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Animals

Inbred C57BL/6 mice (8-12 weeks old) were used throughout this study. Animals were bred and maintained in the animal house facility at JNU, New Delhi or obtained from the National Institute of Nutrition Hyderabad. The animals were housed in positive-pressure air conditioned units (25°C, 50% relative humidity) and kept on a 12 hrs light/dark cycle. Water and mouse chow were provided ad libitum. The JNU Institutional Animal Ethical Committee approved all experimental protocols requiring the use of animals.

Reagents and other supplies

All tissue culture reagents, Collagenase, DNase, L-lysine, Trypsin, Tween-20, and Catalase were purchased from Sigma Aldrich (India). Tween-80, DMEM/F12 and Napthylethylenediamine for preparation of Griess reagent were purchased from Sigma Chemicals (St. Louis, MO, USA). Sources of other reagents were: Fetal Bovine Serum (FBS) Hyclone Laboratories Inc. (South Logan, UT) USA; Middlebrook 7H11 agar and Lip polysaccharides Difco Laboratories, MI, USA; Dispase II, Anti mouse CD16/32, anti mouse Annexin V FITC, anti mouse CD54 FITC, anti mouse CD80 PE, anti mouse CD3 FITC, anti mouse CD45R FITC, anti mouse CD11b FITC, anti mouse CD4 PE, anti mouse CD8 PE, anti mouse IgG2α PE, anti hamster IgG1 PE, anti rat IgG2α FITC, anti rat IgG2β FITC, anti rat IgG2ακ FITC, anti rat IgG2α PE, anti mouse IgG2ακ PE, anti mouse IgG2ακ R-PE from BD Biosciences San Diego, CA, USA; anti mouse NK1.1 PE, anti mouse TLR2 FITC, anti mouse TLR4 PE, anti human TLR2 FITC, anti human TLR4 PE, anti mouse TLR9 FITC, anti mouse MHC class I PE, anti mouse MHC class II FITC, anti mouse F4/80 APC, anti rat IgG2ακ APC, 7AAD, recombinant murine TNFα and IFNγ cytokines, murine IL-2, IFNγ, and TNFα Elisa kit, RBC lysis buffer (1X) from eBiosciences San Diego, CA, USA; mouse epithelial cell enrichment kit and mouse CD4 selection kit from Stem Cell Technology (USA); DiI C18 dye from Molecular probes, USA; Haematoxylin from Qualigens. All the syringes and needles were procured from Becton Dickinson, Singapore. All other chemicals were purchased locally and were of analytical grade. 40 μm cell strainer and 5 ml polystyrene tube were purchased from BD. Costar (Cambridge, MA), USA was the source of all plastic disposable culture ware. All concentrations indicated in
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percentage are in (w/v) basis unless stated otherwise. All solutions were prepared in Milli Q (Billerica, MA, USA) water unless stated otherwise. Autoclaving was done at a pressure of 15lbs per square inch for 20 min.

Particles

Crystalline silica was obtained from Val Vallyathan, NIOSH, CDC, Morgantown, USA. The size of silica particles was <10 μm. Single-walled carbon nanotubes (SWCNTs) were procured from Sigma and were acid functionalized as described (Saxena et al, 2007). The particles were dispersed in saline and sonicated for 2 min in Branson sonicator, before use.

Preparation of Reagents and Media

Sauton’s Medium

For preparing Sauton’s medium, 4.0 g L-asparagine, 2.0 g citric acid, 0.50 g potassium dihydrogen phosphate, 0.50 g magnesium sulphate, 0.05 g ferric ammonium citrate and 60 ml glycerol were added to 950 ml of deionized water obtained from the Milli Q Water System. The pH was adjusted to 7.3 and the volume made upto 1 litre. 0.05% Tween 80 was added whenever required. Medium was dispensed in culture flasks, sterilized by autoclaving for 30 min.

OADC Supplement

To prepare ODAC supplement, 5.0 g BSA, 2.0 g glucose, 0.004 g catalase and 0.85 g sodium chloride were dissolved in 99.94 ml deionized water and 60 μl oleic acid was added to it. The solution was filter sterilized using a 0.22 μm membrane and stored at -20°C till use. 100 ml of the supplement was added per litre of Middlebrook 7H 11 agar.

Griess reagent

To prepare griess reagent, 1% (w/v) sulphanilamide and 0.1% (w/v) napthylethlenediamine dihydrochloride were dissolved separately in 2.5 % ortho phosphoric acid and mixed. The solution was stored in glass bottle at 4°C.
Hanks’ Balanced Salt Solution (HBSS) Modified

To prepare HANK’s BSS, 0.185 g calcium chloride dihydrate, 0.097 g magnesium sulphate (anhydrous), 0.4 g potassium chloride, 0.06 g potassium phosphate monobasic (anhydrous), 8.0 g sodium chloride, 0.048 g sodium phosphate dibasic (anhydrous), 1.0 g D-glucose, 2.380 g HEPES and 0.35 g sodium bicarbonate were added to 950 ml of deionized water obtained from Milli Q Water System. The pH was adjusted to 7.2-7.6 and volume made up to 1 litre. The solution was filter sterilized using a 0.22 μm membrane and stored at 4°C.

Culture Media

Human alveolar epithelial cell line (A549), Mouse lung epithelial cell line (TC1) Mouse alveolar epithelial cell line (LA4) and Mouse alveolar macrophage cell line (MH-S) were used for this study. These cell lines were maintained in RPMI 1640 with 10% FBS (LA4 with 15% FCS) containing 300 μg/ml Glutamine, 20 mM HEPES, 2X10⁻⁵ M 2-Mercaptoethanol and 40mg/ml gentamicin. All cell cultures were incubated at 37°C with 5% CO₂. Primary mouse lung epithelial cells, Bronchoalveolar lavage, (BAL) cells and peritoneal macrophages were also cultured in RPMI 1640 medium with 10% FCS.

Culture of BCG

All inoculations and manipulations of BCG were carried out in a Biosafe Laminar Flow Chamber. Cultures were initiated from frozen stocks. For initial seeding, a loopful of cells were scraped from the surface of frozen stock and inoculated in a culture flask containing 20 ml of Sauton’s medium and incubated at 37°C shaker for three weeks. Cells were then sub cultured in increasing volumes of Sauton’s medium (with 0.05% Tween 80) and harvested in the late log phase. Growth was monitored by measuring absorbance of the bacterial suspension at 600 nm. For rapid estimation of bacterial concentration in a given suspension, absorbance of 0.4 at 600nm corresponded with a concentration of 1.1x10⁸ cfu/ml. When culture reached a cell density of 1.1x10⁸ cells/ml bacteria were harvested by centrifuge at 4000 rpm for 20 min and the pellet was washed twice with PBS. Resulting bacterial cells were either inoculated into fresh medium or aliquots were frozen at -70 °C in medium with
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20% glycerol. Viability and cfu count was determined by plating on 7H11 agar OADC plates.

**DIC 18 staining and flowcytometric analysis of BCG**

BCG were grown in Sauton’s medium containing 0.05% Tween 80 as shaken cultures. A working solution of 100 μM DiI C18 in 300mM sucrose was freshly prepared from stock solution. Bacteria were resuspended in PBS and incubated with DIC 18 dye (10 μM final concentration). The samples were then incubated in dark at 37°C for 2 hrs. The bacterial pellet was washed twice with PBS before being analyzed using a flowcytometer.

**Measurement of Nitric Oxide (NO)**

LA4 and MH-S cells were seeded at density of 25x10^5/ml/well in 24 well plate. After overnight culture cells were treated with silica (50 μg/ml), LPS (10 μg/ml) or BCG (25x10^5 cfu), individually or in combinations. At 24 and 48 hrs, post treatment 100 μl of culture supernatant from each sample was collected and transferred to wells of a 96 well flat bottom microtiter plate. 100 μl of Griess reagent was added in each well and kept in dark for 10 min. NO formed was quantified at 540 nm in a microplate reader. A calibration curve was prepared using increasing amount of NaN02 ranging from 1μM to 50 μM.

**Flowcytometric analysis of cell surface receptors on cell lines and primary cells**

0.2x10^6 cells were incubated with anti mouse CD16/32 Fc block (1μg/10^6 cells) for 20 min on ice prior to staining. Cells were then incubated with mouse anti TLR2 FITC, TLR4 PE, TLR9 FITC, MHC I PE, MHC II FITC, CD54 FITC, CD 80 PE, CD11b FITC, CD4 PE, CD8 PE, CD45R FITC, NK1.1 PE, F4/80 APC, human anti TLR2 FITC, TLR4 PE and their isotype control for 30 min on ice. Fc block (CD16/32 antibody) was used to block Fc receptor on cells for 20 min before staining. Cells were then washed twice with staining buffer (PBS containing 1% FCS and 0.09% Sodium azide) and fixed in 200 μl of 2% paraformaldehyde. Cells were analysed on BD FACS calibur by using Cell Quest software.
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Intracellular staining for FACS analysis

A549, LA4 and MH-S cells were harvested by trypsinisation, washed twice in cold PBS. 0.2x10^6 cells were fixed in 200 μl of 4% paraformaldehyde by incubating at room temperature for 10 min. Following fixation cells were vortexed thoroughly and washed twice in cold PBS. Cell pellate was harvested and suspended in 0.8 ml of SAP buffer (saponin 0.1% w/v, sodium azide 0.05% in HBSS), the cells were centrifuged at 1500 rpm for 5 min. Supernatant was discarded and pellate was resuspended in 50 μl of SAP and incubated with anti mouse. CD16/32 Fc block (1μg/10^6 cells) for 20 min at room temperature prior to staining. Tubes were vortexed thoroughly, fluorescence conjugated antibodies and their isotype control were added and incubated at room temperature for 30-45 min in the dark. Cells were washed twice using SAP buffer and finally resuspended in 200 μl of PBS for flowcytometric analysis.

Determination of Macrophage population in the peritoneal exudates

To detect macrophage population in peritoneal fluid, cells isolated from mice were washed two times in PBS containing 1% FBS. Peritoneal cells were stained with anti mouse F4/80 antibody conjugated with APC and respective isotype control. Fc block (CD16/32 antibody) was used to block Fc receptor on macrophages before staining. For flowcytometery FSC and SSC were kept on the linear scale.

Instillation of BCG or particles in lungs

Mice were anesthetized and exposed either to silica (100 μg/mouse) or BCG (1x10^6 cfu) or phosphate buffer saline vehicle by intratracheal instillation as previously described before by Saxena et al. (2002). Briefly, anesthetized mice were suspended on a 60-degree incline board from their incisors using a rubber band. Gentle pressure was exerted on thoracic cavity to prevent breathing. With the tongue gently extended, the dose of the material to be instilled was delivered onto the oral cavity with a Finn-pipette tip. Pressure from thoracic cavity was then released to allow the mice to inhale with great force. The material in oral cavity would then reach lungs. Using this technique, approximately 95% of BCG is deposited in the lungs of each mouse as calculated by viable cell counting on 7H11-OADC agar plates.
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Bronchoalveolar lavage (BAL)

Mice were sacrificed by chloroform and skin incision made in the neck region. Trachea was exposed by retracting the surrounding tissues. A piece of suture thread was passed under the exposed trachea. A small transverse incision was made in the trachea and an 18 gauge blunt needle in plastic tubing was placed in the opening and pushed through the trachea towards the lung. The needle was secured in place with a thread by ligation. One ml of phosphate buffered saline (PBS) was delivered into lungs and after six influxes and out fluxes; the washing was collected in a tube. The process was repeated 6-8 times and washings were pooled. BAL cells were collected by centrifugation and washed two times in appropriate medium.

Dissociation mixture

Solution of 1X collagenase (300 units/ml) with DMEM /F12 supplemented with 5% FBS was used to prepare single cell suspension from crude lung extract.

Isolation of primary epithelial cells from lung

Epithelial cells were isolated from the lungs of uninfected or BCG infected mice using mouse epithelial cell enrichment kit. Lungs were dissected from mice and transferred to sterile petridishes containing cold PBS. Lung tissues were cut into small pieces and transferred to tube containing dispase solution (2 mg/ml) and incubated for 45 min at room temperature to detach cells from one another. Lung tissue pieces were then transferred to a tube containing dissociation mixture and incubated for three hrs at 37°C. After dissociation, tube was centrifuged at 1500 rpm for 5 min. Pellite was resuspended in dispase solution containing DNase I (40 Kunitz/ml) and pipetted the sample for 1 minute with P1000 sterile disposable plastic tip. Protease action was blocked by adding 10 ml of cold Hank’s Balanced Salt Solution (HBSS) with 2% FBS. Cells were centrifuged at 1500 rpm for 5 min and pellite was again suspended in 10 ml of cold HBSS with 2% FBS. Mouse lung cell suspension was filtered through 40 μm cell strainer into a new tube. Cell suspension was centrifuged at 1500 rpm for 5 min and suspended in 1 ml of HBSS with 2% FBS and counted on a hemocytometer.
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Purification of lung epithelial cells

Mice epithelial cells were purified from the lungs using mouse epithelial cell enrichment kit (Stem Cell Technologies) by negative selection. 1x10⁸ cells/ml were suspended in 1 ml of recommended medium (HBSS+2%FBS) supplemented with DNase I (40 Kunitz/ml). EasySep mouse epithelial cell enrichment cocktail at 50 μl/ml of cells was added, vortexed thoroughly and incubated at 4°C for 15 min. EasySep Biotin selection cocktail at 100 μl/ml of cells was then added vortexed thoroughly and incubated at 4°C for 15 min. EasySep magnetic nanoparticles at 50 μl/ml of cells were then added vortexed thoroughly and incubated at 4°C for 15 min. The cell suspension was brought to a total volume of 2.5 ml by adding recommended medium. Cells were mixed in the tube by gently pipetting up and down and placed the tube (without cap) into the magnet, set aside for 5 min. In one continuous motion magnet and tube were inverted and desired fraction of supernatant was poured off into other polystyrene tube. The magnetically labeled unwanted cells were retained inside the tube, held by the magnetic field. Magnet and tube were left in inverted position left for 2-3 sec, then returned to upright position. To maximize cell recovery, the process of magnetic separation was repeated once more on cells retained in the tube. Pooled, recovered cells were centrifuged at 1500 rpm for 5 min, supernatant was discarded and pellate was resuspended into 2.5 ml of recommended medium. Cell suspension was again kept inside the magnet and set aside for 5 min. Supernatant was poured off into another tube and magnetically labeled unwanted cell were remained bound inside the original tube. Poured off cell suspension was centrifuged at 1500rpm for 5 min, supernatant was discarded and pellate was suspended into 1 ml of complete medium (RPMI+10% FCS).

Purity assessment of primary lung epithelial cells

Primary lung epithelial (PLE) cells recovered from above procedure were stained with anti F4/80, anti-CD3, anti-B220, anti-NK1.1 and anti-CD11b antibodies with respective isotype controls to determine the macrophages, lymphocytes (T, B and NK) and dendritic cells in the single cells suspension before and after the magnetic enrichment process.
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Isolation of CD4^+ T cells by EasySep Magnet

CD4^+ T cells were purified from the spleens of uninfected or BCG infected mice using anti-CD4 immunomagnetic microbeads by positive selection. Mouse spleen cells were aseptically removed and teased in 5 ml of recommended medium (PBS +2% FBS with 1 mM). To make single cell suspension, clumps were dispersed by gently pipetting up and down several times and centrifuged at 1500 rpm for 5 min. Erythrocytes were lysed by using RBC lysis buffer. The cells were washed twice with recommended medium and suspended at desired concentrations in recommended medium. Remaining clumps and debris were removed by passing cell suspension through nylon sieve.

CD4^+ T cells were purified from the spleen cells using mouse CD4 positive selection kit (Stem Cell Technologies) by positive selection. 1x10^8 cells/ml were suspended in 1 ml of recommended medium and placed in a 5 ml (12x75 mm) polystyrene tube to properly fit into the EasySep Magnet. CD4 PE labelling reagent at 50 µl/ml of cells was added vortexed thoroughly and incubated at room temperature for 15 min. EasySep PE selection cocktail at 100 µl/ml of cells was then added vortexed thoroughly and incubated at room temperature for 15 min. EasySep magnetic nanoparticles at 50 µl/ml of cells were then added vortexed thoroughly and incubated at room temperature for 10 min. The cells suspension was brought to a total volume of 2.5 ml by adding recommended medium. Cells were mixed in the tube by gently pipetting up and down and placed the tube (without cap) into the magnet, set aside for 5 min. In one continuous motion magnet and tube were inverted and desired fraction of supernatant was poured off into other polystyrene tube. Magnetically labeled cells were remained inside the tube held by the magnetic field of EasySep magnet. Tube removed from the magnet and added 2.5 ml of recommended medium cell suspension by gently pipetting up and down 2-3 times. The tube was placed back in the magnet and set aside for 5 min. This step was repeated three times and the cells collected were resuspended in 1 ml of RPMI-1640 reconstituted with 10% FCS. Since the positively selected cells were already PE-labeled, purity was assessed directly by flowcytometer.
Staining of lung epithelial cells (Hematoxylin staining)

To examine the morphology of lung epithelial cells and compare it with that of the alveolar lung epithelial cell line LA4, monolayer culture of these cells were washed with PBS and then fixed for 2-5 min in fixing mixture consisting of ethyl alcohol (seven parts), undiluted formalin (two parts), and glacial acetic acid (one part). Fixed cells were washed consecutively with 70% alcohol and distilled water. They were stained for 5-15 min with a solution of hematoxylin (Qualigen) and washed with distilled water and then dehydrated in alcohols of increasing concentration (70% and 100%) and examined under microscope.

TNFα assay

Primary lung epithelial (PLE) cells and peritoneal cells were seeded at a density of 2x10^5 cells/ml in 48-well plates. Cells were treated with silica (100μg/ml), LPS (10μg/ml) and BCG (MOI 100:1) individually or in combination. After 24 hrs supernatants from treated and control cells were harvested and the levels of TNF α was measured by ELISA by using ELISA kit from e-Biosciences.

Cell cycle

Primary lung epithelial cells were cultured in wells of 6-well plate with or without 50 μg/ml of AF-SWCNT preparations. After 24 hrs, cells were washed and isolated by trypsinization. Cells were fixed, treated with RNase and stained with propidium iodide for flow cytometric analysis. Data was analyzed by using Modfit software that enumerated proportion of cells in G1/Go, S and G2/M phase.

Apoptosis measurement

Primary lung epithelial cells and peritoneal macrophages were seeded at a concentration of 25x10^4 cells/ml in 6 well culture plates. After overnight culture cells were washed with complete medium to remove debris and dead cells and allowed to grow for another 2 days (peritoneal cells one day). Cells were treated with sonicated BCG (MOI 10:1, and 100:1) and cultures were continued for 24 hrs. After 24 hrs cells were harvested by trypsinization and washed with PBS containing 1%FCS. Cells were washed again with 1X binding buffer. 0.1x10^6 cells were double stained with 7AAD. 

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and Annexin V FITC conjugated and incubated for 15 min at room temperature. Cells were washed twice in 1X binding buffer and within 1 h samples were analysed on FACS Calibur.

**In vitro antigen presentation assay**

Primary lung epithelial cell and peritoneal macrophages (2x10⁵/well) were seeded on 96 well flat bottom plate. After overnight culture cells were washed with complete medium to remove debris and dead cells and allowed to grow for another 2 days (peritoneal cells one day). Cells were treated with BCG sonicate (MOI 100:1) in presence and absence of silica (100 μg/ml) or AFSWCNTs (50μg/ml) and cultures continued for 24 hrs. After 24 hrs excess antigen was removed by washing and fixation was performed using 0.0125% glutaraldehyde for 30 sec followed by quenching with L-lysine. Splenic CD4⁺T (3x10⁵/well) cells from infected and uninfected mice were added to the wells containing epithelial cells. The amount of IL-2 (after 24hrs) and IFNγ (after 48hrs) released in culture supernatants was quantified using ELISA kits.

**Statistical analysis**

Non-paired Students t-Test was used to test for significance of differences between different sets of data using Sigma plot software. Comparisons were considered significant at p ≤ 0.05.