

The Interleukin 12 p40 Gene Promoter Is Primed by Interferon γ in Monocytic Cells

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Summary

Interleukin (IL) 12 is a proinflammatory cytokine produced by phagocytic cells, B cells, and other antigen-presenting cells that modulates adaptive immune responses by favoring the generation of T helper type 1 cells. IL-12 mediates some of its physiological activities by acting as a potent inducer of interferon (IFN) γ production by T and natural killer cells. IFN- γ enhances the ability of the phagocytic cells to produce IL-12 and other proinflammatory cytokines. Thus, IL-12-induced IFN- γ acts in a positive feedback loop that represents an important amplifying mechanism in the inflammatory response to infections. We show here that IFN- γ enhances IL-12 production mostly by priming phagocytic cells for lipopolysaccharide (LPS)-induced transcription of the IL-12 p40 gene, which encodes the heavy chain of the IL-12 heterodimer; furthermore, IFN- γ directly induces transcription of the IL-12 p35 gene, which encodes the light chain of IL-12, and has at least an additive effect with LPS stimulation in inducing its transcription. The priming effect of IFN- γ on the LPS-induced p40 gene transcription requires preincubation of the cells with IFN- γ for at least 8 h to obtain a maximal effect. The priming effect of IFN- γ for IL-12 production is predominantly at the transcriptional level for both the p40 and the p35 gene, and no evidence for a major role of posttranscriptional or translational mechanisms was found. A 3.3-kb human IL-12 p40 promoter construct transfected into cell lines recapitulated the tissue specificity of the endogenous gene, being silent in two human T cell lines, constitutively active in two human Epstein-Barr virus-positive B lymphoblastoid cell lines, and LPS inducible in the human THP-1 and mouse RAW264.7 monocytic cell lines. Because the RAW264.7 cell line is easily transfectable and regulates the endogenous IL-12 p40 gene in response to IFN- γ or LPS similarly to human monocytes, it was used for analysis of the regulation of the cloned human IL-12 p40 promoter. A requirement for the region between -222 and -204 in both LPS responsiveness and IFN- γ priming was established. This region contains an ets consensus sequence that was shown to mediate activation of the promoter by IFN- γ and LPS, as well as by a cotransfected ets-2. The -222 construct was also regulated in a tissue-specific manner. Two other elements, IRF-1 located at -730 to -719, and NF-IL6 at -520 to -512, were also studied by deletion analysis, which did not result in decreased response to IFN- γ and LPS stimulation.

NK cell stimulatory factor or IL-12 is a heterodimer of 70,000 daltons (p70) formed by two covalently linked glycosylated chains of ~40,000 (p40) and 35,000 (p35) daltons (1). IL-12 is produced by phagocytic cells, B cells, and other types of APC (2). IL-12 mediates several biological activities on T and NK cells, including induction of IFN- γ production, enhancement of cell-mediated cytotoxicity, and mitogenic effects (1). Through these functions IL-12 plays a major role in the early inflammatory response to infections. In addition, APC-produced IL-12 is critically

involved in the generation of Th1 cells (3-9) and is required for optimal differentiation of CTL (10). The early decision toward Th1 or Th2 cells in the immune response is dependent on the balance between IL-12, which favors Th1 responses, and IL-4, which favors Th2 responses (11).

Originally, IL-12 was found to be produced by EBV-transformed human B cell lines either constitutively or in response to phorbol diesters (1, 12). However, the major producer cells of IL-12 are phagocytic cells (monocytes, macrophages, or neutrophils), which do not respond to

phorbol diesters with IL-12 production (2, 13). The most efficient inducers of IL-12 production from phagocytic cells are bacteria, bacterial products, and intracellular parasites (2). The genetic control of the production of IL-12 is complex because of the requirement for expression of two different genes to produce the biologically active heterodimer. The expression of p40 transcripts is highly correlated with the ability of the producer cells to make IL-12, whereas the p35 transcripts are ubiquitously expressed in almost all cell types of both hematopoietic and nonhematopoietic origin (2, 14). As originally observed in B cell lines, stimulated PBMC and neutrophils produce the free p40 chain in a 10- to 50-fold excess over the biologically active p70 heterodimer (2, 13). Both p40 and p35 messenger RNAs (mRNAs) are constitutively expressed at very low levels in unstimulated PBMC and neutrophils. Accumulation of both is upregulated by stimulation with bacteria or bacterial products, although the induction of the p40 gene is much more marked, resulting in an abundance of p40 transcripts in stimulated cells up to 200-fold higher than that of p35 transcripts, which may explain the excess production of the p40 chain (2, 13, 15).

The ability of phagocytic cells to produce IL-12 is regulated by several cytokines with activating or suppressing effects on the producer cells. IFN- γ and GM-CSF, among other cytokines, enhance the production of IL-12 from phagocytic cells (13, 15, 16 and our unpublished data), whereas IL-10, IL-4, IL-13, and TGF- β inhibit IL-12 production (16, 17). The latter inhibit both IL-12 protein secretion and p40 and p35 mRNA accumulation in stimulated phagocytic cells. However, the effect of IL-4 and IL-13 is complex: They inhibit production of IL-12 and TNF- α when added to monocytes simultaneously to the inducing stimulus but prime the monocytes for IL-12 production in response to various stimuli when added to the culture at least 18 h before the stimulus (17).

In this report, we show that p40 and p35 are differentially regulated by IFN- γ and LPS in PBMC; that is, IFN- γ treatment does not directly induce p40 gene expression and protein production, but it primes monocytic cells to produce them in response to LPS stimulation. On the other hand, IFN- γ alone can induce low levels of p35 transcription and mRNA accumulation. Treatment of monocytic cells with IFN- γ before LPS stimulation enhances transcription rates, steady-state mRNA accumulation, and protein synthesis synergistically for p40 and additively for p35. The priming effect of IFN- γ on IL-12 expression is primarily at the transcriptional level. Finally, we demonstrate that the cloned human p40 promoter can recapitulate the behavior of its endogenous counterpart in transient transfection assays in terms of cell type specificity and responsiveness to IFN- γ and LPS.

Materials and Methods

Production and Quantitation of IL-12. PBMC were isolated from four normal donors by Ficoll gradient (Accu-prep lymphocytes; Accurate Chemical & Scientific Corp., Westbury, NY) and

cultured in RPMI-1640 medium supplemented with 10% FCS (Irvine Scientific, Santa Ana, CA), both endotoxin free, at 10^6 cells/ml in 1 ml of culture. After 18 h, cells were treated with nothing or with recombinant human (rh)¹ IFN- γ (1,000 U/ml) for 24, 16, or 8 h before and simultaneously with (0 h) LPS (1 μ g/ml) stimulation for 30 h. Control cultures were treated with IFN- γ alone or LPS alone. Culture supernatants were collected and used for detection of IL-12 p40 and p70 in an RIA that detects both free p40 and p40 bound to p35 in the heterodimeric form (2). The human p70 heterodimer was detected using an ELISA kit (Quantikine Human IL-12 Immunoassay, D1200; R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Reagents. Human IFN- γ was purchased from Genzyme Diagnostics (Cambridge, MA). Murine IFN- γ was a generous gift from Dr. Gianni Garotta (Human Genome Sciences, Inc., Rockville, MD). LPS was purchased from Sigma Chemical Co. (St. Louis, MO).

Immunoprecipitation of IL-12 p40 and p70 from Human Monocytes. PBMC depleted of T cells by rosetting with SRBC treated with AET were incubated with medium alone or with rhIFN- γ (1,000 U/ml). After 24 h, cells were washed with methionine-free RPMI-1640 medium and resuspended in 2 ml of the same medium containing 10% fetal bovine serum and 0.5 mCi [³⁵S]methionine. Cells preincubated with medium were treated with medium alone or with LPS (1 μ g/ml), and cells pretreated with IFN- γ were treated with IFN- γ (1,000 U/ml) or IFN- γ (1,000 U/ml) plus LPS (1 μ g/ml). Supernatants (2 ml) were collected after 18 h, diluted with 1 ml of TBS/NP-40 1%/BSA 0.5% containing leupeptin and antipain as protease inhibitors, and pre-cleared once with 1 μ g of irrelevant mAb and three times with 50 μ l Sepharose-protein A (overnight at 0°C). Each sample was then treated with 10 μ l of anti-human p40 mAb C11.79 bound to CNBr-activated Sepharose (2 mg mAb/ml Sepharose) for 90 min at 0°C. Immunoprecipitated materials were washed four times with 1 ml of 0.1% TBS/NP-40 and twice with 1 ml of 0.1% NP-40. Immunoprecipitates were eluted by boiling samples for 3 min with 40 μ l of 2.3% SDS (Laemmli buffer) in nonreducing conditions. All 40- μ l samples were run on a 10% SDS-polyacrylamide gel. The gel was dried and exposed in a PhosphorImager screen (Molecular Dynamics, Inc., Sunnyvale, CA) for 72 h.

Production of IL-12 by the Murine Cell Line RAW264.7. Mouse macrophagic cell line RAW264.7 (American Tissue Culture Collection [ATCC], Rockville, MD) was cultured in the same conditions and time course as the PBMC, except that the cells were pretreated with 1.2% DMSO 24 h before LPS induction, because DMSO treatment enhances the ability of human and murine myeloid cell lines to produce IL-12 (16), and recombinant murine (rm) IFN- γ was used. p40 was then measured using an RIA specific for murine p40 (18). The coating antibody used was C17.15, and the ¹²⁵I-labeled detection antibody was C15.6.

Electroporation. Murine macrophagic cell line RAW264.7, the human B cell lines RPMI-8866 and CESS, and the T cell lines Molt 13 and Jurkat were grown to confluence (10^6 /ml) in RPMI-1640 medium supplemented with 10% FCS. Cells were transfected with the 3.3-kb p40 promoter-reporter construct or other constructs (see below) prepared by the CsCl procedure. Cells were collected, washed once with RPMI-1640 medium, and resuspended in the same medium at a concentration of $30 \times$

¹Abbreviations used in this paper: IRF, IFN-regulatory factor; mRNA, messenger RNA; NO, nitrous oxide; rh, recombinant human; rm, recombinant murine; TPA, tetradecanoylphorbol 13-acetate.

10^6 cells/700 μ l. 700 μ l of cell suspension and 300 μ g of DNA (24 μ g p40-luc/3.3 kb, 6 μ g CMV- β -galactosidase, 270 μ g BS/KSII⁺) were placed in 0.45-cm electroporation cuvettes (Gene Pulser; Bio-Rad Laboratories, Richmond, CA), and electroporation was carried out at 960 μ F and 400 V. Transfected cells were collected and resuspended to 5×10^6 /ml in RPMI-1640 + 10% FCS, and chloroquine was added to a final concentration of 10 μ M. Cells were placed in wells (2 ml/well) of a 24-well plate and incubated for 16 h at 37°C in a 5% CO₂ atmosphere. Cells were treated with 1.2% DMSO for 24 h and with rmIFN- γ (1,000 U/ml) for 24, 16, 8, or 0 h before the addition of LPS (1 μ g/ml). After an 8-h stimulation with LPS, cells were harvested and lysed by Triton and freeze-thawing. Lysates were used for both luciferase and β -galactosidase assays.

DEAE-Dextran Transfection. THP-1 cells (ATCC) were grown to confluence (10^6 /ml) in RPMI-1640 supplemented with 20% FCS and transfected with the 3.3 kb p40 promoter-reporter construct by the DEAE-dextran method. THP-1 cells were resuspended to 10^6 cells/ml in RPMI-1640 containing 50 mM Tris, pH 7.3, and 20 ml of suspension for each condition was placed in T-25 flasks. To each flask, 40 μ g of plasmid DNA (20 μ l p40-luc/3.3 kb, 8 μ l CMV- β -galactosidase, 12 μ l BS/KSII⁺) and 50 μ l of sterile 100 mg/ml DEAE-dextran were added and gently shaken to ensure even distribution. Flasks were incubated for 1 h at 37°C. Cells were then collected, spun at 1,500 rpm for 5 min, washed once with 20 ml of RPMI-1640 containing 100 mM (1.5 U/ml) heparin, and washed twice more with 20 ml of RPMI-1640. Cells were resuspended to a concentration of 10^6 cells/ml in RPMI-1640 supplemented with 20% FCS, distributed at 10 ml per flask, and incubated for 18 h at 37°C in a 5% CO₂ atmosphere. Cells were treated with 1.2% DMSO for 16 h before LPS (1 μ g/ml) induction for 8 h. After LPS induction, cells were removed by gentle scraping, collected, and spun at 1,500 rpm for 5 min. Supernatants were removed by aspiration, and cells were lysed by Triton and freeze thawing. Lysates were then used for both the luciferase and β -galactosidase assays.

Luciferase and β -Galactosidase Assays. Luciferase assay was performed in a buffer containing 25 mM glycylglycine, 15 mM MgSO₄, and 5 mM ATP. Assay buffer (350 μ l) and 50 μ l of cell lysate were combined before reading in a standard luminometer using automated injection of D-luciferin-potassium salt (#1600; Analytical Luminescence Laboratory, San Diego, CA) at a concentration of 0.33 mg/ml.

β -Galactosidase assay was performed in a buffer containing 1 mM MgCl₂, 50 mM β -ME, 0.88 mg/ml of O-nitrophenyl- β -D-galactopyranoside, and 7.4 mM sodium phosphate buffer, pH 7.3. The cell lysate (30 μ l) was incubated with the buffer in a total volume of 300 μ l for 30 min at 37°C, and then inactivated for 10 min at 60°C. Readings were taken at OD₄₁₀ in a standard spectrophotometer.

RNase Protection and Northern Blot. RNase protection and Northern blot analyses and the preparation of the various probes used were performed as described (15).

Nuclear Run-On Assay. Isolation of nuclei and in vitro nuclear transcription were performed as described (19, 20). In all experiments, the coding region of each probe of interest was PCR amplified and spotted on the filter at 200 ng/slot in denatured form.

Cloning of the Human IL-12 p40 Promoter. The human IL-12 p40 promoter was identified from a λ -phage genomic clone (L26a obtained from a Lambda Fix Library; Stratagene Inc., La Jolla, CA) by screening a PstI digest of this clone subcloned into Bluescript KS(-) using as probe an oligonucleotide correspond-

ing to the first 20 nucleotides of the longest cDNA clone: 5'-GCAGAAGAAACAACATCTGT-3'. A 3.3-kb fragment was positively identified, sequenced, and cloned into the luciferase reporter construct pXP2 (21) at the PstI site. All deletion mutant constructs were generated by PCR and fully sequenced for verification.

Primer Extension. Total RNA (15 μ g) from *Staphylococcus aureus*-stimulated PBMC, 12-O-tetradecanoylphorbol 13-acetate (TPA)-stimulated RPMI-8866 cells, or yeast RNA (negative control) was mixed with an end-labeled antisense primer (25-mer corresponding to nucleotides 25-49 in the published p40 cDNA sequence [1]: 5'-GGAAAACCAAGAGATGACCAACTGC-3') and reverse transcribed. The extension products were electrophoresed on a 6% denaturing polyacrylamide gel (7 M urea) along with size markers (ϕ X 174/HaeIII digests). The precise length of the extension products was determined based on M13/mp19 DNA sequenced with the universal primer and run simultaneously.

Results

Induction of IL-12 Protein Production in PBMC. PBMC from four donors were treated with rhIFN- γ at 1,000 U/ml for 24, 16, or 8 h before and simultaneous with (0 h) LPS (1 μ g/ml) stimulation (30 h). Supernatants were then collected, and IL-12 p40 and p70 were measured by RIA and ELISA, respectively. As shown in Fig. 1, IFN- γ alone did not induce significant p40 production, whereas LPS alone stimulated a modest fivefold increase over that of unstimulated cells. IFN- γ treatment followed by LPS stimulation resulted in a dramatic increase in p40 production. The synergistic effect peaked at 16 h of IFN- γ pretreatment, with a 75-fold stimulation in p40 production. Cells treated simultaneously with IFN- γ and LPS produced only marginally more p40 than those stimulated with either IFN- γ or LPS alone. The profile of p70 production followed that of p40 very closely, with a production peak (400 pg/ml) at \sim 24 h of IFN- γ pretreatment. This represents a 40-fold stimulation over the unstimulated level, resembling the magnitude of the level of induced p40 production. The p40/p70 ratio was \sim 30:1.

An alternative approach was taken to measure p40 and p70 production by adherent monocytes from a normal donor by immunoprecipitation with the anti-p40 mAb C11.79 coupled to CNBr-Sepharose. Immunoprecipitated proteins were analyzed by SDS-PAGE under nonreducing conditions (Fig. 2). By this assay, p40 and p70 were detected only in cells pretreated with IFN- γ for 24 h followed by 18 h of LPS stimulation. The apparently high p70/p40 ratio on autoradiography reflects the fivefold higher methionine content of the p35 chain than the p40 chain (1), corresponding to a calculable p70/p40 protein ratio of \sim 1:13, which is within the normal range observed using immunological assays. The identity of the slower-moving band immunoprecipitated by anti-p40 is not clear but has been consistently observed, even with Chinese hamster ovary cell-produced rhIL-12 p40 (not shown), suggesting that it is a more glycosylated form.

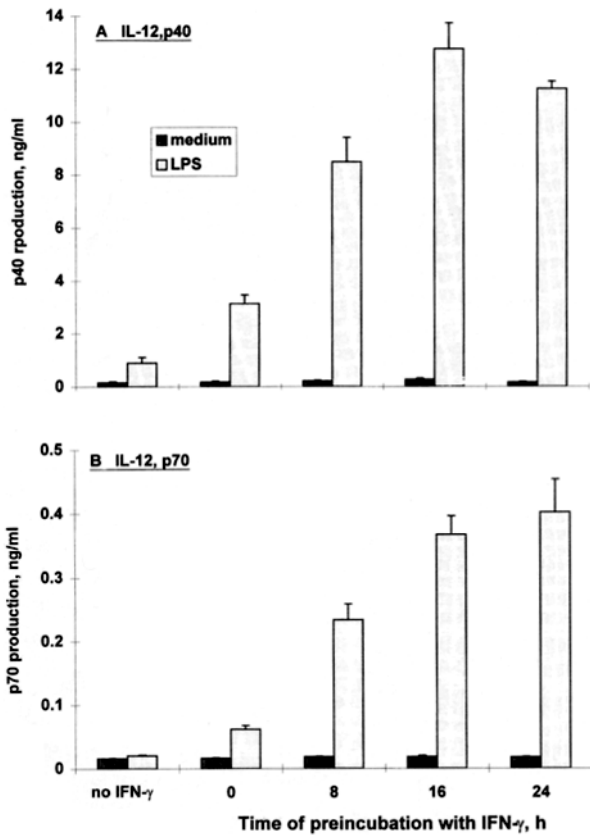


Figure 1. Production of IL-12 p40 (A) and p70 (B) in PBMC stimulated with IFN- γ and LPS. PBMC from four normal donors were cultured in medium only (no IFN- γ) or in the presence of rhIFN- γ (1,000 U/ml) for 24, 16, or 8 h before or simultaneously with (0 h) LPS stimulation (1 μ g/ml, 24 h). Control cultures were pretreated with IFN- γ but not stimulated with LPS. IL-12 p40 (RIA detecting both free and p35-bound forms of the p40 chain, A) or the biologically active p70 heterodimer (ELISA, B) were determined in cell-free supernatant fluids. Results are mean \pm SE of four donors.

Induction of p40 and p35 Steady-State mRNA Accumulation and Transcription in PBMC. Total RNA was isolated from four PBMC preparations treated with IFN- γ 16 h before LPS stimulation (4 h), and accumulation of IL-12 p40 and p35 and β -actin mRNA was evaluated by RNase protection assay. The 4-h stimulation time was previously determined as optimal for LPS stimulation of the IL-12 genes. Fig. 3 A shows results obtained with a representative do-

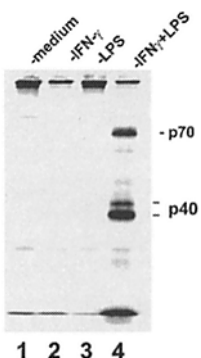


Figure 2. Immunoprecipitation of IL-12 p40 and p70 produced by human monocytes. Immunoprecipitation of [35 S]methionine-labeled IL-12 produced from an enriched monocyte preparation was carried out as described in Materials and Methods using the anti-p40 mAb C11.79 coupled to CNBr-Sepharose. Lane 1, unstimulated monocytes; lane 2, monocytes treated for 24 h with rhIFN- γ (1,000 U/ml); lane 3, monocytes treated with LPS (1 μ g/ml) for 18 h; lane 4, monocytes pretreated with IFN- γ for 24 h before LPS stimulation (18 h).

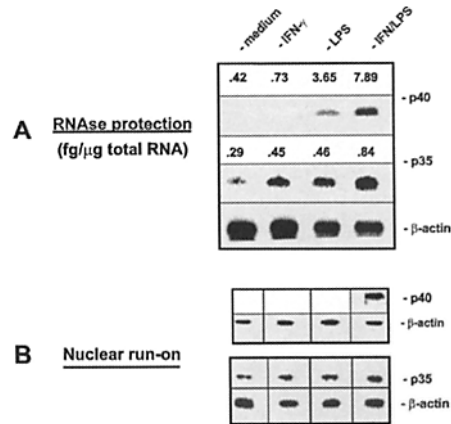


Figure 3. Accumulation of IL-12 p40 and p35 mRNA and gene transcription in human PBMC. Induction of IL-12 p40 and p35 mRNA accumulation (A) was analyzed in human PBMC treated with IFN- γ 16 h before LPS stimulation (4 h). mRNA were isolated by RNazol and subjected to RNase protection assays using the indicated riboprobes. Protected fragments were precipitated and fractionated by PAGE. Gels were dried and exposed to PhosphorImager screens and quantitated as fg of specific mRNA per microgram of total RNA after normalizing against the β -actin message. Transcriptional rate was analyzed by nuclear run-on assays (B) performed on isolated nuclei prepared from human PBMC pretreated with IFN- γ for 16 h followed by LPS stimulation for 4 h. [α - 32 P]UTP-labeled nuclear RNA was hybridized for 72 h to PCR-amplified p40, p35, and β -actin cDNAs (coding regions only, 200 ng/slot) immobilized on nylon membranes. The dried membranes were subjected to PhosphorImager analysis as described above. A representative experiment is shown out of four performed with similar results.

nor. To determine the extent of p40 and p35 mRNA induction, nuclear run-on experiments were performed in isolated nuclei prepared from PBMC pretreated with IFN- γ followed by LPS stimulation for 4 h. Fig. 3 B shows results of a representative experiment. Analyses of transcription rate, steady-state mRNA accumulation, and p40 protein synthesis from two to four donors are summarized in Fig. 4.

The p40 mRNA was synergistically induced by IFN- γ pretreatment followed by LPS stimulation, whereas p35 mRNA was additively stimulated. IFN- γ alone only marginally induced p40 mRNA but stimulated a twofold increase in the p35 transcript. LPS stimulated the accumulation of both transcripts. The transcription rate of p40 was not enhanced by either IFN- γ (16 h) alone or, in most experiments, by LPS (4 h). This apparently differs from the steady-state mRNA accumulation, which was stimulated 10-fold by LPS alone, and from the level of protein production, which was stimulated 5.4-fold (Fig. 4), perhaps because of a posttranscriptional role for LPS in stimulating p40 gene expression, although in other experiments a marginal induction of IL-12 p40 gene transcription by LPS was observed that might account for the enhanced mRNA accumulation (results not shown). Pretreatment with IFN- γ for 16 h before LPS stimulation resulted in a 11.6-fold increase in p40 gene transcription, compared with a 24-fold increase in the steady-state mRNA level, indicating that the effect of IFN- γ priming of LPS-induced p40 mRNA

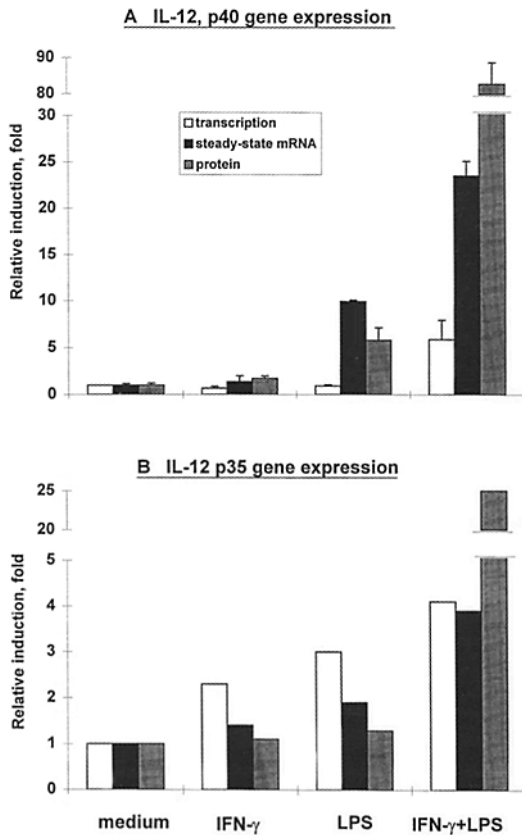
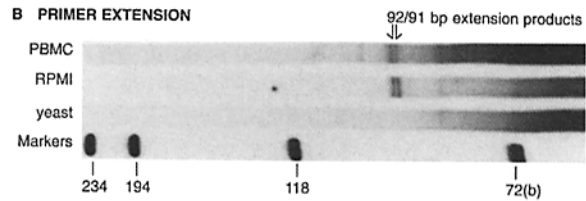
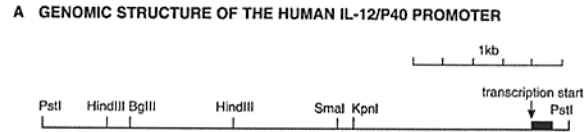


Figure 4. Relative induction rates of transcription, steady-state mRNA accumulation, and protein secretion for IL-12 p40 and p35. Relative rates of transcription, steady-state mRNA accumulation, and protein production are shown for IL-12 p40 and p35 genes in PBMC treated with rhIFN- γ (1,000 U/ml, 18 h) or not treated and followed by LPS stimulation (1 μ g/ml, 4 h for transcription and mRNA analysis, 24 h for protein production) or no stimulation. Values shown for p40 gene expression are the mean \pm SE of three to four experiments, and those for p35 are the average of two experiments. Because free p35 is not detectable in the supernatant fluid, values for the p70 heterodimers were used to evaluate production of the p35 protein.

expression is exerted primarily at the transcriptional level. On the other hand, the transcription rate of p35 enhanced by either LPS, IFN- γ , or both appears to be primarily responsible for the accumulation of its steady-state mRNA because of the close correlation of the induction rates observed at the transcriptional and steady-state mRNA levels.

Cloning of the Human IL-12/p40 Promoter. To study further the regulatory mechanisms of the p40 gene, we identified a 3.3-kb PstI fragment from a λ genomic clone (L26). Primer extension using total RNA obtained from RPMI-8866 cells or *S. aureus*-stimulated PBMC and an antisense primer (25-mer, corresponding to nucleotide positions 25–49 in the p40 cDNA clone [1]) revealed two major extension products of 91 and 92 bp, respectively, allowing placement of the transcription initiation site for the p40 gene near the 3' end of the 3.3-kb PstI genomic fragment (Fig. 5, A and B). The 91-bp product may have derived from an incomplete extension because of difficulties



C THE PROXIMAL PROMOTER REGION OF HUMAN IL-12/p40

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-747 CTGTATGCCTCCTGAGGG TATTCACITTC TGCCTCCATCCGCCCTATGAGCGAGTACCTATGAGCAC
      IRF-1
-677 AGGATGTGCACATATTGAG TCTATTAGT GGTACACGCA GTTTATCATCTCCCAAGT CTGTGCTGT
-607 ATGAAATGTGCATGGGTGTGTGTGTGCACCGTGTGTGCCACTCGGGAAATGGGGAG AGGTGCATGG
-537 AGCCAAGATG GGTGTAATAGTATGT TCTGAAAT TAAAGGACTAATGTGGGAAGGC GCCCCAGATG
      NF-IL6
-467 TACTAAACCCITTCATCTCATCTCTCTGACTTGGGAAGAACAG GATTTTGTIT TTAAGCCCTT
-397 GGGCATACAGTGTTCATCCCGACATGAAGTCAAGCTCC CGTCTGA CCGCCCTTGGCCCTTCCTTCTTC
      SP1
-327 CTCGATCTGTGAACCCAGG GAATCTGCT AGTGTGTCTCCAAGCACT TGGCCATGAT CFAAACCCAG
-257 AGAAATTAGCATCCCATCTCCTCC TTATTCCCACCCAAAGTC TTTCCTCTAGTTCATTAACCTGG
      AP-1 ets
-187 GATTTTGATG TCTATGTTC CTCTCGTATTGATACACACAGAGAGA GACAAACAAA AAAGGAAGCT
-117 CT GAAATTCCTC CAGAAG TTTTGAGAGTGTTTCAATGTGCAACAAGTCAGTTCCTAGTTAAAGTT
      NFkB
-47 TCCATCAGAAAGGAGTAGAG TATATAAGTTCAGTACCAGCAACAGCAGCAGAAAGAACACATCTGTTT
      TATA box
+23 CAGGGCCATGGACTCTCCGCTCCGCCAGAGCAAGTAAGCACTCCCAAGCCCTACC TCCTCCCTC
      exon 1
+93 CCTGTGGGCTGCAG +108

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Figure 5. Restriction map (A), determination of transcription start site (B), and partial sequence (C) of the human IL-12 p40 promoter. The human IL-12 p40 promoter was identified from a λ -phage genomic clone. The restriction map of a fragment containing part of the first intron, the first exon, and \sim 3.3 kb of the 5' upstream region is shown in A. The transcription start site was determined by primer extension using total RNA from *S. aureus*-stimulated PBMC, TPA-treated RPMI-8866 cells, or yeast RNA (B). The sequence of the proximal promoter region of the IL-12 p40 gene is shown in C. The putative motifs IRF-1 (47), NF-IL-6 (48), NFkB (49), SP1 (50), ets (51), and the TATA box identified by computer analysis are underlined and in boldface type. The first exon is underlined. The boxed elements, IRF-1, NF-IL-6, ets, and NFkB, were functionally analyzed in this study (see Results).

encountered by reverse transcriptase in proceeding through the methylated cap site. A 2-kb sequence upstream of the cap site was determined, and Fig. 5 C shows the proximal promoter sequence. This promoter (from +56 to the 5' end of the 3.3-kb clone) was linked to a fruit fly luciferase reporter gene (pXP2; 21) and used for transient transfections in a number of myeloid and lymphocytic cell lines. The 3.3-kb promoter was found to be constitutively active in the RPMI-8866 and CESS B cell lines, and, unlike the endogenous gene in these cells, did not respond to TPA stimulation. The promoter was inducible by LPS in THP-1 (human macrophagic) cells and RAW264.7 (murine macrophagic) cells, but inactive in Jurkat and Molt-13 T cell lines (Fig. 6), suggesting that the cloned promoter region contains adequate sequence information for appropriate cell type-specific expression (Fig. 6).

Induction of p40 Steady-State mRNA and Transcription Rate in the Murine Macrophagic Cell Line RAW264.7. To study

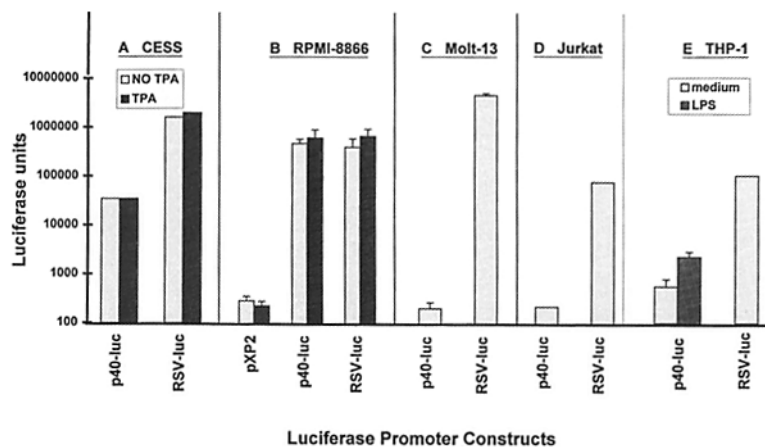


Figure 6. Transfection of B, T, and monocytic cell lines. CESS, RPMI-8866 (both EBV⁺ human B lymphoblastoid cell lines), Molt-13, and Jurkat (both human T cell lines) were transfected by electroporation, and THP-1 (human monocytic cell line) by DEAE-dextran with the 3.3-kb IL-12 p40 promoter-luciferase construct or other constructs. At 18 h after transfection, cells were cultured in medium or treated with TPA (20 ng/ml, 15 h) or LPS (1 μ g/ml, 8 h) as indicated. Luciferase activity was measured in the cell lysate, and values were normalized against the internal control (CMV- β -galactosidase construct). Expression vectors: *p40-luc*, the 3.3-kb p40 promoter linked to the luciferase reporter; *pXP2*, luciferase reporter without a promoter; *RSV-luc*, a luciferase reporter driven by the RSV promoter.

the regulation of IL-12 expression at the molecular level, an *in vitro* system was established using the murine RAW264.7 cell line because of its monocytic origin, its ability to produce physiologically relevant levels of IL-12 in response to LPS and IFN- γ stimulation, and its ready amenability to transfection. Steady-state p40 mRNA accumulation was determined by Northern blot analysis in total RNA samples isolated from cultured RAW264.7 cells treated with rmIFN- γ (1,000 U/ml) for 16 h before LPS stimulation (1 μ g/ml, 8 h). As shown in Fig. 7 A, neither LPS nor IFN- γ alone had any significant effect on p40 mRNA accumulation, whereas a synergistic stimulatory effect was observed when LPS was added after the IFN- γ pretreatment of RAW264.7 cells. IFN- γ pretreatment (16 h) or LPS (8 h) had little effect on IL-12 p40 gene transcription as analyzed by nuclear run-on experiments (Fig. 7 B), whereas the synergy between IFN- γ and LPS in inducing transcription was very pronounced and might account for the increase in the level of steady-state p40 mRNA.

IFN- γ Primes for LPS Induction of the p40 Promoter in RAW264.7 Cells. RAW264.7 cells were transfected with

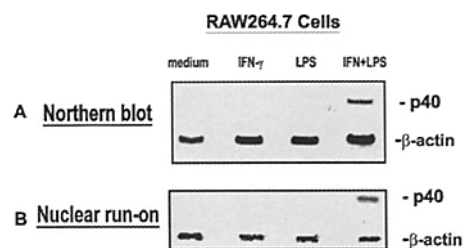


Figure 7. IL-12 p40 mRNA accumulation and transcription induced by IFN- γ and LPS in RAW264.7 murine macrophagic cells. Total RNA was isolated from RAW264.7 cells treated with medium alone, IFN- γ (1,000 U/ml, 16 h), LPS (1 μ g/ml, 8 h), or pretreated with IFN- γ (16 h) followed by LPS stimulation (8 h). Northern blot (15 μ g RNA) and hybridization (A) were performed in 50% formamide at 42°C for 18 h, using a murine p40 cDNA probe. The filter was stripped and reprobed with a murine β -actin probe. Transcription rate was determined by nuclear run-on experiments (B) performed with isolated nuclei from RAW264.7 cells treated as indicated above. Labeled nuclear RNA was hybridized to membrane-immobilized murine p40, p35, and β -actin cDNAs (coding regions only) amplified by PCR (200 ng/slot). The figure shows the PhosphorImager results of one of two experiments with identical results.

the 3.3-kb p40 promoter-luciferase construct by electroporation and pretreated with IFN- γ (1,000 U/ml) for different times, as indicated in Fig. 8 A, followed by LPS stimulation (1 μ g/ml). Luciferase activity was measured in cell lysates prepared from cells collected 8 h after LPS stimulation, and the values were normalized against the internal control (β -galactosidase) to correct for transfection efficiencies. Treatment with IFN- γ alone did not stimulate the promoter, whereas LPS increased the promoter activity \sim 4.5-fold. However, pretreatment of RAW264.7 cells with IFN- γ for 8 h before LPS stimulation had the greatest synergistic effect on the promoter, inducing a 30-fold increase of activity. The magnitude of this effect was similar to the effect of those stimuli on the transcription of the endogenous p40 gene (Fig. 7 B). In addition, the kinetics of the priming effect of IFN- γ was analogous to that observed for production of the endogenous p40 protein under identical culture conditions (Fig. 8 B), and also similar to that observed for production of the p40 protein from human PBMC (Fig. 1 A). The maximum induction in both types of cells was \sim 75-fold, although the IFN- γ -priming effect in RAW264.7 cells appears to be more swift but transient than in PBMC.

5' deletion mutants of the p40 promoter were tested in RAW264.7 cells for their ability to induce transcription of the reporter-luciferase gene in response to LPS and IFN- γ (Fig. 9). When truncated at -222, the p40 promoter was still induced synergistically by the two stimuli, although at a level \sim 50% of the 3.3-kb construct, indicating that upstream elements exist that enhance transcription. Further deletion of the promoter to -204 drastically diminished, but did not completely eliminate, the inducibility by IFN- γ and LPS. A potential ets motif, TTTCCT (or AG-GAAA for the complement), is located between -204 and -222. A 5-bp deletion that eliminated the TTTC sequence in the context of the 3.3-kb promoter resulted in almost complete unresponsiveness to IFN- γ and LPS (Fig. 10). When a CMV-based murine ets-2 expression vector (a gift from Dr. R. Maki) was cotransfected into RAW cells with the wild-type or the deletion mutant, the wild-type construct was activated in all four conditions by ets-2 but not by ets-1, PU-1, or the control vector (data not shown),

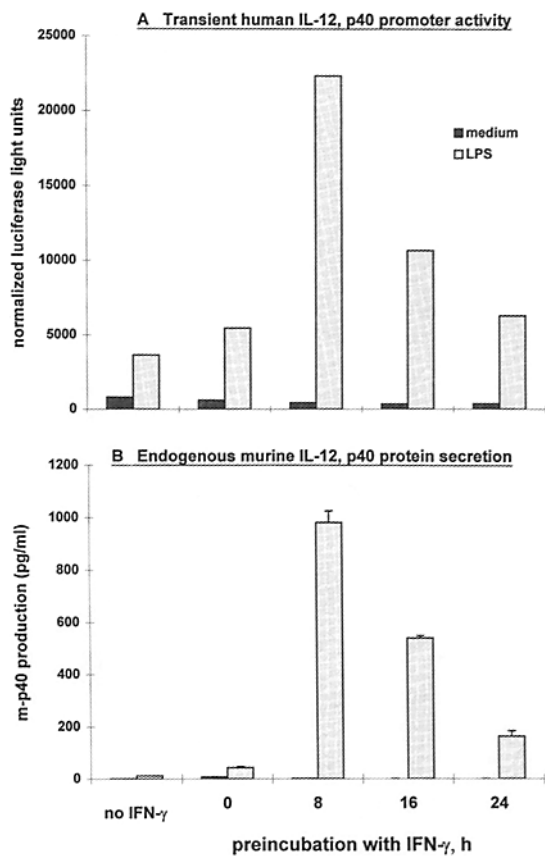


Figure 8. IFN- γ primes the transfected human IL-12 p40 promoter in the murine RAW264.7 monocytic cell line for activation by LPS (A) with a similar kinetics and potency to those of the endogenous murine p40 production (B). RAW264.7 cells were transfected with the 3.3-kb p40 promoter-luciferase construct by electroporation. At 16 h after transfection, cells (5×10^6 cells/well per 2 ml) were treated with 1.2% DMSO for 24 h before LPS stimulation. Cells were pretreated with rIFN- γ (1,000 U/ml) for 24, 16, 8, and 0 h before the addition of LPS (1 μ g/ml, 8 h). Cell lysates were used for both the luciferase assay and β -galactosidase assay. All luciferase values were then corrected against those of their respective β -galactosidase values. The results are representative of two experiments of kinetics of IFN- γ pretreatment and of at least 10 experiments using either 8- or 18-h pretreatment with IFN- γ . To determine secretion of the endogenous murine IL-12 p40 protein, RAW264.7 cells were cultured at 10^6 /ml in 1 ml of medium and treated as above except that LPS stimulation was for 24 h; IL-12 p40 in the cell-free culture supernatants was determined by RIA. All samples were cultured in duplicate, and each duplicate culture was evaluated by RIA in triplicate wells. Mean values are presented with SE.

whereas ets-2 did not induce the deletion mutant, suggesting that the action of ets-2 was indeed mediated by this element.

The -222 construct, although active in both monocytic and B cells, was silent when transfected into T cell lines (data not shown), indicating that it is still regulated in a tissue-specific manner like the 3.3 kb promoter.

The -204 construct was noticed to maintain a low but consistent inducibility by IFN- γ and LPS. A possible candidate responsible for this low level of induction is NF κ B, which indeed binds to a consensus site located at -116 to -107 (CCCTTAAAGT or GGGAATTTTA for the com-

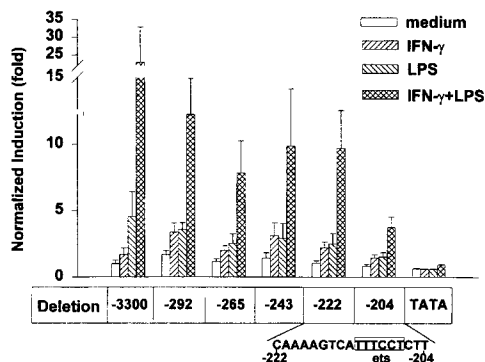


Figure 9. Activation by IFN- γ and LPS of 5' deletion mutants of the human IL-12 p40-luciferase constructs transfected in RAW264.7 cell lines. RAW264.7 cells were transfected by electroporation with the indicated 5' deletion mutants and stimulated with IFN- γ (8 h pretreatment) and LPS (8 h) as described in Fig. 8. The results presented (luciferase activity normalized against an internal cotransfected β -galactosidase standard) are mean \pm SD from six independent experiments. All values were then normalized against the medium condition of the -3,300 construct which was taken as 1.

plement) in response to LPS stimulation in the form of p50 homodimer primarily, but also of p65/C-rel heterodimer and C-rel homodimer (data not shown). However, the functional role of this site remains to be established.

Two other upstream elements, that is, IRF-1, located at -730 to -719, and NF-IL6, at -520 to -512, were also examined by deleting the individual elements from the 3.3-kb promoter. In both cases, no loss of responsiveness to IFN- γ and LPS was observed (data not shown).

Discussion

In this report, we show that the IL-12 p40 and p35 genes are regulated primarily at the transcriptional level in response to IFN- γ and LPS stimulation in human PBMC.

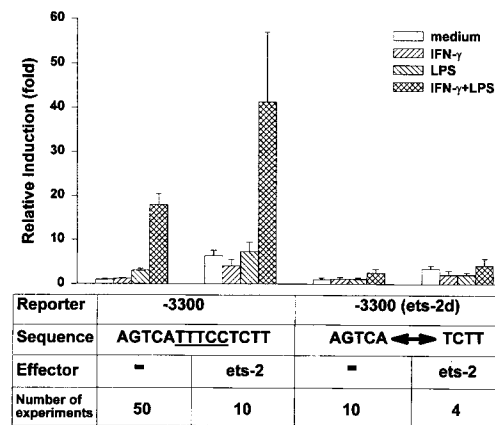


Figure 10. Activation of the IL-12 p40 promoter by ets-2 in RAW264.7 cells. RAW 264.7 cells were transfected by electroporation with the 3.3-kb promoter construct or the same construct with a 5-bp deletion at the ets site. A murine ets-2 expression vector driven by the CMV enhancer/promoter was cotransfected at a molar ratio of 1:1 (reporter/effector). The wild-type and mutant sequences around the ets site are shown. All values were relative to the medium condition of the -3,300 construct, which was set at 1.

IFN- γ alone does not induce p40 mRNA and protein production. This finding confirms our previous results (13, 16), but contrasts with those of Yoshida et al. (22), who detected a marked increase in p40 mRNA levels in the murine macrophage cell line J774 when stimulated with IFN- γ alone. We have been unable to confirm this observation in PBMC or in the murine macrophage-derived cell line RAW264.7. One explanation for the different observations might rest in the use of culture media in the Yoshida et al. (22) study that might not have been endotoxin free. Unlike IFN- γ , LPS alone induced a 10-fold increase in p40 mRNA and a fivefold enhancement in p40 protein production, although minimal transcriptional up-regulation was observed, suggesting that the effects of LPS stimulation could be in part posttranscriptional, for example, at the level of mRNA stability. Direct confirmation of this mechanism is difficult because the unstimulated level of p40 message is too low to quantitate its half-life.

Priming of PBMC with IFN- γ for 16–24 h followed by LPS stimulation (8 h) increased the expression synergistically with an 11.6-fold enhancement in transcription rate, a 24-fold stimulation in mRNA accumulation, a 75-fold induction in the level of secreted p40 chain (both in the free and the heterodimeric forms), and a 40-fold increase in p70 heterodimer production. The varying degrees of enhancement of p40 transcription, mRNA, and protein might be attributed to posttranscription/translational control mechanisms. However, because nuclear run-on assays measure the transcriptional rate at a specific time, RNase protection measures the steady-state levels of stable mRNA accumulation, and protein determination assays measure the accumulation of a stable product in the supernatant fluid, the difference in the degrees of enhancement might simply reflect the detection methods and/or differences in kinetics. Thus, our results point strongly to a transcriptional control of p40 gene expression in response to LPS after IFN- γ priming. The level of p35 mRNA also appears to be regulated primarily transcriptionally, since there is a good correlation between changes in transcription rate and steady-state mRNA accumulation. IFN- γ and LPS each induce IL-12 p35 gene transcription and mRNA accumulation. In addition, an additive effect on p35 gene transcription and mRNA accumulation is observed in PBMC treated with both IFN- γ and LPS. The net result of transcriptional up-regulation of both the p40 and the p35 gene is an observed increase in the production of the biologically active p70 heterodimer. The marked increase in p70 protein is, however, difficult to reconcile with the modest increase in p35 transcripts in response to IFN- γ and LPS. This discrepancy may indicate translational/posttranslational mechanisms or may be due to the constitutive expression of the p35 gene in cell types within the PBMC preparation that do not produce IL-12.

The cloned 3.3-kb p40 promoter transfected in several human and murine cell lines with a construct with the luciferase reporter gene showed both cell type specificity and responsiveness to IFN- γ and LPS, analogous to the endogenous gene, suggesting that it may contain sufficient *cis* ele-

ments to elicit cognate responses to specific stimulation. 5' promoter deletion analysis strongly implicated the region between -222 and -204, which includes a putative ets motif. This region is responsive to IFN- γ and LPS stimulation. Deletion of the ets motif in the context of the entire 3.3-kb promoter resulted in the loss of its responsiveness to both IFN- γ and LPS, as well as to activation by cotransfected ets-2, strongly suggesting the critical role that this site may play in the induction of the p40 promoter by IFN- γ and LPS. However, ets-2 may not be the only factor required to activate the promoter, since the cotransfected ets-2 failed to activate the promoter in unstimulated cells to the level of IFN- γ - and LPS-stimulated cells, but synergized with IFN- γ and LPS in inducing promoter activation. Our unpublished preliminary observations suggest that the -222/-204 and the extended region up to -292 interacts with a series of nuclear factors including ets-2 that responds to IFN- γ and LPS stimulation differentially and may control the regulation of the promoter in a complex manner.

Analysis of p40 promoter regulation in the easily transfected cell line RAW264.7 showed that, as in PBMC, the induction of p40 mRNA is primarily transcriptional since the increase in stimulated p40 transcription is in accord with the increase in its steady-state mRNA accumulation. Like PBMC, these cells are primed by IFN- γ for production of p40 in response to LPS, indicating the suitability of this cell line as an *in vitro* system to study IL-12 gene regulation. The data obtained on the expression of the endogenous p40 gene and the activation of the transfected cloned human p40 promoter using the RAW264.7 homogeneous monocytic cell population as well as PBMC strongly support the notion that IFN- γ and LPS act directly on the IL-12-producing cells. This conclusion is supported by a recent report by Hayes et al. (23) using elutriated monocytes, which responded to IFN- γ treatment followed by LPS stimulation in producing IL-12 p40 message and protein synergistically.

Priming/enhancing effects of IFN- γ on the expression of several other inflammatory cytokines secreted by monocytes/macrophages in response to LPS stimulation, that is, IL-1, IL-6, TNF- α , and G-CSF, have been described (24–30). This priming is not only efficient but also assumes particular physiological relevance in the case of IL-12, because IL-12 itself is a potent inducer of IFN- γ production (1, 31, 32). Thus, after an infective or inflammatory stimulus that provokes IL-12 production, IL-12-induced IFN- γ may enhance the ability of the phagocytic cells to produce IL-12 and other proinflammatory cytokines with a powerful positive feedback mechanism. Self-enhancing mechanisms of proinflammatory cytokine production must be effectively regulated to avoid pathological effects; the production of factors such as IL-10, IL-4, IL-13, TGF- β , and PGE₂, which have been described to inhibit IL-12 production and/or to inhibit IL-12 activity on lymphocytes (15, 17, 33), is probably responsible for the negative feedback regulation of the IL-12/IFN- γ loop. Even in the almost uncontrolled induction of inflammatory cytokines ob-

served in LPS-induced shock, these regulatory mechanisms appear to be effective, because the production of IL-12 subsides before the peak of IFN- γ production is observed at 5–7 h after LPS injection, and anti-IFN- γ antibodies do not prevent the LPS-induced production of IL-12 in vivo (18). However, in the Shwartzman-like reaction in the mouse, the IL-12-induced IFN- γ production in response to a local low-dose priming injection of LPS appears to play an important role in priming the phagocytic cells for production of IL-12 and other proinflammatory cytokines in response to the challenging dose of LPS (34). Furthermore, in response to infectious agents with limited ability to induce IL-12 production, for example, mycobacteria, the priming effect of IFN- γ appears to be required for optimal production of IL-12 both in vitro and in vivo.

The central role of IFN- γ priming in the physiological regulation of IL-12 activity during inflammation and immune response underlines the importance of understanding the molecular mechanisms of such priming. Interestingly, different mechanisms have been found to be responsible for the priming/enhancing effect of IFN- γ on the expression of genes other than IL-12, including proinflammatory cytokines and the nitric oxide (NO) synthase gene in phagocytic cells. In the case of G-CSF, it was shown that IFN- γ enhances LPS-induced transcription and stabilizes LPS-induced mRNA (30). IFN- γ also primes for enhanced transcription of the IL-6 gene induced by LPS or TNF- α (35, 36). The regulation of the NO synthase gene by IFN- γ and LPS is particularly complex (37–41). Unlike the p40 gene, transcription of the NO synthase gene is induced by either IFN- γ or LPS, with the two inducers together showing a modest synergistic transcriptional effect in some studies (39, 40) but not others (41). Two upstream regions in NO synthase promoters appear to be responsible for induction by IFN- γ and LPS, involving binding of the IFN-regulatory factor (IRF-1) and the NF- κ B/Rel transcription factor, respectively (38–40).

Our results suggest that the priming effect of IFN- γ on IL-12 production is due mostly to an enhancement of the ability of LPS to induce transcription of the p40 gene. Although, unlike the p40 gene, IFN- γ alone was shown to induce transcription of the p35 gene, simultaneous stimulation with both IFN- γ and LPS was required for maximal expression of the p35 gene and secretion of the p40/p35 biologically active heterodimer. Our observations raise the possibility that discrete *cis* elements exist in the p40 promoter that respond to IFN- γ and LPS separately and cooperatively. The region –222 to –204 in the p40 promoter appears to be responsible for both LPS responsiveness and IFN- γ priming. A perfect IRF-1-binding site is present in the p40 promoter at –730 to –719; however, this is upstream of the region required for IFN- γ priming, thus sug-

gesting that, unlike in the NO synthase gene (39), IRF-1 may not be responsible for activation of the p40 promoter. Deletion of 5 bp within the element from the 3.3-kb promoter did not decrease responsiveness to IFN- γ (not shown), confirming the lack of activity of the IRF-1 element. An NF κ B element is present at –116 to –107. It was found to bind primarily the NF κ B p50 homodimer and, to a lesser extent, interact with p65/C-rel heterodimer as well as C-rel homodimer in response to LPS stimulation. However, the functional significance of this element awaits confirmation.

IFN- γ pretreatment of cells may induce factors that by themselves do not stimulate the p40 promoter but interact with LPS-induced factors, resulting in the activation of the promoter. The fact that a long preincubation period with IFN- γ (16 h in PBMC, 8 h in RAW264.7 cells) is required to maximize LPS-stimulated IL-12 p40 expression suggests that the effects of IFN- γ priming might not be direct, such as those observed in some immediate-to-early response genes, for example, IL-1 β , TNF- α , and IL-6. This late-acting effect and the absence of clearly identifiable IFN- γ activation sequences (GAS) in the proximal p40 promoter make a role for the JAK-STAT-GAS signal transduction pathway (42) in IL-12 expression unlikely. However, the possibility cannot be ruled out that the initial signal transduction events involve the JAK-STAT pathway, leading to the expression of gene products that are indirectly responsible for the upregulation of IL-12 gene transcription. The –292 to –204 region of the p40 promoter contains several consensus elements for binding of nuclear factors, as identified by a computer search. Particularly interesting are an activator protein 1 (AP1) and an ets element. ets family factors have been shown to be important for transcriptional regulation of other cytokines (e.g., PU-1 for IL-1 β [43] and ets-1 for TNF- α [44]), in some cases forming complexes with AP1-related factors (44). Some ets family factors are also known to be preferentially expressed in phagocytic cells and B cells (45), the cell types expressing the IL-12 p40 gene. The p40 3.3-kb upstream promoter, analogous to the endogenous p40 gene, is constitutively active in B lymphoblastoid cell lines and inducible in monocytic cell lines but not in T cell lines. Thus, analysis of the *cis* and *trans* elements in the p40 promoter should provide information on the control of cell specificity and regulation of expression in monocytic and B cells of one of the genes controlling the production of IL-12, a cytokine that plays an important role in inflammation and regulation of the immune response. This knowledge is also important for the understanding of the mechanism underlying the profound depression of IL-12 p40 and p70 production observed in HIV-infected individuals (46), which may contribute to the immunodeficiency in AIDS patients.

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