INFECTION AND IMMUNITY, Dec. 1997, p. 5149–5156 Vol. 65, No. 12
0019-9567/97/$04.00+0

Induction of Gamma Interferon Production in Human Alveolar Macrophages by *Mycobacterium tuberculosis*

MATTHEW J. FENTON,[1]* MARY W. VERMEULEN,[2] SUE KIM,[1] MARIE BURDICK,[3] ROBERT M. STRIETER,[3] AND HARDY KORNFELD[1]

Pulmonary Center, Boston University School of Medicine, Boston, Massachusetts 02118[1]; Pulmonary Research Laboratory, Massachusetts General Hospital, Charlestown, Massachusetts 02129[2]; and Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan 48109[3]

Received 28 April 1997/Returned for modification 9 June 1997/Accepted 3 September 1997

Gamma interferon (IFN-γ) is a cytokine which plays a critical role in resistance to *Mycobacterium tuberculosis* infection. While T lymphocytes and natural killer cells are a major source of IFN-γ, previous demonstrations that it can be produced by murine macrophages prompted us to examine the capacity of human alveolar macrophages to express IFN-γ. Here we report that in vitro infection of alveolar macrophages with *M. tuberculosis* induces both the release of IFN-γ protein and a transient increase in IFN-γ mRNA levels. The IFN-producing cells were shown to be macrophages by reverse transcription-in situ PCR. We also observed that *M. tuberculosis* stimulation resulted in IFN-γ-dependent expression of the chemokines IFN-γ-inducible protein 10 and monokine induced by IFN-γ, suggesting that macrophage-derived IFN-γ can function in an autocrine and/or paracrine manner. The existence of a positive regulatory loop was suggested by the observation that exogenous IFN-γ protein could induce IFN-γ mRNA expression in uninfected alveolar macrophages. Interleukin-12 was also found to be a potent inducer of IFN-γ production, and *M. tuberculosis*-induced IFN-γ production appears to be mediated, at least in part, by IL-12. In contrast, *M. tuberculosis*-induced IFN-γ production by alveolar macrophages could be blocked by exogenous interleukin-10. These studies are the first to demonstrate an autoregulatory role for IFN-γ produced by alveolar macrophages infected in vitro with *M. tuberculosis*.

Tuberculosis remains an important cause of morbidity and mortality worldwide. *Mycobacterium tuberculosis*, the etiologic agent of tuberculosis, is responsible for more human deaths each year than any other single pathogen. Initially, establishment of a productive infection depends on the bacterium's ability to invade the alveolar space and survive within the alveolar macrophages (AMϕ). Infection of AMϕ by *M. tuberculosis* rapidly leads to the activation of AMϕ, the production of cytokines that serve to limit the growth of ingested organisms, and the recruitment of additional leukocytes from the peripheral circulation (reviewed in reference 17). While innate immune responses predominate initially, the subsequent recruitment of T lymphocytes to the lung is vital to the containment of *M. tuberculosis* within granulomas (reviewed in reference 31). Cytokines produced by T cells, such as gamma interferon (IFN-γ), can activate monocytes and macrophages to become microbicidal (6). Ineffective immunity against mycobacterial infection is associated with a depressed Th1 cytokine response and reduced production of IFN-γ (40, 43).

IFN-γ is thought to be a principal mediator of macrophage activation and resistance to intracellular pathogens (reviewed in reference 41). Exposure of macrophages to IFN-γ results in increased expression of major histocompatibility complex class II molecules and the potentiation of a variety of cellular responses. These responses include cytokine production, nitric oxide (NO) production, and cytolytic activity (reference 41 and references therein). At least in mice, the production of NO is a powerful antimicrobial response to *M. tuberculosis* infection (7). IFN-γ plays a critical role in priming macrophages to generate NO in vitro. The importance of IFN-γ in the resistance to *M. tuberculosis* infection was most clearly shown with mice that are homozygous for a targeted disruption of the IFN-γ gene (9, 10, 18). Upon infection, these mutant mice developed granulomas, failed to produce NO, and were unable to restrict disseminated growth of the bacilli. Treatment of these mice with exogenous IFN-γ delayed, but could not prevent, the fatal course of tuberculosis (18). The importance of IFN-γ in human tuberculosis is suggested by the increased susceptibility to mycobacterial infection in persons with mutations of the IFN-γ receptor 1 that resulted in reduced receptor expression and IFN-γ unresponsiveness (24, 30).

The production of IFN-γ has been generally considered to be restricted to T cells and natural killer (NK) cells. Several investigators have demonstrated that murine macrophages can also express IFN-γ following treatment with either lipopolysaccharide (LPS), cycloheximide, or IFN-γ itself (12, 21). In contrast, IFN-γ has not been shown to upregulate its own expression in murine lymphocytes or in human blood monocytes (12). These studies prompted us to test the possibility that human AMϕ could be induced to express IFN-γ. In this work, we show that *M. tuberculosis* stimulates the production of IFN-γ by AMϕ and that IFN-γ mRNA can also be induced in uninfected cells by IFN-γ protein or interleukin-12 (IL-12) but not by LPS. Data suggesting that macrophage-derived IFN-γ can function in an autocrine and/or paracrine manner toward AMϕ are presented. Lastly, we found that IL-10 could suppress *M. tuberculosis*-induced IFN-γ. Together, our studies suggest that IFN-γ produced by AMϕ may play a role in innate immunity against tuberculosis.

MATERIALS AND METHODS

Antibodies and reagents. A blocking rabbit anti-human IFN-γ antiserum (AHC4734) and normal rabbit serum were purchased from Biosource International (Camarillo, Calif.). Recombinant human IL-10 was a generous gift from Raymond Donnelly (Division of Cytokine Biology, U.S. Food and Drug Administration). Recombinant human IFN-γ and IL-12 and a blocking goat anti-IL-12

* Corresponding author. Mailing address: Pulmonary Center, R-304, Boston University School of Medicine, 80 East Concord St., Boston, MA 02118. Phone: (617) 638-4860. Fax: (617) 536-8093. E-mail: mfenton@bupula.bu.edu.

antiserum (AB-219-NA) were purchased from R & D Systems (Minneapolis, Minn.). DNA oligonucleotide primers were purchased from Gibco-BRL (Grand Island, N.Y.).

Isolation and infection of human AMϕ. Human AMϕ were prepared from bronchoalveolar lavage (BAL) fluids obtained from purified protein derivative-negative, nonsmoking volunteers. BAL was performed after informed consent was obtained according to protocols approved by the Institutional Review Board. After local anesthesia was obtained with topical 2% lidocaine, an Olympus flexible fiberoptic bronchoscope (model BF-4B2) was introduced by the transoral route and wedged into a subsegment of the right middle lobe. Sterile 0.9% NaCl was instilled in 60-ml aliquots and aspirated, with a total of 240 ml of saline. Lavage fluid was filtered through a single layer of sterile gauze and then centrifuged (300 × *g* at room temperature), and cells were suspended at 10^6 cells/ml in RPMI 1640 culture medium (BioWhittaker, Walkersville, Md.) supplemented with 10% heat-inactivated fetal bovine serum (Defined FBS; HyClone Laboratories, Logan, Utah), 10 mM HEPES, 2 mM L-glutamine, and 50 mg of cefotaxime per ml (complete medium). Endotoxin levels of all medium components were less than 20 pg/ml (final concentration). Adherent cells obtained by this procedure were >99% AMϕ, as determined by morphologic examination and α-naphthyl acetate esterase staining. Other controls for lymphocyte contamination are discussed below.

AMϕ (2×10^6 cells) were maintained in 60-mm-diameter dishes containing 3 ml of complete medium in a humidified incubator at 37°C and 5% CO_2 for 6 days prior to use. Before each experiment, the culture supernatants were aspirated and replaced with fresh complete medium. *M. tuberculosis* H37Rv (ATCC 25618) and H37Ra (ATCC 25177) were obtained from the American Type Culture Collection (Rockville, Md.) and were cultured at 37°C in Middlebrook 7H9 medium supplemented with Tween 80 and albumin, dextrose, and catalase under biosafety level 3 conditions. Following vigorous dispersion (25), viable bacilli were added to the macrophage cultures (2×10^6 to 3×10^6 cells) at an infectivity ratio of approximately 10:1 for various times, as indicated in the text. Culture supernatants were harvested, filtered through 0.22-mm-pore-size filters to remove any bacilli and debris, and then stored in frozen aliquots at −30°C. Cytokine levels in these supernatants were measured as described below. Adherent cells were lysed on the dishes by the addition of 2 ml of Tri-Reagent (Molecular Research, Cincinnati, Ohio). Total RNA was purified from these lysates as recommended by the manufacturer.

Measurement of cytokine and nitrite levels. Tumor necrosis factor alpha (TNF-α) and IFN-γ levels in culture supernatants were measured in duplicate by enzyme-linked immunosorbent assay (ELISA) (R & D Systems) as recommended by the manufacturer. Levels of IFN-γ-inducible protein-10 (IP-10) and monokine induced by IFN-γ (MIG) were measured in duplicate by ELISA as described previously (1). Each experiment used AMϕ from a single donor, and at least three experiments were performed. Results from a single experiment are shown in this work. The standard error of the mean was less than 10% in all experiments shown. To assess NO production, the culture supernatants were assayed for the stable metabolite of NO, nitrite. Nitrite levels were measured by using the Greiss reaction, as previously described (23).

Reverse transcription-PCR (RT-PCR). Total RNA was reverse transcribed with murine Moloney leukemia virus reverse transcriptase (Gibco-BRL) and oligo(dT) (Promega, Madison, Wis.) as recommended by the manufacturer. The resulting cDNA was quantitated by spectrophotometry. The cDNA (0.5 to 3 μg per reaction) was amplified by PCR in a 100-μl reaction mixture containing 1 mM primers, 20 mM nucleoside triphosphates, 2.5 mM $MgCl_2$, and 2.5 U of *Taq* polymerase (Promega). Amplifications were carried out for 30 cycles; the parameters for each cycle were 1 min at 95°C, 1 min at 60°C, and 1.5 min at 72°C. The products were then run on a 1.5% agarose gel and stained with ethidium bromide. The sequences of the human β-actin oligonucleotide primers which were used to generate a 504-bp PCR product are 5′-ATGCCATCCTGCGTCTGGA-3′ (sense strand) and 5′-CACATCTGCTGGAAGGTGG-3′ (antisense strand). The sequences of the human glyceraldehyde phosphate dehydrogenase oligonucleotide primers which were used to generate a 983-bp PCR product are 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ (sense strand) and 5′-CATGTGGGCCATGAGGTCCACCAC-3′ (antisense strand). The sequences of the human IFN-γ primers which were used to generate a 470-bp PCR product are 5′-ATGAAATATACAAGTTATATCTTGGCTTTT-3′ (sense strand) and 5′-GATGCTCTTCGACCTTGAAACAGCAT-3′ (antisense strand).

For RT-in situ PCR analysis, 1.5×10^4 AMϕ were fixed onto silane-coated slides (Cell-Line Associates Inc., New Field, Conn.) with 10% buffered formalin. After digestion with proteinase K (100 μg/ml; Sigma, St. Louis, Mo.), RNase-free DNase I (1 U/ml; Boehringer Mannheim, Indianapolis, Ind.) was added and allowed to incubate overnight at room temperature. The RNA was reverse transcribed under the same conditions as described above. The cDNA was then amplified by addition of 25 μl of the PCR mixture described above, supplemented with 2% bovine serum albumin and 1 mM digoxigenin-11-dUTP (Boehringer Mannheim). In some experiments, primers specific for human IL-2 were used as a control for contaminating lymphocytes. PCR was carried out for 30 cycles with the cycle parameters described above. The PCR products were then treated with alkaline phosphatase-labeled antidigoxigenin antibody (Boehringer Mannheim). Final detection of the PCR products was achieved by adding 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Promega). A nuclear fast red counterstain (Shandon Lipshaw, Pittsburgh, Pa.) was then added to each slide.

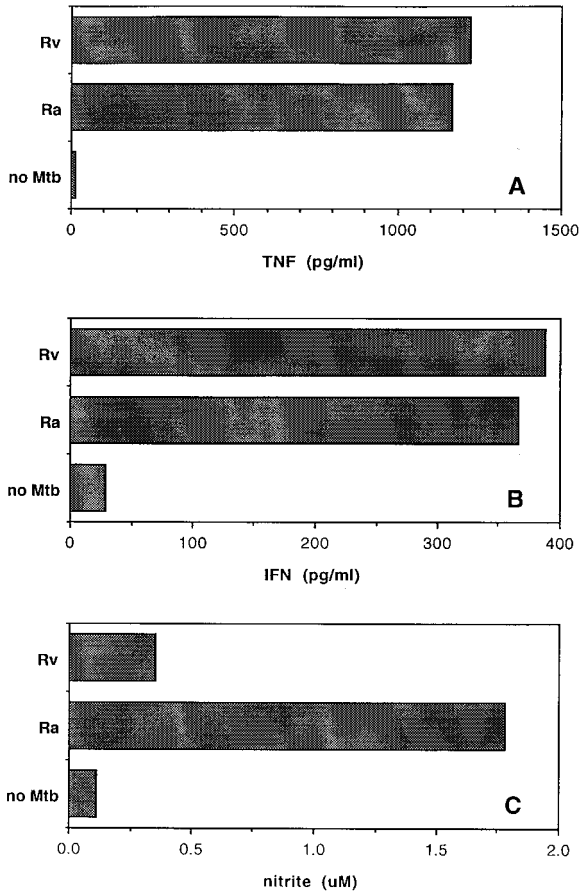

FIG. 1. Responses of human AMϕ to *M. tuberculosis*. Human AMϕ (2×10^6 cells/well) were prepared as described in the text and incubated for 24 h in the presence of a virulent (H37Rv [Rv]) or attenuated (H37Ra [Ra]) strain of *M. tuberculosis* at an infectivity ratio of approximately 10:1 or without *M. tuberculosis* (no Mtb). Following exposure of the AMϕ to *M. tuberculosis*, culture supernatants were collected. TNF-α (A) and IFN-γ (B) levels were then measured by ELISA. Nitrite levels (C) in the same supernatants were measured by the Greiss reagent assay.

RESULTS

Cytokine production by *M. tuberculosis*-stimulated AMϕ. Previous reports that murine macrophages can secrete IFN-γ prompted us to determine if human macrophages could also release this cytokine. Human AMϕ were prepared as described above and incubated for 24 h in the presence of a virulent (H37Rv) or attenuated (H37Ra) strain of *M. tuberculosis* at an infectivity ratio of approximately 10:1. Following exposure of the AMϕ to *M. tuberculosis*, culture supernatants were collected and cytokine levels were measured by ELISA. As shown in Fig. 1, unstimulated AMϕ spontaneously release minimal amounts of TNF-α and IFN-γ (Fig. 1A and B, respectively) into the culture medium. H37Ra and H37Rv appeared to be equally capable of inducing release of both cytokines. In contrast, H37Ra consistently induced nitrite release whereas H37Rv did not induce nitrite release (Fig. 1C). While this nitrite may be generated by the breakdown of *M. tuberculosis*-induced NO, nitrite release could not be blocked by the inducible NO synthase inhibitor *N*-monomethyl arginine or 2-aminoguanidine (data not shown). Substantial donor variability in

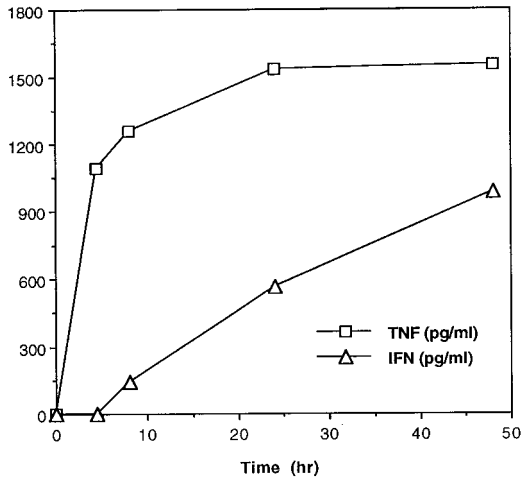

FIG. 2. Kinetics of cytokine release by AMϕ stimulated with *M. tuberculosis*. AMϕ (2×10^6 cells/well) were stimulated with *M. tuberculosis* H37Ra at an infectivity ratio of approximately 10:1 for 4, 8, 24, and 48 h. Following exposure of the AMϕ to *M. tuberculosis*, culture supernatants were collected. TNF-α and IFN-γ levels were then measured by ELISA.

the levels of *M. tuberculosis*-induced IFN-γ was observed. IFN-γ levels measured in six experiments using AMϕ from different donors ranged from 395 to 4,532 pg/ml (after 48 h of stimulation). We subsequently measured the kinetics of IFN-γ and TNF-α release by AMϕ stimulated with H37Ra for 4, 8, 24, and 48 h. As shown in Fig. 2, TNF-α accumulates rapidly in the culture medium, with peak levels observed by 8 h after stimulation. In contrast, IFN-γ levels accumulate in the culture medium at a lower rate. Together, these studies show that IFN-γ is released into the culture supernatants of *M. tuberculosis*-stimulated AMϕ.

***M. tuberculosis* and IFN-γ protein induce the expression of IFN-γ mRNA in AMϕ.** RT-PCR was used to evaluate IFN-γ mRNA expression by AMϕ at various times after stimulation with *M. tuberculosis*. Total RNA was prepared from the adherent AMϕ that were used to generate the culture supernatants described in Fig. 2. This RNA was analyzed by RT-PCR as described above, with IFN-γ- and β-actin-specific primers. As shown in Fig. 3A, cDNA prepared from unstimulated AMϕ RNA (designated 0 h) did not generate a PCR product of the predicted size when the IFN-γ primers were used. A PCR product of the predicted size was observed when cDNA prepared from H37Ra-stimulated cells was used. The specificity of this PCR product was confirmed by DNA sequencing (data not shown). IFN-γ mRNA levels were maximal between 8 and 24 h and were markedly reduced by 48 h. Levels of control (β-actin) mRNA were essentially identical at all time points tested. No PCR products were observed when primers were not included in the RT-PCRs (data not shown). These data demonstrate that IFN-γ mRNA is rapidly induced and transiently expressed in *M. tuberculosis*-stimulated AMϕ.

One earlier study reported that IFN-γ could upregulate its own gene expression in murine resident peritoneal macrophages (12). We used RT-PCR to characterize IFN-γ mRNA expression in unstimulated AMϕ and in cells stimulated with IFN-γ protein (100 U/ml) for 48 h. As shown in Fig. 3B, IFN-γ protein was a potent inducer of IFN-γ mRNA in AMϕ. Levels of control (glyceraldehyde phosphate dehydrogenase) mRNA were essentially identical in unstimulated and IFN-γ-stimulated cells. No PCR products were observed when primers were not included in the RT-PCRs (data not shown). The high level of IFN-γ mRNA observed after stimulation with IFN-γ protein for 48 h contrasted with *M. tuberculosis*-induced IFN-γ mRNA production, in which IFN-γ message levels decreased to near background levels by 48 h. These data demonstrate that, like murine resident peritoneal macrophages, IFN-γ mRNA can be induced in AMϕ by IFN-γ protein. The kinetics of this positive feedback appear to differ from those of IFN-γ mRNA induction by *M. tuberculosis*.

Identification of IFN-γ-producing AMϕ by RT-in situ PCR. Cells from the BAL fluids of normal volunteers are typically ≥90% AMϕ. These BAL cells were further enriched for AMϕ by adherence. Furthermore, the cells were cultured for 6 days prior to the experiment, and any additional nonadherent cells were removed by washing prior to *M. tuberculosis* stimulation. The resulting cells averaged >99% macrophages as judged by nonspecific esterase staining. In order to determine if the source of IFN-γ in these *M. tuberculosis*-stimulated cultures was the AMϕ or the <1% contaminating lymphocytes, we examined IFN-γ mRNA expression in individual cells by RT-in situ PCR. Because of the counterstain used, AMϕ could be easily distinguished from any contaminating lymphocytes by virtue of their characteristic size and morphology. Human peripheral blood T cells stimulated with phorbol esters and ionomycin were used as a positive PCR control for IFN-γ expression (data not shown). As shown in Fig. 4a, unstimulated AMϕ did not contain detectable IFN-γ mRNA. Following 24 h of stimulation with H37Ra, more than 80% of the AMϕ contained IFN-γ mRNA (Fig. 4b). No IL-2 expression was observed in these infected cultures (Fig. 4c), indicating that there was no substantial contamination of the AMϕ by T or NK cells. No signal was observed when digoxigenin-11-dUTP was not included in the PCRs, indicating that endogenous alkaline phosphatase activity was not responsible for the positive IFN-γ signal observed in *M. tuberculosis*-infected cells (Fig. 4d). These studies demonstrate that the predominant cells which express IFN-γ in these cultures are the AMϕ.

IFN-γ-dependent production of chemokines by *M. tuberculosis*-stimulated AMϕ. IP-10 and MIG are chemokines that are secreted by IFN-γ-stimulated monocytes (15, 27, 28). We hypothesized that if IFN-γ could act on AMϕ in an autocrine or paracrine manner, this would result in the expression of these IFN-inducible chemokines. In order to test this possibility, we stimulated AMϕ with H37Ra for 48 h in the presence and

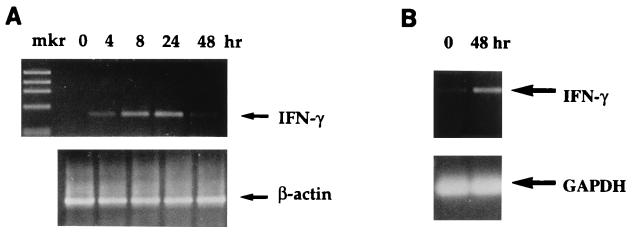

FIG. 3. Expression of IFN-γ mRNA by AMϕ. (A) RT-PCR was used to measure levels of IFN-γ expressed by AMϕ at various times after stimulation with *M. tuberculosis*. Total RNA was prepared from the adherent AMϕ which were used to generate the culture supernatants described in the legend to Fig. 2. This RNA was used to generate reverse-transcribed cDNA, which was analyzed by RT-PCR using IFN-γ- and β-actin-specific PCR primers as described in the text. PCR products were fractionated by agarose gel electrophoresis and visualized by staining with ethidium bromide. mkr, molecular size marker. (B) RT-PCR was used to compare IFN-γ mRNA expression in unstimulated AMϕ (2×10^6 cells/well) and cells stimulated with IFN-γ protein (100 U/ml) for 48 h. Total RNA was used to generate reverse-transcribed cDNA, which was analyzed by RT-PCR using IFN-γ- and GAPDH-specific PCR primers as described in the text. PCR products were fractionated by agarose gel electrophoresis and visualized by staining with ethidium bromide.

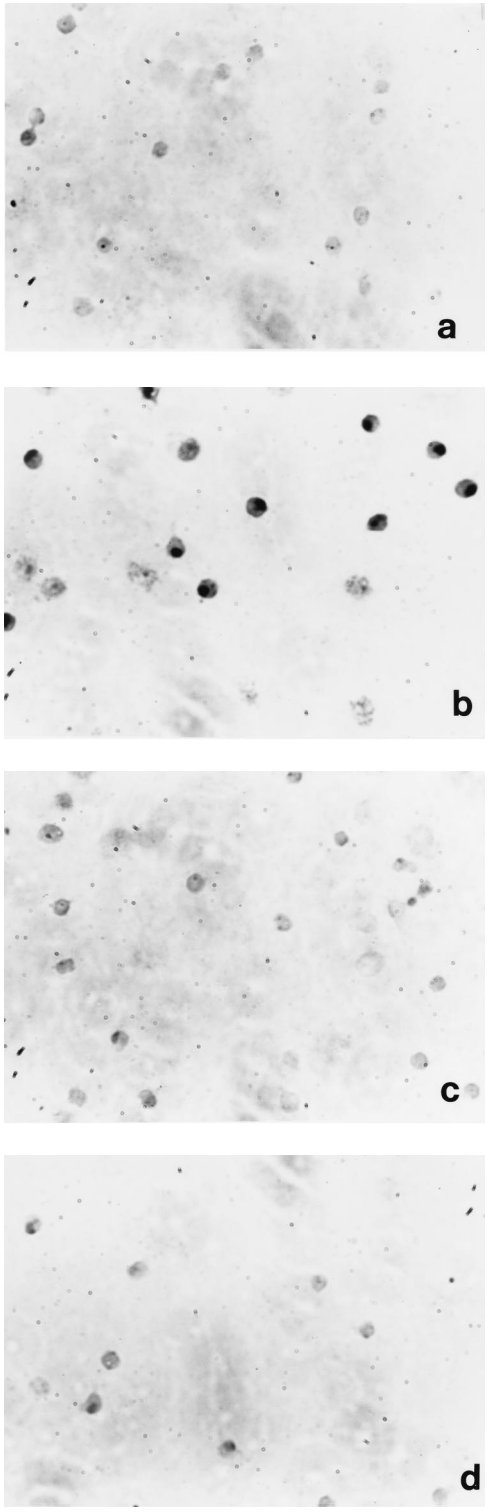

FIG. 4. Identification of IFN-γ-producing AMϕ by RT-in situ PCR. The presence of IFN-γ mRNA was detected in the AMϕ by RT-in situ PCR as described in the text. Unstimulated AMϕ did not contain detectable IFN-γ mRNA (a). Following 24 h of stimulation with *M. tuberculosis* H37Ra, more than 80% of the AMϕ contained IFN-γ mRNA (b). With PCR primers specific for IL-2 mRNA, no IL-2 expression was observed in these infected cultures (c), indicating that there was no substantial contamination of the AMϕ by T or NK cells. As a control for endogenous nonspecific phosphatase activity, additional RT-in situ PCRs were performed with *M. tuberculosis*-stimulated AMϕ in the absence of digoxigenin-labeled UTP (d).

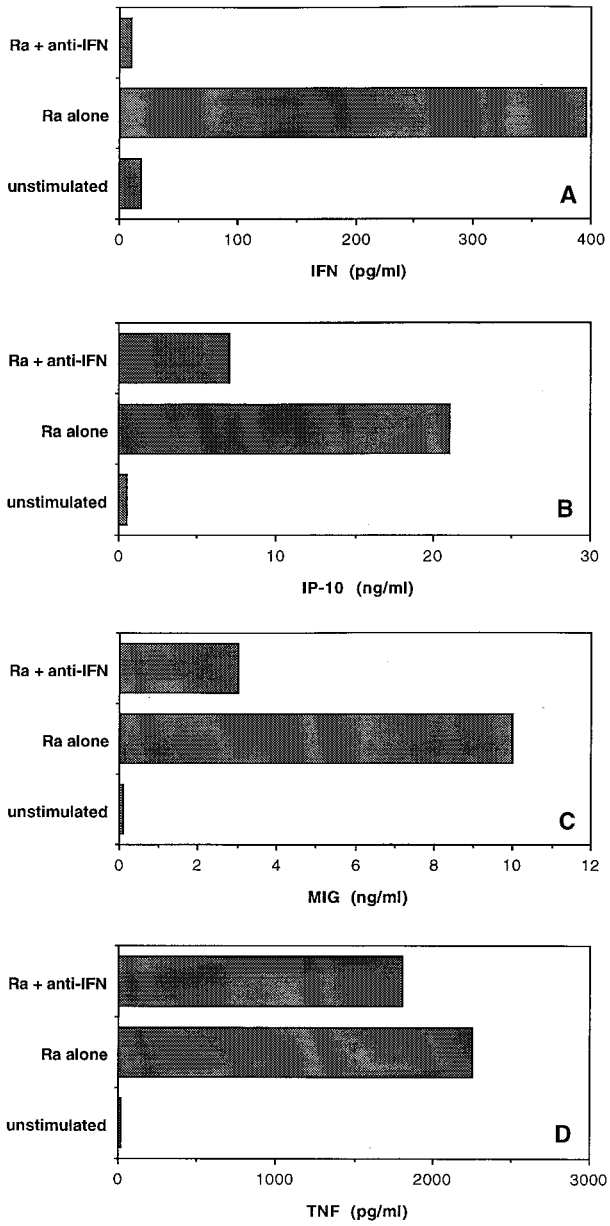

FIG. 5. Effect of a neutralizing anti-IFN-γ antiserum on *M. tuberculosis*-induced chemokine production by AMϕ. AMϕ (2×10^6 cells/well) were stimulated with *M. tuberculosis* H37Ra (Ra) at an infectivity ratio of approximately 10:1 for 48 h in the presence or absence of a neutralizing anti-IFN-γ antiserum (1 μg/ml). Culture supernatants were then harvested and assayed for IFN-γ (A), IP-10 (B), MIG (C), and TNF-α (D) by ELISA.

absence of a neutralizing anti-IFN-γ antiserum (1 mg/ml). Culture supernatants were then harvested and assayed for IFN-γ, IP-10, MIG, and TNF-α by ELISA. As shown in Fig. 5, H37Ra was a potent inducer of each cytokine. A substantial reduction in IFN-γ levels was observed in the supernatants of AMϕ cultures stimulated with H37Ra in the presence of the anti-IFN-γ antiserum (Fig. 5A). Similarly, IP-10 and MIG production was markedly reduced (Fig. 5B and C). The anti-IFN-γ antiserum had no significant effect on TNF-α production (Fig. 5D), and normal rabbit serum had no effect on *M. tuberculosis*-induced cytokine levels (data not shown). These data suggest that *M. tuberculosis*-induced IFN-γ can induce IP-10 and MIG

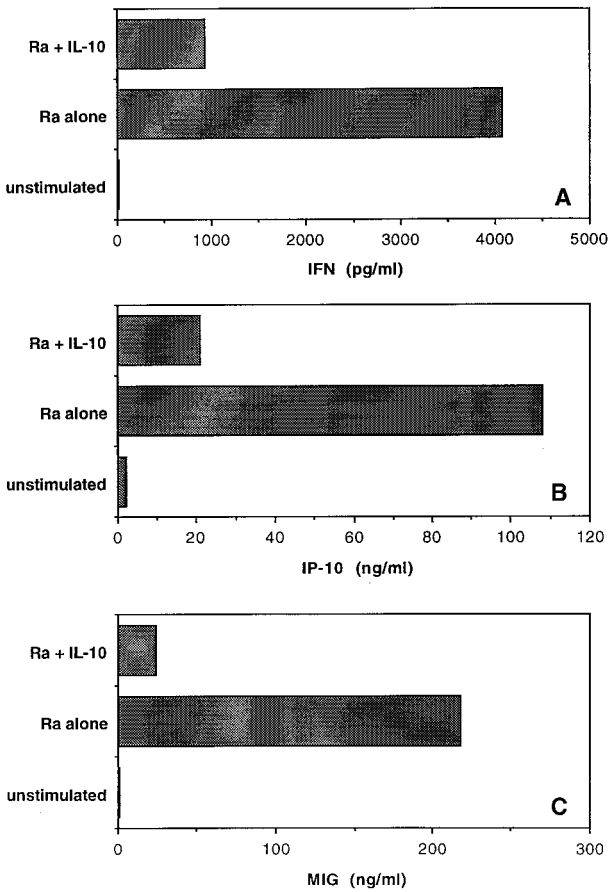

FIG. 6. Effect of IL-10 on *M. tuberculosis*-induced cytokine production by AMϕ. AMϕ (2×10^6 cells/well) were infected in vitro with *M. tuberculosis* H37Ra (Ra) at an infectivity ratio of approximately 10:1 for 48 h in the presence or absence of recombinant human IL-10 (20 ng/ml). Culture supernatants were collected and assayed for IFN-γ (A), IP-10 (B), and MIG (C) by ELISA.

production in an autocrine and/or paracrine manner and that the expression of these chemokines does not result from *M. tuberculosis*-induced TNF-α (32).

Effect of IL-10 on *M. tuberculosis*-induced cytokine production by AMϕ. IL-10 is a likely mediator of the depressed IFN-γ response in diseases caused by intracellular pathogens, including *M. tuberculosis* (22). IL-10 has been previously reported to suppress the production of several LPS-inducible macrophage cytokines, including IL-1β, TNF-α, and IL-6 (17, 29). We questioned whether IL-10 could also suppress *M. tuberculosis*-inducible IFN-γ production by AMϕ. AMϕ were infected in vitro with H37Ra and then incubated for 48 h in the presence or absence of recombinant human IL-10 (20 ng/ml). Culture supernatants were collected and assayed for IFN-γ, IP-10, and MIG as described above. As shown in Fig. 6A, IL-10 was capable of suppressing *M. tuberculosis*-induced IFN-γ production. Chemokine production was similarly suppressed (Fig. 6B and C), perhaps due to the reduction of IFN-γ production. The specificity of these responses was addressed by the finding that a neutralizing anti-IL-10 antiserum could reverse the suppressive effect of IL-10 on *M. tuberculosis*-induced IFN-γ production (data not shown).

Effect of IL-12 on *M. tuberculosis*-induced IFN-γ production by AMϕ. A recent study reported that IL-12 could directly induce IFN-γ production by murine peritoneal macrophages (33). In addition, IL-12 has been shown to be produced by *M. tuberculosis*-stimulated peripheral blood mononuclear cells (PBMC) and is present in the pleural fluids of patients with tuberculous pleuritis (19, 44). We tested the possibility that *M. tuberculosis*-induced IFN-γ might occur as a consequence of *M. tuberculosis*-induced IL-12 production. AMϕ were stimulated with H37Ra in the presence and absence of neutralizing anti-IL-12 antibodies for 48 h. Culture supernatants were collected and assayed for IFN-γ as described above. As shown in Fig. 7A, anti-IL-12 antibodies markedly reduced the levels of IFN-γ produced by the *M. tuberculosis*-stimulated AMϕ. In addition, exogenous IL-12 (20 U/ml) was capable of stimulating these cells to secrete large amounts of IFN-γ (Fig. 7B). Together, these data suggest that *M. tuberculosis*-induced IL-12, as well as exogenous IL-12, can activate IFN-γ production by AMϕ.

LPS fails to induce IFN-γ production by AMϕ. Previous studies have shown that LPS-stimulated murine thioglycolate-elicited peritoneal macrophages and macrophage colony-stimulating factor-derived bone marrow macrophages could express IFN-γ (21). In contrast, resident murine peritoneal macrophages failed to produce IFN-γ in response to LPS alone (12). Our studies showed that LPS stimulation (100 ng/ml; 48 h) failed to induce IFN-γ release by AMϕ (Fig. 8A). As expected, LPS did induce substantial TNF-α release (Fig. 8B). This finding suggests that LPS alone and LPS-inducible cytokines are not sufficient to induce IFN-γ production by human AMϕ. This conclusion is consistent with our observation that the presence of a neutralizing anti-TNF-α monoclonal antibody did not affect IFN-γ production in *M. tuberculosis*-stimulated AMϕ (data not shown).

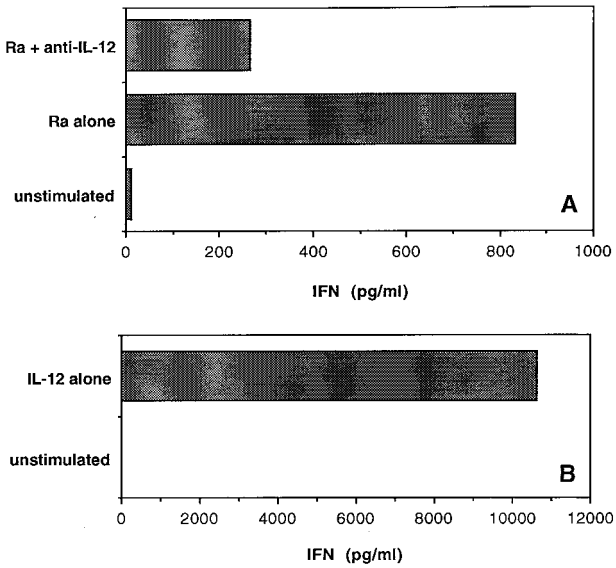

FIG. 7. Effect of a neutralizing anti-IL-12 antiserum on *M. tuberculosis*-induced IFN-γ production by AMϕ. (A) AMϕ (2×10^6 cells/well) were stimulated with *M. tuberculosis* H37Ra (Ra) at an infectivity ratio of approximately 10:1 for 48 h in the presence or absence of a neutralizing anti-IL-12 antiserum (1 μg/ml). Culture supernatants from unstimulated and stimulated AMϕ were then harvested and assayed for IFN-γ by ELISA. (B) AMϕ (2×10^6 cells/well) were stimulated with IL-12 protein (20 U/ml) for 48 h. Culture supernatants from unstimulated and stimulated AMϕ were then harvested and assayed for IFN-γ by ELISA.

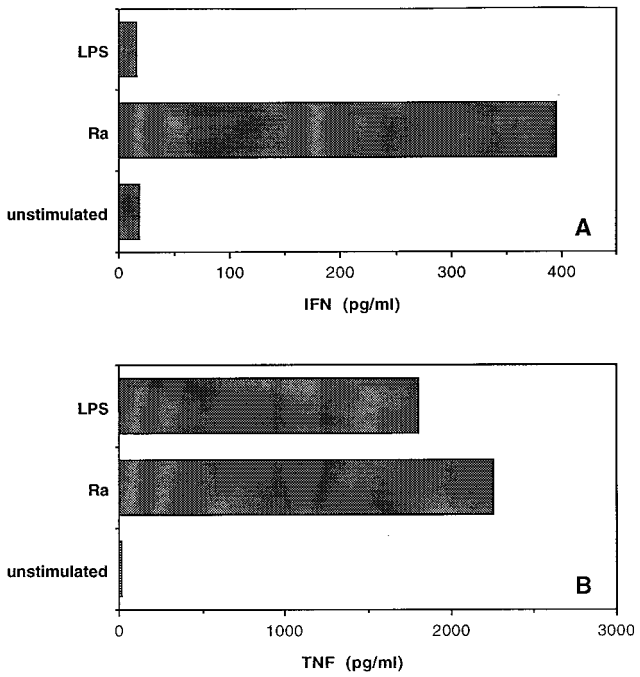

FIG. 8. Effect of LPS on IFN-γ production by AMϕ. AMϕ (2×10^6 cells/well) were stimulated with *Escherichia coli* LPS (100 ng/ml), or *M. tuberculosis* H37Ra (Ra) at an infectivity ratio of approximately 10:1 for 48 h. Culture supernatants from unstimulated and stimulated AMϕ were then harvested and assayed for IFN-γ (A) and TNF-α (B) by ELISA.

DISCUSSION

Here we report that in vitro infection of human AMϕ with *M. tuberculosis* induces both the release of IFN-γ protein and a transient increase in IFN-γ mRNA levels. IFN-γ production could also be induced in uninfected cells by IL-12 and by IFN-γ itself. In contrast, no IFN-γ mRNA or protein was observed when AMϕ were stimulated with LPS. The IFN-γ-producing cells were shown to be macrophages by RT-in situ PCR. Our finding that *M. tuberculosis*-induced production of the IFN-γ-inducible chemokines IP-10 and MIG could be blocked by anti-IFN-γ antibodies suggests that macrophage-derived IFN-γ can function in an autocrine and/or paracrine manner. Furthermore, the ability of anti-IL-12 antibodies to suppress IFN-γ production by *M. tuberculosis*-stimulated AMϕ suggests a role for IL-12 in this induction pathway of IFN-γ expression. Lastly, we found that IL-10 could suppress *M. tuberculosis*-induced IFN-γ and, consequently, the production of IP-10 and MIG. Together, these studies are the first to demonstrate an autoregulatory role for IFN-γ produced by *M. tuberculosis*-infected human AMϕ.

AMϕ were the source of IFN-γ in these studies, based on the absence of lymphocytes in the adherent cell cultures (as judged by morphology and nonspecific esterase staining) and on the presence of IFN-γ mRNA but the absence of IL-2 mRNA in the *M. tuberculosis*-stimulated AMϕ (as judged by RT-in situ PCR). Our data support the earlier findings of Robinson et al. (36), who reported that AMϕ constituted 14% of IFN-γ mRNA-positive cells from BAL fluids of tuberculosis patients. Other investigators have shown that murine macrophages can be induced to express IFN-γ. Fultz et al. (21) showed that LPS-stimulated murine thioglycolate-elicited peritoneal macrophages from C3H/OuJ mice expressed IFN-γ mRNA and immunoreactive IFN-γ protein. These investigators also showed that macrophage colony-stimulating factor-derived murine bone marrow macrophages could also express IFN-γ mRNA following LPS stimulation. In contrast, peritoneal macrophages from LPS-hyporesponsive C3H/HeJ mice did not express IFN-γ following LPS stimulation, whereas peritoneal macrophages from both mouse strains were able to produce comparable levels of IFN-γ mRNA following stimulation with polyinosinic-polycytidylic acid [poly(I)-poly(C)].

Di Marzio et al. (12) later showed that resident murine peritoneal macrophages could produce IFN-γ mRNA following treatment with either cycloheximide or IFN-γ protein. Moreover, IFN-γ did not induce its own expression in murine lymphocytes or in human peripheral blood monocytes. In contrast, our findings demonstrate that IFN-γ protein can induce IFN-γ mRNA production in AMϕ. These differences may reflect the differentiation state of the AMϕ or perhaps previous exposure of AMϕ to modulating signals, such as extracellular matrix proteins encountered during migration from the circulation. Differences in the relative responsiveness of AMϕ and peripheral blood monocytes to stimuli have frequently been reported (3, 4, 26, 34, 35). Our demonstration that exogenous IFN-γ can induce its own expression further supports our conclusion that AMϕ are the source of IFN-γ in these cultures, because lymphocytes do not express this cytokine in response to exogenous IFN-γ. Our studies also revealed that LPS alone could not induce AMϕ to secrete IFN-γ. Similarly, resident murine peritoneal macrophages reportedly failed to produce IFN-γ in response to LPS alone (12). These findings contrast with an earlier study reporting that LPS could stimulate thioglycolate-elicited peritoneal macrophages to produce IFN-γ (21). Furthermore, resident peritoneal macrophages primed in vitro with IFN-γ became capable of producing IFN-γ in response to LPS. Recently, Fultz and Vogel reported that IFN-α could induce IFN-γ expression in elicited murine peritoneal macrophages and vice versa. Furthermore, these investigators showed that in vitro priming of C3H/OuJ peritoneal macrophages with either LPS or IFN-α resulted in a synergistic increase in the amount of IFN-γ produced following stimulation with poly(I)-poly(C). In contrast, priming of C3H/HeJ peritoneal macrophages with IFN-α but not with LPS could augment the production of IFN-γ following poly(I)-poly(C) stimulation. Thus, priming may modulate the ability of macrophages to express IFN-γ in response to LPS (20).

IL-10 and IL-12 are both known to be released by *M. tuberculosis*-stimulated macrophages (19, 22, 32), although these cytokines appear to have opposing effects on IFN-γ production. We have demonstrated that IL-10 suppresses *M. tuberculosis*-induced IFN-γ production by AMϕ, whereas IL-12 can directly induce IFN-γ production by uninfected cells. Furthermore, anti-IL-12 antibodies could suppress *M. tuberculosis*-induced IFN-γ production, suggesting that *M. tuberculosis* may indirectly induce IFN-γ production via the ability of the bacilli to induce IL-12 production. Our data are consistent with a model in which the level of IFN-γ produced by *M. tuberculosis*-stimulated AMϕ is regulated by the balance between the levels of IL-10 and IL-12. Neutralizing antibodies against IL-10 have been shown to enhance IFN-γ production by PBMC from tuberculosis patients by enhancing IL-12 production (22), although IL-10 itself was not overproduced by these cells in vitro relative to PBMC from healthy tuberculin responders (43). IL-12 has been recently reported to induce IFN-γ production directly in murine peritoneal macrophages (33). It should also be noted that IFN-γ is a potent suppressor of IL-10 expression by human monocytes (8, 13). Conversely, IL-10 inhibits the expression of several proinflammatory cytokines by monocytic cells (17, 39, 44). IL-10 has been shown to inhibit the produc-

tion of IL-2 mRNA but not IFN-γ mRNA in activated human peripheral blood T cells (29). This contrasts with our finding that IL-10 can suppress the production of IFN-γ by *M. tuberculosis*-activated AMϕ. Roles for IL-10 and IL-12 in the regulation of IFN-γ production by AMϕ in vivo remain to be determined.

Macrophage cytokines are critically important in the control of tuberculosis (reviewed in reference 40). A central role for IFN-γ as a macrophage activating factor in mycobacterial infections has been clearly demonstrated in the mouse (reviewed in reference 16). Exposure of macrophages to IFN-γ results in increased expression of major histocompatibility complex class II molecules and the augmentation of cytokine production, NO production, and cytolytic activity (reviewed in reference 42). At least in mice, the production of NO is a powerful antimicrobial response to *M. tuberculosis* infection (7), although its role in human tuberculosis remains unclear. The importance of IFN-γ in the resistance to *M. tuberculosis* infection was best shown with mice which are homozygous for a targeted disruption of the IFN-γ gene (9, 18). Upon infection, these mutant mice developed granulomas, failed to produce NO, and were unable to restrict disseminated growth of the bacilli.

In human studies, IFN-γ failed to inhibit mycobacterial growth consistently within monocytic cells in vitro in the absence of other cytokines (12, 37). Some studies have reported that IFN-γ actually enhanced intracellular mycobacterial growth in vitro (5, 14). Levels of IFN-γ were reported to be elevated in the pleural fluid of patients with tuberculous pleuritis (2, 38), yet IFN-γ production by stimulated PBMC from tuberculosis patients was significantly reduced compared with that of healthy tuberculin responders (43). Nonetheless, the enhanced susceptibility to mycobacterial infection in persons with mutations of IFN-γ receptor 1 is powerful evidence that IFN-γ is critically involved in the control of human tuberculosis (24, 30). Thus, IFN-γ production by AMϕ may be part of the normal host response against this pathogen. It is interesting that exposure of AMϕ to *Cryptococcus neoformans*, a distinct lung pathogen, did not induce IFN-γ production (data not shown). This suggests that intracellular pathogens, such as *M. tuberculosis*, induce innate immune responses that are distinct from those induced by extracellular pathogens.

A commonly accepted model of human tuberculosis holds that AMϕ are ineffective at controlling initial infection. After recruitment of lymphocytes and stimulation with lymphocyte-derived IFN-γ, a protective response is developed. The finding that *M. tuberculosis*-infected AMϕ themselves are a source of IFN-γ and that these cells respond in an autocrine and/or paracrine fashion suggests that this model should be revised. Future studies are needed to address the relative contribution of macrophage-derived IFN-γ to the microbicidal activities of *M. tuberculosis*-infected AMϕ and ultimately to the course of tuberculosis disease.

ACKNOWLEDGMENTS

This work was supported by the following grants from the National Heart, Lung, and Blood Institute: HL55681 (M.J.F.), HL43510 (H.K.), and HL51957 (M.W.V.).

REFERENCES

1. **Arenberg, D. A., S. L. Kunkel, P. J. Polverini, S. B. Morris, M. D. Burdick, M. C. Glass, D. T. Taub, M. D. Iannettoni, R. I. Whyte, and R. M. Strieter.** 1996. Interferon-gamma-inducible protein 10 (IP-10) is an angiostatic factor that inhibits human non-small cell lung cancer (NLCLC) tumorigenesis and spontaneous metastases. J. Exp. Med. **184:**981–992.
2. **Barnes, P. F., S. Fong, P. J. Brennan, P. E. Twomey, A. Mazumder, and R. L. Modlin.** 1990. Local production of tumor necrosis factor and IFN-gamma in tuberculous pleuritis. J. Immunol. **145:**149–154.
3. **Becker, S., and H. S. Koren.** 1989. Dual effects of interferon-gamma on the interaction between human alveolar macrophages and *Cryptococcus neoformans*. Chest **95:**172S–174S.
4. **Bernaudin, J. F., K. Yamauchi, M. D. Wewers, M. J. Tocci, V. J. Ferrans, and R. G. Crystal.** 1988. Demonstration by in situ hybridization of dissimilar IL-1 beta gene expression in human alveolar macrophages and blood monocytes in response to lipopolysaccharide. J. Immunol. **140:**3822–3829.
5. **Blanchard, D. K., M. B. Michelini-Norris, C. A. Pearson, S. McMillen, and J. Y. Djeu.** 1991. Production of granulocyte-macrophage colony-stimulating factor (GM-CSF) by monocytes and large granular lymphocytes stimulated with *Mycobacterium avium-M. intracellulare*: activation of bactericidal activity by GM-CSF. Infect. Immun. **59:**2396–2402.
6. **Carvalho de Sousa, J. P., and N. Rastogi.** 1992. Comparative ability of human monocytes and macrophages to control the intracellular growth of *Mycobacterium avium* and *Mycobacterium tuberculosis*: effect of interferon-gamma and indomethacin. FEMS Microbiol. Immunol. **4:**329–334.
7. **Chan, J., Y. Xing, R. S. Magliozzo, and B. R. Bloom.** 1992. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. J. Exp. Med. **175:**1111–1122.
8. **Chomarat, P., M. C. Rissoan, J. Banchereau, and P. Miossec.** 1993. Interferon gamma inhibits interleukin 10 production by monocytes. J. Exp. Med. **177:**523–527.
9. **Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme.** 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J. Exp. Med. **178:**2243–2247.
10. **Dalton, D. K., S. Pitts-Meek, S. Keshav, I. S. Figari, A. Bradley, and T. A. Stewart.** 1993. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. Science **259:**1739–1742.
11. **Denis, M., and E. O. Gregg.** 1990. Recombinant tumour necrosis factor-alpha decreases whereas recombinant interleukin-6 increases growth of a virulent strain of *Mycobacterium avium* in human macrophages. Immunology **71:**139–141.
12. **Di Marzio, P., P. Puddu, L. Conti, F. Belardelli, and S. Gessani.** 1994. Interferon-gamma upregulates its own gene expression in mouse peritoneal macrophages. J. Exp. Med. **179:**1731–1736.
13. **Donnelly, R. P., S. L. Freeman, and M. P. Hayes.** 1995. Inhibition of IL-10 expression by IFN-gamma up-regulates transcription of TNF-alpha in human monocytes. J. Immunol. **155:**1420–1427.
14. **Douvas, G. S., D. L. Looker, A. E. Vatter, and A. J. Crowle.** 1985. Gamma interferon activates human macrophages to become tumoricidal and leishmanicidal but enhances replication of macrophage-associated mycobacteria. Infect. Immun. **50:**1–8.
15. **Farber, J. M.** 1993. HuMIG: a new member of the chemokine family of cytokines. Biochem. Biophys. Res. Commun. **192:**223–230.
16. **Fenton, M. J., and M. W. Vermeulen.** 1996. Immunopathology of tuberculosis: roles of macrophages and monocytes. Infect. Immun. **64:**683–690.
17. **Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard, and A. O'Garra.** 1991. IL-10 inhibits cytokine production by activated macrophages. J. Immunol. **147:**3815–3822.
18. **Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom.** 1993. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. J. Exp. Med. **178:**2249–2254.
19. **Fulton, S. A., J. M. Johnsen, S. F. Wolf, D. S. Sieburth, and W. H. Boom.** 1996. Interleukin-12 production by human monocytes infected with *Mycobacterium tuberculosis*: role of phagocytosis. Infect. Immun. **64:**2523–2531.
20. **Fultz, M. J., and S. N. Vogel.** 1995. Autoregulation by interferons provides an endogenous 'priming' signal for LPS-responsive macrophages. J. Endotoxin Res. **2:**77–84.
21. **Fultz, M. J., S. A. Barber, C. W. Dieffenbach, and S. N. Vogel.** 1993. Induction of IFN-gamma in macrophages by lipopolysaccharide. Int. Immunol. **5:**1383–1392.
22. **Gong, J.-H., M. Zhang, R. L. Modlin, P. S. Linsley, D. Iyer, Y. Lin, and P. F. Barnes.** 1995. Interleukin-10 downregulates *Mycobacterium tuberculosis*-induced Th1 responses and CTLA-4 expression. Infect. Immun. **64:**913–918.
23. **Green, L. C., D. A. Wagner, J. Glogowski, P. L. Skipper, S. Wishnkok, and S. R. Tannenbaum.** 1982. Analysis of nitrate and nitrite and (15N) nitrate in biological fluids. Anal. Biochem. **126:**131–141.
24. **Jouanguy, E., F. Altaire, S. Lamhamedi, P. Revy, J.-F. Emile, M. J. Newport, M. Levin, S. Blanche, E. Seboun, A. Fischer, and J.-L. Casanova.** 1996. Interferon-gamma-receptor deficiency in an infant with fatal bacille Calmette-Guerin infection. N. Engl. J. Med. **335:**1956–1961.
25. **Keane, J., K. Balcewicz-Sablinska, H. G. Remold, G. L. Chupp, B. B. Meek, M. J. Fenton, and H. Kornfeld.** 1997. Infection by *Mycobacterium tuberculosis* promotes human alveolar macrophage apoptosis. Infect. Immun. **65:**298–304.
26. **Kotloff, R. M., J. Little, and J. A. Elias.** 1990. Human alveolar macrophage and blood monocyte interleukin-6 production. Am. J. Respir. Cell Mol. Biol. **3:**497–505.
27. **Luster, A. D., J. C. Unkeless, and J. V. Ravetch.** 1985. Gamma-interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. Nature **315:**672–675.
28. **Luster, A. D., and J. V. Ravetch.** 1987. Biochemical characterization of a

gamma-interferon-inducible cytokine (IP-10). J. Exp. Med. **166:**1084–1097.
29. **Malefyt, R. D., H. Yssel, and J. E. de Vries.** 1993. Direct effects of IL-10 on subsets of human CD4+ T cell clones and resting T cells. J. Immunol. **150:**4754–4765.
30. **Newport, M. J., C. M. Huxley, S. Huston, C. M. Hawrylowicz, B. A. Oostra, R. Williamson, and M. Levin.** 1996. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. N. Engl. J. Med. **335:**1941–1949.
31. **Ohmori, Y., and T. A. Hamilton.** 1995. The interferon-stimulated response element and a kappa B site mediate synergistic induction of murine IP-10 gene transcription by IFN-gamma and TNF-alpha. J. Immunol. **154:**5235–5244.
32. **Orme, I. M., A. D. Roberts, J. P. Griffin, and J. S. Abrams.** 1993. Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. J. Immunol. **151:**518–525.
33. **Puddu, P., L. Fantuzzi, B. Varano, P. Borghi, S. F. Wolf, F. Belardelli, and S. Gessani.** 1996. IL-12 induces IFN-gamma expression and secretion in mouse peritoneal macrophages. Eur. Cytokine Netw. **7:**587.
34. **Pue, C. A., R. F. Mortensen, C. B. Marsh, H. A. Pope, and M. D. Wewers.** 1996. Acute phase levels of C-reactive protein enhance IL-1 beta and IL-1ra production by human blood monocytes but inhibit IL-1 beta and IL-1ra production by alveolar macrophages. J. Immunol. **156:**1594–1600.
35. **Rich, E. A., J. R. Panuska, R. S. Wallis, C. B. Wolf, M. L. Leonard, and J. J. Ellner.** 1989. Dyscoordinate expression of tumor necrosis factor-alpha by human blood monocytes and alveolar macrophages. Am. Rev. Respir. Dis. **139:**1010–1016.
36. **Robinson, D. S., S. Ying, I. K. Taylor, A. Wangoo, D. M. Mitchell, A. B. Kay, Q. Hamid, and R. J. Shaw.** 1994. Evidence for a Th 1-like bronchoalveolar T-cell subset and predominance of interferon-gamma gene activation in pulmonary tuberculosis. Am. J. Respir. Cell Mol. Biol. **149:**989–993.
37. **Rook, G. A., J. Steele, M. Ainsworth, and B. R. Champion.** 1986. Activation of macrophages to inhibit proliferation of *Mycobacterium tuberculosis*: comparison of the effects of recombinant gamma-interferon on human monocytes and murine peritoneal macrophages. Immunology **59:**333–338.
38. **Shimokata, K., H. Saka, T. Murate, Y. Hasegawa, and T. Hasegawa.** 1991. Cytokine content in pleural effusion. Comparison between tuberculous and carcinomatous pleurisy. Chest **99:**1103–1107.
39. **de Waal Malefyt, R., J. Abrams, B. X. Bennett, C. G. Figdor, and J. E. de Vries.** 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J. Exp. Med. **174:**1209–1220.
40. **Wallis, R. S., and J. J. Ellner.** 1994. Cytokines and tuberculosis. J. Leukocyte Biol. **55:**676–681.
41. **Yamamura, M., K. Uyemura, R. J. X. Deans, K. Weinberg, T. H. Rea, B. R. Bloom, and R. L. Modlin.** 1991. Defining protective responses to pathogens: cytokine profiles in leprosy lesions. Science **254:**277–279. (Erratum, **255:**12, 1992.)
42. **Young, H. A., and K. J. Hardy.** 1995. Role of interferon-gamma in immune cell regulation. J. Leukocyte Biol. **58:**373–381.
43. **Zhang, M., Y. Lin, D. V. Iyer, J. Gong, J. S. Abrams, and P. F. Barnes.** 1995. T-cell cytokine responses in human infection with *Mycobacterium tuberculosis*. Infect. Immun. **63:**3231–3234.
44. **Zhang, M., M. K. Gately, E. Wang, J. Gong, S. F. Wolf, S. Lu, R. L. Modlin, and P. F. Barnes.** 1994. Interleukin 12 at the site of disease in tuberculosis. J. Clin. Invest. **93:**1733–1739.
45. **Zhang, M., J. Gong, D. V. Iyer, B. E. Jones, R. L. Modlin, and P. F. Barnes.** 1994. T cell cytokine responses in persons with tuberculosis and human immunodeficiency virus infection. J. Clin. Invest. **94:**2435–2442.

Editor: S. H. E. Kaufmann