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Supplemental Information

Drug Discovery Platform Targeting *M. tuberculosis* with Human Embryonic Stem Cell-Derived Macrophages

Hyo-Won Han, Hyang-Hee Seo, Hye-Yeong Jo, Hyeong-jun Han, Virgínia C.A. Falcão, Vincent Delorme, Jinyeong Heo, David Shum, Jang-Hoon Choi, Jin-Moo Lee, Seung Hun Lee, Hye-Ryeon Heo, Seok-Ho Hong, Mi-Hyun Park, Rajesh K. Thimmulappa, and Jung-Hyun Kim

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Supplementary Experimental Procedures

hPSC culture and macrophage differentiation

The H9 hESC line was obtained from WiCell (Madison, WI, USA) and cultured in feeder-free TeSR-E8 or mTeSR-1 medium (STEMCELL Technologies, Vancouver, Canada) on tissue culture plates coated with Vitronectin (Gibco, Grand Island, NY, USA) or Matrigel hESC-qualified Matrix (Corning Inc., Corning, NY, USA), and were mechanically passaged. The human iPSC cell line (CMC003) was provided by the Korean National Stem Cell Bank (http://kscr.nih.go.kr) and maintained as H9 hESC line. For differentiation into macrophage-like cells, hESCs were transitioned into Growth Factor-Reduced Matrigel (Corning Inc.) in mTeSR-1 medium at a density of 5–8 colonies/35-mm dish. When the size of colonies was approx. 2 mm, differentiation was initiated. Mesodermal differentiation was induced with APEL2 medium supplemented with 1× insulin-transferrin-selenium-X (Gibco) and BMP4. Hematopoietic stem and progenitor cell (HSPC) production was induced by treatment with APEL2 medium containing VEGF4 and SCF for 2 days, followed by SCF, IL-3 and IL-6 and TPO and Flt-3L. After 12-20 days, floating HSPCs were transferred into new 60 mm petri-dishes and further differentiated into iMAC using M-CSF and RPMI 1640 medium supplemented with 10% fetal bovine serum. For scaling-out, 90% confluent iMACs in 60 mm dishes were transferred into 100 mm petri dishes and subsequently transferred into 150 mm petri dishes when the confluency reached to 90%. At every subculture step, cells were split in a ratio of 1:4 onto 150 mm large size petri dishes. For the screening, iMACs originating from the 35 mm dishes of 4 hESC were pooled together.

Phagocytosis assay

Floating iMAC (1×10^6 cells/ml) were cultured with carboxylate-modified fluorescein isothiocyanate-conjugated latex beads with a mean diameter of 1.0 μm (Sigma-Aldrich) at 1:0.2 (cell: beads) to 1:200 for 1.5hrs. After three washes, phagocytic cells were analyzed by fluorescence-activated cell sorting (LSRFortessa; BD Biosciences) and microscopy (Imager A2; Carl Zeiss AG, Oberkochen, Germany).

Influenza H3N2

The seasonal H3N2 influenza virus vaccine strain

A/Switzerland/9715293/2013 was obtained from WHO Collaborating Center, U.S. CDC, and cultured as previously described (Eisfeld et al., 2014).

M. tuberculosis

M. tuberculosis strain H37Rv (ATCC 27294) transformed with an Ms6-based integrative plasmid pNIP48 (Christophe et al., 2009) and harboring GFP (Mtb H37Rv-GFP) was provided by Institut Pasteur Korea. Drug-resistant strains for the extracellular anti-bacterial assay (MDR, 116-47; XDR, 203-60 and 203-101) were provided by the Korean Mycobacterial Resource Center. See more information in (Table S4).

Compound library for screening

The library used for the screen combines 3,716 chemicals obtained from the Prestwick, Prestwick GP, Tocriscreen, Selleck, and National Institutes of Health libraries. Compounds of each library were prepared in an intermediate 384-well polypropylene plate (Greiner Bio-One GmbH, Essen, Germany) at 100 μM in dimethylsulfoxide (DMSO) (v/v). The primary screen was performed at a final concentration of 10 μM in 0.5% DMSO (v/v).

High-throughput phenotypic screen of infected macrophages

Mycobacterium tuberculosis strain H37Rv (ATCC 27294) was transformed using the Ms6-based integrative plasmid pNIP48(Christophe et al., 2009), harboring a green fluorescent protein (GFP), for constitutive expression. Mtb H37Rv-GFP was propagated at 37°C in 7H9 broth medium (BD Biosciences) supplemented with 0.05% Tween 80 (Sigma-Aldrich), 0.2% glycerol (Invitrogen, Carlsbad, CA, USA), 10% oleic acid, albumin, dextrose, catalase enrichment (OADC, BD Biosciences) and 50 μg/mL hygromycin (Invitrogen), referred to as complete 7H9 medium. Macrophages were infected with H37Rv-GFP Mtb at the desired multiplicity of infection (MOI) for 2h with mild shacking (120 rpm) at 37°C. For the high-throughput screening, a MOI of 5 was used. Infected cells were washed twice with fresh medium and seeded at 5×10^5 cells/mL into 384-well assay plates (50 μL per well), containing the test or reference compounds at a final concentration of 10 μM. Plates were incubated at 37°C with 5% CO_2. After 5 days of infection, the macrophages were stained with Hoechst 33342 dye at a final concentration of 5 μM for 20 min at 37°C, 5% CO_2 (Figure S6A-D). Images

were acquired on a fluorescence microscope using a 20X□magnifying objective (Operetta, PerkinElmer). The assay workflow was performed under BioSafety Level 3 regulations, as mandated. Nine fields of view were imaged per well and pictures were analyzed using an in-house software counting the number of nuclei in the well using the blue channel and determining the position and area of the bacteria using the green channel. The number of cells was determined based on the nuclei population, segmented using an in-house seed-growth (or watershed) algorithm. For more details, see: Fenistein *et al.*(Fenistein et al., 2008). Bacteria were detected using a thresholding method, based on support vector machines algorithm, allowing precise segmentation of the pixels positive for a GFP signal versus background signals. A cell was considered infected if at least 2 contiguous pixels of bacteria were found within their cytoplasm.

To analyses the images, two parameters were considered: the total cell number (C) and the ratio of infected cells (R). Data were normalized with the average values from positive (P) and negative controls (N) to give the percentage of cell viability and the percentage of infection, respectively, using the following formulae:

$$\%\ cell\ viability = \frac{C - N}{P - N} \times 100$$

$$\%\ infection = \frac{R - N}{P - N} \times 100$$

In the case of dose-responses, the concentrations of compound required to inhibit 50% of the infection process (IC_{50}) were determined using the percentage of infection as a read-out parameter. The software Prism v6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to fit the data against a sigmoidal dose-response curve model (variable slope, 4 parameters) through non-linear regression.

For the pilot screening (primary screening), positive and negative controls were included in each plate to validate the assay. Columns 1 and 2 were filled with DMSO (0.5% v/v) as a high control (negative control) and columns 23 and 24 with RIF (1 µg/mL) as a low control (positive control). All wells were considered to calculate P_{avg} and $P_{SD,\ avg}$, as well as N_{avg} and $N_{SD,\ avg}$, average and standard deviation of the positive and negative controls, respectively. The z-score was calculated for each plate using the following formula:

$$z = 1 - \frac{3P_{SD,\ avg} + 3N_{SD,\ avg}}{|P_{avg} - N_{avg}|}$$

The average z-score for all the plates of the pilot screening was -0.37 and that of the

Dose-Response Curve (DRC) screening (secondary screening) was 0.35. Despite a low z-score for the pilot screening, positive and negative controls were distinctly separated (Figure S6A-C). The DRC screening showed definite inhibition of Mtb in a dose-dependent manner, within the infectivity window, for both reference drugs RIF and INH. In addition, to compensate for the relatively poor z-score obtained for the pilot screening, we used a 100% inhibition cut off threshold for the selection of positive hits. Both primary and secondary screenings were conducted only once.

In vitro anti-mycobacterial assay using drug-resistant Mtb strains

Clinical isolates were grown at 37°C and 5% CO_2 for 2 weeks in complete 7H9 medium as described above for H37Rv-GFP, while omitting hygromycin. Bacteria were diluted at an optical density at 600 nm ($OD_{600\ nm}$) of 0.02 and seeded in 384-well plates (40 µl/well) containing 10 µl of multiple two-fold serial dilutions of compounds. Plates were incubated at 37°C and 5% CO_2 for 5 days and the $OD_{600\ nm}$ was measured on a microplate reader (Victor 3; PerkinElmer). A 10-µl volume of a freshly prepared and filtered (0.22 µm) solution of 0.01% resazurin in phosphate-buffered saline was added to each well and the plate incubated for 24h at 37°C and 5% CO_2. A change in color from blue (resazurin) to pink (resorufin) was used as an indicator of bacterial cell viability. The fluorescence of resorufin (excitation/emission = 485/535 nm) was measured on the microplate reader. IC_{50} values were calculated by non-linear regression of a sigmoidal dose-response curve using Prism v.6 software (GraphPad Inc., La Jolla, CA, USA).

Flow cytometry analysis

Floating and attached cells were collected and incubated with the following antibodies for 30 min at 4-degree. Antibody-labeled cells were sorted on an LSRFortessa cell analyzer (BD Biosciences) and data were analyzed using FlowJo v.10 software (TreeStar, Ashland, OR, USA). FMO controls were used in experiments (Figure S6D-E). See antibodies information in Table S5.

THP-1 cell and hMDMs cultures

THP-1 pro-monocyte cells were mainlined in RPMI1640 containing 10% FBS and 5% penicillin/streptomycin. For differentiation into macrophages, THP-1 cells were

treated with phorbol 12-myristate 13-acetate (PMA, 100 ng/ml for 48 h). To generate hMDMs, we sorted $CD14^+$ blood monocytes from hPBMCs using magnetic CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured the cells in the presence of M-CSF (100 ng/ml).

RNA isolation, RNA-Seq analysis

RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The library was prepared using Truseq stranded mRNA prep kit (Illumina, San Diego, CA, USA) and run on a HiSeq2500 sequencer (Illumina) with a 101-bp paired-end read length. Raw data were processed using STAR v.2.6 to align genome reads against human reference genome (hg38) and differentially expressed genes were identified using DESeq2(Love et al., 2014) after raw count data were normalized using variance stabilizing transformation (VST) method. Raw data file accession number: GSE138398 (GEO). Multidimensional scaling (MDS) and clustering analysis were calculated based on Poisson model. Spearman correlation coefficients between pairs of samples were computed. Gene ontology (GO) analysis for DEGs were performed on DAVID database. Significant GO term with less than 0.0001 FDR were used for downstream analysis.

Mtb whole-cell broth medium assay

Mtb H37Rv-GFP was grown in static conditions for at least 2 weeks at 37°C, 5% CO_2 in complete 7H9 medium with dilution every week to achieve an $OD_{600\ nm}$ = 0.2. Bacteria were washed twice with PBS and resuspended in fresh complete 7H9 medium to reach $OD_{600\ nm}$ = 0.02, then seeded in 384-well plates (40 µL per well), containing 10 µL of multiple two-fold serial dilutions of compounds. Plates were incubated at 37°C, 5% CO_2 for 5 days and the fluorescence of the GFP (Ex. 485, Em. 535) was measured using a plate reader (Victor 3, PerkinElmer). For clinical isolates, bacteria were grown, washed and seeded in 384-well plates as described above for H37Rv-GFP, omitting hygromycin. Plates were incubated at 37°C, 5% CO_2 for 5 days and the $OD_{600\ nm}$ was recorded using a plate reader. After that, 10 µL of a freshly prepared and filtered (0.22 µm) solution of 0.01% resazurin in PBS was added in each well and the plate incubated for 24h at 37°C, 5% CO_2. A change in color from blue (resazurin) to pink (resorufin) was used as an indicator for bacterial viability.

Fluorescence of resorufin (Ex. 485, Em. 535) was measured using a microplate reader. IC_{50} values were calculated from the raw fluorescence data using Prism software, as described above.

Macrophage infection with drug-resistant Mtb

Clinical isolates were grown in 7H9 complete medium, omitting hygromycin, as described above. Prior to infection, bacteria were washed twice with PBS and resuspended in RPMI1640 medium supplemented with 10% FBS. Cells were infected at a MOI of 20 for 2h under mild stirring (120 rpm) at 37°C, washed twice with fresh RPMI medium, then seeded at 5×10^5 cells/mL into 24-well plates (1 mL/well) containing compounds dilution in DMSO (0.1% final concentration). Plates were incubated at 37°C, 5% CO_2 for 5 days. Cell lysis was achieved by adding 20 µL of a 5% (v/v) Triton X-100 solution in PBS (final concentration 0.1%) and incubation of the cells for 10 min at 37°C, 5% CO_2, followed with mechanical lysis by scrapping the cell monolayer with a pipette tip. The supernatant was serial-diluted 10-fold with PBS (7 dilutions) and 10 µL of each dilution plated in duplicate in 7H11 agar medium supplemented with 0.5% glycerol and 10% OADC. Colonies were enumerated as soon as visible (usually between 14-21 days incubation) and the colony forming units (CFU) were obtained by averaging the number of colonies obtained for each replicate, at each dilution. Bedaquiline (TMC-207) was used as a positive control.

Elisa assay

H3N2 treated iMACs were incubated for 24 hr at 37-degree, 5% CO2 incubator. The supernatants were collected and the secreted cytokine levels were measured by Human IL-6 Quantikine ELISA kit (R&D Systems), Human IFN-gamma Quantikine ELISA Kit (R&D Systems). To measure the secreted cytokine levels of Mtb infected iMAC, cells were treated with Mtb (MOI5) for 5 days, and the supernatants were collected. 8-fold diluted supernatant has been used for measuring TNF-alpha (R&D systems), IL-6 (R&D systems). Elisa assay has been conducted using manufacture's provided protocol.

CFU assay

CFU assays were performed using Methocult M3344 (STEMCELL

Technologies) as manufactures' protocol described.

SEM/TEM

H3N2 treated macrophages were fixed and dehydrated as previously described(Graham and Orenstein, 2007; Nguyen and Harbison, 2017). Zeiss Libra 120 and Zeiss Evo LS10 were used to image the TEM and SEM.

Quantitative real-time PCR (qRT-PCR) and primers information

The qRT-PCR assays were performed as previously described(Kim et al., 2017). Primer sequences are below.

TNFA F: aacctcctctctgccatcaa, R: ggaagacccctcccagatag, IL1B F: ggacaagctgaggaagatgc, R: tcgttatcccatgtgtcgaa, CXCR2 F: gcagggaattcacctcaaga, R: gacaagctttctgcccattc, hHPRT-1 F: gaccagtcaacaggggacat, R:cctgaccaaggaaagcaaag

Author contributions

H.H., H.S. V.D., D.S., and J.K. designed the study and performed the experiments, analyzed the data, and wrote the manuscript. H.J. H.H, V.C., V.D. J.L, J.H, J.C. and S.L. performed the experiments. H.H. S.H, M.P. designed the study, discussed the results, and performed analyses. R.K.T. and V.D. provided conceptual advice and critically revised the manuscript for intellectual content.

Additional information

The authors declare no conflict of interests.

Figure S1

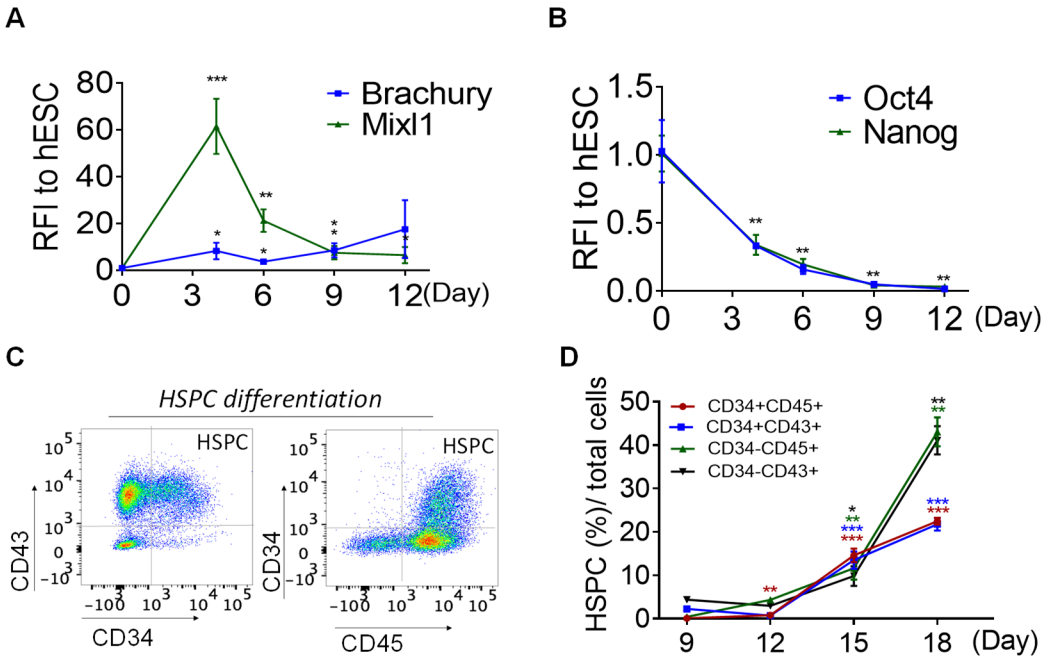

Figure S1. Markers expressions in HSPC differentiation. (A,B) mRNA expression of mesodermal (A) and pluripotency (B) markers at indicated time points shown as relative fold induction (RFI) with respect to hESCs (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 (two-tailed, unpaired, two-sample Student□st test). (C, D) Flow cytometry analysis of cells expressing CD34, CD43, and CD45 markers. Representative dot blots (C) and frequency (%) of specific cell types (D) at indicated time points are shown (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. day 9 (Student□s test).

Figure S2

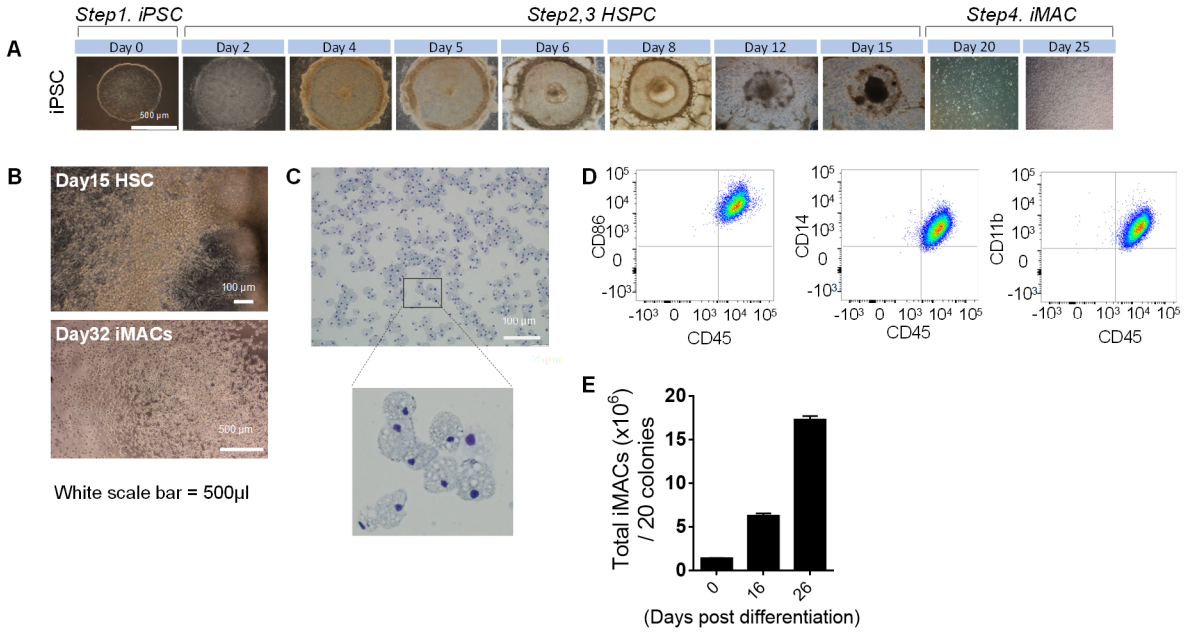

Figure S2. Differentiation and phenotypic characterization of macrophages from hiPSC
(A) Schematic illustration of the step-wise differentiation protocol for induced macrophages from a hiPSC line (CMC003). (B) Representative bright-field image of cells at each step during differentiation from day 15 (HSC) and day 32 (iMAC). (C) Representative image of Wright-Giemsa-stained macrophages 32 days after differentiation from hiPSC). (D) Flow cytometry analysis of iMACs expressing CD11b, CD14, CD86. (E) Accumulated cell number of $CD45^{+}CD14^{+}$ iMACs from 20 colonies of hiPSC in indicated date (n = 3). Results are shown as mean ± SD.

Figure S3

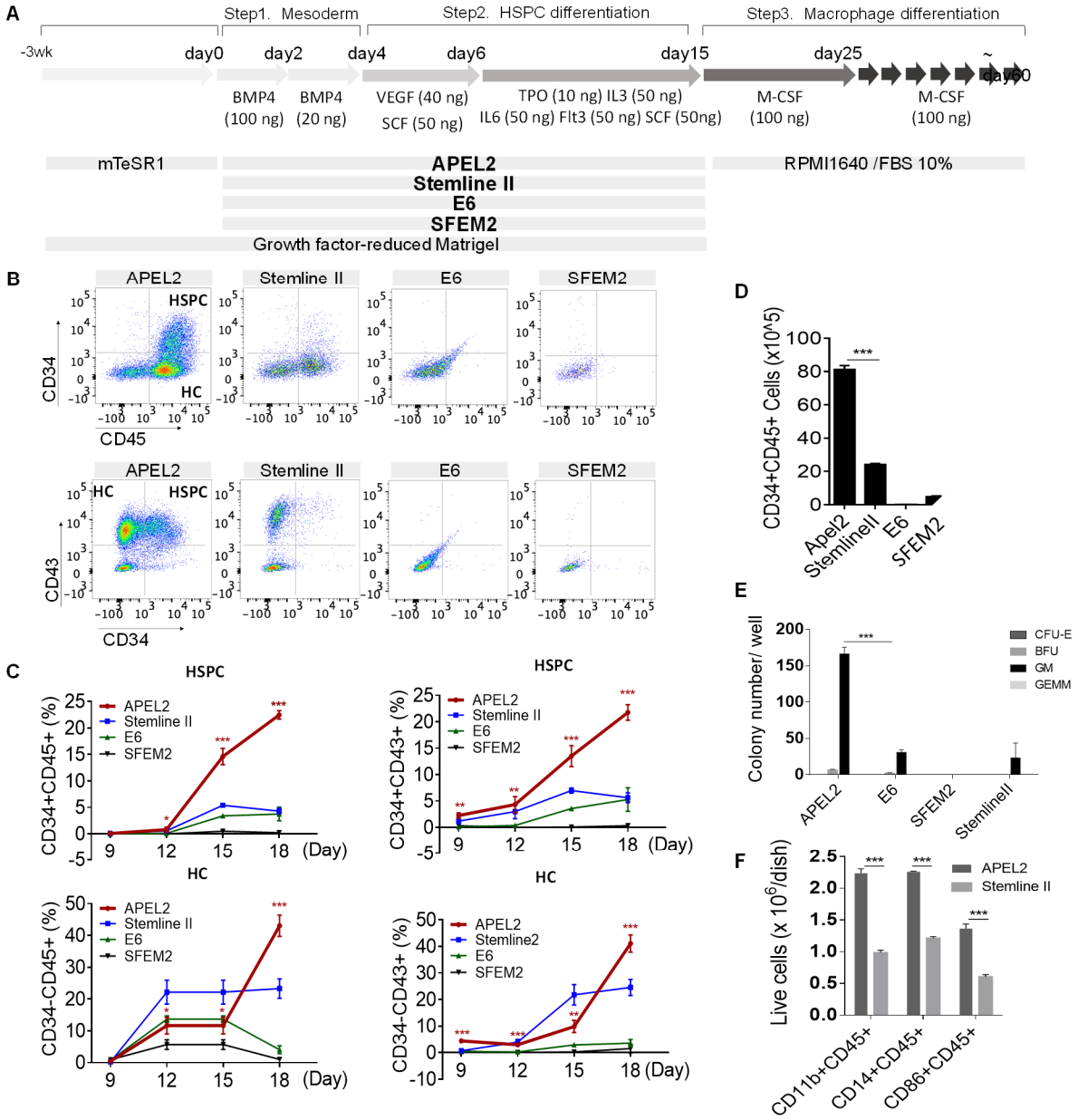

Figure S3. Effect of base medium on hematopoiesis. (A) Schematic illustration of the differentiation protocol. (B) Flow cytometry analysis of cell surface marker expression on day 15 after differentiation. Representative dot blot images. (C) Cell type production yield (n = 3); CD34 and CD45 positive/negative cells were gated for live adherent and floating cells. (D) Cell number of $CD34^{+}CD45^{+}$ floating HSPCs on day 18. (E) Number of colony forming units per well/10^4 input cells obtained on days 15 were differentiated using Methocults 3344. BFU, burst-forming unit, erythroid; CFU-E, colony-forming units, erythroid; GEMM, granulocyte/erythroid/macrophage/megakaryocyte; GM, granulocyte/macrophage.(F) iMAC yield (n=3).Starting HSCP cell number were same in each group as 2.5 x 10^5 per dish *P < 0.05, **P < 0.01, ***P < 0.001 (two-tailed, unpaired, two-sample Student□s t test).

Figure S4

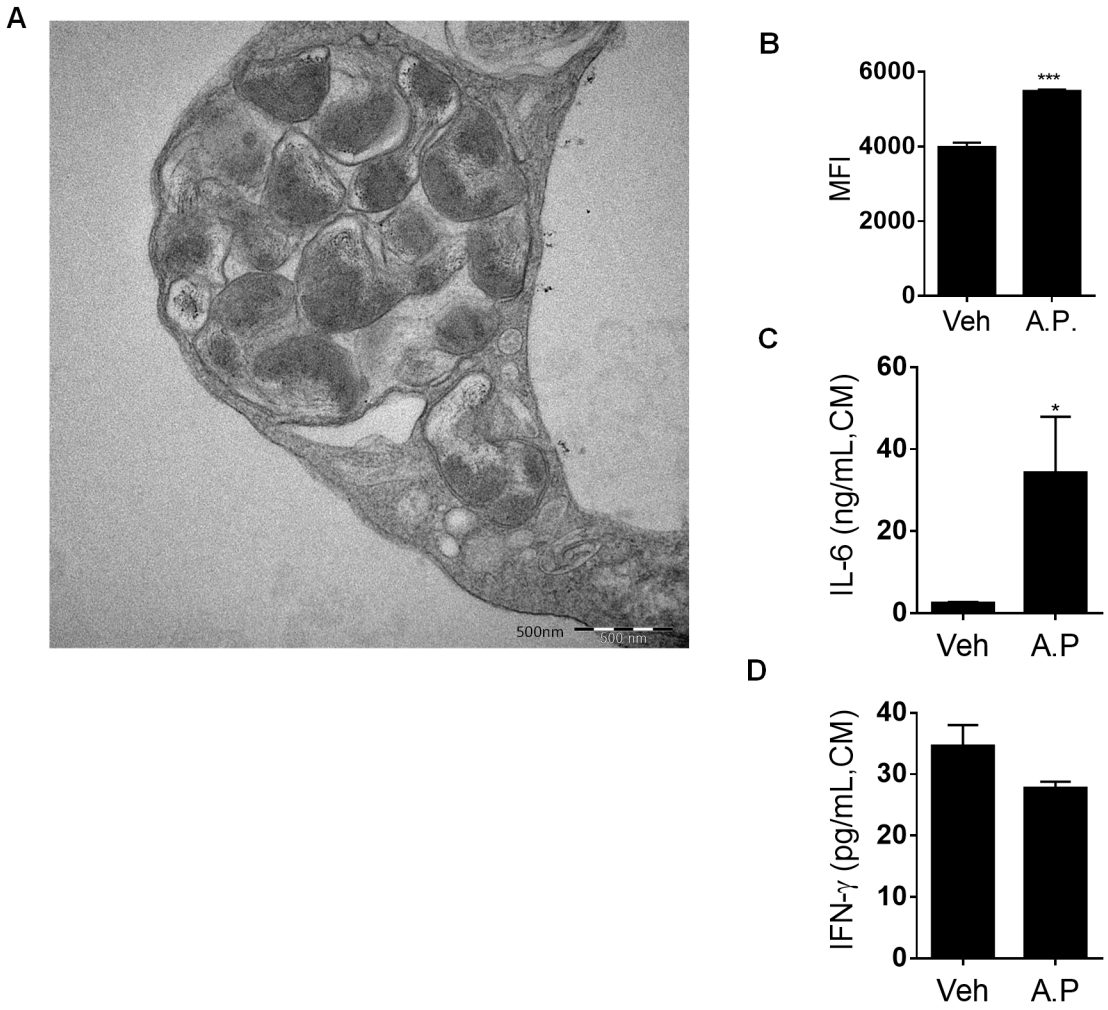

Figure S4. Characterization of innate immune responses of iMACs after bacteria *A. phagocytophilum* infection. (A) TEM images of intracellular *A.phagocytophilum* in iMACs. The image of bacterial morula was captured 5 days post-infection. (B) Flow cytometry analyses of ROS in H2DCFDA stained iMACs 5 days after *A.phagocytophilum* infection or vehicle. Bar graph represents Mitosox mean fluorescence intensity (MFI) in vehicle-treated bacteria infected cells (*n*=3); $P<0.001$. (C-D) Levels of cytokine IL-6 (C) and IFN-γ (D) in culture media (CM) of iMACs 5 days after bacterial infection measured ($P<0.05$).

Figure S5

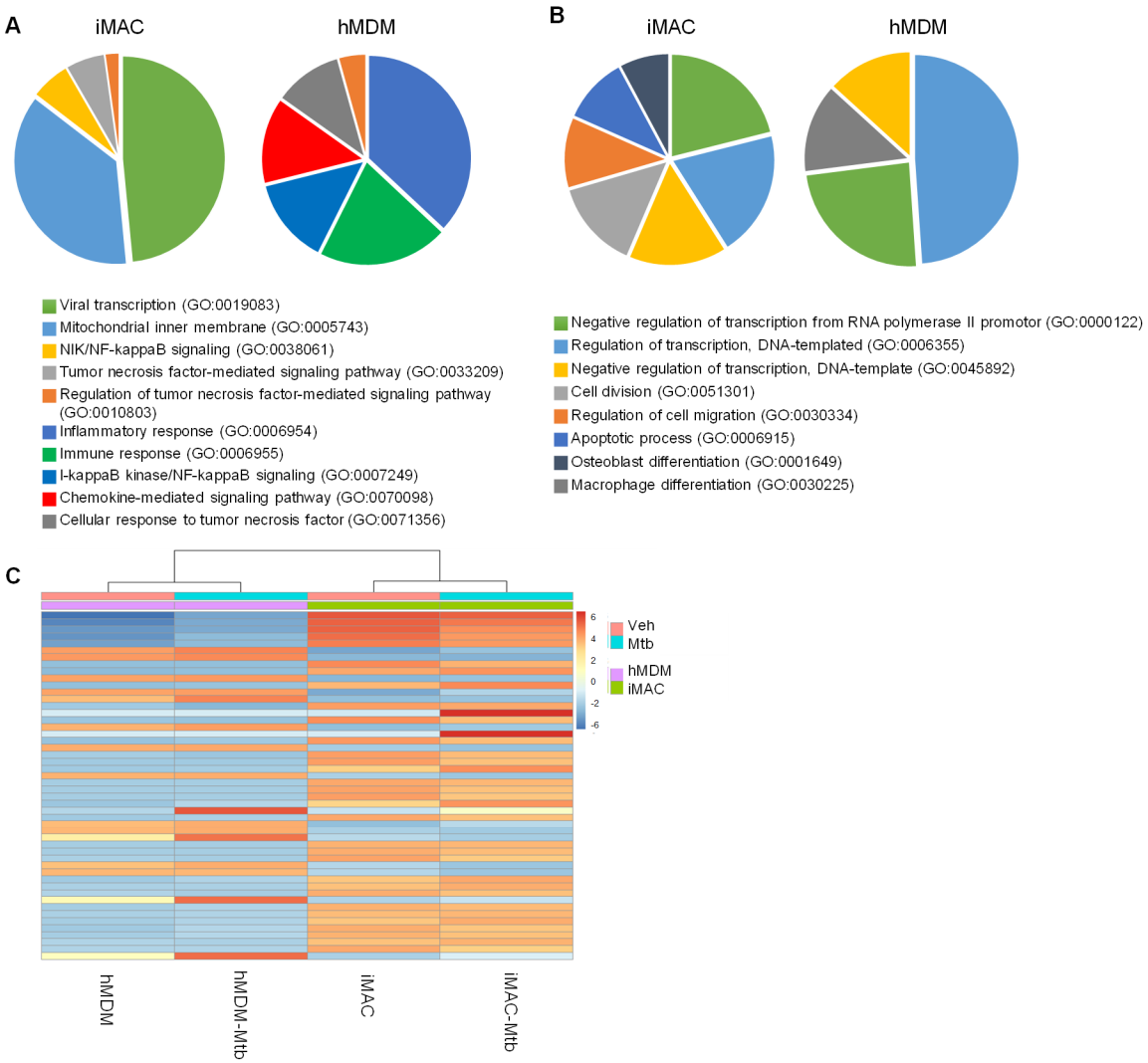

Figure S5. TOP ranked GO pathway of iMAC infected with Mtb (A) up-regulated or (B) down-regulated DEGs (>log2, p<0.05) after 5 days Mtb infection in iMAC and hMDM. GO terms are indicated. Top GO were manually selected. (C) Unsupervised non-hierarchical clustering of samples and heatmap showing variance stabilizing Transformation (VST) - normalized values of the top 50 variably expressed genes.

Figure S6

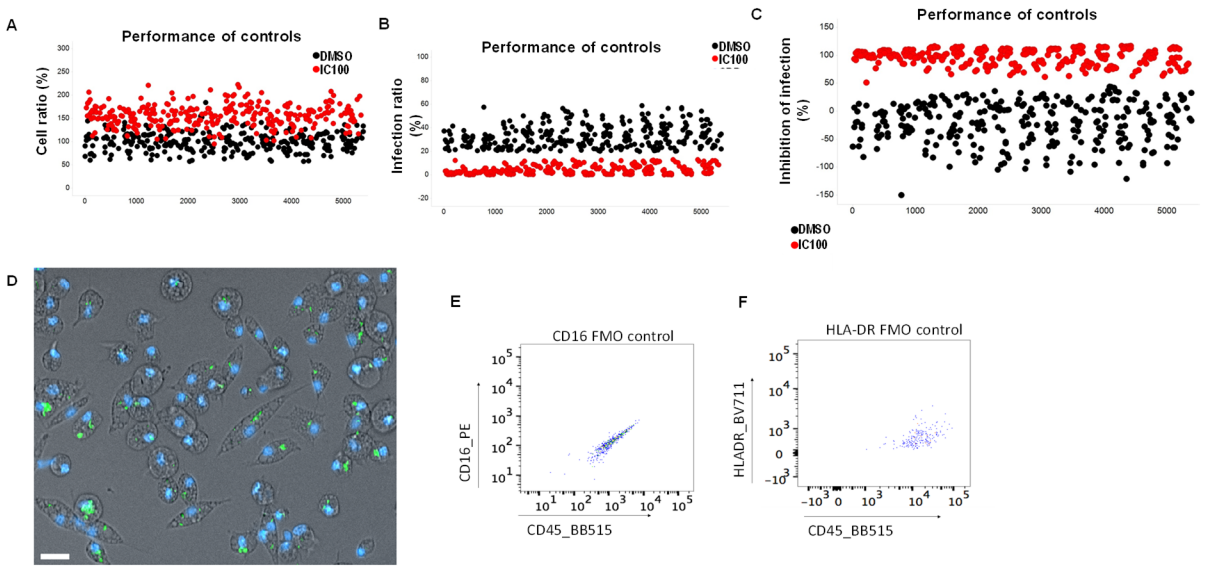

Figure S6. Controls and validations of assays. (A-D) Drug screening assay validation. Scatter plot of DMSO (0.5% v/v) control and 100% inhibitory concentration (IC100) of RIF (1 µg/ml) in the intracellular infection assay. Each dot represents the cell ratio (A), % infection (B), and % inhibition of infection (C) of a single well. (D) Control image of HTS assay. Macrophages were infected for 2h with H37Rv-GFP at MOI 20, then washed twice and plated at 50,000 cells/well in 96-well plates. Cells nuclei were stained with Hoechst 33342 for 30 min (10 µM final concentration) and imaged using a fluorescence microscope with a 20× objective (Operetta, Perkin Elmer). Overlay of three channels (Hoechst, Ex. 405 nm / Em. 450 nm; GFP, Ex. 488 nm / Em. 535 nm; Brightfield) is shown. Scale bar: 25 µm.(E-F) Flow cytometry analysis of iMACs using FMO controls (E) CD45 antibody only for analysis of CD16 and CD45 double stained samples (F) CD45 antibody only for analysis of HLADR and CD45 double stained samples

Figure S7

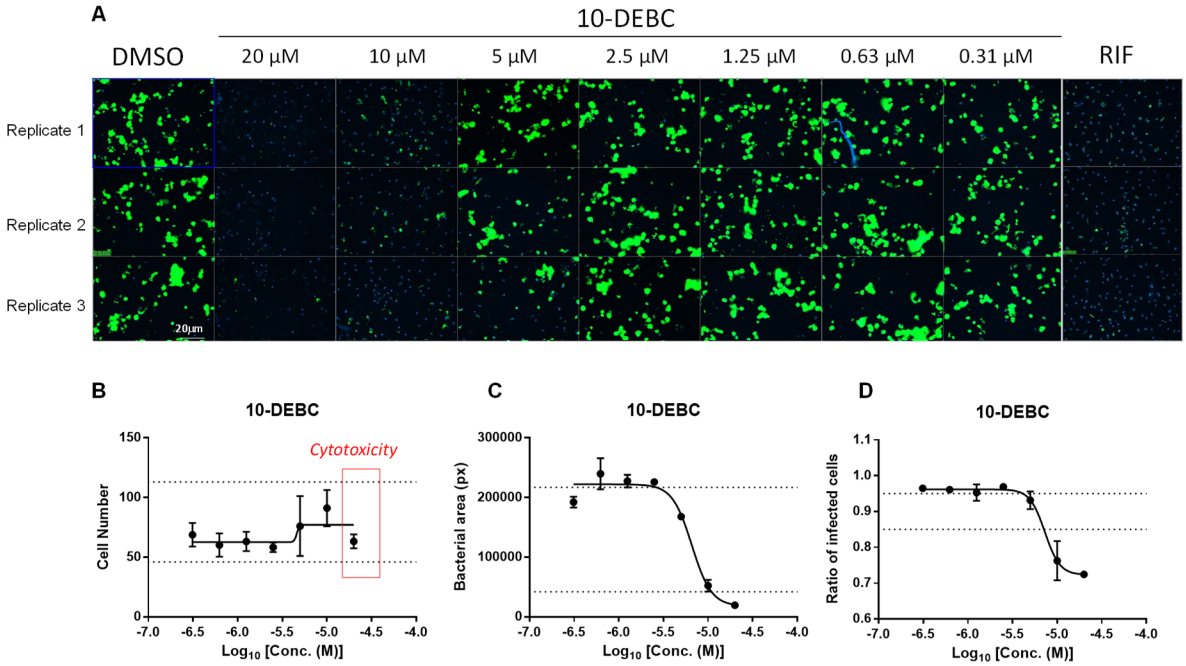

Figure S7. Validation of anti-TB effects of 10-DEBC in hMDMs. Mtb-Rv37-GFP infected hMDMs were incubated with 10-DEBC or RIF for 5 days at indicated concentrations. Infected cells expressing GFP (green) were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). (A) Confocal micrographs showing Mtb (green) in hMDMs treated with indicated concentrations of 10-DEBC or RIF. Triplicated images were shown. (B) Number of hMDMs. (C) Bacterial area (intracellular bacterial load). (D) Ratio of infected cells (GFP/DAPI) at indicated concentrations of 10-DEBC.

Video S1. HSPC differentiation from hESCs. Production of $CD34^{+}CD45^{+}$ HSPCs. Scale bar = 200 μm. The time lapse covers a period of about 24 h, with a 30-min time interval between images. Recordings were made with a Lionheart FX system (BioTek Instruments, Winooski, VT, USA) using a 4× objective lens and data were processed with Gen5 Image+ software (BioTek Instruments).

Video S2. iMAC production from HSPCs derived from hESCs. The video shows macrophages produced from HSPCs. Scale bar = 200 μm. The time lapse covers a period of 48 h, with a 30-min time interval between images. Recordings were made with a Lionheart FX system (BioTek Instruments) using a 4× objective lens and data were processed with Gen5 Image+ software (BioTek Instruments).

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