Author/s:
Bitto, NJ;Baker, PJ;Dowling, JK;Wray-McCann, G;De Paoli, A;Tran, LS;Leung, PL;Stacey, KJ;Mansell, A;Masters, SL;Ferrero, RL

Title:
Membrane vesicles from Pseudomonas aeruginosa activate the noncanonical inflammasome through caspase-5 in human monocytes

Date:
2018-11-01

Citation:
Bitto, N. J., Baker, P. J., Dowling, J. K., Wray-McCann, G., De Paoli, A., Tran, L. S., Leung, P. L., Stacey, K. J., Mansell, A., Masters, S. L. & Ferrero, R. L. (2018). Membrane vesicles from Pseudomonas aeruginosa activate the noncanonical inflammasome through caspase-5 in human monocytes. Immunology and Cell Biology, 96 (10), pp.1120-1130. https://doi.org/10.1111/imcb.12190.

PROFESSOR RICHARD FERRERO (Orcid ID : 0000-0001-6817-6211)

Article type : Original Article

Membrane vesicles from *Pseudomonas aeruginosa* activate the non-canonical inflammasome through caspase-5 in human monocytes.

Natalie J. Bitto[a, *], Paul J. Baker[b,c, *], Jennifer K. Dowling[a], Georgie Wray-McCann[a], Amanda De Paoli[a], Le Son Tran[a], Pak Ling Leung[d], Katryn J. Stacey[d], Ashley Mansell[a], Seth L. Masters[b,c, §], Richard L. Ferrero[a,e, §,1]

[a] Centre for Innate Immunity and Infectious Diseases, Hudson Institute of Medical Research, Clayton, Victoria 3168, Australia;

[b] Inflammation Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, 3052, Australia;

[c] Department of Medical Biology, University of Melbourne, Parkville 3010, Australia;

[d] School of Chemistry and Molecular Biosciences, University of Queensland, Brisbane Qld 4072, Australia.

[e] Biomedicine Discovery Institute, Department of Microbiology, Monash University, Clayton, Victoria 3168, Australia;

[*] These authors contributed equally to this work.

[§] Co-senior authors

Conflict of interest:

The authors declare no conflict of interest.

[1]To whom correspondence should be addressed: Professor Richard Ferrero. 27-31 Wright St, Clayton, Victoria, Australia 3168. Tel.+61385722728 Richard.Ferrero@hudson.org.au

Abstract

Outer membrane vesicles (OMVs) are constitutively produced by Gram-negative bacteria both *in vivo* and *in vitro*. These lipid-bound structures carry a range of immunogenic components derived from the parent cell, which are transported into host target cells and activate the innate immune system. Recent advances in the field have shed light on some of the multifaceted roles of OMVs in host-pathogen interactions. In this study, we investigated the ability of OMVs from two clinically important pathogens, *Pseudomonas aeruginosa* and *Helicobacter pylori*, to activate canonical and non-canonical inflammasomes. *P. aeruginosa* OMVs induced inflammasome activation in mouse macrophages, as evidenced by "speck" formation, as well as the cleavage and secretion of interleukin-1β and caspase-1. These responses were independent of AIM2 and NLRC4 canonical inflammasomes, but dependent on the non-canonical caspase-11 pathway. Moreover, *P. aeruginosa* OMVs alone were able to activate the inflammasome in a TLR-dependent manner, without requiring an exogenous priming signal. In contrast, *H. pylori* OMVs were not able to induce inflammasome activation in macrophages. Using CRISPR/Cas9 knockout THP-1 cells lacking the human caspase-11 homologs, caspase-4 and -5, we demonstrated that caspase-5 but not caspase-4 is required for inflammasome activation by *P. aeruginosa* OMVs in human monocytes. In contrast, free *P. aeruginosa* LPS transfected into cells induced inflammasome responses via caspase-4. This suggests that caspase-4 and caspase-5 differentially recognize LPS depending on its physical form or route of delivery into the cell. These findings have relevance to Gram-negative infections in humans and the use of OMVs as novel vaccines.

Introduction

Extracellular vesicles produced by Gram-negative bacteria, known as outer membrane vesicles (OMVs), are released during all stages of growth. Contrary to the long-held belief that OMVs are bacterial by-products with no biological significance,

recent advances in the field have revealed that OMVs are in fact a form of secretion system, facilitating a range of biological functions, from intra-bacterial communication, to host colonization and immune modulation.[1] It has been shown that OMVs interact with host target cells[2, 3] and are able to transport their cargo into cells. In this way, OMVs serve as vehicles in the long-distance delivery of immunomodulatory molecules and thus act as indirect effectors between the pathogen and its host.

OMVs contain a range of microbial-associated molecular patterns (MAMPs) that have the ability to activate the innate immune response, including lipopolysaccharide (LPS)[3, 4], DNA[5, 6] and peptidoglycan.[2, 7] Several of these MAMPs are recognized by cytoplasmic sensors capable of forming large multiprotein complexes, known as inflammasomes. Inflammasome activation is characterized by rapid macrophage cell death, termed pyroptosis, which is accompanied by the passive release of the mature forms of caspase-1 and interleukin-1β (IL-1β).[8] These responses first require a "priming" signal ("signal one"), generally involving the activation of Toll-like receptor (TLR) signaling, which triggers the activation of the transcription factor nuclear factor-κB (NF-κB) and subsequent production of pro-inflammatory cytokines, including pro-IL-1β.[9] "Signal two" corresponds to a danger signal that activates inflammasome complex formation, resulting in recruitment of the adapter molecule ASC (apoptosis-associated speck-like protein containing CARD) and cleavage of caspase-1 and IL-1β into their mature forms.[10] It is the nature of this second signal that dictates the type of inflammasome activated. Indeed, the absent in melanoma-2 (AIM2) inflammasome is activated in response to foreign cytosolic DNA; the NOD-like receptor family CARD domain-containing protein 4 (NLRC4) inflammasome is activated by cytosolic flagellin or type III effector proteins;[11] and the NACHT, leucine-rich repeat and PYD-domain-containing protein 3 (NLRP3) inflammasome responds to a range of microbial and host stimuli.[12] A non-canonical inflammasome pathway was also recently described in which murine caspase-11 is activated by directly binding to cytosolic LPS from some Gram-negative bacteria but not others. The human homologs were identified as caspase-4 and -5,[13] with caspase-4 required for responses to transfected free LPS, whereas both caspases were required for maximal responses to *Salmonella enterica* Typhimurium infection.[14-16]

OMVs from the opportunistic pathogen *Pseudomonas aeruginosa* harbor multiple MAMPs capable of activating inflammasome signaling pathways, such as LPS, DNA, flagellin. In the current study, we sought to identify the inflammasome pathway(s) induced by *P. aeruginosa* OMV-bound LPS and DNA. We show that *P. aeruginosa* OMVs are able to provide both signal 1 and 2 required for inflammasome activation. Importantly, we identified murine caspase-11, but not AIM2 or NLRC4, as being required for IL-1β responses and cell death in response to *P. aeruginosa* OMVs. Furthermore, for the first time we have demonstrated that caspase-5 and not caspase-4 is required for OMV activation of the non-canonical inflammasome in human monocyte-derived macrophages. Collectively, the findings identify LPS as being the major MAMP responsible for inflammasome activation by *P. aeruginosa* OMVs and suggest that in human macrophages, OMV-associated LPS may be recognized differently to either free LPS or live bacteria.

Results

P. aeruginosa OMVs induce ASC speck formation in macrophages

To investigate inflammasome activation by *P. aeruginosa* OMVs, we first used NLRP3-deficient macrophages stably reconstituted with cerulean-tagged ASC and NLRP3-Flag (ASC-cerulean IMACs[17]), which obviates the need for signal 1 in inflammasome activation.[18] These ASC-cerulean IMACs can therefore be used to visualize the ability of a given stimulus to promote inflammasome activation, as assessed by ASC "speck" formation[18, 19] (Supplementary Movie 1). Live cell imaging of these cells showed uptake of OMVs (Supplementary Movies 2-6), followed by oligomerization of the ASC molecules into discrete specks at approximately 14 h post OMV-treatment (Figure 1a; Supplementary Movie 1). As reported previously[19], one speck was generally observed in each individual cell (Supplementary Movie 7). The number of specks in OMV-treated cells was significantly greater than the untreated control imaged under the same conditions (Figure 1b; $p = 0.0142$; Supplementary Movies 2-6), thus demonstrating that *P. aeruginosa* OMVs are able to induce ASC speck formation in macrophages.

P. aeruginosa OMVs induce caspase-1 and IL-1β processing in macrophages via an ASC-dependent pathway

ASC is required for the formation of inflammasome complexes, which lead to the processing of caspase-1 and IL-1β into their mature forms.[10] Cells were primed with Pam3CSK4 for 4 h, rather than with LPS, to avoid activation of the caspase-11 inflammasome from the priming signal. Stimulation of wild type (WT) and *Asc*$^{-/-}$ primary bone marrow-derived macrophages (BMDMs) with *P. aeruginosa* OMVs for 24 h resulted in loss of IL-1β (Figure 2a) and IL-18 (Supplementary Figure 1a) responses in *Asc*$^{-/-}$ BMDMs, but no changes in TNF-α nor IL-6 production (Supplementary Figure 1b and c). Furthermore, stimulation with *P. aeruginosa* OMVs was shown to promote the maturation of caspase-1 and IL-1β and release into the culture supernatants of WT but not *Asc*$^{-/-}$ BMDMs (Figure 2b). The pro-forms of caspase-1 and IL-1β were detectable in both WT and *Asc*$^{-/-}$ cell lysates, indicating that the difference in processing of the mature forms was not due to varying amounts of pro-form present (Figure 2b). As we have shown that *P. aeruginosa* OMVs contain DNA on their surfaces,[20] we sought to determine whether this DNA may be involved in inflammasome activation. For this, we stimulated cells with DNase-treated and untreated *P. aeruginosa* OMVs. However, no differences were observed in IL-1β, caspase-1 or IL-18 responses for WT BMDMs stimulated with DNase-treated OMVs, suggesting that DNA is unlikely to be involved in inflammasome activation (Figure 2a; Supplementary Figure 1a), although DNA within OMVs and protected from digestion could not be excluded. Consistent with a lack of any role of DNA, we also showed that in contrast to *P. aeruginosa* OMVs, those from *Helicobacter pylori* that also contain DNA (data not shown), were unable to activate the inflammasome (Supplementary Figure 2a and b). *H. pylori* OMVs induced low levels of TNF-α production in mouse BMDMs compared to *P. aeruginosa* OMVs (Supplementary Figure 2c), consistent with the low capacity for *H. pylori* LPS to activate TLR4.[21] These data demonstrate that *P. aeruginosa* OMVs mediate inflammasome activation of pro-inflammatory cytokine responses via an ASC-dependent pathway that is unlikely to involve DNA recognition.

P. aeruginosa OMVs provide signal 1 and 2 for inflammasome activation

P. aeruginosa OMVs induced IL-1β, TNF-α and IL-6 production (Figure 2a; Supplementary Figure 1b and c), as well as pro-IL-1β synthesis (Figure 2b), in the absence of any priming with the TLR2 agonist, Pam3CSK4. This indicates that *P. aeruginosa* OMVs have the ability to prime inflammasome activation without the need for an exogenous signal. To determine the role of TLR signaling in this priming, we used myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) double knockout IMACs (*Myd88*$^{-/-}$/*Tri*$^{-/-}$), which lack the ability to respond to all major TLR agonists.[22] *Myd88*$^{-/-}$/*Trif*$^{-/-}$ IMACs failed to elicit any IL-1β, TNF-α or IL-6 production in response to stimulation with *P. aeruginosa* OMVs (Figure 3), indicating that the priming signal provided by these OMVs is TLR-dependent.

We next sought to determine the TLR agonist(s) that may be responsible for *P. aeruginosa* OMVs priming of the inflammasome. For this, we used IMACs deficient in the Mal adapter molecule (Mal), required for TLR2 and -4 signaling[23] to two major MAMPs of Gram-negative bacteria, lipoproteins[24] and LPS[25], respectively. *Mal*$^{-/-}$ IMACs displayed decreased TNF-α and IL-6 production in response to OMVs when compared with WT IMACs (Supplementary Figure 3). We found that both TLR2 and TLR4 are required for cytokine responses to *P. aeruginosa* OMVs (Supplementary Figure 4). Nevertheless, in common with the situation observed for *Mal*$^{-/-}$ IMACs (Supplementary Figure 3), deletion of either *Tlr2* or *Tlr4* did not completely abrogate cytokine responses to these OMVs. Furthermore, pre-treatment of *Mal*$^{-/-}$ IMACs with an antagonist (ODN 2088) of the DNA sensor TLR9[26] had only a weak effect on cytokine responses to OMVs, yet this treatment completely abrogated responses to a TLR9 agonist (Supplementary Figure 5). DNAse treatment of OMVs had no effect on responses to either WT or *Mal*$^{-/-}$ IMACs (Supplementary Figures 3 and 5), suggesting that DNA is unlikely to account for the priming activity of *P. aeruginosa* OMVs. Taken together, the data suggest that TLR2 and TLR4 agonists, such as lipoproteins and LPS, but not TLR9 recognition of DNA, contribute to the priming observed in response to *P. aeruginosa* OMVs. It is likely that other TLR agonist(s) are also important.

P. aeruginosa OMVs activate the non-canonical inflammasome in mouse macrophages

We have shown that *P. aeruginosa* OMVs activate the inflammasome in mouse macrophages via an ASC-dependent mechanism (Figure 2a,b). To define the specific inflammasome(s) involved, IMACs lacking each of the key sensors required for canonical inflammasome activation by Gram-negative bacteria (that is, *Aim2*, *Nlrp3* and *Nlrc4*) were treated with OMVs (Figure 4). No differences were observed in IL-1β and IL-18 responses in the *Aim2*$^{-/-}$ and *Nlrc4*$^{-/-}$ IMACs when compared with WT cells (Figure 4a,b). In contrast, decreased IL-1β and IL-18 production were observed in the *Nlrp3*$^{-/-}$ IMACs, suggesting that NLRP3 plays a role in inflammasome activation by *P. aeruginosa* OMVs (Figure 4c).

We then examined the role of the non-canonical inflammasome[27] in response to OMVs using *Casp11*$^{-/-}$ primary BMDMs. Compared to WT primary BMDMs, those from *Casp11*$^{-/-}$ mice produced reduced levels of IL-1β and IL-18 in response to either *P. aeruginosa* OMVs or transfected purified *P. aeruginosa* LPS, but responded normally to the canonical inflammasome agonist, polydA:dT (Figure 5a; Supplementary Figure 6a). Significantly reduced levels of cell death occurred in the *Casp11*$^{-/-}$ BMDMs, stimulated with either *P. aeruginosa* OMVs or transfected LPS, but not in those stimulated with polydA:dT (Figure 5b). *Casp11*$^{-/-}$ BMDMs responded normally to TLR stimulation (Supplementary Figure 6b and c) and pro-forms of caspase-1 and IL-1β were observed in both WT and *Casp11*$^{-/-}$ BMDMs treated with *P. aeruginosa* OMVs. (Figure 5c). Importantly, mature forms of caspase-1 and IL-1β were detected in the supernatants of WT but not *Casp11*$^{-/-}$ BMDMs (Figure 5c). Interestingly, *H. pylori* OMVs did not induce production of IL-1β (Figure 5a) or IL-18 (Supplementary Figure 5a), nor caspase-1 and IL-1β processing (Figure 5c) in either WT or *Casp11*$^{-/-}$ BMDMs (Figure 5c). Furthermore, no differences were observed in cell death (Figure 5b), TNF-α and IL-6 responses (Supplementary Figure 5b and c) between WT and Casp11$^{-/-}$ BMDMs treated with *H. pylori* OMVs.

Taken together, the data demonstrate that in common with OMVs from enterohemorrhagic *Escherichia coli*,[3] those from *P. aeruginosa* are recognized by cytosolic caspase-11, which results in the activation of the non-canonical inflammasome. However, as shown by the findings for *H. pylori* OMVs, the ability to activate caspase-11 is not common to OMVs from all Gram-negative bacteria.

P. aeruginosa OMVs activate the caspase-5 inflammasome in human THP-1 monocytes

The human homologs of murine *Casp11* are *CASP4* and *CASP5*, which have been shown to mediate non-canonical inflammasome activation in human monocytes.[13, 16] Although it is thought that there is redundancy between the two human caspases,[13] our recent findings showed that caspase-4 was required for non-canonical inflammasome activation by both free transfected LPS and *S.* Typhimurium bacteria, whereas caspase-5 was required for responses to *S.* Typhimurium and not free LPS.[14] We addressed the respective roles of these caspases in responses to *P. aeruginosa* OMVs using human THP-1 monocytes targeted by CRISPR/Cas9 with guides specific to either *CASP4*, *CASP5* or both *CASP4* and *CASP5*.[14] Cas9-only cells with no CRISPR deletion were used as controls. Consistent with Baker *et al.*[14], we observed decreases in IL-1β production and cell death in response to transfected LPS in both *CASP4*$^{-/-}$ and *CASP4*$^{-/-}$*CASP5*$^{-/-}$, but not in *CASP5*$^{-/-}$ monocytes. As was previously reported for *S*. Typhimurium,[14] stimulation with live *P. aeruginosa* bacteria resulted in decreased IL-1β production and cell death in *CASP4*$^{-/-}$, *CASP5*$^{-/-}$ and double knockout monocytes relative to WT cells (Figure 6a-c). Interestingly, however, significant levels of IL-1β production and cell death were still observed in the double knockout monocytes in response to either *P. aeruginosa* LPS or live bacteria (Figure 6c). This varies with our previous findings for S. Typhimurium[14], in which the effects of the knockout were much greater, and may reflect the differences in the bioactivities of *P. aeruginosa* LPS versus those of Gram-negative bacteria, such as *S.* Typhimurium and *E. coli*.

In response to *P. aeruginosa* OMVs, we observed reduced IL-1β production and cell death in *CASP5*$^{-/-}$ and double knockout monocytes (Figure 6b,c) but not *CASP4*$^{-/-}$ cells (Figure 6a). Similar findings were observed when phorbol 12-myristate 13-acetate (PMA) was used to differentiate the THP-1 monocytes to monocyte-derived macrophages (Supplementary Figure 7). Thus, for the first time we have shown that OMVs from *P. aeruginosa* selectively activate the non-canonical inflammasome in human monocytes through caspase-5.

Discussion

In this study, we have demonstrated the activation of the non-canonical inflammasome by OMVs from the opportunistic pathogen *P. aeruginosa* in both

murine and human systems. Furthermore, we have found that in human cells, OMV-associated LPS is sensed by caspase-5 but not caspase-4, leading to IL-1β secretion and cell death.

We demonstrated that OMVs derived from *P. aeruginosa* provide both signal 1 and 2 required for inflammasome activation, and that signal 1 is TLR-dependent. We next sought to identify the inflammasome activated by *P. aeruginosa* OMVs. Although it has been suggested that *P. aeruginosa* OMVs may be sensed by caspase-11,[3] this has not been thoroughly investigated. *P. aeruginosa* is known to activate the NLRC4 and NLRP3 inflammasomes,[28, 29] whereas DNA carried by OMVs may have the potential to activate the AIM2 inflammasome.[30] Therefore, we investigated the roles of the NLRP3, NLRC4, AIM2 and caspase-11 inflammasomes in response to *P. aeruginosa* OMVs. Our findings demonstrate that *P. aeruginosa* OMVs mediate IL-1β processing in a caspase-11-, NLRP3- and ASC-dependent manner. This is in agreement with the finding that NLRP3/ASC are required for caspase-1 and IL-1β processing upon caspase-11 activation.[31] Further work is required to determine whether NLRP3 is downstream to caspase-11 activation by OMVs.

Given that caspase-11 is cytoplasmic, it is evident that OMVs are present the cytoplasm in macrophages, rather than in the endosomal compartment. Previously, we showed in epithelial cells that *H. pylori* and *P. aeruginosa* OMVs are located in early endosomes within 1 h of cell entry.[2] It is possible that caspase-11 recognition of OMV-associated LPS in macrophages also requires escape from the early endosome into the cytoplasm, suggesting that OMVs or their LPS are not subject to complete lysosomal degradation.

The finding that *H. pylori* OMVs do not activate the inflammasome is consistent with studies showing that *H. pylori* possesses a modified form of LPS with low biological activity[21, 27], resulting in LPS that is likely to be poorly recognized by caspase-11. This finding highlights the differences in the cargo carried by OMVs from different bacterial species, which in turn, have differing effects on innate immune responses.

The sensing of OMVs by the non-canonical inflammasome sensors in human cells, namely caspase-4 and -5,[13] have not been thoroughly examined previously. A recent study showed that aggregates of LPS, as well as OMVs from *Neisseria meningitidis*, bound to caspase-4.[32] We have previously shown that caspase-4 is required for sensing of free LPS from *S.* Typhimurium, however, both caspase-4 and -5 are

involved in the sensing of LPS present in live bacteria.[14] The current study shows that *P. aeruginosa* OMVs activate the non-canonical inflammasome in human cells in a caspase-5-dependent but caspase-4-independent manner. While the reason behind this is unclear, it suggests that OMVs present LPS to the cytosol differently to either free LPS or live bacteria.

This study provides new insights into the previously uncharacterized role of OMVs in non-canonical inflammasome activation in human cells. It also provides a molecular basis for the known "self-adjuvanting" properties of OMVs which makes them effective at inducing cellular and humoral immune responses and thus excellent candidate vaccines. [1, 33] Moreover, we suggest that the findings are likely to be relevant to the pathogenesis of Gram-negative sepsis in human hosts as mice lacking caspase-11, but not those lacking caspase-1 alone, have been shown to be protected in a model for endotoxic shock.[34] Finally, the discovery that OMVs are sensed by caspase-5 raises the potential of investigating therapeutic targets to caspase-5 to prevent the development of sepsis.

Methods

Bacterial culture and OMV isolation

P. aeruginosa PA103 Δ*pilA*[35] and *H. pylori* 251[7] were cultured as described previously. Briefly, OMVs were harvested from mid-exponential phase and cultures pelleted by centrifugation at 4 000 x g, for 40 min at 4 ˚C (Heraeus Multifuge 3SR, ThermoScientific, Melbourne, Australia). Supernatants were filtered through 0.22-µm-pore-size filters (Millipore, Melbourne, Australia). OMVs were isolated by ultracentrifugation, as described previously.[7, 36] The pellets were washed in PBS by ultracentrifugation, re-suspended in PBS and stored at -80 ˚C. OMV protein concentrations were quantified using the Bradford Protein Assay (BioRad, New South Wales, Australia).

Immortalized cell cultures

Stably transfected ASC-cerulean,[17] wild type (WT),[37] *Myd88/Trif*$^{-/-}$,[22] *Mal*$^{-/-}$,[38], *Tlr2*$^{-/-}$, *Aim2*$^{-/-}$,[30] *Nlrp3*$^{-/-}$,[37] and *Nlrc4*$^{-/-}$,[37] C57/BL6 mouse immortalized macrophages were cultured in DMEM (Gibco, Melbourne, Australia) supplemented with 10 % fetal bovine serum (FBS; Gibco) and 1 % penicillin/streptomycin (P/S; Gibco).

CRISPR/Cas9 knockout *CASP4*$^{-/-}$, *CASP5*$^{-/-}$, *CASP4/5*$^{-/-}$ and Cas9-only THP-1 monocytes were generated and confirmed in detail, as described previously.[14] These cells were cultured in RPMI (Gibco) supplemented with 10 % FBS and 1 % P/S. To generate MDMs, monocytes were differentiated with 20 % PMA (phorbol 12-myristate 13-acetate; Sigma, St. Louis, Missouri) for 24 h. IMACs and THP-1 cells were seeded at 1 x 10^6/ml in 96-well plates. The following day, media was replaced with the appropriate antibiotic-free media.

Primary BMDMs

Bone marrow was collected from C57BL/6 WT, *Asc*$^{-/-}$, *Tlr4*$^{-/-}$ and *Casp11*$^{-/-}$ mice and the cells differentiated in DMEM (Gibco) supplemented with 10 % FBS, 1 % P/S and 20 % L929 conditioned media.[39] The media were replenished with a further 20 % L929 conditioned media on day 4. After 7 days, the macrophages were scraped in ice-cold PBS and seeded at 2 x 10^6/ml in 6-well plates.

Live cell imaging

The day prior to imaging, ASC-cerulean cells were seeded at 2 x 10^5/ml in 200 µl in µ-slide 8-well chambers (Ibidi, Munich, Germany). Cells were incubated with *P. aeruginosa* OMVs (20 µg protein per well) that had been labeled with an Alexa Fluor® 647 protein labeling kit (Molecular Probes, Eugene, Oregon) according to the manufacturer's instructions. This technique labels OMV-bound proteins.[7] Imaging was performed on a Lieca TCS Sp5 confocal microscope (Lieca Microsystems, Wetzler, Germany) in a chamber set to 37˚C and 5 % CO_2 for 15-16 h. Images were taken every 15 mins. Images are deconvoluted Z-stacks by overlapping tile scanning processed using Imaris x64 7.6.5.

Cell stimulations

Cells were primed with 1 µg/ml Pam3CSK4 (Pam3CysSerLys4, InvivoGen, San Diego, California) for 4 h, followed by stimulation with OMVs (50 µg protein/ml) for 24 h.[7] OMVs were treated or not with TURBO® DNase I (Ambion, New South Wales, Australia) for 1 h. As controls, the following compounds were transfected into cells using Lipofectamine® 2000 (Invitrogen), according to the following concentrations and times: polydA:dT (1 µg/ml; InvivoGen), 4 h; ODN 2088 TLR9 antagonist (3 µM; InvivoGen), 30 min; CpG (3 µM; InvivoGen), 8 h; or *P. aeruginosa* 10 LPS (100

ng/ml; Sigma), 24 h. Cells were also treated with the following controls: nigericin (20 μM; InvivoGen), 2 h; or flagellin (250 ng/ml; Sigma), 6 h.

Bacterial culture and infection

P. aeruginosa PA103 Δ*pilA*[35] bacteria were grown to mid-log phase, washed in PBS, re-suspended in DMEM and added to cells at a multiplicity of infection (MOI) = 10. After 1 h, 10 μg/ml tetracycline was added to kill any non-internalized bacteria, followed by incubation for a further 23 h.

Enzyme-linked immunosorbent assays (ELISA) and cytotoxicity assays

Cell culture supernatants were assayed for mouse IL-1β, TNF-α or IL-6 according to the manufacturer's instructions (BD Biosciences, Franklin Lakes, New Jersey). Assays for IL-18 and human IL-1β were performed using kits from ELISAkit (Melbourne, Australia). LDH release was measured using the CytoTox96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, Wisconsin). THP-1 monocytes were stained with propidium iodide (PI) (Life Technologies) at 1 μg/mL in a 5 % FCS-PBS solution, followed by quantification of live and dead cells by flow cytometry (FACSCanto™ II, BD Biosciences). Data were analyzed using Kaluza® Flow Analysis Software (Beckman Coulter, Melbourne, Australia).

Immunoblot analysis

Supernatants were concentrated using StrataClean resin (Agilent Technologies, Melbourne, Australia), as per the manufacturer's instructions. Cell lysates were prepared in 200 μl LDS buffer with Reducing Agent (Invitrogen). Proteins were resolved on NuPAGE 4-12 % Bis-Tris Protein Gels (Invitrogen), using NuPAGE MES SDS running buffer (Invitrogen), and transferred onto polyvinylidene difluoride (PVDF) membranes using a wet transfer system at 100 V for 1 h. Membranes were blocked in 5 % (w/v) milk powder in TBS-T (50 mM Tris-HCL, pH 7.6, 150 mM NaCl and 0.1 % (v/v) Tween-20) or Odyssey buffer (LI-COR, Lincoln, Nebraska), as appropriate, for 2 h at room temperature (RT), followed by incubation with primary antibodies diluted in 1 % milk powder in TBS-T or Odyssey buffer (LI-COR) overnight at 4°C. Membranes were then washed in TBS-T and incubated with the appropriate secondary antibody diluted in 1 % milk powder in TBS-T or Odyssey buffer (LI-COR), for 1 h at RT. Mouse monoclonal anti-caspase-1 p20 (1:1 000; Adipogen, San Diego,

California) was added followed by horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody (1:2 000; DAKO, Santa Clara, California) and developed using LumiGLO® ECL detection reagent (Cell Signaling, Danvers, Massachusettes). Biotinylated goat anti-mouse IL-1β p17 (1:2 000; R&D Systems, Northeast, Minneapolis) was added, followed by streptavidin-conjugated Alexa Fluor® 680 (1:2 000; Molecular Probes) and developed on an Odyssey CLx Imager (LI-COR). Rat anti-α-tubulin antibody (1:1 000; Abcam, Cambridge United Kingdom) was added followed by goat anti-rat-IRDye800 secondary antibody (1:3 000; Rockland, Limerick, Pennsylvania) and developed on an Odyssey CLx Imager (LI-COR).

Statistical analysis

Data analysis was performed using Microsoft® Excel 2010 and GraphPad PRISM® 6. All data are presented as means ± SEM. Statistical analyses were performed using the 2-way analysis of variance (ANOVA), unless otherwise stated.

Acknowledgements

P. aeruginosa PA103 Δ*pilA* was kindly provided by Cynthia B. Whitchurch. The WT, *Nlrp3*$^{-/-}$, *Nlrc4*$^{-/-}$, *Aim2*$^{-/-}$, *Mal*$^{-/-}$, *Myd88-Trif*$^{-/-}$, *Tlr2*$^{-/-}$ and ASC-cerulean immortalized macrophage cell lines were generously donated by Douglas Golenbock (University of Massachusetts Medical School, Boston, USA) and Eike Latz (University of Bonn, Bonn, Germany). Bone marrow from *Tlr4*$^{-/-}$ mice was kindly provided by Prof. Matt Sweet and Mr James Curson (Institute for Molecular Bioscience, The University of Queensland, Brisbane, Australia). This project was supported by funding from the Australian Research Council to RLF and KJS (Discovery grant DP120104911) and the National Health and Medical Research Council (NHMRC) to RLF (Project grant APP1030243). Research at the Hudson Institute of Medical Research is supported by the Victorian Government's Operational Infrastructure Support Program. RLF and KJS are supported by NHMRC Senior Research Fellowships (APP1079904 and APP1059729, respectively). RC and NJB were funded by the NHMRC and an Australian Postgraduate Award, respectively.

Conflict of Interests

The authors declare no conflict of interest.

Supplementary information is available at *Immunology & Cell Biology*'s website.

References

1. Kaparakis-Liaskos M, Ferrero RL. Immune modulation by bacterial outer membrane vesicles. *Nat Rev Immunol* 2015; **15:** 375-87.

2. Irving AT, Mimuro H, Kufer TA, Lo C, Wheeler R, Turner LJ *et al.* The immune receptor NOD1 and kinase RIP2 interact with bacterial peptidoglycan on early endosomes to promote autophagy and inflammatory signaling. *Cell Host Microbe* 2014; **15:** 623-35.

3. Vanaja SK, Russo AJ, Behl B, Banerjee I, Yankova M, Deshmukh SD *et al.* Bacterial outer membrane vesicles mediate cytosolic localization of LPS and caspase-11 activation. *Cell* 2016; **165:** 1106-19.

4. Kuehn MJ, Kesty NC. Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes Dev* 2005; **19:** 2645-55.

5. Cecil JD, O'Brien-Simpson NM, Lenzo JC, Holden JA, Chen Y-Y, Singleton W *et al.* Differential responses of pattern recognition rceptors to outer membrane vesicles of three periodontal pathogens. *PLoS ONE* 2016; **11:** e0151967.

6. Vidakovics ML, Jendholm J, Morgelin M, Mansson A, Larsson C, Cardell LO *et al.* B cell activation by outer membrane vesicles - a novel virulence mechanism. *PLoS Pathog* 2010; **6:** e1000724.

7. Kaparakis M, Turnbull L, Carneiro L, Firth S, Coleman HA, Parkington HC *et al.* Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells. *Cell Microbiol* 2010; **12:** 372-85.

8. Aachoui Y, Sagulenko V, Miao EA, Stacey KJ. Inflammasome-mediated pyroptotic and apoptotic cell death, and defense against infection. *Curr Opin Microbiol* 2013; **16:** 319-26.

9. Dowling JK, Mansell A. Toll-like receptors: the swiss army knife of immunity and vaccine development. *Clin Transl Immunology* 2016; **5:** e85.

10. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. *Nat Rev Immunol* 2013; **13:** 397-411.

11. Zhao Y, Yang J, Shi J, Gong Y-N, Lu Q, Xu H *et al.* The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature* 2011; **477:** 596-600.

12. Sagulenko V, Thygesen SJ, Sester DP, Idris A, Cridland JA, Vajjhala PR *et al.* AIM2 and NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC. *Cell Death Differ* 2013; **20:** 1149-60.

13. Vigano E, Diamond CE, Spreafico R, Balachander A, Sobota RM, Mortellaro A. Human caspase-4 and caspase-5 regulate the one-step non-canonical inflammasome activation in monocytes. *Nat Commun* 2015; **6**.

14. Baker PJ, Boucher D, Bierschenk D, Tebartz C, Whitney PG, D'Silva DB *et al.* NLRP3 inflammasome activation downstream of cytoplasmic LPS recognition by both caspase-4 and caspase-5. *Eur J Immunol* 2015; **45:** 2918-26.

15. Schmid-Burgk JL, Gaidt MM, Schmidt T, Ebert TS, Bartok E, Hornung V. Caspase-4 mediates non-canonical activation of the NLRP3 inflammasome in human myeloid cells. *Eur J Immunol* 2015; **45:** 2911-7.

16. Shi J, Zhao Y, Wang Y, Gao W, Ding J, Li P *et al.* Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* 2014; **514:** 187-192.

17. Hett EC, Slater LH, Mark KG, Kawate T, Monks BG, Stutz A *et al.* Chemical genetics reveals a kinase-independent role for protein kinase R in pyroptosis. *Nat Chem Biol* 2013; **9:** 398-405.

18. Pinar A, Dowling JK, Bitto NJ, Robertson AAB, Latz E, Stewart CR *et al.* PB1-F2 peptide derived from avian influenza A virus H7N9 induces inflammation via activation of the NLRP3 inflammasome. *J Biol Chem* 2017; **292:** 826-836.

19. Stutz A, Horvath GL, Monks BG, Latz E. ASC speck formation as a readout for inflammasome activation. *Methods Mol Biol* 2013; **1040:** 91-101.

20. Bitto NJ, Chapman R, Pidot S, Costin A, Lo C, Choi J *et al.* Bacterial membrane vesicles transport their DNA cargo into host cells. *Sci Rep* 2017; **7:** 7072.

21. Praszkier J, Sutton P, Ferrero RL. Virulence Mechanisms of *Helicobacter pylori*: An Overview. In: Backert S, Yamaoka Y (eds). *Helicobacter pylori Research: From Bench to Bedside*. Springer Japan: Tokyo, 2016, pp 57-87.

22. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H *et al.* Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 2003; **301:** 640-3.

513 23. Mansell A, Smith R, Doyle SL, Gray P, Fenner JE, Crack PJ *et al.* Suppressor
514 of cytokine signaling 1 negatively regulates Toll-like receptor signaling by
515 mediating Mal degradation. *Nat Immunol* 2006; **7:** 148-55.

516

517 24. Park BS, Song DH, Kim HM, Choi B-S, Lee H, Lee J-O. The structural basis
518 of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* 2009;
519 **458:** 1191-1195.

520

521 25. Yang RB, Mark MR, Gray A, Huang A, Xie MH, Zhang M *et al.* Toll-like
522 receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature*
523 1998; **395:** 284-8.

524

525 26. Lu C, Ha T, Wang X, Liu L, Zhang X, Kimbrough EO *et al.* The TLR9 ligand,
526 CpG-ODN, induces protection against cerebral ischemia/reperfusion injury via
527 activation of PI3K/Akt signaling. *J Am Heart Assoc* 2014; **3:** e000629.

528

529 27. Kayagaki N, Wong MT, Stowe IB, Ramani SR, Gonzalez LC, Akashi-
530 Takamura S *et al.* Noncanonical inflammasome activation by intracellular LPS
531 independent of TLR4. *Science* 2013; **341:** 1246-9.

532

533 28. Sutterwala FS, Mijares LA, Li L, Ogura Y, Kazmierczak BI, Flavell RA.
534 Immune recognition of *Pseudomonas aeruginosa* mediated by the
535 IPAF/NLRC4 inflammasome. *J Exp Med* 2007; **204:** 3235-3245.

536

537 29. Deng Q, Wang Y, Zhang Y, Li M, Li D, Huang X *et al. Pseudomonas*
538 *aeruginosa* triggers macrophage autophagy to escape intracellular killing by
539 activation of the NLRP3 inflammasome. *Infect Immun* 2015; **84:** 56-66.

540

30. Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR *et al.* AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* 2009; **458:** 514-8.

31. Ruhl S, Broz P. Caspase-11 activates a canonical NLRP3 inflammasome by promoting K(+) efflux. *Eur J Immunol* 2015; **45:** 2927-36.

32. Wacker MA, Teghanemt A, Weiss JP, Barker JH. High-affinity caspase-4 binding to LPS presented as high molecular mass aggregates or in outer membrane vesicles. *Innate Immun* 2017; **23:** 336-344.

33. Bitto NJ, Kaparakis-Liaskos M. The therapeutic benefit of bacterial membrane vesicles. *Int J Mol Sci* 2017; **18:** pii: E1287.

34. Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J *et al.* Non-canonical inflammasome activation targets caspase-11. *Nature* 2011; **479:** 117-21.

35. Whitchurch CB, Beatson SA, Comolli JC, Jakobsen T, Sargent JL, Bertrand JJ *et al. Pseudomonas aeruginosa fimL* regulates multiple virulence functions by intersecting with Vfr-modulated pathways. *Mol Microbiol* 2005; **55:** 1357-78.

36. Turner L, Praszkier J, Hutton ML, Steer D, Ramm G, Kaparakis-Liaskos M *et al.* Increased outer membrane vesicle formation in a *Helicobacter pylori tolB* mutant. *Helicobacter* 2015; **20:** 269-83.

37. Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, Reinheckel T *et al.* The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat Immunol* 2008; **9:** 857-65.

38. Nagpal K, Plantinga TS, Wong J, Monks BG, Gay NJ, Netea MG *et al.* A TIR domain variant of MyD88 adapter-like (Mal)/TIRAP results in loss of MyD88 binding and reduced TLR2/TLR4 signaling. *J Biol Chem* 2009; **284:** 25742-8.

39. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL *et al.* Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 2008; **9:** 847-56.

Figure Legends

Figure 1. *P. aeruginosa* OMVs induce ASC speck formation in mouse macrophages. (**a**) ASC-cerulean IMACs (blue) treated with fluorescently-labeled OMVs (red). OMVs were labeled using the Alexa Fluor® 647 Protein Labeling Kit (Invitrogen) prior to addition to cells. Times post-OMV addition are indicated. Arrows highlight cells that have taken up OMVs and subsequently formed ASC specks. (**b**) Non-stimulated ASC-cerulean IMACs imaged under the same conditions and time points as the OMV-treated sample, showing a lack of ASC speck formation. Cell outline at T=0 is shown in the bright field image (BF). Scale bar = 10 μm. (**c**) Number of ASC specs formed after 14 hours of imaging in non-stimulated (NS) or cells stimulated with OMVs in 16 fields of view; n=2 independent experiments per treatment, ± SEM. *p = 0.0142 (unpaired t-test).

Figure 2. *P. aeruginosa* OMVs activate the inflammasome via ASC in mouse BMDMs. (**a**) IL-1β production in WT vs *Asc*$^{-/-}$ BMDMs stimulated with OMVs ± DNase-treatment for 24 h. Pam3CSK4 priming is indicated with a "–" (unprimed) or "+" (primed) as shown. PolydA:dT was used as a positive control for inflammasome activation. n=3 biological replicates, ± SEM. ****p < 0.0001. (**b**) Western blots show mature and pro-forms of caspase-1 and IL-1β in the supernatant or lysate as indicated. Pam3CSK4 priming is indicated with a "–" (unprimed) or "+" (primed) as

shown. The α-tubulin loading control shown for cell lysates. Western blots are representative of 3 biological replicates.

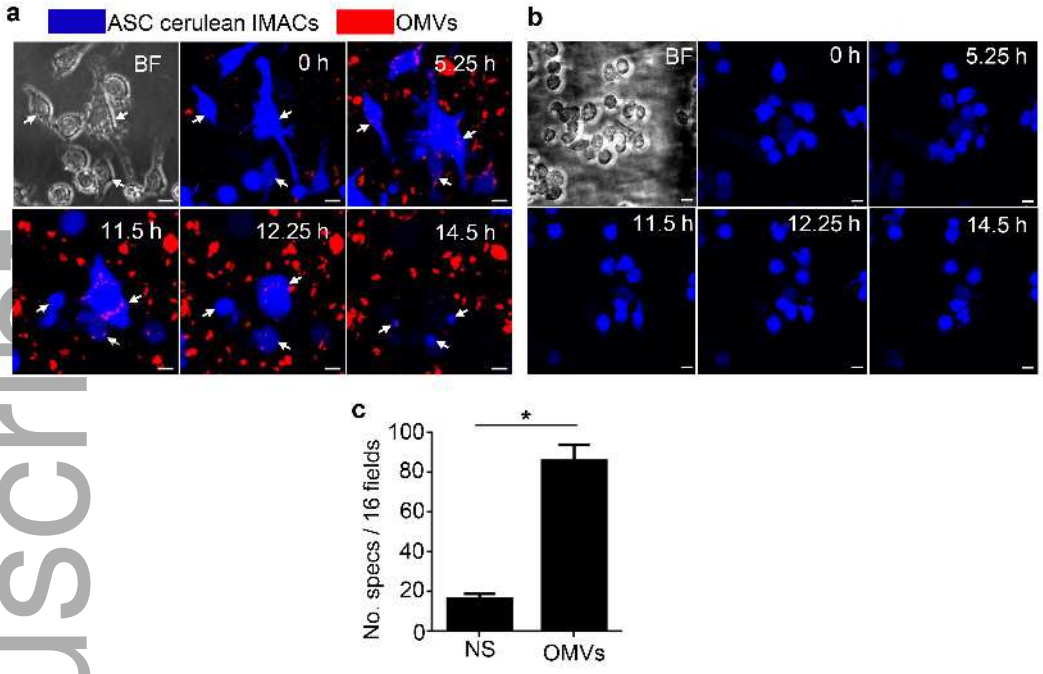
Figure 3. *P. aeruginosa* OMVs are self-priming in a TLR-dependent manner. (**a**) IL-1β, (**b**) TNF-α and (**c**) IL-6 production in non-primed *Myd88-Trif*$^{-/-}$ compared to WT IMACs, in response to OMVs ± DNase-treatment. PolydA:dT-treated cells were primed with Pam3CSK4 for 4 h prior to stimulation. ND = not detected. n=3 biological replicates, ± SEM. ****p < 0.0001.

Figure 4. *P. aeruginosa* OMVs induce inflammasome activation via an NLRP3-dependent but AIM2/NLRC4-independent mechanism. WT versus (**a**) *Aim2*$^{-/-}$, (**b**) *Nlrc4*$^{-/-}$ and (**c**) *Nlrp3*$^{-/-}$ IMACs, showing IL-1β and IL-18 production in response to OMVs. Controls include polydA:dT, flagellin (Flag) or nigericin (Nig) for the AIM2, NLRC4 and NLRP3 inflammasomes, respectively. Pam3CSK4 priming is indicated with a "–" (unprimed) or "+" (primed) as shown. n=3 biological replicates, ± SEM. *p < 0.05, ****p < 0.0001.

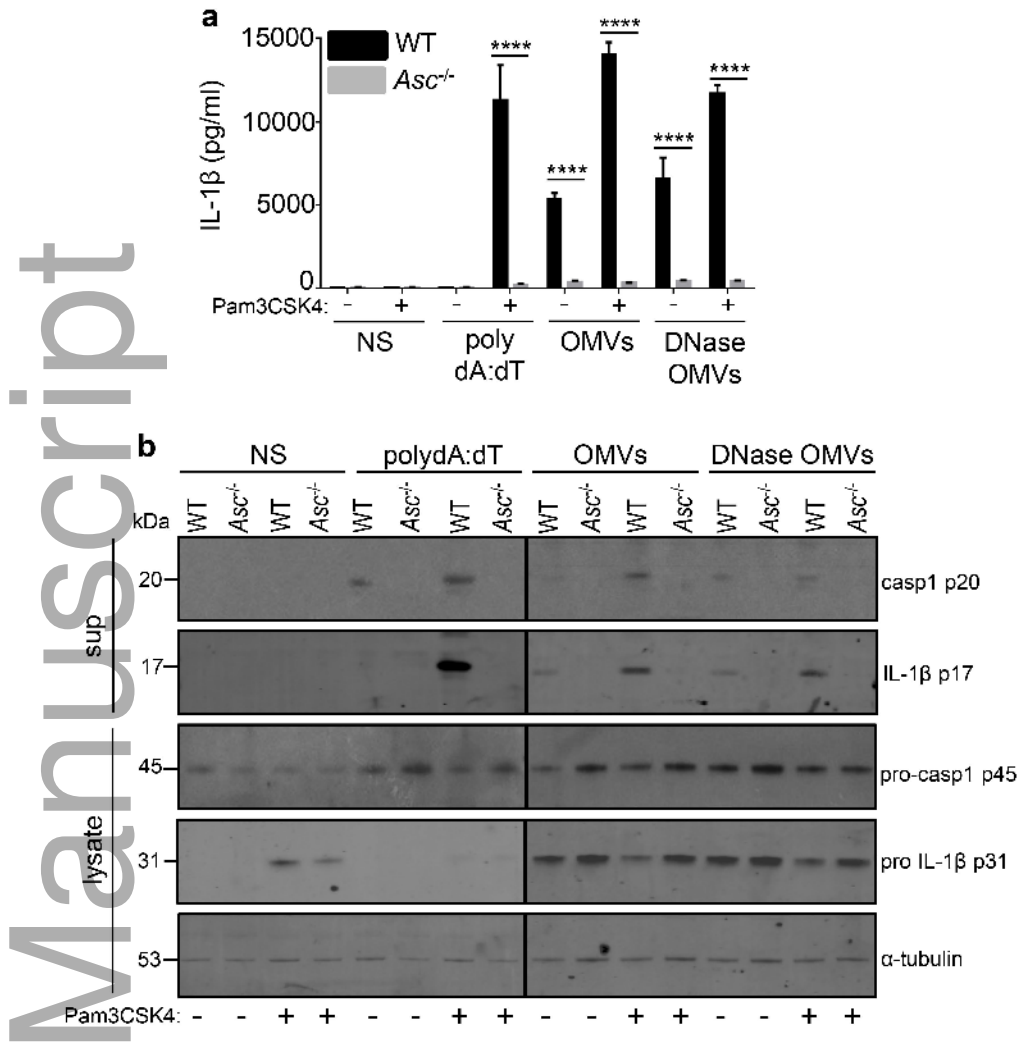
Figure 5. *P. aeruginosa* OMVs induce non-canonical inflammasome activation via caspase-11 in mouse BMDMs. (**a**) WT and *Casp11*$^{-/-}$ BMDMs were stimulated with *P. aeruginosa* OMVs (*P. a* OMVs) or *H. pylori* OMVs (*H. p* OMVs). Controls include polydA:dT and *P. aeruginosa* LPS (*P. a* LPS) to activate the AIM2 or caspase-11 inflammasomes, respectively. Pam3CSK4 priming is indicated with a "–" (unprimed) or "+" (primed) as shown. (**b**) Cell death was determined by % LDH release. (**c**) Western blots show mature and pro-forms of caspase-1 and IL-1β in supernatants or lysates as indicated, with α-tubulin loading control shown for cell lysates. Pam3CSK4 priming is indicated with a "–" (unprimed) or "+" (primed) as shown. n=3 biological replicates, ± SEM. **p < 0.01, ***p < 0.001, ****p < 0.0001. Western blots are representative of 3 biological replicates.

Figure 6. *P. aeruginosa* OMVs activate the non-canonical inflammasome in human cells via caspase 5, but not caspase-4, in THP-1 monocytes. IL-1β and cell death (% PI) are shown for wild type Cas9-expressing THP-1 cells versus (**a**) *CASP4*$^{-/-}$, (**b**) *CASP5*$^{-/-}$ and (**c**) *CASP4*$^{-/-}$*/5*$^{-/-}$ THP-1 monocytes. Pam3CSK4 priming is indicated with a "–" (unprimed) or "+" (primed). PolydA:dT, *P. aeruginosa* LPS (*P. a* LPS) and

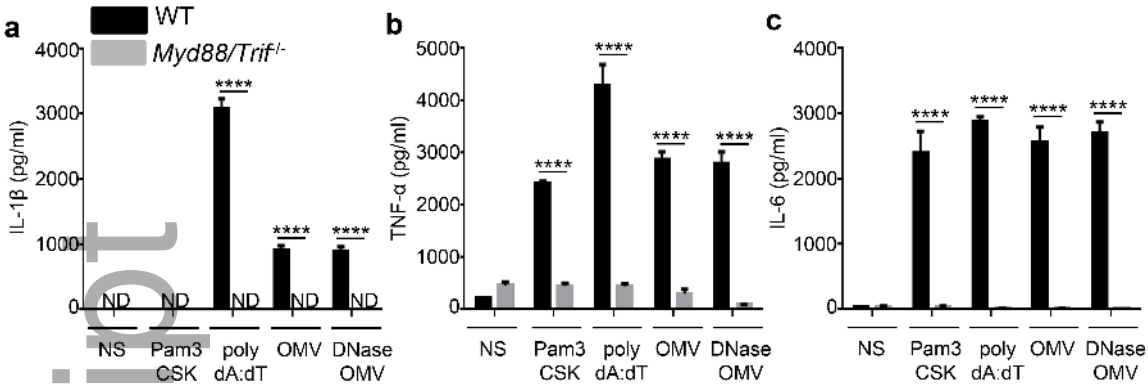
live whole *P. aeruginosa* (live *P. a*) served as controls for the AIM2, capase-4 and caspase-5 inflammasomes, respectively. n=3 biological replicates, ± SEM.



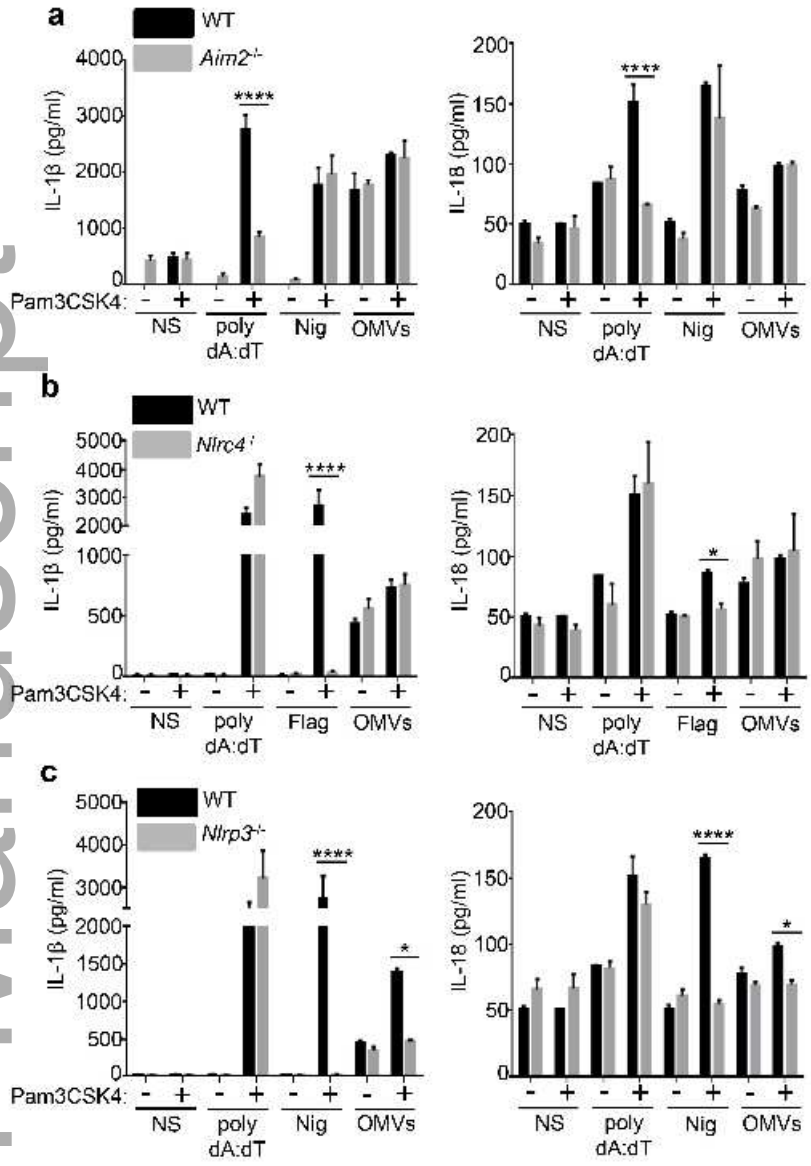
imcb_12190_f1.jpg



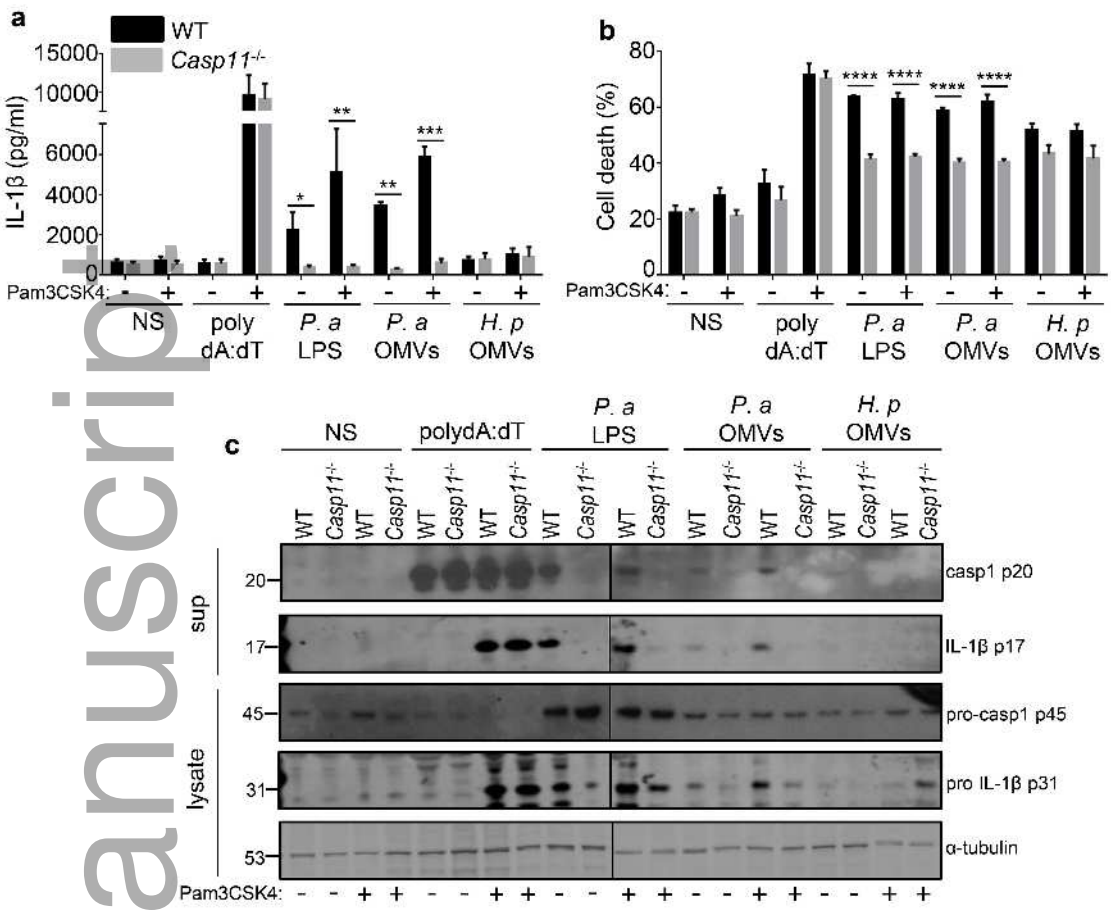
imcb_12190_f2.jpg



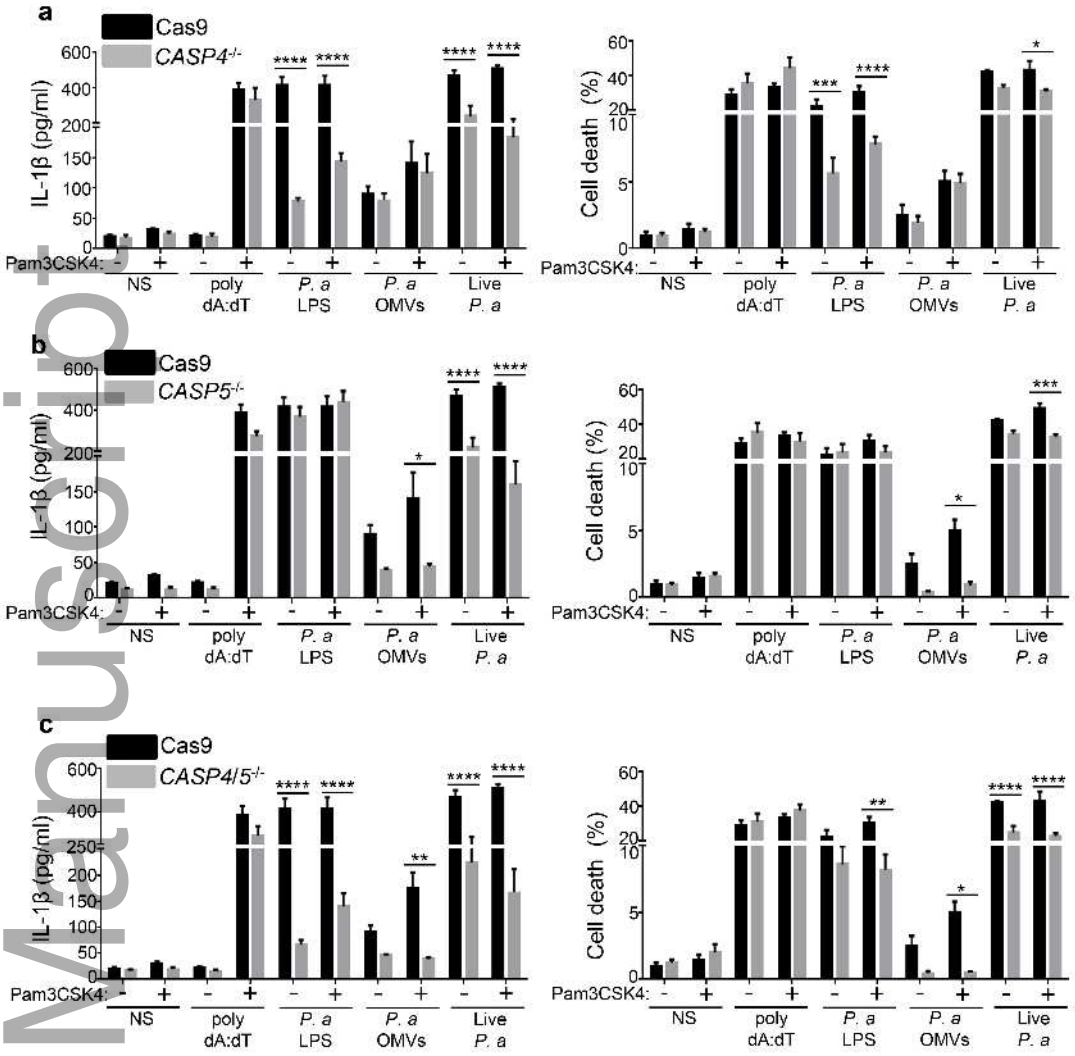
imcb_12190_f3.jpg



imcb_12190_f4.jpg



imcb_12190_f5.jpg



imcb_12190_f6.jpg