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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 JNU Institutional Animal Ethical Committee approved all experimental protocols requiring the use of animals.

Reagents and other supplies:

All tissue culture reagents, Tween-80, Napthylethylenediamine for preparation of Griess reagent, α isonitrosopropiophenone, L-arginine used for arginase assay and α methyl mannoside were purchased from Sigma Chemicals (St. Louis, MO). Sources of other reagents were: Fetal calf serum, Hyclone Laboratories Inc., USA; Middlebrook 7H11 agar, Difco Laboratories, MI, USA; L-asparagine, Hi-Media Labs, India and Urea from Bio-Rad, USA. Anti-mouse CD16/CD32, anti-mouse TCR β PE, anti-mouse CD19 PE, anti-mouse Mac1 FITC, biotinylated anti-mouse IFNγ receptor monoclonal antibody, Streptavidin PE, Streptavidin FITC as well as recombinant murine TNFα and IFNγ were obtained from Pharmingen. DIL C18 stain was purchased from Molecular Probes, USA. All the syringes and needles were procured from Becton Dickinson, Singapore. All other chemicals were purchased locally and were of analytical grade. Costar (Cambridge, MA) was the source of all plastic disposable culture ware.
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Strains of Mycobacteria:

The H37Ra strain (ATCC 2517) of *M. tuberculosis* was provided by Dr. Katoch (Japanese Association for Leprosy Mission Aided Institute, JALMA, Agra). Several clinical isolates of *M. tuberculosis* were originally obtained from the New Delhi TB Center and Lala Ram Swarup TB Hospital, New Delhi and adapted to grow in suspension culture in our laboratory.

In an earlier study done in our laboratory, four clinical isolates of *M. tuberculosis* (JNU 4, JNU 7, JNU 11 AND JNU 22) were selected for CFP (Culture filtrate proteins) preparation and immunogenicity studies (Siddiqui et al 2000). Comparison of the different CFP preparations in their ability to induce immune response showed that JNU7 CFP was the best. So in the present work we selected JNU 7 CFP along with CFP from standard strain H37Ra and H37Rv for different experiments. CFP preparation of H37Rv was gift from Dr. Ian Orme (Colorado State University, USA).

Preparation of Reagents and Media:

Sauton’s Medium:

4.0 g L-asparagine, 2.0 g citric acid, 0.5 g potassium dihydrogen phosphate, 0.5 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 up to 1 litre. 0.05% Tween-80 was added whenever required. Medium was dispensed in culture flasks/vials, sterilized by autoclaving for 30 minutes.
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Bradford's Reagent:

1. 64.35 mg of Coomassie Brilliant Blue G250 was added to 21.6 ml of ethanol in a clean glass container and agitated with the help of a magnetic stirrer till the dye dissolved completely (approx. 20 minutes).

2. 48.6 ml of ortho-phosphoric acid was added to the solution, volume made up to 450 ml with deionized water and stirring continued for 10 minutes.

3. The reagent was passed twice through Whatman filter paper (Grade-1) and stored in brown glass bottles at room temperature.

4. A standard curve using BSA in PBS was plotted using the freshly prepared Bradford's reagent and the K and b values calculated for subsequent protein estimations.

OADC 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 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 7H11 agar.

Griess reagent

1% (w/v) sulfinilamide and 0.1% (w/v) napthylethylenediamine dihydrochloride were dissolved separately in 2.5% ortho phosphoric acid and mixed. The solution was stored in glass bottle at 4°C.
Culture of Mycobacteria:

All inoculations and manipulations involving different strains of *M. tuberculosis* were carried out in a BioSafe Laminar Flow Chamber. Cultures were initiated from Lowenstein-Jenson slants. For initial seeding, a loopful of cells (using a loop of 6 mm diameter) were scraped from the surface of LJ slants and inoculated in a culture flask containing 20 ml of Sauton's medium and incubated at 37°C for two weeks. Cells were then subcultured in increasing volumes (3 passages) of Sauton's medium (with 0.05% Tween 80) and harvested in the late log phase stage. Growth was monitored by measuring absorbance of the bacterial suspension at 600 nm. When culture reached a cell density of 1x10⁸ cells/ml, bacteria were harvested by centrifuging at 4000 rpm for 20 minutes and washing the pellet twice with PBS. Resulting bacterial cells were either inoculated into fresh medium or frozen at -70°C in medium with 20% glycerol. After the vials had been frozen, a sample from the stored set was taken out the following day for determination of viability by plating different dilutions of the bacterial suspension on Middlebrook 7H11 agar (enriched with OADC supplement). Colonies were counted after three to four weeks and CFU/ml of the frozen stock were calculated. For rapid estimation of bacterial concentration in given suspension, absorbance of 0.4 corresponded with a concentration of 1.18 x10⁸ CFU/ml (H37 Ra) and 0.97 x10⁸ CFU/ml (JNU7).

Culture of *E.coli*:

*E.coli* (strain DH 5α) were grown in Luria broth and counted by plating on Luria agar. In spectrometric estimations, an absorbance of 1.0 at 600nm corresponded to 1.12 x10¹⁰ CFU/ml.
Intra peritoneal infection of mice:

A frozen stock of *M. tuberculosis* (H37Ra) of known CFU/ml was thawed and the bacilli diluted in PBS to a concentration of $10^8$/ml. The suspension was slowly dispensed and mixed in order to minimize the possibility of creation of aerosols. The suspension was passed through 24G needle several times to break the clumps and prepare a single cell suspension of bacteria. An aliquot of the suspension was serially diluted and plated on 7H11 agar (supplemented with OADC) to verify the concentration. $50 \times 10^6$ CFUs were injected per mouse (in a volume of 500 µl) from the working stock of $100 \times 10^5$/ml.

**Differential leukocyte count:**

Differential leukocyte count was carried out by making smears of peritoneal cells on glass slides. Cell smears were air dried and the slides were fixed by dipping into absolute methanol for 30 minutes. Commercial Giemsa stain (Qualigens, India) diluted 10 times with PBS were added drop by drop on the fixed smear and kept for 20 minutes. Then the slide was washed with flowing water and air dried. The slide was examined under a light microscope to study the differential staining of various leukocytes and a total of 200 cells from each sample were counted.

**Flowcytometric analysis of cell surface receptors:**

Cells to be stained (0.25 $\times 10^6$ / in 50 µl volume) were incubated with anti mouse CD16 / CD32 Fc block (1 µg/10$^6$ cells) for 10 minutes prior to staining. Cells were then incubated
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with anti mouse TCR β PE or CD19 PE or Mac1 FITC or CD54 FITC antibody (1 μg / 10^6 cells) for 30 minutes on ice. Cells were washed twice with staining buffer (PBS without Ca^{2+} and Mg^{2+} containing 1% FCS and 0.09% Sodium azide) and fixed in 200μl of 0.1% paraformaldehyde. Two step staining procedure was followed for IFN-γ receptor (IFNR) staining; firstly, cells were incubated with anti mouse biotinylated IFNR antibody (1 μg /10^6 cells) for 30 minutes on ice, then washed and stained with streptoavidin-PE (100 ng/10^6 cells) or streptoavidin-FITC (500 ng/10^6 cells) for 30 minutes on ice. Cells were washed with staining buffer and fixed in 200 μl of 0.1% paraformaldehyde. For dual staining the cells were incubated with anti mouse CD16 / CD32 Fc block for 10 minutes and then incubated with anti mouse TCR β PE or CD19 PE and CD54 FITC or biotinylated IFNR antibody for 30 minutes on ice. Streptoavidin-FITC was used in the second step to stain IFNR. Cells were washed with staining buffer, fixed in 200 μl of 0.1% paraformaldehyde and analysed on a flow cytometer (Coulter- EPICS Model and WINMDI software).

DILC 18 staining and flowcytometric analysis of *M. tuberculosis*:

Bacteria were grown in Sauton’s medium (containing 0.05% Tween 80) as shaken cultures. A working solution of 50μM DIL C18 in 300mM sucrose was freshly prepared from stock solution. Bacteria (1 x 10^8) were resuspended in 500 μl of PBS and incubated with DIL C18 (10 μM final concentration). The samples were then incubated in dark at 37°C for 2 hours. The bacterial pellet was washed twice with PBS before being analyzed using a flowcytometer.
Preparation of Culture Filtrate Proteins (CFP):

CFP were prepared as described previously (Roberts et al 1995), with minor modifications. Sauton’s medium was inoculated with *M. tuberculosis* strains at a density of 1 x 10^6 cells/ml and the suspension grown as shaken cultures. After 14 days, culture supernatant was collected upon pelleting the cells at 4000 rpm for 20 minutes. Cells were given three washes in PBS and were either frozen at -70°C or used for further inoculations. The culture supernatant was sequentially filtered through Whatman filter paper (Grade 1) and 0.2 μm pore size membrane (S&S, Germany). The filtrate was subsequently concentrated by ultrafiltration in the Amicon apparatus (Amicon, Columbus, OH) with a 3K MW cutoff membrane. All CFP preparations were filter sterilized, aliquoted and stored at -20°C. The protein content was determined by Bradford’s method (Bradford 1976), using BSA as standard.

Measurement of Nitric oxide:

RAW 264.7 cells cultured in a 24 well plate (0.2 x 10^6 cells / ml / well) were infected with *M. tuberculosis* (H37 Ra or clinical isolate, JNU 7) or co-cultured with 100 ng culture filtrate proteins (CFP) of different isolates of *M. tuberculosis* in presence or absence of IFN γ or TNF α for different time periods. At different time points (24, 48 and 72 hours post infection), 50 μl of culture supernatant from each sample was collected and transferred to wells of a 96 well flat bottom microtiter plate. 50 μl of Griess reagent was added in each well and kept in dark for 10 minutes. NO formed was colorimetrically quantified at 540 nm in a microplate reader (Model multiskan BICHROMATIC of
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Labsystems, Finland). A calibration curve was prepared using increasing amount of NaNO₂ ranging from 1μM to 50 μM.

Measurement of arginase activity:

To evaluate arginase activity in adherent peritoneal cells, 10⁵ control or infected cells were washed twice with PBS and treated with 50 μl of 0.1 % Triton X-100 (E-Merck, Germany) containing 5 μg each of aprotinin and leupeptin (Sigma chemicals, USA). The mixture was then stirred for 30 minutes at room temperature. After the cells were lysed 50 μl of 10 mM MnCl₂, 50 mM Tris-HCl, pH 7.5 were added and enzyme was then activated for 10 minutes at 55°C. Arginine hydrolysis carried out in eppendorf tubes was initiated by the addition of 25 μl of 0.5 M L-arginine (Sigma chemicals, USA), pH 9.7 to a 25 μl aliquot of the previously activated lysate. Incubation was performed at 37°C for 60 minutes and the reaction was stopped by addition of 400 μl of an acid mixture containing H₂SO₄, H₃PO₄ and H₂O (1:3:7). Then 25 μl of 9% α isonitrosopropiophenone (dissolved in 100% ethanol) was added in the mixture and heated at 100°C for 45 minutes. After keeping in dark for 10 minutes, the urea formed was colorimetrically quantified at 540 nm in a microplate reader (Model multiskan BICHROMATIC of Labsystems, Finland) using 200 μl aliquots in 96 well plate. A calibration curve was prepared using increasing amount of urea ranging from 100 μM to 10 mM. In this case, 400 μl of the acid mixture and 25 μl α isonitrosopropiophenone were added and the procedure was followed as described above (Corraliza et al.,1994).
Statistical analyses:

For each set of data, arithmetic mean and standard error were calculated. Data sets were compared by using an appropriate (paired or unpaired) student’s t-test for paired or independent variables.