IRAK-4 is an essential component of the signal transduction complex downstream of the IL-1- and Toll-like receptors. Although regarded as the first kinase in the signaling cascade, the role of IRAK-4 kinase activity versus its scaffold function is still controversial. To investigate the role of IRAK-4 kinase function in vivo, "knock-in" mice were generated by replacing the wild type IRAK-4 gene with a mutant gene encoding kinase-deficient IRAK-4 protein (IRAK-4 KD). IRAK-4 kinase was rendered inactive by mutating the conserved lysine residues in the ATP pocket essential for coordinating ATP. Analyses of embryonic fibroblasts and macrophages obtained from IRAK-4 KD mice demonstrate lack of cellular responsiveness to stimulation with IL-1β or a Toll-like receptor 7 (TLR7) agonist. IRAK-4 kinase deficiency prevents the recruitment of IRAK-1 to the IL-1 receptor complex and its subsequent phosphorylation and degradation. IRAK-4 KD cells are severely impaired in NFκB, JNK, and p38 activation in response to IL-1β or TLR7 ligand. As a consequence, IL-1 receptor/TLR7-mediated production of cytokines and chemokines is largely absent in these cells. Additionally, microarray analysis identified IL-1β response genes and revealed that the induction of IL-1β-responsive mRNAs is largely ablated in IRAK-4 KD cells. In summary, our results suggest that IRAK-4 kinase activity plays a critical role in IL-1 receptor (IL-1R)/TLR7-mediated induction of inflammatory responses.

IL-1 receptor (IL-1R), IL-18 receptor (IL-18R), and Toll-like receptors (TLRs) are important mediators of innate immune responses (1). The signaling cascades initiated by these receptors are involved in host defense mechanisms, fever induction, acute and chronic inflammation, obesity, and immune modulation. IL-1β-mediated activation of IL-1R leads to induction of cellular signaling pathways, involving a cascade of adaptor molecules, kinases, and transcription factors. Although IL-1R and TLRs exhibit structurally distinct extracellular domains, they share an intracellular Toll/IL-1R (TIR) homology domain essential for interactions with downstream signaling components (2). Stimulation of the IL-1R triggers engagement of the IL-1R accessory protein (IL-1RacP) followed by binding of the intracellular adaptor protein myeloid differentiation factor 88 (MyD88) via interactions of TIR domains. This leads to recruitment of IL-1R-associated kinases (IRAKs), IRAK-4, IRAK-1, and tumor necrosis factor-associated factor 6 (TRAF6) to the receptor complex (3, 4). As a consequence, IRAK-1 is phosphorylated (5, 6), and later ubiquitylated and degraded (7). Hyperphosphorylated IRAK-1 together with TRAF6 leaves the receptor complex and interacts with the transforming growth factor β-activated kinase 1 (TAK1) multiprotein signalosome (8). Subsequently, this new complex triggers induction of downstream signaling events, including IkBα degradation and NFκB activation and inhibition of c-Jun and Jun-N-terminal kinases (JNK) (9), leading to activation of transcription factors such as NFκB and AP-1. These transcription factors are involved in control of expression of many pro-inflammatory genes encoding cytokines, chemokines, adhesion molecules, and proteolytic enzymes (10, 11). As TLRs also signal via TIR domains, their signaling is entirely or in part dependent upon IRAK-4 (12).

IRAK-4 has been reported to be pivotal for IL-1R-, IL-18R-, and TLR-induced signaling (13). Analysis of IRAK-4-deficient mice (−/−) revealed that IRAK-4 is essential for mediating the majority of innate immune responses (14). Macrophages and mouse embryonic fibroblasts (MEFs) derived from IRAK-4−/− mice exhibit severe defects in cellular signaling in response to IL-1β and TLRs ligands, namely suppression of IkBα degradation and NFκB activation and inhibition of c-Jun and p38 phosphorylation. Consequently, the production of pro-inflammatory cytokines, such as IL-6, IL-1β, and tumor necrosis factor α (TNFα), is reduced. The animals that lack IRAK-4 also fail to produce cytokines in response to lipopolysaccharide challenge (14). In addition, these mice are sensitive to bacterial infections. Recently, patients with recurrent infections and devoid of IRAK-4 protein due to gene mutations have

---

The abbreviations used are: IL, interleukin; IL-1R, IL-1 receptor; IRAK, IL-1R-associated kinase; TLR, Toll-like receptor; TNF, tumor necrosis factor; MEF, mouse embryonic fibroblast; BMDM, bone marrow-derived macrophage; ES, embryonic stem; WT, wild type; RT, reverse transcription; MyD88, myeloid differentiation factor 88; KD, kinase-deficient; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase.
been described (15, 16). Their phenotype is reminiscent of the phenotype observed in mice devoid of TLR signaling in regard to increased susceptibility for bacterial infections. Although the crucial function of IRAK-4 protein in signaling has been demonstrated, controversial data exist concerning the nature of its kinase activity. On one hand, reconstitution of mouse-derived IRAK-4-deficient cells with kinase-inactive IRAK-4 has been insufficient to rescue IL-1β-induced activation of NFκB and JNK as compared with overexpression of wild type IRAK-4 (17). This implies that the kinase activity of IRAK-4 is a key requirement for the optimal transduction of IL-1R-mediated signaling, suggesting that IRAK-4 acts more as an adaptor protein rather than an essential kinase (18). The latter finding is reminiscent of the observation that IRAK-1, a kinase similar in structure and also present in the signaling complex together with IRAK-4 and MyD88, does not require its kinase activity for signal transduction (19, 20). As it is thought that IRAK-4 phosphorylates IRAK-1 (5, 6) to activate IRAK-1 kinase, the concept of an essential role of the kinase activity of IRAK-4 in TIR domain-mediated signaling is not obvious.

To clarify the biochemical function of IRAK-4 kinase, we have generated genetically engineered mice expressing a kinase-deficient mutant of this protein (IRAK-4 KD). MEFs and bone marrow-derived macrophages (BMDMs) isolated from these mice were characterized for their ability to respond to IL-1R or TLR7. Our analysis demonstrated that IRAK-4 kinase activity is crucial for IL-1R- and TLR7-mediated signaling and expression of pro-inflammatory genes in these cells. Additionally, we identified a set of genes whose expression is dependent upon the presence of IRAK-4 kinase activity.

**EXPERIMENTAL PROCEDURES**

*Generation of IRAK-4 KD Mice*—To generate a targeting construct for homologous recombination in mouse embryonic stem (ES) cells, the mouse IRAK-4 genomic sequence spanning the region from intron 4 to intron 6 (Ensembl ID: ENSMUSG00000059883) was amplified and subcloned into vector pRAY2 (accession number U63120). Site-directed mutagenesis was performed to change K213A/K214A in exon 5. The targeting plasmid was transfected into ES cells from Balb/c mice. Transfected ES cells were selected for neomycin resistance. Homologous recombination was identified by PCR and confirmed by Southern hybridization. The loxp-flanked neomycin cassette was eliminated by co-expression of Cre recombinase. The neomycin-sensitive ES cell clones were amplified and analyzed by PCR and Southern blot. ES cells with homologous recombination were injected into C57Bl/6 host blastocysts. Germline transmission in F1 heterozygous offspring was verified by PCR analysis, and F1 heterozygotes were interbred to obtain homozygous mutant mice. F2 animals were genotyped by PCR using two sets of primers IRAK4/IRAK4 AA and IRAK4/IRAK4 KK (IRAK4, 5′-TTGAGCAGATGTCAGTCAT-GGA-3′; IRAK4 KK, 5′-TTACCGCTCCGAGCTTCTTC-3′; IRAK4 AA, 5′-TTACCGCTCCAGCGCCGG-3′).

*Cell Culture*—Wild type (WT) and IRAK-4 KD MEFs isolated from Balb/c mice were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum (Invitrogen) at 37 °C in a humidified CO2 (5%) incubator. BMDMs were isolated as described previously (21) and maintained in macrophage-SFM medium containing 10% fetal calf serum and 30% L-929-cell-conditioned medium as a source of macrophage colony-stimulating factor. IRAK-4−/− BMDM were derived from respective mice described by Suzuki et al. (14).

*Plasmids*—The plasmids IL-6 and IL-6 (-AP1) were obtained by inserting the PCR-amplified −495 and −225 bp, respectively, human IL-6 promoter fragments into the HindIII/Xhol-digested pGL4.14 luciferase vector (Promega). The IL-6 (-NFκB) construct was made by site-directed mutagenesis on the IL-6 plasmid using an oligonucleotide mutated in the consensus NFκB binding site (5′-AATGTAATATTTTCCCATG-3′, underlined are mutated nucleotides) and QuikChange site-directed mutagenesis kit (Stratagene). The NFκB promoter plasmid was constructed by inserting five repeats of the NFκB consensus binding site into Nhel/BglII of pGL4.14. The pRL-SV40 plasmid was obtained from Promega.

*Transfection-based Reporter Gene Assays*—MEF cells were seeded in 24-well plates 24 h prior to transfection. Transfections of 1 μg/well of one of the pGL4.14 plasmids were carried out with Lipofectamine 2000 (Invitrogen) according to the standard protocol. In each transfection, 10 ng of pRL-SV40 was added. The cells were then serum-starved overnight and subsequently stimulated with 1 ng/ml mouse IL-1β or 10 ng/ml TNFα for 8 h. Cell extracts were assayed for luciferase activity with the Dual-Luciferase reporter assay system (Promega) using a Luminoskan Ascent luminometer (Thermo LabSystems).

*Immunoprecipitation and Western Blot Analysis*—MEF or BMDM cells were plated, and 24 h later, the cultures were serum-starved for 5 h and then stimulated with 1 ng/ml mouse IL-1β (R&D Systems) or 10 ng/ml TNFα (human recombinant TNFα produced within Novartis Pharma AG) or 0.5 μg/ml resiquimod (R-848; Alexis Corp.) and harvested in standard Laemmli buffer. For immunoprecipitations, cell lysates were prepared, and immunoprecipitation analyses were carried out as described previously (22). Western blot analyses were done by standard procedures as described elsewhere (23), and signals were detected by chemiluminescence reagent (PerkinElmer Life Sciences). The IRAK-4 antibody was purchased from ProSci Inc., the IL-1RI antibody was from R&D Systems, the IRAK-1 and IL-1RI antibodies were from Santa Cruz Biotechnology Inc., and antibodies to IκBα, phospho-NFκB p65 (Ser-536), phospho-SEK1/MKK4 (Thr-261), phospho-stress-activated protein kinase (SAPK)/JNK (Thr-183/Tyr-185), phospho-p38 MAPK (Thr-180/Tyr-182) (3D7), phospho-Akt (Ser-473), phospho-p44/42 MAPK (Thr-202/Tyr-204), and p44/42 MAPK were from Cell Signaling Technology Inc.

*Gene Expression Analysis Using DNA Microarrays*—MEF cell cultures were serum-starved overnight and then stimulated with 1 ng/ml mouse IL-1β (R&D Systems). Total RNA was pre-
pared from duplicate samples according to the Qiagen RNeasy protocol (Qiagen). Total RNA was converted to biotin-labeled cRNA according to the NuGEN Ovation protocol (NuGEN Technologies). After purification with Qiagen RNeasy spin columns and chemical fragmentation, the cRNAs were hybridized to Affymetrix mouse genome 430 2.0 microarrays (Affymetrix). Hybridization, washing, and staining of the microarrays were done according to Affymetrix standard protocols. The arrays were scanned using an Agilent Technologies GeneChip Scanner 3000 (Agilent Technologies), and the images were converted to expression values using the Affymetrix Microarray Analysis Suite 5.0 software. Further data analysis was done on per chip normalized expression values using the Silicon Genetics software GeneSpring (version 7.2). When searching for genes with relevant expression profiles, only those probe sets that had absolute expression calls of “Present” in both duplicates of at least one of the conditions examined were taken into consideration. A further requirement was that the duplicate average had to reach an expression value of at least 0.5 in at least one of the conditions examined (chip normalization was to a trimmed average of 1.0). The resulting initial gene lists were further analyzed by filtering for -fold change differences as appropriate. The array data have been deposited in the Gene Expression Omnibus at National Center for Biotechnology Information (NCBI) with GEO accession number GSE6789.

**Quantitative RT-PCR**—MEFs were treated, and RNA was prepared as above. cDNA was synthesized using 750 ng of total RNA samples with the QuantiTect reverse transcription kit (Qiagen). PCR was done using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). 2 μl of diluted cDNA (in three serial dilutions; 1:5, 1:15, and 1:45) were used for each PCR in a final reaction volume of 10 μl. Each primer was used at 200 nM. The PCR was run on a 7500 Fast real-time PCR system (Applied Biosystems) with the following conditions: 50 °C for 2 min hold (uracil-DNA-glycosylase incubation), 95 °C for 2 min hold, 95 °C, 3 s, and 60 °C, 30 s (40 cycles). The assays were run using the absolute quantification profile (standard curve) in the 7500 Fast System SDS software. The three serial dilutions of the non-stimulated WT cDNA were used with arbitrary copy numbers to create the standard curve. The following primers were used: for IRAK-4, 5'-CCTGGATGTCTCTGGAACTT-3' and 5'-CAACAGCGATGCAGACAAGA-3'; for Ccl2, 5'-TGGCTCAGCCAGATGCAGTTA-3' and 5'-TGGTCTGCGGATGCATTCGTCCTT-3'; for Ccl7, 5'-TGCGATAGGGCGCTGGTCTT-3' and 5'-CCGACTACTGTTATCCTTCT-3'; for Cxcl1, 5'-AATGAGCTGGCTGTCAGTGC-3' and 5'-GCTTGCGCACTCGTCTTCTT-3'; for IRAK4, reverse (recognizing the mutated allele), and forward, IRAK4, reverse, IRAK4 AA (recognizing the mutated allele). Numbers indicate the tail biopsy sample: 26, heterozygous; 27, wild type; 30, homozygous; 31, homozygous. C, equal expression of IRAK-4 mRNA, validated by quantitative RT-PCR, in WT and IRAK-4 KD samples isolated from MEF, BMDM, and spleen cells. The y axis represents the expression of IRAK-4 normalized to glyceraldehyde-3-phosphate dehydrogenase expression. The -fold expression in KD samples was calculated as compared with the expression in WT samples (set as 1-fold). The results presented are the means ± S.E. of experiments performed in triplicates.

**Cytokine Analysis**—MEF or BMDM cells were seeded in 12-well plates. The following day, cells were serum-starved for 5 h before stimulation with 1 ng/ml mouse IL-1β (R&D Systems). After the indicated periods of time, supernatant was collected, and cell debris was removed by centrifugation (14 000 × g, 1 min). For detection of secreted IL-6, TNFα, Ccl2, and Cxcl1, cellular supernatants were analyzed using the Bio-Plex cytokine assay method (Bio-Rad Laboratories Inc.) according to the manufacturer’s instructions.
RESULTS

The Kinase Activity of IRAK-4 Is Essential for IL-1β-mediated Signaling Pathways—To investigate the physiological role of IRAK-4 kinase activity, “knock-in” mice were generated by replacing the WT gene with a gene containing a mutation in the IRAK-4 kinase domain (Fig. 1A). The previously described K213A,K214A mutation replaces two conserved lysine residues in the ATP binding site that are essential for the hydrolysis of ATP (5, 24). This mutation abolishes IRAK-4 kinase activity without affecting other protein features, namely interaction with the receptor complex via MyD88 adaptor protein (5). The genotype of mice carrying this mutation in the IRAK-4 gene was confirmed by RT-PCR analysis (Fig. 1B), and the respective homozygous mutant mice (IRAK-4 KD) were used in further studies. IRAK-4 KD mice were viable and reproduced as WT mice. Quantitative RT-PCR confirmed the presence of IRAK-4 mRNA in equal amounts in MEF, BMDM, and spleen cells isolated from both WT and IRAK-4 KD mice.

To determine the effect of IRAK-4 kinase deficiency on the signaling pathways, analysis of receptor complex formation, expression, and phosphorylation of signaling components in response to cytokine stimulation were performed on cellular extracts of MEFs isolated from WT and IRAK-4 KD mice. The analyses clearly demonstrated that the absence of IRAK-4 kinase activity affected the IL-1β-induced pathways. Co-immunoprecipitation experiments showed that IRAK-1 protein interacts with IRAK-4 in both cell lines, even in non-stimulated conditions (Fig. 2A). However, IL-1β promotes increased binding of IRAK-1 to IRAK-4 only in WT but not IRAK-4 KD MEFs. Additionally, the phosphorylated higher molecular weight form and decreased protein levels of IRAK-1 occurred exclusively in WT MEFs as opposed to IRAK-4 KD MEFs upon IL-1β stimulation (Figs. 2A and 3A). From these experiments, we conclude that IRAK-4 KD MEFs lack IRAK-4 kinase activity. To further examine the consequences of disrupting this activity, we compared the responsiveness of the NFκB reporter gene to different stimuli in WT and IRAK-4 KD MEFs. The activation of the promoter by both IL-1β and TNFα via the NFκB element was apparent in WT cells (Fig. 4A). However, the kinase deficiency...
The Role of IRAK-4 Kinase Activity

**A**

**NF\(\kappa\)B promoter**

|            | WT       | IRAK-4 KD |
|------------|----------|-----------|
| IL-1\(\beta\) | **3.0**  | **2.5**   |
| TNF\(\alpha\) | **0.5**  | **0.5**   |

**B**

**IL-6 promoter**

|            | WT       | IRAK-4 KD | + IL-1\(\beta\) | + [IL-1\(\beta\) + TNF\(\alpha\)] |
|------------|----------|-----------|-----------------|----------------------------------|
| IL-6       | **5.0**  | **3.0**   | **2.5**         |                                  |
| IL-6(-AP1) | **5.0**  | **3.0**   | **2.5**         |                                  |
| IL-6(-NF\(\kappa\)B) | **5.0**  | **3.0**   | **2.5**         |                                  |

**FIGURE 4.** Effect of IRAK-4 kinase deficiency on NF\(\kappa\)B and AP-1 regulated promoters. WT or IRAK-4 KD MEF cells were transfected with luciferase reporter plasmid and control Renilla reporter plasmid. After 24 h, cells were left untreated or treated with IL-1\(\beta\) (1 ng/ml) or TNF\(\alpha\) (10 ng/ml) for 8 h and assayed for luciferase and Renilla activities. A, analysis of NF\(\kappa\)B-dependent reporter gene. B, comparison of human wild type IL-6 promoter reporter gene (IL-6), deletion mutant of IL-6 promoter missing AP-1 binding element, IL-6 (-AP1), and promoter with mutation in NF\(\kappa\)B binding element, IL-6 (-NF\(\kappa\)B). The luciferase activity was normalized with the Renilla activity, and fold activation was calculated relative to the activity of WT untreated cells (set as 1-fold). The results presented are the means ± S.E. of two independent experiments performed in triplicates. *, \(p < 0.05\); ***, \(p < 0.001\) indicates a significant difference between WT and IRAK-4 KD samples of the same treatment.

of IRAK-4 prevented IL-1\(\beta\)-mediated, but not TNF\(\alpha\)-mediated, stimulation of the promoter. These data confirmed that the kinase activity of IRAK-4 is essential for NF\(\kappa\)B induction and that the effect we observed is specific for IL-1\(\beta\)-stimulated pathways.

IL-6 cytokine is known to be one of the main targets of IL-1\(\beta\)-mediated NF\(\kappa\)B-dependent transcriptional regulation (25); therefore the induction of the IL-6 reporter gene was also assessed in both WT and IRAK-4 KD MEFs (Fig. 4B). In these experiments, wild type IL-6 promoter (-495 bp) as well as different mutants were analyzed. The results showed that IL-1\(\beta\) only significantly activated the IL-6 promoter when the kinase activity of IRAK-4 was intact. Furthermore, the functional mutation of NF\(\kappa\)B or deletion of AP-1 elements inhibited IL-6 gene transcription in both WT and IRAK-4 KD MEFs, indicating that both elements are essential in regulation downstream of IRAK-4 kinase activity.

**IRAK-4 KD Affects Expression of Pro-inflammatory Genes**—Based on the fact that IL-1\(\beta\)-induced transcriptional activity was disrupted in IRAK-4 KD MEFs, we anticipated that RNA expression of IL-1\(\beta\)-induced genes will also be affected. To identify these genes, we performed a cDNA microarray experiment. After 1 h of IL-1\(\beta\) stimulation as compared with no treatment, expression of 41 genes and expressed sequence tags was induced at least 5-fold in WT cells. In contrast, in IRAK-4 KD cells, 36 of these genes were not induced at all or had a minor increase in expression, less than 50% of WT induction (Fig. 5A and supplemental Table 1). The other five IL-1\(\beta\)-stimulated genes were affected to lesser extend in IRAK-4 KD. Interestingly, the strongly affected genes belong to a group encoding proteins with known relevance to inflammation, including cytokines (IL-6, Lif), chemokines (Cxc1l, Cxcl2, Cxcl5, Ccl2, Ccl7, Ccl20), receptors (interferon \(\gamma\) receptor 1 (IFN\(\gamma\)R1)), other signaling molecules (Gem, Rasa2, Rgs16, Map3k8, Ikb\(\alpha\), TNF\(\alpha\)ip3, Pde4b, Plocr1), transcription factors (Atf3, Irf1, IkB\(\gamma\), FosB), and mRNA stability regulators (Zfp36). Also, NF\(\kappa\)B-dependent anti-apoptotic Birc3 and Btg2 were not induced in IRAK-4 KD cells in contrast to WT cells. Moreover, expression of a further 54, mainly pro-inflammatory, genes was strongly elevated in WT but not in IRAK-4 KD cells after prolonged IL-1\(\beta\) stimulation, measured at 4 h (supplemental Table 2).

To validate the results of microarray hybridization in independent samples, quantitative PCR analyses were performed on six representative mRNAs identified by microarray. IL-6, Cxc1l, Cxcl2, Ccl7, Ikb\(\gamma\), and Fos mRNA expression levels in response to IL-1\(\beta\) stimulation, detected by PCR analysis (Fig. 5B), were similar to those observed in microarray experiments (Fig. 5A and supplemental Tables 1 and 2). It is noteworthy that most of the mRNAs appeared not at all or only marginally induced in IRAK-4 KD MEFs at later time points after IL-1\(\beta\) stimulation, whereas e.g. the Ccl7 mRNA was significantly elevated starting at 2 h of stimulation. Additionally, PCR analyses of expression of Fos mRNA showed that early induced genes, identified in microarray experiment as insignificantly affected in IRAK-4 KD cells, in fact show a considerable difference in expression between WT and IRAK-4 KD when measured at earlier time point. Thus, all these RNA expression analyses allowed us to identify a number of genes whose expression is IRAK-4 kinase activity-regulated.

To confirm results obtained from mRNA quantification, protein levels of IL-6, Cxc1l, and Cxcl2 were measured in the supernatants of IL-1\(\beta\)-stimulated cell cultures (Fig. 6A). As expected, the production of these cytokines was significantly up-regulated in WT MEFs. However, the kinase deficiency of IRAK-4 dramatically reduced the protein expression of these cytokines. The kinase deficiency of IRAK-4 did not affect the TNF\(\alpha\)-activated cytokine production (Fig. 6B). Taken together, all these analyses demonstrate that the kinase activity of
FIGURE 5. Microarray-based identification of IL-1β-induced and IRAK-4 kinase activity-dependent genes. A, WT or IRAK-4 KD MEF cells were either left untreated or stimulated with IL-1β (1 ng/ml) for 1 h. Total RNA was extracted and used for GeneChip microarray analysis. Levels of gene expression are indicated as fold inductions relative to unstimulated controls. Shown are mRNAs induced >5-fold in WT cells. Fold regulations are presented in color code as indicated at the bottom: green for 0-fold, black for 5-fold, and red for 20-fold (or more). Genes with >50% (upper panel) and <50% (lower panel) difference in expression between IRAK-4 KD and WT samples are presented. Gene names or identifiers corresponding to the expressed sequence tags are shown on the left. Data represent averages of assays done in duplicate. B, expression of selected genes was validated by quantitative RT-PCR. Cells were treated with IL-1β (1 ng/ml) for the indicated periods of time, total RNA was extracted, and mRNA expression was determined. Shown is the expression of IL-6, Cxcl1, Ccl2, IκBα, and Fos normalized to glyceraldehyde-3-phosphate dehydrogenase expression. The fold induction was calculated as compared with the expression of WT untreated cells (set as 1-fold). The results presented are the means ± S.E. of experiments performed in triplicates.
IRAK-4 is essential for the IL-1β/H9252-mediated production of most of the pro-inflammatory cytokines in MEFs.

The IRAK-4 Kinase Activity Regulates Signaling Pathways Downstream of TLR7—Macrophages are reactive to microbial products that activate pro-inflammatory responses via TLR-mediated signaling. Individual TLRs transduce signals via divergent pathways: MyD88-dependent pathways, highly similar to the IL-1β/H9252-mediated pathway, or/and MyD88-independent pathways (1). As IRAK-1/4-mediated signaling is confined to the MyD88-dependent pathway, we assessed the role of IRAK-4 kinase activity in TLR-mediated signaling using resiquimod, a known inducer of TLR7, that signals only via MyD88-dependent pathway (1). Stimulation of TLR7 in WT cells led to activation of NFκB and MAPK pathways, reflected by a decrease in levels of IRAK-1 and IκBα, and an increase in JNK, p38, and ERK1/2 phosphorylation (Fig. 7). Conversely, these effects were significantly abolished in IRAK-4 KD and IRAK-4⁻/⁻ BMDMs. These findings indicate that the IRAK-4 kinase activity is necessary for TLR7-mediated activation of the NFκB, JNK, and p38 pathways, similar to what has been observed for IL-1β-mediated signal transduction. In addition, IRAK-4 kinase activity downstream of TLR7 is required for ERK1/2 activation in BMDM.

Since activated macrophages are directly involved in processes of inflammation and produce most of the pro-inflammatory mediators, we examined how kinase deficiency is affecting gene expression of these mediators. mRNA levels of TNFα, IL-1β, IL-6, Cxcl1, IκBα/H9252, and IL-10 were strongly up-regulated in WT BMDMs stimulated with resiquimod (Fig. 8A). Stimulation of IRAK-4 KD and IRAK-4⁻/⁻ cells did not cause a significant increase in the corresponding transcripts. In addition to the mRNAs presented in Fig. 8A, we analyzed the mRNAs for Cxcl10, Ccl7, and Ccl2 (data not shown) and found again no induction in IRAK-4 KD or IRAK-4⁻/⁻ cells, suggesting that the gene expression pattern of these two cell lines is similar. We next studied whether inhibition of mRNA expression in IRAK-4 KD and IRAK-4⁻/⁻ cells did not cause a significant increase in the corresponding transcripts. In addition to the mRNAs presented in Fig. 8A, we analyzed the mRNAs for Cxcl10, Ccl7, and Ccl2 (data not shown) and found again no induction in IRAK-4 KD or IRAK-4⁻/⁻ cells, suggesting that the gene expression pattern of these two cell lines is similar. We next studied whether inhibition of mRNA expression in IRAK-4 KD and IRAK-4⁻/⁻ cells did not cause a significant increase in the corresponding transcripts. In addition to the mRNAs presented in Fig. 8A, we analyzed the mRNAs for Cxcl10, Ccl7, and Ccl2 (data not shown) and found again no induction in IRAK-4 KD or IRAK-4⁻/⁻ cells, suggesting that the gene expression pattern of these two cell lines is similar. We next studied whether inhibition of mRNA expression in IRAK-4 KD and IRAK-4⁻/⁻ cells did not cause a significant increase in the corresponding transcripts. In addition to the mRNAs presented in Fig. 8A, we analyzed the mRNAs for Cxcl10, Ccl7, and Ccl2 (data not shown) and found again no induction in IRAK-4 KD or IRAK-4⁻/⁻ cells, suggesting that the gene expression pattern of these two cell lines is similar. We next studied whether inhibition of mRNA expression in IRAK-4 KD and IRAK-4⁻/⁻ cells did not cause a significant increase in the corresponding transcripts. In addition to the mRNAs presented in Fig. 8A, we analyzed the mRNAs for Cxcl10, Ccl7, and Ccl2 (data not shown) and found again no induction in IRAK-4 KD or IRAK-4⁻/⁻ cells, suggesting that the gene expression pattern of these two cell lines is similar. We next studied whether inhibition of mRNA expression in IRAK-4 KD and IRAK-4⁻/⁻ cells did not cause a significant increase in the corresponding transcripts. In addition to the mRNAs presented in Fig. 8A, we analyzed the mRNAs for Cxcl10, Ccl7, and Ccl2 (data not shown) and found again no induction in IRAK-4 KD or IRAK-4⁻/⁻ cells, suggesting that the gene expression pattern of these two cell lines is similar. We next studied whether inhibition of mRNA expression in IRAK-4 KD and IRAK-4⁻/⁻ cells did not cause a significant increase in the corresponding transcripts. In addition to the mRNAs presented in Fig. 8A, we analyzed the mRNAs for Cxcl10, Ccl7, and Ccl2 (data not shown) and found again no induction in IRAK-4 KD or IRAK-4⁻/⁻ cells, suggesting that the gene expression pattern of these two cell lines is similar. We next studied whether inhibition of mRNA expression in IRAK-4 KD and IRAK-4⁻/⁻ cells did not cause a significant increase in the corresponding transcripts. In addition to the mRNAs presented in Fig. 8A, we analyzed the mRNAs for Cxcl10, Ccl7, and Ccl2 (data not shown) and found again no induction in IRAK-4 KD or IRAK-4⁻/⁻ cells, suggesting that the gene expression pattern of these two cell lines is similar. We next studied whether inhibition of mRNA expression in IRAK-4 KD and IRAK-4⁻/⁻ cells did not cause a significant increase in the corresponding transcripts. In addition to the mRNAs presented in Fig. 8A, we analyzed the mRNAs for Cxcl10, Ccl7, and Ccl2 (data not shown) and found again no induction in IRAK-4 KD or IRAK-4⁻/⁻ cells, suggesting that the gene expression pattern of these two cell lines is similar.
The Role of IRAK-4 Kinase Activity

The importance of IRAK-4 protein in the control of innate immunity responses is well established. However, despite much research since the initial discovery of IRAK-4, the importance of its catalytic function in IL-1/TLR signaling pathways remains elusive. Different reports assigned various functions to IRAK-4, describing it as a kinase and/or an adaptor protein through which downstream kinase cascades get activated. Although Li et al. (5) demonstrated that the kinase activity of IRAK-4 is essential for transmitting the signal from the IL-1R, Qin et al. (18) suggested that the kinase-inactive IRAK-4 is sufficient for IL-1R-mediated signaling. Lye et al. (17) reported both kinase-dependent and partially scaffold-dependent functions of IRAK-4. However, all of these studies relied on reconstitution of IRAK-4-deficient cellular systems by overexpression of IRAK-4 protein. In our studies, we used the IRAK-4 KD knock-in mouse as an experimental model. This approach provides a system where all signaling components, including IRAK-4 KD protein, are present at physiological levels. These mice therefore differ from the previously described IRAK-4−/− mice, which are completely devoid of IRAK-4 protein, and do not allow us to differentiate between the function of IRAK-4 as a scaffold protein or a kinase (14). Our analysis of different cell types derived from IRAK-4−/− mice revealed that the kinase activity of IRAK-4 is critical for IL-1R/TLR-mediated activities. Namely, IRAK-4 kinase is essential for optimal IL-1β-induced receptor complex formation, signaling activation, induction of transcription, and as a consequence, production of pro-inflammatory cytokines and chemokines. Similarly, our data showed an important role of IRAK-4 kinase activity in TLR7-mediated MyD88-dependent signaling. It also appears that IRAK-4 function is not cell type-dependent as suggested by previous studies (17, 18). We observed comparable results in MEF cells as well as adult BMDMs in respect to IRAK-4 involvement in signaling and control of gene expression.

The results we present here are in sharp contrast to conclusions drawn from experiments with human IRAK-4-deficient fibroblasts. Although we believe that these discrepancies might be due to the experimental approach, we cannot formally exclude that species differences between human and mouse IRAK-4-mediated signaling exist. An example for such a difference is tyrosine kinase 2 (Tyk2), which appears to function differently in mice and humans (26). However, such a species disparity appears unlikely for IRAK-4 given the high conservation throughout phylogeny of this pathway. Our analysis of receptor complex formation is compatible with the existing model of IL-1β-mediated interactions of IRAK-1, IRAK-4, and MyD88 (4) but also reveals new features of this system. Here we demonstrate that there is a stable interaction between IRAK-4 and IRAK-1, even in the unstimulated state, which has not been described before. IL-1R activation leads to increased interaction of these two components, accompanied by IRAK-1 phosphorylation and its binding to the IL-1R in WT cells. However, the increased binding of IRAK-1 to IRAK-4 and IRAK-1 recruitment to the IL-1R complex in response to IL-1β is severely impaired in IRAK-4 KD cells, suggesting that the kinase activity of IRAK-4 facilitates or stabilizes the interaction of IRAK-1 with the receptor complex. In line with these findings, cells lacking IRAK-4 show no recruitment of IRAK-1 to IL-1R after IL-1β stimulation (17).

Our observation that IRAK-1 does not undergo phosphorylation and degradation in IRAK-4 KD cells stimulated with IL-1β supports the view of IRAK-4 as IL-1/TLR first proximal kinase, suggested by previous in vitro studies with recombinant enzymes (5, 6) and studies of the IL-1R complex (27). In these studies, IRAK-1 has been proposed as a substrate of IRAK-4 kinase activity. Two crucial sites (Thr-387 and Ser-376) in the activation loop of IRAK-1 have been identified as a potential target for IRAK-4 kinase activity in peptide phosphorylation assays (5, 6). Additional insights into IRAK-4-mediated regulation of IRAK-1 have been gained from studies of co-expression of MyD88s, an alternatively spliced variant of MyD88. MyD88s prevents recruitment of IRAK-4 into receptor complex and thus inhibits IRAK-4-mediated IRAK-1 phosphorylation (27). Moreover, the recently published structure of the human IRAK-4 kinase domain revealed that IRAK-4 contains characteristic structural features of an active serine/threonine kinase (28). Also, work by Cheng et al. (29) suggests autocatalytic phosphorylation of IRAK-4, which strongly supports the concept of IRAK-4 as first proximal kinase in the IL-1R complex.

In our studies, we characterized in detail the IRAK-4 kinase-regulated signaling relevant for MyD88-dependent pathways. We observed complete absence of activation of the NFκB, p38, and JNK pathways in IRAK-4 KD cells in response to stimulation with IL-1β or resiquimod, suggesting that the presence of IRAK-4 protein without a kinase activity is not sufficient to relay signals from the IL-1R or TLR7. We also observed IRAK-4 kinase activity-dependent stimulation of ERK1/2 via TLR7 in BMDMs. This is in contrast to IRAK-4 KD MEFs, where the activation of the ERK1/2 pathway downstream of the IL-1R, previously described as MyD88-independent (22, 30), was not attenuated. This observation may suggest that receptor-specific and/or cell type-specific divergence of ERK1/2 activation exists.

Analysis using a more sensitive IL-6 promoter-driven luciferase reporter system, dependent upon both IkB and stress kinase (JNK and p38) pathways, confirmed the importance of IRAK-4 kinase activity in regulation of these pathways. Importantly, we did not observe any difference in signaling pathways, as well as in mRNA expression and cytokine production between resiquimod-induced IRAK-4−/− and IRAK-4 KD macrophages. The fact that deletion of the whole protein or just elimination of its kinase features results in blocking of pathway activation supports our notion that the kinase activity is crucial for the function of the IRAK-4 protein.

By microarray analysis, we identified 90 genes whose transcription is strongly dependent on IRAK-4 kinase-mediated activity in MEF cells. Not surprisingly, these are mostly chemokines, cytokines, receptors, signaling molecules, and transcription factors known to be involved in inflammatory responses.
The Role of IRAK-4 Kinase Activity

Among them are also negative regulators that control inflammatory responses as part of a negative feedback loop. Atf3, a member of the CREB/Atf family, inhibits IL-6 and IL-12b transcription by altering chromatin structure and restricting access of positive transcription factors (31). Zfp36 encodes tristetraprolin, a zinc-finger protein that modulates TNFα mRNA stability by binding of the AU-rich element in its 3' untranslated region and promotes destruction of the TNFα mRNA (32). In fact, blocking of negative feedback regulators might be responsible for the partial recovery of mRNA and protein expression of some chemokines, such as Ccl7 or Ccl2, observed in our analyses after prolonged IL-1β stimulation in MEF cells (Figs. 5B and 6). We speculate that also the non-disrupted activity of PKB and ERK1/2 pathways might be responsible for low level or delayed expression of some mRNAs, possibly as an indirect effect. This might also be a reason why Lye et al. (17) in their studies observed partial increase in IL-6 production in IRAK-4 KD reconstituted IRAK-4−/− MEFs, measured after 24 h of IL-1β stimulation. However, overall cytokine and chemokine production was severely impaired in IRAK-4 KD cells presented in this study.

Recently published data revealed a new critical role of IRAK-4 in adaptive immune responses (33). IRAK-4-deficient T cells showed impaired T-cell receptor-mediated signaling and blocked NFκB activation and T-cell proliferation following anti-CD3 antibody stimulation. It would be interesting to elucidate whether IRAK-4 kinase activity is also involved in the regulation of T cell function.

In summary, our finding that IRAK-4 kinase activity has a crucial role in pro-inflammatory responses is not only important for understanding the basic mechanisms of IL-1/TLR-mediated gene activation but may also have implications for the development of anti-inflammatory drugs. It would be interesting to study the effect of IRAK-4 kinase deficiency in animal models of inflammatory diseases. Elucidation of the role of IRAK-4 kinase activity in adaptive immunity might also open new opportunities for future treatment modalities in transplantation.

Acknowledgments—We thank Thierry Doll, Marie-Josephe Duriatti, and Elsebeth Andersen for technical assistance in the isolation of MEFs and macrophages and Martin Letzkus and Nicole Hartmann for performing RNA isolation and cDNA microarray hybridization experiments.

REFERENCES

1. Takeda, K., and Akira, S. (2005) Int. Immunol. 17, 1–14
2. Means, T. K., Golenbock, D. T., and Fenton, M. J. (2000) Cytokine Growth Factor Rev. 11, 219–232
3. Martin, M. U., and Wesche, H. (2002) Biochim. Biophys. Acta 1592, 265–280
4. Janssens, S., and Beyaert, R. (2003) Mol. Cell 11, 293–302
5. Li, S., Strelow, A., Fontana, E. J., and Wesche, H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5567–5572
6. Kollewe, C., Mackensen, A. C., Neumann, D., Knop, J., Cao, P., Li, S., Wesche, H., and Martin, M. U. (2004) J. Biol. Chem. 279, 5227–5236
7. Yamin, T. T., and Miller, D. K. (1997) J. Biol. Chem. 272, 21540–21547
8. Jiang, Z., Ninomiya-Tsuji, J., Qian, Y., Matsumoto, K., and Li, X. (2002) Mol. Cell. Biol. 22, 7158–7167
9. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. (2001) Nature 412, 346–351
10. Tak, P. P., and Firestein, G. S. (2001) J. Clin. Investig. 107, 7–11
11. Wisdom, R. (1999) Exp. Cell Res. 253, 180–185
12. Takeda, K., and Akira, S. (2004) Semin. Immunol. 16, 3–9
13. Suzuki, N., Suzuki, S., and Yeh, W. C. (2002) Trends Immunol. 23, 503–506
14. Suzuki, N., Suzuki, S., Duncan, G. S., Millar, D. G., Wada, T., Mirtsos, C., Takada, 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
15. Medvedev, A. E., Thomas, K., Awomoyi, A., Kuhns, D. B., Gallin, J. I., Li, X., and Vogel, S. N. (2005) J. Immunol. 174, 6587–6591
16. 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 Ghonim, A., Al Rayes, H., Al Jumah, S., Al Hajar, S., Al Muhaisen, I. Z., Fryaha, H. H., Rucker, R., Hawn, T. R., Adedem, A., Tufenkenji, H., Haraguchi, S., Day, N. K., Good, R. A., Gougerot-Pocidalo, M. A., Ozinsky, A., and Canasano, J. L. (2003) Science 299, 2076–2079
17. Lye, E., Mirtsos, C., Suzuki, N., Suzuki, S., and Yeh, W. C. (2004) J. Biol. Chem. 279, 40653–40658
18. Qin, J., Jiang, Z., Qian, Y., Canasano, J. L., and Li, X. (2004) J. Biol. Chem. 279, 26748–26753
19. Knop, J., and Martin, M. U. (1999) FEBS Lett. 448, 81–85
20. Mascher, B., Ray, K., Burns, K., and Volpe, F. (1999) Biochem. J. 339, 227–231
21. Comalada, M., Xaus, J., Valledor, A. F., Lopez-Lopez, C., Pennington, D. J., and Celada, A. (2003) Am. J. Physiol. 285, C1235–C1245
22. Davis, C. N., Tabarean, L., Gaidarova, S., Behrens, M. M., and Bartfai, T. (2006) J. Neurochem. 98, 1379–1389
23. Lane, H. A., Beuvink, I., Motoyama, A. B., Daly, J. M., Neve, R. M., and Hynes, N. E. (2000) Mol. Cell. Biol. 20, 3210–3223
24. Zheng, J., Knighton, D. R., ten Eyck, L. F., Karlsson, R., Xuong, N., Taylor, S. S., and Sowadski, J. M. (1993) Biochemistry 32, 2154–2161
25. Libermann, T. A., and Baltimore, D. (1990) Mol. Cell. Biol. 10, 2327–2334
26. Minegishi, Y., Saito, M., Morio, T., Watanabe, K., Agematsu, K., Tsuchiya, T., Takada, H., Hara, T., Kawamura, N., Ariga, T., Kaneko, H., Kondo, N., Tsuge, I., Yachie, A., Sakiyama, Y., Iwata, T., Bessho, O., Ohishi, T., Joh, K., Imai, K., Kogawa, K., Shinohara, M., Fujieda, M., Wakiguchi, H., Pasic, S., Abinun, M., Ochs, H. D., Renner, E. D., Jansson, A., Belohradsky, B. H., Metin, A., Shimizu, N., Mizutani, S., Miyawaki, T., Nonoyama, S., and Karasuyama, H. (2006) Immunity 25, 745–755
27. Burns, K., Janssens, S., Brisoni, B., Olivos, N., Beyaert, R., and Tschopp, J. (2003) J. Exp. Med. 197, 263–268
28. Wang, Z., Liu, J., Sudom, A., Ayres, M., Li, S., Wesche, H., Powers, J. P., and Walker, N. P. (2006) Structure (Lond.) 14, 1835–1844
29. Cheng, H., Addona, T., Keshishian, H., Dahlstrand, E., Lu, C., Dorsch, M., Li, Z., Wang, A., Ocaín, T. D., Li, P., Parsons, T. F., Jaffée, B., and Xu, Y. (2007) Biochem. Biophys. Res. Commun. 352, 609–616
30. Huang, Q., Yang, J., Lin, Y., Walker, C., Cheng, J., Liu, Z. G., and Su, B. (2004) Nat. Immunol. 5, 98–103
31. Gilchrist, M., Thorsson, V., Li, B., Rust, A. G., Korb, M., Kennedy, K., Hai, T., Bolouri, H., and Adem, A. (2006) Nature 441, 173–178
32. Lai, W. S., Carballo, E., Strum, J. R., Kennington, E. A., Phillips, R. S., and Blackshear, P. J. (1999) Mol. Cell. Biol. 19, 4311–4323
33. Suzuki, N., Suzuki, S., Millar, D. G., Umino, M., Hara, H., Calzascia, T., Yamashita, S., Yokosuka, T., Chen, N. J., Elford, A. R., Suzuki, J., Takeuchi, A., Mirtsos, C., Bouchard, D., Ohashi, P. S., Yeh, W. C., and Saito, T. (2006) Science 311, 1927–1932