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# Nitric Oxide Is Involved in *Mycobacterium bovis* Bacillus Calmette-Guérin–Activated Jagged1 and Notch1 Signaling

**Nisha Kapoor,* Yeddula Narayana,* Shripad A. Patil,† and Kithiganahalli N. Balaji***

**Pathogenic mycobacteria have evolved unique strategies to survive within the hostile environment of macrophages. Modulation of key signaling cascades by NO, generated by the host during infection, assumes critical importance in overall cell-fate decisions. We show that NO is a critical factor in *Mycobacterium bovis* bacillus Calmette-Guérin–mediated Notch1 activation, as the generation of activated Notch1 or expression of Notch1 target genes matrix metalloproteinase-9 (MMP-9) or Hes1 was abrogated in macrophages derived from inducible NO synthase (iNOS) knockout ($iNOS^{-/-}$), but not from wild-type, mice. Interestingly, expression of the Notch1 ligand Jagged1 was compromised in *M. bovis* bacillus Calmette-Guérin–stimulated $iNOS^{-/-}$ macrophages, and loss of Jagged1 expression or Notch1 signaling could be rescued by NO donors. Signaling perturbations or genetic approaches implicated that robust expression of MMP-9 or Hes1 required synergy and cross talk between TLR2 and canonical Notch1-PI3K cascade. Further, CSL/RBP-Jk contributed to TLR2-mediated expression of MMP-9 or Hes1. Correlative evidence shows that, in a murine model for CNS tuberculosis, this mechanism operates in vivo only in brains derived from WT but not from $iNOS^{-/-}$ mice. Importantly, we demonstrate the activation of Notch1 signaling in vivo in granulomatous lesions in the brains of *Mycobacterium tuberculosis*-infected human patients with tuberculous meningitis. Current investigation identifies NO as a pathological link that modulates direct cooperation of TLR2 with Notch1-PI3K signaling or Jagged1 to regulate specific components of TLR2 responses. These findings provide new insights into mechanisms by which Notch1, TLR2, and NO signals are integrated in a cross talk that modulates a defined set of effector functions in macrophages.** ***The Journal of Immunology*****, 2010, 184: 3117–3126.**

Mycobacteria species including *Mycobacterium tuberculosis*, *M. bovis*, etc., have evolved diverse sets of sophisticated survival mechanisms to interfere with the ensuing immune response (1). Macrophages are vital components of both innate as well as adaptive immunity, and among macrophages, classically activated macrophages play crucial roles in resisting a range of infections by producing key effector molecules like NO and are known to participate in host control of the spread of various intracellular pathogens including *Mycobacterium* sp. (2, 3).

NO is a product of arginine metabolism and produced by different isoforms of NO synthases. Among three isoforms, inducible NO synthase (iNOS) is expressed in a variety of immune cells, including macrophages, in response to a diverse set of proinflammatory stimuli (4). NO resulting from iNOS enzymatic activity is suggested to integrate fundamental cell fate decisions during immune responses. It is well known that NO or its derivatives like peroxynitrite ($ONOO^-$) modify diverse cellular proteins by processes of nitrosylation, nitrosation, nitration, or oxidation (5). NO mediated *S*-nitrosylation of cysteine residues of target proteins including various signaling molecules or the nitration of key tyrosine residues to nitrotyrosine is implicated as one of the novel strategies to modulate a variety of signaling events during initiation or activation of inflammatory responses (6, 7).

Among various signaling events, Notch signaling pathway is suggested to execute an important function during the onset of inflammatory immune responses (8, 9). Notch signaling is suggested to play an important role in the development of the immune system and seems to act in a significant manner in critical cell-fate decisions among diverse immune cells including macrophages or dendritic cells (9, 10). The binding of Jagged or Delta, specific ligands of Notch receptor, results in the release of Notch intracellular domain (NICD/cleaved Notch) from the membrane by proteolytic cleavage executed by γ-secretase complex. The NICD then translocates to the nucleus and forms a complex with DNA-binding protein CSL/RBP-Jk. In general, CSL/RBP-Jk, along with many corepressors, binds to promoters of Notch target genes, resulting in the inhibition of transcription. Upon nuclear translocation, NICD binds to the CSL/RBP-Jk corepressor complex, resulting in the displacement of the corepressors and recruitment of various coactivators, including Mastermind, p300, and/or CBP, leading to CSL/RBP-Jk–dependent transcription of target genes (11, 12). However, CSL/RBP-Jk–independent activation of its target genes by Notch or activation of CSL/RBP-Jk by signaling cascades other than Notch signaling have been documented (8).

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Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; ChIP, chromatin immunoprecipitation; COX-2, cyclooxygenase-2; DN, dominant-negative; 4EBP1, eukaryotic initiation factor 4E-binding protein 1; ECM, extracellular matrix; GSI-I, γ-secretase inhibitor I; HC, healthy control; IHC, immunohistochemistry; iNOS, inducible NO synthase; Med, medium; MMP-9, matrix metalloproteinase-9; NICD, Notch intracellular domain; PEI, polyethylenimine; SIN1, 3-morpholinosydnonimine; siRNA, small interfering RNA; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SOCS3, suppression of cytokine signaling 3; TBM, tuberculous meningitis; WT, wild-type.

Notch signaling is known to play key roles in the regulation of various developmental stages of T and B cells, T cell activation, or differentiation of Th cells (10). Further, TLR stimulation or various proinflammatory stimuli induce the expression of Notch receptor and ligands on macrophages and dendritic cells (8, 12). In this context, TLR-mediated induction of Notch receptor and ligand expression on myeloid cells has resulted in activation of a noncanonical Notch signaling cascade, leading to NF-κB activation and TNF-α production (8). However, precise molecular details on the TLR-mediated Notch activation remain unexplored.

Further, information on the effects of Notch signaling in myeloid lineage cells still remains limited. In this perspective, matrix metalloproteinase-9 (MMP-9), an important member of $Zn^{2+}$- and $Ca^{2+}$-dependent endopeptidases, participates in a significant manner in several aspects of host immune responses to mycobacterial infection, such as graunloma formation, extracellular matrix (ECM) reorganization, lymphocyte trafficking, infiltrations, and inflammation, etc. MMP-9 is expressed at various clinical categories of tuberculosis disease like active cavitary tuberculosis, meningitis, and pleuritis (13–15). Notably, in the case of pulmonary tuberculosis, breakdown of ECM by MMP-9 forms an integral part of the granuloma formation (16).

The current study demonstrates for the first time that NO, a product of iNOS activity, is responsible for *M. bovis* bacillus Calmette-Guérin (BCG)-triggered activation of Notch1 in macrophages through direct regulation of Jagged1 expression as well as in generation of activated Notch1. We present the evidence that iNOS activity is a critical factor in TLR2-mediated Notch1 activation as macrophages derived from iNOS knockout ($iNOS^{-/-}$) but not from wild-type (WT) mice failed to activate Jagged1 expression as well as Notch1 signaling upon *M. bovis* BCG infection. The loss of TLR2-mediated Jagged1 expression or Notch1 activation in $iNOS^{-/-}$ macrophages could be rescued by treatment with NO donor 3-morpholinosydnonimine (SIN1) or *S*-nitroso-*N*-acetylpenicillamine (SNAP). Signaling perturbations strongly implicated the role for cross talk among members of Notch1-PI3K and MAPK cascades in *M. bovis* BCG-TLR2–mediated activation of Notch1 target genes MMP-9 or Hes1. Chromatin immunoprecipitation (ChIP) experiments demonstrate that *M. bovis* BCG's ability to trigger increased binding of CSL/RBP-Jk to MMP-9 promoter was severely compromised in macrophages derived from $iNOS^{-/-}$ mice compared to WT mice. These results are consistent with the observation that NO-triggered Notch1 signaling-mediated CSL/RBP-Jk recruitment has a positive regulatory role in *M. bovis* BCG-induced MMP-9 transcription. We show correlative evidence that this mechanism operates in vivo by immunohistochemical expression analysis of activated Notch1 or its target gene products Hes1 or MMP-9 in brains of WT or $iNOS^{-/-}$ mice that were intracerebrally infected with *M. bovis* BCG. Further, activation of Notch1 signaling in vivo could be demonstrated only in granulomatous lesions in brains derived from human patients with tuberculous meningitis (TBM) as opposed to healthy individuals validating the role of Notch1 signaling in *Mycobacterium* pathogenesis. Briefly, the current investigation identifies NO as the pathological link between TLR2 and Notch1 signaling, which regulates the relative abundance of various immunopathological parameters including MMP-9 in macrophages.

## Materials and Methods

### *Cells and bacteria*

Peritoneal macrophages were isolated from peritoneal exudates of C57BL/6 or $iNOS^{-/-}$ C57BL/6 mice that were maintained at the Central Animal Facility, Indian Institute of Science, Bangalore, India. RAW 264.7 and HEK 293 cell lines (obtained from National Centre for Cell Science, Pune, India) were cultivated in DMEM supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO). The experiments with mouse macrophages were carried out after approval was obtained from the Institutional Ethics Committee for Animal Experimentation as well as from the Institutional Bioethics Committee, Indian Institute of Science. *M. bovis* BCG Pasteur 1173P2 was grown to midlog phase in Souton's medium, and aliquots were stored as described previously (17, 18). *M. bovis* BCG was used at 10 multiplicity of infection for infection in all of the experiments.

### *Chemicals and reagents*

General laboratory reagents were purchased from Sigma-Aldrich or Merck (Darmstadt, Germany). NO donors, SIN1, and SNAP were obtained from Calbiochem (San Diego, CA).

Anti-Ser473 Akt, anti-Akt, anti-Thr70 eukaryotic initiation factor 4E-binding protein 1 (4EBP1), anti-4EBP1, anti-Thr202/Tyr204 pERK1/2, anti-ERK1/2, Thr180/Tyr182 pp38 MAPK, anti-p38 MAPK, anti-Ser536 NF-κB, anti–NF-κB, anti-Jagged1, and anti-cleaved Notch1 Abs were purchased from Cell Signaling Technology (Beverly, MA). Anti-Hes1 and anti–RBP-Jk Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HRP conjugated anti-mouse IgG Abs were obtained from Calbiochem. Anti–β-actin (AC-15) and anti MMP-9 Abs were obtained from Sigma-Aldrich. Anti-CD16/32 (Fc blocker) Abs were purchased from eBioscience, San Diego, CA. Cy2 or Cy5 or HRP conjugated anti-rat and anti-rabbit IgG Abs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

### *In vivo infection of mice with* M. bovis *BCG*

The experiments involving infection of mice were performed after the approval from the Institutional Ethics Committee for Animal Experimentation as well as from the Institutional Biosafety Committee. C57BL/6 mice and $iNOS^{-/-}$ C57BL/6 mice used in the current investigation were 5 to 6 wk old. For intracranial infection, $1 \times 10^6$ *M. bovis* BCG Pasteur 1173P2 bacteria were washed in PBS, resuspended in 50 μl sterile PBS, and then inoculated intracranially using 1-ml syringes and a 26-gauge needle. One set of $iNOS^{-/-}$ mice received 20 μg SIN1/mice in addition to infection with *M. bovis* BCG. Control mice were injected with 50 μl sterile PBS using the same protocol. Before intracranial inoculation, mice were anesthetized with an i.p. injection of ketamine (6 mg) in 80 μl. In experiments involving TLR2 Ab, WT mice were injected with anti-TLR2 or control IgG Ab (200 μg/kg) 24 h prior to infection with *M. bovis* BCG (19). For in vivo silencing of MyD88, WT mice received 0.6 nmol MyD88 or control small interfering RNA (siRNA) complexed with low m.w. polyethylenimine (PEI) 24 h before intracranial inoculation with *M. bovis* BCG (20–22). After 5 d of inoculations, brains were harvested from experimental mice. Microtome sections (4 μm) were cut from formalin-fixed, decalcified, and paraffin-embedded tissue samples. These paraffin-embedded sections were first deparaffinized followed by Ag retrieval with boiling 10 mM citrate buffer (pH 6.0) in a microwave for 10 min, treated with 1% $H_2O_2$ 10 min, incubated with 0.1 M glycine for 15 min, and blocked with 5% BSA for 1h at room temperature. Primary Abs were incubated overnight, and HRP conjugated secondary Abs for 90 min. The HRP reaction was detected with 0.05% diaminobenzidine and 0.03% $H_2O_2$. Sections were counterstained with hematoxylin, dehydrated, and mounted. Stained tissue sections were analyzed with a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany). All experiments were performed with appropriate isotype-matched control Abs.

### *Human patients with TBM*

Human brain tissue samples were collected from patients with TBM at the National Institute of Mental Health and Neurosciences, Bangalore, India, according to the guidelines of the Institutional Ethics Committee. Each patient provided informed written consent to participation in this study in accordance with institutional and regulatory guidelines. Brain tissue samples from patients that had died due to accident served as controls. Microtome sections (4 μm) of brain tissue samples were stained with indicated Abs for immunohistochemistry (IHC) as described above.

### *Treatment with pharmacological inhibitors*

The pharmacological inhibitors were obtained from Calbiochem and were reconstituted in sterile DMSO and used at following concentrations: 1400W (100 μM), LY294002 (50 μM), wortmannin (100 nM), rapamycin (100 nM), Akt inhibitor II (Akt I-II, 10 μM), γ-secretase inhibitor I (GSI-I) (10 μM), SB203580 (20 μM), U0126 (10 μM), SP600125 (50 μM), manumycin (20 μM), and Bay 11-7082 (20 μM). DMSO at 0.1% concentration was used as the vehicle control. In all experiments with inhibitors, a tested concentration was used after careful titration experiments assessing the viability of the macrophages.

### *Stable transfection of NICD*

RAW 264.7 macrophages stably expressing Notch1 intracellular domain (RAW-NICD) were generated as described previously (17, 18).

### *siRNA transfections*

RAW 264.7 cells were transfected with 100 nM siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Seventy-two hours posttransfection, the cells were infected with 10 multiplicity of infection *M. bovis* BCG for 12 h. Later, protein lysates were prepared and analyzed for the targeted proteins by Western blotting. siRNAs specific to Notch1, Akt, MyD88, CSL/RBP-Jk, control siRNA, and siGLO Lamin A/C were obtained from Dharmacon (Chicago, IL) as siGENOME SMARTpool reagents, which contains a pool of four different dsRNA oligonucleotides (siRNA).

### *Transient transfections and luciferase assays*

RAW 264.7 macrophages were transiently transfected with MMP-9 or Hes1 promoter constructs or TLR2-dominant negative cDNA construct using PEI (Sigma-Aldrich), and assays were carried out as described previously (18).

### *RNA isolation and quantitative real-time PCR*

Macrophages were treated as indicated, and the transcript levels of the target molecules were assessed by quantitative real-time PCR analysis as described previously (17). The forward and reverse primer pairs used were as follows: GAPDH forward 5′-gagccaaacgggtcatcatct-3′, reverse 5′-gaggggccatccacagtctt-3′; MMP-9 forward 5′-gagctgtgcgtcttccccttc-3′, reverse 5′-ggaatgatctaagcccagtgc-3′; Notch1 forward 5′-agaatggcatggtgcccag-3′, reverse 5′-tggtggagaggctgctgtgtag-3′; Jagged1 forward 5′-gaagtcagagttcagaggcgtcc-3′, reverse 5′-agtagaaggctgtcaccaagcaac-3′; and Hes1 forward 5′-gagaggctgccaaggtttttg-3′, and reverse 5′-cactggaaggtgacactgcg-3′. All the primers were purchased from Sigma Genosys (Bangalore, India).

### *Immunoblotting*

Cell lysates were prepared after washing briefly with ice-cold PBS in 1× radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml each aprotinin, leupeptin, and pepstatin, 1 mM $Na_3VO_4$, and 1 mM NaF). An equal amount of protein was subjected to SDS-PAGE, and then proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), which were blocked in TBST buffer (0.02 M Tris-Hcl [pH 7.5], 0.15 M NaCl, and 0.1% Tween 20) containing 5% nonfat dried milk and probed with a primary Ab for overnight at 4°C. After washing with TBST, membranes were incubated with secondary Ab conjugated to HRP (Jackson ImmunoResearch Laboratories). The blots were then visualized with an ECL detection system (PerkinElmer, Wellesley, MA) as per the manufacturer's instructions.

### *Immunofluorescence*

Resident mouse peritoneal macrophages were seeded on coverslips and treated as indicated. The cells were fixed with 3% formaldehyde for 10 min at room temperature. Primary Abs were incubated for 1 h at room temperature following three washes with PBS. Secondary Abs (Cy2-conjugated anti-rabbit IgG or Cy5-conjugated anti-rat IgG) were incubated in the dark for 1 h at room temperature and then washed three times with PBS. Coverslips with cells were mounted on a slide with fluoromount G. Confocal images were taken on Zeiss LSM 510 Meta confocal laser scanning microscope using plan Apochromat 63×/1.4 oil DIC objective (Zeiss, Oberkochen, Germany) and Argon/2 458, 477, 488, and 514 nm and HeNe 543 lasers. During colocalization studies, a series of images at an interval of 0.37-mm focal planes was collected into a z-stack to ascertain the actual colocalization. Every single layer of z-stack was subjected to image analysis by LSM 5 image examiner software to visualize and locate protein expression.

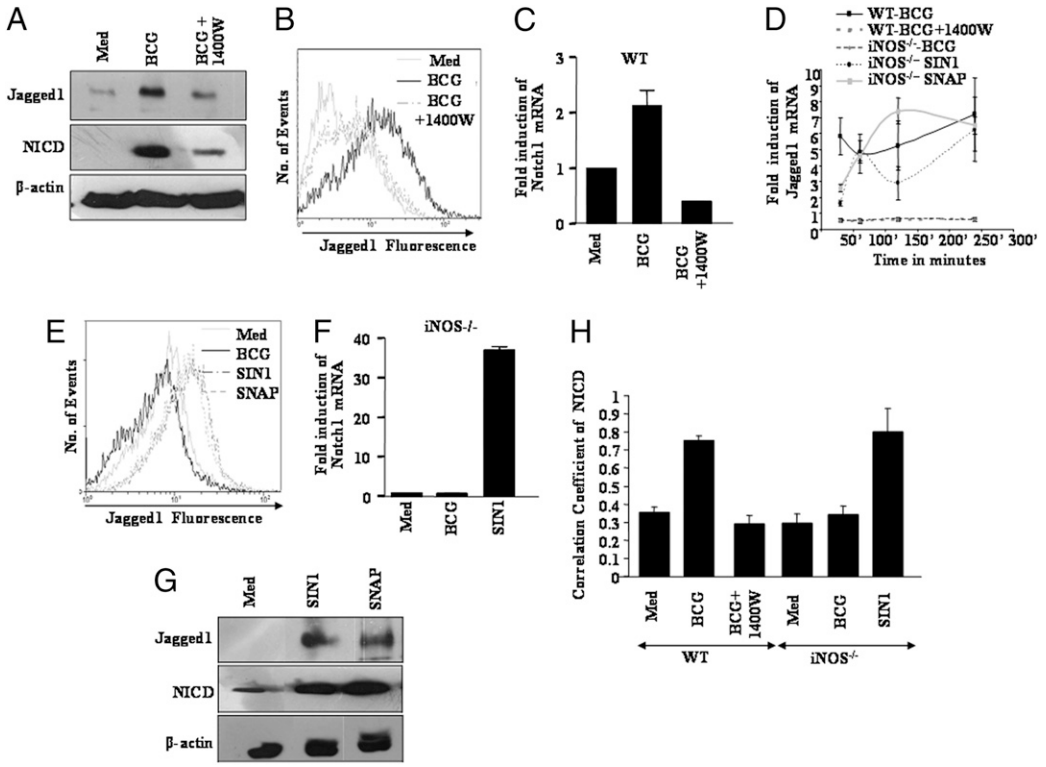


**FIGURE 1.** Involvement of iNOS in *M. bovis* BCG-triggered activation of Notch1 signaling and Jagged1 expression. Macrophages were treated with 1400W prior to infection with *M. bovis* BCG followed by analysis of protein levels of NICD and Jagged1 by immunoblotting (*A*), cell surface expression of Jagged1 by flow cytometry (*B*), and transcript levels of Notch1 by quantitative real-time PCR (*C*). *D*, Macrophages derived from WT mice were pretreated with 1400W or vehicle control followed by infection with *M. bovis* BCG. In case of macrophages derived from iNOS$^{-/-}$ mice, cells were either infected with *M. bovis* BCG or treated with SIN1 or SNAP for different time points and transcript levels of Jagged1 were analyzed by quantitative real-time PCR. iNOS$^{-/-}$ macrophages were either infected with *M. bovis* BCG or treated with SIN1 or SNAP and analyzed for cell surface expression of Jagged1 by flow cytometry (*E*) and transcript levels of Notch1 by quantitative real-time PCR (*F*). *G*, Macrophages derived from iNOS$^{-/-}$ mice were treated with SIN1 and SNAP, and protein level expression of Jagged1 or NICD was evaluated by immunoblotting. *H*, WT and iNOS$^{-/-}$ macrophages were treated as indicated and nuclear translocation of NICD was analyzed by confocal microscopy. The data presented in the figure is representative of three independent experiments. Med, medium.

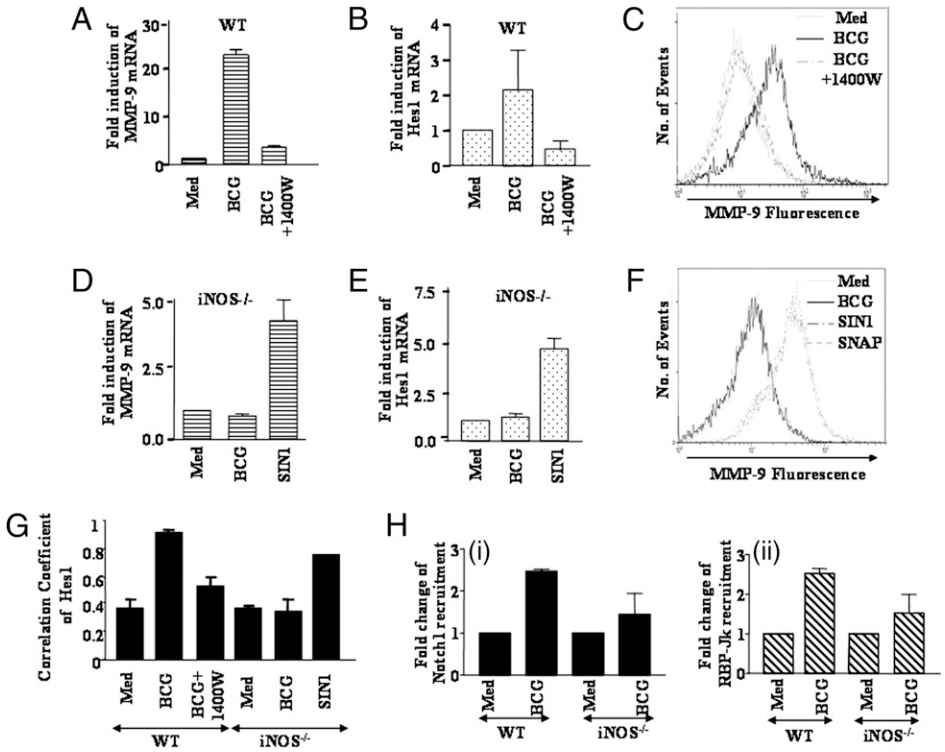


**FIGURE 2.** Essential role of NO in *M. bovis* BCG-induced expression of Notch1 target genes MMP-9 and Hes1. Macrophages derived from WT mice were pretreated with 1400W followed by infection with *M. bovis* BCG, and transcript levels of MMP-9 (*A*) and Hes1 (*B*) were analyzed by quantitative real-time PCR, whereas cell surface expression of MMP-9 was analyzed by flow cytometry (*C*). Macrophages from iNOS$^{-/-}$ mice were either infected with *M. bovis* BCG or treated with SIN1 or SNAP followed by analysis of transcript level expression of MMP-9 (*D*), Hes1 (*E*), and cell surface expression of MMP-9 (*F*). *G*, Nuclear Hes1 expression in WT or iNOS$^{-/-}$ macrophages treated as indicated as analyzed by confocal microscopy. *H*, Recruitment of Notch1 (*i*) and CSL/RBP-Jk (*ii*) at MMP-9 promoter was analyzed by ChIP assay with Abs to Notch1 or RBP-Jk, respectively, in *M. bovis* BCG-infected WT or iNOS$^{-/-}$ macrophages. The recruitment of Notch1 and RBP-Jk at MMP-9 promoter was assessed by quantitative real-time PCR. Data are representative of three independent experiments. Med, medium.

### *Flow cytometry analysis*

The cells were washed with PBS and fixed in 0.1% formaldehyde. To avoid nonspecific binding, cells were incubated with 0.5 μg Fc blocker (per $10^6$ cells) for 30 min on ice followed by staining with rabbit anti-mouse MMP-9/rabbit anti-mouse Jagged1 Ab. This was followed by staining with FITC or Cy2-conjugated anti-rabbit IgG. Cells were analyzed using FACScan (BD Biosciences, San Jose, CA). Dead cells were excluded from the analysis by their forward and sideways light-scattering properties.

### *ChIP assay*

ChIP assays were carried out following a protocol provided by Upstate Biotechnology (Lake Placid, NY), with modifications. Peritoneal macrophages were left uninfected or infected with BCG for 12 h. The cells were fixed with 1.42% formaldehyde for 15 min at room temperature followed by inactivation of formaldehyde with addition of 125 mM glycine. Chromatin extracts containing DNA fragments with an average size of 500 bp were immunoprecipitated using anti-Notch1 or RBP-Jk Ab. Purified DNA was analyzed by quantitative real-time PCR by using the SYBR Green method (Finnzymes, Espoo, Finland). Regions with the RBP-Jk binding site in the mouse MMP-9 promoter were amplified using primer pairs RBP-Jk forward, 5′-atcagtcagggccgtcagac-3′, and RBP-Jk reverse, 5′-gacccacaggaaaccacagaac-3′. 28S rRNA was used as control in the PCR, and the primers were forward, 5′-ctgggtataggggcgaaagac-3′, and reverse, 5′-ggccccaagacctctaatcat-3′. All results were normalized either by respective input values or by amplification of 28S rRNA. All ChIP experiments were repeated at least three times.

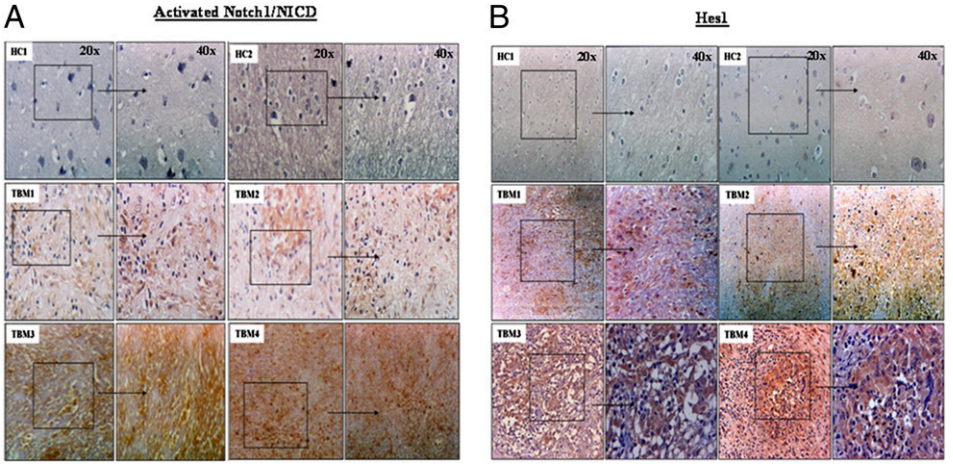


**FIGURE 3.** Activation of Notch1 signaling *in vivo.* Serial sections of human brain tissue samples from patients with TBM or healthy subjects were stained for expression levels of activated Notch1/NICD (*A*) as well as Notch1 target gene Hes1 (*B*) by IHC. Hematoxylin (blue) was used for nuclear staining. HC, healthy control.

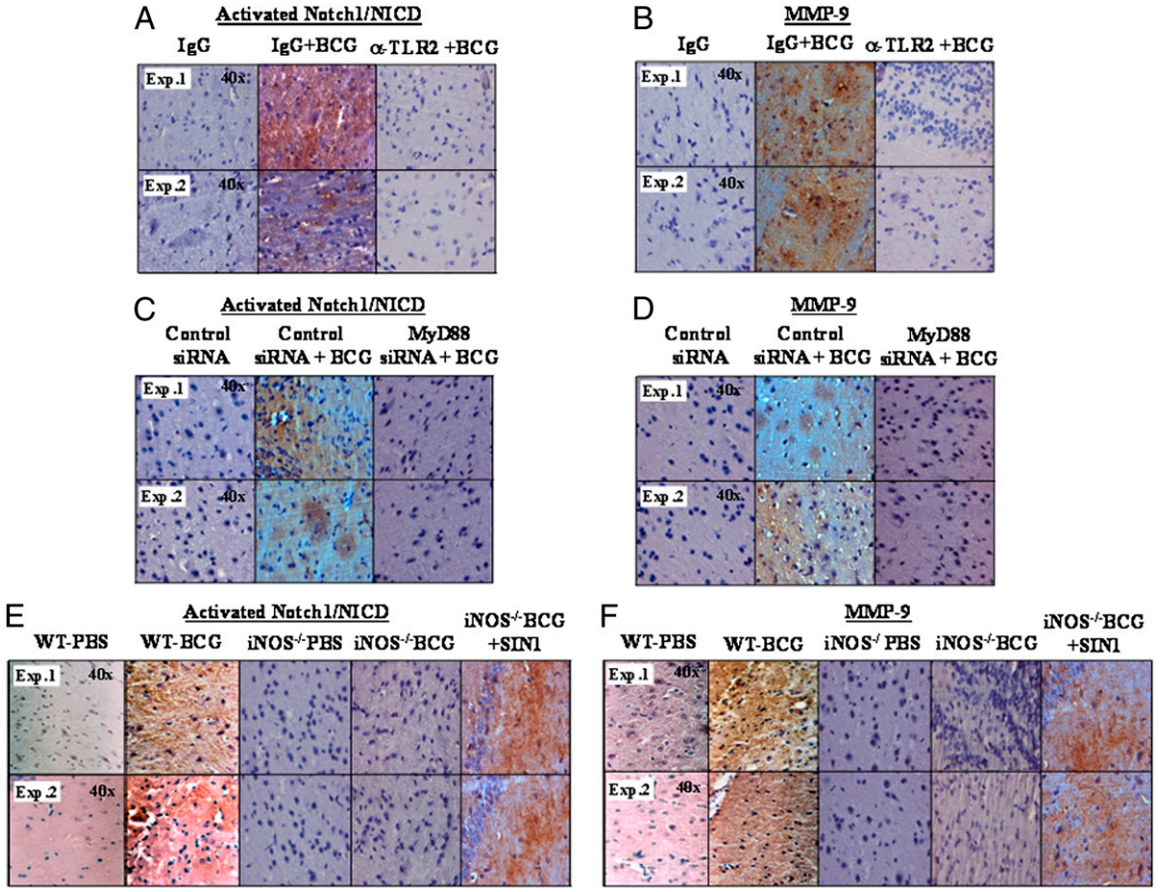


**FIGURE 4.** TLR2-MyD88 axis and NO are essential for activation of Notch1 signaling in vivo. WT mice were injected with anti-TLR2 or control IgG Ab 24 h before intracranial inoculation with *M. bovis* BCG and expression of NICD (*A*) and MMP-9 (*B*) was analyzed by IHC. Expression analysis of NICD (*C*) and MMP-9 (*D*) in brain sections of WT mice injected with MyD88 or control siRNA complexed with PEI 24h prior to intracranial inoculation with *M. bovis* BCG. Brain sections from WT mice infected with *M. bovis* BCG or iNOS$^{-/-}$ mice inoculated with SIN1 in addition to infection with *M. bovis* BCG intracranially were assessed for expression of NICD (*E*) and MMP-9 (*F*) by IHC. Hematoxylin (blue) was used for nuclear staining.

*Statistical analysis*

Levels of significance for comparison between samples were determined by the Student *t* test distribution. The data in the graphs is expressed as the mean ± SEM. Graphpad Prism 3.0 software (GraphPad, San Diego, CA) was used for all the statistical analysis.

## Results

*NO is required for* M. bovis *BCG-induced Notch1 signaling and Jagged1 expression*

*M. bovis* BCG challenge of macrophages could provoke a spectrum of cellular activities including TLR2-MyD88–dependent expression of key Notch1 target genes like MMP-9 (Supplemental Fig. 1*A–D*), an effector molecule that participates in macrophage cell motility during inflammatory responses (16). We show that TLR2 stimulation by *M. bovis* BCG leads to upregulation of Notch1 and activation of the Notch1 signaling pathway by inducing the formation of a cleavage product of Notch1 (NICD) (Fig. 1*A*, Supplemental Fig. 1*E*, 1*F*). Further, *M. bovis* BCG triggers robust activation of Jagged1 expression, a Notch1 receptor ligand (Fig. 1*A*, 1*B*, Supplemental Fig. 1*F*, 1*G*). In this perspective, we addressed whether *M. bovis* BCG-TLR2–mediated expression of iNOS participates in activation of Notch1 signaling. Interestingly, the ability of *M. bovis* BCG to induce the formation of NICD, upregulation of Notch1, or expression of the Notch1 receptor ligand Jagged1 was abrogated in the presence of 1400W, an iNOS-selective inhibitor (Fig. 1*A–C*). To substantiate the importance of iNOS in induction of NICD or Jagged1 expression, macrophages derived from WT and iNOS$^{-/-}$ mice challenged with or without *M. bovis* BCG were analyzed. As shown in Fig. 1*D*, 1*F*, compared with WT macrophages, marked inhibition of Jagged1 or Notch1 expression both at RNA and protein levels evoked by *M. bovis* BCG challenge was observed in iNOS null macrophages. This iNOS deficiency caused defect in the ability of *M. bovis* BCG to trigger Jagged1 or Notch1 expression was not due to the general inability of cells to mobilize, because analogs of iNOS downstream mediators, SNAP, or SIN1 (NO donor) could augment Jagged1, NICD, or Notch1 expression in iNOS-deficient macrophages comparable to that in WT macrophages (Fig. 1*D–G*). Confocal microscopy studies further validated the requirement of NO in *M. bovis* BCG-triggered Notch1 signaling activation as inhibition of iNOS activity or deficiency in iNOS expression resulted in marked reduction in nuclear translocation of NICD in macrophages (Fig. 1*H*). However, treatment of iNOS$^{-/-}$ macrophages with NO donor SIN1 could reverse the inhibition in *M. bovis* BCG-triggered nuclear translocation of NICD (Fig. 1*H*). Accordingly, expression of Notch1 target genes MMP-9 or Hes1 was regulated by NO, as inhibition of iNOS activity by iNOS-selective inhibitor 1400W abrogated the *M. bovis* BCG-induced MMP-9 or Hes1 expression (Fig. 2*A–C*). Further, *M. bovis* BCG failed to induce MMP-9 or Hes1 expression in iNOS null macrophages, which could be rescued by treatment with NO donors, SNAP, or SIN1 (Fig. 2*D–F*). Similar to above

results on NICD nuclear translocation, data derived from confocal microscopy studies suggested that the inhibition of iNOS activity or deficiency in iNOS expression abrogated the ability of *M. bovis* BCG to induce nuclear expression of Hes1 (Fig. 2*G*). However, SIN1 treatment could augment nuclear expression of Hes1 in iNOS null macrophages in contrast to significant reduction triggered by *M. bovis* BCG challenge (Fig. 2*G*).

To further validate, by sequence analysis, we identified three CSL/RBP-Jk–binding consensus in the mouse MMP-9 promoter (23) and showed that the recruitment of CSL/RBP-Jk or NICD was severely compromised in macrophages derived from iNOS$^{-/-}$ mice compared with WT mice by ChIP experiments (Fig. 2*H*). However, total expression levels of CSL/RBP-Jk were unaltered in iNOS$^{-}$/$^{-}$ or WT mice (data not shown). These results are consistent with the observation that NO-triggered Notch1 signaling-mediated CSL/RBP-Jk recruitment has a positive regulatory role in *M. bovis* BCG-induced MMP-9 transcription. These findings suggest the critical participation of the NO pathway in *M. bovis* BCG-induced Jagged1 expression and activation of Notch1 signaling in macrophages.

### *High expression levels of activated Notch1 or its target genes Hes1 or MMP-9 in brains of human TBM patients*

We investigated whether this mechanism for Notch1 activation was relevant to the biology of *Mycobacterium* infection in vivo. Human brain tissue samples of patients with TBM or from healthy subjects were assessed for the expression of activated Notch1 (NICD) or its target genes Hes1 or MMP-9. By immunohistochemical expression analysis, we found that NICD protein levels were significantly increased in the brain of most patients with TBM compared with healthy subjects brains (Fig. 3*A*). Similarly, Hes1 or MMP-9 expression levels were significantly enhanced in brain samples of patients with TBM compared with healthy subjects (Fig. 3*B*, Supplemental Fig. 2).

### *TLR2-MyD88-iNOS/NO axis is a critical link in* M. bovis *BCG-triggered activation of Notch1 signaling in vivo*

To ascertain the role of TLR2-MyD88 and NO signaling in activation of Notch1 signaling in vivo, a suggested murine model for the study of CNS tuberculosis or TBM involving intracranial inoculation of *M. bovis* was used (24). Blocking TLR2 signaling by neutralizing Ab to TLR2 in vivo clearly abrogated *M. bovis* BCG-triggered Jagged1 expression, Notch1 activation, and expression of Notch1 signaling target genes, Hes1 and MMP-9 (Fig. 4*A*, 4*B*, Supplemental Fig. 3).

Further, siRNA-mediated knockdown of MyD88 in vivo abolished *M. bovis* BCG-induced Jagged1 expression, Notch1 activation, and Notch1 target genes Hes1 and MMP-9 expression (Fig. 4*C*, 4*D*, Supplemental Fig. 4), potentiating a critical role of TLR2-MyD88 axis in activation of Notch1 signaling in vivo. Further, in accordance with results obtained with macrophages, IHC data of brain sections demonstrates that iNOS deficiency in iNOS$^{-/-}$ mice severely compromised *M. bovis* BCG potential to trigger augmented expression of NICD and Jagged1 compared with WT mice (Fig. 4*E*, Supplemental Fig. 5). Similarly, expression levels of Nocth1 target genes Hes1 or MMP-9 were markedly reduced in brain sections derived from infected iNOS$^{-/-}$ mice compared with WT mice (Fig. 4*F*, Supplemental Fig. 5). In addition, NO donor (SIN1) treatment of iNOS$^{-/-}$ mice in vivo rescued activation of Notch1 and expression of Jagged1 as well as Notch1 target genes, Hes1 and MMP-9 (Fig. 4*E*, 4*F*, Supplemental Fig. 5). These results imply that NO acts as an essential mediator of *M. bovis* BCG-dependent Notch1 signaling activation and is responsible for expression of specific transcriptional target proinfammatory gene signatures (such as MMP-9) in macrophages.

### *NO-triggered Jagged1 expression and Notch1 signaling requires $Ca^{2+}$*

NO can directly influence diverse signaling cascades through nitrosylation (6) and can exert its capacity to act as a $Ca^{2+}$-mobilizing intracellular messenger (7). This critical interplay between NO and $Ca^{2+}$ might act as key modulator of signaling related processes thus executing important roles in cell-fate decisions. In addition to a number of $Ca^{2+}$-permeable channels, NO modulates upstream components such as cGMP, p21ras, or protein phosphatases (25, 26). In this perspective, blockade of $Ca^{2+}$ mobilization by BAPTA-AM significantly inhibited SNAP/SIN1 (NO donor) or *M. bovis* BCG-triggered activation of Jagged1 expression or Notch1 signaling in iNOS null macrophages and WT macrophages, respectively. (Fig. 5*A*, 5*C*). Accordingly, a Notch1 target gene, MMP-9, expression by SNAP or SIN1 in iNOS null macrophages could be blocked by $Ca^{2+}$ chelator (Fig. 5*B*). Similarly, Hes1 promoter activity triggered by *M. bovis* BCG-mediated TLR2 stimulation could be blocked by intracellular $Ca^{2+}$ chelator BAPTA/AM (Fig. 5*D*). Confocal microscopy analysis further validated the requirement of $Ca^{2+}$ as blocking effects of BAPTA-AM on intracellular $Ca^{2+}$ wave resulted in the inhibition in *M. bovis* BCG-triggered nuclear translocation of NICD (Supplemental Fig. 6*A*) as well as expression of Hes1 (Supplemental Fig. 6*B*). As described subsequently, blockade of $Ca^{2+}$ mobilization by BAPTA-AM abrogated the Notch1-mediated Akt, 4EBP1, or ERK1/2 activation as well as NF-κB nuclear translocation, which ascertains the critical role of NO-$Ca^{2+}$ axis in overall *M. bovis* BCG-TLR2 signaling (Supplemental Fig. 6*C*, 6*D*). However, addition of cGMP could not rescue Jagged1, NICD, or Notch1, MMP-9 expression in

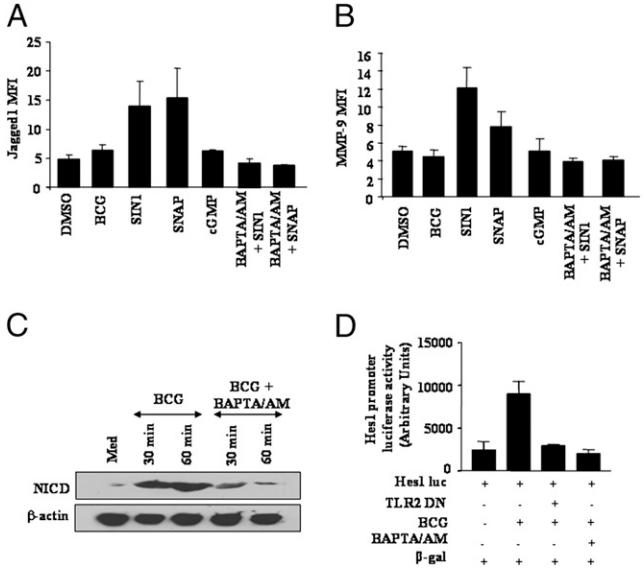


**FIGURE 5.** Requirement of $Ca^{2+}$ in NO-dependent activation of Notch1 signaling. *A* and *B*, Macrophages derived from iNOS$^{-/-}$ mice were pretreated with BAPTA-AM or vehicle control (DMSO) prior to treatment with NO donors. The cell-surface expression levels of Jagged1 (*A*) or MMP-9 (*B*) were analyzed by flow cytometry. Results are represented as mean fluorescence intensity (MFI). Addition of cGMP or infection of iNOS$^{-/-}$ macrophages with *M. bovis* BCG did not alter cell-surface expression levels of Jagged1 (*A*) or MMP-9 (*B*). *C*, WT macrophages were pretreated with BAPTA-AM or DMSO followed by infection with *M. bovis* BCG. The generation of NICD at indicated time points was assessed by immunoblotting. *D*, Chelation of calcium or TLR2 dominant-negative (DN) construct significantly reduced *M. bovis* BCG-triggered Hes1 expression as evaluated by Hes1 promoter activity. The results are expressed as mean ± SEM of three independent experiments, and the blots are representative of three independent experiments. Med, medium.

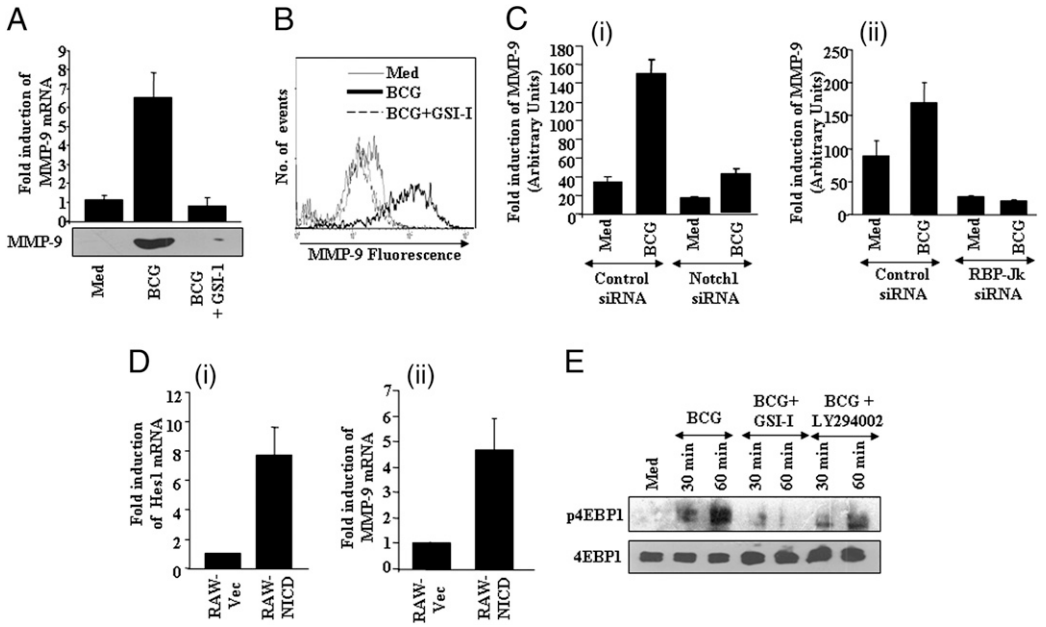


**FIGURE 6.** NO-Notch1-PI3K signaling cross talk involved in infection triggered MMP-9 expression. Notch1 activation inhibitor, GSI-I, abrogated *M. bovis* BCG-induced MMP-9 expression as analyzed by real-time PCR (*A*), immunoblotting (*A*), or flow cytometry (*B*). *C*, RAW 264.7 macrophages were transiently transfected with either control siRNA or Notch1 (*i*) and RBP-Jk siRNA (*ii*). After 3 d of transfection, expression levels of MMP-9 were analyzed. *D*, RAW 264.7 macrophages stably transfected with pCMV-NICD (RAW-NICD) or pCMV (RAW-Vec) were analyzed for expression of Hes1 (*i*) and MMP-9 (*ii*) by quantitative real-time PCR. *E*, Pretreatment of macrophages with GSI-I or PI3K inhibitor, LY294002, abrogated *M. bovis* BCG-induced phosphorylation of 4EBP1. The data presented is representative of three independent experiments. Med, medium.

iNOS-deficient macrophages comparable to that in WT macrophages (Fig. 5*A*, 5*B* and data not shown). These findings rule out the participation of the NO/cGMP pathway in *M. bovis* BCG-mediated activation of Notch1 signaling as well as Jagged1 or MMP-9 expression.

### *Expression of MMP-9 involves iNOS-Notch1–mediated activation of PI3K signaling*

The currently available data on the diverse array of cellular processes under the control of Notch1 clearly illustrate the physiological importance of Notch1 signaling. Notch was described to play pivotal roles in upregulation of antiapototic genes, vasodilatation, etc. (27, 28). The Notch-specific genetic signature is significantly sufficient in the development of immune system and in imparting survival benefits in many cell types (10, 27). An increasing body of evidence indicates that Notch1 signaling-mediated survival effects in many cell types often require participation of the members of PI3K pathway (29, 30). It is known that mycobacteria trigger the activation of PI3K pathway in macrophages (17, 18).

To examine an involvement of Notch1 in NO/Notch1/PI3K-mediated regulation of Notch1 target genes MMP-9 or Hes1 expression, signaling perturbations were carried out with Notch1 activation inhibitor GSI-I, siRNA-mediated knockdown of Notch1 or CSL/RBP-Jk. The inhibitor of Notch1 signaling, GSI-I, abolished NO-induced MMP-9 expression both at RNA level and cell surface expression on macrophages (Fig. 6*A*, 6*B*). Data derived from siRNA-mediated knockdown of Notch1 or CSL/RBP-Jk significantly reduced *M. bovis* BCG-triggered induction of MMP-9 (Fig. 6*C*). Stable expression of NICD in RAW 264.7 macrophages resulted in augmented expression of Notch1 target genes Hes1 or MMP-9 (Fig. 6*D*).

As described, cross talk between Notch1 and PI3K pathways including genetic interactions, the physical binding of Notch1 to PI3K, or their association to common, yet unique cofactors have been described (17, 27). In this perspective, GSI-I abrogated *M. bovis* BCG-triggered 4EBP1 or Akt activation, and attenuation of 4EBP1 activation was quite comparable to LY294002, a known inhibitor of PI3K (Fig. 6*E* and data not shown). In concurrence with these results, inhibition of PI3K by LY294002 or Wortmanin, Akt by Akt inhibitor or siRNA, and mTOR by rapamycin significantly abrogated *M. bovis* BCG-triggered MMP-9 expression (Fig. 7*A–C*). We next studied whether Notch1-PI3K signaling axis regulates MMP-9 expression by activating MAPKs. Our analysis utilizing pharmacological inhibitors revealed that both ERK1/2 and p38 MAPK are involved in Notch1-PI3K–driven MMP-9 expression (Fig. 7*D*). In addition, LY294002 pretreatment downregulated *M. bovis* BCG-triggered ERK1/2 or p38 MAPK activation (Fig. 7*E*). Together, these results strongly suggest that activated Notch1 exert a direct effect in regulating PI3K-MAPK signaling, thus exerting cooperative effects in modulating specific gene transcription.

### *Notch1 signaling inhibition attenuates NF-κB activation*

MMP-9 promoter contains many *cis*-acting NF-κB consensus elements, and Notch1 mediated activation of its target genes often involves the active recruitment of transcription factor NF-κB (8, 11, 17). In this perspective, we demonstrate that besides the physical partitioning of NF-κB, nuclear translocation of NF-κB was abrogated upon inhibition of Notch1 signaling by GSI-I (Fig. 7*F*). Interference in PI3K or MAPK activation reversed *M. bovis* BCG-mediated nuclear translocation of NF-κB from cytosol (Fig. 7*F*). Blocking the NF-κB signaling pathway using Bay 11-7082, a specific inhibitor of IκB-α phosphorylation markedly diminished induced expression of MMP-9 (Fig. 7*G*). These results suggest that NO, upstream of Notch1 signaling, acts as an essential mediator of TLR2-dependent responses and modulates direct cooperation of TLR2 with Notch1-PI3K signaling to regulate a distinct set of effector functions in macrophages.

## Discussion

NO is described to play critical roles during diverse pathophysiological responses associated with inflammation during host-pathogen interactions (31). NO is reported to be microbicidal

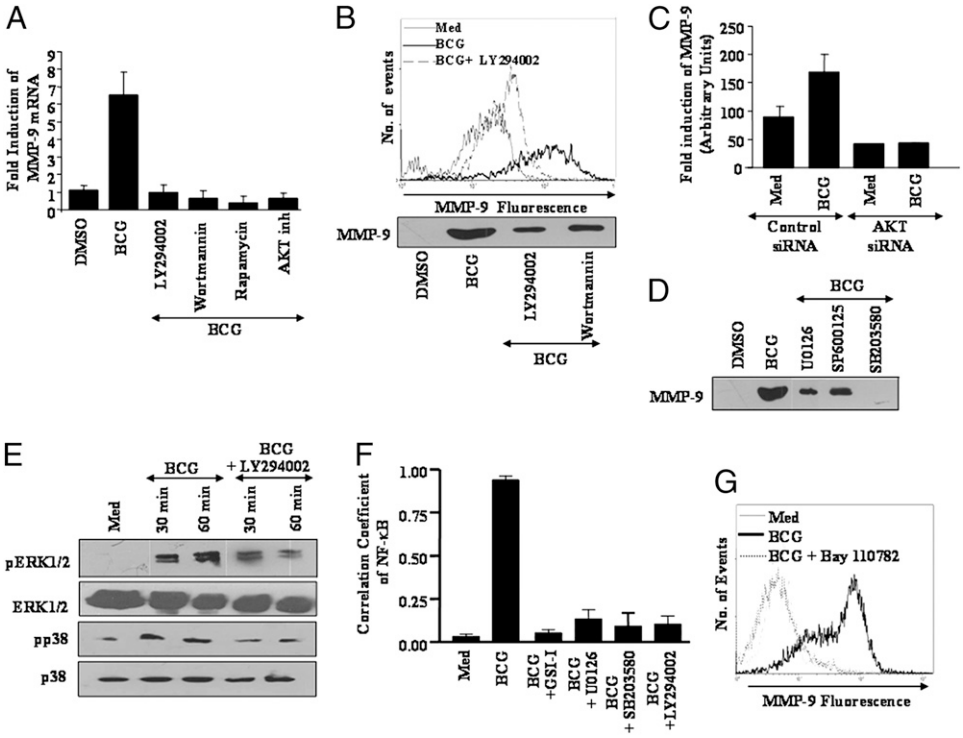


**FIGURE 7.** Notch1-PI3K signaling axis controls MAPK-dependent NF-κB activation. Pretreatment of macrophages with pharmacological inhibitors of PI3K pathway members inhibits *M. bovis* BCG-induced MMP-9 expression as evaluated by real-time PCR (*A*), flow cytometry (*B*), or immunoblotting (*B*). *C*, siRNA targeted to Akt blocks *M. bovis* BCG-induced MMP-9 expression. *D*, Pharmacological inhibition of MAPKs modulates MMP-9 expression. *E*, PI3K inhibitor, LY294002, reduced *M. bovis* BCG-induced ERK1/2 and p38 MAPK activation. *F*, Pharmacological inhibition of Notch1 activation by GSI-I, PI3K by LY294002, MAPKs by U0126, or SB203580 abrogates infection-triggered nuclear translocation of NF-κB as analyzed by confocal microscopy. Colocalization correlation coefficient was generated from scatter plots derived from numerical analysis on 15 cells in each group. *G*, Bay 11-7082 abrogates *M. bovis* BCG-induced cell surface expression of MMP-9. Data represents two independent experiments. Med, medium.

in vitro, which presumed to play a role in antimicrobial action. However, mycobacteria have developed the ability to resist ROI and RNI within the hostile environment of host phagocytes (32–34). In addition, NO represents as a crucial molecular signal, and we had previously reported that NO/iNOS-mediated regulation of proinflammatory gene expression involves multiple pathways in macrophages (18). This could account for elicitation of differential macrophage responses to high-level NO microenvironment at the site of inflammation compared with low NO levels during contraction phase of the inflammatory responses (35). In macrophages, different levels of NO flux or levels could be triggered depending on the state of activation as well as initiation of specific signaling pathways (35, 36), clearly implicating the local concentration and duration of NO exposure as significant elements in regulation of a variety of key genes involved in immune responses and macrophage transition from execution of cellular apoptosis or matrix degradation to cellular proliferation or matrix regeneration, processes required for contraction of inflammation and wound healing (37).

We had earlier reported that *M. bovis* BCG upregulates Notch1 and activates Notch1 signaling pathway, leading to the expression of suppression of cytokine signaling 3 (SOCS3) and cyclooxygenase-2 (COX-2) in macrophages (17, 18). Infection-triggered Notch1-signaling required a role for TLR2-MyD88 axis and cross talk among members of PI3K and MAPK cascades with Notch1 culminating in SOCS3 and COX-2 expression. SOCS3 is known to function as a negative regulator of multiple cytokine and Toll receptor-induced signaling (38) as well as a negative regulator of inflammatory responses (39, 40). One of the critical mechanisms for modulation of host responses by mycobacteria lies in their ability to impart refractoriness of the infected macrophages to many cytokines, including IFN-γ (41–44). In this perspective, the induction of SOCS3 by mycobacteria represents a novel strategy to render unresponsiveness of the infected macrophages to IFN-γ amid robust host immunity. Moreover, mycobacterial infection triggered COX-2, a key enzyme catalyzing the rate-limiting step in the inducible production of $PGE_2$, to act as an important factor influencing the overall host immune response (18). $PGE_2$ is reported to exert immunosuppressive functions such as inhibition of human T lymphocyte activation and proliferation, production of Th1 cytokines (such as IL-2 and IFN-γ, but not of Th2 cytokines IL-4 and IL-5), induction of IL-10 production, etc. (45, 46). These results reveal a crucial role for NO and Notch1 signaling in modulation of immune responses against invading pathogen-like mycobacteria.

In this context, we investigated regulation of a key Notch1-signaling target gene, MMP-9, that codes for ECM protease involved in granuloma formation, lymphocyte trafficking, angiogenesis, inflammation, etc. (47, 48). Detailed understanding of its transcriptional regulation assumes a critical importance for understanding of mycobacterial pathogenesis.

Previous work has shown that stimulation of various TLRs or diverse inflammatory stimuli trigger induced expression of Notch receptors or ligands (8, 49). Similarly, inactivation of the Notch pathway leads to abrogation of TLR2-induced expression of proinflammatory genes (8, 17, 27). In this study, our results define the mechanism that mediates the cross talk between the TLR2 and Notch1 pathway in macrophages and determines its functional relevance. In the current study, we identified NO as a pathological link between TLR2 and Notch1 that may be pertinent to initiation

or expression of key proinflammatory mediators like MMP-9. Upon TLR2 stimulation of macrophages, Notch1 ligand Jagged1 is directly regulated by NO, thus leading to positive regulation of Notch1 activation. Identification of NO as a vital link in triggering Notch1 signaling activation in a TLR2-dependent manner may be of significance in our explant model or in vivo studies and during the activation of inflammation and initiation of granuloma formation. The iNOS deficiency caused a defect in *M. bovis* BCG-triggered expression of Notch1 target genes, MMP-9 or Hes1, which lies in the inability to activate/modulate Notch1-PI3K-MAPK signaling axis in macrophages. We also demonstrate that *M. bovis* BCG-induced expression of MMP-9 requires Notch1-mediated recruitment of suppressor of hairless (CSL/RBP-Jk) and NF-κB to the MMP-9 promoter.

In the perspective of bringing clinical relevance to the biology of *Mycobacterium* infection in vivo, we found that activated Notch1 expression is upregulated in brain samples derived from patients with TBM. As expected, the expression levels of one of the well-documented Notch1 target genes, Hes1, were augmented in TBM brains as opposed to brain samples derived from healthy individuals. Accordingly, similar to Hes1 expression, MMP-9 expression was significantly enhanced in TBM brain samples. Furthermore, data from infection studies in iNOS null mice clearly implicates NO as a critical link in *M. bovis* BCG-TLR2–triggered activation of Notch1 signaling in vivo.

In conclusion, our study demonstrates that NO can regulate Notch1 signaling in a direct cooperative manner with TLR2 by regulating Jagged1 expression and leading to activation of Notch1-PI3K signaling events, which can modulate a defined set of macrophage effector functions. This integrated cross talk of Notch1, TLR2, and NO signals can be of importance during transition of macrophages to classically activated state during inflammatory pathologies of *Mycobacterium* infection. Because our data suggest the involvement of NO-Notch1–specific genetic signature in MMP-9 expression, we believe that our work will extend current mechanistic understanding of inflammatory parameters associated with host-mycobacteria interactions and perhaps might lead to better design and evaluation of therapeutic potential of novel agents targeted at diverse mycobacterial diseases.

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## Disclosures

The authors have no financial conflicts of interest.

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