IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Preclinical and Clinical Evidence of *Mycobacterium tuberculosis* Persistence in the Hypoxic Niche of Bone Marrow Mesenchymal Stem Cells after Therapy

Jaishree Garhyan,^{*†} Seema Bhuyan,^{*} Ista Pulu,^{*} Deepjyoti Kalita,^{*} Bikul Das,^{*‡} and Rakesh Bhatnagar[†]

From the KaviKrishna Laboratory,^{*} Guwahati Biotech Park, Indian Institute of Technology, Guwahati, India; the Molecular Biology and Genetic Engineering Laboratory,[†] School of Biotechnology, Jawaharlal Nehru University, New Delhi, India; and the Department of Immunity and Infectious Diseases,[‡] Forsyth Institute, Cambridge, Massachusetts

Accepted for publication
March 17, 2015.

Address correspondence to
Bikul Das, Ph.D., or Rakesh
Bhatnagar, Ph.D., Forsyth
Institute, 245 First St, Cam-
bridge, MA 01247.
E-mail: bdas@forsyth.org or
rakeshbhatnagar@jnu.ac.in.

Mycobacterium tuberculosis (MTB), the causative agent of pulmonary tuberculosis, is difficult to eliminate by antibiotic therapy. We recently identified CD271⁺ bone marrow—mesenchymal stem cells (BM-MSCs) as a potential site of MTB persistence after therapy. Herein, we have characterized the potential hypoxic localization of the post-therapy MTB-infected CD271⁺ BM-MSCs in both mice and human subjects. We first demonstrate that in a Cornell model of MTB persistence in mice, green fluorescent protein—labeled virulent MTB-strain H37Rv was localized to pimonidazole (an *in vivo* hypoxia marker) positive CD271⁺ BM-MSCs after 90 days of isoniazid and pyrazinamide therapy that rendered animal's lung noninfectious. The recovered CD271⁺ BM-MSCs from post-therapy mice, when injected into healthy mice, caused active tuberculosis infection in the animal's lung. Moreover, MTB infection significantly increased the hypoxic phenotype of CD271⁺ BM-MSCs. Next, in human subjects, previously treated for pulmonary tuberculosis, the MTB-containing CD271⁺ BM-MSCs exhibited high expression of hypoxia-inducible factor 1 α and low expression of CD146, a hypoxia down-regulated cell surface marker of human BM-MSCs. These data collectively demonstrate the potential localization of MTB harboring CD271⁺ BM-MSCs in the hypoxic niche, a critical microenvironmental factor that is well known to induce the MTB dormancy phenotype. (*Am J Pathol* 2015, 185: 1924–1934; <http://dx.doi.org/10.1016/j.ajpath.2015.03.028>)

Pulmonary tuberculosis (PTB) is one of the major global health threats worldwide, causing >2 million deaths each year.¹ *Mycobacterium tuberculosis* (MTB), the causative agent of PTB, has the spectacular ability of long-term persistence despite vigorous host immunity and prolonged chemotherapy.^{2,3} Although effective therapies exist that could eliminate most of the bacteria, some bacteria still persist in secured niches, and then reactivate the disease,⁴ leading to therapy failure (World Health Organization, http://www.who.int/tb/publications/global_report/en/index.html, last accessed January 11, 2014).

We hypothesized that bone marrow—mesenchymal stem cells (BM-MSCs) might serve as a protective intracellular niche for post-therapy MTB. The stem cell niches are immunoprivileged and hypoxic.^{5–7} In humans, CD271^{high}

and CD146^{low} BM-MSCs reside in the hypoxic niche.⁶ Because the hypoxia microenvironment may favor MTB dormancy,^{8,9} leading to marked resistance against therapy,⁹ maintain a low reactive oxygen species niche that may favor the undifferentiated state of stem cells (stemness)¹⁰ required to maintain MTB viability,⁸ and enhance the expression of

Supported by KaviKrishna Foundation (Sualkuchi, Assam, India) grants (J.G., S.B., D.K., and I.P.), Laurel Foundation grants (B.D.), and the Department of Biotechnology, government of India (biosafety level 3 facility of Jawaharlal Nehru University) (R.B.).

B.D. and R.B. contributed equally to this work as senior authors.

Disclosures: B.D. is a founding director and honorary director of the KaviKrishna Foundation (Assam, India), which is a not-for-profit, nongovernmental organization involved in the basic and clinical study described in this work.

drug-efflux pump ABCG2 in stem cells,^{10,11} which might counter intracellular drug availability, it is important to evaluate if post-therapy persistent MTB resides in the hypoxic BM-MSCs.

Accordingly, we recently identified CD271⁺ BM-MSCs as the potential host cell type that could maintain viable bacilli in subjects previously treated for PTB.¹² Herein, we studied the hypoxic niche of MTB harboring CD271⁺ BM-MSCs by using the well-established Cornell model of dormancy.

For decades, the Cornell model of dormancy has been used to study the biology of post-therapy persistent MTB. In this model, mice are first infected with virulent MTB, and treated with drugs for weeks to render lung and spleen sterile to generate drug-induced dormancy.^{13–16} After therapy withdrawal, spontaneous or steroid-induced reactivation of the lung infection occurs, suggesting the existence of yet-to-be-identified secured niches that maintain viable bacteria.¹⁷ Hence, the model could be used to test the hypothesis that CD271⁺ BM-MSCs residing in the hypoxic BM niche harbor viable dormant bacteria after therapy.

Herein, we provide direct evidence that CD271⁺ BM-MSCs serve as a secured niche of persistent MTB, and reside in the hypoxic niche *in vivo*, in a Cornell model of dormancy. Next, in human subjects treated for PTB, we report high hypoxia-inducible factor 1 α (HIF-1 α) expression and decreased CD146 levels in MTB-DNA carrying CD271⁺ BM-MSCs, suggestive of a hypoxic phenotype.⁶ More important, we found that MTB infection increases the hypoxic phenotype of CD271⁺ BM-MSCs. These results highlight the clinical significance of the hypoxic CD271⁺ BM-MSCs as a secured niche for post-therapy persistent MTB.

Materials and Methods

Bacterial Strains and Growth Conditions

MTB H37Rv, green fluorescent protein (GFP) H37Rv, or streptomycin-auxotrophic mutant MTB strain18b¹² (provided by Prof. Stewart T. Cole, Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland) was cultured in 7H9 medium (Difco, BD Biosciences, Franklin Lakes, NJ) supplemented with Middlebrook albumin-dextrose-catalase, 0.2% glycerol, and 0.1% Tween 80 (Sigma, St. Louis, MO) until an OD of approximately 1 was obtained. The medium of streptomycin-auxotrophic mutant MTB strain18b was supplemented with 50 μ g/mL of streptomycin sulfate for growth.¹² Cell suspension was sonicated for 15 seconds, followed by infection of animals with 0.1 mL of bacteria suspension *i.v.* through tail vein. GFP-tagged H37Rv was obtained by electroporation of GFPpUV15⁺ plasmid (donated by Dr. Sabine Ehrt, Weill Cornell Medical College, New York, NY) using the Gene Pulser II Electroporation System (Biorad, Hercules, CA) and selected on 7H10 plates with hygromycin B at 50 μ g/mL. The GFPpUV15⁺ transfected MTB H37Rv maintained its

infective ability, as assessed *in vivo* using BALB/c mice. The mutant 18b strain was cultured as previously described.¹²

Establishing the Cornell Model of Dormancy in Mice

We used a variant of the Cornell model,^{18,19} in which MTB infection was introduced by the *i.v.* route, and mice were treated with isoniazid (INH) and pyrazinamide (PZA) to achieve organ sterility.¹⁸ Briefly, pathogen-free, 6-week-old female BALB/c mice were obtained from the National Institute of Nutrition (Hyderabad, India). They were housed within a biosafety level 3 within microisolator cages, and fed pelleted food and water *ad libitum*. All studies were approved after institutional review and approval by the Institution of Animal Ethics Committee and the Institution of Biosafety Committee at Jawaharlal Nehru University (New Delhi, India) as well as the Biosafety Committee at KaviKrishna Laboratory (Guwahati, India). Mice were infected via tail vein with approximately 2×10^6 colony-forming units (CFUs) of MTB H37Rv or GFPpUV15⁺ H37Rv pathogenic or mutant MTB strain of 18b. Animals were weighed twice monthly and checked daily for clinical symptoms of weight loss and lack of response to stimuli, as displayed in rodent tuberculosis. At 60 days after infection, mice were treated with 0.1 g/L INH and 15 g/L PZA in drinking water *ad libitum* for 90 days. The organ sterilization was confirmed by performing a MTB-CFU study as described below.

Histopathology of Lung Granuloma

For the histopathological detection of lung granuloma, the organ was fixed in 10% buffered formalin, paraffin embedded, and subjected to hematoxylin and eosin and acid-fast bacilli staining for granuloma and presence of bacilli, respectively, using an Olympus inverted microscope 1X71 equipped with a DP71 camera (Olympus, Tokyo, Japan).

In Vivo Pimonidazole Labeling of CD271⁺ BM-MSCs

For determination of the hypoxic niche in BM, a group of mice was injected with saline or 60 mg/kg pimonidazole hydrochloride (Hypoxypore; Hypoxypore, Inc., Burlington, MA) *i.p.* The animals were sacrificed 2 hours after injection, and BM cells were harvested and enriched for CD271⁺ BM-MSCs by flow cytometry, followed by confocal imaging using anti-pimonidazole antibody, as described below.

BM Harvesting of Live CD271⁺ BM-MSCs

The procedure was performed as previously described.¹² Briefly, BM was harvested from femur of each mouse by flushing with buffer [phosphate-buffered saline (PBS) + EDTA], homogenized by passing through a 25-gauge needle four to five times, filtering through a nylon mesh screen

(40 μm thick) to obtain single-cell suspensions, and suspending in 4 mL of buffer (2% PBS + EDTA). Red blood cell lysis was performed by suspending the cells in red blood cell lysis buffer (catalog number R7757; Sigma) for 5 to 10 seconds twice. From each mouse, on average, 4×10^7 nucleated cells were obtained. The BM mononuclear cells were then subjected to magnetic sorting to enrich for a CD45⁺ population by a negative selection process (mouse mesenchymal enrichment kit, catalog number 19771; Stem Cell Technologies, Vancouver, BC, Canada). On average, per mouse, 2.75×10^6 CD45⁺ cells were obtained. Next, the CD45⁺ cells were subjected to positive selection of CD271⁺ cells, as follows. First, mouse CD271 antibody (mouse clone ME20.4, catalog number Ab8877; Abcam, Cambridge, MA) was phycoerythrin (PE) conjugated by SiteClick antibody labeling kit (catalog number S10467; Life Technologies, Grand Island, NY), as per manufacturer instructions. Next, the EasySep PE sorting kit (catalog number 18554; Stem Cells Technologies) was used to perform positive selection of CD271⁺ cells from the CD45⁺ cell population. The purity of the enriched CD271⁺/CD45⁺ BM-MSCs was confirmed by flow cytometry analysis,¹² and found to be 90% enriched. The purified cell population was subjected to MTB-CFU assay and/or fixed and subjected to flow cytometry/confocal immunofluorescence analysis.

Flow Cytometry

Flow cytometry was performed using fluorescence-activated cell sorter calibur (Becton Dickinson, Franklin Lakes, NJ), as described elsewhere. Cell surface staining for CD271, CD45, CD73, and CD44, as well as intracellular staining for pimonidazole and HIF-1 α , was performed. Briefly, the single-cell suspension of CD271⁺ BM-MSCs (enriched by immunomagnetic sorting of CD271⁺/CD45⁺ cells) was fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 minutes on ice, and washed in flow cytometry buffer (PBS containing 0.1% bovine serum albumin and 0.1% sodium azide). Samples were incubated with primary antibody against CD45 (rat clone 30-F11, 1:100; Biolegend, San Diego, CA), CD271 (rat clone 2E3, ab27007, or mouse clone ME20.4, ab8877; Abcam), HIF-1 α (rabbit polyclonal, NB100-449; Novus, Littleton, CO), pimonidazole (mouse clone 4.3.11.3, 1:50; Hypoxypore, Inc.), CD44 [clone IM7; allophycocyanin (APC) conjugated; Biolegend], and CD73 (clone TY/11.8; Pacific blue conjugated; Biolegend) for 2 hours in staining buffer (4% bovine serum albumin plus 0.1% saponin) at room temperature. After washing three times with 0.1% PBS, cells were incubated with appropriate secondary antibodies for 30 to 45 minutes at room temperature, and prepared for flow cytometry analysis. For direct cell surface staining of CD271 in BM mononuclear cells obtained from GFP-H37Rv-infected mice, live cells were incubated with Fc receptor blocking reagent (catalog number 553141; BD Bioscience) for 15 minutes at 4°C, followed by incubation with PE-labeled monoclonal CD271 (clone MLR2; Abcam) and CD45–fluorescein isothiocyanate (catalog number 552772;

BD Bioscience), for 30 minutes in the dark at 4°C in flow cytometry buffer (PBS containing 0.1% bovine serum albumin and 0.1% sodium azide). The antibody-stained cells were washed three times, and then subjected to flow cytometry using fluorescence-activated cell sorter calibur (Becton Dickinson, Franklin Lakes, NJ) within 24 hours of staining.

Confocal Microscopy Imaging for Pimonidazole Staining

Formaldehyde-fixed cells were stained with CD271PE antibody (dilution 1:100) for 20 minutes on ice, washed and stained by DAPI (FluoroshieldDapi; Sigma), and viewed by a confocal microscope (Fluoview FV-1000, model 1X81; Olympus). To visualize the hypoxic marker pimonidazole, the CD271⁺ BM-MSCs were first stained with CD271PE antibody, fixed, and permeabilized by Intraprep Permeabilization

Figure 1 Green fluorescent protein (GFP)-H37Rv detection in CD271⁺ bone marrow (BM)-mesenchymal stem cells in the Cornell model of dormancy. **A:** A schematic diagram of the Cornell model of dormancy. The **arrow** refers to the time point of obtaining flow cytometry data. **B:** A representative flow cytometry profile of BM mononuclear cells depicting the GFP-H37Rv-positive CD271 cells (the left flow cytometry profile depicts isotype staining). **C:** Confocal microscopy images of the same BM cells showing the localization of GFP-positive H37Rv strain intracellular to CD271⁺ cells (**arrows**). The BM cells were obtained from isoniazid (INH)-pyrazinamide (PZA)-treated mice on day 150 after infection + drug treatment, as shown in **A**. For the confocal microscopy, CD45⁺ cells were enriched by immunomagnetic sorting before performing CD271 (phycoerythrin labeled) staining. $n = 3$ independent experiments (**C**). Original magnification, $\times 200$ (**C**).

Figure 2 Recovery of viable bacteria from the CD271⁺ bone marrow—mesenchymal stem cells (BM-MSCs) after therapy. **A:** A schematic diagram to depict the experimental design. **B:** Representative flow cytometry panel of CD271⁺ CD45⁻ cells after enrichment of immunomagnetic sorting. **C:** The sorted CD271⁺ CD45⁻ cells express CD73 and CD44. **D:** The H37Rv—colony-forming units (CFUs) obtained from the immunomagnetically sorted CD271⁺ BM-MSCs on day 150 after infection + drug treatment. H37Rv—CFUs were nondetectable (ND) in lung and spleen. **E:** Injection of CD271⁺ BM-MSCs to healthy mice led to lung granuloma, as depicted in the representative histopathological image; uninvolved alveoli are seen along the left and lower edges. To the right is the acid-fast bacilli (AFB) staining image highlighting an area of the granuloma (G; boxed area near G) showing the presence of AFB-positive bacilli (arrow) in the cytoplasm of a foamy histiocyte. The granuloma was obtained from mice injected with 6×10^4 CD271⁺ BM-MSCs obtained from the *Mycobacterium tuberculosis* (MTB)—H37Rv—infected mice BM cells. $n = 4$ independent experiments (**B** and **C**); $n = 3$ independent experiments (**D** and **E**). AS, alveolar space; INH, isoniazid; PZA, pyrazinamide.

Reagent kit (Beckman Coulter, Fullerton, CA). This was followed by staining with anti-pimonidazole antibody [anti-PIM-RED APC-Mab (dilution 1:100); Hypoxyprobe-Red APC kit, HP8-100Kit; Hypoxyprobe, Inc.] or a mouse IgG1 isotype control antibody at room temperature for 20 minutes. Cells were counterstained with DAPI to visualize nuclear DNA.

Controlled MTB Replication in BM

To regulate the MTB replication in BM, the streptomycin-auxotrophic mutant strain of 18b (henceforth known as m18b) was used, which replicates only in the presence of streptomycin.¹²

The m18b strain was delivered i.v. to 6- to 8-week-old female BALB/c mice at a dose of 2×10^6 CFUs per mouse, as described previously.¹² Mice were divided into two groups ($n = 8$, each group). Although the first group received streptomycin (2 mg per mouse) daily s.c. for 3 weeks, the second group received streptomycin for half a

week (4 days) only. After the injection, mice were observed for 4 weeks without any streptomycin injection. On day 50 (eighth week) of infection, mice were given a pimonidazole injection i.p. and then sacrificed to collect CD271⁺/CD45⁻ and lineage⁺/CD45⁻ cells by magnetic sorting. The enriched cells were either subjected to MTB-CFU assay or fixed in 4% formaldehyde to perform pimonidazole labeling analysis by flow cytometry.

Determination of MTB-CFUs

The assay was performed as previously described.¹² Briefly, lung, spleen, and BM cells were aseptically removed from sacrificed mice, homogenized in PBS with 0.05% Tween 80, and then subjected to CFU assay. For increasing the sensitivity of CFU assay in the antibiotic-treated group, 100 μ L from organ homogenates (in 2 mL) was directly plated onto the 7H11 plates (Difco) without any dilution. For the CFU assay of immunomagnetically sorted cells, the purified population was washed twice, homogenized, and then plated onto 7H11 plates. Data are

presented as the means of $\log_{10}$ CFUs per organ, and the SEM is indicated by error bars ($n = 3$ to 5 mice per group); each experiment was performed three times.

Clinical Study

The detailed procedure of patient selection, immunomagnetic sorting, and mRNA isolation of CD271⁺ BM-MSCs was followed as described previously.¹² The RNAs of post-therapy PTB subjects having confirmed presence of MTB-DNA ($n = 7$) and healthy subjects having no history of PTB ($n = 4$), and without any presence of MTB-DNA in their CD271⁺ BM-MSCs, were subjected to quantitative real-time PCR for HIF-1 α and CD146, as described previously.¹² TaqMan primers, HIF-1 α (Hs00153153), CD146 (Hs00174838_m1), and glyceraldehyde-3-phosphate dehydrogenase (Hs00266705_g1), were obtained from Applied Biosystems, Life Technologies. The fold changes in relative RNA levels of CD146 and HIF-1 α were estimated by comparing the $\Delta\Delta C_T$ values of the clinical subjects (both after PTB and healthy subjects) versus CD271⁺ BM-MSCs obtained from commercially available CD133/CD271 BM cells of healthy volunteers (catalog number ABC026F; Stem Cell Technologies¹²) grown under normoxia for 1 week in the *in vitro* serum-free media, as previously described.¹² For the *in vitro* experiment of hypoxia, the CD271 BM-MSCs obtained from CD133/CD271 cells were treated under 1% hypoxia (hypoxia chamber set to 1% hypoxia) *in vitro* for 1 week or 100 μ mol/L deferoxamine mesylate (Sigma) under normoxia, with three times weekly media change for 1 week in serum-free culture media.¹² The CD271⁺/CD45⁻ cells were magnetically sorted¹² and subjected to real-time PCR for CD146 and HIF-1 α expression. The fold change in relative RNA levels of these two genes was estimated by comparing the $\Delta\Delta C_T$ values of the hypoxia versus normoxia samples.

Statistical Analysis

All experiments, unless otherwise stated, were repeated three times in triplicate. A *t*-test was performed to compare two groups. Cell quest software version 5.1 (BD, San Jose, CA) was used for analysis of flow cytometry data. Data are expressed as means $\pm$ SEM.

Results

GFP-H37Rv Detection in CD271⁺ BM-MSCs in the Cornell Model of Dormancy

To characterize the CD271⁺ BM-MSC niche of MTB after therapy, we first confirmed the intracellular detection of viable MTB in the BM-MSCs in a Cornell model of dormancy. First, we used the well-characterized Cornell model of dormancy, in which GFP-labeled H37Rv mycobacteria were i.v. injected into BALB/c mice to establish infection (Figure 1A). We

Figure 3 *Mycobacterium tuberculosis* (MTB) persistence in CD271⁺ bone marrow—mesenchymal stem cells (BM-MSCs) before and after drug treatment. MTB—colony-forming units (CFUs) obtained from pre-drug- and post-drug-treated H37Rv intracellular to CD271⁺ BM-MSCs. H37Rvs of both lung and spleen exhibit rapid drug-induced toxicity, whereas H37Rv intracellular to CD271⁺ BM-MSCs exhibits no apparent drug-induced toxicity. The CD271⁺ BM-MSCs were obtained by immunomagnetic sorting of BM cells obtained from MTB-H37Rv—infected mice before and after drug treatment. The sorted cells were then subjected to MTB-CFU estimation. Lung and spleen were obtained for MTB-CFU study, as previously described.¹² The arrow indicates the timing of isoniazid (INH)—pyrazinamide (PZA) treatment of MTB-H37Rv—infected animal. Data are expressed as means $\pm$ SEM. $n = 3$ independent experiments.

confirmed granuloma formation in mice 60 days after infection with the GFPpUV⁺-transfected MTB H37Rv strain. Second, the mice were treated with INH-PZA treatment for 90 days (Figure 1A), as previously described.^{18,19} Day 60 after infection was chosen as the start day for INH-PZA treatment, because peak CFU and granuloma formation was typically observed by day 60 after infection in our model, which is consistent with previous reports.^{20,21} Third, the animals were euthanized after 90 days of drug treatment, and BM cells were subjected to flow cytometry and confocal microscopy detection of the GFP-positive CD271⁺ cell population. The flow cytometry (Figure 1B) and confocal microscopy (Figure 1C) results clearly demonstrated the presence of GFP⁺ intracellular bacilli in CD271⁺ cells. Thus, we demonstrated that in a Cornell model of dormancy, intracellular MTB could be detected in the BM CD271⁺ BM-MSC cells. However, we could not test the viability of intracellular MTB by this approach, because live cell flow cytometry sorting was not permitted for cells infected with virulent MTB-H37Rv.

Recovery of Viable Bacteria from the CD271⁺ BM-MSCs with Reactivation Potential

To confirm the viability and reactivation potential of persistent MTB localized in the CD271⁺ BM-MSCs of post-chemotherapy (INH + PZA) treated mice, we took a step-wise experimental approach (Figure 2A). First, we developed an immunomagnetic sorting technique to isolate live MTB-infected CD271⁺ BM-MSCs. Briefly, CD45-depleted BM cells were stained with a CD271-PE—conjugated antibody, and the stained cells were subjected to immunomagnetic sorting using a PE-based positive selection kit.^{22,23} By using this two-step enrichment strategy, we were able to achieve a $90.4\% \pm 3.2\%$ ($n = 4$) enrichment of CD271⁺ CD45⁻ cells, as confirmed by flow cytometry (Figure 2B); the enrichment

Figure 4 *In vivo* pimonidazole labeling of CD271⁺ bone marrow-mesenchymal stem cells (BM-MSCs) containing post-therapy persistent *Mycobacterium tuberculosis* (MTB). **A:** Representative flow cytometry panel showing the pimonidazole labeling of CD271⁺ BM-MSCs obtained from non-MTB-infected healthy mice. The histogram shows hypoxia-inducible factor (HIF)-1α expression of the CD271⁺/pimonidazole⁺ cell fraction. BM CD271⁺/CD45[−] cells (1×10^5) obtained from pimonidazole-injected BALB/c mice (aged 6 to 8 weeks) by immunomagnetic sorting were fixed, and stained against pimonidazole (allophycocyanin), CD271 (phycoerythrin PE), and HIF-1α (fluorescein isothiocyanate) for flow cytometry analysis. **B:** Representative flow cytometry profile showing the pimonidazole staining (APC) of green fluorescent protein (GFP)-H37Rv⁺ CD271⁺ BM-MSCs obtained from day 90 of isoniazid (INH)-pyrazinamide (PZA)-treated mice. After the completion of the INH and PZA treatment, BALB/c mice (aged 6 to 8 weeks) were injected with pimonidazole, and BM cells were recovered to isolate CD271⁺/CD45[−] cells by immunomagnetic sorting. For the isotype controls, pimonidazole-injected healthy mouse BM was used. The **dashed arrows** in **A** and **B** highlight that, although 73% of non-MTB-infected cells were positive for pimonidazole (**A**), most cells were pimonidazole positive in MTB-infected mice (**B**). $n = 6$ mice (**A**); $n = 3$ mice (**B**); $n = 3$ independent experiments (**A** and **B**).

percentage remained similar in MTB-H37Rv-infected mice. The sorted CD271⁺/CD45[−] cells expressed CD44 and CD73 (Figure 2C), which are the additional cell surface markers of the BM-MSCs.¹²

Second, we isolated live CD271⁺ BM-MSCs from the postchemotherapy H37Rv-infected mice, and subjected the cell lysate for MTB-CFU assay. Lung and spleen lysates were also evaluated for viable MTB-CFUs to confirm organ sterilization after therapy. We were able to isolate approximately 80 CFUs from CD271⁺ BM-MSCs obtained from each mouse (Figure 2D), whereas lungs and spleens exhibited absence of viable CFUs (Figure 2D). These results confirmed the recovery of viable H37Rv-CFUs from the CD271⁺ BM-MSCs in the above-described Cornell model of dormancy.

Third, we investigated the reactivation potential of the persistent MTB in CD271⁺ BM-MSCs by performing a transplantation assay (Figure 2E). Briefly, immunomagnetically sorted CD271⁺ BM-MSCs (6×10^4 cells) obtained from postchemotherapy-treated mice were injected to healthy secondary recipient mice (healthy animals without any prior infection) by tail vein injection, and the reactivation potential was confirmed by histopathological detection of lung granuloma formation by hematoxylin and eosin and acid-fast bacilli staining for MTB. MTB noninfected CD271⁺ BM-MSC injection to healthy mice (6×10^4 cells; $n = 3$) was used as the experimental control. We found that two of two mice developed granuloma after 12 weeks of injection of CD271⁺ BM-MSCs (Figure 2E). In contrast, noninfected CD271⁺ BM-MSCs did not form any reactive granuloma or inflammation in the lung of injected mice (Supplemental Figure S1). These results indicated that the postchemotherapy MTB in CD271⁺ BM-MSCs remained viable and maintained potential to generate granulomas *in vivo*.

MTB Persistence in CD271⁺ BM-MSCs before and after Drug Treatment

Our previous *in vitro* data on H37Rv suggest that in the BM-MSC niche, MTB may attain a nonreplicating state, even without anti-TB drug treatment.¹² Hence, we speculated that MTB may also attain a nonreplicating state intracellular to CD271⁺ BM-MSCs *in vivo*. To obtain *in vivo* information about the fate of MTB-H37Rv replication in CD271⁺ BM-MSCs, we obtained the MTB-CFUs of CD271⁺ BM-MSCs from day 30 to day 150 after infection, with or without INH/PZA treatment. The MTB-CFUs of lung and spleen served as experimental controls. The results showed that intracellular MTB in CD271⁺ BM-MSCs increased in CFUs during the first 30 days of infection and then remained unchanged throughout the infection period, suggesting the nonreplicating status of intracellular MTB (Figure 3). Significantly, drug treatment did not exert any visible toxicity to the intracellular MTB pool, because the number of viable MTB-CFUs in drug-treated versus untreated groups did not change. In contrast, as expected, the number of viable CFUs in the lung and spleen of drug-treated mice rapidly declined after drug treatment (Figure 3), as reported previously.^{15,16,19} Our results indicate that in the CD271⁺ BM-MSC niche, MTB-CFUs increase during the first 30 days of infection and thereafter remained stable, with or without drug treatment. The result further demonstrates that MTB inside the CD271⁺ BM-MSCs is protected from INH-PZA-induced toxicity.

Persistent MTB Harboring CD271⁺ BM-MSCs Is Localized in the Hypoxic Niche

One of the main characteristics of the BM stem cell niche is the prevailing hypoxic microenvironment.^{6,7} However, the hypoxic localization of murine CD271⁺ BM-MSCs is not yet studied. Therefore, we characterized the BALB/c mice

Figure 5 *Mycobacterium tuberculosis* (MTB) replication in bone marrow (BM) enhances pimonidazole binding of CD271⁺ BM-mesenchymal stem cells (MSCs). **A:** A schematic representation of the experimental design to regulate the replication of mutant strain of 18b in BM and subsequent pimonidazole injection of the animal to detect hypoxic BM cell. **B:** Histograms show the number of viable MTB-m18b colony-forming units (CFUs) and the percentage of pimonidazole binding cells after streptomycin for 3 versus 0.5 weeks of treatment. CD271⁺ BM-MSCs and lin⁺/CD45⁺ BM cells were obtained by immunomagnetic sorting, whereas lung tissue was obtained as previously described.¹² **C:** A representative flow cytometry analysis result that was used to calculate the percentage of pimonidazole binding data depicted in **B**. Results were obtained from six independent experiments, and *P* value was calculated by *t*-test. **P* < 0.05, ****P* < 0.001.

CD271⁺ BM-MSC hypoxic niche by an *in vivo* pimonidazole labeling assay.^{6,24,25} Pimonidazole is a 2-nitroimidazole compound, which forms stable adducts with cytosolic protein in a hypoxic microenvironment (oxygen concentration of 10 mmHg) when administered *in vivo*; the adducts containing cells are flow cytometry identified with the use of an anti-pimonidazole antibody.²⁴ We also stained the BM-MSCs for HIF-1 α ²⁵ as an additional marker of hypoxia localization. The lineage-positive CD45⁺ cells that reside in the relatively well-oxygenated area were used as a negative control for pimonidazole binding. Briefly, CD271⁺ BM-MSCs obtained (by magnetic sorting) from pimonidazole-injected mice were stained by anti-pimonidazole and anti-HIF-1 α antibodies, and then analyzed by flow cytometry. Of CD271⁺ BM-MSCs, 73% stained positive for pimonidazole and HIF-1 α (Figure 4A). As expected, the lineage⁺/CD45⁺ cells did not stain for pimonidazole, confirming the previous observation

that differentiated cells reside in the well-oxygenated area of BM.⁶ Thus, the selective pimonidazole binding and HIF-1 α expression confirmed the hypoxic localization of CD271⁺ BM-MSCs in BALB/c mice.

To confirm the hypoxic localization of CD271⁺ BM-MSCs in post-drug-treated MTB-H37Rv-infected mice, we collected BM of H37Rv-infected mice after 90 days of INH-PZA regimen (Figure 4A). We detected a 4.3% GFP⁺/pimonidazole⁺ population in the CD271⁺ BM-MSCs (Figure 4B). Most GFP⁺CD271⁺ cells positively stained for pimonidazole. We further confirmed the finding, by confocal microscopy staining, that showed that most cells were strongly positive for pimonidazole (Supplemental Figure S2). These data imply that most CD271⁺ BM-MSCs in the infected mice exhibited pimonidazole binding.

Next, we investigated the potential relation between MTB infection and replication and BM-MSC hypoxia. Although only 73% of CD271⁺ BM-MSCs exhibited pimonidazole binding in noninfected mice, almost all of the BM-MSCs exhibited pimonidazole binding in the MTB-infected mice (Figure 4); however, only a few of the CD271⁺ BM-MSCs (approximately 4.3%) served as a reservoir of MTB. We speculated that initial MTB infection and replication in BM could increase pimonidazole binding of BM-MSCs because recent studies indicate an infection- and inflammation-associated increase in tissue hypoxia.²⁶ To investigate this possibility, we compared pimonidazole binding of BM cells in mice infected with the auxotrophic mutant 18b strain of MTB, which replicates only in the presence of streptomycin.¹² The experimental design is described in Figure 5A.

Briefly, we exploited the streptomycin dependency of this mutant strain to increase or decrease MTB replication in BM,¹² and then compared the pimonidazole binding of BM cells between the MTB-replicating and poorly replicating groups. We found that almost all of the CD271⁺ BM-MSCs in the 3-week streptomycin treatment group exhibited pimonidazole binding. In contrast, mice receiving only half a week of streptomycin and, therefore, limited MTB-CFUs in lung and BM exhibited no significant increase in pimonidazole binding of CD271⁺ BM-MSCs (Figure 5, B and C). Thus, we found that MTB mutant 18b replication was associated with an increase of pimonidazole⁺CD271⁺ BM-MSCs. The lin⁺/CD45⁺ cells also exhibited an increase in pimonidazole binding in 3-week versus 0.5-week streptomycin dose (Figure 5B), indicating that MTB infection and replication in BM may also increase the pimonidazole binding of CD45⁺ differentiated cells. The result suggests that MTB infection and replication in BM may cause a non-specific increase in hypoxia of BM cells.

MTB-Containing CD271⁺ BM-MSCs of Clinical Samples Exhibit Hypoxic Phenotype

To evaluate the clinical significance of the above findings in mice, it is necessary to perform a human-equivalent study to find out if the post-therapy MTB-infected BM-MSC

Figure 6 *Mycobacterium tuberculosis* (MTB)-harboring CD271⁺ bone marrow–mesenchymal stem cells (BM-MSCs) of post-therapy pulmonary tuberculosis (PTB) subjects exhibit hypoxic phenotype. **A:** Histogram showing the high hypoxia-inducible factor (HIF)-1 α /low CD146 gene expression of CD271⁺ BM-MSCs cultured in 1% oxygen for a week. The BM-MSCs were commercially obtained. **B:** Histogram showing the expression of HIF-1 α and CD146 in the CD271⁺ BM-MSCs obtained from seven post-therapy PTB subjects having confirmed MTB-DNA (MTB-DNA + subjects group) intracellular to CD271⁺ BM-MSCs,¹² and four subjects having no history of PTB, and no detectable presence of MTB-DNA intracellular to CD271⁺ BM-MSCs¹³ (healthy subject group). $N = 4$ independent experiments (A). * $P < 0.05$, *** $P < 0.001$, and **** $P < 0.0001$.

population exhibits hypoxia phenotype. However, unlike in mice, it is difficult to assess the hypoxia phenotype of MTB-infected BM-MSCs in post-therapy PTB subjects. Pimonidazole staining–based *in vivo* labeling of BM-MSCs is ethically challenging to perform in humans. Alternatively, HIF-1 α , a general marker of hypoxia, could be used to evaluate the hypoxia phenotype of CD271⁺ BM-MSCs. However, other microenvironmental factors, including reactive oxygen species and growth factors such as vascular endothelial growth factor, may also induce HIF-1 α expression.²⁴ Therefore, we considered an innovative approach to evaluate the hypoxia phenotype of CD271⁺ BM-MSCs obtained from post-therapy PTB subjects. The approach is based on the BM-MSC expression of a cell surface adhesion molecule, the CD146. Recently, it was found that hypoxic BM-MSCs exhibit low expression of CD146, whereas BM-MSCs residing in a relatively well-oxygenated zone exhibited high expression of CD146.⁵ We hypothesized that the combination of low CD146 and high HIF-1 α gene expression could be used to establish the hypoxic phenotype of CD271⁺ BM-MSCs. To test the hypothesis, CD271⁺ BM-MSCs obtained from healthy human volunteers¹² were exposed to 1% hypoxia for 1 week *in vitro*, and the alteration in gene expression of CD146 and HIF-1 α was studied by real-time PCR. Indeed, hypoxia exposure decreased CD146 by twofold while increasing HIF-1 α expression by threefold compared with normoxia culture (Figure 6A). These results confirmed the usefulness of the relative expression of these two genes as the indicator of the hypoxia phenotype of CD271⁺ BM-MSCs.

We then obtained the RNA of MTB-DNA containing CD271⁺ BM-MSCs from seven individuals previously treated for PTB,¹² and subjected the RNA to real-time PCR gene expression for HIF-1 α and CD146. The CD271⁺ BM-MSC RNA obtained from healthy individuals with no MTB-DNA was used as a control. We found that MTB-infected CD271⁺

BM-MSCs exhibited high HIF-1 α and low CD146 (Figure 6B). These results suggest that MTB-infected CD271⁺ BM-MSCs exhibit the hypoxic phenotype of low CD146 and high HIF-1 α expression.

Discussion

The significance of the BM-MSC hypoxic zone as a protective niche of MTB in post-therapy persistence infection has not been studied. By using a variant of the Cornell model of dormancy, we now provide direct evidence that the CD271⁺ BM-MSC hypoxic niche serves as a host that maintains persistent MTB. In human subjects previously treated for PTB, CD271⁺ BM-MSCs positive for MTB-DNA exhibited an enhanced hypoxic phenotype of low CD146 and high HIF-1 α . These results indicate the significance of the BM-MSC hypoxic niche in MTB dormancy after therapy.

Our application of the Cornell model to characterize the MTB niche in hypoxic BM-MSCs was encouraged by our recent observations that in human subjects, viable MTB could be isolated from post-therapy CD271⁺ BM-MSCs.¹² Herein, we used a Cornell model of dormancy, in which MTB-H37Rv–infected mice were treated with INH and PZA for months to render lung and spleen noninfectious. We then performed several experiments to successfully recover viable MTB harboring CD271⁺ BM-MSCs from the BM hypoxic niche. Thus, in the Cornell model of dormancy, this CD271⁺ BM-MSC niche cell type might play a significant role in maintaining MTB persistence after therapy.

The CD271⁺ BM-MSCs constitute a rare cell type of BM niche and, therefore, identifying MTB-harboring CD271⁺ BM-MSCs after therapy could be a major experimental challenge.¹² We tried to overcome the challenge by using GFP-labeled MTB, and then flow cytometry–based analysis of GFP⁺/CD271⁺ BM-MSCs. By using this GFP labeling approach, previously, we showed that 0.6% of total CD271⁺ BM-MSCs contained intracellular GFP-labeled dormant MTB.¹² This possibility of detecting even a few GFP-labeled MTB harboring BM-MSCs by flow cytometry, together with our prior experience to label even a rare fraction of cells by the hypoxia marker pimonidazole in mice,²⁵ encouraged us to identify potentially rare MTB harboring CD271⁺ BM-MSCs that persist in the hypoxic niche of BM after therapy. Thus, we were able to identify a 4% pimonidazole⁺/GFP⁺ fraction in a total of 6×10^4 CD271⁺ BM-MSCs obtained from the two femurs of GFP-H37Rv–infected BALB/c mice treated with INH/PZA. The flow cytometry findings were confirmed by performing confocal microscopy staining of pimonidazole. Although we could not obtain direct evidence of the *in situ* localization of GFP⁺/CD271⁺/pimonidazole⁺ cells in the BM microenvironment, mainly because of high autofluorescence and an extremely rare percentage of the cell population, the flow cytometry and confocal microscopy evidence strongly

indicates the hypoxic localization of post-therapy dormant MTB in the murine CD271⁺ BM-MSCs.

We tested the reactivation potential of the recovered post-INH + PZA-treated MTB in CD271⁺ BM-MSCs by performing a previously described MTB/BM-MSC transplantation assay. The transplantation assay involves tail-vein injection of live CD271⁺ BM-MSCs recovered from MTB-infected mice into naïve mice, and then demonstrates granuloma formation in the lung.¹² To perform this assay, we first developed an immunomagnetic sorting method for CD271⁺ BM-MSCs, because live cell flow cytometry sorting was not allowed in the biosafety level 3 facility. We achieved a 90% purity of CD271⁺ BM-MSCs by immunomagnetic sorting. Our efforts to obtain a highly purified population by using additional cell surface markers, including CD44 and CD73, failed because of lack of appropriate antibodies for immunomagnetic sorting. Hence, we opted to use the CD271⁺ BM-MSC population of limited purity for the transplantation assay. Sixty-thousand CD271⁺ BM-MSCs recovered from post-therapy mice containing nearly 80 viable and persistent MTB (approximately 0.5%) were sufficient to induce lung granulomas in healthy secondary recipient mice. This result is consistent with the previous prediction that <10 persistent MTB-carrying host cells would be able to induce granuloma formation in the Cornell model of dormancy.¹⁷ The results also support our previous findings that only approximately 70 nonreplicating MTB were required to seed granuloma when injected with CD271⁺ BM-MSCs.¹² These results are not confirmatory of the granuloma reactivation ability of dormant MTB, because, unlike the classic granuloma reactivation achieved by immunosuppressive drug treatment of infected mice, we studied the reactivation potential by performing MTB/BM-MSC transplantation assay in naïve mice.

Taken together, we have used flow cytometry, immunomagnetic sorting, and transplantation assays to identify the CD271⁺ BM-MSC hypoxic niche as a protective site in the Cornell model of dormancy. The Cornell model that was used in the study^{18,27} is one of the many variants, in which we used an i.v. route of MTB infection. Although it is the traditional route of infection in the Cornell model,^{14,15,28} it is still not the natural route of infection. We have recently used an aerosol-infected and INH-rifampicin treatment regimen of the Cornell model to demonstrate the persistence of virulent MTB in the BM-MSC compartment.²⁹ Interestingly, in the study, the immunomagnetic sorting and granuloma formation potential of CD271⁺ BM-MSCs harboring MTB were not tested. In addition, direct visualization of MTB intracellular to the CD271 BM-MSC hypoxic niche after rifampicin treatment was not performed. Therefore, our present finding is limited to the INH-PZA treatment variant of the Cornell model. Nonetheless, we suggest that our ability to sort for CD271⁺ cells by immunomagnetic sorting, and then detect GFP⁺/pimonidazole⁺ cells, could be used to characterize the BM-MSC hypoxic niche in other variants of the Cornell model.

Significantly, our study results indicate potential modulation of the BM-MSC hypoxic niche by MTB infection and replication. The pimonidazole binding experiment revealed that unlike noninfected mice, the MTB-H37Rv-infected mice exhibited a significant increase of pimonidazole-positive CD271⁺ BM-MSCs, although only a small fraction of the cells contained intracellular MTB. We speculated that MTB infection and replication in BM might modulate the BM-MSC hypoxic niche. To investigate this possibility, we used a streptomycin-dependent mutant strain of 18b, which replicates only in the presence of streptomycin and infects only approximately 4% of CD271⁺ BM-MSCs.¹² By exploiting the streptomycin dependency of MTB replication of this mutant 18b strain, we found that an increase in MTB replication in BM was associated with an increase of pimonidazole binding of CD271⁺ BM-MSCs. The result indicates potential modulation of the BM hypoxic niche by MTB infection.

We acknowledge that the above results, obtained in a mouse model of MTB infection, may not be clinically relevant. There is a strong rationale to obtain clinical evidence about the putative hypoxic localization of post-therapy MTB harboring CD271⁺ BM-MSCs. However, unlike in mice, a pimonidazole binding assay would be an ethically difficult issue to perform in human subjects. Hence, we tested an innovative approach to assess the hypoxic phenotype of MTB-infected CD271⁺ BM-MSCs in clinical samples isolated from post-therapy PTB subjects. In this approach, we first verified the CD146 and HIF-1 α gene expression values of CD271⁺ BM-MSCs cultured *in vitro* in hypoxia (1% oxygen); as expected, CD146 was down-regulated and HIF-1 α was up-regulated compared with BM-MSCs cultured under normoxia. By using this approach, we found that subjects ($n = 7$) having MTB-DNA in their BM exhibited a low CD146 and a high HIF-1 α phenotype compared with healthy subjects. Although such an approach has its own limitation compared with an *in vivo* pimonidazole binding assay, our findings of low CD146 and high HIF-1 α may reflect the hypoxic localization of CD271⁺ BM-MSCs, as previously suggested.⁵ We also noted a significant increase of HIF-1 α and a decrease of CD146 expression in MTB-infected versus healthy subjects, although a minor population of CD271⁺ BM-MSCs (110 MTB-DNA copies per 10⁴ CD271⁺ BM-MSCs) contained intracellular MTB.¹² Thus, the clinical findings support the animal study results that most CD271⁺ BM-MSCs exhibited the hypoxia phenotype, although only a small fraction of the cells served as a reservoir of MTB, indicating a potential association between MTB infection and dormancy and BM hypoxia. Future studies are required to establish a causative relation between MTB infection and replication and the modulation of the BM hypoxia niche.

Although we identified BM as a protective niche for MTB dormancy, the relation between BM hypoxia and MTB dormancy is not yet clear. Decades of study indicate that the hypoxia microenvironment up-regulates the molecular

pathways of MTB dormancy^{8,9} and also enhances the pathogen's ability to tolerate anti-tuberculosis therapy.⁹ *In vitro* studies indicate that exposure of actively growing MTB to relative hypoxia (approximately 1% O₂ saturation or <10 mmHg) leads to nonreplicating stage 2 (Wayne's dormancy model) that exhibits dormancy and phenotypic resistance to drugs.^{8,9} Considering that pimonidazole binding of cells increases dramatically below an oxygen concentration of 10 mmHg (approximately 1% O₂ saturation), we suggest that this BM-MSC niche may exhibit a relevant hypoxic condition that favors MTB dormancy. However, it could also be hypothesized that MTB infection—modulated BM hypoxia might be an innate defense response against the pathogen. Tissue hypoxia is increasingly found to be an important component of innate immunity defense response.^{30,31} Thus, BM hypoxia may also restrict MTB infection and replication and, therefore, limit MTB spread in the BM-MSC niche. Indeed, our findings that only a minor fraction of pimonidazole-positive CD271⁺ BM-MSCs harbors dormant MTB could indicate that hypoxia might have served as a stem cell niche defense mechanism^{32,33} against MTB infection and replication.

Future studies of CD271⁺ BM-MSC biology, including host/pathogen interaction with MTB, will help us to fully appreciate and interpret the pathological and clinical significance of our recent discovery of BM-MSCs as a putative niche for dormant MTB. Such studies are needed to address important questions, such as the following: i) Why does MTB preferentially target a rare CD271⁺ BM-MSC in the BM hypoxic niche? Is it possible that CD271⁺ BM-MSCs exert defense against MTB invasion to BM stem cell niche? *In vitro* host/pathogen interaction culture between MTB and CD271⁺ BM-MSCs¹² could be useful to study the potential mechanism of BM-MSC defense against MTB infection. ii) How does the MTB-harboring hypoxic CD271⁺ BM-MSC reactivate post-therapy granuloma.¹² Is it possible that intracellular MTB exploits the CD271⁺ BM-MSC defense mechanism to migrate from BM to lung for post-therapy granuloma reactivation? In this regard, the MTB/BM-MSC transplantation assay described herein may be useful to study the dissemination of post-therapy dormant MTB in lung and other tissue niches for disease reactivation. iii) Finally, will MTB-modulated BM hypoxia provide us with a therapeutic opportunity to target the MTB dormancy with hypoxia-activated prodrugs? Thus, exploring the CD271⁺ BM-MSC hypoxia niche may help us gain a novel insight into targeting of MTB dormancy after therapy, a major global health challenge.

In summary, by using the Cornell model of dormancy, herein we demonstrate the intracellular localization of post-drug-treated viable MTB in CD271⁺ BM-MSCs. We then confirmed the hypoxic localization of MTB-infected CD271⁺ BM-MSCs by the pimonidazole labeling method. More important, we provide clinical evidence of the hypoxic phenotype of MTB DNA harboring CD271⁺ BM-MSCs of patients previously treated for TB. Whether the hypoxic

niche provides additional protection to MTB, including escape from immune invasion and drug exposure, remains to be explored. Also, if the degree of hypoxia prevalent in the niche is sufficient enough to induce and maintain MTB dormancy, as well as the stemness of the host stem cells, requires further investigation. We suggest that the study of the *in vivo* hypoxic niche of MTB-infected BM-MSCs will provide the groundwork for understanding the significance of the BM niche for persistent MTB and for developing novel strategies aimed at manipulating the niche to enhance therapy against persistent MTB.

Acknowledgments

We thank the Idu-Mishmi tribe for participation in our clinical study; the government of Arunachal Pradesh (India) for allowing us to conduct the clinical study; Research Institute of World's Ancient Traditions, Cultures and Heritage (Roing, Arunachal Pradesh, India) for supporting the clinical study; Dr. Antonio Campos Neto (Forsyth Institute, Cambridge, MA) for reviewing and making valuable comments on the manuscript; All Advanced Instrumentation Research Facility, Jawaharlal Nehru University, for providing an instrumentation facility for immunofluorescence and histopathology; Dr. Sabine Ehrh (Weill Cornell Medical College, New York, NY) for the pUVGFP+ plasmid; and Prof. Stewart T. Cole (Global Health Institute, Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland) for providing the *M. tuberculosis* H37Rv, green fluorescent protein H37Rv, and streptomycin-auxotrophic mutant *M. tuberculosis* strain 18b.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.03.028>.

References

1. Lonnroth K, Ravigliione M: Global epidemiology of tuberculosis: prospects for control. *Semin Respir Crit Care Med* 2008, 29:481–491
2. Lillebaek T, Dirksen A, Vynnycky E, Baess I, Thomsen VO, Andersen AB: Stability of DNA patterns and evidence of *Mycobacterium tuberculosis* reactivation occurring decades after the initial infection. *J Infect Dis* 2003, 188:1032–1039
3. Lillebaek T, Dirksen A, Baess I, Strunge B, Thomsen VO, Andersen AB: Molecular evidence of endogenous reactivation of *Mycobacterium tuberculosis* after 33 years of latent infection. *J Infect Dis* 2002, 185:401–404
4. Menzies D, Al Jahdali H, Al Otaibi B: Recent developments in treatment of latent tuberculosis infection. *Indian J Med Res* 2011, 133: 257–266
5. Tormin A, Li O, Brune JC, Walsh S, Schutz B, Ehinger M, Ditzel N, Kassem M, Scheduling S: CD146 expression on primary non-hematopoietic bone marrow stem cells is correlated with *in situ* localization. *Blood* 2011, 117:5067–5077
6. Parmar K, Mauch P, Vergilio JA, Sackstein R, Down JD: Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. *Proc Natl Acad Sci U S A* 2007, 104:5431–5436

7. Suda T, Takubo K, Semenza GL: Metabolic regulation of hematopoietic stem cells in the hypoxic niche. *Cell Stem Cell* 2011, 9: 298–310
8. Kumar A, Toledo JC, Patel RP, Lancaster JR Jr, Steyn AJ: Mycobacterium tuberculosis DosS is a redox sensor and DosT is a hypoxia sensor. *Proc Natl Acad Sci U S A* 2007, 104:11568–11573
9. Wayne LG, Sohaskey CD: Nonreplicating persistence of mycobacterium tuberculosis. *Annu Rev Microbiol* 2001, 55:139–163
10. Kobayashi CI, Suda T: Regulation of reactive oxygen species in stem cells and cancer stem cells. *J Cell Physiol* 2012, 227:421–430
11. Martin CM, Ferdous A, Gallardo T, Humphries C, Sadek H, Caprioli A, Garcia JA, Szveda LI, Garry MG, Garry DJ: Hypoxia-inducible factor-2 α transactivates Abcg2 and promotes cytoprotection in cardiac side population cells. *Circ Res* 2008, 102:1075–1081
12. Das B, Kashino SS, Pulu I, Kalita D, Swami V, Yeger H, Felsher DW, Campos-Neto A: CD271(+) bone marrow mesenchymal stem cells may provide a niche for dormant Mycobacterium tuberculosis. *Sci Transl Med* 2013, 5:170ra113
13. McCune RM Jr, McDermott W, Tompsett R: The fate of Mycobacterium tuberculosis in mouse tissues as determined by the microbial enumeration technique, II: the conversion of tuberculous infection to the latent state by the administration of pyrazinamide and a companion drug. *J Exp Med* 1956, 104:763–802
14. McCune RM Jr, Tompsett R: Fate of Mycobacterium tuberculosis in mouse tissues as determined by the microbial enumeration technique, I: the persistence of drug-susceptible tubercle bacilli in the tissues despite prolonged antimicrobial therapy. *J Exp Med* 1956, 104:737–762
15. McCune RM, Feldmann FM, Lambert HP, McDermott W: Microbial persistence, I: the capacity of tubercle bacilli to survive sterilization in mouse tissues. *J Exp Med* 1966, 123:445–468
16. McCune RM, Feldmann FM, McDermott W: Microbial persistence, II: characteristics of the sterile state of tubercle bacilli. *J Exp Med* 1966, 123:469–486
17. Lecoer HF, Lagrange PH, Truffot-Pernot C, Gheorghiu M, Grosset J: Relapses after stopping chemotherapy for experimental tuberculosis in genetically resistant and susceptible strains of mice. *Clin Exp Immunol* 1989, 76:458–462
18. Cheigh CI, Senaratne R, Uchida Y, Casali N, Kendall LV, Riley LW: Posttreatment reactivation of tuberculosis in mice caused by Mycobacterium tuberculosis disrupted in mce1R. *J Infect Dis* 2010, 202: 752–759
19. Miyazaki E, Chaisson RE, Bishai WR: Analysis of rifapentine for preventive therapy in the Cornell mouse model of latent tuberculosis. *Antimicrob Agents Chemother* 1999, 43:2126–2130
20. Garg R, Tripathi D, Kant S, Chandra H, Bhatnagar R, Banerjee N: The conserved hypothetical protein Rv0574c is required for cell wall integrity, stress tolerance, and virulence of Mycobacterium tuberculosis. *Infect Immun* 2015, 83:120–129
21. Copenhaver RH, Sepulveda E, Armitage LY, Actor JK, Wanger A, Norris SJ, Hunter RL, Jagannath C: A mutant of Mycobacterium tuberculosis H37Rv that lacks expression of antigen 85A is attenuated in mice but retains vaccinating potential. *Infect Immun* 2004, 72:7084–7095
22. Paralkar VR, Mishra T, Luan J, Yao Y, Kossenkova AV, Anderson SM, Dunagin M, Pimkin M, Gore M, Sun D, Konuthula N, Raj A, An X, Mohandas N, Bodine DM, Hardison RC, Weiss MJ: Lineage and species-specific long noncoding RNAs during erythro-megakaryocytic development. *Blood* 2014, 123:1927–1937
23. Gordon R, Hogan CE, Neal ML, Anantharam V, Kanthasamy AG, Kanthasamy A: A simple magnetic separation method for high-yield isolation of pure primary microglia. *J Neurosci Methods* 2011, 194: 287–296
24. 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
25. Das B, Yeger H, Tsuchida R, Torkin R, Gee MF, Thorne PS, Shibuya M, Malkin D, Baruchel S: A hypoxia-driven vascular endothelial growth factor/Flt1 autocrine loop interacts with hypoxia-inducible factor-1 α through mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 pathway in neuroblastoma. *Cancer Res* 2005, 65:7267–7275
26. Shehade H, Oldenhove G, Moser M: Hypoxia: an alarm signal during intestinal inflammation. *Nat Rev Gastroenterol Hepatol* 2010, 7: 281–287
27. Dhillon J, Allen BW, Hu YM, Coates AR, Mitchison DA: Metronidazole has no antibacterial effect in Cornell model murine tuberculosis. *Int J Tuberc Lung Dis* 1998, 2:736–742
28. Dhillon J, Dickinson JM, Sole K, Mitchison DA: Preventive chemotherapy of tuberculosis in Cornell model mice with combinations of rifampin, isoniazid, and pyrazinamide. *Antimicrob Agents Chemother* 1996, 40:552–555
29. Beamer G, Major S, Das B, Campos-Neto A: Bone marrow mesenchymal stem cells provide an antibiotic-protective niche for persistent viable Mycobacterium tuberculosis that survive antibiotic treatment. *Am J Pathol* 2014, 184:3170–3175
30. Bartels K, Grenz A, Eltzschig HK: Hypoxia and inflammation are two sides of the same coin. *Proc Natl Acad Sci U S A* 2013, 110: 18351–18352
31. Nizet V, Johnson RS: Interdependence of hypoxic and innate immune responses. *Nat Rev Immunol* 2009, 9:609–617
32. 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
33. Das B: Altruistic stem cell and cancer stem cells. Edited by Rajasekhar VK. *Cancer Stem Cells*. Hoboken, NJ, Wiley Press, 2014, pp 89–105