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**Mycobacterium tuberculosis** LprA Is a Lipoprotein Agonist of TLR2 That Regulates Innate Immunity and APC Function

Nicole D. Pecora,* Adam J. Gehring,2*† David H. Canaday, † W. Henry Boom,3*† and Clifford V. Harding3,4,*

TLR2 recognizes components of *Mycobacterium tuberculosis* (Mtb) and initiates responses by APCs that influence both innate and adaptive immunity. Mtb lipoproteins are an important class of TLR2 ligand, but only two, LpqH and LprG, have been characterized to date. In this study, we characterize a third Mtb lipoprotein, LprA, and determine its effects on host macrophages and dendritic cells. LprA is a cell wall-associated lipoprotein with no homologs outside the slow-growing mycobacteria. Using *Mycobacterium smegmatis* as an expression host, we purified 6× His-tagged LprA both with and without its acyl modifications. Acylated LprA had agonist activity for both human and murine TLR2 and induced expression of TNF-α, IL-10, and IL-12. LprA also induced dendritic cell maturation as shown by increased expression of CD40, CD80, and class II MHC (MHC-II). In macrophages, prolonged (24 h) incubation with LprA decreased IFN-γ-induced MHC-II Ag processing and presentation, consistent with an observed decrease in MHC-II expression (macrophage viability was not affected and apoptosis was not induced by LprA). Reduced MHC-II Ag presentation may represent a negative feedback mechanism for control of inflammation that may be subverted by Mtb for immune evasion. Thus, Mtb LprA is a TLR2 agonist that induces cytokine responses and regulates APC function. The Journal of Immunology, 2006, 177: 422–429.

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*Mycobacterium tuberculosis* (Mtb)3 remains a significant cause of mortality worldwide, especially in the context of antibiotic resistant strains and coinfection with HIV. Host resistance depends on both innate and adaptive immunity. CD4 T cells dominate the adaptive response, but CD8 T cells are also involved. TLRs, which recognize microbial molecules, are major triggers of innate responses (e.g., cytokine production) and modulate adaptive immunity by influencing APCs (1). For example, TLR signaling by Mtb causes dendritic cell maturation and migration to lymph nodes (critical steps for T cell priming) (2–4).

TLRs are important for host responses to Mtb (5–8). Mice deficient in MyD88, an adaptor protein for signaling by many TLRs, have increased susceptibility to mycobacterial infection (9). TLR2, in particular, appears to be critical for sensing mycobacteria (6). Polymorphisms in human TLR2 are associated with enhanced susceptibility to leprosy and tuberculosis (14–16), and TLR2 deficiency increases the susceptibility of mice to mycobacterial infection (17). Several mycobacterial TLR2 ligands have been identified, including lipomannan (19), certain lipoolarabinomannan (LAM) species (5, 20), phosphatidyl-myo-inositol mannoside (20, 21), and two lipoproteins, LpqH (19-kDa lipoprotein, Rv3763) (22–24) and LprG (Rv1411c) (25).

In their mature form, bacterial lipoproteins are characterized by an N-terminal, triacylated cysteine. They are first synthesized as a preprolipoprotein (with an N-terminal signal sequence) that is consecutively processed by prolipoprotein diacylglycerol transferase (Lgt) (26), lipoprotein signal peptidase (LspA) (27, 28) and Lnt, an acyl transferase (28). Lgt catalyzes the thioether linkage of a diacylglycerol moiety to the cysteine immediately C-terminal to the signal sequence, LspA cleaves the signal sequence, and a final N-linked acyl chain is added by Lnt concurrently with transport across the inner membrane (28). Although most of the work characterizing the lipoprotein biosynthetic pathway has focused on *Escherichia coli*, mycobacterial homologs of both Lgt and LspA have been identified (27, 29). Acylation of bacterial lipoproteins and lipopeptides is thought to be important for their ability to signal though TLR2 (4, 22, 24, 30–34), although peptide sequence can also affect TLR2 agonist activity (35–38) and a nonacylated (NA) TLR2 ligand has been reported (36).

In addition to modification with lipids, there is growing evidence that many mycobacterial lipoproteins are also glycosylated. For example, LpqH has been shown to contain carbohydrate modifications on several threonine residues close to the N terminus of the protein (39). It has been proposed that glycosylation of LpqH may be important for regulating proteolytic cleavage of the lipoprotein (39). Glycosylation of bacterial proteins may play multiple roles in bacterial pathogenesis (40), but it is unknown how glycosylation affects interactions between pathogens and TLRs.

Mtb lipoproteins LpqH and LprG have both stimulatory and inhibitory effects on host APCs, all of which are TLR2 dependent. Stimulatory activities include induction of cytokine expression by macrophages and dendritic cells (3, 24, 25), and enhancement of dendritic cell maturation (4). In contrast, prolonged exposure (>18 h) to LpqH inhibits IFN-γ-induced class II MHC (MHC-II) expression and Ag presentation by macrophages (22, 23, 25, 41–45). This response may reflect a homeostatic feedback inhibition mechanism that
limits macrophage activation of effector T cells to prevent excessive inflammation. As a consequence, a subset of infected macrophages with decreased APC function may hypothetically be unable to present Mtb Ags to CD4+ T cells, providing niches in which Mtb persists, evading immune surveillance.

During our characterization of LprG (25), mass spectrometry indicated the presence of LprA (Rv1270c) in Mtb fractions with immunoregulatory activity, but we were unable to purify LprA to assess its activity. The current study was designed to purify Mtb LprA, test whether it is a TLR2 agonist and determine its ability to induce innate immune responses and regulate APC function. LprA, a putative lipoprotein of unknown function, is related to LprG with a similar molecular mass and 34% sequence identity. Like LprG, LprA has no homologs outside of the mycobacteria. Purification of LprA was facilitated by expression of 6× His-tagged LprA in *Mycobacterium smegmatis* and purification of recombinant LprA by affinity chromatography. To explore the role of lipid structures in TLR2 agonist activity, recombinant LprA was expressed in both acylated and NA forms. Acylated LprA was found to signal through TLR2, but NA-LprA lacked activity. LprA was a potent inducer of cytokine expression by both macrophages and dendritic cells. In addition, LprA modulated APC function either positively through TLR2, but NA-LprA lacked activity. LprA was a potent immunoregulatory activity, but we were unable to purify LprA to 20 nM Tris (pH 8.0). Both LprA and NA-LprA were further purified by ion-exchange chromatography with a 1-ml QEP anion exchange column (Amersham Biosciences) and elution in 20 mM Tris (pH 8.0) with 50 mM NaCl. Eluted proteins were concentrated in Amicon Ultra-4 spin columns with 10-kDa cutoff (Millipore). Protein concentration was determined by the BCA assay (Pierce).

### Western blots and SDS-PAGE

Samples were boiled in SDS-PAGE sample buffer under reducing conditions, electrophoresed on 12% polyacrylamide gels, and stained using Bio- 

### Materials and Methods

### Bacterial strains and cloning of recombinant LprA

Cloning was done in *E. coli* DH5α (Invitrogen Life Technologies). LprA was expressed in *M. smegmatis* mc^2^155 (26) (from R. Wilkinson, Imperial College, London, U.K.) cultivated in Middlebrook 7H9 broth (Difco) supplemented with 1% casamino acids, 0.2% glycerol, 0.2% glucose, and 0.05% Tween 80. Kanamycin was used for selections in both *E. coli* and *M. smegmatis* at 30 μg/ml. Full-length lprA (Rv1270c) was amplified from Mtb H37Rv genomic DNA by PCR using the following 5′ primer: 5′-GCA TAT CCA TAT GAA GCA TCC ACC TTG TTC CGT TGT-3′. For Western blotting, protein bands were transferred to polyvinylidene difluoride membranes and blocked in 5% nonfat milk for 30 min at room temperature, and incubated overnight at 4°C with 200 ng/ml mouse anti-6× His primary Ab (Santa Cruz Biotechnology) in PBS with 0.1% Tween 20 and 5% nonfat milk. Blots were washed extensively, incubated with 125 ng/ml HRP-linked horse anti-mouse IgG (Cell Signaling Technology) for 2 h at room temperature, and developed with ECL detection reagents (Amersham Biosciences). Western analysis to rule out ARALAM contamination was similarly performed with 1 μg of LprA or NA-LprA, and blots were stained with 906.4321 (rabbit IgG3 monoclonal anti-arabinofuranosyl-terminated LAM (ARALAM) Ab, Colorado State University TB Vaccine Research and Research Materials Contract NHI N01-AI40091, culture supernatant used at 1/20 dilution) and 0.2 μg/ml HRP-linked goat-anti-rabbit secondary Ab (Cell Signaling Technology). For detection of protein glycosylation, blots were blocked overnight at 4°C in PBS, 0.1% Tween 20, 4% BSA, washed five times in PBS, 0.1% Tween 20, incubated for 1 h at room temperature with Con A-HRP (Sigma-Aldrich) at 0.2 purpurogallin units/ml in PBS, 0.1% Tween 20, 2% BSA, washed several times and developed with ECL reagents.

### Mass spectrometry

To identify proteins, bands were cut from a Coomassie blue-stained SDS-PAGE gel, and washed and destained in 50% ethanol, 5% acetic acid. Gel pieces were dehydrated in acetonitrile, washed in 0.1 M ammonium bicarbonate, dehydrated in acetonitrile, and dried. Trypsin was introduced into gel pieces by rehydrating for 10 min at 4°C in 30 μl of 20 ng/μl trypsin in 50 mM ammonium bicarbonate. Any excess trypsin solution was removed, and 20 μl of 50 mM ammonium bicarbonate was added. Samples were digested overnight at room temperature. Resulting peptides were extracted from polyacrylamide in two 50-μl aliquots of 50% acetonitrile, 5% formic acid. These extracts were combined and evaporated to <20 μl for liquid chromatography/mass spectrometry analysis on a Finnigan LTQ linear ion trap mass spectrometer system.

### Eukaryotic cells and mice

C57BL/6J mice were 8- to 16-wk-old females (The Jackson Laboratory) housed under specific pathogen-free conditions. Unless otherwise specified, mice were inoculated with 37°C in 5% CO2 atmosphere. HEK293 cells transfected with TLR2 (HEK.TLR2) or TLR4 and MD-2 (HEK.TLR4) were provided by D. Golenbock (University of Massachusetts, Worcester, MA) (46, 47) and maintained in DMEM (BioWhittaker) supplemented with 10% heat-inactivated FCS (HyClone), ciprofloxacin (10 μg/ml), and gentamicin (500 μg/ml). Murine macrophages were derived from bone marrow precursors cultured for 7-12 days in medium composed of DMEM (BioWhittaker) supplemented with 10% heat-inactivated FCS (HyClone), 50 μM 2-ME, 2 mM t-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, and antibiotics (standard R10F) with 25% LADMAC cell-conditioned medium (48). Murine dendritic cells were generated by culturing bone marrow precursors in RPMI 1640 (BioWhittaker) supplemented with 10% heat-inactivated FCS (HyClone), 50 μM 2-ME, 2 mM l-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, and antibiotics (standard R10F) supplemented with 25% LADMAC cell-conditioned medium (48). One half of the medium was replaced every 3 days and cells were used on day 9.

### Cytokine ELISAs

HEK293 cells were plated overnight (20,000 cells/well in 96-well plates) and then incubated with TLR ligand for 24 h. Supernatants were tested for IL-8 by ELISA (R&D Systems). Macrophages (800,000 cells/well in 24-well plates) were incubated overnight in D10F and then for 12 h with LprA or a positive control TLR2 agonist peptide, N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl][4'-cytosinyl-3'-seryl][3'-lysyl][5'-lysyl][5'-lysyl][5'-lysine]× 3 HCI (PC5) (EMC Microcollections). Alternatively, dendritic cells (130,000 cells well in 24-well plate) were incubated with LprA or P3C for 24 h. Supernatants were harvested for ELISA for TNF-α, IL-12p70, and IL-10 with OPeTEA ELISA kits (BD Biosciences).

### Purification of LprA

To purify LprA or NA-LprA, a 4-L culture of *M. smegmatis* expressing the 6× His-tagged protein or the pV616 vector control was grown to late log phase and harvested by centrifugation. The pellet was resuspended in 40 ml of lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole (pH 8.0), 2.5% protease inhibitor mixture for His-tagged proteins (Sigma-Aldrich) and 75 U/ml benzamidine (Sigma-Aldrich)). The suspension was passed through a French press four times, and centrifuged for 60 min at 100,000 g to remove cell wall material and unlysed bacteria. In some analyses, a portion of the pellet was solubilized in SDS-PAGE sample buffer, and the supernatant and solubilized pellet were subjected to Western analysis. The supernatant was incubated with nickel-nitriitolriacetic acid (Ni-NTA) agarose beads (Qiagen) for 2 h at 4°C. After washing with wash buffer (50 mM NaH2PO4, 1 M NaCl, 20 mM imidazole, 10% w/v glycerol (pH 8.0)), 6× His-tagged LprA was disassociated from the resin in elution buffer (50 mM NaH2PO4, 300 mM NaCl, 450 mM imidazole (pH 8.0)). PD-10 columns (Amersham Biosciences) were used to exchange the buffer to 20 mM Tris (pH 8.0). Both LprA and NA-LprA were further purified by ion-exchange chromatography with a 1-ml QEP anion exchange column (Amersham Biosciences) and elution in 20 mM Tris (pH 8.0) with 50 mM NaCl. Eluted proteins were concentrated in Amicon Ultra-4 spin columns with 10-kDa cutoff (Millipore). Protein concentration was determined by the BCA assay (Pierce).
**Flow cytometry**

Dendritic cells (500,000 cells/well in 24-well plates) were incubated in R10F for 24 h with or without LprA. Bone marrow-derived macrophages (2 x 10^6 cells/well in a 6-well plate) were treated with LprA or NA-LprA for 48 h with 2 ng/ml IFN-γ present for the last 24 h. Cells were harvested by vigorous pipetting and trypsinization, incubated for 30 min on ice with FcBlock (BD Pharmingen) at 1/100 in PBS with 0.1% BSA and for 1 h on ice with anti-MHC-II-FITC (eBioscience), anti-CD86-PE (BD Pharmingen), anti-CD40-PE-CY5 (eBioscience) or appropriate isotype-matched negative control Ab. Cells were washed three times in PBS with 0.1% BSA, fixed in 1% paraformaldehyde, and analyzed by a BD FACScan flow cytometer.

**Ag-processing assay**

Macrophages (100,000 cells/well in 96-well plates) were incubated overnight in D10F, for 24 h with or without LprA, and for 24 h with 2 ng/ml IFN-γ with or without fresh LprA. Soluble OVA (Sigma-Aldrich) or OVA (323–339) peptide was added for 2 h. Cells were washed in DMEM and fixed in 0.5% paraformaldehyde. DOBW T hybridoma cells, which recognize OVA (323–339) peptide sequence presented by I-A^k, were added to 100,000 cells/well for 24 h. IL-2 was measured in the supernatant by a colorimetric CTL-2 bioassay. Supernatants were incubated with 5000 CTLL-2 cells for 24 h. Alamar Blue (Trek Diagnostic Systems) was added for 24–48 h, and a Bio-Rad Model 550 microplate reader was used to determine OD550-OD595.

**Assays for apoptosis and necrosis**

Macrophages were treated with LprA and NA-LprA as described for the Ag processing assay and then stained with annexin V-biotin (BD Pharmingen) and propidium iodide (Invitrogen Life Technologies) according to the manufacturer’s protocols. Streptavidin-FITC (BD Pharmingen) and propidium iodide (Invitrogen) were used as a secondary Ab at 1/100 to detect annexin V. Fluorescence was measured on a BD Biosciences FACScan flow cytometer.

**Results**

**Purification of LprA**

LprA was produced as a recombinant protein (Fig. 1) expressed in M. smegmatis LprA was produced as a recombinant protein (Fig. 1) expressed in M. smegmatis. Peptide sequence of LprA and NA-LprA. The M. smegmatis genome have not revealed LprG, LpqH, or LprA homologs. The full-length lprA gene (including signal sequence) was cloned from Mtb H37Rv and expressed with a 6X His tag on the C terminus to facilitate purification. M. smegmatis was chosen as an expression host because it grows faster and is easier to manipulate than slow-growing mycobacteria, yet it is phylogenetically closer to Mtb than other potential bacterial hosts, such as E. coli. As such, it is predicted to use correct codon usage and use proper secretion and acylation machinery for expression of LprA derived from Mtb (49–51). M. smegmatis does not produce any endogenous LprA.

M. smegmatis lysate was prepared with a French press and centrifuged to separate insoluble cell wall and membrane material (pellet) from soluble proteins (supernatant). Western blots with anti-6X His Ab indicated that LprA was present in the insoluble fraction (Fig. 2A), consistent with the expected behavior of a lipoprotein. However, a substantial amount of LprA was also present in the soluble fraction (Fig. 2A). To avoid the use of detergents with risks of carryover toxicity or increased release of other contaminating TLR2 ligands, the soluble fraction was used for further purification of LprA by Ni-NTA affinity chromatography, yielding a semipurified preparation. Because several contaminants were present (data not shown), subsequent purification by ion exchange chromatography was performed to yield pure LprA without contaminating proteins detectable by Coomassie staining (Fig. 2B). The average yield from a 4-L culture was 150 μg of purified LprA. Western blot analysis with Abs to LAM and ARALAM demonstrated that the preparations were free from detectable contamination by those species (data not shown). Purified LprA appeared as a main band of ~24 kDa; a fainter band of slightly lower apparent molecular mass was also present to a variable degree (Fig. 2B). The distinction between these bands remained obscure, but both represent LprA, since analysis by mass spectrometry revealed Mtb LprA sequences from both bands, and sequences from other contaminating proteins were not detected. The

**FIGURE 1.** Cloning and expression of LprA and NA-LprA. A, Cloning and expression strategy. Mtb H37Rv DNA was used as a template to amplify lprA, which was cloned into the pVV16 shuttle vector to encode a fusion protein with a 6X His tag at the C terminus. This construct was used to transform E. coli, verified by sequencing, and used to transform M. smegmatis for expression of LprA. A similar approach was used to clone and express NA-LprA. B, Peptide sequence of LprA and NA-LprA. The additional C-terminal KLHHHHHH sequence containing a HindIII site and the 6X His tag is shown in italics. NA-LprA lacks the signal sequence (underlined), and the cytosine that is targeted for acylation in LprA is replaced by methionine.

**FIGURE 2.** Purification and analysis of recombinant LprA-6X His from M. smegmatis. M. smegmatis containing either LprA, NA-LprA, or pVV16 control vector was pelleted from a 4-L culture and lysed with a French press. The lysate was centrifuged to pellet insoluble cell wall debris and unlysed bacteria, leaving soluble species in the supernatant. A, Western analysis of supernatant (Supt.) or detergent solubilized pellet with anti-6X His Ab to detect LprA. B, LprA and NA-LprA were purified from the supernatant by Ni-NTA affinity and ion exchange chromatography, and 1 μg of each was analyzed by SDS-PAGE with Coomassie blue staining. C, LprA and NA-LprA were purified by Ni-NTA affinity and ion exchange chromatography, and 1 μg of each was subjected to SDS-PAGE, transferred to polyvinylidene difluoride membrane and probed with either Con A-HRP or anti-6X His Ab.
lower band may be a cleavage product of the full-length protein. LprA was also found to be glycosylated, as evidenced by binding of Con A to the main upper band (Fig. 2C). The lower molecular mass band, however, may represent a nonglycosylated form of LprA, as it showed little or no binding by Con A (Fig. 2C). As proposed for LpqH (39), it is possible that glycosylation protects proteolytic cleavage of LprA, and nonglycosylated LprA is preferentially cleaved to form a lower molecular mass species, but this remains speculative. In conclusion, the combination of affinity and ion exchange chromatography allowed preparation of highly pure recombinant 6× His-tagged LprA for subsequent analysis.

To produce NA protein, LprA was also cloned without its signal sequence and with its target cysteine residue replaced by methionine (Cys77–Met) to produce NA-LprA (Fig. 1). As there is no signal sequence to direct its export, this protein remains cytosolic, and very little was detected in the insoluble cell wall fraction (data not shown). NA-LprA was purified from the soluble fraction of M. smegmatis and very little was detected in the insoluble cell wall fraction (data not shown). NA-LprA was purified from the soluble fraction of M. smegmatis by chromatography to yield a pure preparation without detectable contamination by Coomassie staining (Fig. 2B) or Western analysis for LAM and ARALAM (data not shown). In some gels, NA-LprA appeared slightly larger than LprA, but this was not always observed. Like LprA, NA-LprA appeared as a doublet with a main upper band and a fainter lower band (Fig. 2B) that sometimes appeared more prominent than observed with LprA. Mass spectrometry again revealed Mtb LprA peptide sequences from both bands, and sequences from other contaminating proteins were not detected. Unlike LprA, NA-LprA does not react with Con A and does not, therefore, appear to be glycosylated (Fig. 2C). This may reflect its cytosolic localization, because mycobacterial protein glycosylation is linked to secretion (52).

We speculate that lack of NA-LprA glycosylation may allow increased proteolytic production of the smaller species. The average yield of purified NA-LprA was 1 mg from a 4-L culture, higher than the yield for LprA. We conclude that M. smegmatis serves as an effective host for expression of 6× His-tagged LprA and NA-LprA, which can then be prepared to high purity.

Acylated LprA is a ligand for human TLR2

LprA has not been studied for TLR agonist activity, although other mycobacterial lipoproteins (LpqH and LprG) are known to signal through TLR2 and MyD88 (22–25). HEK293 cells lack TLRs yet retain downstream components used for TLR signaling. HEK293 cells transfected to express specific TLRs can be used to assess TLR recognition of potential ligands; these cells secrete IL-8 in response to TLR signaling and consequent activation of NF-kB. To test whether LprA can signal through TLR2, we used HEK293 cells transfected with human TLR2 (HEK293.TLR2). Cells were incubated with LprA for 24 h, and supernatants were assessed by ELISA (data not shown). Nonacylated LprA, however, did not enhance expression of IL-8 from HEK293.TLR2 cells transfected with human TLR2 (HEK293.TLR2). Cells were incubated with LprA for 24 h, and supernatants were assessed by ELISA for IL-8. LprA induced IL-8 production by HEK293.TLR2 cells (Fig. 3A) but not untransfected HEK293 cells (Fig. 3B). Significant responses were observed with as low as 5 nM LprA (data not shown), while 2 nM LprA did not produce significant responses and 20 nM LprA produced strong responses (Fig. 3A). A negative control preparation, isolated in parallel by the LprA purification protocol from M. smegmatis, showed no IL-8 agonist activity (data not shown).

The acyl chains of some other lipoproteins or lipopeptides are important determinants of TLR2 agonist activity (22, 24, 30, 31). However, in some cases the peptide portion of a lipoprotein is also able to signal through TLR2 (36). To determine the role of acyl chains in TLR2 signaling by LprA, we tested the ability of NA-LprA to induce IL-8 production by HEK293.TLR2 cells. We found that NA-LprA was not able to signal through TLR2 (Fig. 3A), even at doses of up to 1 μM (data not shown). Thus, TLR2-signaling activity of LprA is acylation dependent.

Acylated LprA induces cytokine production by macrophages in a TLR2-dependent fashion

We tested the ability of LprA to induce responses by macrophages and dendritic cells. First, we tested the ability of LprA to stimulate cytokine production by murine macrophages. Macrophages were incubated for 12 h with or without 50 nM LprA or NA-LprA, and supernatants were analyzed for IL-10 and TNF-α by ELISA. Both cytokines were induced by acylated LprA and the synthetic lipopeptide P3C (a positive control TLR2 agonist) but not by NA-LprA or control medium (Fig. 4). In contrast, macrophages from TLR2−/− mice did not respond to LprA or P3C (Fig. 4). Therefore, LprA is an agonist for murine TLR2 as well as human TLR2, and its ability to induce macrophage production of IL-10 and TNF-α is dependent on TLR2 and requires LprA acylation.

Acylated LprA induces dendritic cells to mature and produce cytokines

Maturation of dendritic cells involves increased expression of surface markers such as MHC-II, CD86, and CD40, and the production of cytokines such as TNF-α, IL-10, and IL-12. To determine whether LprA can induce dendritic cell maturation, Flt3 ligand-derived murine dendritic cells were incubated for 24 h with LprA, NA-LprA, or P3C at a final concentration of 50–500 nM. Expression of surface markers was studied by flow cytometry, and cytokine production was examined by ELISA. LprA induced dendritic cell maturation as indicated by increased expression of CD40, CD86, and MHC-II (Fig. 5, A–C), as observed with P3C (data not shown). Nonacylated LprA, however, did not enhance expression...
of any of these maturation markers (Fig. 5, D–F). LprA also induced production of TNF-α, IL-12, and IL-10 by dendritic cells, whereas NA-LprA was unable to stimulate any cytokine production from dendritic cells (Fig. 6). We conclude that LprA induces maturation of dendritic cells, and this activity is dependent on its acylation.

**Acylated LprA is able to inhibit Ag processing in macrophages**

In contrast to dendritic cell responses, prolonged incubation of macrophages with TLR2 agonists (e.g., LpqH and LprG) inhibits IFN-γ-dependent MHC-II expression and Ag-processing activity (22, 23, 25, 42, 44). To determine whether LprA can similarly inhibit these functions, macrophages were incubated with LprA for 48 h, the last 24 of which were in the presence of IFN-γ (2 ng/ml). To assess MHC-II expression, cells were stained with anti-I-A^b^ or isotype-matched control Ab and analyzed by flow cytometry (Fig. 7A). Induction of MHC-II by IFN-γ was inhibited by LprA but not NA-LprA. To assess Ag processing and presentation, cells were incubated with OVA protein or OVA (323–339) peptide for 2 h and then fixed. The degree of OVA processing and presentation was determined by incubating fixed macrophages with DOBW T hybridoma cells and measuring the amount of IL-2 production by a CTL-L-2 bioassay. In accordance with decreased MHC-II expression, LprA inhibited the ability of macrophages to present OVA (323–339) peptide to T cells (Fig. 7B), whereas NA-LprA had no effect. Furthermore, MHC-II processing and presentation of OVA protein was inhibited by LprA but not NA-LprA (Fig. 7C), and this effect was dependent on TLR2 (Fig. 7D).

**LprA does not induce macrophage apoptosis or necrosis**

TLR signaling has been reported to cause apoptosis in some systems. We have examined this issue extensively with LpqH and LprG in the past, and these TLR2 agonists do not induce detectable apoptosis in the murine bone marrow-derived macrophages (or murine peritoneal or alveolar macrophages, or human THP-1 cells or monocyte-derived macrophages) as indicated by our following unpublished observations. First, viability is consistently as good with MTB-infected or lipoprotein-treated macrophages as with

![FIGURE 5. Dendritic cell expression of MHC-II, CD86, and CD40 is enhanced by LprA in an acylation-dependent fashion. Dendritic cells were incubated with medium, 500 nM LprA (A–C) or 500 nM NA-LprA (D–F) for 24 h (“protein” indicates LprA or NA-LprA). Cells were stained with Abs to MHC-II (A and D), CD86 (B and E), or CD40 (C and F) and analyzed by flow cytometry.](http://www.jimmunol.org/)

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controls. Second, yield of macrophages from MTB-infected cultures is as high or higher than the yield from matched uninfected control cultures. Third, TUNEL assays show no apoptosis in macrophages treated with up to 25 nM LpqH (more than required for the inhibitory effect). Fourth, other active physiological functions of macrophages (e.g., phagocytic uptake) are not inhibited. Fifth, expression of MHC-I (as opposed to MHC-II) is not decreased and the ability to present exogenous MHC-I-restricted peptides to CD8 T hybridoma cells is unchanged or slightly increased. Sixth, RNA yield is not inhibited by LpqH, and expression of many genes is unaltered or increased, not inhibited. These results with LpqH and LprG indicate that apoptosis does not occur in response to lipoprotein-induced TLR2 signaling, and we have confirmed this conclusion specifically with LprA. LprA and NA-LprA both failed to induce apoptosis (by staining with annexin V) or necrosis (by staining with propidium iodide) when used at the same concentrations that were used in the Ag-processing experiments (Fig. 8). In addition, LprA did not reduce macrophage viability or yield in culture (data not shown). These data show that LprA does not induce macrophage apoptosis or necrosis in the system used for our experiments.

Discussion

TLRs play an important role in the course of mycobacterial infection. Macrophages and dendritic cells, both known to harbor Mtb, express TLR2, which appears to be one of the major pattern recognition receptors for detecting this organism. The identification and characterization of mycobacterial TLR2 ligands is, therefore, important for understanding the capacity of the bacillus to interact with APCs in which it resides. Mtb lipoproteins (LpqH and LprG) have been identified previously as TLR2 agonists (22, 24, 25), but the potential TLR agonist activities of other Mtb lipoproteins have not been tested.

Prokaryotic lipoproteins may have diverse functions ranging from roles in bacterial physiology to regulation of host-pathogen interactions and immune responses. For example, prokaryotic lipoproteins serve as solute binding proteins in ATP-binding cassette transport systems or enzymes involved in cell wall synthesis, adhesion, and signaling (29). Lipoproteins are exported outside of the cytoplasmic membrane and, in some cases, are shed into the environment. Thus, they are accessible by receptors on leukocytes that may regulate immune responses. In both Staphylococcus aureus and Mtb, genetic defects that cause global lipoprotein disruptions result in reduced virulence (27), although this may reflect impairments in general bacterial physiology as well as potential alterations in host-pathogen interactions. In a study targeting a specific lipoprotein, deletion of LpgR (a close homolog of LprA) was shown to attenuate the virulence of Mtb H37Rv in a mouse model (53). These observations suggest that further study of Mtb lipoproteins and their activities is important to our understanding of Mtb virulence and host-pathogen interactions in Mtb infection.

The Mtb genome encodes ~100 putative lipoproteins (29). In studies to date with screening of detergent-partitioned, electrophoretically separated Mtb molecules, however, we have identified only four species with sufficient expression and activity to significantly modulate macrophage or dendritic cell function: LpqH (19-kDa lipoprotein) (22), LprG (25), LprA (this study and Ref. 25) and a species of ~38 kDa that is probably PhoS1 (Psts1, 38-kDa lipoprotein) (M. Drage, N. Pecora, and C. Harding, unpublished work).
regulation of MHC-II, CD40, and CD86) and production of cytokines for induction of dendritic cell maturation (as evidenced by up-regulation of MHC-II expression and Ag processing in macrophages (22, 23, 25, 42, 44). We have proposed that this reflects a homeostatic program that limits potentially excessive and damaging responses, but at the same time, this down-regulation of APC function may be a means by which mycobacteria are able to evade immune surveillance. In this study, we have shown that prolonged (24 h) exposure to LprA can inhibit macrophage MHC-II Ag processing, similar to results observed previously with LpqH and LprG.

In conclusion, LprA is a glycosylated lipoprotein with potent TLR2 agonist activity. LprA induces innate immunity (e.g., cytokine responses) and regulates APC functions of dendritic cells and macrophages. Like other TLR agonists, its primary effect may be to help drive immune responses, but it may also induce homeostatic down-regulatory mechanisms, e.g., reduction of macrophage APC function that may allow evasion of immune surveillance by organisms that survive initial microbial mechanisms.

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Disclosures
The authors have no financial conflict of interest.

References

FIGURE 8. LprA does not induce macrophage apoptosis or necrosis. Macrophages were incubated with 50 nM LprA, 50 nM NA-LprA, or control medium for 48 h with IFN-γ (2 ng/ml) present for the final 24 h. Cells were stained with annexin V to assess apoptosis (A and B) or propidium iodide to assess necrosis (B). A positive control for apoptosis/necrosis was provided by macrophages that were exposed to UV light. A. Flow cytometry staining with annexin V. B. Percent of macrophages with positive annexin V staining or positive labeling with propidium iodide.


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