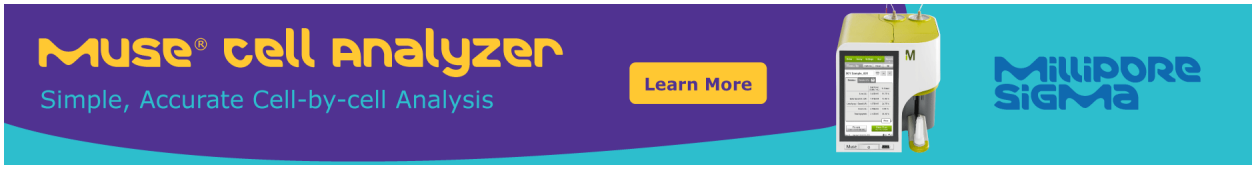


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# *Mycobacterium tuberculosis* LprG (*Rv1411c*): A Novel TLR-2 Ligand That Inhibits Human Macrophage Class II MHC Antigen Processing[1]

**Adam J. Gehring,* Karen M. Dobos[§], John T. Belisle,[§] Clifford V. Harding,[2†] and W. Henry Boom[2,3*†‡]**

**MHC class II (MHC-II)-restricted CD4$^+$ T cells are essential for control of *Mycobacterium tuberculosis* infection. This report describes the identification and purification of LprG (*Rv1411c*) as an inhibitor of primary human macrophage MHC-II Ag processing. LprG is a 24-kDa lipoprotein found in the *M. tuberculosis* cell wall. Prolonged exposure (>16 h) of human macrophages to LprG resulted in marked inhibition of MHC-II Ag processing. Inhibition of MHC-II Ag processing was dependent on TLR-2. Short-term exposure (<6 h) to LprG stimulated TLR-2-dependent TNF-$\alpha$ production. Thus, LprG can exploit TLR-2 signaling to inhibit MHC-II Ag processing in human macrophages. Inhibition of MHC-II Ag processing by mycobacterial lipoproteins may allow *M. tuberculosis*, within infected macrophages, to avoid recognition by CD4$^+$ T cells. *The Journal of Immunology*, 2004, 173: 2660–2668.**

Survival and persistence in macrophages is a hallmark of *Mycobacterium tuberculosis* infection. Adaptive T cell responses control but do not eradicate *M. tuberculosis* in most healthy persons, resulting in a persistent mycobacterial infection that can expand and cause disease when T cell immunity fails. MHC class II (MHC-II)[4] restricted CD4$^+$ T cells are central to the adaptive T cell response required to control *M. tuberculosis* infection (1). CD4 T cells serve as effector cells capable of stimulating macrophage mycobacteriocidal activity or lysing infected macrophages. CD4 T cells also serve as regulators of CD8$^+$ and $\gamma\delta$ T cell responses (2–7). Healthy persons with persistent infection have robust memory CD4$^+$ T cell responses, reflected in strongly positive tuberculin skin test reactions and high precursor frequencies of *M. tuberculosis*-specific T cells. However, little is known about how *M. tuberculosis* evades and resists this active CD4$^+$ T cell response.

*M. tuberculosis*-specific T cells produce IFN-$\gamma$ that is essential for T cell-mediated immunity (1, 8–10). IFN-$\gamma$ up-regulates MHC-II Ag processing in macrophages, propagating a protective immune response, but is inefficient at directly activating human macrophages to kill intracellular bacilli (11, 12). In addition, *M. tuberculosis* has evolved mechanisms to inhibit IFN-$\gamma$ receptor signaling in macrophages, which results in an abrogation of IFN-$\gamma$-regulated MHC-II Ag processing (13, 14). Recent studies from our laboratory determined that the *M. tuberculosis* 19-kDa lipoprotein, LpqH (*Rv3763*), is a mycobacterial product responsible, at least in part, for the inhibition of IFN-$\gamma$ regulated responses, including MHC-II Ag processing, in murine bone marrow-derived macrophages and the human monocytic cell line, THP-1 (15–17). Inhibition of MHC-II Ag processing by LpqH in both murine and human macrophages depended on prolonged stimulation through TLR-2. This finding contrasted with macrophage activation and the inflammatory response associated with short-term signaling through TLR-2 by lipoproteins such as LpqH (18–20).

Lipoprotein-mediated inhibition of MHC-II Ag processing and therefore of CD4$^+$ T cell activation may explain why LpqH overexpression in *M. vaccae* resulted in decreased protection compared with vaccinating with the wild-type (WT) strain, or why its introduction into *M. tuberculosis* strains with mutations in *Rv3763* resulted in increased virulence (21–23). However, inactivation by homologous recombination of the gene for LpqH in *M. bovis*-bacillus Calmette-Guérin (BCG), *Mb3789*, did not improve vaccine efficacy of *M. bovis*-BCG, suggesting the possibility of additional inhibitory mechanisms (24).

As mentioned, LpqH inhibited IFN-$\gamma$-regulated MHC-II Ag processing in THP-1 cells in a TLR-2-dependent manner (17). However, when these studies were extended to primary human macrophages, LpqH was found to be a modest inhibitor of MHC-II Ag processing with substantial variability among donors. These findings led us to determine whether *M. tuberculosis* expressed additional inhibitors of human macrophage MHC-II Ag processing. The availability of a strain of *M. bovis*-BCG in which the gene for LpqH was inactivated (BCG19$^-$) allowed us to directly address this question. Taking advantage of an experimental system using murine T cell hybridomas, specific for microbial Ags and presented by human HLA-DR molecules, we were able to analyze Ag-presenting cell function and screen for novel inhibitors of primary human macrophage MHC-II Ag processing (25).

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[4] Abbreviations used in this paper: MHC-II, MHC class II; BCG, bacillus Calmette-Guérin; BCG19$^-$, *M. bovis* BCG deficient for LpqH; WT, wild type; TT, tetanus toxoid; CHO, Chinese hamster ovary; MDM, monocyte-derived macrophage.

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We identified a 27-kDa fraction from detergent-extracted mycobacterial lysates that contained two lipoproteins, LprA (*Rv1270c*) and LprG (*Rv1411c*), which inhibited MHC-II Ag processing in primary human macrophages. Further purification allowed separation of LprG from the mixture of LprA/LprG. LprG alone was found to be a TLR-2 agonist that upon prolonged stimulation through TLR-2, inhibited MHC-II Ag processing by THP-1 cells and primary human macrophages. Thus, we have identified a novel TLR-2 agonist from *M. tuberculosis* that can inhibit primary human macrophage MHC-II Ag processing. We propose that *M. tuberculosis*, through its lipoproteins, has redundant mechanisms for inhibition of MHC-II Ag processing and thus diminished recognition of infected macrophages by $CD4^+$ T cells. This may be an important mechanism for immune evasion during persistent *M. tuberculosis* infection.

## Materials and Methods

### *Cells and medium*

Unless otherwise specified, cells were cultured at 37°C and 5% $CO_2$. THP-1 cells (American Type Culture Collection, ATCC, Manassas, VA) were maintained in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT), 50 $\mu$M 2-ME, 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES buffer, nonessential amino acids, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin (BioWhittaker). The T cell hybridoma, 1T1A, was generated as previously described and maintained in DMEM (BioWhittaker) supplemented as previously indicated (complete DMEM) (17).

PBMCs were prepared from whole blood with Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ). Monocytes were isolated from PBMC either by adherence or with the Miltenyi monocyte isolation kit (Miltenyi Biotec, Auburn, CA). For adherence-isolated monocytes, PMBCs were incubated for 16–20 h in X-VIVO 15 (BioWhittaker) in 12-well ($10^7$ PBMC/well) or 96-well ($5 \times 10^5$ PBMC/well) tissue culture plates, and nonadherent cells removed by washing three times with RPMI 1640 at 37°. X-VIVO 15 was replaced and monocytes matured for 4 days. Monocytes obtained using Miltenyi monocyte isolation kit were plated on 12-well ($1.5 \times 10^6$ monocytes/well) or 96-well ($5 \times 10^4$ monocytes/well) tissue-culture plates and matured for 4 days in X-VIVO 15. After 4 days, wells were washed to remove nonadherent cells and used for experiments.

3E10 cells were obtained from D. Golenbock (University of Massachusetts Medical School, Worcester, MA). The control 3E10 (3E10-Ctrl) cell line is stably transfected with a membrane CD25 reporter construct under the control of an NF$\kappa$B dependent promoter and the hygromycin vector pCEP4. The 3E10-TLR2 expressing cell line is stably transfected with the described CD25 reporter construct and human TLR-2 in the pFLAG-CMV-1 vector. Chinese hamster ovary (CHO) reporter cell lines were grown and maintained in Ham's F12 nutrient mixture (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin, 50 $\mu$g/ml hygromycin B (Invitrogen Life Technologies). For 3E10-TLR2-expressing cells, 500 $\mu$g/ml geneticin was added (Invitrogen Life Technologies) (26).

### *Culture of Mycobacteria and purification of LprA and LprG*

*M. bovis*-BCG Pasteur (BCG WT) or the *M. bovis*-BCG Pasteur strain deficient for LpqH (19-kDa lipoprotein; $BCG19^-$) (24) were grown in Middlebrook 7H9 broth (Difco, Detroit, MI) with albumin, dextrose, and catalase enrichment (Difco). Cultures were grown to $OD_{600}$ 1.0–1.5 and harvested. Dry pellets were stored at −80°C. Mycobacterial lysates were prepared by passing bacterial pellets resuspended in PBS with 7.5 mM EDTA (Stratagene, La Jolla, CA), 0.7 $\mu$g/ml pepstatin A (Sigma-Aldrich, St. Louis, MO), 200 $\mu$M PMSF (Sigma-Aldrich), 0.7 $\mu$g/ml leupeptin (Sigma-Aldrich), 500 $\mu$g/ml RNase A (Roche Molecular Biochemicals, Indianapolis, IN), 10 U/ml DNase I (Roche Diagnostics) through a French Press four times at 2000 PSI. Mycobacterial lysate was separated from cell wall material by centrifugation of the homogenate at $100,000 \times g$ for 1 h. Bacterial lysate and cell wall material were stored at −80°C.

Purification of lipoproteins from *M. tuberculosis* H37Rv and $BCG19^-$ was performed from the cell wall by biphasic extraction with Triton X-114 (Sigma-Aldrich) followed by a series of preparative electroelutions. Specifically, mycobacterial cell wall was incubated with 4% Triton X-114 in 50 mM Tris, 150 mM NaCl at pH 7.4 for 16 h at 4°C with gentle agitation. The cell wall suspension was centrifuged at $100,000 \times g$ for 1 h to remove insoluble debris and the Triton X-114 solution was subsequently incubated at 37°C for 15 min. The biphase was clarified by centrifugation at $2500 \times g$ at 37°C for 15 min. The aqueous layer was removed and the detergent layer was washed three times with ice-cold Tris-buffered saline. Proteins resolved to the detergent layer were precipitated by addition of 8 volumes of ice-cold acetone followed by incubation at −20°C for 16 h. The Triton X-114 precipitate was harvested by centrifugation at 4°C for 20 min at $2500 \times g$, washed once with cold acetone, and centrifuged as described.

The Triton X-114 precipitate was resuspended in PBS, protein concentration estimated by bicinchoninic acid protein assay (Pierce, Rockford, IL), and sample diluted to 2 mg/ml. Triton X-114 precipitate then was mixed with an equal volume of PBS saturated phenol (Fisher Scientific, Pittsburgh, PA) and mixed at 25°C for 4 h. The mixture was centrifuged at $15,000 \times g$ for 30 min, and the aqueous layer was removed and replaced with fresh PBS. After an additional 4-h incubation, the aqueous layer was removed as previously described and the phenol layer dialyzed (Spectra/Por, 12,000–14,000 Da molecular mass cutoff membrane; Spectrum Laboratories, Rancho Dominguez, CA) against running distilled water for 48 h. The protein precipitate that formed during dialysis was collected and washed once with endotoxin-free $H_2O$. Ammonium bicarbonate (10 mM) was added to the protein precipitate and the suspension was disrupted by bath sonication until a uniform solution was achieved. The protein concentration was estimated by BCA assay, and the sample diluted in 10 mM ammonium bicarbonate to 6 mg/ml. Concentrated (5×) SDS sample buffer (250 $\mu$l) was added to 1 ml of the protein sample and the sample was loaded onto a 15%, 20 cm of SDS-PAGE slab gel. The proteins were resolved by electrophoresis at 37.5 mAmps/gel. Following SDS-PAGE, proteins were eluted into 0.2 M ammonium bicarbonate using a whole gel electro-eluter (Bio-Rad, Hercules, CA). Fractions containing the 27-kDa band were pooled and lyophilized.

The pooled mixture of LprG and LprA were further separated by electrophoresis in NuPAGE LDS loading buffer on a NuPAGE 12% Bis-Tris gel (Invitrogen Life Technologies). Electrophoresis was performed in MOPS running buffer for 50 min at 200 V. Proteins were eluted in 60 mM Tris 40 mM CAPS (3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol v/v, pH 11.0) using a mini whole gel eluter (Bio-Rad). Fractions were collected, dialyzed against $H_2O$, and lyophilized. Lyophilized LprG was then resuspended to a concentration of 50 $\mu$g/ml in PBS with <2% DMSO.

### *Western blot of mycobacterial products*

BCG WT and $BCG19^-$ lysate was separated by SDS-PAGE. Proteins were transferred to nitrocellulose membrane and probed with IT-19, a mouse mAb specific for LpqH, followed by labeling with anti-mouse IgG-HRP (Cell Signaling Technology, Beverly, MA). Detection of LpqH protein was achieved with chemiluminescence using SuperSignal West Pico (Pierce). Purified native LpqH served as positive control.

Western blots of fractions obtained by electroelution were probed with polyclonal rabbit anti-*M. bovis*-BCG serum (Dako, Glostrup, Denmark) followed by labeling with anti-rabbit IgG-HRP (Amersham Biosciences). Chemiluminescence was used for Ag detection as previously indicated.

### *Identification of LprG and LprA by tandem mass spectrometry*

An aliquot of the 27-kDa protein pool purified from electroelution was incubated with modified trypsin (Roche Molecular Biochemicals) in 0.2 M ammonium bicarbonate at an enzyme to substrate ratio of 1:10 (wt:wt) at 37°C for 16 h. The digestion was stopped by addition of 1 $\mu$l of 10% TFA, and the sample desalted using a Microbed C18 Zip-Tip (Millipore, Bedford, MA). Recovered peptides were dried under vacuum and suspended in 15 $\mu$l of 5% acetonitrile, 0.1% acetic acid, and applied to a $0.2 \times 50$ mm C18 reversed phase capillary HPLC column (Michrom BioResources, Auburn, CA). The peptides were eluted with an increasing acetonitrile gradient at a flow rate of 5 $\mu$l per min using an Eldex MicroPro capillary solvent delivery system (Napa, CA). The effluent was introduced directly into a Finnigan LCQ electrospray ion trap mass spectrometer (San Jose, CA). The electrospray needle of the mass spectrometer was operated at 4 kV with a sheath gas flow of nitrogen at 20 PSI and a heated capillary temperature of 200°C. Data dependent tandem mass spectrometry was used to generate fragment ions of individual peptides. The most intense ion from the full mass spectrometry scan was selected for fragmentation. Tandem mass spectrometry was acquired for each precursor ion a maximum of two times before being placed on the dynamic exclusion list for 1 min. Ion fragmentation was achieved using 40% normalized collision energy. Data acquired from tandem mass spectrometry were interrogated against the *M. tuberculosis* genome database using SEQUEST analysis software (27, 28).

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### *Ag processing and presentation assay*

THP-1 cells were incubated in 96-well flat-bottom plates ($1.5 \times 10^5$ cells/well) with 10 ng/ml PMA (Sigma-Aldrich) in complete DMEM for 24 h to promote adherence to plates. Cells were washed once with DMEM and incubated with 50 U/ml recombinant human IFN-γ (Endogen, Woburn, MA) with or without mycobacterial preparations for 24 h. Following 24 h incubation, all media and additions were removed from the cells before Ag pulse. Cells were pulsed with different concentrations of tetanus toxoid (TT) (20 μg/ml for lipoprotein dose response or fraction experiments; or 0.1–50 μg/ml for TT dose response) for 6 h, fixed in 1% paraformaldehyde, and then incubated with 1T1A T hybridoma cells ($1 \times 10^5$ cells/well). Supernatants were harvested after 20–24 h, and IL-2 produced by T hybridoma cells was measured by CTLL-2 assay.

For TLR-2 blocking experiments, THP-1 cells were treated with 20 μg/ml anti-TLR-2 (mAb 2392 graciously provided by Dr. P. Godowski (Genentech, South San Francisco, CA) or isotypic control Ab (IgG1) in complete DMEM plus 50 U/ml IFN-γ for 30 min at room temperature before addition of equal volume of 400 ng/ml LprG ([LprG] = 200 ng/ml). THP-1 cells then were used in the Ag processing assay previously described.

For Ag processing experiments with primary human macrophages, 4-day matured macrophages were exposed to mycobacterial lipoproteins for 48 h, pulsed with TT for 5 h (0.5 μg/ml for lipoprotein dose response; or 0.01–1 μg/ml for TT dose response), and fixed in 1% paraformaldehyde. To measure Ag processing, 1T1A T hybridoma cells were added to fixed macrophages and incubated for 20–24 h. Culture supernatant was harvested and IL-2 production measured by CTLL-2 assay.

### *CTLL-2 assay and cytokine ELISA*

Supernatants from experiments with T hybridoma cells were assessed for IL-2 by bioassay using IL-2-dependent CTLL-2 cell line. Briefly, $5 \times 10^3$ CTLL-2 cells in 50 μl of complete DMEM were incubated with 100 μl of culture supernatant for 16–20 h. Proliferation of CTLL-2 cells in each well was measured by a colorimetric assay using Alamar blue (15 μl; Trek Diagnostics, Westlake, OH) and a Versamax tunable microplate reader. Positive and negative controls were included in every experiment to monitor CTLL-2 cell responsiveness. All data presented for Ag processing assays compared controls with experimental conditions within the same experiment, measuring the effects of lipoprotein exposure relative to macrophages exposed to control buffer. IL-2 production was expressed as OD at 550 nm minus OD at 595 nm. All results of T hybridoma cell experiments represent the mean response of triplicate wells plus SD.

TNF-α and IL-10 ELISA (BioSource International, Camarillo, CA) was performed on supernatants collected from TLR-2 blocking assays using THP-1 cells and 24 and 48 h supernatants harvested from primary human macrophage and THP-1 MHC-II Ag processing assays according to manufacturer's instructions.

### *3E10-CHO cell experiment*

The 3E10 control cell line or 3E10-TLR2 cells were detached using trypsin-versene (BioWhittaker) and seeded at $1 \times 10^5$ cells/well in a 12-well plate for 16 h in Ham's medium. CHO cells then were exposed to 200 ng/ml LprG/LprA mix for 7 h and stained with anti-human CD25 (BD Pharmingen, San Diego, CA) or isotypic control mAb (IgG1; BD Pharmingen). CD25 expression was measured by flow cytometry using a FACSCaliber flow cytometer (BD Biosciences, San Diego, CA).

### *TUNEL assay*

Apoptosis was measured by DNA fragmentation using the TUNEL assay (Roche Diagnostics, Mannheim, Germany). Adhered THP-1 cells ($10^6$/ml) were exposed to 50 U/ml IFN-γ with or without 500 ng/ml LprA/LprG for 24 h. Apoptosis was measured by flow cytometry after TUNEL labeling per manufacturer's instruction. DNase I served as positive control. THP-1 cells were incubated in medium for 24 h, then fixed and permeabilized and incubated with 200 U/ml DNase I for 10 min at 37°C. Following treatment with DNase I, DNA strand breaks were labeled using the TUNEL assay. Cells were considered apoptotic if their fluorescence was higher than that observed in medium alone.

## Results

### M. bovis-*BCG19*$^-$ *inhibits macrophage MHC-II Ag processing*

Earlier studies demonstrated that the 19 kDa lipoprotein, LpqH, from *M. tuberculosis* inhibited IFN-γ-regulated MHC-II Ag processing in murine macrophages and the human THP-1 macrophage cell line (15–17). Extension of these studies to primary human macrophages revealed variable inhibition with substantial variation among donors. The current study was undertaken to determine whether mycobacteria contained additional inhibitors of MHC-II Ag processing, besides LpqH, which would affect Ag processing by human macrophages.

A strain of *M. bovis* BCG-Pasteur was obtained in which *Mb3789* (*M. bovis* LpqH gene) had been inactivated by homologous recombination (BCG19$^-$) (24). Lysates of BCG19$^-$ did not express LpqH protein (Fig. 1*A*). In the first series of experiments, human monocytic THP-1 cells were infected with increasing numbers of either wild-type (WT) or 19$^-$ BCG bacilli. After 24 h of infection, THP-1 cells were tested for their ability to process and present TT protein to HLA-DR1-restricted 1T1A T hybridoma cells (17, 25). Following incubation with TT, macrophages were fixed and T hybridoma cells added in fresh medium. Thus, soluble factors produced by macrophages during infection or incubation with mycobacterial products were removed and could not directly affect T hybridoma function. As shown in Fig. 1*B*, BCG19$^-$ bacilli were equally effective in inhibiting MHC-II Ag processing as BCG WT at multiplicities of infection of 0.3–3 bacteria per THP-1 cell.

Next, we determined whether inhibitors of MHC-II Ag processing were present constitutively or expressed only by viable bacteria. Lysates of WT and 19$^-$ BCG were prepared and THP-1 cells incubated with increasing concentrations (1–80 μg/ml) of lysate

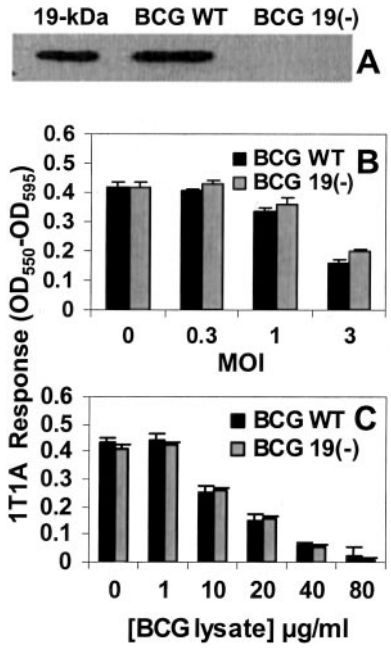


**FIGURE 1.** Macrophage MHC-II Ag processing is inhibited by mycobacterial components other than 19-kDa lipoprotein, LpqH. *A*, BCG19$^-$ does not produce LpqH protein. Western blots were performed on lysates of wild-type BCG (BCG WT) and LpqH deficient BCG (BCG 19$^-$). Purified native LpqH served as positive control. Blots were probed with mAb IT-19, specific for LpqH. *B* and *C*, THP-1 cells were exposed to increasing multiplicities of infection (MOI) (*B*) or lysates (*C*) of BCG WT or BCG 19$^-$ in the presence of 50 U/ml IFN-γ. After 24 h, cells were washed, pulsed with 20 μg/ml TT for 6 h and fixed in 1% paraformaldehyde. 1T1A T hybridoma cells that recognize TT presented by HLA-DR1 were added to fixed THP-1 cells to measure MHC-II Ag processing. After 24 h coincubation of THP-1 and 1T1A T hybridoma cells, supernatants were harvested and assayed for IL-2 production using a colorimetric CTLL assay. T hybridoma IL-2 response is expressed as $OD_{550}$-$OD_{595}$. Data points represent the mean of triplicate samples with SD and are representative of four independent experiments.

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for 24 h before pulsing with TT Ag. Both BCG 19$^-$ and BCG WT lysate inhibited MHC-II Ag processing by THP-1 cells (Fig. 1*C*). Before use in experiments BCG19$^-$ and BCG WT lysates were dialyzed against PBS to remove small m.w. protease inhibitors present in the lysing buffer. Lysing buffer, at concentrations present in bacterial lysates, had no effect on THP-1 MHC-II Ag processing (data not shown). These studies not only established that *M. bovis*-BCG contained additional inhibitors besides LpqH, but also set the stage for identification of novel mycobacterial inhibitor(s) of human macrophage MHC-II Ag processing.

### M. bovis *BCG19$^-$ contains a 27-kDa inhibitor of macrophage MHC-II Ag processing*

Based on our experience isolating mycobacterial inhibitors of MHC-II Ag processing, Triton X-114 was used to separate BCG19$^-$ lysates into aqueous and detergent phases (16). The Triton X-114 detergent fraction was precipitated with acetone and solubilized to produce a detergent-free Triton X-114 fraction. The aqueous and Triton X-114 fractions then were added to THP-1 cells to determine their ability to inhibit processing of TT to HLA-DR1-restricted 1T1A hybridoma cells. When THP-1 cells were incubated for 24 h with different dilutions of the Triton X-114 fraction, marked inhibition of TT processing for 1T1A cells was observed (Fig. 2*A*). Exposure of THP-1 cells to mycobacterial products in the aqueous phase did not affect MHC-II Ag processing by THP-1 cells (data not shown).

Next, the Triton X-114 fraction was separated further by electroelution over a 12% preparative SDS-polyacrylamide gel. Fractions were collected and tested at 1/10,000 dilution for inhibition of Ag processing. A consistent peak of inhibitory activity was observed in fractions 30–40 (Fig. 2*B*). No inhibition was observed by fractions eluted before the dye front (fractions −20 and −10). Eluted fractions were analyzed by Western blot with a polyclonal rabbit serum against *M. bovis*-BCG (Fig. 2*C*). Although some proteins in the Prep-cell elution spread out over multiple fractions, peak inhibitory activity measured in fraction 35 correlated with a prominent band of ±27 kDa. The faint diffuse bands above and below the 27 kDa band are lipomannan and lipoarabinomannan, which did not possess inhibitory activity (Fig. 2*B* and data not shown). Early fractions (peak at fraction 5) had minimal inhibitory activity but did not contain a 27-kDa band, suggesting the possibility of additional mycobacterial inhibitor(s).

### *Mycobacterial LprA (*Rv1270c*)/LprG (*Rv1411c*) inhibit macrophage MHC-II Ag processing*

Analysis of the 27-kDa band for peptide sequence by tandem mass spectrometry revealed that it contained two putative lipoproteins, LprA and LprG, encoded by *Rv1270c* and *Rv1411c*, respectively. A number of other mycobacterial proteins were present in or around the 27-kDa band following the preliminary method of separation; however, based on our previous experience, LprA and LprG were primary candidates. LprA and LprG have predicted masses of 26.2 and 24.5 kDa based on amino acid sequence; however, due to posttranslational modifications, migrate together around 27 kDa in a standard SDS-PAGE gel. To determine whether LprA or LprG were responsible for the inhibitory activity, a number of modifications were made in the purification protocol. First, following Triton X-114 extraction, detergent phase proteins were separated from lipomannan and lipoarabinomannan by extraction in phenol. Proteins were precipitated from phenol by extensive dialysis and then underwent separation by electroelution using a two-step slab gel rather than the one-step columnar system as before. The slab gel method improved separation of proteins and resulted in a fraction from both BCG19$^-$ and *M. tuberculosis*

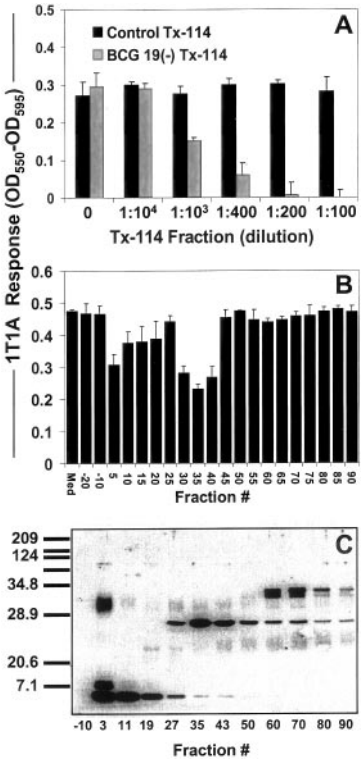


**FIGURE 2.** Triton X-114 extract of BCG19$^-$ contains a 27-kDa inhibitor of MHC-II Ag processing. *A*, THP-1 cells were exposed to decreasing dilutions of material from the detergent phase of Triton X-114 extracted BCG 19$^-$ (BCG 19(−) Tx-114) for 24 h before testing MHC-II Ag processing function. Mock extracted material served as control Triton X-114 (Control Tx-114). *B*) The detergent phase of Triton X-114 extracted BCG19$^-$ was fractionated by SDS-PAGE electroelution and fractions tested for inhibition of MHC-II Ag processing. Fractions −10 and −20 were collected before the dye front to control for nonspecific inhibitory activity. *A* and *B*, Bioactivity of fractions (1/10,000 dilution) was tested in the THP-1 MHC-II Ag processing assay with 1T1A T hybridoma cells as described in Fig. 1. *C*, Representative electroelution fractions of Triton X-114 extracted BCG19$^-$ material were analyzed by Western blot. Representative control fractions (negative fraction, −10) and fractions containing mycobacterial products (positive fractions, no. 3-90) were separated on a 13% polyacrylamide gel, transferred to nitrocellulose membrane, and probed with a polyclonal anti-BCG rabbit serum. Results presented in each panel are representative of at least four separate electroelutions.

H37Rv that contained only LprA and LprG, as determined by liquid chromatography and tandem mass spectrometry (Table I). LprA from *M. bovis*-BCG share 99% and LprG 100% amino acid homology with the same proteins in *M. tuberculosis* H37Rv. LprA/LprG mixture from *M. tuberculosis* H37Rv was used in all subsequent experiments.

Exposure of THP-1 cells to increasing concentrations of the mixture of LprA and LprG from *M. tuberculosis* H37Rv for 24 h inhibited MHC-II Ag processing of TT (Fig. 3*A*). Kinetic experiments determined that, as with LpqH, inhibition of Ag processing by LprA/LprG required at least 16 h of exposure (data not shown). Substantial inhibition was first noted at 100 ng/ml with almost complete inhibition by 300 ng/ml LprG/LprA (Fig. 3*A*). Next, we determined whether monocyte-derived macrophages (MDM) from HLA-DR1 positive donors also were inhibited by 300 ng/ml lipoprotein mixture. Preliminary kinetic studies with MDM revealed that 48 h exposure to LpqH resulted in maximal inhibition of MHC-II Ag processing. When MDMs were exposed to 300 ng/ml lipoprotein mixture for 48 h, processing of TT was reduced with maximal inhibition (>85%) observed at 0.3 $\mu$g/ml TT (Fig. 3*B*).

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Table I. *Mass spectrometry identification of proteins in 27-kDa fraction from* M. tuberculosis *H37Rv electroelution*

| Protein | Peptide Sequence[a] | Observed Mass-to-Charge Ratio | Charge | Predicted $MH^+$ | Peptide AA Position[b] |
|---|---|---|---|---|---|
| LprA[c] | LEGDISNTPQTVATGSATLLVGNK | 1194.2 | 2 | 2387.63 | 57–80 |
| | TVPTTVWIASDGSSHLVQIQIAPTK | 1325.6 | 2 | 2651.01 | 175–199 |
| | LTSEDVK | 791.4 | 1 | 791.87 | 168–174 |
| | VTKLEGDISNTPQTVATGSATLLVGNK | 1358.2 | 2 | 2716.04 | 54–80 |
| | GLANLLANLK | 1027.6 | 1 | 1027.24 | 124–133 |
| | DASVAGSQQADGVATTK | 803.9 | 2 | 1606.67 | 134–150 |
| LprG[d] | GSGNSVQMTLSK | 613.1 | 2 | 1209.36 | 186–197 |
| | IPGLSLK | 727.7 | 1 | 727.92 | 42–48 |
| | DTINGQNTIR | 566.7 | 2 | 1132.21 | 132–141 |
| | SAHM#VLTVNGK | 587.4 | 2 | 1173.37 | 31–41 |

[a] Peptides generated and identified following trypsin digestion of fraction contents. #, Methionine oxidation.
[b] Amino acid position in mature lipoproteins.
[c] Coverage of 35.25% mature LprA was identified by peptide sequence.
[d] Coverage of 19.05% mature LprG was identified by peptide sequence.

*LprA/LprG activate macrophages through TLR-2*

Mycobacterial lipoproteins can activate innate immune responses through recognition by TLR-2 on macrophages. To determine whether LprA/LprG were TLR-2 ligands, CHO cells (3E10), stably transfected with human TLR-2 or control vector and a reporter construct that expresses surface CD25 under control of an NFκB-dependent portion of the human E-selectin promoter, were incubated with the lipoprotein mixture for 7 h. Ligand recognition by TLR-2 leads to the activation of NFκB and subsequently, CD25 expression on the cell surface. 3E10 cells then were analyzed for CD25 expression by flow cytometry. As shown in Fig. 4*A*, only cells transfected with TLR-2 up-regulated CD25 in the presence of the lipoprotein mixture. Control buffer had no effect on either 3E10-TLR-2 or 3E10-control (data not shown).

In light of reports suggesting that TLR-2 signaling may induce apoptosis, we determined whether LprG/LprA induced apoptosis in human macrophages (29, 30). THP-1 cells were exposed to 500 ng/ml LprG/LprA for 24 h (a concentration 200 ng/ml higher than amounts required for maximum inhibition, Fig. 3*A*), and apoptosis was measured by by TUNEL assay. LprG/LprA did not induce apoptosis in THP-1 cells (Fig. 4*B*), consistent with our previously reported results with 19-kDa LpqH (17). DNase I treatment of permeablized THP-1 cells served as positive control for the TUNEL assay (Fig. 4*C*).

TNF-α is rapidly secreted by macrophages after activation through TLR-2. To determine whether lipoproteins could induce TNF-α expression in macrophages, THP-1 cells were incubated with 200 ng/ml LprG/LprA for 6 h and supernatants tested for TNF-α by ELISA. As shown in Fig. 4*D*, the lipoprotein mixture stimulated TNF-α production by THP-1 cells and this production was blocked with the anti-TLR-2 mAb, 2392.

*Purified LprG activates human macrophages to produce TNF-α*

A number of methods were attempted to separate LprA from LprG, including lectin binding and ion exchange chromatography, with little success either due to very low yields or inadequate separation. We were able to separate LprG from the LprA/LprG mixture by NuPAGE Bis-Tris gel electrophoresis in MOPS running buffer. The LDS loading buffer chemistry produces a more uniform negative charge on the protein despite glycosylation and acylation than does standard SDS Laemmli loading buffer. The uniform negative charge in addition to the neutral pH of the gel matrix permits these lipoproteins to migrate close to their actual molecular mass, with less influence by posttranslational modification. Following Bis-Tris gel electrophoresis, fractions were collected using a mini whole gel eluter. Four fractions spanning the molecular mass range from 21 kDa to 30 kDa were analyzed by mass spectrometry. Of the fractions analyzed, one contained LprA and LprG, and the fraction collected immediately following contained only LprG by tandem mass spectrometry (Table II). We were able to purify LprG away from LprA but not vice versa using Bis-Tris gel electrophoresis.

To determine whether purified LprG was bioactive, THP-1 cells and MDMs were tested for TNF-α production in the presence of increasing concentrations of LprG. As shown in Fig. 4*E*, LprG induced TNF-α production in THP-1 cells and MDMs. Thus, LprG is a novel mycobacterial TLR-2 ligand that can activate innate immune mechanisms.

*Purified LprG inhibits MHC-II Ag processing by THP-1 cells and primary macrophages through TLR-2*

In earlier studies, we determined that prolonged signaling (12 h or more) through TLR-2 by LpqH from *M. tuberculosis* interfered with MHC-II Ag processing in murine macrophages and THP-1 cells, in part by interfering with IFN-γ signaling (15). To determine whether LprG contributed to the inhibition of MHC-II Ag processing observed for the LprA/LprG mixture, THP-1 cells were incubated for 24 h with increasing concentrations (1–500 ng/ml) of purified LprG before pulsing with Ag to measure TT presentation to 1T1A cells. As shown in Fig. 5*A*, LprG inhibited IFN-γ-regulated Ag processing by THP-1 cells at 30 ng/ml with maximum inhibition occurring at 300 ng/ml and higher. Inhibition of Ag processing occurred over a wide range of Ag concentrations (Fig. 5*B*) and was reversed in the presence of the blocking anti-TLR-2 mAb 2392 (Fig. 5*E*).

These studies were extended to determine whether LprG inhibited processing and presentation of TT by primary human macrophages to HLA-DR1 restricted T hybridoma cells. As shown in Fig. 5*C*, LprG inhibited MDM MHC-II Ag processing. Primary human macrophages were more sensitive to LprG than THP-1 cells, with significant inhibition of Ag processing observed by 1–10 ng/ml LprG. Similar to THP-1 cells, inhibition was observed along a wide Ag concentration range (Fig. 5*D*). However, unlike THP-1 cells, primary human macrophages did not require IFN-γ to process and present Ag. Therefore, LprG was capable of inhibiting constitutive MHC-II Ag processing in primary macrophages, a finding that has not been demonstrated in previous studies involving mycobacterial lipoprotein, TLR-2-mediated inhibition of Ag processing. Inhibition of MHC-II Ag processing by LprG was not associated with decreased HLA-DR expression (data not shown). Thus, inhibition of Ag processing in primary human macrophages

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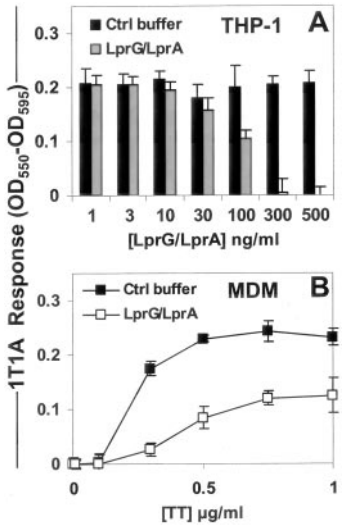


**FIGURE 3.** Mycobacterial LprG/LprA inhibit MHC-II Ag processing by human macrophages. *A*, THP-1 cells were exposed to increasing concentrations of LprG/LprA for 24 h in the presence of 50 U/ml IFN-γ. After 24 h, cells were washed, pulsed with 20 μg/ml tetanus toxoid (TT) for 6 h and fixed in 1% paraformaldehyde. 1T1A T hybridoma cells were added to fixed THP-1 cells to measure MHC-II Ag processing. *B*, Human MDM were isolated from HLA-DR1 positive donors and exposed to 300 ng/ml LprG/LprA or control buffer for 48 h. Following exposure to LprG/LprA, additions were removed and macrophages pulsed with increasing concentrations of TT and fixed in 1% paraformaldehyde before addition of 1T1A T hybridoma cells. After 24 h coincubation of APC and 1T1A T hybridoma cells, supernatants were harvested and assayed for IL-2 production using a colorimetric CTLL assay. Data points represent the mean of triplicate samples with SD. Representative of three experiments (*A*) and of four HLA-DR1 positive donors (*B*).

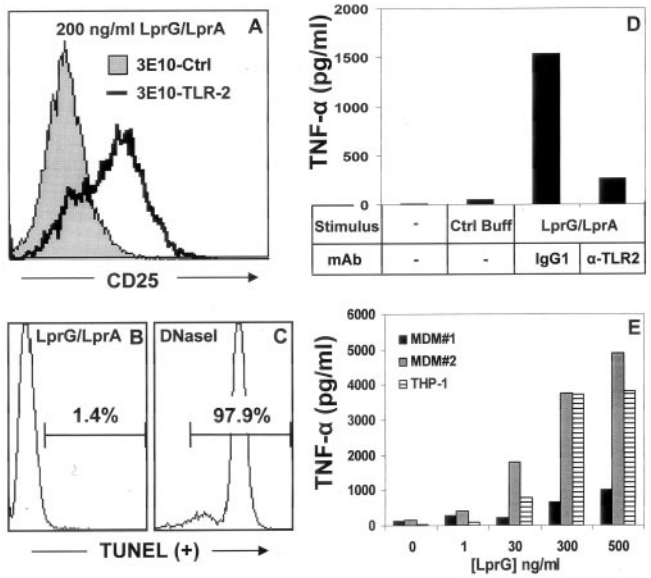


**FIGURE 4.** LprG/LprA and purified LprG induce macrophage TNF-α production but not apoptosis through TLR-2. *A*, CHO cells stably transfected with human TLR-2 and a NFκB-dependent CD25 reporter construct (3E10-TLR2) or with only the reporter construct (3E10-Ctrl) were incubated with LprG/LprA (200 ng/ml). After 7 h, cells were stained for CD25 and analyzed by flow cytometry. Flow histograms for CD25 expression on 3E10-TLR-2 and 3E10-Ctrl after incubation with LprG/LprA are shown. Histograms for CHO cell lines incubated with control buffer did not induce CD25 expression on 3E10-TLR2 cells (data not shown). Experiment is representative of five. *B*, THP-1 cells were incubated with 500 ng/ml LprG/LprA for 24 h and apoptosis was measured by DNA fragmentation using TUNEL assay. *C*, THP-1 cells not exposed to LprG/LprA were incubated with 200 U/ml DNase I and served as positive control for TUNEL assays. *D*, THP-1 cells were preincubated for 30 min. at room temperature with 20 μg/ml anti-TLR-2 mAb 2392 or an IgG1 control mAb. LprG/LprA or control buffer (400 ng/ml) was then added, resulting in final concentrations of 10 μg/ml mAb and 200 ng/ml LprG/LprA. Supernatants were harvested after 6 h and assayed for TNF-α by ELISA. Data are expressed in picogram per milliliter as the mean of duplicate samples. *E*, THP-1 cells and MDM were incubated with increasing concentrations of purified LprG for 24 h and supernatants harvested. TNF-α in supernatants was measured by ELISA and expressed in picogram per milliliter as the mean of duplicate samples.

was independent of inhibition of IFN-γ signaling and HLA-DR expression.

Because inhibition of macrophage Ag processing required prolonged activation through TLR-2, we determined whether LprG-mediated inhibition was a result of IL-10 secretion. Supernatants from THP-1 cells or primary human macrophages incubated for 24 h or 48 h with LprG or the LprA/LprG mixture did not produce detectable levels of IL-10 by ELISA (data not shown).

## Discussion

*M. tuberculosis* produces numerous protein and nonprotein molecules capable of eliciting potent innate and adaptive immune responses. Activation of innate immune responses by *M. tuberculosis* is mediated through recognition of mycobacterial constituents by TLRs expressed on host cells (31). *M. tuberculosis* primarily activates innate immunity through TLR-2 and lipoproteins such as 19-kDa LpqH, which appear to be major mycobacterial ligands for TLR-2. Acute signaling through TLR-2 results in the production of inducible NO synthase and secretion of proinflammatory cytokines such as TNF-α and IL-12, which further activate innate immunity and promote $CD4^+$ T cell activation (19, 20).

In this study, we identified and purified a novel TLR-2 ligand from *M. tuberculosis* and *M. bovis*-BCG, the 24-kDa lipoprotein LprG. Short-term (<6 h) exposure of human macrophages to LprG stimulated TNF-α production that was blocked by an anti-TLR-2 mAb (Fig. 4*B*). In contrast, prolonged exposure (≥16 h) of the same macrophages to LprG resulted in marked inhibition of MHC-II Ag processing in primary human macrophages and THP-1 cells. Inhibition of MHC-II Ag processing by LprG also was dependent on TLR-2 (Fig. 5*E*). Because MHC-II-restricted $CD4^+$ T cells are central for host defense against *M. tuberculosis*, inhibition of macrophage MHC-II Ag processing would be an effective immune evasion strategy.

Signaling through TLR-2 by lipoproteins may represent a double-edged sword for host responses to chronic intracellular pathogens such as *M. tuberculosis*. Short-term signaling through TLR-2 activates macrophages and initiates acute inflammation that may help control initial infection. In contrast, prolonged TLR-2 signaling in macrophages results in down-regulation of certain critical immune functions, such as MHC-II Ag processing. *M. tuberculosis* infects, survives, and persists in macrophages. The ability of *M. tuberculosis* to survive acute inflammation positions the bacilli to take advantage, through secretion of lipoproteins such as LprG and LpqH, of this down-regulation of macrophage immune function.

MHC-II Ag processing refers to the entire process by which APC internalize and degrade Ags to peptides, load peptides onto MHC-II molecules, and transport peptide-MHC-II complexes to the cell surface for presentation to $CD4^+$ T cells. Thus in the absence of decreased surface MHC-II expression, inhibition of MHC-II Ag processing by LprG in primary human macrophages could involve any of the other cellular processes required for

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Table II. *Mass spectrometry protein identification of 24-kDa fraction from Bis-Tris gel electroelution of* M. tuberculosis *H37Rv LprG/LprA*

| Protein | Peptide Sequence[a] | Observed Mass-to-Charge Ratio | Charge | Predicted $MH^+$ | Peptide AA Position[b] |
|---|---|---|---|---|---|
| LprG[c] | TLSGDLTTNPTAATGNVK | 881.0 | 2 | 1761.91 | 49–66 |
| | IPGLSLK | 727.6 | 1 | 727.92 | 42–48 |
| | DTINGQNTIR | 566.5 | 2 | 1132.21 | 132–141 |
| | SAHM#VLTVNGK | 587.0 | 2 | 1173.37 | 31–41 |
| | GSGNSVQMTLSK | 613.2 | 2 | 1209.36 | 186–197 |

[a] Peptides generated and identified following trypsin digestion of fraction contents. #, Methionine oxidation.
[b] Amino acid position in mature LprG.
[c] Coverage of 27.62% of mature LprG was identified by peptide sequences.

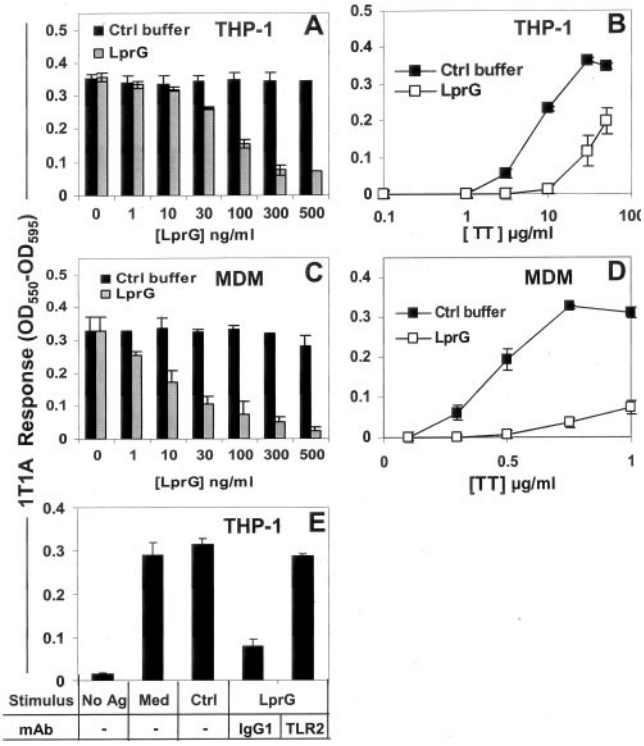


**FIGURE 5.** Purified LprG inhibits MHC-II Ag processing by THP-1 and primary macrophages through TLR-2. *A*, THP-1 cells were incubated with increasing concentrations of LprG or control buffer for 24 h in the presence of 50 U/ml IFN-γ. After 24 h, cells were pulsed with 20 μg/ml TT for 6 h and fixed in 1% paraformaldehyde. 1T1A T hybridoma cells were added to fixed THP-1 cells to measure MHC-II Ag processing. After 24 h coincubation of THP-1 and 1T1A T hybridoma cells, supernatants were harvested and assayed for IL-2 production using a colorimetric CTLL assay. *B*, THP-1 cells were incubated with 300 ng/ml LprG in the presence of IFN-α. After 24 h incubation cells were pulsed with increasing concentrations of TT for 6 h and fixed. Ag processing was measured with 1T1A cells as previously described. *C*, HLA-DR1-positive MDM were incubated with increasing concentrations of LprG or control buffer for 48 h. Following incubation with LprG macrophages were pulsed with 500 ng/ml TT for 5 h and fixed. Ag processing was measured with 1T1A cells as previously described. *D*, HLA-DR1-positive MDM were incubated with 300 ng/ml LprG for 48 h. After 48 h macrophages were pulsed with increasing concentrations of TT for 6 h and fixed. Ag processing was measured with 1T1A cells as previously described. *E*, THP-1 cells were incubated for 30 min at room temperature with either 20 μg/ml anti-TLR-2 mAb 2392 or IgG1 control mAb. After 30 min, purified LprG or control buffer was added to THP-1 cells for 24 h (final concentrations: mAb = 10 μg/ml, LprG = 200 ng/ml). After 24 h, cells were washed, pulsed with 20 μg/ml TT, and fixed in 1% paraformaldehyde. Ag processing was measured with 1T1A cells as previously described. Data points represent the mean of triplicate samples with SD and each panel is representative of at least two independent experiments.

MHC-II Ag processing. Other than changes in MHC-II expression and function, we have found no evidence for other changes in APC surface molecules that might inhibit T hybridoma function.

The ability of multiple mycobacterial lipoproteins (LprG and LpqH) to inhibit MHC-II Ag processing via prolonged signaling through TLR-2 is indicative of a redundant mechanism used by *M. tuberculosis* to inhibit macrophage MHC-II Ag processing. Differences in cell type (bone marrow macrophage vs macrophage cell line vs peripheral blood macrophage) and species (murine vs human TLR-2) may be responsible for the variability in MHC-II inhibitory activity observed for LprG and LpqH. Although, LprG and LpqH likely possess similar acyl modifications, they share no amino acid homology, suggesting that protein structure also may influence recognition by TLR-2 of these lipoproteins.

The *M. tuberculosis* genome encodes over 100 lipoproteins based on the presence of signal peptidase II sequences and amino terminus motifs required for acylation (28, 32). Little research has focused on the role of these lipoproteins in the physiology of *M. tuberculosis*. However, several mycobacterial lipoproteins have been characterized based on their immunogenicity for T cells and B cells. The 38-kDa PstS-1, LpqH, 27-kDa LprA, and LprG are major lipoproteins found in the *M. tuberculosis* cell wall. PstS-1 is a transporter of inorganic phosphate, and was identified as a *M. tuberculosis* complex-specific marker and immunodominant target of the human immune response (33–36). The 19-kDa LpqH has no known function but is a TLR-2 agonist and target of the human immune response (17, 37, 38). Efforts to use LpqH as a vaccine have been unsuccessful. Over-expression of LpqH was detrimental in an animal model of *M. tuberculosis* infection (21–23). LprG also has no described function in *M. tuberculosis* and no homology with other bacterial lipoproteins. LprG from *M. bovis* is recognized by serum from infected cattle suggesting that it is expressed during infection (39). Immunization with recombinant LprG was detrimental in a murine *M. tuberculosis* infection model consistent with the vaccine studies with LpqH (40). Deletion of LprG, *Rv1411c*, from *M. tuberculosis* H37Rv resulted in attenuated growth in mice compared with WT *M. tuberculosis* H37Rv, suggesting that LprG has a role in mycobacterial growth in vivo (41).

Prolonged stimulation of TLR-2 by LpqH inhibited IFN-γ regulated MHC-II mRNA and protein expression in macrophages (16, 17). LpqH inhibited IFN-γ signaling without interfering with Stat-1 phosphorylation, translocation to the nucleus, or binding to IFN-γ-regulated promoters (15). MHC-II Ag processing by THP-1 cells is dependent on IFN-γ and thus LprG likely interferes with IFN-γ signaling in THP-1 cells. However, MHC-II Ag processing in primary human macrophages was not dependent on IFN-γ and inhibition by LprG was not associated with changes in MHC-II surface protein expression (data not shown).

Secretion of soluble mediators or changes in intracellular signaling could play a role in TLR-2-mediated inhibition of MHC-II Ag processing in primary human macrophages. A recent report

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demonstrated that IL-6, produced by *M. tuberculosis* infected murine macrophages, inhibited IFN-γ responses in uninfected macrophages (42). However, inhibition of MHC-II Ag processing in primary macrophages occurred in the absence of exogenous IFN-γ. Nor have we found evidence for soluble factors participating in lipoprotein-mediated inhibition of MHC-II Ag processing. IL-10, known to interfere with MHC-II trafficking in human macrophages, was not detected in supernatants of primary macrophages exposed to LprG for up to 48 h (data not shown) (43).

NFκB, the primary transcription factor activated in TLR signaling, is a dimeric transcription factor formed by the interactions of five Rel family proteins (44). The combination of Rel proteins comprising the NFκB dimer can influence positive or negative regulation of NFκB promoter sites within a given cell. Inhibition of NFκB activation in dendritic cells decreased expression of MHC-II and costimulatory molecules resulting in decreased priming of T cells (45). NFκB binding sites contained within the MHC-II invariant chain promoter were negatively regulated by p50 NFκB homodimers in promonocytic U937 cells (46). Recent evidence suggests that NFκB may also play a role in resolving inflammation. A shift in NFκB subunits from p50-p65 to p50 homodimers was associated with resolution of inflammation (47). The TLR-4 ligand, LPS, and TNF-α can induce p50 Rel protein expression and thus changes in NFκB subunits, as a result of prolonged signaling through TLR-2, could affect MHC-II Ag processing (48–51).

Survival in macrophages in the face of an active MHC-II-restricted $CD4^+$ T cell response is a hallmark of the interaction between *M. tuberculosis* and the human host. The majority of healthy tuberculin skin test positive persons harbor small numbers of viable bacilli within macrophages and have high precursor frequencies of *M. tuberculosis* specific $CD4^+$ T cells. Secretion of lipoproteins resulting in prolonged signaling through TLR-2 molecules on the surface or phagosomal membranes of macrophages may provide a mechanism for *M. tuberculosis* to evade robust effector and memory $CD4^+$ T cell responses of healthy infected persons (52, 53). The abundance of TLR-2 on the surface and phagosomal membranes of macrophages combined with an abundance of redundant lipoprotein ligands for TLR-2, make TLR-2-mediated inhibition of MHC-II Ag processing an attractive mechanism for immune evasion by *M. tuberculosis*.

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