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Inflammasome Priming by Lipopolysaccharide Is Dependent upon ERK Signaling and Proteasome Function

Mohammed G. Ghonime,^{*,†} Obada R. Shamaa,^{*} Srabani Das,^{*} Ramadan A. Eldomany,[†] Teresa Fernandes-Alnemri,[‡] Emad S. Alnemri,[‡] Mikhail A. Gavrilin,^{*} and Mark D. Wewers^{*}

Caspase-1 activation is a central event in innate immune responses to many pathogenic infections and tissue damage. The NLRP3 inflammasome, a multiprotein scaffolding complex that assembles in response to two distinct steps, priming and activation, is required for caspase-1 activation. However, the detailed mechanisms of these steps remain poorly characterized. To investigate the process of LPS-mediated NLRP3 inflammasome priming, we used constitutively present pro-IL-18 as the caspase-1-specific substrate to allow study of the early events. We analyzed human monocyte caspase-1 activity in response to LPS priming, followed by activation with ATP. Within minutes of endotoxin priming, the NLRP3 inflammasome is licensed for ATP-induced release of processed IL-18, apoptosis-associated speck-forming complex containing CARD, and active caspase-1, independent of new mRNA or protein synthesis. Moreover, extracellular signal-regulated kinase 1 (ERK1) phosphorylation is central to the priming process. ERK inhibition and small interfering RNA-mediated ERK1 knockdown profoundly impair priming. In addition, proteasome inhibition prevents ERK phosphorylation and blocks priming. Scavenging reactive oxygen species with diphenylene iodonium also blocks both priming and ERK phosphorylation. These findings suggest that ERK1-mediated posttranslational modifications license the NLRP3 inflammasome to respond to the second signal ATP by inducing posttranslational events that are independent of new production of pro-IL-1 β and NOD-like receptor components. *The Journal of Immunology*, 2014, 192: 3881–3888.

In response to the detection of pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), a multiprotein complex assembles to activate caspase-1 from its zymogen form. This complex has been termed the inflammasome (1). Active caspase-1 cleaves the proinflammatory cytokines IL-1 β and IL-18 from their proforms into their secreted mature forms. In this context, the inflammasome plays a key role in the innate immune response to bacterial and viral infections as well as in responses to tissue damage. It has been implicated in a broad range of common inflammatory diseases such as sepsis (2–4), lung injury (3, 5–7), diabetes mellitus (8, 9), rheumatoid arthritis (10), and rarer inherited disorders such as familial Mediterranean fever (11) and assorted cryopyrin-associated autoinflammatory diseases (12, 13).

Inflammasomes can respond to specific PAMP/DAMPs and are categorized according to their sensor proteins: NLRP1 (NOD-like receptor family, pyrin domain-containing-1), NLRP3 (NOD-like receptor family, pyrin domain-containing-3), NLRC4 (NOD-like receptor family CARD domain-containing protein 4), AIM2 (Absent in melanoma-2), and pyrin (14–18). The NLRP3 inflammasome is the most widely studied and responds to a broad range of PAMP/DAMPs. NLRP3 is part of the NOD-like receptor family, which contains intracellular homologs of the TLR. Similar to the TLRs, NLRP3 contains a leucine-rich repeat region in addition to a nucleotide binding domain and a pyrin domain. It is the adaptor molecule, apoptosis-associated speck-forming complex containing CARD (ASC), that allows caspase-1 to bind to and dimerize in response to NLRP3 activation. How the NLRP3 inflammasome assembles in response to PAMP/DAMPs that have no structural homology is an area of active interest.

It is believed that activation of the NLRP3 inflammasome in macrophages and in dendritic and microglial cells requires two signals. The first signal, termed priming, is commonly induced by a PAMP such as LPS. Current concepts suggest that priming depends upon the de novo synthesis of pro-IL-1 β and the upregulation of NLRP3 (19, 20). However, these concepts were recently challenged by the demonstration that TLR-induced priming of the NLRP3 inflammasome does not require new protein synthesis or upregulation of NLRP3 (21, 22). The second signal induces the assembly and activation of the inflammasome. Signal 2 can be induced by exogenous ATP activation of the P2X7 receptor, as well as by nigericin, silica, and pore-forming bacterial toxins, all of which induce efflux of potassium (23–26). Once cytosolic potassium levels fall, the primed inflammasome becomes functional, caspase-1 becomes catalytically active, and mature IL-1 β and IL-18 are released from the cell.

To understand the mechanism of TLR-mediated priming in the two-step model of the NLRP3 inflammasome in human monocytes,

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Abbreviations used in this article: ASC, apoptosis-associated speck-forming complex containing CARD; BMDM, bone marrow-derived macrophage; DAMP, danger-associated molecular pattern; DPI, diphenylene iodonium chloride; DUB, deubiquitinase; ERK1, extracellular signal-regulated kinase 1; LDH, lactate dehydrogenase; PAMP, pathogen-associated molecular pattern; ROS, reactive oxygen species; siRNA, small interfering RNA; WT, wild-type.

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we sought to study early signaling events that occur before protein translation. We show that procaspase-1, ASC, and pro-IL-18 are constitutively expressed by resting fresh human monocytes. This knowledge allowed us to dissect the kinetics and signaling components of NLRP3 inflammasome priming. Our approach used the standard model of LPS priming followed by ATP. The use of short LPS priming times (5–30 min in duration), as previously described for mouse macrophages (21, 22), eliminated the synthesis of new inflammasome components such as IL-1 β and NLRP3 from contention as necessary components of the ability of LPS to “prime” monocytes for the second signal, ATP. We show that in human monocytes using pro-IL-18 as the caspase-1 substrate, consistent with published findings on mouse macrophages (21, 22), NLRP3 inflammasome priming is independent of new protein synthesis but is dependent on the generation of reactive oxygen species (ROS). We further show that NLRP3 inflammasome priming is dependent upon proteasome function and requires the activation of extracellular signal-regulated kinase 1 (ERK1).

Materials and Methods

Cell culture and chemicals

Human PBMCs were isolated by Histopaque density gradients from fresh-source leukocytes from the American Red Cross. Monocytes were isolated from PBMCs by CD14⁺ selection (Miltenyi Biotec). In brief, blood was layered on lymphocyte separation medium (Cellgro) and spun at 600 \times g for 20 min at room temperature with brakes off. The mononuclear layer was collected and washed three times with RPMI 1640. Monocytes were purified from PBMCs using positive selection with anti-CD14-coated magnetic beads, following the manufacturer's recommendations (Miltenyi Biotec). This method of purification yields >98% pure monocytes based on flow cytometry analysis. Monocytes (1×10^6 /ml) were grown in culture tubes in RPMI 1640 (MediaTech) supplemented with 5% heat-inactivated FBS (Atlanta Biologicals) and 1% penicillin-streptomycin (Invitrogen Life Technologies). FBS lots were prescreened to confirm that they did not induce IL-18 release by ATP in the absence of LPS. Wild-type (WT), *Casp1*^{-/-}, and *Nlrp3*-deficient immortalized mouse bone marrow-derived macrophages (BMDMs) were generated as described earlier (19, 27, 28) and grown in RPMI 1640 supplemented with FBS and penicillin-streptomycin. Priming was provided by LPS (from *Escherichia coli* strain 0111:B4; Alexis Biochemicals) and step 2 activation by ATP (5 mM) from Sigma-Aldrich. MG132 (proteasome inhibitor), U0126 (ERK inhibitor), WP1130 [deubiquitinase (DUB) inhibitor], G5 (ubiquitin isopeptidase inhibitor), and wortmannin (PI3K inhibitor) were from Calbiochem; SB203580 (p38 MAPK inhibitor) and A6355 (another ERK inhibitor) were from Sigma-Aldrich; and SP600125 (JNK inhibitor) was from Invivogen. Diphenylene iodonium chloride (DPI) was from Sigma-Aldrich. The I κ B α inhibitor BAY 11-7082 was purchased from Invivogen, and the AG126 was from Cayman Chemical.

Preparation of cell lysates and Western blotting

Cells were lysed in RIPA buffer (50 mM Tris-HCl [pH7.5], 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% Nonidet P-40, and 0.25% Na-deoxycholate) supplemented with complete protease inhibitor mixture (Sigma-Aldrich), 1 mM PMSF, and 100 μ M *N*-(methoxysuccinyl)-Ala-Ala-Pro-Val chloromethyl ketone. The protein concentrations were determined using the Dc Protein Lowry Assay (Bio-Rad). After SDS-PAGE gel electrophoresis, separated proteins were transferred to a polyvinylidene fluoride transfer membrane, probed with the Ab of interest, followed by HRP-conjugated secondary Ab and developed by ECL (Amersham Biosciences) using autoradiography. Rabbit polyclonal Abs against IL-1 β , ASC, and caspase-1 were developed in our laboratory. Anti-human IL-18 Ab was purchased from MBL, ubiquitin Ab from Santa Cruz Biotechnology, and actin from MP Biomedicals. Mouse anti-phospho-I κ B α , rabbit anti-I κ B α , rabbit anti-phospho-ERK1/2, and rabbit anti-ERK1/2 were purchased from Cell Signaling Technology. Released IL-1 β was quantified with a sandwich ELISA format, using our rabbit polyclonal as previously reported (29) but substituting monoclonal (MAB601) from R&D Systems for the capture Ab. In addition, IL-1 β in the cell culture medium was detected by immunoblot of cell culture medium with rabbit polyclonal IL-1 β Ab (laboratory generated). Released IL-18 was quantified by ELISA using MBL Abs.

Cell death detection by quantification of lactate dehydrogenase release in cell culture medium

Lactate dehydrogenase (LDH) release into cell culture medium was used as an indicator of cell death with the NAD⁺ reduction assay (Roche Applied Science). Monocytes were grown in culture tubes at a density of 10^6 /ml and pretreated with or without inhibitor for 30 min followed by LPS (1 μ g/ml) for 0.5 or 3 h, and finally activated with 5 mM ATP for 30 min. Cell culture medium was collected, clarified by centrifugation, and used for the LDH assay. Total LDH content in cells (positive control) was measured in cells lysed with Triton X-100 (1% final concentration). Cell culture medium alone was used as a blank, and OD values were subtracted from readings of samples and positive control. LDH concentration in the medium was detected at a wavelength of 490 nm. Cell death was calculated by the following formula: cytotoxicity (%) = [(sample – blank)/(positive control – blank) \times 100], as described earlier (30).

Quantitative PCR

Total RNA from monocytes was extracted by the TRIzol method and converted into cDNA by the ThermoScript RT System (both from Invitrogen Life Technologies). Quantitation of *IL1B*, *IL8*, and *TNF* gene expression was performed with SYBR Green I PCR Master Mix in the StepOne Real Time PCR System (both from Applied Biosystems) and expressed in relative copy numbers (RCN) as described earlier (31).

Nucleofection

In knockdown experiments, small interfering RNA (siRNA) against ERK1 and scrambled siRNA were purchased from Sigma-Aldrich, and Signal-Silence p42 MAPK (ERK2) siRNA II was purchased from Cell Signaling Technology. The siRNAs were delivered in monocytes by Amaxa Nucleofector I (Lonza). 5'-GACCGGAUGUUAACCUUUA-3' and 5'-AAGCUGACCCUGAAGUUCA-3' sequences were used to knock down ERK1 and as scrambled control (32, 33). For nucleofection, 5×10^6 monocytes were resuspended in 100 μ l nucleofection solution containing 150 pmol siRNA for ERK1 and scrambled control; siRNA against ERK2 was used according to the manufacturer's protocol. Nucleofection was performed with the Y-01 program. Immediately after nucleofection, monocytes were resuspended in RPMI 1640 medium supplemented with 10% FBS and left to recover overnight in polypropylene culture tubes to avoid adherence. The next morning, monocytes were counted with trypan blue, showing that 90% of cells were viable. Then cells were treated with 1 μ g/ml LPS for 30 min, followed by 5 mM ATP for another 30 min. Released IL-18 in cell culture medium was measured using ELISA, and cells were lysed and analyzed for proteins.

Statistical analysis

All experiments were performed a minimum of three independent times and expressed as mean values \pm SEM. Comparisons of groups for statistical difference were made using the Student *t* test. A *p* value \leq 0.05 was considered significant.

Results

Monocyte priming for IL-1 β versus IL-18

Priming with LPS and subsequent exogenous ATP-induced activation of the P2X7 receptor represents a classic model of NLRP3 inflammasome activation (23–25, 34). However, the mechanistic details of the priming event remain poorly characterized. One proposed function of inflammasome priming is to generate the inflammasome substrate pro-IL-1 β (19). Therefore, to better define priming we first analyzed the kinetics of the two known human monocyte caspase-1 substrates, IL-1 β and IL-18. Human monocytes were purified by CD14⁺ selection and treated with LPS (1 μ g/ml) for intervals up to 180 min. Cells were lysed and analyzed for pro-IL-1 β and pro-IL-18 by immunoblot. As demonstrated in Fig. 1A, whereas purified human monocytes require almost an hour of priming to induce detectable levels of pro-IL-1 β , the other caspase-1 substrate, pro-IL-18, is constitutively present. Thus, pro-IL-18 provides a tool for studying earlier events in inflammasome priming.

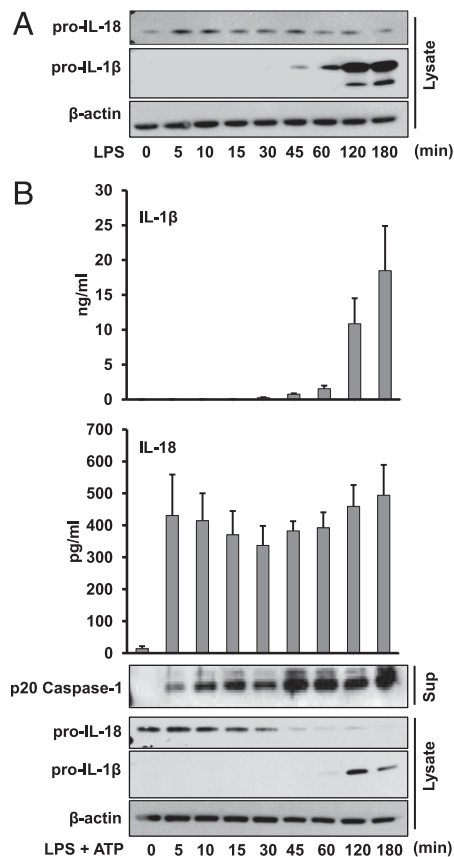


FIGURE 1. IL-18 kinetics differs from that of IL-1β in response to LPS and ATP. **(A)** LPS priming induces expression of monocyte IL-1β, but not IL-18. Fresh human monocytes were stimulated with LPS (1 μg/ml) for the specified times, and then lysates were analyzed for expression of pro-IL-18 and pro-IL-1β by immunoblots, compared with β-actin control. Shown is one representative blot of three. **(B)** LPS stimulates rapid priming for ATP-induced IL-18 and caspase-1 processing and release in monocytes. Monocytes were prepared and primed (step 1) as in **(A)** for the indicated time points, followed by 5 mM ATP for 30 min (step 2). Bar graphs represent released IL-1β and IL-18 from cell supernatants as determined by ELISA. Immunoblots show pro-IL-18, pro-IL-1β, and β-actin in the cell lysates and p20 caspase-1 released into supernatants, respectively. ELISA data represent the mean ± SEM for three separate monocyte donors, and the blots are representative of repeated blots.

IL-18 processing and release are independent of new protein synthesis but require active caspase-1 and NLRP3 inflammasome

It has been presumed that the priming effect of LPS is dependent upon new NLRP3 protein expression, along with generation of the pro-IL-1β substrate (19). However, recent studies have clearly demonstrated that priming of the NLRP3 inflammasome in mouse macrophages does not require new protein synthesis or upregulation of NLRP3 (21, 22). Having shown that pro-IL-18 is constitutively present, we elected to test whether new protein synthesis is required for priming in human monocytes. Monocytes were stimulated with LPS at brief intervals before addition of exogenous ATP. As shown in Fig. 1B, whereas IL-1β processing and release were dependent upon new protein synthesis, this was not true for IL-18 because monocytes primed with LPS for 5 min followed by addition of ATP for 30 min, without removal of LPS, was sufficient to induce IL-18 release. Furthermore, the p20 functional form of caspase-1 was released into the supernatants concurrently with the IL-18 release (Fig. 1B).

To confirm that the released IL-18 was a product of active caspase-1 (i.e., the inflammasome) and not simply the nonspecific release of

pro-IL-18, we analyzed the supernatants by immunoblots. Importantly, the ELISA detection of IL-18 corresponded to the generation of an 18-kDa processed form of IL-18 (Fig. 2A, 2B). This release of the mature form of IL-18 and caspase-1 was inhibited by pretreating the monocytes with caspase-1 inhibitors, YVAD and zVAD. Of note, although caspase-1 inhibition prevented the generation of mature IL-18 and p20 caspase-1, it did not prevent the release of pro-IL-18, procaspase-1, or ASC (Fig. 2A). These results suggest that the inflammasome release pathway does not require functional caspase-1, as we have previously reported (35, 36).

The caspase-1 inhibition effect was further confirmed using BMDMs from mice with caspase-1 knockout (Fig. 2C, Supplemental Fig. 1A), which showed that caspase-1 was essential for the LPS- and ATP-induced IL-18 processing.

Furthermore, extracellular potassium blocked IL-18 release from human monocytes (Supplemental Fig. 1B), which suggests that the human model represented the NLRP3 inflammasome. Rapid LPS priming of BMDMs from the NLRP3 knockout mice (Fig. 2C, Supplemental Fig. 1A) failed to induce IL-18 processing, suggesting that the early LPS priming targets the NLRP3 inflammasome.

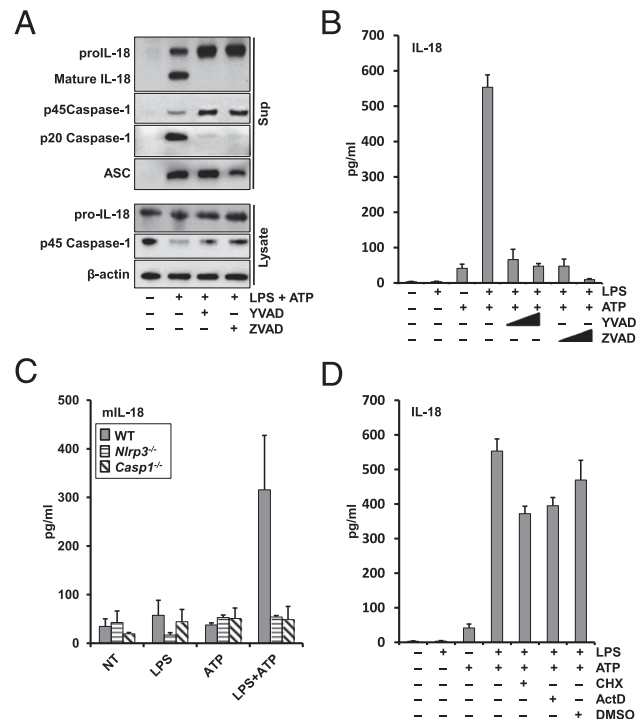


FIGURE 2. IL-18 processing and release are independent of new protein synthesis but require active caspase-1 and NLRP3 inflammasome. Monocytes were pretreated with or without YVAD-cmk, ZVAD-fmk, cycloheximide, or actinomycin D for 30 min prior to stimulation with LPS (1 μg/ml) for 30 min followed by 5 mM ATP for 30 min. **(A)** Immunoblots of IL-18, caspase-1, and ASC expressed in cell lysates (bottom group) and released in supernatants (top group) after pretreatment with YVAD or ZVAD (50 μM). **(B)** Culture media were assayed by ELISA for the released IL-18 after LPS, ATP, and YVAD or ZVAD at 10 or 50 μM, respectively. **(C)** Immortalized BMDMs from WT, *Nlrp3*^{-/-}, and *Casp1*^{-/-} were either left untreated (NT) or were primed with LPS (1 μg/ml) for 30 min followed by 5 mM ATP for 30 min. Bar graphs represent released murine IL-18 from cell supernatants, as determined by ELISA. **(D)** Monocytes were pretreated with cycloheximide (1 μg/ml) or actinomycin D (5 μg/ml), and IL-18 release after LPS/ATP stimulation was measured. ELISA data represent the mean ± SEM for three separate monocyte donors, and the blots are representative of repeated blots.

Although unlikely, it is conceivable that within 5 min of priming and 30 min of ATP, critical inflammasome regulatory proteins could be newly synthesized. To eliminate this possibility, monocytes were treated with cycloheximide, a translation inhibitor, or actinomycin D, a transcription inhibitor, 30 min prior to the priming signal. These inhibitors completely blocked protein synthesis and mRNA transcription (Supplemental Fig. 2). Consistent with the recent finding that priming of the NLRP3 inflammasome is a posttranslational event (21, 22), neither inhibition of protein synthesis nor inhibition of mRNA transcription blocked the ability of LPS to prime for IL-18 processing and release (Fig. 2D).

Priming regulated by proteasome

Having demonstrated that priming is indeed required, even for preformed substrates such as IL-18, and confirmed that this event is independent of new protein synthesis, we next wanted to determine whether posttranslational protein modifications might be a component of priming. One possibility for posttranslational modifications is the proteasome's control of inflammasome-related protein levels. To test this, we inhibited the proteasome using MG132 or bortezomib. Pretreating monocytes with MG132, 30 min prior to LPS priming, induced a significant deficit in ATP-mediated IL-18 and caspase-1 processing and release, while enhancing protein ubiquitination (Fig. 3). In similar experiments, bortezomib also prevented priming of the IL-18 processing inflammasome (data not shown).

Proteasomal blockade also suppressed IL-1 β processing and release. However, because MG132 prevented generation of pro-IL-1 β , it was not possible to distinguish between the NF- κ B dependence of pro-IL-1 β substrate generation and the effects of proteasomal inhibition on priming (Supplemental Fig. 3A). These results document the importance of using preformed pro-IL-18 as the optimum target substrate for studies of inflammasome priming events.

Because the half-life of intracellular constitutive IL-18 may be dependent upon NF- κ B activity, which itself is regulated by the proteasome, it is important to determine that proteasomal inhibition does not induce rapid loss of steady state pro-IL-18. We therefore examined the kinetics of pro-IL-18 in the presence of MG132 and protein synthesis inhibition (cycloheximide) (Supplemental Fig. 3B, 3C). Neither inhibitor decreased intracellular pro-IL-18 levels at 1 h, although pro-IL-18 levels began to decline by 2 h. Because we have shown that the priming and processing and release of IL-18 can occur as early as 35 min (5 min LPS priming and 30 min ATP) (Fig. 1B), these data suggest that proteasomal regulation of pro-IL-18 inflammasome priming was not due to loss of pro-IL-18 substrate. Of note, the rapidity of the priming event (5 min) coincides with the rapidity of the phosphorylation of the IKK substrate I κ B α (Supplemental Fig. 3D), which, as expected, was preserved by MG132.

LPS induced ERK signaling events critical to priming

Having confirmed that inflammasome priming is independent of new protein synthesis, we turned to pathways important to priming upstream of NF- κ B transcriptional activation. Generation of IL-18 was significantly suppressed by the IKK inhibitor Bay 11, but even more powerfully by the ERK and tyrosine kinase inhibitor AG126 (Supplemental Fig. 4A). These data support the concept that priming involves a signaling step upstream of NF- κ B transcriptional activation in the LPS signaling pathway.

To further isolate the location of this priming event, we compared the relative effects of inhibitors targeting MEK1/MEK2, PI3K, JNK, and p38 kinase (Fig. 4). Only MEK1/MEK2 inhibition demonstrated a profound suppression of priming, and this

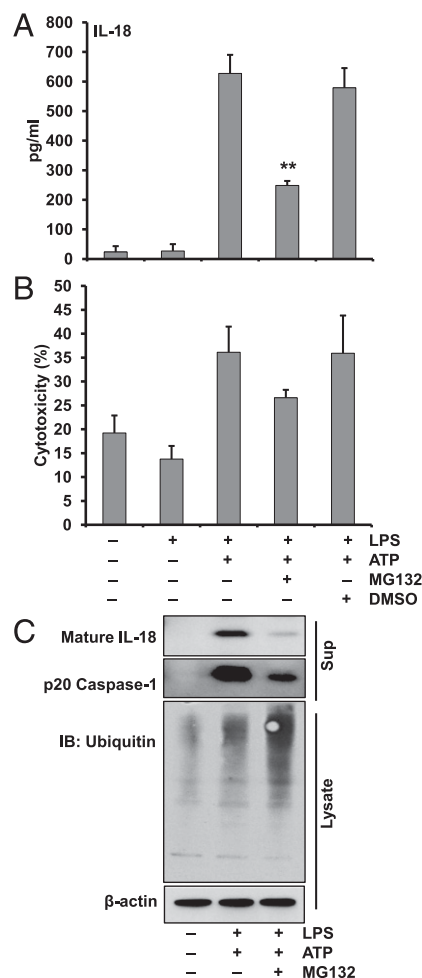


FIGURE 3. Proteasome inhibition suppresses mature IL-18 release by monocytes. Monocytes were pretreated with or without MG132 (20 μ M) for 30 min, then primed with LPS (1 μ g/ml) for 30 min, followed by 30 min with or without 5 mM ATP. (A) Release of IL-18 in supernatants was significantly suppressed with MG132. $**p < 0.01$. (B) LDH release was not affected by MG132. (C) Immunoblots of cell supernatants for mature IL-18 and p20 caspase-1 (upper panels) and cell lysate for ubiquitinated proteins.

finding is confirmed using another ERK inhibitor (A6355) (Supplemental Fig. 4B). To further analyze the specificity of this effect, we found that ERK phosphorylation was induced by LPS priming, but not by the second signal, ATP (Fig. 5A); and ERK phosphorylation, which is downstream of MEK1/MEK2 kinases, was indeed inhibited by U0126 (Fig. 5B). Finally, regarding ERK phosphorylation, we considered whether the proteasomal inhibition also affected ERK signaling. As shown in Fig. 5C, MG132 pretreatment prevented the LPS-induced phosphorylation of ERK, which confirms prior observations linking the proteasome to ERK (37).

To further support the specificity of the ERK inhibitors U0126 and AG126, we downregulated ERK1 and ERK2 with siRNA. As shown in Fig. 6, transient knockdown of ERK1, but not ERK2, was able to inhibit inflammasome priming. Together these data support the concept that ERK signaling is a central event in inflammasome priming. In addition, we examined the role of ERK in priming of murine macrophages using immortalized BMDMs. Surprisingly, we found these cells have ERK MAPK activation at baseline, as shown in Fig. 5D. Hence, these cells may bypass the need for ERK activation as part of the priming effect of LPS.

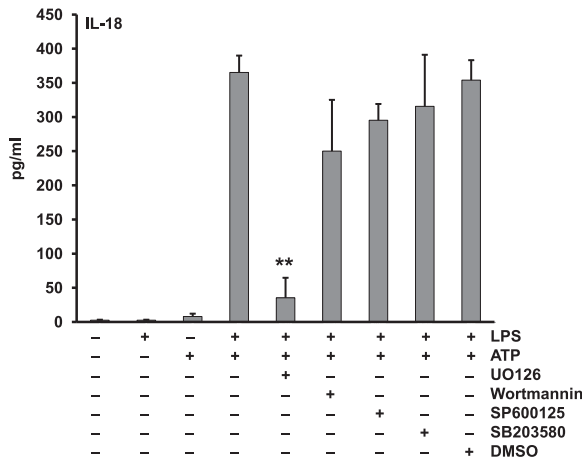


FIGURE 4. ERK plays a crucial role in inflammasome priming. Monocytes were pretreated with UO126 (ERK inhibitor) (10 μ M), wortmannin (PI3K inhibitor) (10 μ M), SB203580 (p38 MAPK inhibitor) (10 μ M), or SP600125 (JNK inhibitor) (10 μ M) for 30 min. Then cells were primed with LPS (1 μ g/ml) for 30 min and activated with ATP (5 mM) for 30 min. Supernatants were assayed for the released IL-18. ** p = 0.0016.

Previous reports suggested a role for DUBs in the posttranslational regulation of the NLRP3 inflammasome (38, 39); therefore, we used the DUB inhibitors WP1130 and G5 to evaluate the role of DUBs in the LPS rapid priming events. Of interest, DUB inhibition diminished the amount of IL-18 released in a dose-dependent manner (Fig. 7), confirming the published results in murine models. However, the DUB inhibitors did not affect ERK phosphorylation, suggesting that these DUB enzymes may act downstream of, or parallel to, the ERK pathway.

ERK signaling and role of oxidants in priming

It has been shown that ROS may activate the inflammasome, but this has not been analyzed from the perspective of ERK-mediated priming (21, 22, 40, 41). We therefore adopted our LPS/ATP IL-18 model to this question. As shown in Fig. 8A and 8B, the inhibitor of the NADPH oxidase DPI was able to inhibit the ability of LPS/ATP to process pro-IL-18 in a dose-dependent manner. Of note, the prevention of IL-18 processing by DPI did inhibit ERK phosphorylation, as shown in Fig. 8C.

These studies suggest a complex interplay between ERK and oxidants in which ERK function may be regulated in part by upstream oxidants that prevent phosphatases from suppressing

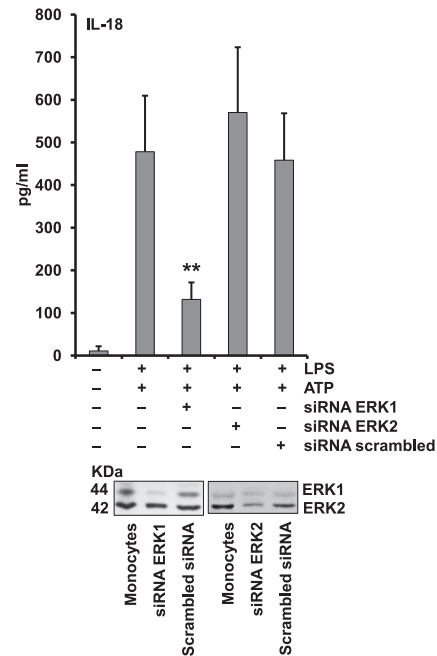


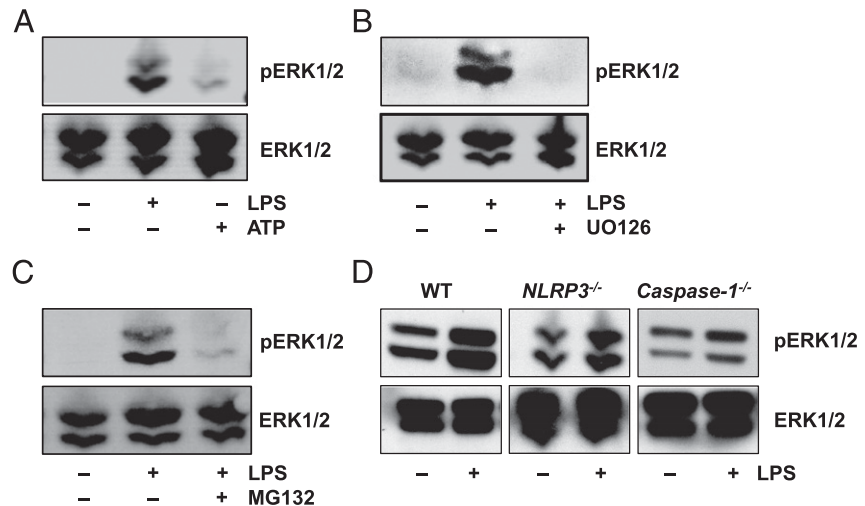
FIGURE 6. ERK1 knockdown suppresses IL-18 processing and release. Monocytes were nucleofected with ERK1- and ERK2-specific siRNA or scrambled control and recovered overnight. Cells were then primed with LPS (1 μ g/ml) for 30 min and activated with ATP (5 mM) for another 30 min. Supernatants were analyzed for IL-18 release, and cell lysates were immunoblotted to confirm ERK protein downregulation using total ERK Ab. IL-18 ELISA represents the mean \pm SEM for three separate monocyte donors, and the blots are representative of repeated blots. ** p < 0.01 compared with scrambled siRNA.

ERK (42, 43). Priming therefore represents a complex of regulated events involving kinases, phosphatases, oxidants, and the proteasome that are linked to ERK signaling in a way that licenses the inflammasome constituents to respond to a second signal (results summarized in Fig. 9).

Discussion

The inflammasome is involved in the pathogenesis of many inflammatory diseases, and a better understanding of how the inflammasome is regulated may identify new therapeutic agents that could be used to treat such diseases. In this context, it has been shown that inflammasome activation requires two distinct steps:

FIGURE 5. ERK phosphorylation is induced by priming, but not by ATP in the absence of priming. ERK phosphorylation was measured in monocyte lysates by immunoblotting with anti-phosphoERK-specific Ab and total ERK Ab, respectively. (A) Comparison of ERK phosphorylation induced by LPS (1 μ g/ml) versus ATP (5 mM), respectively. (B) UO126 (10 μ M) blocks phosphoERK induction by the LPS/ATP combination. (C) MG132 (20 μ M) blocks LPS-induced ERK phosphorylation. (D) ERK phosphorylation in unstimulated versus LPS-primed WT, *Nlrp3*^{-/-}, and *Casp1*^{-/-} BMDMs.



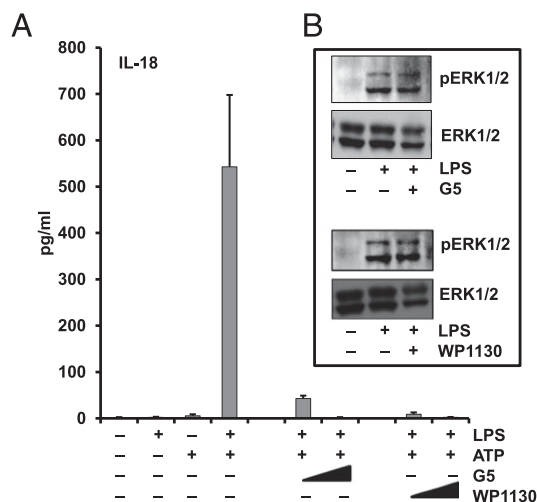


FIGURE 7. DUB inhibitors impair LPS priming of monocytes and IL-18 release. Monocytes were pretreated with either G5 (0.1 and 0.5 μ M) or WP1130 (1 and 5 μ M) for 30 min prior to LPS (1 μ g/ml) priming followed by the ATP (5 mM) activation step. **(A)** Supernatants were analyzed for IL-18 release. **(B)** Cell lysates were analyzed by immunoblot for the effect of G5 (0.1 μ M) and WP1130 (1 μ M) on the ERK phosphorylation.

step 1, priming, and step 2, activation. However, the mechanistic details of priming have been poorly characterized to date. Therefore, to better delineate what inflammasome priming is, we focused on those aspects of priming that do not require new protein synthesis.

First, we show that pro-IL-18 is constitutively expressed in fresh unstimulated monocytes. On the basis of this observation we

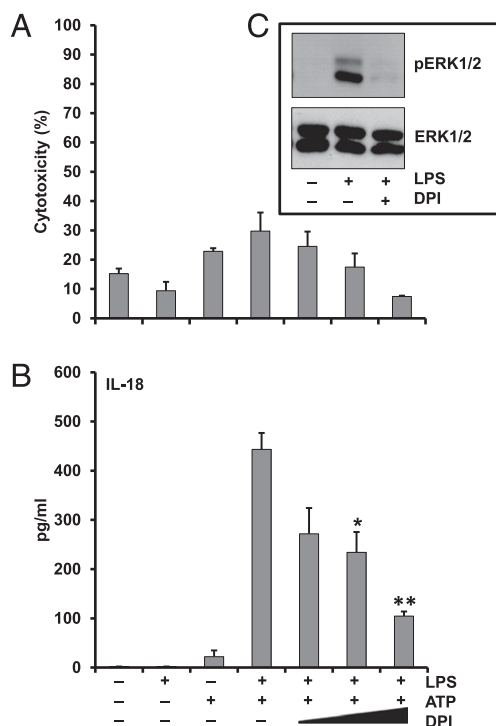


FIGURE 8. Inhibition of ROS generation impairs LPS priming of monocytes and IL-18 release. Monocytes were pretreated with DPI (1, 10, 20, and 50 μ M) for 30 min prior to LPS (1 μ g/ml) priming followed by the ATP (5 mM) activation step. Supernatants were analyzed for **(A)** LDH and **(B)** IL-18 release, respectively. * $p < 0.05$, ** $p < 0.01$, compared with LPS/ATP. **(C)** Cell lysates were immunoblotted for phosphoERK and total ERK.

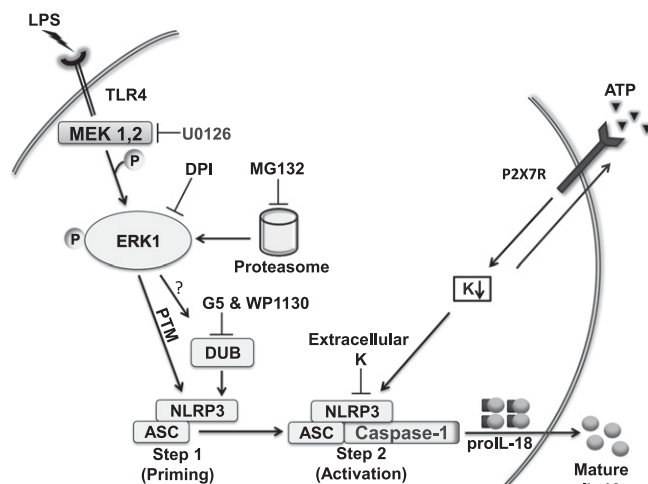


FIGURE 9. Model of the signaling pathways involved in ERK-dependent rapid inflammasome priming. Brief priming with LPS induces ERK1 phosphorylation, which likely induces changes in either the localization or the interaction of ASC with an NLR component of the inflammasome. This step 1 (priming event) can be blocked by U0126 and is controlled by ROS and the proteasome. It seems that LPS-induced ERK phosphorylation leads to an ERK-dependent posttranslational modification (PTM) of one or more of the key proteins involved in inflammasome function. DUBs may work downstream or parallel to the ERK pathway. Step 2 (provided by ATP in our experiments) lowers intracellular potassium levels. Step 2 is dependent upon this rapid signaling process because ATP without priming does not activate caspase-1.

used the LPS/ATP two-step model to identify some of the early regulatory events that occur before protein synthesis. Our approach depended upon a functional readout of inflammasome activation, the cleavage of caspase-1, and the maturation of pro-IL-18. Our results confirm that the widely accepted concept of priming as the induction of NLRP3 and the caspase-1 substrate pro-IL-1 β is incomplete (19, 20). In this regard, we show that the IL-18 processing inflammasome could be primed and activated after 5 min of LPS priming followed by 30 min of ATP. This finding is consistent with recent reports that showed activation of NLRP3 and cleavage of caspase-1 in BMDMs after priming of cells for a short time with LPS, followed by activation with ATP, or costimulation with TLR agonists plus ATP (22). We show that priming can be blocked by inhibitors of the NF- κ B signaling pathway, but this effect was pretranscriptional because kinetic analyses showed the inhibition occurred well before new protein synthesis. Furthermore, inhibitors of protein synthesis had no significant effect on priming of the NLRP3 inflammasome.

Attesting to the validity of the nontranscriptional model of inflammasome activation (21, 22), we confirm by immunoblots that the released IL-18 is the mature processed form and we show that the processed IL-18 is accompanied by released p20 caspase-1 and ASC. This processing was dependent upon active caspase-1 because it was completely inhibited by caspase-1-specific tetrapeptide, YVAD-cmk. This result was further confirmed using BMDMs from caspase-1 knockout mice. However, because these mice were *Casp1/Casp11* double knockout, we cannot exclude the role of caspase 11 in this process.

Of note, however, inhibition of caspase-1 blocked only processing, but not the release of IL-18, suggesting that inflammasome release is not dependent upon active caspase-1, as we have previously observed (35, 36). We interpret these results to mean that inflammasome priming by LPS causes a pretranscriptional modification of the constituent proteins that control inflammasome

readiness for step 2, ATP activation of the P2X7 receptor. Post-translational modifications of the key regulatory proteins by phosphorylation, ubiquitination, and yet to be identified changes play crucial roles in the regulation of signal transduction in the cell. In this context, we previously showed that inhibition of tyrosine phosphatases leads to inflammasome activation and IL-1 β secretion (32).

Recent reports show, as well, that LPS/ATP can induce deubiquitination of NLRP3, affecting inflammasome activation and caspase-1 cleavage (22, 39). In addition, DUB-dependent regulation of signaling is required for assembly of the inflammasome and caspase-1 activation (38). These findings are in concert with our finding that MG132 preserves ubiquitinated protein levels and inhibits inflammasome priming and activation. Moreover, the inhibitors of DUB, G5 and WP1130, inhibited the rapid priming of the inflammasome, suggesting that caspase-1-induced IL-18 release is DUB dependent.

We believe that our model reflects the NLRP3 inflammasome because we were able to block the rapid processing of pro-IL-18 by extracellular potassium, which has previously been shown to be NLRP3 specific (26). In further support of NLRP3 is the abundance of data in murine models that support the LPS/ATP activation pathway as NLRP3 dependent (34, 44). In addition, we used BMDMs for Nlrp3 knockout mice, which showed inhibition of IL-18 release, confirming that NLRP3 is responsible for the rapid priming event in these macrophages.

The posttranslational nature of the priming event supported the idea that priming involves a kinase-mediated event. Addressing the events critical to the LPS-mediated priming, we turned to classic signaling pathways. It is well established that LPS activates the MAPK family, including ERK, p38 MAPK and JNK, as well as PI3K, another important mediator downstream of LPS/TLR4 signaling. To our knowledge, determining the role of these pathways in this post-translational licensing of inflammasome function has not previously been attempted (27). We used specific inhibitors for each of the above kinase pathways. We found that the ERK inhibitor (U0126) could significantly block inflammasome priming and activation. The pharmacological inhibition was further confirmed with the effective siRNA knockdown of ERK1, which is a downstream target of MEK1/2. It seems that ERK1 is the key player in the early events associated with priming, as suggested by the knockdown experiment, but further work will be needed to confirm this finding. In contrast, the p38 MAPK inhibitor (SB203580), the JNK inhibitor (SP600125), and the PI3K inhibitor (wortmannin) did not affect early priming and activation, indicating that these conventional pathways activated by TLR signaling are not mandatory for LPS-dependent inflammasome activation. Also, RIP2 signaling is not obligatory for inflammasome activation, as some recent reports specified RIP2 as another target of SB203580 (45).

It should be noted that after our manuscript was submitted, a report demonstrated that Syk and Jnk are linked to inflammasome activity (46). This work used a 4 h LPS priming step and nigericin model largely in a murine macrophage system. Unlike our current priming model, this work provided the inhibitors after the priming and failed to show an ERK effect. Of interest, we found that ERK is spontaneously phosphorylated in these immortalized, proliferating murine BMDMs. We speculate that this background ERK activation provides "spontaneous" priming that is normally provided by LPS in our nondividing human monocytes, the focus of our model. As we have previously shown, providing ERK inhibition after priming is not able to block LPS/ATP-mediated human monocyte inflammasome activation (36), whereas it does so when added before priming. Thus, methodological, cell type, and species differences likely explain the variances with our current work regarding NLRP3 inflammasome regulation.

To discern whether the ERK activation was addressing the priming phase, we showed that ERK phosphorylation is dependent on LPS priming, as ATP alone did not induce ERK phosphorylation but LPS-induced ERK phosphorylation was blocked by U0126. Of note, ERK has been found to phosphorylate >150 cytoplasmic and nuclear targets (47, 48). On the basis of the rapid kinetics, our findings suggest that ERK phosphorylation of transcription factor targets is not obligatory for the early priming events. It is likely, therefore, that ERK has other targets that play a key role in priming of the NLRP3 inflammasome. For example, it is conceivable that DUBs may be activated downstream of ERK. This posttranslational modification could contribute to a DUB-dependent deubiquitination of NLRP3, leading to inflammasome assembly and caspase-1 cleavage and activation. Further work is needed to identify the target or targets that could provide improved ways to manipulate inflammatory diseases.

One model for inflammasome priming and activation focuses on ROS. Recent reports suggest that ROS interaction with the TXNIP-thioredoxin complex liberates TXNIP, which then causes inflammasome assembly (49). We evaluated the role of ROS in the early events occurring after LPS priming. Inhibition of ROS production by DPI decreased IL-18 release, indicating that ROS plays a major role in early priming events, as shown recently in mouse BMDMs (21, 22, 40, 41). DPI was able to block ERK phosphorylation, suggesting that ROS upstream of ERK can be regulatory. This finding is consistent with previous reports that ROS can initiate ERK activation by inhibition of ERK-directed phosphatases like DUSP1 and DUSP6 by the oxidation of their catalytic cysteine residues (50–52). In addition, ROS can inhibit ERK tyrosine phosphatases, PP1 and PP2A, by inhibiting their cysteine catalytic sites (53). These ERK-directed phosphatases determine the outcome of RAS/Raf/ERK signaling (54, 55). Finally, it also has been shown that some phosphatases are upregulated in proteasome-inhibited cells, including ERK-specific phosphatases (37, 56). Taken together, these prior reports substantiate our recent report that phosphatase inhibition itself can activate the inflammasome in PMA-activated THP-1 cells (32) and provides additional support for the complex role that the proteasome plays in regulating caspase-1 activation (38). It seems that posttranslational modifications are central to regulating inflammasome priming and activation.

In summary, our data reveal that priming (or licensing of the inflammasome) is an early event, independent of transcription and translation and centered upon the ERK signaling pathway. Further characterization of the regulatory mechanisms involved in the early inflammasome priming and activation should enhance our understanding and provide new opportunities for treating inflammasome-dependent disorders.

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Disclosures

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