LprG-Mediated Surface Expression of Lipoarabinomannan Is Essential for Virulence of Mycobacterium tuberculosis

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Abstract

Mycobacterium tuberculosis employs various virulence strategies to subvert host immune responses in order to persist and cause disease. Interaction of M. tuberculosis with mannose receptor on macrophages via surface-exposed lipoarabinomannan (LAM) is believed to be critical for cell entry, inhibition of phagosome-lysosome fusion, and intracellular survival, but in vivo evidence is lacking. LprG, a cell envelope lipoprotein that is essential for virulence of M. tuberculosis, has been shown to bind to the acyl groups of lipoglycans but the role of LprG in LAM biosynthesis and localization remains unknown. Using an M. tuberculosis lprG mutant, we show that LprG is essential for normal surface expression of LAM and virulence of M. tuberculosis attributed to LAM. The lprG mutant had a normal quantity of LAM in the cell envelope, but its surface was altered and showed reduced expression of surface-exposed LAM. Functionally, the lprG mutant was defective for macrophage entry and inhibition of phagosome-lysosome fusion, was attenuated in macrophages, and was killed in the mouse lung with the onset of adaptive immunity. This study identifies the role of LprG in surface-exposed LAM expression and provides in vivo evidence for the essential role of surface LAM plays in M. tuberculosis virulence. Findings have translational implications for therapy and vaccine development.


Editor: Marcel A. Behr, McGill University, Canada

Received: March 22, 2014; Accepted: July 28, 2014; Published: September 18, 2014

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by Stanford University Department of Pathology (NB) and NIH/NIAD AI064798 (MJ). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: I would like to disclose that our study partially overlaps with a manuscript that was submitted to PLoS Pathogens by Clifford V Harding (PPATHOGENS-D-14-00136), on which I am a contributing author. In brief, I had proposed several experiments to Dr. Harding and provided him with unpublished bacterial strains to perform these experiments in his laboratory with the expectation that the results would be published in our manuscript. However, at the end, Dr. Harding decided to publish the findings separately which forced us to perform the proposed experiments in my laboratory. Given this situation, we kindly request Dr. Lalita Ramakrishnan as the contributing author. In brief, I had proposed several experiments to Dr. Harding and provided him with unpublished bacterial strains to perform these experiments in his laboratory with the expectation that the results would be published in our manuscript. However, at the end, Dr. Harding decided to publish the findings separately which forced us to perform the proposed experiments in my laboratory. Given this situation, we kindly request the section editor in order to receive a fair and impartial assessment of our manuscript. This does not alter our adherence to all PLOS policies on sharing data and materials.

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Introduction

Mycobacterium tuberculosis is a human pathogen that has infected one-third of world’s population and causes 8 million new cases and over one million deaths each year [1]. In the absence of a protective vaccine and the global prevalence of drug resistant strains, a greater understanding of virulence mechanisms is needed to facilitate development of anti-tuberculosis drugs with novel mechanisms of action. M. tuberculosis utilizes multiple defensive and offensive strategies to subvert the host immune responses in order to persist and cause disease [2]. Many of the virulence determinants of M. tuberculosis are constituents of the cell envelope [3]. The mycobacterial cell envelope is an impermeable barrier composed of an inner plasma membrane; a cell wall core; an outer mycomembrane; and a surface capsule composed of polysaccharides, lipids, and proteins [4–6]. The constituents of the capsule can be concealed within the ~35 nm capsular layer or they can be exposed on the surface with the ability to interact with the host [6]. Mannose-capped lipoarabinomannan (LAM), an abundant surface-exposed lipoglycan anchored to the inner and outer membranes via a mannosyl phosphate inositol [7], is considered one of the key virulence determinants of...
**Author Summary**

*Mycobacterium tuberculosis* is among the leading infectious causes of human death. A better understanding of its virulence mechanisms is needed to facilitate development of novel therapeutics and a preventative vaccine. Lipoo-abinomannan (LAM), an abundant surface-exposed lipo- glycyan, is believed to be a critical virulence determinant for intracellular survival and latency of *M. tuberculosis*. In vitro experiments with purified LAM have led to a model in which surface-exposed LAM binds to macrophage man- nose receptor and facilitates bacterium entry, inhibition of phagosome-lysocosome fusion, and modulation of innate immune responses. However, confirmation of these findings in vivo has not been possible due to the essentiality of genes involved in the LAM biosynthetic pathway. It was recently shown that LprG, a cell envelope lipoprotein, binds to the acyl groups of lipoglycan, but the role of LprG in LAM biosynthesis and localization remains unknown. Here, using an *M. tuberculosis* *lprG* mutant and a novel cell-imprinting assay, we show that LprG is essential for normal surface expression of LAM and virulence of *M. tuberculosis* attributed to LAM. Our study provides new insights into the mechanism of surface expression of LAM and confirms the essential role surface LAM serves in pathogenesis of *M. tuberculosis*.

*M. tuberculosis* [6,8–11]. Upon contact with macrophages, surface-exposed LAM binds to macrophage mannos receptor (MMR) and facilitates entry, inhibition of Phagosome-Lysosome (P-L) fusion, and modulation of immune responses leading to intracellular survival and persistence [12–16]. However, our understanding of LAM function is derived from in vitro studies using purified LAM. Confirmation of these findings in vivo using *M. tuberculosis* mutants has not been possible due to the essentiality of genes involved in lipoglycan biosynthetic pathway [8,9].

Bacterial lipoproteins are membrane-anchored cell envelope proteins with a broad range of functions including substrate-binding and transport [17]. Using a Toll-like receptor 2 assay to detect agonist activity from mycobacterial lysates, Drage and colleagues unexpectedly found that *M. tuberculosis* lipoprotein LprG binds to LAM and other lipoglycans [18]. Using X-ray crystallography, they showed that LprG forms a hydrophobic pocket that accommodates the alkyl chains of tri-acylated lipoglycans and mutation of the hydrophobic pocket (LprG V91W) abrogates association of LprG with lipoglycans. The genome of *M. tuberculosis* encodes *lprG* (Rv1411c) in an operon that also encodes Rv1410c, a major facilitator superfamily small molecule transporter P55 [19]. Both LprG and P55 are required for the efflux activity of P55 using exogenous substrates suggesting that both proteins mediate the same endogenous function [20,21]. Although *lprG* is essential for survival of *M. tuberculosis* in mice [22,23], the function of LprG in LAM biosynthesis and localization remains unknown.

In this study we used a *M. tuberculosis* *lprG* deletion mutant to investigate the role of LprG in LAM biosynthesis and pathogenesis. We show that (i) LprG is essential for normal expression of surface LAM and (ii) the virulence properties of *M. tuberculosis* attributed to LAM are defective in the *lprG* mutant.

**Results**

**Fitness of *lprG* mutant is unaltered in broth culture**

To investigate the function of LprG in *M. tuberculosis*, we constructed a deletion mutant of *lprG* (*ΔlprG*) in H37Rv and complemented it with *lprG*-Rv1410c (::*lprG*) (Figure S1). Deletion of *lprG* had no effect on the growth of *M. tuberculosis* in Middlebrook 7H9 broth (Figure S2A). On Middlebrook 7H9 agar, the diameter of *ΔlprG* colonies was on average reduced by 47% compared to H37Rv and *ΔlprG* (Figure S2B). To investigate the length and diameter of *ΔlprG* bacilli, scanning electron microscopy (SEM) and atomic force microscopy (AFM) were performed on the bacteria and their imprints on polydimethylsiloxane (PDMS) polymer surface, respectively. SEM and AFM did not show any dimension differences between H37Rv and *ΔlprG* (Figure S3). Both strains measured ~2.4 μm long and 0.27 μm wide by SEM and had an imprinted depth of 0.26 μm.

**lprG mutant has normal LAM content**

To investigate the role of LprG in LAM biosynthesis and transport, the cellular, capsular, and secreted lipoglycan and lipid composition of *ΔlprG* were analyzed in non-shaking and shaking cultures without detergent. As shown in Figure 1A and B, using SDS/PAGE stained with periodic acid/Schiff reagent and thin-layer chromatography, comparable amounts of LAM and its precursors- phosphatidylinositol mannosides and lipomannan- were recovered from H37Rv and *ΔlprG* cells. The phosphatidyli- nositol mannosides composition as determined by MALDI-TOF MS was identical (unpublished data). As a complementary approach, we assessed the cellular LAM content using SDS/PAGE immunoblot. The immunoblot showed no difference between H37Rv and *ΔlprG* (Figure 1C). Consistent with these observations, the mannose, inositol, and arabinose composition of whole delipidated *ΔlprG* cells was not significantly different from that of the H37Rv (Table 1). Likewise, both strains displayed similar glycan contents in their culture filtrate and capsular materials indicating that their capsular lipoglycan compositions were not altered [24]. Biochemical methods also did not reveal any significant quantitative or qualitative differences in the lipid composition between H37Rv and *ΔlprG*, apart from a decrease in trehalose dimycolate in the *lprG* mutant cells growing in shaking broth but not on agar (Figure 1B and Figure S4). We also assessed the major capsular polysaccharide α-glucan by a spot immunoblot assay and did not find a difference between H37Rv and *ΔlprG* (Figure 1D). Altogether, these findings consistently indicate that LprG does not play a role in the biosynthesis of LAM.

**lprG mutant has reduced expression of surface-exposed LAM**

To investigate the surface property of *lprG* and determine the role of LprG in expression of LAM on the surface [10], we employed cell-imprinting technology [25,26]. As illustrated in Figure 2A, cell-imprinting cures PDMS polymer solution around bacteria of interest. During contact, functional groups in the polymer surface serve as receptors for preferential capture of the bacteria of interest. Removing the template cells, the cavities left on the imprinting polymer serve as receptors for preferential capture of template cells based on the chemical property of their surface. When cells of *ΔlprG* were passed through a microfluidic device containing imprints of H37Rv, *ΔlprG*, and *ΔlprG* the mutant cells were selectively captured on imprints of *ΔlprG* compared to imprints of H37Rv and *ΔlprG* (Figure 2B and C). On average 34.0±3.6 (%<0.005) and 35.7±5.5 (%p<0.005) *ΔlprG* cells captured on imprints of H37Rv and *ΔlprG*, respectively (Table S1). In contrast, the cells of H37Rv and *ΔlprG* were preferentially captured on imprints of H37Rv and *ΔlprG* compared to imprints of *ΔlprG* (Figure 2B and C). On average 44.7±4.0 (%p<0.01) and 43.7±4.2 (%p<0.05) H37Rv cells and
findings indicate the cell surface alteration in ΔlprG of H37Rv, a common feature of cell envelope mutants. To determine if the altered surface property of ΔlprG is caused by differential expression of surface LAM, bacteria were pretreated with anti-LAM antibody and then captured on imprints. Blocking surface LAM with two different anti-LAM antibodies, α-LAM and CS-35, abolished selective capture of H37Rv and ΔlprG compared to imprint of ΔlprG (Figure 2D and unpublished data). On average 30.3 ± 3.5, 27.7 ± 3.1 H37Rv cells and 28.3 ± 3.5, 25.3 ± 3.5, and 26.3 ± 3.5 ΔlprG cells were captured on imprints of H37Rv, ΔlprG, and ΔlprG, respectively, after blocking with α-LAM (Table S2). Pretreatment of ΔlprG cells with anti-LAM had no effect on selective capture of ΔlprG cells on imprints of H37Rv and ΔlprG, respectively, after blocking with α-LAM (Table S2). To show that the blocking effect of anti-LAM was specific, bacteria were pretreated with an antibody to Ag85c complex, a cell

Figure 1. The lprG mutant has normal LAM content in the cell envelope. (A) SDS/PAGE analysis of phosphatidylinositol mannosides (PIMs), lipomannan (LM) and LAM prepared from wild-type (H37Rv), lprG mutant (ΔlprG), and ΔlprG complemented with lprG-Rv1410c (ΔlprG). LM and LAM extracted from equal amounts of bacterial cells were separated on a 10–20% Tricine gel and visualized by periodic acid/Schiff reagent staining. (B) Thin-layer chromatograms of total lipids extracted from H37Rv and ΔlprG. The same amounts of total lipids extract from bacilli grown in GAS medium were loaded for each strain. Thin-layer chromatogram plates were run in the solvent system CHCl3/CH3OH/H2O (65:25:4, by vol.) and revealed with α-naphthol. SL, sulfolipid; TMM, trehalose monomycoculates; DAT, diacyltrehaloses; PE, phosphatidylethanolamine; CL, cardiolipin; PIM6, phosphatidylinositol hexamannosides. (C) SDS/PAGE immunoblot for LAM analysis in H37Rv and ΔlprG mutant has normal LAM content in the cell envelope. LprG-Mediated Surface Expression of LAM in M. tuberculosis

42.0 ± 4.0 (P < 0.05) and 43.3 ± 3.5 (P < 0.01) ΔlprG cells were captured on imprints of H37Rv and ΔlprG, respectively, compared to 29.3 ± 3.5 H37Rv and 28.7 ± 4.0 ΔlprG cells captured on imprints of ΔlprG (Table S1). These results confirm that ΔlprG has an altered surface compared to H37Rv and ΔlprG. To determine whether the cell surface alteration in ΔlprG is a common feature of other cell envelope mutants, we measured the capture of M. tuberculosis ΔlspA [27] and ΔwhiB3 [28] on imprints of H37Rv, ΔlprG, and ΔlprG. As shown in Figure 2C, both ΔlspA and ΔwhiB3 cells were captured preferentially on imprints of H37Rv and ΔlprG compared to imprints of ΔlprG. On average 39.3 ± 3.5, 30.3 ± 3.5 (P < 0.05), and 39.7 ± 3.1 ΔlspA cells and 38.0 ± 3.6, 31.7 ± 3.5, and 38.7 ± 3.5 ΔwhiB3 cells were captured on imprints of H37Rv, ΔlprG, and ΔlprG, respectively (Table S1). These findings indicate the cell surface alteration in ΔlprG is not a common feature of cell envelope mutants.
wall antigen not expressed on the surface. Treatment of bacteria with anti-Ag85c complex had no effect on selective capture of H37Rv or ΔprG on H37Rv and ΔprG imprints (Figure 2E and Table S3). Similar results were obtained with an isotype control for CS-35 (Figure S3 and Table S4).

To generate additional evidence for altered expression of surface LAM on ΔprG, we used indirect fluorescent antibody staining to measure the expression of surface-exposed LAM. Surface staining with anti-LAM, but not secondary antibody alone, showed a lower percent of ΔprG cells staining positive with α-LAM and CS-35 compared to H37Rv and ΔprG (Figure S5). On average 45.3±0.18% and 41.3±4.2% of ΔprG cells stained positive with α-LAM and CS-35, respectively, compared to 81.4±3.6% (P<0.001) and 86.0±14.3% (P<0.001) of H37Rv and ΔprG cells (P<0.01) and 73.1±17.8% (P<0.05) of ΔprG cells, respectively. A higher fraction of positively staining ΔprG cells stained dim compared to H37Rv and ΔprG. On average 92.0% and 100% of ΔprG stained dim with α-LAM and CS-35, respectively, compared to 49.9% and 40.7% of H37Rv and ΔprG, respectively. Altogether, a notable difference between H37Rv and ΔprG was the abundance of surface-exposed LAM, indicating LprG is essential for normal expression of LAM.

**Table 1.** The glycosyl composition of the delipidated cells, capsules and culture filtrates of the H37Rv and ΔprG mutant (ΔprG) strains.

<table>
<thead>
<tr>
<th>Source</th>
<th>Strain</th>
<th>Ino+Ar+Man/total sugars</th>
<th>Glc/total sugars</th>
<th>Ino/total sugars</th>
<th>Ara/total sugars</th>
<th>Man/total sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delipidated cells</td>
<td>H37Rv</td>
<td>0.23</td>
<td>0.34</td>
<td>0.02</td>
<td>0.07</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>ΔprG</td>
<td>0.28</td>
<td>0.32</td>
<td>0.03</td>
<td>0.10</td>
<td>0.15</td>
</tr>
<tr>
<td>Capsule</td>
<td>H37Rv</td>
<td>0.17</td>
<td>0.82</td>
<td>0.02</td>
<td>0.02</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>ΔprG</td>
<td>0.14</td>
<td>0.84</td>
<td>0.02</td>
<td>0.02</td>
<td>0.10</td>
</tr>
<tr>
<td>Culture filtrates</td>
<td>H37Rv</td>
<td>0.47</td>
<td>0.30</td>
<td>0.14</td>
<td>0.13</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>ΔprG</td>
<td>0.48</td>
<td>0.28</td>
<td>0.15</td>
<td>0.14</td>
<td>0.18</td>
</tr>
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Ino, myo-inositol; Ara, arabinose; Man, mannos; Glc, glucose.

Values are expressed as molar ratios.

To characterize the kinetics of ΔprG replication in the mouse lung model, we infected wild-type C57BL/6 mice with aerosolized bacteria and assessed bacterial burden and lung pathology during acute and chronic phases of infection. As shown in Figure 6A, ΔprG was attenuated during the acute and chronic phases of infection. Although ΔprG could replicate in the mouse lung, on day 10 the burden of ΔprG was on average 8.3 and 14.5 fold lower than H37Rv (P<0.01) and ΔprG (P<0.01), respectively. With the onset of adaptive immunity [29], the burden of ΔprG started to decline. On day 70, the burden of ΔprG was reduced by 2,805 and 2,721 fold compared to H37Rv (P<0.005) and ΔprG (P<0.005), respectively. Gross examination on day 70 revealed normal sized lungs with no or small lesions in mice infected with ΔprG (Figure 6B). In contrast, mice infected with H37Rv and ΔprG had edematous lungs with numerous large granulomatous lesions.

**lprG mutant burden declines in the mouse lung with the onset of adaptive immunity**

To determine whether the mechanism of in vivo attenuation of ΔprG is due to hypersensitivity to reactive oxygen or nitrogen species, we investigated the growth and survival of ΔprG in BMMΦ.
Figure 2. Cell-imprinting reveals altered cell surface and reduced surface-exposed LAM in the lprG mutant. (A) Illustration of cell-imprinting and cell capture. Equal number of bacteria grown in shaking broth culture with Tween-80 were fixed in paraformaldehyde, washed with PBS, dried on a polystyrene glass slide, and then used as a template for imprinting. The template was pressed into pre-cured PDMS polymer which was then cured at 37 °C for 8 h, followed by 60 °C for 1 h. The template was peeled off and the imprinted polymer film was sonicated in distilled water for 5 min. Samples of bacteria suspended in PBS at an OD_{580} of 0.01 were fluorescently labeled with propidium iodide and 25 μl was flowed at 5 μl/min through a microfluidic device containing the imprints of wild-type (H37Rv), lprG mutant (ΔlprG), and ΔlprG complemented with lprG-Rv1410c (ΔlprG:ΔlprG). The average number of captured bacilli per eight view fields was measured using a fluorescent microscope. (B) Representative images showing captured H37Rv, ΔlprG, ΔlspA mutant (ΔlspA) and ΔwhiB3 mutant (ΔwhiB3). Samples were pretreated with PBS alone (C), with rabbit anti-LAM pAb (α-LAM) at a dilution of 1/20 (D) and with mouse anti-Ag85 complex mAb (CS-90) at a dilution of 1/10 (E). Data is representative of three independent experiments. Capture on imprints of ΔlprG and ΔlprG was compared to H37Rv. *P<0.05; **P<0.01; ***P<0.005.
doi:10.1371/journal.ppat.1004376.g002
from wild-type (C57BL/6), p-47phox−/−, and iNOS−/− mice. Intracellular bacilli in resting and IFN-γ-activated BMMΦ were enumerated on day 0, 2 and 5 post-infection. The lprG mutant was attenuated in resting and IFN-γ-activated wild-type macrophages (Figure 7A, B, E and F). Because ΔlprG is attenuated for cell entry, we determined the fold-change in colony forming units (CFU) between day 0 and 5. On average, fold-change in ΔlprG was 5.2 and 1.1 in resting and IFN-γ-activated macrophages, respectively, compared to 16.5 (P < 0.001) and 3.2 (P < 0.01) in H37Rv and ΔlprG, respectively. As shown in Figure 7, attenuation of ΔlprG was not reversed in p-47phox−/− (Figure 7C and D) or iNOS−/− (Figure 7G and H) macrophages. On day 5, on average, 8,767 and 1,183 ΔlprG were recovered per well from resting and IFN-γ-activated p-47phox−/− macrophages, respectively, compared to 9,667 and 1,683 from wild-type macrophages, respectively.

**Discussion**

Here we provide evidence that LprG is essential for normal surface expression of LAM in *M. tuberculosis*. We showed that the lprG mutant has normal abundance of lipids, lipoglycans, and glycans in the cell wall and the capsule, but it has an altered surface with reduced expression of surface-exposed LAM. This finding is consistent with prior studies showing the lprG mutant of *M. smegmatis* is defective for colony sliding motility [21] and of *M. bovis* BCG displays increased clumping in broth [30], both of which are properties associated with cell surface alteration. The exact mechanism through which LprG and P55 facilitate normal surface expression of LAM remains to be determined. The finding that LAM does not accumulate in the cell envelope or culture filtrate of the lprG mutant rules out the possibility that LprG plays a role in transport or anchoring of LAM, respectively. Furthermore, normal migration of LAM on SDS/PAGE blots together with unchanged capsular glycan content suggests LprG is not involved in elaboration of the LAM side-chains. Drage and colleagues showed that LprG associates with LAM through a hydrophobic pocket that accommodates the alkyl chains of triacylated lipoglycans [18]. Thus, we speculate LprG serves to orient LAM on the cell surface in order to maximize exposure of its mannose cap residues. This would be consistent with the paradigm that *M. tuberculosis* depends on surface-exposed LAM to bind to MMR in order to facilitate cell entry and inhibition of P-L fusion [14–16]. Misorientation of surface LAM in the lprG mutant would also explain reduced but not null expression of

**Figure 3. Surface staining reveals reduced LAM on the lprG mutant cells.** Indirect fluorescent antibody staining of wild-type (H37Rv), lprG mutant (ΔlprG), and ΔlprG complemented with lprG-Rv1410c (::lprG) with anti-LAM antibodies. Bacilli cultured in shaking broth cultures were washed, fixed in 4% paraformaldehyde, and then suspended in 5% bovine serum albumin for 1 h. Bacilli were incubated with primary antibodies (1° Ab) rabbit anti-LAM pAb (α-LAM) and mouse anti-LAM mAb (CS-35), washed, and then incubated with secondary antibodies (2° Ab), goat anti-rabbit IgG Fab2-Alexa fluor and goat anti-mouse IgG IgG-Dylight, respectively. Bacilli were mounted with DAPI-containing medium and visualized using a fluorescent microscope. (A) Confocal images showing positive staining bacteria with α-LAM (arrow). (B and C) Bar graphs show average percent of bacteria staining positive with α-LAM (B) and CS-35 (C) ± SD of three independent experiments. ΔlprG and ::lprG were compared to H37Rv. **P < 0.01; ***P < 0.001. doi:10.1371/journal.ppat.1004376.g003
surface LAM based on anti-LAM staining. Alternatively, LprG may be essential for minor modification of the LAM side-chains, which would impact normal surface chemistry and LAM immunostaining. We also showed that the \textit{lprG} mutant has a smaller colony size on agar. The exact role of surface LAM in colony size remains to be determined but the phenotype may be due to the altered surface property of the \textit{lprG} mutant. Alternatively, LprG could have a secondary function that is unrelated to orienting LAM.

Prior studies using purified mannose-capped LAM from \textit{M. tuberculosis} have identified it as a key virulence determinant for cell entry and inhibition of P-L fusion, which presumably leads to intracellular survival and persistence \cite{8}. However, until now, confirmation of these virulence properties in LAM mutants had not been possible due to the essentiality of genes in the LAM biosynthetic pathway \cite{8,9}. Here we characterized the functional consequences of surface LAM deficiency in the \textit{lprG} mutant. We showed that the \textit{lprG} mutant is in fact less efficient at macrophage entry and inhibition of P-L fusion, and is attenuated for replication in macrophages. These findings are consistent with the attenuated phenotype of the \textit{lprG} mutant reported in mice \cite{22,23}. Our study extends the observations made in vivo by providing a more detailed characterization of the mutant in mice after aerosol infection, which more closely approximates natural infection in humans. We showed that while the \textit{lprG} mutant is able to survive and partially replicate in the mouse lung during the acute phase of infection when innate immunity is in operation, the mutant is killed in the lung with the onset of adaptive immunity \cite{29}. The attenuation during the acute phase may be ascribed to the deficiency of the mutant to enter host cells. We also showed that the attenuation of the \textit{lprG} mutant in resting and IFN-\gamma-activated wild-type macrophages is not rescued in \textit{p47phox}−/− or \textit{iNOS}−/− macrophages, indicating that the \textit{lprG} mutant is not hypersensitive to reactive oxygen and nitrogen species, respectively. This finding is inconsistent with the observation by Chan and colleagues that LAM has the property of scavenging oxygen free radicals \cite{31}. Perhaps the orientation of LAM on the surface is not essential for the scavenging property. However, confirmation of our findings in knockout mice is essential because enhanced replication of H37Rv or \textit{::lprG} in either \textit{p47phox}−/− or \textit{iNOS}−/− macrophages was not observed. The \textit{lprG} mutant also has a cell envelope defect based on increased susceptibility to

\begin{figure}[h]
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\caption{The \textit{lprG} mutant is impaired for macrophages entry. (A) Resting BM-Mφ from C57BL/6 mice were infected with wild-type (H37Rv), \textit{lprG} mutant (\textit{ΔlprG}), and \textit{ΔlprG} complemented with \textit{lprG-Rv1410c (::lprG)} at MOI of 1:10 (Input). At 4 h post-infection cells were washed and intracellular bacteria (Output) were enumerated on agar. Bar graph shows average CFU counts ±SEM of three independent experiments performed in triplicate wells. (B–D) RAW 264.7 macrophages treated with and without 4 mg/ml mannan or 0.5 \textmu g/ml anti-MMR pAb were infected with FITC-labeled bacteria at MOI of 1:100 for 2 h. Cells were washed, mounted and visualized using a fluorescent microscope. Graphs show percentage of infected cells with no treatment (B), treated with mannan (C) and treated with anti-MMR pAb (D). Bars show average percentage ±SEM of two independent experiments performed in triplicates. \textit{ΔlprG} and \textit{::lprG} were compared to H37Rv. *P<0.05. doi:10.1371/journal.ppat.1004376.g004}
\end{figure}
SDS which suggests the in vivo attenuation of the \textit{lprG} mutant may be, in part, due to permeability to immune effectors such as antimicrobial peptides [20]. Other potential mechanisms of attenuation not investigated in this study include loss of LAM-dependent inhibition of protein kinase C activity [31], expansion of regulatory T cells [32], and suppression of a dendritic cell pro-inflammatory program [33]. Furthermore, although our results strongly indicate LAM orientation is the major factor that modulates the surface chemical property of the \textit{lprG} mutant, we are unable to rule out the possibility that interaction of LprG with phosphatidylinositol mannosides, lipomannan, and/or other lipoglycan contributes to the phenotypes observed in the \textit{lprG} mutant.

The bacteria used for cell-imprinting and surface staining experiments in this study were grown under perturbing conditions (in the presence of 0.05% Tween-80 and mechanical agitation).

Using cryo-electron microscopy (EM) and immuno gold-EM, Sani

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{The \textit{lprG} mutant is impaired for inhibition of Phagosome-Lysosome fusion. RAW 264.7 cells were treated with 50 nM LysoTracker-Red with and without 4 mg/ml mannan or 0.5 \mu g/ml anti-MMR pAb. Cells were incubated for 30 min, and then infected with FITC-labeled wild-type (H37Rv), \textit{lprG} mutant (\textDelta\textit{lprG}), and \textDelta\textit{lprG} complemented with \textit{lprG}-Rv1410c (::\textit{lprG}) at MOI of 1:100 for 2 h. Cells were washed, mounted, and visualized using a fluorescent microscope. (A) Confocal images showing bacteria in green, lysosomes in red, and the nucleus in blue. Scale bars, 5 \mu m. 2.5 \times magnification is shown on the right. (B–D) Graphs show average percentage of bacilli co-localized with lysosomes in cells with no treatment (B), treated with mannan (C) and treated with anti-MMR pAb (D). Bars show average percentage \pm SEM of two to three independent experiments performed in triplicates. \textDelta\textit{lprG} and ::\textit{lprG} were compared to H37Rv. *P<0.05.

doi:10.1371/journal.ppat.1004376.g005
\end{figure}
and colleagues showed that under non-perturbing conditions mycobacteria express a thick capsule that labels with anti-LAM but under perturbing conditions mycobacteria shed their capsule [6]. Although anti-LAM surface labeling was reduced under perturbing conditions, as much as 80% of mycobacteria in some experiments stained positive with anti-lipoglycan antiserum. Thus, although we were not able to compare surface LAM properties of the lprG mutant to wild-type bacteria under non-perturbing conditions due to bacterial clumping, our surface LAM staining results in wild-type bacteria are consistent with the above study; perturbing conditions still allowed us to detect a measurable difference in the lprG mutant. Furthermore, although the biological significance of perturbing and non-perturbing conditions in the context of lung environments is unknown, the fact that cell surface alteration has been reported under non-perturbing conditions [21,30] suggests the lprG mutant has a surface defect that manifests itself under both perturbing and non-perturbing conditions.

The findings of this study have translational implications for tuberculosis control and eradication. First, the elimination of the lprG mutant from mouse lung during the adaptive phase of infection makes LprG and P55 attractive drug targets. There has been a growing interest in repurposing of efflux pump inhibitors currently approved for other disorders as a way of increasing the susceptibility of M. tuberculosis to anti-tuberculosis drugs and thus shortening the duration of therapy [34,35]. Using the cell-imprinting assay described in this study, we may be able to identify efflux pump inhibitors that target P55 and chemically achieve an LprG null phenotype. The repurposing of efflux pump inhibitors with activity against P55 may be particularly useful for treatment of strains that are resistant to existing drugs. Secondly, the elimination of the lprG mutant from the mouse lung with the onset of adaptive immunity together with the loss of immunomodulatory effects of surface LAM in the lprG mutant raise the possibility that this mutant elicits an adaptive immune response that is capable of achieving sterilizing immunity. Further studies are underway to determine whether elimination of the lprG mutant is due to a more able adaptive immune response or due to a less resistant mutant. If the former is true, the lprG mutant may have utility as a vaccine candidate for prevention of M. tuberculosis infection.

Materials and Methods

Ethics statement

All animal experiments were done in accordance with procedures approved by the NYU School of Medicine Institutional Animal Care and Use Committee and in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health under the Assurance of Compliance Number A3435-01.

Bacteriologic media

M. tuberculosis was grown in Middlebrook 7H9 broth (DifCo) supplemented with 0.2% glycerol, 10% OADC (DifCo), and

Figure 6. The lprG mutant cannot persist in mouse lung. (A) C57BL/6 mice were infected wild-type (H37Rv), lprG mutant (∆lprG), and ∆lprG complemented with lprG-Rv1410c (::lprG) through the aerosol route and bacterial load in the lung was measured at the indicated time points. Data points show average CFU count ±SD of 4 mice per time point. (B) Representative lungs on day 70 post-infection. Data is representative of two independent experiments. ∆lprG and ::lprG were compared to H37Rv. **P<0.01; ***P<0.005.
doi:10.1371/journal.ppat.1004376.g006
Figure 7. The lprG mutant is attenuated in mouse macrophages. Resting and IFN-γ-activated BMMΦ from C57BL/6 (A, B, E, and F), p47phox−/− (C and D), and iNOS−/− (G, H) mice were infected with wild-type (H37Rv), lprG mutant (ΔlprG), and ΔlprG complemented with lprG-Rv1410c (::lprG) at MOI of 1:10 (A–D) and 1:40 (E–H). Intracellular bacteria were enumerated on Middlebrook 7H9 agar on the indicated days. Data points represent average CFU ±SD of triplicate wells. Data is representative of two to three independent experiments.

doi:10.1371/journal.ppat.1004376.g007

0.05% Tween-80. For lipid and lipoglycan analysis bacteria were grown in Glycerol-Alanine-Salts (GAS) medium with shaking. Middlebrook 7H9 agar with 0.2% glycerol, 10% ADC was used for enumeration of bacterial colonies.

Strain construction

The lprG deletion mutant (ΔlprG) was constructed in the H37Rv strain with conditionally replicating mycobacteriophages as previously described [36]. Primer sequences used in this study are shown in Table S5. To complement the ΔlprG, two PCR-amplified fragments of 1571 bp and 1060 bp, encoding the D

Preparation and analysis of lipids and lipoglycans

Capsular materials were extracted with glass beads as described previously [24]. Culture filtrates (0.22 μm filters) containing the secreted and shed material were similarly prepared. Capsular and culture filtrate materials were concentrated and dialyzed extensively against water prior to analyses. Lipoglycans were prepared from bacterial cells and analyzed by SDS-PAGE on 10–20% gradient Tricine SDS-polyacrylamide gels (Invitrogen) as described previously [39]. Lipids were extracted from bacterial cells, capsular materials and culture filtrates as previously described [40] and analyzed by TLC on aluminum-backed silica gel 60-precoated plates F254 (E. Merck) in a variety of solvent systems [41]. The phosphatidylinositol-glycans polyclonal IgG antibody (Santa Cruz Biotechnology) was used as a positive control.

Surface LAM staining

Mid-log shaking cultures were centrifuged at 800 rpm for 8 min to remove clumps. Bacilli were washed with PBS and fixed in 4% paraformaldehyde, and then resuspended in PBS and incubated in 5% bovine serum albumin for 1 h. Bacilli were sedimented and suspended in 3% bovine serum albumin with α-LAM, at a dilution of 1/100, or mouse anti-LAM monoclonal IgG (CS-35; BEI Resources), at a dilution of 1/150, and incubated overnight at 4°C. Bacilli were washed with PBS-0.02% Tween-20 and resuspended in PBS with goat anti-rabbit IgG Fab2-Alexa fluor (Cell Signalling), at a dilution of 1/200, and goat anti-mouse IgG HRP (Cell Signalling), at a dilution of 1/100, respectively, for 2 h at 24°C. Bacilli were washed, mounted with VECTASHIELD HardSet (Vector Laboratory), and visualized using a fluorescent microscope. The reader was blinded to the identity of each sample and graded each bacillus as positive (dim and bright) or negative staining.

Cell-imprinting

Mid-log shaking cultures were sedimented, washed in PBS, and then fixed in 4% paraformaldehyde. Cell-imprinting was performed as previously described [25,26]. Briefly, one drop of bacteria in PBS at an OD_{600} of 2 was placed on a polylysine slide (Evergreen) and incubated overnight at 4°C. The excess liquid on the slide was removed by spinning the slide at 2000 rpm for 30 sec. The slide was air-dried, heated at 60°C for 2 h, rinsed with deionized water, air-dried, and used as a template for imprinting.

LAM immunoblot

Whole cell lysates were prepared as previously described [27]. Soluble protein concentration was measured (BCA Protein Assay; Pierce Biotechnology). Lysates were normalized to protein content and separated on a 15% SDS/PAGE gel and transferred to a PVDF membrane. The membrane was blocked with 3% BSA in TBS-0.5% Tween-20, and then incubated with rabbit anti-LAM polyclonal IgG (α-LAM; BEI Resources), at a dilution of 1/200, overnight at 4°C. The membrane was washed with TBS-0.5% Tween-20 and incubated with goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology), at a dilution of 1/400, for 90 min at 24°C. The membranes and images were developed as described above.

For cell capture studies, bacterial suspensions in PBS at an OD_{600} of 0.01 were incubated in PBS alone and with α-LAM at a dilution of 1/20, with CS-35 at a dilution of 1/10, rabbit IgG isotype control at a dilution of 1/10, and with mouse anti-Ag85 complex monoclonal IgM (CS-90; BEI Resources) at a dilution of 1/10. Bacilli were fluorescently labeled with propidium iodide (Invitrogen) and 25 μl was passed at 5 μl/min through a microfluidic device containing the imprints of H37Rv, ΔlprG, and ΔlprG. After the infusion process, cells captured on each
imprint were imaged using an iXon+ electron-multiplying CCD camera (Andor Technology) and Andor Solis microscopy imaging software. A custom program written on LabVIEW (National Instruments) was used to control the stage to allow stepwise scanning of 8 adjacent view fields (280 μm x 280 μm). The images were analyzed using ImageJ (National Institutes of Health) and the average number of cells captured per 8 view fields was measured. The operator was not blinded to where the scanning was initiated or scoring of images, however, blinded scoring of H37Rv, ΔlprG, and ΔIP2 sample images from Figure 2C by a blinded reader showed both quantitative and statistical agreement with the unblinded results (interclass correlation coefficient of 0.9).

SEM
Single cell suspensions of bacilli were fixed overnight at 24°C on poly-l-lysine coated glass slides in 0.1 M sodium cacodylate pH 7.3 containing 4% formaldehyde and 2% glutaraldehyde, followed by 1% aqueous osmium tetroxide for 1 h, and then dehydrated in an increasing ethanol concentrations series (50% to 100%), and finally critical-point-dried in liquid carbon dioxide using Tousimis Autosamdr 814. Immediately after the process, the samples were coated with a thin layer (7.5 nm thick) Au/Pt mixture using Denton DeskII sputtering system, and inspected using field emission SEM (Sigma HD, Zeiss) under high vacuum mode with working distance of 5 mm. Cellular measurements were performed using Freehand software (Adobe).

Phagosome-Lysosome co-localization
RAW 264.7 cells were cultivated in Dulbecco’s Modified Eagle Medium (Corning) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% non-essential amino acids and plated on Lab-Tek chamber slides. Lysosomal and bacterial staining were performed as previously described [44,45]. Briefly, RAW cells were treated with 50 nM LysoTracker-Red DND-99 (Life Technology) with and without MMR blocking with 4 mg/ml rabbit IgG isotype control at a dilution of 1/400. Cells were incubated for 30 min, and then infected with FITC-labeled bacteria at MOI of 1:100 for 2 h. Cells were washed with PBS, fixed in 4% paraformaldehyde, mounted with VECTASHIELD HardSet, and visualized using a fluorescent microscope. The reader was blinded to the identity of each sample.

Macrophage infections
Bone marrow-derived macrophages (BMMΦ) were isolated from mice as previously described [38] and plated at a density of 5 x 10⁵ cells per well. Bacterial cultures were sedimented, washed with PBS, and resuspended in BMMΦ media. Monolayers were infected at MOI of 1:10 or 1:40 and activated with recombinant IFN-γ 20 ng/ml. At 4 h post-infection, monolayers were washed with PBS and replaced in fresh BMMΦ media. At the indicated times, monolayers were lysed in 1 ml of PBS with 0.5% triton X-100 and bacterial counts were enumerated.

Mouse infections
C57BL/6 mice were purchased from The Jackson Laboratory and housed under specific pathogen-free conditions. Mice were used in compliance with NYU institutional policies. Aerosol infections were performed in an inhalation exposure device (Glass-Col) as previously described [38]. On designated dates, mice were sacrificed and the lungs were homogenized in 2 ml of PBS with 0.5% tween 80 and the bacterial CFU counts were enumerated on Middlebrook 7H11.

Statistical analysis
The student t-test was used to determine significant differences between groups.

Supporting Information

Figure S1 Construction of the lprG mutant. (A) The genomic map of lprG-Rv1410c operon in M. tuberculosis H37Rv. (B) The allelic exchange substrate used to construct the lprG mutant (ΔlprG). Hyg, hygromycin. (C) The genomic map of lprG-Rv1410c operon in ΔlprG. The black box represents region deleted from lprG. (D) The lprG-Rv1410c genomic fragment used to complement ΔlprG. (E) Real-time PCR amplification plot showing amplification of lprG from genomic DNA. (F) Reverse-transcriptase real-time PCR amplification plot showing amplification of Rv1410c from cDNA.

Figure S2 The lprG mutant has normal growth kinetics in broth. Mid-log shaking broth cultures of wild-type (H37Rv), lprG mutant (ΔlprG), and ΔlprG complemented with lprG-Rv1410c (::lprG) were diluted to OD₅₆₀ of 0.05 in Middlebrook 7H9 broth with Tween-80. (A) The optical density of shaking cultures was measured daily. (B) Images of colonies grown on Middlebrook 7H9 agar for 3 wk. White bars mark the borders of colonies.

Figure S3 The lprG mutant has normal cellular dimensions. SEM micrographs of H37Rv (A and B) and ΔlprG (C and D). Wild-type (H37Rv) and lprG mutant (ΔlprG) bacilli grown in Middlebrook 7H9 broth were fixed with 4% formaldehyde, 2% glutaraldehyde, followed by 1% aqueous osmium tetroxide, and then dehydrated and coated with a thin layer of Au/Pt mixture before inspection using SEM. AFM images of imprints of H37Rv (E) and ΔlprG (F) in PDMS polymer. Pre-fixed bacilli were dried on polystyrene glass slides and used as template stamps to press into pre-cured PDMS polymer. The polymer was cured, the stamp was peeled off, and then the geometrical shape of the imprints was extracted using AFM. (G) Dimensions of H37Rv and ΔlprG obtained with SEM and AFM. Each value is an average of nine bacilli ±SD.

Figure S4 Phosphatidylinositol mannoside and trehalose dimycolate content of the lprG mutant. Thin-layer chromatograms of total lipids extracted from wild-type (H37Rv); lprG mutant (ΔlprG); and ΔlprG complemented with lprG-Rv1410c (::lprG). The same amounts of total lipids were loaded for each strain. TLC plates were run in the solvent system CHCl₃/CH₃OH/H₂O (20:4:0.5, by vol) and revealed with α-naphthol. TDM, trehalose dimycolates; SL, sulfolipid; CL, cardiolipin; PIM2, phosphatidylinositol dimannosides; TDM Std, TDM standard.

Figure S5 Cell-imprinting assay using bacteria pretreated with an isotype control. (A) Samples of wild-type (H37Rv), lprG mutant (ΔlprG), and ΔlprG complemented with lprG-Rv1410c (::lprG) suspended in PBS at an OD₅₆₀ of 0.01 were pretreated with rabbit IgG isotype control at a dilution of 1/10 and then fluorescently labeled with propidium iodide and 25 μl was flowed at 5 μl/min through a microfluidic device containing the imprints of H37Rv, ΔlprG, and ΔIP2. The average number of captured bacilli per eight view fields was measured using a fluorescent microscope. Data is representative of three independent
incubation of samples with anti-LAM isotype control antibody.  

(DOC)

Table S3 Primers used in this study.  

(PDF)

Acknowledgments

We thank Jeffery Glenn for providing reagents, Manuel Anmierva for assistance with imaging, and Christopher Becker for conducting mass spectrometry.

Author Contributions

Conceived and designed the experiments: RLG KR AB MJ NB. Performed the experiments: RLG KR AB SG NB. Analyzed the data: RLG KR MG NB. Contributed reagents/materials/analysis tools: RNZ. Contributed to the writing of the manuscript: RLG KR MJ SE JDE NB.

References


