Materials and methods

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1. Bacteria

A clinical isolate obtained from Lady Hardinge Medical College, New Delhi, India was used as the candidate pathogenic strain of *Salmonella typhimurium*. It has been previously characterized in terms of biochemical and serological characteristics and has been referred to as Stm 754.

Stm SL3235, SL3261 were obtained from Prof. Kenneth Sanderson, (*Salmonella* Genetic Stock Center, Calgary, Canada). These strains are defective for aromatic amino acid production. *Salmonella typhimurium* BRD 150 was used as purine biosynthetic pathway mutant. Stm BRD 150 was a kind gift from Dr. Gordon Dougan, Imperial College, London, UK. *E coli* HB101 was maintained at National Institute of Immunology (NII) for many years. Stm LB5010, a r-m+ strain was obtained from Prof. Kenneth Sanderson (*Salmonella* Genetic Stock Center, Calgary, Canada). It was used as an intermediate strain for transformation of plasmids obtained from *E coli* in Stm754. All the strains mentioned above are sensitive to gentamycin as seen by antibiotic sensitivity assay done at Dept. of Microbiology, AIIMS and at NII.

2. Mice

All the mice that were used in this study were eight to 10 weeks old and were bred at Small animal facility (SAF) of the NII. BALB/c J (H2-d, Ity^s) and C57BL/6 J (H2-b, Ity^s) were maintained for many years in the foundation colony. C3H.SW strain is I-A^b MHC matched with C57BL/6 J and is Ity^r. It was procured from Jackson laboratories, USA and bred at the SAF of NII. Strain C.D2 is congenic to BALB/c J for Ity^r locus. This strain was a kind gift

of Prof. E. Skamene (McGill University, Montreal, Canada). Mice were maintained on standard irradiated pelleted mice feed and autoclaved water with controlled illumination. Six to ten mice were used per group in a standard experimental setup.

3. Cell Lines

CTLL.2 (TIB-214, ATCC) is a mouse cytotoxic T cell clone that is dependent on IL-2 for growth, and can respond to both IL-2 and IL-4 by proliferation. It was maintained on 2U/ml human recombinant IL-2, fed every 48 hours.

1A9.F10 (gift from Dr. Charles A Janeway Jr., Yale University, USA) is a T cell hybridoma that is specific for peptide (Eα52-78) derived from mouse Eα MHC chain in the context of IA^b. Upon activation with appropriate ligand it secretes IL-2 which is then quantitated by CTLL.2 cell line.

9E10 (ATCC, USA) is a mouse clone secreting mouse monoclonal antibody that recognizes *c-myc*. It is used in a Western blot to visualize expressed Eα fusion protein. Eα fusion protein contains *c-myc* as the tag.

T21.2 is a T cell hybridoma, generated by fusion of spleen cells from Stm immunized mice with mouse thymoma BW5147. It recognizes the shared epitope from Stm and *E coli*. It has been described before (Thatte JT, Ph.D. thesis).

4. Tissue Culture Medium

Dulbecco's minimal essential medium (DMEM, Gibco, USA), fortified with L-glutamine (2mM final conc., Sigma, USA) with $5X10^{-5}M$ β-mercapto-ethanol, 50μg/ml gentamicin (Hi-Media, India) was used with 10% heat inactivated fetal calf serum (FCS, Hyclone, USA) for maintenance of cell lines as well as regular tissue culture experiments.

5. Growth of Bacteria

All the bacteria were grown in Luria Bertanni (LB) broth medium (Hi-Media, India) to log phase, washed in PBS (PBS: 1.9mM NaH_2PO_4, 8.1mM Na_2HPO_4, 154mM NaCl, pH 7.2) and adjusted to desired density in 0.85% saline by serial dilution before injection. Bacterial counts were determined by spread plate technique on LB agar plates. Heat killing of bacteria was carried out in boiling water bath for 1 hour, they were cooled at room temperature (RT) and then washed and kept in PBS. Completion of killing was confirmed by absence of bacterial growth on LB agar plates after treatment.

6. Irradiation of bacteria

Bacterial culture was grown to the logarithmic phase of growth in LB medium as described before. Cells were then pelleted by centrifugation at 4000 rpm at RT. They were then washed with 0.05M PBS to remove the salts and other media componants. The density was then adjusted to ~ $1X10^8$ and plated on *Salmonella Shigella* agar (SS agar) plates to determine exact count. This suspension was then put into Gamma Chamber at NII (^{60}Co source, TIFR, India) to receive 1.5 MRad dose (~48 hours). The killing of the bacteria was then confirmed by plating out the suspension on SS agar plates. Another

aliquote of bacterial suspension was kept at RT without radiation treatment as control live bacteria.

7. Sonication of bacteria

The sonication of heat killed or live bacteria (*E coli* or Stm) was done in chilled PBS containing 10mM phenylmethylsulphonyl fluoride (PMSF, Boehringer Mannheim, India) as protease inhibitor using ultrasonicator (Branson, USA, using microtip; 10 cycles of 4 minute each) with intermittent cooling time gaps. This preparation was then spun at 14000 rpm for 30 min. to remove debris and then at 100,000 g_{max} for 60 minutes at 4^0C to remove any insoluble matters as well as lipopolysaccharides. This was then filtered through 0.22μm sterile filter (Sartorius, Germany) and was used as a soluble protein fraction (sonicate) for assays. This is referred to as bacterial sonicate.

8. Protein estimation of sonicate

Protein estimation was done using bicinchonic acid (BCA) method. Reagent A consists of sodium carbonate (2%, Sigma, USA), sodium bicarbonate (0.95%, Merck, India), bicinchonic acid (1%, Sigma, USA) and sodium tartarate (0.16%, Sigma, USA), in 0.1N NaOH. pH of this solution was adjusted to 11.25 using 10N NaOH. This reagent A was mixed with reagent B (0.4% $CuSO_4.5H_2O$ Qualigens, India) in 50:1 ratio. This working mixture was added to titrating amount of Stm sonicate in a ratio of 20:1 in Enzyme immuno-assay (EIA) flat bottom microtitre plate (Nunc, Sweden) in 200μL final volume. Plate was incubated at 37°C for 1 hour and read at 570 nm in a microplate reader (EL340, Biotek Instruments, USA). Amount of protein present was

extrapolated from a standard curve of bovine serum albumin (BSA, Sigma, USA) run in parallel.

9. Transformation of Stm754 with Eα-cmyc-GST plasmid

The plasmid expressing Eα-*cmyc-GST* fusion protein was a kind gift from Dr. AY Rudensky, University of Washington, Seattle, USA. To bypass the restriction system of *E coli*, intermediate strain Stm LB5010 (r^-m^+) was used to facilitate the transformation of Stm with the plasmids obtained from *E coli*. Plasmid obtained from transformed Stm LB5010 using the standard plasmid isolation technique was then used in electrotransformation of wild type Stm 754.

Briefly, Stm was grown in LB in a log phase of growth and washed with ice cold double distilled water twice to remove any carry over of salts that may hamper the eletrotransformation procedure. Cells were resuspended in 10 times less volume in ice cold double distilled water. 0.2 μg pure plasmid DNA was added to the cuvette. Resistance and charging voltage were selected based on the instructions of manufacturer of Electro-cell manipulator™ (BTX, USA). After the expression time of 1 hour in LB at 37^0C, the bacterial cell mixture was plated on LB agar containing 50μg/ml ampicillin (Amp). This method was used to score all transformants except Stm 754 since it is resistant to Amp.

Since the expression of the recombinant protein can be induced by IPTG, nitrocellulose paper saturated with IPTG was placed on the plate with marked orientation. It was kept for one hour to induce the expression of fusion protein

in possible transformants as well as to take the replica print on that paper. The original plate was then stored at 4^0C as a master plate.

The nitrocellulose paper was then put into lysis buffer (1M Tris-HCl, 1M NaCl, 1M $MgCl_2$, 0.1% BSA [Hi-media, India] and a pinch of lysozyme [Boehringer Manheim, Germany], pH7.8) for 1 hour in a stationary condition at 37^0C chamber to lyse the colonies. The paper was then washed with washing buffer twice and was blocked subsequently for 1 hour with 1% BSA at 37^0C. Western blotting was done with anti-*cmyc* antibody (9E10, ATCC, USA) to see the expression of fusion protein. Area showing the colonies expressing fusion protein, was picked up and then that was purified to single colony level using the repeated plating and colony Western blotting. Final Stm754 transformant was checked for plasmid and expression of protein on normal Western blot.

10. Expression of foreign protein in bacteria

E coli transformed with an Amp^r plasmid expressing IPTG inducible fusion protein ($E\alpha_{56-72}$-*cmyc-GST* called Eα fusion protein) was grown in LB overnight. 1mM IPTG (Sigma, USA) in 0.05M PBS was added for 4 hr. to induce the fusion protein. Bacteria were then washed and were sonicated or heat killed. This preparation was used for immunisation of mice or used in 1A9.F10 T cell hybridoma stimulation assay. Same fusion protein expression protocol was also used for Stm expressing Eα fusion protein. Expression of this protein was checked in a Western blot using anti-*c-myc* antibody as in previous section.

11. Kinetics of bacterial clearance

Mice were immunized with 10^4 live Stm754 or SL3235 (Stm *aroA* mutant) in 500µl volume intraperitoneally and monitored for the colony forming units (CFU) in spleen over a period as mentioned. Mice were sacrificed by cervical dislocation and spleens were removed aseptically in 1 ml 0.05M PBS. Spleens were trichurated and 1ml, 1% sterile sodium taurocholate (Hi-media, India) solution was added to lyse the cells and remove the intracellular bacteria. Appropriate dilutions of this were then plated on SS agar (Hi-Media, India) as a selective medium for quantitation of CFU. Plates were incubated at 37°C overnight and colonies were scored manually the next day and CFU were determined.

12. Bleeding of mice and Collection of Sera

Mice were bled from the retro-orbital venous plexus with fine gauged Pasteur pipettes under stabilized diethyl ether (BDH, India) vapors as anesthesia. Around 300 µl blood was removed in the microcentrifuge tubes. It was allowed to clot at room temperature for 1 hour. The sera were separated after centrifugation at 10,000 rpm and were stored at -20 °C till the experimental analysis.

13. Immunisation regimen

Two different regimens were used for immunisation. As it is reported earlier (Thatte J, *et al.* 1993) LD_{50} of Stm in BALB/c J mice was found to be $1.7x10^4$ by intraperitoneal (IP) route for live bacteria. ~10^2 live bacteria followed by ~10^3 live bacteria in PBS intraperitoneally one week apart were given as live

immunisation, ~10^8 heat killed bacteria were given one week apart as killed bacterial immunization in some experiments. In other regimen single dose of ~10^3 to 5 X 10^3 live bacteria was given to mice as live immunisation and 10^8 heat killed bacteria were given as heat killed immunisation. As mentioned, in some experiments a dose of ~10^8 live Stm AroA mutant was given for experimental reasons. All immunizations were done in 500 µl volume intraperitoneally. Heat killing was a natural procedure for killing bacteria, unless mentioned otherwise. After each immunisation live bacteria were plated to determine the actual count, which did not deviate from the range given above.

To see the effect of various drugs on priming *in vivo*, Pentoxifylline (POF, Sigma, USA), a known inhibitor of phosphodiesterase activity, Cholera holotoxin (CT, Sigma, USA) and dibuteryl cAMP (dbcAMP, Sigma, USA) were used. Drugs were given to mice intraperitoneally either on day -1 of immunisation with live or heat killed bacteria or starting on day -1 and given daily till day 7 of immunisation. Pentoxifylline (Sigma, USA) was given 300 µg/mouse, dbcAMP (Sigma, USA), 100 µg/mouse or cholera toxin (Sigma, USA) at 1µg/mouse intraperitoneally in 0.05M PBS for single dose regimen. For daily injections for 8 days, pentoxifylline and dbcAMP were given at 100 µg/mouse in 0.05M PBS.

14. Injections for determining MHC-peptide ligand densities

For the peptide-MHC ligand density tracking experiment, mice were injected with 1X10^3 live or 1X10^8 heat killed Stm754, Stm BRD150 (*purA* mutant) or *E coli*. At different time points after injections, mice were sacrificed and their

spleens were used to prepare the APCs for *in vitro* stimulation of T cell hybridoma, T21.2.

15. Delayed type hypersensitivity (DTH) response

Either Stm sonicate or *E coli* sonicate was injected in one hind footpad (50 μg in 50 μl PBS) of the mouse, the other footpad was injected similarly with PBS as control. Footpad thickness was measured with spring loaded caliper (Mitutoyo, Japan) at 24 and 48 hours. DTH was measured as mean difference in thickness of sonicate-injected footpad over the PBS-injected footpad. It has been reported previously that DTH at 48 hours is optimal (Thatte JT, Ph.D. Thesis). Mean and standard error of at least 5 mice was then calculated.

16. Enzyme linked Immunosorbant Assays (ELISAs)

16.1 Total Anti-Stm and anti-E coli antibody assay

Polyvinyl chloride microtitre plates (Maxisorp™, Nunc, Denmark) were coated with 10 μg/ml sonicate in 0.05M sodium carbonate-bicarbonate buffer (pH9.6) at 37°C for 2 hours in water bath. Plates were washed with 0.05M PBS twice and blocked with 1% Lactogen (Nestle, India) or 1% BSA (Sigma, USA) in PBS at 37°C for two hours in waterbath. Plates were washed at least twice with washing buffer (0.05M PBS containing 0.05% Tween20 [Loba Chemie, India]). Half-log serum dilutions were titrated starting from 1:30 dilution to 1:30,000 in dilution buffer (1% Lactogen or 1% BSA in washing buffer) in 100μl/well final volume and plates were incubated for 1 hour in a waterbath at 37°C. Washing was repeated and plates were incubated with peroxidase coupled goat-anti-mouse immunoglobulin at appropriate dilutions

(Reagent bank, NII, India) and incubated for 1 hour at 37°C. Plates were washed thrice with washing buffer, followed by one or two washes of plain PBS to remove any Tween20 that may hinder in enzyme-substrate reaction. The color was developed using 0.5mg/ml O-phenylenediamine tetrahydrochloride (OPD, Sigma, USA) in citrate-phosphate buffer (0.1M citric acid and 0.2M disodium hydrogen phosphate, pH5.5) containing 1µl/ml of 30%H_2O_2 (Qualigens, India) as a revealing agent. The enzyme reaction was stopped after appreciable color was developed using 1N H_2SO_4. Plates were read at 490nm wavelength in an ELISA plate reader (EL340, Biotek instruments, USA). Concentrations, when calculated were determined from the extrapolations using a standard mouse immunoglobulin (mIg) (Reagent Bank, NII, India) curve run in the same assay in wells which were coated with affinity purified goat anti-mIg instead of Stm sonicate. The sensitivity of detection was often 1ng/ml (0.1 OD). Whenever titres were calculated, extensive titrations were taken into consideration and titres giving half of maximum OD achieved in assay were used for calculations.

16.2 Anti-Stm and anti-E coli IgG subclass assay

Polyclonal goat anti-mouse-IgG1 or anti- mouse-IgG2a (Sigma, USA) were titrated for their optimal use in ELISA, where they did not cross react with the other isotypes of mouse antibody as seen by using culture supernatants of the known isotypes.

For antigen-specific IgG subclass assay, a similar protocol as discussed above in specific antibody ELISA was used. Polyclonal anti-mouse-isotype antibodies were used as secondary antibodies followed by rabbit-anti-goat-Ig coupled to horseradish peroxidase as revealing reagent (Reagent bank, NII).

Half maximal reciprocal titres were determined from an extensive titration of sera for both the isotypes. Ratios of IgG1/IgG2a were calculated using these reciprocal titres at the OD value (half maximal, same for all the samples in IgG1 and same for all the samples in IgG2a assay) that was in linear part of the curve.

17. T cell proliferation assay

Spleens or the lymph nodes (as mentioned in results) from the immunized mice were removed aseptically in 5 ml sterile medium in a Petri dish (Nunc, Denmark). Single cell suspensions were made by gently pressing these organs between two sterile frosted glass slides and suspending the cells in DMEM with 10% FCS (henceforth called complete DMEM). Cells were centrifuged at 1500 rpm for 5 minutes at RT. RBCs were lysed by hypotonic shock by adding water and then immediately 10% v/v 0.5M PBS to suspend the cells in final 0.05M PBS. Cells were then centrifuged again and debris of RBCs was removed after resuspending them in 10 ml complete medium. Since spleens from Stm-infected mice have been known to attract large number of immature macrophages, attempt was made to remove these by plastic adherence. Splenic cells were put in sterile petri dishes and kept in a humidified 37^0C incubator in 7.5% CO_2 atmosphere (Nuaire, USA) for 1 hour for plastic adherence. Two cycles of plastic adherence procedure was done routinely for all the proliferation assays for all the groups of mice.

Cells were counted using a haemocytometer with 50% v/v Trypan Blue as vital dye (0.4% Trypan Blue in PBS, Sigma, USA). These cell suspensions were used to set up the proliferation assays using the graded dose of sonicated soluble extract of the bacteria in flat-bottomed tissue culture grade 96 well

plates (Nunc, Denmark; Corning, USA) with an optimum of $3X10^5$ cells per well in a final 200 µl volume of complete DMEM. Plates were incubated in 7.5% CO_2 atmosphere humidified incubator at 37°C. 100µl of culture supernatant was removed from the wells aseptically after ~60 hours for quantitating the lymphokines. Plates were pulsed with 1µCi/well of ^{3}H-Thymidine (Amersham, UK) for 10-12 hours in total 96 hours assay at 84 hours. The cells were then harvested onto glass fiber filters (Wallac, Finland) and counted on liquid scintillation beta counter (Betaplate™, LKB-Pharmacia, Sweden). The results were expressed as cpm ± standard errors. The standard errors were typically less than 20% .

18. T cell hybridoma stimulation assays

Single cell suspensions were prepared from spleens of C57BL/6 J mice as described before. These cells were irradiated at 3000 rads before using them as APCs. Stm-Eα (Salmonella expressing Eα fusion protein) or *E coli*-Eα were used as a source of antigen for a T cell hybridoma 1A9.F10, that recognizes Ea_{52-78} peptide in the context of I-A^b. Titrating doses of bacteria expressing Eα fusion protein were plated in 96 well flat bottom plates in complete DMEM to which $2X10^5$ irradiated APCs and $1X\ 10^5$ hybridoma cells were added in 200 µl final volume per well. 24 hours later, 100 µl culture supernatants were collected and after one freeze-thaw cycle, to kill any carry over of T cell hybridoma cells, tested on CTLL-2 cell line.

In order to assay the number of specific MHC-peptide ligand density complexes generated *in vivo*, the splenocytes of the mice injected with live or heat killed bacteria a few hours before sacrifice were used to stimulate Stm and *E coli* specific T cell hybrid. The splenocytes were irradiated and used in

titrating doses in a 96 well flat bottom plate in complete DMEM. $1X10^5$/well of T21.2, a representative T cell hybridoma recognizing Stm and E. coli were added to make final volume to 200µl. The stimulation of the hybrid was analyzed as described above with CTLL-2 cell line.

19. Lymphokine Assays

19.1 IL-2 Bioassay

For IL-2 bioassay, cell line CTLL.2 (TIB 214, ATCC, USA) was used as the indicator. CTLL.2 cell line needs IL-2 for proliferation, and hence quantitation of IL-2 can be done using the extent of its proliferation as an index.

Cells were washed twice with complete DMEM and were suspended at $1X10^6$/ml in complete DMEM without IL-2 for 10-12 hours. For assay, $1X10^4$ cells/well were added in 96 well plates containing 100µl of culture supernatant in a final volume of 200 µl. Titrating concentrations of IL-2 were used as reference standards. Plates were incubated at 37^0C in a humidified incubator having 7.5% CO_2 for 24 hours, after which they were pulsed with 1µCi/well ^{3}H-Thymidine for additional 12 hours and then harvested on glass filter mats and counted on Betaplate.

19.2 Assay of IFN-γ and IL-10 using sandwich ELISA

Maxisorp plates (Nunc, Denmark) were coated with monoclonal anti-IFN-γ (Duoset, Genezyme, USA) or monoclonal anti-IL-10 antibody (Pharmingen, USA) at predetermined optimal concentrations following manufacturer's

suggestion in 0.1M carbonate buffer (pH9.6). Plates were incubated at 4^0C overnight, after which they were washed at least thrice with wash buffer as described earlier in total antibody EIAs, then they were blocked with 1% fatty acid free BSA solution in PBS (Sigma, USA) for two hours at 37^0C waterbath. They were washed thrice with wash buffer(0.05M PBS, 0.05% Tween20), after which they were dried on paper towels. 100 µl of culture supernatants from the titrating doses of antigen in proliferation assays were added to each well. IL-10 (Genzyme, USA) and IFN-γ (Duoset, Genzyme, USA) were titrated in parallel as reference. Plates were incubated overnight at 4^0C. They were then washed thrice and then incubated with either biotinylated polyclonal anti-IFN-γ (for IFN-γ ELISA, Duoset, Genzyme, USA) or biotinylated monoclonal anti-IL-10 (for IL-10 ELISA, Pharmingen, USA) at optimal recommended concentrations in washing buffer containing 1% fatty acid free BSA. Plates were then incubated at 37^0C waterbath, for 1 hour. After washing with wash buffer thrice, avidin- horse radish peroxidase (Av-HRP, Duoset, Genzyme, USA) was added in the plates, as a labeling reagent and incubated for 45 minutes at 37^0C in waterbath. The plates were washed thrice with wash buffer and then once with 0.05M PBS to remove traces of Tween20 which may interfere with the HRP enzyme activity followed by drying. Ready-made TMB substrate (tetramethyl benezidine, Sigma, USA) was used to develop color. After optimal color development had taken place, the reaction was stopped with 2N H_2SO_4, and plates were read at 450 nm in ELISA plate reader and results extrapolated from the standard curves.

20. Western Blot Analysis

This was mainly used to detect the expression of *GST-c-myc* fusion protein in bacteria. Bacterial sonicate was boiled with sample loading buffer (0.5M Tris-

HCl, glycerol, 10% SDS, b-mercaptoethanol, and 0.05% bromophenol blue, all BioRad, USA) in a waterbath for 3 minutes. The proteins in the sonicate were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, using 12% acrylamide on a minigel apparatus (BioRad, USA). Prestained molecular weight markers (BioRad, USA) were run in one lane as reference. Separated proteins were either stained by Coomassie blue (Baker chemical, USA) or transferred to nitrocellulose membrane (Biorad, USA) by electrophoresis using Tris-glycine buffer (25mM Tris, 192mM glycine, pH 8.3) containing 20% v/v methanol (ExelR, India). Protein transfer was confirmed by transfer of molecular weight markers at all the molecular weights.

Membranes were washed once with 0.05M PBS containing 0.05% Tween20 (washing buffer) and blocking was done with 1% BSA (Hi-media, India) at 37^0C for one hour. Membranes were washed thrice with washing buffer and incubated with 1:1 diluted anti-*c-myc* culture supernatant (9E10 culture supernatant) and incubated for one hour at 37^0C after which membranes were washed thrice and incubated with goat-anti-mouse Igs-HRP (Reagent bank, NII, India) at appropriate pre-determined concentration as a labeling reagent for half an hour at 37^0C. After extensive washing of membrane with washing buffer (3 times 15min each, followed by plain PBS wash). The color was developed with TMB substrate for Western blot according to manufacturer's instructions (Vector, USA).

21. Statistical Analysis

Statistical analysis was done at relevant places using independent Student's t-test to calculate p values.