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Nitric Oxide and KLF4 Protein Epigenetically Modify Class II Transactivator to Repress Major Histocompatibility Complex II Expression during *Mycobacterium bovis* Bacillus Calmette-Guérin Infection*[S]

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Background: Mycobacteria down-regulates class II transactivator (CIITA)/MHC-II expression and antigen presentation.
Results: During *Mycobacterium bovis* BCG infection, iNOS/NO responsive KLF4 induces EZH2 and miR-150 functions to regulate CIITA expression and thus antigen presentation.
Conclusion: CIITA/MHC-II down-regulation by mycobacteria involves NOTCH/iNOS/NO/KLF4 signaling cross-talk and functions.
Significance: Identification of novel regulators of host-mycobacteria interactions provides promising therapeutic potential.

Pathogenic mycobacteria employ several immune evasion strategies such as inhibition of class II transactivator (CIITA) and MHC-II expression, to survive and persist in host macrophages. However, precise roles for specific signaling components executing down-regulation of CIITA/MHC-II have not been adequately addressed. Here, we demonstrate that *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)-mediated TLR2 signaling-induced iNOS/NO expression is obligatory for the suppression of IFN-γ-induced CIITA/MHC-II functions. Significantly, NOTCH/PKC/MAPK-triggered signaling cross-talk was found critical for iNOS/NO production. NO responsive recruitment of a bifunctional transcription factor, KLF4, to the promoter of CIITA during *M. bovis* BCG infection of macrophages was essential to orchestrate the epigenetic modifications mediated by histone methyltransferase EZH2 or miR-150 and thus calibrate CIITA/MHC-II expression. NO-dependent KLF4 regulated the processing and presentation of ovalbumin by infected macrophages to reactive T cells. Altogether, our study delineates a novel role for iNOS/NO/KLF4 in dictating the mycobacterial capacity to inhibit CIITA/MHC-II-mediated antigen presentation by infected macrophages and thereby elude immune surveillance.

Pathogenic mycobacteria like *Mycobacterium tuberculosis* and *Mycobacterium bovis* have been attributed to induce substantial morbidity and mortality all over the world (1–3). Although many species of mycobacteria elicit T cell-mediated cytokine responses such as IFN-γ, the mounted immune response is able to contain, but not eliminate the infection (4). This is due to a series of immune evasion strategies employed by the pathogenic mycobacteria that strongly interfere in the function of the macrophages, a critical necessity for ensuing robust host innate and adaptive immunity (5–7). IFN-γ, an important cytokine produced during infection with pathogenic mycobacteria induces the expression of diverse sets of immune genes in macrophages (8). Among these, up-regulation of major histocompatibility complex class II (MHC-II) and members of antigen processing machinery by IFN-γ play an important role in resulting $CD4^+$ T cell-dependent adaptive immunity (9, 10). Importantly, IFN-γ null mice are readily susceptible to mycobacterial infections as macrophages display diminished activation and expression of inducible nitric-oxide synthase (iNOS)/nitric oxide (NO) (11, 12). Furthermore, human subjects deficient for IFN-γ receptor or IFN-γ exhibit heightened susceptibility to pathogenic mycobacterial infections (13). However, macrophages infected with mycobacteria are known to become unresponsive to effects of IFN-γ. This selective refractoriness of macrophages involve significant inhibition of IFN-γ-triggered expression of a subset of genes including class II transactivator (CIITA),[3] a crucial transcription factor required for expression of MHC-II as well as H-2M or invariant chain (14, 15). In this context, engagement of Toll-like receptor (TLR) 2 by pathogenic mycobacteria or cell wall antigens could contribute as an early receptor proximal molecular event underlying mycobacteria-mediated inhibition of IFN-γ responses.

iNOS is an immunomodulatory gene regulated by pathogen-induced TLR2 signaling that determines the outcome of infec-

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[3] The abbreviations used are: CIITA, class II transactivator; TLR, Toll-like receptor; DN, dominant-negative; iNOS, inducible nitric-oxide synthase; IP, immunoprecipitation; KLF4, Krüppel-like factor 4; SIN1, 3-morpholinosydnonimine; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance; GSI-I, γ-secretase inhibitor-I; BCG, bacillus Calmette-Guérin.

tion (16, 17). iNOS catalyzing the formation of a reactive nitrogen intermediate, NO, along with other reactive intermediates, mounts a potent immune response against pathogenic mycobacteria, aiding in the effective containment of infection (18, 19). Importantly, in addition to its antimicrobial properties, NO modulates a wide range of signaling cascades in different cell types by nitration, nitrosation, and nitrosylation of key signaling molecules. Such modulations have significant effects on factors that regulate cell-fate decisions of macrophages or dendritic cells during the course of infection with pathogenic mycobacteria (20–24). Thus, mycobacterial infection-triggered expression of iNOS/NO production assumes critical importance. Apart from the numerous functions attributed to NO in the cellular signaling, its role in antigen presentation has rather been context dependent, wherein NO can induce or inhibit antigen presentation depending upon the cytokine milieu (25–28). Nevertheless, the role of NO in regulating a crucial immunological process like IFN-γ-induced CIITA/MHC-II expression remains elusive. Furthermore, the molecular mechanisms involved in mycobacteria-induced iNOS expression and NO production have been inadequately addressed.

In view of these observations, we characterized molecular mechanisms that contribute to mycobacteria responsive down-regulation of IFN-γ-triggered expression of MHC-II and CIITA. This study provides evidence that *M. bovis* bacillus Calmette-Guérin (BCG)-mediated TLR2 signaling triggers iNOS/NO production, which negatively regulates IFN-γ-induced CIITA and MHC-II expression. Deficiency in IFN-γ-induced CIITA or MHC-II expression requires dynamic cross-talk among NOTCH-PKCδ-p38-NF-κB signaling pathways. Importantly, NO-induced expression of KLF4 during *M. bovis* BCG infection acts as a crucial regulatory switch to inhibit CIITA or MHC-II expression by directing epigenetic modifications mediated by EZH2 at the CIITA promoter and post-translational regulation of CIITA mRNA by miR-150. As a consequence, infection-induced expression of KLF4 negatively regulates antigen processing and presentation of ovalbumin by *M. bovis* BCG-infected macrophages to antigen-specific T cells. Collectively, these findings identify novel roles for NO/KLF4 to be what we propose as significant regulators of host-mycobacterial interactions.

EXPERIMENTAL PROCEDURES

Cells and Mice—Macrophages were isolated from peritoneal exudates of BALB/c, C57BL/6, or iNOS$^{-/-}$ mice maintained in a central animal facility at the Indian Institute of Science. The experiments with mice were carried out after the approval from the Institutional Ethics Committee for animal experimentation as well as the Institutional Biosafety Committee. Murine RAW 264.7 macrophage-like cell lines (Obtained from National Center for Cell Sciences, Pune, India) were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated FBS (Sigma).

Bacteria—M. bovis BCG Pasteur 1173P2 was grown to mid-log phase in Sauton's medium. Batch cultures were aliquoted and stored at −70 °C. Representative vials were thawed and enumerated for viable colony forming units on Middlebrook 7H10 agar (Difco) supplemented with OADC (oleic acid, albumin, dextrose, and catalase). Single-cell suspensions of mycobacteria were obtained by short pulses of sonication and used at 10 multiplicity of infection unless indicated.

Reagents and Antibodies—General laboratory chemicals were purchased from Sigma, Promega, or Merck (Germany). Anti-Thr^{180}/Tyr^{182} phospho-p38 MAPK, anti-p38 MAPK, anti-Thr^{183}/Tyr^{185} phospho-SAPK/JNK MAPK, anti-SAPK/JNK MAPK, anti-NF-κB p65, anti-Thr^{505} phospho-PKCδ, anti-PKCδ, anti-cleaved Notch1 (Val^{1744}), anti-EZH2, and anti-H3K27me3 antibodies were purchased from Cell Signaling Technology. Anti-Notch1 (C-20) was procured from Santa Cruz Biotechnology. Anti-β-ACTIN (AC-15), anti-iNOS, and anti-CIITA antibodies were obtained from Sigma, anti-KLF4 antibody was obtained from Imgenex, anti-MHC-II-FITC-conjugated antibody was purchased from Miltenyi Biotec (Germany), 4′,6-diamidino-2-phenylindole (DAPI) was from Sigma. Anti-proliferating cell nuclear antigen was purchased from Calbiochem. IL-2 ELISA kit was purchased from PeproTech.

Treatment with Pharmacological Reagents—All the pharmacological inhibitors were obtained from Calbiochem and reconstituted using sterile DMSO (Sigma) or sterile H_2O and used at the following concentrations: γ-secretase inhibitor-I (GSI-I) (10 μM), chelerythrine (1 μM), PKCα inhibitor (Safingol) (50 μM), PKCδ inhibitor (Rottlerin) (10 μM), PKCζ inhibitor (PKCζ pseudosubstrate inhibitor, myristoylated) (5 μM), PKCϵ inhibitor (V2 peptide) (50 μM), SB203580 (20 μM), U0126 (10 μM), SP600125 (50 μM), BAY 11-7082 (20 μM), HMTase inhibitor (5 μM), and 1400W (100 μM). 0.1% DMSO was used as vehicle control. In all experiments with inhibitors, a tested concentration was used after careful titration experiments assessing the viability of the macrophages using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The cells were treated with inhibitor for 60 min before experimental treatment. NO donor, 3-morpholinosydnonimine (SIN1) (Sigma), was used as indicated in the experiments.

Transfection Studies—RAW 264.7 cells were transfected with 100 nM siRNA or miRNA mimic using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Transfection efficiency was more than 50% throughout all experiments as determined by counting the number of siGLO Lamin A/C positive cells in a microscopic field using a fluorescent microscope. 48 h post-transfection, the cells were treated as indicated and processed for analysis. siRNA specific to *Notch1*, *Klf4*, control siRNA, and siGLO Lamin A/C were obtained from Dharmacon as siGENOME^{TM} SMART pool reagents, which contains a pool of four different double-stranded RNA oligonucleotides. RAW 264.7 cells were transiently transfected with TLR2 DN, PKCδ DN, KLF4 DN, and KLF4 pcDNA3.1 constructs using low M_r polyethylenimine (Sigma). 48 h post-transfection, the cells were treated or infected as indicated and processed for analysis.

RNA Isolation and Quantitative Real-time RT-PCR—Total RNA from infected or treated macrophages was isolated by TRI Reagent (Sigma) as per the manufacturer's protocol. The cDNA synthesis kit (Bioline, UK) was used for reverse transcription according to the manufacturer's protocol. Quantitative real-time RT-PCR amplification (Applied Biosystems) using the SYBR Green PCR mixture (KAPA Biosystems) was performed for quantification of target gene expression. All reactions were

repeated at least three times independently to ensure reproducibility of the result. Amplification of the housekeeping gene *Gapdh* was used as an internal control and the primers utilized are listed in supplemental Table S1.

Quantification of miRNA Expression—For detection of miR-150, total RNA was isolated from infected or treated macrophages using the TRI Reagent (Sigma). Quantitative real-time RT-PCR for miR-150 was performed using TaqMan miRNA assays (Applied Biosystems) as per the manufacturer's instructions. U6 snRNA was used for normalization.

Immunoblotting Analysis—Cells were lysed in RIPA buffer constituting 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25 mM sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml of aprotenin, leupetin, pepstatin, 1 mM Na_3VO_4, and 1 mM NaF. Whole cell lysate was collected and equal amounts of protein from each cell lysate was subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore) by the semi-dry transfer (Bio-Rad) method. Nonspecific binding was blocked with 5% nonfat dry milk powder in TBST (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 0.1% Tween 20) for 60 min. The blots were incubated overnight at 4 °C with primary antibodies in 5% BSA (in TBST). After washing in TBST, blots were incubated with anti-rabbit or anti-mouse IgG secondary antibodies conjugated to HRP in 5% BSA (in TBST) for 2 h. After further washing in TBST, the immunoblots were developed with an enhanced chemiluminescence detection system (PerkinElmer Life Sciences) as per the manufacturer's instruction. β-ACTIN was used as loading control.

Nuclear and Cytosolic Subcellular Fractionation—Cells were treated as indicated, gently lysed in ice-cold Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF). After incubation on ice for 15 min, the cell membrane was disrupted with 10% Nonidet P-40 and nuclear pellets were recovered by centrifugation at 13,000 $\times$ *g* for 15 min at 4 °C. The supernatants from this step were used as cytosolic extracts. The nuclear pellet were lysed with ice-cold Buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA,1 mM EGTA, 1 mM DTT, and 1 mM PMSF) and nuclear extracts were collected after centrifugation at 13,000 {times] *g* for 20 min at 4 °C.

Measurement of NO—To measure the amount of NO produced, macrophages were treated as indicated. At the end of the experiment, culture supernatants were subjected to assay for NO production using Greiss reagent (Promega) according to manufacturer's instructions.

Immunofluorescence—For immunofluorescence studies, peritoneal macrophages or RAW 264.7 macrophages were seeded on coverslips. After the indicated treatment, cells were fixed with 3.7% paraformaldehyde for 15 min and stained with primary antibody anti-MHC class II-FITC in the dark for 1 h at room temperature. Nuclear staining was done using DAPI (1 μg/ml) for 5 min. Coverslips were mounted on a slide with glycerol and confocal images were taken on Zeiss LSM 710 Meta confocal laser scanning microscope using plan-Apochromat $\times$63/1.4 Oil DIC objective and images were analyzed using ZEN 2009 software.

Chromatin Immunoprecipitation (ChIP)—ChIP assays were carried out using the protocol provided by Upstate Biotechnology, with certain modifications. Briefly, macrophages were fixed with 1.42% formaldehyde for 15 min at room temperature followed by inactivation of formaldehyde with addition of 125 mM glycine. Nuclei were isolated from macrophages using modified RIPA buffer containing 1% Triton X-100 and chromatin was sheared using a cup sonicator (Sonics and Materials, Inc.). Chromatin extracts containing DNA fragments with an average size of 500 bp were immunoprecipitated using specific antibodies or rabbit preimmune sera. Purified DNA was analyzed by quantitative real-time RT-PCR. All results were normalized to amplification of 28 S rRNA and all ChIP experiments were repeated at least three times and the primers utilized are listed in supplemental Table S1.

Immunoprecipitation (IP)—IP assays were carried out using protocol provided by Millipore, with certain modifications. Briefly, macrophages were gently lysed in ice-cold RIPA buffer on an orbital shaker. The cell lysates were incubated with anti-KLF4, anti-EZH2, or rabbit preimmune sera at 4 °C for 2 h on an orbital shaker. The immunocomplexes were captured using Protein A-agarose at 4 °C for 2 h. The beads were harvested, washed, and boiled in 5$\times$ Laemmli buffer for 10 min. The samples were separated by SDS-PAGE and further subjected for immunoblotting.

EZH2 Activity in Vivo—Phosphate-buffered saline (PBS) as vehicle control or 10^6 *M. bovis* BCG was injected intravenously into the tail vein of mice. After 4 days of infection, mice were sacrificed, and spleen and lymph nodes were collected. Alternatively, mice were infected intraperitoneally with 10^6 *M. bovis* BCG for 12 h and peritoneal macrophages were isolated. Each *in vivo* experiment involved 4 animals per group. The cells obtained from spleen, lymph node, and peritoneal exudates were utilized for ChIP experiments.

Antigen Presentation Assay—Peritoneal macrophages or RAW 264.7 cells were seeded in 96-well flat-bottom plates (5×10^5 cells/well). In the experiments involving RAW 264.7 cells, transient transfection was carried out using KLF4 pcDNA3.1 or KLF4 DN or pcDNA3.1 alone. After 48 h, cells were infected with 10:1 multiplicity of infection of *M. bovis* BCG for 12 h and then incubated with IFN-γ (100 ng/ml) as indicated. Furthermore, the cells were treated with ovalbumin (500 μg/ml) for 6 h, washed, and incubated with splenocytes (1.5×10^6 cells/well) obtained from C57BL/6 (H-2^b) or BALB/c (H-2^d) mice in case of experiments with peritoneal macrophages from C57BL/6 (H-2^b) mice or RAW 264.7 (H-2^d) macrophages, respectively. The splenocytes used were obtained from mice that were intravenously challenged with 500 μg/ml of ovalbumin for 5 days. Macrophages-splenocytes were co-cultured for 48 h and culture supernatants were utilized for IL-2 ELISA.

Enzyme Immunoassay—Enzyme immunoassays were carried out in 96-well microtiter plates (Nunc, USA) using cell-free culture supernatant. Sandwich ELISA was performed as per the manufacturer's instructions. Briefly, assay plates were incubated with capture antibody at 4 °C overnight. After blocking with 1% BSA for 1 h at 37 °C, wells were incubated with cell-free supernatants for 2 h and then with biotinylated detection antibody for 2 h at 37 °C. The wells were further incubated with streptavidin-HRP for 1 h at 37 °C and developed with 3,3′,5,5′-tetramethylbenzidine. The absorbance was measured at 450 nm using an ELISA reader (Molecular Devices).

Statistical Analysis—Levels of significance for comparison between samples were determined by the Student's *t* test distri-

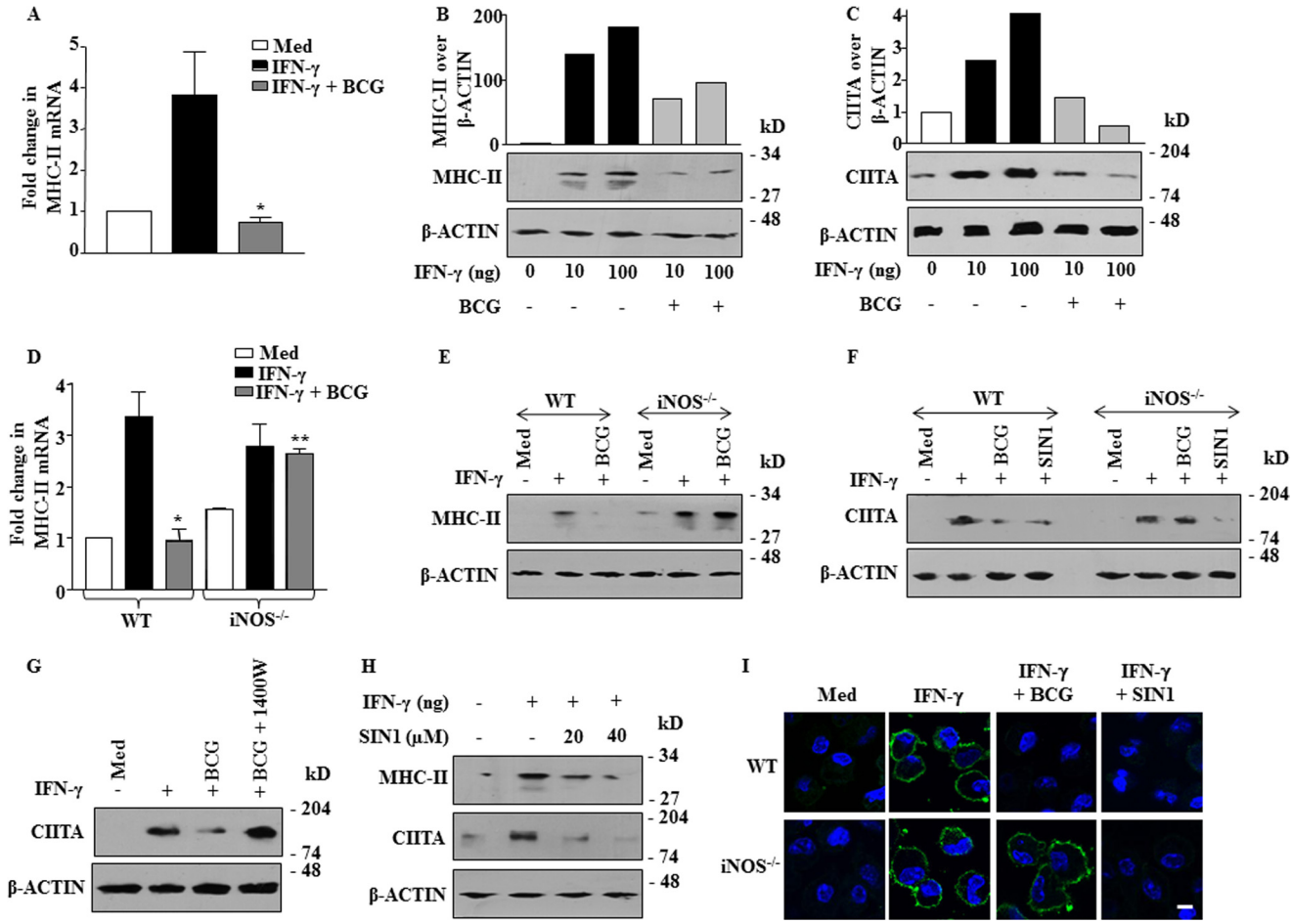

FIGURE 1. ***M. bovis* BCG-mediated inhibition of IFN-γ-induced MHC-II/CIITA requires NO.** *A*, mouse peritoneal macrophages were infected with *M. bovis* BCG for 12 h followed by 100 ng/ml of IFN-γ treatment for 12 h and MHC-II mRNA expression was analyzed by quantitative real-time RT-PCR. *B* and *C*, peritoneal macrophages were infected with *M. bovis* BCG for 12 h prior to treatment with 10 or 100 ng/ml of IFN-γ for 12 h. Expression levels of MHC-II (*B*) and CIITA (*C*) were analyzed by immunoblotting. MHC-II and CIITA expression over β-ACTIN was quantitated. *D-F*, peritoneal macrophages from WT or iNOS$^{-/-}$ mice were infected with *M. bovis* BCG (multiplicity of infection 10), or in *panel F*, treated with NO donor SIN1 followed by treatment of 100 ng/ml of IFN-γ, mRNA (*D*) expression of MHC-II was examined by quantitative real-time RT-PCR and MHC-II (*E*) and CIITA (*F*) protein expression levels were checked by immunoblotting. *G*, macrophages were pretreated with 1400W, an inhibitor of iNOS, for 1 h prior to infection with *M. bovis* BCG for 12 h followed by treatment with 100 ng/ml of IFN-γ for 12 h. CIITA protein levels were examined using immunoblotting. *H*, peritoneal macrophages were treated with SIN1 at the indicated concentration for 12 h, followed by IFN-γ treatment for 12 h. MHC-II and CIITA expression was analyzed by immunoblotting. *I*, WT or iNOS$^{-/-}$ macrophages were infected with *M. bovis* BCG or SIN1 for 12 h followed by treatment of 100 ng/ml of IFN-γ for 48 h and surface expression of MHC-II was assessed by immunofluorescence microscopy. Representative images are shown ($n = 3$). All blots shown are representative of 3 independent experiments. Quantitative real-time RT-PCR data represent mean ± S.E., $n = 3$. DMSO was used as vehicle control. *Med*, medium; *WT*, wild type; *iNOS*$^{-/-}$, iNOS knock out. *, $p < 0.005$, as compared with IFN-γ treated cells and **, $p < 0.005$, as compared with WT IFN-γ + BCG-treated/infected cells (one-way ANOVA). *Bar*, 10 μm.

bution and one-way ANOVA. The data in the graphs are expressed as the mean ± S.E. and $p < 0.05$ were defined as significant. GraphPad Prism 3.0 software (GraphPad Software) was used for all statistical analysis. The representative immunoblots were quantified by the Multi Gauge version 2.3 application (Fujifilm Medical Systems), using profile lane quantification with band detection and subtraction of respective background.

RESULTS

NO Regulates M. bovis BCG-mediated Inhibition of IFN-γ-induced MHC-II Expression—Induced expression of iNOS in macrophages upon infection with various pathogenic microbes often acts as a key mediator to regulate the initiation and maintenance of kinetics of ensuing protective immunity (29). In addition to its antimicrobial attributes, iNOS/NO modulate a wide range of signaling cascades that can have significant bearings on built-in regulatory circuits that control cell-fate decisions of macrophages during the course of infection with pathogenic mycobacteria (17, 30). In view of above mentioned observations, we explored a role for iNOS/NO in the ability of the *M. bovis* BCG to down-regulate MHC-II expression in macrophages. As shown in Fig. 1, *A* and *B*, and supplemental Fig. S1, *A* and *B*, *M. bovis* BCG infection critically abrogated IFN-γ-induced MHC-II expression both at transcript and protein levels in macrophages.

Antigen processing involving MHC-II is tightly regulated by a key transcription factor, CIITA. Mice deficient for CIITA exhibit a marked reduction in MHC-II expression (31). Mycobacteria or its antigens that reduce MHC-II expression have been demonstrated to involve inhibition of CIITA expression (15, 31, 32). As shown, *M. bovis* BCG infection significantly down-regulated IFN-γ-induced CIITA expression levels (Fig. 1*C* and supplemental Fig. S1*C*). Next, we assessed the role for

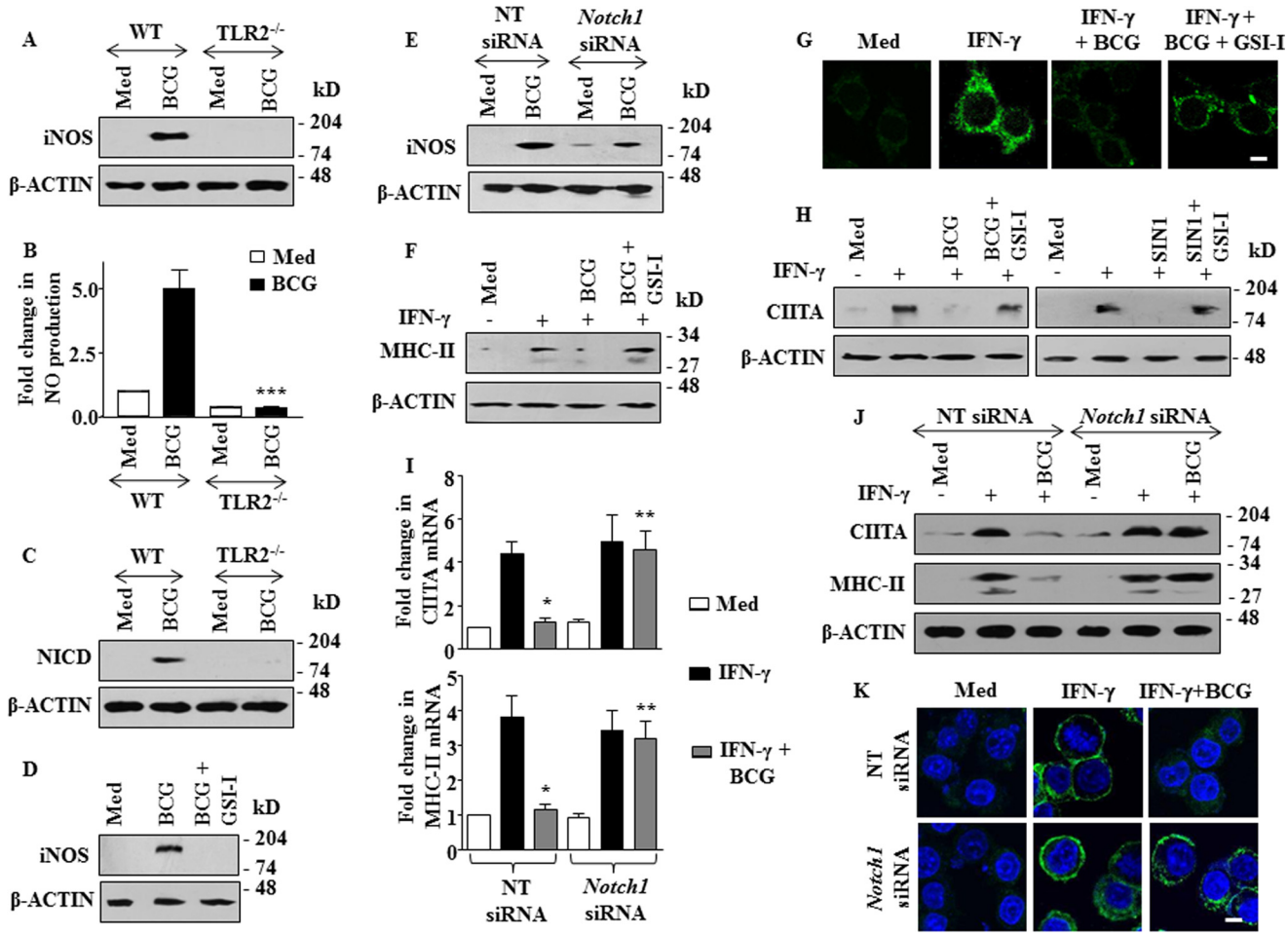

FIGURE 2. ***M. bovis* BCG activates NOTCH1 to mediate iNOS/NO production and suppression of CIITA/MHC-II molecules.** *A* and *B*, WT or TLR2 null peritoneal macrophages were assessed for iNOS expression (*A*) by immunoblotting after infection with *M. bovis* BCG for 6 h and NO production (*B*) by Greiss reagent after infection with *M. bovis* BCG for 12 h. *C*, WT or TLR2 null macrophages were infected with *M. bovis* BCG for 12 h and NOTCH1 activation was analyzed by immunoblotting. *D*, macrophages were pretreated with GSI-I (NOTCH activation inhibitor) for 1 h and infected with *M. bovis* BCG for 6 h to determine iNOS expression by immunoblotting. *E*, immunoblot for iNOS expression with *M. bovis* BCG infection for 6 h in RAW 264.7 macrophages transfected with *Notch1*-specific siRNA. *F*, peritoneal macrophages were pretreated with GSI-I for 1 h and then infected with *M. bovis* BCG for 12 h followed by IFN-γ treatment for 12 h. MHC-II expression was assessed by immunoblotting. *G*, representative immunofluorescence images ($n = 3$) stained for surface MHC-II on murine RAW 264.7 macrophages pretreated with GSI-I for 1 h followed by *M. bovis* BCG infection for 12 h and 100 ng/ml of IFN-γ treatment for 48 h. *H*, peritoneal macrophages were pretreated with GSI-I for 1 h and then infected/treated with *M. bovis* BCG or with SIN1 as indicated for 12 h followed by IFN-γ treatment for 12 h. The expression level of CIITA was determined by immunoblotting. *I* and *J*, murine RAW 264.7 macrophages transfected with NT or *Notch1* siRNA were infected with *M. bovis* BCG for 12 h prior to IFN-γ treatment. Quantitative real-time RT-PCR (*I*) and immunoblotting (*J*) was performed to analyze CIITA and MHC-II expression. *K*, representative immunofluorescence images ($n = 3$) stained for surface MHC-II on murine RAW 264.7 macrophages transfected with *Notch1*-specific siRNA, followed by *M. bovis* BCG infection for 12 h and 100 ng/ml of IFN-γ treatment for 48 h. All blots shown are representative of 3 independent experiments. NO data represent mean ± S.E., $n = 3$. *Med*, medium; *WT*, wild type; *TLR2*$^{-/-}$, TLR2 knock out; *NT*, non-targeting. ***, $p < 0.005$, as compared with *M. bovis* BCG-infected WT cells; *, $p < 0.005$, as compared with IFN-γ-treated NT siRNA-transfected cells; **, $p < 0.05$, as compared with IFN-γ + BCG-treated NT siRNA transfected cells (one-way ANOVA). *Bar*, 10 μm.

iNOS/NO during *M. bovis* BCG-mediated subdued expression of CIITA or MHC-II. Infection of macrophages derived from iNOS null mice, but not WT mice, exhibited a marked deficiency to down-regulate MHC-II and CIITA expression (Fig. 1, *D–F*). Furthermore, pretreatment of macrophages with 1400W, an iNOS activity inhibitor, severely reduced the capacity of the *M. bovis* BCG to inhibit CIITA expression (Fig. 1*G*). Additionally, treatment of macrophages with a NO donor like 3-morpholinosydnonimine (SIN1) was sufficient to abrogate the IFN-γ-induced MHC-II expression, validating the crucial role of NO in CIITA expression (Fig. 1, *F* and *H*). As illustrated in Fig. 1*I*, surface expression of MHC-II during *M. bovis* BCG infection was compromised despite IFN-γ treatment in WT macrophages but iNOS null macrophages failed to do so. Further corroborating the observation that SIN1 failed to induce surface expression of MHC-II with IFN-γ treatment emphasizing the iNOS/NO-dependent CIITA/MHC expression.

M. bovis BCG-triggered TLR2-NOTCH Signaling Pathways Control the Expression of iNOS/NO—Reports have shown that extensive exposure of macrophages to 19-kDa antigens of *M. tuberculosis* inhibits MHC-II expression in a TLR2-dependent manner. Accordingly, macrophages from TLR2 or *Myd88* knock-out mice failed to exhibit mycobacteria or 19-kDa antigen-induced inhibition of MHC-II-mediated antigen processing (14, 15). Interestingly, TLR2-triggered signaling cascades during mycobacterial infection regulates iNOS expression and NO production (17, 33). In this perspective, kinetic analysis demonstrated *M. bovis* BCG-induced expression of iNOS and NO production in a time- or CFU-dependent manner (supple-

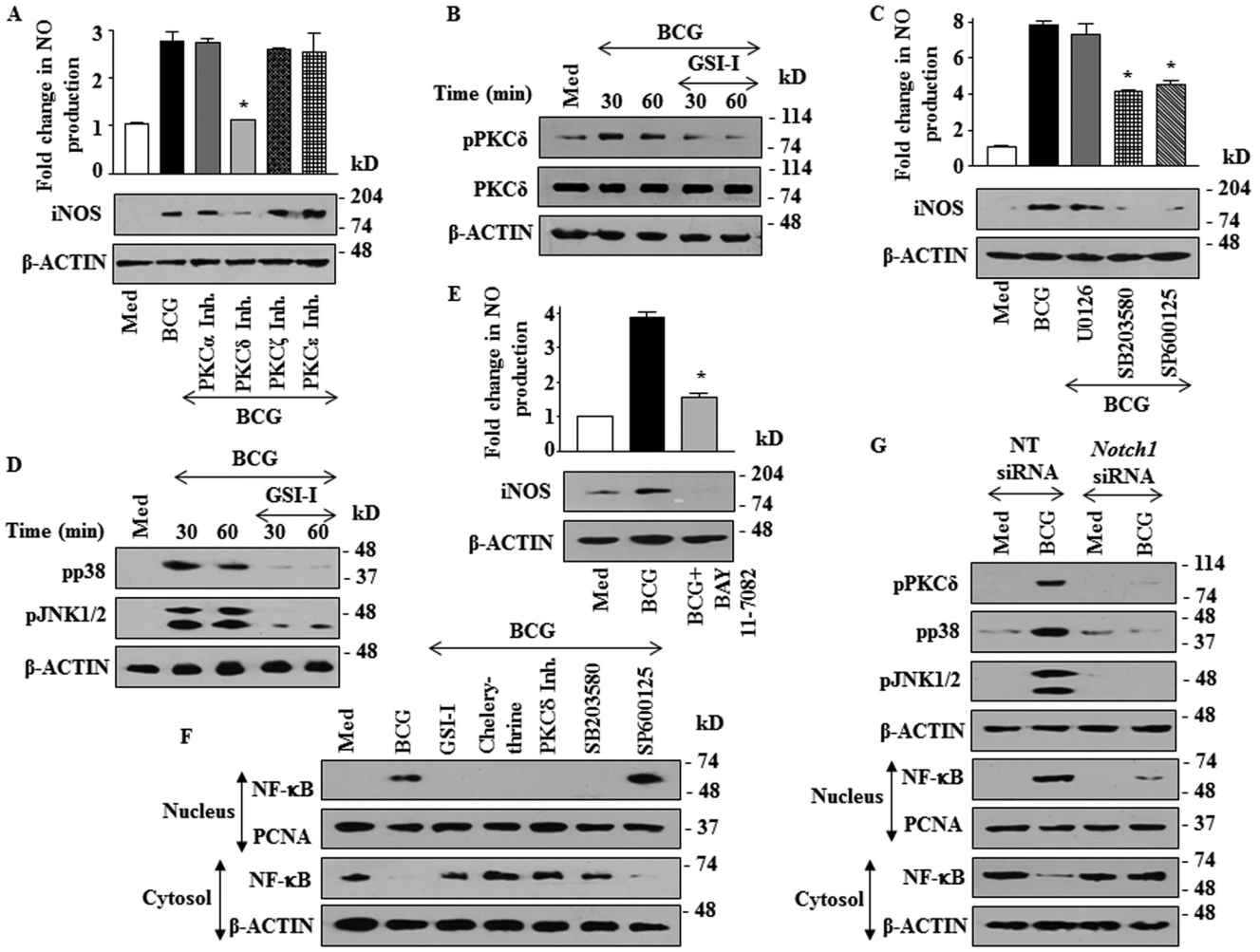

FIGURE 3. **NOTCH/PKC axis regulates MAPK and NF-κB during *M. bovis* BCG-mediated iNOS expression and NO production.** *A*, peritoneal macrophages were treated with PKC isoform inhibitors for 1 h prior to infection with *M. bovis* BCG for 6 and 12 h, iNOS expression was assessed using immunoblotting and NO production in the cell-free supernatant was estimated by Griess reagent, respectively. *B*, inhibition of NOTCH1 activation by GSI-I was carried out 1 h prior to *M. bovis* BCG infection and activation of PKCδ as determined by immunoblotting. *C*, pretreatment of macrophages with MAP kinase inhibitors, U0126 (MEK inhibitor), SB203580 (p38 inhibitor), and SP600125 (JNK1/2 inhibitor) for 1 h followed by infection with *M. bovis* BCG for 6 and 12 h to assay iNOS expression with immunoblotting and NO production in the supernatant by Griess reagent, respectively. *D*, activation of p38 and JNK1/2 MAPK was analyzed by immunoblotting in macrophages treated with GSI-I prior to infection with *M. bovis* BCG. *E*, *M. bovis* BCG-induced iNOS expression as analyzed by immunoblotting and NO production as assayed using Greiss reagent in peritoneal macrophages treated with NF-κB activation inhibitor (BAY 11-7082). *F*, immunoblotting for nuclear localization of p65 NF-κB in peritoneal macrophages infected with *M. bovis* BCG in the presence of GSI-I, chelerythrine, PKCδ inhibitor, SB203580, or SP600125. *G*, murine RAW 264.7 macrophages transfected with *Notch1* siRNA were infected with *M. bovis* BCG and analyzed for PKC-MAPK-NF-κB activation by immunoblotting. All blots shown are representative of 3 independent experiments. NO data represent mean ± S.E., $n = 3$. DMSO was used as vehicle control. *Med*, medium. *, $p < 0.05$, as compared with *M. bovis* BCG-infected cells (one-way ANOVA).

mental Fig. S2, *A–C*). Furthermore, macrophages from TLR2 null mice displayed compromised ability to induce iNOS expression or NO production (Fig. 2, *A* and *B*). Similarly, the TLR2 dominant-negative (DN) construct significantly reduced *M. bovis* BCG responsive iNOS expression or NO production (supplemental Fig. S2, *D* and *E*).

NO often executes key cell-fate decisions in various cellular contexts by modulating several signaling pathways in the host cells (34). Hence, signaling events that regulate iNOS/NO expression could act as a rate-limiting step during *M. bovis* BCG infection-triggered inhibition of MHC-II or CIITA. Interestingly, TLR2- dependent activation of NOTCH1 is known to induce activation of several proinflammatory cytokines and enzymes such as iNOS and COX-2 (17, 35). In this context, we addressed the role for the TLR2-mediated NOTCH1 signaling pathway in regulating iNOS/NO expression. Mycobacterial species, in addition to selected antigens, are known to induce NOTCH1 signaling in macrophages as assessed by the generation of the cleaved product of NOTCH1, notch intracellular domain, or expression of NOTCH1 signaling target genes or cognate ligands (36, 37). As a proof of concept we analyzed the generation of the activation marker of NOTCH1 signaling, the notch intracellular domain (supplemental Fig. S2*F*). Accordingly, *M. bovis* BCG triggered the activation of NOTCH1 signaling in WT, but not in TLR2 null macrophages (Fig. 2*C*). Importantly, inhibition of NOTCH1 signaling activation by the pharmacological inhibitor GSI-I or by siRNA-reduced *M. bovis* BCG-induced iNOS expression and NO production (Fig. 2, *D* and *E*, and supplemental Fig. S2, *G–I*). Supporting these observations, both immunoblotting and immunofluorescence studies demonstrated that abrogation of NOTCH1 signaling significantly compromised the capacity of *M. bovis* BCG to

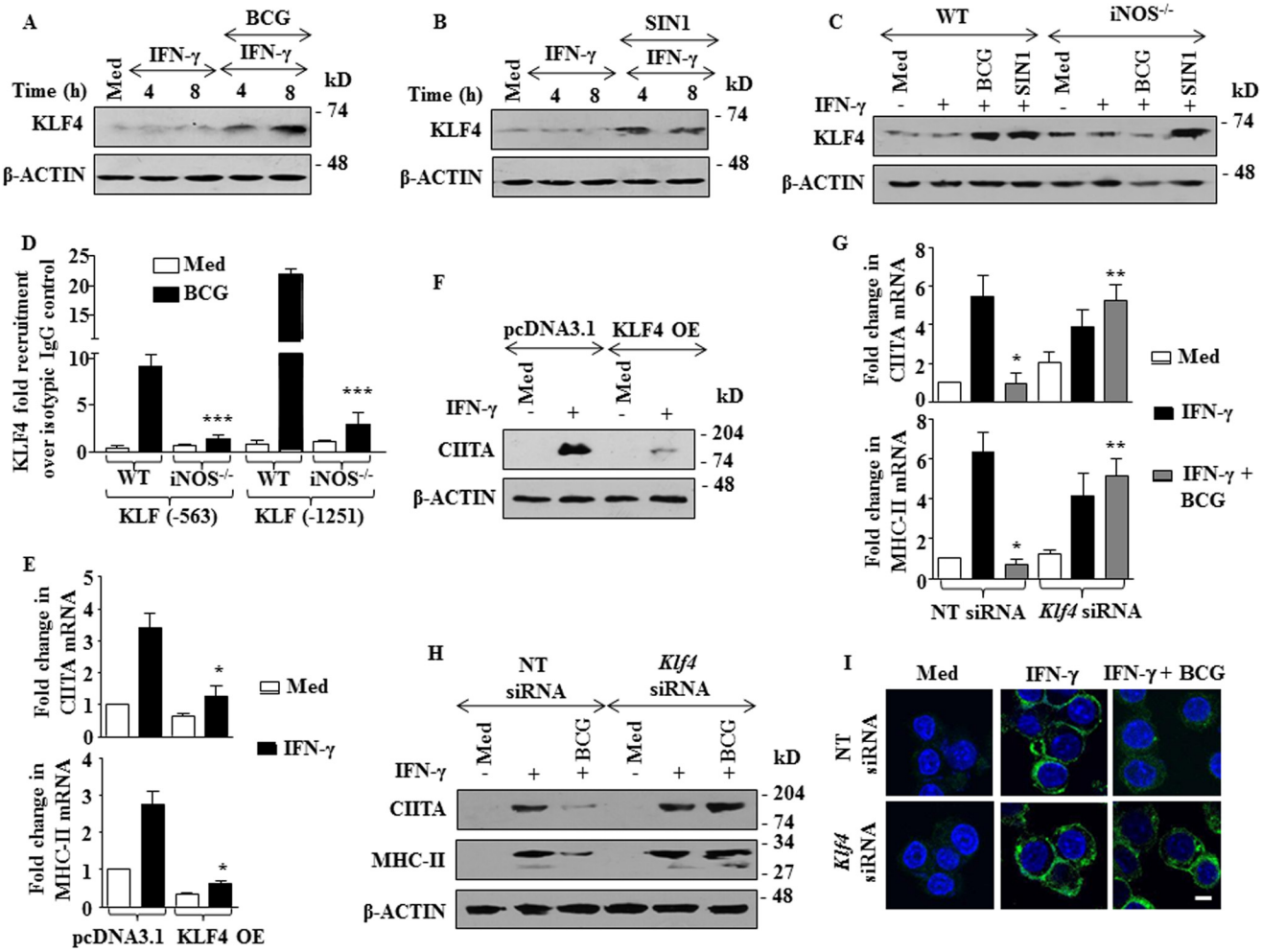

FIGURE 4. **NO-mediated activation of KLF4 suppresses IFN-γ-induced MHC-II/CIITA expression.** *A* and *B*, mouse peritoneal macrophages were infected/treated with *M. bovis* BCG (*A*) or SIN1 (*B*) 12 h prior to IFN-γ treatment at the indicated time points and total expression levels of KLF4 protein were assayed by immunoblotting. *C*, WT or iNOS null macrophages were either infected with *M. bovis* BCG or treated with SIN1 for 12 h followed with treatment with 100 ng/ml of IFN-γ for 12 h. Differential KLF4 expression was analyzed by immmunoblotting. *D*, ChIP assay was performed using anti-KLF4 or anti-IgG antibody using WT or iNOS null macrophages infected with *M. bovis* BCG. Recruitment of KLF4 at two sites at CIITA promoter (−563 and −1251 from +1 site) was determined using quantitative real-time RT-PCR. *E* and *F*, murine RAW 264.7 macrophages were transiently transfected with pcDNA3.1 or KLF4 overexpressed 48 h followed by infection with *M. bovis* BCG for 12 h and IFN-γ treatment for the next 48 or 12 h; *E*, quantitative real-time RT-PCR for CIITA and MHC-II expression, and *F*, immunoblotting for CIITA expression. *G-I*, macrophages were transfected with *Klf4* siRNA and treated similarly to *panel E*. *G* and *H*, CIITA and MHC-II expression analysis by quantitative real-time RT-PCR and immunoblotting. *I*, representative immunofluorescence images are shown ($n = 3$). All blots shown are representative of 3 independent experiments. Quantitative real-time RT-PCR data represent mean ± S.E., $n = 3$. DMSO was used as vehicle control. *Med*, medium; *WT*, wild type; *iNOS*$^{-/-}$, iNOS knock out; *OE*, overexpression; *NT*, non-targeting. ***, $p < 0.005$, as compared with *M. bovis* BCG infected WT cells; *, $p < 0.005$, as compared with IFN-γ-treated pcDNA3.1 or NT siRNA transfected cells; **, $p < 0.05$, as compared with IFN-γ + BCG-treated NT siRNA-transfected cells (one-way ANOVA). *Bars*, 10 μm.

down-regulate MHC-II and CIITA expression (Fig. 2, *F–K*). In accordance with the above mentioned observation, the NO donor SIN1 was unable to inhibit IFN-γ-induced CIITA expression upon blockade of NOTCH1 signaling activation by GSI (Fig. 2*H, right panel*). These results suggest that *M. bovis* BCG-triggered TLR2/NOTCH1 signaling pathways hold the capacity to modulate iNOS/NO production to regulate CIITA/MHC-II expression.

NOTCH1-dependent PKCδ-MAPK-NF-κB Signaling Axis Regulates iNOS Expression and NO Production during M. bovis BCG Infection—To further delineate signaling events involved in *M. bovis* BCG-mediated NOTCH1-NO-triggered regulation of MHC-II and CIITA, we assayed the role for protein kinase C (PKC) isoforms, mitogen-activated protein kinase (MAPK), and transcription factor nuclear factor-κB (NF-κB). PKCs often act as rate-limiting kinases in orchestrating wide ranging functions in immune cells. For example, strong correlates exist between NOTCH1 and PKCs, PKCs in regulating MAPK activations etc. (5, 36). In this perspective, a screen with isoform-specific inhibitors was carried out to identify a role for the specific isoform of PKC in regulating iNOS expression during *M. bovis* BCG infection. As shown, inhibition of PKCδ activity showed profound reduction in iNOS expression and NO production (Fig. 3*A*). Notably, infection with *M. bovis* BCG demonstrated kinetics of PKCδ activation in a time-dependent manner and the PKCδ DN construct critically reduced *M. bovis* BCG-induced iNOS expression or NO production (supplemental Fig. S3, *A–C*). Furthermore, perturbation of *M. bovis* BCG-induced NOTCH1 signaling resulted in a significant inhibition of PKCδ activation (Fig. 3*B*).

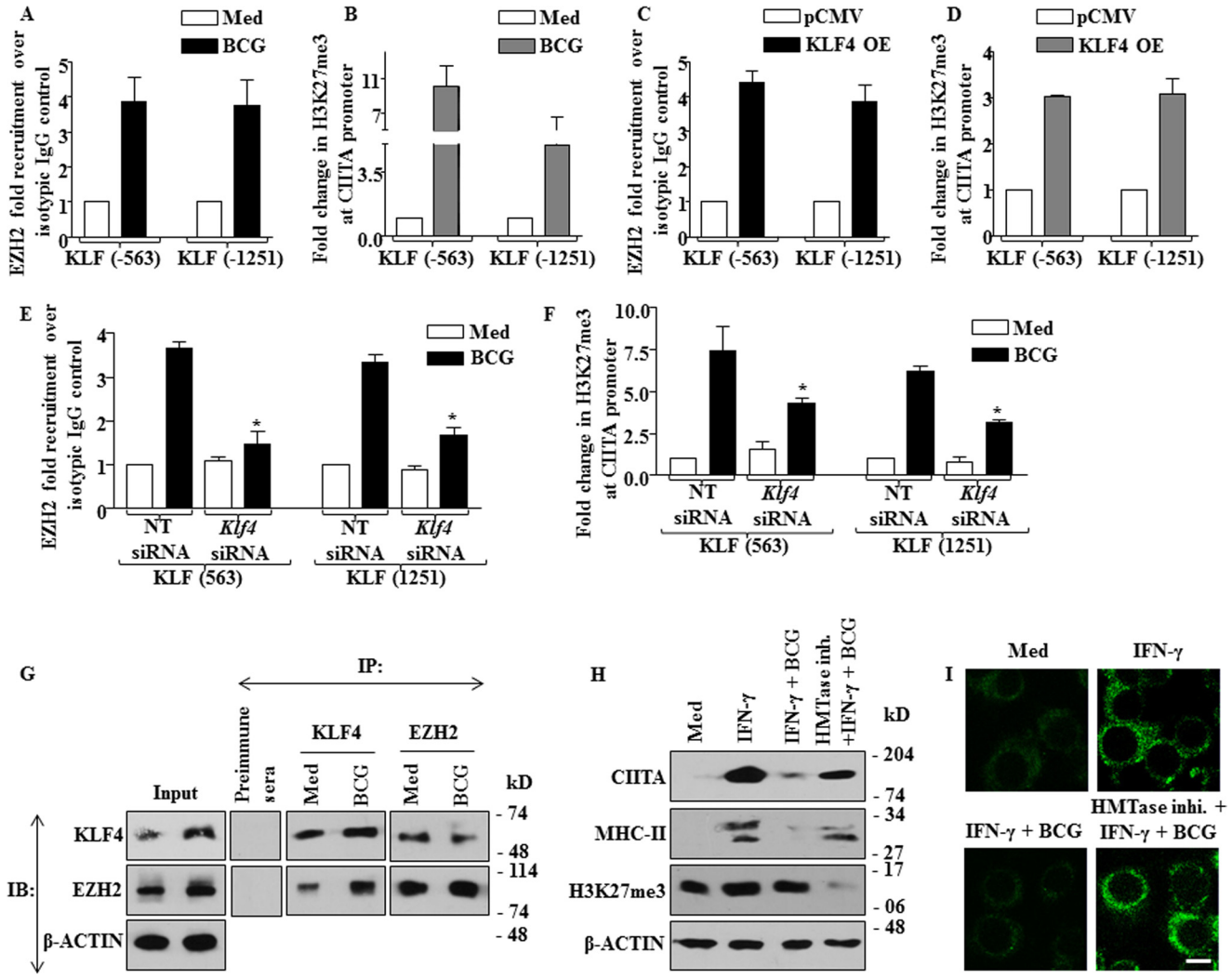

FIGURE 5. **KLF4 mediates histone modification at the CIITA promoter to down-regulate IFN-γ-induced MHC-II/CIITA.** *A–F*, ChIP assays were performed using anti-EZH2 or anti-H3K27me3 or anti-IgG antibody using macrophages infected with *M. bovis* BCG and/or transfected with the KLF4 overexpression construct or *Klf4* siRNA. *A*, *C*, and *E*, recruitment of EZH2; and *B*, *D*, and *F*, presence of H3K27me3 at KLF4 binding sites on the CIITA promoter (−563 and −1251 from +1 site) was determined using quantitative real-time RT-PCR. *G*, peritoneal macrophages were infected with *M. bovis* BCG for 12 h, immunoprecipitated with anti-KLF4 and anti-EZH2 antibodies, and analyzed for interaction using immunoblotting. *H* and *I*, macrophages were pretreated with HMTase (histone methyltransferase) inhibitor for 1 h followed by *M. bovis* BCG infection for 12 h and IFN-γ treatment for the next 12 and 48 h; *H*, immunoblots for the indicated molecules; and *I*, representative immunofluorescence images ($n = 3$) for MHC-II surface expression. All blots shown are representative of 3 independent experiments. Quantitative real-time RT-PCR data represent mean ± S.E., $n = 3$. DMSO was used as vehicle control. *Med*, medium; *OE*, overexpression; *NT*, non-targeting; *IB*, immunoblotting. *, $p < 0.005$, as compared with NT, *M. bovis* BCG infected cells (one-way ANOVA). *Bar*, 10 μm.

TLR2/NOTCH1 signaling pathways often require the activation of MAPKs for modulation of transcription factors that regulate downstream genes to mediate a variety of cellular functions (35). Moreover, several reports have demonstrated PKC as an important regulator of MAPKs (38, 39). Thus, we assayed the role for PKCδ-triggered MAPK-NF-κB activation during infection-induced expression of iNOS/NO. Signaling perturbations with MAPK-specific inhibitors, U0126 (MEK inhibitor), SB203580 (p38 inhibitor), and SP600125 (JNK1/2 inhibitor), ascertained that the inhibition of p38 and JNK1/2, but not ERK1/2 reduced *M. bovis* BCG-induced iNOS expression and NO production (Fig. 3*C*). In these lines, we confirmed the infection-triggered activation of p38 and JNK1/2 in macrophages (supplemental Fig. 3*D*). Furthermore, treatment of macrophages with GSI (NOTCH activation inhibitor), chelerythrine (pan-PKC inhibitor), or Rottlerin (PKCδ inhibitor) impaired the activation of *M. bovis* BCG-mediated p38 and JNK1/2 (Fig. 3*D* and supplemental Fig. 3*C*).

NF-κB acts as a key regulator of important cellular and immunological functions across various cell types. Mycobacterial infection-triggered TLR2/NOTCH1 signaling pathways are known to activate NF-κB (36, 40). Furthermore, the iNOS promoter contains several binding sites for cis acting elements including that of NF-κB. In accordance with published reports, *M. bovis* BCG infection led to time-dependent increments in NF-κB translocation from the cytosol to the nucleus scoring the activation of NF-κB (supplemental Fig. S3*F*). To ascertain the role for NF-κB in inducing iNOS expression or NO production, we utilized the pharmacological inhibitor of NF-κB activation, BAY 11-7082. As shown, BAY 11-7082 significantly reduced *M. bovis* BCG-driven iNOS/NO expression, and inhibition of NOTCH signaling (GSI), pan-PKC (chelerythrine), PKCδ (Rottlerin), p38 (SB203580) but

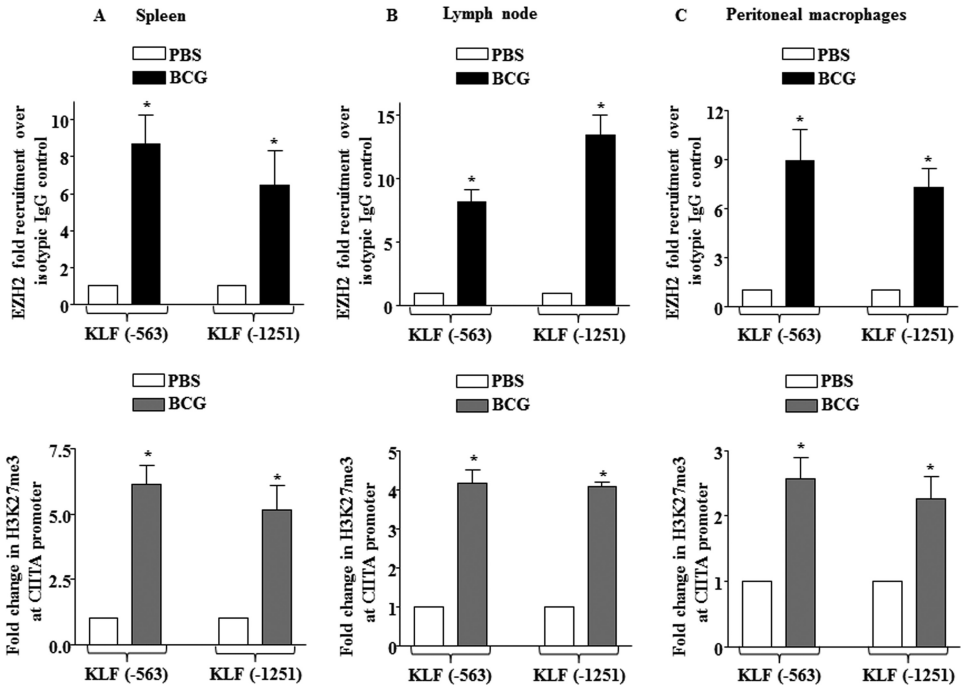

FIGURE 6. ***M. bovis* BCG modulates EZH2 activity *in vivo*.** *A-C*, ChIP analysis to determine the fold-recruitment of EZH2 and presence of H3K27me3 at KLF4 binding sites on the CIITA promoter (−563 and −1251 from +1 site) in spleen (*A*), lymph nodes (*B*), or peritoneal macrophages (*C*) from mice that are intravenously (*A* and *B*) or intraperitoneally (*C*) injected with PBS or *M. bovis* BCG. Quantitative real-time RT-PCR data represent mean ± S.E., $n = 4$. *, $p < 0.005$, as compared with PBS (*t* test).

not JNK1/2 (SP600125) markedly compromised the *M. bovis* BCG driven NF-κB translocation to the nucleus (Fig. 3, *E* and *F*). Substantiating the pharmacological inhibitor data, perturbation of *M. bovis* BCG-induced NOTCH1 signaling by *Notch1*-specific siRNA resulted in a significant decrease in the infection-induced PKCδ, p38, JNK1/2 activation, and NF-κB translocation from cytosol to nucleus (Fig. 3*G*).

Characterization of KLF4-mediated Regulation of CIITA during Infection with M. bovis BCG—As discussed, the *M. bovis* BCG-triggered TLR2/NOTCH1 signaling pathway is necessary for NO production that in turn interferes with IFN-γ-mediated responses in macrophages including the induced CIITA and MHC-II expression. However, the molecular regulatory switch that controls TLR2/NOTCH/iNOS/NO-mediated down-regulation of IFN-γ-induced CIITA expression remains unexplored. In this context, the altered promoter activity of CIITA during *M. bovis* BCG infection of macrophages could critically contribute to abrogation of CIITA expression during infection. Bioinformatic analysis for possible transcription factors binding to the CIITA promoter using the MatInspector program (Software of genomatix, www.genomatix.de) predicted two binding sites for a novel transcription factor, Krüppel-like factor (KLF) 4 in the CIITA promoter. KLF4 belongs to a subclass of the zinc finger family of DNA binding transcriptional factors, which execute varied effects on cellular reprogramming of macrophages either by suppressing or activating promoters of the immune genes (41–44).

To analyze the role of KLF4 in the current study, we evaluated infection-induced expression of KLF4 and as shown in Fig. 4*A*, *M. bovis* BCG significantly induced KLF4 expression in a time-dependent manner. However, IFN-γ treatment alone failed to induce considerable levels of KLF4 expression. Interestingly, SIN1, a pharmacological NO donor, induced KLF4 expression in macrophages in a similar time-dependent manner (Fig. 4*B*). Emphasizing a crucial function of NO in regulating KLF4, macrophages from iNOS null mice exhibited marked deficiency in inducing KLF4 upon infection with *M. bovis* BCG, which could be rescued by addition of SIN1 (Fig. 4*C*). These results strongly advocated a role for NO during *M. bovis* BCG infection-mediated expression of KLF4. To further explore the function for NO-KLF4 signaling, we preformed ChIP experiments and demonstrated that infection with *M. bovis* BCG resulted in significant recruitment of KLF4 at the respective sites of the CIITA promoter (Fig. 4*D*). Corroborating these results, the CIITA promoter in iNOS null macrophages displayed a reduction in KLF4 recruitment. Furthermore, we carried out gain-of-function and loss-of-function studies to validate the role for KLF4 during infection of macrophages with *M. bovis* BCG. Although enforced expression of KLF4 critically reduced IFN-γ-induced MHC-II and CIITA mRNA as well as proteins (Fig. 4, *E* and *F*, and supplemental Fig. S4*A*), repression of KLF4 by DN constructs or siRNA abolished the ability of *M. bovis* BCG to inhibit MHC-II and CIITA expression (Fig. 4, *G–I*, and supplemental Fig. S4, *A* and *B*).

KLF4 Directs Epigenetic Modifications to Fine-tune CIITA Expression—The epigenetic and chromatin modifications provide novel modes of controlling gene expression. These alterations include histone modifications like acetylation, methylation, and DNA methylation. Recently, small non-coding RNAs like miRNAs have been classified as epigenetic modifications that are crucial regulators of gene expression (45–47). Studies with pathogenic mycobacteria and its cell wall antigens have implied infection-induced epigenetic changes at the CIITA promoter (15, 48). In the present study, we hypothesize that

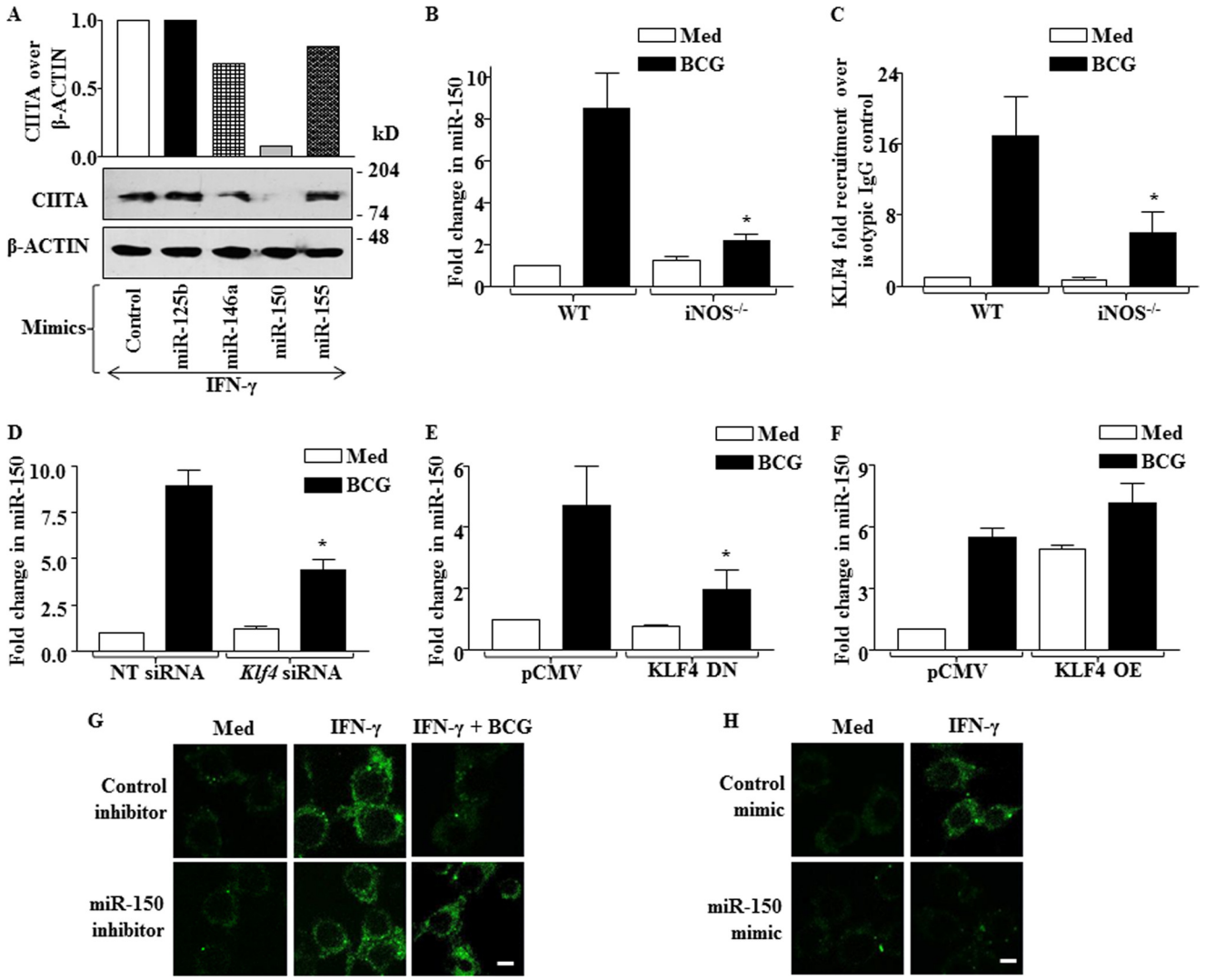

FIGURE 7. **KLF4 responsive miR-150 targets CIITA to subdue IFN-γ-induced MHC-II.** *A*, murine RAW 264.7 cells were transfected with various miRNA mimics as indicated and treated with 100 ng/ml of IFN-γ for 12 h. Immunoblotting was performed to determine the CIITA levels and quantification of the same is shown. *B*, peritoneal macrophages derived from WT and iNOS null macrophages were infected with *M. bovis* BCG for 12 h and assayed for miR-150 using specific primers by quantitative real-time RT-PCR. *C*, recruitment of KLF4 onto the miR-150 promoter in WT and iNOS null macrophages on infection with *M. bovis* BCG was analyzed by ChIP assay. *D–F*, miR-150 levels were estimated in RAW 264.7 macrophages transfected with *Klf4* siRNA (*D*), KLF4 DN (*E*), or KLF4 OE (*F*) and infected with *M. bovis* BCG for 12 h using quantitative real-time RT-PCR. *G* and *H*, RAW 264.7 cells were transfected with miR-150 inhibitor (*G*) or miR-150 mimic (*H*), and infected with *M. bovis* BCG in *panel G*, followed by treatment with IFN-γ for 48 h. Representative immunofluorescence images are shown ($n = 3$). All blots shown are representative of 3 independent experiments. Quantitative real-time RT-PCR data represent mean ± S.E., $n = 3$. *Med*, medium; *WT*, wild type; *iNOS*$^{-/-}$, iNOS knock out; *OE*, overexpression; *, $p < 0.05$, as compared with WT/NT/pCMV *M. bovis* BCG-infected cells (one-way ANOVA). *Bars*, 10 μm.

KLF4 being a novel regulator of CIITA might render repressive epigenetic modifications at the CIITA promoter. Thus, we analyzed the status of H3K27 trimethylation, representing a repressed promoter, at the CIITA promoter under the influence of KLF4. *M. bovis* BCG infection of macrophages led to significantly elevated recruitment of EZH2, a histone methyltransferase specific to H3K27, onto the CIITA promoter at KLF4 binding consensus sequences (Fig. 5*A*). Concordantly, a marked increase in H3K27 trimethylation was observed at corresponding sites (Fig. 5*B*). The role of KLF4 in modulating histone modifications was further validated using gain-of-function and loss-of-function approaches. KLF4-dependent recruitment of EZH2 and subsequent H3K27 trimethylation was affirmed (Fig. 5, *C–F*). As a proof of concept, a IP assay confirmed the KLF4-EZH2 interaction (Fig. 5*G*). Next, the role of EZH2 activity in modulation of IFN-γ-induced CIITA and MHC-II expression was analyzed. As shown in Fig. 5, *H* and *I*, inhibition of histone methyltransferase activity demonstrated a marked rescue of *M. bovis* BCG-mediated suppression of CIITA and MHC-II expression. In accordance to these observations, *in vivo* infection of *M. bovis* BCG through tail vein injection showed significant increments in EZH2 recruitment and trimethylation at the CIITA promoter in cells obtained from spleen as well as lymph nodes (Fig. 6, *A* and *B*). Similarly, *in vivo* peritoneal macrophage infection with *M. bovis* BCG induced increased EZH2 activity at KLF4 binding sites on the CIITA promoter (Fig. 6*C*). Altogether, these observations suggest a significant contribution of KLF4 responsive histone modifications at the CIITA promoter to arbitrate *M. bovis* BCG-infected macrophage refractoriness to IFN-γ.

As discussed, KLF4 exhibits a dual activity of controlling cellular programming either by suppressing or activating the

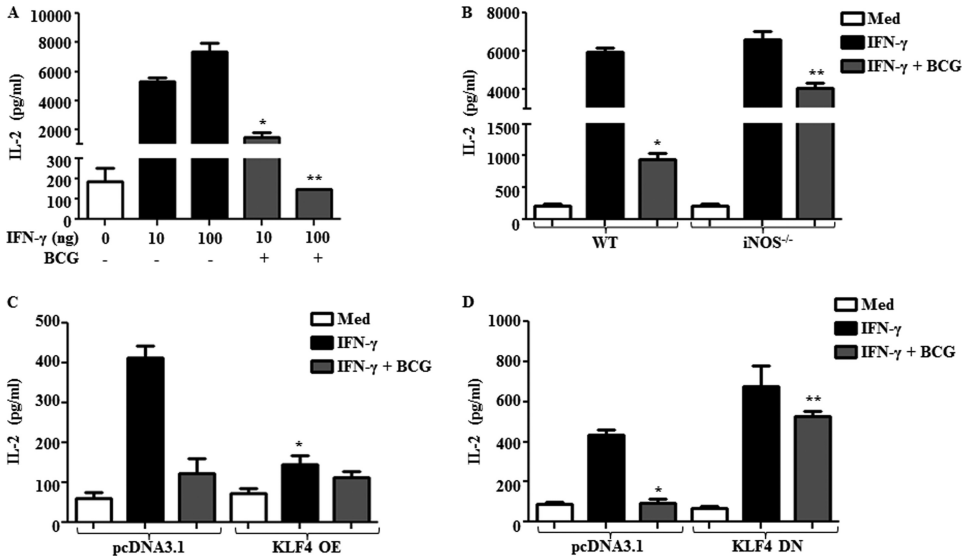

FIGURE 8. **NO-dependent KLF4 regulates antigen presentation in macrophages.** *A*, antigen presentation assay was performed using peritoneal macrophages and splenocytes obtained from C57BL/6 mice and cell-free supernatant was estimated for IL-2 cytokine production using enzyme immunoassay. *, $p < 0.05$; **, $p < 0.005$ as compared with IFN-γ-treated cells (one-way ANOVA). *B*, experimental setup similar to *panel A* was used to assess IL-2 levels in wild type and iNOS null macrophages. *, $p < 0.005$, as compared with IFN-γ-treated cells and **, NS, as compared with wild type *M. bovis* BCG-infected macrophages (one-way ANOVA). *C* and *D*, RAW 264.7 macrophage cells were transiently transfected with KLF4 overexpression (*C*) or KLF4 DN (*D*) for 48 h and used for antigen presentation assay with splenocytes obtained from BALB/c mice. *, $p < 0.05$, as compared with pcDNA3.1-transfected IFN-γ-treated cells; **, NS, as compared with pcDNA3.1-transfected *M. bovis* BCG-infected macrophages (one-way ANOVA). Data represent mean ± S.E., $n = 3$. *Med*, medium; *WT*, wild type; *iNOS*$^{-/-}$, iNOS knock out; *OE*, overexpression.

expression of genes (42, 44). To investigate the dichotomous function of KLF4, we further explored the ability of KLF4 to activate the expression of non-coding miRNAs, which in turn could contribute to maintain the sustained suppression of CIITA. In this perspective, the 3′ UTR of CIITA was analyzed for probable miRNA target sites. Extensive bioinformatics analyses identified the binding sites for miR-125b(1745–1753), miR-146a(909–915), miR-150(347–354), and miR-155(187–194) in the 3′ UTR of CIITA. To confirm the role of miRNA(s) in targeting CIITA mRNA, ectopic expression of these miRNAs was performed. As shown in Fig. 7*A*, miR-150 but not other tested miRNAs, abrogated CIITA expression. In accordance with the previous data, iNOS null macrophages showed a compromised ability to induce elevated levels of miR-150 as compared with WT macrophages (Fig. 7*B*). Furthermore, KLF4-mediated miR-150 expression during *M. bovis* BCG infection was substantiated using ChIP assays. miR-150 promoter analysis using the MatInspector program identified 5 distinct KLF4 binding consensuses sites. Infection induced by increased recruitment of KLF4 at the miR-150 promoter was found to be iNOS/NO-dependent (Fig. 7*C*). Confirming this observation, knockdown of KLF4 activity using specific siRNAs or expression of the KLF4 DN mutant severely reduced *M. bovis* BCG-induced miR-150 expression (Fig. 7, *D* and *E*), whereas in the presence of KLF4 overexpression, heightened miR-150 expression prevailed (Fig. 7*F*). The function of miR-150 during *M. bovis* BCG-induced down-regulation of CIITA was supported with analysis of surface expression of MHC-II. As illustrated in Fig. 7, *G* and *H*, miR-150 inhibition hindered the *M. bovis* BCG responsive suppression of MHC-II surface expression; on the other hand ectopic expression of miR-150 prevented IFN-γ-induced MHC-II expression. Altogether, our data demonstrated that KLF4 directs novel epigenetic mechanisms such as H3K27 trimethylation at the CIITA promoter and miR-150 targeted CIITA expression, which are necessary to regulate macrophage cellular programming.

KLF4 Is a Negative Regulator of IFN-γ-induced Antigen Processing—The adaptive immunity to mycobacterial infections involves activation and expansion of antigen-specific CD4 T cells that often culminates in elevated secretion of IL-2 (49). In this context, modulation of MHC-II expression by *M. bovis* BCG could be a rate-limiting step in ensuing adaptive immunity. In view of above mentioned observation, we assessed the role of KLF4 in regulating antigen processing and presentation by *M. bovis* BCG-infected macrophages to reactive T cells. As shown in Fig. 8*A*, *M. bovis* BCG infection significantly down-regulated IFN-γ-induced MHC-II-mediated processing and presentation of an exogenous antigen, ovalbumin as assessed by IL-2 secretion by ovalbumin reactive T cells. Importantly, macrophages derived from iNOS null mice remained refractory to *M. bovis* BCG-mediated inhibition of ovalbumin presentation (Fig. 8*B*). Furthermore, overexpression of KLF4 in haplotype-matched macrophages inhibited processing and presentation of ovalbumin to reactive T cells (Fig. 8*C*). On the contrary, KLF4 DN abolished the ability of *M. bovis* BCG to inhibit processing and presentation of ovalbumin to T cells (Fig. 8*D*). These results strongly advocate NO/KLF4 as critical regulators of antigen processing and presentation during *M. bovis* BCG infection of macrophages.

DISCUSSION

TLR2 has been implicated as a primary receptor that senses mycobacteria and influences host resistance against the pathogens (50). Nevertheless, recent studies have provided contrasting evidence. The pathogenic mycobacteria-induced TLR2 cascade modulates key signaling and functional molecules

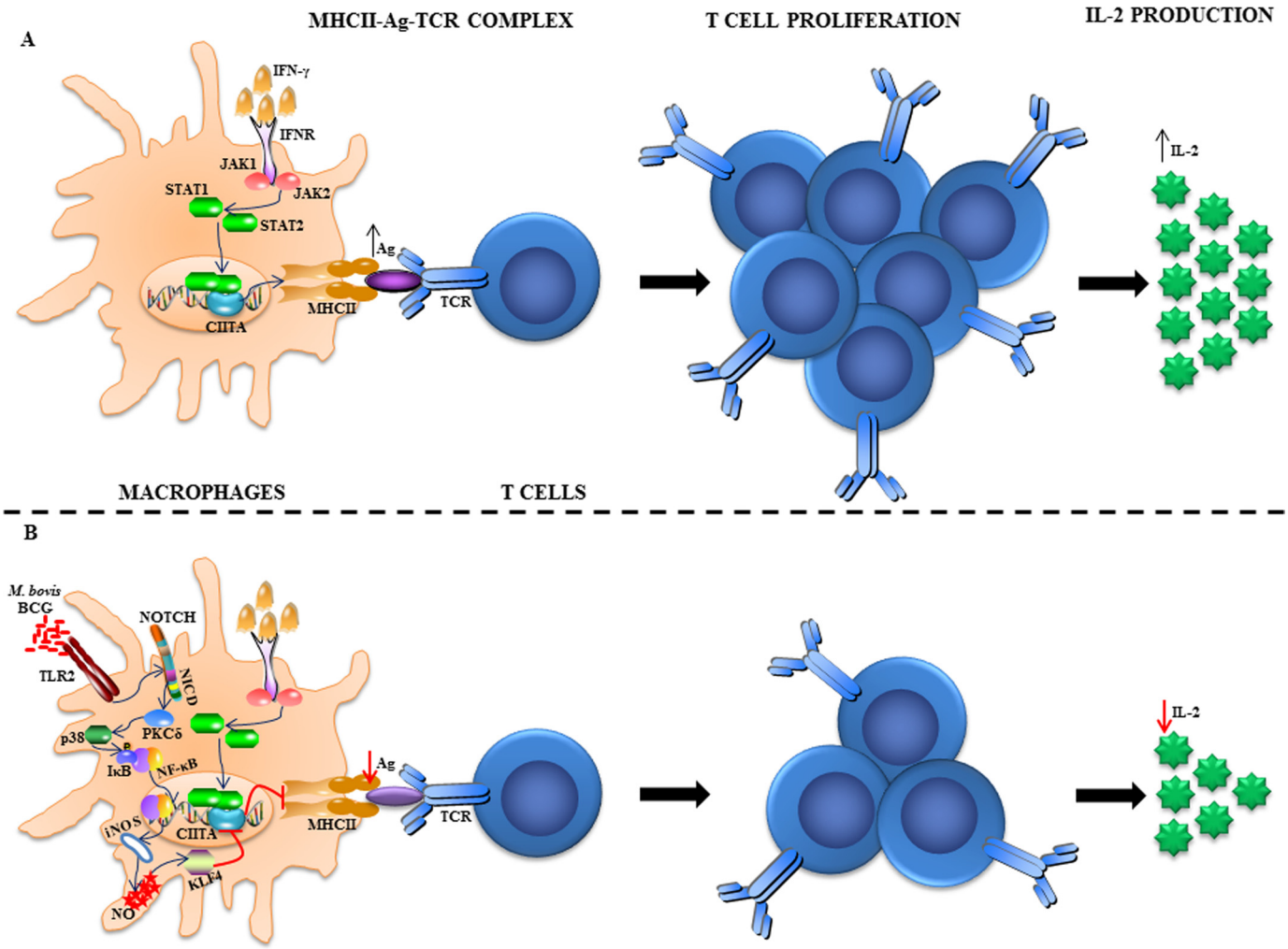

FIGURE 9. **Model.** *A*, IFN-γ triggers up-regulation of CIITA and MHC-II molecules that leads to sustained antigen (*Ag*) presentation, T cells proliferation, and thus increased IL-2 production. *B*, *M. bovis* BCG interacts with TLR2 that cross-talk with the NOTCH1 receptor. TLR2/NOTCH activation transduces the signal to the nucleus through PKCδ-p38/JNK1/2-NFκB culminating in expression of iNOS and consequently NO production. NO in turn, via KLF4, epigenetically suppresses the expression of CIITA, the master co-activator of MHC-II molecules. This leads to inhibition of the macrophage responses to IFN-γ signaling, diminished surface expression of MHC-II molecules, and reduced production of IL-2.

involved in regulating immune responses, creating a niche for its better survival inside the host. One such potential effect of mycobacteria-mediated TLR2 signaling is inhibition of antigen presentation (51). Although, few studies have unraveled the mechanisms involved in mycobacteria-induced TLR2-mediated down-regulation of CIITA and MHC-II expression, the role of NO in such a scenario has not been adequately addressed. Our work herein significantly strengthens the observation as we delineated the role of *M. bovis* BCG-mediated TLR2/NOTCH1-triggered NO in reducing MHC-II and CIITA expression. The NO responsive transcription factor KLF4 was found to orchestrate epigenetic modifications at the CIITA promoter to regulate MHC-II expression.

TLR2-triggered NOTCH1 signaling activates NF-κB, a key transcription factor for numerous immunological processes (36, 37). Interestingly, several reports indicate the involvement of NF-κB in inducing iNOS expression and that NOTCH1 signaling modulates the induction of proinflammatory cytokines (17, 35). In line with these observations, NOTCH1 signaling has emerged as an important regulator of iNOS/NO expression via NF-κB in mouse macrophages.

NOTCH1 signaling has been implicated to activate cellular PKCδ isoforms (36). In the present context, PKCδ, a novel isoenzyme is primarily activated by diacylglycerol. Interestingly, the formation of diacylglycerol is due to the activation of phospholipase C or phospholipase D and their subsequent action on phosphatidylcholine present on the cell membrane (52). Notably, activation of phospholipase D during *M. bovis* BCG infection in macrophages has been addressed in our previous studies (37). Furthermore, PKCδ regulates downstream signaling molecules and transcription factors such as MAPKs and NF-κB (39, 53). In this regard, our current study demonstrates the involvement PKCδ-dependent p38 and JNK1/2, but not ERK1/2 in *M. bovis* BCG-mediated induction of iNOS/NO expression. However, JNK1/2 did not mediate NF-κB activation. We speculate that JNK1/2 could activate other transcription factors like AP-1, known to regulate iNOS expression in other cellular contexts (54, 55).

Role of NO as intracellular messengers has been extensively studied in several cellular contexts. Notably, NO modulates the activity of different transcription factors such as AP-1, EGR-1, HIF-1, and Nrf2 (56–59). However, the ability of NO to regu-

late the KLF family of transcription factors is not documented. In this perspective, the bifunctional nature of KLF4 has been largely attributed to co-activators and co-repressors to which it binds (44). Recent studies have determined the mechanisms by which KLF4 can act as a suppressor or an activator; interaction with co-activators such as p300 and cAMP-response element-binding protein and repressors such as histone deacetylase-3 dictate its function (60–62). The current study underscores the novel functions of *M. bovis* BCG-induced NO-responsive KLF4 to subdue CIITA expression through epigenetic modifications as evidenced by *in vivo* and *ex vivo* experiments. The negative regulation by KLF4 at the CIITA promoter is coordinated by the histone methyltransferase EZH2, a component of the PRC2 complex. Notably, literature evidences for the crucial role of epigenetic regulation of CIITA/MHC expression are available. For example, inhibition of chromatin remodeling factors like Brahma-related gene-1 (*BRG-1*) or histone acetylase recruitment to the CIITApIV promoter by pathogenic mycobacteria or 19-kDa protein suppresses IFN-γ-induced CIITA expression (14, 63, 64). *Mycobacterium avium* inhibits IFN-γ induced functions of CIITA by up-regulating the expression of mSin3A, and the histone deacetylase-1 and -2-associated repressor, which facilitates deacetylation at the CIITA promoter (48). The current investigation also ascribes the function of KLF4 as a positive regulator due to its ability to induce miR-150 that targets CIITA. Epigenetic modification rendered by non-coding RNA like miRNAs have gained tremendous interest. Supporting the role for non-coding RNA, the trophoblast non-coding RNA can suppress endogenous as well as transiently transfected CIITA promoter activity (65, 66). Together, these studies suggest the significance of epigenetic modifications to control CIITA or MHC-II expression and activity. Antigen presentation assays have further highlighted the important role of KLF4 in this process.

Although various studies have implicated the role of kinases and transcription factors in regulating IFN-γ-induced CIITA expression, our study presents a novel mechanism wherein *M. bovis* BCG-mediated TLR2 signaling activates the NOTCH1-PKCδ-p38-NF-κB signaling axis to regulate iNOS expression and NO production; NO-mediated down-regulation of MHC-II and CIITA expression requires KLF4 (Fig. 9). Collectively our results emphasize a novel finding that significantly contributes to the ability of mycobacteria to evade immune surveillance. It is clear from our study that NO scavenger molecules and KLF4 inhibitors would present promising potential in therapeutics as well as immunoprophylaxis of tuberculosis.

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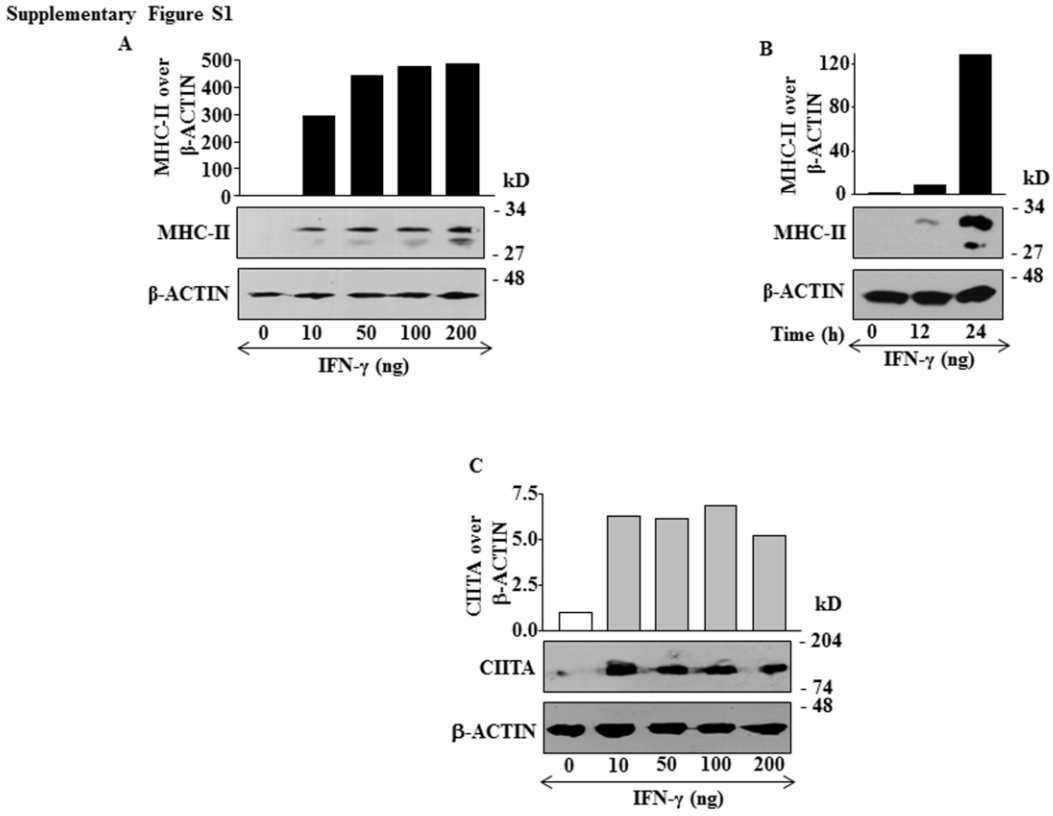
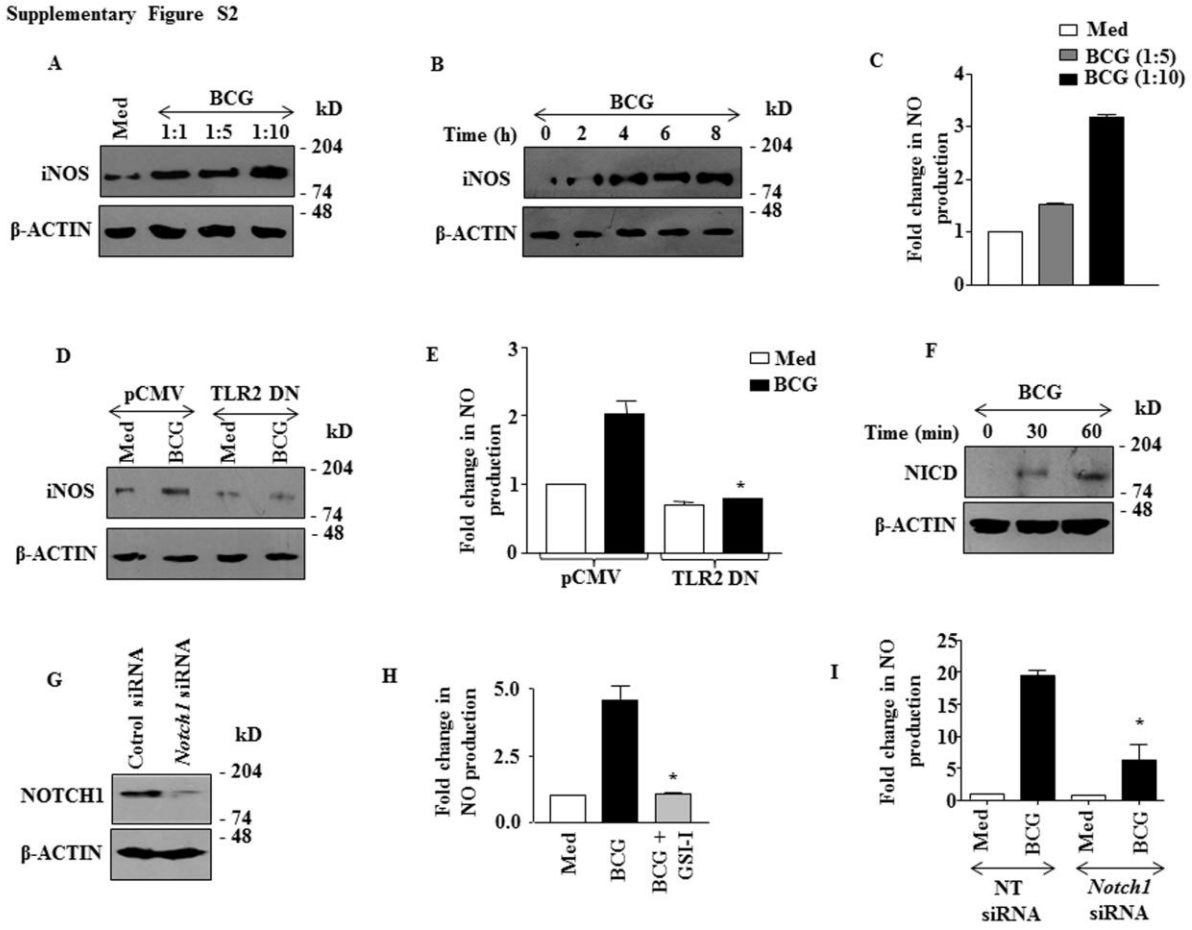
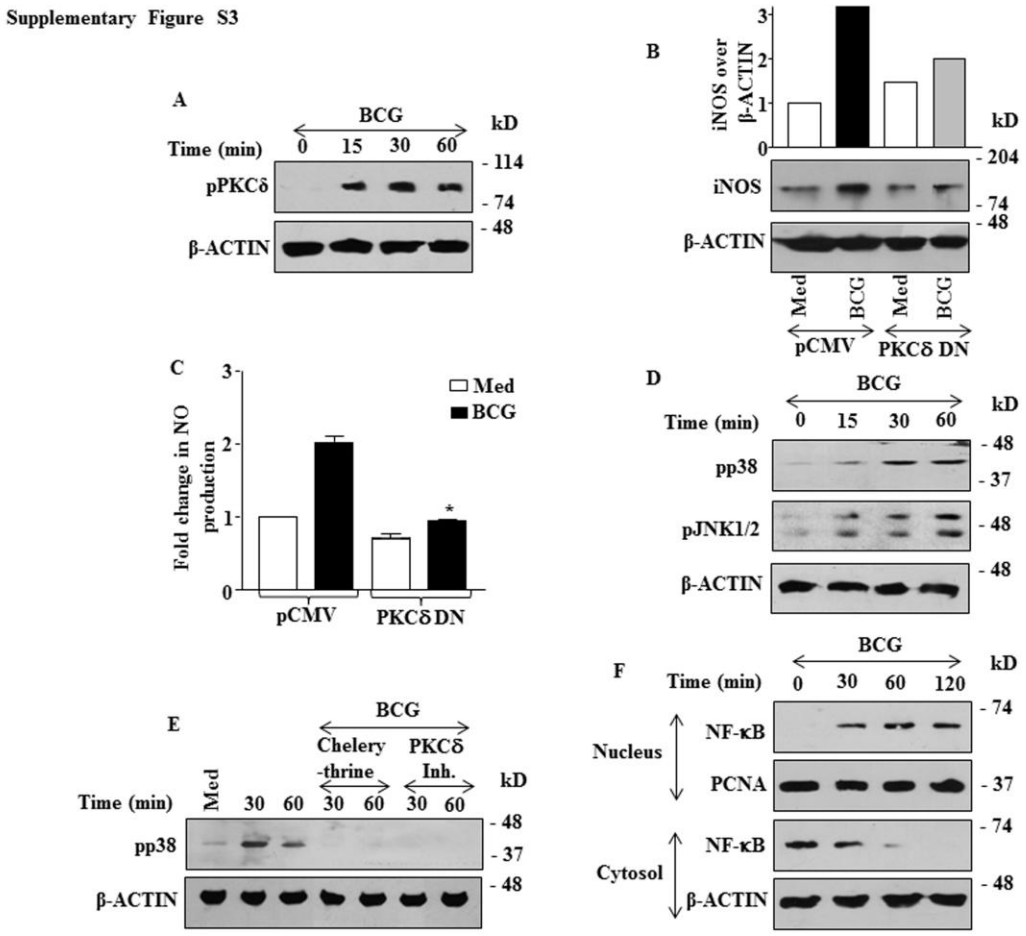
SUPPLEMENTAL DATA



SUPPLEMENTAL FIGURE 1. **IFN-γ induces MHC-II and CIITA expression.** *A*, Mouse peritoneal macrophages were treated with various concentrations of IFN-γ for 12 h and expression levels of MHC-II were assayed using immunoblotting. *B*, The kinetics of MHC-II expression in macrophages treated with 100 ng IFN-γ at indicated time points. *C*, Analysis of CIITA expression in mouse peritoneal macrophages treated with indicated concentrations of IFN-γ. All blots shown are representative of 3 independent experiments and respective quantifications of immunoblots are shown.

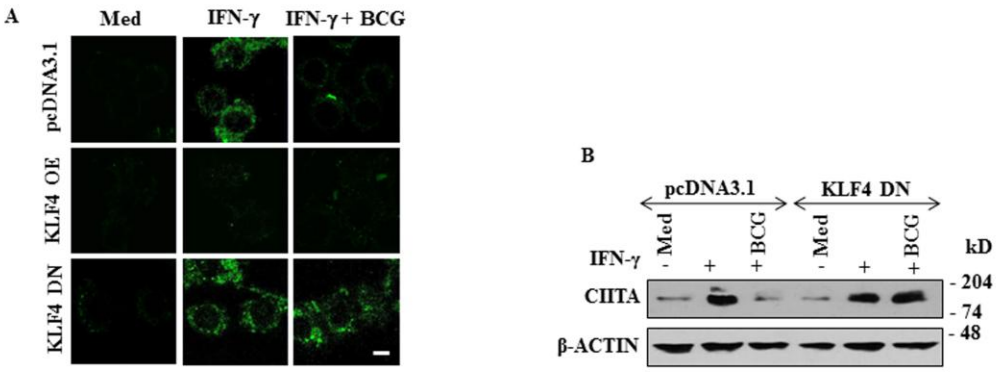


SUPPLEMENTAL FIGURE 2. ***M. bovis*** **BCG-induced iNOS/NO expression is dependent on NOTCH1 activation.** *A* and *B*, Expression levels of iNOS was analysed using immunoblotting in mouse peritoneal macrophages infected with *M. bovis* BCG, (*A*) at various MOIs or (*B*) for various time points. *C*, The cell free supernatants obtained from the above mentioned experiment setup was assayed for NO production using Griess reagent. *D* and *E*, RAW 264.7 macrophages were transfected with pCMV or TLR2 DN constructs for 48 h followed by *M. bovis* BCG infection for 6 h and 12 h, (*D*) iNOS expression and (*E*) NO production was analysed by immunoblotting and using Greiss reagent respectively. *F*, Mouse peritoneal macrophages were infected with *M. bovis* BCG at indicated time points to assess the kinetics of NICD generation by immunobloting. *G*, Validation of the activity of *Notch1* siRNA. *H* and *I*, Macrophages pretreated with GSI-I or transfected with *Notch1* siRNA were infected with *M. bovis* BCG for 12 h and NO production was estimated using Griess reagent using culture supernatants. All blots shown are representative of 3 independent experiments. NO data represent mean ± SE, n = 3. *Med*, Medium; DN, dominant negative; NT, Non-targeting. ***** $p<0.05$, as compared to *M. bovis* BCG infected vector/NT siRNA transfected cells (one-way ANOVA).



SUPPLEMENTAL FIGURE 3. ***M. bovis* BCG induced PKCδ, MAPK and NF-κB are the critical regulators of iNOS expression.** *A*, Peritoneal macrophages were infected with *M. bovis* BCG for indicated time points and PKCδ activation was analysed by immunoblotting. *B* and *C*, RAW 264.7 macrophages were transfected with pCMV or PKCδ DN for 48 h followed by infection with *M. bovis* BCG for 6 h and 12 h; (*B*) iNOS expression was assayed by immunoblotting and quantified and (*C*) NO production in culture supernatant was estimated by Greiss reagent respectively. *D*, The kinetics of p38 and JNK1/2 MAPK activation with *M. bovis* BCG infection of mouse peritoneal macrophages is shown. *E*, Macrophages were treated with Chelerythrine and PKCδ Inhibitor 1 h prior to *M. bovis* BCG infection and activation of p38 MAPK was assessed by immunoblotting. *F*, Cytosolic to nuclear localization of p65 NF-κB in peritoneal macrophages infected with *M. bovis* BCG at indicated time points as assessed by immunoblotting. All blots shown are representative of 3 independent experiments. NO data represent mean ± SE, n = 3. DMSO was used as vehicle control. Med, Medium; ***** $p<0.005$, as compared to *M. bovis* BCG infected pCMV transfected cells (one-way ANOVA).

Supplementary Figure S4



SUPPLEMENTAL FIGURE 4. **KLF4 inhibits IFN-γ induced MHC-II/CIITA expression.** *A* and *B*, Murine RAW 264.7 macrophages were transiently transfected with pcDNA3.1 or KLF4 OE or KLF4 DN as indicated for 48 h followed by infection with *M. bovis* BCG for 12 h and IFN-γ treatment for the next 48 h or 12 h, (*A*) representative immunofluorescence images (n = 3) of MHC-II surface expression and (*B*) immunoblotting for CIITA. All blots shown are representative of 3 independent experiments. Med, Medium; OE, Overexpression; DN, Dominant negative. Bar, 10 µm

SUPPLEMENTAL TABLE 1. **Primers used for quantitative real time RT-PCR and ChIP analysis.**

Sr. No.	Gene name	Sequence
Quantitative real time RT-PCR primers		
1	*Gapdh* forward	5'-gagccaaacgggtcatcatct-3'
	Gapdh reverse	5'-gaggggccatccacagtctt-3'
2	*Mhc-II* forward	5'-tgggcaccatcttcatcattc-3'
	Mhc-II reverse	5'-ggtcacccagcacaccactt-3'
3	*CIITA* forward	5'-gggactctggcaaatctcagg-3'
	CIITA reverse	5'-ctcatttacacgggaggtcagc-3'
Chromatin Immunoprecipitation primers		
1	28S rRNA forward	5'-ctgggtataggggcgaaagac-3'
	28S rRNA reverse	5'-ggccccaagacctctaatcat-3'
	For KLF4 binding at CIITA promoter	
2	KLF(-563) Forward	5'- gctgtcatgaggatgggact-3'
	KLF(-563) Reverse	5'- gcctttgtttccctgtgtgt-3'
3	KLF(-1251) Forward	5'- gccagatactgcttcctgatgtg-3'
	KLF(-1251) Reverse	5'- tcagggttgtggtggtaggca-3'
	For KLF4 binding at miR-150 promoter	
4	KLF(-430) Forward	5'-agacatttccaccgggagagg-3'
	KLF(-430) Reverse	5'-gcagagtctgtgagggaggg-3'

Nitric Oxide and KLF4 Protein Epigenetically Modify Class II Transactivator to Repress Major Histocompatibility Complex II Expression during *Mycobacterium bovis* Bacillus Calmette-Guérin Infection

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