COMPLEMENT EVASION BY S. AUREUS SURFACE PROTEINS

A Dissertation

by

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ABSTRACT

The complement system, a major component of the innate immunity, is critical for combating microbial infections. It can be activated by three distinct pathways: the classical pathway, the lectin pathway and the alternative pathway. Activation of the complement system results in opsonization of pathogens, recruitment of phagocytes, and lysis of bacterial cells. However, successful pathogenic bacteria have evolved strategies to manipulate the complement system, aiming to tip the balance in favor of the bacteria. *Staphylococcus aureus* is an important human pathogen causing more deaths than that of HIV in the United States. It can cause diseases ranging from minor skin infections to life threatening diseases. While most complement evasion molecules from *S. aureus* are secreted proteins and are well characterized, little is known about the effect of surface-anchored *S. aureus* proteins on complement activity. This study aims to bridge this gap and investigate the role of *S.aureus* surface proteins in complement evasion.

S. aureus expresses different kinds of MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) that interact with host extracellular matrix (ECM) proteins. My studies mainly focus on collagen-binding MSCRAMM Cna and fibrinogen-binding MSCRAMM Bbp/SdrE using the hemolysis and the C4b deposition ELISA-type assay to analyze complement activation and a serial biochemical and immunological approaches such as ELISA, biacore and IP assays to investigate the binding and inhibition mechanisms.

I found that Cna specifically interacts with the collagen-like domain of C1q. This binding disturbs C1 complex assembly and causes decrease in C1 binding to the immune complex. Moreover, animal studies revealed that C1q knockout mice are resistant to Cna-expressing *S.aureus* infections and have much less bacterial burdens in liver, compared to that of wild type mice, suggesting that this interaction enhances *S.aureus* infection and dissemination. Furthermore, my study also revealed that Bbp/SdrE blocks all three complement pathways and the binding of Bbp to C3c fragment of C3b blocks the formation of the AP C3 convertase by interfering with the cleavage of factor B.

In summary, I reported that several MSCRAMMs from *S.aureus* not only bind to ECM proteins, but are also complement inhibitors that would benefit *S.aureus* infections.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW*

Recognition of bacterial infection by innate immunity

The immune response consists of two aspects: innate immunity and adaptive immunity. The innate immunity is the first line of defense, which is critical for sensing and killing pathogenic bacteria at early stage (Mogensen, 2009). It contains soluble molecules, as well as various immune cells, such as macrophages, neutrophils, natural killer cells, dendritic cells, all of which express a group of surface receptors or cytosolic receptors known as germline-encoded pattern recognition receptors (PPRs). PPRs interact with pathogen-associated molecular patterns (PAMPs), resulting in the release of proinflammatory and anti-pathogen cytokines via the activation of NF-κB, IFN-I or other signaling pathways (Beutler & Rietschel, 2003). The innate immune soluble molecules include antimicrobial peptides, which have direct antimicrobial activity or immunemodulatory functions (Ganz, 2003), and heat-sensitive complement components in blood, which identify invading microbes and kill them (Gasque, 2004).

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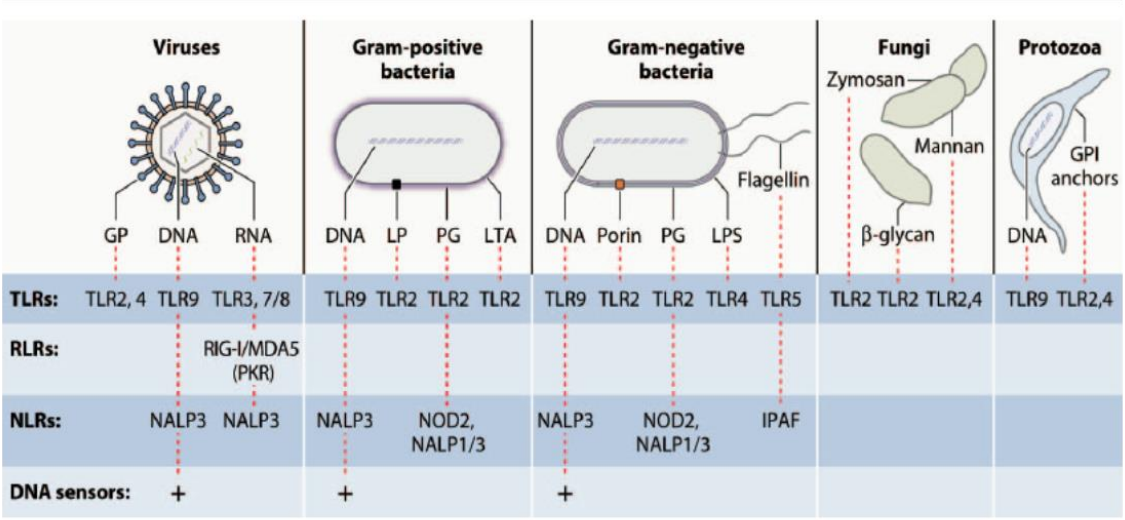

Figure 1. Recognition of PAMPs from pathogens by PRRs. (Reprinted with permission from Mogensen, 2009)

Pattern recognition receptors (PPRs)

PPRs consist of several different families such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs) and DNA-sensing molecules (Figure 1). However only the TLRs and NLRs play important roles in the recognition of bacterial infections (Kumar et al, 2013; Mogensen, 2009).

Compared to other PPR families, TLR family has been characterized in more detail. TLRs are expressed in different immune cells, as well as non-immune cells. Each member contains an extracellular domain containing leucine-rich repeats (LRRs) and a cytosolic Toll/interleukin-1 receptor homology (TIR) domain (Akira & Takeda, 2004). The binding of the ligand to LRR domains induces the dimerization of TLRs in the form of either homodimers or heterodimers, which triggers intracellular signal cascades. So far, 13 different mammalian TLRs with corresponding ligands have been identified (Mogensen, 2009). For instance, multiple ligands of TLR2, such as bacterial peptidoglycans or triacyl lipopeptides, bind the TLR2/TLR1 heterodimer (Liang et al, 2009), while lipoteichoic acid binds the TLR2/TLR6 heterodimer (Irvine et al, 2013). TLR4, the first TLR identified in human, senses LPS from Gram-negative bacteria (Poltorak et al, 1998). TLR 5 and TLR9 recognize Flagellin and CpG DNA, respectively (Bauer et al, 2001; Hayashi et al, 2001; Wolf et al, 2011). Overall, TLR signaling is pivotal in the defense against microbial infections, as TLR knockout mice are significantly more susceptible to microbial challenges, compared to wild type mice

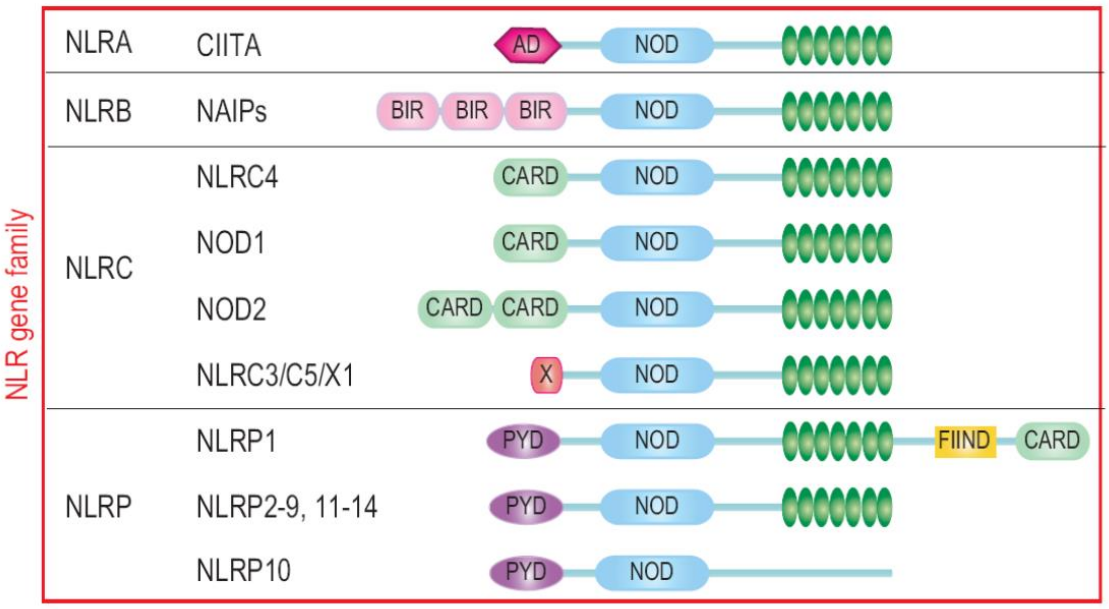

Figure 2. Domain structures of human NLRs. (Reprinted with permission from Zhong et al, 2013)

(Koedel et al, 2003; Lammers et al, 2012; Seibert et al, 2010; Shen et al, 2010; Sing et al, 2003; Takeuchi et al, 2000; Wieland et al, 2011; Wiersinga et al, 2007).

NLRs are cytosolic sensors, which can recognize intracellular bacteria (Kumar et al, 2013; Mogensen, 2009). Most members contain an N-terminal CARD domain which initiates immune signal cascades, a central nucleotide binding domain (NOD) and a C-terminal leucine-rich region (LRR) which mediates ligand binding (Figure 2). Among this family, NOD1 and NOD2 play a role in bacterial infections (Carneiro et al, 2004). NOD1 can be activated by the cell wall component - diaminopimelic acid (DAP) from bacteria (Cardenas et al, 2011; Girardin et al, 2003a). Furthermore, NOD1 deficient mice are more susceptible to bacterial infections (Clarke et al, 2010; Viala et al, 2004). NOD2 senses muramyl dipeptide (Girardin et al, 2003b) and NOD2 deficiency was reported to increase the susceptibility to bacterial infections (Kim et al, 2008; Kobayashi et al, 2005)

Antimicrobial peptides

Antimicrobial peptides (AMPs), also called host defense peptides (HDPs), generally contain 12 to 50 amino acids with a large proportion of positive charges and hydrophobic residues (Hancock & Sahl, 2006). These molecules have different secondary structures (Fjell et al, 2012) and can be abundantly secreted from barrier epithelia, phagocytic cells, fat body and some organs such as liver during infections (Ganz, 2003; Hancock & Sahl, 2006).

The mechanisms of anti-bacterial activity by AMPs vary by bacterial species and AMP. In most cases, the molecular killing mechanism is not well characterized (Nizet,

2006), but it is believed that the major target is the bacterial cytoplasmic membrane. Most of bacterial surface membranes are hydrophobic and carry net negative charges, which might interact with positively charged AMPs. Consequently, bacteria might be killed via three major possible mechanisms: 1. High concentration of AMPs on the bacterial surface may interrupt the equilibrium of ion concentration via a disrupted membrane; 2. AMPs bind to bacterial surfaces and form pores in the membrane, leading to the lysis of the cell; 3. AMPs enter bacterial cell through a disrupted membrane and interact with unknown ligands that are associated with DNA, RNA and protein synthesis or other critical metabolism pathways within the cell (Hancock & Sahl, 2006).

Besides direct bacterial killing, most of AMPs have immunomodulatory properties (Hilchie et al, 2013). It is reported that some AMPs, which lack antibacterial activities in vitro, protect against Gram-positive bacteria or Gram-negative bacteria infection in vivo (Hancock & Sahl, 2006). For example, innate defense–regulator peptide (IDR-1), a well-studied peptide, is known to regulate immune system during bacterial infections (Hilchie et al, 2013; Nijnik & Hancock, 2009). It doesn't have direct antimicrobial activity, but through increasing expression of chemokines (Hilchie et al, 2013), it prevents infections from a variety of bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) (Hou et al, 2013), vancomycin-resistant *Enterococcus* (Scott et al, 2007) and *Salmonella enterica serovar Typhimurium* (Scott et al, 2007).

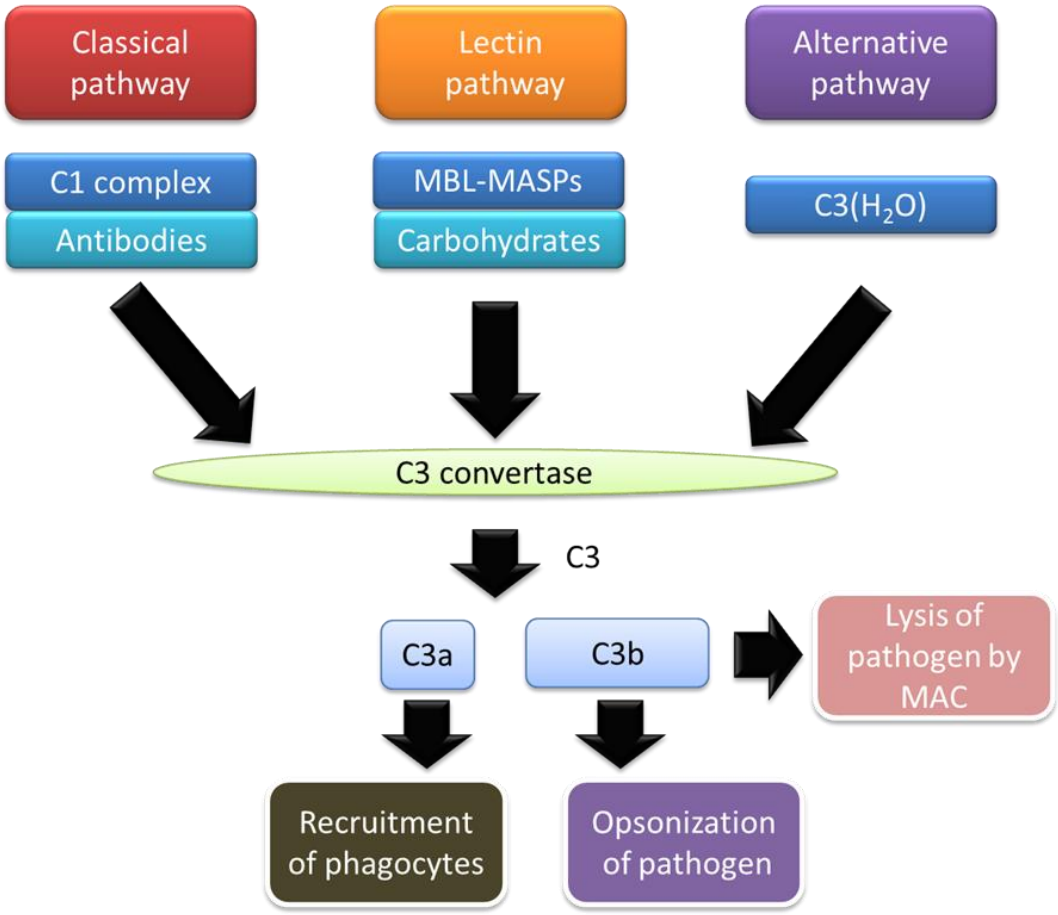

Figure 3. Complement pathway activations and their basic functions.

Complement system

The complement system consists of over thirty different types of heat-sensitive glycoproteins and proteins, most of which are synthesized by the liver and circulating in the blood as protein precursors (Gasque, 2004). When a host is infected by microbes, complement pathways are activated, resulting in opsonization of bacteria by C3b, which can be recognized by C3b receptors presented on macrophages and neutrophils, recruitment of phagocytes by anaphylatoxins C3a and C5a, as well as the lysis of the bacteria mediated by membrane attack complex (MAC) formation.

The complement system was discovered earlier than PPRs and AMPs. In 1896, Jules Bordet found some heat-sensitive components in the serum that had non-specific antimicrobial activity (Laurell, 1990). These components were likely to be a part of the complement system, a term introduced by Paul Ehrlich in the late 1890s. Nowadays, the complement system is known to act through three distinct pathways: the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP), which have different activation mechanisms but will converge to a C3 convertase, followed by the cleavage of the central complement component C3 (Figure 3).

Complement activations

The CP is initiated by the C1 complex, which consists of C1q, C1r and C1s, binding to immobilized antibodies on the surface of bacteria. These bindings will activate the protease C1r, and subsequently cleaves the C1s precursors into active C1s that cleaves C4 and C2 into C4b/C4a and C2b/C2a, respectively. C4b attaches to the cell

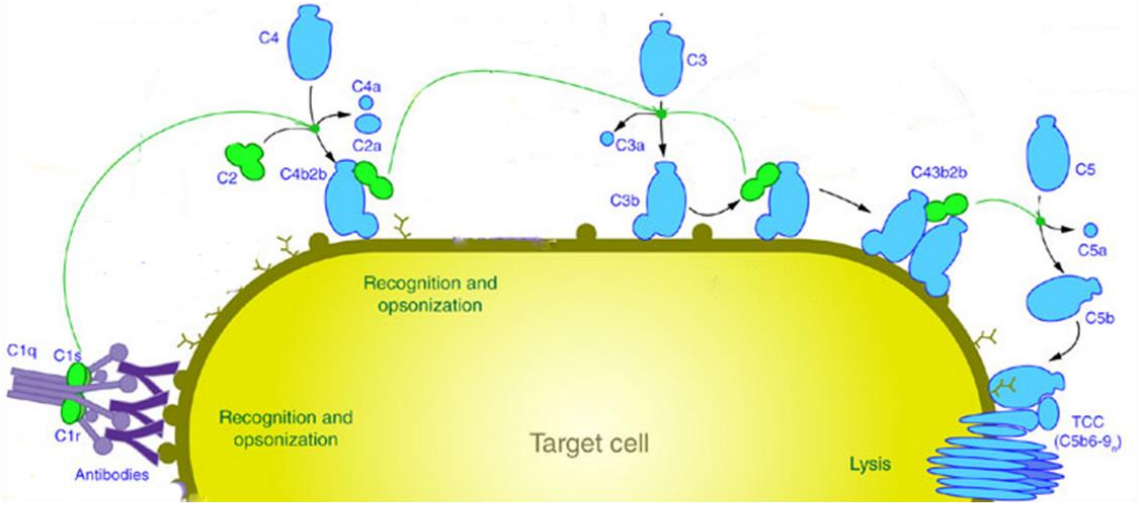

Figure 4. The classical complement pathway. (Reprinted with permission and modified from Ricklin et al, 2010)

surface via a covalent thioester bond and binds to C2a to form C3 convertase, which cleaves C3 into C3a and C3b. Some of the C3b molecules attach to bacterial surfaces, which can be recognized by C3b receptors on macrophages or neutrophils to enhance phagocytosis (Figure 3). Other C3b molecules interact with C3 convertase to form the C5 convertase that binds C5 and cleaves it into C5b and C5a. C5a and C3a are anaphylatoxins that act as chemokines to attract immune cells such as macrophages and neutrophils to the infection sites. On the other hand, C5b attaches to the cell surface and interacts with C6, C7, C8 and C9 to form the membrane attack complex (MAC), leading to the lysis of the cells (Figure 4).

The activation of LP is similar to CP activation (Figure 5), both of which utilize C4b and C2a to form a C3 convertase. However, instead of activation through the binding of C1 complex to an immune complex, LP is activated using mannose-binding lectin (MBL) or ficolin associated with proteases MASP-1 and-2, (MBL-associated serine protease-1 and 2) respectively to form a complex, which interacts with carbohydrates, such as *S. aureus* wall teichoic acid, on the bacterial surface (Kurokawa et al, 2013). This binding will activate MASP-1 and MASP-2, which cleave C4 and C2 to form C3 convertase. Compared to the other two complement pathways, LP is not well characterized and more complicated, based on the controversial structures or functions of MASP and MBL-associated proteins. Previously, scientists demonstrated that MASP-1 or MASP-2 only form a homodimer (Chen & Wallis, 2001). However, Péter G et al found evidence that the MASP-1 and MASP-2 form a heterodimer, which is similar to a C1r and C1s heterodimer in the CP (Parej et al, 2014). Another controversial issue is the

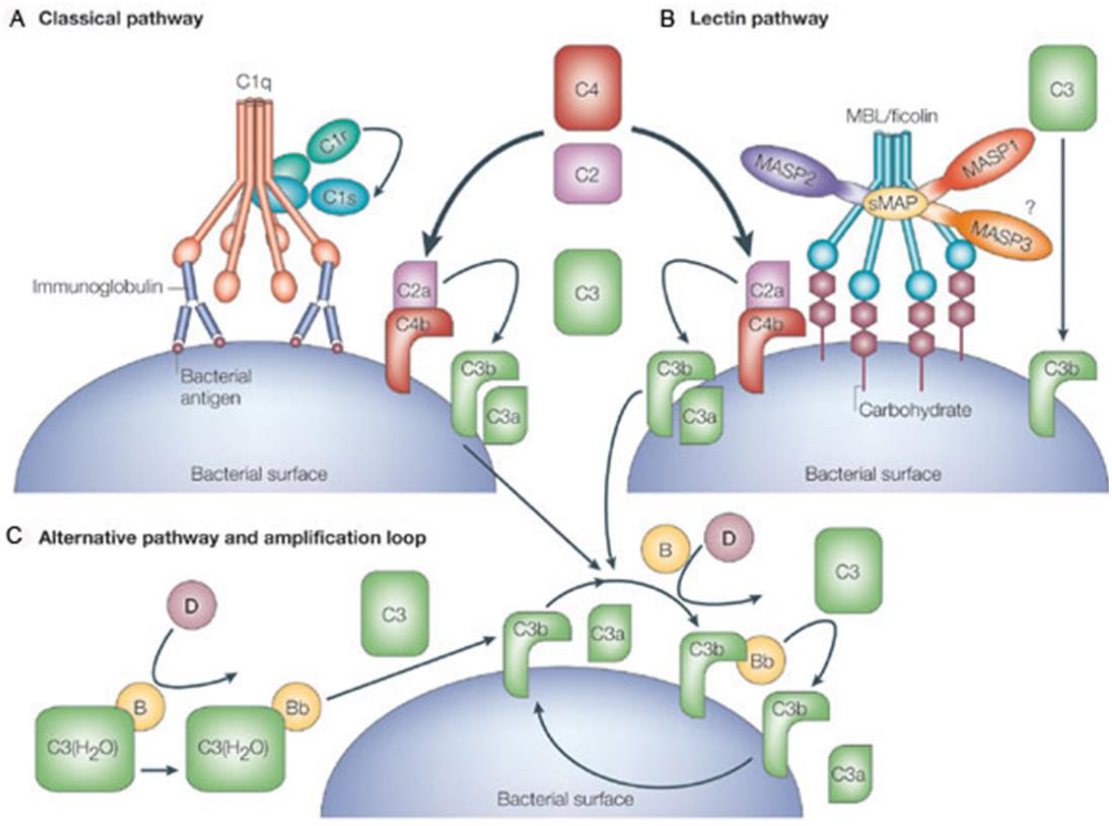

Figure 5. Complement activations. (A) The classical pathway activation. (B)The lectin pathway activation. (C) The alternative pathway activation. (Reprinted with permission from Foster, 2005)

function of MASP-3. Some reports demonstrated that active recombinant MASP-3 cleaved and activated C3 convertase ($C3H_2O$-factor B) and factor D, in order to initiate alternative pathway activation (Iwaki et al, 2011). Meanwhile, functional studies using patient samples with nonsense mutations in MASP or deficiency in both MASP-1 and MASP-3 showed that neither MASP-1 nor MASP-3 affects the alternative pathway (Degn et al, 2012). More interestingly, some reports indicated that MBL-associated proteins act as complement regulators, but the biological significance of this effect hasn't been well determinated (Degn et al, 2013).

AP is distinct from CP and LP (Figure 5). Activation can occur spontaneously through the binding of hydrolyzed C3 ($C3H_2O$) to factor B, which is then cleaved by factor D to form an active C3 convertase (C3bBb). This new complex cleaves C3 into C3a and C3b, which binds to the microbial surface. Opsonized C3b can bind to factor B, followed by cleavage by factor D to form C3bBb. Another C3b fragment binding to the C3 convertase forms C5 convertase (C3bBbC3b), resulting in the cleavage of C5 and the initiation of MAC formation.

Complement regulation

The complement system is a very powerful component of the innate immune defense. Overactivation of the complex system leads to excessive inflammation and damages host tissues and may play a role in many diseases such as Alzheimer's syndrome, schizophrenia, atypical hemolytic-uremic syndrome, angioedema, macular degeneration, and Crohn's disease (Tichaczek-Goska, 2012), Thus proper control of this

system is critical for human health. In order to prevent over-activation of the complement system, human blood contains a high level of complement regulatory proteins such as C1 inhibitor (C1INH), C4-binding protein (C4BP), factor H (fH) and factor I (fI). Some regulatory proteins are present on the membranes of host cells, such as complement receptor 1 (CR1), decay-accelerating factor (DAF) and CD59, to suppress complement activation on host cells. These molecules control and regulate different stages of complement activation using distinct inhibition mechanisms. A general description of the roles of these molecules in the regulation of the complement activation is presented in Table. 1.

C1q and the C1 complex

C1q has a molecular weight of around 460,000 Da (Hu et al, 2010). Unlike most complement proteins, C1q is synthesized by macrophages and dendritic cells, but not hepatocytes (Lu et al, 2008). C1q has six "arms", each of which is assembled from three different poly-peptide chains: A chain (26,000 Da), B chain (25,000 Da) and C chain (24,000 Da). Each arm has a C- terminal globular "head" domain, which interacts with the Fc domain of antibodies to initiate CP activation, followed by a collagen-like triple helix domain, which contains a repeating Gly-Xaa-Yaa sequence (Gaboriaud et al, 2004). These six "arm" collagen-like triple helixes are held together to form an N-terminal "stalk" (Figure 6). Under the electron microscope, C1q exhibits a "bouquet" like structure with an average angle of each arm at about 41 º±19 º, which indicates that C1q is a flexible molecule (Knobel et al, 1975).

Table 1. Complement regulators and their functions

Regulators	Alternative name	Pathways	Ligands	Functions	Refs
C1INH	N/A	CP, LP	C1r,C1s MASP-1, MASP-2	Binds to C1r/C1s and MASP-1/-2; Prevents activation of C1 complex	(Davis et al, 2008)
C4BP	N/A	CP, LP	C4	Binds to C4b and displace C2a; Co-factor for C4b cleavage by fI	(Blom et al, 2004)
FactorH	N/A	AP	C3b and C3d	Binds C3b and displaces Bb; Co-factor for C3b cleavage by fI	(Rodriguez de Cordoba et al, 2004)
Factor I	N/A	CP, LP, AP	C3b and C4b	Cleaves C3b and C4b; Aided by fH,MCP,C4BP or CR1	(Nilsson et al, 2011)
FHL1	N/A	AP	C3b	Co-factor for C3 cleavage by fI	(Zipfel & Skerka, 1999)
Properdin	Factor P	AP	C3	Stabilizes AP convertase	(Hourcade, 2006)
S-protein	Vitronectin	CP, LP, AP	MAC	Prevents MAC insert into cell membrane	(Milis et al, 1993)
Clusterin	SP-40	CP, LP, AP	MAC	Prevents MAC insert into cell membrane	(Tschopp et al, 1993)
CR1	CD35	CP, LP, AP	C3	Binds C4b and C3b; Displace C2a and Bb; Co-factor for C3b and C4b cleavage by fI	(Fearon, 1985)
MCP	CD46	CP, LP, AP	C3	Promotes C3b and C4b inactivation by fI	(Hakulinen et al, 2004)
DAF	CD55	CP, LP, AP	C3	Binds and dissociates C3 convertase (C4bC2a and C3bBb)	(Harris et al, 2007)
CD59	Protectin	CP, LP, AP	MAC	Inhibits MAC formation	(Kimberley et al, 2007)

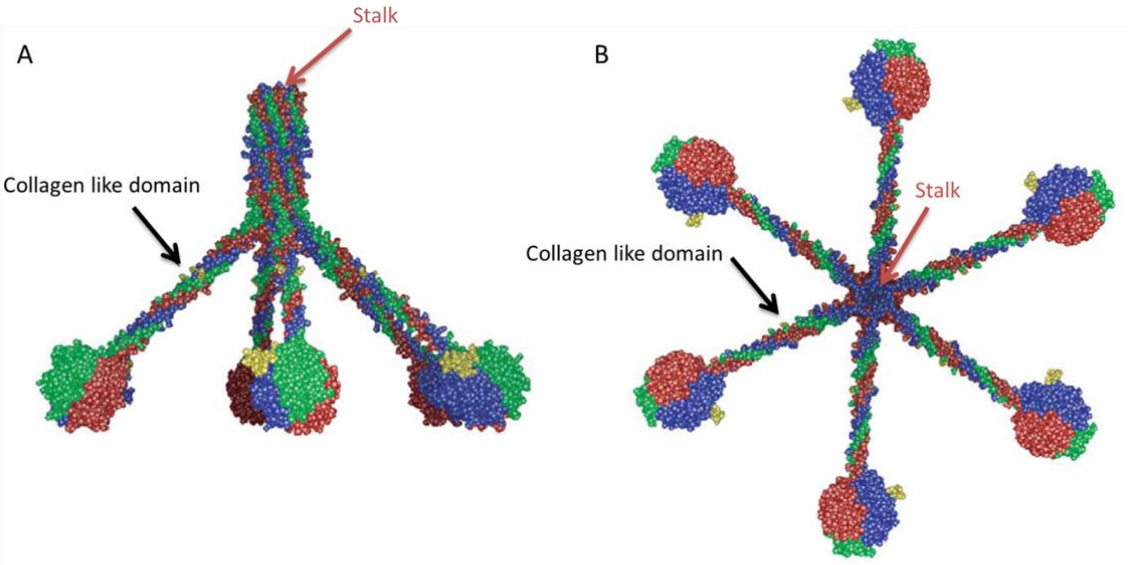

Figure 6. Structure models of C1q. (A) Side view and (B) bottom view of the C1q molecule. (Reprinted with permission and modified from Gaboriaud et al, 2004)

Besides binding to the immune complex, C1q can interact with many additional molecules and these interactions have different biological consequences (Table 2). The $C1r_2/C1s_2$ tetramer interacts with the collagen-like domain of C1q to form the C1 complex (Wallis et al, 2010). This binding will pull "arms" of C1q closer together. After the C1 complex binds to antibodies, the "arms" of C1q will move apart again, which induces the autoactivation of C1r. Active C1r cleaves C1s into the active form, leading to the activation of the downstream C4 and C2 to form C3 convertase (Figure 7). Another well-studied C1q ligand is calreticulin (CRT), which interacts with C1q and the surface receptor CD91 to assemble into a C1q-CRT-CD91 complex that enhances apoptotic cell phagocytosis (Figure 8). C1q also interacts with $\alpha 2\beta 1$ integrin, which will mediate the interaction of C1q with some immune cells and epithelial cells. This system could be hijacked by *Bacillus anthracis* spore surface protein BclA, which mediates spores invasion of epithelial cells (Xue et al, 2011).

In addition to the role in CP and the clearance of apoptotic cell (Figure 8 and Figure 9), C1q is also involved in different immune cell activities including differentiation, cytokines expression, blood coagulations etc (Nayak et al, 2010).

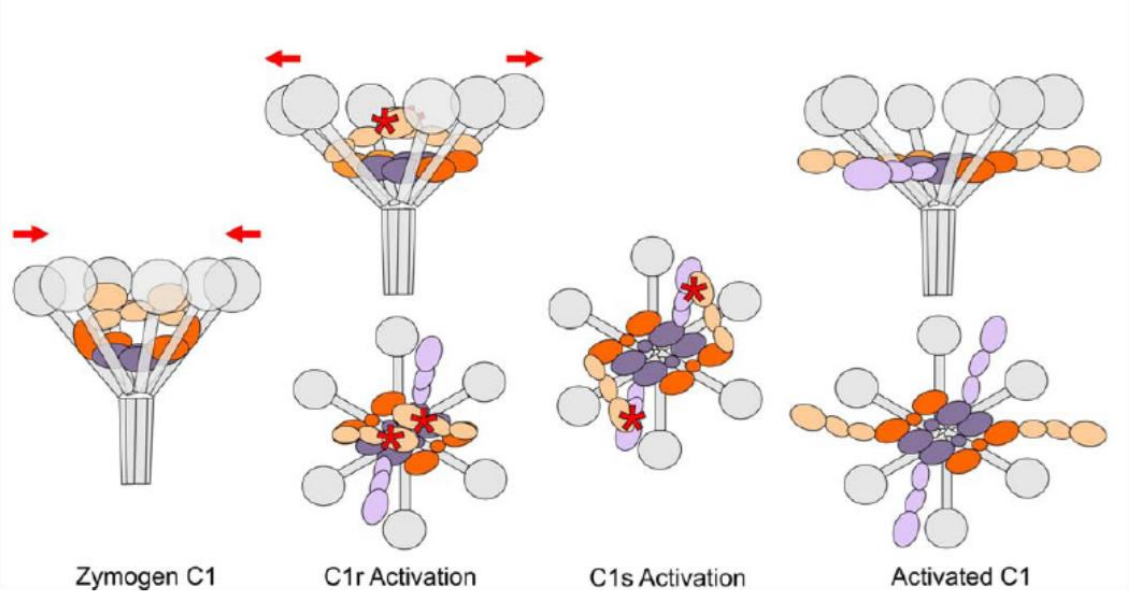

Figure 7. The strained-to-relaxed model of C1 activation. (Reprinted with permission from Wallis et al, 2010)

Table 2. C1q ligands and receptors

C1q ligands or receptors	Binding site(s) on C1q	Effects/ functions	Refs
$C1r_2/C1s_2$	CLR	Form C1 complex	(Wallis et al, 2010)
Antibodies	gC1q	Initiate CP	(Gadjeva et al, 2008)
CRT	Stalk	Enhance AC phagocytosis	(Paidassi et al, 2011)
$\alpha 2\beta 1$ integrin	CLR	Cells adhesion to C1q	(Zutter & Edelson, 2007)
CRP	gC1q	Clearance of apoptotic/necrotic cells	(McGrath et al, 2006)
gC1qR	gC1q	Production of proinflammatory factors and regulation of cell functions	(Peerschke et al, 1994)
CD 91	N/A	Enhance AC phagocytosis	(Duus et al, 2010)
PTX3	gC1q	Inhibition of C1q-mediated complement activation and phagocytosis of apoptotic cells	(Baruah et al, 2006)
Decorin	CLR	Inhibition of C1q-mdicated complement activation	(Krumdieck et al, 1992)

Complement component 3 (C3)

C3 is a central molecule in the complement system. The three pathways converge at C3, which is cleaved into C3a and C3b by C3 convertase (C4bC2a or C3bBb). C3 consists of α (110 kDa) and β (75kDa) chains, which are linked by a single disulfide bond (Figure 10).

C3 is reported to be the most multifunctional molecule in the complement system (Lambris, 1988) and its functions are regulated through a series of cleavages mediated by specific proteases (Figure 10). The cleavage of C3 mediated by C3 convertase will release C3a (9 kDa) from the N-terminus of α chain (figure 10). This subsequently induces a large conformational change in the remaining protein to form C3b, in which the thioester bond is exposed to covalently attach to cells or bacterial surfaces (Gros et al, 2008). C3b can be further cleaved in the α chain into inactive smaller fragments such as iC3b, C3c and C3d, mediated by factor I in complex with co-factors such as factor H, or MCP (Figure 10).

C3a, a small complement peptide that triggers the degranulation of the immune cells, plays an important role in regulating the innate and the adaptive immune responses (Peng et al, 2009). C3a executes their functions by binding to a specific receptor, C3aR, which is a G Protein Coupled Receptor. C3b, another fragment of C3, opsonizes bacteria or apoptotic cells, which can be recognized by many immune cells expressing C3b binding receptors, leading to the enhancement of the phagocytosis (Law, 1988).

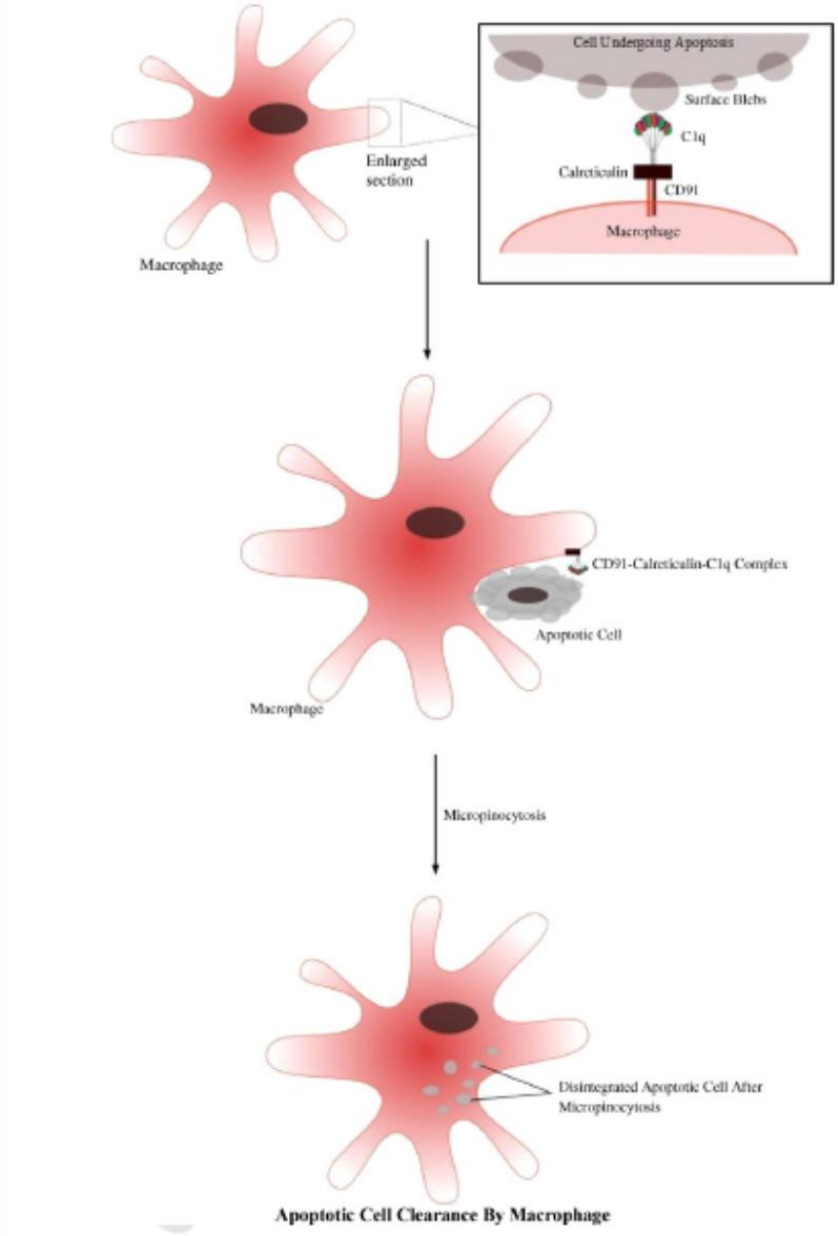

Figure 8. Clearance of apoptotic cells by C1q indirect binding. (Reprinted with permission from Nayak et al , 2010)

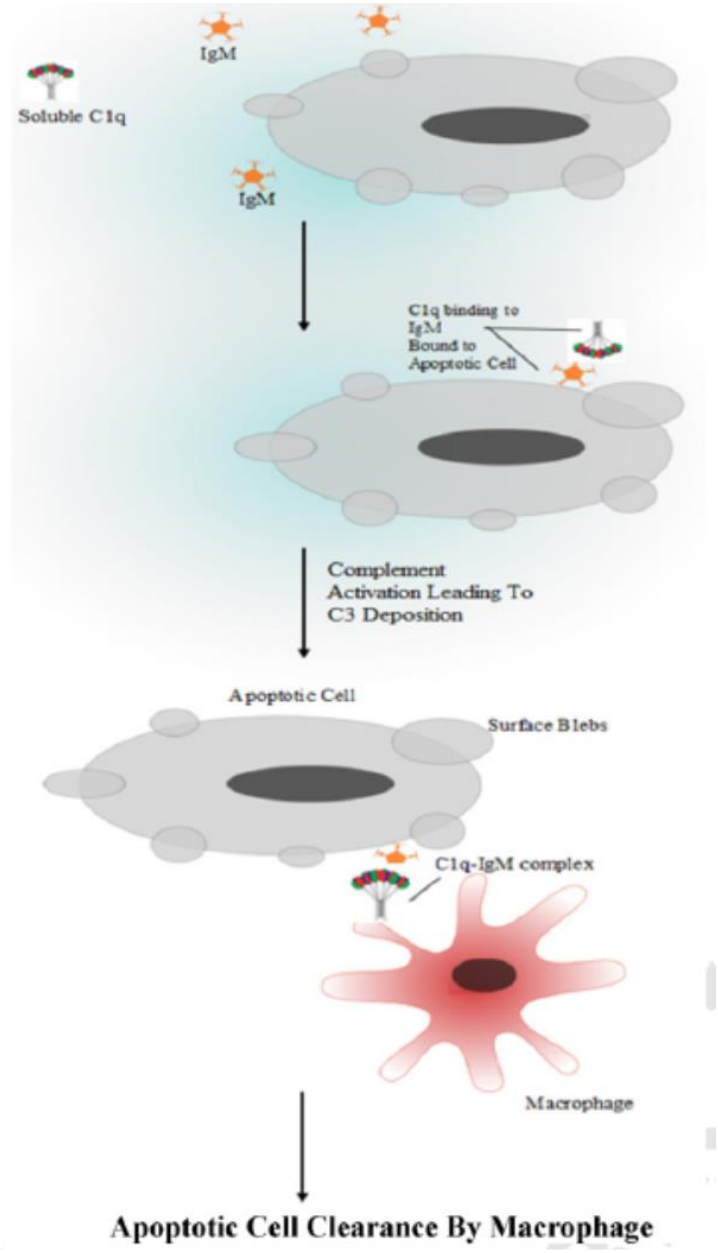

Figure 9. Clearance of apoptotic cells by C1q- mediated CP. (Reprinted with permission from Nayak et al , 2010)

Staphylococcus aureus and diseases

Staphylococcus aureus was first isolated and identified in 1880 by Sir Alexander Ogston (Cheng et al, 2011). It is a Gram-positive commensal bacterium and 80% of the population intermittently or persistently carry it in the nasal cavity (Nouwen et al, 2006). Once the host immune system and/or the physical barrier of external epithelium are compromised, *S.aureus* can enter the body and cause life-threatening diseases. It is reported that *S.aureus* is the most common cause of hospital- acquired infection, ranging from minimal skin infection to serious diseases including bacteremia, endocarditis, arthritis and osteomyelitis (Melzer et al, 2003). In 2005, according to CDC, *S.aureus* killed more people than HIV in the United States (Centers for Disease & Prevention, 2007) and importantly, currently there is no effective vaccine against *S.aureus*.

Bacterial bloodstream infection is also named bacteremia, which will induce cellular and the humoral immune responses, causing increased population of both anti- and pro-inflammatory molecules (Nystrom, 1998). *S.aureus* is one of the major causes of bacteremia with high morbidity and mortality in the United States (Styers et al, 2006). The *clfB* and *fnbA* are two most frequently detected virulence genes in isolates of *S.aureus* obtained from patients with bacteremia. Interestingly, *cna* was also detected in some of these *S.aureus* isolates (Chi et al, 2010).

Infective endocarditis is associated with cardiac endothelium damage caused by several different reasons such as valve implants (Hienz et al, 1996; Patti et al, 1992). These injuries expose the extracellular matrix, which will provide colonization sites for *S.aureus*. At the same time, platelets and fibrin will deposit over the damage sites, in

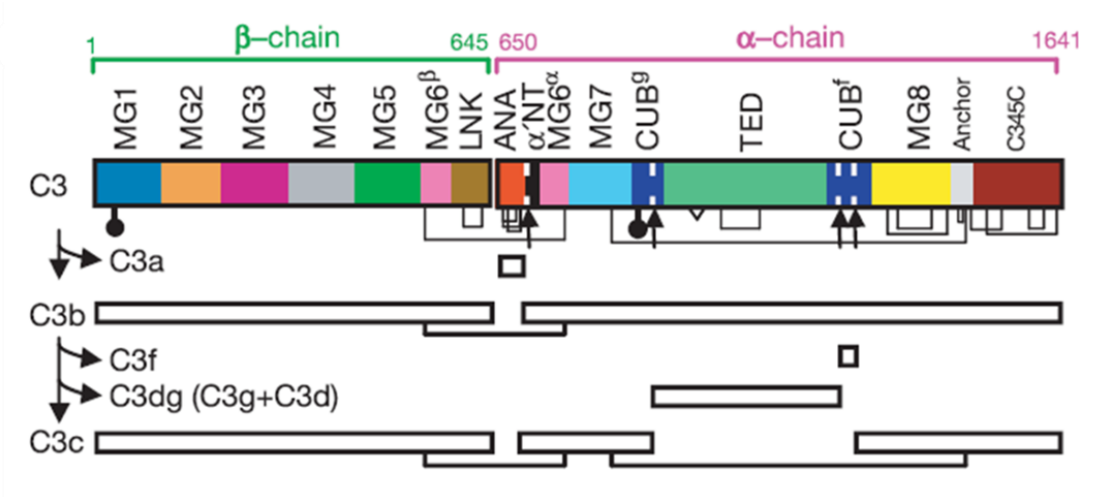

Figure 10. Domain organization of C3 and other C3 small fragments. (Reprinted with permission from Janssen et al, 2005)

order to heal them. These healing systems also provide a "shield" for *S.aureus* to prevent recognition by phagocytes such as macrophage and neutrophil. Previous studies demonstrated that in rat model, Cna, collagen adesin from *S.aureus*, facilitates the adherence of bacteria to endothelia cells in vitro and is a critical virulence factor in infective endocarditis (Hienz et al, 1996).

Even though arthritis and osteomyelitis are two distinct diseases, they might share similar pathogenesis caused by *S.aureus* induced inflammation. Several reports demonstrated that Cna-expressing *S.aureus* specifically attaches to cartilages in vitro and animal studies indicated that Cna plays an important role in experimental arthritis and osteomyelitis by inducing cytokine expression (Elasri et al, 2002; Patti et al, 1994; Xu et al, 2004b). Nevertheless the exact molecular pathogenesis of arthritis and osteomyelitis elicited by *S.aureus* remains unclear. It is believed that once *S.aureus* enters the blood, it can eventually attach to cartilages within the joints. After colonization, *S.aurues* can release many molecules to evade the immune system. In turn, the innate immunity such as the complement system and immune cells will also recognize *S.aurues* and release cytokines and chemokines that attract more immune cells to the infection site, resulting in inflammation. In the end, over-active inflammations will damage cartilages and bones, leading to the erosion of these tissues.

General mechanisms of *S.aureus* infection

S.aureus also employs basic four-step approach to infect human: attachment, colonization, evasion and invasion. Attachment is critical for infection. *S.aureus*

expresses a group of surface protein named MSCRAMMs, which stands for microbial surface components recognizing adhesive matrix molecules (Rivera et al, 2007). This family of surface proteins helps *S.aureus* attach to extracellular matrix such as collagen, fibrinogen and fibronectin. After attachment, *S.aureus* will increase bacterial colony and grow into large clusters. In some circumstance, *S.aureus* will form biofilm, which is a highly organized bacterial community composed of extracellular DNA, proteins, polysaccharides and bacterial cells (Scherr et al, 2013). At the same time, *S.aureus* employs strategies to evade the host immune systems. In turn, immune systems use immune cells, complement system and other components of the host defense to monitor and eliminate *S.aureus*. The reciprocal interaction establishes a balance between *S.aureus* and the host immune system. Once the immune system is compromised, the *S.aureus* virulence factors will cause clinically manifested diseases.

MSCRAMMs

MSCRAMMs play a critical role in the bacterial attachment. So far, various MACRAMMs have been identified in different bacterial species. *S.aureus* has eight different MSCRAMMs, such as collagen- binding MSCRAMMs Cna, fibronectin-binding MSCRAMMs FnBPA and FnBPB, fibrinogen-binding MSCRAMMs ClfA and SdrE (Bbp). Recent studies suggest that MSCRAMMs may also participate in complement evasion.

Table 3. *S.aureus* MACRAMMs and their known ligands

MSCRAMMs	Known ligands
FnBPA	Fibrinogen, Fibronectin, Elastin
FnBPB	Fibrinogen, Fibronectin
Cna	Collagen, C1q, MBL
ClfA	Fibrinogen, Complement factor I
ClfB	Fibrinogen, Keratin
SdrC	Beta-Neurexin
SdrD	Unknown epithelial cell surface component
SdrE(BBp)	Fibrinogen, Bone Sialoprotein, FH, C4BP

Collagen-binding MSCARMMs from Gram-positive bacteria

Collagen, the most abundant ECM molecules in the human body, makes up over 25% of the whole-body protein. To date, over 20 genetically distinct collagen types have been identified and the majority of the collagens are present in different multimeric structures (Lozano et al, 1985). The collagen monomer forms a characteristic triple helix, which consists of three polypeptide chains with repeated "Gly-Xaa-Yaa" motifs in the amino acid sequence (Zong et al, 2005). Bacteria have evolved to express structural related collagen-binding MSCRAMMs, which interact with the triple helix.

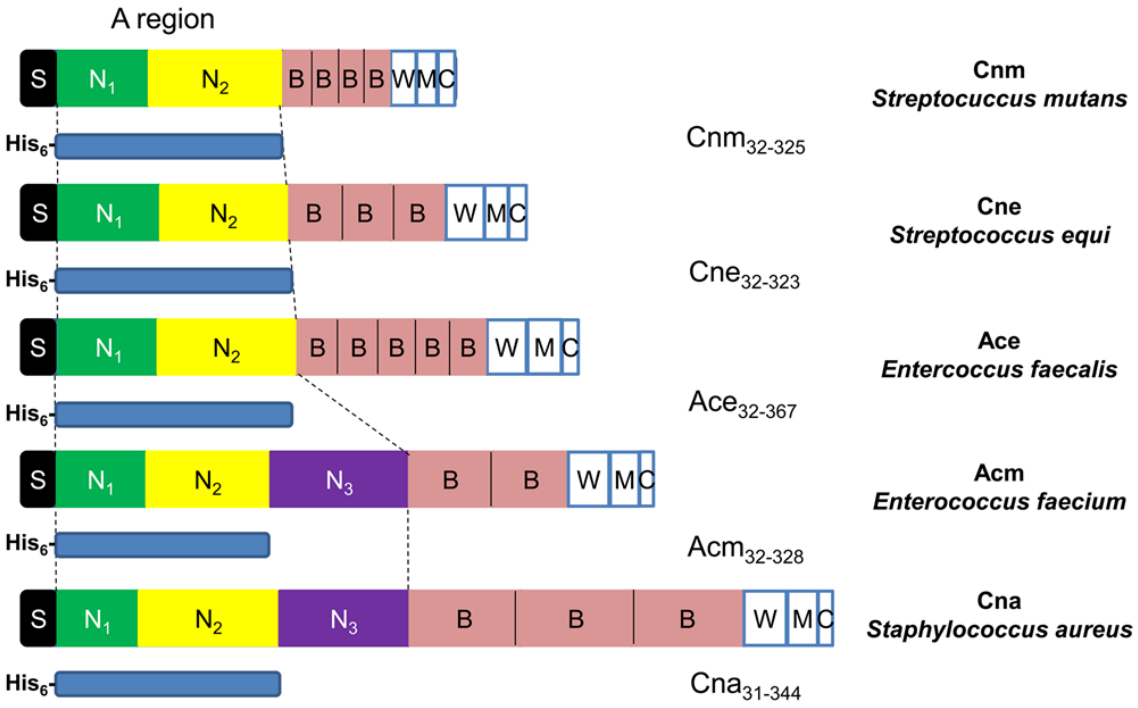

Figure 11. Schematic of collagen-binding MSCRAMM domain organization. S, signal peptide; A region, collagen-binding domain containingN1N2 domains; B, repeated region; W, cell wall-anchoring region; M, transmembrane segment; and C, cytoplasmic tail. The N1N2 regions of these MSCRAMMs were recombinantly expressed as N-terminal His tag fusion proteins, as illustrated. (Kang et al, 2013)

S.aureus Collagen-Binding MSCRAMMs-Cna

Cna, Collagen-binding MSCRAMMs from *Staphylococcus aureus*, was identified in 1994 by our lab (Patti et al, 1994) and has been well characterized for 20 years. Cna contains an N-terminal signal peptide, an A domain which is responsible for collagen binding followed by B-repeats, which are suggested to help A domain display on the surface of bacteria, and the C-terminal cell wall attachment elements, including a cell wall anchoring region, a transmembrane segment and a positively charged cytoplasmic tail (Figure 11). Actually, the collagen binding domain of Cna only contains N1 and N2 subdomains. Previously, our lab crystalized CnaN1N2 domains (Figure 12A) and Cna N1N2 in complex with collagen triple helixes peptide (Figure 12B). These crystal structural data indicate that Cna-collagen interaction follows a multi-step ligand binding mechanism named "collagen hug model" (Figure 13). In this model, the collagen triple helix peptide interacts with Cna N2 subdomain and this binding will redirect N1-N2 linker with N2 subdomain to "hug" the collagen triple helix peptide. In the end, C terminal "latch" domain of N2 subdomain interacts with N1 subdomain, resulting in the stabilization of this complex (Zong et al, 2005). This model also indicates that the interaction is dominated by the rope-like structure of the collagen (Zong et al, 2005).

Previous studies have proved that Cna is a virulence factor in several animal models of infectious diseases such as endocarditis, arthritis, keratitis and osteomyelitis (Hienz et al, 1996; Johansson et al, 2001; Rhem et al, 2000; Xu et al, 2004b) and that the virulence potential is associated with its affinity to collagen (Xu et al, 2004b). In the

septic arthritis mouse model, the role of Cna was examined by comparing the virulence among *S.aureus* expressing wild type CnaN1N2, and Cna mutant with a single point mutation resulting in the loss of collagen binding. These results demonstrated that the virulence of *S.aureus* depends on Cna's collagen-binding ability (Xu et al, 2004b). However, interestingly, the data also showed that Cna doesn't contribute to the early colonization of *S.aureus* (Xu et al, 2004b).

Cna-like Collagen-Binding MSCRAMMs from different species

Collagen-binding MCRAMMs are also found in several different species, such as Ace from *Enterococcus faecalis* (Nallapareddy et al, 2003), Acm from *Enterococcus faecium* (Hubble et al, 2003), Cne from *Streptococcus equi* (Lannergard et al, 2003) and Cnm from *Streptococcus mutan* (Nakano et al, 2010). They all share similar structural domain organizations (Figure 11). The collagen binding mechanism of all these structurally related collagen-binding MCRAMMs seems to be similar and all of them facilitate the adherence of bacteria to the collagen, leading to infections.

Sequence alignment analysis reveals that the amino acid sequences of Cna, Ace, Acm, Cne and Cnm have some similarities, especially on the N2 subdomain containing collagen binding sites. The N2 subdomain of Cna shares 55%, 49% and 48% amino acid sequence identity with those of Cnm, Acm and Cne respectively.

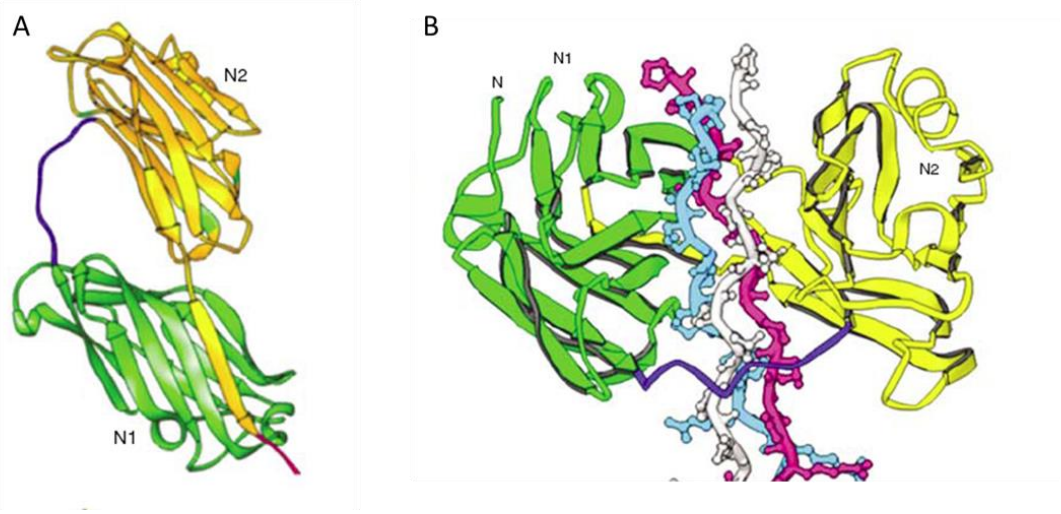

Figure 12. Crystal structures of Cna and Cna-collagen-like peptide complex. (Reprinted with permission and modified from Zong et al., 2005)

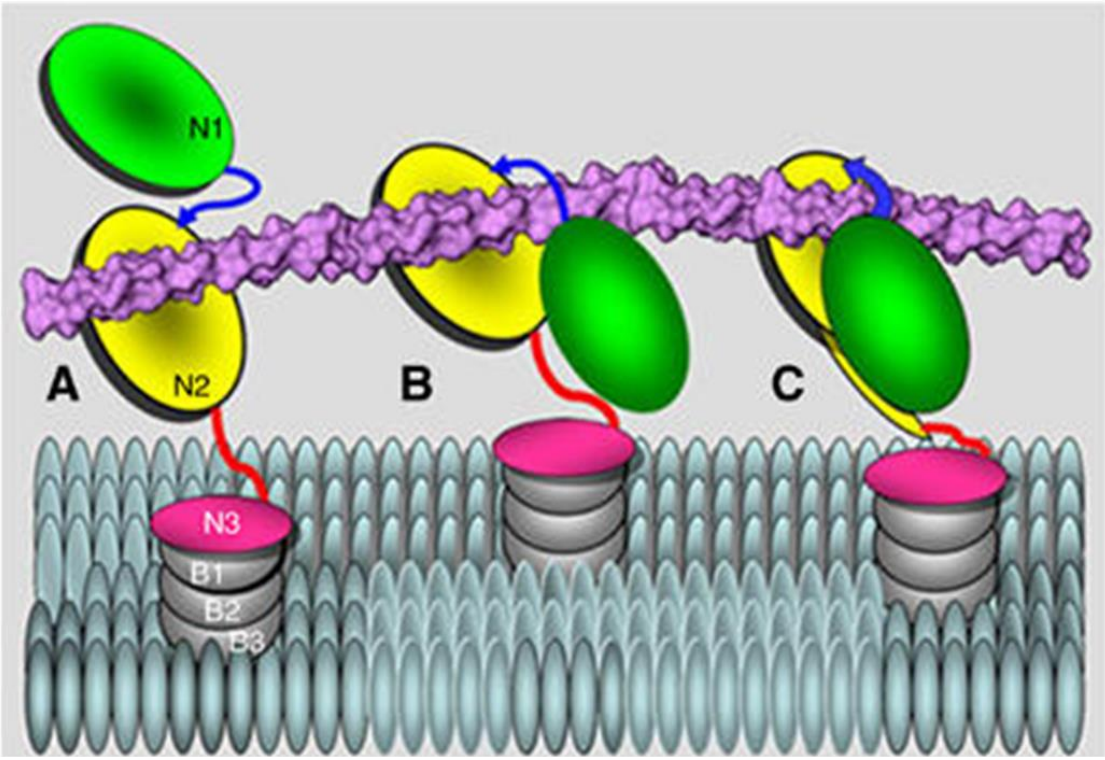

Figure 13. A hypothetical 'Collagen Hug' model. (A) a collagen triple helix peptide interacts with the N2 subdomain. (B) The collagen is hugged by the N1–N2 linker and the N1 subdomain. (C) The C terminal of N1 subdomain interacts with the N2 subdomain to lock the ligand. (Reprinted with permission from Zong et al, 2005)

S.aureus fibrinogen-binding MSCRMMs

S.aureus expresses several fibrinogen (fg) -binding MSCRAMMs, most of which belong to Sdr family of surface proteins, such as ClfA, ClfB, SdrE/Bbp. Although they bind to different sites of fibrinogen (Figure 14), these proteins share similarities in both the domain organizations and the structure of ligand binding domain (Figure 15). Most of them comprise an A region at N-terminus which is responsible for fibrinogen binding, followed by B-repeat region and R domain containing the Ser-Asp (SD) dipeptide repeats. The C terminus contains cell wall attachment elements. Specifically, the A region contains three subdomains: N1, N2 and N3, whereas the fibrinogen binding domain only contains N2N3 subdomain. Crystal structural analysis of N2N3 of ClfA and ClfB showed that the fg-binding domain consists of two Ig-like folds, which are connected by a polypeptide "linker" (Ganesh et al, 2008; Ponnuraj et al, 2003; Xiang et al, 2012).

Like the collagen-binding mechanism, Fg-binding also employs multi-step binding mechanism named "Dock, Lock and Latch" model. Take SdrG from *S. epidermidis* for example: the open form of SdrG allows Fg peptide to "dock" its N2 subdomain binding trench. This binding induces a conformational change at the C-terminal strand, which then interacts with Fg peptide and "locks" it. Further redirection of the C-terminal strand results in a β-sheet of C-terminal complement with the N2 domain (Latch) (Ponnuraj et al, 2003).

Complement evasion by *S.aureus*

The complement system helps the immune system to recognize and kill pathogens. In return, numerous successful pathogenic bacteria, especially *S.aureus*, have evolved many well-defined strategies to evade it. Most bacteria evasion strategies can be categorized into three major mechanisms: direct inhibition, recruitment of complement regulators and the degradation of complement components (Lambris et al, 2008 and Table 4). *S.aureus* expresses several molecules to inhibit complement activation directly. Protein A (SpA), expressed by most of the clinical *S.aureus* strains (Votintseva et al, 2014), is a very critical virulence factor, which has multiple strategies to evade immune system, including complement activation. It binds to the Fc of the antibody, which interrupts the opsonization of antibodies and the formation of immune complexes (Kronvall & Gewurz, 1970). *S. aureus* extracellular fibrinogen-binding protein (Efb), Extracellular complement-binding protein (Ecb) and staphylococcal complement inhibitor (SCIN) directly bind and inhibit C3 convertase activity (Jongerius et al, 2010a; Jongerius et al, 2010b; Lee et al, 2004; Rooijakkers et al, 2005). Chemotaxis inhibitory protein of *S. aureus* (CHIPS) binds C5a receptor and blocks phagocyte recruitment (Postma et al, 2005; Rooijakkers et al, 2006). For the regulator recruitment, *S.aureus* Bbp or SdrE binds to the complement regulators C4b-binding proteins and factor H, which block bacteria phagocytosis by facilitating the decay of C3 convertase (Hair et al, 2013; Sharp et al, 2012); Clumping factor A (ClfA) of *S.aureus* was shown to recruit factor I and enhances the cleavage of C3b to iC3b (Hair et al, 2010). In addition, the

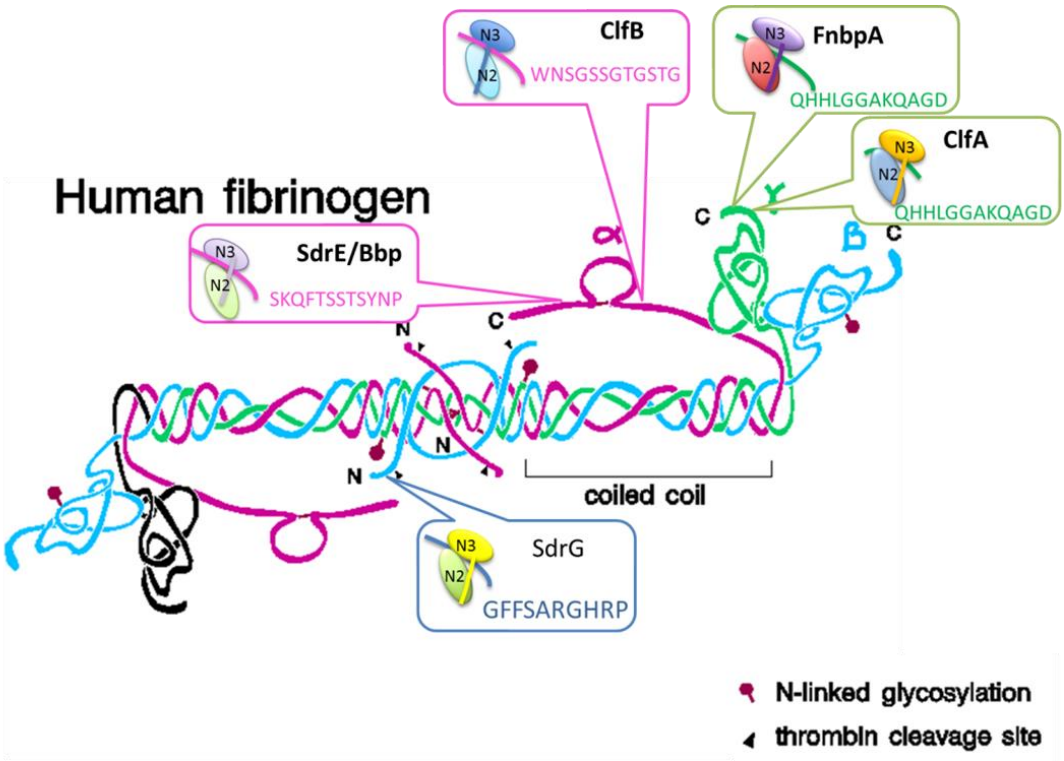

Figure 14. Fg-binding MSCRAMMs bind to different sites of fibrinogen. (Reprinted with permission and modified from Yee et al, 1997)

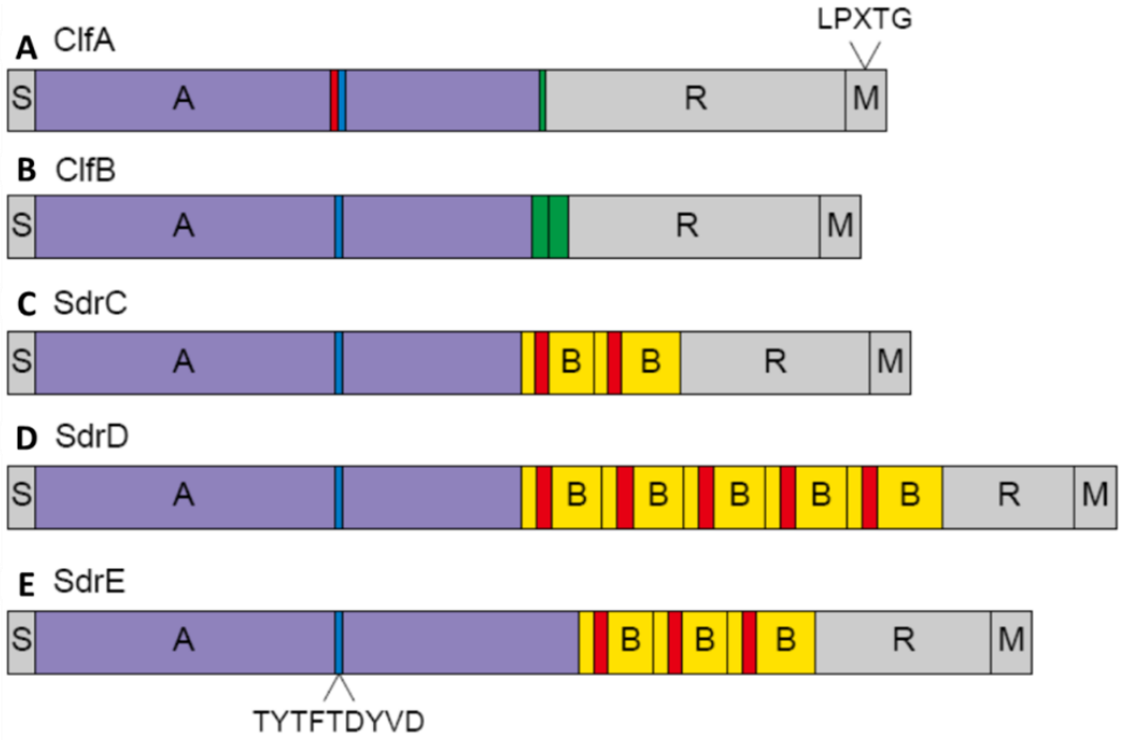

Figure 15. Domain organization of Sdr protein family. (A) ClfA. (B) ClfB. (C) SdrC. (D) SdrD. and (E) SdrE. S, signal peptide; A, A-domain; B, B repeats; R, Ser-Asp dipeptide repeats; M, wall-spanning region; he green region, proline-rich region; red region, EF-hand loops; blue region, TYTFTDYVD motif, overlaps an EF-hand loop in the A-domain of ClfA. (Reprinted with permission from Foster& Hook, 1998)

metalloprotease aureolysin of *S.aureus* degrades C3 (Laarman et al, 2011; Lambris et al, 2008).

Table 4. Complement evasion by *S.aureus*

Complement evasion poteins	Target
Direct inhibition	
Protein A(SpA)	Antibodies
Efb/Ecb	C3 and C3b
SCIN	C3 convertase
CHIPS	C5aR
SSL-7	C5
Recruitment of complement regulators	
ClfA	Factor I
Bbp or SdrE	Factor H and C4BP
Degradation of complement components	
Staphylokinase	C3b and antibodies

CHAPTER II

MATERIALS AND METHODS*

Antibodies

Polyclonal goat anti-human C1q, goat anti-human C4 antibodies and goat anti-human C3 antibodies were purchased from Complement Technology (Tyler, Texas). Monoclonal anti-C1q antibody was purchased from Quidel (San Diego, CA). Goat anti–human C1r and sheep anti-human C1s polyclonal antibodies were from R&D systems (Minneapolis, MN). Rabbit anti-goat and rabbit anti-sheep polyclonal antibodies conjugated with HRP were from Invitrogen (Carlsbad, CA). Monoclonal anti-poly His-conjugated with HRP-antibody was supplied by Alpha Diagnostic (San Antonio, Texas) and human IgM was ordered from EMD Chemical (Philadelphia, PA).

Reagents

Purified human C1q, C1r, C1s, factor B, factor D, C3, C3b, C3c, C3d, C4b, C5 normal human serum (NHS), C1q-Depleted serum (C1q-dpl), factor B depleted serum (fB-dpl), factor H depleted serum(fH-dpl), rabbit erythrocytes (ER) and antibody-sensitized sheep erythrocytes (EAs) were obtained from Complement Technology

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(Tyler, Texas). Recombinant human type I collagen was from FibroGen (San Francisco,CA). Human fibrinogen was from Enzyme Research and o-phenylenediamine dihydrochloride (SigmaFast) was purchased from Sigma.

Expression and purification of recombinant proteins

Expression plasmids were generated as described in the previous studies (Rich et al, 1999b; Symersky et al, 1997). Briefly, the DNA encoding the N1N2 domains of collagen-binding MSCRAMMs or fibrinogen-binding MSCRAMMs Bbp and SdrE were subject to PCR and ligated into pQE30. The primers for cloning PCR-generated N1N2 regions and mutants integrated into expression vector pQE-30 (Qiagen Inc., Chatsworth, CA) are listed in Table 5. The Cna mutants harboring the mutations in N1N2 domains were generated by using QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. E. coli Topp3 (Stratagene) containing the pQE30-derived plasmids were grown overnight at 37℃ in LB containing 100 μg/ml ampicillin. The overnight cultures were diluted 1:50 into fresh LB medium, and the expression of the recombinant proteins was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 hours at 37℃. Bacteria were harvested by centrifugation and lysed using a French press (SLM Aminco). Soluble His6-tagged recombinant proteins were purified through a HisTrap HP Column and a HiTrap Q HP column (GE Healthcare) according to the manufacturer's manual. The Purified proteins were analyzed by SDS-PAGE and appropriate fractions were dialyzed into TBS or PBS

and stored at -20 ℃. Protein concentrations were determined by absorption spectroscopy at 280 nm using calculated molar absorption coefficient values.

Table 5. Oligonucleotides for Cna mutants, Acm, Cne and Cnm constructs

Oligonucleotide[a]	Sequence (5’→3’)
Flanking primers	
Acm 5’	GCGGGATCCGATGCAGGCAGAGATATCAGCAG
Cne 5’	GCGGGATCCGCAACTAATCTTAGTGACAACATC
Cnm 5’	GCGGGATCCAGTGATGTCAGCAGTAACATTTC
Acm 3’	GCGGTCGACTTAAATCTCTGTGTCATTGATATATTT
Cne 3’	GCGGTCGACTTAAAAGCTGGTATAGCGACTGCC
Cnm 3’	GCGGTCGACTTATGTAGTAGTGGTTGTTCTTCC
Mutagenic primers	
QIE forward	GAACGGGACAGATTGAAACAAGTAGTGTTTTCTATTATAAAACG
QIE reverse	GAAAACACTACTTGTTTCAATCTGTCCCGCTTCACTTTTATG
ΔEAG forward	CGGTTCATAAAAGTACAAGTAGTGTTTTCTATTATAAAACG
ΔEAG reverse	GAAAACACTACTTGTACTTTTATGAACCGTAACATTCG
Cna ΔEAGTSS forward	CATAAAAGTGTTTTCTATTAT
Cna ΔEAGTSS reverse	ATAATAGAAAACACTTTTATG
Cna ΔLatch forward 1	GGA TCC GCA CGA GAT ATT TCA TCA AC
Cna ΔLatch reverse 1	GTG CAC AAT ATT AAT GCT GTC GAC
Cna ΔLatch forward 2	GTG CAC AAT ATT AAT GCT TAA GTC GAC CTG CAG CCA AGC
Cna ΔLatch reverse 2	GCT TGG CTG CAG GTC GAC TTA AGC ATT AAT ATT GTG CAC

[a]Oligonucleotides were synthesized by the integrated DNA technologies, INC.

ELISA-type binding assays

Wells on Immulon 4BHX plates (Thermo Scientific) were coated overnight at 4°C with 1 μg of recombinant human collagen or purified human C1q. After blocking with 2% BSA in TBST (0.05% Tween 20, TBS) at RT for 2 hours, recombinant proteins were added to the wells and incubated for 1 hour at RT. Following incubation with HRP conjugated anti-His antibodies (10,000× dilution) at RT for 1 h, the substrate OPD was added. Bound proteins were quantified by measuring the absorbance at 450 nm in a microtiter plate reader (Thermomax) and data were analyzed by GraphPad Prism software.

The competition assay used a protocol similar to that described above, with the following alterations. First, recombinant MSCRAMM was preincubated with C1 complex or C1q (10 nM) in the HBS++ buffer (20 mM Hepes, 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.025% bovine serum albumin (BSA), pH 7.3) at RT for 1 h. The mixture was subsequently added to the microtiter plates previously coated overnight at 4°C with purified IgM (0.5 μg/well), C1r or C1s (0.5 μg/well). The following primary antibodies were used where applicable: goat anti-human C1q antibody (5000× dilution), goat anti-human C1r (2000× dilution) and sheep anti-human C1s (2000× dilution). Secondary antibodies rabbit anti-goat and rabbit anti-sheep and Sigma-fast OPD were used for the detection and visualization of the bound protein.

Table 6. Proteins used in this study (Kang et al, 2013)

Name	Description
Cna_{31-344}	Recombinant protein containing N_1N_2 domains of Cna of *S. aureus* with an N-terminal tail of 6 histidine residues
Cna $Y_{175}K$	Cna_{31-344} with Y175K substitution
Cna Δlatch	Cna_{31-344} without C-terminal 'latch' residual
Cna QIE	Cna_{31-344} with insertion of 3 amino acids (QIE) from Ace linker to linker region (168^169)
Cna ΔEAG	Cna_{31-344} with deletion of 3 amino acids (EAG) from linker region (166-168)
Cna ΔEAGTSS	Cna_{31-344} with deletion of 6 amino acids (EAGTSS) from linker region (168-171)
Cne_{28-323}	Recombinant protein containing N_1N_2 domains of Cne of *S. equi* with an N-terminal tail of 6 histidine residues
Cnm_{32-323}	Recombinant protein containing N_1N_2 domains of Cnm of *S. mutans* with an N-terminal tail of 6 histidine residues
Acm_{29-320}	Recombinant protein containing N_1N_2 domains of Acm of *E. faecium* with an N-terminal tail of 6 histidine residues
Ace_{32-367}	Recombinant protein containing N_1N_2 domains of Ace of *E. faecalis* with an N-terminal tail of 6 histidine residues
Bbp $_{215-544}$	Recombinant protein containing N_2N_3 domains of Bbp of *S. aureus* with an N-terminal tail of 6 histidine residues
SdrE $_{215-544}$	Recombinant protein containing N_2N_3 domains of SdrE of *S. aureus* with an N-terminal tail of 6 histidine residues

In the case of determining C1 complex binding to human IgM or Cna, microtiter wells were coated with 0.5 μg/well of human IgM or of rCna 31-344 and bound C1 complex was detected using goat anti-human C1q (5000× dilution), goat anti-human C1r (2000× dilution) and sheep anti-human C1s (2000× dilution), followed by HRP conjugated rabbit anti-goat or rabbit anti-sheep antibodies (3000× dilution).

To detect the binding of Bbp and SdrE to the immobilized human fibrinogen or purified human C3b, C5 and C3, Immulon 4BHX plates (Thermo Scientific) were coated with 1 μg/well human fibrinogen, C4b C3b, C3 or C5 at 4°C overnight. Coated wells were blocked for 2 hours at room temperature with 2% of BSA in TBST (0.05% Tween-20). Increasing concentrations of Bbp or SdrE were added to the wells and incubated for 1 hour at room temperature. Bound proteins were detected with anti-Bbp or SdrE specific antibodies (3,000 × dilution) followed by goat anti - rabbit antibodies-HRP (3,000 ×dilution, Invitrogen).

Bacterial attachment assay

Adherence assay was performed as described previously (Xu et al, 2004b) with modification. *S. aureus* strain Phillips and correspondence Cna deletion mutant strain (PH100) (Patti et al, 1994) were grown in BHI media overnight and serial dilution of bacteria were made. Subsequently bacteria were incubated in the wells of microtiter plates coated with C1q (1 μg/well). Adhered bacteria were fixed with 4% formaldehyde and stained with 0.5% crystal violet. After washing, 50 μL of 10% acetic acid was added and the absorbance at 590 nm was measured.

Surface plasmon resonance (SPR) analysis

The interactions between C1q and Cna31-344 were characterized using a Biacore 3000 (GE Healthcare/Biacore, Uppsala, Sweden) at 25℃. Sensor chip C1 and amine-coupling kits were obtained from the same company and used to covalently attach ligands onto the sensor surface using amine coupling procedure as recommended by the manufacturer. During immobilization, HEPES-buffered saline (HBST: 10amM HEPES, pH 7.3, 150 mM NaCl and 0.005% Tween-20) was used as running buffer at a flow rate of 5 μl/min. Following the activation of sensor surface, 5 μg/ml of ligand solution (collagen or Cna31-344 in 10 mM sodium acetate, pH5.5; C1q in 100 mM sodium phosphate buffer, pH7.0) was injected and coupled to the flow cell on a C1 chip. A reference flow cell was prepared for each sensor chip with activation and deactivation steps where no protein was coupled. Binding was performed at a flow rate of 30μl/min with TBST (25 mM Tris, pH7.4, 3 mM KCl, 140 mM NaCl, and 0.01% Tween 20) as running buffer. To regenerate the sensor surface, bound Cna proteins were removed from the collagen surface by injecting 10 mM glycine (pH 1.5) for 30 second; bound C1q was removed with 0.5% SDS from the Cna31-344 surface.

To analyze the binding of Bbp or SdrE to complement proteins C3, C3b, C3c, C3d or C5, complement proteins were immobilized onto a CM5 sensor chip, respectively. Various concentrations of Bbp or SdrE in the HBS buffer with 1 mM Mg^{2+} were passed over the sensor chip at a flow rate of 50 μl/min. To regenerate the sensor surface, bound Bbp, SdrE or other complement proteins were removed from the surface

by injecting1M NaCl and 10 mM sodium tetraborae (pH 8.5) for 60 seconds, respectively.

In an on-chip convertase formation assay (Chen et al, 2010), a 1 to 1 ratio mixture of factor B and factor D (250 nM each) in the presence or absence of 50 μM Bbp as premixed solutions was injected onto the immobilized C3b chip for 2 min at 10 μl/min to establish the C3 convertase formation. The association and disassociation rates of the C3 convertases were evaluated and compared.

The analysis of factor B cleavage was detected and performed as described previously (Hourcade, 2006) on the Factor P immobilized sensor chip with C3b, mixture of C3b and factor B or plus factor D in the presence or absence of Bbp for 1 min. The binding of C3bBb was detected by analyzing the association and the disassociation rates.

The baseline corrected SPR response curves (with buffer blank run further subtracted) were used for affinity determination. For the steady-state analysis, the equilibrium response (Req) of each injection was collected and plotted against the concentration (A) of injected protein. A binding isotherm was fitted to the data (GraphPad Prism 4, GraphPad Software, Inc., La Jolla, CA, USA) to obtain the equilibrium dissociation constant (K_D).

The non-equilibrium data were globally fit to a two-state model using BIAevaluation software (Version 4.1). Association and dissociation rate constants (k_{a1}, k_{d1}) for the binding state, and forward and backward rate constants (k_{a2}, k_{d2}) for the conformational change state were obtained from the fitting, and apparent dissociation

constant (K_D^{app}) was calculated from the rate constants: $K_D^{app} = 1 / ((k_{a1} / k_{d1}) * (1+k_{a2} / k_{d2})$.

Immunoprecipitation (IP) assay

Monoclonal anti-human C1q antibodies were captured with protein G beads (Thermo Scientific) at RT for 2h. Subsequently, a mixture of the C1 complex (35 nM) and recombinant MSCRAMMs (80 μM) previously incubated at RT for 1-2 hours in HBS++ buffer were added to protein G beads bearing anti C1q antibodies and allowed to incubate for 2 h at 37 ℃. After washing the beads with HBS++ containing 0.1% Triton X-100 (Sigma), 50 μl of non-reducing buffer was added and proteins were denatured at 95 ℃ for 10 min. After centrifugation at 2500 × g for 5 min, 10 μl of supernatant was subjected to SDS-PAGE and transferred to nitrocellulose (Bio-rad). The component of the C1 complex was detected by polyclonal goat anti-human C1q (10,000× dilution), goat anti-human C1r (8000× dilution), or sheep anti-human C1s (8000× dilution) and followed by HRP-conjugated rabbit anti-goat (10,000× dilution) or rabbit-sheep secondary antibodies (10,000× dilution). Chemiluminescent substrate (SuperSignal West Pico from Thermo Scientific) was used to visualize the cross-reacting material.

CP/LP C4b, C3b and C5b-9 complex deposition on ELISA

C4b, C3b and C5b-9 complex deposition was performed as described ((Laarman et al, 2012; Rooijakkers et al, 2005) with slight modifications. 96-well Immulon 4BHX microtiter plates (Thermo Scientific) were coated with 0.1 μg of human IgM or 1 μg of

mannan (Sigma) overnight at 4℃. Diluted serum (in HBS++) was preincubated with various concentrations of recombinant proteins at RT or 37^{o}C for 1 hour. After blocking the wells with 2% BSA in TBST (0.05% Tween 20, TBS) at RT for 2 hours, the serum-protein mixtures were added and the plates were incubated for 1 hour at RT or 37^{o}C. Bound-C4b, C3b and C5b-9 complex was detected by goat anti-human C4 antibody (10,000 × dilution) goat anti-human C3 antibody (10,000 × dilution) or mouse anti-human C5b-9 antibody (1,000 × dilution), followed by horseradish peroxidase (HRP)-conjugated rabbit anti-goat antibody or goat anti-mouse antibody (10,000 × dilution) for 1hour each and quantified as described above.

AP C3b generation ELISA type assay

Immulon 4BHX plates were coated with 1μg/well LPS at 4°C overnight. Coated wells were blocked with 2% of BSA in 0.5% Tween-PBS buffer for 2 hours at room temperature. Fixed concentrations of serum at 8% in HBS^{o} with 5 mM Mg^{2+} and EGTA plus 0.025% BSA, which were preincubated with various concentrations of recombinant proteins that we want to test, were added to wells and incubated for 1 hour at 37℃. The C3b generation was detected by goat anti human C3 antibody (10,000 × dilution) followed by rabbit anti-goat antibody (4000 × dilution). Color development was performed using SigmaFast OPD (Sigma) and the binding was measured using a microtiter plate reader (Thermomax, Molecular Devices) at 450 nm.

Hemolysis

Complement-mediated hemolysis of EAs was performed as described previously (Itoh et al, 2010) with slight modifications. Briefly, NHS, C1q-dpl, or fB-dpl (final concentration 0.5%) was preincubated with the parent and the mutant collagen-binding MSCRAMMs in a final volume of 260 μl at room temperature (RT) for 1 hour. Subsequently, 40 μl of EAs (5×10^8 /ml) were added to the mixture, which was incubated at 37 ℃ for 45 min. The clear supernatant was transferred to 96-well PVC flat-bottom plate (Becton, Dickinson, BD) after centrifugation (2000 rpm, 10 min). The lysis of EAs was quantified by measuring the absorbance at 405 nm using microtiter plate reader (Thermomax, Molecular Devices). Data was present as the percentages of lysis by dividing with the absorbance value obtained from 100% EAs lysis. The data represent the mean ± SD of three independent experiments performed in triplicates (GraphPad Prism). All serums were diluted in BHepes buffer (20 mM Hepes, 140 mM NaCl, and 0.025% bovine serum albumin (BSA), pH 7.3) containing 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$.

***S.aureus* infection murine model**

All female mice (wild type and C1q knockout) between 8 and 12 weeks old were used in this infection murine model. The strains used were clinical *S. aureus* isolates Phillips strain and its *cna* knockout strain PH100. *S.aureus* were grown overnight in TBS, washed twice and suspended in PBS. Wild type mice and C1q knockout mice were infected with 3.5×10^7 cfu Phillips or PH100 strain / mouse respectively and monitored

for body weight once a day. Mice that lost more than 30% weight were considered moribund and euthanized.

Bacterial load in blood and organs

Mice were killed at day 1 and day 6.5 after the infection. The blood was collected via cardiac puncture blood collection and immediately mixed with 50 µl of 1M sodium citrate. Organs were harvested and homogenized in PBS. Serial dilutions of the blood and the organ homogenates were cultured on TSA plates supplemented with/without proper antibiotic overnight at 37^{o}C. CFUs were calculated as CFU/ml for blood and CFU per organ.

CHAPTER III

THE COLLAGEN-BINDING MSCRAMM FROM S. AUREUS INHIBITS CLASSICAL PATHWAY ACTIVATION*

Introduction

Collagen, the most abundant protein in the human body, plays a critical role in human health and homeostasis. It has a unique triple helix structure which consists of three α chains with repeating Gly-X-Y sequences (Chung et al, 2004). Through this highly regular arrangement of amino acids in each chain and the rope-like structure of collagen, it interacts with many proteins or proteinases. These interactions have essential biological significances, especially for adhesion molecules from pathogenic bacteria (Zong et al, 2005).

Gram-positive bacteria express a group of structurally related collagen adhesins named collagen-binding MSCRAMMs (Figure 11), which are found in many bacterial species, such as Cna in *Staphylococcus aureus* (Patti et al, 1994) , Ace in *Enterococcus faecalis* (Liu et al, 2007), Acm in *Enterococcus faecium* (Nallapareddy et al, 2003), Cne in *Streptococcus equi* (Lannergard et al, 2003) and Cnm in *Streptococcus mutans* (Nakano et al, 2010). These molecules are well-characterized virulence factors in

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several experimental animal models of infectious diseases and mediate the adherence of bacteria to host tissues (Hienz et al, 1996; Johansson et al, 2001; Nakano et al, 2010; Nallapareddy et al, 2008; Rhem et al, 2000; Singh et al, 2010; Xu et al, 2004b).

Cna from *S.aureus* is the prototype of collagen-binding MSCRAMMs. Structural analysis of Cna- triple helix peptide complex demonstrated that the rope-like structure of collagen is critical for this interaction (Zong et al, 2005). Besides collagen, a number of proteins contain collagen-like subdomains, some of which are involved immune system, such as C1q. C1q is an initiator of the CP, which is critical of the host's defense against microbial infection. It is reported that several pathogenic bacterial proteins interact with C1q, leading to the aggravation of bacterial infections, such as BclA from *Bacillus anthracis* (Xue et al, 2011) and PepO from *Streptococcus pneumoniae* (Agarwal et al, 2014). I believe that the collagen-binding MASCRMMs also have similar functions.

In this part of study, I report that the Cna-like family of collagen-binding MSCRAMMs also bind C1q and inhibit the complement classical pathway activation. The molecular bases for this inhibition are dissected and described.

Results

Cna binds to C1q

A recombinant form of Cna, Cna_{31-344}, bound to C1q or collagen type I coated in microtiter wells in processes that were dose dependent and exhibited saturation kinetics (Figure 16A). SPR analyses using C1q and collagen type I immobilized on BIA-

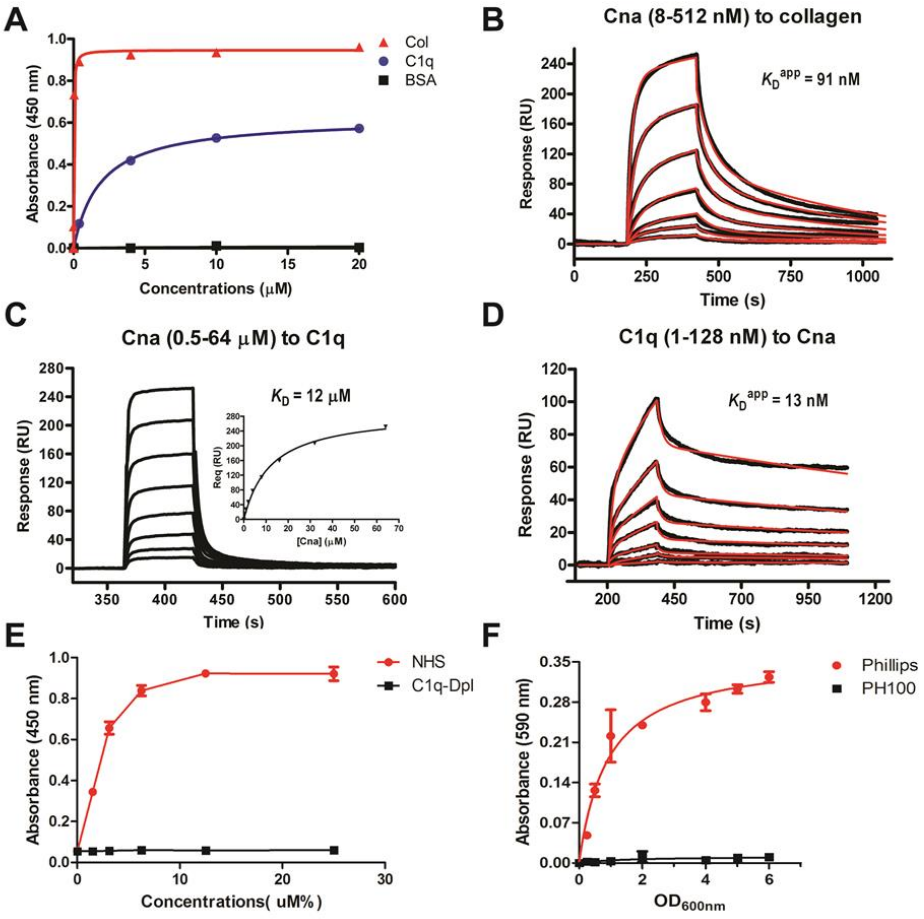

Figure 16. Cna binds to collagen and C1q. (A) ELISA for binding of Cna to immobilized collagen and C1q. (B–D), BIAcore analyses of the interactions between C1q and $Cna_{31–344}$. 2-Fold increasing concentrations (indicated range) of proteins were injected over the immobilized type I collagen (180 RU) (B), C1q (830 RU) (C), and Cna $_{31–344}$ (70 RU) (D). Sensorgrams are shown in black with the lower curve corresponding to the lower concentration of protein injected. Kinetic analyses for (B) and (D) (the fitted curves are shown in *red*) were performed to obtain rate constants and apparent dissociation constants KD_{app}. Association and dissociation rate constants ($ka1$, $kd1$) for the binding state and forward and backward rate constants ($ka2$, $kd2$) for the conformational change state were obtained from the fitting. (B), $ka=17.48\times10^4$ M^{-1} s^{-1}; $k_{d1}=1.71\times10^{-2}$ s^{-1}; $k_{a2}=2.86\times10^{-3}$ s^{-1}; $k_{d2}=1.89\times10^{-3}$ s^{-1}. (D), $k_{a1}=1.66\times10^5$ M^{-1} s^{-1}; $k_{d1}=4.79\times10^{-2}s^{-1}$; $k_{a2}=1.03\times10^{-2}$ s^{-1}; $k_{d2}=4.72\times10^{-4}$ s^{-1}. For steady-state interactions (C), a binding isotherm was created (*inset*) to determine equilibrium *KD*. (E) Immobilized Cna interacts with C1q in the serum.(F) Adherence of *S. aureus* strain Phillips and the correspondence Cna deletion mutant strain (PH100) to C1q. Biacore binding assays were performed by Xiaowen Liang.(Kang et al., 2013)

core chips corroborated our ELISA results and further indicated that the Cna_{31-344} interaction with C1q involves a lower affinity compared to that with collagen type I (calculated K_D of 12 μM and 91 nM, respectively) (Figure 16, C and B). However, when the system was reversed and Cna31-344 was immobilized, C1q bound to Cna_{31-344} with a much higher affinity (K_D = 13 nM) (Figure. 16D). This phenomenon is consistent with what we observed for collagen binding to Cna coated chip, in which a significant higher affinity is also observed (calculated K_D=0.4 pM) (Ross et al, 2012) compared to when Cna binds to collagen immobilized on the chip (calculated K_D= 91 nM). The molecular explanation for this difference is presently unclear; however, the potential for a triple helix collagen monomer and a hexameric C1q to simultaneously bind multiple immobilized Cna_{31-344} molecules likely contribute to the observed differences.

The ability of Cna to interact with C1q in the presence of serum was also evaluated in an ELISA-type assay in which Cna coated wells were incubated with NHS or C1q-dpl serum and bound C1q was detected by polyclonal anti-C1q antibody. The results showed that Cna indeed bound to C1q and/or C1 complex in NHS (Figure 16 E). This observation demonstrated that Cna is able to interact with C1q in its native condition.

A clinical strain of *S. aureus* strain Phillip that expresses full length, native Cna on the bacterial surface and a correspondence Cna deletion mutant strain (PH100) were evaluated for their ability to bind to C1q coated surface. Only wild type Phillip strain that expresses full length Cna bound to C1q whereas the Cna deletion strain PH100 did

not (Figure. 16F). This result demonstrated that bacterial surface anchored native Cna protein interacts with C1q.

Cna mutants with reduced affinity to collagen decrease affinity to C1q

Our previous studies have demonstrated that the Y175 in N2 domain of Cna is important for collagen interaction (Symersky et al, 1997) and the linkers between N1 N2 domain harboring insertions or deletions affect Cna binding to type 1 collagen (Ross et al, 2012). In addition, the latch segment found in the C-terminal extension of N2 is likely important for the interaction (Ponnuraj et al, 2003; Zong et al, 2005). Thus we have generated a panel of Cna mutants including a point mutation in the N2 domain resulting in loss of collagen binding (Y175K), extending or truncating the linker between the N1 and N2 domains in Cna (QIE, ΔEAG and ΔEAGTSS) (Ross et al, 2012) and a deletion of the latch segment (Δlatch) (Figure 17A and Table 3). These mutants have altered collagen-binding activities compared to that of the wild-type Cna protein (Figure 17, B and D). The ability of these mutants to bind to C1q as compared to type 1 collagen was evaluated by ELISA-type binding assays and SPR analyses. Relative binding activities of these mutants to C1q closely tracked those to collagen (Figure 17, B and C), and the mutants that exhibited lower affinities for collagen also showed lower affinities for C1q in SPR experiments (Figure 17, D and E). In the ELISA binding assay, half-maximum binding for QIE, Δlatch, Y175K, ΔEAG, and ΔEAGTSS mutants to C1q was obtained at concentrations of 4.7, 15.3, 20.6, 32.9, and 39.1 μM, respectively, compared to wild-type Cna in which half-maximum binding was observed at a concentration of 1.4

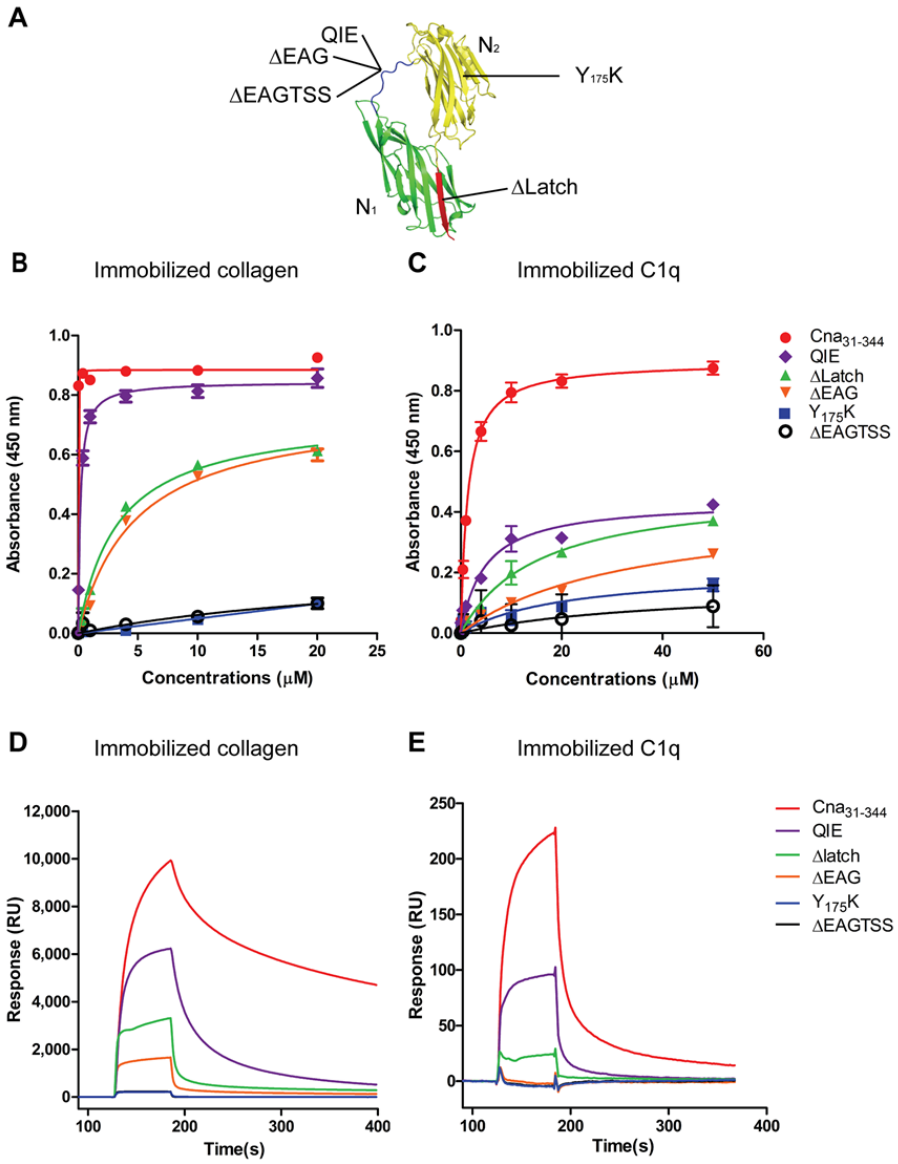

Figure 17. Cna likely binds to the collagenous domain in C1q. (A) A crystal structure of the Cna N1N2 domains with specific mutations indicated. (B-E) Binding of Cna31-344 (red), QIE (purple), Δlatch (green), ΔEAG (orange), Y175K (blue) and ΔEAGTSS (black) to collagen (B, D) and to C1q (C, E) by ELISA binding assays (B, C) and biacore analyses (D, E). Sensograms are shown for comparing the interaction of 10 μM Cna proteins with immobilized (D) collagen (6800 RU) and (E) C1q (7400 RU) on a CM5 chip. Biacore binding assays were performed by Xiaowen Liang. (Kang et al., 2013)

μM (Figure 17 C). Taken together these results strongly suggest that Cna recognizes C1q and collagen type I in similar manners and indicate that Cna likely binds to the collagenous domains in C1q.

Cna inhibits activation of the classical complement pathway

The observed binding of Cna_{31-344} to C1q raised the possibility that the MSCRAMM could modulate complement activation. To explore this possibility, I used functional readouts for activation of the CP including a hemolytic assay and an ELISA-type assay where I quantitated the deposition of C4b on antibody coated surfaces. Hemolysis of antibody-sensitized sheep erythrocytes (EAs) was effective when the EAs were incubated with normal human serum (NHS) but not when the EAs were incubated with C1q-depleted (C1q-dpl) serum (Fig. 18A). Supplementing C1q-dpl serum with C1q restored full hemolytic activity (Fig. 18A), demonstrating that under the conditions used, hemolysis of EAs is primarily the consequence of CP activation and that the presence of C1q is required for the activity. Factor B is a key protease in the activation of the AP. EAs hemolysis in factor B-depleted (fB-dpl) serum is indistinguishable from that in NHS (Figure 18A), suggesting that the AP has a minor (if any) effect on hemolysis under these experimental conditions. However, generation of C3b convertase (C4bC2a) as the result of CP activation may possibly initiate the AP. To exclude any involvement of the AP, I used fB-dpl serum in some experiments.

Addition of purified recombinant His-tagged Cna_{31-344} to NHS or to fB-dpl serum prior to the addition of sensitized erythrocytes inhibited hemolysis of EAs in a dose-

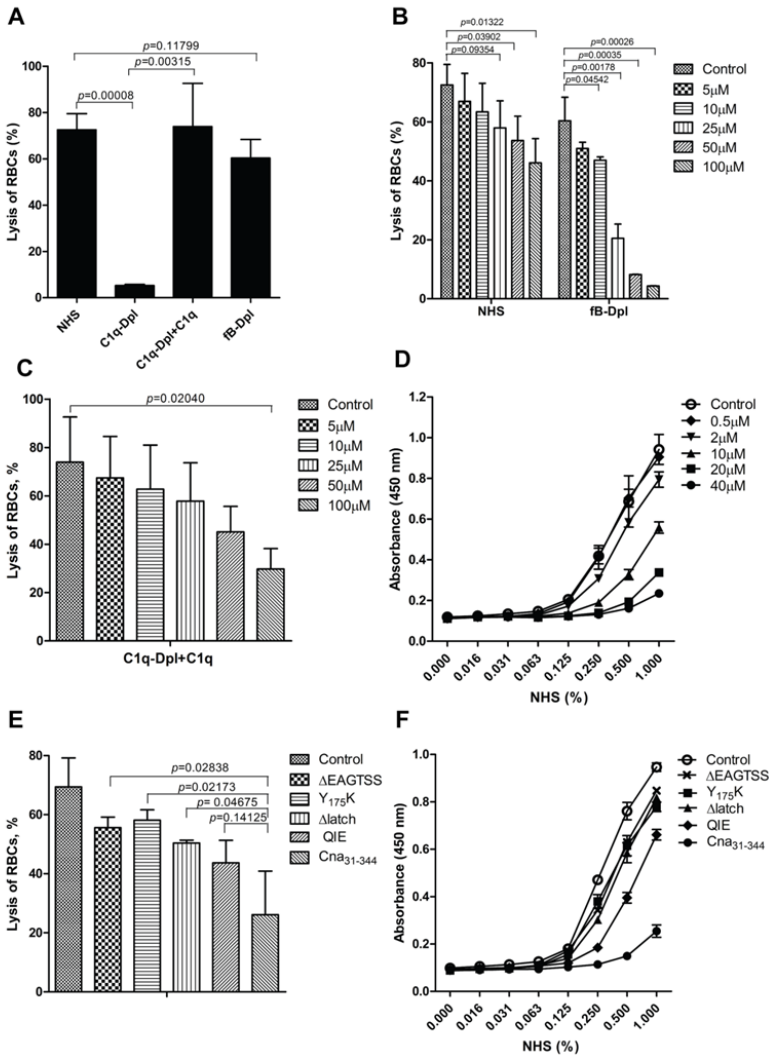

Figure 18. Cna inhibits activation of the CP. (A) Hemolysis of EAs in 0.5% NHS, 0.5% fB-dpl serum, or 0.5% C1q-dpl serum without or with C1q (70 μg/ml). Data represent mean ± s.d. of three separate experiments. (B) Hemolysis of EAs in 0.5% NHS and 0.5% fB-dpl serum with various concentrations of recombinant Cna31-344. Data represent mean ± s.d. of three separate experiments. (C) Cna31-344 dose-dependently inhibits hemolysis of EAs in 0.5% C1q-dpl serum with C1q (70 μg/ml). Data represent mean ± s.d. (n=3). (D) C4b deposition in different concentration of NHS with increasing concentration of Cna31-344. E and F. Effects of Cna mutants on activation of the CP. (E) Classical-pathway mediated hemolysis of EAs in 0.5% fB-dpl serum with 20 μM of Cna mutants. Figures represent mean ± s.d. of three separate experiments. (F) C4b deposition in various concentrations of NHS with 20 μM of Cna31-344 and its mutants. Control, buffer.(Kang et al., 2013)

dependent manner (Figure 18B), demonstrating that Cna_{31-344} blocks the formation of the MAC on the erythrocytes surface. The inhibitory effect of Cna_{31-344} was somewhat more pronounced when fB-dpl serum was used compared to NHS, supporting the hypothesis that Cna inhibits the CP. Cna_{31-344} also inhibited hemolysis in C1q-dpl serum complemented with C1q (Figure 18C).

Next, I determined the relative amounts of C4b generated and deposited on a microtiter plate coated with IgM as a read-out for CP activation. C4b deposition was increased with increasing concentrations of NHS. At a fixed concentration of NHS, pre-incubation of the serum with Cna31-344 decreased C4b deposition, and the effect depended on the amount of added Cna (Figure 18D). In contrast to IgM coated surface, I did not observe C4b deposition on the Cna coated surface, suggesting that Cna did not by itself activate the CP. Thus the observed CP inhibition (Figure 18, C and D) is not due to a consumption of complement components.

Cna mutants lost the ability to inhibit classical pathway activation

I next evaluated the ability of the Cna mutants to inhibit activation of the CP. Compared with wild-type Cna, Cna mutants (QIE, Δlatch, Y175K, and ΔEAGTSS) that had a reduced affinity for C1q, showed a diminished ability to inhibit EAs hemolysis. The percentage of erythrocytes lysed in the presence of wild-type Cna (26%) was increased to 44%, 50%, 58% and 56% in the presence of mutants QIE, Δlatch, Y175K, and ΔEAGTSS, respectively (Figure 18E). The ability of these mutants to inhibit C4b generation and deposition was reduced in a similar manner (Figure 18F). These results

correlated the degree of inhibition of CP activation with the relative C1q-binding affinities (Fig. 17C, 17E, 18E and 18F).

Taken together, our data strongly suggest that Cna_{31-344} interferes with activation of the classical complement pathway and the relative inhibitory activity on Cna mutants correlate with their relative collagen binding activity.

Molecular bases for Cna dependent inhibition of the classical pathway

Formation of the C1 complex is a prerequisite for activation of the CP. The C1r2C1s2 tetramers bind to the collagenous regions in C1q to form the C1 complex (Bally et al, 2009). Our results indicated that Cna31-344 also binds to the collagenous domain in C1q. I therefore asked if Cna31-344 could interfere with the interactions between C1r2C1s2 and C1q. Cna31-344 inhibited C1q from binding to C1r in a competition ELISA where wells coated with C1r were incubated with a constant amount of C1q and increasing concentrations of Cna31-344 (Figure 19A). Cna31-344 had a marginal inhibitory effect on C1q binding to immobilized C1s (Figure 19B). The Y175K mutant, that lost its affinity for C1q, did not affect the C1q-C1r or the C1q-C1s interactions (Figure 19, A and B). These results suggested that Cna interferes with the C1r-C1q interaction by binding to C1q.

The majority of C1q in normal serum is present in the C1 complex (De Bracco & Manni, 1974). I therefore investigated if Cna31-344 could disassemble a preformed C1 complex. Pull down assays were conducted using a monoclonal antibody against C1q and the composition of the pulled down C1 complex was evaluated using specific

antibodies against C1r or C1s. Strikingly, addition of soluble Cna31-344 to the C1 complex significantly decreased the amount of C1r and C1s that was retained in the C1 complex, whereas the amount of C1q pulled-down was similar to that in the control group (Figure 19C), suggesting that Cna displaced C1r and C1s from the C1 complex. Pre-incubation of the C1 complex with the Y175K mutant did not affect the amount of C1r and C1s in the pulled-down complex (Figure 19C). An anti-His antibody detected recombinant His-tagged Cna31-344 protein in the complex that was pulled down by the anti C1q antibody (Figure 19C), demonstrating that Cna31-344 indeed bound to C1q.

I also investigated if surface-bound Cna31-344 is capable of displacing C1r and C1s from the C1 complex. Microtiter wells were coated with either IgM (Figure 19D) or Cna31-344 (Figure 19E) and incubated with increasing concentrations of the C1 complex. The presence of C1q, C1r, and C1s within the bound complex was detected with specific antibodies. In IgM coated wells all three C1 components were successfully detected and the amount of C1q, C1r and C1s detected increased in parallel with the amount of complex added (Figure 19D). Isolated C1r and C1s had negligible IgM-binding activity (data not shown), indicating that the detected C1r and C1s is part of an IgM-bound C1 complex. Intriguingly, C1q was the only C1 complex subunit that could be detected on the Cna31-344-coated surface, demonstrating that surface-bound Cna31-344 also displaced C1r2C1s2 from the C1 complex (Figure 19E). Collectively, these data demonstrate that Cna31-344 binding to C1q interferes with the C1q interaction with C1r and results in dissociation of the C1 complex.

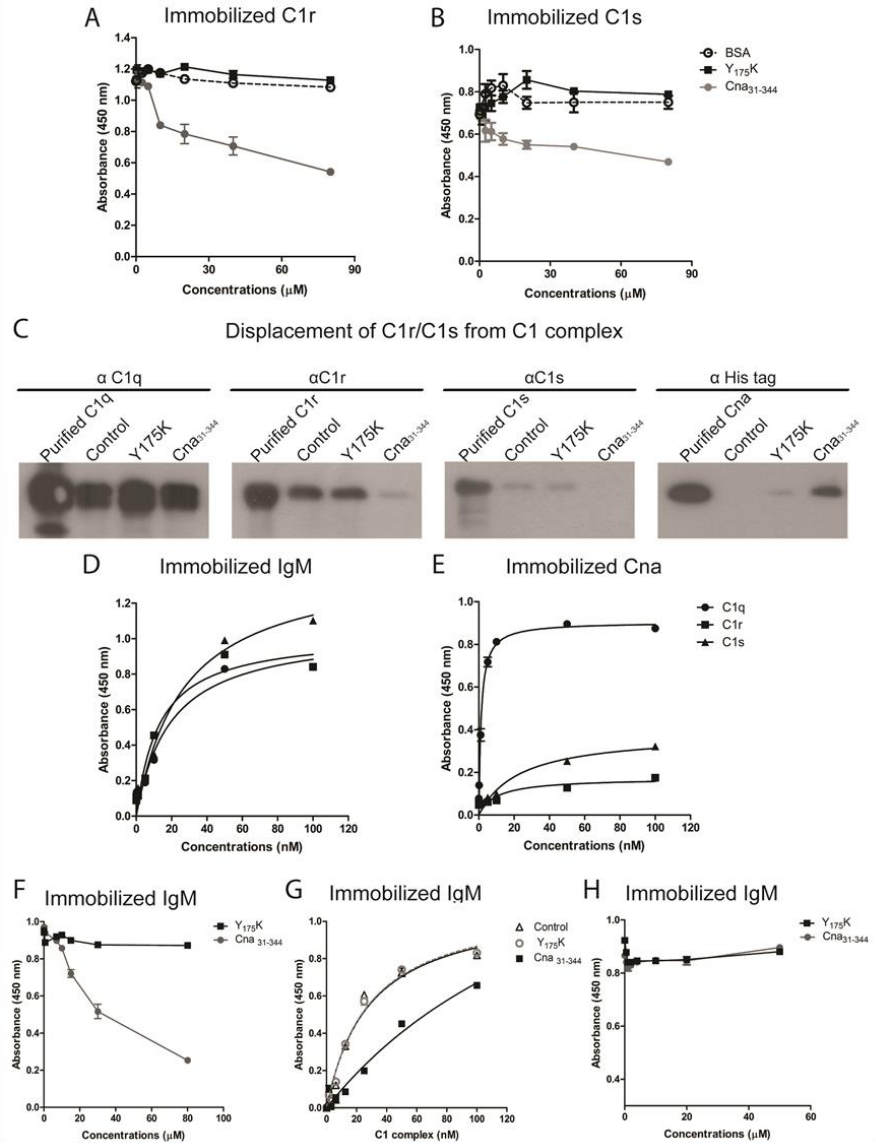

Figure 19. Molecular bases for Cna-dependent inhibition of the classical pathway. A and B**.** Cna prevented C1q from binding to immobilized C1r (A) and C1s (B). Binding of C1q (20 nM) to C1r and C1s in the increasing concentrations of recombinant Cna_{31-344} or $Y_{175}K$. C-E. Cna displaced the $C1r_2C1s_2$ tetramer from the C1 complex. C. Immuno-pull-down assay. C1 complex (35 nM) was pulled down by anti-C1q monoclonal antibody in the presence of 80 μM of recombinant Cna_{31-344} or $Y_{175}K$. Control, buffer. Immunoblots are probed separately with antibody against C1q (αC1q), C1r (αC1r), C1s (αC1s) or anti-His antibody. D and E. Binding of C1 complex (0-100 nM) to IgM (D) or Cna_{31-344} (E) (both at 0.5 μg/well) surface. Each subunit of C1 complex was detected by goat anti- C1q polyclonal, goat anti-C1r polyclonal or sheep anti-C1s polyclonal antibodies. F-H. Cna inhibits C1 complex binding to IgM surface. Binding of C1 complex (F**,** 20 nM) or C1q (H, 20 nM) to human IgM (0.5 μg/well) in increasing concentrations of Cna_{31-344} and $Y_{175}K$. (G) Dose-dependent binding of C1 complex (0-50 nM) to human IgM (0.5 μg/well) in 50 μM of Cna_{31-344}, $CnaY_{175}K$ and buffer control. (Kang et al., 2013)

Recognition and binding to surface-bound antibodies by the C1 complex is required for initiation of the CP. I next investigated if Cna31-344 also affects the recognition of surface-bound antibodies by the C1 complex. This hypothesis was tested in ELISA-type binding experiments using IgM coated microtiter wells. Increasing concentration of Cna31-344 inhibited the C1 complex binding to the IgM-coated surface (Figure 19F). In contrast, Y175K, which does not bind C1q, did not interfere with the C1 complex-IgM interactions (Figure 19F). Consistent with this finding, dose-dependent binding of the C1 complex to the IgM coated surface was significantly attenuated by addition of Cna31-344 (50 μM) (Figure 19G). The half-maximum binding concentration increased from 6 nM (control, in the absence of Cna31-344) to 94 nM (in the presence of Cna31-344), indicating a nearly 17-fold decrease in binding affinity. Addition of Y175K did not alter the binding kinetic of C1 complex to IgM and the kinetic was indistinguishable from that of the control (Figure 19G). These results demonstrated that Cna inhibits the C1 complex binding to the immune complex and that the activity is determined by the C1q-binding affinity. Interestingly, the influence of Cna on binding of isolated C1q to IgM was insignificant (Figure 19H). The fact that the C1 complex exhibits a higher affinity for immune complexes than does isolated C1q (data not shown) supports the idea that two distinct conformational states with different binding properties exist for C1q in these two conditions (Perkins, 1985) and our results indicate that Cna binding to the C1q subunit may stabilize the conformation of C1q that has a low affinity for immune complexes.

In summary, I demonstrated that Cna (and presumably other members of the Cna-like family of collagen-binding MSCRAMMs) inhibits activation of the CP by binding to C1q and interfering with the C1q-C1r interaction thereby disassembling and deactivating the C1 complex.

Other Gram-positive collagen-binding MSCRAMMs bind C1q and block the classical pathway

A number of Gram-positive bacteria express collagen-binding MSCRAMMs that have similar structural organizations (Figure 13) and show a high degree of sequence and structural similarities within the collagen binding segments. Cna from S. aureus is the prototype member of this family of collagen-binding MSCRAMMs that include Ace from *Enterococcus faecalis* (Liu et al, 2007), Acm from *Enterococcus faecium* (Nallapareddy et al, 2003), Cne from *Streptococcus equi* (Lannergard et al, 2003), and Cnm from *Streptococcus mutans* (Nakano et al, 2010) and others (Patti et al, 1994). To determine if these collagen-binding MSCARAMMs bind C1q and function as complement inhibitors, I first compared the collagen- and C1q-binding activities of purified recombinant proteins containing N1N2 domains of these MSCRAMMS (Table 3). These proteins all bound to collagen and C1q in dose-dependent manners although they exhibited different binding capacities in the ELISA-type assays (Figure 20, A and B). Among them, Cna31-344 bound to collagen with the highest affinity, with half-maximum binding observed at a concentration of 0.02 μM, whereas Ace32-367 showed minimal binding activity under the conditions used (Figure 20A). Binding of these

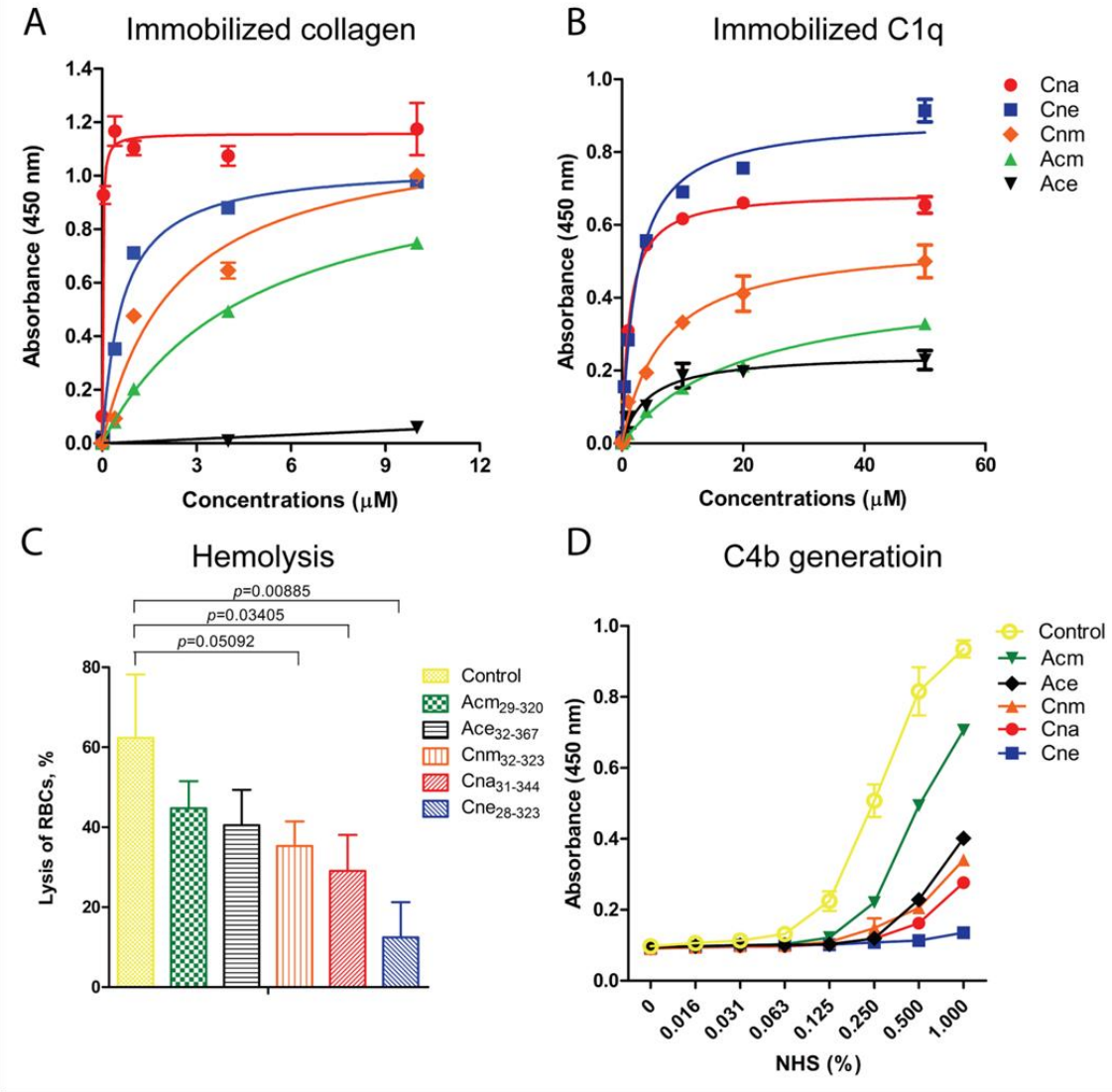

Figure 20. Structurally related collagen-binding MSCRAMMs bind to C1q and inhibit the CP activation. A. and B. ELISA-type binding assays for binding of recombinant N_1N_2 domain of collagen-binding MSCRAMMs to immobilized collagen (A) and C1q (B). C. Classical-pathway mediated hemolysis of EAs in 0.5 % fB-dpl serum with 20 μM of collagen-binding MSCRAMMs. Data represent mean ± s.d. of three separate experiments. D. C4b deposition in various concentrations of NHS with 20 μM of collagen-binding MSCRAMMs. Control, buffer. (Kang et al., 2013)

proteins to C1q largely followed the relative binding activities for collagen type I (Figure 20B). However, there were exceptions; Cne28-323 bound somewhat more effectively to C1q than Cna31-344 (Figure 20B) whereas the reverse was seen when collagen type I was targeted (Figure 20A). Ace32-367 showed noticeable binding to C1q (Fig. 5B) whereas Acm29-320 bound weaker to C1q (Fig. 5B) than to collagen type 1 (Figure 20A) and was the worst C1q binder of the MSCRAMMs tested (Figure 20B).

I next determined the effect of these collagen-binding MSCRAMMs on hemolysis and C4b deposition. Consistent with our observation for Cna31-344, all MSCRAMMs inhibited EAs hemolysis (Figure 20C) and C4b deposition (Figure 20D), and the extent of inhibition was directly related to their relative C1q-binding affinities, in which Cne28-323 shown the strongest and Acm29-320 showed the weakest inhibition (Figure 20, C and D). In summary, our results demonstrated that collagen-binding MSCRAMMs from Gram-positive bacteria are also inhibitors of the classical complement pathway.

Discussion

The Cna-related collagen-binding MSCRAMMs represent a growing family that currently includes almost 20 members from a variety of human and animal Gram-positive (Kreikemeyer et al, 2005; Krishnan & Narayana, 2011; Patti et al, 1994; Sillanpaa et al, 2009; Xu et al, 2004a) and -negative pathogens (Fink et al, 2002; MacKichan et al, 2008; Patti et al, 1994; Wagner et al, 2007). I report that at least five members of this MSCRAMM family bind C1q and inhibit the CP of the complement

system. Cna31-344 appears to bind to the triple helix collagenous domains of C1q and a set of Cna mutants showed similar relative affinities for collagen and C1q. Cna interferes with the interaction of C1r with the collagenous "stems" of C1q. As a result the C1r2C1s2 tetramer cannot dock with C1q or, if Cna encounters a preformed C1 complex, C1s and C1r are displaced resulting in a single isolated hexameric non-active C1q. It is likely that all members of the Cna family of collagen-binding MSCRAMMs inhibit the CP by the same molecular mechanism and there is a direct correlation between the individual MSCRAMM's affinity for C1q and relative CP inhibitory activity. However, there is not a direct correlation between an individual MSCRAMM's relative affinity for type 1 collagen and C1q, suggesting subtle differences in binding specificities.

Cna is a proven virulence factor in several *S. aureus* infectious disease models, such as endocarditis (Hienz et al, 1996), osteomyelitis (Elasri et al, 2002; Johansson et al, 2001), keratitis (Rhem et al, 2000) and arthritis (Xu et al, 2004b). Similarly, Ace and Acm contribute to disease progression in experimental endocarditis (Nallapareddy et al, 2008; Singh et al, 2010), while Cnm is critical for *S. mutans* to adhere to heart endothelial cells (Abranches et al, 2011) and is associated with hemorrhagic stroke (Nakano et al, 2011). It has been presumed that the Cna-like MSCRAMMs in these models act as adhesins and promote bacterial colonization of collagen in host tissues. Undoubtedly, Cna can function as an adhesin and mediate staphylococcal colonization of collagenous substrates (Patti et al, 1994). However, our finding that the Cna-like MSCRAMMs are inhibitors of complement activation points to an alternative

mechanism of action for these virulence factors. An immune evasion mechanism may explain some earlier results using an experimental arthritis model (Xu et al, 2004b), in which I showed that the expression of Cna did not contribute to the initial colonization of the tissue by *S. aureus*. Instead, expression of Cna on *S. aureus* elicited a more severe disease progression over a 28 day period. In the same context, Cna-like MSCRAMMs block the formation of the MAC (Figure 20C), the terminal effector function of the complement activation; it is therefore very likely that the generation of the inflammatory mediators (C3a, C5a) during the process of complement activation is also blocked by presence of these MSCRAMMs and subsequently neutrophil recruitment and phagocytosis could also be possibly inhibited. Future studies will address these questions.

Cna binds collagen by the "collagen-hug" mechanism. An initial interaction of Cna with collagen induces a redirection of the sub-domains in the binding region of the MSCRAMM allowing the N1-N2 domains to encompass the triple helix and "hug" the collagen monomer (Zong et al, 2005). The diameter of the "hole" formed by the N1-N2 domains of Cna when complexed to a collagen triple helix peptide is about 15 Å, as calculated from the crystal structure (Zong et al, 2005). This space can accommodate only one monomeric collagen triple helix structure (diameter ~ 15Å) (Brodsky-Doyle et al, 1976; Chung et al, 2004). Thus Cna can only bind to collagen triple helix monomers but not to collagen fibers or fibrils. This property has recently been taken advantage of where investigators have used a fluorescently tagged form of Cna to detect monomeric collagen product during fibrosis (Chen et al, 2011). This specificity also limits the type

of collagenous tissue that can be targeted in Cna-dependent adhesion since collagen is primarily found in higher orders of fibrous super structures in healthy mature tissues. However, the collagenous "stems" of C1q are always available as monomeric triple helix structures. Hence, the ability of the Cna-like MSCRAMMs to bind to C1q and inactivate the CP may be an important component of the virulence potential of these molecules.

Surface-bound Cna interacts with C1q in solution with high affinity (Fig. 17D), which may be due to the ability of one C1q molecule to interact with multiple MSCRAMMs. This represents the most direct and effective way in which the Cna-like MSCRAMMs encounters and inactivates the CP. However, surface anchored MSCRAMMs such as Ace can be cleaved by protease (McAleese et al, 2001; McGavin et al, 1997; Pinkston et al, 2011) and generated MSCRAMM fragments released into the environment may retain their ligand binding activity. This observation raises the possibility that cell-wall anchored MSCRAMMs may not only function as surface-bound virulence factors but also as a reservoir for the release of biologically active compounds. Thus, the release of C1q-binding fragments of Cna-like MSCRAMMs would allow these fragments to inhibit the CP away from the bacterial colony and could result in a more systemic inhibition of the complement system.

Earlier SPR analyses of Cna binding to type 1 collagen demonstrated that the collagen molecule contains multiple classes of binding sites with different affinities for the MSCRAMM (Rich et al, 1999a). Thus, the high affinity Cna binding sites in type 1 collagen are likely composed of different sequences than the Cna binding sites in C1q, which exhibit moderate affinity for the MSCRAMM. Whereas Cna binds with much

higher affinity to type I collagen compared with C1q, some other members of the Cna-like MSCRAMM family studied do not differ in their binding to collagen and C1q, for example Cne. These observations suggest that, although the different members of the Cna-like family of MSCRAMMs have similar sequences and structural organization, they have evolved to target different triple helix motifs with different affinities.

Previous studies suggest that C1q is a flexible molecule that undergoes conformational changes upon binding to the C1r2C1s2 tetramer and antibodies on the bacterial surface (Perkins, 1985; Schumaker et al, 1981). Our data revealed that the C1 complex and isolated C1q differ in their affinities for immobilized IgM (data not shown), supporting the existence of two distinct C1q conformations (Schumaker et al, 1981). This result also demonstrates that the C1 complex form of C1q is in a state that is favorable for antibody-binding, whereas the isolated C1q has a relatively unfavorable binding conformation. The ability of Cna31-344 to displace C1r2C1s2 from the C1 complex (Figure 19, C-E) and to inhibit C1 complex binding to surface-bound antibodies (Figure 19, F and G) suggests that binding of Cna31-344 to C1q stabilizes the low affinity conformation of the complement component. The observation that Cna31-344 did not affect the interaction of isolated C1q with antibodies supports this hypothesis.

In summary, our data provide evidence for the first time that Cna-like collagen-binding MSCRAMMs are not only adhesins but also are potential complement inhibitors. The dual functions of MSCRAMMs illustrate the elaborated strategies evolved by pathogenic microorganisms to adhere to and colonize host tissues and evade the host defense systems. The molecular mechanism uncovered in this study of how

Cna-like MSCRAMMs interact with C1q and disrupt the complement activation may represent a global mechanism used by collagen-binding MSCRAMMs from different bacterial species.

CHAPTER IV

BIOLOGICAL SIGNIFICANCE OF THE CNA-C1Q INTERACTION IN VIVO

Introduction

Septic arthritis is a disease caused by the invasion of infectious agents e.g., bacteria on a joint, which interact with host immune system, and result in the production of a large amount of inflammation (Wang et al, 2009). *S.aureus* is one of the most common bacteria isolated from clinical septic arthritis (Shirtliff & Mader, 2002). It is believed that the collagen-binding MSCRAMM from *S.aureus*-Cna is a critical virulence factor in experimental mouse model of septic arthritis, based on its collagen binding activity (Patti et al, 1994; Xu et al, 2004b). However, the detailed molecular mechanism on the roles of Cna in pathogenesis of arthritis is not well characterized.

A previous structural study in our lab demonstrated that Cna only binds collagen triple helixes, but not collagen fibril (Zong et al, 2005). It is well known that the majority of the collagen in the healthy human or mouse exists in the form of collagen fibril, which provides few binding sites for Cna. This may be the potential explanation for why Cna doesn't contribute to the initial colonization in *S.aureus* arthritis model (Xu et al, 2004b). These data also indicated that besides collagen binding, Cna might also have other functions during the early stage of infection, which might contribute to the pathogenesis of septic arthritis

In chapter III, I have already demonstrated that Cna interacts with C1q and inhibits CP. C1q is a multi-functional molecule that also contributes to other immune

functions such as the clearance of apoptotic cells via direct/indirect interaction or classical pathway activation (Nayak et al, 2010). Failure to clean apoptotic cells in human body will release dangerous signals such as autoimmune peptides, resulting in inflammation and immune disease, such as arthritis. Therefore, I proposed that binding of Cna to C1q contributes to the pathogenesis of *S.aureus* infectious disease.

In this part of the study, I utilized the C1q knockout mice to investigate the biological significance of C1q-Cna interactions in *S.aureus* infections. I performed the *S.aureus* arthritis model and sepsis model by using *S.aureus* clinical Cna positive strain Phillips and its *cna* mutant PH100 (Patti et al, 1994). The results provided, for the first time, direct evidences that Cna enhances *S.aureus* virulence by binding to C1q.

Results

Cna is not a human-specific complement inhibitor

In chapter III, I demonstrated that Cna inhibits the human CP by interacting with collagen-like domain of C1q. To investigate whether Cna is a human specific inhibitor, I examined the Cna- mouse C1q interaction and effects of Cna on mouse CP by analyzing C4b depositions. In microtiter wells, the immobilized recombinant Cna_{31-344} bound to human C1q or mouse C1q in the human normal serum or mouse normal serum respectively, compared to the control, which used the C1q-Dpl serum from human or mouse (Figure 21A). This observation suggested that Cna also interacts with mouse C1q. Moreover, to evaluate the effect of Cna on mouse CP, I quantitated the deposition of mouse C4b on mouse IgM coated surfaces. Not surprisingly, Cna, as well as the

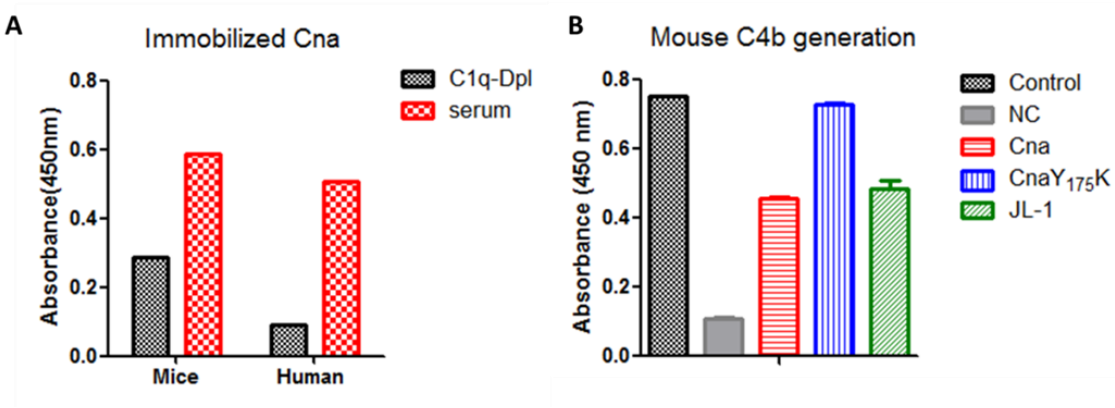

Figure 21. Cna binds mouse C1q and inhibits mouse CP. (A) ELISA for the binding of immobilized recombinant N1N2 domain of Cna to human C1q and mouse C1q in serum condition. (B) C4b deposition at 5% mouse serum with 80 μM Cna. Control, buffer JL-1, monoclonal anti mouse C1q antibody.

monoclonal anti-mouse C1q antibody, strongly inhibited mouse C4b depositions (Figure 21B). In contrast, Cna_{Y175K}, which neither interacts with human C1q nor inhibits human CP, didn't interfere with mouse C4b deposition, compared to the control (Figure 21B). These data suggested that Cna doesn't exclusively inhibit human CP. However, it at least interacts with mouse C1q and blocks the mouse CP.

Cna-C1q interaction enhances *S.aureus* infection

It is well known that the weight loss during infection is usually related to the severity of the disease (Xu et al, 2004b). By exploiting the biological significance of the Cna-C1q interaction in vivo, I compared the weight loss in wild type mice and C1q knockout mice challenged by Cna positive strain Phillips or Phillips *cna* knockout strain PH100 through tail vein IV injection respectively for two weeks. All mice challenged with the bacteria reduced the body weight. However, both wild type mice and C1q knockout mice infected with the Phillips stain reduced weight more rapidly than those of mice infected with PH100 (Figure 22, A and B). These data demonstrated that Cna is a critical virulence factor in the *S.aureus* infection, which validated previous animal experiment results published by our lab (Patti et al, 1994; Xu et al, 2004b). Surprisingly and interestingly, C1q knockout mice lost much less weight than that of wild type mice in both groups challenged by either Phillips strain or PH100 (Figure 22, A and B). In the experimental group infected with Phillips strain, 12 out of 15 wild type mice lost over 30% of weight at day 7, while none of the C1q knockout mice lost more than 30% of weight (Figure 22A). While in the PH100 infection group, none of mice (wild type mice

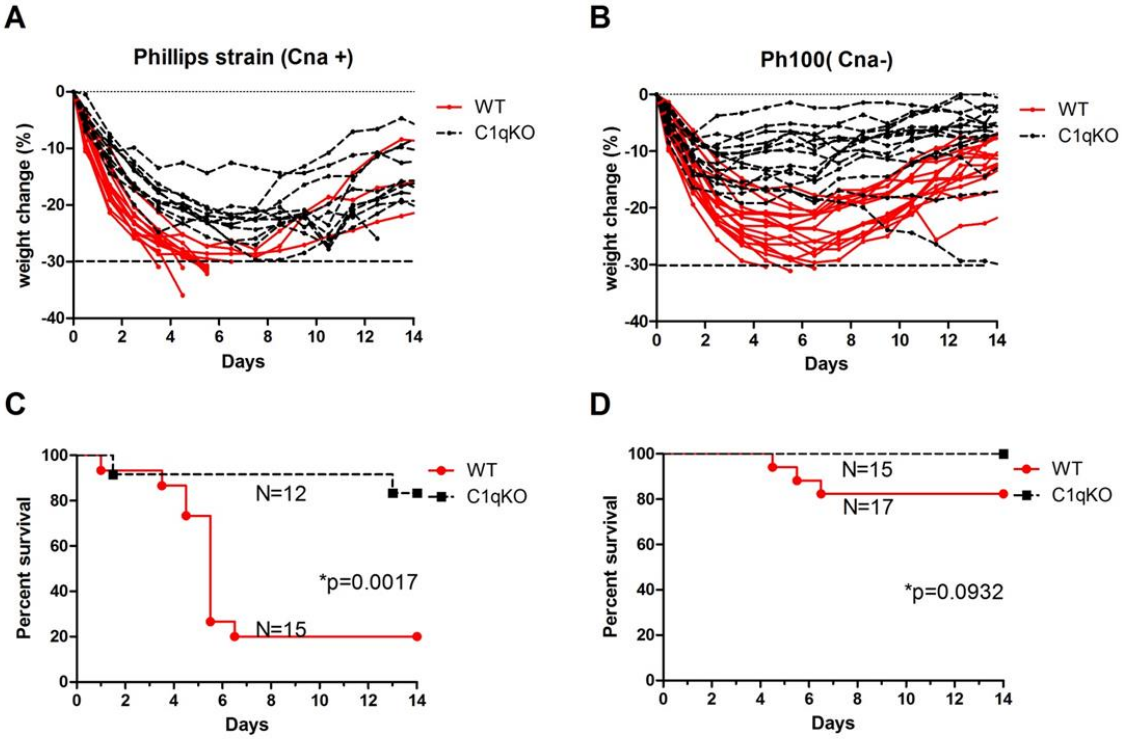

Figure 22. Binding of Cna to mouse C1q contributes *S.aureus* infections. A and B. Body weight change of WT and C1q knockout mice infected with Phillips(A) or PH100 strains (B). *S. aureus* cells (3.5×10^7 cfu) were injected into the tail veins of mice. Weights were recorded for two weeks. C and D. Survival curves for WT mice and C1q knockout mice infected with *S. aureus* Phillips (C) or PH100 (D). Recumbent mice, and mice that lost more than 30% weight, were considered moribund and euthanized.

and C1q knockout mice) lost over 30% of weight (Figure 22B). In order to further interpret the weight loss data, I used 30% of weight loss as the cut-off value to plot the survival curve, including mice that died (Figure 22, C and D). The C1q knockout mice showed significantly more resistance to Phillips strain infection, compared to the wild type mice infected with Phillips strain (Figure 22C), whereas there was no significance in the survival rates between wild type mice and C1q knockout mice infected with PH100 (Figure 22D). These data suggested that Cna-C1q interaction plays a critical role in *S.aureus* virulence.

To further investigate the role of this interaction in vivo, I examined the bacterial burdens in blood and various organs at 24 hours after infection. In blood, *S.aureus* was hardly detected in either wild type or C1q knockout mice infected with Phillips strain or PH100 strain. In contrast, I detected large amounts of *S.aureus* in organs, such as liver, heart, spleen and kidney. These results suggested that *S.aureus* can be easily cleared from blood, be disseminated to and colonize within different organs immediately upon infection. Comparing the bacterial counts between *S.aureus* Phillips strain and PH100 in WT mice, 6 mice challenged with *S. aureus* Phillips had significantly more viable *S. aureus* cells isolated from the heart, kidney and liver, than the mice infected with PH100 strain (Figure 23). Moreover, in kidney and liver from mice infected with Phillips strain, C1q knockout mice exhibited significantly reduced bacterial burden, compared to the WT mice. However, there is no dramatic difference in bacterial burden in kidney and liver between WT mice and C1q knockout mice infected with PH100 (Figure 23). Similarly, I also found that C1q knockout mice infected with Phillips strain showed

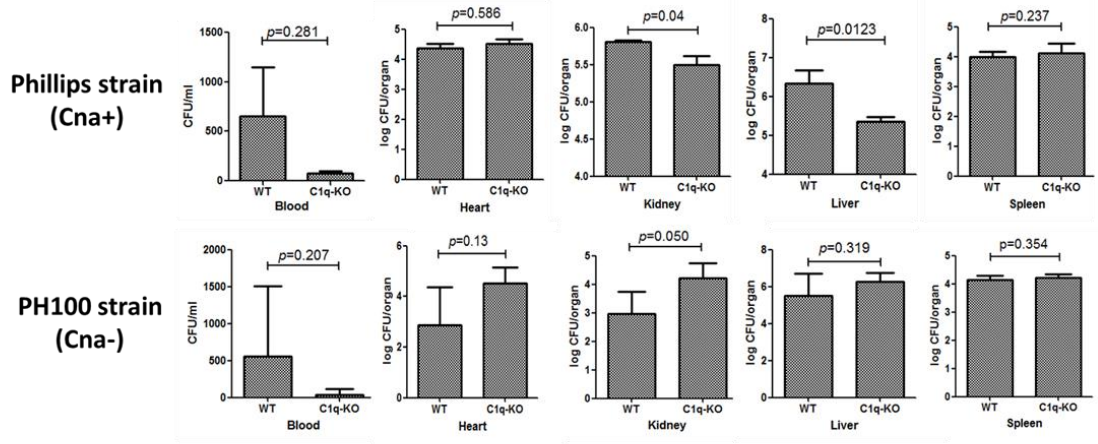

Figure 23. Bacterial burden in the blood and different organs at day 1 after infection. WT or C1q knockout mice infected with 2×10^7 cfu of *Staphylococcus aureus* Phillps and PH100 were killed at 24 hours after infection. Blood, heart, kidney, liver and spleen were taken, homogenized, and plated for bacteria. Statistical significance was calculated by student T- test.

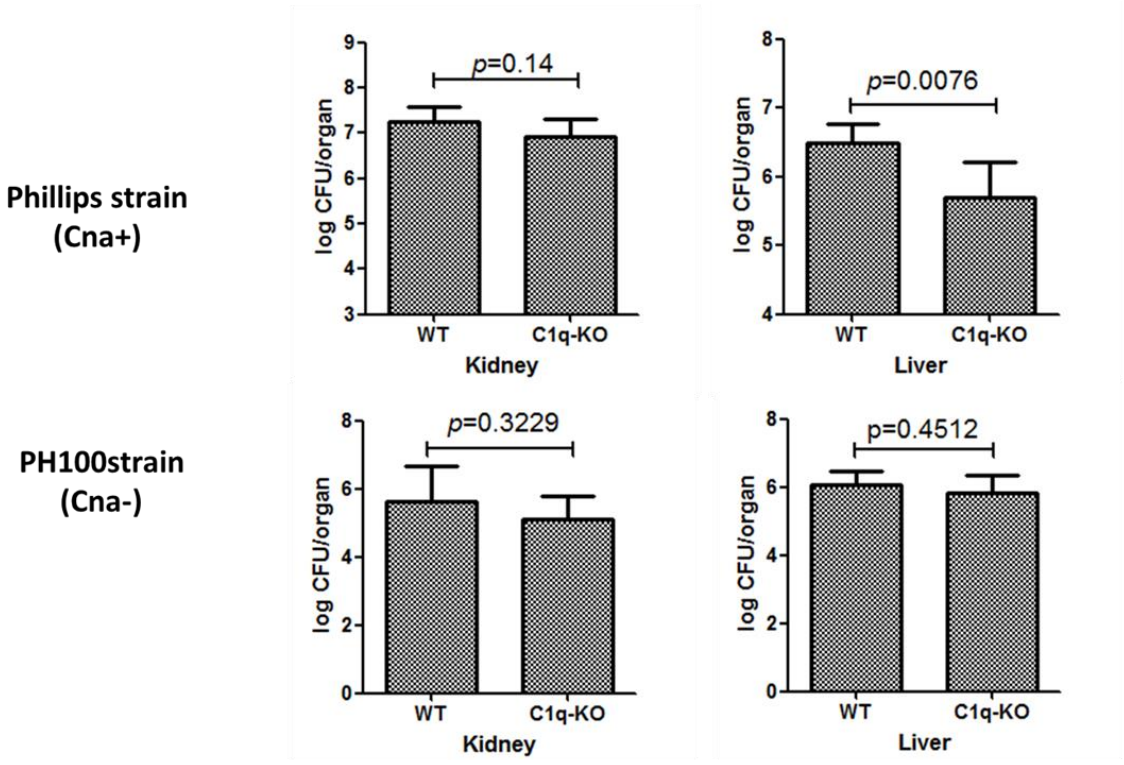

Figure 24. Bacterial burden in kidney and liver at day 6.5 after infection. WT or C1q knockout mice infected with 2×10^7 cfu of *Staphylococcus aureus* Phillps and PH100 were killed at day 6.5 after infection. Kidney and liver were taken, homogenized, and plated for bacteria. Statistical significance was calculated by student T- test.

dramatically reduced bacterial counts in liver than those of the infected WT mice at day 6.5 (Figure 24). These data further indicated that Cna-C1q interaction enhances *S.aureus* infection and dissemination.

Discussion

Cna is a well-known virulence factor in several *S. aureus* infectious disease models such as endocarditis (Hienz et al, 1996), osteomyelitis (Elasri et al, 2002; Johansson et al, 2001), keratitis (Rhem et al, 2000) and arthritis (Xu et al, 2004b) and it is believed that the collagen-binding activity of Cna is a critical determinant for these diseases. Here I report that besides collagen binding, the binding of Cna to C1q may also contribute to the pathogenesis of *S.aureus* infectious diseases. Specifically, our *S.aureus* infection mouse model demonstrates that Cna-C1q interaction enhances *S.aureus* virulence, dissemination to and colonization within the liver in mice.

C1q is highly conserved among different species. The comparison of amino acids of each polypeptide chain between human and mouse C1q showed a high similarity (over 80%) in sequence. Not surprisingly, Cna also interacts with mouse C1q and inhibits mouse CP C4b depositions, while Cna_{Y175K} doesn't inhibit c4b depositions (Figure 21). I also believed that the binding mechanisms and the inhibition mechanisms are similar between human and mouse.

It is reported that C1q itself and the CP play an important role in the recognition and the clearance of microbes and their deficiencies significantly facilitate bacterial infections (Schejbel et al, 2011). Therefore, based on the above studies, C1q knockout

mice should be more susceptible to bacterial infections, such as *Salmonella* infection (Warren et al, 2002). Surprisingly, our data show that C1q knockout mice are more resistant to Cna positive *S.aureus* Phillips infection. During this infection model, only two WT mice and one C1q knockout mice "really died" after Cna positive *S.arueus* infections. Most of the mice challenged with Cna positive bacteria continued to lose the body weight to over 30%. Even though the mice were not in a good condition, they did not die. This phenomenon is dramatically different from that of the *S.aureus* ClfA sepsis model performed in our lab previously (unpublished data), where most of mice died quickly. Because of higher bacterial load in the liver from WT mice infected with Cna positive *S.aureus*, I believe that the body weight loss of mice infected with Cna positive *S.aureus* may be presumably caused by the failure of liver functions, which needs further investigations. More surprisingly, the WT mice infected with Cna negative *S.aureus* (PH100) also experienced significantly more body weight loss than that of the C1q knockout mice, despite that it had much lower significance level compared to the weight loss difference between WT mice and C1q knockout mice infected with Cna-positive *S.aureus* (Figure 22 A and B). Therefore, I proposed that PH100 might secrete another C1q receptor. We used the supernatant of PH100 overnight culture media to pass through immobilized C1q sensor chip and observed the binding responses (Figure 25). Further experiments are needed to identify this novel C1q receptor.

Combining these in vitro and in vivo data, I believe that Cna might have other biological functions, besides acting as a collagen adhesin and complement inhibitor.

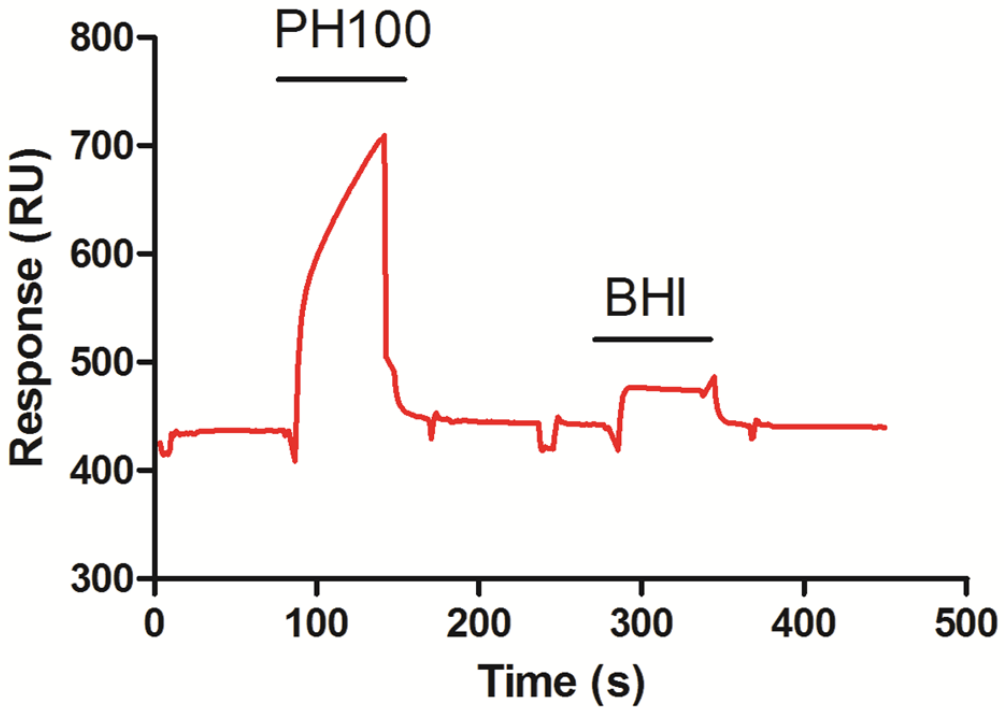

Figure 25. Binding of component(s) from the supernatant of PH100 culture medium to C1q. 2.5 × concentrated supernatant of PH100 overculture media or culture media (BHI) were run through the C1q sensor chip. Biocore assay was performed by Xiaowen Liang.

The most recent reports published from our lab showed that *S.aureus* utilizes Efb to form the fibrinogen "shield", in order to inhibit phagocytosis (Ko et al, 2013). It is implied that membrane-bound Cna might have similar functions. In the blood, membrane-bound Cna or soluble cleaved Cna interacts with C1q and inhibits the CP. Meanwhile membrane-bound Cna will hijack C1q to form the C1q "shield" to block the immune system recognition. Furthermore, our bacteria count data demonstrated that this interaction benefits *S.aureus* dissemination and liver colonization (Figure 23 and 24). It is well known that the epithelial cells of liver and many immune cells located in the liver express C1q receptors. These "coated C1q" on bacterial surface might also interact with C1q receptors on the surface of immune cells and help *S.aureus* dissemination to and colonization of liver.

Aside from the classical pathway activation, C1q also has multiple functions, such as clearance of the apoptotic cell (Nayak et al, 2010). Failure to clear apoptotic cells will lead to inflammation and autoimmunity. Both osteomyelitis and septic arthritis are diseases of inflammation. It is reported that C1q is associated with the pathogenesis of arthritis (Olsen et al, 1991). On the other hand, Cna from *S.aureus* is a virulence factor in experimental animal models of osteomyelitis and arthritis. Therefore, I hypothesized that Cna-C1q interaction contributes to the *S.aureus* pathogenesis, especially septic arthritis. To test this hypothesis, I evaluated this interaction in *S.aureus* arthritis model by using C1q knockout mice. Unfortunately I didn't see any clinical signs of arthritis in both WT mice and C1q knockout mice infected with Cna positive *S.aureus* Phillips strain. This could mainly be due to that the mouse strain (C1q knockout mice are

in a C57BL/6 background) I used is not genetically susceptible to the development of arthritis induced by *S.aureus* (Bremell et al, 1991). In order to continue the study on the role of C1q-Cna interaction in arthritis model, C1q knockout mice in Swiss or BalbC strain background will be used, which is our plan for future studies.

Overall, I have shown that Cna-C1q interaction enhances *S.aureus* infection and dissemination. These data also indicated that Cna is not only a complement inhibitor for classical pathway activation, but also utilizes C1q to evade immune system and help *S.aureus* dissemination. These findings reveal the complicated roles of Cna in *S.aureus* pathogenesis.

CHAPTER V

COMPLEMENT EVAVSION BY BBP (SDRE)

Introduction

The complement system, a major component of the innate immunity, consists of different proteins and glycoproteins that interact with one another leading to cascades of immune response through three distinct pathways. All of these pathways converge to C3 convertase which cleavages C3 into C3a and C3b. There are two different kinds of C3 convertases: C4bC2a and C3bBb. In the CP and the LP, active serine proteases (C1s or MASP2) cleave C4 and C2 to form C4bC2a. Whereas in the AP, the deposited C3b fragment interacts with factor B to form C3bB complex. Binding of factor D to C3bB complex activates factor D, which can cleave factor B and then release the small fragment Ba. The remaining fragment C3bBb is a functional C3 convertase. Since C3 convertase plays an essential role in the complement activation, numerous pathogenic bacteria employ distinct molecules to target C3 converses, especially C3b. As successful pathogenic bacteria, *S.aureus* also utilizes various proteins, most of which are secreted proteins, such as Efb and SCIN, to interfere with the functions of C3 or C3b.

Recently, it is reported that the Fg-binding MSCRAMMs from *S.aureus* - SdrE and Bbp bind to the complement regulators factor H and C4BP to inhibit PMN killing (Hair et al, 2013; Sharp et al, 2012). Bbp and SdrE are allelic variants (Hair et al, 2013), both of which have similar domain organizations containing N-terminal Fg-binding domain A followed by three B repeats and Ser-Asp (SD) dipeptide repeats. The Fg-

binding domain only contains N2N3 subdomains, to which SdrE and Bbp exhibit around 67% amino acid sequence similarity (Francis, 2013). However, in the N1 subdomain and B repeats, 95% sequence similarity is found between Bbp and SdrE. Due to the difference in the Fg-binding domain, SdrE and Bbp show different Fg binding specificity for different species (Francis, 2013). Bbp is more human specific, while SdrE has a weak affinity to human Fg, despite of relatively higher affinity to animal Fg, such as that of cow, pig, dog, sheep and cat.

In this study, I report that Bbp (SdrE) inhibits all three complement pathways and only inhibits C3b depositions of the AP via binding to C3b and interfering with the formation of C3b convertase.

Results

Bbp and SdrE inhibit complement activations

All complement pathways play an important role in anti-bacterial infection. In order to further evaluate the effect of Bbp or SdrE on each complement pathway, I investigated whether Bbp or SdrE inhibits the CP and the AP by using hemolysis assays and the LP using the ELISA-type assay where I quantitated the deposition of C5b-9 on mannan coated surfaces. Hemolysis of antibody-sensitized sheep erythrocytes (EAs) is used to analyze the CP. When the EAs were incubated with NHS, hemolysis of EAs was effective (Figure 26, A and F). The addition of purified recombinant His-tagged and truncated Bbp and SdrE proteins containing N2N3 to the NHS, prior to the addition of sensitized erythrocytes, inhibited the hemolysis of EAs by around 60% at the

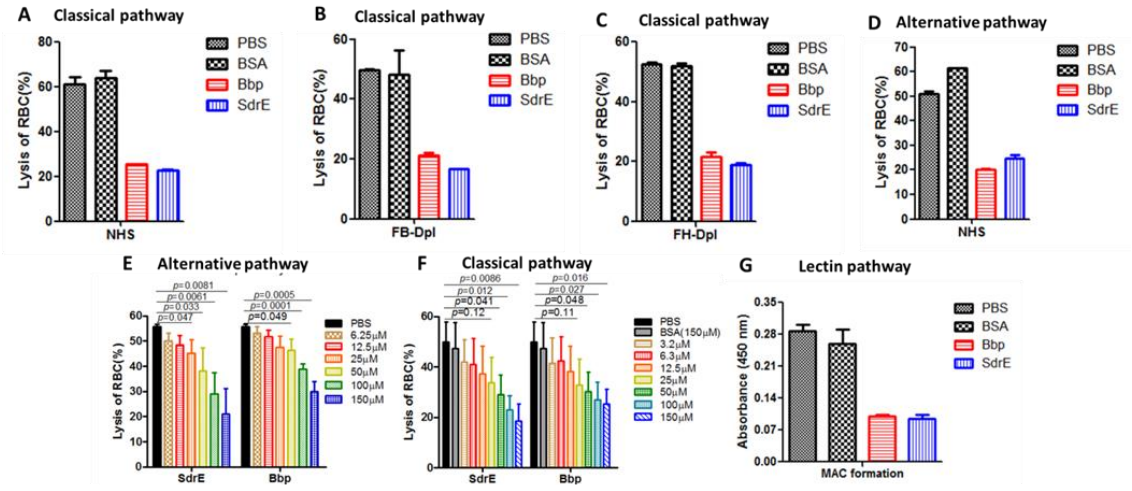

Figure 26. SdrE and Bbp inhibit all three complement pathway activations. A-C and F. Hemolysis mediated by the CP. (A and F) 0.5% normal human serum; (B) 0.5% factor B-depleted serum; (C) 0.5% factor H- depleted serum. D and E. Hemolysis mediated by the AP using 7% normal human serum. (G) C5b-9 deposition via the LP (2.5% normal human serum). Data E and F are mean +S.D. from one representative of three separate experiment and P values were calculated by student T test. Bbp and SdrE, recombinant proteins and concentrations of proteins used in A-D, and G are 150 μM.

concentration of 150 μM. This result demonstrated that Bbp and SdrE block the formation of MAC on the erythrocyte surface. Since the generation of C3b convertase (C4bC2a) in the CP activation might possibly facilitate activation of the AP, I used fB-dpl serum without AP activity to exclusively analyze activation of the CP. The effect of Bbp and SdrE on hemolysis mediated by fB-dpl serum was similar to the NHS (Figure 26B). It is reported that Bbp/SdrE interacts with factor H and inhibits PMN killing (Sharp et al, 2012). To exclude the possibility that the binding of Bbp/SdrE to factor H inhibits complement activation, I tested the hemolysis by using fH-dpl serum and also got similar inhibition results as using NHS (Figure 26C), which indicates that Bbp/SdrE-factor H interaction doesn't affect the inhibition of hemolysis mediated by CP. Similarly, in the AP, hemolysis of rabbit erythrocytes mediated by NHS was also blocked by Bbp and SdrE at the concentration of 150 μM (Figure 26D). To show the specificity, I also performed dose-dependent inhibition assays and observed that Bbp and SdrE inhibited hemolysis in dose-dependent manner for both the CP and the AP. The reduction was statistically significant at protein concentration of 50 μM and above (Figure 26, E and F). The hemolysis assay cannot be used for the LP. To evaluate the effect of Bbp and SdrE on the LP, I detected the deposited MAC (C5b-9) by ELISA. Bbp and SdrE also inhibited the MAC deposition by over 60% at the concentration of 150 μM (Figure 26 G).

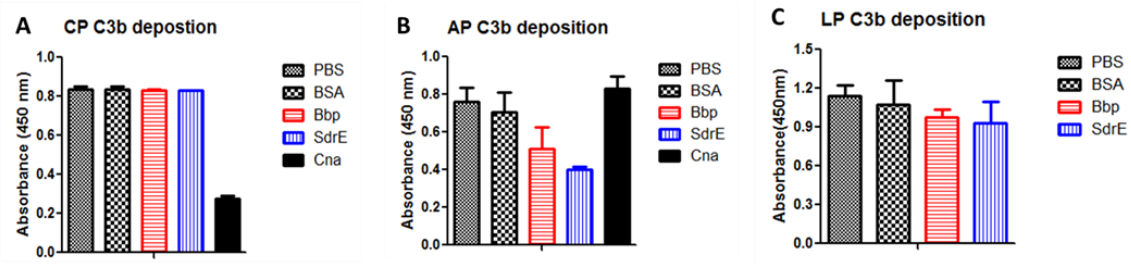

Figure 27. Bbp and SdrE only interfere C3b depositions of AP. A-C. C3b depositions via the CP (A, 1% NHS), the AP (B, 7% NHS) and the LP (2% NHS) in the presence of recombinant Bbp and SdrE at 150 μM.

Bbp and SdrE inhibit C3b deposition of the AP, but not the LP and the CP

My data demonstrated that Bbp and SdrE inhibit all three complement pathways. To narrow down Bbp and SdrE's target(s), I evaluated the complement activation by detecting the deposited C3b. The relative amount of C3b generated and deposited on a microtiter plate coated with IgM, mannan and LPS as a read-out for the CP, the LP and the AP activation respectively. At a fixed concentration of NHS, pre-incubation of the serum with Bbp and SdrE only interfered with the C3b deposition of the AP (Figure 27B), whereas the C3b depositions of the CP and the LP were not affected (Figure 27 A and C).

Bbp or SdrE interacts with C3, C3b and C3c

The observed inhibition of all three complement pathways and the AP C3b deposition mediated by Bbp and SdrE raised the possibility that Bbp and SdrE could act either on C3 or C3 small fragments, which are critical complement components in all complement pathways. To evaluate the direct interaction between C3 or C3b and Bbp or SdrE, I detected the C3b binding by using ELSIA and SPR binding assay. Bbp bound human Fg or C3b coated in microtiter wells in a dose dependent manner and exhibited saturation kinetics (Figure 28). While Bbp has much higher affinity to human Fg, compared to C3b binding. The half maximal bindings are 0.23 μM and 4.1 μM, respectively. ClfB is another fg-binding MSCRAMM from *S.aureus*, which doesn't bind to immobilized C3b at all concentrations tested (Figure 28).

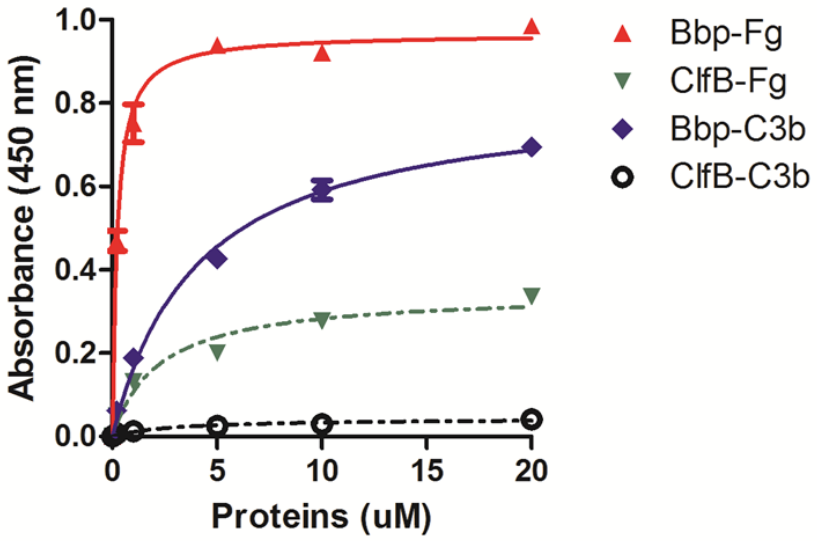

Figure 28. Binding of Bbp to immoblized C3b. Binding of recombinant Bbp and ClfB to fibrinogen and C3b by ELISA binding assays

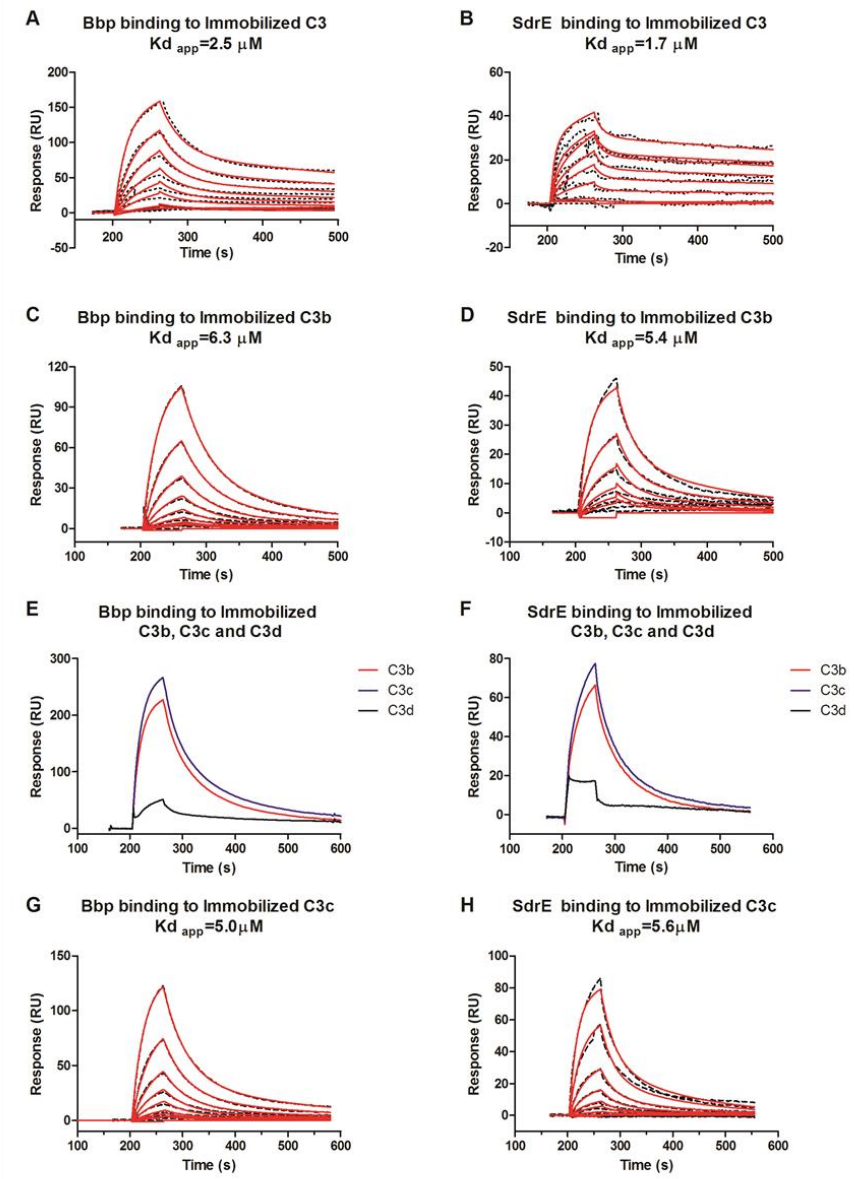

Figure 29. Biacore analysis of the interactions between C3 or C3 small components and the recombinant Bbp or SdrE proteins. A-D. Kinetics analyses of Bbp (0.05- 25 μM, A and C) or SdrE (0.05- 25 μM, B and D) binding to immobilized C3 (5765.9 RUs A and B) or C3b (2909.1 RUs, C and D) sensor chips. E and F. The binding of Bbp (50 μM, E) and SdrE (50μM, F) to immobilized C3b (2909.1RUs), C3c (2855.1 RUs) and C3d (3214.5 RUs). G and H. Kinetics analyses of Bbp (0.05- 25 μM, G) or SdrE (0.05- 25 μM, H) binding to immobilized C3c sensor chips. Kinetic analyses for A-D, H and G (the fitted curves are shown in red) were performed to obtain rate constants and apparent dissociation constants K_D^{app}. Association and dissociation rate constants (*ka1, kd1*) for the binding state and forward and backward rate constants (*ka2, kd2*) for the conformational change state were obtained from the fitting (Table 7).

Table 7. Kinetic profiles of the direct interaction between Bbp or SdrE and C3, C3b or C3c

Bbp/SdrE	Ligands	k_{a1}(1/Ms)	k_{d1}(1/s)	k_{a2}(1/s)	k_{d2}(1/s)	K_D^{app} (μM)
Bbp	C3	1.25×10^{3}	$2.27\times10\text{-}^{2}$	$6.76\times10\text{-}^{3}$	1.06×10^{-3}	2.5
	C3b	2.11×10^{3}	$2.02\times10\text{-}^{2}$	$3.34\times10\text{-}^{3}$	6.34×10^{-3}	6.3
	C3c	2.23×10^{3}	$1.94\times10\text{-}^{2}$	$3.01\times10\text{-}^{3}$	4.1×10^{-3}	5.0
SdrE	C3	1.75×10^{3}	$6.21\times10\text{-}^{2}$	$2.44\times10\text{-}^{2}$	1.24×10^{-3}	1.7
	C3b	3×10^{3}	$3.28\times10\text{-}^{2}$	$6.75\times10\text{-}^{3}$	6.58×10^{-3}	5.4
	C3c	1.44×10^{3}	$2.03\times10\text{-}^{2}$	$2.86\times10\text{-}^{3}$	1.82×10^{-3}	5.6

SPR analyses using C3, C3b, C3c and C3d immobilized on BIA-core CM5 chips were performed to further corroborate the interactions between Bbp or sdrE and C3 or C3 small components. Two-fold dilutions of the recombinant protein Bbp and SdrE (0.05 -25 μM) were run over a sensor chip containing immobilized C3 and C3b (Figure 29 A-D). Recombinant Bbp bound to immobilized C3b in dose-dependent manner with an affinity that appears to be substantially lower than that of C3 binding due to fast dissociation rates (Figure 29, A and C). The apparent K_D for C3 and C3b are 2.5 μM and 6.3 μM respectively.

To further map the binding sites for Bbp on C3b, the recombinant Bbp was run through the immobilized C3c and C3d senor chips. C3c is a large fragment of C3b, both of which interacted with Bbp with similar affinities (Figure 29 E and G). While Bbp weakly bound to immobilized C3d (Figure 29 E). These data demonstrated that the Bbp binding site is located with the C3c fragment, instead of the C3d of C3. Similarly, compared to Bbp binding, SdrE with weaker affinity to human Fg (Francis, 2013), had the same binding patterns and the affinities to C3, as well as to its small fragments such as C3b and C3c with lower binding responses (Figure 29 B, D, F and G and Table 7).

Bbp doesn't compete with the binding of factor B and factor H to C3b

Previous observations demonstrated that factor B bound to partial C3c fragment of C3b and factor H competes with Bb for C3b binding (Forneris et al, 2010; Wu et al, 2009). Therefore I asked whether Bbp, factor B and factor H share the same binding sites on C3b. To achieve this goal, a fixed concentration of the respective factor B or

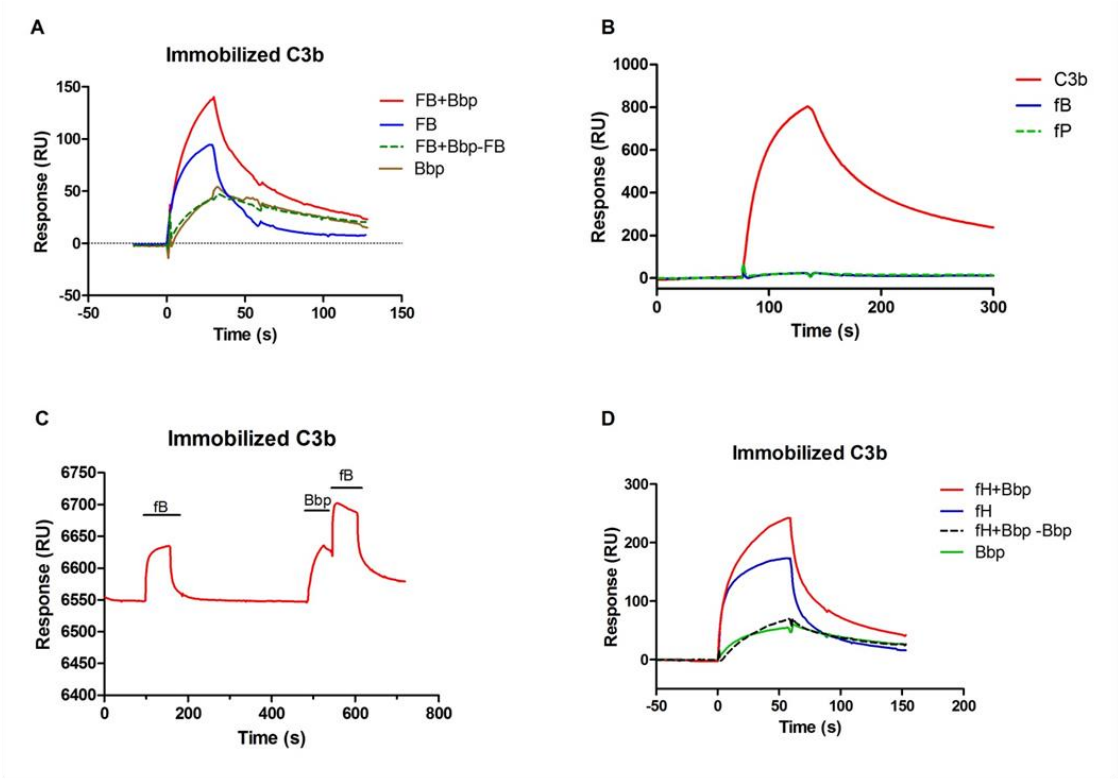

Figure 30. Bbp doesn't share binding sites on C3b with factor B or factor H. (A and D). Constant concentrations 30 µM of Bbp in HBS buffer with 1mM Mg^{2+} were injected to immobilized C3b sensor chip (6280.9 RUs) or as mixtures with either 500 nM factor B (A) or 500 nM factor H (D). The pure factor B or factor H was injected and subtracted from the corresponding response of mixtures. (B) Biacore binding assays for Bbp (50 µM) binding to immobilized C3b (8064.4 RUs), factor B (4801.9 RUs) and factor P (6577.3 RUs). Concentration of Bbp is 50 µM. (C) Co-injection of Bbp (50 µM) and factor B (500 nM) onto immobilized C3b sensor chip (6280.9 RUs).

factor H was mixed with Bbp and injected onto immobilized C3b sensor chip. If Bbp, factor B or factor H bind to C3b independently, the response of Biacore can be additive. In the case of factor B, the subtraction of the pure factor B response from the response of factor B and Bbp mixture revealed a similar binding response as that of pure Bbp (figure 30 A), indicating that Bbp and factor B bind different areas on C3c. Since Biacore binding data shown in Figure 30B demonstrated that Bbp doesn't interact with factor B, co-infection of Bbp and factor B onto immobilized C3b sensor chip also revealed that Bbp and factor B bound to different areas of C3b to form a Bbp-C3b-factor B complex (Figure 30 C). For the factor H binding, I got similar results, but due to the weak interaction between Bbp and factor H (Sharp et al, 2012), the response of the subtracted curve (fH+Bbp-Bbp) is a little bit higher than that of pure factor H (Figure 30 D).

Bbp prevents the assembly of C3b convertase

Our data suggested that Bbp could inhibit the AP C3b deposition, but it doesn't inhibit the C3b-facotor B interaction. Thus Bbp might affect the assembly of C3b convertase. Previous reports demonstrated that the formation of C3b convertase can be detected by Biacore assay directly (Chen et al, 2010; Ricklin et al, 2009). In the immobilized C3b sensor chip, the C3 convertase was formed when a mixture of factor B and factor D were run over the sensor chip and a very stable complex with a notably slow dissociation rate was detected (Figure 31A). The pre-incubation of Bbp with factor B/factor D mixture slowed down and reduced C3 convertase formation (Figure 31 A). Based on the binding of Bbp to the C3b sensor chip, I subtracted the Bbp response from

the responses of the mixtures, in order to obtain the responses of pure factor B and D in the presence of Bbp. Not surprisingly, the rate of the complex formation was significantly decreased, compared to the responses of pure factor B and D (Figure 31A).

Since Bbp doesn't inhibit the binding of factor B to C3b, but blocks the formation of C3bBb, I then hypothesized that Bbp affects the cleavage of factor B. In order to analyze the protease activity, I used the fact that complement factor P binds strongly to C3bBb (C3 convertase) but weakly to C3b and C3bfB (Hourcade, 2006). Indeed, after the factor P sensor chip was injected with C3b, a mixture of C3b and factor B or a mixture of C3b, factor B and factor D respectively, I only detected minimal response from C3b, the mixture of C3b and factor B bound to the factor P-coated surface (less or around 150RUs). In contrast, a mixture of C3b, factor B and factor D resulted in a substantial increase in signal to around 800 RUs which is more than 5 times higher than the response of the mixture of C3b and factor B or pure C3b (Figure 31B). These data demonstrated that binding responses from C3bBb, C3bB and C3b on immobilized factor P sensor surface are distinct and the response of C3bBb also indicates the cleavage of factor B. However, in the presence of various concentrations of recombinant Bbp, the binding response of a mixture of C3b, factor B and factor D decreased (Figure 31C), suggesting that Bbp inhibits the formation of C3 convertase by interfering with the cleavage of factor B.

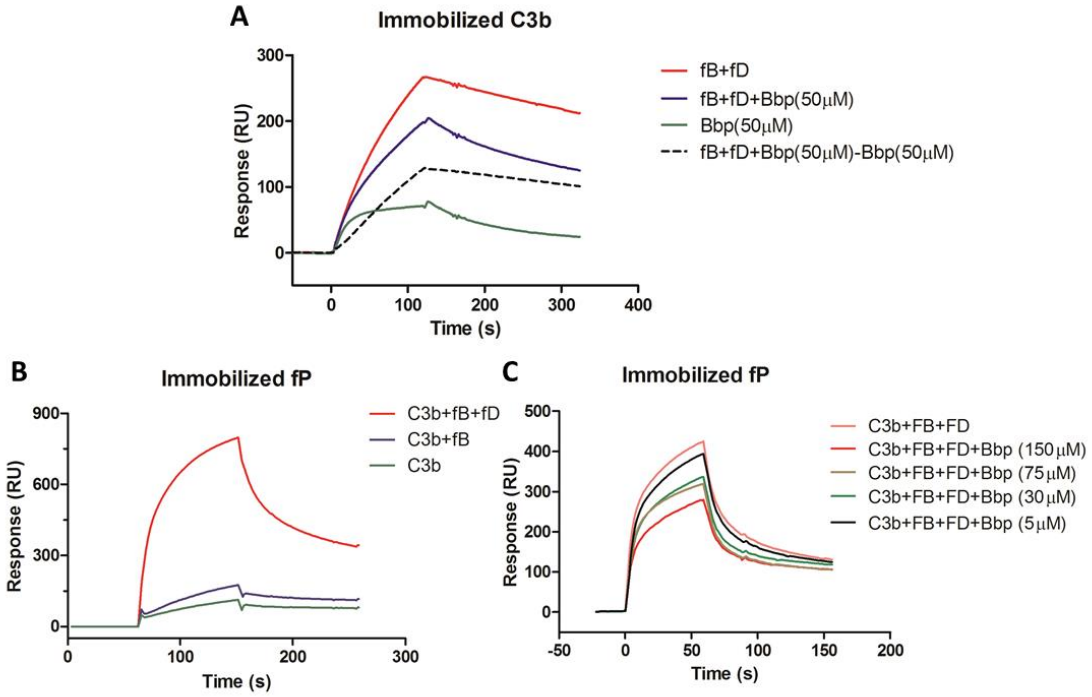

Figure 31. The assembly of C3 convertase on an immobilized C3b or factor P biosensor surface. (A) The formation of C3 convertase on a C3b sensor chip in the presence or absence of Bbp (40 μM). (B) The biosensor surface was treated with C3b (20 μg/ml), mixture of C3b (20 μg/ml) and factor B (20 μg/ml) or plus factor D (1 μg/ml) for 1 min. (C) The biosensor surface were treated with mixture of C3b (10 μg/ml), factorB (10 μg/ml) and factor D (0.5 μg/ml) in the presence of increasing concentration of Bbp.

Discussion

As a successful pathogenic bacterium, *S. aureus* has evolved several strategies to evade or invade the host immune system, including the complement activation pathway. *S.aureus* expresses several well-known secreted complement inhibitors such as SCIN, Efb and Ecb, most of which target the C3b or C3 convertase. In this study, I identified two potential complement inhibitors that target C3b from *S.aureus* cell wall proteins: SdrE and Bbp. Specifically, the binding of Bbp or SdrE to C3c fragment of C3b inhibits the formation of AP C3 convertase by interfering with the cleavage of factor B.

Both Bbp and SdrE belong to the Sdr family, composed of structurally related cell wall-associated proteins. Actually they are allelic variants (Hair et al, 2013{Francis, 2013 #1535)}, both of which have similar domain architectures and share around 87% amino acid sequence in common. Most important thing is that the two genes occur in the same location of the core genome of *S. aureus*, but are found together in less than 1% of *S. aureus* isolates (Francis, 2013). Previous studies showed that Bbp specifically binds to human Fg Aα chain (Vazquez et al, 2011), whereas SdrE binds not only the same area with much lower affinity to human Fg, but also to some animal Fg such as bovine, cow, dog, sheep and cat (Francis, 2013). Surprisingly, the binding affinities of SdrE to human C3 or C3 small components are similar, with little lower binding response than that to the Bbp. It is very interesting to test whether theses interactions are also human specific.

Besides Fg binding activity, recent studies showed that Bbp and SdrE interact with complement regulators factor H and C4BP, leading to the inhibition of phagocytosis killing (Hair et al, 2013; Sharp et al, 2012). Our lab also confirmed the

weak factor H-Bbp/SdrE interaction using SPR assays (Francis, 2013), which is also corroborated by Figure 30D. However, Bbp and SdrE inhibit the hemolysis assay mediated by factor H depleted serum, suggesting that the binding of factor H is not the major contribution to these complement inhibitions. I also believe that the inhibition of neutrophil killing mediated by SdrE/Bbp is also associated with C3b binding. Further experiments are needed to confirm this hypothesis and the analysis of Bbp or SdrE binding to different complement components simultaneously will also need to be investigated.

In the AP, C3 is cleaved by C3 convertase C3bBb into C3a and C3b. This cleavage induces large conformational changes, which expose some critical residues for factor B binding (Schuster et al, 2008). Whereas the conversion from C3b to C3c mediated by factor I and co-factors only induces slight conformational changes (Garcia et al, 2010). This might explain why the binding of Bbp or SdrE to C3 has much slower dissociation rate than that of the C3b or the C3c binding. To further investigate these interactions, I immobilized Bbp on the senor chip to mimic the surface anchored protein, but unfortunately, I didn't observe any binding responses. The possible explanation is that surface-immobilized Bbp may be disrupted by an inactivation during the random immobilization, which was previously observed in other *S.aureus* proteins (Ricklin et al, 2009).

The observation that Bbp or SdrE bound both immobilized C3b and C3c with similar affinity and response, but not C3d indicates that the binding sites must be located at the C3c fragment of C3b. It is well known that factor B and factor H share some

binding sites on C3c (Chen et al, 2010; Forneris et al, 2010). I therefore investigated whether the potential binding of Bbp to C3b would interfere with the activity of factor B and factor H. However, neither factor was substantially inhibited by Bbp on binding to C3b. Interestingly, I found that the effect of Bbp on the assembly of C3 convertase is striking. Preincubation of Bbp with a mixture of factor B and factor D decreased the assembly rate of the C3 convertase on the immobilized C3b sensor chip (Figure 31 A), due to the inhibition of the cleavage of factor B (Figure 31C). As a consequence, I confirmed that Bbp interferes with the formation of C3 convertase at a molecular level.

In summary, my data provide evidence for the first time for direct interaction of Bbp and SdrE with C3b and their inhibition of the AP C3convetase formation by interfering with the cleavage of factor B. The various functions of Bbp or SdrE illustrate the elaborated strategies evolved by pathogenic microorganisms to adhere to and colonize within the host tissues and evade the host defense systems. The molecular mechanism described in this study may also benefit the development of potential anti-complement treatments for other various immune diseases**.**

CHAPTER VI

SUMMARY AND FURTURE DIRECTIONS

Summary

MMSCRAMs and SERAMs are widely expressed by different pathogenic bacteria. It is interesting that most of them have multiple ligands, in order to execute versatile functions. In light of our data, I demonstrated that Cna inhibits the CP by interacting with the collagen-like domain of C1q, while Bbp or SdrE inhibits all three complement pathways. All these studies exploited the novel functions of MSCRAMMs, which were previously implicated only as adhesion molecules with the role of mediating bacteria attachment to the host. Our study advances the knowledge on the role of membrane-bound proteins of bacteria as immune evasion molecules and hopefully will shed light on the discovery of more effective therapeutic strategies against *S. aureus* infections. Moreover, the data from this work may benefit the development of potential anti-complement treatments for other various immune diseases.

Future directions

Cna inhibits C4b deposition on bacterial surface

I already showed solid in vitro data that Cna inhibits the classical pathway activation by interacting with C1q. However, most of my data are created using recombinant Cna. Therefore, I would like to see what could happen on the bacterial surface with Cna expression. One potential experiment is to detect C4b deposition on

bacterial surface. I didn't see significant difference using *S.aureus* strain, which could be due to other known or unknown complement inhibitors form *S.aureus***.** To exclusively analyze the function of Cna, I need to construct nonpathogenic bacteria without any known complement inhibitors to stably express Cna. *Lactococcus lactis* is a Gram-positive bacterium without identified virulence factors. In the previous study in our lab, *L.lactis* has been widely used to express Fg-binding MSCRAMMs such Bbp and ClfA. Therefore, I could construct Cna –expressing *L.lactis* and use it to perform the bacterial C4b deposition assays.

Cna inhibits complement-medicated phagocytosis

MAC formation on the bacterial surface is one strategy for complement system to kill microbes, except for Gram-positive bacteria, due to the thick cell wall. However, complement-medicated phagocytosis plays a crucial role against Gram-positive bacteria, especially *S.aureus*. Cna inhibits the C4b and C3b deposition of the CP, which raises the possibility that Cna interferes with the complement-medicated phagocytosis. I will make use of Cna-expressing *L.lactis* strain to analyze the role of Cna in the complement-medicated phagocytosis

Cna-C1q interaction plays a role in *S.aureus* infection *in vivo*

Preliminary *in vivo* data showed that Cna-C1q interaction enhances *S.aureus* colonization in liver at day 1 and 6.5 but the biological significance for bacteria accumulation in the liver remains unclear. Why Cna-C1q interaction enhances *S.aureus*

colonization in the liver also needs further investigation. While these questions only hint at the possibility that liver endothelial cells and microphages could express some complement receptors such as C1q receptors, which might trap *S.aureus* within the liver. To test this hypothesis, I could use liver cell lines to test whether C1q-Cna interaction mediates *S.aureus* adhesion and invasion to the liver cells.

Bbp/SdrE inhibits CP and LP

My data showed that Bbp or SdrE inhibits all three complement pathways, but they didn't block C3b deposition of the CP and LP . In the CP and LP, C3 convertase (C4bC2a) will interact with C3b to form C5 convertase. It is possible that the binding of Bbp /SdrE to C3b inhibits the assembly of C5 convertase.

Bbp or SdrE interacts with C5

Besides C3 or C3 small components binding, I also found that Bbp and SdrE bind to immobilized C5 using SPR binding assays. Two-fold dilutions of recombinant protein Bbp and SdrE (0.05 -25 μM) were run over a BIA–core chip containing immobilized C5 (Figure 32 A and B). Recombinant Bbp and SdrE bound to immobilized C5 in dose-dependent manner with an affinity that appeared to be substantially lower than that of C3 binding due to a slower dissociation rate (Figure 32, A and B) . The apparent K_D for binding of Bbp and SdrE to C5 were 10.4 μM and 8.3 μM respectively. At present, the biological significance of this interaction remains unknown, which presumably affects the complement activation and C5a generation.

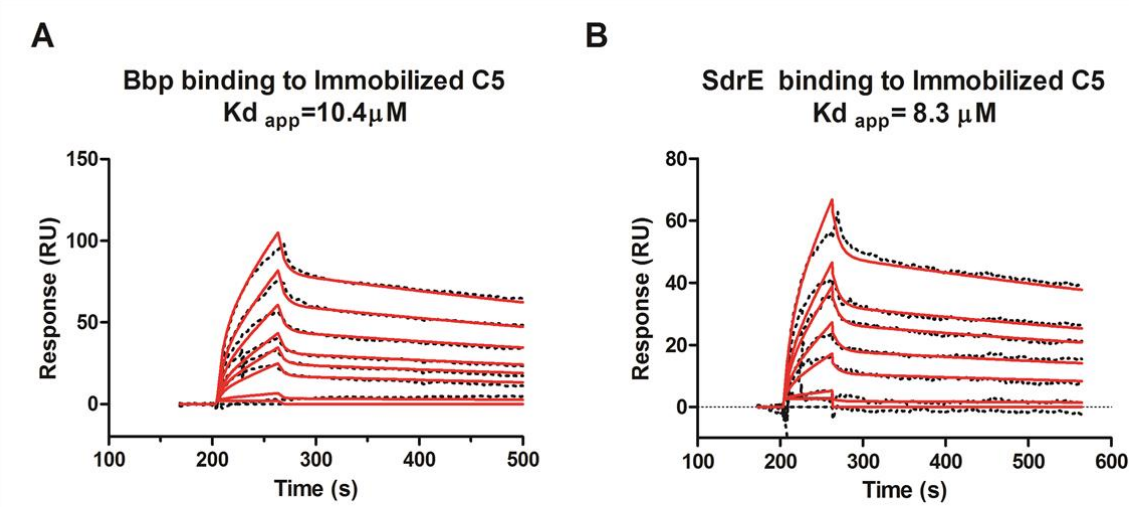

Figure 32. Biacore analysis of the interactions between C5 and Bbp/SdrE. A and B. Kinetics analyses of Bbp (0.05- 25 µM, A) or SdrE (0.05- 25 µM, B) binding to immobilized C5 (7602.6 RUs) sensor chips.

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