Reductive Power Generated by *Mycobacterium leprae* Through Cholesterol Oxidation Contributes to Lipid and ATP Synthesis

Thabatta L. S. A. Rosa1†, Maria Angela M. Marques2†, Zachary DeBoard2, Kelly Hutchins2, Carlos Adriano A. Silva7, Christine R. Montague3, Tianao Yuan4, Julio J. Amaral5, Georgia C. Atella6, Patrícia S. Rosa7, Katherine A. Mattos8, Brian C. VanderVen3, Ramanuj Lahiri9, Nicole S. Sampson4, Patrick J. Brennan2, John T. Belisle2, Maria Cristina V. Pessolani1 and Marcia Berrêdo-Pinho1†

Upon infection, *Mycobacterium leprae*, an obligate intracellular bacillus, induces accumulation of cholesterol-enriched lipid droplets (LDs) in Schwann cells (SCs). LDs are promptly recruited to *M. leprae*-containing phagosomes, and inhibition of this process decreases bacterial survival, suggesting that LD recruitment constitutes a mechanism by which host-derived lipids are delivered to intracellular *M. leprae*. We previously demonstrated that *M. leprae* has preserved only the capacity to oxidize cholesterol to cholestenone, the first step of the normal cholesterol catabolic pathway. In this study we investigated the biochemical relevance of cholesterol oxidation on bacterial pathogenesis in SCs. Firstly, we showed that *M. leprae* increases the uptake of LDL-cholesterol by infected SCs. Moreover, fluorescence microscopy analysis revealed a close association between *M. leprae* and the internalized LDL-cholesterol within the host cell. By using *Mycobacterium smegmatis* mutant strains complemented with *M. leprae* genes, we demonstrated that *ml*1942 coding for 3β-hydroxysteroid dehydrogenase (3β-HSD), but not *ml*0389 originally annotated as cholesterol oxidase (ChoD), was responsible for the cholesterol oxidation activity detected in *M. leprae*. The 3β-HSD activity generates the electron donors NADH and NADPH that, respectively, fuel the respiratory chain and provide reductive power for the biosynthesis of the dominant bacterial cell wall lipids phthiocerol dimycocerosate (PDIM) and phenolic glycolipid (PGL)-I. Inhibition of *M. leprae* 3β-HSD activity with the 17β-[N-(2,5-di-t-butylphenyl)carbamoyl]-6-azaandrost-4-ene-3one (compound 1), decreased bacterial intracellular survival in SCs. In conclusion, our
findings confirm the accumulation of cholesterol in infected SCs and its potential delivery to the intracellular bacterium. Furthermore, we provide strong evidence that cholesterol oxidation is an essential catabolic pathway for M. leprae pathogenicity and point to 3β-HSD as a prime drug target that may be used in combination with current multidrug regimens to shorten leprosy treatment and ameliorate nerve damage.

Keywords: Mycobacterium leprae, cholesterol, cholestenone, PGL-I, PDIM, 3β-HSD, reductive power, oxidation

INTRODUCTION

Leprosy is a chronic infectious disease caused by Mycobacterium leprae, an obligate intracellular bacillus preferentially found in dermal macrophages and Schwann cells (SC) of peripheral nerves. Although effective treatment for leprosy has been a reality for decades, neural damage remains a common outcome of the disease, and is often irreversible (Lockwood and Sauderson, 2012). Despite the sterling ongoing efforts from the World Health Organization (WHO) to eliminate the disease using a 3-drug global control strategy, leprosy remains endemic in many regions of the world with almost 250,000 new cases of leprosy reported yearly. According to the WHO, Brazil represented 93% of all leprosy incidence in the Americas, and together with India and Indonesia, account for 79.6% of all the new cases detected globally (WHO, 2019). Although leprosy is a very ancient disease, an understanding of its pathogenicity is still limited, being hampered by the absence of experimental models and the failure to cultivate the pathogen in vitro. The inability to culture this bacterium in vitro is associated with dramatic reduction of genetic capability of M. leprae. M. leprae underwent reductive evolution throughout the eons losing one-half of its potential coding capacity, compared to other mycobacteria and conserving what is considered the minimal set of genes of a pathogenic Mycobacterium spp. (Cole et al., 2001).

Host lipids play an important role in M. leprae metabolism and mycobacterial infections in general (Kim et al., 2010). M. leprae stimulates lipid droplet (LD) accumulation in the host cell (Tanigawa et al., 2008; Mattos et al., 2010; Mattos et al., 2011a; Mattos et al., 2011b), generating the host cell foamy phenotype, which has been a hallmark of leprosy lesions since Virchow (Virchow, 1863). The host lipid droplets in foamy cells are rich in neutral lipids, such as triacylglycerols, cholesterol, and cholesterol esters and, indeed, cholesterol is one of the main lipids enriched in foamy cells during M. leprae infection (Mattos et al., 2010; Mattos et al., 2011a; Mattos et al., 2014). In fact, M. leprae infection in macrophages increases the expression of key enzymes in the cholesterol biosynthetic pathway, such as 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), as well as an enhanced uptake low-density lipoprotein (LDL) receptor (Mattos et al., 2014). Moreover, LDs are promptly recruited to M. leprae-containing phagosomes in infected SCs. Inhibition of this process decreases bacterial survival, suggesting that LD recruitment constitutes a mechanism by which host-derived lipids are delivered to intracellular M. leprae (Mattos et al., 2011a). Another potential source of cholesterol for M. leprae uptake in nerve cells was recently described. The leprosy bacillus accelerates myelin breakdown, a cholesterol-rich membrane. An inhibition of this phenomenon affected bacterial survival (Mietto et al., 2020). Additionally, inhibition of the host cell cholesterol biosynthesis through treatment with statins markedly decreased M. leprae survival in macrophages (Mattos et al., 2014) and reduced its survival in the Shepard mouse model of leprosy (Lobato et al., 2014), further suggesting a relevant role for cholesterol during in vivo growth of M. leprae.

Mycobacteria in general, including Mycobacterium tuberculosis, catabolize cholesterol as a carbon and energy source (van der Geize et al., 2007; Pandey and Sassetti, 2008; Thomas et al., 2011a; Uhia et al., 2011). This is not the case for M. leprae, as genome sequencing predicted that all genes involved in cholesterol catabolism are non-functional pseudogenes with the one exception: the enzyme responsible for the oxidation of cholesterol to cholest-4-en-3-one (cholestenone), the first step of sterol ring degradation (Cole et al., 2001). Indeed, by using 14C-labeled cholesterol derivatives, it was elegantly demonstrated that the cholesterol carbon atoms from both the sterol framework and the aliphatic side chain are not used by M. leprae for lipid synthesis and CO2 generation (Marques et al., 2015). However, M. leprae avidly incorporates cholesterol, converting it to cholestenone both in vitro and in vivo (Marques et al., 2015). In the same study, 3β-hydroxysteroid dehydrogenase (3β-HSD; encoded by hsd, ml1942) was regarded as the most likely candidate responsible for this step (Marques et al., 2015) based on its high homology with M. tuberculosis 3β-HSD, which displays cholesterol oxidizing activity (Yang et al., 2007; Yang et al., 2011). A second enzyme, cholesterol oxidase (gene is named choD) is annotated as a putative cholesterol oxidase in several mycobacterial genomes based on homology with the well-characterized cholesterol oxidases from Streptomyces and Rhodococcus (Navas et al., 2001; Brzostek et al., 2007). Since choD (ml0389) is also present in the ML genome, this enzyme remained as a possible source of cholesterol oxidation.

3β-HSD represents an extensive family of enzymes that oxidizes steroid substrates using pyridine nucleotides (NAD+ or their phosphorylated forms (NADP+) as electron acceptors (Lachance et al., 1990). In mammals this enzyme is implicated in the generation of steroid hormones, and utilizes as substrates pregnenolone and androstenedione, among others (Simard et al., 2005). The cofactors and their reduced forms, NADH and NADPH, are essential for basic catabolic and anabolic metabolism, respectively. NADPH is a crucial reductant used in lipid anabolism including synthesis of important components
of the mycobacterial cell wall (Minnikin et al., 2002). The 3β-HSD of *M. tuberculosis* is described as using cholesterol as the substrate and NAD*+* as the main enzyme cofactor, and could be inhibited by trilostane [(2R,4R,5R,17α)-4,5-epoxy-17-hydroxy-3-oxoandrostane-2-carbonitrile] (Yang et al., 2007), an androstenediol that is often used as an inhibitor of mammalian 3β-HSD (Potts et al., 1978). More recently, new compounds from a different class of steroids, known as 6-azaandrost-4-en-3-ones (Frye et al., 1993), were generated as specific inhibitors of *M. tuberculosis* 3β-HSD. Some analogs have been shown to effectively inhibit *M. tuberculosis* 3β-HSD, including 17β-[N-(2,5-di-t-butylphenyl)carbamoyl]-6-azaandrost-4-en-3-one (compound 1), which has emerged as a powerful tool for better characterization of mycobacterial 3β-HSD (Thomas et al., 2011b). The capacity of compound 1 to inhibit the enzymatic activity of the recombinant *M. tuberculosis* 3β-HSD (IC50 of 23 µM) was demonstrated (Thomas et al., 2011b).

Accordingly, in this present work, we investigated the biochemical relevance of cholesterol oxidation in *M. leprae*-SC interaction. Firstly, we confirmed that 3β-HSD, not ChoD, is the enzyme responsible for cholesterol oxidation in *M. leprae*. Of note, we showed that 3β-HSD activity generates electron donors that may be used by *M. leprae* for ATP synthesis and for the biosynthesis of key cell wall lipids, and that *M. leprae* 3β-HSD inhibition with the compound 1 decreased bacterial intracellular survival in SCs. We conclude from this study that oxidation of cholesterol to cholestenone is a crucial metabolic activity of *M. leprae* and, in contributing to its intracellular survival in SCs, is implicated in nerve colonization and subsequent damage.

### MATERIAL AND METHODS

#### Mycobacterial Strains and Human Schwann Cell Culture

*M. leprae*, the Thai-53 strain, was obtained from the hind footpad of athymic nu/nu mice, generated at Instituto Lauro de Souza Lima, Bauru, SP, Brazil, and isolated as previously described (Trombone et al., 2014) or prepared at the National Hansen’s Disease Program, Laboratory Research Branch, Louisiana State University, Baton Rouge, LA. Axenic media for *M. tuberculosis* strain mc²6230 culture and for *M. leprae* experiments were previously described (Marques et al., 2015). *M. smegmatis* strain mc²155 and mutant strains were grown at 37°C in an orbital shaker at 250 r.p.m. in 457 minimal salt medium (Uhía et al., 2011) containing 0.05% tyloxapol (457 MSM-Ty) and complemented mutants were grown on LB broth plus 0.05% tyloxapol (LB-Ty) with 100 µg/ml hygromycin and 25 µg/mL kanamycin as required.

Human SC from ST88-14 tumor cell line originated in a malignant schwannoma, isolated from type 1 neurofibromatosis patients, was kindly donated by Dr. J. A. Fletcher (Dana Farber Cancer Institute, Boston, MA). Cells were cultured in RPMI 1640 media (ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal calf serum (FCS) (Crippion Biotecnologia, Andradina, Brazil) and maintained at 37°C in 5% CO₂ atmosphere.

### Genetic Manipulation of *M. smegmatis*

All genetic deletions were generated in *M. smegmatis* mc²155. The *amsmeg_1604* mutant strain was engineered using mycobacterial recombineering as described (Van Kessel and Hatfull, 2008). Briefly, bacteria containing the recombineering plasmid (pJV53) were induced with 0.2% acetamide and electroporated with a linear recombineering substrate to insert a lox-hygromycin-lox cassette into the *msmeg_1604* ORF at 731 bp. The lox-flanked hygromycin resistance cassette was excised by expressing the Cre recombinase from the plasmid (pTL7bv). The *amsmeg_5228* mutant strain was created using the mycobacterial ORBIT method as described (Murphy et al., 2018). Briefly, bacteria containing the ORBIT plasmid (pKM444) were induced with 500 ng/mL anhydrotetracycline and electroporated with a payload vector (pTL11-zeo) and a targeting oligonucleotide to replace the entire *msmeg_5228* ORF sequence (1–1071 bp) with a lox-zeocin-lox cassette. The lox-flanked zeocin resistance cassette was excised by expressing the Cre recombinase from the plasmid (pTL7bv). The *amsmeg_1604Δamsmeg_5228* double mutant was generated by recombineering. The unmarked *amsmeg_5228* mutant containing the plasmid (pJV53) was induced with 0.2% acetamide and electroporated with a linear substrate to insert a lox-hygroycin-lox cassette into the *msmeg_1604* ORF at 731 bp. All engineered mutations were verified with Sanger sequencing.

The *M. leprae* gene *ml1942* was synthesized by GenScript (Piscataway, NJ) generating an optimized sequence containing Ndel and HindIII restriction sites at 5’ and 3’ ends, respectively. The *ml1942* construct was ligated into the expression vector pST-KT (Parikh et al., 2013) digested with the same enzymes generating the recombinant plasmid pMRLB121. All Strains and plasmids used in this study are presented in Table 1.

The *M. leprae* gene *ml0389* (choD) was PCR amplified from *M. leprae* genomic DNA with Q5 DNA polymerase (New England Biolabs Inc., Beverly, MA). PCR amplification was performed with the forward primer 5’-ggagaaggggaggtattctgctc-3’ and a reverse primer 5’-gacagggaggggaggtattctgctc designed to introduce Ndel and HindIII sites to the 5’ and 3’ ends, respectively. The *ml0389* PCR product was ligated into pGEM®-T Easy Vector (Promega, Madison, WI) and transformed into competent *Escherichia coli* TOP10 cells. The *ml0389* gene was subcloned into the expression vector pST-KT resulting in the recombinant plasmid pMRLB122. The sequences of the cloned *ml0389* and *ml1942* genes were confirmed by automated nucleotide sequencing. Freshly made competent *M. smegmatis* cells (200 µL) were electroporated with 200 to 400 ng of plasmid DNA. Transformed cells were plated on LB agar containing 100 µg/ml hygromycin and 25 µg/mL kanamycin and incubated at 37°C for 3 days.

### Metabolic Labeling and Assays

To assess recombinant 3β-HSD activity, *M. smegmatis* strains were cultured in 10 mL of LB-Ty broth or 457 MSM-Ty. *M. smegmatis* mutant strain *Δmsmeg_1604Δmsmeg_5228* complemented with pMRLB121 or pMRLB122 was grown to an optical density at 600 nm of 0.6, induced with 50 ng/mL of anhydrotetracycline (ATc), and further incubated for
TABLE 1 | Bacterial strains and plasmids used in this study.

| Strain or plasmid       | Selectable phenotype | Genotype and/or description                                                                 | Source or reference       |
|-------------------------|----------------------|---------------------------------------------------------------------------------------------|--------------------------|
| **Mycobacterium smegmatis** |                      |                                                                                             |                          |
| None                    |                      | M. smegmatis mc2^155 Δmsme_1604                                                            | This study               |
| None                    |                      | M. smegmatis mc2^155 Δmsme_5228                                                            | This study               |
| Hyg<sup>R</sup>         |                      | M. smegmatis mc2^155 Δmsme_1604/Δ msme_5228 complemented with pMRLB121                     | This study               |
| Hyg<sup>R</sup>, Kan<sup>R</sup> |                      | M. smegmatis mc2^155 Δmsme_1604/Δ msme_5228 complemented with pMRLB122                     | This study               |
| **Escherichia coli**    |                      |                                                                                             |                          |
| Top 10                  |                      | F-mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔm15 ΔacX74 recA1 araD139 ΔaraE4u7697 galU galK rpsL (StrR) endA1 nupG | Invtrogen                |
| **Plasmid**             |                      |                                                                                             |                          |
| pGEM<sup>T</sup>-E Easy Vector | Amp<sup>R</sup> | Cloning vector                                                                             | Promega                  |
| pST-KT                  | Kan<sup>R</sup>     | E. coli-mycobacteria shuttle vector                                                         | Addgene                  |
| pMRLB121                | Kan<sup>R</sup>     | Expression vector, UV15 promoter                                                            | This study               |
| pMRLB122                |                      |                                                                                             | This study               |

approximately 14 h. Cells were washed and suspended with 200 µL 457 MSM-Ty minus glycerol containing 1 µCi/mL of [4,14C] cholesterol (American Radiolabeled Chemicals, Inc., Saint Louis, MO), and incubated at 37°C for 30 min or 2h. Radiolabeled bacteria were separated from spent medium and washed as described previously (Marques et al., 2015). Radiolabeled compounds were resolved by thin-layer chromatography (TLC) using silica gel G60 TLC plates (Millipore, Temecula, CA) developed in petroleum ether-ethyl acetate (1:1) and detected using a Phosphor Imager Typhoon 9400 scanner (GE Healthcare, Sunnyvale, CA). Gene deletion and M. leprae genes complementation were assessed by qRT-PCR. Briefly, RNA was extracted using TRIzol reagent (Thermo) as recommended by the manufacturer after breaking the mycobacteria in tubes extracted using TRizol reagent (Thermo) as recommended by the manufacturer after breaking the mycobacteria in tubes. Genomic DNA was obtained using Genomic DNA isolation kit (Qiagen, Germany). Gene deletion and M. leprae genes complementation were assessed by qRT-PCR. Briefly, RNA was extracted using TRIzol reagent (Thermo) as recommended by the manufacturer after breaking the mycobacteria in tubes. Genomic DNA was obtained using Genomic DNA isolation kit (Qiagen, Germany). The purity of the compound was assessed by NMR and HPLC to be >99% pure.

**Measurement of NAD<sup>+</sup>, NADP<sup>+</sup> and Cytochrome C Reduction**

The impact of 3β-HSD inhibition on NAD<sup>+</sup>, NADP<sup>+</sup> and cytochrome C reduction was investigated using M. leprae whole cell lysate (WCL) (NR-19329) obtained from BEI Resources. Proteins were quantified in the lysates using Bradford protein assay (Biorad, Hercules, CA).
(Bradford, 1976). The equivalent of 50 µg of protein from the WCL was used in the reaction performed in HBSS medium (ThermoFisher Scientific). WCL was incubated with either 200 µM NAD⁺ or 200 µM NADP⁺ (MilliporeSigma, St Louis, MO) in the presence of 200 µM cholesterol (MilliporeSigma) and treatment with or without 100 µM of compound 1. An assay of NAD⁺ and NADP⁺ reduction was performed every 30 sec for 20 min via absorbance measurement at 340 nm in an EON microplate spectrophotometer (BioTek Instruments, Inc. Winooski, VT). Similarly, the reduction of cytochrome C was measured at an absorbance of 550 nm.

**Schwann Cell Cholesterol Incorporation Assay**

SCs were cultured in 24-well plates (Corning, Corning, NY) at a density of 5 x 10⁴ cells per well for flow cytometry and placed onto round 12 mm glass slips for confocal microscopy. Prior to *M. leprae* infection, the medium was replaced by fresh RPMI medium supplemented with 2% fetal calf serum. SCs were infected at a MOI of 50:1 with live *M. leprae* or stimulated with irradiated *M. leprae*, both fluorescently labeled with PKH26 (MilliporeSigma) according to manufacturer’s instructions. Infected or stimulated cultures were maintained at 33°C in 5% CO₂ for 48 h. Cells were washed three times with PBS for 5 min and fresh RPMI medium without serum supplementation containing 0.1 mg/mL [BODIPY-Cho]-LDL (BODIPY 493/503, ThermoFisher) was added for labeling as described previously (Mattos et al., 2014). Flow cytometry was performed with the FACS Calibur Flow cytometer (BD, Holdrege, NE). For confocal microscopy the fixed cells were labeled with 2 µM DAPI (MilliporeSigma) for 1 min, washed with PBS and slides were prepared using Vectashield mounting medium (Vector Laboratories Inc, Burlingame, CA). Images were obtained with a LSM 510 Confocal Microscope (Zeiss, Oberkochen, Germany).

**Measurement of *M. leprae* Viability**

For *M. leprae* intracellular viability, bacilli were incubated in RPMI medium supplemented with 0.05% BSA and 2% DMSO, with or without 100 µM compound 1, for 6 h at 30°C. *M. leprae* was collected by centrifugation at 16,000 x g, suspended in fresh RPMI medium and used to infect 1 x 10⁵ SCs at an MOI of 5:1. The infected SCs were incubated at 33°C for 24 h with 5% CO₂. RNA was extracted using TRIzol reagent (ThermoFisher) as per manufacturer’s instructions. DNA was extracted after RNA isolation using 150 µL of chloroform and 100 µL of Tris-EDTA buffer (200 mM Tris 5 mM EDTA, pH 8) as described (Martinez et al., 2009). Isolated RNA samples were subjected to DNase treatment using the Turbo DNA-free kit (ThermoFisher). cDNA was reverse transcribed from 500 ng of RNA using the GoScript kit (Promega) and diluted to 5 ng/µL. *M. leprae* viability was determined by qRT-PCR using *M. leprae* rRNA 16s gene as molecular target as described (Martinez et al., 2009; De Toledo-Pinto et al., 2016).

To evaluate SC viability in the same experiments, 5 mg/mL of Thiazolyl Blue Tetrazolium Bromide (MTT) (MilliporeSigma) was added to each well 4 h prior to the end of the incubation period. Crystals were suspended in 100 µL 10% SDS and read at absorbance 590 nm using an EON microplate spectrophotometer (BioTek Instruments, Inc).

**RESULTS**

*M. leprae* Increases the Uptake of LDL-Cholesterol by Infected Schwann Cells

Since the extracellular uptake of plasma lipoprotein-derived cholesterol (LDL-Cho) constitutes an important cellular lipid source, we analyzed whether *M. leprae* infection modulates LDL-Cho acquisition by SC. This pathway was investigated by monitoring the cellular internalization of fluorescent LDL. Cells were treated with dead or infected with live PKH-26-labeled *M. leprae*, pulse-labeled with LDL and lipid uptake was analyzed by flow cytometry. In comparison to the uninfected SC or SC treated with dead bacilli, LDL uptake increased in *M. leprae*-infected SC (MFI of 29.44 ± 2.403 in *M. leprae*-infected cultures and 20.05 ± 3.631 in dead *M. leprae*-treated (P=0.0252) (Figure 1A)). Moreover, LDL uptake was significantly higher in SC bearing fluorescent-labeled bacteria in comparison to SC of the same culture that were devoid of bacteria or SC containing dead bacteria (Figure 1B). These data were confirmed by immunofluorescence images that clearly showed intense fluorescence of LDL-[Cho] (in green) in cells bearing live *M. leprae* (in red) when compared to uninfected cells and to cells treated with dead bacilli (Figure 1C, D). These images also revealed a close association between *M. leprae* and the just incorporated LDL-cholesterol in the host cell (Figure 1D).

**3β-HSD Is the Enzyme in *M. leprae* Responsible for Converting Cholesterol to Cholestenone**

The capacity of *M. leprae* to incorporate cholesterol and convert it to cholestenone both in vitro and in vivo was previously demonstrated (Marques et al., 2015). To definitively establish the role of *M. leprae* 3β-HSD in the formation of cholestenone, *M. smegmatis* was used as a surrogate since genetic and functional studies are impaired by the inability to propagate *M. leprae* in vitro. Specifically, the msme₅₂₂₈ and msme₅₁₉₄ genes encoding the *M. smegmatis* 3β-HSD and ChoD homologues, respectively, were knocked out to generate the single and double mutant strains (Δmsme₅₂₂₈, Δmsme₅₁₉₄ and Δmsme₅₂₂₈/Δmsme₅₂₂₈). The utilization of [4-¹⁴C] cholesterol by these strains revealed that, as expected, the production of cholestenone was a function of the msme₅₂₂₈ gene product (3β-HSD), but not msme₅₁₉₄ (ChoD) (Supplementary Figure S1). To confirm that 3β-HSD produced by *M. leprae* also converts cholesterol to cholestenone, the mll₁₉₄₂ gene was expressed in the *M. smegmatis* Δmsme₅₁₉₄/Δmsme₅₂₂₈ double mutant. As shown in Figure 2A, the production of cholestenone by the *M. smegmatis* double mutant was restored by the complementation with mll₁₉₄₂ (3β-HSD). In contrast, cholestenone formation was not restored by complementation with ml₅₀₈₃ (choD). Supplementary...
Figures S2A, B, respectively, show that the *M. smegmatis* double mutant did not express the endogenous 3β-HSD and ChoD proteins, but was successfully complemented with the *ml1942* and *ml0389* genes, expressing the respective *M. leprae* 3β-HSD and ChoD homologues.

To further confirm the involvement of *M. leprae* 3β-HSD in cholesterol oxidation, we tested compound 1 (Figure 2B); an effective inhibitor of *M. tuberculosis* 3β-HSD (Thomas et al., 2011b). Since *M. leprae* 3β-HSD shares 85% similarity to the *M. tuberculosis* homologue, our expectation was that compound 1 would also inhibit the *M. leprae* enzyme. Live *M. leprae* was incubated in axenic medium with 1µCi/mL of [26-14C] cholesterol and 1µCi/mL of [1-14C] palmitic acid in the presence of increasing concentrations of compound 1. After 48 h, [1.14C] palmitic acid β-oxidation was measured by 14CO2 production (radiorespirometry) as an indicator of bacterial viability (Franzblau, 1988) and the impact on cholestenone production was analyzed by TLC. We observed approximately 50% inhibition in cholestenone production in bacilli treated with 100 µM or greater concentrations of compound 1 (Figure 2C), with a minimal effect on bacterial viability up to a concentration of 200 µM (Figure 2D). Compound 1 treatment also decreased cholestenone production by *M. smegmatis* Δmsmeg_1604/Δmsmeg_5228:ml1942 (Supplementary Figure S3). Together these data confirm previous prediction that 3β-HSD is the sole enzyme responsible for cholestenone production in *M. leprae*. Moreover, our data revealed that, like in other mycobacteria, *ml0389* was incorrectly assigned to encode a cholesterol oxidase.

### 3β-HSD Inhibition Affects *M. leprae* Intracellular Survival

To investigate whether 3β-HSD activity is essential for *M. leprae* during infection, the impact of inhibition of the enzyme on bacterial intracellular survival was analyzed. Since mammalian cells also express proteins of the 3β-hydroxysteroid dehydrogenases superfamily, the bacteria were pretreated with compound 1, the inhibitor removed and SCs then infected in order to avoid potential effects on the host cell enzymes. This pretreatment of bacilli with the 3β-HSD inhibitor for 6 h accelerated bacterial killing by 30% after 24 h of infection (Figure 3A) and did not cause SC death (Figure 3B). The assay in axenic medium shown in Figure 3C confirms that bacterial viability is not affected by the drug after 24 h as already demonstrated in Figure 2D for an even longer time.
This assay also gives support to the result observed in Figure 3A. It shows that even removing compound 1 after 6 hours of incubation, inhibition of cholestenone production by *M. leprae* was sustained for the next 24 h (Figure 3D), reinforcing that the decrease in *M. leprae* intracellular viability observed in Figure 3A can be attributed to 3β-HSD inhibition. This data set strengthens the importance of cholesterol metabolism during *M. leprae* host cell interaction and brings attention to 3β-HSD as a new important element of this interaction.

**Cholesterol Oxidation by *M. leprae* 3β-HSD Generates NADH and NADPH**

3β-HSD is a member of a family of enzymes that catalyzes an oxidation reaction with the consequent reduction of NAD⁺ to NADH or NADP⁺ to NADPH. *M. tuberculosis* 3β-HSD uses NAD⁺ instead of NADP⁺ (Yang et al., 2007) and *M. leprae* 3β-HSD has conserved the *M. tuberculosis* NAD⁺ binding motif homologue (Marques et al., 2015), suggesting that it also uses this cofactor. To test whether the catabolism of cholesterol could lead to the reduction of NAD⁺/NADPH in *M. leprae*, a WCL of *M. leprae* was incubated with NAD⁺ or NADP⁺ in the presence or absence of cholesterol and reduction of the cofactors was measured spectrophotometrically by absorbance at 340 nm. As shown in Figures 4A, B, addition of cholesterol (blue curves) resulted in an increased generation of both NADH and NADPH, as compared to the levels observed in the absence of cholesterol (black curves). Moreover, blocking the 3β-HSD activity with compound 1 in the presence of cholesterol decreased both NADH and NADPH to levels close to or below the basal levels (red curves), reinforcing the fact that 3β-HSD is responsible for the increased reduction of these cofactors in the presence of cholesterol. Importantly, compound 1 was unable to affect the generation of NADPH when substrates for other dehydrogenases, such as citrate, acetate, or glyceraldehyde, were added to the cell extract (Supplementary Figure S4), confirming that compound 1 specifically inhibits the oxidation reaction catalyzed by 3β-HSD.

NADH represents a major electron donor feeding the respiratory chain; therefore, the NADH molecules generated by 3β-HSD activity could supply, at least in part, electrons to the oxidative respiratory chain contributing to *M. leprae* ATP synthesis. To check this possibility, the WCL of *M. leprae* was
incubated in the presence or absence of cholesterol plus NAD\(^+\) and the reduction of cytochrome C was measured by absorbance at 550 nm (Figure 4C). An increased level of reduced cytochrome C was observed in the presence of cholesterol plus NAD\(^+\) (blue curve) as compared to baseline levels (black curve). Moreover, a partial decrease in this phenomenon was observed by blocking 3\(\beta\)-HSD activity with compound 1 (red curve). As expected, when NAD\(^+\) was replaced by NADP\(^+\) in identical assays no reduction of cytochrome C was observed (data not shown), emphasizing the specific participation of NAD\(^+\) in this process. These results suggest that cholesterol oxidation with the generation of electron donors can contribute to the \(M.\ leprae\) electron transport chain and, presumably, ATP generation.

**Cholesterol Oxidation by 3\(\beta\)-HSD Increases PGL-I and PDIM Synthesis**

Mycobacteria are enveloped by a notably complex cell wall structure, and many lipids are associated with the external

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**FIGURE 3** | \(3\beta\)-HSD inhibition impacts \(M.\ leprae\) intracellular survival. (A, B) ST88-14 SCs were infected (MOI 5:1) for 24 h at 33°C with \(M.\ leprae\) pre-treated for 6 h with vehicle (2% DMSO) or 100 µM compound 1. (A) \(M.\ leprae\) intracellular viability was determined by qRT-PCR. (*p < 0.05 - by Mann-Whitney test)(n=5) (B) SC viability was measured by MTT assay (n=3). (C, D) \(M.\ leprae\) was incubated in axenic medium with 100 µM compound 1, 1µCi/mL of [26-\(^{14}\)C]cholesterol and 1µCi/mL of [1-\(^{14}\)C]palmitic acid for 6 h at 30°C followed by centrifugation and wash and removal of compound 1 (wash off, +) or replacement with fresh medium containing compound 1 (wash off, -), and incubation for 24 h at 33°C. (C) \(M.\ leprae\) viability was measured by radiorespirometry (n=1). (D) Radiolabeled lipids were resolved by TLC with chloroform-methanol 95:5 (v/v) as mobile phase and observed with a PhosphorImager. The graph indicates corresponding densitometry of cholestenone bands (n=1).

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**FIGURE 4** | \(M.\ leprae\) 3\(\beta\)-HSD is a source of reductive power. (A, B) NAD\(^+\) and NADP\(^+\) reduction to NADH and NADPH, respectively, by 3\(\beta\)-HSD activity was measured in a temporal kinetic curve every 30 s for 20 min at 340 nm. (C) Cytochrome C reduction was determined measuring the reduced form of Cytochrome C at 550 nm every 30 s for 20 min. In all conditions \(M.\ leprae\) WCL was incubated with 200 µM cholesterol, alone (blue) or in the presence of 100 µM compound 1 (red). A condition without cholesterol addition was also included as a control of basal levels (black). Representative of 3 independent experiments.
leaflet. Primary examples of these lipids are the PDIM and PGLs (PGLs are phenolphthiocerol-based glycolipids that contain in its structure PDIM) (Marques et al., 1998). These components are polyketide-derived virulence factors, biosynthesis of which relies on electron donors, mainly NADPH. Since cholesterol oxidation generates reductive power, we investigated a potential link between 3β-HSD activity and bacterial lipid biosynthesis, focusing mainly on PDIM and PGL-I. The initial analysis of lipids produced in the presence of [26-14C]cholesterol and [1-14C]palmitic acid, demonstrated that incubation of M. leprae for 48 h with increasing concentrations of compound 1 resulted in decreased production of PGL-I and PDIM (Figures 2C and 5A). A densitometric analysis of these TLC bands confirmed that compound 1 inhibited lipid biosynthesis in a dose-dependent manner (Figures 5B, C). The inhibition of PDIM biosynthesis, when 3β-HSD was blocked, was further confirmed by two-dimensional TLC analysis of lipid extracts of M. leprae incubated with or without compound 1 (Figure 5D). To evaluate whether compound 1 could act off-target (i.e. decrease lipid biosynthesis by mechanisms independent of 3β-HSD catalyzed cholesterol oxidation and consequent reduction of NADP+), M. tuberculosis was incubated with [1-14C]palmitic acid in the absence of cholesterol and treated or not with 100 µM compound 1, followed by measuring radiolabeled PDIM synthesis by TLC. PDIM synthesis was not affected by the presence of compound 1 (Supplementary Figure S5). Altogether, these results suggest that the reductive equivalents produced by cholesterol oxidation contribute to the biosynthesis of key M. leprae lipids.

**DISCUSSION**

A key aspect of M. leprae pathogenesis is its capacity to exploit host cell lipid metabolism, leading to the accumulation of cholesterol-enriched lipid droplets in the cytoplasm of infected cells (Virchow, 1863; Mattos et al., 2010; Mattos et al., 2011a; Mattos et al., 2014). In contrast to other mycobacteria that can fully degrade cholesterol, M. leprae has preserved only the capacity to oxidize cholesterol to cholestenone, the first step in cholesterol catabolism (Marques et al., 2015). In this study we explored the relevance of cholesterol oxidation in the context of M. leprae-SC interaction. Firstly, we showed that infected SCs display a higher capacity to import LDL, which colocalizes with intracellular bacilli, explaining, at least in part, M. leprae-induced cholesterol accumulation in the host cell. By using M. smegmatis mutant strains complemented with M. leprae genes, we confirmed that 3β-HSD (ml1492), but not ChoD (ml0389), is the enzyme responsible for cholesterol oxidation. Of note, treating M. leprae with compound 1, an inhibitor of 3β-HSD, decreased bacterial intracellular survival. Exploring the potential biochemical roles of this enzyme in M. leprae metabolism, we found that the reductive power generated by cholesterol oxidation can fuel the respiratory chain and potentially impact

**FIGURE 5** | Reductive power generated by 3β-HSD supports PGL-I and PDIM synthesis. (A) TLC of lipid extracts from M. leprae treated with compound 1, representative of 4 experiments. TLC was resolved using chloroform-methanol 95:5 (v/v) as mobile phase. Standards: PDIM, cholesterol, cholestenone, palmitic acid and PGL-I. Corresponding densitometry of PGL-I (B) and PDIM (C) (*p < 0.05 - ANOVA test followed by Bonferroni post-test were performed for statistical analyses (n=4). (D) To confirm the apolar product labeled as PDIM in (A) was truly PDIM, 2-dimensional TLC analyses of the lipid extract from M. leprae treated or not with 80 µM compound 1 was performed.
microbial ATP synthesis. In addition, 3β-HSD inhibition in turn inhibits the biosynthesis of the bacterial cell wall lipids PDIM and PGL-I. Altogether, our data suggest that the reductive power generated by cholesterol oxidation plays an essential role in M. leprae biology inside SCs, contributing to ATP and lipids synthesis.

The higher capacity of infected SCs to incorporate LDL-[Cho] suggests that M. leprae induces exogenous cholesterol uptake via upregulation of LDL receptors on the host cell surface. This same strategy was previously shown to be employed by M. leprae to induce cholesterol accumulation in macrophages (Mattos et al., 2014). Moreover, we observed an intimate physical association between the just incorporated LDL and intracellular M. leprae, in agreement with previous data showing the prompt recruitment and co-localization of LDs with internalized bacteria (Mattos et al., 2011a; Mattos et al., 2014). Altogether, these data reinforce the idea that host-derived lipids, such as cholesterol, promptly delivered to M. leprae containing phagosomes upon infection constitute a nutritional source essential for bacterial survival and persistence inside the host cell.

Our results demonstrated that only 3β-HSD of M. leprae, but not ChoD, was responsible for cholestenone production. These results were consistent with those obtained in the study of M. tuberculosis (Yang et al., 2011) and M. smegmatis (Ivashina et al., 2012). In fact, different studies have suggested an alternative role for ChoD during M. tuberculosis infection, connecting its importance to bacterial virulence with its capacity to down modulate the innate immune response through TLR-2 signaling pathway in infected macrophages (Brzostek et al., 2007; Klink et al., 2013). However, the unknown relevance of M. leprae retaining this single activity of cholesterol catabolism led us to investigate whether inhibition of 3β-HSD affected the viability of M. leprae. The treatment of M. leprae with the 3β-HSD inhibitor, compound 1 decreased cholestenone production; a result corroborated by compound 1 inhibition of cholestenone formation in the M. smegmatis Δmsmeg_1604/Δmsmeg_5228 complemented with 3β-HSD of M. leprae. More importantly, treatment of M. leprae with compound 1 prior to infection of SCs resulted in decreased intracellular survival of the bacilli. These data provide strong support for the hypothesis that M. leprae utilizes cholesterol for intracellular survival (Mattos et al., 2014) and point to 3β-HSD as the linchpin for cholesterol dependent intracellular survival.

We hypothesized that the mechanism of M. leprae cholesterol dependent intracellular survival is rooted in the fact that 3β-HSD utilizes NAD+ as a cofactor. Indeed, the addition of cholesterol to WCL of M. leprae increased the reduction of NAD+, and this NAD+ reduction was inhibited by compound 1, reinforcing the specific involvement of 3β-HSD. Further evidence for the importance of NAD+ reduction by 3β-HSD was the observation that cholesterol plus NAD+ increased cytochrome C reduction when added to WCL of M. leprae, and blockage of 3β-HSD activity with compound 1 inhibited cytochrome C reduction. This suggests that the NADH molecules generated by 3β-HSD activity can fuel M. leprae electron transport chain contributing to ATP synthesis. Based on the genome sequencing data, it was originally suggested that M. leprae was unable to perform oxidative phosphorylation due to the lack of crucial genes of this pathway, especially genes that encode the NADH oxidase complex, which were found to be truncated (Cole et al., 2001). However, more recently it was reported that type II NAD dehydrogenase (NDH-2), encoded by the ndh gene (rv1854c) in M. tuberculosis, is involved in ATP synthesis (Rao et al., 2008). The ndh gene is present in the M. leprae genome (ml2061) and codes for a 466 amino acid protein sharing 90.2% similarity with the M. tuberculosis orthologue (Cole et al., 2001). Therefore, NADH produced by 3β-HSD activity could supply, at least in part, electrons to the oxidative respiratory chain.

Besides reduction of NAD+, M. leprae WCL incubated with cholesterol also generated NADPH, and this could be partially inhibited with compound 1. The M. leprae 3β-HSD shares high homology with the M. tuberculosis ortholog. However, the 3β-HSD of M. tuberculosis exclusively uses NAD+ as a cofactor and does not reduce NADP+. This cofactor-selectivity is suggested to be the result of an aspartate residue in position 45 of the primary amino acid sequence (Yang et al., 2007). Interestingly, M. leprae 3β-HSD also possesses an aspartate residue in the same position, suggesting that this enzyme would also use NAD+ exclusively as a cofactor (Marques et al., 2015). Thus, the inhibition of NADPH formation by compound 1 in the M. leprae extracts, could be an indirect consequence of NADH conversion to NADPH by other enzymes present in the bacterial extract. Prokaryotes present canonical and non-canonical reactions involved in the production and regeneration of NADPH. A NAD kinase (nicotinamide adenine dinucleotide kinase, NADK) is the known enzyme that generates NADP (H) by phosphorylating NAD (H) in almost all living organisms (Mori et al., 2005; Grose et al., 2006). As the major producer of NADP (H), NADK plays vital roles in maintaining the balance between NAD (H) and NADP (H) in NADP (H) -based cellular metabolic pathways (Kawai and Murata, 2008). The functionality of the NADK enzyme, encoded by the ppnK gene, has already been described in M. tuberculosis (Kawai et al., 2000) and a homologous gene, ml3159, is preserved in the M. leprae genome. There are also transhydrogenases that directly catalyze the reversible hydride transfer between NAD (H) and NADP (H). The M. leprae genome encodes two different isoforms of this enzyme, the energy-independent soluble transhydrogenase (STH) encoded by ml1012c and the energy-dependent, or proton-translocating, membrane-bound transhydrogenase (H + -TH) encoded by ml2634c or ptnb. Although ml1012 is a pseudogene in M. leprae, the ptnb is predicted to be functional and could contribute to the formation of NADPH from NADH. In E. coli the H + -TH has been shown to provide about 40% of the total NADPH during growth on glucose (Sauer et al., 2004). Therefore, these enzymes could contribute to the pool of NADPH from the NADH generated by the action of 3β-HSD.

We observed that inhibition of 3β-HSD resulted in decreased synthesis of PGL-1 and PDIM, two major constituents of M. leprae cell envelope, suggesting that the reductive power generated by 3β-HSD activity is being directed to lipid synthesis. This doesn’t seem to be an off-target effect of
compound 1 treatment, since no alteration was observed in PDIM synthesis when *M. tuberculosis* was treated with the drug. This was an expected result as, in contrast to *M. leprae*, *M. tuberculosis* has a more versatile metabolic armament with the ability to generate reducing equivalents from various nutritional sources (Cole et al., 1998). Lipid anabolic pathways rely mainly on NADPH as a cofactor, although in the case of mycobacteria, the enzyme fatty acid synthase II (FAS II) presents one subunit that uses NADH (Marrakchi et al., 2000; Kruh et al., 2008). Despite the drastic reduction of the *M. leprae* genome, the collection of genes necessary for cell wall biosynthesis has been largely conserved; therefore, the reducing equivalents produced by the oxidation of cholesterol to cholestenone may be shifting from this pathway, contributing to the synthesis of PDIM and PGL-I. *M. leprae* has also preserved the gene coding for the transcription factor WhiB3, which may be involved in the effects of cholesterol oxidation on bacterial lipids biosynthesis. WhiB3 is part of a tight redox regulation system that senses variations in the redox balance and controls the anabolism of mycobacterial lipids such as polyacyltrehaloses (PAT), diacyltrehaloses (DAT), sulfolipids (SL-1) and PDIM (Saini et al., 2012; Mehta and Singh, 2019). WhiB3 activates lipid synthesis whenever there is an alteration in the redox state of the mycobacteria (Singh et al., 2009; You et al., 2019). So, it is reasonable to speculate that NADH/NADPH molecules generated upon *M. leprae* oxidation of cholesterol may result in WhiB3 activation and subsequent PGL-I and PDIM biosynthesis.

In conclusion, our findings provide strong evidence that cholesterol oxidation via 3β-HSD is an important source of reductive power for the leprosy bacillus. A model can be proposed describing the key role of this enzyme in facilitating *M. leprae* persistence in the host cell (Figure 6). During reductive evolution, *M. leprae* has lost several oxidoreductases, oxidases and dehydrogenases and, although living in a similar intracellular environment, its capacity to degrade carbon and nitrogen substrates is considered limited when compared to *M. tuberculosis* (Cole et al., 2001; Borah et al., 2019). As a strategy to compensate this metabolic deficiency, *M. leprae* induces cholesterol accumulation and its recruitment to bacterium-containing phagosomes, which combined with the expression of...
of 3β-HSD, turns this lipid into an easily available substrate for bacterial consumption and generation of reducing equivalents. The decrease in bacterial viability observed either by inhibiting cholesterol accumulation in the infected SCs (Mattos et al., 2011a) or by blocking 3β-HSD activity reinforces the hypothesis that cholesterol oxidation is an essential catabolic pathway for M. leprae pathogenesis, pointing to a novel drug target that may be used in combination with current multidrug regimens to diminish leprosy neuropathology.

DATA AVAILABILITY STATEMENT
The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS
TR and MM share first authorship and equally contributed with experiments and rational for the study. TR, MM, JB, MP, and MB-P were responsible for the study design and funding acquisition. TY and NS were responsible compound 1 production and supply. BV and CM were responsible to generate M. smegmatis knock out mutants. TR, MM, KD, KH, CS, JA, and KM were responsible for investigation and performing experiments. GA was responsible for LDL-Cho supply and support with experiments using it. TR, MM, MB-P were responsible for results analysis. RL and PR provided ML for in vitro experiments. Project supervision was performed by MB-P, MP and JB. TR, MM, MB-P, PB, JB and MP were responsible for writing original manuscript draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.709972/full#supplementary-material

Supplementary Figure 1 | Oxidation of Cholesterol to cholestenone by M. smegmatis mutants. M. smegmatis Mc^2^-155 wild type (WT) was mutated by recombinaseering, msme_5228 (hsd) and msme_1604 (choD) genes, coding, respectively, for the M. smegmatis 3β-HSD and ChoD homologues, were knocked out generating the single and double mutant strains Δ5228, Δ1604 and Δ1604/Δ5228. (A, B) M. smegmatis strains were incubated with [4-14C]cholesterol for 2h at 37°C followed by extensive washing with PBS-tyloxapol. Bacilli were extracted with chloroform-methanol (2:1), and the resulting lipids were analyzed by TLC using petroleum ether-ethyl acetate (1:1) as the running solvent. The graph indicates corresponding densitometry (n=2).

Supplementary Figure 2 | Complementation of M. smegmatis choD/hsd double mutant with the hsd and choD M. leprae genes. M. smegmatis MC^2^-155 wild type (WT-gray bar) and double knockout mutant (Δ5228/Δ1604 – blue bar) were cultured in 457 minimal salt medium supplemented with 0,05 tyloxapol and 18 mM glycerol. Double knockout mutant complemented either M. leprae ml0389 (choD) (Δ5228/1604:ml0389 – green bar) or M. leprae ml1942 (hsd) (Δ5228/1604:ml1942 red – bar) were cultured in LB broth with 0,05% tyloxapol at 37°C and induced with 50 ng/mL Atc for 14 h. The cells were disrupted in 1mL of Trizol with 2 cycles in the TissueLyser and relative expression of (A) M. smegmatis genes: msme_1604 (choD) and msme_5228 (hsd) (n=2) or (B) M. leprae genes: ml0389 and ml1942 were determined by qRT-PCR (n=3).

Supplementary Figure 3 | Compound 1 inhibit M. leprae 3β-HSD expressed in double mutant M. smegmatis. M. smegmatis Δ5228/1604:ml1942 was treated with compound 1 for 1h at 37°C with agitation, followed by further incubation in the presence of 1µCi/mL [4-14C]cholesterol for 1h. (A) Lipid extracts obtained from mycobacterial cells by chloroform-methanol 2:1 (v/v) extraction were analyzed by TLC using hexane-ethyl acetate 65:35 (v/v) as mobile phase. Radiolabeled lipids were observed at the PhosphorImager after TLC and corresponding densitometry of cholestenone. (B) Representative of 2 experiments and corresponding densitometry of cholestenone.

Supplementary Figure 4 | Compound 1 does not impact NADP reduction using substrates other than cholesterol. Acetate (A), Glyceroldehyde (B) or Citrate (C), well-known dehydrogenase substrates, were added to M. leprae whole cell sonicate and NADP^+ reduction was determined measuring NADPH generation at 340 nm every 30 s for 20 min. Kinetics was performed in the absence of substrates (black), or with substrate alone (blue) or alongside treatment with 100 µM compound 1 (red).

Supplementary Figure 5 | Absence of off-target effect of compound 1 on PDIM biosynthesis. M. tuberculosis (Mtbc) was pre-incubated with 100 µM compound 1 for 1 h at 37°C followed by 1 µCi/mL [1-14C]palmitic acid addition and incubation for additional 24 h. Radiolabeled lipids were observed at the PhosphorImager after TLC with 2 runs of petroleum ether-ethyl acetate (98:2) as mobile phase. Representative of 2 experiments. Corresponding densitometry of PDIM is presented on the right.

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