JCI The Journal of Clinical Investigation

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J Clin Invest. 2019. https://doi.org/10.1172/JCI128043.

Concise Communication In-Press Preview **Infectious disease**

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Mycobacterium tuberculosis Programs Mesenchymal Stem Cells to Establish Dormancy and Persistence

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Short Title: Adaptation of *M.tb* in MSCs

Keywords: *Mycobacterium tuberculosis*, Latent TB, Lipid-synthesis, Mesenchymal Stem Cells (MSCs).

Conflict of Interest: The authors declare that there is no conflict of interest.

Abstract:

Tuberculosis (TB) remains a major infectious disease worldwide. TB treatment displays a bi-phasic bacterial clearance, in which the majority of bacteria clear within the first month of treatment, but residual bacteria remains non-responsive to treatment and eventually may become resistant. Here, we have shown that *Mycobacterium tuberculosis* (*M.tb)* is taken up by mesenchymal stem cells (MSCs), where it established dormancy and became highly non-responsive to isoniazid, a major constituent of Directly Observed Treatment Short-course (DOTS). Dormant *M.tb* induced quiescence in MSCs and promoted their long-term survival. Unlike macrophages, where *M.tb* 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.tb* gains dormancy in MSCs, which thus serve as a long-term natural-reservoir of dormant *M.tb*. Interestingly, in the murine-model of TB, induction of autophagy eliminated *M.tb* 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 (M.tb),* is the oldest known human infectious disease. Current therapy of 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.tb* (1). The remaining small numbers of organisms are highly non-responsive 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 will 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.tb* organisms. Macrophages are the natural-host for *M.tb,* in which they replicate and survive by employing a variety of host-evasion mechanisms that include inhibition of phagolysosome fusion (5, 6), de-acidification of lysosomal compartments (7), and translocation to the cytosol (8). These bacteria respond to antibiotics and are readily cleared. However, non-replicating bacteria survive within granulomatous structures containing mesenchymal stem cells (MSCs), with limited accessibility to therapeutics (9). Recently, we and others have shown that *M.tb* infects MSCs (9, 10). In some cases *M.tb* was detected in patients who had completed Directly Observed Treatment Short-Course (DOTS) treatment (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.tb*.

The mechanism by which *M.tb* adapts to MSCs, and the targets in MSCs that allow persistence of *M.tb* remain unknown.

M.tb organisms 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 hide out 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.tb* acquires dormancy and thereby drug non-responsiveness in MSCs.

Here, we show that MSCs are a natural-host for dormant *M.tb*. Upon uptake by MSCs, *M.tb* induces the expression of dormancy-related genes and promotes quiescence in MSCs. In contrast, *M.tb* residing in macrophages continues to replicate and causes macrophage necrosis. INH does not affect *M.tb* survival in MSCs but successfully eliminates bacteria from macrophages. In macrophages, most of the organisms are found in early-phagosomal compartments, but in MSCs nearly all bacilli are present in the cytosol. *M.tb* promotes rapid lipid-synthesis in MSCs, which causes lipid-droplets to form that shield the harbored bacteria. Inhibition of lipid-synthesis drastically 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.tb* infection. *M.tb* infection of MSCs is associated with an autophagy-related gene expression signature, and induction of autophagy with rapamycin eliminates *M.tb* 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 non-replicating *M.tb* (9, 10, 13). Therefore, we sought to determine whether MSCs are a natural-reservoir for *M.tb* and dormancy that renders non-responsiveness to antibiotic treatment. We infected human MSCs and peripheral blood mononuclear cell (PBMC)-derived macrophages (**Supplemental Figure 1**) with *M.tb.* We found that, to attain a saturation of infection in macrophages, four hours of infection at 1:10 Multiplicity Of Infection (MOI) was required, whereas six hours at 1:50 MOI attained saturation of infection in MSCs. Under these conditions, similar numbers of bacilli were taken up by these two cell types **(Figure 1A and B).** Thus, it appears that MSCs are less permissive than macrophages for M.tb infection, which might be evolutionary, related to latency of M.tb in MSCs.

With the progression of time, *M.tb* continued to replicate and macrophages became necrotic by 96 hours of infection (**Supplemental Figure 2A-E**)**.** Strikingly, *M.tb* 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.tb* in macrophages and MSCs, we examined the expression of replication and dormancy-related genes in *M.tb* isolated from infected macrophages and MSCs. We found sustained expression of dormancy-related devR/dosR regulon genes in *M.tb* isolated from MSCs (**Figure 1C and Supplemental Figure 3**). However, genes that are involved in various steps of *M.tb* replication were enriched in *M.tb* isolated from infected macrophages **(Figure 1D)**.

To explore in vivo relevance, we sorted $CD45^{-}Sca1^{+}$ MSCs from the bone marrow (BM) and $CD45^{+}CD11b^{+}$ macrophages from the lungs of *M.tb* infected mice. Consistent with the in vitro data, we found that *M.tb* in MSCs express dormancy-related genes whereas *M.tb* that are in macrophages express replication-related genes **(Figure 1E and F)**. Taken together,

these observations strongly suggested that macrophage and MSCs are differentially-programmed for supporting active and dormant infection, respectively.

Our findings showed that MSCs are less permissive to M.tb infection and allow the bacteria to establish dormancy. It will be interesting to determine if other non-permissive cells such as hepatocytes or fibroblasts similarly allow M.tb to establish dormancy. Although it has been reported that M.tb can infect and replicate in fibroblasts (14, 15) we were unable to infect fibroblasts.

Next, we explored whether *M.tb* infection affects MSC replication and found that *M.tb* inhibits MSC replication in a time-dependent fashion. Therefore, we measured expression of quiescence-markers characteristic of stem cells (16, 17). RNA-sequencing analysis revealed upregulation of several quiescence-markers and downregulation of cell cycle progression markers in human MSCs infected with *M.tb* (**Figure 1G**). This was confirmed by qPCR of selected quiescence-markers such as FOXO-3, NOTCH-1 and SOX-9, which were upregulated in MSCs as compared to macrophages (**Figure 1H**). In contrast, cellular proliferation markers, S-phase kinase 2 (SKP2) and CCNA encoding cyclin A2 were highly upregulated in macrophages (**Figure 1H**). Western-blot analysis confirmed enhanced expression of NOTCH-1, FOXO-3 and p-FOXO-3 at Ser318/321 (**Figure 1I and Supplemental Figure 10**). Phosphorylation of FOXO-3a at Ser318 and/or 321 causes its nuclear exclusion and inhibits its transcriptional activity (23). Thus, the increased FOXO-3a phosphorylation might be essential in modifying transcriptional activity to inhibit MSC proliferation. Although phosphorylation of FOXO-3a 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 FOXO-3a at this site. Additionally, there was no significant difference in the protein levels of FOXO-1 and FOXO-4 or p-FOXO-1 **(Supplemental Figure 4)**, suggesting that these quiescence-markers might play a prominent

158 role in attaining a quiescent-state in MSCs. This observation implied that upon infection,
159 *M.tb* acquires dormancy whereas MSCs enter into a quiescent-state. This dual strategy may
160 assist *M.tb* to better shield itself from the host immune system and drugs used for treatment.
161 It is intriguing that macrophages, which are equipped with phagolysosomal killing
162 mechanisms, are permissive to *M.tb* replication, whereas MSCs, which lack a well-defined
163 phagosomal-system compared to macrophages, restrict *M.tb* growth (7, 8). To obtain insight
164 into this apparent paradox of *M.tb* infection, we determined the intracellular localization of
165 GFP-labeled *M.tb* in human macrophages and MSCs. To determine endosomal localization of
166 *M.tb*, we employed an antibody directed against the early-endosomal marker, Rab5, whereas
167 for cytosolic localization, we employed phalloidin, which selectively binds F-actin (19). We
168 observed that in macrophages, most of the *M.tb* localized to early-endosomes immediately
169 after infection, whereas the majority of bacilli in MSCs were found in the cytosol (**Figure 2A**
170 **and B, Supplemental Figure 5 and 6**). Interestingly, we also observed abnormal lipid-
171 droplets in MSCs, which became prevalent over time (**Figure 2C and Supplemental Figure**
172 **7**). *M.tb* co-localized with these lipid-droplets (**Figure 2C and D, Supplemental Figure 8**)
173 and their intensity was significantly higher in MSCs than macrophages (**Figure 2E**). Electron
174 microscopy data revealed that *M.tb* hides within the lipid-droplets (**Figure 2F**). This result is
175 consistent with previous reports that *M.tb* uses lipids as a carbon source (20, 21). To further
176 investigate the pathway of lipid-synthesis in MSCs and to explore the molecular mechanism
177 of *M.tb* adaptation, we performed RNA-seq analyses of infected MSCs. We found that lipid-
178 synthesis pathways, especially genes involved in sphingolipid-synthesis, were highly
179 upregulated in infected MSCs (**Figure 2G**). To examine the relation of lipid-synthesis with
180 dormancy, we employed the lipid-synthesis inhibitor, Triacsin C. Triacsin C is a potent
181 inhibitor of fatty acyl-CoA synthetase that strongly interferes with lipid metabolism by
182 blocking the de novo synthesis of diacylglycerols, triacylglycerols and cholesterol (22).

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

To decipher the mechanism by which MSCs provide a niche for dormancy of *M.tb*, 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.tb* (23, 24). Therefore, we tested if induction of autophagy by rapamycin can eliminate *M.tb* in MSCs. We treated infected human macrophages and MSCs with INH, rapamycin or a combination of both and assessed the viability of *M.tb* 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 macrophages (**Figure 3B and C**). This observation indicated that autophagy can eliminate both active and dormant *M.tb* 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.tb* 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 3D and E)**. We also observed similar trends in lung **(Figure 3F and G)**. We made attempts to culture these bacteria but we were unable to culture them consistently **(Supplemental Figure 9A and B),** which is in agreement with previous reports that dormant M.tb are hard 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 non-replicating *M.tb* 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 to INH treatment alone (**Figure 3I** and **Supplemental 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 vs Supplemental Figure 9A).** Furthermore, dexamethasone did not efficiently reactivate M.tb in bone marrow **(compare Supplemental Figure 9A vs 9B).** 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.tb* 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.

Therefore, our data indicates that MSCs are a natural-reservoir for latent *M.tb* infection, whereas macrophages support the replicating form of *M.tb*. *M.tb* acquires dormancy in MSCs, which, in turn induces MSCs to acquire quiescence. *M.tb* 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 (AIIMS), 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 supervised the experiments. SF, VPD, AR, LVK and GD wrote the manuscript.

Acknowledgements

We thank the Director, CSIR-IGIB and Mr. Manish Kumar for assistance with confocal imaging. The authors also thank National Institute of Biomedical Genomics (NIBMG), India and Dr. Partha P. Majumder for help with the RNA-sequencing studies. SF is supported by the UGC-SRF; SSK is an NPDF, SERB-DST. VPD is DST-INSPIRE Faculty-awardee. DB is supported by CSIR-SRA. We thank ICGEB, Delhi University, AIIMS, and IGIB for generously providing BSL-3 facilities. The work was supported by grants from the Department of Biotechnology, Department of Science and Technology and Indian Council of Medical Research.

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Figures and Legends:

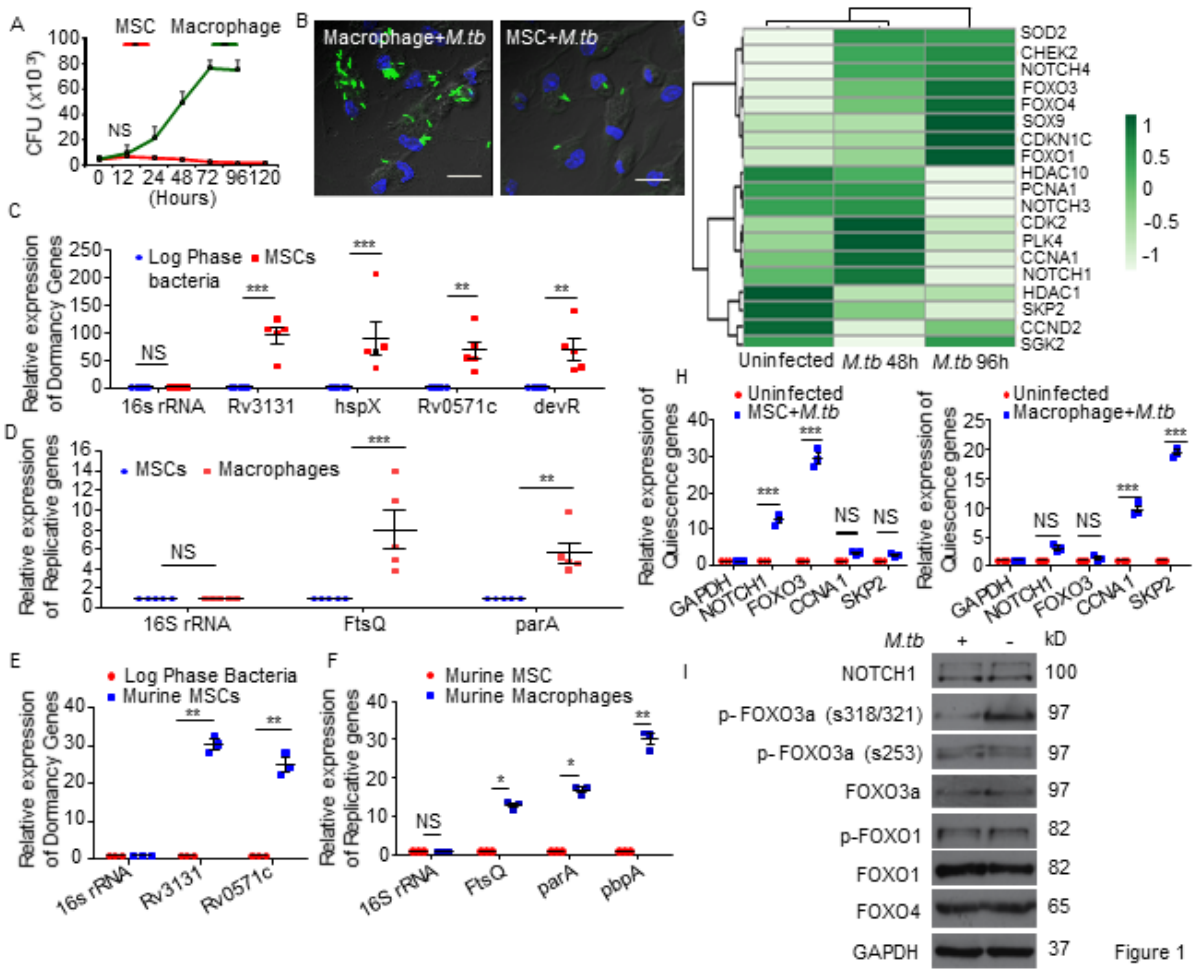

Figure 1: *M.tb* enters dormancy within MSCs through upregulation of DosR regulon while promoting quiescence in mesenchymal stem cells. (A) CFU of *M.tb* in MSCs and PBMC-derived macrophages. **(B)** Confocal microscopy images of macrophages and MSCs infected with *M.tb*-GFP (bar=5µM) at 72 hours post-infection (40X magnification). **(C)** Relative expression of dormancy genes of *M.tb* within human MSCs (derived from 5 donors) at 72 hours post-infection as compared to log-phase bacteria. **(D)** Relative expression of replicative genes of *M.tb* in human MSCs and human macrophages (derived from PBMCs, from 5 donors) at 72 hours post-infection. **(E)** Relative expression of dormancy genes of *M.tb* in $CD45^{-}Sca1^{+}$ MSCs sorted from bone-marrow of infected mice as compared to log-phase bacteria. **(F)** Relative expression of replicative genes of *M.tb* in $CD45^{+}CD11b^{+}$ macrophages sorted from lungs of infected mice as compared to MSCs. **(G)** Heat map showing the relative expression of cell proliferation and quiescence genes in uninfected and *M.tb* 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 to uninfected control at 72 hours. **(I)** Western-blots showing forkhead signaling pathway from uninfected and *M.tb* infected human MSCs at 96 hours. These experiments are representative of three independent experiments with triplicates (n=3). Statistical analyses were conducted using two-way ANOVA followed by Bonferroni post-test. Error bar represent S.E.M.*** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken non-significant (NS).

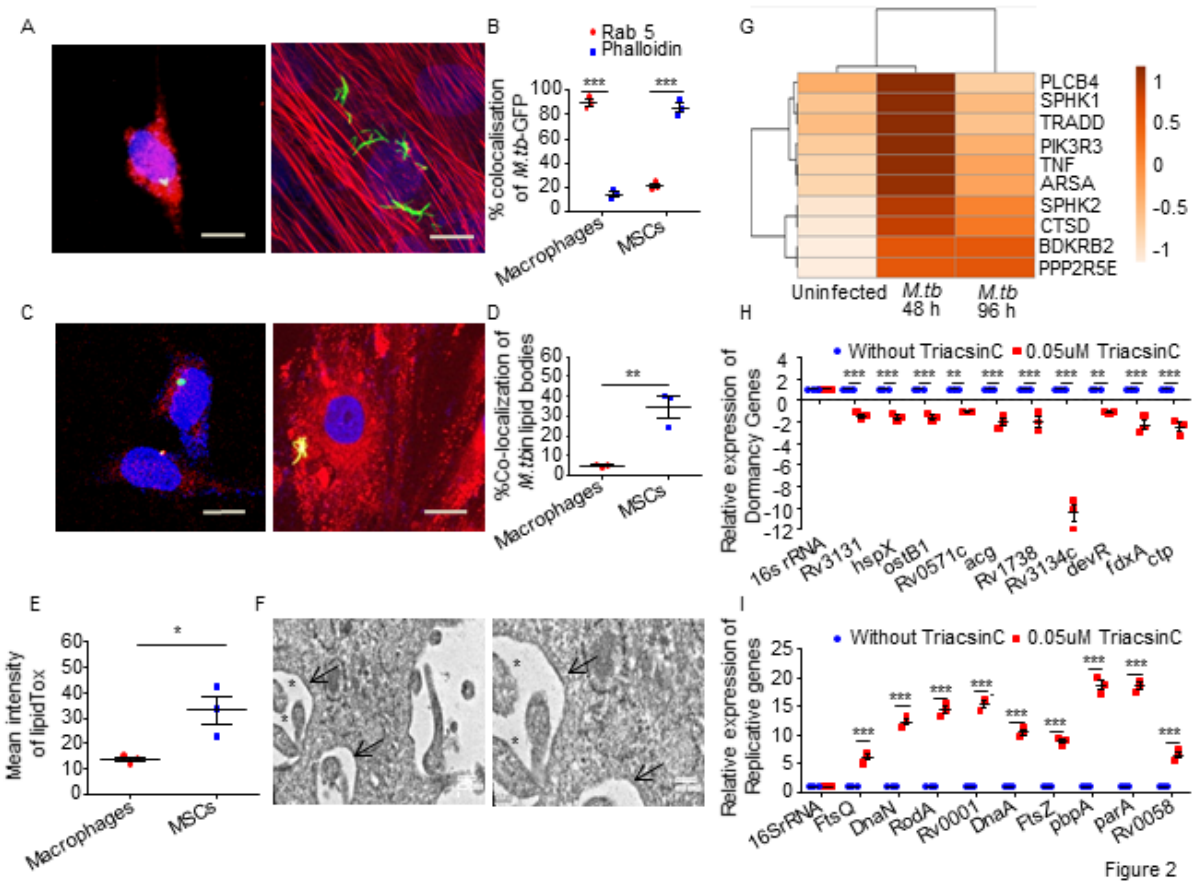

Figure 2

Figure 2: *M.tb* promotes host lipid-synthesis and resides in lipid-bodies, which is essential for maintaining latency in MSCs. (A) Confocal microscopy images showing *M.tb* localization in macrophages (THP-1) (early-endosomes: Rab5) and human MSCs (cytosol: Phalloidin) after 6 hours of *M.tb*-GFP infection. Each image is a representation of at least 30 fields. **(B)** Percentage co-localization of *M.tb*-GFP with Rab5 and Phalloidin in macrophages (THP-1) and human MSCs. Calculation was done by taking the average percent co-localization of *M.tb*-GFP with Rab5 and phalloidin in macrophages (THP-1) and human MSCs (30 fields each). **(C)** Confocal microscopy images showing co-localization of *M.tb*-GFP with lipid-bodies (LipidTox) in macrophages (THP-1) and human MSCs at 72 hours. **(D)** Percentage co-localization of *M.tb*-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 post-infection with *M.tb*-GFP. **(F)** Transmission Electron Microscopy (TEM) images of human MSCs infected with *M.tb*, 72 hours post-infection. Lipid-droplets (arrowheads) and *M.tb* (*asterisk) are indicated. Images were taken at 9900X (left) and 19500X (right). **(G)** Heatmap showing the relative expression of genes involved in the sphingolipid-synthesis in uninfected and *M.tb* infected human MSCs at 48 hours and 96 hours. **(H)** Relative expression of dormancy genes of *M.tb* in infected human MSCs treated with or without Triacsin C (0.05 µM) at 72 hours post-infection**. (I)** Relative expression of replicative genes of *M.tb* inside human MSCs treated with or without Triacsin C (0.05 µM) compared to macrophages (THP-1). These experiments are representative of three independent experiments with triplicates. Human MSCs were derived from 5 donors. For figure, 2B, 2D and 2E, two-tailed unpaired t-test was used, and the remainder data were analysed by two-way ANOVA followed by Bonferroni post-test. Error bar represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken non-significant (NS).

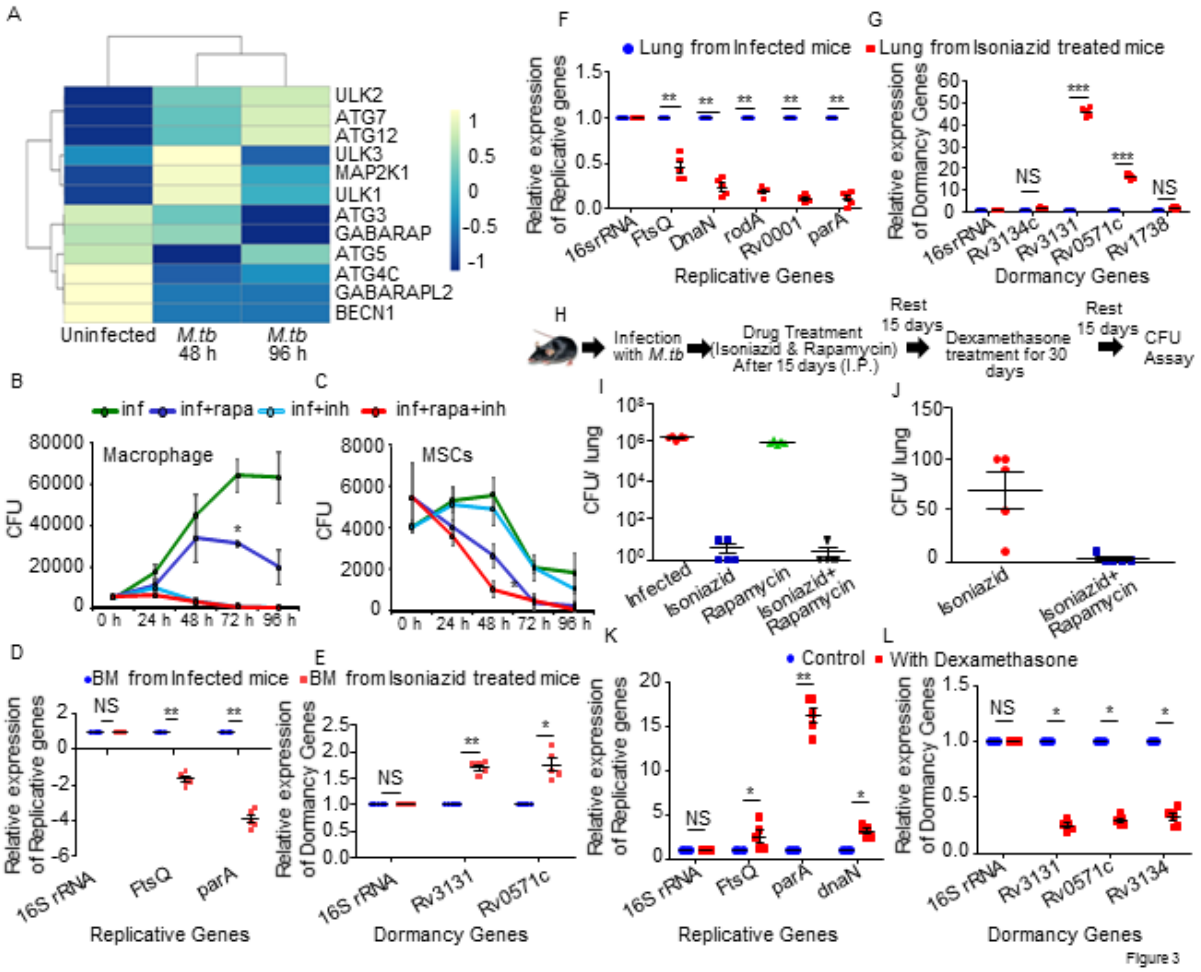

Figure 3: *M.tb* replication inside MSCs is regulated by autophagy and dormant phenotype is reduced upon immune suppression in murine-model. (A) Heat map showing the relative expression of autophagy pathway genes in uninfected and *M.tb* infected human MSCs at 48 hours and 96 hours. **(B and C)** Growth kinetics of *M.tb* in macrophages (5 donors) **(B)** and human MSCs (5 donors) **(C)** either infected alone with *M.tb* and/or treated with rapamycin (1 µM for 3 hours before infection), isoniazid (10 µg/ml) and isoniazid + rapamycin. **(D and E)** Relative expression of replicative genes **(D)** and dormancy genes **(E)** of *M.tb* from bone marrow of isoniazid-treated mice compared to infected control (n=5). **(F and G)** Relative expression of replicative genes **(F)** and dormancy genes **(G)** of *M.tb* from lungs of isoniazid-treated mice compared to infected control (n=5). **(H)** Schematic representation of reactivation experiment in murine-model of TB after treatment with isoniazid and rapamycin. **(I)** *M.tb* burden in lungs isolated from mice treated with or without isoniazid, rapamycin or isoniazid+rapamycin (n=5). **(J)** *M.tb* reactivation in lungs isolated from mice treated with isoniazid or isoniazid+rapamycin followed by dexamethasone treatment (n=5). **(K and L)** Relative expression of replicative genes **(K)** and dormancy genes **(L)** of *M.tb* from bone marrow of dexamethasone-treated mice compared to INH control (n=5). Experiments shown in panels B and C are representative of three independent experiments (n=5). Experiments shown in panels D-L are representative of two independent experiment (n=5). Statistical analyses were conducted using two-way ANOVA followed by Bonferroni post-test. Error bars represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken as non-significant (NS).