Contribution of Interferon-β to the Murine Macrophage Response to the Toll-like Receptor 4 Agonist, Lipopolysaccharide

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Interferon-β (IFN-β) has been identified as the signature cytokine induced via the Toll-like receptor (TLR) 4, “MyD88-independent” signaling pathway in macrophages stimulated by Gram-negative bacterial lipopolysaccharide (LPS). In this study, we analyzed the responses of macrophages derived from wild-type (IFN-β+/+) mice or mice with a targeted mutation in IFN-β (IFN-β−/−) to the prototype TLR4 agonist, Escherichia coli LPS. A comparison of basal and LPS-induced gene expression (by reverse transcription-PCR, real-time PCR, and Affymetrix microarray analyses) resulted in the identification of four distinct patterns of gene expression affected by IFN-β deficiency. Analysis of a subset of each group of differentially regulated genes by computer-assisted promoter analysis revealed putative IFN-responsive elements in all genes examined. LPS-induced activation of intracellular signaling molecules, STAT1 Tyr-701, STAT1 Ser-727, and Akt, but not p38, JNK, and ERK MAPK proteins, was significantly diminished in IFN-β−/− versus IFN-β+/+ macrophages. “Priming” of IFN-β−/− macrophages with exogenous recombinant IFN-β significantly increased levels of LPS-induced gene expression for induction of monocyte chemoattractant protein 5, inducible nitric-oxide synthase, IP-10, and IL-12 p40 mRNA, whereas no increase or relatively small increases were observed for IL-1β, IL-6, monocyte chemoattractant protein 1, and MyD88 mRNA. Finally, IFN-β−/− mice challenged in vivo with LPS exhibited increased survival when compared with wild-type IFN-β+/+ controls, indicating that IFN-β contributes to LPS-induced lethality; however, not to the extent that one observes in mice with more complete pathway deficiencies (e.g., TLR4−/− or TRIF−/− mice). Collectively, these findings reveal unanticipated regulatory roles for IFN-β in response to LPS in vitro and in vivo.

In response to a growing list of conserved microbial structures, Toll-like receptors (TLRs) initiate intracellular signaling cascades that lead to pro-inflammatory gene expression by host cells. The TLRs represent an evolutionarily conserved family of signal transducing receptors (>10 TLRs have been identified in humans) that interact with conserved microbial structures through their N-terminal leucine-rich repeat domains and initiate signaling through the interactions of TLR “Toll-IL-1 resistance” (TIR) domains with one or more intracellular adapter proteins: MyD88, TIRAP/Mal, TRIF/TICAM-1, or TRAM/TIRP/TICAM-2. The adapter molecules enable recruitment of downstream kinases and substrates to the receptor complex, thus initiating intracellular signaling leading to alterations in gene expression.

Not all TLRs activate the pro-inflammatory response to the same extent. Hirschfeld et al. (1) first demonstrated that engagement of different TLRs results in distinct patterns of gene expression. Specifically, Escherichia coli LPS signaling through TLR4 resulted in a much broader pattern of gene expression in murine macrophages than was observed in cells stimulated with the TLR2 agonist, Porphyromonas gingivalis LPS. Toshchakov et al. (2) later showed that TLR4 signaling could be distinguished from TLR2 signaling by the production of IFN-β, which in turn, results in the autocrine/paracrine activation of macrophages, leading to STAT1 activation (and the subsequent induction of STAT1-dependent genes, e.g. MCP-5, IP-10 (CXC110), and iNOS). Both IFN-β gene expression and STAT1 activation were found to be MyD88-independent (2). Activation of the transactivating protein, IRF-3, was shown to be a necessary DNA-binding transcriptional activating protein for induction of IFN-β (3–5), as well as IRF-7, IP-10, and IL-15 (6, 7) after LPS stimulation. It is now widely held that differen-

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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tial utilization of the four adapter molecules by distinct TLRs gives rise to the two major TLR-specific signaling cascades and the corresponding repertoires of gene expression that have been reported (reviewed in Ref. 8). For example, although TLR4 utilizes MyD88 and TIRAP for induction of Tnfa and similarly regulated genes, both TRIF and TRAM are required to stimulate IFN-β production via IRF-3 after LPS stimulation. In contrast, TLR2 appears to be limited to interaction with MyD88 and TIRAP, and therefore, fails to activate genes that are downstream of the MyD88-independent pathway. Conversely, TLR3 appears to be limited to the utilization of TRIF only for induction of IFN-β via the MyD88-independent pathway and does not appear to utilize the other three adapters. Recent microarray analysis has revealed that LPS stimulation of murine macrophages resulted in the induction of a greater proportion of genes induced via the MyD88-independent than the “MyD88-dependent” pathway: of 1055 genes that were statistically induced or repressed by LPS treatment, 74.7% of genes are MyD88-independent as compared with 21.8% that are MyD88-dependent (9). A comparison of LPS and CpG DNA stimulation of murine macrophages revealed that CpG DNA induced or repressed 31.6% of those modulated by LPS (10). Thus, the TLRs evolved not only to recognize distinct pathogens, but also to initiate responses specific to each pathogen. Moreover, recent studies have shown that signaling through the IFN-α/β receptor is required for LPS- and CpG-induced “cross-priming” of ovalbumin-specific cytotoxic T cells (11). Thus, LPS-induced type I IFN contributes to both the innate and adaptive immune responses.

Because IFN-β is one of the “signature” cytokines induced via the MyD88-independent pathway in response to LPS (2), we sought to determine the extent to which IFN-β contributes to LPS-induced signaling. To this end, we compared the capacity of E. coli LPS to induce gene expression, cytokine secretion, and activation of intermediate kinases and transacting factors in macrophages from wild-type, IFN-β knock-out (IFN-β−/−) mice and mice with a targeted mutation in the IFN-β gene (12). We found that IFN-β has a broader impact on TLR4-mediated signaling than would have been predicted based on the simple paradigm of MyD88-dependent versus MyD88-independent signaling pathways. In this report, we have identified previously unappreciated regulatory roles for IFN-β in LPS signaling by comparing not only the induction of gene expression (by real-time PCR and Affymetrix microarray analysis), but also the activation of intracellular signaling molecules in wild-type versus IFN-β−/− macrophages, as well as by studying the response of such cells to LPS after induction “priming” with exogenous IFN-β. In vivo challenge of wild-type and IFN-β−/− mice also revealed that IFN-β contributes significantly to LPS-induced lethality, although not to the extent that one observes in mice with more complete pathway deficiencies (e.g. TLR4 or TRIF knock-out mice).

EXPERIMENTAL PROCEDURES

Reagents—Protein-free (<0.008%) E. coli K235 LPS was prepared by two cycles of hot phenol-water extraction as described previously (13). Polyclonal antibodies directed against phosphorylated Akt, ERK, JNK, p38 MAPK, and STAT1 (Ser-727), along with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G, were obtained from Cell Signaling Technology (Beverly, MA). Polyclonal anti-phospho-STAT1 (Tyrr-701) was purchased from Zymed Laboratories (San Francisco, CA). Anti-β-actin antibody was from Santa Cruz Biotechnolgy (Santa Cruz, CA). Recombinant murine IFN-β was obtained from Toray Industries (Japan).

Mice—Control C57BL/6J (wild-type IFN-β+/+) mice were purchased from Jackson Laboratories (Bar Harbor, ME). IFN-β knock-out (IFN-β−/−) mice (backcrossed ≥N8 onto a C57BL/6 background) (12) were bred homozygously both at the University of Maryland, Baltimore and the Toronto General Research Institute. All animal work was carried out with institutional approval.

Macrophage Cultures—Peritoneal exudate macrophages were obtained from 5- to 6-week old mice by lavage 5 days after intraperitoneal injection of 3 ml of sterile 3% thioglycollate broth. Cells were washed and resuspended in RPMI containing 2% fetal calf serum and standard supplements (14). Macrophages were plated in 24-well tissue culture dishes (4 cells/well) for supernatant collection or 6-well tissue culture dishes (4×10^6 cells/well) for supernatant collection or 6-well tissue culture dishes for supernatant collection or 6-well tissue culture dishes for supernatant collection or 6-well tissue culture dishes. Cells were harvested 1, 3, 5, or 8 h after LPS treatment for mRNA isolation, after 24 h treatment for cytokine production, or after 0.5, 1, or 2 h post-treatment for whole cell lystate preparation. All samples were stored at −70 °C.

Primary bone marrow-derived macrophages (BMMs) were obtained from mice by flushing the marrow from tibia and femurs into 10% Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin (Invitrogen). Cells were allowed to adhere to 24 h at 37 °C and 5% CO₂ and maintained in culture for 6 days in the presence of 10 ng/ml murine colony stimulating factor (R&D Systems, Minneapolis, MN). Cells were treated with 10 ng/ml LPS for 1 or 3 h then harvested using 2.4 units/ml Dispase (Sigma), and total RNA isolation was performed using the Qiagen RNaseq mini kit according to the manufacturer’s protocol for use in Affymetrix GeneChip® Microarray analyses (described below).

Enzyme-linked Immunosorbent Assays—Murine IL-12 p70, IP-10, RANTES, and TNF-α were detected in macrophage culture supernatants using the antibody pairs and standards provided in the Quantikine M enzyme-linked immunosorbent assay kit (R&D Systems).

mRNA Detection by RT-PCR and Southern Blot Analysis—Total RNA was isolated from macrophage cultures and reverse transcription-PCR (RT-PCR) was performed, as described previously (15). PCR was performed under the following conditions. The primers and probe combinations for detection of IRF-3 mRNA were as follows: sense, 5′-AAC AAT GGG AGT TGA CGG TGC-3′; antisense, 5′-TCC TGG ACC TCT CCA TC (annealing temperature, 60 °C); and probe, 5′-TGA CGG ACA AGC TTG TGA AG. The primers and probe combinations for detection of IRF-7 mRNA were as follows: sense,
mRNA Quantification Using Real-time PCR—Real-time PCR was carried out as described elsewhere (22, 23) on additional sets of macrophage mRNAs to confirm results obtained by RT-PCR and Southern blotting. The “fold increase,” the relative increase in gene expression in LPS-treated versus medium-treated control cells, was calculated using the ΔΔCt method as previously described (24).

Affymetrix GeneChip® Microarray Analyses—The preparation of cDNA from RNA derived from BMMs, sample hybridization, and scanning of the MOE430A oligonucleotide microarray GeneChip® Arrays (Affymetrix) was performed at the Centre for Applied Genomics Microarray Facility (Hospital for Sick Children, Toronto, Ontario, Canada) in accordance with the procedures established by Affymetrix. The Affymetrix MOE430A chip contains 11 replicate probe sets per chip (i.e. 11 distinct oligonucleotide probes spanning each gene) that are used to address specifically the issue of reliability of the data generated and to determine a parameter for probe set binding using MAS 5.0 software (Affymetrix). Data were further analyzed using ArrayAssist (Stratagene, La Jolla, CA) software. GC content—robust multiarray average analysis was performed, and hybridization experimental data for each LPS-treated sample were normalized to an intensity cutoff value of 50 was utilized. Hybridization experiments generated and to determine a value of 1. A positive number indicates the fold increase in gene expression, whereas a negative number indicates the fold decrease in gene expression compared with the basal levels of wild-type, untreated controls. Genes exhibiting a 3-fold or greater change in expression compared with the untreated IFN-β+/+ BMM cells were considered to be significant.

Computer-assisted Promoter Analysis—Sequences were obtained from the UCSC Genome Bioinformatics (genome.ucsc.edu). Gene promoters were assessed for potential STAT-binding sites using Gene2Promoter and MatInspector from Genomatix. Sequences 5’ upstream from the Gene2Promoter-predicted transcriptional start site of each gene were analyzed using MatInspector, a software tool that uses the Matrix Family Library Version 5.0 (February, 2005) library of matrix definitions for transcription factor binding sites to locate matches in target sequences (25). The output matrix positions correspond to sense strand numbering, and all sequences were provided in the 5’-3’ direction.

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RESULTS

LPS-induced, IFN-β-dependent Gene Expression—Previous studies have demonstrated that LPS stimulates two distinct signal transduction pathways, MyD88-dependent and MyD88-independent, that induce gene expression (27). The induction of IFN-β by LPS is dependent upon the MyD88-independent pathway. Moreover, the autocrine/paracrine action of IFN-β has been shown to enable the expression of other genes within this pathway, including Mcep5 (Ccl12), IP-10 (Ccr10), and NOS through activation of STAT1 (2). To analyze the contribution of IFN-β to the pro-inflammatory response to LPS, we analyzed the expression of a panel of well characterized, LPS-inducible genes in peritoneal exudate macrophages derived from wild-type C57BL/6 (IFN-β+/+) and IFN-β−/− mice.

Fig. 1A illustrates the results of semi-quantitative RT-PCR with Southern blot analysis for mRNA derived from wild-type, IFN-β−/− C57BL/6 macrophages (left lanes), and IFN-β−/− macrophages (right lanes). Glyceraldehyde-3-phosphate dehydrogenase was used as a “housekeeping” gene and was comparably expressed in all samples. As would be expected, IFN-β mRNA was expressed only in wild-type macrophages and not in the IFN-β−/− macrophages. Our previous studies had demonstrated LPS-induced MCP-5 (Ccl12), iNOS, and IP-10 (Cxcl10) gene expression to be IFN-β-dependent (via induction of activated STAT1) and poorly induced by TLR2 agonists (2). Consistent with these findings, LPS-induced MCP-5 mRNA expression was abrogated in IFN-β−/− macrophages, whereas iNOS and IP-10 mRNA expression were both significantly diminished when compared with the response of control IFN-β−/− macrophages (Fig. 1A). Basal levels of iNOS were undetectable (Fig. 1A), whereas LPS-induced iNOS mRNA message in IFN-β−/− macrophages peaked at 5 h and exhibited a subsequent decline in mRNA levels, in contrast to the sustained signal produced by control macrophages. Similarly, basal levels of steady-state IP-10 mRNA were diminished in IFN-β−/− mac-
rophages, yet in response to LPS, there was a suboptimal peak at 3 h that declined over 5 and 8 h, in contrast to the sustained mRNA levels seen in C57BL/6 macrophages (Fig. 1A). Like IFN-β, RANTES (CCL5) has also been shown to be an immediate early gene that is dependent upon LPS-induced activation of the MyD88-independent pathway (28). However, it is evident from our data that basal levels of RANTES gene expression in IFN-β−/− macrophages are much lower than in control macrophages, whereas levels of RANTES steady-state mRNA appear to be fairly comparably inducible to those seen in wild-type C57BL/6 macrophages at 3, 5, and 8 h after stimulation of macrophages with LPS.

Because RT-PCR with Southern blot analysis is only semiquantitative, we sought to quantify these differences by performing real-time PCR for some of the genes shown in Fig. 1A. As would be expected, there was no detectable mRNA for IFN-β in the IFN-β−/− macrophages. In multiple experiments, the basal levels of all genes examined in this panel (with the exception of the housekeeping gene) were consistently lower in IFN-β−/− macrophages than in control macrophages (not vis-

FIGURE 1. IFN-β regulates basal and LPS-induced expression of genes known to participate in LPS-induced responses. A, RT-PCR with Southern analysis was carried out on RNA derived from thioglycollate-elicited macrophages treated with medium only (0) or for the indicated times with LPS 100 ng/ml. B, real-time PCR analysis of the same samples analyzed in A. The results are derived from one of three independent experiments.
possible on most graphs in Fig. 1B). Fig. 1B confirms significant differences in LPS-inducible levels of iNOS, IP-10, and to a lesser extent, RANTES mRNA, in control versus IFN-β−/− macrophages. Thus, the failure to produce IFN-β also greatly impacts expression of a number of genes induced by LPS shown previously to be dependent upon the MyD88-independent pathway.

Role of IFN-β on MyD88-dependent Gene Expression—To date, examination of gene expression in macrophages obtained from mice with targeted mutations in TRIF or TRAM, the two adapters required for initiation of LPS-induced MyD88-independent pathway, have suggested that the MyD88-dependent pathway is fully intact (reviewed in Ref. 8). To assess the potential contribution of IFN-β to the expression of LPS-inducible genes that have been characterized as MyD88-dependent, similar analyses were performed on the same RNA samples described above. RT-PCR with Southern blot analysis revealed diminished constitutive expression of TNF-α, IL-1β, IL-6, IL-12 p35, and IL-12 p40 mRNA in IFN-β−/− macrophages, as well as a contribution of IFN-β to LPS-induced IL-6, IL-12 p35, and IL-12 p40 mRNA (Fig. 1A). Again, we utilized real-time PCR to obtain more quantitative results for selected genes (Fig. 1B). Although TNF-α mRNA expression was comparable at early time points following LPS, a significant decrease in steady-state mRNA was observed in the IFN-β−/− macrophages at 5 h after LPS stimulation. Induction of IL-12 p40 and MCP-1 mRNA was also markedly decreased in IFN-β−/− macrophages. MyD88 gene expression itself was also shown to be IFN-β-dependent as seen in Fig. 1B; by 5 h after LPS stimulation, there was a 5-fold difference in the levels of MyD88 mRNA in wild-type versus IFN-β−/− macrophages.

These findings were confirmed at the level of cytokine secretion. Specifically, LPS-induced secretion of IL-12 p70 and IP-10 were markedly suppressed in IFN-β−/− macrophages, whereas RANTES and TNF-α secretion were only modestly inhibited (Table 1). Taken collectively, these data suggest that not only does IFN-β contribute to the constitutive and LPS-induced expression of genes normally associated with the MyD88-independent pathway but also those associated with the MyD88-dependent pathway.

Role of IFN-β in Regulation of Interferon Regulatory Factor Genes—A family of closely related transacting factors, the interferon regulatory factors (IRFs), are known to be modulated by stimulation of cells by LPS and by type I and II IFNs. Early studies demonstrated that LPS could induce IRF-1, IRF-2, and IRF-8 (previously referred to as interferon consensus sequence-binding protein or ICSBP) expression in murine macrophages, with IRF-1 being independent of de novo protein synthesis (16). Therefore, we also measured the expression of IRF-1, -3, -7, and -8 genes in wild-type and IFN-β−/− macrophages in response to LPS. LPS is known to activate IRF-3 via the TLR4, MyD88-independent pathway (28, 29), and it has been shown that IRF-3 up-regulates expression of IRF-7 (30). Fig. 1A also shows that basal levels of IRF-1 were decreased in IFN-β−/− macrophages, and LPS-induced mRNA levels were decreased at all time points examined as compared with C57BL/6 macrophages. IRF-3 mRNA levels were IFN-β-independent and not modulated by LPS, and although the basal level of IRF-7 mRNA was IFN-β-dependent, it was not modulated by LPS. IRF-8/ICSBP is essential for IL-12 production (31, 32). Basal levels of IRF-8 mRNA were decreased in the IFN-β−/− macrophages, and interestingly, steady-state mRNA levels decreased with LPS treatment, whereas they increased with LPS treatment of C57BL/6 macrophages (Fig. 1B). Thus, IFN-β modulates basal and LPS-inducible expression of key IRFs.

| Treatment | C57BL/6 | IFN-β+/+ | IFN-β−/− |
|-----------|---------|----------|----------|
| IL-12 p70 | <7.8    | 65 ± 14  | <7.8     | 13 ± 9* |
| IP-10     | <31.3   | 4,406 ± 786 | <31.3 | 631 ± 146* |
| RANTES    | <7.8    | 77,301 ± 13,063 | <7.8 | 56,313 ± 11,117 |
| TNF-α     | <23.4   | 7,927 ± 1,427 | <23.4 | 4,689 ± 1,591 |

* p < 0.01.

**TABLE 1**

Cytokine production in C57BL/6 versus IFN-β−/− macrophages following LPS stimulation

Results represent mean ± S.E. of two independent experiments. All samples measured below the lowest detectable limit were undetected.
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In wild-type IFN-β+/+ macrophages, 150 genes were up-regulated after 1 h of LPS stimulation, and 526 genes were up-regulated after 3 h of stimulation. In contrast, LPS stimulation of IFN-β-/- macrophages for 1 h resulted in 19 genes that were down-regulated and 508 genes were down-regulated after 3 h. In macrophages derived from IFN-β-/- mice, LPS stimulation for 1 and 3 h increased the expression of 111 and 562 genes, respectively. Additionally, LPS treatment down-regulated expression of 47 genes by 1 h and 566 genes in IFN-β-/- macrophages by 3 h. LPS treatment of macrophages resulted in the up-regulation of a common subset of genes (466 genes at 3 h), independent of IFN-β.

Notably, a subset of 62 genes was identified whose expression is LPS-regulated and IFN-β-dependent (supplemental Table S1, sections III and IV). Among these are *Ifnb, Cxcl10 (IP-10), Ccl12 (Mcp5), Il6, Irf1, and Irf7* (identified in Figs. 1 and 2) and other known IFN-regulated genes, including *Mx1, Ifi203, Rsad2, USP18*.

**FIGURE 2.** Affymetrix microarray analysis of gene expression induced by LPS in IFN-β+/+ and IFN-β-/- macrophages. A, depicted here as a heat map are genes that were identified to be differentially expressed (≥3-fold) in supplemental Table S1 (sections I–IV correspond to groupings described in the text and supplemental Table S1). The color-coding is for the intensity of gene expression for each probe set, with red being the highest and green being the lowest. B, Venn diagrams of genes regulated by LPS in macrophages from IFN-β+/+ and IFN-β-/- mice. Only probe sets with ≥3-fold changes of ≥3 (up-regulated (upward arrow) and down-regulated (downward arrow)) in macrophages left untreated (basal levels), or following LPS treatment for 1 or 3 h are shown. The percentages of overlapping genes common to both groups are shown. Genes whose expression was either up-regulated or down-regulated in BMMs derived from wild-type or IFN-β-/- mice were considered for all time points (0, 1, and 3 h), and the numbers of regulated genes common between wild-type and IFN-β-/- BMM were identified. The percentage of common genes were calculated as the number of commonly regulated genes divided by the total number of genes whose expression was significantly (=3-fold) differentially regulated in the BMMs from the IFN-β-/- mice, i.e. following 1 h of LPS stimulation, gene expression was up-regulated in 150 and 111 genes in the BMMs from wild-type and IFN-β-/- mice, respectively. Of these, 89 were common, representing 80.2% of the up-regulated genes identified in the IFN-β-/- BMMs. C, relative gene expression of select genes from groups I–IV as detected by microarray analysis.
Stat1, Gbp2, Trim21, and Daxx (33–36). Consistent with previous reports, many of the genes identified in Fig. 2A and supplemental Table S1 (section III), such as Cxcl10 (IP-10), Ifit1, Ifit2, Tyki, and VHSV1/Rsad2, have been shown to be transcriptionally regulated in a MyD88-independent, but TRIF-dependent fashion (9, 37).

Unexpectedly, we identified a subset of 30 genes for which expression was up-regulated at least \( \geq 3 \)-fold greater following...
LPS treatment of macrophages derived from IFN-β−/− mice compared with macrophages from IFN-β+/+ mice (supplemental Table S1, group IV). These data suggest a negative regulatory role for IFN-β for these LPS-inducible genes. Fig. 2C illustrates the pattern of relative gene expression for select genes from within each subgroup based on the data obtained from the Affymetrix data.

**Computer-assisted Promoter Analysis of IFN-β-dependent Genes**—Two well characterized transcription factor binding sites that are ubiquitously present in the promoters of IFN-sensitive genes and are considered signature elements for IFN induction are the IFN-stimulated response element (ISRE) and the γIFN-activated site (GAS) (38). Accordingly, sequences 1000 bases upstream from the transcriptional start site of selected genes from each of the groups identified in sub-sections I–IV were analyzed for the presence of ISRE and GAS elements: TTN5AA, the palindromic GAS consensus sequence, and (G/A)N4GAAA, the ISRE consensus sequence (Fig. 3). Analysis of these non-coding regions revealed the presence of putative ISRE and/or GAS elements, in addition to other trans-acting factor binding domains (Fig. 3 and Table 2).

**LPS-induced Phosphorylation of Key Intracellular Signaling Proteins**—It has been previously shown that LPS induces phosphorylation of STAT1 on Tyr-701 after 2 h of stimulation and that this is blocked by anti-IFN-β, but not anti-IFN-α, monoclonal antibody (2). We have now confirmed this observation using macrophages derived from IFN-β−/− mice (Fig. 4). Interestingly, phosphorylation of STAT1 on Ser-727 was also partially IFN-β-dependent, as evidenced by a partial reduction in phosphorylation in IFN-β−/− macrophages. LPS-induced phosphorylation of Akt is secondary to activation of phosphatidylinositol 3-kinase (39, 40). Fig. 4 also demonstrates that phospho-Akt levels are markedly lower in IFN-β−/− macrophages than in control macrophages. Therefore, IFN-β is

![FIGURE 3. Promoter analysis reveals ISRE and/or GAS elements in the upstream non-coding regions of selected genes differentially expressed in BMM from IFN-β−/− mice. Elements 1000 bases 5′ upstream from the transcriptional start site were analyzed by MatInspector (www.genomatix.de/products/MatInspector) to identify the presence of transcription factor binding sites. The nucleotide sequences and specific locations of DNA binding elements within these promoter regions are provided with positions relative to the transcriptional start site equal to +1. Symbols indicating distinct DNA binding elements (e.g. Oct1, ISRE, GAS, SPI, NF-kB, GC box), as well as the TATA box, are shown.](image)

### Table 2
**Computer-assisted promoter analysis**

Promoter regions from genes selected from each group were analyzed as described under “Experimental Procedures.”

| Group | Gene     | GAS-like elements | ISRE elements |
|-------|----------|-------------------|---------------|
| Group I | Rpgrip1  | −1893 to −1475 gtagTTCggattgaa  | −97 to −79 agagaaaaGAActgtaaac  |
|        |          | −730 to −712 ctctTTGAAagag  | −623 to −605 tgaataaGAActgtagac  |
|        |          | −517 to −499 ttcaggTTAAGGAAAac  | −663 to −645 ggcaatgGAActgtcct  |
| Group II | Ifi1     | −306 to −288 ccagtttaaGGAagatttt  | −127 to −1256 aatataaGAActgtccac  |
| Group III | Cxcl10   | −1603 to −1585 tttgagttcaGAAacca  | −208 to −190 gtagaAACAActgtac  |
| Ccl12 (Mcp5) |          | −467 to −449 ctagTTCggagscccc  | −139 to −121 tcggaaacGAActtcacc  |
|        |          | −310 to −292 tccagTTAaaaagtgaag  | −1879 to −1861 ggcaaaAGAActgtga  |
| Group IV | Ogn      | −59 to −41 ttctgTTCCggagacttt  | −108 to −90 gtgaatGACActgtgtac  |
|        | Gene E   | −254 to −236 gtagTTCggaggttcctct  |               |
involved in the LPS-induced activation of phosphatidylinositol 3-kinase leading to Akt phosphorylation.

In contrast to activation of STAT1 and Akt, the MAPK pathway was completely unaltered in IFN-β−/− macrophages in response to LPS (Fig. 4). In both wild-type and knock-out macrophages, all three MAPK, p38, JNK, and ERK, exhibited peak phosphorylation at 0.5 h and a subsequent reduction in signal at 1 and 2 h.

**Effect of IFN-β Priming on LPS-induced Gene Expression**—For many decades, it has been recognized that priming of macrophages with IFNs often results in an augmented response to subsequent inflammatory stimuli. Indeed, previous studies have shown that IFN-α/β- or IFN-γ-mediated priming of murine macrophages results in a significant augmentation of LPS-induced soluble mediators, e.g. cytokines, nitric oxide, and the induction of tumoricidal activity (41–43), which has been attributed to the synergistic activation of transacting factors that engage specific promoters, such as the inducible nitric-oxide synthase (iNOS) promoter (44).

We therefore sought to determine if LPS-induced gene expression could also be up-regulated in rIFN-β−primed macromages from mice that lack the ability to produce IFN-β. Therefore, macrophages from wild-type or IFN-β−/− mice were treated with medium only or primed with IFN-β (100 units/ml) for 16 h, followed by stimulation with 100 ng/ml LPS for 0, 1, 3, and 5 h. Fig. 5 illustrates that, for some genes, wild-type responses to LPS were augmented by prior exposure to IFN-β priming (e.g. Ifnb, Mcp5, IP-10, Mcp1, and Il6); however, not all genes exhibited this pattern. Specifically, IFN-β priming of wild-type macrophages resulted in no change in steady-state levels of IL-13, IL-12 p40, and MyD88 mRNA.

Pretreatment of macrophages derived from IFN-β−/− mice with exogenous rIFN-β restored LPS-induced gene expression to near-normal or higher levels for a subset of genes (e.g. MCP-5, iNOS, IL-6, IL-12 p40, and MyD88), whereas IP-10 remained at levels below that exhibited by unprimed, wild-type macrophages. Thus, the lack of endogenous IFN-β not only constrains expression of many LPS-induced genes but also alters the extent to which such cells can be primed exogenously.

**LPS-induced Mortality**—To determine the role of IFN-β in a model of endotoxicity, C57BL/6 mice and IFN-β−/− mice were injected with 35 mg/kg E. coli K235 LPS, a dose that is 90–100% lethal in C57BL/6 mice. As shown in Fig. 6, IFN-β−/− mice are significantly less sensitive to LPS-induced mortality. By Day 4, the mortality rate for C57BL/6 and IFN-β−/− mice was 100% and ~70%, respectively. These data support the hypothesis that IFN-β contributes to LPS-induced lethality in vivo.

**DISCUSSION**

IFN-β−/− mice have been used in the past to elucidate the role of IFN-β in viral infections, due to the pivotal role of IFN in protecting the host against viruses (12). Deonarain et al. found that IFN-β-null mice were much more susceptible to vaccinia virus than WT mice and found that, whereas IFN-β can induce IFN-α mRNA up-regulation, the converse is not true. Deonarain et al. (45) later found that mice lacking IFN-β were significantly more susceptible to coxsackievirus-induced myocarditis. In an independently derived IFN-β−/− colony, wild-type, IFN-β+/−, and IFN-β−/− mice were challenged with Sendai virus and IFN-α was measured. Erlandsson et al. (46) found that IFN-α was comparably inducible in splenocytes and bone marrow to the same extent as WT mice, but was completely abrogated in embryonic fibroblasts derived from IFN-β−/− mice. Only after IFN-β priming of the fibroblasts 2 h prior to viral infection was IFN-α recovered from IFN-β-null cells. These same mice were then used to study Listeria monocytogenes infection. Stockinger et al. (47) stimulated murine macrophages with L. monocytogenes and observed complete abrogation of known "IFN-inducible genes," including iNOS, NOD2, and NOD1 in IFN-β-null cells. A recent study by Treschow et al. (48) revealed that IFN-β-null mice were found to generate greater numbers of osteoclasts in vivo and that adoptive transfer of IFN-β-competent fibroblasts mitigated collagen-induced arthritis in the IFN-β-deficient animals. Thus, in this system, the lack of IFN-β leads to a hyper-inflammatory response. In the study presented herein, we have taken advantage of IFN-β−/− mice to delineate the contribution of this early LPS-inducible cytokine to macrophage gene expression and endotoxicity.

Although it has been recognized for many years that LPS is a potent inducer of IFN-β in vivo and by macrophages in vitro (49), its role in the physiologic response to LPS has been poorly characterized. The data presented herein support a role for IFN-β in endotoxicity as evidenced by the fact that our highly purified E. coli K235 LPS (35 mg/kg) resulted in 100% mortality in 4 days in C57BL/6 mice, whereas ~35% of IFN-β−/− mice were still alive at 6 days after challenge. This is supportive of studies by Karaghiosoff et al. (50) who previously demonstrated that 50 mg/kg E. coli 055:B5 LPS killed 100% of C57BL/6 mice by 6.25 days after challenge, whereas 100% of their independently derived IFN-β knock-out mice survived. The reasons for the relative differences in sensitivity between the two different studies is not obvious; however, differences in LPS purity (highly purified versus commercial) may account for these disparate observations, because the degree of backcrossing of the IFN-β−/− mice to C57BL/6 mice and the age of the mice at the time of challenge appear to be very similar in the two studies.
Nonetheless, our study suggests that IFN-β deficiency results in a less profound phenotype than mice with targeted mutations in TLR4 or TRIF (51, 52).

Nonetheless, our analysis of macrophage gene expression by RT-PCR, real-time PCR, or Affymetrix arrays presented herein provides new insights into the remarkable spectrum of genes that IFN-β regulates both basally and in response to LPS stimulation. IFN-β<sup>−/−</sup> macrophages exhibited lower basal expression of many pro-inflammatory genes as demonstrated in Figs. 1 and 2. Surprisingly, these included a number of genes that had been previously characterized as being MyD88-dependent, such as Tnfa, Il1b, Il6, Il12p40, and Il12p35. This finding implies that IFN-β contributes significantly to the “cross-talk” that is observed between the MyD88-dependent and MyD88-independent signaling pathways induced by TLR4 engagement and that IFN-β provides an important differentiation signal that enables full responsiveness to LPS.

The profound effect of IFN-β deficiency on key signaling pathways confirms and extends our previous observations. Specifically, the failure of LPS to induce STAT1 phosphorylation on tyrosine in IFN-β<sup>−/−</sup> macrophages is consistent with the previous observation that monoclonal anti-IFN-β antibody blocked LPS-induced activation of this key transcription factor and inhibited downstream gene expression (e.g. iNOS, IP-10, and MCP-5) (2, 53). Interestingly, the data presented herein also showed that serine phosphorylation of STAT1 was also partially inhibited in IFN-β-null macrophages. Although previous studies have implicated p38 in the phosphorylation of STAT1 on serine (39), the complete normalcy of p38 activation in response to LPS in IFN-β<sup>−/−</sup> macrophages would strongly support the findings of others that other serine kinases can activate STAT1 on serine (54–57).

FIGURE 5. Effect of rIFN-β priming of IFN-β<sup>+/+</sup> and IFN-β<sup>−/−</sup> macrophages for LPS-induced gene expression. Thioglycollate-elicited macrophages from IFN-β<sup>+/+</sup> and IFN-β<sup>−/−</sup> mice were cultured and pretreated for 16 h with medium (solid or clear bars, respectively) or rIFN-β (100 units/ml) (horizontal and vertical-filled bars, respectively) and were then challenged for the indicated times with LPS (100 ng/ml). The results are representative of one of three independent experiments.
higher than in extracts from pathogen-free or germ-free mice, synthetase and the 67-kDa protein kinase, were significantly suppressed in the latter mice. In contrast, the number of mice challenged in three separate experiments were significantly less expressed in IFN-β−/− macrophages and recently reported that significantly lower levels of STAT1 protein is detected in IFN-β−/− macrophages (58). Thus, both the STAT1 protein levels and the ability to be phosphorylated on tyrosine or serine are deficient in IFN-β−/− mice. This, in turn, would be predicted to account for reduced transcription of many of the genes detected by microarray analysis.

Studies carried out a number of years ago may now be revisited and reinterpreted in the context of TLR4 signaling and the possible role of IFN-β. For example, decreased basal levels of IFN-β in TLR4-defective C3H/HeJ macrophages were originally reported by Fultz and Vogel (43, 59) and by Barber et al. (16), suggesting that TLR4 stimulation by commensals or endogenous TLR4 agonists or exposure of mice to LPS in the environment (e.g. Gram-negative organisms contained in feces or possibly other exogenous TLR4 agonists) provides a necessary signal for the maintenance of basal IFN-β levels in wild-type macrophages. In this regard, DeMaeyer et al. (60) found that macrophages from germ-free mice failed to produce “spontaneous” IFN in vitro, in contrast to macrophages derived from conventionally reared animals, and they postulated that the latter might be the consequence of in vivo exposure to gut bacteria and, more specifically, to their LPS. Galabru et al. (61) found that in spleen and lung extracts of conventionally reared mice, levels of two IFN-inducible enzymes, 2′,5′-oligoadenylate synthetase and the 67-kDa protein kinase, were significantly higher than in extracts from pathogen-free or germ-free mice, or from mice that had been injected with anti-IFN-α/β antibody. Furthermore, Moore and colleagues (62) also showed that CSF-1-derived BMMs were significantly more responsive to LPS than peritoneal exudate macrophages and produced greater levels of type I IFN. This capacity of CSF-1 to prime for LPS-induced IFN production was inhibited by inclusion of anti-IFN-α/β during the expansion and differentiation of the bone marrow-derived precursors into macrophages in CSF-1. The data presented herein strengthen the hypothesis that basal IFN-β levels may contribute to priming, because treatment of IFN-β−/− macrophages with rIFN-β normalized responsiveness to LPS for some, but not all, genes examined (Fig. 5). This may be attributable to the capacity of low levels of IFN-β to induce or activate trans-acting factors that are missing or greatly diminished in IFN-β−/− macrophages (e.g. STAT1, IRF-1, etc.) and normally facilitate optimal transcription when a second inducer, such as LPS, is encountered.

The analysis of the data obtained from the Affymetrix array results and subsequent promoter analysis of genes identified as being IFN-β-dependent (Figs. 2 and 3, Table 2, and supplemental Table S1) reveal that a large number of genes apart from those previously identified as being involved in the inflammatory response to LPS were modulated either basally and/or in response to LPS. Although it is possible to hypothesize about the possible roles of genes such as Mx1, which has been strongly implicated in response to virus infection (34), these data potentially provide new clues as to their possible role in endotoxemia and Gram-negative sepsis. For example, one of the genes identified in our microarray analysis was Usp18, a gene found in subset II that exhibits diminished basal levels of gene expression in IFN-β−/− macrophages, yet is still LPS-responsive. Kim et al. (63) recently reported that the product of this MyD88-dependent gene (also called UBP43) acts as an inhibitor of type I IFN signaling and that UBP43-null mice were hypersensitive to LPS-induced lethality and selective gene expression (e.g. Il6, Mcp1, IP-10, and Mip1α (Ccl3)). The fact that IFN-β-null mice are significantly less sensitive, rather than hypersensitive, to LPS-induced lethality suggests that other IFN-β-dependent genes compensate for diminished expression of this negative regulator. In this regard, Murray and colleagues (64) have shown that SOCS-1−/− mice, like the Usp18−/− mice, are also hypersensitive to LPS in vivo; however, SOCS-1 also blocks IFN-β signaling (64). SOCS-1 was not identified within the microarray analysis as basally or inducibly dysregulated in IFN-β−/− macrophages. Nonetheless, many of the IFN-β-dependent genes selected for subsequent analysis in silico revealed that they contained in their promoters either GAS-like or ISRE-like elements, suggesting that IFN-β may be required to generate many of the cis-acting DNA-binding proteins required for expression of these genes. Moreover, such genes were readily identified in all four groups (i.e. genes whose basal or LPS-inducible levels of mRNA were either 3-fold greater or less than that seen in wild-type macrophages). Fig. 3 illustrates the utility of examining promoter composition in silico for potential commonalities in regulatory motifs within the promoters of similarly regulated genes (as shown in Fig. 3). Confirmation by independent binding assays would be required to confirm the functionality of these putative binding motifs.

In summary, this study reveals that IFN-β regulates both basal and LPS-induced levels of a broad spectrum of cytokines, chemokines, and genes not previously associated with endotoxicity and contributes significantly to endotoxicity in vivo. Thus, it may be possible to target IFN-β or its downstream signaling molecules in hyper-inflammatory syndromes such as Gram-negative sepsis.

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