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Lipoarabinomannan of *Mycobacterium tuberculosis* Promotes Protein Tyrosine Dephosphorylation and Inhibition of Mitogen-activated Protein Kinase in Human Mononuclear Phagocytes

ROLE OF THE Src HOMOLOGY 2 CONTAINING TYROSINE PHOSPHATASE 1*

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Lipoarabinomannan (LAM) is a putative virulence factor of *Mycobacterium tuberculosis* that inhibits monocyte functions, and this may involve antagonism of cell signaling pathways. The effects of LAM on protein tyrosine phosphorylation in cells of the human monocytic cell line THP-1 were examined. LAM promoted tyrosine dephosphorylation of multiple cell proteins and attenuated phorbol 12-myristate 13-acetate-induced activation of mitogen-activated protein kinase. To examine whether these effects of LAM could be related to activation of a phosphatase, fractions from LAM-treated cells were analyzed for dephosphorylation of *para*-nitrophenol phosphate. The data show that LAM induced increased phosphatase activity associated with the membrane fraction. The Src homology 2 containing tyrosine phosphatase 1 (SHP-1) is important for signal termination and was examined as a potential target of LAM. Exposure of cells to LAM brought about (i) an increase in tyrosine phosphorylation of SHP-1, and (ii) translocation of the phosphatase to the membrane. Phosphatase assay of SHP-1 immunoprecipitated from LAM-treated cells, using phosphorylated mitogen-activated protein kinase as substrate, indicated that LAM promoted increased activity of SHP-1 *in vivo*. LAM also activated SHP-1 directly *in vitro*. Exposure of cells to LAM also attenuated the expression of tumor necrosis factor-α, interleukin-12, and major histocompatibility class II molecules. These results suggest that one mechanism by which LAM deactivates monocytes involves activation of SHP-1.

It has been estimated that there are approximately eight million new cases and three million deaths annually, worldwide, from tuberculosis (1). It is also believed that the causative agent of this disease, *Mycobacterium tuberculosis*, infects one-third of the world's population (2). *M. tuberculosis* infects and resides exclusively within mononuclear phagocytes, and its ability to evade being killed within phagocytic cells has likely contributed to its longevity as a highly successful pathogen. Although many other microbes are killed when ingested by phagocytes, *M. tuberculosis* prevents its destruction by impairing critical macrophage functional responses (3–7)[1] using mechanisms that are not well understood.

Following ingestion by macrophages, *M. tuberculosis* modifies the phagosome such that it does not fuse with lysosomes (3), an avoidance tactic that allows it to evade proteolytic destruction. Evidence also suggests that the phagosome membrane is disrupted in a manner that may allow entry of the organism or its products into the host cell cytosol (6, 9, 10). Within the host cell, *M. tuberculosis* is able to replicate (11) and induce a state of diminished responsiveness to further stimulation (3, 4, 6, 7, 12).[1] For example, infected macrophages are poorly responsive to interferon-γ (IFN-γ)[2] as assessed by both the expression of major histocompatibility complex (MHC) class II molecules[1] and intracellular killing (13). Of critical interest is the identity of the virulence factors responsible for macrophage deactivation. One potential candidate is the mycobacterial cell wall glycolipid, lipoarabinomannan (LAM).

LAM is complex molecule consisting of a phosphatidylinositol moiety that anchors a large mannose core to the mycobacterial cell wall (14, 15). The mannose core contains multiple branched, arabinofuranosyl side chains. Comparative analyses of LAMs from different species of mycobacteria have shown that the nonreducing termini of the arabinofuranosyl side chains are differentially modified. For example, *M. tuberculosis* and *Mycobacterium leprae* modify the termini with mannose residues thereby yielding "man-LAM," whereas rapidly growing mycobacterial species use inositolphosphates thereby giving rise to "ara-LAM" (16). It is thought that these contrasting modifications are responsible for the marked differences in the biological activities of man-LAM and ara-LAM (16–18). Studies have shown that, in comparison to man-LAM, ara-LAM is a potent inducer of tumor

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[1] Z. Hmama, R. Gabathuler, W. A. Jeffries, G. de Jong, and N. E. Reiner, submitted for publication.

[2] The abbreviations used are: IFN-γ, interferon-γ; MHC, major histocompatibility complex; LAM, lipoarabinomannan; TNF-α, tumor necrosis factor-α; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; PMA, phorbol 12-myristate, 13-acetate; GST, glutathione *S*-transferase; FCS, fetal calf serum; MFI, mean fluorescence index; LPS, lipopolysaccharide; LM, lipomannan; PIM, phosphatidylinositol mannoside; pNPP, *para*-nitrophenyl phosphate; IL-12, interleukin-12; TGF-β, transforming growth factor-β; erk, extracellular-regulated kinase; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

necrosis factor (TNF)-α and interleukins-1, -6, and -10 (17, 19–22). However, both LAMs appear to be equipotent with respect to induction of transforming growth factor (TGF)-β (22). In addition, the early response genes, c-*fos*, *KC*, *iNOS*, and *JE* are induced by ara-LAM but not by man-LAM (18, 23). These findings suggest that *M. tuberculosis* has evolved mechanisms to "mask" man-LAM such that it has a diminished capacity to induce cytokines and other monocyte gene products that may be detrimental to the organism's survival.

Other studies have revealed that LAM, derived from *M. tuberculosis*, actively inhibits properties of both macrophages and T cells. In macrophages for example, LAM is able to block many actions of IFN-γ, including tumor cell killing (24, 25), intracellular killing of toxoplasma (21, 24, 25), and increased expression of several IFN-γ-inducible genes (26). It has been suggested that these actions of LAM may be related to inhibition of protein kinase C (PKC) to scavenging of cytotoxic oxygen free radicals, or to both (26). In T cells, LAM has been shown to suppress antigen-driven proliferation of a $CD4^+$ T cell clone (27). It has also been shown to inhibit the accumulation of mRNA for interleukins-2 and -3, granulocyte-macrophage colony-stimulating factor, and the interleukin-2 receptor α-chain in Jurkat T cells stimulated with phytohemagglutinin and phorbol esters (28).

This study examined the hypothesis that the inhibitory effects of LAM on mononuclear phagocytes may be related to altered cell signaling. The results show that LAM promotes tyrosine dephosphorylation of multiple proteins including MAPK. In the latter instance, this leads to impaired activation of the enzyme. These effects of LAM may be explained by the action of the Src homology 2-containing tyrosine phosphatase-1 (SHP-1), a tyrosine phosphatase known to be important for attenuating activation signals (29, 30). The results presented show that SHP-1 is activated by LAM both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Reagents—Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma. Protein G-Sepharose was from Pharmacia Biotech Inc. Horseradish peroxidase-conjugated goat anti-rabbit antibodies, protein A-agarose, and electrophoresis reagents and supplies were purchased from Bio-Rad. The THP-1 cell line was obtained from the American Type Culture Collection (Rockville, MD). THP-1 cells, derived from a patient with acute monocytic leukemia, are phagocytic and possess other characteristics of monocytes including expression of Fc and C3b receptors (31). THP-1wt cell line (THP-1 cells stably expressing the glycosylphosphatidylinositol-linked CD14) was kindly provided by Dr. Richard Ulevitch (The Scripps Research Institute, La Jolla, CA). RPMI 1640 and Hank's balanced salt solution were from Stem Cell Technologies (Vancouver, British Columbia). Enhanced chemiluminescence reagents and ECL film were from Amersham International (Oakville, Ontario). Lipopolysaccharide (*Escherichia coli* O1227:B8) was purchased from Difco. Human AB^+ serum was provided by the Canadian Red Cross (Vancouver, British Columbia). Anti-phosphotyrosine monoclonal antibody 4G10, anti-MAPK-ct, and MAPK-glutathione *S*-transferase (GST) were from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-SHP-1 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Recombinant, human IFN-γ was from Genentech Inc. (South San Francisco, CA). Riboprobe systems buffers, RQ1 RNase-free DNase, T7, T3, and SP6 RNA polymerases, and proteinase K were from Promega Corp. (Madison, WI). RNase T1, Trizol, and tRNA were from Life Technologies, Inc. Unless stated otherwise, all reagents were the highest quality available.

Mycobacterial Lipids—Endotoxin-free LAM, lipomannan, and phosphatidylinositolmannoside were generously provided by Dr. P. J. Brennan (Colorado State University, Ft. Collins, CO, through National Institutes of Health Contract NO1-A1–25147). In all experiments, unless otherwise indicated, the LAM (mannose capped) used was derived from the virulent, erdman strain of *M. tuberculosis* (32, 33). Lipomannan is similar to LAM except that it does not contain the arabinofuranosyl side chains (34). Phosphatidylinositolmannoside is similar to lipomannan except that the mannan core contains fewer mannose residues (34). Both of the latter were derived from the virulent H37rv strain of *M. tuberculosis*.

Isolation of Monocytes and Cell Culture—Fractions of peripheral blood enriched in white blood cells were obtained from the Cell Separator Unit (Vancouver Hospital and Health Sciences Center). Monocytes were enriched (85–95% pure) by adherence as described previously (35). Monolayers of adherent cells were treated with either LAM, LPS, or both, rinsed with ice-cold PBS, snap-frozen using liquid nitrogen, and stored at −70 °C prior to analysis. Cell lysates were prepared by lysing cells on ice (20 min) in lysis buffer (20 mM Tris, pH 8.0, 1% Triton X-100, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF, 100 nM microcystin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin). Protein concentrations were determined using Bio-Rad $Protein_{DC}$ and bovine serum albumin as standard. Monocyte cell lines were maintained in RPMI supplemented with 10% heat-inactivated fetal calf serum (FCS). For acute studies, cells were rendered quiescent by culture in RPMI without FCS for 12–16 h at a concentration of 5×10^5 cells/ml. For chronic studies, log phase cells were washed in Hanks' solution and resuspended in RPMI without FCS, followed by the addition of mycobacterial cell wall components. Following treatments, cells were lysed immediately, and detergent-soluble material was frozen at −70 °C until further analysis.

Cellular Fractionation and Translocation Assay—Following incubation of THP-1wt cells with either LAM or LPS, cells were fractionated essentially as described previously (36). In brief, monocytes were scraped into hypotonic fractionation buffer (10 mM Tris, pH 7.4, 4.5 mM EGTA, 2.5 mM EDTA, 1.0 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 100 nM microcystin, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin) and lysed for 20 min at 4 °C while rotating. Lysates were then centrifuged at $100{,}000 \times g$ for 30 min to separate cytosolic from particulate fractions. The resulting pellets were extracted in fractionation buffer containing 1% Triton X-100 for 20 min and centrifuged ($16{,}000 \times g$, 20 min, 4 °C) to separate detergent-insoluble and -soluble material. The resulting supernatant was taken to represent a membrane fraction. Twenty micrograms of cytosolic and membrane fractions were then subjected to SDS-PAGE and immunoblotting using anti-SHP-1 or anti-MAPK-ct antibodies.

Analysis of HLA-DR Cell Surface Expression—THP-1 cells were seeded at a density of 10^5 per cm^2 and allowed to adhere and differentiate in the presence of PMA (20 ng/ml) at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. Cells were then washed three times with Hanks' balanced salt solution and adherent monolayers were replenished with culture medium. LAM or LPS was added to the cells for an additional 24 h followed by treatment with IFN-γ for 24 h more. To measure cell surface expression of HLA-DR, control and treated cells were incubated with anti-HLA-DR monoclonal antibody (clone HL38, Catlag Laboratories, San Francisco, CA) for 30 min and then washed twice and labeled with fluorescein isothiocyanate-conjugated $F(ab)'_2$ sheep anti-mouse IgG (Sigma) for 30 min. All staining and washing procedures were performed at 4 °C in Hanks' balanced salt solution containing 0.1% NaN_3 and 1% FCS. To control for cell viability, cells were incubated with propidium iodide (0.5 μg/ml in staining buffer) for 10 min. Cells were then washed twice and fixed in 2% paraformaldehyde in staining buffer. Fluorescence was analyzed using a Coulter Elite flow cytometer (Hialeah, FL). Viable cells were identified by exclusion of propidium iodide. Relative fluorescence intensities of 5000 cells were recorded as single-parameter histograms (log scale, 1024 channels, 4 log decades), and the mean fluorescence intensity (MFI) was calculated for each histogram. Results are expressed as MFI index which corresponds to the ratio: MFI of cells incubated with specific antibody/MFI of cells incubated with irrelevant isotype-matched IgG.

Western Blotting, Immunoprecipitation, and Densitometry—Whole cell lysates, prepared in lysis buffer, were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine monoclonal antibody (4G10), anti-SHP-1, or anti-MAPK-ct by previously described methods (37). SHP-1 was immunoprecipitated by incubating 0.5–1.5 mg of lysate with anti-SHP-1 for 2 h at 4 °C, followed by the addition of 40 μl of protein G-Sepharose for an additional 2 h. To assess the amount of individual proteins immunoprecipitated in each sample, after detection of bound 4G10 Ab, membranes were stripped and reprobed with anti-SHP-1 or anti-MAPK-ct and developed by enhanced chemiluminescence as described (37). Densitometry was performed using a Howtek Scanner and Quantity One software (PDI Bioscience, Aurora, Ontario, Canada).

RNase Protection Assay—THP-1wt cells were preincubated with either LAM or LPS for 16–20 h, followed by the addition of 1 μg/ml LPS for 2 h. RNA was extracted from cells using Trizol according to the manufacturer's protocol. Equal amounts of RNA were subjected to an RNase protection assay, essentially as described previously (38). Two

cytokine-specific riboprobe template sets were tested, HL-14 and HL-21. These were assembled from *Eco*RI-linearized and purified subclones. The HL-14 template set synthesized riboprobes specific for interleukins-1α, -1β, -6, -10, TNF-β, TNFα, granulocyte-macrophage colony-stimulating factor, TGF-β1, and rpL32. The HL-21 template set synthesized riboprobes specific for interleukins-2, -4, -5, -12 (p40 and p35), and -13, CD4, CD8, and rpL32. The respective nucleotide sequences and GenBank Accession numbers of the individual clones were previously described.[3]

Phosphatase Assays—Assays for protein tyrosine phosphatase activity were carried out using either *para*-nitrophenol phosphate (pNPP) or phosphorylated MAPK-1-GST as substrates. SHP-1 was immunoprecipitated from equal amounts of cell lysate protein as described above. For MAPK-1-GST dephosphorylation, the immunoprecipitates were further washed three times in phosphatase assay buffer (25 mM imidazole, pH 7.0, 1 mM EDTA) and resuspended in phosphatase assay buffer supplemented with 1 mM dithiothreitol (DTT). MAPK-1-GST coupled to glutathione-agarose was autophosphorylated in buffer containing 25 mM β-glycerophosphate, 20 mM MOPS, pH 7.2, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 mM ATP, and 1 mM DTT for 30 min at 30 °C. The agarose beads were washed two times in autophosphorylation buffer without ATP and two times in phosphatase buffer without DTT. After resuspension in phosphatase assay buffer containing 1 mM DTT, the MAPK-1-GST beads were incubated with the SHP-1 immunoprecipitates for 1 h at 30 °C. The reactions were stopped by the addition of an equal volume of 2 × Laemmli buffer followed by immunoblotting with anti-phosphotyrosine antibodies. Blots were then stripped and reprobed with either anti-MAPK-1-GST or anti-SHP-1 to confirm equal loading of substrates and phosphatase enzyme. When pNPP was used as a substrate, the SHP-1 immunoprecipitates were washed further in a buffer containing 50 mM Hepes, pH 7.0, 5 mM EDTA and, 10 mM DTT for 1 h at 37 °C. Immunoprecipitates were ultimately resuspended in the same buffer containing 2 mM pNPP followed by an incubation for 4 h at 30 °C. Absorbances of the samples were read at 405 nM as described previously (39). Activity is expressed as a percent of control. Phosphatase activity in cytosolic and membrane fractions was measured by incubating 25–50 μg of each fraction from control or treated cells in a buffer containing 50 mM imidazole Cl, pH 7.2, 1.0 mM EDTA, and 16 mM pNPP, for 30 min at 30 °C. Absorbances of the samples were measured as described above and expressed as percent of control optical density.

RESULTS

Effects of LAM on PMA-induced Tyrosine Phosphorylation and Activation of MAPK—To examine whether LAM modulates activation of MAPK, THP-1wt cells were preincubated with LAM for 16 h followed by the addition of PMA (100 nM) for 15 min. PMA reproducibly induced the tyrosine phosphorylation of a 42-kDa protein which is positioned exactly with that of $p42^{MAPK2}$ (Fig. 1*A*). Phosphorylation of this band was not evident in cells treated with either 0.1% serum (*lane 1*) or LAM alone (*lane 3*). On the other hand, tyrosine phosphorylation of $p42^{MAPK2}$, in response to PMA, was significantly attenuated in cells preincubated with LAM. Two other parameters of MAPK activation were also assessed. In response to PMA and secondary to the activation of MAPK kinase, MAPK becomes tyrosine-, serine-, and threonine-phosphorylated leading to its activation (40–42). These phosphorylations are associated with retarded mobility (bandshifting) of the kinases as assessed by SDS-PAGE and immunoblotting. As can be seen in Fig. 1*B*, bandshifting in response to PMA was clearly observed for both the $p42^{MAPK2}$ and $p44^{MAPK1}$ isoforms. Of particular interest was the finding that, although LAM markedly attenuated tyrosine phosphorylation of $p42^{MAPK2}$, this did not impair the bandshifting otherwise expected following treatment with PMA. Based on this finding, it would appear that bandshifting is a function of serine/threonine phosphorylations and that LAM selectively affects tyrosine phosphorylation of MAPK. Of interest, while bandshifting was observed for $p44^{MAPK1}$, PMA was unable to induce tyrosine phosphorylation of this isoform, consistent with a previous report of the actions of PMA on $p44^{MAPK1}$ and $p42^{MAPK2}$ (43).

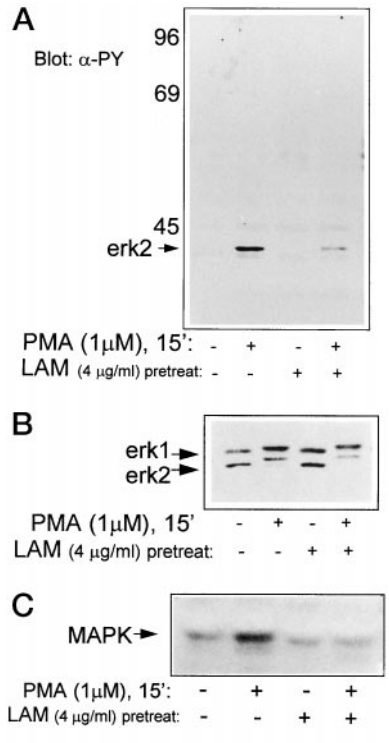

FIG. 1. **Attenuation of PMA-induced activation of $p42^{MAPK2}$ by LAM.** THP-1wt cells were treated for 16 h with either 2 μg/ml LAM or medium alone. Cells were then incubated with either medium or PMA (100 nM) for 15 min. *A*, a cytosolic extract was prepared and analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine (*α-PY*) antibodies. The tyrosine-phosphorylated band corresponds to $p42^{MAPK2}$ (*erk2*). *B*, the blot was stripped and reprobed with anti-MAPK antibodies (erk2 = $p42^{MAPK2}$ and erk1 = $p44^{MAPK1}$). Note that PMA-induced bandshifting was not inhibited by LAM. The blot was subsequently stained with india ink which revealed equal amounts of protein in each lane (data not shown). *C*, equal amounts of cytosol were resolved on 10% SDS-polyacrylamide gels containing 100 μg/ml myelin basic protein. The gel was then subjected to an in-gel kinase assay, dried, and exposed to x-ray film. Results shown are from two independent experiments which yielded similar results.

MAPK activity was also assessed with myelin basic protein using an in-gel assay (Fig. 1*C*). Consistent with previous reports (44) that tyrosine phosphorylation is necessary for activation of MAPK, it was found that treatment of cells with LAM inhibited the increased myelin basic protein phosphotransferase activity otherwise observed with PMA stimulation of (non-LAM-treated) control cells.

Effects of LAM on LPS-induced Tyrosine Phosphorylation of Monocyte Proteins—To determine whether other tyrosine phosphorylation events are regulated by LAM, the effects of LAM on LPS-induced tyrosine phosphorylation of proteins was assessed in normal human monocytes (Fig. 2). As expected, LPS treatment induced increased tyrosine phosphorylation of multiple monocyte proteins. Of interest, pretreatment of cells with LAM (2 μg/ml) for 16 h abrogated the effects of LPS in a manner similar to that observed with tyrosine phosphorylation of $p42^{MAPK2}$ induced by PMA. The autoradiogram shown in Fig. 2*A* was analyzed by densitometry to identify bands that underwent increased phosphotyrosine labeling in response to LPS and to quantitate the effects of LAM on these events. The results of this analysis (Fig. 2*B*) show that LPS induced marked increases in phosphotyrosine content in at least eight proteins. Pretreatment of cells with LAM virtually eliminated

[3] Rochford, R., Cannon, M. J., Sabbe, R. E., Adusimilli, K., Picchio, G., Glynn, J. M., Nonnan, D. J., Mosier, D. E., and Hobbs, M., *Viral Immunol.*, in press.

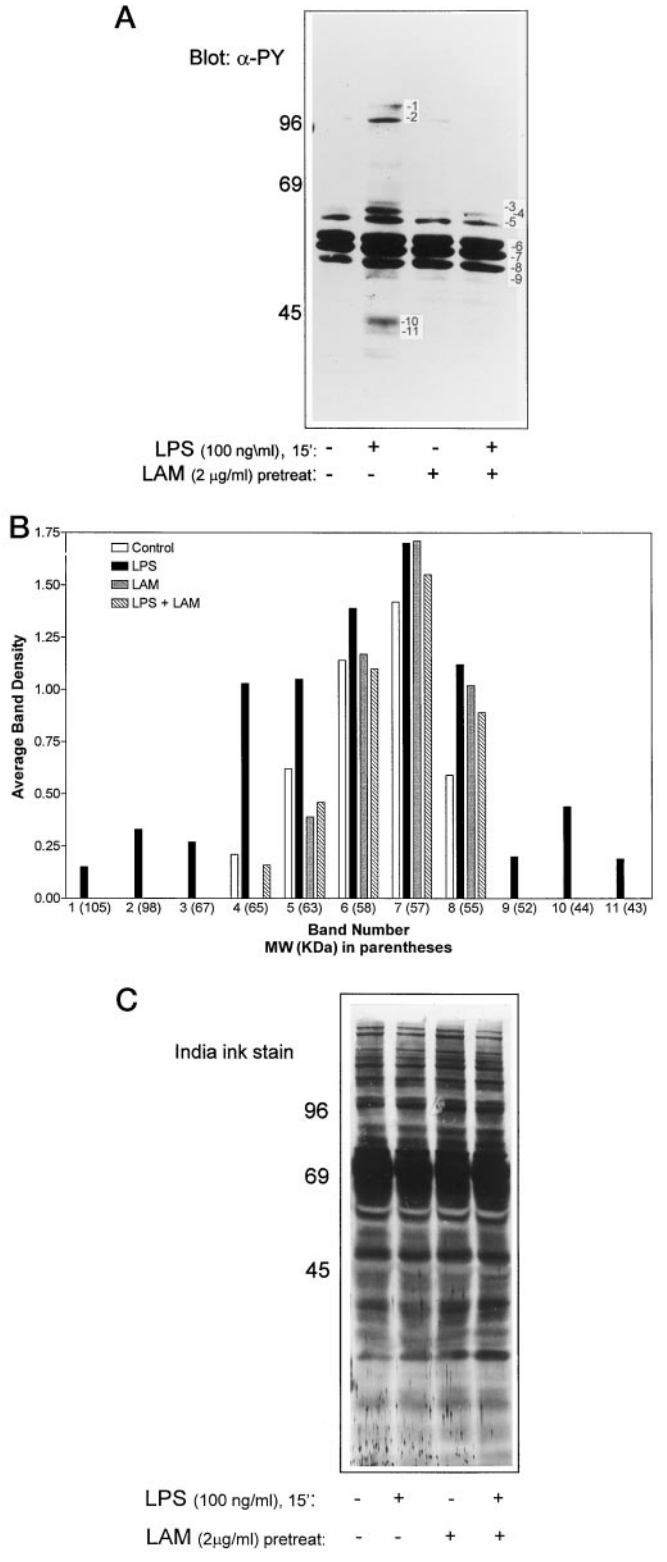

FIG. 2. **Attenuation of LPS-induced tyrosine phosphorylation in human monocytes by LAM.** Monocytes were treated for 16 h with either 2 μg/ml LAM or medium alone. Cells were then treated with either control medium or LPS (100 ng/ml) for an additional 15 min. *A*, detergent lysates were prepared, and equal amounts of protein (50 μg) were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine (*α-PY*) antibodies. The numbers 1–11 correspond to the bands depicted by densitometry in *B*. *B*, the autoradiogram shown in *A* was analyzed by densitometry. Note that where *bars* are absent, there was no detectable tyrosine phosphorylation. *C*, the blot was subsequently stained with india ink which revealed equal protein loading. The data shown are representative of two independent experiments that gave similar results.

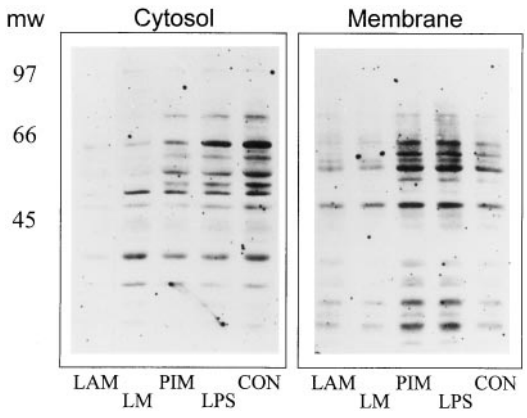

FIG. 3. **Effects of various mycobacterial cell wall components on levels of tyrosine phosphorylation of macrophage cytosolic proteins.** THP-1wt cells were rendered quiescent overnight in RPMI without serum. The cells were treated for 30 min with either LAM (2 μg/ml), lipomannan (2 μg/ml), phosphatidylinositolmannoside (2 μg/ml), LPS (36 pg/ml, representing the maximum contaminating concentration in the lipid preparations), or control medium (*CON*) (RPMI with 10% human AB^+ serum). Cytosol and membrane fractions were prepared, and equal amounts of each fraction were analyzed by SDS-PAGE and immunoblotting with antiphosphotyrosine (*α-PY*) antibodies. The blot shown is representative of two independent experiments. India ink staining of the blot revealed similar levels of protein per lane (not shown).

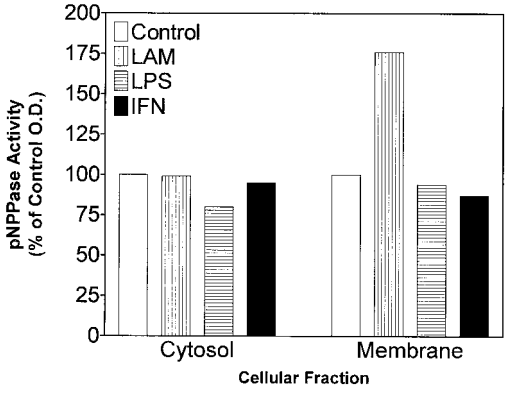

FIG. 4. **Activation of pNPPase activity by LAM.** THP-1wt cells were rendered quiescent overnight in RPMI without serum. The cells were treated for 30 min with either LAM (2 μg/ml), LPS (36 pgml), IFN-γ (200 units/ml), or medium alone (*Control*) (RPMI with 10% human AB^+ serum). Cytosol and membrane fractions were prepared and equal amounts of protein from each fraction were analyzed for pNPPase activity. Shown are data averaged from two independent experiments. Control cytosol, OD = 0.33; control membrane, OD = 0.14.

all of these increases. In contrast to the marked effects of LPS in phosphotyrosine labeling of certain bands (Fig. 2, *A* and *B*), proteins in the 50–60-kDa range were less affected by LPS. Although the overall results of LAM were to promote tyrosine dephosphorylation, occasionally slight, likely insignificant, increases in tyrosine phosphorylation were observed in response to LAM alone. These changes were not consistently reproducible. In parallel experiments, it was also observed that LAM abrogated tyrosine phosphorylation of monocyte proteins induced by IFN-γ (data not shown).

Effects of LAM Precursors on the Tyrosine Phosphorylation of Cytosolic and Membrane Proteins—When precursors of LAM were examined for their abilities to affect levels of tyrosine phosphorylation of cytosolic proteins, apparent structure-dependent effects were observed (Fig. 3). In comparison to control cells, LAM (2 μg/ml) markedly reduced levels of tyrosine phosphorylation of multiple cytosolic proteins. Lipomannan (LM, 2 μg/ml) similarly led to diminished tyrosine phosphorylation of

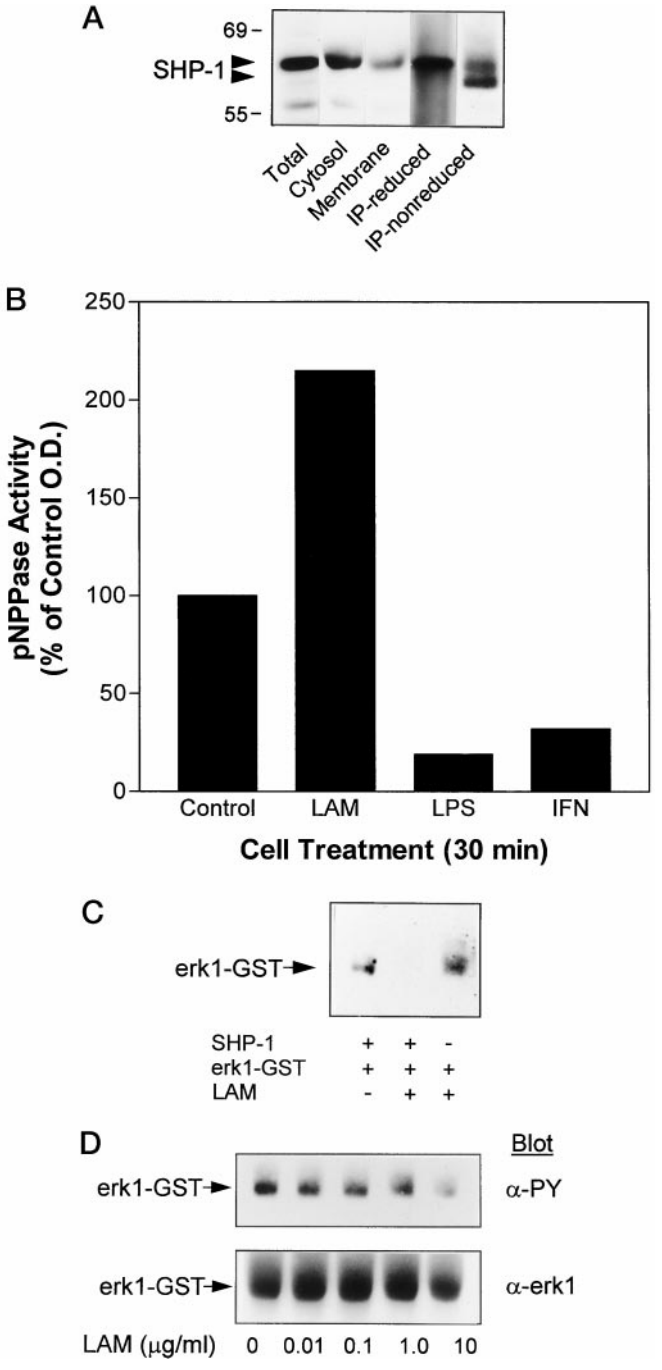

FIG. 5. **Identification of SHP-1 in THP-1wt cells and effects of LAM on phosphatase activity.** *A*, expression of SHP-1 in THP1wt cells. Cells were rendered quiescent overnight in RPMI without serum and were fractionated as described under "Materials and Methods." Twenty-five micrograms of either total lysate, cytosol, or membrane were analyzed by SDS-PAGE and immunoblotting with anti-SHP-1 antibodies. SHP-1 was also immunoprecipitated from 500 μg of cell protein and resolved by SDS-PAGE under both reducing and non-reducing conditions (*two right-hand lanes*). Note that in the basal state SHP-1 is distributed in both fractions with a predominant distribution in the cytosolic fraction. Under non-reducing conditions, SHP-1 is resolved as a doublet which most likely reflects the presence of intramolecular disulfide linkages. Results are representative of at least two independent experiments. *B*, rested THP-1wt cells were exposed to either LAM (2 μg/ml), LPS (36 pg/ml), IFN-γ (200 units/ml), or medium alone for 30 min. Lysates were prepared and SHP-1 was immunoprecipitated from equal amounts of protein. The activity of the immunoprecipitates against pNPP was measured as described under "Materials and Methods." Activity of SHP-1 from treated cells is expressed as a percent of control activity (control OD = 0.022). Similar results were obtained in two independent experiments. Analysis of SHP-1 immunoprecipitates for SHP-1 protein by immunoblotting showed that equal amounts of enzyme were brought down from each treatment. *C*, *in vitro* activation of SHP-1 by LAM. SHP-1 was immunoprecipitated from lysates of quiescent, untreated cells. Either SHP-1, LAM 10 μg/ml, or both were incubated with phosphorylated MAPK-1-GST (erk1-GST). The tyrosine phosphorylation state of the MAPK-1-GST (erk1-GST) was assessed by SDS-PAGE and immunoblotting with anti-phosphotyrosine (*α-PY*) antibodies. *D*, dose-response analysis of activation of SHP-1 *in vitro* by LAM. See *C* for details. The results shown are representative of two independent experiments.

proteins in the cytosol, but not to the same extent as LAM. Diminished levels of tyrosine phosphorylation in response to phosphatidylinositolmannoside (PIM, 2 μg/ml) were also evident, but these changes were less pronounced than those observed with either LAM or LM. Although reductions in protein tyrosine phosphorylation were also seen with LAM and LM in the membrane fraction, it was notable that PIM induced increased tyrosine phosphorylation of proteins in the 50–70-kDa range and the 30–40-kDa range. The effects of PIM in both the cytosolic and membrane fractions were similar to those seen with LPS which was used at a concentration reflecting the maximum potential level of LPS contamination in any of the preparations. In other experiments, ara-LAM, derived from *Mycobacterium smegmatis*, an avirulent environmental species of mycobacterium, was examined for its acute effects on tyrosine phosphorylation. Unlike LAM from *M. tuberculosis*, the arabinofuranosyl side chains of ara-LAM are not capped with mannose residues. Rather, they are capped with inositolphosphates imparting to ara-LAM agonist properties with respect to monocyte activation. In contrast to the results depicted in Fig. 3 for LAM, ara-LAM was observed to increase levels of tyrosine phosphorylation of multiple proteins (data not shown) consistent with its activating properties.

Activation of SHP-1—Attenuation by LAM of tyrosine phosphorylation in monocytes could be explained by inhibition of tyrosine kinases, activation of phosphotyrosine phosphatases, or both. To address whether a phosphatase is involved, initial experiments were done in which cells were exposed to either LAM (2 μg/ml), LPS (36 pg/ml, the maximum potential contaminating concentration in LAM preparations), IFN-γ (200 units/ml), or control medium for 30 min. Cytosolic and membrane fractions were prepared and examined for evidence of phosphatase activation. As shown in Fig. 4, in response to LAM, a membrane-localized increase in pNPPase activity of approximately 1.7-fold was observed. In contrast, no change in cytosolic pNPPase activity was observed. In cells exposed to IFN-γ or LPS, pNPPase activity was similar to control levels in both cytosolic and membrane fractions.

These findings suggested two possibilities to explain an activated membrane-associated phosphatase. First, LAM may activate a cytosol-localized phosphatase that is translocated to the membrane in an activated state. Second, the activated phosphatase could be either an integral or a peripheral membrane protein. The first possibility was explored with SHP-1 since this phosphatase is known to be involved in terminating signals, is abundant in hematopoietic cells, and is known to be activated by phospholipids (45). As shown in Fig. 5*A*, SHP-1 is expressed in resting THP-1wt cells with an apparent subunit size of ~65 kDa and is localized predominantly in the cytosol and to a lesser extent in the membrane fraction (Fig. 5*A*). Using reducing electrophoresis conditions, anti-SHP-1 immunoprecipitates show one band with a subunit size of approximately 65 kDa, but under non-reducing electrophoretic conditions, a fraction of the SHP-1 migrates with an apparent subunit size of 62 kDa.

To examine whether LAM increases the activity of SHP-1 *in vivo*, cells were exposed to either LAM (2 μg/ml), LPS (1 μg/ml), or IFN-γ (200 units/ml) for 30 min. Lysates were prepared, and

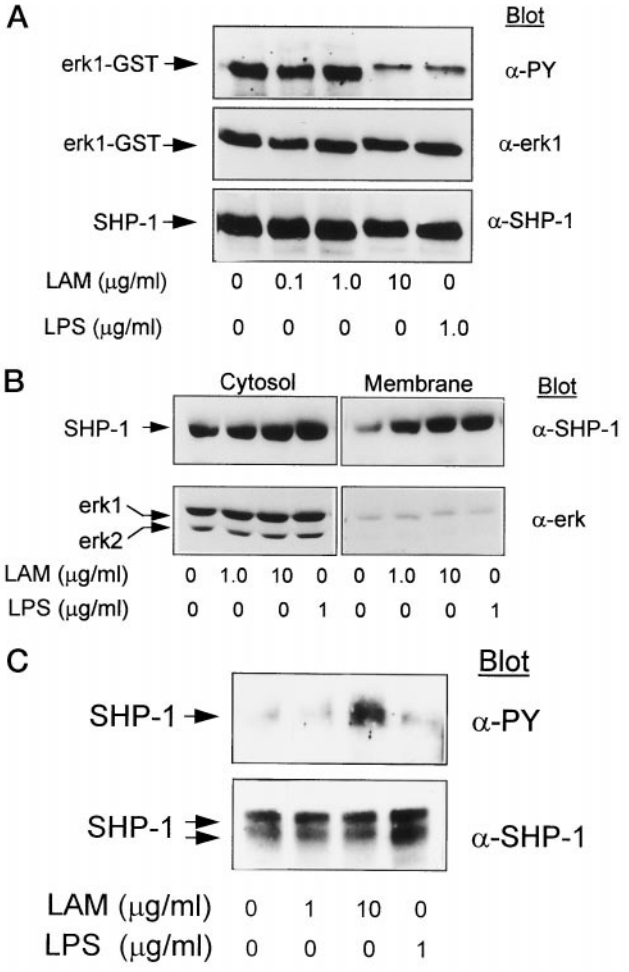

FIG. 6. **Long term, *in vivo* effects of LAM on the phosphatase activity, tyrosine phosphorylation state, and subcellular distribution of SHP-1.** *A*, THP-1wt cells were treated with varying concentrations of LAM, LPS (1 μg/ml), or medium alone for 16 h. SHP-1 was immunoprecipitated from equal amounts of lysate protein, and phosphatase activity was measured using phosphorylated MAPK-1-GST (erk1-GST) as substrate. The tyrosine phosphorylation state of MAPK-1-GST was assessed by SDS-PAGE and immunoblotting with anti-phosphotyrosine (α-*PY*) antibodies. The blot was stripped and re-probed with anti-SHP-1 antibodies and anti-MAPK (α-erk1-GST) antibodies to confirm equal loading of substrate and phosphatase. *B*, LAM-induced redistribution of SHP-1 to the membrane fraction. THP-1wt cells were treated as described above and fractionated into cytosol and membrane as described under "Materials and Methods." Equal amounts of each fraction were analyzed by SDS-PAGE and immunoblotting with anti-SHP-1. The blot was reprobed with anti-MAPK (erk1 and erk2) antibodies to demonstrate that redistribution of SHP-1 was selective. Total levels of SHP-1 did not change with treatment (data not shown). *C*, LAM-induced tyrosine phosphorylation of SHP-1. THP-1wt cells were treated as described above, and SHP-1 was immunoprecipitated from equal amounts of lysate. The immunoprecipitates were analyzed by non-reducing SDS-PAGE and immunoblotting with anti-phosphotyrosine antibodies. The blot was stripped and reprobed with anti-SHP-1 to confirm equal loading of the enzyme. The tyrosine-phosphorylated band corresponds to the faster migrating SHP-1. The blot shown is representative of two independent experiments.

equal amounts of protein were immunoprecipitated with anti-SHP-1 antibody. SHP-1 activity was measured by incubating the immunoprecipitates with pNPP. As shown in Fig. 5*B*, in response to LAM, pNPPase activity was increased 2.3-fold. In contrast, treatment of cells with either IFN-γ or LPS resulted in apparent decreases in phosphatase activity.

Since it has previously been shown that SHP-1 may be activated by phospholipids (45), the ability of LAM to activate SHP-1 *in vitro* was also examined. SHP-1 was immunoprecipitated from resting cells and incubated with either medium only or LAM (10 μg/ml). Phosphatase activity was measured using phosphorylated MAPK-1-GST as a substrate. As shown in Fig. 5*C*, when LAM was incubated with SHP-1 there was enhanced dephosphorylation of MAPK-1-GST, and this required the presence of both SHP-1 and LAM. Shown in Fig. 5*D* is a dose-response analysis of the ability of LAM to enhance SHP-1-mediated dephosphorylation of MAPK-1-GST. As can be seen, enhanced dephosphorylation was dose-dependent when LAM was incubated with SHP-1 immunoprecipitates. The immunoblot of MAPK-1-GST shown in this figure indicates that the decrease in signal intensity for the phosphorylated protein was not related to a decrease in enzyme mass.

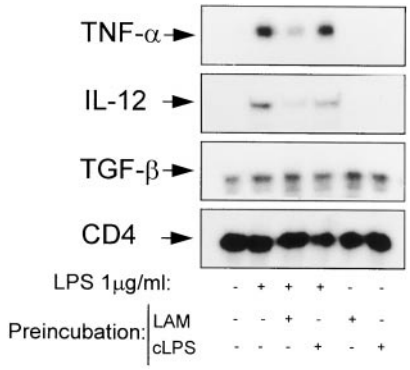

FIG. 7. **Attenuation of LPS-induced TNF-α and IL-12 mRNA production by LAM.** THP-1wt cells were preincubated with either LAM (2 μg/ml), cLPS (36 pg/ml), or medium alone for 16 h, followed by the addition of either LPS (1 μg/ml) or medium alone for 2 h. RNA was isolated, and levels of cytokine or CD4 mRNA were assessed by RNase protection assay as described under "Materials and Methods." cLPS represents the maximum potential contaminating concentration of LPS in the LAM preparation. The results shown are from one of two independent experiments with similar results.

Similar experiments were carried out to examine whether longer term exposure of THP-1 cells to LAM also activates SHP-1. Cells were incubated for 20 h in the presence of varying concentrations of LAM, LPS (1 μg/ml), or medium alone. Lysates were prepared and SHP-1 was immunoprecipitated from equal amounts of protein. Phosphatase activity of SHP-1 was measured against MAPK-1-GST. Fig. 6*A* shows that SHP-1 immunoprecipitated from both LAM- and LPS-treated cells had significantly enhanced activity toward tyrosine-phosphorylated MAPK-1-GST. In addition, Fig. 6*B* shows that SHP-1 redistributed to the membrane fraction when cell were treated chronically with LAM. This effect was detectable at concentrations as low as 1 μg/ml but was not apparent when cells were exposed to LAM for shorter periods (*i.e.* 30 min, data not shown). Under the same conditions, there was no evidence for redistribution of either $p42^{MAPK2}$ or $p44^{MAPK1}$ to the membrane fraction. Thus, this effect of LAM on SHP-1 distribution appears to be selective.

The tyrosine phosphorylation state of SHP-1 in cells treated with LAM was also investigated. Cells were treated with varying concentrations of LAM, LPS (1 μg/ml), or control vehicle. Lysates were prepared, and SHP-1 was immunoprecipitated from equal amounts of protein and immunoblotted with anti-phosphotyrosine antibodies. As shown in Fig. 6*C*, LAM but not LPS induced an increase in the phosphotyrosine content of SHP-1.

Attenuation of LPS-induced Up-regulation of TNF-α and IL-12 p40 mRNA by LAM—To address whether the effects of LAM on monocyte tyrosine phosphorylation correlate with diminished functional responses to LPS, mRNA levels were measured for TGF-β, TNF-α, CD4, and IL-12 p40 by RNase protection assay. As shown in Fig. 7, treatment of THP-1wt cells with LPS (1 μg/mL) up-regulated the expression of both TNFα and IL-12 p40

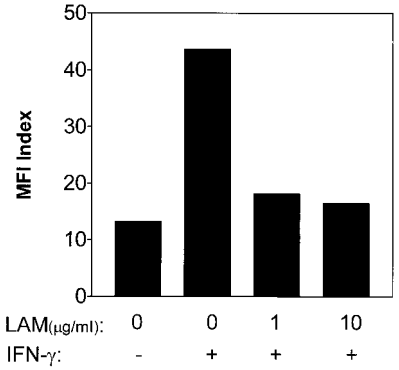

FIG. 8. **Attenuation of IFN-γ-induced MHC class II expression by LAM.** Differentiated THP-1 cells were pretreated with LAM (1 or 10 μg/ml) or with control medium for 24 h. IFN-γ was added for an additional 24 h. Cell surface MHC class II expression was measured by fluorescence-activated cell sorter analysis as described under "Materials and Methods." The results shown are representative of two independent experiments. Similar results were obtained using normal human monocytes (data not shown).

mRNAs within 2 h. In contrast, levels of neither TGF-β1 nor CD4 changed in response to LPS. When cells were preincubated with LAM (2 μg/ml) for 16–20 h, the LPS-induced increases in both TNF-α and IL-12 p40 mRNAs were significantly attenuated. In contrast, LAM had no effects on the steady state levels of TGF-β1 or CD4. Cells were also incubated in 36 pg/ml LPS (cLPS) overnight prior to stimulation with LPS. This preincubation in cLPS represents the apparent level of LPS-like activity contaminating the LAM preparations as assessed by *Limulus amebocyte* assay. Preincubation of cells with this concentration of LPS had negligible effects on the subsequent enhancement of TNFα and IL-12 p40 mRNA levels in response to a second incubation with LPS.

Attenuation of Interferon-γ-induced Up-regulation of Cell Surface MHC Class II Expression by LAM—To examine whether the effects of LAM on IFN-γ-induced protein tyrosine phosphorylation in THP-1 cells (data not shown) correlates with diminished functional responses to this cytokine, cell surface expression of MHC class II expression was analyzed. Differentiated THP-1 cells were treated with various concentrations of LAM or control vehicle for 20 h. Interferon-γ was then added for an additional 24 h. Cell surface MHC class II levels were measured by fluorescence-activated cell sorter analysis. As shown in Fig. 8, treatment of cells with interferon-γ (200 units/ml) produced a 4-fold increase in the level of cell surface MHC class II. Pretreatment of cells with LAM led to a nearly complete abrogation of interferon-γ-induced MHC class II expression. Similar results were obtained with normal human monocytes (data not shown).

DISCUSSION

To maintain itself stably in its human reservoir, *M. tuberculosis* has evolved strategies to undermine both innate host resistance and acquired immune responses. The mechanisms involved most likely include the elaboration of immunosuppressive molecules, one of which is LAM. A great deal of evidence supports the notion that LAM is a potential immunosuppressive molecule (21, 24–26); however, its precise mode of action is still not known. The present study examined the possibility that LAM may affect cell signaling pathways in macrophages and focused on the critical phenomena of protein tyrosine phosphorylation and tyrosine dephosphorylation.

Tyrosine phosphorylation is an important component of all of the signaling pathways examined in this study. PMA is a broad range activator of cells and acts primarily through its ability to activate multiple isoforms of PKC (46). One event that occurs downstream of PKC is activation of the $p42^{MAPK2}$, and this involves its phosphorylation on tyrosine 183 and threonine 185 N-terminal to kinase subdomain VIII (47). MAPK activation is known to be involved in the regulation of a broad range of cellular processes (48). In the present study, the effects of LAM on PMA-induced tyrosine phosphorylation and activation of the $p42^{MAPK2}$ was examined (Fig. 1, *A–C*). As shown, activation of $p42^{MAPK2}$ was markedly attenuated in LAM-treated cells (Fig. 1*C*), and this appeared to be related to diminished tyrosine phosphorylation of the kinase (Fig. 1*A*). The finding that bandshifting of $p42^{MAPK2}$ was unaffected (Fig. 1*B*) suggests that other phosphorylation events, involving either threonine residues, serine residues, or both, regulate bandshifting and that these are unaltered in LAM-treated cells. One possibility to explain these findings is that LAM inhibits activation of PKC by PMA. Three lines of evidence, however, argue against this possibility. First, bandshifting was observed in LAM-treated cells indicating that some phosphorylation of MAPK is nevertheless occurring in response to PMA. Second, LAM only weakly inhibits PKC at the concentrations used (26). Third, it was observed that PMA-induced activation of other renaturable kinases, as assessed by "in-gel" assays, were unaltered by LAM pretreatment (data not shown). On the other hand, the finding of diminished tyrosine phosphorylation of $p42^{MAPK2}$ in the face of apparently normal bandshifting in LAM-treated cells could be explained by selective tyrosine dephosphorylation of MAPK during its activation by PMA. A potential mediator of this effect of LAM is the abundant phosphotyrosine phosphatase SHP-1, which is known to be involved in terminating activation signals. Indeed, it has been reported that MAPK is an *in vitro* substrate for SHP-1 (45), and the experiments reported here provide direct evidence that SHP-1 is activated by LAM (Figs. 5 and 6).

SHP-1 is thought to be an important negative regulator of a variety signaling pathways such as those related to the actions of IFN-γ (29) and insulin (49), and regulation of its activity is complex. It has been reported that phosphorylation on tyrosine 538 increases the activity of SHP-1 (49, 50). In contrast, serine phosphorylation induced by PKC leads to decreased activity (39). It has also been shown that SHP-1 is activated by phospholipids suggesting that it may be regulated by translocation from the cytosol to the membrane (45).

The results of the present study suggest that LAM may regulate SHP-1 by multiple mechanisms. First, it is shown that LAM increases SHP-1 phosphatase activity *in vivo* as assessed by immunoprecipitation phosphatase assays (Figs. 5*B* and 6*A*). The mechanism for this increased activity was investigated by examining SHP-1 tyrosine phosphorylation, direct activation by LAM *in vitro*, and translocation of the phosphatase to the membrane. Following acute exposure of cells to LAM, it was observed that SHP-1 phosphatase activity is increased (Fig. 5*B*). However, under these conditions, LAM did not lead to detectable increases in tyrosine phosphorylation of SHP-1 or to its apparent translocation to the membrane (data not shown). Thus, under conditions of short term exposure, the most likely mechanism is related to direct activation of SHP-1 by LAM or possibly to a decrease in the enzyme's serine phosphorylation state. Activation by a direct interaction is supported by the finding that incubation of SHP-1 with LAM *in vitro* leads to activation of the enzyme (Fig. 5, *C* and *D*). This finding is consistent with prior studies in which activation of SHP-1 *in vitro* by phospholipids has been observed (45).

In contrast to short term incubation, long term exposure of cells to LAM did result in translocation of SHP-1 to the membrane (Fig. 6*B*) and to an increase in its tyrosine phosphorylation (Fig. 6*C*). These effects could explain the increase in SHP-1 activity observed during longer term exposure and sug-

gest the possibility that LAM may regulate SHP-1 by multiple mechanisms including (i) direct interactions, (ii) changes in its phosphorylation state, and (iii) subcellular localization.

In addition to attenuating PMA-induced tyrosine phosphorylation and activation of MAPK, LAM was also observed to inhibit both LPS- (Fig. 2) and IFN-γ (data not shown)-induced protein tyrosine phosphorylation in monocytes. Recent evidence indicates that LPS is a potent inducer of tyrosine phosphorylation (51, 52) and that tyrosine kinase inhibitors block LPS-induced functional changes such as TNF-α production (51) and tumoricidal activity (53). Tyrosine phosphorylation is also known to be a critical element in bringing about functional responses to IFN-γ (54, 55). Indeed, under the conditions of the present study, attenuation by LAM of tyrosine phosphorylation in response to either LPS or IFN-γ correlated with inhibition of functional responses to both agonists (Figs. 7 and 8).

The effects of LAM on levels of tyrosine phosphorylation of macrophage proteins appear to be influenced by specific structural features of the molecule. Thus, the results shown in Fig. 3 indicate that the capacity of LAM to diminish tyrosine phosphorylation of macrophage proteins is dependent on the structure of the polysaccharide region (LAM > lipomannan > phosphatidylinositolmannoside). Lipomannan is identical to LAM except for the absence of arabinofuranosyl side chains, and phosphatidylinositolmannoside is identical to lipomannan except for having fewer mannose residues. Thus, these findings suggest that the action of LAM is at least partially mediated through it polysaccharide region.

The ability of LAM to diminish cytokine induction by LPS and functional responses to IFN-γ through the effects of SHP-1 has the potential to modify the course of infection with *M. tuberculosis*. The importance of TNF-α in the pathogenesis of the *M. tuberculosis* infection has become increasingly evident, notably in granuloma formation. In bacillus Calmette-Guérin infections in mice, TNF-α deficiency results in poor granuloma formation and disseminated disease (56). Furthermore, TNF-α induces macrophages to produce reactive nitrogen oxides that are critical in *M. tuberculosis* killing (57). Similarly, interleukin-12 (IL-12) plays a critical role in innate resistance to *M. tuberculosis* infections by activating natural killer cells that produce IFN-γ, thereby further activating macrophages (8). Thus, impairing TNF-α and IL-12 production and responsiveness to IFN-γ through effects on SHP-1 may be a major mechanism by which LAM promotes intracellular survival of *M. tuberculosis*.

In summary, the data in this study provide support for the hypothesis that LAM is a potential virulence factor that may contribute to the pathogenesis of *M. tuberculosis*. One mechanism by which LAM may exert its effects is by inhibiting signaling pathways that are necessary for macrophage activation and intracellular killing. This inhibitory effect of LAM may be related to its ability to regulate the activity and subcellular distribution of the protein tyrosine phosphatase, SHP-1.

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