

Mycobacterium tuberculosis programs mesenchymal stem cells to establish dormancy and persistence

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Tuberculosis (TB) remains a major infectious disease worldwide. TB treatment displays a biphasic bacterial clearance, in which the majority of bacteria clear within the first month of treatment, but residual bacteria remain nonresponsive to treatment and eventually may become resistant. Here, we have shown that *Mycobacterium tuberculosis* was taken up by mesenchymal stem cells (MSCs), where it established dormancy and became highly nonresponsive to isoniazid, a major constituent of directly observed treatment short course (DOTS). Dormant *M. tuberculosis* induced quiescence in MSCs and promoted their long-term survival. Unlike macrophages, where *M. tuberculosis* resides in early-phagosomal compartments, in MSCs the majority of bacilli were found in the cytosol, where they promoted rapid lipid synthesis, hiding within lipid droplets. Inhibition of lipid synthesis prevented dormancy and sensitized the organisms to isoniazid. Thus, we have established that *M. tuberculosis* gains dormancy in MSCs, which serve as a long-term natural reservoir of dormant *M. tuberculosis*. Interestingly, in the murine model of TB, induction of autophagy eliminated *M. tuberculosis* from MSCs, and consequently, the addition of rapamycin to an isoniazid treatment regimen successfully attained sterile clearance and prevented disease reactivation.

Introduction

Tuberculosis (TB), caused by the obligate intracellular organism *Mycobacterium tuberculosis*, is the oldest known infectious disease in humans. Current therapy for TB consists of multiple antibiotics, is lengthy, and causes toxicity. However, the majority of the bacteria are cleared within 3–4 weeks of treatment, and patients start feeling better and often discontinue treatment, which may promote the generation of drug-resistant variants of *M. tuberculosis* (1). The remaining small numbers of organisms are highly nonresponsive to antibiotic treatment and continue to persist (2). Incomplete treatment may lead to disease reactivation, often associated with drug-resistant variants (3, 4). Therefore, a therapeutic strategy that eliminates persistent bacteria is urgently needed. Addition of such therapeutics along with conventional antibiotics should dramatically reduce the treatment length, and thereby reduce the generation of drug-resistant variants.

The reasons for the unresponsiveness of these persisting organisms to antibiotics remains incompletely understood. Current antibiotic therapy is mostly focused on eliminating replicating *M. tuberculosis*. The natural host for *M. tuberculosis* is macrophages, in which they replicate and survive by employing a variety of host-evasion mechanisms that include inhibition of phagolysosome fusion (5, 6), deacidification of lysosomal com-

partments (7), and translocation to the cytosol (8). These bacteria respond to antibiotics and are readily cleared. However, nonreplicating bacteria survive within granulomatous structures containing mesenchymal stem cells (MSCs), with limited accessibility to therapeutics (9). Recently, we and others have shown that *M. tuberculosis* infects MSCs (9, 10). In some cases *M. tuberculosis* was detected in patients who had completed directly observed treatment short course (DOTS) (11). MSCs express high levels of ABC transporter efflux pumps, which expel a variety of drugs employed to treat TB (12). Thus, MSCs represent a hiding place for *M. tuberculosis*. The mechanisms by which *M. tuberculosis* adapts to MSCs and the targets in MSCs that allow persistence of *M. tuberculosis* remain unknown.

M. tuberculosis within macrophages generally respond to the conventional antibiotic, isoniazid (INH). In contrast, dormant forms of the bacteria generally do not respond to antibiotics, and where and how they evade drugs and detection is incompletely understood. Nevertheless, studies, including our previously published data, have indicated that MSCs represent a major niche for dormant TB (9, 10, 13). Based on these considerations, we hypothesized that *M. tuberculosis* acquires dormancy and thereby drug nonresponsiveness in MSCs.

Here, we show that MSCs are a natural host for dormant *M. tuberculosis*. Upon uptake by MSCs, *M. tuberculosis* induces the expression of dormancy-related genes and promotes quiescence in MSCs. In contrast, *M. tuberculosis* residing in macrophages continues to replicate and causes macrophage necrosis. INH does not affect *M. tuberculosis* survival in MSCs but successfully eliminates bacteria from macrophages. In macrophages, most of the organisms are found in early-phagosomal compartments, but in MSCs

Authorship note: SSK, VPD, and DB contributed equally to this work.

Conflict of interest: The authors have declared that no conflict of interest exists.

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Submitted: February 8, 2019; **Accepted:** October 18, 2019; **Published:** December 17, 2019.

Reference information: *J Clin Invest.* 2020;130(2):655–661.

<https://doi.org/10.1172/JCI128043>.

nearly all bacilli are present in the cytosol. *M. tuberculosis* promotes rapid lipid synthesis in MSCs, which causes lipid droplets to form that shield the harbored bacteria. Inhibition of lipid synthesis dramatically reduces expression of dormancy-related genes while upregulating replication-related genes, which sensitizes the organisms to antibiotic-mediated killing. Thus, our findings establish that MSCs are a reservoir of dormant *M. tuberculosis* infection. *M. tuberculosis* infection of MSCs is associated with an autophagy-related gene expression signature, and induction of autophagy with rapamycin eliminates *M. tuberculosis* from MSCs. Consistent with these findings, addition of rapamycin to a conventional antibiotic treatment regimen successfully attains sterile clearance.

Results and Discussion

Previously, we and others have shown that MSCs are associated with nonreplicating *M. tuberculosis* (9, 10, 13). Therefore, we sought to determine whether MSCs are a natural reservoir for *M. tuberculosis* and dormancy that renders nonresponsiveness to antibiotic treatment. We infected human MSCs and peripheral blood mononuclear cell-derived (PBMC-derived) macrophages with *M. tuberculosis* (Supplemental Figure 1; supplemental material available online with this article; <https://doi.org/10.1172/JCI128043DS1>). We found that, to attain a saturation of infection in macrophages, 4 hours of infection at 1:10 multiplicity of infection (MOI) was required, whereas 6 hours at 1:50 MOI attained saturation of infection in MSCs. Under these conditions, similar numbers of bacilli were taken up by these 2 cell types (Figure 1, A and B). Thus, it appears that MSCs are less permissive than macrophages for *M. tuberculosis* infection, which might be evolutionarily related to latency of *M. tuberculosis* in MSCs.

With the progression of time, *M. tuberculosis* continued to replicate and macrophages became necrotic by 96 hours of infection (Supplemental Figure 2, A–E). Strikingly, *M. tuberculosis* numbers gradually decreased in MSCs, reached a plateau by 72 hours, and remained there in a viable form for an extended time period. To understand this differential behavior of *M. tuberculosis* in macrophages and MSCs, we examined the expression of replication- and dormancy-related genes in *M. tuberculosis* isolated from infected macrophages and MSCs. We found sustained expression of dormancy-related devR/dosR regulon genes in *M. tuberculosis* isolated from MSCs (Figure 1C and Supplemental Figure 3). However, genes that are involved in various steps of *M. tuberculosis* replication were enriched in *M. tuberculosis* isolated from infected macrophages (Figure 1D).

To explore the in vivo relevance, we sorted CD45⁺ Sca1⁺ MSCs from the bone marrow and CD45⁺CD11b⁺ macrophages from the lungs of *M. tuberculosis*-infected mice. Consistent with the in vitro data, we found that *M. tuberculosis* in MSCs express dormancy-related genes, whereas *M. tuberculosis* that are in macrophages express replication-related genes (Figure 1, E and F). Taken together, these observations strongly suggested that macrophages and MSCs are differentially programmed for supporting active and dormant infection, respectively.

Our findings showed that MSCs are less permissive to *M. tuberculosis* infection and allow the bacteria to establish dormancy. It will be interesting to determine if other nonpermissive cells such as hepatocytes or fibroblasts similarly allow *M. tuberculo-*

sis to establish dormancy. Although it has been reported that *M. tuberculosis* can infect and replicate in fibroblasts (14, 15), we were unable to infect fibroblasts.

Next, we explored whether *M. tuberculosis* infection affects MSC replication and found that *M. tuberculosis* inhibits MSC replication in a time-dependent fashion. Therefore, we measured expression of quiescence markers characteristic of stem cells (16, 17). RNA sequencing (RNA-Seq) analysis revealed upregulation of several quiescence markers and downregulation of cell cycle progression markers in human MSCs infected with *M. tuberculosis* (Figure 1G). This was confirmed by quantitative PCR (qPCR) of selected quiescence markers such as FOXO3a, NOTCH1, and SOX9, which were upregulated in MSCs as compared with macrophages (Figure 1H). In contrast, the cellular proliferation markers S-phase kinase 2 (SKP2) and CCNA1 (encoding cyclin A1) were highly upregulated in macrophages (Figure 1H). Western blot analysis confirmed enhanced expression of NOTCH1, FOXO3a, and p-FOXO3a at Ser318/Ser321 (Figure 1I and Supplemental Figure 10). Phosphorylation of FOXO3a at Ser318 and/or Ser321 causes its nuclear exclusion and inhibits its transcriptional activity. Thus, the increased FOXO3a phosphorylation might be essential in modifying transcriptional activity to inhibit MSC proliferation. Although phosphorylation of FOXO3a at Ser253 is known to exert an inhibitory response on its transcriptional activity (18), we did not observe any significant change in the phosphorylation status of FOXO3a at this site. Additionally, there was no significant difference in the protein levels of FOXO1 and FOXO4 or p-FOXO1 (Supplemental Figure 4), suggesting that these quiescence markers might play a prominent role in attaining a quiescent state in MSCs. This observation implied that upon infection *M. tuberculosis* acquires dormancy, whereas MSCs enter into a quiescent state. This dual strategy may assist *M. tuberculosis* to better shield itself from the host immune system and drugs used for treatment.

It is intriguing that macrophages, which are equipped with phagolysosomal killing mechanisms, are permissive to *M. tuberculosis* replication, whereas MSCs, which lack a well-defined phagosomal system compared with macrophages, restrict *M. tuberculosis* growth (7, 8). To obtain insight into this apparent paradox of *M. tuberculosis* infection, we determined the intracellular localization of GFP-labeled *M. tuberculosis* in human macrophages and MSCs. To determine endosomal localization of *M. tuberculosis*, we employed an antibody directed against the early-endosomal marker, Rab5, whereas for cytosolic localization, we employed phalloidin, which selectively binds F-actin (19). We observed that in macrophages, most of the *M. tuberculosis* localized to early endosomes immediately after infection, whereas the majority of bacilli in MSCs were found in the cytosol (Figure 2, A and B, and Supplemental Figures 5 and 6). Interestingly, we also observed abnormal lipid droplets in MSCs, which became prevalent over time (Figure 2C and Supplemental Figure 7). *M. tuberculosis* colocalized with these lipid droplets (Figure 2, C and D, and Supplemental Figure 8) and their intensity was significantly higher in MSCs than in macrophages (Figure 2E). Electron microscopy data revealed that *M. tuberculosis* hides within the lipid droplets (Figure 2F). This result is consistent with previous reports that *M. tuberculosis* uses lipids as a carbon source (20, 21). To further investigate the pathway of lipid synthesis in MSCs and to explore

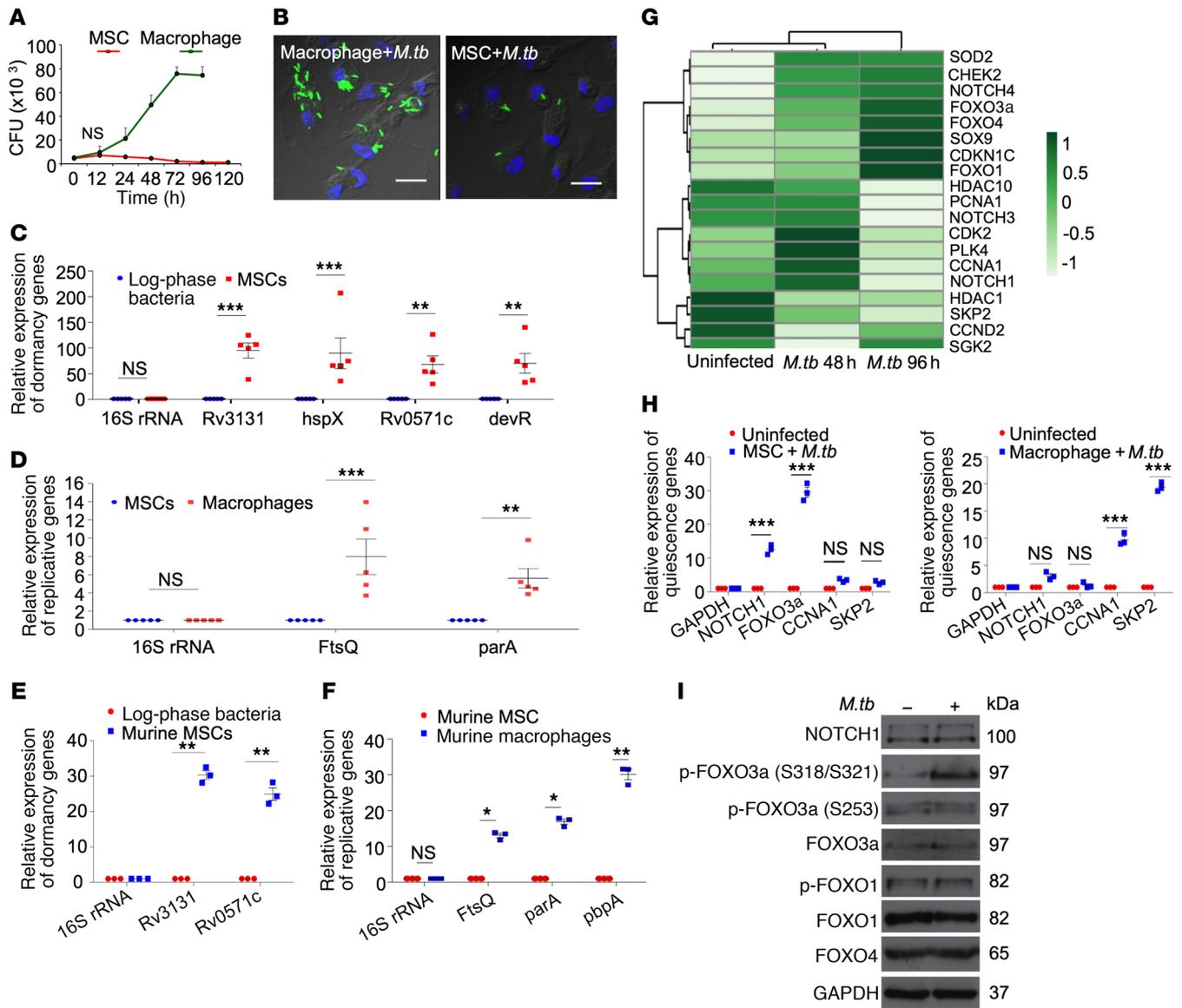


Figure 1. *M. tuberculosis* enters dormancy within MSCs through upregulation of the dosR regulon while promoting quiescence in MSCs. (A) CFU of *M. tuberculosis* (*M.tb*) in MSCs and PBMC-derived macrophages. **(B)** Confocal microscopy images of macrophages and MSCs infected with *M. tuberculosis*-GFP at 72 hours after infection. Scale bars: 5 μ m. Original magnification, $\times 40$. **(C)** Relative expression of dormancy genes of *M. tuberculosis* within human MSCs (derived from 5 donors) at 72 hours after infection as compared with log-phase bacteria. **(D)** Relative expression of replicative genes of *M. tuberculosis* in human MSCs and human macrophages (derived from PBMCs, from 5 donors) at 72 hours after infection. **(E)** Relative expression of dormancy genes of *M. tuberculosis* in CD45⁺Sca1⁺ MSCs sorted from bone marrow of infected mice as compared with log-phase bacteria. **(F)** Relative expression of replicative genes of *M. tuberculosis* in CD45⁺CD11b⁺ macrophages sorted from lungs of infected mice as compared with MSCs. **(G)** Heatmap showing the relative expression of cell proliferation and quiescence genes in uninfected and *M. tuberculosis*-infected human MSCs at 48 and 96 hours. **(H)** Validation of relative expression of cell proliferation and quiescence genes in human MSCs and macrophages (THP-1) as compared with uninfected control at 72 hours. **(I)** Western blots showing forkhead signaling pathway from uninfected and *M. tuberculosis*-infected human MSCs at 96 hours. These experiments are representative of 3 independent experiments with triplicate samples ($n = 3$). Statistical analyses were conducted using 2-way ANOVA followed by Bonferroni's post hoc test. Error bars represent SEM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. NS, $P > 0.05$.

the molecular mechanism of *M. tuberculosis* adaptation, we performed RNA-Seq analyses of infected MSCs. We found that lipid synthesis pathways, especially genes involved in sphingolipid synthesis, were highly upregulated in infected MSCs (Figure 2G). To examine the relationship between lipid synthesis and dormancy, we employed the lipid synthesis inhibitor, triacsin C. Triacsin C is a potent inhibitor of fatty acyl-CoA synthetase that strongly interferes with lipid metabolism by blocking the de novo synthe-

sis of diacylglycerols, triacylglycerols, and cholesterol (22). Inhibition of lipid synthesis resulted in profound downregulation of dormancy-related gene expression in *M. tuberculosis* (Figure 2H), with significant alteration in the expression of replicative genes (Figure 2I). These results imply that *M. tuberculosis* organisms induce lipid synthesis in MSCs and compartmentalize themselves within neolipid droplets, hence thwarting antimicrobial host defense mechanisms.

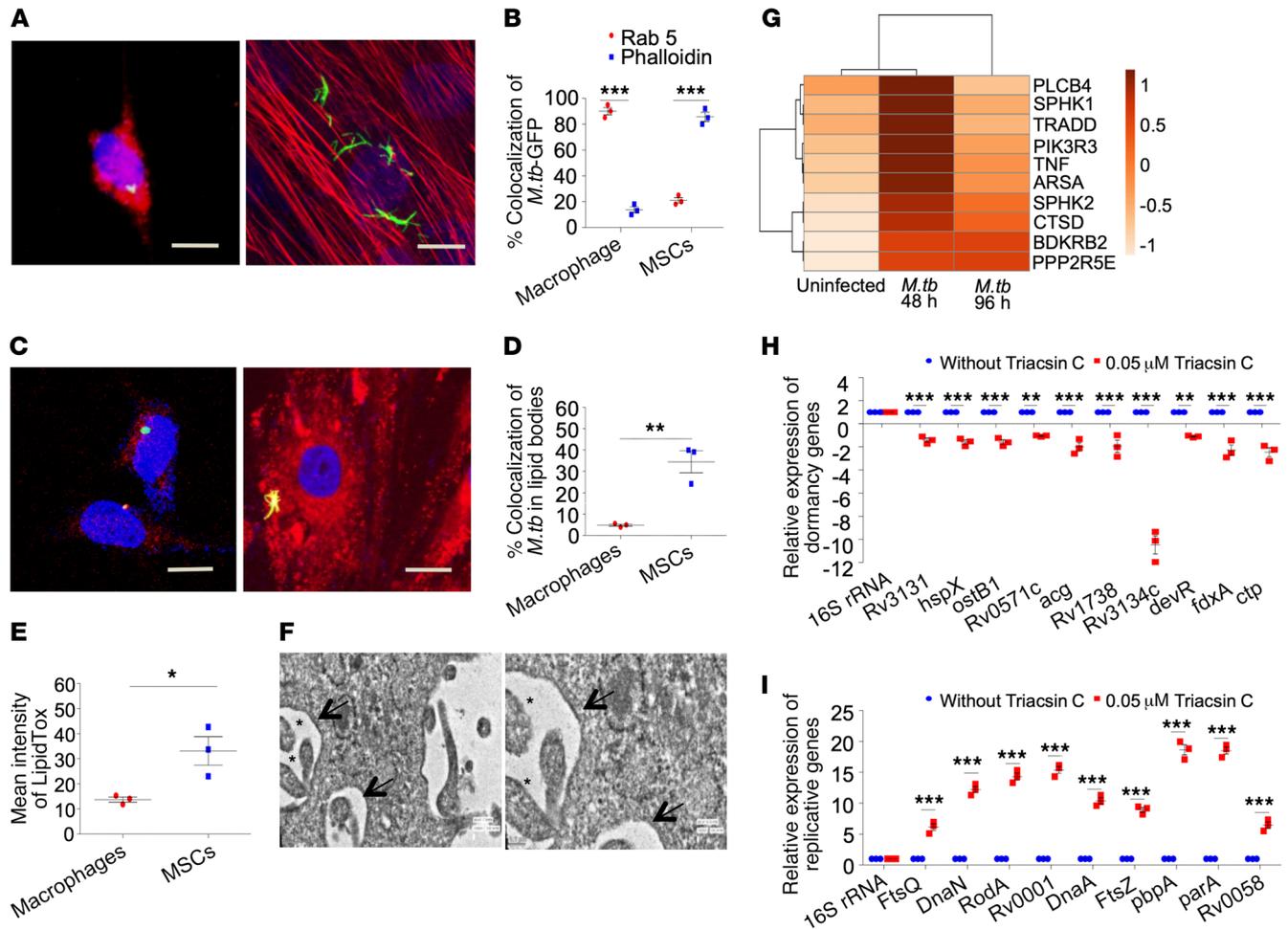


Figure 2. *M. tuberculosis* promotes host lipid synthesis and resides in lipid bodies, which is essential for maintaining latency in MSCs. (A) Confocal microscopy images showing *M. tuberculosis* (*M.tb*) localization in macrophages (THP-1) (early endosomes: Rab5) and human MSCs (cytosol: phalloidin) after 6 hours of *M. tuberculosis*-GFP infection. Each image is representative of at least 30 fields. (B) Percentage colocalization of *M. tuberculosis*-GFP with Rab5 and phalloidin in macrophages (THP-1) and human MSCs. Calculation was performed by taking the average percentage colocalization of *M. tuberculosis*-GFP with Rab5 and phalloidin in macrophages (THP-1) and human MSCs (30 fields each). (C) Confocal microscopy images showing colocalization of *M. tuberculosis*-GFP with lipid bodies (LipidTox) in macrophages (THP-1) and human MSCs at 72 hours. Scale bars (A and C): 25 μ m. (D) Percentage colocalization of *M. tuberculosis*-GFP with lipid bodies in both macrophages (THP-1) and human MSCs. (E) Mean intensity of lipid bodies stained with LipidTox in macrophages (THP-1) and human MSCs after infection with *M. tuberculosis*-GFP. (F) Transmission electron microscopy images of human MSCs infected with *M. tuberculosis*, 72 hours after infection. Lipid droplets (arrowheads) and *M. tuberculosis* (asterisk) are indicated. Original magnification, \times 9900 (left) and \times 19,500 (right). (G) Heatmap showing the relative expression of genes involved in sphingolipid synthesis in uninfected and *M. tuberculosis*-infected human MSCs at 48 hours and 96 hours. (H) Relative expression of dormancy genes of *M. tuberculosis* in infected human MSCs treated with or without triacin C (0.05 μ M) at 72 hours after infection. (I) Relative expression of replicative genes of *M. tuberculosis* inside human MSCs treated with or without triacin C (0.05 μ M) compared with macrophages (THP-1). These experiments are representative of 3 independent experiments with triplicate samples. Human MSCs were derived from 5 donors. Data in B, D, and E were analyzed by 2-tailed unpaired *t* test, and the remaining data were analyzed by 2-way ANOVA followed by Bonferroni's post hoc test. Error bars represent SEM. ****P* < 0.001, ***P* < 0.01, **P* < 0.05. NS, *P* > 0.05.

To decipher the mechanism by which MSCs provide a niche for dormancy of *M. tuberculosis*, we analyzed RNA-Seq data and found that MSCs strongly induce the expression of autophagy-related genes (Figure 3A). Inhibition of autophagy is one of the most widely adopted host-evasion mechanisms used by virulent strains of *M. tuberculosis* (23, 24). Therefore, we tested if induction of autophagy by rapamycin can eliminate *M. tuberculosis* in MSCs. We treated infected human macrophages and MSCs with INH, rapamycin, or a combination of both and assessed the viability of *M. tuberculosis* thereafter. Interestingly, we observed that addition of rapamycin reduced bacterial loads in both macrophages and MSCs in a time-dependent manner. However, effects on MSCs

were more significant than on macrophages (Figure 3, B and C). This observation indicated that autophagy can eliminate both active and dormant *M. tuberculosis* residing in macrophages and MSCs, respectively. Next, we investigated the status of dormancy and replicative gene expression in bacilli from bone marrow and lungs of *M. tuberculosis*-infected mice that were untreated or treated with INH. We found that the bacilli residing in bone marrow of the INH-treated mice were enriched with dormancy-related genes and expressed fewer replication-related genes (Figure 3, D and E). We also observed similar trends in the lung (Figure 3, F and G). We made attempts to culture these bacteria but we were unable to culture them consistently (Supplemental Figure 9, A and B), which

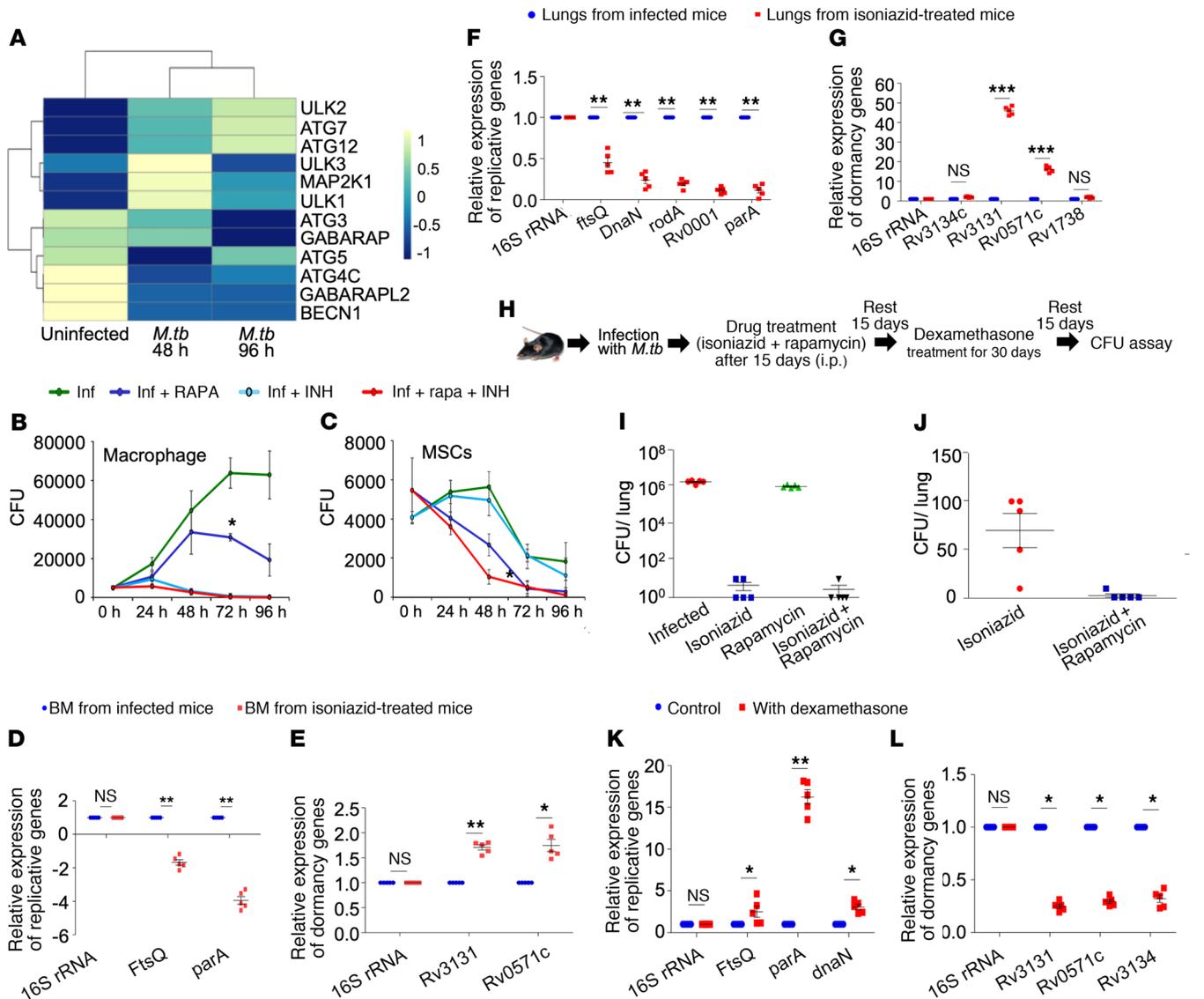


Figure 3. *M. tuberculosis* replication inside MSCs is regulated by autophagy and dormant phenotype is reduced upon immune suppression in a murine model. (A) Heatmap showing the relative expression of autophagy pathway genes in uninfected and *M. tuberculosis*-infected human MSCs at 48 hours and 96 hours. (B and C) Growth kinetics of *M. tuberculosis* in macrophages (5 donors) (B) and human MSCs (5 donors) (C) infected alone with *M. tuberculosis* and/or treated with rapamycin (1 μ M for 3 hours before infection), isoniazid (INH, 10 μ g/mL), and isoniazid plus rapamycin. (D and E) Relative expression of replicative genes (D) and dormancy genes (E) of *M. tuberculosis* from bone marrow (BM) of isoniazid-treated mice compared with infected control ($n = 5$). (F and G) Relative expression of replicative genes (F) and dormancy genes (G) of *M. tuberculosis* from lungs of isoniazid-treated mice compared with infected control ($n = 5$). (H) Schematic representation of reactivation experiment in murine model of TB after treatment with isoniazid and rapamycin. (I) *M. tuberculosis* burden in lungs isolated from mice treated with or without isoniazid, rapamycin, or isoniazid plus rapamycin ($n = 5$). (J) *M. tuberculosis* reactivation in lungs isolated from mice treated with isoniazid or isoniazid plus rapamycin followed by dexamethasone treatment ($n = 5$). (K and L) Relative expression of replicative genes (K) and dormancy genes (L) of *M. tuberculosis* from bone marrow of dexamethasone-treated mice compared with isoniazid control ($n = 5$). Experiments shown in B and C are representative of 3 independent experiments ($n = 5$). Experiments shown in panels D–L are representative of 2 independent experiments ($n = 5$). Statistical analyses were conducted using 2-way ANOVA followed by Bonferroni's post hoc test. Error bars represent SEM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. NS, $P > 0.05$.

is in agreement with previous reports that dormant *M. tuberculosis* are difficult to culture on solid media (25). As our ex vivo data indicated that INH eliminates replicating bacteria in macrophages, whereas induction of autophagy by rapamycin kills nonreplicating *M. tuberculosis* in MSCs, we validated these observations in a mouse model of TB. As expected, addition of rapamycin along with antibiotics was able to achieve sterile cure of TB (Figure 3H), as compared with INH treatment alone (Figure 3I and Supple-

mental Figure 9A). Rapamycin is known to induce autophagy by inhibiting the mTOR pathway (26). To further test if addition of rapamycin along with antibiotics indeed attains sterile cure, we employed dexamethasone to suppress immunity in animals that were previously treated with INH or with the combination of INH and rapamycin. Suppression of the immune response with dexamethasone reactivated TB disease in INH-treated animals but not in animals treated with the combination of INH and rapamycin,

as measured by CFU in the lung (Figure 3J). To our surprise, treatment with INH reduced bacterial burden less efficiently in bone marrow than in lung (compare Figure 3I and Supplemental Figure 9A). Furthermore, dexamethasone did not efficiently reactivate *M. tuberculosis* in bone marrow (compare Supplemental Figure 9, A and B). These apparent differences between lung and bone marrow might be due to differential drug penetration in these organs. In future studies we will seek to identify TB drugs that effectively penetrate bone marrow. Interestingly, dexamethasone treatment strikingly upregulated replicative genes in the harbored *M. tuberculosis* in these animals (Figure 3K) and dramatically reduced expression of dormancy-related genes (Figure 3L), indicating that immune suppression converts dormant bacteria into an active form in these animals. Taken together, these observations strongly imply that a combination of INH and rapamycin can be used to eliminate actively replicating as well as latent bacteria to achieve sterilizing TB cure.

Our data indicate that MSCs are a natural reservoir for latent *M. tuberculosis* infection, whereas macrophages support the replicating form of *M. tuberculosis*. *M. tuberculosis* acquires dormancy in MSCs, which in turn induces MSCs to acquire quiescence. *M. tuberculosis* induces synthesis of lipid droplets, which are employed by the organism to hide from host defense mechanisms. Successful treatment of TB requires elimination of both replicating and dormant bacteria. Dormant bacteria do not respond to conventional antibiotics but can be eliminated by inducing autophagy. Therefore, a combination of antibiotics and inducers of autophagy provides the opportunity for the successful treatment of TB.

Methods

This study was ethically approved by the Institutional Committee for Stem Cell Research, All India Institute of Medical Sciences, New Delhi, India [reference number: IC-SCR/47/16(R)]. Detailed

information regarding materials and methods can be found in Supplemental Methods.

Author contributions

SF, SSK, VPD, DB, and SK performed the experiments and analyzed data. GD conceived the hypothesis and both GD and SM supervised the experiments. SF, VPD, AR, LVK, and GD wrote the manuscript.

Acknowledgments

We thank the Director of the Council of Scientific and Industrial Research Institute of Genomics and Integrative Biology (CSIR-IGIB) and Manish Kumar for assistance with confocal imaging. The authors also thank the National Institute of Biomedical Genomics (NIBMG), India and Partha P. Majumder for help with the RNA-Seq studies. SF is supported by a University Grants Commission Senior Research Fellowship (UGC-SRF). SSK is a recipient of a National Post Doctoral Fellowship (NPDF) from the Science and Engineering Research Board-Department of Science and Technology (SERB-DST). VPD is a DST-INSPIRE faculty awardee. DB is supported by CSIR-Senior Research Associate. We thank the International Centre For Genetic Engineering and Biotechnology (ICGEB) at Delhi University, All India Institute of Medical Sciences, and IGIB for generously providing BSL-3 facilities. This work was supported by grants from the Department of Biotechnology (BT/PR24544/MED/29/1217/2017), Department of Science and Technology (SERB/F/4821/2017-18), and the Indian Council of Medical Research (Special Centre for Molecular Medicine is funded by ICMR).

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