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TLR Agonists Stimulate Nlrp3-Dependent IL-1β Production Independently of the Purinergic P2X7 Receptor in Dendritic Cells and In Vivo

Yuan He,1 Luigi Franchi,1 and Gabriel Nuñez

On the basis of studies in mouse macrophages, activation of the nucleotide-binding oligomerization domain–like receptor (NLR) pyrin domain–containing 3 (Nlrp3) inflammasome is thought to require two signals. The first signal is provided by TLR stimulation and triggers the synthesis of the IL-1β precursor and Nlrp3. The second signal can be mediated by stimulation of the purinergic receptor P2X ligand–gated ion channel 7 (P2X7) by millimolar concentrations of ATP. However, these high concentrations of ATP are not found normally in the in vivo extracellular milieu, raising concern about the physiological relevance of the ATP–P2X7 pathway of inflammasome activation. In this article, we show that unlike macrophages, murine bone marrow–derived and splenic dendritic cells (DCs) can secrete substantial amounts of mature IL-1β upon stimulation with TLR ligands in the absence of ATP stimulation. The differential ability of DCs to release IL-1β and activate caspase-1 was associated with increased expression of Nlrp3 under steady-state conditions and of pro–IL-1β and Nlrp3 after stimulation with TLR agonists. IL-1β secretion from stimulated DCs was largely dependent on the Nlrp3 inflammasome, but independent of P2X7 and unaffected by incubation with apyrase. More importantly, i.p. administration of LPS induced IL-1β production in serum, which was abrogated in Nlrp3-null mice but was unaffected in P2X7-deficient mice. These results demonstrate differential regulation of the Nlrp3 inflammasome in macrophages and DCs. Furthermore, they challenge the idea that the ATP-P2X7 axis is critical for TLR-induced IL-1β production via the Nlrp3 inflammasome in vivo. The Journal of Immunology, 2013, 190: 000–000.

Interleukin-1β is a critical mediator in the induction of immune responses and the development of inflammatory diseases (1). Blood monocytes, tissue macrophages, and dendritic cells (DCs) are the primary sources of IL-1β. Because IL-1β is a potent proinflammatory factor, its production is tightly regulated at both transcriptional and translational levels. Under normal conditions, IL-1β is not constitutively expressed, but its expression is induced in response to stimulation with microbial products, including TLR ligands or certain endogenous stimuli such as TNF-α or IL-1β itself (2, 3). Unlike its family member IL-1α, the IL-1β precursor is synthesized as an inactive precursor (pro–IL-1β) that is cleaved into its biologically active product by activated caspase-1, also known as IL-1β-converting enzyme (4). Caspase-1 activation occurs through autoproteolytic cleavage of pro-caspase-1, which can be initiated by inflammasomes, multi-protein complexes that include a member of the nucleotide-binding oligomerization domain–like receptor (NLR) family as a sensor and the adaptor protein apoptosis-associated specklike protein (Asc) (5).

It has been proposed that, on the basis of studies in mouse macrophages, activation of the NLR pyrin domain–containing 3 (Nlrp3) inflammasome requires two signals. The first signal, referred to as priming, is the NF-κB–dependent production of pro–IL-1β and Nlrp3, through stimulation with microbial products or certain cytokines (6, 7). The second signal activates Nlrp3 and is induced by ATP, certain bacterial toxins, or particulate matter (8). ATP induces Nlrp3 activation through stimulation of the purinergic receptor P2X ligand–gated ion channel 7 (P2X7), which induces K+ efflux (9). In contrast, human monocytes secrete active IL-1β in response to TLR ligands alone, which has been suggested to be dependent on autocrine stimulation by extracellular ATP and the Nlrp3 inflammasome (10, 11). The concentration of ATP required for P2X7-mediated caspase–1 activation is in the millimolar range (12). These high concentrations of ATP are not found normally in the in vivo extracellular milieu, although they could perhaps be reached under certain situations in the context of cell lysis or injury.

Murine bone marrow–derived DCs (BMDCs) have been used for studying inflammasome activation (13, 14), although whether DCs behave like macrophages or monocytes in terms of IL-1β secretion upon stimulation with TLR ligands is largely unknown. In this study, we used murine BMDCs and splenic DCs to investigate IL-1β secretion from DCs in response to TLR ligands. We found that DCs stimulated by TLR ligands can secrete substantial amounts of mature IL-1β (p17), which was dependent on the Nlrp3 inflammasome, but independent of the purinergic P2X7 receptor. Importantly, we demonstrated that P2X7 is not required for IL-1β production in response to LPS administration in vivo.
TLRs INDUCE IL-1β VIA NLRP3 INDEPENDENTLY OF P2X7 IN VIVO

Materials and Methods

Mice

Mice deficient in P2X7, Nlrp3, Nlr4, caspase-1, or Asc on the C57BL6 background have been previously described (15); wild-type (WT) C57BL6 mice were originally purchased from The Jackson Laboratory and bred in our animal facility. All mice were maintained in a specific pathogen–free facility. All protocols of animal studies were approved by the University of Michigan Committee on Use and Care of Animals.

Reagents

Ultrapure LPS from E. coli 0111:B4, synthetic monophosphoryl lipid A (Lipid A), Pam2CSK4, Pam3CSK4, lower m.w. polynosinic/polycydidylic acid (poly[ICL]), R848, and CpG (ODN 1826) were purchased from Invivogen. ATP was from Sigma-Aldrich. Murine IL-1β Ab (AF-401-NA) was purchased from R&D Systems. Gapdh Ab was purchased from GenScript. Caspase-1 Ab (sc-514) was purchased from Santa Cruz Biotechnology. IL-18 Ab (5180R-100) was purchased from BioVision. Ab for mouse Asc has been described (16). Rabbit anti-mouse Nlpr3 Ab was generated by immunizing rabbits with mouse Nlrp3 protein (aa 1–194) expressed in E. coli and purified by affinity chromatography using a nickel column.

Cell culture and splenic DC isolation

Mouse BMDCs and bone marrow–derived macrophages (BMDMs) were prepared as previously described (6). DCs were used for experiments after 7–8 d of culture when CD11c expression, analyzed by flow cytometry, was >90%. Cells were seeded at 4 × 10^5 cells per well in 48-well plates or 2 × 10^6 per well in 12-well plates the day before the experiment. Spleenic DCs (CD11c^+ CD11b^-) and macrophages (CD11c^- CD11b^+) were freshly isolated from mouse spleens by positive and negative selection using MACS beads (Miltenyi Biotec). Cells were suspended in RPMI 1640 medium containing glutamine, sodium pyruvate, and 10% heat-inactivated FBS (Life Technologies–BRL) and were seeded at 2 × 10^5 cells per well in 96-well plates.

Cytotoxicity assay

The percentage of cell death was determined using the LDH release assay (Promega). The absorbance at 490 nm was measured, and the percentage of cytotoxicity was calculated relative to the 100% release value obtained by lysis of cells with a solution of 0.1% Triton X-100.

Measurements of cytokines

Mouse IL-1β and TNF-α in culture supernatants or serum were measured by ELISA kits (R&D Systems). Assays were performed in triplicate for each independent experiment.

Immunoblotting

Cells were lysed in ice-cold PBS buffer containing 1% Nonidet P-40 supplemented with complete protease inhibitor mixture (Roche, Mannheim, Germany). Mature IL-1β in the culture supernatant was precipitated by 7.7% trichloroacetic acid. Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes by electroblotting (Bio-Rad), and membranes were immunoblotted with respective Abs.

cDNA synthesis and real-time RT-PCR

BMDCs and BMDMs were stimulated with LPS (100 ng/ml^-1) for the indicated periods or were left unstimulated. Total RNA extraction, cDNA synthesis, and real-time PCR were carried out as previously described (17). The primer sequences were as follows: IL-1β forward, 5'-TTGAATGA-AAGACGGCACACC-3'; IL-1β reverse, 5'-TCTCTTTGCTATTGCT-TGG-3'; Nlrp3 forward, 5'-CCCTTGGGAGACAGAAGCTC-3'; Nlrp3 reverse, 5'-GAGGCTCGAGTCTAATTCC-3'; TNF-α forward, 5'-TCTTCTATTCTCTGTTGG-3'; TNF-α reverse, 5'-GGTGCTGCGC-CATGAGACGACTC-3'; GAPDH forward, 5'-AGCTTGCTATCAACGGGA-AG-3'; GAPDH reverse, 5'-TGTATGTTAGTGCTGGTCTG-3'; IL-1β, Nlrp3, or TNF-α to GAPDH relative expression was calculated using the 2^(-ΔΔCT) method and normalized to the level of unstimulated BMDCs.

Endotoxemia

For the measurement of cytokines in serum, mice were injected i.p. with 25 mg/kg^-1 LPS (E.coli 0111:B4; Sigma-Aldrich), and mouse serum was collected 1 and 3 h after injection.

Statistical analysis

Student t test was used to determine statistically significant differences between two groups. One-way ANOVA was used to analyze differences among multiple groups. A p value < 0.05 was considered significant.

Results

Activation of TLRs is sufficient for IL-1β secretion in murine DCs

We initially compared the ability of mouse BMDMs and BMDCs to release IL-1β in response to different concentrations of LPS in the absence of exogenous ATP. Consistent with previous results, stimulation with LPS alone did not induce the release of IL-1β in BMDCs, but it triggered secretion of TNF-α (Fig. 1A, 1B). In contrast, BMDCs produced robust amounts of both IL-1β and TNF-α in response to LPS alone (Fig. 1A, 1B). To examine the kinetics of IL-1β release in DCs after LPS exposure, culture supernatants and cell extracts were collected at different time points and immunoblotted for IL-1β (Fig. 1C). After LPS stimulation, production of pro–IL-1β in cell extracts was first detected at 1 h, peaked by 4–8 h, and decreased at later time points (Fig. 1C). In contrast, the mature form of IL-1β (p17) was detected 4 h after LPS stimulation in cell extracts and accumulated over time in culture supernatants (Fig. 1C). Consistently, caspase-1 activation was detected in the cell extract by 1 h after LPS stimulation, as assessed by the presence of the p10 subunit of active caspase-1 (Fig. 1C). Likewise, processing of pro–IL-18 into the mature form of IL-18 (p18) was detected in the cell supernatant at 8 h post LPS stimulation (Fig. 1C). In contrast, neither caspase-1 cleavage nor IL-1β or IL-18 maturation was detected in BMDMs stimulated with LPS alone (Fig. 1C). As expected, stimulation of BMDCs and BMDMs with LPS followed by ATP was associated with toxicity (Supplemental Fig. 1A). However, the levels of LDH release induced by LPS alone were comparable to those observed in cells cultured in medium alone (Supplemental Fig. 1A). We also examined the secretion of IL-1β in DCs stimulated with other TLR ligands. All tested ligands, including Pam2- and Pam3-CSK4 (TLR2 ligand), poly(I:C) (TLR3 ligand), R848 (TLR7 ligand), and CpG (TLR9 ligand), triggered significant release of IL-1β in BMDCs in the absence of exogenous ATP (Fig. 1D). Collectively, these results indicate that unlike in BMDMs, TLR stimulation can trigger caspase-1 activation and IL-1β/IL-18 processing and release in murine BMDCs in the absence of exogenous ATP.

IL-1β secretion from murine splenic DCs

Next we examined whether primary DCs could also secrete IL-1β in response to TLR ligands; we isolated splenic mouse DCs and macrophages and challenged the cells with LPS. Although splenic DCs and macrophages secreted comparable amounts of TNF-α in response to LPS, splenic DCs released about five times more IL-1β (~60 pg/ml) than did splenic macrophages (~10 pg/ml) after LPS stimulation (Fig. 2A, 2B). These results indicate that primary DCs produce more IL-1β than do macrophages after LPS challenge.

DCs express more pro–IL-1β and Nlrp3 proteins than do macrophages in response to LPS

We sought to compare the production of IL-1β and Nlrp3 in DCs and macrophages. In accord with the increased production of IL-1β in the culture supernatant, BMDCs produced higher amounts of both pro–IL-1β and Nlrp3 proteins in response to LPS than did BMDMs (Fig. 3A). Furthermore, Nlrp3 was detected in unstimulated BMDCs, but not BMDMs (Fig. 3A). Analysis of mRNA expression by quantitative PCR showed that the induction of pro–IL-1β by LPS was comparable in BMDCs and BMDMs at
1 h, but was significantly higher in BMDCs than in BMDMs at 4 and 8 h post LPS stimulation (Fig. 3B). In contrast, the induction of Nlrp3 mRNA by LPS was similar in BMDCs and BMDMs (Fig. 3B). Whereas the expression of Nlrp3 mRNA was low in unstimulated BMDCs and BMDMs, it was higher in BMDCs than in BMDMs (Fig. 3B). To determine whether LPS induces differential signaling in BMDCs and BMDMs, we stimulated both cell populations with LPS and assessed NF-κB and MAPK signaling events at different times. Notably, phosphorylation of IκB-α and p38 in response to LPS occurred earlier and was slightly increased in BMDCs when compared with BMDMs (Fig. 3C). Thus, the differential ability of DCs to activate caspase-1 and secrete IL-1β in response to LPS is associated with increased expression of the proteins Nlrp3 and pro–IL-1β as well as enhanced LPS-induced IκB-α and p38 phosphorylation. However, the increased expression of Nlrp3 protein in DCs cannot be explained by differential induction of Nlrp3 mRNA, suggesting that posttranscriptional or other mechanisms can regulate the expression of Nlrp3.

IL-1β secretion by LPS-stimulated DCs requires caspase-1 and Nlrp3 but is insensitive to extracellular K+

We wished to investigate the role of caspase-1 in IL-1β secretion by murine DCs in response to LPS. The release of IL-1β induced by LPS stimulation was impaired in mouse DCs deficient in caspase-1 when compared with WT DCs (Fig. 4A). In contrast, the production of TNF-α was comparable in WT and caspase-1−/− DCs (Supplemental Fig 1B). WT and caspase-1−/− DCs produced similar amounts of cytosolic pro–IL-1β, whereas the mature IL-1β (p17) in the culture supernatant of caspase-1−/− DCs was...
barely detectable after 24 h of LPS stimulation (Fig. 4B). When murine DCs were cocultured with YVAD-cmk, a caspase-1 inhibitor, and the Nlrp3 inflammasome, (A) IL-1β secretion by WT or caspase-1−/− murine DCs in response to different doses of LPS. Culture supernatants were collected 24 h after the treatment of LPS and analyzed for IL-1β by ELISA. (B) WT or caspase-1−/− DCs were stimulated by 1 μg/ml LPS for the indicated time or left unstimulated. Culture supernatants and cell extracts were immunoblotted for IL-1β. Gapdh was used as a loading control. (C) IL-1β secretion by WT, Nlrp3−/−, or Asc−/− DCs in response to different doses of LPS. Culture supernatants were collected 24 h after the treatment of LPS and analyzed for IL-1β by ELISA. (D) WT, Nlrp3−/−, or Asc−/− DCs were stimulated by 1 μg/ml LPS for the indicated time or left unstimulated. Culture supernatants and cell extracts were immunoblotted for IL-1β, Nlrp3, or Asc. Gapdh was used as a loading control. (E) Isolated murine splenic DCs from WT or Nlrp3−/− were stimulated with 1 μg/ml LPS for 24 h or left unstimulated. The culture supernatants were analyzed for IL-1β by ELISA. (F) BMDCs were stimulated with LPS, as indicated in (A), in the presence or absence of 50 mM KCl, and IL-1β in culture supernatants was analyzed by ELISA. Data are representative of three independent experiments. *p < 0.05.

FIGURE 4. IL-1β secretion by LPS-stimulated murine DCs requires caspase-1 and the Nlrp3 inflammasome. (A) IL-1β secretion by WT or caspase-1−/− murine DCs in response to different doses of LPS. Culture supernatants were collected 24 h after the treatment of LPS and analyzed for IL-1β by ELISA. (B) WT or caspase-1−/− DCs were stimulated by 1 μg/ml LPS for the indicated time or left unstimulated. Culture supernatants and cell extracts were immunoblotted for IL-1β. Gapdh was used as a loading control. (C) IL-1β secretion by WT, Nlrp3−/−, or Asc−/− DCs in response to different doses of LPS. Culture supernatants were collected 24 h after the treatment of LPS and analyzed for IL-1β by ELISA. (D) WT, Nlrp3−/−, or Asc−/− DCs were stimulated by 1 μg/ml LPS for the indicated time or left unstimulated. Culture supernatants and cell extracts were immunoblotted for IL-1β, Nlrp3, or Asc. Gapdh was used as a loading control. (E) Isolated murine splenic DCs from WT or Nlrp3−/− were stimulated with 1 μg/ml LPS for 24 h or left unstimulated. The culture supernatants were analyzed for IL-1β by ELISA. (F) BMDCs were stimulated with LPS, as indicated in (A), in the presence or absence of 50 mM KCl, and IL-1β in culture supernatants was analyzed by ELISA. Data are representative of three independent experiments. *p < 0.05.

Discussion

IL-1β is a critical inflammatory mediator of host immune responses. The precursor pro–IL-1β is inactive and requires processing by caspase-1 or serine proteases for maturation and induction of biological activities. The activation of caspase-1 is mediated by inflammasomes, of which Nlrp3 has received significant attention owing to its role in both physiological and pathological conditions. Most studies of the Nlrp3 inflammasome have been performed in macrophages, which showed that stimulation with TLR ligands alone is not sufficient to trigger Nlrp3 inflammasome activation. In contrast, stimulation with TLR ligands can activate Nlrp3 in human monocytes, which may require autocrine P2X7 stimulation by ATP (11). Our results indicate that unlike mouse BMDMs, BMDCs and splenic DCs can produce substantial amounts of IL-1β in the culture supernatant in response to TLR ligands, which depended on the Nlrp3 inflammasome. In contrast to human monocytes, apyrase treatment and P2X7 ablation did not affect IL-1β secretion induced by ATP stimulated in murine DCs. More importantly, Nlrp3, but not P2X7, was required for LPS-induced IL-1β production in vivo.

How might DCs and macrophages differ in the regulation of Nlrp3 inflammasome activation and IL-1β maturation in response to TLR ligands? Under steady-state conditions and after LPS stimulation, we found that DCs express more Nlrp3 protein than do macrophages. Furthermore, the induction of pro–IL-1β by LPS was more marked in DCs than in macrophages. Our findings ap-
Pear to be relevant in vivo because studies with reporter eGFP-Nlrp3 knockin mice revealed that splenic DCs have higher Nlrp3 promoter activity than do splenic macrophages (20). Although downstream signaling events upon TLR4 ligation were more rapid and robust in DCs than in macrophages, the induction of TNF-α and Nlrp3 mRNA was comparable in both cell types. Similarly, the production of pro–IL-1β in response to LPS was comparable at 1 h, but more pro–IL-1β mRNA was accumulated at later points in DCs than in macrophages. Because the latter correlated with the release of IL-1β in DCs, it is possible that the increase in IL-1β mRNA in DCs at later time points is caused by self-induction of pro–IL-1β by mature IL-1β. Although the increased production of pro–IL-1β in DCs contributes to increased production of mature IL-1β, greater release of mature IL-1β is also caused by the induction of caspase-1 activation by LPS in DCs. The latter correlated with processing and release of pro–IL-1β and IL-18 in DCs, indicating that stimulation with LPS is sufficient to trigger caspase-1 activation in DCs, but not macrophages. The increased expression of Nlrp3 protein may explain, at least in part, the differential ability of DCs to activate the Nlrp3 inflammasome in response to TLR agonists. Notably, the induction of Nlrp3 mRNA levels by LPS was comparable in DCs and macrophages. These findings suggest that posttranscriptional mechanisms are important in the regulation of Nlrp3 protein expression. Consistently, recent studies showed that microRNAs— and, in particular, miR-223—control Nlrp3 expression and Nlrp3 inflammasome activation in human monocytes and mouse macrophages and DCs (21, 22). Consistent with our observations, Bauernfeind et al. (22) report that the Nlrp3 protein is expressed at higher levels in DCs than in macrophages, which correlated with higher expression of miR-233 in macrophages. The elevated amounts of Nlrp3 protein in DCs after TLR stimulation might be sufficient to achieve an activation threshold for the Nlrp3 inflammasome. This mechanism of LPS-induced Nlrp3 activation in DCs could be similar to conditions in nonimmune HEK293 cells, in which overexpression of inflammasome components can trigger caspase-1 activation (23). Alternatively, the TLR signaling pathway may be physically linked to Nlrp3 activation in DCs. Regardless of the mechanism, increased Nlrp3 activation in DCs may contribute to their ability to activate certain immune responses against microbial stimuli that involve IL-1β and IL-18.

The amounts of ATP necessary for Nlrp3-mediated caspase–1 activation to be detected in macrophages in vitro are much greater than physiological concentrations (24, 25), and therefore, the relevance of the ATP-P2X7 pathway in vivo is uncertain. Our results indicate that the accepted two-signal model necessary for activation of the Nlrp3 inflammasome in response to TLR ligands in mouse macrophages (8) does not apply to DCs. More importantly, P2X7 signaling was not required for IL-1β production in response to LPS administration in vivo. Thus, DCs and/or other cells can produce mature IL-1β via the Nlrp3 inflammasome in vivo in the absence of P2X7 stimulation. Although our studies do not rule out the possibility of a very minor contribution of P2X7 signaling, they challenge the physiological relevance of

**FIGURE 5.** P2X7 signaling is dispensable for Nlrp3-dependent IL-1β secretion induced by LPS in vivo. (A) IL-1β secretion in WT and P2X7−/− DCs stimulated by indicated doses of LPS. Cell culture supernatants were collected 24 h after LPS stimulation. Data are the mean ± SD of triplicate wells and are representative of three independent experiments. (B) The IL-1β secretion from LPS-stimulated DCs in the presence of apyrase. PBS or apyrase (10 μ/ml) was added to the culture medium of DCs with or without LPS. The amounts of IL-1β in culture supernatant were analyzed 24 h after treatment. Data are the mean ± SD of triplicate wells and are representative of three independent experiments. (C and D) Serum from WT, P2X7−/−, or Nlrp3−/− mice (n = 3–6 per genotype) was collected at 1 and 3 h after i.p. injection of 25 mg/kg−1 LPS, and the amounts of IL-1β and TNF-α were determined by ELISA. Data are representative of two independent experiments.
the ATP-P2X7 axis for the induction of IL-1β production in response to TLR agonists such as LPS.

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Disclosures

Luigi Franchi is an employee of Lycera, a biotechnology company specializing in the area of inflammation. The other authors have no financial conflicts of interest.

References

Supplemental figure 1
**Supplemental Figure 1.** (A) BMDCs or BMDMs were stimulated with LPS (1 μg/ml) for 24 hours, or LPS (1 μg/ml) for 8 hour plus 5mM ATP for 1 h. The LDH release to the medium was measured and normalized to the 100% release value obtained by lysis of cells. (B) TNF-α secretion by WT or caspase-1 deficient murine BMDCs in response to different doses of LPS. Culture supernatants were collected 24 hours after the treatment of LPS and analyzed for TNF-α by ELISA. (C) IL-1β secretion by WT or Nlrc4 deficient murine BMDCs in response to LPS (100 ng/ml). Culture supernatants were collected 24 hours after the treatment of LPS and analyzed for IL-1β by ELISA. (D) TNF-α secretion by WT, Nlrp3-null murine splenic DCs in response to LPS (1 μg/ml). Culture supernatants were collected 24 hours after the treatment of LPS and analyzed for TNF-α by ELISA. (E) BMDCs were left untreated or stimulated with LPS (1 μg/ml, 4 h) plus 5 mM ATP (1 h) in the presence or absence of 50 mM extracellular KCl. IL-1β secretion were analyzed by ELISA. (F) TNF-α secretion by WT or P2X7 deficient murine BMDCs in response to different doses of LPS. Culture supernatants were collected 24 hours after the treatment of LPS and analyzed for TNF-α by ELISA. Bar graphs shown were the mean ± s.d. of triplicate wells. All results shown are mean (± s.d.) of triplicate wells and representative of three separate experiments.