Materials and Methods
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5.1 Animals

Female BALB/c mice, 4-6 week of age, kept in the ACBR animal house under pathogen free environment were used following approval from the Institutional Animal Ethics Committee.

5.2 Materials

Fluorescence-tagged antibodies against mouse CD80, CD86, CD54, CD40 were from BD Biosciences (San Jose, CA, USA). Recombinant mouse GM-CSF was from R&D Systems (Minneapolis, MN). Dichlorofluorescin diacetate (DCFH-DA) and FITC tagged Alexa Flour 488 were obtained from Molecular Probes (Eugene, OR). ELISA kits were from eBioscience (San Diego, CA). Antibodies to Beclin-1, ATG5, β-Actin, SOD1, siRNAs against mouse genes and luminol kits for chemiluminescence detection were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Control siRNAs from Santa Cruz (Catalog sc-37007) was used as a non-specific control. Pathway specific siRNA libraries for primary screening were from Dharmacon (Lafayette, CO). siRNAs for the secondary screen were procured from Santa Cruz Biotechnologies. Recombinant *M. tb* antigens Rv2463 and Rv3416 were expressed and purified as recently described (Gupta et al., 2010). TLR2 ligand, Pam3Csk4, was purchased from Invivogen (San Diego, CA). The following reagent was obtained through BEI Resources (NIAID, National Institutes of Health; purified lipoarabinomannan (LAM) from *Mycobacterium tuberculosis*, strain H37Rv, NR-14848). RPMI-1640 medium, lipofectamine LTX and Trizol were purchased from Invitrogen life technologies (NY, USA). Fetal Calf serum (FCS) was purchased from HyClone (UT, USA). Middlebrook 7H9 liquid media, 7H11 agar, Albumin-Dextrosecatalase (ADC), Oleic acid-Albumin-Dextrose-Catalase (OADC) supplement, Luria Bertani (LB) broth media and LB agar were obtained from Difco-Becton-
Materials and Methods

Dickinson (NJ, USA). Restriction enzymes, T4 DNA ligase and DNA ladder were obtained from MBI Fermentas (USA). Mammalian and bacterial electroporation cuvettes and protein molecular weight markers were from Bio-Rad (Philadelphia, PA, USA). Gel extraction kit, midiprep Plasmid DNA isolation kit, Ni\(^{2+}\)-NTA agarose and RNA protect regent were taken from Qiagen (Valencia, CA). Microbeads, MACS® columns and nylon mesh filters were obtained from Miltenyi Biotech (Germany). Nitrocellulose membrane (Hybond C pure) was procured from Amersham (Arlington Heights, IL). Fine chemicals, primers and antibiotics used in the study were procured from Sigma Aldrich (St. Louis, USA).

5.3 Methodology

5.3.1 Cultivation of *E. coli*

*E. coli* strains were stored at -70°C as glycerol stocks (25% glycerol) and were cultured in Luria Bertani (LB) medium containing either ampicillin (100 µg/ml) or kanamycin (25 µg/ml) or other antibiotic as required.

5.3.2 Preparation of *M. tb* H37Rv stocks for infection studies

*M. tb* H37Rv was grown in Middlebrook 7H9 liquid medium (Difco-Becton-Dickinson, NJ, USA) supplemented with albumin/dextrose/catalase (ADC) (Difco-Becton-Dickinson, NJ, USA) at a final concentration of 5 g/l, 2 g/l and 0.003 g/l, respectively, along with 0.05 % Tween 80. The culture was harvested at an O.D 595 of 0.5, concentrated to small volume and stocks were prepared in small aliquots. Aliquots were frozen at -85°C and viable bacteria were enumerated by plating serial dilutions of stocks on 7H11 agar (Difco-Becton-Dickinson, NJ, USA) supplemented with 10% OADC (oleic acid/albumin/dextrose/catalase) (Difco-Becton-Dickinson, NJ, USA) and 0.5% glycerol. These stocks were then used for all the infection studies.
5.3.3 Enrichment of dendritic cell precursors from the mouse bone marrow

5.3.3.1 Isolation of Bone Marrow cells

Female Balb/c mice, 4-6 weeks of age, were sacrificed by either putting them in a chloroform chamber or by cervical dislocation. Mice were wiped with alcohol to sterilize them. The body wall was cut and mice were pinned on to a dissection board. The tibia and femur bones were carefully dissected out and put into a petridish containing RPMI media. With the help of forceps, bones were teased to remove all the attached tissue. A sharp cut was made at either end of the bones. Following this, 10-15 ml of RPMI was flushed through the bones with a 27-gauge needle to take out the bone marrow into the media. A homogenous suspension of cells was prepared by passing the media containing bone marrow cells through an 18-gauge needle. This suspension of cells was then centrifuged at 700 g for 10 min. After decanting the supernatant, cell pellet was resuspended in RBC lysis buffer (0.9% Ammonium chloride, 10 mM Tris-Chloride – pH 7.5; 5 ml for 2 mice) for 3-4 min. Thereafter, HBSS was added (four times the volume of RBC lysis buffer used) to dilute the cell suspension in RBC lysis buffer and the cell suspension was centrifuged at 700 g for 10 min. The supernatant was discarded and cell pellet was washed twice with the same volume of HBSS. The cell pellet was then suspended in 1 ml of chilled RPMI and passed through a sterile nylon mesh filter (Miltenyi Biotech, Germany) to remove the tissue pieces.

5.3.3.2 Negative selection of leukocyte precursors from bone marrow cells by MACS®

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

5.3.3.2.1 Principle of MACS® Technology

MACS® Technology is based on magnetic labeling of cells with the help of MACS microbeads conjugated to the relevant specific antibody. MACS microbeads are super
Materials and Methods

Paramagnetic 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 column is placed in a MACS separator and a labeled cell suspension is passed through it, the MACS column matrix is provided a magnetic field strong enough to retain cells labeled with minimal amounts of magnetic microbeads. The unlabeled cells pass through the column and can be 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 heterogeneous cell population containing the cells of interest (labeled with microbeads) is 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:** In this, non-target cells are magnetically labeled with MACS microbeads. Undesired cells 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 non-target magnetically labeled cells. To enrich the leukocyte precursors from the bone marrow cells, microbeads conjugated to anti-CD90, anti-CD45R (B220), anti-CD19, anti-MHC class II were added to the cell suspension of bone marrow cells obtained. These beads were added to remove T lymphocytes, B lymphocytes, and all antigen presenting (MHC class II positive) cells from the bone marrow cells. Bone marrow cells together with the microbeads were 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 contained lymphocyte and I-A depleted leukocyte precursors, which were then washed once with HBSS.

5.3.3.3 Generation of Dendritic cells (DCs) from leukocyte precursors

Leukocyte precursor cells, negatively selected from the bone marrow cells, were counted using haemocytometer, after diluting cells with trypan blue dye (1:10). Cells were cultured at a density of 1.5 - 2 x 10^6/ml in RPMI 1640 medium containing 10% FCS, 0.05 M 2-mercaptoethanol, 1mM sodium pyruvate with 15 ng/ml of GM-CSF (R&D Systems, Minneapolis, MN) for 3 days in a 6-well cell culture plate. It has been shown previously in our lab that this method gives a homogenous population of cells that is 99% DCs with negligible contaminating monocytes or macrophages (Latchumanan et al., 2002). These DCs differentiated in the presence of GM-CSF are also referred as GM-CSF DCs.

5.3.4 Enrichment of T lymphocytes from ovalbumin immunized mice

Mice were infected intraperitoneally with chicken egg ovalbumin (50µg/mouse) for 7 days. Mice infected with H37Rv were sacrificed by putting them in a chloroform chamber and dissected to remove the spleen. The spleen was kept in RPMI media in a petridish. It was cut into small pieces using fine forceps and then crushed between the sterile frosted slides to form the homogenate. The spleen homogenate was then centrifuged at 1000 g for 10 min. The pellet obtained was suspended in RBC lysis buffer to remove RBCs and incubated at room temperature for 5 min followed by a spin at 1000 g for 10 min. The cell pellet was washed twice with RPMI. It was then suspended in 5 ml of RPMI and passed through a sterile nylon mesh filter (Miltenyi Biotech, Germany) to remove the tissue pieces. The cell suspension obtained was first depleted of adherent cells by two rounds of panning over plastic plates (5 ml of cell suspension/plate in RPMI medium containing 20% FCS) for 1 hr at 37°C in a humidified incubator. The top layer containing non-adherent cells was collected from the plates while adherent cells on the plate were discarded. Splenic T cells from the non-adherent cell population were enriched
using MACS. Non-adherent cell suspension was incubated with mouse anti-CD11c, anti-CD11b, anti-I-Ad, anti-CD19 and anti-CD45R coated magnetic beads for 30 min at 4°C on a nutator to remove contaminating DCs, macrophages, MHC-II⁺ cells and B lymphocytes, respectively followed by separation through MACS columns. The purity of the resulting negatively selected population of T cells obtained in the flow-through was 95-98% as determined by the surface staining with CD90. The percentage of I-A⁺ cells was less than 0.05%.

5.3.5 Transfection of DCs with siRNA and stimulation

For siRNA transfections, 4 x 10⁶/ml bone marrow precursors were transfected with 60 pmoles of siRNA for 72h using the Hiperfect transfection reagent (Qiagen) in OPTIMEM medium (Invitrogen). GM-CSF was added 5h following transfection and incubation continued for 72h for DC differentiation. Knockdown was verified by RT-PCR, following which, cells were stimulated either with 1 μg/ml Pam₃Csk₄ or 5 μg/ml ManLAM and/or either with 15 μg/ml Rv2463 or Rv3416 for 24h. For some experiments, siRNA transfected DCs were infected with M. tuberculosis H37Rv at 2.5 MOI for indicated times. Cells were processed for monitoring colony forming units (CFU), Reactive oxygen Species (ROS) measurement or western blotting as described below.

5.3.6 Analyses of cell surface markers by FACS

Approximately 0.5-1 x 10⁶ dendritic cells were harvested at 700 g for 10 min and the culture supernatant was discarded. All the further steps were carried out at 4°C and in dark. The cell pellet was washed once with FACS wash buffer and then suspended in 100 μl of wash buffer containing either a respective primary antibody directly conjugated to a fluorochrome such as Fluorescein isothiocyanate (FITC). The cells were then incubated on ice for 30 min and washed twice with 1 ml of FACS wash buffer. In case of fluorochrome tagged primary antibody, cells were fixed with 300 μl of fixing buffer for 15 min followed by washing with wash buffer and acquired on FACS Calibur (BD Biosciences). The data were plotted and analyzed using CellQuest software.
5.3.7 Analyses of signaling intermediates by western blotting

5.3.7.1 Preparation of cytosolic extracts

At the end of incubation of respective time point, DCs were harvested at 10,000 g for 10 seconds and the supernatant was discarded. Cell pellet was chilled on ice and washed once with ice-cold PBS followed by lysis in a cytoplasmic lysis buffer (buffer containing 10 mM HEPES (pH 7.9); 10 mM KCl; 0.1 mM EDTA (pH 8.0) and 0.1 M EGTA with freshly added Sodium vanadate, 0.1 M PMSF and 2 µg/ml each of aprotinin, leupeptin and pepstatin). Approximately 100 µl of buffer was used to dissolve a cell pellet of about 3 x 10^6 cells. Cells suspended in the lysis buffer were incubated on ice for 30 min. Following incubation, 0.5% Nonidet P-40 was added to the cell suspension in lysis buffer. Cells were then vortexed briefly twice within the interval of 2-3 min. The suspension was centrifuged at 10000 g for 5 min at 4°C. The supernatant was designated as the cytosolic extract. All the reagents used and steps carried out were strictly at 4°C to prevent any denaturation of proteins in the extract.

5.3.7.2 Polyacrylamide Gel Electrophoresis of Proteins

The Polyacrylamide gel electrophoresis (PAGE) of proteins was performed in the presence of 0.1% SDS in the gels in a Mini Protean III™ apparatus (Biorad). Total protein concentration in cytoplasmic extracts was estimated using Bradford reagent. 25 µg of protein or as required was mixed with an equal volume of 2X SDS-PAGE sample buffer (4% SDS, 0.2% Bromophenol blue, 20% glycerol, 100 mM Tris-Cl (pH 6.8), 4% β-mercaptoethanol) and denatured in a heating block at 100°C for 10 min. The boiled extracts were then separated on appropriate percentage of SDS-Polyacrylamide gel. A parallel set of samples were run simultaneously on a separate SDS-PAGE and probed for control antibodies (e.g. GAPDH) for normalizing loading errors. Following the run, gels were electro-blotted using the Bio-Rad Mini-transblot apparatus.
5.3.7.3 Western Blotting

For western blotting of proteins, mini Trans-blot Electrophoretic Cell (Bio-Rad) was used to transfer the proteins from gel onto nitrocellulose membrane (Hybond C pure, Amersham, IL). The apparatus for electro-blotting was assembled according to the manufacturer’s instructions. Electro-blotting was performed at a constant current of 150 mA for 2 hours. The membrane was subsequently immersed in blocking buffer and kept for overnight incubation at 4°C. Following blocking, blot was washed twice with the wash buffer with gentle shaking for 10 min each at room temperature. The blot was incubated with an appropriate dilution of the respective primary antibody for 2 hours at room temperature with gentle shaking. Thereafter, the blot was washed three times with wash buffer for 5 min each with shaking. After washing, the blot was incubated with horseradish peroxidase conjugated secondary antibody for 2 hours. The blot was again washed three times with wash buffer as done before. Blot was then processed using Luminol Chemiluminescence kit (Santa Cruz Technologies, USA) for the detection of the bands. The blot was exposed to Kodak Xomatic autoradiography film (eBioscience, San Diego, CA) for different times (15 sec to 3 min) till a clear profile of the specific band of interest was visible followed by developing and fixing of the exposed film in Kodak ready-made solutions available for the specific purpose.

5.3.8 Antigen specific T-cell response: Co-culture of DCs with enriched T lymphocytes

For measuring antigen specific T cell responses, 3 x 10^6 DCs were transfected with siRNAs and stimulated by the addition of chicken egg ovalbumin for 24 hours. These DCs were then co-cultured with T cells (1:1) enriched from the spleen of chicken egg ovalbumin immunized mice for 48 h. Following incubation, the culture supernatants were collected and cytokines levels were monitored using ELISA.
5.3.9 Estimation of cytokines: ELISA

Culture supernatants from stimulated DCs were analyzed for IL-12p40, IL-17 and IL-6 levels. DC-T cell co-cultures were analyzed for IFN-γ and IL-10 levels employing a sandwich ELISA (eBiosciences). Protocols followed were as per manufacturer’s instructions. Samples were diluted to obtain values within the linear range of the standards.

5.3.10 Measurement of Intracellular Reactive Oxygen Species

Intracellular ROS levels were measured by flow cytometry, as described previously, using the redox-sensitive dye DCFH-DA (Gupta et al., 2010; Sinha et al., 2006). The nonfluorescent DCFH-DA readily diffuses into the cells where it is hydrolyzed to the polar derivative nonfluorescent dichlorofluorescin, which is oxidized in the presence of H$_2$O$_2$ to the highly fluorescent dichlorofluorescein. Thirty minutes prior to the end of each incubation period, 1 x 10$^6$ cells/ml were incubated with 10 µM DCFH-DA in the dark. Cells were thoroughly and quickly washed with pulse spin and immediately acquired for analyses in FACS Calibur (BD Biosciences). The data were plotted and analyzed using CellQuest Pro software.

5.3.11 Confocal Microscopy

2 x 10$^6$/ml siRNA-transfected DCs were stimulated with ManLAM along with Rv2463 for 4 h. Cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% saponin, and incubated with antibodies against Beclin-1 or ATG5 followed by anti-rabbit FITC-tagged Alexa Fluor 488. Cells were again fixed with 4% paraformaldehyde. Confocal imaging was performed with Nikon A1 laser scan confocal microscope with 60X objective magnification, numerical aperture 1.4, refractive index 1.5, Plan Apo optics equipped with an argon laser, using excitation and emission wavelength of 488 and 525, respectively. Data were analyzed using the NIS Elements AR software. Expression levels were quantified as average of sum intensity of each fluorescent field.
5.3.12 Intracellular survival of *M. tb* H37Rv in DCs

siRNA transfected DCs were infected with 2.5 MOI of *M. tb* H37Rv for 48 hours. Extracellular bacteria were removed by treatment with gentamycin (100µg/ml) for 2 hours after infection. Infected cells were then scraped and harvested. The supernatant was removed and the pellet was resuspended in PBS for washing. Cells were again pelleted down and were resuspended in PBS (100 µl PBS/ 3 x 10^6 cells). 1% NP-40 was added to the cells for the lysis following which they were vortexed for 2 min after every 5 min. This was done three times to ensure the complete lysis of host cells. This lysed solution containing intracellular mycobacteria was serially diluted and plated onto 7H11 agar culture plates. Three-four weeks later, plates were scored for Colony Forming Units (CFU).

5.3.13 Statistical Analysis

The *in-vitro* cultures were in triplicates and a minimum of four mice were used per group for *in vivo* experiments. The data, represented as mean standard deviation (SD), is from one experiment, which was performed at least three times. Student’s t test was employed to assess the significance of the differences between the mean values of control and experimental groups. A *P*-value of less than 0.05 was considered significant and less than 0.01 was considered highly significant.