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A Novel Mode of Action of C-reactive Protein in Protecting Against Streptococcus pneumoniae Infection and Synergy with Antibiotics

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A Novel Mode of Action of C-reactive Protein in Protecting Against *Streptococcus pneumoniae* Infection and Synergy with Antibiotics

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by

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ABSTRACT

A Novel Mode of Action of C-reactive Protein in Protecting Against *Streptococcus pneumoniae* Infection and Synergy with Antibiotics

by

Donald Neba Ngwa

C-reactive protein (CRP) is a part of the innate immune system, is synthesized in the liver, its blood level increases in inflammatory states, and it binds to Streptococcus pneumoniae. The conformation of CRP is altered under conditions mimicking an inflammatory milieu and this non-native CRP also binds to immobilized/aggregated/pathogenic proteins. Experiments in mice have revealed that one of the functions of CRP is to protect against pneumococcal infection. For protection, CRP must be injected into mice within two hours of administering pneumococci, thus, CRP is protective against early-stage infection but not against late-stage infection. It is unknown how CRP protects or why CRP does not protect against late-stage infection. The hypotheses are that the protection requires complement activation by CRP-pneumococci complexes and that CRP cannot protect if pneumococci have time to recruit complement inhibitor factor H on their surface to become complement attack-resistant. To test these hypotheses, we generated CRP mutants by site-directed mutagenesis: a mutant that binds to pneumococci but does not activate complement and a mutant that binds to immobilized factor H. We found that mutant CRP incapable of activating complement was not protective against infection and that mutant CRP capable of binding to factor H was protective against both early and late stage infections. Additional experiments showed that CRP enhances the effects of the antibiotic clarithromycin in reducing bacteremia in infected mice. Moreover, we observed that mutant CRP capable of binding to factor H bound to several proteins immobilized on plastic,

suggesting that CRP recognizes a pattern, probably an amyloid-like structure, on immobilized proteins. Indeed, mutant CRP, after binding to amyloid β peptides, prevented the formation of pathogenic amyloid fibrils. Lastly, employing a hepatic cell line, we investigated the mechanism of CRP expression in response to pro-inflammatory cytokines. We found that the transcription factor C/EBPβ and two C/EBP-binding sites on the CRP promoter were critical for inducing CRP expression. We conclude that complement activation is necessary for CRP-mediated protection against infection, that CRP functions in two structural conformations, that CRP and clarithromycin act synergistically, that CRP has anti-amyloidogenic properties, and the increased CRP expression requires C/EBPβ.

DEDICATION

This manuscript is dedicated to my father Mr. David Neba and my mother Mrs. Esther Manka'a, who both encouraged and supported me all through my education to this point. They went out of their way financially to make all my education endeavors possible. A special thanks for all their hard work and belief in me. I also dedicate this work to 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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LIST OF ABBREVIATIONS

Aβ	Amyloid-beta
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer-binding protein
CFU	Colony forming units
CHO	Chinese hamster ovary
CRP	C-reactive protein
ELISA	Enzyme linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
EU	Endotoxin units
FH	Factor H
h	Hour
HNF	Hepatocyte nuclear factor
HRP	Horseradish peroxidase
H_2O_2	Hydrogen peroxide
IL-1	Interleukin-1
IL-6	Interleukin-6
i.v.	Intravenous
Luc	Luciferase
mAb	Monoclonal antibody
Mut	Mutant
NF-κB	Nuclear factor kappa B
OCT	Octamer

OD	Optical density
Oligo	Oligonucleotide
O/N	Overnight
Ox-LDL	Oxidized low density lipoprotein
PCh	Phosphocholine
PEt	Phosphoethanolamine
PnC	Pneumococcal C-polysaccharide
RLU	Relative luciferase units
SAP	Serum amyloid P component
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STAT3	Signal transducer and activator of transcription 3
TBS	Tris buffered saline
WT	Wild-type

CHAPTER 1: INTRODUCTION

When the body is faced with injury or infection, inflammation is a defense mechanism it employs to begin the healing process. Part of this inflammatory response involves the production and release of an innate immune molecule called C-reactive protein (CRP) into the blood-stream at very high levels by the liver (Du Clos and Mold 2004). CRP was discovered in the 1920s in the laboratory of Oswald T. Avery during studies to develop therapies against pneumococcal pneumonia. A substance in fraction C was found to strongly precipitate a protein in the serum taken from patients during the early, acute stage of infection. The substance was later determined to be pneumococcal C-polysaccharide (PnC) and the protein CRP (Tillett, Francis, and Jr. 1930; Tillett, Goebel, and Avery 1930; Abernethy and Avery 1941). Due to the surge in CRP levels during inflammation, it is also used as a marker of acute general inflammation.

Structure of CRP

Wild-type (WT) CRP is a pentamer, with each of its 5, ~23 KDa subunits held in the same orientation by non-covalent bonds around a central pore (Fig. 1.1) (Thompson, Pepys, and Wood 1999). This orientation specific property of CRP gives it two faces (sometimes referred to as recognition and effector faces), each performing a distinct role. The ligand recognition face of CRP houses' the binding site for CRP's native ligand phosphocholine (PCh). Each of the five homopentameric subunits of CRP bear a PCh binding site which sits very close to its Ca^{2+}-binding site. For the interaction between PCh and CRP to occur, the phosphate group of PCh has to interact with two calcium ions in the Ca^{2+}-binding site of CRP and the three methyl groups of PCh accommodated in the hydrophobic pocket of the PCh-binding site of CRP. The hydrophobic pocket of the PCh-binding site is made up of Leu^{64}, Phe^{66}, Thr^{76} and Glu^{81} among other amino acids. Two Ca^{2+} are constantly bound to WT CRP, and this is very crucial for the stability of the

molecule. The two Ca^{2+} are coordinated by several amino acids in a loop (the amino acids include; Asp^{60}, Asn^{61}, Glu^{138}, Gln^{139}, Asp^{140}, Glu^{147}, and Gln^{150}) (Shrive et al. 1996; Thompson, Pepys, and Wood 1999; Ramadan et al. 2002). When the calcium binding site is vacant, this loop with a proteolytic site folds outward and becomes exposed to proteolysis (Thompson, Pepys, and Wood 1999).

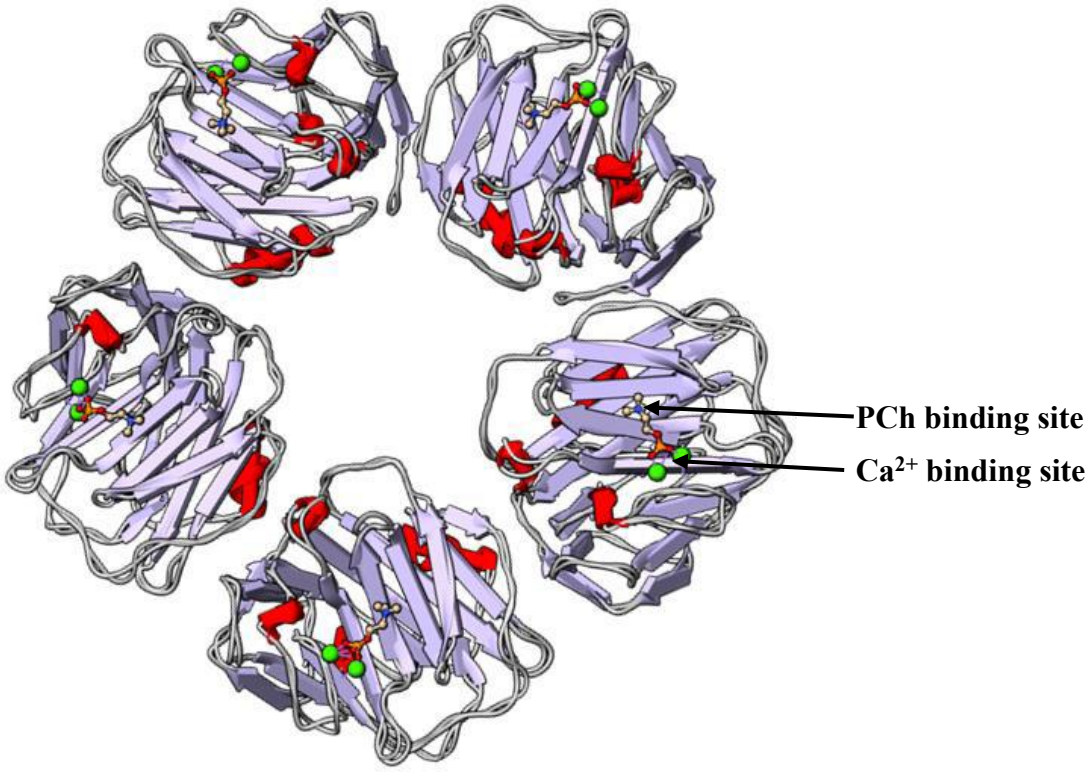

Figure 1. 1: Structure of the CRP-PCh complex. The phosphocholine moiety is shown in ball-and-stick and the Ca^{2+} ions as green spheres. (modified from (Thompson, Pepys, and Wood 1999))

The opposite face, or the effector face of CRP has a deep cleft necessary for the interaction between CRP and complement protein C1q as well as Fcγ receptors (A Agrawal and Volanakis 1994; A Agrawal et al. 2001; Bang et al. 2005), two molecules necessary for CRP's core effector functions.

Functions of CRP

In humans, CRP is a unique plasma protein, displaying functional characteristics specific for the conformational state adopted (Donald N. Ngwa and Agrawal 2019). It is a key acute phase protein and mediates several inflammatory responses. CRP in its native conformation binds substances with exposed PCh groups like, pneumococcal C-polysaccharide (PnC) on bacterial cell walls, low-density lipoprotein (LDL), and apoptotic or damaged cells in a Ca^{2+}-dependent manner (A Agrawal et al. 1997; Bhakdi et al. 1999; Volanakis 2001; Chang et al. 2002). The CRP-PCh complex is then recognized by complement component C1q, the first component of the classical complement pathway, binding on the opposite side of CRP and activating the classical complement cascade leading to the clearance of the PCh bearing particle (Kaplan and Volanakis 1974). The clearance could be due to the formation of the membrane-attack complex or due to opsonophagocytosis by macrophages. The effector Fcγ receptors on macrophages have been shown to bind ligand complexed CRP inducing phagocytosis (Marnell et al. 1995; Bang et al. 2005).

Human CRP is protective against lethal *Streptococcus pneumoniae* infections in mice by decreasing bacteremia and increasing survival of infected animals (Mold et al. 1981; Yother, Volanakis, and Briles 1982; Szalai, Briles, and Volanakis 1995; Suresh et al. 2007; Alok Agrawal et al. 2008; Paul Simons et al. 2014). Passively administered human CRP is only protective when injected 6 h before to 2 h after infecting mice with lethal pneumococci, but not when administered 24 h post infection. The PCh-binding pocket of CRP has been shown to be important for the CRP-mediated initial protection of mice against lethal pneumococcal infection (Suresh et al. 2006; Alok Agrawal et al. 2008; Gang et al. 2012). However, mutating the PCh binding site on CRP surprisingly resulted in mutants still capable (at higher dosages) of

providing protection against lethal pneumococcal infection. This suggest both PCh-dependent and independent mechanisms of protection (Suresh et al. 2007; Gang, Hanley, and Agrawal 2015). The reason why CRP is not protective when administered during the late stages (beyond 2 h post infection) of infection is not known. It is however thought to be related to the bacteria acquiring the complement inhibitory protein factor H.

Factor H

Factor H is a soluble 150 kDa single chain plasma glycoprotein and is one of the regulators of complement activation (Rodríguez de Córdoba et al. 2004). It is made up of 20 short consensus repeats of 60 amino acids each, stabilized by two internal disulfide bonds (Sharma and Pangburn 1996). Factor H is not only constitutively expressed in the liver but also produced by monocytes, fibroblasts, endothelial cells, keratinocytes, and platelets (Devine and Rosse 1987; Katz and Strunk 1988; Mullenix and Mortensen 1994) and has a circulating plasma concentration of 200-300 μg/ml (Hakobyan et al. 2008, 2010). Factor H inhibits complement activation on surfaces by acting as a cofactor for factor I in the cleavage and inactivation of C3b. It also accelerates the decay of C3 convertase of the alternative pathway of complement (Weiler et al. 1976; M K Pangburn, Schreiber, and Müller-Eberhard 1977). By interacting with polyanionic molecules such as sialic acid or glycosaminoglycans usually present on the surface of host cells but absent on surfaces of pathogens, it allows the complement system to selectively target non-host particles for complement activation (Michael K Pangburn 2000; Józsi et al. 2004; Rodríguez de Córdoba et al. 2004). There is no evidence of an interaction between native wild-type (WT) CRP and factor H under conditions of infection and/or inflammation (M Mihlan et al. 2009). However, structurally modified CRP has been shown *in vitro* to bind to immobilized factor H but not to fluid-phase factor H (Donald Neba Ngwa n.d.; Hammond et al. 2010).

Streptococcus pneumoniae is one bacterium known to recruit factor H to its surface to evade immune system recognition and attack, and therefore increasing its virulence. They make use of the Hic protein expressed on their cell surface to bind factor H (Jarva et al. 2002).

Pneumococcus

Streptococcus pneumoniae (pneumococcus) is a Gram-positive bacterium sometimes observed in pairs and called diplococci for that reason (Winslow et al. 1920). They are mostly arranged in chains (streptococcus) but can also be found singly. Pneumococcus remains the most common cause of community-acquired pneumonia world-wide (van der Poll and Opal 2009) and colonizes the respiratory tract, sinuses and the nasal cavity of healthy individuals asymptomatically. In immunocompromised individuals such as children and the elderly, they can move into the blood stream and cause disease (Hughes et al. 2014). Infections caused by these bacteria include bronchitis, otitis media, conjunctivitis and peritonitis to name a few (Tuomanen, Robert, Austrian, and Robert, Masure 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. Some pneumococci species have a capsule and the level of encapsulation is directly related to species pathogenicity (virulence) (Jackson and Pilishvili 2015). Present on the cell wall of pneumococci, are protrusions of PnC with PCh moieties attached (Fig. 1.2). CRP is thought to bind these exposed PCh groups and activate the complement system. Another protein, the factor H inhibitor of complement (Hic) protein, that is also exposed on the surface of these bacteria is known to recruit factor H, a complement inhibitory protein known to help pneumococci escape complement attack (Dagan 2000).

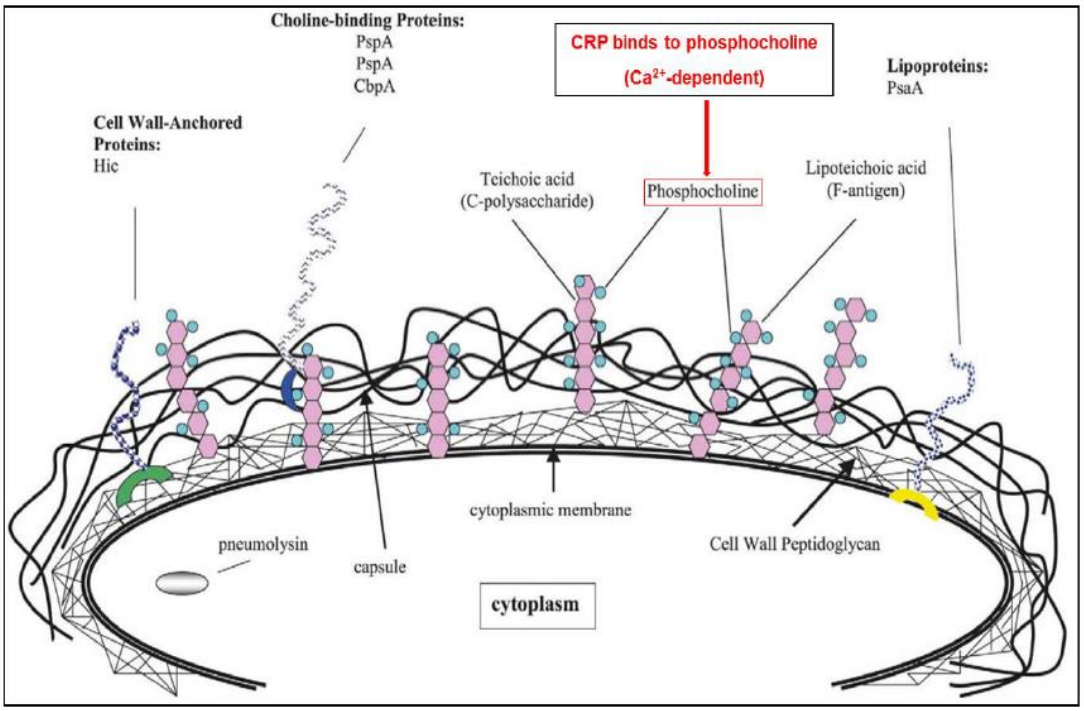

Figure 1.2: Structural representation of selected immunogenic proteins of *S. pneumoniae*. The figures illustrates the binding of CRP to PCh (modified from (DeLano W. L. 2010)).

CRP and Antibiotics in Pneumococcal Infections

The mechanism of the anti-pneumococcal function of CRP is not fully known (Alok Agrawal et al. 2008; van der Poll and Opal 2009). In mice, CRP is only a trace serum protein and, contrary to man, not an acute phase protein (Whitehead et al. 1990). This makes the mouse model very suitable to explore the *in vivo* functions of human CRP. Passively administered human CRP has been shown to be protective against lethal pneumococcal infection as determined by increased survival of and decreased bacteremia in the infected mice (Suresh et al. 2007; Gang, Hanley, and Agrawal 2015; Szalai, Briles, and Volanakis 1995). However, CRP was most effective in protecting mice from infection only when injected within the range of 6 h

before to 2 h after administering pneumococci to mice (Suresh et al. 2006). The protective function of CRP was not observed when mice received CRP 24 h or 36 h post-infection (Suresh et al. 2007, 2006). Thus, CRP-mediated protection of mice requires the presence of CRP in the early stages of infection. Mice transgenic for human CRP were also protected from lethal pneumococcal infection and showed both decreased bacteremia and increased survival (Szalai, Briles, and Volanakis 1995). Taken together, this suggests that WT CRP has only a prophylactic role in pneumococcal infection. Why this is the case is not known. However, pneumococci are known to recruit factor H to their surface through the Hic protein, and are thus able to inhibit and prevent the attack by the complement system (D. Ngwa 2016). WT CRP does not bind to factor H. However, structurally modified CRP prepared *in vitro* is capable of binding to immobilized factor H (Donald N. Ngwa and Agrawal 2019).

CRP may exist in more than one structural form in humans. This will include native and structurally altered pentameric forms. We propose that under inflammatory conditions, CRP is structurally modified, and this structurally altered CRP is capable of binding to even those pneumococci which have recruited factor H on their surface. Binding of structurally altered CRP to factor H-covered pneumococci will relieve the inhibitory effects of factor H leading to the activation of the complement system and subsequent killing of pneumococci.

Antibiotics are usually employed in treatment measures against pneumococcal infections. Some antibiotics that were commonly used included penicillins, macrolides, clindamycin, cephalosporins, rifampin, vancomycin, and trimethoprim-sulfamethoxazole (“Pneumococcal Infections (Streptococcus Pneumoniae) Medication: Antibiotics, Vaccines” n.d.). However, from the 1990s many pneumococcal isolates showed decreased susceptibility to penicillin and other commonly used antibiotics (Fenoll et al. 1998; Postma et al. 2015). This gave rise to a rush to

develop new antibiotics to combat resistant strains. However, the past two decades has seen the introduction of only two new classes of antibiotics to which resistance is already emerging and therefore requiring the development of new treatment strategies. One of these strategies involves the use of drug combination therapies (antibiotic-antibiotic combinations and antibiotic and non-antibiotic adjuvant molecule combinations) (Caballero and Rello 2011; Worthington and Melander 2013). This has been shown not only to be more effective against resistant bacteria but also significantly reduces any risk of bacteria developing resistance as seen in monotherapy (Fox, Sutherland, and Daniels 1954; Caballero and Rello 2011).

Rationale and Hypothesis

Pneumococci have been demonstrated to recruit factor H onto their surface (D. Ngwa 2016). We hypothesize that the bacteria recruit factor H *in vivo* and exploit its complement regulatory property to prevent killing through complement activation. It has been determined that the administration of native CRP into mice later during infection does not provide protection (Suresh et al. 2007). Factor H has been shown to bind to modified forms of CRP (Bíró et al. 2007; Hakobyan et al. 2008; M Mihlan et al. 2009; Michael Mihlan et al. 2009; Okemefuna et al. 2010; Hammond et al. 2010). We have described the E42Q/F66A/T76Y/E81A CRP mutant which has lost its binding ability to PCh but has acquired factor H binding ability (D. Ngwa 2016). We suggest that a CRP mutant that does not bind to PCh but binds to factor H will allow investigation of the potential involvement of factor H in bacterial resistance. If the above hypothesis is supported, we propose that this CRP mutant will bind to factor H on the bacterial surface and neutralize its complement regulating activity. This would allow WT CRP bound to the bacteria to facilitate complement activation and deposition (Fig. 1.3). However, if complete protection is not achieved by using a combination of WT and mutant CRPs in the late stages of

infection, this would lead to the hypothesis that using an antibiotic at low dose in combination with mutant CRP may confer complete protection.

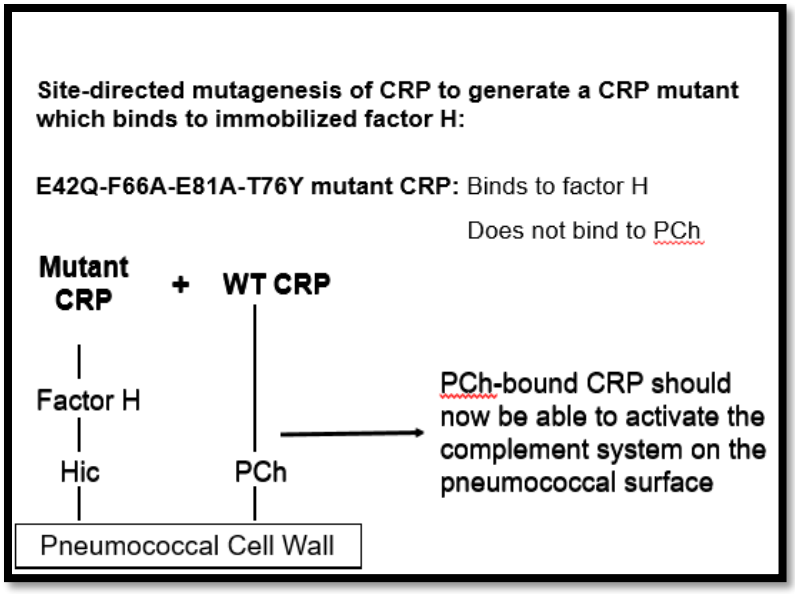

Figure 1. 3: Site-directed mutagenesis of CRP to generate a CRP mutant which binds to immobilized factor H.

Structure/Function Relationship of CRP

The dissociation of native pentameric CRP to monomeric CRP goes through a third non-native pentameric state or conformation (Thiele et al. 2014; Braig et al. 2017; Singh et al. 2017; McFadyen et al. 2018). Therefore, CRP can adopt either native pentameric, non-native pentameric or monomeric forms. Each of these forms of CRP show different ligand binding functions *in vitro* (Suresh, Singh, and Agrawal 2004; Alok Agrawal, Gang, and Rusiñol 2014; Wu et al. 2015; Donald N. Ngwa and Agrawal 2019). These structural modifications arise when CRP is exposed to acidic pH, high salt concentration, and oxidation (Singh et al. 2012, 2017).

CRP in its native conformation does not bind immobilized proteins including factor H, however, acidic pH-induced structurally modified CRP does (Hammond et al. 2010). CRP is not monomerized at acidic pH, but assumes a loosened pentameric conformation (Mold, Kingzette, and Gewurz 1984; Sjöberg et al. 2007; Hakobyan et al. 2008; Hammond et al. 2010). A single sequence motif known as the cholesterol binding sequence (CBS; amino acids 35-47) has been shown to mediate the interactions of monomerized CRP with multiple protein ligands (Li et al. 2016). However, monomeric CRP is not free in circulation and always found deposited. This implies an intermediate state exists between native pentameric and monomeric CRP that can freely circulate and has the binding characteristics of monomeric CRP. The structural changes introduced to CRP by an acidic milieu or oxidation are lost upon neutralization as the molecule reverts to its native conformation. This makes acidic pH-treated CRP unsuitable for *in vivo* experiments due to the buffering effects of blood. Hence, stable CRP mutants generated by mutagenesis suitable for *in vivo* experiments are used (Singh et al. 2012; D. Ngwa 2016). Some of the ligands non-native pentameric CRP is known to interact with include Ox-LDL, IgG, amyloid-β (Aβ) and factor H to name a few (Hammond et al. 2010; D. Ngwa 2016; Singh et al. 2017).

Rationale and Hypothesis

WT CRP does not bind to factor H, but structurally modified mutant CRP or acidic pH-treated CRP have been reported to do so (D. Ngwa 2016; Hammond et al. 2010). The binding of modified CRP to factor H has been shown to be inhibited by a CRP derived, 9-amino acid long, peptide known as the cholesterol binding sequence (CBS). The property of modified CRP to bind to immobilized proteins has also been seen with CRP mutants with mutations in the Ca^{2+}-binding site, in the intersubunit region, and in the lysophosphatidylcholine (LPC)-binding site. It is

possible that certain mutations in CRP expose an otherwise hidden binding site for protein ligands on CRP. Therefore, mutating residues in this otherwise hidden multiple ligand binding site should abolish the binding property of CRP even in an acidic environment. Hence, we aimed at generating a CRP mutant which does not bind to protein ligands in acidic conditions.

We hypothesize that the interaction of modified CRP with its ligands is specific and that mutating one or more amino acids in the CBS region of CRP will abolish this interaction. We also hypothesize that immobilized proteins expose amyloid-like structures which are the moieties on aggregated and immobilized proteins recognized by modified CRP.

CRP Gene Expression

The healthy median CRP serum concentration in humans is 0.8 μg/ml (Pepys and Hirschfield 2003). The level is seen to increase in individuals with chronic inflammation and to rise by several hundred fold in acute inflammation (Pepys and Hirschfield 2003). CRP levels have also been seen to rise in some non-inflammatory conditions such as stress (Gabay and Kushner 1999; Shivpuri et al. 2012). This has made measuring CRP levels a diagnostic tool to determine systemic inflammation. As with the rapid rise of CRP levels following inflammation, there equally is a rapid reduction to basal levels after the inflammation is resolved (Fig. 1.4) (Gabay and Kushner 1999).

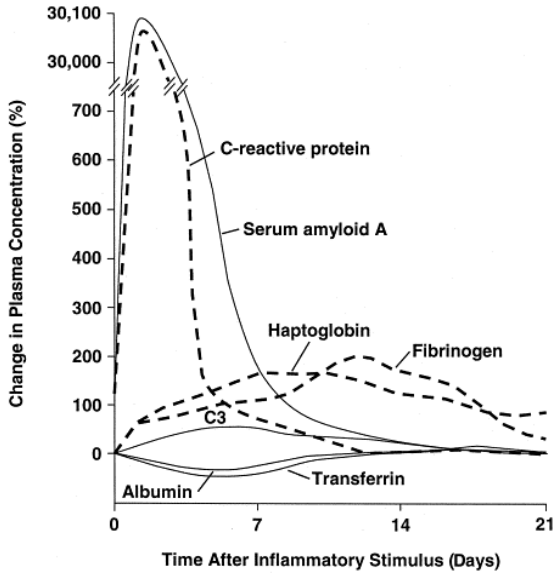

Figure 1. 4: Changes in plasma concentration of several acute phase proteins including CRP, following an inflammatory stimulus (adapted from (Gabay and Kushner 1999)).

Due to the involvement of CRP in various pathological conditions, its gene regulation becomes highly significant to understand how its expression is modulated during and after inflammation. CRP is produced mainly by hepatocytes primarily in response to inflammatory cytokines (Gabay and Kushner 1999). The cytokines come from a variety of cell types, but the most common sources are macrophages and monocytes at inflammatory sites. Cytokines that are produced during and which participate in the inflammatory process are the main stimulants in the production of acute phase proteins like CRP. Some of these inflammation-associated cytokines include interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor α (TNF- α), interferon-γ (INF- γ), and interleukin-8 (IL-8) (Wigmore et al. 1997; Kushner 1993). In the human hepatoma Hep3B cell line, the cytokine IL-6 induces CRP gene expression by activating the transcription factors C/EBPβ and STAT3 (Ochrietor et al. 2000; Poli and Cortese 1989; Ramji et al. 1993; Wang et al. 1999). Cytokine IL-1β alone cannot induce CRP expression, but synergistically enhances the effects of IL-6 by activating the transcription factor NF-κB

(Ganapathi et al. 1988; Darlington, Wilson, and Lachman 1986; Alok Agrawal et al. 2003). It has been shown that the proximal 157 bp of the CRP promoter are sufficient for the synergistic effects of IL-6 and IL-1β (Zhang et al. 1995). This region of the CRP promoter has binding sites for multiple transcription factors, and previous studies have identified the binding sites at which the transcription factors C/EBPβ, STAT3 and NF-κB bind and induce CRP gene expression (Cha-Molstad et al. 2007; Alok Agrawal et al. 2003).

The 157 bp proximal promoter region of the CRP gene also has binding sites for constitutively expressed transcription factors HNF-1, HNF-3, C/EBPζ and Oct-1 (Fig. 1.5). IL-6 activates the C/EBP family of transcription factors. There are six members in the C/EBP family of transcription factors that homodimerize or heterodimerize with each other and influence gene transcription (Artavanis-Tsakonas, Rand, and Lake 1999). C/EBPβ binds to its site centered at -52 and -219 on the CRP promoter. The binding site positioned at -52 overlaps with the binding sites for NF-kB (p50-p50) and HNF-3 transcription factors (Fig. 1.5). In this context, it is not known how C/EBPβ regulates CRP gene transcription and also not much is known about how the C/EBPβ site at -219 works to regulate the transcription of the CRP gene. Therefore, the mechanisms involved in C/EBPβ mediated regulation are not properly understood.

Rationale and Hypothesis

The 300 bp promoter region of the CRP gene shows a significantly better induction of CRP gene expression than the 157 bp promoter region even though the 157 bp region is sufficient for the synergy seen between IL-6 and IL-1β (Voleti and Agrawal 2005). Therefore, investigating the role of the C/EBPβ site at -219 is of importance. Given that the C/EBPβ site at -52 overlaps with other constitutively activated transcription factors (Fig. 1.5), we hypothesize that the relative concentrations of constitutively active and cytokine-activated transcription factors determine the

activity of the CRP promoter and that the additional C/EBP site at -219 is responsible for the enhanced induction of the CRP gene seen with the 300 bp CRP promoter.

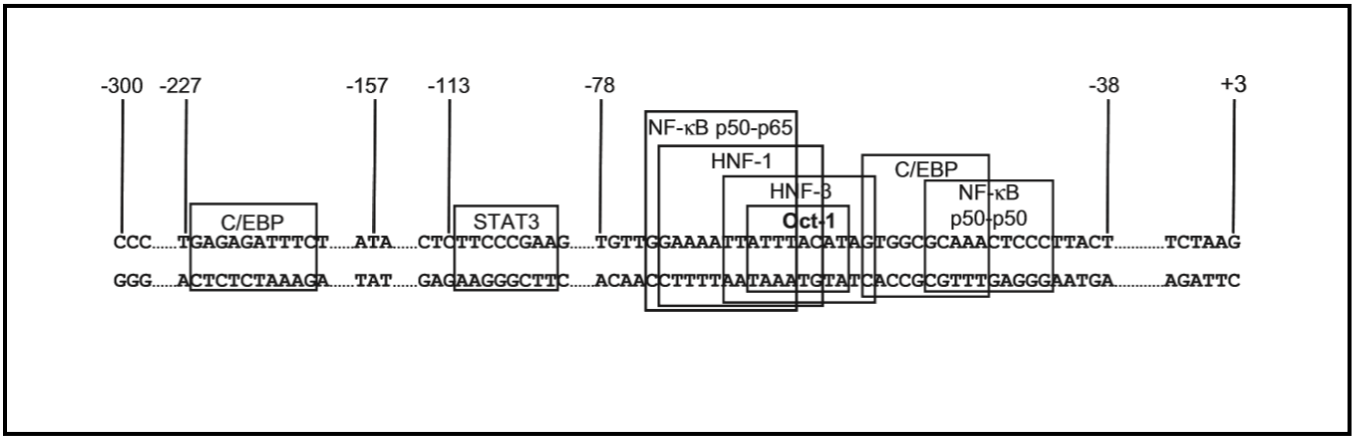

Figure 1. 5: The -300 to +3 region of the proximal promoter region of the CRP gene is shown. The binding sites of various transcription factors on the promoter are boxed (adapted from (Voleti and Agrawal 2005))

In order to test these hypotheses, the following specific aims were developed:

Specific Aims

1. To determine the role of complement in CRP mediated protection against pneumococcal infection in mice.
    a. Identify a CRP mutant that does not activate the mouse complement system.
    b. Investigate the protective effects of this CRP mutant in a mouse model of pneumococcal infection.

2. To determine the efficacy of a CRP mutant capable of binding to factor H in protection against pneumococcal infection.

a. Investigate the efficacy in a late-stage infection model in which native CRP has been shown to be ineffective.
b. Investigate the protective effects of the CRP mutant when combined with an antibiotic in both early-stage and late-stage infection models.

3. To investigate the mechanism of binding of CRP to aggregated and immobilized proteins including factor H.
    a. To define the ligand-binding site on CRP when CRP is in its alternate pentameric structural conformation.
    b. To evaluate the significance of the interaction of modified CRP with aggregated and immobilized protein ligands.

4. To define the role of the transcription factor C/EBPβ in IL-6-induced CRP expression in hepatic cells.
    a. Investigate the role of the C/EBP-binding sites located at positions -52 and -219 on the CRP promoter.
    b. Investigate the interactions of C/EBPβ with other transcription factors bound to the nearby and overlapping sites on the promoter.

CHAPTER 2: C-REACTIVE PROTEIN PROTECTS MICE AGAINST PNEUMOCOCCAL INFECTION BY ACTIVATING THE COMPLEMENT SYSTEM

Running Title: Complement activation by CRP in infection

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Keywords: Acute phase reactants, C-reactive protein, Complement, Inflammation

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Abstract

C-reactive protein (CRP), a component of the innate immune system, is an anti-pneumococcal plasma protein. It has been shown that human CRP protects mice against infection with lethal doses of *Streptococcus pneumoniae*; the protection is due to decrease in bacteremia and increase in survival time. CRP binds to phosphocholine-containing substances, such as pneumococcal C-polysaccharide, in a Ca^{2+}-dependent manner. Phosphocholine-complexed human CRP activates the complement system in both human and murine sera. The mechanism of anti-pneumococcal action of CRP *in vivo*, however, has not been defined. In this study, we tested a decades-old hypothesis that the complement-activating property of ligand-complexed CRP contributes to protection of mice against pneumococcal infection. We employed site-directed mutagenesis of CRP, guided by its three-dimensional structure, and identified a mutant H38R which, unlike wild-type CRP, did not activate complement C3 in murine serum. Substitution of His^{38} with Arg in H38R CRP did not affect the Ca^{2+}-dependent binding of CRP to pneumococci, did not affect the stability of CRP in vivo, and did not affect the overall pentameric structure of CRP. Employing a murine model of pneumococcal infection, we found that passively administered H38R CRP failed to protect mice against infection. Infected mice injected with H38R CRP showed no reduction in bacteremia and did not survive longer, as opposed to infected mice treated with wild-type CRP. Thus, the hypothesis that complement activation by ligand-complexed CRP is an anti-pneumococcal effector function was supported. We conclude that complement activation by ligand-complexed CRP is essential for CRP-mediated protection against pneumococcal infection.

Introduction

C-reactive protein (CRP) is a multifunctional component of the acute phase response and innate host defense machinery (1, 2). CRP is composed of five identical subunits arranged as a cyclic pentamer (3, 4). Each subunit has a phosphocholine (PCh)-binding site through which CRP binds to PCh-containing substances such as C-polysaccharide (PnC) of the cell wall of *Streptococcus pneumoniae*, in a Ca^{2+}-dependent manner (3-6). After complexing with a ligand such as PnC, CRP activates the complement system (7, 8). Human CRP activates complement in both human and murine sera (9, 10). In human serum, CRP binds to C1q and activates the classical pathway of complement (7). Since human CRP does not interact with murine C1q, it is not known which pathway is utilized by human CRP to activate murine complement (9).

The C1q-binding site of CRP is formed in and around a cleft that is located on the opposite side of the PCh-binding site of the CRP pentamer (3, 4). The amino acid residues which contribute to the formation of the C1q-binding site of CRP are His^{38}, Glu^{88}, Asp^{112}, Asn^{158} and Tyr^{175} from one subunit and Lys^{114} from the neighboring subunit. Mutational analysis of these amino acids revealed that His^{38}, Asp^{112} and Tyr^{175} were critical for binding to C1q and activating C3 in human serum (11, 12). Asp^{112} and Tyr^{175} appeared to be the C1q contact residues. Three CRP mutants, H38R, D112N and Y175A have been previously identified as the mutants which displayed reduced binding to C1q and did not activate C3 in human serum (12).

Human CRP has been shown to protect mice against lethal pneumococcal infection (13-16). Although a functioning complement system is required for full CRP-mediated protection, the exact mechanism of action of CRP in protecting mice against pneumococcal infection is not known (17-19). Decades ago, it was hypothesized that complement activation by pneumococcus-bound CRP was responsible for CRP-mediated protection of mice against pneumococcal

infection (20). This hypothesis could not be tested experimentally at the time due to the unavailability of a CRP mutant which would bind to pneumococci but would not activate complement in murine serum.

Previously, we tested the Y175A CRP mutant for activation of murine C3. We reported that Y175A CRP did not activate human C3 but activated murine C3 (9). Other CRP mutants, H38R and D112N, that did not activate human C3 were not tested for murine C3 activation earlier. Here, we report that the CRP mutant H38R does not activate murine C3 either. The availability of H38R CRP provided us with the needed tool to test the hypothesis that complement activation by ligand-complexed CRP is critical for CRP for protection against pneumococcal infection.

Materials and Methods

Construction and expression of CRP mutants

The construction of H38A and H38R CRP mutants has been described earlier (12). CRP mutants were expressed in CHO cells using the ExpiCHO Expression System (ThermoFisher Scientific) according to the manufacturer's instructions. In brief, non-adherent ExpiCHO-S cells (Gibco) were cultured in a shaker flask at 37°C with 5% CO_2. Cells (6 x 10^6 cells/ml) were then transfected with mutant CRP cDNA (1 µg) using Expifectamine reagent (3.2 µl/ml). Transfected cells were cultured for 20 h at 37°C with 5% CO_2. At 20 h post-transfection, ExpiCHO enhancer (6 µl/ml) and ExpiCHO feed (240 µl/ml) were added to the transfected cells and the culture was then transferred to 32°C with 8% CO_2. The culture media containing expressed CRP mutants were harvested 14 d post-transfection.

Purification of CRP

WT CRP was purified exactly as described previously, and the same method was used to purify CRP mutants H38A and H38R (21). In brief, CRP was purified by Ca^{2+}-dependent affinity chromatography on a PCh-Sepharose column (Pierce), followed by gel filtration on a Superose12 column (GE Healthcare) using the Biologic Duo Flow Protein Purification System (Bio-Rad). Purified CRP was stored in TBS containing 2 mM $CaCl_2$ at 4°C and was used within 10 d. The purity and pentameric structure of CRP mutants were determined by SDS-PAGE and gel filtration.

For use in mice, purified CRP was treated with the Detoxi-Gel Endotoxin Removing Gel (ThermoFisher Scientific) according to the manufacturer's instructions. The concentration of

endotoxin in all CRP preparations, as determined by using the Limulus Amebocyte Lysate kit QCL-1000 (Lonza), was <2.2 endotoxin units per 25 μg CRP.

Murine C3 activation assay

First, poly-L-lysine-PnC (P-PnC) was synthesized, as described previously (22), with slight modifications. Briefly, 200 μl of 1 mg/ml PnC (Statens Serum Institute, 3459) was slowly added to 10 ml of 10 mM NaOH. Then, 10 mg of cyanuric chloride (Sigma, C95501) was added, followed by the addition of 2 ml of poly-L-lysine (200 μg/ml in H_2O), to the mixture. After adjusting the pH to 8.2 using NaOH, the mixture was incubated for 2 h at 4°C with occasional stirring. The resulting P-PnC (poly-L-lysine ~20 μg/ml and PnC ~100 μg/ml) was stored at 4°C; a 1:4 dilution of this preparation was used to coat microtiter wells for the following assays.

Binding of CRP to the PCh ligand P-PnC was evaluated as follows: Microtiter wells were coated with P-PnC in 100 μl TBS, overnight at 4ºC. The unreacted sites were blocked with TBS containing 0.5% gelatin for 1 h at room temperature. CRP, diluted in TBS containing 2 mM $CaCl_2$, 0.1% gelatin and 0.02 % Tween 20 (TBS-Ca), was then added in duplicate wells and incubated for 2 h at 37°C. After washing the wells with TBS-Ca, bound CRP was detected by using anti-CRP mAb HD2.4 diluted in TBS-Ca. HRP-conjugated goat anti-mouse IgG diluted in TBS-Ca was used as the secondary antibody. Color was developed using ABTS substrate and the OD was read at 405 nm in a plate reader.

Deposition of activated murine C3 on P-PnC-complexed CRP was evaluated as follows: Microtiter wells were coated with P-PnC in 100 μl PBS overnight at 4°C. The unreacted sites were blocked with PBS containing 1% BSA for 1 h at room temperature, followed by rinsing the wells with buffer A (PBS containing 0.1% BSA and 1 mM $CaC1_2$). CRP diluted in buffer B

(buffer A containing 0.01% Tween 20) was then added in duplicate wells and incubated for 1 h at 37°C. The wells were washed with buffer B and then with buffer C (PBS containing 1% BSA, 0.15 mM $CaC1_2$ and 0.5 mM $MgC1_2$). Normal mouse serum (Innovative Research, IGMSC57SER)**,** diluted 1/30 in chilled buffer C, was added to each well and incubated for 30 min at 37°C, followed by washing with buffer C. Goat anti-mouse C3 antibody (Cappel; 1/750), diluted in buffer C, was added to each well. After 1 h at 37°C, the wells were washed, and developed with HRP-conjugated bovine anti-goat IgG (Santa Cruz Biotechnology). Color was developed using ABTS substrate and the OD was read at 405 nm in a plate reader.

Pneumococcus binding assay

The pneumococcus binding assay was performed exactly as described previously (16, 23). Briefly, microtiter wells were coated with 10^7 CFU of pneumococci overnight at 4ºC. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin. CRP, diluted in TBS-Ca, was then added to the wells for 2 h at 37 °C. After washing the wells with TBS-Ca, bound CRP was detected by using anti-CRP mAb HD2.4. HRP-conjugated goat-anti mouse IgG was used as the secondary antibody. Color was developed using ABTS substrate and the OD was read at 405 nm in a plate reader.

Clearance of H38R CRP from mouse circulation

The clearance rate of H38R CRP from the mouse blood was determined as described previously (23). Briefly, five mice were injected i.v. with 50 µg of H38R CRP in 100 µl TBS containing 2 mM $CaCl_2$ through the tail vein. Blood samples were collected from the tip of the tail after 12,

16, 20 and 24 h, and sera were separated. The concentration of CRP in the sera was measured by ELISA.

Mice

Male C57BL/6J mice, 8-10 wk old, were purchased from Jackson Laboratories and used in the protection experiments. All animal studies have been reviewed and approved by the University Committee on Animal Care.

Pneumococci

Virulent *S. pneumoniae* type 3, strain WU2 (obtained from Dr. David Briles, University of Alabama, Birmingham, AL), was cultured as described previously (23). A single use bacterial aliquot (1 ml) of virulent stock was prepared and stored at -80°C. For each experiment, an aliquot of frozen pneumococci was thawed in 50 ml Todd-Hewitt broth containing 0.5% yeast extract and incubated at 37°C with shaking at 125 rpm for 4.5 h and collected from mid-log phase cultures. The culture was centrifuged at 7,500 rpm for 15 min. The bacterial pellet was washed and resuspended in 10 ml normal saline and the volume adjusted to an absorbance A_{600} = 0.29 (3.5×10^8 CFU/ml). The concentration, purity, and viability of pneumococci was confirmed by plating on sheep blood agar plates.

Mouse protection experiments

Mouse protection experiments were performed exactly as described previously (24). In brief, mice were injected i.v. with 25 μg CRP. After 30 min, 100 μl of 3.4×10^8 CFU/ml of pneumococci was injected. Survival of mice was recorded three times per day for 7 d. To

determine bacteremia (CFU/ml), blood samples were collected from each surviving mouse twice daily for the first 3 d, followed by once daily for next 2 d. Blood was diluted and plated on blood agar plates, and incubated for 18 h at 37°C before the colonies were counted. The plotting and statistical analyses of the data were done using the GraphPad Prism 4 software. Statistical significance for survival among the groups was determined by Log-rank test and differences in bacteremia were analyzed by Mann-Whitney test.

Results

H38R CRP does not activate murine C3

Previously, for murine C3 deposition assays, we used CRP-PnC complexes to activate complement (9). However, we failed to generate a reliable C3 deposition assay using commercially available batch of PnC at this time. Instead of using CRP-PnC complexes, we used CRP-P-PnC complexes for murine C3 activation. As shown (Fig. 2.1A), H38A and H38R CRP mutants bound to P-PnC as well as WT CRP did. In the C3 activation assay (Fig. 2.1B), WT CRP activated murine C3 in a CRP concentration-dependent manner. Like WT CRP, H38A CRP also activated murine C3 in a CRP concentration-dependent manner. Even if the binding of WT CRP and H38A CRP to P-PnC did not differ from each other, H38A CRP was more efficient than WT CRP in activating murine C3. However, H38R CRP did not result in any C3 deposition on CRP-P-PnC, suggesting that H38R CRP did not activate murine C3.

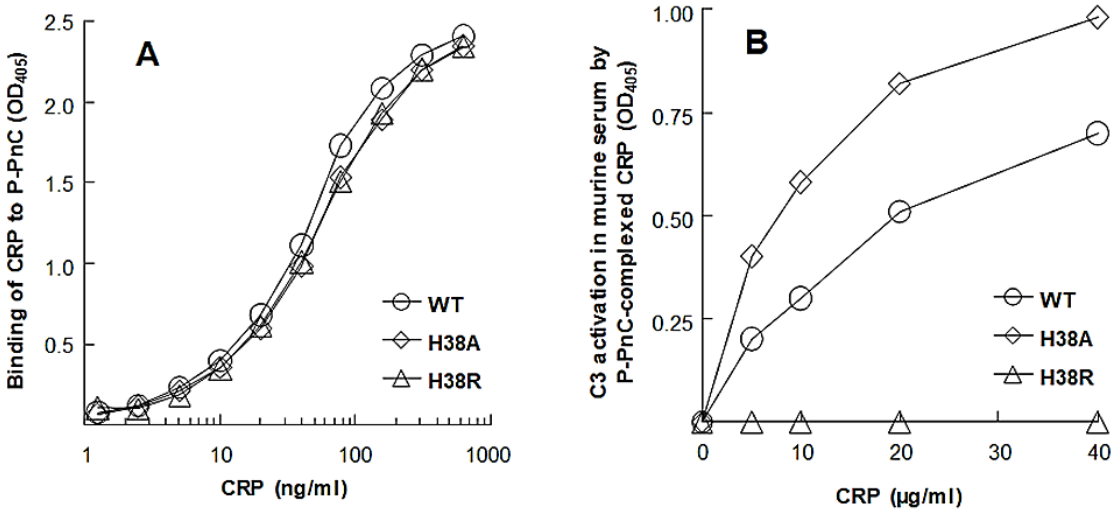

Figure 2. 1. Activation of C3 in murine serum by P-PnC ligand-bound CRP. A representative of three experiments is shown. **(A)** Binding of CRP to P-PnC. Microtiter wells were coated with P-PnC. CRP diluted in TBS-Ca was added to the wells. Bound CRP was detected by using anti-CRP mAb HD2.4. Color was developed and the OD was read at 405 nm. **(B)** Activation of C3 by CRP complexed with P-PnC. Microtiter wells were coated with P-PnC. CRP diluted in TBS-Ca was added to the wells. Normal mouse serum was then added to the wells. Deposited C3 was detected by using goat anti-mouse C3 antibody. Color was developed and the OD was read at 405 nm.

H38R CRP is pentameric and binds to pneumococci

The elution volume of H38R CRP from the gel filtration column was identical to that of WT CRP (Fig. 2.2A), indicating that the m.w. of H38R CRP was same as WT CRP. Thus, H38R CRP was pentameric. SDS-PAGE analysis (Fig. 2.2B) of H38R CRP confirmed the purity of the preparation and showed that there was no difference in the m.w. of the subunuits of WT and H38R CRP. Also, the Ca^{2+}-dependent binding of H38R CRP to pneumococci was similar to that of WT CRP. We have reported previously that the Ca^{2+}-dependent binding of H38R CRP to PnC and PCh-BSA was also similar to that of WT CRP (12).

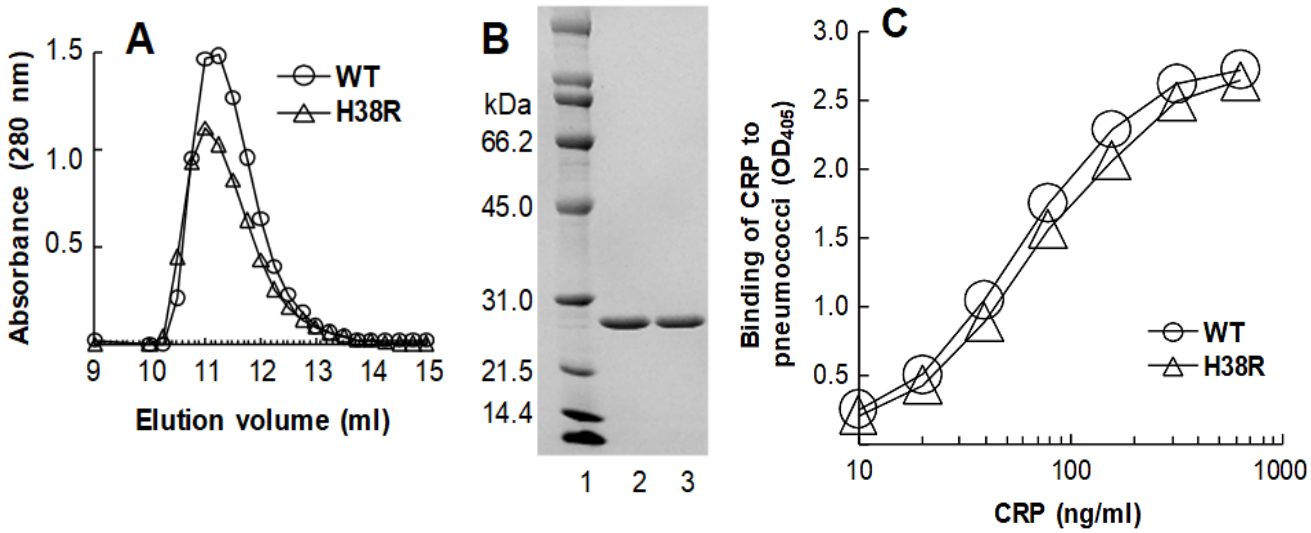

Figure 2. 2. Overall pentameric structure of H38R CRP. A representative of three experiments is shown. **(A)** Elution profiles of CRP from the gel filtration column are shown. CRP in TBS containing 2 mM CaCl2 was applied to the column and eluted with the same buffer. Sixty fractions (0.25 ml) were collected and protein measured (A280) to determine the elution volume of CRP. **(B)** Denaturing SDS-PAGE of CRP. A Coomassie brilliant blue-stained gel (lane 2, WT CRP; lane 3, H38R CRP), is shown. **(C)** Binding of CRP to pneumococci. Microtiter wells were coated with pneumococci. CRP diluted in TBS-Ca was added to the wells. Bound CRP was detected by using anti-CRP mAb HD2.4. Color was developed and the OD was read at 405 nm.

Clearance rate of H38R CRP is similar to that of WT CRP

We have reported previously that the the rate of clearance of WT CRP from mouse circulation was 0.67 μg/ml/h (23). To determine the dose of H38R CRP for *in vivo* use, we evaluated the rate of clearance of H38R CRP from mouse circulation (Fig. 2.3). The clearance rate of H38R CRP was found to be 0.20 μg/ml/h, suggesting that the the clearance of H38R CRP was not faster than that of WT CRP and that the substitution of His^{38} with Arg did not reduce the stability of H38R CRP *in vivo*.

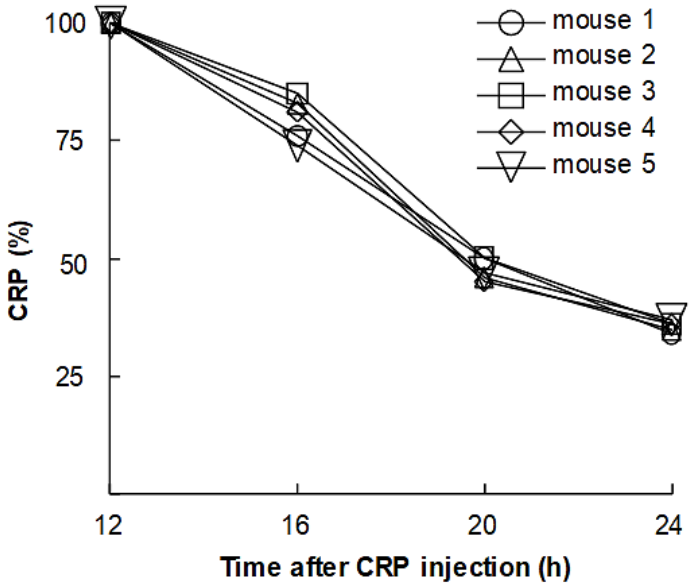

Figure 2. 3. Clearance of H38R CRP from mouse circulation. Mice were injected with 50 μg of CRP. Blood was collected at various time points, sera separated, and the concentration of CRP measured.

H38R CRP does not protect mice against pneumococcal infection

Fig. 2.4 shows the combined results from two separate mouse protection experiments. H38A CRP, which was not different from WT CRP in activating murine C3, was included as a control in the experiment.

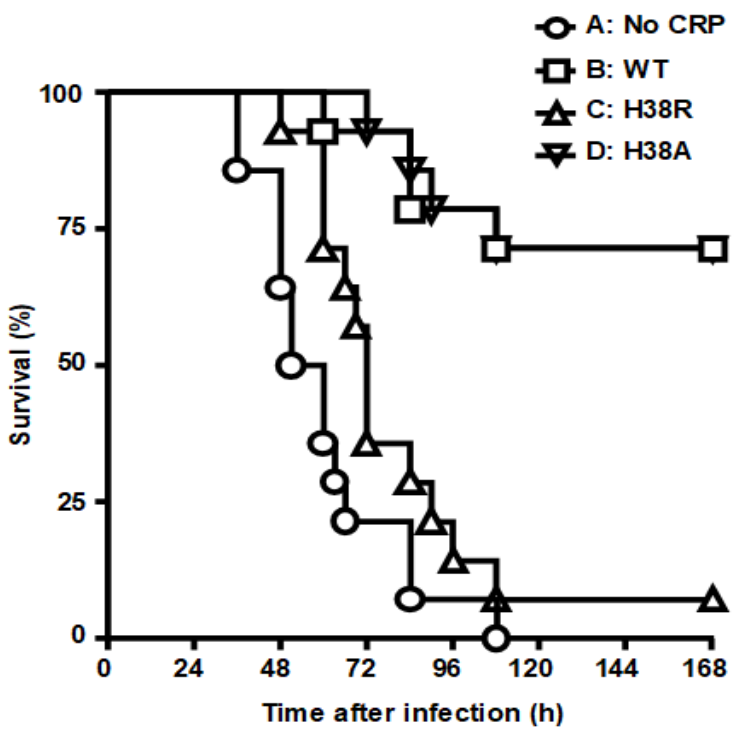

Figure 2. 4. Survival curves of mice infected with pneumococci with and without CRP. CRP was injected first; pneumococci were injected 30 min later. The data are combined from two separate experiments with 7 mice in each group in each experiment. The *p* values for the differences in the survival curves between groups A B, A D, B C and C D were <0.05. The *p* values for the differences in the survival curves between groups A C and B D were >0.05.

The median survival time (MST, the time taken for the death of 50% of mice) for mice injected with bacteria alone was 56 h. The MST for mice injected with H38R CRP was 72 h. There was no statistically significant difference between mice receiving H38R CRP and mice not receiving any CRP. The MST for mice injected with either WT CRP or H38A CRP could not be determined since >50% mice survived in both groups. There was no statistically significant difference between mice receiving either WT or H38A CRP. Next, we determined bacteremia in each surviving mouse (Fig. 2.5). In mice receiving H38A CRP, bacteremia decreased, like in WT

CRP-treated mice. There was no statistically significant difference in bacteremia in WT CRP-treated and H38A CRP-treated mice. However, bacteremia continued to increase in H38R CRP-treated mice, like in untreated mice, and mice died once bacteremia was $>10^8$ CFU/ml. There was no statistically significant difference in bacteremia in untreated and H38R CRP-treated mice. Combined data from survival of mice and bacteremia suggested H38R CRP was not protective against infection and that the lethality of H38R CRP-treated mice was due to increased bacteremia.

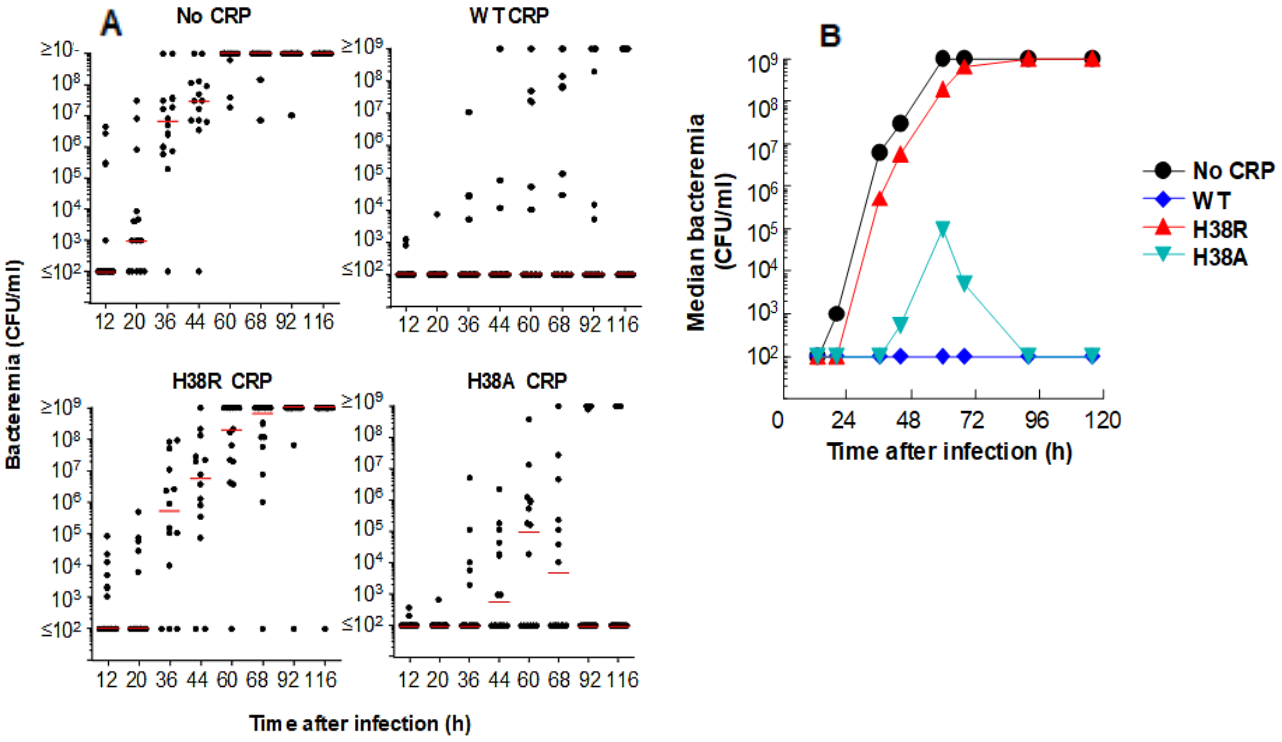

Figure 2. 5. Bacteremia in mice infected with pneumococci with and without CRP. (A) Blood was collected from each surviving mouse shown in Fig. 2.4. Bacteremia was determined by plating. Each dot represents one mouse. The horizontal line in each group of mice represents median bacteremia. A bacteremia value of $>10^8$ indicates a dead mouse. The *p* values for the differences between groups A B and A D were <0.05. The *p* value for the difference between groups A C was >0.05. **(B)** The median bacteremia values for each group shown in A are plotted.

Discussion

Our major findings in this study were: 1. The H38R CRP mutant, which did not activate complement in human serum as reported previously (12), did not activate complement in murine serum either. 2. The H38R CRP mutant incapable of activating murine complement failed to protect mice against lethal pneumococcal infection. These findings confirm that complement activation by CRP-PCh complexes constitute the mechanism of CRP-mediated protection (decrease in bacteremia and increase in survival time) of mice against lethal pneumococcal infection.

Human CRP does not interact with murine C1q and, therefore, the activation of murine C3 by human CRP was not through the classical pathway (9). The pathway through which human CRP activates murine C3 remains unknown (9, 10). Based on the known crosstalk among CRP, lectins, ficolins and pneumococci, it has been proposed earlier that human CRP activates murine complement through the lectin pathway (9, 10, 25, 26). Irrespective of the pathway through which human CRP activated murine C3, our data suggest that the cleft on CRP, that accommodates the C1q-binding site, was critical for human CRP to activate murine C3. However, all three amino acid residues, His^{38}, Asp^{112} and Tyr^{175}, critical for the formation of the C1q-binding site and human C3 activation, were not critical for murine C3 activation. The Y175A CRP does not activate human C3 but activates murine C3. The H38R does not activate C3 in both human and murine sera. The D112N CRP does not activate human C3, and has not been tested for murine C3 activation yet. The role of the other amino acid residues, Glu^{88} and Asn^{158}, present in the CRP cleft in activating murine C3 is also unknown.

Previously, employing CRP mutants, we investigated the role of the PCh-binding site, and indirectly the role of CRP-dependent complement activation, in protection of mice against

pneumococcal infection (15, 22, 23, 27). We used two CRP mutants, F66A/E81A and F66A/T76Y/E81A, both incapable of binding to PCh. Different animal models provided different results (24). Later, we found out that the F66A/T76Y/E81A CRP mutant, like acidic pH-treated WT CRP, had inadvertently gained the capability to bind to immobilized complement factor H which is an inhibitor of complement activation (28-31) (unpublished observations). Our current finding that complement activation is the mechanism through which CRP is protective suggests that in all the previously published protection experiments, at some point in time, endogenous murine CRP participated in protection by binding to PCh on pneumococci and activating the complement system (9, 16, 23, 24). Since endogenous murine CRP has been shown to be protective against pneumococcal infection, we propose that the experiments on structure-function relationships of CRP in pneumococcal infection should be conducted employing CRP knockout mice (2, 32).

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Footnotes

[1] S.K.S. and D.N. contributed equally to this work.

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Abbreviations used in this article: CRP, C-reactive protein; PCh, phosphocholine; PnC, pneumococcal C-polysaccharide; P-PnC, poly-L-lysine-conjugated PnC; TBS-Ca, TBS containing 0.1% gelatin, 0.02% Tween-20 and 2 mM $CaCl_2$; WT, wild-type.

CHAPTER 3: TREATMENT OF PNEUMOCOCCAL INFECTION BY USING ENGINEERED C-REACTIVE PROTEIN

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Summary

C-reactive protein (CRP), whose blood level increases in inflammatory states in humans, binds to several species of bacterial pathogens[1-3]. Experiments in mice have revealed that one of the functions of CRP is to protect against *Streptococcus pneumoniae* infection[4-6]. For protection, however, CRP must be injected into mice within two hours of administering pneumococci, that is, CRP is protective against early-stage infection but not against late-stage infection[7]. It is not known why CRP is not protective against late-stage pneumococcal infection. The hypothesis is that the protection requires activation of the complement system by CRP-pneumococci complexes and that CRP cannot protect if pneumococci have time to recruit complement inhibitor factor H on their surface to become complement attack-resistant[8,9]. We have reported previously that the conformation of CRP is altered under conditions mimicking an inflammatory milieu and that CRP, in a non-native conformation, also binds to immobilized factor H[10,11]. Accordingly, we engineered CRP molecules (E-CRP), which bind to factor H on pneumococci but do not bind to factor H or any other host cell in the blood. Here, we show that E-CRP is protective against both early-stage and late-stage infections by reducing bacteremia. We also show that E-CRP functions synergistically with the antibiotic clarithromycin and reduces bacteremia drastically. The preclinical results presented here suggest that pre-modified CRP, such as E-CRP, is therapeutically beneficial to enhance survival. Our findings also have implications for infections with antibiotic-resistant pneumococcal strains and possibly for infections with other bacterial species that use host proteins to evade complement-mediated killing[12-14].

Introduction

C-reactive protein (CRP) is a component of the acute phase response and a critical host defense molecule of the innate immune system against *Stretococcus pneumoniae* infection[15,16]. CRP binds to pneumococci by recognizing the phosphocholine (PCh) molecules present on the pneumococcal cell wall C-polysaccharide (PnC)[17]. The binding of CRP to PCh requires two Ca^{2+} ions. CRP is made of five identical subunits arranged as a cyclic pentamer[18,19]. Each subunit has a PCh-binding site consisting of Phe^{66}, Thr^{76} and Glu^{81}. Through the PCh-binding site, CRP can also interact with phosphoethanolamine (PEt)[20,21]. It has been shown that the structure of CRP is altered in a reversible manner under conditions mimicking an inflammatory milieu and that non-native CRP can bind to immobilized complement inhibitor factor H[10,11,22].

In mouse models of pneumococcal infection, human CRP has been shown to be protective against lethality; however, the mechanism of anti-pneumococcal action of CRP remains undefined[4-6,16,23]. Interestingly, CRP is protective against pneumococcal infection only when injected 6 h before to 2 h after administering pneumococci into mice[7]. CRP was not protective against late-stage infection in murine models. This 36-year old observation[7] provided us with an experimental strategy to define the mechanism of anti-pneumococcal functions of CRP. It is assumed that CRP is protective because of the ability of CRP-PCh complexes to activate the complement system and is not protective against late-stage infection because, by then, pneumococci recruit the complement inhibitory protein factor H on their surface to escape the attack of complement[8,9,24-29]. We hypothesize that in order to protect mice against late-stage infection, a structural change in CRP is needed, followed by the interaction between structurally altered CRP and factor H-bound pneumococci, and that was not happening in mice. We further hypothesize that non-native CRP prepared exogenously would bind to factor H on pneumococci

in vivo, mask its complement inhibitory activity, and therefore should be able to protect against complement-resistant pneumococci. To test this hypothesis, we engineered CRP (E-CRP) by site-directed mutagenesis and produced two types of E-CRP: One, E42Q/F66A/T76Y/E81A, that binds to immobilized factor H but does not bind to PCh (E-CRP-1) and another, Y40F/E42Q, that binds to both immobilized factor H and to PCh (E-CRP-2).

E-CRP-1 and E-CRP-2 have desired ligand-binding properties

We previously reported a triple mutant of CRP, F66A/T76Y/E81A, which does not bind to PCh, and a single mutant of CRP, E42Q, which, unlike wild-type (WT) CRP, binds to immobilized factor H[11,21,30]. By employing site-directed mutagenesis, we constructed a quadruple mutant of CRP (E-CRP-1), E42Q/F66A/T76Y/E81A, in which the E42Q/F66A/T76Y mutations were introduced to abolish PCh-binding and E42Q mutation was added to insert the factor H-binding ability. E-CRP-1 was expressed in CHO cells and purified by PEt-affinity chromatography. The elution profiles of WT CRP and E-CRP-1 from the gel filtration column were almost overlapping; both proteins eluted at 11 ml (Fig. 3.1a). SDS-PAGE of purified E-CRP-1 showed a single band and the molecular weight of the E-CRP-1 subunits was same as WT CRP (Fig. 3.1b). We assessed the PCh-binding ability of E-CRP-1 by using PnC and broth-grown pneumococci (Pn-broth). WT CRP bound to both ligands. However, for equivalent binding to either PnC or Pn-broth, ~100-times more of E-CRP-1 was required compared to that of WT CRP, indicating that the PCh-binding ability of E-CRP was ~99% less than that of WT CRP (Fig. 3.1c, d). In contrast, E-CRP-1 bound to PEt more efficiently than WT CRP (Fig. 3.1e), which facilitated purification of E-CRP-1 by PEt-affinity chromatography. In factor H-binding assays, unlike WT CRP, E-CRP-1 bound readily to immobilized human and murine factor H (Fig. 3.1f).

Surprisingly, triple mutant CRP, which was not investigated before for factor H binding[21], also bound to factor H.

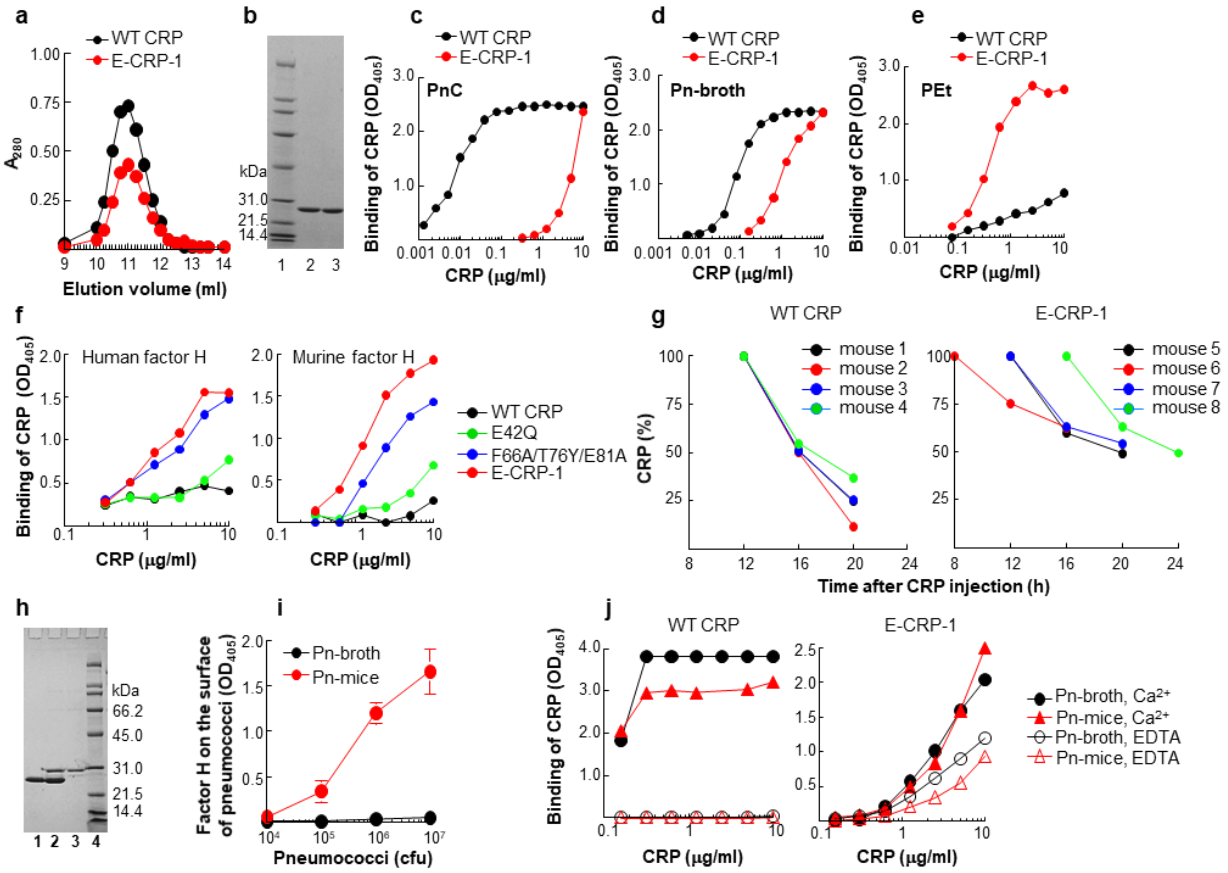

Figure 3. 1: Characterization of E-CRP-1. **a**. Elution profiles of CRP from gel filtration column. **b.** SDS-PAGE of WT CRP (lane 2) and E-CRP-1 (lane 3). **c.** Binding of CRP to PnC. **d.** Binding of CRP to broth-cultured pneumococci (Pn-broth). **e.** Binding of CRP to PEt. **f.** Binding of CRP to human (left) and murine (right) factor H immobilized on microtiter wells. **g.** Clearance of CRP from mouse circulation. **h.** Re-purification of E-CRP-1 from purified E-CRP-1-spiked mouse serum. SDS-PAGE of re-purified E-CRP-1 is shown. Lane 1, purified E-CRP-1 (5 µg); Lane 2, EDTA eluate (25 µl, A_{280} 1.13) from the PEt-affinity chromatography column through which mouse serum containing E-CRP-1 was passed in the presence of Ca^{2+}; Lane 3, EDTA eluate (25 µl, A_{280} 0.29) from the PEt-column through which mouse serum alone was passed. **i.** Presence of murine factor H on pneumococci isolated from the blood of infected mice (Pn-mice). **j.** Binding of CRP to Pn-broth and Pn-mice in the presence and absence of Ca^{2+}. A representative of three experiments is shown for **a-j**, except for **i**.

Similar results were obtained for E-CRP-2, except that E-CRP-2 retained its PCh-binding property (Extended Data Fig. 3.1). Thus, the overall structure of WT CRP, E-CRP-1 and E-CRP-2 were similar and both E-CRP-1 and E-CRP-2 had the desired ligand-binding properties to test our hypothesis.

E-CRP-1 and E-CRP-2 are suitable for in vivo use

We determined the $T_{1/2}$ of CRP from mouse circulation. Based on the data obtained from four mice (Fig. 3.1g), the average $T_{1/2}$ of WT CRP and E-CRP-1 were 4.9 and 8.0 h, respectively. Thus, the clearance of E-CRP-1 was not markedly faster than that of WT CRP. In another approach to confirm that E-CRP-1 was free in the mouse serum, we performed an experiment where E-CRP-1 could be re-purified from E-CRP-1-spiked mouse serum (Fig. 3.1h). As shown, E-CRP-1 present in the mouse serum bound to PEt in a Ca^{2+}-dependent manner and could be eluted with EDTA (lane 2). The recovery of E-CRP-1 was 96%. Besides CRP, no additional protein bands were found when compared with the nonspecific bands seen with the serum alone control (lanes 2 and 3). Thus, E-CRP-1 stayed free in the mouse serum, was not sequestered by any other serum protein, and the mutations did not confer instability to E-CRP-1 *in vivo*.

Next, we tested whether E-CRP-1 binds to pneumococci which have recruited factor H. We isolated pneumococci (Pn-mice) from infected mice and first tested for the presence of murine factor H on pneumococci. As shown (Fig. 3.1i), factor H was present on Pn-mice. WT CRP bound to both Pn-broth and Pn-mice but only in the presence of Ca^{2+}, suggesting that the binding of WT CRP to Pn-mice was through PCh (Fig. 3.1j). In contrast, E-CRP-1 bound to Pn-mice in the absence of Ca^{2+} also, suggesting that E-CRP-1 bound to a molecule other than PCh, and that molecule could be factor H recruited *in vivo*. Surprisingly, in EDTA, E-CRP-1 also bound to Pn-

broth suggesting that E-CRP-1 bound to a pneumococcal surface protein. Similar results were obtained for E-CRP-2 (Extended Data Fig. 3.1). The $T_{1/2}$ of E-CRP-2 was 7.5 h. Thus, both E-CRP-1 and E-CRP-2 were suitable for use in mouse models of infection to test our hypothesis.

E-CRP-1 protects mice against late-stage infection

All the data presented in this study show the combined results of two separate protection experiments using six to eight mice in each group in each experiment. Protection experiments shown in Fig. 3.2a, c and e were performed together. Fig. 3.2a shows the results of experiments in which CRP was injected into mice within 30 min of administering pneumococci. The median survival time (MST, the time taken for the death of 50% of mice) for mice injected with bacteria alone (group A) was 60 h. The MST for mice injected with bacteria and either WT CRP (group B) or E-CRP-1 (group C) could not be calculated because >50% of mice survived. WT CRP and E-CRP-1 were not significantly different in protecting mice against lethality. Increase in survival was due to decrease in bacteremia (Fig. 3.2b, Extended Data Fig. 3.2). By 44 h, in group A, median bacteremia increased dramatically, and mice died once bacteremia reached 10^9 cfu/ml; however, in groups B and C, median bacteremia reached only ~10^5 cfu/ml and then decreased dramatically afterwards. There was >99% reduction in bacteremia in CRP-treated mice. Since E-CRP-1 does not bind to PCh, these results indicated that the increased resistance to infection in E-CRP-1-treated mice was due to combined actions of E-CRP-1 and endogenous mouse CRP. E-CRP-1 bound to a protein ligand on the pneumococcal surface and, once bacteremia was already lower, endogenous mouse CRP bound to PCh to activate the complement system to reduce bacteremia. Since the dose of E-CRP-1 was same as that of WT CRP, it is unlikely that the protection depended upon the residual PCh-binding activity of E-CRP-1.

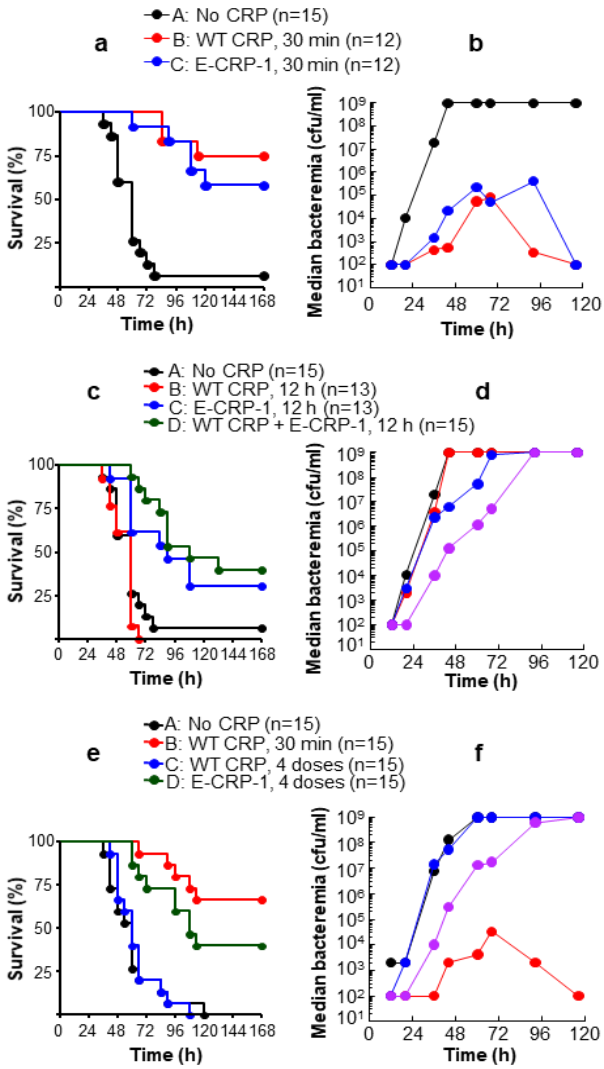

Figure 3. 2: Unlike WT CRP, E-CRP-1 is protective against late-stage infection. The data are combined from two separate experiments with 6-8 mice in each group in each experiment. **a.** Survival curves. CRP was injected first; pneumococci were injected 30 min later. The *p* values for the differences in the survival curves between groups A B, A C, and B C were <0.001, <0.001 and 0.43, respectively. **b.** Bacteremia. Blood was collected from each surviving mouse shown in **a**. The median bacteremia values are plotted. For 36-116 h, the *p* values for the difference between groups A B and A C were <0.001. **c.** Same as **a**, except that CRP was injected 12 h after injecting pneumococci. The *p* values for the difference in the survival curves between groups A B, A C, A D, B C, B D and C D are 0.28, <0.01, <0.001, <0.001, <0.001 and 0.31, respectively. **d.** Bacteremia. Blood was collected from each surviving mouse shown in **c**. The median bacteremia values are plotted. For 44-92 h, the *p* values for the difference between groups B C and B D were <0.001. **e.** Same as **a**, except that CRP was injected four times: 6, 12, 24 and 48 h after injecting pneumococci. The *p* values for the differences in the survival curves between groups A B, A D, B C, and C D were <0.001. **f.** Bacteremia. Blood was collected from each surviving mouse shown in **e**. The median bacteremia values are plotted. For 36-116 h, the *p* values for the difference between groups A B, B C, and A D were <0.01.

Next, we injected E-CRP-1 into mice 12 h after administering pneumococci, a time point for CRP injection when WT CRP does not confer protection (Fig. 3.2c). A gap of 12 h is clinically significant because all strategies for a sepsis drug have so far failed in human clinical trials[31,32]. We included WT CRP, 30 min regimen, in all experiments to ensure that the animal model was comparable from experiment to experiment. The MST for mice injected with either bacteria alone or with bacteria and WT CRP was 60 h. In contrast, the MST for mice injected with bacteria and E-CRP-1 was 90 h and the MST for mice injected with bacteria and both WT CRP and E-CRP-1 was 108 h. In the WT CRP-treated group, all mice died by 66 h. However, in the E-CRP-1-treated groups, it took 4 days until 60-70% mice died, and 30-40% mice survived up to 7 days. As reported previously[7], WT CRP was not protective. These data again suggested that endogenous mouse CRP participated and that's why E-CRP-1 alone was not different from the combination of E-CRP-1 and WT CRP. In groups receiving E-CRP-1, median bacteremia was reduced by ~99% as early as 44 h and lower bacteremia was maintained for up to 92 h (Fig. 3.2d, Extended Data Fig. 3.2). Also, the injection of E-CRP-1 at 12 h was as effective as it was when administered within 30 min (Extended Data Fig. 3.3a, b). These data raise the possibility that E-CRP-1 may protect mice against infection regardless of the time point of injecting E-CRP-1, as long the time falls within 44 h in our animal model.

Next, we injected CRP into mice four times, at 6, 12, 24, and 48 h after administering pneumococci, to determine whether multiple injections of E-CRP-1 were better than a single injection at 12 h (Fig. 3.2e). The MST for mice injected with either bacteria alone or with bacteria and four doses of WT CRP was 60 h. Like a single dose of WT CRP at 12 h, multiple doses of WT CRP were also not protective. In contrast, the MST for mice injected with bacteria and multiple doses of E-CRP-1 was 108 h. In E-CRP-1-treated mice, median bacteremia was

reduced by ~99% as early as 36 h and the reduction lasted for up to 72 h. There was ~48 h gain over WT CRP for bacteremia to reach the deadly levels (Fig. 3.2f, Extended Data Fig. 3.2). Four injections of E-CRP-1 were more effective than one injection of E-CRP-1 in reducing bacteremia (Extended Data Fig. 3.3c, d), although there was no significant difference in survival of these two groups of mice. It took another 24 h for bacteremia to reach the highest level in mice receiving four injections and we think, based on the half-life of CRP, that the fourth injection was critical.

E-CRP-2 also protects against late-stage infection

Fig. 3.3a shows the results of protection experiments with E-CRP-2. The MST for mice injected with bacteria alone was 54 h. The MST for mice injected with E-CRP-2, 12 h after administering pneumococci, was extended to 132 h. The MST for mice injected with E-CRP-2, 30 min after administering pneumococci, could not be calculated because >50% of mice survived, as expected. There was >99% reduction in bacteremia even when E-CRP-2 was given to mice 12 h after administering pneumococci and the lower bacteremia stayed as such for >96 h (Fig. 3.3b, Extended Data Fig. 3.4). Since E-CRP-2 binds to PCh, like WT CRP does, we do not know the involvement of mouse endogenous CRP in this case. However, the animal model we employed in this study indicates that mouse endogenous CRP is enough to protect if mice were otherwise administered with only ~10^7 cfu of pneumococci (Fig. 3.3c). These data also suggest that if mice are administered with, for example, >10^9 cfu bacteria, mouse CRP can be protective once bacteremia is lowered by ~99% and E-CRP is present to cover the virulence factors.

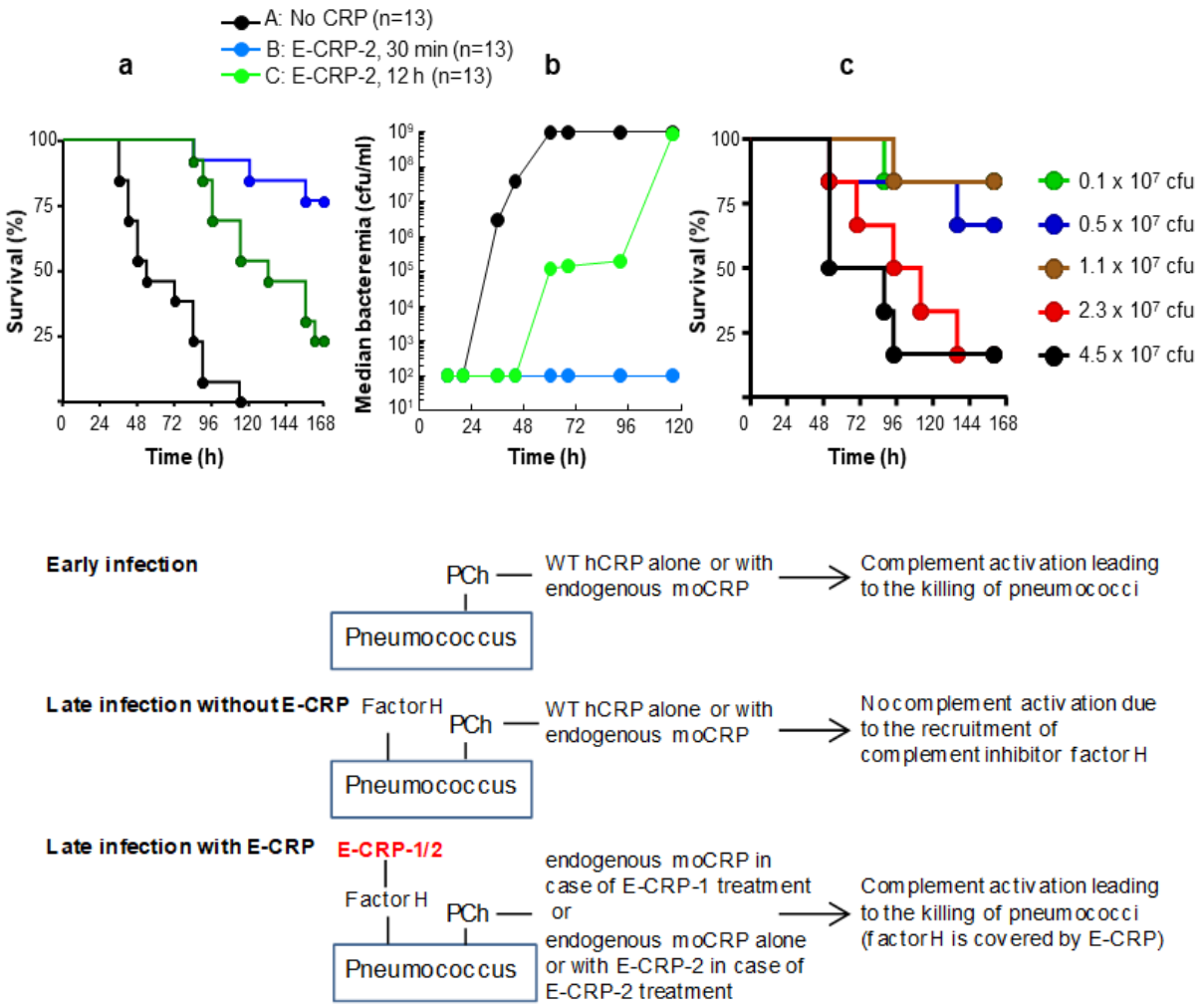

Figure 3. 3: Like E-CRP-1, E-CRP-2 also protective against late-stage infection. a-c. The data are combined from two separate experiments with 6-8 mice in each group in each experiment. **a.** Survival curves. CRP was injected first; pneumococci were injected either 30 min or 12 h later. The *p* values for the difference in the survival curves between groups A C and A B were <0.001 and between groups B C was 0.01. **b.** Bacteremia in each surviving mouse shown in **a**. The median value of bacteremia are plotted. For 36-116 h, the *p* values for the difference between groups A B and A C are <0.001. The *p* values for the difference between groups B C was >0.05 till 60 h and <0.05 after 60 h. **c.** The animal model. Survival curves of mice infected with different doses of pneumococci. **d.** A proposed model for the mechanism of action of CRP in pneumococcal infection. moCRP, mouse CRP; hCRP, human CRP.

Based on these results, we propose a model for how WT CRP and E-CRP work together to protect mice against lethality (Fig. 3.3d): The killing of bacteria is due to the binding of WT CRP (human or murine or both) to PCh on pneumococci and subsequent activation of the complement

system. If bacteremia is overwhelming, then WT CRP is not enough to protect and, as a result, bacteria have time to recruit host proteins to become resistant to complement attack. The role of E-CRP is to bind and cover the recruited proteins, or to bind and cover the surface virulence factors themselves, so that WT CRP can activate complement to continue killing bacteria. Previously[21,30,33], unaware of the fact that the CRP triple mutant can also bind to immobilized factor H, we proposed that CRP protects mice against pneumococcal infection without binding to PCh and, therefore, without binding to pneumococci. However, the current data suggest that in all previously published experiments, endogenous murine CRP had also participated in protection.

E-CRP acts synergistically with clarithromycin

To compare the effects of E-CRP with that of an antibiotic, clarithromycin[34,35], in protecting mice, we first titrated the dose of clarithromycin in our animal model. As seen in Fig. 3.4a, it was clear that a dose of 0.02 mg/mouse should be used to evaluate the effects of the combination of clarithromycin and E-CRP. Fig. 3.4b shows the results of experiments on clarithromycin and E-CRP-1. The MST for mice injected with either E-CRP-1 alone or clarithromycin alone were 84 h and 96 h, respectively. The MST for mice injected with both E-CRP-1 and clarithromycin could not be calculated because >50% of mice survived. Addition of WT CRP to clarithromycin did not change the efficacy of clarithromycin. The dramatic increase in survival by the combination of E-CRP-1 and clarithromycin was due to drastic decrease in bacteremia (Fig. 3.4c, Extended Data Fig. 3.5). Bacteremia could not rise beyond 10^5 cfu/ml in mice treated with both E-CRP-1 and clarithromycin. Similar results were seen with the combination of clarithromycin and E-

CRP-2 (Fig. 3.4d, Extended Data Fig. 3.5). We were not prepared for the data suggesting that an antibiotic can potentiate the effects of E-CRP; we were not testing any hypothesis.

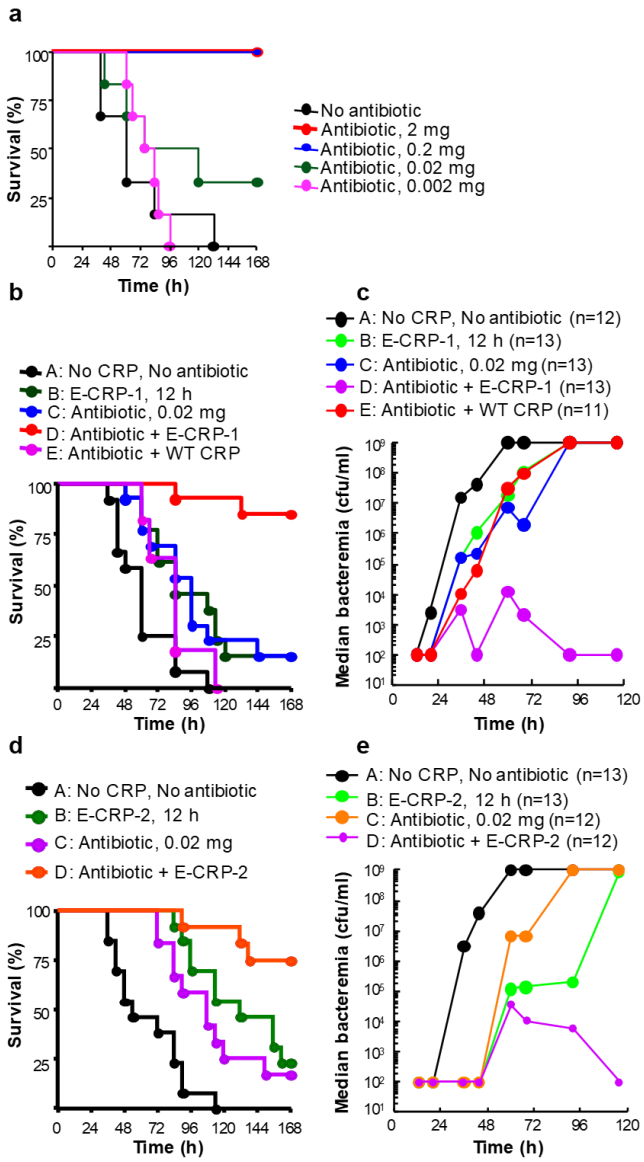

Figure 3. 4: E-CRP and clarithromycin act synergistically. The data are combined from two separate experiments with 6-8 mice in each group in each experiment. **a.** Survival curves of mice treated with clarithromycin. **b.** Survival curves of mice with E-CRP-1 and clarithromycin. The *p* values for the difference in the survival curves between groups B D and C E were <0.001 and 0.23, respectively. **c.** Bacteremia. Blood was collected from each surviving mouse shown in **b**. The median bacteremia values are plotted. For 44-116 h, the *p* values for the difference between groups B D and C D were <0.001. **d.** Survival curves of mice with E-CRP-2 and clarithromycin. The *p* values for the difference in the survival curves between groups B D was 0.01. **e.** Bacteremia. Blood was collected from each surviving mouse shown in **d**. The median bacteremia values are plotted. The *p* values for the difference between groups B D and C D were <0.01.

Topological differences between WT CRP and E-CRP-1

Data obtained from ligand inhibition experiments (Extended Data Fig. 6a-d, Extended Data Fig. 3.7) suggested that the residual binding of E-CRP-1 to PCh at higher concentrations of E-CRP-1 was probably due to the Ca^{2+}-dependent binding of E-CRP-1 to the phosphate group of PCh. The inability of PCh and dAMP to inhibit E-CRP-1-Pn-broth interaction suggested that the binding of E-CRP-1 to Pn-broth was not via PCh but mainly due to a surface protein. Data obtained from MonoQ ion-exchange chromatography experiments suggested that the overall charge on WT CRP was different from E-CRP-1 (Extended Data Fig. 3.6e). The two proteins also had different mobility in a native PAGE gel (Extended Data Fig. 3.6f). The migration of E-CRP was slower than that of WT CRP and not affected by the absence or presence of Ca^{2+}. Protease cleavage experiment suggested that E-CRP-1 might also have lost one of the two Ca^{2+} (Extended Data Fig. 3.6g)[36,37]. We performed molecular modelling of E-CRP-1; the model suggested that T76Y mutation affects Ca^{2+}-binding and the E42Q mutation affects intersubunit contact region. We have not performed similar studies with E-CRP-2 yet, and the binding site for factor H on E-CRP-1 remains undefined.

Discussion

Our data provide an explanation for the mechanism of CRP-mediated protection of mice against pneumococcal infection. Our data also provide a proof of concept that the structure of CRP is subtly modified *in vivo* to execute full anti-pneumococcal activities (Fig. 3.3d): native CRP binds to pneumococci through PCh groups and activates the complement system while structurally-modified CRP binds to factor H recruited by pneumococci[8,38]. Indeed, it has been shown that the presence of modified pentameric CRP, designated as either $pCRP_m$ or pCRP*, is a feature of the

inflamed sites[39,40]. We hypothesize that in individuals in whom the conformation of CRP remains unchanged, perhaps due to inappropriate inflammatory conditions around CRP, CRP is not fully functional during infection. If this hypothesis is correct, then our data provides a new strategy to treat infection by injecting exogenously prepared pre-modified CRP. A single dose of E-CRP combined with a tiny amount of clarithromycin was the best prescription among all others in this study for nearly complete protection of our experimental mice. Neither E-CRP nor clarithromycin could do it singly; indicating a previously unknown pathway through which the innate immune system responds to antibiotic-resistant bacteria. The approach to use E-CRP may also result in reducing the antibiotic treatment and preventing the development of antibiotic resistance.

Since E-CRP binds to a variety of different proteins immobilized on microtiter wells (data not shown), and not just to immobilized factor H, and because many other pathogens, including pneumococci recruit serum proteins to their surface, such as factor H, C1q and C4BP, E-CRP these findings may be applicable to infections with a wide range of bacterial pathogens which evade the immune system by recruiting host proteins[12]. In addition, because our strategy is dependent on the recruited proteins and not on the serotype, and since it is also likely that E-CRP binds to pneumococcal surface virulence factors (Fig. 3.1j), such as PspC itself, the proposed strategy should also work against other strains of pneumococci[13,14]. Our data is also relevant to other inflammatory diseases, such as age-related macular degeneration, where both CRP and factor H have been implicated[41-43].

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Methods

Construction of mutant CRP cDNAs

The template for construction of the CRP quadruple mutant E42Q/F66A/T76Y/E81A (E-CRP-1) was a cDNA for the CRP triple mutant F66A/T76Y/E81A cDNA (substitution of Phe^{66} with Ala, Thr^{76} with Tyr, and Glu^{81} with Ala). Mutagenic oligonucleotides, 5'-C CAC TTC TAC ACG ***CAA*** CTG TCC TCG ACC-3' and 5'-GGT CGA GGA CAG ***TTG*** CGT GTA GAA GTG G-3', to substitute Glu^{42} with Gln (codons shown in bold and italicized letters), were designed according to the sequence of the template cDNA and obtained from Integrated DNA Technologies. Mutagenesis was conducted using the QuickChange site-directed mutagenesis kit (Stratagene). Mutations were verified by nucleotide sequencing, utilizing the services of the Molecular Biology Core Facility of the university. The construction of cDNAs for CRP mutants E42Q, F66A/T76Y/E81A and Y40F/E42Q (E-CRP-2) has been reported earlier[11,21,44].

Expression and purification of CRP

All CRP mutants were expressed in CHO cells using the ExpiCHO Expression System (ThermoFisher Scientific). Purification of E-CRP-1 from culture supernatants involved Ca^{2+}-dependent affinity chromatography on a PEt-conjugated Sepharose column, followed by ion-exchange chromatography on a MonoQ column, and gel filtration on a Superose12 column, as reported previously for F66A/T76Y/E81A CRP mutant[21]. PEt-conjugated Sepharose was prepared as described previously[21]. Briefly, CHO cell culture media was diluted (1:1) in 0.1 M borate buffer saline, pH 8.3, containing 3 mM $CaCl_2$, and passed through the PEt-sepharose column. After collecting the flow-through and washing the column with the same buffer, bound E-CRP was eluted with 0.1 M borate buffer saline, pH 8.3, containing 5 mM EDTA. E-CRP-1

was then subjected to ion-exchange chromatography and bound E-CRP-1 was eluted with an NaCl gradiant. E-CRP-1 containing fractions were pooled, concentrated, and further purified by gel filtration. The gel filtration column was equilibrated and eluted with TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.2) containing 5 mM EDTA. Eluted E-CRP-1 was immediately dialyzed against TBS containing 2 mM $CaCl_2$, stored at 4°C, and was used within a week. WT CRP and all other CRP mutants including E-CRP-2 were purified as described previously[45]. The purity of CRP preparations was confirmed by denaturing 4-20% SDS-PAGE under reducing conditions.

For *in vivo* experiments, purified CRP was treated with Detoxi-Gel Endotoxin Removing Gel (ThermoFisher Scientific). The concentration of endotoxin in CRP preparations was determined by using the Limulus Amebocyte Lysate kit QCL-1000 (Lonza).

Determination of pentameric structure of E-CRP

The pentameric structure of E-CRP was confirmed by employing gel filtration and denaturing SDS-PAGE. The gel filtration column was equilibrated with TBS containing 5 mM EDTA. E-CRP was injected into the column and eluted with TBS containing 5 mM EDTA at a flow rate of 0.3 ml/min. Fractions (60 fractions, 250 µl each) were collected and absorbance at 280 nm measured to locate the elution volume of E-CRP. Gel filtration of WT CRP was carried out on the same column to determine the elution volume of pentameric CRP.

Pneumococci (Pn-broth)

Streptococcus pneumoniae type 3, strain WU2 (obtained from Dr. David E. Briles, University of Alabama at Birmingham, USA), were made virulent by sequential i.v. passages in mice, and were stored in 1 ml aliquots at -80 °C in Todd-Hewitt broth containing 0.5% yeast extract and

10% glycerol, as described previously[30]. For each experiment, a separate 1 ml aliquot of pneumococci was thawed. Pneumococci were then grown in 50 ml Todd-Hewitt broth containing 0.5% yeast extract and incubated at 37 °C with 125 rpm for 3 h (mid-log phase culture). The culture was centrifuged at 7,500 rpm for 15 min. The bacterial pellet was washed and resuspended in 5 ml normal saline and adjusted the volume until OD_{600} was 0.29 to give a concentration of 3.5×10^8 cfu/ml ($OD_{600} = 1.00 = 1.2 \times 10^9$ cfu/ml). This preparation of pneumococci cultured in broth was called as Pn-broth. The concentration, purity, and viability of pneumococci were confirmed by plating on sheep blood agar plates.

Isolation of pneumococci (Pn-mice) from infected mice

Mice were injected i.v. with 3.5×10^7 cfu of Pn-broth. After 40 h, blood was collected by cardiac puncture, in tubes containing 10% EDTA (1% v/v of blood). Blood was diluted with an equal volume of normal saline and centrifuged at 2,200 rpm for 2 min. The supernatant was recovered. The bacterial pellet was washed four times with normal saline, centrifuged at 2,200 rpm for 2 min after each wash, and continued to recover the supernatant. All recovered supernatants were then pooled and centrifuged at 11,000 rpm for 5 min. This time the supernatant was discarded, and the pellet was resuspended in normal saline for immediate use or resuspended in Todd-Hewitt broth containing 0.5% yeast extract and 10% glycerol for storage at -80 °C. This preparation of pneumococci isolated from infected mice was called as Pn-mice. The concentration, purity, and viability of pneumococci were confirmed by plating on sheep blood agar plates.

PCh-binding, PEt-binding, and pneumococcus-binding assays

Binding activity of CRP for PCh was evaluated by using pneumococcal C-polysaccharide (PnC, from Statens Serum Institut) as the ligand, exactly as described previously[33]. Binding activity of CRP for PEt was evaluated by using biotinylated-PEt as the ligand, exactly as described previously[21]. Binding activity of CRP for whole pneumococci, Pn-broth, was evaluated exactly as described previously[21]. Binding activity of CRP for whole pneumococci, Pn-mice, was evaluated both in the presence and absence of Ca^{2+}.

Factor H-binding assay

The binding activity of CRP for factor H was evaluated by using both human factor H (Complement Technology) and murine factor H (R&D). Microtiter wells were coated with 2 μg/ml of factor H in TBS (100 μl/well), overnight at 4 °C. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin for 45 min at room temperature. CRP diluted in TBS-Ca (TBS containing 2 mM $CaCl_2$, 0.1% gelatin and 0.02% Tween 20) was added in duplicate wells. After incubating the plates for 2 h at 37 °C, the wells were washed with TBS-Ca. Polyclonal rabbit anti-human CRP antibody (1μg/ml) (EMD Millipore Corp., 235752), diluted in TBS-Ca, was used (1 h at 37°C) to detect bound CRP. HRP-conjugated donkey anti-rabbit IgG (GE Healthcare), diluted in TBS-Ca, was used (1 h at 37 °C) as the secondary antibody. Colour was developed and the OD_{405} read in a microtiter plate reader (Molecular Devices).

Detection of factor H on the surface of Pn-mice

Microtiter wells were coated with Pn-mice in TBS (10^7 cfu/100 μl/well) overnight at 4 °C. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin for 45 min at room

temperature. Murine factor H present on the surface of Pn-mice was detected by using sheep polyclonal anti-mouse factor H antibody (R&D, AF4999) diluted in TBS-Ca. HRP-conjugated rabbit anti-sheep IgG (ThermoFisher Scientific), in TBS-Ca, was used as the secondary antibody. Colour was developed and the OD_{405} read in a microtiter plate reader.

Determination of $T_{1/2}$ of E-CRP in mouse circulation

Mice were injected i.v. with 100 μg of CRP in TBS containing 2 mM $CaCl_2$ through the tail. Four mice were used for WT CRP and four mice were used for E-CRP. After 8 h, blood was collected from the tip of the tail vein at four different time points up to 24 h. The concentration of CRP in the serum was measured by ELISA. The concentration of CRP in the serum at the first bleed was plotted as the 100% value.

Repurification of E-CRP from E-CRP-spiked mouse serum

Purified E-CRP (400 μg) was added to 2 ml C57BL/6 mouse serum (Innovative Research) and the final volume was made to 10 ml by adding 0.1 M borate buffered saline, pH 8.3, containing 3 mM $CaCl_2$. The mixture was incubated for 30 min at 37 °C. E-CRP was repurified by Ca^{2+}-dependent affinity chromatography on PEt-Sepharose beads whose capacity to bind E-CRP was >400 μg. After collecting the flow-through and washing the column with the same buffer, bound E-CRP was eluted with 0.1 M borate buffered saline, pH 8.3, containing 5 mM EDTA. To control the experiment, mouse serum alone (2 ml), without spiking with E-CRP, was used. The EDTA eluates were subjected to SDS-PAGE. The concentration of CRP in the EDTA eluates was measured by ELISA to calculate percent recovery.

Sequestration of E-CRP in mouse blood

To test the possibility that E-CRP could be sequestered by cells in the mouse blood, E-CRP-spiked mouse blood was centrifuged at 8000 rpm for 5 min and the serum recovered. The concentration of E-CRP in the recovered serum was determined by ELISA.

Mice

Male C57BL/6J mice (Jackson ImmunoResearch Laboratories) were brought up and maintained according to protocols approved by the University Committee on Animal Care. Mice were 8-10 weeks old when used in experiments.

Mouse protection experiments with CRP

Separate mouse protection experiments were performed using at least two different preparations of purified WT CRP and E-CRP. The endotoxin content in 25 μg all CRP preparations was <1.5 endotoxin units. Mice were first injected i.v. with 3.5×10^7 cfu (based on A_{600}) of pneumococci in 100 μl normal saline. The actual number of pneumococci injected, based on the plating results obtained on the next day, for all *in vivo* experiments, was $3.53 \pm 0.21 \times 10^7$ cfu. In the first set of experiments, mice were injected i.v. with either WT CRP or E-CRP, 30 min after the administration of pneumococci. In the second set of experiments, mice were injected i.v. with either WT CRP or E-CRP or both (WT CRP first and, an hour later, E-CRP), 12 h after the administration of pneumococci. In the third set of experiments, mice were injected i.v. with either WT CRP or E-CRP, four times (6, 12, 24 and 48 h) after the administration of pneumococci. CRP (25 μg) was injected in 100 μl TBS containing 2 mM $CaCl_2$. The dose of 25 μg of CRP with 3.5×10^7 cfu bacteria was chosen because, under these conditions, the protection

of mice with WT CRP injected 30 min apart from the administration of pneumococci was same as reported previously[30]. Survival of mice was recorded three times per day for 7 days. To determine bacteremia (cfu/ml) in the surviving mice, blood was collected daily for 5 days from the tip of the tail vein, diluted in normal saline, and plated on sheep blood agar for colony counting. The bacteremia value for dead mice was recorded as 10^9 cfu/ml because mice died when the bacteremia exceeded 10^8 cfu/ml.

Mouse protection experiments with clarithromycin

The antibiotic clarithromycin (Santa Cruz Biotechnology, sc-205634) was reconstituted in acetone to a stock concentration of 50 mg/ml and stored at 4 °C for a maximum of 5 days. To evaluate the efficacy of clarithromycin, the stock solution was diluted in acetone to final concentrations of 40, 4, 0.4 and 0.04 mg/ml, and 50 µl of each dose was injected i.v. per mouse at 12, 36, 60 and 84 h after the administration of pneumococci. For protection experiments involving the combination of CRP and clarithromycin, the stock clarithromycin solution was diluted in normal saline to a final concentration of 0.2 mg/ml, and 100 µl was injected i.v. per mouse at 12, 36, 60 and 84 h after the administration of pneumococci.

PCh inhibition assays

Microtiter wells were coated with either 10 µg/ml of PCh-BSA or PnC, or with 10^7 cfu of Pn-broth, in TBS, overnight at 4 °C. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin for 45 min at room temperature. In one set of inhibition assays, CRP diluted at a fixed concentration in TBS-Ca was added to duplicate wells. To determine the effects of PCh (Sigma-Aldrich) and dAMP (Chem-Implex International, 00406) on the binding of CRP

to PCh-BSA, PnC and Pn-broth, CRP was added in the presence of increasing concentrations of either PCh or dAMP. In another set of inhibition assays, CRP diluted in TBS-Ca, at increasing concentrations, was added to duplicate wells. To determine the effects of PCh and dAMP on the binding of CRP to ligands, CRP was added in the presence of either 10 mM PCh or 100 mM dAMP. After incubating the plates for 2 h at 37 °C, unbound CRP was aspirated, followed by washing the wells with TBS-Ca. The plates were then processed exactly as described previously.

Ca^{++}-site proteolytic cleavage assay

The Ca^{++}-binding site-dependent proteolytic cleavage assay of CRP was conducted as described previously with modifications[37]. CRP (4 μg) in TBS was incubated with 1 μg protease (Sigma-Aldrich, P6911), with and without 5 mM $CaCl_2$, for 2 h at 37 °C, and subjected to 4-20% denaturing SDS-PAGE under reducing conditions. The gels were stained with Coommassie Brilliant Blue. BioRad's broad-range marker was used as the molecular weight standard.

Molecular modeling of CRP

Molecular modeling was based on the X-ray crystal structure of WT CRP-PCh complex. The PDB file, 1BO9.pdb, was imported into Swiss-PdbViewer (also known as DeepView) and used to substitute the mutated amino acids. The *in silico* mutated structure was saved as a PDB file and opened with the help of PYMOL graphics software for measuring distances and for creating the figure. On-screen images were captured with Snagit 10 (TechSmith) and saved as Portable Network Graphic files.

Statistical analysis

All experiments were performed three times and comparable results were obtained each time. Results of a representative experiment are shown in the figures where the raw data (OD_{405}) were used to plot the curves. Survival curves were generated using the GraphPad Prism 4 software. To determine *p* values for the differences in the survival curves among various groups, the survival curves were compared using the software's Logrank (Mantel-Cox) test. For plotting bacteremia data, bacteremia values of 0-100 were plotted as 100 and bacteremia values of $>10^8$ were plotted as 10^9. The calculation of the median values for bacteremia in each group of mice and the statistical analyses of the bacteremia data were performed by using the GraphPad Prism 4 software and Mann-Whitney test.

Data availability

Source Data for all figures are available from the corresponding author.

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Author contributions: D.N., S.K.S. and T.B.G. performed the experiments. A.A. designed the experiments and wrote the manuscript with input from D.N.

Competing interests: The authors declare no competing interests.

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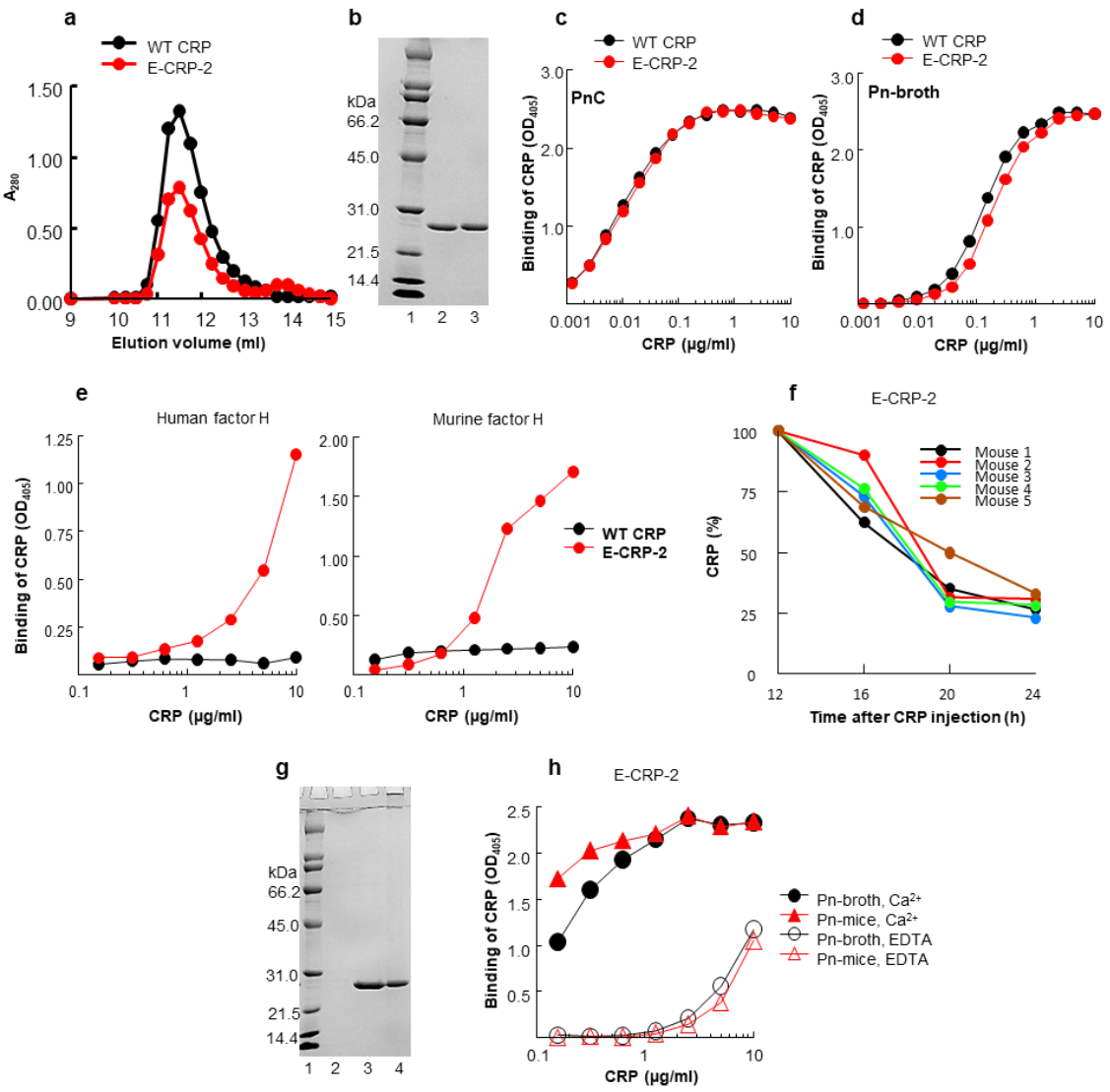
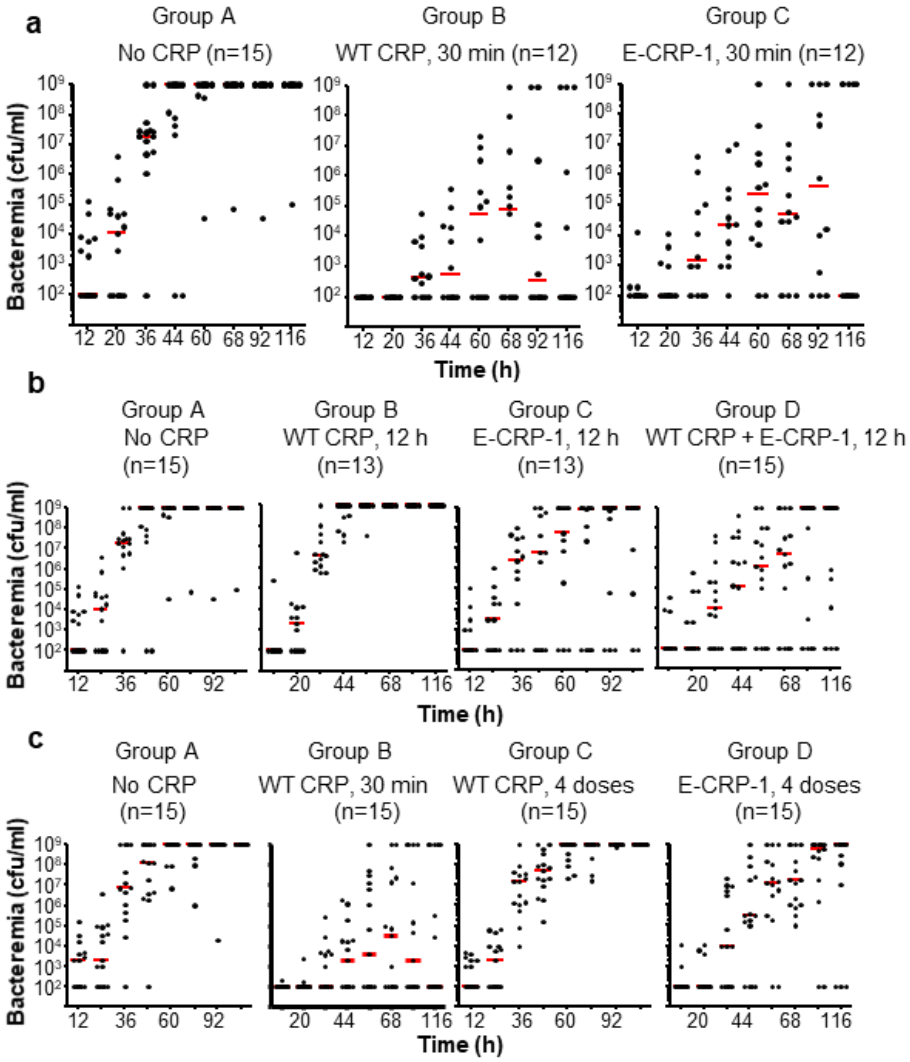
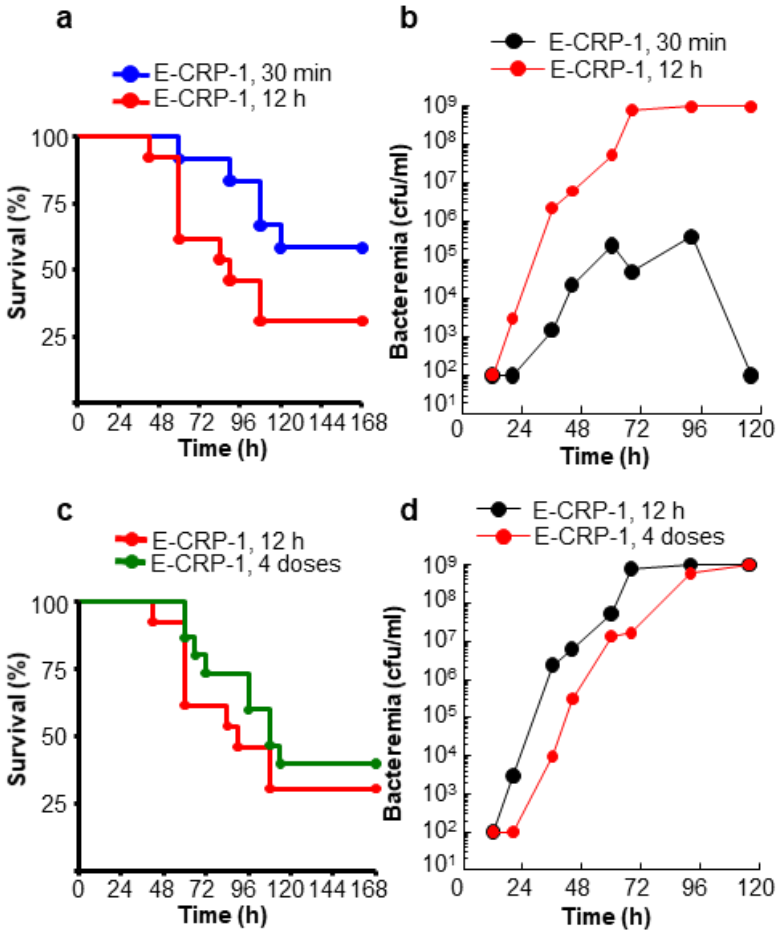
Extended Data Figures



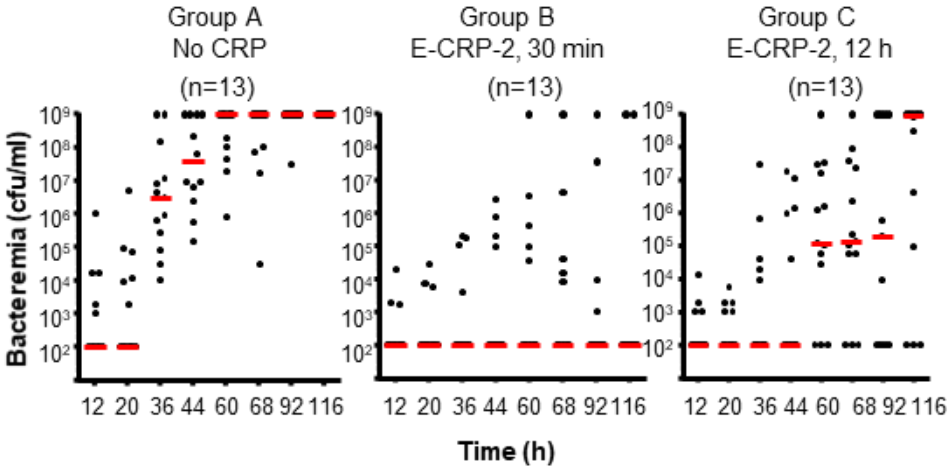
Extended Data Figure 3. 1: Characterization of E-CRP-2. a. Elution profiles of CRP from the gel filtration column. **b.** SDS-PAGE of WT CRP (lane 2) and E-CRP-2 (lane 3). **c.** Binding of CRP to PnC. **d.** Binding of CRP to broth-cultured pneumococci (Pn-broth). **e.** Binding of CRP to human (left) and murine (right) factor H. **f.** Clearance of CRP from mouse circulation. **g.** Re-purification of E-CRP-2 from purified E-CRP-2-spiked mouse serum. SDS-PAGE of re-purified E-CRP-2 is shown. Lane 1, purified E-CRP-2 (5 µg); Lane 2, EDTA eluate from the PCh-affinity column through which mouse serum containing E-CRP-2 was passed in the presence of Ca^{2+}; Lane 3, EDTA eluate from the PCh-column through which mouse serum alone was passed; Lane 4, mol. wt. markers. **h.** Binding of CRP to Pn-broth and Pn-mice in the presence and absence of Ca^{2+}. A representative of three experiments is shown for **a-h**, except for **g**.



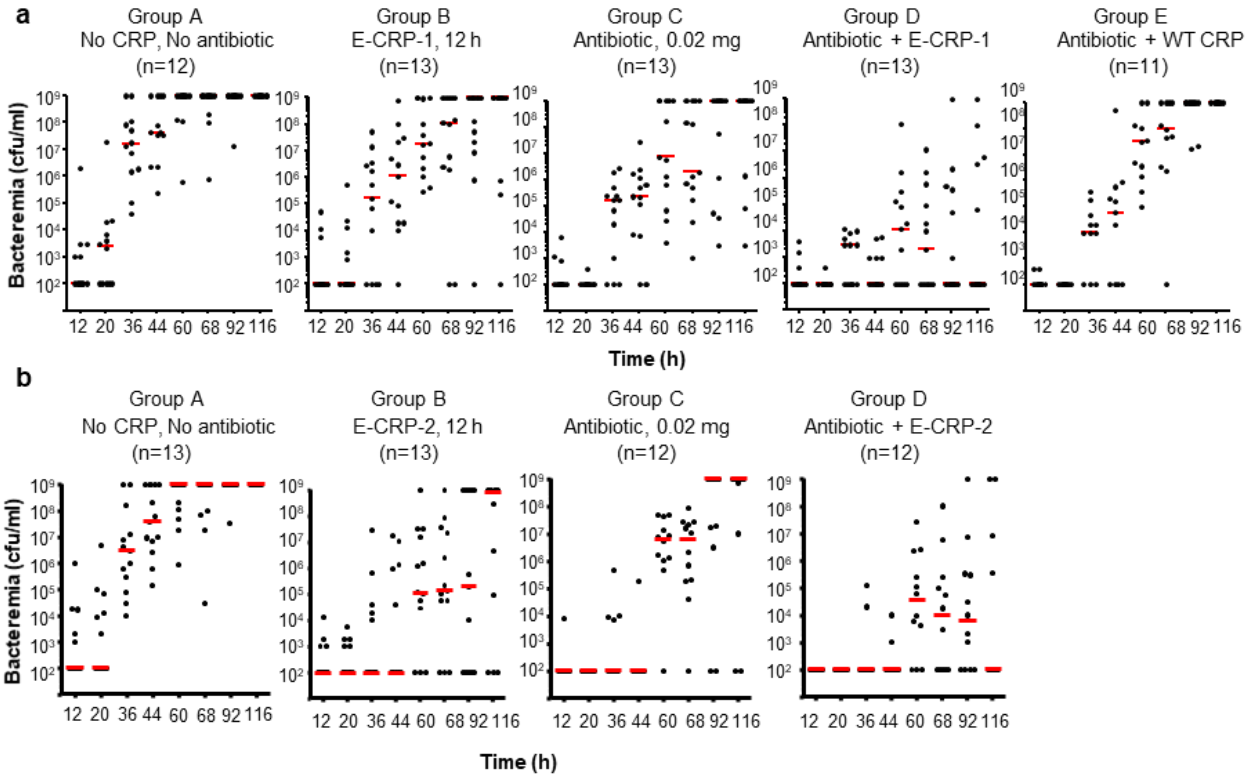
Extended Data Figure 3. 2: Scatter plots of the bacteremia data shown in Fig. 3.2b, d and f. The horizontal line in each group of mice represents the median value of bacteremia. **a.** CRP was injected first; pneumococci were injected 30 min later. For 36-116h, the *p* values for the difference between groups A B and A C are <0.001. The *p* values for the difference between groups B C are >0.05 at all time points. **b.** Same as a, except that CRP was injected 12 h after injecting pneumococci. The *p* values for the difference between groups A B and C D are >0.05 at all time points. For 44-92 h, the *p* values for the difference between groups B C and B D were <0.01. **c.** Same as **a**, except that CRP was injected four times (6, 12, 24 and 48 h after injecting pneumococci). The *p* values for the difference between groups A C was >0.05 at all time points. For 36-116 h, the *p* values for the difference between groups B C are <0.001. For 44-116 h, the *p* values for the difference between groups B D and C D were <0.05. For 44-116 h, the *p* values for the difference between groups A D was <0.01.



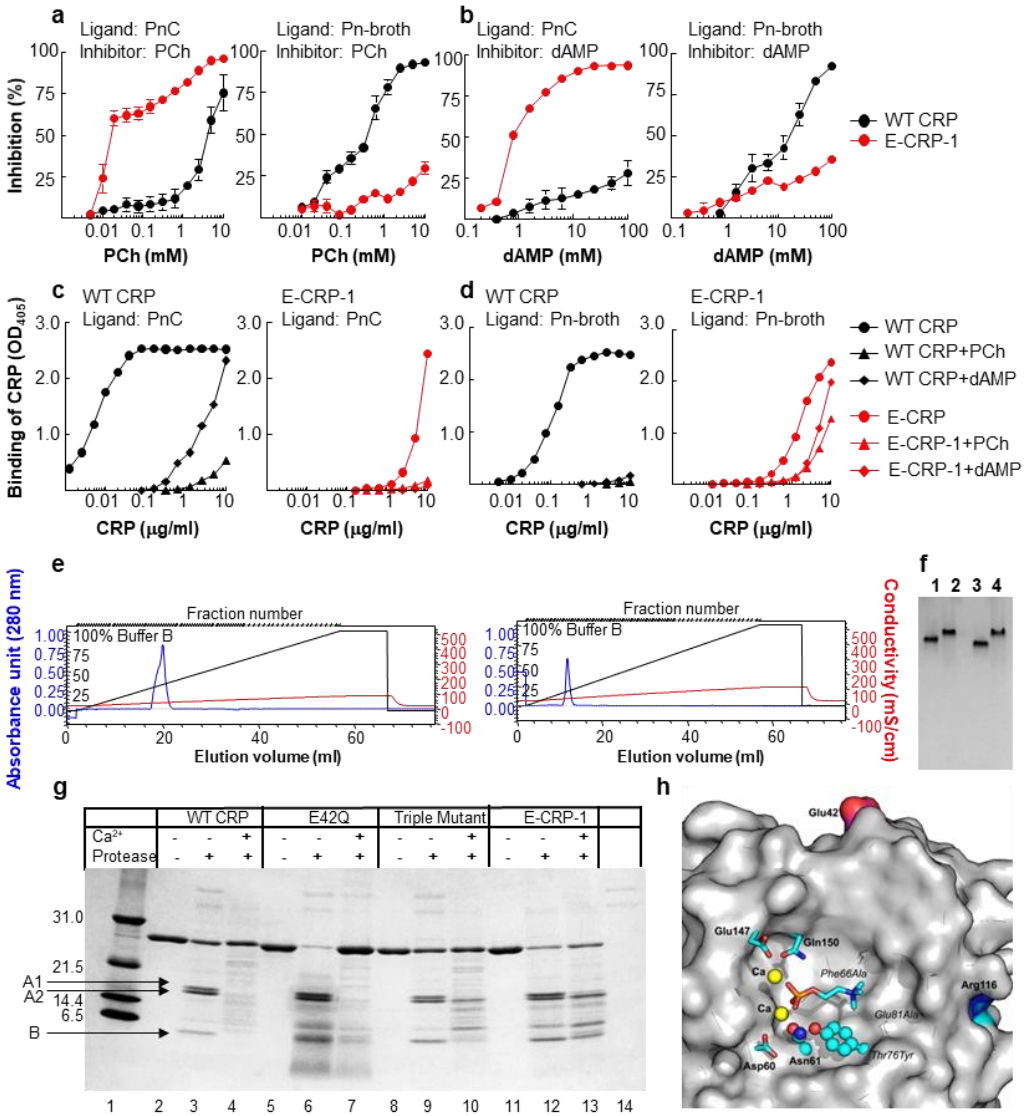
Extended Data Figure 3. 3: Additional comparisons of the data shown in Fig. 3.2. a, Survival curves. The *p* values for the difference between 30 min and 12 h was 0.09. **b,** Bacteremia in each surviving mouse shown in **a**. The median bacteremia values are plotted. For 36-116 h, the *p* values for the difference either between groups A B or A C were <0.001. **c,** Survival curves. The *p* values for the difference between one dose and four doses survival curves was 0.41. **d,** Bacteremia in each surviving mouse shown in **c**. The median bacteremia values are plotted. For 36-116 h, the *p* values for the difference either between groups A B or A C were <0.001.



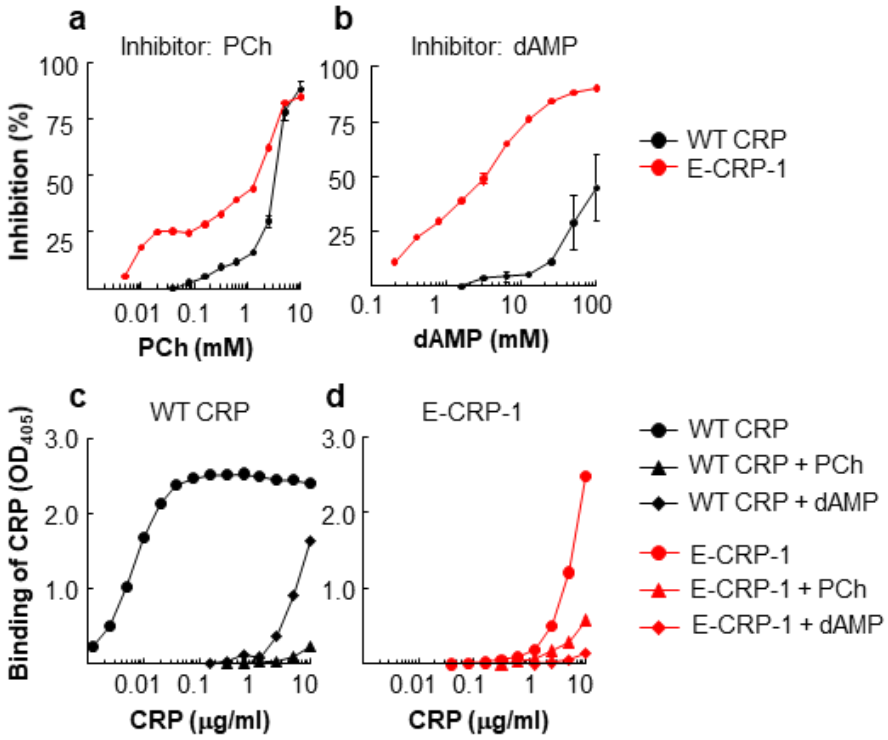
Extended Data Figure 3. 4: Scatter plots of the bacteremia data shown in Fig. 3.3b. The horizontal line in each group of mice represents median bacteremia. For 36-116 h, the *p* values for the differences between groups A B and A C were <0.001. The *p* values for the difference between groups B C was >0.05 till 60 h and <0.05 after 60 h.



Extended Data Figure 3. 5: Scatter plots of the bacteremia data shown in Fig. 3.4. The horizontal line in each group of mice represents median bacteremia. **a.** For 44-116 h, the *p* values for the difference between groups B D was <0.001. The median bacteremia values are plotted. For 44-116 h, the *p* values for the difference between groups C D was <0.005. **b,** The *p* values for the difference between groups B D was <0.05 only at 116 h. The median bacteremia values are plotted. For 44-116 h, the *p* values for the difference between groups C D was <0.05.



Extended Data Figure 3. 6: Further characterization of E-CRP-1. a. Inhibition of binding of CRP to PnC (left panel) and Pn-broth (right panel) by PCh. **b.** Inhibition of binding of CRP to PnC (left panel) and Pn-broth (right panel) by dAMP. Average ± SEM of three experiments is shown in **a, b. c.** Inhibition of binding of WT CRP (left panel) and E-CRP-1 (right panel) to PnC by PCh and dAMP. **d.** Inhibition of binding of WT CRP (left panel) and E-CRP (right panel) to Pn-broth by PCh and dAMP. A representative of three experiments is shown in **c, d. e.** MonoQ anion exchange chromatography of WT CRP (left) and E-CRP-1 (right). **f.** Native PAGE of CRP (lane 1; WT CRP in the presence Ca^{2+}, lane 2; E-CRP-1 in the presence Ca^{2+}, lane 3; WT CRP in the absence Ca^{2+}, lane 4; E-CRP-1 in the absence Ca^{2+}). A representative of two Coomassie blue-stained gels is shown. **g.** Ca^{++}-site-dependent proteolytic cleavage of CRP. Reducing SDS-PAGE of CRP treated with protease in the absence and presence of Ca^{++}. **h.** Molecular modelling of E-CRP-1. The side chains of Phe^{66}, Thr^{76} and Glu^{81} involved in the formation of the PCh-binding pocket and E42 are highlighted. Ca^{++} are shown as yellow balls. The PDB coordinates of E-CRP-1 were generated from the PDB file 1B09 using SYBYL (Tripos, Inc.). The side chains of Phe^{66}, Thr^{76} and Glu^{81} are substituted with Ala, Tyr and Ala, respectively. One of the five subunits is shown.



Extended Data Figure 3. 7: Further characterization of E-CRP-1 (continued). a. Inhibition of binding of CRP to PCh-BSA by PCh. **b.** Inhibition of binding of CRP to PCh-BSA by dAMP. **c.** Inhibition of binding of WT CRP to PCh-BSA by PCh and dAMP. **d.** Inhibition of binding of E-CRP-1 to PCh-BSA by PCh and dAMP. A representative of three experiments is shown in **c, d**.

CHAPTER 4: BIOLOGY OF THE INTERACTION BETWEEN C-REACTIVE PROTEIN AND AMYLOID β

Running title: Investigations of the interaction between Amyloid β and C-reactive protein

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Abstract

CRP is a prominent pattern recognition molecule of the innate immune system, recognizing phosphocholine (PCh) containing substances and activating the classical complement pathway. Mild acidic or redox conditions modify WT CRP into a non-native pentameric form (modified CRP) with new binding properties to various immobilized and denatured protein ligands. The binding of modified CRP to these many denatured and aggregated protein ligands has raised questions about the specificity of these interactions. Based on the affinity of modified CRP for amyloid β (Aβ), we hypothesized that immobilized proteins expose common amyloid-like structures that are recognized by CRP in its modified pentameric state. Therefore, there must exist a site (Aβ binding site) on CRP responsible for its interaction with these many denatured protein ligands. Our attempts to identify this site was unfruitful. However, further investigations into the significance of modified CRP interaction with Aβ in the formation of amyloid fibrils were done. Mutant CRP which has similar binding characteristics as acidic pH-treated or H_2O_2-treated CRP was used. We found that the mutant CRP prevents the formation of amyloid fibrils by Aβ 1-42. Taken together, we conclude that immobilizing proteins denatures them and cause them to present common (amyloid-like) structures which are recognized by CRP in its modified pentameric conformation. Therefore, modifications of CRP into its non-native pentameric conformation causes it to expose an otherwise hidden (Aβ) site giving it anti-amyloidogenic properties.

Key Words: C-reactive protein, Amyloid β, Amyloid fibrils

Introduction

Human C-reactive protein (CRP) is a soluble, pentameric, pattern recognition molecule of the innate immune system. Each of the 5 subunits of CRP are held together in a symmetric planar conformation by noncovalent bonds and possess two binding sites for calcium ions (1,2). CRP is a potent acute phase protein with levels that rise dramatically during infections or inflammation by up to a thousand fold. At physiological pH, CRP recognizes and binds phosphocholine containing substances of pathogenic bacteria and damaged cells in a Ca^{2+}-dependent manner (1,4,3). The ligand-binding properties of CRP have previously been shown to be regulated by pH and redox conditions (5–7). CRP acquires enhanced or novel binding capabilities under mild oxidative and acidic conditions to a multitude of immobilized/denatured ligands including oxidized LDL (ox-LDL), factor H, and amyloid β (Aβ) (9,8,5–7).

Amyloid β are peptides of 36-43 amino acids long and are normal components of both plasma and cerebrospinal fluid (10,11). The aggregation of these peptides into oligomers or fibrils is implicated with the development and progression of Alzheimer's disease (AD) (12,13). Two main variants of this peptide exist in humans and include Aβ40 and Aβ42 which are 40 and 42 amino acids long respectively (14). The most prevalent circulating amyloid β variant is Aβ40, however, Aβ42 (Aβ) has been shown to nucleate faster, forming fibrils more rapidly (15) and was the species used in this study.

CRP is synthesized primarily in the liver, but can also be produced in other organ cells such as neurons (16). Denatured (monomeric) CRP has been shown to be present (deposited) at the sites of many types of inflammatory diseases including in senile plaques and neurofibrillary tangles of AD (18,17,20,21,19). The role of CRP at these sites remains unknown but likely involve its non-native state, as native WT CRP does not interact with fluid phase or immobilized Aβ (5).

Clinical studies have linked high levels of plasma CRP with the risk of developing AD (23,22). However, these elevated CRP levels may just be indicative of inflammation and not necessarily a causal factor because CRP does not cross the blood brain barrier. Inflammatory sites are generally characterized by acidic conditions and increased presence of reactive oxygen species (ROS) which have been linked with the generation of amyloid plaques and the development of AD (24–26,28,27). As earlier stated, CRP can be modified by these conditions (or by mutations of the protein) causing it to acquire a non-native pentameric structure with new ligand binding properties (5–7). These changes in the conformation of CRP can occur at sites of inflammation (29).

We have previously reported that H_2O_2-treated (H_2O_2 serving as a prototype for ROS) and acidic pH-treated pentameric CRP bound many different types of immobilized proteins including Aβ in an H_2O_2 concentration and pH dependent manner (8,5,7). Native WT CRP did not bind to these immobilized protein ligands even when the immobilized proteins were treated with acidic pH (5). This suggests that CRP acquires this new binding capability only when CRP is exposed to acidic or redox conditions, meaning when CRP is in its modified pentameric conformation and should involve a site which is hidden on WT CRP but exposed on modified CRP. The interaction of CRP with many diverse protein ligands at inflammatory sites may therefore be due to the ROS rich and acidic microenvironment at inflammatory sites that cause modifications to proteins in the area including to CRP itself. This gives CRP the role of protecting against the toxicity of denatured proteins in inflammatory microenvironments. However, the binding of modified CRP to these many protein ligands may seem nonspecific unless they all exposed a common structure or pattern that is recognized by modified CRP. We therefore hypothesize that denatured, aggregated and immobilized proteins expose amyloid-like structures recognized by modified CRP.

In this study we investigate whether immobilizing proteins, which denatures them (30), also causes them to expose amyloid-like structures recognized and bound by acidic pH treated or mutated CRP. We also try to identify the site on modified CRP responsible for theses novel ligand binding capabilities by screening our library of CRP mutants with mutations in the Ca^{2+}-binding site, the intersubunit region and the cholesterol-binding site (CBS). Using mutant CRP, which binds to Aβ without the need for acidic pH, we explored the significance of this interaction in fibril formation assays. While we were unable to identify the site on modified CRP responsible for interacting with immobilized protein ligands, we found that the interaction between mutant CRP and Aβ prevented the formation of amyloid fibrils *de novo* and stopped further fibril growth.

Mutant	Mutagenic Oligo
Y40A	5′-GTGTGCCTCCACTTC**GC**CACGGAACTGTCCTC-3′ 3′-CACACGGAGGTGAAG**CG**GTGCCTTGACAGGAG-5′
L37A/H38A	5′-TTCACTGTGTGC**GC**C**GC**CTTCTACACGGAACTG-3′ 3′-AAGTGACACACG**CG**G**CG**GAAGATGTGCCTTGAC-5′
L37F/H38A	5′-TTCACTGTGTGC**T**TC**GC**CTTCTACACGGAACTG-3′ 3′-AAGTGACACACG**A**AG**CG**GAAGATGTGCCTTGAC-5′
H38A/F39A	5′-TTCACTGTGTGCCTC**GC**C**GC**CTACACGGAACTG-3′ 3′-AAGTGACACACGGAG**CG**G**CG**GATGTGCCTTGAC-5′
H38A/Y40A	5′-GTGTGCCTC**GC**CTTC**GC**CACGGAACTGTCCTC-3′ 3′-CACACGGAG**CG**GAAG**CG**GTGCCTTGACAGGAG-5′
H38A/Y40F	5′-GTGTGCCTC**GC**CTTCT**T**CACGGAACTGTCCTC-3′ 3′-CACACGGAG**CG**GAAGA**A**GTGCCTTGACAGGAG-5′
H38A/Y40F/E42Q	5′-GTGTGCCTC**GC**CTTCT**T**CACG**C**AACTGTCCTC-3′ 3′-CACACGGAG**CG**GAAGA**A**GTGC**G**TTGACAGGAG-5

Figure 4. 1: Pairs of mutagenic oligonucleotides used in constructing CRP mutants. The sequences were designed based on the published sequence for WT CRP (51). Triplet codons of the mutated amino acids are boxed. Mutated bases are in red and bold.

Methods

Construction and expression of mutant CRP cDNA

We constructed CRP cDNA encoding the Y40A, L37A/H38A, L37F/H38A, H38A/F39A, H38A/Y40F, H38A/Y40A, and H38A/Y40F/E42Q mutants. The WT human CRP cDNA in the expression vector p91023 (31) was used as the template for the construction of Y40A CRP cDNA. H38A mutant CRP cDNA was used as a template to construct cDNAs for L37A/H38A, L37F/H38A, H38A/F39A, H38A/Y40F, and H38A/Y40A CRP mutants and Y40F/E42Q mutant CRP cDNA was used as a template to construct the cDNA for H38A/Y40F/E42Q. Site-directed mutagenesis was conducted using the QuikChange mutagenesis kit (Stratagene). Mutagenic oligonucleotides (Fig. 4.1) were designed according to the kit instructions and obtained from Integrated DNA Technologies. Mutations were verified by sequencing performed in our Core Facility. Two clones for each mutant were purified using the maxiprep plasmid isolation kit (Eppendorf). Mutant CRP cDNA constructs H38A, Y40F, E42Q, E147A, E147K, E147Q, Y40F/E42Q, F66A/E81A, and Y40F/P115A have been reported previously (33,34,32,5). CRP was expressed using the ExpiCHO-S expression system (Gibco) and following manufacturer's instructions. Purification of mutant CRP from the culture media supernatant was done as described below.

Purification of CRP

Native WT CRP was purified from discarded human pleural fluid in a three step process previously described (35). Briefly, the first step involved a Ca^{2+}-dependent affinity chromatography on a phosphocholine-Sepharose column (Pierce), followed by anion-exchange chromatography on a MonoQ column and gel filtration on a Superose12 column (GE Healthcare)

using the Biologic Duo Flow Protein Purification System (Bio-Rad). CRP mutants were purified from cell culture supernatants in two steps which involved a Ca^{2+}-dependent affinity chromatography on a PCh-Sepharose column (Pierce) followed by gel filtration on a Superose12 column (GE Healthcare). Purified CRP was stored frozen and on the day of the experiment CRP was repurified by gel filtration on a Superose12 column to remove any monomeric CRP that might have been generated due to storage. Repurified CRP was stored in 10 mm TBS (pH 7.2), containing 2 mm $CaCl_2$ at 4 °C, and was used within a week.

pH dependent protein ligand-binding assay

To evaluate the binding activity of WT and mutant CRP to various ligands under different pH conditions, microtiter wells were coated either with 10 μg/ml amyloid β (fragment 1–42, catalog no. H-1368 1000, Bachem) or 2 μg/ml complement factor H (Catalog no. A137, Complement Technology, inc.) diluted in TBS (100 μl/well) overnight at 4 °C. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin. Purified CRP was diluted to 10 μg/ml in TBS (pH 7.2 to 4.6) containing 0.1% gelatin, 0.02% Tween 20, and 2 mm $CaCl_2$ (TBS-Ca) and added in duplicate wells (100 μl/well), then incubated for 2 h at 37 °C. The wells were washed with TBS-Ca and Rabbit anti-CRP antibody (Sigma), diluted 1/1000 in TBS-Ca, was used (100 μl/well, 1 h at 37 °C) to detect bound CRP. HRP-conjugated donkey anti-rabbit IgG (GE Healthcare), diluted in TBS-Ca, was used as the secondary antibody (100 μl/well, 1 h at 37 °C). Color was developed, and the absorbance was read at 405 nm in a microtiter plate reader (Molecular Devices).

Amyloid-β / factor H binding assay

This assay was used to determine the dose dependent binding of CRP to amyloid β and factor H. It was performed as described for the pH dependent protein ligand-binding assay, except that CRP was diluted either in pH 7.2 (WT and mutant CRP) or pH 4.6 (WT CRP) TBS-Ca buffers.

Preparation of amyloid-β monomers

The vial of lyophilized stock of synthetic amyloid β stored at -20 °C was equilibrated at room temperature for 30mins. Then a 1 mM amyloid β stock was prepared by adding hexafluoroisopropanol (HFIP) and incubating the solution at 37 °C for 1 h (221.7 µl of HFIP was used per mg amyloid β powder). After incubation, aliquots of 25 µl (0.1128 mg) where made in glass vials and HFIP removed by evaporation inside a fume hood overnight. The resulting peptide film was stored with desiccant at -20 °C until needed.

Amyloid-β fibril formation and thioflavin T (ThT) Assay

To begin, the amyloid β peptide film was taken out from -20 °C and allowed to come to room temperature. Then the peptide was resuspended in 50 µl of 10 mM Tris-HCl (0.5 mM amyloid β) and used to prepare an 800 µl reaction mix (30 µM amyloid β final concentration) by adding 10 mM Tris-HCl, 150 mM NaCl, and 10 µM ThT with or without 4.3 µM CRP on ice. The reaction mix was then vortexed and 260 µl aliquots transferred in triplicate wells in a 96-well plate (266, Corning Costar, Immunochemistry Technologies). An initial reading was taken using the Synergy H1 microplate reader (BioTek) with excitation at 440 nm and emission at 480 nm. Then the plate was incubated at 37 °C with 300 rpm shaking for 24 h, taking additional readings every hour.

Results

All experiments were performed at least three times with comparable results obtained each time. A representative figure is shown where the data used to plot the figures was in A_{405} absorbance units.

Binding of CRP mutants to immobilized Aβ as a function of pH

In our quest to find CRP mutants that have lost the ability to bind immobilized protein ligands (Aβ as prototype) even at acidic pH; implying mutations to the multiple ligand binding (Aβ) site, or CRP mutants that are able to bind Aβ at physiological pH; to study the significance of this interaction, we screened our library of CRP mutants with mutations in the Ca^{2+}-binding site, the intersubunit region, and the cholesterol-binding site (CBS). It is believed that CRP circulates in the body in its Ca^{2+}-bound form and this helps protect the molecule from proteolytic cleavage as in the absence of Ca^{2+}, a loop it hides that is prone to proteolysis moves out and is exposed to cleavage (36,37). Equally, the other putative sites were selected because they are buried within the WT CRP molecule and can only be exposed when CRP is modified.

Fig. 4.2 shows the results of protein ligand-binding assays in which we determined the binding of CRP to immobilized Aβ as a function of pH (7.0–4.6). As seen in Fig. 4.2A, neutralizing the negative charge with a switch from Glu^{147} to Ala (E147A) or maintaining the charge with a switch from Glu^{147} to Gln (E147Q) did not affect the binding of mutant CRP as much as when the mutation involved changing the negatively charged Glu^{147} to a positively charged Lys (E147K). The binding of E147K mutant CRP at pH 6.4 and lower was much reduced when compared to WT CRP. The double mutant F66A/E81A gave intermediate results. Meanwhile,

mutating amino acids in the intersubunit region of CRP did not yield mutants with binding deficiency to immobilized Aβ (Fig. 4.2B).

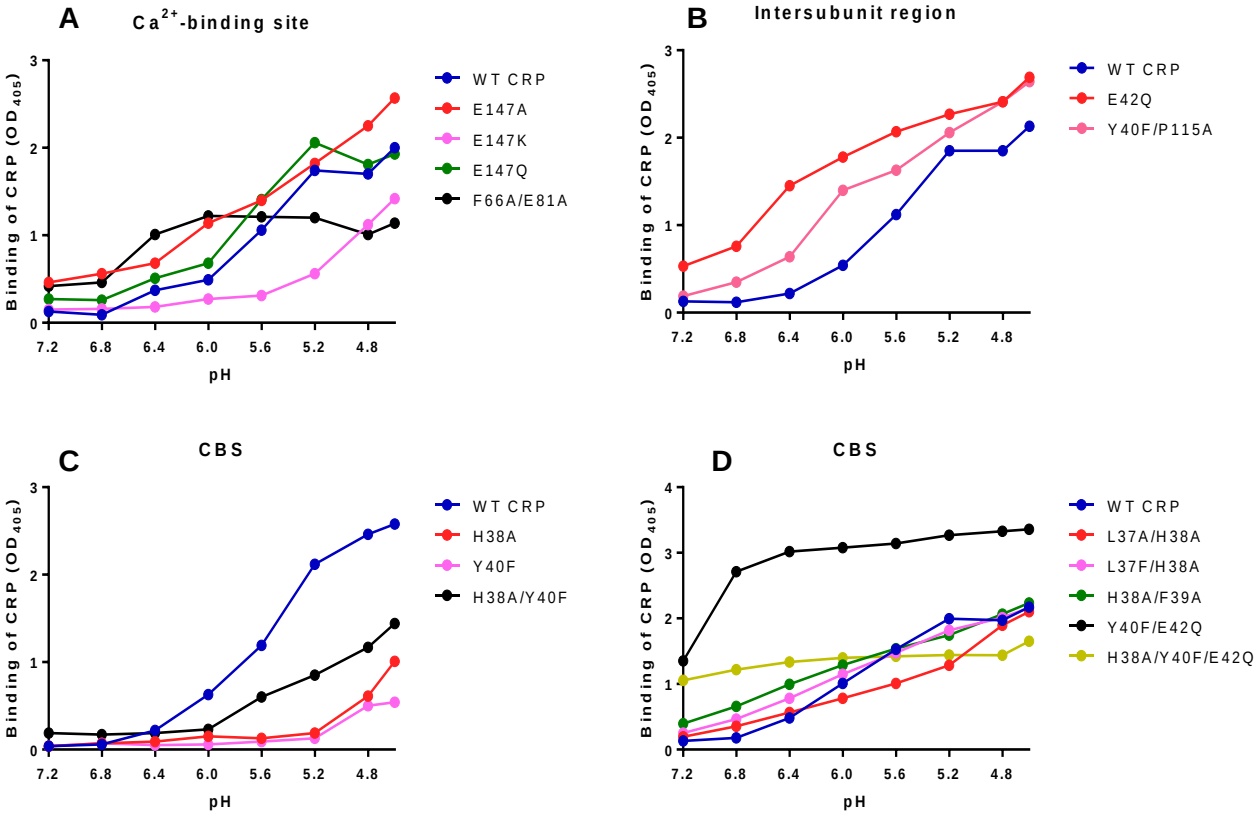

Figure 4. 2: Binding of CRP to immobilized Aβ as a function of pH. Microtiter wells were coated with factor H, Ox-LDL and Aβ. The unreacted sites in the wells were blocked with gelatin. CRP (10 µg/ml), diluted in TBS-Ca, pH 7.0–4.6, was then added to the wells and incubated at 37 °C, for 2 h. Bound CRP was detected by using a rabbit polyclonal anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. The absorbance of the developed color was read at 405 nm and plotted.

However, when mutations where carried out in CBS (Fig. 4.2C and D), the resulting mutants showed a drastic lost in binding with comparative binding of WT CRP at pH 6.0 being similar to the binding of H38A and Y40F at pH 4.8. Binding to immobilized Aβ was not initiated until the pH dropped below 5.2 for both mutants. The double H38A/Y40F mutant with both mutations

surprisingly bound better than the single mutants. Albeit, it still bound less compared to WT CRP (Fig. 4.2C). In Fig. 4.2D, other CRP mutants with mutations to CBS were screened. They all bound to a similar degree as WT CRP except for Y40F/E42Q and the triple mutant H38A/Y40F/E42Q. CRP mutant Y40F/E42Q bound immobilized Aβ at physiological pH (pH 7.2) 10 times better compared to WT CRP. Its binding was even more pronounced with decreasing pH, plateauing at pH ranges of 6.4 to 4.6. Additional mutation of His^{38} to Ala inhibited some of the binding and also reduced the pH sensitivity of H38A/Y40F/E42Q, resulting in about the same binding at all pHs. Amidst our failure to identify the binding site on CRP for Aβ, we identified mutant Y40F/E42Q which binds immobilized Aβ at physiological pH and can be used to study the significance of these interactions between CRP and multiple ligands.

Binding of CRP to immobilized factor H and Aβ

We tested the binding of Y40F/E42Q CRP to another ligand, factor H, as a function of pH (Fig. 4.3A). The binding of Y40F/E42Q to immobilized factor H was similar to its binding to Aβ (Fig. 4.2D). This confirmed that the multiple ligand binding site of CRP was accessible on Y40F/E42Q CRP, making the molecule suitable for downstream studies as the requirement for an acidic environment was eliminated. Also, performing a CRP dose-response assay to compare the efficiency of binding of various CRP species to factor H and Aβ revealed that Y40F/E42Q CRP binds as efficiently as pH 4.6-treated CRP to these immobilized ligands (Fig. 4.3B and C). This further confirms the usability of Y40F/E42Q CRP to study the significance of CRP's interaction with multiple immobilized ligands. WT CRP did not bind to either of these ligands.

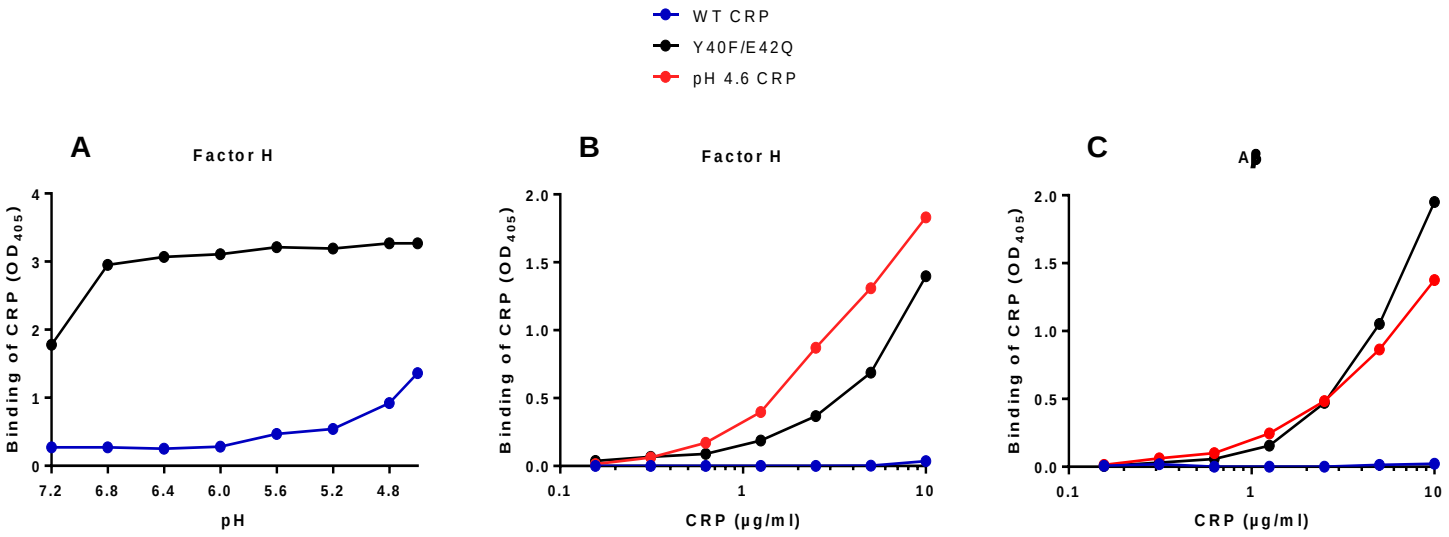

Figure 4. 3: pH and dose dependent binding of CRP to immobilized factor H and Aβ. Microtiter wells were coated with factor H and Aβ. The unreacted sites in the wells were blocked with gelatin. (A) CRP (10 µg/ml), diluted in TBS-Ca, pH 7.0–4.6 or (B and C) CRP, in increasing concentrations diluted in TBS-Ca, pH 7.2 or 4.6, was then added to the wells and incubated at 37 °C, for 2 h. Bound CRP was detected by using a rabbit polyclonal anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. The absorbance of the developed color was read at 405 nm and plotted.

Amyloid fibrillation assay with CRP

After determining that Y40F/E42Q CRP was suitable for use in downstream studies, we investigated the significance of this interaction in the formation of fibrils by Aβ. Amyloid fibrillation is a distinctive feature in the development of Alzheimer's disease. The results obtained from a thioflavin (ThT) assay showed that Y40F/E42Q mutant CRP prevents fibrillation. A slow build up was initially observed when WT CRP was used, but this effect was lost as time went on.

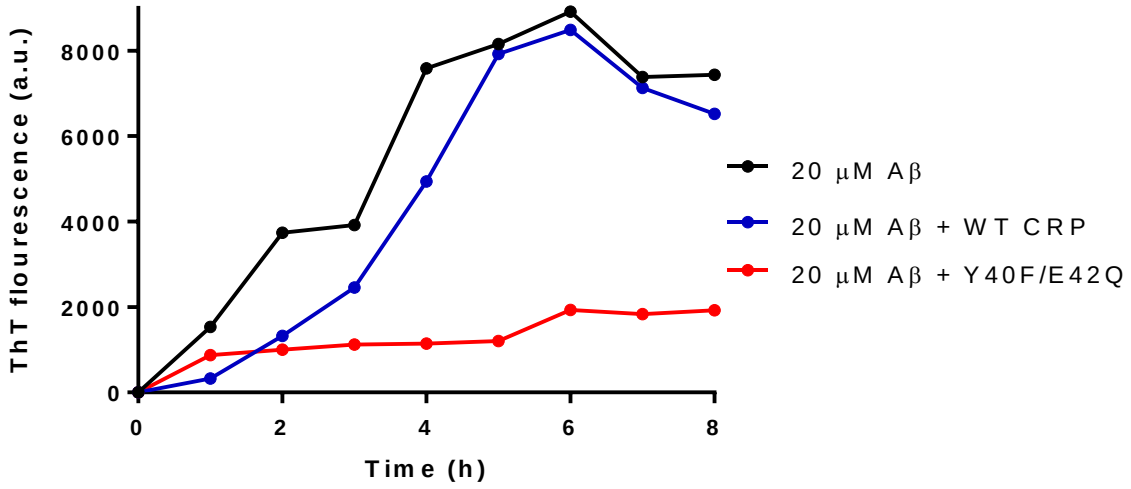

Figure 4. 4: The effect of CRP on Aβ amyloid fibril formation in Tris buffered saline. Amyloid fibril formation time course monitored by ThT fluorescence in the absence (black) or presence of 0.4 µM CRP (red and blue).

Discussion

The ligand binding properties of WT CRP can be expanded beyond its ability to bind molecules and cells bearing exposed phosphocholine (PCh) at physiological pH to include immobilized, denatured and aggregated proteins when it is modified by acidic/redox conditions and mutagenesis (5–7,38). Given that acidic and redox conditions are associated with the microenvironment at various types of inflammatory sites (45,43,42,44,39–41), and that CRP has been shown to be localized at these sites of inflammation (18,17,20,21,19), suggest that this novel binding property is an innate characteristic of WT CRP only exploited in its non-native/modified pentameric state. We have previously demonstrated that for the interaction between CRP and these various protein ligands to occur, both CRP and the protein ligands have to be subjected to the same mild denaturing conditions (5). Native WT CRP does not bind to these immobilized, denatured and aggregated protein ligands at physiological pH. However,

there has been debate regarding this multiple ligand binding property of modified CRP within the scientific community. Some speculated that the binding of CRP to these many protein ligands may be due to CRP being sticky and therefore the binding artefactual. However, as shown in Fig. 4.3B and C, the interaction between modified pentameric CRP and immobilized protein ligands is dose dependent, suggesting specificity for the protein ligands and leading to our hypothesis that immobilized proteins present common unique structures with which modified CRP interacts. This means that CRP in its alternate pentameric conformation exposes a multiple ligand (Aβ) binding site which binds amyloid-like structures of immobilized denatured proteins. This is in contrast with the argument that the multiple ligand binding property of CRP could be artefactual and gives modified pentameric CRP specificity of interaction with these new ligands.

We investigated the significance of the binding between CRP and these immobilized protein ligands. Knowing that CRP carries out general housekeeping functions in the body which include getting rid of misfolded, denatured and conformationally altered proteins to prevent the toxicity they generate, and that the interaction of CRP is probably with common amyloid-like structures of denatured proteins, we investigated the significance of this interaction with respect to amyloid fibril formation. We found that the interaction of modified CRP with Aβ prevents progression of amyloid fibrillation (Fig. 4.4).

The location of the Aβ binding site on CRP is not known. However, studies have shown CRP to dose dependently inhibit both Aβ40 and D76N β_2 -microglobulin fibril formation in a Ca^{2+}- independent manner (46). CRP has also been shown to bind fibronectin (Fn) with high affinity Ca^{2+}- independently (32) and site directed mutagenesis of the amino acids involved in coordinating Ca^{2+} greatly reduced the affinity of CRP for Fn. This suggested that the Ca^{2+}-binding site formed the Fn-binding site and therefore a putative Aβ binding site. However, acidic

pH conditions known to chelate Ca^{2+} bound CRP, cause the exposure of an otherwise buried loop prone to proteolytic cleavage (47). This renders Ca^{2+}-free CRP very unstable. A second more stable and promising putative binding site is the cholesterol binding region (CBS; a.a. 35–47). This region is said to be intrinsically disordered and responsible for the binding of CRP to diverse protein ligands. Using a synthetic CBS peptide, the binding of CRP to all tested immobilized protein ligands was inhibited (48,49) suggesting that this region could be the multiple ligand (Aβ) binding site. However, the mutations we carried out to this site did not completely prevent the binding of modified CRP to immobilized Aβ. Given the reduction in binding seen especially with the single mutants H38A and Y40F, suggests the implication of this site. More studies are required to definitively identify this site as the multiple ligand or Aβ binding site.

The deposition of amyloid fibrils as plaques is a key feature of many neurodegenerative diseases like Parkinson's and Alzheimer’s disease. The continuous accumulation of Aβ peptides trigger secondary pathogenic events leading to progressive cognitive impairment (50). Therapies geared at preventing the aggregation of Aβ into fibrils or methods of increasing the dissociation of these amyloid fibril plaques are potential strategies for combatting amyloid diseases. Modified pentameric CRP is a potential candidate for not only preventing the formation of amyloid fibrils but also stopping the progression of amyloid β deposition.

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CHAPTER 5: THE CONTRIBUTION OF CCAAT-ENHANCER-BINDING PROTEINS IN THE TRANSCRIPTIONAL REGULATION OF THE C-REACTIVE PROTEIN GENE

Running title: The role of the two CCAAT-enhancer-binding protein promoter sites in mediating C-reactive protein gene expression

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Abstract

Human C-reactive protein (CRP) is mainly produced by hepatocytes and its levels go up in both acute and chronic inflammation. In Hep3B cells, the cytokine IL-6 induces CRP gene expression by activating the transcription factors C/EBPβ and STAT3. Cytokine IL-1β alone cannot induce CRP expression, but synergistically enhances the effects of IL-6 by activating the transcription factor NF-κB. This synergy can be seen with the first 157 bp proximal CRP promoter region. However, an increased expression of the CRP gene is seen when the longer 300 bp proximal CRP promoter is used compared to the shorter 157 bp proximal promoter. The reason for this enhanced expression is not known. We hypothesize that a second C/EBP site centered at position -219 of the CRP promoter is responsible for this enhanced expression. Therefore, with the use of mutagenic probes in transactivation (Luc) assays we were able to show that this site was not only critical for the increased expression seen with the longer 300 bp promoter but also that it worked in synergy with the downstream C/EBP site at -52 to fully activate CRP gene expression. We also found that the upstream C/EBP site worked independent of both NF-κB and STAT3, two transcription factors known to induce CRP gene expression.

Introduction

C-reactive protein (CRP) is a member of the pentraxin family of proteins and a major acute-phase protein (1–3). It is synthesized primarily by liver hepatocytes and maintained in circulation at a median serum concentration of 0.8 mg/L. Following an inflammatory insult or bacterial infection, serum CRP levels surge sometimes several hundred fold above basal levels (4,5). Serum CRP levels rise in both acute and chronic inflammation (6). After the resolution of inflammation, CRP levels immediately fall back to basal levels (4). Thus, levels of CRP are measured to evaluate the general inflammatory state of the body (7,8). Due to the nature of the CRP gene to cause fluctuations in CRP expression, it has become a point of intrigue to understand how it is regulated.

At the transcriptional level, where the regulation of the CRP gene occurs, cytokines IL-1β, IL-6, IL-17, TGFβ, and TNFα have been shown to modulate its induction (9,10,19,11–18). In human hepatoma Hep3B cells which are the most commonly used model to study CRP gene expression, expression of the CRP gene is primarily regulated by cytokines IL-6 and IL-1β (20). IL-6 activates transcription factors STAT3 and C/EBPβ to induce a modest expression of the CRP gene while IL-1β, which alone does not affect the expression of CRP, together with IL-6 synergistically enhances the induction of CRP gene expression (9,17,21–24). However, the mechanism by which IL-1β synergistically enhances the effects of IL-6 induction of the CRP gene remains elusive but may involve the IL-1β response element Rel protein/NF-κB family of transcription factors (25). We have previously showed that the binding of Rel proteins p50 and p52 to a nonconsensus κB site overlapping the proximal C/EBP binding site on the CRP promoter is a requirement for C/EBPβ to bind to its cognate site (10). This association may be

key to the synergistic action of the two cytokines. Also, the first 157 bp of the CRP promoter have been shown to be sufficient for the synergistic actions of IL-6 and IL-1β (9,11,12,26).

In addition to the improved induction of the CRP gene seen with a combination of IL-6 and IL-1β observable with only the first 157 bp of the CRP promoter, Hep3B cells consistently show better induction of CRP gene expression when the 300 bp proximal CRP promoter is used compared to the proximal 157 bp (9,27). Most of the binding sites for transcription factors involved in regulating CRP gene expression identified so far are located within the 157 bp proximal CRP promoter (Fig 5.1a). C/EBP has two identified sites on the CRP promoter; one centered at position -52 and a second at -219 (23,28). Therefore, in this study, we decided to evaluate the contribution of the C/EBP site at position -219 given that a better induction is seen with the 300 pb promoter compared to the 157 pb promoter which lacks this upstream C/EBP site (at -219). We focused on understanding the role of the -219 C/EBP site in CRP gene expression, as well as investigating the mechanism of action of this site in inducing CRP gene expression all in the context of the 300 bp CRP promoter. We found that the -219 C/EBP site was not only crucial for the enhanced expression of the CRP gene seen with the 300 pb promoter, but also that it worked in synergy with the downstream C/EPB site at -52 to induce this CRP expression. No direct crosstalk was seen with the C/EPB site at -219 and NF-κB or STAT3 sites of the CRP promoter (Fig 5.1A) known to be involved in CRP gene expression. We therefore conclude that the C/EPB site at -219 is critical for the full activation of the CRP gene, working in synergy with the C/EBP site at -52 but independent (at least directly) of the NF-κB and STAT3 sites at -69 and -108 respectively known to promote CRP gene expression.

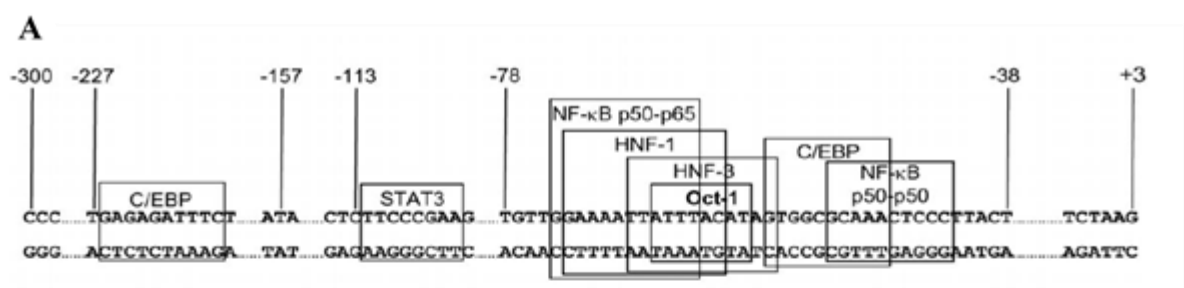

Figure 5. 1: CRP promoter and oligos used to study the role of C/EBPβ in CRP gene regulation. (A) The −300 to +3 region of the CRP gene is shown. The binding sites of various transcription factors on the promoter are boxed. (B) Sequences of the WT and mutagenic oligos derived from the CRP promoter and used as probes in EMSA. (C) Sequences of the mutagenic oligos used for mutagenesis of the CRP promoter and used as probes for Luc assays. Mutated bases are in red and binding sites underlined.

Methods

Electrophoretic mobility shift assays (EMSA)

Hep3B cells cultured in 100 mm dishes were subjected to overnight serum starvation and then treated with IL-6 for 18 h, as described previously (10). The confluency of cells was approximately 60% at the time of treatment. IL-6 (R&D) was used at a concentration of 10 ng/ml. Nuclear extracts were prepared by using NE-PER nuclear and cytoplasmic kit (Pierce), as described previously (21). The sequences of the oligonucleotides (Integrated DNA Technologies) used in EMSA are shown in Fig. 5.1B. Probes were prepared by annealing complementary oligos and labelling with [γ ^{32}P] ATP. The probe-nuclear extract reaction buffer contained 16 mM HEPES (pH 7.9), 40 mM KCl, 1 mM EDTA, 2.5 mM DTT, 0.15% Nonidet P-40, 8% Histopaque, and 1 μg of poly dI-dC. In super shift experiments, antibodies to C/EBPβ (C19, Santa Cruz Biotechnologies) used at 2 μg, were added to the reaction mixture and incubated on ice for 15 min before adding the probe. Then, 150 ng of unlabeled oligos was added to the reaction mixture before addition of the antibody and probe in oligo competition experiments. DNA-protein complexes were resolved in native 5% polyacrylamide gels containing 2.5% glycerol and visualized in a phosphorimager using Image-Quant software (GE Healthcare).

Construction of CRP promoter-luciferase (Luc) reporter vectors

Making the WT CRP promoter constructs, Luc 157 WT (−157/+3 of CRP gene) and Luc 300 WT (−300/−1 of CRP promoter), has been reported previously (9,10,29). These two WT

constructs were used as templates for mutagenesis of the CRP promoter by using the QuickChange site-directed mutagenesis kit (Stratagene). The mutagenic primers are shown in Fig. 5.1C. Double mutants of C/EBP (-52) and NF-κB (-69) or C/EBP (-52) and STAT3 (-108) where made using Luc 300/157 mut C/EBP (-52) as template while the double mutant of NF-κB (-69) and STAT3 (-108) was made using Luc 300/157 STAT3 (-108) as template. As for the triple mutant with mutations to C/EBP (-52), NF-κB (-69), and STAT3 (-108), the template used was Luc 300/157 mut C/EBP (-52) & NF-κB (-69). Mutations were verified by sequencing and plasmids were purified using maxiprep plasmid isolation kit (Eppendorf).

Luciferase transactivation assays (Luc assays)

Hep3B cells were cultured in 6-well plates. Transient transfections were done on cells at 60% confluency using FuGENE 6 reagent (Promega) according to manufacturer's instructions. The CRP promoter-Luc reporter constructs were used at 1 μg plasmid per well in 10 μl FuGENE 6 and 125 μl RPMI-1640, and the mixture incubated for 20 mins at RT before adding to wells. After transfection, cells were left in serum-free medium and 16 h later, the transfected cells were treated with IL-6 and/or IL-1β for 24 h or left untreated. IL-6 (R&D) was used at a concentration of 10 ng/ml and IL-1β was used at a concentration of 1 ng/ml. 40 h post transfection, Luc assays were performed following the protocol supplied by the manufacturer (Promega). Luc activity was measured in a luminometer (Molecular Devices), as described previously (21).

Results

The binding of C/EBPβ to the site centered at -219 on the CRP

To demonstrate that C/EBPβ binds to its cognate site centered at -219 upstream of the CRP promoter, we performed an EMSA using a 29 bp oligo (WT C/EBP upstream oligo; Fig.5.1B). Nuclear extract from Hep3B cells treated with IL-6 for 18 h was used as the source of C/EBPβ. A faint C/EBPβ complex was observed (Fig. 5.2; lane 1).

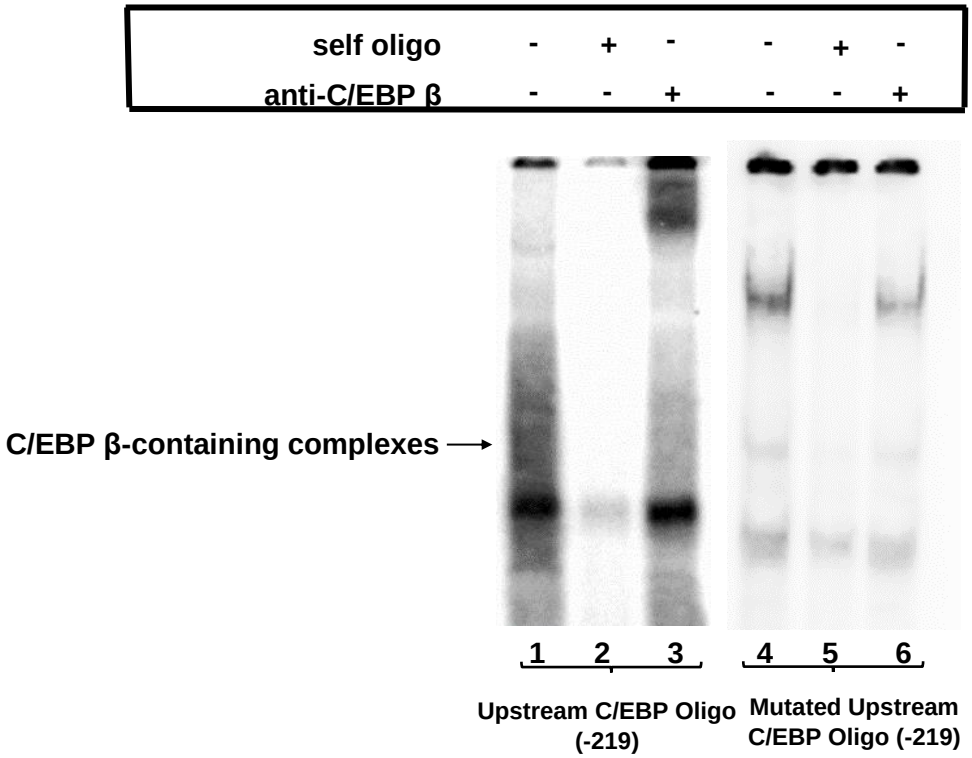

Figure 5. 2: Mutations to the upstream C/EBP site (-219) results in the abolition of C/EBPβ binding. A representative EMSA using the upstream WT and mutagenic C/EBP oligo as probe is shown. Nuclear extract from 18h, IL-6 treated Hep3B cells was used.

The faint complex contained C/EBPβ because it was super shifted when C/EBPβ specific antibodies were used (Fig. 5.2; lane 3). Mutating the C/EBP site on this oligo (m-C/EBP upstream oligo; Fig. 5.1B) resulted in the abolition of C/EBPβ binding to the probe (Fig. 5.2;

lanes 4 and 6). This confirmed that the mutations done to this site prevents the binding of C/EBPβ and therefore that repeating these mutations on probes to be used in the subsequent functional transactivation assays (Luc assays) would be sufficient to investigate how this site affects the induction of the CRP gene.

The C/EBP site at -219 is critical for the enhanced response with Luc 300

A better induction of the CRP gene has been reported in Hep3B cells when the proximal 300 bp CRP promoter was used compared to the proximal 157 bp promoter (9,27). Therefore, to investigate the contribution of the C/EBP site at -219 in this better induction of CRP gene expression, we mutated this upstream C/EBP site (m-C/EBP upstream; Fig. 5.1C) exactly as with the EMSA probe aforementioned. This probe together with the WT probe was then used to test CRP gene induction in a Luc assay (Fig. 5.3). For maximum induction of the gene, Hep3B cells were treated with a combination of IL-6 and IL-1β. We found that mutating the upstream C/EBP site resulted in a drastic loss of CRP gene expression (approximately 67% drop). However, the Luc 300 mC/EBP reporter construct was still 2 times better at inducing CRP gene expression compared to the Luc 157 WT reporter construct, suggesting that there may be another site contributing to CRP gene expression upstream of the proximal 157 CRP promoter. We conclude from these results that the upstream C/EBP site is very critical for the full activation of the CRP gene.

The two C/EBP sites at -219 and -52 work in synergy to activate CRP gene expression

To investigate the mechanism of the upstream C/EBPβ-mediated activation of CRP expression, we used mutated C/EBP promoter constructs with mutations to the upstream and/or downstream C/EBP sites.

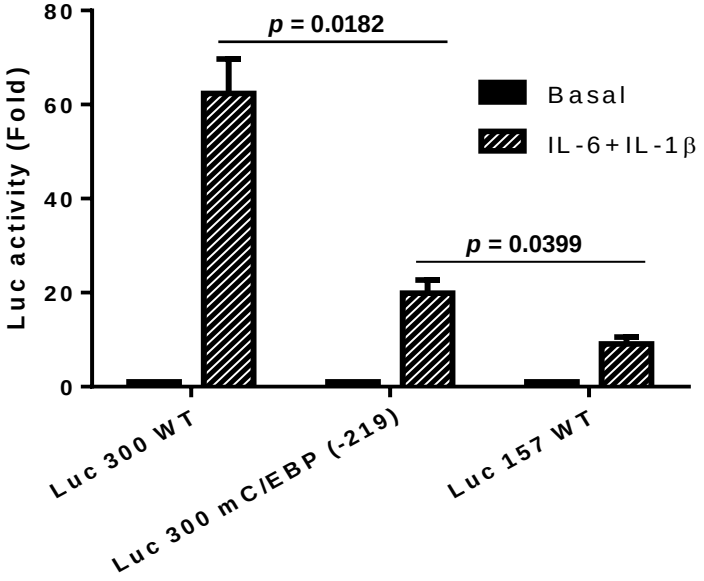

Figure 5. 3: The C/EBP site at -219 is critical for the enhanced response with Luc 300. Hep3B cells were transfected with Luc 300 WT, Luc 300 mC/EBP, and Luc 157 WT CRP promoter constructs. After 40 h, CRP transcription was measured as Luc activity and plotted on the y-axis. Average ± S.E.M. of three experiments are shown. Unpaired two-tailed Students t-test was used to calculate p values.

Unlike the upstream C/EBP site mutations which resulted in about 67% drop in CRP gene activation, results were even more drastic when the downstream C/EBP site (-52) was mutated (about 83% drop) (Fig. 5.4). Suggesting that the downstream C/EBP site is more critical for the activation of CRP gene expression than the upstream -219 C/EBP site. Conversely, the results also indicate that these two sites work in synergy to activate CRP gene expression as the additive results of single C/EBP site mutations was lower than the combined effect when both sites were

present. Mutating both C/EBP sites resulted in a complete loss of CRP gene expression (Fig. 5.4) indicating unequivocally that C/EBPβ is the principal activator of the CRP gene.

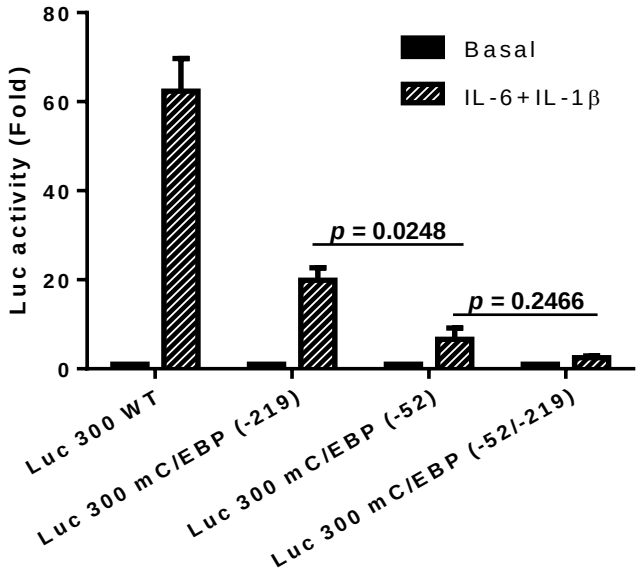

Figure 5. 4: The two C/EBP sites of CRP promoter work in synergy to induce CRP expression. Hep3B cells were transfected with Luc 300 WT, Luc 300 mC/EBP (-219), Luc 300 mC/EBP (-52), and Luc 300 double mC/EBP (-52 and -219) CRP promoter constructs. After 40 h, CRP transcription was measured as Luc activity and plotted on the y-axis. Average ± S.E.M. of three experiments are shown. Unpaired two-tailed Students t-test was used to calculate p values.

<u>CRP gene expression due to the C/EBP site at -219 is not affected by other sites known to induce CRP gene</u>

CRP gene expression with the Luc 300 promoter construct is always higher than with the Luc 157 promoter construct. This, as shown earlier, is due to the synergy between the two C/EBP sites at -52 and -219 (Fig. 5.4). Likewise, CRP gene expression was always better with mutant Luc 300 promoter constructs compared to matching mutations on Luc 157 promoter constructs (Fig. 5.5A and B). Mutations were introduced to sites of the CRP promoter known to directly participate in CRP gene expression. These included double and triple mutations of

C/EBP (-52), NF-κB (-69), and STAT3 (-108) sites, referred to with single letters C, N, and S respectively (Fig. 5.5A and B). Interestingly, the trend in CRP gene expression was very similar with both Luc 300 and Luc 157 promoter constructs (Fig. 5.5A and B). However, when the NF-κB (-69) and STAT3 (-108) sites where mutated (mNS), a peak was observed with Luc 300 (Fig. 5.5A) compared to a drop with Luc 157 (Fig. 5.5B). This could be due to the synergistic effect of the two C/EBP sites present on the Luc 300 promoter constructs. Given that a similar trend or drop in CRP gene activation relative to respective WT Luc promoter constructs was seen with or without contributions from the upstream C/EBP site (-219), suggest that the upstream C/EBP site works independent of the NF-κB (-69), and STAT3 (-108) sites of the CRP promoter. *P* values of 0.0317 and 0.0635 where obtained when comparing the trend of both graphs with and without Luc 300 mNS respectively.

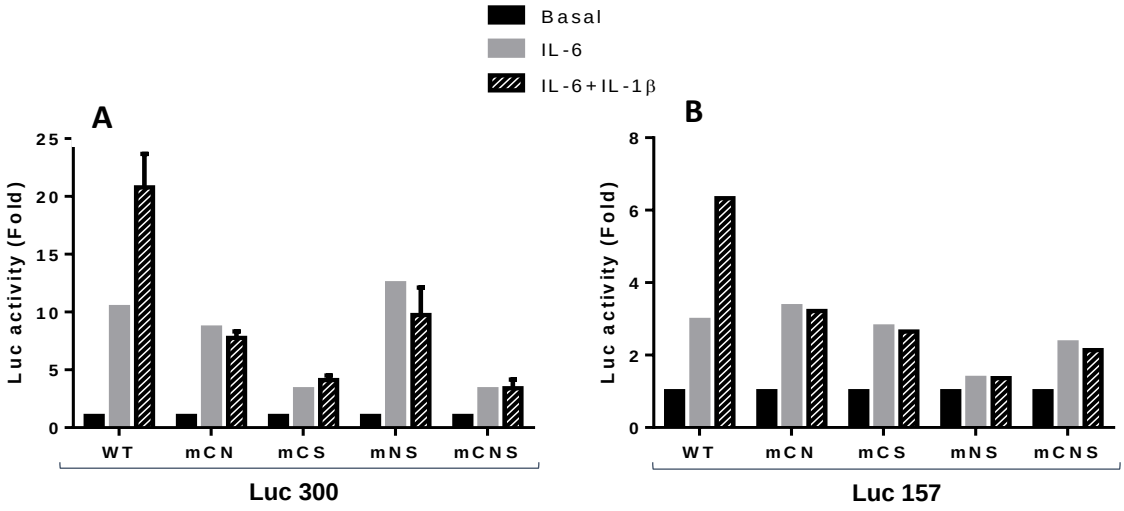

Figure 5. 5: C/EBP site at -219 works independent of NF-κB (-69), and STAT3 (-108) sites of the CRP promoter. Hep3B cells were transfected with (A) Luc 300 WT and mutant CRP promoter constructs, and (B) Luc 157 WT and mutant CRP promoter constructs. After 40 h, CRP transcription was measured as Luc activity and plotted on the y-axis. Unpaired two-tailed Students t-test was used to calculate p values.

Discussion

CRP is a very important molecule of the human immune system. Its expression is tightly regulated by a combination of both constitutively and cytokine induced transcription factors. In this study, we investigate one of these transcription factors, C/EBPβ, which is believed to play a central role along with STAT3 in CRP gene expression. We focused on the C/EBP site centered at position -219 of CRP promoter region -300/+3. We found that 1) The C/EBP site at -219 was critical for the full activation of CRP gene expression. 2) The two C/EBP sites, one centered at -219 and the other at -52 of the CRP promoter work in synergy to activate the CRP gene 3) C/EBPβ bound to its site at -219 work independent of NF-κB, and STAT3 bound to their respective sites at -69 and -108 of the CRP promoter. Taken together, our data show that for the full induction of CRP gene expression, both C/EBP sites have to participate.

The C/EBP site centered at -219 was initially identified by Li and Goldman in 1996 (23) and said to participate in CRP gene expression. Consistent with their findings, we observed that CRP gene expression was increased when the proximal 300 bp CRP promoter containing this upstream C/EBP site was used compared to using the proximal 157 bp promoter which lacked this site (9,27). The reason for the increase expression was not known but thought to be in part due to the upstream C/EBP site at position -219. However, given the nature of the CRP gene promoter with several crowded and overlapping binding sites within the proximal 157 bp (Fig. 5.1A) and how far upstream the second C/EBP site is (at -219), it is posible that other sites yet to be identified between this crowded region and the upstream C/EBP site could be an additional reason for the better expression seen with the 300 bp promoter. We decided to abolish by mutagenesis the influence of the -219 C/EBP site in the induction of CRP gene to determine how much this site actually contributes to CRP gene expression. Using transfected Hep3B cells in

transactivation (Luc) assays with CRP promoter constructs, we noticed a 67% drop in CRP expression when the upstream C/EBP site was mutated. However, given that the drop in expression was still better than the overall expression seen with Luc 157 WT promoter construct, suggest that there may be other site(s) upstream of the proximal 157 bp promoter that participate in CRP gene expression. IL-6 is known to induce CRP gene expression modestly, but in combination with IL-1β synergistically induce CRP gene expression (16). IL-6 activates both C/EBPβ and STAT3 and therefore we propose that other contributing site may be a STAT3 site as no other C/EBP site but for the one at -219 is found upstream of the proximmal 157 bp CRP promoter. When the downstream C/EBP site at -52 was mutated, the loss in CRP gene expression was greater than seen with the abolition of upstream C/EBPβ binding (83% drop). This suggests that the downstream C/EBP site is of greater importance for CRP gene expression. However, it is not known whether this loss in CRP gene expression was due solely to the loss of C/EBPβ binding to this downstream site or because mutations to this site also affected nearby binding of other transcription factors important for CRP expression, given the crowded nature of the region. The double mutation of both C/EBP sites resulted in a complete loss of CRP gene expression and this highlights the predominance of C/EBPβ in CRP gene expression. Mutating other sites like the NF-κB and STAT3 sites known to induce CRP gene expression resulted in the loss of CRP gene expression but did not seem to be influenced by the presence or absence of the upstream C/EBP site and therefore it seems that this upstream C/EBP site works independent of the NF-κB and STAT3 sites. In conclusion, we found that the full activation of the CRP gene was mainly due to the synergy between the two C/EBP sites at -52 and -219 of the CRP promoter.

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CHAPTER 6: SUMMARY

This work was conducted under the following specific aims:

1. To determine the role of complement in CRP mediated protection against pneumococcal infection in mice.
    a. Identify a CRP mutant that does not activate the mouse complement system.
    b. Investigate the protective effects of this CRP mutant in a mouse model of pneumococcal infection.
2. To determine the efficacy of a CRP mutant capable of binding to factor H in protection against pneumococcal infection.
    a. Investigate the efficacy in a late-stage infection model in which native CRP has been shown to be ineffective.
    b. Investigate the protective effects of the CRP mutant when combined with an antibiotic in both early-stage and late-stage infection models.
3. To investigate the mechanism of binding of CRP to aggregated and immobilized proteins including factor H.
    a. To define the ligand-binding site on CRP when CRP is in its alternate pentameric structural conformation.
    b. To evaluate the significance of the interaction of modified CRP with aggregated and immobilized protein ligands.
4. To define the role of the transcription factor C/EBPβ in IL-6-induced CRP expression in hepatic cells.

a. Investigate the role of the C/EBP-binding sites located at positions -52 and -219 on the CRP promoter.
b. Investigate the interactions of C/EBPβ with other transcription factors bound to the nearby and overlapping sites on the promoter.

Our major findings from investigating the importance of complement activation in CRP-mediated protection against pneumococcal infection were:

1. Mutating His^{38} → Arg resulted in a CRP mutant that neither activates human nor mouse complement as determined by measuring C3 deposition. Meanwhile, His^{38} → Ala mutation did not produce a mutant with the same deficiency in complement activation as H38R CRP.
2. H38R mutant CRP did not protect mice from pneumococcal infection as seen with H38A and WT CRP, implying the activation of complement is critical for CRP-mediated protection against pneumococcal infection in mice.

AIM 1 Limitations

A drawback in this study is that we do not know for certain what specific pathway of complement is involved in CRP-mediated protection against pneumococcal infection in mice. It was shown earlier that human CRP does not bind mouse C1q, meaning that the classical pathway of complement is probably not used in CRP mediated protection of mice. The alternative pathway is always on and can be activated by bacteria alone. Therefore, the lectin pathway remains the primary suspected pathway to be involved. H38R does not activate complement and therefore cannot be used to investigate what pathway of complement is activated in CRP-

mediated protection. However, mice deficient in specific complement components corresponding with specific complement pathways should eventually answer this question.

The main findings from our work to understand the anti-pneumococcal properties of CRP in late stage infection models were:

1. E-CRPs bind immobilized factor H at physiological pH. Binding to immobilized factor H requires CRP to be in its modified conformation.
2. Both E-CRPs are not sequestered by serum components and their rate of clearance was not faster than seen with WT CRP. Therefore, E-CRPs were readily available in mice circulation during infection experiments.
3. Pneumococci recruit factor H to their surface in the later stages of infection, making them complement resistant. *In vivo* isolated bacteria tested positive for factor H whereas broth grown bacteria did not.
4. E-CRP-1 with its PCh binding site mutated was very effectively inhibited from binding to PnC by either PCh or dAMP as might be expected. WT CRP required approximately 10 times higher concentration of inhibitors to achieve the same level of inhibition. In contrast, E-CRP-1 was found to bind with greater affinity to broth grown bacteria requiring levels of inhibitor greater than required for WT CRP for similar inhibition. This suggests, that E-CRP does not interact with the PCh on bacteria. This was confirmed when E-CRP-1 bound broth grown bacteria in the absence of calcium (a requirement for PCh binding by CRP).
5. E-CRPs protect against pneumococcal infections in both early and late stages of infection. Multiple injections of E-CRP given in the late stage were no more protective than a single injection. However, the level of protection provided by early administration of E-CRP was

higher than when E-CRP was administered in the late stages as determined by both survival and bacteremia data.

6. Both E-CRPs work synergistically with the antibiotic clarithromycin to protect mice against late stage pneumococcal infection.

AIM 2 Limitations

The main drawback in this study was our inability to show E-CRP on the surface of *in vivo* isolated bacteria (Pn-mice). This was because of the cross reactivity of anti-human/mouse CRP antibodies for both human and mouse CRPs. So, all *in vivo* isolated bacteria, even those from infected control mice (no exogenous CRP injections), tested positive for human CRP. Nonetheless, in the *in vitro* assays, we were able to show that E-CRP binds to both Pn-mice and Pn-broth allowing us to proceed with our experiments. Our future experiments will include the use of CRP knockout mice, to completely nullify any contributions made by mouse CRP in the protection against pneumococcal infection and making it possible to study exclusively the anti-pneumococcal properties of exogenously administered CRP.

The major findings from our work to investigate the multiple ligand binding properties of CRP were:

1. CRP can assume three different structural conformations and all three forms; native pentameric, non-native pentameric, and monomeric CRP display different ligand recognition functions *in vitro.*

2. Immobilizing proteins may cause them to expose amyloid-like structures recognized by non-native (modified) pentameric CRP.
3. E-CRP-2, prevents the formation of amyloid fibrils suggesting that CRP is an anti-amyloidogenic protein.

AIM 3 Limitations

The main difficulty in this study was identifying the Aβ binding site on CRP. The most promising putative site was the cholesterol binding site. However, mutant CRPs generated with mutations to this site or other putative sites did not abrogate the multiple ligand binding ability of modified CRP. Some mutants did show reduced binding, but we were not able to completely block the multiple ligand binding ability of modified CRP. Even if we were able to prevent the binding of modified CRP to immobilized ligands by mutagenesis, it still might not mean that the mutations were at the Aβ binding site, since mutations at other regions can cause modifications to this binding site. We could also have a situation where the binding site is formed by different amino acids in the different CRP subunits. In which case, finding the binding site by mutagenesis will be extremely difficult with current technology.

Our major findings investigating the transcriptional regulation of the CRP gene were;

1. The C/EBP site at -222 is critical for the full activation (induction) of the CRP gene expression as determined by luciferase activity.
2. The upstream C/EBP site at -222 does not work with other known sites of the CRP promoter except for the C/EBP site at -53, where they work in synergy to induce CRP gene expression.

AIM 4 Limitations

The major drawback of this study is that only a fragment of the CRP promoter is used to investigate its gene regulation. This approach cannot give a complete picture of the regulation of CRP gene expression. Therefore, other techniques such as chromatin immunoprecipitation (ChIP) are needed for a more accurate understanding of how the CRP gene is regulated. One other drawback is the use in this study of only the Hep3B cell line. The results should be confirmed using other cell lines such as the HepG2 cell line as well as primary human hepatocytes.

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1. **Ngwa D. N.**, Agrawal A. (2019). Structure-Function Relationships of C-Reactive Protein in Bacterial Infection. *Front Immunol*. 10:166.

2. Singh, S. K., Thirumalai A., Pathak A., **Ngwa D. N.,** and Agrawal A. (2017). Functional transformation of C-reactive protein by hydrogen peroxide. *J. Biol. Chem.* 292: 3129-3136.

3. Thirumalai, A., Singh S. K., Hammond Jr. D. J., Gang T. B., **Ngwa D. N.**, Pathak A., and Agrawal A. (2017). Purification of recombinant C-reactive protein mutants. *J. Immunol. Methods* 443: 26-32.

Awards:

The American Association of Immunologist (AAI) Trainee Abstract Award, 2016

Professional Affiliations:

The American Association of Immunologist (AAI), 2016 - present

The Biomedical Science Graduate Student Association, 2016 - present

Shades of Africa-ETSU, 2014 - present