Pannexin-1 promotes NLRP3 activation during apoptosis but is dispensable for canonical or non-canonical inflammasome activation

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Abstract

Inflammasomes are multimeric protein complex that assemble in the cytosol upon microbial infection or cellular stress. Upon activation, inflammasomes drive the maturation of proinflammatory cytokines interleukin (IL)-1β and IL-18, and also activate the pore-forming protein gasdermin D to initiate a form of lytic cell death known as 'pyroptosis'. Pannexin-1 is channel-forming glycoprotein that promotes membrane permeability and ATP release during apoptosis; and was implicated in canonical NLRP3 or non-canonical inflammasome activation. Here, by utilising three different pannexin-1 channel inhibitors and two lines of *Panx1*$^{-/-}$ macrophages, we provide genetic and pharmacological evidence that pannexin-1 is dispensable for canonical or non-canonical inflammasome activation. Instead, we demonstrate that pannexin-1 cleavage and resulting channel activity during apoptosis promotes NLRP3 inflammasome activation.

Introduction

The innate immune system provides the first line of defence against microbial pathogens but also drives inflammatory diseases. Inflammasomes, multimeric protein complexes that assemble in the cytosol in response to infection or cellular stress, are now emerging as key molecular drivers of both processes [1]. The NLRP3 inflammasome comprises the NLRP3 sensor protein, the adaptor protein ASC and the protease caspase-1. NLRP3 is an unusual sensor protein because it has a unique ability to sense a wide variety of structurally-unrelated molecules ranging from whole pathogens, bacterial toxins, ATP released from damaged cells, particulate crystals or misfolded protein aggregates such as α-synuclein generated during neurodegenerative disease [2]. The precise mechanism by which NLRP3 senses these structurally diverse agonists remains unclear, however, it appears that the vast majority of NLRP3 agonists elicit a common potassium efflux stress pathway that is sensed by NLRP3 [3]. Upon activation, NLRP3 oligomerises and recruits the adaptor protein ASC into large filamentous structure called the 'ASC speck', which creates a multitude of binding sites for the caspase-1 zymogen [4, 5]. Caspase-1 recruited within the inflammasome undergoes proximity-induced activation and autoprocessing to generate an active p33/p10 fragment, and is further processed to an inactive p20/p10 fragment that is released from the inflammasome [6]. Active caspase-1 cleaves the pore-forming protein gasdermin D (GSDMD) to induce a form of lytic cell death known as pyroptosis [7, 8]. In parallel, caspase-1 cleaves interleukin (IL)-1 family members such as pro-IL-1β and triggers IL-1β relocation to the plasma membrane to enable GSDMD-dependent and independent secretion [9-11].

The non-canonical inflammasome pathway is activated by cytoplasmic lipopolysaccharide (LPS) from Gram-negative bacteria [12]. Unexpectedly, LPS sensing by the non-canonical inflammasome does not require a classical pattern recognition receptor. Instead, cytoplasmic LPS appears to directly bind the caspase recruitment domain (CARD) of caspase-11, triggering caspase-11 oligomerization and activation [13]. In macrophages, active caspase-11 cleaves

its substrate, GSDMD to trigger pyroptosis [7, 8], while in neutrophils, active GSDMD promotes plasma and nuclear membrane damage and the extrusion of neutrophil extracellular traps [14]. Of note, caspase-11 is unable to directly process pro-IL-1β, but does so indirectly by triggering GSDMD pores, potassium efflux and NLRP3 inflammasome activation in a cell-intrinsic manner [7, 8, 12, 15].

Pannexin-1 is a channel-forming transmembrane protein with that is expressed in most cell types, including macrophages. Under resting state, the pannexin-1 channel is autoinhibited by its cytoplasmic C-terminal tail. During apoptosis, effector caspase-3 and -7 cleave pannexin-1 at its C-terminus to promote pannexin-1 channel opening and drive membrane permeability [16, 17]. In agreement with this model, we recently demonstrate that pannexin-1 channels promote NLRP3 inflammasome assembly during apoptosis [18]. Interestingly, exposure of macrophages to pannexin-1 inhibitor carbenoxolone or the pannexin-1 inhibitory peptide ^{10}PANX were reported to suppress canonical NLRP3 inflammasome activation [19-21]. More recently, pannexin-1 channel was also implicated to promote pyroptosis and NLRP3 activation following the assembly of the non-canonical inflammasome [22]. However, this finding is at odds with the observation that caspase-11 drives NLRP3 inflammasome activation through GSDMD pores [7, 8]. Whether pannexin-1 is required for optimal GSDMD processing and NLRP3 inflammasome activation after cytoplasmic LPS recognition is unclear and a focus of this study. Here, by using two independent lines of *Panx1*-deficient cells, we provide genetic evidence that pannexin-1 is only required for NLRP3 assembly during apoptosis but is dispensable for canonical NLRP3 or non-canonical inflammasome activation.

Results and discussion

Pannexin-1 channel activity is required for NLRP3 inflammasome activation during apoptosis

The requirement for pannexin-1 in canonical and non-canonical inflammasome activation is controversial [19-21, 23]. To examine the function of pannexin-1 in inflammasome activation, we first tested probenecid, trovafloxacin and spironolactone, three well-established pharmacological inhibitors of pannexin-1 channel activity [24-26]. We recently demonstrated that pannexin-1 promotes potassium efflux and NLRP3 inflammasome activation during intrinsic and extrinsic apoptosis in macrophages [18]. In agreement with that, we observed robust processing of full length pannexin-1 into the cleaved p19 fragment when bone marrow-derived macrophages (BMDMs) were exposed to the BH3-mimetic ABT-737 and MCL1 inhibitor S63845 to induce intrinsic apoptosis **(Fig. 1A)**. Probenecid, trovafloxacin and spironolactone had little to no effect on pannexin-1 cleavage, but completely abrogated caspase-1 processing in apoptotic BMDMs, indicating that pannexin-1 channel activity and resultant cellular permeability drives NLRP3 inflammasome assembly during apoptosis **(Fig. 1A)**. Next, we investigated whether pannexin-1 inhibitors similarly block IL-1β secretion during intrinsic apoptosis. For this, we primed BMDMs with LPS for 3 h to induce pro-IL-1β expression, and stimulated the cells for another 16 h with the ABT-737 and S63845 for to induce intrinsic apoptosis in the presence of absence of pannexin-1 inhibitors. Pannexin-1 inhibitors had little to no effect on macrophage cytotoxicity **(Fig. 1B)**. Consistent with our recent finding [18], we observed that IL-1β secretion was significantly reduced in apoptotic *Panx1*$^{-/-}$ macrophages compared to WT cells. In agreement with this, the pannexin-1 inhibitor, spironolactone similarly reduced IL-1β secretion in apoptotic wild type macrophages **(Fig. 1C)**. Of note, spironolactone also further reduced IL-1β secretion in *Panx1*$^{-/-}$ BMDMs, indicating that spironolactone additionally inhibits IL-1β secretion through pannexin-1-independent mechanisms. Surprisingly, while probenecid and trovafloxacin blocked caspase-1 processing in unprimed apoptotic macrophages over a 5 h period **(Fig. 1A)**, they were ineffective in blocking IL-1β secretion in LPS-primed apoptotic macrophages over 16 h **(Fig. 1C).** Next, we investigated if pannexin-1 channel activity similarly promotes NLRP3 activation during extrinsic apoptosis. Indeed, probenecid, trovafloxacin and spironolactone similarly reduced caspase-1 cleavage but not pannexin-1 processing when BMDMs were treated with TNF

and the SMAC-mimetic AZD5582 to trigger extrinsic apoptosis **(Fig. 1D)**. Taken together, these data indicate that during apoptosis, pannexin-1 channel opening promotes NLRP3 inflammasome activation, most likely by triggering membrane permeability.

Pannexin-1 is not required for canonical NLRP3 inflammasome activation

Having established that pannexin-1 channel inhibitors suppress NLRP3 activation during apoptosis **(Fig. 1A, D)**, we next investigated the function of pannexin-1 in canonical NLRP3 inflammasome signalling. For this, we primed wild type (WT) or *Panx1*$^{-/-}$ BMDMs with LPS for 4 h to induce NLRP3 and pro-IL-1β expression, and stimulated the cells with the soluble NLRP3 agonists nigericin and ATP, or the insoluble particulate agonist monosodium urate (MSU). In contrast to apoptotic triggers **(Fig. 1A, D)**, probenecid, trovafloxacin and spironolactone had no effect on caspase-1 processing in nigericin-stimulated macrophages **(Fig. 2A)**. In agreement with that, cleavage of the caspase-1 substrate GSDMD was unaffected by pannexin-1 inhibitors, although a minor reduction in pro-IL-1β processing was consistently observed **(Fig. 2A)**. Importantly, caspase-1 processing and cleavage of the caspase-1 substrates, pro-IL-1β and GSDMD were comparable between WT and *Panx1*$^{-/-}$ BMDMs **(Fig. 2A, Supplementary Fig. 1A)**. IL-1β secretion and macrophage pyroptosis, as measured by lactate dehydrogenase (LDH) release, were similar between WT and *Panx1*$^{-/-}$ BMDMs **(Fig. 2B-C, Supplementary Fig. 1B)**. In contrast, nigericin-induced IL-1β secretion and pyroptosis were completely dependent on GSDMD **(Fig. 2D-E)**, consistent with previous reports (ref). Next, we investigated whether pannexin-1 is required for ATP-induced NLRP3 inflammasome activation. Consistent with before, we observed comparable levels of caspase-1, pro-IL-1β and GSDMD processing between WT and *Panx1*$^{-/-}$ BMDMs upon ATP stimulation **(Fig. 2D, Supplementary Fig. 1A)**. As expected, IL-1β secretion and pyroptosis were similar between ATP-stimulated WT and *Panx1*$^{-/-}$ BMDMs, but completely abrogated in *Gsdmd*$^{-/-}$ cells **(Fig. 2G-J, Supplementary Fig. 1B)**. Interestingly, probenecid but not trovafloxacin or spironolactone reduced caspase-1 processing and cleavage of caspase-1

substrates in both WT and *Panx1*$^{-/-}$ BMDM, indicating that probenecid inhibits ATP-induced inflammasome activation in a pannexin-1-independent manner. In support of this, a recent study revealed that probenecid directly blocks the ATP-gated ion channel P2X7 [27]. Next, we investigated whether pannexin-1 is required for NLRP3 inflammasome upon exposure to the particulate agonist, MSU. In agreement with our findings with soluble NLRP3 agonists, we found that pharmacological or genetic blockade of pannexin-1 had no impact on caspase-1, pro-IL-1β and GSDMD processing in BMDMs following MSU stimulation **(Fig. 2K).** In line with that, IL-1β secretion and pyroptosis were similarly unaffected by *Panx1*-deficiency **(Fig. 2L-M)**. Consistent with a recent report [28], we observed that MSU-induced cell lysis and IL-1β secretion occurs independently of the pyroptotic effector GSDMD **(Fig. 2N-O)**. Lastly, we examined pannexin-1 cleavage following canonical NLRP3 inflammasome activation. In line with our earlier observations **(Fig. 2A-C, F-H, K-M)**, we found no evidence of pannexin-1 cleavage during canonical inflammasome activation, although treatment with ABT-737/S63845 to trigger apoptosis induced robust pannexin-1 cleavage in macrophages as expected **(Fig. 2P)**. Taken together, these data indicate that while pannexin-1 is required for driving NLRP3 inflammasome assembly during apoptosis, it is dispensable for canonical NLRP3 inflammasome activation in macrophages.

GSDMD but not pannexin-1 promotes cell death and NLRP3 assembly following non-canonical inflammasome activation

Two recent landmark studies revealed that caspase-11 cleaves its substrate GSDMD to drive pyroptosis, potassium efflux and NLRP3 activation [7, 8]. By contrast, another study proposed that caspase-11-dependent pyroptosis and NLRP3 assembly requires pannexin-1 channel activity [22], raising the possibility that pannexin-1 channels potentiate GSDMD pore activity. To investigate whether GSDMD processing, cell death or NLRP3 inflammasome activation require pannexin-1 channel activity, we prepared WT and *Gsdmd*$^{-/-}$ BMDMs and activated the non-canonical inflammasome by LPS transfection in the presence or absence of the pannexin-1 inhibitors probenecid, trovafloxacin

and spironolactone. In agreement with previous reports [8, 12], we observed that cytoplasmic LPS induced cleavage of the caspase-11 substrate GSDMD into the active p30 fragment and resulted in pyroptosis **(Fig. 3A-B)**. In addition, GSDMD was required to drive caspase-1 processing, pro-IL-1β maturation and secretion **(Fig. 3A, C).** Exposure of LPS-transfected BMDMs to probenecid, trovafloxacin and spironolactone had no effect on GSDMD, caspase-1 and pro-IL-1β processing **(Fig. 3A)**, while all three pannexin-1 inhibitors suppressed caspase-1 processing in apoptotic macrophages **(Fig. 1A, D)**. Next, we compared LPS transfection in WT and *Panx1*$^{-/-}$ BMDMs. In line with our inhibitor data, we observed comparable levels of GSDMD, caspase-1 and pro-IL-1β processing in WT and *Panx1*$^{-/-}$ BMDMs **(Fig. 3C, Supplementary Fig. 1C)**. And as expected, IL-1β secretion and pyroptosis were unaffected by *Panx1*-deficiency compared to WT BMDM **(Fig. 3D-E, Supplementary Fig. 1D-E)**. However, all three pannexin-1 inhibitors triggered a mild, but non-significant reduction in IL-1β secretion in both WT and *Panx1*$^{-/-}$ macrophages **(Fig. 3F)**. Lastly, we investigated the status of pannexin-1 cleavage upon non-canonical inflammasome activation. In agreement with our observation that pannexin-1 is dispensable for non-canonical inflammasome signalling **(Fig. 3D-F)**, we did not find any evidence of pannexin-1 cleavage during non-canonical inflammasome activation; in contrast, full length pannexin-1 was completely converted into the cleaved p19 fragment in apoptotic macrophages **(Fig. 3H)**. Taken together, our data support the conclusion that non-canonical inflammasome drives pyroptosis, NLRP3 activation and IL-1β secretion through GSDMD pores but not pannexin-1 channels.

Concluding remarks

Pannexin-1 is a channel-forming transmembrane protein with that is widely expressed in most cell types, including in transformed cells [29]. Our finding that pannexin-1 channel opening during apoptosis promotes NLRP3 inflammasome activation has major clinical relevance, especially under conditions where IL-1β signalling has been implicated to impair tumour clearance [30]. While our study only examined apoptosis induced by SMAC-

mimetic, BH3-mimetic and MCL1 inhibitor, it is likely that other commonly used apoptosis-inducing chemotherapies such as cisplatin and doxorubicin would similarly activate the NLRP3 inflammasome through pannexin-1 channels. The pannexin-1 channel inhibitor spironolactone is an inexpensive, orally-available drug that is already approved for human use [26]. Therefore, spironolactone could potentially offer rapid clinical translation and be administered in combination with standard chemotherapeutics to reduce NLRP3 and IL-1β signalling to promote tumour clearance.

Pharmacological pannexin-1 inhibitor or siRNA knockdown of pannexin-1 were reported to suppress canonical NLRP3 inflammasome activation [19-21]. Based on these observations, it was proposed that pannexin-1 channels promote NLRP3 inflammasome assembly. However, this concept was subsequently challenged as NLRP3 inflammasome signalling were found to be similar between WT and *Panx1*$^{-/-}$ BMDMs [23]. By using two independent lines of *Panx1*$^{-/-}$ BMDMs and three different pannexin-1 channel inhibitors, we confirmed previous reports and conclude that pannexin-1 is indeed dispensable for canonical NLRP3 inflammasome activation in murine macrophages.

Recognition of cytosolic Gram-negative bacteria by caspase-11 promotes pyroptosis and NLRP3 inflammasome activation, and this pathway confers susceptibility to murine models of endotoxin shock (ref). However, the mechanism by which caspase-11 drives cell death and NLRP3 activation remains debated. While two landmark studies identified that caspase-11 cleaves GSDMD to trigger pyroptosis and NLRP3 activation [7, 8], another study proposed that caspase-11-dependent pyroptosis and NLRP3 activation requires the membrane glycoprotein pannexin-1 [22]. In this model, the authors proposed that caspase-11 cleaves pannexin-1 to drive membrane permeability and NLRP3 inflammasome activation, while pannexin-1-dependent ATP release promotes pyroptosis via engagement of the purinergic receptor P2X7. Here, by using three different pannexin-1 channel inhibitors and 2 lines of *Panx1*$^{-/-}$ BMDMs, we provide evidence that pannexin-1 is not processed upon non-canonical inflammasome activation and demonstrate that pannexin-1 is

dispensable for non-canonical inflammasome signalling in BMDM. In contrast, our study supports the conclusion that active caspase-11 promotes cell lysis and NLRP3 activation via GSDMD pores. While the report from Yang *et al.* and our study both utilised *Panx1*$^{-/-}$ lines generated from C57BL/6 background, it remains possible, but unlikely, that passenger mutation and genetic variation between the *Panx1*$^{-/-}$ lines might influence the outcome of the study. This is because *Panx1*$^{-/-}$ BMDMs used in the study of Yang *et. al* expressed comparable levels of caspase-11 compared to WT cells; and are also likely to express normal levels of GSDMD since *Panx1*-deficiency did not affect pyroptosis upon NLRP3 and AIM2 activation in that study (ref). While we provide clear evidence that pannexin-1 is dispensable for non-canonical inflammasome signalling in BMDMs, at this moment, we cannot rule out the possibility that that pannexin-1 may indeed confer susceptibility to murine models of endotoxin shock *in vivo.* Since apoptotic caspases contribute to endotoxin-induced lethality (ref), it is tempting to speculate that pannexin-1 may potentially contribute to lethality by promoting NLRP3 activation, pyroptosis and IL-1β secretion in apoptotic cells (ref). Future studies should confirm the role of pannexin-1 in endotoxin-induced lethality and explore the therapeutic function of pannexin-1 inhibitors in such disease.

Materials and methods

Mice

C57BL/6 and *Gsdmd*$^{-/-}$ mice were housed in specific-pathogen free facilities in the University of Lausanne. *Panx1*$^{-/-}$ were previously described [31, 32] and kindly provided by Prof. Marc Chanson and Prof. Nathalie Rouach.

Cell culture

Murine bone marrow-derived macrophages (BMDM) were prepared by differentiating bone marrow progenitor cells for 7 days in DMEM (Gibco) supplemented with 20% MCSF (3T3 supernatant), 10% heat-inactivated FCS (Bioconcept), 10 mM HEPES (Bioconcept), penicillin/streptomycin (Bioconcept) and non-essential amino acids (Gibco). Inflammasome and apoptosis assays were performed on mature BMDMs on day 7–9 of differentiation. All WT versus *Panx1*$^{-/-}$ data presented in the main manuscript were generated using *Panx1*$^{-/-}$ and WT control BMDM provided by Prof Marc Chanson [32], while all WT versus *Panx1*$^{-/-}$ data in the supplementary data were generated using *Panx1*$^{-/-}$ and WT controls provided by Prof Nathalie Rouach [31].

Apoptosis assay

BMDMs were plated in 96-well plates at a density of 5×10^4 cells per well in complete media a day prior to stimulation. BMDMs were stimulated with a combination of ABT-737 and S63845 (all 0.5 μM; Selleckchem) or a combination of recombinant murine TNF (100 ng/ml; Enzo) and the SMAC-mimetic AZD5582 (0.5 μM; Selleckchem) in Opti-MEM for 5 h to induce intrinsic or extrinsic apoptosis respectively. To measure IL-1β secretion, BMDMs were primed for 4 h in Opti-MEM and stimulated with ABT-737 and S63845 (all 1 μM; Selleckchem) for 16 h. Where indicated, cells were treated with probenecid (1 mM; Sigma), trovafloxacin (10 μM; Sigma) or spironolactone (20 μM; Sigma) 30 min prior to cell stimulation.

Inflammasome assay

BMDMs were plated in 96-well plates at a density of 5 × 10^4 cells per well in complete media a day prior to stimulation. To activate the canonical NLRP3 inflammasome, cells were primed with ultrapure *E. coli* 055:B5 LPS (100 ng/ml; InvivoGen) for 4 h in Opti-MEM and stimulated with nigericin (5 μM; Invivogen) or ATP (2.5 mM; Sigma) for 90 min, or MSU (150 μg/ml; Invivogen) for 4 h. To activate the non-canonical inflammasome, cells were primed with Pam3CSK4 (1 μg/ml; Invivogen) for 4 h in Opti-MEM and transfected with ultrapure *E. coli* 0111:B4 LPS (2 μg/ml) using 0.25% Fugene HD (Promega) and centrifuged for 500 *g* for 10 min at 37°C. BMDMs were harvested 16 h post LPS transfection. Where indicated, cells were treated with probenecid (1 mM; Sigma), trovafloxacin (10 μM; Sigma) or spironolactone (20 μM; Sigma) at the last 20–30 min of priming.

Immunoblotting

Cell extracts were lysed in in boiling lysis buffer (66 mM Tris–Cl pH 7.4, 2% SDS, 10 mM DTT, 1x NuPage LDS sample buffer; Thermo Fisher) and resuspended with methanol/chloroform-precipitated supernatant. Mixed supernatant and extracts were separated on 14% polyacrylamide gels and transferred onto nitrocellulose membrane using Trans-blot Turbo (Bio-rad). Antibodies for immunoblot were against caspase-1 p20 (casper-1; Adipogen; 1:1000), GSDMD (EPR19828; Abcam; 1:1000), pro-IL-1β (AF-401-NA, R&D; 1:1000), pannexin-1 (D9M1C; Cell Signaling; 1:1000) and alpha-tubulin (DM1A; Abcam; 1:5000).

LDH assay

Lactate dehydrogenase (LDH) release into the cell culture supernatant was quantified using the TaKaRa LDH cytotoxicity detection kit (Clontech) according to the manufacture's protocol.

IL-1β ELISA

IL-1β release into the cell culture supernatant was quantified using the mouse IL-1 beta DuoSet ELISA Kit (R&D systems) according to the manufacture's protocol.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 7 software. All data sets were analysed using the parametric t-test. Data were considered significant when *P ≤ 0.05, **P ≤ 0.01.

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Figure legends

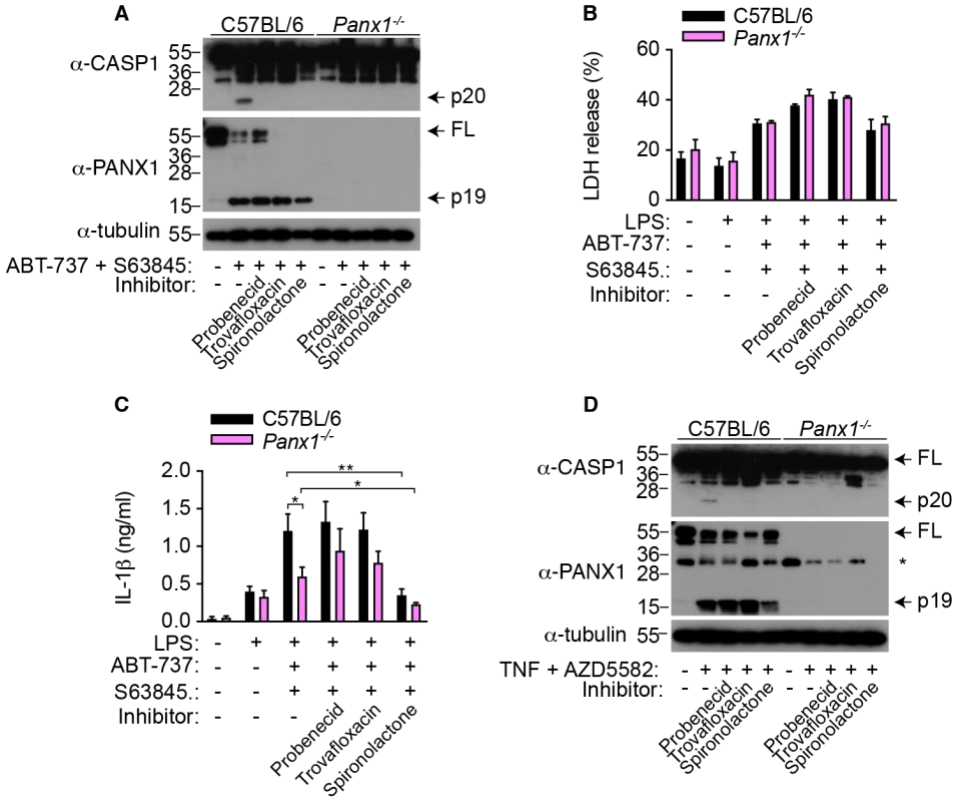


Figure 1. Channel activity of pannexin-1 is required for NLRP3 inflammasome activation during apoptosis. (A) BMDMs were treated stimulated with ABT-737 (0.5 μM) and S63845 (0.5 μM) for 5 h to induce intrinsic apoptosis. (B-C) BMDMs were primed with LPS (100 ng/ml) for 3 h and stimulated with ABT-737 (1 μM) and S63845 (1 μM) for 16 h. (D) BMDMs were stimulated with TNF (100 ng/ml) and AZD8882 (0.5 μM) for 5 h to induce intrinsic apoptosis. Where indicated, pannexin-1 channel inhibitors probenecid (1 mM), trovafloxacin (10 μM) and spironolactone (20 μM) were added 30 min prior to cell stimulation. (B, C) Data are mean ± S.E.M of pooled data from 3 independent experiments. Data were considered significant when *P < 0.05, **P < 0.01. Immunoblots show mixed supernatant and cell extracts and are representative of 3 independent experiments. * indicates a cross-reactive band.

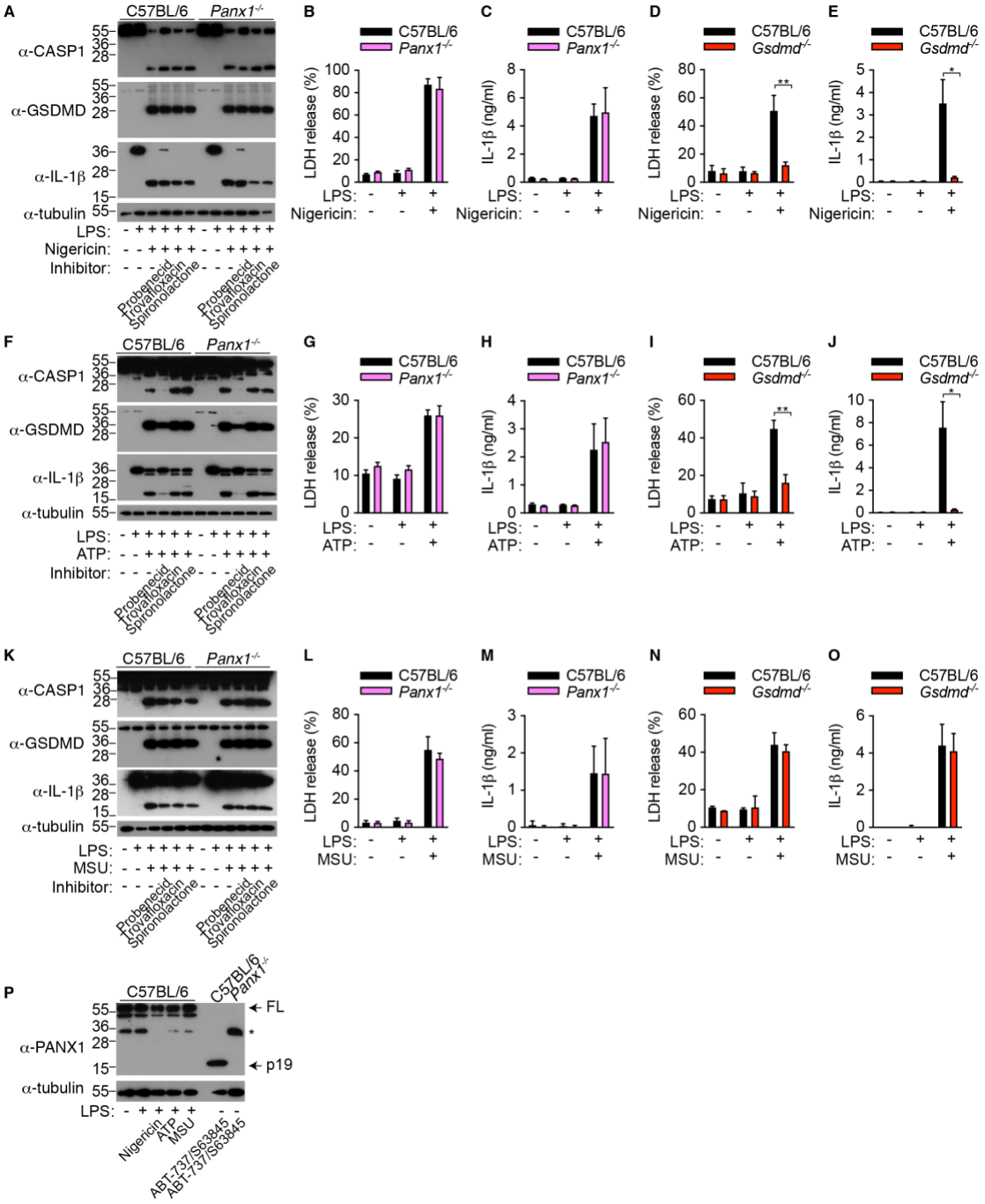


Figure 2. Pannexin-1 is dispensable for canonical NLRP3 inflammasome activation. (A-P) BMDMs were primed with 100 ng/ml ultrapure LPS for 4 h and stimulated with nigericin (0.5 μM; 90 min), ATP (2.5 mM; 90 min) or MSU (150 μg/ml; 4 h), or stimulated with ABT-737 (0.5 μM) and S63845 (0.5 μM) for 5 h. Where indicated, pannexin-1 channel inhibitors probenecid (1 mM), trovafloxacin (10 μM) and spironolactone (20 μM) were added 30 min prior to cell stimulation. (A, F, K, P) Mixed supernatant and cell extracts were examined

by immunoblotting and are representative of at 2-3 independent experiments. Data are mean ± S.D. of triplicate cell stimulation, representative of 3 (B, C, G, H, L, M) or 2 (N, O) independent experiments; (D, E, I, J) data are mean ± S.E.M of pooled data from 3 independent experiments. Data were considered significant when *P < 0.05, **P < 0.01.

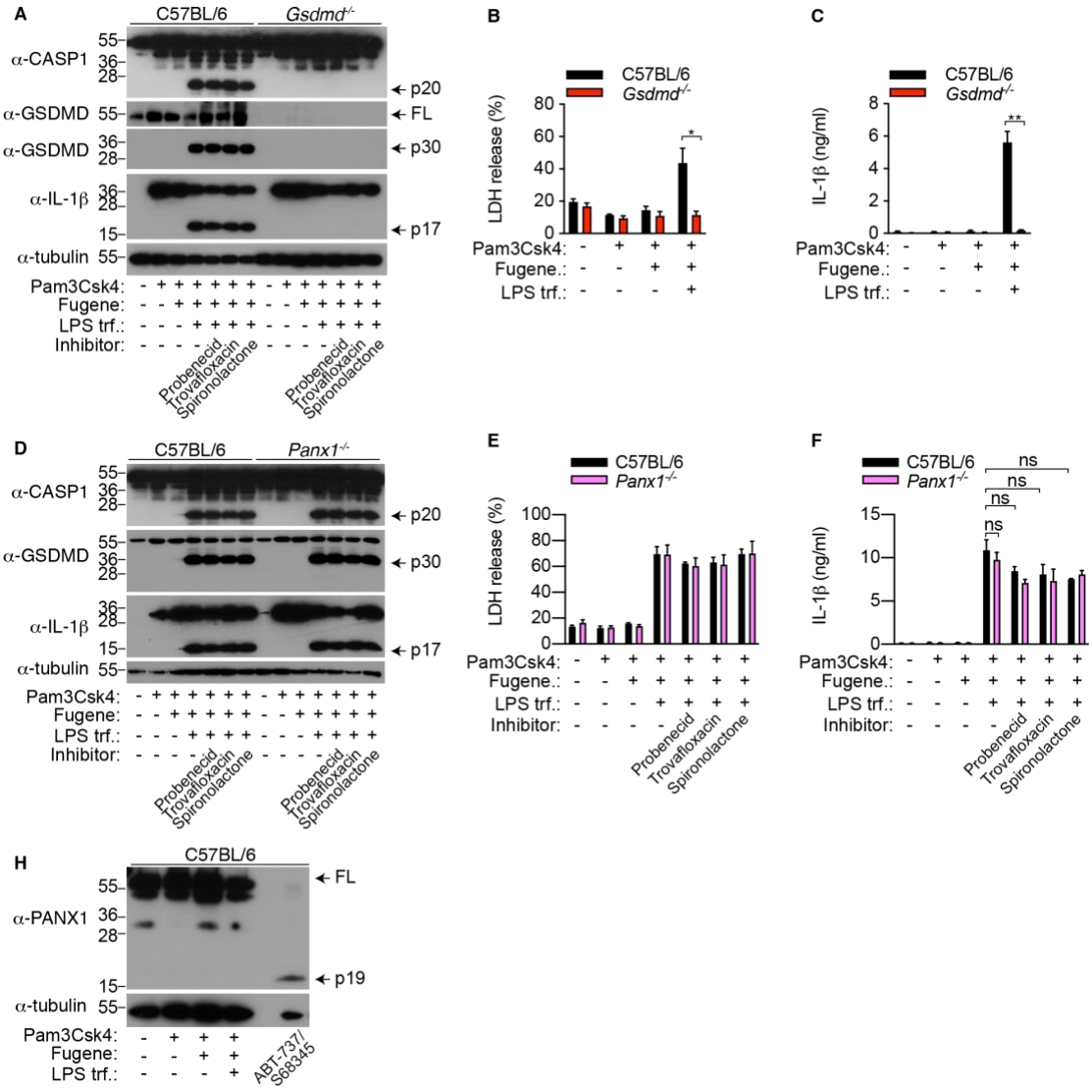
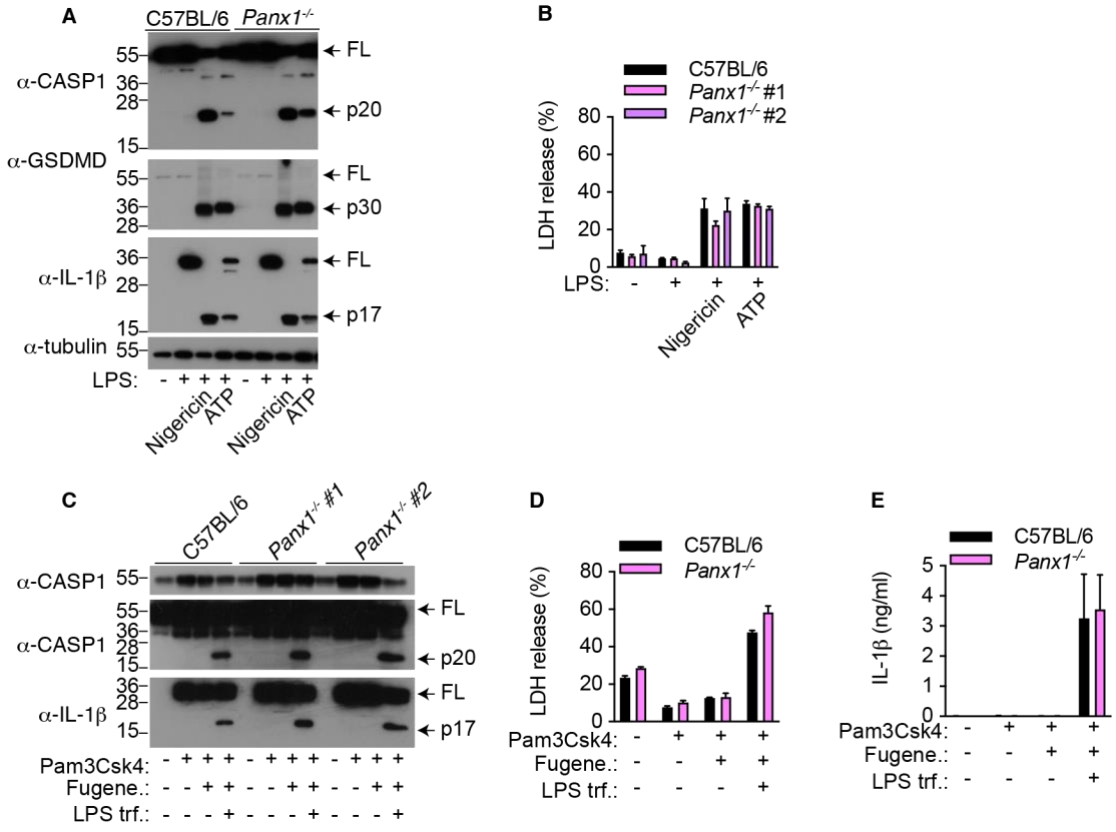


Figure 3. Non-canonical inflammasome drives pyroptosis and NLRP3 activation via GSDMD but not pannexin-1. (A-G) BMDMs were primed with 1 μg/ml Pam3CSK4 for 4 h and transfected with 2 μg/ml ultrapure 0111:B4 LPS using Fugene for 16 h, or treated stimulated with ABT-737 (0.5 μM) and S63845 (0.5 μM) for 5 h. (A, D, G) Mixed supernatant and cell extracts were examined by immunoblotting and are representative of at 2-3 independent experiments. (B-C) Data are mean ± S.E.M of pooled data from 3 independent experiments

or (E-F) mean ± S.D of triplicate cell stimulation representative of 3 independent experiments. Data were considered significant when *P < 0.05, **P < 0.01.



Supplementary Figure 1. Pannexin-1 is dispensable for canonical or non-canonical inflammasome signalling. (A-B) BMDMs were primed with 100 ng/ml ultrapure LPS for 4 h and stimulated with nigericin (0.5 μM; 90 min) or ATP (2.5 mM; 90 min). (C-E) BMDMs were primed with 1 μg/ml Pam3CSK4 for 4 h and transfected with 2 μg/ml ultrapure 0111:B4 LPS using Fugene for 16 h. (B, D, E) Data are mean ± S.D of triplicate cell stimulation representative of 2-3 independent experiments.