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SHORT COMMUNICATION

Bone Marrow Mesenchymal Stem Cells Provide an Antibiotic-Protective Niche for Persistent Viable *Mycobacterium tuberculosis* that Survive Antibiotic Treatment

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During tuberculosis (TB), some *Mycobacterium tuberculosis* bacilli persist in the presence of an active immunity and antibiotics that are used to treat the disease. Herein, by using the Cornell model of TB persistence, we further explored our recent finding that suggested that *M. tuberculosis* can escape therapy by residing in the bone marrow (BM) mesenchymal stem cells. We initially showed that *M. tuberculosis* rapidly disseminates to the mouse BM after aerosol exposure and maintained a stable burden for at least 220 days. In contrast, in the lungs, the *M. tuberculosis* burden peaked at 28 days and subsequently declined approximately 10-fold. More important, treatment of the mice with the antibiotics rifampicin and isoniazid, as expected, resulted in effective clearance of *M. tuberculosis* from the lungs and spleen. In contrast, *M. tuberculosis* persisted, albeit at low numbers, in the BM of antibiotic-treated mice. Moreover, most viable *M. tuberculosis* was recovered from the bone marrow $CD271^{+}CD45^{-}$-enriched cell fraction, and only few viable bacteria could be isolated from the $CD271^{-}CD45^{+}$ cell fraction. These results clearly show that BM mesenchymal stem cells provide an antibiotic-protective niche for *M. tuberculosis* and suggest that unraveling the mechanisms underlying this phenomenon will enhance our understanding of *M. tuberculosis* persistence in treated TB patients. *(Am J Pathol 2014, 184: 3170–3175; http://dx.doi.org/10.1016/j.ajpath.2014.08.024)*

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is an intracellular pathogen known to infect primarily macrophages and dendritic cells.[1–3] However, the viability of *M. tuberculosis* in these intracellular niches is poor,[4] and no evidence exists indicating that these cells can maintain live nonreplicating *M. tuberculosis* for long periods of time. Therefore, it is unlikely that these cell populations can harbor viable *M. tuberculosis* during the chronic phase of the disease, which lasts for months or years, as well as during the latent TB infection, which can last for decades.

We have recently shown that *M. tuberculosis* can reside in bone marrow (BM) within the $CD271^{+}CD45^{-}$ mesenchymal stem cells (BM-MSCs) of individuals treated for pulmonary TB and in mice experimentally infected with *M. tuberculosis*.[5] MSCs can provide an ideal protective niche for *M. tuberculosis* because these cells have several properties that may promote the pathogen's long-term persistence and survival: i) MSCs are present in TB granulomas of infected mouse and human lung tissue[6]; ii) stem cells possess the capacity for self-renewal[7]; iii) stem cells express drug efflux pumps, such as ABCG2, that could contribute to drug evasion by *M. tuberculosis*[5]; iv) stem cells have low production of reactive oxygen species,[8] which may favor the viability of nonreplicating *M. tuberculosis*; v) although

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MSCs have the capacity of self-renewal, they are relatively quiescent,[9] and reside in the immune-privileged niche of the BM[10,11]; and vi) MSCs do not normally express major histocompatibility complex (MHC) class II on their cell surface and their MHC class I molecules are not functionally active (ie, these molecules do not trigger effector functions of cytotoxic T lymphocytes).[12] Therefore, it is logical that BM-MSCs constitute a host cell capable of supporting long-term persistence of viable nonreplicating *M. tuberculosis*. However, many fundamental questions regarding the survival of virulent *M. tuberculosis* in BM-MSCs remain unanswered. Herein, we confirmed *in vivo* in the mouse model of TB that virulent *M. tuberculosis* disseminates rapidly to the BM within 2 weeks after infection with aerosolized organisms and preferentially resides within BM-MSCs. In addition, and more important, we show that antibiotics appeared to efficiently clear the infectious process within the lungs and spleens but fail to do so in the BM.

Materials and Methods

Six- to eight-week-old C57BL/6 female mice (Charles River Laboratories, Kingston, NY) were exposed (nasally only) to virulent *M. tuberculosis* Erdman strain using a CH Technologies (Westwood, NJ) aerosol-generating machine contained within a class III biosafety cabinet in the New England Regional Biosafety Laboratory (Grafton, MA). The low-dose exposure deposited approximately 80 bacilli in the lungs. After 28 days of infection, half of the mice received 100 mg/L rifampicin (RIF) with 250 mg/L isoniazid (INH) in the drinking water, and the other half received sterile acidified water. At the time points indicated, mice were euthanized by CO_2, and lungs, spleens, and BM were harvested, homogenized in sterile phosphate-buffered saline (PBS), and plated onto oleate-albumin-dextrose-catalase–supplemented 7H11 agar. BM was harvested from both femurs and tibia from each mouse by flushing with sterile PBS, homogenized by pressing through 70-μm nylon mesh screens to yield single-cell suspensions, and resuspended in 2 mL of PBS. In initial experiments, the entire 2 mL of BM from each mouse was plated onto agar for colony-forming unit (CFU) recovery. In subsequent experiments, 500 μL of the 2-mL BM from each mouse was plated onto agar. The remaining 1.5 mL from four to six mice was pooled, and red blood cells were lysed, counted (yielding a calculated average of approximately 3.5×10^7cells per mouse), and enriched for BM-MSCs. The enriched/depleted/unfractioned cells were plated onto agar in duplicate or triplicate at the maximum number possible for BM-MSCs or 1 to 1.25×10^7 cells for the depleted and unfractioned cells. In some experiments, single-cell suspensions were obtained from the BM, and enriched for $CD271^+CD45^-$ BM-MSCs using Stemcell Technologies (Vancouver, BC, Canada) EasySep Mouse Mesenchymal Stem/Progenitor Cell Enrichment Kit (catalog number 19771) for mouse MSCs, and fractions plated onto agar. Lung and spleen homogenates were serially diluted in sterile PBS before plating. *M. tuberculosis* CFUs were counted after at least 3 weeks at 37°C. To confirm the quality control of the kit before using it inside the biosafety level 3 facility, BM fractions were obtained from noninfected mice (outside the biosafety level 3 facility) by flushing the marrow out of femur and tibia, followed by softening of bone chips by incubation in solution containing 0.25% collagenase type I in PBS + 20% fetal bovine serum for 45 minutes at 37°C. The mononuclear cells were then filtered through a 70-μm cell strainer and washed twice in PBS containing 2% fetal bovine serum. The $CD45^-$ fractions were obtained by performing magnetic sorting, and the isolated cell population was stained for anti-CD45 (17-0451-83, activated protein C labeled; eBiosciences, San Diego, CA), anti-CD271 (ab62122, fluorescein isothiocyanate labeled; Abcam, Cambridge, MA), or isotype-matched controls. Flow cytometry analysis was performed on an AccuriC6 cytometer (BD Biosciences, San Jose, CA). We were able to collect a calculated average of 2.8×10^6 of $CD45^-$ cells from 3×10^7 BM cells ($n = 5$); 33.5% of $CD45^-$ cells showed expression of CD271. This analysis confirmed $CD271^+$ cell enrichment using the Stemcell Technologies EasySep Mouse Mesenchymal Stem/Progenitor Cell Enrichment Kit (catalog number 19771) for mouse MSCs. All experiments were approved by Tufts University (Grafton, MA) Institutional Biosafety Committee (GRIA04) and Institutional Animal Care and Use Committee (G2012-151).

Results

Virulent *M. tuberculosis* Disseminates to the BM within 2 Weeks of Infection

Our initial observation that showed that *M. tuberculosis* could infect and reside *in vivo* in the mouse BM-MSCs was done using the i.v. route of infection and an avirulent strain of *M. tuberculosis*.[5] To confirm those findings after a natural route of *M. tuberculosis* transmission, mice were initially infected with a low dose (approximately 80 CFUs) of aerosolized virulent *M. tuberculosis* (Erdman strain). Mice were then euthanized at different time points after the infection, and the BM target cells of the infectious process were evaluated. After infection, typical bacillary growth for C57BL/6 mice was observed, peaking at 3 to 4 weeks and then declining by approximately 10-fold to a stable CFU burden (Figure 1).[13,14] The sharp slowing of the bacterial growth rate and decline in bacterial burden between days 28 and 42 in the lungs correspond to the timing for the generation and manifestation of acquired anti-*M. tuberculosis* type 1 helper T-cell immunity.[1,14] Interestingly, between days 7 and 14 after aerosol exposure to *M. tuberculosis*, the bacteria disseminated from the lungs to the BM (Figure 1). This dissemination is similar to that observed in spleen (not shown) and liver.[1,15] Thus, it seems that *M. tuberculosis* enters the BM before the initiation of acquired immunity.

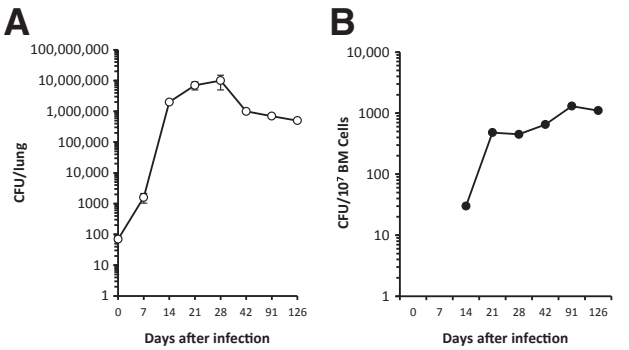

Figure 1 The *M. tuberculosis* disseminates, grows, and survives in bone marrow (BM) during early and chronic infection. Mice were infected with 82 ± 52 virulent *M. tuberculosis* Erdman bacilli by aerosol. At the indicated time points, viable *M. tuberculosis* CFUs were recovered from the lungs (**A**) and BM (**B**). Data are combined from two independent experiments each with four to six mice per time point (n = 8 to 12 mice) and shown as means ± SEM.

Moreover, and in contrast to the lungs, spleen, and liver (not shown in this study), the kinetics of the *M. tuberculosis* burden in the BM do not follow the pattern of a striking peak and decline in these organs, but instead, in the BM, the bacterial burden increases early and remains stable for at least 120 days (Figure 1). These results are consistent with our former observation pointing to BM as a secondary target organ of the infectious process that follows the lung colonization of virulent aerosolized *M. tuberculosis*.

Virulent *M. tuberculosis* that Disseminate from Lung to BM Preferentially Reside within the MSC Subpopulation

To verify the BM cell target of *M. tuberculosis*, mice were challenged with aerosolized bacteria and their BM cells were subsequently harvested at different time points. These experiments aimed to confirm that viable *M. tuberculosis* preferentially target BM-MSCs. Harvested BM cells were separated by negative selection (Stemcell Technologies EasySep Mouse Mesenchymal Stem/Progenitor Cell Enrichment Kit) into two subpopulations of cells, one that expresses $CD271^+CD45^-$ (BM-MSCs) and a second population of cells that are $CD271^-CD45^+$. To verify which cell population was preferentially infected with *M. tuberculosis*, the fractions of BM cells were independently inoculated in oleate-albumin-dextrose-catalase–enriched Middlebrook agar plates and incubated at 37°C for at least 3 weeks, followed by enumeration of the bacterial CFUs. Throughout the infection process, the burden of *M. tuberculosis* infection (CFU) was ≥10-fold higher in the $CD271^+CD45^-$-enriched BM cell fraction (BM-MSCs) than in the CD271-depleted cell population (Figure 2). This preferential BM targeting by *M. tuberculosis* occurs as early as 14 days after lung challenge and is maintained throughout the chronic phase of the infection. As expected, the $CD271^+CD45^-$ depleted cell population also contains a relatively high level of infection because this population of cells comprises other cell types, including macrophages, a well-known population of cells that are normally infected by *M. tuberculosis*. Moreover, the depletion procedure did not non-specifically remove infected $CD271^-$ cells because the CFUs recovered from the unfractionated population of cells did not differ from the $CD271^+$-depleted cell population (Figure 2). On the other hand, it is possible that the depleted $CD271^+$ cells still contain $CD271^+CD45^-$ cells because enrichment procedure does not fully deplete the latter cells. Nonetheless, these results clearly confirm that virulent *M. tuberculosis* enters the mammalian host through the respiratory tract and subsequently infects MSCs in a remote tissue like the BM.

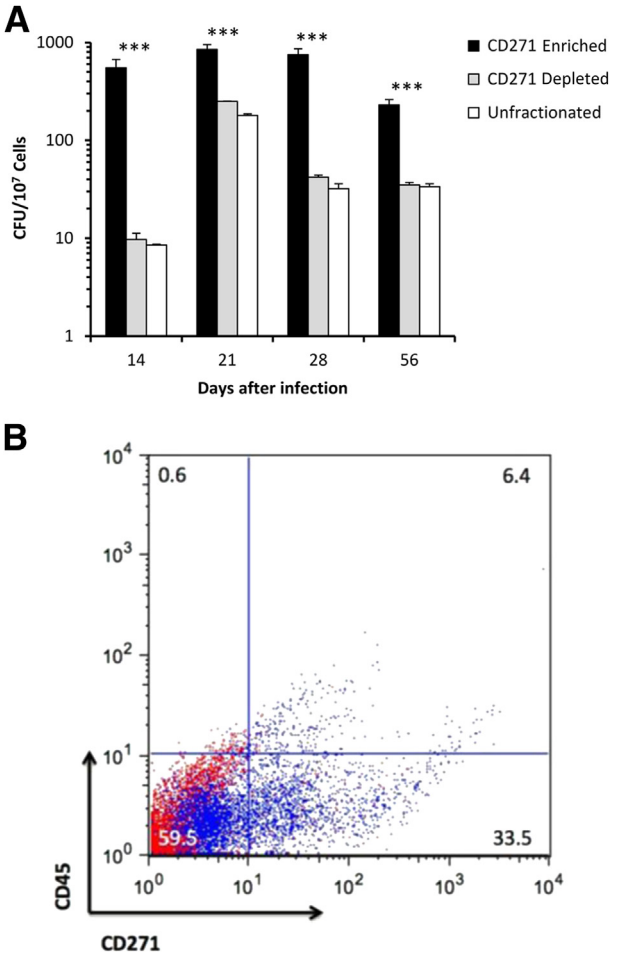

Figure 2 The *M. tuberculosis* disseminates to bone marrow (BM) during early and chronic infection and preferentially infects $CD271^+$ cells. **A:** Mice were infected with 72 ± 21 virulent *M. tuberculosis* Erdman bacilli by aerosol. At the indicated time points, BM cell fractions were obtained after negative selection for BM-mesenchymal stem cell. Viable *M. tuberculosis* colony forming units were recovered from each cell fraction. Data are from four to five mice per time point and shown as means ± SEM. Results were analyzed by one-way analysis of variance with Tukey's post test. **B:** Representative flow cytometry panel shows the presence of $CD271^+/CD45^-$ cells in the enriched population for mouse MSCs (blue dots). Isotype controls are indicated in red. ***$P < 0.001$.

Antibiotics Are Not as Effective at Treating Virulent *M. tuberculosis* in the BM Compared with Lungs and Spleens

Our initial observation in humans indicated that TB patients successfully treated and considered cured still harbored viable *M. tuberculosis* in their BM-MSCs.[5] It is possible that this biological state of resistance to therapy could be a consequence of efflux pump transporters present in these cells.[5,16,17]

To begin to evaluate a role for BM-MSCs as a protective niche allowing *M. tuberculosis* to evade therapy by antibiotic drugs, we took advantage of a well-established animal model of dormant TB. In this model, known for >50 years as the Cornell model,[18–20] mice are infected with virulent *M. tuberculosis* and the resultant infection is typically treated for 12 weeks with the anti-mycobacterial drugs, which eliminates cultivable tubercle bacilli from the lungs, spleen, and livers. However, the condition can revert spontaneously or be induced by immune-suppressive drugs, and *M. tuberculosis* can again be cultured from lungs and spleens of approximately 50% of the animals. This indicates that in some mice, tubercle bacilli were present somewhere and somehow protected from the antibiotics. Because BM is a major niche of MSCs, we hypothesized that viable *M. tuberculosis* could still be present in BM even after successful sterilization of the lungs and spleen. In *M. tuberculosis*-infected mice, which received continuous treatment with RIF and INH starting 28 days after infection, viable *M. tuberculosis* were still present in BM for at least 100 days after no more viable *M. tuberculosis* could be recovered from the lungs and spleens (Figure 3). Interestingly, after the initiation of the antibiotic therapy, there was a steep and equal reduction of the viable *M. tuberculosis* that could be recovered from lungs and spleens, which lead

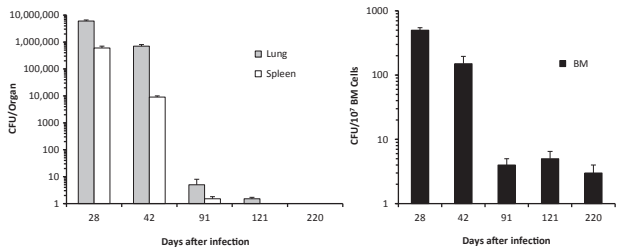

Figure 3 The *M. tuberculosis* survives in the bone marrow (BM) after anti-tuberculosis therapy. Mice were infected with 82 ± 52 *M. tuberculosis* Erdman bacilli by aerosol. Starting at day 28 of *M. tuberculosis* infection, mice received rifampicin and isoniazid continuously in the drinking water. At the indicated time points, viable *M. tuberculosis* colony forming unit (CFU) were recovered from lungs, spleens, and BM. Data are from two independent experiments each with four to six mice per time point ($n = 8$ to 12 mice) and shown as the means ± SEM. The last time points that *M. tuberculosis* could be recovered from the spleens and lungs were after 65 and 93 days, respectively, of continuous antibiotic therapy (days 121 and 220 after infection, respectively). In contrast, viable *M. tuberculosis* CFUs could be recovered from BM at all of the time points tested. Control CFU yields for mice receiving sterile water (instead of antibiotics) are depicted in Figure 1 (experiments were performed concomitantly).

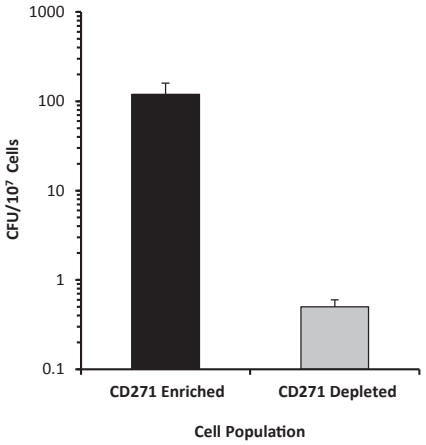

Figure 4 The *M. tuberculosis* bacilli are preferentially recovered from $CD271^{+}CD45^{-}$ bone marrow cells after anti-tuberculosis therapy. Mice were infected with 72 ± 21 *M. tuberculosis* Erdman bacilli by aerosol. Starting at day 28 of *M. tuberculosis* infection, mice received rifampicin and isoniazid in the drinking water. After 4 weeks of antibiotic therapy, BM was harvested, followed by separation of $CD271^{+}CD45^{-}$ and $CD271^{-}CD45^{+}$ cell fractions by negative selection. Cells were dispersed for *M. tuberculosis* culture, and CFU was determined after at least 3 weeks of incubation at 37°C. Data are from four to six mice per group and shown as the means ± SEM. Control colony forming unit (CFU) yields for mice receiving sterile water (instead of antibiotics) are depicted in Figure 2 (experiments were performed concomitantly).

to undetectable levels of viable *M. tuberculosis* (CFU). In contrast, no sterilization occurred in the BM, although there was a reduction in viable *M. tuberculosis* that reached the maximum at day 91 after infection (7 weeks of continuous antibiotic therapy). From then on, *M. tuberculosis* bacilli plateaued at low numbers until the end of the experiment at day 220 after infection (after >6 months of continuous antibiotic therapy). The level of detection in our assays was equivalent for the lungs, spleens, and BM.

To identify the cell population in the BM that hosts and protects the *M. tuberculosis* from RIF/INH, mice were infected for 28 days, followed by treatment with the antibiotics for 4 weeks. BM cells were obtained and separated as described above into $CD271^{+}CD45^{-}$ cells and $CD271^{-}CD45^{+}$ cells. *M. tuberculosis* bacilli were 100 times more numerous in the $CD271^{+}CD45^{-}$ cell population than in the $CD271^{-}CD45^{+}$ cell fraction (Figure 4). These results clearly indicate that BM-MSCs constitute a unique cellular niche, which, in the Cornell model of *M. tuberculosis* persistence, protects the bacteria from therapeutic properties of antibiotics that are used to treat TB.

Discussion

Our previous work established that BM cells, in particular, the $CD271^{+}CD45^{-}$ BM-MSCs, harbor viable *M. tuberculosis* bacilli.[5] However, these studies did not address the kinetics of dissemination from the lungs and persistence of virulent *M. tuberculosis* in the BM or explore temporal relationships between growth of the *M. tuberculosis* in the BM, compared

with the primary site of infection and disease (lungs) or other secondary sites (spleen). Understanding these fundamental processes *in vivo* is important because the results have mechanistic implications for *M. tuberculosis* latent TB infection, which affects billions of people across the globe.[21] Because these experiments cannot be performed in humans, we used the most common inbred mouse strain in *M. tuberculosis* research: C57BL/6 mice. C57BL/6 mice are well-known to be relatively resistant to low-dose aerosol infection of virulent *M. tuberculosis* (as we used herein) with survival >1 year,[22] attributed to strong antimycobacterial immunity due to numerous and potent antigen-specific T cells.[13,23] Thus, we used C57BL/6 mice as a model for studying *M. tuberculosis* in the BM of hosts capable of mounting strong type 1 helper T-cell–polarized anti-*M. tuberculosis* immunity.

Herein, we define that, after lung infection of mice with virulent *M. tuberculosis*, the infectious process disseminates not only to organs like spleen and liver but to BM as well. Interestingly, the pattern of growth and control of the *M. tuberculosis* in the lungs, spleen, and liver[1,13–15] is not the same to that occurring with *M. tuberculosis* in the BM. In contrast to the lung, spleen, and liver, where growth is limited and burden declines because of robust immunity, the growth of the bacteria in the BM does not decline during the chronic phase of infection. Although we have not yet formally investigated the biological and molecular mechanisms that contribute to this phenomenon, it is possible that *M. tuberculosis* within the BM is protected from the host immune response because this tissue is generally considered an immune-privileged niche,[10,11] and this is supported by our observation that no granulomas formed within the BM (not shown).

More important, the present studies clearly demonstrate that *M. tuberculosis*, in addition to the $CD45^+$ cells, also resides in the BM within the unique MSC population ($CD271^+CD45^-$ cells). The results that supported this conclusion (Figure 2) indicated that proportionally the $CD271^+CD45^-$ cells were more infected than the $CD45^+$ population of cells. However, it is important to keep in mind that during the *in vivo* infectious process, the $CD45^+$ cell population constitutes a major target of the *M. tuberculosis* infection because these cells are disparately more numerous than the $CD271^+CD45^-$ cell population. Nonetheless, because of the unique properties of the MSCs, the results strongly support the proposed hypothesis that infection of these cells constitutes an important *M. tuberculosis* escape mechanism of survival within the host. At this point, nothing is known about the mechanism of internalization of *M. tuberculosis* into the BM-MSCs. It is possible that the final localization of the bacteria within these cells may be a stepwise process (ie, the *M. tuberculosis* may be initially internalized into conventional host cells, like macrophages, followed by subsequent dedifferentiation into stem cells). Indeed, this set of events has recently and elegantly been demonstrated to be the case for *Mycobacterium leprae*–infected Schwann cells.[24] This report shows that the leprosy bacterium induces the macrophage-like adult Schwann cells, the preferred host cells for *M. leprae*, to reprogram their differentiation to a stage of progenitor/stem-like cells by down-regulating cell lineage/differentiation-associated genes and up-regulating genes mostly of mesoderm development. Although hypothetical, this is an attractive mechanism to explain our observations that *M. tuberculosis* can be found internalized and likely persist within BM-MSCs.

The persistence of *M. tuberculosis* may represent an important escape mechanism from both the host immune response and may mask them from the reach of anti-TB drugs. The evasion of the immune response is likely to occur within the BM-MSCs because these cells do not normally express MHC class II on their cell surface and their MHC class I molecules are not functionally active and do not trigger effector functions of cytotoxic T lymphocytes.[12] Protection from drugs is likely to be achieved because BM-MSCs have potent efflux pump transporters of ABCG2,[5] an ATP-binding cassette family member that can pump a diversity of organic molecules, including the anti-TB antibiotic RIF, out of the cells.[16,17] Indeed, our data lend support to this hypothesis. By using the well-established Cornell mouse model to study *M. tuberculosis* persistence after drug therapy, for the first time, our results clearly showed that even with prolonged and continuous treatment for >6 months with INH and RIF, the bacterium could still be recovered from the BM. In contrast and as expected from the Cornell model, no *M. tuberculosis* could be isolated from the lungs or spleen. These results identify a hidden location of the residual and viable *M. tuberculosis* present in mice in the Cornell model, which has been elusive for >50 years. More important, our results clearly identified that within the BM cells of antibiotic-treated mice, the pathogen was isolated primarily from the $CD271^+CD45^-$ (BM-MSCs). In contrast, only few *M. tuberculosis* could be isolated from the $CD271^-CD45^+$ cells (monocytes, macrophages, and other phagocytic cells). However, because the $CD271^+CD45^-$ enriched cell population was obtained by negative selection, other cells besides BM-MSCs may be present in this BM cell fraction. Therefore, other possible targets of infection by *M. tuberculosis* that could also help the pathogen escape antibiotic treatment cannot be excluded. However, the fact that the depleted fraction contained few organisms strongly supports the hypothesis that the BM-MSCs can protect *M. tuberculosis* from long-term administration of RIF/INH. These observations are highly relevant, because they may explain why it can be exceedingly difficult to achieve complete microbial sterilization of TB patients with antibiotics.

In conclusion, by using a stringent *in vivo* model of TB, these results strongly support the proposal that BM-MSCs are important target cells of the infection process that provides a robust antibiotic-protective niche for *M. tuberculosis* to survive therapy within its host. In addition, these studies

suggest that unraveling the mechanisms underlying the *M. tuberculosis* and BM-MSC interactions will enhance our understanding of *M. tuberculosis* persistence under immune and antibiotic pressures in TB patients.

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