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IL-1R-Associated Kinase 4 Is Required for Lipopolysaccharide-Induced Activation of APC

Nobutaka Suzuki,*† Shinobu Suzuki,* Urs Eriksson,‡ Hiromitsu Hara,‡ Christine Mirtosis,* Nien-Jung Chen,* Teiji Wada,* Denis Bouchard,* Irene Hwang,§ Kiyoshi Takeda,** Takashi Fujita,¶ Sandy Der,§ Josef M. Penninger,‡ Shizuo Akira,** Takashi Saito,†† and Wen-Chen Yeh2* The Journal of Immunology, 2003, 171: 6065–6071.

Lipopolysaccharide is a key component of the bacterial cell walls that triggers host immune responses against the invading pathogen (1, 2). LPS can stimulate macrophages to produce proinflammatory cytokines that are critical for the innate immune response (3). It can also induce the maturation of dendritic cells (DCs),3 which in turn produce more cytokines and express costimulatory molecules to activate T lymphocytes (4). Macrophage activation and DC maturation are critical for the subsequent establishment of the adaptive immune response.

LPS signals are received and transmitted through Toll-like receptor (TLR) 4 (5), a member of the TLR family proteins that are germline-encoded receptors for various pathogen-associated molecular patterns (6, 7). The downstream signaling pathways triggered by LPS and TLR4 are complex (8, 9). LPS signaling can lead to the activation of the transcription factor NF-κB and various mitogen-activated protein kinases. These signals contribute critically to the induction of a variety of inflammatory cytokines, including TNF, IL-1, IL-6, and IL-12 p70, and the expression of costimulatory molecules such as CD80 and CD86 on the cell surface (9, 10). In addition, LPS can trigger a pathway leading to the activation of another transcription factor, IFN regulatory factor 3 (IRF-3), that contributes to the expression of genes such as IFN-γ-inducible protein 10 (IP-10) and IFN-β (11, 12). LPS also induces other signaling pathways such as, for example, one leading to the activation of phosphoinositol-3 kinase (PI-3-K) and subsequently Akt, although the contribution of this particular pathway to the induction of gene transcription remains unclear (13). Finally, the Janus kinase (JAK)/STAT signaling pathway is also activated after LPS stimulation and leads to another wave of gene induction (14, 15). However, JAK/STAT activation is most likely secondary to the expression of IFN-β and subsequent triggering of the IFN-β receptor (16, 17).

The current understanding of LPS and TLR4-associated signaling complexes is modeled after the signal transduction cascade shared by IL-1R and other TLRs (18, 19), where, upon specific ligand binding, the receptor associates with the intracellular adaptor protein myeloid differentiation factor 88 (MyD88) (20, 21), which recruits IL-1R-associated kinases (IRAKs) (22–25) to the receptor complex. IRAKs then depart from the receptor complex and associate with TNF-associated factor 6 (TRAF-6) (26) to trigger downstream signaling events.

An interesting question that remains is how this TLR4 receptor-proximal signaling complex contributes to the many aforementioned downstream events induced by LPS. Studies using MyD88-deficient mice and cells have provided many insights into this question (27). It is clear that in cells lacking MyD88 the production of inflammatory cytokines in response to LPS is severely impaired, but the induction of IP-10 and IFN-β remain unchanged (11, 28). LPS-induced IRF-3 activation is normal in MyD88-deficient cells.
However, interestingly, LPS-induced activation of NF-κB and JNK are delayed in cells lacking MyD88, although the peak activities achieved at later time points are comparable to wild-type cells (11, 28). MyD88-deficient DCs remain capable of expressing costimulatory molecules and stimulating allogeneic T cell proliferation (29). These results clearly differentiate downstream signaling events that are MyD88 dependent and MyD88 independent, and also raise additional issues that are specific to LPS signaling. For example, how exactly does LPS transduce signals to activate NF-κB and mitogen-activated protein kinases? What are the roles of NF-κB and other transcription factors in trans-activating inflammatory cytokines, IP-10, IFN-β, and costimulatory molecules that are inducible by LPS? Toll-IL-1R domain containing adapter region (TIRAP; Mal), a MyD88 homologue, was originally thought to play a role in at least some MyD88-independent pathways (30, 31). However, recent gene targeting studies suggest that TIRAP and MyD88 are not redundant in most signaling pathways examined thus far and appear to cooperate in the same signaling axis downstream of TLR4 and TLR2 signaling (32, 33). Instead, TIRAM (Ticam-1), another MyD88 family member appears to play a critical role in MyD88-independent pathways (34–37).

Receiving signals downstream of MyD88 and TIRAP are the four IRAK family members. Interestingly, these seemingly redundant IRAK proteins may each have specific functions in the signaling cascade of IL-1R/TLR. From gene targeting studies, IRAK-4 appears to be critical for the transduction of IL-1R/TLR signals (38), and IRAK-1 is also required for the optimal transduction of positive signals (39–42). IRAK-M, an inducible IRAK member without apparent kinase activity, appears to play a role as a negative regulatory signal (43). Studying the signaling mechanism at the IRAK level therefore provides a new dimension in which to further understand the connection between LPS/TLR4 and key downstream signaling events leading to innate immunity and linking to adaptive immunity.

We have previously demonstrated that IRAK-4-deficient mice are resistant to LPS-induced shock and cytokine responses in vivo (38). Macrophages lacking IRAK-4 fail to produce inflammatory cytokines in response to LPS, but residual activation of NF-κB and JNK can nevertheless be detected. In this report, we investigate further the LPS-induced events in IRAK-4-deficient macrophages and DCs. We show that IRAK-4 is required for the induction of IP-10 and IFN-β, but is dispensable for IRF-3 activation. LPS-activated IRAK-4-deficient DCs can still stimulate allogeneic T cell proliferation but fail to induce cytokine production from Th cells. Our results demonstrate that blocking IRAK-4 specifically affects certain LPS signaling pathways and certain aspects of DC activation.

Materials and Methods

Mice

IRAK-4-deficient mice, a mixed 129/Ola;B6 background, were established as described previously (38). BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Bone marrow (BM)-derived macrophage and DC cultures

To obtain BM-derived macrophages, BM cells from femurs and tibias were plated at 5 × 10⁶ cells per 10-cm dish with 6 ml of RPMI 1640 medium containing 10% FBS and 25 ng/ml murine M-CSF. After a 1-day culture, 3 ml of the above medium was added to the culture. After 3 or 4 days, floating cells were discarded and adherent macrophages were used for experiments. For the preparation of DCs (44–46), BM cells were plated at 2 × 10⁶ cells per 10-cm Petri dish to avoid adhesion of cells to the plates. Cells were cultured with 10 ml of RPMI 1640 plus 10% FBS and 20 ng/ml GM-CSF. After 3 days of culture, 5 ml of the above medium was added. After 6 and 8 days, 5 ml of the cell suspension was taken from each dish and centrifuged for 5 min at 1100 rpm. The cell pellets were cultured in 5 ml of fresh medium (with 10 ng/ml GM-CSF) and brought back to their original plates. On day 9 after the culture was initiated, loosely adherent cells were harvested with gentle pipetting, plated at 5 × 10⁷ cells/ml in 24-well plates, and cultured with or without 1 μg/ml LPS (Escherichia coli O111:B4; Sigma-Aldrich, St. Louis, MO), 10 μg/ml peptidoglycan (PGN), 10 μM oligodeoxyribonucleotides that contain unmethylated CpG motifs (CpG-ODN; 5'-TCG TCG TTT TGT CGT GTC GTT-3'; phosphorothioate stabilized), or 1 μg/ml poly(I:C) in fresh medium for 2 more days.

Northern blot analysis

Total RNA was extracted from wild-type and IRAK-4-deficient cells that were untreated or treated with LPS (1 μg/ml) for the indicated times, using TRIzol reagent, following the protocol supplied by the manufacturer (Invitrogen, Carlsbad, CA). Five micrograms of each RNA sample was electrophoresed through a 1% denaturing formaldehyde-agarose gel, transferred to a nylon membrane (Hybond-N Membrane; Amersham, Arlington Heights, IL) and hybridized to cDNA fragments specific for gene encoding IP-10, glucocorticoid attentuated response gene 16 (GARG16), IFN-β, or GAPDH. The probes were radioactively labeled using a multiprime DNA labeling system (Amersham) according to the manufacturer’s instructions.

Flow cytometry

DCs were treated with various stimuli and then harvested for staining of surface molecules, including CD11c, MHC class II, CD40, CD80, and CD86 are expressed on activated DCs (29, 48). Fluorescent signals on cell surfaces were analyzed with a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) and CellQuest software (BD Biosciences).

Allogeneic T cell stimulation

BM-derived DCs derived from either wild-type or IRAK-4-deficient mice were left untreated or stimulated with various ligands for TLRs (LPS or CpG-ODN (5'-TCG TCG TTT TGT CGT GTC GTT-3'; phosphorothioate stabilized)) for 2 days. Cell supernatants were harvested after 48 h of treatment to measure TNF-α, IL-6, IFN-γ, and IL-12 p70 production by ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Measurement of IL-6, TNF-α, IL-1, IL-12 p70, and IFN-γ

On days 7–9 of the DC culture, cells were left untreated or stimulated with various ligands for TLRs (LPS or CpG-ODN) for 2 days. Cell supernatants were harvested after 48 h of treatment to measure TNF-α, IL-6, IFN-γ, and IL-12 p70 production by ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Protein samples were fractionated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane. Western blots were then incubated with Abs according to the manufacturer’s instructions. Briefer, 2 × 10⁵ gamma-irradiated (15 Gy) DCs from wild-type and IRAK-4-deficient mice on 129/Ola;B6 mixed backgrounds were cocultured with 5 × 10⁷ cells/ml of various ligands for TLRs (LPS or CpG-ODN (5'-TCG TCG TTT TGT CGT GTC GTT-3'; phosphorothioate stabilized)) for 2 days. Cell supernatants were harvested after 48 h of treatment to measure TNF-α, IL-6, IFN-γ, and IL-12 p70 production by ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Gel mobility shift assay

Nuclear extracts were prepared from mouse embryonic fibroblasts (MEFs) following treatment with or without 1 μg/ml LPS for various time periods. The Bio-Rad protein assay (Bio-Rad, Hercules, CA) was used to adjust for equal amounts of nuclear proteins in each sample. Gel mobility shift assay
Expression of GAPDH previously described protocol (50). MEFs were stimulated with 1 μg/ml LPS for the indicated time periods to examine the expression of genes other than inflammatory cytokines, we examined LPS-induced IFN-β and IP-10 gene expression in IRAK-4-deficient cells. As shown in Fig. 1A, both IFN-β and IP-10 induction are impaired in LPS-stimulated macrophages lacking IRAK-4. IP-10 and another gene, GARG16, both inducible by IFN and LPS, are also abundantly expressed in MEFs. We therefore examined the induction of IP-10 and GARG16 by LPS in MEFs and found that both were severely defective in IRAK-4-deficient cells (Fig. 1B). Coincidentally, LPS-induced NF-κB activation was also severely impaired in MEFs lacking IRAK-4 (Fig. 1A, C and D). In contrast, LPS-induced IP-10, GARG16, and NF-κB activation was less affected in MyD88-deficient MEFs (Fig. 1A, B and C).

The induction of IP-10 and IFN-β could also be dependent on the activation of IRF-3 by LPS. Thus, we examined the LPS-induced dimerization of IRF-3, which is an indicator of IRF-3 activation, in IRAK-4-deficient cells. We found that IRAK-4 is dispensable for LPS-induced activation of IRF-3 (Fig. 1A). LPS is also capable of inducing the activation of the JAK/STAT pathway, which also contributes to the induction of gene transcription. We therefore examined the phosphorylation status and activation of STAT1 in macrophages and showed that LPS-induced activation of STAT1 was also independent of IRAK-4 (Fig. 2B). Taken together, these results suggest that unlike MyD88, IRAK-4 is required for LPS-mediated induction of IFN-β and IP-10 and activation of the peak activity of NF-κB. On the other hand, IRAK-4 is similar to MyD88 in that it is dispensable for other signaling pathways such as those involving IRF-3 and STAT1.

The stimulation of cells with LPS induces activation of the PI-3-K pathway and strong phosphorylation of the downstream target Akt. In this study, we show that this pathway does not require the presence of IRAK-4 (Fig. 2C). Quite likely the pathway of PI-3-K runs in parallel to the MyD88→IRAK→TRAF6 pathway, although it remains to be confirmed that in MyD88- or TRAF6-deficient cells PI-3-K is activated in a normal fashion by LPS. Consistent with this possibility, IL-1, which employs a cellular signaling system similar to that used by LPS, is also capable of inducing mild activation of Akt, and this induction is also independent of IRAK-4 (Fig. 2D).

**IRAQ-4-deficient DC maturation is partially impaired after LPS stimulation**

Having established the specific defects in LPS signaling in IRAK-4-deficient cells, we next investigated the effects of IRAK-4 deficiency on DC maturation and function. We used BM-derived DCs that were unstimulated or stimulated with LPS or other TLR ligands. First of all, surface expression of costimulatory molecules was examined. As shown in Fig. 3, wild-type DCs respond to LPS or CpG-ODN (a TLR9 ligand) by up-regulating CD40, CD80, CD86, and MHC class II. IRAK-4-deficient DCs, however, exhibited a partial defect in response to LPS, in that although the costimulatory molecules and MHC class II were still somewhat up-regulated, their levels of expression were reduced compared with those of wild-type controls. Markedly, IRAK-4-deficient DCs failed to respond to CpG-ODN at all. These results suggest that IRAK-4 is partially required for LPS-induced DC maturation.

In sharp contrast, when unstimulated or LPS-stimulated DCs were cultured to evaluate their production of inflammatory cytokines, we found that IRAK-4-deficient DCs failed to produce any

**FIGURE 1.** Impaired induction of LPS-inducible genes in IRAK-4-deficient mice. A, BM-derived macrophages from wild-type and IRAK-4-deficient mice were stimulated with 1 μg/ml LPS for the indicated time periods to examine the expression of LPS-inducible genes, including IFN-β, IP-10, and GARG16, by Northern blot analyses. Expression of GAPDH was also measured to control for protein loading. B, Wild-type (WT), IRAK-4 knockout (KO), and MyD88 knockout MEFs were stimulated with 1 μg/ml LPS for the indicated time periods, and mRNA expression of IP-10 and GARG16 were examined. C, Wild-type, IRAK-4 knockout, and MyD88 knockout MEFs were stimulated with 1 μg/ml LPS for the indicated time periods, and NF-κB activation was measured by the degradation of IκB by Western blot analysis. D, Wild-type and IRAK-4-deficient MEFs were stimulated with 1 μg/ml LPS for 1, 2 and 3 h. Nuclear extracts were then prepared for gel mobility shift assay to measure NF-κB activation.
cytokines including IFN-γ, IL-12 p70, TNF, and IL-6 (Fig. 4). These results are consistent with what we previously reported in LPS-challenged IRAK-4-deficient macrophages (38). Taken together, our results suggest that a proportion of LPS-induced signals controlled by IRAK-4 are absolutely required for the induction of inflammatory cytokines in different types of APCs.

LPS-stimulated IRAK-4-deficient DCs are capable of triggering alloimmune T cell proliferation, but unable to stimulate Th1 cytokine production

One way to evaluate the function of LPS-maturated DCs is to assess their ability to induce alloreactive T cell proliferation. Wild-type and IRAK-4-deficient BM-derived DCs, either unstimulated or stimulated with LPS or CpG-ODN, were irradiated and incubated with allologeneic T cells. Wild-type DCs without TLR ligand treatment ("none" in Fig. 5A) were already able to stimulate T cell proliferation, while LPS or CpG-ODN further enhanced this allostimulatory activity of the DCs (Fig. 5A). IRAK-4-deficient DCs also exhibited such allostimulatory capacity, and LPS, but not CpG-ODN, was able to augment the activity of mutant DCs to a similar degree as compared with wild-type controls (Fig. 5A). These results are consistent with the expression levels of costimulatory molecules (Fig. 3) and suggest that partial expression of the costimulatory molecules, as seen on the surface of LPS-stimulated IRAK-4-deficient DCs, may be sufficient to stimulate T cell proliferation to allogenic stimuli. However, the level of T cell activation, as characterized by CD69 expression, stimulated by allogeneric IRAK-4-deficient DCs was less than that induced by allogenic wild-type DCs (Fig. 5B).

We next investigated the ability of LPS-stimulated DCs to induce Th differentiation of CD4⁺ T cells as another measurement of DC function. As shown in Fig. 5C, wild-type DCs primed with TLR ligands including LPS and CpG-ODN were capable of inducing IFN-γ, but not IL-4 production by allogenic CD4⁺ T cells under these culture conditions. On the other hand, LPS- or CpG-ODN-stimulated IRAK-4-deficient DCs failed to stimulate Th1 cytokine production from allogeic T cells (Fig. 5C). Thus, IRAK-4 is required for both the LPS-induced production of inflammatory cytokines from DC as well as the LPS-mediated priming of DC to stimulate Th1 cell differentiation. Taken together, our results suggested that IRAK-4-deficient DCs exhibit specific defects in LPS signaling. Cytokine secretion and Th cell instruction by DCs use IRAK-4-dependent pathways, while the stimulation of T cell proliferation is IRAK-4 independent.

Discussion

In this article, we investigated various signaling events induced by LPS that are critical for the activation of APCs such as macrophages and DCs. We showed that IRAK-4, a key molecule in LPS signaling, plays a crucial role in the induction of inflammatory cytokines and Th1 cell differentiation. These findings provide insights into the mechanisms by which IRAK-4 regulates the functions of APCs in response to LPS stimulation.
NF-κB/H9260 expression of each surface marker by CD80, anti-CD86, and anti-MHC class II mAbs and analyzed for the expression of each surface marker by flow cytometry.

Cells were then stained with FITC- or PE-conjugated anti-CD40, anti-IP-10, and anti-GARG16 by Cheng and colleagues (15) that cells expressing an undegradable form of IκB, in which NF-κB activity is attenuated, fail to respond to LPS by IP-10 and IFN-β induction. Intriguingly, IRAK-4-deficient MEF shows more severe defects in LPS-induced NF-κB activation and IP-10 and GARG16 expression than the mutant macrophages. Further investigations are required to elucidate the difference in LPS signaling between macrophages and MEFs.

It is intriguing to observe that in IRAK-4 knockout cells, there is a defect in LPS-induced IFN-β induction but STAT1 activation is normal. Activation of the STAT1 and JAK pathway by LPS is believed to occur by the induction of IFN-β, IP-10, and GARG16 in response to LPS. Interestingly, macrophages lacking both MyD88 and TIRAP displayed a mild defect of IP-10 and GARG16 induction by LPS (32). This is consistent with the possibility that IRAK-4 receives signals from both MyD88 family members and plays a critical role in transducing the signals from these proteins. The induction of IFN-β, IP-10, and GARG16 likely depends on both NF-κB and IRF-3 (15). Other reports also suggest that LPS-induced STAT1 activation contributes significantly to IP-10 and GARG16 induction (17). Although IRF-3 and STAT1 activation appear normal in IRAK-4 knockout, LPS-mediated NF-κB activation appears to be defective. This defect in NF-κB activation induced by LPS appears to be more severe in IRAK-4-deficient cells than in cells lacking MyD88. It is quite possible that the intact activity of NF-κB is required for the full induction of IFN-regulated genes. This possibility is consistent with the results by Cheng and colleagues (15) that cells expressing an undegradable form of IκB, in which NF-κB activity is attenuated, fail to respond to LPS by IP-10 and IFN-β induction. Intriguingly, IRAK-4-deficient MEF shows more severe defects in LPS-induced NF-κB activation and IP-10 and GARG16 expression than the mutant macrophages. Further investigations are required to elucidate the difference in LPS signaling between macrophages and MEFs.

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 ROLE OF IRAK-4 IN LPS SIGNALING

It has been clearly demonstrated that LPS-stimulated DC maturation depends on the TLR4 signaling pathway. However, the involvement of MyD88 in this process is questionable, aside from the critical role in inflammatory cytokine production (29). In this study, we showed that IRAK-4 is not only required for inflammatory cytokine production but also is involved in the optimal expression of costimulatory molecules on DCs in response to LPS signaling. Importantly, LPS-activated IRAK-4-deficient DCs can still stimulate allogeneic T cell proliferation but fail to induce Th cell differentiation.

We previously demonstrated that animals lacking IRAK-4 are resistant to LPS-induced endotoxic shock but are more susceptible to infection by intact bacteria (38). Therefore, an IRAK-4 inhibitor, while useful in treating life-threatening septic shock, may result in a severe risk for patients not being able to control the concurrent infection. Along the same line, a beneficial effect could be expected from blocking IRAK-4 signaling in chronic inflammatory disorders, but the long-term usage of such an inhibitor may expose patients to opportunistic infections. In this context, the specific defect of DC function induced by LPS stimulation in the absence of IRAK-4 is interesting, since certain adaptive immune responses triggered by IRAK-4-deficient APCs remain intact. Further investigation into the impact of IRAK-4 on various signaling pathways and the molecular mechanisms of IRAK-4 function are required before a practical therapeutic design can be conceived.

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