Chapter 4

The Cross-protective Efficacy of Recombinant GroEL (Hsp60) of S. Typhi Against Multiple Pathogens Including Shigella species
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THE CROSS-PROTECTIVE EFFICACY OF RECOMBINANT GroEL (Hsp60) OF S.TYPHI AGAINST MULTIPLE PATHOGENS INCLUDING SHIGELLA SPECIES

4.1. RESULTS

4.1.1. Hsp 60 sequence homology

Multiple sequence alignment reveals that Hsp 60 from S.Typhi shares 99 % sequence similarity with Hsp60 of Shigella Spp and E.coli. 96% with Hsp60 of K.pneumoniae and 79 % sequence similarity with P.aeruginosa (Figure 4.1.1).

![Figure 4.1.1: Hsp 60 (GroEL) sequence homology. Sequence similarity between Hsp60 of S.Typhi with Hsp60 of S.flexneri, S.dysenteriae type I, S.boydii, E.coli, K.pneumoniae, P.aeruginosa.](image)

4.1.2. *In vitro* Bactericidal Assay

The sera obtained 7 days after last immunization from GroEL immunized and control groups of mice were tested for their bactericidal activity against *S. flexneri*, *S. dysenteriae* type I, *S. boydii*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*. Absorbance in all
different control wells were in the range of 0.4 - 0.5. However, in wells containing test sera, lower OD values were observed in the range of 0.2- 0.3 indicating the bactericidal activity of the test serum (GroEL). Further, a significant decrease in CFU in test sera group (GroEL) when compared to the control group at different sera dilutions was observed ($p < 0.05$). No significant difference was observed between the various control groups (Data not shown). So only one control was considered for each pathogen for comparison. The sera dilution which showed $> 50 \%$ inhibition of the bacterial growth was considered as bactericidal titre. GroEL sera dilution of 1: 64 inhibited 50-55 % CFU of *Shigella* Sp., 65-75 % CFU of *E.coli*, sera dilution of 1: 128 gave 60-65% inhibition of *K.pneumoniae*, sera dilution of 1: 32 inhibited approximately 45-50 % CFU of *P.aeruginosa* (Figure 4.1.2).

*Figure 4.1.2: In vitro bactericidal effect of GroEL antisera.* Control groups contain adjuvant immunized serum and the respective pathogens. Test groups contain GroEL immunized serum and the respective pathogens. Results are representative of three independent experiments (Control Vs Test - * $p < 0.01$, # $p < 0.001$ by t-test).
4.1.3. *In vivo* Challenge Studies

15 days after the last immunization, mice were challenged with *S.flexneri, S.dysenteriae* type I, *S.boydii, E.coli, K.pneumoniae, P.aeruginosa*. There was significant difference in the number of mice survived between the control and immunized groups. All the control mice died within 5 days of challenge with the above pathogens whereas GroEL immunized group showed 60-65 % protection against the lethal infection by *S.flexneri* (*p*<0.01), *S.dysenteriae* type I (*p*<0.05), *S.boydii* (*p*<0.01), 75-80 % cross-protection (*p*<0.05) was observed against enteropathogenic *E.coli*, 70-80 % survival against *K.pneumoniae* (*p*<0.01) and 50 % was recorded against *P.aeruginosa* (*p*<0.05) (Fig. 3). Statistical significance in the survival of mice was determined by *t* test between control and GroEL immunized group from the three independent experiments done (Figure 4.1.3).

4.1.4. Organ burden

Bacterial organ load was estimated from spleen, liver, lung and intestine collected from different groups of mice challenged with different pathogens. There was significant decrease in CFU in liver, spleen and intestine of the GroEL immunized mice challenged with *S.flexneri, S.dysenteriae* type I, *S.boydii* and *E.coli* when compared to the control (Figure 4.1.4 a, *p*<0.05). Similarly mice challenged with *K.pneumoniae* showed significant differences in the bacterial burden between immunized and control group (Fig. 4.1.4 b, *p*<0.05). Reduction in *P.aeruginosa* cells was observed in liver and spleen samples of immunized group when compared to the control group (Figure 4.1.4 b, *p*<0.05).
Figure 4.1.3: Effect of GroEL immunization on survival of mice. Groups of mice (6) were immunized on day 0 i.p. with 40 µg GroEL/mouse emulsified in complete Freund’s adjuvant followed by two booster injections using 40 µg GroEL/mouse emulsified in incomplete Freund’s adjuvant on the 7th and 28th days. After 15 days from the last immunization, i.e., 43rd day, the mice were challenged with (a) S. flexneri, (b) S. dysenteriae type I, (c) S. boydii, (d) E. coli, (e) K. pneumoniae, (f) P. aeruginosa. Graph shows the percent survival of mice and statistical significance was determined in the number of mice survived between control and GroEL immunized group by t test (* represents $p < 0.05$, ** represents $p < 0.01$).
Figure 4.1.4: Organ burden estimated by the average CFU/ml. (a) liver, spleen and intestine of control (Adjuvant immunized) and GroEL immunized mice challenged with *S. flexneri*, *S. dysenteriae* type I and *S. boydii*, (b) *E. coli*, *K. pneumoniae* and *P. aeruginosa*. Statistical significance was determined by *t* test between control and GroEL immunized group (* represents *p* < 0.05, ** represents *p* < 0.01, # represents *p* < 0.001).
4.1.5. Histopathology

Intestinal sections of control mice challenged with *S. flexneri, S. dysenteriae* type I, *S. boydii* cells showed infected villi with inflammatory exudate in the intestinal lumen while the GroEL immunized group showed intact villi in the intestinal lumen without any inflammatory cells (Figure 4.1.5. a-f). Intestinal sections of control mice challenged with *E. coli* cells showed loss of intestinal epithelial cells and dilated blood vessels at the tip of the villi and presence of inflammatory cells in the intestinal lumen while the immunized mice showed intact intestinal epithelium and no inflammatory cells in the lumen (Figure 4.1.5. g-h). Spleen cells of control mice infected with *P. aeruginosa* showed pronounced atrophy of the lymphoid cells in the white pulp and vascular congestion in the red pulp while in the immunized group better red and white pulp areas were seen in the splenic parenchyma (Figure 4.1.5. i-j). Section of lung was seen infected with the *K. pneumoniae* cells in the control group, while the immunized group showed improved lung parenchyma with uniform alveoli. No inflammatory cell infiltrate was seen (Figure 4.1.5. k-l).
**Figure 4.1.5: Comparative histology of different tissues of control (adjuvant immunized) and GroEL immunized mice.**

(a) Control mice challenged with *S. flexneri* showing villi with inflammatory exudate in the intestinal lumen. (b) GroEL immunised mice challenged with *S. flexneri* showing intact villi with intestinal lumen without any inflammatory cells. (c) Control mice challenged with *S. dysenteriae* type 1 showing necrosis of villi with collection of inflammatory cells in intestinal lumen. (d) GroEL immunized mice shows intact villi with intestinal lumen as compared to control. (e) Control mice challenged with *S. boydii* showing loss of intestinal epithelial cells and presence of inflammatory cells in the intestinal lumen. (f) Mice immunised with GroEL shows intact intestinal epithelium and no inflammatory cells in the lumen. (g) Intestinal section of control mice challenged with *E. coli* showing loss of intestinal epithelial cells and dilated blood vessels at the tip of the villus and presence of inflammatory cells in the intestinal lumen. (h) GroEL immunised mice showing intact intestinal epithelium and no inflammatory cells in the lumen. (i) Spleen section of control mice challenged with *P. aeruginosa* shows a pronounced atrophy of the lymphoid cells in the white pulp and vascular congestion in the red pulp. (j) GroEL immunized mice showing better red and white pulp areas in the splenic parenchyma as compared to control. (k) Lung section of control mice challenged with *K. pneumoniae* showing a terminal bronchiole and adjacent alveoli. The alveolar septae show vascular congestion with numerous RBC filling the septal blood vessels. (l) GroEL immunized mice showing lung parenchyma with uniform alveoli. No inflammatory cell infiltrate is seen. Images shown at 100X magnification.
4.2. DISCUSSION

Increased antimicrobial resistance among the bacterial pathogens has limited the efficacy of traditionally used antibiotics against various microbes (Taylor 2003; Dupont 2004). So, the best way to combat infections by these pathogens is to prevent them in the first place, by developing an effective vaccine using a common immunodominant molecule. Since HSP elicits both humoral as well as cell-mediated immune responses, they have the potential to be developed as new generation prophylactic and therapeutic vaccines against infectious agents (Mountzouros et al., 2000; Murshid et al., 2001; Hosseinzadeh 2012). During infection, HSPs, as molecular chaperones, associate with unfolded or newly synthesized or denatured antigenic microbial proteins. As a result, B cells with cell surface B-cell receptors specific for a microbial antigen can internalize the microbial HSP together with the microbial antigen, process them both, and present peptides derived from the two proteins in the context of MHC class II molecules for recognition by T helper cells (Kaufmann 1990; Eden et al., 2005).

Earlier we have reported significant protection (70-90%) elicited by recombinant HSPs of S.Typhi against lethal challenge by S.Typhi and S.typhimurium (Sagi et al., 2006; Paliwal et al., 2008; Bansal et al., 2010; Paliwal et al., 2011). Since HSPs are evolutionarily conserved molecules, this cross protection indicates that the immune response is directed at shared epitopes between these Salmonella serovars, suggesting the use of HSPs in prevention of diseases caused by other pathogens viz; S. flexneri, S. dysenteriae type I, S. boydii, E. coli, P. aeruginosa and K. pneumoniae. Presently no effective vaccine is available against these pathogens, therefore, the present study was undertaken to evaluate the cross-protective efficacy of recombinant
GroEL of S.Typhi against these microbes. Hsp60 specific antibodies have been detected in patients with Tuberculosis and Leprosy, and also in mice after infection with *Mycobacterium tuberculosis* (Young *et al.*, 1988; Shinnick, 1991). Similarly increased antibody levels to Hsp70 have been identified in sera of patients suffering from Malaria, Leishmaniasis, Schistosomiasis, Filariasis and Candidiasis (Shinnick, 1991). Antibodies specific for both Hsp60 and Hsp70 of *Chlamydia trachomatis* have been detected in the sera of patients infected with *C.trachomatis* (Sanchez-Campillo *et al.*, 1999). Taken together, these findings indicate that HSPs are important immunogenic antigens in infection.

Due to the homology of HSPs between the species, these were considered as candidates causing autoimmune diseases and hence were thought to be a poor vaccine candidate (Elías *et al.*, 1990; Boog *et al.*, 1992; Abulaﬁa-Lapid *et al.*, 2003; Puga Yung *et al.*, 2009). However, most people don’t develop dangerous autoimmune responses to self-HSPs, although they do possess T-cells which recognize these self HSPs, suggesting that these cells are highly regulated (Rees *et al.*, 1988; Munk *et al.*, 1989; Schawartz, 1989). HSPs represent unique targets for γδ T cells and these cells are considered to contribute to the first line of defence. A minimal peptide of mycobacterial Hsp60 which is not homologous to the mammalian Hsp60 allows recognition by Hsp60 reactive γδT cells (Fu *et al.*, 1994). Further, detailed analysis of HSP induced immune responses in experimental model shows that reactivity to self-HSPs can down regulate the disease process rather than promoting the disease (Eden *et al.*, 2005; de Graeff-Meeder *et al.*, 1995).
There are numerous studies reporting Hsp60 as a potent candidate vaccine molecule against various diseases (Lee et al., 2006; Wilhelm et al., 2005; Noll et al., 1996; Lowrei et al., 1997). Vaccination of mice with recombinant Hsp60 from *Histoplasma capsulatum* induced protection against pulmonary histoplasmosis (Gomez et al., 1995). Immunization of mice with recombinant GroES-GroEL from *Helicobacter pylori* protected the animals against subsequent infection and development of gastroduodenal disease (Ferrero et al., 1995).

The *in vitro* bactericidal assay was performed in the present study to assess the protective activity of the anti-sera from GroEL immunized mice against other microorganisms. There was significant decrease (55-75%) in the number of CFU of all the pathogens studied in the sera group (GroEL) as compared to the control and the sera dilutions showing more than 50% inhibition of bacterial growth was considered as bactericidal titer. The serum bactericidal assay (SBA) is a functional measure of the ability of antibodies in conjunction with complement to kill bacteria and is considered the assay of choice for measurement of functional antibodies *in vitro*. This assay relies upon conditions in which antibody recognizes the surface exposed antigens and binds to the complement (activation via the classical pathway), resulting in the bacteriolysis and death of the target organisms (Mountzouros and Howell, 2000; Rodriguez et al., 2002; Romero-Stainer et al., 2004).

The abundance of GroEL combined with its surface expression makes it a major antigen and its highly conserved nature makes it a common antigen providing some degree of cross-protection between different infections (Kaufmann, 1990). We determined the *in vivo* cross-protective efficacy of GroEL of *S. Typhi* in GroEL...
immunized mice by challenging them with the lethal dose of \textit{S. flexneri}, \textit{S. dysenteriae} type I, \textit{S. boydii}, \textit{E. coli}, \textit{K. pneumoniae} and \textit{P. aeruginosa}. The results revealed that immunization of mice with \textit{S.Typhi} GroEL conferred 60-65\% protection against all \textit{Shigella} Spp., 75-80\% protection against \textit{E. coli}, 70-80\% protection observed against \textit{K. pneumoniae}, 50\% protection against \textit{P. aeruginosa}. It was reported that \textit{Porphyromonas gingivalis} GroEL cross-protected against periodontal disease induced by multiple pathogenic bacteria (Lee \textit{et al.}, 2006). However, to the best of our knowledge, we report for the first time the efficacy of \textit{S.Typhi} GroEL immunization in according cross-protection against \textit{S. flexneri}, \textit{S. dysenteriae} type I, \textit{S. boydii}, \textit{E. coli}, \textit{K. pneumoniae} and \textit{P. aeruginosa} infections. The protective mechanism could be mediated by induction of both humoral and cellular immune responses as revealed by our earlier studies (Sagi \textit{et al.}, 2006; Paliwal \textit{et al.}, 2008; Bansal \textit{et al.}, 2010; Paliwal \textit{et al.}, 2011). Antibody isotyping showed production of both IgG1 and IgG2a antibodies indicating the stimulation of both Th1 and Th2 type of immune responses. We also reported that the passive immunization with anti-GroEL sera provided only partial protection (50\%) against \textit{S.Typhi} infection in mice (Paliwal \textit{et al.}, 2008). In the present study also, \textit{in vitro} bactericidal assay using anti-GroEL sera showed partial inhibition of bacterial growth providing evidence for the requirement of both the arms of immunity for protection against various pathogens.

The organ burden studies further revealed the reduction in the colonization of various pathogens in different tissues of mice immunized with \textit{S.Typhi} GroEL. The bacterial count was significantly decreased in the liver, spleen and intestine of immunized animals challenged with \textit{S. flexneri}, \textit{S. dysenteriae} type I, \textit{S. boydii}, \textit{E. coli} and \textit{P. aeruginosa} as compared to unimmunized animals. Similarly, decreased bacterial burden
was observed in liver, spleen, lung of immunized mice infected with *K. pneumoniae*. Histopathological studies also showed improved tissue morphology in GroEL immunized mice challenged with different pathogens as compared to controls.

In conclusion, our findings reveal that immunization of mice with recombinant GroEL of *S.*Typhi is not only protective against *Salmonella* infections but elicits cross-protection against other bacterial infections also, indicating the immense potential of GroEL to be developed as a single vaccine candidate protective against multiple pathogens.