THE JOURNAL OF IMMUNOLOGY

Mycobacterium avium Inhibition of IFN-γ Signaling in Mouse Macrophages: Toll-Like Receptor 2 Stimulation Increases Expression of Dominant-Negative STAT1 β by mRNA Stabilization

Gail R. Alvarez, Bruce S. Zwilling and William P. Lafuse

J Immunol 2003; 171:6766-6773; ;
http://www.jimmunol.org/content/171/12/6766

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Mycobacterium avium Inhibition of IFN-γ Signaling in Mouse Macrophages: Toll-Like Receptor 2 Stimulation Increases Expression of Dominant-Negative STAT1β by mRNA Stabilization[1]

Gail R. Alvarez,* Bruce S. Zwilling,† and William P. Lafuse[2]*

Mycobacterial infections of macrophages have been shown to inhibit the ability of the macrophage to respond to IFN-γ. We previously reported that *Mycobacterium avium* infection of mouse macrophages decreases IFN-γ-induced STAT1 tyrosine phosphorylation and STAT1 DNA binding. Because macrophages respond to *M. avium* through Toll-like receptor 2 (TLR2), we determined whether TLR2 stimulation inhibits the response to IFN-γ. Treatment of mouse RAW264.7 macrophages with TLR2 agonists inhibited the induction of IFN-γ-inducible genes by IFN-γ. In contrast to *M. avium* infection, TLR2 agonists did not inhibit the IFN-γ induction of DNA-binding activity of STAT1 and the tyrosine phosphorylation of STAT1α. Instead, IFN-γ induction of RAW264.7 cells treated with TLR2 agonists resulted in an increase in the tyrosine phosphorylation of the dominant-negative STAT1β. TLR2 stimulation of RAW264.7 cells increased both STAT1β protein and mRNA expression, suggesting that the increased STAT1β phosphorylation results from increased STAT1β expression. Because STAT1α and STAT1β mRNA have different 3′ untranslated regions, and 3′ untranslated regions can regulate mRNA stability, we examined the effects of TLR2 stimulation on mRNA stability. TLR2 stimulation of RAW264.7 cells increased the stability of STAT1β mRNA, while not affecting the stability of STAT1α mRNA. The ability of STAT1β to function as a dominant negative was confirmed by overexpression of STAT1β in RAW264.7 macrophages by transient transfection, which inhibited IFN-γ-induced gene expression. These findings suggest that *M. avium* infection of mouse macrophages inhibits IFN-γ signaling through a TLR2-dependent increase in STAT1β expression by mRNA stablization and a TLR2-independent inhibition of STAT1 tyrosine phosphorylation. *The Journal of Immunology,* 2003, 171: 6766–6773.

Interferon-γ, a cytokine produced by activated T and NK cells, plays a key role in host defense mechanisms. Mice with a disrupted IFN-γ gene are more susceptible to intracellular pathogens such as *Leishmania major* (1), *Listeria monocytogenes* (2), and *Mycobacterium tuberculosis* (3, 4). IFN-γ exerts its effects on immunity to intracellular pathogens by activating antimicrobial resistance mechanisms of macrophages (5). IFN-γ induces gene expression by the Janus kinase (JAK)[3]/STAT signaling pathway (6–8). Binding of IFN-γ to its receptor results in phosphorylation of STAT1 by Janus kinases, JAK1 and JAK2. The phosphorylated STAT1 is then translocated to the nucleus to activate gene transcription. STAT1 exists in two forms as the result of alternative RNA splicing, STAT1α (p91) and STAT1β (p84) (9). STAT1α has 38 aa at the C terminus that are absent in STAT1β. Only STAT1α is able to activate transcription of IFN-γ-inducible genes (10, 11), as the C-terminal region of STAT1α is required for interaction with transcriptional coactivator CREB-binding protein (CBP)/p300 (12). Thus, STAT1β is thought to act as a dominant-negative inhibitor of IFN-γ.

Mononuclear phagocytes infected with mycobacteria have reduced ability to respond to IFN-γ, resulting in low expression of MHC II genes and other IFN-γ-induced genes (13–18). Studies from this laboratory (17) have investigated the mechanism involved in the inhibition of IFN-γ signaling by infection with *Mycobacterium avium*. Infected mouse macrophages stimulated with IFN-γ were found to have decreased STAT1 DNA binding and tyrosine phosphorylation. Decreased STAT1 activation was correlated with decreased tyrosine phosphorylation of JAK1, JAK2, and IFN-γ receptor α-chain. We also observed a decrease in the expression of the IFN-γ receptor in *M. avium*-infected macrophages. However, infection of human monocytes with *M. tuberculosis* was shown by Ting et al. (18) to inhibit IFN-γ-induced FcγR1 gene expression, but had no effect on the activation of STAT1 by the JAK/STAT signaling pathway. They observed a reduction in the interaction of STAT1 with the transcriptional coactivator CBP/p300, suggesting that *M. tuberculosis* is affecting the ability of activated STAT1 to induce gene transcription. These studies suggest that mycobacterial infection can interfere with IFN-γ signaling at multiple steps in the JAK/STAT pathway.

The interaction of mycobacterial products with Toll-like receptors (TLR) expressed by the macrophage initiates the proinflammatory response of macrophages and induces antimicrobial activity (19–23). *M. avium* stimulates macrophages through TLR2, while *M. tuberculosis* stimulates through both TLR2 and TLR4 (23–25). The mycobacterial products that interact with TLR2 include lipoarabinomannan, phosphatidylinositolmannan, and a 19-kDa lipoprotein from *M. tuberculosis* (23, 26–28). In the current

Departments of *Molecular Virology, Immunology, and Medical Genetics, and †Microbiology, Ohio State University, Columbus, OH 43210

Received for publication January 30, 2003. Accepted for publication October 7, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants HL59795 and AI45673.

[2] Address correspondence and reprint requests to Dr. William P. Lafuse, Department of Molecular Virology, Immunology, and Medical Genetics, Ohio State University, 333 West 10th Avenue, Columbus, OH 43210. E-mail address: lafuse.1@osu.edu

[3] Abbreviations used in this paper: JAK, Janus kinase; GAS, IFN-γ activation site; IRF1, IFN regulatory factor 1; TGTP, T cell GTP-binding protein; TLR, Toll-like receptor; CBP, CREB-binding protein.

0022-1767/03/$02.00

study, we examined the effect of stimulation of TLR2 on the IFN-γ activation of mouse RAW264.7 macrophages. We found that prior TLR2 stimulation reduces gene expression induced by IFN-γ. TLR2 stimulation did not alter IFN-γ-induced STAT1 DNA binding and phosphorylation of STAT1α. Our studies suggest that TLR2 stimulation instead inhibits IFN-γ-induced gene expression by increasing the expression of the transcriptionally inactive STAT1β through mRNA stabilization. Upon IFN-γ activation, higher levels of STAT1β are tyrosine phosphorylated in the TLR2-stimulated macrophages, reducing the transcriptional response of the macrophage to IFN-γ. Furthermore, the ability of STAT1β to act as a dominant negative was confirmed by overexpressing STAT1β by transient transfection of RAW264.7 cells, which reduced gene expression induced by IFN-γ.

Materials and Methods

Reagents

FBS was purchased from Harlan Bioproducts for Science (Indianapolis, IN). Mouse IFN-γ and DNA polymerase (Klenow fragment) were obtained from Invitrogen (Carlsbad, CA). Actinomycin D was obtained from Sigma-Aldrich (St. Louis, MO). [^{32}P]dCTP (3000 Ci/mmol) was obtained from Amersham (Piscataway, NJ). Pam_3CSK_4 was acquired from EMC Microcollections (Tübingen, Germany). Lipoarabinomannan purified from *Mycobacterium smegmatis* and 19-kDa lipoprotein purified from *M. tuberculosis* were obtained from the National Institutes of Health Tuberculosis Research Materials Contract AI-75320, Colorado State University (Fort Collins, CO).

Mycobacteria

M. avium (ATCC 35713) was initially passed once in mice and then cultured in Middlebrook 7H9 broth (Difco, Detroit, MI) to mid-log phage, aliquoted in 1-ml amounts at 2×10^8 CFU/ml, and stored frozen in 10% glycerol at −80°C.

Mice

C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) at 4–6 wk. The mice were used as macrophage donors when 6–10 wk of age.

Cell culture

Murine RAW264.7 macrophages, obtained from the American Type Culture Collection (Manassas, VA), were cultured in DMEM supplemented with 10% FBS, 5 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Mouse peritoneal macrophages were obtained by lavage of mice that had been injected with 4% thioglycolate broth (Difco) 4 days previously. RAW264.7 cells and peritoneal macrophages were seeded in six-well plates at 5×10^6 cells/well. RAW264.7 cells were cultured for 4 h before addition of *M. avium* and TLR2 agonists. Peritoneal macrophages were cultured overnight, nonadherent cells were removed by washing with HBSS, and adherent cells were treated with *M. avium* and TLR2 agonists. The expression of IFN-γ-inducible genes MHC class II Eb and IFN regulatory factor 1 (IRF1) was determined by Northern blot hybridization. RAW264.7 macrophages were cultured for 16 h with TLR2 agonists (lipoarabinomannan, 19-kDa *M. tuberculosis* lipoprotein, Pam_3CSK_4) and *M. avium* (10:1) and stimulated with IFN-γ (200 U/ml) for 20 h. RNA was isolated by the method of Chomczynski and Sacchi (29). The effect of TLR2 agonists on STAT1 mRNA expression was examined by treating RAW264.7 macrophages with TLR2 agonists and isolating RNA at the indicated times. For EMSA and Western blotting, cells treated with TLR2 agonists for 16 h were stimulated with IFN-γ (200 U/ml) for 45 min.

Northern blot hybridization

RNA (15 μg) was separated in 1% formaldehyde agarose gels and transferred to Hybond-N$^+$ membranes by capillary blotting. Northern blot hybridization was performed, as previously described (30). The cDNA probe to IRF1 was obtained by RT-PCR of IFN-γ-stimulated RAW264.7 macrophages using primers 5′-CCAAGAGGAAGCTGTGTGGAG-3′ (sense) and 5′-CAGCAGGCTGTCCATCCACATG-3′ (antisense) (31). cDNA clones of MHC class II Eb, STAT1α, T cell GTP-binding protein (TGTP), and G3PDH were isolated from a cDNA library of IFN-γ-stimulated macrophages, and identity was confirmed by DNA sequencing. The probes were labeled with [^{32}P]dCTP by the High Prime labeling system.

Measurement of STAT1 mRNA stability

To measure the effects of TLR2 agonists on STAT1 mRNA stability, RAW264.7 cells were cultured in six-well tissue plates with/without 2 μg/ml Pam_3CSK_4. After 16 h, transcription was inhibited by the addition of actinomycin D (2 μg/ml). RNA was then isolated at 0, 1, 2, 4, 6, and 8 h. STAT1α and STAT1β mRNA were detected by Northern blot hybridization, as described. Autoradiographs were scanned with a Epson scanner and quantified using SigmaScan Pro 4 (SPSS, Chicago, IL). To account for differences in loading, blots were hybridized with the G3PDH cDNA probe, and STAT1 mRNA signals were normalized with the G3PDH signal. Linear regression analysis of semilogarithmic plots of percentage of STAT1 mRNA remaining vs time after addition of actinomycin D was used to determine mRNA $t_{1/2}$.

EMSA

Nuclear extracts were prepared from 10^7 RAW264.7 cells, as described previously (32). EMSAs were done in 20 μl binding reactions containing 7.5 μg of nuclear protein, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM DTT, 5 mM $MgCl_2$, 10% glycerol, 0.20% Nonidet P-40, 1 μg of poly(dI-dC), and 100,000 cpm of ^{32}P-labeled IFN-γ activation site (GAS) probe. The GAS probe used (5′-AGCCATTTCCAGGAATCGAAA-3′) contains the optimum GAS sequence (TTCCSGGAA) for STAT1 DNA binding (32). The double-stranded GAS oligonucleotide probe was radiolabeled with [^{32}P]dCTP by fill-in reaction with Klenow DNA polymerase. Binding reactions were incubated for 20 min at room temperature and then separated on 5% polyacrylamide gels in 0.50× Tris-borate-EDTA. Gels were dried and analyzed by autoradiography.

Western blot analysis

RAW264.7 cells and peritoneal macrophages were incubated with TLR2 agonists and IFN-γ, as described above. Following treatment with IFN-γ, cells were solubilized in lysis buffer containing 20 mM Tris-HCl, pH 8, 1% Trition X-100, 137 mM NaCl, 10% glycerol, and 20 μl/ml Protease Arrest (Calbiochem, San Diego, CA). Cell debris was removed by centrifugation at 4°C at $14,000 \times g$ for 15 min. Protein concentrations were determined by the Bradford method using Bio-Rad protein assay reagent (Bio-Rad, Richmond, CA). Samples (25 μg) were separated by SDS-PAGE using 10% Tris-glycine gels (Invitrogen), followed by transfer to Immobilon P membranes (Millipore, Bedford, MA). Membranes were blocked in 5% Quickblocker (GenoTech, St. Louis, MO) in TBS containing 0.05% Tween 20 for 1 h and incubated with primary Abs overnight. The detection step was performed with peroxidase-coupled anti-mouse IgG and anti-rabbit IgG Abs (GenoTech; 1:7500). Primary Abs were monoclonal anti-phosphoSTAT1 (Zymed, South San Francisco, CA; 1:2000) and STAT1 p84/p91 rabbit Ab (Santa Cruz Biotechnology, Santa Cruz, CA; 1:3000). Blots were developed with the femtoLucent detection system (GenoTech).

Transient transfection

A STAT1β expression vector was constructed by PCR amplification of the coding region of a STAT1β plasmid obtained from the American Type Culture Collection (I.M.A.G.E. clone 358731) and cloning of the PCR product into the pcDNA 3.1$^+$ expression vector (Invitrogen) using *Bam*HI and *Xba*I sites. Sequence of the pcDNA-STAT1β plasmid was confirmed by DNA sequencing. RAW264.7 cells (2×10^6 cells/well) in six-well plates were transfected with varying amounts of the pcDNA-STAT1β plasmid or pCDNA3.1 vector by lipofection using Lipofectamine Plus (Invitrogen), according to manufacturer's instructions. After overnight incubation, duplicate wells were stimulated with 200 U/ml IFN-γ. After 45 min, cellular lysates were prepared from one set of wells, and phospho-STAT1 was determined by Western blotting, as described above. RNA was isolated from the second set of wells after 8 h, and gene induction by IFN-γ was determined by Northern blotting.

Results

TLR2 stimulation inhibits IFN-γ-induced gene expression

To examine the effects of TLR2 activation on gene induction by IFN-γ, RAW264.7 macrophages were stimulated for 16 h with TLR2 agonists, lipoarabinomannan (26), 19-kDa lipoprotein from *M. tuberculosis* (28), and a synthetic lipoprotein Pam_3CSK_4 (19). The macrophages were then activated for 20 h with IFN-γ. IFN-γ-induced gene expression was assessed by Northern blot hybridization (Fig. 1). Each of the TLR2 agonists completely inhibited IFN-γ induction of MHC class II Eb mRNA, while dramatically

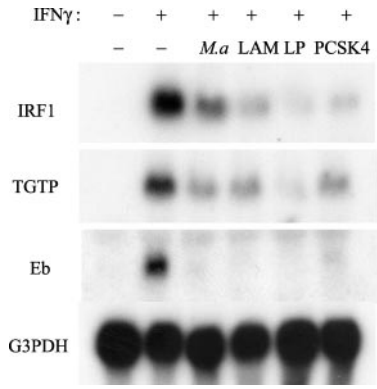

FIGURE 1. Stimulation of TLR2 inhibits gene expression induced by IFN-γ. RAW264.7 macrophages were incubated with *M. avium (M.a*) and TLR2 agonists lipoarabinomannan (LAM), 1 μg/ml; 19-kDa lipoprotein from *M. tuberculosis* (LP), 1 μg/ml; and Pam_3CSK_4, 1 μg/ml (PCSK4) for 16 h and then stimulated with IFN-γ (200 U/ml) for 20 h, as indicated. RNA was isolated and gene induction by IFN-γ was determined by Northern blot hybridization with IRF1, TGTP, Eb, and G3PDH probes. Results represent one of three similar experiments.

inhibiting induction of IRF1 and TGTP, a putative GTP-binding protein (30, 33). The level of inhibition is identical with that observed in *M. avium*-infected macrophages. Eb expression in macrophages is regulated by IFN-γ induction of class II transactivator by STAT1 and IRF1 activation (5). Both IRF1 and TGTP expression are regulated directly by STAT1 activation (5–30).

TLR2 stimulation does not inhibit STAT1 DNA binding

The effects of TLR2 stimulation on IFN-γ-induced STAT1 activation were assessed by EMSA using a double-stranded oligonucleotide containing the consensus GAS sequence for STAT1 DNA binding (34). RAW264.7 macrophages were stimulated with lipoarabinomannan and Pam_3CSK_4 for 16 h and then activated with IFN-γ for 45 min. As shown in Fig. 2*A*, neither of the TLR2 agonists inhibited the STAT1 DNA-binding activity. In contrast, infection with *M. avium* inhibited STAT1 binding, as previously reported (17). Specificity for STAT1 was confirmed by Ab supershift using a STAT1 p84/p91 Ab (Fig. 2*B*).

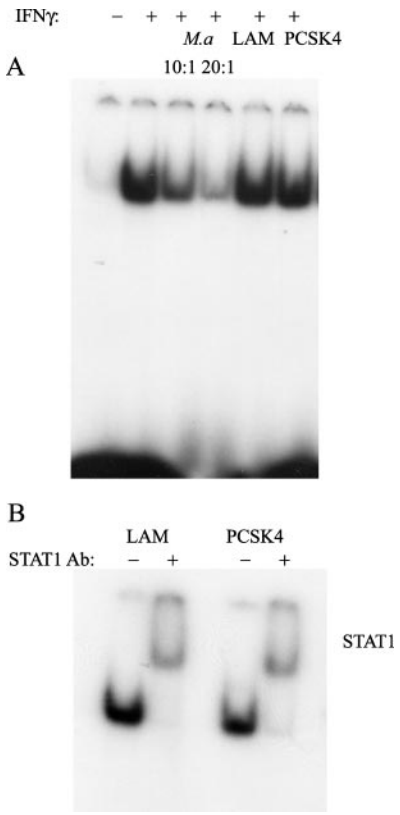

FIGURE 2. Stimulation of TLR2 does not inhibit STAT1 DNA-binding activity. *A*, RAW264.7 macrophages were infected with *M. avium* (*M.a*) at 10:1 and 20:1 bacteria/macrophage or stimulated with TLR2 agonists lipoarabinomannan (LAM), 1 μg/ml, and Pam_3CSK_4 (PCSK4), 1 μg/ml, for 16 h. Cells were then stimulated for 45 min with IFN-γ (200 U/ml). Nuclear extracts were prepared and STAT1 DNA binding was determined by EMSA. Results are representative of three experiments. *B*, Ab supershift experiment with anti-STAT1 Ab and nuclear extracts from RAW264.7 stimulated with lipoarabinomannan (LAM), Pam_3CSK_4 (PCSK4), and IFN-γ.

TLR2 stimulation up-regulates expression and phosphorylation of STAT1β

We used Western blot analysis to study the tyrosine phosphorylation of STAT1α and STAT1β induced by IFN-γ in RAW264.7 macrophages stimulated for 16 h with TLR2 agonists. As shown in Fig. 3*A*, IFN-γ activation of unstimulated RAW264.7 macrophages resulted in phosphorylation of predominantly STAT1α. The ratio of phosphorylated STATα to phosphorylated STAT1β was 5:1 (Fig. 3*C*). In RAW264.7 macrophages stimulated with the TLR2 agonists, IFN-γ induced the same level of STAT1α phosphorylation as unstimulated RAW264.7 macrophages (Fig. 3, *A* and *B*). In contrast, there was a 5-fold increase in STAT1β phosphorylation in TLR2 agonist-stimulated RAW264.7 cells. This reduced the ratio of phosphorylated STAT1α/STAT1β to ~1:1 (Fig. 3*C*). A similar increase in STAT1β phosphorylation was also observed in mouse peritoneal macrophages stimulated with Pam_3CSK_4 and lipoarabinomannan (data not shown). In RAW264.7 cells infected with *M. avium* at 10:1, the ratio of phosphorylated STAT1α/STAT1β induced by IFN-γ was also reduced compared with RAW264.7 treated with IFN-γ alone. At the higher ratio of 20:1 bacteria/macrophage ratio, inhibition of tyrosine phosphorylation of both STAT1α and STAT1β was observed (Fig. 3, *A* and *B*), as previously reported. These data provide evidence that TLR2 stimulation shifts the phosphorylation of STAT1 from a predominantly STAT1α phosphorylation in unstimulated cells to equal phosphorylation of the transcriptionally active STAT1α and transcriptionally inactive STAT1β. To further study the effect of TLR2 stimulation on STAT1 phosphorylation, RAW264.7 macrophages were stimulated with increasing concentrations of lipoarabinomannan (Fig. 4). Lipoarabinomannan stimulation had no effect on phosphorylation of STAT1α induced by IFN-γ, but dose dependently increased phosphorylation of STAT1β (Fig. 4, *A* and *B*). The blot was then stripped and reprobed with a p91/p84 STAT1 Ab to detect total STAT1 protein levels. Lipoarabinomannan stimulation increased the protein level of STAT1β by ~2-fold, while only slightly increasing STATα protein levels (Fig. 4, *A* and *C*).

TLR2 stimulation increases STAT1β mRNA levels by stabilizing STAT1β mRNA

Because the Western blot analysis showed that protein levels of STAT1β are increased by TLR2 stimulation, we examined the mRNA levels of STAT1α and STAT1β in RAW264.7 macrophages stimulated for 16 h with Pam_3CSK_4. RNA was isolated, and Northern blots were hybridized with a STAT1 cDNA probe that detects both STAT1α and STAT1β mRNA. As shown in Fig. 5, Pam_3CSK_4 increased STAT1β mRNA levels to a greater extent

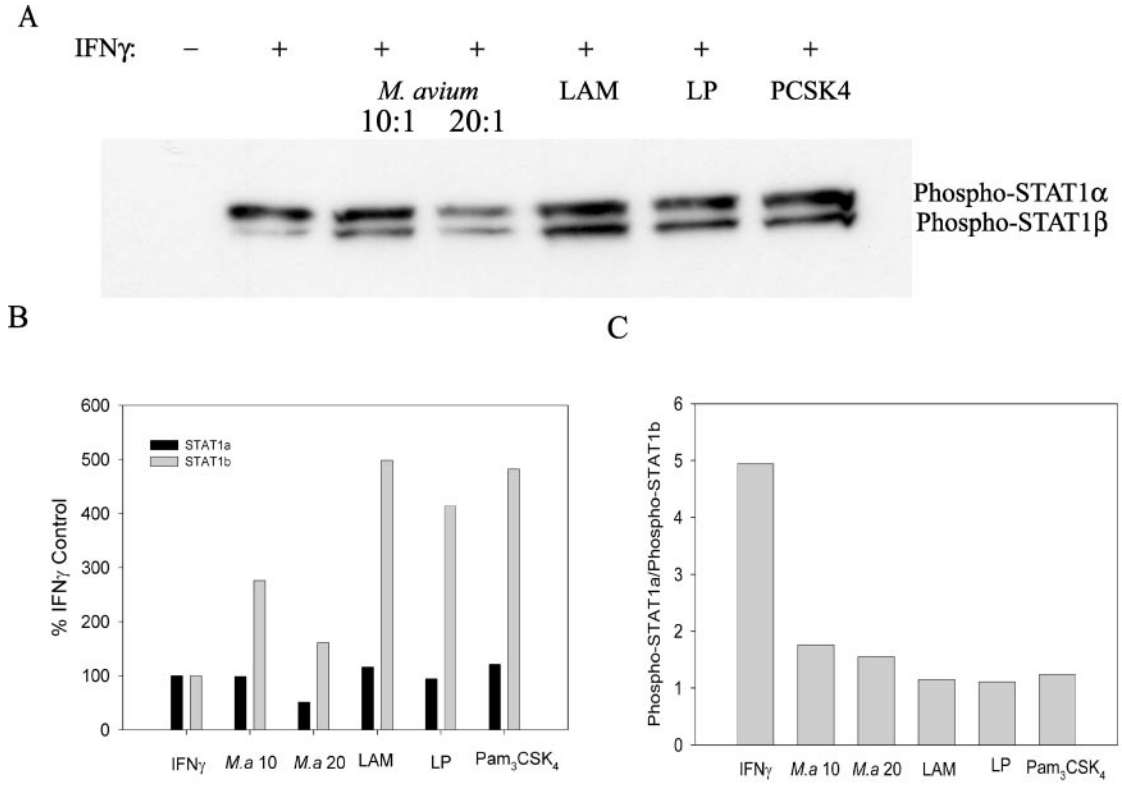

FIGURE 3. TLR2 stimulation increases phosphorylation of STAT1β. *A*, RAW264.7 macrophages were infected with *M. avium* (*M.a*) at 10:1 and 20:1 bacteria/macrophage or stimulated with TLR2 agonists lipoarabinomannan (LAM), 1 μg/ml; 19-kDa lipoprotein (LP), 1 μg/ml; and Pam_3CSK_4 (PCSK4), 1 μg/ml, for 16 h. Cells were then stimulated for 45 min with IFN-γ (200 U/ml). Cell lysates were analyzed for phospho-STAT1 by Western blot. *B*, Densitometry analysis was plotted as percentage of increase in phospho-STAT1α, and phospho-STAT1β levels relative to control cells were stimulated with IFN-γ. *C*, Densitometry analysis plotted as ratio of phospho-STAT1α/phospho-STAT1β. This experiment is representative of four separate experiments.

than STAT1α mRNA. Identical results were obtained using lipoarabinomannan (data not shown).

STAT1α and STAT1β are transcribed from the same gene and result from alternate splicing of the transcript (9). Thus, differences in levels of STAT1α and STAT1β mRNA must be posttranscriptionally regulated. As a result of the alternate splicing, STAT1α and STAT1β mRNA differ in the 3′ untranslated region. Because mRNA stability can be regulated by sequences in the 3′ untranslated region of mRNA (35), we examined whether TLR2 stimulation influences the stability of STAT1α and STAT1β mRNA. RAW264.7 macrophages were activated by incubation with Pam_3CSK_4 for 16 h. Actinomycin D was then added to the TLR2-stimulated and control-unstimulated RAW264.7 macrophages. At various times following addition of actinomycin D, RNA was isolated and STAT1 mRNA decay was analyzed by Northern blot hybridization. In control-unstimulated RAW264.7 cells, STAT1α mRNA was more stable than STAT1β mRNA (Fig. 6). TLR2 stimulation did not change the stability of STAT1α mRNA. However, TLR2 stimulation greatly increased stability of STAT1β mRNA. As the result, STAT1β mRNA was more stable in TLR2-stimulated cells than STAT1α.

Transient transfection of RAW264.7 cells with STAT1β inhibits IFN-γ-induced gene expression

The above studies suggest that transcriptionally inactive STAT1β may be acting as dominant-negative inhibitor of IFN-γ in TLR2-stimulated macrophages. However, although transfection studies have shown that STAT1β is transcriptionally inactive, studies have not been done to determine whether STAT1β acts as dominant negative when both STAT1α and STAT1β are expressed. To test whether STAT1β can act as a dominant negative in RAW264.7 cells, RAW264.7 cells were transiently transfected with increasing concentrations of a STAT1β pcDNA expression vector. The transfected cells were stimulated with IFN-γ, and the effect on STAT1 phosphorylation and IFN-γ-induced gene expression was examined (Fig. 7). As shown in Fig. 7, *A* and *B*, transfection with the STAT1β expression vector increased STAT1β phosphorylation, resulting in reduced phosphorylated STAT1α/STAT1β ratios with increasing amounts of the STAT1β expression vector. Transfection with the empty pcDNA3.1 vector had only a minimal effect on STAT1β phosphorylation. Transfection with STAT1β expression inhibited IFN-γ-induced IRF1, TGTP, and MHC class II Eb gene expression in a dose-dependent manner (Fig. 7*C*). At 2 μg of STAT1β expression vector, inhibition was 33% for IRF1, 46% for TGTP, and 47% for Eb (Fig. 7*D*).

Discussion

Studies (13–18) have demonstrated that mycobacterial infections of mouse macrophages and human monocytes inhibit the ability of the macrophage to respond to IFN-γ. In the current study, we report that prior stimulation with TLR2 agonists also inhibits IFN-γ gene induction of several IFN-γ-inducible genes, including the MHC class II Eb gene. Similar inhibition of IFN-γ-induced MHC class II expression by TLR2 agonists 19-kDa lipoprotein and lipoarbinomannan have also been previously reported (36–38). We began these studies to determine whether TLR2 stimulation might account for our previous observation that *M. avium* infection of mouse macrophages inhibits IFN-γ activation of the JAK/STAT

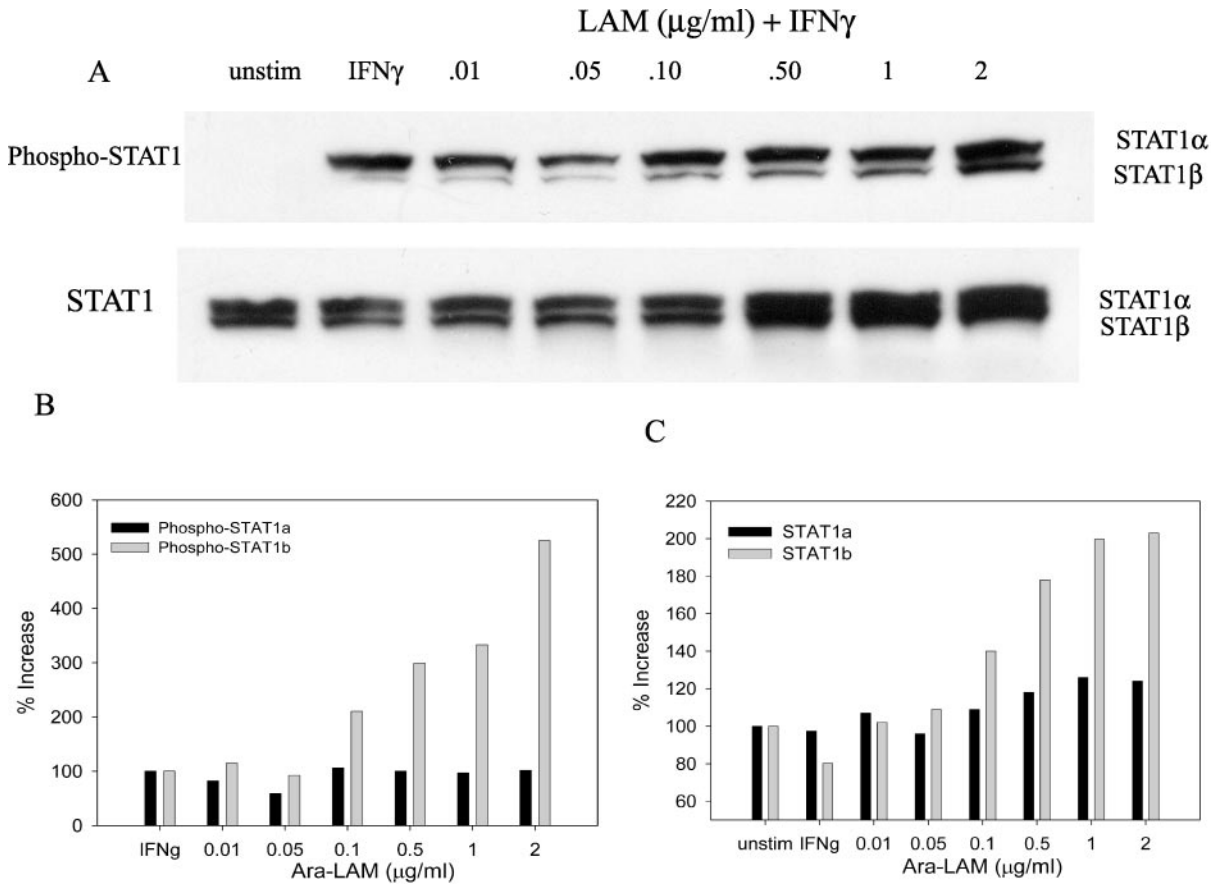

FIGURE 4. Concentration-dependent effect of TLR2 stimulation on phosphorylation of STAT1α and STAT1β. RAW264.7 cells were stimulated with the indicated concentrations of lipoarabinomannan for 16 h and stimulated with 200 U/ml IFN-γ for 45 min. *A*, Cell lysates were analyzed for phospho-STAT1 and STAT1 by Western blots. *B*, Densitometry analysis of the phospho-STAT1 blot plotted as percentage of increase relative to control cells stimulated with IFN-γ. *C*, Densitometry analysis of the STAT1 blot plotted as percentage of increase relative to control-unstimulated cells. Results are representative of three separate experiments.

signaling pathway (17). Studies have shown that TLR2 stimulation accounts for the *M. avium* induction of proinflammatory cytokines in mouse macrophages (19–21). However, in contrast to *M. avium* infection, which inhibited STAT1α tyrosine phosphorylation and DNA binding, TLR2 stimulation did not inhibit STAT1 DNA binding or STAT1α tyrosine phosphorylation. Instead, we found that TLR2 stimulation increased the level of tyrosine-phosphorylated STAT1β. In control RAW264.7 cells stimulated with IFN-γ, the ratio of phosphorylated STAT1α/STAT1β was 5:1. In TLR2 agonist-treated RAW264.7 cells stimulated with IFN-γ, the ratio decreased to near 1:1. STAT1β lacks 38 aa at the C terminus that are required for transcriptional activation. The C terminus is required for interaction of STAT1 with transcriptional coactivator CBP/p300 (12). Overexpression of STAT1β by transient transfection of STAT1-deficient cells results in cells that are not responsive to IFN-γ (10, 11), indicating that STAT1β may act as a dominant negative. This was confirmed in our studies by transient tranfection of RAW264.7 cells with STAT1β, which resulted in partial inhibition of IFN-γ-induced IRF1, TGTP, and class II Eb expression. Thus, the increase in STAT1β tyrosine phosphorylation in TLR2-stimulated RAW264.7 cells is a mechanism by which IFN-γ-induced gene expression is inhibited. However, while overexpression of STAT1β results in partial inhibition of IFN-γ-induced MHC class II Eb expression, stimulation by TLR2 agonists results in almost complete inhibition of IFN-γ-induced Eb gene expression. This suggests another mechanism may also be involved in TLR2 inhibition of MHC class II expression. More in-depth studies of the effects of TLR2 agonists on MHC class II expression are in progress.

M. avium infection at 10:1 also increased STAT1β phosphorylation and lowered the ratio of phosphorylated STAT1α/STAT1β. This suggests that this TLR2 pathway is activated by *M. avium* infection. Inhibition of tyrosine phosphorylation of STAT1α and STAT1β and inhibition of STAT1 DNA-binding activity were observed in RAW264.7 macrophages infected with *M. avium* at 20:1, which is consistent with our previous studies. This inhibition of phosphorylation appears to be independent of TLR2 stimulation and requires a high level of infection. The mechanism responsible for this second pathway of inhibiting IFN-γ-induced gene expression remains unknown, but is also currently under investigation.

Our results in this study with TLR2 agonists are very similar to studies of Ting et al. (18), who showed that infection of human monocytes with *M. tuberculosis* inhibited IFN-γ-induced gene expression. Similarly, JAK/STAT signaling was not inhibited. They observed, using in vitro pull-down experiment with a GAS oligonucleotide, that STAT1 from infected cells bound less of the transcriptional coactivator CBP/p300 than STAT1 from control cells. They concluded that *M. tuberculosis* infection is inhibiting the interaction of STAT1 with the transcriptional coactivator. Because STAT1β is unable to interact with CBP/p300 (12), their observation can be explained by an increase in phosphorylation of STAT1β. Examination of their Western blots shows an increase in STAT1β expression and tyrosine phosphorylation that is comparable to the increase induced in mouse macrophages with TLR2

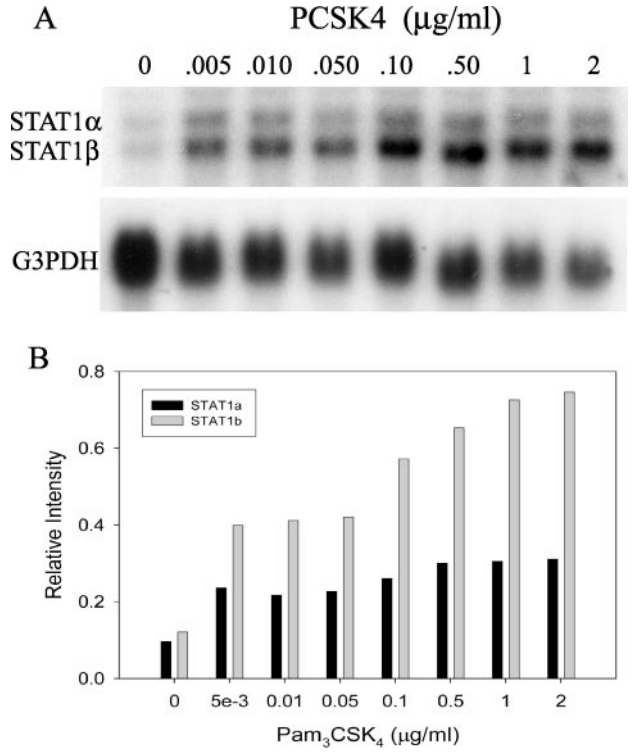

FIGURE 5. TLR2 stimulation increases mRNA level of STAT1β. *A*, RAW264.7 macrophages were stimulated with the indicated concentrations of Pam_3CSK_4 for 16 h. RNA was isolated, and expression of STAT1α and STAT1β mRNA was determined by Northern blot hybridization. The blot was then stripped and probed with G3PDH. *B*, Densitometry analysis plotted as relative intensity after normalization with the G3PDH blot. These data are representative of three separate experiments.

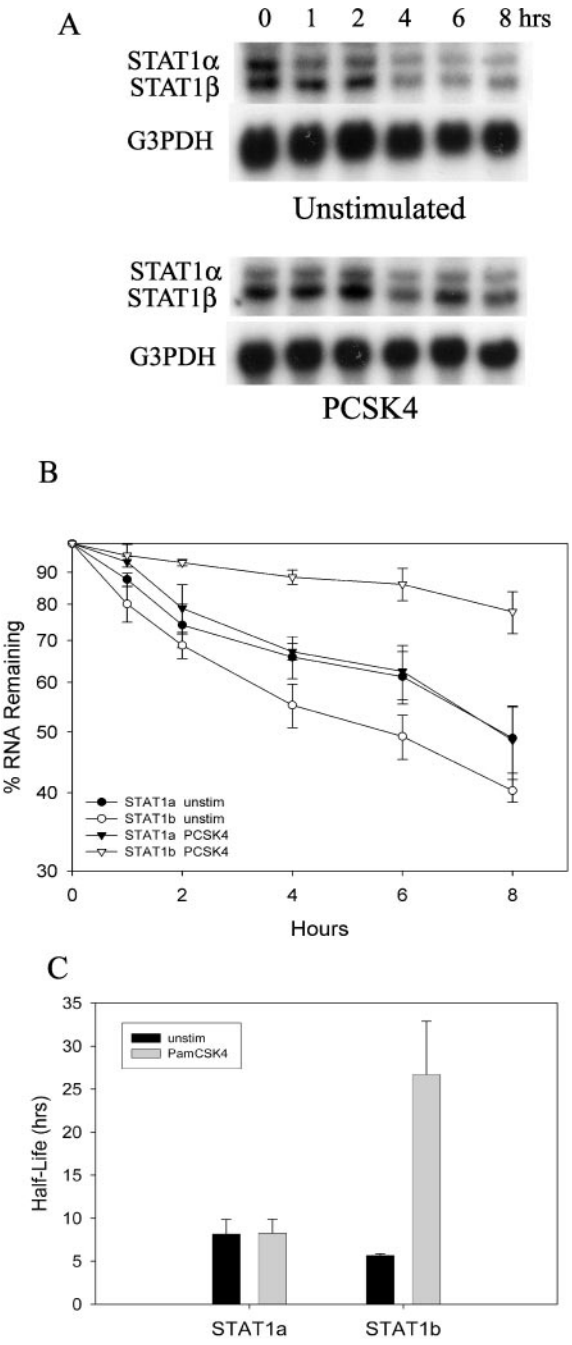

FIGURE 6. TLR2 stimulation increases mRNA stability of STAT1β. *A*, RAW264.7 macrophages were stimulated for 16 h with 2 μg/ml Pam_3CSK_4. Actinomycin D was added to Pam_3CSK_4-stimulated cells and control-unstimulated RAW264.7 cells. RNA was isolated at the indicated times, and mRNA expression was determined by Northern blot hybridization with STAT1 and G3PDH probes. *B*, Decay curves of STAT1α and STAT1β mRNA were determined from densitometry analysis of Northern blots hybridized with STAT1 and G3PDH. The data represent the means $\pm$ SEM of four separate experiments. *C*, $t_{1/2}$ of STAT1α and STAT1β mRNA was determined from the decay curves. The data represent the means $\pm$ SEM of the four experiments. TLR2 stimulation significantly increased STAT1β mRNA $t_{1/2}$ ($p < 0.05$, Student's *t* test).

agonists. Thus, we suggest that TLR2 stimulation of human monocytes by *M. tuberculosis* is also increasing STAT1β expression and that this accounts for the inhibition of IFN-γ-induced gene transcription by *M. tuberculosis* in human monocytes.

We also found in this study that TLR2 stimulation preferentially increases STAT1β mRNA and protein expression. Thus, it is likely that the increased levels of tyrosine-phosphorylated STAT1β, in TLR2 agonist-treated cells, are due to the increase in STAT1β expression. However, STAT1α and STAT1β result from alternative splicing of the same gene transcript (9), thus eliminating transcriptional regulation as a possible mechanism for the differential expression of STAT1α and STAT1β mRNA. We therefore explored whether the differential expression is the result of posttranscriptional regulation. There are two possible pathways by which STAT1α and STAT1β mRNA could be posttranscriptionally regulated. TLR2 stimulation could be affecting the level of expression or activity of the splicing factor involved in generating the STAT1β mRNA transcript. However, the splicing factor has not been identified. As a consequence of the alternative splicing, STAT1α and STAT1β mRNA have different 3′ untranslated regions. Because mRNA stability is often regulated through the 3′ untranslated region (35), the differential expression could result from differences in mRNA stability. In fact, our results show that TLR2 stimulation dramatically increases stability of the STAT1β mRNA, while not affecting the stability of STAT1α mRNA. We suggest that TLR2 stimulation regulates STAT1β mRNA stability by regulating the expression or activity of proteins that bind to the STAT1β mRNA and stabilize the mRNA. Our results do not preclude the possibility that TLR2 stimulation is regulating activity of the splicing factor, because splicing factor ASF/SF2 has been shown to also regulate mRNA stability (39).

In conclusion, infection of macrophages with mycobacteria inhibits the ability of the macrophage to respond to IFN-γ. Several diverse pathways are involved in the inhibition of IFN-γ signaling

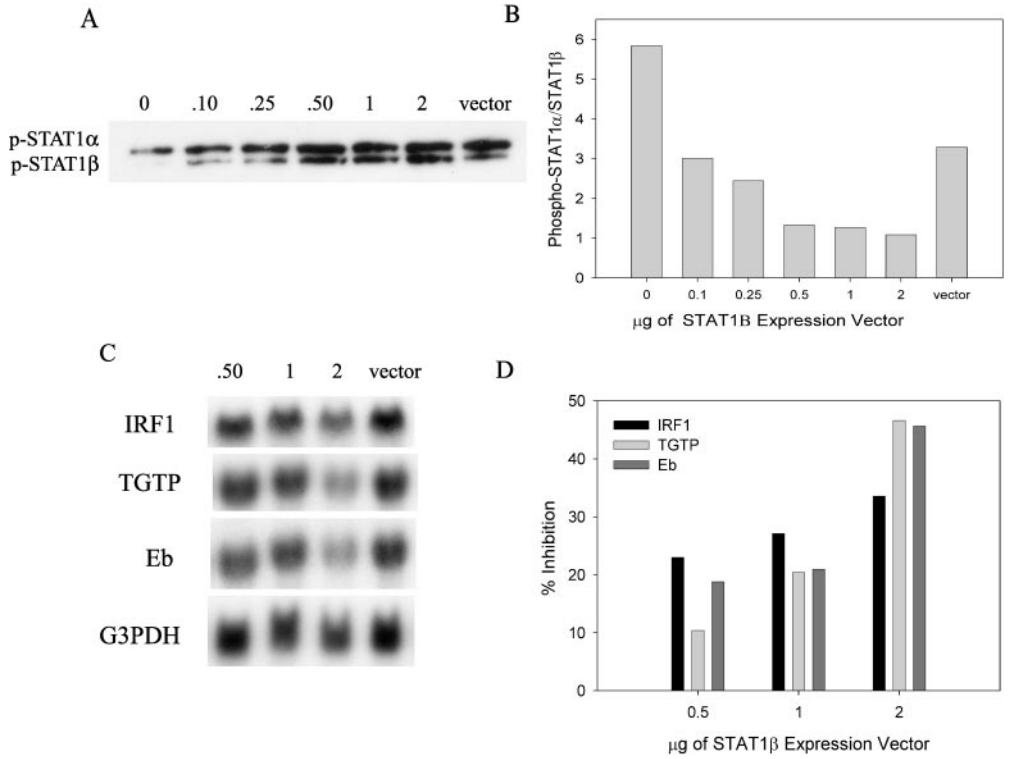

FIGURE 7. Overexpression of STAT1β by transient transfection of RAW264.7 cells results in inhibition of IFN-γ-induced gene expression. RAW264.7 cells were transiently transfected overnight with the indicated amounts of a STAT1β-pcDNA 3.1 expression plasmid or 2 μg of empty pcDNA 3.1 vector. The transfected cells were stimulated with IFN-γ (200 U/ml), and cell lysates were prepared after 45 min and analyzed for phospho-STAT1 by Western blotting (*A*). *B*, Densitometry analysis plotted as ratio of phospho-STAT1α/phospho-STAT1β. *C*, RNA was isolated from a duplicate set of wells after 8 h of stimulation, and gene induction by IFN-γ was determined by Northern blot hybridization with IRF1, TGTP, Eb, and G3PDH probes. *D*, Data plotted as percent inhibition relative to vector alone control. Results represent one of three similar experiments.

by *M. avium* infection of mouse macrophages. One of these pathways is the increase in the expression of the dominant-negative STAT1β by TLR2 stimulation. This occurs through preferential stabilization of STAT1β mRNA. These studies indicate that STAT1β is not just a curiosity of the JAK/STAT pathway, but is a pathway through which IFN-γ-induced gene expression can be down-regulated by pathogen interaction with TLR2. Such a down-regulation of IFN-γ signaling would be to the advantage of a pathogen because it would depress the IFN-γ induction of antimicrobial pathways.

References

1. Wang, Z. E., S. L. Reiner, S. Zheng, D. K. Dalton, and R. M. Locksley. 1994. CD4$^+$ effector cells default to the Th2 pathway in interferon γ-deficient mice infected with *Leishmania major*. *J. Exp. Med. 179:1367*.
2. Harty, J. T., and M. J. Bevan. 1995. Specific immunity to *Listeria monocytogenes* in the absence of IFN γ. *Immunity 3:109*.
3. Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme. 1993. Disseminated tuberculosis in interferon γ disrupted mice. *J. Exp. Med. 178:2243*.
4. Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom. 1993. An essential role for interferon γ in resistance to *Mycobacterium tuberculosis* infection. *J. Exp. Med. 178:2249*.
5. Boehm, U., T. Klamp, M. Groot, and J. C. Howard. 1997. Cellular responses to IFN-γ. *Annu. Rev. Immunol. 15:749*.
6. Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark. 1994. Jak-STAT pathways of transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science 264:1415*.
7. Ihle, J. N. 1996. STATs: signal transducers and activators of transcription. *Cell 84:331*.
8. Schindler, C., and J. E. Darnell, Jr. 1995. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu. Rev. Biochem. 64:621*.
9. Schindler, C., X.-Y. Fu, T. Improta, R. Aebersold, and J. E. Darnell, Jr. 1992. Proteins of transcription factor ISGF-3: one gene encodes the 91- and 84-kDa ISGF3 proteins that are activated by interferon α. *Proc. Natl. Acad. Sci. USA 89:7836*.
10. Muller, M., C. Laxton, J. Briscoe, C. Schindler, T. Improta, J. E. Darnell, Jr., G. R. Stark, and I. M. Kerr. 1993. Complementation of a mutant cell line: central role of the 91 kDa polypeptide of ISGF3 in the interferon-α and -γ signal transduction pathways. *EMBO J. 12:4221*.
11. Bromberg, J. F., C. M. Horvath, Z. Wen, R. D. Schreiber, and J. E. Darnell, Jr. 1996. Transcriptional active Stat1 is required for the antiproliferative effects of both interferon α and interferon γ. *Proc. Natl. Acad. Sci. USA 93:7673*.
12. Zhang, J. J., U. Vinkemeier, W. Gu, D. Chakravarti, C. M. Horvath, and J. E. Darnell, Jr. 1996. Two contact regions between Stat1 and CBP/p300 in interferon γ signaling. *Proc. Natl. Acad. Sci. USA 93:15092*.
13. Kaye, P. M., M. Sims, and M. Feldman. 1986. Regulation of macrophage accessory cell activity by mycobacteria. II. In vitro inhibition of Ia expression by *M. microti*. *Clin. Exp. Immunol. 64:28*.
14. Mshana, R. N., R. C. Hastings, and J. C. Krahenbuhl. 1988. Infection with live mycobacteria inhibits in vitro detection of Ia antigen on macrophages. *Immunobiology 177:40*.
15. Mohagheghpour, N., D. Gammon, A. V. Vollenhoven, Y. Horing, L. E. Bermudez, and L. S. Young. 1997. *Mycobacterium avium* reduces expression of costimulatory/adhesion molecules by human monocytes. *Cell. Immunol. 176:82*.
16. Wojciechowski, W., J. Desanctis, E. Skamene, and D. Radzioch. 1999. Attenuation of MHC class II expression in macrophages infected with *Mycobacterium bovis* bacillus Calmette-Guerin involves class II transactivator and depends on the *Nramp1* gene. *J. Immunol. 163:2688*.
17. Hussain, S., B. S. Zwilling, and W. P. Lafuse. 1999. *Mycobacterium avium* infection of mouse macrophages inhibits IFN-γ Janus kinase-STAT signaling and gene induction by down-regulation of the IFN-γ receptor. *J. Immunol. 163:2041*.
18. Ting, L. A., C. Kim, A. Cattamanchi, and J. D. Ernst. 1999. *Mycobacterium tuberculosis* inhibits IFN-γ transcriptional responses without inhibiting activation of STAT1. *J. Immunol. 163:3898*.
19. Aliprantis, A. O., R.-B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Radolf, G. R. Klimpel, P. Godowski, and A. Zychlinsky. 1999. Cell activation and apoptosis by bacterial lipoproteins through Toll-like receptor-2. *Science 285:736*.
20. Underhill, D. M., A. Ozinsky, K. D. Smith, and A. Aderem. 1999. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. *Proc. Natl. Acad. Sci. USA 96:14459*.
21. Heldwein, K. A., and M. J. Fenton. 2002. The role of Toll-like receptors in immunity against mycobacterial infection. *Microbes Infect. 4:937*.
22. Thoma-Uszynksi, S., S. Stenger, O. Takeuchi, M. T. Ochoa, M. Engele, P. A. Sieling, P. F. Barnes, M. Röllinhoff, P. L. Bölcskei, M. Wagner, et al. 2001.

Induction of direct antimicrobial activity through mammalian Toll-like receptors. *Science 291:1544*.

23. Stenger, S., and R. L. Modlin. 2002. Control of *Mycobacterium tuberculosis* through mammalian Toll-like receptors. *Curr. Opin. Immunol. 14:452*.
24. Means, T. K., S. Wang, E. Lein, A. Yoshimura, D. T. Golenbock, and M. J. Fenton. 1999. Human Toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis*. *J. Immunol. 163:3920*.
25. Lein, E., T. J. Sellati, A. Yoshimura, T. H. Flo, G. Rawadi, R. W. Finbeg, J. D. Carroll, T. Espevik, R. R. Ingalls, J. D. Radolf, and D. T. Golenbock. 1999. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J. Biol. Chem. 274:33419*.
26. Means, T. K., E. Lein, A. Yoshimura, S. Wang, D. T. Golenback, and M. J. Fenton. 1999. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. *J. Immunol. 163:6748*.
27. Jones, B. W., T. K. Means, K. A. Heldwein, M. A. Keen, P. J. Hill, J. T. Belisle, and M. J. Fenton. 2001. Different Toll-like receptor agonists induce distinct macrophage responses. *J. Leukocyte Biol. 69:1036*.
28. Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, et al. 1999. Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. *Science 285:732*.
29. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinum thiocyanate-phenol-chloroform extraction. *Anal. Biochem. 162:156*.
30. Lafuse, W. P., D. Brown, L. Castle, and B. S. Zwilling. 1995. Cloning and characterization of a novel cDNA that is IFN-γ-induced in mouse peritoneal macrophages and encodes a putative GTP-binding protein. *J. Leukocyte Biol. 57:477*.
31. McDowell, M. A., D. M. Lucas, C. M. Nicolet, and D. M. Paulnock. 1995. Differential utilization of IFN-γ responsive elements in two maturationally distinct macrophage cell lines. *J. Immunol. 155:4933*.
32. Ohmori, Y., and T. A. Hamilton. 1993. Cooperative interaction between interferon stimulus response element and κβ sequences motifs controls interferon-γ and lipopolysacharide-stimulated transcription from the mouse IP-10 promoter. *J. Biol. Chem. 268:6667*.
33. Carlow, D. A., J. Marth, I. Clark-Lewis, and H.-S. Teh. 1995. Isolation of a gene encoding a developmentally regulated T cell-specific protein with a guanine nucleotide triphosphate-binding motif. *J. Immunol. 154:1724*.
34. Horvath, C. M., Z. Wen, and J. E. Darnell, Jr. 1995. A STAT protein domain that determines DNA sequence recognition suggests a novel DNA-binding domain. *Genes Dev. 9:984*.
35. Misquitta, C. M., V. R. Iyer, E. S. Werstiuk, and A. K. Grover. 2001. The role of 3′-untranslated region (3′-UTR) mediated mRNA stability in cardiovascular pathophysiology. *Mol. Cell. Biochem. 224:53*.
36. Sibley, L. D., S. W. Hunter, P. J. Brennan, and J. L. Kraenbuhl. 1998. Mycobacterial lipoarbinomannan inhibits γ interferon-mediated activation of macrophages. *Infect. Immun. 56:1232*.
37. Noss, E. H., R. K. Pai, T. J. Sellati, J. D. Radolf, J. Belisle, D. T. Golenbock, W. H. Boom, and C. V. Harding. 2001. Toll-like receptor-2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*. *J. Immunol. 167:910*.
38. Pai, R. K., M. Convery, T. A. Hamilton, W. H. Bloom, and C. V. Harding. 2003. Inhibition of IFN-γ-induced class II transactivator expression by 19-kDa lipoprotein from *Mycobacterium tuberculosis*: a potential mechanism for immune evasion. *J. Immunol. 171:175*.
39. Lemaire, R., J. Prasad, T. Kashima, J. Gustafson, J. L. Manley, and R. Lafyatis. 2002. Stability of a *PKCI-1*-related mRNA is controlled by the splicing factor ASF/SF2: a novel function for SR proteins. *Genes Dev. 16:594*.