

Mycobacterial persistence requires the utilization of host cholesterol

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A hallmark of tuberculosis is the ability of the causative agent, *Mycobacterium tuberculosis*, to persist for decades despite a vigorous host immune response. Previously, we identified a mycobacterial gene cluster, *mce4*, that was specifically required for bacterial survival during this prolonged infection. We now show that *mce4* encodes a cholesterol import system that enables *M. tuberculosis* to derive both carbon and energy from this ubiquitous component of host membranes. Cholesterol import is not required for establishing infection in mice or for growth in resting macrophages. However, this function is essential for persistence in the lungs of chronically infected animals and for growth within the IFN- γ -activated macrophages that predominate at this stage of infection. This finding indicates that a major effect of IFN- γ stimulation may be to sequester potential pathogens in a compartment devoid of more commonly used nutrients. The unusual capacity to catabolize sterols allows *M. tuberculosis* to circumvent this defense and thereby sustain a persistent infection.

bacterial pathogenesis | *mce* | metabolism | tuberculosis | carbon

Tuberculosis is a chronic bacterial infection that causes millions of deaths each year (1). The causative agent, *Mycobacterium tuberculosis*, is an intracellular pathogen thought to reside primarily within a phagosome-like compartment of macrophages during infection (2). Despite the apparent homogeneity of this environment, existing evidence indicates that the challenges faced by the bacterium change dramatically as the disease progresses. Early in infection, resting macrophages are quite permissive of bacterial growth and bacteria replicate relatively rapidly (3). Later, after the onset of adaptive immunity, macrophages are stimulated by T cell-derived cytokines, such as IFN- γ , to elaborate a variety of antibacterial effectors. Although this response effectively controls the replication of *M. tuberculosis* (4, 5), the bacteria are able to resist eradication. The result of this biological impasse is chronic tuberculosis, which is characterized by slowly replicating bacteria, and progressive immunopathology. In these chronic tuberculosis lesions, both intra- and extracellular bacteria are apparent (6). Thus, to maintain a persistent infection, *M. tuberculosis* must adapt to a heterogeneous and continually changing host environment.

IFN- γ -activated macrophages are critical for controlling mycobacterial infections, and the antimicrobial mechanisms of this cell are generally thought to rely on the production of directly toxic oxidative radicals such as nitric oxide (7, 8). However, the antimicrobial effect of IFN- γ cannot be solely attributed to these responses (9), and activated macrophages also restrict the growth of intracellular bacteria by depriving them of essential nutrients (10). The inability of many different auxotrophic mutants to replicate *in vivo* attests to the scarcity of nutrients in this environment (11–15). Although the mechanisms by which *M. tuberculosis* resists host-derived oxidative and nitrosative stresses have been intensively studied (16, 17), it is entirely unclear how this pathogen acquires the nutrients necessary for replication in this isolated compartment.

Not surprisingly, genetic experiments indicate that success after the onset of adaptive immunity requires distinct bacterial adaptations that are not initially important (18–20). The dichot-

omous use of the four homologous *mce* operons of *M. tuberculosis*, *mce1–mce4*, provides one example. Mutations in the *mce1* operon cause a growth defect in the spleens of infected animals during the earliest phase of infection. In contrast, mutations in the *mce4* operon have little effect for the first 2 weeks of infection and only attenuate bacterial growth in this organ after the onset of adaptive immunity (20, 21).

Each *mce* operon contains 9–13 genes that encode two transmembrane proteins with homology to the permease subunits of ABC transporters, along with several putative secreted or cell-surface proteins (22). These features suggest that the *mce* loci may encode ATP-driven transport systems, and we have proposed that energy for substrate translocation by all four systems is generated by a common ATPase, MceG (21).

To define the function of the Mce4 system more precisely, we performed a synthetic lethality screen and identified a number of genes located near the *mce4* operon that appeared to function in concert with this transporter (21). Many of these genes were predicted to be involved in lipid metabolism, leading to the hypothesis that the *mce* operons encode lipid transporters. Recently, similarities have been noted between this chromosomal region and genes required for cholesterol catabolism in a soil-dwelling relative, *Rhodococcus* RHA1 (23). These observations suggested that *M. tuberculosis* might share the ability to use cholesterol as a carbon source and that the Mce4 proteins may be important for this function. However, the ability to use sterols as a source of carbon is a very unusual trait. Although cholesterol has been shown to stimulate the growth of *M. bovis* bacillus Calmette-Guérin *in vitro* (23), multiple reports suggested that *M. tuberculosis* might not be able to use this compound (24, 25).

In this work, we demonstrate that *M. tuberculosis* can indeed degrade cholesterol and derive both carbon and energy from this compound. Furthermore, we show that the Mce4 transporter is critical for this ability and represents the major cholesterol import system of the bacterium. The loss of this function impairs the growth of the bacterium predominantly during the chronic phase of murine infection and in IFN- γ -activated macrophages *in vitro*. Thus, the ability of *M. tuberculosis* to maintain a chronic infection is critically linked to its ability to acquire cholesterol from the host.

Results

***M. Tuberculosis* Can Use Cholesterol as a Carbon Source.** To determine conclusively whether *M. tuberculosis* could catabolize cholesterol, we followed the fate of ^{14}C -radiolabeled cholesterol added to bacterial cultures. We found that carbon at the 4 position of the A sterol ring (Fig. 1A) was efficiently converted to CO_2 by *M. tuberculosis* but was poorly incorporated into the extractable lipid

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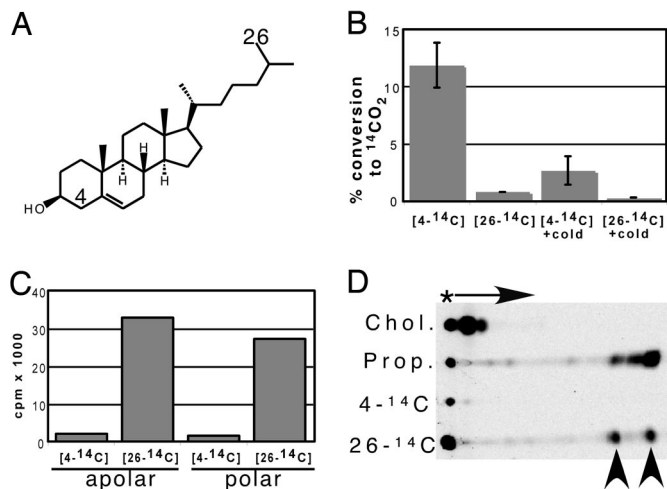


Fig. 1. *M. tuberculosis* catabolizes cholesterol. (A) Structure of cholesterol. The locations of the ¹⁴C label in substrates used in this study are indicated. (B) The conversion of [4-¹⁴C] or [26-¹⁴C]cholesterol to ¹⁴CO₂ by *M. tuberculosis* was quantified. Data are presented as the percentage of input radioactivity recovered as ¹⁴CO₂ after 4 h of culture. "Cold" indicates the addition of a 100-fold excess of unlabeled cholesterol. Error bars indicate standard deviation. (C) Cholesterol-derived carbon is assimilated into mycobacterial lipids. Bacteria were grown with cholesterol labeled at the indicated position, and the amounts of radioactivity recovered in the apolar or polar lipid extracts were determined. (D) The apolar lipid extracts of metabolically labeled bacteria were resolved by TLC. [3-¹⁴C]Propionate (Prop.) was used to specifically label branched-chain lipids such as PDIM (arrowheads). Labeled PDIM was detected in the [26-¹⁴C]cholesterol-labeled cells, but not in the [4-¹⁴C]cholesterol-labeled sample. The migration of intact cholesterol is shown (Chol.). The origin (asterisk) and direction of migration are indicated. The identity of PDIM was confirmed by purifying the species marked by arrowheads and subjecting them to atmospheric pressure chemical ionization mass spectrometry (APCI-MS). Major ions characteristic of PDIM (45) were detected at *m/z* = 914, 928, 942, 956, and 970.

fraction (Fig. 1C). This finding suggested that this carbon was preferentially used for energy generation via the trichloroacetic acid cycle. Similar results were obtained by using *M. smegmatis* (data not shown), a nonpathogenic relative of *M. tuberculosis* that is known to metabolize sterols (24, 26). In contrast, [26-¹⁴C]cholesterol, which is labeled on the side chain, was not converted into ¹⁴CO₂. Instead, this carbon was efficiently assimilated into mycobacterial lipids, including a major virulence-associated lipid, phthiocerol dimycocerosate (PDIM) (27). Thus, both the sterol rings and the side chain of cholesterol could be catabolized by *M. tuberculosis*, although the bacterium appeared to preferentially use different portions of the molecule for energy generation and biosynthetic purposes.

We also found that *M. tuberculosis* could grow by using cholesterol as a primary source of carbon (Fig. 2A). Using a defined medium that supported no growth without the addition of a carbon source, we found that cholesterol sustained bacterial replication with a doubling time of 39.8 h. This rate was nearly as rapid as the growth of glycerol-supplemented cultures (35.3 h). The maximal cell density observed with cholesterol supplementation was lower than glycerol, but this result is likely due to the relatively low concentration of cholesterol that could be added (which is limited by its solubility).

The Mce4 Transporter Is Required for Cholesterol Utilization. To determine whether the *mce4* genes contributed to cholesterol utilization, we compared the ability of wild-type and mutant bacteria to grow by using cholesterol. Mutations in either the *mce4* operon or in the gene encoding the common ATPase subunit, *mceG*, caused a severe restriction of cholesterol-

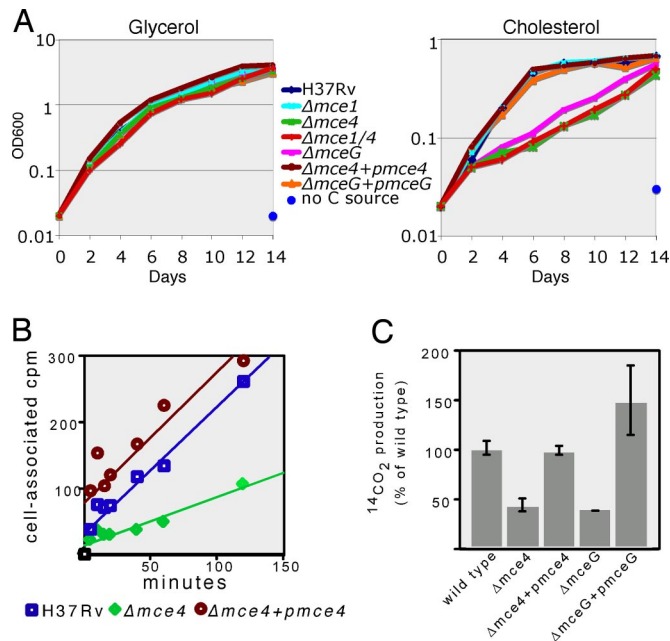


Fig. 2. Cholesterol utilization requires Mce4 and MceG. (A) The indicated strains were cultured in defined medium containing 0.1% glycerol, 0.01% cholesterol, or no added carbon source. The OD₆₀₀ of each culture over time is plotted on a log scale. H37Rv is the parental strain of all mutants. (B) *M. tuberculosis* strains were incubated with [4-¹⁴C]cholesterol for the indicated times, and the cell-associated radioactivity remaining after extensive washing was quantified. Curves were fit by linear least squares regression. (C) The indicated strains were incubated with [4-¹⁴C]cholesterol for 4 h, and the relative production of ¹⁴CO₂ is plotted.

dependent growth but had no effect in cultures supplemented with either glycerol (Fig. 2) or fatty acid (polyoxyethylenesorbitan monooleate) (ref. 28 and data not shown). The growth defects of both mutants could be complemented by the addition of the deleted genes on a plasmid vector. This defect was specific to *mce4* mutations because deletion of the *mce1* operon had no effect in either a wild-type or an *mce4*-deficient background.

The homology between Mce4 and ABC transporters suggested that this system might be involved in cholesterol uptake. Indeed, the rate at which cholesterol became associated with *mce4*-deficient bacteria was 3-fold lower than wild-type or complemented strains (Fig. 2B). *mce4* and *mceG* mutations also caused a decrease in the rate at which *M. tuberculosis* converted [4-¹⁴C]cholesterol to ¹⁴CO₂ (Fig. 2C), which is consistent with an import defect.

The Acquisition of Host Cholesterol Is Critical for Persistence During Chronic Infection. We reported that Mce4 was specifically required for bacterial survival during the later stages of infection in the mouse spleen (21, 29). Because tuberculosis is predominantly a disease of the lung, we assessed the importance of *mce4* in this organ. Consistent with previous findings, we observed that the *Δmce4* mutant initially grew relatively well in the mouse lung. However, this strain displayed a growth or survival defect that became apparent at 4 weeks after infection and became more pronounced as the disease progressed (Fig. 3A).

At this later stage of infection, the bacteria are primarily associated with activated macrophages, implying that cholesterol utilization may be specifically required for growth in these cells. To test this hypothesis, we compared the growth of wild-type and *mce* mutants in resting or IFN- γ -stimulated macrophages. As anticipated, *mce4* was not required for replication in resting macrophages (Fig. 3B). However, IFN- γ stimulation completely

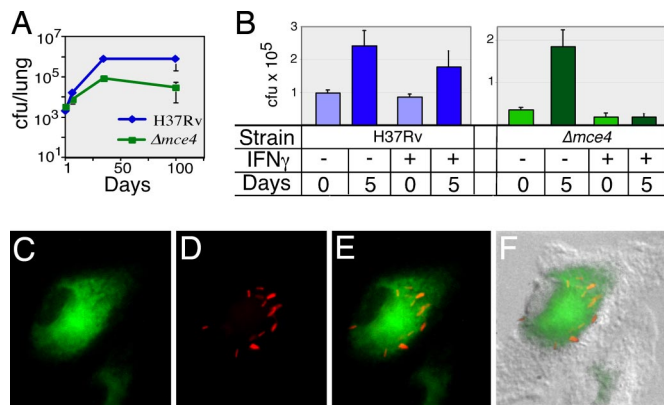


Fig. 3. Cholesterol utilization is required for mycobacterial persistence in chronically infected mice and replication of IFN- γ -activated macrophages. (A) The abilities of wild-type and $\Delta mce4$ mutant bacteria to persist in mouse lungs were compared. C57BL/6 mice were infected i.v. with a 1:1 mixture of wild-type (H37Rv) and $\Delta mce4$ mutant bacteria, and the abundance of each strain in lung homogenates was quantified at the indicated times by plating. (B) The abilities of wild-type and mce mutants to replicate in bone marrow-derived macrophages (BMM) were compared. The number of cfu recovered from cell lysates at 0 and 5 days after infection are presented. The indicated macrophages were stimulated with IFN- γ 24 before infection. The data in A and B are representative of three or four independent experiments, respectively. Error bars indicate standard deviation. (C–F) Colocalization of cholesterol and *M. tuberculosis* in IFN- γ -activated macrophages. Filipin-stained cholesterol (C) and RFP-expressing bacteria (D) were simultaneously visualized in infected BMMs by using confocal microscopy. These images were merged to demonstrate colocalization (E, orange) and overlaid on a DIC image (F). In representative optical sections, 53–85% of RFP-positive pixels colocalized with filipin. The three fluorescent images shown represent a single optical section.

inhibited the growth of *mce4* mutants while only reducing the replication rate of wild-type *M. tuberculosis* by ≈ 2 -fold. Thus, the ability to metabolize cholesterol was essential for growth in IFN- γ -activated cells. This result likely explains the specific requirement for *mce4* during the chronic phase of disease.

The proposal that Mce4 imports cholesterol *in vivo* requires that the bacterium have access to this compound as it grows intracellularly. Although *M. tuberculosis* is initially surrounded by cholesterol-rich plasma membrane after phagocytosis (30), it is unclear whether this membrane composition is maintained in the relatively mature compartment in which replication occurs. To identify potential sources of cholesterol, we used confocal microscopy to simultaneously visualize bacteria and the cholesterol-binding fluorophore, filipin (31). As reported in ref. (32), intracellular cholesterol was highly concentrated in a discrete perinuclear region (Fig. 3C). This distribution is consistent with the known concentration of intracellular cholesterol in the endoplasmic reticulum in which it is synthesized and the secretory pathway through which it is transported to the plasma membrane (33). Bacteria were invariably associated with this cholesterol-rich region of the cell and were colocalized with filipin in virtually every optical section analyzed. IFN- γ treatment did not noticeably affect the distribution of cholesterol or its association with bacteria, indicating that *M. tuberculosis* likely has access to an abundant supply of this compound regardless of the activation state of the macrophage.

Discussion

This work demonstrates that both the sterol rings and side chain of cholesterol can be degraded by *M. tuberculosis* to provide energy and carbon to the bacterium. Based on homology, the pathway used by the pathogenic mycobacteria to degrade cholesterol appears similar to their environmental relatives (23). Our observations regarding the flux cholesterol-derived carbon support this model. Sterol-degrading environmental organisms

cleave the 4 carbon of ring A from the rest of the molecule as a pyruvate unit (34). Under aerobic conditions, pyruvate is expected to be oxidized to CO₂ via the tricarboxylic acid (TCA) cycle, as observed in our studies. In contrast, the 26 carbon of the side chain is removed from cholesterol as a propionyl-CoA unit in other organisms (35). This compound is efficiently assimilated into branched chain lipids (including PDIM) in *M. tuberculosis* (36), possibly explaining the preferential flux of this carbon into lipid synthesis.

The production of propionyl-CoA during cholesterol catabolism also could explain previously described effects of central metabolic mutations on pathogenesis. The inactivation of one of the two isocitrate lyase enzymes of *M. tuberculosis*, Icl1, leads to a chronic persistence defect that is very similar to the *mce4* deletion (19). Although the two Icl isozymes appear to serve redundant roles under most circumstances, *icl1* mutants have a specific growth defect when propionate is the primary carbon source (28). It has been proposed that Icl1 might be important *in vivo* for the metabolism of odd chain fatty acids. However, because propionyl-CoA also is likely to be produced during cholesterol degradation, it is tempting to speculate that the persistence defect of *icl1*-deficient bacteria might instead be due to an inability to fully metabolize cholesterol.

We conclude that the Mce4 transporter likely represents the major cholesterol import system of *M. tuberculosis* because strains lacking either Mce4 proteins or MceG have a drastically reduced ability to acquire cholesterol *in vitro*, to metabolize this compound, and to grow when cholesterol is the primary source of carbon. Thus, it appears that Mce transporters represent essential sterol importers in both *M. tuberculosis* and *Rhodococcus* RHA1. However, $\Delta mce4$ mutants of *M. tuberculosis* retain some ability to use cholesterol (Figs. 1 and 2). At least under *in vitro* conditions, this residual uptake does not appear to involve the Mce1 transporter because the loss of this system has no effect on any of these abilities, even in a *mce4*-deficient strain. Thus, the residual cholesterol uptake seen in $\Delta mce4$ mutant bacteria could be due to another less efficient import system or may simply reflect passive diffusion into the cell.

For cholesterol transport to be important during intracellular growth, bacteria must have access to this compound. Our results indicate that bacteria reside predominantly in a cholesterol-rich region of the macrophage. Although the resolution of these studies precludes us from determining whether cholesterol-containing membranes are directly adjacent to the pathogen, it seems likely that mycobacteria have access to adequate local stores of cholesterol during intracellular growth. This compound may be even more abundant in tuberculosis lesions *in vivo*, where *M. tuberculosis* is often found within cholesterol-laden foamy macrophages or in extracellular spaces in which cholesterol is deposited as insoluble crystals (6).

Why is cholesterol import only required for growth in IFN- γ -stimulated macrophages? We hypothesize that, although cholesterol may be available in resting cells, it is not needed because other sources of carbon are available. Indeed, the mycobacterial phagosome resides in the recycling endocytic network in these cells, and the bacteria likely have access to numerous carbon sources. The requirement for cholesterol could result from the IFN- γ -induced maturation of the mycobacterial phagosome, which excludes the compartment from recycling endosomes (9, 37) and may deny the bacteria access to these more commonly used carbon sources. These observations support the concept that nutrient restriction is a specific IFN- γ -mediated defense mechanism and indicate that cholesterol utilization is one of the major strategies used by *M. tuberculosis* to circumvent this response.

Although no other pathogens are known to catabolize cholesterol, intracellular parasites interact with this compound for a variety of different purposes (38). Similarly, the degradation of

cholesterol by *M. tuberculosis* might play important roles other than providing carbon and energy. For example, the metabolism of cholesterol could alter the flux of carbon in the bacterium, which has important effects on the structure and abundance of bacterial components that are critical for virulence. Carbon sources that provide propionyl CoA units promote an increase in both the abundance and chain length of mycobacterial branched chain lipids, including PDIM and sulfollipid-1, by increasing the abundance of the biosynthetic precursor, methyl malonyl-CoA (36). The preferential incorporation of cholesterol side chain carbon into PDIM suggests that the flux of carbon into these lipids may be increased when the bacteria use this compound. In addition, sterol metabolism also could have important effects on the host by reducing the local concentration of membrane cholesterol (39), altering immunoregulatory sterols such as vitamin D3 (40) or corticosteroids (41), or producing novel secondary metabolites.

The ability to use sterols as a sole source of carbon is an unusual attribute that is known to be shared by only a handful of environmental organisms. *M. tuberculosis* clearly inherited this capacity from its saprophytic ancestors (25). Our data indicate that this trait was retained because it enabled the bacteria to acquire carbon and energy in an intracellular compartment that effectively starves other potential pathogens. Thus, the distinct lifestyles of different pathogens appear to be a reflection not only of the sum of their acquired virulence systems, but also of the metabolic legacy left by their nonpathogenic ancestors.

Materials and Methods

Bacterial Strains and Culture. *M. tuberculosis* mutants were derived from strain H37Rv and maintained on Middlebrook 7H10 agar or 7H9 broth supplemented with 10% OADC enrichment. Kanamycin or hygromycin was added at 20 or 50 $\mu\text{g/ml}$, respectively. The $\Delta mce1$, $\Delta mce4$, $\Delta mce114$, $\Delta mceG$, and $\Delta mceG + pmceG$ strains have been described (21). To complement the *mce4* mutation, the lox-flanked chromosomal hygromycin-resistance gene was excised by the expression of Cre recombinase. This strain was transformed with *pmce4* (GenBank accession no. DQ823233). For red fluorescent protein (RFP) expression, H37Rv was transformed with pMSP12::RFP (42). For growth on defined carbon sources, strains were grown in minimal medium (0.5 g/liter asparagine, 1 g/liter KH_2PO_4 , 2.5 g/liter Na_2HPO_4 , 50 mg/liter ferric ammonium citrate, 0.5 g/liter $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 mg/liter CaCl_2 , and 0.1 mg/liter ZnSO_4) containing either 0.1% glycerol (vol/vol) or 0.01% cholesterol (wt/vol). Before addition, cholesterol was dissolved at 100 mg/ml in a solution of Triton WR1339:ethanol (1:1) at 80°C. *M. tuberculosis* cannot use ethanol (43), and the combination of Triton WR1339 and ethanol did not support significant growth (Fig. 2, "no C source"). Cell densities were determined by using a Biowave cell density meter (ISC BioExpress) and reported after the subtraction of the OD₆₀₀ of the culture medium.

Mineralization Assay. *M. tuberculosis* strains grown in 7H9 media were washed twice with PBS and diluted in minimal medium containing 20 nCi/ml of [4-¹⁴C] or [26-¹⁴C]cholesterol at an OD₆₀₀ of 0.6 in a final volume of 5 ml in a sealed serum vial. Vials were incubated at 37°C for 4 h, and the release of ¹⁴CO₂ was quantified by using BACTEC TB-460 Instrument. Total activity of ¹⁴CO₂ was calculated by using the following formula: growth index \times 0.00025 = nCi ¹⁴C produced (as described by the manufacturer).

Cholesterol Import Assay. Strains were washed and diluted to an OD₆₀₀ of 1.0 in minimal medium containing 0.02 $\mu\text{Ci/ml}$ [4-¹⁴C]cholesterol. Aliquots were incubated at 37°C for various times, after which [¹⁴C]cholesterol uptake was terminated by adding PBST (PBS containing 0.1% Triton X-100) containing a 100-fold excess of unlabeled cholesterol. Cells were collected on 0.4 μm MCE filters (Millipore) and washed three times with PBST and once with 50% ethanol. [¹⁴C]cholesterol remaining on the filter was quantified by using a scintillation counter.

Lipid Analysis. Labeling, extraction, and analysis of lipids were performed essentially as described (44). Log-phase cultures were washed in PBS and resuspended in minimal medium containing either 0.1 μCi of [4-¹⁴C] or [26-¹⁴C]cholesterol or [3-¹⁴C]propionate. Cultures were incubated for 16 h at 37°C. Cell pellets were washed twice with water and extracted with either petroleum ether for apolar lipids (largely consisting of PDIM) or chloroform:methanol (2:1) for polar lipids. Extracts were dried under nitrogen and redissolved in their respective solvents. PDIM was resolved by thin-layer chromatography using glass-baked 250- μm -thick silica gel plates (Whatman) with three developments of petroleum ether:ethyl acetate (98:2). Radiolabeled species were detected by using a PhosphorImager.

Mouse and Macrophage Infections. C57BL/6 mice were infected with a 1:1 mixture of H37Rv and $\Delta mce4$ bacteria; $\approx 10^5$ total cfu were injected into the lateral tail vein in 200 μl of PBS containing 0.05% Tween 80 (PBST). At the indicated time points, groups of four mice were killed, the lungs were homogenized in PBST, and dilutions were plated on 7H10 agar to enumerate mutant (hygromycin-resistant cfu) or wild type (total cfu minus hygromycin-resistant cfu). Bone marrow-derived macrophages (BMMs) were isolated by culturing bone marrow cells from C57BL/6 mice in DMEM containing 10% FBS, 2 mM glutamine, 10% L929-conditioned medium, and 10 $\mu\text{g/ml}$ ciprofloxacin for 5 days; ≈ 24 h before infection, differentiated BMMs were detached and seeded on a 24-well tissue culture plate at 5×10^5 cells per well in the same medium lacking antibiotic. Macrophages were infected with different strains of *M. tuberculosis* at an moi of 1 for 4 h at 37°C. Extracellular bacteria were killed by incubating the cells with DMEM containing 100 $\mu\text{g/ml}$ amikacin for 1 h followed by washing three times with warm PBS. Intracellular bacteria were quantified by lysing the cells with PBST at the indicated time points and plating dilutions on 7H10 agar. The indicated samples were treated with 100 units/ml murine IFN- γ for 24 h before infection.

Confocal Microscopy. BMMs were plated in a Lab-Tek tissue culture chamber slides at 2×10^5 cells per well. When necessary, cells were stimulated with 100 units/ml murine IFN- γ for 24 h before infection. BMMs were infected with RFP-expressing H37Rv at an moi of 5 for 4 h. Subsequently, the cells were washed to remove extracellular bacteria and then incubated for another 24 h. Cells were washed and fixed in 4% paraformaldehyde (PFA) for 1 h at 25°C. After incubating with 0.15% glycine in PBS for 10 min at 25°C to quench the PFA, the cells were stained with 0.05% filipin for 2 h at 25°C. Images were acquired by using a Nikon TE2000-E2 microscope equipped with a Yokogawa CSU10 spinning disk confocal scan head. The resulting images were colored and merged by using ImageJ software (<http://rsb.info.nih.gov/ij/>).

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