THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 52, pp. 42794–42803, DECEMBER 30, 2005

Mycobacterium tuberculosis Lipoarabinomannan-mediated IRAK-M Induction Negatively Regulates Toll-like Receptor-dependent Interleukin-12 p40 Production in Macrophages*

Received for publication, June 14, 2005, and in revised form, August 22, 2005 Published, JBC Papers in Press, November 1, 2005, DOI 10.1074/jbc.M506471200

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Mannose-capped lipoarabinomannans (Man-LAMs) are members of the repertoire of *Mycobacterium tuberculosis* modulins that the bacillus uses to subvert the host innate immune response. Interleukin-12 (IL-12) production is critical for mounting an effective immune response by the host against *M. tuberculosis*. We demonstrate that Man-LAM inhibits IL-12 p40 production mediated by subsequent challenge with lipopolysaccharide (LPS). Man-LAM inhibits LPS-induced *IL-12 p40* expression in an IL-10-independent manner. It attenuates LPS-induced NF-κB-driven luciferase gene expression, suggesting that its effects are likely directly related to inhibition of NF-κB. This is probably because of dampening of the Toll-like receptor signaling. Man-LAM inhibits IL-1 receptor-associated kinase (IRAK)-TRAF6 interaction as well as IκB-α phosphorylation. It directly attenuates nuclear translocation and DNA binding of c-Rel and p50. Man-LAM exerts these effects by inducing the expression of *Irak-M*, a negative regulator of TLR signaling. Knockdown of *Irak-M* expression by RNA interference reinstates LPS-induced IL-12 production in Man-LAM-pretreated cells. The fact that *Irak-M* expression could be elicited by yeast mannan suggested that ligation of the mannose receptor by the mannooligosaccharide caps of LAM was the probable trigger for IRAK-M induction.

Mycobacterum tuberculosis is the causative agent of tuberculosis, which leads to an estimated 2 to 3 million deaths worldwide each year. The organism evades the immune response of the host by manipulating host cell signaling pathways (1). One of the important mechanisms is the suppression of interleukin-12 (IL-12)[3] production. IL-12 is a heterodimeric cytokine composed of two disulfide-linked subunits of 35 (p35) and 40 (p40) kDa encoded by two separate genes (2–4). It is produced by macrophages and dendritic cells in response to infection with bacteria or exposure to bacterial constituents such as lipopolysaccharide (LPS) (2, 4). It is required for cell-mediated immunity and host defense against intracellular microbes (5). IL-12 is critical for the generation of Th1 responses against *M. tuberculosis* infection. In support of this view are the observations that individuals with mutations affecting the genes of *IL-12* (6) or its receptor (7, 8) have increased susceptibility to mycobacterial infections. IL-12-deficient mice are susceptible to *Mycobacterium bovis* bacillus Calmette-Guerin infection (9). This underscores the need to understand how *M. tuberculosis* dampens IL-12 production. Intracellular bacteria and bacterial products regulate *IL-12* production at the transcriptional level (10). The best studied transcriptional regulation of the *IL-12 p40* promoter is mediated by binding of Rel family members effecting induction of a Th1 immune response (11–13). We therefore considered the possibility of *M. tuberculosis* modulins dampening this pathway.

Lipoarabinomannan (LAM) is a cell wall lipoglycan. LAMs have been considered modulins because of their capacity to manipulate the host immune system. To date, three families of LAMs have been described (14). A glycosylphosphatidylinositol anchor, a D-mannan core, and a large terminal D-arabinan represents the core structure. The arabinan domain is capped by either mannosyl (15–18) or phosphoinositol residues (19, 20). The mannose-capped LAMs (Man-LAMs) are found in pathogenic species including *M. tuberculosis* (14). Phosphoinositol-capped LAMs have been isolated from non-pathogenic species such as *Mycobacterium smegmatis*. Phosphoinositol-capped LAMs activate macrophages in a Toll-like receptor (TLR) 2-dependent manner by activating NF-κB-dependent pathways (21). Mycobacterial Man-LAMs on the other hand, modulate the immune response by dampening IL-12 production in macrophages and dendritic cells (22, 23) and attenuating apoptotic signaling (24, 25). The emerging consensus is that Man-LAM contributes to the capacity of *M. tuberculosis* to survive inside macrophages by mediating immunosuppressive effects. Recent studies have demonstrated that lipomannans induce IL-12 production (26) in a TLR-dependent manner and may also inhibit IL-12 production in a TLR-independent manner (27), suggesting that the ratio of lipomannans to Man-LAM may govern the capacity of the organism to circumvent IL-12 production. The anti-inflammatory effects of Man-LAM have been attributed to their binding to the mannose receptor (23) or to DC-specific intercellular adhesion molecule 3 grabbing nonintegrin (28, 29).

Stimulation through TLRs by pathogen-associated molecular patterns, such as LPS triggers production of various cytokines, including IL-12, which is crucial for induction of Th1 responses (10, 31). The resultant inflammatory response is essential for the eradication of infectious microorganisms. However, excessive and prolonged activation of innate immunity is harmful to the host. To prevent such an undesirable outcome, the innate immune system has dampening mechanisms to prevent continued inflammatory signaling in response to repeated challenge by pathogenic stimuli. Dampening of the proinflammatory response may occur by multiple processes, which often lead to limited activation of NF-κB. Four different IL-1 receptor-associated kinases (IRAKs) have been identified. IRAK-M is devoid of kinase activity and is restricted to monocytes/macrophages. It negatively regulates TLR signaling (32, 33). Suppressor *of* cytokine signaling-1 is another inducible

* This work was supported in part by a National Bioscience Award for Career Development grant of the Department of Biotechnology, Government of India (to J. B.). 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] Supported by a fellowship from the Council of Scientific and Industrial Research, Government of India.

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[3] The abbreviations used are: IL-12, interleukin-12; LPS, lipopolysaccharide; LAM, lipoarabinomannan; Man-LAM, mannose-capped lipoarabinomannan; TLR, Toll-like receptor; IRAK, IL-1 receptor-associated kinase; TRAF6, tumor necrosis factor receptor-associated factor-6; TBS, Tris-buffered saline; RT, reverse transcriptase; IRAK-M, interleukin-1 receptor-associated kinase; MR, mannose receptor.

negative regulator of TLR signaling, although its induction occurs only through TLR4 (34, 35). Additional safety systems to control the magnitude of NF-κB activation involve phosphatidylinositol 3-kinase, which negatively regulates TLR signaling and likely functions at the early stages of encounter to pathogens (36, 37).

In this study, we have demonstrated that Man-LAM from *M. tuberculosis* suppresses LPS-induced IL-12 p40 protein production, mRNA expression, and promoter activity in murine RAW264.7 macrophages by inhibiting LPS-induced IRAK-TRAF6 interaction, IκB-α phosphorylation, and nuclear translocation of c-Rel and p50. Silencing of *Irak-M* by RNA interference supports our view that Man-LAM dampens IL-12 p40 production through an IRAK-M-mediated pathway. Whereas the inhibitory effect of Man-LAM on IL-12 production is widely acknowledged, the mechanism has remained obscure. Our studies provide mechanistic insight into how a mycobacterial modulin functions to dampen TLR signaling.

EXPERIMENTAL PROCEDURES

Reagents—Anti-IRAK-M, anti-β-actin, anti-FLAG antibody, anti-FLAG-agarose, cycloheximide, and *Escherichia coli* LPS were from Sigma. Anti-TRAF6 and anti-IRAK1 antibody were from Santa Cruz Biotechnology, and anti-phospho-IκB-α antibody was from Cell Signaling Technology, Beverley, MA.

Cell Culture—The murine macrophage cell line RAW264.7 was obtained from the National Center for Cell Science, Pune, India, and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics.

Mycobacterial Lipids—Endotoxin free Man-LAM from *M. tuberculosis* H37Rv, and phosphoinositol-capped LAM were kindly provided by Dr. John Belisle, Colorado State University, Fort Collins, CO. Lipids were dissolved at a concentration of 1 mg/ml in pyrogen-free water and diluted with medium before each experiment.

Plasmid Constructs—4× NF-κB-luc containing four NF-κB sites in tandem fused to the luciferase reporter gene, was a gift from Dr. A. Brent Carter of the University of Iowa. Murine *IL-12 p40* promoter (−350 to +55) was a gift from Prof. Stephen Smale, Howard Hughes Medical Institute, University of California, Los Angeles, CA.

RNA Isolation and Reverse Transcription (RT)-PCR—Total RNA was prepared from cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. 100 ng of RNA was reverse transcribed using the Titanium One-Step RT-PCR Kit (Clontech). 363 bp of *Irak-M* mRNA and 417 bp of *IL-12* p40 mRNA were amplified by using the primer pairs 5′-TCTTCAGGTGTCCTTCTCCACTGT-3′(sense) and 5′-CCTCTTCTCCATTGGCTTGCTCCCAT-3′(antisense) for *Irak-M*, and 5′-GGAGACCCTGCCCATTGAACT-3′ (sense) and 5′-CAACGTTGCATCCTAGGATCG-3′ (antisense) for *IL-12* p40. Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) was amplified using the primers 5′-CCATCAATGACCCCTTCATTGACC-3′ (sense) and 5′-GAAGGCCATGCCAGTGAGCTTCC-3′ (antisense) to generate a 604-bp product.

Western Blotting—Proteins were separated by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes. The blots were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature and subsequently incubated overnight at 4 °C with primary antibodies in TBS-Tween 20 (1%, v/v) (TBST) with 5% (w/v) bovine serum albumin. Following three washes of 5 min each with TBST, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc.) at a dilution of 1:1,000 in blocking buffer for 1 h at room temperature. After 3 washes with TBST, the blots were developed by chemiluminescence using the phototope-horseradish peroxidase Western Detection Kit (Cell Signaling Technology) and exposed to x-ray film (Kodak X-AR5).

Luciferase Reporter Assays—RAW264.7 cells were transfected with luciferase reporter plasmid constructs using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Transfected cells after treatment were lysed in 25 mM Tris-HCl, pH 7.8, containing 2 mM dithiothreitol, 1% Triton X-100, 4 mM EGTA, 10% glycerol, 20 μg/ml aprotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin, centrifuged, and the supernatant assayed for luciferase activity. Results were normalized for transfection efficiencies by assay of β-galactosidase activity.

Enzyme-linked Immunoassay for IL-12 p40—6×10^4 RAW264.7 cells (plated in each well of a 96-well plate) were either left untreated or treated with Man-LAM for 16 h. Man-LAM was then removed and cells were incubated in fresh medium without or with LPS for a further 24 h. The conditioned medium was removed and assayed for IL-12 p40 by enzyme-linked immunosorbent assay using the murine IL-12 p40 assay kit (BD Biosciences).

Co-immunoprecipitation of TRAF6 and IRAK1—RAW264.7 cells transfected with FLAG-TRAF6 were washed with cold phosphate-buffered saline and lysed for 15 min on ice in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 8, containing 280 mM NaCl, 0.5% Nonidet P-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM dithiothreitol, 1 mM Pefabloc, and 1 μg/ml aprotinin). Cell lysates were clarified by centrifugation for 10 min at 15,000 × *g* in a Microfuge and incubated with 2 μg of anti-FLAG-agarose overnight at 4 °C with gentle rocking. Immunoprecipitates were washed, denatured in SDS gel denaturing buffer, proteins were separated electrophoretically, transferred on to polyvinylidene difluoride membranes, and immunoblotted with anti-IRAK1 antibody.

Western Blotting for Phospho-IκB-α—RAW264.7 cells after treatment were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 5 mM EGTA, 50 mM NaF, 12 mM sodium β-glycerophosphate, 1 mM dithiothreitol, 1 mM Na_3VO_4, 1 mM Pefabloc, and 1 μg/ml aprotinin). Lysates after clarification were separated by 10% SDS-PAGE, electrotransferred on to polyvinylidene difluoride, and immunoblotted with phospho-IκB-α-specific antibody. When required, blots were reprobed with anti-β-actin antibody to confirm equal loading in all lanes.

Generation and Transfection of Small Interfering RNA Constructs for Irak-M—A vector-based system was used to knock down *Irak-M* by RNA interference using the pSuppressor Neo vector (Imgenex Corporation). The target sequences for murine *Irak-M* were 5′-TGTCCCAAGTATTCCAGTA-3′ (S1) and 5′-GTCCTACTGTGATCAGTTT-3′ (S2). Cloning was carried out between the SalI and XbaI sites of the vector. All constructs were verified by sequencing. RNA interference expression vectors or control vector (containing a scrambled sequence from Imgenex that did not show significant sequence homology to rat, mouse or human gene sequences) were transfected into RAW264.7 cells using Lipofectamine (Invitrogen).

NF-κB Assay—Nuclear extracts from cells were prepared using the TransFactor extraction kit (Clontech). After centrifugation for 5 min at 20,000 × *g* at 4 °C, supernatants were assayed for the presence of c-Rel and p50. An equal amount of nuclear lysate was added to incubation wells precoated with the DNA-binding consensus sequence. The presence of translocated NF-κB c-Rel or p50 subunit was then assessed by using the Mercury TransFactor kit (Clontech) according to the manufacturer's instructions. Plates were read at 655 nm.

Statistical Analysis—Statistical significance between groups was evaluated by Student's *t* test. Differences were considered significant at the level of $p < 0.05$.

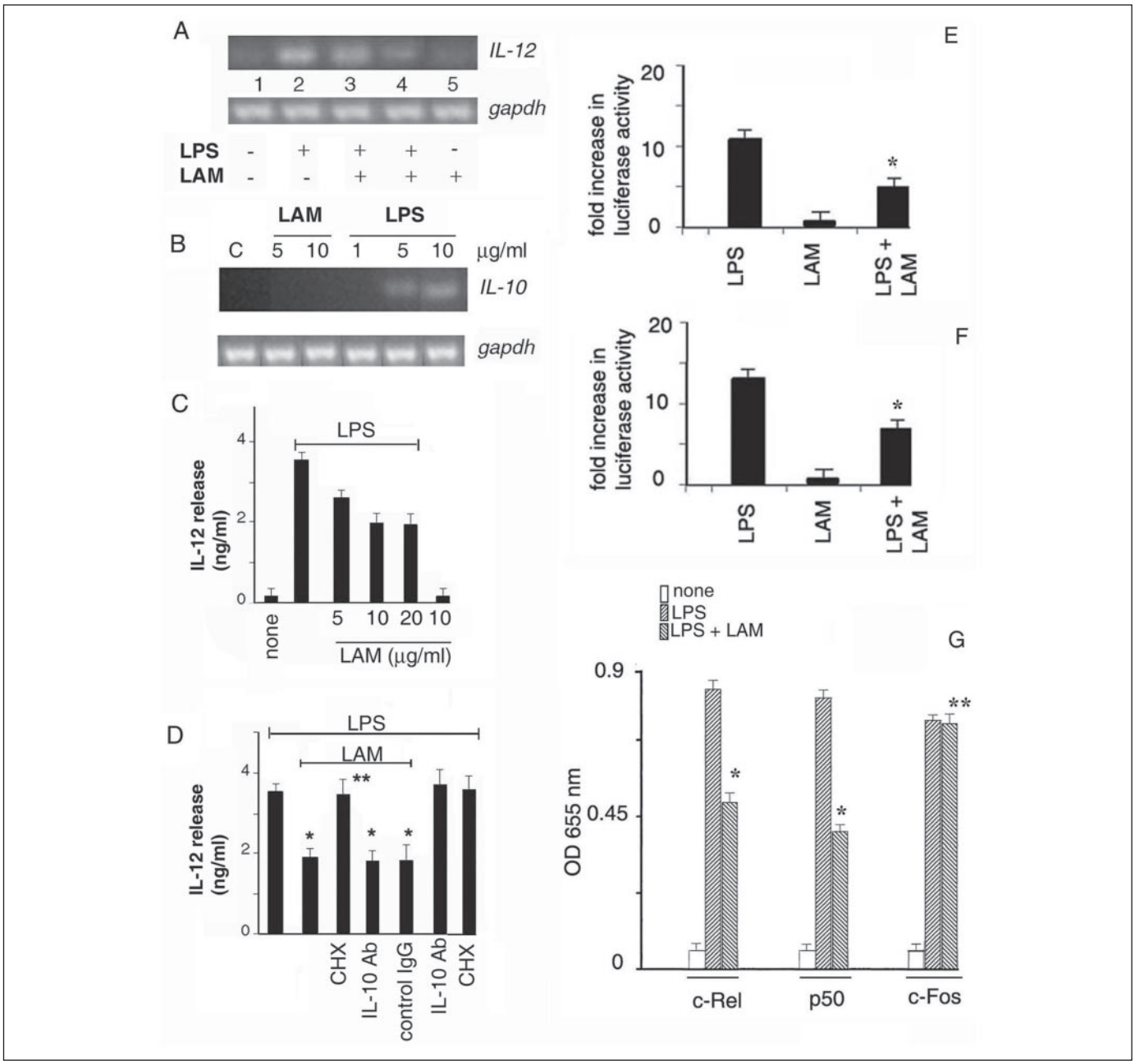

FIGURE 1. **Man-LAM modulates LPS-induced *IL-12 p40* production and inhibits LPS-induced *IL-12* promoter-driven luciferase reporter expression and nuclear translocation of c-Rel/p50 family members in RAW264.7 macrophages.** *Panel A*, *IL-12 p40* mRNA was analyzed by RT-PCR using RNA isolated from cells incubated without (*lane 1*) or with Man-LAM at concentrations of 5 (*lane 3*) and 10 μg/ml (*lanes 4* and *5*) for 16 h followed by incubation without (*lane 5*) or with LPS (1 μg/ml) (*lanes 3* and *4*). Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was used as a control. *Panel B*, cells were left untreated (*C*) or treated either with Man-LAM or with LPS at the indicated concentrations. IL-10 mRNA was analyzed by RT-PCR as described. *Panel C*, RAW264.7 cells were incubated without or with the indicated concentrations of Man-LAM for 16 h followed by treatment without or with LPS(1 μg/ml). *Panel D*, cells were preincubated without or with cycloheximide (*CHX*, 10 μg/ml) for 15 min followed by incubation without or with Man-LAM (10 μg/ml) alone, or Man-LAM in combination with neutralizing IL-10 monoclonal antibody (10 μg/ml) or control IgG for 16 h prior to treatment with LPS as described under *panel B*. Cell-free supernatants were collected and IL-12 p40 was measured by enzyme-linked immunosorbent assay for *panels C* and *D*. Results under *panels A* and *B* are representative of the results obtained in three independent experiments. Results in *panels C* and *D* represent the mean $\pm$ S.D. of three independent experiments. *, $p < 0.001$; **, $p > 0.5$ *versus* cells treated with LPS alone. RAW264.7 cells were transfected with the *IL-12* promoter (-350 to $+55$)-luciferase reporter (*panel E*) or 4$\times$ κB-luciferase (*panel F*) and β-galactosidase constructs. Transfected cells were left untreated, or treated with Man-LAM (10 μg/ml) for 16 h. Cells were subsequently incubated without or with LPS (1 μg/ml) for 16 h. Following incubations, luciferase reporter activities in the cell extracts were measured using luciferin as substrate. In each case, the level of induction of luciferase activity is expressed relative to that of untreated cells. The activities shown represent data that have been normalized with β-galactosidase activity. Results represent the mean $\pm$ S.D. of three independent experiments. *, $p < 0.001$, *versus* cells treated with LPS alone. *Panel G*, cells were left untreated or pretreated with Man-LAM, followed by incubation without or with LPS as described above. Nuclear extracts were prepared, and p50, c-Rel, or c-Fos translocation was measured according to the manufacturer's protocol. Results represent mean $\pm$ S.D. of three independent experiments. *, $p < 0.001$; **, $p > 0.5$ *versus* cells treated with LPS alone.

RESULTS

Man-LAM Inhibits LPS-induced IL-12 p40 mRNA Expression and Protein Release in RAW 264.7 Cells—To determine whether Man-LAM affects *IL-12 p40* expression and release in RAW264.7 murine macrophages, cells were pretreated with Man-LAM, followed by challenge with LPS (1 μg/ml) for 4 (for mRNA expression) or 24 h (for IL-12 release). Pretreatment of cells with Man-LAM inhibited LPS-induced *IL-12 p40* mRNA induction in a dose-dependent manner (Fig. 1*A*) as assessed by RT-PCR. Pretreatment

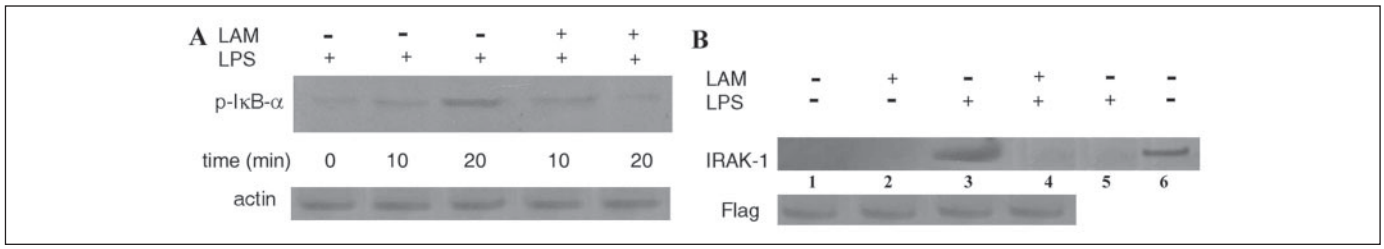

FIGURE 2. **Man-LAM attenuates LPS-induced IκB-α phosphorylation and IRAK-TRAF6 interaction in RAW264.7 macrophages.** *A*, RAW264.7 cells were preincubated without or with Man-LAM (10 μg/ml) for 16 h followed by treatment without or with LPS (1 μg/ml) for the indicated time. The cell lysates were probed with anti-phospho-IκB-α antibody and then reprobed with anti-actin antibody to ensure equal loading of gels. *B*, RAW 264.7 cells were transfected with FLAG-tagged TRAF6 and preincubated with or without Man-LAM (10 μg/ml) followed by treatment with LPS (1 μg/ml). Cell lysates were incubated with anti-FLAG-agarose (*lanes 1–4*) or Protein A/G-agarose alone (*lane 5*). Agarose-bound proteins were separated by SDS-PAGE and immunoblotted with anti-IRAK1 antibody (*lanes 1–6*). Blots were probed with anti-FLAG (*lanes 1–4*) to show loading of gels. *Lane 6* represents a positive control with immunoprecipitated IRAK1 only. Results are representative of those obtained in three independent experiments.

of cells with Man-LAM also resulted in a dose-dependent inhibition of IL-12 p40 release (Fig. 1*C*), in harmony with previous reports (23). Phosphoinositol-capped LAM did not inhibit either LPS-induced IL-12 protein release or *IL-12 p40* mRNA expression (data not shown). The effect of Man-LAM on LPS-induced IL-12 release could be abrogated when cells were pretreated with the protein synthesis inhibitor cycloheximide followed by treatment with Man-LAM (Fig. 1*D*), suggesting that the inhibitory effect of Man-LAM was dependent on *de novo* protein synthesis.

Inhibition of LPS-induced IL-12 p40 Induction by Man-LAM Is Independent of IL-10—IL-10 is a potent inhibitor of *IL-12* gene expression and protein production (38). To assess whether the inhibitory effect of Man-LAM on *IL-12 p40* production was because of IL-10-dependent effects, induction of *IL-10* mRNA was analyzed. As a positive control, *IL-10* mRNA expression was observed by RT-PCR in RAW264.7 cells treated with LPS at concentrations of 5 and 10 μg/ml (Fig. 1*B*), in accordance with the observations of Brightbill *et al.* (39). However, Man-LAM (at concentrations up to 10 μg/ml) did not induce IL-10 release in RAW264.7 macrophages in agreement with previous reports (40). In addition, when RAW264.7 macrophages were treated with Man-LAM in the presence of neutralizing antibody to IL-10, no effect was observed on the ability of Man-LAM to suppress LPS-induced IL-10 production (Fig. 1*D*). We therefore concluded that down-regulation of LPS-induced IL-12 production by Man-LAM is not dependent on IL-10-mediated effects.

Man-LAM Inhibits LPS-induced IL-12 p40 Promoter Activity—To analyze the molecular mechanisms of inhibition of *IL-12 p40* gene expression by Man-LAM, the ability of Man-LAM to modulate LPS-induced *IL-12 p40* promoter-driven luciferase gene expression was determined. Cells were transfected with an *IL-12 p40* promoter (−350 to +55)-luciferase construct, pretreated with Man-LAM for 16 h and then stimulated with LPS for 16 h. Man-LAM inhibited LPS-induced *IL-12* promoter activity by ~50% (Fig. 1*E*), suggesting that Man-LAM attenuated LPS-induced activation of the *IL-12* promoter.

LPS-induced cytokine gene expression in macrophages is the result of signaling through TLR4, a member of the family of pattern recognition receptors. NF-κB is a target of TLR signaling (41–43). This signaling pathway commonly involves MyD88-dependent IRAK activation (44, 45), its phosphorylation and association with TNF receptor-associated factor-6 (TRAF6) leading to IκB kinase activation, IκB-α phosphorylation and degradation, culminating in release of NF-κB facilitating its translocation to the nucleus. NF-κB binding to the *IL-12* promoter synergizes with the binding of other transcription factors to activate the *IL-12* promoter (12). We asked the question whether Man-LAM inhibits NF-κB activation. Cells were transfected with a multimerized NF-κB luciferase reporter plasmid to study the effect of Man-LAM on LPS-induced NF-κB-driven luciferase gene expression. Pretreatment of cells with Man-LAM prior to stimulation with LPS led to a consistent reduction in luciferase reporter activity (Fig. 1*F*), lending support to the view that the inhibitory effect of Man-LAM is likely exerted at the level of NF-κB-dependent gene expression.

Inhibition of DNA Binding of Rel Family Proteins by Man-LAM—LPS stimulates the translocation of p50/c-Rel heterodimers to the nucleus in RAW264.7 macrophages (47) with c-Rel being essential for activation of the p40 gene (48). Considering that Man-LAM inhibits LPS-induced IκB-α phosphorylation, we asked the question whether Man-LAM inhibits nuclear translocation and consequent DNA binding of the Rel family of proteins. Nuclear extracts from cells left untreated or pretreated with Man-LAM followed by challenge with LPS were tested for binding to consensus NF-κB sequences. Binding of c-Rel and p50 was expectedly inhibited in cells pretreated with Man-LAM (Fig. 1*G*). On the other hand, Man-LAM did not influence LPS-induced c-Fos translocation into the nucleus, suggesting that the effect of Man-LAM was specific for Rel family members.

Man-LAM Inhibits IκB-α Phosphorylation in LPS-treated RAW264.7 Cells—In view of the fact that TLR signaling leads to the phosphorylation of IκB-α, prior to its degradation and the release of IκB-α-sequestered NF-κB, we assessed the effect of Man-LAM on LPS-induced IκB-α phosphorylation. LPS-induced IκB-α phosphorylation was found to be significantly reduced in cells pretreated with Man-LAM (Fig. 2*A*), supporting the view that Man-LAM inhibits TLR signaling resulting in decreased IκB-α phosphorylation.

Man-LAM Attenuates the Interaction of IRAK with TRAF6—In the classical pathway of TLR4 signal transduction, the adaptor molecule MyD88 is recruited to the cytoplasmic tail of TLR4 following activation with LPS, followed by recruitment of IRAK. IRAK is then phosphorylated, and leaves the TLR4-MyD88 complex prior to its interaction with TRAF6, a downstream transducer that finally leads to IκB kinase activation, IκB-α phosphorylation, its degradation, and release of NF-κB facilitating its translocation to the nucleus. Considering that Man-LAM was capable of inhibiting LPS-induced IκB-α phosphorylation, we explored the possibility of Man-LAM inhibiting an upstream event along the cascade. The interaction of IRAK and TRAF6 was tested after transfecting cells with FLAG-TRAF6. Transfected RAW264.7 cells were pretreated with Man-LAM prior to challenge with LPS. After treatment, proteins were immunoprecipitated from whole cell lysates with anti-FLAG antibody-bound agarose, and immunoblotted with IRAK antibody. The physical interaction between IRAK and TRAF6 was abrogated in cells pretreated with Man-LAM (Fig. 2*B*). No band corresponding to IRAK was obtained when immunoprecipitation was performed with Protein A/G-agarose only. This result argued in favor of a

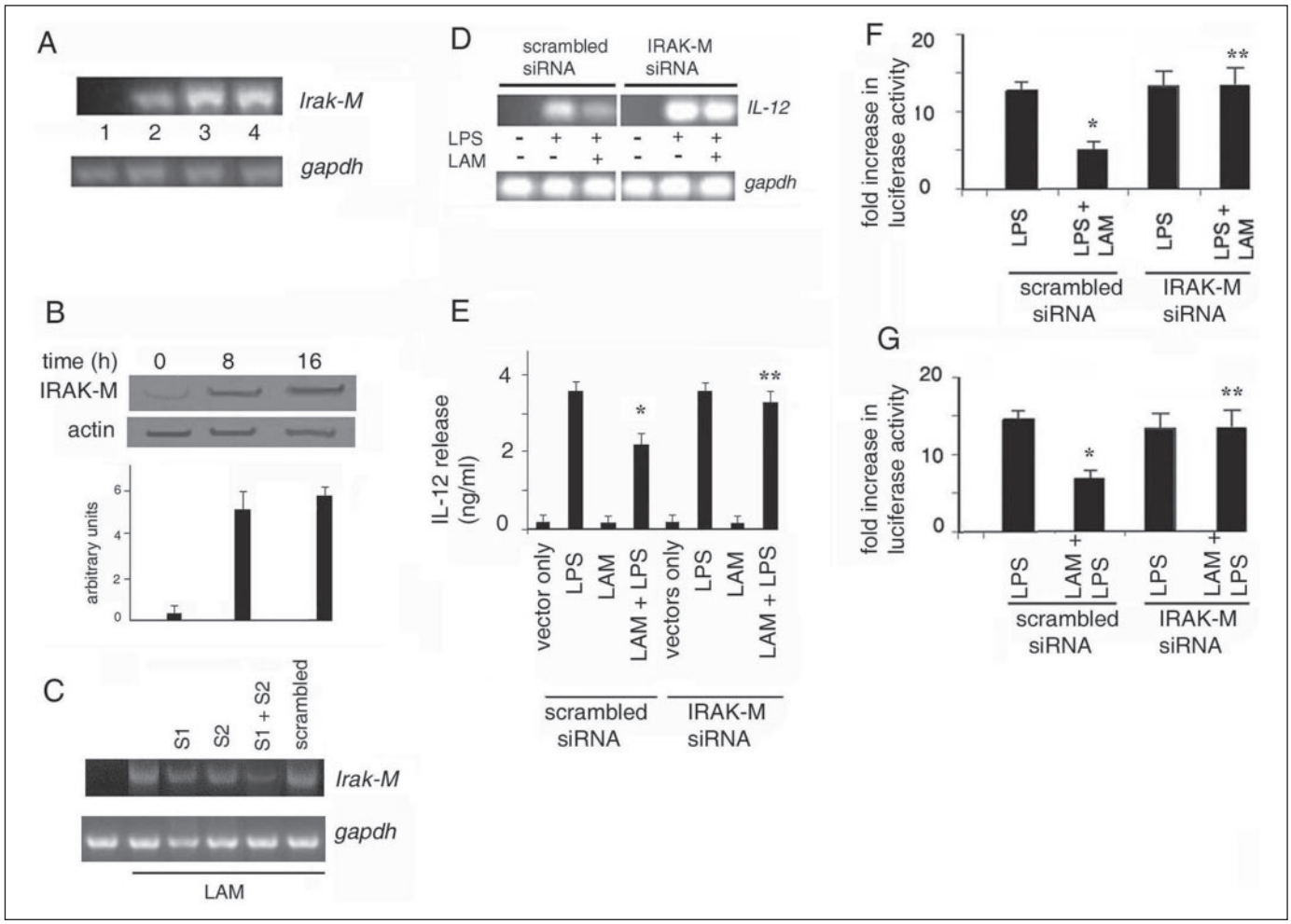

FIGURE 3. **Man-LAM-induced *Irak-M* expression in RAW264.7 macrophages and effects of silencing its expression by RNA interference.** *Panel A*, RAW264.7 cells were either left untreated or treated with Man-LAM at concentrations of 2.5 (*lane 2*), 5 (*lane 3*), and 10 (*lane 4*) μg/ml. *Irak-M* mRNA was quantitated by RT-PCR. *Panel B*, RAW264.7 cells were treated with Man-LAM (5 μg/ml) for the indicated time periods. Total cell lysates were blotted with an antibody specific for IRAK-M. To ensure equal loading, cell lysates were also probed with anti-β-actin antibody. Results are representative of those obtained in three independent experiments. *Bars* represent mean $\pm$ S.D. of three independent experiments. *Panel C*, RAW264.7 cells were either left untreated or transfected with constructs carrying inserts of small interfering RNAs directed against different (S1, S2) sequences of *Irak-M*, alone or in combination; or a construct carrying a scrambled control sequence. Cells were then incubated without or with Man-LAM (5 μg/ml) for 16 h prior to isolation of RNA and analysis of *Irak-M* expression by RT-PCR. *Panels D* and *E*, RAW264.7 cells were transfected with constructs carrying inserts of small interfering RNA (*siRNA*) (S1 + S2) for *Irak-M* or control scrambled sequence. Cells were then pretreated without or with Man-LAM (5 μg/ml) followed by treatment with LPS (1 μg/ml). *IL-12 p40* mRNA was analyzed by RT-PCR (*panel D*) as described under *panel A* and IL-12 p40 release was measured by enzyme-linked immunosorbent assay (*panel E*). *Panels F* and *G*, RAW264.7 cells were transfected with the *IL-12* promoter (-350 to $+55$)-luciferase reporter (*panel F*) or 4$\times$ κB-luciferase (*panel G*) and β-galactosidase constructs; along with constructs harboring inserts of small interfering RNA (*siRNA*) of *Irak-M* (*S1* and *S2*) or scrambled sequence as indicated. Transfected cells were left untreated, or treated with Man-LAM. Cells were subsequently incubated without or with LPS. Following incubation, luciferase reporter activities in the cell extracts were measured using luciferin as substrate. In each case, the level of induction of luciferase activity was compared with that of untreated cells. The activities shown represent data that have been normalized with β-galactosidase activity. Results represent mean $\pm$ S.D. of three independent experiments. *, $p < 0.001$; **, $p > 0.5$ *versus* cells treated with LPS alone.

specific interaction between IRAK and TRAF6 that could be inhibited by Man-LAM.

Man-LAM Induces Irak-M Expression—Because Man-LAM inhibited the IRAK-TRAF6 interaction, we searched for possible involvement of negative modulators of this interaction in Man-LAM-mediated inhibition of NF-κB activation. IRAK-M is a negative modulator of TLR4 signaling that is restricted to monocytes and macrophages (32, 33). Previous studies have reported that IRAK-M can compete with IRAK1 for binding to TRAF6 (33). Because pre-exposure to Man-LAM dampens the release of IL-12 by secondary stimulation with an inducer such as LPS, we reasoned that Man-LAM likely acts in a manner akin to the mechanism of induction of endotoxin tolerance where IRAK-M plays a central role (32). There was a dose-dependent increase in *Irak*-M mRNA levels in cells treated with Man-LAM for 3 h (Fig. 3*A*). In addition, there was a time-dependent increase of IRAK-M protein in cells treated with Man-LAM (Fig. 3*B*).

Specific Down-regulation of Irak-M Abolishes the Inhibitory Effect of Man-LAM on LPS-induced IL-12 Production—We tested whether experimental modulation of *Irak-M* expression would alter Man-LAM-mediated suppression of LPS-induced IL-12 production. RNA interference using a vector-based system was employed for knocking down IRAK-M expression. RAW264.7 cells were transfected with each of the two plasmids containing the *Irak-M* silencing sequences (S1 and S2) individually or in combination, or with a scrambled sequence bearing no homology to any human gene. *Irak-M* gene expression (Fig. 3*C*) and LPS-induced *IL-12* promoter-driven luciferase expression (Fig. 3*F*) were down-regulated in cells transfected with plasmids containing the S1 and S2 inserts, but not with the plasmid containing the scrambled insert. Down-regulation was more pronounced in cells cotransfected with both S1 and S2 bearing inserts, compared with either expression vector used alone. Both the expression vectors were therefore cotransfected in subsequent experiments. The inhibitory effects of Man-LAM

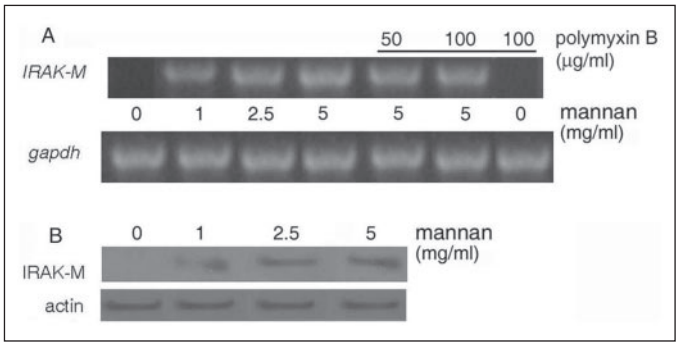

FIGURE 4. **Mannan induces *Irak-M* in RAW264.7 cells.** Cells were left untreated, or treated with polymyxin B (*panel A*, *last lane*), or with different concentrations of yeast mannan in the absence or presence of different concentrations of polymyxin B (where indicated), for 16 h. The *Irak-M* mRNA was quantitated by RT-PCR (*A*) as described above. Total cell lysates were blotted with an antibody specific for *Irak-M* (*B*). To ensure equal loading, cell lysates were also probed with anti-β-actin antibody. Results are representative of those obtained in three independent experiments.

on LPS-induced *IL-12* gene expression (Fig. 3*D*) and IL-12 release (Fig. 3*E*) were abrogated in cells cotransfected with both expression vectors. RNA interference also showed that the effect of Man-LAM in inhibiting NF-κB-driven luciferase gene expression was abrogated by silencing *Irak-M*, suggesting that LAM-induced IRAK-M dampens NF-κB activation (Fig. 3*G*).

Effect of Mannan on IRAK-M Production in RAW264.7 Cells—Man-LAM targets the mannose receptor (MR) in macrophages (48, 49). The MR recognizes glycosylated molecules with terminal mannose, fucose, and *N*-acetylglucosamine moieties (50). Its role in pathogen-triggered signaling is beginning to be appreciated (51). Nigou *et al.* (23) have reported that *Saccharomyces cerevisiae* mannan could inhibit LPS-induced IL-12 release from dendritic cells. To test whether ligation of the MR is involved in IRAK-M production, cells were treated with *Saccharomyces cerevisiae* mannan, a known ligand of the MR. Mannan elicited a dose-dependent increase in *Irak-M* gene (Fig. 4*A*) and protein (Fig. 4*B*) expression. Polymyxin B did not have any bearing on the effect of mannan (Fig. 4*A*), confirming that LPS contamination was not responsible for the observed effect of mannan. This result suggested that interaction of the MR with mannooligosaccharide caps of Man-LAM was likely partly responsible for Man-LAM-mediated *Irak-M* expression. The involvement of receptors other than the MR, as well as moieties of Man-LAM other than the mannooligosaccharide caps, in this process, remains to be explored.

DISCUSSION

Dampening of the Th1 response favors survival of pathogenic mycobacteria within macrophages. It is therefore not surprising that *M. tuberculosis* has evolved strategies to dampen release of cytokines such as IL-12, which are crucial for a Th1 response. A variety of effectors likely acts in a spatially and temporally controlled manner to manipulate host signaling pathways to the benefit of the bacterium. One of the better studied of these mycobacterial modulins is Man-LAM of *M. tuberculosis.* Man-LAMs have clearly emerged as anti-inflammatory molecules inhibiting the production of TNF-α and IL-12 by human macrophages/dendritic cells (52). However, little is known about the underlying mechanisms. In this paper we provide mechanistic insight into Man-LAM-mediated suppression of IL-12 production in RAW264.7 macrophages.

Biologically active IL-12 is a heterodimer composed of two subunits, p40 and p35. The *p35* gene is constitutively expressed, whereas the *p40* gene is expressed only in cells that produce bioactive IL-12 (4). We therefore focused our attention on understanding the regulation of IL-12 p40 production. *IL-12 p40* expression is inducible in macrophages and dendritic cells (53, 54) and is regulated primarily at the transcription level in monocytic cells (55). Using LPS as an agonist of IL-12 production in RAW264.7 cells, we observed that Man-LAM inhibited both the production of IL-12 p40 protein and expression of the *IL-12 p40* mRNA. Multiple control elements have been demonstrated to regulate *IL-12 p40* transcription in response to microbial products including LPS (11, 12, 56, 57). Man-LAM did not induce *IL-10* gene expression. Using neutralizing antibodies against IL-10 we have demonstrated that the inhibitory effect of Man-LAM is not dependent on IL-10. Pretreatment with Man-LAM inhibited LPS-induced *IL-12* promoter-driven luciferase gene expression. Given the important contribution of NF-κB in innate immunity, its direct role in production of cytokines as well as induction of apoptosis as a response of the macrophage encountering a pathogen, microbes have evolved strategies to limit NF-κB activation. The mechanisms through which activation of NF-κB is counteracted has been best studied in viruses (58, 59). We evaluated the possibility of Man-LAM attenuating NF-κB activation. Pretreatment with Man-LAM was indeed found to attenuate LPS-induced 4× κB-driven luciferase gene activation. At the same time, we could demonstrate attenuation of nuclear translocation of p50 and c-Rel, the principal players in LPS-induced NF-κB activation. One of the mechanisms of attenuation of NF-κB activation involves negative regulation of this pathway through the induction of negative regulators such as IRAK-M. In the present study we observed that Man-LAM could elicit *Irak-M* expression in RAW264.7 cells, suggesting that the inhibitory effect of Man-LAM was likely being mediated through this negative regulator of TLR signaling. RNA interference is a mechanism for sequence-specific post-transcriptional inhibition of gene expression via double-stranded RNA molecules. Small interfering RNAs are 21–23-nucleotide long RNA duplex molecules that when transfected into mammalian cells cause inhibition of a variety of signaling molecules (30, 46). This technique was used to specifically knock down the expression of *Irak-M*. The reinstatement of LPS-induced IL-12 production in cells pre-exposed to Man-LAM suggested that induction of *Irak-M* was crucial to the inhibitory effect of Man-LAM.

Repression of IL-12 production may enhance the survival of *M. tuberculosis* against the innate immune response or the developing adaptive immune response. Understanding the mechanism of attenuation of TLR agonist-stimulated IL-12 expression by Man-LAM provides new insight into tuberculosis pathogenesis. Man-LAM triggered induction of IRAK-M could likely contribute to the suppression of a second wave of TLR signaling by giving rise to a tolerant phenotype thereby preventing excessive production of NF-κB-dependent proinflammatory cytokines. Although it has been established for some time that *M. tuberculosis* uses various effectors such as Man-LAM to attenuate IL-12 induction, this is the first mechanistic insight into how this is achieved. Detailed understanding of these processes could help in developing novel therapeutic strategies against tuberculosis.

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Mycobacterium tuberculosis Lipoarabinomannan-mediated IRAK-M Induction Negatively Regulates Toll-like Receptor-dependent Interleukin-12 p40 Production in Macrophages

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J. Biol. Chem. 2005, 280:42794-42800.
doi: 10.1074/jbc.M506471200 originally published online November 1, 2005
