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. 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 defined 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/IL-1R domain-containing adapter inducing IFN-β (TRIF)-KO, MyD88-KO, and TRIF-related adapter molecule (TRAM)-KO (S. Akira, Osaka University, Suita, 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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Franz G. Bauernfeind,* Gabor Horvath,* Andrea Stutz,* Emad S. Alnemri,‡ Kelly MacDonald,* David Speert,* Teresa Fernandes-Alnemri,‡ Jianghong Wu,‡ Brian G. Monks,* Katherine A. Fitzgerald,* Veit Hornung,‡ and Eicke Latz*‡

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2 V.H. and E.L. contributed equally to this study.

3 Address correspondence and reprint requests to Dr. Eicke Latz, University of Massachusetts Medical School, Department of Infectious Diseases and Immunology, 364 Plantation Street, Lazare Research Building 308, Worcester, MA 01605, E-mail address: eicke.latz@umassmed.edu or Dr. Veit Hornung, University of Bonn, Department of Clinical Chemistry and Pharmacology, Sigmund-Freud-Strasse 25, 53127 Bonn, Germany. E-mail address: veit.hornung@uni-bonn.de

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:polyribocytidylic acid, ultra-pure LPS, R848, and N-γ-glutamyl- meso-diaminopimelic acid (E-DAP) were from InvivoGen. Anti-TLR4 Abs (UT18 and MT5510) were from eBioscience. The anti-NLRP3 polyclonal Ab was raised against the NLRP3 pyrin domain that was expressed in Escherichia coli. The mAbs against mouse pro-IL-1β was from the National Cancer Institute and the mouse anti-actin mAb was from Sigma-Aldrich.

Cell stimulation and analysis

Human PBMCs were isolated by density-gradient centrifugation, stimulated in complete RPMI 1640, and the caspase-1 carboxyfluorescein-YVAD-fluoromethyl ketone FLICA (fluorescent labeled inhibitors of caspase) 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 pGEM-T Easy (Promega) and the plasmids were transfected into Escherichia coli DH5α. The mAb against human ASC was raised against a recombinant human ASC fusion protein (a gift from Dr. S. Emili, University of Pittsburgh, PA). 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 into pGL3-basic. HEK293T cells were transfected with luciferase reporter plasmid and expression plasmids (100 ng each) using Lipofectamine (Invitrogen).

Microscopy

A Leica SP2 AOB (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-dependently 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. 1B 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 MT5510), 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
stimulated with ATP (1 h).

Primed macrophages were subsequently activated.

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 Aec 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 (3,000 to 0 bp upstream of the start site and constructed a luciferase reporter gene construct. We made use of the fact that heterologous overexpression of ASC is not sufficient to overcome the priming requirement for NLRP3 activation 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.


dependent signals regulate NLRP3 expression

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NF-κB activation in macrophages treated with LPS (200 ng/ml) for 4 h and ATP or nigericin for 1 h. Caspase-1 activity was assessed by immunoblotting after GFP immunoprecipitation. (C) Confocal microscopy of caspase-1 cleaved and mCitrine-stained NLRP3-mCherry cells. The images show the localization of caspase-1 and NLRP3 in cells treated with LPS (200 ng/ml) for 4 h and ATP or nigericin for 1 h. Caspase-1 cleavage was visualized using a caspase-1-specific antibody, and NLRP3 expression was detected using an NLRP3-specific antibody. A representative experiment of three is shown.

To determine whether enhanced expression of NLRP3 was not only required but also sufficient for NLRP3 inflammasome activation by a "bona fide" NLRP3 stimulus, we next generated macrophage cell lines expressing NLRP3 controlled by a constitutively active viral promoter (Fig. 4). In contrast to cell lines overexpressing ASC alone, cells that overexpressed fluorescent NLRP3 together with fluorescent ASC responded to NLRP3 stimuli in the absence of a prior priming step with the formation of NLRP3/ASC pyroptosomes (Fig. 4, A, and C). We next assessed caspase-1 activity in wild-type and NLRP3-deficient macrophages with and without overexpressed NLRP3. Consistent with earlier results, LPS priming led to significant induction of NLRP3 protein in wild-type cells, and LPS was required for caspase-1 cleavage in combination with an NLRP3 activator. NLRP3-mCherry-overexpressing wild-type cells, however, responded to nigericin (or ATP; data not shown) in the absence of prior priming (Fig. 4D). Hence, overexpression of NLRP3 per se had no influence on caspase-1 cleavage in the absence of nigericin stimulation, demonstrating the need for an NLRP3 activator at this level of expression. Altogether, these results establish that the requirement for priming of the NLRP3

NLRP3 expression is a limiting factor for NLRP3 inflammasome activation

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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