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Statins Increase Rifampin Mycobactericidal Effect

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***Mycobacterium leprae* and *Mycobacterium tuberculosis* antimicrobial resistance has been followed with great concern during the last years, while the need for new drugs able to control leprosy and tuberculosis, mainly due to extensively drug-resistant tuberculosis (XDR-TB), is pressing. Our group recently showed that *M. leprae* is able to induce lipid body biogenesis and cholesterol accumulation in macrophages and Schwann cells, facilitating its viability and replication. Considering these previous results, we investigated the efficacies of two statins on the intracellular viability of mycobacteria within the macrophage, as well as the effect of atorvastatin on *M. leprae* infections in BALB/c mice. We observed that intracellular mycobacteria viability decreased markedly after incubation with both statins, but atorvastatin showed the best inhibitory effect when combined with rifampin. Using Shepard's model, we observed with atorvastatin an efficacy in controlling *M. leprae* and inflammatory infiltrate in the BALB/c footpad, in a serum cholesterol level-dependent way. We conclude that statins contribute to macrophage-bactericidal activity against *Mycobacterium bovis*, *M. leprae*, and *M. tuberculosis*. It is likely that the association of statins with the actual multidrug therapy effectively reduces mycobacterial viability and tissue lesion in leprosy and tuberculosis patients, although epidemiological studies are still needed for confirmation.**

Tuberculosis (TB) and leprosy are chronic infections caused by *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the most important facultative pathogen and an obligate intracellular pathogen, respectively; they target macrophages for replication and persistence, while *M. leprae* also infects Schwann cells in nerves. Tuberculosis is the most frequent bacterial infection in humans, resulting in 1.4 million deaths around the world in 2011 (1). Leprosy may evolve to cause permanent nerve damage and incapacities, and in 2011, it was diagnosed in 219,075 patients worldwide, but it is usually not fatal. On the other hand, *M. leprae* infection was responsible for 12,225 cases of motor disability and deformity, which are the hallmarks of the infection (2).

Leprosy and TB are bacterial infections treatable with effective combined multidrug therapy, but very long treatment is necessary: 12 or 6 months for leprosy (depending on the clinical form) and 6 months for TB. In fact, the introduction of multidrug therapy (MDT) represented a great achievement in the control of the disease, which has been declining in incidence in the past 20 years around the world (2). Nevertheless, adherence to treatment is an important issue where the emergence of multidrug resistance (MDR) cases is increasing quickly, especially with TB. However, *M. leprae* resistance has been followed with great concern during the last few years (3). Over the last decades, the WHO has identified clinical isolates of *M. leprae* resistant to rifampin, dapsone, or ofloxacin and has reported that the number of these isolates is growing (4).

It is well known that after *M. leprae* or *M. tuberculosis* phagocytosis by host cells, an alteration in cellular lipid metabolism takes place, resulting in an increase in cholesterol and aliphatic lipid uptake and *de novo* synthesis (5–7). Parihar and colleagues (8) recently demonstrated that cholesterol reduction by statins reduces *M. tuberculosis* viability *in vitro* and *in vivo*, a strategy already proposed in the literature (9). This metabolic remodeling by the mycobacterial infection is responsible for an increase in different classes of lipids in a systemic way, as demonstrated in leprosy patient serum metabolomics studies, in which a correlation was established between the bacilloscopy index (BI) (the bacillary load in skin biopsy samples expressed in a logarithmic scale from 0 to 6) and the abundance of some polyunsaturated fatty acids and phospholipids, such as arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid (10). Our group also demonstrated an increase in cholesterol ester in multibacillary skin biopsy samples compared to paucibacillary ones (11). Additionally, it has been shown that *M. leprae* suppresses lipid degradation through the inhibition of hormone-sensitive lipase expression, contributing to lipid accumulation in infected cells (12), and this process plays a central role in bacterial survival (11). Although only two lipases and one phospholipase were encoded by its genome, recent proteomic analyses indicate an active glyoxylate cycle in *M. leprae*, in which fatty acid beta oxidation generates succinate for the synthesis of carbohydrates (13, 14). For that reason, the metabolism of host lipids represents an important mechanism for this bacterium to cause and sustain infection. This is best illus-

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trated by *M. tuberculosis*, which presents a large number of coding genes involved with lipogenesis and lipolysis (15). Controversially, many of these genes are pseudogenes in the *M. leprae* genome (14). Most likely, enzymes from the host cells complement the bacterial genes, as suggested by a study describing induction by the infection of cellular lipases and phospholipases in the tissues of lepromatous patients (16). Although cholesterol metabolism is still not completely understood in *M. leprae*, accumulation in the *M. tuberculosis* cell wall was previously shown to be responsible for decreasing tritiated rifampin permeability *in vitro* (17), as well as being involved in mycobacterial avoidance of macrophage vacuolar fusion and, consequently, the immune response (18); this represents the most important carbon source inside gamma interferon (IFN-γ)-activated macrophages *in vitro* (19).

Based on an accumulation of data involving the essential role of cholesterol and lipids in the intracellular survival of mycobacteria, in this study, we investigated the use of statins to control *M. leprae* infection both *in vitro* and *in vivo*. Statins are a class of drugs largely used in the treatment of cholesterol-induced atherosclerotic cardiovascular disease. They are structural analogs of mevalonate that are able to inhibit the rate-limiting enzyme 3-hydroxy-3-methyl-glutaryl–coenzyme A (HMG-CoA) reductase, which is responsible for cholesterol synthesis in mammals. Mainly used in the treatment of hypercholesterolemia, statins are now recognized as immunomodulatory drugs, presenting satisfactory pleiotropic effects in immune disorders (20, 21). By reducing cholesterol availability in the intracellular environment, we anticipate a reduction in mycobacterial persistence and multiplication in host cells.

In our present work, we tested the activity and additive effect of rifampin treatment in association with two statins, atorvastatin and simvastatin, in the control of *M. leprae* and *M. tuberculosis* in a macrophage *in vitro* infection model and *in vivo* in Shepard's mouse footpad *M. leprae* infection model. We demonstrated that both statins induce a bactericidal effect in *M. tuberculosis* and *M. leprae* infections. The bactericidal effect observed in cells infected by *M. leprae* is related to phagosomal arrest, and its combination with rifampin drastically reduces cellular infiltration in the leprosy mouse footpad model.

MATERIALS AND METHODS

Cell culture. THP-1 cells were obtained from the American Type Culture Collection (ATCC) and maintained in RPMI medium 1640 (LCG Bioscience, São Paulo, Brazil), supplemented with 10% fetal bovine serum (Cultilab, Campinas, São Paulo, Brazil), without antibiotics. The cultures were kept at 37°C or 33°C within a humidified 5% CO_2 atmosphere. The differentiation of monocytes to macrophages was achieved by exposing the cells to 200 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma, St. Louis, MO) for 24 h.

Mycobacterial strains and staining. Live *M. leprae* prepared from the footpads of athymic nude (nu/nu) mice was produced at the Lauro de Sousa Lima Institute, Bauru, São Paulo, Brazil. *M. leprae* was purified from iodine-disinfected hind footpads. Briefly, the skin and bones were removed, and the tissue was minced into small pieces with scissors and digested with a solution of 170 units of collagenase type I, 2 units of dispase (Life Technologies, NY), 5 mg/ml ampicillin (Sigma, St. Louis, MO), and 150 units of DNase (Life Technologies) for 2 h at 33°C. The digested tissue was homogenized by vortex and washed three times in water, one time in 0.1 N NaOH, and one time in RPMI medium, centrifuged at 10,000 × *g* for 5 min, and counted by acid-fast staining (Ziehl-Neelsen kit; Becton Dickinson). Part of the *M. leprae* suspension was sterilized by gamma irradiation at the Acelétron Facility (Rio de Janeiro, Brazil). *M. bovis* strain BCG Pasteur (ATCC 35734) and *M. tuberculosis* strain H37Rv were grown at 37°C in Middlebrook 7H9 base ADC enrichment medium (Becton Dickinson, Franklin Lakes, NJ), supplemented with 0.2% glycerol and 0.05% Tween 80 (Sigma).

***M. tuberculosis* and *M. bovis* BCG Pasteur viability determination.** *M. tuberculosis* and *M. bovis* BCG viability was measured after 2×10^5 THP-1 cells were infected and differentiated into macrophages, and after 24 h, these cells were infected with *M. tuberculosis* or *M. bovis* BCG at a multiplicity of infection (MOI) of 10:1 or 50:1, respectively, for 72 h at 37°C. The cultures were washed and lysed by 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 10 min. The number of intracellular live bacteria was assessed by serial dilution of the lysate and seeding on Middlebrook 7H10 medium with 10% oleic acid-albumin-dextrose-catalase (OADC), with a determination of the CFU after 1 month of incubation at 37°C.

***M. leprae* viability determination.** We used two methods to determine *M. leprae* viability. The first one is a fluorimetric LIVE/DEAD staining protocol described elsewhere (22) using the LIVE/DEAD *Bac*Light bacterial viability kit (Life Technologies, CA), performed according to the manufacturer's instructions, to be sure that the viability of bacilli from the nude mouse preparation was always >85%; otherwise, it was discarded. The second method is a molecular approach described elsewhere (23) to determine *M. leprae* viability in cellular cultures, in which the levels of labile *M. leprae* mRNA were normalized by its highly stable DNA. Briefly, 2×10^5 THP-1 cells were differentiated into macrophages within 24 h of incubation with 200 nM PMA. After this time, the cells were washed and allowed to rest. After 24 h of rest, the cells were infected with *M. leprae* at an MOI of 10:1. After testing at different times of infection, we observed that 7 days of infection generated the most consistent results on viability analysis. After 1 week of infection, *M. leprae* RNA and DNA were extracted using the TRIzol reagent (Invitrogen, CA) in FastPrep-24 tubes (MP Biomedicals, CA), as described previously (23). In RNA preparation, DNA was removed using the Turbo DNA-free kit (Ambion, CA). *M. leprae* RNA was reverse transcribed using random primer and SuperScript III according to the manufacturer's instructions (Invitrogen). The levels of *M. leprae* 16S rRNA, mRNA, and DNA were determined from cultured macrophages by real-time reverse transcription-PCR (RT-PCR), using the same primer pair: sense, 5′-GCA TGTCTTGTGGTGGAAAGC-3′, and antisense, 5′-CACCCCACCAACAAGCTGAT-3′. All samples presented cycle threshold (C_T) values between 20 and 28. One hundred percent viability was arbitrarily assumed as $2^{-\Delta CT}$ of the infected control samples, and all other values were normalized as a percentage of this. The reactions were performed in an ABI StepOnePlus sequence detection system (Applied Biosystems, CA).

Lipid extraction and analysis. A total of 8×10^5 THP-1 cells were differentiated into macrophages as described above, infected with *M. leprae* at an MOI of 10:1, and exposed to 2 μM simvastatin for 1 week. The cultures were washed twice with PBS, detached, and homogenized by three freeze-thaw cycles. The total cholesterol content was determined using the Amplex red cholesterol assay kit (Invitrogen, CA), according to the manufacturer's instructions. The total cholesterol levels were represented as μg of total cholesterol per mg of protein.

Fluorescence microscopy. THP-1 cells were plated in 24-well plates containing coverslips at a density of 2×10^5 cell/well and treated with statins for 24 h. To measure the infection rate in the statin-treated cells, the cultures were exposed to irradiated *M. leprae* stained with PKH26 red fluorescence, as described elsewhere (24), at an MOI of 10:1 for 5 h, a time known to be sufficient to infect one-third of the THP-1 cells with *M. leprae* at this MOI. After that, the medium was removed by washing in PBS, and the cells were fixed in 4% (wt/vol) paraformaldehyde at 4°C for 20 min. Images were taken in a Zeiss Axio Observer fluorescence microscope, where 10 fields from three biological replicates were analyzed.

For immunocytochemistry by confocal microscopy, the cultures were infected with live *M. leprae* at an MOI of 1:10 for 24 h, a time known to be sufficient to observe *M. tuberculosis* escape from the phagosome (25). The

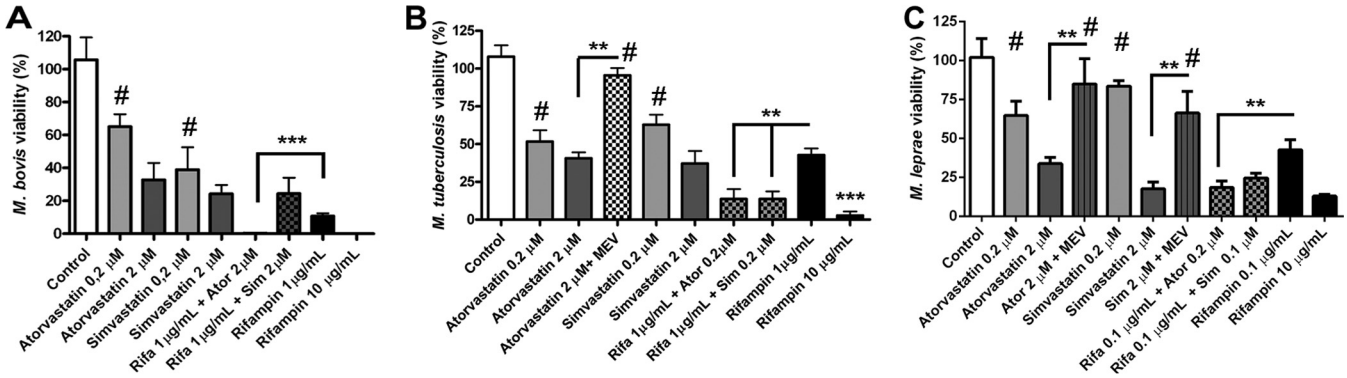

FIG 1 Statins increase rifampin mycobactericidal effect. THP-1 cells were differentiated to macrophages with PMA, and after 24 h of rest, the cultures were treated with different regimes and were concomitantly infected with *M. bovis* (BCG Pasteur) (A), *M. tuberculosis* H37Rv (B), and *M. leprae* strain Thai-53 (C). The viability in relation to the controls was measured after 72 h from the infection by CFU determination (A and B) or after 1 week by real-time PCR (C). It was observed that despite simvastatin displaying a stronger mycobactericidal effect, only atorvastatin showed an additive effect when associated with rifampin against all three mycobacteria tested. The effects were reverted by mevalonate at 150 μM (MEV). Means and standard errors of the mean (SEM) were generated from four normalized independent biological replicates, where two and three asterisks mean $P < 0.01$ and $P < 0.001$, respectively. #, treatment was not statistically different from control. Rifa, rifampin; Ator, atorvastatin; Sim, simvastatin.

slides were permeabilized and blocked by 30 min incubation with 0.01% Triton X-100 and 10% fetal bovine serum in PBS (pH 7.2). The cells were incubated for 2 h with rabbit IgG antilipoarabinomannan (anti-LAM) antibodies (1:50 [vol/vol]), which were kindly donated by John S. Spencer (BEI Resources Repository, NIAID, NIH), to identify *M. leprae*, and mouse IgG anti-human Rab7 (1:500 [vol/vol]; Abcam, MA) to identify late endosomes. Secondary antibodies conjugated with Alexa Fluor 488 (IgG anti-mouse) and Alexa Fluor 555 (IgG anti rabbit) (Invitrogen, CA) were incubated with the samples for 2 h. The slides were observed in an LSM 710 confocal laser scanning microscope (Carl Zeiss, Heidenheim, Germany). We analyzed 100 cells from three biological replicates, plotting the percentage of late phagosomes ($Rab7^+$ vesicles) containing *M. leprae*.

***In vivo* atorvastatin efficacy test against *M. leprae* in Shepard's BALB/c mouse model.** A suspension containing 1×10^4 live *M. leprae* cells in 10 μl was inoculated in each hind footpad of the BALB/c mice, as described in Shepard's model. One month after inoculation, the mice were divided into 6 groups. The control group was inoculated and not treated; two other groups were treated with atorvastatin added to the daily feed at doses of 40 and 80 mg/kg of body weight/day. The other three groups received rifampin at 10 mg/kg, rifampin at 1 mg/kg by gavage weekly, or rifampin at 1 mg/kg of body weight and atorvastatin at 80 mg/kg of body weight/day in the feed. The mice were treated for 5 months. Six months after inoculation, each mouse was sacrificed and both footpads were excised one was macerated for bacillary counting, and the contralateral was fixed in 10% buffered formalin, paraffinized, and sectioned for histopathological examination with hematoxylin and eosin and Fite-Faraco staining for acid-fast bacilli (AFB).

Determination of cholesterol and transaminase activity in mouse plasma. Each BALB/c mouse was sacrificed under anesthesia, and plasma was collected in heparin directly from the heart. Cholesterol, aspartate aminotransferase (AS), and alanine aminotransferase (ALT) were measured using the assay kit Bioclin/Quibasa (Belo Horizonte, Minas Gerais, Brazil), according to the manufacturer's instructions.

Statistical analysis. All numerical data were statistically analyzed using nonparametric Kruskal-Wallis test and Dunn's posttest to compare relevant groups, with the GraphPad Prism software.

Ethics statement. The animal protocols were in agreement with the animal care guidelines of the National Institutes of Health and were approved by the Animal Welfare Committee of Sagrado Coração University (São Paulo, Brazil).

RESULTS

Intracellular mycobacterial viability is reduced by statins. Since host cholesterol accumulation in the *M. tuberculosis* cell wall is involved in the decrease in rifampin permeability *in vitro* (17), we hypothesize that cholesterol *de novo* synthesis inhibition might not only kill mycobacteria by carbon deprivation but also make them more vulnerable to rifampin.

We first observed the potential of atorvastatin and simvastatin in the control of *in vitro* THP-1 mycobacterial infection. After 72 h of infection, both atorvastatin and simvastatin were able to inactivate intracellular BCG in a dose-dependent fashion (Fig. 1A). With the higher dose (2 μM), both drugs were able to reduce the number of viable intracellular bacilli by about 75%, and atorvastatin showed an additive effect in combination with 1 μg/ml rifampin. *M. tuberculosis* displayed a very similar response to statins as that of BCG, presenting a reduction in viability in a dose-dependent manner (Fig. 1B). One distinction between BCG and *M. tuberculosis* was that in the pathogenic strain, the additive effect of statins with rifampin was seen in both combinations (1 μg/ml rifampin plus 0.2 μM atorvastatin or simvastatin), with a *P* value of <0.05.

We determined *M. leprae* viability by RT-PCR, the most sensitive method available, being chosen due to its capacity to determine the viability of a small amount of bacilli (10^6) (23). After 1 week of infection at 33°C, the *M. leprae* RNA copy number, which is rapidly degraded after bacillus inactivation, was quantified after normalization. As shown in Fig. 1C, both atorvastatin and simvastatin caused a bactericidal effect in a dose-dependent fashion. Here again, only atorvastatin showed an additive effect when combined with rifampin (0.1 μg/ml rifampin plus 0.2 μM atorvastatin). None of these effects are related to the cytotoxicity of statins (see Fig. S1 in the supplemental material).

Statin treatment does not alter mycobacterial invasion. To observe if statins were able to reduce cholesterol levels in our model and through that mechanism prevent mycobacterial infectivity instead of reducing mycobacterial viability, we treated infected THP-1 cells for 1 week with 2 μM simvastatin. Our group has already shown that *M. leprae*-infected macrophages accumu-

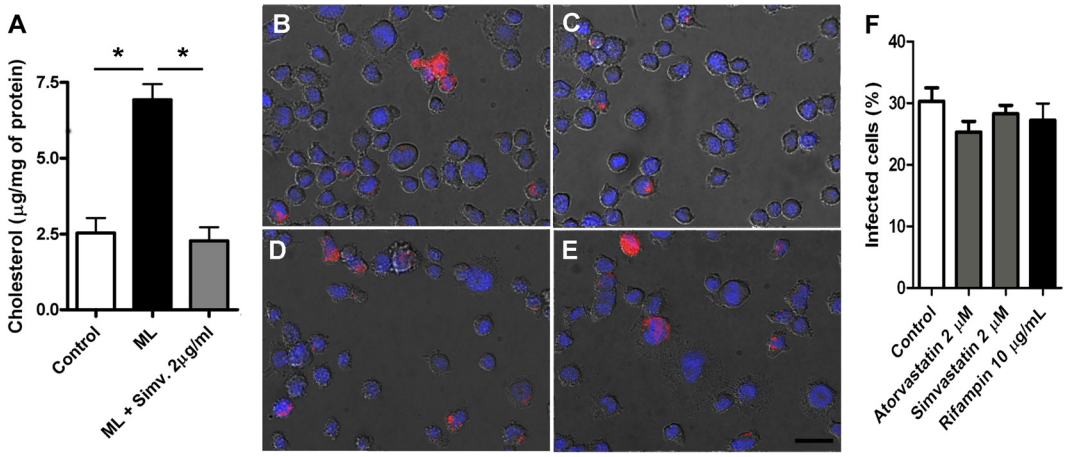

FIG 2 THP-1 mycobacterial infection and cholesterol levels after treatment with statins. (A) Total cellular free cholesterol and cholesteryl ester levels were measured by the fluorometric method after 1 week of infection with concomitant treatment. Atorvastatin treatment decreased cholesterol levels even in the presence of *M. leprae* (ML). Means and SEM are representative of the results from three independent biological replicates (* indicates $P < 0.01$). PKH-stained *M. leprae* was used to infect THP-1 cells during 5 h without treatment (B) or after 24 h pretreatment with 2 μM atorvastatin (C) or simvastatin (D) or 10 μg/ml rifampin (E). The nuclei were stained in blue by 4′,6-diamidino-2-phenylindole (DAPI). (F) Quantification of the percentage of infected cells, where it can be observed that the treatments did not interfere with infection rate. Forty representative images from three biological replicates were used to perform this analysis. The scale bar corresponds to 20 μm.

late cholesterol as lipid bodies through Toll-like receptor 2/6 (TLR2/6) signaling (7). In this study, we observed that simvastatin was able to prevent *M. leprae*-infected cells from accumulating cholesterol, restoring normal cholesterol levels (Fig. 2A). Since the importance of 9-*O*-acetyl GD3, a cholesterol-anchored ganglioside located in cellular lipid rafting, to *M. leprae* invasion in Schwann cells was already demonstrated by our group (26), we investigated if the changes in cholesterol levels observed in the treated cells could interfere in the ability of *M. leprae* to infect cells. We observed the rates of infection between the untreated and statin-treated cells using irradiated PKH26-stained *M. leprae* and determined that there was no difference (Fig. 2B to F). Thus, we conclude that the lower number of viable mycobacteria after treatment with statins is not due to either THP-1 cell mortality or differences in the infection rate.

Statins increase association between *M. leprae* and late endosomes. Cholesterol also plays a role in the ability of mycobacteria to escape the phagosome, as it serves as an anchor for early secretory antigenic target 6 (ESAT-6), a secreted mycobacterial protein able to disrupt cellular membranes and to consequently release engulfed mycobacteria to the cytosol (A. B. Robottom-Ferreira et al., unpublished data) (27). Other authors showed that only in cholesterol-depleted macrophages did *Mycobacterium avium*-containing phagosomes fuse with lysosomes, generating phagolysosomes (18). In addition, it was recently demonstrated that macrophages isolated from simvastatin-treated mice more efficiently kill *M. tuberculosis* through phagosomal maturation and autophagy (8). It has been shown that the dissociation of Rab7 from *M. tuberculosis* phagosomes, which occurs around 6 h, is important for the maintenance of the phagosomal environment, which matures to a phagolysosome after fusion with lysosomes (25). Corroborating the literature, we show a close association of the transient phagosomal membrane protein Rab7 with *M. leprae* lipoarabinomannan (LAM) within the phagosomal space 24 h after statin treatment of the infected cells. We observed that Rab7 and LAM were colocalized within the phagosome 24 h after *M. leprae* infection only in THP-1 cells treated with statins (Fig. 3A, arrows and insets). Simvastatin induced higher mycobacterial LAM/Rab7 colocalization than did atorvastatin, in agreement with its higher bactericidal activity against *M. leprae*, as shown in Fig. 1C. However, since atorvastatin had a similar activity when used at a higher dose (2 μM) and this drug was the only one to present an additive effect in association with rifampin, we chose it for the *in vivo* tests.

Atorvastatin synergizes with rifampin in its antibacterial effect *in vivo*. Due to the previous studies showing that host cholesterol deposition in the mycobacterial cell wall inhibits rifampin permeability (17), our hypothesis is that the application of statins in tuberculosis and leprosy treatment involves its association with the actual multidrug therapy. For this reason, we evaluated whether atorvastatin was able to potentiate the effect of rifampin *in vivo*. To test this, we infected BALB/c mouse footpads and, after 1 month of infection, the mice were subjected to five different treatments for 5 months (Fig. 4). After 6 months, atorvastatin alone at the higher dose (80 mg/kg/day) effectively reduced *M. leprae* replication (Fig. 4A, triangles). In addition, only groups treated with the higher dose of atorvastatin showed a significant reduction in plasma cholesterol levels (Fig. 4B). More interestingly, atorvastatin combined with rifampin (1 mg/kg/week) induced a larger decrease than rifampin alone ($P < 0.01$). We also demonstrate that none of the treatment schemes increased muscle tissue damage or led to detectable hepatotoxic effects (Fig. 4C and D). We examined if atorvastatin at the higher dose could not only prevent infection but also eliminate an established 4-month Foxn1nu (nude) mouse infection. After 1 month of treatment, we observed that 2 of 3 treated nude mice had a higher number of dead bacilli recovered from their footpads (data not shown).

The representative histological characteristics from contralateral footpads from the same groups of mice analyzed in Fig. 4 are shown in Fig. 5. As seen at a higher magnification, the untreated group of animals (Fig. 5A and B, asterisks) presents a predominantly macrophage inflammatory infiltrate in the dermis, around

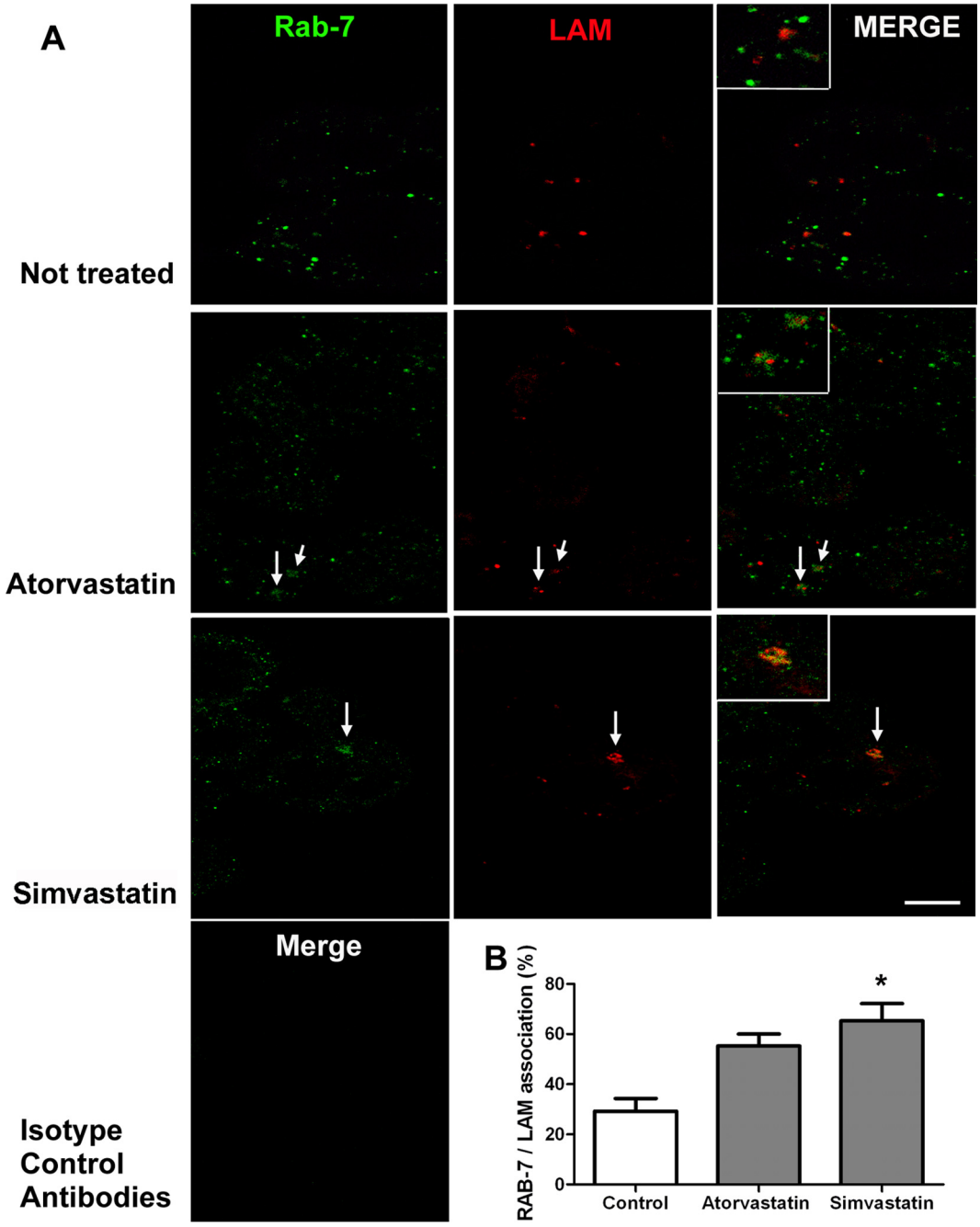

FIG 3 Confocal analysis of *M. leprae* phagosomal arrest in THP-1 statin-treated cells. (A) Immunolocalization of Rab7, a late-endosome marker (Alexa Fluor 488) and *M. leprae* lipoarabinomannan (LAM) (Alexa Fluor 633), in THP-1 cells after 24 h of *M. leprae* infection with 2 μM atorvastatin or simvastatin treatment, demonstrating antigen association by arrows and in higher magnification by insets. Merge images of both channels' signals produced by isotype control antibodies are also presented. (B) The percentage of intracellular LAM signal associated with Rab7 is shown as mean and SEM, demonstrating a higher mycobacterial arrest in mature phagosomes due to exposure to simvastatin. Images are representative from 360 randomly chosen cells of four experiments from two independent biological replicates. The scale bar corresponds to 10 μm in panels and 5 μm in insets. Asterisk indicates a difference with a P value of <0.05.

blood vessels, and involving skeletal muscle fibers. The macrophages showed features of activated cells, with eosinophilic cytoplasm and round or oval nuclei containing one or more nucleoli. There were a small number of lymphocytes, rare neutrophils, and eosinophils mixed with the macrophages. The bacilloscopic index (determined by Fite-Faraco stain) was high, at 5/6+, with the fragmented bacilli inside irregularly distributed macrophages in the entire extension of the inflammatory infiltrate. In some areas of Fig. 5F (1 mg/kg/week rifampin), it can be seen that the infiltrate was composed of granulomas in regression, characterized by vacuolated macrophages and nuclei without nucleoli, as well as lymphocytes. Figure 5G and H (1 mg/kg/week rifampin plus 80 mg/kg/day atorvastatin) and I and J (10 mg/kg/week rifampin) show a small and discrete inflammatory infiltrate, consisting pre-

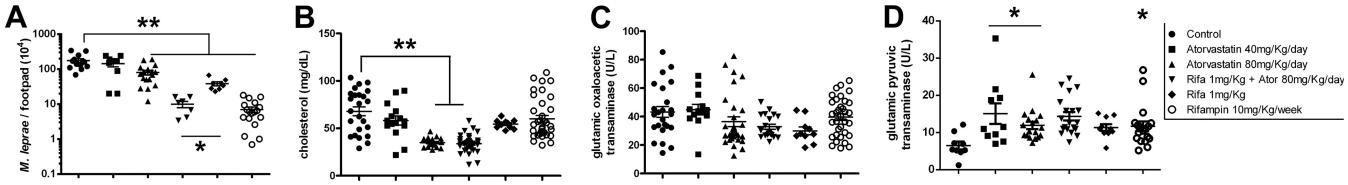

FIG 4 Atorvastatin activity in BALB/c mouse *M. leprae* infection. BALB/c mouse footpads were infected with 10^4 *M. leprae* bacilli. After 1 month, different groups of animals were untreated (control) or treated with 40 mg/kg/day or 80 mg/kg/day of atorvastatin, the combination of 80 mg/kg/day of atorvastatin (Ator) with 1 mg/kg/week of rifampin (Rifa), 1 mg/kg/week of rifampin alone, or 10 mg/kg/week of rifampin. (A) Number of bacilli in footpad suspensions after 6 months of infection, followed by 5 months of treatment. Plasma cholesterol (B), glutamic oxaloacetic (C), and pyruvic (D) transaminase activities were measured in the same groups of mice. Means and SEM were generated from two independent biological replicates, where one and two asterisks mean $P < 0.05$ and $P < 0.0001$, respectively.

dominantly of loosely arranged macrophages located in the dermis and perivascular area, and involving the skeletal muscle fibers. The granulomas are more regressive, and some muscle fibers show recovered areas. The bacilloscopic index (Fite-Faraco stain) was lower, at only 2+, with multifragmented weakly stained bacilli inside the macrophages. This supports an important and previously described beneficial pleiotropic effect of statins and their possible immunomodulatory role, which might be involved in the reduction in tissue damage promoted by statin treatment in tuberculosis animal infections (8, 21), a desired effect in the control of immune-related tissue inflammation and damage in leprosy.

DISCUSSION

Our group recently described the mechanism by which *M. leprae* induces lipid droplet formation in macrophages and Schwann cells (5, 7, 28), showing that host-derived cholesterol represents the major lipid component inside these organelles. The importance of lipids in *M. leprae* energetic metabolism is further supported by the high prevalence of genes involved in lipid anabolism and catabolism, despite the huge reductive evolution that occurred in its genome, producing essentially a minimal gene set for survival (14). It has already been shown in *in vitro* studies that host cholesterol accumulation in the *M. tuberculosis* cell wall is related to the decrease in rifampin permeability (17); this is a possible explanation for the additive effect observed between statins and rifampin. The hypothesis that mycobacterial HMG-CoA reductase would be inhibited by statins is not valid, since the similarity of *M. tuberculosis* to the human enzyme is <40%. Moreover, this enzyme is not expressed in *M. leprae*, which displayed a similar sensitivity to statins as those of *M. tuberculosis* and BCG. More importantly, the higher concentrations of atorvastatin and simvastatin used in this work (2 μM) failed to kill *M. tuberculosis* H37Rv cultivated in 7H9 medium. On the other hand, the results observed in the infected THP-1 cells could not be ascribed to the cytotoxicity of statins, since cellular viability was not altered under all conditions employed in the study (see Fig. S1 in the supplemental material). Concerning the isolated effect of statins against mycobacteria inside macrophages, other events must be taken into consideration. For example, the metabolism of *M. tuberculosis* isolated from infected mouse lungs is stimulated by fatty acids and is unresponsive to carbohydrates (29), and the generation of *M. tuberculosis* mutants with knockouts of some enzymes involved in cholesterol metabolism, such as KshA, KshB, FadA5, ChoD, and KstD, leads to inefficient mouse and macrophage infection, demonstrating the pivotal relevance of this pathway to tuberculosis infection and persistence inside the host (30–33). Since statins were already described as generators of oxidative stress in hepatocytes (34), we measured nitrite production and observed no significant difference between all conditions used in our study (see Fig. S2 in the supplemental material). Therefore, we also discarded the possibility that statins would kill mycobacteria by increasing the oxidative stress in our model. Another hypothesis, different from carbon restriction, might explain the mycobactericidal effect of statins, such as the capacity of a statin to disrupt signaling cascades originated at lipid rafts, a phenomenon already described in T lymphocytes (35). Recently, Parihar and collaborators (8) showed that statins can control *M. tuberculosis* infection, and they suggested that this effect is due to phagolysosomal arrest of *M. tuberculosis*. We conclude that atorvastatin and simvastatin display similar effects in our study, achieving effective mycobactericidal activities against BCG, *M. tuberculosis*, and *M. leprae* reverted by mevalonate, the product of HMG-CoA reductase, as shown in Fig. 1. We successfully observed *M. leprae* and mature endosome association increasing after statin treatment (Fig. 3). It has been clearly shown that the mechanism used by *M. tuberculosis* to escape from the endosome compartment involves the expression of a complex of proteins, including ESX-1, which is involved in the transport of proteins able to bind cholesterol-rich membranes, such as ESAT-6, which is involved in membrane destabilization and rupture (27). Statins efficiently prevent *M. tuberculosis*-induced inhibition of macrophage phagosomal maturation (8). ESAT-6 is also expressed by *M. leprae* during THP-1 infection (Robottom-Ferreira et al., unpublished data). Although simvastatin was not able to reduce the cholesterol levels of the infected cell below that of the control, we hypothesize that the avoidance of cholesterol accumulation induced by *M. leprae* in treated THP-1 cells is sufficient to inhibit ESAT-6 disruption of the phagosome, which had matured in the late endosome with the mycobacteria arrested inside (Fig. 3, insets).

Our *in vivo* study corroborates the *in vitro* findings, with significant differences from the control groups observed only in mice receiving the highest dose of statin (80 mg/kg) in their daily diet. An equivalent dose in humans is 390 mg/day for a 60-kg adult (36). This very high dose of atorvastatin could be avoided if we were to use daily gavage or intraperitoneal injections as the administration route, but this would be impractical for a 5-month period of treatment. This subject will be addressed in a future work.

Taken together, our results show that statins are able to inactivate *M. leprae* and *M. tuberculosis in vitro*, as well as potentiate the antimicrobial effect of rifampin against both pathogens. We can see that the atorvastatin-rifampin combination presents very

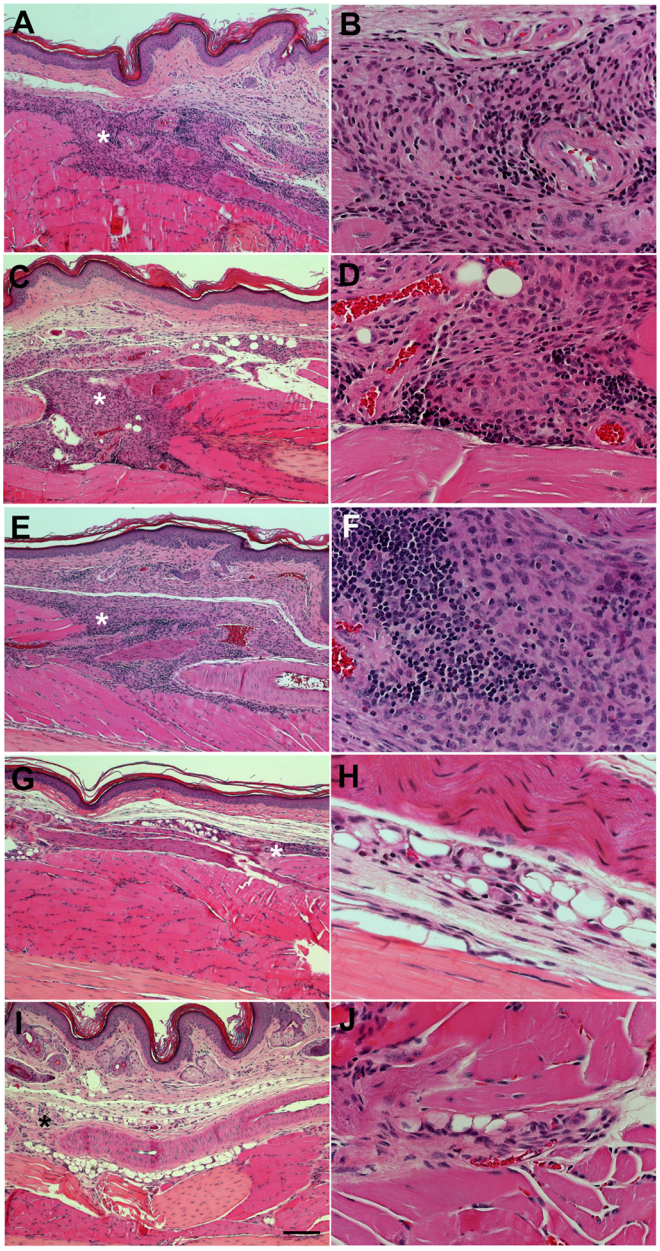

FIG 5 Histopathological analysis of footpads from *M. leprae*-infected mice after different treatment regimes. Contralateral footpads from the same groups of mice analyzed in Fig. 4 were fixed, paraffinized, sectioned, and stained with hematoxylin and eosin after 6 months of infection and 5 months of treatment. (A and B) Untreated controls. (C and D) Representative images of a lesion area from an animal treated with 80 mg/kg/day of atorvastatin. (E and F) Treatment with 1 mg/kg/week rifampin (subtherapeutic dose). (G and H) Combination of 80 mg/kg/day atorvastatin and 1 mg/kg/week rifampin. (I and J) Rifampin at 10 mg/kg/week. The asterisks show inflammatory cellular infiltrates, which were virtually absent in panels G and I. The scale bar represents 100 μm in panels A, C, E, G, and I and 25 μm in panels B, D, F, H, and J.

different results than those with each drug used separately, increasing rifampin mycobactericidal activity (Fig. 1 and 4A) while decreasing the inflammatory response and tissue damage (Fig. 5G and H). We believe that this decrease in *M. leprae* viability contributed to the reduction in the inflammatory infiltrate observed in Fig. 5, but this decrease alone does not explain everything, since the viability observed under both conditions, rifampin alone and the rifampin-atorvastatin combination, shows only a small difference, while the inflammatory infiltrate reduction is much more evident.

The inflammatory infiltrate reduction observed in Fig. 5 can be explained by the well-known pleiotropic effect of the statins already described in other models (20, 21, 37–39). Recent studies have demonstrated that statins may be beneficial in the treatment of T-cell–mediated autoimmune diseases (40), due to the involvement of cholesterol in the maintenance of lipid raft structures and its importance in T-cell activation (41). By reducing isoprenoids, statins also inhibit protein prenylation, blocking small GTPase Ras superfamily tethering and activity (42), leading to the inhibition of T-cell activation (43), antigen uptake, processing, and presentation by antigen-presenting cells, as well as their maturation (44).

The immunomodulatory pleiotropic effect of the combination of statin and rifampin might be particularly beneficial to the inhibition of leprosy reactional episodes during treatment. The combination of statins with MDT might reduce the occurrence of reactional episodes, which are characterized by an intense and sudden activation of the host immune response with high levels of tumor necrosis factor alpha (TNF-α), affecting about half of the patients under treatment (45). The use of statins to control mycobacteriosis might be a low-cost, efficient, and fast way to provide an entirely new class of drugs to aid tuberculosis and leprosy treatment efforts, being of extreme importance if used in association with the MDT regime against MDR or extensively drug-resistant (XDR) mycobacterial strains.

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