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Immune regulation and Fc(alpha)RI Recognition and activation by C-reactive protein

Kristopher Marjon

Kristopher David Marjon
Candidate

Department of Molecular Genetics and Microbiology
Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Terry W. Du Clos , Chairperson

Carolyn Mold

Hattie Gresham

Eric Prossnitz

IMMUNE REGULATION AND FcαRI RECOGNITION AND ACTIVATION BY C-REACTIVE PROTEIN

by

KRISTOPHER DAVID MARJON

B.S., BIOCHEMISTRY, UNIVERSITY OF NEW MEXICO, 2006

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Kristopher David Marjon

B.S., Biochemistry, University of New Mexico, 2006

Ph.D., Biomedical Sciences, University of New Mexico, 2012

ABSTRACT

The acute phase serum protein C-reactive protein (CRP) is one of the major responding proteins during inflammation response in humans. CRP is a serum pattern recognition molecule that can bind to dead or damaged cells and bacteria to initiate their clearance through interaction with immunoglobulin receptors expressed on the surface of immune cells. Immunoglobulin receptors are traditionally thought to bind exclusively to the fragment crystallizable (Fc) region of immunoglobulins and are consequently called Fc receptors (FcRs). FcRs have been shown to play an immunoregulatory role by either exacerbating inflammatory states or potently inhibiting or reversing inflammation. CRP has been proposed to act through FcRs either promoting inflammation or resolving an inflammatory insult.

Immunosuppressive properties have been demonstrated for CRP in mouse models of immune complex mediated inflammation and autoimmune disease. However, the initiating and downstream mechanisms of this process have not been investigated. This dissertation uses an adoptive cell transfer model in mice to address the immunosuppressive capacity of CRP. In this model, treatment of donor spleen cells with CRP suppresses the induced platelet clearance in recipient mice and indicates that protection by CRP is dependent on expression of FcγRI on donor macrophages and FcγRIIb in the recipient mouse. As an extension of the work characterizing CRP-FcR interactions, this dissertation also describes the IgA Fc receptor I (FcαRI) as a novel receptor for CRP. The interaction between CRP and FcαRI induces cytokine production, enhances phagocytosis and alters surface expression of FcαRI on human neutrophils. Collectively, these findings highlight key components in CRP-mediated immunosuppression and a novel receptor for CRP, indicating the complex role this ancient molecule has in the human immune system.

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CHAPTER 1

INTRODUCTION

IMMUNE SYSTEM

The human body has two primary systems utilized for protection against pathogens, adaptive and innate immunity. These systems work in synergy with each other to make up the immune system, which is able to respond quickly and retain memory against most pathogens. A dysfunction in any part of the immune system will increase susceptibility to infections, lead to disease states like autoimmunity or both. Therefore, both adaptive and innate immunity are critical to maintain homeostasis and protection against pathogens.

The adaptive immune system is comprised of lymphocytes, B and T cells, and the products they produce. Lymphocytes respond to invading pathogens by recognizing foreign, or non-self antigens, which leads to clonal B and T cell expansion as well as the release of their immunoregulatory products. Once the infection has resolved, select B and T cells become memory cells allowing the immune system to respond more quickly to the invading pathogen during the next exposure. During an immune response, B cells secrete antibodies that specifically recognize unique antigenic determinates. Secreted antibodies are synthesized as different immunoglobulin (Ig) isotypes (IgG, IgM, IgE and IgA). Once secreted, each isotype is then recognized by its specific isotype receptor expressed primarily on innate immune cells including macrophages, dendritic cells, neutrophils, basophils and mast cells. The receptors interact with the Fc portion of the antibodies and, therefore, are called Fc receptors (FcRs). The antibody-FcR interaction increases the phagocytosis and clearance of the antibody-targeted molecules and promotes other downstream effector functions such as phagocytosis, cytokine production, reactive oxygen species generation and chemokine secretion (Nimmerjahn and Ravetch 2008).

Each antibody class has a specific function and is found in unique areas of the body. For example, secretory IgA is exclusively found at mucosal sites to bind and clear pathogens before they can enter the body, whereas IgG makes up the majority of immunoglobulin in the serum responding to pathogens that have already invaded (Chorny, Puga et al. 2010; Bakema and van Egmond 2011). The adaptive immune system is very effective at responding to and clearing pathogens. However, the generation of specific T cells to a new antigen takes several days, and antibody production by B cells requires 1-2 weeks. Therefore, the innate immune system is critical in providing protection to the host during the period when the adaptive immune response is being activated.

The innate immune system consists of four components: epithelial barriers, innate immune cells, innate cytokines, and pattern recognition proteins. The epithelial cells provide a physical barrier to keep out microbes, and they produce anti-microbial peptides that help kill microbes (Hiemstra 2007). If microbes are able to bypass the epithelial barrier, they will encounter innate immune cells. Innate immune cells include phagocytic cells such as macrophages and neutrophils and non-phagocytic natural killer (NK) cells. The phagocytic cells ingest microbes aiding in clearance of the pathogens, and NK cells patrol the body and kill infected cells or cells that have lost expression of MHC class-I (Silva 2010; Paust and von Andrian 2011). Innate immune cells release cytokines, which modulate the immune response. Cytokines such as TNF-α, IL-1β and IL-6 are examples of inflammatory cytokines because they promote activation of immune cells. Anti-inflammatory cytokines including TGF-β, IL-10 and IL-1Ra dampen the activation of immune cells (Calcagni and Elenkov 2006; Weber, Wasiliew et al. 2010). The last component of the innate immune system, pattern recognition proteins, promotes

recognition and clearance of microbes, viruses, and dead or damaged cells, all containing specific inherent repeating patterns. Examples of pattern recognition proteins include the family of toll-like receptors and soluble circulating proteins such as complement, C-reactive protein and serum amyloid P component (Litvack and Palaniyar 2010; Kawai and Akira 2011; Moalli, Jaillon et al. 2011). These four main components of the innate immune system act in coordination with each other to rapidly neutralize and induce the clearance of an invading pathogen.

The focus of this dissertation is the interaction of the innate pattern recognition protein, C-reactive protein, with Fc receptors and the functional outcomes of these interactions.

History of C-Reactive Protein

C-reactive protein (CRP) was identified in the 1930s by Tillet and Francis who observed that patients who were admitted to the hospital with a pneumococcal infection had markedly elevated levels of a serum protein that precipitated the cell wall fraction of *Streptococcus pneumoniae* (Tillett and Francis 1930). However, as the infection resolved the serum concentration of this protein returned to undetectable levels. The serum protein precipitated out the C-fraction of the bacterial cell wall and, therefore, it was named C-reactive protein (Macleod and Avery 1941). It was later discovered that CRP bound to phosphocholine residues on the repeating teichoic acid structures in the C polysaccharide on the bacteria (Brundish and Baddiley 1968). The change in levels of CRP during the course of infection is hallmark for acute phase serum proteins, which rapidly increase due to inflammatory insults then wane as the insult subsides (Cray, Zaias et al. 2009).

Although the initial discovery of CRP was made in pneumococcal infection where it specifically binds to the cell wall of the bacteria, later studies showed that CRP was elevated non-specifically in other infectious and inflammatory conditions. CRP has since been used as a clinical marker for inflammation and to monitor the efficacy of treatment (Pepys and Berger 2001).

CRP is a highly conserved protein that can be traced back to the horseshoe crab, *Limulus polyphemus,* one of the oldest living species on the planet dating back 445 million years ago (Nguyen, Suzuki et al. 1986; Nguyen, Suzuki et al. 1986; Liu, Minetti et al. 1994). These animals lack an adaptive immune system, so they rely exclusively on pattern recognition proteins like CRP, serum amyloid P component (SAP) and other innate proteins for defense against infections or clearance of dead or damaged cells. These highly regulated pattern recognition proteins are critical in recognizing potentially harmful insults and helping to neutralize them before the insult exacerbates. Therefore, it is believed CRP and SAP have anti-inflammatory properties by promoting the clearance of harmful or inflammatory material resulting in protection against infection or autoimmunity (Kravitz, Pitashny et al. 2005).

PENTRAXIN STRUCTURE AND REGULATION

CRP is a member of the pentraxin family and is composed of five identical non-covalently assembled subunits each having the signature pentraxin sequence motif HxCxS/TWxS in the C terminal domains (Deban, Jaillon et al. 2011). A structurally similar family member is SAP with 53% homology to CRP (Anderson and Mole 1982;

Woo, Korenberg et al. 1985). Both CRP and SAP have similar chromosomal locations, 1q21-q23, and are classified as short pentraxins. Other potential members of this family are the long pentraxin, PTX3 and neuronal pentraxins (Martinez de la Torre, Fabbri et al. 2010). Although they share some sequence homology with the short pentraxins, they are structurally distinct. For example, while the structure of PTX3 has not been solved, it is thought to be composed of ten subunits that form dimers, in contrast to the pentameric short pentraxins. (Bottazzi, Vouret-Craviari et al. 1997). Long pentraxins are also found in distinct chromosomal locations from the short pentraxins. Both short and long pentraxins have been demonstrated to have immunomodulatory properties; however, this dissertation is focused on CRP and SAP.

Structurally, CRP and SAP are nearly identical. The subunits of CRP and SAP are aligned in a ring-formation with each subunit oriented in the same manner. The ring orientation yields two distinct sides or faces for CRP and SAP, shown in Figure 1A (Thompson, Pepys et al. 1999; Kolstoe, Ridha et al. 2009). The faces are identified as the A or B face, which interact with unique receptors and ligands, respectively. Compared to SAP, the subunits of CRP on the A face are rotated 15-20 degrees towards the central axis (Volanakis 2001).

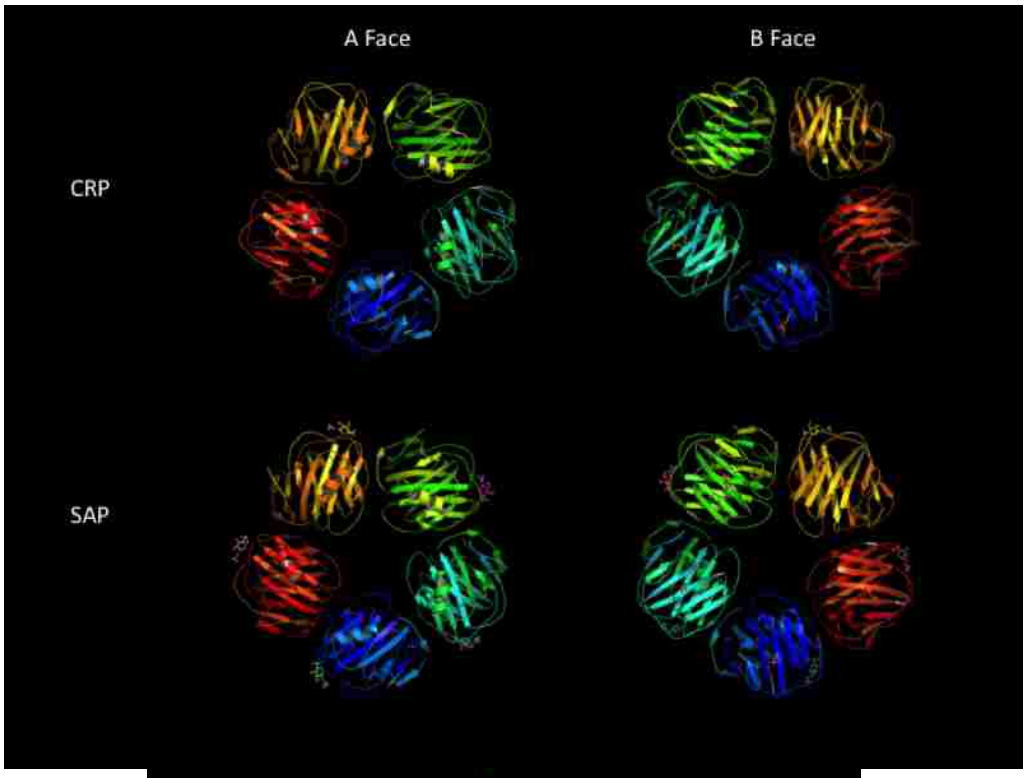
The B face of CRP has been described to bind to small nuclear ribonuclear proteins (snRNPs), histones, oxidized lipids, and phosphocholine. These binding partners allow CRP to interact with bacteria and dead or damaged cells (Du Clos, Zlock et al. 1988; Schwalbe, Dahlback et al. 1992; Chang, Binder et al. 2002; Du Clos and Mold 2004). Phosphocholine is the most well described ligand for CRP, and each subunit of CRP has a calcium dependent phosphocholine-binding pocket on the B face (Figure 1B).

Although the affinity between each subunit and phosphocholine is low, the avidity is high due to the multiple ligand binding sites on CRP.

The A face of CRP interacts with the classical initiating complement factor (C1q) (Bharadwaj, Stein et al. 1999; Bang, Marnell et al. 2005; McGrath, Brouwer et al. 2006) and recruits and binds factor H, a complement serum protein that inhibits C3b, to abrogate the amplification of complement activation (Mold, Kingzette et al. 1984; Okemefuna, Nan et al. 2010). The A face of CRP also binds Fcγ receptors, which are expressed on immune cells and recognize the fragment crystallizable (Fc) region on immunoglobulins or antibodies. Fc receptors are responsible for mediating phagocytosis of immunoglobulin coated, or opsonized, particles and initiate activation of immune cells. Therefore CRP can opsonize dead cells, inflammatory antigens or bacteria and promote their clearance or phagocytosis through FcRs or complement receptors (Figure 2).

In humans, the serum concentration of CRP is elevated in response to inflammatory insults such as burns, sepsis, myocardial infarction or cancer, which increase the systemic production of the inflammatory cytokine, IL-6 (Majello, Arcone et al. 1990; Toniatti, Arcone et al. 1990). Hepatocytes respond to the systemic increase of IL-6 and rapidly synthesize CRP; however, there are also reports of non-hepatic production of CRP at sites of inflammation (Dong and Wright 1996; Gabay and Kushner 1999). Baseline serum concentrations for CRP are approximately 1 μg/ml and markedly increase to levels of 500 μg/ml peaking within 36 hours in response to IL-6 (Ramji, Vitelli et al. 1993). CRP can be quickly cleared from the body with a half-life of 19 hours (Vigushin, Pepys et al. 1993). This rapid response to inflammation allows CRP to be a

A.



B.

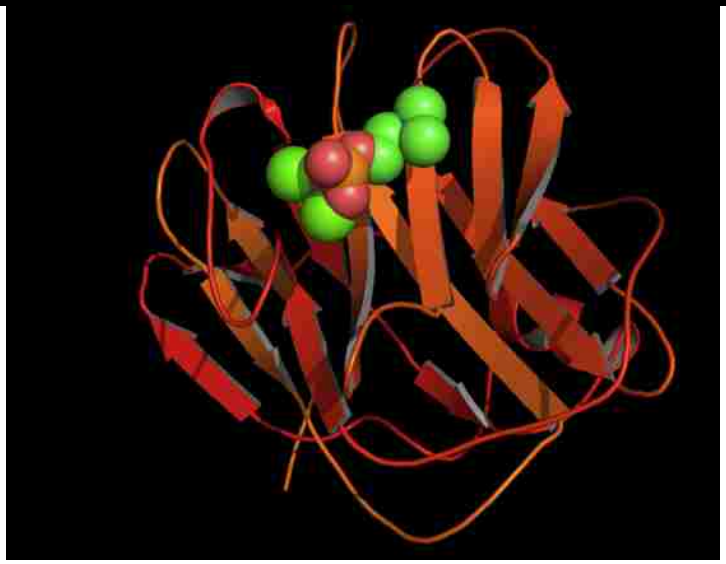


Figure 1. The structural components of C-reactive protein and Serum Amyloid P component. (A) CRP and SAP consists of five identical subunits assembled in a ring formation. Each Pentraxin has an A face and a B face that interacts with Fc receptors or ligands respectively. (B) Subunit of CRP interacting with phosphocholine in a calcium dependent manner on the B face.

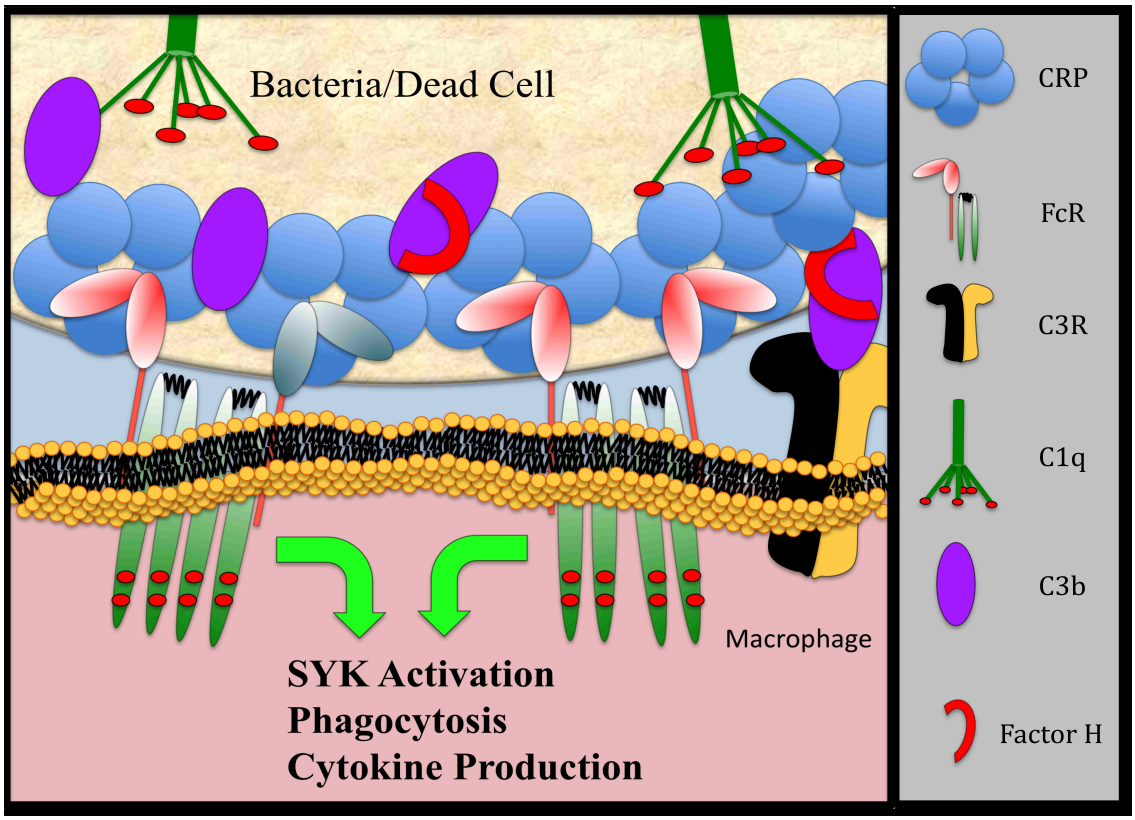


Figure 2. CRP recognized dead or damage cells and initiates clearance though FcγRs and Complement receptors. CRP binds to targets on the B face allowing the binding to FcRs or complement activation. Complement activation leads to C3b deposition on the target, which can also interact with complement receptors. Once receptors are crosslinked activation (syk) is initiated and phagocytosis and cytokine generation takes place.

first responder to inflammatory insults helping to control infection or possibly dampen an excessive immune response.

SAP has similar functions to CRP, such as opsonizing particles including invading bacteria and dead or damage cells to promote their clearance though FcRs. SAP interacts with phosphoethanolamine and DNA on the B face, while the A face can bind all Fc receptors described to interact with CRP. Unlike CRP, SAP is not an acute phase serum protein because the SAP serum concentration is not acutely affected during an inflammatory insult (Bijl, Bootsma et al. 2004). However, SAP is the major serum pentraxin under non-inflammatory conditions with serum concentrations of 40 μg/ml. Therefore, SAP is thought to aid in the clearance of dead or damaged cells and DNA during homeostasis (Pepys and Butler 1987; Ciurana and Hack 2006) with CRP executing similar functions during an inflammatory response.

Fc RECEPTORS

The most well characterized ligands for FcRs are immunoglobulins. There are five major classes of immunoglobulins with three of the immunoglobulins (IgG, IgE, IgA) with well-characterized receptors that have unique structures (FcγR, FcεR, FcαR, respectively). Immunoglobulins all have the same basic structure of two heavy chains and two light chains forming a "Y" shape, except IgA, which has a "T" shape (Boehm, Woof et al. 1999). The heavy and light chains combine to form two arms with the antigen recognition sites at each end. The stalk of the antibody forms the constant domain, which

binds to Fc receptors and is unique to each class of antibody. This dissertation focuses on the IgG receptors, FcγRs, and the IgA receptor, FcαRI.

Immunoglobulins carry out their function when bound to their respective antigen by two different mechanisms: complement activation and recognition by its cognate Fc receptor (Lutz and Jelezarova 2006; Daha, Banda et al. 2011). Classical complement activation is initiated when antigens are opsonized with immunoglobulins, allowing them to interact with C1q and induce activation of other complement factors (C3, C3b, C5, C5 convertase, C5a, MAC complex). The activation of this cascade ultimately leads to the lysis or phagocytosis of the antigenic material (Sarma and Ward 2011). The alternative mechanism for clearing the immunoglobulin-opsonized antigen (immune complex) is recognition by Fc receptors on immune cells such as macrophages, dendritic cells, monocytes, and neutrophils. Classically, FcγRs and FcαRI bind their immunoglobulin in a 1:1 ratio and require dimerization of the receptor to initiate signaling. The crystal structure of FcαRI with the Fc portion of IgA demonstrates a 2:1 (receptor:IgA) binding ratio; however, the physiological relevance of this binding ratio is unclear. As Fc receptors bind immune complexes and more receptors cluster in close proximity together on the cell membrane, intracellular signaling occurs. Signaling ultimately induces phagocytosis, cytokine production, antibody-dependent cell-mediated cytotoxicity or the production of reactive oxygen species (Ravetch and Bolland 2001). Therefore, Fc receptors play an integral role in the clearance of a pathogen, mediating protection and orchestrating the immune response.

The FcRs can be either activating or inhibitory receptors. The classification of a receptor as an activating or inhibitory receptor depends on the signaling domain inherent

to or associated with the FcR. FcRs are composed of extracellular domains that bind to antibodies and a cytoplasmic tail. The tail contains either an immunoreceptor tyrosine-based activation motif (ITAM), an immunoreceptor tyrosine-based inhibition motif (ITIM), or is associated with an FcR γ chain which contains an ITAM. Once the receptor is cross-linked with another receptor, the ITAM or ITIM motifs are in close proximity to each other, promoting phosphorylation by kinases (Agarwal, Salem et al. 1993; Salcedo, Kurosaki et al. 1993; Ghazizadeh, Bolen et al. 1994). Signaling from ITAMs leads to further downstream signaling such as SYK activation, Ca^{2+} signaling and gene regulation, which promote activation of the cell (Young, Ko et al. 1984; Anderson, Shen et al. 1990). Signaling from ITIMs leads to the blunting or inhibition of signaling by recruiting SH2 domain-containing inositol-polyphosphate 5'-phosphatase (SHIP), which inhibits other kinases or signaling pathways (Ono, Bolland et al. 1996; Bolland, Pearse et al. 1998). Therefore the inhibitory receptors are thought to play a critical role in setting the threshold for activation of immune cells that express FcRs (Nimmerjahn and Ravetch 2006).

Humans and mice have several activating FcγRs and one inhibitory receptor, FcγRIIb, shown in Figure 3. Each receptor binds to its immunoglobulin with different affinity (Nimmerjahn and Ravetch 2011). FcγRI has the highest affinity, in the nanomolar range, for IgG in both humans and mice while the other FcγRs have micromolar affinity for IgG (Ravetch and Kinet 1991). FcγRI is structurally different than the other members of the FcγRs as it is the only FcR with three extracellular domains while the other FcγRs have two domains (Lu, Ellsworth et al. 2011). Regardless of this difference, FcγRs interact with IgG between their D1 and the D2 domains. FcαRI also has two extracellular

domains, but interacts differently with IgA at the most distal portion of the D1 domain (Herr, Ballister et al. 2003). Although FcαRI shares general structural similarities with the FcγRs, it is structurally more similar to the Killer-cell immunoglobulin-like receptors, KIRs, as shown in Figure 4 (Morton and Brandtzaeg 2001). For example, the orientation of the D1 and D2 domains of FcαRI are inverted with respect to the D1 and D2 domains for FcγRII. The FcγRs are located on chromosome 1q21-23, while the FcαRI is located in the same area as the leukocyte receptor complex and KIR receptors on chromosome 19q13.

FcγRs have been studied extensively in murine models, allowing the genetic deletion of each receptor or signaling component to elucidate its function. This approach has given insight into how these receptors function during both homeostasis and in disease states. For example FcγRIIb deficient mice spontaneously develop autoimmune-like symptoms and have heightened or exacerbated immune responses ultimately harming the host since the activating signals cannot be shut off efficiently (Takai, Ono et al. 1996; Nimmerjahn and Ravetch 2008). Until recently the role of FcαRI in the immune system could not be extensively studied, as mice do not express the FcαRI receptor. The role of this receptor in the immune system is just beginning to be understood with the aid of FcαRI transgenic mice (van Egmond, van Vuuren et al. 1999; Launay, Grossetete et al. 2000). Although FcαRI has an ITAM motif, it has been shown to have both activating and inhibitory properties *in vitro* and *in vivo* (Pasquier, Launay et al. 2005; Kanamaru, Arcos-Fajardo et al. 2007). FcαRI is the primary receptor on infiltrating neutrophils, and activation of this receptor can lead to the generation of reactive oxygen species, cytokine production, lactoferrin release and leukotriene C_4 and B_4 release ((Monteiro and Van De

Winkel 2003)). Monomeric targeting of this receptor using non-crosslinking IgA or anti-receptor antibody has shown to be a potent inhibitor of other FcR signaling *in vitro* and *in vivo*. For example, monomeric targeting of FcαRI on cells leads to the abrogation of FcεRI-mediated degranulation or inhibition and reversal of inflammation in a nephrotoxic nephritis model (Kanamaru, Pfirsch et al. 2008). The inhibitory signaling initiated through an ITAM is termed ITAM*i* and is thought to occur for other ITAM receptors (Barrow and Trowsdale 2006; Blank, Launay et al. 2009).

Together FcγRs and the FcαRI play important roles in the human immune system such as protection against pathogens. However, when deregulation of FcRs occurs, it results in autoimmune diseases or inefficient immune protection demonstrating these receptors are critical in all aspects of the human immune system (Takai, Li et al. 1994; Takai 1996; Schmidt and Gessner 2005; Nimmerjahn and Ravetch 2007).

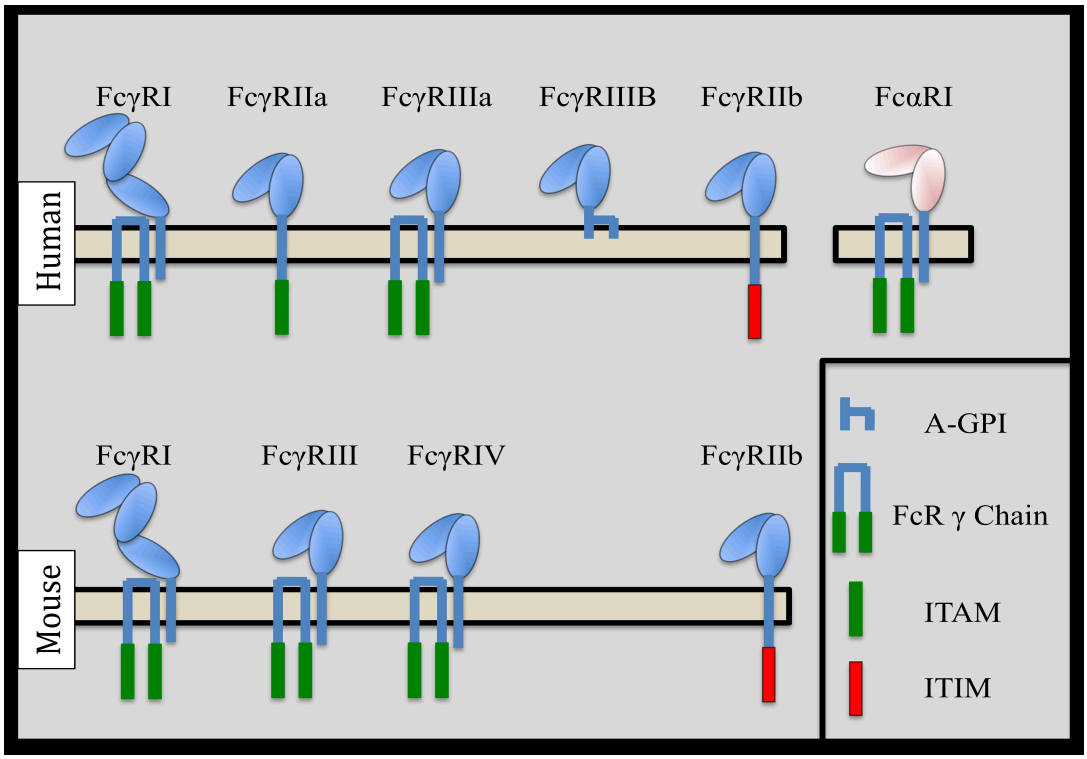


Figure 3. Human and Mouse FcRs. In humans and mice FcγRI consists of three domains while all the other FcRs displayed here have two domains. All of the activating receptors in mice associate with the gamma chain and humans have two activating receptors that associate with the gamma chain. FcγRIIa and FcγRIIb intrinsically have an ITAM or an ITIM, respectively and FcγRIIIB is GPI linked to the cell membrane. FcαRI associates with the gamma chain similarly to the activating FcγRs.

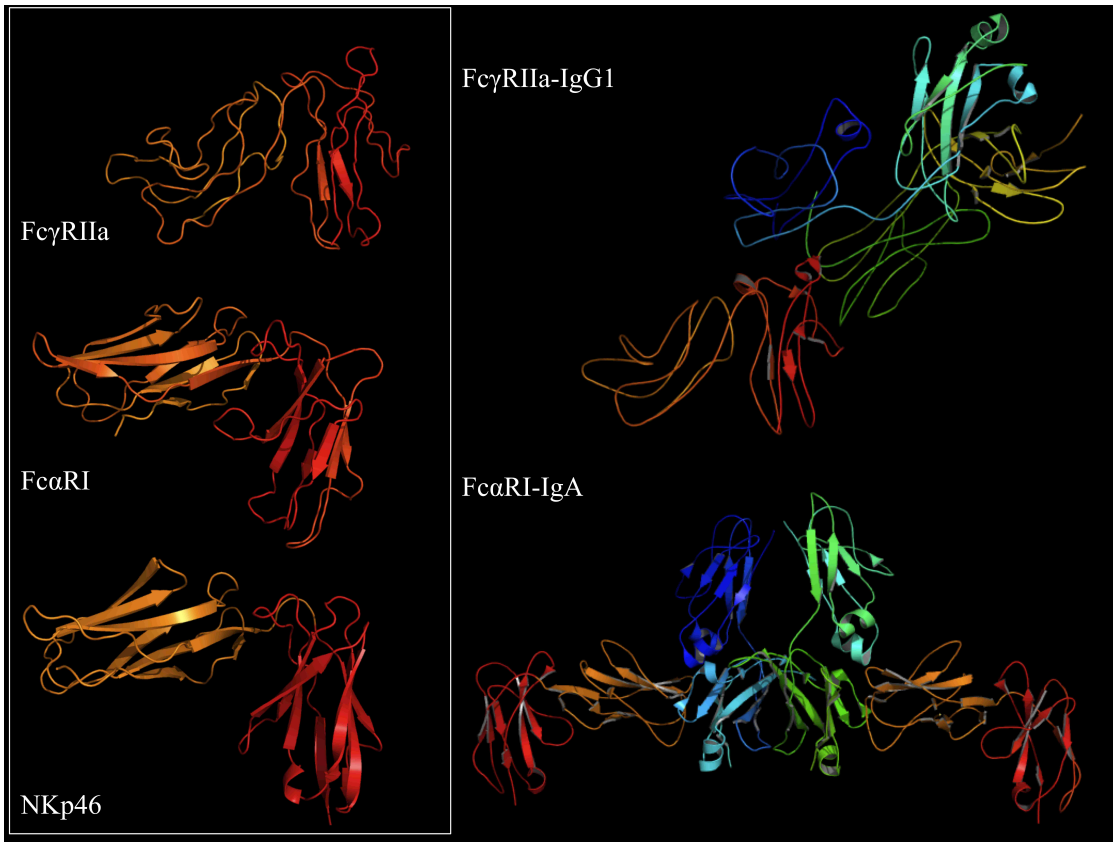


Figure 4. FcγRs and FcαRI bind their ligands differently. The overall structure of the FcRs and KIR (NKp46) appear similar but are unique to each other. The D1 domain is colored orange and the D2 domain is red. The D1-D2 hing of FcγRIIa binds to IgG where as the most distal portion of the D1 domain of FcαRI binds IgA. The immunoglobulins are blue and green.

Pentraxins and Fc Receptors

The binding of CRP and SAP to FcγRs and characterization of the functional outcomes of the interactions have been described by Du Clos (Marnell, Mold et al. 1995; Stein, Mold et al. 2000; Bharadwaj, Mold et al. 2001; Mold, Gresham et al. 2001; Mold, Baca et al. 2002). These data introduced a paradigm shift in the understanding of the role pentraxins and FcγRs in the immune system. CRP and SAP bind with micromolar affinities to FcγRs, similar to IgG (Lu, Marnell et al. 2008). Therefore, pentraxins act as innate antibodies and can carry out similar functions to immunoglobulins including opsonization, complement activation and induction of phagocytosis (Figure 5) (Mold, Du Clos et al. 1982; Du Clos and Mold 2001). Recently the crystal structure of SAP interacting with FcγRIIa was solved, providing insight into how pentraxins interact with FcγRs (Lu, Marnell et al. 2008). The crystal structure shows pentraxins bind to FcγRs on the same domains as IgG, indicating competition for binding and complementing previous studies showing aggregated IgG inhibited CRP binding to FcγRs (Mortensen and Duszkiewicz 1977). The crystal structure also demonstrated that pentraxins interact with the receptor in a 1:1 (pentraxin: receptor) ratio, similar to the interaction between IgG and FcγRs. Because FcRs must be cross-linked to induce activation, the finding that CRP interacts with the receptor in a 1:1 ratio implies that aggregation of pentraxins, like CRP-opsonized particles, must take place to initiate signaling through the FcγRs. The crystal structure revealed how pentraxins bind to FcγRs and also provided data that both CRP and SAP can bind all of the FcγR family members. Together this study and others reveal that CRP and SAP bind FcγRs to induce effector functions such as phagocytosis,

induction of Ca^{2+} signaling, cytokine production, and generation of reactive oxygen species in a similar manner to IgG.

Understanding that IgG and FcγRs play a central role in regulating the immune response and have a role in autoimmunity led to the investigation of CRP and FcγRs role in these processes. Protective effects mediated by FcRs have been under investigation since the clinical observation that high concentrations of IgG given intravenously (IVIg) suppress inflammatory cascades. Different inflammatory models suggest that CRP suppresses inflammation through the interactions with FcγRs (Mold, Rodriguez et al. 2002; Rodriguez, Mold et al. 2005; Rodriguez, Mold et al. 2006; Hu, Wright et al. 2011). In an immune complex mediated disease model, nephrotoxic nephritis, in which the glomeruli in the kidneys are targeted with antibodies, mice progressively lose kidney function from inflammation in the kidneys. In this model, CRP protects against the initiation of inflammation or reverses ongoing kidney inflammation (Rodriguez, Mold et al. 2005; Rodriguez, Mold et al. 2006). Protection was dependent on macrophages and expression of FcγRI. This model demonstrates that CRP regulates the immune response through the interactions with FcγRs.

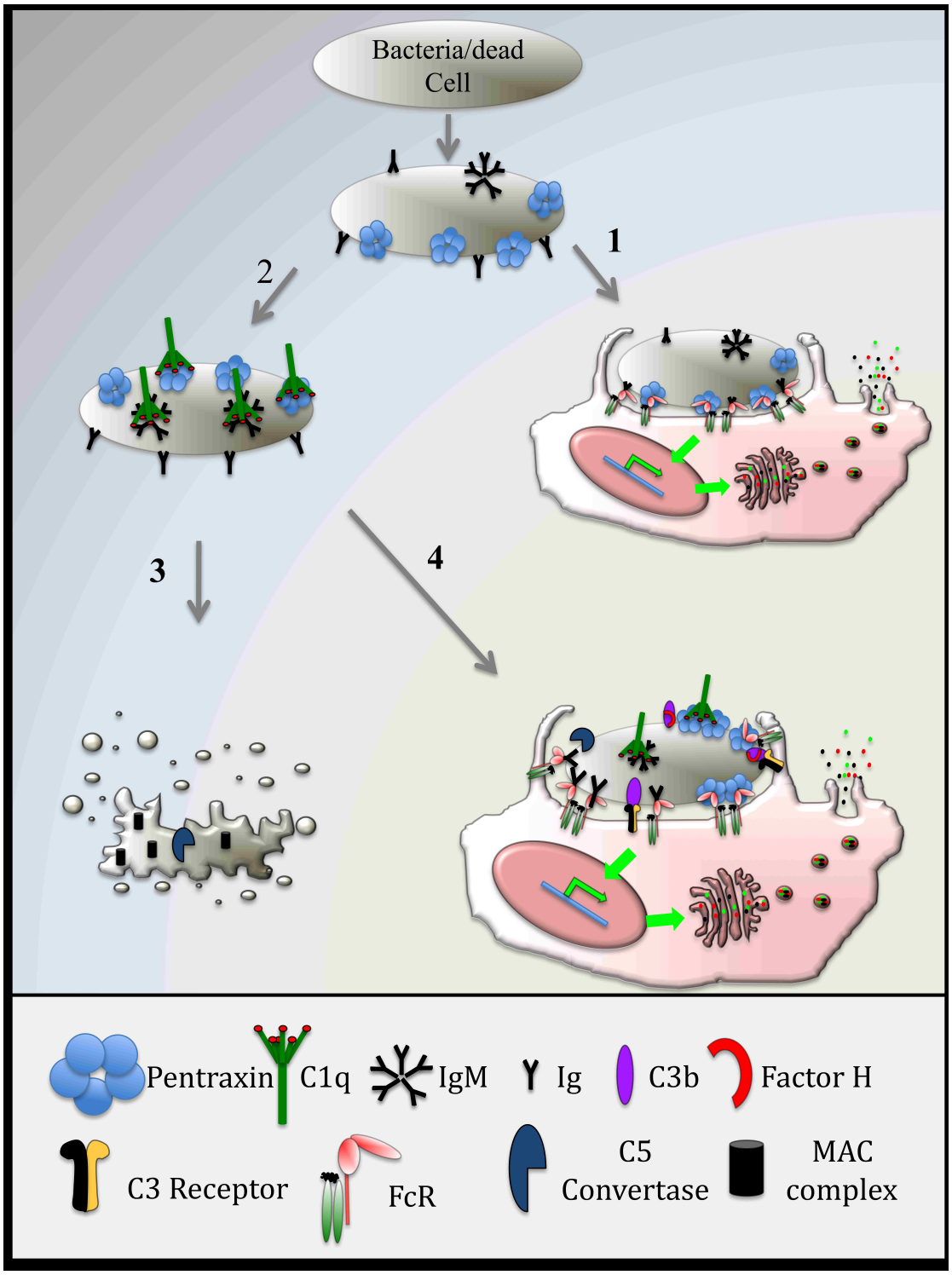


Figure 5. Pentraxins and Immunoglobulins Induce Clearance of Pathogens. Bacteria, dead or damaged cells are first recognized and opsonized by pentraxins or immunogloulins. (1) The opsonized bacteria then are phagocytosed by immune cells expressing FcRs or (2) activates complement leading to (3) lysis of the target by complement factors. (4) CRP recruits Factor H, halting complement activation at C3b and aids in phagocytosis by immune cells expressing FcRs and complement receptors. Signal transduction occurs via receptor crosslinking and leads to gene regulation and cytokine production.

C-REACTIVE PROTEIN IN AUTOIMMUNITY

The role of CRP in inflammation is controversial (Prasad 2006; Pepys 2008; Danesh and Pepys 2009; Nordestgaard and Zacho 2009). Patients who have ongoing inflammation generally have a high serum concentration of CRP. Thus, CRP is associated with inflammatory states and is thought to contribute to the inflammation. However, little is known about the function of CRP during these states only that its expression is increased during an inflammation. There are many disease states that lead to an inflammatory response, one of them being autoimmune disease. Autoimmune diseases (lupus, glomerulonephritis, thrombocytopenia, diabetes, etc.) are complex and occur when the immune system recognizes components of the host (DNA, basement membrane components, histones, etc.) as foreign and initiates activation of the immune system. The immune system clears the self-antigens using autoantibodies and autoreactive T cells, promoting inflammation. While CRP is generally increased during inflammatory reactions, there are examples of decreased CRP expression in some autoimmune diseases as exemplified by lupus. CRP levels are decreased in lupus for multiple reasons. One reason is a lupus-associated polymorphism that decreases CRP serum levels (Edberg, Wu et al. 2008; Rhodes, Furnrohr et al. 2011). Another reason is that patients with lupus have a propensity to produce auto-antibodies, and some of these auto-antibodies recognize innate molecules such as CRP, SAP and C1q, which all play a role in removal of dead or damaged cells and nuclear material (Shoenfeld, Szyper-Kravitz et al. 2007; Sjowall and Wettero 2007). A recent study found that CRP synthesis may be decreased in SLE due to inhibition by type I interferon, which is oftern elevated in patients with SLE (Enocsson,

Sjowall et al. 2009). It is hypothesized that patients who have low circulating CRP levels may have a higher risk of developing an autoimmune disease because the removal or clearance of nuclear antigenic material may be decreased.

There are no known cases of CRP or SAP deficiency in humans, but murine models lacking the expression of pentraxins develop autoimmune like symptoms or have heightened inflammation when challenged with an immune complex-mediated disease (Bickerstaff, Botto et al. 1999; Jones, Pegues et al. 2011). Protective properties of CRP have also been observed with single injections in mice induced to develop nephrotoxic nephritis and in mice that spontaneously develop autoimmune like symptoms (Rodriguez, Mold et al. 2006). Although the mechanism of protection in these models is unclear, it demonstrates that short pentraxins may play a larger role in maintaining homeostasis than previously recognized.

Summary and Hypothesis

Pentraxins have been studied for over 80 years and within the last 20 years investigators have identified receptors and novel ligands for CRP and SAP. High concentrations of CRP in the serum during inflammation have been correlated with many diseases, but the functional role of CRP in these diseases remains unclear. However, in murine inflammatory disease models, CRP is protective and effective in modulating the immune response by shifting the balance to a more anti-inflammatory state. The necessity for macrophages and FcγRI expression in CRP-mediated protection has been identified; however, the temporal mechanism of these components is elusive. Therefore, we

hypothesize that CRP mediates protection though the interaction of FcγRI on macrophages that induce suppression in an immune complex mediated disease. To address this hypothesis we use an adoptive cell transfer model in an immune complex-mediated disease model of immune thrombocytopenia (ITP), a disease that leads to antibody-mediated clearance of platelets (Siragam, Crow et al. 2006). The adoptive transfer model uses donor spleen cells treated with reagents such as CRP or IVIg. The treated cells are transferred to recipient mice, which are then induced to clear their platelets by administration of anti-platelet antibody. The unique property of this model is that the donor and recipient mice can differ in gene expression facilitating the identification of the receptors, cell types and other components that are essential for protections in the donor and recipient mice. Using this model, the following aims were addressed: 1) to determine which Fc receptors are involved in mediating protection against TIP and if the receptor expression is necessary on the donor cell or in recipient mice; 2) to identify the donor cell type CRP is acting on to induce suppression; and 3) to determine soluble key mediators that aid in suppression. We demonstrate CRP mediated protection is dependent on macrophages expressing FcγRI in the donor cell population and the expression of FcγRIIb in recipient mice. We also observed no role for the anti-inflammatory cytokine IL-10 or complement in mediating the protection against depleting platelets in mice. Using a unique system, this study identified which receptors and cell type directly mediate CRP-dependent immunosuppression. These observations imply CRP acts as an anti-inflammatory agent in the immune system and may help clarify its functions when its serum concentrations are high.

The potent immune regulation of CRP through FcγRs combined with the observations that CRP and SAP can bind all of the members of the FcγR family led to the investigation of potential interactions between CRP and other FcRs. Dr. Peter Sun's laboratory screened binding interactions between short pentraxins and FcRs using surface plasmon resonance and found that both CRP and SAP bound to FcαRI with micromolar affinity. Therefore, we hypothesized that CRP binds to FcαRI expressed on cells and functionally activates this receptor. To approach this hypothesis we focused on the following aims: 1) to identify and characterize how CRP and FcαRI interact with each other; 2) to determine if CRP induces signaling and phagocytosis though FcαRI in transfected or primary cells; and 3) to determine if CRP induces cytokine production through activation of FcαRI. We demonstrate that CRP binds to FcαRI expressed on cells and interacts with both the D1 and D2 domains. We also establish that the interaction of CRP with FcαRI alters cellular distribution of FcαRI on the surface of human neutrophils, initiates phosphorylation of ERK, induce phagocytosis of opsonized cells, and production of IL-4 and TNF-α. Together these data demonstrate a novel ligand for FcαRI and a new functional receptor for CRP. The interaction between CRP and FcαRI broadens the immunological implications of CRP and demonstrates the complex integration of the adaptive and innate immune systems.

CHAPTER 2

METHODS

Reagents

CRP was purified from human pleural fluids as described previously (30). All preparations were examined on overloaded SODS-PAGE gels stained with SYPRO Ruby protein gel stain (Invitrogen) to ensure purity. No additional bands other than the major band at about 23 kDa were seen. All preparations used contained < 3 endotoxin units (0.3 ng) of endotoxin per mg of protein determined by a quantitative chromogenic Limulus amebocyte lysate assay (Lonza). IVIg purchased from Bayer was used for most experiments. In some experiments IVIg (Baxter) provided as a generous gift from Dr. Ralph C. Williams was used with equivalent results. Chicken OVA, piceatannol, egg phosphatidylcholine, BSA and cholesterol were purchased from Sigma-Aldrich. Clodronate was provided by Boehringer Mannheim. Recombinant mIFN-γ was purchased from R&D Systems. Antibodies were obtained as follows: rabbit IgG anti-OVA from MP Biomedical; rat mAb specific for mouse CD41 (IgG1k, clone MWReg 30), FcγRI (CD64) and CD11b from BD Biosciences; rat mAb specific for F4/80 and FcγRIIb (clone MM7301) from Caltag; rat mAb specific for CD11c from Miltenyi. Rat mAb 2.4G2 was used as an FcγR blocking agent. Corresponding isotype control antibodies were used in all flow cytometry experiments. Magnetic microbeads along with separations columns were purchased from Miltenyi. IC were prepared by mixing chicken OVA with a 10-fold molar excess of anti-OVA for 30 min at room temperature.

Mice

C57BL/6 (B6) mice were obtained from the National Cancer Institute. FcγRIIB$^{-/-}$ mice and IL-10$^{-/-}$ mice were obtained from Jackson Laboratories and backcrossed to 10 generations on B6 mice. FcγRI$^{-/-}$ mice were obtained from J. S. Verbeek and further backcrossed onto B6 mice (31). Female mice at 6–12 wk of age were used in all experiments. All procedures involving animals were approved by the Institutional Review Board of the Department of Veterans Affairs Medical Center.

Induction of ITP

Mice were injected i.v. with 4 μg of anti-CD41 in 0.2 ml PBS pH 7.2. Blood was taken from the retroorbital plexus and diluted in Unopettes (Unopette, BD Bioscience). Platelets were counted using a hemocytometer. Baseline platelet counts were obtained from mice before treatment. In the adoptive transfer experiments, the average baseline platelet counts for each mouse strain were determined and are shown as normal platelet counts on the graphs.

Treatment of ITP in vivo

Mice were injected i.p. with 50 mg IVIg 24 h before the injection of anti-CD41 (20). For CRP, mice were injected i.p. with 200 μg of CRP 24 h before treatment with anti-CD41 or i.v. with 200 μg of CRP 4 h before treatment with anti-CD41.

Treatment of spleen cells in vitro and adoptive transfer

Splenocytes were prepared from donor mice and erythrocytes lysed with ammonium chloride (ACK) lysis buffer. In the standard protocol splenocytes were suspended in

serum free RPMI 1640 at a concentration of 1.4 x 10^6 cells/ml and treated with 200 µg/ml of CRP, 200 µg/ml BSA or 18 mg/ml of IVIg for 0.5 h at 37^{o}C. After treatment, cells were washed twice in RPMI-1640 and resuspended at a concentration of 5 x 10^6 cells/ml and 0.2 ml of the suspension was injected i.v. into recipient mice 24 h before induction of ITP.

Culture of bone marrow macrophages (BMM)

Bone marrow cells were cultured for one week in DMEM (Sigma-Aldrich, high glucose) with 20% FBS and 30% L-cell conditioned medium, as a source of M-CSF. BMM were > 95% positive for the F4/80 macrophage marker by flow cytometry. BMM were treated with 100 U/ml IFN-γ for 24 h before treatment with CRP or IVIg. Cells were harvested using 10 mM EDTA for 5 min at 4^{0}C, washed and suspended to desired concentration in RPMI for treatment, as described for splenocytes.

Macrophage depletion and enrichment

Liposomes containing dichloromethylene bisphosphonate (Clodronate) were prepared as previously described (32). The liposomes were washed by ultra-centrifugation and mice were injected i.v. with 0.2 ml of liposomes containing Clodronate or control liposomes prepared with PBS. Spleens were taken from mice 48 h after injection of liposomes. We previously found that treatment with Clodronate liposomes depletes 99% of Kupffer cells and 95% of splenic macrophages (33). Clodronate liposome treatment of mice resulted in an 80% decrease in F4/80+ spleen cells by flow cytometry. F4/80+ cells were enriched from adherent splenocytes using biotin anti-F4/80 and streptavidin beads (Miltenyi). The

target cell population was selected, following the manufacturer's instructions, and was verified by analysis using flow cytometry.

Immunoprecipitation and Western Blot Analysis of Syk phosphorylation

BMM (10×10^6) in 1 ml were stimulated with 200 μg/ml CRP for the desired time, then immediately put on ice and subsequently washed twice with PBS. Cells were lysed with lysis buffer (1% NP-40, 0.15 M NaCl, 0.05 M Tris, pH 7.2 with Halt Protease Inhibitor 2X (Thermo Scientific), and 100 μM activated NaVO4). The protein concentration was determined by a BCA assay (Thermo Scientific). *Syk* was immunoprecipitated with anti-*Syk* (N-19) antibody overnight with protein A/G PLUS-agarose beads (Santa Cruz Biotechnology). Beads were washed five times with lysis buffer then boiled for 5 min in 2X loading buffer. Samples were loaded on a 4-20% SDS polyacrylamide precast gel (Invitrogen) then SDS PAGE was carried out for 3 hours at 120 V. Proteins were transferred to a PVDF membrane with a wet transfer system (NOVEX) at 25 V for 90 min. The membrane was blocked with 5% BSA in TBST (TBS + 0.1% Tween-20) for 1 h then probed for Phospho-*Syk* (Cell Signaling Technology) with polyclonal Phospho-*Syk* antibody (1:1000) in TBST + 5% BSA over night at 4^{o}C. The membrane was washed three times for 5 min in TBST. HRP conjugated secondary anti-rabbit IgG (Cell Signaling Technologies) (1:2000) was incubated with membrane for 1 h at room temperature. The blot was washed three times with TBST before detection with ECL (SuperSignal West Dura Extended Duration Substrate, Thermo Scientific). The blot was imaged using a CCD camera (Gene Gnome, Syngene Bio Imaging System) and analyzed

with ImageJ (NIH). Optical density was determined and normalized for each sample using the IgG heavy chain as an internal loading control.

Data analysis

Graphical and statistical analyses were performed using GraphPad Prism software (GraphPad). Data are presented as the means ± SEM for the number of mice indicated in the figure legends. Comparisons between means were done using Student's t test or one way ANOVA. p values are indicated in the figure legends as follows: * = $p<0.05$, ** = $p<0.01$; *** = $p<0.001$.

BIAcore binding experiments

SPR studies were performed using a BIAcore 3000 with BIAevaluation 4.1 software in 10 mM HEPES (pH 7.4), 0.15 M NaCl, 1.0 mM CaCl2 at a flow rate of 50 µl/min. For affinity analysis, FcγRIIa and FcαRI were immobilized on carboxylated dextran CM5 sensor chips using primary amine coupling. Serial dilutions of SAP, CRP, and PTX3 from 7.2 to 0.04 µM were added. For C1q competition binding experiments, the analytes consisted of 2.72 µM CRP with 0.4 mg/ml of C1q. To measure the competition between human IgA and CRP on FcαRI binding, a CM5 chip was coupled with IgA at levels of 6000 - 9000 RU. The analytes consisted of 2.9 µM of CRP with or without 4.7 µM of refolded FcαRI. The dissociation constants were obtained by either steady state or kinetic curve fittings.

Cell surface binding by FACS analysis

A rat basophilic leukemia cell line (RBL) was stably transfected with a Gly 248 variant of human FcαRI(16). Human FcαRI exists in two common alleles (Gly and Ser) as a result of a single nucleotide polymorphism (SNP) at amino acid 248 in the cytoplasmic domain of the receptor gene. The G248 variant of FcαRI produced a more robust proinflammatory cytokines than the S248 variant in transfected cells as well as in human neutrophils. RBL cells and G248 cells were harvested with trypsin, and washed into PBS containing 0.1 % BSA and 0.05% sodium azide (PAB). Cells were incubated with CRP or Cy3-IgA for 30 min at 4^{o}, washed twice with PAB. CRP binding was detected with an anti-CRP mAb (FITC-2C10). Data were acquired using a FACScan or Accuri flow cytometer and analyzed with FlowJo Software.

Homology modeling of the FcαRI-CRP complex

An initial complex between FcαRI (1OW0) and CRP (1GNH) was prepared by manual superposition of the corresponding components onto FcγRIIa and SAP in the SAP-FcγRIIa complex (3D5O). Docking was performed by tumbling FcαRI over CRP, but was largely constrained to the contact interface in the SAP-FcγRIIa complex using the shape-only correlation in Hex5 with, standard parameters. After clustering three lowest energy orientations (-371.4 to -392.4KJ/mol) were selected as the final model.

ERK phosphorylation assay

RBL or 9.4 RBL cells were harvested with trypsin then washed into Tyrode's buffer (130 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5.6 mM Glucose, 10 mM HEPES, 0.5% BSA, pH 7.4) and resuspended at 5.0 x 10^{6} cells/ml. One hundred µl aliquots of

cells were incubated with buffer or CRP (200 μg/ml) for 1 h at 37˚. Cells were incubated with 2C10 (40 μg/ml) or buffer. For flow cytometry analysis, cells were fixed with 2% formaldehyde, followed by 90% methanol, and washed with PBS + 4 % FBS. Cells were stained with P-p44/42 MAPK (T202/Y204) (1:100) rabbit Ab (Cell Signaling Technologies) for 15 min, washed twice and then stained with a secondary Alexa Fluor 488 F(ab')2 goat anti-rabbit IgG (1:500)(Invitrogen) for 15 min. For western blotting, RBL or RBL 9.4 cells were seeded at 1.5 $x10^6$ cells in 60 mm dishes overnight in complete medium. After treatment, cells were washed with ice cold HBSS and then lysed with HBSS containing 1% Triton X-100 with protease and phosphatase inhibitors (Thermo Scientific). Lysates were incubated 20 min on ice, centrifuged at 20,000 x g for 25 min, separated by 10% SDS PAGE and transferred to PVDF. Membranes were probed with P-p44/42 MAPK (T202/Y204) rabbit Ab then probed with anti-rabbit IgG HRP (Cell Signaling Technologies). Membranes were stripped with Restore (Thermo Scientific) and probed for total ERK using p44/42 MAPK.

Degranulation and IL-4 production assays

RBL cells or transfected-RBL cells (G248 or 9.4) were cultured overnight in 48 well plates then washed into Tyrode's buffer. Some cells were incubated with 200 μg/ml IgA or CRP for 1 h at 37˚C. Buffer was removed and buffer or 40 μg/ml of F(ab')2 anti-IgA or 2C10 was added and incubated at 37˚C. In the case of G248 cells, 50 μg/ml AggCRP was added at time 0 and activity was measured over time. Supernatants were collected and β-hexosaminidase activity measured with respect to total release determined by lysis with 1% Triton X-100. Activity was measured by incubation with substrate, 1.4 mg/ml 4-

nitrophenyl-N-acetyl ß-D-glucosaminide in 75 mM sodium citrate, pH 4.5 for 1 h at 37˚. Reactions were stopped by addition of 0.2 M lysine, pH 10.7 and activity calculated from the A405 (% release = 100 x supernatant A405/A405 of detergent lysed cells). To assay for IL-4 production, G248 or untransfected RBL cells were seeded into 96 well plates at a density of $2 \bullet 10^4$ and pre-incubated with or without biotin labeled CRP (100 µg/ml) and/or piceatannol (25 µg/ml, Sigma) for 30 min, followed by streptavidin (20 µg/ml, Sigma) cross-linking of CRP. After 20 hours incubation at 37°C, the supernatant were assayed for rat IL-4 production using ELISA (R&D Systems, Inc.) according to manufacturer's instructions. Data shown were mean ± SEM of triplicate wells from one representative experiment.

Confocal microscopy

Human neutrophils were incubated in chamber slides (Thermo Scientific) for 2 h. PnC-SRBC were incubated with 150 µg/ml of CRP for 45 min at 37^o, washed and added to PMN at an 8:1 ratio for 10 min. Cells were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100 for 5 min. Slides were treated with Image-iT (Invitrogen). Cells were stained with anti-FcαRI antibody A59, washed, stained with a goat anti-mouse antibody labeled with Alexa Fluor 488 (Invitrogen), washed and mounted in ProLong Gold Antifade (Invitrogen). Images were acquired using a Zeiss LSM 510 inverted laser scanning microscope.

Neutrophil phagocytosis and cytokine secretion assays

Neutrophils were purified by Ficoll-Hypaque centrifugation and resuspended at $2x10^6$ cells/ml in RPMI-1640+10%FCS. *S. pneumoniae* serotype 27 (Pn27) (ATCC) was grown to log phase, washed in PBS, heat-killed, and FITC-conjugated. Neutrophils were combined with FITC-Pn27 and 100 μg/ml CRP, centrifuged briefly and incubated for 30 min at 37˚. Phagocytosis was measured by mean FITC-fluorescence on gated neutrophils after washing and adding trypan blue to quench uningested bacteria as Pn27 ingested/100 neutrophils. For cytokine secretion, neutrophils were treated for an hour with CRP or IgA (200 μg/ml) with or without the Fab fragment of MIP8a (15 μg/ml). Medium was removed, then 2C10 or anti-IgA (40 μg/ml) was added, and cells were incubated overnight at 37^o. Supernatants were analyzed for TNF-α using an R&D systems ELISA kit.

CHAPTER 3

MACROPHAGES ACTIVATED BY C-REACTIVE PROTEIN THROUGH FcγRI TRANSFER SUPPRESSION OF IMMUNE THROMBOCYTOPENIA

Kristopher Marjon*, Lorraine Marnell, Ph.D.†, Carolyn Mold, Ph.D.*, and Terry W. Du Clos, M.D., Ph.D*†.

*University of New Mexico, †Department of Veterans Affairs Medical Center, 1501 San Pedro SE, Albuquerque, NM 87108.

Keywords: C-reactive protein, Fc receptors, autoimmunity, acute phase reactants, inflammation.

Address correspondence and reprint requests to Dr. Terry W. Du Clos, VA Medical Ctr. 1501 San Pedro, S.E., Albuquerque, NM 87108. 505-265-1711 ext. 4140 E-mail address: tduclos@unm.edu

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ABSTRACT

C-reactive protein (CRP) is an acute phase protein with therapeutic activity in mouse models of systemic lupus erythematosus (SLE) and other inflammatory and autoimmune diseases. To determine the mechanism by which CRP suppresses immune complex (IC) disease, an adoptive transfer system was developed in a model of immune thrombocytopenic purpura (ITP). Injection of 200 μg of CRP 24 h before induction of ITP markedly decreased thrombocytopenia induced by anti-CD41. CRP-treated splenocytes also provided protection from ITP in adoptive transfer. Splenocytes from B6 mice were treated with 200 μg/ml of CRP for 30 min, washed and injected into mice 24 h before induction of ITP. Injection of 10^6 CRP-treated splenocytes protected mice from thrombocytopenia, as did intravenous immunoglobulin (IVIg)-treated but not BSA-treated splenocytes. The suppressive cell induced by CRP was found to be a macrophage by depletion, enrichment and the use of purified bone marrow-derived macrophages. The induction of protection by CRP-treated cells was dependent on FcR γ-chain and *Syk* activation, indicating an activating effect of CRP on the donor cell. Suppression of ITP by CRP-treated splenocytes required FcγRI on the donor cell and FcγRIIb in the recipient mice. These findings suggest that CRP generates suppressive macrophages through FcγRI, which then act through an FcγRIIb dependent pathway in the recipient to decrease platelet clearance. These results provide insight into the mechanism of CRP regulatory activity in autoimmunity and suggest a potential new therapeutic approach to ITP.

Introduction

C-reactive protein (CRP)[ii] is an acute phase serum protein and a classical pentraxin (1, 2). Serum levels of CRP may increase up to a thousand fold in response to inflammation, infection or trauma. Pentraxins are pattern recognition molecules composed of five identical subunits arranged in cyclic pentameric configuration (3). CRP interacts with the immune system through interaction with FcγR and activation of the classical complement pathway. The functions of CRP that have been best described include protection from bacterial infection, clearance of damaged tissue and regulation of the inflammatory response (1, 4). We have been particularly interested in the role of CRP in protection from autoimmunity, which was suggested by its ability to bind to nuclear antigens that are targets of autoantibodies in connective tissue diseases, including chromatin, histones and small nuclear ribonucleoproteins (5-7).

Studies in our laboratory first showed that CRP prevented accelerated autoimmune disease in (NZB x NZW) F1 female mice injected with chromatin (8). Subsequent studies by our laboratory and others have confirmed that CRP treatment suppresses glomerulonephritis and delays mortality in both the (NZB x NZW) F1 and the MRL/Fas^{lpr} mouse models of systemic lupus erythematosus (SLE) (9-11). CRP transgenic mice are also protected from experimental autoimmune encephalomyelitis, a predominantly T cell-mediated autoimmune disease (12). Others established potent anti-inflammatory activity of CRP in endotoxin and cytokine-induced shock (13), and we showed that this activity of CRP was dependent on FcγR (14). Our results in the SLE models suggested that the anti-inflammatory activity of CRP was also central to its effectiveness in treating ongoing lupus nephritis (9, 11). Using a nephrotoxic nephritis model, we established that CRP-mediated suppression of IC-induced renal disease

required FcγRI and macrophages (15). However, identification of the mechanisms involved in CRP-mediated regulation of inflammation has been limited by the requirements for direct administration of CRP into the animal model or the expression of CRP as a transgene product. Therefore, we sought to develop a model in which CRP would induce a suppressive cell *in vitro* with activity after adoptive transfer. This approach would allow for the identification of the active cell, the receptors required for its generation and the mechanism by which it induces regulation of the inflammatory response.

Immune thrombocytopenic purpura (ITP) is a common human autoimmune disease, which is caused by the production of anti-platelet antibodies (16, 17). These autoantibodies opsonize platelets for splenic clearance, resulting in low levels of circulating platelets. The disease is characterized by recurring episodes of severe thrombocytopenia and is associated with a bleeding diathesis. Treatments for ITP include splenectomy, high dose corticosteroids and intravenous immunoglobulin (IVIg). Over the last decade, the mechanism by which IVIg modulates ITP has been under intense investigation but no consensus exists for a single protective mechanism (18, 19). The development of an adoptive transfer approach to study the mechanism of IVIg-mediated protection from ITP was recently reported (20).

FcγR on splenic macrophages are the primary mediators of ITP in man and mouse (16, 21). FcγRI, FcγRIII and FcγRIV are activating receptors in mice that signal through ITAMs on the associated γ-chain (22). FcγRIIb is the only inhibitory FcγR and contains an ITIM in its intracellular domain. The balance between activating and inhibitory FcγR is critical in the ability of cells to not only respond but also to regulate the intensity of the

response (23). For example, mice that are normally resistant to collagen-induced arthritis become susceptible when FcγRIIb is genetically eliminated (24). CRP binding to FcγR was first demonstrated for FcγRI (25). Additional reactivity of CRP with FcγRII was later established in both man and mouse (26-28). Recently, we defined the crystal structure of the related pentraxin serum amyloid P component in complex with FcγRIIa as a model for CRP-FcγR interaction (29). These studies confirmed the interaction of CRP with FcγR and defined the binding characteristics of the human pentraxins for all three classes of human FcγR.

In these experiments, we examine the effect of CRP treatment *in vitro* and *in vivo* in a mouse model of ITP. Adoptive transfer of CRP-treated cells was used in this model to examine the role of activating and inhibitory FcγR in CRP-mediated suppression. A primary role for macrophages in transfer of suppression was established using depletion and enrichment strategies. The results demonstrate that activation of macrophages by CRP through FcγRI produces a cell capable of suppressing IC disease in the absence of the continued presence of CRP. We propose that induction of this suppressive macrophage is the initial event in CRP suppression of a range of inflammatory and autoimmune diseases.

Material and Methods

Reagents

CRP was purified from human pleural fluids as described previously (30). All preparations were examined on overloaded SDS-PAGE gels stained with SYPRO Ruby protein gel stain (Invitrogen) to ensure purity. No additional bands other than the major

band at about 23 kDa were seen. All preparations used contained < 3 endotoxin units (0.3 ng) of endotoxin per mg of protein determined by a quantitative chromogenic Limulus amebocyte lysate assay (Lonza). IVIg purchased from Bayer was used for most experiments. In some experiments IVIg (Baxter) provided as a generous gift from Dr. Ralph C. Williams was used with equivalent results. Chicken OVA, piceatannol, egg phosphatidylcholine, BSA and cholesterol were purchased from Sigma-Aldrich. Clodronate was provided by Boehringer Mannheim. Recombinant mIFN-γ was purchased from R&D Systems. Antibodies were obtained as follows: rabbit IgG anti-OVA from MP Biomedical; rat mAb specific for mouse CD41 (IgG1k, clone MWReg 30), FcγRI (CD64) and CD11b from BD Biosciences; rat mAb specific for F4/80 and FcγRIIb (clone MM7301) from Caltag; rat mAb specific for CD11c from Miltenyi. Rat mAb 2.4G2 was used as an FcγR blocking agent. Corresponding isotype control antibodies were used in all flow cytometry experiments. Magnetic microbeads along with separations columns were purchased from Miltenyi. IC were prepared by mixing chicken OVA with a 10-fold molar excess of anti-OVA for 30 min at room temperature.

Mice

C57BL/6 (B6) mice were obtained from the National Cancer Institute. FcγRIIB$^{-/-}$ mice and IL-10$^{-/-}$ mice were obtained from Jackson Laboratories and backcrossed to 10 generations on B6 mice. FcγRI$^{-/-}$ mice were obtained from J. S. Verbeek and further backcrossed onto B6 mice (31). Female mice at 6–12 wk of age were used in all experiments. All procedures involving animals were approved by the Institutional Review Board of the Department of Veterans Affairs Medical Center.

Induction of ITP

Mice were injected i.v. with 4 µg of anti-CD41 in 0.2 ml PBS pH 7.2. Blood was taken from the retroorbital plexus and diluted in Unopettes (Unopette, BD Bioscience). Platelets were counted using a hemocytometer. Baseline platelet counts were obtained from mice before treatment. In the adoptive transfer experiments, the average baseline platelet counts for each mouse strain were determined and are shown as normal platelet counts on the graphs.

Treatment of ITP in vivo

Mice were injected i.p. with 50 mg IVIg 24 h before the injection of anti-CD41 (20). For CRP, mice were injected i.p. with 200 µg of CRP 24 h before treatment with anti-CD41 or i.v. with 200 µg of CRP 4 h before treatment with anti-CD41.

Treatment of spleen cells in vitro and adoptive transfer

Splenocytes were prepared from donor mice and erythrocytes lysed with ammonium chloride (ACK) lysis buffer. In the standard protocol splenocytes were suspended in serum free RPMI-1640 at a concentration of 1.4×10^6 cells/ml and treated with 200 µg/ml of CRP, 200 µg/ml BSA or 18 mg/ml of IVIg for 0.5 h at 37^{o}C. After treatment, cells were washed twice in RPMI-1640 and resuspended at a concentration of 5×10^6 cells/ml and 0.2 ml of the suspension was injected i.v. into recipient mice 24 h before induction of ITP.

Culture of bone marrow macrophages (BMM)

Bone marrow cells were cultured for one week in DMEM (Sigma-Aldrich, high glucose) with 20% FBS and 30% L-cell conditioned medium, as a source of M-CSF. BMM were > 95% positive for the F4/80 macrophage marker by flow cytometry. BMM were treated with 100 U/ml IFN-γ for 24 h before treatment with CRP or IVIg. Cells were harvested

using 10 mM EDTA for 5 min at 4C, washed and suspended to desired concentration in RPMI for treatment, as described for splenocytes.

Macrophage depletion and enrichment

Liposomes containing dichloromethylene bisphosphonate (Clodronate) were prepared as previously described (32). The liposomes were washed by ultra-centrifugation and mice were injected i.v. with 0.2 ml of liposomes containing Clodronate or control liposomes prepared with PBS. Spleens were taken from mice 48 h after injection of liposomes. We previously found that treatment with Clodronate liposomes depletes 99% of Kupffer cells and 95% of splenic macrophages (33). Clodronate liposome treatment of mice resulted in an 80% decrease in F4/80+ spleen cells by flow cytometry.

F4/80+ cells were enriched from adherent splenocytes using biotin anti-F4/80 and streptavidin beads (Miltenyi). The target cell population was selected, following the manufacturer's instructions, and was verified by analysis using flow cytometry.

Immunoprecipitation and Western Blot Analysis of Syk phosphorylation

BMM (10×10^6) in 1 ml were stimulated with 200 μg/ml CRP for the desired time, then immediately put on ice and subsequently washed twice with PBS. Cells were lysed with lysis buffer (1% NP-40, 0.15 M NaCl, 0.05 M Tris, pH 7.2 with Halt Protease Inhibitor 2X (Thermo Scientific), and 100 µM activated NaVO4). The protein concentration was determined by a BCA assay (Thermo Scientific). *Syk* was immunoprecipitated with anti-*Syk* (N-19) antibody overnight with protein A/G PLUS-agarose beads (Santa Cruz Biotechnology). Beads were washed five times with lysis buffer then boiled for 5 min in 2X loading buffer. Samples were loaded on a 4-20% SDS polyacrylamide precast gel (Invitrogen) then SDS PAGE was carried out for 3 hours at 120 V. Proteins were

transferred to a PVDF membrane with a wet transfer system (NOVEX) at 25 V for 90 min. The membrane was blocked with 5% BSA in TBST (TBS + 0.1% Tween-20) for 1 h then probed for Phospho-*Syk* (Cell Signaling Technology) with polyclonal Phospho-*Syk* antibody (1:1000) in TBST + 5% BSA over night at 4^{o}C. The membrane was washed three times for 5 min in TBST. HRP conjugated secondary anti-rabbit IgG (Cell Signaling Technologies) (1:2000) was incubated with membrane for 1 h at room temperature. The blot was washed three times with TBST before detection with ECL (SuperSignal West Dura Extended Duration Substrate, Thermo Scientific). The blot was imaged using a CCD camera (Gene Gnome, Syngene Bio Imaging System) and analyzed with ImageJ (NIH). Optical density was determined and normalized for each sample using the IgG heavy chain as an internal loading control.

Data analysis

Graphical and statistical analyses were performed using GraphPad Prism software (GraphPad). Data are presented as the means ± SEM for the number of mice indicated in the figure legends. Comparisons between means were done using Student's t test or one way ANOVA. p values are indicated in the figure legends as follows: * = $p<0.05$, ** = $p<0.01$; *** = $p<0.001$.

Results

Injection of CRP protects mice from ITP.

An experimental model of ITP was established based on the previous studies of Siragam et al (20). B6 mice and FcγRIIB$^{-/-}$ mice were injected with a mAb to CD41 and platelet counts were performed over time. At 24 h after injection, platelet levels were found to be at their lowest (Fig. 1A). In all subsequent experiments, platelets were counted 24 h after

injection of the anti-platelet antibody. To determine whether CRP would suppress ITP, mice were injected i.p. with 200 μg of CRP 24 h before injection of anti-CD41. An additional set of mice was injected with 50 mg of IVIg i.p. as described (20). IVIg was administered i.p. to avoid fluid overload due to rapidly increasing the intravascular volume. As shown in Fig. 1B, i.p. injection of 200 μg of CRP protected mice from developing ITP to a similar degree as did administration of 50 mg of IVIg (Fig. 1C). In data not shown, injection of 200 μg of CRP i.v. 4 h before induction of ITP was equally effective to injection of CRP 24 h prior to induction of ITP.

CRP protects mice from ITP in the adoptive transfer system

CRP inhibited the induction ITP to a similar degree as, but at a much lower dose than IVIg when injected directly. To determine whether CRP was acting to block FcγR-mediated uptake or whether an effector cell was generated, adoptive transfer of suppression was tested. Previous studies indicated that treatment of splenocytes *in vitro* with 18 mg/ml of IVIg rendered them capable of adoptive transfer of suppression of ITP (20). Spleen cells were treated with 0-500 μg/ml of CRP or 18 mg/ml IVIg as a positive control and then transferred to mice, which were subsequently treated with mAb to CD41. As seen in Fig. 2A and Fig. 2B**,** CRP treated splenocytes were as effective as IVIg

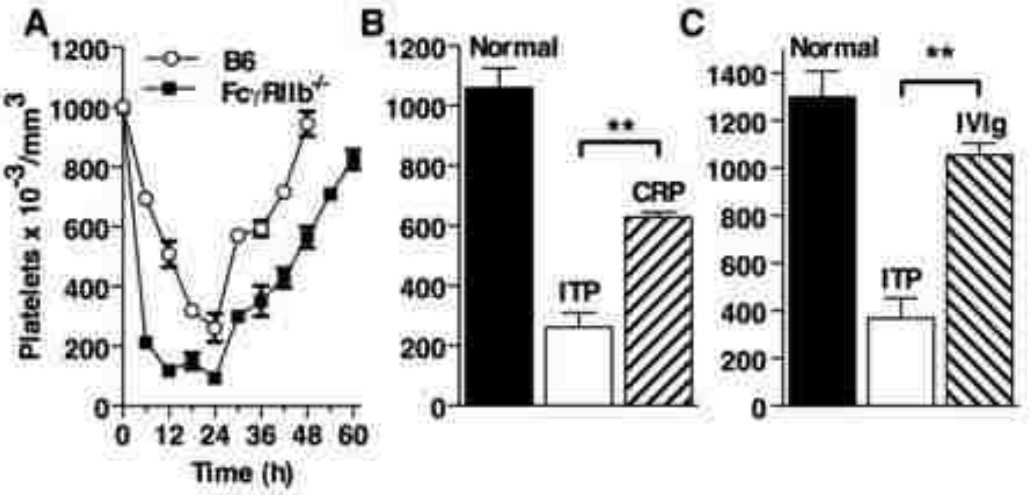

FIGURE 1. Injection of CRP or IVIg protects mice from ITP. *A.* ITP model, B6 and FcγRIIB$^{-/}$ mice were injected with mAb to CD41 and platelets were counted every 6 h. n=3. *B.* B6 mice were injected i.p. with 200 μg of CRP 24 h before injection with anti-CD41. Twenty-four h later platelets were counted. n=3. *C.* B6 mice were injected i.p. with 50 mg of IVIg 24 h before injection with anti-CD41. Twenty-four h later platelets were counted. Normal indicates platelet counts in mice before treatment. n=3.

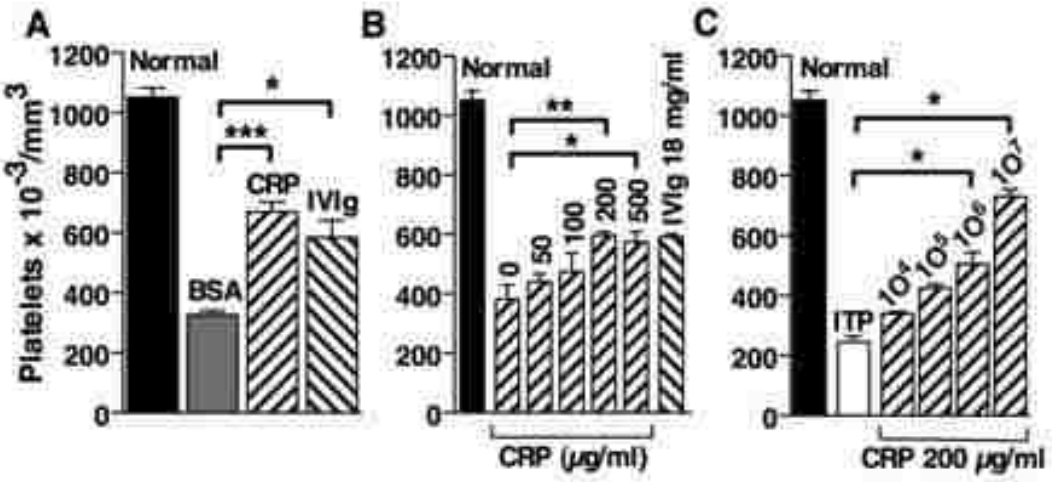

FIGURE 2. Transfer of CRP-treated spleen cells protects mice from ITP. Spleen cells from B6 mice were treated with CRP, BSA or IVIg for 30 min, washed and injected i.v. into B6 mice. Twenty-four h after cell transfer mice were injected i.v. with anti-CD41 to induce ITP and 24 h later platelets were counted. *A*. Spleen cells were treated with 200 μg of CRP or BSA or with 18 mg/ml of IVIg and 10^6 cells were transferred. *B*. Spleen cells were treated with 0-500 μg/ml of CRP or 18 mg/ml of IVIg before transfer. *C*. Spleen cells were treated with 200 μg/ml of CRP before transfer of the indicated number of cells. ITP indicates that no spleen cells were transferred. n = 3.

treated splenocytes with maximum effectiveness at a concentration of approximately 200 μg/ml, a dose that was ninety times less than IVIg. Subsequent experiments used a CRP dose of 200 μg/ml for adoptive transfer studies. We next determined the number of splenocytes that were required to transfer suppression of ITP. Splenocytes treated with 200 μg/ml of CRP were transferred at numbers ranging from 10^4-10^7 cells. The effect of cell transfer was seen with as few as 10^4 cells but increasing numbers of cells gave progressively more suppression of thrombocytopenia (Fig 2C).

Induction of the suppressive phenotype requires signaling through the γ-chain and Syk activation

Our previous studies indicated that many of the effects of CRP on inflammation *in vivo* are due to interaction with FcγR (15). We first examined whether the effects of CRP were mediated by activating FcγR using γ-chain$^{-/-}$ mice. Mouse FcγRI, III and IV are expressed in association with the γ-chain, and use this chain to initiate signaling. FcγR signaling also requires *Syk,* which is recruited to phosphorylated ITAMs and activated. Syk then phosphorylates adaptor proteins, which are necessary for downstream signaling events (23). Splenocytes from γ-chain$^{-/-}$ mice were treated with CRP and transferred to B6 mice after which ITP was induced. Splenocytes from γ-chain$^{-/-}$ mice provide no protection from ITP (Fig. 3A). Therefore, to determine whether the γ-chain is needed simply for CRP to interact with the cell or if signaling downstream of the γ-chain is also required, inhibition of FcγR signaling was examined. Splenocytes from B6 mice were treated with CRP after

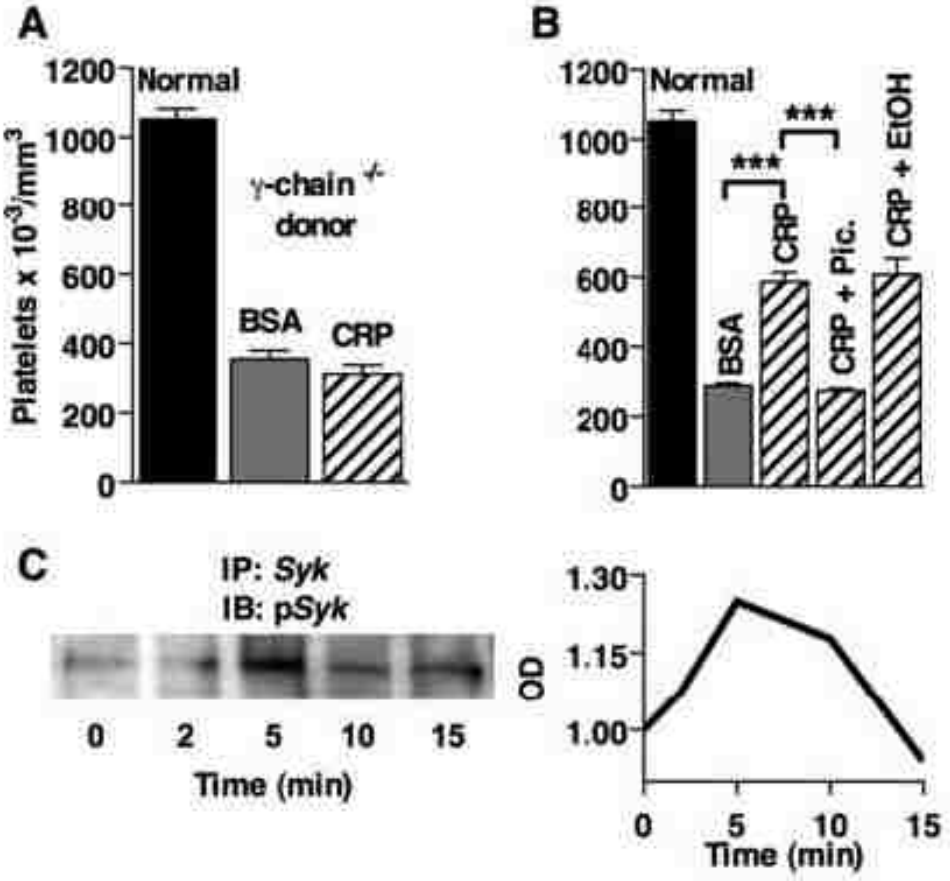

FIGURE 3. CRP-mediated protection from ITP requires the FcR γ-chain and *Syk* activation. Spleen cells were treated with 200 μg/ml of CRP or BS*A* for 30 min, washed and 10^6 cells were injected i.v. into B6 recipient mice. Twenty-four h after spleen cell transfer, mice were injected with anti-CD41 and 24 h later platelets were counted. *A*. Spleen cells from FcR γ-chain$^{-/-}$ mice injected into B6 mice. n = 3. *B.* Spleen cells from B6 mice were treated with 10 μM piceatannol or ethanol (vehicle control) before CRP treatment and transfer into B6 mice. n = 3. *C.* BMM from B6 mice were stimulated with CRP 200 μg/ml for 0, 2, 5, 10 and 15 min then immunoprecipitated with anti-*Syk*. The blot was probed for phospho-*Syk* and the bands were normalized to IgG heavy chain in each respective lane.

incubation with the *Syk* inhibitor, piceatannol or ethanol (vehicle) before transfer. Inhibition of *Syk* eliminated the suppressive effect of CRP-treated cells whereas vehicle alone did not. Thus, generation of the suppressive phenotype required signaling through a γ-chain associated receptor and *Syk* activation. To verify that signaling was initiated, BMM from B6 mice were stimulated with CRP for various time points then lysed and immunoprecipitated with anti-*Syk*. Proteins were transferred and probed for phosphorylated *Syk*. Fig. 3C demonstrates that CRP induced maximum phosphorylation at 5 min. Together these results indicate that CRP induces intracellular signaling events through *Syk* to mediate protection in recipient mice.

FcγRI is required for CRP-mediated protection

Our previous studies of CRP-mediated suppression of inflammation suggested a central role for FcγRI (15). FcγRI is the high affinity receptor for IgG and binds CRP with moderate to high affinity. Spleen cells from FcγRI$^{-/-}$ mice were treated with CRP *in vitro*, washed and then transferred to B6 mice before induction of ITP. No protective effect on the development of ITP was seen (Fig. 4A), indicating that FcγRI expression on the donor cell is required for CRP-mediated suppression. The requirement for FcγRI in the recipient mouse of adoptive transfer was also examined. In contrast to the results using FcγRI$^{-/-}$ donor cells, FcγRI was not required for development of ITP or for suppression by CRP-treated B6 splenocytes (Fig. 4B). These results suggest that FcγRI is an integral part of the protective effect of CRP in the donor cell but that its presence is not required in the recipient. These results indicate that the initial event in the suppressive cell is an activating event, which induces the suppressive phenotype.

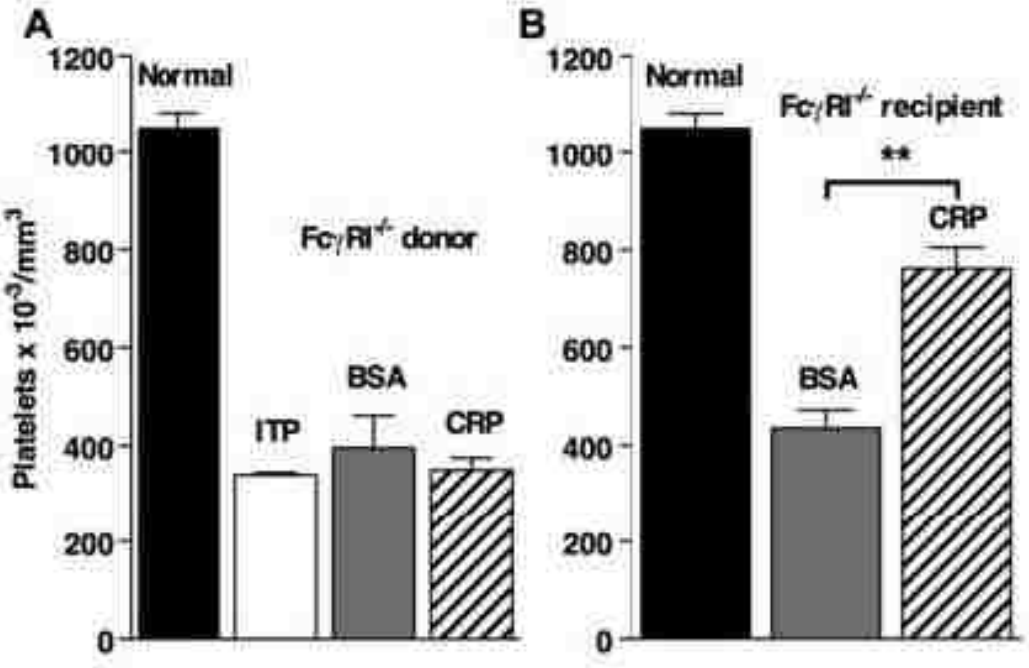

FIGURE 4. CRP-mediated protection from ITP requires FcγRI on donor splenocytes. Spleen cells were treated with 200 μg/ml of CRP or BSA for 30 min, washed and 10^6 cells injected i.v. into recipient mice. Twenty-four h after spleen cell transfer, mice were injected with anti-CD41 and 24 h later platelets were counted. *A.* Spleen cells from FcγRI$^{-/-}$ mice injected into B6 mice. *B.* Spleen cells from B6 mice were injected into FcγRI$^{-/-}$ mice. ITP indicates that no spleen cells were transferred. n = 3.

CRP interaction with FcγRIIb is not required for its protective role

FcγRIIb is an inhibitory receptor and has been shown to regulate phagocytosis through activating FcγR (34). It has also been shown that FcγRIIb is required in recipients for the adoptive transfer of suppression of ITP by IVIg-treated spleen cells (20). To test the requirement for FcγRIIb expression in CRP-mediated suppression, FcγRIIB$^{-/-}$ mice were used either as a source of splenocyte suppressor cells or as recipients undergoing ITP. Spleen cells from FcγRIIB$^{-/-}$ mice were fully active in adoptive transfer of suppression (Fig. 5A). However, CRP-treated splenocytes from B6 mice were unable to suppress ITP in FcγRIIB$^{-/-}$ mice (Fig. 5B). These findings indicate a crucial role for FcγRIIb in the recipient mouse as previously found for IVIg (19, 20).

IL-10 is not required for suppressor cell activity

Previous studies in our laboratory on the suppression of nephrotoxic nephritis indicated a requirement for IL-10 in CRP-mediated suppression. We therefore tested IL-10$^{-/-}$ mice as donors and recipients in the ITP model. Unlike the case of nephrotoxic nephritis, IL-10 was not required in either the donor or the recipient for suppression of ITP by CRP-treated splenocytes (Fig. 6).

CRP-treated splenocytes from IL-10$^{-/-}$ donor mice showed a small decrease in the ability to transfer suppression of ITP compared to wild type cells. Thus suppression by CRP-treated splenocytes does not rely completely on IL-10.

The adoptive transfer of CRP-mediated suppression requires macrophages

Previous adoptive transfer studies of IVIg-mediated suppression suggested that dendritic cells were the major cell population responsible for suppression (20). Preliminary studies

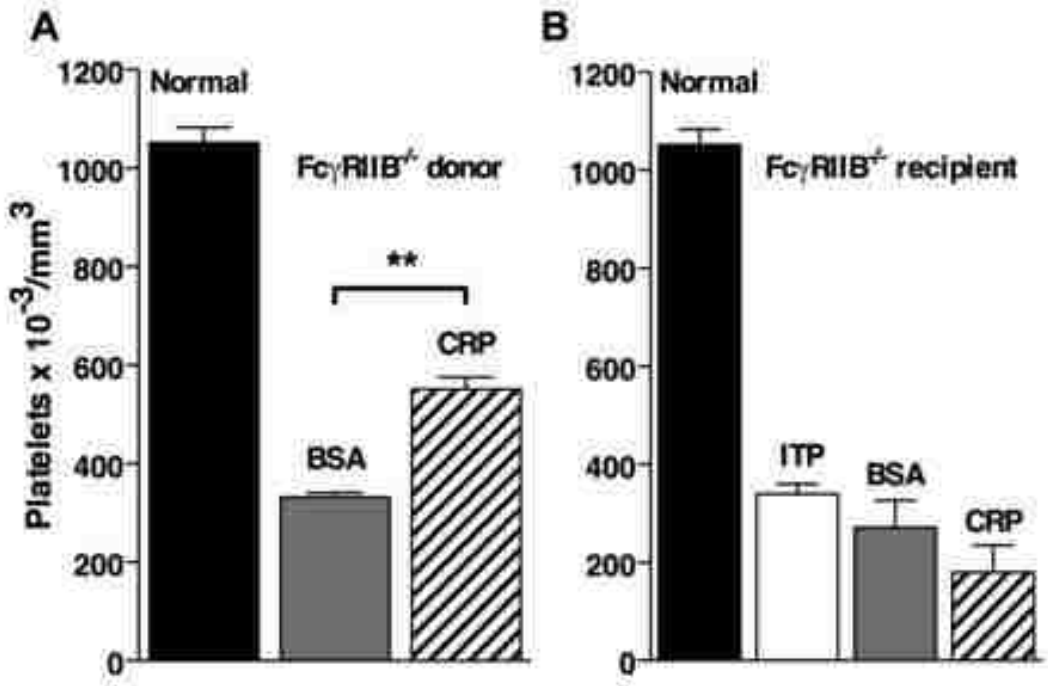

FIGURE 5. CRP-mediated protection from ITP requires FcγRIIb in recipient mice. Spleen cells were treated with 200 μg/ml of CRP or BSA for 30 min, washed and 10^6 cells injected i.v. into recipient mice. Twenty-four h after spleen cell transfer, mice were injected with anti-CD41 and 24 h later platelets were counted. *A.* Spleen cells from FcγRIIB$^{-/-}$ mice injected into B6 mice. *B.* Spleen cells from B6 mice injected into FcγRIIB$^{-/-}$ mice. n = 3.

were performed to determine whether dendritic cells were responsible for CRP-mediated suppression. CD11c+ cells were isolated using magnetic bead separation, treated with CRP and tested in the adoptive transfer system. CRP-treated $CD11c^+$ dendritic cells were less effective than $CD11c^-$ cells in transferring suppression (not shown). To determine whether macrophages were the cells in the splenocyte population that mediates the protective effect of CRP, mice were treated with Clodronate-containing liposomes 2 days before harvesting spleens. Mice treated with Clodronate containing liposomes lack splenic macrophages for 5-6 days (32). Spleen cells from these mice were then treated with CRP *in vitro* and tested for adoptive transfer of suppression. CRP-treated splenocytes from Clodronate-treated mice were unable to transfer suppression of thrombocytopenia (Fig. 7A). However, CRP-treated spleen cells from control mice were effective (Fig. 7A) as were spleen cells from mice that were pretreated with PBS-liposomes (not shown). In contrast, IVIg mediated protection was not eliminated by Clodronate pretreatment of mice (Fig. 7A). We next used magnetic bead separation with anti-F4/80 to enrich for splenic macrophages. F4/80 enriched spleen cells transferred suppression of ITP after CRP treatment, but were less effective after IVIg treatment (Fig. 7B). These studies suggest that macrophages are the predominant cell type required for the transfer of CRP-mediated suppression of ITP and that different cells are essential for transfer of suppression following CRP and IVIg treatment.

BMM transfer the protective effect from CRP

The previous data suggested that splenic macrophages are required to transfer the protective effect of CRP. To determine if macrophages alone could mediate this effect,

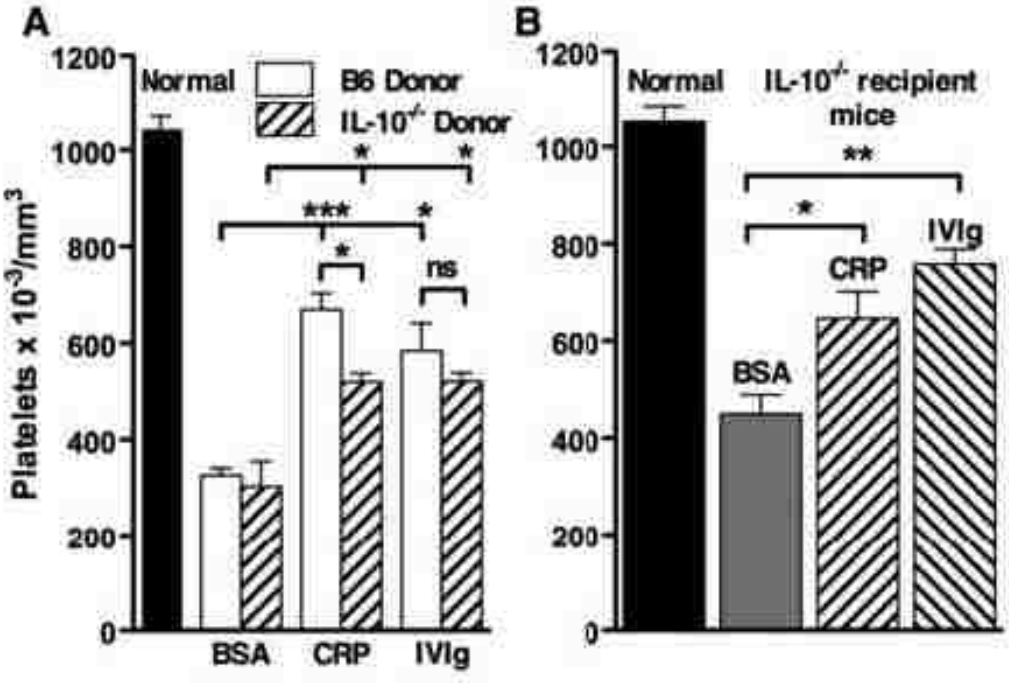

FIGURE 6. CRP-mediated protection from ITP does not require IL-10. Spleen cells were treated with 200 µg/ml of CRP or BSA or 18 mg/ml IVIg for 30 min, washed and 10^6 cells were injected i.v. into recipient mice. Twenty-four h after spleen cell transfer, mice were injected with anti-CD41 and 24 h later platelets were counted. *A*. Spleen cells from B6 or IL-$10^{-/-}$ mice were injected into B6 mice. ***B.*** Spleen cells from B6 mice injected into IL-$10^{-/-}$ mice. n = 3.

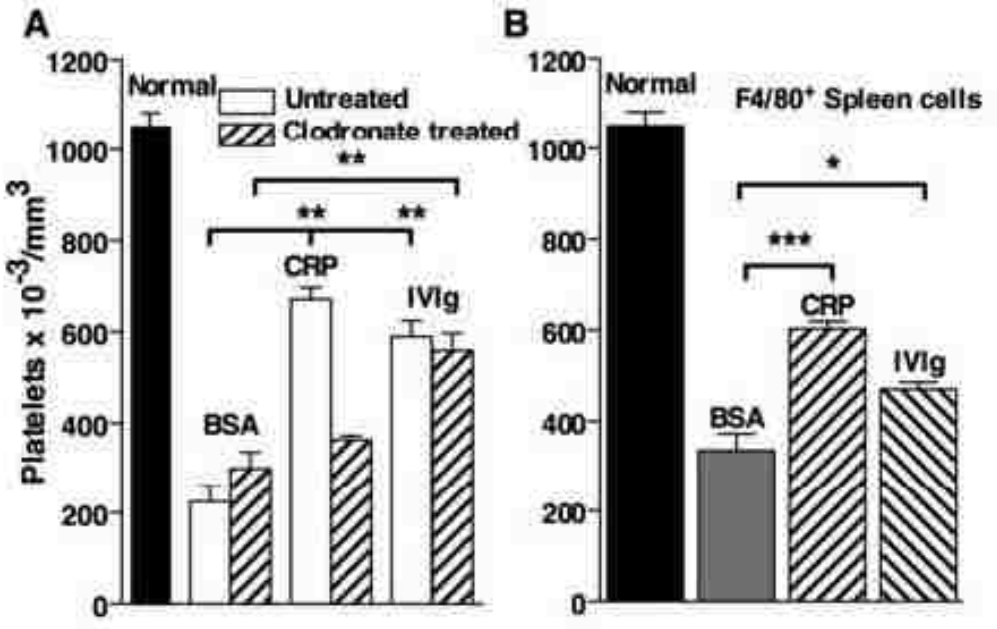

FIGURE 7. Splenic macrophages are required for the transfer of protection from ITP by CRP-treated splenocytes. *A*. B6 mice were untreated or injected with liposomes containing Clodronate to deplete macrophages. After 48 h, spleens were removed and cells were treated with 200 μg/ml of CRP or BSA or with 18 mg/ml of IVIg for 30 min, washed, and 10^6 cells were injected i.v. into recipient mice. Twenty-four h after spleen cell transfer, mice were injected with anti-CD41 and 24 h later platelets were counted. n=3. ***B.*** Spleen cells from B6 mice were enriched for macrophages by selection for F4/80+ cells. These cells were treated with CRP, BSA or IVIg, washed, and then 1 x 10^6 cells were injected into B6 mice. Twenty-four h after treatment mice were injected with anti-CD41 to induce ITP. Twenty-four h later blood was taken and platelets were counted. n=5.

purified BMM were used as the donor cell population. BMM from B6 mice were primed with IFN-γ for 24 h, then treated with CRP and transferred to B6 mice 24 h before induction of ITP (Fig. 8A). BMM treated with 18 mg/ml IVIg or 200 μg/ml IC prior to transfer were able to protect mice from developing ITP. BMM are greater than 95% F4/80+, arguing against a requirement for other cell types. A dose response experiment was done using BMM treated with 200 μg/ml CRP (Fig. 8B). BMM were maximally effective when 10^5 or 10^6 cells were transferred and 10^5 BMM were as protective as 10^7 spleen cells (Fig. 2C), further suggesting that macrophages are the active cell population in the spleen. To examine the role of FcγRI on the generation of the suppressive phenotype of macrophages by CRP, BMM from FcγRI$^{-/-}$ or B6 mice were treated with 200 μg/ml CRP then transferred to B6 mice. BMM that lack expression of FcγRI$^{-/-}$ were not able to protect mice from developing ITP (Fig. 8C).

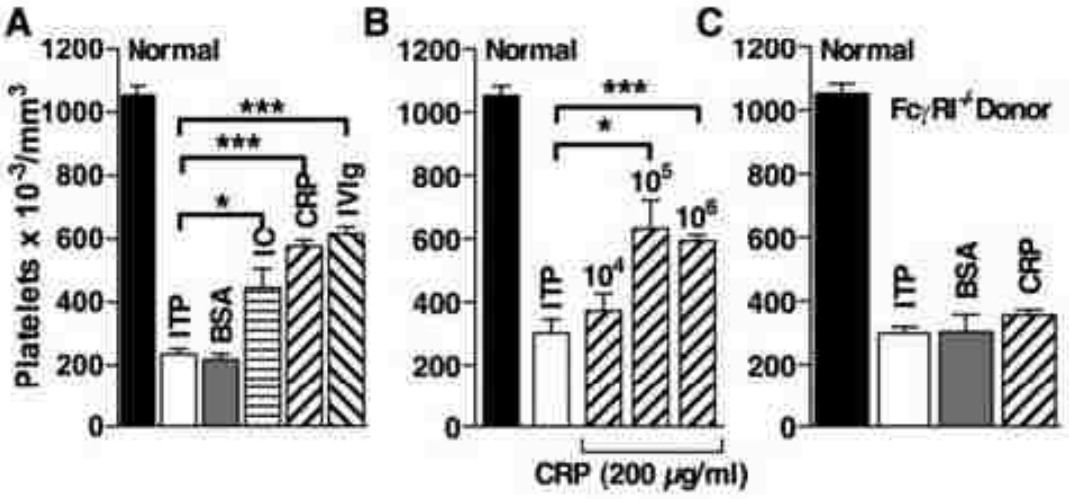

FIGURE 8. BMM reproduce the protective effect of CRP-treated splenocytes on ITP. BMM from B6 mice were prepared as described and treated with IFN-γ for 24 h before treatment with 200 μg/ml of CRP, BSA or IC or with 18 mg/ml of IVIg for 30 min. IC were prepared as described in methods. Cells were then washed and injected into B6 mice. Twenty-four h after transfer mice were injected with anti-CD41 to induce ITP and 24 h later platelets were counted. *A*. BMM were treated 200 μg/ml of CRP, BSA and IC or with 18 mg/ml of IVIg and 10^6 cells were injected per mouse. n = 3. *B*. BMM were treated with 200 μg/ml of CRP and 10^4, 10^5 or 10^6 cells were injected per mouse. n=5 combined from 2 experiments. *C.* BMM from FcγRI$^{-/-}$ mice were treated with 200 μg/ml of CRP and 10^6 cells were injected per mouse. n=3. ITP indicates that no BMM were transferred.

Discussion

We, and others, have previously reported that CRP suppresses inflammation in mouse models (13-15). However, these activities were dependent on the endogenous expression of CRP from a transgene or the direct injection of CRP. These requirements made identification of the cell type responsible for immune regulation and the mediators involved difficult to determine. The major new finding presented here is that CRP can be used in an adoptive transfer system to induce immunomodulation in a non-treated host. This approach allowed us to determine that macrophages activated by CRP through FcγRI are responsible for this activity.

CRP injection markedly suppressed the induction of ITP by anti-CD41. The degree of protection from ITP was similar to that provided by IVIg. However, CRP suppressed ITP at a much lower dose. IVIg is a heterogeneous preparation composed of multiple IgG isotypes with broad antigenic reactivity. The mechanism by which IVIg suppresses ITP remains unresolved. It is unknown whether the high dose of IVIg required is due to the low activity of the IgG or whether a minor fraction of the IgG is active based on, e.g. glycosylation status, aggregation or antigenic reactivity (35). Most recently, it has been suggested that the interaction of IVIg occurs through a receptor for sialylated Fc and not through FcγR (35, 36). However, dendritic cells activated through FcγR suppressed ITP in the adoptive transfer model studied here (20).

CRP binds to a limited group of determinants and is essentially homogenous with respect to binding specificity. CRP is a non-glycosylated molecule with no sequence variation or isotypes and thus binding to FcγR is expected to be homogeneous. CRP binding to FcγR occurs with 1:1 stoichiometry and monomeric CRP is therefore unlikely to induce

receptor crosslinking or signaling (29). The preparations used did not contain aggregates before injection, as determined by analytical gel filtration, but it is possible that ligands for CRP exist *in vivo*, which could result in receptor crosslinking. CRP was effective up to 24 h before induction of ITP suggesting that CRP need not be present throughout the course of ITP. Human CRP is known to have a short half-life in mice, 4 h (37).

CRP was also found to be active in a transfer model of suppression. This finding represents the first model in which one can transfer suppression using CRP-treated cells. The effect was very unlikely to be due to CRP carryover as splenocytes were washed before transfer to recipient mice. The dose response curve would suggest that a much higher dose of CRP would be required than any amount carried over in the washed cells. In addition, cells lacking FcγRI or γ-chain or cells treated with a tyrosine kinase inhibitor were inactive. FcγRI was not required in the recipient suggesting that at least two different cells could be involved in the adoptive transfer of suppression of ITP. The suppressive cell generated in the donor by CRP treatments must act on the effector cell in the recipient undergoing ITP that is responsible for platelet clearance. However, it is likely that, in the case of direct injection of CRP into mice undergoing ITP, no second cell is involved and CRP acts directly on the effector cell. The spleen cell dose response in adoptive transfer suggested that the suppressive cell was active at relatively low numbers or represented activity in a broad population of cells.

The results of additional experiments indicate that the active cell is a macrophage. This was somewhat unexpected as previous studies using IVIg indicated a central role for dendritic cells in this adoptive transfer model of ITP (20). Our experiments, using enrichment and depletion of splenic macrophage and dendritic cell populations, are

consistent with a requirement for macrophages for CRP-induced suppression, but not for IVIg-induced suppression. BMM treated with IFN-γ were however sufficient to transfer suppression of ITP after treatment with CRP, IVIg or IC. CRP-treated BMM were active at 10-fold lower numbers than splenocytes, consistent with the numbers of macrophages in the spleen. Although the overall effect of CRP appears similar to IVIg, the receptor interactions undoubtedly differ between the two agents.

Suppressive macrophages with different phenotypes have been described in several systems (38). Mosser's group has characterized a macrophage that he has called type II activated macrophages. These macrophages produce IL-10 and down regulate IL-12 production, but are distinguished from alternatively activated macrophages by their expression of nitric oxide rather than arginase and other characteristics (39). Activation of this phenotype is induced by crosslinking activating FcγR together with TLR stimulation (40). Type II activated macrophages were active in the regulation of endotoxin-induced inflammation and altered T cell responses to antigen, but have not been studied in IC inflammation models.

Our previous studies have suggested an important role for the suppressive effect of CRP on inflammatory models in mice (15). The results indicated a central role for FcγRI, the high affinity receptor for IgG and a receptor of intermediate affinity for CRP. The ability of CRP to induce the suppressive macrophage was strictly dependent on FcγRI. Ongoing studies indicate that CRP binds FcγRI with lower affinity than IgG (29). FcγRI is an activating, γ-chain associated receptor suggesting that the initial event involved in generating the suppressive phenotype is also activating. FcγRI has been implicated in the

suppressive activities of IC (41) and FcγRI$^{-/-}$ mice have enhanced antibody production in response to immunization (42).

In the recipient mice undergoing ITP, the presence of FcγRIIb was required for suppression. A requirement for FcγRIIb was shown previously in both direct treatment and adoptive transfer models of IVIg suppression of ITP (20). However, it is possible that the somewhat greater efficacy of the mAb in induction of thrombocytopenia makes the FcγRIIB$^{-/-}$ mouse more resistant to suppression by CRP.

Because the protective effect of CRP in nephrotoxic nephritis required IL-10 and not FcγRIIb, we tested the requirement for IL-10 in the ITP transfer model. Unlike the case of nephrotoxic nephritis, no requirement for IL-10 was seen, as IL-10 deficient mice were fully responsive to CRP treatment. The effect of the CRP-induced suppressive macrophage population was not limited to ITP as these cells were also effective in the Arthus model (data not shown). The Arthus reaction is a well-established and defined model of IC-mediated inflammation. Neutrophil recruitment to the peritoneal cavity of the mouse is mediated primarily by two chemokines that are produced by resident macrophages.

We cannot conclusively determine that the mechanism involved in the adoptive transfer corresponds to the *in vivo* protection from ITP shown by CRP. However, these studies establish an initial common pathway for anti-inflammatory effects of CRP in two distinctly different models. We have defined a population of macrophages induced by CRP acting through FcγRI that is active in an adoptive transfer model. The mechanisms by which these cells mediate suppressive effects are currently incompletely understood,

but they are likely to play an initial role in many of the antiinflammatory activities of CRP.

CRP could be considered as an alternative therapeutic agent to IVIg for several reasons. CRP is a homogeneous well-defined molecule of consistent properties. It was active at a much lower dose than IVIg *in vitro* and *in vivo*. IVIg can contain antigenic specificities that are potentially deleterious. The supply of IVIg is limited due to the current increasing range of therapeutic indications and the inherent requirement for very large numbers of donors.

Acknowledgments

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Footnotes

[i] This work was supported by the Department of Veterans Affairs and the University of New Mexico RAC.

[ii] Abbreviations used in this paper: B6, C57BL/6; BMM, bone marrow macrophage; CRP, C-reactive protein; Clodronate, dichloromethylene bisphosphonate; IC, immune complex; ITP,

immune thrombocytopenic purpura; IVIg, intravenous immunoglobulin; SLE, systemic lupus erythematosus.

CHAPTER 4

RECOGNITION AND FUNCTIONAL ACTIVATION OF HUMAN FcαRI BY C-REACTIVE PROTEIN

Jinghua Lu*[1], **Kristopher D. Marjon***[2], Lorraine L. Marnell[2], Ruipeng Wang[1], Carolyn Mold[2], Terry W. Du Clos[2,3] and Peter D. Sun[1]

[1]Structural Immunology Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852, USA; [2]Department of Internal Medicine and Department of Molecular Genetics and Microbiology, University of New Mexico, Albuquerque, NM 87131, USA and [3]VA Medical Center, Albuquerque, NM 87108, USA.

*Both authors contributed equally to the manuscript

Corresponding authors: Peter D. Sun (psun@nih.gov); or Terry W. Du Clos (tduclos@unm.edu)

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ABSTRACT

C-reactive protein (CRP) is an important biomarker for inflammatory diseases. However, its role in inflammation beyond complement-mediated pathogen clearance remains poorly defined. Here, we identified the major IgA receptor, FcαRI, as a new ligand for pentraxins. CRP recognized FcαRI both in solution and on cells, and the pentraxin binding site on the receptor appears distinct from that recognized by IgA. Further competitive binding and mutational analysis showed that FcαRI bound to the effector face of CRP in a region overlapping with C1q and FcγR binding sites. CRP crosslinking of FcαRI resulted in ERK phosphorylation, cytokine production and degranulation in FcαRI transfected RBL cells. In neutrophils, CRP induced FcαRI surface expression, phagocytosis and TNF-α secretion. The ability of CRP to activate FcαRI defines a novel function for pentraxins in inflammatory responses involving neutrophils and macrophages. It also highlights the innate aspect of otherwise humoral immunity-associated antibody receptors.

INTRODUCTION

C-reactive protein (CRP), a member of the pentraxin family, is a major acute phase protein in human and a clinical marker of infection (1). Interest in the biological activities of CRP has increased dramatically in recent years due to its association with inflammatory diseases like atherosclerosis and autoimmune diseases like systemic lupus erythematosus. Other pentraxins include serum amyloid P component (SAP), PTX3, NPTX1 and NPTX2. They are innate pattern recognition molecules targeted to various microbial and self-determinants including polysaccharides, phosphocholine and

phosphoethanolamine on the surface of microorganisms, apoptotic or necrotic cells, and nuclear autoantigens. CRP and SAP are produced in the liver in response to inflammatory cytokines such as IL-6 and IL-1. While the role of CRP in pathogen clearance through complement activation has been established (2), the participation of pentraxins in activating cellular immune functions is poorly understood due to lack of knowledge of their cellular receptors. CRP and SAP have been shown to bind and activate FcγR on monocytes and macrophages (1, 3-6). In addition, CRP suppressed immune complex-mediated nephrotoxic nephritis in a mouse model (7). Despite their distinct folds, both antibody and pentraxins bind FcγR in a 1:1 stoichiometry, obligating pathogen opsonization or immune complex formation as the mechanism for receptor clustering and activation (6, 8-9). In addition, they share an overlapping binding site on FcγR, predicting a mutually exclusive FcγR association between antibodies and pentraxins.

Human macrophages and neutrophils express a major receptor for IgA, FcαRI/CD89, which activates through the common FcR γ-chain. FcαRI activation by IgA immune complexes leads to phagocytosis, antigen presentation and the release of cytokines, superoxide and other inflammatory mediators (10). Despite sharing the common γ-chain for signaling, IgA and IgG antibodies recognize their own receptors and do not cross-react. The structural recognition of IgA by FcαRI is distinct from that of IgG by FcγRs (8, 11). Nevertheless, the ability of pentraxins to bind FcγRs with broad specificities and the functional similarity between FcγRs and FcαRI prompted us to investigate if pentraxins recognize the receptor for IgA. Here we identify FcαRI, for the first time, as a receptor for pentraxins. The establishment of specific interactions of pentraxins with Fc

receptors provides insight into the mechanism for these soluble pattern recognition molecules to activate macrophages and neutrophils. The finding also reveals a novel role of FcαRI in the innate immune response.

RESULTS

Pentraxins recognize FcαRI in solution

CRP and SAP were first shown to bind FcγRI-transfected cells and activate phagocytosis through FcγRI and FcγRIIa (4-5). More recently, a systematic solution binding study revealed a broader recognition between pentraxins and all isoforms of FcγRs (6). This broader recognition between pentraxins and FcγR is supported by their closely related structures in that CRP and SAP share identical structural folds and form similar pentamers. Similarly, FcγRs consist of homologous tandem Ig-like domains with IgG binding sites located in the two structurally similar membrane proximal domains (8, 12-13). The permissive pentraxin-FcγR recognition led us to investigate further pentraxin recognition of other Fc receptors, including an IgA receptor, FcαRI, and an IgE receptor, FcεRI. Both FcαRI and FcεRI consist of two tandem Ig-like domains. Functionally, FcαRI and FcεRI share a common signaling γ-chain with FcγRs, and participate in antibody-mediated inflammation, phagocytosis and cytokine release. To examine whether pentraxins interact with these Fc receptors, recombinant FcαRI and FcεRI were immobilized on CM5 BIAcore sensor chips together with FcγRIIa (CD32A) as a control. The binding with various dilutions of CRP, SAP or PTX3 as the analytes showed that CRP and SAP, but not PTX3 bound to immobilized FcαRI, with affinities of 2.8±0.2 and 3.2±0.2 μM, respectively (Figure 1), similar to their binding to FcγRs (6). It is worth

noting that the kinetic rate constants for CRP and SAP binding to FcαRI are quite different. While CRP binding to FcαRI (ka=3.1 ±1.4 x10^{5} $M^{-1}s^{-1}$, kd=0.35±0.02 s^{-1}) resembles the pentraxin and IgG binding to the low affinity Fcγ receptors (6), the SAP binding to FcαRI (ka=1.5 ±0.5 x10^{4} M^{-1} s^{-1}, kd=0.031±0.008 s^{-1}) displays slower kinetic association and dissociation rates. The molecular basis for the observed differential rate constants between CRP and SAP binding to FcαRI is not clear. Interestingly, FcαRI is located genetically on human chromosome 19 in a region close to the leukocyte receptor complex (LRC) that encodes KIR, ILT/LILR and NKp46 (14-15). FcαRI is most homologous to NKp46 and KIR sharing 30-35% in sequence identity. Structurally, FcαRI also resembles KIR and NKp46 with a similar juxtaposition in its two Ig-like domains, which is opposite to those in FcγRs and FcεRI (Figure 1B). However, CRP failed to bind immobilized FcεRI, KIR and NKp46 (Figure S1), suggesting that the pentraxin recognition of FcαRI is specific. The ability of pentraxins to recognize both FcαRI and FcγRs receptors further extends the functional similarity between them.

CRP recognizes FcαRI on transfected RBL cells

To determine whether the observed solution binding between pentraxins and FcαRI also occurred on cell surfaces, we investigated CRP binding to RBL cells, a rat basophilic leukemia cell line, stably transfected with a Gly 248 variant of FcαRI (referred to as G248 cells)(16). The expression of FcαRI on G248 cells can be readily detected by an anti-FcαRI antibody, MIP8a, (Figure 2). G248 cells and untransfected RBL cells were incubated with CRP, followed by a FITC-labeled anti-CRP antibody (2C10) for FACS analysis (Figure 2C). Greater CRP binding was observed to G248 cells than to

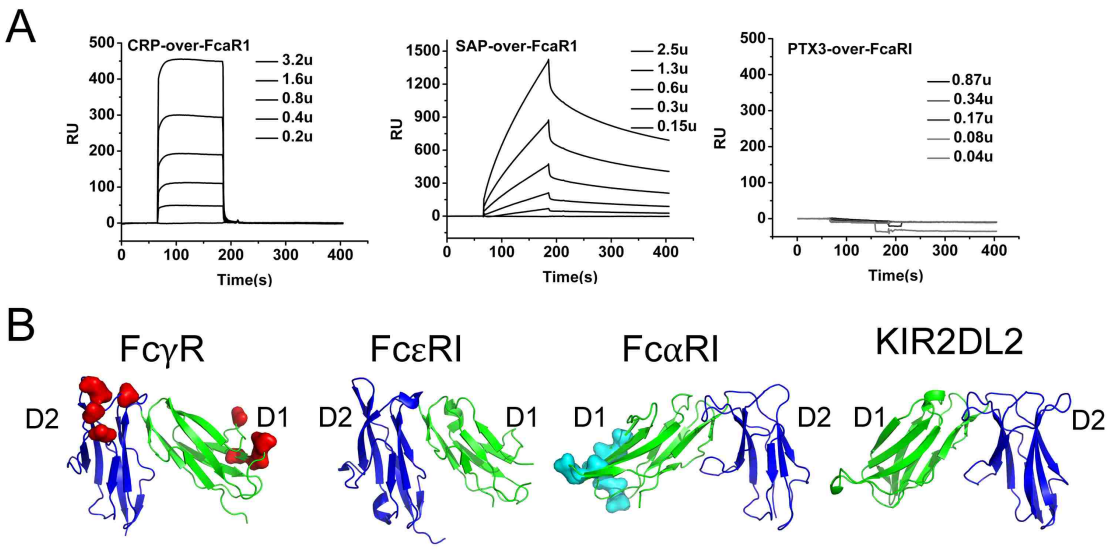

Figure 1. CRP and SAP bind to FcαRI in solution. (A) The binding between serial dilutions of CRP, SAP or PTX3 in micromolar concentrations (u) and immobilized FcαRI on a CM5 sensor chip. (B) The D1 (green) and D2 (blue) domains of FcγRIIa (3D5O), FcεRI (1F2Q), FcαRI (1OW0) and KIR2DL2 (2DL2) are shown in respective D2 orientations. The CRP binding sites on FcγRIIa and the IgA binding site on FcαRI are shown as surface patches.

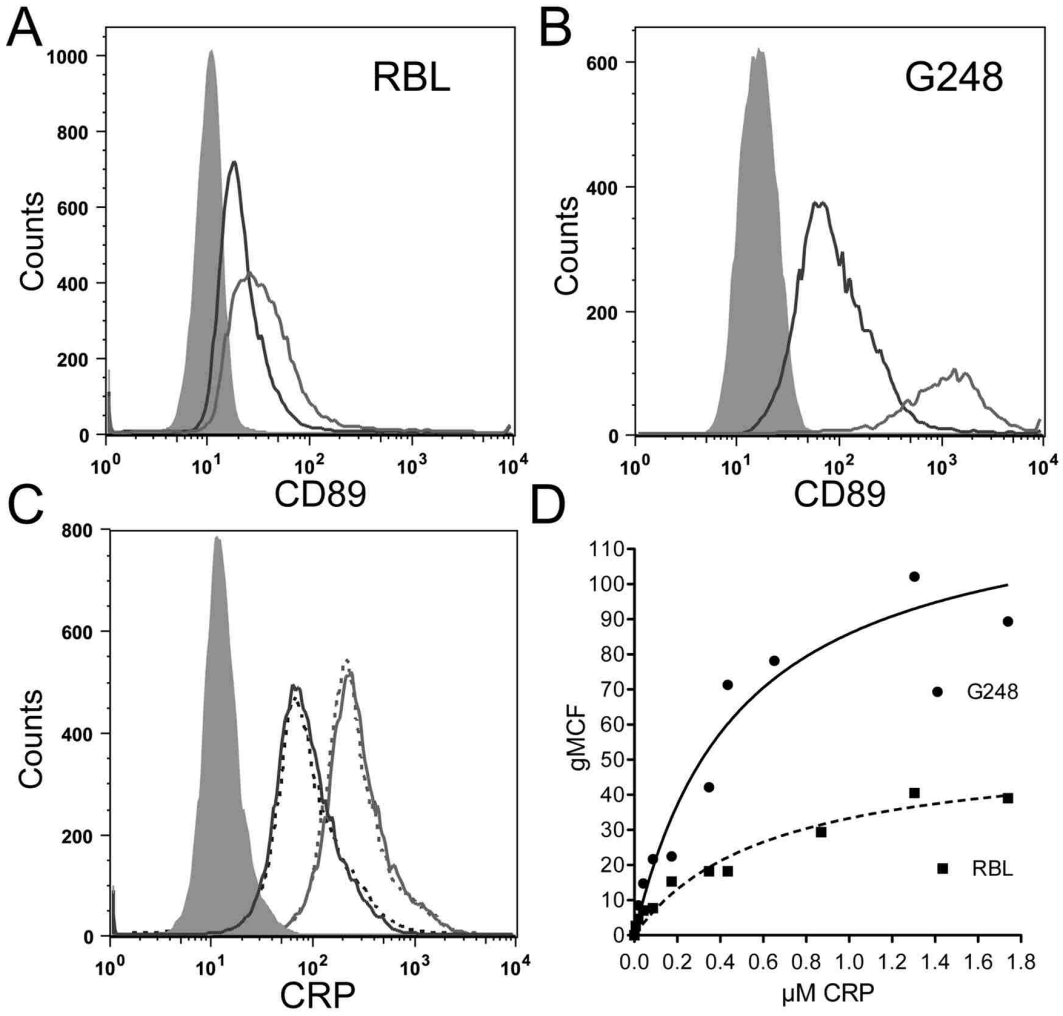

Figure 2. CRP binds to FcαRI on transfected RBL cells. (A, B) Anti-FcαRI (MIP8a)(gray) or isotype control (black) staining of RBL (A) or FcαRI-transfected RBL (G248) (B) cells. (C) CRP (150 μg/ml) bound to G248 (gray) better than RBL cells (black). The CRP binding to G248 cells was blocked by MIP8a (heavy dashed) but not by its isotype control (thin dashed). Filled histograms are unstained cells. Horizontal axes are fluorescence intensities. (D) Dose-dependent CRP binding to G248 or RBL cells detected using FITC-2C10. Data are representative of at least three experiments.

untransfected RBL cells, although significant binding to the untransfected cells was also observed, probably due to the binding of CRP to rat FcγRs on RBL cells (17-18). More importantly, the binding of CRP to G248 cells was reduced to the level of RBL cells in the presence of MIP8a but not its isotype control. The binding of CRP to G248 cells was dose dependent with an apparent KD of 0.3 μM (Figure 2D). These results are in agreement with the data obtained by surface plasmon resonance (SPR) measurements and showed that CRP recognized specifically FcαRI both in solution and on transfected cells.

The pentraxin binding site on FcαRI is distinct from that of IgA

The crystal structure of FcαRI in complex with the Fc region of IgA showed that IgA recognizes the N-terminal Ig-like domain of FcαRI (D1) (Figure 1B) (11). This recognition is very different from that of IgG binding to FcγR, which involves both the N- and C-terminal domains of the receptor (D1 and D2) (8-9). The stoichiometry of these interactions is also different with IgG binding to FcγR at 1:1 and IgA binding to FcαRI at 1:2. The IgG binding site on FcγR partially overlaps with that of the pentraxins resulting in pentraxins competing with IgG for FcγR binding (6). To determine whether pentraxins and IgA share a common binding site on FcαRI, a solution binding competition experiment between CRP and IgA was carried out using soluble FcαRI as analyte on an IgA-immobilized sensor chip. If CRP shares a common binding site with IgA, the addition of CRP to the analyte is expected to block the receptor binding to immobilized IgA. In contrast, the addition of CRP to FcαRI enhanced the receptor binding to IgA (Figure 3A). The enhanced binding response is likely due to the binding of the higher molecular mass of CRP-FcαRI complex to IgA, suggesting that CRP and IgA bind to

distinct regions of FcαRI. Using the FcαRI-transfected RBL cells (G248), we observed that while MIP8a blocked both IgA and CRP binding to the transfected RBL cells (Figure 3C, 2B), a second anti-FcαRI mAb (A59), which binds to the D2 domain of FcαRI away from the IgA binding site, partially inhibited CRP but not IgA binding to the FcαRI-transfected cells (Figure 3B, 3C). Similarly, preincubation with IgA failed to block CRP binding to FcαRI on the transfected G248 cells (Figure 3B). Conversely, unlabeled CRP at a concentration of 1.3 μM failed to inhibit IgA (Cy3 labeled) binding to G248 cells at 0.03 or 0.125 μM of IgA (Figure 3C). These results are consistent with CRP and IgA binding to distinct regions of FcαRI and raise the possibility that CRP and IgA could simultaneously interact with FcαRI and potentially co-stimulate cells.

FcαRI binds to a similar region on CRP as FcγRIIa

The pentameric ring of pentraxins has two faces, a ligand-binding face known to recognize microbial ligands in a calcium-dependent manner, and an effector face to interact with complement C1q and FcγRs. To determine whether FcαRI also binds to the effector face of the pentraxins, a competitive CRP binding between C1q and FcαRI was carried out using BIAcore with immobilized recombinant FcαRI and FcγRIIa. CRP, when present at 2.7 μM in the analyte, displayed similar binding to immobilized FcαRI and FcγRIIa (Figure 4A). In contrast, C1q did not bind either receptors. Since both C1q and FcγRIIa interact with the effector face of CRP, their binding to CRP is mutually exclusive. As expected, the CRP binding to FcγRIIa was partially reduced with the addition of 0.25 μM of C1q to the CRP containing analyte, and eliminated when the concentration of C1q was increased to 1 μM (Figure 4A). Similarly, the presence of 1

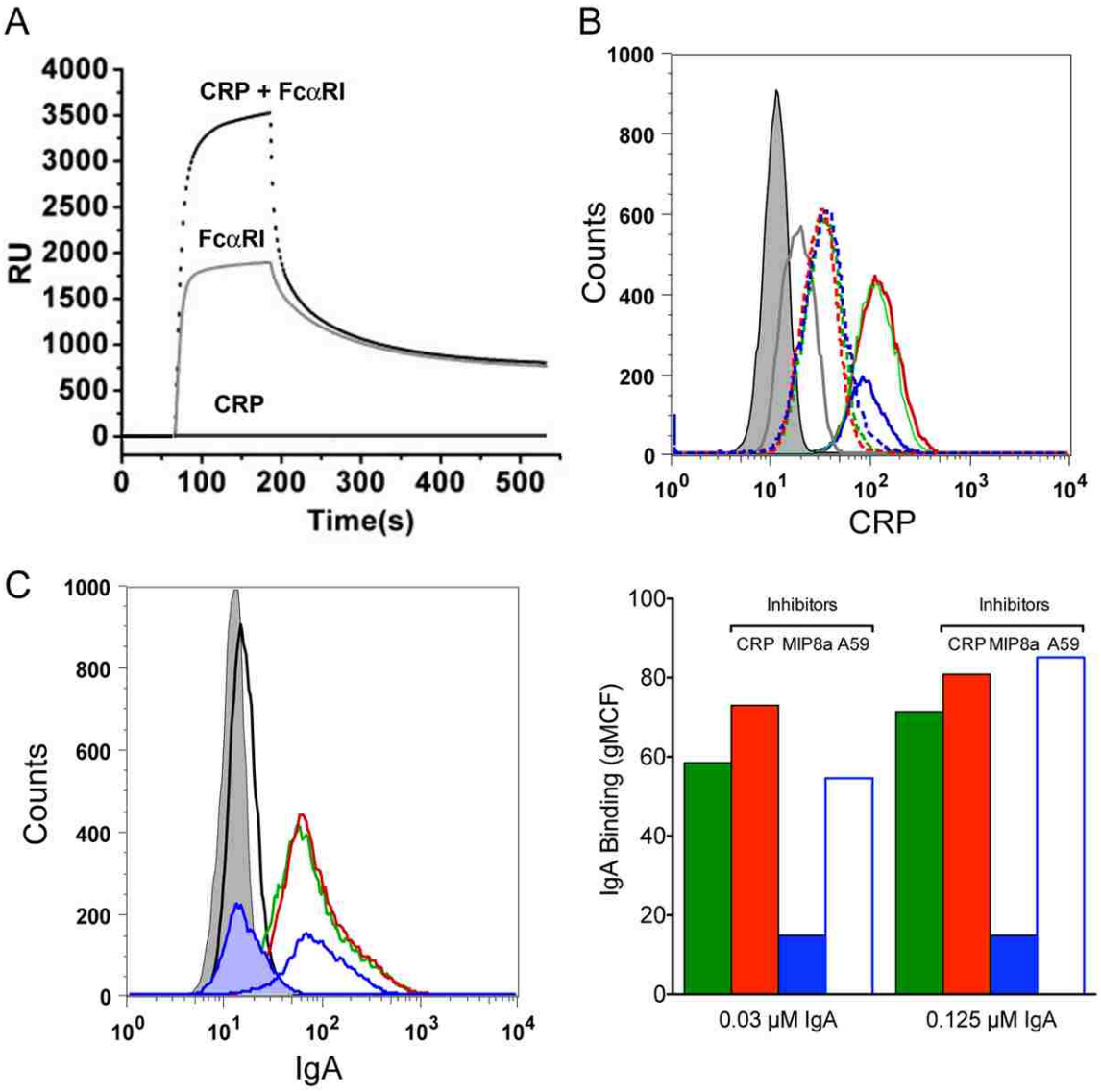

Figure 3. CRP and IgA bind at nonidentical sites on FcαRI. (A) The solution binding response for CRP (2.9 μM), recombinant FcαRI (4.7 μM) or their combination onto an IgA immobilized CM5 sensor chip. CRP alone did not bind IgA. (B) The binding of CRP alone (red) in the presence of IgA (1000 μg/ml) (green), or mAb A59 (blue) to G248 (solid lines) and RBL (dashed lines) cells. Unstained G248 (gray line) and RBL cells (shaded gray) are shown. Horizontal axes of the histograms are fluorescence intensities. (C) Cy3-IgA binding to G248 cells alone (green) or in the presence of 150 μg/ml CRP (red), blocking MIP8a (blue shaded), and non-blocking anti-FcαRI mAb A59 (blue line). Unstained G248 (black line) and Cy3-IgA stained RBL cells (shaded gray) are shown. Bar graph shows the geometric mean channel fluorescence for Cy3-IgA binding.

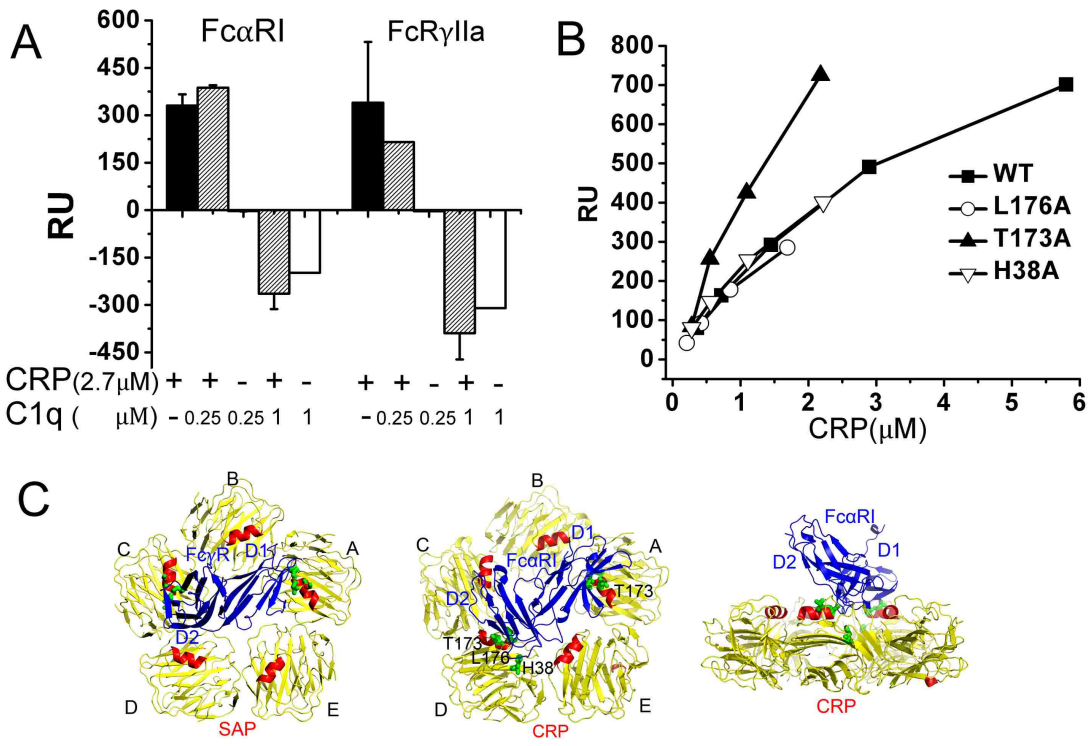

Figure 4. FcαRI recognizes the CRP effector face. (A) Competitive solution binding between C1q and immobilized FcαRI or FcγRIIa to CRP using BIAcore. Recombinant FcαRI and FcγRIIa were immobilized individually on CM5 chips. The analytes were CRP (2.7 μM) in the presence or absence of 0.25 or 1 μM C1q. C1q alone resulted in close to zero response at 0.25 μM and negative responses at 1 μM, likely due to higher binding to the dextran sulfate surface in the reference cell. (B) The equilibrium binding responses of wild type and mutant CRP to immobilized FcαRI (see supplemental Figure S2 for sensorgrams). (C) The structure of SAP-FcγRIIa complex (3D5O) and the docked CRP- FcαRI model in two orthogonal views. The mutation sites used in (B) are shown by green sticks. The putative interface residues in CRP-FcαRI model are listed in supplemental Table S1.

μM but not 0.25 μM of C1q blocked the CRP binding to immobilized FcαRI. Since C1q exists as a hexamer of trimer with each trimeric head capable of binding to one pentameric CRP (19), 0.25 and 1 μM of C1q are expected to titrate a maximum of 1.5 and 6 μM of CRP, respectively. This is consistent with the observed partial or no inhibition to CRP (2.7 μM) binding to FcRs at the lower concentration of C1q but complete blockage to the CRP binding at the higher concentration of C1q. The stoichiometric inhibition of CRP binding to FcαRI by C1q suggests that FcαRI also interacts with the effector face of CRP. Thus, CRP recognition of C1q, FcαRI, and FcγRs are mutually exclusive.

To determine whether FcγRs and FcαRI recognize similar sites on pentraxins, we examined the receptor binding of CRP mutants that were previously identified as defective in FcγR binding. His 38, Thr 173 and Leu 176 form part of the putative FcR binding site on CRP and mutations of each one reduced both FcγR and C1q binding significantly (6, 20). When these CRP mutants were assayed for FcαRI binding using BIAcore, H38A and L176A bound to the receptor similarly to wild type CRP, while T173A showed increased FcαRI binding compared to the wild type (Figure 4B). These mutational data suggest that while both FcαRI and FcγRs recognize the same face of CRP, the specific interface residues are likely to differ. This is not surprising as FcαRI adopts an opposite three dimensional domain arrangement from FcγRs (Figure 1B). The T173A mutant of CRP will provide a useful reagent to look at differential effects of the two receptor classes.

Based on the assumption that FcαRI binds to a similar site on pentraxins as FcγRs, a docking model for FcαRI binding to CRP was generated using the crystal structure of the

SAP-FcγRIIa complex (Figure 4C). Despite its opposite domain orientation, FcαRI could be docked onto CRP based on the FcγRIIa complexed SAP structure due to the pentameric symmetry of pentraxins. The model shows that it is possible for FcαRI to interact with pentraxins in a diagonal orientation similar to FcγRIIa in the SAP complexed structure. However, unlike FcγRIIa, which contacts the A and C subunits of SAP, the opposite D1-D2 hinge angle of FcαRI results in the receptor contacting the A and D subunits of pentraxins (Figure 4C). Interestingly, the structural model of the CRP-FcαRI complex shows T173 and L176 but not H38 as the immediate receptor contacting residues (supplemental Table 1), consistent with the mutant binding data showing that the binding of Thr 173 but not His 38 was affected compared to the wild type CRP.

CRP crosslinking of FcαRI leads to the activation of cellular functions

Similar to FcγR and FcεRI, FcαRI associates with the common FcR γ-chain and signals through the γ-chain ITAM motif (10). Crosslinking of FcαRI leads to activation of several kinases including Syk and ERK (21). We have previously shown that pentraxin recognition of FcγRs results in phagocytosis and cytokine secretion by monocytes and macrophages (1, 5-6, 2223). To investigate whether CRP recognition of FcαRI results in receptor activation, we examined both ERK phosphorylation and degranulation in FcαRI-transfected RBL cells (9.4) upon CRP crosslinking. Since RBL cells express FcεRI, which associates to and can compete with FcαRI for the FcR γ-chain, and FcαRI is known to exist in a γ-chain-free form (10), we obtained an FcαRI-transfected RBL cell line, referred to as RBL 9.4, that expresses a chimeric FcαRI with the cytosolic domain of the receptor replaced by that of the FcR γ-chain (24). ERK phosphorylation was readily

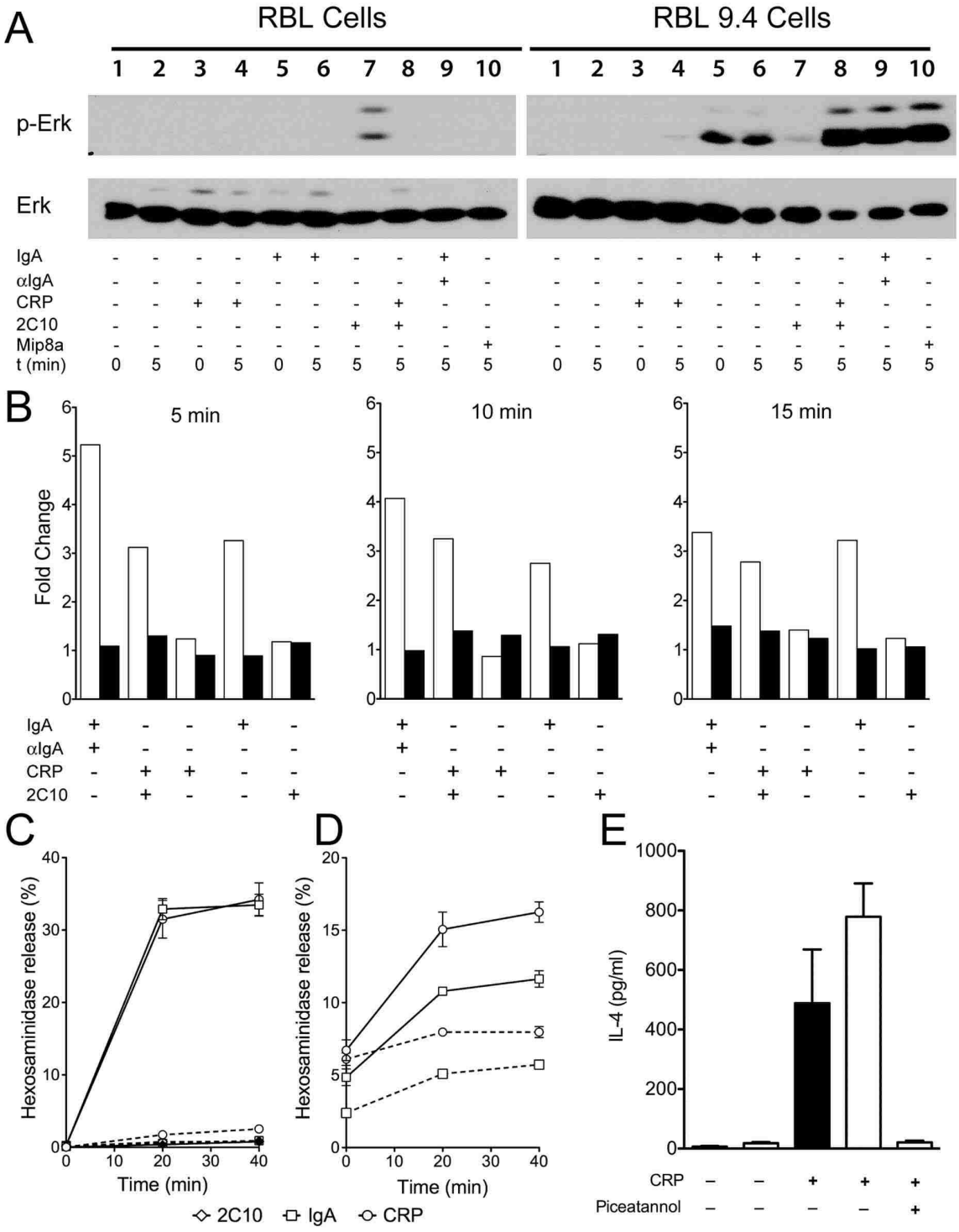

Figure 5. CRP induces ERK phosphorylation, degranulation and cytokine secretion in FcαRI transfected RBL cells. (A) FcαRI-transfected (9.4) or untransfected RBL cells were preincubated with CRP or IgA (200 µg/ml), then incubated with 2C10, anti-IgA or MIP8a at time 0. Cells were lysed at time 0 and 5 min,

detected for phospho-ERK by Western blot. Blots were stripped and reprobed for total ERK. Results are representative of 3 experiments. (B) RBL 9.4 (open bars) or untransfected RBL cells (solid bars) were treated as in panel A and ERK phosphorylation was determined at 5, 10 and 15 min by flow cytometry. (C) FcαRI-transfected 9.4 (solid lines) or RBL (dashed lines) cells were preincubated with CRP (circle) or IgA (square), then crosslinked with 2C10 or anti-IgA. No significant ß-hexosaminidase release was detected in 9.4 cells treated with 2C10 alone (diamond). ß-hexosaminidase release was measured and expressed as the percentage of total activity. Means ± SEM of triplicate wells from one experiment are shown. (D) Similar ß-hexosaminidase release was measured as in (C) except on FcαRI-transfected G248 cells with streptavidin crosslinked biotin-CRP. (E) IL-4 secretion in G248 (open) or untransfected RBL (solid) cells treated with streptavidin crosslinked CRP with or without piceatannol.

detectable in these RBL 9.4 cells upon crosslinking of the receptor either by using MIP8a or by binding IgA followed by anti-IgA crosslinking (Figure 5A). More importantly, the binding of CRP to FcαRI-expressing RBL 9.4 cells followed by crosslinking with anti-CRP antibody (2C10) induced higher levels of ERK phosphorylation by both western blot and FACS analysis than the same treatment of untransfected RBL cells (Figure 5A, B). CRP-induced ERK phosphorylation was detectable up to 15 min after the crosslinking. RBL cells express the high affinity IgE receptor, FcεRI, and the FcR γ-chain, and crosslinking of FcεRI by IgE leads to potent degranulation as measured by the release of ß-hexosaminidase. This γ-chain dependent degranulation was also observed in G248 cells upon antibody crosslinking of FcαRI (16). Importantly, significant release of β-hexosaminidase was observed in both RBL 9.4 and G248 but not untransfected cells upon CRP crosslinking (Figure 5C,D). The level of CRP-induced degranulation was comparable to IgA. In addition to degranulation, activated mast cells also produce IL-4 (25-26). As RBL is a mast cell line, we tested the secretion of IL-4 upon crosslinking of FcαRI by CRP in G248 cells. A significant amount of IL-4 was detected upon CRP-crosslinking of G248 cells. Further, the cytokine production was inhibited by piceatannol, a known Syk inhibitor of FcRγ-chain signaling in mast cells (27-28) (Figure 5E). These results suggest that CRP crosslinking activates FcRγ-chain signaling pathway through FcαRI.

CRP induces neutrophil surface expression of FcαRI, phagocytosis and TNF-α production

IgA crosslinking of FcαRI induced the receptor surface redistribution into lipid raft like domains in FcαRI transfected A20 cells (29). In neutrophils, FcαRI is rapidly mobilized from intracellular granules to the surface by chemokines and other mediators (30). We examined the effect of CRP binding on FcαRI surface expression on neutrophils using confocal microscopy. Labeled RBC coated with PnC as CRP ligands were incubated with neutrophils and the expression distribution of FcαRI was measured with mAb A59 and AF488 labeled secondary antibody. Interestingly, FcαRI was diffusely distributed on neutrophils with a significant amount residing in the intracellular compartment (Figure 6A). Binding of CRP-opsonized SRBC resulted in a sharp thin layer of the receptor entirely distributed on the surface of treated neutrophils with no detectable intracellular localization of the receptor (Figure 6B). This suggests that CRP binding induces the surface expression of FcαRI. We next examined the role of CRP binding to FcαRI in phagocytosis of bacteria by neutrophils. *Streptococcus pneumoniae* serotype 27 (Pn27) was used because it expresses the CRP ligand phosphocholine in its capsule as well as its cell wall. FITC-conjugated Pn27 were opsonized with CRP and incubated with neutrophils. Phagocytosis was determined from the FITC intensity associated with neutrophils after quenching extracellular fluorescence. Phagocytosis of Pn27 was increased with CRP opsonization and preincubation of the neutrophils with the anti-FcαRI (MIP8a) significantly inhibited the phagocytosis of CRP-opsonized Pn27 (Figure 6C). Pretreatment with A59 or an IgG1 isotype control did not inhibit the phagocytosis. Activated neutrophils produce type 1 inflammatory cytokines, including TNF-α (31-32). We then examined whether CRP activation of FcαRI induces TNF-α production. The result showed that crosslinking CRP with an anti-CRP antibody (2C10) induced the

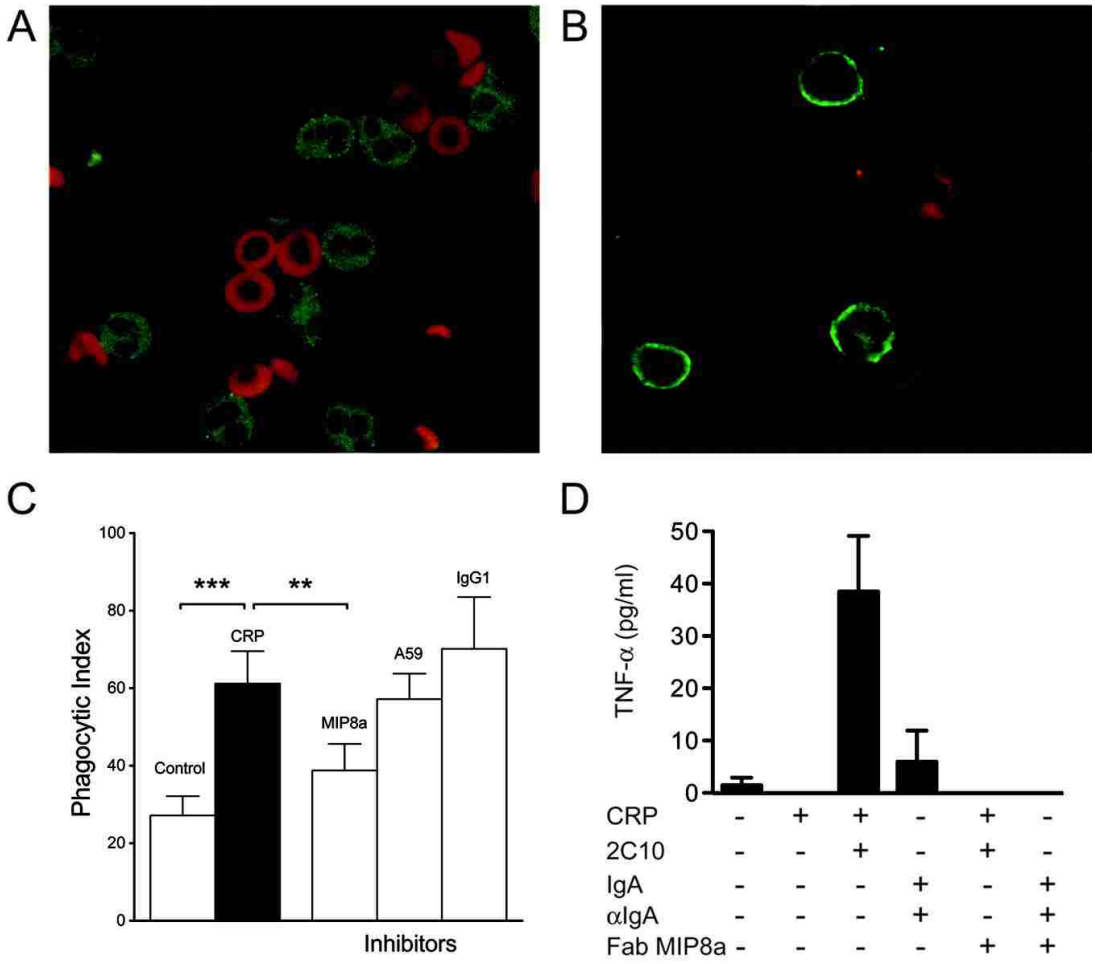

Figure 6. CRP induces neutrophil FcαRI surface expression, phagocytosis and TNF-α secretion. (A, B) Confocal images of neutrophils stained with anti-FcαRI (A59) and AF488 goat anti-mouse (green). Cells were incubated for 30 min at room temperature with PnC-SRBC (A) or CRP-opsonized PnC-SRBC (B) labeled with PKH26 (red). (C) Uptake of CRP-opsonized FITC *S. pneumoniae* by neutrophils expressed as phagocytic index (bacteria/100 neutrophils) with or without inhibitors. Mean ± SEM of four experiments. (*** $p<0.001$; ** $p<0.01$). (D) CRP or IgA (200 μg/ml) crosslinking induced TNF-α release in human neutrophils. The TNF-α secretion upon either CRP or IgA treatment was inhibited by Fab fragment of MIP8a.

neutrophils to secrete TNF-α and the TNF-α production upon CRP crosslinking was blocked by a Fab fragment of the anti-FcαRI antibody (MIP8a), similar to that by IgA (Figure 6D). These data suggest that CRP can effectively activate neutrophils through FcαRI.

DISCUSSION

The fact that pentraxins recognize both FcαRI and FcγRs is counter-intuitive since the two receptors have opposite D1, D2 domain structural arrangements. In addition, IgA and IgG bind their receptors in distinctly different modes and the two isotypes of antibodies do not cross- react. The ability of pentraxins to recognize both families of Fc receptors is most likely a result of their pentameric structure, which makes it possible to contact the same secondary structure elements from the two receptors with opposite domain arrangement using symmetrical but different pentraxin subunits. However, pentraxin recognition of FcαRI is not entirely due to its permissive ligand binding as they failed to bind FcεRI despite its closer sequence and structural homology than FcαRI to FcγRs. Likewise, pentraxins did not recognize other 'FcαRI-like' receptors, such as KIR and NKp46. In addition to binding, we showed that CRP crosslinking of FcαRI lead to the activation of ERK, degranulation and cytokine production in FcαRI-transfected cells, as well as the induction of cell surface FcαRI expression, phagocytosis of bacteria and TNF-α release in neutrophils.

FcαRI is primarily expressed on cells of the myeloid lineage, including monocytes, macrophages, neutrophils, and eosinophils (10). Similar to FcγRs, the expression of FcαRI is up regulated by LPS, TNF-α and other proinflammatory stimulators (33), but

down regulated by polymeric IgA (34). The regulation of Fc receptor expression by inflammatory mediators coincides with increased serum levels of CRP during the acute phase response suggesting their potential involvement in pentraxin-mediated innate immunity, especially early in infections prior to effective antibody responses. The expression of FcαRI on the surface of neutrophils was increased rapidly in response to chemoattractants and this increase was shown to be due to its release from intracellular storage granules (30). Similarly, we found CRP treatment induces redistribution of the receptor to the cell membrane, potentially contributing to the activation of the receptor on macrophages and neutrophils during infection. Since cells expressing FcαRI often express FcγRs, it remains to be seen if pentraxins can co-engage FcαRI and FcγRs and whether such co-engagement synergistically activates their functions. Alternatively, it is not clear whether the structural difference between FcαRI and FcγRs would result in a different functional outcome in pentraxin-mediated FcR activation, and thus contributing to cell-type dependent pathogen responses.

Macrophages and neutrophils are major innate inflammatory responders to infection. Their effector functions are initiated primarily through the activation of Toll like receptors (TLR) by microbial and endogenous TLR ligands and Fc receptors by antibody immune-complexes. The recent characterization of pentraxins as ligands for FcγRs and currently for FcαRI adds a novel dimension, an Fc receptor mediated innate immune response, as a potential contributor parallel to the TLR pathway to host defense against pathogens. There are both similar and contrasting features in TLR- and FcR-mediated innate immune responses. TLRs and FcRs are often co-expressed on myeloid immune cells. Both TLR and FcR expression are regulated by inflammation and infection. While

there are more TLRs than FcRs, the larger number of TLRs presumably reflects their direct recognition of diverse microbial and pathogenic ligands and the activation of TLRs is directly in response to the concentration increase of these ligands. In contrast, FcRs recognize a small number of conserved pentraxins and achieve ligand diversity through the pattern recognition of the pentraxin-ligand binding. The activation of FcRs would then depend on the concentration increase of pentraxins during infection. It is possible that both microbial activation of TLR pathways and CRP-opsonized microbial pathogen activation of FcR pathways occur concurrently resulting in synergistic and complementary innate immune responses and together they provide a powerful first line of host immune defense.

MATERIALS AND METHODS

The reagents are listed in the supplemental information.

BIAcore binding experiments. SPR studies were performed using a BIAcore 3000 with BIAevaluation 4.1 software in 10 mM HEPES (pH 7.4), 0.15 M NaCl, 1.0 mM CaCl2 at a flow rate of 50 μl/min. For affinity analysis, FcγRIIa and FcαRI were immobilized on carboxylated dextran CM5 sensor chips using primary amine coupling. Serial dilutions of SAP, CRP, and PTX3 from 7.2 to 0.04 μM were added. For C1q competition binding experiments, the analytes consisted of 2.72 μM CRP with 0.4 mg/ml of C1q. To measure the competition between human IgA and CRP on FcαRI binding, a CM5 chip was coupled with IgA at levels of 6000 - 9000 RU. The analytes consisted of 2.9 μM of CRP with or without 4.7 μM of refolded FcαRI. The dissociation constants were obtained by either steady state or kinetic curve fittings.

Cell surface binding by FACS analysis. A rat basophilic leukemia cell line (RBL) was stably transfected with a Gly 248 variant of human FcαRI(16). Human FcαRI exists in two common alleles (Gly and Ser) as a result of a single nucleotide polymorphism (SNP) at amino acid 248 in the cytoplasmic domain of the receptor gene. The G248 variant of FcαRI produced a more robust proinflammatory cytokines than the S248 variant in transfected cells as well as in human neutrophils. RBL cells and G248 cells were harvested with trypsin, and washed into PBS containing 0.1 % BSA and 0.05% sodium azide (PAB). Cells were incubated with CRP or Cy3IgA for 30 min at 4^{o}, washed twice with PAB. CRP binding was detected with an anti-CRP mAb (FITC-2C10). Data were acquired using a FACScan or Accuri flow cytometer and analyzed with FlowJo Software.

Homology modeling of the FCαRI-CRP complex. An initial complex between FcαRI (1OW0) and CRP (1GNH) was prepared by manual superposition of the corresponding components onto FcγRIIa and SAP in the SAP-FcγRIIa complex (3D5O). Docking was performed by tumbling FcαRI over CRP, but was largely constrained to the contact interface in the SAP-FcγRIIa complex using the shape-only correlation in Hex5 with, standard parameters. After clustering three lowest energy orientations (-371.4 to -392.4KJ/mol) were selected as the final model.

ERK phosphorylation assay. RBL or 9.4 RBL cells were harvested with trypsin then washed into Tyrode's buffer (130 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5.6 mM Glucose, 10 mM HEPES, 0.5% BSA, pH 7.4) and resuspended at 5.0 x 10^{6}

cells/ml. One hundred µl aliquots of cells were incubated with buffer or CRP (200 µg/ml) for 1 h at 37˚. Cells were incubated with 2C10 (40 µg/ml) or buffer. For flow cytometry analysis, cells were fixed with 2% formaldehyde, followed by 90% methanol, and washed with PBS + 4 % FBS. Cells were stained with P-p44/42 MAPK (T202/Y204) (1:100) rabbit Ab (Cell Signaling Technologies) for 15 min, washed twice and then stained with a secondary Alexa Fluor 488 F(ab')2 goat anti-rabbit IgG (1:500)(Invitrogen) for 15 min. For western blotting, RBL or RBL 9.4 cells were seeded at 1.5 x10^6 cells in 60 mm dishes overnight in complete medium. After treatment, cells were washed with ice cold HBSS and then lysed with HBSS containing 1% Triton X-100 with protease and phosphatase inhibitors (Thermo Scientific). Lysates were incubated 20 min on ice, centrifuged at 20,000 x g for 25 min, separated by 10% SDS PAGE and transferred to PVDF. Membranes were probed with P-p44/42 MAPK (T202/Y204) rabbit Ab then probed with anti-rabbit IgG HRP (Cell Signaling Technologies). Membranes were stripped with Restore (Thermo Scientific) and probed for total ERK using p44/42 MAPK.

Degranulation and IL-4 production assays. RBL cells or transfected-RBL cells (G248 or 9.4) were cultured overnight in 48 well plates then washed into Tyrode's buffer. Some cells were incubated with 200 µg/ml IgA or CRP for 1 h at 37˚C. Buffer was removed and buffer or 40 µg/ml of F(ab')2 anti-IgA or 2C10 was added and incubated at 37˚C. In the case of G248 cells, 50 µg/ml AggCRP was added at time 0 and activity was measured over time. Supernatants were collected and β-hexosaminidase activity measured with respect to total release determined by lysis with 1% Triton X-100. Activity was measured

by incubation with substrate, 1.4 mg/ml 4nitrophenyl-N-acetyl ß-D-glucosaminide in 75 mM sodium citrate, pH 4.5 for 1 h at 37˚. Reactions were stopped by addition of 0.2 M glycine, pH 10.7 and activity calculated from the A405 (% release = 100 x supernatant A405/A405 of detergent lysed cells). To assay for IL-4 production, G248 or untransfected RBL cells were seeded into 96 well plates at a density of $2 \bullet 10^4$ and pre-incubated with or without biotin labeled CRP (100 µg/ml) and/or piceatannol (25 µg/ml, Sigma) for 30 min, followed by streptavidin (20 µg/ml, Sigma) cross-linking of CRP. After 20 hours incubation at 37°C, the supernatant were assayed for rat IL-4 production using ELISA (R&D Systems, Inc.) according to manufacturer's instructions. Data shown were mean ± SEM of triplicate wells from one representative experiment.

Confocal microscopy. Human neutrophils were incubated in chamber slides (Thermo Scientific) for 2 h. PnC-SRBC were incubated with 150 µg/ml of CRP for 45 min at 37^o, washed and added to PMN at an 8:1 ratio for 10 min. Cells were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100 for 5 min. Slides were treated with Image-iT (Invitrogen). Cells were stained with anti-FcαRI antibody A59, washed, stained with a goat anti-mouse antibody labeled with Alexa Fluor 488 (Invitrogen), washed and mounted in ProLong Gold Antifade (Invitrogen). Images were acquired using a Zeiss LSM 510 inverted laser scanning microscope.

Neutrophil phagocytosis and cytokine secretion assays. Neutrophils were purified by Ficoll-Hypaque centrifugation and resuspended at $2x10^6$ cells/ml in RPMI-1640+10%FCS. *S. pneumoniae* serotype 27 (Pn27) (ATCC) was grown to log phase,

washed in PBS, heat-killed, and FITC-conjugated. Neutrophils were combined with FITC-Pn27 and 100 μg/ml CRP, centrifuged briefly and incubated for 30 min at 37˚. Phagocytosis was measured by mean FITC-fluorescence on gated neutrophils after washing and adding trypan blue to quench uningested bacteria as Pn27 ingested/100 neutrophils. For cytokine secretion, neutrophils were treated for an hour with CRP or IgA (200 μg/ml) with or without the Fab fragment of MIP8a (15 μg/ml). Medium was removed, then 2C10 or anti-IgA (40 μg/ml) was added, and cells were incubated overnight at 37^{o}. Supernatants were analyzed for TNF-α using an R&D systems ELISA kit.

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CHAPTER 5

DISCUSSION AND CONCLUSIONS

SUMMARY AND FUTURE DIRECTIONS

This dissertation focuses on the interactions between FcRs and CRP and the functional consequences of these interactions. Although it has been demonstrated that CRP inhibits the induction of autoimmune disease in mice, the temporal mechanism involving cell types and receptors mediating the protection were previously unknown. Therefore, an adoptive cell transfer model was used to determine the cellular targets of CRP-mediated protection in autoimmune disease. In this model, splenocytes from a donor mouse were treated with CRP and transferred to a recipient mouse. The recipient mouse was then injected with an anti-platelet antibody to induce platelet clearance. It was found that CRP-mediated protection against ITP is dependent on macrophages expressing FcγRI in the donor cell population. Further, the expression of FcγRIIb in the recipient is essential for CRP-mediated suppression of ITP. Understanding which receptors are responsible for CRP-mediated protection in autoimmune disease is paramount in elucidating the mechanism of CRP and potentially targeting the downstream pathways in treatment of disease. CRP promiscuously binds to all members of the FcγR family, but it was unknown if CRP binds and modulates the immune system through other related receptors. Surface plasmon resonance was used to screen for binding between short pentraxins and FcRs. FcαRI was found to be a novel receptor for CRP and SAP. To further characterize the interaction between CRP and FcαRI, blocking antibodies to the D1 and D2 domains of FcαRI and the classical FcαRI ligand, IgA, were used to compete for binding with CRP. CRP binding to FcαRI is inhibited by the D1 blocking antibody and modestly by the D2 blocking antibody. CRP and IgA do not compete for binding to

FcαRI with the potential to interact simultaneously with the receptor. Crosslinking FcαRI with CRP initiates signaling events, phagocytosis, cytokine generation and cellular redistribution of FcαRI. These observations expand the current understanding regarding the role of short pentraxins in immune regulation.

Lupus is strongly correlated with low serum levels of CRP indicating that CRP may play a protective role in this disease (Rhodes, Furnrohr et al. 2011). Autoimmune diseases, such as lupus, are exacerbated when immune complexes against self-antigens interact with FcRs on immune cells. This interaction initiates inflammatory signaling cascades and promotes the recruitment of inflammatory cells. Neutrophils and macrophages are two cell types responsible for immune complex-mediated inflammation. These cells promote inflammation and tissue destruction by phagocytosing opsonized particles and releasing reactive oxygen species and other inflammatory products. The results from the ITP model described in this dissertation demonstrate CRP is able to inhibit immune complex-mediated macrophage activation through interaction with FcγRI on macrophages; however, a full understanding of CRP-mediated immune modulation requires clarification of several aspects of these observations.

Although it is now clear that CRP is interacting with FcγRI on macrophages, the exact mechanism of CRP-mediated inhibition of immune complex disease is unknown. Macrophages are dynamic cells, able to either activate or inhibit the immune system depending on signals received from the extracellular environment. FcR activation has been shown to dampen inflammatory responses (Anderson, Gerber et al. 2002; Lucas, Zhang et al. 2005; Monteiro 2010). Therefore, CRP may induce inhibitory macrophages through activation of FcγRI increasing the production of soluble suppressive mediators.

Changes in gene regulation must occur to generate inhibitory macrophages so they are able to produce immunomodulatory cytokines, such as TGF-β and IL-10. One method to analyze which genes are differentially regulated by the CRP-FcγRI interaction is to use a gene array. If CRP stimulation generates suppressive macrophages, anti-inflammatory cytokines TGF-β and IL-10 gene expression will increase while pro-inflammatory cytokine gene expression will decrease. These data will help determine if CRP treated macrophages have characteristics of suppressive macrophages responsible for the inhibition of immune complex-mediated disease.

High-doses of IVIg are administered to patients that suffer from immune complex-mediated disease because of its anti-inflammatory properties (Nimmerjahn and Ravetch 2008). It has been demonstrated that IVIg will stimulate the production of IL-33, which induces IL-4 release from basophils and mast cells. IL-4 upregulates the expression of FcγRIIb on macrophages, increasing the threshold for cell activation and protecting mice from immune complex-mediated diseases (Anthony, Kobayashi et al. 2011). In the adoptive cell transfer ITP model, the recipient mouse expression of FcγRIIb was required for CRP-mediated protection, consistent with the mechanism of IVIg-mediated protection. Therefore, the release of IL-33 and IL-4 is a potential mechanism of CRP-dependent suppression of immune complex-mediated disease. The production and release of these cytokines will be analyzed by intracellular fluorescence staining by flow cytometry and ELISA. The role of these cytokines in CRP-mediated suppression of immune complex-mediated disease will be confirmed using the adoptive transfer model with IL-33$^{-/-}$ or IL-4$^{-/-}$ mice. One would expect that IL-33$^{-/-}$ donor splenocytes and IL-4$^{-/-}$ recipient mice would be unable to transfer protection from ITP because, without the

release of these cytokines, the expression of FcγRIIb would not be increased in the recipient mouse.

Monomeric activation of FcγRI by CRP to induce ITAM*i* in donor macrophages is another possible mechanism of ITP suppression. It is possible to induce ITAM*i* by using a Fab directed against the receptor, which will bind but not crosslink the receptor. A goat anti-FcγRI Fab will be used to investigate the signaling pathways downstream of monomeric activation of FcγRI. Downstream signaling events will be compared to a receptor bound by goat anti-FcγRI Fab and a chicken anti-goat antibody to induce receptor crosslinking. The monomeric activation of FcRs initiates SYK activation, leading to SHP-1 recruitment. The SHP-1 is a phosphatase that blunts activating signals from ITAMs and is responsible for ITAM*i* (Pfirsch-Maisonnas, Aloulou et al.). Therefore, the activation of SYK and SHP-1 will be analyzed in monomeric targeting of FcγRI. This experiment would determine if FcγRI has the capability to induce inhibitory signaling. Second, it would clarify the necessity of receptor crosslinking in the CRP-induced suppression of immune complex disease. Together these results would contribute to understanding the role of FcγRI in mediating suppression and how CRP utilizes FcγRI for suppression.

The extent of suppression observed in the recipient mouse upon transfer of CRP treated cells is unclear. The data from the adoptive transfer model suggest that CRP treated splenocytes decrease phagocytosis of IgG opsonized platelets, but the degree of inhibition is unknown. If overall phagocytosis is decreased it will give rise to a bacterial or viral infection. Therefore, it is important to identify precisely which immune functions are suppressed in animals given CRP treated splenocytes. A bacterial challenge after

transfer of CRP-treated splenocytes would indicate if CRP is completely suppressing the immune functions or just suppressing FcγR mediated phagocytosis.

This dissertation also focused on the discovery of a novel interaction between FcαRI and CRP. The blocking and modeling studies suggest CRP interacts with both the D1 and D2 domains of FcαRI, in contrast to IgA binding to the most distal portion of the D1 domain. Therefore, it is believed that IgA and CRP do not compete for binding to FcαRI. Simultaneous binding may have important consequences on downstream signaling. Elucidating how CRP binds to FcαRI is critical in understanding its potential physiological role. Since the crystal structure of the CRP-FcαRI complex has not been determined, mutational analysis will highlight key residues involved in the interaction between the two proteins. A docking model has been proposed for the CRP-FcαRI interaction that identifies six major sites on FcαRI that interact with three subunits of CRP (APPENDIX Table S1). Mutational studies are in progress and initial data suggest that the docking model is correct and that CRP interacts with both D1 and D2 domains. Individual mutations at each major site of FcαRI predicted in the model decreases CRP binding as measured by surface plasmon resonance; however, loss of binding is not observed for IgA except for the mutations in the IgA binding region on the D1 domain. Complete abrogation of CRP binding to FcαRI has not been observed, most likely due to the repeating structure of CRP and the multiple contact sites involved on FcαRI. These studies are in progress to elucidate how CRP interacts with FcαRI.

As previously mentioned, CRP and IgA do not compete for binding to FcαRI and potentially can bind simultaneously to the same receptor. To verify this possibility, fluorescence resonance energy transfer (FRET) will be used. CRP and IgA would be

labeled with a donor and an acceptor fluorophore, respectively, and incubated with cells expressing FcαRI. If energy transfer occurs, CRP and IgA are likely interacting with the receptor simultaneously. We propose that simultaneous binding of CRP and IgA to FcαRI provides a form of receptor regulation that has the potential to increase FcαRI function. In order to determine the functional outcomes of IgA and CRP binding to FcαRI, cytokine production, phagocytosis, reactive oxygen species generation and signaling cascades would be measured in the presence of both ligands, as well as the ligands individually. Finally, inside out regulation of FcαRI may be initiated by one ligand and, in doing so, would change the binding capability or downstream signaling events of the other ligand. Inside out regulation of ligand binding is common across FcRs and has been observed for FcαRI. If this type of regulation is occurring, it will help elucidate why CRP and IgA would act on the same receptor and how dual binding affects receptor function. Together, these experiments will begin to clarify the interaction of CRP and IgA with FcαRI and would begin to describe the physiological consequences of these interactions.

One of the main functions of secretory IgA is protection at mucosal surfaces. CRP could promote similar protection because it is reported to be present in mucosal secretions of the lung during infection and is proposed to opsonize bacteria at mucosal surfaces. Alveolar macrophages express FcγRs and FcαRI and, therefore, may promote the clearance of CRP opsonized bacteria before antigen-specific antibodies are able to reach these sites. To determine the role of CRP at these sites, a lung mucosal infection model will be induced in either FcαRI transgenic mice or control WT or FcR γ-chain$^{-/-}$ mice to monitor the clearance of the bacteria in the absence or presence of CRP. Neutrophil lung influx as well as cytokines will be analyzed from lung lavages to

determine if the presence of CRP alters the inflammatory response. This would aid in determining the role of CRP in mucosal immunity, broaden the functionality of pentraxins and increase the understanding of the immune surveillance capabilities for CRP.

Conclusions

The data and interpretations presented in this manuscript describe a role for CRP-mediated inhibition of an immune complex-mediated disease and characterize FcαRI as a new receptor for CRP. These finding shed light on potential functions and interactions that may take place in the human body during inflammation. CRP may serve two functions when the serum concentration levels increase 1000 fold during an acute phase response. One function is to bind and opsonize potential inflammatory material (bacteria, dead cells, and nuclear material) and interact with FcγRs and FcαRI to induce phagocytosis of opsonized material. This action would protect the host from the exacerbation of an insult by inducing the clearance of the inflammatory material. Another potential function during the acute phase response or chronic inflammation is initiate ITAM*i* by saturating FcRs to promote low level signaling to decrease overall activation of innate immune cells. ITAM*i* reduces the propensity to over-respond to immune complexes or other receptor activation.

The ability of pentraxins to act on different immunoglobulin receptors broadens the diversity of targets cleared by FcRs. This ligand diversity also allows innate immune cells to respond quickly to an insult before antibody production occurs. Therefore, pentraxins are critical during homeostasis (SAP) and during acute or chronic

inflammation (CRP) to mediate host defense and immune regulation through the interaction with FcRs.

APPENDIX

Supplemental Material

Reagents.

C-reactive protein (CRP) was purified by affinity and ion exchange chromatography from human pleural fluid (1). CRP ran as a single band on overloaded SDS/PAGE gels with less than one unit endotoxin per milligram protein. Recombinant pentraxin 3(PTX3) was obtained from Barbara Bottazzi (Institututo di Ricovero e Cura a Carattere Scientifico-Istituto Clinico Humanitas, Rozzano, Italy). Serum amyloid P (SAP) was provided by Walter Kisiel (University of New Mexico, Albuquerque, NM) and further purified by FPLC on Mono Q (2). For some degranulation experiments, CRP aggregates (aggCRP) were prepared. CRP was biotinylated to a biotin:CRP ratio of 3:1 (Chromalink; Solulink) and aggregated with 2-M equivalents of streptavidin (Pierce). Recombinant human Fc IgE receptor I (FcεRI) was provided by Theodore Jardetzky (Stanford University, Stanford, CA). Mouse anti-Fc IgA receptor I (FcαRI) mAb, MIP8a, and A59 were purchased from Serotec and BD. Human IgA, Cy3-conjugated IgA, and $F(ab')_2$ goat anti-IgA were purchased from Jackson ImmunoResearch. Fab fragments of MIP8a were prepared using a kit from Thermo Scientific. Anti-human CRP (2C10), a gift from Lawrence Potempa (Roosevelt University, Chicago, IL), was purified and FITC-conjugated. Human IgG was purchased from BD. Mouse monoclonal anti-2,4-dinitrophenylated (DNP) IgE, DNP conjugated BSA, and RBL-2H3 cells were from Bridget Wilson (University of New Mexico, Albuquerque, NM). RBL-2H3 cells transfected with FcαRI (G248 cells) were from Jeffrey Edberg (University of Alabama, Birmingham AL). RBL cells transfected with FcαRI and γ-chain fusion construct (RBL 9.4 cells) were obtained from Renato Monteiro (Institut National de la Santé et de la Recherche Médicale U699, Paris, France). RBL cells were grown in complete DMEM with G418 added for transfectants. Recombinant natural killer cell p46 (NKp46), killer cell Ig-like receptor, two domains, long cytoplasmic tail, 2 (KIR2DL2), FcαRI, and Fc IgG receptor IIa (FcγRIIa) were from R&D Systems. Recombinant ectodomains of FcαRI (1–208) were expressed in Escherichia coli (BL21DE3) using a pET30a vector with a His_6-tag at the C terminus and refolded (3). Human C1q was purified from EDTA plasma using BioRex 70, ammonium sulfate precipitation, and SP-Sepharose (4). Pneumococcal C (PnC) polysaccharide (Statens Serum Institut) was conjugated to sheep red blood cells

(SRBC) using chromium chloride as previously described (5). PnC-SRBC were labeled with PKH26 (Sigma) according to the manufacturer's instructions.

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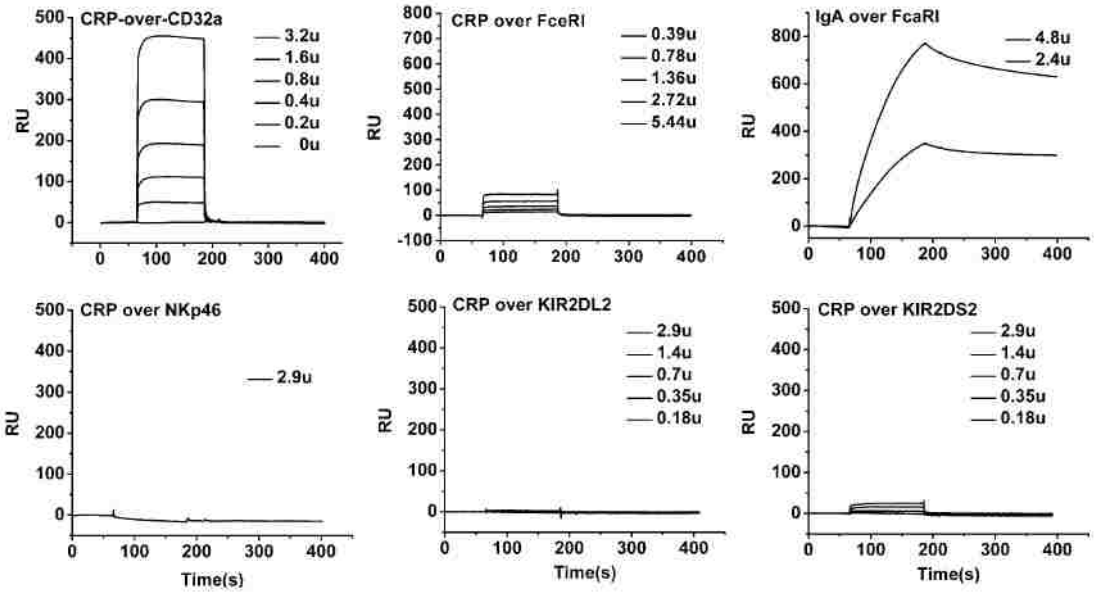

Fig S1. Binding of CRP to individually immobilized recombinant soluble receptors including FcγRIIa, FcεRI, NKp46, KIR2DL2, and killer cell Ig-like receptor, two domains, short cytoplasmic tail, 2 (KIR2DS2) on CM5 sensor chips. The binding of IgA to immobilized FcαRI was used as a control. The concentrations of the analytes (CRP or IgA) are indicated in micromolar (u). RU, resonance units.

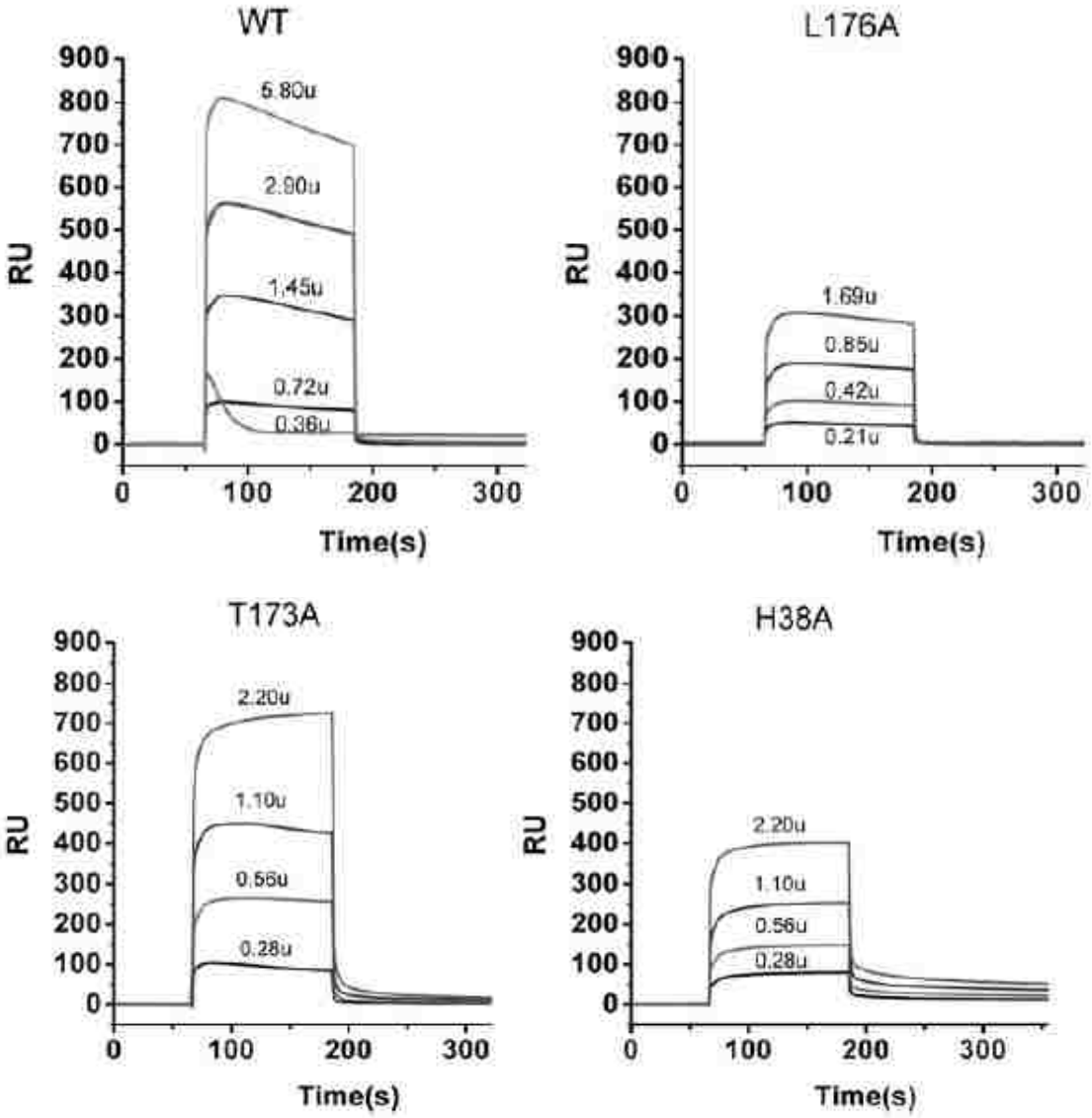

Figure S2. Biacore sensorgrams of wild-type (WT) and mutant CRP binding of FcαRI.

CD89–CRP subunit A interface

CRP A subunit		CD89		Interaction type	Distance (Å)
Asp^{169}	OD2	Arg^{49}	NH1	Salt Bridge	3.1
Asn^{172}	OD1	Glu^{50}	OE1	H-bond	2.7
Thr^{173}	N	Glu^{50}	OE1	H-bond	3.3
Thr^{173}	O	Gln^{39}	NE2	H-bond	4.0
Thr^{173}	CG2	Gln^{39}	OE1	VDW	3.5
Thr^{173}	CG2	Glu^{50}	OE2	VDW	2.8
Leu^{176}	O	Gln^{39}	NE2	H-bond	3.9
Leu^{176}	CD2	Glu^{50}	OE1	VDW	2.8
Gly^{178}	N	Gln^{39}	NE2	H-bond	3.0
Pro179	O	Arg^{83}	NH1	H-bond	3.0
Pro179	CB	Tyr^{36}	CZ	VDW	2.8
Pro179	CG	Leu^{37}	CD2	VDW	2.6

CD89–CRP subunit D interface

CRP D subunit		CD89		Interaction type	Distance (Å)
Arg^{6}	CZ	Ile^{131}	CG1	VDW	3.0
Thr^{173}	OG1	Lys^{105}	NZ	H-bond	3.7
Leu^{176}	O	Gly^{104}	N	H-bond	3.8
Leu^{176}	O	Lys^{105}	N	H-bond	3.4
Gly^{177}	O	His^{130}	ND1	H-bond	2.4
Pro^{179}	CB	Ala^{129}	CB	VDW	3.0

CD89–CRP subunit E interface

CRP E subunit		CD89		Interaction type	Distance (Å)
Asp^{169}	O	Arg^{179}	NH2	H-bond	3.1
Glu^{170}	N	Arg^{179}	NH1	H-bond	3.9
Thr^{173}	OG1	Arg^{179}	NH2	H-bond	3.4
Thr^{173}	CG2	Arg^{179}	NH2	VDW	2.4

H-bond, hydrogen bond; VDW, Van der Waals interaction.

Table S1. Putative interface contacts between CD89 and CRP

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