Short Analytical Review

C-reactive protein: Ligands, receptors and role in inflammation ☆

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Abstract

C-reactive protein (CRP) is the prototypical acute phase serum protein, rising rapidly in response to inflammation. CRP binds to phosphocholine (PC) and related molecules on microorganisms and plays an important role in host defense. However, a more important role may be the binding of CRP to PC in damaged membranes. CRP increases clearance of apoptotic cells, binds to nuclear antigens and by masking autoantigens from the immune system or enhancing their clearance, CRP may prevent autoimmunity. CRP binds to both the stimulatory receptors, FcγRI and FcγRIIa, increasing phagocytosis and the release of inflammatory cytokines; and to the inhibitory receptor, FcγRIIb, blocking activating signals. We have shown that, in two animal models of systemic lupus erythematosus (SLE), the (NZB × NZW)F1 mouse and the MRL/lpr mouse, a single injection of CRP before onset of proteinuria delayed disease development and late treatment reversed proteinuria. Thus, in these models, CRP plays an anti-inflammatory role.
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Introduction

C-reactive protein (CRP) is the prototypical acute phase protein in humans. Tillet and Francis discovered CRP over 70 years ago in the blood of patients with *Streptococcus pneumoniae* infection [1], as a substance that precipitated the "C" polysaccharide of the cell wall of the pneumococcus and they called it C-reactive substance, which was later changed to C-reactive protein [2]. The acute phase nature of CRP was observed in these early studies. It was undetectable in normal blood and appeared at high concentrations very early during the course of the infection. If the patient recovered, the substance again became undetectable. This substance was also found in the blood of patients acutely ill with other febrile diseases, such as hemolytic streptococcus infection, rheumatic fever and staphylococcus infection. Clinically, CRP has been used to detect acute infections and to assess the response to treatment. It has also been used to evaluate the inflammatory response in chronic diseases, such as vasculitis and rheumatoid arthritis. In addition, CRP levels slightly above normal have been put forth as an indicator of mild inflammation associated with atherosclerotic vascular disease [3].

Synthesis

CRP is primarily made in the liver [4] in response to IL-6, and this synthesis is enhanced synergistically by IL-1β [5]. IL-1β enhances IL-6 induction through NF-κB p50 and p65 [6]. IL-6 induction is mediated by transcription factors STAT3 acting through a response element at position −108 [7], and C/EBPβ acting through response elements at positions −52 [8] and −219 [9]. Transcription is also controlled through E-box elements [10] in the promoter at positions −412 to −407 (E-box1) and −394 to −389 (E-box2). Upstream stimulating factor 1 (USF1) was shown to bind to E-box1. Single nucleotide polymorphisms (SNPs) (Fig. 1) were found within each of these E-boxes that influence baseline CRP levels in the individuals studied, i.e., individuals with the −409G/−390T
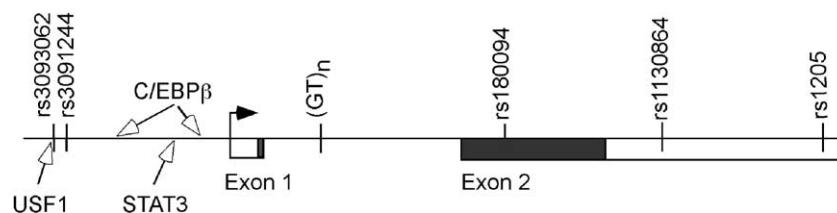
Fig. 1. Structure of the CRP gene. Exons are shown as boxes, introns and intergenic regions as lines. Binding sites in the promoter for transcription factors STAT3, C/EBPβ and USF1 are shown by open arrows. Exon 1 contains the leader sequence and the first two amino acids of the mature protein. The coding regions of the mature protein are denoted by dark boxes. SNPs and the dinucleotide repeat $(GT)_n$ are labeled above the gene. The transcription start site is shown by a black arrow. SNPs rs3093062 and rs3091244 are E-box1 and E-box2, respectively. SNP rs1800947 is within the coding region but does not change the amino acid sequence. SNPs rs1130864 and 1205 are in the 3′ untranslated region.

haplotype had the highest baseline CRP levels and individuals with the −409A/−390T haplotype had the lowest. These are the first SNPs that have been shown by in vitro studies to influence baseline CRP levels as well as influence functionality of the promoter.

Baseline levels of CRP are being used by some as a predictor of inflammation leading to atherosclerotic vascular disease [3]. Levels of CRP may also influence whether it induces a pro-inflammatory or anti-inflammatory response. Therefore, it is important to understand how genetic polymorphisms in the CRP gene influence these levels in a given population. Several SNPs (Fig. 1), in addition to the SNPs discussed above, have been shown to be associated with differences in baseline CRP levels in human populations. As discussed below, CRP may play an important role in the clearance of nuclear antigens from apoptotic cells. As a result, populations have been studied for associations between CRP polymorphisms and the risk of developing systemic lupus erythematosus (SLE). Szalai et al. identified a polymorphic dinucleotide repeat in exon 2, with 13 alleles ranging from nine to 25 repeats (GT_9 to GT_{25}) [11]. They found that individuals who were homozygous for either 18 or 20 repeats had significantly higher baseline levels of CRP than those of the most common alleles with 16 or 21 repeats. None of the alleles of this dinucleotide have been found to be associated with SLE [11,12]. However, in a separate study, Szalai et al. found that non-Caucasian SLE patients with the GT_{20} allele had increased risk of vascular arterial events [13]. The authors speculate that factors other than CRP levels could be causing the increased risk because this association is more apparent in African–American and Hispanic patients than for Caucasians [13]. What is striking about CRP is that no SNPs that cause a change in its amino acid sequence have been identified, indicating there is strong evolutionary pressure against change.

A synonymous leucine/leucine SNP (rs1800947) in exon 2 has been associated with changes in CRP levels in 4 studies [12,14–16], but not in another [17]. Men homozygous for a combination of the G-allele of this SNP and the C-allele of IL-1β + 3954 had almost a 3-fold higher baseline CRP value [16]. Two SNPs, rs1130864 [17] and rs1205 [12], in the 3′ untranslated region are also associated with changes in CRP levels. The rare T allele at SNP rs1205 is not only associated with low levels of CRP, but also with SLE and anti-nuclear autoantibody production in families with SLE [12]. The authors propose that reduced levels of CRP allow the

development of anti-nuclear antibodies, which leads to the development of SLE.

Structure

CRP is a member of the pentraxin protein family and consists of a cyclical arrangement of 5 identical non-covalently bound subunits (protomers). The crystal structure has been determined with binding sites for 2 calcium ions and one phosphocholine (PC) molecule per protomer on the B face [18,19], and binding sites for C1q and Fc receptors hypothesized to be on the A face. Two regions in IgG have been identified as important for binding to FcγRI, [234]LLGGPS[239] for human IgG_1 (FLGGPS in IgG_4) and a second region [327]ALPAPI[333]. Sequence homologies between CRP and these regions are shown in Table 1. Amino acids 175–185 form part of the edge of a long deep cleft in each protomer on the A face, shown as purple on one subunit of CRP in Fig. 2. The other subunits in Fig. 2 show the residues around this cleft that have been implicated by site-directed mutagenesis experiments as important for binding to C1q [20] and Fc receptors [21,22] and that these binding sites overlap. Modeling of CRP binding to C1q predicted that each globular domain of C1q would bind one CRP pentamer in the central pore on the A face [23]. Binding to C1q causes the activation of the classical complement cascade and several of the effects of CRP require complement.

Ligands

The most well characterized ligand of CRP is PC (Fig. 3) [18]. This interaction requires calcium and is responsible for

Table 1
Sequence homology between CRP and IgG binding region[a]

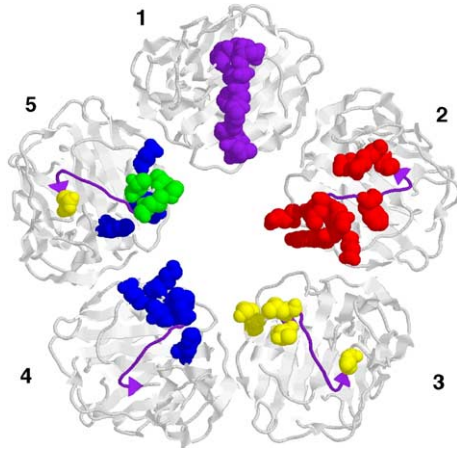| CRP sequence	175	176	177	178	179	180	181	182	183	184	185
HUMAN CRP	Y	**L**	**G**	**G**	**P**	F	S	P	N	V	L
Human IgG1	L	**L**	**G**	**G**	**P**	L	P	A	P	I	E
Human IgG3	L	**L**	**G**	**G**	**P**	L	P	A	P	I	E
Human IgG4	F	**L**	**G**	**G**	**P**	L	P	S	S	I	E
Human IgG2	V	A	-	**G**	**P**	L	P		P	I	E
IgG sequence (EUsystem)	234	235	236	237	238	328	329	330	331	332	333

[a] Amino acid identities are represented by bold letters.

Fig. 2. Structure of CRP with proposed FcγR and C1q binding sites. The CRP pentamer is shown with residues 175–185 as space-filled in purple on subunit 1, as purple ribbon on the other subunits. The residues investigated by us [21] are indicated in red on subunit 2. On subunit 3, the residues directly implicated by mutagenesis experiments to be involved in binding to FcγR are shown in yellow. The proposed C1q binding site is shown on subunit 4 in blue, based on data presented by Agrawal et al. [20] and our laboratory [21]. The subunit 5 shows the residues from third and fourth subunits with those that are important for both C1q and FcγR binding shown in green. The structure was rendered in RasMol.

the binding to several microorganisms including the C-polysaccharide of pneumococcus [24], the repeating phosphorylated disaccharide on *Leishmania donovani* [25] and the lipopolysaccharide of *Hemophilus influenzae* [26]. This activity has very ancient roots since a homologous protein can be found in the horseshoe crab, *Limulus polyphemus*, which has been in existence for 70 million years. The limulus homologue of CRP also functions in host defense and can protect against infection with *Pseudomonas aeruginosa* [27].

Although CRP recognition of PC or related molecules on microorganisms plays an important role in our defense against them, a more important role may be the binding of CRP to PC in damaged membranes. PC is not normally exposed on the surface of cells but is exposed by damage due to complement [28], or certain phospholipases [29]. CRP binds to both apoptotic and necrotic cells and deficiencies in the clearance of apoptotic cells have been associated with autoimmune disease [30,31]. CRP binding to the apoptotic cell surface in a calcium-dependent manner promotes the early classical complement pathway, by increasing binding of C1q and C3b/bi, but reducing the amount of the membrane attack complex. CRP binding improves opsonization and phagocytosis of apoptotic cells by macrophages, and promotes an anti-inflammatory response [32,33].

CRP also binds to nuclear antigens that are prominent autoantigens. In 1977, Gitlin et al. [34] reported CRP localized in nuclei of cells from the synovium of rheumatoid arthritis patients. Robey et al. [35] extended this work and showed rabbit CRP bound in a spotty pattern in the nuclei and this binding could be inhibited by excess CRP or PC. They hypothesized that the binding was to chromatin and showed that CRP did bind chromatin, but not histones or DNA. Later

studies showed that CRP bound chromatin through interactions with the histones and does not bind naked DNA [35,36]. CRP binds the histones H1 and H2A most strongly, with considerably less binding to H2B, H3 and H4 [36]. The binding of CRP to chromatin has been reported to cause its solubilization in the presence of complement [37,38]. The speckled binding pattern seen by Gitlin et al. [34] and Robey et al. [35] was more characteristic of binding to extractable nuclear antigens than to chromatin. Therefore, we investigated CRP binding to nuclear antigens and showed by two color fluorescence microscopy that the binding of antibodies to nuclear RNP particles (snRNP) colocalized with CRP staining [39]. Blotting experiments showed that CRP bound the D protein of Sm and the 70 kDa protein of snRNPs [40]. These antigens are major targets of autoantibodies in patients with SLE. The ability of CRP to increase the clearance of apoptotic cells as well as its ability to bind to nuclear antigens has led to the theory that by masking autoantigens from the immune system or enhancing their clearance CRP could prevent autoimmunity.

Complement activation

A very important property of CRP is the ability to bind C1q to activate the classical complement cascade. Activation of complement is a factor in the killing of microorganisms and mediates protection by CRP from pathogenic bacteria such as *S. pneumoniae* [41] and *H. influenzae* [26]. Complement activation by CRP differs from activation by antibody in that there is selective activation of early components without the formation of the membrane attack complex (MAC). CRP
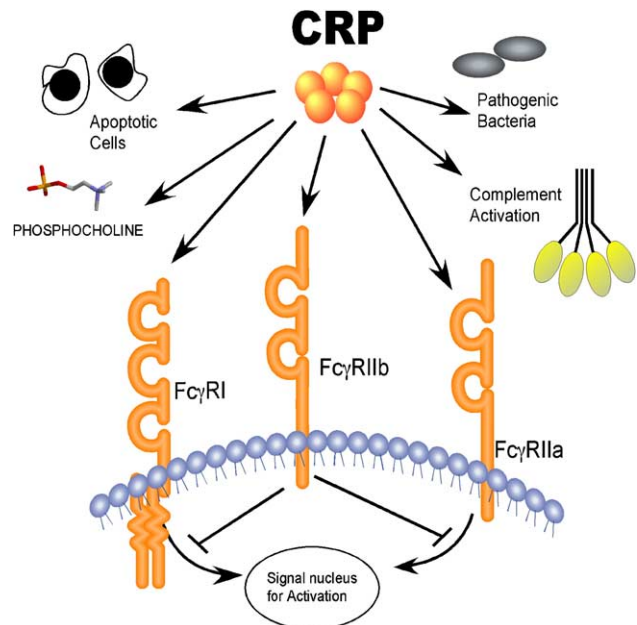

Fig. 3. CRP ligands. The prototypical ligand of CRP is PC, which is present on some bacterial cell walls and damaged cell membranes. CRP binding activates complement and opsonizes them for phagocytosis. It also binds to FcγRI and FcγRII on the surface of leukocytes. FcγRI and FcγRIIa are stimulatory receptors and crosslinking activates them, increasing phagocytosis and release of inflammatory cytokines. FcγRIIb is an inhibitory receptor and crosslinking of it blocks activating signals.

recruits factor H to the cell surface, inhibiting the alternative complement pathway amplification loop and C5 convertases [42]. The C5 convertase cleaves C5 to start the late steps of complement activation, leading to recruitment of neutrophils and formation of MAC. It is hypothesized that CRP deposited at sites of tissue damage could recruit factor H, and thereby reduce inflammation. CRP has been found complexed with complement activation products in the sera of patients after renal allograft, but not normal donors [43]. CRP can also be found colocalized with C4d in infarcted human myocardium, indicating complement activation [44]. However, although CRP colocalizes with modified lipoproteins in early athero-sclerotic lesions and activates complement, the MAC does not form [45], indicating that the contribution of CRP to the tissue damage may be limited.

Binding to receptors

The literature concerning the receptor for CRP on leuko-cytes was confusing for many years (reviewed in [46]). We were able to show specific binding of CRP using COS-7 cells transfected with plasmids expressing FcγRI [21]. This was confirmed by others using surface plasmon resonance [47] and they showed that CRP actually bound to FcγRI with a 3-fold higher affinity than IgG. This binding was inhibited by IgG1 but not by PC. Binding to FcγRI increased phagocytosis of erythrocytes opsonized with CRP and resulted in activation of phospholipase D signaling [47].

CRP binding to cell lines, such as K562, which do not express FcγRI, indicated the existence of another receptor [48]. We showed that CRP bound FcγRIIa expressed by transfection on COS-7 cells [49] and that it was allele specific, with stronger binding to the allele with an arginine at amino acid 131 (R-131), than to the allele with histidine 131 (H131) [50]. Binding of CRP to PMN from a homozygous R-131 donor caused a transient rise in intracellular Ca^{2+} but no change was seen in PMN from a homozygous H-131 donor. The difference in allelic binding has been confirmed by others [51]. They showed that COS-7 cells transfected with FcγRIIa R-131 were more efficient at CRP-dependent phagocytosis than those transfected with FcγRIIa H-131. Binding to FcγRIIa R-131 was also shown by direct labeling of CRP with Cy3 and detection by confocal microscopy [52]. Since FcγRIIa, b and c have similar extracellular domains, CRP probably binds to all three, allowing it to bind to a wide variety of leukocytes.

The binding of CRP to FcγR is expected to have similar effects as the binding of IgG. FcγR containing immunor-eceptor tyrosine-based activation motifs (ITAM), such as FcγRI, FcγRIIA/C and FcγRIIIA, are activated by clustering on the cell surface caused by ligand binding. This is followed by phosphorylation of the two tyrosines in the ITAM motif by Src-related tyrosine kinases, such as Lyn, Fgr and Hck. This leads to recruitment of Src homology 2-containing molecules such as Syk tyrosine kinase which leads to a cascade of events: (1) phosphorylation of phosphatidylinositol 3-kinase (PI-3K) with the generation of PI(3,4,5)P₃, which promotes downstream signaling events, including phosphorylation of phospholipase Cγ2 (PL Cγ2) which produces (a) DAG which activates phosphokinase C which activates p38 transcription factor, (b) calcium mobilization through IP₃; (2) activation of Raf which binds Ras, phosphorylates MEK which in turn phosphorylates ERK. ERK stimulates transcription from promoters controlled by c-Myc, Ets, CREB, NF-kappaB and AP-1. ERK can also be activated by Rac/Cdc42. The Rho family GTPases Cdc42 and Rac are also activated after FcγR crosslinking and are crucial to the regulation of phagocytosis [53].

Similar to IgG, binding of CRP to HL-60 cells caused tyrosine phosphorylation of FcγRIIa in a dose-dependent manner and recruitment of Syk [54]. Also, like IgG binding, some of the downstream events involve MEK and ERK. Treatment of human umbilical vein endothelial cells (HUVECs) with CRP caused an up-regulation of several mRNAs including IL-8. The increase in IL-8 synthesis was confirmed by RT-PCR and was blocked by an inhibitor of MEK, which is upstream of ERK. CRP treatment was also shown to stimulate phosphorylation of ERK-1/2 [55]. IL-8 stimulated promyelocytic HL-60 cells also show increased -2 phosphorylation upon CRP treatment [56]. ERK can stimulate several transcription factors; however, NF-κB appears to be involved in the IL-8 up-regulation by CRP [57]. CRP also stimulated U937 cells to secrete matrix metalloproteinase (MMP-1) through the ERK pathway and this stimulation could be inhibited by anti-CD32, but not anti-CD64, indicating that the signaling was through FcγRII [58]. Also, similar to IgG, binding of CRP to PNM increased their PI-3K activity [56] and we showed that phagocytosis of CRP-opsonized zymosan was inhibited by the PI-3K inhibitor, wortmannin and the syk kinase inhibitor, piceatannol [59]. CRP was also shown to induce phospholipase D activity in U937 cells [47]. In HL-60 cells, CRP treatment causes tyrosine phosphorylation of PLCγ2 with the mobilization of calcium stores [54]. We also showed that CRP induced a rise in intracellular calcium in PMN and this effect was dependent on FcγRIIa [50]. Therefore, CRP works similarly to IgG in causing events downstream of FcγR binding.

Protection from bacterial infection

CRP was first shown to be protective from bacterial infection in an in vivo mouse model of *S. pneumoniae* infection by our laboratory [41]. This has been confirmed by others also using passive inoculation [60] and in transgenic animals [61]. The mechanism is presumably through binding of CRP to the pneumococcal C-polysaccharide and opsonization of the bacteria for phagocytosis and thereby killing. The opsonization of *S. pneumoniae*, in the presence of CRP, primarily activates the classical complement pathway and complement is required for the protection by CRP [62,63]. However, CRP protection does not require FcγR, indicating that CRP is not primarily protective by direct opsonization but by activation of complement and subsequent opsonophagocy-tosis [63]. CRP and complement were also shown to be necessary for serum bactericidal activity against *H. influenzae*
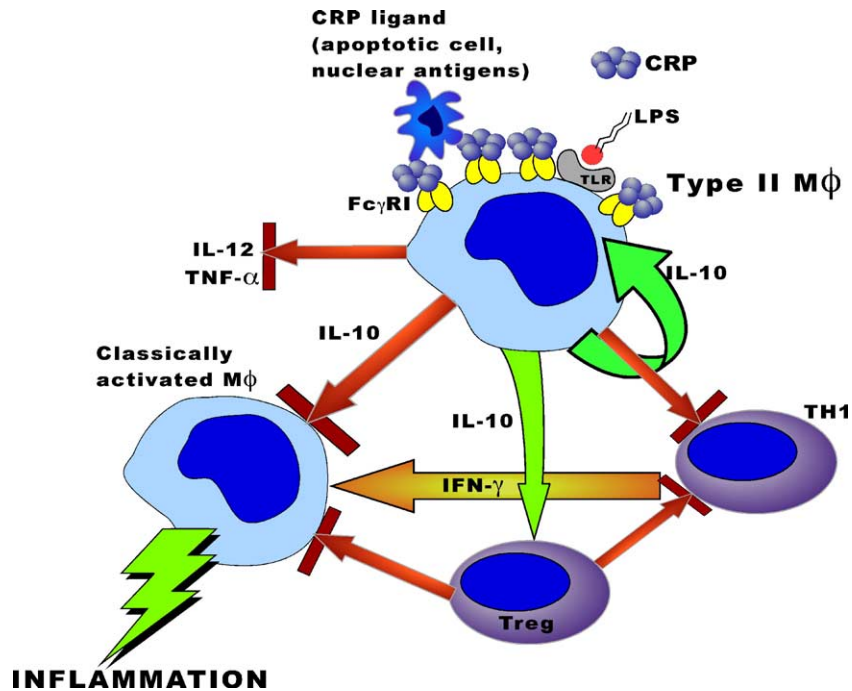
Fig. 4. Possible mechanism of CRP modulating inflammation. CRP binds and crosslinks FcγRI on macrophages, inducing the production of IL-10. This causes the macrophages to become Type II immunosuppressive macrophages, turning off the production of IL-12 and TNF-α. The IL-10 then down-regulates TH1 T cells and classically activated macrophages. Another anti-inflammatory consequence of IL-10 could be the activation of regulatory T cells (Treg).

[64]. Using a CRP transgenic mouse model [65], CRP has also been shown to be protective against a gram-negative bacterium, *Salmonella enterica*. Therefore, although CRP binds to FcγR and requires them for opsonization of zymosan in vitro [66], the in vivo experiments suggest that complement activation plays a greater role in *S. pneumoniae* clearance.

Anti-inflammatory effects

Humans and mice differ significantly in the control of CRP synthesis. In humans, CRP is the prototypical acute phase protein, while in mice, it is expressed constitutively at low levels and increases only slightly during the acute-phase response [67]. Therefore, to study the effect of CRP in the mouse requires the addition of CRP from another species, either passively or through a transgene. Samols and colleagues [68] developed a transgenic mouse expressing rabbit CRP under control of the rat phosphoenolpyruvate carboxykinase (PEPCK) promoter in response to dietary signals. These transgenic mice, when expressing high levels of CRP, are partially protected from a lethal challenge of bacterial lipopolysaccharide (LPS), platelet-activating factor (PAF) and the combination of tumor necrosis factor alpha plus interleukin 1beta, but not with TNF-α alone. The mechanism of this protection was not determined. We have studied the protective mechanism using passively administered human CRP in a mouse endotoxin shock model [69]. These studies showed that CRP-mediated protection from lethal LPS challenge required FcγR. Mice that are γ-chain deficient, therefore lacking the IgG receptors, FcγRI and FcγRIII, were not protected by CRP. In addition, CRP increased LPS lethality in mice deficient in

FcγRIIb, the inhibitory IgG receptor. The interaction of CRP with FcγRI induces anti-inflammatory substances, IL-10 [69] and IL-1 receptor antagonist (IL-1RA) [70], while the interaction with FcγRIIb reduces FcγRI activation and production of the pro-inflammatory cytokine IL-12 [69]. Therefore, it appears that the administered human CRP alters the equilibrium between pro-inflammatory and anti-inflammatory cytokines.

In a study using passively administered human CRP in an accelerated model of SLE in (NZB × NZW)F1 mice injected with chromatin, we showed that CRP had a protective effect [71]. We have extended this to show that, in the (NZB × NZW)F1 model of SLE, a single injection of CRP before onset
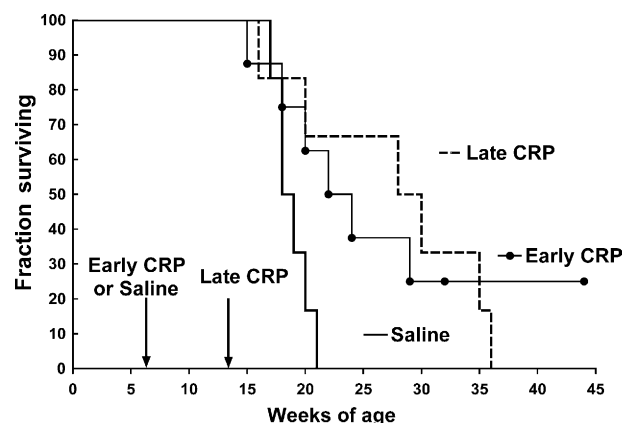

Fig. 5. CRP enhances survival of MRL/*lpr* lupus prone mice. Injecting mice with human CRP either early (at 6 weeks before the onset of disease) or late (13 weeks) increased survival.

of proteinuria delayed its development and late treatment actually reversed proteinuria [72]. The treatment did not lower the levels of anti-double-stranded (ds) DNA antibodies indicating that it is not working solely through clearance of autoantigens but by a more generalized anti-inflammatory mechanism. In order to gain insight into the mechanism, we used CRP treatment in an accelerated nephrotoxic nephritis model. In this model, mice were pre-immunized with rabbit IgG, and several days later, nephritis was induced by rabbit anti-mouse glomerular basement membrane serum. We were able to show reversal of proteinuria in C57BL/6 mice but not in IL-10 deficient mice indicating a role for this cytokine. This work adds to that of Szalai and colleagues [73] who used a transgenic (NZB × NZW)F1 mouse expressing human CRP as a transgene with the human CRP promoter. Expression of CRP in these mice is that of an acute phase protein and during the experiment the mice only expressed < 1 μg/ml human CRP. Even with these low CRP levels, the mice with the transgene survived longer and had less proteinuria than those without and accumulation of immune complexes in the renal glomeruli was delayed, despite a higher serum IgG anti-dsDNA titers. Again, this points to a generalized anti-inflammatory role for CRP, not simply removal or masking of autoantigens. A summary of our model of how CRP might cause long term modulation of inflammation is shown in Fig. 4.

We are currently studying the effect of CRP treatment in the MRL/*lpr* mouse (manuscript submitted). This mouse provides a rapid onset model of SLE, with the autoimmune prone MLR mouse exacerbated by homozygosity of a mutant form of the apoptosis gene Fas. MRL/*lpr* mice develop anti-nuclear antibodies and glomerulonephritis, as well as lymphadenopathy and vasculitis [74]. We have found that when these mice are treated with a single dose of human CRP before disease onset, the development of proteinuria and lymphadenopathy is delayed significantly, with increased survival and lower levels of anti-dsDNA antibodies. Treatment of mice after they develop active disease reverses proteinuria and prolongs survival (Fig. 5). Renal pathology in the treated mice correlates with the decrease in proteinuria and azotemia. These findings demonstrate systemic suppression of autoimmunity initiated by a single injection of CRP. To determine whether the mechanism for protection involved macrophages, we used treatment with Clodronate containing liposomes to deplete macrophages, but this did not reverse the protection by CRP. However, treatment of the mice with anti-CD25 antibody caused a return to high levels of proteinuria, suggesting a role for regulatory T cells. The ability of CRP to induce IL-10 synthesis and suppress inflammation may create the appropriate environment for induction of regulatory T cells [75,76].

Conclusion

During the acute phase, where CRP plays an anti-inflammatory role, CRP is produced very rapidly and at high levels. In contrast, CRP levels slightly higher than normal have increasingly been used as an indicator of inflammation associated with cardiovascular disease. However, the same factors that are shown to increase CRP levels, i.e., age, obesity, smoking and diet, are also risk factors for cardiovascular disease. There is also a genetic influence, not only on CRP levels, but in addition, the ability of CRP to bind to its receptors is influenced by genetic polymorphisms in the receptors. These differences in genetic backgrounds could be affecting the risk of vascular disease. It is possible that at low levels CRP could cause localized vascular damage by activating complement or inducing pro-inflammatory cyto-kines. The possible role of CRP in inducing vascular inflammation is beyond the scope of this review. It is clear that CRP can be both pro-inflammatory and anti-inflammatory and the primary role of CRP is likely to be the regulation of acute inflammation.

Acknowledgments

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