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Inhibition of IFN-γ-Induced Class II Transactivator Expression by a 19-kDa Lipoprotein from *Mycobacterium tuberculosis*: A Potential Mechanism for Immune Evasion[1]

Rish K. Pai,* Marilyn Convery,* Thomas A. Hamilton,‡ W. Henry Boom,[2†] and Clifford V. Harding[2,3]*

Mycobacterium tuberculosis **(MTB) persists inside macrophages despite vigorous immune responses. MTB and MTB 19-kDa lipoprotein inhibit class II MHC (MHC-II) expression and Ag processing by a Toll-like receptor 2-dependent mechanism that is shown in this study to involve a defect in IFN-γ induction of class II transactivator (CIITA). Exposure of macrophages to MTB or MTB 19-kDa lipoprotein inhibited IFN-γ-induced MHC-II expression, but not IL-4-induced MHC-II expression, by preventing induction of mRNA for CIITA (total, type I, and type IV), IFN regulatory factor-1, and MHC-II. MTB 19-kDa lipoprotein induced mRNA for suppressor of cytokine signaling (SOCS)1 but did not inhibit IFN-γ-induced Stat1 phosphorylation. Furthermore, the lipoprotein inhibited MHC-II Ag processing in SOCS1$^{-/-}$ macrophages. MTB 19-kDa lipoprotein did not inhibit translocation of phosphorylated Stat1 to the nucleus or Stat1 binding to and transactivation of IFN-γ-sensitive promoter constructs. Thus, MTB 19-kDa lipoprotein inhibited IFN-γ signaling independent of SOCS1 and without interfering with the activation of Stat1. Inhibition of IFN-γ-induced CIITA by MTB 19-kDa lipoprotein may allow MTB to evade detection by CD4$^+$ T cells.** ***The Journal of Immunology,*** **2003, 171: 175–184.**

The cellular immune response to tuberculosis involves macrophages, CD4$^+$ T cells, CD8$^+$ T cells, and γδ T cells. Macrophages are critical to control of *Mycobacterium tuberculosis* (MTB)[4] infection, because they harbor the bacteria in intracellular compartments and present MTB Ags via class II MHC (MHC-II) to CD4$^+$ T cells. CD4$^{-/-}$ and MHC-II$^{-/-}$ mice have increased susceptibility to MTB (1). CD4$^+$ T cells secrete IFN-γ to activate infected macrophages and induce microbicidal function (2–4). IFN-γ$^{-/-}$ mice are extremely susceptible to MTB, and infection leads to unchecked growth of MTB in the organs of these mice. Macrophage activation is defective, and NO synthase 2 expression is low in these animals (3). In humans, IFN-γ also appears to play a pivotal role, because children defective in the genes for IFN-γ or the IFN-γR are prone to serious mycobacterial infections (5).

Despite robust CD4$^+$ T cell responses, live MTB bacilli can persist inside the host for many years. The mechanisms that allow prolonged survival of MTB in the face of vigorous CD4$^+$ T cell responses are not understood. We have previously shown that a constitutive component of MTB can inhibit MHC-II expression and Ag processing in murine macrophages (6). This component was purified and found to be a 19-kDa lipoprotein (7), a previously defined lipoprotein Ag of MTB (8, 9) (lpqH/Rv3763; see http://www.sanger.ac.uk/Projects/M_tuberculosis/Gene_list/CDS/Rv3763.shtml). Recognition of MTB 19-kDa lipoprotein and other microbial pathogen-associated molecular patterns (PAMPs) by Toll-like receptors (TLRs) produces proinflammatory host responses that help combat infection (10), but PAMPs may also induce counterregulatory or inhibitory effects, e.g., inhibition of MHC-II expression and Ag processing (6, 7, 11). Although MTB 19-kDa lipoprotein appears to be the predominant MTB PAMP involved in inhibiting MHC-II expression and Ag processing (7), MTB contains multiple other PAMPs that may also contribute. In this study, we further characterize the mechanism whereby MHC-II expression and Ag processing are inhibited. MTB and MTB 19-kDa lipoprotein are shown to inhibit IFN-γ induction of mRNA for class II transactivator (CIITA), IFN regulatory factor-1 (IRF-1), and MHC-II. The inhibition of IFN-γ-induced responses may contribute to the ability of intracellular MTB to evade immune surveillance and maintain chronic infection.

Materials and Methods

Cells and reagents

The standard medium was DMEM (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT), 50 μM 2-ME, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, and antibiotics. The following were housed under specific pathogen-free conditions: C3H/HeJ (H-2^k) and C57BL/6 (H-2^b) mice (The Jackson Laboratory, Bar Harbor, ME), TLR2$^{-/-}$ and MyD88$^{-/-}$ knockout mice (generously provided by Drs. O. Takeuchi and S. Akira (Osaka University, Osaka, Japan) (12, 13) and bred onto the C57BL/6 background), and suppressor of cytokine signaling (SOCS)1$^{+/-}$IFN-γ$^{-/-}$ mice (from J. Ihle (St. Jude Hospital, Memphis, TN); bred to produce SOCS1$^{-/-}$, SOCS1$^{+/-}$,

*Department of Pathology, †Division of Infectious Diseases and ‡Tuberculosis Research Unit, Case Western Reserve University and University of Hospitals of Cleveland, Cleveland, OH 44106; and Department of Immunology, Cleveland Clinic Foundation, Cleveland, OH 44195

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[2] W.H.B. and C.V.H. shared senior authorship.

[3] Address correspondence to Dr. Clifford V. Harding, Department of Pathology, Biomedical Research Building 925, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106-4943. E-mail address: cvh3@po.cwru.edu

[4] Abbreviations used in this paper: MTB, *Mycobacterium tuberculosis*; MHC-II, class II MHC; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor; CIITA, class II transactivator; IRF-1, IFN regulatory factor-1; ODN, oligodeoxynucleotide; CTLL, cytotoxic lymphoid line; SOCS, suppressor of cytokine signaling; p, promoter; IP, immunoprecipitation; JAK, Janus-activated kinase; GAS, γ-activated sequence; USF-1, upstream stimulation factor-1; CBP, CREB binding protein; RNase, bovine RNase A; MFV, mean fluorescence value.

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and SOCS1$^{+/+}$ mice). Macrophages were derived from femur marrow precursors differentiated in bacterial grade dishes for 7 days in standard medium with 20% LADMAC conditioned medium (14). Resulting macrophages were used the following week. Bovine RNase A (RNase) and OVA were from Sigma-Aldrich (St. Louis, MO). RNase$_{42–56}$:I-A^k complexes were detected with TS12 T hybridoma cells (P. Allen and E. Unanue (Washington University, St. Louis, MO)). OVA:I-A^b complexes were detected with DOBW T hybridoma cells (15). MTB Ag85B$_{241–256}$:I-A^b complexes were detected with BB7 T hybridoma cells (7). RAW 264.7 cells were from American Type Culture Collection (Manassas, VA). Murine IFN-γ and IL-4 were from R&D Systems (Minneapolis, MN). Phosphorothioate-modified CpG oligodeoxynucleotide (ODN) 1826 (TCCATGACGTTCCTGACGTT) was provided by Coley Pharmaceutical Group (Wellesley, MA).

MTB culture and purification of MTB 19-kDa lipoprotein

MTB H37Ra (American Type Culture Collection) was grown to log phase in Middlebrook 7H9 medium (Difco, Detroit, MI) with albumin, dextrose, and catalase enrichments (Difco), harvested, and frozen at −70°C (16). Bacterial titer was determined by counting CFU on 7H10 medium (Difco). MTB 19-kDa lipoprotein was purified as described (7) except that the source was MTB cell wall instead of MTB lysate. MTB cell wall was obtained by suspending MTB H37Ra in deionized water containing 7.5 mM EDTA, 0.7 μg/ml leupeptin, 0.2 mM PMSF, 0.7 μg/ml pepstatin A, 10 U/ml DNase, and 25 U/ml RNase, passing the suspension through a French press two to three times, centrifuging it for 1–2 h at 100,000 × *g*, and harvesting the pellet. MTB cell wall was rotated at 4°C overnight with ice-cold 17% TX114 (Sigma-Aldrich) in 50 mM Tris-Cl (pH 7.4) and centrifuged for 2 h at 100,000 × *g*. The pellet was discarded. The solution was warmed to 37°C for 15 min. The detergent layer was washed three times with buffer (50 mM Tris-Cl; pH 7.4) and precipitated overnight in acetone at −20°C. MTB 19-kDa lipoprotein was purified from the resulting pellet by SDS-PAGE electroelution using a Bio-Rad (Hercules, CA) Model 491 Prep Cell (7). Fractions containing MTB 19-kDa lipoprotein were pooled, extracted into TX114, and precipitated by acetone at 20°C. The protein was resolubilized in 90% DMSO. SDS-PAGE with silver staining or Western analysis using a polyclonal rabbit anti-BCG serum (that detects many mycobacterial Ags) revealed a single major band of appropriate size that stained with mAb to MTB 19-kDa lipoprotein.

Ag processing and presentation assays

Macrophages were removed from dishes with trypsin-versene (Life Technologies) and plated in 96-well flat-bottom plates (5 × 10^4 cells/well). Cells were incubated with or without MTB 19-kDa lipoprotein or CpG ODN for 20–24 h, or infected by incubation with MTB H37Ra for 2 h, and then washed and incubated for 20–24 h. Cells were incubated for 24 h with 2 ng/ml rIFN-γ or 10 ng/ml rIL-4 (in the continued presence or absence of MTB 19-kDa lipoprotein or CpG ODN), incubated with RNase or OVA for 2 h, fixed in 0.5–1% paraformaldehyde, washed, and incubated with T hybridoma cells (10^5/well) for 20–24 h. Supernatants were assessed for IL-2 by cytotoxic lymphoid line (CTLL)-2 bioassay with colorimetric determination using Alamar Blue (Alamar Biosciences, Sacramento, CA) and a Bio-Rad 550 microplate reader.

Flow cytometry

Macrophages were removed with trypsin plus 0.02% EDTA (Life Technologies), placed in V-bottom 96-well plates (2 × 10^5/well), incubated with 10% normal mouse serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 1% FCS in PBS, and then stained with biotinylated 10.3.6-2 anti-I-A^k, anti-IFN-γRI (BD PharMingen, San Diego, CA), or biotinylated IgG2a isotype control Ab (BD PharMingen) at 10 μg/ml. Cells were washed, incubated with streptavidin-CyChrome (1:100; BD PharMingen), resuspended in 1% paraformaldehyde, and analyzed with a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA).

RNA purification, cDNA synthesis, and real-time quantitative PCR

Macrophages (4 × 10^6) were plated in 60-mm Petri dishes and incubated with or without MTB 19-kDa lipoprotein for 24 h and then for various periods with IFN-γ in the continued presence or absence of lipoprotein. To study the induction of SOCS1 and -3, macrophages were similarly plated, allowed to rest overnight, and incubated with MTB 19-kDa lipoprotein for 6 h. Macrophages were removed with trypsin plus 0.02% EDTA and lysed with a QiaShredder (Qiagen, Valencia, CA). Total RNA was purified using the RNeasy kit (Qiagen). Residual genomic DNA was removed with RNase-free DNase (Qiagen). RNA was stored in RNase-free water at −80°C. RNA (1 μg) was converted to cDNA using the SuperScript preamplification system (Life Technologies) for first-strand cDNA synthesis. The cDNA mixture was diluted 1/5 with PCR-grade water. Ten percent (10 μl) of the cDNA product was used per reaction for real-time quantitative PCR using a high-speed thermal cycler (LightCycler; Roche Diagnostics, Indianapolis, IN). Product was detected by FastStart Master SYBR Green I (Roche Molecular Biochemicals, Indianapolis, IN). The amplification cycle was 95°C for 15 s, 50°C for 5 s (57°C for GAPDH primers), and 72°C for 20 s. The following primers were designed using OLIGO version 6.4 (Molecular Biology Insights, Cascade, CO): MHC-II (I-A$_\beta^k$ chain), sense, 5′–3′ GCGACGTGGGCGAGTACC, and antisense, 5′–3′ CATTCCG GAACCAGCGCA (predicted size, 276 bp); total CIITA mRNA, sense, 5′–3′ ACGCTTTCTGGCTGGATTAGT, and antisense, 5′–3′ TCAAC GCCAGTCTGACGAAGG (predicted size, 342 bp); antisense primer for types I and IV CIITA, 5′–3′ GGTCGGCATCACTGTTAAGGA; sense primer type I CIITA, 5′–3′ AAGAGCTGCTCTCACGGGAAT (predicted size, 264 bp); sense primer type IV CIITA, 5′–3′ GAGACTGCATGCAG GCAGCA (predicted size, 129 bp); IRF-1 mRNA, sense, 5′–3′ ACCCGT TCTTGCCCTCCTG, and antisense, 5′–3′ CAGCCCAGCGATAAGAC CCTC (predicted size, 316 bp); SOCS1 mRNA, sense, 5′–3′ TCCGC TCCCACTCCGATTACC, and antisense 5′–3′ CTGAGCGCGAAGAA GCAGTTC (predicted size, 175 bp); and SOCS3 mRNA, sense, 5′–3′ AGAGCTGGCAGGACCTGGAAT, and antisense, 5′–3′ AAACCAAAT CAAAGCGCAAAC (predicted size, 268 bp). GAPDH primer sequences were as published (17): GAPDH, sense, 5′–3′ AACGACCCCTTCATT GAC, and antisense, 5′–3′ TCCACGACATACTCAGCAC (predicted size, 191 bp). Specific cDNA was quantified by standard curves based on known amounts of product. Standards were generated from amplified cDNA purified on agarose gels by QiaQuick gel extraction (Qiagen). Melting curve analysis confirmed that only one product was amplified. Specificity was confirmed by centrifugation of capillaries to obtain PCR products for electrophoresis through 1.5% agarose gels (stained with ethidium bromide). Only one product was observed with each primer set, and product size matched that predicted from published cDNA sequences. Normalized copy number = (copy number of mRNA of interest/copy number of GAPDH) × 1000. Fold induction = (normalized copy number of treated cells)/(normalized copy number of untreated cells).

Preparation and analysis of whole-cell and nuclear lysates

Cells were washed with cold PBS, scraped into PBS, and pelleted. For whole-cell lysates, cell pellets were resuspended in modified radioimmunoprecipitation analysis buffer (50 mM Tris (pH 7.5), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 2 mM EDTA, 50 mM NaF, 1 mM $NaVO_4$, 1 mM PMSF, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin), and insoluble material was removed by centrifugation at 12,000 rpm for 10 min at 4°C. For nuclear lysates, cell pellets were resuspended in buffer A (10 mM HEPES (pH 7.9); 10 mM KCl; 0.1 mM EDTA; 1 mM EGTA; 1 mM $NaVO_4$; 1 mM PMSF; 10 μg/ml each of leupeptin, antipain, and aprotinin; and 5 μg/ml pepstatin A) for 10 min at 4°C. Nonidet P-40 was added to 0.2% final concentration. The suspension was passed through a 26-gauge needle. Nuclei were pelleted by centrifugation for 1 min at 12,000 rpm, washed once in buffer A, lysed by resuspension in buffer B (20 mM HEPES (pH 7.9); 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM $NaVO_4$; 1 mM PMSF; 10 μg/ml each of leupeptin, antipain, and aprotinin; and 5 μg/ml pepstatin A) at 4°C for 1 h. Insoluble material was removed by centrifugation for 10 min at 12,000 rpm. Protein concentrations were determined with Bio-Rad or DC protein assay (Bio-Rad) and BSA standards.

For precipitation of nuclear lysate with promoter (p)IV CIITA ODN, an equal amount of protein (70 μg) from each condition was diluted 3-fold in immunoprecipitation (IP) buffer (0.1% Triton X-100; 10 mM HEPES (pH 7.3); 2 mM EDTA; 1 mM EGTA; 1 mM DTT; 10% glycerol; 1 mM NaF; 1 mM $NaVO_4$; 1 mM PMSF; 10 μg/ml each of leupeptin, aprotinin, and antipain; and 5 μg/ml pepstatin A) and then diluted to 500 μl in IP buffer with 0.1 M KCl. Double-stranded pIV CIITA ODN (150 pmol; upperstrand 5′–3′ biotin-GGATCCGTGGCAGCTTCTGAGAAAGCACGTG GTGGA) was incubated with 80 μl of 50% slurry of streptavidin-agarose beads (Amersham Pharmacia, Piscataway, NJ) for 4 h with rotation at 4°C. Beads were pelleted, washed four times in IP buffer with 0.1 M KCl, and resuspended in SDS-PAGE buffer.

SDS-PAGE and Western blot analysis

Samples were boiled in SDS-PAGE sample buffer under reducing conditions and electrophoresed on 8–10% SDS polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membrane, incubated for 1 h at room temperature in blocking buffer (5% Carnation nonfat dry milk (Nestle, Solon, OH) in PBS with 0.1% Tween 20) and probed with primary

Ab in blocking buffer overnight at 4°C. Blots were washed, incubated with HRP-labeled secondary Ab (Amersham Pharmacia) for 1 h at room temperature, and developed with ECL detection kit (Pierce, Rockford, IL). Blots were stripped with ReBlot Strong Western blot recycling kit (Chemicon, Temecula, CA) and reprobed. Abs included anti-Stat1-Y701 and anti-Stat1-S727 (Upstate Biotechnology, Lake Placid, NY), and anti-Stat1α, anti-actin, anti-USF-1 and anti-IRF-1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Luciferase reporter gene assay

RAW 264.7 cells were transiently transfected with 8 μg of control plasmid (pGL2-Basic) or pIVCIITA.Luc (luciferase reporter construct containing CIITA pIV, -360 to $+50$ (18, 19); generously provided by J. Ting (University of North Carolina, Chapel Hill, NC)) and 2 μg of reference plasmid (pRL-TK; Promega, Madison, WI) using the Superfect transfection system (Qiagen). Some experiments used the IRFGAS2X luciferase reporter construct containing Stat1 binding sites from the IRF-1 promoter (20). Cells were rested for 2 h and incubated for 24 h with or without 30 nM MTB 19-kDa lipoprotein, and for 20 h with or without IFN-γ in the continued presence or absence of the lipoprotein. Cells were harvested, and luciferase activity was measured using a luminometer with the Dual-Luciferase Reporter assay system (Promega).

Results

Inhibition of IFN-γ-induced MHC-II Ag processing by MTB and MTB 19-kDa lipoprotein involves TLR2 and MyD88

We have previously shown that prolonged exposure to MTB 19-kDa lipoprotein inhibits macrophage MHC-II expression and Ag processing by a TLR2-dependent mechanism (7). Because MyD88 is required for signaling by TLR2 and most other TLRs (with the exception of a MyD88-independent component of TLR4 signaling), we compared the effects of MTB and the 19-kDa lipoprotein in MyD88$^{-/-}$, TLR2$^{-/-}$, and wild-type macrophages. Consistent with prior results, exposure of macrophages to MTB 19-kDa lipoprotein for 24 h impaired the ability of IFN-γ to induce MHC-II Ag processing in C57BL/6 macrophages (Fig. 1*A*), but TLR2$^{-/-}$ macrophages were completely resistant to inhibition by MTB 19-kDa lipoprotein (Fig. 1*B*). Furthermore, inhibition of MHC-II Ag processing by MTB 19-kDa lipoprotein was completely dependent on MyD88 (Fig. 1*C*).

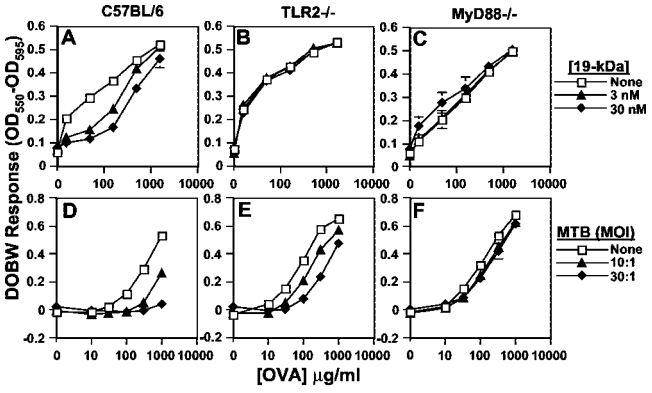

FIGURE 1. MTB and MTB 19-kDa lipoprotein inhibit Ag processing by signaling through TLRs. Macrophages were prepared from TLR2$^{-/-}$, MyD88$^{-/-}$, and wild-type C57BL/6 mice. Macrophages were incubated for 24 h with or without MTB 19-kDa lipoprotein (*A–C*) or infected with MTB (*D–F*). Macrophages were then stimulated for 24 h with IFN-γ (2 ng/ml) in the continued presence or absence of MTB 19-kDa lipoprotein or MTB, exposed to OVA for 2 h, fixed, and incubated with DOBW T hybridoma cells. IL-2 secretion was determined using a CTLL-2 bioassay with colorimetric readout. Results are expressed as the means of triplicate wells ± SD. When error bars are not visible, they are smaller than the symbol width. These results are representative of three independent experiments.

Infection of wild-type macrophages with live MTB H37Ra also inhibited IFN-γ-induced MHC-II Ag processing (Fig. 1*D*). As previously described (6), infection of TLR2$^{-/-}$ macrophages produced a lesser or partial inhibition (Fig. 1*E*), suggesting that signaling for MTB-mediated inhibition involved both TLR2-dependent and TLR2-independent mechanisms. In this study, we show that MyD88$^{-/-}$ macrophages were completely resistant to MTB-mediated inhibition of IFN-γ-dependent MHC-II Ag processing (Fig. 1*F*). In summary, TLR2 is a major contributor to recognition of MTB PAMPs (e.g., the 19-kDa lipoprotein) and induction of MTB-mediated inhibition, but MTB appears to contain other PAMPs that are distinct from the 19-kDa lipoprotein and signal via different receptor(s). Nonetheless, MTB-mediated inhibition is completely dependent on MyD88 for transduction of signals arising from all MTB PAMPs and their receptors.

During the course of MTB infection, macrophages may become infected and exposed to MTB PAMPs (including the 19-kDa lipoprotein) before they are activated by IFN-γ. As Fig. 1 suggests, infected macrophages may be unable to process MTB Ags for MHC-II presentation even when subsequently stimulated with IFN-γ due to MTB-mediated inhibition of IFN-γ-induced MHC-II expression and Ag processing. Furthermore, this inhibition may involve MTB 19-kDa lipoprotein (which is shed from bacilli) and TLR2-mediated signaling. To test these hypotheses, we infected wild-type or TLR2$^{-/-}$ macrophages with MTB for 48 h and then added IFN-γ for 24 h. Processing of Ag from intracellular MTB was measured using BB7 T hybridoma cells (specific for MTB Ag85B). Consistent with our hypothesis, TLR2$^{-/-}$ macrophages were better able to process internalized MTB than were wild-type macrophages (Fig. 2). Thus, infection and exposure of macrophages to intracellular MTB resulted in a TLR2-dependent inhibition of subsequent induction of MHC-II Ag processing by IFN-γ. These results suggest that continued activation of TLR2 by PAMPs (e.g., 19-kDa lipoprotein) from intracellular MTB allows this pathogen to inhibit MHC-II presentation of its own Ags and escape immune detection by CD4$^+$ T cells.

MTB 19-kDa lipoprotein inhibits IFN-γ-induced but not IL-4-induced MHC-II expression and Ag processing

To determine whether inhibition of MHC-II Ag processing is due to defective IFN-γ responses, we tested the ability of MTB 19-kDa lipoprotein to inhibit IL-4-induced Ag processing activity. IL-4 induces MHC-II expression in macrophages (21, 22), although less

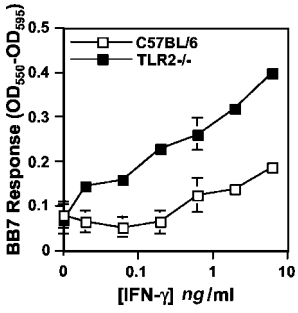

FIGURE 2. Intracellular MTB are processed more efficiently in the absence of TLR2. TLR2$^{-/-}$ and wild-type C57BL/6 macrophages were infected with MTB (multiplicity of infection of 10:1) and then incubated for 48 h. IFN-γ was added for 24 h, and the cells were fixed and incubated with BB7 T hybridoma cells. T hybridoma responses were assessed as in Fig. 1. Results are expressed as the means of triplicate wells ± SD. When error bars are not visible, they are smaller than the symbol width. These results are representative of four independent experiments.

effectively than IFN-γ and by mechanisms that remain unclear. Macrophages were incubated for 24 h with or without MTB 19-kDa lipoprotein, incubated for 24 h with IFN-γ, IL-4, or control medium in the continued presence or absence of the lipoprotein, and tested for ability to process Ag and present peptide:MHC-II complexes to T hybridoma cells. MTB 19-kDa lipoprotein dramatically reduced the ability of IFN-γ to induce MHC-II Ag processing (Fig. 3*A*). In contrast, induction of MHC-II Ag processing by IL-4 was enhanced by MTB 19-kDa lipoprotein (Fig. 3*B*). When IFN-γ and IL-4 were both absent, MHC-II Ag processing was essentially undetectable in the absence of MTB 19-kDa lipoprotein, although the lipoprotein induced a very low level of Ag presentation at high Ag doses (Fig. 3*C*). Flow cytometry showed that MTB 19-kDa lipoprotein inhibited IFN-γ-induced MHC-II protein expression (Fig. 3*D*) but enhanced IL-4-induced MHC-II expression (Fig. 3*E*), consistent with the Ag presentation assays. Treatment with MTB 19-kDa lipoprotein alone led to a small increase in MHC-II expression (Fig. 3*E*) consistent with its ability to slightly induce MHC-II Ag processing (Fig. 3*C*). In summary, MTB 19-kDa lipoprotein selectively inhibited IFN-γ induction of MHC-II expression and Ag processing.

MTB and MTB 19-kDa lipoprotein inhibit IFN-γ induction of MHC-II and CIITA mRNA

MHC-II Ag processing requires expression of MHC-II, invariant chain, and H-2M, which are all controlled by the CIITA (23, 24). CIITA$^{-/-}$ mice have greatly reduced MHC-II expression, because CIITA is the master regulator of MHC-II expression (25, 26). CIITA does not bind directly to the promoter of genes that it regulates; rather, it coordinates the actions of numerous transcription factors, e.g., members of the regulatory factor X family, NF-Y, and CREB (27–31). We investigated the ability of MTB 19-kDa lipoprotein to inhibit IFN-γ-induced expression of mRNA for MHC-II and CIITA. Macrophages were incubated with MTB 19-kDa lipoprotein for 24 h before addition of IFN-γ for 24 or 48 h, and mRNA was measured by real-time quantitative RT-PCR. MTB 19-kDa lipoprotein dramatically inhibited IFN-γ-induced expression of mRNA for MHC-II (Fig. 4*A*) and CIITA (Fig. 4*B*).

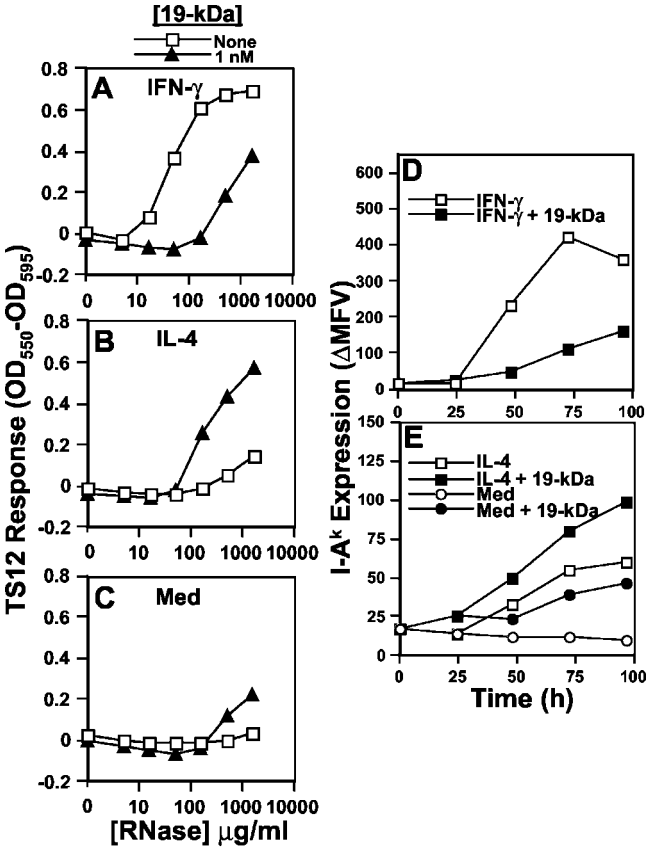

FIGURE 3. MTB 19-kDa lipoprotein inhibits induction of MHC-II expression and Ag processing by IFN-γ but not IL-4. *A–C*, C3H/HeJ macrophages were incubated for 24 h with or without MTB 19-kDa lipoprotein (1 nM) and for 24 h with IFN-γ (*A*), IL-4 (*B*), or no cytokine (*C*) in the continued presence or absence of the lipoprotein. Macrophages were then incubated with RNase for 2 h, fixed, and incubated with TS12 T hybridoma cells. Results are expressed as the means of triplicate wells ± SD. When error bars are not visible, they are smaller than the symbol width. These results are representative of four independent experiments. *D* and *E*, Macrophages were incubated with or without MTB 19-kDa lipoprotein (5 nM) and for 0, 24, 48, and 72 h with IFN-γ, IL-4, or control medium (Med) in the continued presence or absence of the lipoprotein. I-A^k expression was assessed by flow cytometry. Data are represented as Δmean fluorescence value (MFV) (ΔMFV = $MFV_{specific\ Ab} - MFV_{isotype\text{-}matched\ control\ Ab}$). These results are representative of three independent experiments.

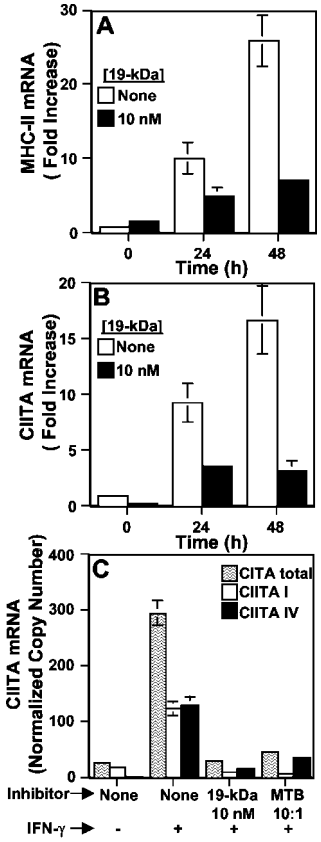

FIGURE 4. MTB 19-kDa lipoprotein inhibits IFN-γ induction of MHC-II and CIITA (total, type I, and type IV) mRNA. *A*, C3H/HeJ macrophages were incubated with or without 10 nM MTB 19-kDa lipoprotein for 24 h and then with 2 ng/ml IFN-γ in the continuing absence or presence of the lipoprotein. RNA was isolated at the indicated time points after addition of IFN-γ and analyzed for I-A_β^k expression by quantitative real-time RT-PCR. Data are represented as fold increase in expression relative to control (no lipoprotein, $t = 0$; i.e., no IFN-γ). *B*, The same RNA samples analyzed in *A* were similarly analyzed for total CIITA. *C*, C3H/HeJ macrophages were infected with MTB or incubated with 19-kDa lipoprotein for 24 h. IFN-γ (2 ng/ml) was added for 6 h. RNA was isolated, and quantitative real-time RT-PCR was used to analyze expression of total, type I, and type IV CIITA. Results are expressed as means ± SD of triplicate samples; all panels are representative of three independent experiments.

Transcriptional activation of CIITA occurs through the action of three promoters (pI, pIII, and pIV), leading to types I, III, and IV CIITA (32). pI and pIV are induced by IFN-γ in murine macrophages (33, 34), although with different kinetics, suggesting differences in their regulation (34). IFN-γ induction of mRNA for both type I and type IV CIITA was suppressed in macrophages incubated with MTB 19-kDa lipoprotein or infected with MTB (Fig. 4*C*). Thus, inhibition of MHC-II expression and Ag processing by MTB or the 19-kDa lipoprotein involves inhibition of both types I and IV CIITA.

Dissection of IFN-γ signaling and the roles of SOCS: MTB 19-kDa lipoprotein induces SOCS1 and SOCS3 mRNA but does not prevent Stat1 phosphorylation

The results in Fig. 3 suggested that MTB and MTB 19-kDa lipoprotein may selectively inhibit IFN-γ signaling. A previous study reported that infection of macrophages with *Mycobacterium avium* decreased expression of IFN-γR (35), but our studies showed that treatment of macrophages with MTB 19-kDa lipoprotein had no effect on levels of IFN-γRI (measured by flow cytometry; data not shown). Therefore, we investigated subsequent stages of the IFN-γ signaling pathway.

After IFN-γ binds to its receptor, Janus-activated kinase (JAK)-1 and -2 mediate phosphorylation of Stat1 on tyrosine 701 (Fig. 5*A*). This leads to Stat1 homodimerization, translocation to the nucleus, DNA binding, and transcriptional activation (36). In some systems, SOCS proteins can inhibit JAK-mediated phosphorylation of substrates (e.g., Stat1), thereby inhibiting cytokine signaling (37–39). SOCS1 has been reported to inhibit IFN-γ signaling in some systems (40, 41). Furthermore, other PAMPs (e.g., LPS and CpG ODN) can induce SOCS proteins (42, 43). Real-time quantitative RT-PCR showed that SOCS1 and SOCS3 were induced in bone marrow-derived macrophages by both MTB 19-kDa lipoprotein and CpG ODN (Fig. 5*B*).

If inhibition of IFN-γ signaling were mediated by SOCS1 and/or SOCS3, IFN-γ-induced phosphorylation of Stat1 on Y701 should be impaired. In contrast, immunoblot analysis of whole-cell lysates after stimulation with IFN-γ for 30 min revealed normal phosphorylation of Stat1 on Y701 in macrophages treated with MTB 19-kDa lipoprotein or CpG ODN (Fig. 5*C*). In addition, prior incubation of macrophages with MTB 19-kDa lipoprotein for 24 h also did not affect the ability of IFN-γ to induce phosphorylation of Stat1 on Y701 after 6 h of IFN-γ stimulation (data not shown), a time point at which 19-kDa lipoprotein-mediated inhibition of CIITA mRNA is present (Fig. 4*C*). Phosphorylation of Y701 is critical for the *trans*-acting activity of Stat1, and phosphorylation on serine 727 can further increase the activity of Stat1. MTB 19-kDa lipoprotein and CpG ODN also failed to reduce the amount of Stat1 phosphorylated on S727 in IFN-γ-treated macrophages. MTB 19-kDa lipoprotein and CpG ODN induced phosphorylation of Stat1 on S727 in the absence of IFN-γ (Fig. 5*C*). Interestingly, both MTB 19-kDa lipoprotein and CpG ODN dramatically increased the amount of Stat1α in macrophages (Fig. 5*C*). In summary, macrophages exposed to MTB 19-kDa lipoprotein maintain IFN-γR complex function, including JAK-mediated phosphorylation of Stat1. Thus, despite expression of mRNA for SOCS1 and -3, SOCS proteins do not appear to block JAK activity or IFN-γ signaling in macrophages.

These data are discrepant with a previous report that treatment of RAW 264.7 cells with CpG ODN induced SOCS1 that then prevented IFN-γ-induced tyrosine phosphorylation of Stat1 (42). Accordingly, we analyzed the ability of MTB 19-kDa lipoprotein

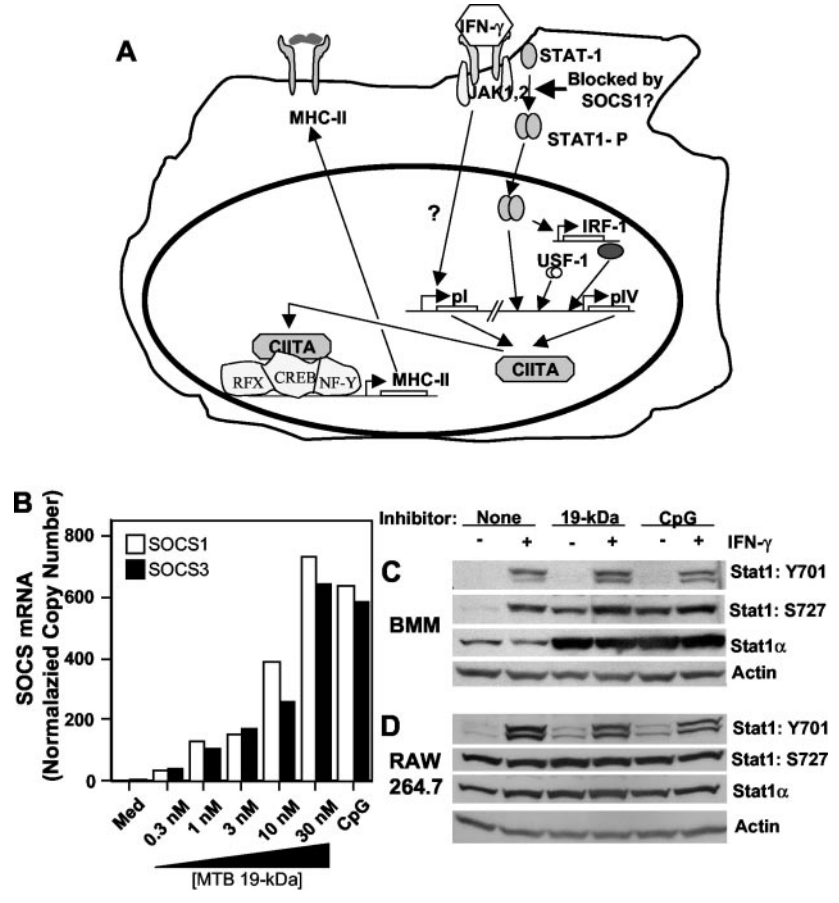

FIGURE 5. MTB 19-kDa lipoprotein induces SOCS1 and -3 but does not prevent Stat1 phosphorylation on tyrosine 701 or serine 727. *A*, Model of CIITA induction by IFN-γ. After ligation of IFN-γR, Stat1 becomes tyrosine phosphorylated, homodimerizes, and translocates into the nucleus, where it activates IRF-1 transcription. IRF-1, activated Stat1, and the constitutively expressed transcription factor USF-1 bind to elements in pIV of CIITA to induce transcription. It is unclear how pI becomes activated by IFN-γ. CIITA then coordinates the action of numerous transcription factors at the *MHC-II* locus to induce transcription. In some systems and under certain conditions, SOCS proteins may inhibit IFN-γ signaling by preventing Stat1 tyrosine phosphorylation. *B*, Macrophages were plated overnight and then incubated with MTB 19-kDa lipoprotein or 1 μg/ml CpG ODN for 6 h. Quantitative real-time RT-PCR quantification of SOCS1 and -3 mRNA is expressed as normalized copy number (see *Materials and Methods*). *C*, Macrophages were incubated with or without 30 nM MTB 19-kDa lipoprotein or 1 μg/ml CpG ODN for 24 h, stimulated with 2 ng/ml IFN-γ for 30 min, harvested, and lysed. Equal amounts of protein were analyzed by Western blot for Stat1(Y701). The blot was stripped and reprobed for the following proteins: Stat1(S727), Stat1α, and actin. *D*, RAW 264.7 cells were analyzed as in *C*. The results in *B* and *C* are representative of three independent experiments; the results in *D* are representative of two independent experiments.

and CpG ODN to inhibit Stat1 tyrosine phosphorylation in RAW 264.7 cells. Incubation of these cells with MTB 19-kDa lipoprotein or CpG ODN inhibited IFN-γ-induced MHC-II Ag processing (data not shown), but IFN-γ-induced Stat1 tyrosine phosphorylation occurred normally under these conditions (Fig. 5*D*). In RAW 264.7 cells, unlike bone marrow-derived macrophages, Stat1 was constitutively phosphorylated on S727, and MTB 19-kDa lipoprotein, CpG ODN, and IFN-γ had no appreciable effect on the levels of serine-phosphorylated Stat1 (Fig. 5*D*). In addition, Stat1α levels did not change in RAW 264.7 cells upon exposure to MTB 19-kDa lipoprotein and CpG ODN (Fig. 5*D*). In summary, Stat1 activation was normal in both RAW 264.7 cells and bone marrow-derived macrophages after exposure to MTB 19-kDa lipoprotein and CpG ODN, despite expression of SOCS1 and -3 mRNA. Thus, inhibition of MHC-II expression and Ag processing are not mediated by a SOCS-mediated blockade of JAK activity.

To further examine the role of SOCS1, we tested the ability of MTB 19-kDa lipoprotein to inhibit MHC-II Ag processing in SOCS1-deficient macrophages. Of all SOCS proteins, SOCS1 is critical for negative regulation of IFN-γ signaling (38–41), and SOCS1 is induced by IFN-γ to produce a negative feedback mechanism (44). SOCS1$^{-/-}$ mice have elevated serum levels of IFN-γ, increased numbers of activated T cells, extensive tissue damage, and perinatal death (41, 45). In contrast, mice that are deficient in both SOCS1 and IFN-γ survive into adulthood (40, 41) and can be used to produce cells for in vitro study. Bone marrow-derived macrophages were prepared from SOCS1$^{-/-}$, SOCS1$^{+/-}$, or SOCS1$^{+/+}$ mice that were also IFN-γ$^{-/-}$ (but with normal IFN-γR). Both MTB 19-kDa lipoprotein and CpG ODN inhibited IFN-γ-induced MHC-II Ag processing by SOCS1$^{-/-}$, SOCS1$^{+/-}$, and SOCS1$^{+/+}$ macrophages (Fig. 6). However, SOCS1$^{-/-}$ macrophages were slightly more resistant than control macrophages to inhibition by MTB 19-kDa lipoprotein and CpG DNA. This may be due to the fact that IFN-γ signaling is sustained for longer periods in SOCS1$^{-/-}$ macrophages than in SOCS1$^{+/+}$ macrophages, because SOCS1 is induced by IFN-γ and serves to limit IFN-γ signaling (44). If IFN-γ signaling is sustained in SOCS1$^{-/-}$ macrophages, the ability of MTB 19-kDa lipoprotein to inhibit IFN-γ-induced MHC-II Ag processing might be slightly reduced. Nevertheless, these data clearly indicate that MTB 19-kDa lipoprotein mediates inhibition of MHC-II Ag processing in the absence of SOCS1.

MTB 19-kDa lipoprotein does not prevent Stat1 nuclear translocation or DNA binding

After tyrosine phosphorylation, Stat1 is transported into the nucleus where it binds γ-activated sequences (GAS). We tested whether MTB 19-kDa lipoprotein interferes with these steps in the IFN-γ signaling pathway. MTB 19-kDa lipoprotein did not prevent translocation of activated Stat1 to the nucleus, as illustrated by Western blot analysis of nuclear lysates (Fig. 7*A*). Nuclear lysates were not contaminated with cytoplasmic protein, because Grx1, a protein predominately confined to the cytoplasm (46), was not detected in our nuclear extracts (data not shown). Upstream stimulation factor-1 (USF-1) is also critical for CIITA induction (47) (Fig. 5*A*), and a decrease in USF-1 could contribute to decreased MHC-II Ag processing. However, USF-1 expression in the nucleus was not altered by MTB 19-kDa lipoprotein (Fig. 7*A*). To test DNA binding activity of Stat1 and USF-1, nuclear lysates were prepared, and Stat1 and USF-1 were precipitated with a biotinylated dsDNA sequence containing the GAS and E-box (binding Stat1 and USF-1, respectively) present in CIITA pIV. MTB 19-kDa lipoprotein did not decrease binding of activated Stat1 or USF-1 to the CIITA pIV DNA sequence (Fig. 7*B*). In summary,

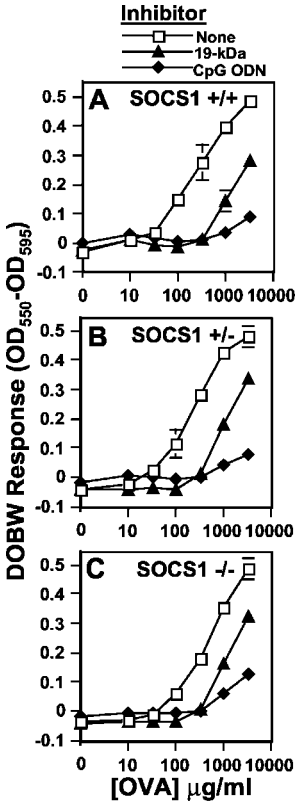

FIGURE 6. MHC-II Ag processing is inhibited by MTB 19-kDa lipoprotein in SOCS1$^{+/+}$, SOCS1$^{+/-}$, and SOCS1$^{-/-}$ macrophages. Macrophages were prepared from SOCS1$^{-/-}$, SOCS1$^{+/-}$, and SOCS1$^{+/+}$ mice and incubated with or without 30 nM MTB 19-kDa lipoprotein or 1 μg/ml CpG ODN for 24 h. Macrophages were then stimulated with 2 ng/ml IFN-γ for 24 h, exposed to OVA for 2 h, washed, fixed, and incubated with DOBW T hybridoma cells. IL-2 secretion was determined using a CTLL-2 assay as in Fig. 1. Results are expressed as the means of triplicate wells ± SD. When error bars are not visible, they are smaller than the symbol width. These results are representative of three independent experiments.

MTB 19-kDa lipoprotein did not inhibit Stat1 activation (Fig. 5, *C* and *D*), nuclear translocation (Fig. 7*A*), or DNA binding (Fig. 7*B*).

In addition to a GAS and E-box, pIV of CIITA contains an IRF-1 binding site (Fig. 5*A*). To obtain maximal activation of pIV, all three sites must be occupied (47). We tested the ability of MTB 19-kDa lipoprotein to inhibit IRF-1 induction by IFN-γ. Treatment of macrophages with MTB or MTB 19-kDa lipoprotein inhibited IFN-γ induction of IRF-1 mRNA (Fig. 7*C*). Expression of IRF-1 protein in the nucleus was also slightly inhibited by MTB 19-kDa lipoprotein (Fig. 7*D*). Thus, decreased IRF-1 may contribute to decreased expression of CIITA, although the inhibition of IRF-1 mRNA and protein is not complete and is unlikely to explain the strong inhibition of CIITA expression by MTB 19-kDa lipoprotein.

MTB 19-kDa lipoprotein does not inhibit Stat1 activation of a CIITA pIV reporter construct

To assess the ability of Stat1 and IRF-1 to activate IFN-γ-sensitive promoters, RAW 264.7 cells were transiently transfected with pGL2-Basic control vector or pIVCIITA.Luc vector containing the promoter sequence from −346 to +50 nt upstream of exon 1 of human type IV CIITA (containing Stat1, USF-1, and IRF-1 binding sites) (18, 19) driving expression of luciferase. IFN-γ induced luciferase activity in cells that were transfected with the pIVCIITA.Luc vector (Fig. 7*C*) but not those transfected with control vector (data not shown). Treatment of RAW 264.7 cells with MTB 19-kDa

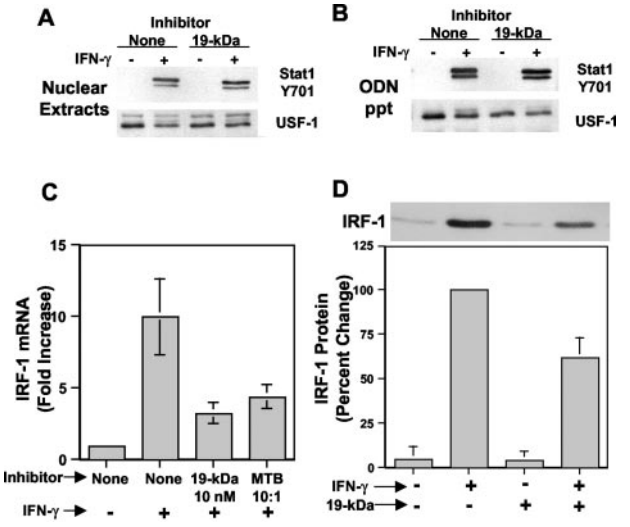

FIGURE 7. MTB 19-kDa lipoprotein does not inhibit Stat1 nuclear translocation or DNA binding, but slightly decreases IFN-γ-induced IRF-1 expression. *A*, C3H/HeJ macrophages were incubated with or without 30 nM MTB 19-kDa lipoprotein for 24 h before the addition of 2 ng/ml IFN-γ for 30 min. Nuclei were isolated, washed, and lysed. Equal amounts of protein were analyzed by Western blot for Stat1(Y701) and USF-1. *B*, Nuclear lysates from samples analyzed in *A* were incubated with a biotinylated dsDNA sequence containing the CIITA pIV binding sites for Stat1 and USF-1. The dsDNA:protein complexes were precipitated with streptavidin beads and analyzed by Western blot for Stat1 (Y701) and USF-1. The results in *A* and *B* are representative of two independent experiments. *C*, C3H/HeJ macrophages were incubated for 24 h with or without 10 nM MTB 19-kDa lipoprotein and then stimulated with IFN-γ for 6 h in the continued presence or absence of the lipoprotein. RNA was isolated and analyzed for IRF-1 mRNA by quantitative real-time RT-PCR. Results are expressed as means ± SD of triplicate samples and are representative of four independent experiments. *D*, C3H/HeJ macrophages were incubated for 24 h with or without 30 nM MTB 19-kDa lipoprotein and then stimulated with IFN-γ for 6 h in the continued presence or absence of the lipoprotein. Nuclear extracts were analyzed for IRF-1 protein by Western blot. This panel shows a representative blot and composite densitometry data (mean percent change observed in four independent experiments ± SD). Densitometry values were normalized to the intensity observed in IFN-γ-treated macrophages (defined as 100%).

lipoprotein did not inhibit IFN-γ induction of luciferase activity from pIVCIITA.Luc (Fig. 8) despite decreased IRF-1 mRNA and protein (Fig. 7, *C* and *D*). It is possible that the remaining IRF-1 protein is sufficient to activate this promoter contruct. In addition, MTB 19-kDa lipoprotein did not inhibit IFN-γ-induced expression of luciferase driven by a simplified Stat1-dependent promoter construct containing two GAS sites (IRFGAS2X, containing portions of the IRF-1 promoter (20)) (data not shown). These results confirm that Stat1 can be phosphorylated in response to IFN-γ in the presence of MTB 19-kDa lipoprotein and that the activated Stat1 is functional. In summary, all functions of Stat1 that were tested were unaffected by treatment with MTB 19-kDa lipoprotein, despite dramatic inhibition of CIITA mRNA.

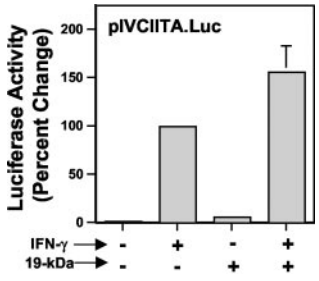

FIGURE 8. MTB 19-kDa lipoprotein does not inhibit the ability of IFN-γ to activate a type IV CIITA reporter construct. RAW 264.7 cells were cotransfected with 8 μg of pIVCIITA.Luc and 2 μg of pRL-TK, rested for 2 h, incubated with MTB 19-kDa lipoprotein for 24 h, and stimulated with IFN-γ for 20 h in the continued presence or absence of the lipoprotein. Luciferase activity was measured and normalized to the activity observed with IFN-γ alone (the latter value was defined as 100%). This panel shows the mean percent change in four different experiments ± SD.

Discussion

IFN-γ is critically important to the control of MTB infection, and it is not surprising that MTB has developed mechanisms to suppress IFN-γ responses. In this study, we show that chronic (>16–24 h) stimulation of TLR2 by MTB 19-kDa lipoprotein inhibits IFN-γ-dependent induction of CIITA, IRF-1, MHC-II, and MHC-II Ag processing. IFN-γ-dependent MHC-II Ag processing is specifically targeted for inhibition, because IL-4-induced MHC-II Ag processing is augmented (Fig. 3). This observation is important to dissect signaling mechanisms, but it is not clear whether IL-4-induced MHC-II expression is of any significance in vivo. IL-4 induces only low levels of MHC-II expression and is not known to play a significant role in the pathogenesis of tuberculosis. IFN-γ is the primary inducer of CIITA (types I and IV) in macrophages, so the ability of MTB 19-kDa lipoprotein to inhibit IFN-γ signaling should dominate and lead to reduced MHC-II expression. This mechanism reduces MHC-II presentation of MTB Ags expressed by intracellular bacilli and may allow MTB to evade immune surveillance by $CD4^+$ T cells.

MTB or MTB 19-kDa lipoprotein inhibits multiple IFN-γ-dependent responses. As shown in Figs. 4 and 7, MTB 19-kDa lipoprotein inhibits IFN-γ induction of MHC-II, CIITA, and IRF-1. In addition, Ting et al. (48) showed that infection of macrophages with MTB inhibited induction of FcγRI by IFN-γ, and the inhibitory component was present in the cell wall of MTB, of which MTB 19-kDa lipoprotein is a major constituent (49). We have also observed that MTB 19-kDa lipoprotein inhibits induction of FcγRI by IFN-γ in human THP-1 cells (64). Furthermore, preliminary experiments with microarray analysis of gene expression indicate that a large number of IFN-γ-dependent genes are inhibited by MTB 19-kDa lipoprotein (R. K. Pai, unpublished observations). Together, these observations indicate that chronic stimulation of TLR2 by MTB 19-kDa lipoprotein inhibits IFN-γ signaling. This may result in inhibition of multiple IFN-γ-dependent host defense mechanisms, including MHC-II expression and Ag processing.

MTB 19-kDa lipoprotein is one of several PAMPs demonstrated to inhibit MHC-II Ag processing by macrophages after long-term stimulation (others include CpG DNA and LPS) (7, 11). Thus, the inhibitory mechanisms of several PAMPs, mediated by chronic TLR signaling, may overlap. Many acute bacterial pathogens may fail to exploit this inhibitory mechanism, but MTB is particularly well adapted to exploit this mechanism in vivo. MTB selectively infects macrophages, a cell type that expresses TLRs and is susceptible to inhibition of IFN-γ-dependent MHC-II Ag processing. In contrast, other APCs (e.g., B cells and dendritic cells) express MHC-II independent of IFN-γ, and dendritic cells respond to PAMPs by increasing MHC-II expression. Most importantly, MTB has other mechanisms to resist innate microbicidal mechanisms, allowing it to persist inside macrophages for a sufficient period to promote chronic exposure to PAMPs. The 19-kDa lipoprotein is shed from live, intracellular mycobacteria (50), making it available to stimulate TLR2 (7, 8, 10), in association with TLR1 (51), which are both recruited to phagosomal compartments

(52). Intracellular persistence of MTB and shedding of MTB 19-kDa lipoprotein provide effective means for chronic stimulation of TLR2 and consequent inhibition of MHC-II expression and Ag processing.

Inhibition of macrophage MHC-II Ag processing by TLR agonists, including LPS (11), bacterial CpG DNA (11), and lipoproteins (7), seems discordant with the ability of TLRs to enhance microbicidal functions and stimulate innate and acquired immunity. However, inhibition of MHC-II Ag processing by MTB 19-kDa lipoprotein and other PAMPs occurs only after 16 h of stimulation (data not shown). Thus, our results are consistent with a model wherein PAMPs, e.g., MTB 19-kDa lipoprotein, initially activate microbicidal mechanisms and innate immunity (also impacting on specific immunity), but later down-regulate certain aspects of immune responses.

Once IFN-γ binds its receptor, activation of JAK1 and JAK2 leads to phosphorylation of Stat1 on tyrosine 701. Dalpke et al. (42) reported that treatment of RAW 264.7 cells with CpG ODN prevented Stat1 phosphorylation, and SOCS1 was implicated in the inhibitory mechanism. In contrast, our results show that both MTB 19-kDa lipoprotein and CpG ODN fail to inhibit IFN-γ-induced phosphorylation of Stat1 on tyrosine 701 in both bone marrow-derived macrophages and RAW 264.7 cells, despite induction of SOCS1 by these PAMPs. Moreover, IFN-γ-induced MHC-II Ag processing was suppressed by MTB 19-kDa lipoprotein and CpG ODN even in SOCS1-deficient macrophages. These results clearly indicate that SOCS1 plays little or no role in TLR-mediated suppression of IFN-γ-induced MHC-II expression and Ag processing in macrophages.

It is not clear why expression of SOCS1 was not associated with inhibition of Stat1 phosphorylation. One factor may be the dramatic increase in Stat1 protein induced by both CpG ODN and MTB 19-kDa lipoprotein. Because SOCS1 is thought to be a competitive inhibitor of JAKs (38), an increase in concentration of substrate (Stat1) might overcome inhibition by SOCS1. However, RAW 264.7 cells treated with CpG ODN or MTB 19-kDa lipoprotein did not have increased Stat1α (Fig. 5*D*), yet still showed inhibition of IFN-γ-induced MHC-II Ag processing (data not shown). We found that SOCS1 mRNA expression peaked between 6 and 8 h and declined sharply over the next 12 h (data not shown), and it has been shown that SOCS1 protein has an extremely short half-life (~1.5 h) (53). Thus, SOCS1 expression may be too transient to contribute to the long-term inhibition of IFN-γ signaling addressed in our experiments.

Several possibilities remain to explain the exact mechanism whereby IFN-γ signaling is inhibited. We did not observe a defect in Stat1 phosphorylation (Fig. 5), nuclear translocation (Fig. 7*A*), DNA binding (Fig. 7*B*), or activation of type IV CIITA promoter construct in transiently transfected cells (Fig. 8). Posttranscriptional regulation is one possibility, but extreme mRNA destabilization would be necessary to explain the essentially complete loss of IFN-γ-induced CIITA mRNA. Furthermore, numerous IFN-γ-dependent genes are inhibited by MTB 19-kDa lipoprotein, and it is unlikely that all of these mRNA species could be coordinately regulated in a posttranscriptional manner. Alternatively, inhibition may be achieved by transcriptional regulation by one of several mechanisms. First, the existence of regulatory sequences outside of the promoter sequences present in the pIVCIITA.Luc construct used in our studies, and induction of negative regulators or repression of positive regulators that bind to these sequences is a possibility; this remains a theoretical possibility for which there is no support. Second, there is the possibility of regulation of factors that bind to Stat1 or Stat1-associated proteins to modulate Stat1 activity. However, known factors in this category do not fit with our data showing lack of inhibition of Stat1 binding to DNA constructs and Stat1-driven expression of pIVCIITA.Luc. For example, PIASy and PIAS1 can block IFN-γ signaling in some systems, but PIAS1 blocks the ability of Stat1 to bind DNA whereas PIASy does not block the ability of Stat1 to bind to DNA but does block IFN-γ-dependent activation of luciferase reporter constructs (54, 55). Furthermore, expression of PIAS1 and PIASy was detected in macrophages by real-time quantitative RT-PCR and was not altered by MTB 19-kDa lipoprotein at any concentration or time point reported in this paper (data not shown). Third, regulation of CIITA expression by DNA methylation is a possibility. Although this mechanism has been reported for pIV (56, 57), it has not been demonstrated for pI, which also drives CIITA expression in macrophages in a manner that is inhibited by the MTB 19-kDa lipoprotein (Fig. 4), and it may not explain inhibition of numerous other IFN-γ-dependent genes by MTB 19-kDa lipoprotein. Fourth, regulation of CIITA expression by chromatin remodeling remains an interesting possibility (58, 59).

As suggested by Ting et al. (48), one possible inhibitory mechanism used by MTB may involve CREB binding protein (CBP) and p300, related transcription factors that bind Stat1 and promote Stat1-mediated gene transcription. Binding of CBP and p300 to Stat1 promotes chromatin remodeling to enhance gene expression by histone acetylation (CBP and p300 have intrinsic histone acetylase activity and associate with pCAF, another protein with histone acetylase activity (60); histone acetyltransferase activities have been associated with CIITA activation of MHC-II expression (61)). Inhibition of this mechanism by MTB 19-kDa lipoprotein could prevent the expression of endogenous genes but not plasmid-borne genes, a pattern of results consistent with our results. In contrast, CBP and p300 also enhance gene transcription by recruiting factors associated with the basal transcriptional machinery such as RNA polymerase II (58). Indeed, overexpression of CBP enhances IFN-γ-dependent induction of transiently transfected luciferase reporter constructs, and the introduction of E1A, a molecule that prevents the association of Stat1 with CBP/p300, inhibits the activation of these reporter constructs (62, 63). If MTB 19-kDa lipoprotein were to inhibit association of Stat1 with CBP/p300, then recruitment of RNA polymerase II and activation of Stat1-responsive genes would be inhibited. Inhibition of this mechanism by MTB 19-kDa lipoprotein would lead to decreased IFN-γ induction of both endogenous genes and genes introduced on plasmids (e.g., luciferase reporter constructs), a pattern of results not consistent with our observations. In summary, MTB 19-kDa lipoprotein may inhibit association of Stat1 with CBP/p300 under circumstances in which chromatin remodeling activity is the critical regulatory effect of CBP/p300 on Stat1 induction of endogenous CIITA transcription (inhibiting expression of endogenous CIITA but not affecting pIVCIITA-driven luciferase expression).

In this study, we determined that MTB 19-kDa lipoprotein inhibits IFN-γ induction of MHC-II expression and Ag processing by inhibiting IFN-γ-induced CIITA expression. The mechanism of this inhibition involves inhibition of IFN-γ signaling distal to Stat1 activation and may involve regulation of chromatin remodeling. This inhibition has important implications for tuberculosis pathogenesis. Macrophages that are chronically infected with MTB may be unable to process and present MTB Ags, even when stimulated with IFN-γ, preventing recognition by MTB-specific $CD4^+$ T cells and promoting chronic infection. The observation that MTB and MTB 19-kDa lipoprotein inhibit the induction of multiple IFN-γ-responsive genes (including *CIITA*, *IRF-1*, and *FcγRI*) is intriguing for two reasons. First, it suggests that the inhibitory mechanism may involve steps in IFN-γ signaling common to the regulation of a wide variety of IFN-γ-induced genes. Second, it suggests that

MTB may inhibit multiple IFN-γ-dependent macrophage host-defense mechanisms, not just MHC-II expression and Ag processing. IFN-γ clearly plays a central role in host defense in tuberculosis, and suppression of IFN-γ-induced macrophage responses by MTB may greatly impact the course of infection.

Acknowledgments

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