

## RESEARCH ARTICLE

# Co-administration of rIpaB domain of *Shigella* with rGroEL of *S. Typhi* enhances the immune responses and protective efficacy against *Shigella* infection

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*Shigella* species cause severe bacillary dysentery in humans and are associated with high morbidity and mortality. The Invasion plasmid antigen (IpaB) protein, which is conserved across all *Shigella* spp., induces macrophage cell death and is required to invade host cells. The present study evaluates the immunogenicity and protective efficacy of the recombinant (r) domain region of IpaB (rIpaB) of *S. flexneri*. rIpaB was administered either alone or was co-administered with the rGroEL (heat shock protein 60) protein from *S. Typhi* as an adjuvant in a mouse model of intranasal immunization. The IpaB domain region (37 kDa) of *S. flexneri* was amplified from an invasion plasmid, cloned, expressed in BL21 *Escherichia coli* cells and purified. Immunization with the rIpaB domain alone stimulated both humoral and cell-mediated immune responses. Furthermore, robust antibody (IgG, IgA) and T-cell responses were induced when the rIpaB domain was co-administered with rGroEL. Antibody isotyping revealed higher IgG1 and IgG2a antibody titers and increased interferon-gamma (IFN- $\gamma$ ) secretion in the co-administered group. Immunization of mice with the rIpaB domain alone protected 60%–70% of the mice from lethal infection by *S. flexneri*, *S. boydii* and *S. sonnei*, whereas co-administration with rGroEL increased the protective efficacy to 80%–85%. Organ burden and histopathological studies also revealed a significant reduction in lung infection in the co-immunized mice compared with mice immunized with the rIpaB domain alone. This study emphasizes that the co-administration of the rIpaB domain and rGroEL protein improves immune responses in mice and increases protective efficacy against *Shigella* infection. This is also the first report to evaluate the potential of the GroEL (Hsp 60) protein of *S. Typhi* as an adjuvant molecule, thereby overcoming the need for commercial adjuvants.

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## INTRODUCTION

Shigellosis or bacillary dysentery is an acute diarrheal disease caused by the Gram-negative bacillus *Shigella*. It is a major health problem worldwide, with more than 163 million cases reported annually.<sup>1</sup> *Shigella* spp. invade and destroy the host intestinal epithelium, leading to watery diarrhea that contains blood and mucus.<sup>2–4</sup> The genus *Shigella* includes four species, namely, *Shigella dysenteriae* (13 serotypes), *Shigella flexneri* (15 serotypes), *Shigella boydii* (18 serotypes) and *Shigella sonnei* (1 serotype).<sup>1</sup> The emergence of antibiotic resistance and the presence of multiple serotypes emphasize the need for a safe and effective vaccine against *Shigella* that is protective across all of

the serotypes. To the best of our knowledge, there is no available vaccine against *Shigella*, and only a few vaccines are under development at different phases of clinical trials.<sup>5</sup>

All *Shigella* spp. contain a large 220 kb virulence plasmid for invasion. The virulence plasmid encodes a 30 kb Mxi-Spa type III secretion system (T3SS). The T3SS includes a needle tip that acts as a molecular syringe and is comprised of several effector proteins. Of these effector proteins, the invasion plasmid antigens (IpaA, IpaB, IpaC and IpaD) have been reported to be essential for the invasion of epithelial cells and for the release of the bacteria into the host cell cytosol.<sup>3,6,7</sup> These Ipa proteins are reported to be the dominant antigens responsible for inducing

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humoral immune responses in the convalescent sera of infected monkeys and humans.<sup>8</sup> The IpaB molecule is conserved in all pathogenic *Shigella* spp. and plays a major role in the bacterial invasion of epithelial cells.<sup>9</sup> In particular, IpaB performs unique functional roles within the T3SS during pathogenesis. For example, IpaB induces apoptosis in host macrophages and dendritic cells, which leads to inflammation and forms a translocon complex that is inserted as a pore into the targeted host cell membranes to direct the entry of other effector proteins into the host cell cytoplasm.<sup>3,10–13</sup> Recent studies in a mouse lung model have also indicated the immunogenicity and protective efficacy of the invasion complex (IpaB, IpaC and lipopolysaccharide) of *Shigella* against *Shigella* infections.<sup>14</sup> The interaction between IpaB and IpaD at the needle tip mediates host cell sensing and allows translocon insertion into the host cell membrane.<sup>15–20</sup> Recently, the crystal structure of a protease-stable fragment that was identified within the N-terminal region of IpaB from *S. flexneri* was presented; this fragment has substantial similarity to the coiled-coil regions of pore-forming proteins from other Gram-negative pathogens.<sup>15</sup> Early studies predicted that the domain regions of IpaB were necessary for invasion, phagosome escape, caspase-1 binding and cytotoxicity.<sup>21–26</sup> In the IpaB domain, amino acids (aa) 44–310 have been reported to contain the regions necessary for the process of invasion. Among aa 44–310 of the IpaB domain, aa 51–72 contain the cognate chaperone invasion plasmid gene C binding site which provides stability to the IpaB protein and prevents its degradation in the cytoplasm; aa 75–310 contain the region responsible for cytotoxicity, invasion, lysing phagosome and macrophage death.<sup>15–19</sup> Therefore, in this study, the IpaB domain region (44–310 aa) was explored for its immunogenicity and protective efficacy against *Shigella* infection.

Heat shock proteins (HSPs) are evolutionarily conserved intracellular molecules that are found in all forms of life. They function as molecular chaperones in numerous processes, such as protein folding and transport, and are induced under stressful conditions. They are potent inducers of the immune system, inducing both innate and adaptive immune responses.<sup>27,28</sup> Many microbial HSPs are reported to be dominant antigens that elicit host immune responses to a variety of pathogens<sup>29–34</sup> and act as effective adjuvants.<sup>35</sup> Previously, we developed recombinant (r) Hsp 60 (GroEL) and rHsp70 (DnaK) from *Salmonella enterica* serovar Typhi, and we reported that these candidate vaccine molecules stimulate both arms of immunity against *S. Typhi* and *S. typhimurium* infection in mice.<sup>36–39</sup> HSPs share high sequence homology among various spp., and we have previously reported the *in vitro* and *in vivo* cross-protective efficacy of rGroEL of *S. Typhi* against multiple bacterial pathogens.<sup>40</sup>

With this background, the present study was designed to evaluate the immunogenicity and protective efficacy of the rIpaB domain of *Shigella* alone (without any adjuvant) and to investigate the adjuvant effect of rGroEL of *S. Typhi* when co-administered with the rIpaB domain in mice against infection with *S. flexneri*, *S. boydii* and *S. sonnei*. Because IpaB is conserved in all serotypes of *Shigella* spp., it would be a potent

cross-protective antigen for the development of a safe and effective vaccine against all *Shigella* serotypes.

## MATERIALS AND METHODS

### Animals

Four- to six-week-old female BALB/c mice were used in all experiments. All of the animals were reared and housed in the Experimental Animal Facility, Defence Institute for Physiology and Allied Sciences, Delhi, India, under standard laboratory conditions. Food and sterile water were provided *ad libitum*. All animal protocols were approved by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Government of India, and were carried out according to the guidelines of the Institutional Ethical Committee.

### Growth conditions and maintenance of bacterial strains and vectors

*Shigella flexneri* 2a, *Shigella boydii* and *Shigella sonnei* were clinically isolated pathogens collected from the All India Institute for Medical Sciences, New Delhi, India. All *Shigella* strains were grown in Tryptic Soy Broth (Difco, Sparks, MD, USA) at 37 °C. Virulent plasmid bearing *Shigella* colonies were isolated by growing the strains regularly in Tryptic Soy Agar with 0.02% Congo red. *Escherichia coli* DH5 $\alpha$  cells were maintained in LB medium, *E. coli* BL21 (DE3) pLysS cells were maintained in LB medium with 35  $\mu$ g/ml chloramphenicol and pRSET A vector was maintained in LB medium with 50  $\mu$ g/ml ampicillin at 37 °C.

Plasmid DNA was isolated using GenElute Plasmid DNA Isolation kits according to the manufacturer's instructions (Sigma, St. Louis, MO, USA), and isolated plasmid DNA was stored at –20 °C.

### Cloning, expression and purification of recombinant the IpaB domain region of *S. flexneri*

The 220 kb invasion plasmid of *S. flexneri* was isolated and used as a template to amplify the 801 bp domain region of IpaB using the following IpaB primers:

IpaB forward primer: 5'-agttctc**gagg**atcttactgctaacaaaat-3'

IpaB reverse primer: 3'-gg**tactgcaga**acacaaccattactctgt-5'

The primers were designed such that the amplified gene contained XhoI and PstI restriction sites at the 5' and 3' ends, respectively. The PCR reaction was carried out using the following conditions: 95 °C for 5 min (initial denaturation), 95 °C for 1 min (denaturation), 57 °C for 1 min (annealing) and 72 °C for 2 min (extension). This cycle was repeated 37 times, followed by a final extension step of 72 °C for 10 min. The 801-bp amplified PCR product was digested with XhoI and PstI, gel eluted and purified. The digested product was then ligated into the expression vector pRSET A, which was further digested and purified with XhoI and PstI. The 801-bp IpaB domain was in frame with histidine<sub>6</sub> tag sequence at the N-terminal end. The ligated, cloned product was transformed into DH5 $\alpha$  cells to screen for the recombinants by CaCl<sub>2</sub> transformation. The presence of the cloned product was confirmed by colony

PCR and double digestion. The recombinant pRSET A IpaB was finally transformed into *E. coli* BL21 pLysS cells.

Transformed *E. coli* BL21 pLysS cells were inoculated into LB broth (500 ml) and incubated until the OD<sub>600</sub> reached 0.5. Then, the cells were induced with 1 mM isopropylthiogalactoside (IPTG) for 4 h at 37 °C, followed by centrifugation at 5000g. The resulting pellet was suspended in lysis buffer containing 8 M urea, 200 mM NaCl, 2 mM imidazole and 50 mM Tris-Cl, and the pellet was sonicated using a sonicator (Vibra, Sonics and Materials Inc, CT, USA) under cold conditions. The sonicated sample was purified by NiNTA slurry according to the manufacturer's instructions (Qiagen, Hilden, Germany). The recombinant protein was eluted from the NiNTA slurry using elution buffer with 0.5 M imidazole. The purification of recombinant protein through Ni-NTA yielded a highly purified protein without any bacterial contaminants. The purified protein was refolded *in vitro* using 1 M arginine at 4 °C. Using a dialysis membrane, the recombinant protein was dialyzed in dialysis buffer containing 50 mM Tris and 1 mM EDTA for 48 h. The dialysis buffer was changed every 6 h to remove the urea and imidazole residues. The purified and dialyzed recombinant protein was concentrated using Amicon filtration columns (Millipore, Billerica, MA, USA). The purity of the protein was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The expressed rIpaB domain protein was confirmed by western blotting using an IpaB antibody (Santacruz, CA, USA). LPS content in the purified protein was found to be negligible (<1 EU/mg protein) as determined by limulus amoebocyte lysate (Sigma).

### Expression and purification of recombinant GroEL protein of *S. Typhi*

The *S. Typhi* GroEL gene was cloned, expressed and purified previously in our laboratory. Briefly, transformed *E. coli* BL21 cells were grown in LB medium (500 ml) and induced with 0.5 mM IPTG. The expressed protein was analyzed by SDS–PAGE. The expressed recombinant GroEL protein was purified by Ni-NTA chromatography under denaturing conditions according to the manufacturer's instructions (Qiagen). The purified protein was refolded *in vitro* using 1 M arginine and then dialyzed and concentrated using Amicon filtration columns.<sup>37</sup>

### Immunization of mice

Four groups of female BALB/c mice ( $n=6$ /group) were taken for immunization studies. Group 1 was immunized intranasally (i.n.) with rIpaB alone without any adjuvant (40 µg/mouse dissolved in 25 µl sterile phosphate-buffered saline (PBS)). Group 2 was immunized with rIpaB (i.n., 40 µg/mouse in 25 µl sterile PBS) along with rGroEL as an adjuvant molecule (i.n., 40 µg/mouse in 25 µl sterile PBS). The third group of mice (Group 3) was immunized with rGroEL adjuvant alone (i.n., 40 µg/mouse in 25 µl sterile PBS). The fourth group of mice (Group 4) was immunized with an equal amount of sterile PBS (25 µl/mice i.n.) and served as the control group. Subsequent booster doses were given on both the seventh and twenty-eighth days.

### Determination of antibody titers

Seven days after the last booster dose, blood was drawn from both the control and immunized mice *via* the retro-orbital sinus. Serum was collected and stored at –20 °C for further immunological studies. Additionally, BAL fluid (BALF) was collected 1 week after the last immunization by inflating the lungs with 1.0 ml of ice cold PBS into the trachea and aspirating through a 25-G needle. The lavage fluid was then separated from the tissue debris by centrifugation at 2700g for 10 min at 4 °C. The clarified BAL samples were stored at –20 °C for further analysis. The levels of IpaB-specific IgG and IgA were measured in serum, and IgA was measured in BALF by enzyme-linked immunosorbent assay. Briefly, 200 µl of the coating buffer (0.1 M bicarbonate, pH 9.3) containing 1 µg of rIpaB domain alone/rGroEL adjuvant alone was added to 96-well microtiter plates (Grenier, Frickenhausen, Germany) and incubated overnight at 4 °C. The plates were washed 3 times with PBS–0.05% Tween-20 (PBST) and blocked with 5% BSA in PBS for 2 h. Again, the plates were washed three times with PBST and then incubated with serially diluted mice sera/BALF at room temperature for 2 h. The plates were then washed three more times with PBST. The bound immunoglobulins were detected by incubating the plates with diluted anti-mouse IgG/IgA HRP conjugate (1 : 2000) for 1 h at room temperature. To measure IgG isotypes in the serum, bound antibodies were detected by incubation with anti-mouse IgG1/IgG2a HRP conjugate (1 : 3000) for 1 h at room temperature. Then, the plates were washed three times with PBST, and a color change was elicited by adding 200 µl of TMB/H<sub>2</sub>O<sub>2</sub> substrate (BD Biosciences, San Diego, CA, USA). The plates were incubated in the dark for 30 min, and the reaction was stopped by adding 50 µl of 2 N H<sub>2</sub>SO<sub>4</sub>. The absorbance was read at 450 nm using a multimode reader (Molecular devices, Sunnyvale, CA, USA).

### Lymphocyte proliferation

The control and immunized mice were killed on the seventh day after the last booster dose was administered; splenocytes were then collected from the spleen. Briefly, 200 µl of splenocytes was cultured in RPMI 1640 medium ( $1 \times 10^5$  cells/well) in 96-well microtiter plates and stimulated with 5 µg of rIpaB domain/rGroEL adjuvant separately. The plates were incubated for 72 h at 37 °C in a 5% CO<sub>2</sub> incubator. Lymphocyte proliferation was determined by adding 10 µl of MTT reagent (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (5 mg/ml) to each well, and the plates were incubated for 3 h in the dark. The plates were centrifuged at 1500 r.p.m. for 10 min. The supernatant was stored for cytokine estimation, and the pellet was incubated for 15 min in 100 µl of dimethyl sulphoxide. The absorbance was measured at 570 nm.

### Cytokine estimation

Splenocytes from control and immunized mice were isolated as described above. Cells were stimulated with 5 µg of rIpaB domain alone/rGroEL adjuvant alone as described above, and the supernatant was collected. Interferon-gamma (IFN- $\gamma$ ) was estimated from the culture supernatants collected using

enzyme-linked immunosorbent assay kits (BD Biosciences, CA, USA) according to the manufacturer's instructions. The absorbance was read at 450 nm.

### Challenge studies

To study the protective efficacy of rIpaB domain against *Shigella* infection, four groups of mice ( $n=10$ /group) were used for the challenge studies. Group 1 was immunized with rIpaB domain alone, group 2 was immunized with rIpaB domain+rGroEL protein, group 3 was immunized with rGroEL alone and group 4 was administered sterile PBS as a control. The lethal dose of *Shigella* spp. was determined by performing serial dilutions in agar plates and counting colonies after incubation overnight. Protective efficacy was evaluated 15 days after the last booster dose was administered. Each group of mice was challenged i.n. with a lethal dose ( $1 \times 10^7$  colony forming units (CFU)/mouse) of *S. flexneri*, *S. boydii* and *S. sonnei*. The mice were observed for mortality for 30 days.

### Passive immunization

To assess antibody-mediated protection, immune sera collected from all four groups of mice (7 days after the last booster was administered, as described above) was heated at 56 °C for 30 min to inactivate the complement system.<sup>41</sup> Another four groups of mice ( $n=10$ /group) were passively immunized (i.n.) with 30  $\mu$ l of this heat-inactivated sera. After 24 h, the mice were challenged with a lethal dose of *S. flexneri*, *S. boydii* and *S. sonnei* (i.n.,  $1 \times 10^7$  CFU/mouse). The mice were monitored for mortality for 30 days.

### Organ burden

To assess the bacterial load in the control and immunized mice, lungs were collected from individual animals aseptically after 30 days. Tissues were homogenized in 5 ml of ice-cold PBS using a tissue homogenizer (Kinematica, AG, Luzern, Switzerland). The resulting homogenates were plated in 10-fold serial dilutions on LB agar plates followed by incubation at 37 °C for 16–18 h. The number of CFU were counted in each plate and expressed as the number of CFU/ml.

### Histopathology

Lung tissue from control and immunized groups after challenge was excised, fixed in 10% formalin and embedded in paraffin blocks. Sections were stained with hematoxylin and eosin and analyzed by microscopic examination.

### Statistical analysis

All experimental data were statistically analyzed using one-way analysis of variance in SPSS, the Statistical Package for the Social Sciences (SPSS Inc. Version 16.0, Chicago). A  $P$ -value of  $<0.05$  was considered significant in all experiments, and the data are presented as the mean  $\pm$  s.d. Protection studies were expressed using Kaplan–Meier survival curves. All experiments were performed in triplicate.

## RESULTS

### Cloning, expression and purification of the *S. flexneri* IpaB domain

The 801-bp IpaB domain was ligated into a pRSET A vector and transformed into *E. coli* BL21 cells. The transformants were confirmed by amplification of the IpaB domain by colony PCR and by restriction digestion. The 801-bp IpaB domain gene was identified by PCR amplification. The generation of restriction fragments on a 1% agarose gel of 2.9 kb pRSET A vector and 801-bp IpaB provided confirmation of the cloned products in BL21 cells.

The transformed cells were induced with 1 mM IPTG and purified by Ni-NTA chromatography. The rIpaB domain protein (Figure 1a) was refolded, dialyzed and concentrated using Amicon filtration columns. The expression of the purified rIpaB domain (37 kDa) was confirmed by SDS–PAGE (Figure 1b), followed by western blotting with IpaB antibody (Figure 1c).

### rGroEL improves humoral immune responses generated by rIpaB

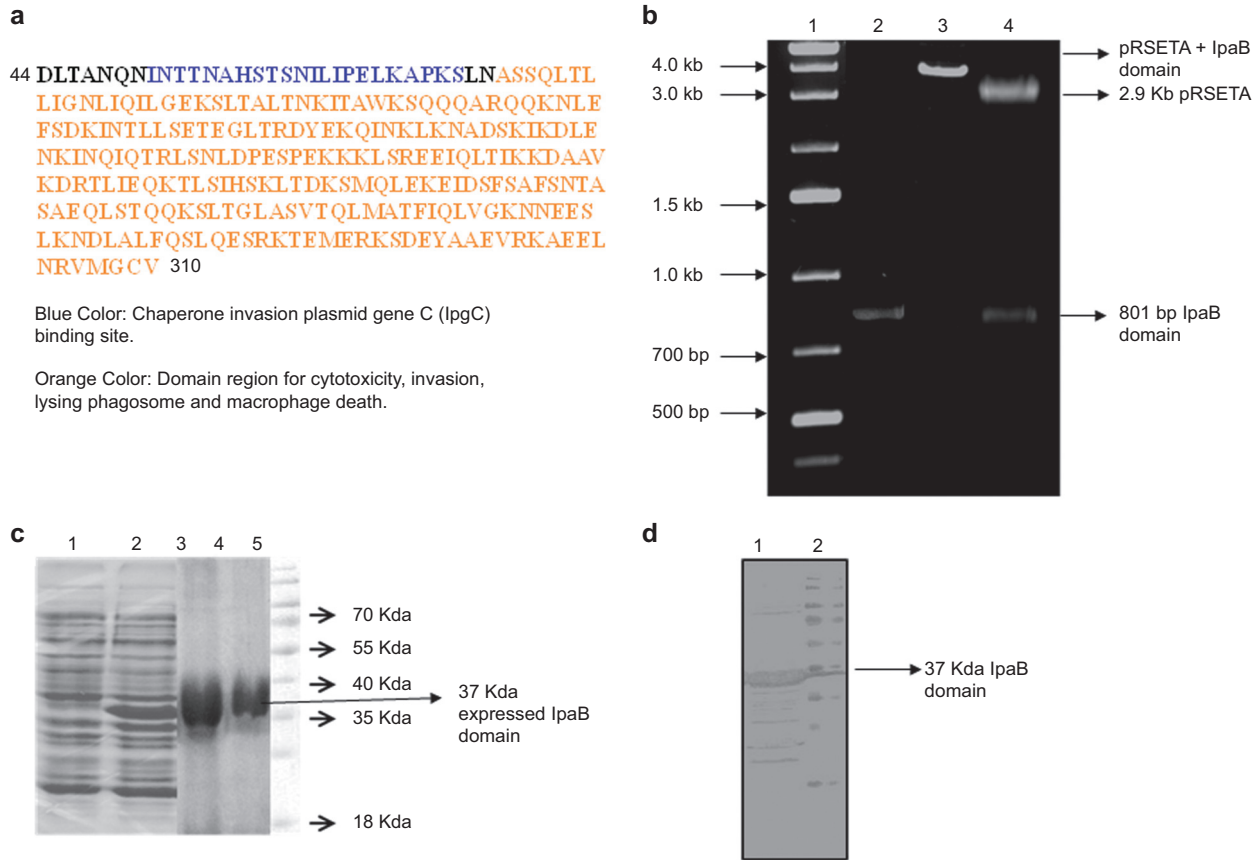
**Antibody titers.** To assess humoral immune responses, sera collected from all immunized and control groups of mice were screened for the presence of the antigen-specific antibodies IgA and IgG. All three immunized groups showed high antibody titers (IgG, IgA) compared with the control groups; however, there was a significant difference in the magnitude of antibody response when rIpaB was administered either alone or with rGroEL. The highest antibody response was generated when rIpaB was co-administered with rGroEL. Maximum titers were observed 1 week after the last immunization in all groups (Figure 2a and b).

**IgG isotyping.** The isotype profile of antibody responses depends on the cytokines produced by antigen-specific T cells and is an indirect measure of Th1 and Th2 type cytokines. The effect of rIpaB/rIpaB+rGroEL/rGroEL immunization was studied on the Th1 and Th2 profile by determining IgG1 and IgG2a antibody levels. High IgG1 and IgG2a levels in the immunized groups compared with the control groups indicate the induction of both Th1 and Th2 immune responses. However, the increase was more pronounced in the group co-administered rIpaB+rGroEL compared with the groups administered only rIpaB or rGroEL (Figure 2c). High levels of IgA were observed in BALF of the immunized groups compared with the control groups but the maximum level of IgA was observed when rIpaB was administered along with rGroEL, suggesting a robust induction of mucosal immunity (Figure 2d).

### Cell-mediated immune responses

To analyze T-cell responses in immunized mice, lymphocyte proliferation was measured by isolating the splenocytes from individual animals stimulated *in vitro* with rIpaB alone/rIpaB+rGroEL/rGroEL adjuvant 1 week after the last immunization. Splenocytes of all groups of immunized mice showed





**Figure 1** Cloning, expression and purification of the rlpA domain region. **(a)** Schematic diagram representing the aa 44–310 domain region of IpaB. Blue indicates aa residues 51–72, the cognate chaperone IpgC binding site, and orange indicates the 75–310 aa region for cytotoxicity, invasion, lysing phagosome and macrophage death. **(b)** Cloning of the rlpA domain region in pRSET A, Lane 1: 1 kb DNA ladder. Lane 2: 801 bp IpaB domain region amplified by colony PCR. Lane 3: XhoI digested plasmid showing 3.7 kb product (2.9 kb pRSET A + 801 bp IpaB). Lane 4: XhoI and PstI digested plasmid showing 2.9 kb pRSET A and 801 bp IpaB gene. **(c)** SDS–PAGE showing the expression and purification of the rlpA domain region in *E. coli* cells. The transformed cells were induced with 1 mM IPTG for 4 h at 37 °C, and the expressed recombinant protein was purified by Ni-NTA affinity chromatography. Lane 1: un-induced cells; Lane 2: IPTG-induced cells with over-expressed 37 kDa protein; Lane 3: eluted protein; Lane 4: dialyzed and purified protein; Lane 5: molecular mass marker. **(d)** Western blot. Lane 1: expression of the 37 kDa rlpA domain protein when probed with IpaB antibody was confirmed by western blotting; Lane 2: Molecular mass marker. aa, amino acid; IpgC, invasion plasmid gene C; IPTG, isopropylthiogalactoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

significant lymphocyte proliferation, even in the absence of external stimulation, compared with the control groups. A further increase in the proliferation was observed upon *in vitro* stimulation in the rIpaB immunized group compared with the rGroEL adjuvant/control groups, and significantly higher proliferation was observed in the rIpaB+rGroEL co-administered group than in the rIpaB immunized group ( $P<0.001$ ) (Figure 3a).

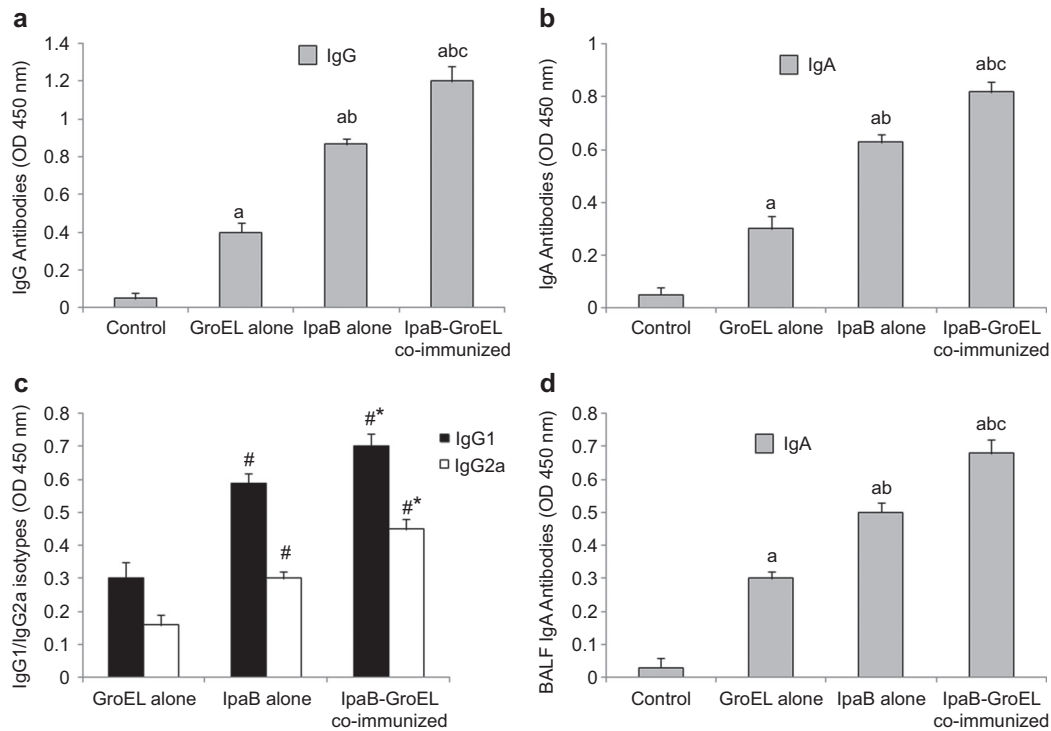
To further characterize the type of immune response generated, splenocytes from all of the groups were cultured *in vitro* for 72 h and IFN- $\gamma$  was measured in the culture supernatants. There was a significant increase in the IFN- $\gamma$  level in all of the immunized groups compared with the control group. The level of IFN- $\gamma$  was appreciably higher in the co-administered group stimulated *in vitro* with the rGroEL+ rIpaB domain compared to the group stimulated with the rIpaB domain alone, confirming the shift toward a Th1 immune response ( $P<0.001$ ) which was also revealed by IgG isotyping (Figure 3b).

### rIpaB domain co-administered with rGroEL improves protective efficacy

The significant increase in antibody titers and IFN- $\gamma$  led us to evaluate the protective efficacy of the rIpaB domain against *S. flexneri*, *S. boydii* and *S. sonnei*. Fifteen days after the last immunization, control/rIpaB domain immunized/co-administered/rGroEL adjuvant alone mice ( $n=10$ /group) were challenged with the lethal dose of  $1 \times 10^7$  CFU/mouse i.n. All of the control mice died within 3–4 days, whereas 50% of the rGroEL immunized mice survived and 60%–70% of the rIpaB domain immunized mice survived when challenged with all three of the *Shigella* spp. Interestingly, rIpaB domain co-administered with rGroEL protein protected 80%–85% of the mice challenged with *Shigella* spp. (Figure 4a–c).

### Passive immunization

Antibody-mediated protection against *Shigella* infection was assessed by immunizing the mice with heat inactivated



**Figure 2** Effects of rIpaB and rIpaB+rGroEL (co-administration) immunization on antibody levels in mice. Groups of female BALB/c mice ( $n=6$ ) were immunized i.n. with 40  $\mu\text{g}/\text{mouse}$  of recombinant GroEL alone (adjuvant control)/rIpaB alone/rIpaB+rGroEL. Subsequent booster doses were given on the seventh and twenty-eighth days. The control group of mice ( $n=6$ ) was immunized with sterile PBS. Mice were killed 1 week after the last booster, and sera were collected to estimate the antibody titers by ELISA. (a) Serum IgG, (b) Serum IgA, (c) IgG1 and IgG2a and (d) BALF IgA antibody titers. No detectable IgG1/IgG2a isotope antibodies were observed in the control group. The sera samples which gave an o.d. value  $>0.3$  were measured for scoring. Data were represented as the mean  $\pm$  s.d.  $P<0.001$ , a vs. Control, b vs. GroEL, c vs. IpaB.  $P<0.001$ , # vs. GroEL, \* vs. IpaB. BALF, BAL fluid; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

anti-IpaB and anti-IpaB+GroEL antiserum. The mice were challenged after 24 h with *S. flexneri*, *S. boydii* and *S. sonnei* i.n. Fifty percent of the animals passively immunized with rIpaB antisera survived against lethal infection by all of the *Shigella* spp., whereas the survival rate was higher (60%) in the mice passively immunized with the rIpaB domain co-administered with rGroEL antisera (Figure 4d–f).

### Organ burden and histopathology

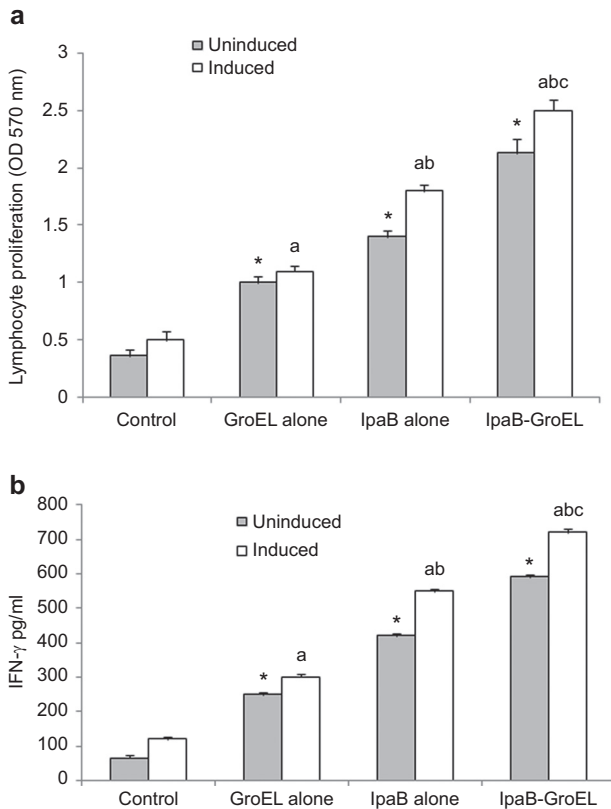
Bacterial load was determined from the lungs of control and immunized mice challenged with *Shigella* spp. Organ burden studies showed a decrease in CFU in the lungs of rIpaB domain immunized mice compared to the controls ( $P<0.0001$ ). However, the maximum reduction in CFU was observed in the rIpaB+rGroEL co-administered group compared with the control/rIpaB domain groups ( $P<0.0001$ ) (Figure 5).

Histopathology of lung tissues immunized with the rIpaB domain protein revealed that these tissues had a more normal morphology with uniform alveoli compared with the controls. The lung sections of the rIpaB+rGroEL co-administered group challenged with *Shigella* spp. showed normal lung parenchyma with no inflammatory infiltrates or edema in the alveolar spaces or bronchial lumina compared with the rIpaB group (Figure 6a–l).

### DISCUSSION

The development of an effective vaccine against *Shigella* is of global importance; however, such a vaccine has yet to be successfully developed. The emergence of antibiotic resistance among *Shigella* serotypes limits the number of drugs available for the treatment of shigellosis.<sup>42–46</sup> The vaccine strategies currently being developed against this pathogen include live attenuated, conjugate, broad spectrum, LPS-based and proteasome-based vaccines.<sup>47</sup> However, broad protection against all four *Shigella* spp. and their serotypes must be considered in the development of vaccines against shigellosis. Such a vaccine could be developed by exploiting a common molecule that is conserved in all the *Shigella* spp. Therefore, in our present study, we focused on the IpaB domain protein, which is a conserved molecule across all *Shigella* spp. that plays a major role in the pathogenesis of the bacteria. Furthermore, we tested the adjuvanticity of rGroEL of *S. Typhi* that we previously developed.<sup>37,38</sup>

We evaluated the immunogenicity and protective efficacy of the domain region of the IpaB protein (37 kDa) against *Shigella* spp. It has been reported that these IpaB domains are recognized by human convalescent sera and that they are found to be immunogenic during natural infection.<sup>16</sup> The antibodies produced against these IpaB antigens efficiently prevent the colonization of *Shigella* in the host cells,



**Figure 3** Effects of rIpaB and rIpaB+rGroEL (co-administration) immunization on lymphocyte proliferation and IFN- $\gamma$  production. **(a)** Groups of female BALB/c mice ( $n=6$ ) were immunized i.n. with 40  $\mu\text{g}/\text{mouse}$  of rGroEL/rIpaB alone/rIpaB+rGroEL. Subsequent booster doses were given on the seventh and twenty-eighth days. The control group of mice ( $n=6$ ) was immunized with sterile PBS. After the last immunization, the splenocytes were isolated and cultured ( $1 \times 10^5$  cells/well) either in the absence (unstimulated) or in the presence (stimulated) of rIpaB/rGroEL for 72 h in a  $\text{CO}_2$  incubator at 37  $^\circ\text{C}$ . The absorbance was measured at 570 nm. **(b)** After the last immunization, the splenocytes were isolated and cultured for 72 h in a  $\text{CO}_2$  incubator at 37  $^\circ\text{C}$  to estimate the IFN- $\gamma$  production. The absorbance was measured in culture supernatants at 450 nm.  $P < 0.001$ , \* vs. control UI, a vs. control-induced, b vs. IpaB-induced, c vs. IpaB-GroEL-induced. IFN- $\gamma$ , interferon-gamma; i.n., intranasally.

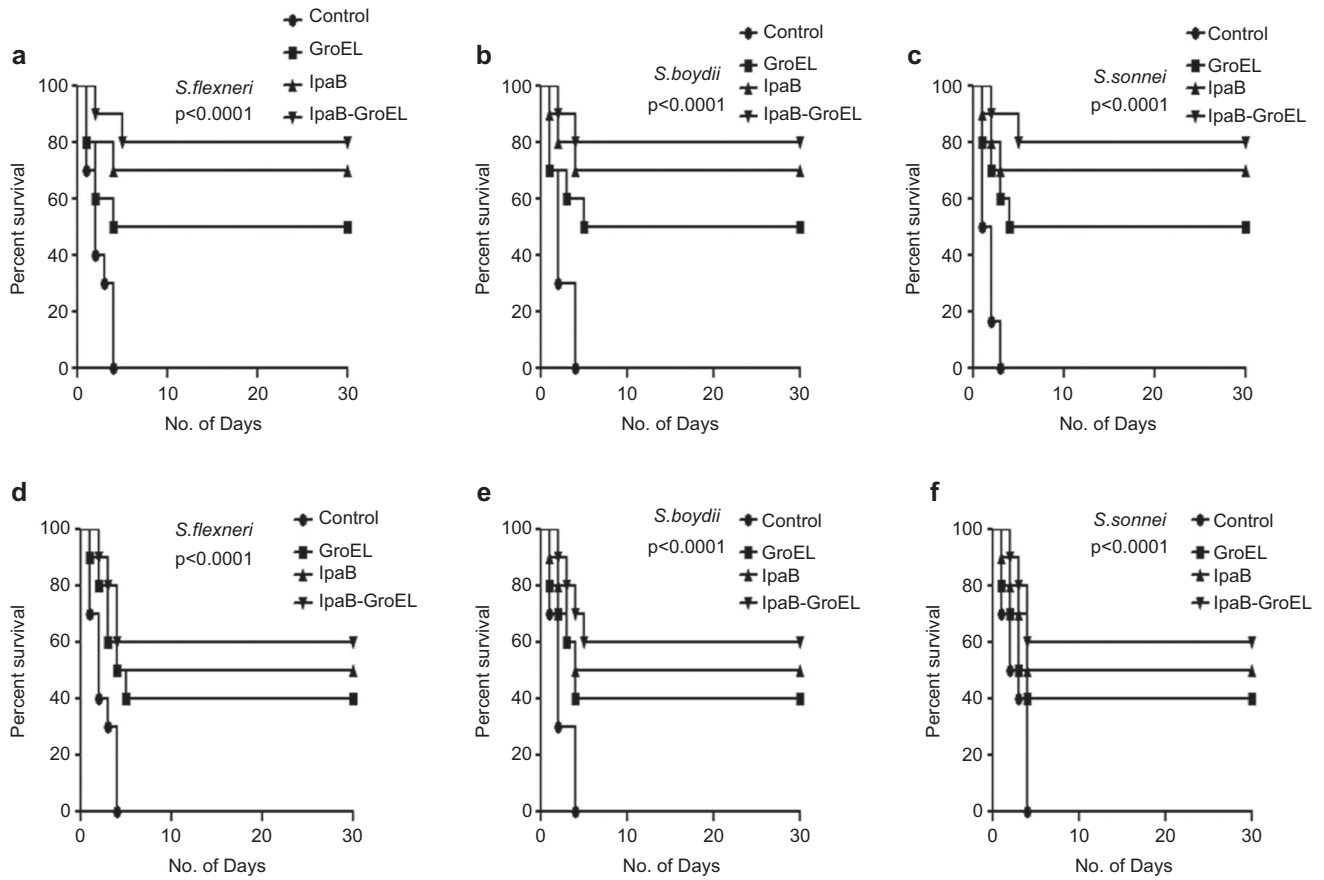
making them effective as a candidate molecule for vaccine development.

We cloned and expressed the IpaB domain region in *E. coli* BL21 cells and purified the recombinant protein. Our study showed that immunization of mice i.n. with the rIpaB domain region resulted in an increase of both IgG and IgA antibody titers in the sera compared with the control. Antibody isotyping revealed elevated levels of both IgG1 and IgG2a antibodies, indicating the induction of both Th1 and Th2 immune responses. It has been reported that intranasal immunization stimulates a higher IgA antibody response in the mucosal surfaces and also primes the systemic immune responses with the development of serum antibodies.<sup>48</sup> We also observed an increase in IgA antibodies in BALF among all of the immunized groups compared with the control groups, but the maximum

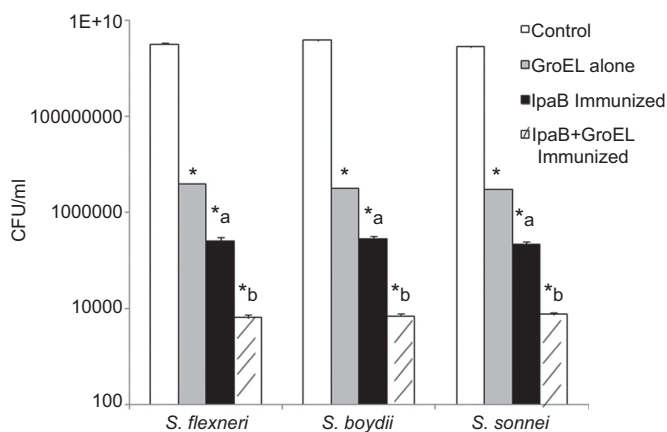
titer was observed in the group co-administered rIpaB+rGroEL. The secretion of IgA is important because it is necessary to prevent mucosal enteric infection in humans. Significantly higher circulating IgA antibody secreting-cells have been reported in patients infected with *S. flexneri* after the onset of the disease.<sup>49</sup> Although the mechanisms of protection against *Shigella* are not well defined, mucosal immune defenses may play an important role in neutralizing the *Shigella* T3SS interactions in the epithelial barrier, preventing pathogenic attachment and thereby preventing colonization of the host epithelial cells by the bacteria. The protection provided by the recombinant IpaB domain was 60%–70% against a lethal infection with *S. flexneri*, *S. boydii* and *S. sonnei*. To the best of our knowledge, we are the first to report the induction of protective immunity against *Shigella* infection by the rIpaB domain region in mice.

Furthermore, to increase the immunogenicity and protective efficacy of the rIpaB domain, the mice were co-administered both rIpaB and the recombinant *S. Typhi* GroEL. Co-administration of the rIpaB domain with rGroEL in mice resulted in an increase in IgG, IgG1 and IgG2a compared with the administration of IpaB alone, indicating the stimulation of both the Th1 and Th2 immune responses. The increased production of IFN- $\gamma$  observed in the splenocytes isolated from all of the immunized groups suggests that T cells also contribute to the protection against *Shigella* infection. Cell-mediated immunity favors the production of IFN- $\gamma$ , which boosts the production of the IgG2a isotype by murine B cells.<sup>50</sup> Interestingly, the titers were higher in the co-administered group than they were in the other groups. Moreover, the ratio of IgG1 and IgG2a decreased significantly in the co-administered group, suggesting a shift toward the Th1 response. This finding is in line with the observation that higher production of IFN- $\gamma$  was observed in the rIpaB+rGroEL co-administered group compared with the IpaB group. This finding is also supported by the observation of higher lymphocyte proliferation in the splenocytes isolated from the co-administered group compared with the other groups. The requirement of IFN- $\gamma$  for protection against *Shigella* infection in mice has been reported previously.<sup>51,52</sup> IFN- $\gamma$  knockout mice lacking this cytokine showed an increased susceptibility to *Shigella* infection compared with wild-type controls, and the *Shigella* infection was not cleared in the IFN- $\gamma$ -deficient mice.<sup>53</sup> In shigellosis patients, the expression of gamma interferon was twofold higher during convalescence than during the acute stage.<sup>54</sup> It has been reported that both humoral and T-cell immune responses were induced when *Shigella* IpaB/IpaD was co-administered with double mutant heat labile toxin and that this protected the mice from *Shigella* challenge.<sup>20,55–57</sup>

Furthermore, we observed that the rIpaB domain region alone conferred 60%–70% protection against *Shigella* spp. Interestingly, when rGroEL was administered along with the rIpaB domain, the survival percentage of the mice increased to 80%–85% protection against the lethal infection by *Shigella* spp., indicating an adjuvant effect of rGroEL. Because



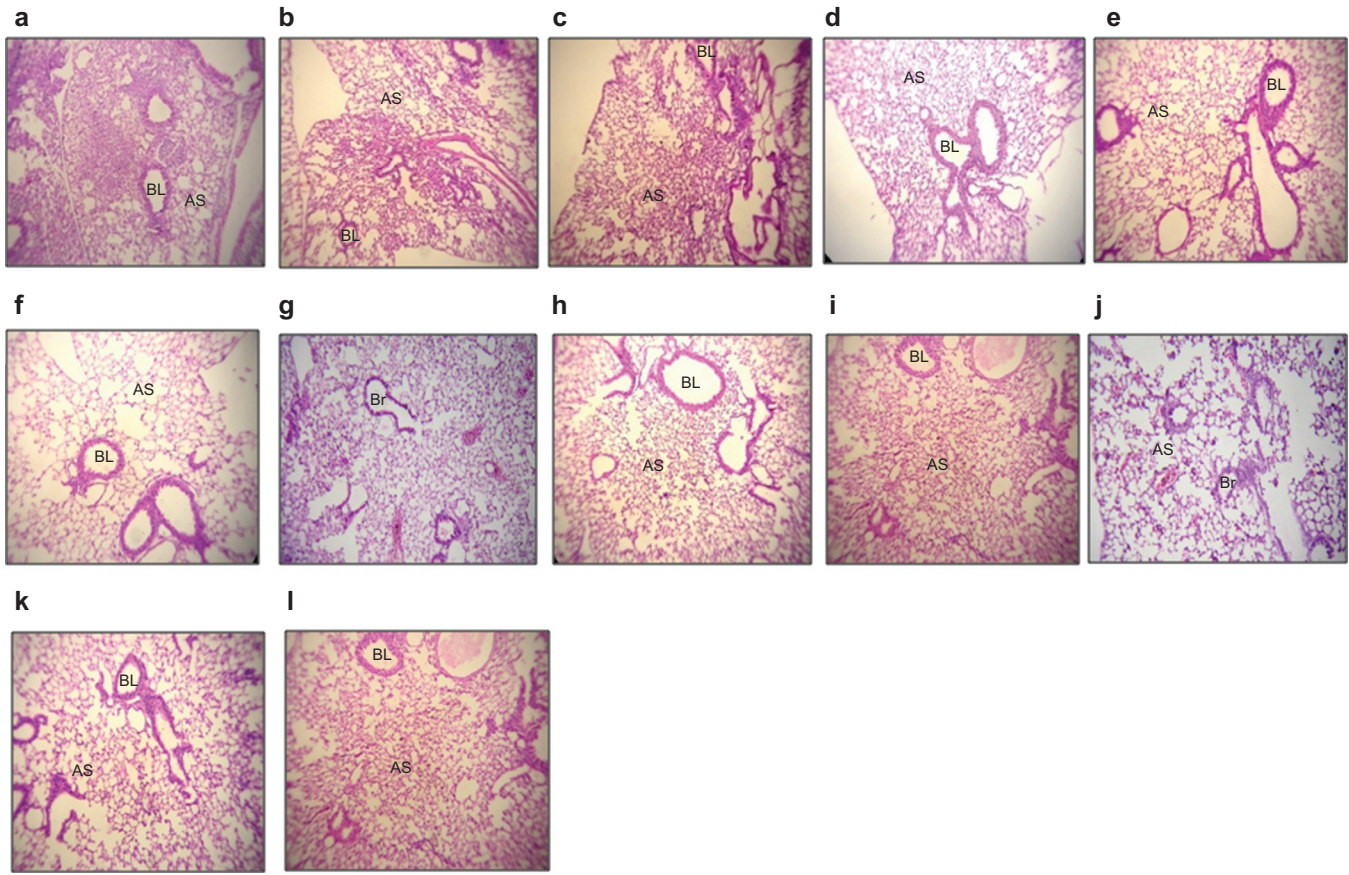
**Figure 4** Protective efficacy of rIpaB and rIpaB+rGroEL immunization on survival. Groups of female BALB/c mice ( $n=10$ ) were immunized i.n. with  $40 \mu\text{g}/\text{mouse}$  of rGroEL/rIpaB/rIpaB+rGroEL. Subsequent booster doses were given on the seventh and twenty-eighth days. The control group of mice ( $n=10$ ) was immunized with sterile PBS. Fifteen days after the last booster dose, mice were challenged with a lethal dose of  $1 \times 10^7$  CFU/mouse i.n. of (a) *S. flexneri*, (b) *S. boydii* or (c) *S. sonnei*. Another group of 10 mice was passively immunized i.n. with  $30 \mu\text{l}$  of heat inactivated immune sera raised from the mice immunized with rGroEL/rIpaB/rIpaB+rGroEL and these mice were challenged with a lethal dose of  $1 \times 10^7$  CFU/mouse i.n. of (d) *S. flexneri*, (e) *S. boydii* or (f) *S. sonnei*. All groups of mice were observed for mortality for 30 days. CFU, colony forming units; i.n., intranasally.



**Figure 5** Organ burden studies after challenge of immunized mice with a lethal dose of  $1 \times 10^7$  CFU/mouse i.n. of *S. flexneri*, *S. boydii* and *S. sonnei*. Lung tissues were homogenized in 5 ml of ice-cold PBS. The resulting homogenates were plated in 10-fold serial dilutions on LB agar plates followed by incubation at  $37^\circ\text{C}$  for 16–18 h. CFU were counted and recorded. \* vs. control,  $P < 0.0001$ ; a vs. rGroEL,  $P < 0.0001$ ; b vs. a,  $P < 0.0001$ . CFU, colony forming units; i.n., intranasally; PBS, phosphate-buffered saline.

*Shigella* is an intracellular pathogen, cell-mediated immunity is also required to fight off infection through the secretion of pro-inflammatory mediators that further promote innate immune cells and T helper cytokines that support antibody production.<sup>55</sup> Passive immunization with anti-IpaB or anti-GroEL+anti-IpaB immune sera conferred 50%–60% protection in the mice, demonstrating the importance of the humoral response for protection against shigellosis. This antibody-mediated protection observed in the present study could be due to the surface localization of IpaB, which is an important constituent of T3SS, an essential virulence determinant of many Gram-negative bacterial pathogens.<sup>23–26</sup> The *Shigella* T3SS consists of a cytoplasmic bulb, a transmembrane region and a hollow ‘needle’ protruding from the bacterial surface. The needle is directly involved in sensing the host cells and leads to the secretion and assembly of pore that are formed by IpaB and IpaC in the host membrane, through which the proteins that facilitate host cell invasion are translocated. The localization of IpaB at the surface of needle tips has been demonstrated by many researchers, thus facilitating the recognition of IpaB antigen by antibodies.<sup>17–20</sup>





**Figure 6** Histopathology of control and immunized (rIpaB alone and rIpaB+rGroEL co-administered) mouse lung tissue. Sections of lung tissue were stained with hematoxylin and eosin and analyzed by microscopic examination. (a–c) Lung sections of control mice challenged with *S. flexneri*, *S. boydii* or *S. sonnei*, respectively, showing lung parenchyma with two large areas of consolidation with heavy neutrophilic cell infiltration into the lung parenchyma. (d–f) Lung sections of adjuvant control GroEL immunized mice challenged with *Shigella* spp. showing improved lung histology. (g–i) IpaB immunized lung section from a mouse challenged with *Shigella* spp. showing improved lung parenchyma with no edema in the alveolar spaces or bronchial lumina. (j–l) Lung section of a mouse co-immunized with IpaB-GroEL and challenged with *Shigella* spp. showing normal lung parenchyma with no inflammatory infiltrates or edema in the alveolar spaces or bronchial lumina. Images shown are at  $\times 100$  magnification. AS, alveolar space; BL, bronchial lumen.

In vaccine development, the choice of the adjuvant is as vital as the selection of the vaccine antigen. HSPs are conserved immunodominant molecules, and we have previously reported that an *in vitro* serum bactericidal assay with *S. Typhi* rGroEL antisera exhibited 50%–55% inhibition of cells of *Shigella* spp. and that *in vivo* cross-protection studies with rGroEL of *S. Typhi* demonstrate 60% protection against *Shigella* spp. i.p.<sup>40</sup> In this study, we have shown that immunization with rGroEL i.n. also confers 50% protection by inducing immune responses. An ideal vaccine adjuvant should induce both humoral and cell-mediated immunity and should be non-toxic and without any side effects.<sup>58</sup> rGroEL boosted the immune responses and the protection rate of rIpaB. In this regard, recombinant *S. Typhi* GroEL can be considered an effective adjuvant because it induces both arms of immunity, as we reported previously.<sup>35</sup> In the present study, co-administration of the rIpaB domain with rGroEL augmented the immune responses and the protective efficacy against *Shigella* infection in mice. Recent studies have reported that the double mutant heat labile

toxin increases the efficacy of *Shigella* IpaB/IpaD.<sup>21,55–57</sup> This is the first report evaluating the use of recombinant GroEL of *S. Typhi* as an adjuvant, surmounting the side effects of conventional adjuvants.

The organ burden studies presented here revealed a significant reduction in the number of colonies of *S. flexneri*, *S. boydii* and *S. sonnei* in the lung tissues of mice that were co-administered both the rIpaB domain and rGroEL compared with mice administered the rIpaB domain alone. Histopathological studies of lung tissue also showed improved tissue morphology in the co-administered mice challenged with *Shigella* spp. compared with the rIpaB domain-immunized group.

In conclusion, we report the recombinant IpaB domain of *S. flexneri* to be immunogenic and protective against *Shigella* species and the recombinant GroEL of *S. Typhi* to be an effective adjuvant. When co-administered, rIpaB+rGroEL showed robust humoral and cellular immune responses and provided protection against *Shigella* infections. Therefore, this study highlights the potential of the recombinant IpaB domain along

with rGroEL for the development of broadly protective subunit vaccines against Shigellosis.

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