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IL-1 Receptor-Associated Kinase Modulates Host Responsiveness to Endotoxin

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Endotoxin triggers many of the inflammatory, hemodynamic, and hematological derangements of Gram-negative septic shock. Recent genetic studies in mice have identified the Toll-like receptor 4 as the transmembrane endotoxin signal transducer. The IL-1 intracellular signaling pathway has been implicated in Toll-like receptor signal transduction. LPS-induced activation of the IL-1 receptor-associated kinase (IRAK), and the influence of IRAK on intracellular signaling and cellular responses to endotoxin has not been explored in relevant innate immune cells. We demonstrate that LPS activates IRAK in murine macrophages. IRAK-deficient macrophages, in contrast, are resistant to LPS. Deletion of IRAK disrupts several endotoxin-triggered signaling cascades. Furthermore, macrophages lacking IRAK exhibit impaired LPS-stimulated TNF-α production, and IRAK-deficient mice withstand the lethal effects of LPS. These findings, coupled with the critical role for IRAK in IL-1 and IL-18 signal transduction, demonstrate the importance of this kinase and the IL-1/Toll signaling cassette in sensing and responding to Gram-negative infection. *The Journal of Immunology, 2000, 164: 4301–4306.*

Septicemia kills 20,000 hospitalized patients in the United States each year, often as a result of shock leading to multiple organ failure (1). However, the pathogenesis of septic shock remains poorly understood. LPS, a membrane glycolipid from Gram-negative bacteria, elicits many of the signs of septic shock, including vasodilatation, myocardial dysfunction, and disseminated intravascular coagulation. LPS mediates these effects by inducing production of proinflammatory cytokines, in particular TNF-α and IL-1. Purified TNF-α can recapitulate aspects of LPS-induced shock in vivo (2), and IL-1 potentiates these actions of TNF-α (3). Furthermore, passive immunization against TNF-α protects animals from the lethal effects of LPS (4) and IL-1 antagonism improves survival in some models of Gram-negative septic shock (5).

LPS induces cytokine secretion by binding CD14, which in turn triggers intracellular signal transduction cascades in many different cell types (reviewed in Ref. 6). LPS-mediated TNF-α production is subject to both transcriptional and posttranscriptional controls and requires intact signaling through at least three of these pathways. LPS-induced TNF-α transcription depends on NF-κB pathway activation (7, 8), while the Jun N-terminal kinase (JNK)4/stress-activated protein kinase (SAPK) and p38 mitogen-activated protein kinase (MAPK) pathways regulate TNF mRNA translation (9, 10). Furthermore, recent positional cloning of the murine Lps locus and demonstration that Lpsδ alleles that cause impaired LPS responsiveness are mutant forms of the Toll-like receptor 4 (Tlr4) gene have begun to clarify the molecular basis for LPS signal transduction to the cell interior (11).

TLR4 belongs to the Toll/IL-1R family. Members share a conserved cytoplasmic domain required for signal transduction to the cell interior (12, 13). Receptors for IL-1 and IL-18 activate a common intracellular pathway composed of the IL-1R-associated kinase (IRAK), MyD88, and TNF receptor-associated factor 6 (TRAF6) (14–18). Identification of TLR4 as the murine LPS signal transducer prompted us to ask whether LPS activated IRAK in relevant immune effector cells and to determine the role of this kinase in cellular and in vivo responses to LPS.

**Materials and Methods**

**Animals and reagents**

IRAK-deficient mice were generated as described (19). Sib-sib intercrosses between hybrid 129/B6 animals produced wild-type mice. LPS from *Escherichia coli* K-12 (LDC25, from Robert Munford, University of Texas Southwestern Medical Center) was used for in vitro experiments and from *E. coli* O111:B4 (Sigma, St. Louis, MO) for in vivo challenges. Polyclonal anti-IRAK antiserum (from Zhaodan Cao, Tularik, South San Francisco, CA) was generated as described (16). Abs recognizing extracellular signal-regulated kinase 2 (ERK2), p38α, and JNK/SAPK were previously described (9, 20). Anti-IκB kinase (IKK) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Macrophage isolation and stimulation**

Thioglycollate-elicited macrophages were obtained as described (21). LPS stock solution was thawed, sonicated, and diluted to appropriate concentrations in medium. For TNF-α production experiments, macrophages were stimulated with the indicated concentrations of LPS for 20 h before harvesting supernatants. For in vitro kinase and EMSA studies, macrophages were treated with LPS (10 ng/ml) for different time periods.

**In vitro kinase assays**

Endogenous IκB-α, IκB-β, JNK/SAPK, and p38α were immunoprecipitated from LPS-stimulated macrophages, and assays determining kinase activity were performed as described (8, 9). For IRAK and ERK2, LPS-treated wild-type (WT) or knockout (KO) macrophages were lysed and cleared by centrifugation. IRAK or ERK2 was immunoprecipitated using...
polyclonal rabbit sera. After washing, immunoprecipitated IRAK or ERK2 was incubated in kinase buffer containing myelin basic protein (MBP) (0.5 mg/ml) and [γ-32P]ATP (~10 μCi/sample) for 45 min. Supernatants containing substrate were removed, added to SDS-loading dye, boiled, and fractionated using SDS-PAGE. Once completed, gels were processed and analyzed as previously described (8, 9).

**Northern analysis**

Total RNA was isolated from thioglycollate-elicited macrophages stimulated with LPS (1 ng/ml) for indicated times. One microgram of total RNA was fractionated, blotted, and probed with a radiolabeled murine TNF-α cDNA (from Bruce Beutler, University of Texas Southwestern Medical Center), according to standard procedures. The membrane was stripped and reprobed with a rat GAPDH cDNA (from Brett Giroir, University of Texas Southwestern Medical Center) to control for RNA loading.

**EMSA**

Nuclear extracts were prepared from saline- and LPS-treated macrophages at different times following LPS stimulation as previously described (22). After determination of total protein, 2.5 μg of extract protein was incubated for 30 min with a 32P-labeled double-stranded oligonucleotide probe containing the NF-κB binding site from the murine Ig κ-chain promoter. Samples were then electrophoresed on a 5% acrylamide/0.5X TBE non-denaturing gel. The gel was dried and autoradiography performed.

**In vivo LPS challenge**

Statistical power analysis indicated a requirement for 45 animals per test group to detect a 25% mortality difference with 80% probability. Accordingly, 48 WT and 48 KO mice were injected with 500 μg/25 g body weight LPS. Animals were observed throughout the 7-day test period. Mortality and time of death were recorded.

**Statistics**

Two-way ANOVA followed by Bonferroni-corrected t tests were used to determine significant differences in TNF-α production between WT and KO macrophages at each dose. Mortality from in vivo LPS challenges was analyzed using a two-tailed Fisher’s exact test.

**Results**

**LPS activates IRAK**

To demonstrate that LPS activates IRAK, we stimulated WT thioglycollate-elicited macrophages with LPS, immunoprecipitated endogenous IRAK, and measured IRAK catalytic activity. Although physiologic IRAK substrates are unknown, the activated kinase can phosphorylate permissive substrates such as MBP (23). As shown in Fig. 1, LPS activates IRAK in macrophages. Activation occurs rapidly: it is detectable within 7.5 min and reaches maximum activity by 15 min after LPS stimulation. Catalytic activity declines quickly thereafter. LPS also triggers IRAK activity in the murine macrophage cell line RAW 264.7, eliciting a catalytic profile similar to that seen in primary macrophages (data not shown). We also performed similar immunoprecipitation (IP)-kinase reactions in LPS-treated KO macrophages. Anti-IRAK antiserum also precipitated a weaker, signal-dependent kinase activity in cells lacking IRAK (Fig. 1). This catalytic activity peaked at 15 min after LPS stimulation, exhibiting a 2-fold induction over baseline (compared with 4- to 5-fold seen in WT macrophages). Non-immune serum does not precipitate kinase activity in this assay (data not shown).

**IRAK mediates optimal LPS-induced IKK/NF-κB pathway activation**

We then wished to learn whether IRAK influenced LPS-triggered downstream signaling cascades. We first examined signal transduction in the pathway leading to NF-κB activation, a critical event in the initial host response to infection. NF-κB activation occurs following signal-dependent serine phosphorylation of the inhibitor proteins, IκB-α and IκB-β by the Ikkks, IKK-α and IKK-β (24–28). Therefore, we tested the capacity of LPS to activate IKK-α and IKK-β in IRAK-deficient and WT macrophages. LPS-triggered IKK-α activity is unaffected in KO macrophages (Fig. 2A). In contrast, IRAK exerts a striking effect on LPS-mediated IKK-β activity. IKK-β reaches peak catalytic activity more slowly in IRAK-deficient macrophages: 30 min vs 7.5 min in WT cells (Fig. 2B). Furthermore, in IRAK-deficient cells, maximal LPS-induced IKK-β activity is reduced compared with WT macrophages. The differences in maximal catalytic activity cannot be explained by reduced IKK concentrations in KO macrophages, as both WT and IRAK-deficient cells contain equivalent amounts of immunoreactive proteins (data not shown).

Because IKK activation leads to 1κB degradation and NF-κB nuclear translocation, promoter binding, and transcriptional up-regulation of responsive genes, we tested whether LPS-induced NF-κB DNA binding was altered in IRAK-deficient macrophages. LPS-treated KO macrophages exhibit reduced NF-κB DNA binding activity compared with their WT counterparts at each time point assayed (Fig. 2C), although binding activity at later time points in KO cells approaches that seen in WT cells at earlier time points. This attenuation parallels the retardation of peak IKK-β activity in LPS-treated KO macrophages. Thus, the decrease in IKK activity in KO cells results in defective NF-κB function. Therefore, IRAK optimizes LPS-triggered signaling to NF-κB activation through its effect on both timing and maximal activation of downstream pathway members.

**Optimal LPS-induced activation of multiple MAPK pathways is IRAK dependent**

LPS activates several MAPK pathways, at least two of which directly regulate macrophage production of TNF-α (8, 9, 29), an
early mediator of the innate immune response to Gram-negative infection. We examined IRAK’s influence on LPS-induced MAPK signaling by stimulating WT and IRAK-deficient macrophages with LPS and assessing the catalytic function of endogenous JNK/SAPK, p38α, and ERK2. As shown in Fig. 3, A and B, activation of JNK/SAPK and p38α is delayed in KO cells. Peak SAPK and p38α kinase activities are also decreased in KO macrophages (Fig. 3, A and B). Furthermore, LPS-dependent ERK2 activity is attenuated in macrophages without IRAK (Fig. 3C). The differences in maximal catalytic activity are not due to reduced SAPK, p38α, or ERK2 concentrations in IRAK-deficient macrophages, as both WT and KO cells contain similar quantities of immunoreactive proteins (data not shown). Thus, IRAK is required for appropriate timing of downstream kinase activity in two MAPK pathways and maximal catalytic function in all three cascades.

**FIGURE 2.** NF-κB activation is impaired in LPS-treated IRAK-deficient macrophages. Macrophages were isolated from WT and KO mice, pooled by genotype, and treated as above. In vitro kinase reactions were performed following immunoprecipitation of endogenous IKK-α (A) or IKK-β (B) using IκB-α as a substrate. Samples were treated as described in Fig. 1. Values beneath the autoradiograms represent fold activity increases over unstimulated cells. C, Nuclear extracts were prepared at the indicated times following treatment with LPS and tested for the ability to retard the electrophoretic mobility of a 32-P-radiolabeled oligonucleotide containing an NF-κB binding site from the mouse Ig κ L chain promoter. Results are representative of at least three independent experiments.

**FIGURE 3.** Attenuated LPS-triggered MAPK signaling in macrophages lacking IRAK. Endogenous MAPKs (A, JNK/SAPK; B, p38α; C, ERK2) were immunoprecipitated from peritoneal macrophages treated with LPS (10 ng/ml) for the indicated times. Immunoprecipitated kinase activity was measured using in vitro kinase assays with indicated substrates (arrows). Kinase reactions were treated as described in preceding figures. Values beneath the autoradiograms represent fold activity increases over unstimulated cells. Results are representative of three independent experiments.

**IraK is essential for LPS-induced TNF-α production**

Demonstration of a requirement for IRAK in optimal LPS-induced signaling to NF-κB and the MAPK pathways led us to ask whether this kinase mediated a relevant biologic response to LPS. Because NF-κB, JNK/SAPK, and p38 MAPK activation regulate TNF-α biosynthesis (8–10), we tested whether IRAK regulated LPS-induced TNF-α production. We first examined TNF-α mRNA in macrophages at different times after LPS treatment and saw no discernible difference in net accumulation and decay between WT and KO cells (Fig. 4A). We then examined secretion of TNF-α protein. Macrophages lacking IRAK exhibit significant impairment in LPS-induced TNF-α production at 0.1 ng/ml, 0.5 ng/ml, and 1 ng/ml (Fig. 4B). This reduction in TNF-α secretion is not due to IL-1β secreted into the medium, as this latter cytokine was undetectable at these LPS doses (data not shown). The difference in TNF-α secretion between WT and IRAK-deficient macrophages persists at LPS concentrations up to 1.6 ng/ml (data not shown). Higher LPS doses (>2 ng/ml) overcome the effect of IRAK deletion on TNF-α production (Fig. 4B and data not shown), even though defective signaling still occurs at 10 ng/ml (see above). Thus, IRAK mediates optimal LPS-stimulated macrophage TNF-α production, a critical host response to Gram-negative infection through mechanisms independent of net mRNA accumulation.
**Discussion**

Our results demonstrate that IRAK contributes to the host response to endotoxin. We show for the first time that LPS activates IRAK in macrophages, innate immune cells that serve both sensor and effector functions during infection by Gram-negative bacteria. Furthermore, we define an essential role for IRAK in optimal LPS-induced activation of several signaling cascades. IRAK-deficient macrophages display marked reductions in LPS-triggered IKK-β activity and NF-κB DNA binding, both of which are required for signal-dependent NF-κB transcriptional activation (8). Macrophages lacking IRAK also exhibit defective LPS-induced activation of three MAPK pathways. IRAK appears to exert two major functions on the activity of these downstream pathways. First, it enhances the kinetics of subsequent kinase cascade activation. Second, it boosts peak catalytic activity of these kinases. This dual effect on downstream kinase activation is also seen in MyD88-deficient macrophages (30). Macrophages without IRAK produce less TNF-α in response to LPS than WT cells. The reduction in TNF-α may be related to a selective IRAK effect on translation of TNF-α mRNA, a process regulated by SAPK/JNK and p38α (9, 10, 31), as elimination of IRAK has no effect on the net accumulation and decay of TNF-α message. The diminished production of this key proinflammatory cytokine persists over a >10-fold dose range. Furthermore, mice without IRAK respond abnormally to high doses of LPS, dying at a lower frequency than their WT counterparts. This difference is detectable despite the engagement of multiple mechanisms to compensate for genetic deletion of IRAK.

LPS-activated TLR4 may recruit MyD88 and IRAK to the activated receptor complex, as occurs with the IL-1R1 (14, 15) (see Fig. 6). Several lines of evidence support this notion. First, the cytoplasmic domain of TLR4 conserves residues from the IL-1R1 homology domain (11), still respond to LPS with NF-κB activation (33). Third, cells from MyD88-deficient macrophages and mice are hyporesponsive to LPS, exhibiting a more pronounced resistance to LPS than IRAK-deficient cells and animals (30). Furthermore, dominant negative versions of MyD88, IRAK, and TRAF6 also inhibit NF-κB activity triggered by a constitutively activated human TLR4 (34, 35). Finally, in a single case report, cells from a patient with multiple life-threatening Gram-negative and Gram-positive bacterial infections exhibit discrete hyporesponsiveness to LPS and IL-1 (36). Therefore, TLR4 may engage many of the same signaling components as the IL-1R1, but documentation of a signal-dependent interaction between TLR4, MyD88, and IRAK awaits confirmation.

Although attenuated, LPS-triggered responses persist in IRAK-deficient macrophages and mice. Residual responsiveness to IL-1 and IL-18 also takes place in cells and animals without IRAK (19, 37). At least two possible explanations may account for this persistent signaling. First, other IRAK-related molecules may assume the signaling burden in the absence of IRAK. Two such IRAK-like proteins, called IRAK-M and IRAK-X, display high levels of homology with IRAK and are thought to serve as functional substitutes under conditions of IRAK deficiency. However, the physiological relevance of these genes remains to be established. Second, the IRAK-driven pathways may be part of a larger complex that contains multimeric signaling molecules. This complex may include additional IRAK family members, as well as adapter proteins and transcription factors, which could interact with IRAK in a cell-type-specific manner to determine the magnitude and duration of the immune response.

**IRAK-deficient mice exhibit resistance to LPS-induced mortality**

Having established the insensitivity of IRAK-deficient macrophages to LPS and linked diminished TNF-α production to disrupted LPS-induced signal transduction, we then asked if IRAK-deficient mice were resistant to the lethal effects of endotoxin. As seen in Fig. 5, 19 of 48 (40%) WT mice died when administered 500 μg/25 g body weight, whereas only nine of 48 (19%) IRAK-deficient mice succumbed to the same LPS dose. Thus, IRAK also participates in the response to acute lethal endotoxemia.

**FIGURE 4.** Impaired TNF-α production in IRAK-deficient macrophages. A. Peritoneal exudate macrophages from WT and KO mice were stimulated with LPS (1 ng/ml). Total RNA was collected at the indicated time points, fractionated, blotted, and probed with a radiolabeled murine IRAK cDNA. The blot was stripped and reprobed with a rat GAPDH cDNA to ensure approximately equal loading. One of three representative blots is shown here. B. Elicited peritoneal macrophages from WT (open bars) and KO (closed bars) mice were stimulated with LPS at the indicated concentrations. Twenty hours later, supernatants were removed, cleared by centrifugation, and frozen at −80°C. Total cellular protein was determined after direct lysis of adherent cells in wells. TNF-α concentrations in supernatants were measured using ELISA (R&D Systems, Minneapolis, MN). Values represent the mean ± SEM from four wells treated with the same LPS dose. The results of one of seven representative experiments are depicted here (*, p < 0.005; †, p < 0.01).

**FIGURE 5.** IRAK-deficient mice are resistant to LPS. WT and KO mice (48 animals/group) were injected i.p. with LPS (E. coli O111:B4; Sigma) at a dose of 500 μg/25 g body weight. Animals were monitored every 12 h for death or moribund state over the 7-day test period, and mortality and time of death recorded (*, p < 0.05).
molecules have been described: IRAK2 and IRAK-M (38, 39). These molecules share 40% overall sequence similarity with IRAK. They lack conserved residues in the kinase domain normally required for catalytic function, but may nonetheless account for the residual LPS-induced catalytic activity precipitated by the IRAK antiserum from IRAK-deficient cells. Both molecules partially restore IL-1β signaling to NF-κB in one IRAK-deficient cell line, supporting the notion that they may substitute for IRAK in KO cells and mice (39). A second possible explanation for residual signal transduction could be that optimal LPS-induced signal transduction requires that the activities of two or more proximal signaling molecules be detected by their downstream targets. As our results indicate, IRAK may be required for proper kinetics of kinase activation or assembly of signaling molecules. Thus, in cells lacking IRAK, exposure to endotoxin leads to suboptimal downstream effector activation.

Differences in TNF-α production between WT and KO macrophages are greatest at the lower end of the dose range tested (0.1–1 ng/ml), whereas the distinction disappears at doses higher than 5 ng/ml. Clinical studies suggest that the concentration range in which IRAK functions is physiologically relevant. Patients with meningococcal sepsis—a disease caused by the Gram-negative bacterium Neisseria meningitidis and characterized by severe septic shock and coagulation abnormalities—had serum LPS levels that ranged from 10 to 970 pg/ml in one study (40) and from 800 pg/ml to 3000 ng/ml in another series with sicker patients (41). In the second study, patients with serum LPS concentrations >3.8 ng/ml died within minutes or hours of hospital admission, their normal host defenses already overwhelmed by the infection. Although conclusions from these clinical data must be tempered by several caveats (bioactivity of LPS not determined, serum is not usual compartment where LPS/bacteria and innate immune cells interact, and so forth), these studies suggest that the upper limits of “normal” LPS concentrations at the site of Gram-negative infection. If this is indeed the case, IRAK clearly plays a critical role in transducing the LPS signals that result in TNF-α production, one of the earliest and most important host responses to Gram-negative infection.

IRAK may be an important target for treatment of severe Gram-negative infections, because even complete inhibition (or genetic deletion) of IRAK down-regulates, but does not obliterate, the host response to endotoxin. Complete blockade of endotoxin responsiveness is undesirable. Maintenance of immune competence is critical to contain and eliminate invasive bacteria. This point is highlighted by the finding that the recent increase in human sepsis-related mortality in the U.S. is due to the increasing numbers of immunodeficient patients (1). Thus, inhibiting IRAK activity could moderate an overexuberant inflammatory response, as occurs in septic shock, while retaining the host’s ability to fight infection. IRAK could also represent an attractive therapeutic target because its participation in at least three different steps in the response to Gram-negative infection. Inhibiting IRAK activity could temper the host response to Gram-negative infection at three sequential control points, obviating the need for three different extracellular agents—against LPS, IL-1, and IL-18—to accomplish a similar goal.

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