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Comparison of Anti-Pneumococcal Functions of Native and Modified Forms of C-Reactive Protein

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by

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ABSTRACT

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The anti-pneumococcal function of native C-reactive protein (CRP) involves its binding to phosphocholine molecules present on *Streptococcus pneumoniae* and subsequent activation of the complement system. However, when pneumococci recruit complement inhibitory protein factor H on their surface, they escape complement attack. Non-native forms of CRP have been shown to bind immobilized factor H. Accordingly, we hypothesized that modified CRP would bind to factor H on pneumococci, masking its complement inhibitory activity, allowing native CRP to exert its anti-pneumococcal function. As reported previously, native CRP protected mice from lethal pneumococcal infection when injected 30 minutes before infection but not when injected 24 hours after infection. However, a combination of native and mutant CRP was found to protect mice even when administered 24 hours after infection. Therefore, it is concluded that while native CRP is protective only against early-stage infection, a combination of native and mutant CRP offers protection against late-stage infection.

DEDICATION

This manuscript is dedicated to my father Mr. Neba David; my mother Manka'a Esther, who both encouraged and supported me in every way possible. Especially to my mother who also took the role of a father in my life. I really appreciate her strength and belief in me. My siblings Sheila Sirri, Kelvin Ngwa, Lynn Bih and Rosella Asoh for their unending moral support. They all helped me build the strength and stamina to succeed.

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CHAPTER 1

INTRODUCTION

C-reactive protein (CRP) belongs to the pentraxin family of proteins. The other key member of this family is serum amyloid P component (SAP) (Osmand et al. 1977). CRP is an evolutionarily conserved protein and an acute phase protein produced by the liver in humans (Volanakis 2001). During inflammation or microbial infection, the serum level of CRP rises several hundred fold (Ramamoorthy et al. 2012). CRP exhibits calcium-dependent binding to phosphocholine (PCh) and phosphoethanolamine (PEt), while SAP exhibits calcium-dependent binding to PEt only (Emsley et al. 1994; Agrawal et al. 1997; Thompson et al. 1999; Gang et al. 2012). CRP binds to PCh residues exposed on the surface of dead or dying cells and in the cell wall C-polysaccharide (PnC) of *Streptococcus pneumoniae* (Du Clos 1989; Marnell et al. 1995; Szalai et al. 1995; Suresh et al. 2007). Once CRP is bound to a PCh-containing particle, it activates the complement system (Mold et al. 1999; Bíró et al. 2007). Both CRP and complement are components of the innate immune system, and thus CRP plays an important role in the humoral arm of the innate immune system (Bíró et al. 2007). Given that no CRP polymorphism or deficiency has been reported in humans, CRP is considered as an important host defense protein (Thompson et al. 1999; Simons et al. 2014).

Structure of CRP

Native or wild type (WT) CRP is made up of five identical subunits arranged in a pentameric ring and held together by non-covalent bonds, with all five subunits having the same orientation in the pentamer (Thompson et al. 1999). It has two faces, a recognition face and an

effector face, and all five subunits have their PCh-binding site on the recognition face of the molecule.

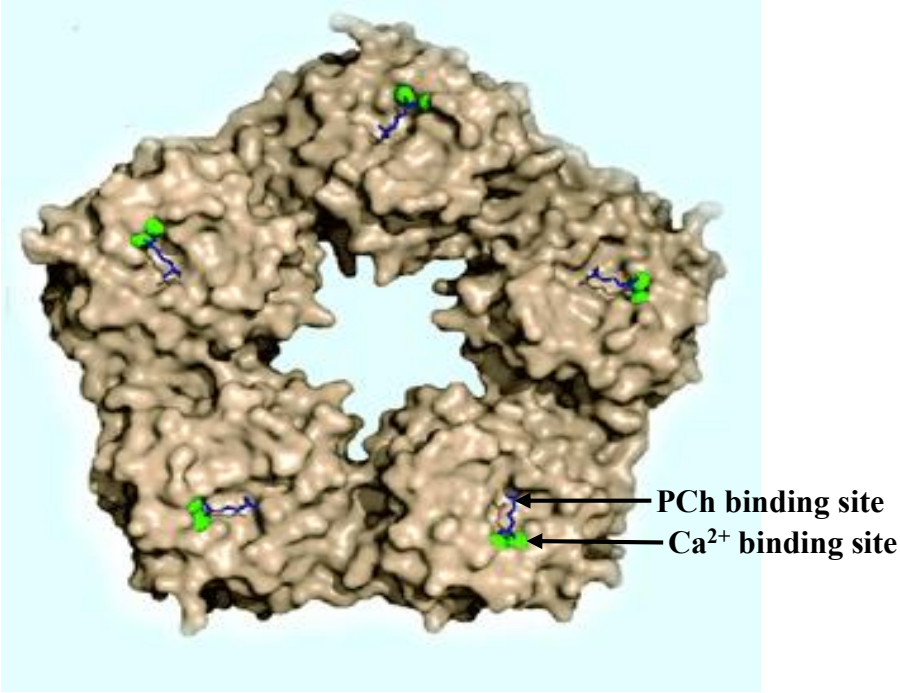

Figure 1. Structure of the CRP-PCh complex. The figure shows the binding sites for Ca^{2+} (green) and PCh (blue) on the recognition face of the CRP molecule. (Modified from Peisajovich et al. 2008)

The recognition face of CRP binds PCh residues and two calcium ions (Figure 1). The binding of PCh to CRP requires its phosphate group to interact with the two calcium ions present in the calcium-binding site of CRP, and its three choline methyl groups interacting with Phe^{66} in the exposed face of the hydrophobic binding pocket made up of Leu^{64}, Phe^{66}, Thr^{76} and Glu^{81} among other amino acids. Glu^{81} forms part of the side chain interacting with the choline nitrogen, increasing the avidity of PCh binding (Shrive et al. 1996; Thompson et al. 1999). The calcium-binding and PCh-binding sites sit close to each other. Calcium binding is additionally very important for the stability of CRP because when the calcium binding site is vacant, a large

loop with a proteolytic site folds outward and becomes exposed to proteolysis (Thompson et al. 1999). Thr^{76} is interestingly conserved in most mammals and it is replaced by Tyr^{74} at the same position in the closely related SAP. Phe^{66} has been shown by site directed mutagenesis analysis to be a key residue for PCh binding (Thompson et al. 1999). However, Tyr^{74} in SAP confers PEt binding capacity to this molecule. The molecular mass of each subunit of CRP is about 23 KDa and is made up of 206 amino acids (Emsley et al. 1994; Agrawal et al. 1997).

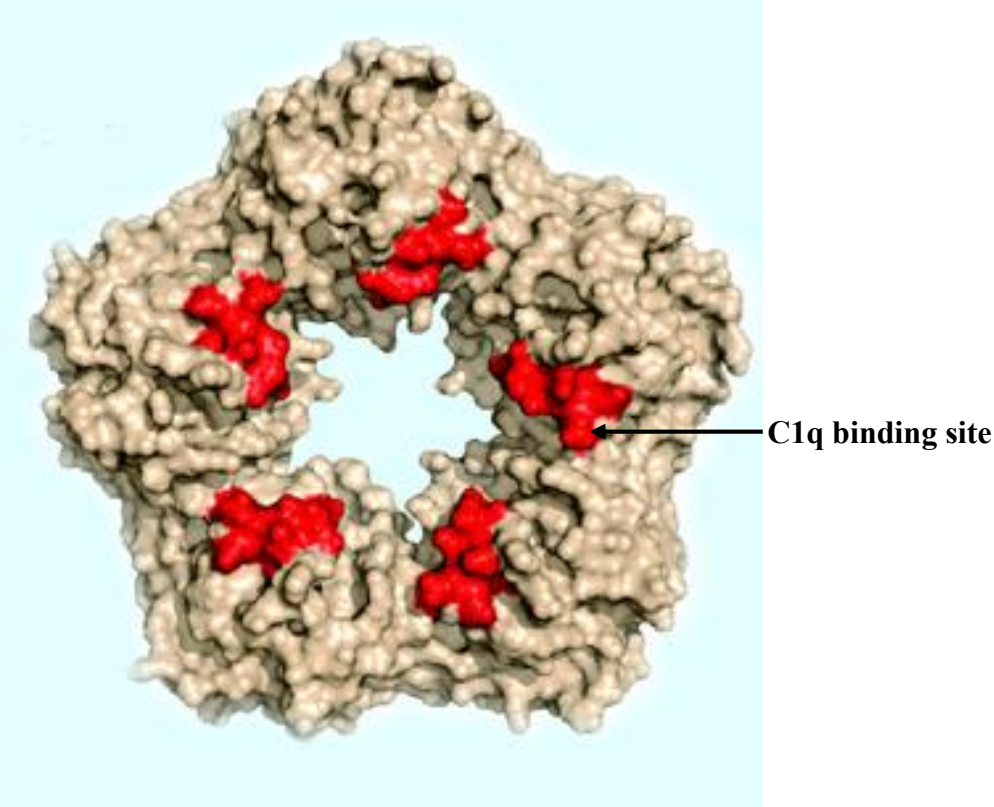

Figure 2. The C1q-binding site on CRP. The figure shows the binding sites for C1q on the effector face of the CRP molecule. (Modified from Peisajovich et al. 2008)

The effector face of CRP interacts with Fcγ receptors on phagocytic cells (Shrive et al. 1996) and complement component C1q (Bang et al. 2005) (Figure 2). From the relative sizes of

the C1q globular head and CRP, it was suggested that only one C1q globular head could bind to the central pore of the CRP pentamer (Agrawal et al. 2001)

Pneumococcus

Streptococcus pneumoniae (pneumococci) are Gram-positive bacteria generally observed in pairs and were earlier called diplococci for that reason (Winslow et al. 1920). But they can also be found in either short chain arrangements or singly. Pneumococci colonize the respiratory tract, sinuses and the nasal cavity of healthy individuals asymptomatically. They easily progress from the lungs to the pleural cavity then to blood in individuals with weak immune systems, causing disease (Hughes et al. 2014). It is the main pathogen involved in community acquired pneumonia and meningitis in children, the elderly and in immunocompromised individuals (Tuomanen et al. 1995; Dagan 2000; Lim et al. 2001; Berkley et al. 2005). Other pneumococcal infections caused by these bacteria include bronchitis, otitis media, conjunctivitis and peritonitis to name a few (Tuomanen et al. 1995; Dagan 2000; Jackson and Pilishvili 2015). The modes of transmitting these bacteria include coughing, sneezing and direct fluid transfer from an infected individual to a healthy one.

Pneumococci are encapsulated and the level of encapsulation is a pathogenic (virulence) determinant (Jackson and Pilishvili 2015). The cell wall is composed of teichoic acid which contains C-polysaccharide. Attached to the C-polysaccharide are PCh moieties to which CRP binds and activates the complement system (Figure 3). Also, on the surface of these bacteria, factor H inhibitor of complement (Hic) protein is expressed which recruits factor H, a complement inhibitory protein. Factor H helps pneumococci in escaping complement attack (Jarva et al. 1999).

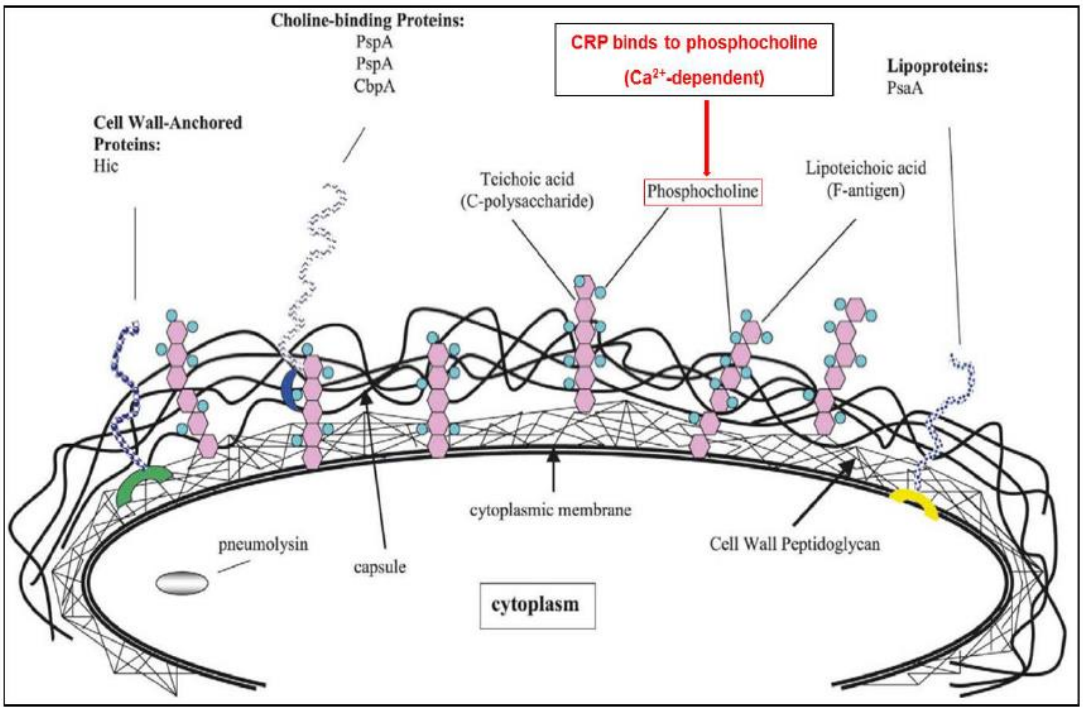

Figure 3. Structural representation of selected immunogenic proteins of S. pneumoniae. The figures illustrates the binding of CRP to PCh (Modified from DeLano W. L. 2010)

Functions of CRP

CRP is an acute-phase plasma protein in humans with high levels measured in individuals with autoimmune disorders, such as rheumatoid arthritis, and vasculitis and chronic vascular inflammatory diseases like atherosclerosis. Based on the levels measured, the inflammatory response can be assessed (Ramamoorthy et al. 2012).

CRP functions as the first line of defense in the humoral arm of the innate immune system. Two major functions have been suggested for CRP (Mold et al. 1981; Szalai et al. 1995). The first is the recognition function. The most acknowledged ligand which CRP recognizes and binds to is PCh, which is found associated with most cell membranes and the polysaccharide part

of the cell walls of most bacteria and fungi (Volanakis and Kaplan 1971). CRP also binds to certain nuclear materials which do not have PCh, such as ribonucleoproteins (Weiser et al. 1998). The second function is the effector function: when CRP binds to one of its ligands, the complex is recognized by C1q, the first component of the classical pathway of complement, which then activates the complement cascade resulting in the clearance of the particle bearing the CRP ligand (Kaplan and Volanakis 1974). The binding of CRP to its ligand could also trigger phagocytosis by phagocytic cells working through the Fcγ receptors (Marnell et al. 1995).

It has been shown previously that human CRP protects mice from lethal doses of *Streptococcus pneumoniae.* The data suggest that CRP binds to pneumococci and activates the complement system (Mold et al. 1981; Szalai et al. 1995; Suresh et al. 2007; Agrawal et al. 2008). The mouse model was used because mouse CRP is not an acute phase protein and is found only in trace amounts in mouse circulation, hence making the mouse model most suitable to study the *in vivo* functions of human CRP. Human CRP was found to be protective in mice injected with a lethal dose of pneumococci judging from the increased survival and decreased bacteremia (Suresh et al. 2007; Agrawal et al. 2008). However, this protection was observed only if human CRP was injected 6 hours before infection with a lethal pneumococci dose to 2 hours after infection. Mice were not protected if CRP was administered at the late stages of infection, that is, when administered beyond 2 hours. Therefore, the early presence of CRP was required for protection (Agrawal et al. 2008). CRP binds to PCh and then activates the complement system leading to the clearing of the bacteria from the host system (Mold et al. 1981; Gang et al. 2012). The reason why this protective function is lost in the late stages of infection is unknown, but is thought to be related to the bacteria acquiring the complement inhibitory protein factor H.

Factor H

Factor H is a 150 KDa soluble plasma glycoprotein. It is made up of 20 short consensus repeats (SCR) and each SCR has 60 amino acids (Sharma and Pangburn 1996). Factor H regulates the alternative pathway of complement in two ways: acting as a cofactor for factor I, in the proteolytic cleavage of C3b and accelerating the decay of C3 convertase of the alternative pathway (Weiler et al. 1976). It possesses three binding sites for C3b which are equally important for C3b binding. They include SCR1-4, SCR6-10, and SCR16-20. However, only the site in SCR1-4 is necessary for its cofactor function (Sharma and Pangburn 1996).

For its proteolytic activity with factor I and its decay acceleration of C3 convertase, factor H is a complement down-regulator. This down-regulating property of factor H is very important in protecting host cells from complement attack and directing complement attack to pathogens and foreign material (Sánchez-Corral et al. 2002). This is achieved by its interaction with polyanionic molecules such as sialic acid or glycosaminoglycans generally present on the surface of host cells but absent on the surface of pathogens (Pangburn 2000; Józsi et al. 2004; Rodríguez de Córdoba et al. 2004) to which it binds and shields host cells from attack.

Streptococcus pneumoniae is one of the bacteria known to recruit factor H on its surface to evade immune system recognition and attack, therefore increasing its virulence. They make use of the Hic protein expressed on their cell surface to bind factor H (Jarva et al. 2002). As factor H is a complement inhibitory protein, this interaction works to their favor in escaping the complement system. This was suspected because CRP protection of pneumococcal infected mice was lost in late stages of infection (Szalai et al. 1995; Suresh et al. 2007; Agrawal et al. 2008).

CRP can exist in more than one structural form (WT and structurally-altered form). These structural modifications arise when CRP is exposed to acidic pH, high salt concentration,

and oxidation (Singh et al. 2012). CRP in its native conformation does not bind to factor H, however structurally modified CRP in acidic pH have been reported to do so (Hammond et al. 2010). CRP was not monomerized at acidic pH, but was assumed to acquire a loosened pentameric conformation (Mold et al. 1984; Sjöberg et al. 2007; Hakobyan et al. 2008; Hammond et al. 2010). Generating a modified CRP molecule which binds to immobilized factor H at physiological pH, mimicking the factor H binding ability of WT CRP in acidic pH will be useful in evaluating the *in vivo* functions of factor H coated pneumococci.

Mutant CRP

WT CRP binds many ligands especially those having the PCh moieties. However, structurally altered CRP molecules are thought to bind even more ligands. This has given rise to the concept of structure-based functions of this molecule. Nonetheless, structurally altered CRP molecules obtained from acidic pH, high salt concentration or oxidation modifiers, cannot be used in *in vivo* studies because of the buffering capacity of living systems. Therefore stable mutants bearing the binding capacities of the transiently modified CRP are needed for *in vivo* studies.

Using site directed mutagenesis, mutant CRP was generated and the mutation was confirmed by sequencing followed by the investigation of loss or gain of functions targeted (Gang 2013). The E42Q-F66A-T76Y-E81A (quadruple) CRP mutant was used in this study which is a mutant generated by the combination of the cDNA templates of two different mutants (E42Q and F66A-T76Y-E81A) previously used in the lab (Gang et al. 2012; Singh et al. 2012).

E42Q Mutant CRP

Based on the gain of binding function observed with WT CRP in acidic pH, it was hypothesized that the gain of function was due to the loosening of the CRP pentamer in acidic pH, exposing the amino acids otherwise hidden at physiological pH responsible for this gain in function (Singh et al. 2012); this was further supported by the fact that CRP was not monomerized at acidic pH (Hammond et al. 2010). Therefore, generation of a mutant CRP with a loose pentameric arrangement that mimics the functions of acidic pH-treated WT CRP was the goal of this study. This loose acidic pH modified CRP maintained the pentameric structure suggesting that the modifications to this CRP was likely at the level of inter-subunit interaction.

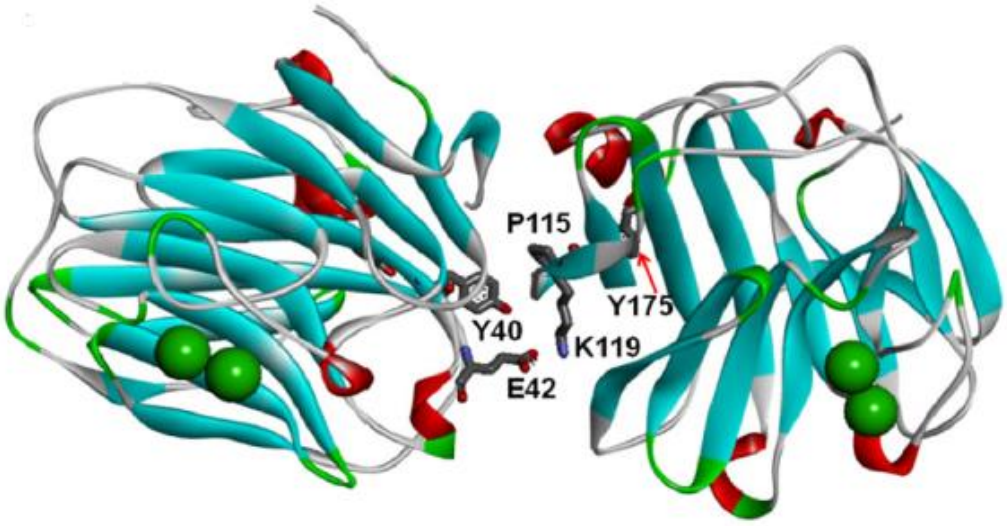

Figure 4. Two of the five subunits of pentameric CRP showing some amino acids involved in the inter-subunit interactions and bound calcium ions in green. Glu 42 was mutated to Gln to generate the E42Q mutant. (Singh et al. 2012)

Hence, these interactions were the target for mutation (Singh et al. 2012). It has been documented that the following amino acids take part in the inter-subunit interactions; Val^{10}-Ile^{104}, meaning, Val^{10} of one subunit interacts with Ile^{104} of the adjacent subunit; Pro^{12}-Ser^{118}; Tyr^{40}-Pro^{115}; Tyr^{40}-Val^{117}; Glu^{42}-Glu^{85}; Ser^{46}-Glu^{85}; Glu^{42}-Lys^{119}; Ser^{44}-Lys^{69}; Val^{90}-Pro^{87};

Gly^{101}-Lys^{201}; Ser^{120}-Tyr^{197}; Pro^{115}-Trp^{205}; and Arg^{118}-Asp^{155} (Shrive et al. 1996; Singh et al. 2012). Different mutations were introduced into the CRP molecule, and the CRP mutant E42Q showed the desired binding properties at physiological pH otherwise observed only in acidic pH with WT CRP (Figure 4).

The E42Q CRP mutant binds to immobilized factor H at physiological pH, a property not observed with WT CRP (Figure 5) and the binding is further enhanced as the pH drops. It shows better binding to factor H compared to WT at whatever pH used. The E42Q mutant had acquired the factor H binding property at physiological pH only found with WT at pH levels of 5.0 (Figure 5).

However, this mutant also binds to PCh just like WT CRP and a CRP mutant with factor H binding capacity but lacking the PCh binding property was required. Therefore, the inability of F66A-T76Y-E81A (triple) mutant CRP to bind PCh was translated to the E42Q mutant.

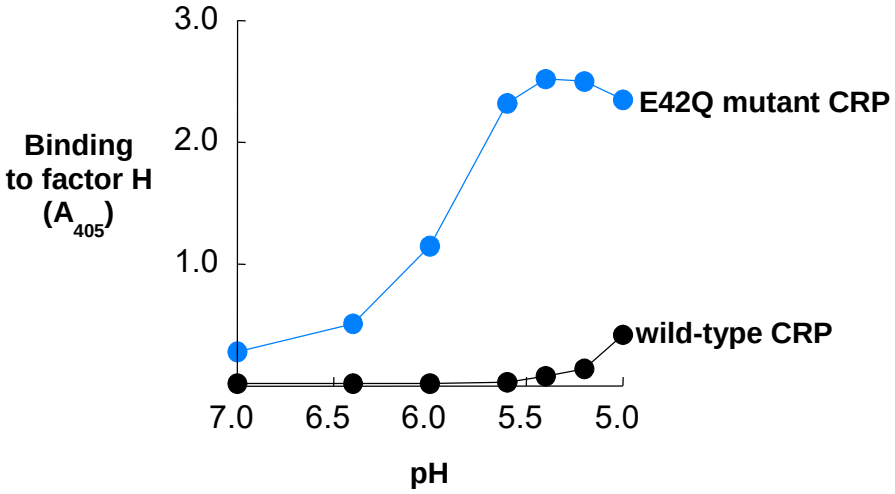

Figure 5. Binding of mutant and WT CRP to factor H in acidic pH. The figure shows increased binding with decreasing pH (Singh et al. 2012)

F66A-T76Y-E81A Mutant CRP

The F66A-T76Y-E81A (triple) mutant CRP was generated when the PCh binding pocket was investigated for its importance in the initial protection of mice against pneumococcal infections (Gang et al. 2012). The mutations introduced in this CRP were directed to the PCh binding pocket made up of the following amino acids of interest Phe^{66}, Thr^{76}, and Glu^{81}. Phe^{66} is involved in hydrophobic interactions with the three methyl groups of choline, Thr^{76} is vital for creating the right pocket size to accommodate PCh, and Glu^{81} interacts with the positively charged nitrogen atom of choline (Thompson et al. 1999). These properties altogether are critical for CRP binding to PCh not forgetting the two calcium ions required for binding (Shrive et al. 1996; Thompson et al. 1999). The amino acids Phe^{66}, Thr^{76}, and Glu^{81} were replaced with Ala, Tyr, and la, respectively (Figure 6). These mutations made the triple mutant incapable of binding PCh (Gang et al. 2012).

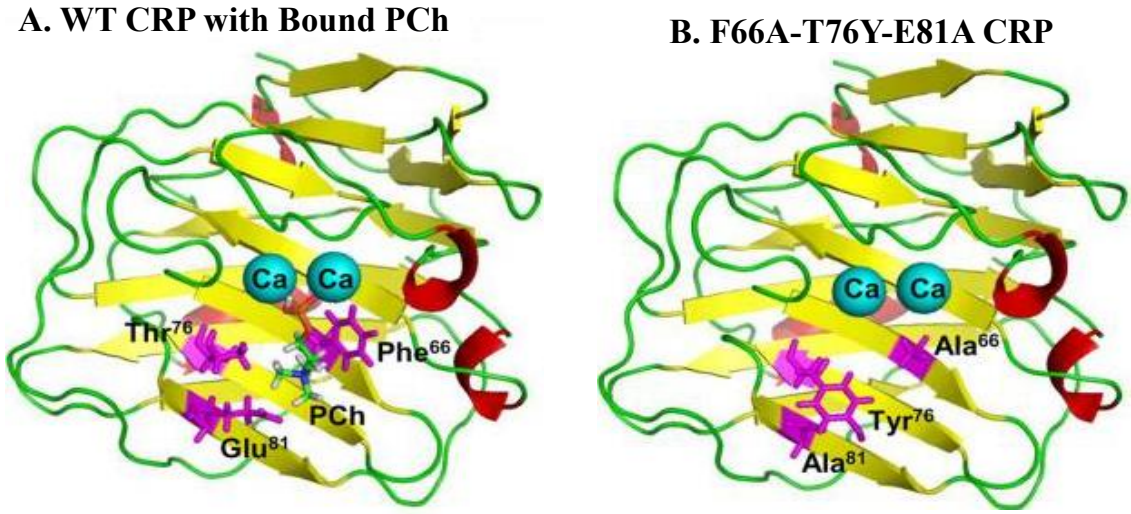

Figure 6 A single CRP subunit showing the PCh binding pocket. (A) Shows a WT CRP subunit bound to PCh, amino acids and calcium ions (cyan) involved in this interaction (B) Shows the triple mutant CRP with mutated amino acids. (Modified from Gang et al. 2012)

E42Q-F66A-T76Y-E81A Mutant CRP

With the ability of E42Q mutant to bind factor H and the triple mutant not being able to bind to PCh, a mutant with a combination of both characteristics was made. Using as template the cDNA of the F66A-T76Y-E81A (triple) mutant in conjugation with that of E42Q, the E42Q-F66A-T76Y-E81A (quadruple) mutant was made. The quadruple mutant being a combination of the E42Q and the triple mutant acquired the properties of both mutants; it can bind factor H but not PCh (Gang 2013).

Rationale and Hypotheses

As stated previously, passively administered human CRP has been shown to protect mice against lethal pneumococcal infection only if injected 6 hours before to 2 hours after injecting pneumococci (Agrawal et al. 2008). The anti-pneumococcal mechanism of early protection has been shown to involve CRP binding to the PCh moieties present in pneumococcal cell wall C-polysaccharide (Gang et al. 2012), then the pathogen-bound CRP activates the complement system and the bacteria are cleared by opsonophagocytosis (Chudwin et al. 1985). The classical pathway of the complement system has been shown not to be involved in the anti-pneumococci function of CRP in mice (Suresh et al. 2006). However, it is not known why the protection conferred by CRP in the early stages of infection is lost in the late stages.

The cell wall of pneumococci presents several structures. One of them is the factor H-binding inhibitor of complement (Hic protein). This protein expressed on the surface of virulent pneumococci has been shown to recruit factor H helping the bacteria evade complement attack even if CRP is bound to PCh on the cell wall of the pneumococci (Jarva et al. 2002). It was

therefore hypothesized that in the late stages of infection, pneumococci would have had ample time to express the Hic protein on its surface which in turn will recruit factor H and factor H will prevent the building of the complement cascade proteins initiated by the bound CRP, by cleaving C3b and accelerating the decay of C3 convertase, hence preventing complement activation. To test this hypothesis, it has been shown that factor H binds to modified forms of CRP (Singh et al. 2012). We suggest therefore that by using a mutant CRP which binds to factor H but not PCh, we will be able to investigate the role of factor H in the late stages of pneumococcal infection. Using site-directed mutagenesis, a mutant capable of binding factor H but not PCh was generated. From the hypothesis, we will inject this mutant CRP expecting that it will bind to the recruited factor H on the bacteria surface and inhibit its complement down-regulating activity allowing the WT CRP bound to the bacteria PCh moieties present in the cell wall C-polysaccharide to activate the complement system leading to the clearance of the bacteria.

This research may help elucidate how pneumococci gains virulence at the late stages of infection. A similar condition is also observed in humans, where high serum levels of CRP are seen during established pneumococcal infections, but with no improvement in the condition of the individual. We however think that before sepsis, the host CRP was not in a suitable microenvironment to undergo the necessary conformational changes required to enable it prevent the progression of the infection. The results from this research could be useful in determining methods of tackling bacteria virulence acquired by the recruitment of factor H.

CHAPTER 2

MATERIALS AND METHODS

Material and Reagents

Chemicals purchased from Sigma-Aldrich: polyclonal anti-CRP from rabbit, bovine serum albumin, gelatin from bovine. Material purchased from Fisher Scientific: boric acid, $CaCl_2$, $MgCl_2$, Borax ($Na_2B_4O_7 \cdot 10H_2O$), sodium citrate, tween 20, TEMED, NaCl, tris base, Todd Hewitt broth, buffer solution, polyethylene glycoldeoxycholic acid, ethylenediamine tetraacetic acid, weigh dishes, RPMI-1640, Fetal Bovine Serum, Dulbecco's phosphate-buffered saline, Antibiotic, G418 (Geneticin), dailysis bags, SFM4CHO, Remel tryptic soy agar, capillary tubes, tissue culture dishes, petri dishes, trypsin, pipettes, gloves, kimwipes, Donkey anti-rabbit HRP, pipet tips, corning tubes, vortex, filters, sterile pipets, clear tubes, centrifuge tubes, incubators, liquid nitrogen facility, centrifuge, spectrophotometer, Water Bath and incubator shaker New Brunswick Scientific; Classic Series, 12-well plates, Labconco safety cabinet. Mouse Complement Factor H (R and D Systems), Defibrinated sheep blood (Quad Five), Human Factor H (Complement Technologies). Equipment purchased from BioRad: centrifuge, HPLC system and fraction collector.

Transfections

Two types of transfections were performed: Transient transfections using COS7 cells and stable transfections using CHO cells (both cell types from ATCC).

For transient transfections, COS7 cells split a day before with confluency of approximately 60% at the time of transfection were used. To 180 µl of RPMI1640 (Gibco), 20 µl of Fugene6 reagent (Promega) was added directly into the RPMI1640, mixed and incubated for 5

min at RT. Then 5 µg of CRP cDNA construct in plasmid p91023 (Agrawal et al. 2002) was added and incubated for 20 min at RT. The spent media from the COS7 cell dish was aspirated and fresh 6 ml of complete media (90% RPMI1640, 10% FBS (Atlanta Biology), 1% antibiotic-antimycotic 100x (Gibco)) added. The DNA-Fugene6 mixture was then pipetted dropwise and equally distributed around the dish. The cell media was collected after 96 h of incubation at 37º C, 5% CO_2.

For stable transfection, CHO cells split a day before with confluency of approximately 60% at the time of transfection were used. To 360 µl of RPMI1640, 40 µl of Fugene6 reagent was added directly into the RPMI1640, mixed and allowed to sit for 5 min at RT. In another tube, 8 µg of CRP cDNA-p91023 and 2 µg of pSV2neo selection vector (Invitrogen Life Technologies) were mixed and transferred to the Fugene6 reagent tube. The mixture was incubated for 20 min at RT. The spent media from the CHO cell dish was aspirated and fresh 6 ml of complete media added. The DNA-Fugene6 mixture was then added dropwise, equally distributing it around the dish and the dish incubated at 37º C, 5% CO_2 for 72 h after which G418 selection was done to remove cells which had not incorporated the pSV2neo plasmid. For selection, the spent media from the dish was aspirated and 10 ml of complete media containing 1 mg/ml G418 (Geneticin) was added and incubated for 10 days at 37º C, 5% CO_2. After 10 days, the surviving cells are the stable transfected pool cells. From this stable transfected pool, a cell line was generated as described below.

To generate a cell line, the spent media was collected and the cells washed with DPBS. Then the cells were detached using 2 ml trypsin (0.25%) and the trypsin neutralized with 4 ml complete media. To a 6 well dish containing 1 ml complete media in each well, 1 ml of the cell suspension was added and incubated at 37º C, 5% CO_2 for 4 days followed by CRP ELISA on

samples from each of the 6 wells as described below. The highest CRP producing well was selected and the number of viable cells counted. The cell concentration was adjusted to 4 cells per ml media. Using 10 such 96 well plates, 200 µl of this diluted suspension was added per well (0.8 cell/well) and incubated at 37^{o} C, 5% CO_2 for 10 days. CRP ELISA was done on wells with single colonies and the top 4 wells with highest levels of CRP selected. The cells from these wells were collected and re-plated in 12 well, then 6 well and finally in 100 x 20 mm dishes (Falcon). These are the high CRP producing sub-clones. These cells were grown and frozen in freezing medium (complete medium containing 15% DMSO) at -80^{o} C, then transferred to liquid nitrogen the next day.

Cell Culture

CHO cells were thawed rapidly in a 37^{o} C water bath, seeded in 10 ml complete media (90% RPMI1640, 10% FBS, 1% antibiotic-antimycotic 100x) and incubated at 37^{o} C, 5% CO_2 over night or for 5 h. After which the DMSO containing media was aspirated and either fresh complete media added or the cells split depending on how confluent the cells were. To split, the spent media was aspirated and the cells washed. Then 2 ml of trypsin was added to detach the cell which was neutralized with 4 ml complete media. Then 150 µl of this neutralized cell suspension was added to 5 ml complete media in 100 x 20 mm tissue culture dishes for a 1 to 40 split. The samples were incubated at 37^{o} C, 5% CO_2 and the protein-rich spent media collected after 5 days.

Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of CRP was determined by ELISA. Microtiter wells were coated with

rabbit anti-CRP polyclonal antibody (IgG, Sigma) diluted 1/1000 in Tris Buffer Saline (TBS; 10 mM Tris, 150 mM NaCl), pH 7.2, overnight at 4^{o} C. The antibody was then aspirated and the wells blocked using block buffer (TBS + 0.5% gelatin) at RT for 45 min. Block was aspirated and the standard CRP sample added (two fold dilutions of a 100 ng/ml, 600 µl purified CRP made ranging from 100-1.56 ng/ml) in duplicate wells. Then dilutions of samples with unknown CRP concentrations in ELISA buffer (TBS + 0.1% gelatin + 0.02% Tween-20) were made and added to the wells. The plate was incubated for 2 h at 37^{o} C or overnight at 4^{o} C. Then the wells were aspirated and washed 3 times with ELISA buffer and 100 µl of 0.5 µg/ml monoclonal anti-CRP antibody HD2.4 (affinity purified) diluted in ELISA buffer was added to each well. The plate was incubated for 1 h at 37^{o} C. After which the wells were aspirated and washed 3 times with ELISA buffer and 100 µl of HRP-labeled goat anti-mouse IgG (H+L) antibody diluted 1/1000 in ELISA buffer added into each well and incubated for 1 h at 37^{o} C. Then 100 µl of Horseradish Peroxidase (HRP)-working substrate solution (10 ml Citrate buffer pH 4.0, 200 µl ABTS solution and 10 µl 30% hydrogen peroxide) was added to each well after the wells had been aspirated and washed 3 times with ELISA buffer. The plate was read at OD_{405} using the Molecular Devices Plate Reader.

HD2.4 Reactivity

The epitope for the anti-CRP mAb HD2.4 was evaluated by the HD2.4 reactivity assay. Microtiter wells were coated with anti-CRP mAb HD2.4 in TBS overnight at 4^{o} C. The unreacted sites in the wells were blocked with block buffer (TBS + 0.5% gelatin) for 45 min at RT. Then purified WT and mutant CRP diluted in TBS + 2 mM Ca^{2+} was added to the wells and incubated for 1 h at 37^{o} C. The wells were then washed and Rabbit polyclonal anti-CRP Ab (Sigma-

Aldrich) diluted in TBS + 2mM Ca^{2+} added and again incubated for 1 h at 37º C. Then the wells were washed and HRP-conjugated donkey anti-rabbit IgG (Thermo) diluted in TBS + 2 mM Ca^{2+} added. Incubated for 1 h at 37º C and color developed using HRP-working substrate solution and read at OD_{405} using the microtiter plate reader (Molecular Devices). OD_{405} obtained from the WT CRP coated wells was then compared to that obtained from the mutant CRP wells.

Factor H Binding Assay

To evaluate the binding of CRP to factor H, microtiter wells were coated with 2 µg/ml of mouse factor H (R & D Systems) and human factor H (Complement Technology) in TBS and incubated at 4º C overnight. The uncoated sites of the wells were blocked with block buffer (TBS + 0.5% gelatin) for 45 min at RT. Then CRP diluted in TBS + 2 mM Ca^{2+} from 10 µg/ml to 0.156 µg/ml was added in duplicate wells and the plate incubated for 2 h at 37º C. The wells were then washed with TBS + 2 mM Ca^{2+} and rabbit polyclonal anti-CRP antibody (Sigma-Aldrich) diluted in the same wash buffer added. Plate was incubated for 1 h at 37º C and the secondary antibody, HRP-conjugated donkey anti-rabbit IgG (Thermo), diluted in TBS + 2 mM Ca^{2+} added. The plate was again incubated for another 1 h at 37º C and color developed using HRP-working substrate solution and read at OD_{405} using the microtiter plate reader (Molecular Devices)

Purification of WT CRP

WT CRP was purified from human pleural fluid in three steps. First, the fluid was centrifuged and the supernatant diluted in equal volumes of borate buffer saline plus calcium (BBS + Ca; Boric acid 100 mM, Borax 25 mM, NaCl 225 mM, $CaCl_2$ 3 mM,) pH 8.3, and purified by affinity chromatography on a PCh-sepharose column (Pierce). Bound CRP was eluted using borate buffer saline plus EDTA (BBS + EDTA; Boric acid 100 mM, Borax 25 mM,

NaCl 225 mM, EDTA 5 mM) pH 8.3. The CRP eluted fractions were pooled and further purified by ion-exchange chromatography. The CRP sample was syringe filtered then diluted 1:4 with Mono Q buffer A (Tris 20 mM, NaCl 150 mM, EDTA 0.1 mM, pH 7.8) and applied to the Mono Q column (GE Healthcare). Elution was done using Mono Q buffer B (Tris 20 mM, NaCl 1 M, EDTA 0.1 mM, pH 7.8). The CRP fractions collected were pooled and dialyzed against TBS + 2 mM Ca^{2+} overnight at 4^{o} C, then concentrated and injected to a Superose 12 column (GE Healthcare) using TBS + 2 mM Ca^{2+} as equilibration and elution buffer. This final step of purification was gel filtration (size exclusion chromatography). CRP fractions were stored frozen. On the day of the experiment, the CRP was purified by gel filtration to remove any modified forms of CRP (monomers) which might have formed after storage. CRP was stored in TBS + 2mM Ca^{2+} and used within a week. The purity of CRP was further verified by denaturing SDS-PAGE.

Purification of Quadruple Mutant CRP

Mutant CRP was purified from cell culture supernatant in two steps. The first step involved purification by affinity chromatography on a PEt-conjugated sepharose column. Here, the cell culture supernatant was diluted in equal volumes of BBS + 3 mM Ca^{2+} at pH 8.3 and applied to a column with PEt-conjugated sepharose beads. The bound mutant CRP was eluted with BBS + 5 mM EDTA, pH 8.3. The eluted mutant CRP fractions were pooled and concentrated. In the second step of purification, the concentrated affinity pure mutant CRP was injected to a Superose 12 column for purification by gel filtration chromatography. Purification was carried out in the same way as for the WT CRP with the exception that the equilibration and elution buffer was TBS + 5 mM EDTA. This was because mutant CRP binds to Superose beads

in the presence of Ca^{2+}. After elution, the mutant CRP fractions were immediately dialyzed against TBS + 2 mM Ca^{2+} and stored at 4º C. Mutant CRP was used within a week of its purification and its purity was further verified by denaturing SDS-PAGE. The concentration of both mutant and WT CRP was determined by spectrophotometry at 280 nm using 1.95 as extinction coefficient. For mouse protection experiments, the WT and mutant CRP were treated with Detoxi-Gel Endotoxin Removing Gel (Thermo) according to the manufacturer's protocol. The concentration of endotoxin in the CRP samples was evaluated using the Limulus Amebocyte Lysate Kit QCL-1000 (Lonza) according to manufacturer's instructions.

Pneumococci

Streptococcus pneumoniae type 3 of the WU2 strain (obtained from Dr. David Briles, University of Alabama, Birmingham, AL) were rendered virulent by 3 times intravenous passage in mice. The bacteria collected from the final passage was cultured on tryptic soy agar plates supplemented with 5% sheep blood, incubated overnight at 37° C and a single colony picked and grown in Todd-Hewitt broth containing 0.5% yeast extract. At the mid-log growth phase, after 4 hours of growth, 10% glycerol was added to the culture and the bacteria were aliquoted (1 ml single use aliquots) and frozen at -80° C. When needed, one aliquot was thawed and grown in 50 ml Todd-Hewitt broth containing 0.5% yeast extract and the bacteria were harvested from mid-log phase cultures. They were centrifuged and resuspended in saline. The concentration of the bacteria used was 5×10^8 CFU/ml obtained at $OD_{600} = 0.45$. The viability, purity and concentration of the bacteria were confirmed by plating on tryptic soy agar plates supplemented with 5% sheep blood.

Mice

C57BL/6J mice (Jackson ImmunoResearch Laboratories) were maintained according to protocols approved by the ETSU Committee on Animal Care. Mice were 8-10 weeks old when used in experiments and included only males or only females.

Isolation of Pneumococci from Infected Mice

Mice were injected 5 x 10^7 CFU of broth-grown pneumococci at the tail vein. Blood was collected from mice by cardiac puncture after 40 hours using EDTA containing tubes to prevent clotting and sequestration of the bacteria. The blood was then mixed with an equal volume of saline and centrifuged at 2,200 rpm for 3 min. The recovered pellet was washed 4 times in the same conditions, collecting the bacteria pellet each time. The pellet was finally centrifuged at 13,000 rpm for 5 min and any supernatant discarded. The remaining pellet was then resuspended in saline and plated on tryptic soy agar plates supplemented with 5% sheep blood. The plates were incubated at 37° C overnight and the bacteria concentration determined.

Mouse Protection Experiments

Mice were injected intravenously with either WT CRP or mutant CRP or both in 100 µl TBS + 2 mM calcium, 30 min before or 12-24 hours after administering a lethal dose of 5 x 10^7 CFU of pneumococci in 100 µl saline. The survival was determined 3 times daily for 6 days and survival curves generated using the GraphPad Prism 6 software. To determine *p* values for the differences in the survival curves between the different groups, the survival curves were compared using the software's Logrank test. Blood was also collected from the tail vein of surviving mice, diluted in saline and plated on tryptic soy agar plates supplemented with 5% sheep blood to determine bacteremia. Blood was collected either once or twice daily for 5 days.

The bacteremia value for dead mice was assumed to be $>10^8$ CFU/ml. The plotting and statistical analysis of the bacteremia data was done using the GraphPad Prism 6 software and *p* values determined using the Mann-Whitney two-tailed test.

CHAPTER 3

RESULTS

Quadruple Mutant CRP Exist as a Pentamer

The epitope for the anti-CRP monoclonal antibody HD2.4 was evaluated in an HD2.4 reactivity assay. HD2.4 anti-CRP mAb binds to its epitope on the effector face of the CRP molecule (the face opposite the PCh-binding face). It binds only to CRP in its pentameric configuration (Kilpatrick et al. 1982; Ying et al. 1989). Both WT CRP and mutant CRP were recognized by mAb HD2.4 (Figure 7), meaning that the overall pentameric structure of mutant CRP was not affected by the four mutations (Glu^{42}, Phe^{66}, Thr^{76} and Glu^{81} for Gln, Ala, Tyr and Ala, respectively)

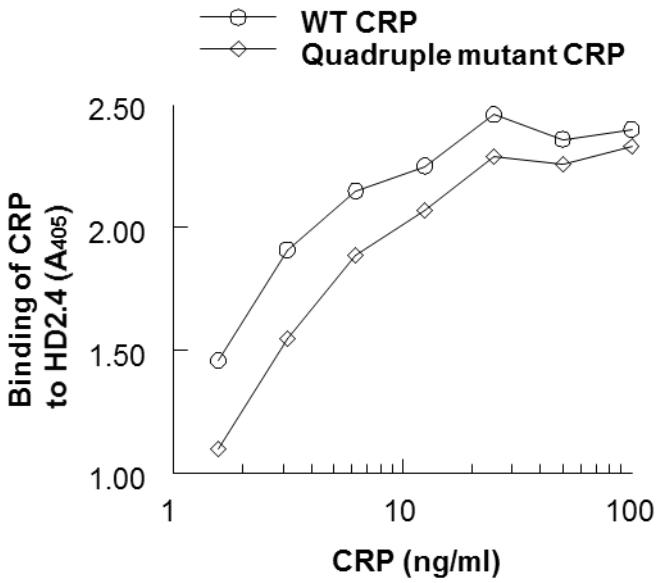

Figure 7. Quadruple mutant CRP is a pentamer. Microtiter wells were coated with anti-CRP mAb HD2.4 and the unreacted sites in the wells blocked with gelatin. Purified CRP diluted in TBS-Ca buffer was then added to the wells. Bound CRP detection was done using rabbit polyclonal anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. Color was developed, and the absorbance read at 405 nm.

The pentameric structure of the quadruple mutant was evaluated by gel filtration, where WT CRP and mutant CRP showed similar elution profiles (Figure 8A) and denaturing SDS-PAGE of WT CRP and mutant CRP showed single bands at the same molecular weight of their subunits (Figure 8B). The data showed that mutant CRP was a pentamer and that the four mutations did not affect the overall structure of CRP.

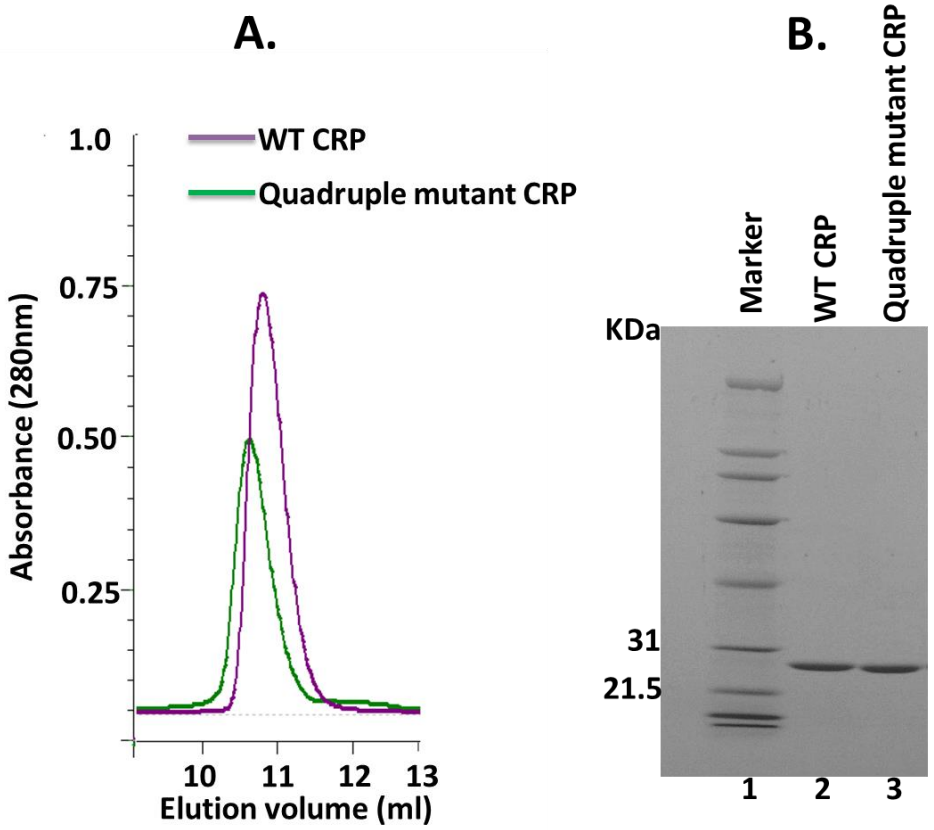

Figure 8. Overall pentameric structure of CRP quadruple mutant. A. Elution profiles of WT and mutant CRP from the Superose12 gel filtration column are shown. WT CRP in TBS containing 2 mM $CaCl_2$ was applied to the equilibrated column and eluted with the same buffer. Mutant CRP in TBS containing 5 mM EDTA was applied to the equilibrated column and eluted with the same buffer. Sixty 0.25 ml fractions were collected and protein measured (OD_{280}) to determine the elution volume of CRP from the column. A representative experiment is shown. B. Denaturing SDS-PAGE (4%-20% gel) of CRP (5 µg). A representative gel is shown.

Other characterization experiments generated data suggesting that this mutant does not bind to PCh or broth-grown pneumococci, it is free and functional in mouse serum, and its rate of clearance from mouse circulation is similar to that WT CRP (Gang 2013). Mutant CRP cDNA was successfully expressed in CHO cells. It was purified from cell culture supernatant by PEt-affinity chromatography followed by gel filtration chromatography.

Quadruple Mutant CRP Binds Readily to Immobilized Factor H

The binding of quadruple mutant CRP to immobilized factor H was carried out because we hypothesized that pneumococci recruit factor H to their surface and by so doing can evade the complement system. It has already been shown that this mutant CRP binds to factor H-coated

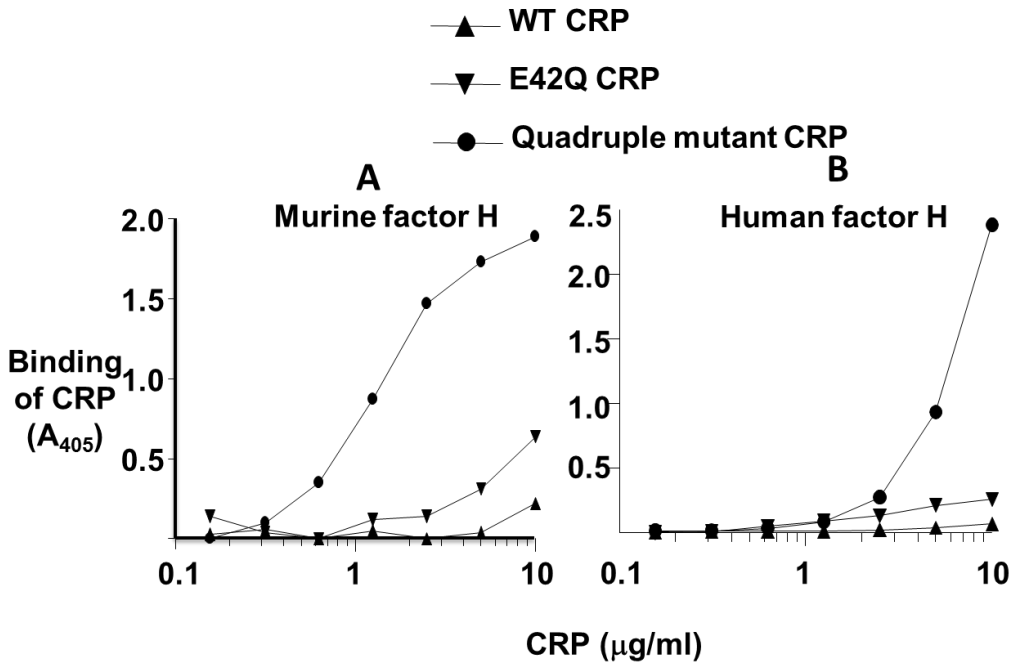

Figure 9. Quadruple mutant CRP binds purified immobilized factor H. Microtiter wells were coated with 2μg/ml of mouse factor H (A) and human factor H (B) in TBS, overnight at 4 ºC. The unreacted sites in the wells were blocked with TBS containing 0.5% BSA for 45 min at RT. TBS-Ca buffer diluted CRP was the added in duplicate wells and incubated at 37 ºC for 2 h. Bound CRP was detected using polyclonal rabbit anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG all diluted in TBS-Ca buffer. Color was developed and read at OD 405nm.

pneumococci (Gang 2013). To test whether the binding observed was due to factor H and not to some other ligand, a binding assay was set up with purified factor H as the ligand. Results showed that, quadruple mutant CRP was in fact binding to immobilized factor H (Figure 9). E42Q mutant CRP was included in this binding assay because it also binds to immobilized factor H. It shows some binding especially at higher doses compared to WT CRP. But, quadruple mutant CRP showed the highest binding indicating that the additional mutations on the E42Q mutant to generate the quadruple mutant had synergistically improved its binding to immobilized factor H.

Quadruple Mutant CRP does not Protect Mice against Pneumococcal Infection when Administered during the Late Stages of Infection

Given that quadruple mutant CRP binds to factor H as demonstrated in (Figure 9) above, and given that it was proposed that in late stages of pneumococcal infections, pneumococci will recruit factor H to its surface and inhibit the activation of the complement system, we injected mutant CRP in the late stages of infection to determine if it will bind to recruited factor H on the surface of these bacteria and lead to their destruction. However, no protection was observed. There was no significant difference in survival for mice injected with 100 µg WT or mutant CRP at 24 hours post infection. The median survival time (time it takes for 50% of mice to die in a particular group) for these groups was 76 hours for mice injected with 100 µg WT (Group D) and 70 hours for those injected with mutant CRP (Group E) (Figure 10). The control groups (A and B) which include mice injected with bacteria alone and mice injected with bacteria plus WT CRP at time of infection, had median survival time 52 hours and 85 hours, respectively (p value = 0.026). Results also showed that, administering mutant CRP at time of infection was

protective. The median survival time for this group was 109 hours and there was no significant difference between this group and the control Group B which received WT CRP at the time of infection (*p* value = 0.489).

The bacteremia values for (Figure 10) can be seen in (Figure 11). After 24 hours of infection with pneumococci, the bacteria alone group had a median bacteria load of 1.1×10^3 CFU/ml of blood which went up to 1.6×10^7 CFU/ml of blood on the second day. The median bacteria load for the group which received WT CRP at the time of infection was 200 CFU/ml of blood after 24 hours and, although it rose to 1.5×10^6 CFU/ml of blood by 48 hours, it was ten times less than what was seen in the bacteria alone group. Comparing the Group B that received WT CRP at the time of infection with Group C which received mutant CRP at the time of infection, there was no significant difference between them (*p* value = 0.545, 24 h; and *p* value = 0.288, 48 h). The median bacteria load for Group B was 100 CFU/ml of blood at 24 hours post infection and 1.6×10^5 CFU/ml of blood on the second day. There was also no significant difference between the two groups of mice (D and E) which received either WT CRP or mutant CRP at 24 hours after infection. Both had mean bacteria loads of approximately 10^3 CFU/ml of blood on the first day after infection which is expected because at this time these groups have not been administered any CRP and therefore should show bacteremia levels which are similar to the bacteria alone group (1.1×10^3 CFU/ml of blood). However, on the second day post infection, these two last groups (D and E) showed about a 10 fold difference of bacteria load between them; 2.3×10^7 CFU/ml and more than 10^8 CFU/ml of blood, respectively. It was therefore clear that mutant CRP does not protect when injected in the late stages of infection. (*p* value = 0.933, 24 hours; and *p* value = 0.041, 48 hours between these last two groups). Combining the data in (Figure 10) and (Figure 11), shows that the observed deaths were due to high bacteremia values

as the median bacteria load was observed to be concurrent with the death of mice from the second day when bacteremia increased exponentially.

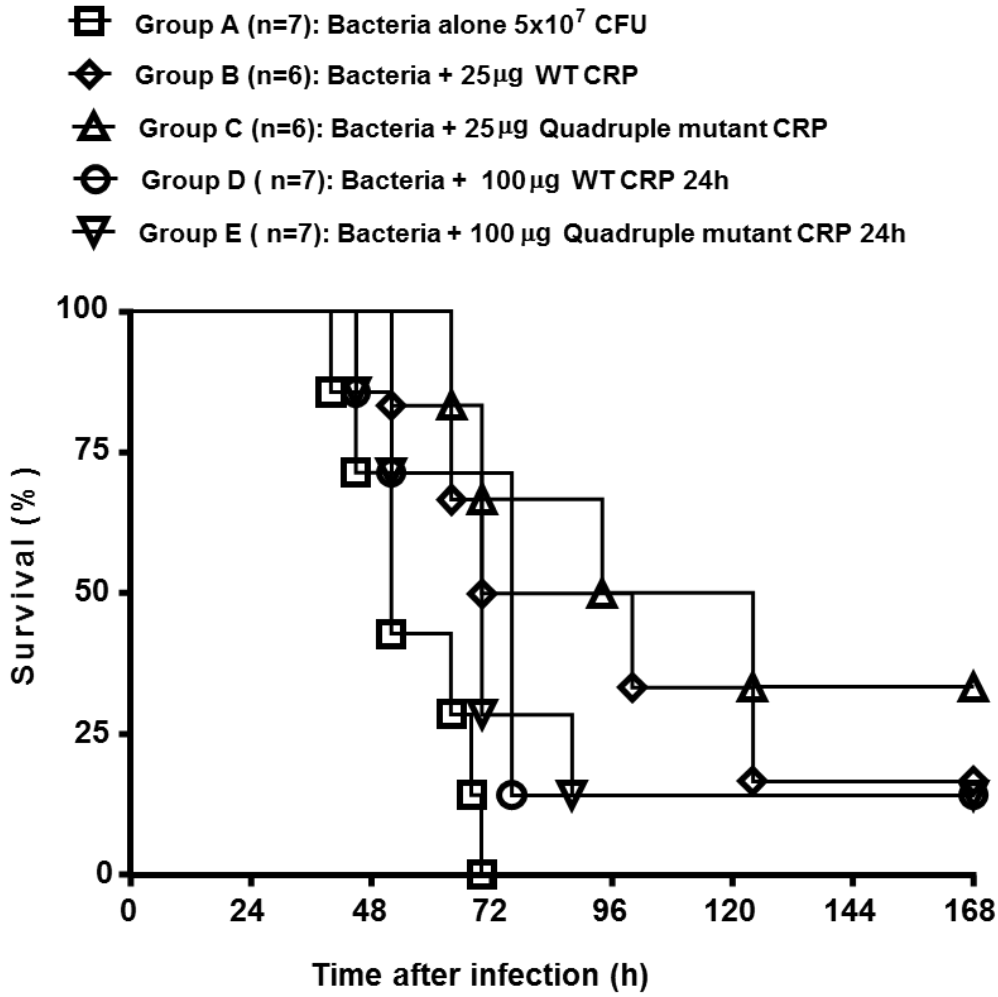

Figure 10. Survival curves of mice infected with *S. pneumoniae* with or without CRP. Mice were injected with 5 x 10^7 CFU pneumococci, with or without 25 µg of either WT or quadruple mutant CRP at time of infection or 100 µg after 24 h post infection. CRP was injected first; bacteria was injected 30 min later in Groups B and C. Deaths were recorded 3 times a day for 7 days.

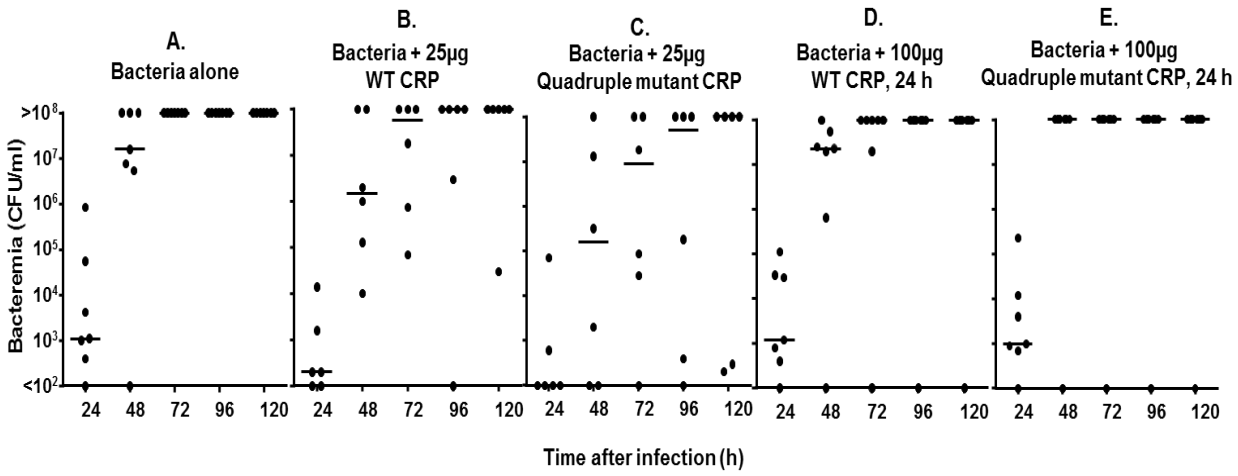

Figure 11. Bacteremia in of mice infected with *S. pneumoniae* with or without 25 µg WT or quadruple mutant CRP at time of infection or 100 µg WT or quadruple mutant CRP administered 24 h after infection. Blood samples were collected from each surviving mouse for the first 5 days after infection. Bacteremia was determined by plating on blood agar plates. Each dot represents 1 mouse. The horizontal line in each group of mice represents the median bacteremia value in that group. A bacteremia value of $>10^8$ CFU/ml indicates a dead mouse. Groups B and C received 25 µg CRP at the time of infection. Groups D and E received 100 µg CRP 24 h post infection.

It was proposed that mutant CRP will bind to factor H on the pneumococci surface in the late stage of infection and either by complement system activation or by opsonophagocytosis, clear the infection. This was on the assumption that it maintained or expressed the structures necessary for such immune activities. Since no protection was seen in the later stages of infection which should have reflected the use of either pathways (complement system or opsonophagocytosis), it was possible that the bound mutant CRP to factor H did not have the right conformation necessary for activation of these immune processes. However, these bacteria still expose the PCh moieties on their surface and given that the mechanism of protection has been shown to involve the binding of WT CRP to PCh (Gang et al. 2012). Therefore, a combination of WT CRP and mutant CRP was used expecting that mutant CRP will bind to factor H obstructing its inhibitory activity towards the activation of the complement system and

WT CRP bound to PCh on the surface of the bacteria would then be able to activate the complement system.

Quadruple Mutant CRP Protects Mice against Pneumococcal Infection when Administered in Combination with WT CRP during the Late Stages of Infection

The effects of injecting both WT and mutant CRP in late stage pneumococcal infection was next investigated. As expected, a combination of WT and mutant CRP was found to confer protection to mice in late stages of infection. Groups A and D which include bacteria alone and bacteria and WT CRP after 12 hours (late stage of infection) where not different in terms of their median survival times as expected. They both had a median survival time of 60 hours (Figure 12). As observed in the previous experiment, mutant CRP protects in the early stages of infection. There was no significant difference between this group and the positive control group (Group B) which received WT CRP at the time of infection (p value = 0.61) and in both groups more than 50% of mice survived to the end of the experiment. This suggests that mutant CRP is as protective as WT CRP in the early stages of infection. The median survival time for Group D, that received only WT CRP after 12 hours post infection was 60 hours and 90 hours for Group E that received both WT and mutant CRP at 12 hours post infection. There was a significant difference between these two groups (p value = 0.0003), suggesting that the protection seen was due to the additional mutant CRP injected. This protection was also observed in Group F which received the combination of WT and Mutant CRP much later than Group E, at 24 hours post infection. The median survival time for mice in Group F was 79.5 hours and, importantly, there was no significant difference between this group and Group E (p value = 0.58), but there was a

significant difference between this group and Group D (*p* value = 0.001). These results suggest that a combination of WT and mutant CRP is protective in the late stages of infection.

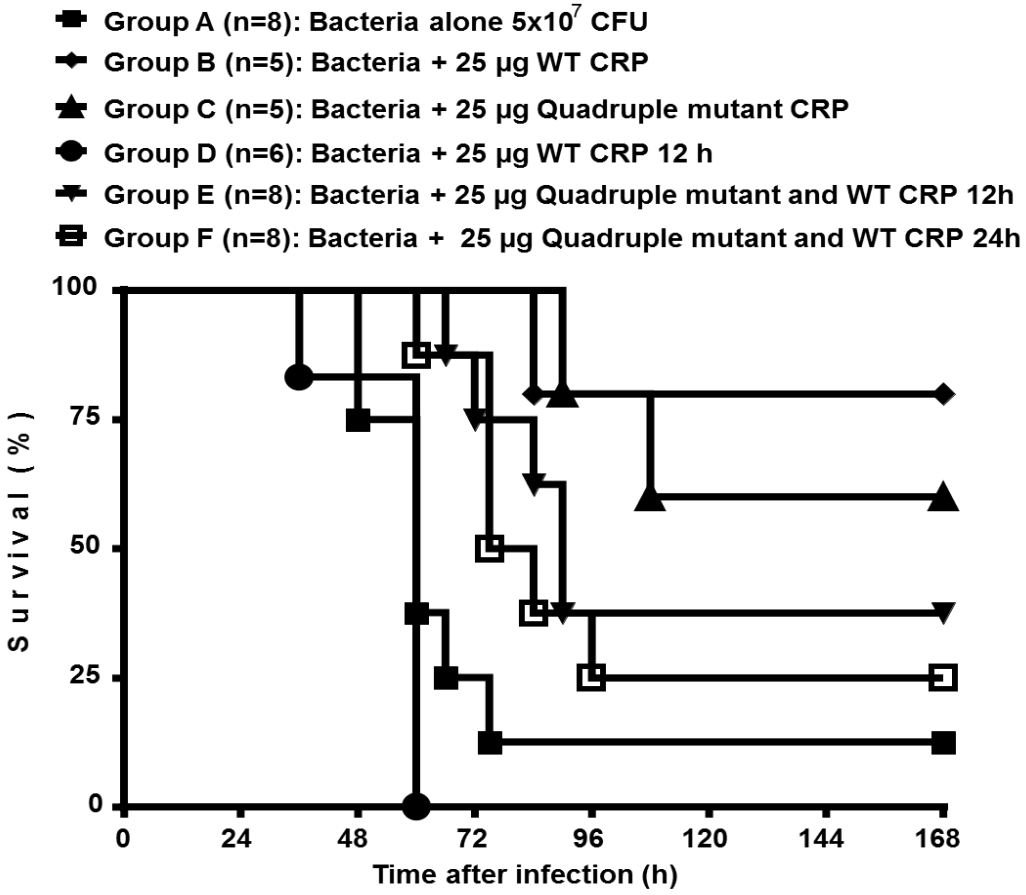

Figure 12. Survival curves of mice infected with *S. pneumoniae* with or without CRP. Mice were injected with 5 x 10^7 CFU pneumococci, with or without 25 µg of either WT or quadruple mutant CRP at time of infection or both after 12 and 24 h post infection or just WT CRP at 12 h after injection. CRP was injected first; bacteria was injected 30 min later in Groups B and C. Deaths were recorded 3 times a day for 7 days.

Bleeding and plating of blood collected from the tail vein of the mice were done twice daily in this experiment. This was to provide a more detailed view of the progression of the infection and to see the physiological changes that might not be seen by observing just mortality in the animals. However, there was no further information acquired from doing this. On the first

day following administration of pneumococci, the mice in the bacteria alone group (Group A) had a median bacteria load of 1550 and 5550 CFU/ml of blood at 12 and 20 hours, respectively (Figure 13). This increased to 1.5 x 10^7 and greater than 10^8 CFU/ml of blood at 36 and 44 hours,

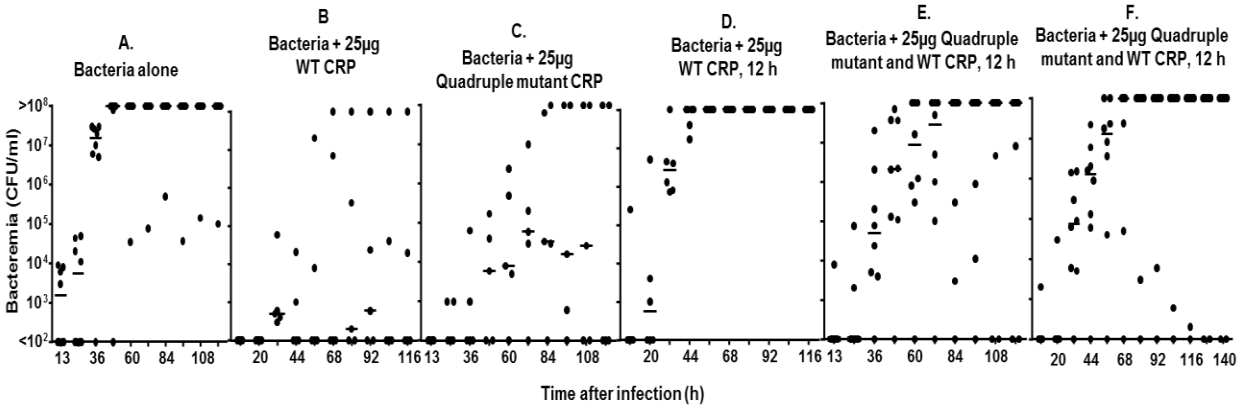

Figure 13. Bacteremia in mice infected with *S. pneumoniae* with or without 25 µg WT and/or quadruple mutant CRP at time of infection, 12 h and 24 h post infection. Blood samples were collected from each surviving mouse for the first 5 days after infection and 6 days for mice in Group F. Bacteremia was determined by plating on blood agar plates. Each dot represents 1 mouse. The horizontal line in each group of mice represents the median bacteremia value in that group. A bacteremia value of >10^8 CFU/ml indicates a dead mouse.

respectively, on the second day and stayed at 10^8 CFU/ml of blood throughout the experiment. In Group B, which received WT CRP at time of infection, the median bacteria load was less than 100 CFU/ml of blood at both 12 and 20 hours on day one. This was significantly different to the bacteria alone group. Throughout the experiment, the bacteremia in Group B was never found to reach 1000 CFU/ml and the majority of time it was lower than 100 CFU/ml of blood. This was similar with Group C which received mutant CRP at the time of infection. Even though the median bacteria load in Group C was generally higher than for Group B, it was still lower compared to the bacteria alone group. Hence, it could be said that WT CRP was a more efficient

anti-bacteriae agent than was mutant CRP and also suggest that two different pathways or mechanism of action are involved.

There was no significant difference in the survival of mice in Groups B and C (Figure 12). Therefore, even though there is an overall observed higher bacteria load in Group C compared to Group B, it was still within the acceptable bacteremia limits the animals could harbor without entering sepsis and dying. The bacteria load of mice in Group D was very similar to that seen in Group A, which was expected because WT CRP protects only when injected in the early stages of infection. Groups E and F also had a fairly similar pattern of median bacteria load as time went on. Interestingly, these two last groups had median loads higher than 10^8 CFU/ml of blood (at which bacteria load the mice die) after 68 and 60 hours, respectively, that was much later than 44 hours observed for Groups A and D. In summary, these data suggest that administering both WT and mutant CRP have the combined effect of reducing bacteremia opposed to administering only WT CRP or only mutant CRP (Figure 10).

CHAPTER 4

DISCUSSION

CRP is known to protect mice challenged with the lethal doses of *Streptococcus pneumoniae* (Suresh et al. 2006; Suresh et al. 2007; Agrawal et al. 2008) and this protection is seen only if CRP is injected 6 hours before to 2 hours after infection, requiring binding to PCh (Gang et al. 2012). This protective function of CRP is lost in late stages of infection (beyond 2 hours) and the reason is not known. However pneumococci has been shown to recruit factor H to its surface, and this ability helps it gain virulence as it inhibits the activation of the complement system when it does so (Jarva et al. 2002; Rodríguez de Córdoba et al. 2004). Therefore, in this study we hypothesized that in the late stages of infection, pneumococci would have had ample time to recruit factor H to its surface and that it was for this reason that CRP lost its ability to protect since it could no longer activate the complement system due to the inhibitory effect of factor H on the bacteria surface. To test this hypothesis, the effects of factor H had to be shielded. A quadruple mutant CRP was generated that does not bind to PCh but binds to factor H. It was constructed based on the information obtained from two other mutants, the E42Q mutant that moderately binds factor H and the triple mutant that does not bind PCh. This combination gave the quadruple mutant a synergistic ability to bind factor H (Figure 9) and made it incapable of binding to PCh. This mutant CRP was used to test the hypothesis in two conditions: Injecting mice challenged with lethal dose of pneumococci in the late stage of infection with either mutant CRP only or a combination of both WT CRP and mutant CRP.

Under the first condition where mutant CRP was injected alone, it was hypothesized that mutant CRP will possess the conformational structure necessary to activate the complement

system after binding to factor H recruited on the surface of pneumococci. However, the results did not support the hypothesis as analysis of the survival data show that there was no significant difference in the median survival time of mice that received the mutant CRP at the late stages of infection compared to the negative control group that received WT CRP at that time or with the group that received no protein (Figure 10). The observed survival was correlated with the bacteremia data obtained (Figure 11).Taken together, the data indicate that injecting mutant CRP alone in the late stages of infection does not confer any protection. Therefore, it could be interpreted that even though this mutant CRP binds to the recruited factor H on the pneumococci surface (Gang 2013), it does not possess the necessary conformation or expose the necessary structures needed to stimulate an immune response.

This result therefore prompted a combination experiment where both the mutant and WT CRP were injected. The hypothesis at this time was that the mutant CRP would bind to the recruited factor H on the bacteria surface and block its inhibitory function on the complement system and the WT CRP bound to the PCh exposed moieties would then be able to activate the complement system or by opsonophagocytosis lead to the clearance of the bacteria. The combination of mutant and WT CRP was found to be protective at the two different times it was injected (12 hours and 24 hours post infection). The bacteremia values obtained (Figure 13) showed that both groups that had received the combination of proteins had lower bacteremia compared to either the bacteria alone group or the group which received only WT CRP. These data indicate that the combination of these proteins was able to reduce the bacteria load in the mice not seen when only WT CRP was injected or when only the mutant was injected (Figure 11), therefore supporting our hypothesis that a combination of WT and mutant CRP would be protective in the later stages of infection. The reduced bacteremia observed was matched with improved survival in these two groups. Even though the median survival time of both groups was

lower than in Group B which received WT CRP at the time of infection, there was no significant difference between them.

The results showed that during the late stages of infection, protection could be acquired by administering a combination of WT and mutant CRP. Administering only WT CRP or just the mutant CRP did not improve survival. However, injecting mutant CRP at the time of infection was found to be protective. The mechanism of early protection had previously been established by the laboratory as requiring PCh (Gang et al. 2012) and later it was found that protection could be through PCh-dependent as well as PCh-independent mechanisms (Gang et al. 2015). Hence, the early protection seen with quadruple mutant CRP goes to confirm the later finding (Gang et al. 2015) because mutant CRP does not bind to PCh. Therefore, the mutant may be binding to some other ligand exposed on the surface of these bacteria. This remains to be investigated.

Mutant CRP binds to factor H and this property is the reason it was used in this study. However, this property could be a reason why it should not be used. That is, if this mutant binds to factor H which is also recruited by host cells to prevent the activation of host immune system against host cells giving rise to autoimmune diseases, then it will bind to and shield this very important function of factor H leading to attack directed to host cells by the immune system. However, protein-protein interactions are different from protein-sugar interactions. Factor H interacts with polyanionic molecules like sialic acid or glycosaminoglycans on host cells and with the Hic protein on pneumococci surface. We think that the conformational changes are different when factor H interacts with the Hic protein (Protein-protein interactions) compared to when it interacts with polyanionic molecules (protein-sugar interactions) and this difference in conformational change is exploited by the mutant to bind specifically to the factor H recruited on the surface of pneumococci.

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

This research was aimed at identifying the reason why late passive administration of WT CRP did not confer any protection to mice injected with lethal doses of pneumococci. The ability of these bacteria to recruit factor H was thought to be the reason for the late stage virulence. Therefore, using site directed mutagenesis, a mutant CRP was made using the cDNAs of two other mutants; the E42Q mutant and the F66A-T76A-E81A (triple) mutant as template. This quadruple mutant CRP (E42Q-F66A-T76Y-E81A) was able to bind factor H and was not able to bind PCh, therefore making it suitable for use to understand the importance of factor H in the late stages of pneumococcal infections in mice. HD2.4 reactivity analysis showed that this mutant maintained its pentameric structure. We were able to show in this work that factor H does play an important role in the gain of virulence seen in the late stages of infections. Quadruple mutant injected at the late stages of pneumococcal infection in mice, in combination with WT CRP shows protection as indicated by the survival and bacteremia results. However, injecting only the mutant CRP in the late stages of infection showed no protection. Protection was also observed when mutant CRP was injected at the time of infection.

Further studies could include performing complement activation assays with this mutant using factor H as ligand to determine if it can activate the complement system. However this will not help us understand why mutant protein is protective in the early stages of infection and not in the late stages where it requires the presence of WT CRP. The results suggest a mechanism that is available in the early stages of infection but not in the late stages. Furthermore, the bacteremia results obtained from the group of mice injected with WT CRP and those injected with mutant

CRP suggest the involvement of two different mechanism. It has not been possible to show by *in vivo* studies the mechanism or mechanisms involved in the clearance of bacteria with WT CRP, but assumed from *in vitro* studies to involve the activation of the complement system. This same difficulty will certainly be faced when attempting to define what mechanism or mechanisms are involved in early-stage protection with mutant CRP. Given that it could involve two or more mechanisms, makes identification all the more difficult. One thing common between this mutant and WT CRP is that they are both protective when administered at the time of infection and are both not protective when administered later unless administered together.

The protection conferred when both WT and mutant CRP were administered together at the later stages of infection was lower judging from the survival data, compared to administering either WT or mutant CRP at the time of infection. This could be because of several factors including the presence of more bacteria in the late stages of infection. Therefore, to improve the survival of animals in this group, further work will have to be done with the regimen to improve the availability of CRP in the system of these animals. To achieve this, increasing the dose of CRP injected and multiple injections could be done.

CRP is very important in the innate immune system. It recognizes and helps in the elimination of foreign material and dead cells from the body. For most of its innate immune functions, CRP has to undergo a conformational change. Some people lack the required microenvironment for CRP to undergo these changes. Therefore the long term goal of this research is to develop a mutant or a particle that can cause a conformational change in WT CRP to this functional form. This mutant CRP or particle could be administered to help against various disease conditions. In the processes, the mechanism or mechanisms underlying the protective function of CRP will be unveiled.

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APPENDICES

Appendix A: List of Abbreviations

BSA	Bovine serum albumin
CFU	Colony forming units
CHO	Chinese hamster ovary
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
EU	Endotoxin units
h	Hour
HRP	Horseradish peroxidase
i.v.	Intravenous
mAb	Monoclonal antibody
Min	Minute
OD	Optical density
PCh	Phosphocholine
PEt	Phosphoethanolamine
PnC	Pneumococcal C-polysaccharide
RT	Room temperature
SAP	Serum amyloid P component
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
WT	Wild-type

Appendix B: Recipe for Media and Agar

Complete media

(For 1 L): In a 1 L filtration flask,

900 ml	RPMI-1640
100 ml	Fetal Bovine Serum (FBS), heat inactivated (56°C, 30 min)
10 ml	Penicillin-Streptomycin stock (or Pn-Str-Fungizone)

Filter sterilized

Stored at 4°C

Warmed in 37 °C water bath for 30-45 minutes before use.

Freezing medium (culture medium containing 15% DMSO)

In a 100 ml filtration flask,

85 ml	culture medium
15 ml	DMSO

Filter sterilized; Stored at -10°C as 25 ml aliquots in 50 ml tubes.

G418 media

G418 sulfate stock was made at 50 mg/ml in water

Dissolved 0.5 g G418 sulfate powder in 10 ml of water

Filter sterilized and stored at -20°C or added all 10 ml of this stock to 500 ml of complete RPMI media for a final concentration of 1 mg/ml

Todd Hewitt broth plus 0.5% yeast extract (TH-YE)

Bacto Todd Hewitt broth	15 g
Yeast Extract	2.5 g

Dissolved in 450 ml of dH_2O, adjusted volume to 500 ml with water

Divide into 5 bottles (100 ml in each of 250 ml bottle), autoclave at 121°C for 15 minutes

Store at room temperature

*Before use, a sterilized 250 ml flask was used into which 50 ml of the Todd Hewitt broth plus yeast extract (TH-YE) was aseptically transferred for cultivation of the bacteria.

5% Sheep blood agar plating media

Tryptic soy agar (TSA) 20 g

Added 20 g of TSA into 475 ml of water in a 1 L flask

Autoclaved at 121°C for 15 minutes (liquid cycle) and allowed the media to cool in a 55°C water bath for 1-2 hours.

500 ml of the media makes approximately 25 plates (roughly 5 ml/plate)

Placed the number of petri dishes out on the bench top with the flame burning

When the flask is warm to the touch (but not hot), aseptically transfered 25 ml of defibrinated sheep blood to 475 ml of TSA media (500 ml total)

Aseptically poured ~5ml/dish, covered the plates, left them to solidify O/N at RT

Normal saline

(0.9% NaCl) – 0.9 g NaCl per 100 ml of water

Autoclaved and stored at RT

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