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Mycobacterium tuberculosis 19-kDa Lipoprotein Inhibits IFN-γ-Induced Chromatin Remodeling of MHC2TA by TLR2 and MAPK Signaling

Meghan E. Pennini,* Rish K. Pai,* David C. Schultz,† W. Henry Boom,‡ and Clifford V. Harding*–3

During infection of macrophages, prolonged signaling by Mycobacterium tuberculosis (Mtb) or its 19-kDa lipoprotein (LpqH; Rv3763) inhibits IFN-γ-induced expression of several immune function genes, including class II transactivator (CIITA), which regulates class II MHC. Mtb does not inhibit early IFN-γ signaling events, e.g., Stat1α activation. This study analyzed downstream mechanisms that regulate the transcription of MHC2TA, the gene encoding CIITA. Chromatin immunoprecipitation showed that IFN-γ-induced acetylation of histones H3 and H4 at the CIITA promoter IV (pIV). In contrast, IFN-γ-dependent histone acetylation at CIITA pIV was inhibited by Mtb or 19-kDa lipoprotein. Mtb 19-kDa lipoprotein also inhibited IFN-γ-dependent recruitment of Brahma-related gene 1, a chromatin remodeling protein, to CIITA pIV. Mtb 19-kDa lipoprotein did not inhibit histone acetylation in TLR2-/- macrophages. Furthermore, 19-kDa lipoprotein did not inhibit CIITA expression or IFN-γ-dependent histone acetylation of CIITA pIV in macrophages treated with inhibitors of MAPKs p38 or ERK. Thus, CIITA expression was inhibited by TLR2-induced MAPK signaling that caused histone hypoacetylation at CIITA pIV and suppression of CIITA transcription. Chromatin remodeling at MHC2TA is a novel target of inhibition by Mtb. These mechanisms may diminish class II MHC expression by infected macrophages, contributing to immune evasion by Mtb. The Journal of Immunology, 2006, 176: 4323–4330.

Mycobacterium tuberculosis (Mtb)4 elicits T cell responses and production of IFN-γ that are essential for control of infection, but are insufficient to eradicate the pathogen, which persists in long-term chronic infection, harbored by macrophages (1). Mice that are deficient in IFN-γ or the IFN-γR develop fatal, disseminated, mycobacterial disease (2, 3). Similarly, human mutations linked to IFN-γ signaling are associated with disseminated infection after Mtb bacillus Calmette-Guérin vaccination and increased susceptibility to mycobacterial infection (4–6).

The persistence of Mtb may involve inhibition of some IFN-γ-dependent responses. Prolonged (>18 h) infection of macrophages with mycobacteria or exposure to Mtb 19-kDa lipoprotein or lipooligosaccharide (both cell wall components) inhibits the IFN-γ-induced expression of some genes (7–16), but not others (8, 11). Mtb inhibits macrophage expression of class II transactivator (CIITA), genes that are regulated by CIITA, including class II MHC (MHC-II), H2-M, and invariant chain (11–15), and other accessory proteins required for Ag presentation (11), potentially inhibiting responses by CD4+ T cells that produce IFN-γ. Inhibition of MHC-II Ag presentation by Mtb or 19-kDa lipoprotein is dependent on prolonged signaling by TLR2 (12). The ability of Mtb to diminish both T cell production of IFN-γ and macrophage responses to IFN-γ may help this pathogen evade immune surveillance and establish chronic infection. However, the mechanism by which CIITA expression is inhibited remains unclear.

The expression of CIITA is regulated by multiple promoters. Each promoter contains specific regulatory sequences and initiates transcription of a unique exon 1, followed by transcription of common exons 2–19 (17). CIITA promoters I and III (pl and pIII) are the predominant regulators of constitutive CIITA expression in dendritic cells and B cells, respectively. CIITA pIV controls IFN-γ-induced CIITA expression in macrophages. Recent data indicated CIITA pl activity in macrophages stimulated with IFN-γ (18), although the mechanism by which this occurs is not fully understood. CIITA pIV contains several IFN-γ-responsive elements, including STAT1α and IFN regulatory factor-1 (IRF-1) binding sites.

Eukaryotic DNA transcription is controlled by chromatin structure, which is organized around nucleosomes, which are composed of 147 bp of DNA wrapped around an octamer core of histone proteins. Unstructured histone tails are the target of various post-translational modifications, including acetylation, phosphorylation, and methylation, which can alter nucleosome structure to increase or decrease gene transcription. Of these modifications, histone acetylation has emerged as a major determinant of transcriptional activation. MHC2TA is silenced by deacetylation of histones located within its promoter regions in various circumstances (e.g., in certain carcinomas and mature dendritic cells) (19, 20).

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4 Abbreviations used in this paper: Mtb, Mycobacterium tuberculosis; Brg1, Brahma-related gene 1; ChIP, chromatin immunoprecipitation; CIITA, class II transactivator; HAT, histone acetyl transferase; HDAC, histone deacetylase; iNOS, inducible NO synthase; IRF-1, IFN regulatory factor-1; MHC-II, class II MHC; pIV, CIITA promoter IV; TX114, Triton X-114; USF-1, upstream stimulating factor-1.
Several studies have addressed the mechanism by which Mtb may inhibit the expression of some IFN-γ-induced genes. IFN-γ-induced STAT1 activation, translocation to the nucleus, and binding to DNA are not inhibited by Mtb or its 19-kDa lipoprotein (8, 13, 21). Mtb causes a slight decrease in IRF-1 expression (13), but this does not seem sufficient to explain the strong inhibition of CIITA expression. Furthermore, only a subset of IFN-γ-induced genes are inhibited by Mtb or 19-kDa lipoprotein, whereas the induction of many others by IFN-γ remains uninhibited or enhanced, indicating that proximal IFN-γ signaling mechanisms remain active.

In this study we went beyond analysis of Stat1 activation to addressing signaling branches (e.g., signaling by MAPKs) and chromatin remodeling mechanisms that may explain the inhibition of a specific subset of IFN-γ-induced genes by Mtb. We focus on Mtb and its 19-kDa lipoprotein, because previous studies showed that extracted Mtb cell wall fractions containing 19-kDa lipoprotein had the greatest inhibitory effect on IFN-γ-induced Ag presentation (12). Our studies indicate for the first time that inhibition of IFN-γ-induced CIITA expression by Mtb or 19-kDa lipoprotein is linked to histone deacetylation at MHC2TA. Furthermore, we used inhibitors to specifically target several MAPKs and define their role in the regulation of CIITA. SB203580 (a p38 kinase inhibitor) inhibitors to specifically target several MAPKs and define their role in the regulation of CIITA. SB203580 (a p38 kinase inhibitor) but this does not seem sufficient to explain the strong inhibition of (8, 13, 21). Mtb causes a slight decrease in IRF-1 expression (13), and stained using the Bio-Rad Silver Stain Plus kit. Samples were then transferred onto model 491 membranes (Millipore). Membranes were incubated overnight at 4°C in 0.1% Tween 20 in PBS supplemented with 5% nonfat dry milk, and incubated for 1 h at room temperature with a polyclonal rabbit anti-M. bovis antiserum (DakoCytomation) that recognizes many mycobacterial constituents, including 19-kDa lipoprotein. Membranes were washed repeatedly, incubated for 1 h with HRP-labeled donkey anti-rabbit secondary Ab (Amersham Biosciences), and developed with an ECL detection kit (Amersham Biosciences). Fractions determined to contain the 19-kDa lipoprotein were pooled, extracted with TX114, precipitated in acetone, resuspended to 30–55 μg in DMSO, and stored at −80°C.

Luciferase reporter assay RAW264.7 were transfected with 1 μg of CITAapII-luc construct (23, 24), provided by J. Ting (University of North Carolina, Chapel Hill, NC), consisting of murine CITA pIV −360 to +50 using the Superfect transfection system (Qiagen). For transient transfection studies, cells were treated with 19-kDa lipoprotein with or without IFN-γ 3 h after transfection. For stable transfections, cells were transfected with the pIV reporter plasmid (25). Single colonies were isolated and expanded in standard medium supplemented with 5 μg/ml puromycin. Luciferase activity was measured using the Dual-Luciferase Reporter System (Promega).

Quantitative real-time PCR Macrophages (3–4 × 10⁶) were incubated with or without 19-kDa lipoprotein (30 nM) and then with IFN-γ (2 ng/ml) in the continued absence or presence of IFN-γ was isolated. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) as described by the manufacturer’s protocol. Total RNA yield was determined by spectrophotometer, and 1 μg of total RNA was used in a reverse transcriptase reaction (SuperScript First Strand Synthesis System; Invitrogen Life Technologies) to convert RNA to double-stranded cDNA. One tenth of the cDNA template was used for real-time PCR analysis with SYBR Green and the Bio-Rad iCycler fluoroscence detection system. A standard curve for each gene was generated by serial dilution of the amplified product standard of known starting concentration. The following primers were designed using Oligo 6.0 software (MBI): CITA sense, 5′-ACG CTT TCT GCC GCG TGG ATT AGT-3′; CITA antisense, 5′-TCA ACG CCA GTC TCA CGA AAG-3′; inducible NO synthase (iNOS) sense, 5′-GAG ACT CAT GCT TTA GAG AC-3′; iNOS antisense, 5′-AAC GAC CAT ACC ATT GAC-3′; GAPDH sense, 5′-GTC TCA GCA C-3′. Quantification was determined based on a standard curve of known concentration for each gene and was normalized to GAPDH.

Chromatin immunoprecipitation (ChIP) Macrophages (7–8 × 10⁶) were plated in 100-mm petri dishes and incubated with or without 19-kDa lipoprotein (30 nM) or were infected with Mtb (multiplicity of infection, 30:1) for 18–24 h. IFN-γ (2 ng/ml) was added for 4–20 h in the continued presence or absence of Mtb or 19-kDa lipoprotein. ChIP was performed with reagents and protocol (item 17–295) as described by the manufacturer’s protocol. Total RNA yield was determined by spectrophotometer, and 1 μg of total RNA was used in a reverse transcriptase reaction (SuperScript First Strand Synthesis System; Invitrogen Life Technologies) to convert RNA to double-stranded cDNA. One tenth of the cDNA template was used for real-time PCR analysis with SYBR Green and the Bio-Rad iCycler fluoroscence detection system. A standard curve for each gene was generated by serial dilution of the amplified product standard of known starting concentration. The following primers were designed using Oligo 6.0 software (MBI): CITA sense, 5′-ACG CTT TCT GCC GCG TGG ATT AGT-3′; CITA antisense, 5′-TCA ACG CCA GTC TCA CGA AAG-3′; inducible NO synthase (iNOS) sense, 5′-GAG ACT CAT GCT TTA GAG AC-3′; iNOS antisense, 5′-AAC GAC CAT ACC ATT GAC-3′; GAPDH sense, 5′-GTC TCA GCA C-3′. Quantification was determined based on a standard curve of known concentration for each gene and was normalized to GAPDH.

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Materials and Methods

Cells and media RAW264.7 cells (American Type Culture Collection) were maintained in standard medium composed of DMEM (BioWhittaker) with 10% heat-inactivated FBS, 50 μM 2-ME, 1 mM sodium pyruvate, 10 mM HEPES buffer, penicillin, and streptomycin. Unless otherwise stated, all primary macrophages were isolated from femur marrow from C57BL/6 mice (The Jackson Laboratory). TLR2−/− mice (provided by Drs. O. Takeuchi and S. Akira, Osaka University, Osaka, Japan) were bred onto a C57BL/6 background. Bone marrow was cultured in standard medium supplemented with 20–25% LADMAC (22) cell-conditioned medium. After 5 days, nonadherent cells were removed. Adherent cells were harvested after 8–14 days in culture and replated for experimental use. Unless otherwise indicated, macrophages were activated with 2 ng/ml IFN-γ, and 19-kDa lipoprotein was used at 30 nM. All experiments were conducted at 37°C in 5% CO₂.

Macrophages (7–8 × 10⁶) were plated in 100-mm petri dishes and incubated with or without 19-kDa lipoprotein (30 nM) or were infected with Mtb (multiplicity of infection, 30:1) for 18–24 h. IFN-γ (2 ng/ml) was added for 4–20 h in the continued presence or absence of Mtb or 19-kDa lipoprotein. ChIP was performed with reagents and protocol (item 17–295) as described by the manufacturer’s protocol. Total RNA yield was determined by spectrophotometer, and 1 μg of total RNA was used in a reverse transcriptase reaction (SuperScript First Strand Synthesis System; Invitrogen Life Technologies) to convert RNA to double-stranded cDNA. One tenth of the cDNA template was used for real-time PCR analysis with SYBR Green and the Bio-Rad iCycler fluoroscence detection system. A standard curve for each gene was generated by serial dilution of the amplified product standard of known starting concentration. The following primers were designed using Oligo 6.0 software (MBI): CITA sense, 5′-ACG CTT TCT GCC GCG TGG ATT AGT-3′; CITA antisense, 5′-TCA ACG CCA GTC TCA CGA AAG-3′; inducible NO synthase (iNOS) sense, 5′-GAG ACT CAT GCT TTA GAG AC-3′; iNOS antisense, 5′-AAC GAC CAT ACC ATT GAC-3′; GAPDH sense, 5′-GTC TCA GCA C-3′. Quantification was determined based on a standard curve of known concentration for each gene and was normalized to GAPDH.
CCA ACA CGC CCA GG-3; GAPDH sense, 5'-AGA CAA AAT GGT GAA GGT CGG-3'; and GAPDH antisense, 5'-AGG TCA ATG AAG GGG TCG TT-3'. Quantification was determined based on a standard curve of known concentration for each gene and was normalized to GAPDH. Normalization for Brg1 was based on the GAPDH quantity of input DNA for each sample.

Preparation of whole-cell lysates and Western blots
Cells (3–4 × 10⁶) were plated in 60-mm petri dishes and incubated for 1 h with or without MAPK inhibitor (SB203580, U0126, or SP600125; all from Calbiochem; stocks at 20 mM in DMSO). Cells were then exposed to 30 nM 19-kDa lipoprotein in the continued presence or the absence of MAPK inhibitor for 15–40 min. Cells were washed with ice-cold PBS, pelleted, and resuspended in 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 sodium fluoride, and 1 mM NaVO₄ with a protease inhibitor mixture (P8340, Sigma-Aldrich). When required, the protein concentration was determined using the Bio-Rad protein detection kit. Lysates were boiled for 5 min in reducing sample buffer (39001; Pierce). Equal quantities of total protein were electrophoresed on 7 or 12% SDS polyacrylamide gels and transferred onto membrane 491 membranes. Membranes were washed in PBS with 0.1% Tween 20 (PBST), incubated for 1 h at room temperature in 5% BSA or 5% nonfat milk in PBST, incubated overnight at 4°C with primary Ab in 5% BSA or 5% nonfat milk in PBST, washed, incubated for 1 h at room temperature with HRP-labeled donkey anti-rabbit (Amersham Biosciences) or donkey anti-goat (Santa Cruz Biotechnology) secondary Ab, and developed with the ECL detection kit (Pierce). Anti-p-JNK, anti-p-ERK, anti-Brg1, and anti-actin Abs were purchased from Santa Cruz Biotechnology. Anti-p-p38 was purchased from Cell Signaling Technology.

Results
Prolonged stimulation with Mtb 19-kDa lipoprotein inhibits IFN-γ-induced expression of CIITA, but not iNOS
Mtb 19-kDa lipoprotein is known to inhibit the expression of a subset of IFN-γ-induced genes in murine macrophages (8, 11). To extend these findings, we treated bone marrow-derived macrophages with 19-kDa lipoprotein for 18 h, then stimulated the cells with IFN-γ for various times. Previous incubation with 19-kDa lipoprotein inhibited induction of CIITA by IFN-γ from 2 to 24 h (Fig. 1A). This effect was not produced by negative control fractions from the electrophoretic lipoprotein purification procedure (data not shown). In contrast, IFN-γ induction of iNOS mRNA was enhanced by 19-kDa lipoprotein and IFN-γ to a far greater extent than by IFN-γ alone (Fig. 1B). In addition, CIITA expression was inhibited by 19-kDa lipoprotein when macrophages were treated simultaneously with both lipoprotein and IFN-γ or when macrophages were treated first with IFN-γ, then exposed to lipoprotein (data not shown). We chose to continue our studies with exposure to 19-kDa lipoprotein, followed by IFN-γ, because in vivo infection with Mtb and lipoprotein exposure may occur before the up-regulation of IFN-γ. This protocol also allowed us to assess rapid events in the induction of CIITA by IFN-γ under lipoprotein-influenced conditions. Because inhibition of CIITA expression by Mtb 19-kDa lipoprotein has important effects on the expression of MHC-II and other molecules involved in Ag presentation, we examined the mechanism by which Mtb 19-kDa lipoprotein inhibits IFN-γ-dependent CIITA expression.

19-kDa lipoprotein inhibits IFN-γ-induced expression of CIITA promoter-luciferase constructs in stably transfected RAW 264.7 cells, but not transient transfecants
Despite its ability to inhibit endogenous CIITA expression, we observed that Mtb 19-kDa lipoprotein did not inhibit IFN-γ-induced expression of a luciferase reporter construct driven by the murine CIITA pIV (CIITApIV.Luc) in transiently transfected macrophages (13) (Fig. 2A). However, previous studies demonstrated that macrophages stably transfected with a similar, but nonidentical, CIITApIV.Luc construct are subject to inhibition in response to Mtb (8). To reconcile the discrepancy between results with transient vs stable transfection with CIITApIV.Luc, we considered the potential role of chromatin remodeling. Transiently transfected plasmids do not generally integrate into the genome of recipient cells, and the expression of genes by transient transfection may not be controlled by chromatin remodeling (26). In contrast, stably transfected plasmids integrate into chromosomal DNA and may then be subject to control by chromatin structure. We generated stable transfecants of RAW264.7 macrophages with the same CIITApIV.Luc plasmid and selected puromycin-resistant clones. Of 19 clones, 15 showed IFN-γ-dependent expression of CIITApIV.Luc that was inhibited by 19-kDa lipoprotein. Although some of these clones were not stable over time and showed variable degrees of both CIITApIV.Luc induction in response to IFN-γ and inhibition by 19-kDa lipoprotein, at least three had a stable phenotype and showed consistent degrees of both. Thus, in contrast to results with transient transfecants, 19-kDa lipoprotein inhibited IFN-γ-induced luciferase expression in stably transfected macrophages (Fig. 2B). These results suggest that the mechanism

![FIGURE 1](http://www.jimmunol.org/)

Mtb 19-kDa lipoprotein inhibits IFN-γ-induced expression of CIITA, but enhances expression of iNOS. Macrophages were incubated with or without 30 nM 19-kDa lipoprotein for 18 h, then with 2 ng/ml IFN-γ in the continued presence or absence of 19-kDa lipoprotein for the indicated times. RNA was isolated, and quantitative real-time PCR was used to analyze the expression of CIITA (A) and iNOS (B). The expression of CIITA and iNOS was normalized to GAPDH expression. Data are shown as the fold change in gene expression compared with cells from the same time point that were incubated only with medium (labeled Med). Data are expressed as the mean of triplicate samples ± SD. Certain data points in B are not visible due to their small value depicted on a large scale. These data are representative of at least five independent experiments.

![FIGURE 2](http://www.jimmunol.org/)

Mtb 19-kDa lipoprotein inhibits IFN-γ-induced expression of CIITA-driven luciferase in stably, but not transiently transfected, RAW264.7 cells. RAW264.7 macrophages were transfected either transiently (A) or stably (B) with a luciferase construct driven by the murine CIITA pIV promoter. Transfected cells were treated with or without 30 nM 19-kDa lipoprotein for 18–24 h, and IFN-γ (2 ng/ml) was added for an additional 20 h. –. Samples that were not exposed to IFN-γ or 19-kDa lipoprotein. Cells were lysed, and luciferase activity was measured in samples with equal amounts of total protein. Data are representative of results from at least three separate experiments. A. Treatment with 19-kDa lipoprotein alone did not increase luciferase expression (data not shown).
by which Mtb 19-kDa lipoprotein inhibits IFN-γ-induced gene expression involves regulation of chromatin remodeling, perhaps by control of histone acetylation.

**Sodium butyrate partially reverses 19-kDa lipoprotein-mediated inhibition of CIITA**

We used sodium butyrate to examine the role of histone acetylation in 19-kDa lipoprotein-mediated inhibition of CIITA expression. Sodium butyrate inhibits histone deacetylases (HDACs), causing widespread, nonspecific increases in histone acetylation throughout the genome and increased accessibility of DNA to transcriptional machinery. Therefore, sodium butyrate can be used to relieve suppression of transcription due to histone deacetylation. Treatment of macrophages with sodium butyrate diminished the inhibition of IFN-γ-dependent CIITA expression by 19-kDa lipoprotein (Fig. 3), suggesting that decreased histone acetylation contributed to transcriptional inhibition of CIITA. This result is consistent with previous data indicating that 19-kDa lipoprotein does not negatively affect the activation or binding of STAT1, IRF-1, or upstream stimulating factor-1 (USF-1) to the CIITA promoter in vitro (8, 13, 21), indicating that even in the presence of the required transcriptional activators, a lack of histone acetylation can cause transcriptional suppression.

**Mtb and its 19-kDa lipoprotein inhibit IFN-γ-induced acetylation of histones H3 and H4 at the CIITA pIV locus**

To directly address the possibility that 19-kDa lipoprotein inhibits IFN-γ-dependent chromatin remodeling of the MHC2TA locus, we performed ChIP assays. After preparation of sheared chromatin, immunoprecipitation was performed with Abs specific for acetylated histones H3 and H4, followed by quantitative real-time PCR with primers specific for CIITA pIV. IFN-γ increased acetylation of histones H3 and H4 at CIITA pIV, but this increase was inhibited when cells were infected by Mtb or exposed to Mtb 19-kDa lipoprotein (Fig. 4, A and B). As a control for the specificity of these results, we performed ChIP analysis of histone acetylation at the iNOS promoter, because Mtb and 19-kDa lipoprotein enhance, rather than inhibit, iNOS gene transcription (Fig. 1B). At the iNOS promoter, acetylation of histones H3 and H4 was only slightly increased by IFN-γ alone, but was greatly increased by IFN-γ plus either Mtb or 19-kDa lipoprotein (Fig. 4, C and D). With the exception of Fig. 4, E and F, all data are normalized to GAPDH to ensure equal recovery of DNA from each ChIP sample. GAPDH levels changed minimally in response to our stimuli (Fig. 4, E and F, insets). Moreover, the same patterns of results and conclusions were reached when data were analyzed without being normalized to GAPDH. Data are expressed as the mean of triplicate samples ± SD. *p < 0.05 compared with untreated cells; #, p < 0.05 compared with IFN-γ-treated cells (as determined by Welch’s Student’s t test). N.S., not significant compared with IFN-γ alone (p > 0.05). Results are representative of three or more independent experiments.
inhibit IFN-γ-induced enrichment of acetylated histones at the CIITA locus (and, hypothetically, perhaps at other genes with regulation similar to that of CIITA).

Because Mtb 19-kDa lipoprotein has been shown to signal through TLR2 (12, 27), we used macrophages from TLR2−/− mice to determine whether inhibition of histone acetylation at CIITA pIV requires signaling through TLR2. RT-PCR studies confirmed that the ability of 19-kDa lipoprotein to inhibit IFN-γ-induced expression of CIITA mRNA was lost in TLR2−/− macrophages (Fig. 5A). Furthermore, ChIP studies showed that the 19-kDa lipoprotein inhibited IFN-γ-induced histone acetylation at CIITA pIV in wild-type macrophages (Fig. 5B), but not in TLR2−/− macrophages (Fig. 5C). Thus, 19-kDa lipoprotein requires TLR2 to mediate its inhibitory effect on IFN-γ-dependent chromatin remodeling of MHC2TA.

**Mtb 19-kDa lipoprotein inhibits IFN-γ-induced recruitment of Brg1 to the MHC2TA promoter**

Brg1, a component of the larger Swi/Snf remodeling complex, has recently been identified as a mediator of IFN-γ-dependent induction of CIITA (28). To further examine 19-kDa lipoprotein-mediated defects in chromatin remodeling, we performed ChIP assays to detect the presence or the absence of Brg1 at CIITA pIV. Macrophages were treated with the 19-kDa lipoprotein for 18 h, and IFN-γ was added for an additional 6–20 h. Chromatin was sheared and immunoprecipitated with anti-Brg1. Quantitative real-time PCR was used to determine the amount of CIITA pIV that coprecipitated with Brg1. Brg1 was recruited to CIITA pIV in response to IFN-γ, and 19-kDa lipoprotein inhibited this recruitment (Fig. 6A). These results cannot be explained by a reduction of Brg1 expression in response to 19-kDa lipoprotein, because Western blot analysis demonstrated equal levels of Brg1 under all experimental conditions (Fig. 6B). We therefore conclude that the recruitment of Brg1 to CIITA pIV is inhibited by the 19-kDa lipoprotein.

**Mtb 19-kDa lipoprotein activates multiple MAPKs**

To better elucidate the mechanism by which 19-kDa lipoprotein inhibits chromatin remodeling, we examined downstream branches of TLR2 signaling. MAPK signal cascades are known mediators of TLR responses. To determine whether MAPKs play a role in Mtb-mediated responses through TLR2, we treated macrophages with 19-kDa lipoprotein for 5–40 min and probed for phosphorylation of p38, ERK, and JNK MAPKs by Western blot. Mtb 19-kDa lipoprotein strongly activated ERK1/2 and p38, but induced very little or no activation of JNK (Fig. 7A). To determine which of

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**FIGURE 5.** Mtb 19-kDa lipoprotein does not inhibit IFN-γ-induced histone acetylation of MHC2TA in TLR2−/− macrophages. A, Mtb 19-kDa lipoprotein does not inhibit IFN-γ-induced CIITA mRNA in TLR2−/− macrophages. Macrophages were treated with or without 30 nM 19-kDa lipoprotein for 20 h, then stimulated with 2 ng/ml IFN-γ for 5 h. Quantitative RT-PCR was performed to assess CIITA mRNA expression (results were normalized to GAPDH in the same sample and expressed as the fold change compared with untreated samples). B and C, Inhibition of IFN-γ-induced histone acetylation of MHC2TA is dependent on TLR2. Wild-type (B) or TLR2−/− (C) macrophages were incubated with or without 30 nM 19-kDa lipoprotein for 20 h, and IFN-γ (2 ng/ml) was added for an additional 20 h. * Samples not exposed to IFN-γ or 19-kDa lipoprotein; No Ab, samples (+ IFN-γ, no lipoprotein) for which primary Ab was omitted during ChIP. Cross-linked DNA was sheared to an average length of 600 bp and immunoprecipitated with anti-acetyl histone H4. DNA was isolated from the precipitates, and quantitative real-time PCR was used to determine the amount of CIITA pIV that coprecipitated with acetylated histone H4. Data are expressed as the mean of triplicate samples ± SD. All samples were first normalized to GAPDH ChIP values, expressed as the fold change compared with untreated samples. (*, p < 0.05 compared with untreated cells; #, p < 0.05 compared with IFN-γ-treated cells (as determined by Welch’s Student’s t test). N.S., not significant compared with IFN-γ alone (p > 0.05). Results are representative of two independent experiments.

**FIGURE 6.** Mtb 19-kDa lipoprotein inhibits IFN-γ-induced recruitment of Brg1 to CIITA pIV. Macrophages were treated with or without 30 nM 19-kDa lipoprotein for 20 h, and IFN-γ (2 ng/ml) was added for an additional 20 h. * Samples not exposed to IFN-γ or 19-kDa lipoprotein. A, ChIP assay for association of Brg1 with CIITA pIV. Cross-linked DNA was sheared to an average length of 600 bp and immunoprecipitated with anti-Brg1. Quantitative real-time PCR was used to determine the amount of CIITA pIV that coprecipitated with Brg1. Brg1 was recruited to CIITA pIV in response to IFN-γ, and 19-kDa lipoprotein inhibited this recruitment (Fig. 6A). These results cannot be explained by a reduction of Brg1 expression in response to 19-kDa lipoprotein, because Western blot analysis demonstrated equal levels of Brg1 under all experimental conditions (Fig. 6B). We therefore conclude that the recruitment of Brg1 to CIITA pIV is inhibited by the 19-kDa lipoprotein.
These pathways, if any, is important in mediating CIITA inhibition. We used inhibitors specific for each kinase signaling cascade; U0126, a MAPK kinase 1/2 inhibitor, inhibited phosphorylation of ERK1/2 at 10 μM, but did not affect activation of p38 (Fig. 7B). SB203580, a selective inhibitor of p38 kinase activity, did not inhibit the phosphorylation of ERK (Fig. 7), but did inhibit MARCO mRNA expression, which is p38 dependent (data not shown). We treated macrophages with these inhibitors for 1 h, followed by addition of 19-kDa lipoprotein for 6 h and then addition of IFN-γ for a final 5 h (in the continued presence of inhibitors). Both SB203580 and U0126 completely reversed 19-kDa lipoprotein-mediated inhibition of CIITA mRNA expression, as determined by quantitative real-time PCR analysis (Fig. 8A). As expected, the JNK inhibitor, SP600125, had no effect on CIITA inhibition by the lipoprotein. As an additional negative control, the inactive SB203580 analog, SB202474, was tested and had no effect on CIITA inhibition (data not shown). These data indicate that signaling through ERK1/2 and p38 both contribute to inhibition of CIITA by 19-kDa lipoprotein. Moreover, inhibition of either p38 or ERK1/2 was sufficient to block Mtb-mediated suppression of CIITA, suggesting that this effect requires signaling by both p38 and ERK1/2.

To support our hypothesis that 19-kDa lipoprotein inhibits IFN-γ-induced CIITA by inhibiting histone acetylation, we performed ChIP assays with chromatin isolated from cells treated with the p38 inhibitor, SB203580. Macrophages were treated with SB203580, followed by treatment with 19-kDa lipoprotein and IFN-γ. ChIP was performed with anti-acetyl histone H3 and anti-acetyl histone H4 Abs. Quantitative real-time PCR with primers specific for CIITA pIV was used to determine the presence of acetylated histones at this locus. In the presence of the p38 inhibitor, 19-kDa lipoprotein did not inhibit histone acetylation of CIITA pIV (Fig. 8B), correlating with the ability of this inhibitor to prevent inhibition of CIITA expression by 19-kDa lipoprotein.

We conclude that CIITA promoter activity is inhibited by 19-kDa lipoprotein by a mechanism that involves inhibition of chromatin remodeling that is dependent on TLR2 and MAPK signaling.

Discussion

Mtb and its 19-kDa lipoprotein inhibit the macrophage expression of a subset of IFN-γ-induced genes, including CIITA, but enhance the expression of others (8, 11, 13). CIITA regulates the expression of MHC-II and other genes involved in Ag processing and presentation, which are inhibited by Mtb or 19-kDa lipoprotein (11). In this study we explored the mechanism by which IFN-γ-induced CIITA expression is inhibited by Mtb and 19-kDa lipoprotein. TLR2-dependent signaling by 19-kDa lipoprotein inhibited IFN-γ-induced acetylation of histones H3 and H4 at CIITA pIV, but not the iNOS promoter. Mtb 19-kDa lipoprotein also inhibited IFN-γ-dependent recruitment of the ATP-dependent chromatin-remodeling protein, Brg1, to CIITA pIV. The HDAC inhibitor sodium butyrate reversed 19-kDa lipoprotein-mediated inhibition of CIITA, restoring IFN-γ-induced CIITA mRNA expression. Together, these data suggest that Mtb-mediated inhibition of chromatin remodeling and histone acetylation at the CIITA promoter accounts for the inhibition of CIITA expression.
Previous studies suggested that Mtb inhibits IFN-γ-induced CIITA expression without affecting proximal IFN-γ signaling events, e.g., Stat1α activation or IRF-1 expression (13). In the presence of Mtb, IFN-γ still induces the expression of many other genes (8, 11), consistent with a persistence of proximal IFN-γ signaling. Stat1β, a dominant negative isofrom of the protein, is induced by 19-kDa lipoprotein (29), but our previous studies indicated that the lipoprotein had no effect on IFN-γ-induced activation of Stat1β in RAW264.7 cells (13). In addition, IFN-γ-induced expression of a IFN-γ activation site-driven luciferase construct was not affected by Mtb components in two previous studies (8, 13), suggesting that our results were not explained by dominant negative effects of Stat1β. Some studies have reported a slight decrease in IRF-1 expression in macrophages exposed to Mtb or 19-kDa lipoprotein (8, 13), but this modest change does not seem to be sufficient to account for the dramatic decrease in CIITA expression. In the current studies we found that 19-kDa lipoprotein did not inhibit IFN-γ-induced transcription of CIITAplIV.Luc, which contains binding sites for Stat1α, IRF-1, and USF-1, when this construct was transiently expressed in macrophages (Fig. 2A). These studies confirm that the mechanism for Mtb-mediated inhibition of CIITA expression lies distal to Stat1α, IRF-1, and USF-1.

We present several experiments that implicate the regulation of chromatin remodeling as a mechanism for Mtb-mediated inhibition of CIITA expression. Inhibition of CIITA by Mtb 19-kDa lipoprotein was not observed in CIITAplIV.Luc transient transfectants, but was observed in stable transfectants (Fig. 2B). Stable transfectants, unlike transient transfectants, can stably integrate transfected genes within the chromatin structure, where expression may be controlled by chromatin remodeling. The experiments comparing transient and stable transfection with CIITAplIV.Luc were insufficient by themselves to prove regulation by chromatin remodeling. Their importance was to support the development of subsequent, more definitive experiments. Moreover, we present them as an important reconciliation of our current data with previously published results with transient transfection (8, 13). The chromatin remodeling hypothesis was supported by experiments with sodium butyrate, an HDAC inhibitor. Sodium butyrate reversed the inhibition of CIITA expression, although not fully, suggesting that the main defect lies in histone acetylation at MHC2TA and is not completely explained by a lack of activating transcription factors. The inability of sodium butyrate to fully restore CIITA expression (at doses up to those causing toxicity; data not shown) suggests that it does not fully restore histone acetylation, that histone acetylation alone is not sufficient to sustain maximum chromatin remodeling of CIITA plIV, or that Mtb modulates other transcription factors that also contribute to the regulation of CIITA expression. Finally, ChIP studies provided definitive proof of the ability of 19-kDa lipoprotein to control histone acetylation at MHC2TA. IFN-γ-dependent histone acetylation and recruitment of Brg1 to CIITA plIV were both inhibited by 19-kDa lipoprotein. These data suggest that Brg1 and other chromatin-remodeling proteins are required for the complete transcriptional activation of CIITA and indicate that inhibition of histone acetylation is the mechanism by which 19-kDa lipoprotein inhibits CIITA expression.

TLRs are known to signal through MAPKs, and we demonstrate for the first time the activation of MAPKs by 19-kDa lipoprotein (Fig. 7A), a known TLR2 agonist (12, 13, 27). Mtb 19-kDa lipoprotein activated both p38 and ERK1/2 MAPKs (Fig. 7A). Using inhibitors selective for each of these MAPKs, we determined that inhibition of either p38 or ERK1/2 is sufficient to restore IFN-γ-dependent expression of CIITA (Fig. 8A). These data suggest that 19-kDa lipoprotein-mediated inhibition of CIITA requires distinct contributions from both the p38 and ERK signaling pathways. Alternatively, CIITA inhibition may require a threshold of MAPK activation that is not achieved in the presence of either inhibitor. This explanation seems less likely due to the observation that the presence of the p38 inhibitor, SB203580, enhances activation of ERK (our unpublished observations). Although 19-kDa lipoprotein produced a low level of JNK activation (Fig. 7A), a JNK-selective inhibitor had no effect on CIITA expression (Fig. 8A), indicating that JNK activation is not significant in this context. Importantly, the restoration of CIITA expression in the presence of either the p38 or ERK inhibitor correlated with the restoration of IFN-γ-dependent CIITA plIV histone acetylation. We confirmed the involvement of TLR2 signaling in the control of chromatin remodeling at MHC2TA, because 19-kDa lipoprotein inhibited histone acetylation of CIITA plIV in wild-type, but not TLR2−/−/− macrophages (Fig. 6). Together, these data indicate that 19-kDa lipoprotein must activate p38 and/or ERK to inhibit IFN-γ-dependent histone acetylation at CIITA plIV, which results in the inhibition of CIITA mRNA expression. IFN-γ-induced expression of CIITA was also inhibited by Pam3Cys lipopeptid (data not shown), and ligands of various TLRs (TLR2, TLR4 and TLR9) share the ability to inhibit macrophage Ag presentation and MHC-II expression (12, 14, 30), although we have not directly demonstrated that all TLRs use the same mechanism to inhibit CIITA.

These studies demonstrate the ability of the 19-kDa lipoprotein, a TLR2 agonist expressed by virulent Mtb, to inhibit IFN-γ-induced histone acetylation at one locus (MHC2TA), but not another (NOS2). However, the mechanism for targeted inhibition of a subset of IFN-γ-dependent genes is still uncertain. Our data focus primarily on CIITA plIV, and we have observed similar results at CIITA pl (data not shown). Additional studies are needed to determine whether other genes that are inhibited by 19-kDa lipoprotein also show defects in chromatin remodeling.

There are at least two models to explain Mtb-induced inhibition of chromatin remodeling. The first involves decreased expression of histone acetyltransferases (HATs) or increased expression of HDACs. HATs are known to catalyze acetylation of histones H3 and H4 and are generally considered to promote transcriptional activation, whereas HDACs suppress transcriptional activity through deacetylation of histones (31, 32). Therefore, 19-kDa lipoprotein could either inhibit the expression or activity of a particular HAT that is required at the CIITA promoter or increase the expression or activity of a HDAC that regulates the CIITA promoter. Microarray gene expression studies (11) revealed no differences in HAT or HDAC expression (our unpublished observations), but we cannot definitively exclude this possibility. A second mechanism to explain Mtb-induced defects in chromatin remodeling lies in the promoter-specific recruitment of remodeling proteins. In this model, it is not the expression of chromatin-remodeling proteins that is altered by 19-kDa lipoprotein, but the recruitment of these proteins to the CIITA promoter. This model is supported by the inhibition of Brg1 recruitment to CIITA plIV in response to 19-kDa lipoprotein despite unaltered Brg1 protein expression. However, the identity of putative sequence-specific DNA binding protein(s), which is responsible for recruiting a particular HAT or Brg1 to CIITA plIV, is still unknown. It is likely that prolonged TLR2 signaling induced by 19-kDa lipoprotein modulates the expression and/or activation of unidentified transcription factors or repressor proteins to suppress transcription of target genes, but additional studies are required to answer these questions.

Mtb is a highly successful pathogen due to its ability to establish chronic, latent infection despite a vigorous host immune response.
We propose that Mtb, which resists microbicidal mechanisms, provides prolonged TLR2 signaling that down-regulates Ag processing and presentation in a subset of infected macrophages, providing niches for Mtb to evade immune surveillance by CD4+ T cells. It is possible that this down-regulation represents a homeostatic regulatory mechanism (e.g., to protect the host from excessive inflammatory responses) that has been subverted by this successful intracellular pathogen. As the master regulator of expression of MHC-II and other genes associated with Ag processing, CIITA is required for macrophages to present Mtb Ags to CD4+ T cells, making it a central target for down-regulation. To achieve this down-regulation, mycobacteria disrupt IFN-γ-dependent chromatin remodeling at MHC2TA, inhibiting CIITA transcriptional activity. Insight into the mechanisms by which Mtb establishes and maintains latent infection may improve our chance of combating tuberculosis.

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Disclosures
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References