Results
Capsular polysaccharide is the main virulence factor of *S. pneumoniae* as it shields it from immune assaults. The protective immunity against pneumococcus is primarily antibody mediated. Serotype 1 is a significant contributor to the global burden of pneumococcal diseases. PCP1 is a zwitterionic polysaccharide. Zwitterionic polysaccharides are known to activate T cells in an MHC class II dependent manner; therefore there is a need to study how PCP1 modulates responses from immune cell.

3.1 PCP1 binds to the surface of immune cells

PCP has been detected in respiratory secretions, serum and urine of pneumococcal patients thus raising the possibility that this molecule could interact with cells of the immune system during systemic dissemination of *S. pneumoniae*. We were interested in studying the possible role of PCP1 in modulation of immune response by pneumococcus, given that immune responses are typically initiated by the physical interaction of the antigen with the immune cells. We investigated the possible physical interaction of PCP1 with various cell lines (RAW264.7, GG2EE, A-20, DC2.4 and Jurkat cells). Cells were incubated in a 96 well plate with 50 µg/ml of PCP1 for 2 hr at 4°C. The surface bound PCP1 was detected using rabbit type sera against PCP1 as primary antibody followed by FITC labelled goat anti rabbit Ig antisera as secondary antibody. Flow cytometric analysis revealed that PCP1 bound to the surface of RAW264.7 (Figure 8a) and GG2EE cells (Figure 8b) as observed by the rightward shift compared to primary antibody control (blue peak) and unstained cells (shaded histogram). Binding was also observed
Figure 8: PCP1 binds to the surface of various immune cells in vitro. Cells were incubated with 50 μg/ml of PCP1 at 4°C for 2 hr followed by staining with anti-PCP1 rabbit sera for 1 hr. Subsequently the cells were stained with FITC conjugated goat anti rabbit Ig antisera for 1 hr and analyzed by flow cytometry. Flow cytometric analysis of PCP1 binding with RAW264.7 (a), GG2EE (b), A-20 (c), DC2.4 (d) and Jurkat (e). Shaded histogram represents unstained cells.
with A-20 cells (Figure 8c) and dendritic cell line DC2.4 (Figure 8d) although to a lesser extent as compared to binding with RAW264.7 and GG2EE. PCP1 did not bind with T cell line Jurkat (Figure 8e). To investigate whether this binding was true for ex vivo cells, splenocytes from C57BL/6 mice were fractionated into adherent (Figure 9a) and non-adherent fractions (Figure 9b) and analyzed flow cytometrically. It was observed that PCP1 preferentially bound to the adherent fraction which is known to comprise primarily of monocytes and macrophages. This data suggested that PCP1 physically interacts with certain cell types like macrophages.

Since macrophages are known to form an important link between innate and acquired immune systems, and play a crucial role in shaping the adaptive immune response, we chose RAW264.7 as a model macrophage cell line for further experiments. RAW264.7 cells were incubated with 50 to 500 µg/ml of PCP1 for 1 hr at 4°C and analyzed for surface binding using flow cytometry. PCP1 exhibited a dose dependent binding to the surface of RAW264.7 cells (Figure 10).

3.2 Interaction of PCP1 with different immune cell types leads to pro-inflammatory responses

The above data suggests that PCP1 binds to the surface of various immune cell types. We were interested in finding out the functional consequence of these interactions. We incubated RAW264.7 cells with 100, 250 and 500 µg/ml of PCP1 for 24 hr and assessed the culture supernatants for TNF-α and
Figure 9: PCP1 preferentially binds to splenocyte adherent fraction. Cells were incubated with PCP1 at a concentration of 50 μg/ml at 4°C for 2 hr followed by staining with anti-PCP1 rabbit sera for 1 hr. Subsequently cells were stained with FITC conjugated goat anti rabbit Ig antisera for 1 hr and analyzed by flow cytometry. Surface binding of PCP1 with adherent (a) and non-adherent fraction (b) of splenocytes isolated from C57BL/6 mice. Shaded histogram depicts unstained cells.
Figure 10: PCP1 binds to the surface of RAW264.7 cells in a dose dependent manner. RAW264.7 cells were incubated with various concentrations of PCP1 (50 to 500 µg/ml) for 2 hr at 4°C followed by staining with anti-PCP1 rabbit sera for 1 hr. Subsequently, cells were stained with FITC conjugated goat anti rabbit Ig antisera for 1 hr and analyzed by flow cytometry. Shaded histogram represents unstained cells.
IL-6 production by ELISA. PCP1 induced RAW264.7 cells to produce TNF-α in a dose dependent manner (Figure 11a), but no IL-6 was detected in these culture supernatants (Figure 11b). Since we were using PCP1 at concentrations up to 500 µg/ml, we checked whether PCP1 had any cytotoxicity at these concentrations. We assessed this using MTT assay. Cells pre-stimulated with PCP1 for 24 hr were incubated with MTT dye for 4 hr after which DMSO was added to stop the reaction. Data indicates that incubation of RAW264.7 cells with PCP1 for 24 hr did not induce cell death (Figure 11c). This implies that PCP1 is not cytotoxic to RAW264.7 cells at the concentrations used.

Commercial preparations of PCP are known to contain about 5% (w/w) CWPS as a contaminant (Skovsted et al., 2007). CWPS is known to be pro-inflammatory in nature. To rule out the possibility that the observed inflammatory response was due to contaminating CWPS, we incubated RAW264.7 cells with PCP1 at concentrations ranging from 50 to 500 µg/ml or CWPS at concentrations ranging from 5 to 250 µg/ml. Culture supernatants were collected 24 hr post stimulation and assayed for TNF-α by ELISA (Figure 12). The data indicated that PCP1 induced TNF-α production from RAW264.7 cells is primarily due to PCP1 with a small contribution from CWPS. For example, 500 µg/ml of PCP1 induced 882 ± 95 ng/ml of TNF-α, while 50 µg/ml of CWPS induces only 184 ± 44 ng/ml of TNF-α. Assuming PCP1 preparation is contaminated with 10% (w/w) CWPS (as opposed to 5% reported in the literature), the contribution of CWPS to PCP1 induced TNF-α
Figure 11: PCP1 induces the production of pro-inflammatory cytokine from RAW264.7 cells. RAW264.7 cells were incubated with media alone, LPS (100 ng/ml) or with PCP1 at 100, 250 and 500 μg/ml for 24 hr. Supernatants were assessed for TNF-α (a) and IL-6 (b) levels by ELISA. LPS served as a positive control. (c) MTT assay was done to assess for cytotoxicity of PCP1. Data is presented as average ± SD (standard deviation). Statistical significance was determined by Student’s t test where * denotes p < 0.05; ** denotes p ≤ 0.001 when compared with unstimulated (US) sample.
Figure 12: Comparison of PCP1 and CWPS induced TNF-α production by RAW264.7 cells. RAW264.7 cells were incubated with media alone (US, unstimulated), LPS (100 ng/ml) or with the indicated concentrations of PCP1 (a) and CWPS (b) for 24 hr. Culture supernatants were assayed for TNF-α levels by ELISA. Data is presented as average ± SD. Statistical significance was determined by Student's t test where * denotes p < 0.05; ** denotes p ≤ 0.001 when compared with unstimulated (US) sample.
Results

is ~21%. We next investigated whether this was a cell line specific phenomenon. Incubation of THP-1, a human monocyctic cell line with PCP1 for 8 hr resulted in a dose dependent production of TNF-α (Figure 13a) and IL-8 (Figure 13b). DC2.4 cells when stimulated with various concentrations of PCP1 for 24 hr resulted in the production of TNF-α (Figure 13c). The data indicates that the pro-inflammatory activity of PCP1 was not restricted to a particular cell line or cell type or cytokine. Incubation of human PBMCs with PCP1 led to the dose dependent production of TNF-α (Figure 13d), pointing to the importance of PCP1 during infection.

3.3 PCP1 engages TLR2 to activate immune responses

Toll-like receptors have been implicated in the modulation of immune responses induced by various bacterial molecules. For example, TLR2, expressed by macrophages, is known to interact with bacterial polysaccharides like PSA from *B. fragilis* and Vi from *S. typhi*. Besides TLR2 plays a vital role in pneumococcal pathogenesis. This prompted us to analyze the role of TLR2 in PCP1 mediated immune responses. We use the human embryonic kidney cell line HEK293T as it is unresponsive to TLR ligands like LPS and Pam3Csk.

Untransfected, and TLR1 and TLR2 double transfected HEK293T cells were incubated with 100, 250 and 500 μg/ml of PCP1 for 8 hr, after which the culture supernatants were assayed for IL-8 by ELISA. TLR1 and TLR2 double transfected HEK293T cells secreted IL-8 upon stimulation with PCP1 (Figure
Figure 13: PCP1 induced cytokine production is not cell line or cytokine specific. THP-1 cells were incubated with media alone (US, unstimulated), LPS (50 ng/ml) or with indicated concentrations of PCP1 for 8 hr and resultant supernatants were assessed for TNF-α (a) and IL-8 (b) levels by ELISA. Supernatants from DC2.4 (c) and hPBMCs (d) incubated with media, LPS or PCP1 for 24 hr were assessed for TNF-α levels. LPS was used as a positive control. Data is presented as average ± SD. Statistical significance was determined by Student's t test where * denotes p < 0.05; ** denotes p ≤ 0.001 when compared with US. hPBMCs, human PBMCs.
but untransfected HEK293T cells did not produce IL-8 (Figure 14b) suggesting a role of TLR2/1 heterodimer complex in PCP1 mediated immune response.

To confirm the role of TLR2 in PCP1 mediated inflammatory responses, RAW264.7 cells were pre-incubated with either TLR2 blocking antibody or an isotype matched control antibody at 30 μg/ml for 2 hr after which the cells were stimulated with 100, 250 or 500 μg/ml of PCP1 for 24 hr and supernatants were assessed for TNF-α production (Figure 15). Pre-incubation with TLR2 blocking antibody or isotype control antibody did not affect RAW264.7 cells in media alone. TNF-α production from Pam3Csk (a synthetic TLR2 ligand) treated cells was reduced by pre-incubation of the cells with TLR2 blocking antibody but not with the isotype control antibody. More importantly PCP1 (used at 100, 250 and 500 μg/ml) failed to stimulate cells to produce TNF-α when cells were pre-incubated with TLR2 blocking antibody. However, responses from these cells were not affected when they were pre-incubated with an isotype matched control antibody indicating that PCP1 engaged TLR2 on macrophages to generate inflammatory responses. TLR2 blocking antibody did not affect TNF-α produced from LPS (a TLR4 ligand) treated cells (Figure 15). This data suggests that engagement of TLR2 by PCP1 is important for production of proinflammatory cytokine from RAW264.7 cells.
Figure 14: PCP1 activates HEK293T cells expressing TLR1 and TLR2. HEK293T cells transfected with TLR1 and TLR2 (a) and untransfected HEK293T cells (b) were incubated with media (US, unstimulated), Pam$_3$Csk (Pam) or PCP1 at the indicated concentrations for 8 hr after which supernatants were assayed for IL-8 by ELISA. Pam$_3$Csk, a synthetic TLR2 ligand, was used as a positive control. Data is presented as average ± SD. Statistical significance was determined by Student’s t test where * denotes p < 0.05; ** denotes p ≤ 0.001 when compared with unstimulated sample. hIL-8, human IL-8.
Figure 15: PCP1 engages TLR2 on macrophages to mediate its responses. RAW264.7 cells were pretreated with medium, isotype control (IC) antibody or anti-TLR2 blocking antibody (30 μg/ml) for 2 hr and stimulated with Pam3Csk (Pam), LPS (50 ng/ml) or PCP1 (100 μg/ml, P100; 250 μg/ml, P250; 500 μg/ml, P500) for 24 hr. Culture supernatants collected were assayed for TNF-α production by ELISA. Pam3Csk and LPS were used as positive and specificity control, respectively. Data is presented as average ± SD. Statistical significance was determined by Student's t test where * denotes $p < 0.05$; ** denotes $p \leq 0.001$ when compared with respective PCP1 stimulated group.
3.4 PCP1 modulates intracellular signalling events to induce pro-inflammatory cytokines

Engagement of TLR2 on monocytes and macrophages triggers MAP kinase and NF-κB signalling pathways resulting in the secretion of cytokines. To identify the intracellular signalling events triggered by PCP1, RAW264.7 cells were incubated with PCP1 at a concentration of 250 μg/ml for the indicated time periods following which cell lysates were prepared, run on SDS PAGE and blotted for phospho ERK, phospho JNK, I-κB and total ERK signalling molecules (Figure 16). In PCP1 stimulated cells, we detected ERK phosphorylation at 30 min with maximum activation being observed at 60 min as compared to unstimulated cells (Figure 16a). Upon probing for NF-κB pathway, we observed a time dependent degradation of I-κB in PCP1 treated cells with maximum degradation being observed at 60 min time point in comparison to unstimulated cells (Figure 16b). We observed that PCP1 does not activate p38 MAP kinase pathway in RAW264.7 cells as no significant change in the phosphorylation of p38 was observed (Figure 16d). The same blots were reprobed for total ERK as loading control (Figure 16c and e). Our data indicates that PCP1 induces the NF-κB and MAP kinase pathways in RAW264.7 cells.

To confirm the role of phospho ERK kinase pathway in PCP1 activity, we used a MAP kinase specific inhibitor PD98059. PD98059 binds to the inactive form of MEK1 and prevents its activation by upstream activators thereby blocking MAP Kinase pathway. RAW264.7 cells were incubated with
Figure 16: PCP1 activates Phospho ERK and NF-κB signaling pathway. Lysates of RAW264.7 cells stimulated with 250 μg/ml of PCP1 for indicated time points (5, 15, 30 or 60 min) were run on SDS-PAGE and transferred to NC membrane. The membrane was probed for Phospho ERK (a) and then reprobed for I-κB (b) and total ERK (c). Lysates of stimulated RAW264.7 cells were immunoblotted for Phospho p38 (d) and then reprobed for total ERK (e). US, unstimulated cells. In panel a, b and c water has been used as a vehicle control.
PD98059 or medium (RPMI-10) for 2 hours after which the cells were stimulated with 100, 250 and 500 µg/ml of PCP1. Twenty four hr post stimulation, culture supernatants were collected and assayed for TNF-α by ELISA. We observed that RAW264.7 cells incubated with medium were not affected by pre-treatment with PD98059 (Figure 17). Pre-treatment of cells with the inhibitor at 50 and 200 µM PD98059 led to an abrogation of TNF-α production from LPS stimulated RAW264.7 cells. Pre-incubation of RAW264.7 cells with the inhibitor for 2 hr resulted in a dose dependent reduction in TNF-α production from PCP1 treated RAW264.7 cells (Figure 17). These data together indicate that PCP1 induces increase in the phosphorylation of ERK thereby activating MAP kinase pathway and this activation can be blocked by the use of Map Kinase inhibitor PD98059.

3.5 Acetyl groups are important for PCP1 induced inflammatory responses

Structural analysis of PCP1 has shown that it contains both O and N-linked acetyl groups. Acetyl groups of capsular polysaccharide of *Neisseria meningitidis* have been shown to be important for generating protective antibody responses in BALB/c mice. We wanted to assess whether acetyl groups present on PCP1 were important for its activity. PCP1 was treated with 2 M NaOH to remove acetyl groups. Deacetylation was confirmed using a biochemical assay. Compared to PCP1, the treated sample displayed negligible absorbance at 540 nm, suggesting deacetylation of PCP1 (Figure 18a). In contrast to PCP1, deacetylated PCP1 failed to induce RAW264.7
Figure 17: PCP1 activates phospho ERK MAP kinase pathway in macrophages. RAW264.7 cells were incubated with medium (RPMI-10), 50 or 200 μM PD98059 for 2 hr and stimulated with LPS at 50 ng/ml or PCP1 (100 μg/ml, P100; 250 μg/ml, P250; 500 μg/ml, P500) for 24 hr. LPS was used as a positive control. The culture supernatants were collected and assayed for TNF-α by ELISA. Data is presented as average ± SD. Statistical significance was determined by Student's t test where * denotes p < 0.05; ** denotes p ≤ 0.001 when compared with respective PCP1 stimulated group.
Figure 18: Acetyl groups are important for activity of PCP1. (a) PCP1 was deacetylated by treatment with NaOH and deacetylation was confirmed by reaction with hydroxylmine. (b) RAW264.7 cells were incubated with the indicated concentrations of PCP1 and deacetylated PCP1 for 24 hr after which culture supernatants were collected and assayed for TNF-α by ELISA. Data is presented as average ± SD. (c) Flow cytometric assessment of surface binding of PCP1 and deacetylated PCP1 with RAW264.7 cells. Cells were incubated with PCP1 or deacetylated PCP1 (50 μg/ml) at 4°C for 2 hr followed by anti-PCP1 rabbit sera for 1 hr. Subsequently, cells were stained with FITC conjugated goat anti-rabbit Ig antisera for 1 hr and analyzed by flow cytometry. Shaded histogram represents unstained cells.
cells to produce TNF-α (Figure 18b). The complete loss of inflammatory potential of PCP1 upon deacetylation was consistent with loss in the ability of deacetylated PCP1 to bind RAW264.7 cells (Figure 18c). These data suggest that acetyl groups present on PCP1 are important for binding RAW264.7 cells and TNF-α production.

3.6 Deacetylation of PCP1 compromises its ability to induce PCP1-specific antibody responses

To check whether deacetylation of PCP1 affected the anti-PCP1 antibody responses, PCP1 and deacetylated PCP1 were injected in BALB/c mice and serum antibody titres were assessed by ELISA. Deacetylated PCP1 immunized mice evoked poor total Ig, IgM and IgG anti-PCP1 antibody compared to PCP1 (Figure 19) suggesting that antibody responses are generated against the acetyl groups of PCP1. Comparative analysis suggested that a major portion of the anti-PCP1 antibody response is directed towards the backbone shared by PCP1 and deacetylated PCP1 (Figure 19a versus c, e versus g, i versus k, a versus b, e versus f and i versus j). This indirectly suggests that a small but significant proportion of the anti-PCP1 antibody response is directed against its acetyl groups also.

3.7 Kinetics of anti-PCP1 antibody responses depends on the form in which PCP1 is administered

We were interested in studying the kinetics of anti-PCP1 antibody response when PCP1 was administered as a soluble antigen or in context of the whole
Figure 19: Antibodies are generated against the acetyl groups present in PCP1. BALB/c mice (n = 6) were injected with 5 μg of PCP1 (a, c, e, g, i and k) or deacetylated PCP1 (b, d, f, h, j and l) and sera was obtained on day 0, 4, 8 and 12. Total lg antibodies against PCP1 (a and b) and deacetylated PCP1 (c and d); IgM against PCP1 (e and f) and deacetylated PCP1 (g and h); IgG against PCP1 (i and j) and deacetylated PCP1 (k and l) (diluted 1 in 100) were determined by ELISA. Each dot represents data from individual mouse. Horizontal bar denotes median of the respective group. Statistical significance was determined by Student's t test where * denotes p < 0.05; ** denotes p ≤ 0.001 when compared with the preimmune control.
Figure 19 contd.
Immunized with PCP1

Immunized with deacetylated PCP1

Figure 19 contd.
Figure 20: The kinetics of anti-PCP1 antibody response is faster when PCP1 is administered as a soluble antigen compared to when given as hk 6301. 5 μg of PCP1 (a) or 10⁹ cfu hk 6301 (b) was injected intraperitoneally into BALB/c mice (n = 6). Sera collected on day 0, 8, 16, 28 and 42 were analyzed at 1 in 100 dilution for PCP1 specific total Ig production by ELISA. Each dot represents data from individual mouse. Horizontal bar indicates median of the respective group. Statistical significance was determined by Student’s t test where * denotes p < 0.05; ** denotes p ≤ 0.001 when compared with the preimmune control.
heat-killed *S. pneumoniae* strain ATCC 6301 (hk 6301). BALB/c mice were immunized intraperitoneally with 5 μg of PCP1 or $10^9$ cfu of hk 6301 and the sera obtained was analyzed for anti-PCP1 antibodies by ELISA (Figure 20). We observed that anti-PCP1 antibody response peaked at day 8 and 28 (or later) when PCP1 was given as a soluble antigen and as whole hk 6301, respectively (Figure 20). We observed that PCP1 and hk 6301 immunized mice generated anti-PCP1 IgM (Figure 21a and b) and IgG antibodies (Figure 21c and d). IgG subtyping data is shown in Figure 22. Interestingly PCP1 when administered as a soluble antigen induced class switching similar to that observed in the case of PCP1 when administered in context of hk 6301 with IgG1 and IgG2b being the predominant antigen-specific subtypes detected in both the sera.

Recent studies have shown that zwitterionic polysaccharides like PSA and PCP1 induce T cell activation in an MHC class II dependent manner. We were interested in investigating whether MHC class II mediated presentation of PCP1 had any effect on the anti-PCP1 antibody production. We used C57BL/6 mice, Tap1 (transporter associated antigen processing subunit 1) deficient and li (invariant chain) deficient mice for this experiment. These groups of mice were immunized with 5 μg of PCP1 and serum obtained on day 0, 7 and 14 was assessed for anti-PCP1 antibody titers by ELISA. As expected, intraperitoneal immunization of C57BL/6 mice induced production of anti-PCP1 antibodies (Figure 23a). PCP1 induced antibody production in Tap1 deficient mice (Figure 23b). This data demonstrated that a defect in the
Figure 21: PCP1 and whole hk 6301 induce anti-PCP1 IgM and IgG antibodies. 5 µg of PCP1 (a and c) or 10^8 cfu hk 6301 (b and d) was injected intraperitoneally into BALB/c mice (n=6). Sera collected on day 0, 8, 16, 28 and 42 were analyzed (at 1 in 100 dilution) for anti-PCP1 IgM (a and b) and IgG (c and d) antibodies by ELISA. Each dot represents data from individual mouse. Horizontal bar denotes median of the respective group. Significance was determined by Student's t test where * denotes p < 0.05; ** denotes p ≤ 0.001 compared to preimmune.
Figure 22: IgG subtyping of PCP1-specific antibodies generated in response to immunization with PCP1 or whole hk 6301. 5 µg of PCP1 (a, c, e and g) or 10^9 cfu hk 6301 (b, d, f and h) was injected intraperitoneally into BALB/c mice (n = 6). Sera collected on day 0, 8, 16, 28 and 42 were analyzed (at 1 in 10 dilution) for anti-PCP1 IgG1 (a and b) and IgG2b (c and d) and (at 1 in 20 dilution) for IgG2a (e and f) and IgG3 (g and h) antibodies by ELISA. Each dot represents data from an individual mouse. Horizontal bar represents median of the respective group. Statistical significance was determined by Student's t test where * denotes p < 0.05; ** denotes p ≤ 0.001 when compared with the preimmune control.
Figure 22 contd.
Figure 23: MHC class II is required for anti-PCP1 antibody production. C57BL/6 (a), Tap1 knockout (b) and li knockout (c) mice were immunized intraperitoneally with 5 µg PCP1 while li knockout mice (d) were immunized with 10 µg Salmonella typhi capsular polysaccharide Vi antigen (n = 6). Sera collected on day 0, 7 and 14 were analyzed (at 1 in 100 dilution) for anti-PCP1 or anti-Vi Ig production by ELISA. Each dot represents data from individual mouse. Horizontal bar indicates median of the respective group. Statistical significance was determined by Student's t test where * denotes p < 0.05; ** denotes p ≤ 0.001 when compared with the preimmune control.
stable assembly and intracellular transport of class I molecules did not hamper the production of anti-PCP1 antibodies. In contrast to Tap1^{-/-} mice, li deficient mice when immunized with PCP1 failed to produce anti-PCP1 antibodies (Figure 23c). This observation demonstrated that MHC class II mediated processing and presentation of PCP1 is critical for generation of anti-PCP1 antibody responses. Remarkably, MHC class II deficiency did not prevent li knockout mice from producing antibodies against Vi, a non-zwitterionic polysaccharide (Figure 23d).

3.8 Generation of PCP1 deficient mutant of S. pneumoniae ATCC 6301

Data from figures 11, 12 and 13 demonstrated that PCP1 when given as a soluble antigen showed isotype class switching to IgG subtypes similar to that observed for PCP1 when administered as whole hk 6301. We wanted to investigate whether there were functional differences in the anti-PCP1 antibodies generated by these two different forms of the antigen using opsonophagocytosis and mouse protection assays. As immunization with whole hk 6301 leads to the production of anti-protein antibodies besides anti-PCP1 antibodies, therefore there was a need to isolate anti-PCP1 antibodies from serum obtained from mice immunized with hk 6301. To generate anti-PCP1 sera devoid of anti-protein antibodies, we generated a PCP1 deficient mutant of ATCC 6301 and used it for depleting anti-protein antibodies from the sera. The strategy used for disrupting the operon encoding the capsular biosynthesis genes is depicted in Figure 24. PCP1 biosynthesis involves the Wzx/Wzy-dependent pathway. The capsular polysaccharide synthesis operon
Figure 24: Schematic representation of the strategy used for construction of capsule deficient mutant of pneumococcal strain ATCC 6301. Flippase gene of the cps operon was amplified from ATCC 6301 genomic DNA and cloned into pJY4163 vector which carries an erythromycin resistance gene (Erm). The recombinant construct was electroporated into ATCC 6301 electrocompetent cells. The cps locus was inactivated by the insertion of the recombinant plasmid in the flippase gene.
is flanked by genes \textit{dexB} and \textit{aliA}. Besides various regulatory and processing genes, the cps operon contains \textit{wzx} gene (also known as flippase) which is responsible for transportation of the sugar repeat units across the cytoplasmic membrane for attachment. Disruption of the flippase gene and indirectly the downstream genes would not allow the synthesized sugars to be linked to form a capsular layer. We PCR amplified the flippase gene. The size of the PCR product obtained was found to be in keeping with the expected size of 1086 bp (Figure 25a). The PCR amplified fragment was cloned into XbaI and SacI restriction sites of plasmid pJY4163 (4407 bp) and transformed into DH 5\(\alpha\) \textit{E. coli} competent cells. The recombinant construct was confirmed by restriction analysis (Figure 25b) and sequencing. The expected restriction pattern of the recombinant construct for restriction enzymes used in the analysis is mentioned in Table 4. After confirming the clone, the recombinant construct was electroporated into ATCC 6301 electrocompetent cells and erythromycin resistant colonies were selected. The absence of the capsule in the mutant was confirmed by immunofluorescence microscopy using rabbit anti-PCP1 sera. It was observed that wild type cells stained for the capsule but no staining was observed in the case of PCP1 deficient strain suggesting absence of the capsule (Figure 26a). As loss of capsule is known to render the pneumococci avirulent, we further characterized the mutant by administering it intraperitoneally to BALB/c mice. We observed that all mice injected with 500 cfu of ATCC 6301 died within 36 hr. In contrast, all the mice injected with capsule deficient mutant (500 cfu) survived as on day 7, our last observation time point (Figure 26b). We absorbed the sera generated by
Figure 25: Production of capsular deficient polysaccharide mutant of ATCC 6301. (a) The PCR amplified product of flippase gene was resolved on a 0.8% agarose gel. Molecular mass marker (in Kb) is shown in lane 1. (b) Recombinant construct digested with XbaI and SphI (lane 1), HindIII (lane 2), AlwNI (lane 3) and EcoRV (lane 4). The molecular mass markers (in Kb) are in lanes 5 and 6.
Table 4: Expected band sizes upon restriction digestion of recombinant construct (pJY4163 containing flippase)

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Expected band sizes (bp)</th>
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<tbody>
<tr>
<td>Xbal and Sacl</td>
<td>4386 and 1082</td>
</tr>
<tr>
<td>HindIII</td>
<td>4613 and 855</td>
</tr>
<tr>
<td>AlwN1</td>
<td>4033 and 1435</td>
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<tr>
<td>EcoRV</td>
<td>4728 and 750</td>
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Figure 26: Characterization of capsule deficient mutant of ATCC 6301. (a) Immunofluorescent staining of ATCC 6301 and capsule deficient mutant. Wild type and capsule deficient strain were stained with anti-PCP1 rabbit sera followed by FITC labelled goat anti-rabbit lg antisera and observed under a confocal microscope. (b) BALB/c mice (n = 6) were challenged intraperitoneally with 500 cfu of ATCC 6301 and capsule deficient mutant. Mice survival was monitored every 12 hr for 7 days. Statistical significance was determined by using Mann-Whitney Rank Sum test where * denotes p < 0.05; ** denotes p ≤ 0.001.
immunizing mice with hk 6301, with PCP1 deficient mutant thrice. The depletion of anti-protein antibodies was confirmed by blotting for a surface accessible conserved pneumococcal protein, PpmA using normal and depleted sera (Figure 27) (Cron et al., 2009). Immunoblotting with day 28 sera raised in hk 6301 immunized mice revealed a band at ~36 KDa for PpmA (Figure 27a). The same blot when reprobed with anti-hk 6301 sera depleted of anti-protein antibodies, did not show the band for PpmA, suggesting loss of anti-protein antibodies after depletion (Figure 27b). The same blot was reprobed with anti-His antibody to shows the band at ~36 KDa for PpmA (Figure 27c).

3.9 Anti-PCP1 antibodies induced upon immunization with PCP1 or whole hk 6301 are opsonic in nature

Serum from BALB/c mice immunized with PCP1 or whole hk 6301 and subsequently depleted of anti-protein antibodies was assayed for its opsonophagocytic activity. Extracellular ATCC 6301 were counted on TSA plates following infection of peritoneal exudates cells with opsonized ATCC 6301 for 1 hr. We observed that anti-PCP1 antibodies generated in PCP1 immunized mice reduced the extracellular bacterial count to ~50% (Figure 28). Whereas, anti-PCP1 sera obtained from mice immunized with hk 6301 and subsequently depleted for anti-protein antibodies reduced the extracellular bacterial numbers to ~65%. This data suggests that anti-PCP1 antibodies induced upon immunization with both soluble as well as whole bacteria associated form are opsonic in nature.
Figure 27: Immunoblot for PpmA using sera from mice immunized with hk 6301 and hk 6301 sera depleted of anti-protein antibodies. N-terminal His tagged recombinant PpmA was run on SDS PAG and transferred on to NC membrane which was probed with sera raised in BALB/c mice against hk 6301 (a), blot reprobed with anti-hk 6301 sera depleted of anti-protein antibodies (b) and anti-His antibody (c).
Figure 28: Sera generated from PCP1 and whole hk 6301 immunized mice promotes phagocytosis of ATCC 6301. 1000 cfu of ATCC 6301 were incubated with preimmune, day 8 sera from PCP1 immunized mice or day 28 sera from hk 6301 immunized mice (depleted for anti-protein antibodies) at 4°C for 1 hr. Peritoneal exudate cells were infected with the treated pneumococci for 1 hr at 37°C. Surviving extracellular pneumococci were enumerated by plating. Data is presented as average ± SD of three independent experiments. Numbers are represented relative to the extracellular pneumococcal count of the control group which contained only pneumococci (taken as 100). Statistical significance was determined by using One way ANOVA where * denotes $p < 0.05$; ** denotes $p \leq 0.001$. Preimmune was used as the control group for statistical analysis.
3.10 Anti-PCP1 antibodies generated by PCP1 or hk 6301 immunization are protective in mice

To further characterize the functional relevance of anti-PCP1 antibodies, serum from PCP1 immunized mice and anti-hk6301 (depleted of anti-protein antibodies) was incubated with 500 cfu of ATCC 6301. Six to eight week old BALB/c mice were administered opsonized pneumococci and survival of mice was monitored for 7 days (Figure 29). All mice challenged with ATCC 6301 alone or ATCC 6301 preopsonized with preimmune sera died within 36 hr. Mice challenged with ATCC 6301 preopsonized with day 8 sera from PCP1 immunized mice or day 28 sera from whole hk 6301 immunized mice (depleted of anti-protein antibodies), survived till day 7, our last observation time point. This data indicates that anti-PCP1 antibodies generated against soluble and whole hk 6301 are protective in mice.
Figure 29: Sera generated from PCP1 and whole hk 6301 immunized mice protects mice against pneumococcal challenge. 500 cfu of ATCC 6301 were opsonized with preimmune sera, day 8 sera from PCP1 immunized mice or day 28 sera from hk 6301 immunized mice (subsequently depleted for anti-protein antibodies). Opsonization was done at 4°C for 1 hr followed by intraperitoneal challenge of BALB/c mice (n = 12). Mice were observed for survival every 12 hr for 7 days. Statistical significance was determined by using Mann-Whitney Rank Sum test where * denotes p < 0.05; ** denotes p ≤ 0.001. Preimmune was used as the control group for statistical analysis.