The Kinase Activity of IL-1 Receptor-associated Kinase 4 Is Required for Interleukin-1 Receptor/Toll-like Receptor-induced TAK1-dependent NFκB Activation*

JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 283, No. 46, pp. 31697–31705, November 14, 2008
© 2008 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

Jerzy Frańczek1,2, Tae Whan Kim3,4, Hui Xiao5, Jianhong Yao6, Qian Wen1, Yali Li3, Jean-Laurent Casanova2,*, Juliusz Pryjma2,6, and Xiaoxia Li1,2

From the 1Department of Immunology, Cleveland Clinic Foundation, Cleveland, Ohio 44195, the 2Department of Immunology, Faculty of Biotechnology, Jagiellonian University, Gronostajowa 7, Kraków 30-387, Poland, the 3Unite d’Immunologie et d’Hematologie Pediatriques, Laboratoire de Genetique Humaine des Maladies Infectieuses Unite Mixte de Recherche Universite Rene Descartes-INSERM U550, Paris 75270, Cedex 06, France, the 4Department of Pathology, Case Western Reserve University, Cleveland, Ohio 44106, and the 5Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, Ohio 44106

Two parallel interleukin-1 (IL-1)-mediated signaling pathways have been uncovered for IL-1R–TLR-mediated NFκB activation: TAK1-dependent and MEKK3-dependent pathways, respectively. The TAK1-dependent pathway leads to IKKα/β phosphorylation and IKKβ activation, resulting in classic NFκB activation through IκBα phosphorylation and degradation. The TAK1-independent MEKK3-dependent pathway involves IKKγ phosphorylation and IKKα activation, resulting in NFκB activation through dissociation of phosphorylated IκBα from NFκB without IκBα degradation. IL-1 receptor-associated kinase 4 (IRAK4) belongs to the IRAK family of proteins and plays a critical role in IL-1R/TLR-mediated signaling. IRAK4 kinase-inactive mutant failed to mediate the IL-1R-TRL-induced TAK1-dependent NFκB activation pathway, but mediated IL-1-induced IRAK4 death domain-dependent NFκB activation and retained the ability to activate substantial gene expression, indicating a structural role of IRAK4 in mediating this alternative NFκB activation pathway. Deletion analysis of IRAK4 indicates the essential structural role of the IRAK4 death domain in receptor proximal signaling for mediating IL-1R-TRL-induced NFκB activation.

Toll-like receptors (TLRs)3 play a critical role in innate immune responses in mammals through the recognition of conserved molecular patterns associated with different microorganisms (1–6). Upon binding of TLR ligands, all of the TLRs except TLR3 recruit the adaptor molecule MyD88 through the TIR domain, mediating the so-called MyD88-dependent pathway (7). MyD88 then recruits serine-threonine kinases IRAK4 (IL-1 receptor-associated kinase 4) and IRAK (8–12). Although IRAK4 is the kinase that functions upstream of and phosphorylates IRAK, the phosphorylated IRAK mediates the recruitment of TRAF6 to the receptor complex (13, 14). Upon phosphorylation of IRAK, the IRAK-TRAF6 complex dissociates from the receptor complex to interact with and activate downstream kinases, leading to the activation of NFκB and JNK (14, 15).

We recently reported the co-existence of the two parallel IL-1-mediated TAK1-dependent and MEKK3-dependent signaling pathways for NFκB activation (Fig. 1) (16). These two pathways are regulated at the level of IRAK modification. The TAK1-dependent pathway causes IKKα/β phosphorylation and IKKβ activation, leading to classic NFκB activation through IκBα phosphorylation and degradation. The TAK1-independent MEKK3-dependent pathway induces IKKγ phosphorylation and IKKα activation, resulting in NFκB activation through IκBα phosphorylation and subsequent dissociation from NFκB but without IκBα degradation. It is important to note that we recently found that TLR8-mediated NFκB and JNK activation are TAK1-independent and MEKK3-dependent, suggesting a regulatory mechanism at the level of receptor complexes that determines the usage of TAK1-dependent versus MEKK3-dependent pathways in IL-1R/TLR signaling.

IRAK4 has been shown to play an essential role in TLR-mediated signaling (9,10). IRAK4 kinase-inactive knock-in mice were completely resistant to LPS- and CpG-induced shock, due to impaired TLR-mediated induction of pro-inflammatory cytokines and chemokines (17–20). Although inactivation of IRAK4 kinase activity did not affect the levels of TLR/IL-1R-mediated NFκB activation, a reduction of LPS-, R848-, and IL-1-mediated mRNA stability contributed to the reduced cytokine and chemokine production in bone marrow (BM)-derived macrophages from IRAK4 kinase-inactive knock-in mice (18,20). These in vivo studies indicate that IRAK4 kinase activity plays a critical role in TLR-dependent immune responses (21).

* This work was supported, in whole or in part, by National Institutes of Health GM060020 (to X. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: Dept. of Immunology, Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Tel.: 216-445-8706; Fax: 216-444-9329; E-mail: lix@ccf.org.

3 The abbreviations used are: TLR, Toll-like receptor; IRAK4, IL-1 receptor-associated kinase 4; TRAF, TNF receptor-associated factor; JNK, c-Jun N-terminal kinase; MEKK1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1; IL-1R, interleukin-1 receptor; LPS, lipopolysaccharide; BM, bone marrow; DD, death domain; TAK1, TGF-β-activated kinase 1.
Previous studies suggest that IRAK4 is required for the recruitment and activation of IRAK at the signaling complex. Interestingly, IRAK4 kinase-inactive mutant had similar ability as the wild-type IRAK4 in restoring IL-1-mediated NFκB in human IRAK4-deficient cells (22). On the other hand, by reconstituting IRAK4-deficient mouse embryonic fibroblasts, Lye et al. (23) showed that the kinase activity of mouse IRAK4 is required for the optimal transduction of IL-1-induced signals, although they found that IRAK4 is capable of mediating some NFκB activation. In support of these previous findings, IL-1-, LPS-, and R848-induced NFκB activation was not reduced in the BM-derived macrophages from IRAK4 kinase-inactive knock-in mice as compared with that in the wild-type control cells. Therefore, the kinase activity of IRAK4 seems to be dispensable for TLR/IL-1R-mediated NFκB activation.

We recently further examined the role of kinase activity of IRAK4 in IL-1-induced NFκB activation. Whereas NFκB was activated, IκBα was only phosphorylated but not degraded in human IRAK4-deficient cells transfected with the IRAK4 kinase-inactive mutant in response to IL-1 stimulation, suggesting that kinase activity of IRAK4 might be important for TAK1-dependent NFκB activation pathway. Similar results were observed in BM macrophages from IRAK4 kinase-inactive knock-in mice. IRAK4 kinase-inactive mutant failed to mediate IL-1R-TLR-induced TAK1-dependent NFκB activation pathway, evident by greatly reduced IL-1R-TLR-induced TAK1 phosphorylation and activation, IKKα/β phosphorylation, and IκBα degradation. The fact that the kinase-inactive IRAK4 mutant can still mediate IL-1-induced TAK1-independent NFκB activation indicates a structural role of IRAK4 in mediating this alternative NFκB activation pathway. Deletion analysis of IRAK4 demonstrates the essential structural role of IRAK4 death domain in receptor proximal signaling for mediating IL-1R-TLR-induced NFκB activation.

**MATERIALS AND METHODS**

Biological Reagents and Cell Culture—Recombinant human IL-1 was provided by the NCI, National Institutes of Health. CpG oligodeoxynucleotide was purchased from Invivogen. LPS (Escherichia coli 055:B5) was purchased from Sigma-Aldrich, and R848 was obtained from GLSynthesis Co. Antibodies against phosphorylated IκBα (Ser-32/S36), IRAK4 from Stressgen (recognizing C-terminal region). JNK, IKKα/β (Ser-176/180), IKKγ (Ser-376), and total IκBα, JNK, IKKα, and IKKβ were purchased from Cell Signaling. Antibody to FLAG (anti-FLAG) was purchased from Sigma. Antibodies against ubiquitin, NFκB, IRAK, and TAK1 were from Santa Cruz Biotechnologies (Santa Cruz, CA). 293 cells and IRAK-deficient cells (derived from parental 293 cells) (12), and IRAK4-deficient dermal fibroblasts derived from skin biopsies of the human patient (10) were maintained in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serine, penicillin G (100 μg/ml), and streptomycin (100 μg/ml). BM-derived macrophages were obtained from the bone marrow of tibia and femur by flushing with Dulbecco’s modified Eagle’s medium. The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum, and 30% L.929 supernatant for 5 days.

Transfection and Luciferase Assay—Transfection of the human IRAK4-deficient fibroblasts was performed using the FuGENE 6 transfection reagent as recommended by the manufacturer (Roche Applied Science). After 24 h, the cells were stimulated with IL-1 or left untreated the next day for 6 h before harvest. Luciferase activity was determined by using the luciferase assay system and chemiluminescent reagents from Promega (Madison, WI).

Plasmids—IRAK4 deletion mutants and IRAK4/IRAK chimeric constructs were generated by overlapping PCR and cloned in pcDNA 3.1 expression vector. For all PCR reactions high fidelity Pfu Turbo polymerase was used (Stratagene).

Immunoblotting—Cell were harvested and lysed in a Triton-containing lysis buffer (0.5% Triton X-100, 20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl₂, 10 mM NaF, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor mixture from Roche Applied Science). For immunoblotting, cell lysates were separated by 10% SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and analyzed by immunoblotting.

**Gel Shift Assays**—An NFκB binding site (5’-GGACGAGGGAAATTCCGTAAACTT-3’) from the IP-10 gene was used as a probe (24). Complementary oligonucleotides, end-labeled with polynucleotide kinase (Boehringer Mannheim) and γ-32P-labeled ATP, were annealed by slow cooling. Approximately 20,000 cpm of probe was used per assay. Cytoplasmic extracts were prepared as described by Kessler et al. (25). The binding reaction was carried out at room temperature for 20 min in a total volume of 20 μl containing 20 mM Hepes buffer, pH 7.0, 10 mM KCl, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, and 10% glycerol.

Kinase Assays—Cell lysates were immunoprecipitated with anti-TAK1 and collected on protein A-Sepharose beads. Kinase reactions were performed in 50 μl of buffer containing 20 mM Hepes (pH 7.0), 20 mM MgCl₂, 1 mM ATP, 10 μCi of [γ-32P]ATP at 30 °C for 30 min. The substrate for TAK1 kinase assay was 2 μg of His-MKK6. Samples were analyzed by 10% SDS-PAGE, followed by autoradiography.

Quantitative Real-time PCR—Total RNA was isolated using TRIzol reagent (Invitrogen). 3 μg of total RNA was then used for reverse transcription reaction using SuperScript-reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed in an ABI 7300 RealTime PCR System, and the gene expression of human IL-6-, IL-8-, and β-actin was examined by SYBR® GREEN PCR Master Mix (Applied Biosystems). PCR amplification was performed in triplicate, and water was used to replace cDNA in each run as a negative control. The reaction protocol included preincubation at 95 °C to activate FastStart DNA polymerase for 10 min, amplification of 40 cycles that was set for 15 s at 95 °C, and annealing for 60 s at 60 °C. The results were normalized with the housekeeping gene mouse β-actin. Primer sequences were designed using online tools from GeneScript as follows. Human IL-6 forward, 5’-AATGAGGAGAC-TTGCCCTGGT-3’; human IL-6 reverse, 5’-GCAGGAACTGGATCAGGACT-3’; human IL-8 forward, 5’-AAGACATACTC-CAAACCTTTCCA-3’; human IL-8 reverse, 5’-CCAGACA-GAGCTCTCTTCCA-3’; human β-actin forward, 5’-CCTGG-
CACCACGACAAAT-3'; human β-actin reverse, 5’-GCCGATCCACAGGACTACT-3'; mouse IRAK2 forward, 5’-GGACATCCTCAGCTGGAATTGTA-3'; mouse IRAK2 reverse, 5’-CTGAGAAGCCAATCTCTCAAGTAAAC-3'; mouse IRAK3 forward, 5’-GGGCGCTTTCGAGAACGACA-3'; mouse IRAK3 reverse, 5’-ACGCGTGAAGTGTGTTGCAAGTA-3'; mouse CCL2 forward, 5’-CTCAGCCAGATGCTGTTGGTGGTAACCC-3'; mouse CCL2 reverse, 5’-GGTGGTCTGAAGACCTTAGGGCAGAT-3; mouse CCL7 forward, 5’-AAATGATCCACACTGCTGTA-3'; mouse CCL7 reverse, 5’-ATGCGGCTTACAGACACAGACT-3'; mouse CLEC9 forward, 5’-CTCAGTGCTTTCACCACTACC-3'; mouse CLEC9 reverse, 5’-TCCAGTTCAAGGACACAGCA-3'; mouse CEBPβ forward, 5’-CAAGCTGAGGAGCAACAAGACA-3'; mouse CEBPβ reverse, 5’-GAGCACTGCTCCACCTTCTT-3'; mouse ICAM1 forward, 5’-GTGATGCTCAGTATCCATCCA-3'; mouse ICAM1 reverse, 5’-CACAGTTCACCAAGTACAGG-3'; mouse IkBα forward, 5’-CAAGTCACTGAGGCTTTCAAC-3'; mouse IkBα reverse, 5’-ACTGAACTG-CTGTTGGTCC-3'; mouse CCL4 forward, 5’-CTccccacctctcctcttcctc-3'; mouse CCL4 reverse, 5’-GCTCTGAGGTAGCCTGCTC-3; mouse GRP84 forward, 5’-CTCCAGGTTGAGGTAGTCTGCT-3'; mouse GRP84 reverse, 5’-CGCTGGACATGGTGGTCTG-3'; mouse β-actin reverse, 5’-ACGGATGTCAACGTCACACT-3.

Enzyme-linked Immunosorbent Assay—Supernatants from cell cultures were collected and measured for the level of human cytokines IL-6 and IL-8 using OptEIA ELISA kits II (BD Biosciences) according to the manufacturer’s instructions.

Illumina Beadchip Microarray Analysis—250 ng of RNA was reverse transcribed into cRNA and biotin-UTP labeled using the Illumina (San Diego, CA) TotalPrep RNA amplification kit (Ambion, Austin, TX). cRNA was quantified using a nanodrop spectrophotometer, and the cRNA quality (size distribution) was further analyzed on a 1% agarose gel. cRNA was hybridized to the Illumina MouseRef8 v1.1 Expression BeadChip using standard protocols (provided by Illumina).

RESULTS

The Kinase Activity of IRAK4 Is Required for IL-1R-induced TAK1-dependent NFκB Activation in Human IRAK4-deficient Fibroblasts—IRAK is phosphorylated after it is recruited to the receptor, subsequently ubiquitinated, and eventually degraded upon IL-1 stimulation. Through the study of IRAK modification, we uncovered two IL-1-mediated signaling pathways for NFκB activation: TAK1-dependent and MEKK3-dependent, respectively (Fig. 1) (16). As depicted in Fig. 1, IL-1R mediates NFκB activation through TAK1- and MEKK3-dependent pathways. The TAK1-dependent pathway leads to IKKα/β phosphorylation and IKKβ activation, resulting in classic NFκB activation through IκBα phosphorylation and degradation. The TAK1-independent MEKK3-dependent pathway involves IKKγ phosphorylation and IκKα activation, resulting in NFκB activation through IκBα phosphorylation and subsequent dissociation from NFκB but without IκBα degradation. Impairment of IL-1-induced IRAK phosphorylation and ubiquitination shifts receptor proximal signaling from the TAK1-dependent to the MEKK3-dependent pathway. Previous studies suggest that IRAK4 is required for the recruitment and activation of IRAK at the signaling complex. Interestingly, IRAK4 kinase-inactive mutant (lysine 213 to methionine, which is part of the ATP binding pocket of the kinase domain) had similar ability as the wild-type IRAK4 in restoring IL-1-mediated NFκB activation in IRAK4-deficient fibroblasts derived from human patient (Fig. 2, A and B) (10, 22). In the light of the new discovery of TAK1- and MEKK3-dependent NFκB activation pathways (Fig. 1), we reexamined the ability of IRAK4 kinase-inactive mutant in mediating intermediate signaling events of IL-1-induced NFκB activation in human IRAK4-deficient fibroblasts. Whereas IRAK4 kinase-inactive mutant had similar ability as the wild-type IRAK4 in mediating IL-1-induced IκBα phosphorylation and NFκB activation, the mutant failed to restore IL-1-induced TAK1 phosphorylation and activation, IKKα/β phosphorylation, and IκBα degradation (Fig. 2, C and D). These results suggest that IRAK4 kinase activity may be only required for TAK1-dependent NFκB activation. Thus, the kinase-inactive IRAK4 mutant probably activates NFκB through a TAK1-independent pathway. Based on our previous findings on the link between TAK1-independent NFκB activation and MEKK3 (16), we propose that inactivation of IRAK4 kinase activity might shift receptor proximal signaling from the TAK1-dependent to the MEKK3-dependent pathway, which is further addressed below.

FIGURE 1. Model of the IL-1 signaling pathway.
Inactivation of IRAK4 Kinase Activity Abolished TLR4/7/9-mediated TAK1-dependent NFκB Activation—To further determine the role of the kinase activity of IRAK4 in IL-1R- and TLR-mediated signaling, we generated IRAK4 kinase-inactive knock-in mice by mutating lysine 213 and 214 residues to methionines (20). The inactivation of the kinase activity of the mutant IRAK4 from the knock-in mice was confirmed by immunoprecipitation kinase assay in vitro (20). BM-derived macrophages from wild-type, IRAK4-deficient, and IRAK4 kinase-inactive knock-in mice were examined for IL-1R- and TLR4/7/9-mediated NFκB activation. IL-1, LPS, R848, and CpG induced phosphorylation of IkBα and NFκB activation in both wild-type and IRAK4-deficient knock-in BM-derived macrophages (Fig. 3, A and B) (20). However, TLR4/7/9-mediated TAK1 phosphorylation (slower migration abolished by phosphatase treatment (16)), IKKα/β phosphorylation, and IkBα degradation were significantly attenuated in IRAK4 kinase-inactive knock-in macrophages as compared with that in wild-type cells (Fig. 3, B and C, and data not shown). The impact of inactivation of IRAK4 kinase activity on NFκB activation echoes our published results of the TAK1-deficient mouse embryonic fibroblasts for the IL-1-induced signaling, including abolished IL-1-induced TAK1 activation, reduced IL-1-induced IKKα/β and IkBα degradation, retained IkBα phosphorylation, and NFκB activation (16). Taken together, these findings suggest that the impact of IRAK4 kinase activity on NFκB activation might be due to a lack of TAK1 activation. In support of this, TAK1 inhibitor blocked TLR4/7-induced IkBα degradation and retained IkBα phosphorylation and NFκB activation in BM-derived macrophages (Fig. 3E), which is very similar to the impact of IRAK4 kinase activity on IkBα and NFκB activation upon TLR4/7 stimulation in BM-derived macrophages. These results confirm that IRAK4 kinase-inactive mutant probably activates NFκB through a TAK1-independent pathway.

IRAK4 Kinase Activity Is Required for IL-1R- and TLR4/7/9-mediated IRAK Modification and Degradation—We recently reported that, although a point mutation changing lysine 134 to arginine (K134R) in IRAK abolished IL-1-induced IRAK ubiquitination and degradation, mutations of serines and threonines adjacent to Lys-134 to alanines (S/T)A (131–144)) reduced IL-1-induced IRAK phosphorylation and abolished IRAK ubiquitination (16). The IRAK modification mutants failed to form complex with TAK1 and were unable to mediate TAK1 phosphorylation and activation. Interestingly, we now found that IRAK4 kinase activity is also required for TAK1-dependent NFκB activation. These results suggest that IRAK4 and IRAK might mediate TAK1-dependent pathway in a sequential fashion, through a kinase-substrate relationship. Importantly, TLR4/7/9-induced IRAK modification and degradation were indeed abolished in BM macrophages from IRAK4 kinase-inactive knock-in mice as compared with that of wild-type mice, demonstrating that the kinase activity of IRAK4 is required for IRAK modification (17,19) (Fig. 3F and data not shown).

The Kinase-inactive IRAK4 Mutant Protein Mediates TLR7/9-induced TAK1-independent NFκB Activation—The above results indicate that TLR4/7/9 can still mediate TAK1-independent NFκB activation in the absence of IRAK4 kinase activity. One important question was whether this TAK1-independent NFκB activation pathway requires the presence (structural role) of IRAK4 protein. To address this question, we examined TLR2/4/7/9-mediated NFκB activation and related signaling events in IRAK4-deficient macrophages. Whereas TLR2/4-mediated NFκB activation and related signaling events were attenuated, TLR7/9-mediated signaling events were abolished or greatly reduced in BM-derived macrophages from IRAK4-deficient mice (Fig. 3G and H). It should be noted that TLR7/9 can sometimes still slightly induce NFκB activation in IRAK4-deficient macrophages (which was previously observed by Kawagoe et al. (17). However, the level of NFκB activation was much lower in IRAK-deficient macrophages compared with that in IRAK4 kinase-inactive knock-in or wild-type macrophages. These results clearly indicate that most of the TLR7/9-mediated signaling pathways are IRAK4-dependent, although TLR7/9-mediated TAK1-independent NFκB activation does not require IRAK4 kinase activity. In other words, the kinase-inactive IRAK4 protein probably plays a structural role for the TLR7/9-mediated TAK1-independent NFκB activation.
Our previous studies have suggested a potential role of MEKK3 in IL-1-induced TAK1-independent NFκB activation (16). The IRAK modification mutants that failed to interact with TAK1 retained the ability to interact with MEKK3 and were also able to mediate the interaction between TRAF6 and MEKK3, implicating a specific role of MEKK3 in IL-1-induced TAK1-independent signaling. Furthermore, although the TAK1 inhibitor (5Z-7-oxozeaenol) had partial inhibition on NFκB activation in wild-type mouse embryonic fibroblasts, the inhibitor completely impaired IL-1-induced NFκB activation in MEKK3-deficient mouse embryonic fibroblasts. We indeed found that, although TLR7-induced TAK1 phosphorylation was abolished in the absence of IRAK4 kinase activity (Fig. 3C), TLR7 stimulation induced MEKK3 phosphorylation in macrophages from both wild-type and IRAK4 kinase-inactive knock-in mice (Fig. 3D). TLR7-induced TAK1 and MEKK3 phosphorylation was confirmed by treatment with calf intestinal phosphatase (Ref. 16 and data not shown). Taken together, these results suggest the possible participation of MEKK3 in TLR7-induced TAK1-independent NFκB activation.

IRAK4 in TAK1-dependent and -independent NFκB Activation

FIGURE 3. Inactivation of IRAK4 kinase activity abolished IL-1R-TRL-mediated TAK1-dependent NFκB activation. A–D, cell lysates from bone marrow-derived macrophages from wild-type, IRAK4 kinase-inactive knock-in or IRAK4-deficient mice that were either untreated or treated with LPS (1 μg/ml), R848 (1 μg/ml), or CpG (4 μg/ml) for the indicated times were analyzed by electrophoretic mobility shift assay with an NFκB-specific probe (A) or by Western analysis with antibodies against pJNK, IRAK4 and actin (A) and antibodies against phospho-IκBα, IκBβ, phospho-IKKα/β, and IKKα/β (B), TAK1 (C), and MEKK3 (D). E, bone marrow-derived macrophages were pretreated with 600 nm TAK1 inhibitor for 3 h prior to stimulation with LPS (1 μg/ml) and R848 (1 μg/ml) for the indicated times. The cell lysates were analyzed by electrophoretic mobility shift assay with an NFκB-specific probe or by Western analysis with antibodies against phospho-IκBα, IκBβ, and actin. F, cell lysates from bone marrow-derived macrophages from wild-type and IRAK4-deficient mice that were either untreated or treated with R848 (1 μg/ml) or CpG (4 μg/ml) for the indicated times were analyzed by Western analysis with antibodies against IRAK and β-actin. G and H, cell lysates from bone marrow-derived macrophages from wild-type and IRAK4-deficient mice that were either untreated or treated with LPS (1 μg/ml), R848 (1 μg/ml), or Malp2 or CpG (4 μg/ml) for the indicated times were analyzed by electrophoretic mobility shift assay with an NFκB-specific probe (G) or by Western analysis with antibodies against pJNK, phospho-IKKα/β, phospho-IκBα, IκBβ, and IRAK4 (H). The levels of NFκB activation were analyzed by Scion Image 1.62C alias and presented as -fold induction of the untreated samples.
would predict inactivation of IRAK4 kinase activity should have minimum impact on mRNA levels of the genes that are not regulated at the post-transcription levels. We recently examined gene expression profiles of macrophages from wild-type and IRAK4 kinase-inactive knock-in mice in response to TLR7 and TLR9 stimulation using the Illumina Microarray with probes for 46,000 transcripts. Bone marrow-derived macrophages from wild-type and IRAK4 kinase-inactive knock-in mice were treated with TLR7 (R848) and TLR9 (CpG) ligands for 30 and 240 min. The majority of the genes was induced at similar levels in wild-type and IRAK4 kinase-inactive knock-in macrophages at early time (stimulated for 30 min) (supplementary Fig. S1A and data not shown). Some of those genes were then up-regulated at higher levels in wild type than that in kinase-inactive knock-in macrophages at late time (stimulated for 240 min) (supplementary Fig. S1B and C), which is consistent with the previously reported role of IRAK4 kinase activity in post-transcriptional regulation (20). Importantly, we have also identified a group of genes that were induced similarly in wild-type and IRAK4 kinase-inactive knock-in macrophages at both early and late time in response to TLR7 or TLR9 ligands (Fig. 4, A – D), which was confirmed by real-time PCR (Fig. 4, C and D). The expression of these IRAK4 kinase-independent genes was abolished in IRAK4-deficient macrophages, indicating a structural
role of IRAK4 in the induction of these genes (Fig. 4, C and D). Based on these findings, we propose that the TAK1-independent pathway in IRAK4 kinase-inactive macrophages can mediate substantial expression of TLR7/9-dependent genes, especially on the genes that are not regulated at post-transcriptional levels.

The Death Domain of IRAK4 Is Essential for IL-1R-induced NFκB Activation—The above results showed that the kinase-inactive IRAK4 mutant can still mediate IL-1R- and TLR7/9-induced TAK1-independent NFκB activation and gene expression, indicating a structural role of IRAK4 in mediating this alternative NFκB activation pathway. We were then interested in determining the structural domain of IRAK4 important for this activity. As shown in Fig. 5A, IRAK4 contains several functional domains, including a death domain at the N terminus (DD, residues 1–100), followed by an undetermined region (UD, residues 100–179) and a kinase domain at the C terminus (residues 179–460). A set of IRAK4 deletion mutants were generated to assess the function of these different domains in IL-1R-induced NFκB activation (Fig. 5A). The deletion mutants were co-transfected with NFκB-dependent luciferase construct into IRAK4-deficient cells, followed by IL-1 treatment and luciferase reporter assay. The IRAK4 deletion mutants without UD (d101–179) or kinase domain (d179–460) could still restore IL-1-induced NFκB activation in IRAK4-deficient cells, indicating the essential structural role of the IRAK4 death domain in receptor proximal signaling (Fig. 5B).

The DD of IRAK4 Distinguishes IRAK4 from IRAK in Mediating IL-1R-induced NFκB Activation—We hypothesize that the IRAK4 DD participates in the formation of receptor complex (Complex I, Fig. 1) to facilitate the recruitment of IRAK. To test this hypothesis, we swapped the death domain between IRAK and IRAK4 and tested the resulting chimeric IRAK4/IRAK constructs (DD4 + 1 and DD1 + 4) for their ability to restore IL-1-induced NFκB activation in IRAK-deficient cells. Interestingly, although DD4 + 1 restored IL-1-induced NFκB activation in IRAK-deficient cells, DD1 + 4 failed to activate NFκB in these cells (Fig. 5, C, E, and F). These results indicate that the death domain of IRAK4 is essential and sufficient in the recruitment of IRAK to mediate IL-1-induced NFκB activation. Interestingly, the DD4 + 1 also restored IL-1-induced NFκB activation in IRAK-deficient cells, confirming the critical role of the death domain of IRAK4 for the recruitment of IRAK in mediating IL-1-induced NFκB activation (Fig. 5D).

DISCUSSION

Herein, we report that the kinase activity of IRAK4 is required for IL-1R-TLR-induced TAK1-dependent NFκB activation pathway, evident by greatly reduced IL-1R-TLR-induced TAK1 phosphorylation and activation, IKKα/β phosphorylation, and IκBα degradation. The fact that the kinase-inactive IRAK4 mutant can still mediate IL-1-induced TAK1-indepen-
ent NFκB activation and gene expression indicates a structural role of IRAK4 in mediating this alternative NFκB activation pathway. Deletion analysis of IRAK4 indicates the essential structural role of IRAK4 death domain in receptor proximal signaling for mediating IL-1R- and TLR-induced NFκB activation.

Significant levels of IL-1, LPS-, and R848-induced NFκB activation were retained in the BM-derived macrophages from IRAK4 kinase-inactive knock-in mice as compared with that in the wild-type control cells (17, 20). We previously uncovered two parallel IL-1-mediated signaling pathways for NFκB activation: TAK1-dependent and MEKK3-dependent, respectively (16). These two pathways bifurcate at the level of IRAK modification. The TAK1-dependent pathway leads to IKKα/β phosphorylation and IKKβ activation, resulting in classic NFκB activation through IkBα phosphorylation and degradation. The TAK1-independent MEKK3-dependent pathway involves IKKγ phosphorylation and IKKα activation, resulting in NFκB activation through IkBα phosphorylation and subsequent dissociation from NFκB but without IkBα degradation. These results provide significant insight to our understanding of NFκB activation data from the IRAK4 kinase-inactive knock-in cells. LPS and R848 stimulation led to IkBα phosphorylation; therefore, NFκB activation with attenuated IkBα degradation in IRAK4 kinase-inactive knock-in cells suggests that the kinase activity of IRAK4 is likely to play a more critical role in TLR/IL-1R-induced NFκB activation pathway. In this report, our results showed that TLR-IL-1R-induced IKKα/β and TAK1 phosphorylation and activation were indeed abolished in the absence of IRAK4 kinase activity. Consistent with the fact that IRAK modification is required for the TAK1-dependent but not the MEKK3-dependent pathway (16), IRAK is not modified in IRAK4 kinase-inactive knock-in macrophages in which TAK1-dependent NFκB activation is abolished. Therefore, we concluded that IRAK4 and IRAK function in a sequential fashion, through a kinase-substrate relationship, to mediate TLR-induced TAK1-dependent but not MEKK3-dependent NFκB activation.

Importantly, we previously reported that IRAK4 kinase-inactive mutant (K213M) had similar ability as the wild-type IRAK4 in restoring IL-1-mediated NFκB activation in human IRAK4-deficient fibroblasts (22). In light of the new discovery of TAK1- and MEKK3-dependent NFκB activation pathways, we re-examined the ability of IRAK4 kinase-inactive mutant to mediate intermediate signaling events of IL-1-induced NFκB activation in human IRAK4-deficient fibroblasts. Consistent with our findings in IRAK4 kinase-inactive knock-in macrophages, human IRAK4 kinase-inactive mutant also failed to activate TAK1-dependent NFκB activation in human IRAK4-deficient cells. It should be noted that the human fibroblasts are not responsive to TLR ligands. Therefore, one cannot directly compare the role of IRAK4 kinase activity for TLR-mediated TAK1-mediated NFκB activation in primary mouse macrophages with that in human fibroblasts. It is intriguing that, although IRAK4 kinase activity is required for TRAF7-induced JNK activation, JNK is still activated in IRAK4-deficient cells transfected with IRAK4 kinase-inactive mutant in response to IL-1 stimulation. Overexpression of IRAK4 protein in these human IRAK4-deficient cells might cause hyperactivation of the JNK pathway.

It is interesting that, whereas TLR2/4-mediated NFκB activation and related signaling events were attenuated, TLR7/9-mediated signaling events were completely abolished in BM-derived macrophages from IRAK4-deficient mice. TLR2/4 were able to mediate some NFκB activation through one or more IRAK4-independent pathways. Although the TLR4-induced MyD88/IRAK4-independent signaling is known to be mediated by the TRIF-dependent pathway (26–28), the TLR2-induced IRAK4-independent pathway still needs to be defined. Our results are consistent with the studies presented by Kawagoe et al. (17), in which TLR2 signaling was the major focus. Unlike TLR4, TLR2 ligand mediates a unique MyD88-dependent IRAK4-independent pathway. On the other hand, our results clearly indicate that most of the TLR7/9-mediated signaling pathways are IRAK4-dependent, although TLR7/9-mediated TAK1-independent NFκB activation does not require IRAK4 kinase activity. IL-1R- and TLR7/9-mediated NFκB activation are completely abolished in human patients defective in IRAK4 expression (10), which is consistent with our study in IRAK4-deficient mice.

Although we previously reported that the kinase activity of IRAK4 is essential for IL-1R-TLR-mediated mRNA stabilization of cytokines and chemokines in mouse bone marrow-derived macrophages (20), we now find that IRAK4 kinase activity is required for IL-1R- and TLR4/7/9-mediated TAK1-dependent, but not TAK1-independent NFκB activation. We propose that IRAK4 mediates IL-1R-TLR-induced receptor proximal signaling events through its kinase activity to coordinate NFκB activation and mRNA stabilization to ensure robust production of cytokines and chemokines during inflammatory response. In support of this, Illumina Microarray experiments indeed identified a group of inflammatory genes that were induced at similar levels in wild-type and IRAK4 kinase-inactive knock-in macrophages at early time, but with reduced expression in kinase-inactive knock-in macrophages at late time. One important issue is whether the observed TAK1-independent NFκB activation results in any substantial expression of TLR-dependent genes in the IRAK4 kinase-inactive knock-in mice. Based on the fact that mRNA stabilization is abolished in the absence of IRAK4 kinase activity, one would predict that the TAK1-independent pathway in IRAK4 kinase-inactive macrophages probably mediates the expression of genes that are not regulated at post-transcriptional levels. Illumina Microarray experiments have now revealed a group of IRAK4 kinase-independent genes that were induced similarly in wild-type and IRAK4 kinase-inactive knock-in macrophages at both early and late time. However, the TLR-induced expression of these genes was abolished in IRAK4-deficient macrophages, indicating a structural role of IRAK4 in the induction of this group of genes.

The fact that the kinase-inactive IRAK4 mutant can still mediate IL-1-induced TAK1-independent NFκB activation and gene expression indicates a structural role of IRAK4 in mediating this alternative NFκB activation pathway. Importantly, although the IRAK4 death domain (either alone of fused to IRAK as DD4 + 1) restored IL-1-induced NFκB activation in

\[ \text{IRAK4 in TAK1-dependent and -independent NFκB Activation} \]
IRAK4-deficient cells, DD1 + 4 failed to activate NFκB in these cells. These results indicate that the death domain of IRAK4 plays a key role in mediating receptor proximal signaling, which cannot be replaced by the death domain of IRAK. On the other hand, DD4 + 1 was able to restore IL-1-induced NFκB activation in IRAK-deficient cells, which indicates the essential and sufficient role of the death domain of IRAK4 for recruitment of IRAK in mediating IL-1-induced NFκB activation. The specific functions of IRAK4 and IRAK death domain are probably due to their structural differences. The crystal structure for the IRAK4 death domain reveals a six-helical bundle with a prominent loop (29). Comparing with other IRAK family members (including IRAK), the highly structured loop contained between helices two and three (an 11-amino acid stretch, residues 39–49), is unique to IRAK4 and may be responsible for its different function in mediating the formation of receptor signaling complex.

Although the detailed signaling mechanism and physiological impact of the TLR-induced IRAK4 kinase-independent pathway are still unclear, the ability of IRAK4 kinase-inactive mutant to mediate signaling is critical for pursuing IRAK4 as a drug target. It has been controversial whether IRAK4 is a feasible drug target for anti-inflammatory therapy. The total blockage of TLR-IL-1R signaling is likely to severely impair innate immunity, which may lead to immune deficiency with a dysfunctional host defense. Importantly, our studies indicate that, although IRAK4 kinase activity is essential for TLR-IL-1R-mediated production of cytokines and chemokines, not all of the TLR-IL-1R signaling events are ablated in IRAK4 kinase-inactive knock-in mice. Significant levels of NFκB activation and substantial gene expression are retained upon TLR7/9 and IL-1R stimulation in the absence of IRAK4 kinase activity. The residual TLR7/9-induced signaling event allows production of some cytokines and chemokines at early times after stimulation and induction of a group of TLR7/9-mediated IRAK4 kinase-independent genes in IRAK4 kinase-inactive knock-in cells. Therefore, it is likely that pharmacological inhibition of IRAK4 kinase activity will not completely impair host defense, while alleviating the levels and duration of inflammatory responses.

Acknowledgment—The Illumina gene expression profiling Microarray experiments were performed by the Cleveland Clinic Genomics Core (www.lerner.ccf.org/services/gc/).

REFERENCES
1. Medzhitov, R., Preston-Hurlburt, P., and Janeway, C. A., Jr. (1997) Nature 388, 394–397
2. Rock, F. L., Hardiman, G., Timans, J. C., Kastelein, R. A., and Bazan, J. F. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 588–593
3. Takeuchi, O., Kawai, T., Sanjo, H., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Takeda, K., and Akira, S. (1999) Gene (Annu.) 231, 59–65
4. Chuang, T. H., and Ulevitch, R. J. (2000) Eur. Cytokine Netw. 11, 372–378
5. Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sat0, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000) Nature 408, 740–745
6. Zhang, D., Zhang, G., Hayden, M. S., Greenblatt, M. B., Bussey, C., Flavell, R. A., and Ghosh, S. (2004) Science 303, 1522–1526
7. Akira, S., Takeda, K., and Kaisho, T. (2001) Nat. Immunol. 2, 675–680
8. Li, S., Strelow, A., Fontana, E. J., and Wesche, H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5567–5572
9. Suzuki, N., Suzuki, S., Duncan, G. S., Millar, D. G., Wada, T., Mirsots, C., Akita, H., Wakeham, A., Itie, A., Li, S., Penninger, J. M., Wesche, H., Ohashi, P. S., Mak, T. W., and Yeh, W. C. (2002) Nature 416, 750–756
10. Picard, C., Puel, A., Bonnet, M., Ku, C. L., Bustamante, J., Yang, K., Soudais, C., Dupuis, S., Feinberg, J., Fieschi, C., Elbim, C., Hitchcock, R., Lammas, D., Davies, G., Al Ghonaiaum, A., Al Rays, H., Al Jumaah, S., Al Hajjar, S., Al Mohsen, I. Z., Frayha, H. H., Rucker, R., Hawn, T. R., Adrem, A., Tufkenkji, H., Haraguchi, S., Day, N. K., Good, R. A., Gougerot-Pocidalo, M. A., Ozinsky, A., and Casanova, J. L. (2003) Science 299, 2076–2079
11. Cao, Z., Hezel, W. J., and Gao, X. (1996) Science 271, 1128–1131
12. Li, X., Commene, M., Burns, C., Vithalani, K., Cao, Z., and Stark, G. R. (1999) Mol. Cell Biol. 19, 4643–4652
13. Cao, Z., Xiong, J., Takeuchi, M., Kuruma, T., and Goeddel, D. V. (1996) Nature 383, 443–446
14. Jiang, Z., Ninomiya-Tsuji, J., Qian, Y., Matsumoto, K., and Li, X. (2002) Mol. Cell Biol. 22, 7158–7167
15. Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999) Nature 398, 252–256
16. Yao, J., Kim, T. W., Qin, J., Jiang, Z., Qian, Y., Xiao, H., Lu, Y., Qian, W., Gilen, M. F., Sizemore, N., DiDonato, J., Sato, S., Akira, S., Su, B., and Li, X. (2007) J. Biol. Chem. 282, 6675–6689
17. Kawagoe, T., Sato, S., Jung, A., Yamamoto, M., Matsu, K., Kato, H., Uematsu, S., Takeuchi, O., and Akira, S. (2007) J. Exp. Med. 204, 1013–1024
18. Kozyczak-Holbro, M., Gluck, A., Tschopp, C., Mathison, J. C., and Gram, H. (2008) Eur. J. Immunol. 38, 788–796
19. Kozyczak-Holbro, M., Joyce, C., Gluck, A., Kinzel, B., Muller, M., Tschopp, C., Mathison, J. C., Davis, C. N., and Gram, H. (2007) J. Biol. Chem. 282, 13552–13560
20. Kim, T. W., Stachke, K., Bulek, K., Yao, J., Peters, K., Oh, K. H., Vandenburg, Y., Xiao, H., Qian, W., Hamilton, T., Min, B., Sen, G., Gilmour, R., and Li, X. (2007) J. Exp. Med. 204, 1025–1036
21. Li, X. (2008) Eur. J. Immunol. 38, 614–618
22. Qin, J., Jiang, Z., Qian, Y., Casanova, J. L., and Li, X. (2004) J. Biol. Chem. 279, 26748–26753
23. Lye, E., Mirsots, C., Suzuki, N., Suzuki, S., and Yeh, W. C. (2004) J. Biol. Chem. 279, 40653–40658
24. Majumder, S., Zhou, L. Z., Chaturvedi, P., Babcock, G., Aras, S., and Ransohoff, R. M. (1998) J. Neurosci. Res. 54, 169–180
25. Kessler, D. S., Veals, S. A., Fu, X. Y., and Levy, D. E. (1990) Genes Dev. 4, 1753–1765
26. Hoebe, K., Du, X., George, P., Janssen, E., Tabeta, K., Kim, S. O., Goode, J., Lin, P., Mann, N., Mudd, S., Crozat, K., Sovath, S., Han, J., and Beutler, B. (2003) Nature 414, 743–748
27. Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., and Akira, S. (2003) Science 301, 640–643
28. Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K., and Akira, S. (2003) Nat. Immunol. 4, 1144–1150
29. Lasker, M. V., Gajjar, M. M., and Nair, S. K. (2005) J. Immunol. 175, 4175–4179