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Lipopolysaccharide Synergizes with Interferon-γ to Induce Expression of *Mig* mRNA in Mouse Peritoneal Macrophages

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Abstract Lipopolysaccharide (LPS) is responsible for the tissue injury that occurs following the invasion of multicellular organisms by Gram-negative microbes. The effect of LPS on IFN-γ-induced chemokine *Mig* gene expression in mouse peritoneal macrophages was investigated. Very little *Mig* mRNA was detectable upon exposure to LPS without IFN-γ. Although LPS alone is only minimally effective, LPS plus IFN-γ synergized to produce a high level of *Mig* mRNA in the peritoneal macrophages. This synergy was not dependent on a new protein synthesis, and was not controlled at the level of the gene transcription. Furthermore, LPS did not increase IFN-γ-induced *Mig* mRNA stability. Accordingly, it is suggested that LPS may synergize the expression of IFN-γ-induced *Mig* mRNA through a process that depends on a pretranscriptional level or concurrent *Mig* mRNA translation.

Key words: *Mig*, LPS, IFN-γ

Gene regulation during macrophages activation represents a complex response of enhanced and suppressed transcription and mRNA stability, the precise pattern of which depends on the stimuli given to the macrophages and gene examined [2, 12, 17, 18, 19, 24, 25]. This heterogeneity may reflect differences in the signaling events induced by the stimuli that promote their expression. Macrophages play an important role in acute and chronic inflammation [1]. The behavioral potential of macrophages during an inflammatory process is mediated by multiple signals encountered in the tissue microenvironment. These include lipopolysaccharide (LPS) as a kind of bacterial cell wall product and secreted cytokines such as interferon-γ (IFN-γ).

The inflammatory response to systemic LPS administration is mediated, in part, by the secretion of chemokines at sites of incipient inflammation [11, 34]. Based upon studies with various cell types, virtually every cell type has been known to have potential to generate large amounts of many chemokines. Some studies have provided data indicating that individual chemokine genes are differentially regulated in reponse to LPS [15, 16, 27].

The biologic properties of chemokine *Mig* include the chemoattraction of activated T cells, inhibition of endothelial cell chemotaxis, and inhibition of growth factor-induced angiogenesis *in vivo* [31, 33]. Studies *in vivo* and *in vitro* have indicated that IFN-γ is the only inducer of *Mig* in monocyte/macrophages, fibroblasts, and keratinocytes [7]. IFN-γ is generally considered to be a potent macrophage activator that interacts synergistically with LPS to induce inflammatory mediators like TNF-α and iNOS, in addition to enhancing LPS-induced lethality [8, 9, 32]. Ohmori *et al.* [23, 26] provided data that TNF-α and IFN-γ synergistically induce the expression of the mRNAs of monokine induced by IFN-γ (*Mig*) and IFN-γ-inducible protein of 10 kDa (IP-10) in fibroblasts, and that the synergy between IFN-γ and TNF-α in transcriptional activation is mediated by a cooperation between STAT-1 and NFkB. The present study examined the role of LPS on IFN-γ-inducible *Mig* mRNA expression in murine macrophages. A marked synergy was found between LPS and IFN-γ in *Mig* mRNA expression.

Materials and Methods

Materials

Brewer's thioglycollate broth was purchased from Difco Laboratories (Detroit, U.S.A.). RPMI 1640, Dulbecco's phosphate-buffered saline (PBS), Hank's balanced salt solution (HBSS), L-glutamine, trypsin, and agarose were all purchased from Life Techologies Inc. (Gaithersburg, U.S.A.). The fetal bovine serum (FBS), phenol, guanidine isothiocyanate, cesium chloride, and formamide were obtained from Gibco BRL (Gaithersburg, U.S.A.). The Magna nylon transfer membrane was obtained from Micron

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Separation Inc. (Westboro, U.S.A.). The high prime kits were purchased from Boehringer Mannheim (Indianapolis, U.S.A.). [α-^{32}P]dCTP and [α-^{32}P]dUTP were purchased from Dupont -New England Nuclear (Boston, U.S.A.). Trihydroxymethyl aminomethane (Tris), sodium dodecyl sulfate (SDS), and *Escherichia coli* LPS (0111:B4) were obtained from Sigma Chemical Co. (St. Louis). The recombinant mouse IFN-γ (5×10^5 U/mg) was purchased from Genzyme (Cambridge, U.S.A.). The plasmid encoding genes for *Mig*, IP-10, and GAPDH were kindly provided by Dr. Hamilton, Department of Immunology, Lerner Research Institute, Cleveland Clinic Foundation, USA.

Mice

Specific pathogen free, inbred C57BL/6, BALB/c mice, 9 to 12 weeks of age, were purchased from Hyeuchang Sci., Corp., (Taegu, Korea). Utmost precautions were taken to ensure that the mice remained free from infection by environmental pathogens, thereby confirming that the degree of spontaneous activation of tissue macrophages would be minimal.

Mouse Peritoneal Macrophages

Thioglycollate (TG)-elicited macrophages were obtained by Tannenbaum's method [34]. Peritoneal larvage was performed using 10 ml of cold HBSS (Hank's balanced salt solution) containing 10 U/ml heparin. Macrophages in a complete medium were plated in 60-mm tissue culture dishes, incubated for 2 h at 37°C in an atmosphere of 5% CO_2, and then washed three times with HBSS to remove any nonadhering cells. The macrophages were cultured overnight in a complete medium at 37°C in 5% CO_2, and then cultured in the presence or absence of stimuli for the indicated times.

Preparation of RNA and Northern Hybridization Analysis

Total cellular RNA was extracted using the guanidine thiocyanate-cesium chloride method [3]. An equal amount of RNA (10 μg/ml) was used in each lane of the gel. The RNA was denatured, separated by electrophoresis in a 1% agarose/2.2 M formaldehyde gel, and transferred to a nylon membrane, as previously described [13]. The blots were prehybridized for 6 h at 42°C in 50% formamide, 1% SDS, 5× saline sodium citrate, 1× Denhardt's solution (0.02% bovine serum albumin and 0.02% polyvinylpyrrolidone), 0.25 mg/ml denatured herring testis DNA, and a 50 mM sodium phosphate buffer, pH 6.5. Hybridization was carried out at 42°C for 18 h with 1×10^7 cpm of denatured plasmid DNA containing appropriate specific cDNA inserts. The blots were rinsed with a solution of 0.1% SDS-0.2× SSC, washed at 42°C for 1 h and then at 65°C for 15 min. The blots were dried and exposed to XAR-5 X-ray film (Eastman Kodak Co., Rochester, U.S.A.) at -70°C. The blots were quantified with computer analysis using BIO-1D version 6.

Nuclear Transcription Assay

Cultures of 2×10^7 macrophages were treated as indicated in the text and the nuclei were isolated as described previously [13]. The transcription initiated in intact cells was allowed to complete in the presence of [α-^{32}P]dUTP, and the RNA was isolated and hybridized to slot-blotted plasmids containing specific cDNA (7 μg DNA/slot), essentially as described elsewhere [8, 13]. The blots were hybridized for 72 h and exposed to X-ray film for 2 to 4 days. The α-tubulin gene was used as the internal standard. The expression of specific transcripts was quantified by computer analysis using BIO-ID version 6. Numerical values for the specific transcripts were normalized to an α-tubulin transcript level in the same sample. This ratio in untreated samples was arbitrarily set to unity. The experimental values were presented as the fold induction relative to the untreated samples.

RESULTS

Induction of *Mig* Gene Expression by LPS was Strain-Specific and Independent of LPS Dosage

Initially, the current study was planned to assess the relative capacity of LPS in inducing *Mig* mRNA expression in mouse peritoneal macrophages from different strains. After the thioglycollate-elicited C57BL/6 and Balb/c peritoneal macrophages were stimulated with 0.01, 0.1, and 1 μg/ml of LPS for 4 h, a Northern analysis was performed. While the LPS-stimulated peritoneal macrophages from C57BL/6 expressed *Mig* mRNA, the LPS-stimulated macrophages from Balb/c did not, and the induction of *Mig* mRNA in the peritoneal macrophages stimulated with LPS was not dependent upon the dose of LPS. All doses of LPS used in the macrophages from C57BL/6 showed the same effect in inducing *Mig* mRNA expression (Fig. 1). Although LPS was able to induce *Mig* mRNA in the peritoneal macrophages of C57BL/6, the expression was very weak and was independent of the LPS dosage.

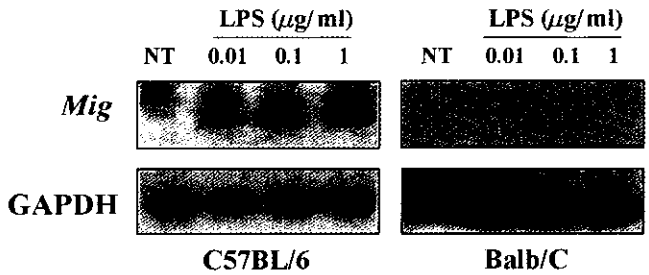

Fig. 1. Strain-specific and dose-independent response of LPS in *Mig* gene expression in mouse peritoneal macrophages.
Northern analysis of mRNA derived from thioglycollate-elicited (TG) mouse peritoneal macrophages stimulated with varying doses of LPS for 4 h. These data are the representatives of three experiments.

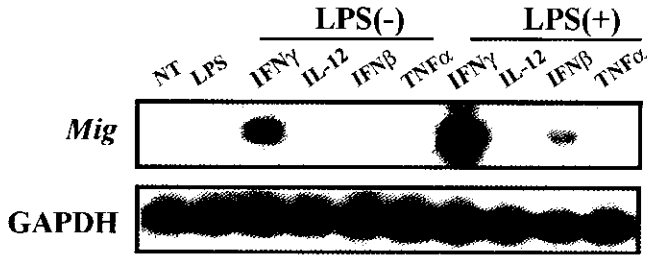

Fig. 2. Effect of LPS on stimulus-specific expression of *Mig* mRNA.

Confluent monolayers of TG-elicited mouse peritoneal macrophages were untreated (NT) or treated with LPS (100 ng/ml) and/or IFN-γ (50 U/ml), IL-12 (25 ng/ml), IFN-β (500 U/ml), and TNF-α (10 ng/ml) for 4 h. Thereafter, total RNA was prepared and the *Mig* mRNA levels were analyzed by Northern hybridization. These data are representative of three experiments.

LPS Synergized with IFN-γ to Induce *Mig* mRNA Expression in Mouse Macrophages

To gain a further insight into LPS-induced *Mig* mRNA expression in macrophages by various stimuli, the macrophages were stimulated for 4 h with various combinations of cytokines, and the total RNA was analyzed by a Northern blot analysis. As shown in Fig. 2, when IL-12, IFN-β, and TNF-α were used alone, they failed to induce *Mig* gene expression. In contrast, a considerable accumulation of *Mig* mRNA was observed in macrophages when treated with IFN-γ or IFN-γ plus LPS, and there was an especially high induction in the case of IFN-γ plus LPS-treated cells.

Next, the question of whether the synergistic effect on IFN-γ-induced *Mig* mRNA expression by LPS was temporarily related to the time of macrophages exposure to IFN-γ was examined. As shown in Fig. 3, the synergy between IFN-γ and LPS in *Mig* mRNA accumulation was only observed when LPS plus IFN-γ were present together during the stimulation period. This result indicates that the synergistic effect of LPS needs a simultaneous stimulation with IFN-γ.

This time dependency of the LPS-mediated synergistic effect of the IFN-γ-induced *Mig* mRNA expression was determined in macrophages treated for various time periods (Fig. 4). The results demonstrated that the kinetics of the *Mig* mRNA expression were comparable in the IFN-γ-treated macrophages with and without added LPS. The IFN-γ-induced *Mig* mRNA expression reached a maximum as early as 4 h after treatment and then gradually declined up to 24 h. Thus, the treatment of macrophages with LPS had no effect on the time course of the response to IFN-γ, yet it markedly increased the quantity of *Mig* mRNA detected at any given time. Many of the effects of LPS treatment are believed to be mediated by the induction of new genes. If the ability of LPS synergy on IFN-γ-induced *Mig* mRNA expression was due to the induction of a new protein, the synergistic effect in macrophages might be blocked by co-treatment with a protein synthesis inhibitor such as cycloheximide. To test this possibility, macrophages were treated with only LPS or in combination with IFN-γ in the presence or absence of cycloheximide. The LPS-induced synergistic effect was not attenuated in the presence of cycloheximide (Fig. 5).

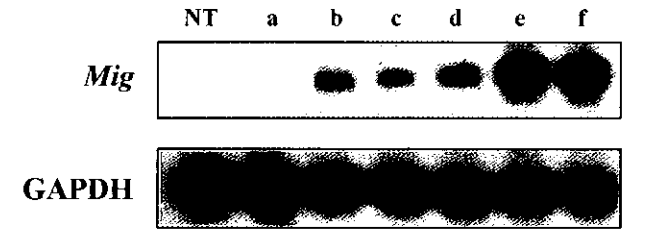

Fig. 3. Effect of LPS on IFN-γ-induced *Mig* mRNA expression in mouse peritoneal macrophages.

TG-elicited mouse peritoneal macrophages were treated as follows: (NT) no treatment; (a) treated with LPS (100 ng/ml) for 4 h; (b) treated with IFN-γ (50 U/ml) for 4 h; (c) pretreated with IFN-γ for 15 min, change of medium, and then treated with LPS for 4 h; (d) pretreated with LPS for 15 min, change of medium, and then treated with IFN-γ for 4 h; (e) pretreated with IFN-γ for 15 min, then treated with LPS for 4 h; (f) pretreated with LPS for 15 min, then treated with IFN-γ for 4 h. Total cellular RNA was prepared and used to determine levels of *Mig* and GAPDH mRNA by Northern analysis. These data are representative of two similar experiments.

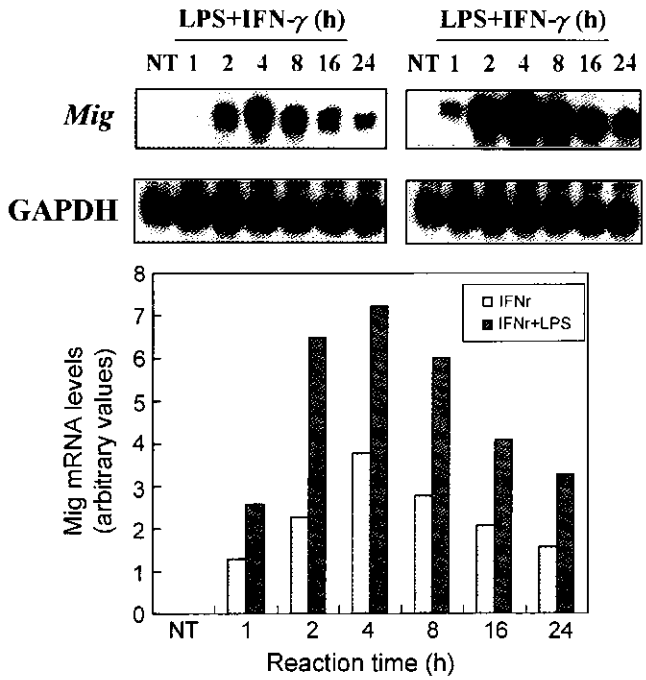

Fig. 4. Time kinetics of LPS/IFN-γ-induced *Mig* mRNA expression in mouse peritoneal macrophages.

Confluent monolayers of TG-elicited mouse peritoneal macrophages were untreated (NT) or treated with LPS (100 ng/ml) and/or IFN-γ (50 U/ml) for the indicated times. The mRNA levels were determined by Northern hybridization and quantified by a computer analysis using Bio-1D version 6. The levels of *Mig* and *IP-10* mRNA in each sample were normalized for the levels of GAPDH mRNA. Similar results were obtained in two separate experiments.

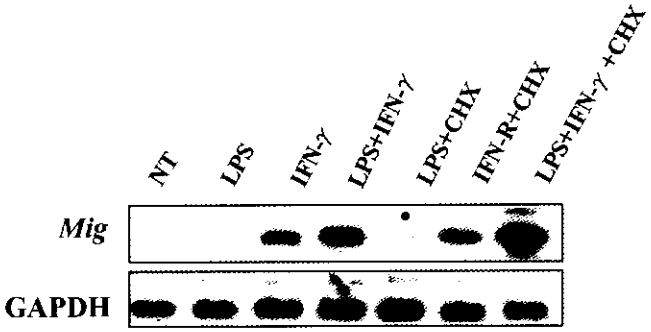

Fig. 5. LPS-mediated synergy of IFN-γ-induced *Mig* mRNA expression is not prevented by an inhibitor of protein synthesis.
TG-elicited mouse peritoneal macrophages were untreated (NT) or treated with LPS (100 ng/ml) with IFN-γ (50 U/ml), and/or with cycloheximide (CHX) (10 μg/ml) for 4 h. The mRNA was isolated, and a Northern analysis was performed. These data are representative of three similar experiments.

Mechanisms of LPS-Mediated Synergy of IFN-γ-Induced *Mig* mRNA Expression

Alternations in specific mRNA levels can be caused by modulating the transcriptional activity of the gene and/or mRNA degradation. To ascertain which mechanism(s) is involved, the half-life of IFN-γ-induced *Mig* mRNA was first measured in the presence or absence of LPS. Macrophages were then stimulated with IFN-γ in the presence or absence of LPS for 4 h before treatment with actinomycin D to prevent any further transcription. After an additional incubation of up to 90 min, the specific mRNA levels were assessed by Northern hybridization (Fig. 6). Although the process of chemokine *IP-10* mRNA decaying was observed, the process of *Mig* mRNA decaying was not seen, and the steady state levels of *Mig* mRNA were not different in the IFN-γ and LPS-treated cells vs those treated with IFN-γ. The half-life of *Mig* mRNA was not comparable. This result indicates that LPS does not affect the stabilitiy of IFN-γ-induced *Mig* mRNA. To determine if the synergy of IFN-γ-induced *Mig* mRNA by LPS involves an increased transcription,

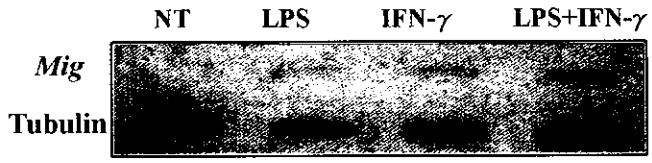

Fig. 7. The synergistic effect of LPS on the expression of IFN-γ-induced *Mig* mRNA is not controlled at the level of transcription.
Thioglycollate-elicited macrophages were treated with IFN-γ (50 U/ml) for 4 h in the absence or presence of LPS (100 ng/ml) before the isolation of the nuclei and an analysis of transcription by a nuclear run-on. Radiolabeled nuclear RNA was hybridized with nylon membranes containing equivalent amounts of denatured plasmid DNA encoding Mig and tubulin.

nuclear run-on experiments were performed. Cultures of peritoneal macrophages were treated with IFN-γ for 4 h in the absence or presence of LPS, the nuclei were then harvested, and the RNA transcripts initiated *in vivo* were allowed to elongate *in vitro* in the presence of [α^{32}P]-UTP. The radiolabeled RNA product was hybridized to slot-blotted cDNAs encoding *Mig* and α-tubulin, and the resulting blots were quantified by computer analysis using Bio-1D version 6 (Fig. 7). The transcriptional activity of the *Mig* gene was not enhanced by treatment with IFN-γ plus LPS. Accordingly, LPS seemed to have no effect on *Mig* gene transcript levels.

DISCUSSION

During active infection, there is an expression of inflammatory cytokines such that the superimposition of LPS in this milieu can enable the host to mount a more vigorous response to the pathogen [28]. Thus, the synergy between LPS and inflammatory cytokines such as IFN-γ may represent an important regulatory mechanism by which the host is sure of a significant ongoing infection before it activates potent effector responses [4, 6, 10]. The purpose of this study was to examine the effects of LPS on the

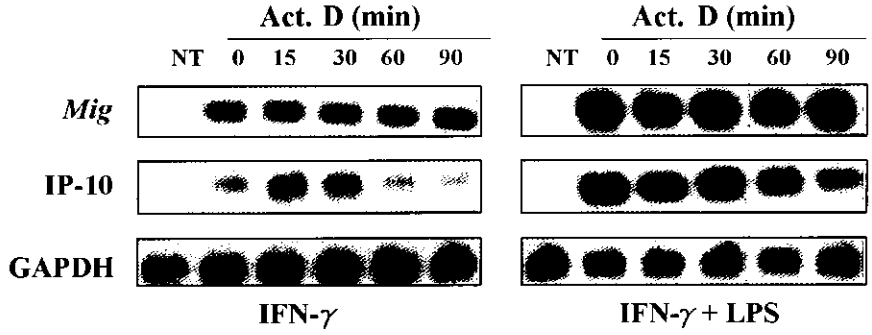

Fig. 6. Effect of LPS on the stability of INF-γ-induced *Mig* mRNA.
TG-elicited macrophages were untreated (NT) or treated with IFN-γ (50 U/ml) in the absence or presence of LPS (100 ng/ml) for 4 h. Actinomycin D (5 μg/ml) was added to all cultures and incubated continuously for the indicated times before an analysis of the *Mig* and GAPDH mRNA levels by Northern hybridization.

expression of the inflammatory chemokine *Mig* in the presence of IFN-γ. *Mig* is unique in that no stimulus other than IFN-γ has been shown to induce its expression in macrophages.

LPS alone induce minimally induced *Mig* mRNA with a species-specific pattern. However, LPS enhanced the IFN-γ-induced steady state mRNA levels of *Mig* in both primary mouse peritoneal macrophages and RAW 264.7 macrophage cell line (data not shown).

To further characterize the mechanism by which LPS and IFN-γ synergize to induce *Mig* gene expression in macrophages, dose-response, time course and *Mig* mRNA stability experiments were performed. The peak synergy between LPS and IFN-γ occurred after 4–8 h of stimulation, however, LPS did not affect the stability of IFN-γ-induced *Mig* mRNA. In an attempt to delineate whether a new protein synthesis was necessary for the synergy, an experiment was perfomed in the presence of the protein synthesis inhibitor, cycloheximide. An evaluation of this experiment revealed that LPS- and IFN-γ-induced *Mig* expression was not blocked by CHX, thereby suggesting that the synergy did not require the synthesis of a secondary mediator. In the mRNA stability experiment, upto 90 min of exposure after Act. D treatment was examined. In this case, LPS did not affect the stabiltiy of IFN-γ-induced *Mig* mRNA.

The selective regulation of chemokine gene expression may result from the differential response of macrophages to various stimuli and to specific cell-type patterns of stimulus sensitivity. IFN-γ and LPS have been shown to enhance chemoattractant cytokine gene expression in mononuclear phagocytes [8, 9, 14, 29, 32]. However, others showed that IFN-γ suppressed LPS-induced chemokine mRNA expression in a cell-type and gene-specific fashion. Ohmori *et al.* [22] demonstrated that the expression of *JE* and *KC* mRNA in macrophages stimulated with LPS was markedly suppressed by IFN-γ in a dose and time dependent fashion. Gasperini *et al.* [30] investigated *Mig* production from neutrophils and compared it with PBMC. In their studies, a considerable accumulation of *Mig* was observed in neutrophils stimulated with IFN-γ plus LPS, yet LPS down-regulated the up-regulatory effect of IFN-γ on *Mig* mRNA in PBMC. Other investigations have determined that TNF-α and IFN-γ synergize to induce *Mig* mRNA expression in a fibroblast [26], and hyaluronan plus IFN-γ also synergize to induce a *Mig* gene in macrophages [11]. Therefore, in the present study, the role of other cytokines in mediating LPS-inducible *Mig* gene expression was examined in mouse peritoneal macrophages. TNF-α did not affect LPS-inducible *Mig* gene expression, yet IFN-β did show a synergistic effect on LPS-inducible *Mig* gene expression. These findings clearly indicate that the mechanisms governing the expression of *Mig* in various cell types are specific and subject to distinct regulatory pathways, however, the physiologic significance of these diverse patterns of *Mig* mRNA expression has not yet been fully understood.

Chemokine gene expression in macrophages appears to be regulated by both transcriptional and post-transcriptional mechanisms [2, 15, 21, 24]. In this study, the LPS-mediated synergistic effect of IFN-γ-induced *Mig* gene expression was not regulated at the transcriptional level. Nuclear run-on studies on macrophages showed that LPS did not induce *Mig* mRNA transcription. IFN-γ alone had a minimal effect on *Mig* transcription compared with unstimulated cells, and cells stimulated with LPS plus IFN-γ had almost the same effect on *Mig* gene transcription as IFN-γ alone.

The LPS-induced transcriptional activation of other chemokine genes has been linked with the presence of NFκB binding motifs in the region of the gene flanking the transcriptional start site [35]. LPS activates the NFκB family of transcription factors [20]. In contrast, IFN-γ employs the JAK-STAT pathway for its signal transduction [5]. Studies by Held *et al.* [9] showed that IFN-γ strongly augments LPS-induced NFκB activation and accerelates the binding of NFκB, and that LPS also enhances the activation of the signal-transducing activator of transcription 1 (STAT-1) by IFN-γ in the synergistic induction of inducible nitric oxide synthetase mRNA and nitric oxide production. Therefore, they suggested novel mechanisms for the synergy between IFN-γ and LPS by which the signal-transducing molecules are cross regulated. Another recent study suggested that the IFN-γ induction of *Mig* was mediated by the transcription factor γRF-1 [36]. γRF-1 has recently been shown to consist of a complex containing STAT-1, and purified γRF-1 does not bind to the STAT-1 site but, rather, is specific to the imperfect palindrome resembling a STAT binding site. These studies have been further supported by data from other investigations [26]. An analysis of the *Mig* promoter reveals that there are three NF-kB binding sites on the 5' promoter downstream from the γRE-1. Thus, LPS may synergize with IFN-γ to induce *Mig* gene expression through interactions between a STAT-1α like protein and/or NFκB for *Mig* gene expression.

In summary, evidence are presented that the IFN-γ-induced *Mig* mRNA expression of macrophages is increased by LPS, yet the synergy is not controlled at the level of the gene transcription or mRNA stability. Since the murine *Mig* promoter contains three conserved kB binding motifs in the 5' regulatory region, which motif is necessary for the transcriptional response to LPS is still unknown. Futhermore, the molecular mechanism(s) by which LPS synergizes the IFN-γ-induced transcription of the *Mig* gene is also currently unknown. Therefore, the pretranscriptional role of the LPS synergistic effect on IFN-γ-induced *Mig* gene expression requires further analysis.

Acknowledgments

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