LppM impact on the colonization of macrophages by Mycobacterium tuberculosis

Nathalie Deboosère, Raffaella Iantomaso, Christophe J. Queval, Gaspard Deloison, Samuel Jouy, Anne-Sophie Debrie, Mathias Chamaillard, Jérôme Nigou, Martin Cohen-Gonsaud, Anne-Sophie Debrie, Camille Locht, Priscille Brodin and Romain Veyron-Churlet

1 Univ. Lille, U1019 – UMR 8204 – CIIL – Centre d’Infection et d’Immunité de Lille, Lille F-59000, France.
2 CNRS, UMR 8204, Lille F-59000, France.
3 Inserm U1019, Lille F-59000, France.
4 CHU Lille, Lille F-59000, France.
5 Institut Pasteur de Lille, Lille F-59000, France.
6 institut de Pharmacologie et de Biologie Structurale, Université de Toulouse CNRS, Toulouse F-31077, France.
7 Centre de Biochimie Structurale, CNRS UMR 5048, Inserm U1054, Université de Montpellier, Montpellier F-34090, France.

Introduction

Tuberculosis (TB) remains a leading infectious disease around the world with 1.5 million deaths and 9.6 million new cases per year. Approximately one-third of the world population is considered to have latent TB, and although they show no clinical symptoms, they are at risk to progress to active disease (Barry et al, 2009; Young et al, 2009). The treatment of active TB requires chemotherapy to be taken for at least 6 months, which often leads to a poor compliance because of the toxicity of the treatment and thereby to the emergence of multi-drug-resistant or extremely-drug-resistant strains of Mycobacterium tuberculosis. The emergence of totally drug-resistant strains of M. tuberculosis has become a major global threat (Klopper et al., 2013), urging the need to better understand TB pathogenesis in order to develop new and more potent drugs. TB pathogenesis is linked to a tight interplay between the host immune response and M. tuberculosis, which produces factors that contribute to its successful colonization and its persistence within host cells. Tubercle bacilli are able to infect macrophages and to actively replicate within their phagosomes. After interaction with several cell-host receptors and subsequent phagocytosis, the bacilli are able to block phagosomal maturation, to escape from the phagosome and to subvert the host immune response (for reviews (Killick et al., 2013; Stanley and Cox, 2013)).

The cell colonization and intracellular trafficking of M. tuberculosis involve several distinct bacterial effectors, and deciphering the contribution of each of the different actors at the host-pathogen interface is essential to understand the intracellular persistence of M. tuberculosis. The bacterial virulence factors that have been extensively studied to impact on the invasion process are the ESX type VII secretion system, the serine protein kinase PknG, the lipid phosphatase SapM and components of the mycobacterial cell wall (Walburger et al., 2004; Vergne et al., 2005; Brodin et al., 2006; Cambier et al., 2014). Using a non-biased global screening approach on an 11 000 M. tuberculosis transposon mutant library, we identified additional mycobacterial genes whose expression impairment impacts the intracellular trafficking (Brodin et al., 2010). Among these genes, rv2171 (lppM) was shown to affect colocalization of M. tuberculosis Beijing GC1237 with LysoTracker, a marker of the acidic lysosomal compartment, suggesting that LppM is involved in preventing early phagosomal maturation in infected macrophages (Brodin et al., 2010).

Summary

Mycobacterium tuberculosis produces several bacterial effectors impacting the colonization of phagocytes. Here, we report that the putative lipoprotein LppM hinders phagocytosis by macrophages in a toll-like receptor 2-dependent manner. Moreover, recombinant LppM is able to functionally complement the phenotype of the mutant, when exogenously added during macrophage infection. LppM is also implicated in the phagosomal maturation, as a lppM deletion mutant is more easily addressed towards the acidified compartments of the macrophage than its isogenic parental strain. In addition, this mutant was affected in its ability to induce the secretion of pro-inflammatory chemokines, interferon-gamma-inducible protein-10, monocyte chemoattractant protein-1 and macrophage inflammatory protein-1α. Thus, our results describe a new mycobacterial protein involved in the early trafficking of the tubercle bacillus and its manipulation of the host immune response.

Received 25 August, 2015; revised 10 May, 2016; accepted 12 May, 2016. *For correspondence. E-mails priscille.brodin@inserm.fr (P. Brodin); romain.veyron@yahoo.fr (R. Veyron-Churlet); Tel. + 33 320 87 11 84; Fax + 33 320 87 11 92
†Equal contribution of the second authors.
Interestingly, \textit{lppM} expression has been shown to be induced during macrophage infection (Dubnau et al., 2002), and it was recently selected by bioinformatic analysis as a potential \textit{M. tuberculosis} effector involved in host–pathogen interaction (Li et al., 2015).

In this study, we demonstrate that LppM limits the early step of \textit{M. tuberculosis} entry into the macrophage. This phenotype can be reversed by the exogenous addition of recombinant LppM and is dependent on toll-like receptor 2 (TLR2). We further characterized the involvement of \textit{lppM} in \textit{M. tuberculosis} macrophage colonization by showing its implication in the blocking of the phagosomal maturation using different cellular markers: LysoTracker, lysosomal-associated membrane protein-1 (LAMP-1) and cathepsin D. Furthermore, we show that the \textit{lppM} deletion mutant is affected in its ability to induce the secretion of the pro-inflammatory chemokines interferon-gamma-inducible protein-10 (IP-10) (CXCL10), monocyte chemoattractant protein-1 (MCP-1) (CCL2) and macrophage inflammatory protein-1α (MIP-1α) (CCL3). Altogether, these data suggest that LppM is an additional bacterial effector implicated in the early steps of \textit{M. tuberculosis} infection.

Results

\textit{LppM} hinders mycobacterial uptake into macrophages

To investigate the contribution of LppM in the colonization of macrophages, a \textit{lppM} deletion mutant (\textit{MtbH37RvΔlppM}) was constructed in \textit{M. tuberculosis} H37Rv by substituting the \textit{lppM} gene with a cassette coding for hygromycin resistance (Fig. S1A). Allelic exchange was confirmed by polymerase chain reaction (PCR) with the amplification of a 2.9 kb fragment (Fig. S1B) and checked by sequencing to confirm the insertion of the hygromycin-resistance cassette at the expected locus (data not shown). In axenic cultures of \textit{M. tuberculosis} H37Rv, LppM is constitutively expressed, and its apparent migration weight around 28 kDa after SDS-PAGE is above its predicted molecular weight (Fig. S1C). As expected, the production of LppM was totally abolished in \textit{M. tuberculosis} H37RvΔlppM (Fig. S1C). The deletion mutant was complemented with a copy of \textit{lppM} under the control of its own promoter (\textit{prom\textsubscript{lppM}}:\textit{lppM}) or the strong \textit{hsp60} promoter (\textit{prom\textsubscript{hsp60}}:\textit{lppM}) in the integrative vector pMV306 (Fig. S1D).

DsRed-expressing \textit{M. tuberculosis} H37Rv and \textit{M. tuberculosis} H37RvΔlppM were used to infect murine bone marrow-derived macrophages (BMDM) at multiplicities of infection (MOI) of 5 and 10, and uptake of fluorescent mycobacteria was determined 2 h post-infection. The percentage of BMDM containing bacteria was largely increased for \textit{M. tuberculosis} H37RvΔlppM in comparison with \textit{M. tuberculosis} H37Rv (Fig. 1A). As the use of a third antibiotic marker was detrimental to the normal growth of \textit{M. tuberculosis} H37RvΔlppM, preventing complementation by an additional plasmid, we checked complementation by covalently staining the bacteria with the red fluorescent dye CypHer5E. Although labelling and detection were not as strong as DsRed-labelled bacteria, complementation of CypHer5E-labelled \textit{M. tuberculosis} H37RvΔlppM with \textit{prom\textsubscript{lppM}}:\textit{lppM} vector restored the infectivity to levels similar to those of \textit{M. tuberculosis} H37Rv (Fig. 1B). To discard any impact of clumping bacteria, we assessed the median of the bacterial area in infected BMDM. In these conditions, there was no difference between \textit{M. tuberculosis} H37Rv and \textit{M. tuberculosis} H37RvΔlppM, indicating that the deletion of \textit{lppM} did not increase clumping of the culture (Fig. S2A). On the contrary, heat-killed \textit{M. tuberculosis} H37Rv and \textit{M. tuberculosis} H37RvΔlppM displayed a significantly higher bacterial area reflecting their tendency to form bigger aggregates (Fig. S2A).

To obtain insight into the fate of \textit{M. tuberculosis} H37RvΔlppM after macrophage uptake, we assessed the bacterial area at different time points (from Days 0 to 4) after BMDM infection, using a lower MOI of 2. As \textit{M. tuberculosis} H37RvΔlppM uptake was higher in comparison with \textit{M. tuberculosis} H37Rv, each bacterial area was standardized by the corresponding bacterial area obtained at Day 0. In these conditions, \textit{M. tuberculosis} H37RvΔlppM growth in the macrophage was slower than that of \textit{M. tuberculosis} H37Rv during the first 3 days of infection. However, \textit{M. tuberculosis} H37RvΔlppM growth was identical to that of the wild-type at Day 4 (Fig. S2B). These data illustrate the importance of LppM for the early steps of macrophage colonization and less so at later time points.

\textit{LppM} hinders phagosomal maturation

To determine whether \textit{M. tuberculosis} H37RvΔlppM is affected in a subsequent early trafficking process, we investigated the effect of the \textit{lppM} deletion on phagosomal maturation. DsRed-labelled \textit{M. tuberculosis} H37Rv-infected or \textit{M. tuberculosis} H37RvΔlppM-infected BMDM were stained with LysoTracker, a fluorescent marker of acidified cellular compartments and analysed by confocal imaging using a dedicated multiparameter automated image analysis system (Brodin et al., 2010). This analysis showed that the percentage of bacteria colocalizing with LysoTracker was higher for \textit{M. tuberculosis} H37RvΔlppM than for \textit{M. tuberculosis} H37Rv, indicating increased targeting of the mutant to the acidified compartment (Fig. 1C and D). The percentage of LysoTracker-positive cells was higher for \textit{M. tuberculosis} H37RvΔlppM than for \textit{M. tuberculosis} H37Rv (Fig. S2C), in agreement with our previous observation on a Δ\textit{lppM} transposon mutant in the \textit{M. tuberculosis} Beijing GC1237 background (Brodin et al., 2010). Complementation by \textit{lppM} under the dependence of its own promoter restored the wild-type phenotype (Fig. S2C). However, as CypHer5E-staining is pH sensitive, we used DsRed-expressing bacteria to standardize the LysoTracker intensity to quantify the
bacteria (bacterial area) present in the macrophages. This ratio highlights the increase of Lysotracker intensity in the case of *M. tuberculosis* H37RvΔlppM compared with *M. tuberculosis* H37Rv, regardless of the number of intracellular bacteria, which reflects an enhanced acidification of the vacuoles containing *M. tuberculosis* H37RvΔlppM (Fig. 1E). These results indicate that *lppM* partly contributes to the blocking of the phagosomal maturation by the bacilli.

This was further characterized by using other cellular markers: the LAMP-1 and the endosomal protease, ...
cathepsin D. Similarly to LysoTracker staining, LAMP-1 and cathepsin D colocalized at a significantly higher extent with *M. tuberculosis* H37RvΔlppM compared with *M. tuberculosis* H37Rv (Fig. 2A–D), confirming the role of LppM in the early steps of macrophage infection.

Recombinant ⁶His-LppM₂₆₋₁₈₅ limits phagocytosis

Recombinant soluble ⁶His-LppM₂₆₋₁₈₅, starting right after the residue Cys²⁵ and devoid of the putative C-terminal trans-membrane domain (Barthe et al., unpublished), was produced in *Escherichia coli* (Fig. 3A). When ⁶His-LppM₂₆₋₁₈₅

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**Fig. 2.** *Mycobacterium tuberculosis* H37RvΔlppM is more easily addressed towards the degradative compartments of macrophages than *M. tuberculosis* H37Rv.

A. DsRed-producing bacteria (red), lysosomal-associated membrane protein-1 (LAMP-1) staining (green) and Hoechst staining (blue) of bone marrow-derived macrophages (BMDM) infected with *M. tuberculosis* H37Rv or *M. tuberculosis* H37RvΔlppM. The bar corresponds to 20 μm. Zoomed-in inset (right panel) corresponds to the area indicated by the white arrow.

B. LAMP-1 mean intensity per bacteria after infection of BMDM with *M. tuberculosis* H37Rv or *M. tuberculosis* H37RvΔlppM.

C. DsRed-producing bacteria (red), Cathepsin D (CatD) staining (green) and Hoechst staining (blue) of BMDM infected with *M. tuberculosis* H37Rv or *M. tuberculosis* H37RvΔlppM. The bar corresponds to 20 μm. Zoomed-in inset (right panel) corresponds to the area indicated by the white arrow.

D. Cathepsin D mean intensity per bacteria after infection of BMDM with *M. tuberculosis* H37Rv or *M. tuberculosis* H37RvΔlppM.
was added to the medium of M. tuberculosis H37RvΔlppM, the phagocytosis level of the mutant strain was reduced to that of M. tuberculosis H37Rv (Fig. 3B). This complementation of the uptake defect did not depend on LppM acylation, as Cys25 was not present in 6His-LppM26–185, inferring that LppM action is mediated by another structural element. However, the addition of 6His-LppM26–185 did not complement the M. tuberculosis H37RvΔlppM phenotype regarding the bacterial colocalization with acidified compartments (Fig. 3C) or LysoTracker intensity (Fig. 3D).

Phenotype of Mycobacterium tuberculosis H37RvΔlppM is mediated by toll-like receptor 2-dependent signalling

Toll-like receptor 2 operates as a primary sensor of a large variety of mycobacterial components and thereby triggers innate immune responses (Underhill et al., 1999; Harding and Boom, 2010). Therefore, we examined the effect of the lppM deletion on murine TLR2−/−BMDM. Interestingly, no difference was observed between M. tuberculosis H37Rv and M. tuberculosis H37RvΔlppM in bacterial uptake by TLR2−/−BMDM (Fig. 4A). Bacterial colocalization in the acidified compartments of TLR2−/−BMDM was also similar between M. tuberculosis H37Rv and M. tuberculosis H37RvΔlppM (Fig. 4B and C). In addition, there was no significant difference in LysoTracker intensity when TLR2−/−BMDM were infected with M. tuberculosis H37Rv or M. tuberculosis H37RvΔlppM (Fig. 4D). These results indicate that LppM modulates bacterial uptake through TLR2 signalling.

Mycobacterium tuberculosis H37RvΔlppM induces reduced secretion of pro-inflammatory chemokines over Mycobacterium tuberculosis H37Rv

We used the Luminex technology to further characterize the cytokine signatures induced by M. tuberculosis H37RvΔlppM. M. tuberculosis H37RvΔlppM was affected in its ability to induce the secretion of the pro-inflammatory chemokines IP-10 (Fig. 5A), MCP-1 (Fig. 5B) and MIP-1α (Fig. 5C), and this phenotype was (partially) reversed with the complemented strain. In contrast to the phagocytosis phenotype, the defect in chemokine secretion was not mediated by TLR2, as the profile of chemokines was almost identical between wild-type and TLR2−/−BMDM infected by M. tuberculosis H37RvΔlppM (Fig. 5A–C). As LppM is
implicated in cytokine production in a TLR2-independent manner, it appears that LppM displays its action through a mechanism different from that of other mycobacterial lipoproteins.

**Discussion**

In this study, we show that LppM limits *M. tuberculosis* uptake by macrophages and phagosomal maturation. 6His-LppM26–185 is able to complement the phenotype of *M. tuberculosis* H37RvΔlppM with respect of phagocytosis, arguing for a direct involvement of LppM in macrophage uptake, regardless of LppM acylation. However, the addition of 6His-LppM26–185 to *M. tuberculosis* H37RvΔlppM did not complement the phagosomal maturation phenotype, suggesting that the exogenous protein is not phagocytosed to the same extend as the live bacilli that is unstable once internalized by macrophage or that additional partners of LppM are required for its intracellular complementation. It has been previously reported that LprG, another

![Fig. 4. Mycobacterium tuberculosis H37RvΔlppM mutant phenotype depends on toll-like receptor 2 (TLR2)-signalling.](image-url)
mycobacterial lipoprotein, is able to control phagolysosomal fusion and that its action is mediated by the binding of LipoArabinoMannan (LAM) in its hydrophobic pocket (Gaur et al., 2014; Shukla et al., 2014), a lipoglycan known to be involved in this process. Therefore, it is tempting to hypothesize that the presence of ligands is required to fully complement the intracellular phenotype of Mycobacterium tuberculosis H37RvΔlppM, similarly to LprG with LAM. Consistent with this contention, we found that 6His-LppM26–185 produced by Mycobacterium smegmatis is able to bind PhosphatidylInositol Mannosides (PIM) derivatives, such as PI, PIM2Ac2 and PIM6Ac2 (Barthe et al., unpublished), suggesting a role of these PIM derivatives in the LppM-mediated phagosomal maturation defect. Therefore, a defect in PIM presentation by M. tuberculosis H37RvΔlppM could be directly responsible for the increased phagocytosis phenotype. Alternatively, the absence of LppM may have perturbed the general architecture of the mycobacterial cell wall and may have favoured the recognition of some other mycobacterial ligands by macrophages.

Several mycobacterial lipoproteins have been shown to be TLR2 agonists (Brightbill et al., 1999; Gehring et al., 2004; Pecora et al., 2006; Drage et al., 2009; Drage et al., 2010; Lancioni et al., 2011). Here, we show that LppM inhibits macrophage uptake of TB bacilli through a TLR2-dependent signalling, as M. tuberculosis H37Rv and M. tuberculosis H37RvΔlppM show similar colocalization with acidified compartments within TLR2+/− BMDM. There is previous evidence that phagosomal maturation may be controlled by TLR-dependent signals, as E. coli-containing or Staphylococcus aureus-containing phagosomes can fuse with lysosomes in wild-type macrophages, whereas the phagosomes of MyD88−/− or TLR2x4−/− macrophages cannot (Blander and Medzhitov, 2004). TLR2 has also been shown to be expressed on the surface of phagosomes (Underhill et al., 1999). Therefore, LppM would be able to trigger a TLR2 signalling while the bacilli reside in the extracellular environment and during its prolonged stay in the phagosomal compartment.

Mycobacterium tuberculosis H37RvΔlppM induces lower levels of pro-inflammatory chemokines IP-10, MCP-1 and MIP-1α than M. tuberculosis H37Rv, reflecting a potential attenuation of the mutant on the very first hours of the infection. Indeed, IP-10 is expressed in very high amounts in patients with active TB and is one of the most promising alternative markers to interferon-gamma to detect patients with active TB (Ruhwald et al., 2007). Furthermore, high levels of MCP-1 are correlated with the disease severity in TB patients (Hasan et al., 2009). Moreover, infection of macrophages results in the induction of MIP-1α, which is required for the inhibition of M. tuberculosis growth (Saukkonen et al., 2002). Interestingly, it was shown that non-capped lipoarabinomannan plays a direct role in inflammation by inducing the expression of MCP-1 and MIP-1α (Moller et al., 2003). This could be in direct correlation with the binding of PIM derivatives by LppM in M. smegmatis mc2155 (Barthe et al., unpublished), considering that PIM are precursors of LAM. Consequently, for M. tuberculosis H37RvΔlppM, a defect in the presentation of PIM derivatives by LppM or in the recognition of other mycobacterial ligands may potentially drive a decreased expression of both MCP-1 and MIP-1α.

It appears that mycobacterial lipoproteins display a large panel of cellular functions, ranging from phagosome-
Experimental procedures

Bacterial strains

Escherichia coli TOP10 (Invitrogen) and E. coli BL21(DE3) (Stratagene) were used for cloning and expression of recombinant proteins (Table S1). All strains were grown in LB medium (Difco) at 37°C supplemented with ampicillin (100 μg ml⁻¹), hygromycin (100 μg ml⁻¹) or kanamycin (25 μg ml⁻¹), when required. M. tuberculosis H37Rv, M. tuberculosis H37Rv ΔlppM and complemented strains were grown in Middlebrook 7H9 plus 0.05% Tween 80 or on Middlebrook 7H11 agar plates, with OADC enrichment (Difco) supplemented with hygromycin (50 μg ml⁻¹) or kanamycin (25 μg ml⁻¹), when required. (Table S1).

Generation of a lppM deletion mutant in Mycobacterium tuberculosis H37Rv

To generate M. tuberculosis H37RvΔlppM, we followed the protocol previously described by van Kessel et al. (van Kessel et al., 2008). Briefly, upstream and downstream regions of lppM were amplified from M. tuberculosis H37Rv genomic DNA using [Up_lppM_dir and Up_lppM_rev] and [Down_lppM_dir and Down_lppM_rev] as primers (Table S2). Both fragments were inserted at either side of a hygromycin resistance cassette. After linearization, the resulting construction was electroporated in competent M. tuberculosis H37Rv containing pJV53 (Table S1) after acetamide induction. Several clones were selected and tested for the insertion of the hygromycin resistance cassette by PCR using Seq_lppM_dir and Seq_lppM_rev (Fig. S1A and Fig. S1B, Table S2). The resulting PCR fragment was further checked by sequencing to confirm the insertion at the expected M. tuberculosis locus. In addition, M. tuberculosis H37Rv and M. tuberculosis H37RvΔlppM were transformed by pMRF1 (Table S1), a derivative of pMV261 carrying a kanamycin resistance cassette, which allows the expression of the red fluorescent protein DsRed.

Plasmid constructions

Fragments of lppM were amplified by PCR using M. tuberculosis H37Rv genomic DNA as a template and [pET15b_lppM26-227_dir and pET15b_lppM26-227_rev] or [pET15b_lppM26-227_dir and pET15b_lppM26-185_rev] (Table S2), containing Ndel and Nhel sites, as primers. The resulting fragments were digested with Ndel and Nhel and ligated into pETPhos, generating pETPhos::lppM26-227 and pETPhos::lppM26-185 (Table S1). The same approach was undertaken to insert lppM into pMV306 and pVV16, using the following pairs of primers [pMV306_lppM_dir and pMV306_lppM_rev] and [pVV16_lppM_dir and pVV16_lppM_rev] (Table S2), together with the relevant restriction enzymes, to obtain pMV306::prom hbox{hsp60}::lppM and pVV16::lppM (Table S1). Finally, prom hbox{hsp60}::lppM was subcloned from pVV16::lppM into pMV306 using XbaI and Clal to yield pMV306::prom hbox{hsp60}::lppM (Table S1). All plasmids were checked by sequencing.

Purification of recombinant 6His-LppM

Escherichia coli BL21(DE3) strains containing pETPhos::lppM26-227 or pETPhos::lppM26-185 were used to inoculate 11 of LB medium supplemented with ampicillin (100 μg ml⁻¹) and cultured at 37°C with shaking until the A600 reached ~0.5. Then, 1 ml (final concentration) of isopropyl β-D-thiogalactopyranoside was added, and growth was continued for 3 h at 37°C. Soluble 6His-LppM26-227 and 6His-LppM26-185 were purified using Ni-NTA agarose beads (Qiagen) as previously described (Veyron-Churl et al., 2010).

Generation of anti-LppM antibodies and Western-blot analysis

Recombinant 6His-LppM26-227 purified from E. coli was used to generate specific rabbit antibodies against LppM according to the Speedy protocol (Eurogentec). Proteins were resolved by SDS-PAGE using 4–15% acrylamide gels (BioRad) and then transferred onto a polyvinylidene difluoride membrane. The membrane was saturated with TBS-Tween-5% milk and probed overnight with anti-LppM antibodies diluted 1:1000 with TBS-Tween-3% milk. The membrane was then incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) for 1 h at room temperature, followed by detection using Immobilon kit (Millipore). Chemiluminescence was determined using the ImageQuant LAS 4000 (GE Healthcare).

Cell culture

Wild-type and TLR2⁻/⁻ bone marrow progenitors were obtained from tibias and femur bones of 7 to 11 week-old C57BL/6 mice and frozen at −80°C. The BMDM were obtained by seeding 10⁷ frozen bone marrow cells in 75 cm² flasks in RPMI 1640 Glutamax medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and 10% L929-conditioned medium containing macrophage colony-stimulating factor. After 7 days of incubation at 37°C in 5% CO₂, BMDM were rinsed with Dulbecco’s phosphate-buffered saline (D-PBS) and harvested with Versene (Gibco) for further use.

Mycobacterial preparation and labelling

Bacteria were harvested, washed three times and resuspended in PBS. The bacteria were allowed to stand for 30 min to allow residual aggregates to settle. The bacterial suspensions were then aliquoted and frozen at −80°C for further use. When needed, M. tuberculosis H37Rv, M. tuberculosis H37RvΔlppM and the complemented strain (prom hbox{hsp60}::lppM) were covalently labelled with CypHer5 mono ester.
dye, which is a red-excitable cyanine dye derivative, available as an N-Hydroxysuccinimide ester that can be directly coupled to a broad range of cell surface receptors (Sigma Aldrich, Saint-Louis, MO). 10⁶ bacteria were diluted in 300 µl of 0.1 mg ml⁻¹ CypHer5E in 0.1 m sodium carbonate buffer (pH 9). The bacterial suspensions were incubated at 30°C under stirring at 600 r.p.m. and washed three times with PBS before use.

**Infection for intracellular growth and LysoTracker assays**

The bacteria were quantified by measuring the optical density (OD₅₃₀) and/or DsRed-fluorescence on a Victor Multilabel Counter (Perkin Elmer). The heat-killed bacteria were used as a positive control for infection in LysoTracker assays. Twenty microliter of bacterial suspensions and LppM₂₆-₁₈₅-coated beads was seeded in 384-well plates (Greiner) at MOI ranging from 2 to 10 bacteria per cell. Then, 2.10⁶ cells in a 30 µl volume were seeded per well. The plates were incubated at 37°C in 5% CO₂ for 2 h. To follow the mycobacterial replication in the BMDM, the cells were washed and treated for 1 h with 50 ng ml Amikacin (Sigma-Aldrich) to remove extracellular bacteria. Cells were finally incubated at 37°C with 5% CO₂ in complete culture medium containing a non-cytotoxic concentration of Hoechst (Sigma-Aldrich), corresponding to 300 ng ml⁻¹, to stain the cell nuclei. Image acquisition was performed daily. To study the phagosomal maturation/acidification, 50 µl of RPMI containing 1 µM of LysoTracker green DND-26 (Invitrogen) was added per well, and cells were incubated at 37°C with 5% CO₂ for 1.5 h. Cell nuclei were stained using 10 µl of Hoechst for 30 min at 37°C with 5% CO₂. Finally, cells were fixed with 10% neutral buffered Formalin solution (Sigma-Aldrich), containing paraformaldehyde at 4%, for 30 min at room temperature, washed in PBS and kept at 4°C in D-PBS 1% FBS until image acquisition.

**Immunofluorescence assays**

Macrophage infection was performed as described earlier. After 2 h of infection, cell monolayers were fixed with Formalin and permeabilized with D-PBS 0.2% Triton X100 for 5 min at room temperature. Cells were incubated for 30 min with blocking buffer, corresponding to 10% Donkey serum in D-PBS, prior to overnight incubation at 4°C with mouse anti-LAMP-1 [H4A3] (Abcam, Cambridge, UK) or goat anti-Cathepsin D (C-20) (Santa Cruz Biotechnology, Texas, USA) antibodies. Then, cells were incubated for 1 h at room temperature with Alexa-Fluor 488-conjugated goat anti-mouse and donkey anti-goat (Life technologies) secondary antibodies respectively. Cell nuclei were fluorescently labelled using 5 µg ml DAPI (Sigma-Aldrich) for 10 min. Cell monolayers were washed in PBS and kept in D-PBS 1% FBS until further use.

**Image acquisition by automated confocal microscopy**

Images were acquired using an automated fluorescent confocal microscope OPERA (PerkinElmer), with a 20X (Nuramic Aperture 0.7) or 63X (NA 1.2) water immersion lens. The microscope was equipped with 405, 488, 561 and 640 nm excitation lasers. The emitted fluorescence was captured using three cameras associated with a set of filters covering a detection wavelength ranging from 450 to 690 nm. Nucleus staining with Hoechst was detected using the 405 nm laser (blue channel) with a 450/50 emission filter. LysoTracker-positive, LAMP-1-positive and cathepsin D-positive compartments (green channel) and mycobacteria (red channel) were detected on 488 and 561 nm laser with a 540/75 and 600/40 emission filters respectively. CypHer5E dye was detected with laser and filter that match the 644 nm absorbance and 663 nm emission wavelengths respectively.

**Image-based analysis**

For bacteria replication and acidic compartment colocalization, images were analysed using a multi-parameter script developed with Columbus system (version 2.3.1; PerkinElmer). Cells were detected and counted using internal methods provided by the software. The cell number and the bacterial area were determined by well. Intracellular bacterial growth was daily quantified by the ratio of bacterial area per cell divided by the bacterial area at Day 0. Acidic compartments stained by LysoTracker and bacteria were individually localized by a manual threshold method, using non-infected wells to determine the minimum threshold required to discard any background signals. The bacterial area was then stretched from 2 pixels to define the bacterial ring region. LysoTracker fluorescence area was detected in the red channel by keeping only pixels with a higher intensity than a manually defined threshold. Cells were split into four groups based on the presence (infected) or the absence (non-infected) of an intracellular bacterial signal and a LysoTracker positive or negative signal. Results are expressed as the average of all images in four different wells in at least three independent experiments. The percentage of infection and bacteria/LysoTracker colocalization was calculated as follows:

\[
\%_{\text{population}} = \frac{\text{Nb of cells in the population}}{\text{Total Nb of cells}} \times 100.
\]

Lysosomal-associated membrane protein-1-positive and cathepsin D-positive compartments were identified according to an arbitrary threshold of fluorescence intensity, determined on non-infected cells, associated with anti-LAMP-1 or cathepsin D immunolabelling. The same thresholds were then applied to infected cells. The LAMP-1 and cathepsin D mean intensities were measured for each bacterium.

**Infection for the determination of the cytokine profile**

Murine macrophages were seeded at a density of 3.8 × 10⁵ cells per well in 24-well plates. After 24 h, at 37°C in 5% CO₂, supernatants were removed, and cells were infected with 950 µl of *M. tuberculosis* H₃₇Rv, *M. tuberculosis* H₃₇Rv/LppM or complemented strain (prompák/lppM) suspensions to reach MOI of 1, 2 and 3. The plates were incubated at 37°C in 5% CO₂ for 5 or 24 h. The supernatants were then filtered using a 0.22 µm PVDF filter, sampled and stored at −80°C until analysis. Mouse cytokine concentrations were quantified using Cytokine Mouse Magnetic 20-Plex Panel kit (Life Technologies) and Luminex® platform, accordingly to the manufacturer's protocol.
Statistics
For bacteria replication and acidic compartment colocalization using the LysoTracker probe, the statistical significance (P-value) was tested with a two-tailed unpaired t-test (GraphPad Prism version 5.04), and P-values ≤ 0.05 were considered to show significant differences. For the comparison of the LAMP-1 and cathepsin D mean intensities obtained for wild-type and mutant bacteria, the statistical significant value was obtained using a Wilcoxon unpaired test (or Mann–Whitney U test).

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Generation of M. tuberculosis H37RvΔlppM. (A) Genomic organization of the lppM locus in M. tuberculosis H37Rv and schematic representation of the insertion of the hygromycin cassette resistance. Primers 1 and 2 used for PCR are symbolized by arrows. (B) PCR using primers 1 and 2 with genomic DNA extracted from M. tuberculosis H37Rv or M. tuberculosis H37RvΔlppM. (C) Western-blot analysis on crude lysates of M. tuberculosis H37Rv and M. tuberculosis H37RvΔlppM cultures using polyclonal antibodies against LppM and anti-Hsp65 monoclonal antibodies, used as a control. (D) Western-blot analysis using anti-LppM antibodies on crude lysates of M. tuberculosis H37RvΔlppM transformed by integrative empty vector (pMV306), vector containing lppM under the control of its own promoter (promlppM::lppM), vector containing lppM under the control of the strong hsp60 promoter (promhsp60::lppM) and the recombinant mature 6His-LppM26-227 purified from E. coli.

Fig. S2. Analysis of M. tuberculosis H37RvΔlppM phenotype after infection of BMDM. (A) Median of the bacterial area after infection of BMDM by M. tuberculosis H37Rv, M. tuberculosis H37RvΔlppM or respective heat-killed bacteria (MOI 10) as a control. (B) Fold change of the bacterial area after infection of BMDM by M. tuberculosis H37Rv or M. tuberculosis H37RvΔlppM (using a MOI of 2) from Day 0 to Day 4. (C) Percentage of Lysotracker-positive BMDM 2h post-infection, using M. tuberculosis H37Rv, M. tuberculosis H37RvΔlppM or the complemented strain (promlppM::lppM) labelled with CypHer5E.

Table S1. List of strains and plasmids used in this study.
Table S2. List of primers used in this study.