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

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

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 stabilization 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)/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 FcyR1 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
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). [32P]dCTP (3000 Ci/mmol) was obtained from Invitrogen (Carlsbad, CA). 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, 100 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM DTT, 5 mM MgCl2, 10% glycerol, 0.20% Nonidet P-40, 1 μg of poly(dI-dC), and 100,000 cpm of 32P-labeled IFN-γ activation site (GAS) probe. The GAS probe used (5'-AGCCATTCGAGGAACTGAAA-3') contains the optimum GAS sequence (TTCCSGGAA) for STAT1 DNA binding (32). The double-stranded GAS oligonucleotide probe was radiolabeled with [32P]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 0.5× Triton X-100. EMSA blots were developed with the femtolumincent detection system (GenoTech).

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, 1% Triton 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 × 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 femtolumincent 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 (Manassas, VA) and cloned into the pcDNA 3.1+ expression vector (Invitrogen) using BamHI and XhoI 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 μl 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 Pam3CSK4 (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
phosphorylated STAT1

M. avium

RAW264.7 cells infected with phosphorylation of both STAT1 ratio of 20:1 bacteria/macrophage ratio, inhibition of tyrosine

A

16 h and then stimulated with IFN-

H9253

TLR2 agonists lipoarabinomannan (LAM), 1 µg/ml; and Pam3CSK4 (PCSK4), 1 µg/ml 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 Pam3CSK4 for 16 h and then activated with IFN-γ for 45 min. As shown in Fig. 2A, 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. 2B).

**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. 3A, IFN-γ activation of unstimulated RAW264.7 macrophages resulted in phosphorylation of predominantly STAT1α. The ratio of phosphorylated STAT1α to phosphorylated STAT1β was 5:1 (Fig. 3C). 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. 3C). A similar increase in STAT1β phosphorylation was also observed in mouse peritoneal macrophages stimulated with Pam3CSK4 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 STAT1α 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 Pam3CSK4. 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, Pam3CSK4 increased STAT1β mRNA levels to a greater extent
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 Pam3CSK4 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. 7C). At 2 μg of STAT1β expression vector, inhibition was 33% for IRF1, 46% for TGTP, and 47% for Eb (Fig. 7D).

**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 lipoarabinomannan 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
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 STAT1/α tyrosine phosphorylation and DNA binding, TLR2 stimulation did not inhibit STAT1 DNA binding or STAT1/α tyrosine phosphorylation. Instead, we found that TLR2 stimulation increased the level of tyrosine-phosphorylated STAT1/β. In control RAW264.7 cells stimulated with IFN-γ, the ratio of phosphorylated STAT1/α/STAT1/β was 5:1. In TLR2 agonist-treated RAW264.7 cells stimulated with IFN-γ, the ratio decreased to near 1:1. STAT1/β 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 STAT1/β by transient transfection of STAT1/α-deficient cells results in cells that are not responsive to IFN-γ (10, 11), indicating that STAT1/β may act as a dominant negative. This was confirmed in our studies by transient transfection of RAW264.7 cells with STAT1/β, which resulted in partial inhibition of IFN-γ-induced IRF1, TGTP, and class II Eb expression. Thus, the increase in STAT1/β tyrosine phosphorylation in TLR2-stimulated RAW264.7 cells is a mechanism by which IFN-γ-induced gene expression is inhibited. However, while overexpression of STAT1/β 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 STAT1/β phosphorylation and lowered the ratio of phosphorylated STAT1/α/STAT1/β. This suggests that this TLR2 pathway is activated by M. avium infection. Inhibition of tyrosine phosphorylation of STAT1/α and STAT1/β 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 STAT1/β is unable to interact with CBP/p300 (12), their observation can be explained by an increase in phosphorylation of STAT1/β. Examination of their Western blots shows an increase in STAT1/β expression and tyrosine phosphorylation that is comparable to the increase induced in mouse macrophages with TLR2

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**FIGURE 4.** Concentration-dependent effect of TLR2 stimulation on phosphorylation of STAT1/α and STAT1/β. 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.
agonists. Thus, we suggest that TLR2 stimulation of human monocytes by *M. tuberculosis* is also increasing STAT1/H9252 expression and that this accounts for the inhibition of IFN-\(\gamma\)-induced gene transcription by *M. tuberculosis* in human monocytes.

We also found in this study that TLR2 stimulation preferentially increases STAT1/H9252 mRNA and protein expression. Thus, it is likely that the increased levels of tyrosine-phosphorylated STAT1/H9252 in TLR2 agonist-treated cells, are due to the increase in STAT1/H9252 expression. However, STAT1/H9251 and STAT1/H9252 result from alternative splicing of the same gene transcript (9), thus eliminating transcriptional regulation as a possible mechanism for the differential expression of STAT1/H9251 and STAT1/H9252 mRNA. We therefore explored whether the differential expression is the result of posttranscriptional regulation. There are two possible pathways by which STAT1/H9251 and STAT1/H9252 mRNA could be posttranscriptionally regulated. TLR2 stimulation could be affecting the level of expression or activity of the splicing factor involved in generating the STAT1/H9252 mRNA transcript. However, the splicing factor has not been identified. As a consequence of the alternative splicing, STAT1/H9251 and STAT1/H9252 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 the stability of the STAT1/H9252 mRNA, while not affecting the stability of STAT1/H9251 mRNA. We suggest that TLR2 stimulation regulates STAT1/H9252 mRNA stability by regulating the expression or activity of proteins that bind to the STAT1/H9252 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-\(\gamma\). Several diverse pathways are involved in the inhibition of IFN-\(\gamma\) signaling...
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**


