Title
Differential role of the pannexin-1/ATP/P2X7 axis in IL-1β release by human monocytes

Running title
TLR4 driven IL-1β release is pannexin-1 independent

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Abstract
IL-1β release is integral to the innate immune system. The release of mature IL-1β depends on two regulated events; (i) the de novo induction of pro-IL-1β, generally via NFκB-dependent transduction pathways and (ii) the assembly and activation of the NLRP3 inflammasome. This latter step is reliant on active caspase-1, pannexin-1 and P2X7 receptor activation. Pathogen associated molecular patterns in Gram-positive and Gram-negative bacteria activate IL-1β release from immune cells via TLR2 and TLR4 receptors respectively. Here, we show that pro-IL-1β and mature IL-1β release from human monocytes is stimulated by the TLR2 agonists, Pam3CSK4 or FSL-1, and the TLR4 agonist, LPS, in the absence of additional ATP. TLR2 agonists required pannexin-1 and P2X7 receptor activation to stimulate IL-1β release. By contrast, IL-1β release stimulated by the TLR4 agonist, LPS, is independent of both pannexin-1 and P2X7 activation. In the absence of exogenous ATP, P2X7 activation requires endogenous ATP release, which occurs in some cells via pannexin-1. In line with this, we found that LPS-stimulated human monocytes released relatively low levels of ATP, whereas cells stimulated with TLR2 agonists released high levels of ATP. These findings suggest that, in human monocytes, TLR2 and TLR4 signalling both induce pro-IL-1β expression, but the mechanism by which they activate caspase-1 diverges at the level of the pannexin-1/ATP/P2X7 axis.
**Introduction**

IL-1β is a pro-inflammatory cytokine with a pivotal role in innate immunity and is a mono-therapeutic target for a number of inflammatory conditions such as gout (1). Unlike other pro-inflammatory cytokines such as TNFα and IL-6, IL-1β is not processed in the traditional manner through the Golgi apparatus, but is instead cleaved from its precursor molecule pro-IL-1β. In this fashion, mature IL-1β production requires two distinct steps; (i) a priming stage where pro-IL-1β is induced followed by (ii) the assembly and activation of the NLRP3 inflammasome, which enzymatically cleaves caspase-1 to its active form.

The ‘priming’ step in IL-1β production is triggered by many pro-inflammatory stimuli, including bacterial pathogen associated molecular patterns (PAMP), which activate pattern recognition receptors (PRRs) (2), such as the Toll like receptors (TLRs). Production of mature IL-1β from pro-IL-1β is less well understood, particularly the processes leading to activation of the NLRP3 inflammasome, which seem to vary between cell types and particular stimuli. Current knowledge indicates that NLRP3 inflammasome activation is mediated by pannexin-1, a large-pore channel that facilitates the release of ATP and potassium ions. ATP released via pannexin-1 activates the purine receptor, P2X7, resulting in additional potassium ion efflux (3). ATP/P2X7 signaling can then further activate pannexin-1, resulting in an amplification loop for potassium efflux and cellular hyperpolarization (4). This triggers NLRPS inflammasome assembly and activation.

Potassium efflux and cell hyperpolarization facilitated by the pannexin-1/ATP/P2X7 axis is probably the best-studied mechanism by which NLRP3 inflammasome and caspase-1 activation occurs. There are, however, pannexin-1-independent pathways that lead to NLRP3 inflammasome. For example, physical perturbation of the cell membrane by nano-particles or crystals of cholesterol or monosodium urate can cause hyperpolarization of cells through direct membrane damage (5-7).

In many studies designed to investigate IL-1β release from cells, the assembly and activation step in the process is artificially modelled by simply adding very high levels of ATP, in the mM range (8). This has proved useful in determining aspects of cell priming and the function of pannexin-1 and P2X7. However, exogenous ATP in mM concentrations is unlikely to occur in the body, even at sites of inflammation. So, to understand how IL-1β release occurs in more physiological settings requires the inclusion of protocols in which endogenous pathways are compared without an artificially large exogenous ATP challenge (9).

Monocytes release ATP when stimulated with LPS (10), while human macrophages and dendritic cells differ from monocytes in their ability to release both ATP and mature IL-1β after stimulation with TLR ligands. For example, human monocytes, but not macrophages or dendritic cells, have been shown to have constitutively active caspase-1, meaning that they release mature IL-1β after stimulation with TLR2 or TLR4 agonists, without the requirement of exogenous ATP(9). However, the role of pannexin-1 and/or P2X7 in endogenous release of IL-1β stimulated by TLR4 versus TLR2 agonists has not been addressed.

Thus, in the present study, we compared the relative ability of TLR2 and TLR4 agonists to activate caspase-1, express pro-IL-1β, and release ATP and mature IL-1β from human monocyctic cells. We also...
determined the relative role that pannexin-1 and P2X₇ may have in IL-1β production induced by TLR2 versus TLR4.
Materials and Methods

Cell culture
Human acute monocytic leukemia cell line THP-1 were obtained from ECACC and cultured in RPMI 1640 supplemented with 10% filtered heat-inactivated Foetal Calf Serum (FCS), 2mM glutamine and 100U/ml penicillin/streptomycin and maintained at 37°C containing 5% CO₂. Cells were seeded in 96 well plates at 1x10^5 cells/well for 12h prior to treatment. Cell viability was assessed using Alamar Blue™ (Invitrogen, Paisley, UK) after all treatments and IL-1β and pro-IL-1β release was measured by ELISA (R&D systems, Abingdon, UK).

Inhibitor treatments
Human monocytes were treated with TLR4 agonist, LPS (0.001-1µg/ml) or TLR2 agonists, Pam₃CSK₄ (0.001-1µg/ml) and/or FSL-1 (0.001-1µg/ml) for 3 or 24h. In some experiments cells were pulsed for 30mins with ATP (1-5mM) post activation. In the inhibition studies cells were pre-treated for 30mins with inhibitors of caspase-1 (Z-VAD-FMK; 0.01-1µg/ml) or pannexin-1 (carbenoxolone; 0.03-30µg/ml) or P2X₇ (AZ11645373; 0.01-1µg/ml) or Maxi-K⁺ channel (paxilline; 5-20 µM or TEA; 5-20 mM) after which they were stimulated with LPS, Pam₃CSK₄ or FSL-1.

siRNA transfection
THP-1 cells were re-suspended in solution V (VCA–1003). siRNA was added (20nM-100nM) and cells electroporated using standard methods (Amaza Nucleofector transfection system, Lonza, Basel, Switzerland). After incubation in pre-warmed media for 10min at 37°C, cells were transferred onto the 96 well plate. After 48h, cells were treated as above. siRNA sequence used were, AllStars Negative Control siRNA (Qiagen, Crawley, UK) and ON-TARGET plus SMARTpool, Human Panx1 (Dharmacon, Fisher Scientific, Loughborough, UK):

UAAGUGAGGUCAAGUCAUA
CGGCAGAGCUCCAAGGUAU
CAUAUUUGCUCAGACUUGA
CACUGUGGCUGCAUAAGUU

Western blotting
Western blot was performed 48h after gene knock-down. Whole cell protein extracts were prepared on ice using a lysis buffer containing 50mM Hepes ph7.5, 50mM Sodium Fluoride, 5mM sodium Pyrophosphate, 1mM EDTA, 1mM DTT, 10% glycerol, 1% Triton, and complete EDTA-free Protease Inhibitor Cocktail (Roche). Protein extracts were loaded on SDS-PAGE gel, transferred to PVDF membrane and subjected to the incubation with individual antibodies. Primary antibodies used were: pannexin-1 (Abcam), P2X₇
(Alomone labs), beta-tubulin (Cell Signalling). The PageRuler Plus Prestained Protein Ladder (Pierce Biotechnology) was used as a molecular weight Marker.

**Capase-1 activity**

Caspase-1 activity was measured by FAM-FLICA in vitro caspase detection kit according to the manufacturer instruction (Abcam, Cambridge, UK). Briefly, A549 cells were treated with either DMSO or LPS (0.1µg/ml) or Pam3CSK4 (0.1µg/ml) for 24h. Cells were labelled with FAM-FLICA for 60min at 37C. After washing cells were read on fluorescent plate reader (excitation wavelength at 488nm, emission wavelength 530 nm).

**ATP release**

ATP release from THP-1 cells was measured using the ChronoLUME luciferase assay system (Chrono-log Corporation, Havertown, PA, USA). THP-1 cells, cultured in 96 well microtitre plates, were stimulated with LPS (0.1µg/ml) or Pam3CSK4 (0.1µg/ml). Cells were incubated for 30 mins then ChronoLUME reagent containing firefly luciferase (16µg/ml final) and D-luciferin (1,760U/ml final) was added to each well. After brief mixing the luminescence read using a Mithras LB940 multimode plate reader (Berthold Technologies, Bad Wildbad, Germany). ATP release was calculated with reference to the luminescence of wells containing ChronoLUME and 40µM exogenous ATP standard (Chrono-log Corporation).
Results and Discussion

To assess the effects of TLR2 and TLR4 ligands on the release of IL-1β by human monocytes, cells were treated for 24h with FSL-1 or Pam3CSK4 (TLR2), or LPS (TLR4). In each case, Pam3CSK4, FSL-1 or LPS caused a concentration-dependent release of mature IL-1β into the conditioned media without the requirement of exogenous ATP (Figures 1A). In line with this, both Pam3CSK4 and LPS increased the release of unprocessed Pro-IL-β (Figure 1B). As expected, and as others have shown, when cells were primed with Pam3CSK4, FSL-1 or LPS and challenged with a brief (20 minutes) exposure to mM concentrations of exogenous ATP, mature IL-1β was released (Figure 1C and D). IL-1β release in response to exogenous ATP is known to require pannexin-1 and P2X7 cooperation (11). However, in the absence of exogenous ATP, the role these pathways play in TLR4 vs TLR2 mediated IL-1β release is incompletely understood.

The processing of IL-1β from pro-IL-1β requires for caspase-1 (12). Thus, as expected, we found that IL-1β release stimulated by either LPS or Pam3CSK4 and FSL-1 was inhibited in a concentration dependent manner by the pan-caspase inhibitor, Z-VAD-FMK (Figure 2A). However, whilst IL-1β release stimulated by the TLR2 ligands, Pam3CSK4 and FSL-1, was inhibited by the pannexin-1 inhibitor, carbenoxolone, release stimulated by the TLR4 agonist LPS was completely unaffected (Figure 2B). Activation of pannexin-1 results in ATP release and subsequent activation of P2X7 (13). In line with what we observed with pannexin-1, inhibition of P2X7 using pharmacological concentrations of AZ11645373 inhibited IL-β release by Pam3CSK4 and FSL-1, but did not affect release by LPS (Figure 2C). It has been shown in human macrophages, that the large conductance potassium channel MaxiK plays a crucial role in the cellular activation by TLRs (14). This may therefore be an alternative way for potassium efflux to occur in human monocytes, leading to the consequent assembly and activation of the NLRP3 inflammasome after TLR4 stimulation. Impeding this process by using the MaxiK inhibitor paxilline, resulted in an inhibition of IL-1β from both LPS and Pam3CSK4 stimulated cell (Figure 4D). Suggesting that this pathway plays an important role in both TLR2 and TLR4 activation of the NLRP3 inflammasome.

Using pharmacological tools to dissect signalling pathways can be subject to compromise by non-specific drug effects. AZ11645373 is a well-validated drug and is highly specific to human P2X7 receptors when used at mid-high nM concentrations (15). Carbenoxolone, whilst widely used as a pannexin-1 inhibitor, is not specific and has well documented effects on other biological pathways (16). Thus, in order to validate our observation – that TLR4-induced IL-1β production occurs independently of pannexin-1 and P2X7 – we performed gene knock down of pannexin-1. Selected siRNA sequences for pannexin-1 reduced its expression at both the transcriptional and translation level (Figure 3A and B), and, in direct corroboration of our data using pharmacological inhibitors, also reduced IL-1β release induced by the TLR2 agonist, Pam3CSK4 (Figure 3C), but not by the TLR4 agonist, LPS (Figure 3D). By way of a functional control, production of CXCL8, which is stimulated by TLR2 or TLR4 agonists in parallel with IL-1β and
independently of caspase-1, was not affected by any of the inhibitor drugs (Supplementary Figure 1). Similarly, cell viability was not compromised in any of the protocols used (Supplementary Figure 2).

Pannexin-1 activation and gap junction formation is one of the recognised mechanisms by which endogenous ATP is actively released from cells (9). Once released from cells primed for pro-IL-1β induction, ATP acts on P2X₇ receptors to amplify potassium ion efflux (17). Since our data suggests that TLR2, but not TLR4, receptor activation involves pannexin-1, we measured release of ATP from cells stimulated with LPS versus Pam₃CSK₄. We found that monocytes stimulated with Pam₃CSK₄ released significantly higher levels of ATP than monocytes stimulated with LPS (Figure 4A). These observations, together with our data implicating involvement of P2X₇ in TLR2-, but not TLR4-, mediated IL-1β release, suggest that LPS-induced ATP release from monocytes is not sufficient to initiate NLRP3 inflammasome assembly, whereas levels of ATP released by Pam₃CSK₄ constitute a pharmacologically relevant event.

The function of pannexin-1 and P2X₇ in IL-1β release is to (ultimately) mediate potassium ion efflux; this is required for assembly and activation of the NLRP3 inflammasome and subsequent activation of caspase-1. Netea and co-workers (9) showed that, in primary monocyes, but not in THP-1 cells, caspase-1 is ‘constitutively’ active. Thus, we next sought to determine if TLR4 signalling with LPS differed from TLR2 signalling with Pam₃CSK₄ at the level of caspase-1 activation. We found that caspase-1 was activated at identical levels when cells were treated with either Pam₃CSK₄ or LPS (Figure 4B). Suggesting that the extent of NLRP3 activation and hence IL-1β release is similar with both TLR2 and TLR4 activation. However, it seems that the monocytes has different ways of processing these stimuli.

Taken together, our observations, using a simple monocytic cell line model, show that TLR2- and TLR4-mediated activation of the NLRP3 inflammasome and subsequent activation of caspase-1 can occur by completely different signalling pathways. For TLR2, we have defined these pathways and hypothesise that the sequence of events are (i) activation of pannexin-1, (ii) release of ATP, (iii) activation of P2X₇, (iv) efflux of potassium ions, (v) activation of NLRP3 (Figure 5A). For TLR4, a full characterisation of the steps leading to activation of the NLRP3 inflammasome and caspase-1 is beyond the scope of this study, but our results show that it is independent of either pannexin-1 or P2X₇. Possible pathways include the activation of Maxi-K channels, which are activated by LPS (18, 19) and facilitate potassium efflux without involvement of the pannexin-1/ATP/P2X₇ axis (Figure 5B).
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Authorship contributions
Katarzyna Parzych - Contributed to the design of experiments, carried out experiments and contributed to the writing of the manuscript
Anna Zetterqvist - Contributed to the design of experiments, carried out experiments and contributed to the writing of the manuscript
William R. Wright - Carried out experiments
Nicholas S. Kirkby - Contributed to the design of experiments, carried out experiments and contributed to the writing of the manuscript
Jane A. Mitchell - Contributed to the design of experiments and writing of the manuscript
Mark J. Paul-Clark - Contributed to the design of experiments, carried out experiments and contributed to the writing of the manuscript

Conflict of interest/disclosures
No author has any commercial arrangements or funding that would have direct influence on the data in this paper.
References


Figure 1. Production of pro-IL-1β and release of mature IL-1β from human monocytes in response to Gram +ve and Gram -ve PAMPs. THP-1 monocytes were treated with (A) concentration responses (0.001-1µg/ml) to LPS, Pam3CSK4 and FSL-1 for 24h and IL-1β release measured by ELISA. (B) Intracellular levels of pro- IL-1β were measured by ELISA after 24h stimulation with LPS (0.1µg/ml) and Pam3CSK4 (0.1µg/ml). In addition, monocytes were treated with, LPS (1 µg/ml) or Pam3CSK4 (1 µg/ml) for 3 or 24h after which media was removed and cells were pulsed for 30min with either ATP (5mM) or fresh media. IL-1β release was measured after (C) 3h and (E) 24h. Data represents mean ± SEM of a total of at least n=9 replicates. *Denotes p≤ 0.05 as assessed using a one-way ANOVA followed by a Dunnett’s post-hoc test.
Figure 2. The effect inhibiting inflammasome components on TLR2 and TLR4 induced IL-1β release from human monocytes. THP-1 monocytes were stimulated for 24h with LPS (0.1µg/ml), Pam$_3$CSK4 (0.1µg/ml) and FSL-1 (0.1µg/ml) after 30min pre-treatment with (A) pan-caspase inhibitor Z-VAD-FMK (0.01-1µM), (B) pannexin-1 inhibitor carbenoxolone (0.3-30µM), (C) P2X$_7$ inhibitor AZ 11645373 (0.01-10µM) and (D) MaxiK channel inhibitor paxilline (5-20 µM) and IL-1β levels measured by ELISA. Data represents mean ± SEM of a total of at least n=9 replicates. *Denotes p<0.05 as assessed using a one-way ANOVA followed by a Dunnett’s post-hoc test.
Figure 3. Effect of pannexin-1 gene knockdown on TLR2 and TLR4 induced IL-1β release from human monocytes. THP-1 cells were transfected with 100µM of pannexin-1 siRNA or scrambled siRNA using Amaxa Nucleofector system. After 48h (A) gene expression was measured by quantitative RT-PCR and (B) protein levels were measured by western blot analysis. Transfected THP-1 monocytes were stimulated with (C) Pam3CSK4 (0.01-1µg/ml) and (D) LPS (0.01-1µg/ml). PCR transfection data is representative of n=3 separate transfections and Western blot analysis is representative of these transfections. *denotes p≤ 0.05 as assessed using a one-way ANOVA followed by a Dunnett’s post-hoc test. Stimulation data represents mean ± SEM of n=9 replicates. *Denotes p≤ 0.05 as assessed using a two-way ANOVA followed by a Bonferoni post-hoc test.
**Figure 4.** ATP release and caspase-1 activity in human monocytes stimulated with TLR2 and TLR4 ligands. THP-1 monocytes were stimulated with LPS (0.1 µg/ml) and Pam3CSK4 (0.1 µg/ml) and (A) ATP release assessed after 30min and (B) caspase-1 activity after 24h. Data represents mean ± SEM of n=9 replicates. *Denotes p≤ 0.05 as assessed using a one-way ANOVA followed by a Dunnett’s post-hoc test.

**Figure 5.** Putative signaling for panexin-1/ATP/P2X7 dependent and independent release of IL-1β by TLR2 and TLR4 agonists respectively. IL-1β release induced by the TLR2 and TLR4 ligands from human monocytes. TLR2 agonists induce expression of pro-IL-1β via NFkB and activate ATP release via panexin-1. ATP activates P2X7, which mediates potassium ion efflux and hyperpolarization leading to activation of caspase-1 which cleaves pro-IL-1β resulting in the release of mature IL-1β. The TLR4 agonist, LPS activates TLR4 and NFkB pathways resulting in increased expression of pro-IL-1β. LPS evades pannexin activation resulting in reduced ATP release but activates MaxiK channels resulting in potassium ion efflux and hyperpolarization resulting in the release of mature IL-1β.