements for active IL-1β secretion, we sought to establish a tractable cellular system for inducing robust m-IL-1β secretion uncoupled from cell rupture. We, as well as others (Evavold et al., 2018; Fink and Cookson, 2006; Pelegrin et al., 2008; Verhoef et al., 2004), used the osmoprotectant glycine to delay caspase-1-dependent macrophage cell rupture and resultant passive protein release and monitored IL-1β maturation and release upon inflammasome activation (e.g., nigericin, ATP) in lipopolysaccharide (LPS)-primed macrophages. We confirmed that maturation is a prerequisite for regulated m-IL-1β secretion in glycine-cultured cells by ectopically expressing V5-tagged wild-type (WT) p-IL-1β and the caspase1-uncleavable D117A mutant cytokine in RAW264.7 macrophage-like cells stably expressing ASC (ASC-RAW, used here because parental RAW264.7 are naturally deficient in ASC; Figure S2A). Thus, the first function of caspase-1 in UPS is to cleave p-IL-1β, a process that endows the cytokine with properties that facilitate its regulated secretion. To determine whether IL-1β maturation was not only necessary but sufficient for secretion, we ectopically expressed V5-tagged IL-1β (p- versus m-IL-1β-V5) in cells. Intriguingly, ectopically expressed m-IL-1β-V5, but not p-IL-1β-V5, was secreted in inflammasome-unstimulated macrophages (±LPS controls, "slow UPS"), while a caspase-1-activating stimulus further boosted the pace of ectopic m-IL-1β-V5 secretion ("rapid UPS") in both primary murine macrophages (Figures 2A and 2B) and ASC-RAW (Figure S2B). The finding that ectopic m-IL-1β, but not p-IL-1β, was slowly secreted from macrophages in the absence of inflammasome stimulation indicates that maturation is not only necessary but sufficient for IL-1β release, and it provides an excellent cellular system for studying m-IL-1β release uncoupled from cell death induction.

We next characterized the requirements for m-IL-1β release. In primary macrophages, both caspase-1 and GSDMD were required for nigericin-induced cell lysis by pyroptosis (Figures S2C and S2D) and nigericin-induced rapid UPS of m-IL-1β-V5 (Figures 2A and 2B). By contrast, caspase-1, caspase-1 activity (VX765-treated cells), and GSDMD were dispensable for slow UPS of ectopic m-IL-1β-V5 from inflammasome-unstimulated, non-pyroptotic cells (Figures 2A, 2B, and S2E). Punicalagin was recently reported to stabilize plasma membrane lipids and, thus, suppress pyroptosis-associated plasma membrane changes, blocking IL-1β release in pyroptotic cells (Martín-Sánchez et al., 2016). We show here that punicalagin ablates both slow and rapid m-IL-1β UPS secretion in ASC-RAW and primary macrophages (Figures 2C and S3A), suggesting that the plasma membrane is the exit point in both settings, regardless of whether the cell dies by pyroptosis. Autophagy pathways can mediate m-IL-1β release under starvation conditions (Dupont et al., 2011; Zhang et al., 2015) but were not required for m-IL-1β secretion in unstarved conditions (Figures S3B and S3C).

## IL-1β Maturation Triggers Its Relocation to Plasma Membrane Projections

The observation that IL-1β maturation is sufficient for its slow UPS in unstimulated cells suggests that IL-1β cleavage endows the cytokine with properties that facilitate UPS. We noted that the predicted isoelectric points of human and murine IL-1β undergo substantial changes upon maturation (e.g., predicted isoelectric points for murine p-IL-1β = 4.6, and m-IL-1β = 8.8). Thus, at neutral pH, p-IL-1β will be negatively charged, and likely repelled by the negatively charged plasma membrane, while processing liberates positively charged m-IL-1β, allowing for electrostatic interaction with membranes. Indeed, p-IL-1β and m-IL-1β exhibit strikingly distinct subcellular locations by cryo-electron microscopy (EM) of transfected RAW macrophages (Figure 3) and immunofluorescence microscopy of transduced primary macrophages (Figure 4A). The plasma membrane is enriched for phospholipids such as phosphatidylserine (PS) and phosphatidylinositol 4,5-bisphosphate (PIP2), particularly in cell-surface projections and ruffles, rendering these membrane domains highly negatively charged (Bohdanowicz and Grinstein, 2013). To examine whether positively charged m-IL-1β is recruited to plasma membrane projections and ruffles, IL-1β constructs were co-expressed alongside the PIP2 probe, PLCδ-PH-GFP (Bohdanowicz and Grinstein, 2013), which was detected by GFP immunostaining (Figure 3) or by fluorescence imaging (Figures 4B and 4C). At the EM level, immunogold labeling for PLCδ-PH-GFP shows that PIP2 decorates plasma membrane ruffles, which are also V5 immunolabeled for m-IL-1β-V5 but not for p-IL-1β-V5, which is, instead, found at intracellular locations, away from the plasma membrane but not consistently on any other organelle membranes (Figures 3A, 4B, and 4C). Quantification of gold labeling reveals the preponderance of m-IL-1β-V5 on the plasma membrane and its 9-fold enriched density on these domains compared to p-IL-1β-V5 (Figures 3B and 3C). These data suggest that IL-1β maturation enables its relocation to specific domains of the plasma membrane and that this may be a prerequisite for UPS.

## A Polybasic Motif Directs IL-1β Plasma Membrane Relocation and Secretion

Sequence or structural determinants directing m-IL-1β location and secretion are unknown. To identify such motifs, we aligned the sequences of murine and human m-IL-1β with m-IL-18, which is also cleaved and unconventionally secreted upon caspase-1 activation. m-IL-1β and m-IL-18 exhibit low sequence identity (e.g., 16% for murine proteins), despite their conserved

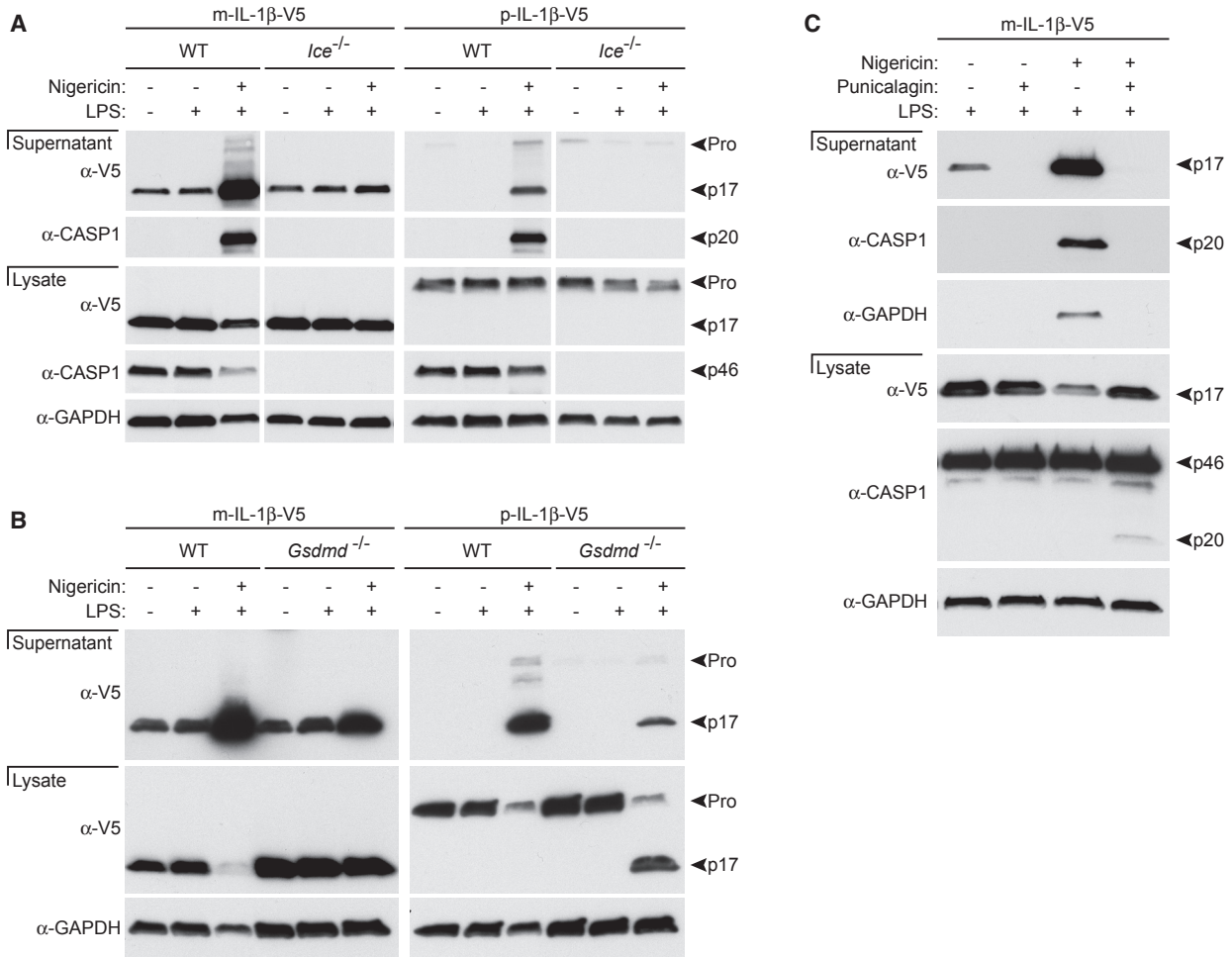



**Figure 2. IL-1β Maturation Is Sufficient for Slow IL-1β Secretion, while Caspase and GSDMD Are Required for Rapid IL-1β Secretion**

(A and B) Wild-type (WT) versus (A) *Casp1*-deficient (*Ice*$^{-/-}$) or (B) *Gsdmd*-deficient primary macrophages were transduced to express IL-1β-V5 (pro-IL-1β versus mature IL-1β). Cells were LPS primed for 4 hr and then stimulated with nigericin for 1 hr (A) or 45 min (B). WT and *Ice*$^{-/-}$ data for each construct were cropped from the same film.

(C) ASC-RAW cells were transiently transfected to express m-IL-1β-V5, LPS primed for 4 hr and stimulated with nigericin for 1 hr in the presence and absence of prior punicalagin exposure (25 μM, added together with LPS for nigericin-untreated cells, and 30 min prior to nigericin for nigericin-treated cells). Cell culture supernatant and cell lysates were analyzed by immunoblot. Data are representative of at least 5 biological replicates.

See also Figures S2 and S3.

β-trefoil structure. One area of high conservation between both proteins in both species is a polybasic region (Figure 4D) that resembles characterized charge-based phospholipid-interacting motifs. We mutated the positively charged amino acids within this region to assess their potential contribution to UPS. Indeed, the m-IL-1β polybasic motif was necessary for both slow and rapid UPS in both ASC-RAW and primary macrophages (Figures 4E and S4A), and the polybasic mutant was no longer targeted to the cell periphery or co-localized with PIP2 (Figures 4Aii, 4Bii, and 4C). Since m-IL-1β appears unable to directly bind PIP2 (Martín-Sánchez et al., 2016), precise mechanisms of charge-based attraction to these domains requires further characterization. The cleavage-induced relocation of endogenous m-IL-1β to the macrophage plasma membrane is extremely challenging to image in wild-type cells, because caspase-1 cleavage of p-IL-1β to generate m-IL-1β is coupled to its rapid GSDMD-dependent exit from the cell. We thus characterized the location of endogenous IL-1β in *Gsdmd*-deficient macrophages that cannot rapidly secrete m-IL-1β. Primary *Gsdmd*$^{-/-}$ macrophages exhibited nigericin-induced ASC specks and plasma-membrane-localized IL-1β (Figure S4B). Further, IL-1β co-localized with the PIP2 probe in *Gsdmd*-deficient macrophages with ASC specks (Figure S4C). Some *Gsdmd*$^{-/-}$ macrophages displayed characteristics of early apoptosis, such as nuclear condensation and plasma membrane bulges (e.g., Figure S4C), in keeping with recent reports that *Gsdmd* deficiency in macrophages enables inflammasome-mediated apoptotic signaling to proceed (He et al., 2015; Taabazuing et al., 2017). In all, these data indicate that IL-1β maturation enables IL-1β relocation to plasma membrane projections and surface ruffles via a

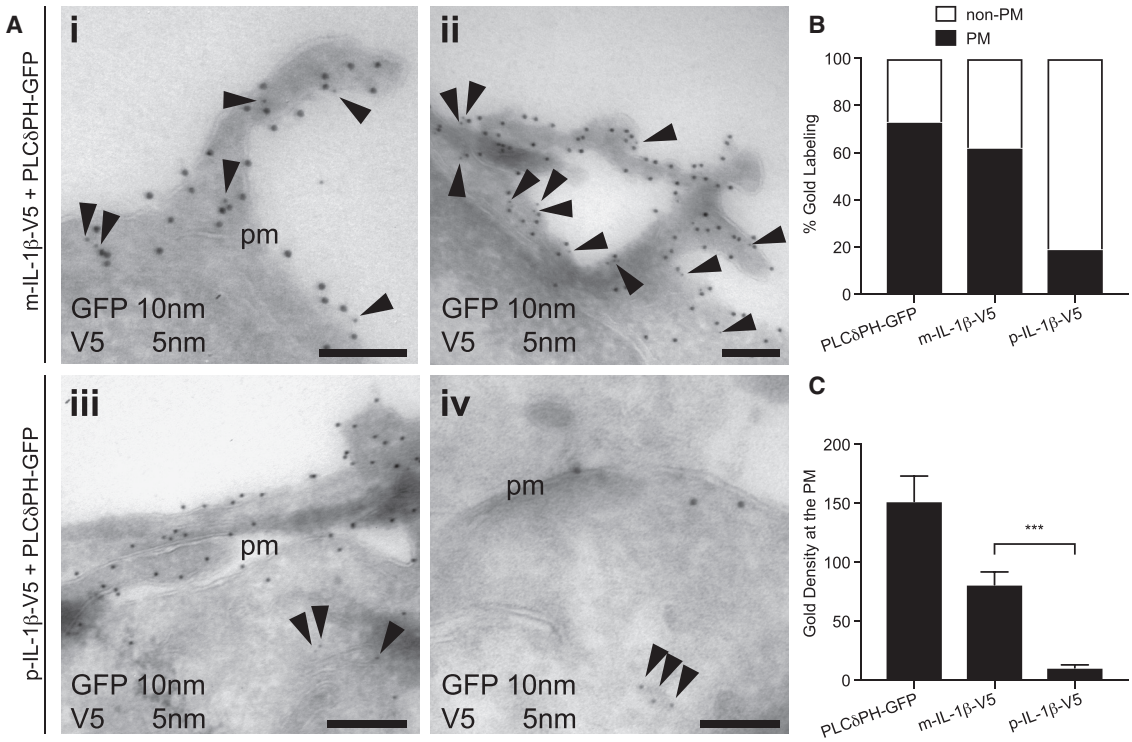



**Figure 3. IL-1β Maturation Triggers Its Relocation to the Plasma Membrane for Secretion**

RAW264.7 macrophages co-expressing a PIP2 probe (PLCδ-PH-GFP) and IL-1β-V5 constructs. Cryo-EM sections were immunolabeled for GFP (10 nm gold) and V5 (5 nm gold).

(A) Arrowheads indicate labeling of m-IL-1β-V5 in two cell areas (panels i and ii) and of p-IL-1β-V5 in two cell areas (panels iii and iv). pm, plasma membrane. Scale bars, 100 nm.

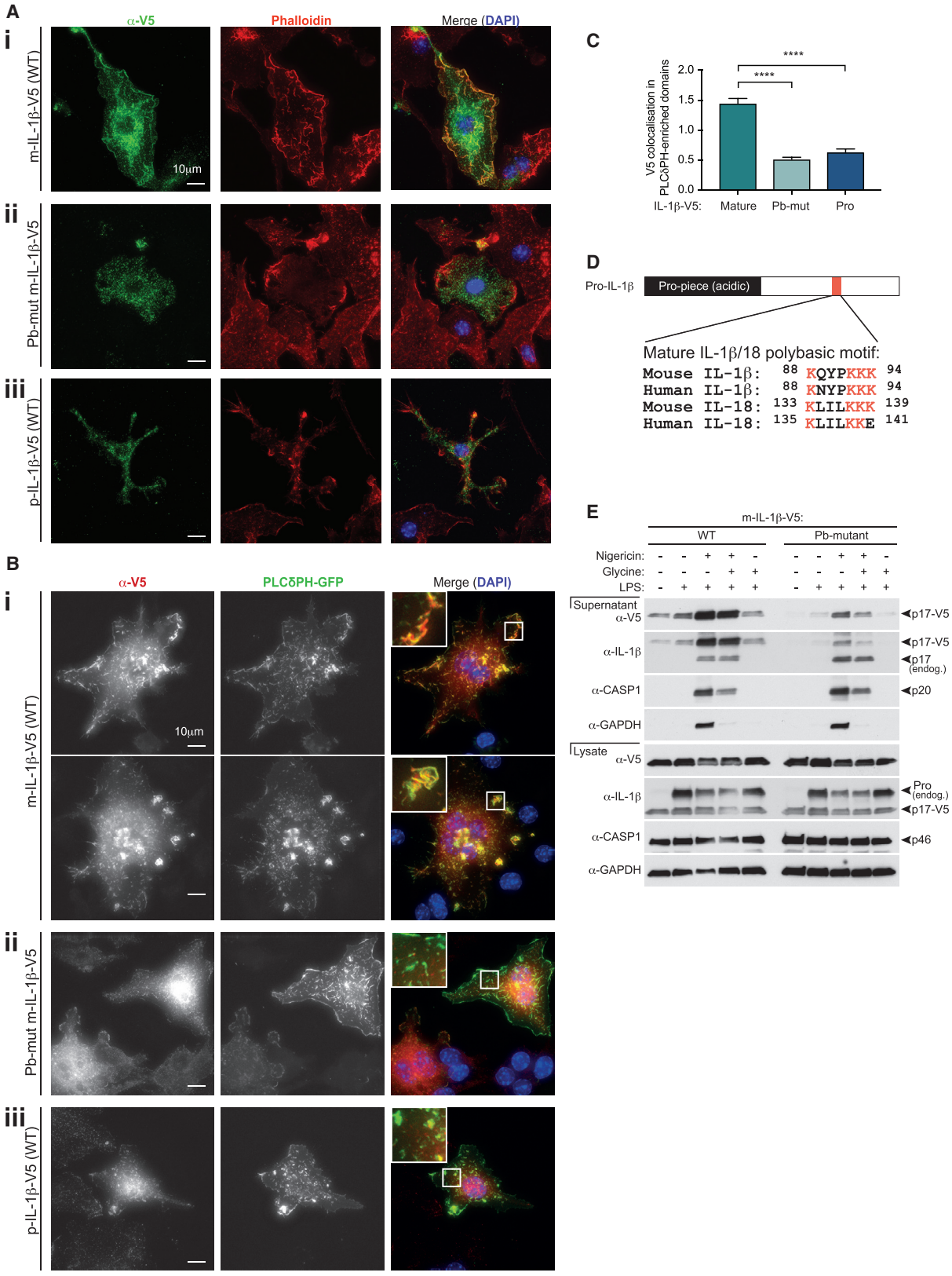
(B and C) Gold labeling was counted to determine the proportions of each probe on plasma membrane (PM) versus non-PM (intracellular) regions (B), and labeling density over the PM was calculated for each probe (C), where data are mean + SEM of 10–20 PM regions, and significance was determined by Mann-Whitney non-parametric test (***$p < 0.001$). Data are representative of 2 biological replicates.

polybasic motif that supports both GSDMD-dependent and GSDMD-independent secretion from the cell.

## DISCUSSION

Caspase-1 controls the unconventional secretion of multiple IL-1 family members (e.g., IL-1β, IL-18, and IL-37), but the underlying mechanism has remained unclear. The cell-rupture model for IL-1β exit is often favored in the literature, but the present study and a very recent study (Evavold et al., 2018) provide compelling evidence against this model. We demonstrate that IL-1β maturation is sufficient for its slow UPS in resting, non-pyroptotic macrophages. Further, protein secretion cannot be passive if a motif within the cargo directs or inhibits its exit; we discovered that a polybasic motif within m-IL-1β promotes its association with plasma membrane domains enriched for PIP2 for both slow and rapid UPS, while an intact pro-piece in the precursor cytokine suppresses p-IL-1β recruitment to the plasma membrane and ablates its regulated exit.

Our data indicate that IL-1β enrichment at the cell periphery and subsequent secretion are regulated by multiple sequential events. p-IL-1β cleavage by caspase-1 removes the negatively charged pro-piece, allowing m-IL-1β to relocate from the cytosol to plasma membrane projections via its polybasic motif. We propose that IL-1β relocation facilitates plasma membrane exit via GSDMD-independent (slow) and GSDMD-dependent (rapid) mechanisms, regardless of immediate cell fate (continued viability, apoptosis, pyroptosis, and necroptosis). GSDMD-dependent IL-1β release likely reflects IL-1β exit through GSDMD pores themselves (Evavold et al., 2018), regardless of whether the number of GSDMD pores reach the required threshold for pyroptosis induction, osmotic cell lysis, and resultant passive protein release. The GSDMD-independent secretion pathway we describe here may be partially or fully responsible for IL-1β secretion from non-pyroptotic cells such as neutrophils, or, indeed, cells that do not express GSDMD. The discovery here of a previously uncharacterized mechanism for slow IL-1β secretion from non-pyroptotic cells may begin to explain the chronicity of IL-1β production in many human autoinflammatory, autoimmune, and arthritic diseases. In revealing the signal-dependent relocation of IL-1β to domains of the plasma membrane in non-pyroptotic cells, our study delivers insight into the fundamental mechanisms of unconventional protein secretion that are likely relevant to other cargo (e.g., IL-18) and may, ultimately, lead to the development of strategies for therapeutic intervention in IL-1β-driven diseases.

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## EXPERIMENTAL PROCEDURES

### Mice

C57BL/6, *Ice*$^{-/-}$ (*Casp1*$^{-/-}$/*Casp11*$^{null/null}$) (Kuida et al., 1995) and *Gsdmd*$^{-/-}$ (Kayagaki et al., 2015) mice were housed in specific pathogen-free facilities at the University of Queensland (UQ). The UQ's animal ethics committee approved all experimental protocols, which were performed with age- and sex-matched 6- to 12-week-old animals.

### Myeloid Cell Preparation

Primary bone-marrow-derived macrophages were differentiated with 150 ng/mL CSF-1 (endotoxin-free, produced in insect cells by the UQ Protein Expression Facility), as previously described (Schroder et al., 2012a) and used for experiments on day 7 of differentiation. Bone marrow neutrophils were purified using an α-Ly6G-FITC (fluorescein isothiocyanate) antibody (1A8, BioLegend) and α-FITC beads by magnetic-assisted cell sorting (MACS), as previously described (Chen et al., 2014), and achieved >98% purity, as assessed by flow cytometry. Parental RAW264.7 cells were transfected with a pEF6 vector (Invitrogen) encoding murine ASC by electroporation and maintained under blasticidin selection for stable ASC expression.

### Macrophage Transduction and Transfection

The coding sequence of murine IL-1β was cloned into the pEF6 mammalian expression vector and a replication-defective mouse stem cell retroviral construct (pMSCV). The following IL-1β coding sequences were used: pro-IL-1β (matching the full coding sequence of NP_032387), mature IL-1β (residues 118–269 of NP_032387, mimicking the caspase-1 cleavage product), mature IL-1β with the polybasic motif mutated (KQYPKKKMEKR to AQY PAAAMEAA at residues 205–215 of the pro-protein), and p-IL-1β with the capase-1 cleavage site mutated (D117A). All constructs encoded a C-terminal V5 tag. pEF6 plasmids were transiently transfected into ASC-RAW or parental RAW264.7 by either electroporation (for secretion analysis) or lipofectamine (for microscopy), either singly or in combination with the plasmid for the PLCδ-PH-GFP probe that labels PIP2 microdomains (Bohdanowicz and Grinstein, 2013). The retroviral packing cell line, PlatE, was lipofectamine-transfected with pMSCV vectors encoding IL-1β or the PLCδ-PH-GFP probe and incubated at 32°C for 2 days for virus production (Schroder et al., 2012b). Cell-free retrovirus was prepared and used to spinfect primary bone marrow cells on day 2 of their colony-stimulating factor 1 (CSF-1)-directed differentiation into macrophages.

### Inflammasome and Cell Lysis Assays

Differentiated murine bone-marrow-derived macrophages were plated in tissue culture plates at a density of 1 × $10^6$ cells per milliliter in full media (RPMI-1640, 10% fetal bovine serum, 1× Glutamax, 150 ng/mL recombinant human CSF-1). Immediately prior to experiments, the cell culture media was replaced with Opti-MEM (supplemented with 150 ng/mL human CSF-1). Bone marrow neutrophils were purified, plated at 1 × $10^7$ cells per milliliter in Opti-MEM (Life Technologies) supplemented with 4 μg/mL aprotinin (Sigma-Aldrich), and used immediately for experiments. Cells were primed with ultrapure *E. coli* K12 LPS (100 ng/mL) or Pam3CSK4 (1 μg/mL) for 4 hr before exposure to 5 μM nigericin (Sigma-Aldrich) or ATP (1.25 mM; Sigma-Aldrich), infected with *Salmonella* Typhimurium (SL1344, log phase at an MOI of 10 for macrophages or 25 for neutrophils), or transfected with ultrapure flagellin (1 μg/mL [InvivoGen] plus FuGENE HD) by centrifugation at 700 × *g* for 10 min at room temperature. In some experiments, cells were exposed to additional agents: glycine (5 mM [Sigma-Aldrich], added 60 min prior to inflammasome agonist), VX765 (50 μM [InvivoGen], added 60 min prior to inflammasome agonist), 3-methyladenine (3MA; 5–10 mM [Sigma-Aldrich], freshly made and added 30 min prior to nigericin), or punicalagin (25 μM [Sigma-Aldrich], added 30 min prior to nigericin or together with LPS for slow UPS). LPS-unprimed macrophages were also exposed to peptidoglycan (10 and 20 μg/mL; Sigma-Aldrich) for 18 hr. ELISA quantified IL-1β in cell-free supernatants (eBioscience). Cellular lysis was quantified using the Cytox96 non-radioactive cytotoxicity assay (Promega) and displayed as a percentage of total cellular lactate dehydrogenase (LDH) (100% cell lysis control). Western blots of supernatants precipitated with methanol and chloroform and cell extracts were performed using standard procedures (Gross, 2012). Antibodies for immunoblot were IL-1β (polyclonal goat, R&D Systems), caspase-1 (mouse monoclonal Casper-1, AdipoGen Life Sciences), V5 (mouse monoclonal SV5-Pk1, Serotec), and GADPH (polyclonal mouse, BioScientific).

### Microscopy

RAW264.7 macrophages were transfected with plasmids and incubated overnight for immunoelectron microscopy. Cells were fixed in 4% paraformaldehyde, embedded in warm gelatin before solidifying on ice, and frozen onto cryo-stubs. Ultra-thin sections were cut on a Reicherdt Cryo Ultramicrotome and immunolabeled by placing on sequential drops of glycine, BSA, and primary and secondary Protein A-Gold antibodies before final fixation in 1% glutaraldehyde. Sections were viewed on a transmission electron microscope (JEOL 1011, JEOL Australasia) at 80 kV, and images were captured using iTEM software (Soft Imaging System, Olympus) (Yeo et al., 2016). Primary antibodies were rabbit anti-V5 (Merck) and rabbit anti-GFP (Life Technologies Australia). Protein A-Gold was from Jan Slot (University Medical Center Utrecht, Utrecht, the Netherlands), and it was applied to sections labeled with antibodies or to no antibody sections as a control. For image quantification, 10 images were analyzed per condition. The numbers of 5- or 10-nm gold particles were counted to determine the proportion of each label on the plasma membrane (taken as a 100-nm band centered on the membrane) or in the rest of the cell, and gold densities over the plasma membrane were calculated per square micron.

Immunofluorescence staining was performed as previously described (Yeo et al., 2016). Briefly, cells were plated on glass coverslips and, after treatment, fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were permeabilized in 0.1% Triton X-100 for 10 min and, after blocking in 0.5% BSA, incubated with primary and secondary antibodies for 1 hr. Primary antibodies used were IL-1β (polyclonal goat; R&D Systems), ASC (N15; Santa Cruz Biotechnology), and V5 (mouse monoclonal SV5-Pk1; Serotec). Alexa Fluor 488-, 594-, and 647-conjugated secondary antibodies, Alexa Fluor 594-phalloidin, and wheat germ agglutinin (WGA-647, stained before cell permeabilization) were purchased from Molecular Probes. Coverslips were mounted in ProLong Gold Reagent (Life Technologies) and imaged using a DeltaVision Olympus IX80 inverted wide-field deconvolution microscope equipped with an Olympus Plan-Apochromat 60×/1.35 oil lens and a Lumencor 7 line LED

**Figure 4. The Polybasic Motif of Mature IL-1β Is Required for Recruitment to PIP2-Enriched Plasma Membrane Domains and Slow and Rapid Secretion**

(A) Primary WT macrophages were transduced to express IL-1β-V5 (pro-, mature, and polybasic motif mutant [Pb-mut]), LPS primed for 4 hr, and imaged by deconvolution immunofluorescence microscopy. Images are representative of data from 4 biological replicates. Scale bars, 10 μm.

(B) RAW264.7 macrophages co-expressing PLCδ-PH-GFP and IL-1β-V5 constructs (pro-, mature- and polybasic motif mutant) were stimulated with LPS for 2 hr and imaged by deconvolution immunofluorescence microscopy. Scale bars, 10 μm.

(C) The extent of co-localization between PLCδ-PH-GFP and IL-1β-V5 constructs was quantified in 19–30 line scans per construct from at least two biological replicate experiments. Data are mean + SEM and significance was assessed using an unpaired non-parametric test (****$p < 0.0001$, Mann-Whitney test).

(D) Alignment of the polybasic (Pb) regions of murine and human mature IL-1β and IL-18 (residues are numbered according to the mature cytokine).

(E) ASC-RAW cells were transiently transfected to ectopically express m-IL-1β-V5 (WT versus polybasic motif mutant). Cells were LPS primed for 4 hr and then stimulated with nigericin for 1 hr. Cell culture supernatant and cell lysates were analyzed by immunoblot. All data are representative of at least 3 biological replicates.

See also Figure S4.

lamp. Images were captured using a Photometrics Coolsnap HQ2 camera. Images were deconvolved using 10 cycles of iterative deconvolution using SofWorx. Image analysis was performed using ImageJ software (NIH). For quantification of V5 co-localization with PLCδ-PH-GFP, multiple GFP-enriched membrane microdomains within individual cells were selected for analysis in ImageJ. Briefly, 6 line scans were taken within each subcellular region, and maxima values corresponding to peak fluorescence intensity were measured for both PLCδ-PH and V5 labeling. Data are represented as a ratio of V5 fluorescence levels against PLCδ-PH.

### *In Vivo* Salmonella Challenge

Mice were challenged by intraperitoneal injection with $10^6$ colony-forming units (CFUs) of log-phase *Salmonella* Typhimurium (SL1344 strain). Mice were sacrificed at 6 hr post-injection, and the peritoneal cavity was flushed with 10 mL ice-cold Dulbecco's phosphate-buffered saline (DPBS). IL-1β in the lavage fluid was quantified by ELISA.

### Statistical Analysis

Statistical analyses were performed using the non-parametric Mann-Whitney t test.

Data were considered significant when $p \leqslant 0.05$ (*), 0.01 (**), 0.001 (***), or 0.0001 (****).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.07.027.

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## AUTHOR CONTRIBUTIONS

Conceptualization, K.S.; Methodology and Investigation, M.M., A.C.S., K.W.C., D.L.B., J.S.B., J.B.v.P., M.R.S., and R.K.; Visualization, M.M., K.W.C., D.L.B., J.S.B., and K.S.; Resources, C.L.H., D.B., V.R., and M.J.S.; Writing – Original Draft, K.S.; Writing – Review & Editing, M.M. and K.S., with input from all authors; Supervision, M.M., J.L.S., and K.S.; Project Administration, K.S.; Funding Acquisition, J.L.S. and K.S.

## DECLARATION OF INTERESTS

K.S. holds a full-time associate professorial research fellow appointment at the University of Queensland and conducts research focused on inflammation and inflammasomes. K.S. is a co-inventor on patent applications for NLRP3 inhibitors that have been licensed to Inflazome, a company headquartered in Dublin, Ireland. Inflazome is developing drugs that target the NLRP3 inflammasome to address unmet clinical needs in inflammatory disease. K.S. served on the scientific advisory board of Inflazome in 2016–2017.

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