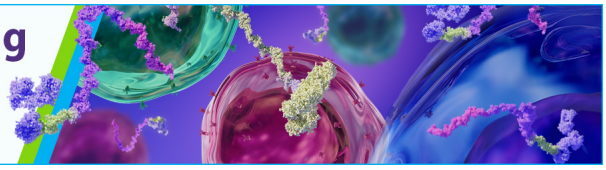

The Journal of Immunology

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

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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

Noemí Marina-García,* Luigi Franchi,* Yun-Gi Kim,* Douglas Miller,† Christine McDonald,[2]* Geert-Jan Boons,† and Gabriel Núñez[3]*

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 protease 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 $P2X_7R$ 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,

*University of Michigan Medical School, Department of Pathology and Comprehensive Cancer Center, Ann Arbor, MI 48109; and †Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602

Received for publication December 10, 2007. Accepted for publication January 16, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grants DK61707 and AI06331 (to G. N.). L.F. was supported by a fellowship from the Arthritis Foundation and Y.-G.K. by a fellowship from the University of Michigan Comprehensive Cancer Center. MDP derived compounds synthesis was supported by Grant GM065248 (to G.-J. B.). C.M. was supported by a Career Development Award from the Crohn's and Colitis Foundation of America.

[2] Current address: Department of Pathobiology, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195.

[3] Address correspondence and reprint requests to Dr. Gabriel Núñez, University of Michigan Medical School, Department of Pathology and Comprehensive Cancer Center, 1500 East Medical Center Drive, Ann Arbor, MI 48109. E-mail address: bclx@umich.edu

[4] Abbreviations used in this paper: Nod, nucleotide-binding oligomerization domain; KO, knockout; MDP, muramyl dipeptide; MAPK, mitogen-activated protein kinase; NLR, Nod-like receptors; $P2X_7R$, $P2X_7$ receptor; WT, wild type; BMDM, bone marrow-derived macrophages; DMF, dimethylformamide; DAPI, 4′,6-diamidino-2-phenylindole; Ipaf, ICE-protease activating factor.

pannexin-1 was found to be critical for Cryopyrin-dependent caspase-1 activation and IL-1β secretion induced by a diverse array of microbial stimuli (32). However, the mechanism by which pannexin-1 regulates activation of caspase-1 via Cryopyrin in response to MDP or other microbial molecules remains unclear. We 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 demonstrate 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 background 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 Invivogen. MDP (Ac-muramyl-Ala-D-Glu-NH_2 and Ac-(6-*O*-stearoyl)-muramyl-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 AlexaFluor 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 rhodamine B isothiocyanate (2.4 mg, 4.48 μmol, Sigma-Aldrich) in dry DMF (0.2 ml) under an Argon atmosphere. Diisopropylethylamine (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 $C_{53}H_{73}N_9O_{14}S_2$ 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 tetrafluorphenol 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 reaction 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 $C_{45}H_{53}N_7O_{21}S_3$ 1147.12 m/z (M plus Na), found m/z 1147.33 (M plus Na). The pannexin-1 mimetic blocking peptide ^{10}panx1 (WRQAAFVDSY) was synthesized by Sigma-Genosys (33).

Western blot

For caspase-1 activation, cells were stimulated with MDP (50 μg/ml) for 3h, incubated for 30 min with 500 μM ^{10}panx1 blocking peptide when indicated, and pulsed with ATP (5 mM, Sigma-Aldrich) for 30 min. Extracts were prepared from cells and culture supernatant supplemented with 0.1% Nonident P-40, complete protease inhibitor mixture (Roche), and 2 mM DTT. Samples were clarified, denatured with SDS buffer, and boiled for 5 min. Immunoblotting was performed using Ab for caspase-1, a gift from Peter Vandenabeele (Gent University, Belgium). For analysis of Iκ-Bα, p38, ERK, and JNK phosphorylation, cells were stimulated with MDP (10 μg/ml) for the periods indicated in the figures. Immunoblotting was performed using specific Abs (Cell Signaling Technology) as described (22).

Measurement of cytokines

IL-1β secretion was measured by ELISA (R&D Systems) in culture supernatants 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 experiments, 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 synthesized using iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions. Real-time PCR was performed using Sybr Grenn master mix (Applied Biosystems) at the University of Michigan Comprehensive Cancer Center Affymetrix and Microarray Core Facility (University 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, CAACCAACAAGT GATATTCTCCATG; β-actin forward, AGAGGGAAATCGTGCGTG GAC; and β-actin reverse, CAATAGTGATGACCTGGCCGT. IL-1β to β-Actin relative expression was calculated using the $2^{(-\Delta Ct)}$ method (36).

Fluorescence microscopy

MDP-Alexa488 and MDP-Rhodamine cellular localization was detected by fluorescence confocal microscopy. For MDP-labeled localization experiments, 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-Rhodamine (20 μg/ml) for 3 h, incubated with 500 mM ^{10}panx1 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% paraformaldehyde. 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 Student'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 activation 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 fluorescent-labeled MDP molecules and assessed their uptake in mouse BMDM 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. 1*A*). At 30 min, the intracellular MDP-labeling pattern was fine punctuate but changed to coarse granular after 3 h of MDP incubation (Fig. 1*B*). This observation is consistent with the notion that early acidified endosomes fuse to form large acidified vesicles (37). Similar results were observed when another MDP derivative, MDP-Alexa488 (green), was used to assess the uptake of MDP (Fig. 1*C*). Colabeling experiments revealed that MDP-Alexa488 (green) colocalized with LysoTracker-D99 (red), a marker that labels acidified endosomal compartments (Fig. 1*C*), but not with MitoTracker, a mitochondrial marker (Fig. 1*C*). Similarly, MDP-Rhodamine (red) colocalized with DQ-OVA, a self-quench OVA conjugate that exhibits bright green fluorescence upon proteolytic degradation in acidified endosomal compartments (Fig. 1*C*). These studies

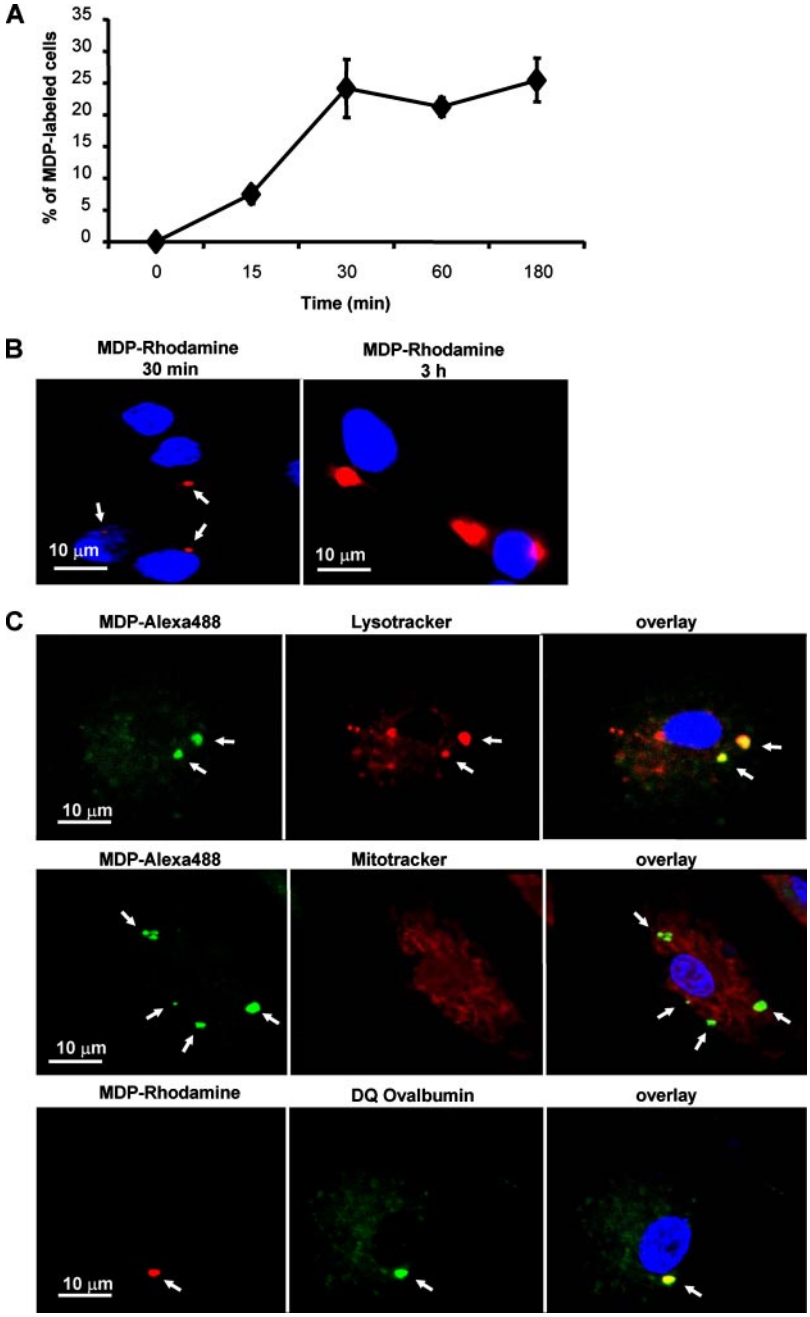

FIGURE 1. Intracellular MDP localizes to acidic compartments in macrophages. *A*, Percentage of MDP-labeled macrophages derived from WT mice after incubation with MDP-Rhodamine (20 μg/ml, red) for the indicated periods of time. Results are given as the mean ± SD based on analysis of 8–10 confocal Z-stacks (15–20 optical sections). *B*, Fluorescence confocal images of WT mouse macrophages incubated for 30 min or 3 h with MDP-Rhodamine (20 μg/ml, red). *C*, Fluorescence images of mouse macrophages derived from WT mice incubated with MDP-Alexa488 (20 μg/ml, green) or MDP-Rhodamine (20 μg/ml, red) for 3 h, and acidic organelle marker dye (LysoTracker Red DND-99, red), mitochondria marker dye (MitoTracker, red), or soluble fluorescent OVA (DQ OVA, green). In all cases, the nuclei were counterstained with DAPI (blue), and cells were fixed and imaged by fluorescence confocal microscopy.

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. 2*A*). To quantify this effect, we analyzed confocal Z-stacks images of macrophages incubated with MDP-Rhodamine before and after 2-min 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. 2*B*). In contrast, the majority of macrophages displayed a diffuse MDP-labeling pattern after the 2-min pulse with ATP (Fig. 2*B*). 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. 2*B*). 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

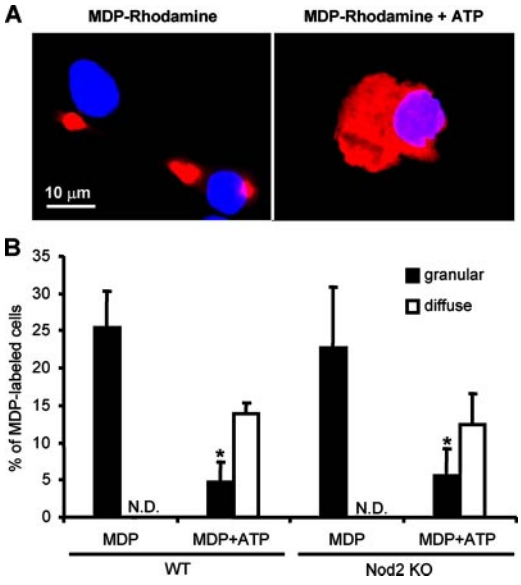

FIGURE 2. Intracellular MDP is released into the cytosol upon ATP stimulation independently of Nod2. *A*, Macrophages derived from WT and Nod2-KO mice were incubated for 3 h with MDP-Rhodamine (20 μg/ml, red) and pulsed with ATP for 2 min when indicated. In all cases, the nuclei were counterstained with 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.

activation, macrophages were incubated with MDP-Rhodamine and the intracellular localization of MDP was determined in the presence and absence of ^{10}panx1, a peptide that inhibits the function of pannexin-1 (33). As it was shown in Fig. 2*A*, ATP induced rapid re-localization of intracellular MDP-Rhodamine to the cytosol (Fig. 3*A*). 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. 3*A*). 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. 3*B*). 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. 3*B*). 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-1β secretion. Recent studies have shown that caspase-1 processing induced by MDP and ATP was reduced in Nod2-null macrophages (24), suggesting that IL-1β 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. In addition, we performed control experiments in parallel to assess the activation of NF-κB and MAPK in response to MDP stimulation. Consistent with previous studies (14), Nod2 deficiency was associated with impaired NF-κB and MAPK activation in response to MDP as determined by immunoblotting with Abs recognizing phosphorylated forms of IκBα, p38, Erk, and Jnk (Fig. 4*A*). In WT macrophages, proteolytic activation of caspase-1 was induced by incubation with MDP followed by a brief pulse with ATP, but not by MDP (Fig. 4*B*) or ATP alone (data not shown). Importantly, the activation of caspase-1 triggered by MDP and ATP was unimpaired in Nod2-deficient macrophages (Fig. 4*B*). Furthermore caspase-1 activation induced by MDP and ATP in both WT and Nod2-deficient macrophages was abrogated by incubation with the pannexin-1

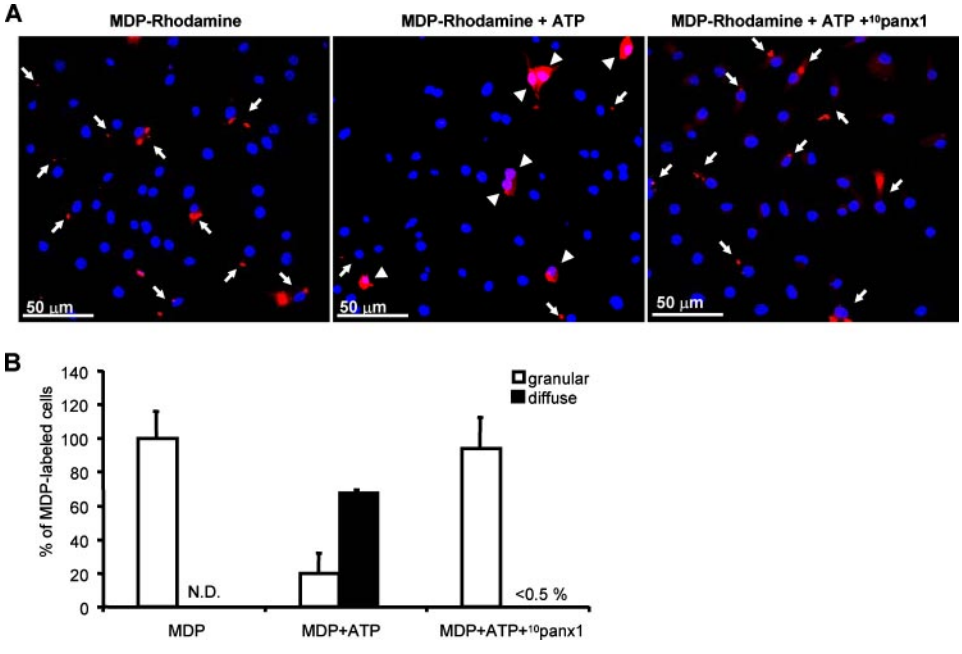

FIGURE 3. ATP-induced re-localization of labeled MDP is regulated by pannexin-1. *A*, Macrophages derived from WT mice were incubated for 3 h with MDP-Rhodamine (20 μg/ml, red), incubated for 30 min with 500 μM ^{10}panx1 blocking peptide, and pulsed with ATP for 2 min, when indicated. In all cases, 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.

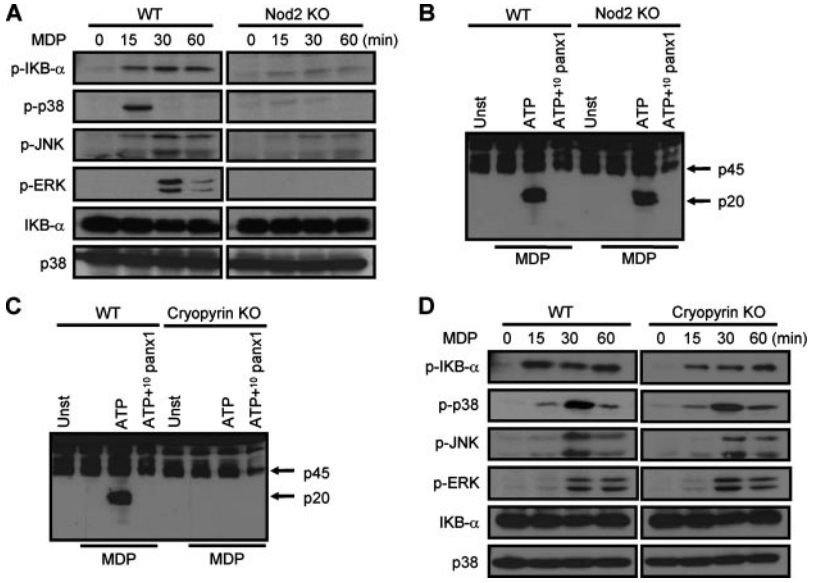

FIGURE 4. Role of Nod2 and Cryopyrin in MDP-induced MAPK and caspase-1 activation. BMDM from WT and Nod2-KO mice (*A*) or WT and Cryopyrin-KO mice (*D*) were stimulated with the MDP derivative (Ac-(6-*O*-stearoyl)-muramyl-Ala-D-Glu-NH_2, 10 μg/ml) for the indicated periods. Cell lysates were prepared 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 ^{10}panx1 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-NH_2, induced caspase-1 activation, which was equally inhibited by the ^{10}panx1 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-NH_2 were required for signaling. Arrows denote procaspase-1 (p45) and its processed large subunit (p20). Unst, Not stimulated.

inhibitory peptide (Fig. 4*B*). 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. 4*C*). Unlike Nod2-deficient macrophages, NF-κB and MAPK activation was unimpaired in Cryopyrin-deficient macrophages (Fig. 4*D*). These results indicate

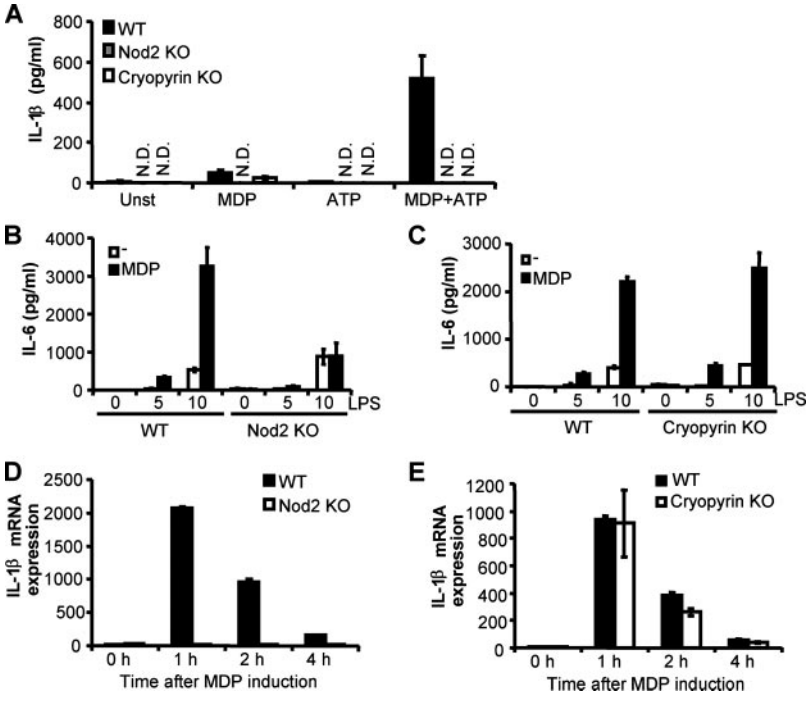

FIGURE 5. Nod2, but not Cryopyrin, is required for mRNA IL-1β expression in response to MDP stimulation. *A*, Macrophages from WT, Nod2-KO and Cryopyrin-KO were stimulated with MDP (50 μg/ml) for 16 h and then pulsed with ATP for 1 h. Culture supernatants were analyzed for IL-1β production by ELISA. 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 ($\times 10^6$) and are the mean of triplicates ± SD and representative of two separate experiments. N.D., Not detected.

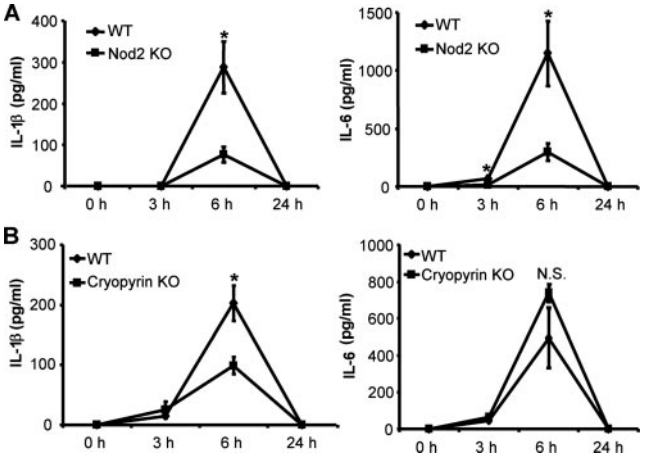

FIGURE 6. Nod2 and Cryopyrin regulation of IL-1β and IL-6 secretion in response to MDP stimulation in vivo. WT and Nod2-KO mice (*A*) or WT and Cryopyrin-KO mice (*B*) ($n = 5$) were injected i. p. with 300 μg MDP. Blood was collected at indicated time points and serum was prepared. Serum levels of IL-1β and IL-6 were measured by ELISA. Results are presented as the mean $\pm$ SEM. $*$, $p < 0.05$; statistically significant differences between WT and mutant macrophages. N.S., Not significant.

that Cryopyrin is 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. 5*A*). Importantly, induction of IL-1β secretion by MDP and ATP was abrogated in macrophages lacking Nod2 or Cryopyrin (Fig. 5*A*). 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. 5, *B* 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. 5, *D* and *E*). Significantly, the induction of IL-1β mRNA by MDP was abolished in macrophages deficient in Nod2 (Fig. 5*D*) but unimpaired in cells lacking Cryopyrin (Fig. 5*E*). 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. 6*A*). The levels of IL-1β and IL-6 induced by MDP administration were reduced greater than 85% in Nod2-deficient mice (Fig. 6*A*). 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. 6*B*). 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 $P2X_7R$ 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 $P2X_7R$ 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 $P2X_7R$ activation, and is mediated via Ipaf (32, 38–40, 45). In the case of *Listeria monocytogens* or *Bacillus anthrax*, 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/$P2X_7R$ appears to represent an alternative endogenous conduit to deliver microbial molecules, such as MDP, to the host cytosol triggering Cryopyrin-dependent caspase-1 activation.

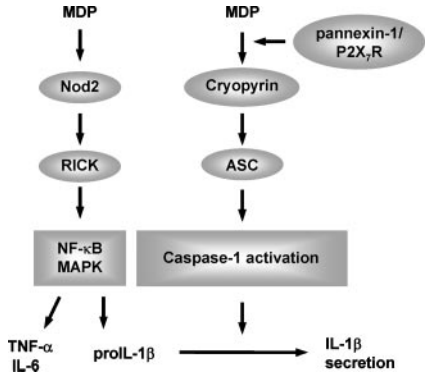

FIGURE 7. Proposed model for the regulation of MDP-induced IL-1β secretion. The model is based on experiments presented in the current manuscript and work by Q. Pan et al. (24). There is also evidence for Nod2- and Cryopyrin-independent regulation of IL-1β production after MDP administration in vivo (see Fig. 6), which is not depicted in the figure.

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 $P2X_7R$/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 is 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 $P2X_7R$/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 $P2X_7R$ 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 $P2X_7R$/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 $P2X_7R$/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 show 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 monocytic 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 fluorescently 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

1. Dinarello, C. A. 1996. Biologic basis for interleukin-1 in disease. *Blood* 87: 2095–2147.
2. Dinarello, C. A. 2006. Interleukin 1 and interleukin 18 as mediators of inflammation and the aging process. *Am. J. Clin. Nutr.* 83: 447S–455S.
3. Cerretti, D. P., C. J. Kozlosky, B. Mosley, N. Nelson, K. Van Ness, T. A. Greenstreet, C. J. March, S. R. Kronheim, T. Druck, L. A. Cannizzaro, et al. 1992. Molecular cloning of the interleukin-1 β converting enzyme. *Science* 256: 97–100.
4. Kuida, K., J. A. Lippke, G. Ku, M. W. Harding, D. J. Livingston, M. S. Su, and R. A. Flavell. 1995. Altered cytokine export and apoptosis in mice deficient in interleukin-1 β converting enzyme. *Science* 267: 2000–2003.
5. Li, P., H. Allen, S. Banerjee, S. Franklin, L. Herzog, C. Johnston, J. McDowell, M. Paskind, L. Rodman, J. Salfeld, et al. 1995. Mice deficient in IL-1 β-converting enzyme are defective in production of mature IL-1 β and resistant to endotoxic shock. *Cell* 80: 401–411.
6. Thornberry, N. A., H. G. Bull, J. R. Calaycay, K. T. Chapman, A. D. Howard, M. J. Kostura, D. K. Miller, S. M. Molineaux, J. R. Weidner, J. Aunins, et al. 1992. A novel heterodimeric cysteine protease is required for interleukin-1 β processing in monocytes. *Nature* 356: 768–774.
7. Martinon, F., K. Burns, and J. Tschopp. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-β. *Mol. Cell.* 10: 417–426.
8. Kanneganti, T. D., M. Lamkanfi, and G. Núñez. 2007. Intracellular NOD-like receptors in host defense and disease. *Immunity* 27: 549–559.
9. Franchi, L., J. Park, M. Shaw, N. Marina-Garcia, G. Chen, Y. Kim, and G. Núñez. 2008. Intracellular NOD-like receptors in innate immunity, infection and disease. *Cell. Microbiol.* 10: 1–8.

10. Inohara, N., Y. Ogura, A. Fontalba, O. Gutierrez, F. Pons, J. Crespo, K. Fukase, S. Inamura, S. Kusumoto, M. Hashimoto, et al. 2003. Host recognition of bacterial muramyl dipeptide mediated through NOD2: implications for Crohn's disease. *J. Biol. Chem.* 278: 5509–5512.
11. Chamaillard, M., M. Hashimoto, Y. Horie, J. Masumoto, S. Qiu, L. Saab, Y. Ogura, A. Kawasaki, K. Fukase, S. Kusumoto, et al. 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat. Immunol.* 4: 702–707.
12. Girardin, S. E., I. G. Boneca, J. Viala, M. Chamaillard, A. Labigne, G. Thomas, D. J. Philpott, and P. J. Sansonetti. 2003. Nod2 Is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* 278: 8869–8872.
13. Girardin, S. E., I. G. Boneca, L. A. Carneiro, A. Antignac, M. Jehanno, J. Viala, K. Tedin, M. K. Taha, A. Labigne, U. Zahringer, et al. 2003. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 300: 1584–1587.
14. Kobayashi, K. S., M. Chamaillard, Y. Ogura, O. Henegariu, N. Inohara, G. Nunez, and R. A. Flavell. 2005. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 307: 731–734.
15. Hugot, J. P., M. Chamaillard, H. Zouali, S. Lesage, J. P. Cézard, J. Belaiche, S. Almer, C. Tysk, C. A. O'Morain, M. Gassull, et al. 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411: 599–603.
16. Kanazawa, N., I. Okafuji, N. Kambe, R. Nishikomori, M. Nakata-Hizume, S. Nagai, A. Fuji, T. Yuasa, A. Manki, Y. Sakurai, et al. 2005. Early-onset sarcoidosis and CARD15 mutations with constitutive nuclear factor-B activation: common genetic etiology with Blau syndrome. *Blood* 105: 1195–1197.
17. Miceli-Richard, C., S. Lesage, M. Rybojad, A. M. Prieur, S. Manouvrier-Hanu, R. Hafner, M. Chamaillard, H. Zouali, G. Thomas, and J. P. Hugot. 2001. CARD15 mutations in Blau syndrome. *Nat. Genet.* 29: 19–20.
18. Ogura, Y., D. Bonen, N. Inohara, D. Nicolae, F. Chen, R. Ramos, H. Britton, T. Moran, R. Karaliuskas, R. Duerr, et al. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411: 603–606.
19. Chin, A. I., P. W. Dempsey, K. Bruhn, J. F. Miller, Y. Xu, and G. Cheng. 2002. Involvement of receptor-interacting protein 2 in innate and adaptive immune responses. *Nature* 416: 190–194.
20. Kobayashi, K., N. Inohara, L. D. Hernandez, J. E. Galan, G. Nunez, C. A. Janeway, R. Medzhitov, and R. A. Flavell. 2002. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 416: 194–199.
21. Abbott, D. W., A. Wilkins, J. M. Asara, and L. C. Cantley. 2004. The Crohn's disease protein, NOD2, requires RIP2 in order to induce ubiquitinylation of a novel site on NEMO. *Curr. Biol.* 14: 2217–2227.
22. Park, J.-H., Y. Kim, C. McDonald, T.-D. Kanneganti, M. Hasegawa, M. Body-Malapel, N. Inohara, and G. Nunez. 2007. RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs. *J. Immunol.* 178: 2380–2386.
23. Maeda, S., L. Hsu, H. Liu, L. Bankston, M. Iimura, M. Kagnoff, L. Eckmann, and M. Karin. 2005. Nod2 mutation in Crohn's disease potentiates NF-κactivity and IL-1β processing. *Science* 307: 734–738.
24. Pan, Q., J. Mathison, C. Fearns, V. V. Kravchenko, J. Da Silva Correia, H. M. Hoffman, K. S. Kobayashi, J. Bertin, E. P. Grant, A. J. Coyle, et al. 2007. MDP-induced interleukin-1β processing requires Nod2 and CIAS1/NALP3. *J. Leukocyte Biol.* 82: 177–183.
25. Kanneganti, T. D., M. Body-Malapel, A. Amer, J. Park, J. Whitfield, L. Franchi, Z. Taraporewala, D. K. Miller, J. Patton, N. Inohara, and G. Núñez. 2006. Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J. Biol. Chem.* 281: 36560–36568.
26. Mariathasan, S., D. S. Weiss, K. Newton, J. McBride, K. O'Rourke, M. Roose-Girma, W. P. Lee, Y. Weinrauch, D. M. Monack, and V. M. Dixit. 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440: 228–232.
27. Martinon, F., V. Petrilli, A. Mayor, A. Tardivel, and J. Tschopp. 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440: 237–241.
28. Sutterwala, F. S., Y. Ogura, M. Szczepanik, M. Lara-Tejero, G. S. Lichtenberger, E. P. Grant, J. Bertin, A. J. Coyle, J. E. Galan, P. W. Askenase, and R. A. Flavell. 2006. Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity* 24: 317–327.
29. Agostini, L., F. Martinon, K. Burns, M. F. McDermott, P. N. Hawkins, and J. Tschopp. 2004. NALP3 forms an IL-1β-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* 20: 319–325.
30. Dowds, T. A., J. Masumoto, L. Zhu, N. Inohara, and G. Nunez. 2004. Cryopyrin-induced interleukin 1β secretion in monocytic cells: enhanced activity of disease-associated mutants and requirement for ASC. *J. Biol. Chem.* 279: 21924–21928.
31. Kanneganti, T. D., N. Ozoren, M. Body-Malapel, A. Amer, J. H. Park, L. Franchi, J. Whitfield, W. Barchet, M. Colonna, P. Vandenabeele, et al. 2006. Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* 440: 233–236.
32. Kanneganti, T. D., M. Lamkanfi, Y. Kim, G. Chen, J. Park, L. Franchi, P. Vandenabeele, and G. Núñez. 2007. Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. *Immunity* 26: 433–443.
33. Pelegrin, P., and A. Surprenant. 2006. Pannexin-1 mediates large pore formation and interleukin-1β release by the ATP-gated P2X7 receptor. *EMBO J.* 25: 5071–5082.
34. Celada, A., P. W. Gray, E. Rinderknecht, and R. D. Schreiber. 1984. Evidence for a γ-interferon receptor that regulates macrophage tumoricidal activity. *J. Exp. Med.* 160: 55–74.
35. Siriwardena, A., M. R. Jorgensen, M. A. Wolfert, M. L. Vandenplas, J. N. Moore, and G. J. Boons. 2001. Synthesis and proinflammatory effects of peptidoglycan-derived neoglycopeptide polymers. *J. Am. Chem. Soc.* 123: 8145–8146.
36. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 22DDCT method. *Methods* 25: 402–408.
37. Knapp, P. E., and J. A. Swanson. 1990. Plasticity of the tubular lysosomal compartment in macrophages. *J. Cell Sci.* 95: 433–439.
38. Amer, A., L. Franchi, T. D. Kanneganti, M. Body-Malapel, N. Ozoren, G. Brady, S. Meshinchi, R. Jagirdar, A. Gewirtz, S. Akira, and G. Nunez. 2006. Regulation of *Legionella* phagosome maturation and infection through flagellin and host Ipaf. *J. Biol. Chem.* 281: 35217–35223.
39. Franchi, L., A. Amer, M. Body-Malapel, T. D. Kanneganti, N. Ozoren, R. Jagirdar, N. Inohara, P. Vandenabeele, J. Bertin, A. Coyle, et al. 2006. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1β in *Salmonella*-infected macrophages. *Nat. Immunol.* 7: 576–582.
40. Miao, E. A., C. M. Alpuche-Aranda, M. Dors, A. E. Clark, M. W. Bader, S. I. Miller, and A. Aderem. 2006. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1β via Ipaf. *Nat. Immunol.* 7: 569–575.
41. Locovei, S., E. Scemes, F. Qiu, D. C. Spray, and G. Dahl. 2007. Pannexin1 is part of the pore forming unit of the P2X(7) receptor death complex. *FEBS Lett.* 581: 483–488.
42. Abeele, F. V., A. Zholos, G. Bidaux, Y. Shuba, S. Thebault, B. Beck, M. Flourakis, Y. Panchin, R. Skryma, and N. Prevarskaya. 2006. Ca^{2+}-independent phospholipase A(2)-dependent gating of TRPM8 by lysophospholipids. *J. Biol. Chem.* 281: 40174–40182.
43. Pelegrin, P., and A. Surprenant. 2006. Pannexin-1 couples to maitotoxin and nigericin-induced IL-1β release through a dye-uptake independent pathway. *J. Biol. Chem.* 282: 2386–2394.
44. Franchi, L., J. Stoolman, T. D. Kanneganti, A. Verma, R. Ramphal, and G. Nunez. 2007. Critical role for Ipaf in *Pseudomonas aeruginosa*-induced caspase-1 activation. *Eur. J. Immunol.* 37: 3030–3039.
45. Franchi, L., T. D. Kanneganti, G. R. Dubyak, and G. Nunez. 2007. Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria. *J. Biol. Chem.* 282: 18810–18818.
46. Boyden, E. D., and W. F. Dietrich. 2006. Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat. Genet.* 38: 240–244.
47. Duncan, J. A., D. T. Bergstralh, Y. Wang, S. B. Willingham, Z. Ye, A. G. Zimmermann, and J. P. Ting. 2007. Cryopyrin/NALP3 binds ATP/dATP, is an ATPase, and requires ATP binding to mediate inflammatory signaling. *Proc. Natl. Acad. Sci. USA* 104: 8041–8046.
48. Ferrari, D., C. Pizzirani, E. Adinolfi, R. M. Lemoli, A. Curti, M. Idzko, E. Panther, and F. Di Virgilio. 2006. The P2X7 receptor: a key player in IL-1 processing and release. *J. Immunol.* 176: 3877–3883.
49. Lecine, P., S. Esmiol, J. Y. Metais, C. Nicoletti, C. Nourry, C. McDonald, G. Nunez, J. P. Hugot, J. P. Borg, and V. Ollendorff. 2007. The NOD2-RICK complex signals from the plasma membrane. *J. Biol. Chem.* 282: 15197–15207.
50. Kufer, T. A., E. Kremmer, A. C. Adam, D. J. Philpott, and P. J. Sansonetti. 2008. The pattern-recognition molecule Nod1 is localized at the plasma membrane at sites of bacterial interaction. *Cell. Microbiol.* 10: 477–486.
51. Vavricka, S. R., M. W. Musch, J. E. Chang, Y. Nakagawa, K. Phanvijhitsiri, T. S. Waypa, D. Merlin, O. Schneewind, and E. B. Chang. 2004. hPepT1 transports muramyl dipeptide, activating NF-κB and stimulating IL-8 secretion in human colonic Caco2/bbe cells. *Gastroenterology* 127: 1401–1409.
52. Ismair, M. G., S. R. Vavricka, G. A. Kullak-Ublick, M. Fried, D. Mengin-Lecreulx, and S. E. Girardin. 2006. hPepT1 selectively transports muramyl dipeptide but not Nod1-activating muramyl peptides. *Can. J. Physiol. Pharmacol.* 84: 1313–1319.
53. Ogihara, H., H. Saito, B. C. Shin, T. Terada, S. Takenoshita, Y. Nagamachi, K.I. Inui, and K. Takata. 1996. Immuno-localization of H+/peptide cotransporter in rat digestive tract. *Biochem. Biophys. Res. Commun.* 220: 848–852.
54. Charrier, L., A. Driss, Y. Yan, V. Nduati, J. M. Klapproth, S. V. Sitaraman, and D. Merlin. 2006. hPepT1 mediates bacterial tripeptide fMLP uptake in human monocytes. *Lab. Invest.* 86: 490–503.
55. Bruey, J., N. Bruey-Sedano, F. Luciano, D. Zhai, R. Balpai, C. Xu, C. Kress, B. Bailly-Maitre, X. Li, A. Osterman, et al. 2007. Bcl-2 and Bcl-XL regulate proinflammatory caspase-1 activation by interaction with NALP1. *Cell* 129: 45–56.