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Prolonged Exposure to Lipopolysaccharide Induces NLRP3-Independent Maturation and Secretion of Interleukin (IL)-1β in Macrophages

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Upon sensing of microbial infections or endogenous danger signals in macrophages, inflammasome signaling plays a significant role in triggering inflammatory responses via producing interleukin (IL)-1β. Recent studies revealed that active caspase-1, a product of the inflammasome complex, causes maturation of inactive pro-IL-1β into the active form. However, the underlying mechanism by which this leaderless cytokine is secreted into the extracellular space remains to be elucidated. In this study, we demonstrated that prolonged lipopolysaccharide (LPS) treatment to macrophages could trigger the unexpected maturation and extracellular release of IL-1β through a nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3 (NLRP3)-independent manner. Short-term treatment (less than 6 h) of LPS induced robust production of the IL-1β precursor form inside cells but did not promote the maturation and secretion of IL-1β in bone marrow-derived macrophages or peritoneal macrophages. Instead, prolonged LPS treatment (more than 12 h) led to a significant release of matured IL-1β with no robust indication of caspase-1 activation. Intriguingly, this LPS-triggered secretion of IL-1β was also observed in NLRP3-deficient macrophages. In addition, this unexpected IL-1β release was only partially impaired by a caspase-1 and NLRP3 inflammasome inhibitor. Collectively, our results propose that prolonged exposure to LPS is able to drive the maturation and secretion of IL-1β in an NLRP3 inflammasome-independent manner.

Keywords: Inflammasome, interleukin-1β, lipopolysaccharide, NLRP3

Introduction

Interleukin (IL)-1β is the key proinflammatory cytokine to trigger or promote the inflammatory responses at sites of microbial infection or tissue injury [1]. Unlike other proinflammatory cytokines, IL-1β contains two unique features, including expression as the immature form pro-IL-1β [1] and lack of the endoplasmic reticulum-targeting leader sequence [2]. In this regard, the production of active IL-1β requires both posttranslational maturation and unconventional secretion to the extracellular space. It has been well known that active caspase-1 mediates the proteolytic cleavage of pro-IL-1β into the active form [3]. Furthermore, previous study demonstrated that caspase-1 is required for the non-classical secretion of IL-1β [4], although the detailed mechanism of IL-1β secretion remains to be further elucidated.

In this context, caspase-1 activation is the most critical event for the maturation and secretion of IL-1β. Recent studies revealed that caspase-1 is activated by the formation of an inflammasome multiprotein complex mainly in myeloid cells such as macrophages [5, 6]. Recognition of microbe- or tissue damage-derived factors by inflammasome sensor molecules, such as nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3 (NLRP3), triggers assembly of the inflammasome sensor molecule, adaptor molecule, and inactive procaspase-1 [7]. The formation of the inflammasome complex then leads to the activation of caspase-1 by proximity-induced autoprocessing of procaspase-1.

In particular, NLRP3 recognizes a wide range of pathogen- or danger signal-associated molecular patterns, including microbial toxins and endogenous abnormal metabolites [8]. Although the detailed molecular mechanism by which NLRP3 is activated in response to diverse stimuli is still poorly understood, full activation of the NLRP3 inflammasome generally requires two independent signals; a Toll-like receptor (TLR)-mediated signal 1 such as lipopolysaccharide (LPS) for the transcriptional induction of NLRP3, and a signal 2 such as ATP for the activation of NLRP3 [9]. However, recent studies further revealed that LPS, a signal 1 stimulus, induces a non-transcriptional priming step, required for assembly of the NLRP3 inflammasome [10–12]. Additionally, it was also reported that LPS stimulation in the absence of signal 2 could promote the secretion of IL-1β in certain cell types, such as dendritic cells, monocytes, and PMA-differentiated THP-1 macrophages [2, 13–15]. Furthermore, LPS administration to mice led to a robust increase in the inflammatory phenotype, such as serum or bronchiolar lavage fluid level of IL-1β [16, 17]. These findings raised a possibility that LPS stimulation alone can trigger the activation of IL-1β in certain contexts. In the present study, we thus attempted to examine the prolonged exposure to LPS for the maturation and secretion of IL-1β in macrophages.

Materials and Methods

Mice

C57BL/6 and *Nlrp3*$^{-/-}$ (C57BL/6 background) mice were obtained from Jackson Laboratory and bred at Yonsei University College of Medicine. All mice were maintained under specific pathogen-free conditions. Seven- to 10-week-old male mice were used for all experiments. Protocols for the animal experiments were approved by the Institutional Ethical Committee, Yonsei University College of Medicine (2014-0257). All experiments were performed in accordance with the approved guidelines of the Institutional Ethical Committee.

Reagents and Antibodies

LPS, ATP, poly dA:dT, nigericin, and glibenclamide were purchased from Sigma-Aldrich (USA). Ultrapure LPS was obtained from Invivogen (USA). YVAD-chloromethylketone and z-VAD-fluoromethylketone were purchased from Bachem (Switzerland). Anti-mouse IL-1β antibody was obtained from R&D Systems (USA). Anti-mouse caspase-1 (p20) and anti-NLRP3 antibodies were from Adipogen (Switzerland). Anti-ASC antibody was obtained from Santa Cruz (USA).

Cell Cultures

Mouse bone marrow cells were isolated from mouse femurs and differentiated into bone marrow-derived macrophages (BMDMs) with L929-conditioned medium. Immortalized *Nlrp3*-deficient and *Nlrp3*-reconstituted BMDMs were provided by E.S. Alnemri (Thomas Jefferson University, Philadelphia, PA, USA). All BMDMs were maintained in L929-conditioned DMEM supplemented with 10% FBS and antibiotics. Mouse peritoneal macrophages were prepared as described below. In brief, mice were injected with 3% BBL thioglycollate medium (BD, USA). After 4 days, the mice were euthanized and injected with 10 ml of cold PBS into the peritoneal cavity. The peritoneal fluid was restored by aspiration and centrifuged, and the cell pellets were maintained in DMEM.

Assay of Inflammasome Activation and Cytokine Production

To induce classical activation of the NLRP3 inflammasome as a positive control, cells were primed with LPS (0.25 μg/ml) for 3 h, followed by treatments with ATP or nigericin. To stimulate the absent in melanoma 2 (AIM2) inflammasome, poly dA:dT was transfected into macrophages using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instruction. Inflammasome activation was determined by the detection of active caspase-1 p20 and active IL-1β in culture supernatants using immunoblots, and by quantification of extracellular IL-1β using the mouse IL-1β Quantikine ELISA kit (R&D Systems). The level of IL-6 was determined by ELISA (R&D Systems).

Assay of ASC Oligomerization

To determine the oligomerization of ASC, disuccinimidyl suberate (DSS)-mediated cross-linking assay was performed as described previously [18]. Briefly, cells were lysed and centrifuged at 3,500 ×*g* for 10 min. The pellets were washed with PBS and then resuspended in PBS. The resuspended pellets were cross-linked with 1 mM DSS (Pierce, USA) for 30 min. The cross-linked pellets and soluble lysates were fractionated by SDS-PAGE and immunoblotted with anti-ASC antibody.

Immunoblot Analysis

Cells were lysed in buffer containing 20 mM HEPES (pH 7.5), 0.5% Nonidet P-40, 50 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, and protease inhibitors. Soluble lysates were fractionated by SDS-PAGE and then transferred to PVDF membranes. In some experiments, cell culture supernatants were precipitated by the addition of a methanol/chloroform mixture as described previously [6] and then immunoblotted. All the blots shown are representative images of at least three-independent experiments. Images have been cropped for presentation.

Statistical Analysis

All values are expressed as the mean and standard error of individual samples. Data were analyzed using two-way analysis of variance followed by Bonferroni post hoc testing. The level of statistical significance was set at $p \leq 0.05$. Analyses were performed with GraphPad Prism.

Results

Prolonged Treatment of Lipopolysaccharide Leads to the Maturation and Secretion of IL-1β in Macrophages

To examine whether extensive exposure to LPS alone could drive the activation of IL-1β in macrophages, LPS was treated into BMDMs for up to 24 h. LPS plus ATP or nigericin treatment was used as a positive control to stimulate NLRP3 inflammasome signaling. The 3 h treatment of LPS to BMDMs promoted a strong production of pro-IL-1β inside the cells, but did not promote the secretion of IL-1β into the culture medium (Fig. 1A). Instead, a longer exposure of LPS for more than 12 h caused a slight secretion of matured IL-1β in the culture supernatants of BMDMs (Fig. 1A). To exclude the possibility that any contaminants in the LPS could induce this unexpected release of IL-1β, we also tested the effect of ultrapure (up)-LPS as well. Similar to LPS, prolonged treatment of up-LPS triggered a sharp release of IL-1β in BMDMs (Fig. 1A) and in murine peritoneal macrophages, as determined by immunoblots (Fig. 1B). Up-LPS also caused robust activation of IL-1β, as determined by the presence of mature IL-1β in the culture supernatants, in immortalized BMDMs (Fig. 1C). Of note, prolonged treatment with up-LPS led to the release of mature IL-1β, but not of active caspase-1, in peritoneal macrophages and immortalized BMDMs (Figs. 1B and 1C). These findings suggest that extensive LPS exposure is highly likely to promote the maturation and secretion of IL-1β irrespective of caspase-1 activation.

Prolonged Treatment of Lipopolysaccharide Causes the Activation of IL-1β in an NLRP3-Independent Manner

To examine whether the observed LPS-triggered activation of IL-1β is associated with NLRP3 inflammasome activation, we performed similar experiments in *Nlrp3*$^{+/+}$ and *Nlrp3*$^{-/-}$ BMDMs. Similar to Fig. 1, 12 h treatment of LPS, but not 3 h treatment, promoted robust secretion of mature IL-1β in the culture supernatants of NLRP3-expressing wild-type BMDMs (Fig. 2A). Of note, this LPS-induced activation of IL-1β was also clearly found in NLRP3-deficient BMDMs, whereas LPS/nigericin-stimulated IL-1β secretion was completely abolished in *Nlrp3*$^{-/-}$ cells (Fig. 2A). We also examined this NLRP3-independent IL-1β release from BMDMs using up-LPS. Prolonged treatment of up-LPS clearly induced the secretion of IL-1β in NLRP3-deficient BMDMs as well as in wild-type cells (Fig. 2B). Consistent with this data, prolonged up-LPS treatment triggered a considerable production of IL-1β, as quantified by ELISA, in both *Nlrp3*$^{+/+}$ and *Nlrp3*$^{-/-}$ BMDMs (Fig. 2C). Of note, the secreted level of IL-1β was significantly less in NLRP3-deficient BMDMs than that in wild-type cells (Fig. 2C). These data raised a possibility that LPS-triggered activation of IL-1β is partly dependent on the presence of NLRP3 in BMDMs. Indeed, weak caspase-1 processing was found in the culture supernatants of wild-type BMDMs upon treatment with LPS for 24 h, but not of *Nlrp3*$^{-/-}$ cells (Fig. 2B). Unlike IL-1β, the production of IL-6 was similar

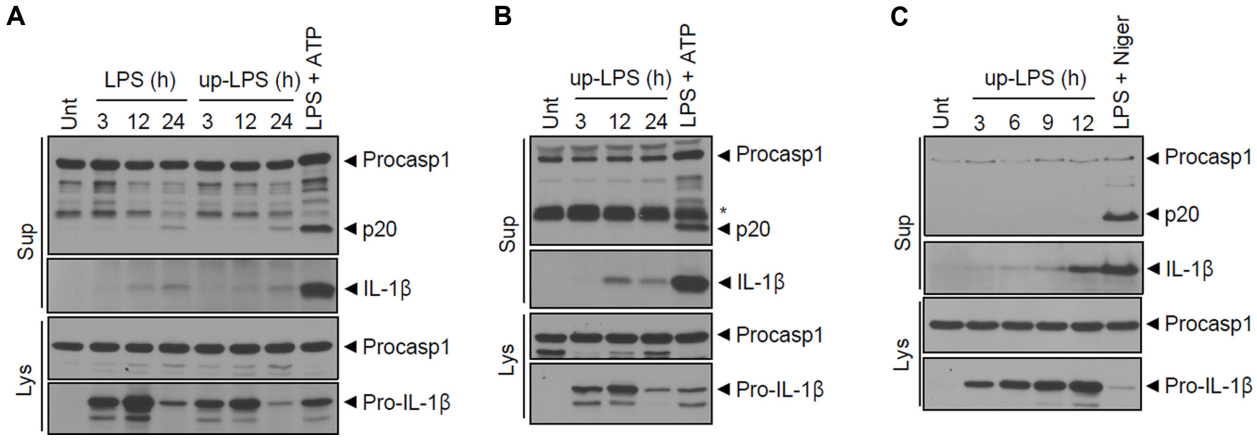

Fig. 1. Prolonged LPS treatment triggers the release of IL-1β.

(**A**) Mouse bone marrow-derived macrophages (BMDMs) were treated with LPS (0.25 μg/ml) or ultrapure-LPS (up-LPS, 0.25 μg/ml) for the indicated times, or primed with LPS (0.25 μg/ml) for 3 h, followed by the treatment of ATP (2 mM) for 30 min. (**B**, **C**) Mouse peritoneal macrophages (**B**) or immortalized BMDMs (**C**) were treated with up-LPS (0.25 μg/ml) for the indicated times, or primed with LPS (0.25 μg/ml) for 3 h, followed by the treatment of ATP (2 mM, 30 min, B) or nigericin (niger, 5 μM, 30 min, C). (**A**–**C**) Culture supernatants (Sup) or cellular lysates (Lys) were immunoblotted with the indicated antibodies. Unt, untreated. Procasp 1, procaspase-1.

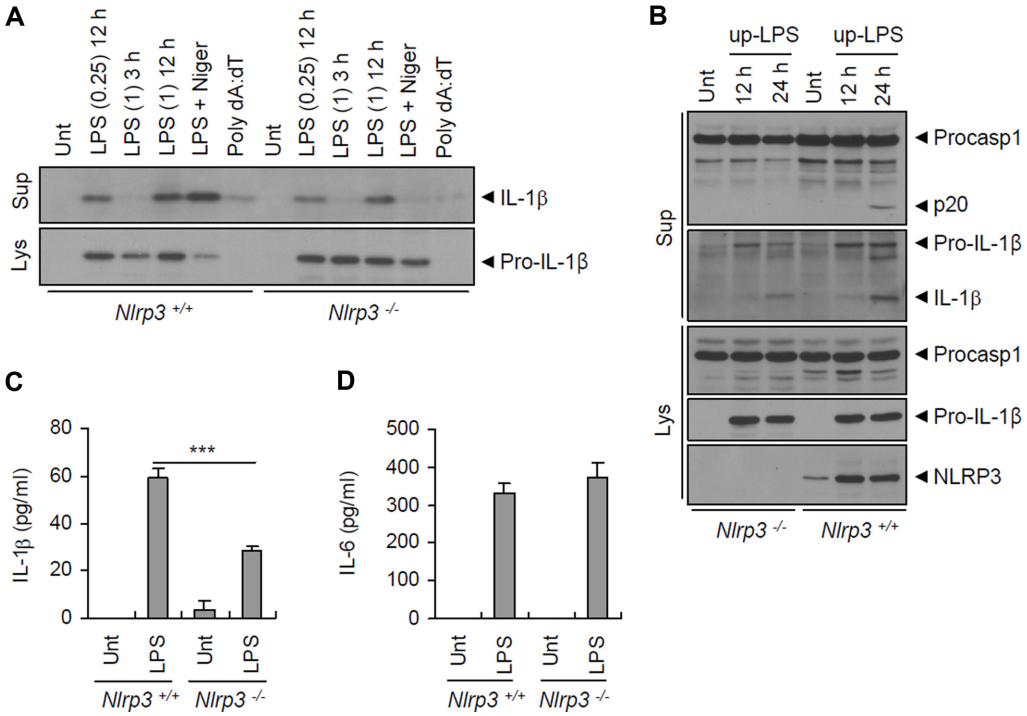

Fig. 2. Prolonged LPS treatment promotes NLRP3-independent release of IL-1β in bone marrow-derived macrophages (BMDMs). (**A**) *Nlrp3*$^{+/+}$ or *Nlrp3*$^{-/-}$ mouse BMDMs were treated with LPS (0.25 or 1 μg/ml) for 3 or 12 h as indicated, or primed with LPS (0.25 μg/ml, 3 h), followed by the treatment with nigericin (5 μM, 30 min), or transfected with poly dA:dT (1 μg/ml, 6 h). (**B**) *Nlrp3*$^{+/+}$ or *Nlrp3*$^{-/-}$ mouse BMDMs were treated with ultrapure (up)-LPS (0.25 μg/ml) for 12 or 24 h. (**A**, **B**) Culture supernatants (Sup) or cellular lysates (Lys) were immunoblotted with the indicated antibodies. (**C**, **D**) *Nlrp3*$^{+/+}$ or *Nlrp3*$^{-/-}$ mouse BMDMs were treated with up-LPS (0.25 μg/ml) for 24 h. Culture supernatants were assayed for the quantification of IL-1β (C, $n = 3$) or IL-6 (D, $n = 3$) by ELISA. Asterisks indicate significant differences (***$p < 0.001$).

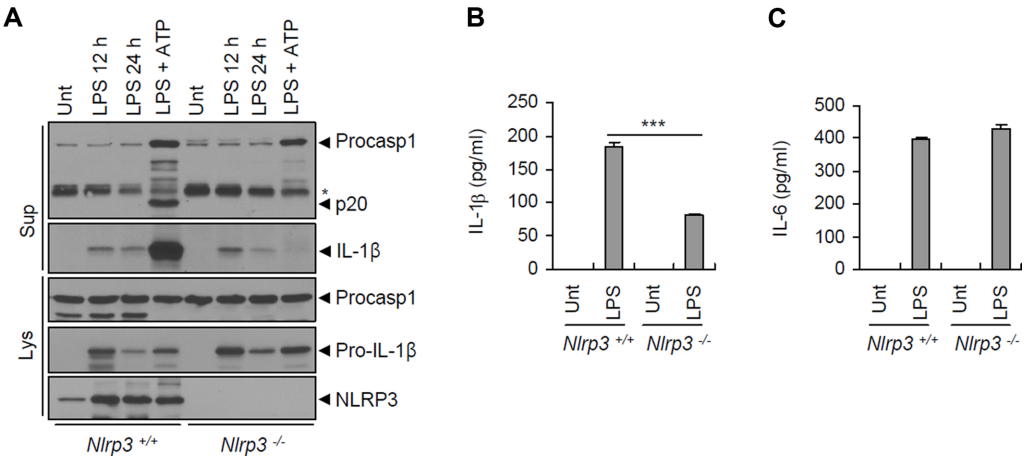

Fig. 3. Prolonged LPS treatment promotes NLRP3-independent release of IL-1β in peritoneal macrophages.
(**A**) *Nlrp3*$^{+/+}$ or *Nlrp3*$^{-/-}$ mouse peritoneal macrophages were treated with ultrapure (up)-LPS (0.25 μg/ml, 12 or 24 h), or primed with up-LPS (0.25 μg/ml) for 3 h, followed by the treatment of ATP (2 mM) for 30 min. Culture supernatants (Sup) or cellular lysates (Lys) were immunoblotted with the indicated antibodies. (**B**, **C**) *Nlrp3*$^{+/+}$ or *Nlrp3*$^{-/-}$ mouse peritoneal macrophages were treated with up-LPS (0.25 μg/ml) for 12 h. Culture supernatants were assayed for the quantification of IL-1β (B, $n = 3$) or IL-6 (C, $n = 3$) by ELISA. Asterisks indicate significant differences (***$p < 0.001$).

in both *Nlrp3*$^{+/+}$ and *Nlrp3*$^{-/-}$ BMDMs upon LPS treatment (Fig. 2D).

We also checked the implication of NLRP3 in IL-1β activation by prolonged LPS treatment in peritoneal macrophages. Similar to that observed in BMDMs, up-LPS treatment caused robust secretion of mature IL-1β in wild-type and NLRP3-deficient peritoneal macrophages (Fig. 3A). Of interest, the LPS-driven production level of IL-1β in *Nlrp3*$^{-/-}$ peritoneal macrophages was significantly less than that in wild-type cells, similar to BMDMs (Fig. 3B), suggesting that extensive exposure to LPS could promote the activation of IL-1β through NLRP3-dependent and -independent pathways. In addition, prolonged LPS treatment produced a similar level of IL-6 in peritoneal macrophages (Fig. 3C). These findings clearly support that LPS stimulation is able to trigger, at least in part, the NLRP3-independent maturation and secretion of IL-1β.

Prolonged Treatment of Lipopolysaccharide Causes Inflammasome-Independent Activation of IL-1β

To further examine whether prolonged LPS exposure could activate inflammasome signaling in macrophages, we checked for ASC oligomerization, an essential event of inflammasome activation [19]. LPS priming followed by

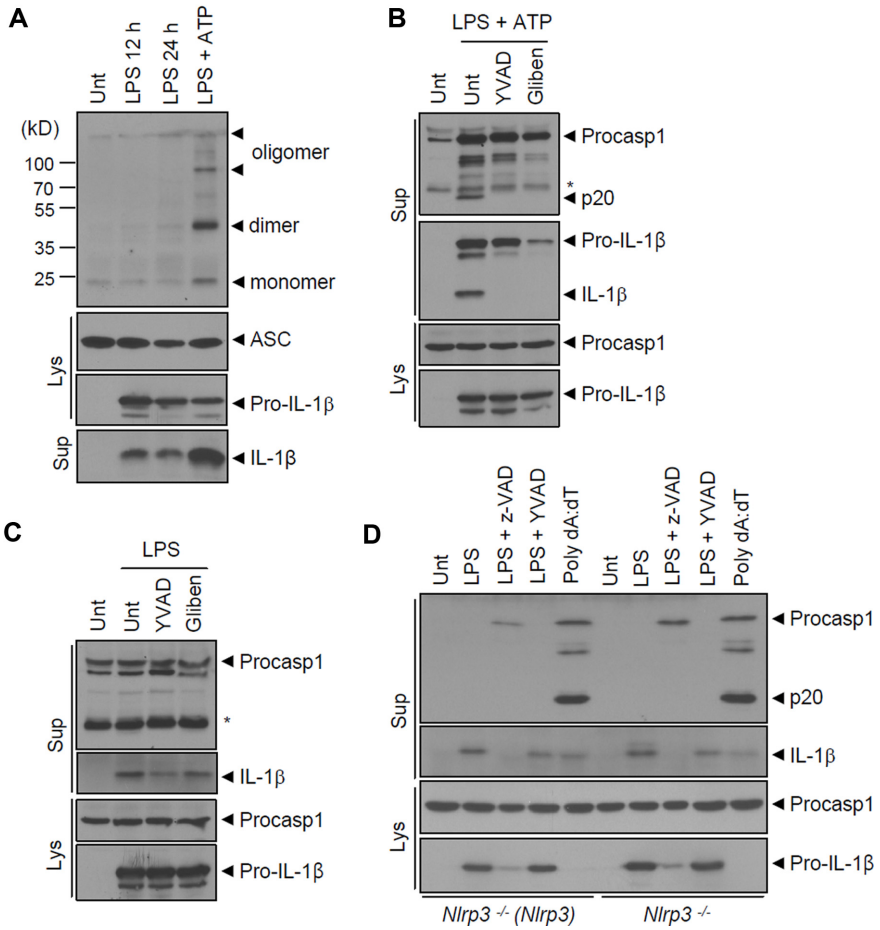

Fig. 4. Prolonged LPS treatment does not induce ASC oligomerization but promotes inflammasome-independent IL-1β release. (**A**) Mouse peritoneal macrophages were treated with ultrapure (up)-LPS (0.25 μg/ml, 12 or 24 h), or primed with up-LPS (0.25 μg/ml) for 3 h, followed by the treatment of ATP (2 mM) for 30 min. Disuccinimidyl suberate-crosslinked pellets (upper panel), culture supernatants (Sup), or cellular lysates (Lys) were immunoblotted with the indicated antibodies. (**B**) Mouse peritoneal macrophages were treated with up-LPS (0.25 μg/ml) for 3 h in the presence of YVAD (20 μM) or glibenclamide (gliben, 50 μM), followed by the treatment of ATP (2 mM) for 30 min. (**C**) Mouse peritoneal macrophages were treated with up-LPS (0.25 μg/ml) for 12 h in the presence of YVAD (20 μM) or glibenclamide (gliben, 50 μM). (**D**) Immortalized *Nlrp3*-reconstituted or *Nlrp3*-deficient bone marrow-derived macrophages were treated with LPS (0.25 μg/ml) for 12 h in the presence of z-VAD or YVAD (20 μM), or transfected with poly dA:dT (1 μg/ml, 6 h). (**B**–**D**) Culture supernatants (Sup) or cellular lysates (Lys) were immunoblotted with the indicated antibodies.

ATP, a classical NLRP3 inflammasome stimulation, promoted robust oligomerization of ASC in peritoneal macrophages (Fig. 4A). On the contrary, 12 or 24 h treatment of LPS failed to induce ASC oligomerization, but drove the secretion of mature IL-1β in peritoneal macrophages (Fig. 4A). This observation indicates that LPS-triggered activation of IL-1β is not associated with the assembly of the ASC-mediated inflammasome.

Then, we further examined the implication of the inflammasome in LPS-triggered IL-1β activation using a caspase-1-specific inhibitor, YVAD, or NLRP3 inflammasome inhibitor, glibenclamide, a blocking reagent of potassium efflux. Both YVAD and glibenclamide clearly abolished caspase-1 activation and IL-1β secretion in peritoneal macrophages upon LPS/ATP stimulation (Fig. 4B). However, the secretion of mature IL-1β from peritoneal macrophages was still evident in the presence of YVAD and glibenclamide in response to prolonged LPS treatment (Fig. 4C), although there was a slight decrease in IL-1β by cotreatment with YVAD. To further provide molecular insights, we performed similar experiments with z-VAD, a pan-caspase inhibitor. Intriguingly, z-VAD, but not YVAD, treatment completely abrogated the secretion of mature IL-1β from NLRP3-expressing and NLRP3-deficient BMDMs upon stimulation with LPS (Fig. 4D), indicating that caspases other than caspase-1 may be involved in the LPS-triggered IL-1β activation in macrophages. Collectively, our data propose that prolonged LPS stimulation seems likely to induce the maturation and secretion of IL-1β in a caspase-1- or NLRP3 inflammasome-independent manner.

Discussion

Despite the significance of the NLRP3 inflammasome involving the pathogenesis of inflammatory and metabolic disorders, the mechanistic basis regarding the activation of NLRP3 remains to be further determined [20]. Initially, the role of the TLR-engaged priming stimulus, called signal 1, for the inflammasome activation was considered to induce the expression of pro-IL-1β and NLRP3 [21]. However, recent studies revealed that LPS stimulation could mediate the posttranslational modifications of NLRP3 to facilitate the activation [10, 11, 22]. In addition, LPS treatment alone was shown to activate the inflammasome pathway in certain cellular contexts [2, 13]. In this regard, we attempted to revisit the role of LPS, a signal 1 stimulus, for activation of the NLRP3 inflammasome, for the maturation and secretion of IL-1β in macrophages.

Unexpectedly, we found that extensive LPS exposure could induce only modest activation of IL-1β in BMDMs and peritoneal macrophages. As this LPS-driven IL-1β activation was also found in NLRP3-deficient cells, our results indicate that extensive LPS stimulation is able to trigger NLRP3-independent maturation and secretion of IL-1β in macrophages. The secreted level of IL-1β by ELISA was significantly less in *Nlrp3*-knockout macrophages than that in wild-type macrophages (Figs. 2C and 3B). These data claim a possibility that prolonged LPS exposure also mediates NLRP3-dependent activation of IL-1β. However, the quantification of IL-1β by ELISA could not discriminate premature pro-IL-1β and active IL-1β form. It is thus possible that the reduced level of IL-1β based on ELISA in NLRP3-deficient cells upon LPS exposure reflects less production of pro-IL-1β rather than IL-1β. Therefore, further studies need to be done to evaluate whether LPS stimulation could induce NLRP3-dependent activation of IL-1β in macrophages.

Furthermore, the potential implication of caspase-1 during LPS-triggered IL-1β activation remains to be determined. There is likely to be a slight reduction in the secreted level of mature IL-1β in the presence of caspase-1-selective inhibitor YVAD (Fig. 4C). However, the maturation and secretion of IL-1β was still observed in the presence of YVAD, as determined by immunoblots. On the other hand, the LPS-triggered secretion of IL-1β was completely abrogated by pan-caspase inhibitor z-VAD. These findings suggest that extensive LPS stimulation could promote, at least in part, caspase-1-independent, but possibly other caspase-mediated activation, of IL-1β in macrophages.

The underlying mechanism of LPS-promoted maturation and secretion of IL-1β is not fully understood at present. Intracellular LPS has been shown to promote caspase-11-mediated noncanonical activation of caspase-1 [23, 24]. In addition, Eltom *et al*. [17] showed that LPS challenge in mice could release IL-1β in the lung via the NLRC4-dependent, but caspase-1-, caspase-11-, and caspase-8-independent, pathway. Moreover, a recent study presented that LPS plus histone deacetylase inhibitors promoted the caspase-1-independent, but caspase-8-dependent, activation of IL-1β in dendritic cells and in macrophages [25]. To correlate with these previous findings, future studies will be required to clarify whether NLRC4, caspase-8, or caspase-11 is implicated in the activation of IL-1β by extensive LPS stimulation of macrophages. At present, our data propose that prolonged LPS exposure in the absence of NLRP3 agonists could potentially allow noncanonical NLRP3- and caspase-1-independent maturation and secretion of IL-1β.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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