Chapter 6

Immunogenicity and Protective Efficacy of Recombinant Fusion Protein (IpaB-GroEL) Against Lethal Infection by S. flexneri, S. boydii and S. sonnei in Mice
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IMMUNOGENICITY AND PROTECTIVE EFFICACY OF RECOMBINANT FUSION PROTEIN (IpaB-GroEL) AGAINST LETHAL INFECTION BY S. FLEXNERI, S. BOYDII AND S. SONNEI IN MICE.

6.1. RESULTS


2.4 Kb fusion gene (801 bp IpaB and 1.6 Kb GroEL) was observed on the agarose gel after Overlap extension PCR. After ligation and transformation, cloned product was confirmed by colony PCR which showed 2.4 Kb fusion gene. The recombinant plasmid was isolated and showed 5.3 Kb fragment by single digestion (2.9 Kb pRSET A + 2.4 Kb fusion gene). Double digestion resulted in two fragments of size 2.9 Kb pRSET A and 2.4 Kb fusion gene which confirms the cloned fusion construct in BL-21 cells (Figure 6.1.1).

![Figure 6.1.1: Cloning of fusion gene (IpaB domain with GroEL). Lane 1: 1 Kb DNA ladder. Lane 2: 801 bp IpaB domain gene. Lane 3: 1.6 Kb GroEL gene Lane 4: IpaB - GroEL fusion gene. Lane 5: 2.9 Kb pRSET A expression plasmid digested with XhoI and HindIII. Lane 6: Cloned fusion gene confirmed by XhoI digestion of plasmid showing 5.3 Kb product (2.9 Kb pRSET A + 2.4 Kb fusion gene). Lane 7: Xho I and HindIII digested plasmid showing two bands of 2.9 Kb pRSETA and 2.4 Kb fusion gene which shows the cloned fusion gene.](image-url)
6.1.2. Expression, isolation and purification of the fusion protein (IpaB-GroEL).

The transformed cells were induced with 1mM IPTG and purified by Ni-NTA chromatography. The fusion protein (90Kda) was isolated and purified by Ni-NTA chromatography followed by refolding and dialysis. Finally purified fusion protein was concentrated using Amicon filters. The expression and the purity of fusion protein was detected by SDS-PAGE followed by western blotting (Fig. 6.1.2 a,b).

Figure 6.1.2: (a) Expression and purification of Fusion protein. Lane 1: Uninduced cells, Lane 2: IPTG induced cells with 90 KDA expressed protein, Lane 3: Protein MW marker, Lane 4&5: Eluted and purified protein. (b) Western blot confirming the expression of 90 KDa fusion protein. Lane 1) Expressed 90 KDa fusion protein. Lane 2) MW marker.
6.1.3. Determination of Antibody Titers and Isotyping

To assess the antibody mediated immune responses, mice were immunized with fusion protein. IgA, IgG, IgG1, IgG2a levels were determined in the sera of immunized mice by ELISA. Immunization (i.n.) with recombinant fusion protein showed robust antibody titers (IgG, IgA) as compared to control (Figure 6.1.3 a,b). Th1 and Th2 profile was studied in the immunized group by determining IgG1 and IgG2a antibodies. Increased levels of IgG1 and IgG2a were observed in the fusion protein immunized group as compared to control indicating the strong induction of both Th1 as well as Th2 type of immune responses (Figure 6.1.3 c). However fusion immunized mice showed higher serum IgG/IgA/IgG1/IgG2a antibody titers than the co-immunized group. Significant increase was observed in the IgG1 and IgG2a ratio of fusion immunized group as compared to control (p<0.01) (Figure 6.1.3 d) but the ratio was less than that of IpaB+GroEL co-immunized mice. This result indicates the presence of both Th1 and Th2 immune response to fusion protein but predominantly Th1 type of immune response. Mucosal type of immune response was observed by determining the IgA titers in the BAL fluid. Increased levels of the BAL IgA was observed in the fusion protein immunized group as compared to the control but more than co-immunized group suggesting the role of mucosal immunity also (Figure 6.1.3 e).
**Figure. 6.1.3: Antibody titers in mice.** (a) anti-mouse IgG, (b) anti-mouse IgA, (c) anti-mouse IgG1 and IgG2a, (d) IgG1/ IgG2a ratio, (e) IgA in BAL fluid. Sera samples which gave absorbance (OD) value >0.3 were measured for scoring. *Control vs Immunized groups p<0.01, # IpaB/GroEL vs IpaB+GroEL coimmunized groups p<0.01, ** IpaB/GroEL/IpaB+GroEL vs Fusion immunized group p<0.01.
6.1.4. Lymphocyte Proliferation and Cytokine responses

T cell responses were identified in the mice immunized with fusion protein by measuring the lymphocyte proliferation. Significant increase in lymphocyte proliferation was observed in the fusion protein group as compared to control (p<0.01) (Figure 6.1.4 a). The proliferation was appreciably higher in fusion group than IpaB/GroEL alone/IpaB+GroEL immunized groups (p<0.05).

To further characterize the type of immune response generated, splenocytes from both immunized and control mice were cultured in vitro for 72 hr and cytokines were measured in the culture supernatants. There was significant increase in IFN-γ, IL-4 and IL-10 level in immunized group as compared to control (Figure 6.1.4 b,c,d) IFN-γ mediates Th1 immune response while IL-4 and IL-10 confers Th2 response. This suggests the predominance of both Th1 and Th2 type of immune response in the fusion protein immunized mice (p<0.01) as revealed by IgG isotyping also.

6.1.5. Protective Efficacy of Fusion Protein against Shigella spp. in Mice.

Protective efficacy was determined in the fusion protein immunized mice against the lethal infection of *S. flexneri, S. boydii and S. sonnei*. All the control mice died within 3-4 days, whereas 90% protection was observed in the fusion protein immunized mice which confirms the efficacy of fusion protein (IpaB-GroEL) (Figure 6.1.5 a-c). The protection was significantly higher than other groups; IpaB (60-70%), GroEL (50-60%) alone, IpaB+GroEL co-administered (80%).
Figure. 6.1.4: Lymphocyte proliferation and cytokine levels in fusion protein immunized mice. (a) Lymphocyte proliferation. (b) IFN γ. (c) IL-4. (d) IL-10. *Control vs Immunized p< 0.01, # IpaB/GroEL vs IpaB+GroEL coimmunized groups p<0.05, ** IpaB/GroEL/IpaB+GroEL vs Fusion immunized group p<0.05.
Figure 6.1.5: Protection elicited by different recombinant proteins (IpaB, GroEL alone, IpaB+GroEL, IpaB-GroEL fusion). (n=10/group) 15 days after the last booster dose, mice were challenged with a lethal dose of (a) *Shigella flexneri* (1x10^7 CFU/ mouse), (b) *Shigella boydii* (1x10^7 CFU/ mouse) , (c) *Shigella sonnei* (1x10^7 CFU/ mouse) i.n. # Fusion vs Control/IpaB/ GroEL alone, * Fusion vs Co-immunized group, p<0.05.

6.1.6. Passive Immunization

To study the role of humoral immune response, mice were immunized with the heat inactivated anti fusion protein anti serum. After 24 hrs, mice were challenged with lethal dose of *S. flexneri, S. boydii* and *S. sonnei* as mentioned earlier. All the control mice challenged with the *Shigella* spp. died within 3-4 days, whereas 60% of passively immunized mice survived the lethal infection. As mentioned earlier, 50% mice survived after IpaB/GroEL immunization and 60% in co-administered group. This study confirms that only partial protection was conferred by the antibodies against the *Shigella* infection in mice (Figure 6.1.6 a-c).
Figure 6.1.6: Protective efficacy of passive transfer of immune sera on the survival of mice. Mice were challenged with 1x10^7 CFU/mouse (a) *S. flexneri* (b) *S. boydii* (c) *S. sonnei.* *p*<0.05 Fusion vs Control/ IpaB/ GroEL alone.

### 6.1.7. Organ Burden Studies

To assess the bacterial load in control and immunized mice, lungs were collected from individual animals. Tissues were homogenized in 5 ml PBS. Resulting homogenates were plated in 10-fold serial dilutions on LB agar plates followed by incubation at 37°C for 16-18 hrs. The number of colony forming unit (CFU) was counted. There was significant decrease in the CFU in the fusion immunized mice as compared to the control mice *p*<0.01 (Figure 6.1.7). The organ burden in other immunised groups was observed to be higher than fusion protein immunized group.
Figure 6.1.7: Bacterial burden in different immunized group and control groups. * Control vs Immunized p<0.05, # Fusion vs IpaB/ GroEL/ IpaB+GroEL immunized groups p<0.01.

6.1.8. Histopathology

Histopathology of lung tissues immunized with fusion protein showed normal morphology with uniform alveoli with no inflammatory infiltrates or edema in the alveolar spaces or bronchial lumina when compared to the control. This study confirms the protective efficacy of the fusion protein in all the groups challenged with *Shigella* spp. Control mice challenged with *Shigella* spp. showed edema and area of consolidation with heavy neutrophilic cell infiltration into the lung parenchyma (Figure 6.1.8 a-c) Fusion protein immunized group challenged with *S. flexneri* showed normal lung parenchyma with no inflammatory infiltrates or edema in the alveolar spaces or bronchial lumina (Figure 6.1.8 d-f).
Figure 6.1.8. Histopathology of Control and fusion protein immunized lung tissue of mice. The lungs tissues were excised, fixed in 10% formalin and embedded in paraffin blocks. Sections were stained with hematoxylin and eosin and analyzed through microscope. BL = Bronchial Lumen, AS = alveolar Space. Images shown at 400X magnification.

6.2. DISCUSSION

The immunogenicity and protective efficacy of IpaB, GroEL alone and when co-administered in mice have been reported so far in the present study. To further improve the immune responses, IpaB and GroEL were fused, the fusion protein was expressed, isolated and purified. The immunogenicity and protective efficacy of fusion protein (90 kDa) was then evaluated against Shigella spp. in mice. Fusion gene was constructed by overlap extension PCR, cloned and expressed in E.coli BL-21 cells. The fusion protein was further purified by Ni-NTA chromatography. Upon immunization, the antibodies produced against these conserved antigens IpaB and GroEL will efficiently prevent the colonization of Shigella in the host cells making them an effective vaccine candidate molecule. Studies also support the induction of serum antibody immune response to Shigella plasmid protein antigens in the
convalescent sera of the infected monkey and humans (Oaks et al., 1986). The present study revealed that immunization of mice with fusion protein i.n. showed a significant increase in both IgG and IgA antibody titers in sera as compared to the control and other immunized groups. Studies demonstrated the potential of the intranasal challenge mouse model for characterization of immune responses protecting against Shigella infection in mucosal epithelia (Van De Verg et al., 1995).

For a potential vaccine molecule, the type of immune response induced is very important which is determined by the IgG1 to IgG2a ratios. Antibody isotyping revealed elevated levels of IgG1 and IgG2a antibodies indicating the induction of both Th1 and Th2 type of immune responses though the ratio of IgG1 to IgG2a was IpaB > GroEL > IpaB+GroEL > Fusion IpaB-GroEL, indicating shift towards Th1 immune response. Mucosal immune response is determined by the increased IgA antibodies in BALF in the fusion protein immunized mice as compared to the control. It has been reported that a protein antigen administered through mucosal route can induce both mucosal immune response with IgA antibody and systemic immune response with the development of serum IgG inducing specific cellular immunity (Mewat and Weiner, 1999). The simultaneous increase of serum IgA, mucosal IgA has been reported in Shigella infected patients suggesting the massive increase in the local IgA production, giving an increase in systemic IgA (Islam et al., 1995).

Further, the type of immune response induced is confirmed by the cytokines produced. Increased level of IFN-γ suggests a robust Th1 type of immune response in the fusion immunized group as compared to the control. It has been reported that IFN-γ is required for protection from Shigella infection in vivo and that gamma interferon-mediated activation of host cells leads to killing of Shigellae in primary macrophages.
from wild-type and gamma interferon-deficient mice as well as in macrophage- and fibroblast-derived cell lines in vitro (Way et al., 1998). High levels of IL-4 and IL-10 indicates Th2 type of immune response in the immunized group. IL-10 plays a role as a stimulant of antibody production and as an anti-inflammatory agent reported to limit the consequences of the inflammatory response during shigellosis (Samandari et al., 2000). Immunization of mice with recombinant S. Typhi GroEL elicited both humoral and cellular immune responses as evidenced by the increased production of IFN-γ and IL-4 (Paliwal et al., 2008). It has also been reported that HIV-1 p24 antigen fused to the amino-terminus of mycobacterial hsp70 elicited both B cell and T cell responses in mice (Suzue and Young, 1996). In our study also, intranasal immunization of fusion protein also stimulated both humoral and cellular immune responses in mice. But as compared to the co-immunized group, the IgG1 to IgG2a ratio decreases gradually in the fusion protein immunized group which implies the switching towards the Th1 type phenotype from Th2 type. This result well correlates with the higher lymphocyte proliferation in the splenocytes isolated from the fusion immununized group as compared to the co-immunized group. This increased immunogenicity of fusion protein over co-administered proteins may due to the single polypeptide with the functional properties derived from both of the proteins (IpaB-GroEL).

Following the effect of immune responses in the fusion immunized mice protective efficacy was also studied. 90% of the mice immunized with the fusion protein survived against the i.n. lethal infection by S. flexneri, S. boydii and S. sonnei. The protection was significantly higher than other groups; IpaB (70%), GroEL (50-60%) alone, IpaB+GroEL co-administered (80%) indicating that fusion protein is more effective than either of recombinant protein alone or co-administered. Further
passive immunization with anti-fusion protein anti-serum conferred 60% protection against lethal infection by *Shigella* spp. 50% mice survived after IpaB/GroEL immunization and 60% in co-administered group. This result suggests that humoral immune response gave only partial protection in mice against *Shigella*. As *Shigella* is an intracellular pathogen, it has been hypothesized that cell-mediated immunity may be essential for defense against shigellosis (Sellge *et al.*, 2010; Samandari *et al.*, 2000), thus suggesting the necessity for both cellular and antibody mediated immunity to overcome *Shigella* infection.

Further, organ burden was estimated after the lethal challenge with the *Shigella* spp. in the fusion immunized and control mice. Significant decrease in the bacterial load was observed in the lungs of fusion protein immunized mice as compared to the control and other immunized groups. It has been reported that heat shock proteins especially Hsp 60 and Hsp 70 have an important cytoprotective role during lung inflammation and injury (Wheeler and Wong, 2007). Histopathological examination of these lung tissues also exposed normal lung parenchyma with no inflammatory infiltrates or edema in the immunized mice whereas control group revealed infected lung morphology with edema and neutrophilic infiltration.

In conclusion, recombinant fusion protein (IpaB-GroEL) was a potent vaccine molecule as revealed from their immunogenic and protective efficacy against *Shigella* species. Recombinant GroEL of *S.*Typhi, as an adjuvant molecule, improved the immune response to vaccine antigen IpaB effectively by modulating its immunogenicity eliciting both the humoral and cellular immune responses and increased the protective efficacy of mice against *Shigella* infection. Therefore, this
study strongly emphasizes the possible use of recombinant fusion protein (IpaB-GroEL) as a better subunit vaccine candidate molecule widely protective across all species of Shigella.