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Salmonella Typhi outer membrane protein STIV is a potential candidate for vaccine development against typhoid and paratyphoid fever

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ABSTRACT

Enteric fever, caused by *Salmonella enterica* serovars, Typhi (*S.* Typhi) and Paratyphi (*S.* Paratyphi) is a major public health challenge for the developing nations. Globally, the disease affects ~15-30 million individuals every year, resulting in > 200,000 deaths. Multidrug-resistant *S.* Typhi H58 strain has emerged as the dominant circulating strain in a large part of the world and an extensively drug-resistant (XDR) subclade of the strain was recently reported. Many believe that vaccination of the susceptible populations is urgently needed and the best option to control the infection. However, the commercial live attenuated (Ty21a) vaccine is not recommended for children below six years of age while the Vi-polysaccharide-based vaccine has poor long-term efficacy against typhoid fever. Moreover, no vaccines are available against *S.* Paratyphi infection. Thus, a new formulation capable of providing long term protection against both the pathogens and safe for all age groups is immediately required. We show that recombinant, *S.* Typhi outer membrane protein STIV (rSTIV) is immunogenic in mice and elicits high serum titers of different immunoglobulin subtypes. STIV antibodies opsonize *S.* Typhi and *S.* Paratyphi A to promote antibody-dependent cellular cytotoxicity and complement-mediated lysis. Immunization with rSTIV also induces robust cell-mediated immunity, including antigen-specific T cell proliferation and cytotoxic T lymphocyte response. Finally, mice immunized with rSTIV are significantly protected against *S.* Typhi and *S.* Paratyphi A challenge, with reduced visceral bacterial load. Our results underscore the potential of rSTIV as a novel vaccine candidate for enteric fever.

1. Introduction

Enteric fever refers to a systemic febrile illness, caused by *Salmonella enterica* serovar Typhi (*S.* Typhi) and *Salmonella enterica* serovars Paratyphi (*S.* Paratyphi A, B and C) infection of humans and remains a major public health threat to the developing countries. While *S.* Typhi causes 9.9–24.2 million new infections with disease manifestations and 75,000–208,000 deaths annually around the world (Lee et al., 2016; Mogasale et al., 2014; Buckle et al., 2012; Mogasale et al., 2016; Ali et al., 2009), *S.* Paratyphi infection has milder symptoms with an annual incidence of 5.4 million, as reported in the year 2000 (Crump et al., 2004). However, a recent study found significant mortality due to paratyphoid fever, which caused 13.7–56.3 thousand deaths in 2015 (Mortality GBD, Causes of Death C., 2016). Enteric fever accounts for ~21.6 million disability adjusted life years, reflecting high economic and public health impact (Crump et al., 2015). If left untreated, patients may develop severe complications like bacteraemia, hypotensive shock, perforation of the intestines, meningoencephalitis, multi-organ abscesses and osteomyelitis, leading to high (~20%) mortality (WHO, 2008). Chronic carriage of *S.* Typhi (reported in 3 ~ 5% of all infections) has been associated with higher risks for the development of gall bladder cancer (Gonzalez-Escobedo et al., 2011). Despite the availability of effective antibiotics, multidrug resistance (resistance to all the first line drugs like chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole), which was first reported in late 1980s (Rowe et al., 1997) frequently contributes to treatment failures (Zaki and Karande, 2011; Yan et al., 2016; Baltazar et al., 2015). Recent spread of fluoroquinolone resistant H58 strain of *S.* Typhi (Wirth, 2015) and the identification of an extensively drug resistant (XDR) H58 subclade have signalled public health emergency (Klemm et al., 2018). Incidence of multidrug resistance is also increasing for *S.* Paratyphi, both in the endemic regions and the developed countries (Sahastrabuddhe et al.,

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2013).

Enteric fever is spread by faecal contamination of food and water. Experience from the developed world suggests that improvement of personal and food hygiene, sanitation and access to clean water can effectively control the infection. However, this may not be achieved in the low income and lower-middle income countries in the near future, due to financial constraints and the lack of education. Instead, vaccination remains the most effective interim prevention strategy for these regions, not only to control infections, but also to check the emergence and spread of multidrug resistance (Crump et al., 2015).

Currently available, FDA-approved vaccines for typhoid fever include a live, attenuated strain of *S*. Typhi Ty21a for oral administration and a parenteral preparation, based on the outer polysaccharide coat (Vi polysaccharide or Vi-PS) of *S*. Typhi (Crump and Mintz, 2010). However, there are no recommended vaccines for children < 2 years of age, despite reports of increasing incidence of *S*. Typhi infection in this age group (Owais et al., 2010). Polysaccharide, being a T cell independent antigen fails to generate memory response. On the other hand, the live vaccine raises safety concerns, due to the previous reports of bacteraemia in IFN-γ- or T cell-deficient mice (Sinha et al., 1997; Hess et al., 1996). Although rapid seroconversion was observed in large proportions of the vaccinated individuals, protective antibody titers drop significantly after 2 years, leading to modest long term efficacy of the available vaccines (Crump et al., 2015). Currently, there is no vaccine available for *S.* Paratyphi infection (Martin et al., 2016). However, there are a few candidate vaccines in various stages of development.

To overcome the above limitations, several new approaches were adopted. Among them, typhoid conjugate vaccines (Vi-polysaccharide-protein conjugates) appear most promising. One such formulation, called Vi-TT (Vi-polysaccharide conjugated to tetanus toxoid) was recently pre-qualified by WHO and made available to the Indian consumers. Despite higher efficacy compared with the Vi-PS vaccine in limited human trials, significant induction of cell-mediated immune response by the conjugate vaccine that is critical for late clearance of intracellular *Salmonella* needs to be demonstrated (Arya and Agarwal, 2014; Shah, 2009). Inclusion of a non-*Salmonella* carrier molecule in Vi-TT precludes the generation of *Salmonella*-specific T cells (MacLennan, 2014). Additionally, this vaccine will be ineffective against Vi-negative *S.* Typhi strains, which are already circulating in south-east Asia (Ali et al., 2009; Baker et al., 2005). These strains, along with non-capsulated *S.* Paratyphi A and B may eventually replace the capsulated strains, if mass vaccination strategy with Vi or Vi based conjugate vaccine is adopted (Baker et al., 2005; Saha et al., 2000).

In addition, preparation of cost-effective conjugate vaccines with minimal batch variation is a challenge in itself (Szu, 2013). Contrastingly, recombinant protein vaccines derived from *Salmonella* proteins, which are conserved across different serovars, including *S*. Typhi and *S*. Paratyphi may overcome the existing challenges. These vaccines would be safer than the live vaccines, while being more immunogenic compared with the Vi-polysaccharide vaccine and may provide long term protection in younger children by elicitingcell-mediated immune response (CMIR) and memory response.

S. Typhi invasin (STIV) is a constitutively expressed outer membrane protein that is required for bacterial invasion of intestinal epithelial cells. As a result, *S*. Typhi devoid of STIV was significantly less pathogenic in a mouse model of infection (Chowdhury et al., 2015, 2018). Here, we report that an identical gene is present in *S*. Paratyphi serovars and the protein encoded by *STIV* is expressed by *S*. Paratyphi A and the clinical isolates of *S*. Typhi. Recombinant STIV induces strong humoral and cell-mediated immune response in mice, conferring protection against *S*. Typhi and *S*. Paratyphi A challenge.

2. Materials and methods

2.1. Cells and bacterial strains

EL4 cells (ATCC TIB-39 TM) were cultured in DMEM (GIBCO), supplemented with 10% horse serum (GIBCO). *S*. Typhi Ty2, a gift from J. Parkhill (Sanger Institute, UK) was cultured at 37°C in Luria Bertani broth (LB) or agar (LA) containing streptomycin (50 μg/ml). *S*. Paratyphi A (ATCC 9150) was maintained in Hektoen-Enteric agar (HEA) or LA broth (both from BD DIFCO) at 37°C. Details of the clinical *S*. Typhi strains used in this study (cultured in HEA) are described in Supplementary information.

2.2. Reagents

Vi-TT and Desferal were purchased from Bharat Biotech (Typbar-TCV) and Novartis India, respectively. Ferric chloride, guinea pig complement sera and other chemicals were purchased from Sigma, unless otherwise mentioned. Lactate Dehydrogenase (LDH) assay kit was from Takara Inc and IFN-γ ELISA kit was procured from eBiosciences, USA.

2.3. Recombinant protein expression and purification

Recombinant STIV (rSTIV) was expressed and purified as mentioned previously (Chowdhury et al., 2018). Briefly, chemically-competent *E. coli* BL21 (DE3) was transformed with pET28a-*STIV* plasmid. Transformants were grown overnight in Luria-Bertani broth and transferred to 200 ml of fresh medium at a ratio of 1: 400 (v/v). When OD_{600} of the bacterial culture reached 0.6, STIV expression was induced by treatment with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma) for 5 h. Bacteria were precipitated by centrifugation, resuspended in lysis/ binding buffer (6 M Guanidine Hydrochloride, 0.1 M Phosphate buffer, 10 mM Tris − HCl, 30 mM Imidazole, pH 8.0) and incubated for 2 h at room temperature for complete lysis. Lysates were cleared by centrifugation at 14,000 x *g* for 20 min. rSTIV was purified from the supernatants by affinity purification using Ni-NTA agarose columns (Qiagen). Agarose beads were washed (wash buffer contained 6 M Urea, 0.1 M Phosphate buffer, 10 mM Tris − HCl, 30 mM Imidazole, pH 6.5) and bead-bound proteins were eluted with a buffer containing 6 M Urea, 0.1 M Phosphate buffer, 10 mM Tris − HCl, 30 mM Imidazole, pH 4.0. The eluates were subjected to dialysis against a buffer with the composition of 0.1 M Phosphate buffer, 10 mM Tris − HCl, 20% Glycerol, 0.03% Triton-X100, pH 7.5 and 6 M urea. The buffer was replaced every 12 h with decreasing concentrations of urea to ensure proper folding. Molecular weight and purity of the protein was checked by resolving it in 12% SDS-PAGE (**Fig. S1**).

2.4. Western blot

Bacterial cultures were grown till OD_{600} reached ˜1.00. Equal culture volumes (1 ml each) of different experimental samples were centrifuged at 5000 x *g* and the pellets were lysed by 1x Lamelli buffer. Lysates were run in 12% SDS-PAGE and transferred to PVDF membranes. After blocking with 3% BSA for 1 h, membranes were incubated overnight at 4 °C with STIV antiserum (1:1000 dilutions). Membranes were washed (15 ml each) with TBS-T [Tris Buffered Saline pH 7.5, containing 0.1% Tween-20 (v/v)] for 5 times and incubated with HRP-conjugated anti-mouse IgG (1:2500 dilutions) for 1 h at room temperature. After 3 washes with TBS-T in an orbital shaker, membranes were developed by adding chemiluminescent substrates (SuperSignal West Pico, Thermo Fisher) and the signals were captured by autoradiography.

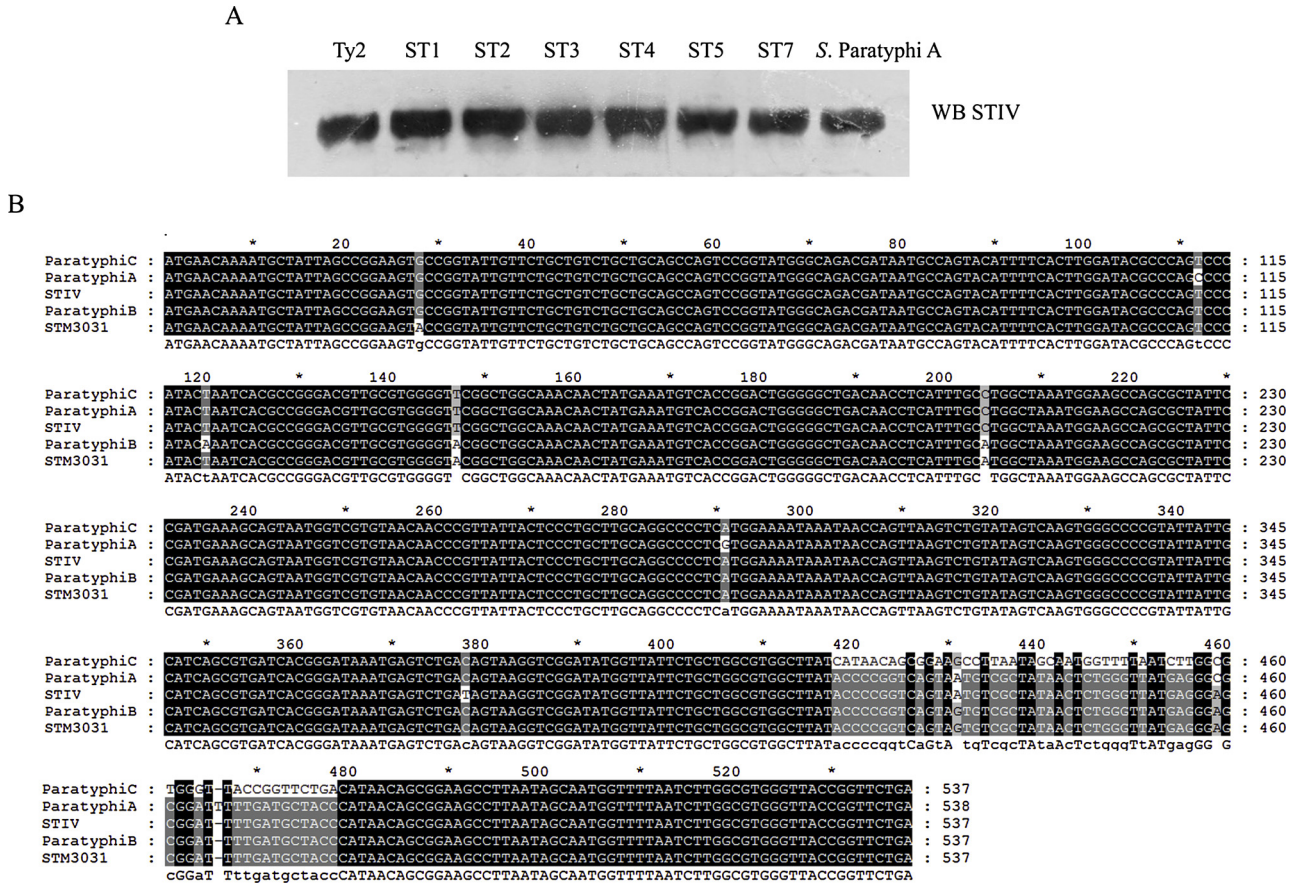

Fig. 1. STIV is expressed by clinical isolates of *Salmonella*. **A.** Bacterial lysates were prepared from the log phase cultures of *S*. Typhi reference strain and clinical isolates and *S*. Paratyphi A and the expression of STIV was checked by western blotting. The experiment was repeated 3 times and blot of a representative experiment is shown. **B.** Alignment of nucleotide sequence of *STIV* from *S.* Typhi Ty2, *S.* Paratyphi A, *S.* Paratyphi B, *S.* Paratyphi C and *S*. Typhimurium (*stm3031*).

2.5. Detection of STIV specific antibody titer by ELISA

96 well microtiter plates were coated with 100 µl of 10 ng/ml rSTIV (in 0.1 M Sodium carbonate buffer, pH 9.0) and incubated overnight at 4 °C. Wells were washed 3 times with 200 µl each of PBS and blocked by 1% BSA in phosphate buffered saline (PBS, pH 7.5) for 1 h at room temperature. Wells were then incubated with serially-diluted mouse anti-serum for 2 h, followed by three washes with PBS containing 0.1% Tween-20 (PBS-T). Wells were incubated with HRP-conjugated anti-mouse IgG for 1 h and again washed with PBS-T for three times. Wells were developed by adding o-phenylenediamine dihydrochloride (OPD) substrate (Sigma). The reaction was stopped after 15 min by adding 2 N sulphuric acid (Sigma) and the colour developed was measured by spectrophotometry (Shimadzu, Japan) at 492 nm.

To detect various immunoglobulin subtypes (IgA, IgG1, IgG2b, IgG3 and IgM), mouse anti-serum was added to the rSTIV-coated wells. Biotin-conjugated antibodies against the individual immunoglobulin subtypes (1:2500 dilution) were used as secondary antibodies followed by the addition of streptavidin-conjugated HRP. The plates were developed and read as described above. Absolute values of the immunoglobulin subtypes were calculated from the respective standard curves using GraphPad Prism V5.0.

2.6. IFN-γ ELISA

IFN-γ released in the culture supernatants of antigen-pulsed $CD8^+$ cells was measured by ELISA (eBiosciences) according to the manufacturer's instructions. Ninety six well plates were coated with IFN-γ capture antibody. After washing with PBS for three times, the wells were blocked with 1% BSA in PBS followed by incubation with the culture supernatants for 2 h. The wells were washed for three times with PBS-T and incubated with the detection antibody (1:2500). Colour development was monitored after addition of the TMB substrate and measured by spectrophotometry (Shimadzu, Japan) at 450 nm, after adding 2N sulphuric acid (Sigma) to stop the reaction.

2.7. Antibody dependent cellular cytotoxicity (ADCC) assay

ADCC assay was performed as described previously (Das et al., 2017). Briefly, 10^4 CFU of bacteria (target) were opsonized for 30 min at 4 °C with pre-immune serum or STIV antiserum. Mouse splenocytes were isolated by homogenising spleens and RBCs were lysed by a buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA followed by resuspension of the cells. NK cells (effectors) were isolated from the remaining cells resuspended in RPMI medium supplemented with 10% FBS using NK cell isolation kit (BD Biosciences, USA) and following the manufacturer's protocol. Briefly, cells were stained with PE-conjugated anti-CD49b antibody. After washing, anti-PE magnetic particles were added to the cells and NK cells were isolated by placing the cell-containing tubes on a magnet. Isolated NK cells were incubated with the opsonised bacteria at various target to effector (T: E) ratios in 96 well plates. After centrifugation (400 x *g*, 5 min) to synchronise the targets and the effectors, plates were incubated at 37 °C for 2 h. Dilutions of the reaction mixtures were spread on LB Agar plates containing streptomycin selection (50 µg/ml) for *S.* Typhi or HEA plates for *S.* Paratyphi A. Percentage antibacterial activity (index) was calculated by the following formula:

100 − 100[(CFU of experimental wells)/ (CFU of control wells

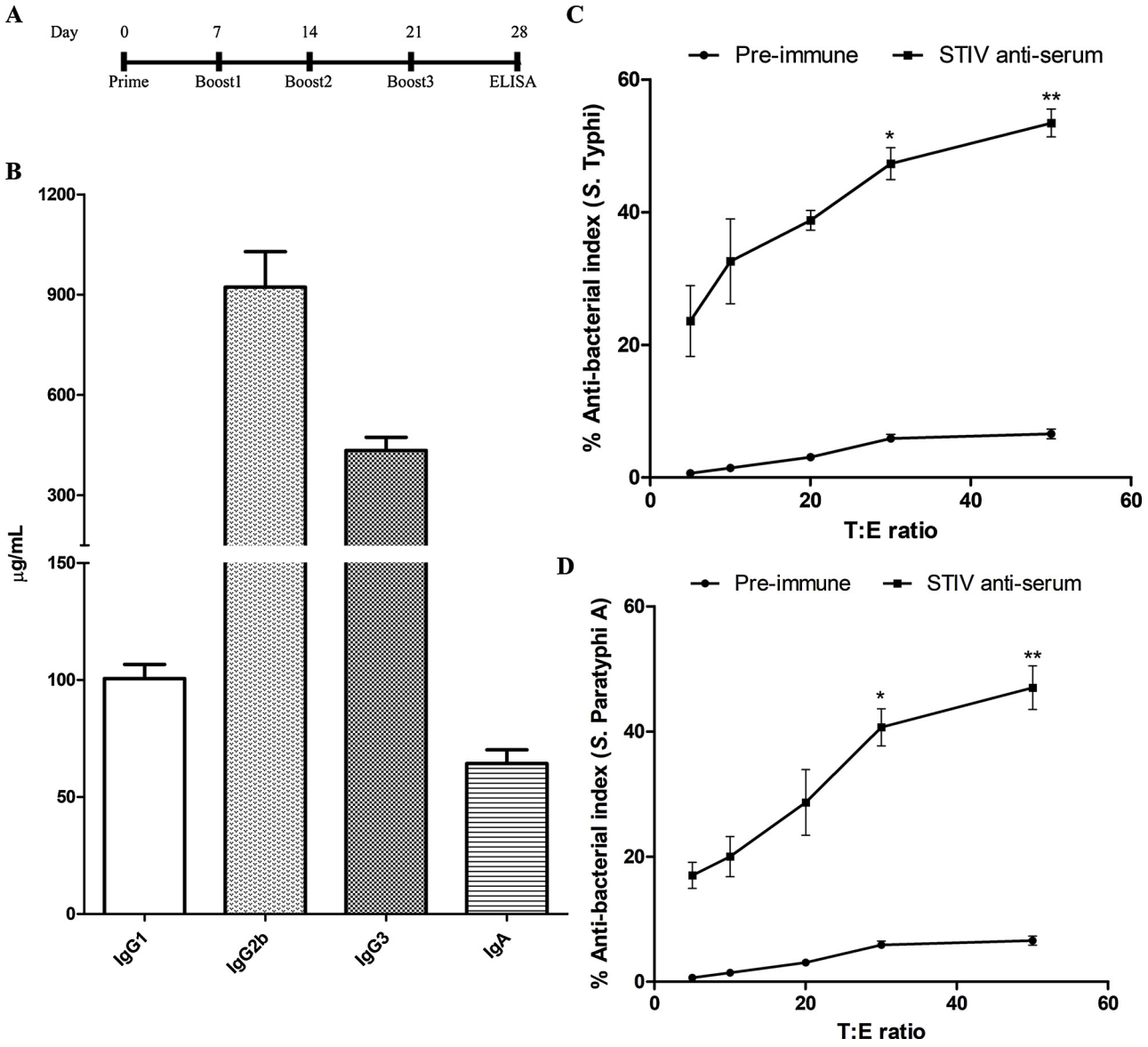

Fig. 2. STIV is immunogenic and induces humoral response. **A.** Summary of the immunization scheme. **B**. 6–8 weeks old BALB/c mice (n = 6/group) were immunized with rSTIV (5 µg) or PBS (control) for 4 weeks. Seven days after last immunization, mice were bled and serum was collected. Concentrations of STIV specific antibody isotypes IgG1, IgG2b, IgG3, and IgA in the antisera were measured by ELISA and the respective quantities were derived from the standard curves after negating respective values obtained in control immunized mice. **C-D**. ADCC assay. *S.* Typhi (**C**) or *S.* Paratyphi A (**D**) was incubated with STIV antiserum or pre-immune serum, followed by addition of NK cells from the spleens of control immunized mice. Antibacterial index was calculated as described in Materials and Methods. Significance was calculated by comparing antibacterial indices of rSTIV antiserum with pre-immune serum by Kruskal-Wallis test and Dunn's post-test comparison. Experiments were repeated 3 times and data from one representative experiment is shown.

without lymphocytes)].

2.8. Cytotoxic T lymphocyte (CTL) assay

For *in vitro* CTL assay, $CD8^+$ T lymphocytes were isolated from the spleens of immunized C57BL/6 mice using Anti-Mouse $CD8^+$ magnetic particles (BD Biosciences) according to the manufacturer's protocol. Isolated $CD8^+$ T cells were activated by culturing them with rSTIV-pulsed EL4 cells (EL4 cells incubated with 1 µg/ml rSTIV for 30 min) at the ratio of 20:1 (Hess et al., 1996). Viable T lymphocytes in the co-cultures were separated by ficoll density gradient and used as effector cells. These cells were incubated with the target cells (rSTIV pulsed EL4 cells) at various ratios in 96 well plates for 5 h at 37 °C. LDH activity of the cell supernatants was measured by LDH assay kit (Takara, Japan) and the percentage specific lysis was calculated by the following formula:

(ER – TS – ES)/ (MR – ES) X 100

ER represents experimental LDH release (activity in the supernatants of the target cells, incubated with the effector cells), TS represents spontaneous LDH release from the target cells (activity in the supernatants of the target cells alone), ES represents spontaneous release from the effector cells (activity in the supernatants of the effector cells alone) and MR represents maximum release from target cells (activity in the supernatants of the target cells lysed with 1% Triton-X 100).

2.9. BMDC isolation

BMDCs were generated as described earlier (Szu, 2013). Briefly, bone marrows were flushed from the femurs and tibia of 6–8 weeks old

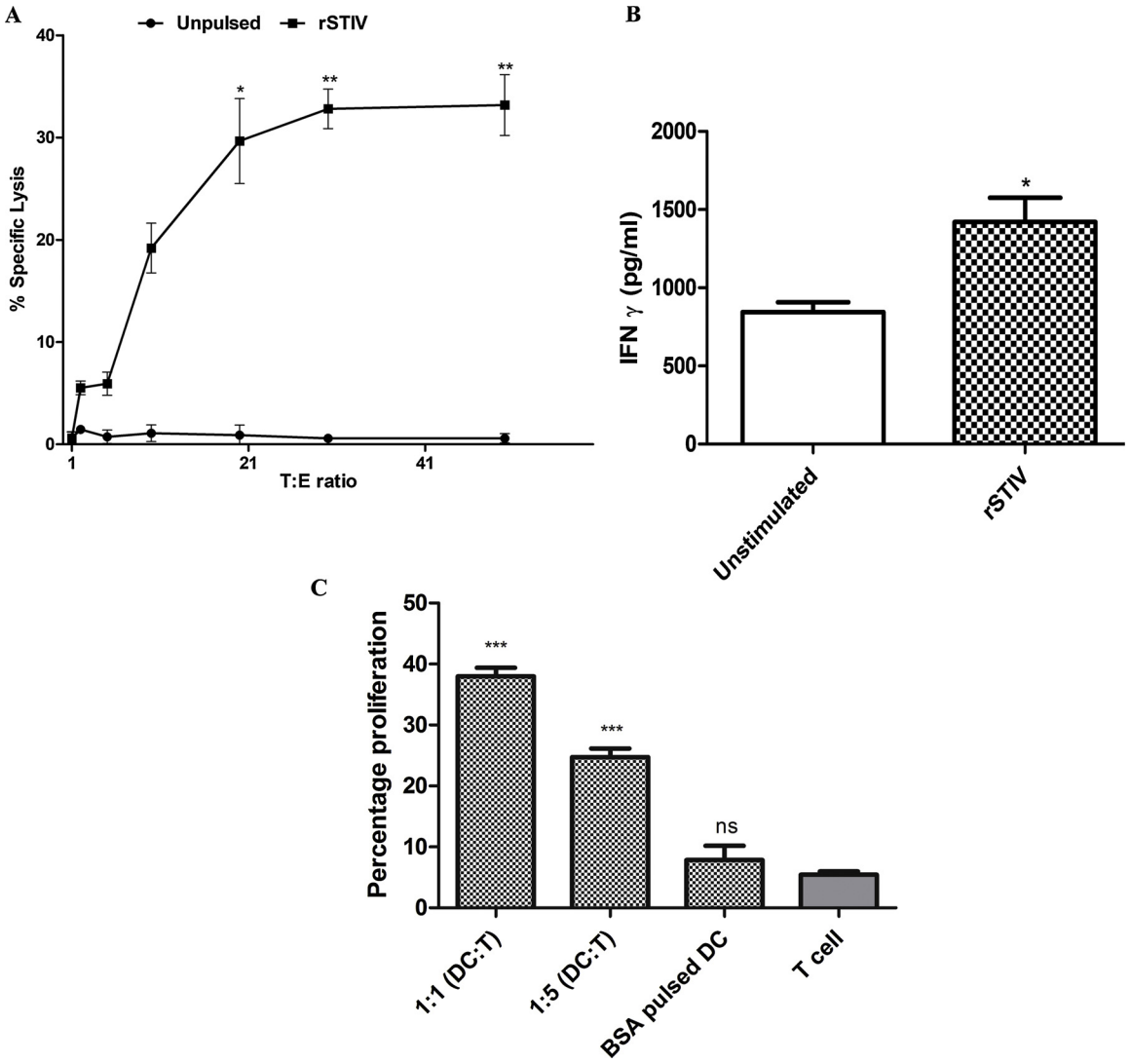

Fig. 3. STIV can induce cell mediated immune response. **A.** Cytotoxic T Lymphocyte Assay. $CD8^+$ T lymphocytes isolated from the spleen of mice immunized with rSTIV were used as effector cells. Target cells were generated by pulsing EL4 cells with rSTIV. Different ratios of target and effector cells were incubated and percent specfic lysis were calculated. Significance of lysis was calculated by comparing the percent specific lysis of the pulsed and unpulsed target cells using Kruskal-Wallis test followed by Dunn's post-test comparison. **B.** $CD8^+$ T cells isolated from the spleens of rSTIV immunized mice were co-cultured for 24 h with BMDCs pulsed with rSTIV. IFN-γ released in the culture supernatants was analysed by ELISA. Significance was calculated by comparing IFN-γ release by unstimulated and STIV stimulated $CD8^+$ cells by Kruskal-Wallis test followed by Dunn's post-test comparison. **C.** T cell proliferation assay. $CD4^+$ T cells isolated from the spleens of rSTIV immunized mice and labelled with CFSE were co-cultured with rSTIV-pulsed BMDCs. Proliferation of the T cells was quantified by CFSE dilution in the progenitor cells using flow cytometry. Significance was calculated by comparison with the unstimulated T cells (co-cultured with unpulsed DCs). Experiments were repeated 3 times and data from one representative experiment is shown.

BALB/c mice with RPMI media containing 10% FBS. Bone marrow cells collected were cultured in 90 mm tissue-culture dishes in complete RPMI media supplemented with 10% FBS and recombinant mouse GM-CSF (20 ng/ml). 60% of the culture medium was replaced every 3 days and the generation of immature dendritic cells on day 7 was confirmed by flow cytometry.

2.10. T cell proliferation assay

Mice were immunized with rSTIV as described previously. Animals were euthanized and $CD4^+$ T lymphocytes were isolated from the spleens using anti-$CD4^+$ magnetic beads (BD Biosciences). Cells were labelled with 5 μM CFSE [5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester] at 37 °C for 30 min in presence of 5% CO_2]. After washing, cells were co-cultured with antigen-pulsed BMDCs (BMDC's incubated with 1ug/ml rSTIV for 30 min) at various ratios for 5 days in 96 well tissue culture plates. Cells were harvested and T cell proliferation was studied by CFSE dilution in the progenitor cells (Das et al., 2017) using flow cytometry.

2.11. In vitro bactericidal assay

This assay was performed as described earlier (Ho et al., 2010) with modifications. Sera collected from the immunized mice were heat inactivated for 1 h in a 65 °C water bath. 10^6 CFU of bacteria from log-phase cultures (OD_{600} = 1.00) were opsonized by incubating them with various dilutions of the heat-inactivated sera containing 25% guinea pig complement in a 50 μL reaction for various durations. The reaction was stopped by adding Luria broth (950 μL) and various dilutions of the reaction mixtures were spread on Luria Agar plates containing streptomycin (50 μg/ml) for *S.* Typhi or on HEA plates for *S.* Paratyphi A. Reactions containing unopsonized bacteria were used as the control. Percent Lysis was quantified by the following formula:

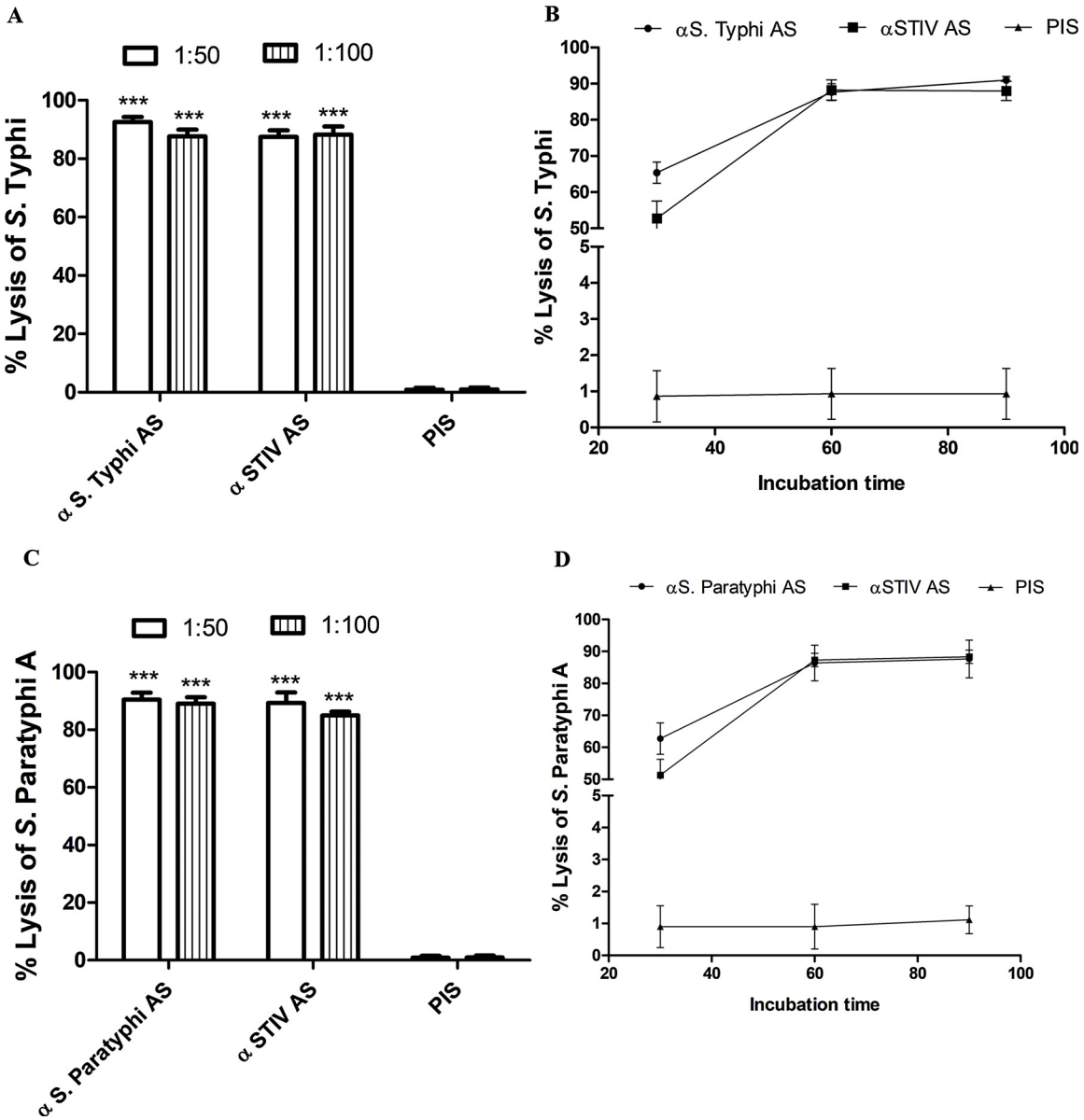

Fig. 4. STIV antibodies are capable of complement mediated lysis of *Salmonella*. *S.* Typhi or *S.* Paratyphi A was incubated with the indicated dilutions of heat-inactivated antiserum (AS) or pre-immune serum (PIS) in the presence of guinea pig complement for 2 h (**A, C**). In parallel experiments, fixed dilution of antiserum in the presence of guinea pig complement was incubated for different time points (**B, D**). Live bacteria were enumerated by plating on LB Agar with streptomycin selection (50 μg/ml; for *S.* Typhi) or HEA (*S.* Paratyphi A). Significance was calculated by comparing lysis by pre-immune and STIV antiserum by Kruskal-Wallis test with Dunn's post-test comparison. Experiments were repeated 3 times and data from one representative experiment is shown.

100 − 100[(CFU of experimental wells)/(CFU of control wells without antisera)]

2.12. Animal experiments

All animal experimental protocols and procedures were approved by the Institutional Animal Ethics Committee of National Institute of Cholera and Enteric Diseases, Kolkata. Animals were housed in negative pressure micro-isolator enclosures and 12-hour light and dark cycle was maintained. Temperature and relative humidity were maintained at 18–23 °C and 40–60%, respectively. Food and water was provided *ad libitum*.

2.13. Immunization of mice

Inbred, 6–8 weeks old BALB/c mice, weighing 18–25 g m were immunized as described previously (Das et al., 2017). Briefly, antigen was prepared by adding 2% Alhydrogel (Invivogen) to rSTIV at 1:1 (v/v) ratio. Mouse were immunized with 4 subcutaneous injections of rSTIV (5 μg) administered over the flank at intervals of 7 days. Equal doses of Vi-TT (Typbar TCV) [5 μg of Vi polysaccharide conjugated to tetanus toxoid] was used as the immunogen for separate groups of mice. The control groups were immunized with PBS mixed with 2% Alhydrogel.

2.14. Survival and bacterial load determination assay

An Iron overload mouse model of *S.* Typhi developed in the laboratory was used for intra-gastric infections as described previously (Ghosh et al., 2011). After immunization as per the protocol described above, different groups of BALB/c mice (Vi-TT immunized, rSTIV immunized and control) were injected intra-peritoneally with Desferal

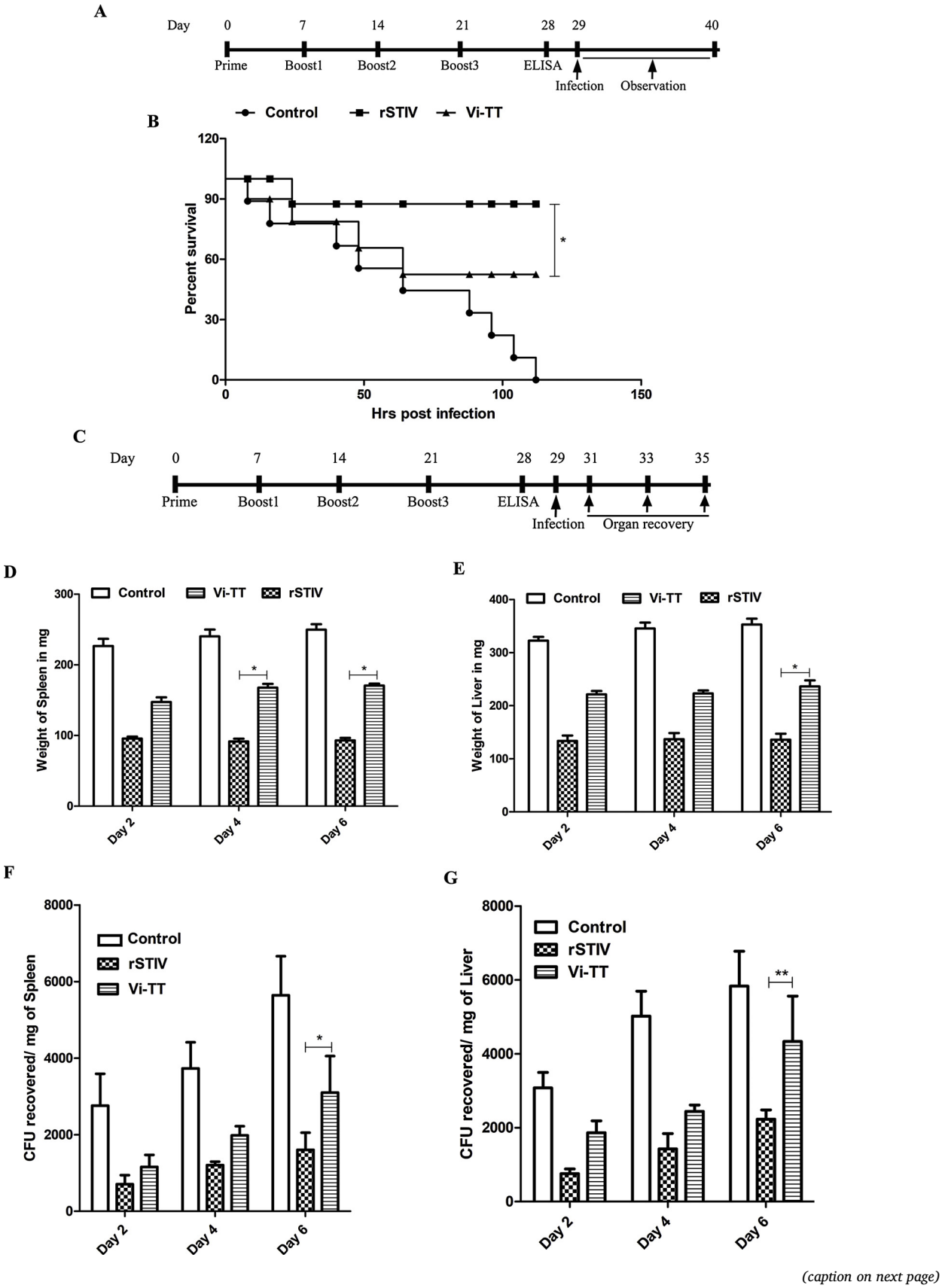

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Fig. 5. STIV immunization induces protection from *S*. Typhi challenge. **A.** Summary of the experimental scheme of survival assay. **B.** BALB/c mice (n = 10/group) were immunized with rSTIV or Vi-TT or PBS and challenged with 5×10^6 CFU of *S*. Typhi through intragastric route. Animals were monitored every 6 h. Significance was calculated by comparing survival of the mice immunized with rSTIV and Vi-TT by Log-Rank Mantel Cox test. **C.** Summary of experimental scheme of organ recovery. **D-E**. Immunized BALB/c mice (n = 9/group) were challenged with 10^4 CFU of *S*. Typhi Ty2. Mice were euthanized at the indicated time-points and weights of the visceral organ were measured. Significance was calculated by comparing organ weights of rSTIV and Vi-TT immunized mice by Kruskal Wallis test with Dunn's post-test comparison. **F-G**. Bacterial load in visceral organs were determined by spreading the organ homogenates on LB Agar plates with streptomycin selection (50 μg/ml). Significance was calculated by comparing CFU of bacteria from rSTIV and Vi-TT immunized mice by Kruskal Wallis test with Dunn's post-test comparison. Experiments were repeated 3 times and data from one representative experiment is shown.

(Novartis; 0.025 mg/gm body weight) and Ferric chloride (0.32 mg/gm body weight). Five hours later, mice were infected with *S*. Typhi or *S*. Paratyphi A through the intragastric route after neutralization of the gastric acids with 5% $NaHCO_3$ prior to bacterial challenge. A similar mouse model was used for infection as well.

For the survival assay, mice were challenged with the log phase cultures of 5×10^6 CFU ($10xLD_{50}$) of *S*. Typhi or 5×10^5 CFU ($10xLD_{50}$) of *S*. Paratyphi A. and monitored for clinical signs of infection (fever, reduced locomotion, piloerection), morbidity and mortality.

To determine organomegaly and visceral bacterial load, mice were infected with sub-lethal doses of *S*. Typhi (10^4 CFU) or *S*. Paratyphi A (10^3 CFU) as were previously optimised for maximal recovery of the bacteria from the visceral organs. Liver and Spleen were harvested on day 2, 4 and 6 post infection and weighed. Organs were homogenized and lysed with 1% Triton-X100. To enumerate the bacterial load, lysates were serially diluted and spread on Luria Agar plates containing streptomycin (50 μg/ml) for *S*. Typhi or HEA plates for *S*. Paratyphi A.

2.15. Statistical analysis

Data were analyzed using GraphPad Prism Version 5. Statistical significance was calculated using Kruskal Wallis test with Dunn's post-test comparison unless otherwise mentioned (One way analysis of variance; non-parametric method). The difference were considered significant at $p < 0.05$. *, **, *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. Data represents mean +/- SD values for individual experiments.

3. Results

3.1. STIV is expressed by clinical Salmonella isolates

STIV was previously reported as constitutively expressed and encoded by a chromosomal gene of *S*. Typhi Ty2 strain. STIV expression was independent of environmental stimulations, such as pH and temperature changes (Chowdhury et al., 2015). To investigate if STIV was conserved in the *S*. Paratyphi serovar and the circulating *S*. Typhi isolates, we performed western blots with the bacterial lysates using STIV anti-serum. The results showed STIV expression by all the stains tested (Fig. 1A, Table S1). *S*equence homology searches by nBLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that *S*. Paratyphi A, B and C genomes also encoded genes identical to *STIV* (99% identity and 100% sequence coverage). Multiple sequence alignment performed by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) is shown in Fig. 1**B**. Given wide distribution of STIV in the typhoidal serovars of *Salmonella*, including the clinical strains and constitutive expression of the protein in the bacterial outer membrane, we hypothesized that STIV could be a potential candidate for vaccine development against typhoid and paratyphoid fever.

3.2. STIV is immunogenic and induces humoral immune response

Earlier studied reported that the magnitude of the humoral immune response against *S*. Typhi correlates with protection (MacLennan, 2014). To check for the induction of protective antibody response by STIV, BALB/c mice were immunized with rSTIV or PBS (control) in a prime-boost regimen over 4 weeks, as described under the Material and Methods (schematised in Fig. 2A). STIV-specific IgG and IgA antibodies were detected in the immunized mice sera, with the maximum induction observed for IgG2b (922.66 μg/ml), followed by IgG3 (434 μg/ml) (Fig. 2B).

Mouse IgG2 antibodies have higher efficacy than the other antibody isotypes to promote antibody-dependent cellular cytotoxicity (ADCC) (Overdijk et al., 2012). We checked if STIV antiserum that contained high titers of IgG2b antibody isotype was capable to opsonize *S*. Typhi and *S*. Paratyphi A for their lysis by ADCC. To this end, target *S*. Typhi or *S*. Paratyphi A strain, pre-opsonized with pre-immune serum or STIV antiserum was incubated with naïve NK cells, isolated from the mouse spleen and used as the effectors. We found progressively increasing lysis of the opsonised bacteria with rising effector to target ratios. Nearly 50% of the bacteria were lysed when the effector cell number was 50-folds higher than the target (Fig. 2**C-D**). Opsonisation with the pre-immune serum resulted in negligible lysis, indicating specificity of the antibody effects. These results indicated that immunization with rSTIV induces opsonic antibodies, which promote bacterial clearance by ADCC.

3.3. STIV induces cell-mediated immune response

Salmonella Typhi and Paratyphi are intracellular pathogens, which survive and replicate inside the phagosomal vacuoles of host macrophages. CMIR is considered to be essential for complete cure of intracellular bacterial infections (Kirimanjeswara et al., 2008). To study if STIV immunization mounts CMIR, $CD8^+$ T cells (Cytotoxic T Lymphocytes or CTLs) isolated from the spleens of the immunized mice were expanded *in vitro* by antigenic stimulation and co-cultured with the unpulsed or rSTIV-pulsed EL4 cells (target cells). Lysis of the antigen-presenting target cells, as measured by LDH release assay showed 35% specific lysis of rSTIV-pulsed EL4 cells, as opposed to negligible lysis of the unpulsed cells **(**Fig. 3A**),** suggesting a strong CTL response.

To further characterize the above response induced by STIV, splenic $CD8^+$ T cells from the immunized mice were stimulated *in vitro* by co-culturing with rSTIV-pulsed BMDCs and the amounts of IFN-γ released in the culture supernatants were quantified by ELISA. We found significant increase in the IFN-γ levels in rSTIV-stimulated compared with the un-stimulated $CD8^+$ CTLs (Fig. 3B).

$CD4^+$ T cell proliferation is considered a hallmark of CMIR (Luckheeram et al., 2012). To study $CD4^+$ T cell response to immunization with rSTIV, cells separated from the splenocytes of immunized mice using the magnetic beads were labelled with CFSE and co-cultured with rSTIV-pulsed BMDCs. Cell proliferation was analysed by CFSE dilution in the progenitor $CD4^+$ T cells using flow cytometry. The results showed 37.96% proliferation of T cells when mixed with BMDCs at the ratio of 1:1 (Fig. 3C). This was reduced to 24.7% when 5-folds less BMDCs than $CD4^+$ T cells were present. Unstimulated T cells and cells co-cultured with BSA-pulsed BMDCs displayed minimal proliferation. Together the above data suggested that immunization with rSTIV induced robust cell-mediated immune response.

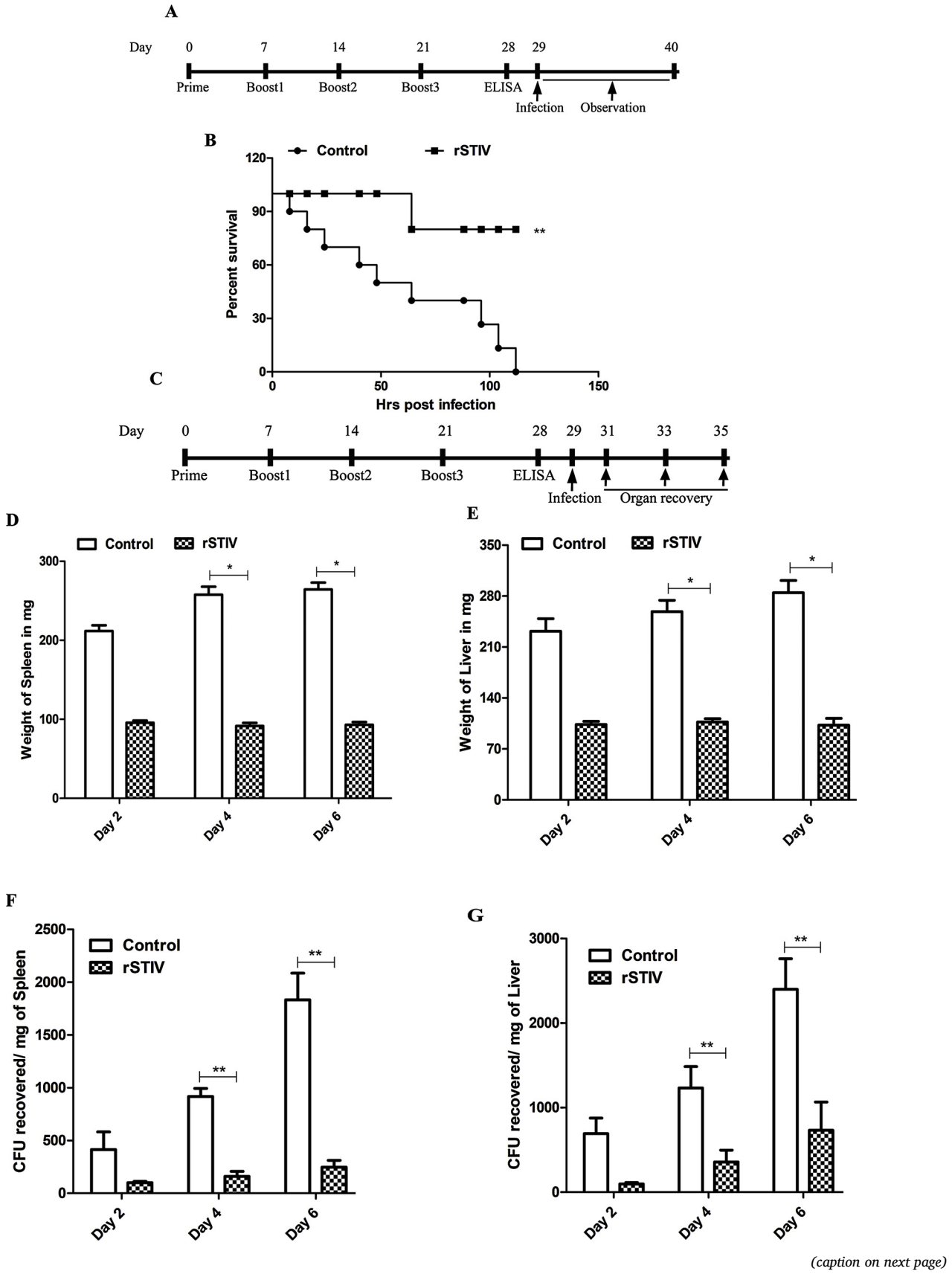

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Fig. 6. STIV immunization provides protection against *S.* Paratyphi challenge. **A.** Summary of experimental scheme of survival assay. **B.** Groups of BALB/c mice (n = 10/group) were immunized with rSTIV or PBS and challenged with 5×10^5 CFU of *S.* Paratyphi A. Animals were observed every 6 h and Significance was calculated by comparing survival of control immunized and rSTIV immunized mice by Log-Rank Mantel Cox test.
C. Summary of experimental scheme of organ recovery. **D-E.** BALB/c mice (n = 9/ group) were immunized with 10^3 CFU of *S.* Paratyphi A. Mice were euthanized at the indicated time-points and visceral organ were weighed. Significance was calculated by comparing organ weights of control immunized and rSTIV immunized mice by Kruskal Wallis test with Dunn's post-test comparison. **F-G.** Bacterial load in the visceral organs were determined by spreading the organ homogenates on HEA plates. Significance was calculated by comparing CFU of bacteria from control immunized and rSTIV immunized mice by Kruskal Wallis test with Dunn's post-test comparison. Experiments were repeated 3 times and data from one representative experiment is shown.

3.4. STIV antibodies are capable of complement-mediated lysis of Salmonella

Complement activation in the circulation is a critical component of the innate arm of antibacterial immunity. Given high levels of induction of IgG3 isotype following immunization with STIV, we checked for complement activation by mouse immune sera. To this end, *S.* Typhi and *S.* Paratyphi A were incubated with the heat inactivated pre-immune or immune serum, along with guinea pig complement. Significant lysis of *S.* Typhi opsonized with the immune serum, which was comparable to the lysis induced by commercial *S.* Typhi antiserum was observed, as opposed to minimal lysis in the presence of pre-immune serum (Fig. 4**A-B**). Bactericidal effects of 1:100 dilution of STIV antiserum on *S.* Paratyphi A reached almost 90% (Fig. 4**C**) and was higher with longer incubation periods (Fig. 4D). The above results suggested strong complement activation after immunization with rSTIV, which might contribute to anti-bacterial immunity.

3.5. STIV immunization induces protection against S. Typhi challenge

To investigate the protective efficacy of rSTIV as a candidate vaccine, we challenged the immunized mice with 10xLD_{50} dose (5×10^6 CFU) of *S.* Typhi Ty2 strain. Bacteria were administered by the intragastric route following iron overload, as described under Materials and Methods, one week after the last dose of immunization (Fig. 5A). Vi-TT and PBS were used as comparator vaccine and control, respectively in this experiment. All mice in the control group succumbed to infection within 7 days, while < 60% survival was noted after immunization with Vi-TT. In contrast, the survival percentage (89%) was higher for the rSTIV-immunized mice (Fig. 5B). This suggested strong protective efficacy of STIV against *S.* Typhi challenge in mice.

To investigate if the protection of the animals in the above experiment was due to reduced bacterial load in the visceral organs, mice were infected with a sub-lethal dose of *S.* Typhi (10^4 CFU). Immunized mice suffered from less severe hepatosplenomegaly compared with the control group, suggesting lower visceral bacterial load in the former. However, least organomegaly was observed in the group of mice that received rSTIV (Fig. 5**D-E**), suggesting that systemic infection was perhaps the lowest in this group. This was supported by bacterial recovery from the visceral organs, which showed significantly fewer live bacteria from the spleen and liver of the rSTIV-immunized mice compared with the un-immunized and Vi-TT-immunized mice (Fig. 5**F-G**). Together these results suggested that rSTIV conferred significant protection of mice against *S.* Typhi challenge.

3.6. STIV immunization provides protection against S. Paratyphi A challenge

To check if immunization with rSTIV was also protective against *S.* Paratyphi A infection, we challenged the immunized mice with 10xLD_{50} dose (5×10^5 CFU) of *S.* Paratyphi A (Fig. 6**A**). Eighty percent of the immunized mice survived the bacterial challenge, while all the animals in the control group succumbed to infection (Fig. 6B). As observed earlier with *S.* Typhi (Fig. 5**D-E**), organomegaly (Fig. 6**C-D**) and visceral bacterial load (Fig. 6**E-G**) after sub-lethal infection with *S.* Paratyphi A was less for mice immunized with rSTIV compared with Vi-TT. Together the above results suggested that rSTIV protects against *S.* Typhi and *S.* Paratyphi A infections.

4. Discussion

Intracellular pathogen *Salmonella* survives and replicates inside the phagosomes of host macrophages (Ernst et al., 1999). As humans are the only reservoir for *S.* Typhi and *S.* Paratyphi, a successful vaccination strategy will limit the spread of infection and decrease the burden of infection in the endemic areas. Although serum antibody titer is generally considered a good indicator of protection, no such correlation was found in some studies (Maitta et al., 2004; Patarroyo et al., 2006; Hess et al., 2013). Natural infection with *Salmonella* sp induces serum IgM and IgG response, but fails to provide long term protection (Sarasombath et al., 1987). Entry through the intestinal mucosa and intracellular life of *Salmonella* suggest that intestinal sIgA and CMIR would be important attributes to a highly effective vaccine. However, Vi-based vaccines do not induce gut homing IgA secreting cells (Kantele et al., 2013), while the live Ty21a vaccine fails to provide better protection despite the generation of sIgA secreting cells and CMIR (Fraser et al., 2007). As of today, actual correlates of protection following immunization against enteric fever remain unknown.

The new generation typhoid vaccines are aimed at ensuring greater protection and better safety profiles over the existing ones. Vi-rEPA conjugate vaccine demonstrated high efficacy in children who belong to the age group of 2–5 years (Szu et al., 2014; Lin et al., 2001; Parry et al., 2002; Thiem et al., 2011; Canh et al., 2004), but safety concerns related to EPA precluded its licensing so far. Vi-TT is the most recently introduced typhoid conjugate vaccine with significantly higher seroconversion rates that Vi-TT (Voysey and Pollard, 2018; Mohan et al., 2015). However, the only efficacy trial was a single cluster-randomized study conducted in India in children aged 6 months to 12 years, which was described as a very low-certainty evidence (Milligan et al., 2018). Several *Salmonella* antigens, such as flagella, SseB and OmpD have been used as vaccine candidates. However, they are poorly conserved across the serovars and confer only partial immunity in mouse typhoid fever model (McSorley et al., 2000; Gil-Cruz et al., 2009; Rollenhagen et al., 2004). Consequently, a recent study concluded that immunization with OmpC, OmpD and OmpF is not protective against *S.* Typhi (Barat et al., 2012). In contrast, STIV is a highly conserved gene that is widely distributed among *Salmonella* serovars, including *S.* Typhi and *S.* Paratyphi strains. This underscores the potential of STIV as a successful vaccine candidate. We have shown that STIV activates both the innate and adaptive arms of the host immune system and provides significant protection against *S.* Typhi and *S.* Paratyphi A challenge in mouse models of infection. Being a recombinant protein-based subunit vaccine, it would be inexpensive to manufacture and safer than the live vaccine for younger children and immunocompromised individuals.

Immunization with rSTIV generates antigen-specific antibody isotypes in mice. High levels of IgG1, IgG2b, IgG3 and IgA were detected by ELISA in the mouse antisera. Mouse IgG2 has similar activities as human IgG1 and promotes ADCC and immune effector functions (Overdijk et al., 2012). High IgG2b levels in STIV antiserum might have contributed to efficient lysis of the antibody-opsonised *S.* Typhi and *S.* Paratyphi A by ADCC. ADCC induced by rSTIV was comparable to that of Ty21a antisera (Tagliabue et al., 1986). On the other hand, immune

effector functions of mouse IgG2 is mediated by complement activation through the classical pathways (Seino et al., 1993). Thus, STIV immunization not only induced strong systemic antibody response, but the antibodies were protective in nature in the *in vitro* experiments. The extent to which this contributes to the actual protection of the host against *Salmonella* infection remains to be determined. Given the transient nature of the freely circulating bacteria during *Salmonella* infection and rapid lysis of *S*. Typhi by serum (Sztein et al., 2014), antibodies may function as a surrogate marker, rather than conferring actual protection. However, protective serum antibodies may be crucial for individuals with T cell deficiency and under conditions of significant bacteraemia during the late stages of the disease.

It is widely believed that a functional CMIR is essential to achieve a high level of protection against *S*. Typhi and *S*. Paratyphi. CMIR is indispensable for clearance of intracellular bacteria and complete resolution of the disease (Pham and McSorley, 2015). However, Vi-PS vaccine does not generate a memory T cell response (MacLennan, 2014). Ty21a vaccine, on the other hand, encounters the mucosal tolerogenic environment that precludes the induction of a strong immune response. Vi-conjugate vaccines efficiently convert the polysaccharide to a T cell-dependent antigen and are expected to mount CMIR and memory response (Mitra et al., 2016). However, these vaccines would still perhaps work by augmenting the levels of anti-Vi antibodies compared with the unconjugated Vi-PS vaccine. Due to the absence of intrinsic *Salmonella* proteins in the vaccine formulations, no *Salmonella*-specific CTL response will be generated by the conjugated vaccines that use non-*Salmonella* proteins as carrier molecules. However, earlier studies reported that CTLs that lyse *S*. Typhimurium-infected cells contribute to protection from mouse infection (Bumann, 2014; Lo et al., 1999). As reported earlier with Ty21a vaccine, we observed the elicitation of pathogen-specific $CD8^+$ CTLs following rSTIV immunization. CTLs respond to viral and bacterial infections by recognizing the pathogen-derived peptides, presented by the major histocompatibility complex class I molecules on the surface of the antigen presenting cells (Germain, 1994). CTL mediated lysis of the infected macrophage cells will release intracellular *S*. Typhi and *S*. Paratyphi from the protected cellular niche and render them vulnerable to destruction by soluble immune effectors, such as opsonic antibodies that will promote ADCC and the complement as well as phagocytosis and neutralization by activated macrophages. Re-stimulation of CTLs from the STIV-immunized mice induced IFN-γ secretion, which is known to activate macrophages, leading to increased bacterial killing and antigen presentation (Vazquez-Torres and Fang, 2001).

$CD4^+$ T cells from the rSTIV-immunized mice displayed enhanced proliferation upon antigen-specific stimulation. These cells will activate macrophages and CTLs on one hand and induce B cells to produce antibodies on the other. This would further enhance the protective efficacy of the vaccine.

Overall, the evidence presented here suggests that the spectrum of protection imparted by rSTIV subunit vaccine will not only be wider but also more efficacious than the Vi-PS based vaccines, including Vi-TT. This is further proved by lower bacterial load of the visceral organs and better protection of the immunized mice against *S*. Typhi and *S*. Paratyphi A challenge compared with Vi-TT immunization. Our study indicates that STIV may serve as an excellent vaccine candidate against the pathogens causing enteric fever.

Animal ethics statement

All experiments were approved by the Institutional Animal Ethics committee and carried out following guidelines set by the committee.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.02.011.

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