Pannexin-1-Mediated Intracellular Delivery of Muramyl Dipeptide Induces Caspase-1 Activation via Cryopyrin/NLRP3 Independently of Nod2

Noemí Marina-García, Luigi Franchi, Yun-Gi Kim, Douglas Miller, Christine McDonald, Geert-Jan Boons and Gabriel Núñez

*J Immunol* 2008; 180:4050-4057; doi: 10.4049/jimmunol.180.6.4050

http://www.jimmunol.org/content/180/6/4050

---

**References**  This article cites 55 articles, 22 of which you can access for free at: [http://www.jimmunol.org/content/180/6/4050.full#ref-list-1](http://www.jimmunol.org/content/180/6/4050.full#ref-list-1)

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

**Permissions**  Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
N. Marina-García,* Luigi Franchi,* Yun-Gi Kim,* Douglas Miller,† Christine McDonald,2* Geert-Jan Boons,† and Gabriel Núñez3*

Muramyl dipeptide (MDP), the microbial activator of nucleotide-binding oligomerization domain 2 (Nod2), induces NF-κB and MAPK activation, leading to the production of multiple anti-bacterial and proinflammatory molecules. In addition, MDP has been implicated in IL-1β secretion through the regulation of caspase-1. However, the mechanisms that mediate caspase-1 activation and IL-1β secretion in response to MDP stimulation remain poorly understood. We show here that fluorescent MDP molecules are internalized in primary macrophages and accumulate in granular structures that colocalize with markers of acidified endosomal compartments. The uptake of MDP was Nod2-independent. Upon ATP stimulation, labeled MDP was rapidly released from acidified vesicles into the cytosol, a process that required functional pannexin-1. Caspase-1 activation induced by MDP and ATP required pannexin-1 and Cryopyrin but was independent of Nod2. Conversely, induction of pro-IL-1β mRNA by MDP stimulation was abolished in Nod2-deficient macrophages but unimpaired in macrophages lacking Cryopyrin. These studies demonstrate a Nod2-independent mechanism mediated through pore-forming pannexin-1 that is required for intracellular delivery of MDP to the cytosol and caspase-1 activation. Furthermore, the work provides evidence for distinct roles of Nod2 and Cryopyrin in the regulation of MDP-induced caspase-1 activation and IL-1β secretion. The Journal of Immunology, 2008, 180: 4050–4057.

Interleukin-1β (IL-1β) plays an important role in the induction of immune responses and in the development of inflammatory disease, fever, and septic shock (1). In response to proinflammatory stimuli, including pathogenic bacteria, the IL-1β precursor is induced in monocytes and macrophages and processed into the biologically active IL-1β molecule by caspase-1 (2–6). The pro tease caspase-1 is expressed in monocyte/macrophages as an inactive zymogen that is activated by self-cleavage by large multiprotein complexes named “inflammasomes” (7). Recent studies have implicated members of the nucleotide-binding oligomerization domain (Nod)4-like receptor (NLR) family in the activation of caspase-1 via the inflammasome (8). NLRs are intracellular pattern-recognition receptors that sense microbial structures that are common to a large number of microorganisms (9). Two NLR family members, Nod1 and Nod2, induce the activation of several host defense signaling pathways, including the NF-κB transcription factor and the MAPK, in response to bacterial molecules produced during the synthesis and/or degradation of peptidoglycan (10–14). The importance of Nod2 in inflammatory homeostasis is underscored by the association of specific mutations in the NOD2 gene with inflammatory disorders, including Crohn’s disease and Blau syndrome (15–18). Nod2 recognizes peptidoglycan-related molecules containing muramyl dipeptide (MDP) that are produced by both Gram-negative and Gram-positive bacteria (10, 12). Once activated, Nod2 induces gene transcription through NF-κB and MAPK signaling pathways by interacting with the kinase RIP-like interacting CLARP kinase/RIP2 (10, 19–22). Nod2 has also been implicated in the regulation of caspase-1 and IL-1β secretion (23, 24). However, the mechanism by which Nod2 regulates IL-1β secretion is poorly understood.

Several NLR family members, including ICE-protease activating factor (Ipaf) and Cryopyrin (also called NLRP3/Nalp3/PYPAF1), have been shown to play a critical role in the regulation of caspase-1 activation in response to specific microbial stimuli (25–28). The relevance of Cryopyrin-mediated caspase-1 activation is underscored by the finding that missense Cryopyrin mutations cause three periodic fever syndromes characterized by dysregulated IL-1β production (29, 30). Cryopyrin is critical for caspase-1 activation induced by bacterial RNA, synthetic purine-like compounds, and endogenous urate crystals (25, 27, 31). In addition, Cryopyrin regulates caspase-1 activation triggered by several microbial products, including MDP in the presence of ATP (26, 28, 32). Stimulation of the P2X7-R with ATP induces a rapid opening of the potassium-selective channel, followed by the gradual opening of a larger pore mediated by pannexin-1 (33). Notably,
pammyl-Ala-D-Glu-NH$_2$ was used. MDP labeled with Alex-
labeled MDP, amino spacer modified MDP (35) (1.0 mg, 1.64
trifluoracetic acid. HRMS (Maldi-Tof) m/z calc. for C$_{45}$H$_{53}$N$_7$O$_{21}$S$_3$
column and a linear gradient of water to acetonitrile containing 0.05%
analysis of NF-

provide, here, evidence for a Nod2-independent mechanism that
mediates intracellular delivery of MDP to the cytosol through
pannexin-1 in response to ATP stimulation. Our studies also dem-
onstrate distinct roles for Cryopyrin and Nod2 in mediating MDP-
induced caspase-1 activation and IL-1β secretion.

Materials and Methods
Mice and macrophages
Nod2 knockout (KO) and Cryopyrin-KO mice have been previously described (22, 31). Nod2-KO mice were backcrossed seven times to C57BL/6 mice. Cryopyrin-KO mice were in mixed 129-C57BL/6 background. Mixed back-
129-C57BL/6 wild-type (WT) or C57BL/6 WT mice were used in experiments involving Cryopyrin-KO mice or Nod2-KO mice, respectively. C57BL/6 WT mice were purchased from The Jackson Laboratory. Mice were housed in a pathogen-free facility. Bone marrow-derived macrophages (BMDM) were prepared as described before (34). The animal studies were conducted under approved protocols by the University of Michigan Committee on Use and Care of Animals (Ann Arbor, Michigan).

Reagents
Ultrapure LPS from Escherichia coli 0111:B4 was purchased from Invivi-
ogen. MDP (Ac-muramyl-Ala-D-Glu-NH$_2$ and Ac-(6-O-stearoyl)-mu-
ramyl-Ala-D-Glu-NH$_2$) were purchased from Bachem. Ac-muramyl-Ala-
D-Glu-NH$_2$ was used in all the experiments except for the Western blot
analysis of NF-κB and MAPK activation experiments, where Ac-(6-O-
stearoyl)-muramyl-Ala-D-Glu-NH$_2$ was used. MDP labeled with Alex-
fluor 488 (MDP-Alexa488) and MDP labeled with rhodamine B (MDP-
Rhodamine) were prepared as follows. For the synthesis of Rhodamine B labeled MDP, amino spacer modified MDP (35) (1.0 mg, 1.64 μmol) was dissolved in dry dimethylformamide (DMF) (0.3 ml) and added to rhoda-
mine B isothiocyanate (2.4 mg, 4.48 μmol, Sigma-Aldrich) in dry DMF
(0.2 ml) under an Argon atmosphere. Dissopropylethylamine (5.7 μl, 32.80
μmol) was then added, and the reaction was stirred in the dark for 18 h.
Upon completion of the reaction, the solvent was removed under high vacuum and the crude product purified by HPLC using a C8 column and a linear gradient of water to acetonitrile containing 0.05% trifluoracetic acid.
HRMS (Maldi-Tof) m/z calc. for C$_{53}$H$_{73}$N$_9$O$_{14}$S$_2$ 1110.32, found m/z
1109.43. For the synthesis of AlexaFluor 488 labeled MDP, triethylamine (5 μl, 36 μmol) was added to amino spacer modified MDP (35) (0.90 mg, 1.5 μmol) under an atmosphere of Argon. The tetrahydrophosphol ester of AlexaFluor 488 (1.0 mg, 1.13 μmol, Invitrogen Life Technologies) was dissolved in dry DMF (500 μl) and added to the MDP solution. The re-
action was stirred in the dark for 18 h after which the solvent was removed under high vacuum and the crude product purified by HPLC using a C8 column and a linear gradient of water to acetonitrile containing 0.05% trifluoracetic acid. HRMS (Maldi-Tof) m/z calc. for C$_{45}$H$_{53}$N$_7$O$_{21}$S$_3$
1147.12 m/z (M + Na$^+$), found m/z 1147.33 (M + Na$^+$). The pannexin-1 mimetic blocking peptide (RQAAFLVDYS) (33).

Western blot
For caspase-1 activation, cells were stimulated with MDP (50 μg/ml) for 3 h, incubated for 30 min with 500 μM p3panx1 blocking peptide when indicated, and pulsed with ATP (5 mM) for 2 min. In all experiments, nuclei were stained with nucleic acid dye 4,
-diamino-2-phenylindole (DAPI; Molecular Probes) and cells were fixed with 2.5% paraformalde-

Measurement of cytokines
IL-1β secretion was measured by ELISA (R&D Systems) in culture su-
pernatants collected after 16 h of MDP stimulation (50 μg/ml) followed by a pulse with 5 mM ATP for 1 h. In NF-κB and MAPK activation ex-
periments, IL-6 was measured by ELISA in culture supernatants after 24 h of
MDP stimulation (10 μg/ml) in the presence of the indicated amounts of
LPS.

In vivo induction of cytokines
Mice (6–8-wk old) were injected i.p. with 300 μg of MDP, and blood
samples were collected at 3, 6, and 24 h after injection. Sera were obtained by centrifugation at 3000 rpm for 10 min and submitted to ELISA for analysis of IL-1β and IL-6.

cDNA synthesis and real-time RT-PCR
Total RNA was extracted from cultured macrophages after stimulation with MDP (10 μg/ml) for the indicated periods, using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthe-
sized using iScript cDNA synthesis kit (Bio-Rad) according to manufac-
turer’s instructions. Real-time PCR was performed using Sybr Green master mix (Applied Biosystems) at the University of Michigan Comprehensive Cancer Center Affymetrix and Microarray Core Facility (Uni-
versity of Michigan, Ann Arbor). The PCR conditions were as follows: initial denaturation for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The primers sequence was: IL-1β forward, GATCCACACTCTCCAGCTGCA; IL-1β reverse, CAACCAACAGT
GATATTTCCTCATG; β-actin forward, AGAGGGAAATGCTGGCTG
GAC; and β-actin reverse, CAAATGTGATAGCTGCGGCT. IL-1β to
B-Actin relative expression was calculated using the 2$^{-}\Delta\Delta$Ct method (36).

Fluorescence microscopy
MDP-Alexa488 and MDP-Rhodamine cellular localization was detected by fluorescence confocal microscopy. For MDP-labeled localization ex-
periments, cultured macrophages were stimulated with MDP-Alexa488 (20 μg/ml) or MDP-Rhodamine (20 μg/ml) for 3 h and additionally incubated with LysoTracker Red DND-99 (Molecular Probes; 30 min), MitoTracker (Molecular Probes; 30 min), or DQ OVA (Molecular Probes; 20 μg/ml, 3 h). Alternatively, cultured macrophages were stimulated with MDP-Rho-
damine (20 μg/ml) for 3 h, incubated with 500 mM p3panx1 for 30 min when indicated, and pulsed with ATP (5 mM) for 2 min. In all experiments, nuclei were stained with nucleic acid dye 4', 6-diamino-2-phenylindole (DAPI; Molecular Probes) and cells were fixed with 2.5% paraformalde-
hyde. Samples were imaged using an Olympus Fluoview-500 Confocal laser scanning microscope at the University of Michigan Microscopy & Image Analysis Facility. Magnifications varied, and scale bars are included in the pictures.

Statistical analysis
Statistical significance between groups was determined by two tailed Stu-
dent’s t test. Differences were considered significant when p < 0.05.

Results
Fluorescent-labeled MDP is internalized into an acidified endosomal compartment
Several microbial molecules, including MDP, induce the activa-
tion of caspase-1 in mouse macrophages after a brief pulse with
ATP (32). To study the mechanism by which ATP triggers MDP-
mediated caspase-1 activation, we first generated two fluorescently-
labeled MDP molecules and assessed their uptake in mouse BM
MDM by fluorescence confocal microscopy. In the absence of
ATP stimulation, ∼25% of the macrophages displayed intracellular
localization of MDP-Rhodamine (red) in a punctate granular pattern that is consistent with vesicular localization (Fig. 1A). At
30 min, the intracellular MDP-labeling pattern was fine punctuate
but changed to coarse granular after 3 h of MDP incubation (Fig.
1B). This observation is consistent with the notion that early acid-
ified endosomes fuse to form large acidified vesicles (37). Similar results were observed when another MDP derivative, MDP-Al
exa488 (green), was used to assess the uptake of MDP (Fig. 1C).
Colabeling experiments revealed that MDP-Alexa488 (green) co-
localized with LysoTracker-D99 (red), a marker that labels acidified
endosomal compartments (Fig. 1C), but not with MitoTracker, a mitochondrial marker (Fig. 1C). Similarly, MDP-Rhodamine (red) colocalized with DQ-OVA, a self-quench OVA conjugate that exhibits bright green fluorescence upon proteolytic degrada-
tion in acidified endosomal compartments (Fig. 1C). These studies
suggest that fluorescent MDP is internalized and localizes to acidified vesicles after incubation in macrophages.

**Uptake and ATP-induced intracellular re-localization of fluorescent-labeled MDP is independent of Nod2**

To assess the effect of ATP in the intracellular localization of MDP, macrophages were incubated with MDP-Rhodamine followed by a 2-min pulse with 5 mM ATP, and the cells were immediately fixed for microscopic evaluation. Upon ATP stimulation, the intracellular labeling pattern of MDP rapidly changed from granular to diffuse, which is consistent with cytosolic re-localization of MDP (Fig. 2A). To quantify this effect, we analyzed confocal Z-stacks images of macrophages incubated with MDP-Rhodamine before and after ATP stimulation. In the absence of ATP stimulation, ~25% of macrophages were MDP-labeled, of which 100% exhibited a granular pattern consistent with vesicular localization (Fig. 2B). In contrast, the majority of macrophages displayed a diffuse MDP-labeling pattern after the 2-min pulse with ATP (Fig. 2B). To determine whether Nod2 regulates the uptake or intracellular localization of MDP, WT, and Nod2-deficient macrophages were incubated with MDP-Rhodamine and the intracellular localization of MDP was determined before and after ATP stimulation. The analysis revealed a similar labeling pattern of MDP in WT and Nod2-deficient macrophages before and after incubation with ATP (Fig. 2B). These results suggest that ATP stimulation induces the rapid release of labeled MDP from a vesicular compartment to the cytosol, and this process is Nod2 independent.

**Functional pannexin-1 is required for ATP-induced cytosolic re-localization of labeled MDP**

Pannexin-1 has been shown to be required for ATP-mediated caspase-1 activation induced by microbial molecules including MDP (32), but how pannexin-1 regulates this process is unknown. To determine the role of pannexin-1 in the regulation of caspase-1...
activation, macrophages were incubated with MDP-Rhodamine and the intracellular localization of MDP was determined in the presence and absence of 10\textsuperscript{panx1}, a peptide that inhibits the function of pannexin-1 (33). As it was shown in Fig. 2A, ATP induced rapid re-localization of intracellular MDP-Rhodamine to the cytosol (Fig. 3A). Notably, the change from a granular to a cytosolic labeling pattern induced by ATP was abrogated by incubation of macrophages with the pannexin-1 inhibitory peptide (Fig. 3A). Quantification of the results revealed that ~100% of the MDP-positive macrophages exhibited a granular labeling pattern after incubation of the macrophages with MDP-Rhodamine in the absence of ATP (Fig. 3B). After 2-min incubation with ATP, ~60% of the macrophages displayed a diffuse labeling pattern and the switch from granular to diffuse MDP-labeling in response to ATP stimulation was abrogated by preincubation with the pannexin-1 inhibitory peptide (Fig. 3B). Taken together, these experiments revealed that the rapid re-localization of labeled MDP from acidified vesicles to the cytosol induced by ATP requires a functional pore-forming pannexin-1 protein.

Nod2 is required for MDP-induced NF-κB and MAPK signaling, but not caspase-1 activation

We next investigated the role of cytosolic Nod2 and Cryopyrin in MDP-induced caspase-1 and IL-1\beta secretion. Recent studies have shown that caspase-1 processing induced by MDP and ATP was reduced in Nod2-null macrophages (24), suggesting that IL-1\beta secretion in response to MDP involves caspase-1 activation regulated via Nod2. However, the interpretation of these previous studies is difficult because MDP- and ATP-induced caspase-1 activation was assessed in macrophages preincubated with LPS and cyclohexamide (24). To determine more directly whether Nod2 is involved in caspase-1 activation, macrophages from WT and Nod2-deficient mice were stimulated with MDP alone followed by a brief pulse with ATP, and cellular extracts were immunoblotted with an Ab that recognizes the p20 subunit of caspase-1. Importantly, the activation of caspase-1 triggered by MDP and ATP in both WT and Nod2-deficient macrophages was unimpaired in Nod2-deficient macrophages (Fig. 4B). Furthermore caspase-1 activation induced by MDP and ATP in both WT and Nod2-deficient macrophages was abrogated by incubation with the pannexin-1

FIGURE 2. Intracellular MDP is released into the cytosol upon ATP stimulation independently of Nod2. A, Macrophages derived from WT and Nod2-KO mice were incubated for 3 h with MDP-Rhodamine (20 μg/ml, red) and pulsed with ATP for 2 min when indicated. In all cases, the nuclei were counterstained with DAPI (blue), and cells were fixed and imaged by fluorescence confocal microscopy. B, Percentage of MDP-labeled cells that presented granular pattern and diffuse pattern. The bars represent the mean of 8–10 confocal Z-stacks (15–20 optical sections) ± SD and are representative of at least three different experiments. *p < 0.05; statistically significant differences in the granular pattern between MDP-labeled cells treated or not treated with ATP. N.D., Not detected.

FIGURE 3. ATP-induced re-localization of labeled MDP is regulated by pannexin-1. A, Macrophages derived from WT mice were incubated for 3 h with MDP-Rhodamine (20 μg/ml, red), incubated for 30 min with 500 μM 10\textsuperscript{panx1} blocking peptide, and pulsed with ATP for 2 min, when indicated. In all cases, the nuclei were counterstained with DAPI (blue), and cells were fixed and imaged by fluorescent confocal microscopy. B, Percentage of MDP-labeled cells that exhibited granular (arrow) or diffuse (arrowhead) labeling pattern, based on the percentage of fluorescent cells detected after MDP-Rhodamine incubation. The bars represent the mean of 8–10 confocal Z-stacks (15–20 optical sections) ± SD and are representative of three different experiments. N.D., not detected.
inhibitory peptide (Fig. 4B). These results indicate that activation of caspase-1 induced by MDP and ATP requires functional pannexin-1 but proceeds normally in the absence of Nod2.

Cryopyrin is required for caspase-1 activation but not NF-κB and MAPK signaling induced by MDP stimulation

Cryopyrin has been shown to be required for caspase-1 activation induced by several microbial stimuli in the presence of ATP (26, 28). We tested, next, the role of Cryopyrin in the activation of caspase-1 in macrophages stimulated with MDP and ATP. The analysis revealed that proteolytic activation of caspase-1 induced by stimulation with MDP and ATP was abolished in macrophages lacking Cryopyrin when compared with that observed in WT macrophages (Fig. 4C). Unlike Nod2-deficient macrophages, NF-κB and MAPK activation was unimpaired in Cryopyrin-deficient macrophages (Fig. 4D). These results indicate

FIGURE 4. Role of Nod2 and Cryopyrin in MDP-induced MAPK and caspase-1 activation. BMDM from WT and Nod2-KO mice (A) or WT and Cryopyrin-KO mice (D) were stimulated with the MDP derivative (Ac-(6-O-stearoyl)-muramyl-Ala-D-Glu-NH₂, 10 μg/ml) for the indicated periods. Cell lysates were prepared and blotted with indicated Abs. Results are from one representative experiment of three independent experiments. p, Phosphorylated. BMDM from WT and Nod2-KO mice (B) or WT and Cryopyrin-KO mice (C) were stimulated with MDP (50 μg/ml) for 3 h, then incubated for 30 min with medium or 500 μM 10panx1 blocking peptide as indicated, and pulsed with ATP (5 mM) for 30 min. Finally, cell extracts were immunoblotted for caspase-1 activation. Both MDP and its derivative, Ac-(6-O-stearoyl)-muramyl-Ala-D-Glu-NH₂, induced caspase-1 activation, which was equally inhibited by the 10panx1 blocking peptide (data not shown). Similarly, NF-κB and MAPK activation was induced by both MDP and its Ac-(6-O-stearoyl) derivative, although lower concentrations of Ac-(6-O-stearoyl)-muramyl-Ala-D-Glu-NH₂ were required for signaling. Arrows denote procaspase-1 (p45) and its processed large subunit (p20). Unst, Not stimulated.

FIGURE 5. Nod2, but not Cryopyrin, is required for mRNA IL-1β expression in response to MDP stimulation. A, Macrophages from WT, Nod2-KO and Cryopyrin-KO were stimulated with MDP (50 μg/ml) for 16 h and then pulsed with ATP for 1 h. Culture supernatants were analyzed for IL-1β production by ELISA. Bars represent the mean ± SD of triplicates and are representative of three independent experiments. BMDM from WT and Nod2-KO (B) or WT and Cryopyrin-KO (C) mice were stimulated with the indicated amount of LPS in the absence or presence of MDP (10 μg/ml). Cells supernatants were collected 24 h after stimulation, and IL-6 was measured by ELISA. Results are presented as the mean of triplicate wells ± SD and correspond to one representative experiment of three independent experiments. BMDM from WT and Nod2-KO (D) or WT and Cryopyrin-KO (E) mice were stimulated with MDP (10 μg/ml) for the indicated periods, and IL-1β mRNA amount was measured by real time RT-PCR. Results are expressed as the IL-1β mRNA to β-actin mRNA relative expression (×10⁶) and are the mean of triplicates ± SD and representative of two separate experiments. N.D., Not detected.
Nod2 and Cryopyrin are required for caspase-1 processing induced by MDP and ATP but is dispensable for NF-κB or MAPK activation induced by MDP.

**Nod2, but not Cryopyrin, is critical for induction of IL-1β mRNA after MDP stimulation**

To further assess the role of Nod2 and Cryopyrin in mediating MDP-induced caspase-1 activation, we determined the secretion of IL-1β, an event that requires the processing of pro-IL-1β by active caspase-1 (5, 6). In these experiments, macrophages from WT, Nod2-KO, and Cryopyrin-KO mice were stimulated with MDP, ATP, or MDP plus ATP and the production of IL-1β was assessed in culture supernatants by ELISA. Secretion of IL-1β was induced by MDP stimulation after a brief pulse with ATP in WT macrophages (Fig. 5A). Importantly, induction of IL-1β secretion by MDP and ATP was abrogated in macrophages lacking Nod2 or Cryopyrin (Fig. 5A). MDP is a poor inducer of cytokines in mouse macrophages but it enhances the production of cytokines such as IL-6 in response to low doses of LPS (22). Control experiments revealed that enhancement of LPS-induced IL-6 secretion by MDP was abrogated in macrophages deficient in Nod2 but was normal in macrophages lacking Cryopyrin (Fig. 5B and C). To further understand the role of Nod2 and Cryopyrin in IL-1β secretion, we stimulated WT and mutant macrophages with MDP, and the levels of IL-1β mRNA were determined by quantitative PCR analysis. IL-1β mRNA levels were expressed at very low levels in unstimulated macrophages but they were rapidly induced greater than 1000-fold after MDP stimulation (Fig. 5D and E). Significantly, the induction of IL-1β mRNA by MDP was abolished in macrophages deficient in Nod2 (Fig. 5D) but unimpaired in cells lacking Cryopyrin (Fig. 5E). Taken all together, our results indicate that Nod2 regulates MDP-induced IL-1β secretion by controlling the transcriptional activation of pro-IL-1β via NF-κB/MAPK while Cryopyrin is critical for caspase-1 activation.

**Nod2 and Cryopyrin are important for MDP-induced production of IL-1β in vivo**

We next examined the contribution of Nod2 and Cryopyrin in mice injected with MDP. In these experiments, MDP was injected i. p. into WT and mice deficient in Nod2 or Cryopyrin in the absence of LPS prestimulation, and the levels of serum IL-1β and IL-6 were measured at several times following MDP administration. By 6-h post injection, IL-1β was detected in the serum of WT mice, and this response returned to baseline levels 24 h after injection of MDP (Fig. 6A). The levels of IL-1β and IL-6 induced by MDP administration were reduced greater than 85% in Nod2-deficient mice (Fig. 6A). In contrast, IL-1β production triggered by MDP injection was reduced ~50% whereas that of IL-6 was unimpaired in animals deficient in Cryopyrin (Fig. 6B). Taken together, these experiments revealed Nod2 and Cryopyrin are important for MDP-induced IL-1β secretion in vivo. Furthermore, the results suggest the existence of mechanisms independent of Nod2 and Cryopyrin that contribute to the production of MDP-induced IL-1β production in the animal.

**Discussion**

Several studies have demonstrated a critical role for NLR proteins, such as Ipaf and Cryopyrin, in caspase-1 activation and IL-1β secretion induced by specific microbial stimuli (25, 26, 31, 38–40). Furthermore, recent experiments suggested a role for pannexin-1 in caspase-1 activation induced via Cryopyrin, but the mechanism by which bacterial molecules, such as MDP together with ATP, trigger the activation of caspase-1 via pannexin-1 and NLR family members remains poorly understood. Using fluorescent MDP molecules, we show that MDP is internalized to acidified vesicles independently of Nod2 and is rapidly released to the cytosol upon stimulation of macrophages with ATP. This process required functional pannexin-1, a recently described protein that associates with the P2X7R upon ATP stimulation and induces the formation of a large non-selective pore (33, 41). Together, these studies support a model whereby ATP stimulation via P2X7-R leads to the formation of a functional pore through pannexin-1 that allows the passage of certain molecules, such as MDP, to the cytosol triggering caspase-1 activation. Because pannexin-1 is a transmembrane protein located at the cell surface and the endoplasmic reticulum (42, 43), it is likely that pannexin-1 acts at the membrane of acidified vacuolar structures, such as lysosomes, to regulate MDP release into the cytosol. It is also formally possible that pannexin-1 controls the passage of MDP from acidified vesicles to the cytosol by an indirect mechanism. For example, pannexin-1 could function at the plasma membrane and regulate an intracellular event that impacts on the release of MDP into the cytosol. Together, these results suggest a unifying model for caspase-1 activation induced by bacteria, whereby the presence of bacterial molecules in the cytosol triggers the activation of caspase-1 via NLR family members. For example, intracellular bacteria such as *Salmonella*, *Pseudomonas*, or *Legionella* activate caspase-1 through a pathway that requires a functional type III/IV secretion system to deliver microbial molecules, such as flagellin, into the cytosol of infected macrophages (38–40, 44). This pathway is independent of pannexin-1, occurs in the absence of P2X7-R activation, and is mediated via Ipaf (32, 38–40, 45). In the case of *Listeria monocytogenes* or *Bacillus anthracis*, these pathogens produce pore-forming molecules that enable the pathogen to enter the host cytosol or to deliver microbial molecules to the cytosol, respectively, leading to the induction of caspase-1 activation (32, 45, 46). Thus, the pannexin-1-mediated pore activated by ATP/P2X-R appears to represent an alternative endogenous conduit to deliver microbial molecules, such as MDP, to the host cytosol triggering Cryopyrin-dependent caspase-1 activation.
Recent studies have examined the role of Nod2 and Cryopyrin signaling in MDP-induced caspase-1 activation and IL-1β secretion (23, 24). In agreement with these studies, we show that MDP-induced caspase-1 activation and IL-1β secretion requires Cryopyrin. In contrast to these authors, however, we found no role for Nod2 in MDP-induced caspase-1 activation. Rather, our results demonstrate a critical role for Nod2 in the secretion of IL-1β through the induction of pro-IL-1β mRNA that was found to be abrogated in Nod2-deficient macrophages but not in cells lacking Cryopyrin. Although the reason for the discrepancy in results between the current and previous studies remains unclear, it could be explained, at least in part, by differences in the experimental approach. Although previous authors incubated the macrophages with low doses of LPS and cyclohexamide before MDP and ATP stimulation, we studied the role of Nod2 in the presence of MDP and ATP alone. Together, the results suggest a model for IL-1β secretion (Fig. 7) in which MDP induces Nod2-dependent expression of pro-IL-1β via NF-κB and MAPK activation whereas ATP stimulation triggers the entry of MDP into the cytosol via the P2X7R/pannexin-1 pore leading to caspase-1 activation through the Cryopyrin inflammasome independently of Nod2. NLRs, including Cryopyrin, contain an ATP/GTPase domain that binds nucleotides that are critical for NLR oligomerization and signaling (47). The latter event is likely mediated by intracellular nucleotide, whereas extracellular ATP appears to be required for MDP-induced Cryopyrin activation via P2X7R/pannexin-1. Because exogenous ATP alone does not activate caspase-1, the results are more consistent with a model in which extracellular ATP acts through the P2X7R to deliver microbial molecules via the pannexin-1 pore rather than inducing direct activation of Cryopyrin. We and others have found that MDP activates Nod2 signaling, but not caspase-1 activation, in the absence of extracellular ATP (10, 14, 32). These results suggest that events mediated via ATP and P2X7R/pannexin-1 are critical for the induction of MDP-induced caspase-1 activation. The latter may involve intracellular ionic fluxes or other events regulated by P2X7R/pannexin-1 that have been suggested to be play a role in caspase-1 activation (48). An alternative and non-exclusive possibility is that Nod2 activation via MDP may rely on cellular mechanisms that are distinct from those involved in Cryopyrin-mediated caspase-1 activation. For example, Nod2 and its Nod1 homologue have been shown to be closely associated with the plasma membrane in certain cells (49, 50), and, therefore, Nod2 activation through MDP could rely on a different route of MDP internalization or be induced via surface receptors or another mechanism independent of cytosolic recognition. It has been reported that the plasma membrane transporter, PepT1, can translocate MDP into the cytosol of host cells (51, 52). PepT1 is expressed primarily at the apical membrane of intestinal epithelial cells (53), although some expression has been reported in human monocytes (54). However, there is no evidence that PepT1 functions as a transporter of MDP in monocyte cells. Furthermore, PepT1 is probably not the physiologically relevant transporter in this system, as it is unable to transport larger MDP-containing molecules, such as our fluorescein labeled MDP (52). Clearly, additional studies are needed to understand the recognition of MDP by Nod2. The studies in vivo confirmed a critical role for Nod2 and Cryopyrin in IL-1β secretion induced by administration of MDP. Unlike the macrophage studies, however, we found significant reduction but not abrogation of IL-1β production in Nod2 or Cryopyrin-deficient mice after administration of MDP in vivo. Nevertheless, the studies revealed that Nod2 is the dominant NLR involved in MDP recognition as the levels of IL-1β and IL-6 induced by MDP administration were reduced greater than 85% in Nod2-deficient mice. However, the levels of IL-1β induced by MDP were only reduced by ~50% in animals deficient in Cryopyrin when compared with WT mice. These studies suggest that the presence of a Cryopyrin-independent mechanism that mediates MDP induced caspase-1 activation in vivo. Although the mechanism involved remains unclear, it is tempting to speculate that a NLR family member other than Cryopyrin regulates caspase-1 activation in response to MDP in vivo. For example, there is evidence that human Nalp1, a NLR protein involved in caspase-1 regulation, is activated by MDP (55). However, there are several Nalp1 homologues in the mouse and it is unknown whether any of them responds to MDP. Further studies are needed to understand the regulation of MDP-induced caspase-1 by NLR proteins.

**Acknowledgments**

We are grateful to Richard Flavell and Millennium Pharmaceuticals for mutant mice, and Joel Whitfield from the Cellular Immunology Core Facility of the University of Michigan Cancer Center for ELISA.

**Disclosures**

The authors have no financial conflict of interest.

**References**


