Chapter 4

Materials and Methods
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4.1 Mice

C57BL/6 and OT-II TCR transgenic mice (6–8 weeks of age) were initially purchased from The Jackson Laboratories, USA. TLR-2 and MyD-88 knock-out mice (6–8 weeks of age), both on a C57BL/6 background, were the kind gift of Prof. Ruslan Medzhitov, Yale University, New Haven, USA. All animals were subsequently bred and maintained in the animal facility of the International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India. All animal experiments were performed according to the guidelines of institutional animal ethic committee of ICGEB (approval ID; ICGEB/IAEC/IMM-13/2007).

4.2 Bacteria

*Mycobacterium tuberculosis* strain H37Rv was a kind gift from the Colorado State University repository. H37RvΔRD1 and BCG were a kind gift from Prof. David Sherman, (SBRI, Seattle, WA, USA). The integrative cosmid vector pYUB412 (control vector) and the recombinant cosmid vector RD1-2F9 harbouring RD1 locus of *M. tb* (Pym et al., 2002) were kind gifts from Prof. Stewart Cole of the École Polytechnique Fédérale de Lausanne (EPFL), Switzerland. The control and recombinant cosmids were electroporated individually into electrocompetent cells of BCG (Danish) to obtain BCG::YUB412 and BCG::RD1 strains, essentially as described previously (Pym et al., 2002). Briefly, 100 ml of bacilli suspension (OD<sub>600nm</sub>, 0.4) from a 7-day-old Middlebrook 7H9 (Difco) culture, supplemented with albumin-dextrose-catalase (ADC; Difco), was pelleted by centrifugation at 2500g for 15 min at 16°C, washed twice with 10% glycerol and finally resuspended in 3 ml of 10% glycerol. 200 µl of the electrocompetent bacilli were mixed with 5 µl of the control vector pYUB412 (85 ng µl<sup>-1</sup>) or recombinant vector RD1-2F9 (100 ng µl<sup>-1</sup>) and electroporated using the Gene Pulser Xcell Electroporation System (Bio-Rad Pacific, Hong Kong) with settings of 2.5 kV, 25 µF and 1000 Ω. After electroporation, cells were resuspended in 5 ml of 7H9 medium supplemented with ADC, and kept overnight at 37°C. The cells were then pelleted by centrifugation, resuspended in 100 µl of 7H9 medium, and plated on Middlebrook 7H11 medium supplemented with oleic acid-albumin-dextrose-catalase (OADC, Difco), hygromycin
(200 μg ml⁻¹) and ampicillin (100 μg ml⁻¹). After three to four weeks of incubation at 37°C, hygromycin-resistant clones were selected. BCG::RD1-2F9 (BCG::RD1) clones were characterized for secretion of ESAT-6 by immunoblotting using mouse anti-ESAT-6 antibody (unpublished data).

4.3 Recombinant ESAT-6 protein:

Detailed procedures for preparation and characterization of recombinant ESAT-6 have been described in earlier publication (Ganguly et al., 2008a). Briefly, *Escherichia coli* BL21(plysS) transformed with pET23b+ vector (Novagen) carrying esat6 gene of *M. tb* was grown to mid-log phase, induced with IPTG (0.4mM final conc.) for 4 hrs, and the recombinant ESAT-6 protein was extracted from the inclusion bodies in 8M urea. The recombinant ESAT-6 protein was then purified by Nickel -nitrilotriacetic acid (Ni-NTA) chromatography, checked for LPS contamination by LAL (*Limulus* amebocyte lysate) tests, and characterized for purity by SDS-PAGE, immunoblotting and N-terminal amino acid sequencing as described previously (Ganguly et al., 2008a). The purified and LPS-free recombinant ESAT-6 protein was aliquoted and kept at -80°C until further use.

4.4 Bacterial cultures:

All mycobacterial strains were grown in 7H9 (Middlebrook, Difco, USA) medium supplemented with 10% ADC and with 0.05% Tween 80 and 0.2% glycerol, and cultures were grown to mid-log phase. Aliquots of the cultures in 20% glycerol were preserved at -80°C and these cryo-preserved stocks were used for infections.

4.5 *M. tb* infection of mice and estimation of Colony Forming Units (CFU)

Mice were infected with various mycobacterial strains (namely H37Rv, H37RvΔRD1, BCG, or BCG::RD1) via the aerosol route using a Madison aerosol chamber (University of Wisconsin, Madison, WI) with its nebulizer pre calibrated to deposit a total of ~110 to the lungs of each mouse as previously described (Raghuvanshi et al., 2010; Tousif et al., 2011). Briefly, mycobacterial stocks recovered from a -80°C freezer were quickly thawed and subjected to light ultra-sonication to obtain a single cell suspension. 15 ml of the bacterial cell suspension (10×10⁶ cells per ml) was placed in the nebulizer of the Madison aerosol chamber pre-calibrated to deliver via aerosol route the desired number of CFUs to the lungs of animals placed
inside the chamber. A day after the aerosol exposure procedure, three randomly selected mice were sacrificed at various time points and organs were harvested, homogenised in 0.2 μm filtered PBS containing 0.05% Tween 80 and plated onto 7H11 Middlebrook (Difco USA) plates containing 10% oleic acid, albumin, dextrose and catalase (OADC) (Difco USA). Undiluted, ten-fold diluted and one hundred-fold diluted lung and spleen cell homogenates were plated in duplicate on the above 7H11 plates and incubated at 37°C for 21-28 days. Colonies were counted and CFUs were estimated. Mice from various groups were euthanized at the indicated time points in various experiments; their organs were harvested for obtaining CFU counts and/or immune cell subpopulations for immunological studies as described under other subsections.

4.6 Reagents

Luminex kits were purchased from Millipore and Bio-Rad. GM-CSF and IL-4 were obtained from R&D Biosystems, USA. Purified or fluorescently-conjugated monoclonal antibodies against mouse CD11c (N418), CD11b (M1/70), CD80 (16-10A1), CD86 (GL1), and MHC-II (NIMR-4) were purchased from eBioscience, USA, and fluorescently-conjugated anti-mouse IgG2a (R19-15) was purchased from BD Pharmingen. LPS was obtained from Sigma-Aldrich (L-2654).

4.7 Solutions for Flow Cytometric Analysis

**Phosphate Buffer Saline (PBS, pH 7.4):** 13.7 mM sodium chloride (NaCl), 0.27 mM potassium chloride (KCl), 10 mM Di-Sodium hydrogen phosphate (Na₂HPO₄) and 0.2 mM Sodium di-hydrogen phosphate (NaH₂PO₄).

**Wash Buffer:** 0.5% Bovine serum albumin (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% Fetal calf serum (FCS) in PBS.

**RBC lysis buffer:** 0.826 % ammonium chloride (NH₄Cl), 0.1 % potassium bicarbonate (KHCO₃) and 0.0037 % tetrasodium ethylene-diaminetetraacetic acid (EDTA) in double distilled water, pH adjusted to 7.2, filtered and autoclaved.
4.8 Generation of Dendritic Cells (DCs)

Mice were euthanized and the femurs were isolated. Bone marrow was flushed out with RPMI-1640 medium using a 2.0 ml syringe (26G). The cells were washed twice with PBS at 600g for 5 minutes at room temperature and then cultured in RPMI-1640 (Gibco, UK) medium supplemented with 10% v/v heat inactivated FCS, 100mM HEPES, 100U/ml penicillin, 100mg/ml streptomycin, 2mM L-glutamine (all Invitrogen) denoted as complete RPMI. To differentiate bone marrow cells into myeloid DCs, bone marrow cells (1x10^6 cell/ml in each well) were cultures into 24 well plate (Nunc, USA) in the presence of complete RPMI media further supplemented with GM-CSF (40 ng/ml) and IL-4 (10 ng/ml) (R&D systems, USA), 1mM sodium pyruvate (Invitrogen) and 50μM 2-mercaptoethanol (Sigma; Gillingham, Dorset, UK). On the third day, 75% of the medium was replaced with fresh DC culture medium. On day 5, the suspended cells were removed and the loosely adherent cells were used as immature DCs (CD11c-positive cells were >90%). Flow cytometric analysis by using anti-CD11c, -CD11b, -CD80, -CD86, -MHC class II, and - IgG2a (isotype control) antibodies suggested that >95% of the cells were conventional DCs.

4.9 Bacterial infections and co-culture of CD4+ T cells with OT-II TCR transgenic mice

BM cells were isolated from different mouse strains (C57BL/6, TLR-2−/− and MyD-88−/−) and differentiated into immature DCs as described above and cultured in 24-well plates (1x10^6 cells per well). DCs were infected with H37Rv, H37RvΔRD1, BCG or BCG::RD1 as described below.

Frozen stocks of bacteria were taken out and washed once with complete media at 2000g for 10 minutes at room temperature. Bacteria were resuspended again in complete RPMI media and to avoid clump formation, the bacterial suspension was passed ten times through a 24 gauge needle and then ten times through a 26 gauge needle. Infection experiments were performed in 24 well plates (Nunc, USA). DCs were infected in complete RPMI medium without antibiotics with a final multiplicity of infection (MOI) of 1:10 (number of infecting bacteria were ten times the number of DCs). After inoculation, the culture plates were centrifuged for 5 min at 600xg in order to sediment the bacteria and facilitate phagocytosis. Infected DCs were cultured
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four hours in humidified CO₂ incubator at 37°C after which the supernatant was removed and DCs were washed with complete medium with antibiotics. After the washing step, fresh RPMI medium was added with 100µg/ml gentamicin (Sigma, USA) and DCs were kept for another hour in humidified CO₂ incubator at 37 0C. Finally DCs were washed three times with complete media and added 1ml complete RPMI media per well and were incubated at 37°C in 5% CO₂ humidified incubator.

Similarly, 1x10⁶ DCs were cultured in 24-well plates in the presence or absence of LPS at 1 µg/ml and co-stimulated with ESAT-6 protein at a final concentration of 5 µg/ml. Supernatants from cells were collected at 24, 48 and 72 hours for cytokine profiling. For Th1 and Th17 cell differentiation, CD4⁺ T cells (1x10⁶) were purified by MACS method (CD4⁺ T cell isolation beads kit; Miltenyi Biotech, Germany) from OT-II TCR transgenic mice and cultured with immature DCs (1x10⁶) infected with H37Rv, H37RvΔRD1, BCG or BCG::RD1 (MOI of 1:10) in the presence of ovalbumin (10 µg/ml) peptide (Thermo Scientific, USA) for 72 hours. Then, CD4⁺ T cells were harvested and subjected to intracellular staining for IL-17 and IFN-γ expression.

4.10 Antibiotic treatment of mice

Thirty days post infection, groups of mice were treated with 0.1 g/L rifampicin and 0.1 g/L isoniazid (Sigma-Aldrich, St. Louis, MO, USA) administered in the drinking water (changed daily) for 4 weeks. M. tb-infected control mice received plain drinking water. A control group of infected mice was sacrificed at the start of treatment (early control group). A second group of infected but untreated mice was sacrificed 4 weeks after therapy was initiated (late control group).

4.11 Isolation of lymphocytes from infected animals

Lungs from infected or uninfected animals were harvested and washed by swirling in PBS. The thoracic cavity of the sacrificed mouse was opened up by cutting longitudinally and then the lungs were cut into ~0.5-cm pieces. These lung pieces were agitated in 25 ml of extraction buffer (PBS, 3% FCS, 1 mM dithiothreitol, 1 mM EDTA) for 30 min at 37°C. This slurry was passed through a loosely packed nylon wool column to remove the aggregates. The filtrate was layered on a discontinuous
Percoll gradient (Amersham Pharmacia Biotech, USA). This gradient was then centrifuged at 900g for 20 minutes. Cells at the interface of the 40/70 % layer were collected and washed in staining buffer (PBS, 3% FCS). Cells were cultured for intracellular staining as described below. Bronchoalveolar lavage (BAL) fluid was collected from lungs by intratrachial infusion of PBS and cell-free BAL was used for cytokine assay (Devadas et al., 2006).

4.12 Intracellular cytokine staining

For intracellular cytokine staining, cells were treated with 50 ng/ml phorbol myristate acetate (PMA) and 500 ng/ml ionomycin in the presence of 1μg/ml brefeldin A (Sigma-Aldrich or eBiosciences, USA) added during the last 6 h of culture. Cells were washed twice with PBS and resuspended in a permeabilization buffer (Cytofix/Cytoperm kit; BD), and stained with the following fluorescently conjugated monoclonal antibodies: anti-CD4 (clone: GK1.5)-APC, anti- IFN-γ (clone: XMG1.2)-FITC, and anti-IL-17 (clone: 17B7)-PE (all from eBiosciences, USA). Fluorescence intensity was measured by flow cytometry (FACS Calibur; BD) and data were analysed with FlowJo (Tree star, USA).

4.13 [3H]-Thymidine incorporation assay of splenocytes

Spleens were macerated by frosted slides in complete RPMI 1640 (Gibco, Invitrogen, UK) and made into a single cell suspension. Red blood cells (RBCs) were lysed with RBC lysis buffer and washed with complete RPMI 1640. Splenocytes were counted and plated at 0.1 X 10⁶ cells/well in 96-well plates and stimulated with different concentrations of M. tb complete soluble antigen (CSA). Cells were cultured for 48 hours and then pulsed with tritiated thymidine (³H-TdR, 1.0 μCi per well; Amersham Biosciences UK). 16 hours later, incorporation of ³H-TdR was measured by means of a cell harvester and liquid scintillation counter (Wallac Trilux, Perkin Elmer, UK).
4.14 q-PCR analysis

Bone marrow derived DC were isolated and infected with different bacterial strains (H37Rv, H37RvΔRD1, BCG and BCG::RD1) as described above and cultured for 24 hours for RNA isolation. Total RNA, including miRNAs was isolated by miRNAeasy isolation kit (QIAGEN, Germany) according to the manufacturer's instructions. Real-time quantitative RT-PCR analysis was performed using BioRad Real-Time thermal cycler (BioRad, USA) and miRCURY LNA universal reverse transcriptase microRNA PCR SYBR green master mix (EXIQON, Denmark) for miRNA amplification and IQ BioRad SYBER green master mix (BioRad, USA) for IL-6 expression, respectively. cDNA was synthesized by the miRCURY LNA universal reverse transcriptase microRNA cDNA synthesis kit (EXIQON) and the reaction was set up according to the manufacturer’s protocol. For amplification of miR146a, LNA PCR miR146a and reference 5S rRNA primer sets were used and the reaction was set up as recommended by EXIQON. The relative expression level of miRNAs was normalized to that of internal control 5S rRNA by using 2-ΔΔCt cycle threshold method. Furthermore, for amplification of IL-6 or IL-22, cDNA was synthesized by Omniscript RT kit (QIAGEN) using oligo(dT)₁₆ primers (Fermentas, Maryland, USA). For IL-6 mRNA expression analysis primer sequences were IL-6F, 5’-TGGAGTCACAGAGGAGTGCTAAG-3’ and IL-6R, 5’-TCTGACCACAGTGGAGGATGTCCAC-3’, and control GAPDH-F, 5’-CGTCCCTAGCATAATGGT-3’ and GAPDH-R, 5’-TTGATGGCAACAATCTCCAC-3’ and for IL-22 mRNA expression analysis primer sequences were IL-22F 5’-GTGACGACCAGAACATCCAG-3’ and IL22R 5’-ATCTCTCGCTCTCTCCAAG-3’. Data were normalized by the level of GAPDH expression in samples as described above.

4.15 Knock-down of miR146a using anti-miR146a

For transfection of anti-miR146a and scramble control (EXIQON) into DCs, cells were transfected at day 4 of culture in antibiotic free media using Lipofectamine2000 (Invitrogen, UK) reagents. Briefly, at day 4 DCs were washed once with antibiotics free RPMI media and cultured in 500µl of antibiotics free RPMI media for four hours in humidified CO₂ incubator at 37°C. Four hours later, 100nM of antisense and scramble control separately were added into 50µl of opti-MEM medium
(Invitrogen, UK) in eppendorf tube (A). In another eppendorf tube (B) 1µl of Lipofectamine 2000 was added into 50µl of opti-MEM medium. After 5 minutes, both eppendorf tube A and B contents were mixed gently together. After 20 minutes incubation at room temperature, this mixture was added to the cultured DCs by swirl mixing. Next day, the media were changed and the DCs were infected with H37Rv, H37RvΔRD1, BCG or BCG::RD1. After 24 hours of bacterial infection, cells were harvested for RNA preparation and analyzed for miR146a, IL-6 and IL-22 expression by quantitative real-time PCR as described above.

4.16 Detection of cytokines

Cytokines in the culture supernatant samples were assayed by a Luminex microbead-based multiplex assay using commercially available kits according to the manufacturer’s protocol (Milliplex kit, Millipore and BioPlex, Bio-Rad, USA).

The Bio-Plex or Milliplex suspension array system is built around three core technologies. The first is the technique to internally colourcode microspheres with two fluorescent dyes. Through precise concentrations of these dyes, 100 distinctly coloured bead sets can be created, each of which is coated with a specific capture antibody. The second is a flow cytometer with two lasers and associated optics to measure biochemical reactions that occur on the surface of the microspheres. The microspheres are allowed to pass rapidly through a laser which excites the internal dyes marking the microsphere set. Another laser excites PE, the fluorescent dye on the reporter molecule. The third is a high-speed digital signal processor that efficiently manages the fluorescent output. The Bio-Plex or Milliplex suspension array system employs multiplexing technology that uses up to 100 colour-coded bead sets, each of which can be conjugated with a specific reactant. Each reactant is specific for a different target molecule. Bio-Plex or Milliplex cytokine assays are designed in a capture sandwich immunoassay format. Antibody specifically directed against the cytokine of interest is covalently coupled to colour-coded 5.6 µm polystyrene beads. The antibody-coupled beads are allowed to react with a sample containing an unknown amount of cytokine, or with a standard solution containing a known amount of cytokine. After performing a series of washes to remove unbound protein, a biotinylated detection antibody specific for a different epitope on the cytokine is
added to the beads. The result is the formation of a sandwich of antibodies around the cytokine. The reaction mixture is detected by the addition of streptavidin-phycoerythrin (streptavidin-PE), which binds to the biotinylated detection antibodies. The constituents of each well are drawn up into the flow-based Bio-Plex or Luminex suspension array system, which identifies and quantitates each specific reaction based on bead colour and fluorescence. The magnitude of the reaction is measured using fluorescently labelled reporter molecules associated with each target protein. Unknown cytokine concentrations are automatically calculated by Bio-Plex Manager or Luminex IS 2.3 software using a standard curve derived from a recombinant cytokine standard.

4.17 Histology

Lung tissues were stained with Hematoxylin and Eosin (H&E) dyes. The hematoxylin and eosin stain uses two separate dyes, one staining the nucleus and the other staining the cytoplasm and connective tissue. Hematoxylin is a dark purplish dye that will stain the chromatin (nuclear material) within the nucleus, leaving it a deep purplish-blue colour. Eosin is an orangish-pink to red dye that stains the cytoplasmic material including connective tissue and collagen, and leaves an orange-pink counterstain. The lung tissues were dissected out and fixed in 4% paraformaldehyde. Then the tissues were processed through alcohol grades and xylene and embedded in paraffin. After paraffin embedding, the tissues were sectioned, placed on slides and the slides were dried in an oven. The dried slides were taken through brief changes of xylene, alcohol and water to ‘hydrate’ the tissue (this process is called ‘running the slides down to water’ and must be done to give the cells an affinity for the dyes). The slides were then stained with the nuclear dye (hematoxylin) and rinsed, then stained in the counter-stain (eosin). After staining, the slides were rinsed, run in the reverse manner from the run down (taken back through water, alcohol, and xylene), then coverslipped and dried. Finally, the slides were viewed under microscope.
4.18 Statistical analysis

All data were derived from at least three independent experiments. Statistical analyses were conducted using SPSS software and values were presented as mean ± SD. Significant differences between the groups were determined by ANOVA followed by Tukey's multiple comparison test (SPSS software). A value of p<0.05 was accepted as an indication of statistical significance.