Published in final edited form as:
*Sci Transl Med*. 2013 January 30; 5(170): 170ra13. doi:10.1126/scitranslmed.3004912.

# CD271+ Bone Marrow Mesenchymal Stem Cells May Provide a Niche for Dormant *Mycobacterium tuberculosis*

**Bikul Das**[1,*], **Suely S. Kashino**[2], **Ista Pulu**[3], **Deepjyoti Kalita**[4,5], **Vijay Swami**[3], **Herman Yeger**[6], **Dean W. Felsher**[1,*,†], and **Antonio Campos-Neto**[2,†]

[1]Division of Oncology, Departments of Medicine and Pathology, Stanford University School of Medicine, Stanford, CA, USA

[2]Global Infectious Disease Research Center, Forsyth Institute, Cambridge, MA, USA

[3]Research Institute of World's Ancient Traditions, Cultures and Heritages (RIWATCH), Roing, Arunachal Pradesh, India

[4]KaviKrishna Laboratory, Guwahati Biotech Park, Technology Complex, Indian Institute of Technology, Guwahati, India

[5]Department of Microbiology, Gauhati Medical College, Guwahati, India

[6]Department of Pediatric Laboratory Medicine, Hospital for Sick Children, Toronto, Ontario, Canada

## Abstract

*Mycobacterium tuberculosis* (*Mtb*) can persist in hostile intracellular microenvironments evading immune cells and drug treatment. However, the protective cellular niches where *Mtb* persists remain unclear. We report that *Mtb* may maintain long-term intracellular viability in a human bone marrow (BM)–derived $CD271^+/CD45^-$ mesenchymal stem cell (BM-MSC) population in vitro. We also report that *Mtb* resides in an equivalent population of BM-MSCs in a mouse model of dormant tuberculosis infection. Viable *Mtb* was detected in $CD271^+/CD45^-$ BM-MSCs isolated from individuals who had successfully completed months of anti-*Mtb* drug treatment. These results suggest that $CD271^+$ BM-MSCs may provide a long-term protective intracellular niche in the host in which dormant *Mtb* can reside.

## INTRODUCTION

Tuberculosis (TB) infects nearly 2.2 billion people worldwide (1, 2). Effective drugs that can target replicating *Mycobacterium tuberculosis* (*Mtb*) have been available for 50 years. Yet, the eradication of this disease remains elusive. This has been largely attributed to the ability of *Mtb* to maintain a latent or dormant infection in a host despite the evidence for a vigorous host immune response (1, 2).

*To whom correspondence should be addressed. bikuldas@stanford.edu (B.D.); dfelsher@stanford.edu (D.W.F.).
†These authors contributed equally to this work.

**Author contributions:** B.D. initiated, designed, and supervised the study. B.D. and S.S.K. performed the in vitro and in vivo experiments. B.D., D.K., V.S., and I.P. performed the clinical study. H.Y. contributed reagents/analytic tools. B.D., A.C.-N., and D.W.F. analyzed the data. B.D., H.Y., A.C.-N., and D.W.F. wrote the paper.

**Competing interests:** B.D. is a founding director and now the honorary director of KaviKrishna Foundation. The Foundation is a not-for-profit, nongovernmental organization based in Assam, India. The Foundation is involved in the clinical study described in the manuscript. The other authors declare that they have no competing interests.

Dormant *Mtb* may remain in a nonreplicating state during asymptomatic infection (1). In addition, dormant *Mtb* can tolerate the extreme hypoxic environment present in the tuberculous granulomas in lung tissue (3, 4). Dormant *Mtb* remains sensitive to antitubercular drugs like rifampicin (4, 5), as illustrated by their reduced viability in dormant, fibrotic granulomas after drug treatment (6). However, it is not yet clear how *Mtb* retains viability during the asymptomatic infection phase as well as during post-chemotherapy dormancy, such that it can be reactivated causing clinical disease (7).

Nonreplicating *Mtb* may reside in a protective intracellular niche to maintain viability (1). Therefore, identification of the protective intracellular niches that enable *Mtb* to remain in a viable nonreplicating dormant state is urgently needed to understand the pathogenesis of this disease and to enhance our ability to develop better drugs and vaccines.

Macrophages and dendritic cells have been known for decades to serve as host cells for *Mtb* growth (8). However, the viability of *Mtb* in these intracellular niches is poor (9), and no evidence exists indicating that these cells can maintain live nonreplicating *Mtb*. Other cell types such as epithelial cells, fibrocytes, adipocytes, and endothelial cells distributed in pulmonary and extrapulmonary niches have been described as possible host cells for nonreplicating *Mtb* (1, 10, 11). These possibilities have been suggested on the basis of human autopsy studies that demonstrated the presence of *Mtb* DNA in these cell types (10, 11). However, viable nonreplicating *Mtb* in these cell types during infection in vivo have not been demonstrated. Hence, to date, although *Mtb* is known to infect many cell types, the evidence that any of these host cells may serve as a reservoir of live nonreplicating *Mtb* in vivo has as yet not been shown.

We postulated that bone marrow stem cells (BMSCs), comprising both hematopoietic and mesenchymal stem cells (MSCs), might provide an ideal protective niche for nonreplicating *Mtb* because these cells have several properties that are ideal for the pathogen's long-term persistence and survival. First, these cells are present in the TB granulomas of infected mouse and human lung tissue (12). Second, they have the capacity for self-renewal (13–15). Third, they express drug efflux pumps such as ABCG2 that could contribute to drug evasion by *Mtb* (16). Fourth, stem cells produce only low levels of endogenous reactive oxygen species, which might benefit the viability of nonreplicating *Mtb*. Fifth, they are relatively quiescent (17) and reside in the immune-privileged niche in the BM (18, 19). Therefore, we set out to examine whether BMSCs could be a host cell for the long-term persistence of viable nonreplicating *Mtb*.

Here, we report that *Mtb* may persist in a mesenchymal subpopulation of human BMSCs from patients previously treated for pulmonary TB and of mouse BMSCs from a mouse model of nonreplicating *Mtb* infection. Our results suggest that a BMSC subpopulation, $CD271^+/CD45^-$ mesenchymal BMSCs (19–25), may provide an intracellular niche for *Mtb* persistence. $CD271^+/CD45^-$ cells purified from the BM of mice infected with nonreplicating *Mtb* maintained the ability to reseed infection when injected into healthy mice. Viable *Mtb* could be recovered from the$CD271^+/CD45^-$ cells obtained from individuals who had completed antitubercular treatment. Our observations suggest that a BM cellular niche may be important for the maintenance of the nonreplicating phase of the *Mtb* life cycle.

## RESULTS

### *Mtb* infects and survives in human $CD271^+/CD133^+$ BMSCs

The general stem cell marker CD133 of BMSCs provided the starting point for further subfractionation of human BM-derived stem cells. To determine whether *Mtb* can infect specific $CD133^+$ BMSC populations, we isolated the $CD271^+/CD133^+$, $CD271^-/CD133^+$,

and $CD34^+/CD133^+$ populations from healthy human donors by magnetic sorting (20). The cells were then cultured in vitro in serum-free medium containing growth factors that can maintain $CD133^+$ BMSCs in their undifferentiated state (table S1) (14, 26). Next, these purified subpopulations were exposed to either the virulent strain of *Mtb* H37Rv or the avirulent strain of *Mtb* H37Ra. The *Mtb*-exposed cells were then briefly treated with amikacin to kill residual extracellular bacteria. Subsequently, viable intracellular *Mtb* were measured by colony-forming unit (CFU) assay using Middlebrook 7H11 agar plates (27). The $CD271^+/CD133^+$ BMSC fraction exhibited higher *Mtb* CFUs than did the other BMSC fractions exposed to either *Mtb* H37Rv or H37Ra ($P < 0.05$; Fig. 1A). These results indicated that BMSCs can be infected in vitro with *Mtb* and that the $CD271^+/CD133^+$ BMSC fraction was the most permissive for *Mtb* infection.

In the next step, the long-term viability of both the host cells and *Mtb* from *Mtb*-infected $CD271^+/CD133^+$ BMSCs was measured after 2 weeks of in vitro culture (Fig. 1B). The infection of $CD271^+/CD133^+$ human BMSCs with either *Mtb* H37Ra or *Mtb* H37Rv did not change the viability of the host cells as measured by Alamar blue assay (28). The number of *Mtb* CFUs increased only two- to threefold between 0 and 4 days of infection ($P < 0.05$; Fig. 1C) and then remained unchanged. These results suggest that the internalized and viable avirulent and virulent *Mtb* strains can replicate, albeit slowly, without impeding the growth of $CD271^+/CD133^+$ BMSCs.

To further examine and quantify *Mtb* infection of $CD271^+/CD133^+$ human BMSCs, we used PKH26-labeled *Mtb* (27) to visualize internalized mycobacteria by fluorescence microscopy (Fig. 2A) and to isolate them by fluorescence-activated cell sorting (FACS). Internalized H37Ra *Mtb* were clearly visible (Fig. 2A), and viable *Mtb* could be isolated from the FACS-sorted $CD271^+/PKH26^+$ cells (Fig. 2, B and C). As a control, labeled heat-killed *Mtb* or rifampicin-killed *Mtb* were confirmed to show markedly reduced intracellular internalization with fewer *Mtb* DNA copies associated with $CD271^+/CD133^+$ BMSCs (fig. S1). Thus, viable *Mtb* H37Ra can be internalized and retained in $CD271^+/CD133^+$ BMSCs.

$CD271^+/CD133^+$ BMSCs contain both hematopoietic stem cells (HSCs; $CD34^+/CD45^+$) and MSCs ($CD271^+/CD45^-$) (20–22). To define which subpopulation was preferentially infected by *Mtb*, we examined FACS-sorted $CD271^+/PKH26^+$ cells for HSC and MSC markers by real-time quantitative polymerase chain reaction (qPCR) gene expression and FACS analysis. Gene expression analysis revealed that the *Mtb*-infected cells expressed MSC markers CD105, CD73, and CD90, whereas the hematopoietic markers CD45 and CD11b were not expressed (fig. S2A).Moreover, expression of the HSC marker CD34 and endothelial progenitor marker VEGFR2 (vascular endothelial growth factor receptor 2) was negligible in the $CD271^+/PKH26^+$ cells (fig. S2A). Also, the $CD271^+/PKH26^+$ cells expressed ABCG2 (fig. S2A), a drug efflux pump expressed in the undifferentiated $CD271^+/CD133^+$ cells (table S1) (20). The FACS analysis of the $CD271^+/PKH26^+$ cells confirmed the expression of the MSC markers CD105, CD73, and CD90, and the absence of the hematopoietic marker CD45 (fig. S2B). We conclude that *Mtb* preferentially infects $CD271^+/CD45^-$ BM MSCs (henceforth called $CD271^+$ BM-MSCs).

Finally, we examined if *Mtb* H37Rv also preferentially infects the $CD271^+$ BM-MSCs. Magnetic sorting was performed to isolate the $CD271^+/CD45^-$ fraction from the infected $CD271^+/CD133^+$ BMSCs grown in serum-free culture for 2 weeks (see Supplementary Materials and Methods). Purity of the $CD271^+/CD45^-$ cells was confirmed ($89.2 \pm 4.4\%$; $n = 4$), as shown in Fig. 2D. Most of the *Mtb* H37Rv CFUs were obtained from the $CD271^+/CD45^-$ versus $CD271^-/CD45^-$ fraction ($P < 0.0001$; Fig. 2E). We conclude that *Mtb* preferentially infects $CD271^+$ BM-MSCs.

### Undifferentiated CD271+ BM-MSCs are needed to maintain *Mtb* viability

Next, we investigated whether the undifferentiated state of CD271+ BM-MSCs (20) is required to maintain the viability of intracellular *Mtb*. FACS-sorted CD271+/PKH26+ cells were cultured in a serum-rich medium that induces their differentiation as illustrated by the loss of the CD271/CD133 markers (table S1) (20). *Mtb*-infected CD271+ BMMSCs were treated with adipogenic differentiation medium to induce their differentiation from BM-MSCs to adipocytes (28). The results (Fig. 3A) illustrate that *Mtb* viability in the differentiated CD271+ BM-MSCs was reduced by fourfold ($P < 0.05$). Similar findings were obtained with *Mtb* H37Rv infection of undifferentiated versus serum-rich and adipogenic differentiation medium–treated CD271+ BM-MSCs ($P < 0.001$; Fig. 3B). Hence, *Mtb* viability appears to require the undifferentiated state of CD271+ BM-MSCs.

### Dissemination of *Mtb* H37Rv from lungs to BM in mice infected with aerosolized bacteria

To translate our in vitro results to an in vivo infectious process, we used a well-established mouse model of TB. In this model, mice are infected via the respiratory route with a low number of aerosolized virulent *Mtb* (~100 CFUs per mouse) (29). *Mycobacterium* grows in the murine lungs and subsequently is disseminated to organs such as spleen and liver. To evaluate whether the infectious process would also enable dissemination of *Mtb* from the lung to BM, we infected mice with aerosolized virulent *Mtb* (strain H37Rv), and 4 weeks later, the animals were sacrificed and the presence of *Mtb* in the animals' lungs and BM (femur) was enumerated using Middlebrook 7H11 agar plates to grow the mycobacteria. *Mtb* was found in mouse BM and lung (Fig. 4). Our results support the possibility that BM cells can be a target of *Mtb* infection in vivo as well as in vitro.

### Viable nonreplicating *Mtb* resides in lung and BM MSCs of mice

We then evaluated whether mouse MSCs present in lung and BM could maintain the viability of nonreplicating *Mtb*. To perform these experiments, we used a unique mouse model of nonreplicating *Mtb* infection. In this model, an auxotrophic mutant of *Mtb* (strain 18b) replicates only in the presence of streptomycin (30–32). After streptomycin withdrawal, the mutant *Mtb* persists in a nonreplicating viable state, and a small number of viable *Mtb* CFUs can be obtained from the mouse lung and spleen even after 6 months after infection (31). Hence, this model provided the opportunity to test whether nonreplicating *Mtb* retained viability in MSCs in vivo.

Using this model, we measured the recovery of viable *Mtb* from the MSC fractions in the lung and BM. In the mouse lung, the MSCs are enriched in a population of cells, the side population (SP) (33), which is defined by their ability to exclude Hoechst 33342 dye as detected by FACS gating and by their expression of the drug efflux pump ABCG2 (16). As depicted in Fig. 5A, using Hoechst dye exclusion (34) and FACS (Fig. 5A), we could purify a population that did not retain the Hoechst dye (SP) as well as a population that retained the dye (non-SP fraction). The SP fraction was positive for ABCG2 and showed an increase in expression of MSC markers Sca-1, CD105, CD44, and CD271 (more than fourfold) in the SP versus the non-SP fraction (Fig. 5B). In contrast, neither CD45 nor the macrophage-associated CD11b cell markers were detected in the SP fraction (Fig. 5B). Hence, lung SP cells express CD271 and are enriched in MSCs, as has been suggested previously (33). In the mouse BM, MSCs are enriched in CD271+/CD45− cells (fig. S3). Therefore, we used lung SP cells and BM CD271+/CD45− cells of infected mice (6 months after streptomycin withdrawal) to measure the recovery of viable nonreplicating *Mtb* strain 18b.

First, we examined the lung SP and non-SP cells for viable *Mtb* by culturing lysates of these cells in Middlebrook agar plates containing streptomycin. Viable *Mtb* strain 18b could be retrieved from the lung SP fraction even after spending a prolonged period of time in a non-

replicating state (Fig. 5C).When the infected SP cells ($5 \times 10^4$ SP cells, containing on average 70 *Mtb* CFUs, $n = 4$) were injected by tail vein injection into recipient mice (female C57BL/6 mice, 6 to 8 weeks old), the mice developed lung granulomas. Viable CFUs (1000 ± 430 per lung, $n = 4$) were obtained after 4 weeks of infection, suggesting that *Mtb* in the SP cells maintained their ability to reinfect host lung tissue. In contrast, few viable *Mtb* were recovered from the non-SP population of cells. Thus, nonreplicating *Mtb* sequestered in mouse MSCs in the lung SP population retain in vivo viability and reinfection capabilities.

Second, in examining the BM fraction, we observed that viable *Mtb* could be recovered from $CD271^+$ BM-MSCs (Fig. 5, D and E). Figure 5E shows that on average 32,182 viable CFUs were recovered per $10^7$ $CD271^+/CD45^-$ cells. This implies that ~0.32% of $CD271^+/CD45^-$ cells contained viable and nonreplicating *Mtb*. These intracellular non-replicating *Mtb* maintain reinfection capabilities similar to those of lung SP cells. Similar to lung SP cells, the tail vein injection of *Mtb*-infected $5 \times 10^4$ $CD271^+$ BM-MSCs (Fig. 5D) led to lung granuloma development in the recipient mice 4 weeks after injection (Fig. 5, E and F). Notably, $CD271^+$ BM-MSCs recovered from the recipient mice contained *Mtb* CFUs (Fig. 5E). Thus, viable nonreplicating *Mtb* could be obtained from both BM and lung MSCs of mice infected with *Mtb* strain 18b.

To directly examine if $CD271^+$ mouse BM-MSCs can serve as a host of infectious *Mtb*, the auxotrophic mutant *Mtb* strain 18b was labeled with the green fluorescent protein (GFP)–expressing plasmid $GFPpUV15^+$(35). The $GFPpUV15^+$-transfected *Mtb* strain 18b (GFP-*Mtb* 18b) maintained its infective ability as assessed in vitro using the mouse macrophage RAW cell line (fig. S4). Mice were then infected with the GFP-*Mtb* 18b followed by daily administration of streptomycin sulfate for 3 weeks. Mice were sacrificed, and BM cells were obtained and analyzed by both confocal microscopy and multicolor flow cytometry. By confocal microscopy examination, we observed the presence of intracellular GFP-labeled *Mtb* strain 18b in $CD271^+$ cells among the heterogeneous BM mononuclear cell population (Fig. 6A). By FACS analysis, 0.6 ± 0.2% ($P < 0.02$; $n = 5$) of the $CD271^+$ cells stained double positive for both the CD271 MSC marker and GFP (Fig. 6B). Our results further suggest that $CD271^+$ BM-MSCs can be infected with *Mtb* strain 18b in vivo.

### Viable *Mtb* persist in $CD271^+/CD45^-$ BM-MSCs of treated TB patients

To examine whether *Mtb* could infect human $CD271^+$ BM-MSCs and maintain *Mtb* long-term viability, we obtained BM cells from individuals previously treated for pulmonary TB. Nine subjects who had under-gone complete Directly Observed Treatment Short-Course (DOTS) were studied. After completion of treatment, these individuals had negative sputum for *Mtb* culture and *Mtb* DNA, which is consistent with a therapeutically successful treatment. However, such individuals are known to potentially still harbor residual *Mtb* infection (1, 7, 36, 37). $CD271^+$ BM-MSCs were obtained from BM biopsies from these individuals by an immunomagnetic cell sorting procedure (20–22). The purity and phenotype were evaluated by performing FACS analysis, as well as gene expression analysis as described (20–22). Using an immunomagnetic technique, we could obtain an 89.2 ± 5.8% ($n = 2$) pure population of $CD271^+$ BM-MSCs ($3.2 \pm 2 \times 10^4$ $CD271^+/CD45^-$ cells from $5 \times 10^7$ total BM mononuclear cells) as confirmed by FACS analysis (Fig. 7A). From these sorted $CD271^+$ BM-MSCs, the presence of *Mtb* DNA was investigated by qPCR using *Mtb*-specific primers (38). As a negative control, DNA was obtained from $CD271^+$ BM-MSCs and $CD271^-/CD45^-$ cells of healthy volunteers ($n = 6$) from nonendemic areas (ABC026F, Stem Cell Technologies; details in the Supplementary Materials and Methods). We detected on average 110 *Mtb* DNA copies per $10^4$ cells in eight of nine subjects ($P < 0.0001$; Fig. 7B). In two individuals, *Mtb* DNA was found in the $CD271^-CD45^-$ cell population (25 and 10 copies, respectively, per $10^6$ cells). In one individual, *Mtb* DNA was found in the $CD11b^+/CD45^+$ cell population (15 copies per $10^7$ cells). No measurable *Mtb* DNA was

associated with $CD34^+/CD45^+$ cells. The DNA obtained from $CD271^+/CD45^-$ cells of healthy volunteers from nonendemic areas did not exhibit measurable *Mtb* DNA. Although these results do not exclude other cell types as potential hosts for dormant *Mtb*, they do suggest that *Mtb* can infect and persist in human $CD271^+$ BM-MSCs.

To examine the presence of viable *Mtb* in the sorted $CD271^+/CD45^-$ cells from these human subjects, we used a liquid culture method that facilitates the replication of "difficult to culture" *Mtb* (9, 39). We identified two positive *Mtb* cultures (black circles, Fig. 7B) of eight *Mtb* DNA–positive subjects. Notably, positive cultures were obtained from individuals with a higher copy number of *Mtb* DNA (245 and 206 *Mtb* DNA copies per $10^4$ cells; Fig. 7B). In contrast, the microbial cultures from the $CD271^-/CD45^-$ and $CD11b^+/CD45^+$ subpopulations were all negative. Thus, our observations suggest that viable *Mtb* could persist in $CD271^+/CD45^-$ BM-MSCs obtained from TB patients who have completed anti-*Mtb* therapy.

## DISCUSSION

The protective cellular niche where *Mtb* persists to maintain long-term viability is not yet clearly defined, complicating the development of effective vaccines and therapeutic strategies to eliminate TB (1). Here, we demonstrate in vitro and in vivo in human subjects previously treated for TB and in a mouse model of *Mtb* infection that *Mtb* can infect and persist in $CD271^+$ BM-MSCs.

Specifically, we identified that in vitro human $CD271^+$ BM-MSCs can be infected and maintain viable *Mtb*. Then, using an in vivo mouse model designed to study nonreplicating *Mtb*, we found that $CD271^+$ BM-MSCs purified from the BM of *Mtb*-infected mice maintain viable nonreplicating *Mtb* with reinfection capabilities. In humans, we identified *Mtb* DNA as well as viable *Mtb* in the $CD271^+$ BM-MSCs from patients who had successfully completed antitubercular therapy. Our results suggest that $CD271^+$ BM-MSCs are a possible reservoir for nonreplicating live *Mtb* that potentially is a source for reinfection of the host.

Although BM-MSCs have been studied for several decades in an in vitro setup by culture expansion and selection methods (40), only recently has it been possible to study this cell type in their micro-environment in vivo (15, 17, 24, 40). This success is mainly attributable to the identification of markers that define BM-MSCs including CD271, CD146, Stro-1, and SSEA-4 (19–25, 40). This has enabled direct isolation and in vivo study of primary $CD271^+$ BM-MSCs including their self-renewal and differentiation into multiple lineages (19–25). In addition, $CD271^+$ BM-MSCs express the drug efflux pump ABCG2 (20) and exhibit immunosuppressive activity (21). In mouse BM, we found a $CD271^+/CD45^-$ BM cell population that expresses $Sca\text{-}1^+$ and exhibits an MSC phenotype. Others have reported that BM $Sca\text{-}1^+$ cells are enriched in MSCs (41, 42). However, it is not yet clear whether the mouse and human $CD271^+$ BM-MSCs are necessarily equivalent. Nevertheless, we were able to isolate viable *Mtb* from both human and mouse $CD271^+$ BM-MSCs. Thus, recent advances in the characterization of primary BM-MSCs enabled us to identify the $CD271^+$ BM-MSCs as a host cell type that can maintain viable *Mtb*. Our work suggests that BM-MSCs, identified many decades ago (43), could be involved in the pathogenesis of an infectious disease.

Our results introduce a new possible route for TB pathogenesis (fig. S5), whereby a subpopulation of BM cells, the $CD271^+$ BM-MSCs, could be responsible for themaintenance of viable nonreplicating *Mtb*. The $CD271^+$ BM-MSCs are localized in the BM niche (19). The BM niche (including both calvarial and trabecular BM sites) is a niche

that provides immune privilege to stem cells and protects stem cells from immune attack (17, 18). Therefore, this niche is an attractive site where *Mtb* could persist in CD271$^+$ BM-MSC host cells while evading the host immune response.

For several reasons, host cells have been thought to be important in the dormant infection phase of TB pathogenesis (1). First, primary granulomas of asymptomatic subjects are mostly sterile, whereas viable *Mtb* has been recovered from the normal part of lung (44–46), indicating the potential existence of an extragranuloma niche for *Mtb*. Second, human autopsy findings report *Mtb* DNA within macrophages, epithelial cells, and fibroblasts distributed throughout normal lung tissues without any sign of visible granuloma lesions (10). *Mtb* DNA has also been detected in adipocyte-like cells of extrapulmonary tissues (11). Thus, it appears that *Mtb* may reside within a variety of host cells during the dormant infection phase. However, the data supporting epithelial cells and fibroblasts as host cells of *Mtb* infection are based solely on the detection of *Mtb* DNA in these cells (10). Therefore, it is not clear whether these various cells could maintain viable nonreplicating *Mtb* for a prolonged period of time such as would occur during the dormant infection phase.

Our in vitro experiments suggest that *Mtb* can infect, multiply, and maintain viability intracellularly in undifferentiated human CD271$^+$ BM-MSCs. To further evaluate whether *Mtb* could infect MSCs in vivo, we used a mouse model of nonreplicating dormant *Mtb*. First, we showed that viable *Mtb* could be obtained in the BM of mice infected with a low dose of aerosolized virulent *Mtb* H37Rv (~100 CFUs). However, technical restrictions imposed by working with virulent organisms inside BSL-3 facilities precluded us from performing flow cytometry characterization of the target BM cells infected by the virulent *Mtb*. Magnetic sorting, which can be done in a BSL-3 facility, did not work to isolate murine CD271$^+$ BM-MSCs. Briefly, the anti-CD271 magnetic sorting antibody was not able to enrich the population of mouse CD271$^+$ BM-MSCs. There is no other available mouse anti-CD271 magnetic sorting antibody. Therefore, to investigate the BM target cells infected by *Mtb* in vivo, we opted to use a less virulent streptomycin auxotrophic mutant of *Mtb* that has been used to study the persistence of nonreplicating *Mtb* in mice and guinea pigs. In this model, which we (30, 31) and others (32) have previously characterized, inoculation of mice with this unique streptomycin auxotrophic mutant of *Mtb* recapitulates several features of a dormant or latent infectious process. Although in this model the bacilli stop replicating because of a lack of streptomycin rather than in response to host defenses, this model has the advantage that it can be manipulated to stringently ensure that the microorganisms remain in a dormant nonreplicating state for long periods during the infectious process (31). This is an important condition enabling demonstration that infection of the CD271$^+$ BM-MSCs with *Mtb* is due to long-term persistence and not due to recent infection caused by replicating organisms. Hence, data from the mouse model demonstrate that the nonreplicating infection with viable *Mtb* strain 18b is due to dormant *Mtb* (32).

Using this mouse model, we showed that dormant *Mtb* could be isolated from the MSC populations present in both lungs and BM of infected mice that had not been treated with streptomycin for ~6 months. In BM, viable *Mtb* were obtained directly from the CD271$^+$ BM-MSCs. Also, we confirmed the infection of this cell type by using GFP-labeled *Mtb*. In lung, viable *Mtb* was obtained from the SP cell population that is enriched in MSCs. We found that when these infected MSCs were reinjected into healthy mice, the animals developed lung granulomas, suggesting that the nonreplicating *Mtb* maintained intracellularly in the MSCs had reinfection capabilities. At this time, it is not clear to what extent mouse lung CD271$^+$ SP cells and BM-derived CD271$^+$ BM-MSCs are phenotypically equivalent. Nevertheless, our data are consistent with our in vitro findings that *Mtb* specifically infected and remained viable in an undifferentiated CD271$^+$ BM-MSC population.

Our observations suggest the possibility that CD271$^+$ BM-MSCs may be an alternative host cell population for supporting the long-lasting nonreplicating phase of the *Mtb* life cycle. It is intriguing that CD271$^+$ BM-MSCs, members of the heterogeneous MSC population, do have features that could maintain the nonreplicating *Mtb* for a prolonged period of time. In particular, adult undifferentiated stem cells including MSCs are characterized by their self-renewal and quiescent properties (15–19). In contrast, quiescent, but differentiated, cells including macrophages, endothelial cells, and fibroblast are characterized by a shorter life span. Our results from differentiating MSCs suggest that such differentiated cells do not support the persistence of viable *Mtb*. Nonreplicating *Mtb* seems to persist for long periods of time in CD271$^+$ BM-MSCs without being compromised or needing to change their host cells.

Validation of these findings in mice was obtained using BM cells from subjects previously treated for pulmonary TB, who did not exhibit evidence of active pulmonary TB (Supplementary Materials and Methods). We found that specific *Mtb* DNA was present or associated with the CD271$^+$ BM-MSCs from most of these individuals. We confirmed the presence of viable *Mtb* in the CD271$^+$ BM-MSCs of two of eight subjects having *Mtb* DNA copies. The negative culture results in the other *Mtb* DNA–positive subjects are likely in part due to the limitation of the current culture methods for growth of dormant or nonreplicating *Mtb*.

Epidemiological studies indicate that disease recurrence after drug treatment for TB remains a major challenge (36, 37). TB recurrence could occur due to either endogenous reactivation of persistent *Mtb* infection or exogenous reinfection with a new *Mtb* strain (47). Several studies indicate that reactivation could be a significant source of recurrent TB (48–50). Therefore, prevention and management of the reactivation process could reduce the incidence of recurrent TB (36, 49). However, the sources and mechanisms of reactivation are not clearly known (1, 7), which compromises our ability to eradicate TB (7, 51). Although our results do not yet address whether *Mtb*-infected BM-MSCs are a major source of TB reactivation, our findings suggest a new potential route for TB reactivation that should be studied further.

Our results from in vitro studies of human BMSCs, an in vivo mouse model of nonreplicating *Mtb* infection, and the analysis of human CD271$^+$ BM-MSCs from patients with pulmonary TB suggest that CD271$^+$ BM-MSCs are a cellular niche that may be important for the maintenance of the nonreplicating phase of *Mtb* in humans. CD271$^+$ BM-MSCs have many features that could be potentially advantageous for *Mtb* persistence including their ability to self-renew, expression of the ABCG2 drug efflux pump, and the immune-privileged nature of the BM stem cell niche (19–25). We suggest that human BM-derived MSCs may participate in TB pathogenesis with potentially important clinical implications.

## MATERIALS AND METHODS

### Stem cell sorting and culture

All studies conducted in the various institutions were approved by Stanford Stem Cell Research Oversight Panel (protocol number 289; principal investigator: B.D.). Human BM–derived CD133$^+$ (ABC026F) and CD34$^+$ (ABM016F) were cultured in serum-free medium with appropriate growth factors, and CD271$^+$/CD133$^+$ cells were obtained from the CD133$^+$ BM cells with the EasySep Human CD271 Positive Selection kit (18659). The sorted cells were cultured in the serum-free medium with the growth factor cocktails (TPO, SCF, and Flt3 ligand) to maintain the CD271$^+$/CD133$^+$ phenotype (Supplementary Materials and

Methods), and used for *Mtb* infection and growth. The purity and the phenotype of the sorted cells are described in table S1.

### *Mycobacterium* strains, growth conditions, and stem cell infections

The *Mtb* strains H37Ra [American Type Culture Collection (ATCC) 25177] and H37Rv (ATCC 27294) and a streptomycin-auxotrophic mutant *Mtb* strain 18b (provided by S. Cole, Global Health Institute, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) were grown and maintained in BBL Middlebrook 7H9 broth with glycerol (BD Biosciences, 221832) at 37°C and 5% $CO_2$. Single-cell suspension was obtained to infect stem cells with standard protocol used to infect macrophages with an MOI of five bacteria per cell for 8 hours. For the immunofluorescence visualization and flow cytometry sorting of *Mtb*-infected stem cells, *Mtb* was tagged with PKH26 red dye (PKH26 red fluorescent cell linker mini kit, Sigma) (27) and subsequently used for stem cell infection. GFP-tagged *Mtb* strain 18b was obtained as described (35). The viable intracellular *Mtb* from the *Mtb*-infected stem cells was measured by *Mtb* CFU assay (27).

### Mesenchymal characterization of human and mouse $CD271^+/CD45^-$ BM cells

The phenotypic characterization of the human and mouse $CD271^+/CD45^+$ BM cells was performed as described (28).

### mRNA and DNA extraction, and qPCR assay

To isolate mammalian mRNA and bacterial DNA from the infected cells, we used the μMACS technology (Miltenyi Biotec). Methods and various primers used in the article are described in the Supplementary Materials and Methods.

### Mice infection with *Mtb*

Six- to 8-week-old female C57BL/6 mice were obtained from Charles River Laboratories. The streptomycin-auxotrophic mutant 18b strain was delivered intravenously at $2 \times 10^6$ CFUs per mouse (31), and after 24 weeks of streptomycin starvation, the $CD271^+/CD45^-$ cells were obtained by FACS. The lung SP and non-SP cells were isolated by dissociating the lung tissue (33), followed by FACS as described (34). The FACS-sorted cells from lung and BM were subjected to *Mtb* CFU assay. A portion of the sorted cells was subjected to qPCR study by the μMACS technique described above. For the aerosolized TB infection, phosphate-buffered saline/Tween–suspended Mtb H37Rv was delivered to mice (~100 CFUs per mouse; $n = 5$) with an aerosol generation device (Inhalation Exposure System; Glas-Col).

### Clinical study

The clinical study was approved by Stanford Stem Cell Research Oversight Panel, Stanford University (protocol number 289; principal investigator: B.D.), and the government of Arunachal Pradesh, India (MSTB/STDC/IRL/20/2010). Subjects were recruited from the Idu-Mishmi tribe living in a remote mountain range of Roing, Arunachal Pradesh, India. The population represents an extremely homogeneous background (Supplementary Materials and Methods). Previously treated subjects (DOTS II) were prospectively recruited on the basis of the local TB control program data and chest x-ray findings. After proper consent, 6 to 7 ml of BM were taken from the posterior superior iliac crest, and then, the immunomagnetically sorted cells were lysed and subjected to mRNA and *Mtb* DNA isolation by qMACS technology as described above. The magnetically sorted cells obtained from healthy volunteers ($n = 6$) were subjected to both FACS and qPCR analysis to confirm the purity and phenotype of the $CD271^+$ BM-MSCs, and also served as a negative control for *Mtb*

DNA. Part of the lysate was subjected to liquid culture to obtain viable *Mtb*, as described (9, 39), with modifications.

### Statistics

Mean values ± SEM are shown. Student's *t* test was used for comparisons (GraphPad Prism version 4.0a for Macintosh). Data are expressed as means ± SEM; $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank the Idu-Mishmi people, volunteers, and the government of Arunachal Pradesh, India, for allowing us to conduct the clinical study in the Idu-Mishmi land. Correspondence for this clinical study should be addressed to B.D. at bikuldas@stanford.edu. We also thank L. Qin and M. Bigos for flow cytometry assay and analysis.

Funding: The work was funded by a grant from the Bill & Melinda Gates Foundation through the "Grand Challenges Exploration Initiative" (B.D.). Additional funding was obtained from the Canadian Cancer Society (B.D.); the KaviKrishna Foundation, Sualkuchi, Assam, India (D.K. and I.P.); Laurel Foundation (B.D. and D.W.F.); NIH grants R01AI076425 (A.C.-N.), R01 CA105102, CA89305-0351, and CA112973; and Department of Defense grant PR080163 (D.W.F.).

## REFERENCES AND NOTES

1. Gomez JE, McKinney JD. *M. tuberculosis* persistence, latency, and drug tolerance. Tuberculosis. 2004; 84:29–44. [PubMed: 14670344]
2. Young DB, Gideon HP, Wilkinson RJ. Eliminating latent tuberculosis. Trends Microbiol. 2009; 17:183–188. [PubMed: 19375916]
3. Via LE, Lin PL, Ray SM, Carrillo J, Allen SS, Eum SY, Taylor K, Klein E, Manjunatha U, Gonzales J, Lee EG, Park SK, Raleigh JA, Cho SN, McMurray DN, Flynn JL, Barry CE III. Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates. Infect. Immun. 2008; 76:2333–2340. [PubMed: 18347040]
4. Wayne LG, Hayes LG. An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. Infect. Immun. 1996; 64:2062–2069. [PubMed: 8675308]
5. Filippini P, Iona E, Piccaro G, Peyron P, Neyrolles O, Fattorini L. Activity of drug combinations against dormant *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 2010; 54:2712–2715. [PubMed: 20350948]
6. Vandiviere HM, Loring WE, Melvin I, Willis S. The treated pulmonary lesion and its tubercle bacillus. II. The death and resurrection. Am. J. Med. Sci. 1956; 232:30–37. [PubMed: 13326887]
7. Bloom BR, McKinney JD. The death and resurrection of tuberculosis. Nat. Med. 1999; 5:872–874. [PubMed: 10426305]
8. Agarwal N, Lamichhane G, Gupta R, Nolan S, Bishai WR. Cyclic AMP intoxication of macrophages by a *Mycobacterium tuberculosis* adenylate cyclase. Nature. 2009; 460:98–102. [PubMed: 19516256]
9. Biketov S, Mukamolova GV, Potapov V, Gilenkov E, Vostroknutova G, Kell DB, Young M, Kaprelyants AS. Culturability of *Mycobacterium tuberculosis* cells isolated from murine macrophages: A bacterial growth factor promotes recovery. FEMS Immunol. Med. Microbiol. 2000; 29:233–240. [PubMed: 11118902]
10. Hernández-Pando R, Jeyanathan M, Mengistu G, Aguilar D, Orozco H, Harboe M, Rook GA, Bjune G. Persistence of DNA from *Mycobacterium tuberculosis* in superficially normal lung tissue during latent infection. Lancet. 2000; 356:2133–2138. [PubMed: 11191539]

11. Neyrolles O, Hernández-Pando R, Pietri-Rouxel F, Fornès P, Tailleux L, Barrios Payán JA, Pivert E, Bordat Y, Aguilar D, Prévost MC, Petit C, Gicquel B. Is adipose tissue a place for *Mycobacterium tuberculosis* persistence? PLoS One. 2006; 1:e43. [PubMed: 17183672]
12. Raghuvanshi S, Sharma P, Singh S, Van Kaer L, Das G. *Mycobacterium tuberculosis* evades host immunity by recruiting mesenchymal stem cells. Proc. Natl. Acad. Sci. U.S.A. 2010; 107:21653–21658. [PubMed: 21135221]
13. Cheshier SH, Morrison SJ, Liao X, Weissman IL. In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proc. Natl. Acad. Sci. U.S.A. 1999; 96:3120–3125. [PubMed: 10077647]
14. Gallacher L, Murdoch B, Wu DM, Karanu FN, Keeney M, Bhatia M. Isolation and characterization of human CD34−Lin− and $CD34^{+}Lin^{-}$ hematopoietic stem cells using cell surface markers AC133 and CD7. Blood. 2000; 95:2813–2820. [PubMed: 10779426]
15. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M, Bianco P. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell. 2007; 131:324–336. [PubMed: 17956733]
16. Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, Lagutina I, Grosveld GC, Osawa M, Nakauchi H, Sorrentino BP. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat. Med. 2001; 7:1028–1034. [PubMed: 11533706]
17. Aria, F.; Suda, T. Quiescent stem cells in the niche, in StemBook. Cambridge, MA: Harvard Stem Cell Institute; 2008.
18. Fujisaki J, Wu J, Carlson AL, Silberstein L, Putheti P, Larocca R, Gao W, Saito TI, Lo Celso C, Tsuyuzaki H, Sato T, Côté D, Sykes M, Strom TB, Scadden DT, Lin CP. In vivo imaging of $T_{reg}$ cells providing immune privilege to the haematopoietic stem-cell niche. Nature. 2011; 474:216–219. [PubMed: 21654805]
19. Tormin A, Li O, Brune JC, Walsh S, Schütz B, Ehinger M, Ditzel N, Kassem M, Scheding S. CD146 expression on primary nonhematopoietic bone marrow stem cells is correlated with in situ localization. Blood. 2011; 117:5067–5077. [PubMed: 21415267]
20. Bakondi B, Shimada IS, Perry A, Munoz JR, Ylostalo J, Howard AB, Gregory CA, Spees JL. CD133 identifies a human bone marrow stem/progenitor cell sub-population with a repertoire of secreted factors that protect against stroke. Mol. Ther. 2009; 17:1938–1947. [PubMed: 19690521]
21. Kuçi S, Kuçi Z, Kreyenberg H, Deak E, Pütsch K, Huenecke S, Amara C, Koller S, Rettinger E, Grez M, Koehl U, Latifi-Pupovci H, Henschler R, Tonn T, von Laer D, Klingebiel T, Bader P. CD271 antigen defines a subset of multipotent stromal cells with immunosuppressive and lymphohematopoietic engraftment-promoting properties. Haematologica. 2010; 95:651–659. [PubMed: 20179086]
22. Quirici N, Soligo D, Bossolasco P, Servida F, Lumini C, Deliliers GL. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. Exp. Hematol. 2002; 30:783–791. [PubMed: 12135677]
23. Bühring HJ, Battula VL, Treml S, Schewe B, Kanz L, Vogel W. Novel markers for the prospective isolation of human MSC. Ann. N. Y. Acad. Sci. 2007; 1106:262–271. [PubMed: 17395729]
24. Jones E, McGonagle D. Human bone marrow mesenchymal stem cells in vivo. Rheumatology. 2008; 47:126–131. [PubMed: 17986482]
25. Qian H, Le Blanc K, Sigvardsson M. Primary mesenchymal stem and progenitor cells from bone marrow lack expression of CD44 protein. J. Biol. Chem. 2012; 287:25795–25807. [PubMed: 22654106]
26. Baksh D, Davies JE, Zandstra PW. Soluble factor cross-talk between human bone marrow-derived hematopoietic and mesenchymal cells enhances in vitro CFU-F and CFU-O growth and reveals heterogeneity in the mesenchymal progenitor cell compartment. Blood. 2005; 106:3012–3019. [PubMed: 16030193]
27. Kelly DM, ten Bokum AM, O'Leary SM, O'Sullivan MP, Keane J. Bystander macrophage apoptosis after *Mycobacterium tuberculosis* H37Ra infection. Infect. Immun. 2008; 76:351–360. [PubMed: 17954721]

28. Das B, Antoon R, Tsuchida R, Lotfi S, Morozova O, Farhat W, Malkin D, Koren G, Yeger H, Baruchel S. Squalene selectively protects mouse bone marrow progenitors against cisplatin and carboplatin-induced cytotoxicity in vivo without protecting tumor growth. Neoplasia. 2008; 10:1105–1119. [PubMed: 18813359]
29. Orme I, Gonzalez-Juarrero M. Animal models of *M. tuberculosis* infection. **Chapter 10**, Unit 10A. 5. Curr. Protoc. Microbiol. 2007
30. Kashino SS, Napolitano DR, Skobe Z, Campos-Neto A. Guinea pig model of *Mycobacterium tuberculosis* latent/dormant infection. Microbes Infect. 2008; 10:1469–1476. [PubMed: 18817888]
31. Kashino SS, Ovendale P, Izzo A, Campos-Neto A. Unique model of dormant infection for tuberculosis vaccine development. Clin. Vaccine Immunol. 2006; 13:1014–1021. [PubMed: 16960113]
32. Sala C, Dhar N, Hartkoorn RC, Zhang M, Ha YH, Schneider P, Cole ST. Simple model for testing drugs against nonreplicating *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 2010; 54:4150–4158. [PubMed: 20679505]
33. Martin J, Helm K, Ruegg P, Varella-Garcia M, Burnham E, Majka S. Adult lung side population cells have mesenchymal stem cell potential. Cytotherapy. 2008; 10:140–151. [PubMed: 18368593]
34. Das B, Tsuchida R, Malkin D, Koren G, Baruchel S, Yeger H. Hypoxia enhances tumor stemness by increasing the invasive and tumorigenic side population fraction. Stem Cells. 2008; 26:1818–1830. [PubMed: 18467664]
35. Gandotra S, Schnappinger D, Monteleone M, Hillen W, Ehrt S. In vivo gene silencing identifies the *Mycobacterium tuberculosis* proteasome as essential for the bacteria to persist in mice. Nat. Med. 2007; 13:1515–1520. [PubMed: 18059281]
36. Panjabi R, Comstock GW, Golub JE. Recurrent tuberculosis and its risk factors: Adequately treated patients are still at high risk. Int. J. Tuberc. Lung Dis. 2007; 11:828–837. [PubMed: 17705947]
37. Millet JP, Orcau A, de Olalla PG, Casals M, Rius C, Caylà JA. Tuberculosis recurrence and its associated risk factors among successfully treated patients. J. Epidemiol. Community Health. 2009; 63:799–804. [PubMed: 19179367]
38. Zumárraga M, Bigi F, Alito A, Romano MI, Cataldi A. A 12.7 kb fragment of the *Mycobacterium tuberculosis* genome is not present in Mycobacterium bovis. Microbiology. 1999; 145:893–897. [PubMed: 10220168]
39. Sun Z, Zhang Y. Spent culture supernatant of *Mycobacterium tuberculosis* H37Ra improves viability of aged cultures of this strain and allows small inocula to initiate growth. J. Bacteriol. 1999; 181:7626–7628. [PubMed: 10601224]
40. Bianco P, Robey PG, Simmons PJ. Mesenchymal stem cells: Revisiting history, concepts, and assays. Cell Stem Cell. 2008; 2:313–319. [PubMed: 18397751]
41. Morikawa S, Mabuchi Y, Kubota Y, Nagai Y, Niibe K, Hiratsu E, Suzuki S, Miyauchi-Hara C, Nagoshi N, Sunabori T, Shimmura S, Miyawaki A, Nakagawa T, Suda T, Okano H, Matsuzaki Y. Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. J. Exp. Med. 2009; 206:2483–2496. [PubMed: 19841085]
42. Vranken I, De Visscher G, Lebacq A, Verbeken E, Flameng W. The recruitment of primitive $Lin^{-}$ $Sca\text{-}1^{+}$, $CD34^{+}$, $c\text{-}kit^{+}$ and $CD271^{+}$ cells during the early intraperitoneal foreign body reaction. Biomaterials. 2008; 29:797–808. [PubMed: 18022690]
43. Goujon E. Recherches experimentales sur les proprietes physiologiques de la moelle des os. J. Anat. Physiol. 1869; 6:399–412.
44. Aronson JD, Whitney CE. The types of tubercle bacilli found in tuberculous lesions and in nontuberculous tissue in man. J. Infect. Dis. 1930; 47:30–55.
45. Balasubramanian V, Wiegeshaus EH, Taylor BT, Smith DW. Pathogenesis of tuberculosis: Pathway to apical localization. Tuber. Lung Dis. 1994; 75:168–178. [PubMed: 7919306]
46. Medlar EM. The pathogenesis of minimal pulmonary tuberculosis; a study of 1,225 necropsies in cases of sudden and unexpected death. Am. Rev. Tuberc. 1948; 58:583–611. [PubMed: 18099839]
47. van Rie A, Warren R, Richardson M, Victor TC, Gie RP, Enarson DA, Beyers N, van Helden PD. Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. N. Engl. J. Med. 1999; 341:1174–1179. [PubMed: 10519895]

48. Farnia P, Masjedi MR, Varahram M, Mirsaeidi M, Ahmadi M, Khazampour M, Tabarsi P, Baghei P, Marjane M, Bahadori M, Zarifi AZ, Velayati AA. The recent-transmission of *Mycobacterium tuberculosis* strains among Iranian and Afghan relapse cases: A DNA-fingerprinting using RFLP and spoligotyping. BMC Infect. Dis. 2008; 8:109. [PubMed: 18681980]
49. Jasmer RM, Bozeman L, Schwartzman K, Cave MD, Saukkonen JJ, Metchock B, Khan A, Burman WJ. Tuberculosis Trials Consortium, Recurrent tuberculosis in the United States and Canada: Relapse or reinfection? Am. J. Respir. Crit. Care Med. 2004; 170:1360–1366. [PubMed: 15477492]
50. Dobler CC, Crawford AB, Jelfs PJ, Gilbert GL, Marks GB. Recurrence of tuberculosis in a low-incidence setting. Eur. Respir. J. 2009; 33:160–167. [PubMed: 18829676]
51. Brewer TF, Heymann SJ. To control and beyond: Moving towards eliminating the global tuberculosis threat. J. Epidemiol. Community Health. 2004; 58:822–825. [PubMed: 15365106]
52. Charrier S, Boiret N, Fouassier M, Berger J, Rapatel C, Pigeon P, Mareynat G, Bonhomme J, Camilleri L, Berger MG. Normal human bone marrow $CD34^{+}CD133^{+}$ cells contain primitive cells able to produce different categories of colony-forming unit megakaryocytes in vitro. Exp. Hematol. 2002; 30:1051–1060. [PubMed: 12225797]
53. Mehta PK, King CH, White EH, Murtagh JJ Jr, Quinn FD. Comparison of in vitro models for the study of *Mycobacterium tuberculosis* invasion and intracellular replication. Infect. Immun. 1996; 64:2673–2679. [PubMed: 8698494]
54. Wei J, Dahl JL, Moulder JW, Roberts EA, O'Gaora P, Young DB, Friedman RL. Identification of a *Mycobacterium tuberculosis* gene that enhances mycobacterial survival in macrophages. J. Bacteriol. 2000; 182:377–384. [PubMed: 10629183]
55. Takii T, Abe C, Tamura A, Ramayah S, Belisle JT, Brennan PJ, Onozaki K. Interleukin-1 or tumor necrosis factor-α augmented the cytotoxic effect of mycobacteria on human fibroblasts: Application to evaluation of pathogenesis of clinical isolates of *Mycobacterium tuberculosis* and *M. avium* complex. J. Interferon Cytokine Res. 2001; 21:187–196. [PubMed: 11331042]
56. Mack E, Neubauer A, Brendel C. Comparison of RNA yield from small cell populations sorted by flow cytometry applying different isolation procedures. Cytometry A. 2007; 71:404–409. [PubMed: 17431883]
57. Summer R, Fitzsimmons K, Dwyer D, Murphy J, Fine A. Isolation of an adult mouse lung mesenchymal progenitor cell population. Am. J. Respir. Cell Mol. Biol. 2007; 37:152–159. [PubMed: 17395889]
58. Kashino SS, Vallerskog T, Martens G, Troudt J, Keyser A, Taylor J, Izzo A, Kornfeld H, Campos-Neto A. Initiation of acquired immunity in the lungs of mice lacking lymph nodes after infection with aerosolized *Mycobacterium tuberculosis*. Am. J. Pathol. 2010; 176:198–204. [PubMed: 20008132]
59. McMurray DN. A coordinated strategy for evaluating new vaccines for human and animal tuberculosis. Tuberculosis. 2001; 81:141–146. [PubMed: 11463235]
60. Arankalle VA, Gandhe SS, Borkakoty BJ, Walimbe AM, Biswas D, Mahanta J. A novel HBV recombinant (genotype I) similar to Vietnam/Laos in a primitive tribe in eastern India. J. Viral Hepat. 2010; 17:501–510. [PubMed: 20059669]
61. Thomas A, Gopi PG, Santha T, Chandrasekaran V, Subramani R, Selvakumar N, Eusuff SI, Sadacharam K, Narayanan PR. Predictors of relapse among pulmonary tuberculosis patients treated in a DOTS programme in South India. Int. J. Tuberc. Lung Dis. 2005; 9:556–561. [PubMed: 15875929]
62. Das B, Yeger H, Baruchel H, Freedman MH, Koren G, Baruchel S. In vitro cytoprotective activity of squalene on a bone marrow versus neuroblastoma model of cisplatin-induced toxicity. Implications in cancer chemotherapy. Eur. J. Cancer. 2003; 39:2556–2565. [PubMed: 14602142]
63. Muschler GF, Boehm C, Easley K. Aspiration to obtain osteoblast progenitor cells from human bone marrow: The influence of aspiration volume. J. Bone Joint Surg. Am. 1997; 79:1699–1709. [PubMed: 9384430]

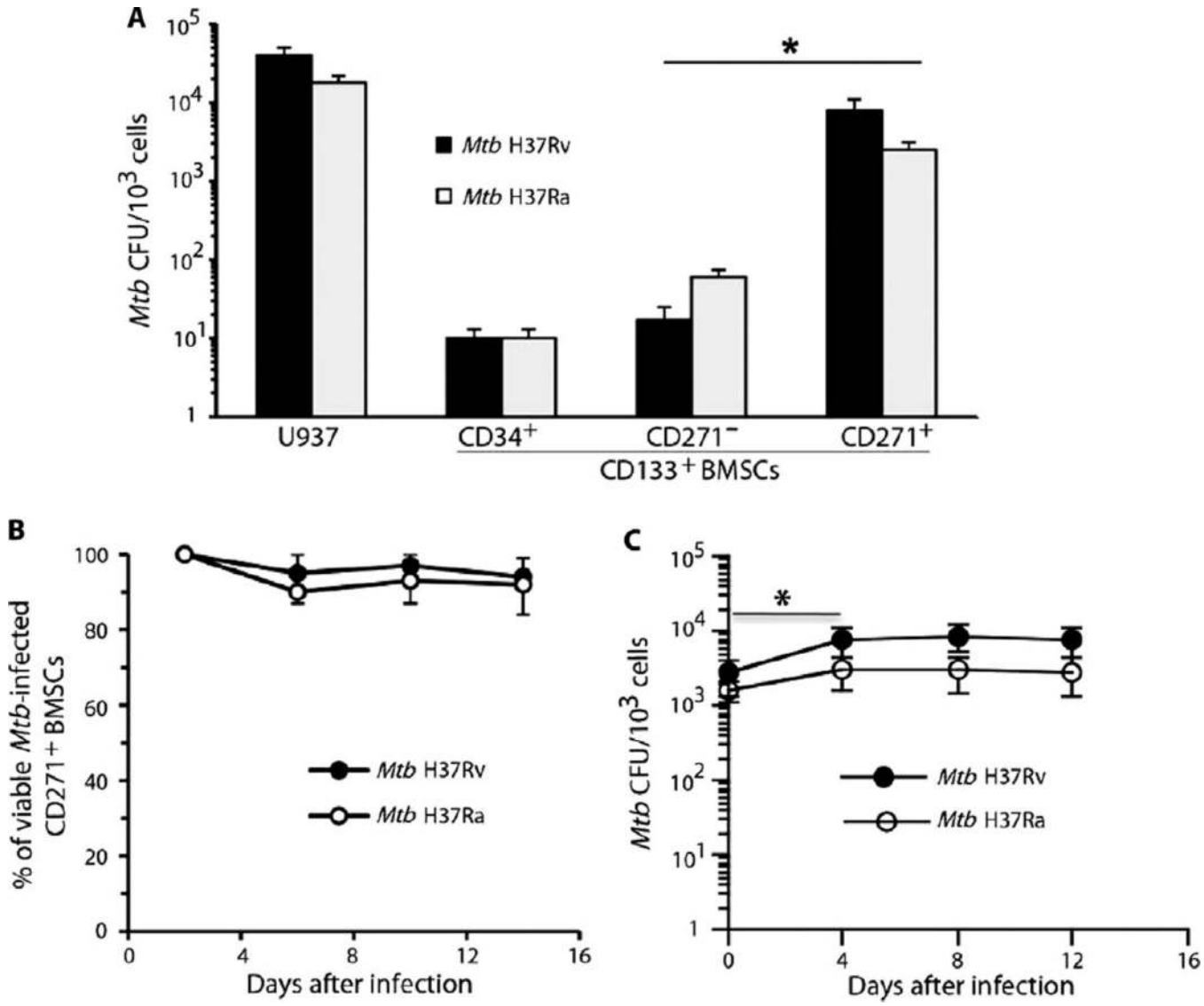


**Fig. 1.**

*Mtb* infects human CD271$^+$/CD133$^+$ BMSCs. (**A**) In vitro infection of human CD271$^+$/CD133$^+$ BMSCs with *Mtb* H37Ra or *Mtb* H37Rv mycobacterial strains resulted in highest CFU production in the CD271$^+$ fraction of CD133$^+$ BMSCs. Infection of U937 cells was the control. The cells were infected with *Mtb* [multiplicity of infection (MOI), 5:1], and CFUs were counted after 4 days of in vitro culture. (**B**) *Mtb* infection did not reduce CD271$^+$/CD133$^+$ BMSC viability as revealed by the Alamar blue assay (28). (**C**) *Mtb* CFUs derived from infected CD271$^+$/CD133$^+$ BMSCs cultured in serum-free medium for 2 weeks (table S1). *$P < 0.05$, Student's $t$ test; $n = 4$ independent experiments.

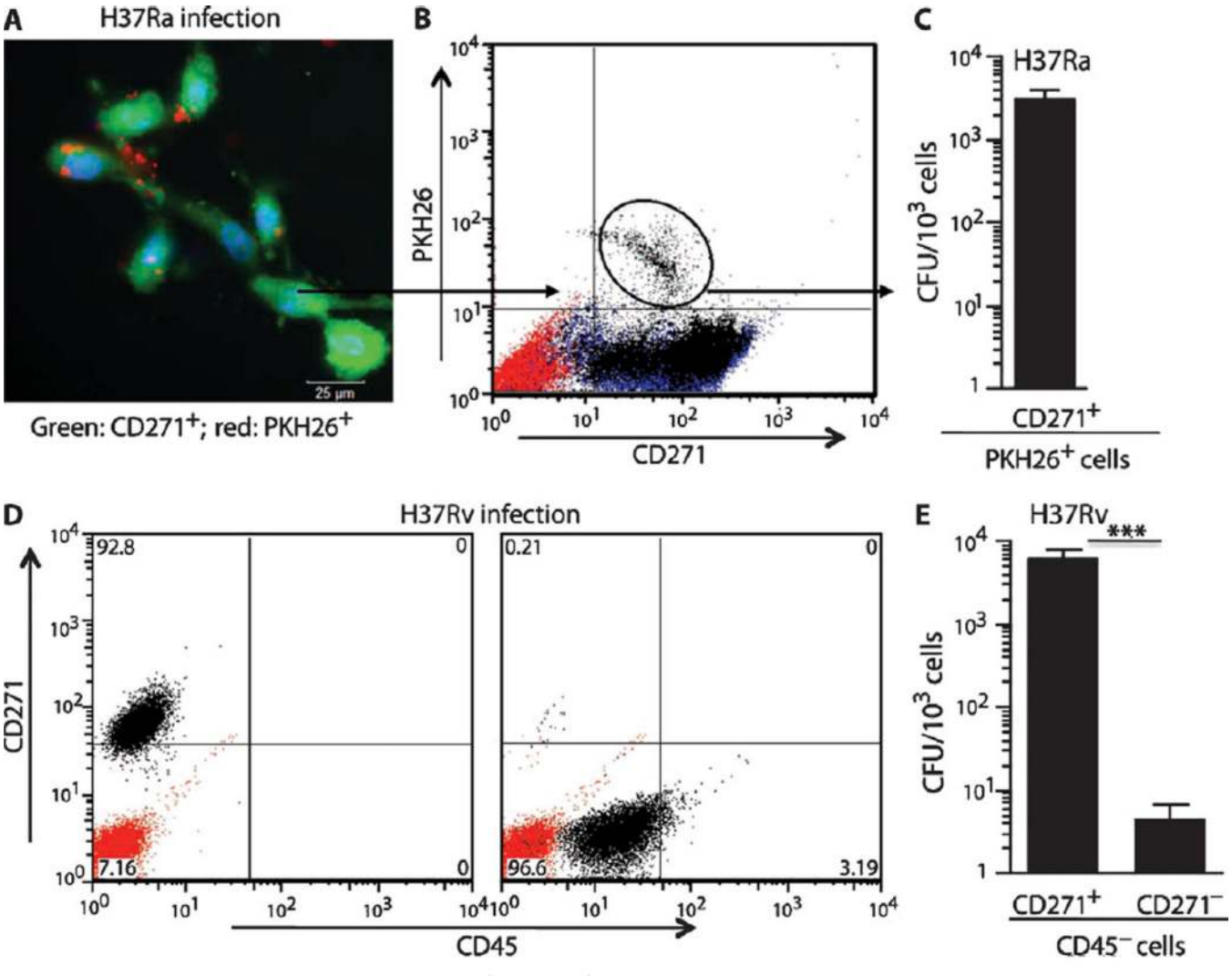


**Fig. 2.**

*Mtb* resides inside human CD271+/CD133+ BMSCs. (**A**) Epifluorescence image showing amikacin-treated CD271+/CD133+ BMSCs (green) containing intracellular PKH26-labeled *Mtb* H37Ra strain (red). (**B** and **C**) Flow cytometry analysis of infected CD271+/CD133+ BMSCs, and CFUs of PKH26-labeled Mtb H37Ra bacteria derived from infected CD271+/CD133+ BMSCs. (**D**) Purity of the magnetically sorted CD271+/CD45− and CD271−/CD45− cells obtained from H37Rv-infected CD271+/CD133+ BMSCs. (**E**) *Mtb* H37Rv CFUs obtained from both of the cell populations depicted in (D). The CD271+/CD133+ BMSCs were infected with *Mtb* (MOI, 5:1) and grown for 2 weeks in serum-free medium (table S1) before FACS analysis (B) or immunomagnetic sorting (D). The red-stained population in flow cytometry panels (B and D) represents isotype controls. Before harvesting for the *Mtb* CFU study, infected cells were treated with amikacin (200 μg/ml) for 3 hours to kill extracellular *Mtb*. **P* < 0.05, Student's *t* test; *n* = 4 independent experiments.

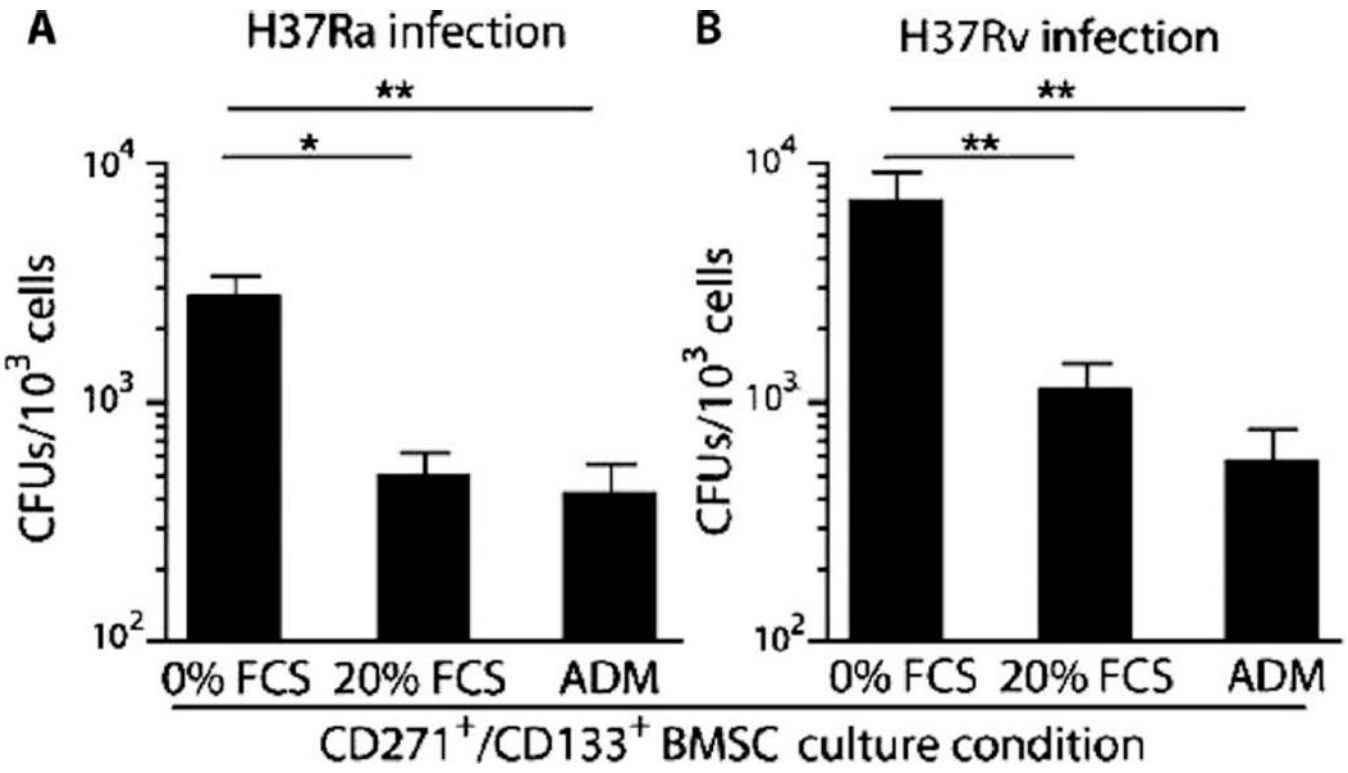


**Fig. 3.**

Viable *Mtb* reside in undifferentiated CD271$^+$/CD133$^+$/CD45$^-$ BM-MSCs. The PKH26$^+$/CD271$^+$ (Fig. 2B) or CD271$^+$/CD45$^-$ BM-MSCs, which were obtained from CD271$^+$/CD133$^+$ BMSCs (Fig. 2D), were cultured in vitro in high-serum medium [fetal calf serum (FCS); table S1] or adipogenic differentiation medium (ADM) and cultured for 2 weeks before harvesting. Shown are the numbers of *Mtb* CFUs grown from each cell population. *$P < 0.05$, **$P < 0.001$, Student's $t$ test; $n = 5$ independent experiments.

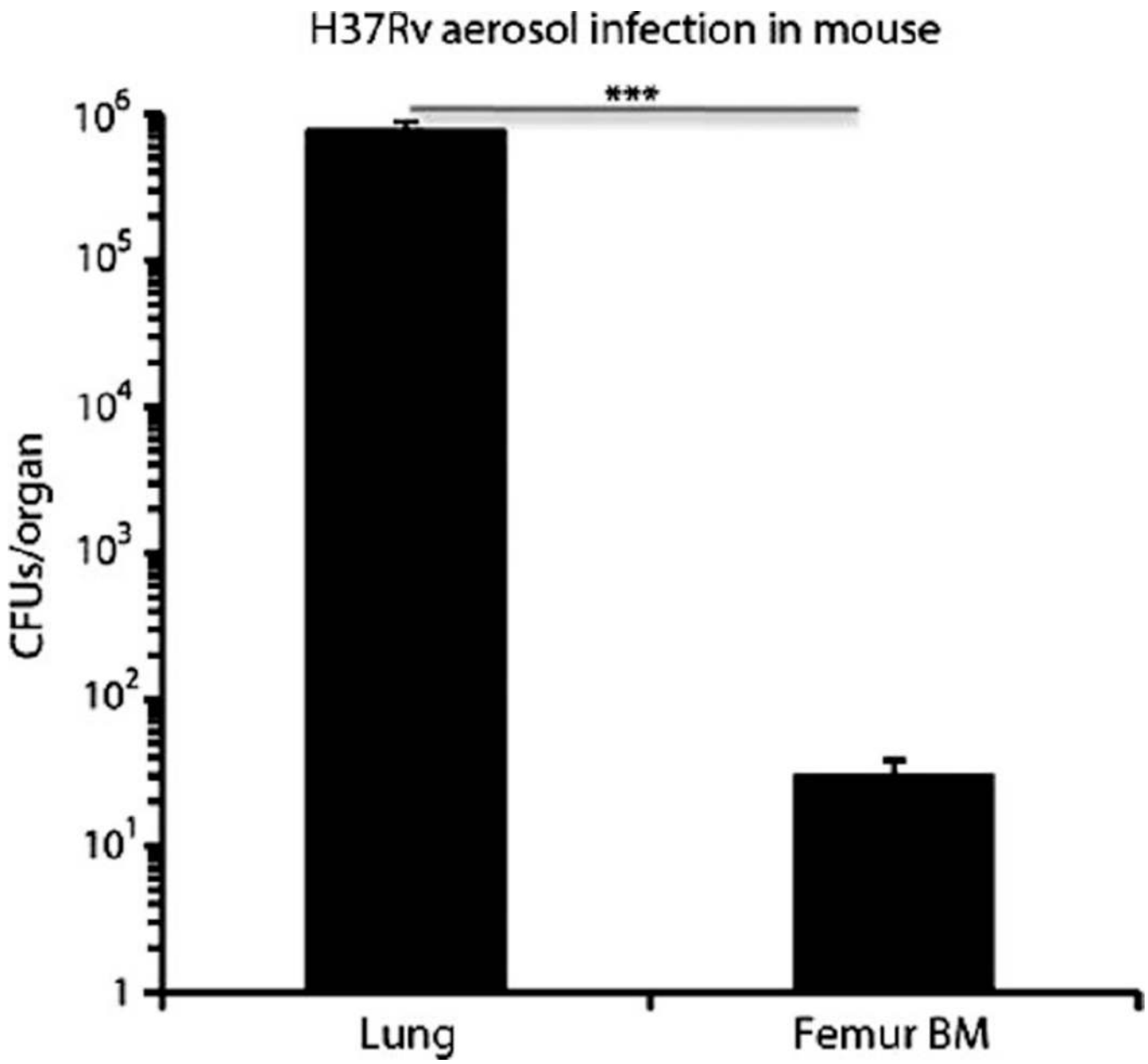


**Fig. 4.**

Dissemination of *Mtb* H37Rv to BM in a murine aerosolized model of infection. BM cells ($10^7$ mononuclear cells) obtained from mice (female BALB/c) infected with *Mtb* H37Rv (~100 CFUs) for 4 weeks contained viable *Mtb*. Shown are numbers of *Mtb* CFUs grown from mouse femur BM and from lung as a positive control. $P < 0.0001$, Student's $t$ test; $n = 5$ independent experiments.

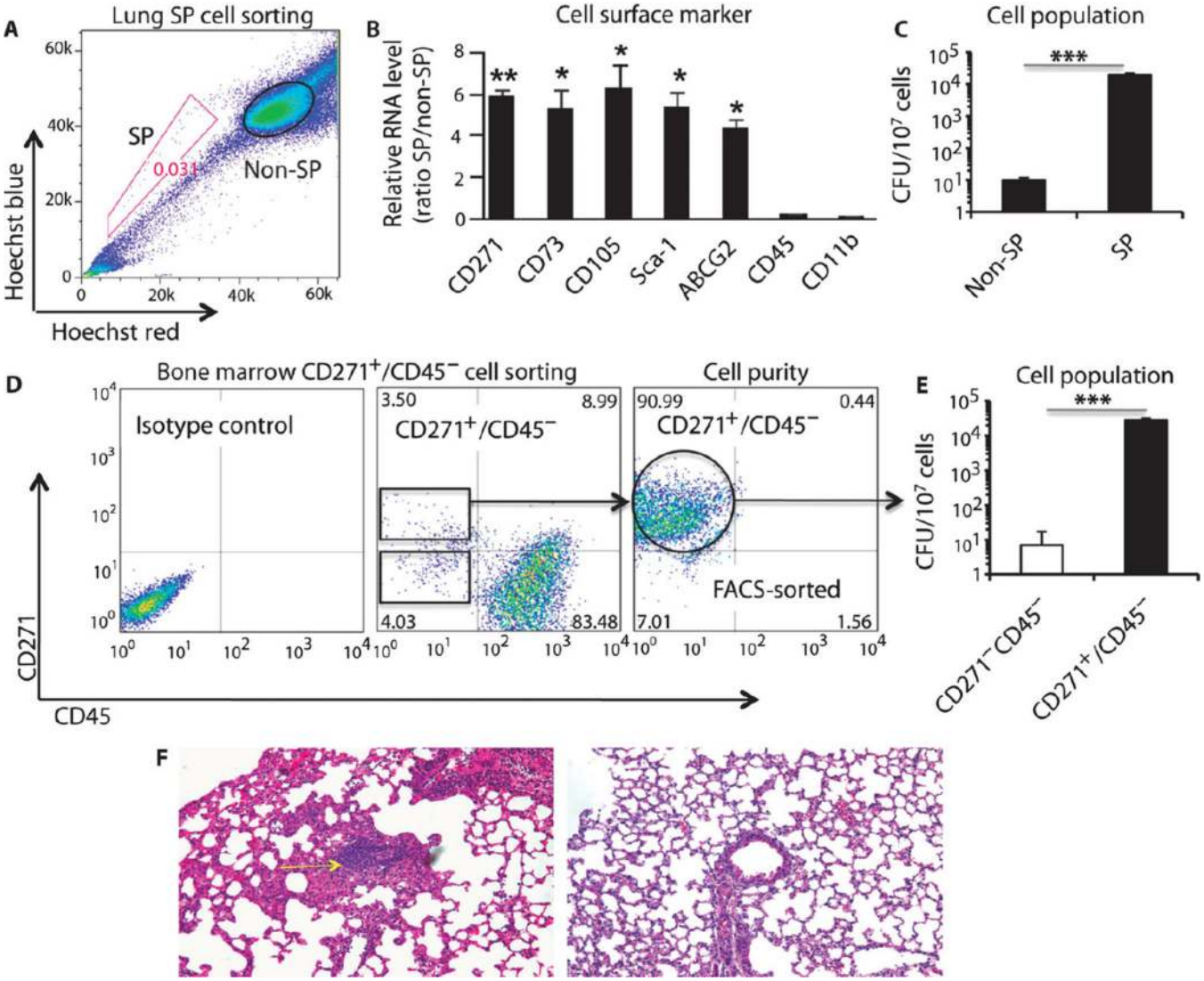


**Fig. 5.**

Recovery of viable nonreplicating *Mtb* from MSCs of infected mice. (**A**) Flow cytometry profile of infected lung SP and non-SP cells after staining with Hoechst 33342 dye. SP gatingwas performed with verapamil treatment. (**B** and **C**) Expression ofMSC markers (B) and detection of viable *Mtb* CFUs (C) in the FACS-sorted SP and non-SP cellpopulations from infected lungs. (**D**) Flow cytometry profiles of BM $CD271^+/CD45^-$ cells from infectedmice. The far right flow cytometry panel represents the purity of the FACS-sorted $CD271^+/CD45^-$ mouse BM-MSCs. (**E**) Viable *Mtb* CFUs were detected in mouse BM-MSC $CD271^+/CD45^-$ cells (see fig. S3 for characteristics of the $CD271^+/CD45^-$ BM-MSCs). (**F**) Granuloma development in the lungs of mice injected with $CD271^+$ BM-MSCs [see (D)] harboring viable dormant *Mtb* (infected lung, left panel, yellow arrow; the right panel depicts normal lung tissue). Hematoxylin and eosin section was prepared as described (30, 31). Magnification, ×20. $*P < 0.05$, $**P < 0.001$, Student's *t* test; $n = 3$ independent experiments.

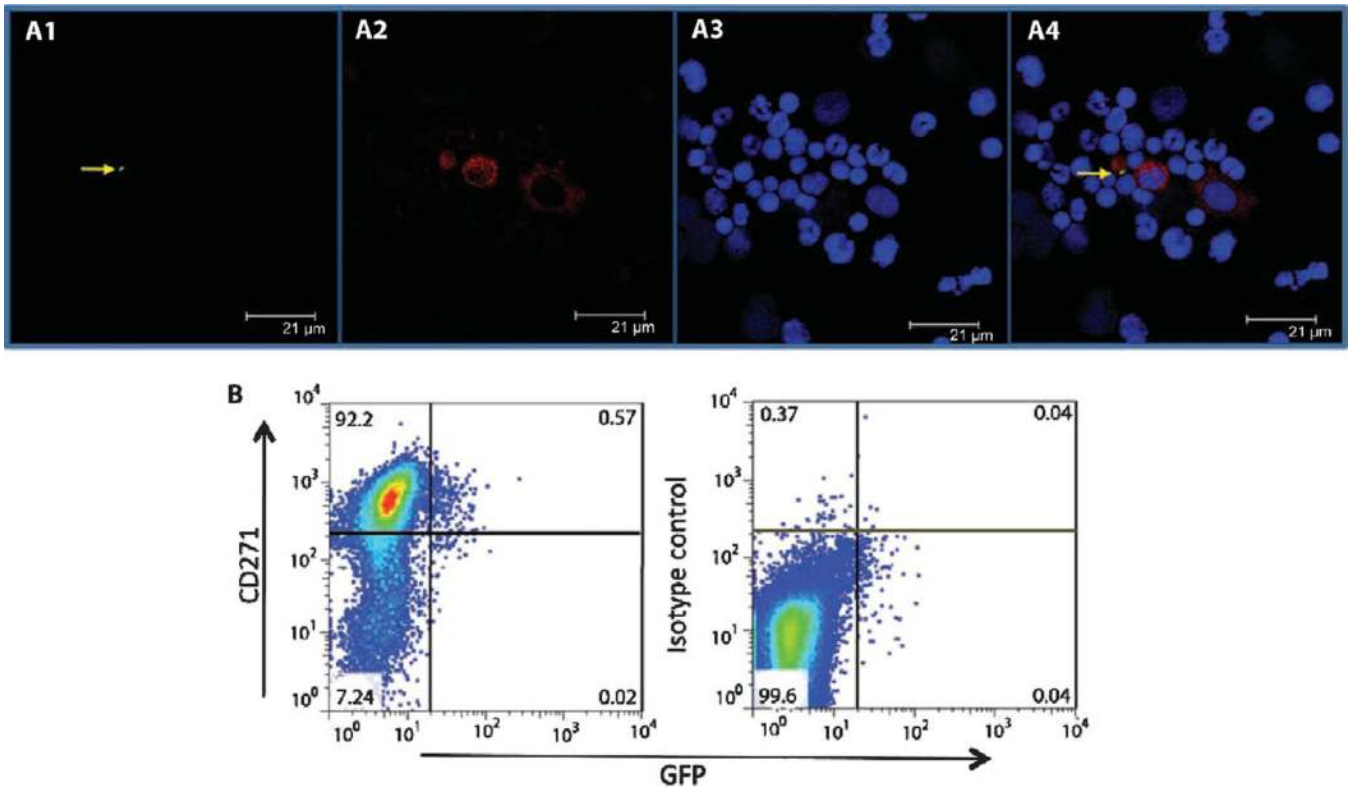


**Fig. 6.**

*Mtb* strain 18b resides in the CD271$^+$/CD45$^-$ BM-MSCs of infected mice. (**A**) Confocal image of flow cytometry–sorted mouse CD271$^+$ BM-MSCs depicting (A1) GFP-tagged *Mtb* strain 18b (green; indicated by the yellow arrow), (A2) BM cells stained with anti-CD271 monoclonal antibody (red), and (A3) BM nuclei stained with Draq5, a nuclear staining dye. A4, merged image. Images are representative of a typical experiment ($n = 3$). (**B**) FACS analysis of the CD271$^+$ BM-MSC population in (A). Representative data are shown for cells staining double positive for CD271 and GFP. Five independent experiments were performed to obtain the percentage of CD271$^+$/GFP$^+$ cells.

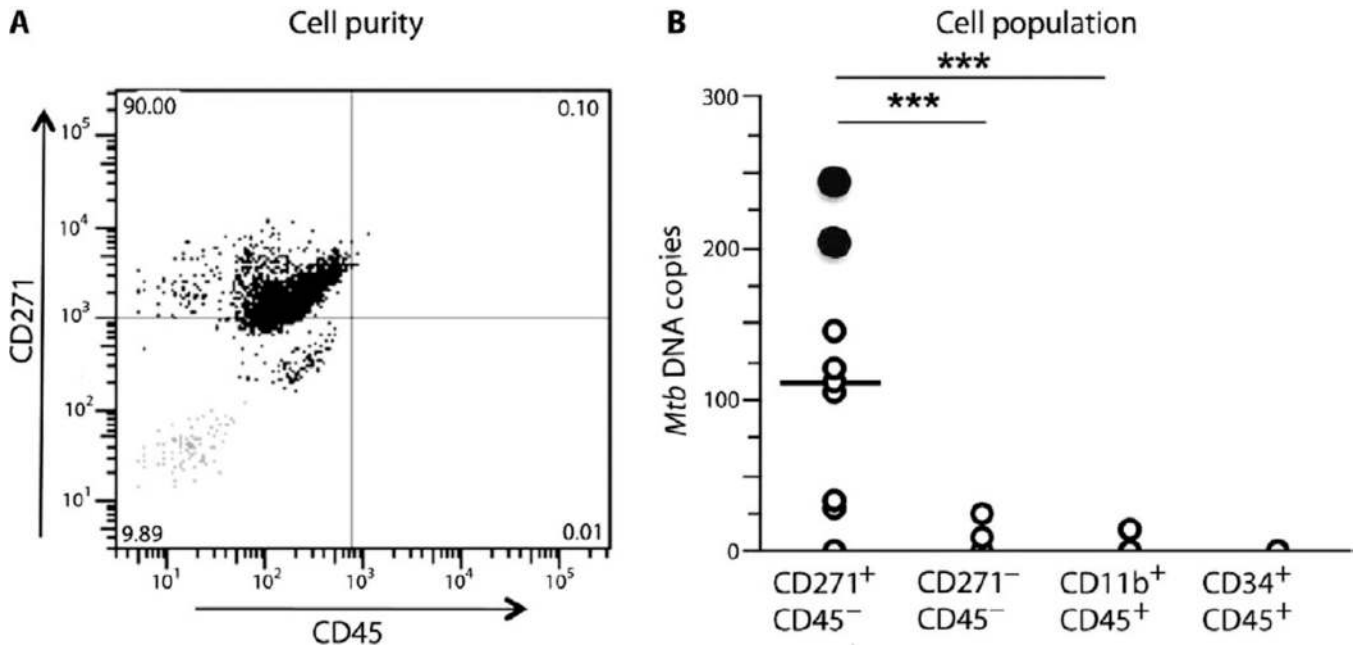


**Fig. 7.**

Viable *Mtb* persist in the CD271$^+$/CD45$^-$ BM-MSCs of treated TB patients. (**A**) Representative flow cytometry panel showing the purity of the magnetically sorted CD271$^+$/CD45$^-$ cells obtained from fresh human BM from subjects previously treated for pulmonary TB. The gray dots (bottom left square) represent the isotype control. (**B**) CD271$^+$/CD45$^-$ BM-MSCs were obtained from previously treated TB patients (DOTS II) who did not show evidence of pulmonary TB. This cell population is enriched for *Mtb* DNA in eight of nine subjects. The black circles denote two subjects for which viable *Mtb* could be cultured from their BM-MSCs. A total of 11 post-therapy TB patients were studied. BM from two subjects was verified for enrichment by magnetic sorting (A). The nine other subjects were examined for *Mtb* DNA and for viable *Mtb* mycobacteria. *Mtb* DNA results were analyzed by Student's *t* test; ***$P < 0.0001$.