

# Effect of Interleukin-10 on Lipopolysaccharide/Interferon- $\gamma$ -Induced Chemokine *Mig* Gene Expression

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## ABSTRACT

Interferon- $\gamma$  (IFN- $\gamma$ ) is well known as a potent inducer in monokine induced by IFN- $\gamma$  (*Mig*) mRNA expression. Although lipopolysaccharide (LPS) alone is weakly effective on *Mig* mRNA expression, the stimulation of LPS and IFN- $\gamma$  (LPS/IFN- $\gamma$  simultaneously) has been shown to synergize to produce a high level of *Mig* mRNA in mouse peritoneal macrophages. In this study, interleukin-10 (IL-10) was found to suppress the LPS/IFN- $\gamma$ -induced *Mig* mRNA expression in cell type- and mouse strain-specific fashion, but IFN- $\gamma$  alone-induced *Mig* mRNA was unaffected by IL-10 under identical experimental conditions. The IL-10-mediated suppression of LPS/IFN- $\gamma$ -stimulated *Mig* mRNA expression was dependent on the concentration of IL-10, and was prevented when the agent was added 2 hours after LPS/IFN- $\gamma$  treatment. The suppressive action of IL-10 was dependent on a protein synthesis. However, IL-10 did not reduce the stability of LPS/IFN- $\gamma$ -induced *Mig* mRNA. These data may have important implications for a previously unrecognized role for IL-10 as a regulator of synergistic effect of LPS on the IFN- $\gamma$ -induced expression of the *Mig* gene in macrophages. (**Immune Network 2002;2(1):12-18**)

**Key Words:** IL-10, *Mig*, LPS, IFN- $\gamma$ , gene expression

## Introduction

The biologic properties of monokine induced by Interferon- $\gamma$  (*Mig*) include the chemoattraction of activated T cells, the inhibition of endothelial cell chemotaxis, and the inhibition of growth factor-induced angiogenesis in vivo (1,2). Studies in vivo and in vitro have indicated that IFN- $\gamma$  is the only inducer of *Mig* in monocyte/macrophages, fibroblast and keratinocytes (3). IFN- $\gamma$  is generally considered to be a potent macrophage activator that interacts synergistically with lipopolysaccharide (LPS) to induce inflammatory mediators like tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and inducible nitrogen oxide synthetase (iNOS), in addition to enhancing LPS-induced lethality (4-6). In a previous study (7), the role of LPS on IFN- $\gamma$ -inducible *Mig* expression in murine macrophages was examined, and a marked synergy was

found between LPS and IFN- $\gamma$  in terms of inducing *Mig* mRNA expression.

Interleukin-10 (IL-10) has an important regulatory role as it limits the duration and extent of acute inflammatory response. It has been shown to have profound effects on monocytes and macrophages by down-regulating the expressions of a number of cytokine genes (8-13). Although IL-10 is a well documented negative regulator of LPS-induced macrophage gene expression, the mechanisms have not been clearly defined. Multiple studies into the effects of IL-10 on cytokine gene expression have demonstrated diverse mechanisms, including the modulation of transcription, mRNA stability, and mRNA translation (11,14-17).

In this study, we investigated the inhibitory mechanism of the action of IL-10 on LPS plus IFN- $\gamma$  (LPS/IFN- $\gamma$ )-induced *Mig* gene expression in thio-glycollate (TG)-elicited mouse peritoneal macrophages. Our results show that IL-10 inhibits LPS/IFN- $\gamma$ -induced *Mig* mRNA expression in a cell type, and a mouse strain specific fashion, and that this inhibitory action of IL-10 does not reduce the stability of LPS/IFN- $\gamma$ -induced *Mig* mRNA.

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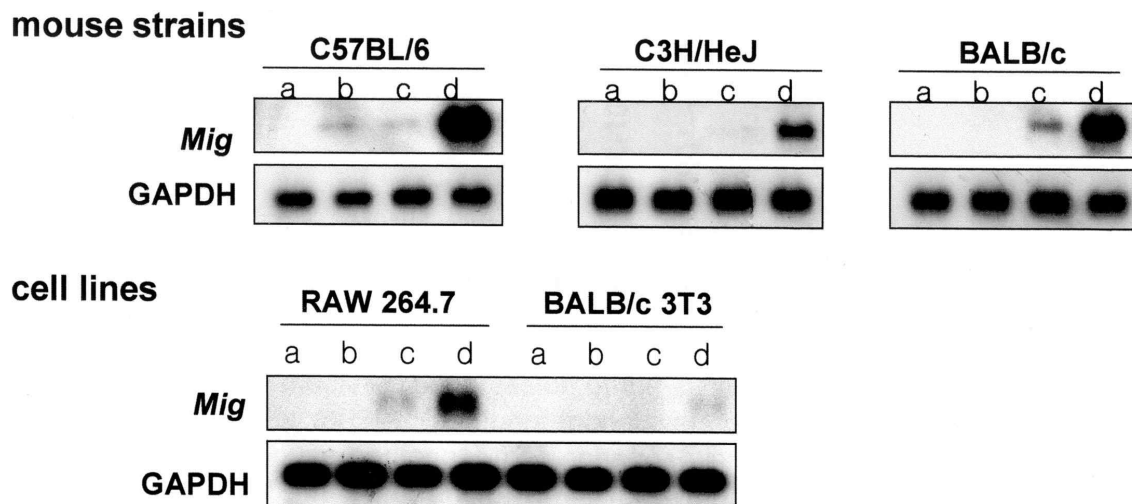
## Materials and Methods

**Reagents.** Brewer's thioglycollate broth was purchased from Difco Laboratories (Detroit, USA). RPMI 1640, Dulbecco's phosphate-buffered saline (PBS), Hank's balanced salt solution (HBSS) and L-glutamin, trypsin, and agarose were all purchased from Life Technologies Inc. (Gaithersburg, USA). The fetal bovine serum (FBS), phenol, guanidine isothiocyanate, cesium chloride, and formamide were obtained from Gibco BRL (Gaithersburg, USA), the Magna nylon transfer membrane from Micron Separation Inc. (Westboro, USA), the high prime kits from Boehringer Mannheim (Indianapolis, USA), [ $\alpha$ - $^{32}$ P]dCTP from Dupont-New England Nuclear (Boston, USA), trihydroxymethyl aminomethane (Tris), sodium dodecyl sulfate (SDS), and *Escherichia coli* LPS (O111:B4) from Sigma Chemical Co. (St. Louis), and the recombinant mouse IFN- $\gamma$  ( $5 \times 10^5$  units/mg) and IL-10 were purchased from R&D Systems (Minneapolis, USA). The plasmid encoding *Mig*, and the GAPDH genes were kindly provided by Dr. Hamilton, at the Department of Immunology, Lechner Research Institute, Cleveland Clinic Foundation, USA.

**Mice.** Specific pathogen free, inbred C57BL/6, BALB/c, and C3H/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 Tannenbaum's method (18). 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<sub>2</sub>, and then washed three times with HBSS to remove any non-adhering cells. The macrophages were cultured overnight in a complete medium at 37°C in 5% CO<sub>2</sub>, 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 (19). An equal amount of RNA (10  $\mu$ g/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 (16). The blots were prehybridized for 6 h at 42°C in 50% formamide, 1% SDS, 5 $\times$  saline sodium citrate, 1 $\times$  Denhardt's solution (0.02% bovine serum albumin and 0.02% polyvinylpyrrolidone), 0.25 mg/mL denatured herring testis DNA, and a 50 mM of sodium phosphate buffer, pH 6.5. Hybridization was carried out at 42°C for 18 h with  $1 \times 10^7$  cpm of denatured plasmid DNA containing *Mig* and GAPDH cDNA inserts. The blots were rinsed with a solution of 0.1% SDS-0.2 X SSC, washed at 42°C for 1 h and at 65°C for 15 min. The blots were then dried and exposed using XAR-5 X-ray film (Eastman Kodak Co. Rochester USA) at -70°C.



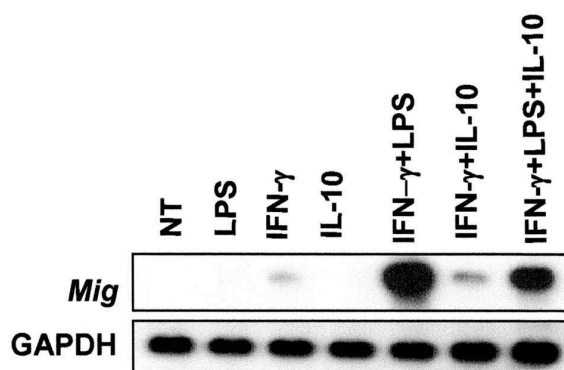
**Figure 1.** Mouse strain- and cell type-specific expression of LPS and IFN- $\gamma$ -induced *Mig* mRNA. Confluent monolayers of peritoneal macrophages from each species, RAW 264.7 macrophages, and BALB/c 3T3 fibroblasts were either (a) untreated or treated with (b) LPS (100 ng/mL), (c) IFN- $\gamma$  (50 U/mL), or (d) LPS plus IFN- $\gamma$  for 4 h. Total RNA was prepared and the levels of *Mig* mRNA were analyzed by Northern blot hybridization.

## Results

*Cell type- and mouse strain-specific expression of chemokine Mig mRNA.* Initially, it was planned to assess the relative capacity of LPS/IFN- $\gamma$  to induce *Mig* mRNA expression in mouse peritoneal macrophages using different strains and two cell types (Fig. 1). After the TG-elicited C57BL/6, BALB/c and C3H/HeJ peritoneal macrophages were stimulated with LPS (100 ng/mL), IFN- $\gamma$  (50 U/mL) or LPS plus IFN- $\gamma$  (LPS/IFN- $\gamma$ ) for 4 h, a Northern analysis was performed. The LPS/IFN- $\gamma$ -induced *Mig* mRNA expressions in C57BL/6 and BALB/c macrophages were significantly higher than in C3H/HeJ macrophages. However, LPS alone-induced *Mig* mRNA expression in BALB/c macrophages was undetectable. RAW 264.7 cells responded more effectively to LPS/IFN- $\gamma$  stimulation than BALB/c 3T3 cells.

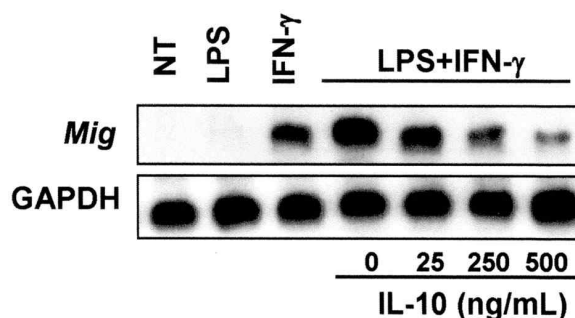
*IL-10 acts to suppress LPS/IFN- $\gamma$ -induced *Mig* mRNA expression in primary mouse macrophages.* To assess the effect of IL-10 on LPS and IFN- $\gamma$ -induced *Mig* mRNA expression, TG-elicited C57BL/6 peritoneal macrophages were treated with LPS, IFN- $\gamma$ , and LPS/IFN- $\gamma$  in the presence or absence of IL-10 for 4 h (Fig. 2). IL-10 was found to suppress LPS/IFN- $\gamma$ -induced *Mig* mRNA expression. However, IL-10 did not inhibit IFN- $\gamma$  induced *Mig* mRNA expression.

In order to determine whether the inhibition of *Mig* expression by IL-10 was dependent on the IL-10 level, we investigated the effect of various IL-10 concentrations by adding IL-10 at 25 ng/mL, 250 ng/mL, and 500 ng/mL to C57BL/6 peritoneal macrophages at the same time as LPS and IFN- $\gamma$ . Ac-

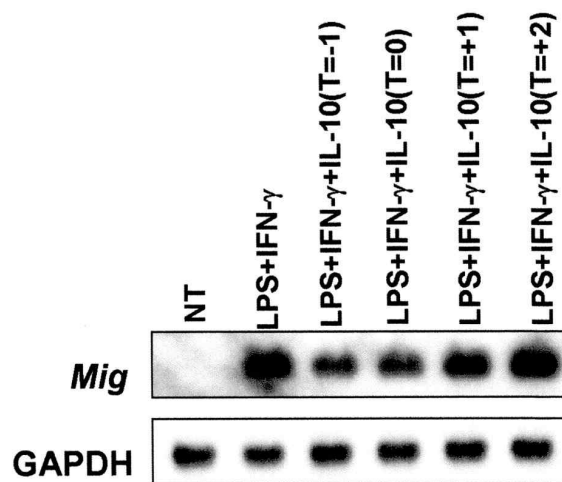


**Figure 2.** IL-10-mediated suppression of the synergistic effect of LPS on IFN- $\gamma$ -induced *Mig* mRNA. Confluent monolayers of Thioglycollate (TG)-elicited C57BL/6 mouse peritoneal macrophages were untreated (NT) or treated with LPS (100 ng/mL) and/or IFN- $\gamma$  (50 U/mL), IL-10 (50 ng/mL) for 4 h. Total RNA was then prepared and *Mig* mRNA levels were analyzed by Northern blot hybridization. The data shown are representative of three experiments.

cordingly, we found that the suppression of LPS/IFN- $\gamma$ -induced *Mig* mRNA expression was dependent upon the dose of IL-10. The higher concentration of IL-10 showed a greater suppressive effect on *Mig* mRNA expression (Fig. 3). In order to determine whether the inhibition of *Mig* gene expression by IL-10 was dependent on the duration of macrophage exposure to IL-10, macrophages were treated with IL-10 either 1 h before, or 1 h or 2 h after, or at the same time as the LPS and IFN- $\gamma$  additions. *Mig* mRNA levels were measured 4 h after adding the LPS/IFN- $\gamma$  (Fig. 4). The suppressive ac-



**Figure 3.** Dose-dependence of IL-10-mediated suppression of LPS/IFN- $\gamma$ -induced *Mig* mRNA expression in macrophages. TG-elicited C57BL/6 mouse macrophages were treated with LPS (100 ng/mL) or IFN- $\gamma$  (50 u/mL) in the presence or in the absence of increasing concentrations of IL-10, as indicated, 4 h before *Mig* mRNA levels were determined, as described in Materials and Methods.

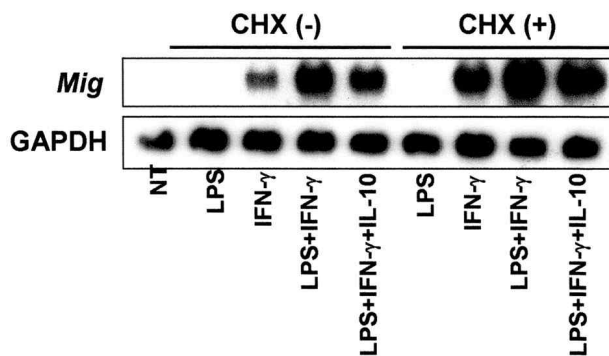


**Figure 4.** Effect of IL-10 treatment time on LPS/IFN- $\gamma$ -induced *Mig* mRNA expression. TG-elicited C57BL/6 mouse peritoneal macrophages were untreated (NT) or treated with IFN- $\gamma$  (50 U/mL) and LPS (100 ng/mL) for a period of 4 h. Samples were exposed to IL-10 (50 ng/mL) 1 h before (T=-1), simultaneously (T=0), 1 h after (T=+1), or 2 h after (T=+2) the addition of LPS (100 ng/mL) and IFN- $\gamma$  (50 U/mL). Total RNA was prepared and analyzed for *Mig* and GAPDH mRNA levels as described in Materials and Methods.

tion of IL-10 was observed when it was added 1 h before and at the same time as LPS/IFN- $\gamma$ . No effect was observed when it was added IL-10 2 h after the LPS/IFN- $\gamma$  addition. These results indicate that the suppression of the expression of *Mig* mRNA is dependent on the time of exposure to IL-10.

**Mechanisms of IL-10-mediated suppression of LPS/IFN- $\gamma$ -induced *Mig* mRNA expression.** If the suppressive action of IL-10 on LPS/IFN- $\gamma$ -induced *Mig* mRNA expression is due to the induction of a new protein, this suppression may be blocked in macrophages co-treated with a protein synthesis inhibitor, such as cycloheximide (CHX). To test this possibility, macrophages were treated with only IL-10 or with IL-10 in combination with LPS, IFN- $\gamma$ , or LPS/IFN- $\gamma$  in the presence or in the absence of CHX. It was found that when protein synthesis was inhibited with CHX, the suppressive effect of IL-10 was fully abrogated (Fig. 5).

Alterations in specific mRNA levels can be caused



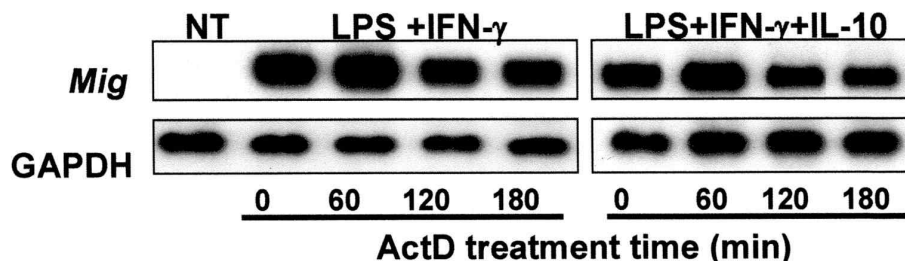
**Figure 5.** Effect of cycloheximide on IL-10-mediated suppression of LPS/IFN- $\gamma$ -induced *Mig* mRNA expression. TG-elicited C57BL/6 mouse peritoneal macrophages were untreated (NT) or treated with LPS (100 ng/mL) or IFN- $\gamma$  (50 U/mL), or LPS and IFN- $\gamma$  simultaneously in the presence or absence of cycloheximide (CHX, 10  $\mu$ g/mL) for 4 h before analysis of *Mig* mRNA levels as described in Materials and Methods.

by the modulation of the gene transcriptional activity and/or by mRNA degradation. To ascertain which mechanism(s) was involved, the half-life of LPS/IFN- $\gamma$ -induced *Mig* mRNA was measured in the presence or in the absence of IL-10. Macrophages were then stimulated with LPS plus IFN- $\gamma$  simultaneously in the presence or absence of IL-10 for 4 h before treatment with actinomycin D, which prevents any further transcription. After an additional incubation of up to 180 min, the specific mRNA levels were assessed by Northern blot hybridization (Fig. 6). The steady state levels of *Mig* mRNA were similar in the IL-10-treated cells and those treated with only LPS/IFN- $\gamma$ . This result indicates that IL-10 does not affect the stability of LPS/IFN- $\gamma$ -induced *Mig* mRNA.

## Discussion

IFN- $\gamma$  and LPS have been shown to enhance chemoattractant cytokine gene expression in mononuclear phagocytes (4-7,20,21). The selective regulation of *Mig* gene expression may result from the differential response of macrophages to various stimuli and to cell type stimulus sensitivities (22-24). However, the physiologic significance of these diverse patterns of *Mig* mRNA expression is not well understood.

The purpose of this study was to identify the IL-10 mechanisms that regulate chemokine *Mig* gene expression by LPS/IFN- $\gamma$ . IL-10 suppressed LPS/IFN- $\gamma$ -induced *Mig* mRNA expression in a cell type- and mouse strain-specific fashion, but IFN- $\gamma$  alone-induced *Mig* mRNA was not affected by IL-10. The LPS-induced transcriptional activation of chemokine genes has been previously linked with the presence of NF- $\kappa$ B binding motifs in the region of the gene flanking the transcriptional start site (25,26), and an analysis of the *Mig* promoter reveals that there are three NF- $\kappa$ B binding sites on the 5' promoter downstream of  $\gamma$ RE-1 (24). In contrast, a recent study suggested that the IFN- $\gamma$  induction of *Mig* is me-



**Figure 6.** IL-10 does not reduce the stability of LPS/IFN- $\gamma$ -induced *Mig* mRNA. TG-elicited C57BL/6 mouse peritoneal macrophages were untreated (NT) or treated with LPS (100 ng/mL) and IFN- $\gamma$  (50 U/mL) simultaneously in the presence or absence of IL-10 (50 ng/mL) for 4 h. Actinomycin D (ActD, 5  $\mu$ g/mL) was added to all cultures except NT and the incubation continued for the indicated times before analysis of *Mig* and GAPDH mRNA levels by Northern blot hybridization.

diated by the transcription factor  $\gamma$ RF-1 (27). Indeed, IL-10 has been reported to reduce or inhibit LPS-induced activation of the transcription factor NF- $\kappa$ B (28,29), which is likely to be a contributor to the control of *Mig* gene expression in mouse peritoneal macrophages.

The mechanisms of the suppressive effects of IL-10 are incompletely understood. Many studies have demonstrated that IL-10 decreases the level of gene transcription, and the stability of the mRNAs, and/or reduces their translation. Several studies have demonstrated that the inhibitory action of IL-10 is predominantly at the level of decreased mRNA stability (11, 30,31). In this study, however, IL-10 did not produce a decrease in the stability of LPS/IFN- $\gamma$ -induced *Mig* mRNA. Repeated AU-rich sequence elements (AREs) in the 3'-untranslated region (3'-UTR) of several mRNAs have been shown to be responsible for a short mRNA half-life (32-34). Moreover, mRNAs encoding TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , and GM-CSF, and the chemokine genes KC, MIP-1 $\alpha$ , MIP-1 $\beta$ , and IL-8 have all been reported to be destabilized in IL-10-treated cells (8,10,11,16,35,36). A common feature of all of these mRNAs is the presence of multiple clusters of AU-rich sequences in the 3'UTR of their mRNAs. Thus, the IL-10-mediated inhibition of various cytokine gene expressions can be mediated by an AREs in the 3'UTR of sensitive genes. However, *Mig* mRNA lacks AREs in the 3'UTR.

Some reports have also identified gene transcription as a target for the anti-inflammatory action of IL-10 (13,37,38). However, transcription does not appear to be the predominant mode of IL-10 suppression of the *Mig* gene induced by LPS/IFN- $\gamma$  in this study (data not shown). In a previous study upon the mouse KC gene (11), IL-10 was found not to inhibit KC gene transcription, however, Horton et al (23) showed that IL-10 appeared to inhibit hyaluronan-induced chemokine mRNAs expression, by altering both mRNA stability and gene transcription. These findings indicate that the mechanisms governing the expression of *Mig* due to various stimulators and in different cell types are specific and subject to distinct regulatory pathways.

The mechanisms of the inhibitory action of IL-10 appear to be diverse and depend on the gene of interest, the nature of the stimulus, and the cell type. In this study, the suppressive effect of IL-10 on the expression of LPS/IFN- $\gamma$  induced *Mig* mRNA does not act upon the stability of *Mig* mRNA and requires new protein synthesis. The inhibitory actions of IL-10 in regulating *Mig* gene expression may have an important role in determining the physiologic response induced by LPS/IFN- $\gamma$ . Further work is warranted to determine which mechanisms are linked with the

inhibitory action of IL-10 on LPS/IFN- $\gamma$ -induced *Mig* mRNA expression.

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