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*Full Length Research Paper*

# *Mycobacterium tuberculosis* TLR2 agonists LprA, LM and Man-LAM *induce notch1 and socs3* transcription

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***Mycobacterium tuberculosis* employs a number of strategies to subvert host signaling events, leading to its persistence within macrophages. Upon infection, *Mycobacterium bovis* BCG induce the expression of suppressor of cytokine signaling 3 (*socs3*), in a Toll-like receptor 2 (TLR2)-*Notch1*-dependent manner. Purified phosphatidyl inositol di-mannosides (a TLR2 agonist) act as an inducer for the *Notch1-socs3* pathway. This prompted us to analyze other TLR2 agonists seeking for additional molecules that may affect this pathway. We found that lipoprotein LprA, as well as glycolipids lipomannan (LM), and mannose-capped lipoarabinomannan (ManLAM) treatment of murine macrophages resulted in stimulation of *notch1* and *socs3* transcription**.

**Key words:** Mycobacterium tuberculosis, Toll-like receptor 2 (TLR2), *notch1, socs3*, innate immunity.

## INTRODUCTION

*Mycobacterium tuberculosis* employs several strategies in order to survive within infected macrophages, including interference of host signaling pathways (Meena and Rajni, 2010). From macrophage-mycobacteria interaction experiments, it became evident that human and murine macrophages require different stimuli tocontrol mycobacterial growth *in vitro*, with nitric oxide being highly relevant to restrict bacterial replication in murine models (Liu and Modlin, 2008). Recently, the capacity of nitric-oxide-mediated signaling in controlling mycobacterial induction of *Notch1* expression has been documented (Bansal et al., 2009b; Kapoor et al., 2010). Notch1 induction occurs via a Toll-like receptor 2 (TRL2)-MyD88 pathway when murine macrophage cell line RAW264.7 were infected with *Mycobacterium bovis* BCG (Narayana and Balaji, 2008), and it modulates gene expression (Monsalve et al., 2006), including that of supressor of cytokyne signaling 3 (*SOCS3*) (Narayana and Balaji, 2008), a molecule required to control inflammatory signals triggered by IL6 (Yasukawa et al., 2003). In humans, differences in IL6 secretion in response to mycobacterial lipopeptide antigens or whole bacteria due to TLR6 polymoprphisms may be associated with variation in innate immune responses (Shey et al., 2010). To date, Phosphatidyl Inositol di Mannoside

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**Abbreviations:** ***SOCS3***, Suppressor of cytokine signaling 3; **TLR2,** toll-like receptor 2; **LM,** lipomannan; **LprA,** lipoprotein; **ManLAM,** mannose-capped lipoarabinomannan; **PIM2,** phosphatidyl inositol di mannoside; **COX2,** cyclooxygenase 2; **MMP9,** matrix metalloproteinase 9; **cDNA,** cdeoxyribonucleic acid; **mRNA,** mribonucleic acid; **LSD,** least significant difference; **NICD,** *notch1* intracellular domain; **IL2RA,** interleukin-2 receptor alpha chain.

(PIM2) is the only isolated molecule that has been shown to induce *Notch1* in murine macrophages, activating *SOCS3* (Narayana et al., 2009), as well as cyclooxygenase 2 (COX2) and matrix metalloproteinase 9 (MMP9) (Bansal et al., 2009a). Therefore, considering that PIM2 might not be the only molecule responsible for mycobacterial-mediated induction of *SOCS3* expression in a TLR2-Notch1 dependent-manner, we tested the known TLR2 agonists LprG (Gehring et al., 2004; Drage et al., 2010), LprA (Pecora et al., 2006), PIM2, lipomannan (LM) and mannose-capped lipoarabinomannan (ManLAM) (Drage et al., 2010) for their capacity to stimulate *notch1* and *SOCS3* transcription in murine macrophage.

## MATERIALS AND METHODS

We cultured the murine macrophage-like cell line RAW264.7 (ATCC number TIB-71) in DMEM supplemented with 10% heated-FBS, 200 mM L-glutamine and antibiotics at 37°C in 5% $CO_2$ until confluence was reached. Cells were stimulated for 24 h with *Mycobacterium tuberculosis* antigens PIM2, LM and ManLam (kindly donated by Dr. Germain Puzo) at a concentration of 0, 10 and 20 ng per $1x10^6$ cells/mL/well. Likewise, cells were stimulated with 0, 50 and 500 ng of lipoproteins LprA and LprG [produced as Hig-tag proteins in *E. coli* from either pMRLB43 (LprG) or pMRLB50 (LprA) plasmids]. Samples were negative to LPS content using the E-toxate kit (Sigma Aldrich), which has a sensitivity of 0.015 U/mL of endotoxin. Controls consisted of untreated cells and 0.1% DMSO (antigen diluent for PIM2, LM and Man-LAM), as well as different amounts of LPS. All experiments were performed by in triplicates. After RNA isolation, complementary deoxyribonucleic acid (cDNA) was synthesized using AffinittyScript QPCR cDNA Synthesis kit (Agilent Technologies), and random primers, from total RNA (1-2 µg) for 50 min at 42°C and a 5 min inactivation step at 95°C. messenger ribonucleic acid (mRNA) levels were determined using iQ™ Supermix (Bio-Rad Laboratories) with EvaGreen™ Dye (Biotium) according to manufacturer instructions, on an ABI 7500 Real Time PCR System (Applied Biosystems). The primers used for detecting mRNA were as follows: 18S-F 5'-GCGGTTCTATTTTGTTGGTTTT-3', 18S-R 5'-AGTCGGCATCGTTTATGGTC-3', Notch1-F 5'-TCAGGGTGTCTTCCAGATCC-3', Notch1-R 5'-GTTCTTCAGGAGCACAACAGC-3', SOCS3-F 5'GGACCAAGAACCTACGCATC-3' and SOCS3-R 5' CGCCCCCAGAATAGATGTAG-3'. The 18S amplification was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. Relative expression was calculated and expressed as $2^{-\Delta\Delta Ct}$ as described (Livak and Schmittgen, 2001). On the other hand, RAW264.7 cells stimulated for 24 h were homogenized and 30 µg of protein extract of each condition were separated in 10% SDS polyacrylamide gel, transferred onto nitrocellulose membranes (Amersham) using a Bio-Rad Semi-Dry Electrophoretic Transfer Cell. The membranes were incubated with a panel of primary antibodies [Sigma Aldrich: rabbit polyclonal antibody to actin (residues 20-33), rabbit polyclonal to *Notch1* (residues 2170-2187), mouse monoclonal to NF-kB (clone NF-12, ascites fluid); Abcam: rabbit polyclonal *SOCS3* (residues 200 to the C-terminus of Human SOCS3), *SOCS3* peptide (ab16199), pan-AKT antibody (residues 466-480), rabbit polyclonal to MyD88 (residues 233 to 248) and rabbit polyclonal to c-Jun (N terminal region)] followed by incubation with the secondary antibody anti-mouse/rabbit IgG alkaline phosphatase-conjugated (Sigma Aldrich), and the membranes were visualized by color development with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (USB). As shown in Figure 1A, treatment with the solvent (DMS 0.1%) stimulated to some extent transcription of n*otch1* compared to the 18S *rna* gene. Therefore, in order to consider mycobacterial antigens as inducers of the *notch1-socs3* pathway, fold-change in gene expression must be above this threshold.

### Statistical analysis

Data concerning the mean gene expression in the various groups were analyzed statistically by one-way ANOVA using the Stat Graphics v 7.0 (Statistical Graphics Corp., USA) and Kaleida Graphs v 4.1 (Sinergy software, USA) for Windows. The level of significance was set at p<0.001 at 95% the confidence level for two-tailed tests. Data concerning the mean gene expression in the various groups were analyzed statistically by one-way ANOVA using the Stat Graphics v 7.0 (Statistical Graphics Corp., USA) and Kaleida Graphs v 4.1 (Sinergy software, USA) for Windows. The level of significance was set at p<0.001 at 95% the confidence level for two-tailed tests. After performing a One-way ANOVA test, we conducted a least significant difference (LSD) test at 95% confidence using 0.1% DMSO as reference. after performing a One-way ANOVA test, we conducted a least significant difference (LSD) test at 95% confidence using 0.1% DMSO as reference.

## RESULTS AND DISCUSSION

In this study, we observed that *notch1* transcription was significantly different (*P*=0.05) in the presence of Man-LAM (10 ng/mL, Figure 1A). Similarly, transcription of *socs3* compared to the *18S rna* gene was significantly stimulated (*P*=0.001) in the presence of LprA (50 ng/mL) LM (10 and 20 ng/mL), and Man-LAM (10 ng/mL, Figure 1B). In some instances, increased antigen concentration (LM and Man-LAM for *Notch1*, and Man-LAM for *SOCS3*) resulted in decreased stimulation in gene transcription, although with no statistical meaning. We observed greater induction with mycobacterial lipid antigens than that produced when stimulation was performed with lipoproteins. As our lipoproteins were produced in *Escherichia coli*, they may lack full and mycobacterial-like acylation, as has been demonstrated for LprA (Pecora et al., 2006). When cells were treated with lipid antigens LM and Man-LAM, *Notch1* transcription was highly induced (15 to 50-fold depending of dose and compound used, Figure 1), whereas *SOCS3* transcription did not change as dramatically (10 to 20-fold), results in agreement with those obtained by Narayana et al (Narayana and Balaji, 2008). The expression of the genes under study showed normal distribution in the various groups.

On the other hand, at the protein level, MyD88 was found expressed during the induction with 50 and 500 ng of LprA (Figure 2, lanes 5 and 6). In fact, this result suggests that as occurs with PIM2 (Figure 1B, Narayana

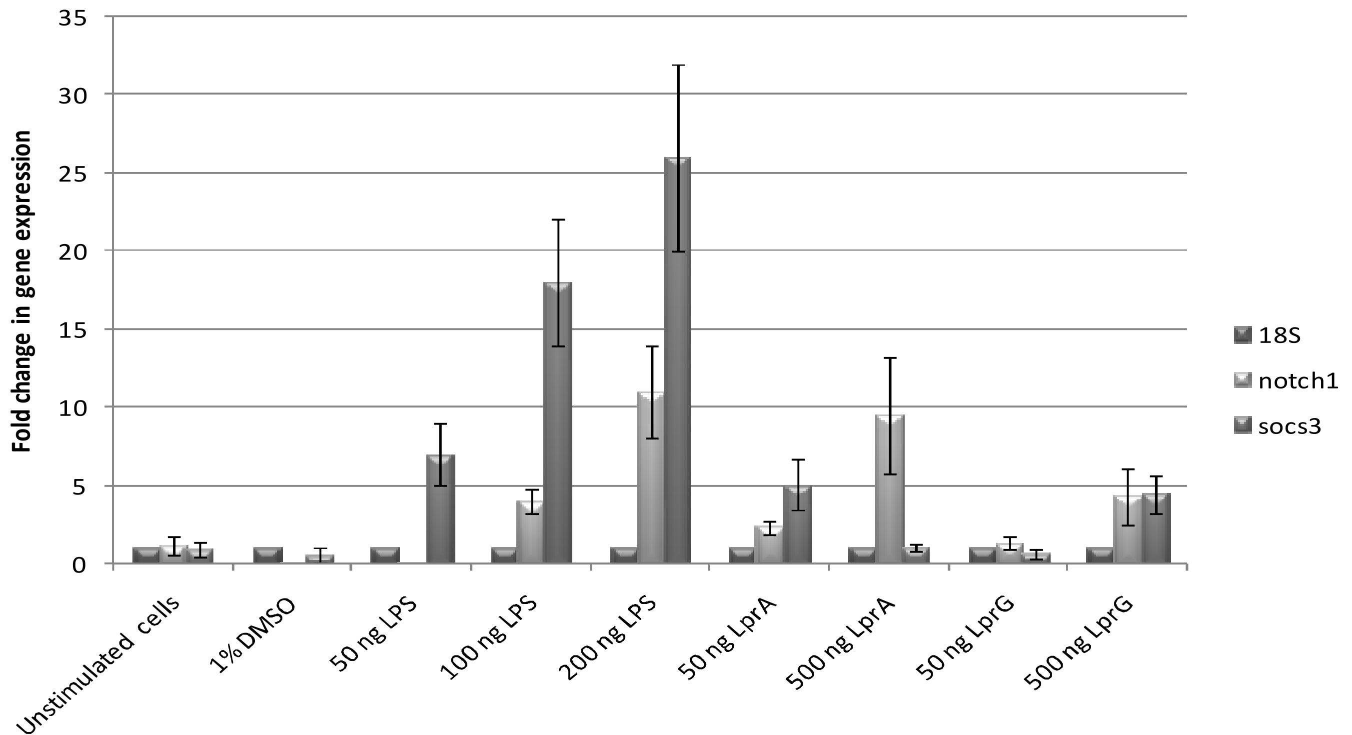


**Figure 1A.** *Mycobacterium tuberculosis* antigens stimulate *notch1* and *socs3* transcription. 18S RNA was used as a reference. The fold-change in expression related to this gene is depicted in the Y-axis. Average of triplicate wells, with corresponding standard deviation results is shown.

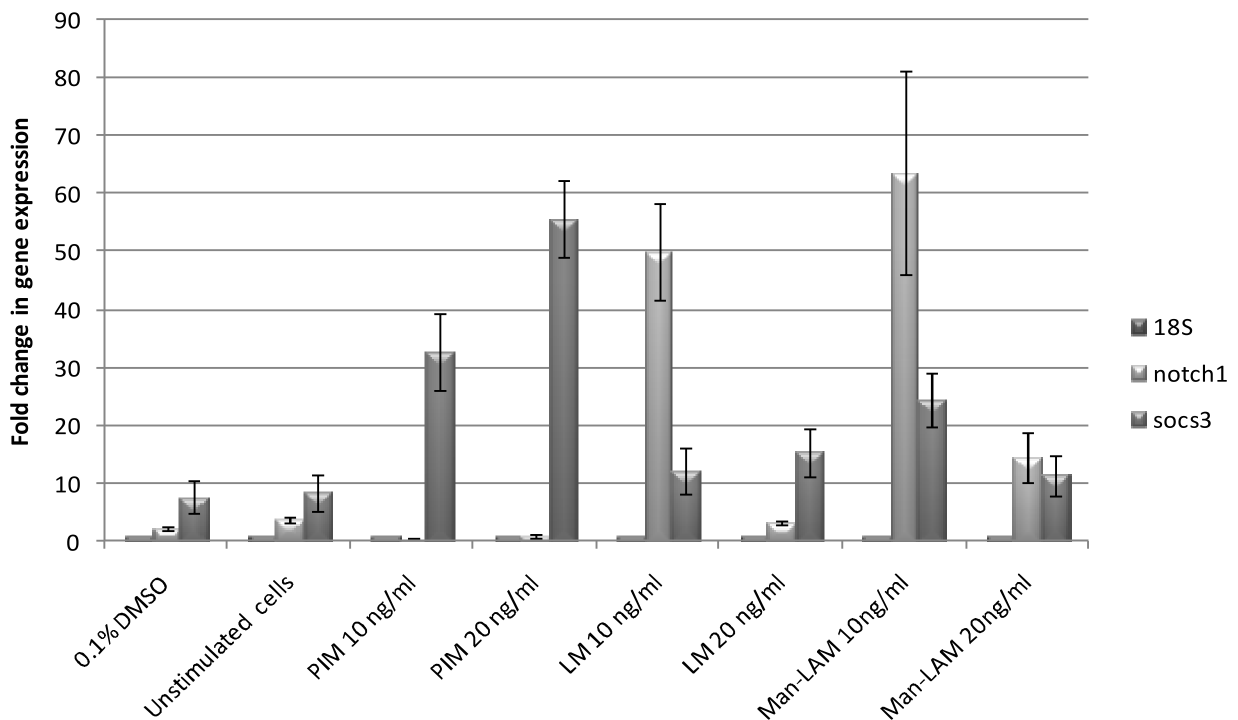


**Figure 1B.** *Mycobacterium tuberculosis* antigens stimulate *notch1* and *socs3* transcription. 18S RNA was used as a reference. The fold-change in expression related to this gene is depicted in the Y-axis. Average of triplicate wells, with corresponding standard deviation results is shown.

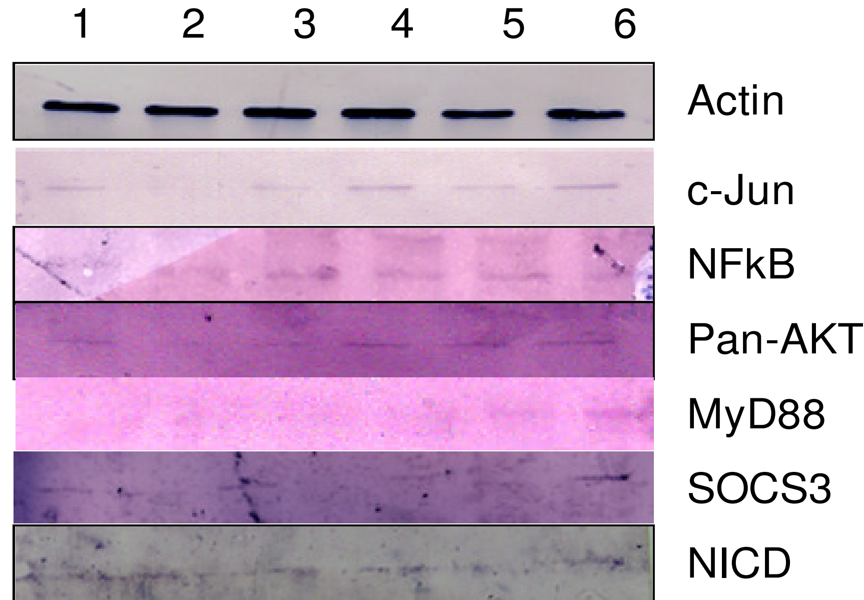


**Figure 2.** Expression profiles of TLR2-downstream molecules in RAW264.7 cells. Lanes 1-3 correspond to cells treated with LprG (0, 50, 500 ng/mL respectively) and lanes 4-6 are cells treated with LprA (0, 50, 500 ng/mL respectively) for 24 h. The β-actin was used as loading control. The results shown represent independent experiments in triplicate

et al., 2009), LprA induced *SOCS3* transcription possibly in a TLR2-MyD88 dependent-manner, an observation deserving to be experimentally tested. On the other hand, no significant change was detected by Western blot for *Notch1* intracellular domain (NICD), NF-kB, AKT or c-Jun when cells were treated with LprA or LprG (Figure 2) nor PIM2, LM or ManLAM (data not shown. We found a sustained basal protein level of NICD when cells were stimulated with LprA or LprG (Figure 2). Transcription levels similar to those obtained in the present study were found when the gene *gapdh* was used as a reference in real time PCR experiments (Narayana and Balaji, 2008). The lack of correlation between transcript and protein levels in our results could be explained by the fact of *Notch-1* mRNA being likely labile, given it was apparent in BCG-infected macro-phages after 15 min to 2 h, while levels decayed after 4 h of infection (Narayana and Balaji, 2008). We performed all experiments with cells stimulated by 24 h in order to monitor simultaneously transcriptional and translational effects of antigens addition. As for *SOCS-3*, it appears to be more stable because it was detected in BCG-infected cells after 12 and 24 h (Narayana and Balaji, 2008), so unexpectedly we did not detect it in our experiments. This may be due to either difference in the primary antibodies we used or because incomplete stimulation with purified antigens occurs, as opposed to whole bacteria. The first scenario looks very likely in light of *SOCS3* being stimulated in response to 2 or 4 μg/mL of PIM2 (Narayana et al., 2009) (we used 20 ng/mL as maximal concentration), or the 3 μg/mL of LM required for suppressing LPS-induced TNF-α production(Doz et al., 2007).

The relevance of *SOCS3* in tuberculosis (TB) in clinical settings has recently been emphasized by the finding of its lower abundance during TB lymphadenitis in Brazilians (Nicol et al., 2008), as well as having its expression along with that of Janus kinase 3 (Jnk3), interleukin-2 receptor alpha chain (IL2RA), and the proto-oncogene serine/threonine-protein kinase (PIM-1) in peripheral blood T-cells, as possible biomarkers to discriminate TB infection status (Jacobsen et al., 2010). Our work provide evidence of LprA, LM, and ManLAM as modifiers of *notch1-SOCS3* signaling, suggesting their participation in multiple host defense events, which may ultimately lead to the successful residence of mycobacteria in their hosts. We found that LprA, LM, and ManLAM have capacity to induce *notch1* and *socs3* transcription after 24 h stimulation, with no effect at the protein level.

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