Brief Definitive Report

Interferon γ Upregulates Its Own Gene Expression in Mouse Peritoneal Macrophages

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Summary

Interferon γ (IFN-γ) exerts a variety of immunoregulatory effects on several cell targets. It is generally assumed that IFN-γ is specifically produced by T and large granular lymphocytes. In this study, we show that IFN-γ is constitutively expressed in resting mouse peritoneal macrophages (PM). Treatment of PM with cycloheximide results in a significant accumulation of IFN-γ mRNA, suggesting that a short-lived IFN-γ mRNA accumulates when protein synthesis is inhibited. Moreover, treatment of PM with IFN-γ also results in a clear-cut accumulation of this mRNA. This effect is not observed in murine lymphocytes from mesenteric lymph nodes (which instead produce IFN-γ after phytohemagglutinin treatment) and in mouse cell lines. The treatment of PM with IFN-γ also results in secretion of IFN-γ after 24–48 h. The upregulation of IFN-γ expression is also found in PM from anti-asialo GM_1–treated nude mice. We suggest that the ability of PM to produce this IFN-γ is indicative of an autocrine mechanism. The macrophage IFN-γ may play a role in the regulation of cell differentiation and immune response.

IFN-γ displays potent immunomodulatory effects on a variety of immune cells in vitro and in vivo (1). In macrophages, IFN-γ is involved in cell activation (2) as well as in the induction of MHC class II antigens (3, 4). Moreover, IFN-γ can modulate the proliferation and function of T lymphocytes (5, 6) and enhance the cytolytic activity of NK cells (7, 8).

The expression of IFN-γ is generally considered to be restricted to T cells and LGL (9). There are a limited number of reports on the expression of IFN-γ in other cell types (10–13), but the consistency and biological relevance of these observations are still unclear (9). The production of IFN-γ is generally considered to be strictly controlled, and significant amounts of this cytokine are produced only after specific stimulation (9) or in the course of certain pathologic conditions (12).

We have previously demonstrated that murine peritoneal macrophages (PM) constitutively transcribe low levels of IFN-β mRNA and secrete active IFN-β, which is responsible for their antiviral state (14, 15). In these cells, IFN-β mRNA is rapidly degraded, but its accumulation is markedly increased by treatment with LPS and IFN-γ. This type of response appears to be characteristic of macrophages, since it does not occur in other cell types (15).

In this study, we report that IFN-γ mRNA is constitutively expressed in PM and its accumulation is markedly increased after treatment with cycloheximide (CHX). Moreover, treatment of PM with IFN-γ results in overexpression of its gene and in IFN-γ secretion. In contrast, IFN-γ does not upregulate its own expression in mouse lymphocytes and in other cell types. These results suggest that PM exhibit specific autocrine mechanisms for the regulation of the IFN-γ gene. These mechanisms may play important biological roles in vivo, in the regulation of cell differentiation and immune response.

Materials and Methods

Mice. Male C3H/HeN and Swiss *nu/nu* mice (5–8-wk-old) were obtained from Charles River, Italia S.p.A. (Milan, Italy). Mice were kept under either pathogen-free (C3H/HeN mice) or germ-free (nude mice) conditions and used within 1 wk.

Peritoneal Macrophages and Lymphocyte Cultures. PM were harvested by washing the peritoneal cavity with RPMI 1640 medium containing 10% FCS and seeded in plastic dishes. After 1 h, nonadherent cells were removed by three washes with medium. Experiments were undertaken when the cells were firmly adherent to the culture wells after vigorous washing. More than 95% of the cells stained for nonspecific esterase and were positive in immunofluorescence studies with a rat mAb (F4/80) specific for mouse macrophages, as previously described (16). Freshly isolated lymphocytes were obtained by sieving mesenteric lymph nodes into RPMI 1640 medium supplemented with 10% FCS and seeded at a density of 4×10^6 cells/ml.

Reagents. Murine recombinant IFN-γ was a gift of Dr. G. Adolf (Boehringer, Vienna, Austria). RPMI 1640 medium (M. A. Bioproducts, Walkersville, MD) was supplemented with penicillin (100

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U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 10% heat-inactivated FCS. All tissue culture reagents were purchased as endotoxin-free lots, as assessed by the Limulus amebocyte assay. LPS from *Escherichia coli* (serotype 026:B6) and CHX was purchased from Sigma Chemical Co. (St. Louis, MO). PHA was obtained from Murex Diagnostics Ltd. (Dartford, England). Anti-asialo GM_1 rabbit antibody (17) was purchased from Wako (Neuss, FRG). Spleen cells taken from Swiss *nu/nu* mice 24 h after injection of 50 μg of antibody to asialo GM_1 showed no significant NK cell cytotoxicity for mouse YAC cells in vitro.

RNA-PCR of IFN-γ mRNA. Total cellular RNA, prepared by the method of Chirgwin et al. (18), was reverse transcribed in a 20-μl reaction containing 0.5 μg of total RNA, 1 μg oligo-dt (12–18 mer; Pharmacia, Piscataway, NJ), 20 U/μl Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD), 0.5 mM each of dATP, dCTP, dGTP, and dTTP, 10 mM DTT, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$. After incubation at 37°C for 1 h, 3 μl of the cDNA product was amplified in a 20-μl reaction containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 5 ng/μl each of the gene-specific upstream and downstream primers, and 0.025 U/μl of Taq polymerase by using a DNA thermal cycler (Perkin Elmer Cetus Corp., Norwalk, CT).

The cycler program consisted of an initial denaturation of 3 min at 95°C followed by 30 repeated cycles of denaturation for 40 s at 90°C, primer annealing for 40 s at 62°C, and extension for 60 s at 72°C. A negative control lacking template RNA or RT was included in each experiment. The PCR products were run on an 2.5% agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled IFN-γ, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA probes.

The sequences for the primers used are as follows: GAPDH sense primer CCATGGAGAAGGCTGGGG, antisense primer CAAAGTTGTCATGGATGACC, and probe CTAAGCAGTTGGTGGTGCA; IFN-γ sense primer AACGCTACACACTGCATCTTGG, antisense primer GACTTCAAAGAGTCTGAGG, and probe GGAGGAACTGGCAAAAGGA. The IFN-γ probe recognizes a 19-bp sequence internal to the 237 bp IFN-γ amplification product. These sequence data are available from GenBank under accession number M28621.

In Situ Hybridization. Firmly adherent PM were washed with cold PBS and detached by using a rubber scraper. 5×10^4 cells were plated onto acetylated glass microscope slides by cytocentrifuge and processed for in situ hybridization as previously described (19). Samples were hybridized for 16 h at 50°C with 80 pg/μl of an antisense IFN-γ RNA probe synthetized in vitro using T7 RNA polymerase (Promega Corp., Madison, WI), 2 μg of linearized IFN-γ plasmid DNA, and 1 mCi of lyophilized α-[^{35}S]UTP (1,000 Ci/mmol, Amersham Corp., Arlington Heights, IL) as described elsewhere (20). The IFN-γ probe was derived from a 330-bp HindIII-ClaI fragment of the first exon of the mouse IFN-γ gene (19). Slides were examined in a Dialux 20 microscope as previously described (19).

IFN-γ Assay. The IFN-γ released in the culture medium was quantitated by ELISA (INTERTEST-gamma®, Genzyme Corp., Cambridge, MA).

Results

Expression of IFN-γ mRNA in PM. Upregulation by IFN-γ. We have recently demonstrated that treatment of PM with CHX increases the expression of IFN-β mRNA as a consequence of mRNA stabilization (21). In the course of these experiments, we noticed that low levels of IFN-γ gene transcription could be detected in unstimulated PM after a longer exposure of the autoradiographs. As shown in Fig. 1, a 3-h treatment of these cells with CHX resulted in marked accumulation of IFN-γ mRNA. These data indicated that the IFN-γ gene was constitutively expressed in resting PM, but that its mRNA was rapidly degraded under normal conditions.

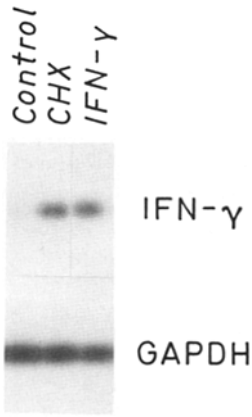

Figure 1. RNA-PCR analysis of IFN-γ gene expression in PM treated with CHX or IFN-γ. 3×10^6 peritoneal cells were seeded as described in Materials and Methods. Adherent cells were treated with IFN-γ (100 IU/ml) or CHX (50 μg/ml) and total RNA was extracted after 3 h of incubation at 37°C. 0.5 μg of total RNA was used for the RNA-PCR as described in Materials and Methods.

The addition of IFN-γ to PM resulted in a significant accumulation of its mRNA (Fig. 1). As little as 10 IU/ml of IFN-γ was sufficient to induce a clearly detectable accumulation of IFN-γ mRNA (data not shown). Similar results were also observed after IFN-γ treatment of PM from LPS-hyporesponsive C3H/HeJ mice (data not shown).

In situ hybridization experiments were then carried out to detect the expression of IFN-γ mRNA in individual cells from IFN-γ–treated PM. Fig. 2 illustrates the results of a representative experiment with PM from C3H/HeN mice. IFN-γ mRNA–producing cells were found in IFN-γ–treated PM, whereas no positive cells could be detected in unstimulated PM (Fig. 2). The specificity of the labeled cells was checked in concomitant experiments in which the in situ hybridization was performed in the presence of a 200-fold excess of the unlabeled IFN-γ probe. Under these conditions, no IFN-γ mRNA positive cells were detected in IFN-γ–treated PM (data not shown).

Secretion of IFN-γ by IFN-γ–treated PM. To assess whether the IFN-γ mRNA that accumulated in IFN-γ–treated PM was translated and the corresponding protein secreted, we measured the kinetics of the IFN-γ release in the culture medium. In a typical experiment, PM were treated with IFN-γ for 3 h, extensively washed, and then incubated in fresh medium (Table 1, Exp. 1). Culture supernatants were harvested at 6, 24, and 48 h and IFN-γ was measured by ELISA. No IFN-γ was found at 6 h, whereas considerable amounts were detected at 24 and 48 h. 10 IU/ml of IFN-γ was sufficient to induce some IFN-γ secretion, even though maximal production was obtained with 100 IU/ml of IFN-γ (Table 1, Exp. 2). The addition of 1 IU/ml of IFN-γ to PM did not result in IFN-γ secretion.

It is noteworthy that when PM were treated with 10 IU/ml of IFN-γ and 3 h later with LPS (0.1–10 μg/ml), there was a synergistic increase in the production of IFN-γ in the cul-

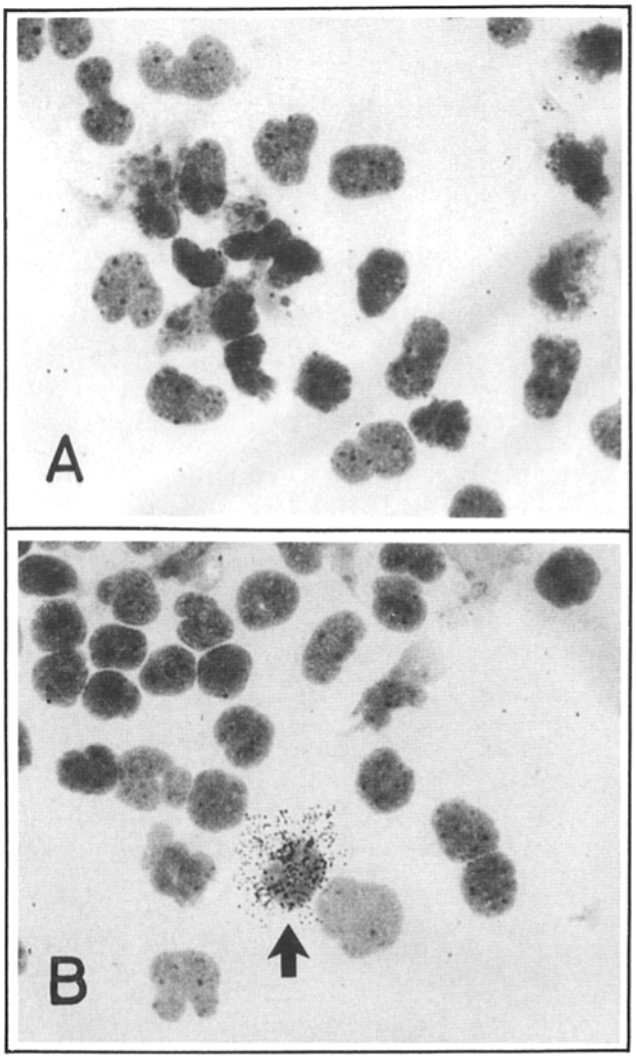

Figure 2. Detection by in situ hybridization of IFN-γ mRNA in individual IFN-γ–treated PM. PM from C3H/HeN mice were prepared as described in Materials and Methods. Firmly adherent PM were incubated with or without IFN-γ (100 IU/ml) for 3 h at 37°C. Cells were then processed for in situ hybridization as described in Materials and Methods. (*A*) Untreated PM. (*B*) IFN-γ–treated PM. An example of a IFN-γ mRNA–expressing cell is shown. ×800.

ture medium (Fig. 3), as compared with single treatments. Under these conditions, no IFN-γ was secreted by PM treated with LPS alone (Fig. 3). Further experiments were performed to determine the minimal LPS concentration required for inducing an increased secretion of IFN-γ in PM pretreated with 10 IU/ml of IFN-γ. As low as 1 ng/ml of LPS was sufficient to induce a twofold increase in IFN-γ secretion as compared with PM treated with IFN-γ alone (data not shown).

We then performed experiments to evaluate the residual IFN-γ in supernatant of PM shortly after IFN-γ treatment. PM cultures were treated with 100 IU/ml of IFN-γ (mean value by ELISA, 12,720 pg/ml). After a 15-min incubation at 37°C, equal amounts of IFN-γ (mean value by ELISA, 12,716 pg/ml) were still found in the culture medium, suggesting that very few IFN-γ molecules were bound and/or

Table 1. *IFN-γ Production in IFN-γ–treated PM*

Exp.	Treatment	Secreted IFN-γ (pg/ml) at 6 h	24 h	48 h
1	None	<128	<128	<128
	IFN-γ	<128	1,125	400
2	None		<128	
	IFN-γ (1 IU/ml)		<128	
	IFN-γ (10 IU/ml)		147	
	IFN-γ (100 IU/ml)		4,085	

10^6 peritoneal cells were seeded in 24-well cluster plates, as described in Materials and Methods. Adherent PM were treated with IFN-γ (100 IU/ml) for 3 h at 37°C and then extensively washed to remove any residual IFN-γ. An aliquot of the culture medium was collected at the end of the wash and checked by ELISA for the presence of any residual IFN-γ. After 6, 24, and 48 h, cell supernatants were collected and the IFN-γ measured by ELISA (Exp. 1). Some cultures (Exp. 2) received different concentrations of IFN-γ. Cell supernatants were collected after 24 h for IFN-γ titration. Values represent the mean of duplicate samples. Standard deviation did not exceed 15%.

internalized in PM. Cells were then extensively washed three times with fresh medium and cultured for 24 h at 37°C. No IFN-γ was detected in the culture fluids 15 min after cell washings. However, a clear-cut production of IFN-γ (mean value by ELISA, 1,522 pg/ml) was found 24 h later in the supernatants, suggesting that newly synthesized IFN-γ was secreted after IFN-γ stimulation.

Specificity of the IFN-γ–induced Upregulation of IFN-γ Gene Expression in PM. Figs. 4 and 5 illustrate the results of two sets of experiments aimed at investigating the cell specificity of the IFN-γ–induced upregulation of IFN-γ expression. In a first set of experiments, we compared macrophages with different cell types for the response to IFN-γ. In lymphocytes freshly harvested from mesenteric lymph nodes, the addition of IFN-γ did not result in IFN-γ mRNA accumulation, whereas upregulation of IFN-γ gene expression was observed in PHA-treated cultures (Fig. 4). IFN-γ did not

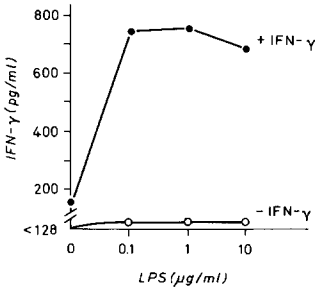

Figure 3. LPS potentiates IFN-γ production in IFN-γ–treated PM. 10^6 peritoneal cells were seeded in 24-well cluster plates as described in the legend to Table 1. Adherent cells were treated with IFN-γ (10 IU/ml) for 3 h at 37°C. Cells were washed three times and subsequently treated with different concentrations of LPS. Cell supernatants were collected for IFN-γ titration after 24 h at 37°C. Values represent the mean of duplicate samples. Standard deviations did not exceed 15%.

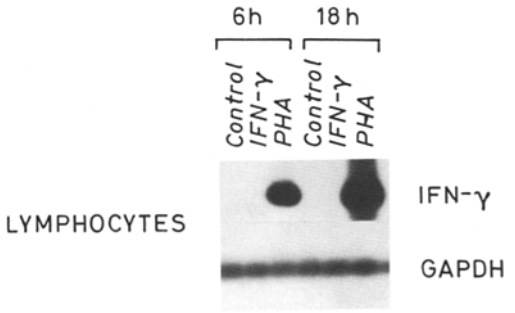

Figure 4. Effect of IFN-γ or PHA on the expression of IFN-γ mRNA in primary lymphocytes. Primary lymphocytes, seeded as described in Materials and Methods, were treated with IFN-γ (100 IU/ml) or PHA (2.5 μg/ml). Total RNA was extracted after 6 and 18 h of treatment at 37°C and analyzed by RNA-PCR.

induce accumulation of its own mRNA in mouse cell lines such as ANA-1, Eb lymphoma cells, and Friend erythroleukemia cells or human peripheral blood monocytes (data not shown). In a second set of experiments, we further confirmed that IFN-γ–induced upregulation of IFN-γ mRNA expression was a specific response of macrophages by using PM from either untreated or anti-asialo GM_1–treated T cell–deficient nude mice. As shown in Fig. 5, IFN-γ treatment of PM harvested from nude mice resulted in a marked accumulation of IFN-γ mRNA as well as in IFN secretion. Similar results were obtained in PM harvested from anti-asialo GM_1–treated nude mice (Fig. 5).

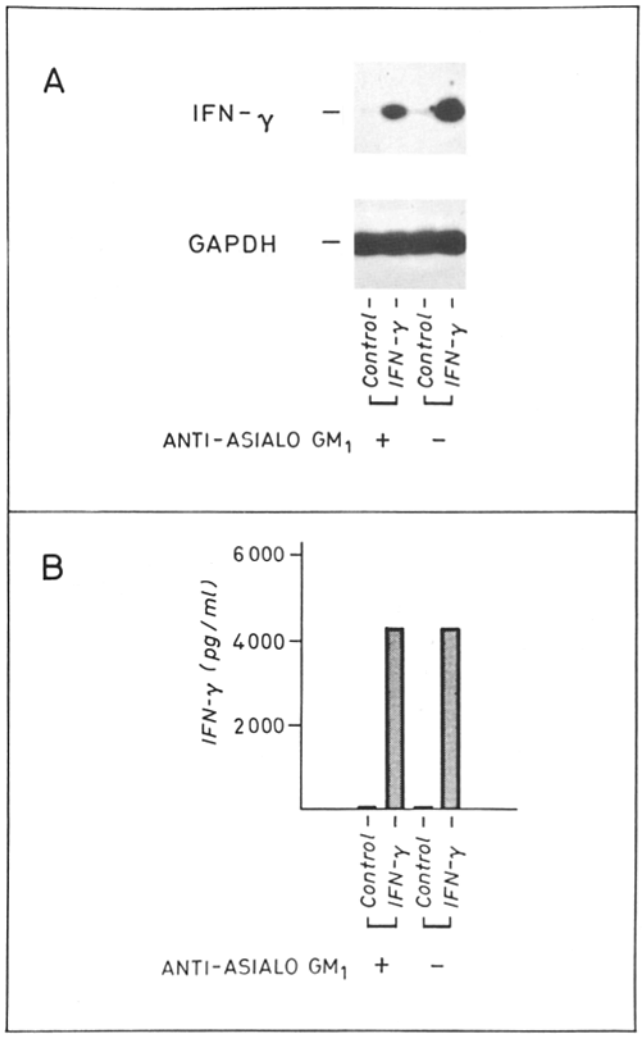

Figure 5. IFN-γ–induced upregulation of IFN-γ gene expression in PM from untreated or anti-asialo GM_1–treated nude mice. (*A*) 3×10^6 peritoneal cells harvested from either untreated or anti-asialo GM_1 inoculated nude mice were seeded as described in Materials and Methods. RNA extraction and RNA-PCR were performed as described in the legend to Fig. 1. (*B*) 10^6 peritoneal cells harvested from either untreated or anti-asialo GM_1–treated nude mice were seeded as described in the legend to Table 1. Adherent PM were treated with IFN-γ (100 IU/ml) for 3 h at 37°C and then extensively washed. Cell supernatants were collected for IFN-γ titration after 24 h.

Discussion

The two major findings reported in this article can be summarized as follows: (*a*) IFN-γ mRNA is constitutively expressed at very low levels in unstimulated PM and accumulates after treatment with CHX; and (*b*) IFN-γ upregulates the expression of its own gene and induces IFN-γ secretion in PM, but not in other cell types (i.e., lymphocytes and several mouse cell lines).

It is generally assumed that T cells and LGL are the only two cell types capable of producing IFN-γ (9). To our knowledge, IFN-γ production by mouse cells other than T cells and LGL has been reported in only two studies: by peritoneal cells cocultivated with syngeneic tumor cells (22) and by lymphokine-activated spleen macrophages (23). The finding that IFN-γ mRNA is expressed in resting PM and is upregulated by IFN-γ itself indicates that macrophages should be regarded as potentially important producers of IFN-γ in vivo. Cockfield et al. (24) recently reported that IFN-γ upregulates the expression of its own mRNA in normal and LPS-treated mice. Since this also occurs in T cell–deficient nude mice, these authors conclude that "the cell population responsible for IFN-gamma secretion is probably the LGL or NK cell fraction, the only non-T cells able to express IFN-gamma after stimulation in vitro or in vivo" (24). In light of our data on the upregulation of IFN-γ expression by IFN-γ itself in PM, we can assume that Cockfield et al. (24) may have neglected a possible role of macrophages in the IFN-γ–mediated amplification of the IFN-γ production observed in their in vivo studies. In this study, we observed a reproducible upregulation of IFN-γ mRNA by IFN-γ (by RNA-PCR and in situ hybridization) only using firmly adherent macrophages. The specificity of this autostimulatory loop of IFN-γ production is indicated by the fact that lymphocytes from mesenteric lymph nodes, as well as different mouse cell lines and human monocytes, did not show any evidence of IFN-γ mRNA accumulation after treatment with IFN-γ.

It is noteworthy that this response was also found in PM from either untreated or anti-asialo GM_1–treated T cell–deficient nude mice, further indicating that the upregulation

of IFN-γ expression was not due to any possible contamination of PM with residual T or NK cell populations.

In the last few years several reports have indicated that one cytokine may regulate the expression of other cytokines or of their receptors in an autocrine or paracrine manner (1). Additional complexity of the cytokine network is suggested by the observation that certain cytokine genes may be regulated in vitro and in vivo by their own products (24–27). Concerning IFN-γ expression, Hardy and Sawada (28) reported that IFN-γ can upregulate its own gene expression in lectin-activated human PBMC. The data reported here represent the first example that the simple addition of IFN-γ alone can upregulate its own gene expression in vitro. This type of response appears to be a unique property of macrophages.

Recent studies in mice with disrupted IFN-γ genes (29) as well as in mice lacking the IFN-γ receptor (30) have underlined the relevance of the IFN-γ system in mice. IFN-γ plays a crucial role in a number of immune responses and is the major cytokine involved in the regulation of macrophage activation and differentiation (9). We suggest that autocrine mechanisms of regulation of IFN-γ expression may play important biological roles in vivo for maintaining certain macrophage functions. The ability of macrophages to produce suitable amounts of IFN-γ after stimulation with IFN-γ itself suggests a specific role of macrophage IFN-γ in the regulation of cell differentiation and immune response.

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Note added in proof: Fultz et al. (31) have recently reported the induction of IFN-γ expression in mouse peritoneal exudate macrophages after LPS stimulation.

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