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Interleukin-12 Production by Human Monocytes Infected with *Mycobacterium tuberculosis*: Role of Phagocytosis

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***Mycobacterium tuberculosis* and its antigens are potent inducers of cytokine expression by mononuclear phagocytes. In this study, the ability of live *M. tuberculosis* to stimulate interleukin-12 (IL-12) expression by human monocytes was examined. Monocytes were purified from peripheral blood mononuclear cells by adherence and either infected with *M. tuberculosis* or exposed to soluble protein antigens of *M. tuberculosis* (purified protein derivative [PPD]). Live *M. tuberculosis* (10^6 to 10^7 CFU/ml) was a potent stimulus for interleukin-12 (IL-12). By using reverse transcription-PCR, p40 mRNA was detected at 3 h, peaked at 6 to 12 h, and decayed to baseline levels at 18 to 24 h following infection. Bioactive IL-12 (p70) was measured by the phytohemagglutinin blast proliferation assay and confirmed the p40 mRNA results. In contrast, soluble PPD at concentrations known to readily induce IL-1 and tumor necrosis factor alpha expression by monocytes (10 to 100 μg/ml) was a poor stimulus for IL-12 p40 mRNA expression. The different efficiencies of *M. tuberculosis* bacilli and PPD for IL-12 expression by monocytes was in part due to a requirement for phagocytosis. Induction of IL-12 in response to *M. tuberculosis* was reduced by cytochalasin D. Furthermore, phagocytosis of dead *M. tuberculosis* or inert 2-μm-diameter polystyrene beads by monocytes induced IL-12 p40 mRNA. In contrast, 0.5-μm-diameter beads, which can enter cells through pinocytosis, did not stimulate IL-12 expression. Functionally, IL-12 readily enhanced PPD-stimulated IFN-γ production and $CD4^+$ T-cell-mediated cytotoxicity by peripheral blood mononuclear cells from healthy tuberculin-positive donors but induced less enhancement when live *M. tuberculosis* was the antigen. These results suggest that IL-12 is upregulated as part of the early cytokine response of mononuclear phagocytes to *M. tuberculosis* and that the cellular events associated with phagocytosis are themselves a potent signal for IL-12 production. IL-12 released by infected macrophages in turn can further upregulate *M. tuberculosis*-specific $CD4^+$ T-cell effector function.**

Mycobacterium tuberculosis is an intracellular bacterial pathogen, which readily survives within human mononuclear phagocytes. The majority (>90%) of healthy individuals infected with *M. tuberculosis* develop a cellular immune response to this pathogen and successfully control its growth (10). The interaction of T cells and mononuclear phagocytes is critical in this acquired, protective immune response to *M. tuberculosis* and is associated with a proinflammatory cytokine environment, which includes interleukin-1 (IL-1) IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) (38).

Recent studies have demonstrated that upon exposure to *M. tuberculosis* bacilli or its constituents, monocytes/macrophages can secrete both proinflammatory cytokines such as IL-1, IL-6, and TNF-α and inhibitory cytokines such as IL-10 and transforming growth factor β (TGF-β) (4, 49, 54). Cytokines secreted by monocytes/macrophages thus play a major role in the regulation of the cellular immune response to *M. tuberculosis.*

IL-12 is a heterodimeric 70-kDa protein composed of two subunits (p35 and p40) linked by a disulfide bond (30, 47, 56). IL-12 is produced primarily by mononuclear phagocytes, B cells, and polymorphonuclear leukocytes, and its expression is elicited by microbial antigens (7, 14). Although the p40 subunit is secreted in excess over the p70, only the heterodimer is biologically active (22, 56). Immunological properties of IL-12 include the enhancement of IFN-γ production and cytotoxic activity of NK and T cells, including αβ ($CD4^+$ and $CD8^+$) and γδ T-cell receptor-bearing cells (5, 8, 17, 20, 32, 41, 57). In addition, IL-12 favors the development of Th-1 like T-cell responses by enhancing IFN-γ and antagonizing IL-4 and IL-10, thereby down-regulating Th-2 responses (9, 12, 13, 29, 31, 33, 40, 51). In murine models, the administration of exogenous IL-12 enhances immunity against *Mycobacterium tuberculosis* (11), *Listeria monocytogenes* (52), *Leishmania major* (26), *Toxoplasma gondii* (19), *Shistosoma mansoni* (39, 58), viruses (18, 37), and tumors (36). Recent studies with human subjects have demonstrated IL-12 in the pleural fluid of patients with tuberculous pleuritis (60) and in skin lesions of patients with tuberculoid leprosy (46). Little is known, however, about the ability of mycobacteria to induce IL-12 production by human mononuclear phagocytes, the principal host target for mycobacteria. The present study was undertaken to determine the ability of *M. tuberculosis* and its antigens to induce IL-12 expression by monocytes and to assess the role of phagocytosis in the regulation of IL-12 expression.

MATERIALS AND METHODS

Bacteria and antigens. *M. tuberculosis* H37Ra was cultured in Middlebrook 7H9 medium enriched with albumin, glucose, and catalase (ADC [Difco, Detroit, Mich.]) at 37°C for 3 to 4 weeks as previously described (24). Mid-log-phase cultures were pelleted, resuspended in 7H9 medium, and frozen in 1.0-ml aliquots at −70°C. Bacterial titers (viable counts) of freshly thawed samples were determined by counting CFU on 7H10 agar (Difco) after 2 to 3 weeks of incubation. The viability was routinely greater than 50%. For each experiment, mycobacteria were thawed, washed in RPMI 1640 (Bio Whittaker, Walkersville, Md.), pelleted (14,000 × *g* for 10 min), resuspended in RPMI 1640 (8×10^5 to

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8×10^7/ml), and sonicated for 40 s (90 W; 20 kHz; Heat Systems-Ultrasonics, Farmingdale, N.Y.) to disrupt clumps.

Purified protein derivative (PPD) of *M. tuberculosis* H37Ra was a generous gift from Lederle Laboratories, Pearl River, N.Y. Formalin-fixed *Staphylococcus aureus* Cowan 1 strain (Pansorbin; Calbiochem, La Jolla, Calif.) was washed in RPMI 1640, pelleted (14,000 × *g* for 10 min), and resuspended to 0.12% (wt/vol) in RPMI 1640. Working dilutions of *S. aureus* Cowan 1 (0.03 to 0.06%) were made in RPMI 1640.

Polystyrene beads 0.50, 2.07 and 3.71 μm in diameter (Duke Scientific, Palo Alto, Calif.) were washed in RPMI 1640, pelleted (14,000 × *g* for 10 min), and resuspended at concentrations ranging from 8×10^5 to 8×10^9/ml. All antigen preparations, polystyrene beads, and culture components were analyzed for lipopolysaccharide (LPS) contamination by the *Limulus* lysate enzyme-linked immunosorbent assay (ELISA; Bio Whittaker) and contained less than 50 pg/ml.

Isolation of PBMC and monocytes. Venous blood from healthy volunteers was drawn into heparinized syringes, diluted 1:2 in RPMI 1640, layered onto Ficoll-Paque (Pharmacia, Piscataway, N.J.), and centrifuged for 45 min at 1,000 rpm (200 × *g*). Peripheral blood mononuclear cells (PBMC) were isolated from the interface and washed three times in RPMI 1640. Approximately 50×10^6 PBMC were then placed in 100-mm polystyrene tissue culture plates (no. 25020; Corning, Corning, N.Y.) that had been precoated with heat-inactivated pooled human serum (PHS) for 30 min at 37°C. After a 1-h incubation at 37°C (with approximately 20% [vol/vol] PHS), nonadherent cells were removed by four washes. Adherent cells were refrigerated for 15 min in Ca^{2+}-Mg^{2+}-free phosphate-buffered saline (PBS [Bio Whittaker]) and collected by scraping with a plastic policeman (no. 3010; Costar Cambridge, Mass.). The adherent cells were washed once with RPMI 1640 and resuspended in complete medium (CM) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), penicillin-streptomycin (100 IU/ml and 100 μg/ml, respectively), and PHS (5 or 20% [vol/vol]). At least 95% of the adherent cells were monocytes as determined by both Wright-Giemsa staining (Diff-Quik Stain [Baxter Scientific Products, McGaw Park, Ill.]) and measurement of myeloperoxidase activity (Sigma, St. Louis, Mo.). The remaining 1 to 5% of cells consisted primarily of $CD3^+$ T lymphocytes. Each experiment was carried out with monocytes isolated from a single donor.

Monocyte stimulation and isolation of RNA. Adherence-purified monocytes (1.5×10^6 to 2.0×10^6/ml of CM with 20% PHS) were incubated overnight without stimulation in 1.5-ml polypropylene tubes (Sarstedt, Newton, N.C.) at 37°C in an atmosphere of 5% CO_2. After this overnight rest, the monocytes were stimulated with a variety of antigens and incubated for an additional 1 to 24 h. The monocytes were pelleted (8,000 × *g* for 5 min), and the medium was removed. The pellets were dispersed and solubilized with RNAzol B (200 μl/10^6 cells [TEL-TEST Inc., Friendswood, Tex.]). The homogenate was shaken for 15 s at room temperature, frozen on dry ice, and stored at −70°C until further use. Total RNA was extracted with chloroform-isoamyl alcohol (24:1) and precipitated with isopropanol as specified by the manufacturer. The RNA was resuspended in sterile diethylpyrocarbonate (Sigma)-treated, deionized, double-distilled H_2O, dissolved for 15 min at 65°C, and quantitated spectrophotometrically. RNA integrity was assessed by denaturing gel electrophoresis. Briefly, 1 to 2 μg was dried under vacuum for 5 min, suspended in 10.8 μl of sample buffer consisting of 20 mM MOPS (3-*N*-morpholinepropanesulfonic acid [Sigma]), 12.5 M formamide (Sigma), and 2.2 M formaldehyde (Fisher, Pittsburgh, Pa.) and heated for 15 min at 65°C. The RNA was electrophoresed (50 mA for 2 to 3 h) through a 1.0% agarose (Gibco BRL, Gaithersburg, Md.) gel (20 mM MOPS, 0.7 M formaldehyde) in 20 mM MOPS running buffer (pH 7.0). Only samples with intact 28S and 18S rRNA were used for reverse transcription (RT) reactions.

To inhibit the phagocytosis of particulate antigens, 1.5×10^6 to 2.0×10^6 rested monocytes were incubated with antigens (2×10^7 CFU of M. tuberculosis) in the presence of cytochalasin D (Sigma). Cytochalasin D was present during the entire stimulation period of 6 h. Total cellular RNA was isolated as described previously.

To quantitate phagocytosis, 2×10^5 monocytes were placed on sterile coverslips after 5 h of antigen stimulation. The monocytes were allowed to adhere to the glass coverslip for 1 h and then rinsed gently with sterile PBS. Adherent monocytes were fixed with absolute methanol for 1 min at room temperature. The monocytes were stained by either the cold Kinyoun method to enumerate acid-fast bacilli or the Wright-Giemsa (Diff-Quik Stain [Baxter Scientific Products]) method to enumerate *S. aureus* Cowan 1. The coverslips were mounted with Permount (Fisher Scientific) and viewed at a magnification of ×100 by either planar or phase-contrast light microscopy. The number of cells with ingested or closely associated organisms was recorded. The mean percentage of infected cells from four experiments was determined.

Semiquantitative RT-PCR. For each experiment, equivalent amounts of intact RNA (1 μg) were primed (70°C for 5 min, 0°C for 5 min, and 25°C for 5 min) with random hexamer oligonucleotides (100 mU [Boehringer-Mannheim, Indianapolis, Ind.]). cDNA was synthesized for 60 min at 42°C in a 25-μl reaction mixture containing 150 μM deoxynucleoside triphosphates (Boehringer-Mannheim), 1× buffer, 8 mM dithiothreitol, 200 U of Moloney murine leukemia virus reverse transcriptase (Superscript II; Gibco-BRL), 4 mM $MgCl_2$ (Promega, Madison, Wis.), and 20 U of rRNasin (Promega). The reaction was terminated by heating at 95°C for 5 min, and the mixture was diluted 1:4 with diethylpyrocarbonate-treated H_2O. Equivalent amounts of cDNA in 5.0-μl aliquots were amplified by PCR (Omnigene Temperature Cycler; National Labnet Co., Woodbridge, N.J.) in a 25-μl reaction mixture containing 1 mM $MgCl_2$, 1× buffer, 0.5 U of *Taq* polymerase (Promega), and 1.0 μM sense and 1.0 μM antisense primers (National Biosciences, Plymouth, Minn.). Semiquantitative conditions for amplification were determined experimentally for each primer pair. Using 1 μg of total RNA from *S. aureus* Cowan 1-stimulated monocytes as a control, we determined the optimal number of cycles whereby the PCR product was detectable in an amount that was proportional to the input quantity of cDNA. In addition, nonsaturating conditions were verified by using undilute and serially diluted cDNA mixtures (18, 46). All samples were first denatured at 95°C for 30 s and cycled with the following log-linear parameters: (i) for HPRT (hypoxanthine phosphoribosyltransferase), 35 cycles of denaturation (94°C for 10 s), annealing (55°C for 20 s), and extension (72°C for 2 min 40 s), and (ii) for TNF-α, IL-12 p40, and IL-12 p35, respectively, 25, 32, and 35 cycles of denaturation (95°C for 30 s), annealing (60°C for 40 s), and extension (72°C for 2 min 35 s). A final extension of 10 min at 72°C was performed for all samples. Positive cDNA controls and negative controls (reverse-transcribed diethylpyrocarbonate-treated H_2O) were included. The primer sequences were as follows: (i) for HPRT, 5′-GGA GAT GTG ATG AAG GAG ATG G-3′ (sense) and 5′-GGA TTA TAC TGC CTG ACC AAG G-3′ (antisense); (ii) for IL-12 p40, 5′-CCA AGA ACT TGC AGC TGA AG-3′ (sense) and 5′-TGG GTC TAT TCC GTT GTG TC-3′ (antisense); (iii) for IL-12 p35, 5′-CCT CAG TTT GGC CAG AAA CC-3′ (sense) and 5′-GGT CTT TCT GGA GGC CAG GC-3′ (antisense); and (iv) for TNF-α, 5′-TCT CGA ACC CCG AGT GAC AA-3′ (sense) and 5′-TCC CAG ATA GAT GGG CTC AT-3′ (antisense). PCR product mixture (20 μl) was electrophoresed (110 V for 1 h) through a 1.5% agarose gel (in 0.5× Tris-boric acid-disodium EDTA [Bio Whittaker]), denatured (1.5 M NaCl, 0.5 M NaOH), neutralized (1.5 M NaCl, 1.0 M Tris-HCl), and transferred onto nylon (Hybond-N^+ [Amersham, Arlington Heights, Ill.]) with 10× SSC (0.15 M trisodium citrate and 1.5 M NaCl). The membranes were UV exposed (120 J [UV-Stratalinker; Stratagene, La Jolla, Calif.]), dried (80°C for 4 h), hybridized overnight with fluoresceinated probes, and processed as recommended by the manufacturer (3′-oligonucleotide-labeling system and enhanced chemiluminescence [Amersham]). The probe sequences were as follows: (i) for HPRT, 5′-GCT GAC CTG GAT TAC AT-3′; (ii) for IL-12 p40, 5′-TCT GCC GCA AAA ATG CCA GC-3′; (iii) for IL-12 p35, 5′-CCA AAA CCT GCT GAG GGC CG-3′; and (iv) for TNF-α, 5′-GTG GAG CTG AGA GAT AAC-3′ (National Biosciences). X-ray film (X-Omat AR [Kodak, Rochester, N.Y.]) was exposed for minimal times (0.5 to 20 min) and developed with an automatic film processor (M35A X-Omat processor [Kodak]).

IL-12 protein measurement. IL-12 p70 was measured by a modification of the phytohemagglutinin (PHA) blast proliferation assay described by Stern et al. (47). PBMC were isolated as described above and cultured at a density of 5×10^5/ml in supplemented medium containing 0.5% (vol/vol) phytohemagglutinin-M (Gibco). After 3 days at 37°C, PHA blasts were split 1:2 with fresh medium and incubated for an additional 24 h. Four-day-activated PHA blasts were washed four times and resuspended at 10^6/ml. Cell stocks were stored in liquid N_2 for further use.

PHA blasts (25 μl; 2.5×10^4/ml) were cultured for 48 h in 96-well plates (Falcon no. 3072 [Becton Dickinson, Lincoln Park, N.J.]) containing 25 μl of culture supernatant (diluted 1:2 to 1:8), 25 μl of polyclonal rabbit anti-human IL-2 antibody (final concentration, 1:100 [no. 40012; Collaborative Biomedical Products, Bedford, Mass.]), and either 25 μl of polyclonal anti-human IL-12 (1 μg/ml, capable of neutralizing 50 to 100 U of IL-12 per ml) or 25 μl of CM (plus 5% PHS) to measure nonneutralized proliferation. An IL-12 dose-response standard curve was established by culturing PHA blasts with recombinant human IL-12 (0.1 to 500 U/ml). After an overnight pulse with 1 μCi of [^{3}H]thymidine (ICN Radiochemical, Irvine, Calif.), cultures were harvested and counted with the Matrix-96 β-counter system (Packard Instruments Co., Downers Grove, Ill.). Neutralizable counts per minute (cpm) (i.e., cpm without anti IL-12 minus cpm with anti-IL-12) were used to determine the IL-12 concentration from the standard curve. The sensitivity of the assay was approximately 10 U/ml. PHA blast responsiveness was confirmed by incubation of PHA blasts with 12.5 U of recombinant IL-2 (Proleukin; Chiron Co., Emoryville, Calif.) per ml. IL-2 proliferation was not inhibited by coincubation with anti-IL-12 antibody. The neutralizing polyclonal rabbit serum did not stimulate PHA blasts.

Some culture supernatants were also tested by ELISA for p40 and p70. Immulon 4 plates (Dynatech Laboratories, Chantilly, Va.) were coated with monoclonal anti-human p40 (2-4A1) or anti-p70 (20C2) in 0.1 M carbonate buffer overnight at room temperature. The plates were washed, blocked with PBS–1% bovine serum albumin for 1 h at 37°C, and incubated with recombinant protein or culture supernatants for 3 h at room temperature. Biotinylated secondary antibody (4D6; 500 ng/ml) was added for 1 h at 37°C, and the samples were reacted with streptavidin-peroxidase for 30 min at 37°C (Sigma) and developed with ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)]. The A_{405} was measured with an automated ELISA plate reader (Molecular Device, Menlo Park, Calif.).

Measurement of IL-2, IFN-γ, and TNF-α. Supernatants were harvested from monocyte or PBMC cultures after 24 h for IL-2 and TNF-α and after 48 h for IFN-γ. Samples were clarified through prewetted 0.2-μm-pore-size filters and stored at −70°C. IL-2 concentrations in culture supernatants were determined by

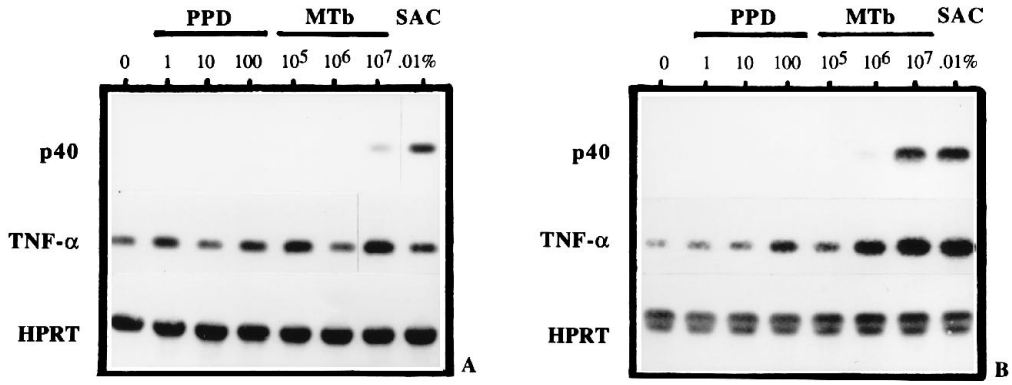

FIG. 1. Induction of IL-12 p40 mRNA in monocytes stimulated with PPD, *M. tuberculosis* (MTb), and *S. aureus* Cowan 1 (SAC). Adherence-purified monocytes (1.5×10^6) were isolated, incubated overnight, and stimulated for 6 h with RPMI 1640, PPD (1.0, 10.0 or 100 μg/ml), *M. tuberculosis* (10^5/ml, 10^6/ml, or 10^7/ml), or SAC. Total cellular RNA (1 μg) was isolated, reverse transcribed, and PCR amplified for HPRT, IL-12 p40, and TNF-α as described in Materials and Methods. Then 20 μl of each PCR mixture was separated on a 1.5% agarose gel and transferred to nylon. The Southern blots were developed by enhanced chemiluminescence, and the results of two representative experiments are shown.

the standard CTLL proliferation assay. Briefly, 10^4 CTLL cells were mixed with equal volumes of either culture supernatant or recombinant IL-2 (0.1 to 100 U/ml) and incubated for 24 h. After a 6- to 8-hour pulse with 1 μCi of [^{3}H]thymidine, cpm were determined as described above. The IL-2 concentrations were determined from the standard curve, where 1.0 U/ml was defined as one-half maximal stimulation.

IFN-γ and TNF-α concentrations were determined by ELISA. Briefly, Immulon 4 plates were coated with mouse anti-human TNF-α (Endogen, Boston, Mass.) or mouse anti-human IFN-γ (Interferon Sciences, New Brunswick, N.J.) overnight at 4°C. The plates were washed three times, blocked with PBS–1% bovine serum albumin for 60 min at 37°C, and then incubated with culture supernatants at 37°C for 2 h (IFN-γ) or 1 h (TNF-α). Culture supernatants were used either straight (for IFN-γ) or diluted 1:2 (for TNF-α). After three washes, rabbit anti-human IFN-γ (1:1,000 [Interferon Sciences]) or rabbit anti-human TNF-α (1:1,000 [Genzyme, Cambridge, Mass.]) was added for 60 min at 37°C. After four washes, bound antibody was detected with peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) diluted 1:1,000 for IFN-γ or 1:5,000 for TNF-α. Peroxidase substrates (ABTS for IFN-γ and *o*-phenylenediamine dihydrochloride for TNF-α) were added, and the A_{405} and A_{490} respectively, were measured.

Proliferation and cytotoxicity assays. Proliferation and cytoxicity assays were performed as described previously (6, 25). For proliferation assays, PBMC were cultured with antigen in complete medium for 5 days and then pulsed with [^{3}H]thymidine for 18 h before being harvested. For cytotoxicity assays, fresh autologous monocytes were incubated overnight with PPD (20 μg/ml) or no antigen. After incubation, the monocytes were washed three times and labeled for 1 h at 37°C with 100 μCi of ^{51}Cr (New England Nuclear, Boston, Mass.). After being washed, 5×10^3 ^{51}Cr-labeled monocyte targets were added to 96-well round bottom plates. PBMC stimulated with PPD for 7 to 10 days were used (2.5×10^5 to 5×10^3) as effector cells (50:1 to 1:1 effector-to-target-cell ratio). Assays were performed in triplicate for each effector-to-target-cell ratio. After a 4-h incubation, culture supernatants were harvested, and ^{51}Cr release was measured by gamma counting. Spontaneous release was measured in wells containing target cells and medium alone. Maximum release was determined by treating target cells with 1% sodium dodecyl sulfate. The percent specific release was calculated for each experiment group by the following equation: $100 \times$ [(mean cpm experimental − mean cpm spontaneous release)/(mean cpm maximum − mean cpm spontaneous release)]. A specific release of 10% or more was considered to indicate significant cytotoxicity. Spontaneous ^{51}Cr release from monocytes was ≤15% for unpulsed macrophages and PPD-pulsed monocytes.

RESULTS

Induction and kinetics of IL-12 p40 mRNA expression in monocytes stimulated with *M. tuberculosis*. Initial studies were designed to determine if infection with live *M. tuberculosis* or exposure to mycobacterial antigens stimulated IL-12 production by monocytes. The expression of IL-12 p40 mRNA was measured by RT-PCR and used as an indicator of IL-12 expression. Log-linear RT-PCR conditions for IL-12 p40 mRNA detection were established with RNA isolated from monocytes stimulated with *S. aureus* Cowan 1, a known stimulus of IL-12 (14). Initially, we found that freshly isolated adherence-purified monocytes at times expressed p40 and p35 mRNA in the absence of antigen, suggesting that plastic adherence itself stimulated IL-12 expression. Therefore, to study antigen-specific IL-12 induction, monocytes were rested overnight (16 to 18 h) before exposure to antigen. Under these conditions, unstimulated rested monocytes revealed no IL-12 p40 mRNA expression (see Fig. 1 to 4).

Induction of IL-12 p40 mRNA was examined in monocytes from 11 donors, and although there was variability in the amount of p40 expression, all donors were inducible for IL-12 p40 mRNA expression in response to live *M. tuberculosis* bacilli. The results of two representative antigen dose-response experiments are shown in Fig. 1. Optimal IL-12 p40 mRNA expression was measured at 10^7 CFU of *M. tuberculosis* per ml (Fig. 1), and in some experiments, upregulated mRNA transcription was noted at 10^6 CFU/ml (Fig. 1B). *S. aureus* Cowan 1 served as a positive control for IL-12 p40 mRNA expression. There was a correlation between the IL-12 p40 mRNA signal and the degree of monocyte infection, which typically ranged from 60 to 80% after a 6-h incubation at a 10:1 *M. tuberculosis*-to-monocyte ratio (10^7 CFU/ml). Although IL-12 p35 mRNA expression was present in both stimulated and unstimulated cultures, upregulation of IL-12 p35 mRNA was noted in response to *M. tuberculosis* (data not shown).

Surprisingly, PPD at concentrations (1.0 to 100 μg/ml) known to induce IL-1, IL-10, TNF-α, and TGF-β expression by monocytes failed to induce IL-12 p40 mRNA (50, 54). Low levels of IL-12 p40 mRNA were detected in some donors when higher concentrations of PPD were used to stimulate monocytes (data not shown). To ensure that the mycobacterial antigen preparations were stimulating monocytes, TNF-α mRNA expression was measured. TNF-α mRNA (Fig. 1) and protein (123 to 3,156 pg/ml) (data not shown) were readily upregulated by low concentrations of PPD or small numbers of *M. tuberculosis* cells, suggesting that the threshold for IL-12 p40 mRNA induction was higher than TNF-α mRNA. We cannot exclude the possibility that contaminating T cells enhance TNF-α more efficiently than they enhance IL-12. However, ongoing studies suggest that the addition of increasing numbers of nonadherent cells to infected monocytes does not enhance IL-12 p40 protein expression.

To characterize the kinetics of IL-12 p40 mRNA induction, resting monocytes were stimulated for 1, 3, 6, 12, and 24 h with either no antigen, *M. tuberculosis*, PPD, or *S. aureus* Cowan 1.

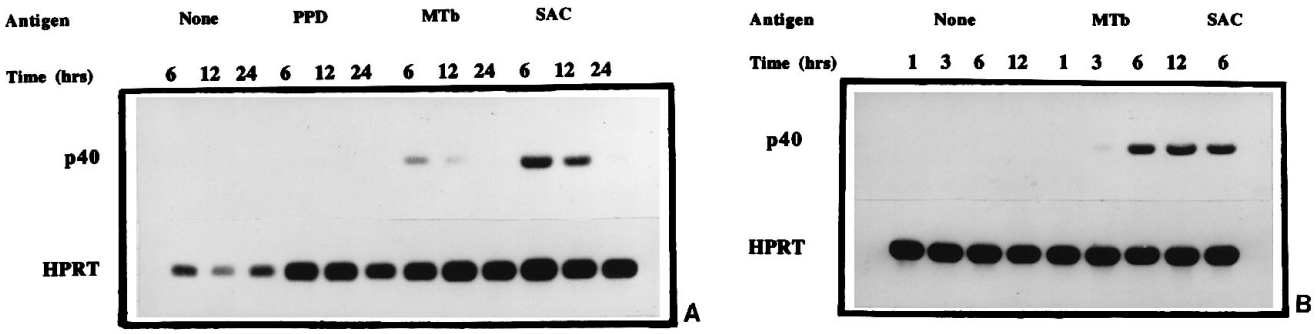

FIG. 2. Kinetics of IL-12 p40 mRNA expression in monocytes stimulated with *M. tuberculosis* (Mtb) and PPD. Monocytes (1×10^6 [A] or 1.5×10^6 [B]) were isolated from two donors, incubated overnight, and stimulated with PPD (10 μg/ml), *M. tuberculosis* (2×10^7/ml), or *S. aureus* Cowan 1 (SAC) for 1, 3, 6, 12, or 24 h as designated. Total cellular RNA (1 μg) was isolated, reverse transcribed, and PCR amplified for HPRT and IL-12 p40 as described in Materials and Methods. The Southern blots displayed were developed by enhanced chemiluminescence and are representative of five experiments.

The results of two representative experiments are shown in Fig. 2. IL-12 p40 mRNA was measured as early as 3 h after exposure to *M. tuberculosis*, and maximal stimulation was observed after 6 to 12 h. By 18 to 24 h, p40 mRNA levels were markedly diminished or had returned to baseline for both *M. tuberculosis* and *S. aureus* Cowan 1, despite continued exposure of the monocytes to these two bacterial preparations. As noted before, PPD (10 μg/ml) did not stimulate IL-12 p40 mRNA during the 24-h stimulation period.

To exclude LPS contamination as a stimulus for IL-12 induction, LPS levels were measured by the *Limulus* lysate ELISA in PPD, *M. tuberculosis*, *S. aureus* Cowan 1, and culture media and were consistently less than 50 pg/ml. Furthermore, in experiments in which polymyxin B (5 μg/ml) was added as an inhibitor of LPS (27), no inhibition of *M. tuberculosis*-induced p40 expression was noted (data not shown).

IL-12 protein (p70) secretion by monocytes infected with *M. tuberculosis*. To confirm that p40 mRNA expression measured by RT-PCR reflected biologically active IL-12 (p70) secretion, 24-h culture supernatants from infected monocytes were tested for IL-12 p70 in the PHA-activated lymphocyte proliferation assay (47). The cumulative data from six experiments in which monocytes were stimulated with various numbers of *M. tuberculosis* show that consistent with the p40 mRNA results (Fig. 1 and 2), substantial amounts of IL-12 (439.1 ± 320.9 U/ml [range, 80 to 1,490 U/ml]) were produced in the presence of 2×10^7 CFU of *M. tuberculosis* per ml of culture supernatant, whereas no IL-12 was produced in the absence of antigen and only 11.6 ± 15.7 and 70.2 ± 42.7 U/ml were produced in the presence of 2×10^5 and 2×10^6 CFU/ml, respectively. When 0.0075% *S. aureus* Cowan 1 was used as the antigen, 2,199.7 ± 1,052.5 U of IL-12 per ml was produced. A 35-fold (12.0 to 440.0 U/ml) increase in IL-12 p70 production was measured over the range of *M. tuberculosis* concentrations examined. (1.0 U/ml is equivalent to 5.86 pg/ml). As observed for p40 mRNA (Fig. 1 and 2), IL-12 p70 was not measurable in response to low concentrations of PPD. In some experiments, however, IL-12 p40 and p70 were measurable at 100 μg of PPD per ml or above (data not shown). Independent measurement of IL-12 by p70 capture ELISA from selected experiments corroborated the results observed by the PHA blast proliferation assay (data not shown). These results demonstrate that measurement of IL-12 p40 mRNA by RT-PCR correlated with bioactive IL-12 p70 protein secretion by monocytes.

Inhibition of IL-12 p40 expression by cytochalasin D. The results described above suggested that particulate microbial antigens such as live *M. tuberculosis* and formaldehyde-fixed *S. aureus* Cowan 1 were potent stimuli for the induction of IL-12 p40 mRNA. In contrast, stimulation with a soluble mycobacterial antigen (PPD) was found to be a weak signal for both p40 mRNA and bioactive p70 protein. These observations suggested that phagocytosis of particulate antigen itself might be a potent signal for the induction of IL-12 expression. First, we determined if IL-12 p40 mRNA expression in monocytes was dependent on mycobacterial viability. Treatment of *M. tuberculosis* with 1.5% formaldehyde (28) reduced final viability to less than 1 CFU/ml but did not affect the induction or kinetics of IL-12 p40 mRNA and protein expression (data not shown). Next, we examined IL-12 p40 mRNA expression in the presence of cytochalasin D, an inhibitor of phagocytosis (23). Monocytes were stimulated with live *M. tuberculosis* for 6 h in the presence of cytochalasin D, and IL-12 p40 mRNA levels were compared. As shown in Fig. 3, 5.0 μg of cytochalasin D per ml decreased *M. tuberculosis*-induced IL-12 p40 mRNA expression. At this concentration of cytochalasin D, phagocytosis of *M. tuberculosis* was reduced by >80% as determined by light microscopy. In a representative experiment, IL-12 p40 protein was decreased by 18 to 40% when monocytes were incubated with 5 to 10 μg of cytochalasin D per ml (the p40 levels were 1,010.7, 1,039.5, 819.7, and 599.5 pg/ml for cytocha-

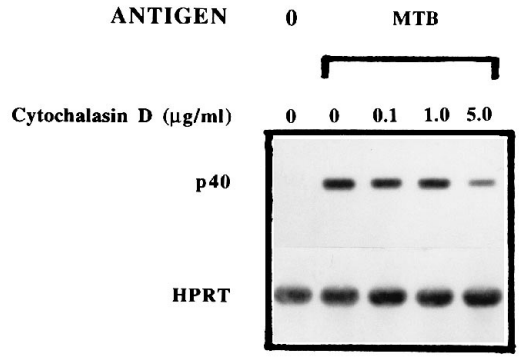

FIG. 3. IL-12 p40 mRNA expression in monocytes stimulated with *M. tuberculosis* (MTB) in the presence of cytochalasin D. Adherence-purified monocytes (2×10^6) were isolated and incubated overnight as described in the text. The monocytes were stimulated without antigen or *M. tuberculosis* (2×10^7/ml) for 6 h in the absence and presence of cytochalasin D (0.1, 1.0, and 5.0 μg/ml). Total cellular RNA (1 μg) was isolated, reverse transcribed, and PCR amplified for HPRT and IL-12 p40 as described in Materials and Methods. The PCR products were transfered to nylon membranes and visualized by enhanced chemiluminescence. The results are representative of four experiments.

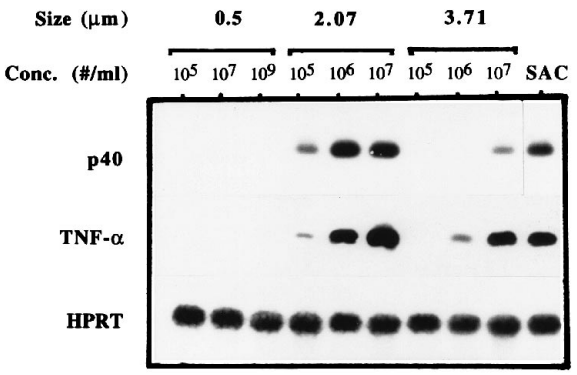

FIG. 4. IL-12 p40 mRNA expression in monocytes stimulated with polystyrene microspheres. Monocytes (1.5×10^6) were isolated, incubated overnight, and stimulated for 6 h with either 0.50-μm (2×10^5, 2×10^7, or 2×10^9), 2.07-μm (2×10^5, 2×10^6, 2×10^7), or 3.7-μm (2×10^5, 2×10^6, 2×10^7) polystyrene microspheres or *S. aureus* Cowan 1 (SAC). RNA (1 μg) was isolated, reverse transcribed, and cDNA PCR amplified for HPRT, IL-12 p40, and TNF-α as described in Materials and Methods. A Southern blot representing four experiments is shown. Unstimulated monocytes were negative for p40 mRNA under the described conditions.

lasin D concentrations of 0, 1.0, 5.0, and 10.0 μ/ml, respectively. In four experiments, the percent inhibition ranged from 28 to 52% (data not shown). Monocytes infected with *M. tuberculosis* in the absence of cytochalasin D and unstimulated monocytes served as the respective positive and negative controls. The effect was not due to nonspecific cytotoxicity, since the expression of HPRT (Fig. 3) was unaffected by cytochalasin D. These observations suggested that phagocytosis was an important signal for the induction of IL-12 p40 mRNA expression by *M. tuberculosis* and that mycobacterial viability was not required. Since the process of phagocytosis begins with particle-membrane interactions, we cannot formally exclude the possibility that adherence of the organism is the actual initiating event.

IL-12 p40 mRNA induction in monocytes by polystyrene microspheres. To extend the observation that phagocytosis of particulate antigen was a signal for IL-12 expression, IL-12 expression in monocytes exposed to inert, polystyrene microspheres was examined. As shown in Fig. 4, at particle-to-cell ratios similar to those used for *M. tuberculosis*, 2.07-μm and 3.71-μm-diameter beads induced IL-12 p40 mRNA and TNF-α mRNA expression. Preincubation of the beads with polymyxin B did not reduce IL-12 p40 mRNA expression (data not shown). In addition, stimulation of monocytes with 2.07-μm beads resulted in the secretion of bioactive IL-12 p70 (2,000 to 4,000 U/ml) of the same level as that observed for *S. aureus* Cowan 1 (see above).

By light microscopy, complete and incomplete ingestion of the 3.71-μm beads occurred less frequently than that observed for the 2.07-μm beads (30 to 40% versus 50 to 60%). These differences could explain the lower efficiency of the 3.71-μm beads in inducing IL-12 expression. In contrast, 0.50-μm beads induced neither IL-12 p40 mRNA nor TNF-α even at concentrations up to 2×10^9/ml (ratio of beads to monocytes, 1,000:1). By phase-contrast light microscopy, 0.50-μm beads were observed within 30 to 40% of monocytes after the 6-h stimulation. However, particles of this size can enter cells by pinocytosis and do not require phagocytosis (42). These results indicated that the size of the particle ingested by the monocyte was an important factor in triggering IL-12 and that particles requiring phagocytosis were effective stimuli for IL-12 production. The requirement for particles of optimal size also was

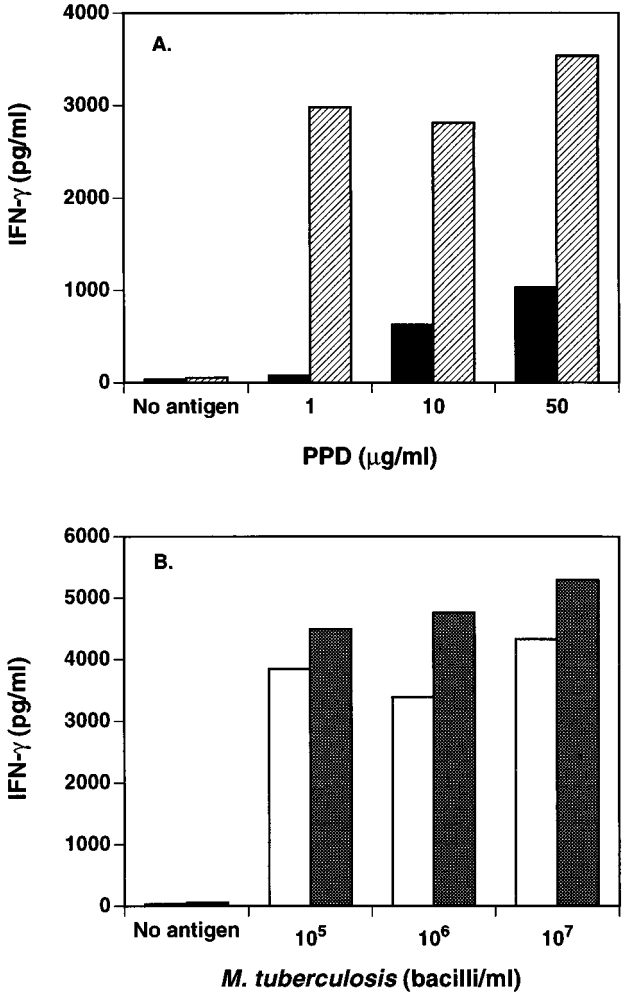

FIG. 5. IL-12 enhancement of IFN-γ production by PBMCs stimulated with PPD and *M. tuberculosis*. PBMCs (2×10^5 cells per 0.2-ml well) from a healthy tuberculin-reactive individual were stimulated with either medium alone, PPD (A), or live *M. tuberculosis* (B) in the absence or presence of human IL-12 (100 U/ml). IFN-γ was measured in supernatants harvested at 48 h as described in Materials and Methods. Symbols in panel A: ■, no IL-12; ▨, IL-12; symbols in panel B: □, no IL-12; ▩, IL-12.

demonstrated in experiments with *M. tuberculosis* bacilli which were fragmented by repeated freeze-thawing and sonication. Fragmentation and a reduction in the multiplicity of infection (see above and Fig. 1.) both contributed to the diminished expression of IL-12 p40 mRNA (data not shown).

IL-12 enhancement of IFN-γ secretion and cytotoxic effector function. The major biological activities of IL-12 involve cellular immune responses and include enhancement of IFN-γ production by and cytotoxic effector function of NK and T cells. Given the results demonstrating that live *M. tuberculosis* was a more efficient stimulus for IL-12 production than was PPD, we tested whether the addition of exogenous IL-12 could enhance IFN-γ secretion by T cells stimulated by either PPD or *M. tuberculosis*. In these experiments, PBMC from healthy tuberculin-positive donors were stimulated with either PPD or live *M. tuberculosis* in the presence or absence of 100 U of IL-12 per ml and supernatants were tested for IFN-γ secretion. IL-12 enhanced IFN-γ secretion in response to PPD to a far greater extent than to *M. tuberculosis*: 5.1-fold for PPD and 1.8-fold for *M. tuberculosis* ($n = 6$). Results from a represen-

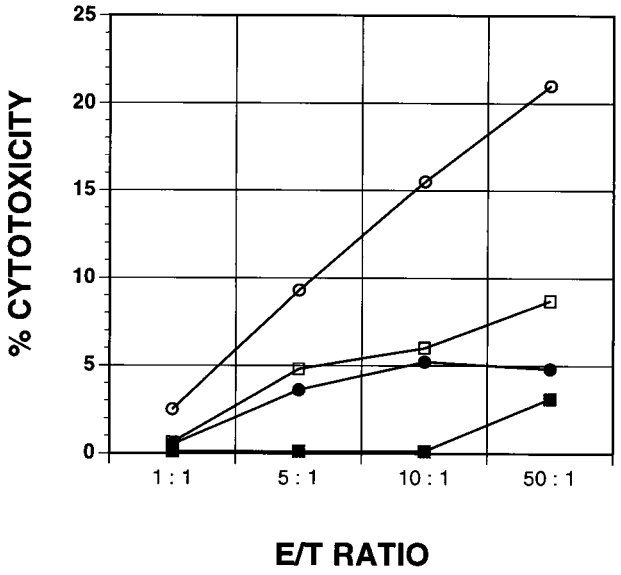

FIG. 6. Enhancement by IL-12 of PPD-specific cytotoxicity by PBMCs for autologous monocytes. PBMC (2×10^6 per 2-ml well) from a healthy tuberculin-reactive individual were stimulated with PPD with or without the addition of exogenous IL-12 (100 U/ml). After 10 days, PPD-stimulated PBMCs were used as effector cells with autologous monocytes as targets, as described in Materials and Methods. Symbols: ■, no antigen; ●, no antigen, IL-12 present; □, PPD; ○, PPD plus IL-12; E/T ratio, effector-to-target-cell ratio.

tative experiment are shown in Fig. 5. Proliferative responses of PBMCs to mycobacterial antigens were not increased by addition of IL-12 (data not shown). IL-12 also was able to enhance antigen-specific cytotoxicity for monocytes pulsed with PPD, as shown in Fig. 6. PBMCs from tuberculin-positive donors were stimulated with PPD with and without IL-12 (100 U/ml) for 10 days before use as cytotoxic effector cells with monocytes pulsed with *M. tuberculosis*. The predominant cytotoxic effector cell is $CD4^+$ when PPD is the antigen (53). These functional results with PBMC in which responses to PPD were readily enhanced by adding exogenous IL-12 correlated with the minimal IL-12 expression by monocytes after stimulation with PPD. Thus, the IL-12 produced by monocytes infected with *M. tuberculosis* would play an important role in the development of the subsequent protective immune response.

DISCUSSION

Monocytes and macrophages play a critical role in the protective cellular immune response to *M. tuberculosis*. Mononuclear phagocytes serve not only as effector cells for control of mycobacterial growth but also as antigen-presenting cells responsible for initiation and activation of T-cell responses. Protective T-cell responses to *M. tuberculosis* are characterized by IFN-γ production and cytotoxicity against macrophages infected with tubercle bacilli, and they are consistent with a Th-1-like response. Bacterial antigens, including mycobacterial protein and nonprotein antigens, are potent activators of mononuclear phagocytes, resulting in the secretion of a large number of proinflammatory (IL-1, IL-6, IL-8, IL-12, and TNF-α) and inhibitory (IL-10 and TGF-β) cytokines. The kinetics and balance of cytokines secreted by mononuclear phagocytes after exposure to microbial antigens regulate subsequent T-cell responses. On the basis of murine studies with model antigens and infectious pathogens, IL-12 is thought to play an important role in the initiation and regulation of Th-1-like responses. These studies have used mostly exogenous IL-12 or, to a lesser extent, blocking antibodies to determine the biological or pharmacologic effect of IL-12 in vivo and in vitro. Few studies have evaluated the ability and mechanisms of microbial pathogens, including *M. tuberculosis*, to stimulate IL-12 production by human mononuclear phagocytes. We have found that IL-12 is induced readily and rapidly after infection with *M. tuberculosis*, that phagocytosis is a potent signal for IL-12 production by monocytes, and that IL-12 secreted by *M. tuberculosis*-infected monocytes can modulate IFN-γ production and cytotoxicity by $CD4^+$ T cells. Thus, IL-12 is produced by mononuclear phagocytes as part of cellular events associated with phagocytosis and can regulate early cellular immune responses to intracellular pathogens such as *M. tuberculosis.*

Infection of monocytes with live *M. tuberculosis* induced IL-12 p40 mRNA within 3 to 6 h, and the p40 mRNA levels remained elevated for 6 to 12 h before returning to baseline by 18 to 24 h. The kinetics for p40 mRNA were similar to those observed for mouse peritoneal macrophages infected with *Toxoplasma gondii* tachyzoites and human epidermal keratinocytes exposed to soluble contact allergens in vivo (19, 34). The expression of IL-12 in keratinocytes (34), however, may represent the cumulative effect of antigen on neighboring cells present in the skin prior to the isolation of the keratinocytes. In contrast, soluble mycobacterial antigens were a much weaker stimulus for IL-12 production and required much higher concentrations than those necessary to stimulate IL-1, TNF-α, IL-10, and TGF-β production by monocytes (50, 54). Our novel finding that phagocytosis was a potent stimulus for IL-12 production provides one explanation for the difference in the efficiency of IL-12 induction between intact particulate and soluble mycobacterial antigens. A similar distinction was suggested by Scheicher et al. in studies on the processing of soluble and bead-bound conalbumin in phagocytic, dendritic murine cells, although in their study, no difference in p40 mRNA induction between soluble and bead-bound antigen was noted (44).

The importance of phagocytosis as a signal for IL-12 induction was demonstrated by three lines of evidence. First, the ingestion of very different particulate antigens was associated with IL-12 expression. *S. aureus*, a non-LPS-containing gram-positive bacterium, was used as a formaldehyde-fixed preparation, which was readily phagocytosed by monocytes. *M. tuberculosis*, characterized by a complex glycolipid cell wall containing the LPS analog lipoarabinomannan, was used as a live preparation. Inert polystyrene beads were the third stimulus. All three particulate stimuli, each with unique surface characteristics and without obvious common ligands for phagocytes, stimulated IL-12 production. Functional differences among particulate stimuli for monocyte activation were predicted based on studies by Schnyder and Baggiolini, which demonstrated that phagocytosis of zymosan (*Saccharomyces cerevisiae*) or formaldehyde-fixed sheep erythrocytes by murine resident peritoneal cells induced β-glucuronidase, lactate dehydrogenase, and plasminogen activator activity whereas latex beads did not (45). Therefore, both ligand-specific receptor-mediated (e.g., C3b, FcR) and nonspecific (latex) phagocytosis by monocytes may be dominant proximal signals for IL-12 expression, which does not depend completely upon later metabolic activation events. Second, intact nonviable mycobacteria induced IL-12 p40 mRNA as efficiently as live organisms did, while fragmentation of mycobacteria reduced IL-12 p40 mRNA expression. The lack of IL-12 p40 mRNA induction by 0.5-μm-diameter beads, which did not induce IL-12 p40 mRNA (Fig. 4), further supports the hypothesis that a phagocytic signal is important for the induction of IL-12 by *M. tu-*

berculosis infection. Third, cytochalasin D reduced IL-12 p40 mRNA production.

The relationship between phagocytosis and cellular signalling events is poorly understood. Using zymosan as a model antigen, Zaffran et al. demonstrated that phagocytosis induced protein tyrosine kinase activity which was associated with cytoskeletal actin filaments adjacent to phagocytic cups (59). Inhibition of protein tyrosine kinase activity with genestein or herbimycin A reduced zymosan ingestion. Similarly, zymosan binding resulted in TNF-α production by an LPS-independent pathway, which was regulated in part by protein kinase C and was associated with the cytoskeleton (43). Similar results were reported for Fc receptor-mediated uptake of opsonized sheep erythrocytes by mouse peritoneal exudate cells (21). Furthermore, granulocyte-macrophage colony-stimulating factor mRNA was upregulated in murine macrophages by particulate antigens such as opsonized sheep erythrocytes and polystyrene beads, as well as by soluble LPS (48). The association of cytoskeletal proteins and tyrosine kinase activity during phagocytosis might account for the ability of disparate particulate antigens to regulate cytokines such as IL-12, TNF-α, and granulocyte-macrophage colony-stimulating factor.

Our results do not exclude the possibility of alternative mechanisms for IL-12 expression in monocytes exposed to mycobacterial antigens, since soluble mycobacterial antigens were able to induce some IL-12, albeit at high concentrations. A number of defined mycobacterial antigens, including lipoarabinomannan and protein antigens such as 30-kDa fibronectin-binding antigen and a 58-kDa secreted antigen, induce TNF-α production by mononuclear phagocytes (2, 3, 55). Ongoing studies will determine if these defined antigens can stimulate IL-12 production as efficiently as phagocytosis. Soluble LPS can induce both TNF-α and IL-12 p40 mRNA, with the TNF-α concentration peaking at 1 h and the IL-12 p40 concentration peaking at 4 to 8 h (13). LPS also can induce expression of IL-12 in polymorphonuclear leukocytes, with p40 mRNA first observed at 3 h and maximal responses being observed at 20 h (7). These and our studies demonstrate that exposure of phagocytic cells to LPS and live *M. tuberculosis* results in early expression of IL-12 soon after TNF-α. Whether phagocytosis and LPS signalling for IL-12 involve common pathways of cellular activation will require further investigation.

In murine macrophages, IL-12 expression in response to *M. bovis* BCG infection required priming with IFN-γ and coexpression of TNF-α (16). Since TNF-α expression precedes IL-12 p40 mRNA accumulation by 1 to 3 h, we cannot exclude the possibility that priming by TNF-α is required for IL-12 induction by *M. tuberculosis*. Neutralization of TNF-α did not affect IL-12 induction by heat-killed *Listeria monocytogenes* (35) and affected IL-12 induction only minimally in our preliminary experiments with *M. tuberculosis* (data not shown). The finding that PPD, a potent stimulus for TNF-α, was not a strong stimulus for IL-12 production also suggests that the presence of TNF-α was not sufficient for IL-12 expression.

Both defined mycobacterial antigens and whole mycobacteria activate mononuclear phagocytes to produce multiple cytokines. For example, PPD and lipoarabinomannan stimulate mononuclear phagocytes to produce IL-1, TNF-α, and IL-10 (3, 54). Recent studies have demonstrated that TGF-β also is readily produced by monocytes stimulated with mycobacterial antigens (49). However, the mechanisms of how these defined antigens induce cytokine expression remain poorly defined and probably involve different pathways. IL-1, TNF-α, and IL-12 enhance cellular responses, whereas IL-10 and TGF-β downmodulate responses. Thus, both the kinetics and balance of cytokines produced by mononuclear phagocytes influence the net cytokine effect of *M. tuberculosis*-infected cells. IL-10 can downregulate IL-12 (12). However, IL-10 expression by monocytes stimulated with mycobacterial antigens is maximal at 24 to 48 h and thus is delayed compared with TNF-α and IL-12 expression (48a), similar to the kinetics of IL-10 expression in response to LPS (15). Delayed expression of inhibitory cytokines such as IL-10 may explain the downregulation of IL-12 mRNA observed by 18 to 24 h despite the continued presence of *M. tuberculosis* bacilli.

The expression of IL-12 in response to phagocytosis may represent an innate defense mechanism against intracellular microorganisms. The ability of intracellular pathogens to enter without triggering phagocytosis and/or to modulate cytokines secreted by mononuclear phagocytes is probably an important mechanism for deviation of and evasion from cellular immune responses. Unravelling the mechanisms used by host and microbial pathogen to trigger or modulate cytokine responses of macrophages is necessary to understand how intracellular bacterial pathogens such as *M. tuberculosis* elicit and evade acquired immune responses. In light of studies suggesting that IL-12 may have a role as an adjuvant for vaccine administration (1), our results imply that the adjuvant effects of IL-12 may be particularly important for subunit vaccines. Subunit vaccines administered to humans may not elicit sufficient endogenous IL-12 to stimulate Th-1 development. In contrast, vaccines involving expression of heterologous antigens in bacterial vectors, such as *Salmonella* spp. and BCG, will probably elicit sufficient endogenous IL-12 upon phagocytosis to initiate protective T-cell responses.

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