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Synergistic Effect of Lipopolysaccharide and Interferon-β on the Expression of Chemokine *Mig* mRNA

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Abstract Expression of monokine induced by IFN-γ (*Mig*) mRNA is well-known to strictly depend on Interferon-γ (IFN-γ). Lipopolysaccharide (LPS) alone is weakly effective on *Mig* mRNA expression in mouse peritoneal macrophages. This study was undertaken to investigate the synergistic effect of LPS and IFN-β on chemokine *Mig* gene expression in mouse peritoneal macrophages. Although IFN-β alone was minimally effective, LPS plus IFN-β synergized to produce a high level of *Mig* mRNA. The synergistic effect of LPS and IFN-β (LPS/IFN-β) on *Mig* mRNA expression was strain-specific. The most effective synergistic effect of LPS/IFN-β on the mRNA expression was found in simultaneous stimulation of LPS/IFN-β. This synergy was modulated at the level of the gene transcription and was not dependent on a new protein synthesis. Synergistic effect of LPS/IFN-β also required the activation of NF-κB. Accordingly, these data suggest that LPS/IFN-β synergizes the expression of *Mig* mRNA through a process that depends on a pretranscriptional level and/or coincident *Mig* mRNA transcription.

Key words: Interferon-β, lipopolysaccharide, chemokine *Mig*

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 signals can include lipopolysaccharide (LPS) and secreted proinflammatory cytokines. The inflammatory response to systemic LPS administration is mediated in part by the secretion of chemokines at sites of incipient inflammation. Virtually every cell type has the 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 [10, 11, 20].

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The biologic properties of chemokine *Mig* include chemoattraction of tumor infiltrating lymphocytes and activated T cells, the inhibition of endothelial cell chemotaxis, and the inhibition of growth factor-induced angiogenesis *in vivo* [22, 23]. A study *in vivo* and *in vitro* has indicated that IFN-γ is the only inducer of *Mig* in monocyte/macrophages, fibroblasts, and keratinocytes [3]. IFN-β is a major contributor to the first line of antiviral defense and is used in the clinic as an antiviral and antitumor agent, whereas IFN-γ is well known as a major macrophage activating factor and an important modulator of the immune system. However, IFN-β also has antiproliferative, antibacterial, and immunomodulatory functions [24]. Spontaneous IFN-β production has frequently been observed in cultures of some hemopoietic cells without viral infections. Compared to IFN-γ, there are relatively few studies on the mechanisms of IFN-β as an inducer of cytokines. Hamilton *et al.* [5] demonstrated that IFN-γ and IFN-β independently stimulate the expression of LPS-inducible genes in murine peritoneal macrophages.

The purpose of this study was to examine the effects of IFN-β on the expression of the inflammatory chemokine *Mig* in the presence of LPS in mouse peritoneal macrophages. A marked synergy was found between LPS and IFN-β (LPS/IFN-β) in *Mig* mRNA expression.

Materals 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, agarose, fetal bovine serum (FBS), phenol, guanidine isothiocyanate, cesium chloride, and formamide were purchased from Gibco BRL (Gaithersburg, U.S.A.). The Magna nylon transfer membrane was obtained from Micron Separation Inc. (Westboro, U.S.A.), the high prime kits was obtained from Boehringer

Mannheim (Indianapolis, U.S.A.), and [α-^{32}P]dCTP and [α-^{32}P]dUTP were from Dupont-New England Nuclear (Boston, U.S.A.). Trihydroxymethyl aminomethane (Tris), sodium dodecyl sulfate (SDS), pyrrolidine dithiocarbamate (PDTC), and *Escherichia coli* LPS (O111:B4) were obtained from Sigma Chemical Co. (St. Louis, U.S.A.), and the recombinant mouse IFN-β and ELISA kit for IFN-γ were purchased from R&D Systems (Minneapolis, U.S.A.). The plasmid encoding *Mig* and the *GAPDH* genes were kindly provided by Dr. Hamilton at the Department of Immunology, Lehner Research Institute, Cleveland Clinic Foundation, U.S.A.

Mice

Specific pathogen free inbred C57BL/6, BALB/c, and C_3H/HeJ mice, 8 to 12 weeks of age, were purchased from Hyeuchang Science (Taegu, Korea). The utmost precautions were taken to ensure that the mice remained free from infection by environmental pathogens, thereby ensuring that the degree of spontaneous activation of tissue macrophages would be minimal.

Mouse Peritoneal Macrophages

Thioglycollate (TG)-elicited macrophages were obtained by the method of Lee *et al.* [12]. Briefly, 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 period of time.

Preparation of RNA and Northern Hybridization Analysis

Total cellular RNA was extracted using the guanidine thiocyanate-cesium chloride method [2]. 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 [8]. 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 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 *Mig* and *GAPDH* cDNA inserts. The blots were rinsed with 0.1% SDS-0.2×SSC, washed at 42°C for 30 min and at 65°C for 15 min. The blots were then dried and exposed using XAR-5 X-ray film (Eastman Kodak Co. Rochester, U.S.A.) at −70°C.

Nuclear Transcription Assay

Cultures of 2×10^7 macrophages were treated as indicated in the text, and the nuclei were isolated as described previously [8]. The transcription initiated in intact cells was allowed to be completed 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 [5, 8]. 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 ration in untreated samples was arbitrarily set to unity. The experimental values were presented as the fold induction relative to the untreated samples.

Enzyme-Linked Immnunosorbent Assay (ELISA)

ELISA for IFN-γ were performed according to the manufacturer's instruction using kit from R&D Systems (Minneapolis, U.S.A.). Briefly, 100 μl of standards or patient serum were incubated in anti-human IFN-γ coated well plates at room temperature for 2 h. After 200 μl of prepared biotinylated antibody was added, the plates were incubated at room temperature for 1 h. Two hundred μl of tetramethylbenzidine solution were added, and the plates were developed in the dark state at room temperature for 20 min. The reaction was ceased by adding 50 μl of stop solution. Absorbency of serum was by measured at 450 nm for IFN-γ.

RESULTS

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

After the thioglycollated (TG)-elicited C57BL/6 peritoneal macrophages were stimulated either with LPS (100 ng/ml), IFN-β (500 U/ml), or LPS plus IFN-β (LPS/IFN-β) for 4 h, Northern analysis was performed. Lipopolysaccharide (LPS) alone was very weakly effective on *Mig* mRNA expression in macrophages, and IFN-β alone was also

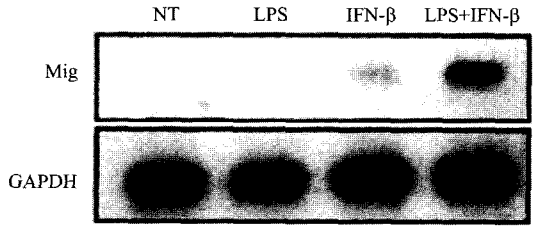

Fig. 1. Synergistic effect of LPS and IFN-β on *Mig* mRNA expression.
Thioglycollate (TG)-elicited C57BL/6 mouse peritoneal macrophages were untreated (NT) or treated with LPS (100 ng/ml) and/or IFN-β (500 U/ml) for 4 h. The mRNA was isolated, and Northern blot analysis was performed. Similar results were obtained in three separate experiments.

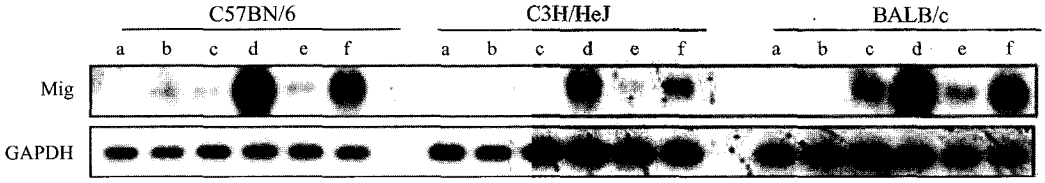

Fig. 2. Mouse strain-specific expression of *Mig* mRNA.
Confluent monolayers of peritoneal macrophages from each strain were either a) untreated or treated with b) LPS (100 ng/ml), c) IFN-γ (50 U/ml), d) LPS+IFN-γ, e) IFN-β (500 U/ml), or f) LPS+IFN-β for 4 h. Total RNA was prepared and the levels of *Mig* were analyzed by Northern blot hybridization.

minimally effective. But, the LPS plus IFN-β (LPS/IFN-β)-induced *Mig* mRNA expression was significantly higher than LPS or IFN-β alone-induced *Mig* mRNA expression (Fig. 1).

Next, we assessed the relative capacity of LPS/IFN-β to induce *Mig* mRNA expression in mouse peritoneal macrophages, using strains of C57BL/6, BALB/c and C3H/HeJ mice macrophageas. C57BL/6 and BALB/c were chosen because they are commonly used in LPS-induced cytokine studies, and C3H/HeJ was used as a LPS-resistant strain. After TG-elicited, C57BL/6, BALB/c, and C3H/HeJ peritoneal macrophages were stimulated with LPS, IFN-β, or LPS/IFN-β for 4 h, and Northern blot analysis was performed. The LPS/IFN-β-induced *Mig* mRNA expressions in C57BL/6 and BALB/c macrophages were higher than in C3H/HeJ macrophages (Fig. 2).

The time dependence for LPS/IFN-β-induced *Mig* mRNA expression was determined in peritoneal macrophages at various times. The maximum synergistic effect occurred within 2 h after simultaneously adding LPS/IFN-β, and then it gradually declined up to 12 h (Fig. 3). Next, it was examined whether the synergistic effect of LPS/IFN-β on *Mig* mRNA expression was dependent on the duration of the macrophages exposure to IFN-β or LPS. Macrophages were treated with LPS (or IFN-β) at either 1 h or 2 h before or simultaneously with the IFN-β (or LPS) additions. *Mig* mRNA levels were measured 4 h after adding them. As shown in Fig. 4, the synergy between LPS and IFN-β in *Mig* mRNA accumulation was observed only in the presence of LPS plus IFN-β during the stimulation period. This result indicates that the synergy with LPS needs a simultaneous stimulation with IFN-β.

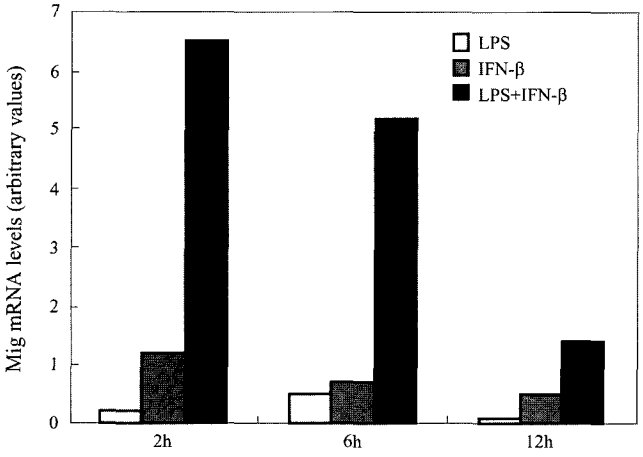

Fig. 3. Time course of LPS/IFN-β-induced *Mig* mRNA expression in C57BL/6 peritoneal macrophages.
Confluent monolayers of TG-elicited C57BL/6 peritoneal macrophages were treated with LPS (100 ng/ml) and/or IFN-β (500 U/ml) for the indicated times. The mRNA levels were determined by Northern hybridization and quantified by a computer analysis using Bio-1D version 6.32. The levels of *Mig* mRNA in each sample were normalized for the levels of *GAPDH* mRNA. Similar results were obtained in two separate experiments.

Mechanisms of Synergistic Effect of LPS/IFN-β on *Mig* mRNA Expression

Although the main producers of IFN-γ are natural killer cells and T lymphocytes, to gain a further insight into the synergistic effect of LPS/IFN-β on *Mig* mRNA expression in macrophages, we examined the possible production of IFN-γ from mouse peritoneal macrophages stimulated with LPS or/and IFN-β by ELISA. Although the production level of IFN-γ from peritoneal macrophage was very low, the IFN-γ level was enhanced by LPS plus IFN-β stimulation (Fig. 5).

Alternations in specific mRNA levels can be caused by modulating the transcriptional activity of the gene. To determine if the synergy of LPS/IFN-β on *Mig* mRNA expression involved an increased transcription, nuclear run-on experiments were performed. Thus, cultures of peritoneal macrophages were treated with LPS for 4 h in the absence or presence of IFN-β, the nuclei were

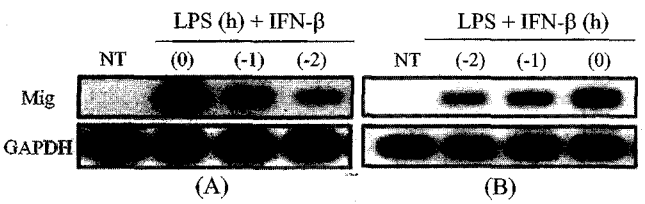

Fig. 4. Pretreatment with LPS or IFN-β is not necessary for the synergistic effect of LPS and IFN-β on *Mig* mRNA expression.
TG-elicited C57BL/6 peritoneal macrophages were pretreated with LPS (100 ng/ml, A) or IFN-β (500 U/ml, B) for 1 or 2 h before stimulation with IFN-β (A) or LPS (B) or treated with LPS and IFN-β simultaneously for 4 h. Total RNA was isolated, and Northern analysis was performed. Similar results were obtained in three separate experiments.

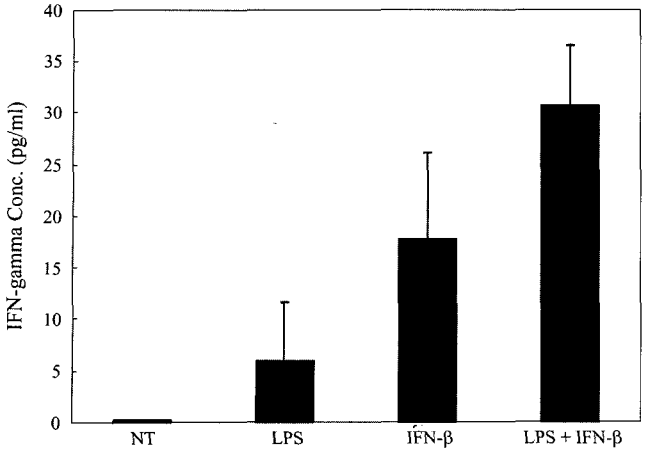

Fig. 5. IFN-γ production from the mouse peritoneal macrophages induced by LPS and IFN-β.
TG-elicited C57BL/6 peritoneal macrophages were untreated (NT) or treated with LPS (100 ng/ml) and/or IFN-β (500 U/ml) for 4 h prior to harvesting supernatants for IFN-γ ELISA. Values represent the mean±SE of three independent experiments conducted in duplicate.

harvested, and the RNA transcripts initiated *in vivo* were allowed to elongate *in vitro* in the presence of [α^{32}P]-dUTP. The radiolabeled RNA product was hybridized to slot-blotted cDNAs encoding *Mig* and α-tubulin. The transcriptional activity of the *Mig* gene was enhanced by treatment with LPS plus IFN-β (Fig. 6), demonstrating a synergistic effect of LPS/IFN-β on *Mig* gene transcript levels.

If the ability of LPS/IFN-β synergy on *Mig* mRNA expression was due to induction of a new protein, the synergistic effect might be blocked in macrophages co-treated with a protein synthesis inhibitor such as cycloheximide (CHX). To test this possibility, macrophages were treated with LPS alone or in combination with IFN-β in the presence or absence of CHX. The LPS/IFN-β-induced synergistic effect was not attenuated in the presence of CHX (Fig. 7). Next, we investigated the role of NF-κB activation in LPS/IFN-β-induced *Mig* expression. Although pyrrolidine dithiocarbamate (PDTC) also inhibits the action of other transcription factor, as a selective inhibitor of cytosolic NF-κB activation, it blocked the synergistic effect

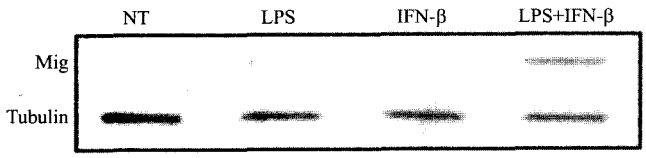

Fig. 6. The synergistic effect of LPS and IFN-β on the expression of *Mig* mRNA is controlled at the level of transcription.
TG-elicited C57BL/6 peritoneal macrophages were untreated (NT) or treated with LPS (100 ng/ml) and/or IFN-β (500 U/ml) for 4 h before the isolation of the nuclei and an analysis 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. Similar results were obtained in two separate experiments.

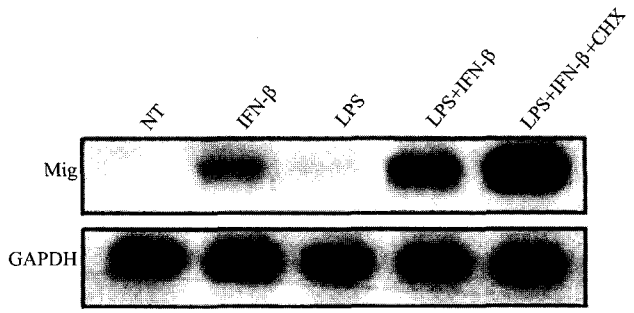

Fig. 7. Synergistic induction of *Mig* mRNA expression induced by LPS and IFN-β is not prevented by protein synthesis inhibitor.
TG-elicited C57BL/6 peritoneal macrophages were untreated (NT) or treated with LPS (100 μg/ml) and/or IFN-β (500 U/ml) in the absence or presence of cycloheximide (CHX) (10 μg/ml) for 4 h. Total mRNA was isolated, and Northern analysis was performed. Similar results were obtained in two separate experiments.

of LPS/IFN-β-induced *Mig* mRNA expression (Fig. 8). This finding suggests that the synergistic effect of LPS/IFN-β-induced *Mig* mRNA expression may be mediated via activation of NF-κB.

DISCUSSION

The selective regulation of *Mig* gene expression may result from the differential response of macrophages to various stimuli and cell type sensitivities to stimulus [4, 7, 21]. *Mig* has been well-known, in that no stimulus other than IFN-γ was shown to induce its expression in macrophages. In our previous study [9], LPS alone minimally induced *Mig* mRNA in a mouse strain-specific pattern, and LPS enhanced the IFN-γ-induced steady state *Mig* mRNA levels in both primary mouse peritoneal macrophages and RAW 264.7 cells. However, TNF-α did not affect LPS-inducible *Mig* gene expression. Gesperini *et al.* [4] investigated *Mig* production in neutrophils and compared this with peripheral

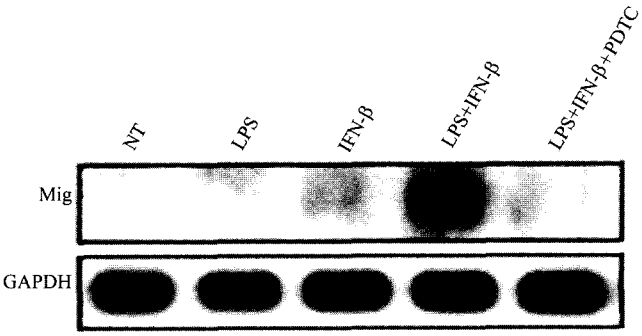

Fig. 8. Involvement of NF-κB activation in synergistic effect of LPS and IFN-β on the expression of *Mig* mRNA expression.
TG-elicited peritoneal macrophages were treated with LPS (100 ng/ml) and/or IFN-β (500 U/ml) in the absence or presence of PDTC (10 mM/ml) for 4 h. Total mRNA was isolated and Northern blot analysis was performed. Similar results were obtained in two separate experiments.

blood mononuclear cells (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 found that TNF-α and IFN-γ synergize to induce *Mig* mRNA expression in fibroblasts [21], and hyaluronan plus IFN-γ also synergistically to induce a *Mig* gene in macrophages [7]. These findings clearly indicate that the mechanisms governing the expression of *Mig* in various cell types are specific and subject to distinct regulatory pathways, but the physiologic significance of these diverse patterns of *Mig* mRNA expression is not yet fully understood.

In the present study, the synergistic effect of LPS/IFN-β in mediating *Mig* gene expression was examined in mouse peritoneal macrophages. Low levels of *Mig* mRNA was detected in macrophages stimulated with IFN-β, and LPS alone was also able to induce *Mig* mRNA. However, LPS/IFN-β enhanced the expression of *Mig* mRNA expression. The maxium synergy between LPS and IFN-β occurs within 2 h of simultaneous stimulation. Furthermore, the production of IFN-γ from peritoneal macrophages was detected in simultaneous stimulation of LPS/IFN-β. Macrophages produce interferons (IFNs) *in vitro* when treated with LPS and polysaccharides, which are ineffective in inducing IFN synthesis in other cells [6]. Both LPS and IFN-β induce transcription of mRNAs for various cytokines. These cytokines, including IFN-γ, may synergistically stimulate *Mig* expression by autocrine or paracrine mechanisms. However, we were unable to confirm the autocrine effect of IFN-γ in the synergistic effect of LPS/IFN-β on *Mig* mRNA expression, because of the low level of IFN-γ production.

Chemokine gene expression in macrophages appears to be regulated by both transcriptional and post-transcriptional mechanisms [10, 16–19]. In this study, the synergistic effect of LPS/IFN-β on *Mig* gene expression was found to be regulated at the transcriptional level. Nuclear run-on studies on macrophages showed that LPS or IFN-β alone minimally induced *Mig* transcription, but cells stimulated with both LPS plus IFN-β had marked enhancement of *Mig* gene transcription. A study with hyaluronan (HA) and IFN-γ [7] also showed that the synergistic induction of *Mig* by HA and IFN-γ was due to the up-regulation of *Mig* mRNA transcription. The synergistic effect of LPS/IFN-β on *Mig* mRNA expression was not blocked by protein synthesis inhibitor CHX, thereby suggesting that the synergy does not require the synthesis of a secondary mediator.

PDTC, a selective inhibitor of cytosolic NF-κB activation, blocked the synergistic effect of LPS/IFN-β on *Mig* mRNA expression. IFNs generate cytoplasmic signals by the JAK-STAT pathway [13], and IFN-activated STATs translocate into the nucleus to induce gene transcription. Yang *et al.* [26] reported that IFNs also activated another transcription factor, NF-κB. LPS activates the NF-κB family for its signal transduction [14]. LPS promotes dissociation of inactive NF-κB/IκB complexes, allowing active NF-κB to enter the nucleus and bind to *cis*-acting κB sites in the promoters and enhancers of genes. The LPS-induced transcriptional activation of chemokine genes has been linked with the presence of NF-κB binding motifs in the region of the gene flanking the transcriptional start site [25]. An analysis of the *Mig* promoter reveals that there are three NF-κB binding sites on the 5' promoter downstream from the IFN-γ responsive element-1 (γRE-1). Ohmori *et al.* [17, 21] showed that TNF-α and IFN-γ synergistically induce the expression of *Mig* and *IP-10* mRNA in fibroblasts, and that the synergy between IFN-γ and TNF-α in transcriptional activation is mediated by a cooperation between STAT-1 and NF-κB. Thus, LPS may synergize with IFN-β to induce *Mig* gene expression through interactions between an NF-κB and/or a STAT protein for *Mig* gene expression. Mahalingam *et al.* [14] investigated the transcription of the *Mig* in IFN-γ deficient mice infected with vaccinia virus, and found that IFN-α/β were able to induce *Mig* in response to a viral infection *in vivo*, and that *Mig* mRNA expression was mediated through the interaction between γRE-1 and IFN-α/β-induced STAT-1 complex, referred to as IFN-γ response factor 2 (γRF-2).

Except for Mahalingam *et al.* [14], studies for the mechanisms of IFN-β-inducible *Mig* gene expression at the pretranscriptional level have not been carried out. The molecular mechanisms by which LPS and IFN-β synergize in transcription of *Mig* mRNA are currently unknown, and the pretranscriptional role of the synergistic effect of LPS and IFN-β on *Mig* gene expression requires further analysis.

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