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ORIGINAL ARTICLE

Streptolysins are the primary inflammasome activators in macrophages during *Streptococcus pyogenes* infection

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Abstract

Group A *Streptococcus* (GAS) is a Gram-positive bacterial pathogen that causes an array of infectious diseases in humans. Accumulating clinical evidence suggests that proinflammatory interleukin (IL)-1β signaling plays an important role in GAS disease progression. The host regulates the production and secretion of IL-1β via the cytosolic inflammasome pathway. Activation of the NLR family pyrin domain-containing 3 (NLRP3) inflammasome complex requires two signals: a priming signal that stimulates increased transcription of genes encoding the components of the inflammasome pathway, and an activating signal that induces assembly of the inflammasome complex. Here we show that GAS-derived lipoteichoic acid can provide a priming signal for NLRP3 inflammasome activation. As only few GAS-derived proteins have been associated with inflammasome-dependent IL-1β signaling, we investigated novel candidates that might play a role in activating the inflammasome pathway by infecting mouse bone marrow-derived macrophages and human THP-1 macrophage-like cells with a panel of isogenic GAS mutant strains. We found that the cytolysins streptolysin O (SLO) and streptolysin S are the main drivers of IL-1β release in proliferating logarithmic phase GAS. Using a mutant form of recombinant SLO, we confirmed that bacterial pore formation on host cell membranes is a key mechanism required for inflammasome activation. Our results suggest that streptolysins are major determinants of GAS-induced inflammation and present an attractive target for therapeutic intervention.

INTRODUCTION

Group A *Streptococcus* (GAS; *Streptococcus pyogenes*) is a human-adapted, Gram-positive bacterial pathogen that causes significant morbidity and mortality worldwide.[1] Diseases caused by GAS range from mild infections of epithelial or mucosal tissues such as impetigo and pharyngitis (strep throat), to severe invasive diseases such as necrotizing fasciitis and streptococcal toxic shock-like syndrome. Repeated infections with GAS can trigger the development of chronic autoimmune complications, or poststreptococcal sequelae such as rheumatic heart disease.[2] The abundant M surface protein is the major antiphagocytic virulence factor found in all GAS isolates. The nucleotide sequence of the *emm* gene, which encodes this protein, displays extensive variability and is conventionally used to serotype clinical isolates of GAS. More than 220 *emm* types have been identified.[3] Over the past decades, a globally disseminated serotype M1T1 subclone has been the leading cause of severe invasive GAS infections,[4] whereas GAS *emm12* clones have been responsible for recent scarlet fever outbreaks in Northeast

Asia.[5] Despite extensive research effort, a vaccine to protect against GAS infection is not yet available.[6]

Recent evidence suggests that the inflammatory marker interleukin (IL)-1β plays an important role in GAS disease progression, presenting a potential target for therapeutic intervention.[7–12] In host cells, the production and secretion of IL-1β is controlled by the inflammasome pathway. Inflammasomes are multimeric cytosolic protein complexes that provide a platform for the activation of caspase-1, an inflammatory caspase that proteolytically processes pro-IL-1β and pro-IL-18 into mature cytokines.[13] Caspase-1 also cleaves the protein gasdermin D to release the p30 subunit with pore-forming activity.[14] The formation of gasdermin D membrane pores enables the secretion of IL-1β and IL-18 and can lead to a proinflammatory form of cell death termed "pyroptosis," which is characterized by plasma membrane rupture and the release of cytosolic contents.[15] Pyroptosis restricts replication of intracellular bacterial pathogens,[16] and IL-1β signaling promotes immune resistance to several microbes including GAS.[7,8]

Among various inflammasomes,[15] the NLR family pyrin domain-containing 3 (NLRP3) inflammasome is the most intensively studied and is implicated as the only inflammasome that responds to GAS infection.[17–19] Activation of the NLRP3 inflammasome typically requires two signals: a "priming" signal that mediates the nuclear factor (NF)-κB-dependent upregulation in NLRP3 and pro-IL-1β protein expression, and an "activation" signal that triggers the assembly of the inflammasome complex, leading to caspase-1 activation and the production of mature IL-1β and IL-18.[15] Priming can be initiated by pattern recognition receptor [including Toll-like receptors (TLRs)] signaling and although it is known that GAS-derived molecules are detected by pattern recognition receptors,[20,21] it remains unclear which isolated components can contribute to inflammasome priming. The activation signal can be provided by a wide variety of stimuli, many of which induce the efflux of cytosolic K^+; however, the precise mechanisms of NLRP3 activation remain incompletely understood.[15]

As a human-adapted pathogen, GAS expresses numerous surface-bound and secreted virulence factors that subvert innate immune defenses. Several GAS virulence factors have been reported to activate the NLRP3 inflammasome pathway and induce IL-1β production in macrophages, including the M surface protein, the ADP-ribosyltransferase SpyA and streptolysin O (SLO).[17–19] SLO is a 69-kDa cholesterol- and glycan-dependent cytolysin that plays a central role in the pathogenesis of GAS infections. SLO pores in host cell membranes are around 30 nm in diameter, causing rapid and nonselective ion fluxes and efflux of cytosolic proteins.[22] Because cytosolic K^+ efflux is a trigger for NLRP3 activation, SLO pores are sufficient for triggering inflammasome signaling. Recombinant SLO induced IL-1β secretion from mouse bone marrow-derived macrophages (BMDMs), and this was blocked by treatment with extracellular KCl.[22,23] The THP-1 monocytic leukemia cell line is another model frequently used to study inflammasome activation. THP-1 cells can be differentiated into a macrophage-like phenotype through treatment with phorbol 12-myristate 13-acetate (PMA).[24] This model of human macrophages has previously been used to study GAS-induced activation of the inflammasome pathway.[19]

In this study, we used both mouse BMDMs and THP-1 monocyte-derived macrophages to study inflammasome activation by GAS. We found that GAS-derived lipoteichoic acid (LTA) is a potent cell type-specific priming agent for the NLRP3 inflammasome in mouse BMDMs. A GAS isogenic mutant screen revealed that streptolysins [SLO and streptolysin S (SLS)] are the major determinants for GAS-induced inflammasome signaling in both cell types. Furthermore, we demonstrate that pore formation is essential for recombinant SLO to induce a strong IL-1β response and pyroptosis in THP-1 cells.

RESULTS

Lipoteichoic acid from *Streptococcus pyogenes* is a cell-specific priming agent

Experimental priming of the NLRP3 inflammasome is routinely achieved by TLR4 stimulation with lipopolysaccharide (LPS),[25] a major component of the outer membrane of Gram-negative bacteria. As a pattern recognition molecule, LTA provides a functional equivalent to LPS in Gram-positive bacteria that is detected by both TLR2[26] and Mincle.[21] GAS infection of mouse BMDMs was previously shown to be sufficient for inflammasome activation,[17] which suggests that GAS provides both priming and triggering stimuli for inflammasome signaling. We therefore tested whether LTA derived from GAS can induce an inflammasome priming effect similar to LPS. Mouse BMDMs derived from wild-type C57BL/6 mice were stimulated with either LPS, which served as positive control for priming, or LTA for 4 h. Cells were then infected with the GAS M1T1 isolate 5448 at a multiplicity of infection of 20 for 1 h. As a positive control for the NLRP3 activating signal, cells were treated with 5 μM nigericin, an ionophore and established canonical NLRP3 inflammasome activator.[27] In uninfected mouse BMDMs, the addition of either LPS or LTA as a priming signal was alone insufficient to induce an IL-1β response (Figure 1a). As expected, significantly elevated levels of IL-1β were

observed in cells infected with GAS 5448 following priming with LPS at 100 ng mL^{-1} (Figure 1b). We observed a dose-dependent IL-1β response with increasing concentrations of LTA. At a concentration of 1 μg mL^{-1} LTA, IL-1β levels were comparable to those in samples primed with 100 ng mL^{-1} LPS. A similar pattern was observed in nigericin-treated cells, where 10-fold more LTA was required to prime a significant IL-1β response compared with LPS (Figure 1c). Nigericin-stimulated mouse BMDMs secreted higher levels of IL-1β than GAS 5448-infected cells, underlining the potency of this ionophore in activating the NLRP3 inflammasome. To further assess priming of the NLRP3 inflammasome, we undertook immunoblot analysis of whole cell lysates of uninfected cells to determine pro-IL-1β and NLRP3 expression levels after stimulation with either 100 ng mL^{-1} LPS or 1 μg mL^{-1} LTA. Both compounds strongly induced pro-IL-1β expression to a similar degree (Figure 1d). Likewise, NLRP3 expression levels were increased in the presence of either priming agent, confirming that in mouse BMDMs both substances can serve as a priming signal for NLRP3 inflammasome activation.

Next, we examined whether LTA can also provide a priming signal for NLRP3 inflammasome activation in PMA-differentiated human THP-1 cells (Figure 2). Based on observations in mouse BMDMs (Figure 1), we used LTA at a concentration of 1 μg mL^{-1}. Similar to mouse BMDMs, the addition of either 100 ng mL^{-1} LPS or 1 μg mL^{-1} LTA alone was insufficient to induce IL-1β secretion in uninfected THP-1 cells (Figure 2a). LPS-primed THP-1 cells infected with GAS strain 5448 or challenged with nigericin released substantial IL-1β, as did mouse BMDMs (Figure 2b, c). However, 1 μg mL^{-1} LTA failed to promote IL-1β release in THP-1 cells infected with GAS 5448 or challenged with nigericin (Figure 2b, c), suggesting a cell-specific role for LTA as a priming agent. Of note, we detected substantial IL-1β release in unprimed THP-1 cells either infected with 5448 or treated with nigericin. This is in line with previous findings that THP-1 cell differentiation with PMA itself serves as a priming signal, resulting in the upregulation of pro-IL-1β expression.[19] Immunoblotting of whole-cell lysates of uninfected cells further confirmed this observation, showing that unprimed and LTA-treated THP-1 cells express pro-IL-1β in cell lysates following PMA differentiation (Figure 2d). In agreement with the ELISA data (Figure 2b, c), pro-IL-1β levels were strongly induced by LPS priming (Figure 2d). Interestingly, despite the induction of pro-IL-1β expression, priming with LPS had no significant effect on NLRP3 protein levels (Figure 2d). It is possible that PMA differentiation already induces a maximum level of NLRP3 expression that cannot be further increased by additional stimulation. Taken together, these experiments demonstrate that LTA derived from GAS is a cell-specific priming agent for NLRP3 inflammasome activation in mouse BMDMs, but provided no additional priming effect in PMA-differentiated human THP-1 cells.

GAS 5448 isogenic mutant screen reveals that SLO is the major determinant for GAS-induced inflammasome activation

Three secreted or surface-associated GAS virulence factors have been identified as triggers of NLRP3 inflammasome activation: the ADP-ribosyltransferase SpyA, the M

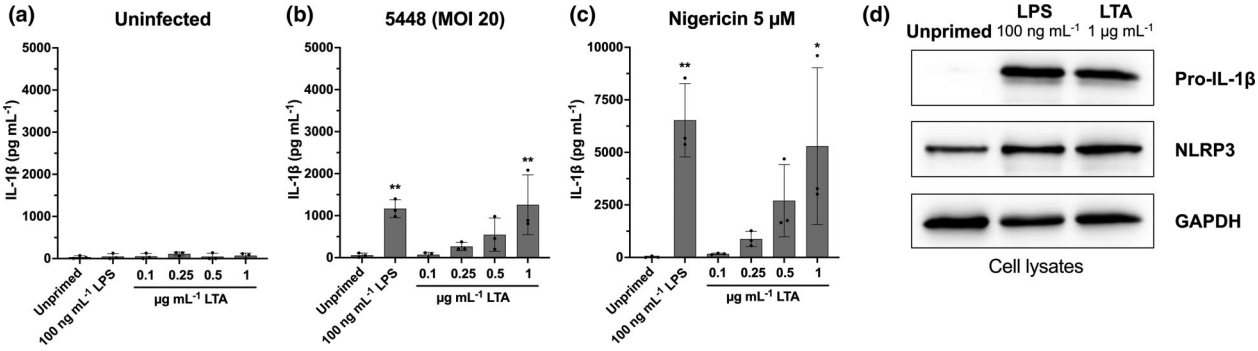

Figure 1. Lipopolysaccharide (LPS) and lipoteichoic acid (LTA) prime the NLR family pyrin domain-containing 3 (NLRP3) inflammasome for activation in mouse bone marrow-derived macrophages (BMDMs). Interleukin-1β (IL-1β) release of mouse BMDMs that were primed with either 100 μg mL^{-1} LPS or indicated concentrations of LTA for 4 h and then **(a)** cultured in assay medium only, **(b)** infected with GAS 5448 at a multiplicity of infection (MOI) of 20 or **(c)** treated with 5 μM nigericin for 1 h. Immunoblot detection of **(d)** pro-IL-1β and NLRP3 in whole-cell lysates was performed to compare the priming effect of 100 ng mL^{-1} LPS or 1 μg mL^{-1} LTA. ELISA data shown in **a**–**c** are plotted as the mean ± s.d. of three independent experiments performed in technical triplicates. Values were compared with the unprimed control of each group, and significance was analyzed by one-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAS, group A *Streptococcus*.

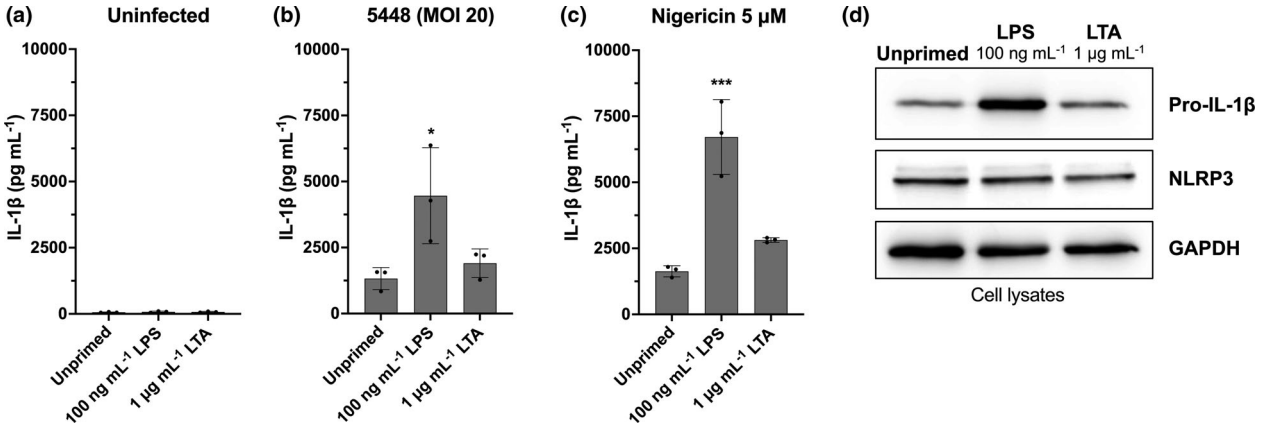

Figure 2. Lipoteichoic acid (LTA) does not enhance priming of the NLR family pyrin domain-containing 3 (NLRP3) inflammasome in differentiated THP-1 cells. Interleukin-1β (IL-1β) release of phorbol 12-myristate 13-acetate-differentiated THP-1 cells that were primed with either 100 ng mL^{-1} lipopolysaccharide or 1 μg mL^{-1} LTA for 4 h and then **(a)** cultured in assay medium only, **(b)** infected with GAS 5448 at a multiplicity of infection (MOI) of 20 or **(c)** treated with 5 μM nigericin for 2 h. Immunoblot detection of **(d)** pro-IL-1β and NLRP3 in whole-cell lysates was performed to compare the priming effect of 100 ng mL^{-1} LPS or 1 μg mL^{-1} LTA. ELISA data are plotted as the mean ± s.d. of three independent experiments. Values were compared with the unprimed control of each group, and significance was analyzed by one-way ANOVA. *$P < 0.05$ and ***$P < 0.001$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

surface protein and SLO.[17–19] In addition, the broad-spectrum GAS protease SpeB, produced abundantly in late logarithmic and stationary phases, amplifies inflammatory signaling by cleaving and activating pro-IL-1β in an inflammasome-independent manner.[28] To investigate additional GAS-derived proteins that may modulate the inflammasome pathway, we screened a panel of 17 isogenic GAS 5448 mutant strains (Table 1) for their ability to trigger inflammasome-induced IL-1β production in differentiated THP-1 cells, chosen to model human macrophages (Figure 3). Primed THP-1 cells were infected with GAS 5448 and isogenic mutant strains at a multiplicity of infection of 40. As expected, wild-type GAS 5448 stimulated substantial IL-1β secretion in primed THP-1 cells, reaching 67% of the nigericin control (Figure 3a). When compared with wild-type 5448, 3 of the 17 tested isogenic mutants had a significant reduction in IL-1β secretion. Infection with GAS 5448△*slo* reduced IL-1β levels by 56%, whereas cells infected with GAS 5448△*emm1* secreted 20% less IL-1β than the wild-type strain. These results validate previous studies that identified SLO and M1 protein as triggers of inflammasome activation in THP-1 cells.[19] IL-1β secretion was also significantly reduced by 20% in cells infected with GAS 5448△*sagA*, revealing an additional virulence determinant involved in triggering IL-1β secretion in THP-1 cells. The *sagA* gene encodes the second cytolysin produced by GAS (SLS),[29] which was previously shown to induce IL-1β secretion in human keratinocytes and in a murine *in vivo* model of subcutaneous infection.[30] For all the other isogenic mutants tested (14 out of 17), bacterial gene knockout did not affect IL-1β secretion in infected THP-1 cells. These included mutants deficient in the ADP-ribosyltransferase SpyA and the protease SpeB, which were previously implicated in the production of IL-1β in mouse BMDMs,[18,28] and the NAD^+-glycohydrolase NADase, which was reported to counteract inflammasome-dependent IL-1β secretion.[31,32]

Because inflammasome activity can induce pyroptosis, we also assessed the levels of cytotoxicity caused by the GAS 5448 wild-type and isogenic mutants (Figure 3b). The observed pattern of lactate dehydrogenase (LDH) release was consistent with that of IL-1β secretion, with wild-type GAS 5448 triggering 76% LDH release compared with the 100% cell lysis control. Significant reductions in LDH release were observed in cells infected with the GAS 5448△*slo* (29%), 5448△*emm1* (48%) and 5448△*sagA* (50%) mutants, confirming the involvement of these virulence factors in the induction of pyroptotic cell death.[17,19,33] A minor but significant increase in LDH release (88%) was detected in cells infected with the GAS 5448△*gloA* mutant, which is deficient in glyoxalase I that enables GAS to break down antimicrobial methylglyoxal produced by neutrophils and macrophages during infection.[34,35] However, because IL-1β secretion was not altered by this mutant (Figure 3a), this effect on LDH release appears to be inflammasome independent.

To validate the observations made in THP-1 cells, we compared GAS wild-type 5448 and 5448△*slo*, 5448△*sagA* and 5448△*emm1* mutants using mouse BMDMs. IL-1β and LDH levels were significantly reduced in wild-type

Table 1. Group A *Streptococcus* (GAS) strains and isogenic mutants used in this study

Mutant	Virulence determinant	Function	References
5448			48
△*sagA*	Streptolysin S	Cytolysin	56
△*slo*	Streptolysin O	Cytolysin	47
△*emm1*	M1 protein	Cell adhesion and inhibition of phagocytosis	57
△*sda1*	Streptodornase	DNase	58
△*mac*	CD11b homolog	Inhibition of opsonophagocytosis	59
△*ska*	Streptokinase	Fibrinolytic enzyme	60
△*hasB*	Hyaluronic acid capsule	Capsule biosynthesis	61
△*speB*	Cysteine protease	Broad-spectrum protease	62
△*pepO*	Endopeptidase	Complement inhibition and regulation of *speB* gene expression	63
△*spyA*	ADP-ribosyltransferase	Modulation of host cell proteins	18
△*cepA*	Cell envelope protease	CXC chemokine protease	64
△*gloA*	S-lactoylglutathione lyase (glyoxalase I)	Antimicrobial resistance	34
△*oatA*	Peptidoglycan-O-acetyltransferase	Peptidoglycan synthesis	this study
△*lsa*	Large surface-anchored protein	Epithelial cell interaction and anti-microbial resistance	65
△*fctA*	GAS pili	Cell adhesion	66
△*nga*	NAD-glycohydrolase	Manipulation of host cell metabolism	47
△*sic*	Streptococcal inhibitor of complement	Complement inhibition	67
HKU16			38
△*slo*	Streptolysin O	Cytolysin	49

BMDMs infected with GAS 5448△*slo* and 5448△*sagA*, similar to the data for THP-1 cells. However, there was no detectable difference in IL-1β and LDH release for 5448△*emm1*-infected cells (Supplementary figure 1). IL-1β and LDH release triggered by infection with GAS required NLRP3 under our experimental conditions (Supplementary figure 1b, d).

Inflammasome activation in *emm1* and *emm12* GAS

A recent report suggests that the ability of GAS to stimulate the inflammasome pathway is *emm* serotype specific.[19] While purified M1, M2, M4, M5 and M6 proteins were shown to stimulate inflammasome signaling in THP-1 cells, the cells were unresponsive to purified M28.[19] GAS *emm1* and *emm12* are among the most prevalent *emm* serotypes globally,[36] and have been linked to invasive disease and scarlet fever outbreaks.[37,38]

To investigate the contribution of SLO expression to inflammasome activation in *emm12* GAS, we used the scarlet fever isolate HKU16 as an *emm12* reference strain.[38] We infected PMA-differentiated and LPS-primed THP-1 cells with either GAS 5448 or HKU16, as well as their respective △*slo* mutants, at a multiplicity of infection of 40. Both GAS wild-type strains induced robust IL-1β secretion in infected THP-1 cells (Figure 4a). As expected, *slo* knockout significantly reduced IL-1β levels by approximately 50% in cells infected with either HKU16 or 5448 strains, emphasizing a conserved role for SLO in inflammasome activation in these *emm* serotypes. THP-1 cells infected with either HKU16△*slo* or 5448△*slo* showed a marked reduction of LDH release (Figure 4b), underscoring the critical role of SLO in GAS-induced inflammasome signaling. To confirm inflammasome activation by HKU16, immunoblotting analysis was performed to validate cleavage of critical components of the inflammasome pathway, including caspase-1, gasdermin D and pro-IL-1β. Cleaved and active forms of caspase-1 (caspase-1 p33), gasdermin D (gasdermin D p30) and mature IL-1β (IL-1β p17) were readily detectable in the supernatants of GAS 5448- and HKU16-infected THP-1 cells (Figure 4c). By contrast, these proteins were diminished or absent in the supernatants of cells infected with the respective △*slo* isogenic mutant strains, indicative of reduced inflammasome activity and cell lysis. We also noticed a strong abundance of uncleaved, inactive precursors of caspase-1, gasdermin D and pro-IL-1β in the supernatants of GAS 5448- and HKU16-infected cells, likely reflecting their release during cell lysis. Altogether, our data demonstrate that two of the most prevalent *emm* serotypes can induce a robust inflammasome response that is strongly dependent on the production of SLO.

Pore formation is required for SLO-induced inflammasome activation in THP-1 cells

To confirm the mechanism by which SLO induces inflammasome activation in THP-1 cells, we treated THP-1 cells with recombinant wild-type SLO protein, as well as a $SLO_{P427L+W535F}$ mutant protein which is

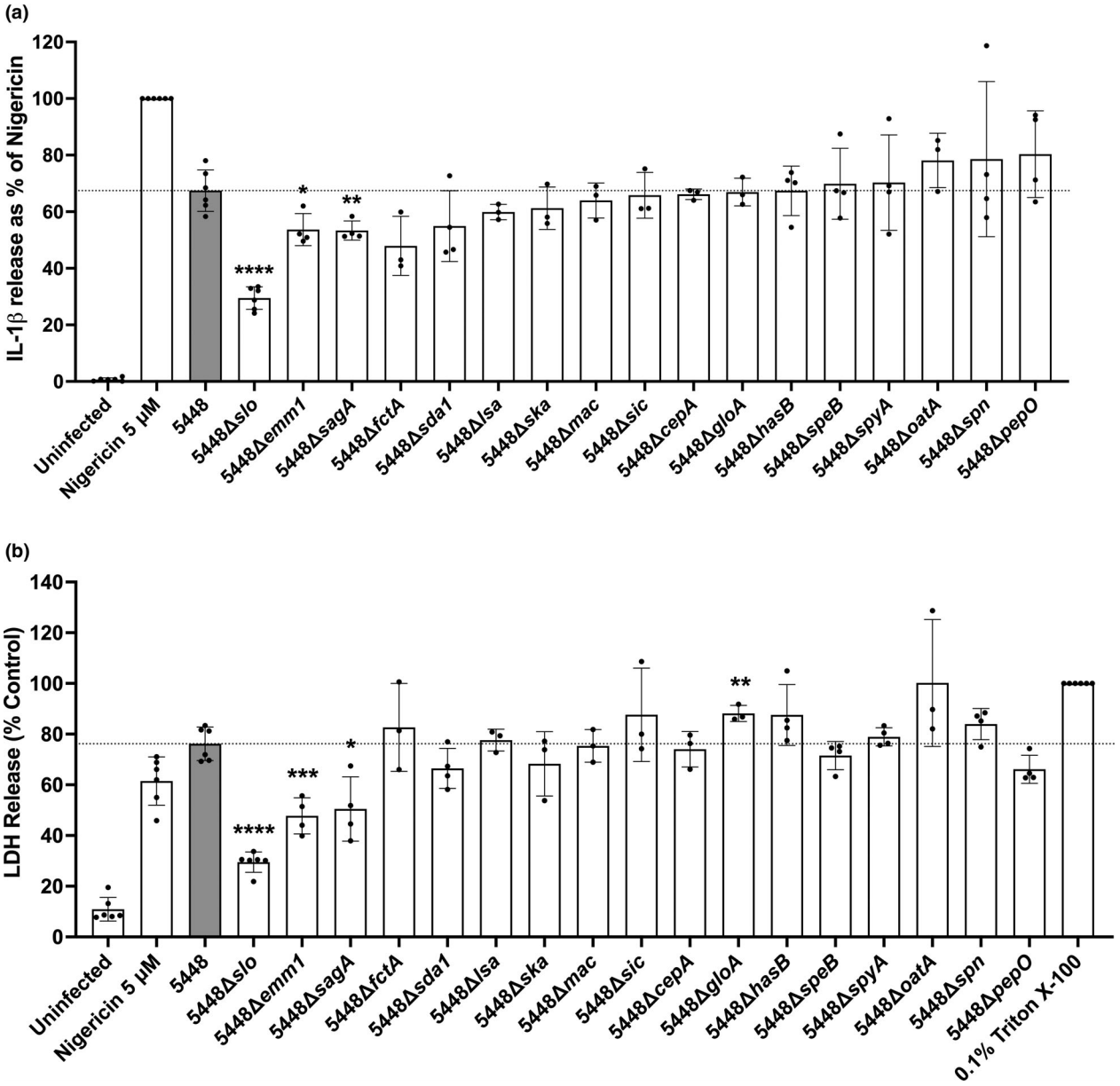

Figure 3. Group A *Streptococcus* (GAS) 5448 isogenic mutant screen in differentiated THP-1 cells. Cells were primed with 100 ng mL^{-1} lipopolysaccharide (LPS) for 4 h and then infected with either wild-type GAS 5448 or indicated isogenic mutant strains at a multiplicity of infection of 40. Uninfected THP-1 cells were used as a negative control. After 2 h, assay supernatants were collected and analyzed for **(a)** interleukin-1β (IL-1β) and **(b)** lactate dehydrogenase (LDH) release. Data are plotted as the mean ± s.d. of three or six independent experiments. Values of isogenic mutants were compared with the GAS 5448 control, and significance was analyzed by the unpaired Student's *t*-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$.

incapable of membrane pore formation.[39] Wild-type SLO significantly triggered IL-1β secretion in a concentration-dependent manner (Figure 5a). Wild-type SLO already induced a marked increase in IL-1β secretion at a concentration of 0.1 μg mL^{-1}; however, significant differences in IL-1β levels were detected at a concentration of 0.25 μg mL^{-1}, reaching a plateau at 0.5 μg mL^{-1}. A further increase in SLO concentration up to 4 μg mL^{-1} had no additive effect on IL-1β secretion from THP-1 cells. At the highest concentration tested (4 μg mL^{-1}), the $SLO_{P427L+W535F}$ mutant protein failed to induce IL-1β secretion, confirming that SLO pore formation is required for this protein to stimulate inflammasome activation.[22,23]

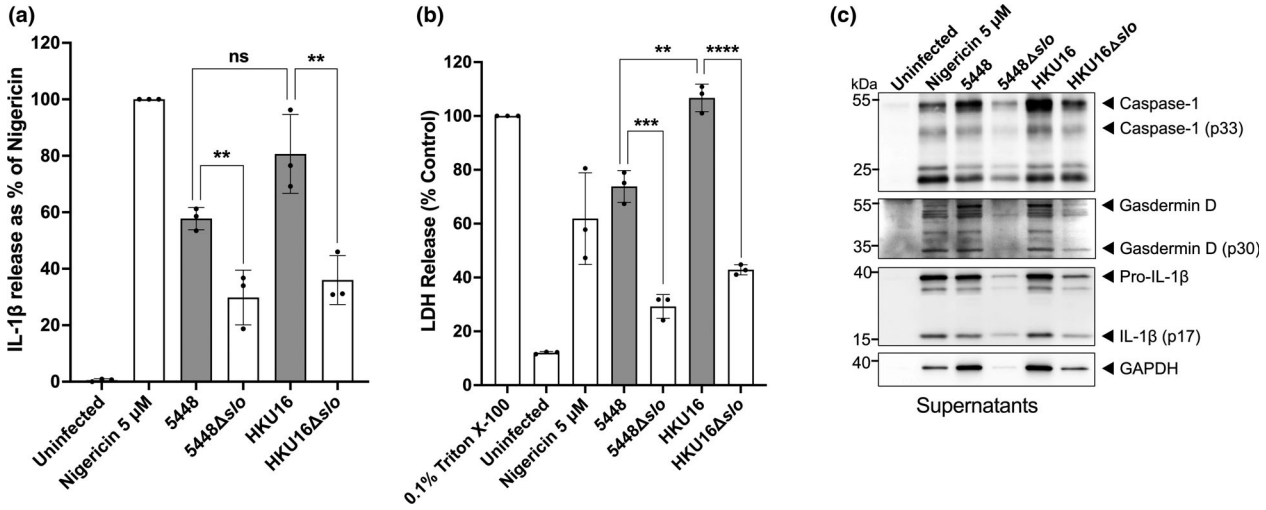

Figure 4. Streptolysin O (SLO) drives inflammasome activation in *emm1* and *emm12* group A *Streptococcus* (GAS). Differentiated THP-1 cells were primed with 100 ng mL^{-1} lipopolysaccharide for 4 h and then infected with either GAS 5448, HKU16 or their respective △*slo* mutants at a multiplicity of infection of 40. After 2 h, samples from the assay supernatants were collected and analyzed for **(a)** interleukin-1β (IL-1β) and **(b)** lactate dehydrogenase (LDH) release. The remaining supernatants were prepared for **(c)** immunoblot detection of caspase-1, gasdermin D and IL-1β. Note: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) does not act as loading control, but indicates cytotoxicity. Data are plotted as the mean ± s.d. of three independent experiments. Groups were compared as indicated. Significance was analyzed by the unpaired Student's *t*-test. $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

Next, we analyzed LDH release from SLO-treated THP-1 cells as an indicator of pyroptosis. As with IL-1β secretion, LDH release was significantly elevated at 0.25 μg mL^{-1} of wild-type SLO (Figure 5b). However, LDH concentrations in culture supernatants gradually increased further in a SLO dose-dependent manner, reaching 100% of the lysis control at 4 μg mL^{-1} (Figure 5b). At the same concentration, the $SLO_{P427L+W535F}$ mutant protein again failed to trigger LDH release. To further elucidate how different concentrations of SLO affect inflammasome signaling, we performed immunoblotting analysis to assess cleavage of the inflammasome pathway components caspase-1 (p33), gasdermin D (p30) and IL-1β (p17; Figure 5c). The amount of mature gasdermin D and IL-1β increased in the supernatants of SLO-treated THP-1 cells up to a concentration of 0.5 μg mL^{-1} SLO. Higher SLO concentrations had an inverse effect on inflammasome signaling with decreasing band intensities of the cleaved caspase-1 substrates, which were almost absent at 4 μg mL^{-1} SLO. This suggests that THP-1 cells were lysed rapidly in the presence of high concentrations of SLO, preventing efficient inflammasome signaling. This hypothesis is supported by the detection of an increasing abundance of uncleaved, inactive precursors of inflammasome components as well as the control protein glyceraldehyde-3-phosphate dehydrogenase in the assay supernatants with rising SLO concentrations resulting from increased SLO cytotoxicity. As expected, cleavage of inflammasome components was undetectable in samples treated with the $SLO_{P427L+W535F}$ mutant protein.

DISCUSSION

Inflammasome activation and IL-1β production are critical processes of the host innate immune defense against invading pathogens, such as GAS. IL-1β signaling ensures efficient recruitment of innate immune cells, such as macrophages and neutrophils, to the site of infection, which is essential for GAS clearance *in vivo*.[9] Inhibition of IL-1β signaling can have dramatic consequences; while mice deficient in the IL-1β receptor IL-1R were more susceptible to severe GAS infection,[8] human patients with rheumatoid arthritis treated with the IL-1R blocker anakinra experienced invasive GAS disease at an alarming ~330-fold increased rate.[28] However, high IL-1β production can also be a risk factor for increased disease severity and mortality during invasive GAS infection.[10–12] In host cells, the production and secretion of IL-1β are controlled by the inflammasome pathway. Previous studies have established the importance of the NLRP3 inflammasome during infection with GAS.[17–19] Using a combination of isogenic mutant strains and purified proteins, these studies revealed that the GAS virulence factors SLO,[17] SpyA[18] and M protein[19] induce IL-1β secretion from macrophages in an NLRP3- and caspase-

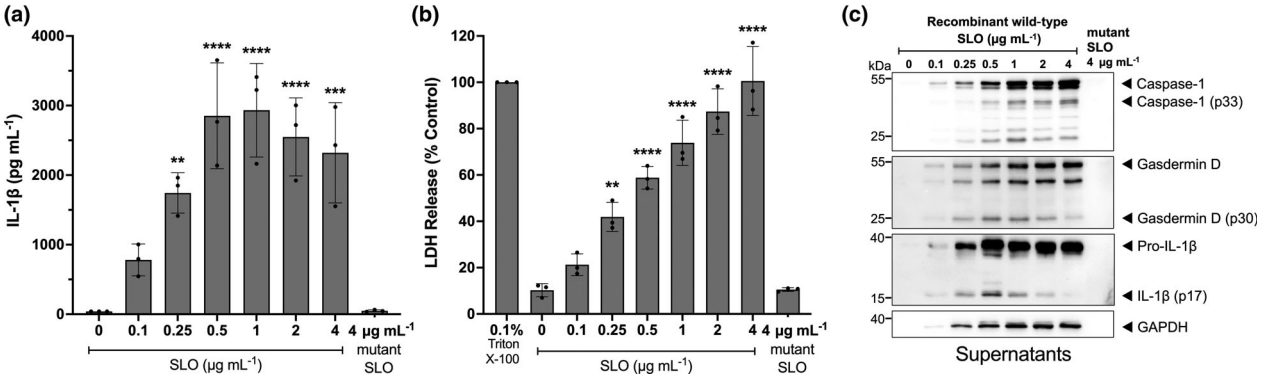

Figure 5. Pore-forming activity of recombinant streptolysin O (SLO) is required to trigger inflammasome signaling in differentiated THP-1 cells. Cells were primed with 100 ng mL^{-1} lipopolysaccharide for 4 h before being treated with increasing concentrations of recombinant wild-type SLO or 4 µg mL^{-1} mutant SLO$_{P427L+W535F}$ protein. After 1 h, assay supernatants were collected and analyzed for **(a)** interleukin-1β (IL-1β) and **(b)** lactate dehydrogenase (LDH) release. The remaining supernatants were prepared for **(c)** immunoblot detection of caspase-1, gasdermin D and IL-1β. Note: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) does not act as loading control, but indicates cytotoxicity. Data are plotted as the mean ± s.d. of $n = 3$ independent experiments. Values were compared with the untreated control of each group, and significance was analyzed by one-way ANOVA. $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

1-dependent manner. In particular, both SLO and M1 protein triggered the cytosolic efflux of K^+,[19,22,23] an important upstream trigger for NLRP3 inflammasome activation. Although these findings provide insight into the modulation of inflammasome signaling by secreted toxins, less is known about GAS–inflammasome interactions. Here, we sought to further characterize the interaction of GAS with the inflammasome pathway in mouse and human macrophages.

The NLRP3-dependent production of IL-1β typically requires a priming signal that induces the NF-κB-dependent upregulation of pro-IL-1β. Priming is frequently accomplished *in vitro* using LPS as a microbial TLR4 ligand. Here, we report that the TLR2/Mincle ligand LTA derived from GAS is an effective priming signal with physiological relevance for NLRP3 signaling in mouse BMDMs. By contrast, differentiated human THP-1 cells were efficiently primed by the addition of LPS, but not LTA. PMA-differentiated THP-1 cells predominantly respond to TLR4 stimulation,[40] possibly because they downregulate TLR2 expression during their differentiation into macrophages,[41,42] which may explain this cell-specific role of LTA as a priming agent. Differentiated THP-1 cells may therefore not be a suitable model to study the effectiveness of LTA as a priming agent of human innate immune cells.

To identify additional virulence factors which may contribute to IL-1β signaling during GAS infection, we screened a panel of isogenic GAS mutants for their potential to trigger IL-1β secretion and pyroptosis in THP-1 macrophages and mouse BMDMs. Our results confirmed the importance of SLO and the M1 protein for inflammasome activation, and demonstrated that the majority of GAS-induced IL-1β and LDH release in mouse BMDMs is NLRP3 dependent, validating findings from previous studies.[17,19] The cytolysin SLS, which is hypothesized to assert its lytic activity through interaction with specific cell surface proteins,[43] has been reported to induce IL-1β secretion in human keratinocytes.[30] Our finding that SLS also triggers IL-1β signaling in mouse and human macrophages supports the view that the functional disruption of host cell membranes represents a key mechanism of inflammasome activation. SLS is a 2.7-kDa oxygen-stable cyclic peptide with hemolytic activity that is structurally unrelated to the oxygen-sensitive SLO, but that can also induce an ionic imbalance in target cells.[43,44] In addition, SLS triggers endoplasmic reticulum stress and consequently activates the unfolded protein response pathway,[45] which has been linked to NLRP3 signaling[46]; however, the precise mechanism of SLS-induced inflammasome activation is yet to be clarified.

Our findings also suggest that SLO is the main driver of GAS-induced IL-1β signaling, and that SLO is equally critical for inflammasome activation in *emm1* and *emm12* GAS. Similar results were previously reported for GAS *emm3*, where deletion of *slo* dampened IL-1β secretion.[17] Combined, these data suggest a conserved and critical role for SLO in GAS-induced inflammasome signaling among different GAS *emm* serotypes. Further investigation of the mechanism by which SLO activates the inflammasome pathway revealed that the pore-forming activity of recombinant SLO was both required and sufficient to trigger IL-1β secretion in human THP-1 cells, which validates observations made in mouse

BMDMs.[22,23] The potent ability of SLO to activate the NLRP3 inflammasome was previously shown to be dependent on its induction of cytosolic K^+ efflux.[22,23]

In conclusion, this work provides evidence that streptolysins are key contributors to GAS-induced inflammasome activation in macrophages. Given that inflammasome-dependent IL-1β signaling facilitates inflammation observed in invasive GAS infections,[10–12] the cytolytic properties of streptolysins constitute a potential target for therapeutic intervention.

METHODS

Reagents

Ultrapure LPS from *E. coli* K12 was purchased from InvivoGen (Catalog number tlrl-peklps; San Diego, CA, USA). LTA from *S. pyogenes* and nigericin were purchased from Sigma-Aldrich (Catalog number L3140-5MG; St. Louis, MO, USA). Recombinant SLO and the mutant $SLO_{P427L+W535F}$ protein were produced as previously described.[39,47]

Bacterial strains and dose preparation for infection of cells

All GAS strains and isogenic mutants used in this study are listed in Table 1. The GAS M1T1 strain 5448[48] and GAS M12 strain HKU16[38] were routinely grown at 37°C on 5% horse blood agar (Biomérieux, Sydney, NSW, Australia). For preparation of the infection dose, bacterial cultures were grown statically in a chemically defined medium to the mid-logarithmic growth phase at OD_{600} = 0.3.[49] The cultures were collected by centrifugation for 5 min at 8000*g* and washed in Dulbecco's phosphate-buffered saline (Thermo Fisher Scientific, Waltham, MA, USA). Bacterial cell pellets were then resuspended in assay medium and diluted to the indicated multiplicity of infection.

Group A *Streptococcus* 5448△*oatA* mutant construction

The GAS 5448△*oatA*::*aad9* mutant strain was constructed using a modified version of the protocol described previously.[50] All PCR primer sequences are provided in Supplementary table 1. First, DNA fragments flanking *oatA* (SP5448_RS00280 in NCBI Accession number NZ_CP008776) from 5448 were amplified by PCR using primers oatA-1202S and aad9-oatA5-A (upstream fragment), and aad9-oatA3-S and oatA+3044A (downstream fragment). The promoter-less *aad9* spectinomycin-resistance gene from pUCSpec[51] was amplified using primers oatA-aad9+1S and oatA-aad9+750A. The purified PCR products were used as templates and fused together by virtue of their complementary sequences in a second-round PCR, using primers oatA-1165S and oatA+3000A. The resulting PCR product was gel purified and cloned into pCR-Blunt II-TOPO (Thermo Fisher Scientific). Approximately 100 μg of the resulting plasmid was linearized by digestion with *Sma*I and electroporated into GAS strain 5448 followed by selection for spectinomycin-resistant colonies. Allelic replacement of *oatA* with *aad9* was confirmed by PCR.

Isolation and differentiation of murine bone marrow-derived macrophages

All mice were housed in a specific pathogen-free facility at the Australian Institute for Bioengineering and Nanotechnology (The University of Queensland, Australia). NLRP3-knockout mice were generated on a C57BL/6 background.[52] Both male and female C57BL/6 and NLRP3-deficient mice were used for experiments at 7–14 weeks of age. Mouse bone marrow cells were isolated as previously described.[53] Briefly, mice were killed using a CO_2 chamber according to the "humane killing of mice and rats" guidelines by the National Health and Medical Research Council of Australia, and femurs and tibias from each mouse were collected immediately. Using a 27-gauge needle, the bone marrow was flushed from the bone cavities through a cell strainer with complete BMDM medium, which was composed of Roswell Park Memorial Institute-1640 supplemented with 10% low-endotoxin fetal bovine serum and 1% GlutaMAX (all purchased from Thermo Fisher Scientific). Isolated bone marrow cells were frozen in liquid nitrogen before being prepared for macrophage differentiation. Typically, one-third of the isolated bone marrow cells were cultured in two square cell culture dishes (100 cm^2; Thermo Fisher Scientific) in 12 mL culture medium, which was complete BMDM medium supplemented with 150 ng mL^{-1} recombinant human macrophage colony-stimulating factor 1 (Protein Expression Facility, The University of Queensland), for 5 days at 37°C and 5% CO_2. On day 5, 5 mL of fresh culturing medium was added to each plate, and on day 6, cells were harvested with Dulbecco's phosphate-buffered saline (Thermo Fisher Scientific). Cell numbers were quantified using a Countess automated cell counter (Thermo Fisher Scientific), before seeding 100 000 viable cells in 100 μL culturing media per well in 96-well plates.

THP-1 cell culture and differentiation

A variant of the human THP-1 acute monocytic leukemia cell line with integrated Cas9[54] was maintained in Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum (Bovogen, Melbourne, VIC, Australia), 10 mM HEPES (Sigma-Aldrich) and 1 mM sodium pyruvate (Thermo Fisher Scientific). For all experiments, THP-1 cells were seeded at 1×10^6 cells mL^{-1} in 12-well cell culture plates and then differentiated for 24 h by addition of 25 nM phorbol myristate acetate (PMA, Sigma-Aldrich) to the culture medium.

Inflammasome activation assay

For mouse BMDM experiments, the assay medium was composed of serum-free Opti-MEM (Thermo Fisher

Scientific) supplemented with 150 ng mL^{-1} recombinant human macrophage colony-stimulating factor 1 (Protein Expression Facility, University of Queensland). For THP-1 experiments, Roswell Park Memorial Institute-1640 supplemented with 2% fetal bovine serum (Bovogen) was used as assay medium, with the exception of experiments in which supernatant protein was extracted for immunoblotting, where the THP-1 assay medium was replaced with Opti-MEM.

To investigate inflammasome signaling, differentiated BMDMs or THP-1 cells were primed with either 100 ng mL^{-1} LPS or indicated concentrations of LTA at 37°C. For unprimed cells, the differentiation medium was replaced with fresh medium instead. After 4 h of priming, the cell media were removed and replaced with the indicated treatments or infection doses diluted in assay medium. The NLRP3-activating ionophore nigericin, at a final concentration of 5 μM, served as a positive control. As a negative control, cells were treated with assay medium only. Cell culture plates were centrifuged for 10 min at 300*g* to bring bacteria into contact with the cells, before being placed back into the incubator for 1 h (BMDMs) or 2 h (THP-1 cells). Following the assay, plates were centrifuged for 5 min at 300*g*. Finally, supernatants were separated from the cells and frozen at −20°C before processing. Cells were lysed in a Tris–sodium dodecyl sulfate buffer and stored at −20°C before being prepared for immunoblotting.

Lactate dehydrogenase assay and IL-1β ELISA

LDH is a cytosolic enzyme that is released into the culture supernatant during cellular lysis or loss of cell membrane integrity, and can therefore act as an indirect measure of cellular toxicity.[55] As a control for complete cell lysis, THP-1 cells were fully lysed with 0.1% Triton X-100. Of note, while LDH release is commonly presented as an indicator of pyroptosis, it may also occur through inflammasome-independent mechanisms, such as toxin-induced cell membrane damage. Following the inflammasome activation assay, frozen assay supernatants were thawed at room temperature and analyzed with the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) according to manufacturer's directions. LDH release was calculated as percentage of a 100% cell lysis control that was treated with 0.1% Triton-X 100 (Sigma-Aldrich) for 20 min at 37°C. Assay supernatants were also analyzed for human or murine IL-1β using the respective DuoSet IL-1β ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Sample absorbance at 450 nm was measured using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Sample readings were analyzed with Prism 9 (GraphPad Software, San Diego, CA, USA) software, and IL-1β concentrations were determined by sigmoidal nonlinear regression of samples against a standard curve. Because of considerable variability between experiments in absolute IL-1β concentrations, values in Figures 3a, 4a, 5a and Supplementary figure 1a and b are presented as percentages of the nigericin control of each individual experiment.

Immunoblotting

Protein extraction from infection assay supernatants was performed as previously described.[53] Briefly, frozen infection assay supernatants were removed from the −20°C freezer and left to thaw at room temperature. Cellular debris was then removed by centrifugation at 16 000*g* for 2 min. An equal volume of methanol and one-third volume of chloroform were added before mixing thoroughly by vortexing at high speed for 5 s. Centrifugation at 16 000*g* for 10 min led to phase separation; the upper aqueous layer was removed and discarded, leaving the middle protein layer and bottom chloroform layer behind. Next, an amount of methanol equating to 1.6 times the initial assay supernatant volume was added to all samples, which were again mixed by vortexing for 5 s. Total protein was pelleted by centrifugation at 16 000*g* for 10 min. The remaining supernatant was removed completely, and protein pellets were left to dry at room temperature.

Finally, protein pellets from assay supernatants or whole cell lysates were resuspended in a sample buffer containing lithium dodecyl sulfate (NuPAGE LDS buffer; Thermo Fisher Scientific) and 50 mM dithiothreitol. Samples were boiled at 99°C for 5 min before being loaded onto 12% acrylamide gels. Proteins were separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (120 V, ~1.5 h) and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) using a wet transfer system (100 V, 1 h). Membranes were blocked in 5% milk in Tris-buffered saline + 0.1% Tween-20 for 1 h at room temperature and then incubated overnight at 4°C with the indicated primary antibodies (Table 2) diluted in 2.5% milk in Tris-buffered

Table 2. Antibodies used in this study

Target	Specificity	Manufacturer (location) and catalog number
NLR family pyrin domain-containing 3	Mouse/Human	Adipogen (San Diego, CA, USA; AG-20B-0014-C100)
Caspase-1	Mouse	Adipogen (San Diego, CA, USA; AG-20B-0042-C100)
Caspase-1	Human	Abcam (Cambridge, United Kingdom; ab207802)
IL-1β	Mouse/Human	R&D Systems (Minneapolis, MN, USA; AF-401-SP)
Gasdermin D	Mouse	Abcam (Cambridge, United Kingdom; ab209845)
Gasdermin D	Human	CUSABIO (Houston, TX, USA; CSB-PA009956LA01HU), Sigma-Aldrich (St. Louis, MO, USA; 126-138)
Glyceraldehyde-3-phosphate dehydrogenase	Mouse/Human	Abcam (Cambridge, United Kingdom; ab181602)

[a]Human-specific gasdermin D antibodies were used based on availability.

saline + 0.1% Tween-20. Membranes were washed with Tris-buffered saline + 0.1% Tween-20 prior to incubation with appropriate horseradish peroxidase-conjugated secondary antibodies for 70 min at room temperature. Following final membrane washes in Tris-buffered saline + 0.1% Tween-20, protein bands were revealed using the Pico or Femto enhanced chemiluminescence reagents (Thermo Fisher Scientific) according to kit instructions. Finally, membranes were imaged using the Fusion Solo S chemiluminescence imager (Vilber, Collégien, France).

Ethics statement

All experimental protocols were approved by the Ethics Committee of The University of Queensland (IMB/324/17).

Statistical analysis

All statistical analyses were carried out using GraphPad Prism version 9. Data shown were collected from at least three independent experiments and were represented as mean ± s.d. unless otherwise indicated. Results were either analyzed by the unpaired Student's *t*-test or by one-way ANOVA. *P*-values < 0.05 were considered statistically significant.

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CONFLICT OF INTEREST

Kate Schroder is a co-inventor on patent applications for NLRP3 inhibitors which have been licensed to Inflazome Ltd, a company headquartered in Dublin, Ireland. Inflazome is developing drugs that target the NLRP3 inflammasome to address unmet clinical needs in inflammatory disease. Kate Schroder served on the Scientific Advisory Board of Inflazome in 2016–2017, and serves as a consultant to Quench Bio, USA and Novartis, Switzerland. No other conflicts of interest are reported.

AUTHOR CONTRIBUTIONS

Johanna Richter: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Visualization; Writing-original draft; Writing-review & editing. **Mercedes M Monteleone:** Methodology; Resources; Supervision. **Amanda J Cork:** Methodology; Resources. **Timothy C Barnett:** Methodology; Resources; Writing-review & editing. **Victor Nizet:** Conceptualization; Resources; Writing-review & editing. **Stephan Brouwer:** Conceptualization; Formal analysis; Methodology; Resources; Supervision; Writing-original draft; Writing-review & editing. **Kate Schroder:** Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing-review & editing. **Mark J Walker:** Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing-review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.