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Complete Dependence on IRAK4 Kinase Activity in TLR2, but Not TLR4, Signaling Pathways Underlies Decreased Cytokine Production and Increased Susceptibility to \textit{Streptococcus pneumoniae} Infection in IRAK4 Kinase–Inactive Mice

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IRAK4 is critical for MyD88-dependent TLR signaling, and patients with \textit{Ira}k4 mutations are extremely susceptible to recurrent bacterial infections. In these studies, mice homozygous for a mutant IRAK4 that lacks kinase activity (IRAK4\textsuperscript{KD}KI) were used to address the role of IRAK4 in response to TLR agonists or bacterial infection. IRAK4\textsuperscript{KD}KI macrophages exhibited diminished responsiveness to the TLR4 agonist LPS and little to no response to the TLR2 agonist Pam3Cys compared with wild-type macrophages as measured by cytokine mRNA, cytokine protein expression, and MAPK activation. Importantly, we identified two kinases downstream of the MAPKs, MNK1 and MSK1, whose phosphorylation is deficient in IRAK4\textsuperscript{KD}KI macrophages stimulated through either TLR2 or TLR4, suggesting that IRAK4 contributes to TLR signaling beyond the initial phosphorylation of MAPKs. Additionally, IRAK4\textsuperscript{KD}KI macrophages produced minimal cytokine mRNA expression in response to the Gram-positive bacteria \textit{Streptococcus pneumoniae} and \textit{Staphylococcus aureus} compared with WT cells, and IRAK4\textsuperscript{KD}KI mice exhibited increased susceptibility and decreased cytokine production in vivo upon \textit{S. pneumoniae} infection. Treatment of infected mice with a complex of polyinosinic-polycytidylic acid with poly-L-lysine and carboxymethyl cellulose (Hiltonol), a potent TLR3 agonist, restored survival to both WT and IRAK4\textsuperscript{KD}KI mice, thereby providing a potential treatment strategy in both normal and immunocompromised patients. \textit{The Journal of Immunology}, 2013, 190: 307–316.

A major component of the mammalian immune system relies on the recognition of certain microbial components, which are absent in host cells, called pathogen-associated molecular patterns (PAMPs) (1, 2). Host cells recognize PAMPs through pattern recognition receptors that initiate a signal cascade that results in the upregulation of proinflammatory cytokines and the clearance of invading microbes. Of the pattern recognition receptors, TLRs play a critical role in controlling microbial infection in both mice and humans. Mutations that occur in the TLR signaling pathway result in increased susceptibility to viral, bacterial, and/or fungal infections, depending on the specific signaling component affected. In the past decade, an increasing number of innate immune deficiencies has been identified in genes that encode TLR pathway components (3–7).

In humans, 10 TLRs have been identified. TLR2 heterodimerizes with TLR1 or TLR6, leading to the recognition of Gram-positive bacterial components, such as lipoproteins (8). TLR4, the first human TLR cloned (9, 10), is engaged by LPS found on Gram-negative bacteria, whereas TLR3, TLR5, TLR7, TLR8, and TLR9 recognize other bacterial and/or viral components, such as dsRNA (TLR3), flagellin (TLR5), ssRNA (TLR7 and TLR8), and CpG DNA (TLR9) (1). All TLRs activate NF-κB and MAPKs, but the intermediate signaling molecules used by a particular TLR can vary. Although some proteins are common to all TLR signaling pathways, others are unique to a particular TLR or are shared among only a subset of the TLRs. For example, cells deficient in the adapter protein MyD88 are completely refractory to signaling through nearly all TLRs, with the notable exceptions of TLR3 and TLR4 (11, 12). TLR3 is completely independent of MyD88, whereas TLR4 has two signaling pathways: one is MyD88 dependent, and the other is MyD88 independent. Upon TLR engagement, MyD88 is recruited to the cytosolic TIR domain of a TLR and facilitates recruitment of the IRAK kinases, IRAK4, IRAK1, and IRAK2. Each of these proteins contributes to the assembly of a multiprotein structure, called the “myddosome,” which is critical for TLR-mediated signal transduction (13).

IRAK1 and IRAK2 are thought to have redundant roles in myddosome formation and signal transduction, but the unique contributions, if any, of IRAK2 have not been thoroughly addressed (14). Once IRAK4 complexes with MyD88, it phosphorylates IRAK1, allowing IRAK1 to autophosphorylate and recruit the ubiquitin ligase TRAF6. TRAF6 ubiquitinates both itself and...
IRAK1, enabling interaction of the complex with TAK1, the kinase responsible for IκB phosphorylation and degradation, and leading to the translocation of activated NF-κB to the nucleus. Another adapter protein, TIRAP (also called Mal), facilitates MyD88 recruitment to TLR2. Like TLR2, TLR4 uses TIRAP/MyD88 to initiate signaling through the MyD88-dependent pathway; however, TLR4 also uses another “bridging adapter,” TRAM, to recruit TRIF to the receptor complex and initiate TRIF-dependent signaling. TLR3 signals exclusively through the adapter TRIF.

Because of its role in propagating MyD88-dependent signaling, IRAK4 is presumably a critical component in most TLR-signaling pathways, with the exception of TLR3 (15–18). Mice deficient in IRAK4 (IRAK4−/−) show characteristics reminiscent of MyD88−/− mice in that they are resistant to doses of certain TLR agonists, such as LPS or CpG, which are lethal to wild-type (WT) mice (19, 20). However, there are discrepancies in the literature with regard to whether the kinase activity of IRAK4 is required for TLR signaling (19–23). Interestingly, cytokine expression induced by LPS in macrophages expressing a mutant IRAK4 that lacks kinase activity was diminished but not to the same extent as IRAK4−/− cells (20). These data suggest that IRAK4 plays a role in propagating TLR signaling independent of its ability to phosphorylate IRAK1. In contrast, a separate study demonstrated diminished gene expression in both IRAK4−/− and IRAK4 kinase–inactive cells, thus demonstrating a need for further exploration into the necessity of IRAK4 kinase activity in the propagation of TLR signaling (19).

Patients with mutations in IRAK4 present with recurrent bacterial infections but show no impaired defense against viral infections (presumably due to their retained ability to signal through TLR3 and other non-TLR viral receptors). Streptococcus pneumoniae is the most common infection found in these patients, followed by Staphylococcus aureus and Pseudomonas aeruginosa (4, 6). Although other investigators observed impaired gene expression in IRAK4-deficient cells stimulated with synthetic TLR agonists, there is a paucity of data to address how this deficiency fares against infection with whole bacteria. In the studies presented in this article, we sought to clarify the literature with regard to impaired TLR signaling in IRAK4−/− macrophages and to develop a mouse model of infection to address the importance of IRAK4 kinase activity in controlling bacterial infection. We found that TLR4 signaling is partially deficient in macrophages derived on a C57BL/6J background and were bred homozygously at the University of Maryland, School of Medicine. Age- and sex-matched C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Peritoneal macrophages were obtained by performing a peritoneal lavage 5 d after i.p. administration of sterile thioglycollate (Remel, Lenexa, KS). For bone marrow–derived macrophages, bone marrow was cultured in standard medium supplemented with 20–25% LADMAC cell–conditioned medium (24). After 6 d, nonadherent cells were removed. Adherent cells were harvested using 0.25% Trypsin-EDTA (Life Technologies, Grand Island, NY) after 7–14 d in culture and replated for experimental use in the absence of a CSF-1 source. All animal experiments were conducted under the guidelines and approval of the Institutional Animal Care and Use Committee. All cellular experiments were conducted using DMEM (Life Technologies) supplemented with 5% FCS, 2 mM l-glutamine, and 1% penicillin–streptomycin (penicillin–streptomycin was omitted in experiments using live or heat-killed bacteria) at 37°C and 5% CO2.

Agonists for TLR2 (Pam3Cys) and TLR3 (polynosinic-polycytidylic acid [poly I:C]) were purchased from InvivoGen (San Diego, CA). Poly IC:LC (Hiltonol) was generously provided by Dr. Andres Salazar (Oncovir). Protein-free, phenol water–extracted Escherichia coli K235 LPS was prepared as described elsewhere (25).

Quantitative real-time PCR

Macrophages were cultured in 12-well plates at a density of 2 × 106 cells/well. After stimulation, RNA was extracted using Trizol reagent (Roche, Indianapolis, IN), and 1 μg of total RNA was reversed transcribed (RT-PCR) using iScript (Bio-Rad, Hercules, CA), according to the manufacturer’s instructions. The resulting cDNA template was used for real-time PCR analysis with Power SYBR Green and the AB 7900HT Fast Time fluorescence detection system. The following primers were used: IL-1β sense, 5′-TCTTGGATACCATCTGCCAG-3′; IFN-β sense, 5′-TTCCACACCCAGGTTTCCTC-3′ and antisense, 5′-CTTCACTCATGCTTCCAG-3′; IL-12p40 sense, 5′-TCTTTGTGTCATACCCACCGG-3′ and antisense, 5′-GGAACGCCACTTCTGTTGTA-3′; IFN-γR sense, 5′-GACTTGACCGCAGGTTATGTC-3′ and antisense, 5′-ATCTCTGATCAGAGCCTTTGC-3′; TNFα sense, 5′-AAATGACCTGATATTTCAATTTTCCATC-3′ and antisense, 5′-GAGACAGCAGAGGATGTGGA-3′; IL-10 sense, 5′-ATTGAA-TTCCCTGTGGGAGAAG-3′ and antisense, 5′-CACAGGGGAGAAATGCTGACA-3′; COX-2 sense, 5′-ACTGGGCGCTAGGTGAGGAC-3′ and antisense, 5′-AATGACTCTGATTATTTCTCATCATTCCAT-3′; PAI2 sense, 5′-TACAAGCGCAACAAGCGGATTGATGAAA-3′ and antisense, 5′-GAGCTAGAGAGGAGAAGCTGTA-3′; IL-6 sense, 5′-TGCTATACACTTACCAACAAGCTGGAG-3′ and antisense, 5′-GCACAACCTCTTCTTCCAT-3′; IP-10 sense, 5′-GATTCCAGACCTCGGAGTGG-3′ and antisense, 5′-CTTATGCCACCGCCGGTGT-3′. Relative gene expression was calculated using the ΔΔCt calculation method, in which all samples were normalized to the housekeeping gene HPRT (26).

Cytokine protein measurements

Macrophages were cultured in 12-well plates at a density of 2 × 106 cells/well. Six hours after stimulation, supernatants were collected and sent to the University of Maryland Cytokine Core Laboratory (Baltimore, MD) for cytokine analysis using the Luminex Multianalyte System (R&D Systems, Minneapolis, MN). For in vivo measurements, serum samples were collected from mice 48 h postinfection and processed as stated above.

Cell lysate preparation and Western blot analysis

Macrophages (3 × 106) were plated in six-well plates and incubated with the indicated concentrations of TLR agonists or bacteria. Cells were washed with ice-cold PBS and resuspended in lysis buffer (50 mM Tris [pH 7.5], 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 2 μM EDTA with a protease inhibitor mixture [P8340; Sigma-Aldrich, St. Louis, MO]) and a phosphatase inhibitor mixture [PhosStop; Roche]). Protein concentrations were determined (Bio-Rad), and lysates were reduced for 5 min by boiling the sample buffer (Thermo Scientific). Anti–p-JNK, anti–p-ERK, anti–p-p38, anti-p38 total, and anti-MNK1 were purchased from Cell Signaling Technology (Danvers, MA); anti-H3ser10 and anti-H3 were purchased from Cell Signaling Technology (Danvers, MA); anti-H3ser10 and anti-H3 were purchased from Cell Signaling Technology (Danvers, MA).

Materials and Methods

Mice, macrophage isolation, and reagents

IRAK4−/− mice, generously provided by Dr. Kirk A. Staschke and Dr. Raymond Gilmour (Lilly Research Laboratories, Indianapolis, IN), were derived on a C57BL/6J background and were bred homozygously at the University of Maryland, School of Medicine. Age- and sex-matched IRAK4−/− mice, which were maintained in the specific pathogen-free animal facility at the University of Connecticut, were purchased from Iffa Credo (Maclean, VA). IRAK4−/− mice were genotyped by genotyping PCR using the following primers: IRAK4−/− sense, 5′-GGAATCTCTACAATTCCTC-3′ and antisense, 5′-TTCTGGATACCATCTGCCAG-3′; IFN-β sense, 5′-TTCCACACCCAGGTTTCCTC-3′ and antisense, 5′-CTTCACTCATGCTTCCAG-3′; IL-12p40 sense, 5′-TCTTTGTGTCATACCCACCGG-3′ and antisense, 5′-GGAACGCCACTTCTGTTGTA-3′; IFN-γR sense, 5′-GACTTGACCGCAGGTTATGTC-3′ and antisense, 5′-ATCTCTGATCAGAGCCTTTGC-3′; PAI2 sense, 5′-TACAAGCGCAACAAGCGGATTGATGAAA-3′ and antisense, 5′-GAGCTAGAGAGGAGAAGCTGTA-3′; IL-6 sense, 5′-TGCTATACACTTACCAACAAGCTGGAG-3′ and antisense, 5′-GCACAACCTCTTCTTCCAT-3′; IP-10 sense, 5′-GATTCCAGACCTCGGAGTGG-3′ and antisense, 5′-CTTATGCCACCGCCGGTGT-3′. Relative gene expression was calculated using the ΔΔCt calculation method, in which all samples were normalized to the housekeeping gene HPRT (26).
Abcam (Cambridge, MA); and anti-action was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-elf4e and anti-\(\gamma\)-elf4e Abs were generously provided by Dr. Nahum Sonenberg (McGill University, Montreal, QC, Canada).

**Nuclear extract preparation and oligodeoxynucleotide coprecipitation**

Macrophages (\(8 \times 10^6\)) were plated in 10-cm dishes and treated with TLR agonists. The cells were washed one time with PBS and pelleted by centrifugation, and nuclear extracts were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA), according to the manufacturer’s protocol. Briefly, biotinylated oligodeoxynucleotide (ODN) containing the NF-\(\kappa\)B consensus sequence (5'-AGTTGAGGGACCTTTCCAGGC-3') was annealed to the complementary strand for 1 h at room temperature. This procedure was also performed with a negative control ODN with a mutated sequence (5'-AGTTGAGGGGACCTTTCCAGGC-3'). Nuclear extract samples prepared from cells were diluted to 900 \(\mu\)l with coprecipitation buffer (0.1% Triton X-100, 10 mM HEPES [pH 7.5], 2 mM EDTA, 1 mM EGTA, 10% glycerol, supplemented with a protease inhibitor mixture [P8340; Sigma-Aldrich]) and precleared with 60 \(\mu\)l salmon sperm–agarose beads (Millipore, Billerica, MA) for 30 min at 4°C. Beads were removed by centrifugation, and the supernatant was incubated overnight at 4°C with 30 nM NF-\(\kappa\)B–ODN. Streptavidin-agarose beads (Sigma-Aldrich) were added for 1 h, and ODN–protein complexes were pelletted, washed three times, and diluted in SDS-PAGE sample buffer. Boiled samples were centrifuged to remove beads, and the entire supernatant was loaded onto an 12% polyacrylamide gel. Western blots were performed as described above.

**Mouse infections and bacteria**

*S. pneumoniae* (ATCC 6303) was grown to log phase in BHI nutrient broth (Teknova, Hollister, CA), supplemented with 10% sterile glycerol, and stocks were stored at \(-80^\circ\)C. Mice were lightly anesthetized using isoflurane (VetOne), and bacteria were administered intranasally (i.n.) at the appropriate concentration in 30 \(\mu\)l total saline. For studies using poly IC: LC, bacteria were administered i.n. in 50 \(\mu\)l saline or poly IC:LC solution (100 \(\mu\)g/mouse). Bacterial concentration was verified for each experiment by performing serial dilutions of the inoculum plated on Mueller–Hinton Agar supplemented with 5% sheep’s blood (Teknova). Mice were monitored twice a day for survival studies. For mRNA analysis of cytokine production, mice were sacrificed 24 or 48 h postinfection, and a portion of the left lobe of the lung was excised and homogenized in TriPure RNA extraction reagent (Roche). RT-PCR and quantitative real-time PCR (qPCR) were performed as described above.

**Results**

**IKAR4 kinase activity is required for optimal gene induction in response to TLR2 and TLR4, but not TLR3, agonists**

IKAR4 was reported to be a critical kinase for propagating TLR-mediated signaling events leading to proinflammatory gene induction (14). To assess the requirement for IKAR4 kinase activity in MyD88-dependent TLR signaling, macrophages from WT and IKAR4\(^{KDKI}\) mice were isolated and treated with the TLR2 agonist Pam3Cys (MyD88 dependent) or the TLR4 agonist LPS (MyD88 and TRIF dependent). mRNA expression for MyD88-dependent and MyD88-independent genes was measured 1 or 3 h poststimulation by qPCR. Induction of IL-\(\beta\) and KC (Fig. 1A) mRNA in Pam3Cys-treated IKAR4\(^{KDKI}\) macrophages was minimal compared with the robust induction induced in WT cells. In contrast, LPS upregulated MyD88-dependent gene expression in IKAR4\(^{KDKI}\) macrophages, although the levels of IL-\(\beta\) and KC mRNA were significantly less compared with the full expression observed in WT cells. Because the TLR3-signaling pathway is MyD88 and IKAR4 independent, we also stimulated macrophages with the TLR3 agonist poly I:C to ensure that our IKAR4\(^{KDKI}\) macrophages were capable of normal gene expression in response to an IKAR4- and MyD88-independent stimulus. Very little difference in mRNA induction in response to poly I:C was observed in WT macrophages compared with IKAR4\(^{KDKI}\) macrophages, although the magnitude of gene expression was less than that induced by TLR2 or TLR4 signaling (Fig. 1A). IFN-\(\beta\) mRNA expression, which is induced by both LPS and poly I:C in a MyD88-independent, TRIF-dependent fashion, was not inhibited in IKAR4\(^{KDKI}\) macrophages stimulated with LPS or poly I:C (Fig. 1B). To examine whether these observations extend to additional genes, we performed qPCR analysis for the MyD88-dependent genes that encode IL-10, COX-2, PAI-2, and IL-6, as well as the MyD88-independent gene IP-10 (Fig. 1C). These data further support the observation that TLR2-induced gene expression is completely dependent on the kinase activity of IKAR4, whereas TLR4-induced gene expression is only partially dependent on the presence of this functional kinase. In contrast, LPS induction of IFN-\(\beta\) and IP-10 (Fig. 1B, 1C) is independent of IKAR4 kinase activity. Additionally, we measured protein levels in the supernatants of both WT and IKAR4\(^{KDKI}\) macrophages treated with Pam3Cys, poly I:C, or LPS (Fig. 1D). In agreement with the mRNA data, Pam3Cys-treated IKAR4\(^{KDKI}\) macrophages secreted very little IL-6 or KC, whereas LPS induced some IL-6 and KC secretion in these cells, but not to the same extent as in WT macrophages. Although poly I:C was not a potent inducer of either protein, it is interesting to note that KC protein expression was enhanced in IKAR4\(^{KDKI}\) macrophages compared with WT macrophages (Fig. 1D, right panel, inset). This confirms, at both the mRNA and protein levels, that the IKAR4\(^{KDKI}\) macrophages are capable of responding to some TLR agonists (i.e., TLR3, TLR4) but not to others.

We also sought to determine whether IKAR4 kinase activity is required to establish endotoxin tolerance in macrophages. As expected from previous studies (27), LPS-treated macrophages from WT mice that were previously exposed to LPS demonstrated diminished induction of both MyD88-dependent (i.e., KC, Fig. 1E, LPS/LPS) and MyD88-independent (i.e., IFN-\(\beta\), Fig. 1E, LPS/LPS) genes compared with macrophages not previously exposed to LPS (Fig. 1E, Med/LPS). Consistent with the data in Fig. 1A and 1B, stimulation of medium-pretreated IKAR4\(^{KDKI}\) macrophages with LPS resulted in reduced KC, but normal IFN-\(\beta\), mRNA levels (Fig. 1E, Med/LPS). However, like WT macrophages, IKAR4\(^{KDKI}\) macrophages that were previously exposed to LPS exhibited reduced expression of both KC and IFN-\(\beta\) mRNA compared with cells that were not. We conclude that, although IKAR4 kinase activity contributes to the MyD88-dependent gene induction response to both TLR2 and TLR4 agonists, but it is not required for all macrophage responses because endotoxin tolerance and the response to TLR3 remain intact in the absence of kinase-active IKAR4.

**MAPK activation is severely deficient in TLR2-stimulated IKAR4\(^{KDKI}\) macrophages, whereas it is only partially deficient in response to TLR4 stimulation**

TLR signaling mediates activation of the MAPKs p38, ERK1/2, and JNK that ultimately promote proinflammatory gene expression. Therefore, we assessed whether IKAR4 kinase activity is required for activation of MAPKs. Macrophages from WT and IKAR4\(^{KDKI}\) mice were treated with Pam3Cys to activate TLR2 or with LPS to activate TLR4, and the cells were lysed after 30 min. Activated MAPK proteins were measured by Western blot analysis using Abs against phosphorylated (activated) proteins p38, ERK1/2, and JNK. Although LPS-treated IKAR4\(^{KDKI}\) macrophages showed diminished activation of p38 and ERK1/2 compared with WT macrophages, activation of JNK was minimally affected (Fig. 2A). In contrast, Pam3Cys treatment of IKAR4\(^{KDKI}\)
macrophages yielded essentially no activation of any of the three MAPKs compared with WT cells (Fig. 2A), p38, ERK, and JNK were all activated in response to the TLR3 agonist poly I:C in both WT and IRAK4KDKI macrophages, although to a lower extent than was detected with Pam3Cys or LPS (Fig. 2A, right panel). Interestingly, p38 and JNK activation were slightly augmented in IRAK4KDKI cells compared with WT cells. Membranes were reprobed with total p38 or actin to ensure equal loading.

MNK1 is a kinase that is activated by both p38 and ERK (28, 29). Nearly complete inhibition of MNK1 phosphorylation was observed in IRAK4KDKI macrophages (Fig. 2B), suggesting that the TLR4-signaling cascade is defective despite detectable, albeit reduced, upstream activation of p38 and ERK1/2. As expected, because of the lack of p38 and ERK activation observed in Fig. 2A, Pam3Cys stimulation in IRAK4KDKI macrophages also failed to activate MNK1 in IRAK4KDKI macrophages but not in WT cells (Fig. 2B). Membranes were reprobed with anti-MNK1 to ensure that the lack of activation is not attributable to a loss of protein expression in IRAK4KDKI cells. Interestingly, MNK1 appears to be differentially expressed in IRAK4KDKI macrophages, as observed in IRAK4KDKI macrophages (Fig. 2B).
demonstrated by the presence of lower molecular weight bands. These bands may represent degradation products of the protein, because treatment with the proteasome inhibitor MG-132 reduced their expression (data not shown).

We also sought to determine whether our TLR agonists activate MSK1, a protein kinase that is similarly activated by ERK1/2 (30). As demonstrated by Western blot analysis, MSK1 is slightly activated by the TLR2 agonist Pam3Cys and is robustly activated by LPS in WT macrophages, but phosphorylation of MSK1 was not detectable in IRAK4<sup>KDki</sup> macrophages (Fig. 2B). Together, these data suggest that, despite the ability of IRAK4<sup>KDki</sup> macrophages to retain the capacity to elicit some early MAPK activation, downstream activation is significantly impaired in response to both TLR2 and TLR4 stimuli.

eIF4e, a protein involved in translational regulation (31), is a known substrate for MNK1 (32). Phospho-eIF4e was increased in response to Pam3Cys and LPS in WT macrophages, albeit with different kinetics, but neither Pam3Cys nor LPS induced significant activation of eIF4e in the IRAK4<sup>KDki</sup> cells (Fig. 2C). Therefore, we conclude that a lack of MNK1 activation disrupts downstream signaling events that may ultimately contribute to reduced gene expression in IRAK4<sup>KDki</sup> macrophages.

MSK1 phosphorylation of histone H3 at serine 10 leads to upregulation of early immediate genes that promote cytokine transcriptional activation (i.e., c-jun, c-fos) (33–35). Therefore, we tested whether a lack of MSK1 activation in IRAK4<sup>KDki</sup> macrophages led to a decrease in H3ser10 phosphorylation. Indeed, H3ser10 phosphorylation was decreased in IRAK4<sup>KDki</sup> macrophages compared with WT cells in response to both Pam3Cys and LPS (Fig. 2C).

Previous reports demonstrated IRAK4-independent NF-κB activation in response to TLR2 and TLR4 agonists in mice (19, 20), whereas other studies showed reduced NF-κB activity in response to LPS or IL-1β stimulation in a human patient with IRAK4 deficiency (36). Therefore, we used both TLR2 and TLR4 agonists to explore the role of IRAK4 kinase activity in NF-κB activation in murine macrophages. Because activation of NF-κB prompts its translocation into the nucleus, nuclear lysates from macrophages treated with Pam3Cys or LPS for 1 h were analyzed by Western blot using anti-p65 and anti-p50 Abs. We found no deficiency of p65 in the nucleus of IRAK4<sup>KDki</sup> macrophages in response to LPS. In contrast, there was a slight p65 deficiency in the nucleus of IRAK4<sup>KDki</sup> macrophages compared with WT cells in response to Pam3Cys (Fig. 2D, left panel). NF-κB p50 expression in the nucleus was diminished in IRAK4<sup>KDki</sup> macrophages compared with WT cells in response to both Pam3Cys and LPS (Fig. 2D, left panel).

To determine whether NF-κB binding to its cognate DNA sequence was impaired in IRAK4<sup>KDki</sup> macrophages, we performed a coprecipitation assay using a biotinylated ODN containing the NF-κB consensus-binding sequence. The same nuclear extract preparations that were analyzed by Western blot in Fig. 2D were incubated with biotinylated NF-κB–specific ODN or a mutated sequence that served as a negative control (LPS-stimulated nuclear extracts were incubated with the mutated sequence to control for nonspecific binding). Streptavidin-conjugated beads were used to precipitate the biotinylated ODNs, and coprecipitated proteins were resolved by Western blot and probed with anti-p65 and anti-p50 Abs (left panel). Similarly, binding of NF-κB p50 was not impaired in Pam3Cys- or LPS-treated IRAK4<sup>KDki</sup> cells, despite the diminished quantity of p50 in the lysates used. Interestingly, binding of both p65 and p50 was slightly augmented in IRAK4<sup>KDki</sup> macrophages in response to LPS compared with WT cells. This may reflect augmented activation of p65 and p50 (independent of its translocation) and/or the activation of other transcription factors that enhance their DNA binding in IRAK4<sup>KDki</sup> cells. Importantly, these data demonstrate that, unlike the complete attenuation of MAPK signaling in response to the MyD88-dependent TLR2 stimulus, Pam3Cys, NF-κB binding remains intact in IRAK4<sup>KDki</sup> macrophages. Altogether, these data suggest that, although TLR2-induced MAPK activation is completely dependent on IRAK4 kinase activity, TLR2-induced NF-κB activation is only partially dependent on the presence of a functional IRAK4 kinase (as
demonstrated by decreased translocation of p65). In contrast, TLR4 activation of MAPKs is only partially dependent on IRAK4 kinase activity, yet the activation of NF-κB is independent of a functional IRAK4 kinase. To examine whether the NF-κB activation in IRAK4KDKI macrophages is a result of activation of an alternative pathway, such as activation of PI3K, we repeated our ODN precipitation in the presence of the PI3K inhibitor LY294002. We found that the inhibitor did not decrease NF-κB activation at a dose that inhibited activation of PI3K (data not shown). Thus, the mechanism by which NF-κB achieves full activation, despite the lack of IRAK4 kinase activity, remains unclear but is independent of PI3K.

**IRAK4 kinase activity is required for complete gene induction in macrophages in response to heat-killed and live bacteria**

Although previous work largely examined the role of IRAK4 kinase activity in response to purified TLR agonists, few studies addressed the importance of this kinase in response to whole bacteria. Therefore, we cultured macrophages from WT and IRAK4KDKI mice and stimulated them with heat-killed *S. aureus* (HKSA; a Gram-positive bacterium) or heat-killed *P. aeruginosa* (HKPA; a Gram-negative bacterium) for 1, 3 or 5 h. Gene expression was measured by qPCR. As positive controls, we also treated these cells with the purified TLR agonists, Pam3Cys or LPS. We observed differences in the macrophage response to bacteria that were species dependent. Fig. 3A shows that IL-1β, KC, and IL-6 mRNA expression in response to HKSA was completely dependent on IRAK4 kinase activity (similar to Pam3Cys) and was weaker than responses induced by LPS or HKPA. The responses to HKPA were much more robust than even those induced by LPS and were only partially dependent on the presence of a functional IRAK4 kinase (Fig. 3A). To determine whether Pam3Cys and LPS cotreatment elicits a more robust response than either agonist alone, we treated WT macrophages with Pam3Cys, LPS, or a combination of the two for 3 h and measured gene expression by qPCR (Fig. 3B). Macrophages treated with both Pam3Cys and LPS exhibited slightly higher levels of gene expression but did not synergize. Therefore, the extent to which responses to whole heat-killed Gram-positive and Gram-negative bacteria are IRAK4 dependent appears to reflect the predominant TLR agonist in the bacteria itself, because HKPA expresses both TLR2 and TLR4 PAMPs, whereas HKSA expresses predominantly TLR2 agonists. The relative amounts of these agonists are likely to play a role in the magnitude of response.

*S. pneumoniae* is the leading killer of patients with deficiencies in IRAK4 (4, 6). Therefore, we sought to determine what response, if any, was induced in IRAK4KDKI macrophages infected with increasing numbers of *S. pneumoniae*. Strikingly, there was virtually no mRNA induction of the proinflammatory cytokines IL-1β, KC, or IL-12 p40 in IRAK4KDKI macrophages in response to infection with live bacteria, despite robust responses observed in WT cells (Fig. 3C).

**IRA4KDKI** mice are more susceptible to *S. pneumoniae* infection than WT mice

To assess whether IRAK4 kinase activity plays a role in controlling *S. pneumoniae* infection, WT and IRAK4KDKI mice were infected i.n. with *S. pneumoniae*, and survival was assessed over time. Although the majority of both WT and IRAK4KDKI mice succumbed to *S. pneumoniae* infection within 7 d, there was a significant increase in the rate of death of IRAK4KDKI mice compared with their WT counterparts (Fig. 4, *p* = 0.022, log-rank test). To determine whether this increased susceptibility was due to a deficiency in cytokine production, lungs were isolated from
S. pneumoniae–infected mice 48 h postinfection, RNA was extracted, and qPCR was used to quantify gene induction. As observed in vitro using isolated macrophages, there was a significant decrease in cytokine mRNA production in the IRAK4KDKI lungs compared with WT lungs in response to S. pneumoniae infection (Fig. 5A). Interestingly, in contrast to stimulation of macrophages with Pam3Cys, which does not induce IFN-β, infection with S. pneumoniae in vivo induced IFN-β in both WT and IRAK4KDKI mice, suggesting that there are non-TLR2, IRAK4-independent ligands present on S. pneumoniae that can induce IFN-β mRNA, an observation that is consistent with other recent studies (37, 38). To characterize further differences in the course of infection between WT and IRAK4KDKI mice, we also measured serum cytokine levels (Fig. 5B) and bacterial burden (Fig. 5C) 48 h postinfection. IRAK4KDKI mice produced consistently lower levels of IL-6 and KC protein while demonstrating significantly higher bacterial burdens in their lung tissue. Interestingly, we did not observe any striking differences in tissue histology between WT and IRAK4KDKI mice when sacrificed at 48 or 72 h postinfection (data not shown). Both groups of mice showed low levels of inflammatory infiltration at both 48 and 72 h (a time point when 40% of the IRAK4KDKI mice had already expired because of infection). Together, these data suggest that the absence of a functional IRAK4 kinase decreases proinflammatory cytokine production in response to S. pneumoniae infection while facilitating bacterial replication and, thereby, increasing the susceptibility of IRAK4KDKI mice to pneumococcal infection.

Poly IC:LC (Hiltonol) improves survival of both WT and IRAK4KDKI mice infected with S. pneumoniae

Our in vitro data demonstrate that TLR3 signaling remains intact in IRAK4KDKI macrophages. Therefore, we sought to determine whether administration of the TLR3 agonist poly IC:LC (Hiltonol), a stabilized poly I:C, could improve survival in IRAK4KDKI mice infected with a lethal dose of S. pneumoniae. In these studies, vehicle only or 100 μg of poly IC:LC was administered i.n. at the time of infection in both WT and IRAK4KDKI mice. There was a significant increase in the survival of infected mice treated with poly IC:LC compared with those treated with vehicle only (Fig. 6; WT, p = 0.0097, log-rank test; IRAK4KDKI, p = 0.0001, log-rank test). Thus, stimulation of TLR3, a MyD88- and IRAK4-independent innate immune pathway, provides a possible therapeutic strategy to boost immunity against bacterial pathogens in patients lacking key signaling components of MyD88- and/or IRAK4-dependent signaling pathways.

**FIGURE 4.** IRAK4KDKI mice are more susceptible than WT mice to S. pneumoniae infection. WT or IRAK4KDKI mice were infected i.n. with 1 × 10^5 S. pneumoniae (S.p.) in a total volume of 30 μl or mock infected with an equal volume of saline. Survival was assessed over the course of 14 d. Graph shows the combined data of four separate experiments with a total of 19 mice/mouse strain (S.p. infected) or 4 mice/mouse strain (mock infected) in each group. *p = 0.022, log-rank test.

**FIGURE 5.** Gene induction is impaired in the lungs of S. pneumoniae–infected IRAK4KDKI mice. (A) WT or IRAK4KDKI mice were infected i.n. with 5 × 10^5 S. pneumoniae (SP) in a total volume of 30 μl or mock infected with an equal volume of saline. After 48 h, mice were sacrificed, lungs were harvested, RNA was extracted, and gene expression was measured by qPCR. Each sample was normalized to HPR. Data shown are combined from at least three separate experiments using a total of four mice (saline) or at least nine mice (SP infected) per group. (B and C) WT or IRAK4KDKI mice were infected as in (A). After 48 h, serum cytokines (B) or bacterial rRNA (C) was measured. Data are combined from two separate experiments using a total of nine (B) or six (C) mice/group. '*' p ≤ 0.05, '**p < 0.01, Student t test.
FIGURE 6. Poly IC:LC (Hiltonol) treatment improves survival of mice infected with S. pneumoniae. WT (A) or IRAK4KDKI (B) mice were infected i.n. with 1 x 10^5 S. pneumoniae in a total volume of 50 µl of poly IC:LC (100 µg/mouse, ○) or saline (vehicle, ▲). Survival was assessed over the course of 14 d. The data are combined from three separate experiments with a total of 11 mice/group (10 mice for WT vehicle group). *p = 0.0097, **p = 0.0001, log-rank test.

Discussion

TLR signaling is a critical activator of immune defense during infection. In this study, we clarified the importance of IRAK4 kinase activity in generating full responses to Pam3Cys and LPS, agonists for TLR2 and TLR4, respectively. Importantly, cytokine production in IRAK4KDKI macrophages was also impaired in response to both heat-killed and live bacteria. We observed the most severe deficiency in IRAK4KDKI macrophages when these cells were infected with S. pneumoniae, reminiscent of the phenotype observed in patients with IRAK4 mutations (4, 6, 36). Although early signaling events, such as phosphorylation of p38, ERK1/2, and JNK, were completely abrogated in response to Pam3Cys, IRAK4KDKI macrophages retained some ability to activate these proteins in response to LPS. However, activation of MNK1 and MSK1 in response to LPS was diminished in IRAK4KDKI macrophages, suggesting that IRAK4 also plays a role downstream of p38, ERK1/2, and JNK activation in response to TLR4 agonists. Proinflammatory cytokine mRNA and protein expression remained partially intact in IRAK4KDKI macrophages in response to LPS, suggesting that early MAPK signaling is sufficient for upregulation of gene expression, despite diminished downstream activity. However, our data also indicate that LPS activation of MAPKs is at least partially IRAK4 dependent, because we observed diminished activation of their downstream substrates (such as MNK1 and MSK1, Fig. 2B).

Our data support the hypothesis that IRAK4 kinase activity is most essential for MyD88-dependent TLR signaling. TLR2 is entirely MyD88 dependent, and we show a nearly complete loss of signaling in response to the TLR2 agonist Pam3Cys, as demonstrated by decreased cytokine mRNA expression (Figs. 1, 3) and loss of MAPK activation (Fig. 2). Similar results were reported by Kawagoe et al. (19) using the TLR2 agonist MALP2, a TLR2/6 agonist (Pam3Cys is a TLR1/2 agonist). However, NF-κB activation in response to a TLR2 agonist was not diminished in IRAK4KDKI macrophages compared with WT cells (Fig. 2D), suggesting that IRAK4 kinase-dependent activation of MAPKs is essential for upregulation of proinflammatory cytokine gene expression. We also report that TLR3 signaling, which is completely independent of MyD88, is not affected by the loss of IRAK4 kinase activity, as demonstrated by equal cytokine mRNA expression in both WT and IRAK4KDKI macrophages in response to the TLR3 agonist poly I:C, a result that agrees with previous studies (19, 20). However, currently published data do not agree on the role of IRAK4 kinase activity in response to the TLR4 agonist LPS. Because LPS can propagate both MyD88-dependent and MyD88-independent (TRIF-dependent) signaling pathways, it is of critical importance that induction of genes that are pathway dependent be considered separately. Our data confirm that LPS signaling is dependent on IRAK4 kinase activity, because IRAK4KDKI macrophages exhibited diminished MyD88-dependent cytokine production in response to LPS (Fig. 1). However, MyD88-independent genes, such as IFN-β and IP-10, are not affected by the loss of IRAK4 kinase activity. Furthermore, the loss in gene expression cannot be attributed to a loss of early signaling events, such as activation of p38, ERK1/2, or JNK, as is the case for TLR2 signaling. Our results show LPS-induced activation of MAPK in the absence of IRAK4 kinase activity, a result that agrees with previous studies showing MAPK activation in both IRAK4KDKI and IRAK4KDKI macrophages (20, 39, 40).

Together, these data support a model in which LPS can induce MAPK activation completely independently of IRAK4 protein. This may occur via the LPS-induced MyD88-independent/TRIF-dependent pathway, because MAPK activation by poly I:C, a TLR3 agonist that also signals in a MyD88-independent/TRIF-dependent fashion, is not affected in cells lacking IRAK4 kinase activity (Fig. 3A). Indeed, even MyD88−/− macrophages demonstrate MAPK activation in response to LPS (39, 41, 42). Importantly, both MNK1 and MSK1, kinases that are activated downstream of activated MAPKs, were deficient in IRAK4KDKI macrophages in response to LPS. To confirm a functional consequence for this loss of kinase activation, we demonstrated diminished activation of their downstream substrates as well (i.e., eIF4e for MNK1 and histone H3 serine 10 for MSK1). Of particular note, the observation that LPS-induced tolerance was not affected in IRAK4KDKI macrophages, despite the observed decrease in MyD88-dependent signaling, suggests either that the partial response to LPS observed in such macrophages achieves a threshold sufficient to elicit the chromatin remodeling suggested to underlie tolerance (43) or that tolerance induction is independent of IRAK4 activity. Studies are ongoing to determine the extent to which IRAK4 contributes to endotoxin tolerance.

Kim et al. (20) suggested that, although LPS-induced mRNA expression was not greatly affected in IRAK4KDKI macrophages, protein levels were severely deficient as the result of a loss of mRNA stability and, thus, a reduction in translation of these cytokines. Although our data clearly demonstrate diminished mRNA expression, our observation that eIF4e phosphorylation is also severely diminished in response to LPS (Fig. 2C) suggests that there may also be a secondary loss of translational activity for LPS-induced, MyD88-dependent genes. eIF4e plays a role in maintaining mRNA stability, leading to efficient mRNA translation (31). If eIF4e activation were lost, as we found in response to both TLR2 and TLR4 agonists (Fig. 2C), then it would be expected that translational activity would also be reduced. Interestingly, a similar pattern of MAPK activation (i.e., no defect in activation of p38 or ERK with downstream impairment of MNK1 and eIF4e activation) was demonstrated in IRAK2-deficient macrophages in response to LPS (44). This suggests that activation of MNK1 and its downstream target, eIF4e, requires the presence of IRAK2, as well as the kinase activity of IRAK4 (potentially to phosphorylate/activate IRAK2). In addition, we demonstrate little activation of MSK1 in response to LPS in IRAK4KDKI macrophages. MSK1 phosphorylates histone H3 serine 10, leading to increased gene expression, most notably of early
Infection is not inhibited in IRAK4KDKI mice (Fig. 5) suggests that showing that IFN-β increases poly IC:LC was administered less frequently. Interestingly, the determination of the survival rate of mice receiving poly IC:LC treatment, and there was no significant increase in colonization when poly IC:LC was administered less frequently. Interestingly, the increased S. pneumoniae colonization was dependent on poly IC:LC-induced IFN-β. Because poly IC:LC is a potent inducer of IFN-β in vivo (46), we are investigating the requirement of IFN-β to confer poly IC:LC protection in our model. However, our data showing that IFN-β production in response to S. pneumoniae infection is not inhibited in IRAK4KDKI mice (Fig. 5) suggests that the mechanism may be independent of IFN-β induction. With further studies, such intervention could prove useful in patients with mutations in the MyD88/IRAK-4-dependent pathway, because our in vitro data suggest that activation of MAPKs with stimuli that are TLR/MyD88/IRAK-4 independent might compensate for deficiencies in early signaling proteins of the TLR cascade. With NF-κB signaling relatively intact in TLR2- or TLR4-stimulated IRAK4KDKI macrophages, our data support the concept of increasing activation of the MAPK pathway and targeting its complete restoration in the absence or mitigation of IRAK4 kinase activity.

Disclosures
A.S. works for Oncovar, which manufactures poly IC:LC, a reagent used in this study. The other authors have no financial conflicts of interest.

References


