The innate immune system senses pathogen-specific molecular patterns via pattern recognition receptors, such as Toll-like receptors (TLRs; references 1–3). 12 TLR family members have been identified in mammals, and the pathogen-specific molecular patterns recognized by these TLRs have been mostly identified. The cytoplasmic portion of TLRs, called TIR (Toll/IL-1R [IL-1R]) domain, resembles that of IL-1R family members, and these two receptor families in part share intracellular signaling machineries. Stimulation with TLR ligands or IL-1 family cytokines recruits a TIR domain–containing adaptor, MyD88, to the receptors. IL-1R–associated kinases (IRAKs) are recruited to MyD88 through a homophilic interaction of the death domains and associate with TNF receptor–associated factor 6 (TRAF6), which acts as an ubiquitin protein ligase. TRAF6 catalyzes the formation of a K63-linked polyubiquitin chain on TRAF6 itself and on IκB kinase γ (IKK-γ)/NF-κB essential modulator. TGF-β–activated kinase 1 is also recruited to TRAF6 and then phosphorylates IKK-β and mitogen-activated protein (MAP) kinase kinase 6. Phosphorylation of IκB by the IKK complex leads to its degradation, and freed NF-κB translocates into the nucleus, resulting in induction of genes involved in inflammatory responses as well as increase in the surface expression of costimulatory molecules on innate immune cells. The activation of MAP kinase cascade is responsible for AP-1–induced gene expression. In addition to the MyD88–dependent signaling pathway, the TLR4 signaling also activates a MyD88–independent signaling cascade via another TIR domain–containing adaptor protein inducing IFN-β, TRIF (4). It triggers the signaling cascade leading to the production of type I IFNs.

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Interleukin-1 receptor–associated kinase 4 (IRAK-4) was reported to be essential for the Toll–like receptor (TLR)– and T cell receptor (TCR)–mediated signaling leading to the activation of nuclear factor κB (NF-κB). However, the importance of kinase activity of IRAK family members is unclear. In this study, we investigated the functional role of IRAK-4 activity in vivo by generating mice carrying a knockin mutation (KK213AA) that abrogates its kinase activity. IRAK-4−/− mice were highly resistant to TLR-induced shock response. The cytokine production in response to TLR ligands was severely impaired in IRAK-4−/− as well as IRAK-4−/− macrophages. The IRAK-4 activity was essential for the activation of signaling pathways leading to mitogen–activated protein kinases. TLR–induced IRAK-4/IRAK-1–dependent and –independent pathways were involved in early induction of NF-κB–regulated genes in response to TLR ligands such as tumor necrosis factor α and IκBα. In contrast to a previous paper (Suzuki, N., S. Suzuki, D.G. Millar, M. Unno, H. Hará, T. Calzascia, S. Yamasaki, T. Yokosuka, N.J. Chen, A.R. Elford, et al. 2006. Science. 311:1927–1932), the TCR signaling was not impaired in IRAK-4−/− and IRAK-4−/− mice. Thus, the kinase activity of IRAK-4 is essential for the regulation of TLR–mediated innate immune responses.
via IKK-related kinases, TANK-binding kinase 1 (TBK1) and IKK-i. The TLR3 signaling also entirely relies on TRIF to activate NF-κB and IFN-regulatory factors.

The IRAK family is comprised of four members and is characterized by the presence of an N-terminal death domain and a serine/threonine kinase domain (5). IRAK-1 was initially identified as a kinase that is coprecipitated with IL-1R in response to IL-1 stimulation (6). IRAK-1 associates with MyD88 through a homophilic interaction of the death domains (7). Whereas IRAK-1 has a nonredundant role in the production of type I IFNs in response to TLR9 ligands in plasmacytoid DCs (pDCs; reference 8), IRAK1-deficient (IRAK-1−/−) macrophages show modest impairment in IL-1R– and TLR-mediated proinflammatory cytokine production (9, 10). IRAK-2 is suggested to be involved in the signaling via TIRAP/Mal, an adaptor protein responsible for TLR2 and TLR4 responses (11). In contrast, IRAK-M was identified as the negative regulator of the TLR/IL-1R signaling (12). The fourth member of IRAK family members, IRAK-4, has been discovered by a database search (13). Generation of IRAK-4−/− mice revealed its essential role in IL-1R/TLR–mediated responses (14, 15). Furthermore, the poor defenses against bacterial infection were observed in patients having autosomal recessive amorphic mutations in IRAK-4 (16, 17). It was suggested that IRAK-4 can directly phosphorylate IRAK-1 for the signaling. Recently, IRAK-4 has been reported to be a requisite for TCR-induced NF-κB activation by associating with ZAP-70 (18).

Although IRAK family members are involved in TLR/IL-1R signaling, the role of their kinase activity is still controversial. Among IRAK family members, IRAK-1 and -4 were shown to possess intrinsic kinase activity (13, 19). Nevertheless, it has been shown that IRAK-1 kinase activity is dispensable for its ability to activate NF-κB (20). IRAK-1 could act as a scaffold protein recruiting MyD88 and TRAF6 for the signaling (13). Second, a critical aspartate residue in the catalytic domain has changed to an asparagine or a serine in IRAK-2 or -M, and their kinase domains have been shown to be inactive (21). Regarding the requirement of IRAK-4 activity for the IL-1R signaling, two controversial observations have been reported to date (22, 23). One paper showed that the reconstitution with the kinase-inactive mutant IRAK-4 fully restored IL-1 responsiveness (22), whereas the other showed that the same reconstitution was capable of restoring only a partial cytokine response to IL-1β (23). Therefore, the requirement of kinase activity in IRAK family members has not been well understood.

In the present study, we generated mice carrying a knockin mutation that abrogated IRAK-4 activity. For the assessment of the roles of kinase activity, we also generated IRAK-4−/− mice. The analysis of these mice revealed that the kinase activity of IRAK-4 is essential for the physiological function of IRAK-4, and the TLR-mediated proinflammatory responses are severely impaired in these mice. Nevertheless, we did not observe any defects in the T cell responses in either IRAK-4−/− or IRAK-4Kn/Kn mice. This study demonstrates
that IRAK-4 functions as an actual kinase for relaying the TLR signaling.

RESULTS

Generation of IRAK-4<sup>KN/KN</sup> and IRAK-4<sup>−/−</sup> mice

It has been shown that a mutation in ATP binding pocket (K239S) of IRAK-1 abrogated its kinase activity. Nevertheless, overexpression of this mutant IRAK-1 still efficiently induced NF-κB activation. Corresponding mutations in IRAK-4 (KK213AA) was capable of inducing activation of NF-κB in response to IL-1β stimulation. These results suggested that IRAK family members function as adaptor molecules for the signaling, and the IRAK kinase activity was dispensable for their function. To identify the role of IRAK-4 activity in TLR signaling, we inserted a mutation (KK213AA) of IRAK-4. To replace serines 213 and 214 of IRAK-4 with alanines, a loxP-flanked Neo cassette was inserted. Serine to alanine substitutions were introduced by site-directed mutagenesis (Fig. 1 A). A targeting vector containing these mutations were electroporated into embryonic stem (ES) cells, clones with homologous recombination at the IRAK-4 locus were obtained, and IRAK-4–mutated mice were generated. The mice were crossed with CAG-Cre transgenic mice to excise the neo resistant gene. Homologous recombination of IRAK-4 locus was confirmed by Southern blotting, and the sequencing analysis revealed that the mutations were correctly introduced (Fig. 1, B and E). The Northern blot and immunoblot analysis showed that IRAK-4 messenger RNA (mRNA) and protein were expressed in wild-type and IRAK-4<sup>KN/KN</sup> macrophages, although the expression of IRAK-4 protein was slightly reduced in IRAK-4<sup>KN/KN</sup> macrophages (Fig. 1, C and D).

We also generated IRAK-4<sup>−/−</sup> mice by homologous recombination to compare the importance of kinase activity of IRAK-4 (Fig. S1, A and B, available at http://www.jem.org/cgi/content/full/jem.20061523/DC1). For generation of IRAK-4<sup>−/−</sup> mice, we targeted exon 2 of mouse IRAK-4 gene with the neo cassette in ES cells and established IRAK-4<sup>−/−</sup> mice. Absence of IRAK-4 protein in IRAK-4<sup>−/−</sup> macrophages was confirmed by Northern blotting and immunoblotting (Fig. 1, C and D).

In vitro kinase assay revealed that IRAK-4 autophosphorylation was induced in wild-type macrophages in response to TLR2 stimulation (Fig. 2 A). In contrast, the autophosphorylation of IRAK-4 was not observed either in IRAK-4<sup>KN/KN</sup> or IRAK-4<sup>−/−</sup> cells. To further investigate whether the mutant IRAK-4 can phosphorylate IRAK-1, we isolated IRAK-4 cDNA from wild-type and IRAK-4<sup>KN/KN</sup> cells. The IRAK-4 proteins were expressed using the rabbit reticulocyte lysate system, and in vitro kinase assay was performed with mouse IRAK-1 protein (aa 301−500), which contains an activation loop, as a substrate. As shown in Fig. 2 B, the wild-type IRAK-4, but not IRAK-4 (KK213AA), phosphorylated IRAK-1. Immunoblot analysis revealed that the amounts of wild-type and IRAK-4 (KK213AA) expressed were not altered (Fig. 2 B).

We next examined whether IRAK-4 activity was required for the recruitment of IRAK-4 in the complex of...
MyD88 and IRAK-1 in response to IL-1R stimulation. When mouse embryonic fibroblasts (MEFs) were stimulated with IL-1β, IRAK-4 was coprecipitated with MyD88 in both wild-type and IRAK-4KN/KN cells (Fig. 2 C). In contrast, interaction between IRAK-4 and -1 was not induced in IRAK-4KN/KN macrophages (Fig. 2 D). These results indicate that IRAK-4 activity is dispensable for the recruitment of IRAK-4 to MyD88, although the kinase activity is essential for the recruitment of IRAK-1 to -4.

The role of IRAK-4 activity in TLR-induced responses

We first examined the role of IRAK-4 activity in response to TLR ligand stimulation in vivo. After challenge with LPS or CpG-DNA together with d-galactosamine, wild-type mice succumbed to shock and died, whereas all IRAK-4−/− and IRAK-4KN/KN mice survived, indicating that the kinase activity of IRAK-4 is critical for the TLR-induced shock in vivo (Fig. 3, A and B). We examined cytokine production of macrophages possessing mutated IRAK-4 against TLR ligands, including macrophage-activating lipopeptide-2 (MALP-2; TLR6/TLR2), poly I:C (TLR3), LPS (TLR4), R-848 (TLR7), and CpG-DNA (TLR9). Thioglycollate-elicited peritoneal macrophages were stimulated with each TLR ligand and the production of proinflammatory cytokines was measured by ELISA. In accordance with a previous paper, production of IL-6, TNF-α, and IL-12 p40 in response to these TLR ligands except poly I:C was severely impaired in IRAK-4−/− macrophages compared with wild-type cells (14). The production of IL-6, TNF-α, and IL-12 p40 was also profoundly impaired in IRAK-4KN/KN cells, and the extent of reduction was similar to that of IRAK-4−/− cells (Fig. 3, C–E). In contrast, IL-6 and TNF-α production in response to poly I:C was not altered between wild-type, IRAK-4−/−, and IRAK-4KN/KN macrophages. DCs from IRAK-4KN/KN mice also showed defective cytokine production in response to these TLR ligands (unpublished data). Thus, the IRAK-4 activity is important for evoking cytokine production in response to various TLR ligands, except for TLR3 ligand.

Not only proinflammatory cytokines but also type I IFNs are strongly induced in response to TLR7 and TLR9 stimulation in pDCs (8, 17, 24). It has been shown that IRAK-4 and -1 are essential for TLR-9-induced type I IFN production in pDCs. To examine the role of IRAK-4 activity in type I IFN response, we generated pDCs by cultivating bone-marrow cells in the presence of Flt3 ligand. Whereas wild-type Flt3L-DCs produced IFN-α and IL-6 in response to A/D-type LPS, IRAK-4−/− and IRAK-4KN/KN Flt3L-DCs produced IFN-α and IL-6 in response to A/D-type CpG-DNA. Concentrations of IL-6 (C), TNF-α (D), and IL-12 p40 (E) in the culture supernatants were measured by ELISA. Data are shown as mean ± SD of triplicates. Similar results were obtained in three independent experiments.

Figure 3. Essential role of IRAK-4 activity in TLR-mediated cytokine responses. (A and B) Age-matched wild-type (n = 10), IRAK-4−/− (n = 5), and IRAK-4KN/KN (n = 5) mice were challenged with 2 mg LPS (A) and 20 nmol CpG-DNA together with 20 mg d-galactosamine (B). The survival of mice was monitored for 5 d. (C–E) Thioglycollate-elicited peritoneal macrophages from wild-type, IRAK-4−/−, and IRAK-4KN/KN mice were stimulated with MALP-2, poly I:C, LPS, R-848, and CpG-DNA for 24 h. For measuring IL-12 p40 concentration, macrophages were stimulated with the indicated TLR ligands in the presence of 30 ng/ml IFN-γ.
CpG-DNA, cells from neither IRAK-4<sup>−/−</sup> nor IRAK-4<sup>KN/KN</sup> mice produced both IFN-α and IL-6 (Fig. 3, F and G).

Next we investigated the proliferation of B cells in response to these TLR ligands. When we stimulated purified B cells with MALP-2, poly I:C, LPS, R-848, and CpG-DNA, wild-type B cells proliferated in a dose-dependent manner (Fig. 4). In contrast, IRAK-4<sup>−/−</sup> as well as IRAK-4<sup>KN/KN</sup> B cells failed to proliferate in response to MALP-2, LPS, R-848, and CpG-DNA. Stimulation with poly I:C induced proliferation of B cells even in IRAK-4<sup>−/−</sup> and IRAK-4<sup>KN/KN</sup> mice, suggesting that TLR3 signals independent of IRAK-4 in B cells. The proliferative responses against anti-IgM, anti-CD40 antibodies (Abs) were not altered in wild-type, IRAK-4<sup>−/−</sup>, and IRAK-4<sup>KN/KN</sup> mice, indicating that the MyD88-dependent responses were specifically impaired in IRAK-4<sup>−/−</sup> or IRAK-4<sup>KN/KN</sup> cells. Collectively, the kinase activity of IRAK-4 is essential for the pleiotrophic effects in response to TLR stimulation.

Expression of TLR-mediated gene expression in IRAK-4 mutated mice

We examined whether the defects in cytokine response to TLR stimulation in IRAK-4<sup>−/−</sup> mutation were regulated in a gene expression level by Northern blot analysis. We chose TLR2 ligands as the stimulus because TLR2 signals only via the MyD88–dependent pathway. In response to MALP-2 stimulation, wild-type macrophages induced expression of IL-6, TNF-α, IkBζ, and cyclooxygenase-2 (COX-2) genes (Fig. 5 A). In contrast, IRAK-4<sup>−/−</sup> and IRAK-4<sup>KN/KN</sup> macrophages failed to express IL-6 and COX-2 in response to MALP-2 stimulation. However, TNF-α and IkBζ were expressed even in the absence of IRAK-4, albeit the expression was weaker than wild-type cells. Thus, the kinase activity of IRAK-4 is critical for regulating IRAK-4–mediated controlling of gene expression. Indeed, TNF bioassay revealed that a subtle amount of TNF activity was induced 1 and 2 h after MALP-2 stimulation in IRAK-4<sup>−/−</sup> and IRAK-4<sup>KN/KN</sup> macrophages, although the amount was much smaller than in wild-type cells (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20061523/DC1). Interestingly, MyD88<sup>−/−</sup> macrophages failed to induce any detectable amount of these genes in response to MALP-2 stimulation (Fig. 5 A). In addition, PAM<sub>3</sub>CSK<sub>4</sub>, a synthetic lipopeptide known to be recognized by TLR1/TLR2 heterodimer, also induces expression of TNF-α and IkBζ even in the absence of IRAK-4 (Fig. 5 B; reference 25). These results indicate that the early expression of TNF-α and IkBζ genes in response to TLR2 ligands is regulated in part in a IRAK-4–independent fashion, although IRAK-4 plays a major role in the expression of TLR2-inducible genes.

Figure 4. IRAK-4 activity is critical for TLR-mediated proliferation of splenocytes. Splenocytes were cultured with the indicated concentrations of MALP-2, poly I:C, LPS, R-848, CpG-DNA, anti-IgM, or anti-CD40 for 48 h. Samples were pulsed with 1 μCi [3H]thymidine for the last 16 h. [3H]thymidine incorporation was measured by a scintillation counter. Data are representative of three independent experiments. Indicated values are mean ± SD of triplicates.

Figure 5. IRAK-4–independent induction of gene expression in TLR signaling. (A and B) Peritoneal macrophages were stimulated with 10 ng/ml MALP-2 (A) and 10 ng/ml PAM<sub>3</sub>CSK<sub>4</sub> (B) for the indicated periods. Total RNA was extracted and subjected to Northern blot analysis for expression of IL-6, TNF-α, IkBζ, and COX-2. The same membrane was rehybridized with a β-actin probe.
The role of IRAK-4 activity in the TLR-mediated signaling pathway

These observations prompted us to examine intracellular signaling pathways in response to TLR stimulation. It has been shown that IRAK-4 is recruited to IL-1R in response to ligand stimulation, where it phosphorylates and activates IRAK-1. It was also shown that IRAK-4 is essential for the initiation of IL-1R-mediated signaling pathways leading to the activation of NF-κB and MAP kinases in MEFs (14).

TLR2-mediated autophosphorylation of IRAK-1 was completely abrogated in IRAK-4−/− and IRAK-4 KN/KN macrophages (Fig. 6 A). TLR/IL-1R stimulation induces not only phosphorylation but also degradation of IRAK-1. MALP-2 stimulation decreased the IRAK-1 expression in wild-type macrophages (Fig. 6 B). However, TLR2-mediated degradation of IRAK-1 was not observed in IRAK-4−/− and IRAK-4 KN/KN macrophages. Thus, IRAK-4 activity is essential for IRAK-1 activation in response to TLR2 activation.

We examined the role of IRAK-4 activity in the activation of MAP kinases and NF-κB in macrophages. First, activation of c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) induced by MALP-2 was profoundly impaired in IRAK-4−/− as well as IRAK-4 KN/KN macrophages (Fig. 6, C–E). These results indicate that IRAK-4 activity is critical for the activation of MAP kinases in the TLR signaling.

We analyzed activation of NF-κB. Phosphorylation and degradation of IκBα were also severely impaired in IRAK-4−/− and IRAK-4 KN/KN macrophages (Fig. 7, A and B). MALP-2-induced phosphorylation of NF-κB p65 was not observed in IRAK-4−/− and IRAK-4 KN/KN macrophages (Fig. 7 C). Nevertheless, an electrophoretic mobility shift assay (EMSA) revealed that the NF-κB–DNA binding activity was clearly induced even in the absence of IRAK-4, although the activation was ~10 min delayed compared with wild-type cells (Fig. 7 D). Consistent with our previous study, MyD88−/− macrophages failed to induce NF-κB–DNA binding activity in response to MALP-2 (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20061523/DC1; reference 26). These results indicate that TLR2 activates a MyD88-dependent and IRAK-4–independent signaling pathway leading to the activation of NF-κB. To assess the subunits of the NF-κB complexes observed in response to MALP-2 stimulation, we performed supershift assays using anti-p65 or anti-p50 Ab and nuclear extracts from macrophages stimulated with MALP-2 for 40 min (Fig. 7 E). In wild-type and IRAK-4–mutated cells, the bands were supershifted with anti-p65 and anti-p50 Ab, suggesting that the NF-κB complex is mainly composed of p65/p50 heterodimers in both wild-type and IRAK-4–mutated cells. These findings indicate that TLR2 activates a MyD88-dependent and IRAK-4–independent signaling pathway leading to the

Figure 6. IRAK-4 is critical for TLR-mediated activation of IRAK-1 and MAP kinases. (A) In vitro kinase assay for IRAK-1 activation. Peritoneal macrophages were stimulated with 10 ng/ml MALP-2 for the indicated periods. The cell lysates were prepared and immunoprecipitated with anti-IRAK-1 Ab, and the kinase activity of IRAK-1 was measured by in vitro kinase assay. The data shown are representative of three independent experiments. Auto, autophosphorylation. (B) Immunoblot analysis for the change in IRAK-1 expression in response to MALP-2 stimulation. Peritoneal macrophages from wild-type, IRAK-4−/−, and IRAK-4 KN/KN mice were stimulated with MALP-2 for the indicated periods. Whole cell lysates were subject to immunoblot analysis using anti-IRAK-1. ERK1/2 levels are shown as loading control. (C–E) Macrophages from wild-type, IRAK-4−/−, and IRAK-4 KN/KN mice were stimulated with MALP-2 for 40 min (min) and whole cell lysates were subject to immunoblot analysis using anti-phospho-JNK (C), anti-phospho-p38 (D), and anti-phospho-ERK (E). The blots of JNK, p38, and ERK are shown as loading controls. Similar results were obtained in three independent experiments.
activation of NF-κB. Stimulation with R-848 and CpG-DNA also induced NF-κB activation in an IRAK-4–independent manner without degrading IκBα (Fig. S4). When IRAK-4–mutated cells were stimulated with LPS, degradation of IκBα as well as NF-κB–DNA binding was delayed as observed in MyD88−/− macrophages (Fig. S4). These data indicate that the IRAK-4–independent pathway is activated downstream of various TLRs.

**Figure 7. IRAK-4–independent activation of NF-κB in TLR2 signaling.** (A–C) Macrophages from wild-type, IRAK-4−/−, and IRAK-4KN/KN mice were stimulated with MALP-2 for the indicated periods. Whole cell lysates were subject to immunoblot analysis using anti-IκBα (A), anti–phospho-IκBα (B), and anti–phospho-NF-κB p65 Abs (C). The blots of ERK1/2 and NF-κB p65 are shown as loading controls. The data are representative of three independent experiments. (D) Wild-type, IRAK-4−/−, and IRAK-4KN/KN macrophages were stimulated with 10 ng/ml MALP-2 for the indicated periods. Nuclear extracts were then prepared, and NF-κB–DNA binding activity was determined by EMSA using an NF-κB–specific probe. (E) Nuclear extracts from macrophages stimulated with MALP-2 for 40 min were incubated with Abs specific to NF-κB p65 and p50 before addition of NF-κB probe. The single and double asterisks indicate the supershifts induced by Ab to p65 and p50, respectively. (F) IRAK-1/IRAK-4–independent activation of NF-κB in response to TLR stimulation. Wild-type, IRAK-4−/−, IRAK-1−/−, and IRAK-4−/− IRAK-1−/− doubly deficient macrophages were stimulated with 10 ng/ml MALP-2 for the indicated periods. NF-κB–DNA binding activity was determined by EMSA.

As shown in Fig. 7 F, the activation of NF-κB–DNA binding activity was induced even in the absence of both IRAK-1 and −4. Furthermore, TLR2–induced TNF-α gene induction was still observed in IRAK-1−/−IRAK-4−/− macrophages (unpublished data). Thus, IRAK-1– and IRAK-4–independent mechanisms are responsible for the signaling pathway leading to the activation of NF-κB.

**Normal TCR responses in IRAK-4−/− and IRAK-4KN/KN T cells**

A recent study has shown that deficiency in IRAK-4– 4 results in the impaired responses to TCR stimulation (18). IRAK-4 interacts with ZAP-70 in the cells and regulates TCR–mediated activation of NF-κB. We then analyzed responses of IRAK-4−/− and IRAK-4KN/KN mice to TCR stimulation. Surprisingly, proliferation of purified T cells in response to either immobilized or soluble anti-CD3 was not impaired in either IRAK-4−/− or IRAK-4KN/KN mice compared with wild-type mice.
In addition, production of IL-2 in response to TCR stimulation was not impaired in T cells from these mice (Fig. 8 B). Furthermore, wild-type, IRAK-4−/−, and IRAK-4<sup>KN/KN</sup> T cells have equivalent ability to proliferate in response to allogenic DCs either untreated or treated with various TLR ligands, including MALP-2, LPS, and CpG-DNA (Fig. 8 C). Moreover, TCR-mediated activation of NF-κB as well as MAP kinases was also not altered between wild-type, IRAK-4−/−, and IRAK-4<sup>KN/KN</sup> T cells (Fig. 8, D and E). We investigated whether IRAK-4 was involved in adaptive T cell responses in vivo. Wild-type and IRAK-4−/− mice were infected with lymphocytic choriomeningitis virus (LCMV). Splenocytes were prepared 8 d after infection, and induction of LCMV-specific CD8<sup>+</sup> T cells was analyzed by tetramer staining. As shown in Fig. 9 A, LCMV-specific CD8<sup>+</sup> T cells were induced both in wild-type and IRAK-4−/− mice in a similar manner after infection. Similarly, wild-type and IRAK-4−/− mice induced comparable ex vivo CTL responses as determined in a 51Cr release assay (Fig. 9 B). These results indicate that IRAK-4 is not involved in the TCR signaling leading to the activation of NF-κB as well as T cell responses in vivo.

**DISCUSSION**

In the present study, we analyzed the role of IRAK-4 activity in vivo by generating mice with knockin mutation KK213AA and with null mutation. In agreement with previous papers, IRAK-4−/− macrophages showed severe defects in TLR-mediated cytokine responses (14, 15). Although the expression of IRAK-4 protein was slightly lower than wild-type cells, IRAK-4<sup>KN/KN</sup> macrophages also showed profound defects in the responses to various TLR ligands to the same extent as IRAK-4−/− cells. These results clearly indicate that the kinase activity of IRAK-4 is essential for the function of IRAK-4 in vivo. Previous in vitro studies implicated that the IRAK family members could activate NF-κB and inflammatory responses even in the absence of their kinase activity (19, 20). In the case of IRAK-4, one group has shown that the mutant IRAK-4 (KK213AA) restored IL-1β responsiveness (22), and the other group reported that the same mutation could restore the response only partially (23). It has been shown that expression of kinase-inactive IRAK-1 could also restore IL-1β-induced NF-κB activation. It may be possible that overexpressed IRAK-4 behaved differently compared with the physiological expression. In the physiological level of expression, the kinase activity of IRAK-4 is critical for its function. So far IRAK-4 substrates responsible for the signaling
have not been well understood. Although it was shown that IRAK-4 phosphorylated IRAK-1 for activating TRAF6, TLR-mediated production of proinflammatory cytokines in IRAK-1−/− cells was not impaired in peritoneal macrophages. Further studies are required for identifying substrates other than IRAK-1 that are responsible for the TLR signaling pathway. Nevertheless, this is the first paper showing that the kinase activity of IRAK family members plays a critical role in their function in vivo.

The association between MyD88 and IRAK-4 was induced in response to IL-1β stimulation in both wild-type and IRAK-4−/− cells. A previous study showed that MyD88 interacted with kinase-negative, but not with wild-type, IRAK-4 when they were overexpressed in human embryonic kidney 293 cells (13). In contrast, it was reported that IL-1 stimulation induced an interaction between endogenous MyD88 and wild-type IRAK-4 (16). In that study, the kinase-truncated mutant of IRAK-4 was shown to constitutively interact with MyD88 even before IL-1 stimulation. Given that overexpression of wild-type IRAK-4 immediately activates NF-κB without further stimulation, the localization of overexpressed IRAK-4 may be different from endogenous protein. Based on our observation and that study (16), the endogenous IRAK-4 is probably recruited to MyD88 in response to stimulation, and IRAK-4 with KK213AA point mutation behaves similarly to wild-type IRAK-4 regarding association with MyD88.

Although IRAK-4 deficiency profoundly affected TLR2-mediated cytokine production, TNF-α gene induction was impaired, but not abrogated, as observed in MyD88 deficiency. TLR2-mediated expression of TNF-α and IkBα genes was induced even in the absence of IRAK-4, though the expression in IRAK-4−/− and IRAK-4−/−/− cells was reduced and transient compared with wild-type cells. Furthermore, induction of NF-κB–DNA binding activity was also induced in IRAK-4−/− and IRAK-4−/−/− macrophages, although the activation was ~10 min delayed compared with wild-type cells. This finding is in contrast to the complete abrogation of TLR2 signaling in MyD88−/− macrophages and indicates the existence of an IRAK-4–independent signaling pathway. Stimulation with R-848 and CpG-DNA also induced NF-κB activation in an IRAK-4–independent manner without degrading IκBα, suggesting that the IRAK-4–independent pathway is not TLR2 specific. Given that the death domain of MyD88 is responsible for downstream signaling, other IRAK family members that contain an N-terminal death domain are candidates for mediating IRAK-4–independent signaling. Nevertheless, the activation of NF-κB in response to a TLR2 ligand was observed even in the absence of both IRAK-1 and −4. Because it was shown that IRAK-2 also positively regulated the IL-1β–signaling pathway, IRAK-2 may be responsible for the signaling pathway. Future studies will clarify if IRAK family members redundantly function in IL–1R/TLR responses in vivo. Although we clearly detected NF-κB–DNA binding activity, which is supershifted by anti-p50 and p65 Ab, we failed to detect activation of IκKs and phosphorylation of IκBα in the absence of IRAK-4 or its kinase activity in response to TLR2 stimulation. Induction of NF-κB activation without degradation of IκBα is quite unique, although the mechanism of activation is enigmatic. It has been reported that TLR2 stimulation leads to the recruitment of active Rac1 and phosphatidylinositol-3 kinase to the TLR2 cytosolic domain (27). Therefore, it is possible that the signaling is mediated through the small G protein–phosphatidylinositol-3 kinase pathway.

The TLR/IL-1R and antigen-receptor signaling share signaling molecules for activating NF-κB. In addition to IKK complex, TRAF6 was also reported to be involved in TCR-mediated NF-κB activation (28). TRAF6 can associate with MALT1, which forms a complex with BCL10 and CARMA1/CARD11. TRAF6 is oligomerized by the complex and activates IKKs by inducing polyubiquitination of IKK-γ/NF-κB essential modulator and activation of TGF-β–activated kinase 1. A recent paper showed that IRAK-4 was also involved in TCR responses via suppressing NF-κB activation by associating with ZAP-70 (18). However, the newly generated IRAK-4−/− mice did not show any defects in the T cell response as well as the TCR signaling pathway. Furthermore, IRAK-4 was not required for LCMV-induced CTL responses. IRAK-4−/− T cells also showed normal responses against TCR stimulation. We do not have a clear explanation for the discrepancy, and it may be due to the difference in the genetic background of the strains. However, it is unlikely that the critical TCR signaling components are different between mouse strains, suggesting that IRAK-4 is not critically involved in TCR signaling.

In summary, this study demonstrates that IRAK-4 activity plays a critical role in the physiological function of IRAK-4. Macrophages and DCs from IRAK-4−/−/− mice as well as IRAK-4−/− mice were profoundly defective in TLR-mediated...
proinflammatory cytokine production. In addition, IRAK-4\(^{-/}\) mice were highly resistant to LPS-induced shock response. The exploration of small compounds targeting kinase activity of IRAKs has been challenged by the fact that expression of even kinase-inactive IRAK-4 mutant results in the activation of the intracellular signaling pathway. However, this study clearly indicates that the kinase activity of IRAK-4 is essential for the physiological functions, and the kinase activity of IRAK-4 is a good therapeutic target for inflammatory diseases and septic shock, without affecting acquired immune responses.

**MATERIALS AND METHODS**

**Generation of IRAK-4\(^{-/}\) mice.** The IRAK-4 gene was isolated from genomic DNA extracted from ES cells (GSI) by PCR. A genomic fragment containing exon 2 of IRAK-4 was cloned into a pT7Blue vector (Nugen), and point mutations resulting in the KK213AA conversion in the kinase domain were introduced by a site-directed mutagenesis. A targeting vector has a neomycin-resistance gene cassette (neo) flanked with two loxp sites, and a HSV thymidine kinase driven by PGK promoter was inserted into the genomic fragment for negative selection. The targeting vector was linearized and electroporated into ES cells (GSI). G418 and gancyclovir doubly resistant clones were selected and screened by PCR and further confirmed by Southern blotting. Three clones with homologous recombination were injected into blastocysts from C57BL/6 female mice, and the obtained chimeric males were crossed with C57BL/6 females, and the obtained F1 generations with mutated IRAK-4 mice were crossed with CAG-Cre transgenic mice to excise the neo cassette. CAG-Cre transgenic was removed from IRAK-4\(^{-/}\) mice without a neo cassette by crossing the mice with C57BL/6 mice. IRAK-4\(^{-/}\) mice were further intercrossed to obtain IRAK-4\(^{-/-}\) mice. The IRAK-4\(^{-/}\) mice used were under 129Sv × C57BL/6 background. Mice were maintained in our animal facility and treated in accordance with the guidelines of Osaka University.

**Generation of IRAK-4\(^{-/-}\) mice.** The IRAK-4 gene was isolated from genomic DNA extracted from ES cells (GSI) by PCR. The targeting vector was constructed by replacing a 4.3-kb fragment encoding the IRAK-4 ORF with a neo cassette, and a HSV thymidine kinase driven by PGK promoter was inserted into the genomic fragment for negative selection. After the targeting vector was transfected into ES cells, G418 and gancyclovir doubly resistant colonies were selected and screened by PCR and further confirmed by Southern blotting. Homologous recombinants were microinjected into blastocysts from C57BL/6 female mice, and heterozygous F1 progenies were intercrossed to obtain IRAK-4\(^{-/-}\) mice. The IRAK-4\(^{-/-}\) mice used were under 129Sv × C57BL/6 background.

**Cells.** Peritoneal exudate cells were isolated from the peritoneal cavity of mice 3 d after injection with 2 ml of 4.0% thioglycollate medium (Sigma-Aldrich) by washing with ice cold Hank’s balanced salt solution (Invitrogen). Bone-marrow DCs were prepared by culturing either in the presence of 100 ng/ml human Flt3 ligand (PeproTech) or 10 ng/ml mouse GM-CSF (PeproTech) as described previously (29). Splenic T cells were isolated using 10 ng/ml human Flt3 ligand (PeproTech) or 10 ng/ml mouse GM-CSF (PeproTech) and electroporated into ES cells (GSI). G418 and gancyclovir doubly resistant colonies were selected and screened by PCR and further confirmed by DNA sequencing. A genomic fragment extracted from ES cells (GSI) by PCR. The targeting vector has a neomycin-resistance gene cassette (neo) flanked with two loxP sites, and a HSV thymidine kinase driven by PGK promoter was inserted into the genomic fragment by recombination. CAG-Cre transgene was removed from the mice with C57BL/6 mice. The mice were then further incubated with a specific probe for NF-κB DNA binding sites, and the same membrane was rehybridized with a β-actin probe.

**Northern blot analysis.** Peritoneal macrophages were treated with 10 ng/ml MALP-2 for 0, 1, 2, 4, and 8 h, and total RNA was extracted using TRIzol reagent (Invitrogen). RNA was electrophoresed, transferred to nylon membranes, and hybridized with the indicated cDNA probes. To detect the expression of IRAK-4 mRNA, a 394 bp fragment (707–1,101) was used as a probe. The same membrane was rehybridized with a β-actin probe.

**Western blot analysis.** Peritoneal macrophages were treated with 10 ng/ml MALP-2 for the indicated times. Cells were then lysed in a lysis buffer containing 0.5% NP-40, 150 mM NaCl, 20 mM Tris-Cl, pH 7.5, 1 mM EDTA, and protease inhibitor cocktail (Roche). Cell lysates were dissolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with the specific Ab to indicated proteins and visualized with an enhanced chemiluminescence system (NEN Life Science Products). For immunoprecipitation, 10\(^{7}\) MEFs were treated with 10 ng/ml IL-1β for the indicated periods, and cell lysates were immunoprecipitated with anti-MyD88 or anti-IRAK-4 Ab, followed by immunoblotting with the indicated Abs.

**EMSAs.** The nuclear extracts were prepared from peritoneal macrophages (5 × 10\(^{6}\)) stimulated with MALP-2 as described previously (4). Nuclear extracts were incubated with or without Abs against NF-κB p65 or p50, and then further incubated with a specific probe for NF-κB DNA binding sites, electrophoresed, and visualized by autoradiography.

**Allogenic T cell response assay.** The allogenic T cell responses were analyzed as described previously (32). In brief, bone marrow–derived DCs stimulated with 10 ng/ml MALP-2, 1 μg/ml LPS, or 100 nM CpG-DNA from Santa Cruz Biotechnology, Inc. Anti-MyD88 Ab was purchased from ProSci, and anti–IRAK-1 Ab was made as described previously (25). Rabbit anti–IRAK-4 polyclonal Ab was raised against a peptide corresponding to aa 436 to 450 of mouse IRAK-4. Specificity of this Ab was tested on overexpressed IRAK-4 (unpublished data) and on IRAK-4\(^{-/-}\) cells (Fig. 1 D).
for 48 h from BALB/c mice were harvested at day 8, irradiated at a dose of 30 Gy, and plated at threefold serial dilutions in 96-well round-bottom plates. These DGs were incubated for 3 d with 5 × 10⁴/well of splenic CD4⁺ T cells from wild-type, IRAK-4⁻/⁻, and IRAK-4⁺/⁻ KN/KN mice isolated using MACS with CD4 microbeads (Miltenyi Biotec). [3H]thymidine was added for the last 16 h. [3H]thymidine incorporation was measured by a scintillation counter.

**LCMV infection and analysis of T cell responses.** LCMV-WE strain was obtained from T. Otheki (Akita University, Akita, Japan). Wild-type and IRAK-4⁻/⁻ mice were intravenously infected with 5 × 10⁴ PFU of LCMV-WE and spleenocytes were harvested at day 8 after infection. To investigate the induction of LCMV-specific T lymphocytes, spleenocytes were incubated with T-select H-2Db LCMV tetramer-KAVYNFATC-PE (MBL International Corporation) and CD8a-APC Ab. Samples were acquired on a FACS Calibur (BD Biosciences) and analyzed with FlowJo software (TreeStar). For assessment of cytotoxicity of LCMV-specific T cells, spleenocytes prepared from LCMV-infected mice were incubated for 5 h with EL-4 target cells that had been loaded with a peptide (GP33; KAVYNFATC; Peptide Institute) and labeled with ⁵¹Cr. The percentage of specific lysis was calculated as [(experimental release – spontaneous release)/(maximal release – spontaneous release)] × 100.

**TNF bioassay.** TNF activity was measured in macrophage culture supernatant after stimulation with MALP-2 for 1 and 2 h by cytotoxicity on L929 fibroblasts. L929 cells were plated on 96-well plates in RPMI 1640 medium supplemented with 2% FCS. Serial twofold dilutions of supernatants in 8 mg/ml actinomycin D were added to each well and incubated for 20 h. Viability of cells was determined using CellTiter-Glo (Promega) according to the manufacturer’s instructions. Mouse recombinant TNF-α (R&D systems) was used to derive a standard curve, and the concentration of TNF-α was determined based on the standard curve.

**Online supplemental material.** Fig. S1 shows the generation of IRAK-4⁻/⁻ mice. Fig. S2 shows the induction of TNF activity in response to MALP-2 stimulation. Fig. S3 shows that activation of NF-κB in response to MALP-2 was dependent on MyD88. Fig. S4 shows the activation of NF-κB in IRAK-4⁻/⁻ and IRAK-4⁺/⁻ KN/KN macrophages in response to LPS, R-848, and CpG-DNA. Fig. S5 shows the proliferative responses of T cells from wild-type, TCR-α, γ/δ, CD4⁻/⁻, and CD8⁻/⁻ mice isolated using MACS with CD4 microbeads (Miltenyi Biotec). [3H]thymidine was added for the last 16 h. [3H]thymidine incorporation was measured by a scintillation counter.

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