Suppressor of cytokine signaling (SOCS) 3 is a critical negative regulator of cytokine signaling and is induced by Mycobacterium bovis Bacille Calmette-Guérin (M. bovis BCG) in mouse macrophages. However, little is known about the early receptor proximal signaling mechanisms underlying mycobacteria-mediated induction of SOCS3. We demonstrate here for the first time that M. bovis BCG up-regulates NOTCH1 and activates the NOTCH1 signaling pathway, leading to the expression of SOCS3. We show that perturbing Notch signaling in infected macrophages results in the marked reduction in the expression of SOCS3. Furthermore, enforced expression of the Notch1 intracellular domain in RAW 264.7 macrophages induces the expression of SOCS3, which can be further potentiated by M. bovis BCG. The perturbation of Toll-like receptor (TLR) 2 signaling resulted in marked reduction in SOCS3 levels and expression of the NOTCH1 target gene, Hes1. The down-regulation of MyD88 resulted in a significant decrease in SOCS3 expression, implicating the role of the TLR2-MyD88 axis in M. bovis BCG-triggered signaling. However, the SOCS3 inducing ability of M. bovis BCG remains unaltered also upon infection of macrophages from TLR4-defective C3H/HeJ mice. More importantly, signaling perturbation data suggest the involvement of cross-talk among members of the phosphoinositide 3-kinase and mitogen-activated protein kinase cascade with NOTCH1 signaling in SOCS3 expression. Furthermore, SOCS3 expression requires the NOTCH1-mediated recruitment of Suppressor of Hairless (CSL) and nuclear factor-κB to the Socs3 promoter. Overall, these results implicate NOTCH1 signaling during inducible expression of SOCS3 following infection of macrophages with an intracellular bacillus-like M. bovis BCG.

Mycobacterium tuberculosis is the etiologic agent of tuberculosis and a major cause of morbidity and mortality worldwide, with one-third of the world’s population estimated to be infected with this microorganism (1, 2). Despite many species of mycobacteria that elicits robust host T cell responses as well as production of interferon-γ (IFN-γ)3 that are essential for the control of infection, the mounted immune responses contain, but does not eliminate the infection (2). One potential mechanism by which mycobacteria may achieve a state of long-term persistence amid a robust host immune response is by modulating the signaling cascades leading to macrophage activation (3–7). Activation of proinflammatory responses by the host macrophages upon infection with mycobacteria requires involvement of a variety of signaling events (8–13). Studies have indicated that macrophages infected with pathogenic mycobacteria produce significantly less tumor necrosis factor-α and other proinflammatory molecules compared with infection with nonpathogenic mycobacteria, which likely play a role in enhancing M. tuberculosis survival in vivo (9–10). Furthermore, macrophages infected with mycobacteria become refractory to many cytokines including IFN-γ (13–17). In this regard, modulation of host cell signaling responses is critical for the suppression of a generalized inflammatory response and the persistence of mycobacteria within the host.

In this context, suppressor of cytokine signaling (SOCS) 3, a member of SOCS family, function as negative regulators of multiple cytokine and Toll receptor-induced signaling (18). The SOCS3 has been shown to specifically inhibit signaling by IFN-γ, the IL-6 family of cytokines (19–22), and can act as a negative regulator of inflammatory responses (23, 24). In this regard, many species of mycobacteria (25–27) including Mycobacterium bovis BCG (26) triggers the inducible expression of SOCS3. Furthermore, it has been suggested that M. bovis BCG-triggered SOCS3 and SOCS1 proteins leads to the inhibition of IFN-γ-stimulated JAK/STAT signaling in macrophages (26). Albeit the JAK/STAT signaling pathway is generally believed to be involved, STAT-independent signals are suggested to take part in the induction of SOCS proteins in many systems signi-
M. bovis BCG Activates NOTCH1 Signaling

fying the involvement of multiple signaling pathways in regulation of SOCS expression (28). Furthermore, little is known about the early, receptor proximal signaling mechanisms underlying mycobacteria-mediated induction of SOCS3.

During initial phases of the current study, preliminary data suggested that M. bovis BCG up-regulated the expression of different members of the NOTCH family upon infection of mouse macrophages. In this context, we present evidence here that M. bovis BCG infection of macrophages leads to the activation of NOTCH signaling, which in turn leads to SOCS3 expression. Many recently reported studies have suggested significant roles for NOTCH signaling in the immune system; in cells involved in lymphoid and myeloid differentiation (29). The diverse functions of NOTCH are mediated through a conserved signaling pathway in which four transmembrane NOTCH receptors, NOTCH-1, -2, -3, and -4, undergo regulated proteolysis after productive interaction with transmembrane ligands JAGGED 1 and 2 or three delta-like ligands, DIL-1, -3, and -4. The Notch proteolysis is carried out by the γ-secretase complex to release notch intracellular domain (NICD), which then translocates to the nucleus and associates with the DNA-binding protein CSL/RBP-Jk. CSL/RBP-Jk is normally bound to promoters of a variety of Notch target genes along with corepressors resulting in transcriptional inhibition of target genes including that of Hes1. NICD binding results in displacement of corepressors with concomitant recruitment of coactivators leading to activation of target genes in a CSL/RBP-Jk-dependent transcription (30, 31).

Even though implications of NOTCH signaling in the development of the immune system have been well documented, functional significance of NOTCH receptors in critical cell-fate decisions among diverse immune cells like macrophages or dendritic cells remain unclear. For example, it has been reported that macrophages during culture express Notch1 to Notch4 and among Notch, NOTCH3 is suggested to be selectively increased during differentiation of macrophages (33–35). Furthermore, another study has suggested that up-regulated NOTCH1 and JAGGED1 during macrophage activation modulates the expression of gene expression involved in antigen presentation and cytotoxic activity (36). More importantly, a recent report suggested that stimulation of macrophages through the TLR signaling cascade triggered activation of Notch signaling, which in turn regulated gene expression patterns involved in pro-inflammatory responses, through activation of NF-kB (49).

However, implications of NOTCH signaling on immunological parameters associated with the host macrophages and intracellular pathogen-like mycobacteria have not been reported. In the current study, we show for the first time that infection of macrophages with M. bovis BCG activates NOTCH1 signaling events that leads to activation of SOCS3. Furthermore, the current study demonstrates that M. bovis BCG-triggered NOTCH1 signaling involves the activation of PI3K and MAPK, but not the JAK/STAT pathway as downstream regulators of SOCS3 expression. The M. bovis BCG initiates the signaling events at Toll-like receptor 2 (TLR2) and downstream signaling requires the involvement of MyD88 adaptor protein. More importantly, data from NOTCH1 signaling perturbation clearly suggest the involvement of the cross-talk with members of PI3K and the MAP kinase pathway in the induction of SOCS3 expression. Taken together, our results suggest that Notch1 signaling is an obligatory early proximal signal in activating SOCS3 expression upon M. bovis BCG infection of macrophages.

EXPERIMENTAL PROCEDURES

Cells, Bacteria, and Reagents—Mouse peritoneal macrophages were isolated from peritoneal exudates of C57BL/6 mice (maintained at the central animal facility, Indian Institute of Science) and cultured in Dulbecco’s modified Eagle’s medium (Sigma) with 10% heat-inactivated fetal bovine serum (Sigma). The experiments with mouse macrophages derived from C57BL/6 mice were carried out after the approval from the institutional ethics committee for animal experimentation as well as from the institutional biosafety committee. The RAW 264.7 mouse macrophage cell line (obtained from the National Center for Cell Sciences, Pune, India) was cultivated in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum. M. bovis BCG Pasteur 1173P2 was grown to mid-log phase in Souton’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, catalase). Single-cell suspensions of mycobacteria were obtained with short pulses of sonication. Bacteria were used at 10 multiplicity of infection for all experiments. General laboratory reagents were purchased from Sigma and Merck. The anti-Ser473 pAKT, anti-AKT, anti-Ser468 p4EBP1, anti-4EBP1, anti-Thr180/Tyr182 pp38 MAPK, anti-p38 MAPK, anti-Thr202/Tyr204 pERK1/2, anti-ERK1/2, anti-Ser536 pNF-κB, anti-NF-κB, anti-Tyr701 pSTAT1, and anti-STAT1 were purchased from Cell Signaling Technology. Anti-NOTCH1 (C-20), anti-MyD88, anti-TLR2, and anti-SOCS-3 (H-103) were purchased from Santa Cruz Biotechnology, and anti-β-actin antibody (AC-15) purchased from Sigma.

Treatment with Pharmacological Reagents—The pharmacological inhibitors were obtained from Calbiochem, and were reconstituted in sterile DMSO and used at the following concentrations: LY294002 (50 μM), wortmannin (100 nM), AKT inhibitor II (AKT I-II, 10 μM), rapamycin (100 nM), γ-secretase inhibitor I (GSI I) (10 μM), SB203580 (20 μM), UO126 (15 μM), SP600125 (50 μM), and AG490 (10 μM). DMSO at 0.1% concentration was used as the vehicle control. In all experiments with inhibitors, a treated concentration was used after careful titration experiments assessing the viability of the macrophages. In addition, the chosen concentrations of inhibitors are in agreement with published reports (48). Additionally, when a given inhibitor was tested, its efficacy in terms of inhibition of phosphorylation of the intended signaling molecule as well as a non-intended signaling molecule was also tested (for example, U0126 inhibited only BCG-triggered ERK1/2, but not of p38 phosphorylation). In experiments with inhibitors, the macrophages were treated with a given inhibitor for 45 min before infection with M. bovis BCG.

Stable Transfection of NICD—NICD stable transfectants (RAW-NICD) were generated by transfecting RAW 264.7 cells with the pCMV-NICD (37) using Lipofectamine 2000 (Invitrogen). RAW 264.7 cells stably transfected with pCMV vector
M. bovis BCG Activates NOTCH1 Signaling

RAW-Vec alone were harvested as control. Cells were selected in G418 sulfate (400 μg/ml) and screened for NICD expression by immunoblotting as well as assessed for the expression of Hes1 mRNA, a transcriptional target for Notch1 by quantitative RT-PCR.

Transient Transfections and Luciferase Assays—RAW 264.7 cells were transfected with promoter constructs of Socs3 (Socs3 Luc) and Hes1 (Hes1 Luc) using polyethylenimine (Sigma). After 36 h of transfection cells were infected with M. bovis BCG for 12 h. Cells were harvested and lysed in reporter lysis buffer (Promega) and luciferase activity was assayed using luciferase assay reagent (Promega). The results were normalized for transfection efficiencies by assay of β-galactosidase activity.

Quantitative Real-time PCR—Total RNA from macrophages was isolated by the TRIzol method (Sigma) as per the manufacturer’s protocol, and treated with RNase-free DNase (Promega). The cDNA synthesis kit (Fermentas) was used for reverse transcription according to the manufacturer’s protocol. A real-time PCR amplification (Applied Biosystems) was used for SYBR Green PCR mixture (Finnzymes, Finland) was used for quantification of Socs3, Notch1–4, Jagged 1–2, Dll-1, -3, and -4, lunatic fringe, manic fringe, radical fringe, kuzbanian, preselinin, Hes1, and glyceraldehyde-3-phosphate dehydrogenase with the following conditions: 45 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. All reactions were repeated at least three times independently to ensure the reproducibility of the results. Primer sequences used in the study are: Gapdh forward, 5′-gagcgaacaggttcatctc-3′, reverse, 5′-gaagggcctcatccagtct-3′; Socs3 forward, 5′-gcgaagaagtaaggctggt-3′, reverse, 5′-ccgtagctctccgcaaa-3′; Notch1 forward, 5′-aagaatgccatggtccgca-3′, reverse, 5′-tgtcgagggctgtctggag-3′; Notch2 forward, 5′-gatgtagggcttgctctcc-3′, reverse, 5′-ctgcttgctatccctggtgc-3′; Notch3 forward, 5′-gattccctacaactcctgg-3′, reverse, 5′-ttgctgaatcataacccctg-3′; Notch4 forward, 5′-gtagaagaattgatcgcacgcc-3′, reverse, 5′-agcaagaaccgtgttttg-3′; Jagged 1 forward, 5′-aagaatccagatgagcagcttc-3′, reverse, 5′-agtagaagctgtctcacta-3′; Jagged 2 forward, 5′-tctgtgctaggtgctagttc-3′, reverse, 5′-ttgctgcctctgctgctgg-3′; Dll-1 forward, 5′-gacgctctgactagcagcag-3′, reverse, 5′-ggtgagctgctgctgtggag-3′; Dll-3 forward, 5′-agttgctctccatcgcggc-3′, reverse, 5′-acggcctctagtttggc-3′; Dll-4 forward, 5′-gtagaagaattgatcgcacgcc-3′, reverse, 5′-gtagaagaattgatcgcacgcc-3′; Jagged 2 forward, 5′-tctgtgctaggtgctagttc-3′, reverse, 5′-ttgctgcctctgctgctgg-3′; Jagged 3 forward, 5′-ggtgagctgctgctgtggag-3′; Jagged 4 forward, 5′-gtagaagaattgatcgcacgcc-3′, reverse, 5′-gtagaagaattgatcgcacgcc-3′.

Lysate Preparation and Immunoblotting Analysis—Cells were harvested after washing briefly with ice-cold phosphate-buffered saline and lysed in buffer (50 mm Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mm NaCl, 1 mm EDTA, 1 mm phenylmethylsulfonyl fluoride, 1 μg/ml each aprotinin, leupeptin, and pepstatin, 1 mm Na3VO4, 1 mm NaF). Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis and then proteins were transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked in TBST buffer (0.02 m Tris-HCl, pH 7.5, 0.15 mm NaCl, and 0.1% Tween 20) containing 5% nonfat dried milk and probed with a primary antibody overnight at 4°C. After washing with TBST, membranes were incubated with secondary antibody linked to horseradish peroxidase (Jackson Immunologicals). The blots were then visualized with an enhanced chemiluminescence detection system (PerkinElmer Bioscience) as per the manufacturer’s instructions.

Immunoprecipitation—Cells were washed with ice-cold phosphate-buffered saline and lysed in RIPA buffer (50 mm Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mm NaCl, 1 mm EDTA, 1 mm phenylmethylsulfonyl fluoride, 1 μg/ml each aprotinin, leupeptin, and pepstatin, 1 mm Na3VO4, 1 mm NaF). The protein concentrations of the cellular extracts were measured with the Bradford reagent. To avoid nonspecific binding, 400 μg of protein was incubated with 2 μg of rabbit IgG and precipitated with protein G coupled to agarose beads (Amersham Biosciences). The immunoprecipitation of NOTCH1 or TLR2 was performed using 2 μg of goat anti-rabbit anti-NOCTH1 (Santa Cruz Biotechnology) or rat anti-mouse anti-TLR2 (eBioscience) followed by electrophoresis and immunoblotting as described above.
as siGENOMETM SMARTpool reagent, which contains a pool of four different double-stranded RNA oligonucleotides.

**Nuclear and Cytosolic Subcellular Fractionation**—After treatment, macrophages were harvested by centrifugation and gently resuspended in Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). After incubation on ice for 15 min, membranes were disrupted with 10% Nonidet P-40, and the nuclear pellets were recovered by centrifugation at 13,000 × g for 15 min at 4 °C and the supernatant was used as the cytosolic extract. Nuclear pellets were lysed with Buffer C (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and nuclear extracts were collected after centrifugation at 13,000 × g for 20 min at 4 °C.

**Electrophoretic Mobility Shift Assay (EMSA)**—Mouse peritoneal macrophages were infected with M. bovis BCG for the indicated time points and gently lysed in buffer containing Nonidet P-40. After the cells were washed, nuclear proteins were extracted with a high-salt buffer. Nuclear proteins (5 μg) were incubated for 30 min at 25 °C with 32P-labeled NF-κB or CSL/RBP-Jk probe in binding buffer for NF-κB, 10 mM Tris-Cl, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10 μg/ml bovine serum albumin, 1% Nonidet P-40, 5% glycerol, and 1 μg of poly(dI-dC) and for CSL/RBP-Jk, 20 mM HEPES, pH 7.9, 60 mM KCl, 1.33 mM MgCl2, 1 mM dithiothreitol, 4% glycerol, and 1 μg of poly(dI-dC), and separated by electrophoresis through 6% Tris borate-EDTA gels. Gels were dried and analyzed by phosphorimaging (Fujiﬁlm, Japan). The consensus binding sequences of both wild-type and mutant, used for CSL/RBP-Jk and NF-κB, were CSL/RBP-Jk wild-type, 5′-TTGTTGTAACACGCCTGTGGAAAAATTTA-3′; CSL/RBP-Jk mutant, 5′-TTGTTGTAACACGCCTGTGGAAAAATTTA-3′; NF-κB wild type, 5′-AGTTAGGGAGCATTCTCCAGGC-3′; and NF-κB mutant, 5′-AGTTAGGGAGCATTCTCCAGGC-3′.

**Statistical Analysis**—Levels of significance for comparison between samples were determined by the Student’s t test distribution. The data in the graphs are expressed as the mean ± S.D. Graphpad Prism 3.0 software (Graphpad Software) was used for all the statistical analysis.

## RESULTS

**M. bovis BCG Up-regulates NOTCH1 and Activates NOTCH Signaling in Induced SOCS3 Expression in Macrophages**—The infection of mouse peritoneal macrophages with M. bovis BCG triggers expression of SOCS3 both at mRNA and protein levels (Fig. 1, A–C). To assess the role of receptor proximal signaling in M. bovis BCG-triggered expression of SOCS3, the expression levels of NOTCH1 were assessed. Fig. 1, D and E, shows that infection with M. bovis BCG leads to up-regulation of NOTCH1, both at mRNA levels quantified by real-time PCR and protein by Western blot analysis. As indicated by immunoblotting, a similar level of induction of NICD, a cleavage product of NOTCH1, as well as full-length NOTCH1 was induced in macrophages upon infection with M. bovis BCG (Fig. 1E and supplemental Fig. S1A) signifying the activation of NOTCH1 signaling. Accordingly, the NOTCH1 target gene Hes1 (38) was also induced by M. bovis BCG (Fig. 1D). As mentioned under the Introduction, preliminary experiments during the initial phases of the current investigation on the expression profiles of genes involved in macrophage survival suggested that M. bovis BCG up-regulated the expression levels of different members of the Notch family. In this perspective, expression levels of Notch3 in addition to Notch1, Notch ligands Jagged 1, Dll-1, and Dll-3 as well as that of modiﬁers presenilin and lunatic fringe were up-regulated upon infection of macrophages with M. bovis BCG (Fig. 1D and data not shown).

To assess whether up-regulated NOTCH1 or NICD was involved in induction of SOCS3, chemical inhibitors that affect the NOTCH processing were tested for their effect on SOCS3 expression. As shown in Fig. 1, F and G, the GSI-I, a γ-secretase inhibitor, blocked the induction of SOCS3 by M. bovis BCG. In these experiments, treatment with the GSI resulted in a loss of processed NOTCH1-NICD, which preceded the induction of SOCS3 expression as well as reduction in the NOTCH1 target gene; Hes1 expression levels (supplemental Fig. S1, B and C). This was accompanied by a minimal change if any in expression of total NOTCH1 protein (data not shown). Furthermore, perturbation of NOTCH1 signaling by knockdown siRNA (Fig. 1H) resulted in marked reduction (59% decrease in SOCS3 to actin protein ratio) in SOCS3 expression (Fig. 1I). Evidence in the literature suggests that multiple effects of NOTCH signaling often require both transcription-dependent and transcription-independent processes (29–32). In this perspective, we investigated a potential role of CSL/RBP-Jk in NOTCH-induced expression of SOCS3. The down-regulation of CSL/RBP-Jk (supplemental Fig. S1D) by siRNA in M. bovis BCG-infected macrophages resulted in a significant decrease in SOCS3 expression (Fig. 1J) clearly implicating that NOTCH signaling regulates SOCS3 expression to a significant extent in a transcription-dependent manner. To exclude the autocrine effect of macrophage-produced IL-6 or IL-10 upon infection of M. bovis BCG as opposed to direct activation of SOCS3 expression by M. bovis BCG, mouse macrophages were infected with M. bovis BCG in the presence or absence of cycloheximide. The data presented in supplemental Fig. S6A clearly demonstrates that M. bovis BCG-triggered Socs3 mRNA expression was not affected by cycloheximide clearly ruling out the autocrine effect of bacilli-induced cytokines, such as IL-10 or IL-6 in SOCS3 expression. Furthermore, addition of neutralizing antibody to IL-10 did not affect the M. bovis BCG-triggered Socs3 expression (supplemental Fig. S6, B and C). In contrast to SOCS3 expression, cycloheximide blocked the expression of IL-6 and II-10 transcripts (supplemental Fig. S6D and data not shown).

Furthermore, various studies have reported that IL-10- and IL-6-induced SOCS3 expression requires STAT3 activation (18) and as presented in supplemental Fig. S5, M. bovis BCG does not trigger either STAT3 or STAT1 activation. More importantly, a recent report suggests that NOTCH signaling in macrophages unlike in T cells, inhibits IL-10 production (49). These results are in agreement with our data presented in supplemental Fig. S6E that demonstrates that NOTCH activation inhibitor GSI-1 increases the II-10 mRNA to significant levels in M. bovis BCG-infected macrophages. Overall, these results in our opinion clearly rule out the possible involvement of IL-10 or II-6 in M. bovis BCG-induced SOCS3 expression.
Enforced Expression of NOTCH1 Intracellular Domain Induces the Expression of SOCS3—To further assess NOTCH function, RAW 264.7 stable transfectants constitutively expressing the NOTCH1 intracellular domain (RAW-NICD) were derived (Fig. 1M). The constitutively active NICD protein induced SOCS3 expression at both mRNA and protein levels (Fig. 1K) as well as the NOTCH target gene, Hes1 (Fig. 1N). The ability of constitutively active NICD to induce Socs3 was further potentiated by M. bovis BCG infection (Fig. 1L).

Involvement of TLR2 in M. bovis BCG-triggered NOTCH1 Signaling and SOCS3 Expression—TLRs are implicated as sensors during mycobacterial infections and many studies have suggested that whole mycobacteria bacilli or mycobacterial components can act as TLR agonists leading to the activation of inflammatory signaling cascades (4). Among the TLRs, TLR2 is a major receptor on macrophages utilized by mycobacterium to initiate a variety of signaling events. In this regard, the involvement of TLR2 was tested during M. bovis BCG-induced NOTCH1 activation as well as SOCS3 expression. The inhibition of TLR2 signaling by a TLR2 dominant-negative construct resulted in marked inhibition of SOCS3 expression as well as levels of Hes1, a marker gene for NOTCH1 activation (Fig. 2A and supplemental Fig. S1E). Furthermore, knockdown siRNA to MyD88, a downstream adaptor molecule among the majority of TLR signaling pathways, resulted in substantial reduction (78% decrease in the SOCS3 to actin protein ratio) in M. bovis BCG-induced SOCS3 and Hes1 expression (Fig. 2B and data not shown). To assess the involvement of the TLR4-MyD88 mediated pathway in SOCS3 induction, macrophages derived from TLR4-defective C3H/HeJ mice were infected with M. bovis BCG. The data presented in supplemental Fig. S1F clearly demonstrates that M. bovis BCG-triggered SOCS3 expression levels remain unaltered in the absence of TLR4 signaling. In this perspective, to address whether TLR2 directly activates NOTCH1 or PI3K, immunoprecipitation experiments demonstrate that TLR2 can associate with both PI3K and NOTCH1. This implicates M. bovis BCG-mediated TLR2 triggering in the activation of PI3K and NOTCH1 (Fig. S4, A and B).
M. bovis BCG Activates NOTCH1 Signaling

FIGURE 2. NOTCH intracellular domain-triggered expression of SOCS3 requires PI3K- and MAPK-mediated signaling. A, RAW 264.7 macrophages were transfected with either empty vector (pcDNA3.1) or dominant-negative TLR2 (TLR2-DN) along with Socs3-Luc, then left uninfected or infected with M. bovis BCG at a multiplicity of infection of 10 for 12 h. Cell lysates were prepared and assayed for luciferase reporter activity as described under “Experimental Procedures.” B, RAW 264.7 cells were transfected with 100 nm of either siRNA directed to MyD88 or control siRNA. Three days post-transfection, cells were infected with M. bovis BCG for 12 h and protein levels of SOCS3 were assessed. C, analysis of Socs3 mRNA expression in RAW-NiCD cells or in RAW-Vec cells upon treatment with LY294002 (50 μM) or U0126 (10 μM). DMSO was used as a vehicle control. D, expression analysis of SOCS3 protein in cell lysates prepared under the conditions as described in C. E, activation of AKT in stable RAW-NiCD and RAW-Vec cells. F, phospho-4EBP1 versus total 4EBP1 in RAW-NiCD or RAW-Vec cells that were treated either with LY294002 (50 μM) or rapamycin (100 nM). G, activation of ERK1/2 in stable RAW-NiCD and RAW-Vec cells. The graph represents fold activation of ERK1/2 calculated by densitometric quantitation of bands obtained from immunoblotting. H, RAW 264.7 macrophages were transfected with either empty vector (pcDNA3.1) or dominant-negative STAT1 (STAT1-DN) along with Socs3-Luc, then left uninfected (UI) or infected with M. bovis BCG at a multiplicity of infection of 10 for 12 h. Cell lysates were prepared, and assayed for luciferase reporter activity as described under “Experimental Procedures.” I, mouse macrophages were treated with AG490 (10 μM), or DMSO, prior to infection with M. bovis BCG for 12 h and SOCS3 protein levels were analyzed.

option, this is the first report demonstrating the physical association of NOTCH1 with TLR2 in addition to PI3K. More importantly, in agreement with many of our results, a recent study suggested that stimulation of TLR signaling cascades leads to activation of NOTCH signaling and was dependent on the MyD88 pathway when stimulated with TLR2, but not with TLR4 (49).

As mentioned under the Introduction, the JAK/STAT signaling pathway is generally believed to be involved in the case of IFN-γ-mediated activation of SOCS3. In this perspective, a recent study (36) suggested that NICD can augment IFN-γ-mediated activation of certain immune genes in a STAT1-dependent transcription manner. In this regard, when tested, either the STAT-1 dominant-negative construct (Fig. 2H) or JAK inhibitor, AG490 (Fig. 2I), did not perturb the SOCS3 inducing ability of M. bovis BCG. Furthermore, experiments presented in supplemental Fig. S5 demonstrates that M. bovis BCG fails to trigger either STAT3 or STAT1 phosphorylation, when compared with cytokine IFN-γ. Whereas AG490 efficiently blocked IFN-γ-triggered JAK-mediated STAT3 or STAT1 phosphorylation (supplemental Fig. S5), AG490, as discussed earlier, did not block M. bovis BCG-triggered SOCS3 expression (Fig. 2J). Additionally, we were unable to find any reports describing STAT1 or STAT3 activation by M. bovis BCG. Furthermore, in this regard, many reports have suggested that STAT-independent signals can take part in the induction of SOCS proteins in many systems signifying the involvement of multiple signaling pathways in regulation of SOCS expression (28). These results, we believe, demonstrate that M. bovis BCG, unlike cytokines such as IFN-γ, can trigger the expression of SOCS3 in a TLR2-mediated (through MyD88) signaling in a JAK-STAT independent manner.

M. bovis BCG-triggered Activation of PI 3-Kinase Signaling Requires NOTCH1 Activation—Even though data in Fig. 1 suggest the role of NOTCH1-dependent transcription in SOCS3 expression, we attempted to assess the role of NOTCH1 in transcription-independent expression of SOCS3. In this regard, reports have suggested that NOTCH1 signaling-induced survival effects in many cell types require activation of PI 3-kinase and MAPK pathways (38–40). Even though many of the functions of NOTCH1 requires PI3K, the precise nature of the signaling downstream of PI3K is not very clear. To check whether similar mechanisms are operative in triggering the expression of SOCS3, we used pharmacological inhibitors of PI3K, AKT, and MAPK to test the functional interactions between NOTCH signaling and the PI3K or MAPK pathway in infected macrophages. In this context, Fig. 3, A–C, clearly demonstrates that inhibitors of PI3K, LY294002 and wortmannin, AKT inhibitor, mTOR inhibitor, and rapamycin clearly reversed the M. bovis BCG-triggered SOCS3 expression. Furthermore, to assess whether M. bovis BCG-mediated activation of the PI 3-kinase signaling pathway requires the involvement of NOTCH1, mouse peritoneal macrophages were first assessed for the phosphorylation of AKT and 4E-BP1 upon infection with M. bovis BCG. As shown in Fig. 3, D and E (and supplemental Fig. S2A), M. bovis BCG infection leads to phosphorylation of both AKT and 4E-BP1, clearly demonstrating activation of the PI3K pathway. To assess whether NOTCH1 is involved in activation of...
**M. bovis BCG Activates NOTCH1 Signaling**

4E-BP1, peritoneal macrophages were treated with the GSI-I, a inhibitor of γ-secretase, or with LY294002, a PI3K inhibitor, before infection and Fig. 3E clearly demonstrates the reversal of *M. bovis* BCG-induced phosphorylation of 4E-BP1. In addition, perturbing the PI 3-kinase signaling by siRNA to AKT (inset) resulted in marked reduction in SOCS3 expression (Fig. 3F). In addition to direct activation of PI3-AKT-mTOR kinases, NOTCH was suggested to activate PLD1 (41), which in turn can activate mTOR through its metabolic product, phosphatidic acid. In this context, when assessed, propranolol, a known PLD1 inhibitor significantly diminished the NOTCH-mediated SOCS3 expression (Fig. 3C). Furthermore, treatment with phosphatidic acid induced SOCS3 expression (supplemental Fig. S1G) clearly implicating the role of the NOTCH-PLD1 axis in *M. bovis* BCG-induced SOCS3 expression. In addition, perturbation of PI 3-kinase and MAPK pathways in RAW 264.7 stable cells expressing NICD, by inhibitors LY294002 (PI3K), rapamycin (mTOR) (Fig. 2, C and D, and data not shown), and U0126 (MEK1/2 inhibitor) reversed the SOCS3 inducing function of NICD (Fig. 2, C and D), further strengthening the above mentioned results. In addition, we detected increased phosphorylation of AKT-Set473 with no change in levels of total AKT (Fig. 2E), and increased levels of ERK1/2 and p38 in RAW-NICD stable cells (Fig. 2F, and data not shown). Furthermore, increased phosphorylation of the downstream mTOR target 4E-BP1 in RAW-NICD stable cells could be blocked by LY294002 and rapamycin (Fig. 2F). The experiments presented in Figs. 1E and 3A were part of the same experiment and hence the same controls have been utilized in both representations. Similarly, Figs. 1F and 3B were part of the same experiment and
M. bovis BCG Activates NOTCH1 Signaling

the same controls have been used in these figures. Taken together, these experiments suggest that NICD-triggered expression of SOCS3 involves the cross-talk of PI3K and MAPK signaling cascades.

NOTCH1 Is Involved in M. bovis BCG-mediated Activation of ERK1/2 and p38 MAPK—As described earlier, NOTCH1 signaling is often involved to activate MAPK in this context, both U0126 and SB203580, but not SP600125 (JNK inhibitor) reduced the M. bovis BCG-induced SOCS3 mRNA and protein expressions (Fig. 3, G and H). Furthermore, exposure of mouse peritoneal macrophages to M. bovis BCG resulted in an increase in the phosphorylation of ERK1/2 and p38 MAPK, apparent within minutes of M. bovis BCG interaction with macrophage (Fig. 3, I and J, and supplemental Fig. S2, B and C). However, the SOCS3 expression level remained unperturbed (Fig. 3, G and H), when the M. bovis BCG-triggered activation of JNK (supplemental Fig. S3D) was inhibited. To delineate whether activation of ERK1/2 and p38 MAPK by M. bovis BCG involves activation of NOTCH1 and PI 3-kinase pathways, the phosphorylation of ERK1/2 and p38 MAPK by M. bovis BCG were assessed in the presence of GSI-I, a γ-secretase inhibitor, LY294002, or with AKT I-II, an inhibitor of AKT. The M. bovis BCG-triggered activation of ERK1/2 or p38 MAPK was markedly reduced, when the activation of NOTCH or PI3K or AKT signaling was perturbed (Fig. 3, I and J). These results suggest that ERK1/2 and p38 MAPK represents important links in the NOTCH1-Pi3K signaling partnership by which M. bovis BCG induces SOCS3 expression.

CSL/RBP-Jk and NF-κB Bind the Socs3 Promoter in Vivo—NOTCH1-mediated activation of many of its target genes involves the active recruitment of transcription factors CSL/RBP-Jk and NF-κB (30, 42, 43). To confirm the role of CSL/RBP-Jk and NF-κB in M. bovis BCG-induced SOCS3 transcriptional regulation, the mouse Socs3 promoter was computationally analyzed for the possible binding sites for a variety of transcription factors including that of RBP-Jk and NF-κB. The analysis of the region spanning 2000 bp upstream of the Socs3 transcription start site was carried out using MatInspector, a potential transcription factor-binding sites prediction program (44). The program predicted the binding site for RBP-Jk between −948 and −962 bp and for NF-κB between −464 and −476 bp relative to the +1 transcription start site. ChIP assays were performed to determine whether these proteins interact with the Socs3 promoter in vivo. The infection of mouse peritoneal macrophages with M. bovis BCG increased the binding of CSL/RBP-Jk to the Socs3 promoter, consistent with the observation of CSL/RBP-Jk having a positive regulatory role in M. bovis BCG-induced Socs3 transcription (Fig. 4, E and F). Furthermore, data presented in Fig. 4, A, C, and D, shows M. bovis BCG increased binding of NF-κB to the Socs3 promoter as demonstrated by ChIP, EMSA, and cell fractionation assays, supporting the idea that M. bovis BCG stimulus utilizes both transcription factors to regulate SOCS3 expression. The nuclear translocation of NF-κB, as shown in Fig. 4D, is dependent on activation of Notch, as treatment with GSI-I, a γ-secretase inhibitor, significantly reduced the nuclear translocation of NF-κB upon M. bovis BCG infection. Furthermore, inhibitors of either PI3K or MAPK pathways reversed infection-mediated nuclear translocation of NF-κB from the cytosol (supplemental Fig. S3). In addition, an IκB phosphorylation inhibitor, BAY 11-7082 markedly diminished the infection-induced SOCS3 protein levels clearly implicating NF-κB as a key regulator of M. bovis BCG-induced SOCS3 expression (Fig. 4B).

DISCUSSION

One of the critical modulations of host signaling response by mycobacteria is the refractoriness of the infected macrophages to many cytokines including IFN-γ (13–17). In this perspective, the induction of SOCS3 by mycobacteria represents a novel

FIGURE 4. CSL/RBP-Jk and NF-κB bind the Socs3 promoter in vivo. A, NF-κB recruitment at the Socs3 promoter was analyzed by the chromatin immunoprecipitation assay with the NF-κB antibody in M. bovis BCG-infected cell lysates and the extent of NF-κB binding was assessed by real time PCR. B, pretreatment of macrophages with BAY 1170782 (20 μM) abrogates M. bovis BCG-induced SOCS3 expression. C, EMSA was performed using [γ-32P]ATP-labeled NF-κB binding oligo in nuclear lysates prepared from M. bovis BCG-infected mouse macrophages. The specificity of NF-κB binding was assessed with mutant NF-κB binding oligo or competition with cold probe. D, nuclear localization of NF-κB was analyzed by immunoblotting of nuclear or cytosolic extracts from M. bovis BCG-infected macrophages with or without treatment with GSI-I prior to infection. Assessment of purity of the nuclear fraction was carried out using marker protein proliferating cell nuclear antigen (PCNA). E, CSL/RBP-Jk recruitment at Socs3 promoter in M. bovis BCG-infected cells as assessed by the chromatin immunoprecipitation assay using NOTCH1 antibody. The recruitment of NOTCH1 complexed with CSL/RBP-Jk to the Socs3 promoter was assessed as fold induction by quantitative RT-PCR. F, EMSA was performed using γ-32P-labeled CSL/RBP-Jk binding oligo in nuclear lysates prepared from M. bovis BCG-infected mouse macrophages. The specificity of CSL/RBP-Jk binding was assessed with mutant CSL/RBP-Jk binding oligo or competition with cold probe. Further confirmation of specificity was carried out by pretreatment with anti-NICD antibody (2 μg), which abolished the specific binding of CSL/RBP-Jk to the labeled CSL/RBP-Jk binding oligo. UI, uninfected.
strategy to render unresponsiveness of the infected macrophages to IFN-γ and the current study for the first time high-
lights the role of NOTCH1 signaling as an obligatory early proximal signal in *M. bovis* BCG-induced expression of SOCS3 in macrophages.

In recent years, experimental evidence have suggested the significant roles for NOTCH signaling in a variety of systems, including in cells involved in lymphoid and myeloid differenti-
ation (29). Nevertheless, it is our conviction that very little attention has been placed on the role of NOTCH signaling dur-
ing infection of macrophages with an intracellular bacillus like *M. bovis* BCG, albeit macrophages are known to express to varied levels of the NOTCH receptors and ligands (33–36). Our studies show that *M. bovis* BCG infection leads to up-regulation of NOTCH1 expression and activates the NOTCH signaling pathway leading to the expression of SOCS3. Moreover, ectopic expression of the NICD in RAW 264.7 macrophages induces the expression of SOCS3, which involved PI3K and MAP kinases as possible downstream signaling components.

TLRs in general play important roles in immune responses and are implicated in the activation of inflammatory immune responses by mycobacteria. Furthermore, many TLR agonists activate SOCS proteins that in turn act as a negative feedback loop of the TLR signaling (18, 19). Our study clearly indicates the involvement of TLR2-MyD88, but not TLR4 in *M. bovis* BCG-triggered SOCS3 expression. In accordance with our results, a recent report implicated TLR stimulation to activation of NOTCH signaling that was dependent on the Myd88 pathway in the case of TLR2 stimulation, but not with TLR4. As described under "Results," when we assessed, immunoprecipitation experiments clearly suggest that TLR2 can associate with both PI3K and NOTCH1, which implicates TLR2 triggering by *M. bovis* BCG in the activation of PI3K and NOTCH1. Even though TLR2 association with PI3K has been reported (57, 58), we are demonstrating for the first time the physical association of NOTCH1 with TLR2 in addition to PI3K. Based on the reported studies (50, 51), it possible that association of PI3K and NOTCH1 with the GRB2-DELTEX complex might be involved in the overall interaction with TLR2 in macrophages. Interestingly, inhibition of the TLR2 pathway by the dominant negative construct reduced Hes1 expression, which further strengthens the possible participation of TLR2 in activation of NOTCH signaling. However, extensive investigation is necessary to broaden the understanding of the above finding.

We have observed very low levels of SOCS3 transcript in unstimulated peritoneal macrophages. Furthermore, the data derived from various studies clearly indicates significant cell-
specific differences in the basal level of untranslabeled SOCS3 between a variety of cell types including RAW 264.7 cells, peripheral blood mononuclear cell-derived macrophages etc., even though the reasons remains unclear. Similarly, we are not clear about the possible roles for the low levels of SOCS3 trans-
scripts in our studies as the regulation of SOCS3 expression is complex, which is known to occur on at least at four different levels: 1) at the level of transcription; 2) on the level of SOCS3-mRNA stability; 3) at the level of protein degradation suggested to be by tyrosine phosphorylation of SOCS3 or possible cross-talk with SOCS2; and 4) by means of alternative translation start points under stress conditions (18, 52–55). In addition, reports have suggested that low-level basal expression of SOCS3 may play a role in dampening activation of signaling molecules including that of STAT3 under unstimulated conditions (56). This could constitute a system of checks and balances that can ensure the strength of immune responses during the basal state and during inflammation.

Even though the JAK/STAT signaling cascade is generally thought to be involved, STAT-independent signals are sug-
gested to take part in the induction of SOCS proteins in many systems signifying the involvement of multiple signaling path-
ways in regulation of SOCS expression (28). In this context, as discussed earlier, we have carried out an extensive literature search for possible STAT3 or STAT1 phosphorylations by *M. bovis* BCG and so far we have not been able to find any reports describing STAT1 or STAT3 activation by *M. bovis* BCG. Fur-
thermore, *M. bovis* BCG fails to trigger either STAT3 or STAT1 phosphorylation, when compared with cytokine IFN-γ and inhibition of JAK-mediated STAT3 or STAT1 phosphorylation by AG490, as discussed earlier, did not block *M. bovis* BCG-triggered SOCS3 expression. Based on these observations, we believe *M. bovis* BCG triggers the expression of SOCS3 in a TLR2-mediated signaling in a JAK-STAT independent man-
ner. Furthermore, mouse macrophage infection experiments with cycloheximide or neutralizing antibody to IL-10 demon-
strated that the SOCS3 inducing ability of *M. bovis* BCG was unaffected clearly ruling out the autocrine effect of bacilli-in-
duced cytokines like IL-10 or IL-6 in SOCS3 expression.

The earlier reported studies have suggested that NOTCH modulates the activity of signaling pathways through transcriptional regulation of its target genes and NOTCH-mediated sur-
vival pathways in many cell types often require the involvement of PI3K-AKT, ERK1/2, and NF-κB (39–43), as possible down-
stream regulators of NOTCH signaling. In this context, the inhibition of NOTCH1 signaling resulted in a significant decrease in phosphorylation of 4E-BP1, ERK1/2, and p38 MAPK clearly implicating the involvement of PI3K and MAPK cascades downstream to the NOTCH1 signaling. This observation raises an important issue of how precisely does NOTCH1 interact with the PI3K/AKT/mTOR pathway. One possibility is that NOTCH positively regulates mTOR through PI3K/AKT as reported in certain cases of T cell developmental studies in the face of NOTCH1 deficiency (38, 41, 45). In this context, we show that the siRNA to AKT as well as inhibitors to PI3K, AKT, and mTOR abrogates not only the expression of *M. bovis* BCG-induced SOCS3, but also the activation of ERK1/2 and p38 MAPK.

Another possibility is the involvement of PLD1, which acti-
vates mTOR activity through its metabolic product, phospha-
tidic acid. Even though the molecular details of PLD1 regul-
ation by NOTCH remain obscure, it is interesting to note that PLD1 activity or trafficking is reported to be modulated by a direct physical interaction with γ-secretase (46). Further treatment of leukemic cells with γ-secretase inhibitors reduced the PLD1 phosphorylation (41) suggesting that the NOTCH and PLD1 pathways may be coordinately regulated. In this regard, when tested, propranolol, a known PLD1 inhibitor significantly diminished the NOTCH1-mediated SOCS3 expression, phos-
The expression of SOCS3 gene is regulated by the Notch signaling pathway. The schematic diagram depicted in Fig. 5 shows the cross-talk between the Notch and PI3K-MAPK signaling pathways during infection of macrophages with M. bovis BCG, eventually resulting in regulation of specific gene expressions, such as SOCS3. The schematic diagram presented in Fig. 5 depicts the overall findings of the study and these observations lead to a possibility of differential effects of NOTCH1 signaling activated upon infection by an intracellular bacillus, which could be involved in modulating macrophage functions depending on a local immunological milieu.

Acknowledgments—We thank Drs. Eugene Chin, Brown University School of Medicine Providence, RI, Flavia Bazzoni, University of Verona, Verona, Italy, and Douglas Golenk bock, University of Massachusetts Medical School, Worcester, MA, for the kind gift of reagents. We also thank Drs. Kumaravel Somasundaram, Apurva Sarin, Annapoorni Rangarajan, and Dipshika Chakravorty for help during the course of the current investigation.

REFERENCES

1. World Health Organization (2007) WHO Report 2007 WHO/HTM/TB/2007.376
2. Kaufmann, S. H. (2005) N. Engl. J. Med. 353, 2423–2426
3. Koul, A., Herget, T., Klebl, B., and Ullrich, A. (2004) Nat. Rev. Microbiol. 2, 189–202
4. Jo, E. K., Yang, C. S., Choi, C. H., and Harding, C. V. (2007) Cell. Microbiol. 9, 1087–1098
5. Shchery, I. S., and Cooper, A. M. (2003) Cell. Microbiol. 5, 133–142
6. Pathak, S. K., Basu, S., Basu, K. B., Banerjee, A., Pathak, S., Bhattacharyya, A., Kaisho, T., Kundu, M., and Basu, J. (2007) Nat. Immunol. 8, 610–618
7. Nigou, J., Gilleron, M., Rojas, M., Garcia, L. F., Thurnher, M., and Puzo, G. (2002) Microbes Infect. 4, 945–953
8. Roach, S. K., and Shchery, J. S. (2002) Infect. Immun. 70, 3040–3052
9. Falcone, V., Bassey, E. B., Toniolo, A., Conalpri, P. G., and Collins, F. M. (1994) FEMS Immunol. Med. Microbiol. 8, 225–232
10. Beltan, E., Hofgen, L., and Rastogi, N. (2000) Microb. Pathog. 28, 313–318
11. Balowitz-Sablinska, M. K., Keane, J., Kornfeld, H., and Remold, H. G. (1998) J. Immunol. 161, 2636–2641
12. Reiling, N., Blumenthal, A., Flad, H. D., Ernst, M., and Ehrler, S. (2001) J. Immunol. 167, 3339–3345
13. Pennini, M. E., Pai, R. K., Schultz, D. C., Boom, W. H., and Harding, C. V. (2006) J. Immunol. 176, 4323–4330
14. Banaiee, N., Kincaid, E. Z., Buchwald, U., Jacobs, W. R., Jr., and Ernst, J. D. (2006) J. Immunol. 176, 3019–3027
15. Nagabhushanam, V., Solache, A., Ting, L. M., Escaron, C. J., Zhang, Y. J., and Ernst, J. D. (2003) J. Immunol. 171, 4750–4757
16. Fortune, S. M., Solache, A., Jaeger, A., Hill, P. J., Belisle, J. T., Bloom, B. R., Rubin, E. J., and Ernst, J. D. (2004) J. Immunol. 172, 6272–6280
17. Pai, R. K., Pennini, M. E., Tobian, A. A., Canaday, D. H., Boom, W. H., and Harding, C. V. (2004) Infect. Immun. 72, 6603–6614
18. Yoshimura, A., Naka, T., and Kubo, M. (2007) Nat. Rev. Immunol. 7, 454–465
19. Wormald, S., Zhang, J. G., Krebs, D. L., Mielke, L. A., Silver, J., Alexander, W. S., Speed, T. P., Nicola, N. A., and Hilton, D. J. (2006) J. Biol. Chem. 281, 11135–11143
20. Karlsen, A. E., Ronn, S. G., Lindberg, K., Johannesen, J., Galsgaard, E. D., Pociot, F., Nielsen, J. H., Mandrup-Poulsen, T., Nerup, J., and Billestrup, N. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12191–12196
21. Stoiber, D., Kvarik, P., Cohn, S., Johnston, J. A., Steinlein, P., and Decker, T. (1999) J. Immunol. 163, 2640–2647
22. Lang, R., Paulau, A. L., Pargas, E., Takahashi, Y., Mages, J., Ihle, J. N., Rutschman, R., and Murray, P. J. (2003) Nat. Immunol. 4, 546–550
23. Yasukawa, H., Ohishi, M., Mori, H., Murakami, M., Chinen, T., Aki, D., Hanada, T., Takeda, K., Akira, S., Hoshijima, M., Hirano, T., Chien, K. R., and Yoshimura, A. (2003) Nat. Immunol. 4, 551–556
24. Yoshimura, A., Mori, H., Ohishi, M., Aki, D., and Hanada, T. (2003) Curr.
