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Induction of In Vitro Human Macrophage Anti-Mycobacterium tuberculosis Activity: Requirement for IFN-γ and Primed Lymphocytes

M. Gloria Bonecini-Almeida, Sadhana Chitale, Iosif Boutsikakis, Jiayuan Geng, Howard Doo, Suhui He, and John L. Ho1,2

Mycobacterium tuberculosis (Mtb) is the world’s leading infectious cause of mortality. Despite the overwhelming data supporting the critical role of cellular immunity, little is known of the early microbial and immune cell interactions and whether human macrophages can be activated to express anti-Mtb activity. We report the reconstitution of an in vitro system whereby human macrophages express anti-Mtb activity only in coculture with PBL and with IFN-γ. Omission of IFN-γ in the cocultures or Mtb lysate/IFN-γ-primed lymphocytes was associated with high growth of Mtb, high IL-10 and IL-12 p40, nearly undetectable IL-12 p70 levels, and the highest percentages of CD4 and CD8 T cells. In contrast, IFN-γ treatment of cocultures containing Mtb lysate/IFN-γ-primed PBL reduced bacilli count by ~2.5 log, decreased the production of IL-10 by 5.7-fold, increased IL-12 p70 by ~50-fold, and reduced the percentages of CD4 and CD8 T cells. Activation of anti-Mtb activity was time and dose dependent. At 2000 U/ml of IFN-γ, bactericidal activity was achieved (10-fold reduction from initial inoculum). Anti-Mtb activity against several strains of M. tuberculosis (H37Ra and H37Rv, and C, a clinical isolate) was observed and was associated with expression of inducible nitric oxide synthase. These data suggest that induction of human macrophage anti-Mtb activity required dual signaling from PBL and IFN-γ. Thus, the development of an in vitro human system may greatly facilitate studies to delineate immune cells, cytokines, and effector functions/genes critical in controlling Mtb. Defining the mechanisms may also provide novel treatment strategies for tuberculosis. The Journal of Immunology, 1998, 160: 4490–4499.

One-third of the world’s population is infected with Mycobacterium tuberculosis (Mtb), a facultative intracellular bacillus. After infection with Mtb, the lifetime risk of developing tuberculosis is approximately 10%, while 90% of infected persons have latent infection with viable bacilli (1). This 10% rate of tuberculosis accounts for the 8 million persons reported annually with active tuberculosis, and the resultant 3 million deaths. Therefore, tuberculosis is the leading cause of death from any infectious agent. The increased incidence of tuberculosis over the last decade has made more urgent the need to delineate host factors that control susceptibility to tuberculosis (2).

Little is known of the early interactions of microbes and immune cells that result in either restricted infection or dissemination and disease, nor of the reasons why some individuals reactivate latent infection. However, cell-mediated immunity is critical for restricting Mtb infection; this is highlighted by the increased risk of tuberculosis associated with decreased cellular immunity such as by immunosuppressive drugs, certain cancers, and the acquired immunodeficiency syndrome. Anti-tubercular cellular immunity involves the critical interplay of T lymphocytes, macrophages, and cytokines (3, 4). Mycobacterium-specific CD4 and CD8 T lymphocytes have been identified that have cytolytic activity against mycobacteria-harboring macrophages (4–8). In mice infected with Mtb complex, depletion of CD4 T lymphocytes results in disseminated disease, while competent mice have restricted infection (4, 5). Similarly, when CD4 T cell counts decrease in HIV-1-infected persons, the risk of tuberculosis is increased whether from primary infection or from reactivation of latent Mtb infection (9). In contrast to CD4 T lymphocytes, the role of Mtb- or Mycobacterium avium-specific CD8 T cells in experimental murine infection and in humans remains undefined (3, 4).

In vitro human and murine Mycobacterium Ag-specific CD4 T cells produce plentiful amounts of IFN-γ (7, 8). The production of IFN-γ by CD4+ T cells is thought to activate macrophages to control intracellular microbes (3, 5, 7–12). Homologous knockout of IFN-γ ligand or receptor genes in mice results in defective macrophage antimicrobial effector function, as exemplified by impaired expression of inducible nitric oxide synthase (iNOS, Nos2), MHC class II Ag, and disseminated mycobacterial infection (13–15). In man, mutations of the IFN-γ receptor rendering it functionless are associated with fulminant tuberculosis and disseminated mycobacteremia after vaccination with Mycobacterium.
oxidation of in vitro murine macrophage anti-Mtb activity by IFN-γ has been observed, whether human macrophages can achieve anti-Mtb activity in vitro via induction by IFN-γ remains controversial (18, 19).

The purpose of this study is to develop an in vitro system to delineate the host modulators, cellular elements, and effector functions critical for control of Mtb infection. We report the induction of in vitro human macrophage anti-Mtb activity and the requirement for coculture with autologous lymphocytes primed with IFN-γ plus Mtb Ag and additional treatment with IFN-γ. Studies using this model system may facilitate the characterization and delineation of host and microbial factors critical for controlling Mtb.

Materials and Methods

Materials and reagents

The materials and reagents used and the respective manufacturers are as follows: RPMI 1640, gentamicin sulfate, M199 medium, and L-glutamine (BioWhittaker, Walkersville, MD); penicillin, streptomycin, and HEPES (Life Technologies, Grand Island, NY); Triton X-100 (Sigma Chemical, St. Louis, MO); normal saline (Baxter Health Care, Deerfield, IL); BSA (Boehringer Mannheim Biochemicals, Indianapolis, IN); purified protein derivative (PPD) of Mtb; Pasteur-Mérieux-Connaught, Ontario, Canada); L929 broth, Tween-80, and Middlebrook 7H11 agar (Difco, Detroit, MI); a 24-well flat-bottom plate and 96-well round-bottom plates (Corning Glass, Corning, NY); 13-mm diameter Gold Seal glass coverslips (Clay Adams/Becton Dickinson Labware, Lincoln Park, NJ); IFN-γ, IFN-β (Genentech, San Francisco, CA); Kinyoun Carbol Fuchsin (Becton Dickinson, Cockeysville, MD); [3H]thymidine (New England Nuclear, Boston, MA); IL-12 p40 EIA kit (R&D Systems, Minneapolis, MN); IL-10 and IL-12 p70 EIA kits (Immunotech, Coulter, Hialiala, FL); phycocerythrin-labeled anti-CD3 and FITC-labeled anti-CD4 and anti-CD8 mAbs (Coulter); CCL64 mink lung fibroblasts (Mv1Lu; American Type Culture Collection (ATCC), Bethesda, MD); and Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR). To remove endotoxin, tissue culture medium (composed of RPMI 1640, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM HEPES, and 5 mM L-glutamine) was ultrafiltered through Hemo-Flow F-40 (MTS Medizin-Technischl System, Fresenius, Germany) and is herein designated as ultrafiltered medium (UFM).

Enumeration of mycobacteria and preparation of mycobacterial Ag

Mtb strains H37Ra and H37Rv (ATCC nos. 25177 and 27294, respectively) and a New York City clinical isolate, designated as the C strain (20), were grown in 7H9 broth supplemented with 0.5% Tween-80. Log phase cultures were pelleted, washed three times in RPMI 1640, and resuspended in L929 broth. The cell suspension was homogenized by vortexing (3 min), and aggregates were allowed to sediment at 1 x g for 3 to 5 min. A bacterial suspension (in some instances in 20% glycerol) was vortexed (3 min), and aggregates were allowed to sediment at 1 x g for 3 to 5 min. Detached immune cells (and potential non-cell-associated bacilli) pelleted by a microfuge (12,000 rpm, 5 min) were lysed in 0.5% Triton X-100 (normal saline, 2 to 5 min). Detached immune cells (and potential non-cell-associated bacilli) pelleted by a microfuge (12,000 rpm, 5 min) were lysed in 0.5% Triton X-100 (normal saline, 2 to 5 min). Detached immune cells (and potential non-cell-associated bacilli) pelleted by a microfuge (12,000 rpm, 5 min) were lysed in 0.5% Triton X-100 (normal saline, 2 to 5 min). Detached immune cells (and potential non-cell-associated bacilli) pelleted by a microfuge (12,000 rpm, 5 min) were lysed in 0.5% Triton X-100 (normal saline, 2 to 5 min). Detached immune cells (and potential non-cell-associated bacilli) pelleted by a microfuge (12,000 rpm, 5 min) were lysed in 0.5% Triton X-100 (normal saline, 2 to 5 min).

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The Mtb input inoculum used to infect macrophages was established on the day of each experiment because storage of Mtb at −70°C may decrease viability. For each experiment, uptake of Mtb at 2 h was enumerated as follows: at 2 h after Mtb infection, Mtb-exposed MDM were washed free of unassociated bacilli, lysed in 0.5% Triton X-100 (NS, 2 to 5 min), and CFU were enumerated. Growth of Mtb in macrophages or macrophages cocultured with PBL was enumerated as follows: at 24 to 96 h. Adherent macrophages and PBL were lysed in 0.5% Triton X-100 (normal saline, 2 to 5 min). Detached immune cells (and potential non-cell-associated bacilli) pelleted by a microfuge (12,000 rpm, 5 min) were lysed in 0.5% Triton X-100 (NS, 2 to 5 min) after the supernatant had been removed for storage at −70°C for cytokine assays. Lysed adherent immune cells and lysed detached cells were pooled and viable Mtb was enumerated directly and from serial dilutions of the lysate. Preliminary experiments showed that <10% of the total CFU of Mtb was contributed by the supernatant fraction of the infected cell culture. Transmission electron microscopy showed that macrophages exposed to Mtb for 2 h, washed free of unassociated bacilli, and studied for 24 h or longer showed no cell surface-associated tubercule bacilli.
Mtb growth in 7H9 mycobacterial medium, RPMI 1640 tissue culture medium, and binding and growth in macrophages

To establish growth rates of Mtb, macrophages were infected with Mtb (0.5 CFU/macrophage or 10 × 10^4 CFU/ml) while 7H9 broth and tissue culture medium were inoculated with 1 × 10^4 CFU/ml. In replicate cultures, the numbers of viable tubercle bacilli were quantitated in these conditions at designated times, and assessed for binding. To assess for binding of Mtb to macrophages, macrophages on coverslips after 2 h of infection and washed (three times) were removed, fixed for 10 min in 100% methanol, and stained with Kinyoun Carbol Fuchsin. To enumerate the number of mycobacteria bacilli that are macrophage associated, we counted a minimum of 100 macrophages, and assessed the distribution of acid-fast bacilli within the macrophages as the number of macrophages with 0, 1 to 5, 6 to 9, or 10 bacteria. In preliminary experiments, macrophages pretreated or not with IFN-γ had similar visual distribution of cell-associated mycobacteria.

Measurement of immune cell viability and profile of T cell subsets

To assess whether changes in bacillary counts may be due to killing of mycobacteria-laden macrophages, cell viability was determined in Mtb-infected macrophages or cocultures of macrophages and PBL. Using the ability of fluorescent compounds, calcein AM, and ethidium homodimer (EthD-1) to differentiate, respectively, viable or dead/dying cells, monolayers washed (one time) were treated with fluorescent dyes, 2 μM calcein AM, and 4 μM EthD-1 as instructed by the manufacturer (Molecular Probes) for 30 min. Monolayers were washed (two times) with PBS and retained dyes were quantitated by Cytofluor Reader (Millipore, Bedford, MA) using excitation wavelengths of 485 nm for calcein and 530 nm for EthD-1, and emission wavelength of 530 nm for calcein and 645 nm for EthD-1. Preliminary experiments to establish the assay used adherent monocytes and cells permeabilized with 0.2% Triton X-100.

T cell subset profiles were performed in freshly isolated PBMC, or detached cells from Mtb-infected cocultures were washed, incubated with anti-CD3 and anti-CD4 or anti-CD8 mAb, washed, fixed in 1% paraformaldehyde in PBS, and subjected to FACS analysis.

Detection of biologically active TGF-β and IL-10, IL-12 p40, and IL-12 p70 by EIA

TGF-β activity in cell supernatant was assayed by determining the inhibition of tritiated thymidine incorporation in CCL64 mink lung fibroblasts. An aliquot (20 μl) was diluted into 980 μl of CCL64 culture medium (medium 199 plus 10% FBS and 50 μg/ml gentamicin) and added to CCL64 cells plated 24 h previously at 1 × 10^6 cells per well of a 96-well plate (100 μl/well with triplicate) (21). After 18 h at 37°C, the medium was carefully decanted and 100 μl/ml tritiated thymidine in M199 medium was applied for an additional 4 h. Incorporation of tritiated thymidine into DNA was quantitated by semiautomated techniques (22). The reduction of >60,000 cpm is maximally inhibited by as little as 5 ng/ml TGF-β to <3000 cpm. Half-maximal inhibition is typically observed at 50 pg/ml with a detection threshold of 1 pg/ml or less of TGF-β, and anti-TGF-β mAb, which neutralized the inhibitory activity, served as a control. For assay of IL-10, IL-12 p40, and IL-12 p70, commercial kits were used as directed by the manufacturer, and OD was measured by EIA plate reader (Bio-Tek Instruments, Winooski, VT) at 450 nm with 590 nm as a reference.

RT-PCR for human inducible nitric oxide synthase (iNOS, NOS2)

Macrophages, Mtb-infected macrophages, or cocultures containing Mtb-infected macrophages and primed PBL were harvested and suspended in RNA Stat-60 (Tel Test, B, Friendswood, TX) at −70°C. Human 293 cells transfected with NOS2 cDNA or vector alone (gifts of Drs. Carl Nathan and Qiao-wen Xie, Cornell University Medical College) constituted positive and negative controls (23–25). The methods for detection of iNOS were as previously described (23). In brief, total RNA (1 μg) was reverse-transcribed using oligo(dT)16 and PCR performed by standard methods (GeneAmp System 9600; Perkin-Elmer, Norwalk, CT). First-round PCR was conducted with 20% of the cDNA using oligonucleotide with primers from exon 1 (5′-CAC CTT TGA TGA GGC GAC-3′) and exon 4 (5′-GCA TCC ACC TTT ACC AG-3′) of the human NOS2. For nested PCR, 4% of the first-round product was amplified with primers from exon 1 (5′-ATG AGG GGA CTG GGC AGT TC-3′) and exon 4 (5′-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3′) (Clontech, Palo Alto, CA). The β-actin amplicons were resolved by electrophoresis in 1.5% agarose and directly stained with ethidium bromide.

Statistical analysis

Results were analyzed using either Student’s paired t test (two-tailed), Kruskall Wallis test, and ANOVA with a p value <0.05 considered significant.

Results

Anti-mycobacterial activity of human macrophages

We first sought to determine intrinsic rates of Mtb replication, and to evaluate whether human macrophages possess antimycobacterial activity. Illustrated in Figure 1 is the kinetics of growth of Mtb in 7H9 broth and RPMI 1640 tissue culture medium, and by day 4 of culture, bacilli growth had reached, respectively, ~140-fold and ~7.0-fold above the initial inoculum. Incremental growth of Mtb in macrophages was also noted over 4 days regardless of postinfection treatment with IFN-γ. As shown in Figure 1, intracellular growth of Mtb in macrophages or IFN-γ-treated macrophages after 4 days of culture was five- and six-fold higher than at 2 h after infection, respectively (p < 0.01), despite the finding that the initial number of Mtb associated with IFN-γ-treated macrophages was slightly lower than untreated macrophages (2.6 ± 2.0 × 10^4 CFU/ml vs 5.2 ± 2.1 × 10^4, respectively, p > 0.05).

Induction of anti-mycobacterial activity by coculture with primed PBL and IFN-γ

Control of Mtb infection in vivo is associated with granuloma formation. Histologically, the tuberculous granuloma is composed of...
a central area of macrophages within a palisade of lymphocytes. We reason that the inability to induce in vitro anti-mycobacterial activity in IFN-γ-treated macrophages is due to the requirement for activation by lymphocytes primed with Ags of Mtb and IFN-γ. To reconstitute the in vivo milieu, we assessed anti-mycobacterial activity in Mtb-infected macrophages cocultured with autologous naïve PBL or PBL primed with IFN-γ/M. tuberculosis H37Ra lysate, and we also evaluated the additional contribution by IFN-γ treatment of the infected cocultures. Cocultures of macrophages infected with Mtb H37Ra and naïve PBL showed a 26-fold increase in bacilli count above the bacilli count at 2 h after infection (135 ± 14.3 × 10⁴ CFU/ml vs 5.2 ± 2.1 × 10⁴ CFU/ml). Cocultures of Mtb-infected macrophages and PBL primed with IFN-γ (500 U/ml) showed a similar although slightly lower bacilli count (110 ± 33.8 × 10⁴ CFU/ml, p > 0.05). In contrast, cocultures of macrophages and PBL primed with IFN-γ/Mtb lysate significantly enhanced the growth of Mtb H37Ra, which after 4 days of culture was 58-fold greater than at 2 h of infection (301 ± 30.5 × 10⁴ CFU/ml, p < 0.01). Cocultures of macrophages and PBL primed with Mtb lysate showed similar growth of Mtb (data not shown).

We next evaluated whether IFN-γ treatment of cocultures containing PBL and Mtb-infected macrophages induced antimiycobacterial activity. (Fig. 2). Compared with cocultures without IFN-γ, IFN-γ treatment of cocultures resulted in a significant reduction of Mtb H37Ra. The bacilli count in IFN-γ-treated cocultures containing naïve PBL, PBL primed with IFN-γ, or IFN-γ/Mtb lysate were, respectively, 25.4 ± 6.5, 16.2 ± 4.4, and 7.2 ± 1.9 × 10⁴ CFU/ml (p < 0.03 for each condition with IFN-γ vs no IFN-γ (n = 8 to 26). Thus, IFN-γ treatment of cocultures containing PBL primed with IFN-γ/Mtb lysate reduced mycobacterial growth by 42-fold (~1.8 log reduction) when compared with cocultures not treated with IFN-γ. Moreover, IFN-γ treatment of cocultures containing PBL primed with IFN-γ/Mtb lysate restricted bacterial growth, resulting in a bacillus count at 4 days that was similar to the count at 2 h after infection (7.2 × 10⁴ CFU/ml vs 5.2 × 10⁴ CFU/ml), and significantly lower than cocultures containing naïve PBL (25.4 ± 6.5 CFU/ml, p < 0.05 (Student’s t test)).

Kinetics of induction of anti-mycobacterial activity by coculturing with primed PBL and IFN-γ
As shown in Figure 3, a significant time-dependent growth of Mtb was observed in macrophage cultures or cocultures of macrophages and naïve PBL (p < 0.05, Kruskall Wallis test). In cocultures of macrophages and naïve PBL, we observed a 10-fold increase in mycobacterium count (from 4.2 ± 1.3 × 10⁴ CFU/ml at 2 h to 43.7 ± 6.4 × 10⁴ CFU/ml at day 4 (n = 5, p < 0.004, Kruskall Wallis test). The bacilli count at day 4 in cocultures of macrophages and naïve PBL was slightly higher than that in macrophages or macrophages primed with IFN-γ (Fig. 3). In contrast, additional IFN-γ treatment of macrophages cocultured with IFN-γ/Mtb lysate-primed PBL restricted the growth of Mtb, permitted only a 2.4-fold increase in bacilli count at 4 days (6.3 × 10⁴ CFU/ml vs 2.6 × 10⁴ CFU/ml at 2 h after infection, p > 0.05). Importantly, omission of IFN-γ treatment from cocultures containing PBL primed with IFN-γ/Mtb lysate resulted in a 115-fold increase in bacilli growth (compared with 2 h after infection and to IFN-γ-treated cocultures containing PBL primed with IFN-γ/Mtb lysate, p < 0.01, Student’s t test, Fig. 3).

Effect of IFN-γ dose on intracellular growth of Mtb
We next examined the effect of varying doses of IFN-γ treatment of cocultures of Mtb-infected macrophages and PBL on antimycobacterial activity. As seen in Figure 4, a dose-dependent induction of anti-mycobacterial activity was observed. Compared with no IFN-γ treatment of the cocultures, IFN-γ (2000 U/ml and...
showed a striking inhibition in Mtb reduction, 140-3. Bacterial survival was assessed after 4 days and expressed in semilog as g with IFN-

PBL were cultured in medium while cocultures containing PBL primed with IFN-

Mtb, we evaluated the C strain, a clinical isolate, and H37Rv, an attenuated strain, H37Ra, can be extended to virulent strains of M. tuberculosis naı ¨ve PBL not additionally treated with IFN-

g

17.5 than bacillus uptake at 2 h (87.6
tericidal activity. Thus, compared with uptake of the tubercle bacillus at 2 h (2.6 ± 0.4 × 10^7 CFU/ml), IFN-γ at 1000 and 2000 U/ml reduced the bacilli count by ~4- and ~10-fold, respectively, (0.7 × 10^8 CFU/ml and 0.2 × 10^4 CFU/ml, p = 0.07 and p < 0.05, respectively, n = 4) (Fig. 4). In contrast, at lower doses (100 and 500 U/ml) IFN-γ achieved bacteriostasis but significantly reduced mycobacterial growth by 21- and 40-fold, respectively, when compared with cocultures of Mtb-infected macrophages and naïve PBL not additionally treated with IFN-γ (p < 0.05 for all IFN-γ doses; n = 6).

Anti-mycobacterial activity against M. tuberculosis C strain and H37Rv

To determine whether the in vitro cellular capacity to kill Mtb-attenuated strain, H37Ra, can be extended to virulent strains of Mtb, we evaluated the C strain, a clinical isolate, and H37Rv, a virulent strain of Mtb. As shown in Figure 5A, human macrophages stimulated for 4 days with IFN-γ (500 U/ml) were infected with the C strain was observed. Compared with macrophages cultured in medium, a 36-fold and a 4.5-fold growth increase of Mtb H37Rv was noted, respectively, in cocultures of macrophages and naïve (nonprimed) PBL (p < 0.03). In contrast, the addition of IFN-γ to cocultures of macrophages and PBL primed with IFN-γ/Mtb lysate resulted in a 4-fold and 30-fold reduction in bacillary count when compared with cocultures of macrophages and naïve PBL, or PBL primed with IFN-γ/Mtb lysate, respectively (Fig. 5B). Similar uptake of bacilli at 2 h was noted in cocultures of macrophages and naïve PBL or PBL primed with IFN-γ/Mtb lysate.

FIGURE 4. The effect of IFN-γ dose on intracellular survival of Mtb. Macrophages cultured with medium or medium containing IFN-γ (500 U/ml) for 4 days were infected with Mtb H37Ra and cocultured with naïve PBL or PBL primed with IFN-γ/Mtb lysate. Cocultures containing naïve PBL were cultured in medium while cocultures containing PBL primed with IFN-γ/Mtb lysate were treated with IFN-γ from 100 to 2000 U/ml. Bacterial survival was assessed after 4 days and expressed in semilog as Mtb CFU × 10^6/ml (n = 6 separate experiments).

1000 U/ml)-treated cocultures of Mtb-infected macrophages showed a striking inhibition in Mtb growth of 470-fold (~2.5 log reduction, 140 × 10^4 CFU/ml vs 0.3 × 10^9 CFU/ml) and 186-fold, respectively. Treatment with higher doses of IFN-γ triggered bactericidal activity. Thus, compared with uptake of the tubercle bacillus at 2 h (2.6 ± 0.4 × 10^7 CFU/ml), IFN-γ at 1000 and 2000 U/ml reduced the bacilli count by ~4- and ~10-fold, respectively, (0.7 × 10^8 CFU/ml and 0.2 × 10^4 CFU/ml, p = 0.07 and p < 0.05, respectively, n = 4) (Fig. 4). In contrast, at lower doses (100 and 500 U/ml) IFN-γ achieved bacteriostasis but significantly reduced mycobacterial growth by 21- and 40-fold, respectively, when compared with cocultures of Mtb-infected macrophages and naïve PBL not additionally treated with IFN-γ (p < 0.05 for all IFN-γ doses; n = 6).

FIGURE 5. Anti-mycobacterial activity against Mtb C strain and H37Rv. Four-day-old macrophages primed or not primed with IFN-γ (500 U/ml) were infected with (A) Mtb C strain, a clinical isolate, or (B) Mtb H37Rv, a virulent strain, cocultured or not with naïve PBL or PBL primed with IFN-γ/Mtb C strain or H37Rv lysate, and additionally treated or not with IFN-γ (500 U/ml). Bacterial recovery was monitored at 2, 24, 48, 72, and 96 h (n = 3 to 4 separate experiments). Mtb H37Rv uptake at 2 h by macrophages pretreated with medium or medium containing IFN-γ was 8.7 ± 0.8 × 10^6 CFU/ml and 7.6 ± 1.4 × 10^6 CFU/ml, respectively (n = 4).
Assessment of cell viability and the profile of CD4 and CD8 T cells

We assessed whether changes in bacilli count could be solely explained by death of macrophages laden with mycobacteria. Figure 6A shows the fluorescence emission values representing the number of live and dead cells. The proportion of live and dead cells was similar between \textit{Mtb}-infected macrophages and \textit{Mtb}-infected macrophage cocultures containing PBL primed with IFN-\(\gamma\)/\textit{Mtb} lysate and between infected cultures not treated or additionally treated with IFN-\(\gamma\). Four days after infection, the immune cells were washed, stained with dyes, and the uptake of these dyes was quantitated using Cytosluor Reader. The relative fluorescence values for live or dead cells were summed and presented as percentage of fluorescence. A similar result was observed in cells treated with 500 U/ml or 2000 U/ml IFN-\(\gamma\). B. In similar cell cultures, cells were washed, incubated with phycoerythrin- or FITC-labeled anti-CD3, CD-4, or CD8 mAbs, and fixed with 1% paraformaldehyde. Cells were examined by FACS analysis by gating on the lymphocyte population. Illustrated is a representation of three separate experiments.

Cytokine network in \textit{Mtb}-infected cocultures

The induction of TGF-\(\beta\) by \textit{Mtb} infection of human macrophages has been reported, and its ability to down-modulate macrophage effector functions is thought to play a role in the pathogenesis of tuberculosis (26–28). We assessed TGF-\(\beta\) levels in the in vitro system. A biologic assay was used to detect active TGF-\(\beta\) because the regulation of this ligand is by activation of an inactive precursor molecule rather than by up-regulation of ligand production. As

![Graph](https://example.com/graph.png)
shown in Figure 7A, macrophage infection by *Mtb* rapidly produced active TGF-β (*n* = 3). The presence of PBL primed with *Mtb* lysate/IFN-γ reduced TGF-β to a lower level. Treatment of the coculture with IFN-γ further lowered the amount of active TGF-β.

Macrophage-produced cytokines, such as IL-10 and IL-12, regulate T cells and the responsive T cells can feed back to modulate macrophages (29–38). The production of IL-10, IL-12 p40 subunit, and IL-12 (p40/p35) heterodimer p70 was measured in the culture supernatant of *Mtb* H37Ra-infected macrophage cocultures (*n* = 3). As shown in Figure 7B, high levels of IL-10 and IL-12 p40 were detected in *Mtb*-infected macrophage cultures and in cocultures containing infected macrophages and PBL primed with *Mtb* lysate/IFN-γ, although excessively low levels of IL-12 p70 were detected in both conditions. IFN-γ treatment significantly reduced IL-10, induced moderate levels of IL-12 p70, and increased the amounts of IL-12 p40 production in cocultures containing *Mtb*-infected macrophages and PBL primed with *Mtb* lysate/IFN-γ or in *Mtb*-infected macrophage cultures. The induction of IL-10 was similarly seen in macrophages infected with either *Mtb* virulent strains H37Rv or C (data not shown). The induction of IL-10 is specific to *Mtb*-infection because uninfected macrophages and macrophages cultured with PPD of *Mtb* produced very low amounts of IL-10.

We next correlated the level of IL-10 and IL-12 production with the bacillary counts obtained from each of the cell coculture conditions. Similar to earlier experiments, *Mtb*-infected macrophages cocultured with PBL primed with *Mtb* lysate/IFN-γ showed the highest bacilli count (310 ± 80 X 10^4 CFU/ml) and the highest IL-10, but nearly undetectable levels of IL-12 p70 (Fig. 7B). In contrast, IFN-γ treatment of cocultures containing infected macrophages and PBL primed with *Mtb* lysate/IFN-γ reduced bacilli

**FIGURE 7.** Cytokine production in *Mtb*-infected macrophages and in *Mtb*-infected cocultures. A, Active TGF-β production by cultures containing *Mtb* H37Ra-infected macrophages cultured in medium or cocultured with *Mtb* lysate (Ag)/IFN-γ-primed PBL and treated with IFN-γ (500 U/ml) or medium. Cell culture supernatant was obtained at 2, 24, and 48 h after infection and measured for active TGF-β using a biologic assay. B, Cytokine production was measured in similar macrophage cultures infected with *Mtb* H37Ra cultured in medium or cocultured with *Mtb* Ag/IFN-γ-primed PBL and treated with IFN-γ (500 U/ml) or medium. Uninfected macrophages or macrophages treated with PPD (5 μg/ml) served as a controls. Supernatants from these cultures were obtained at 96 h, and immune cells were harvested to enumerate CFU of *Mtb*. EIA for each cytokine were measured as indicated by the manufacturer’s protocol. Uninfected macrophages or macrophages treated with PPD produced very low levels of IL-10 (<200 pg/ml) and no detectable IL-12 p40 or p70.
We report the development of an in vitro model of *Mtb* infection whereby human macrophages are activated to reduce the growth of *Mtb* by ~2.5 logs CFU or >40-fold. Anti-*Mtb* activity in this model required coculturing infected macrophages with autologous PBL and the additional treatment of the coculture with IFN-γ. Omission of *Mtb* Ag-primed PBL or further treatment of the infected coculture with IFN-γ resulted in unrestricted growth of *Mtb* (Figs. 2 and 4). The requirement of IFN-γ is further supported by the finding that the restriction of bacillus growth by IFN-γ is dose dependent in that the reduction of bacillus growth by 2000 U/ml of IFN-γ reached 2.5 logs when compared with similar cocultures lacking IFN-γ (Fig. 4). Moreover, we found that in response to doses of 2000 and 1000 U/ml of IFN-γ, bacterial activity in cocultures containing primed PBL was achieved (10- and 4-fold reduction from initial inoculum, respectively) while lower doses were bacteriostatic (Fig. 4).

Our experiments indicated that the highest growth of *Mtb* was found in cocultures of macrophages and PBL primed with IFN-γ plus *Mtb* lystate (Fig. 2). One potential explanation may be that one or more *Mtb* Ags triggered the production of cytokines, such as IL-10, and facilitated the development of T cells. We therefore examined the production of IL-10 by *Mtb*-infected macrophages or by cocultures containing primed PBL. Surprisingly, high levels of IL-10 and nearly undetectable amounts of IL-12 p70 were found in culture supernatant from *Mtb*-infected macrophages and in cocultures containing macrophages and PBL primed with *Mtb* lystate/IFN-γ (Fig. 7B). The production of IL-10 appears to be induced by *Mtb* infection because neither macrophages cultured in medium nor medium containing PPD of *Mtb* produced significant amounts of IL-10. We suspected that high levels of IL-10 in the context of low IL-12 p70 triggered by *Mtb* facilitated the expansion of a subset of T cells. This is supported by our finding that the highest percentage of CD4 and CD8 T cells in association with high IL-10 levels was seen in cocultures containing *Mtb*-infected macrophages and PBL primed with *Mtb* lystate/IFN-γ (Fig. 6B).

In contrast to *Mtb*-infected cocultures not treated with IFN-γ, additional treatment of the cocultures with IFN-γ reduced IL-10 and enhanced IL-12 p70 production (Fig. 7B). This change in cytokine profile resulting from IFN-γ is associated with lowering of the percentages of CD4 and CD8 T cells in cocultures containing *Mtb*-infected macrophages and PBL, primed with *Mtb* lystate/IFN-γ (Fig. 6B).

In the absence of additional cellular and host immune modulators, this reasoning is based on the histology finding that the tuberculous granuloma in which *Mtb* has been contained is composed of centrally located tissue macrophages harboring tubercle bacilli surrounded by a palisade of lymphocytes and other mononuclear cells. We therefore reconstituted such a picture by coculturing *Mtb*-infected macrophages with *Mtb* lystate/IFN-γ-primed PBL and further activating these cells by IFN-γ.

Discussion

We confirmed that in vitro IFN-γ activation is insufficient to induce anti-*Mtb* activity of human macrophages (19, 40). We speculated that the lack of in vitro anti-*Mtb* activity of human macrophages shown by earlier investigators and by this study was due to the absence of additional cellular and host immune modulators. This reasoning is based on the histology finding that the tuberculous granuloma in which *Mtb* has been contained is composed of centrally located tissue macrophages harboring tubercle bacilli surrounded by a palisade of lymphocytes and other mononuclear cells. We therefore reconstituted such a picture by coculturing *Mtb*-infected macrophages with *Mtb* lystate/IFN-γ-primed PBL and further activating these cells by IFN-γ.

FIGURE 8. Detection of iNOS or NOS2. Macrophages infected with *Mtb* strain H37Rv and cultured in medium or cocultured with *Mtb* lystate (Ag)/IFN-γ-primed PBL were treated with medium or medium containing IFN-γ (500 U/ml). Cells harvested at 24 and 48 h after infection were extracted for total RNA. RT-PCR for human (hu-iNOS) and IFN-γ (8.7 10^4 CFU/ml). Cells harvested at 24 and 48 h after infection were extracted for total RNA. RT-PCR for human (hu-iNOS) and IFN-γ (8.7 10^4 CFU/ml). Cells harvested at 24 and 48 h after infection were extracted for total RNA. RT-PCR for human (hu-iNOS) and IFN-γ (8.7 10^4 CFU/ml). Cells harvested at 24 and 48 h after infection were extracted for total RNA. RT-PCR for human (hu-iNOS) and IFN-γ (8.7 10^4 CFU/ml). Cells harvested at 24 and 48 h after infection were extracted for total RNA.
interpretation is corroborated by the recent report that IL-10 transgenic mice are susceptible to disseminated *M. bovis* BCG while wild-type mice restricted the infection (31). Of note is that spleen cells from IL-10 transgenic mice compared with cells from wild-type mice upon activation expressed normal levels of IL-2 and IFN-γ and only ~4-fold higher IL-10. This amount of IL-10 overproduction was sufficient to transform a BCG resistant to a susceptible phenotype (31). Such a case can also be made for human tuberculosis, in that we have observed the coexpression of IL-10 with IL-2, IFN-γ, and nitric oxide synthase by lung cells from patients with active pulmonary tuberculosis (23, 41). In addition to IL-10, we also found high amounts of IL-12 p40. Although we cannot exclude a differential sensitivity in the ability of the assay to detect IL-12 p40 and IL-12 p70, IL-12 p40 homodimer has been shown to bind to IL-12R and to antagonize IL-12 p70 activation (42, 43). Moreover, in mice, IL-12 p40 homodimers were reported to block the tumoricidal activity of IL-12 p70 (44). Thus, should the detected high IL-12 p40 exist as a homodimer in this in vitro model, it may also have a pathologic function. In addition, as previously reported, active TGF-β from *Mtb*-infected macrophages likely also contributed to macrophage deactivation (26–28). However, in this model, coculture of infected macrophages with PBL appeared to suppress active TGF-β levels.

In this study, high level expression of iNOS was observed in association with anti-*Mtb* activity found in IFN-γ-treated cocultures containing *Mtb*-infected macrophages and *Mtb* lysate/IFN-γ-primed PBL. In the absence of IFN-γ treatment of the *Mtb*-infected cocultures, the low level nitric oxide synthase detected was ineffective as indicated by the finding that these cocultures had the highest bacillus load (Figs. 2 and 8). Although we observed that IFN-γ-activated *Mtb*-infected macrophages expressed nitric oxide synthase, significant anti-mycobacterial activity was not demonstrated (Figs. 1 and 8). In contrast, lung macrophages from patients with lung disease already primed by inflammatory mediators, such as in idiopathic pulmonary fibrosis, required only the in vitro signal provided by *M. bovis* BCG infection to trigger iNOS gene expression and enzymatic activity (39). These data suggest that human iNOS gene expression and/or its activity may be regulated by several signals, thus accounting for the detection of iNOS activity in lung macrophages from patients with active tuberculosis; whereas the induction of iNOS in alveolar macrophages from inflamed lung needed only in vitro BCG infection, and in peripheral blood macrophages iNOS expression required *Mtb* infection and coculture with IFN-γ and primed PBL (i.e., the in vitro model). Prior reports did not demonstrate iNOS in macrophages (23, 39, 40). Whether the in vitro anti-*Mtb* activity found by this report is solely mediated by iNOS, and whether dual signaling induced the expression of iNOS gene with functional activity were not examined. Similarly, what T cell subsets and how they transmit the signal to activate macrophages were not examined. However, the establishment of an in vitro model will facilitate our understanding of the complex cellular and cytokine network required for maximal anti-*Mtb* activity.

We believe that our present findings represent primary immune response following infection because the New York blood donor population is predominantly middle to upper middle class and white (Dr. Celso Bianco, New York Blood Center, unpublished observations). In such a population, the overall incidence of tuberculosis is ~16 per 100,000 which translates to a PPD-positive rate of less than 2% (assuming infection to disease ratio of 1 to 50) (45). Thus, the consistent finding of inducible antimycobacterial activity in all experiments in which donors have a high likelihood of no prior exposure to *Mtb* and in which 12 NYBC donors tested showed lack of in vitro recall response to PPD (e.g., proliferation and IFN-γ production) strongly suggests that our findings reflect the induction of primary immune response. Moreover, the requirement for IFN-γ and high IL-10 production also argue for a primary response because if the donors’ cells contained memory cells, repriming with Ag should have expanded these memory CD4 + T cells and increased the in vitro production of IFN-γ, and thus should have obviated the need for additional treatment of the cocultures with IFN-γ.

Our finding that IFN-γ is required for induction of anti-*Mtb* activity in cocultures of macrophages and lymphocytes confirms the critical role of IFN-γ in defense against *Mtb*. For example, administration of IFN-γ to mice leads to an increase in resistance to challenge with *Mtb* and *M. bovis* (18). In contrast, mice with homologous knockouts of IFN-γ ligand or receptor genes infected with a sublethal dose of *M. bovis* showed increased mortality and bacterial numbers in infected organs (14, 15). Recently, mutation of the IFN-γ receptor that led to a functionless receptor was similarly associated with disseminated mycobacteria or BCG infection (16, 17). Moreover, IFN-γ treatment has been shown to improve clinical outcome in persons with nontuberculous mycobacterial disease (46). Furthermore, HIV-1-negative patients with disseminated or chronic cavitary pulmonary infection caused by atypical mycobacteria have now had IFN-γ added to their chemotherapeutic regimens (47), and IFN-γ is being tested as an adjuvant for therapy of patients with drug-resistant tuberculosis (S. Holland, unpublished observations).

The availability of an in vitro model will facilitate the delineation of mechanisms by which cytokine-modulators, in addition to IFN-γ, activate anti-mycobacterial activity. We speculate that IL-12 in this system may have an additional spectrum of activity not shared by IFN-γ alone because of its ability to enhance Th1 cell, NK cell, and CD8+ activity and development, and to increase the level of IFN-γ production of these cells (34). As seen with the expression of nitric oxide synthase, we believe that additional genes involved in effective control of *Mtb* will be highly expressed in conditions in which infected macrophage cocultures containing IFN-γ/Mtb Ag-primed PBL are further treated with IFN-γ. The reconstitution of effective in vitro immune response to *Mtb* will enable the delineation of cell constituents, host modulators, and cell functions/effector genes critical to restrict *Mtb*. Defining the mechanisms involved using such an in vitro human system may provide novel treatment strategies for tuberculosis. Moreover, an in vitro system may also facilitate a systematic evaluation of mycobacterial Ag for its ability to induce effective in vivo immunity, and thus provide a screening method for evaluating vaccine candidates.

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References


