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Innate Inhibition of Adaptive Immunity: *Mycobacterium tuberculosis*-Induced IL-6 Inhibits Macrophage Responses to IFN-γ[1]

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In humans and in mice, control of the intracellular pathogen, *Mycobacterium tuberculosis* (Mtb), requires IFN-γ. Although the adaptive immune response results in production of substantial amounts of IFN-γ in response to Mtb, the immune response is unable to eradicate the infection in most cases. We have previously reported evidence that Mtb inhibits macrophage responses to IFN-γ, suggesting that this may limit the ability of IFN-γ to stimulate macrophages to kill Mtb. We have also observed that uninfected macrophages, adjacent to infected macrophages in culture, exhibit decreased responses to IFN-γ. Here we report that IL-6 secreted by Mtb-infected macrophages inhibits the responses of uninfected macrophages to IFN-γ. IL-6 selectively inhibits a subset of IFN-γ-responsive genes at the level of transcriptional activation without inhibiting activation or function of STAT1. Inhibition of macrophage responses to IFN-γ by IL-6 requires new protein synthesis, but this effect is not attributable to suppressor of cytokine signaling 1 or 3. These results reveal a novel function for IL-6 and indicate that IL-6 secreted by Mtb-infected macrophages may contribute to the inability of the cellular immune response to eradicate infection. *The Journal of Immunology,* 2003, 171: 4750–4757.

I nfection with *Mycobacterium tuberculosis* (Mtb)[3] induces a cellular immune response including CD4+ and CD8+ T cells that secrete IFN-γ (1, 2). IFN-γ, the predominant activator of microbicidal functions of macrophages (3), is detectable at sites of Mtb infection (4) and is essential for prevention of uncontrolled progression of infection (5, 6). However, despite the development of a cellular immune response and local production of IFN-γ, the immune response is unable to eradicate the infection in humans or mice. Insight into one mechanism by which a cellular immune response might be unable to eradicate Mtb infection was gained by in vitro experiments that demonstrated that IFN-γ, while capable of activating macrophages to kill diverse intracellular pathogens such as *Toxoplasma gondii*, *Leishmania donovanii*, and *Legionella pneumophila*, was unable to activate macrophages to kill virulent strains of Mtb (7–10). We and others (11–14) have previously presented evidence that this may be due at least in part to the ability of Mtb to selectively inhibit macrophage responses to IFN-γ.

In addition to a direct effect of Mtb infection of macrophages on responses to IFN-γ, we have also noted an apparent bystander effect in which uninfected macrophages adjacent to Mtb-infected macrophages were also unable to respond normally to IFN-γ. Here we report that Mtb-infected macrophages synthesize and secrete one or more polypeptides that inhibit the responses of uninfected macrophages to IFN-γ. Moreover, we provide evidence that this activity is not attributable to IL-10 or to TGF-β, but rather to IL-6, indicating that the secretion of IL-6 by infected macrophages may contribute to the inability of IFN-γ to eradicate Mtb infection.

Materials and Methods

Mice

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions.

Cell lines

RAW264.7 cells and L929 cells (both obtained from American Type Culture Collection, Manassas, VA) were maintained in complete DMEM (DMEM supplemented with 10% FCS and 2 mM L-glutamine; Life Technologies, Gaithersburg, MD). L cell-conditioned medium was collected from confluent L929 cultures.

Abs, cytokines, primers, and other reagents

PE-labeled anti-IA/IE (BD PharMingen, San Diego, CA), FITC-labeled anti-FcγRI (Ancell, Bayport, MN), anti-TGF-β (1D11; R&D Systems, Minneapolis, MN), anti-IL-10 (JES5-2A5; BD PharMingen), anti-IL-6 (MP5-20F3; BD PharMingen), anti-STAT1α (Zymed, South San Francisco, CA), and anti-phospho-STAT1 (Tyr701; Zymed) were obtained from the respective manufacturers. IFN-γ was obtained from BD PharMingen, and IL-6 from BioSource (Camarillo, CA). The IL-6 ELISA was purchased from R&D Systems. Cycloheximide was obtained from Sigma-Aldrich (St. Louis, MO). Oligonucleotide primers were purchased from Invitrogen (Carlsbad, CA). The sequences of primers used for real-time RT-PCR analysis are shown in Table I.

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[3] Abbreviations used in this paper: Mtb, *Mycobacterium tuberculosis*; BMDM, bone marrow-derived macrophage; CIITA, IFN-γ-inducible class II *trans*-activator; CM, conditioned medium; C_T, threshold cycle; JAK, Janus kinase; MFI, mean fluorescence intensity; RLU, relative light units; SOCS, suppressor of cytokine signaling; TLR, Toll-like receptor.

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Table I. *Sequences of primers used for quantitative RT-PCR*

Gene	Sense	Antisense
GAPDH	5′-CAATGCATCCTGCACCACCAA-3′	5′-GTCATTGAGAGCAATGCCAGC-3′
IP-10	5′-CCTATCCTGCCCACGTGTTG-3′	5′-CGCACCTCCACATAGCTTACA-3′
MIG	5′-ATGAAGTCCGCTGTTCTTTTCC-3′	5′-TTATGTAGTCTTCCTTGAACGAC-3′
IGTP	5′-TCAAGCGCCTCATCAGCCCGTGGT-3′	5′-TGGCCAGCTTCACATGATTTGAG-3′
LRG-47	5′-TTTAAGAGAAGGAAAACTACTGGA-3′	5′-AGCTTGGTCCAGACAATATAGAA-3′
CIITA	5′-TGCAGGCGACCAGAGGGAGACA-3′	5′-GAAGCTGGGCACCTCAAAGAT-3′
ICSBP	5′-GATCAAGGAACCTTCTGTGG-3′	5′-GAAGCTGATGACCATCTGGG-3′
IRF-1	5′-CAGAGGAAAGAGAGAAAGTCC-3′	5′-CACACGGTGACAGTGCTGG-3′
SOCS-1	5′-CACCTTCTTGGTGCGCG-3′	5′-AAGCCATCTTCACGCTGAGC-3′
SOCS-3	5′-TGCGCCATGGTCACCCACAGCAAGTTT-3′	5′-GCTCCTTAAAGTGGAGCATCATACTGA-3′

Isolation and culture of bone marrow-derived macrophages (BMDM)

Mice were euthanized using CO_2 narcosis and cervical dislocation, and femurs and tibias were removed aseptically. Bone marrow was flushed from either end with cold, complete DMEM (Life Technologies), and the crude cell suspension was filtered through a cell strainer (Falcon; BD Biosciences, Franklin Lake, NJ) and centrifuged at $500 \times g$ to pellet cells, which were then washed twice in complete DMEM. Cells were plated in 150×25-mm bacterial grade petri dishes (Falcon) at 4×10^6/plate, in DMEM supplemented with 20% FCS, 30% L929-cell conditioned medium, 1 mM sodium pyruvate (Life Technologies), and 2 mM L-glutamine (Life Technologies). The cells were incubated at 37°C in 5% CO_2 for 4 days, after which nonadherent cells were gently washed away, and the medium was replaced. On day 6 adherent cells were scraped, washed, and replated at 5×10^5/well in 12-well tissue culture plates for further use.

Isolation and culture of human monocyte-derived macrophages

Human monocytes were isolated from buffy coats as previously described (14) with a minor modification: to improve adherence, cells were cultured in RPMI 1640 with 2 mM L-glutamine (Life Technologies) and 1% autologous serum for 1 h after plating. Culture medium and nonadherent cells were removed by aspiration, and monolayers were subsequently incubated with fresh culture medium supplemented with 2.5% autologous serum. Human monocyte-derived macrophages were infected with *Toxoplasma gondii* (RH strain) as previously described (14).

Infection of macrophages and preparation of conditioned medium

Mtb suspensions were prepared as described previously (14). Cells were incubated with the bacterial suspension, calculated to yield a multiplicity of infection of 10, for 4 h and then were washed to remove uningested bacteria. Alternatively, cells were treated with gamma-irradiated Mtb (500 μg/ml) for 4 h. Cells were then washed three times and cultured in fresh medium for 48 h. Conditioned medium (CM) was collected 48 h following infection, filtered through a 0.22-μm pore size filter (Millipore, Bedford, MA), and stored at −20°C for further analysis.

Assay of conditioned medium

CM was serially diluted and added to macrophages cultured at 5×10^5 in 12-well plates (Corning, Corning, NY). Macrophages were incubated in CM for 24 h and stimulated with IFN-γ (20 ng/ml), and surface expression of MHC class II (mouse macrophages) or FcγRI (CD64; human macrophages) was measured 24 h later by flow cytometry as described below.

Neutralization of cytokines

RAW264.7 cells were plated at a concentration of 5×10^5 in 12-well plates and treated with serial dilutions of CM from either infected or uninfected BMDM. To neutralize cytokines, mAbs to IL-6 (MP5-20F3), IL-10 (JES5-2A5), TGF-β (1D11), or their respective isotype controls were added at 2 μg/ml. Following 24-h incubation, IFN-γ (20 ng/ml) was added, and surface expression of MHC class II was determined by flow cytometry as described below.

Flow cytometry

Mtb-infected and uninfected macrophages were left untreated or were treated with IFN-γ (20 ng/ml) for 24 h at 37°C. Cell monolayers were washed twice on ice with cold PBS containing 0.5 mM EDTA, scraped from the wells, and transferred to FACS tubes (Falcon). The cells were washed, resuspended in PBS with 2.5% FCS and 0.1% NaN_3, and incubated for 45 min on ice with PE-conjugated anti-I-A/I-E (anti-MHC class II; mouse macrophages) or FITC-conjugated anti-FcγRI (human macrophages) using the concentrations recommended by the manufacturer. Cells were then washed with PBS and fixed overnight in 1% paraformaldehyde, if infected, or resuspended in PBS. Ten thousand cells were analyzed for MHC class II or CD64 expression on a FACSort flow cytometer and analyzed using CellQuest software (BD Biosciences, Mountain View, CA). The experimental data were expressed as the mean fluorescence intensity (MFI) of triplicate samples ± SD unless otherwise stated.

Quantitative RT-PCR

Total RNA was extracted using RNeasy (Qiagen, Valencia, CA), treated with DNase (Ambion, Austin, TX) to remove contaminating genomic DNA, and quantitated fluorometrically using the RiboGreen assay (Molecular Probes, Eugene, OR). RNA (1 μg) was reverse transcribed to cDNA under standard protocols using the RT system (Promega, Madison, WI). Standard cDNA, prepared from RNA extracted from RAW264.7 cells following an 8-h stimulation with IFN-γ was used at 50, 10, 2, and 0.4 ng in PCR reactions. For the amplification of individual genes, samples were used at a concentration of 10 ng, with the exception of IFN-γ-inducible class II *trans*-activator (CIITA; 100 ng) and the housekeeping gene, GAPDH (1 ng). Reactions were set up using SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA), and samples were subjected to thermal cycling on an ABI 7700 thermal cycler (PE Applied Biosystems) under the following conditions: stage 1, 95°C for 10 min; and stage 2, 94°C for 45 s, 60°C for 15 s, and 72°C for 30 s. Stage 2 was repeated for 40 cycles. Controls included a control without template to determine the presence of primer-dimers as well as controls without RT to determine the potential presence of residual genomic DNA. Dissociation curve analysis was performed to evaluate potential contribution to fluorescence from primer dimers or nonspecific amplicons. The data were analyzed with the Sequence Detector version 1.7a software (PE Applied Biosystems). To maintain consistency, the baseline was set automatically by the software, using data collected from cycles 3–15. In some cases it was necessary to over-ride this setting to optimize analysis. The increase in fluorescence intensity of SYBR Green (ΔRn) was plotted against cycle number. The threshold cycle (C_T) was calculated by the software as the cycle number at which ΔRn crosses the baseline. For each gene examined, a standard curve was generated by plotting C_T values against input cDNA concentration, and samples were quantified by interpolation from the standard curve. Data obtained for individual genes were normalized to the corresponding mean GAPDH levels. Fold increases were calculated taking the untreated sample as the baseline reference.

Production of pGrl-CIITApD1 stable transfectants and assay for luciferase activity

RAW 264.7 cells were stably cotransfected with 1 μg of pGrl-CIITApD1 (15) and 0.1 μg of pcDNA3.1-neo (Invitrogen)/10^6 cells, using the Trans-IT reagent (Mirus Technologies, Madison, WI) and protocols recommended by the manufacturer. Cells were selected in complete DMEM supplemented with 400 μg/ml G418 (Invitrogen), and clones stably expressing pGrl-CIITApD1 were selected for utilization in additional experiments on the basis of a >5-fold increase in luciferase activity in response to IFN-γ. To determine the effect of IL-6 on CIITA promoter activity, stably transfected RAW264.7 cells were treated with 4 ng/ml IL-6 and stimulated with 20 ng/ml of IFN-γ 24 h later. Cells were lysed 8 h later using passive lysis buffer (Promega), and the lysates were examined for luciferase activity using the luciferase reporter system assay (Promega).

Firefly luciferase induction driven by the CIITA promoter was normalized to the input protein levels as determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Immunoblots for STAT1 and phosphorylated-STAT1

For detection of total and phosphorylated STAT1, RAW264.7 cells were pretreated with IL-6 (4 ng/ml) for 24 h and then with 20 ng/ml IFN-γ for various time points as determined in pilot experiments. Total cellular protein lysates were prepared by washing cells with ice-cold PBS and then scraping them in ice-cold RIPA buffer (50 mM Tris (pH 7.4), 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml pepstatin A) supplemented with protease inhibitors (Roche, Mannheim, Germany) and phosphatase inhibitors (Calbiochem, San Diego, CA). The lysates were transferred to microfuge tubes, incubated on ice for 20 min, and clarified by centrifugation at $10,000 \times g$ at 4°C for 20 min. Proteins were separated by electrophoresis on a 12% reducing polyacrylamide gel, transferred to nitrocellulose membranes, blocked with 5% skim milk in Tris-buffered saline with 0.05% Tween 20, and incubated with either anti-STAT1 (Zymed) at a 1/250 dilution or anti-phospho-STAT1 (Tyr701; Zymed) at a 1/1,000 overnight at 4°C. Blots were washed, incubated with the appropriate HRP-labeled secondary Ab, and developed using ECL (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistical analysis

Statistical analysis was conducted using the software application PRISM (GraphPad, San Diego, CA). The statistical significance of experimental data was determined by two-tailed, unpaired, Student's t test. Differences with $p < 0.05$ were considered significant.

Results

CM from M. tuberculosis-*infected macrophages inhibits human and mouse macrophage responses to IFN-γ*

IFN-γ activation of macrophages effectively controls the growth of the intracellular parasite, *Toxoplasma gondii*. However, when macrophages are infected first with Mtb and subsequently with *T. gondii*, activation with IFN-γ fails to control the replication of *T. gondii* (14). This effect of Mtb is not limited to directly infected macrophages. Uninfected macrophages in culture with Mtb-infected macrophages were also unable to control *T. gondii* replication on activation with IFN-γ (L.-M. Ting and J. D. Ernst, unpublished observations), suggesting that infected macrophages secrete one or more factors that block IFN-γ-induced responses. To further investigate this observation, human macrophages were treated with fresh medium, medium from uninfected macrophages, or medium from macrophages infected with Mtb for 48 h, and then infected with *T. gondii*. As shown in Fig. 1A, macrophages treated with CM from Mtb-infected macrophages were unable to control replication of *T. gondii* on activation with IFN-γ, while addition of CM from uninfected macrophages had no such inhibitory effect. The effect of Mtb-CM on IFN-γ-inducible responses was not confined to inhibition of restriction of *T. gondii* growth. Mtb-CM also inhibited IFN-γ-induced surface expression of FcγRI (CD64) in a dose-dependent manner (Fig. 1B), suggesting a broader effect on IFN-γ-inducible responses. We also found that murine BMDM exposed to live (not shown) or gamma-irradiated (Fig. 2A) Mtb exhibit reduced responses to IFN-γ up-regulation of surface MHC class II, and in addition, CM from Mtb-infected BMDM inhibited IFN-γ-induced MHC class II expression in a dose-dependent manner (Fig. 2B). Inhibition of IFN-γ-induced MHC class II by CM was also observed in the murine macrophage cell line, RAW 264.7. As RAW264.7 cells are easily maintained and manipulated, additional experiments were performed using these cells.

IL-6 contributes to inhibition of responses to IFN-γ

Infection of macrophages with Mtb induces the production of multiple cytokines, including IL-10 (16), TGF-β (17), and IL-6 (18). To determine whether any or all of these cytokines were respon-
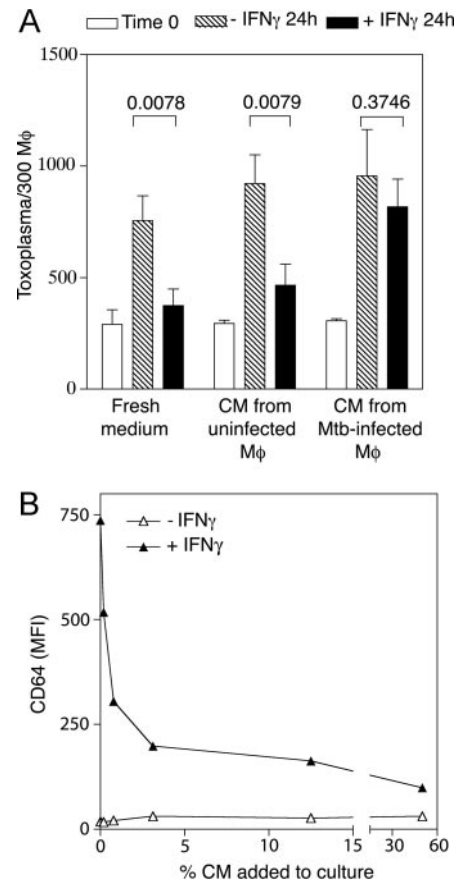

FIGURE 1. Mtb-CM inhibits IFN-γ-induced responses in human monocyte-derived macrophages. *A*, Monocyte-derived macrophages were treated with fresh medium, CM from uninfected macrophages, or CM from Mtb-infected macrophages for 48 h, and then infected with *T. gondii*. One hour after infection, some cells were fixed for time zero enumeration, while others were treated with IFN-γ (100 ng/ml) or medium. After an additional 24 h the remaining cells were fixed with methanol/acetone, and *Toxoplasma* were enumerated after staining with 4′,6-diamido-2-phenylindole hydrochloride. Values shown are the mean ± SD of the number of *T. gondii* nuclei per 300 macrophage nuclei, counted in triplicate. The *p* values shown are the comparison of the number of parasites in untreated vs IFN-γ-treated cells. *B*, Human monocyte-derived macrophages cultured in 96-well nonadherent plates were treated with CM from Mtb-infected monocyte-derived macrophages for 24 h, then stimulated with 100 ng/ml IFN-γ or left untreated. Surface expression of CD64 was analyzed by flow cytometry. Data shown are representative of three experiments, using monocytes isolated from different donors.

sible for the inhibition of IFN-γ-induced responses, neutralizing Abs to IL-6, TGF-β and IL-10 or their respective isotype controls were added to CM when used to treat macrophages before stimulation with IFN-γ. Neutralization of IL-6 resulted in a significant recovery of IFN-γ induction of MHC class II expression (Fig. 3A), while neutralization of neither IL-10 nor TGF-β had a measurable effect (Fig. 3, *B* and *C*). To determine whether IL-6 alone could antagonize macrophage responses to IFN-γ, RAW264.7 cells were incubated with increasing concentrations of recombinant murine IL-6 for 24 h and then stimulated with IFN-γ. As shown in Fig. 4, IL-6 inhibited the up-regulation of MHC class II by IFN-γ in a dose-dependent manner, with half-maximal inhibition occurring at 2 ng/ml. BMDM showed similar responses, although inhibition of IFN-γ-induced expression of MHC class II occurred at higher concentrations of IL-6, with half-maximal inhibition occurring at 8 ng/ml (data not shown).
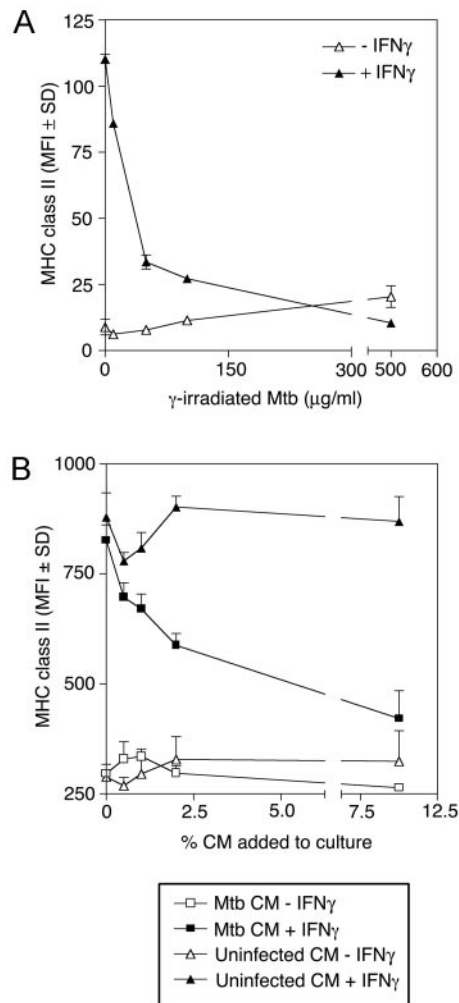
FIGURE 2. Gamma-irradiated Mtb and Mtb-CM inhibit IFN-γ induction of MHC class II on murine macrophages. BMDM were treated with increasing concentrations of gamma-irradiated Mtb for 48 h (A) or with increasing dilutions of CM for 24 h (B) and then stimulated with IFN-γ (20 ng/ml) or left untreated. Surface expression of MHC class II was measured 24 h later.

FIGURE 3. Neutralization of IL-6 partially restores IFN-γ induction of MHC class II expression in Mtb-CM-treated macrophages. IFN-γ induction of MHC class II expression was measured in RAW264.7 cells treated with increasing dilutions of CM and a constant concentration (2 μg/ml) of neutralizing Abs to IL-6 (A), IL-10 (B), or TGF-β (C).

To determine whether Mtb induces the production of sufficient IL-6 to account for the activity in Mtb-CM, we assayed IL-6 by ELISA. This revealed that 5×10^5 BMDM treated with 500 μg/ml gamma-irradiated Mtb for 24 h produced 12 ± 3 ng/ml of IL-6, a concentration sufficient to account for substantial inhibition of responses to IFN-γ.

IL-6 inhibits induction of selected IFN-γ-responsive genes

Since IL-6 and/or Mtb could inhibit surface expression of MHC class II by disrupting transcription, translation, post-translational modification, or intracellular trafficking, we first investigated whether IL-6 inhibits IFN-γ-induced gene expression. As the expression of MHC class II is under the control of the CIITA (19), we examined the effect of IL-6 on steady state levels of CIITA mRNA in IFN-γ-stimulated RAW264.7 cells. Treatment with IL-6 alone had no effect on CIITA mRNA levels. Pretreatment with IL-6 reduced IFN-γ-induced CIITA mRNA by 50–80% in separate experiments, at peak levels of CIITA expression (8 h; Fig. 5A). The reduction in mRNA levels was not limited to CIITA, but was also observed for the chemokine monokine induced by γ (55–70% reduction at peak expression) and the transcription factors, IFN regulatory factor-1 and IFN consensus sequence binding protein
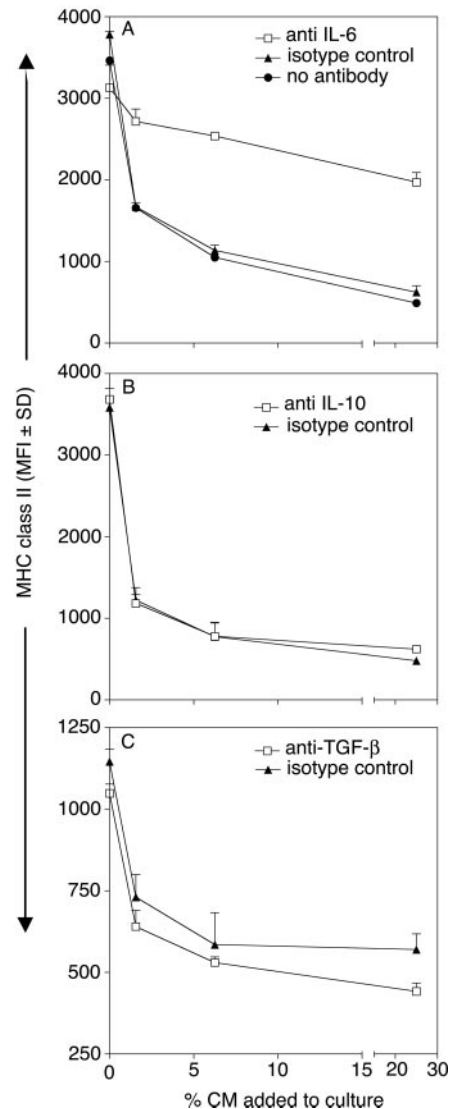
(50% reduction; Fig. 5B). IL-6 did not inhibit induction of all IFN-γ-responsive genes examined; the IFN-γ-inducible GTPases IFN-inducible GTPase and LRG-47, and the chemokine inducing protein-10 were marginally affected or unaffected by pretreatment of macrophages with IL-6 (Fig. 5B), indicating that the inhibitory effects of IL-6 are exerted on a subset of IFN-γ-responsive genes.

IL-6 inhibits transcription of the class II trans-*activator*

Although IFN-γ induction of CIITA depends on the induction of IFN regulatory factor-1 as well as on a γ-activated sequence element that binds STAT1 (15, 20), we performed additional studies of the mechanism of IL-6 inhibition by focusing on CIITA, because of its more marked inhibition. As IFN-γ induction of CIITA mRNA in macrophages is predominantly under the control of CIITA promoter IV (21), we examined the effect of IL-6 on RAW264.7 cells stably transfected with a plasmid containing a 560-bp promoter IV fragment driving luciferase. Pretreatment with IL-6 inhibited IFN-γ-induced activity of the CIITA promoter IV
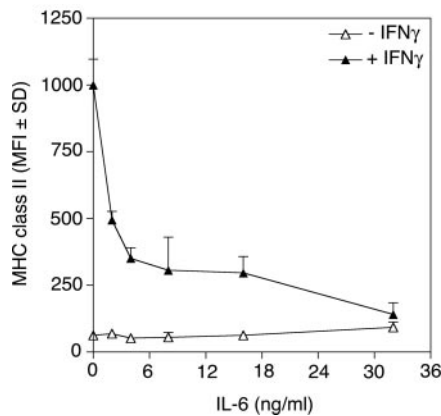
FIGURE 4. Recombinant IL-6 inhibits IFN-γ induction of MHC class II. RAW264.7 cells were treated with increasing concentrations of IL-6 for 24 h, then stimulated with IFN-γ or left unstimulated. Surface expression of MHC class II was assayed by flow cytometry 24 h later.

(Fig. 6*A*). Although promoter IV is the predominant IFN-γ-responsive CIITA promoter, CIITA promoters I and III are also IFN-γ-inducible in RAW264.7 cells (21, 22). We also found that CIITA promoters I and III are IFN-γ-responsive in RAW264.7 cells, and that IL-6 decreased IFN-γ-induced CIITA mRNA driven by all three promoters (Fig. 6*B*).

To determine whether IL-6 also affected the stability of CIITA mRNA, we examined the rate of decay of IFN-γ-induced CIITA mRNA. RAW264.7 cells, treated for 24 h with either IL-6 or control medium, were stimulated with IFN-γ. At the point of maximal expression of CIITA mRNA (8 h), actinomycin D was added to halt further RNA synthesis, and CIITA mRNA transcripts were quantitated by real-time RT-PCR at 2-h intervals over the course of 6 h. The rate of decay of CIITA was the same in IL-6-treated and control RAW264.7 cells (data not shown), indicating that IL-6 does not affect the stability of CIITA transcripts.
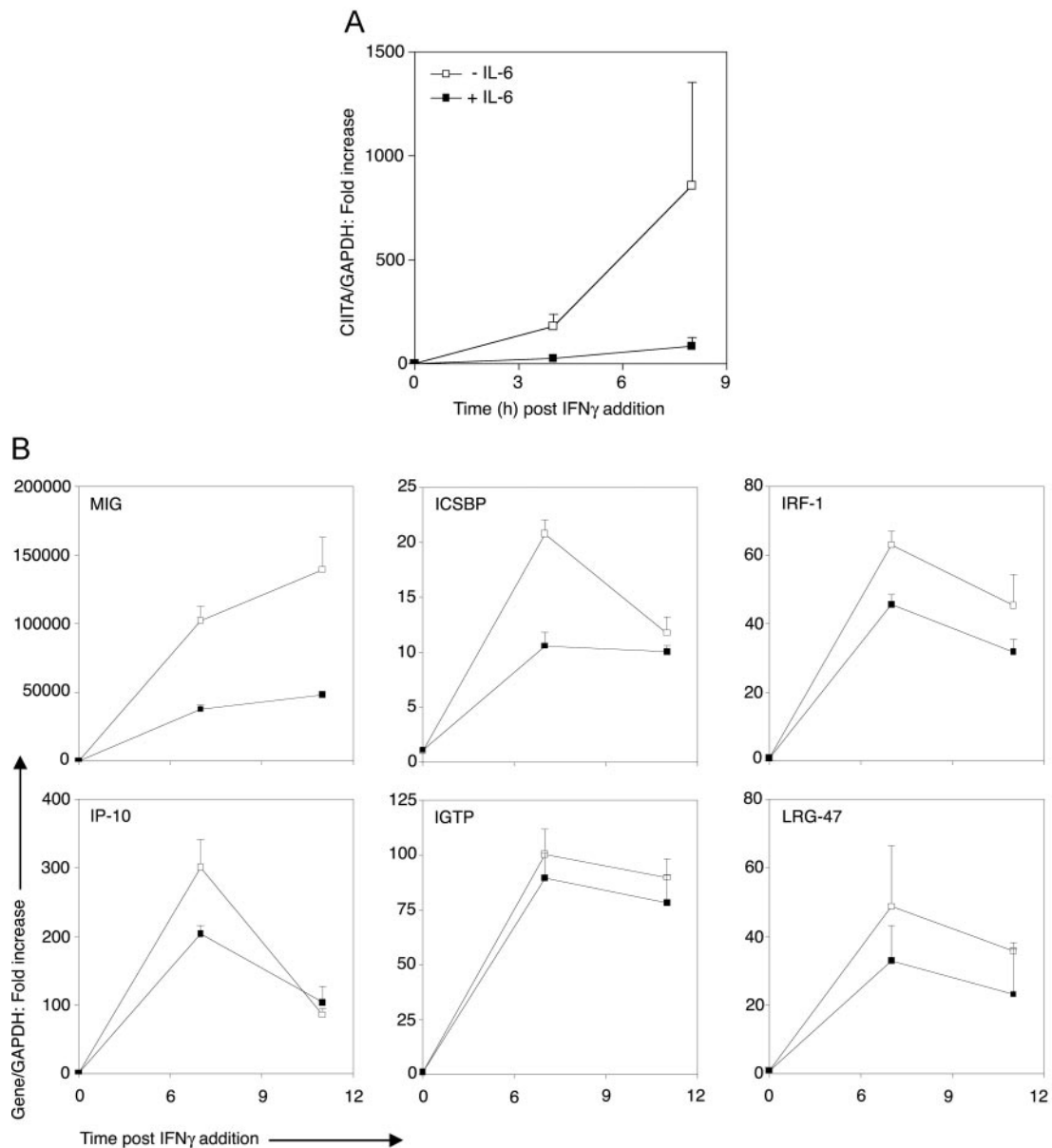

FIGURE 5. Pretreatment with IL-6 inhibits a subset of IFN-γ-responsive genes. RAW264.7 cells, treated with IL-6 or left untreated, were stimulated with IFN-γ. Total RNA was extracted at intervals as indicated, and transcripts of CIITA (*A*) or other IFN-γ-regulated genes (*B*) were quantitated by real-time RT-PCR as described in *Materials and Methods*.
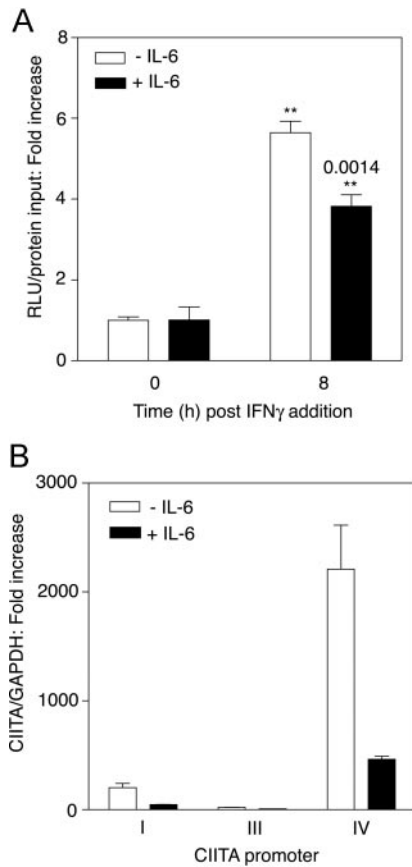
FIGURE 6. IL-6 inhibits transcriptional control of CIITA. *A*, RAW264.7 cells stably transfected with pGrl-CIITApD1 were treated with IL-6 or medium alone. Following stimulation with IFN-γ 24 h later, the cells were lysed at the time points indicated for detection of luciferase activity. Log luminescence units driven by the CIITA promoter for each sample was normalized to the respective protein input. Data are expressed as the fold increase over the untreated sample and represent the mean ± SD of triplicate samples. *B*, RAW264.7 cells were either treated with IL-6 for 24 h or were left untreated. Total RNA was extracted 0 or 8 h following the addition of IFN-γ, and transcripts of CIITA driven by promoters I, III, and IV were quantitated by real-time RT-PCR.

IL-6 inhibition of macrophage responses to IFN-γ requires new protein synthesis

To further understand the mechanism of IL-6, we examined the time course of its effect on macrophages. Brief (15–60 min) pretreatment of macrophages with IL-6 had no effect on subsequent IFN-γ induction of MHC class II (Fig. 7*A*). However, after 24 h, inhibition by IL-6 was apparent (Fig. 7*A*), which suggests that the effect of IL-6 on responses to IFN-γ requires the synthesis of new protein, rather than inhibition of post-transcriptional modification of constitutive proteins. Indeed, cycloheximide, a reversible inhibitor of protein synthesis, blocked the effect of IL-6 on IFN-γ induction of surface MHC class II (Fig. 7*B*) and CIITA mRNA (Fig. 7*C*).

Suppressors of cytokine signaling (SOCS) proteins do not account for the effects of IL-6 in macrophages

SOCS proteins are inducible by IFNs and other cytokines and mediate the inhibition of responses to IFN-γ by blocking phosphorylation of STAT1 (23, 24). Since IL-6 inhibition of macrophage responses to IFN-γ required new protein synthesis, we examined the possibility that SOCS-1 and/or SOCS-3 accounted for the effects of IL-6. As shown in Fig. 8*A*, LPS induced the expression of
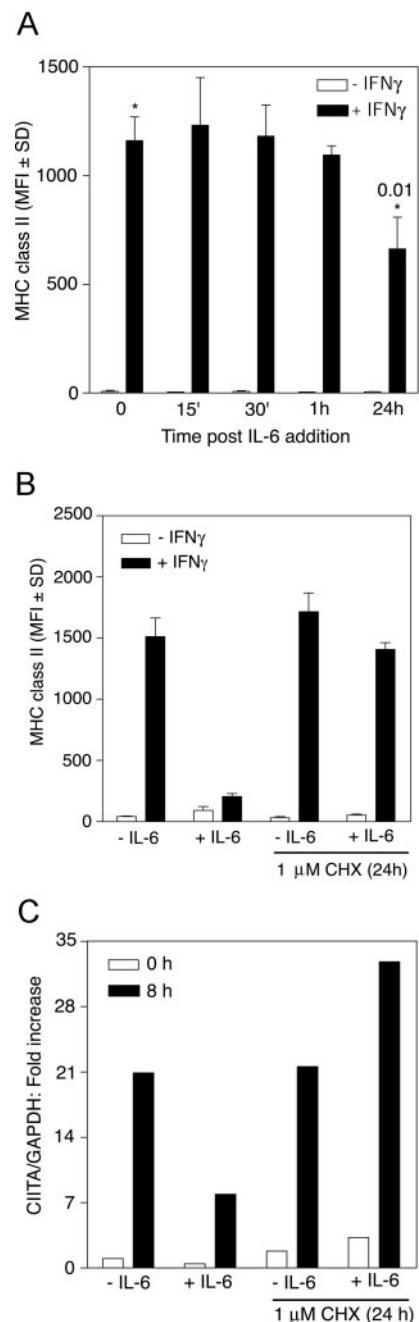

FIGURE 7. Inhibition of IFN-γ responses by IL-6 requires the synthesis of new protein(s). *A*, RAW264.7 cells were treated with IL-6 for varying lengths of time as indicated. The cells were either treated with IFN-γ or left untreated, and the surface expression of MHC class II was assayed 24 h later. *B*, RAW264.7, treated with IL-6 or medium alone were either simultaneously treated with 1 μM cycloheximide or left untreated. Cells were washed 24 h later, allowed to recover for 1 h, and then stimulated with IFN-γ or incubated in medium in the absence of cycloheximide. The surface expression of MHC class II was measured 24 h later. *C*, Alternatively, total RNA was extracted at 0 and 8 h poststimulation with IFN-γ. CIITA transcripts were quantitated by real-time PCR.

SOCS-1 and SOCS-3 mRNA in RAW264.7 macrophages. However, IL-6 did not induce SOCS-1 expression and induced only marginal amounts of SOCS-3 at concentrations that markedly inhibited IFN-γ induction of CIITA and surface MHC class II (Fig. 8*A*). IL-6 was also unable to induce SOCS-1 or significant SOCS-3 expression in BMDM from C57BL/6 mice (data not shown). As
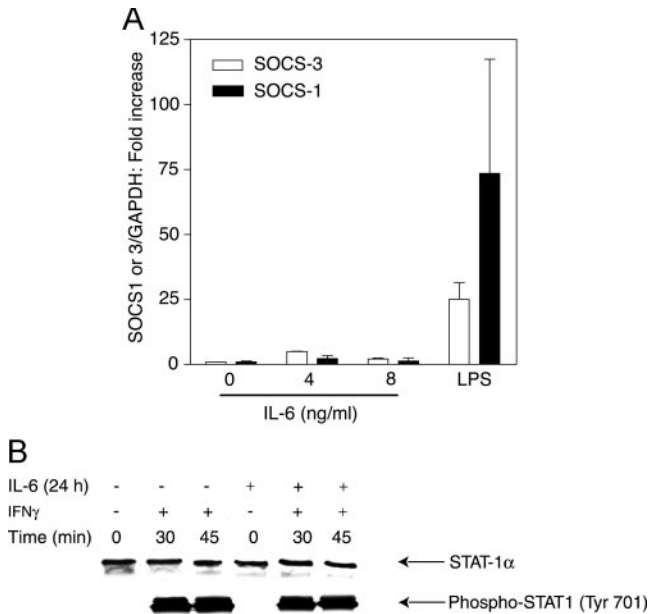
FIGURE 8. IL-6 does not induce SOCS-1 or SOCS-3. *A*, RAW264.7 cells were treated with IL-6 (4 or 8 ng/ml) or LPS (100 ng/ml) or were left untreated. Total RNA was extracted 5 h later, and SOCS-1 or SOCS-3 mRNA were quantitated by real-time RT-PCR. *B*, RAW 264.7 cells, either treated with IL-6 or medium alone, were stimulated with IFN-γ, and the cells were lysed at intervals as indicated. The presence of total STAT1 or phosphorylated-STAT1 was detected by Western blotting.

further evidence that the action of IL-6 is not mediated by SOCS proteins, IL-6 did not inhibit tyrosine phosphorylation of STAT1 in response to IFN-γ (Fig. 8*B*).

Discussion

Mtb is a highly successful intracellular pathogen that is able to survive and cause disease despite the induction of innate and adaptive immune responses. While IFN-γ is clearly required for the prevention of rapidly progressive Mtb infection (5, 6), even the development of an immune response with abundant local production of IFN-γ is unable to reliably eradicate Mtb infection. We have previously reported that Mtb is able to block macrophage responses to IFN-γ by inhibiting the transcription of IFN-γ-responsive genes, indicating that this phenomenon may contribute to the inability of an adaptive immune response to provide complete protection against tuberculosis (14). We extend our observations to report here that one mechanism for Mtb to render macrophages refractory to activation by IFN-γ is to stimulate macrophages to secrete an inhibitory factor(s), one of which is IL-6. Furthermore, our observations indicate a novel role for IL-6, in that it inhibits IFN-γ signaling in macrophages.

IL-6 is a pleiotropic cytokine that is produced by a variety of cells, including macrophages (25), T cells (26), endothelial cells (27), and fibroblasts (28), and also exerts its effects on multiple cell types. IL-6, recognized for its role as a proinflammatory cytokine, induces the production of acute phase proteins in response to infection and injury (29) and synergizes with IL-1 in inducing acute phase responses such as pyrexia and leukocytosis (30). In addition, IL-6 is both a B cell growth and differentiation factor (25, 31). In vivo, IL-6 has also been shown to play a role in the initiation of T cell activation (32). More recently, IL-6 induced by microbial components has been observed to support effector T cell proliferation in vivo by suppressing regulatory T cells (33).

In contrast to these observations, IL-6 has also been implicated in inhibiting T cell responses. VanHeyningen and coworkers (34) observed that exposure to macrophages infected with *M. bovis* BCG or *M. avium* inhibited Ag-specific proliferation of a CD4^{+} T cell hybridoma. In addition, they noted that both CM from mycobacterium-infected macrophages and purified IL-6 were capable of inhibiting T cell hybridoma proliferation. As this phenomenon was observed even when APCs were fixed with paraformaldehyde, T cells were probably the predominant target of the action of IL-6 in their studies. Additionally, IL-6 has been been found to block IFN-γ induction of Th1 differentiation in naive CD4^{+} T cells (35). In these cells IL-6 induced expression of SOCS-1, thereby inhibiting IFN-γ-induced tyrosine phosphorylation of STAT1 and, therefore, IFN-γ-dependent Th1 differentiation. In contrast, our studies of macrophages reveal that IL-6 can also inhibit cellular responses to IFN-γ by a SOCS-1- and SOCS-3-independent mechanism, implying that the effects of IL-6 on IFN-γ responses can be mediated by distinct mechanisms in different cell types.

IL-6 has been previously observed to have an inhibitory effect on macrophage function. It is capable of suppressing the production of LPS-induced TNF-α in human peripheral blood monocytes (36). Additionally, murine peritoneal macrophages pretreated with IL-6 were found to be incapable of restricting *Toxoplasma* replication in the presence of IFN-γ (37). We observed that IL-6 was induced by infection of macrophages with Mtb and contributed to inhibition of macrophage responses to IFN-γ. Our findings thus concur with these observations and additionally provide insight into the mechanism of action of IL-6 in inhibiting the effects of IFN-γ.

Although infection with Mtb induces IL-6, the role of IL-6 in the immune response to Mtb in vivo is not well understood. Significant quantities of IL-6 are produced in response to Mtb infection; human and murine macrophages secrete IL-6 in response to Mtb in vitro (38, 39), and elevated concentrations of IL-6 are present in plasma of patients with tuberculosis (40). Studies of Mtb infection of IL-6^{−/−} mice suggest that IL-6 may play a role in the early stages of T cell activation (41), in contrast to our results indicating that IL-6 inhibits macrophage activation. We have also observed, however, that Mtb inhibits IFN-γ induction of MHC class II on BMDM from both wild-type and IL-6^{−/−} mice and that CM from *M. tuberculosis*-infected macrophages from IL-6^{−/−} mice contains some residual inhibitory activity (data not shown), indicating that Mtb inhibits IFN-γ activation of macrophages in both an IL-6-dependent and independent manner.

The induction of IL-6 production is not unique to Mtb. Indeed, stimulation of TLR4 is a predominant mechanism for the induction of IL-6 (42), and numerous bacteria possess components that activate TLR4 signaling. For instance, bacteria such as *Escherichia coli* (43) and *Neisseria* species (44) induce the production of significant quantities of IL-6. However, these bacterial species are responsible for acute infections and have not developed mechanisms to survive intracellularly. Consequently, the effects of IL-6 on the immune system do not impact the progress of infection induced by these bacteria in a manner similar to that observed in tuberculosis. Mtb, on the other hand, has developed multiple survival strategies, such as prevention of phagosome maturation (45, 46), prevention of phagosomal acidification (45), and alteration of the capacity of dendritic cells to serve as APCs (46). Our results suggest Mtb has also taken advantage of the induction and activity of IL-6 as another strategy to ensure its survival in the face of an apparently robust cellular immune response. In addition, our observations reveal a novel inhibitory function for IL-6 with respect to IFN-γ signaling, and we suggest that intracellular organisms such as Mtb that lead to chronic infection exploit this inhibitory property of IL-6 to evade eradication by a cellular immune response.

Acknowledgments

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