Differential Regulation of Interleukin (IL)-12 p35 and p40 Gene Expression and Interferon (IFN)-γ–primed IL-12 Production by IFN Regulatory Factor 1

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Abstract

Interleukin (IL)-12 is a heterodimeric cytokine consisting of the p40 and p35 chains encoded on separate chromosomes. Coordinated expression of the two constituent genes is crucial for appropriate immune responses in timing, location, and magnitude. Interferon (IFN)-γ priming of IL-12 production by macrophages represents an important physiological process in vivo for escalated cellular response to microbial infections. We provide evidence that IFN regulatory factor (IRF)-1–deficient macrophages have a selective impairment in mRNA synthesis of IL-12 p35 but not the p40 gene, and a strong deficiency in the production of IL-12 p70 but not p40. We demonstrate that the levels of IL-12 p35 protein stimulated by IFN-γ and lipopolysaccharide (LPS) correspond to those of its mRNA, and that the nuclear factor κB signaling pathway is essential for the induction of IL-12 p35 transcription by LPS. IRF-1 plays a major role in the transcriptional activation of the IL-12 p35 gene, but not of the p40 gene, by physically interacting with an inverted IRF element within the IL-12 p35 promoter upon IFN-γ activation. Moreover, IRF-1–mediated transcriptional activation of the p35 promoter requires the cooperation of two adjacent Sp1 elements. Thus, IRF-1 acts as a critical component of IFN-γ signaling in the selective activation of IL-12 p35 transcription in synergy with LPS-mediated events.

Key words: IL-12 • IFN-γ • IRF-1 • macrophage • transcription

Introduction

IL-12 is produced primarily by macrophages and DCs in both innate and adaptive immune responses. It is a key factor in the induction of T cell–dependent and –independent activation of macrophages, NK cells, generation of Th1 cells, and CTLs, induction of opsonic, complement-fixing antibodies, and resistance to intracellular infections (1). Pathogens and tumor cells, on the other hand, often evade immune activation by producing immunosuppressive agents such as IL-10, TGF-β, and prostaglandin E2 that can inhibit IL-12 production, among other things, resulting in much weakened immune recognition and /or activation (2).

The control of IL-12 production by APCs is complicated by the fact that IL-12 is composed of two heterologous chains, p40 and p35. The genes encoding IL-12 p40 and p35 are located on different human and mouse chromosomes (3). The highly coordinated expression of p40 and p35 genes to form IL-12 (also called p70) in the same cell type at the same time is essential for the initiation of an effective immune response. The molecular mechanisms by which IL-12 p40 gene expression is regulated in APCs have been studied extensively in the last 7 yr and some important mediators and effectors have been identified (4–8). In contrast, the regulation of the p35 gene expression has not been given sufficient attention for a number of practical reasons. First, there was a misimpression that the p35 gene was "constitutively" and "ubiquitously" expressed and therefore not as interesting to study as the more inducible p40 gene. Second, the induction of p35 in APCs requires two signals typically provided by LPS and IFN-γ, respectively, making it more challenging to sort out the intermingled processes (5, 9). Third, there has been a lack of useful reagents with which to probe the pathway that leads to the induction of the p35 gene. Several recent studies, however, have shed

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new light on the transcriptional regulation of this gene and stirred reinvigorated enthusiasm for further exploration of the control of IL-12 p35 gene expression in immune responses. Among these studies that used gene knockout mice and overexpression approaches, IFN regulatory factor (IRF)-1 and nuclear factor (NF)-κB c-Rel are strongly implicated to be important transcription factors for IFN-γ–primed and LPS-induced p35 transcription, respectively (10–13). However, the molecular details of how IRF-1 regulates the p35 gene transcription remain to be elucidated.

IRF-1 is the first member of the IRF family of nine cellular genes identified as a transcription factor induced during a viral infection that bound to the IFN-β promoter (14, 15). Intensive analyses focused on IRF-1 because its initial discovery has revealed a remarkable functional diversity of this molecule in the regulation of cellular response in host defense. IRF-1 selectively targets different sets of genes in various cell types in response to diverse cellular stimuli and evokes appropriate innate and adaptive immune responses (16). IRF-1 has been firmly established as a critical effector molecule in IFN-γ–mediated signaling and in the development and function of NK and NKT cells, and CTLs (10, 17–21). The first demonstration of the importance of IRF-1 in IL-12 production and IL-12–mediated induction of Th1 immune responses was provided by the work of Taki et al. (10) who showed that T cells from mice lacking IRF-1 failed to mount Th1 responses and instead exclusively underwent Th2 differentiation in vitro. Compromised Th1 differentiation was associated with defects in IL-12 production by macrophages and hyporesponsiveness of CD4$^+$ T cells to IL-12. This study provides a mechanistic explanation for the IFN-γ–priming effect, i.e., IFN-γ–mediated activation of APCs that is required for vigorous IL-12 production to generate robust cellular immunity.

In this study, we mainly address the following question: how does IRF-1 regulate IL-12 p40 and p35 gene expression?

Materials and Methods

Mice. IRF-1$^{-/-}$ mice and their control, C57BL/6J mice, were obtained from The Jackson Laboratory. All mice were female and ~6–8 wk old. Mice were housed in cages with filter tops in a laminar flow hood and fed food and acid water ad libitum.

Cells. The murine macrophage cell line RAW 264.7 (RAW cells hereafter) was obtained from American Type Culture Collection and maintained in RPMI 1640 supplemented with 2 mM glutamine, 100 units/ml of penicillin and streptomycin, and 10% FBS (endotoxin <1 ng/ml; Hyclone). Resident peritoneal macrophages were obtained directly from the peritoneum cavity, and inflammatory peritoneal exudate macrophages were obtained by lavage 4 d after injection of sterile 3% thioglycollate broth (1 ml i.p.). Cells were washed and resuspended in RPMI containing 10% FCS and standard supplements. Macrophages were plated in 24-well tissue culture dishes (0.5 × 10^6 cells/well). After 2 h of incubation to allow for adherence of macrophages, monolayers were washed three times to remove nonadherent cells and incubated with RPMI containing 10% FCS and standard supplements. The next day some wells were treated with 1 μg/ml LPS in a final volume of 1 ml, or 10 ng/ml IFN-γ first for 16 h (priming) followed by LPS.

Human monocytes were isolated from fresh blood by Ficoll/Hypaque gradient centrifugation. Mononuclear cells were incubated for 1 h in polystyrene tissue culture flasks (Falcon; Becton Dickinson). To generate macrophages, adherent cells, typically >85% CD14$^+$ by FACS® analysis, were cultured in complete media supplemented with 100 ng/ml human M-CSF (PeproTech) for 6 d. The resulting cells were >95% F408$^+$ macrophages.

Reagents. All antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. The anti–human IL-12 p35 rabbit IgG from Santa Cruz Biotechnology, Inc. that was used in IL-12 p35 Western blot is H-197. Expression vectors pAct-1 (IRF-1), pAct-2 (IRF-2), and control pAct-C were provided by T. Taniguchi (University of Tokyo, Tokyo, Japan). The "dominant positive mutant" of IκBα was originally generated in the laboratory of H.C. Liou at Weill Medical College of Cornell University. It was made by deleting the first 38 amino acids of IκBα that contained the two critical serine residues in the phosphorylation sites, making this mutant resistant to intracellular degradation mediated through phosphorylation. According to H.C. Liou (personal communication), this truncated IκBα has a potent inhibitory effect on NF-κB activity in B cells. The expression vectors for NF-κB p50, p65, and c-Rel were provided by K. Murphy (University of Washington, St. Louis, MO) and have been described (4). All plasmid DNA were prepared with QIAGEN Endo-free Maxi-Prep kits (QIAGEN). Murine TNF-α was purchased from Genzyme. Recombinant human and mouse IL-12 were provided by the Genetics Institute.

ELISAs. Supernatants from macrophage cultures were harvested at 6, 12, and 24 h after LPS stimulation and stored at −70°C. Mouse and human IL-12 p70 and p40 were detected by using the OPT-EIA ELISA kit (BD Biosciences) according to the manufacturer's instructions. Concentrations were calculated by regression analysis of a standard curve. Data are expressed as picograms per milliliter.

RNase Protection Assay (RPA). Mouse macrophages and human peripheral blood monocytes were pretreated with IFN-γ for 16 h followed by treatment with LPS for an additional 4 h. 10 μg total RNA for each determination was subjected to multiprobe RNase protection kit (BD Biosciences), mCK2b for mouse and hCK2 for human samples, according to the manufacturer's instructions.

Nuclear Extract Preparation. Nuclear extracts for Western blot and DNA affinity binding assays were prepared according to the methods of Schreiber et al. (22).

Western Blotting. SDS-PAGE was performed according to Laemmli (23). Gels were electroblotted to nitrocellulose membranes and blocked in 5% milk in Tris buffer, pH 8.0. Primary antibody was added at the concentration of 1 μg/ml in Tris buffer containing 1% milk powder and left overnight at 4°C. After extensive washing, secondary antibody conjugated to horseradish peroxidase was added at a 1:5,000 dilution in 5% milk. After extensive washing, blots were subjected to enhanced chemiluminescence detection (PerkinElmer).

Transfection Assay. Transient transfections were performed by electroporation as previously described (5).

DNA Affinity Binding Assay. Complementary biotinylated oligonucleotides encompassing the human IL-12 p35 promoter IRF-E site (−229/−249) or the mutant IRF-E were synthesized and annealed. 2 μg biotinylated dsDNA were conjugated to 100 μl streptavidin-bound magnetic beads (Dynabeads, M280; Dynal) in binding/washing buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 M NaCl) for 30 min at room temperature. Unconjugated DNA was collected with a magnetic particle concentrator.

DNA-conjugated beads were then blocked by 0.5% BSA in TGEDN buffer (120 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 M NaCl, 1 mM DTT, 0.1% Triton X-100, and 10% glycerol) at room temperature for 1 h. Beads were washed once in TGEDN buffer and resuspended in 50 μl TGEDN. 10 μl beads conjugated to 2 μg DNA were equilibrated with TGEDN buffer and incubated with 500 μg RAW cell nuclear extracts and 20 μg herring sperm DNA (Sigma-Aldrich) at 4°C for 2 h. Beads were washed in TGEDN buffer and bound materials were eluted in 20 μl the same buffer supplemented with 0.5% SDS and 1 M NaCl. Eluted materials and unconjugated protein were separated by 12% SDS-PAGE and detected by immunoblot analysis using rabbit anti–IRF-1 or anti–PU.1 antibody with the enhanced chemiluminescence kit (PerkinElmer). Nuclear extracts were prepared from RAW cells treated with 10 ng/ml IFN-γ for 16h followed by incubation with LPS plus IFN-γ for an additional 4 h.

RT-PCR. RT reactions were performed as follows: 2 μg total RNA were mixed with 2 μl oligo dT primers (16 mer, 0.5 mg/ml) and ddH$_2$O to equalize volumes of all samples at 8.5 μl. The mix was heated at 65°C for 10 min, quenched on ice, spin down briefly, and 11.5 μl of a Master Mix was added. The RT Master Mix consisted of 4 μl 5× first stand buffer (GIBCO BRL), 4 μl 2.5 mM dNTPs, 2 μl 0.1 M DTT, 0.5 μl RNase inhibitor (40 U/μl; GIBCO BRL), and 1 μl Superscript II (200 μl/μl; GIBCO BRL). The action was incubated at 37°C for 90 min, and then 95°C for 10 min, followed by 4°C soak. To each sample (in 20 μl total volume) 80 μl ddH$_2$O were added. 3 μl were used for each PCR reaction of 25 μl in volume. The following primers were used for PCR amplification: human IL-12 p40 sense primer: CCAAGAACTTGCAGCTGAAG; antisense primer: TGGGTCTATTCCGTTGTGTC; human IL-12 p35 sense primer: GATGAGCTGATGCAGGCC; antisense primer; AGTCCTCCACCTCGTTGTCCGTGA; human IRF-1 sense primer: CAAATCCCGGGGCTCATCTGG; antisense primer: CTGGCTCCTTTTCCCCTGCTTTGT; human HPRT sense primer: CCTGCTGGATTACATCAAAGCACTG; antisense primer: TCCAACACTTCGTGGGGTCCT.

Chromatin Immunoprecipitation (ChIP) Assay. The ChIP procedure was performed using an assay kit according to the manufacturer's instructions (Upstate Biotechnology). In brief, 10^7 glycollate-elicited peritoneal macrophages from C57BL/6 mice or human peripheral blood monocytes were stimulated with 10 ng/ml IFN-γ priming and 1 μg/ml LPS or alone, and cross-linked by 1% formaldehyde for 10 min at 37°C. Nuclei were prepared and subjected to sonication to obtain DNA fragments ranging from 200–1,000 bp. Chromatin fractions were precleared with protein A agarose beads followed by immunoprecipitation overnight at 4°C with 3 μg anti–IRF-1 antibody and control antibody. Crosslinking was reversed for 4 h at 65°C and was followed by proteinase K digestion. DNA was purified and subjected to PCR. The input DNA were diluted 200 times before PCR. The input and precipitated DNA were PCR amplified by primers encompassing the IRF-1 site in the mouse IL-12 p35 promoter (5′ primer: TTGCTTTCGCTCTGAGTGTG and 3′ primer: GCTGAC-CTTGGGAGACACAT) and human IL-12 p35 promoter (5′ primer: GCGAACATTTCGCTTTCATT and 3′ primer: ACTTTCCCGGGACTCTGGT) in a buffer containing 2 mM MgCl$_2$. The samples were amplified for 34 cycles by PCR and analyzed by electrophoresis on a 1.2% agarose gel.

Adenoviral Vectors and Their Propagation. We integrated pTRE into pAdeno-X express system (CLONTECH Laboratories, Inc.). First, we amplified the mouse IRF-1 cDNA (mIRF-1) by RT-PCR and subcloned it into the T/A cloning vector PCR2.1 (In-

vitrogen), and then transferred the mIRF-1 fragment (NotI and KpnI) into pShuttle vector (NotI and KpnI). Finally, the mIRF-1 expression cassette was ligated with the pAdeno-X backbone by I-CeuI and PI-sceI digestion. At the same time we constructed the LacZ expression vector as a control by transferring the LacZ expression cassette into pAdeno-X using the same construction strategy as described for the mIRF-1 vector. Viruses were propagated in the HEK293 cell line (human embryonic kidney) and purified by ultracentrifugation through two cesium chloride gradients. Titers of viral stocks were determined by plaque assay in HEK293 cells after exposure to virus for 1 h in serum-free DMEM and by OD$_{260}$ reading. Approximately 25 optical viral particles were equivalent to 1 plaque-forming unit. Freshly isolated human monocytes were cultured in 100 ng/ml human M-CSF for 6 d. The resulting macrophages were exposed to recombinant virus for 4–8 h in serum-free RPMI 1640 medium followed by an equal volume of RPMI 1640 supplemented with 10% fetal calf serum for an additional 24 h. The transfection medium was replaced with fresh RPMI 1640 medium with 10% FCS. 24 h later the transfection efficiency was monitored by β-galactosidase staining (24) under reverse-phase fluorescence microscope.

Statistical Analysis. Student's *t* test was performed wherever applicable. Standard deviation of the mean is shown unless otherwise indicated.

Results

Differential Impact of IRF-1 Deficiency on IL-12 p40 and p70 Production. To determine the role of IRF-1 in the regulation of IL-12 production we obtained inflammatory peritoneal macrophages from IRF-1$^{-/-}$ mice and control wild-type animals, and then stimulated them in vitro with LPS or primed them with IFN-γ followed by LPS stimulation. IL-12 p40 and p70 production over a period of 24 h was measured by specific ELISA. Because of the lack of a p35-specific ELISA due to the fact that it is secreted only as a heterodimer, measurement of p70 production is a generally accepted indicator of IL-12 p35 production due to the stoichiometric nature of the p40-p35 dimerization. As shown in Fig. 1, IL-12 p40 production stimulated by LPS alone (Fig. 1 A) or IFN-γ plus LPS (Fig. 1 B) was only marginally lower in IRF-1$^{-/-}$ macrophages than in control cells (no statistical significance). On the other hand, IL-12 p70 production induced by LPS (Fig. 1 C) or IFN-γ plus LPS (Fig. 1 D) was strongly decreased in IRF-1$^{-/-}$ cells. Because IL-12 p70 consists p40 and p35 subunits in a 1:1 molar ratio, these data likely suggest that IRF-1 differentially regulates IL-12 p40 and p35 gene expression. This differential impact of IRF-1 deficiency on IL-12 p40 and p70 production is not restricted to inflammatory macrophages as resident and bone marrow–derived macrophages also exhibited very similar patterns (unpublished data).

To resolve the differences between our observation and that of Taki et al. (10), which showed a defect of IL-12 p40 production in IRF-1$^{-/-}$ macrophages stimulated by IFN-γ and LPS, we compared the timing of IFN-γ addition to the cell culture, which is the main difference in the technical protocol of the two studies. When IFN-γ was added simultaneously with LPS, IL-12 p40 production was substantially reduced in IRF-1$^{-/-}$ cells (Fig. 1 E) whereas p40 synthesis
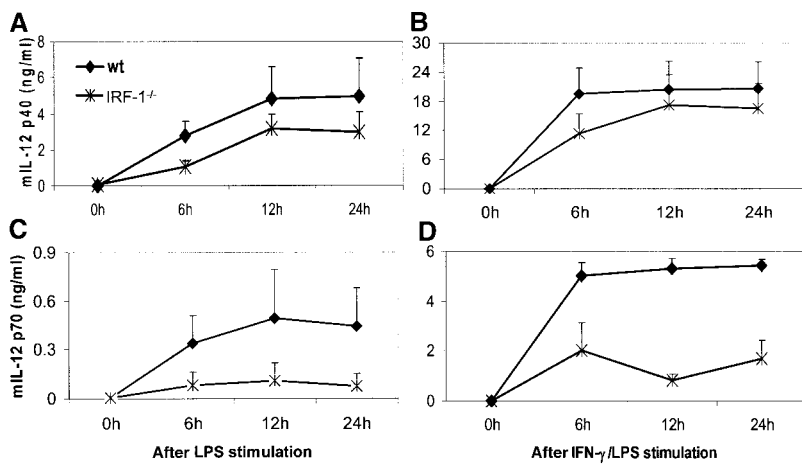
Figure 1. Differential impact of IRF-l deficiency on IL-12 p40 and p70 production. IL-12 p40 (A, B, and E) and p70 (C, D, and F) were measured by ELISA from cell-free supernatants of thioglycollate-elicited inflammatory mouse peritoneal macrophage cultures (0.5×10^6 cells in 1 ml) stimulated with LPS (A and C) or primed with IFN-γ for 16 h followed by LPS (B and D) or LPS with IFN-γ at same time (E and F) for the indicated times in hours. ◆, wild-type macrophages; *, IRF-1–deficient cells. Results shown are mean plus SD of four independent experiments.

was not impacted when IFN-γ was used as a priming agent (Fig. 1, A and B). IL-12 p70 production, however, was reduced in IRF-1$^{-/-}$ cells regardless of the timing of IFN-γ addition (Fig. 1, C, D, and F). This indicates that the length of the exposure of macrophages to IFN-γ before seeing LPS determines their ability to produce IL-12 p40 whereas it is not able to rescue IL-12 p35 expression in the absence of IRF-1.

Selective Impairment of IL-12 p35 mRNA Expression by IRF-1 Deficiency. To determine the level of the differential effects of IRF-1 deficiency on IL-12 p40 and p35 gene expression we examined their steady-state mRNA expression by RPA. Fig. 2 A shows that the level of IL-12 p40

mRNA induced by LPS alone or IFN-γ plus LPS was comparable between wild-type and IRF-1$^{-/-}$ peritoneal macrophages. So were most of the other monokines produced under these conditions. However, the IL-12 p35 mRNA expression was severely impaired in IRF-1$^{-/-}$ cells in LPS or IFN-γ/LPS–stimulated cells. These results suggest that IRF-1 is preferentially involved in the transcriptional regulation of the IL-12 p35 gene. In human peripheral blood monocytes, the IFN-γ priming effect is highly pronounced for IL-12 p35 and p40 mRNA expression (Fig. 2 B, compare lanes 3 and 4), and the p35 gene appears to be even more dependent on IFN-γ priming than the p40 gene. Moreover, in these cells, the mRNA expression
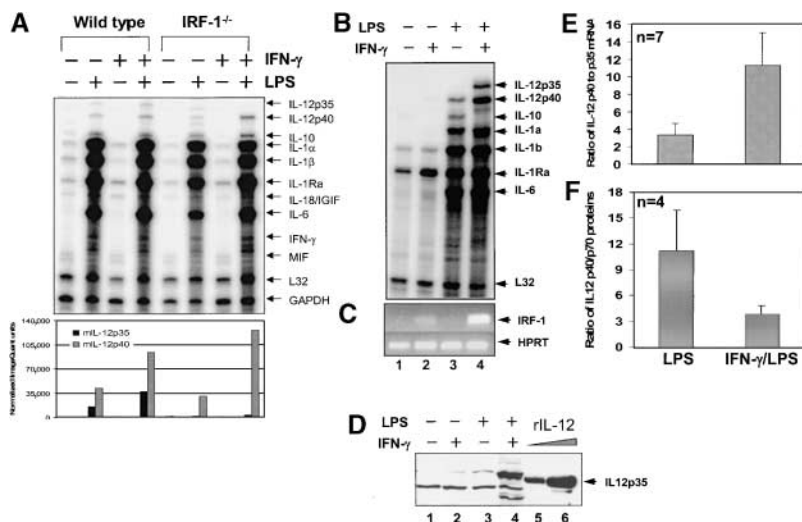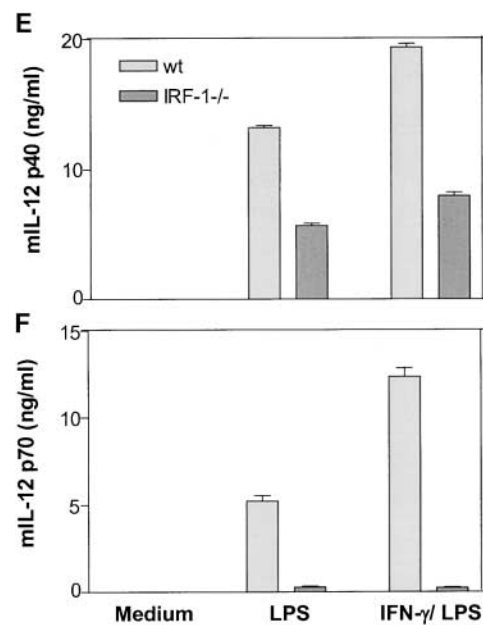

Figure 2. Selective impairment of IL-12 mRNA synthesis in IRF-1$^{-/-}$ macrophages. (A and B) RPA. Total RNA was isolated from peritoneal macrophages (A) or human peripheral blood monocytes (B) and subjected to RPA. 10 μg RNA were used for the analyses. The intensities of mIL-12 p35 and p40 mRNA bands in the top of A were quantified using the ImageQuant software and normalized to the internal loading controls L32 and GAPDH (A, bottom). Data are representative of four separate experiments with very similar results. (C) The IRF-1 mRNA expression was examined by RT-PCR under the same conditions together with the determination of mRNA expression of the housekeeping gene HPRT. (D) IL-12 p35 protein expression was analyzed by Western blot under reducing conditions using an anti–IL-12 p35 antibody with whole cell lysates (150 μg per lane) isolated from human blood–derived monocytes stimulated with LPS for 20 h with or without IFN-γ priming. The control used was reduced recombinant human IL-12 in two amounts: 200 and 510 pg. Note that the IFN-γ/LPS–induced band in lane 4 ran slightly slower than the control (rIL-12), possibly because

of the cell lysate overloading effect. Other bands that reacted with the antibody in lanes 1–4 are of undetermined nature. (E) The ratio of mIL-12 p40 versus p35 mRNA was derived from seven independent RPAs after normalization against the internal controls L32 and GAPDH. (F) The ratio of mIL-12 p40 to p70 proteins was derived from four separate experiments.

of IRF-1 is highly consistent with that of the IL-12 p35 as shown in Fig. 2 C in that IFN-γ and LPS synergistically induced IRF-1 transcript synthesis (Fig. 2 C).

It has not been established if p35 production is directly related to p35 mRNA levels. To demonstrate the relationship between IL-12 p35 mRNA expression and its protein synthesis, we analyzed the level of IL-12 p35 protein in whole cell lysates derived from human monocytes stimulated with IFN-γ, LPS, or both. As shown in Fig. 2 D, the protein expression of IL-12 p35, detected by an anti-p35 antibody, was most induced by IFN-γ and LPS together. The level of this induction was comparable to that seen in the IL-12 p35 mRNA level, indicating that there is no major regulatory step between the level of p35 mRNA and its translation. To test the assumption whether the discrepancy between p40 and p70 released into the culture supernatant is due to regulation of p35 production, we quantified the ratio of IL-12 p40 versus p35 mRNA levels from seven independent RPAs in wild-type murine peritoneal macrophages stimulated with LPS or IFN-γ plus LPS (Fig. 2 E). The p40/p35 mRNA ratio was 3.4 in LPS-stimulated cells and 11.2 in IFN-γ/LPS–stimulated cells. The p40/p70 protein ratio calculated from the four experiments in Fig. 1, A–D, was 11.2 in LPS-stimulated cells and 3.8 in IFN-γ/LPS–stimulated cells (Fig. 2 F). Taken together, these results suggest that in macrophages activated by LPS, there might be additional regulatory steps in the total output of p70 other than transcription and translation of IL-12 p35, such as the assembly of p40 and p35 to form p70 and its secretion. In IFN-γ– and LPS-stimulated cells the p40 and p35 proteins might be more efficiently assembled into p70 or secreted.

Regulatory Regions of the IL-12 p35 Promoter in IFN-γ/ LPS Response. To further explore the molecular mechanism whereby IFN-γ and LPS regulate IL-12 p35 transcription through IRF-1, we analyzed the human IL-12 p35 promoter with respect to the important regions that mediate transcriptional response to IFN-γ/LPS stimulation by sequentially deleting the full-length promoter, a 1,143-bp genomic fragment (8), from the 5′ end. The transcriptional start site of the human IL-12 p35 gene in macrophages has been mapped (25). We used a well-established transient transfection cell model in the murine myeloid cell line RAW264.7 using the firefly luciferase reporter gene (8). The full-length IL-12 p35 promoter (+61 to −1,082, C1 construct) was highly responsive to stimulation by the combination of IFN-γ priming and LPS treatment whereas LPS alone had little effect in the activation of p35 transcription (Fig. 3 A). Sequential deletion of the full-length promoter down to −392 (C2 and C3 constructs) had no visible impact on the inducibility of the p35 promoter by IFN-γ and LPS. A further deletion of 152 bp (to −240, C4 construct) resulted in a complete loss in the response, indicating the presence of a critical cis element(s) in this region involved in response to IFN-γ and LPS. By this approach, we identified the C3 construct as a minimal promoter (+61 to −392) that retained full IFN-γ/LPS response, and it was used for most of the subsequent experiments. In this cell line, IFN-γ, not LPS, greatly induced nuclear IRF-1 protein expression (Fig. 3 B). Again, this illustrates that IRF-1 is necessary but not sufficient to activate IL-12 p35 transcription. Additional LPS-induced factors, such as NF-κB (12), have also been shown to be required to stimulate the transcriptional response.

Role of LPS and NF-κB in the Regulation of IL-12 p35 Transcription. The role of LPS in the induction of IL-12 p35 transcription has been addressed in a previous study by Grumont et al. (12). This study demonstrated that the NF-κB signaling pathway is necessary for the induction of IL-12 in response to microbial stimuli. In particular, expression of IL-12 p35 induced by *Staphylococcus aureus*, DNA, or LPS is c-Rel dependent and regulated directly by c-Rel complexes binding to the IL-12 p35 promoter (12). We overexpressed NF-κB p50, p65, and c-Rel by cotransfection
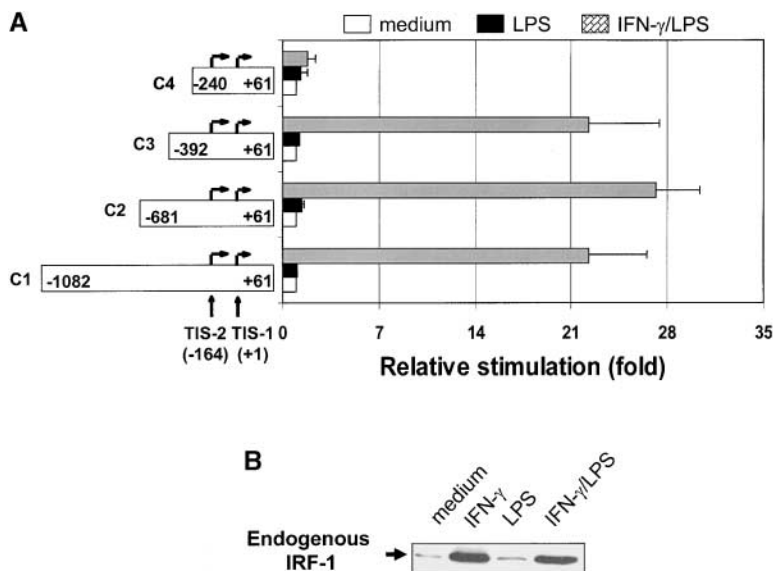
Figure 3. Critical regions of IL-12 p35 promoter in IFN-γ/LPS response. (A) Various truncation constructs of the human IL-12 p35 promoter luciferase reporter were transiently transfected into RA W264.7 cells by electroporation. Cells were stimulated with LPS alone (7 h), or IFN-γ (16 h) followed by LPS. Luciferase activity from each construct was measured from cell lysates. Results shown are mean plus SE of three to four independent experiments. The promoter coordinates all refer to the first, monocyte-specific transcription initiation site (TIS-1) located at +1 (reference 25). The second, B cell–specific transcription initiation site (TIS-2) located at −164 (reference 25) is also indicated. (B) The endogenous IRF-1 protein expression in RAW264.7 cells was analyzed by Western blot using an affinity-purified polyclonal anti–IRF-1 antibody under the same cellular stimulations.

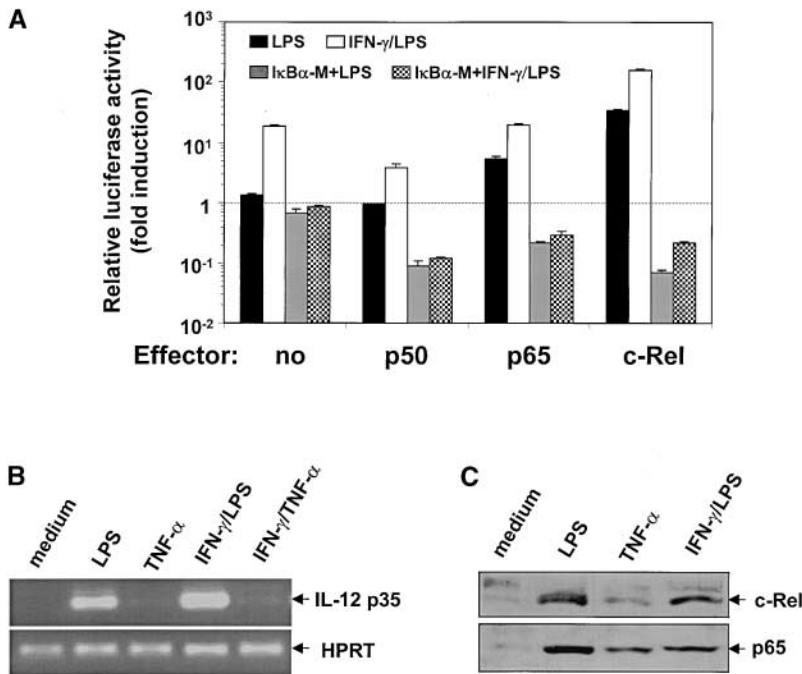**Figure 4.** Role of LPS and NF-κB in the regulation of IL-12 p35 transcription. (A) c-Rel can activate IL-12 p35 transcription. RAW264.7 cells were transiently transfected with the full-length IL-12 p35 promoter, as described in Fig. 3, together with an expression vector for NF-κB p50, p65, or c-Rel at a 1:1 molar ratio (effector to reporter) with or without the IκBα mutant, also at a 1:1 molar ratio. Data are representative of four independent experiments (mean plus SE), expressed as relative induction over the baseline (medium) of each cotransfection, which was set as 1 (indicated by a dotted line). (B) TNF-α cannot substitute for LPS. Mouse peritoneal macrophages were treated with LPS, 10 ng/ml mTNF-α for 4 h, or in combination with IFN-γ (priming). Total RNA was subjected to RT-PCR to measure IL-12 p35 mRNA expression. HPRT mRNA was also measured as a loading control. Data are representative of two independent experiments. (C) TNF-α activates poorly the nuclear translocation of c-Rel. Nuclear extracts were obtained from mouse peritoneal macrophages used in the experiment described in B, and analyzed by Western blot using anti–c-Rel and anti-p65 polyclonal antibodies (20 μg nuclear extract per lane).

with the IL-12 p35 promoter reporter in RAW264.7 cells stimulated with LPS or IFN-γ plus LPS (Fig. 4 A). Expression of p50 inhibited the p35 promoter activity stimulated by LPS or IFN-γ plus LPS whereas c-Rel strongly stimulated the p35 transcription. NF-κB p65 enhanced the p35 promoter activity in LPS-stimulated but not in IFN-γ plus LPS-stimulated cells. The essential role of the NF-κB pathway in the transcriptional activation of the IL-12 p35 gene in this system was also confirmed by using a dominant positive mutant of IκBα, in which the two important serine phosphorylation sites have been deleted such that the mutant becomes resistant to phosphorylation-triggered degradation, thus remaining constantly active in the cell. Blocking NF-κB activation using this IκBα mutant resulted in strong inhibition of the IL-12 p35 promoter activity induced by LPS, or IFN-γ plus LPS, and of the p35 promoter activity enhanced by p65 and c-Rel (Fig. 4 A).

Given the importance of LPS-induced NF-κB c-Rel in the transcriptional regulation of IL-12 p35, it was intriguing to ask this question: What is the role of TNF-α in the regulation of IL-12 p35 gene expression since it can also activate the NF-κB pathway? We first analyzed IL-12 p35 mRNA expression in freshly isolated mouse peritoneal macrophages after stimulation with LPS, TNF-α, IFN-γ plus LPS, or IFN-γ plus TNF-α. As shown in Fig. 4 B, TNF-α, when used alone or in combination with IFN-γ, only slightly induced IL-12 p35 mRNA. In the same cells, TNF-α, unlike LPS, failed to induce significant nuclear expression of c-Rel although it strongly activated NF-κB p65 (Fig. 4 C). Thus, TNF-α cannot substitute for LPS for the induction of IL-12 p35 gene expression potentially because TNF-α lacks the ability to activate the critical c-Rel in these macrophages. This is consistent with the finding in our previous study in human macrophages (26).

Differential Regulation of Transcription of IL-12 p40 and p35 by IRF-1. To study the role of IRF-1 in transcriptional activation of the p35 promoter we took advantage of the observation that in RAW264.7 cells, IRF-1 expression is solely induced by IFN-γ but not by LPS treatment (Fig. 3 B). This allows measurement of the response of the IL-12 p35 promoter to exogenously introduce IRF-1 expression by transient transfection in LPS-stimulated cells without the concern of the presence of significant amounts of endogenous IRF-1. It also provides a unique setting to assess the synergism between IFN-γ–induced signals, which are mimicked by the activities of IRF-1, and those mediated by LPS.

The full-length IL-12 p35 promoter (+61 to −1,082) was transiently transfected into RAW264.7 cells together with a vector constitutively expressing mouse IRF-1 or IRF-2, the antagonistic repressor of IRF-1 (17), under the β actin promoter, or the parental control vector. The transfected cells were subsequently stimulated with LPS. Fig. 5 A shows that the p35 promoter was not activated in LPS-stimulated cells by the control or IRF-2 expression vector at various molar ratios of effector (IRF-1, IRF-2, or control vector) to reporter (p35 promoter luciferase construct). Cotransfection with the IRF-1 effector plasmid induced a dose-dependent activation of the IL-12 p35 promoter. In contrast, cotransfection of the same amounts of IRF-1 with the human IL-12 p40 promoter (−3,300 to +108; reference 5) resulted in little transcriptional activation (Fig. 5 B). Taken together, these results indicate that IRF-1 has a differential ability to regulate IL-12 p35 and p40 transcription in synergy with LPS.

Localization of IRF-1 Response Element in IL-12 p35 Promoter. To further localize the putative IRF-1 response element in the IL-12 p35 promoter, a computer-assisted
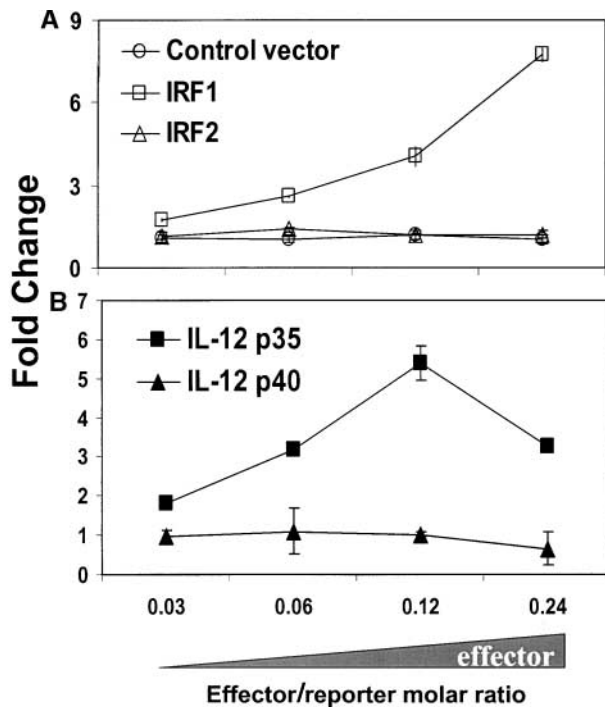
Figure 5. Differential regulation of transcription of IL-12 p35 and p40 by IRF-l. (A) The full-length IL-12 p35 promoter (+61/−1082) reporter construct was cotransfected with an IRF-1, IRF-2, or the parental expression vector into RAW264.7 cells at various effector/reporter molar ratios as indicated. After LPS stimulation (7 h), luciferase activity was measured and expressed as fold change over that of the unstimulated cells within each cotransfection. Results are mean plus SE from four independent experiments. (B) The full-length IL-12 p35 and p40 promoter reporter constructs were cotransfected, respectively, with IRF-1 expression vector into RAW264.7 cells at various effector/reporter molar ratios. After LPS stimulation, luciferase activity was measured and expressed as fold change induced by IRF-1 over that by the control vector within each cotransfection. Results are mean plus SE from three independent experiments.

scanning was performed and a sequence, GCTTTC-ATTTT located at −235 to −243 that matched with the consensus IRF-1 sequence in reverse, AAAATGAAAGC, was identified (Fig. 6 A).

To determine if this region was critical for the IRF-1 response we compared the ability of the C3 and C4 constructs (Fig. 3 B) to be activated by IRF-1 in LPS-stimulated cells (Fig. 6 B). The C4 construct truncates in the middle of the putative IRF-E site at −240. The minimal p35 promoter construct C3 (Fig. 3 B) appeared to harbor the potential IRF-1 response element because a further deletion of 152 bp from the 5′ end resulted in the loss of response to IRF-1. Thus, the IFN-γ/LPS response element and the IRF-1 element were both placed in the same region of the p35 promoter (between −240 and −392).

To further establish if the putative IRF-E was important for the response of the p35 promoter to IFN-γ and LPS, point mutations (4 bp) were introduced into this site in the context of the minimal promoter (C3 construct, +61 to −392). The wild-type and mutant promoter constructs were transiently transfected into RAW264.7 cells followed by stimulation with LPS or IFN-γ or a combination of the

two (Fig. 6 C). The response of the mutant promoter to IFN-γ alone or IFN-γ plus LPS was severely diminished compared with the response of the wild-type promoter, indicating that the putative IRF-1 site is indeed critical for the IFN-γ response of the p35 promoter.

To find out if this site was required for IRF-1 response, the wild-type and mutant constructs were cotransfected with the IRF-1 expression vector into RAW264.7 cells at various molar ratios of effector to reporter followed by LPS activation. The mutant promoter construct completely lost its ability to respond to transcriptional activation by IRF-1 (Fig. 6 D), suggesting that this site indeed mediates the IRF-1 response.

A recent study by Goriely et al. (27) showed that the two Sp1 elements located at −352/−349 and −297/−294, respectively, were essential for the response of the IL-12 p35 promoter to LPS and IFN-γ–induced transcription. We confirmed this finding by mutating the two sites in the context of the −392/+61 construct, both of which resulted in significant reduction of the p35 promoter activation by LPS and IFN-γ (Fig. 6 E). Furthermore, mutations at these sites also affected negatively their response to transcriptional activation by IRF-1 (Fig. 6 F). These results suggest that there might be interactions between the SP1 elements and the IRF-E site through transcription factors that bind to them, and that these interactions produce a synergistic effect on the p35 promoter.

Specific Binding of IRF-1 to IRF-E In Vitro and In Vivo upon Cellular Activation. To determine if IRF-1 could physically interact with the IRF-E in the p35 promoter, DNA affinity binding assays were performed. Fig. 7 A demonstrates that upon IFN-γ activation, a portion of the endogenous IRF-1 specifically bound to this site (Fig. 7 A, top, lanes 2 and 4), but not to the mutant site (Fig. 7 A, top, lanes 6 and 8). The unbound fractions (Fig. 7 A, two bottom panels) contained similar amounts of IRF-1 and PU.1 regardless of the DNA ligand (wild-type or IRF-1 mutant) they interacted with. These results strongly indicate that the IRF-E located at −235 to −243 is essential for IFN-γ/IRF-1 response of the IL-12 p35 promoter and it specifically interacts with the endogenously produced IRF-1 upon IFN-γ stimulation. Interestingly, unlike the regulation of IL-12 p40 gene transcription (22), we did not find ICSBP in the IRF-1 complex (unpublished data).

To determine if the endogenous IRF-1 interacts with this region of the promoter of the chromosomal IL-12 p35 gene, we performed ChIP analysis, which allows for the examination of specific protein–DNA interactions in the context of living cells (23). Fig. 7 B depicts a pair-wise alignment of the human and mouse IL-12 p35 promoters in the region harboring the IRF-E and the primers used to analyze the DNA–protein interaction by genomic PCR. As shown in Fig. 7 C, specific interaction between the endogenous IRF-1 and IL-12 p35 promoter was detected in LPS- or IFN-γ–stimulated mouse peritoneal macrophages (Fig. 7 C, lanes 10–12) but not in unstimulated cells (Fig. 7 C, lane 9). In the presence of a control antibody (rabbit IgG), there was no specific amplification of the chromo-
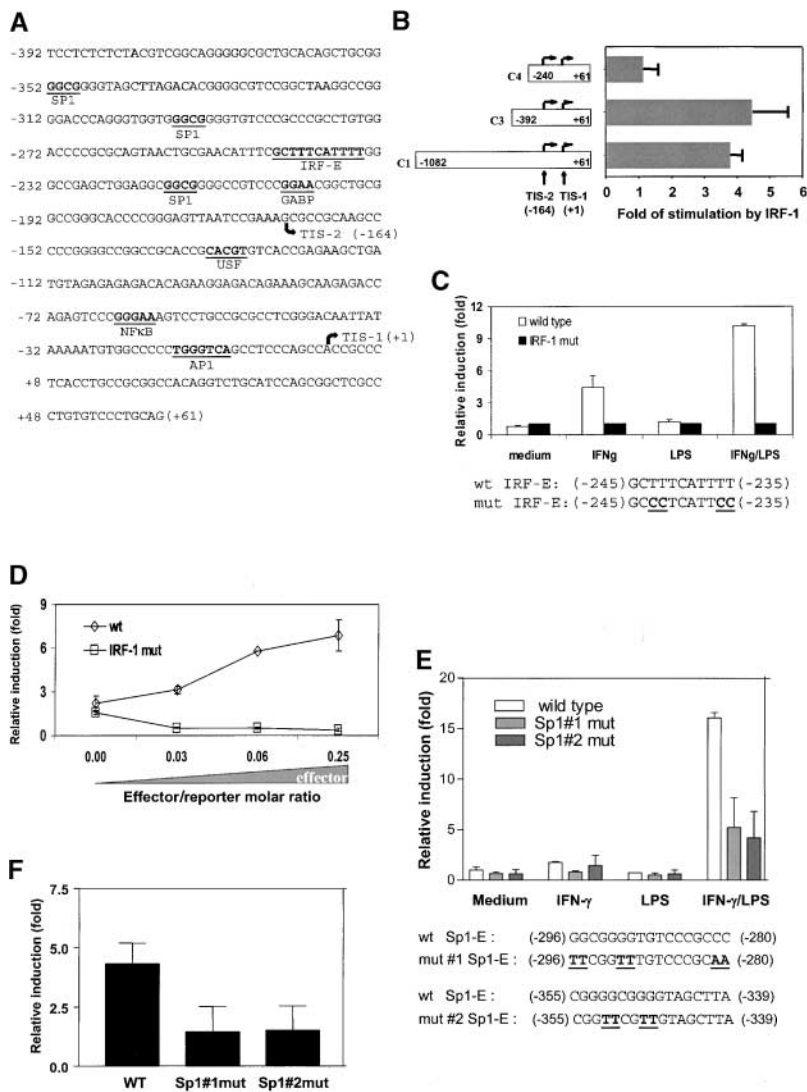
A

```
-392  TCCTCTCTCTACGTCGGCAGGGGGCGCTGCACAGCTGCGG

-352  GGCGGGGTAGCTTAGACACGGGGCGTCCGGCTAAGGCCGG
      SP1

-312  GGACCCAGGGTGGTGGGCGGGGTGTCCCGCCCGCCTGTGG
                    SP1

-272  ACCCCGCGCAGTAACTGCGAACATTTCGCTTTCATTTTGG
                              IRF-E

-232  GCCGAGCTGGAGGCGGCGGGGCCGTCCCGGAACGGCTGCG
          SP1            GABP

-192  GCCGGGGCACCCGGGAGTTAATCCGAAAGCGCCGCAAGCC
                         TIS-2 (-164)

-152  CCCGGGGCCGGCCGCACCGCACGTGTCACCGAGAAGCTGA
                            USF

-112  TGTAGAGAGAGACACAGAAGGAGACAGAAAGCAAGAGACC

-72   AGAGTCCCGGGAAAGTCCTGCCGCGCCTCGGGACAATTAT
             NFKB

-32   AAAAATGTGGCCCCTGGGTCAGCCTCCCAGCCACCGCCC
                       AP1          TIS-1(+1)

+8    TCACCTGCCGCGGCCACAGGTCTGCATCCAGCGGCTCGCC

+48   CTGTGTCCCTGCAG(+61)
```

B

C

D

E

F

Figure 6. Localization of the IRF-1 response element. (A) The sequence of the minimal IL-12 p35 promoter (+61/−392). Putative cis elements were predicted using MatInspector 3.3. The two transcriptional initiation sites, TIS-1 and TIS-2, are indicated. (B) Three of the IL-12 p35 promoter reporter constructs were cotransfected with the IRF-1 expression vector at the molar ratio of 0.12 (effector/reporter), stimulated with LPS, and luciferase activity was measured as above. (C) Effects of mutations in the IRF-E site on the IFN-γ and LPS response of the IL-12 p35 promoter. Site-specific mutations (4 bp) were introduced into the IRF-E site at −235 to −245 in the context of the minimal promoter (+61 to −392) as indicated below the graph. The wild-type and mutant promoter constructs were transfected into RA W264.7 cells, stimulated with IFN-γ, LPS, or a combination of the two. Luciferase activity was measured from cell lysates. The data were normalized against the values obtained with the mutant construct, which was set as 1. (D) Effects of mutations in the IRF-E site on the IRF-l response. The wild-type and IRF-E mutant p35 promoter constructs were transiently cotransfected into RAW264.7 cells with the IRF-1 expression vector at various effector to reporter molar ratios as indicated. Luciferase activity was measured after LPS stimulation and expressed as fold of stimulation over that of unstimulated cells. (E) The C3 IL-12 p35 promoter construct (+61 to −392) was mutated at the two Sp1 sites as indicated (the underlined nucleotides are substitutions). The mutant constructs were transfected and luciferase activity was measured after appropriate stimulation of the cells. All data are normalized to the activity of the wild-type, unstimulated (medium) p35 promoter, which is set as 1. Results shown are combined from three separate experiments with SEM. (F) The wild-type and Sp1 mutant constructs were cotransfected with the IRF-1 expression vector at a molar ratio of 0.12 (effector/reporter) and luciferase activity was measured after LPS stimulation. Data are normalized against non–LPS–stimulated wild-type promoter activity, which is set as 1.

somal DNA derived from the IL-12 p35 promoter (Fig. 7 C, lanes 5–8), whereas amplification of the input DNA was efficient and constant in all four samples (Fig. 7 C, lanes 1–4). Note that in mouse peritoneal macrophages, unlike RAW264.7 cells, IFN-γ as well as LPS induce IRF–1 expression (Fig. 7 E). Likewise, in human peripheral blood–derived monocytes, specific IRF-1 binding to this region of the endogenous IL-12 p35 promoter was also detected albeit primarily in IFN-γ and LPS–costimulated cells (Fig. 7 D, lane 12). This is consistent with the observation that unlike in mouse peritoneal macrophages (Fig. 7 E) or RAW264.7 cells (Fig. 3 B), IRF-1 nuclear expression in human monocytes was principally induced by a combination of IFN-γ and LPS, rather than by either stimulus alone (Fig. 7 F, lane 4). Thus, the ChIP patterns match perfectly with those of the IRF-1 expression in both mouse and human macrophages. These results demonstrate a specific and physiological association of activation-induced IRF-1 with the IL-12 p35 promoter at the IRF-E site.

IRF-1 Expression Enhances IL-12 p35 Expression and p70 Production. Our working model at this point was that IRF-1 serves as a critical transcription factor for high levels of IL-12 production in macrophages by selectively activating p35 in the presence of a microbial signal. We have demonstrated this by the reduced IL-12 p35 mRNA expression and p70 protein production in IRF-1$^{-/-}$ macrophages, and by the ability of exogenous IRF-1 to activate IL-12 p35 promoter reporter activity in transient transfections in a cell line. To further demonstrate the effect of IRF-1 on the endogenous IL-12 p35 gene transcription in primary macrophages, we constructed adenovirus vectors expressing the murine IRF-1 or the β-galactosidase gene (LacZ), transduced human monocyte–derived macrophages with these viral expression vectors at the multiplicity of infection (MOI) of 160 and 320 for 48 h, and measured IL-12 p70 production after stimulation with LPS (Fig. 8 A). The LacZ-expressing virus did not stimulate IL-12 production in the absence or presence of LPS. Adenovirus-mediated IRF-1 expression induced IL-12 p70 pro-
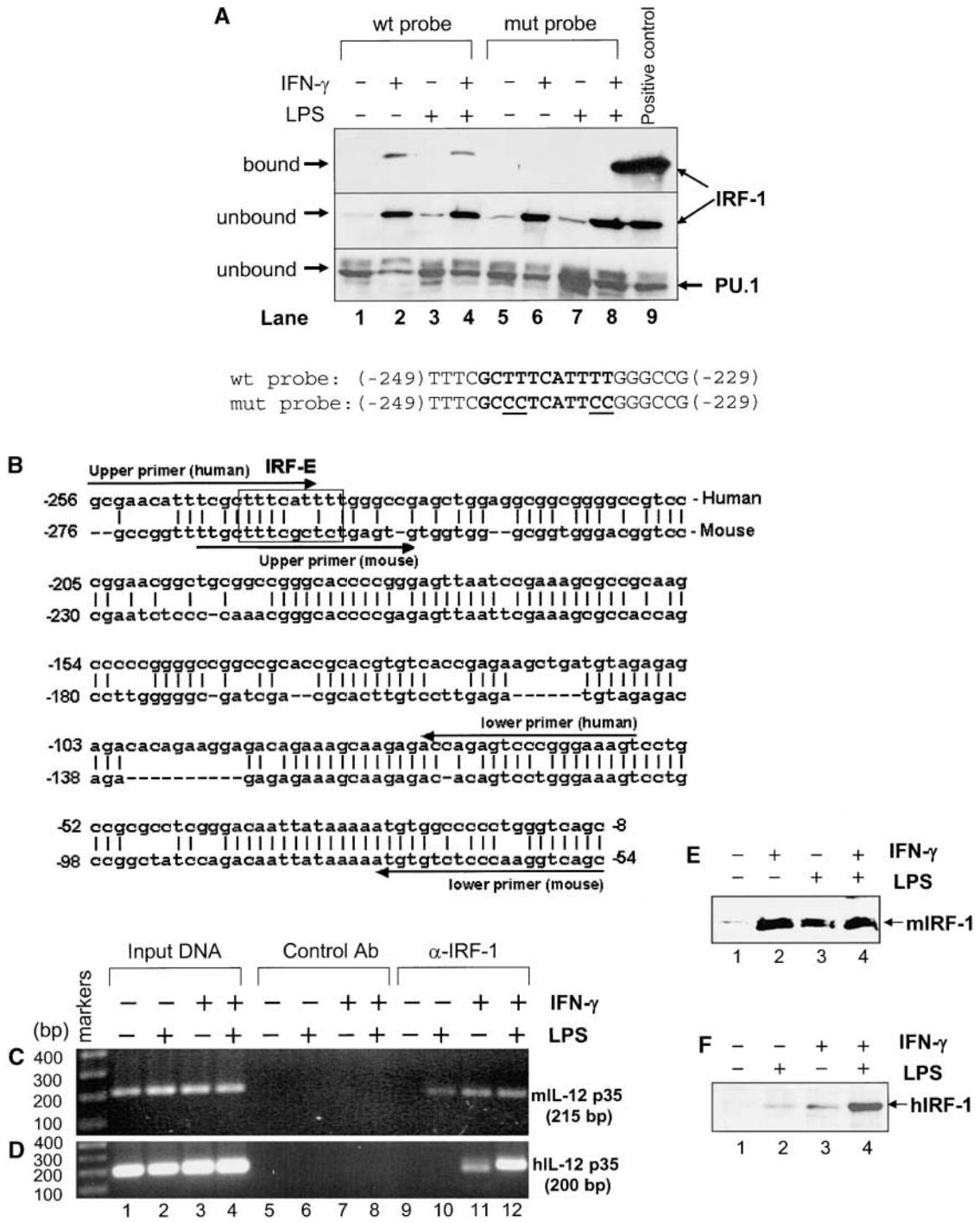
A

wt probe | mut probe | Positive control

IFN-γ − + − + − + − +
LPS   − − + + − − + +

bound → IRF-1
unbound → IRF-1
unbound → PU.1

Lane 1 2 3 4 5 6 7 8 9

wt probe: (-249)TTTC**GCTTTCATTTT**GGGCCG(-229)
mut probe:(-249)TTTC**GCCCTCATTCC**GGGCCG(-229)

B

Upper primer (human)   IRF-E

-256 gcgaacatttcgctttcattttgggccgagctggaggcggcggggccgtcc -Human
     | ||| |||||| | ||| | | || || || | | ||||
-276 --gccggtttgctttcgctctgagt-gtggtgg--gcggtgggacggtcc -Mouse
              Upper primer (mouse)

-205 cggaacggctgcggccgggcaccccgggagttaatccgaaagcgccgcaag
     || | | | |||||||||||| |||||||| |||||||| | ||
-230 cgaatctccc-caaacgggcaccccgagagttaattcgaaagcgccaccag

-154 cccccggggccggccgcaccgcacgtgtcaccgagaagctgatgtagagag
     || |||||| | ||| || | | |||| || | |||||||
-180 ccttgggggc-gatcga--cgcacttgtccttgaga------tgtagagac

                               lower primer (human)
-103 agacacagaaggagacagaaagcaagagaccagagtcccgggaaagtcctg
     |||| | ||||||||||||| | |||| ||||||||||||
-138 aga--------------gagagaaagcaagagac-acagtcctgggaaagtcctg

-52 ccgcgcctcgggacaattataaaaatgtggccccctgggtcagc -8
    ||| || |||||||||||||||||||| | ||| |||||||
-98 ccggctatccagacaattataaaaatgtgtctcccaaggtcagc -54
                  lower primer (mouse)

E − + − + IFN-γ
   − − + + LPS
   .... ← mIRF-1
   1 2 3 4

F − − + + IFN-γ
   − + − + LPS
   .... ← hIRF-1
   1 2 3 4

Input DNA | Control Ab | α-IRF-1

       − − + + − − + + − − + + IFN-γ
       − + − + − + − + − + − + LPS

(bp) markers
C 400
  300
  200 .... mIL-12 p35
  100   (215 bp)

D 400
  300
  200 .... hIL-12 p35
  100   (200 bp)

  1 2 3 4 5 6 7 8 9 10 11 12

Figure 7. Specific binding of IRF-1 to the IRF-1 response element. (A) A 21-bp oligonucleotide harboring the IRF-E site of the human IL-12 p35 promoter (−229 to −249) and its mutational derivative containing 4-bp changes within the IRF-E site were used as DNA ligand in affinity binding assays with nuclear extracts isolated from RAW264.7 cells stimulated as indicated. The bound and unbound (washes) fractions were analyzed by Western blot using an anti–IRF-l antibody. The unbound fractions were also stripped and reprobed with an anti-PU.l antibody to have an independent parameter. The sequences of the two oligos are described with the IRF-E in bold and the mutations are underlined. The positive control used was a nuclear extract isolated from IFN-γ–stimulated RAW264.7 cells. The results shown are representative of four independent tries with very similar outcomes. (B) Sequence alignment of human and mouse IL-12 p35 promoter regions. The human (top line) and mouse (bottom line) promoters that contain the IRF-E site are aligned with respect to the IRF-E site (boxed). The promoter coordinates with respect to the transcription start sites are shown. The lined arrows indicate the PCR primers used in ChIP analysis shown in C and D. (C and D) IRF-1 binding in vivo. ChIP analysis was performed according to the procedure described in Materials and Methods in mouse peritoneal macrophages (C) and in human blood–derived monocytes (D). The amplified mouse and human genomic fragments derived from the endogenous IL-12 p35 promoter encompassing the IRF-E are indicated. Gel size markers are labeled in basepairs. The control antibody was an isotype-matched rabbit IgG. (E and F) Western blot analysis of IRF-1 nuclear expression in mouse peritoneal macrophages (E) and human monocytes (F) after appropriate stimulation as indicated.
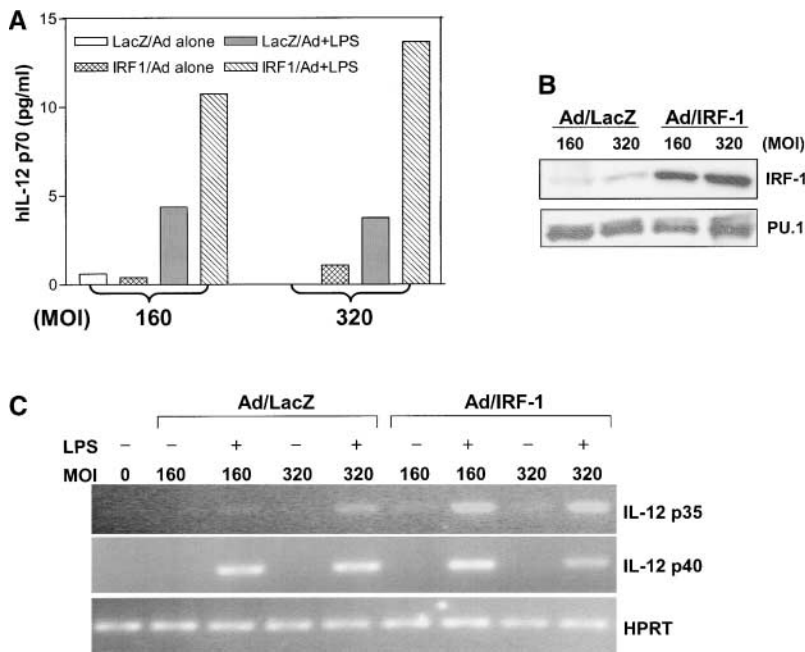
Figure 8. IRF-1 expression enhances IL-12 p35 expression and p70 production. (A) Human monocyte–derived macrophages were transduced with Ad/IRF-l or Ad/LacZ at an MOI of 160 or 320 plaque-forming units per cell followed by stimulation with LPS (24 h). IL-12 p70 production was measured from the culture supernatant by ELISA. The transduction rate assessed by LacZ staining indicated that ∼40% at 160 MOI and 70% at 320 MOI. Data shown are representative of two independent experiments with similar results. (B) IRF-1 protein expression in adenovirus-transduced macrophages. Resting human macrophages were transduced with an adenovirus expression vector containing mIRF-l cDNA (Ad/IRF-l) or LacZ cDNA (Ad/LacZ) for 24 h. Nuclear extracts were isolated and analyzed by Western blot using an anti–IRF-l antibody (top). 33 μg cytoplasmic and nuclear extracts were used in each lane. The membrane was stripped and reblotted with an anti-PU.1 antibody to ensure equal protein loading (bottom). (C) IL-12 p35 and p40 mRNA expression in the above experiment in A was measured by conventional RT-PCR under nonsaturating conditions. HPRT mRNA expression from the same samples was measured as a loading control.

duction at an MOI of 160 or 320 in the absence of LPS, and further enhanced p70 in the presence of LPS. Consistent with the induction of IL-12 production by the introduction of IRF-1–expressing adenovirus, IRF-1 protein expression in the nucleus was strongly enhanced in Ad/IRF-1– but not Ad/Laz-transduced macrophages (Fig. 8 B). Moreover, IL-12 p35 mRNA but not p40 was also slightly induced by IRF-1 alone, and greatly enhanced in the presence of LPS (Fig. 8 C). The pattern of p35 mRNA expression affected by IRF-1 matches quite well with that of p70 production, further demonstrating the selective role of IRF-1 in IL-12 p35 regulation.

Discussion

IRF-1 and IL-12 are both crucial players in immune defense against infectious and malignant diseases. The regulatory relationship between them is a subject of great interest. A previous study by Grumont et al. (12) investigated the regulation of the IL-12 p35 gene in response to microbial stimulation using c-Rel knockout mice. This study shows that NF-κB c-Rel is essential for the transcriptional response of the p35 gene but not the p40 gene to microbial stimuli such as *S. aureus*, CpG DNA, or LPS. We confirm that the NF-κB signaling pathway is indeed essential for the transcriptional activation of IL-12 p35 by LPS (Fig. 4 A). TNF-α, which is able to activate the NF-κB pathway, is nonetheless unable to activate c-Rel particularly (Fig. 4 C), thus explaining to some extent its inability to substitute for LPS in the transcriptional regulation of IL-12 p35 (Fig. 4 B). It is noted that in our transient transfection system, NF-κB p65, when overexpressed, is also able to activate LPS-stimulated p35 promoter activity (Fig. 4 A). This observation is inconsistent with the ability of TNF-α to activate NF-κB p65 (Fig. 4 C). The explanation for this apparent contradiction may lie in the fact that in LPS-stimulated RAW264.7 cells, the endogenous c-Rel is activated, thus making it possible for the p65-mediated induction of the IL-12 p35 transcription. This explanation does not apply to the effect of NF-κB p50 because its overexpression could lead to formation of p50/p50 homerdimers, which are transcriptionally inhibitory as we have shown previously on the IL-12 p40 transcription (28).

In this study, we demonstrate the molecular mechanisms whereby IRF-1, primarily induced by IFN-γ, selectively regulates the transcriptional activation of the IL-12 p35 gene (Fig. 5 B). IRF-1 does so by directly binding to the p35 promoter at the IRF-E site (Fig. 7) and activates the promoter possibly in synergy with other essential factors such as Sp1 (Fig. 6, E and F; reference 27) and NF-κB induced by LPS (12). These findings clearly provide the mechanistic basis of IFN-γ priming for potent IL-12 production in immune responses and illustrate the complexity of coordination and discordance in the regulation of multisubunit, heterologous molecules such as IL-12.

The lack of a strong deficiency in IL-12 p40 mRNA expression in IRF-1$^{-/-}$ macrophages shown in this study is in apparent disagreement with the results of two previous studies (10, 29). The possible reason(s) for this discrepancy was investigated in our study, which indicates that prolonged exposure of macrophages to IFN-γ before LPS stimulation is able to rescue the deficiency in IL-12 p40 production in IRF-1$^{-/-}$ cells (Fig. 1, A and B) via an alternative, uncharacterized mechanism. This unknown alternative pathway is unlikely to play a role in IL-12 p35 and p70 production because they are not rescued by the IFN-γ pretreatment. The rescue effect of IFN-γ pretreatment on IL-12 p40 expression in IRF-1$^{-/-}$ macrophages was also observed in a previous study by Salkowski et al. (11). The differential impact of the length of IFN-γ pre-

treatment on p40 but not on p70 production supports the notion that IRF-1 contributes to the transcriptional regulation of IL-12 p40 and p35 genes through different mechanisms.

The discrepancy in the degree of IFN-γ dependency of the IL-12 p35 gene for its mRNA expression between human monocytes and mouse macrophages has been noted by us for some time. The lesser IFN-γ dependency of mouse peritoneal macrophages for p35 mRNA expression is not due to preactivation of these cells by thioglycollate as we observed the same patterns in resident macrophages (unpublished data). It is not due to the differences between human monocytes and macrophages generated from freshly isolated monocytes in M-CSF because the latter cells are just as dependent on IFN-γ as the former for IL-12 p35 mRNA expression (30). It is correlated, however, with the ability of the cells to express IRF-1 in response to IFN-γ or LPS. As we show in Fig. 7 E, either LPS or IFN-γ is able to induce nuclear IRF-1 expression whereas in human monocytes abundant IRF-1 is induced only by a combination of LPS and IFN-γ (Fig. 7 F). These patterns of IRF-1 expression correlate with the degree to which the cells are dependent on IFN-γ for the activation of IL-12 p35 transcription.

We provide conclusive evidence that the total amount of IL-12 p35 protein synthesized in IFN-γ and LPS-stimulated cells correlates with that of its mRNA (Fig. 2, B and D), indicating that there is no major regulatory step involved between mRNA and translation. However, the differences in IL-12 p40/p35 mRNA and p40/p70 protein ratios indicate that the previous general assumption that p35 mRNA levels correspond to those of p70 synthesis is an oversimplification. Posttranscriptional mechanisms may exist that regulate the assembly of p35 into the heterodimeric complex with p40 or the secretion of p70 (31, 32).

The results derived from our study may have significant impact on the regulation of immune responses exerted by IFN-γ. It could be speculated that during a microbial infection, the infectious agent such as bacteria stimulates innate immune cells such as macrophages to produce the initial, small amount of IL-12, which then activates NK cells to produce IFN-γ. This IFN-γ production would act as a costimulatory factor for macrophages in the presence of the microbial agent to induce much larger amounts of IL-12 production via transcriptional activation of IL-12 p40 primarily by ICSBP (33), and p35 via IRF-1, both induced by IFN-γ. This second wave, large amount of IL-12 production can exert both local and systemic effects on APCs and lymphocytes in the ensuing adaptive phase of the immune response.

In summary, this study has established the molecular basis and elucidated the operational details of the selective regulation of IL-12 p35 gene transcription by IRF-1 in its control of IL-12 production in response to pathogenic perturbation. It provides a mechanistic explanation for the synergistic activation of IL-12 production and enhanced immune activation by the perpetual stimulation delivered by cellular factors such as IFN-γ and microbial agents such as LPS.

References

1. Trinchieri, G. 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol.* 13:251–276.

2. Ma, X., and G. Trinchieri. 2001. Regulation of interleukin-12 production in antigen-presenting cells. *Adv. Immunol.* 79:55–92.

3. Sieburth, D., E.W. Jabs, J.A. Warrington, X. Li, J. Lasota, S. LaForgia, K. Kelleher, K. Huebner, J.J. Wasmuth, and S.F. Wolf. 1992. Assignment of genes encoding a unique cytokine (IL12) composed of two unrelated subunits to chromosomes 3 and 5. *Genomics.* 14:59–62.

4. Murphy, T.L., M.G. Cleveland, P. Kulesza, J. Magram, and K.M. Murphy. 1995. Regulation of interleukin 12 p40 expression through an NF-kappa B half-site. *Mol. Cell. Biol.* 15:5258–5267.

5. Ma, X., J.M. Chow, G. Gri, G. Carra, F. Gerosa, S.F. Wolf, R. Dzialo, and G. Trinchieri. 1996. The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. *J. Exp. Med.* 183:147–157.

6. Plevy, S.E., J.H. Gemberling, S. Hsu, A.J. Dorner, and S.T. Smale. 1997. Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. *Mol. Cell. Biol.* 17:4572–4588.

7. Ma, X., M. Neurath, G. Gri, and G. Trinchieri. 1997. Identification and characterization of a novel Ets-2-related nuclear complex implicated in the activation of the human interleukin-12 p40 gene promoter. *J. Biol. Chem.* 272:10389–10395.

8. Grazia Cappiello, M., F.S. Sutterwala, G. Trinchieri, D.M. Mosser, and X. Ma. 2001. Suppression of Il-12 transcription in macrophages following Fc gamma receptor ligation. *J. Immunol.* 166:4498–4506.

9. Hayes, M.P., J. Wang, and M.A. Norcross. 1995. Regulation of interleukin-12 expression in human monocytes: selective priming by interferon-gamma of lipopolysaccharide-inducible p35 and p40 genes. *Blood.* 86:646–650.

10. Taki, S., T. Sato, K. Ogasawara, T. Fukuda, M. Sato, S. Hida, G. Suzuki, M. Mitsuyama, E.H. Shin, S. Kojima, et al. 1997. Multistage regulation of Th1-type immune responses by the transcription factor IRF-1. *Immunity.* 6:673–679.

11. Salkowski, C.A., K. Kopydlowski, J. Blanco, M.J. Cody, R. McNally, and S.N. Vogel. 1999. IL-12 is dysregulated in macrophages from IRF-1 and IRF-2 knockout mice. *J. Immunol.* 163:1529–1536.

12. Grumont, R., H. Hochrein, M. O'Keeffe, R. Gugasyan, C. White, I. Caminschi, W. Cook, and S. Gerondakis. 2001. c-Rel regulates interleukin 12 p70 expression in CD8$^+$ dendritic cells by specifically inducing p35 gene transcription. *J. Exp. Med.* 194:1021–1032.

13. Kollet, J., C. Witek, J.D. Gentry, X. Liu, S.D. Schwartzbach, and T.M. Petro. 2001. Deletional analysis of the murine IL-12 p35 promoter comparing IFN-gamma and lipopolysac-

charide stimulation. *J. Immunol.* 167:5653–5663.

14. Weissmann, C., and H. Weber. 1986. The interferon genes. *Prog. Nucleic Acid Res. Mol. Biol.* 33:251–300.

15. Taniguchi, T. 1988. Regulation of cytokine gene expression. *Annu. Rev. Immunol.* 6:439–464.

16. Taniguchi, T., K. Ogasawara, A. Takaoka, and N. Tanaka. 2001. IRF family of transcription factors as regulators of host defense. *Annu. Rev. Immunol.* 19:623–655.

17. Matsuyama, T., T. Kimura, M. Kitagawa, K. Pfeffer, T. Kawakami, N. Watanabe, T.M. Kundig, R. Amakawa, K. Kishihara, A. Wakeham, et al. 1993. Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development. *Cell.* 75:83–97.

18. Duncan, G.S., H.W. Mittrucker, D. Kagi, T. Matsuyama, and T.W. Mak. 1996. The transcription factor interferon regulatory factor-1 is essential for natural killer cell function in vivo. *J. Exp. Med.* 184:2043–2048.

19. Ogasawara, K., S. Hida, N. Azimi, Y. Tagaya, T. Sato, T. Yokochi-Fukuda, T.A. Waldmann, T. Taniguchi, and S. Taki. 1998. Requirement for IRF-1 in the microenvironment supporting development of natural killer cells. *Nature.* 391:700–703.

20. Ohteki, T., H. Yoshida, T. Matsuyama, G.S. Duncan, T.W. Mak, and P.S. Ohashi. 1998. The transcription factor interferon regulatory factor 1 (IRF-1) is important during the maturation of natural killer 1.1$^+$ T cell receptor-α/β^+ (NK1$^+$ T) cells, natural killer cells, and intestinal intraepithelial T cells. *J. Exp. Med.* 187:967–972.

21. Lohoff, M., G.S. Duncan, D. Ferrick, H.W. Mittrucker, S. Bischof, S. Prechtl, M. Rollinghoff, E. Schmitt, A. Pahl, and T.W. Mak. 2000. Deficiency in the transcription factor interferon regulatory factor (IRF)-2 leads to severely compromised development of natural killer and T helper type 1 cells. *J. Exp. Med.* 192:325–336.

22. Schreiber, E., P. Matthias, M.M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* 17:6419.

23. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.

24. Cepko, C. 2001. Preparation of a Specific Retrovirus Producer Cell Line. John Wiley & Sons, Inc., New York. 749 pp.

25. Hayes, M.P., F.J. Murphy, and P.R. Burd. 1998. Interferon-gamma-dependent inducible expression of the human interleukin-12 p35 gene in monocytes initiates from a TATA-containing promoter distinct from the CpG-rich promoter active in Epstein-Barr virus-transformed lymphoblastoid cells. *Blood.* 91:4645–4651.

26. Ma, X., J. Sun, E. Papasavvas, H. Riemann, S. Robertson, J. Marshall, R.T. Bailer, A. Moore, R.P. Donnelly, G. Trinchieri, et al. 2000. Inhibition of IL-12 production in human monocyte-derived macrophages by TNF. *J. Immunol.* 164:1722–1729.

27. Goriely, S., D. Demonte, S. Nizet, D. De Wit, F. Willems, M. Goldman, and C. Van Lint. 2003. Human IL-12(p35) gene activation involves selective remodeling of a single nucleosome within a region of the promoter containing critical Sp1-binding sites. *Blood.* 101:4894–4902.

28. Gri, G., D. Savio, G. Trinchieri, and X. Ma. 1998. Synergistic regulation of the human interleukin-12 p40 promoter by NFkappaB and Ets transcription factors in Epstein-Barr virus-transformed B cells and macrophages. *J. Biol. Chem.* 273:6431–6438.

29. Maruyama, S., K. Sumita, H. Shen, M. Kanoh, X. Xu, M. Sato, M. Matsumoto, H. Shinomiya, and Y. Asano. 2003. Identification of IFN regulatory factor-1 binding site in IL-12 p40 gene promoter. *J. Immunol.* 170:997–1001.

30. Cao, S., J. Liu, M. Chesi, P.L. Bergsagel, I.C. Ho, R.P. Donnelly, and X. Ma. 2002. Differential regulation of IL-12 and IL-10 gene expression in macrophages by the basic leucine zipper transcription factor c-Maf fibrosarcoma. *J. Immunol.* 169:5715–5725.

31. Murphy, F.J., M.P. Hayes, and P.R. Burd. 2000. Disparate intracellular processing of human IL-12 preprotein subunits: atypical processing of the P35 signal peptide. *J. Immunol.* 164:839–847.

32. Carra, G., F. Gerosa, and G. Trinchieri. 2000. Biosynthesis and posttranslational regulation of human IL-12. *J. Immunol.* 164:4752–4761.

33. Wang, I.M., C. Contursi, A. Masumi, X. Ma, G. Trinchieri, and K. Ozato. 2000. An IFN-gamma-inducible transcription factor, IFN consensus sequence binding protein (ICSBP), stimulates IL-12 p40 expression in macrophages. *J. Immunol.* 165:271–279.