MATERIALS

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4. Material and Method

4.1 Animals

C57BL/6, TGFβRIIDN, and iNOS2-/− mice were initially purchased from The Jackson laboratories, and maintained in the specific pathogen free animal facility of the International Center for Genetic Engineering and Biotechnology (New Delhi, India). Fox-P3 knock in animals are the kind gift from Prof. Vijay Kuchroo, Harvard Medical School, USA. In all experiments, 4-8 weeks old female mice were used.

4.2 Patients

Female patients (n=5) with active tuberculosis at the age of 25-45 years were included in the study group. X-ray studies of these patients were consistent with the diagnosis of active tuberculosis. These patients were positive for the Mantoux test, and biopsies of Lymph Node (LN) showed live bacilli. Granuloma-containing LNs were surgically removed, and were processed for further studies as per institutional guidelines constituted by the ethics committee of All India Institute of Medical Sciences (New Delhi).

4.3 Materials

Purified or fluorescently-conjugated monoclonal antibodies against CD11b (M1/70), CD11c (N418), CD19 (MB19-1), CD29 (TS2/16), CD34 (RAM34), CD44 (IM7) CD45 (C363.16A) and B220 (D7) were purchased from eBiosciences; antibodies against CD3 (145-2C11) and Gr1 (RB6-8C5) were purchased from BD Biosciences. Antibodies to Flk-1 (89B2A5), CD28 (37.51) and CD29 (610467) were purchased from Biolegend. Ovalbumin, ConA, PHA, alum, CFSE, Oil Red O, Griess reagent and L-NMMA were purchased from Sigma Chemicals.
RPMI-1640 medium, Dulbecco’s Modified Eagle’s medium (DMEM) were purchased from Invitrogen life technologies (NY, USA). Fetal Calf serum (FCS) was purchased from Hyclone, (UT, USA). Middlebrook 7H9 liquid media, 7H10 agar and Oleic acid-Albumin-Dextrose-Catalase (OADC) supplement were obtained from Difco-Becton-Dickinson (NJ, USA).

*M. tb* whole cell lysate/extract (CE) was obtained from Dr. John Belisle, Colorado state University, USA under the National Institutes of Health, National Institute of Allergy and Infectious diseases Contract AI-75320, entitled “Tuberculosis Research Materials and Vaccine Testing”, Colorado State University. This preparation is a filtrate of log phase *M. tb* H37Rv culture sonicate. Additional details of its preparation and composition can be viewed at http://www.cvmbs.colostate.edu/microbiology/tb/top.

### 4.3.1 Bacterial growth media and solutions:

**LB** (Luria-Bertani) medium (1000 ml): 10g tryptone, 5g yeast extract, 10g NaCl.

Autoclaved at 1 kg/cm² (15 psi), 1210 °C, for 20 minutes.

**LB** agar: LB medium containing 15g/liter agar. Sterilized by autoclaving at 1 kg/cm² (15 psi), 1210 °C, for 20 minutes.

Kanamycin stock solution: 25 mg/ml in double-distilled water, filter sterilized and stored in aliquots at -200°C until use.

Ampicillin stock solution: 100 mg/ml in double-distilled water, filter sterilized and stored in aliquots at -200°C until use.

Chloramphenicol stock solution: 34 mg/ml in 100% ethanol, filter sterilized

and stored in aliquots at -200°C until use.

### 4.3.2 Solutions for Flow Cytometric Analysis

Wash Buffer: 0.5% BSA, 0.1% sodium azide in PBS, filtered to remove particulates.
Fixing Solution: 2% Paraformaldehyde solution in PBS, filtered to remove particulates.

Permeabilization Buffer: 0.1% saponin, 1% FCS in PBS

RBC lysis buffer: 0.9% Ammonium chloride, 10mM Tris-Chloride-pH 7.5, 5 ml for 2 mice

4.4 Methods

4.4.1 Enrichment of MSCs precursors from bone marrow

Isolation of Bone Marrow cells

Female BALB/c mice, 4-6 weeks of age were sacrificed in a chloroform chamber. Mice were wiped with 70% alcohol, pinned on to a dissection platform facing ventral side upwards. The body wall was cut and pinned. The tibias and femurs were carefully dissected out and put into a petri dish containing HBSS. With the help of forceps, the bones were teased to remove attached tissue. A sharp cut was made at either ends of the bones. Following this, 10-15 ml HBSS was flushed through the bones with a 27 gauge needle to take out the bone marrow into the medium. A homogenous suspension of cells was prepared by passing the medium containing bone marrow cells through a 18 gauge needle. Cell suspension was centrifuged at 1200 rpm for 10 min. After decanting the supernatant, cell pellet was suspended in 3 ml RBC lysis buffer for 3-4 min. Thereafter, 12 ml HBSS was added to dilute the RBC lysis buffer and the cell suspension was centrifuged at 1200 rpm for 10 min. The supernatant was discarded and the pellet was washed twice with the same volume of HBSS. The cell pellet was suspended in 1 ml of chilled RPMI and passed through a sterile nylon mesh filter (Miltenyi Biotech) to remove the tissue pieces.

4.4.2 Generation of MSCs from bone marrow precursors

Precursor cells were counted on a haemocytometer. Cells were cultured at a density of 3 x 10^6/ml in RPMI 1640 medium containing 10% FCS, 0.05 M 2-mercaptoethanol, MSCs were cultured in 10% FCS
supplemented stem cell medium (MACS, Miltenyi Biotec). For assays or sub-cultures cells were detached with trypsin/EDTA, re-seeded and grown to confluency as required. A homogeneous population of MSCs was obtained after 4 wks of culture. The MSCs expanded in culture showed surface staining for CD29, Flk-1, and CD44, but were negative for CD19, CD34, and CD45 surface expression. Vascular endothelial growth factor receptor 2 (VEGFR2 or Flk-1) is a major receptor for VEGF-induced signaling in endothelial cells. The receptors contain seven immunoglobulin like repeats in their extracellular domains and kinase insert domains in their intracellular regions. Flk-1 marker has been found on the surface of MSCs in previous studies (Fang, Shi et al. 2004). These receptors are likely to play essential roles in vasculogenesis and angiogenesis. The expanded MSCs retained the capacity to differentiate into adipocytes.

Mesenchymal Stem Cell (MSCs) Isolation from Murine Bone Marrow Cells

1. Euthanized mice by CO₂ asphyxiation. It is preferable to work with 2-3 mice at one time which are between 6-10 weeks of age.

2. Harvested hind limbs. Removed skin and as much muscle and connective tissue as possible. Cut the limb above the hip and below the ankle joint (including some of the foot); it was important to maintain the bone ends to ensure sterility of the bone marrow. After severing the limb, carefully broke apart the knee joint and stripped remaining connective tissue from both ends of femur and tibia if ends remain intact.

3. Placed the bones in dish of sterile 1X PBS on ice. Collect all bones in pool in same dish.

4. Washed bones by transferring through sterile PBS 7-8 times.

5. Snipped off ends of each bone with scissors (keeping as close to end as possible to extract more bone marrow) and gently placed in sterile PBS.

6. Filled 10cc syringe with prewarmed complete conditioned media and attached 25 gauge needle. With forceps, grabbed one bone. Used syringe to force media through bone shaft to extract all red marrow into 150mm plate. Flipped bone over and did the same from opposite side. Repeated a few times to ensure all marrow is removed till bone looked very white. Continued until all bones were demarrowed.

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7. Pipetted cell mixture up and down a few time so as to dissociate cells. Further used syringe to pull large marrow pieces through needle to dissociate it further.

8. Passed cell suspension through cell strainer (70μm size) to remove any large cell clumps or bone particles. Counted cell suspension. Added 10μm cells to 10μl trypan blue. Used hemacytometer to count.
   - For plating for antibody staining or other experiment directly, plated at 1x10⁶ per well of 6-well dish.
   - For maintaining for future experiments, brought total volume to 25-30mL and plated entire volume in 150mm plate.

9. Incubated cells until cells adhered and looked nearly confluent—usually around day 4 of culture for a pool from 4 mice. Removed media and wash once with PBS to remove nonadherent cells. Trypsinized and split 1:3-1:5.

10. Expanded cells until 70%-90% confluent, changing media every 3-4 days. At this point, either split further for experiments of freeze in liquid nitrogen for further use.

11. At this time, cells can be plated for differentiation assays.

4.4.3 MSC enrichment from spleen and adoptive transfer

Single cell suspension of splenocytes was prepared from mice, 30 days *M. tb* post-infection. These cells were stained with dye conjugated anti-CD3 and anti-B220 antibodies conjugated with the anti-Sca-1 and anti-CD29 antibodies. CD3^+^B220^−^Sca-1^−^ cells were sorted on a FACSaria. Cells were cultured overnight, washed and adoptively transferred (2-4 X10⁶ cells/mouse) to syngeneic mice. Groups of reconstituted animals were infected intravenously with *M. tb* as described above. For long term cultureMSCs were cultured in stem cell medium (MACS, Miltenyi Biotec).

4.4.4 MSC differentiation assay

Trans-differentiation of MSCs and staining of adipocytes were performed as described elsewhere (Doucet, Ernou et al. 2005). When adipoblasts treated with a combination of dexamethasone,
isobutylmethylxanthine (IBMX or MIX) and insulin, cells start to adopt a rounded phenotype and within 5-8 days begin to accumulate lipids intracellularly in the form of lipid droplets. Treatment of cells with dexamethasone activates the transcription factor CCAAT/enhancer-binding protein b (C/EBPb). IBMX inhibits soluble cyclic nucleotide phosphodiesterases and results in increased intracellular cAMP levels. At the nuclear level, treatment with IBMX results in activation of the related transcription factor C/EBPd. C/EBPb and d in turn induce transcription of C/EBPa and (peroxisome proliferator-activated receptors) PPAR. Within 3 days of exposure to inducers, the cells undergo two rounds of mitosis, termed mitotic clonal expansion, which are required for differentiation. Insulin or insulin-like growth factor-1 promote adipocyte differentiation by activating (Phosphoinositide Kinase-3) PI3K and (protein kinase B) Akt activity. Modulation of the activity of the forkhead transcription factor Fox01 appears to be necessary for insulin to promote adipocyte differentiation. C/EBPa and PPAR direct the final phase of adipogenesis by activating expression of adipocyte-specific genes, such as fatty acid synthetase, fatty acid binding protein, leptin and adiponectin.

Following solutions were made for the differentiation assay:

10% Calf Serum/DMEM
60 mL : Calf Serum
6 mL : 100 mM MEM Sodium Pyruvate
6 mL : 100x P/S/G
500 mL : DMEM

10% FBS/DMEM
60 mL : Fetal Bovine Serum (Filter Sterilized)
6 mL : 100 mM MEM Sodium Pyruvate
6 mL : 100x P/S/G
500 mL : DMEM

IBMX Solution (make fresh)
Dissolve IBMX in a solution made of 0.5N KOH to a final concentration of 0.0115 g/mL.
Filter sterilize through a 0.22 mm syringe filter.
Insulin Stock Solution
167 mM (1 mg/mL) in 0.02 M HCl
Filter sterilized through 0.22 mm filter
Can store at -20°C for long term, 4°C short term.

Dexamethasone Stock Solutions
Freezer Stock: 10 mM of Dex in 100% ethanol (store at -20°C)
Working Stock: Dilute Freezer stock to 1mM in PBS
Filter sterilize and store at 4°C.

MDI Induction Media (10 mL/ 10cm plate; 5 mL/ 6 cm plate)
To required volume of 10% FBS/DMEM add:
1:100 IBMX
1:1000 Insulin
1:1000 Dexamethasone working stock

Insulin Media (10 mL/ 10 cm plate; 5 mL/6 cm plate)
To required volume of 10% FBS/DMEM add:
1:1000 Insulin

Oil red O stock solution (0.5 g/ 100 ml isopropanol)
Just before staining: mix 60 ml of stock with 40 ml of H2O, let it sit for 1 hr at RT and filter through whatman paper 3mm.

Initially the cells were plated 10% Calf Serum/ Fetal Bovine Serum-DMEM on culture dishes and incubated them at 37 °C in 10% CO2 and fed after every couple of days. They can be split as far as 1:15, while passaging to avoid confluency more than 70%.

Adipocyte Differentiation Protocol

1. Grew preadipocytes/ fibroblast to confluency in 10% FBS-DMEM.

2. Two days after the required confluency (which is counted as day 0), stimulated the preadipocytes with MDI induction media.
3. After two days of MDI an induction medium (which is called as day 2) replaced the MDI induction media with Insulin Media. The media began to get more viscous as free fatty acids were produced by the cells and secreted into the media.

4. Two days later (which is called as day 4) changed media to 10% FBS-DMEM. Fed the cells with 10% FBS-DMEM every two days. Full differentiation was achieved by day 8.

**Staining procedure:**

1. Aspirated the media, added formaldehyde slowly and was made to sit for 30 minutes.

2. Aspirated formaldehyde and added Oil red O solution to cover the well, left for 1 hour at room temperature.

3. Removed the stain and was washed with distilled water twice.

4. Finally was dried for pictures.

Briefly, cells grown at fifty percent confluency were cultured with medium containing 115 μg/ml of isobutylmethylxanthine, 1 μg/ml insulin and 1 μM dexamethasone for two days. After two days medium was replaced with 10% FBS-DMEM containing 1 μg/ml insulin. Finally, cells were cultured for an additional two days in 10% FBS-DMEM. Cells were then stained with Oil Red O solution to visualize adipocytes.

4.5 Principle of MACS

Leukocyte precursors in the bone marrow were negatively enriched by negative selection using Magnet Assisted Cell Sorting (MACS).

**MACS Technology:** MACS Technology is based on labelling of cells with the help of MACS microbeads conjugated to the specific antibodies. MACS microbeads are superparamagnetic particles of 50 nm in diameter, composed of biodegradable matrix. The magnetically labeled cells are separated over a MACS column placed in a MACS Separator, a strong permanent magnet. When MACS columns are
placed in a MACS Separator and a labeled cell suspension is passed through it, the MACS column matrix is provided with a magnetic field strong enough to retain cells labeled with minimal amounts of magnetic microbeads. The unlabeled cells pass through and collected as the unlabeled fraction while the retained cells are eluted from the MACS Column after removal from the magnet.

The desired cell population can be purified from a heterogeneous cell suspension in two ways:

**Positive Selection:** Cells of interest are magnetically labeled with MACS microbeads. The cells are passed through a MACS column placed in a MACS separator. The flow-through fraction contains the unlabeled cells as the negative fraction depleted of labeled cells. The column is removed from the separator and the retained cells are eluted as the enriched positively selected fraction.

**Negative Selection:** Non-target cells are magnetically labeled with MACS microbeads, are retained in a MACS column placed in a MACS separator. The target cells pass through the column as the enriched, unlabelled fraction, depleted of undesired cells.

**Negative selection of leukocyte precursors by MACS**

To the cell suspension was added microbeads conjugated to anti-CD90, anti-CD45R (B220), anti-CD19, anti-I-A (MHC class II) to remove T lymphocytes, B lymphocytes, and all antigen presenting (MHC class II') cells. The resulting suspension was incubated at 4°C on a nutator. After 30 min of incubation the cell suspension was passed through a pre-wet MACS column (MS column, Miltenyi Biotech). The flow-through containing lymphocyte and I-A depleted leukocyte precursors was washed once with HBSS.

**4.6 M. tb infections and measurement of CFUs in organs**

Animals were infected with *M. tb* as described before (Dezawa, Ishikawa et al. 2005). H37Rv were grown in Middlebrook 7H9 medium containing 0.05% Tween 80 at 37 °C until mid-log phase of growth.
Bacteria were harvested by centrifugation and resuspended in saline containing 0.04 % Tween 80. The bacterial suspensions were briefly sonicated to disrupt the bacterial clumps, diluted, and stored in aliquots at -70 °C until use. Intravenous infection with *M. tb* strain H37Rv was performed through the tail lateral vein with 1-2x10^6 CFU of bacteria. Mice were infected by the aerosol route with ~110 CFU of *M. tb* H37Rv using an aerosol chamber. Mice were sacrificed at different time points and the organs were harvested in aseptic conditions, homogenized, and serial dilutions were prepared in distilled sterile water with 0.05% Tween 80 for counting of CFUs. Single cell suspensions were made and filtered with a cell strainer. Serial dilutions were plated on 7H11 agar medium containing Oleic Acid Albumin Dextrose Catalase (OADC), and incubated in a 37°C incubator for 21 days and the numbers of CFU were counted.

4.7 Measurement of T cell responses

Spleens were harvested from infected and uninfected animals, and single cell suspensions were made using frosted glass slides. RBCs were lysed with lysis buffer. Cells (4x10^5 cells/well/200μl) were seeded in 96-well plates. Cultures were supplemented with different dosages of ConA as indicated in the figure. For proliferation of purified CD4+ T cells, cells were incubated with Dyna beads (Dynal Biotec) as described by the supplier. Cells (2x10^5/well/200 μl) were cultured with plate-bound anti-CD3 ( 1 μ g/ml) and anti-CD28 (2 μ g/ml) antibodies. For co-cultures of T cells and accessory cells, T cells were removed from splenocytes following incubation with anti-CD3, anti-CD4 and anti-CD8, beads. The resultant cells were considered as accessory cells. Accessory cells from infected animals were mixed with purified CD4+ T cells at a ratio of 2:1 from infected or uninfected animals and vice-versa. These cells were cultured in 96-well plates (4 x10^5 cells/well/200μl) in the presence of ConA. All cultures were pulsed with 1μCi 3[H]-thymidine after 48 hrs. One day later cells were harvested on filter mats using a semi-automated cell harvester (Perkin-Elmer). Thymidine incorporation was determined by using a plate β-counter (Perkin-Elmer).

4.8 Immunization

For antigen-specific immune responses, mice were immunized subcutaneously with 100 μg chicken OVA (Sigma-Aldrich) in 100 μl of PBS emulsified with 100 μl of alum. Nine days later draining lymph nodes
were harvested. Single cell suspensions were made and challenged with different dosages of OVA as indicated in the figures. Proliferation of T cells was determined by $^3$H-thymidine incorporation, as described above.

4.9 Analyses of cell surface markers by FACS

Approximately 0.5-1 X 10$^6$ DC precursor cells or DCs were centrifuged at 2500 rpm for 5 min and the culture supernatant was discarded. Further steps were carried out at 4°C and in dark. The cell pellet was suspended in 100 μl of FACS Wash Buffer containing either a primary antibody directly conjugated to a fluorochrome such as Fluorescein isothiocyanate (FITC) or Phycoerythrin (Dominici, Le Blanc et al.); or a biotin conjugated primary antibody at a dilution of 1:500. This was incubated on ice for 30 min and washed twice with 1 ml of FACS Wash Buffer at 2500 rpm for 5 min. Following this, the cells were either fixed in 300 μl of Fixing buffer and taken for acquisition. Ab-biotin labeled cells were further processed with streptavidin-PE or streptavidin-FITC beads, diluted 1:500 in FACS Wash Buffer and fixed. The data were plotted and analyzed using CellQuest software.

4.10 Determination of cytokines and NO production

Cytokines produced in the culture supernatants were measured using multiplex system (Luminex XLP, CA). Cytokines were detected using detection beads (Millipore), following the manufacturer’s protocol.

The mouse cytokine assays helped to estimate the concentration in culture supernatant. The capture antibody coupled beads provided in the kit were first incubated with antigen standards or samples for 30 minutes followed by incubation with biotinylated detection antibodies for 30 minutes. After washing away the unbound biotinylated antibodies, the beads were incubated with a reporter streptavidin-phycoerythrin (SA-PE) conjugate for 10 minutes. Following the removal of excess of SA-PE, the beads were passed through the Bio-Plex array reader, which reads the fluorescence of the bead and of the bound SA-PE. All incubations were performed at room temperature.

Nitric oxide production was measured by using Griess reagent kit, as described by the supplier.
Following Solutions are required for nitric oxide determination:

1. NED Solution

(0.1% N-1-naphthylethlenediamine dihydrochloride in water)

2. Nitrite Standard

(0.1M sodium nitrite in water)

3. Sulfanilamide Solution

(1% sulfanilamide in 5% phosphoric acid)

A Nitrite Standard reference curve was prepared for each assay for accurate quantitation of NO$_2^-$ levels in experimental samples.

1. Prepared 1ml of a 100μM nitrite solution by diluting the provided 0.1M Nitrite Standard 1:1,000 in the matrix or buffer used for the experimental samples.

2. Dispensed appropriate matrix or buffer into the wells.

3. Added 100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0μM nitrite solution (50μl/well) in triplicate in 96 well plate for preparing standard reference curve so that final volume of solution in each well is not more than 50 μl.

4. Allowed the Sulfanilamide Solution and NED Solution to equilibrate to room temperature (15–30 minutes).

5. Added 50μl of each experimental sample to wells in duplicate or triplicate.
6. Using a multichannel pipettor, dispensed 50µl of the Sulfanilamide Solution to all experimental samples and wells containing the dilution series for the Nitrite Standard reference curve.

4. Incubated for 5–10 minutes at room temperature, protected from light.

5. Using a multichannel pipettor, dispensed 50µl of the NED Solution to all wells.

6. Incubated at room temperature for 5–10 minutes, protected from light. A purple/magenta color began to form immediately.

7. Measured absorbance within 30 minutes in a plate reader with a filter between 520 nm and 550 nm.

8. To generate a Nitrite Standard reference curve, plotted the average absorbance value of each concentration of the Nitrite Standard as a function of "Y" with nitrite concentration as a function of "X".

9. Determined the average absorbance value of each experimental sample.

10. Determined its concentration by comparison to the Nitrite Standard reference curve.

Preparation of reference curve(s) was done in the same matrix or buffer as used for experimental samples.

4.11 Immunohistochemical analysis

Immunohistochemical analyses were performed as described by (Das, Eynott et al. 2006). Briefly, slices of tissues (5 µM) mounted on slides were incubated with normal mouse serum to block non-specific binding. The slides were then incubated overnight with primary antibody diluted with 0.5% BSA, followed by fluorescently-labeled secondary antibodies (Santa Cruz Biotechnology, catalogue no).

4.12 Infection of mice with M. tb and adoptive transfer of MSCs

Naïve mice were infected with 1 x 10⁶ M. tb H37Ra via the tail vein and aerosol. 24h later one group of mice was sacrificed and lung homogenates were plated onto 7H10 agar plates for confirming establishment of infection. Seven days post infection, 10 x 10⁶ MSCs were injected into the tail vein of mice. Seven days following the 2nd transfer, mice were sacrificed and lung and spleen cells were enriched
using a homogenizer. An aliquot of the homogenate was lysed and plated onto 7H10 agar plates in serial dilutions for CFU monitoring.