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Lipoarabinomannans Derived from Different Strains of *Mycobacterium tuberculosis* Differentially Stimulate the Activation of NF-κB and KBF1 in Murine Macrophages

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The inflammatory cytokine tumor necrosis factor alpha (TNF-α) is rapidly induced in macrophages after exposure to *Mycobacterium tuberculosis*. Recently it was shown that lipoarabinomannan (LAM) derived from an attenuated (H37Ra) strain of *M. tuberculosis* (AraLAM) was capable of macrophage activation and induction of TNF-α production, whereas LAM derived from the virulent Erdman strain (ManLAM) was considerably reduced in this activity. A critical component in the regulation of many genes central to immune function is the transcription factor NF-κB. Lipopolysaccharide (LPS)-mediated induction of TNF-α expression in murine macrophages has been demonstrated to be regulated in part by NF-κB. In this study, we demonstrate that AraLAM is capable of rapid activation of NF-κB- and KBF1-binding activities in C3H/HeN bone marrow-derived macrophages and the J774.A and RAW264.7 murine macrophagelike cell lines, whereas ManLAM is considerably less potent at stimulating NF-κB. Treatment of RAW264.7 cells with AraLAM or LPS results in the stimulation of DNA binding of both forms within 7.5 min, which peaks within 30 min and 1 h, respectively. Interestingly, treatment of RAW264.7 macrophage-like cells with AraLAM, LPS, or ManLAM for greater than 2 h resulted in significant accumulation of KBF1. Inhibition of protein synthesis blocked the transient nature of NF-κB activation as well as the accumulation of KBF1. Using Western immunodetection of the NFκB1 p50 subunit, we also show that AraLAM and LPS stimulate the loss of the NFκB1 p105 precursor. These results demonstrate that NF-κB and KBF1 are rapidly induced in response to AraLAM and may play a role in avirulent *M. tuberculosis* activation of TNF-α expression in macrophages. The differential temporal regulation of κB element DNA-binding activities and the transient stimulation of NF-κB followed by the sustained accumulation of KBF1 may serve as a feedback switch ensuring transient induction of TNF-α transcription.

After nearly three decades of decline, the number of tuberculosis cases in the United States has increased by 18.4% since 1985, prompting renewed interest in *Mycobacterium tuberculosis* (9). Changes in production of acute-phase proteins, onset of fever, and weight loss characterize this disease. Hemorrhagic tumor necrosis activity due to *Mycobacterium* infection has also been demonstrated, suggesting the involvement of tumor necrosis factor alpha (TNF-α) in the response to *M. tuberculosis* challenge (8). In an elegant study, Kindler et al. (21) determined that localized production of TNF-α at sites of *Mycobacterium bovis* BCG infection was necessary for granuloma formation and subsequent bacterial containment and clearance. Ensuing studies have firmly supported the role of macrophage activation as an integral component in the management of mycobacterial infection, and it has been established that mycobacteria stimulate TNF-α production in mononuclear cells, a function critical if not essential to the elimination of *M. tuberculosis* infection (7, 14, 40, 43, 44).

Analysis of the mycobacterial components mediating the activation of mononuclear cells revealed that lipoarabinomannan (LAM), a cell wall-associated glycolipid produced by *M. tuberculosis*, was capable of inducing TNF-α production and secretion from macrophages (27). More recently, the structural basis of LAM-induced TNF-α production has been investigated (11). With arabinofuranosyl-terminated LAM (AraLAM) derived from the attenuated H37Ra strain of *M. tuberculosis* and heavily mannosylated LAM (ManLAM) derived from the virulent Erdman strain, it was observed that the absence of mannosyl capping of AraLAM translated into a dramatically increased capacity to induce TNF-α. Further analysis of the functional components of LAM with simpler forms of LAM (lipomannan [LM] and phosphoinositol mannan [PIM]) revealed that the critical structure required for modulation of TNF-α mRNA levels was lipid in nature. Such a property was very similar to that of the lipid A component of the gram-negative lipopolysaccharide (LPS) molecule (4, 27).

Mycobacterial AraLAM and gram-negative LPS have been shown to have many overlapping effects, including the induction of reactive oxygen intermediate production, inhibition of gamma interferon stimulation of major histocompatibility complex class I, induction of the chemotactic cytokines JE and KC, and the stimulation of interleukin 1β and TNF-α production (10, 13, 22, 31, 36, 45). It was recently reported that induction of interleukin 1β and TNF-α transcription by both AraLAM and LPS in a macrophagelike cell-line was blocked by monoclonal antibodies against CD14, an LPS receptor, suggesting a shared mechanism of macrophage activation (47). LPS induces transcription of TNF-α in the RAW264.7 macrophagelike cell line (39). Furthermore, it has been clearly shown that LPS induction of TNF-α transcription is mediated in part by the transcription factor NF-κB (12, 35, 42).

NF-κB is a member of the NF-κB/REL/Dorsal (NRD) fam-

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ily of transcription factors and is composed of two subunits, RelA (p65) and NFκB1 (p50) (for recent reviews, see references 2 and 18). NFκB1 p50 is produced as an inactive 105-kDa precursor which must be proteolytically processed into a 50-kDa form that is then capable of DNA binding (17, 25). We have demonstrated that LPS stimulates p105 processing to the active p50 subunit in the RAW264.7 macrophagelike cell line (48). Additionally, LPS rapidly stimulates the nuclear localization of two forms of κB-binding activity in RAW264.7 cells (42, 48). Form I, NF-κB, is a heterodimer composed of p50:p65, whereas form II, KBF1, is a homodimer of p50:p50 initially described as a factor binding to a κB element in the *H-2K*^{b} promoter (20). NF-κB is maintained in the cytosol as an inactive complex coupled to IκB, the inhibitor of NF-κB. Upon LPS stimulation, IκB is posttranslationally modified and rapidly degraded, allowing NF-κB to translocate into the nucleus and modulate transcription. It has been speculated that LPS-stimulated KBF1 is generated by the stimulus-coupled processing of the 105-kDa precursor (48). It is generally supported that NF-κB is the mediator of induced transactivating potential, whereas KBF1 serves in a basal transcription capacity or acts as a repressor by blocking the κB element (18). However, it has been demonstrated that KBF1 can be transcriptionally active in some in vitro transcription assays (15, 19, 23, 33).

Because LPS-mediated induction of TNF-α is modulated in part through the activation of NF-κB, we sought to determine if LAM from attenuated or virulent *M. tuberculosis* would induce nuclear localization of NF-κB. In this report, we demonstrate that AraLAM is capable of rapid and transient induction of NF-κB to the −510 TNF-α κB element at a time course of activation similar to that of LPS. LPS, ManLAM, and AraLAM induced a significant accumulation of KBF1 after 4 h of stimulation. ManLAM derived from the virulent Erdman strain of *M. tuberculosis* was significantly less effective in its ability to activate NF-κB DNA binding. We also demonstrate that AraLAM shares with LPS the ability to enhance depletion of NFκB1 p105.

MATERIALS AND METHODS

Cell culture and reagents. The RAW264.7 and J774.A murine macrophage-like cell lines were maintained in culture as previously described (49). Murine bone marrow-derived macrophages (BMDMs) were cultured from precursor cells obtained from C3H/HeN femur bone marrow by flushing excised bones with a 25-gauge needle attached to a 10-ml syringe containing Dulbecco's modified Eagle's medium. Extracted cells were centrifuged for 10 min, resuspended at 10^5 cells per ml, plated in 100-mm^2 petri dishes, and grown to confluence for 7 days in Dulbecco's modified Eagle's medium supplemented with 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), penicillin-streptomycin, 5% horse serum (Sigma, St. Louis, Mo.), 5% supplemented calf serum (HyClone, Logan, Utah), and 15% L cell-conditioned medium as a crude source of colony-stimulating factor 1. BMDMs were colony-stimulating factor starved for 20 h prior to stimulation by culture in Dulbecco's modified Eagle's medium with 5% supplemented calf serum. For cell stimulation, 10 ng of phenol-extracted, chromatographically purified LPS O111:B4 (Sigma) per ml was resuspended in pyrogen-free water and added for the time period specified. Cycloheximide (CHX [Sigma]), which was processed through Detoxi-Gel (Pierce Chemical Co., Rockford, Ill.) to remove contaminating endotoxin, was used at 25 μg/ml alone or added 5 min prior to stimulation with 5 μg of AraLAM per ml or 10 ng of LPS per ml. Highly purified, pyrogen-free AraLAM, ManLAM, and other *M. tuberculosis* antigens were generously provided by Delphi Chatterjee and Patrick Brennan (Department of Microbiology, Colorado State University, Fort Collins, Colo.), resuspended at 1 mg/ml in endotoxin-free water, and used at the concentrations indicated (generally 5 μg/ml) for the time specified. Prior to experimental use, all reagents were determined to be free of contaminating endotoxin by the E-Toxate assay (Sigma). Cell stimulation was performed by addition of the stimulus to the cells in Dulbecco's modified Eagle's medium with 5% supplemented calf serum followed by incubation at 37°C in 5% CO_2-humidified air for the time specified.

Nuclear and cytoplasmic extract preparation. Nuclear proteins were prepared as described by Schreiber et al. (34) with minor modifications (48). After the treatment indicated, cells were scraped, centrifuged and washed in cold Tris-buffered saline (pH 7.9), and then resuspended in 400 μl of cold buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, 14 μM leupeptin, 1 μM pepstatin, 80 μg of benzamidine per ml). After the cells were allowed to swell on ice for 15 min, the cells were lysed by the addition of Nonidet P-40 (final concentration of 0.6%). Lysis was completed by vigorous vortexing for 10 s. The homogenate was centrifuged for 30 s in a microcentrifuge (13,000 × *g*), and the nuclear pellet was resuspended in 50 μl of cold buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, 14 μM leupeptin, 1 μM pepstatin, 80 μg of benzamidine per ml). This suspension was agitated at 4°C for 15 min with a Vortex-Genie 2 (Scientific Industries, Inc., Bohemia, N.Y.) with a multiple sample head at setting 5, followed by microcentrifugation for 5 min at 4°C. The resulting supernatant was stored in small aliquots at −80°C. Protein concentrations were determined by the method of Bradford (5). For whole-cell preparations, 1.5×10^7 cells were lysed with hot (95°C) 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and placed in a boiling water bath for 10 min.

Gel mobility shift assays. Electrophoretic mobility shift assays (EMSAs) were performed as described by Vincenti et al. (42). The oligonucleotides were end labelled with [α-^{32}P]dCTP with the Klenow fragment of DNA polymerase I, and 30,000 cpm of the probe was used to assess DNA binding. The reaction mixture for DNA-protein interactions contained 5 to 10 μg of nuclear extract in 25 mM HEPES–0.5 mM EDTA–0.5 mM dithiothreitol–0.1 M NaCl–2 μg of poly(dI-dC)–1 μg of bovine serum albumin–1 μg of high-molecular-weight eukaryotic DNA–10% glycerol in a final volume of 25 μl. The radioactive DNA probe was added to this mixture and allowed to preincubate on ice for 15 min. After a subsequent incubation at 37°C for 15 min, the samples were loaded onto a 4% polyacrylamide gel (1× Tris-borate-EDTA) and electrophoresed at 150 V for 4 h. The gel was then dried and analyzed by autoradiography.

The 27-mer probe for the NF-κB EMSAs consisted of the κB site located −510 bp from the start of transcription of the TNF-α gene. The 28-mer TNFiENH probe contains an enhancer located at +1991 in the third intron of the TNF-α gene (38) that consists of the following sequence:

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5′-GATCCAGAGGGTGCAGGAACCGGAAGTG-3′
3′-    GTCTCCCACGTCCTTGGCCTTCACTCGA-5′
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Western immunoblot analysis. Whole-cell lysates were prepared as described above, and samples were fractionated by SDS-PAGE (4% stacking, 8% resolving) and transferred to 0.45-μm-pore-diameter nitrocellulose (Schleicher and Schuell, Keene, N.H.) with Towbin transfer buffer (41). Membranes were processed as described in detail by Zheng et al. (48) with a rabbit anti-50-kDa subunit antibody purified against amino acids 350 to 500, a region sharing no homology with other NRD family members.

RESULTS

Differential dose-dependent activation of NF-κB and KBF1 by attenuated and virulent *M. tuberculosis*-derived LAM treatment of macrophages. LPS-mediated induction of TNF-α is modulated in part through the activation of NF-κB (12, 35). Because LAM and LPS have many overlapping effects on macrophage function, including TNF-α induction, we sought to determine if LAM from attenuated or virulent *M. tuberculosis* would induce nuclear localization of NF-κB. RAW264.7 cells were treated with 10 ng of LPS per ml or increasing concentrations (1, 5, or 10 μg/ml) of AraLAM derived from an attenuated strain (H37Ra) or ManLAM derived from a virulent strain (Erdman) of *M. tuberculosis*. The concentrations of LPS and LAM were chosen on the basis of previously reported optimal doses for maximal production of TNF-α (4, 11). After a 1-h treatment, nuclear extracts were prepared and assayed by EMSA for their ability to bind to the −510 κB element of the murine TNF-α gene (Fig. 1A). A detectable level of nuclear form II, KBF1, was observed in untreated cells. Compared with untreated cells, nuclear extracts from cells stimulated with LPS had significant κB element-binding activity, detected as two distinct forms (Fig. 1A, lanes 2 and 3). We had previously shown that these were the optimal conditions for LPS-mediated activation of the NRD transcription factor family members NF-κB and KBF1 (42, 48). Nuclear extracts from cells treated with increasing concentrations of AraLAM showed similar activation of these two transcription factors (Fig. 1A, compare lane 2 with lanes 4 to 6). In our preliminary studies,

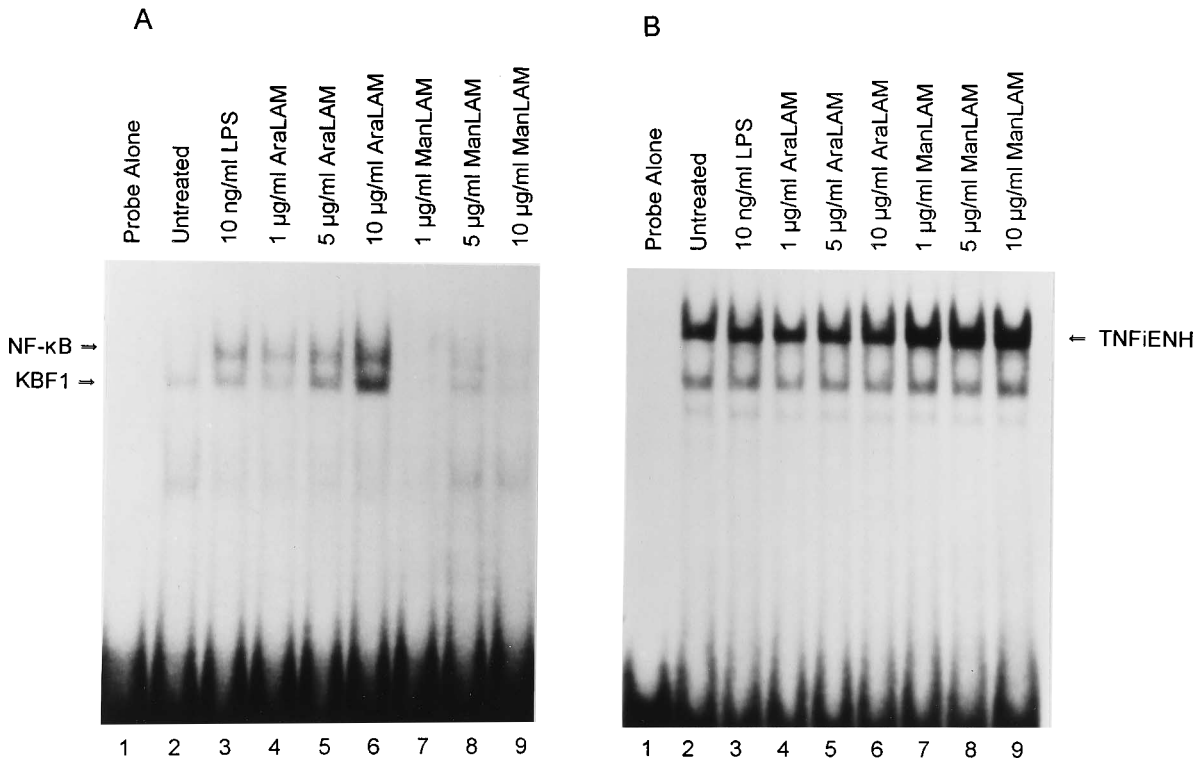

FIG. 1. Differential dose-dependent activation of NF-κB and KBF1 by AraLAM and ManLAM in RAW264.7 cells. Nuclear extracts were prepared from untreated RAW264.7 cells or cells treated with 10 ng of LPS per ml or increasing concentrations of AraLAM or ManLAM for 1 h at 37°C. Nuclear extracts were tested by EMSA with an oligonucleotide containing the −510 κB element of the murine TNF-α gene (A) or an intronic TNF-α enhancer-containing oligonucleotide (B). Lanes: 1, probe alone; 2, untreated; 3, LPS (10 ng/ml); 4, AraLAM (1 μg/ml); 5, AraLAM (5 μg/ml); 6, AraLAM (10 μg/ml); 7, ManLAM (1 μg/ml); 8, ManLAM (5 μg/ml); 9, ManLAM (10 μg/ml). The band migrating between KBF1 and free probe is nonspecific as determined by competition experiments (data not shown). The positions of NF-κB and KBF1 are marked. EMSA reaction mixtures contained 5 μg of nuclear extract and 30,000 cpm of radiolabelled probe. One of four comparable experiments is shown.

stimulation of DNA-binding protein activities with 5 μg of AraLAM per ml was routinely similar to that with 10 ng of LPS per ml, and this concentration was chosen for ensuing studies. Interestingly, ManLAM, although able to activate the two forms of κB-binding activity, was a significantly less-potent stimulus (Fig. 1A, compare lane 3 with lanes 7 to 9). Treatment of RAW264.7 cells with 5 μg of ManLAM per ml resulted in κB-binding activity comparable to that from treatment with 1 μg of AraLAM per ml (Fig. 1A, compare lanes 4 and 8). We repeated this experiment with another murine macrophage-like cell line, J774.A (Fig. 2). As with RAW264.7 cells, AraLAM was a significant activator of κB element DNA-binding activities, with 1 μg of AraLAM per ml stimulating greater activity than 10 ng of LPS per ml. Interestingly, treatment of J774.A cells with ManLAM resulted in a significant activation of κB element DNA-binding activities compared with RAW264.7 cells, greater than 10 ng of LPS per ml (Fig. 2, compare lanes 9 and 8). To determine if ManLAM was capable of NF-κB activation in "real" murine macrophages, we treated BMDMs with 10 ng of LPS per ml, 5 μg of AraLAM per ml, or 5 μg of ManLAM per ml for 1 h and analyzed the nuclear extracts with EMSAs (Fig. 3B). In a manner similar to that of the RAW264.7 cell line, ManLAM was a less-potent activator of NF-κB activity, while 5 μg of AraLAM per ml stimulated DNA-binding activity at a level similar to 10 ng of LPS per ml (Fig. 3B, compare lanes 3, 4, and 5 with lane 2).

To determine if the virulent *M. tuberculosis*-derived ManLAM was toxic to the RAW264.7 cells or in some way interfered with nuclear integrity, we performed a control EMSA with an intronic TNF-α enhancer. The enhancer-binding protein is constitutively active in RAW264.7 macrophage-like cells (6a). When nuclear extracts from LPS-, AraLAM-, or ManLAM-treated cells (Fig. 1A) were analyzed for intronic enhancer-binding activity, no significant difference was detectable (Fig. 1B, lanes 2 to 9), although ManLAM may actually slightly increase the amount of TNFiENH-binding activity. In addition, RAW264.7 cells treated with 5 μg of ManLAM per ml for 30 min and then treated with 10 ng of LPS per ml for 1 h were not refractory to stimulation of both forms of κB element-binding activities (data not shown). These results suggested that the impaired ability of LAM derived from virulent *M. tuberculosis* to stimulate κB-binding activity was not due to toxicity to macrophages.

Analysis of the structural composition of LAM and the relationship to the functional activation of NF-κB and KBF1. Previous studies of the capacity of various mycobacterial cell wall components to stimulate cytokine production revealed the importance of the lipid portion for TNF-α induction, a property shared with the lipid A portion of gram-negative LPS (4, 27). To further characterize the correlation between the ability to stimulate TNF-α production and the capacity to activate κB element DNA binding, RAW264.7 cells and BMDMs were treated with 10 ng of LPS per ml for 1 h or 5 μg each of AraLAM, ManLAM, deacylated AraLAM (dAraLAM), LM (lacks arabinose motifs), or PIM (lacks arabinose and part of the mannan core) per ml for 30 min and nuclear extracts were prepared and analyzed by EMSA (Fig. 3). Treatment of RAW264.7 cells with LPS induced significant binding of both

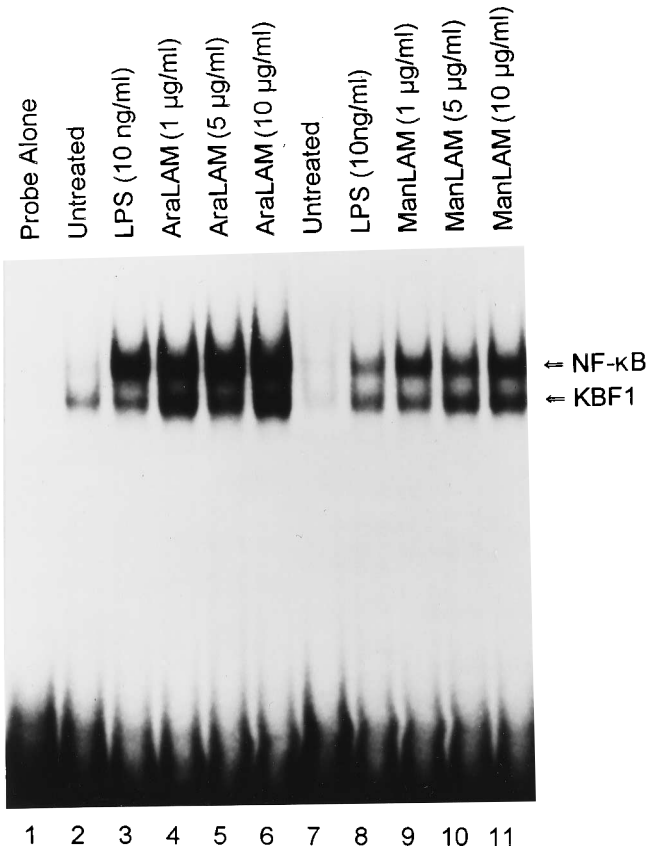

FIG. 2. Differential dose-dependent activation of NF-κB and KBF1 by attenuated and virulent *M. tuberculosis*-derived LAM in J774.A macrophagelike cells. Nuclear extracts were prepared from untreated J774.A cells or from cells treated with 10 ng of LPS per ml or increasing concentrations of AraLAM or ManLAM for 1 h. Nuclear extracts were tested by EMSA with an oligonucleotide containing the −510 κB element of the murine TNF-α gene. Lanes: 1, probe alone; 2 and 7, untreated; 3 and 8, LPS (10 ng/ml); 4, AraLAM (1 μg/ml); 5, AraLAM (5 μg/ml); 6, AraLAM (10 μg/ml); 9, ManLAM (1 μg/ml); 10, ManLAM (5 μg/ml); 11, ManLAM (10 μg/ml). The AraLAM and ManLAM experiments were performed independently. The positions of NF-κB and KBF1 are marked. EMSA reaction mixtures contained 5 μg of nuclear extract and 30,000 cpm of radiolabelled probe. One of two comparable experiments for each LAM is shown.

forms compared with the untreated control (Fig. 3A, lanes 2 and 3). Similarly, treatment with AraLAM stimulated significant binding activity over that of control cells, as did LM and PIM, although these LAM components were less-potent agonists (Fig. 3A, compare lane 2 with lanes 4, 6, and 8). ManLAM and dAraLAM were less-effective inducers of κB element-binding activities (Fig. 3A, compare lane 2 with lanes 5 and 7). These results correlated strongly with those from previous studies demonstrating the ability of AraLAM and, to a lesser extent, LM and PIM to induce cytokine production as measured indirectly by PCR amplification of TNF-α cDNA (4). When extracts from BMDMs treated similarly were examined, a comparable pattern of stimulation of κB element DNA binding was revealed (Fig. 3B). Treatment with AraLAM stimulated significant binding activity over that of control cells, as did LM and PIM, although these LAM components were less-potent agonists (Fig. 3B, compare lane 2 with lanes 4, 6, and 8). ManLAM and dAraLAM were less-potent inducers of κB element-binding activities (Fig. 3B, compare lane 2 with lanes 5 and 7). Interestingly, in stimulated BMDMs, only one form of κB element DNA-binding activity was detected. The demonstration of this ability to induce κB element DNA-binding activities which correlated with the capacity to induce TNF-α transcription with these *M. tuberculosis* antigens was supportive of a role for NF-κB activation in their induction of TNF-α gene expression.

Differential rate of NF-κB and KBF1 activation by H37Ra- and Erdman-derived mycobacterial LAMs and gram-negative LPS. The rate of NF-κB and KBF1 activation by ManLAM, AraLAM, and gram-negative LPS (Fig. 4) was investigated. Nuclear extracts from RAW264.7 cells treated with 5 μg of ManLAM per ml were analyzed for κB element-binding activities (Fig. 4A). Within 7.5 min of treatment (the earliest time tested), activation of both forms was detectable. Binding activity of both forms began to decline at 1 h, although form II, KBF1, began to increase at 2 h and continued to increase through 6 h, which was the last time tested. Treatment with 5 μg of AraLAM per ml stimulated DNA-binding activities, with a kinetics of activation similar to that of ManLAM treatment (Fig. 4B), although the magnitude of the response was significantly greater than in response to ManLAM. Within 15 min of stimulation, the amount of NF-κB was greater than that of KBF1, whereas at 30 min, a maximal amount of both NF-κB and KBF1 was present in the nucleus. By 60 min, binding activity was reduced, with NF-κB activity rapidly declining and KBF1 activity accumulating. Cells were also treated with 10 ng of LPS per ml for up to 6 h, followed by preparation of nuclear extracts and analysis by EMSA (Fig. 4C). Activation of both forms of κB element DNA binding was rapid and detectable at the earliest time tested, 7.5 min. A reproducible initial increase in the stimulation of KBF1 at 7.5 min was followed by a proportionally greater amount of NF-κB over KBF1 from 15 to 30 min. However, compared with AraLAM activation of κB binding, which peaked at 30 min, κB-binding activities in response to LPS activation did not peak until 1 h. In a manner similar to those of both AraLAM and ManLAM, but with slightly slower kinetics, LPS-activated κB binding decreased by 2 h, followed by a substantial accumulation of form II, KBF1, between 4 and 6 h.

These observations demonstrated that AraLAM rapidly activated NF-κB- and KBF1-binding activities, with a maximal response greater than and a time course faster than those of LPS, as tested with the respective optimal (TNF-α-inducing) concentrations. Although the concentration of LAM used was 500 times that of LPS, treatment of macrophages with more than 10 ng of LPS per ml did not result in any additional activation of NF-κB (48). As such, AraLAM routinely appears to be a more-potent stimulus of NRD activities as measured by EMSA. AraLAM and LPS activation of NF-κB peaked between 30 and 60 min, followed by a decline in binding. Interestingly, there was an accumulation of KBF1 that followed this initial decline that continued to increase until 6 h, the last time point tested. The maximal activation of binding activities induced by ManLAM was significantly less than that induced by AraLAM, which was consistent with the data presented in Fig. 1. DNA-binding activities induced by ManLAM were consistently less intense than those of LPS and AraLAM, necessitating overexposure of the autoradiogram; however, the kinetics of activation were similar, although the magnitude of the maximal NF-κB response was significantly decreased. The accumulation of KBF1 at longer time points nevertheless was generally equivalent. The impaired ability of ManLAM to induce NF-κB binding to the −510 κB element in RAW264.7 cells correlated with the inability of ManLAM to induce TNF-α (4, 11, 31, 47).

Dependence of protein synthesis on the differential time course of NF-κB and KBF1 activation. The expression kinetics

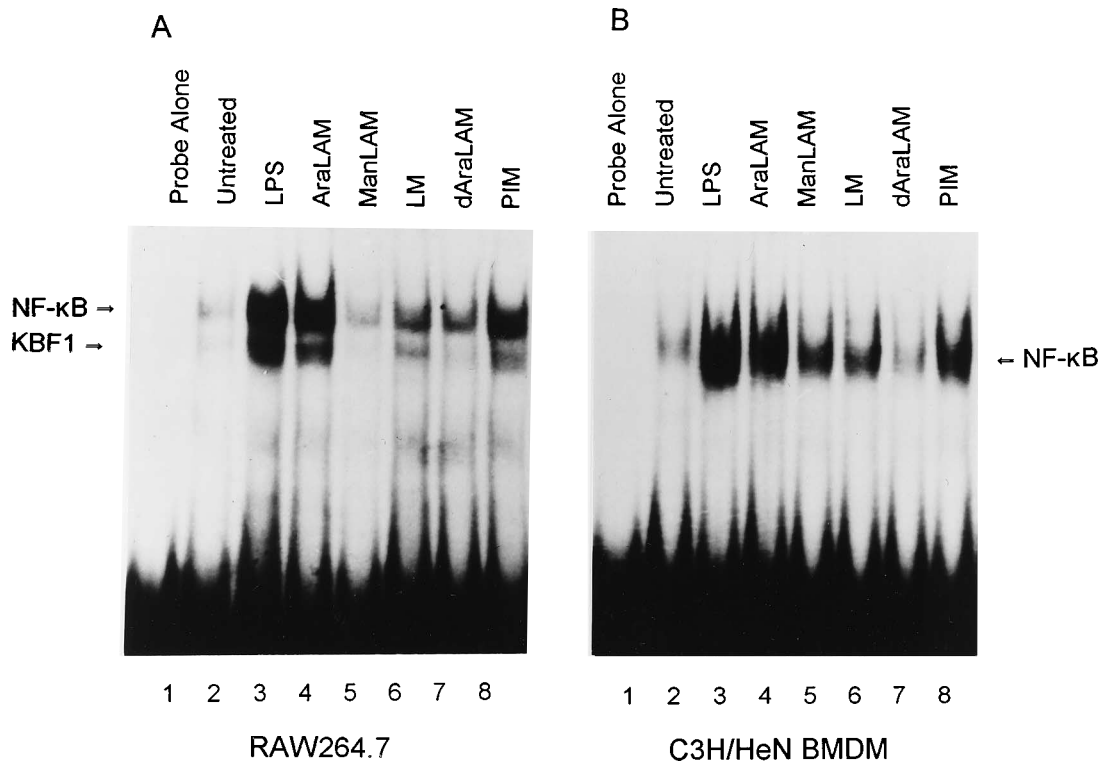

FIG. 3. Analysis of the structural composition of LAM and its relationship to the functional activation of NF-κB and KBF1. RAW264.7 (A) or BMDM (B) cells were left untreated (lane 2), treated with 10 ng of LPS per ml for 1 h (lane 3), or treated with 5 μg of AraLAM (lane 4), ManLAM (lane 5), LM (lane 6), dAraLAM (lane 7), or PIM (lane 8) per ml for 30 min at 37°C. The band migrating between KBF1 and free probe is nonspecific as determined by competition experiments (data not shown). The positions of NF-κB and KBF1 are marked. EMSA reaction mixtures contained 5 μg of nuclear extracts and 30,000 cpm of radiolabelled probe. One of three comparable experiments is shown.

of NF-κB activation, a rapid increase in binding followed by a decrease in activity over time, was consistent with recent reports examining the regulation of IκB, the inhibitor of NF-κB (6, 37). Upon cell stimulation, IκB is rapidly degraded, followed by an NF-κB-dependent induction of IκB mRNA levels and IκB protein production. The renewal of the pool of inhibitor results in the inhibition of NF-κB DNA-binding activity and the inhibition of κB-dependent transcription. To determine whether de novo protein synthesis was required for the transient stimulation of NF-κB DNA-binding activity, we pretreated RAW264.7 cells for 5 min with 25 μg of the protein synthesis inhibitor CHX per ml, followed by treatment with 5 μg of LPS or AraLAM per ml for 1 or 4 h (Fig. 5). One-hour LPS and AraLAM treatments resulted in the stimulation of NF-κB and KBF1 activities (Fig. 5, lanes 3 and 4). Incubation with CHX alone for 1 h resulted in a slight enhancement in nuclear NF-κB DNA-binding activities (Fig. 5, lane 8) which has been speculated to be a result of turnover of the labile inhibitor IκB. Nuclear extracts from RAW264.7 cells stimulated with LPS or AraLAM for 4 h contained predominantly KBF1-binding activity (Fig. 5, lanes 6 and 7), which was consistent with data presented in Fig. 4. When nuclear extracts from cells pretreated with CHX followed by LPS or AraLAM for 4 h were examined, the reduction in NF-κB activity was not observed (Fig. 5, lanes 12 and 13). Unexpectedly, the accumulation of KBF1 after 4 h of treatment with LPS or AraLAM was also blocked. This effect was most striking when the KBF1 accumulation in response to 4 h of LPS treatment was compared with the accumulation in response to 4 h of CHX-LPS and when 4-h AraLAM treatment was compared with 4-h CHX-AraLAM treatment (Fig. 5, compare lanes 12 and 6 and 13 and 7). These data demonstrated that de novo protein synthesis was required for both the reduction in NF-κB activity that follows the original increase as well as the long-term accumulation of KBF1. Such an observation was consistent with LPS stimulating the destruction of IκB, with CHX blocking the replacement of the inhibitor, thereby impeding the usual removal of NF-κB from the nucleus by 4 h of LPS stimulation.

Depletion of the NFκB1 p105 precursor in response to LPS and AraLAM. During the course of our analysis of the composition and regulation of the two forms of LPS-stimulated κB element DNA-binding activities, we demonstrated that LPS could stimulate processing of the NFκB1 p105 precursor (48). We speculated that this was the source of LPS-activatable KBF1. Because the regulation of κB-binding proteins by AraLAM and LPS had been similar to this point, a Western immunoassay was employed to determine whether AraLAM was capable of stimulating p105 loss. RAW264.7 cells were treated with 10 ng of LPS per ml for 1 h or 5 μg of AraLAM or ManLAM per ml for 30 min, followed by the preparation of whole-cell lysates for analysis of NFκB1 (p105 or p50) by Western immunoblotting (Fig. 6). With an antibody generated against the p50 subunit and purified against amino acids 350 to 500, a region with no similarity to other members of the highly conserved NRD family, two proteins were detected. In unstimulated RAW264.7 cells (Fig. 6, lane 1), the 105-kDa precursor protein and the 50-kDa active subunit were detected. After activation of cells with LPS for 1 h, a reduction in the amount of the 105-kDa protein was observed (Fig. 6, compare

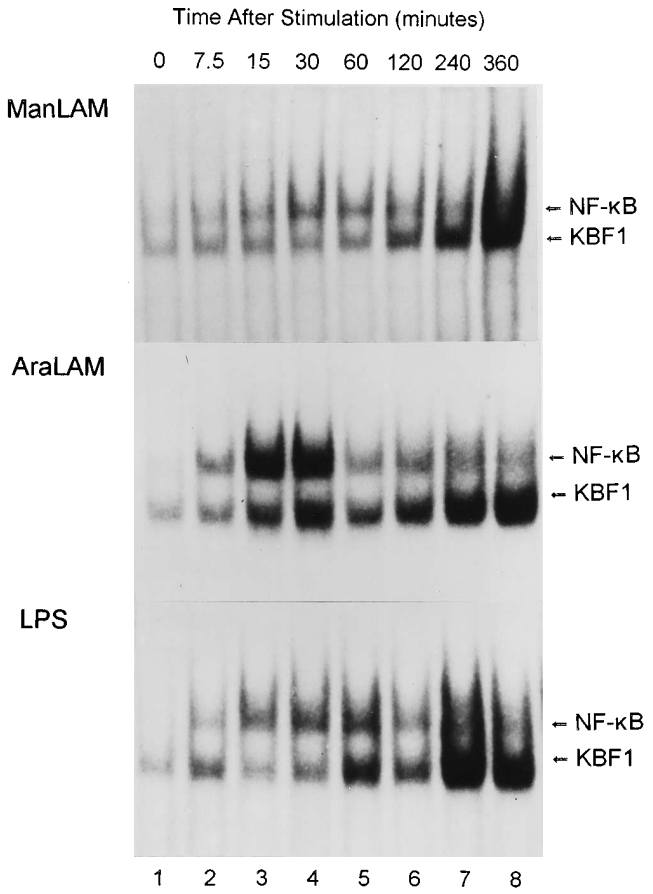

FIG. 4. Differential kinetics and maximal activation of NF-κB and KBF1 by *Mycobacterium*-derived LAMs and gram-negative LPS. EMSAs were performed with the −510 κB element from the murine TNF-α gene with extracts from untreated RAW264.7 cells (lane 1) or cells treated with 5 μg of ManLAM (A) or AraLAM (B) per ml or 10 ng of LPS (C) per ml for 7.5, 15, 30, 60, 120, 240, or 360 min (lanes 2 to 9). Free probe not shown. The positions of NF-κB and KBF1 are marked. EMSA reaction mixtures contained 10 μg of nuclear extracts and 30,000 cpm of radiolabelled probe. One of three comparable experiments is shown.

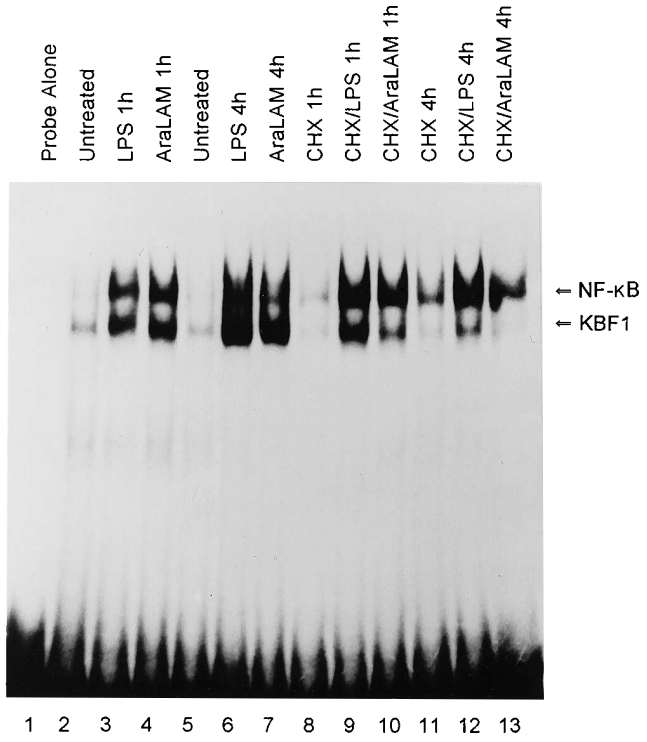

FIG. 5. Dependence of the differential kinetics of NF-κB and KBF1 activation on continued protein synthesis. EMSA was performed with the −510 κB element from the murine TNF-α gene with extracts from untreated RAW264.7 cells (lanes 2 and 5) or cells treated with 25 μg of CHX per ml for 1 h (lane 8) or 4 h (lane 11). Cells were also treated with 10 ng of LPS per ml for 1 h (lane 3) or 4 h (lane 6) or 5 μg of AraLAM per ml for 1 h (lane 4) or 4 h (lane 7), CHX and LPS for 1 h (lane 9) or 4 h (lane 12), or CHX and AraLAM for 1 h (lane 10) or 4 h (lane 13). The positions of NF-κB and KBF1 are marked. EMSA reaction mixtures contained 10 μg of nuclear extracts and 30,000 cpm of radiolabelled probe. One of three comparable experiments is shown.

lanes 1 and 2). Similarly, there was a reduction in the amount of 105-kDa precursor protein after a 30-min AraLAM treatment (Fig. 6, lane 3). Treatment with ManLAM did not result in a significant alteration in the amount of the 105-kDa protein compared with the untreated control (Fig. 6, lane 4), consistent with the impaired ability of ManLAM to modulate κB element DNA-binding activities in RAW264.7 macrophage-like cells. A more comprehensive examination of the processing of the 105-kDa precursor in response to AraLAM is required to definitively demonstrate stimulus-coupled processing; however, on the basis of the considerable overlap in the activities of AraLAM and LPS, we believe that these bacterial antigens are functioning in a similar manner, and as such, their respective activities can be generalized.

DISCUSSION

The data presented here demonstrate that AraLAM was capable of significant activation of NF-κB and KBF1 in murine BMDMs and macrophage-like cell lines. Interestingly, ManLAM was significantly less effective at stimulating NF-κB-binding activity in RAW264.7 cells and BMDMs. Reports on the dif-

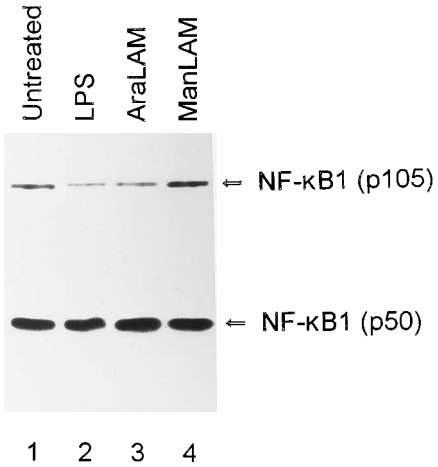

FIG. 6. Stimulus-coupled processing of NFκB1 precursor p105 in response to LPS and AraLAM. Western blotting was performed with affinity-purified anti-p50 antiserum (1:700). RAW264.7 cells were lysed in 95°C sample solubilizing buffer, separated by discontinuous SDS-PAGE, and analyzed by enhanced chemiluminescence blotting. The positions of p105 and p50 are marked. One of four comparable experiments is shown.

ferential ability of these LAMs to induce TNF-α are consistent with our finding of a reduced capacity of NF-κB induction by ManLAM compared with AraLAM and gram-negative LPS (31, 47). The impaired ability of ManLAM to activate significant NF-κB DNA binding was not due to an adverse effect on the nucleus or, more specifically, transcription factors as assessed by EMSA analysis with a constitutive enhancer (Fig. 1B), the ability of LPS to activate NF-κB in ManLAM-pretreated cells, and morphologic analysis by phase-contrast microscopy (data not shown). Also arguing against the possibility of ManLAM toxicity is the fact that ManLAM was effective at stimulating the accumulation and maintenance of KBF1 in RAW264.7 cells. ManLAM is also capable of activating NF-κB-binding activity in the J774.A cell line. Additionally, ManLAM has been shown to be capable of modulating some macrophage functions. It was reported that ManLAM synergized with gamma interferon to induce the chemokine JE (31) as well as substituted for LPS as a triggering signal for primed macrophages measured by toxoplasma killing (1).

As reported by Chatterjee et al. (11), ManLAM from the virulent Erdman strain of *M. tuberculosis* has mannosyl caps on the nonreducing termini. This difference from the potent, attenuated *M. tuberculosis*-derived AraLAM translates into an inability of the virulent LAM to induce TNF-α. However, it has been determined that avirulent BCG also produces ManLAM, arguing against mannose capping being a primary determinant of virulence, whereas the presence of AraLAM may play other roles (30). Indeed, this structural alteration creates differences in macrophage phagocytosis (32). Binding of the attenuated H37Ra strain of *M. tuberculosis* (AraLAM) to macrophages is mediated through complement receptors, whereas the binding of the virulent Erdman strain of *M. tuberculosis* (ManLAM) to macrophages is mediated by complement receptors and mannose receptors and may affect intracellular survival (32). Such differences in LAM structure may also impart differences to the growth of mycobacteria in macrophages. Analyses of the growth kinetics of Erdman, H37Ra, and BCG strains revealed a significantly faster intracellular doubling time for the virulent Erdman strain than for the avirulent mycobacterial strains (28). Additionally, a similar series of studies investigating virulence demonstrated the ability of virulent mycobacteria to escape from fused phagolysosomes, whereas H37Ra and BCG were unable to (24).

In a recent study examining the mechanism of LPS and AraLAM activation of TNF-α, it was reported that antibodies against the LPS receptor CD14 blocked both LPS and AraLAM induction (47). Because it appears that these two agents act through the same receptor, mannosyl capping of ManLAM may structurally interfere with a productive CD14 receptor interaction and therefore be ineffective in NF-κB activation and subsequent TNF-α induction. Alternatively, mannose receptors may compete with the LPS receptor for ManLAM binding. Interestingly, the J774.A macrophage-like cell line response to stimulation with ManLAM was equivalent to or greater than that of LPS (Fig. 2). The molecular basis of this observation, whether receptor or second messenger related, is currently under investigation, and the significance of this response to macrophage activation or the modulation of cytokine production is not known. Future studies should determine the molecular mechanism for the differential effects of AraLAM and ManLAM on TNF-α production.

Our results demonstrate that LAM and gram-negative bacterial LPS regulate NRD family transcription factor DNA binding in a similar fashion. Both stimulate rapid nuclear translocation of NF-κB and KBF1 according to similar kinetics and maxima of binding, as well as stimulating the loss of the NFκB1 p105 precursor. Such a considerable overlap in the characteristics of LAM and LPS regulation of NF-κB and KBF1 supports the view that both antigens function through the same receptor and suggests that AraLAM activation of NF-κB may play a role in the induction of TNF-α transcription. Additionally, the demonstration of an impaired ability of ManLAM to stimulate NF-κB binding to the TNF-α −510 κB element while stimulating KBF1 accumulation extends knowledge about the differential induction of TNF-α by the H37Ra and Erdman strains of *M. tuberculosis* at the molecular level. Furthermore, the demonstration of mycobacterial LAM and LPS activation of κB-element binding and the differential activation of NF-κB and KBF1 further contribute to our understanding of the complexity of NRD regulation.

The initial rapid increase and subsequent decrease in NF-κB binding at later time points were consistent with the findings of other reports which have demonstrated the expression kinetics of IκB, the inhibitor of NF-κB (6, 37). In macrophages and many other cells, NF-κB is maintained in the cytosol in an inactive complex with IκB (3, 26). After stimulation by LPS, IκB undergoes a posttranslational modification resulting in dissociation from NF-κB and subsequent nuclear localization and DNA binding of NF-κB (16, 46). Rapid activation of NF-κB DNA binding correlated with the disappearance of IκB, whereas inhibition of NF-κB activity correlated with the reappearance of newly synthesized IκB at 8 to 24 h posttreatment. It has not been definitively demonstrated whether IκBα is present or modulated by AraLAM or LPS in RAW264.7 cells. We have been able to demonstrate that in RAW264.7 cells, NF-κB, but not KBF1, is present in untreated cytoplasm in a deoxycholate-activatable form and that once activated this DNA binding is then inhibitable by recombinant IκBα (6b). Furthermore, pretreatment of RAW264.7 cells with 25 μg of CHX per ml followed by treatment with AraLAM or LPS for 4 h results in the stabilization of NF-κB and the blocking of KBF1 accumulation (Fig. 5). The maintenance of NF-κB may be explained by the blocking of IκB production and replacement, after LPS-stimulated destruction, resulting in the obstruction of NF-κB removal from the nucleus. Additionally, this finding suggests that the accumulation of KBF1 requires de novo protein synthesis and that the initial NF-κB activation events may be uncoupled from the long-term KBF1 accumulation. The inhibition of KBF1 accumulation may be due to the depletion of the 105-kDa precursor. Western blotting of CHX-pretreated LPS- or AraLAM-treated cell cytoplasmic extracts with the anti-NFκB1 antibody (p105 or p50) revealed the presence of little immunodetectable p105 (6b). These results are consistent with IκB being the primary regulator of NF-κB in RAW264.7 cells.

We have previously reported that LPS stimulates rapid processing of the p105 precursor to the p50 subunit (48), an event which may serve as a major regulatory point in the dynamics of κB-binding subunits. In this study, we observe that AraLAM, ManLAM, or LPS treatment for 4 h or more leads to a significant accumulation of KBF1. It has been shown that KBF1 has the capability of downregulating class I gene expression, with the ratio of NF-κB to KBF1 critical for the level of expression (29). More recently, it was determined that the induction of tolerance to LPS (hyporesponsiveness of macrophages to LPS stimulation of TNF-α) involves an accumulation of KBF1 (50). We observed that virulent *M. tuberculosis*-derived ManLAM, although ineffective at induction of NF-κB, is capable of stimulating the longer-term accumulation of KBF1 (Fig. 3A). ManLAM then bypasses the stimulation of a transcriptionally active complex, NF-κB, while stimulating the accumulation of a potential repressor, KBF1. This may be a mechanism of

preventing TNF-α production and macrophage activation, thereby averting clearance and facilitating the persistence of virulent *M. tuberculosis*. Furthermore, the phenomenon of the accumulation and maintenance of KBF1 after the transient induction of NF-κB may serve as a feedback switch and therefore a means to turn off transcription of the TNF-α gene.

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