**IRF-8/Interferon (IFN-γ) Consensus Sequence-binding Protein Is Involved in Toll-like Receptor (TLR) Signaling and Contributes to the Cross-talk between TLR and IFN-γ Signaling Pathways**

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Toll-like receptor (TLR) and interferon-γ (IFN-γ) signaling pathways are important for both innate and adaptive immune responses. However, the cross-talk between these two signaling pathways is incompletely understood. Here we show that IFN-γ and LPS synergistically induce the expression of proinflammatory factors, including interleukin-1 (IL-1), IL-6, IL-12, NO, and tumor necrosis factor-α (TNF-α). Comparable synergism was observed between IFN-γ and peptidoglycan (PGN; a TLR2 ligand) and poly(I:C) (a TLR3 ligand) in the induction of IL-12 promoter activity. IFN-γ enhanced lipopolysaccharide (LPS)-induced ERK and JNK phosphorylation but had no effect on LPS-induced NF-κB activation. Interestingly, we found that IRF-8+/− macrophages were impaired in the activation of LPS-induced ERK and JNK and the production of proinflammatory cytokines induced by LPS or IFN-γ plus LPS. Retroviral transduction of IRF-8 into IRF-8−/− macrophages rescued ERK and JNK activation. Furthermore, co-immunoprecipitation experiments show that IRF-8 physically interacts with TRAF6 at a binding site between amino acid residues 356 and 305 of IRF-8. Transfection of IRF-8 enhanced TRAF6 ubiquitination, which is consistent with a physical interaction of IRF-8 with TRAF6. Taken together, the results suggest that the interaction of IRF-8 with TRAF6 modulates TLR signaling and may contribute to the cross-talk between IFN-γ and TLR signaling pathways.

IRF-8/interferon consensus sequence-binding protein (ICSBP) is a transcription factor that belongs to the IRF family and plays a role in the innate immune response. IRF-8 is dispensable for the development of macrophages and dendritic cells but is critical for the control of the Th1 immune response during infection. IRF-8 engages in multiple protein-protein interactions and is involved in diverse biological processes such as cell proliferation, differentiation, and apoptosis. Despite its importance, the mechanisms by which IRF-8 regulates the immune response are not fully understood.

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1 The abbreviations used are: TLR, Toll-like receptor; IL, interleukin; TNF, tumor necrosis factor; TRAF6, tumor necrosis factor receptor-associated factor 6; MAP, mitogen-activated protein; Tlr1, T, helper 1; IFN, interferon; LPS, lipopolysaccharide; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; HA, hemagglutinin; RT, reverse transcription; INOS, inducible nitric oxide synthase; GFP, green fluorescent protein; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; PGN, peptidoglycan; Ub, ubiquitin.

**Response to IFNα/β and IFN-γ are vital for both innate and adaptive immunity**

IFN signaling involves a variety of trans- and cis-acting factors and is mediated through DNA motifs, designated the IFN-stimulated response element and IFN-γ-activated sequence, found in promoters of IFN-inducible genes (10). These genes play prominent roles in the control of growth, differentiation, and activation of the immune system. The IRF-8/interferon consensus sequence-binding protein belongs to the IFN regulatory factor (IRF) family of transcription factors, which has 10 members (11, 12). Early reports suggested that IRF-8 is a negative regulator of gene promoters containing an IFN-stimulated response element (13–15). However, recent studies indicate a more complex role in both immunity and cell-cycle regulation (16–19). IRF-8-deficient mice are highly susceptible to several pathogens, including Listeria monocytogenes and lymphocytic choriomeningitis virus, due to defects in both innate and adaptive immunity (20–22). Macrophages from IRF-8−/− mice do not produce IL-12 in response to IFN-γ and LPS. IL-12 production is controlled by multiple transcription factors. The loss of IRF-8 binding to an IFN-stimulated response element site in the IL-12 promoter region in IRF8-deficient macrophages results in a defect in IL-12 production. However, this evidence does not necessarily rule out the possibility that IRF-8 may also affect upstream signaling pathways leading to IL-12 production. In addition, other members of the IRF family such as IRF3, IRF5, and IRF7 are involved in TLR signaling pathways (23–26). Therefore, it is reasonable to speculate that IRF-8 response (4). The TLRs are type I integral membrane glycoproteins, and on the basis of considerable homology in the cytoplasmic region, they are members of a superfamily that includes the interleukin-1 receptor. Eleven members of the TLR family have been identified in mammals (3). The signal pathway activated by TLR stimulation involves, including TIR domain-containing adaptors, IL-1 receptor-associated kinases, and tumor necrosis factor receptor-associated factor 6 (TRAF6) (5). TLR stimulation results in the activation of NF-κB and MAP kinases, leading to production of proinflammatory factors, including IL-1, IL-6, NO, IL-12, and TNF-α. Although the innate immune response triggered by TLRs is necessary for host defense against various pathogens, it is not sufficient to clear infection by intracellular bacteria such as Listeria monocytogenes. Either a Th1 immune response or the Th1 cytokine, IFN-γ, is required to eliminate intracellular bacterial pathogens (6, 7). This suggests that the TLR signaling pathway works synergistically with other pathways to arm macrophages for efficient clearance of pathogens. However, the cross-talk of the TLR pathway with other signaling pathways is incompletely understood.

Responses to IFNα/β and IFN-γ are vital for both innate and adaptive immunity (8, 9). IFN signaling involves a variety of trans- and cis-acting factors and is mediated through DNA motifs, designated the IFN-stimulated response element and IFN-γ-activated sequence, found in promoters of IFN-inducible genes (10). These genes play prominent roles in the control of growth, differentiation, and activation of the immune system. The IRF-8/interferon consensus sequence-binding protein belongs to the IFN regulatory factor (IRF) family of transcription factors, which has 10 members (11, 12). Early reports suggested that IRF-8 is a negative regulator of gene promoters containing an IFN-stimulated response element (13–15). However, recent studies indicate a more complex role in both immunity and cell-cycle regulation (16–19). IRF-8-deficient mice are highly susceptible to several pathogens, including Listeria monocytogenes and lymphocytic choriomeningitis virus, due to defects in both innate and adaptive immunity (20–22). Macrophages from IRF-8−/− mice do not produce IL-12 in response to IFN-γ and LPS. IL-12 production is controlled by multiple transcription factors. The loss of IRF-8 binding to an IFN-stimulated response element site in the IL-12 promoter region in IRF8-deficient macrophages results in a defect in IL-12 production. However, this evidence does not necessarily rule out the possibility that IRF-8 may also affect upstream signaling pathways leading to IL-12 production. In addition, other members of the IRF family such as IRF3, IRF5, and IRF7 are involved in TLR signaling pathways (23–26). Therefore, it is reasonable to speculate that IRF-8
Interacts with the TLR signaling pathways resulting in the cross-talk between TLR and IFN-γ signaling pathways.

In this study, we show that IFN-γ and LPS synergistically activate ERK and JNK kinases resulting in the synthesis of macrophage proinflammatory factors. The activation of ERK and JNK kinases is significantly impaired in macrophages from IRF-8-deficient mice. Furthermore, IRF-8 binds to TRAF6 and regulates TRAF6 ubiquitination. These results show that IRF-8 is an important molecule mediating inflammatory factors. The activation of ERK and JNK kinases resulting in the synthesis of macrophage proinflammatory factors. The activation of ERK and JNK kinases resulting in the synthesis of macrophage proinflammatory factors.

Materials and Methods

Reagents and Antibodies—Lipopolysaccharide (LPS) was from Sigma and IFN-γ was obtained from R&D System (Minneapolis, MN). Antibodies against IRF-8, TRAF6, MyD88, ubiquitin, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Hemagglutinin (HA) monoclonal antibody 12CA5 was from Roche Applied Science; the FLAG monoclonal antibody and antibody against FLAG was from Sigma; antibody against AU1 was from Imgenex. Antibodies against ERK, JNK, and phosphorylated ERK and JNK were obtained from Cell Signaling. Protein G-Sepharose beads were from Amersham Biosciences. Recombinant mouse macrophage-colony stimulating factor and granulocyte macrophage-colony stimulating factor were from Peprotech.

Plasmid Constructs—IRF-8 full-length cDNA and IRF-8 C-terminal deletion mutants were inserted into the mammalian expression vector pcDNA3.1. The HA-ubiquitin plasmid was kindly provided by Dr. Ze’ev Ronai (Mount Sinai School of Medicine). The TRAF6 expression plasmid was from Dr. Adrian Ting (Mount Sinai School of Medicine).

Cell Lines and Peritoneal Macrophage—The RAW264.7 murine macrophage cell line and 293T cell line were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin-streptomycin. CL-2 cells (IRF-8 macrophage cell line and 293T cell line were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, antibiotics, recombinant mouse macrophage-colony stimulating factor (6 ng/ml), and recombinant mouse granulocyte macrophage-colony stimulating factor (6 ng/ml). Peritoneal macrophages were isolated from C57/OUJ mice 3 days after intraperitoneal injection of 2 ml of thioglycollate medium. Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics.

RT-PCR—Total RNA was isolated from tumor cells using TRIzol reagent (Invitrogen). 0.4 μg of RNA was used for each reaction. An RT-PCR protocol was used to determine relative quantities of mRNA after 28 PCR cycles (One-step RT-PCR kit, Qiagen), and quantified relative to glyceraldehyde-3-phosphate dehydrogenase as an internal control. The primers protocol was used to determine relative quantities of mRNA after 28 PCR cycles (One-step RT-PCR kit, Qiagen), and quantified relative to glyceraldehyde-3-phosphate dehydrogenase as an internal control. The primers for all genes tested were analyzed by BLAST search and synthesized by Amersham Biosciences. Recombinant mouse macrophage-colony stimulating factor and granulocyte macrophage-colony stimulating factor were from Peprotech.

IL-12 p40 Promoter Analysis—RAW264.7 cells were transiently transfected using SuperFect (Qiagen). For each transfection, 2.5 μg of plasmid was mixed with 100 μl of Dulbecco’s modified Eagle’s medium (without serum and antibiotics) and 10 μl of SuperFect reagent, incubated at room temperature for 10 min, mixed with 600 μl of Dulbecco’s modified Eagle’s complete medium, and immediately added to the cells in 6-well plates. Luciferase activity was measured 16–24 h later. When indicated, LPS (1 μg/ml) was added to the culture for 6–12 h before harvest. The cells were extracted with reporter lysis buffer (Promega), and 20 μl of extract was assayed for luciferase as described. Cells were co-transfected with a constitutively active cytomegalovirus promoter-β-galactosidase reporter plasmid to normalize experiments for transfection efficiency.

Western Blotting—Cell lysates and precipitated molecular weight markers were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in TBST (Triton-containing Tris-buffered saline), incubated with various antibodies (1:2000) for 1–2 h, washed with TBST, and stained with anti-rabbit, or anti-goat, or anti-mouse IgG conjugated to peroxidase (1:5000). Immunoreactivity was visualized by enhanced chemiluminescence (ECL kit, Santa Cruz Biotechnology).

Electrophoretic Mobility Shift Assay—RAW264.7 cells nuclear extracts were prepared as described previously (18, 27). Electrophoretic mobility shift assay probes were prepared by annealing complementary single-stranded oligonucleotides with 5′-GATC overhangs (Genosys Biotechnologies, Inc.) and were labeled by filling in with [α-32P]dGTP and [α-32P]dCTP using Klenow enzyme. Labeled probes were purified with Nuctrap purification columns (Roche Molecular Biochemicals). Electrophoretic mobility shift assays were performed as described previously, using 105 cpm of probe and 5 μg of nuclear extracts per reaction. DNA-binding complexes were separated by electrophoresis on a 5% polyacrylamide-Tris-glycine-EDTA gel, which was dried and exposed to x-ray film.

Co-immunoprecipitation—Cells were lysed in 0.5 ml of ice-cold buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 0.2 mM EDTA, 1% Nonidet P-40, 1 mM dithiothreitol) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin, 10 μg/ml chymostatin, and leupeptin). Lysates were clarified by centrifugation (14,000 rpm, 15 min at 4 °C). Aliquots of lysate (500 μg of protein) were incubated with 2 μg of normal rabbit IgG or normal goat IgG for 4 h with intermittent agitation at 4 °C, and the lysates were precleared by the addition of 15 μl of protein G-Sepharose (Amersham Biosciences).
Biosciences) for 2 h at 4 °C. After centrifugation, the resulting supernatant was incubated with 2 μg of anti-TRAF6 antibody or anti-IRF-8 antibody overnight at 4 °C with gentle rocking. Immune complexes were absorbed on protein G-Sepharose and washed five times with lysis buffer. Finally immunoblotting was performed as described above.

RESULTS

IFN-γ and TLR Ligands Synergistically Induce the Production of Proinflammatory Factors in Macrophages—To analyze the cross-talk between IFN-γ and TLR signaling, we incubated peritoneal macrophages for 6 h (mRNA) or 24 h (protein release) with IFN-γ (10 ng/ml), LPS (1 μg/ml), or IFN-γ (10 ng/ml) and LPS (1 μg/ml). The results showed that IFN-γ and LPS synergistically induced the mRNAs for IL-1β, TNF-α, iNOS, IL-12 p40, and IL-6 (Fig. 1A). To ascertain if the synergism was at the transcriptional level, we transfected RAW264.7 cells with an IL-12 p40 promoter luciferase reporter construct for 12–18 h, and activated with IFN-γ, LPS, and IFN-γ and LPS for 12 h. IFN-γ and LPS synergistically activated the IL-12 p40 promoter (data not shown).

We next wanted to determine if IFN-γ synergizes with PGN (TLR2) and poly(I:C) (TLR3). We transfected RAW264.7 cells with an IL-12 p40 promoter luciferase reporter plasmid for 12–18 h, and activated with IFN-γ, PGN, and poly(I:C) plus LPS (Fig. 1B). The results showed that IFN-γ and LPS synergistically induced the IL-12 p40 promoter activation (18). We also found that IFN-γ alone did not induce ERK and JNK phosphorylation, but there was no synergism between IFN-γ and LPS (Fig. 2A). Phosphorylation of ERK, JNK, and IκBα was extracted by electrophoresis mobility shift assay. 5 μg of nuclear protein was added to the 32P-labeled NF-κB consensus sequence. Extract and probe were incubated with 0.5 μg of Poly(dI:dC) at room temperature for 30 min. C, effect of IFN-γ and LPS on NF-κB activation. RAW264.7 cells were transfected with NF-κB reporter luciferase plasmid for 12–18 h, and the transfected cells were activated with IFN-γ (10 ng/ml), LPS (1 μg/ml), or IFN-γ plus LPS for 12 h. Extracts were analyzed by luciferase activity. Results are expressed as the luciferase activity (normalized for β-galactosidase activity).

MAP Kinase Activation Is Impaired in IRF-8 −/− Macrophages—We previously reported that IRF-8 is important for IL-12 and iNOS gene activation (18). We also found that IFN-γ and LPS acted synergistically to induce IRF-8 protein expression (18). To extend these observations, we analyzed the synthesis of IRF-8 in RAW264.7 macrophages incubated with IFN-γ alone and in combination with TLR ligands PGN and poly(I:C). IRF-γ acted synergistically with PGN and poly(I:C) to induce IRF-8

FIGURE 1. IFN-γ and TLR ligands induce proinflammatory factors. A, expression of cytokine and iNOS mRNAs in peritoneal macrophages after IFN-γ and LPS stimulation. Thioglycollate-elicited peritoneal macrophages from C57Bl/6 mice were activated with LPS (1 μg/ml) or IFN-γ (10 ng/ml) plus LPS (1 μg/ml) for 6 h, and cytokine and iNOS mRNAs were analyzed by RT-PCR. B, the synergistic effect of IFN-γ with PGN and poly(I:C) in the activation of IL-12 p40 promoter. RAW264.7 cells were transfected with an IL-12 p40 promoter luciferase reporter for 18–24 h. The transfected cells were activated with IFN-γ, PGN, and poly(I:C) as indicated for 12 h. Extracts were analyzed for luciferase activity. Results are expressed as the luciferase activity (normalized for β-galactosidase activity).

FIGURE 2. Effect of IFN-γ on LPS-induced MAP kinase and NF-κB activation in macrophages. A, synergistic effect of IFN-γ and LPS on ERK and JNK activation. RAW264.7 cells were activated with IFN-γ (10 ng/ml), LPS (1 μg/ml), or IFN-γ plus LPS for various time intervals (10, 20, 30, and 60 min). Phosphorylation of ERK, JNK, and IκBα was analyzed by immunoblotting with phosphorylation-specific and control antibodies. B, IFN-γ has no effect on LPS-induced NF-κB DNA binding activity. RAW264.7 cells were activated with LPS (1 μg/ml) or IFN-γ (10 ng/ml) plus LPS for 4 h, and nuclear protein was extracted for electrophoretic mobility shift assay. 5 μg of nuclear protein was added to the 32P-labeled NF-κB consensus sequence. Extract and probe were incubated with 0.5 μg of Poly(dI:dC) at room temperature for 30 min. C, effect of IFN-γ and LPS on NF-κB activation. RAW264.7 cells were transfected with NF-κB reporter luciferase plasmid for 12–18 h, and the transfected cells were activated with IFN-γ (10 ng/ml), LPS (1 μg/ml), or IFN-γ plus LPS for 12 h. Extracts were analyzed by luciferase activity. Results are expressed as the luciferase activity (normalized for β-galactosidase activity).
IRF-8 was synergistically induced by IFN-γ with TLR ligands. RAW264.7 cells were activated for 4 h with IFN-γ (10 ng/ml), LPS (1 μg/ml), PGN (5 μg/ml), and poly(I:C) (1 μg/ml) as noted. Cell lysates were analyzed for IRF-8 protein expression by immunoblotting. Thioglycollate-elicited peritoneal macrophages (5 × 10^6) from wild-type and IRF-8−/− mice were activated with IFN-γ (10 ng/ml), LPS (1 μg/ml), or IFN-γ plus LPS for 24 h. The production of IL-6 (B), IL-1β, TNF-α (D), and IL-12 p70 (E) in the supernatants was measured by enzyme-linked immunosorbent assay.

(Fig. 3A). These results parallel the synergistic induction of proinflammatory factors by IFN-γ and LPS. Therefore we hypothesize that IRF-8 is pivotal for the cross-talk between IFN-γ and TLR signaling pathways. To address this question directly, we assayed cytokines released by thioglycollate-elicited peritoneal macrophages from wild-type and IRF-8−/− mice activated with IFN-γ (10 ng/ml), LPS (1 μg/ml), or IFN-γ plus LPS for 24 h. The production of IL-6, IL-1β, TNF-α, and IL-12 p70 were lower in all cases from IRF-8−/− macrophages (Fig. 3, B–E).

We next analyzed if MAP kinase activation was impaired in IRF-8−/− macrophages. Thioglycollate-elicited peritoneal macrophages from IRF-8−/− and wild-type mice activated with LPS (1 μg/ml) were assayed for phosphorylation of ERK and JNK by Western blot. Activation of ERK and JNK by LPS was strongly impaired in macrophages derived from IRF-8−/− mice and the IRF-8-deficient macrophage cell line CL-2 (Fig. 4, A and B). To confirm further the importance of IRF-8, we transduced CL-2 cells with IRF-8 or GFP for 2 days, activated the...
cells with LPS (1 μg/ml) at intervals, and analyzed ERK and JNK phosphorylation. Transduction of IRF-8 into IRF-8−/− macrophages rescued LPS-induced ERK and JNK activation compared with the GFP control (Fig. 4C). Similar results were found using the TLR ligands, PGN and poly(I:C) (Fig. 5, A and B). The inhibition of both MAP kinase activation and production of proinflammatory factors in IRF-8−/− macrophages highlight the central role for IRF-8 in TLR signaling.

To further analyze whether transactivation of IRF-8 is important for MAP kinase activation, we transduced the IRF-8 DNA binding domain mutant (K79E) (16, 17) or GFP into CL-2 cells for 2 days, activated the cells with LPS (1 μg/ml) at intervals, and analyzed ERK phosphorylation as above. The results showed that transduction of IRF-8 DNA binding domain mutant still rescued ERK activation (Fig. 5C), suggesting that the transactivation of IRF-8 is not essential for MAP kinase activation.

**IRF-8 Interacts Physically with TRAF6**—To investigate how IRF-8 regulates TLR signaling, we performed co-immunoprecipitation experiments between IRF-8 and MyD88 or TRAF6, which revealed an interaction between IRF-8 and TRAF (Fig. 6A) but not MyD88 (data not shown). However, co-immunoprecipitation experiments showed that MyD88 interacted with IRF-7 (Fig. 6B). In addition, gel filtration of lysates from 293T cells co-transfected with IRF-8 and TRAF6 showed that the two proteins co-eluted in a high molecular weight complex (data not shown). To confirm the interaction of IRF-8 with TRAF6 without protein overexpression, we incubated thioglycollate-elicited peritoneal macrophages with IFN-γ (10 ng/ml) and LPS (1 μg/ml) for 4 h and then collected cell lysates for co-immunoprecipitation experiments. Significantly we found that the physical interaction between IRF-8 and TRAF6 existed in peritoneal macrophages only after activation with IFN-γ and LPS (Fig. 6C). On a functional level, when IRF-8 and TRAF6 were co-transfected into RAW264.7 cells with an IL-12 p40 promoter luciferase reporter plasmid, we observed synergism in IL-12 p40 promoter activation (Fig. 6D).

To map the domains of IRF-8 necessary for TRAF6 binding, we made several N-terminal FLAG epitope-tagged C-terminal truncation mutants, including IRF-8-(1–253) and IRF-8-(1–356), and IRF-8-(1–390). We co-transfected TRAF6 and IRF-8 or the IRF-8 truncation mutants into 293T cells and 2 days later prepared lysates for co-immunoprecipitation experiments. TRAF6 was co-immunoprecipitated with IRF-8, IRF-8-(1–390), and IRF-8-(1–356) but not with IRF-8-(1–305) or IRF-8-(1–253), indicating that the region between residues 305 and 356 of IRF-8 is required to interact with TRAF6 (Fig. 6E).

**IRF-8 Enhances TRAF6 Ubiquitination**—TRAF6, an E3 ubiquitin ligase, plays a pivotal role in the activation of TLR signaling (1, 3) by catalysis of Lys-63-coupled polyubiquitination. Signal transduction by the IL-1 receptor, which shares many signaling components with TLR4, including TRAF6, is inhibited by a ubiquitin K63R mutant (28). Because we find that IRF-8 and TRAF6 interact, we next analyzed the effect of IRF-8 on TRAF6 ubiquitination. Cell lysates from 293T cells co-transfected with TRAF6, HA-ubiquitin, and IRF-8 for 48 h were immunoprecipitated with anti-TRAF6 and immunoblotted with anti-HA or anti-ubiquitin. Co-transfection of IRF-8 significantly enhanced TRAF6 ubiquitination visualized by staining HA-ubiquitin (Fig. 7A). Similarly, transfection of IRF-8 also enhanced TRAF6 ubiquitination visualized by anti-ubiquitin staining (Fig. 7B). To analyze the region in IRF-8 that is required for enhancement of TRAF6 ubiquitination, we co-transfected various IRF-8 C-terminal truncation mutants into 293T cells with TRAF6 and HA-Ub plasmids for 48 h. In agreement with the experiments to map IRF-8 TRAF6 interaction (Fig. 6E), IRF-8-(1–390), and IRF-8-(1–356) clearly enhanced TRAF6 ubiquitination, but IRF-8-(1–305) and IRF-8-(1–253) were without effect. Thus the enhancement of TRAF6 ubiquitination by IRF-8 is dependent on physical interaction of IRF-8 with TRAF6 (Fig. 7C). To confirm these experiments, we co-transfected IRF-8, TRAF6, and HA-Ub into RAW264.7 and CL-2 cells for 48 h and analyzed TRAF6 ubiquitination. In contrast to RAW264.7 cells, TRAF6 ubiquitination was significantly reduced in CL-2 cells. However, transduction of IRF-8 restored TRAF6 ubiquitination in both cell types to comparable levels (Fig. 7D). To investigate the relationship of the enhancement of TRAF-6 ubiquitination by IRF-8 with MAP kinase activation, we transduced CL-2 cells for 2 days with GFP or the IRF-8 mutant (1–390), which can enhance TRAF6 ubiquitination. Cells were activated with LPS (1 μg/ml) at intervals, and ERK phosphorylation was analyzed as above. The results showed that transduction of
IRF-8-(1–390) still rescued ERK activation (Fig. 7E), suggesting TRAF6 ubiquitination enhanced by IRF-8 is important for MAP kinase activation. These results, in aggregate suggest that the interaction of IRF-8 with TRAF6 modulates TRAF6 ubiquitination and MAP kinase activation.

**DISCUSSION**

In this study we investigated the role of IRF-8 in the cross-talk between IFN-γ and TLR signaling. We have shown that the activation of TLR4 by LPS to induce the expression of proinflammatory factors, including IL-1, IL-6, IL-12, NO, and TNF-α is synergized by IFN-γ. Comparable results were observed using PGN (a TLR2 ligand) and poly(I:C) (a TLR3 ligand) and IFN-γ to activate the IL-12 promoter. IFN-γ augmented only LPS-induced ERK and JNK phosphorylation and had no effect on NF-κB activation induced by LPS. Interestingly, in IRF-8−/− macrophages both the LPS-stimulated activation of ERK and JNK as well as expression of proinflammatory factors was inhibited. We found by co-immunoprecipitation experiments, that IRF-8 interacts physically with TRAF6 and TRAF6 at a binding site between residues 305 and 356 of IRF-8. Furthermore, transfection of IRF-8 enhanced TRAF6 ubiquitination, which was dependent on the physical interaction between these two proteins. Taken together, the results suggest that the interaction of IRF-8 with TRAF6 modulates TLR signaling and may contribute to the cross-talk between IFN-γ and TLR signaling.
We initially hypothesized that IFN-γ would enhance LPS-induced NF-κB activation, because NF-κB is an essential downstream target for TLR signaling (1, 3). Unexpectedly, IFN-γ had no effect on LPS-induced NF-κB activation and had only a marginal effect on NF-κB activation.

The synergistic activation by IFN-γ and LPS of ERK and JNK suggests that MAP kinases mediate IFN-γ and TLR cross-talk.

The IFN-γ signal transduction pathway is composed of primary and secondary phases. In the primary phase, after IFN-γ binding to IFN-γR, STAT1 mediates the primary signaling response. After ligand engagement, STAT1 is first phosphorylated by tyrosine kinases JAK1 and JAK2, then dimerizes, and subsequently translocates to the nucleus (29).

In the nucleus, STAT1 binds to IFN-γ-activated sequence sequences and activates transcription of target genes including IRF-1 and IRF-8, which are important in both innate and adaptive immune responses (17, 21). IRF-3, IRF-5, and IRF-7 have all been implicated in the TLR signaling pathway. Several viruses activate the TLR3 signal pathway (30–32), suggesting that TLR3 may play an important role in anti-viral host defense. IRF-5-deficient mice are severely impaired in TLR ligand stimulation of proinflammatory cytokine synthesis (24). Furthermore, IRF-5 interacts with and is activated by MyD88 and TRAF6. TLR activation results in the nuclear translocation of IRF-5 to activate cytokine gene expression. IRF-7 is also reported to play a role in TLR signaling. IRF-7 interacts with MyD88 and the TLR stimulated synthesis of IFN-γ requires the formation of a complex consisting of MyD88, TRAF6, and IRF-7 (33).

IRF-8, an essential transcription factor in the induction of Th1 immune responses, is also involved in the TLR signaling pathway. CpG DNA-induced NF-κB activation was impaired in IRF-8−/− dendritic cells, suggesting IRF-8 is involved in TLR9 signaling. However, the molecular mechanism was not elucidated (34). We report that macrophages from IRF-8−/− mice produced sharply decreased levels of...
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IL-12, TNF-α, IL-1, and IL-6 in response to LPS. Furthermore, we have found that LPS-induced ERK and IKK MAP kinase activation was severely impaired in peritoneal macrophages from IRF-8−/− mice. Unlike IRF-7, IRF-8 binds to TRAF6, but not to MyD88 (33). IRF-8 resides predominantly in the nucleus but, upon stimulation, moves to the cytoplasm. Because signal transmission occurs in a short time frame, it is reasonable to postulate that, upon TLR activation, IRF-8 interacts with TRAF6 in the cytosol. IRF-3 is produced constitutively in most cell types and is present in the cytoplasm in the absence of activation. Both IRF-5 and IRF-7 are expressed constitutively at very low levels mainly in lymphoid tissues, but their expression is enhanced by IFN-α/β treatment (35–37). Unactivated macrophages express very low levels of IRF-8. However, IFN-γ stimulation induces IRF-8 synthesis, which is further augmented by TLR ligands LPS, PGN, and poly(I:C).

The present study implicates TRAF6 as a target for IRF-8. TRAF6 is a ring domain-containing ubiquitin ligase, essential for the activation of NF-κB and MAP kinases downstream of the TLR signaling pathway (3). TRAF6 forms a complex with an E2 ubiquitin-conjugating enzyme complex, consisting of Ubc13 and Uev1A, which catalyze the synthesis of lysine 63 polyubiquitin chains. Lysine 63 ubiquitination regulates protein-protein interaction rather than serving as a degradation signal. TRAF6, acting as an E3 ligase, activates signal transduction through association with downstream proteins, and TRAF6 itself is polyubiquitinated after oligomerization or dimerization. We find that IRF-8 interacts with TRAF6 and regulates TRAF6 ubiquitination. Because IRF-8 is not an E3 ligase, we suggest that IRF-8 either is part of the signaling complex, consisting of Ubc13-UEV1a complex, or is involved in recruitment of a different E3 ligase.

Our study provides an insight into the mechanism of the gene induction program downstream of TLR signaling. The observation that IRF-8, whose synthesis is stimulated by IFN-γ and augmented by TLR ligands, is required for MAP kinase but not NF-κB activation clarifies the complex regulation of the inflammatory responses. Thus, IRF-8 is not only involved in TLR signaling, but also contributes to the cross-talk between IFN-γ and the TLR signaling pathways, resulting in the maximal production of proinflammatory cytokines.

The interaction of IRF-8 with the TLRs signaling pathway is a secondary signal required to boost the immune response, especially during intracellular bacteria or viral infection. IRF-8, therefore, is a potential target for therapeutic intervention aimed at controlling harmful immune responses.

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