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Cutting Edge: NF-κB Activating Pattern Recognition and Cytokine Receptors License NLRP3 Inflammasome Activation by Regulating NLRP3 Expression

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The IL-1 family cytokines are regulated on transcriptional and posttranscriptional levels. Pattern recognition and cytokine receptors control pro-IL-1β transcription whereas inflammasomes regulate the proteolytic processing of pro-IL-1β. The NLRP3 inflammasome, however, assembles in response to extracellular ATP, pore-forming toxins, or crystals only in the presence of proinflammatory stimuli. How the activation of gene transcription by signaling receptors enables NLRP3 activation remains elusive and controversial. In this study, we show that cell priming through multiple signaling receptors induces NLRP3 expression, which we identified to be a critical checkpoint for NLRP3 activation. Signals provided by NF-κB activators are necessary but not sufficient for NLRP3 activation, and a second stimulus such as ATP or crystal-induced damage is required for NLRP3 activation. The Journal of Immunology, 2009, 183: 787–791.

Members of the TLR and C-type lectin receptor families signal in response to microbial or altered endogenous molecules when presented extracellularly or in endo-lysosomal compartments (1–3). In the cytoplasm, Nod-like receptors and Rig-I-like helicases respond to modified microbial components gaining access to the cytosol (3).

Most innate signaling receptors respond to a relatively restricted ligand spectrum (4). In contrast, diverse molecular entities including bacteria, viruses, purified microbial products, components of dying cells, small molecule immune activators, and crystalline or aggregated materials can activate the Nod-like receptor protein NLRP3 (5). The molecular mechanisms of how NLRP3 can recognize such a diverse array of activators and the role of transcriptionally active signaling receptors for the activation of the NLRP3 inflammasome are controversial and mechanistically poorly understood (2, 5–8). Upon activation, NLRP3 forms a so-called inflammasome complex with the adaptor molecule ASC, which controls the activation of caspase-1. Activated caspase-1, in turn, cleaves pro-IL-1β and pro-IL-18 into the biologically active, secreted forms (9).

In this study, we demonstrate that the expression of NLRP3 itself is tightly controlled by the activity of multiple signaling receptors. We reveal that enhanced expression of NLRP3 in response to NF-κB is sufficient for NLRP3 inflammasome activation by ATP or pore-forming toxins or crystals. Thus, macrophages need to acquire a licensing signal provided by a transcriptionally active signaling receptor that enables them to respond to NLRP3 activators.

Materials and Methods

Mice

The following mice were provided as indicated: NLRP3-knockout (KO)4 and ASC-KO (Millenium Pharmaceuticals); TLR2-KO, TLR4-KO, TLR7-KO, IL-1R-associated kinase 4 (IRAK4)-KO, MyD88 adaptor-like (MAL)-KO, Toll-like receptor 1 (TLR1)-domain-containing adapter inducing IFN-β (TRIF)-KO, MyD88-KO, and TRIF-related adapter molecule (TRAM)-KO (S. Akira, Osaka University, Saitama, Japan); TLR3-KO (R. Flavell, Yale University, New Haven, CT); and MD-2-KO (K. Miyake, Tokyo University, Tokyo, Japan). C57BL/6 mice were purchased from Jackson Laboratories. All animal experiments were approved by the University of Massachusetts Animal Care and Use Committee (Worcester, MA).

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4 Abbreviations used in this paper: KO, knockout; IRAK4, IL-1R-associated kinase 4; MDP, muramyl dipeptide; TRIF, Toll/IL-1R domain-containing adapter inducing IFN-β; YFP, yellow fluorescent protein.

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Reagents
ATP, poly(dA-dT), muramyl dipeptide (MDP), nigericin, cycloheximide, and Bay11-7082 were from Sigma-Aldrich. Pam2CysK4, polyinosinic-polycytidylic acid, ultra-pure LPS, R848, and γ-glutamyl-β-D-glutamyl-L-2-aminoethylketone FLICA (fluorescent labeled inhibitors of caspases) kit (Immunochemistry Technologies) was used to stain active caspase-1. CD14-positive cells were analyzed for FLICA positivity by flow cytometry. The respective local ethics committees approved experiments involving PBMCs. Immortalized macrophage cell lines were generated as described (10). Caspase-1 was detected in serum-free cell supernatants or cell lysates by SDS PAGE using caspase-1 polyclonal Ab (catalog no. sc-514; Santa Cruz Biotechnology). Human ASC and NLRP3 were cloned from cDNA into the lentiviral plasmid FugW and immortalized macrophages were transduced as described (11). Quantitative real-time PCR was performed as described (12). Primer sequences are available upon request. The mouse NLRP3 promoter (~3000 bp to 0 bp upstream of the transcription start site) was cloned from cDNA into pGL3-basic. HEK293T cells were transfected with luciferase reporter plasmid and expression plasmids (100 ng each) using Lipofectamine (Invitrogen).

Microscopy
A Leica SP2 AOBS (acousto-optical beam splitter) confocal laser scanning microscope was used. ASC pyroptosomes were quantified by epifluorescence microscopy and ImageJ software.

Results and Discussion
Assembly of the NLRP3 inflammasome requires priming signals functioning upstream of ASC
Fluorescent ASC forms speck-like structures, termed pyroptosomes, upon activation (13). We generated immortalized mouse macrophages expressing ASC-yellow fluorescent protein (YFP) to test whether the NLRP3 inflammasome can assemble upstream of caspase-1 in the absence of priming. Resting cells or cells treated with ATP or LPS alone uniformly expressed ASC-YFP throughout the cells. However, when LPS-primed cells were treated with ATP, ASC-YFP formed large, irregularly shaped pyroptosomes, indicating that LPS signaling was also required for ASC pyroptosome formation upon NLRP3 activation (Fig. 1A). Thus, mouse macrophages require two stimuli for NLRP3 inflammasome activation even at the level of ASC, similar to what is observed at the level of caspase-1 (supplemental Fig. 1A and Ref. 6). Furthermore, pyroptosomes dose-responsively formed in response to LPS and ATP and caspase-1 cleavage closely correlated with the number of pyroptosomes (Fig. 1, B and C, and supplemental Fig. 1B). Synthetic LPS or TLR2 and TLR7 activators also induced pyroptosomes together with ATP (supplemental Fig. 1, C and D) suggesting that TLR activators and not an undefined contaminant were responsible for pyroptosome formation in response to ATP. In line with previous observations, pyroptosome formation in response to the AIM2 inflammasome activator, transfected dsDNA (poly(dA-dT)), did not require LPS priming (supplemental Fig. 1, B and Refs. 12 and 14).

Different signaling receptor family members are able to license NLRP3 inflammasome activation
We next aimed at dissecting the influence of TLR4 signaling on NLRP3 inflammasome activation from TLR-independent mechanisms. We made use of an activating anti-TLR4/MD-2 Ab (clone UT18) and tested whether the activation of TLR4 in the absence of LPS was sufficient for priming of the NLRP3 inflammasome. Preincubation with an activating Ab, but not with a blocking TLR4/MD-2 Ab (clone MTS510), led to the cleavage of caspase-1 in wild-type macrophages when stimulated with ATP. Furthermore, activation by the stimulating Ab was dependent on TLR4 and MD-2, consistent with the fact that it fails to bind and activate TLR4 or MD-2 alone (15) (Fig. 2A). Furthermore, activation of TLR2, TLR3, and TLR7 also induced priming of the NLRP3 inflammasome, and macrophages lacking TLRs for the respective stimuli failed to activate caspase-1 after activation via ATP (Fig. 2B).

We next analyzed the requirement of TLR signaling for NLRP3 priming. Macrophages deficient in MyD88 or TRIF responded normally to LPS and ATP with cleavage of caspase-1, whereas macrophages doubly deficient in both MyD88 and TRIF or TLR4 failed to respond (supplemental Fig. 2A). These results show that both TRIF- and MyD88-dependent signaling pathways can compensate for each other in
their ability to induce priming for NLRP3 activation. Of note, the priming of macrophages was also a necessary step for NLRP3 inflammasome activation by other established NLRP3 activators such as the pore-forming toxin nigericin or crystalline activators (supplemental Fig. 2, B and C), suggesting that priming is generally required for NLRP3 inflammasome activation.

MDP, which engages NOD2, also mediated priming for ATP responsiveness, and the activity was dependent on RIP2, the downstream signaling transducer of this signaling pathway (Fig. 2C). We further observed that priming with a cytokine stimulus (TNF-α) was sufficient to induce caspase-1 activation by ATP. Notably, TNF-α failed to prime cells obtained from mice doubly deficient in TNFR1 and TNFR2, whereas LPS was able to prime these cells (Fig. 2D). Collectively, these results suggest that multiple transcriptionally active signaling receptors can prime macrophages for subsequent NLRP3 inflammasome activation.

**Priming is required for NLRP3 activation in human monocytes**

IRAK4 is essentially required for signal transduction to NF-κB downstream of MyD88. Indeed, ligands for TLRs that exclusively signal via the adapter MyD88 also failed to prime IRAK4-deficient macrophages for NLRP3-mediated ATP activation, whereas signaling cascades that operate independently of MyD88 induced priming of IRAK4 independently (supplemental Fig. 3A). This differential requirement for priming allowed us to address the role of priming for NLRP3 inflammasome activation in the human system. We obtained monocytes from healthy volunteers and from a patient with a loss-of-function mutation (Q293X) in IRAK4 and tested their ability to induce caspase-1 upon ATP stimulation after priming with IRAK-4-dependent and -independent stimuli. Only primed monocytes displayed a robust caspase-1 activation upon ATP stimulation, whereas the AIM2 inflammasome activation was independent of this type of priming (supplemental Fig. 3B). When primed with IRAK-4-independent ligands, monocytes from the IRAK4-mutant patient responded robustly with caspase-1 activation after ATP stimulation. In contrast and consistent with IRAK4 deficiency in the murine system, we failed to observe priming activity toward ATP after TLR2 or TLR7/8 stimulation (supplemental Fig. 3B). Thus, these data indicate that NLRP3 activation is also critically dependent on priming activity by signaling receptors in human cells.

**NF-κB-dependent signals regulate NLRP3 expression**

Treatment of macrophages with the protein synthesis inhibitor cycloheximide dose-dependently led to reduced caspase-1 activation obtained by the combination of LPS and ATP, indicating that protein de novo synthesis was functionally limiting in mouse macrophages (Fig. 3A). In addition, priming of the NLRP3 inflammasome was dose-dependently reduced by a specific inhibitor of NF-κB (Bay11-7082), suggesting a key role for NF-κB in priming (Fig. 3B).

Overexpression of ASC is not sufficient to overcome the priming requirement for NLRP3 activation (Fig. 1), suggesting that the NF-κB-induced activity was acting upstream of ASC. Consistent with this idea, we found that LPS stimulation did not change Asc mRNA levels but led to strong, NF-κB-dependent increases in Nlrp3 mRNA in mouse macrophages (Fig. 3C). These studies are in line with a report demonstrating NLRP3 induction by TNF and TLR ligands in human cells (16). To analyze the putative Nlrp3 promoter activity, we cloned the promoter region entailing −3,000 to 0 bp upstream of the Nlrp3 transcription start site and constructed a luciferase reporter gene construct. We made use of the fact that heterologous overexpression of
MyD88 activates downstream signaling and therefore expressed the Nlrp3 promoter-luciferase construct alone or together with MyD88. Indeed, coexpression of the Nlrp3 promoter-luciferase construct with MyD88 led to a ~10-fold induction of luciferase activity, indicating that MyD88-mediated signaling can activate the promoter of Nlrp3. Notably, ~40% of patients with autoinflammatory diseases present with classical clinical symptoms without carrying any mutations in the coding region of NLRP3 (17). Recently, unique NLRP3 promoter sequence variants leading to enhanced NLRP3 promoter activity were identified in patients with autoinflammatory diseases that lack NLRP3 coding sequence mutations. This suggests that dysregulated NLRP3 expression could evoke autoinflammatory symptoms (18).

We further found that LPS stimulation led to both NLRP3 and pro-IL-1β protein induction in a dose- and time-dependent manner (Fig. 3, E and F). In addition, LPS failed to induce NLRP3 or pro-IL-1β in cells lacking TLR4 or doubly deficient in MyD88 and TRIF, and NF-κB inhibition led to a dose-dependent reduction of NLRP3 protein induction by LPS (supplemental Fig. 4, A and B). Collectively, these data indicate that NLRP3 expression is tightly controlled by signals culminating in the activation of NF-κB.

FIGURE 3. NLRP3 induction involves NF-κB activity. A and B, Immunoblotting (IB) of caspase-1 from supernatants of wild-type macrophages pretreated with cycloheximide (A) or Bay11-7082 (B) as indicated for 1 h followed by LPS (200 ng/ml, 4 h) and stimulated with ATP (1 h). C, Messenger RNA expression of ASC or NLRP3 in LPS-primed or untreated macrophages. Cells were pretreated with Bay11-7082 for 1 h where indicated. D, HEK293T cells were transfected with pcDNA3-MycD88 or control (pcDNA3) together with a NLRP3 promoter reporter and assessed for luciferase activity after 20 h (error bars, SD). E and F, Immunoblot for NLRP3, pro-IL-1β, and β-actin in lysates from C57BL/6 macrophages treated with LPS for 6 h as indicated, or treated with LPS (200 ng/ml) (P) for the indicated periods of time. Controls are lysates from NLRP3-KO macrophages with and without heterologous NLRP3 expression. Data are from one representative experiment of three (A–D) or two (E and F) experiments.
inflammasome can be explained by the restricted expression of NLRP3 itself and that the NLRP3 expression level is a limiting step for the NLRP3 inflammasome activation in macrophages (2, 5). We revealed that the NLRP3 inflammasome is, in fact, rather restricted in its ability to directly recognize microbial-derived substances. We found that the activation of the NLRP3 inflammasome requires two steps that are controlled by different mechanisms. First, NLRP3 expression itself needs to be transcriptionally induced, and a second, posttranscriptional step, leads to the activation of NLRP3, allowing for NLRP3 inflammasome assembly. The key mediator of immunity, NF-κB, plays a critical role for the priming of the NLRP3 inflammasome. We thus speculate that other NF-κB activators such as UV light or reactive oxygen species can also induce NLRP3 priming.

The fact that priming is a necessary step for NLRP3 inflammasome assembly suggests that macrophages need to acquire a signal that indicates either the presence of infection (via activation of pattern recognition receptors by microbial products) or the activation of other cells (via the presence of proinflammatory cytokines) to commit to sense danger signals in their immediate environment via the activation of the NLRP3 inflammasome. This dual stimulation requirement may operate to prevent accidental or uncontrolled NLRP3 activation, which can have devastating consequences for the host as exemplified by the clinical presentation of patients with autoinflammatory diseases (5, 17).

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Disclosures
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References


