Discussion
Capsular polysaccharide of *S. pneumoniae* has been detected in patient sera, urine, sputum and saliva (Burman et al., 1991; Dominguez et al., 2001; Kenny et al., 1972). Bacteraemic patients with pneumonia have an approximate concentration of circulating capsular polysaccharide in the range of 0.1 to 100 µg/ml of serum and the concentration appears to be stable for at least 10 to 15 days. In this scenario, it is highly likely that the capsular polysaccharide can interact with immune cells.

Using flow cytometry we observed that PCP1 bound to the surface of B cell line A-20, macrophage cell lines RAW264.7 and GG2EE and dendritic cell line DC2.4. PCP1 did not display any binding to T cell line Jurkat (Figure 8). The binding of PCP1 to the surface of RAW264.7 was dose dependent, but was observed at relatively high concentrations of PCP1 (Figure 10). Bacterial capsular polysaccharides are known to interact with immune cells via various receptors. Studies have demonstrated binding of PCP3 and PCP14 to the surface of dendritic cells via DC-SIGN receptor (Meltzer and Goldblatt, 2006) and other pneumococcal polysaccharides type 3, 9V, 23F, 6B and 2 engage macrophage mannose receptor to bind to macrophage cells (Zamze et al., 2002). Our data suggested that PCP1 interacts with some immune cell types though the binding partner for PCP1 on surface of immune cells still remains to be elucidated.

Interaction of various molecules with immune cells typically leads to their activation which is manifested in the form of production of cytokines. Pneumococci have been shown to induce various pro-inflammatory molecules
like IL-6, TNF-α and IFN-γ (Arva and Andersson, 1999; Jagger et al., 2002; Rubins and Pomeroy, 1997). PCP14 has been shown to induce IL-6 and TNF-α from whole blood (Jagger et al., 2002). We checked for production of cytokines in response to incubation of cells with PCP1. We observed that PCP1 induces the production of TNF-α but not IL-6 from RAW264.7 cells (Figure 11). We observed pro-inflammatory activity of PCP1 at relatively high concentrations (100 to 500 μg/ml). Polysaccharides have been used at concentrations in this range by other groups (Iwata et al., 2005; Segura et al., 1999). Kasper and colleagues claim that PCP1 failed to stimulate TNF-α or IL-12p40 (Wang et al., 2006). The authors may have failed to detect TNF-α or IL-12p40 as they used PCP1 at a concentration of 100 μg/ml. Besides they treat PCP1 with NaOH for removal of CWPS contaminant. In our experience NaOH treatment of PCP1 leads to its deacetylation and our data shows that deacetylated form of PCP1 is non-inflammatory. A recent report observed that Pneumo-23 induced TNF-α, IL-6 and IFN-γ production from macrophages (Hong et al., 2010). The reason for this difference could be that Pneumo-23 contains polysaccharides from 23 different pneumococcal serotypes which differ in their structure, composition and properties.

Our immunoblot data suggests activation of NF-κB and ERK MAP kinase pathway in PCP1 stimulated RAW264.7 cells (Figure 16). MAPK specific inhibitor PD98059 reduced TNF-α secretion from PCP1 treated RAW264.7 cells in a dose dependent manner (Figure 17). LPS induced TNF-α production from macrophages has been shown to involve NF-κB and ERK MAP kinase pathway (Means et al., 2000). S. pneumoniae has been shown to activate NF-
Discussion

κB and c-JNK pathway in lung epithelial cells (Quinton et al., 2007; Schmeck et al., 2006) and this activation has been shown to be important for release of inflammatory cytokines.

TLRs are an important class of PRRs present on or in immune cells that are responsible for bacterial sensing. TLR2, TLR4 and TLR9 have been shown to play an important role in protection against pneumococcal infection (Albiger et al., 2007; Koedel et al., 2003; Malley et al., 2003). TLR2 has been implicated in immune cell activation by various zwitterionic and non-zwitterionic polysaccharides (Garg and Qadri, 2010; Wang et al., 2006). We observed that PCP1 activates macrophages to produce TNF-α via a TLR2 dependent mechanism as pre-incubation of RAW264.7 cells with TLR2 blocking antibody abolished the production of TNF-α (Figure 15). PCP1 treated HEK293T transfectants expressing TLR1 and TLR2 produced IL-8 (Figure 14). These data led us to conclude that TLR2 is engaged by PCP1 to activate macrophages. There exists a possibility that TLR2 may be the membrane receptor for PCP1 on cells, though it needs to be investigated. It is documented that TLR2 deficient mice are more susceptible to S. pneumoniae induced death (Echchannaoui et al., 2002). TLR2 has been shown to affect anti-protein and anti-polysaccharide Ig production in response to immunization with S. pneumoniae type 14 (Khan et al., 2005). Our data suggests that TLR2 is involved in modulation of immune responses to PCP1.

PCP1 is a linear polymer of trisaccharide repeating units and it is known to be variably acetylated (Stroop et al., 2002). Acetyl groups have been shown to
be important for immunogenicity of *Neisseria meningitides* serogroup A polysaccharide (Berry et al., 2002). On the contrary O-linked acetyl side groups of *S. pneumoniae* 9V were not essential for generation of functional antibodies (McNeely et al., 1998). The inability of deacetylated PCP1 to induce TNF-α from RAW264.7 cells correlated with its inability to bind RAW264.7 cells (Figure 18). Our data suggests that a small but significant proportion of anti-PCP1 antibodies are directed against the acetyl groups (Figure 19). Felton and Prescott noted that acetyl groups on type 1 pneumococcus are not important for the antigenicity of PCP1 (Felton and Prescott, 1939). Their observation was based on precipitin reactions which are not as sensitive as ELISA to detect subtle differences in antibody titers (Heidelberger et al., 1930). Our observation has implication in pneumococcal vaccine production and development. Since antibodies are directed against acetyl groups present in PCP1, care should be taken so that chemical processing of the polysaccharide required for glycoconjugate production does not lead to the loss of acetyl groups.

It is well documented that antibodies directed against pneumococcal capsular polysaccharide confer protection against invasive pneumococcal disease. During pneumococcal infection, the immune system is likely to encounter the pneumococcal components like PCP1 in free soluble form and associated with pneumococci. It is known that soluble and particulate antigens exhibit distinct immunological properties (Ziegler et al., 1987). It has been observed that PCP14 and PCP19F were not immunogenic in BALB/c mice while their conjugates with CRM_{197} induced significant IgG antibodies (Mawas et al.,
Pneumococcal polysaccharides when conjugated to carrier proteins predominantly induce IgG1 antibodies (Uddin et al., 2006). Subcutaneous immunization and boosting of mice with PCP1 emulsified with Quil A adjuvant led to preferential switching to IgG1 and IgG2b subclasses (Groneck et al., 2009). Anti-PCP1 IgG responses have also been reported by Jakobsen and coworkers (Jakobsen et al., 1999). They observed predominance of IgG3 antibodies in the serum of mice immunized subcutaneously with soluble PCP1, but switched to IgG1, IgG2a and IgG3 antibodies upon subcutaneous immunization of PCP1 conjugated to tetanus toxoid when administered with *E. coli* heat labile enterotoxin as an adjuvant (Jakobsen et al., 2001). IgG subclass analysis of anti-PCP1 antibodies induced in response to immunization with PCP1 and whole hk 6301 revealed that both forms of PCP1 predominantly induced IgG1 and IgG2b antibodies. Our data differs from results reported by Jakobsen et al. and a possible explanation for this can be the difference in the route of immunization and the fact that we were studying the primary response while Jakobsen et al. analyzed tertiary response. The IgG subtype profiles of the anti-pneumococcal surface protein A (PspA) antibody response induced following immunization with recombinant PspA and PspA in the context of heat killed pneumococcal strain R36A were comparable (Rohatgi et al., 2009). The predominant IgG subtype was IgG1.