JBC Papers in Press. Published on April 2, 2019 as Manuscript RA119.008009
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.RA119.008009

The three cytokines IL-1β, IL-18, and IL-1α share related but distinct secretory routes

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Running title: *Mechanisms of IL-1 secretion*

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Keywords: inflammasome, inflammation, interleukin 1 (IL-1), NLRP3, caspase 1 (CASP1), calpain, cytokine, immunology, immune cell, neutrophil

ABSTRACT

Interleukin (IL)-1-family cytokines potently regulate inflammation, with the majority of the IL-1 family proteins being secreted from immune cells via unconventional pathways. In many cases, secretion of IL-1 cytokines appears to be closely coupled to cell death, yet the secretory mechanisms involved remain poorly understood. Here, we studied the secretion of the three best characterized members of the IL-1 super-family, IL-1α, IL-1β, and IL-18, in a range of conditions and cell types, including murine bone marrow derived and peritoneal macrophages, human monocyte derived macrophages, HeLa cells, and mouse embryonic fibroblasts. We discovered that IL-1β and IL-18 share a common secretory pathway that depends upon membrane permeability, and that can operate in the absence of complete cell lysis and cell death. We also found that the pathway regulating the trafficking of IL-1α is distinct from the pathway regulating IL-1β and IL-18. Although the release of IL-1α could also be dissociated from cell death, it was independent of the effects of the membrane stabilizing agent punicalagin which inhibited both IL-1β and IL-18 release. These results reveal that in addition to their role as danger signals released from dead cells, IL-1 family cytokines can be secreted in the absence of cell death. We propose that models used in the study of IL-1 release should be considered context dependently.

Understanding mechanisms of unconventional protein secretion is a fundamental question of cell biology. Its importance is underscored by the biomedical relevance of many unconventionally secreted

proteins. This is typified by the unconventionally secreted members of the interleukin(IL)-1 cytokine family which have established roles in host-defence responses and in inflammatory responses that contribute to disease (1). The ancestral IL-1 family consists of IL-1β, IL-1α, IL-1Ra, IL-36Ra, IL-36α, IL-36β, IL-36γ, IL-37, and IL-38, with IL-18 and IL-33 having a distinct ancestry, but based on structural homology, receptor binding and immunomodulatory function remain part of the IL-1 super-family (2). The best characterised members of the IL-1 family are IL-1α, IL-1β, and IL-18, and all are released via unconventional pathways.

IL-1β, IL-1α and IL-18, are initially produced as precursor pro-forms. In cells of hematopoietic lineage such as macrophages expression of precursor forms of IL-1β and IL-1α occurs after stimulation of membrane pattern recognition receptors (PRRs) such as TLR4, which can be activated by bacterial endotoxin for example, while pro-IL-18 is constitutively expressed. Both precursor forms of IL-1β and IL-18 are cleaved directly by the protease caspase-1, which then also, indirectly, influences Ca^{2+} and calpain dependent processing of pro-IL-1α (3). Activation of caspase-1 occurs after the assembly of macro-molecular protein complexes called inflammasomes upon which caspase-1 is activated. Inflammasomes are formed by a cytosolic PRR, the best studied of which is NLRP3, which nucleates oligomerisation of an adaptor protein ASC into an inflammasome complex (4). A consequence of inflammasome activation is an inflammatory form of cell death called pyroptosis (5). Thus, a consequence of studying IL-1 release after inflammasome activation has been the concomitant death of the secreting cell and so it has long been considered that IL-1β release occurred through membrane rupture and lysis (6, 7). However, there are numerous examples, namely in human monocytes and neutrophils, where inflammasome activation can drive the release of IL-1β in the absence of cell death (8, 9). We, and others, have reported that the mechanism of secretion of IL-1β may depend on an alteration in membrane permeability (10, 11). Furthermore, the recent discovery that caspase-1 also cleaves gasdermin D which subsequently forms pores in the plasma membrane which could allow passage of IL-1β (12, 13), and that a polybasic motif in the mature IL-1β domain could target it to phosphatidylinositol 4,5-bisphosphate (PIP2) rich domains in the plasma membrane (14), have established that the mechanism of secretion is more complicated than simply membrane rupture. The mechanism through which IL-18 and IL-1α are secreted, and whether they are common with IL-1β, remains under explored. Here we show that IL-1β, IL-18 and IL-1α can be secreted when cell lysis is prevented and that IL-1β and IL-18 share a common mechanism that relies on gasdermin D-dependent plasma membrane permeabilisation.

RESULTS

Secretion of IL-1β is dependent on membrane permeability

Here we set out to test the initial hypothesis that release of IL-1β following NLRP3 inflammasome activation was dependent on a change in membrane permeability and not cell lysis. To interrogate this we used the membrane stabilizing reagent punicalagin. Punicalagin is a complex polyphenolic compound isolated from pomegranate extract that we previously reported to inhibit ATP-induced IL-1β release and uptake of the dye Yo-PRO-1 (as a measure of membrane permeability) with comparable potency and kinetics. Punicalagin also inhibits Yo-PRO-1 uptake and LDH release in response to membrane detergents digitonin and triton X-100. Punicalagin also inhibits release of IL-1β independent of the inflammasome (10), strongly suggesting that under these conditions it is acting at the plasma membrane. However, punicalagin is also reported to have additional effects, including potent anti-oxidant activity in macrophages (15), and so some care must be taken when interpreting its effects. We also used the cytoprotectant glycine, which does not inhibit IL-1β release but limits cell lysis (16). We initially tested the effects of punicalagin (50 μM) and glycine (5 mM) directly on membrane permeability. LPS primed (1 μg/mL, 2h) immortalized mouse bone marrow derived macrophages (iBMDMs) were incubated with CellTox Green dye which would label cells after permeabilisation, and were then incubated with vehicle, the NLRP3 activating stimulus nigericin (10 μM), or nigericin and either punicalagin or glycine, with the effects on dye uptake monitored by

microscopy (for 100 min). In untreated cells there was no dye uptake, whereas nigericin treatment caused a robust increase in fluorescence (Figure 1A). Glycine had no effect on nigericin-induced dye uptake, whereas punicalagin significantly delayed it, suggesting that these two reagents were having significantly different effects on the plasma membrane (Figure 1A). Sixty minutes of nigericin treatment of LPS primed iBMDMs caused significant cell lysis, as measured by release of the cytoplasmic protein lactate dehydrogenase (LDH) (Figure 1B). Nigericin induced LDH release at 60 min was completely inhibited by both punicalagin and glycine. At 90 min nigericin treatment there was more LDH released and this was still significantly decreased by punicalagin and glycine (Figure 1B), suggesting that both reagents had a protective effect.

We next assessed the effects of punicalagin and glycine on nigericin induced IL-1β release. Sixty minutes of nigericin treatment caused significant release of IL-1β that was partially inhibited by both punicalagin and glycine (Figure 1C). At 90 min of nigericin treatment punicalagin still decreased IL-1β release to some degree, but the inhibitory effect of glycine observed at 60 min was absent (Figure 1C). These data suggest that while punicalagin was an effective inhibitor of IL-1β release, glycine slowed its release, likely by preventing release due to cell lysis. We then tested the effects of punicalagin and glycine on inflammasome dependent processing of pro-IL-1β and gasdermin D by western blotting of combined cell lysates and supernatants after nigericin treatment. Neither punicalagin nor glycine blocked the caspase-1 dependent processing of pro-IL-1β or gasdermin D, suggesting they did not inhibit the inflammasome (Figure 1D). Incubation with the NLRP3 inflammasome inhibitors NBC6 (17), or MCC950 (18) inhibited pro-IL-1β and gasdermin D processing as would be expected (Figure 1D).

The iBMDM cells are useful for measuring inflammasome responses in general. However, it is important to determine whether the same mechanisms occur in cells which may experience tissue specific disease conditions. Recent research has demonstrated that microglia form inflammasomes during Alzheimer's disease (19), and inflammasomes are implicated in the progression of a number of other neurological and neurodegenerative conditions (20). Thus we investigated the relationship between IL-1β release and membrane permeability and cell death in cultures of glial cells (astrocytes and microglia) isolated from the brains of mice. These mixed glial cultures from the brains of neonates have microglial inflammasome responses very similar to those from primary microglia isolated from adult mice (21). LPS-primed (1 μg/mL, 3h) glial cells were treated with the NLRP3 inflammasome activator nigericin (10 μM, 1h) which caused robust cell death, and this was significantly reduced by punicalagin and by glycine (Figure 1E). Nigericin also induced release of IL-1β from glial cells, which was inhibited by punicalagin (50 μM) but not by glycine (5 mM) (Figure 1F). These data confirm the data obtained using the iBMDM cultures which suggested that the release of IL-1β is dependent on a change in membrane permeability rather than cell lysis. These data also suggest that mechanisms of inflammasome activation and IL-1β release under these conditions are common between macrophages and microglia.

Activation of the NLRP3 inflammasome often forms an ASC speck, which itself can be released and is known to have pro-inflammatory effects in the extracellular space (22, 23). Using iBMDMs stably expressing ASC-mCherry (24) we observed that the number of visible ASC specks increased with nigericin treatment (10 μM), and that the number of visible specks increased further when the cells were treated with nigericin plus punicalagin (50 μM) or glycine (5 mM) (Figure 2A). We then repeated this experiment, except we included the pan-caspase inhibitor zVAD which allowed ASC-speck formation, but inhibited potential ASC speck loss through caspase-1-dependent pyroptotic cell death. Incubation of the cells with zVAD increased the number of specks produced by nigericin treatment but had no further effect on the increase of ASC specks observed in nigericin treated cells in the presence of punicalagin or glycine (Figure 2B). These data suggest that ASC specks are released by pyroptotic cell lysis and that both punicalagin and glycine inhibited this release. This further supports a regulated mechanism for IL-1β release dependent upon a change in membrane permeability as its release was unaffected by glycine (Figure 1). We

investigated the release of ASC further by analysing the oligomeric forms released into the cell supernatant using cross-linking and western blotting. LPS treated iBMDMs did not release any ASC into the supernatant (Figure 2C). Nigericin treatment caused the release of a monomeric and a range of oligomeric ASC forms, which was reduced by punicalagin treatment (Figure 2C). Glycine treatment inhibited the release of high molecular weight oligomeric ASC, but lower molecular weight forms were released (Figure 2C). This suggested that monomeric ASC may follow the same pathway out of the cell as IL-1β. The inflammasome inhibitor NBC6 inhibited the release of all forms of ASC confirming that its release was inflammasome dependent (Figure 2C). These data suggest that oligomeric ASC and ASC specks are released by pyroptotic cell lysis, but that changes in membrane permeability allow the release of monomeric ASC. Western blotting of combined cell lysates and supernatants after nigericin treatment confirmed this showing that punicalagin and glycine did not inhibit ASC oligomerisation in response to nigericin while the inflammasome inhibitor NBC6 did (Figure 2D).

IL-18 and IL-1β follow a similar secretory route

IL-18 is also produced as a precursor pro-form and is directly regulated by the inflammasome and caspase-1. However, the mechanisms underpinning release of IL-18 are poorly understood. In these studies we used primary human monocyte derived macrophage cultures (MDMs) which also allowed us to interrogate the release pathway in human cells. MDMs were primed with LPS (1μg/mL, 4h) and then stimulated with nigericin (10 μM, 45 min) to induce release of IL-1β and IL-18. Under these conditions nigericin induced release of both IL-1β and IL-18, and in both cases release was inhibited by a 15 min pre-incubation with punicalagin (25 μM)(Figure 3A, B). Under these conditions punicalagin did not significantly reduce nigericin induced LDH release (Figure 3C). Under the same conditions, incubation with glycine (5mM) did not inhibit nigericin induced IL-1β or IL-18 release from MDMs (Figure 3D, E) but did inhibit nigericin induced LDH release (Figure 3F), suggesting that the release of IL-18 was also dependent upon a change in membrane permeability. These data also suggest that IL-1β release after NLRP3 inflammasome activation is common in mouse and human macrophages highlighting that under these conditions the mouse BMDMs are representative of a range of macrophage IL-1 release models.

IL-1α is secreted via an alternative pathway

Release of IL-1α can also be regulated by inflammasomes, albeit indirectly (3). Release of IL-1α is thought to be dependent upon Ca^{2+} activated calpain proteases (3), though a number of other proteases are known to also cleave pro-IL-1α (25). In primary mouse peritoneal macrophages primed with LPS (1 μg/mL, 2h) and treated with NLRP3 inflammasome activators ATP (30 min), or MSU or CPPD crystals (250 μg/mL, 1h), there was a release of both mature IL-1α and mature IL-1β into the culture supernatants (Figure 4A). Incubation of the cells with calpain inhibitor III (50 μM), or in Ca^{2+} free buffer, with subsequent NLRP3 inflammasome activation by ATP, or MSU or CPPD crystals, inhibited the release of mature IL-1α but not IL-1β (Figure 4A), highlighting the divergence of the secretory signalling pathways. Whilst the pharmacological data produced by us, and others, has strongly suggested that a calpain protease is required for pro-IL-1α processing, genetic proof for this is lacking. To address this we generated a reconstituted cellular model of IL-1α secretion. Macrophage cells are difficult to transfect, and respond to DNA with inflammasome activation. Thus we investigated whether we could model IL-1α release in easy to transfect HeLa cells. HeLa cells were transfected to express pro-IL-1α-GFP, and were then treated with the Ca^{2+} ionophore ionomycin (10 μM, 1h) to induce calpain activation and IL-1α processing and release (Figure 4B). In this model, release of mature IL-1α was also inhibited by calpain inhibitor III (40 μM), and by the removal of extracellular Ca^{2+}, suggesting that the pathways of IL-1α processing and release in our reconstituted HeLa cell model are representative of the pathways of IL-1α release in primary macrophages (Figure 4B). There are 14 members of the calpain family in mammals with calpains 1 and 2 the best characterised (26). We therefore knocked down expression of calpain 1, calpain 2, or both calpains 1 and 2 in HeLa cells using

siRNA that were transfected to express pro-IL-1α-GFP and then treated them with ionomycin to induce calpain activation and mature IL-1α release (Figure 4C). We were able to selectively knockdown expression of calpain 1 and calpain 2 (Figure 4C). Knockdown of either calpain inhibited release of mature IL-1α as determined by western blot (Figure 4C), confirming the importance of calpain to this pathway and suggesting that both calpains-1 and 2 can process pro-IL-1α. This was further confirmed by ELISA analysis of the HeLa cell supernatants after ionomycin treatment, which contained significantly less IL-1α after calpain knockdown (Figure 4D-F). Calpain knockdown also significantly reduced ionomycin-induced cell death (Figure 4G-I).

IL-1α may be released and processed as a consequence of cell death (27, 28). Whilst IL-1β secreting cells often undergo a pyroptotic, or pyronecrotic cell death (6, 29), there are examples where IL-1β is secreted in the absence of cell death (9, 30). Our data above suggest that IL-1β release is dependent upon membrane permeability, but not cell lysis *per se*. We therefore sought to determine whether Ca^{2+}/calpain dependent release of IL-1α requires cell lysis. We previously published that punicalagin does not inhibit IL-1α release, suggesting a separate pathway of secretion from IL-1β (10). To directly compare the effects of punicalagin and glycine on the release of IL-1α to IL-1β and IL-18 we treated primary mouse BMDMs with LPS (1μg/mL, 4h), then incubated them with punicalagin (50 μM), or glycine (5 mM), and then stimulated with ionomycin (10 μM, 1h) to activate calpain, or with nigericin (10 μM, 1h) to activate the NLRP3 inflammasome. Both punicalagin and glycine inhibited ionomycin and nigericin-induced cell death (Figure 5A). However, neither punicalagin nor glycine inhibited release of IL-1α in response to ionomycin or nigericin (Figure 5B, C). Ionomycin did not cause release of IL-1β (Figure 5D) or cleavage of gasdermin D (Figure 5E), and nigericin-induced cleavage of gasdermin D was not prevented by calpain inhibition, but was by NLRP3 inflammasome inhibition with NBC6 (Figure 5E). Together these data suggest that, while in many cell types close associations between the release of related cytokines IL-1α and IL-1β and cell death are observed, these processes can be dissociated from each other. Release of IL-1β appears to rely on a change in membrane permeability dependent upon gasdermin D cleavage (31, 32). Calpain-dependent IL-1α release appears to be independent of cell lysis, but relies on alternative, but seemingly parallel, pathways to IL-1β.

The above data suggested that IL-1α could be secreted from cells independently of plasma membrane rupture. We next examined the release of IL-1α in an alternative cell model. Cellular senescence is a barrier to tumorigenesis in response to oncogenic stresses by forcing cells to permanently exit from the cell cycle (33). Whilst beneficial early in an organisms life, at older age cellular senescence can promote tissue disruption and can paradoxically be pro-tumorigenic by virtue of the senescence associated secretory phenotype (SASP) (33). The SASP is the secretion of pro-inflammatory cytokines from senescent cells that can promote paracrine senescence (34). A defining feature of cellular senescence is the expression of IL-1α, which is critical for the development of the SASP, and in some cases can drive senescence (34), whilst in other cases blockade of IL-1α reduces the SASP but the cell still senesces (35, 36). It is not currently understood whether IL-1α secretion, required for SASP development, results in death of the secreting cell. To address this we used immortalised mouse embryonic fibroblast (MEF) cells (37) as cellular senescence is often studied with fibroblasts. To recapitulate a SASP-like phenotype MEF cells were transfected to express pro-IL-1α-GFP. IL-1α was released from transfected MEF cells when assayed 48 h after transfection (Figure 6A). IL-6 and CXCL1 levels were also significantly increased after 48 h in pro-IL-1α-GFP transfected groups but not in GFP transfected, or non-transfected groups (Figure 6B, C). In addition, 48 h after treatment with the IL-1 receptor antagonist IL-1Ra (1 μg/mL added every 24 h for the duration of the experiment), CXCL1 and IL-6 secretion were significantly reduced while levels of IL-1α were not affected (Figure 6A-C). These data suggest that IL-1α is secreted over time to induce the release of IL-6 and CXCL1. Importantly, there was no significant cell death over the duration of this experiment, and glycine (5mM) added for the last 24 h of incubation had no effect on IL-1α release (Figure 6D, E). These data confirm the data above that IL-1α can be

released in the absence of cell lysis and through a separate pathway to IL-1β and IL-18 in a variety of cell types. The release of IL-1α by these MEF cells was independent of gasdermin D cleavage as it was not expressed (Figure 6F), again highlighting the difference between IL-1α and IL-1β release.

DISCUSSION

The IL-1 family have been described as canonical DAMPs, and indeed there is evidence to support this (38). For an IL-1 family member to act like a canonical DAMP, it would be released as a result of cellular death or injury. However, there is also evidence to suggest that some IL-1 family members can be released in the absence of cell death, and may therefore also act as actively secreted cytokines. For example, there is evidence that caspase-1-dependent processing and secretion of IL-1β from macrophages and neutrophils can occur in the absence of cell lysis (9, 30), although from these studies the measurements are from cell populations rather than single cells which may mask any correlations with cell death and IL-1β release at the single cell level (10). An often reported consequence of inflammasome activation in macrophages is cell death, and though release of IL-1β can be temporally separated from release of a lytic marker such as LDH, it seems that a complete loss of cell integrity is inevitable in many cases (29). However, use of cytoprotectants such as glycine can be used to prevent cell lysis after inflammasome activation in macrophages, but still allow the release of mature IL-1β (16, 39).

The release of IL-1β from macrophages can also be blocked by the membrane stabilizing agent punicalagin (10). Here we showed that glycine, while blocking cell lysis and ASC speck/oligomer release, did not inhibit NLRP3-dependent release of IL-1β, and that punicalagin was at least partially effective against both cell lysis and IL-1β release. Pyroptosis leads to release of active IL-1β and concomitant release of ASC specks capable of being taken up by other cells and propagating an inflammatory response (22, 23). This process of ASC speck release was recently implicated in models of Alzheimer's disease where ASC specks were shown to seed amyloid-β plaques (19). Here, we report conditions allowing release of IL-1β and monomeric ASC from cells with active inflammasomes, but where release of ASC oligomers and specks is blocked. By dissociating IL-1β secretion from ASC speck release we have provided conditions that allow for novel insights to be made into the individual roles played by these inflammatory factors in future studies.

The IL-1β release inhibitor punicalagin influenced the permeability of the plasma membrane to the dye CellTox Green, suggesting that it is a specific change in membrane permeability rather than cell lysis *per se* that is allowing release of IL-1β. This was also the case for NLRP3-dependent IL-1β release in human macrophages, and for the related cytokine IL-18, suggesting that they may share a common exit route from the cell. Identifying that, under the stated conditions, the pathway of IL-1β release is common between mouse and human macrophages, and different subtypes of macrophage, allows us to further reliably interpret and compare studies in different cell types and from different species. Although the secretion of IL-1α and IL-1β from macrophages in response to NLRP3-inflammasome activating stimuli were previously suggested to follow a common secretory route based on kinetics and inhibitor sensitivity (40), our data suggest that in fact the secretory mechanisms are distinct. IL-1α and IL-1β are closely related molecules with IL-1α arising as a result of a gene duplication event of IL-1β (2). Significant divergence between IL-1α and IL-1β has occurred since the duplication event at the amino acid level, particularly within the pro- domain, although there is very little evidence of divergence in mechanisms of secretion. Here we provide evidence in macrophages that the secretion of IL-1α is independent from IL-1β and IL-18. We have also modelled the IL-1α release pathway in easy to transfect cell lines (HeLa and MEF) allowing us to further conclude that IL-1α may be actively secreted from cells which may be important for development of the SASP and thus cellular senescence. This discovery opens further avenues of research where we can now address the other contexts in which IL-1α is actively secreted from living cells. Our studies in the MEF cells suggest that IL-1α secretion is independent of gasdermin D. It should be noted however that IL-1α release from BMDMs infected with a mutant strain of *S. aureus* was less from gasdermin D KO cells compared to WT (32). Also, while it

is now becoming well accepted that release of IL-1β is gasdermin D dependent, a delayed gasdermin D-independent mechanism of IL-1β release has also been described (14).

Overall, these data have broad implications and suggest that IL-1 family members behave as both DAMPs and as actively secreted cytokines. Our use of a senescence-like model to study IL-1α secretion highlights the value of using context specific models when studying IL-1 release pathways. Cellular senescence, a process in which there is no overt cell death, now provides a context for the non-lytic release of IL-1α. Likewise, DAMP-dependent release of IL-1 from macrophages may not present us with a unifying pathway to describe IL-1β secretion in all circumstances, and we are learning that activation of canonical and alternative inflammasomes have very different effects on ASC speck formation and cell death (8, 41).

EXPERIMENTAL PROCEDURES

Antibodies and reagents

Antibodies used targeted mouse IL-1β (AF-401-NA, R&D Systems), mouse gasdermin D (ab209845, Abcam), ASC (AL177, Adipogen), mouse IL-1α (AF-400-NA, R&D Systems), human calpain-1 (ab39170, Abcam), human calpain-2 (ab39165, Abcam) and β-actin-HRP (A3854, Sigma). Pharmacological agents used were punicalagin (Sigma), glycine (Sigma), NBC6 (synthesised in house(17)), MCC950 (CP-456773, Sigma), Z-VAD-FMK (Merck), calpain inhibitor III (Merck), nigericin (Sigma), adenosine triphosphate (Sigma), ionomycin (Sigma), mono-sodium urate crystals (InVivoGen), calcium pyrophosphatedihydrate crystals (InVivoGen) and IL-R1a (Kineret®, Amgen). All other materials were from Sigma Aldrich unless specified.

Cell culture

Mouse iBMDMs, obtained from Claire Bryant (Department of Veterinary Medicine, University of Cambridge), and ASC-mCherry iBMDMs (23), were cultured in Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS, Life Technologies), 100 U/mL penicillin and 100 μg/mL penicillin-streptomycin (PenStrep). The iBMDMs were seeded overnight at a density of 0.75 x 10^6 cells/mL. Murine mixed glia cultures were prepared from brains isolated from 3-4 day old C57BL/6 mouse pups (Envigo). All animal procedures were performed with appropriate personal and project licenses in place, in accordance with the Home Office (Animals) Scientific Procedures Act (1986), and approved by the Home Office and the local Animal Ethical Review Group, University of Manchester. As described previously (20), brain tissue was mechanically digested and cells were maintained in DMEM, 10% FBS, PenStrep until 80% confluency was reached (10-13 days *in vitro*). Cultures were then reseeded at a density of 1.7 x 10^5 cells/mL. After a further 2-3 days, cells were ready for experimentation.

Murine peritoneal macrophages were isolated from C57BL/6 mice. Mice were anaesthetized with isoflurane (induced at 3–4% in 33% O_2, 67% NO_2, maintained at 1–2%) and peritoneal cavities lavaged with 6 mL RPMI. Peritoneal macrophages were cultured in DMEM, 10% FBS, PenStrep and seeded overnight at a density of 1×10^6 cells/mL before experiment the following day.

Murine primary BMDMs were prepared by flushing femurs of C57BL/6 mice. Red blood cells were lysed with ACK lysis buffer (Lonza) and BMDMs were generated by culturing the resulting marrow cells in 70% DMEM (containing 10% FBS, PenStrep) and 30% L929 mouse fibroblast-conditioned media for 7-10 days. Primary BMDMs were seeded overnight at a density of 1 x 10^6 cells/mL before the experiment.

The mouse embryonic fibroblast (MEF) cell line was derived from primary MEFs and immortalised with retroviral introduction of shRNA against p53 (pSuperRetro-sh53) (37). Cells were cultured in DMEM, 10% FBS, PenStrep. Before experiments MEFs were seeded overnight at a density of 5 x 10^4 cells/mL.

Human monocyte-derived macrophages (MDM) were generated as previously descried (17). Briefly, peripheral blood mononuclear cells (PBMC) were obtained from leukocyte cones from healthy donors (Service Blood and Transplant, Manchester, UK) with full ethical approval from the Research Governance, Ethics, and Integrity Committee at the University of Manchester (ref. 2018-2696-5711). After a density centrifugation step using a Ficoll gradient the PBMC layer was carefully

removed and monocytes were obtained from the PBMCs by positive selection with CD14 magnetic MicroBeads and a LS column (Miltenyi) for 15 min. Monocytes were differentiated into macrophages for 6 days (5 x 10^5 cells/mL) in RPMI (containing 10% FBS, PenStrep) and in the presence of 0.5 ng/mL M-CSF (Peprotech). At day 3, half of the media was removed and replaced with fresh media to foster proliferation.

Human HeLa cells were cultured in DMEM, 10% FBS, PenStrep. HeLa cells were cultured overnight after seeding at a density of 5 x 10^4 cells/mL.

NLRP3 and calpain activation protocol

Cells were seeded in 96 or 48-well plates and primed with LPS (1 µg/mL) for 2 (iBMDMs, peritoneal macrophages), 3 (mixed glia), or 4 h (primary BMDMs, MDMs). After priming, the media was changed to FBS-free DMEM, calcium-free buffer, or calcium-containing buffer (42). Calcium-free buffer was composed of 121 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 6 mM $NaHCO_3$, 25 mM HEPES, 5 mM glucose and 5 mM EGTA. Calcium-containing buffer was composed of 121 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 6 mM $NaHCO_3$, 25 mM HEPES and 5 mM glucose. Cells were then treated with punicalagin (25 or 50 µM), glycine (5 mM), NBC6 (20 µM), MCC950 (10 µM) or calpain inhibitor III (40 and 50 µM) for 15 min. Cells were then stimulated by adding the NLRP3 activating stimuli ATP (5 mM), nigericin (10 µM), MSU (250 µg/mL), CPPD (250 µg/mL) or the Ca^{2+} ionophore ionomycin (10 µM).

Live cell imaging

Real-time membrane permeabilization assays were performed using iBMDMs seeded in 96-well plates. Cells were primed with LPS (1 µg/mL, 2h). After priming, cells were incubated with CellTox Green (Promega), treated with punicalagin (50 µM), glycine (5 mM), zVAD (50 µM) and stimulated with nigericin (10 µM, 100 min). Real-time ASC speck assays were performed in the same conditions, using ASC-mCherry iBMDMs. Images were captured every 10 or 15 min using an IncuCyte ZOOM System (Essen Bioscience) with a 20×/0.61 S Plan Fluor objective. Excitation and emission wavelengths were 440-480 and 504-544 nm for permeability assays, and 565-605 nm and 625-705 nm for speck assays.

Cell death analysis

Cell death was measured by assessing lactate dehydrogenase (LDH) release into cell culture supernatants, using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to manufacturer's instructions. Samples were quantified by reading absorbance at 490 nm in a Synergy HT (Biotek instruments). LDH release was expressed as a percentage normalized to a total cell lysis control, subtracting the background signal from cell culture media.

Cytokine release analysis

IL-1α, IL-1β, CXCL1, and IL-6 measurements were made in cell supernatants, by using Duoset ELISA kits (R&D systems) following manufacturer's instructions. Human IL-18 was measured using the e-bioscience kit (IL-18, BMS267/2MST). Samples were quantified using corrected values of 450 and 570 nm, reading absorbance in a Synergy HT (Biotek instruments).

Western blot analysis

Western blot analysis was performed on supernatants and lysates for IL-1β, gasdermin D, ASC, IL-18, IL-1α, calpain-1, calpain-2 and β-actin. Samples were run on 10% (calpain-1 and calpain-2), 12% (ASC and pro-IL-1IL-1α-GFP) or 15% (IL-1α, IL-1β, IL-18, and gasdermin D) sodium dodecyl sulphate (SDS) polyacrylamide gels. Gels were transferred using a Trans-Blot® Turbo™ Transfer System (BioRad) at 25 V for 7 min before blocking with 2.5% bovine serum albumin (BSA) in phosphate-buffered saline, 1% Tween 20 (PBST) for 1h at RT. Membranes were washed and incubated (4 °C) overnight in primary antibody in PBST 0.1% BSA. Following this, membranes were washed and incubated with HRP-conjugated secondary antibodies (Dako) in PBST, 0.1% BSA for 1h at RT. Finally, membranes were washed and incubated in Amersham ECL Western Blotting Detection Reagent (GE Life Sciences) before exposure using a G:BOX gel doc system (Syngene).

ASC oligomerization assay

iBMDMs were seeded into 24-well plates. Cells were primed with LPS (1 µg/mL,

2h), then incubated with punicalagin (50 μM), glycine (5 mM), or NBC6 (20 μM) for 15 min and stimulated with nigericin (10 μM, 60 min). For total oligomerization, cells were directly lysed in the well by addition of 1% Triton-X100. Cell lysates were separated into a Triton-X100 soluble fraction and insoluble fraction by centrifugation at 6800 x g for 20 min at 4 °C. The insoluble pellets were crosslinked with DSS (2 mM, Thermo Fisher) for 30 min. Crosslinked pellets were further spun down at 6800 x g for 20 min and eluted in Laemmli buffer for SDS/PAGE. For detection of released ASC oligomers, supernatants were collected and detached cells were removed, the supernatants then concentrated by centrifugal filtering (Amicon 10K centrifugal filters) according to manufacturer's instructions. The concentrated supernatants were chemically crosslinked with DSS for 30 min at RT before Laemmli buffer was added in preparation for western blotting.

IL-1α release from HeLa cells

HeLa cells were seeded into 24-well plates. Cells were transfected with plasmids expressing pro-IL-1α-GFP, or GFP only control (0.5 μg/well, 24h) using Lipofectamine 3000 according to manufacturer's instructions. For RNAi studies, HeLa cells were transfected with siRNA for calpain-1, calpain-2 or scrambled control (Santa Cruz, 40 nM, 72h) using Lipofectamine 3000. On the day of the ionomycin stimulus, media was changed to DMEM, calcium-free buffer, or calcium-containing buffer and cells were incubated with or without calpain inhibitor III (40 μM, 15 min) before stimulation with ionomycin (10 μM, 1h).

SASP-like IL-1α release model

MEF cells were seeded into 24-well plates. Cells were transfected with plasmids for pro-IL-1α-GFP or GFP expression (0.5 μg DNA/well, 48h) using Lipofectamine 3000. Transfected groups were treated with and without IL-1Ra (1 μg/mL, added at 0 and 24h), and with or without glycine (5 mM, 24h).

Statistics

Data are presented as scatter plots with the mean ± SD indicated. Statistical analysis was performed using GraphPad Prism 7 software. Accepted levels of significance were *P < 0.05, **P < 0.01, ***P <0.001, ****P < 0.0001. Data with comparisons against a vehicle control were analysed using a one-way ANOVA followed by Dunnet´s *post hoc* analysis, or with multiple comparisons with Sidak´s *post hoc* analysis. Time courses were compared against a vehicle control by a two-way ANOVA with with Sidak's *post hoc* analysis. In one-way ANOVA equal variance was evaluated with the Brown-Forsythe test and transformations were performed where necessary. Experimental replicates (n) were defined as experiments performed on different passages of immortal cell lines (iBMDMS, HeLa or MEF cells) or individual animal/human donors (primary BDMDs or MDMs, respectively).

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council (MRC) (grant ref no: MR/N029992/1 to D.B.) and a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (Grant number 104192/Z/14/Z to G.L-C). J.R-A. was funded by the BBSRC (BB/P01061X/1). M.J.D.D. was funded by a MRC DTP studentship (MR/K501311/1). V.S.T. was funded by CONICYT (Becas Chile 721704488). P. P-R. was funded by the Manchester Collaborative Centre for Inflammation Research, as a joint initiative of the University of Manchester, AstraZeneca, and GlaxoSmithKline. We would like to thank Dr Jonathan Worboys (UoM) for help with the preparation of MDMs.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

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FIGURE LEGENDS

Figure 1: IL-1β release after inflammasome activation is dependent on plasma membrane permeability. (A-D) LPS-primed

(1 µg/mL, 2h) iBMDMs were incubated with vehicle, punicalagin (Pun, 50 µM) or glycine (Gly, 5 mM) 15 min prior to activation with nigericin (Nig, 10 µM). (A) Membrane permeability was measured in real time by CellTox Green uptake (n=4). Supernatants were assayed for (B) cell death, measured as LDH release and normalized to a total cell lysis control, and for (C) IL-1β release by ELISA (n=4). (D) 1h after nigericin stimulation combined supernatant and cell lysate were analysed for pro-IL-1β (31 kDa), m-IL-1β (17 kDa), Gasgermin D (GSDMD) full length (FL, 53 kDa), GSDMD N-terminal fragment (NT, 31 kDa) and β-actin (42 kDa) by western blot. (E-F) LPS-primed (1 µg/mL, 3h) mixed glia cultures were incubated with vehicle, punicalagin (50 µM), glycine (5 mM) or MCC950 (10 µM) 15 min prior to activation with nigericin (10 µM, 1h). Supernatants were assayed for (E) cell death, measured as LDH release, and (F) IL-1β release by ELISA (n=4). * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$; n.s. non-significant, determined by a two-way ANOVA with Sidak's *post hoc* analysis and compared to nigericin treated group (A-C), or a one-way ANOVA with Dunnet's *post hoc* analysis compared to nigericin treated group (E-F). Western blots are representative of 3 independent experiments.

Figure 2: ASC speck release is dependent on cell lysis. (A-B) LPS-primed (1 µg/mL, 2h) ASC-mCherry iBMDMs were incubated with vehicle, punicalagin (Pun, 50 µM) or glycine (Gly, 5 mM) 15 min prior to activation with nigericin (Nig, 10 µM) and ASC speck formation was measured in real time (A) without or (B) with incubation of zVAD (50 µM) (n=4). (C-D) Wild type iBMDMs were treated as in (A-B) and activated with nigericin for 1h. (C) Supernatants or (D) combined supernatant and lysate were cross-linked and analysed for ASC or β-actin by western blot. ASC monomers (22 kDa), dimers (44 kDa) and oligomers are indicated. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, determined by a two-way ANOVA with Sidak's *post hoc* analysis and compared to nigericin treated group. Western blots are representative of 3 independent experiments.

Figure 3: IL-18 release during inflammasome activation is dependent on plasma membrane permeability. (A-C) LPS-primed (1 µg/mL, 2h) MDMs were incubated with vehicle or punicalagin (25 µM) 15 min prior to activation with nigericin (10 µM, 45 min). Supernatants were assayed for IL-1β (A) and IL-18 (B) by ELISA (n=8-9). (C) Cell death was measured as LDH release normalized to a total cell lysis control. (D-F) LPS-primed (1 µg/mL, 2h) MDMs were incubated with vehicle or glycine (5 mM) 15 min prior to activation with nigericin (10 µM, 45 min). Supernatants were assayed for IL-1β (D) and IL-18 (E) by ELISA (n=8-9). (F) Cell death was measured as LDH release. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.001$; n.s. non-significant, determined by a one-way ANOVA with Dunnet's *post hoc* analysis and compared to nigericin treated group.

Figure 4: IL-1α processing and release is dependent on calpains 1 and 2. (A) LPS-primed (1 µg/mL, 2h) peritoneal macrophages were incubated with calpain inhibitor III (50 µM), or in calcium containing (+Ca) or free (0Ca) buffers 15 min prior to activation with ATP (5 mM, 1h), MSU (250 µg/mL, 1h) or CPPD (250 µg/mL, 1h). Supernatants were analysed for IL-1α (pro - 31 kDa, mature 17 kDa), and IL-1β (pro - 31 kDa, mature – 17 kDa) by western blot. (B) HeLa cells were transfected with pro-IL-1α-GFP (24h), then incubated with calpain inhibitor III (40 µM) or in calcium containing (+Ca) or free (0Ca) buffers 15 min prior to activation with ionomycin (10 µM, 1h). Supernatants were analysed for IL-1α-GFP (pro - 58 kDa, mature – 44 kDa) by western blot. (C-I) HeLa cells were transfected with Calpain 1, Calpain 2 or scrambled siRNA (48h), transfected with pro-IL-1α-GFP (24 h) and treated as in (B). (C) Cell lysates were analysed for Calpain 1 (CPN1, 75 kDa), Calpain 2 (CPN2, 75 kDa) and β-actin (42 kDa), and supernatants for m-IL-1α-GFP (44 kDa) by western blot. Supernatants were assayed for (D-F) IL-1α release measured by ELISA and (G-I) cell death, measured as LDH release (n=4). * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$; n.s non-significant, determined by a one-way ANOVA with Dunnet's *post hoc* analysis compared to ionomycin treated group. Western blots are representative of at least 3 independent experiments.

Figure 5: IL-1α release can occur independently of cell lysis. (A-D) LPS-primed (1 µg/mL, 4h) primary mouse BMDMs were incubated with vehicle, punicalagin (Pun, 50 µM) or glycine (Gly, 5 mM) 15 min prior to activation with ionomycin (10 µM, 1h) or nigericin (10 µM , 1h). (A) Cell death was measured as LDH release (n=5). (B) IL-1α release was assayed by ELISA (n=5) or (C) analysed for pro-IL-1α (31 kDa) and m-IL-1α (17 kDa) by western blot. (D) IL-1β release was assayed by ELISA (n=5). (E) BMDMs were treated as previously, but incubated with calpain inhibitor III (40 µM) or NBC6 (20 µM) prior to ionomycin or nigericin treatment. Combined supernatants and lysates were analysed for Gasdermin D (GSDMD) full length (FL, 53 kDa), GSDMD N-terminal fragment (NT, 31 kDa) and β-actin (42 kDa) by western blot. * = $P < 0.01$; ** = $P < 0.001$; *** = $P < 0.0001$; n.s non-significant, determined by a one-way ANOVA with Dunnet's *post hoc* analysis compared to ionomycin or nigericin treated groups. Western blots are representative of 2 independent experiments.

Figure 6: IL-1α is released from viable MEF cells. (A-C) MEF cells were transfected with pro-IL-1α-GFP or with GFP alone (48h) and treated with IL-1Ra (10 µg/mL, 48h). Supernatants were assayed for (A) IL-1α, (B) IL-6 and (C) CXCL1 release by ELISA (n=6). (D-E) MEF cells transfected as previously were treated with glycine (5 mM, 24h). Supernatants were assayed for (D) cell death, measured as LDH release, and (E) IL-1α release measured by ELISA (n=4). (F) Cell lysates from MEFs, transfected as previously, and LPS-primed iBMDMs, incubated with NBC6 (20 µM) and stimulated with nigericin (10 µM, 1h) were analysed for gasdermin D (GSDMD) full length (FL, 53 kDa), GSDMD N-terminal fragment (NT, 31 kDa) and β-actin (42 kDa) by western blot. **** = $P < 0.0001$; n.s non-significant, determined by a one-way ANOVA with Sidak's *post hoc* analysis with multiple comparisons (A-B), or one-way ANOVA with Dunnet's *post hoc* analysis compared to pro-IL-1α-GFP transfected group (D-E). n.d. not detected. Western blots are representative of 2 independent experiments.

Figure 1

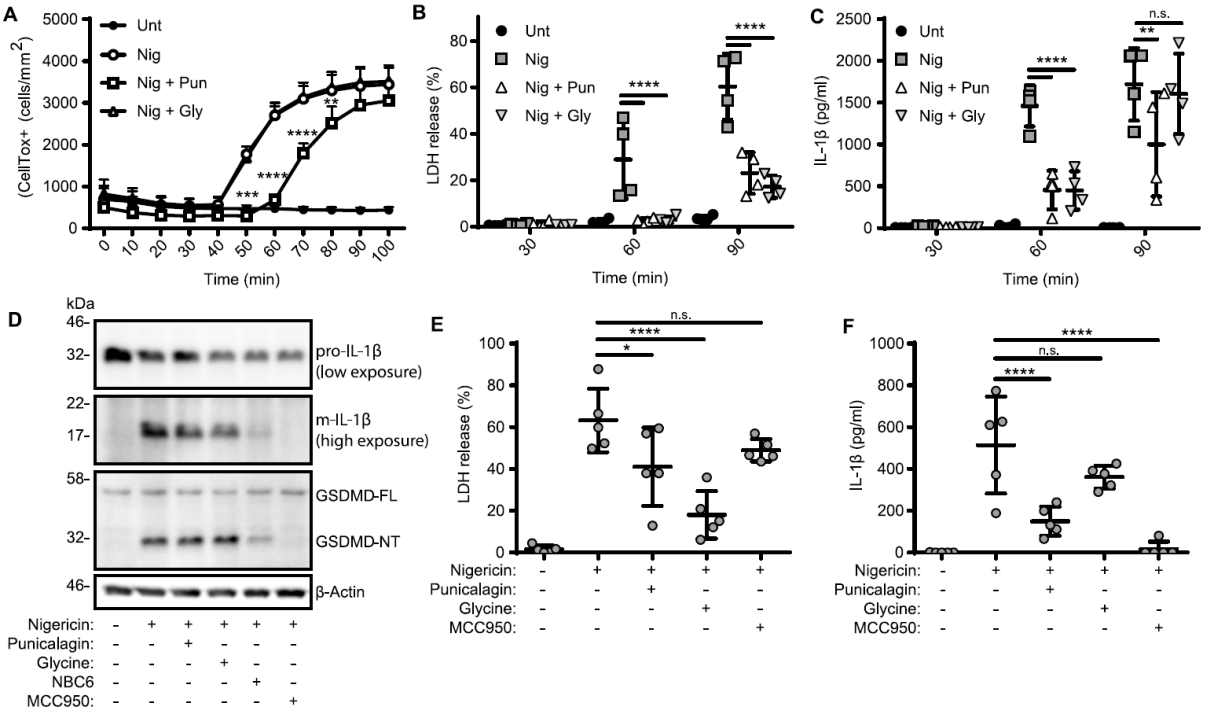


Figure 2

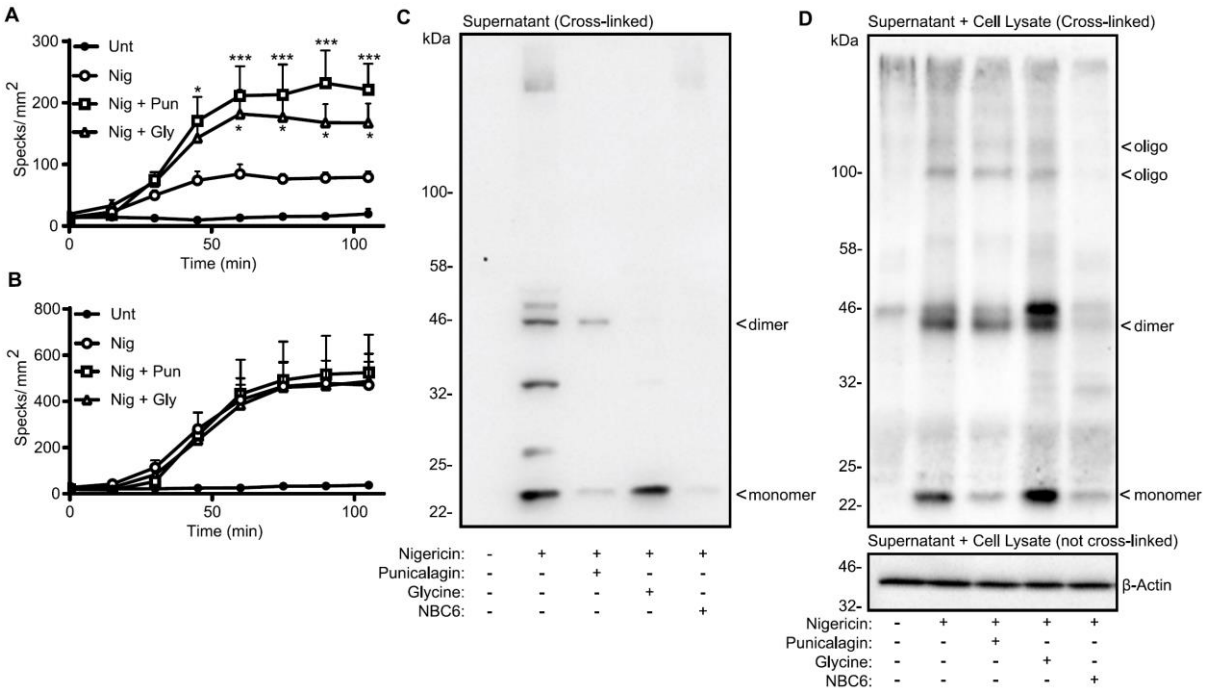


Figure 3

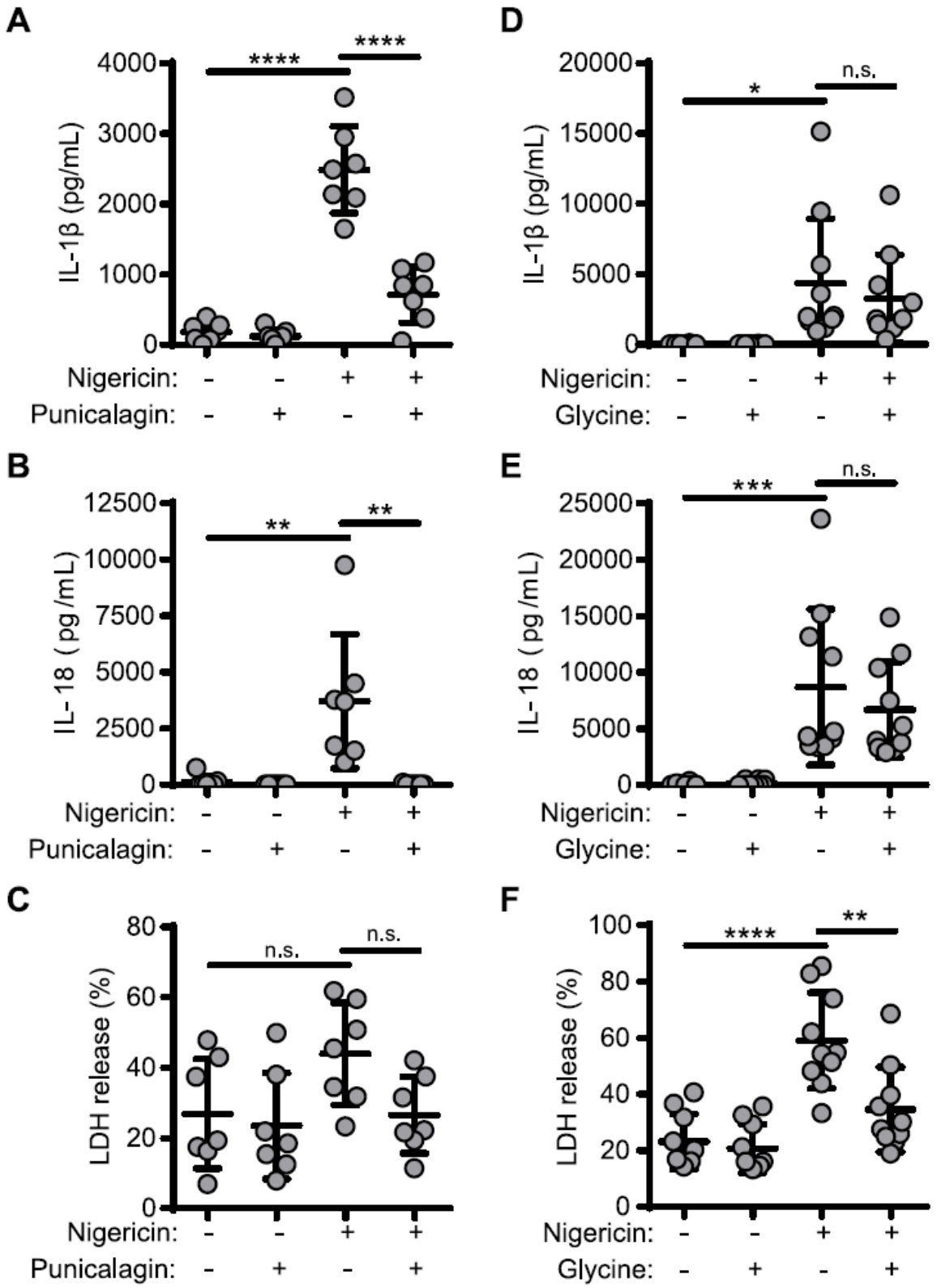


Figure 4

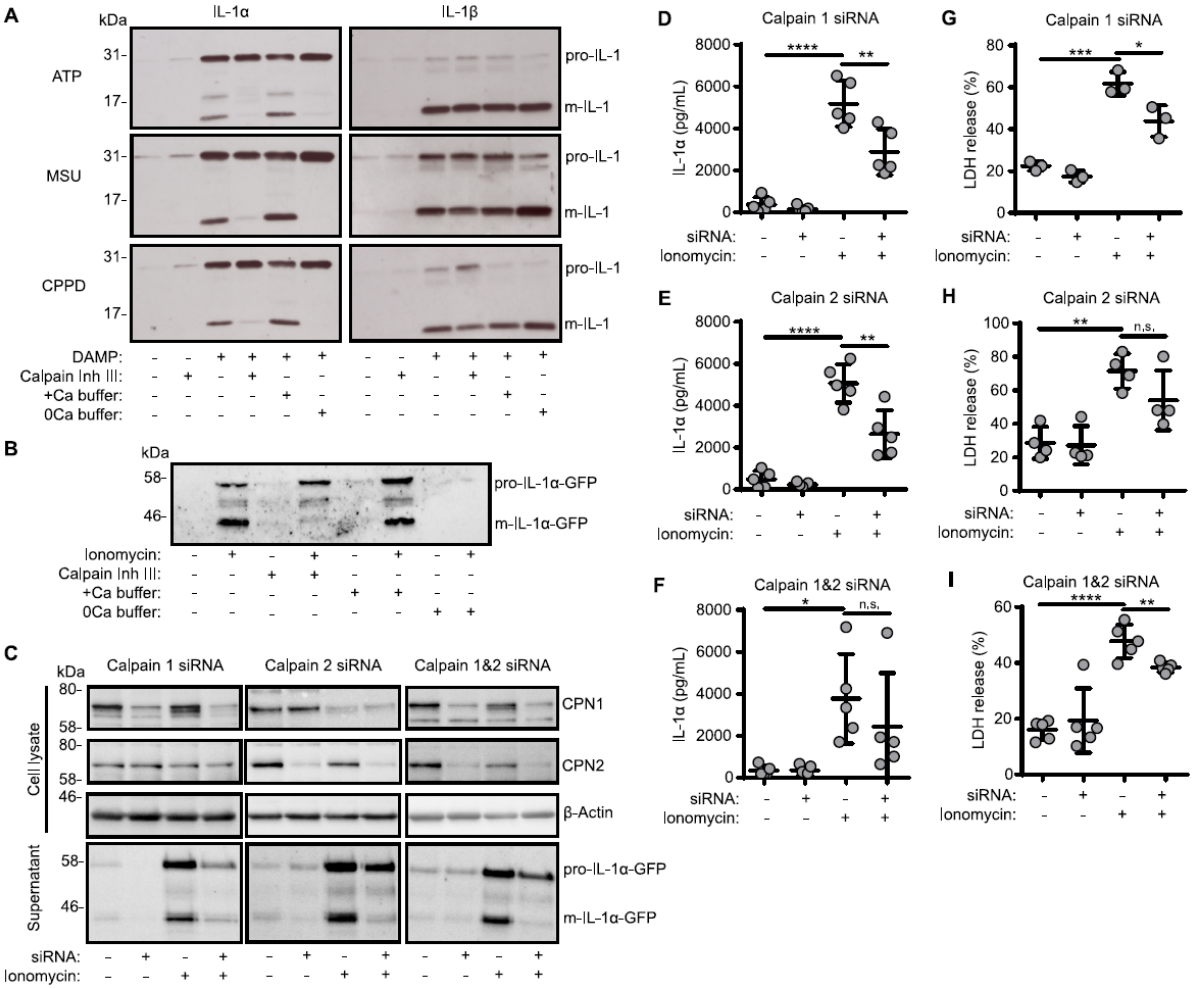


Figure 5

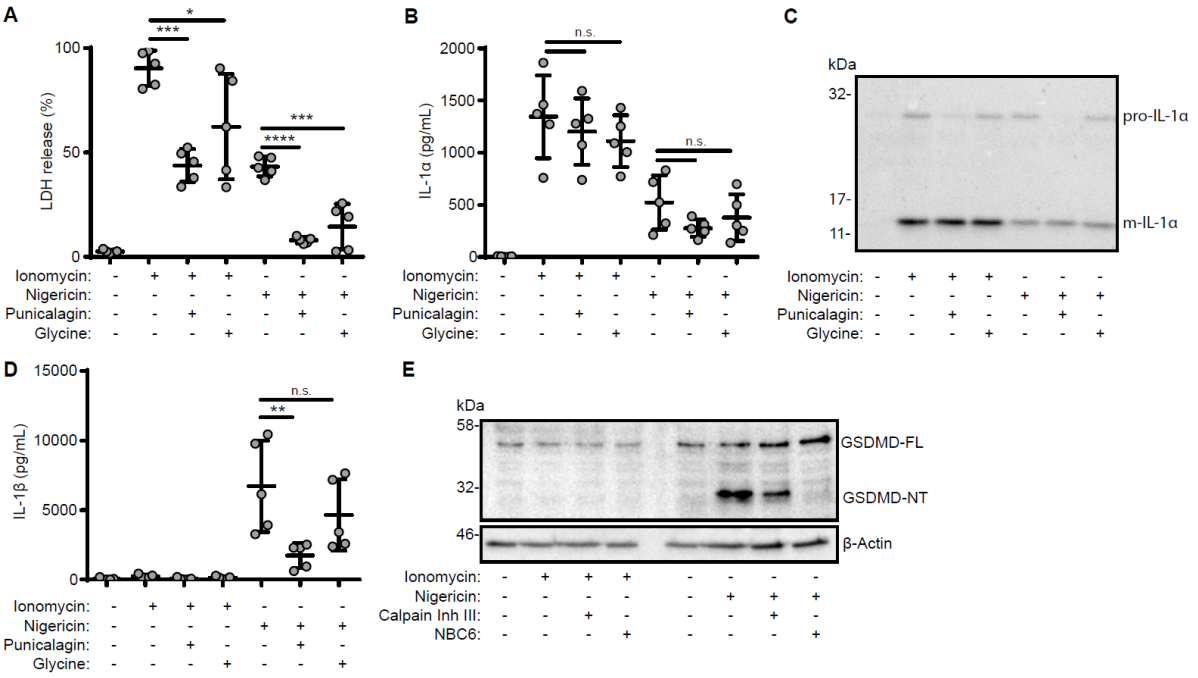
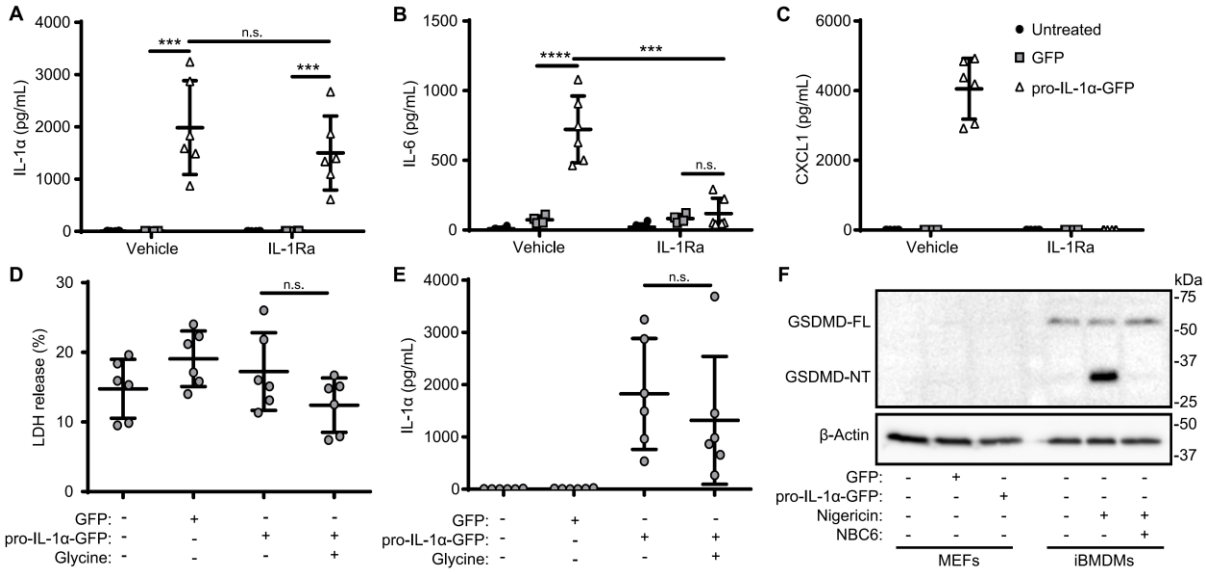


Figure 6



The three cytokines IL-1β, IL-18, and IL-1α share related but distinct secretory routes

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J. Biol. Chem. published online April 2, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA119.008009