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Regulation of Lipopolysaccharide Sensitivity by IFN Regulatory Factor-2

Natalia Cuesta, Cindy A. Salkowski, Karen E. Thomas, and Stefanie N. Vogel

IFN regulatory factors (IRFs) are a family of transcription factors and include several members that regulate expression of pro- and anti-inflammatory genes. Mice with a targeted mutation in IRF-2 (IRF-2-/-) were studied after injection of LPS to evaluate the importance of IRF-2 in the regulation of endotoxicity. IRF-2-/- mice were highly refractory to LPS-induced lethality. Although hepatic TNF-α mRNA and circulating TNF-α were significantly elevated in LPS-challenged IRF-2-/- mice, levels of IL-1, IL-12, and IFN-γ mRNA and protein, as well as IL-6 protein, were significantly lower than levels seen in LPS-challenged IRF-2 +/- mice. IRF-2-/- mice were also more refractory to TNF-α challenge than were control mice, which was consistent with their diminished sensitivity to LPS, yet no significant difference in the mRNA expression of TNFRs was observed. IL-12Rβ2 mRNA levels from LPS-challenged IRF-2-/- mice were significantly different after 1, 6, and 8 h, suggesting that both diminished IL-12 and altered IL-12R expression contribute to the paucity of IFN-γ produced. IRF-2 knockout mice also failed to sustain LPS-inducible levels of IRF-1 and IFN consensus sequence binding protein mRNA expression, two transacting factors required for IL-12 transcription, perhaps as a result of diminished IL-1β, IL-6, and IFN-γ levels. Liver sections from IRF-2-/+ and IRF-2-/- mice were analyzed 6 h after a typically lethal injection of LPS. IRF-2-/- mice exhibited greater numbers of apoptotic Kupffer cells than did wild-type mice, suggesting a novel anti-apoptotic role for IRF-2. Collectively, these findings reveal a critical role for IRF-2 in endotoxicity, and point to a previously unappreciated role for IRF-2 in the regulation of apoptosis. The Journal of Immunology, 2003, 170: 5739–5747.

Interferons constitute a family of multifunctional cytokines that have been strongly implicated as primary mediators of the host defense against viral infection (1). The response to IFNs results in the transcriptional activation of target genes that are regulated by their interaction with DNA-binding proteins generated through the Janus kinase-STAT signaling pathway and/or the IFN regulatory factor (IRF). The best characterized members of the IRF family of transcription factors, IRF-1 and IRF-2, were originally found to activate and repress transcription of the IFN-β gene, respectively, by competing for the same DNA element, now termed the IRF-E (2, 3). IRFs may also interact to form protein complexes with other IRF family members, as well as other trans-acting factors. For example, IFN consensus sequence binding protein (ICSBP) interacts with both IRF-1 and IRF-2 (4–6). IRF-1 also interacts with other DNA-binding proteins, such as NF-κB and p53 (7–10), and IRF-2 has been reported to bind to NF-κB in vitro (11). Thus, these transcription factors have the potential to mediate diverse functions in response to extracellular stimuli through their interactions with various promoter elements.

In addition to their opposing actions on IRF-β gene expression, IRF-1 and IRF-2 have been shown to exert opposing effects on cyclooxygenase 2 gene expression (12). However, within the last few years, this relatively simplistic model of positive/negative regulation has been modified to account for the findings that IRF-2 has more recently been found to function as a transcriptional activator of the VCAM-1 (13) and histone H4 genes (14). Conversely, IRF-1 has been reported to act as a negative regulator of c-myb at the level of transcription (15).

Studies from mice with targeted mutations have revealed some other relevant IRF functions (16, 17). NK and Th1 cell development is impaired in IRF-1 knockout mice (18–20), and IL-12 is dysregulated in macrophages derived from both IRF-1 and IRF-2 knockout mice (21). Although induction of the inducible nitric oxide synthase gene is IRF-1-dependent (22), IRF-2 does not seem to have any effect in the transcriptional activation of this gene in macrophages, but it clearly affects release of NO (23, 24). IRF-1-/- mice are more susceptible to infection with Mycobacterium bovis (25), Brucella abortus (26), Leishmania major (18), and Toxoplasma gondii (27) than are wild-type mice, and IRF-2-/- mice are more susceptible to Listeria monocytogenes (24) and L. major (20). IRF-1 has also been implicated in the regulation of apoptosis (28–30); for example, macrophages from IRF-1 knockout mice are more resistant to LPS- and IFN-γ-induced apoptosis (31, 32). In addition, IRF-1 regulates DNA damage-induced cell cycle arrest (8).

Some of these studies have focused on the cells in the liver, given the important immunological role that this organ plays in the first line of host defense. Many bacterial agents enter the liver via the portal vein (33), and most bacteria are entrapped by Kupffer cells and hepatocytes (34). In response to these stimuli, hepatocytes produce acute phase proteins and proteins of the complement cascade, whereas Kupffer cells produce many cytokines including IL-12, IL-10, and TNF-α (33, 35). IL-12 and other ILs, in turn, activate NK and Th1 cells to produce IFN-γ (36). Both TNF-α and IFN-γ are proinflammatory cytokines involved in LPS-induced toxicity (37–40). Reporting that IRF-1 plays an important role in the pathogenesis of disease models mediated by TNF and IFN-γ is evidenced by experiments in...
IRF-1/−/− mice. IRF-1/−/− mice are highly refractory to a dose of LPS that is lethal for wild-type mice (41), and the production of TNF-α and IFN-γ is strikingly impaired, which is secondary to a down-regulation of gene expression in the liver and spleen or in the macrophages of IRF-1/−/− mice (21, 41, 42). In light of the close relationship that exists between IRF-1 and IRF-2 in many physiological responses, we sought to extend our original findings on the role of IRF-1 in LPS-induced toxicity to investigate the role of IRF-2 in a similar model of endotoxemia. Therefore, IRF-2/−/− mice were studied after injection of LPS or TNF-α with the purpose of exploring the importance of IRF-2 in the pathogenesis of LPS-induced mortality. We have found that IRF-2/−/− mice are more resistant to both LPS and recombinant TNF-α challenge than are IRF-2+/+ mice, and IRF-2/−/− mice exhibit a significant inhibition of the expression of IL-12, IL-12R, and IFN-γ, as well as IL-1β and IL-6, while TNF-α levels are increased significantly. In contrast to IRF-1/−/− macrophages that are resistant to LPS- and IFN-γ-induced apoptosis, the number of apoptotic Kupffer cells is markedly increased (44). Breeding pairs of IRF-2/−/−mice could result in decreased production of specific inflammatory cytokines and attenuation of LPS-induced toxicity. Taken collectively, our data demonstrate the importance of IRF-2 as yet another critical transacting factor that regulates in vivo responses to LPS and have provided products were electrophoresed and transferred to Hybond N+ by standard Southern blotting techniques. DNA was cross-linked by exposure to UV light, baked onto the nylon membrane, and hybridized with an internal oligonucleotide probe. Labeling of the probe and subsequent detection of bound probe was conducted using an ECL system (Amersham).

**Real-time PCR for mRNA quantification**

For certain experiments, mRNA levels were measured using real-time PCR. The PCR was performed in a Sequence Detector System (ABI Prism 7900 Sequence Detection System and software; Applied Biosystems, Foster City, CA). Amplification was performed in a final volume of 25 μL containing 30 ng cDNA from the reverse transcribed reaction, primer mixture (0.3 μM each of sense and antisense primers), and 12.5 μL 2 X SYBR Green Master Mix (Applied Biosystems). The oligonucleotide primers were designed using Primer Express 1.5 software (Applied Biosystems). In addition, the sense and antisense sequences of each pair of primers were designed to overlap adjacent exon boundaries to exclude detection of genomic DNA.

The standard amplification program included 40 cycles of two steps each, comprised of heating to 95°C and heating to 60°C. Fluorescent product was detected at the last step of each cycle. The final mRNA levels of the gene studied were normalized according to the HPRT concentration of each sample. mRNA levels were reported as fold changes over background levels detected in control tissues.

The primers used were: IL-10 sense (5′-ATTGGAATTCCCTGGGTGAGAAAG-3′); IL-10 antisense (5′-CACAGGGGAAATCTGATGACAC-3′); HPRT sense (5′-GCTGACCTGCTGGATTACATTAA-3′); HPRT antisense (5′-AGATCTTACATGCTAGCCTTTCG-3′); IL-1β sense (5′-ACAGAATATACACAAACAGTATTTCTC-3′); IL-1β antisense (5′-GATTCTTTCCTTGAGGGCCA-3′); IL-6 sense (5′-TCCAGAAATTTGCTATTGGAATT-3′); IL-6 antisense (5′-TCTTGTTCCTTCTTATCTTTTTATGTT-3′).

**Immunohistochemistry**

In vivo apoptosis was assessed using the TUNEL assay (49, 50). After the indicated treatment, livers were dissected and immersed overnight in 10% buffered formalin (Sigma-Aldrich, St. Louis, MO) and embedded in paraffin. Five micrometer thick tissue sections were adhered to Superfrost Plus slides (VWR, West Chester, PA). Sections were deparaffinized by heating at 60°C for 20 min and clearing in xylene for 30 min. The slides were then rehydrated through a graded series of ethanol concentrations (96, 70, and 50% ethanol), ending with a wash in distilled water. To inactivate endogenous peroxidase, sections were treated with 3% H2O2 for 30 min at room temperature. After washing thoroughly in water, the slides were incubated in 10 mM Tris-HCl, pH 7.4 for 5 min and digested with 10 μg/ml protease K for 10 min at 37°C. washed in 10 mM Tris-HCl, then incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min at 4°C. After three washes in PBS, slides were incubated at 37°C in culture medium with 1 μM Stock Cell Death Detection, POD kit; Roche Biomedical Laboratories, Mannheim, Germany) as instructed by the manufacturer. They were subsequently rinsed in PBS and incubated with converted POD (Roche Biomedical Laboratories) for 30 min at room temperature. The slides were washed in PBS and stained with the chromogens 3,3′-diaminobenzidine tetrachloride (Vector Laboratories, Burlingame, CA).

The TUNEL assay was followed by immunostaining with a rat monoclonal anti-F4/80 Ab to identify Kupffer cells. For this purpose, TUNEL-stained sections were incubated with normal rabbit serum (5% in PBS; Vector Laboratories) for 30 min at room temperature and then with a primary rat anti-F4/80 Ab at 4°C overnight (1:200 dilution; Accurate Chemical and Scientific, Westbury, NY). Normal rabbit serum was used instead of the primary Ab as a negative control. After washing in PBS, biotinylated rabbit anti-rat Abs were applied for 30 min at room temperature (1:200 dilution; Vector Laboratories), followed by avidin-biotin alkaline phosphatase complexes for 30 min (1:100 dilution; Vector Laboratories). Red alkaline phosphatase substrate was used as the chromogen (Vector Laboratories). The slides were counterstained with Harris hematoxylin (LR, dehydrated, cleared in xylene, and mounted in Permount (Fisher Scientific, Fair Lawn, NJ). Double staining was considered positive when the cells displayed a brown nuclear staining in the context of a surrounding red immunoreactive pattern.

Hepatic cells were counted under ×1000 magnification. The areas scored were always selected randomly, and counts were conducted in a blinded fashion, revealing the code at the end of the study. The “apoptotic index” was determined by dividing the total number of Kupffer cells (F4/
assess whether loss of the transcription factor IRF-2 altered susceptibility to LPS. As shown in Fig. 1, IRF-2<sup>+/+</sup> mice were less susceptible to the lethal effects of LPS. By 48 h after LPS challenge, the mortality rate was 80% in IRF-2<sup>+/+</sup> mice and only 10% in IRF-2<sup>−/−</sup> mice. Despite their enhanced resistance, IRF-2<sup>−/−</sup> mice developed clinical signs of endotoxemia, including lethargy, diarrhea, piloerection, cachexia, and conjunctivitis; these symptoms, however, were milder in IRF-2<sup>−/−</sup> than in IRF-2<sup>+/+</sup> mice.

**FIGURE 1.** IRF-2<sup>−/−</sup> mice exhibit enhanced resistance to LPS. IRF-2<sup>+/+</sup> and IRF-2<sup>−/−</sup> mice were injected i.p. with 35 mg/kg LPS (10 mice/strain) and monitored for 72 h. 

80-positive cells) that were exhibiting apoptosis (TUNEL-positive) by the total number of F4/80-positive cells in the fields examined (i.e., double-positive cells/total F4/80-positive cells) × 100.

**Statistics**

Results were analyzed using Student’s t test for comparisons between two groups or by one-way ANOVA, with Tukey’s post-hoc tests for comparisons between multiple treatment groups. Values for p < 0.05 were accepted as the level of significance. All experiments were repeated at least twice with similar results. For immunohistochemical data, a minimum of 350 cells were enumerated per slide for analysis, with four slides per treatment derived from four separate mice.

**Results**

**Enhanced resistance of IRF-2<sup>−/−</sup> mice to LPS-induced lethality**

IRF-2<sup>+/+</sup> and IRF-2<sup>−/−</sup> mice were injected with 35 mg/kg LPS to assess whether loss of the transcription factor IRF-2 altered susceptibility to LPS. As shown in Fig. 1, IRF-2<sup>−/−</sup> mice were less susceptible to the lethal effects of LPS. By 48 h after LPS challenge, the mortality rate was 80% in IRF-2<sup>+/+</sup> mice and only 10% in IRF-2<sup>−/−</sup> mice. Despite their enhanced resistance, IRF-2<sup>−/−</sup> mice developed clinical signs of endotoxemia, including lethargy, diarrhea, piloerection, cachexia, and conjunctivitis; these symptoms, however, were milder in IRF-2<sup>−/−</sup> than in IRF-2<sup>+/+</sup> mice.

**IL-12 and IFN-γ production are impaired in LPS-challenged IRF-2<sup>−/−</sup> mice**

Numerous studies have demonstrated that lethality following LPS challenge is associated with overproduction of TNF-α, for which production is, in part, amplified by IL-12-induced IFN-γ. In murine models of endotoxemia, loss of any of these principal mediators typically results in enhanced resistance to LPS-induced lethality (37–39, 51, 52). Because IRF-2<sup>−/−</sup> mice were more resistant to the lethal effects of LPS (Fig. 1), we next examined the in vivo regulation of IL-12 p35, IL-12 p40, IFN-γ, and TNF-α mRNA expression in the livers of LPS-challenged IRF-2<sup>+/+</sup> and IRF-2<sup>−/−</sup> mice (Fig. 2). Levels of circulating IL-12 p40, bioactive IL-12 p70, IFN-γ, and TNF-α protein also were measured (Fig. 3). As shown in Fig. 2, IRF-2<sup>−/−</sup> mice had significantly heightened (~1.5- to 10-fold) levels of basal and LPS-induced IL-12 p40 mRNA expression in the liver than did IRF-2<sup>+/+</sup> control mice. Whereas untreated IRF-2<sup>−/−</sup> mice had an ~2.5-fold higher level of circulating IL-12 p40, levels of circulating IL-12 p40 were not significantly different in the sera of IRF-2<sup>+/+</sup> and IRF-2<sup>−/−</sup> mice after LPS challenge (Fig. 3). In contrast to IL-12 p40 mRNA expression, significantly lower levels of IL-12 p35 mRNA expression were observed in the livers of IRF-2<sup>−/−</sup> mice at 6 and 8 h (50% and 90% reduction, respectively) after LPS challenge (Fig. 2). These reductions in the level of IL-12 p35 mRNA were consistent with significantly lower levels of bioactive IL-12 p70 production in IRF-2<sup>−/−</sup> mice (Fig. 3). Specifically, IRF-2<sup>−/−</sup> mice produced 60–75% less circulating IL-12 p70 at 3, 6, and 8 h after LPS challenge than did IRF-2<sup>+/+</sup> mice. Also consistent with the mitigated IL-12 p70 response was a profound reduction in hepatic
Induction of IL-10 mRNA is not dysregulated in IRF-2\textsuperscript{/−/−} mice

Because IL-10 is a potent anti-inflammatory cytokine and an inhibitor of TNF-α, IL-12, and IFN-γ production (53–56), we also measured IL-10 gene expression in IRF-2\textsuperscript{/−/−} mice in response to LPS. Fig. 4 illustrates that there were no significant differences in IL-10 mRNA levels between IRF-2\textsuperscript{+/+} and IRF-2\textsuperscript{/−/−} mice at early time points. At 8 and 12 h after LPS i.p., IRF-2\textsuperscript{/−/−} mice produced slightly elevated levels of IL-10 mRNA, but this difference failed to achieve statistical significance (p = 0.059 and 0.054, respectively). Thus, IRF-2-dependent regulation of IL-10 gene expression cannot account for decreased early expression of IL-12 or IFN-γ mRNA.

**TNFRI1 and TNFR2 hepatic mRNA expression is not dysregulated in IRF-2\textsuperscript{/−/−} mice**

The heightened levels of both TNF-α mRNA and circulating TNF-α in IRF-2\textsuperscript{/−/−} mice were unexpected because IRF-2\textsuperscript{+/+} mice were significantly more resistant to the lethal effects of LPS. This suggested the possibility that some component of the TNF signaling pathway might be disrupted in IRF-2\textsuperscript{/−/−} mice. Thus, hepatic TNFRI1 and TNFR2 mRNA expression were examined to ascertain whether IRF-2\textsuperscript{/−/−} mice exhibited altered TNFR mRNA levels in vivo. By 1 h after LPS challenge, TNFR2 mRNA expression in the liver had increased ~4-fold in both IRF-2\textsuperscript{+/+} and IRF-2\textsuperscript{/−/−} mice and remained heightened (6- to 12-fold increase) for 12 h (Fig. 5). In contrast, no increase in TNFR1 mRNA expression in IRF-2\textsuperscript{+/+} and IRF-2\textsuperscript{/−/−} mice was observed until 12 h after LPS challenge (~2-fold increase). Importantly, no significant differences in either TNFR1 or TNFR2 mRNA levels were observed between IRF-2\textsuperscript{+/+} and IRF-2\textsuperscript{/−/−} mice.

Enhanced resistance of IRF-2\textsuperscript{/−/−} mice to TNF-α-induced mortality

The refractory response of IRF-2\textsuperscript{/−/−} mice to LPS was surprising, given the elevated levels of TNF-α exhibited by IRF-2\textsuperscript{+/−} mice.
IL-6 and IL-1β expression are dysregulated in IRF-2−/− mice after LPS challenge

Both IL-1 and IL-6 have long been implicated in endotoxemia. Moreover, both have been shown to induce IRF-1 gene expression (59–61). Therefore, we sought to investigate whether impaired production of these two cytokines could be responsible for the lowered levels of IRF-1 mRNA expression in LPS-challenged IRF-2−/− mice. In response to LPS, IRF-2−/− mice exhibited a significant decrease in both circulating IL-1β and hepatic IL-1β mRNA (Fig. 9, A and B). Although IL-6 mRNA gene expression was not dysregulated in IRF-2−/− mice, IL-6 protein levels were substantially reduced in IRF-2−/− mice after LPS treatment when compared with IRF-2+/+ mice (Fig. 9, C and D). The latter observation suggests a role for IRF-2 in the posttranscriptional regulation of IL-6.

IRF-2+/+ and IRF-2−/− mice exhibit differential sensitivity to LPS- and TNF-α-induced Kupffer cell apoptosis

Previous studies proposed that LPS-induced lethality is secondary to TNF-α-induced endothelial apoptosis that is detectable in various organs within 6 h of LPS administration (62). Macrophages derived from IRF-1−/− mice were found to resist LPS-induced apoptosis (45). Therefore, we next analyzed wild-type and IRF-2 knockout mice that had been injected with an LD100 (in wild-type mice) of LPS or TNF-α. Although IL-6 mRNA gene expression was not dysregulated in IRF-2−/− mice, IRF-2 protein levels were substantially reduced in IRF-2−/− mice after LPS treatment when compared with IRF-2+/+ mice (Fig. 9, C and D). The latter observation suggests a role for IRF-2 in the posttranscriptional regulation of IL-6.

Discussion

Results from this study indicate that loss of the transcription factor IRF-2 renders mice more resistant to both LPS and TNF-α challenge (Figs. 1 and 6). Transfection experiments have shown that IRF-1 normally functions as an activator of transcription, whereas IRF-2 antagonizes the function of IRF-1 by working as a repressor (6). Any variation in the intracellular concentration of these transcription factors alters the ratio of IRF-1 to IRF-2 and may produce a dramatic change in the transcription pattern of cells (63). Our data show that LPS-induced IL-12, IL-12R, and IFN-γ expression are all dysregulated in IRF-2−/− mice (Figs. 2, 3, and 6), indicating that IRF-2 plays a crucial role in endotoxemia by contributing to the regulation of both IL-12 and IL-12R expression, and subsequently, IFN-γ production. Previous studies have shown that mice given Abs to IFN-γ (39) are also refractory to LPS. Although levels of circulating IL-12 p40 were not significantly different in IRF-2+/+ and IRF-2−/− mice after LPS challenge, decreased IL-12 p70 levels in IRF-2−/− mice may be explained, in part, by significantly lower IL-12 p35 mRNA expression because IL-12 p35 is limiting in the formation of the bioactive IL-12 p40/p35 heterodimer (64). In addition, because IL-12 p40 homodimers have been previously shown to be competitive inhibitors of apoptosis (62), this was not observed in livers of control or knockout mice.

Table 1. Recombinant TNF-α fails to induce IL-12 p70 and IFN-γ in IRF-2+/+ and IRF-2−/− mice in vivo

<table>
<thead>
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<th>C57BL/6</th>
<th>IRF-2−/−</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>rTNF-α</td>
</tr>
<tr>
<td>IL-12 p70b</td>
<td>&lt;125.0 ± 0.0 (6)</td>
<td>&lt;125.0 ± 0.0 (7)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>&lt;62.5 ± 0.0 (6)</td>
<td>&lt;62.5 ± 0.0 (9)</td>
</tr>
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*Mice were injected i.p. with recombinant TNF-α (2 mg/kg) or LPS (35 mg/kg) and were bled 6 h after injection. Serum was collected from individual mice and assayed for IL-12 p70 or IFN-γ by ELISA as described in Materials and Methods. The number in parentheses indicates the number of mice injected per treatment. Mean ± SEM.

The lowest detectable amount for the BD PharMingen OptEIA IL-12/70 ELISA was 125.0 pg/ml.

The lowest detectable amount for the BD PharMingen OptEIA IFN-γ ELISA was 62.5 pg/ml.
DNA-binding proteins, including NF- and p35 promoters contain consensus sequences for a number of levels in normally LPS-responsive macrophages. Both IL-12 p40 and p35 promoters contain consensus sequences for a number of DNA-binding proteins, including NF-κB consensus sequences, NF-IL-6 binding sites, and others (68–70), and quite recently Maruyama et al. (71) demonstrated an IRF-1 binding site in the NF-IL-6 binding sites, and others (68). The IRF-1 promoter contains a potential IFN-stimulated regulatory element only weakly, but upon interactions with IRF-1 or IRF-2, its DNA-binding activity is dramatically increased (4). Other authors have also demonstrated that ICSBP may repress IRF-1-induced transcription (4, 74). Given the functional diversity of the IRFs, their capacity for interaction with other members of the family, and the changing levels of these proteins in stimulated cells, our data point to a more complex regulation of the production of IL-12 in vivo than in vitro in response to LPS.

A possible explanation for the down-regulation of IRF-1 mRNA expression after LPS challenge in IRF-2−/− mice is the significantly lower levels of serum IFN-γ, IL-1β, and IL-6. The IRF-1 promoter contains a potential IFN-γ-activated sequence (75). The profound reduction that is seen in IFN-γ levels in IRF-2−/− mice when compared with IRF-2+/+ after LPS challenge would be predicted to result in an attenuation of IRF-1 transcription in the IRF-2-deficient mice. Both IL-1 and IL-6 have also been shown to induce IRF-1 (59–61), and our data show clearly that both are significantly reduced at the protein level in IRF-2−/− mice challenged with LPS. Therefore, diminished IRF-1 mRNA levels may well be secondary to decreases in these cytokines. It is interesting to note that work by Marecki et al. (76) showed that concurrent expression of IRF-1, IRF-2, and the Ets-like protein PU.1 with either IRF-4 or ICSBP synergize for maximal IL-1β transcription. Thus, diminished levels of LPS-inducible IRF-1 mRNA are likely, in turn, to contribute to the diminished IL-1 expression. Taken collectively, our results reveal that IRF-2 is normally required for optimal expression of LPS-induced IL-1 and IL-6, and that there is cross-regulation between these cytokines and IRF-1.

In the same way, the down-regulation of ICSBP mRNA expression after LPS challenge in IRF-2−/− mice may be explained by the significant low levels of IFN-γ, because it has been shown to induce ICSBP (74, 77). Our in vivo data indicate that the IL-12Rβ2 mRNA expression is down-regulated between 6 to 8 h after studies showing that macrophage-like cells from ICSBP−/− mice could not induce IL-12 p40 transcripts (68), leading to the conclusion that ICSBP acts as a principal activator of this gene in macrophages. ICSBP binds to the IFN-stimulated regulatory element only weakly, but upon interactions with IRF-1 or IRF-2, its DNA-binding activity is dramatically increased (4). Other authors have also demonstrated that ICSBP may repress IRF-1-induced transcription (4, 74). Given the functional diversity of the IRFs, their capacity for interaction with other members of the family, and the changing levels of these proteins in stimulated cells, our data point to a more complex regulation of the production of IL-12 in vivo than in vitro in response to LPS.

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LPS challenge in IRF-2<sup>−/−</sup> mice, whereas the IL-12Rβ1 mRNA levels are not affected. The expression of these two genes is independently regulated (78–80), and although IRF-1 has been implicated in the regulation of both of them (42), our findings would suggest that IRF-2 normally contributes to the expression of the IL-12Rβ2 subunit. IL-12Rβ2 serves as the signal transduction component of this IL-12R heterodimeric receptor (79, 80). Disruption in the IL-12 pathway in IRF-2<sup>−/−</sup> mice was paralleled by a significant impairment in the ability of IRF-2 knockout mice to produce IFN-γ. Although we have provided strong evidence for a disruption in this pathway at the levels of IL-12 p70 and IL-12Rβ2 receptor mRNA expression, Lohoff et al. (20) also showed that NK and Th1 cell development is compromised in IRF-2<sup>−/−</sup> mice. As NK cells are the dominant IFN-γ-secreting cell population in response to LPS (81), the combined paucity of IL-12 p70 and IL-12R expression in cells with mitigated NK function could readily account for the profound inhibition of IFN-γ that we observed in the IRF-2<sup>−/−</sup> mice. Future studies will be required to demonstrate this directly.

The observed decrease in IFN-γ expression might have been predicted to have resulted in lowered levels of TNF-α (82). Rather, circulating TNF-α was actually significantly higher in IRF-2<sup>−/−</sup> than IRF-2<sup>+/+</sup> mice early after LPS injection, with no apparent TNFR mRNA dysfunction. In addition, heightened levels of TNF-α should have stimulated an increase in IFN-1 mRNA expression, as IRF-1 is also induced by many LPS-inducible cytokines (2, 83), as well as in LPS-challenged mice and macrophages (5, 42). Taken collectively,
these data suggest that IRF-2 normally regulates expression of one or more components of the TNF-α-inducible signaling pathway.

Another factor that may contribute to the refractory response of IRF-2−/− mice to LPS is the observation that the number of apoptotic Kupffer cells is significantly higher than in control mice (both after saline injection and in response to LPS or TNF-α). Interestingly, it has been shown that Kupffer cell depletion results in an increase in circulating TNF-α (84). As TNF-α mediates apoptosis in Kupffer and endothelial cells (85, 86), increased levels of TNF-α in IRF-2−/− mice after LPS treatment may contribute to the significantly increased number of apoptotic Kupffer cells measured at 6 h after LPS challenge. This notion is supported by our observation that injection of recombinant TNF-α recapitulates this effect. However, IRF-2−/− mice also exhibited more apoptotic Kupffer cells after saline injection, suggesting that IRF-2 regulates basal levels of apoptosis as well. IRF-1 has been implicated in the regulation of cell cycle and apoptosis, and the target genes critical for apoptotic response may include caspases 1 and 7 (28, 87).

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These findings point to IRF-2 as a novel attenuator of apoptotic events in Kupffer cells. In addition, it has been reported that LPS-induced proinflammatory cytokine production by macrophages is significantly suppressed by coculture with apoptotic cells (88). Therefore, it is likely that the enhanced resistance of IRF-2−/− mice to LPS challenge may depend both on the removal of Kupffer cells and the active suppression of inflammatory mediators such as IL-12 and IFN-γ by remaining cell types.

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References


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