Chapter 5

Immunogenicity and Protective Efficacy of Recombinant IpaB Protein (Domain)/ IpaB-GroEL Co-administration Against Lethal Infection by S. flexneri, S. boydii and S. sonnei in Mice.
CHAPTER 5

IMMUNOGENICITY AND PROTECTIVE EFFICACY OF RECOMBINANT IpaB PROTEIN (DOMAIN)/IpaB-GroEL CO-ADMINISTRATION AGAINST LETHAL INFECTION BY S. FLEXNERI, S. BOYDII AND S. SONNEI IN MICE

5.1. RESULTS

5.1.1. Cloning of IpaB domain region

5.1.1.1. Isolation of Invasion plasmid from S. flexneri by Congo red binding assay

The 220 Kb virulent plasmid bearing Shigella strains were identified by growing them in tryptic soy agar with 0.02% congo red. TSA congo red plates were incubated overnight at 37°C. Red colonies grown on the plates were isolated (Figure 5.1.1.1a). From the isolated red colonies invasion plasmid was isolated and purified. The purified plasmid loaded on the agarose gel showed 220 Kb size Shigella invasion plasmid (Figure 5.1.1.1b).

![Figure 5.1.1.1: (a) Plasmid bearing red Shigellae colonies in TSA congo red agar. (b) Invasion Plasmid (220 Kb) isolated from the red Shigellae colonies. Lane 1. Lambda DNA ladder, Lane 5. Isolated 220 Kb plasmid.](image)
5.1.1.2. Cloning of IpaB domain region into pRSETA expression vector

IpaB domain region was amplified from isolated invasion plasmid as template using the designed IpaB primers. Amplified IpaB was purified, restricted with Xho I and Pst I and showed 801 bp band in the agarose gel electrophoresis. Isolated and purified pRSETA expression vector was also restricted digested with Xho I and Pst I. 801 bp IpaB domain was ligated in pRSET A vector and transformed into *E.coli* BL-21 cells. The transformants were confirmed by amplification of IpaB domain by colony PCR and also by restriction digestion. PCR amplification showed 801 bp IpaB domain gene. Generation of restricted fragments of 2.9 Kb pRSET A vector and 801 bp IpaB confirmed the cloned product in BL21 cells (Figure 5.1.1.2).

![Image showing agarose gel electrophoresis with bands for various DNA lengths and IpaB](image)

*Figure 5.1.1.2: Cloning of IpaB domain region. Lane1: 1 Kb DNA ladder. Lane 2: 801 bp IpaB domain region amplified by colony PCR. Lane3: XhoI digested plasmid showing 3.7 Kb product (2.9 Kb pRSETA + 801 bp IpaB). Lane 4: Xho I and Pst I digested plasmid showing 2.9 Kb pRSETA and 801 bp IpaB gene.*

5.1.2. Expression, Isolation and Purification of the IpaB Domain

The transformed cells were induced with 1mM IPTG and purified by Ni-NTA chromatography. The expression of purified IpaB (37 KDa) was confirmed by SDS-
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PAGE (Figure 5.1.2 a), followed by western blotting (Figure 5.1.2 b). Further, the IpaB protein was refolded, dialysed and concentrated by Amicon filter.

5.1.2: (a) SDS-PAGE showing the expression and purification of IpaB domain region in E.coli cells. The transformed cells were induced with 1 mM IPTG for 4 hrs at 37⁰C and the expressed recombinant protein was purified by Ni-NTA affinity chromatography. Lane 1: Uninduced 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. (b) Western Blot of IpaB. Expression of 37 Kda IpaB protein confirmed by Western blotting.

5.1.3. Antibody Titers and IgG Isotyping

To assess the antibody mediated humoral immune responses, mice were immunized with IpaB/GroEL/IpaB+GroEL. IgA, IgG, IgG1, IgG2a levels were determined in the sera of immunized mice by ELISA. Immunization (i.n.) with recombinant IpaB (Domain) protein/GroEL showed high antibody titers (IgG, IgA) as compared to control but significantly lower than co-immunized group (Figure 5.1.3 a,b). Effect of GroEL/IpaB/IpaB+GroEL immunization was also studied on Th1 Th2 profile by determining IgG1 and IgG2a antibodies. All the three groups showed high IgG1 and IgG2a levels as compared to control indicating induction of both Th1 as well as Th2 immune responses. But the increase was more dominant in co-immunized group than GroEL or IpaB immunized animals (p<0.01) (Fig. 5.1.3 c). The ratio of IgG1 and IgG2a showed decline from IpaB to co-immunized groups which is
indicative of shift towards Th1 response ($p<0.01$) (Figure 5.1.3 d). High levels of the IgA were observed in BALF of all the immunized groups compared to control but maximum level was in the co-immunized group suggesting the robust induction of mucosal immunity (Figure 5.1.3 e).

![Figure 5.1.3: Effect of IpaB, GroEL, IpaB-GroEL (co-immunization) immunization on antibody titers in mice.](image)

Effect of GroEL, IpaB, IpaB-GroEL (co-administration) immunization on antibody titers in mice. Groups of female BALB/c mice (n=6) were immunized i.n. with 40 µg/mouse of recombinant IpaB / GroEL alone/ IpaB+GroEL. Subsequent booster doses were given on the 7th day and 28th day. Control group of mice (n=6) were immunized with sterile PBS. Mice were sacrificed 1 week after the last booster and sera was collected to estimate the antibody titers by ELISA (a) serum IgG antibody titer, (b) serum IgA. (c) IgG1 and IgG2a. (d) IgG1/IgG2a ratio. (e) BALF IgA titers. * Control vs Immunized p<0.01, # IpaB/GroEL alone vs IpaB+GroEL p<0.01. The sera samples which gave OD value $>0.3$ were measured for scoring.
5.1.4. **Lymphocyte Proliferation and Cytokine Responses**

To analyze T cell responses in immunized mice, lymphocyte proliferation was measured by isolating the splenocytes from individual animals. Lymphocyte proliferation showed significant increase in the IpaB immunized group as compared to control. Similarly, immunization with recombinant GroEL protein also showed high lymphocyte proliferation than control. Still higher proliferation was observed in co-immunized mice as compared to the IpaB/ GroEL alone (p<0.01) (Figure 5.1.4 a).

To further characterize the type of immune response generated, splenocytes from all the groups were cultured *in vitro* for 72 hrs and IFN-γ was measured in culture supernatants. There was significant increase in IFN-γ level in all the three groups as compared to control. However, the level was appreciably higher in mice co-immunized with IpaB and GroEL than in mice immunized with either GroEL or IpaB alone confirming the shift towards Th1 immune response (p<0.01) as revealed by IgG isotyping also (Figure 5.1.4 b).

*Figure 5.1.4: (a) Effect of GroEL, IpaB, IpaB-GroEL (co-immunization) immunization on lymphocyte proliferation in mice spleen cells by MTT assay. Groups of female BALB/c mice (n=6) were immunized i.n. with 40 µg/ mouse of recombinant IpaB /GroEL alone/ IpaB+GroEL. Subsequent booster doses were given on the 7th and 28th day. Control group of mice (n=6) were immunized with sterile PBS. After the last immunization, the splenocytes were isolated and cultured (1x 10⁵ cells/ well) either in the absence (uninduced) or in the presence (induced) of IpaB/ GroEL / IpaB + GroEL for 72 hrs in a CO₂ incubator at 37°C. The absorbance was measured at 570 nm. * indicates, Control vs Immunized p<0.01, # IpaB/GroEL alone vs IpaB+GroEL p<0.01. (b) IFN-γ production from the splenocytes supernatant of control and immunized (GroEL, IpaB, IpaB-GroEL co-immunized) mice. The absorbance was measured at 450 nm. *Control vs Immunized p<0.01, # IpaB/GroEL alone vs IpaB+GroEL p<0.01.*
5.1.5. Protective Efficacy Studies

The significant increase in antibody titres and IFN-γ led us to evaluate the protective efficacy of IpaB against *S.flexneri*, *S. boydii* and *S. sonnei*. All the control mice died within 3-4 days, whereas 60-70% protection was observed in the IpaB immunized mice whereas 50-60% in GroEL immunized group challenged with all the three *Shigella* spp. Interestingly IpaB co-administered with GroEL protein protected 80% of mice as compared with control challenged with *Shigella* spp. (Figure 5.1.5 a-c).

*Figure 5.1.5: Protective efficacy of IpaB, GroEL, IpaB-GroEL (co-immunization) immunization on the survival of mice.* Groups of female BALB/c mice (n=10) were immunized i.n. with 40 µg/mouse of recombinant IpaB /GroEL alone/ IpaB+GroEL. Subsequent booster doses were given on the 7th day and 28th day. Control group of mice (n=10) were immunized with sterile PBS, 15 days after the last booster dose, mice were challenged with a lethal dose of (a) *S. flexneri* (1x10^7 CFU/ mouse), (b) *S. boydii* (1x10^7 CFU/ mouse), (c) *S. sonnei* (1x10^7 CFU/ mouse) i.n. The mice were observed for morbidity and mortality for 30 days. Statistical significance was determined in the number of mice survived between control and immunized group *, IpaB/GroEL vs co-immunized group # (p<0.05).
5.1.6. Passive immunization

Antibody mediated protection against *Shigella* infection was assessed by immunizing the mice with heat inactivated, anti-IpaB, anti-GroEL and anti-IpaB+GroEL antiserum. The mice were challenged after 24 hrs with *S. flexneri*, *S. boydii* and *S. sonnei* i.n. 50% animals passively immunized with IpaB/GroEL survived against lethal infection by all *Shigella* spp. whereas in mice passively co-administered with IpaB and GroEL, the survival rate was higher (60%) (Figure 5.1.6 a-c).

*Figure 5.1.6: Protective efficacy of passive transfer of immune sera on the survival of mice.* Groups of 10 mice was immunized i.n. with 30 µl heat inactivated immune sera raised from the mice immunized with IpaB and IpaB+GroEL coimmunized. Mice were challenged i.n. with 1x10^7 CFU/mouse (a) *S. flexneri*, (b) *S. boydii*, (c) *S. sonnei*. Statistical significance was determined in the number of mice survived between control and immunized group *p*, IpaB/GroEL vs co-immunized group # (*p* < 0.05).
5.1.7. Organ Burden

Organ burden studies showed decreased CFU in the lungs of IpaB immunized mice as compared to the control (p<0.05). However, maximum reduction in the CFU was observed in IpaB-GroEL co-immunized group as compared to the control/IpaB/GroEL groups (p<0.05) (Figure 5.1.7).

Figure. 5.1.7: Organ burden Studies. Organ burden determined after challenge of immunised mice with the lethal dose of *S. flexneri* (1x10^7 CFU/ mouse), *S. boydii* (1x10^7 CFU/ mouse) and *S. sonnei* (1x10^7 CFU/ mouse) i.n. Lung tissues were homogenized in 5 ml of ice cold phosphate-buffered saline (PBS). Resulting homogenates were plated in 10-fold serial dilutions on LB agar plates followed by incubation at 37°C for 16-18 hrs. Colony forming unit (CFU) was counted and recorded. * Control vs Immunized p<0.01, # IpaB/GroEL vs IpaB+GroEL p<0.01.

5.1.8. Histopathology

The lungs sample were excised, fixed in 10% formalin and embedded in paraffin blocks. Sections were stained with hematoxylin and eosin and analysis by microscopic examination. Lung section of control mice challenged with *S. flexneri, S. boydii* and *S. sonnei* showed lung parenchyma with two large areas of consolidation with heavy neutrophilic cell infiltration into the lung parenchyma (BL- Bronchial Lumen, AS- alveolar Space) and infected morphology (Figure 5.1.8 a-c).
Figure 5.1.8: Histopathology of Control and Immunized (GroEL, IpaB, IpaB+GroEL co-immunized) lung tissue of mice. Lung sections were stained with hematoxylin and eosin and analyzed by microscopic examination. 

a-c) Lung sections of control mice challenged with *S. flexneri*, *S. boydii*, *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 GroEL immunized mice challenged with *Shigella* spp., shows better lung histology. 

g-i) IpaB immunized lung section challenged with *Shigella* spp. showing improved lung parenchyma with no edema in the alveolar spaces or bronchial lumina. 

j-l) Lung section of the IpaB-GroEL co-immunized group challenged with *Shigella* spp. showing normal lung parenchyma with no inflammatory infiltrates or edema in the alveolar spaces or bronchial lumina. Images shown at 100x magnification.
Improved lung parenchyma was observed in the GroEL immunized group challenged with *Shigella* spp. (Figure 5.1.8 d-f). IpaB immunized lung section challenged with *Shigella* spp. showed improved lung parenchyma (Figure 5.1.8 g-i). Lung section of the IpaB+GroEL co-immunized group challenged with *Shigella* spp. showing normal lung parenchyma with no inflammatory infiltrates or edema in the alveolar spaces or bronchial lumina as compared to other immunized groups (Figure 5.1.8 j-l).

5.2. DISCUSSION

The development of an effective vaccine against *Shigella* remains an unmet need of global importance. The emerging antibiotic resistance among *Shigella* serotypes limits the number of drugs available for the treatment of Shigellosis (Taylor, 2003; Dutta *et al*., 2003; Seidlein *et al*., 2006; Pazhani *et al*., 2008; Antoine *et al*., 2010). The vaccines strategies against this pathogen under development include live attenuated, conjugate, broad spectrum, LPS based and proteosome based vaccines (Kweon, 2008). But, the vaccine development against shigellosis must consider the broad protection against all the four species and their serotypes. Such type of vaccine can be achieved by exploiting a common molecule conserved in all the *Shigella* spp. Therefore, in our present study, we focused on IpaB protein, a conserved molecule across all *Shigella* spp, that plays a major role in the pathogenesis of the bacteria. Further, we tested the adjuvanticity of recombinant GroEL of S.Typhi which we developed earlier (Paliwal *et al*., 2008, 2011; Bansal *et al*., 2010) and reported to be cross protective against multiple pathogens (Chittradevi *et al*., 2013).

We evaluated the immunogenicity and protective efficacy of 44 - 310 aa region of IpaB protein (30 kDa) against *Shigella* spp. The domain region of IpaB has
cognate chaperone Invasion plasmid gene C (IpgC) binding site (aa 51-72) which prevents degradation and gives IpaB stability in the cytoplasm, aa 75-310 contains region for cytotoxicity, invasion, lysing phagosome and macrophage death (Menard et al., 1994; Mills et al., 1988; Barzu et al., 1993; Guichon et al., 2001; Shen et al., 2010). These IpaB domains are recognized by human convalescent sera and found to be immunogenic during natural infection (Barzu et al., 1993). The antibodies produced against these IpaB antigen will efficiently prevent the colonization of *Shigella* in the host cells making them effective vaccine candidate molecule.

We cloned and expressed the IpaB domain in *E.coli* BL-21 cells and purified the recombinant protein. Our study showed that immunization of mice with IpaB domain region i.n. resulted in a significant increase both in the IgG and IgA antibody titers in sera as compared to the control. Antibody isotyping revealed elevated levels of both IgG1 and IgG2a antibodies indicating induction of both Th1 and Th2 type of immune responses. It has been reported that intranasal immunization stimulates higher IgA antibody response in the mucosal surfaces and also primes the systemic immune responses with the development of serum antibodies (Ogra et al., 2001). We also observed increase in the IgA antibodies in BALF of all the immunized groups as compared to control but the titer was maximum in the co-immunized group. Secretion of IgA is important as it is necessary to prevent mucosal enteric infection in humans. Significantly higher circulating IgA antibody secreting-cells (ASC) were reported in the *S. flexneri* infected patients after the onset of the disease (Rasolofo-Razanamparany et al., 2001). Although the mechanisms of protection against *Shigella* are not well defined, mucosal immune defences play an important role in neutralizing the *Shigella* Type III secretion system (TTSS) interactions in the epithelial barrier,
preventing pathogenic attachment, thereby preventing their colonization of the host epithelial cells.

The protection accorded by recombinant IpaB domain was 70% against 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 recombinant IpaB domain region in mice.

Further to increase the immunogenicity and protective efficacy of IpaB domain, the mice were co-administered with both IpaB and recombinant S.Typhi GroEL. Immunization with GroEL alone and co-administration in mice also showed increase in the IgG, IgG1, IgG2a as compared to the control indicating the stimulation of both Th1 and Th2 immune responses. Increased production of IFN-γ in the splenocytes isolated from all the immunized groups suggest that T-cells also contribute to the protection against *Shigella* infection. Cell mediated immunity favours the production of IFN-γ, which boosts the production of IgG2a isotype by murine B cells (Finkelmen *et al.*, 1988). Interestingly, the titres were higher in co-immunized group as compared to other groups. Moreover, the ratio of IgG1 and IgG2a decreased significantly in co-immunized group, suggesting shift towards Th1 response which is in line with the highest production of IFN-γ in co-immunized group as compared to GroEL or IpaB groups. This is also evidenced by the higher lymphocyte proliferation in the splenocytes isolated from the co-immunized group as compared to the other groups. Requirement of IFN-γ for protection against *Shigella* infection in mice has been reported by Barillec *et al* (Le- Barillec *et al.* 2005, Jhel *et al.*, 2012). IFN-γ knockout mice lacking this cytokine showed increased susceptibility
to *Shigella* infection compared with mice not lacking this cytokine. Also the *Shigella* infection was not cleared in IFN-γ deficient mice (Way *et al*). In shigellosis patients, the expression of gamma interferon was twofold higher during convalescence than during the acute stage (Raqib *et al*., 1996).

IpaB domain region alone conferred 60-70% protection against *Shigella* Spp. Interestingly when GroEL was administered along with IpaB the survival percentage of mice increased to 80% against the lethal infection of *Shigella spp* indicating the adjuvant effect of GroEL. However, passive immunization studies showed 50% protection in mice immunized either with anti-IpaB or anti-GroEL and 60% with anti-GroEL+anti IpaB immune sera, demonstrating that antibodies alone are not sufficient for protection against Shigellosis. Since *Shigella* is an intracellular pathogen, cell mediated immunity is also required to fight against the infection through the secretion of proinflammatory mediators that will further promote innate immune cells and T helper cytokines that will support antibody production (Martinez Becerra *et al*., 2012).

In vaccine development, the choice of the adjuvant is as vital as the selection of vaccine antigen. An ideal vaccine adjuvant should induce both humoral and cell-mediated immunity, non-toxic and without any side-effects (Kurella *et al*., 2000). In this regard, *S*.Typhi GroEL can be considered as an effective adjuvant since it induces both arms of immunity as we reported earlier (Paliwal *et al*., 2008). This is the first report on the use of recombinant GroEL of *S*.Typhi as an adjuvant. Becerra *et al* also reported the induction of humoral and T cell immune responses when *Shigella* IpaB/IpaD was co-administered with double mutant heat labile toxin (dmLT) in mice (Martinez Becerra *et al*., 2012).
Further, organ burden studies revealed significant reduction in the number of colonies of *S. flexneri, S. boydii* and *S. sonnei* in the lung tissues of co-immunized mice as compared to IpaB or GroEL alone. Histopathological studies of lung tissue also showed improved tissue morphology in co-immunized mice challenged with *Shigella* spp than IpaB immunized group.

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