IRAK-1 bypasses priming and directly links TLRs to rapid NLRP3 inflammasome activation

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Pathogenic infections and tissue injuries trigger the assembly of inflammasomes, cytosolic protein complexes that activate caspase-1, leading to cleavage of pro-IL-1β and pro-IL-18 and to pyroptosis, a proinflammatory cell death program. Although microbial recognition by Toll-like receptors (TLRs) is known to induce the synthesis of the major caspase-1 substrate pro-IL-1β, the role of TLRs has been considered limited to up-regulation of the inflammasome components. During infection with a virulent microbe, TLRs and nucleotide-binding oligomerization domain-like receptors (NLRs) are likely activated simultaneously. To examine the requirements and outcomes of combined activation, we stimulated TLRs and a specific NLR, nucleotide binding and oligomerization, leucine-rich repeat, pyrin domain-containing 3 (NLRP3), simultaneously and discovered that such activation triggers rapid caspase-1 cleavage, leading to secretion of presynthesized inflammatory molecules and pyroptosis. This acute caspase-1 activation is independent of new protein synthesis and depends on the TLR-signaling molecule IL-1 receptor-associated kinase (IRAK-1) and its kinase activity. Importantly, *Listeria monocytogenes* induces NLRP3-dependent rapid caspase-1 activation and pyroptosis, both of which are compromised in IRAK-1–deficient macrophages. Our results reveal that simultaneous sensing of microbial ligands and virulence factors by TLRs and NLRP3, respectively, leads to a rapid TLR- and IRAK-1–dependent assembly of the NLRP3 inflammasome complex, and that such activation is important for release of alarmins, pyroptosis, and early IFN-γ production by memory CD8 T cells, all of which could be critical for early host defense.

Interleukin-18 | ASC | HMGB-1

Toll-like receptors (TLRs) recognize conserved molecules from pathogens and initiate signaling that activates NF-κB, MAP kinases, and IFN response factor proteins (1, 2). This signaling induces proinflammatory cytokines, chemokines, adhesion molecules, and inflammasome components, all of which facilitate effector responses (1, 2). A second family of receptors, nucleotide-binding oligomerization domain-like like receptors (NLRs), reside in the cytosol and are activated in response to either microbial ligands that gain access to the cytosol or virulence factors, such as bacterial toxins (3, 4).

Activation of NLRs leads to assembly of an inflammasome complex, leading to activation and cleavage of cysteine protease, caspase-1, which in turns cleaves IL-1β and IL-18, leading to their secretion (5). The widely studied nucleotide binding and oligomerization, leucine-rich repeat, pyrin domain-containing 3 (NLRP3) inflammasome, composed of NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and procaspase-1, undergoes assembly in response to stimulation by various stimuli, including ATP, nigericin, maitotoxin, uric acid crystals, silica, asbestos, and such pathogens as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Listeria monocytogenes*, and *Salmonella typhimurium* (6).

Inflammasome-mediated caspase-1 activation promotes inflammation and host defense by two principal avenues: secretion of mature cytokines (IL-1β and IL-18) and activation of pyroptosis (7), a proinflammatory cell death pathway that eliminates the infected cell and removes the niche for intracellular microbial replication (8). The current understanding of the biology of IL-1β synthesis and secretion holds that the TLR signaling pathway induces synthesis and accumulation of pro-IL-1β in the cytosol, and inflammasome ligands cause assembly of the respective inflammasome complexes, leading to cleavage of pro-IL-1β by active caspase-1. The role of TLR signaling is thus considered limited to synthesis of the substrates or up-regulation of levels of the components of the inflammasome complexes themselves.

In the present study, we investigated whether TLRs play a direct role in activation of the NLRP3 inflammosome and discovered that there are at least two phases of NLRP3 inflammasome activation. The early phase, acute inflammasome activation, is independent of new protein synthesis, depends on simultaneous activation of TLRs and NLRP3, and is directly regulated by TLR signaling via the TLR-signaling molecule IL-1 receptor-associated kinase (IRAK-1). The late phase, involving priming-dependent activation of the NLRP3 inflammasome, occurs independent of direct participation of IRAK-1. We also found that the acute IRAK-1–dependent NLRP3 inflammasome activation pathway is critical for pyroptosis and secretion of inflammatory proteins presynthesized by the cell. Our findings provide evidence supporting a direct link between TLR signaling and NLRP3 inflammasome activation and ascribe a unique function to IRAK-1 in early innate responses.

Significance

Toll-like receptors recognize conserved molecules that are expressed by both harmless (commensal) and harmful (virulent) microbes. Another set of receptors, nucleotide-binding oligomerization domain-like receptors (NLRs), are expressed in the cytosol and recognize virulence factors and toxins from pathogenic microbes. Previous studies on TLRs and NLRs have suggested that TLR signaling primes the NLR inflammasome pathway. Here we discovered that TLRs, via the signaling molecule IL-1 receptor-associated kinase, directly regulate activation of a specific NLR, nucleotide binding and oligomerization, leucine-rich repeat, pyrin domain-containing 3 (NLRP3). This is important because when infection occurs, the virulent/pathogenic microorganisms activate both of these receptors. We also found that simultaneous activation of TLRs and NLRP3 is important for rapid innate immune response by the host.

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Results

Simultaneous Stimulation of TLRs and NLRP3 Leads to Acute Inflammasome Activation. Traditional inflammasome studies use sequential stimulation in which cells are first primed using a TLR ligand and hours later a second stimulus is added to trigger NLRP3 inflammasome assembly and caspase-1 activation (9). Host cells at the site of infection are likely to be exposed to both TLR and NLR ligands simultaneously or in rapid succession, however. Thus, to mimic natural infection with a pathogenic microbe, we tested the responses of bone marrow-derived macrophages (BMDMs) to simultaneous stimulation of TLRs and NLRP3. WT BMDMs stimulated simultaneously with a TLR ligand and ATP for 30 min activated caspase-1 (Fig. 1*A*). Ligands for TLR4, TLR9, TLR7, and TLR2—LPS, CpG, R837, and Pam3CSK4—triggered rapid caspase-1 cleavage in BMDMs costimulated with ATP, but poly I:C, a TLR3 ligand, did not (Fig. 1*A*). Kinetically, stimulation of BMDMs with ATP and LPS for as little as 15 or 20 min led to rapid caspase-1 activation (*SI Appendix*, Fig. S1).

Rapid inflammasome activation was abolished in both TLR- and NLRP3-deficient BMDMs, suggesting a necessary role for both TLRs and NLRP3 (*SI Appendix*, Fig. S2 *A–C*). Interestingly, TLR4-driven rapid caspase-1 activation occurred only in Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) KO BMDMs (Fig. 1*B*), and was absent in both myeloid differentiation primary response gene 88 (MyD88) KO (Fig. 1*C*) and MyD88/TRIF double-KO BMDMs (*SI Appendix*, Fig. S2*D*). As noted previously, TLR3 signaling did not trigger rapid caspase-1 activation (Fig. 1*A*), suggesting that TRIF and its downstream components do not directly activate the NLRP3 inflammasome. Thus, rapid caspase-1 activation downstream of all TLRs depends entirely on the adapter MyD88. Previous studies have shown that TLR signaling in both MyD88- and TRIF- dependent pathways (10) leads to NF-κB–dependent up-regulation of inflammasome components, particularly NLRP3 (11), suggesting the need for inflammasome "priming" before activation (11, 12).

Combined stimulation of BMDMs with LPS and ATP, pretreated with cycloheximide (Fig. 1*D*) or an NF-κB inhibitor (Fig. 1*E*), led to caspase-1 cleavage comparable to that seen in untreated cells, suggesting that rapid NLRP3 inflammasome activation is independent of "priming," given that both NF-κB activation and new protein synthesis are not necessary (*SI Appendix*, Fig. S3). Collectively, these data suggest that constitutive expression of NLRP3 (10) is sufficient to activate caspase-1 when cells receive signals from both TLR and NLRP3 ligands simultaneously.

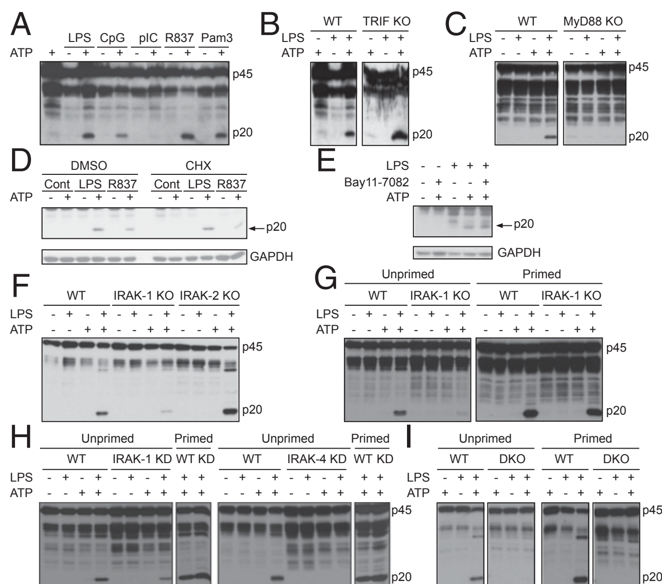

Fig. 1. Simultaneous TLR and NLRP3 signaling induces IRAK-1 dependent acute inflammasome activation. (*A*) BMDMs were stimulated with LPS, CpG, Poly I:C (pIC), R837, or Pam3CSK4 (Pam3) together with ATP for 30 min, after which cell lysates were evaluated for caspase-1 cleavage by Western blot analysis. (*B* and *C*) BMDMs of indicated genotypes stimulated with LPS and ATP for 30 min were evaluated for caspase-1 cleavage by Western blot analysis. (*D* and *E*) BMDMs were treated with 50 ng/mL cycloheximide (CHX) (*D*) or 10 μM Bay11-7082 (*E*) for 60 min before stimulation with LPS or R837 together with ATP for 30 min, followed by evaluation for caspase-1 activation by Western blot analysis. (*F*) BMDMs from WT, IRAK-1 KO, and IRAK-2 KO mice were stimulated with LPS together with ATP for 30 min and evaluated for caspase-1 activation by Western blot analysis. (*G–I*) BMDMs from the indicated mouse strains were left unprimed or were primed with LPS for 4 h, followed by stimulation with ATP for 30 min. Lysates were probed for caspase-1 activation by Western blot analysis. Data are representative of three to five independent experiments. DKO, IRAK-1 and IRAK-2 double KO.

Rapid NLRP3 Inflammasome Activation Is Dependent on IRAK-1. To examine the mechanism of priming-independent caspase-1 activation, we focused on the role of signaling components directly downstream of MyD88, in particular the IRAK family of molecules. This was also prompted by our finding that IL-1β, but not TNF-α, induced rapid caspase-1 activation (*SI Appendix*, Fig. S4). Although MyD88-dependent signaling requires IRAK-4 (13), the functions of IRAK-1 and IRAK-2 are less clearly understood. IL-1R and TLR signaling seem to be only mildly affected in the absence of either IRAK-1 or IRAK-2 (14, 15). IRAK-1 KO BMDMs and peritoneal cavity resident macrophages, but not IRAK-2 KO BMDMs, exhibited a severe defect in rapid caspase-1 cleavage (Fig. 1 *F* and *G* and *SI Appendix*, Fig. S5). These data suggest the existence of a TLR- and IRAK-1–dependent pathway that leads to rapid NLRP3 inflammasome assembly and caspase-1 activation. Interestingly, priming of macrophages for 4 h with LPS abolished the requirement of IRAK-1 for caspase-1 cleavage (Fig. 1*G*).

We next tested whether the kinase activity of IRAK-1 is important for the activation of this pathway. We found that rapid caspase-1 activation was compromised in BMDMs from IRAK1 kinase-dead (KD) knock-in mice (16) (Fig. 1*H*). Given that the kinase function of IRAK-1 is dependent on the kinase activity of IRAK-4 (17), we tested whether rapid NLRP3 inflammasome activation requires the kinase activity of IRAK4, and found that caspase-1 cleavage is completely abolished in BMDMs from IRAK-4 KD knock-in mice (18) (Fig. 1*H*). Priming-dependent caspase-1 activation was independent of the kinase activities of both IRAK-1 and IRAK-4 (Fig. 1*H* and *SI Appendix*, Fig. S6). Strikingly, TLR priming-dependent late-phase caspase-1 activation was abolished when BMDMs lacked both IRAK-1 and IRAK-2 (Fig. 1*I*). This suggests a critical role for IRAK-1 and IRAK-2 in canonical NLRP3 activation, considering the previous report of intact inflammasome activation after LPS priming in the absence of MyD88 (11). Among the other NLRP3 activators (6), nigericin induced IRAK-1–dependent rapid inflammasome activation, whereas other stimuli, such as crystals (monosodium urate and silica), failed to induce rapid inflammasome activation and activated priming-dependent NLRP3 inflammasome in an IRAK-1–independent manner (*SI Appendix*, Fig. S7). Taken together, these findings suggest that TLR-mediated priming induces a mechanistically different, IRAK-1–independent caspase-1 activation pathway.

IRAK-1 Associates with Inflammasome Components and Regulates NLRP3 Inflammasome Assembly. Given the clear genetic evidence for IRAK-1 involvement in rapid NLRP3-mediated caspase-1 activation, we further tested whether IRAK-1 interacts with inflammasome components. We observed association of IRAK-1 with ASC when cells were stimulated with either ATP or a combination of LPS and ATP (Fig. 2*A*). Previous results indicate that BMDM activation with TLR ligands leads to the disappearance of IRAK-1 (17). It also has been shown that TLR7 activation leads to a slowly migrating species of IRAK-1 that is polyubiquitinated and phosphorylated and becomes undetectable (19). Consistent with this, we found that stimulation

of WT BMDMs with LPS led to lack of IRAK-1 detection within 15 min of TLR4 activation, but simultaneous exposure of BMDMs to LPS and ATP prevented polyubiquitination and/or degradation of IRAK-1 (Fig. 2*B*), allowing it to associate with ASC. Interestingly, failure of IRAK-1 detection coincided with the absence of IκBα degradation at 15 min (Fig. 2*B*). Further kinetic analysis of IRAK-1 detection and IκBα phosphorylation/degradation revealed that these events were delayed by simultaneous stimulation with LPS and ATP (Fig. 2*C*). These results suggest that combined activation of TLR and NLRP3 favors IRAK-1–ASC interaction and inflammasome activation, thereby delaying in NF-κB activation.

The foregoing findings prompted us to further examine the signal requirements for possible interactions among IRAK-1, ASC, and NLRP3. We observed that in WT BMDMs, treatment with LPS or ATP for 15 min led to relocalization of ASC from the nucleus to the cytosol (*SI Appendix*, Fig. S8), as has been reported previously (20). In IRAK-1 KO BMDMs, LPS stimulation failed to induce nuclear–cytoplasm translocation of ASC, whereas ATP treatment induced this relocalization (*SI Appendix*, Fig. S8). On inflammasome activation, oligomerized ASC forms specks in the cells, representing assembled inflammasome complexes (20). We observed speck formation by ASC only when cells were exposed to both LPS and ATP, and this formation was significantly more pronounced in WT BMDMs compared with IRAK-1 KO BMDMs (Fig. 2 *D* and *E* and *SI Appendix*, Fig. S8). LPS and ATP treatment induced more cells with specks in WT BMDMs compared with IRAK-1 KO BMDMs (Fig. 2*F*), and the specks formed in IRAK-1 KO BMDMs were of much smaller size and lower intensity (*SI Appendix*, Fig. S8). More importantly, we found that IRAK-1 colocalized in the specks of inflammasome complexes that contain both ASC and NLRP3 (Fig. 2*D*), but IRAK-2 was not part of the specks during rapid NLRP3 activation (*SI Appendix*, Fig. S9). Importantly, the speck formation and colocalization of IRAK-1 with NLRP3 were abolished in the presence of a kinase inhibitor of IRAK-4 and IRAK-1 (*SI Appendix*, Fig. S10), as well as in IRAK-1 KD knock-in BMDMs (Fig. 2*G*).

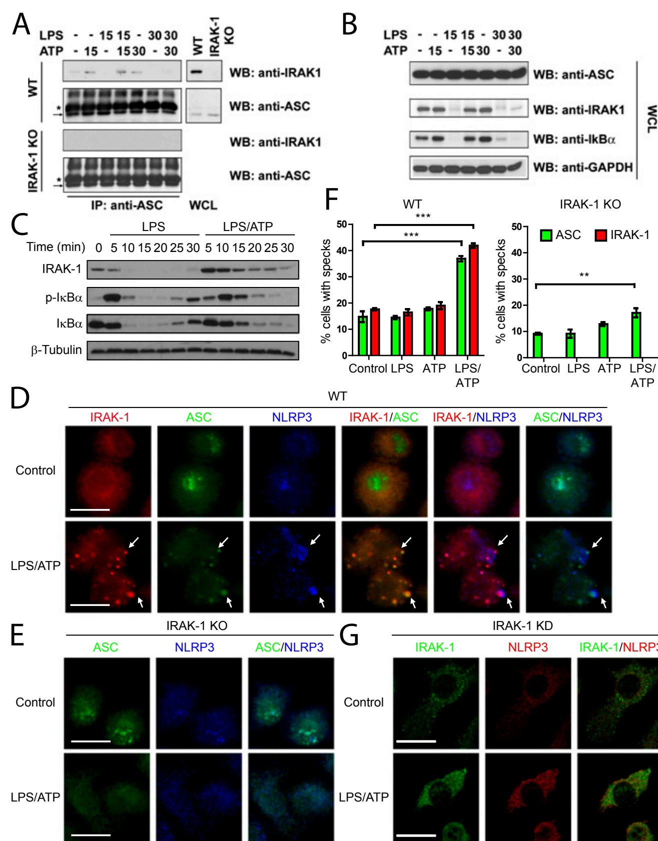

Fig. 2. IRAK-1 associates with NLRP3 inflammasome components. (*A*) WT or IRAK-1 KO BMDMs were stimulated with LPS, ATP, or LPS plus ATP for the indicated times, and coimmunoprecipitation was performed by precipitating with anti-ASC, followed by Western blot analysis for IRAK-1 and ASC. *Light chain of the antibody. The arrow indicates the position of ASC. WCL, whole-cell lysates. (*B*) Whole-cell lysates from WT BMDMs stimulated as described in *A* were blotted for the indicated proteins. (*C*) Whole-cell lysates from WT BMDMs stimulated with LPS alone or LPS together with ATP for the indicated times were immunoblotted for the indicated proteins. Data are representative of three independent experiments. (*D* and *E*) Immunostaining of endogenous ASC, IRAK-1 and NLRP3 in WT (*D*) and IRAK-1 KO (*E*) BMDMs stimulated with LPS and ATP for 15 min. Arrows point to specks formed by the indicated proteins. (Scale bar: 20 μm.) (*F*) Quantification of percentages of cells containing ASC or IRAK-1 specks in WT and IRAK-1 KO BMDMs. (*G*) Immunostaining of endogenous IRAK-1 and NLRP3 in IRAK-1 KD knock-in mouse BMDMs stimulated with LPS and ATP for 15 min. Bar graphs show mean ± SEM from three experiments, with at least 100 cells counted in each condition. $^{**}P < 0.01$; $^{***}P < 0.005$, one-way ANOVA.

Rapid IRAK-1–Dependent NLRP3 Inflammasome Activation Is Important for Secretion of Presynthesized IL-18. We next investigated the physiological relevance of the rapid NLRP3 inflammasome activation pathway. Although they do not express pro-IL-1β, BMDMs are known to express pro-IL-18 without any TLR stimulation (Fig. 3*A*). In addition, caspase-1 activation triggers pyroptosis, a proinflammatory cell death program that could play a major role in host defense by eliminating infected cells and releasing inflammatory cellular contents (7). We hypothesized that early inflammasome activation may allow the cells to secrete presynthesized caspase-1 substrates and undergo pyroptosis, and that these events would be compromised in the absence of IRAK-1 owing to defective caspase-1 activation. Confirming this model, we found that WT BMDMs secreted IL-18 but IRAK-1 KO BMDMs did not, despite similar pro-IL-18 expression levels (Fig. 3*A*). WT BMDMs failed to secrete IL-18 when stimulated only with LPS or ATP, suggesting the importance of simultaneous activation of TLR4 and NLRP3. IRAK-1 deficiency did not hinder the ability of TLR-primed BMDMs to secrete either IL-1β or IL-18, consistent with their ability to cleave caspase-1 when primed by TLR ligands (Fig. 3*B*).

IRAK-1 Is Important for Pyroptosis Induced by Acute Inflammasome Activation. We also found that WT BMDMs underwent rapid pyroptosis, as indicated by propidium iodide (PI) uptake, whereas caspase-1 KO and MyD88 KO BMDMs did not (Fig. 3*C*). IRAK-1 deficiency severely compromised the ability of cells to undergo pyroptosis (Fig. 3*C*), suggesting that IRAK-1 is critical for triggering pyroptotic cell death through regulation of caspase-1 activation. We were able to detect cleaved caspase-1 and the proinflammatory mediator high-mobility group box 1 (HMGB-1) (Fig. 3*D*), a hallmark of pyroptosis (21, 22), in the supernatants of WT BMDMs. These proteins were considerably reduced or absent in the supernatants of IRAK-1 KO, caspase-1 KO, and MyD88 KO BMDMs (Fig. 3*D*). Consistently, cell lysis, as measured by lactate dehydrogenase (LDH) release, was reduced in the absence of IRAK-1, caspase-1, and MyD88 (Fig. 3*E*). Microscopic examination of cells revealed that WT BMDMs treated with LPS and ATP had lost HMGB-1 staining in the nuclei, suggesting its release, whereas IRAK-1 KO BMDMs treated with LPS and ATP were indistinguishable from untreated BMDMs (Fig. 3*F*).

***L. monocytogenes* Induces Rapid IRAK-1–Dependent NLRP3 Inflammasome Activation and Pyroptosis.** Given that many pathogens express both TLR and inflammasome activators, we hypothesized that infection with a live pathogen would activate TLRs and NLRs simultaneously or in rapid sequence and induce acute inflammasome activation. The pathogen *L. monocytogenes* has been implicated in the activation of several inflammasome complexes,

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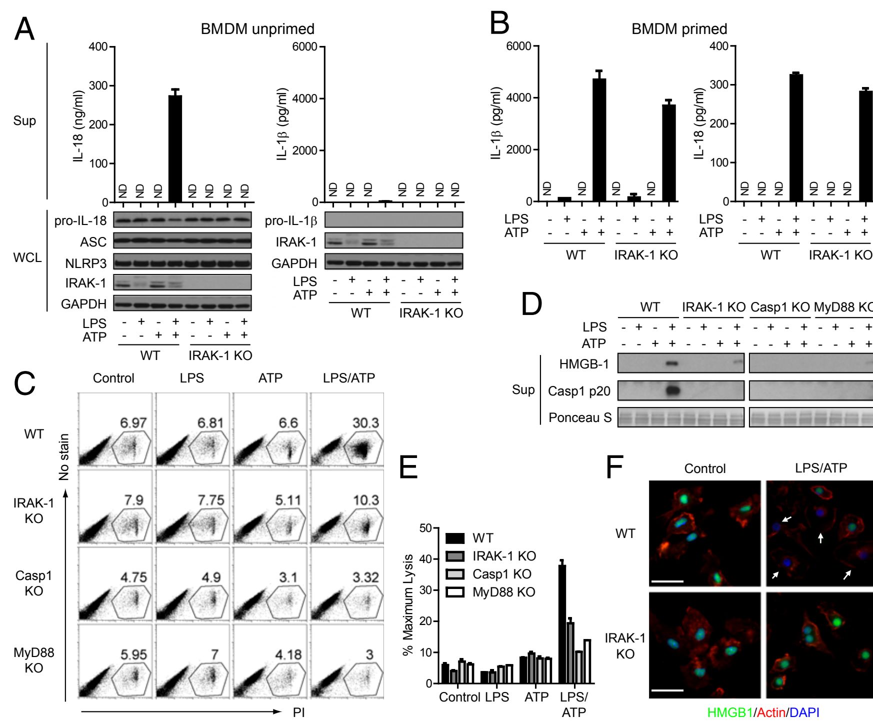

Fig. 3. Acute inflammasome activation leads to IRAK-1–dependent pyroptosis and release of presynthesized alarmins. (*A*) BMDMs of the indicated genotypes were stimulated with LPS alone, ATP alone, or both LPS and ATP for 60 min, and supernatants were analyzed for IL-18 and IL-1β by ELISA. Cell lysates were subjected to Western blot analysis to detect indicated proteins. (*B*) BMDMs were primed for 4 h with LPS and then pulsed with ATP for 30 min. Supernatants were collected after 4 h of ATP stimulation for measurement of IL-1β and IL-18. (*C*–*F*) BMDMs of the indicated genotypes were stimulated with LPS alone, ATP alone, or both LPS and ATP for 60 min, after which cells were stained with propidium iodide (PI) and analyzed by flow cytometry for pyroptosis (*C*). The supernatants were blotted for caspase-1 and HMGB-1 (*D*), LDH activity in the supernatants was determined to measure cell lysis (*E*), and cells were fixed and stained for endogenous HMGB-1 and actin (*F*). Arrows indicate cells that have lost HMGB-1 in the nuclei. (Scale bar: 20 μm.) Ponceau S staining served as the loading control. Data in the bar graph are mean ± SEM (n = 3) and are representative of five independent experiments. ND, not detectable.

including the NLRP3 inflammasome (9, 23, 24). When exposed to *L. monocytogenes* for 1 h (without previous priming by TLR ligands), WT BMDMs underwent pyroptosis, as evidenced by PI uptake, caspase-1 cleavage, HMGB-1 release, and LDH release, all of which were absent in NLRP3 KO BMDMs (*SI Appendix*, Fig. S11). Although we found that rapid activation of caspase-1 by *L. monocytogenes* is induced in an NLRP3-dependent manner, prolonged infection of BMDMs as demonstrated previously (23) using *L. monocytogenes* leads to absent in melanoma 2 (AIM2)-dependent cleavage of caspase-1. This effect possibly could be related to the induction of AIM2 protein by type I IFNs induced by *L. monocytogenes*.

Consistent with our earlier findings, caspase-1 activation, pyroptosis, and HMGB-1 release were absent in MyD88 KO and caspase-1 KO BMDMs and severely compromised in IRAK-1 KO cells infected with *L. monocytogenes* (Fig. 4 *A*–*C*). *L. monocytogenes* caused clear speck formation (containing both ASC and IRAK-1) in infected WT BMDMs (Fig. 4*D* and *SI Appendix*, Fig. S12); however, infection of IRAK-1 KO BMDMs by *L. monocytogenes* did not induce speck formation (Fig. 4*D* and *SI Appendix*, Fig. S13). In addition, *L. monocytogenes*-infected WT BMDMs lost nuclear HMGB-1 staining, whereas nuclear HMGB-1 staining of IRAK-1 KO BMDMs remained intact (*SI Appendix*, Fig. S14), consistent with the lack of HMGB-1 in the supernatants of infected cells (Fig. 4*C*). Taken together, these data suggest that acute *L. monocytogenes* infection causes NLRP3-dependent and TLR-MyD88-IRAK-1–dependent activation of caspase-1 and pyroptosis. However, *Salmonella typhimurium*- and flagellin (25)-mediated NLRC4 inflammasome activation (*SI Appendix*, Fig. S15) and poly dA:dT-mediated AIM2 (26) inflammasome activation (*SI Appendix*, Fig. S16) were equivalent in WT and IRAK-1-deficient BMDMs, suggesting that these inflammasomes can be activated independent of IRAK-1.

IRAK-1 Promotes Host Response to *L. monocytogenes*. We reasoned that the early inflammasome activation pathway promotes host defense against infections. Indeed, injection of *L. monocytogenes* into the peritoneal cavity resulted in rapid disappearance of resident BMDMs in WT mice but no change in both caspase-1 and IRAK-1 KO mice (Fig. 5*A*). A higher proportion of WT BMDMs incorporated PI, suggesting that the peritoneal BMDMs from WT mice were depleted because of pyroptotic cell death (Fig. 5*A*).

IFN-γ has been shown to play important roles in host defense against *L. monocytogenes* infection (27). The innate source of IFN-γ is an important part of host defense during the early stage of infection (28). Previous studies have shown that memory CD8 T cells can secrete IFN-γ in response to IL-18 and IL-12 in the absence of cognate antigen (29). When we examined in vivo IFN-γ production 12 h after *L. monocytogenes* infection, we found that memory CD8 T cells from IRAK-1 and caspase-1–deficient mice were severely compromised in IFN-γ production (Fig. 5 *B* and *C*). Consistent with previous reports (29), IL-18 receptor deficiency abrogates the ability of memory CD8 T cells to produce IFN-γ (Fig. 5 *B* and *C*). These data suggest that IRAK-1/capsase-1–dependent IL-18 production through acute inflammasome activation is a critical inducer of early innate IFN-γ. IL-18 is also known to be important for innate induction of IFN-γ from NK cells after infection (30). Indeed, we observed reduced IFN-γ production by NK cells from IRAK-1–, caspase-1–, and IL-18R–deficient mice after infection (*SI Appendix*, Fig. S17). Serum IFN-γ levels in both IRAK-1 and caspase-1 KO mice were also severely compromised (Fig. 4*D*). Interestingly, IL-18R KO mice were slightly more defective than both IRAK-1 and caspase-1 KO mice, suggesting that some IL-18 could be produced independent of caspase-1. IFN-γ production by memory T cells was unaffected in IL-1R KO mice, however (*SI Appendix*, Fig. S18), suggesting that the reactivation of memory CD8 T cells is controlled by IL-18 rather than by IL-1.

To uncouple the effects of IRAK-1 in regulating IL-18R–mediated induction of IFN-γ, we transferred ovalbumin (OVA)-specific WT OT-I T cells into WT and IRAK-1–deficient mice. The mice were exposed to OVA-expressing vesicular stomatitis virus (VSV-OVA), followed by 45 d of rest, and then were rechallenged with non–OVA-expressing *Listeria* to measure IFN-γ production by OVA-specific memory T cells (Fig. 5*E*). A recent study demonstrated that IL-1 receptor signaling in dendritic cells (DCs) is important for productive priming of CD8 T cells in response to live influenza A virus infection ((31)). Although IRAK-1 is downstream of IL-1R, we detected no defect in

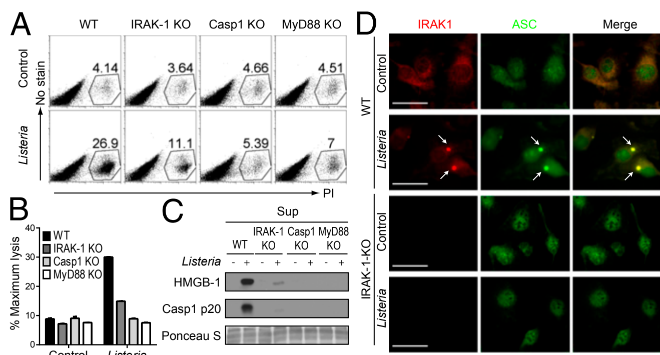

Fig. 4. *L. monocytogenes* induces rapid IRAK-1–dependent NLRP3 inflammasome activation and pyroptosis. (*A*–*C*) BMDMs of the indicated genotypes were infected with *L. monocytogenes* for 1 h and then analyzed for pyroptosis by PI incorporation (*A*), LDH release (*B*), and cleaved caspase-1 release and HMGB-1 release (*C*). Data in the bar graphs are mean ± SEM (n = 3). (*D*) Immunostaining of endogenous ASC and IRAK-1 in WT or IRAK-1 KO BMDMs infected with *L. monocytogenes for* 1 h. Arrows show specks formed by the indicated proteins. (Scale bar, 20 μm.) Data are representative of four independent experiments.

expansion or survival of antigen-specific CD8 T cells (*SI Appendix*, Fig. S19), suggesting that IRAK-1 downstream of IL-1R in DCs does not play an obligatory role in the expansion of antigen-specific CD8 T cells to systemic VSV infection. Importantly, although there was no difference in the ability of IRAK-1 KO recipients to produce IL-12 or IL-6 (*SI Appendix*, Fig. S20), there was a significant defect in the ability of memory OT-I T cells from IRAK-1 KO mice to produce IFN-γ (Fig. 5*F*). These findings suggest that IRAK-1 plays a significant role in the ability of infected cells to sense *L. monocytogenes*, induce NLRP3 inflammasome activation, trigger pyroptosis, and release IL-18 in vivo, which may influence the subsequent inflammatory response, such as IFN-γ production by memory T cells.

Discussion

Our findings reveal a critical early cellular response pathway in BMDMs induced by simultaneous engagement of TLRs and NLRP3. This previously uncharacterized rapid pathway of NLRP3 inflammasome activation is distinct from the commonly studied priming-dependent NLRP3 inflammasome activation pathway, in which TLR and NLRP3 are engaged in a sequential manner. Traditionally, TLRs have only been implicated in the sensitization of NLRP3 inflammasome activation through a priming phase involving up-regulation of inflammasome components, such as NLRP3, as a result of NF-κB activation and ROS generation. TLR signaling per se was not considered to be directly involved in delivering signals to trigger assembly of the inflammasome complex. Our findings expand the role of TLR signaling beyond that of inflammatory gene induction through NF-κB. Our data show that during early NLRP3 inflammasome activation, MyD88-dependent TLRs play a direct role via the MyD88-IRAK1 signaling axis, and that dual signals from TLRs and NLRP3 synergistically provoke inflammasome activation.

The early inflammasome activation pathway appears to operate mechanistically differently from the late priming-dependent pathway (*SI Appendix*, Table S1). We discovered that the TLR signaling molecule IRAK-1 plays a unique role in rapid inflammasome activation and seems to regulate NLRP3 inflammasome assembly and activation at several different levels. The kinase activity of IRAK-4 is important for activation of IRAK-1, and KD IRAK-4 completely abrogates the rapid inflammasome activation pathway. On the other hand, abrogation of kinase activity of IRAK-1 does not completely prevent rapid inflammasome activation, suggesting that IRAK-1 might have additional kinase-independent functions that regulate NLRP3 inflammasome activation. We also observed that cellular sensing of either a TLR ligand or a NLRP3 ligand causes relocalization of ASC from the nucleus into the cytosol, but these signals are insufficient to induce inflammasome complex formation. TLR-induced ASC relocalization is IRAK-1–dependent, but ATP-induced ASC relocalization is IRAK-1–independent. Inflammasome complex formation as measured by ASC speck formation is IRAK-1 dependent, however, and occurs only when cells are stimulated by LPS and ATP simultaneously. It is possible that TLR activation through IRAK-1 induces some posttranslational modification of one or more inflammasome components, which allows the inflammasome complex to stabilize, and this stability could be dependent on the kinase activity and adapter functions of IRAK-1. Further work is needed to tease out the exact biochemical nature of this inflammasome assembly.

Interestingly, when both TLRs and NLRP3 were activated, compared with TLR activation alone, IRAK-1's disappearance was delayed, suggesting redistribution of IRAK-1 between the NF-κB and inflammasome pathways. Similarly, IκBα phosphorylation and degradation was also delayed and reduced in magnitude, indicating that the use of IRAK-1 by the inflammasome pathway reduced the availability of IRAK-1 for activating NF-κB. Thus, when encountering TLR and inflammasome activators at the same time, as might occur in the case of a virulent pathogen, IRAK-1 serves as a controlling node for the cells to choose between the prosurvival NF-κB pathway, which will lead to new gene synthesis that takes time, and the prodeath inflammasome pathway, which immediately eliminates the niche for

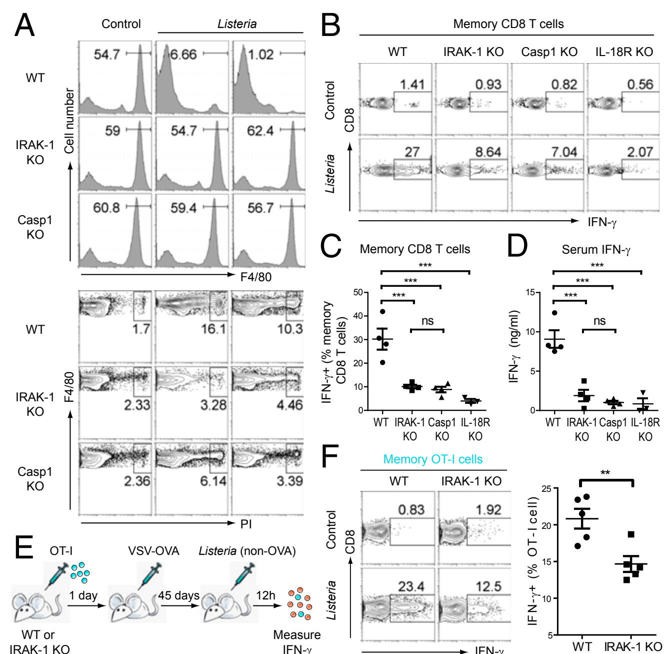

Fig. 5. IRAK-1–dependent inflammasome activation promotes early host responses to *L. monocytogenes* infection. (*A*) WT, IRAK-1 KO, and caspase-1 KO mice were injected i.p. with *L. monocytogenes* (1×10^7 CFU/mouse), and 20 min later, cells in the peritoneal cavity were stained with anti-CD45, anti-F4/80, and PI. (*Upper*) Proportions of $CD45^+$ cells staining positive for F4/80. (*Lower*) Proportions of $CD45^+$ $F4/80^+$ cells staining positive for PI. (*B* and *C*) IFN-γ production by polyclonal memory CD8 T cells from mice infected i.v. with 1×10^6 CFU of *L. monocytogenes* for 12 h. Representative plots (*B*) and combined data (*C*) are shown. (*D*) Scatterplot showing mean ± SEM IFN-γ in the sera of mice infected i.v. with *L. monocytogenes* for 12 h (*n* = 3–4 mice). (*E*) Experimental procedure for assessing IFN-γ production by memory OT-I T cells after *Listeria* infection. (*F*) Scatterplot showing mean ± SEM IFN-γ production by memory WT OT-I T cells generated in WT or IRAK-1 KO mice (*n* = 5 mice). Data are representative of two or three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$; ns, nonsignificant, one-way ANOVA (*C* and *D*) or *t* test (*F*).

survival and replication of pathogens and secretes proinflammatory cell contents.

Our findings also reveal that although rapid NLRP3 inflammasome activation is defective in IRAK-1–deficient BMDMs, priming of BMDMs with LPS abrogates the requirement for IRAK-1 for inducing inflammasome activation. It is possible that elevated NLRP3 protein levels bypass additional regulators of inflammasome activation. Of note, IRAK-1 and IRAK-2 combined are still necessary for late inflammasome activation. Whether this is related to a failure of NLRP3 up-regulation or to a lack of other signals transduced by these two IRAKs remains to be investigated.

The rapid NLRP3 inflammasome pathway that we describe here could enable the host to mount a "true innate" response immediately on pathogen invasion, before the transcriptional induction of inflammatory cytokines and chemokines. The rapidity of caspase-1 cleavage and pyroptosis suggests that this pathway contributes to detection and limitation of early infection by depriving pathogens such as *Listeria* of an intracellular sanctuary for survival and replication, and by initiating local inflammation through the release of presynthesized IL-18 and other proinflammatory mediators, such as HMGB-1. These rapid events are likely critical for decreasing the early pathogen burden (8) and do not depend on new protein synthesis that could be targeted by virulence factors (32). Of note, an earlier study found no defect in the clearance of *L. monocytogenes* by IRAK-1–deficient mice (33). It is possible that the late inflammasome activation pathway induced in IRAK-1–deficient mice might compensate for the lack of early activation. Future work with lower doses of *Listera* using natural routes of infection should provide insight into the importance of early-phase and late-phase inflammasome activation in protecting against *Listeria*.

The differential requirement for IRAK-1 in the early and late pathways allowed us to investigate the importance of rapid NLRP3 inflammasome pathway in vivo using IRAK-1–deficient animals. In particular, we found that IL-18 secretion resulting from early inflammasome activation plays an important role in inducing IFN-γ production by memory CD8 T cells, which occurs early in the course of infection (12 h). In contrast, the late inflammasome pathway induced after priming of cells by TLR ligands leads to de novo synthesis of IL-1 family members, such as pro-IL-1β, and subsequent processing and secretion, which could be important for a powerful systemic inflammatory response.

Materials and Methods

Mice. Mice strains (described in *SI Appendix*, SI Text) were bred and maintained at the animal facility of University of Texas Southwestern Medical Center. IRAK-4 KD (18) and IRAK-1 KD knock-in mice (16) and their littermates were bred and housed at the animal facility of Cleveland Clinic and University of Dundee, respectively. All mouse experiments were conducted in accordance with protocols approved by Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center. All strains were on a C57BL/6 background.

Caspase-1 Activation Assays. BMDMs were plated in 12-well tissue culture plates at 5×10^5 cells per well and allowed to attach. For acute caspase-1 activation studies, BMDMs were stimulated simultaneously with TLR ligands and ATP (5 mM) for 30 min. For delayed activation studies, BMDMs were incubated with LPS for 2–4 h and then pulsed with ATP (5 mM) during the last 30 min of incubation. Western blot analysis for caspase-1 and all other assays were performed as described in *SI Appendix*, SI Text.

In Vivo *Listeria* Infection. Mice were injected with 1×10^7 CFU of *L. monocytogenes* i.p. or with with 1×10^6 CFU i.v., and various assays were performed as described in *SI Appendix*, SI Text.

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