Structural biochemistry of the pentraxins

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4 An acidic microenvironment causes CRP to bind to and agglutinate apolipoproteins in human serum

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Abstract

Atherosclerotic cardiovascular disease is a leading cause of mortality worldwide, and is characterised by lipid containing plaques, inflammation and leukocyte infiltration. Serum levels of the acute phase component C-reactive protein (CRP) has been linked to several cardiovascular pathologies, suggesting it may play a role in disease progression. Furthermore, CRP is known to modify its properties in an acidic microenvironment, as found at sites of inflammation, indicating that CRP may execute biological functions locally at the sites of atheromatous plaques. However, it is unknown if or how CRP contributes to disease progression in cardiovascular disorders. Here we show that CRP associates with elements of the complement cascade and apolipoproteins at an acidic pH. CRP agglutinates lipid nanoparticles in human serum via binding to their apolipoprotein content and may result in cleavage of an unidentified protein factor by both the classical and alternative pathways. This work suggests that CRP may have a causative role in foam cell formation and may subsequently participate in a positive feedback loop characterised by greater complement activation and leukocyte infiltration, producing further localised acidification. This presents CRP as a promising therapeutic target to produce better clinical outcomes for cardiovascular disorders such as atherosclerosis.

Introduction

Atherosclerotic cardiovascular disease affects countries across the globe at various stages of economic development (159). A primary cause of atherosclerosis is the lipid vesicle content of normal human serum (NHS), such as low density lipoprotein (LDL) that contain apolipoproteins A and B (Apo A and B)(160, 161). Excessive uptake of these lipids by phagocytes, such as macrophages, leads to foam cell formation(162) and ultimately an atheromatous plaque(163, 164). Pharmaceutical interventions that lower these lipids result in better clinical outcomes(165). However, increasingly inflammation and immunity is being linked with the development of cardiovascular disorders(166), reinforced by the fact that risk factors relating to lifestyle and lipid metabolism appear to be associated with a heightened inflammatory state(167, 168). In support of this are reports that show the acute phase inflammatory protein, C-reactive protein (CRP), is a better prognostic predictor for cardiovascular risk than both blood pressure or cholesterol(125).

CRP is a disc-shaped homopentameric pattern recognition receptor (PRR) with two faces(34). CRP can bind to phosphocholine head groups (PC) in lipid membranes via binding face (B-face)(104) coordinated Ca^{2+}, as well as elements of the complement cascade or phagocytic receptors via the activating face (A-face)(91, 136). During the acute phase, CRP serum levels increase(13), and CRP is deposited at the sites of inflammation(62, 93). Furthermore, the inflammatory microenvironment is characterised by non-homeostatic conditions such as localised acidosis(75-77, 82), where CRP has been shown to have altered biochemical properties(30, 35, 85). In addition, CRP has been shown to increase tissue damage in rat models of myocardial infarction via CRP mediated complement activation(169), suggesting CRP may play an active role in the development of these pathologies.

Given the modulated properties of CRP under inflammatory conditions and the link between CRP(125, 170, 171), complement activation(73, 172) and inflammation to the progression of atherosclerosis(166), we sought to establish whether there exists a mechanistic connection between these factors.

Here, we observed greater binding of CRP to complement proteins C3 and soluble membrane attack complex (sMAC) at pH 5.0, as has been previously reported for CRP binding to complement activators such as C1q (***chapter 1***)(35). Additionally, as CRP is present in NHS *in-vivo*, CRP was incubated with NHS at pH 7.4 and 5.0, which revealed that large aggregates containing CRP formed at an acidic pH that was dependent on both CRP binding to apolipoproteins in NHS and the formation of a decameric species of CRP(35, 87). This also resulted in the hydrolysis or membrane deposition of as unidentified protein factor by the classical and alternative complement pathways.

Taken together, these observations present a model whereby chronic inflammation results in acidification and increases CRP binding to apolipoproteins, which are then agglutinated and activate complement. This in turn would result in greater inflammation, leukocyte infiltration and foam cell formation, leading to further acidification, starting a positive feedback loop contributing to disease progression. This work represents first steps towards deconvoluting the mechanisms of CRP in cardiovascular diseases and has wide ramifications for cardiovascular diseases as it indicates targeting CRP as a potential therapeutic strategy.

Results

CRP has been found to have modified biochemical properties under acidic conditions(30, 35, 85), including greater association with complement activators such as C1q and IgG (chapter 2)(35). To test if this property extended to other complement proteins, pH dependent CRP binding to the globular head domain of C1q (gC1q), C3 and sMAC was assessed via enzyme-linked immunosorbent assay (ELISA), [as well as the globular region of C1q (gC1q)] (Fig. 1A). This showed an increased association between CRP and these ligands at pH 5.0 compared to 7.5. Moreover, this increase was higher than for the other serum proteins tested; human serum albumin (HSA) and bovine serum albumin (BSA) (Fig. 1B), indicating that CRP associates with these immunological ligands in a pH dependent manner, as has been previously postulated (chapter 2)(35).

As the lipid content of NHS plays a central role in the progression of cardiovascular pathologies(160, 161), CRP was incubated with NHS (10%) at pH 7.4 or pH 5.0. After incubation, larger structures or aggregates present in NHS were separated from fluid phase protein by centrifugation. The protein content of the pelleted fraction was then analysed via reducing SDS-PAGE (Fig. 1C&D), revealing that incubation of CRP at pH 5.0 resulted in the sedimentation of protein compared to CRP with NHS at pH 7.4 (Fig. 1C). Additionally, NHS or CRP incubated alone at pH 5.0 did not result in the sedimentation of detectable protein (Fig. 1D), showing the sedimentation of protein was dependent on the interaction between CRP and unidentified factors in NHS. Western blot revealed that much of the signal could be attributed to CRP, even bands corresponding to higher molecular weights than monomeric

CRP (23 kDa, Fig. 1C). This data illustrates that an acidic pH CRP causes the sedimentation of large CRP-containing aggregates in NHS compared to pH 7.4.

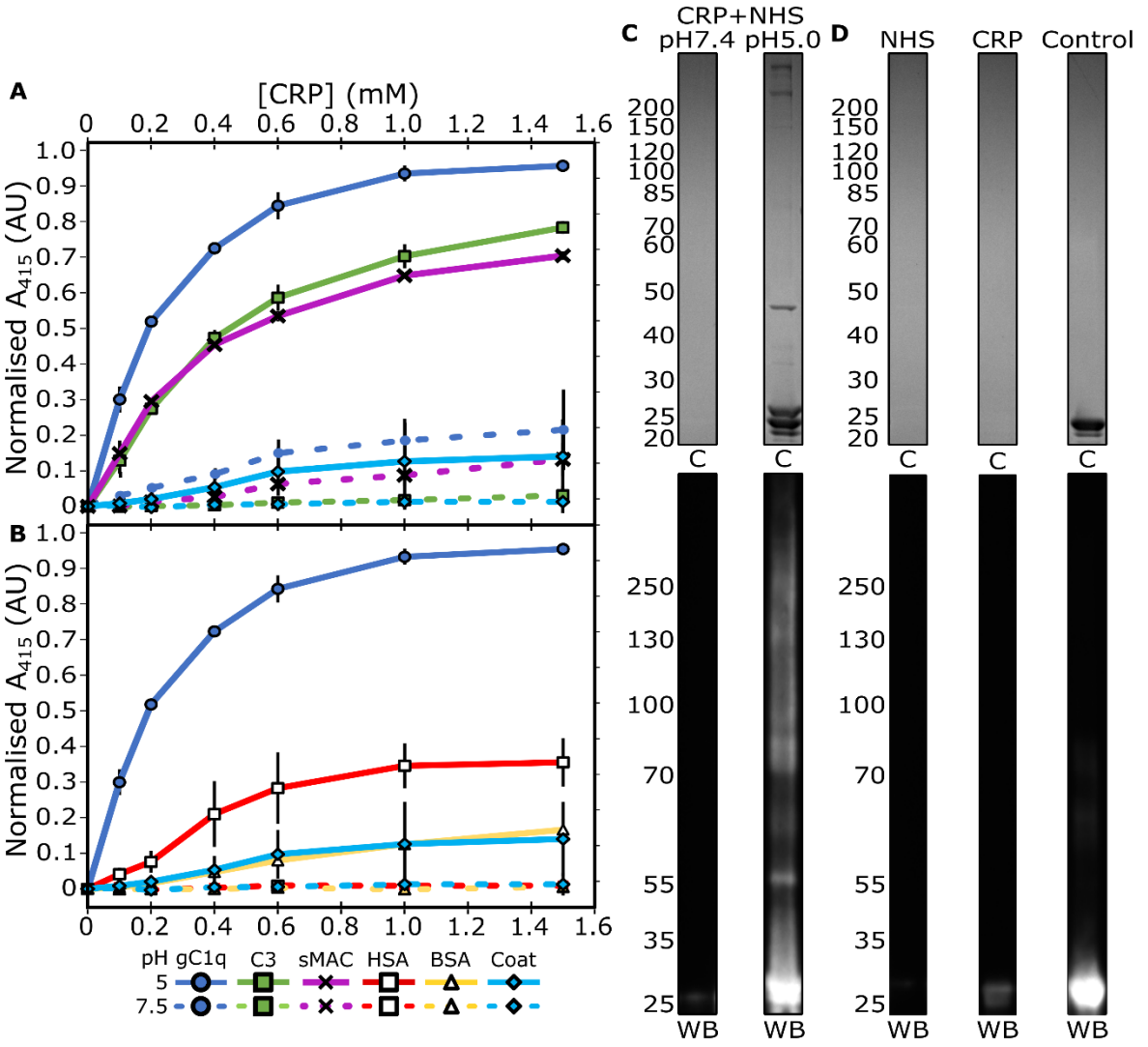

Figure 1. C-reactive protein (CRP) binds to protein ligands associated with the classical complement cascade and elements of normal human serum in a pH dependent manner*. (**A** & **B**) Ligands were adhered to the wells and CRP detected by ELISA. Ligands tested were: (**A**) complement components gC1q, C3 and sMAC; (**B**) HSA, BSA and coating agent only. CRP binding to gC1q at pH 5 and spermidine (coat) is present in all panels for reference. Error bars show the standard deviation of 3 independent replicates. (**C**) SDS-PAGE either stained with Coomassie (C) and Western blot (WB) analysis of the effect of incubation of CRP (2000 nM) with normal human serum (10%) separated from the fluid phase at pH 7.4 and pH 5.0. (**D**) CRP incubated without NHS or NHS incubated without CRP and a well containing pure CRP (1 µg, control). Western blots were stained with a mouse αCRP polyclonal antibody.*

The complement cascade comprises three activation routes; the classical, the lectin and the alternative pathways(7). CRP is able to bind to the C1 complex in order to activate the classical complement cascade (***chapter 2***)(136), which in turn can cause activation of the alternative pathway(7). As NHS contains components of the complement cascade(173) and CRP can activate the complement cascade(13), it was hypothesised that the bands corresponding to higher molecular weight species in **Fig. 1C** could relate to complement activation. To interrogate this further, CRP incubated with NHS was compared to CRP with C1q depleted serum or factor B depleted serum to knock out the classical and alternative pathways respectively. Incubation of each of the sera at pH 7.4 or without CRP at pH 5.0 did not result in the detection of any protein (**Fig. 2**). However, whilst both C1q and Factor B dep sera also resulted in a largely similar pattern of sedimented protein, the two high molecular weight bands (>200 kDa) exhibited different patterns. The highest molecular weight band was present in all three types of serum, but the lower band of the two did was not detected after incubation with depleted sera (**Fig. 2**). This suggests that the higher molecular weight band relates to a substrate of the complement cascade and the lower band relates to a proteolytically cleaved or membrane deposited version of this substrate, implying complement activation.

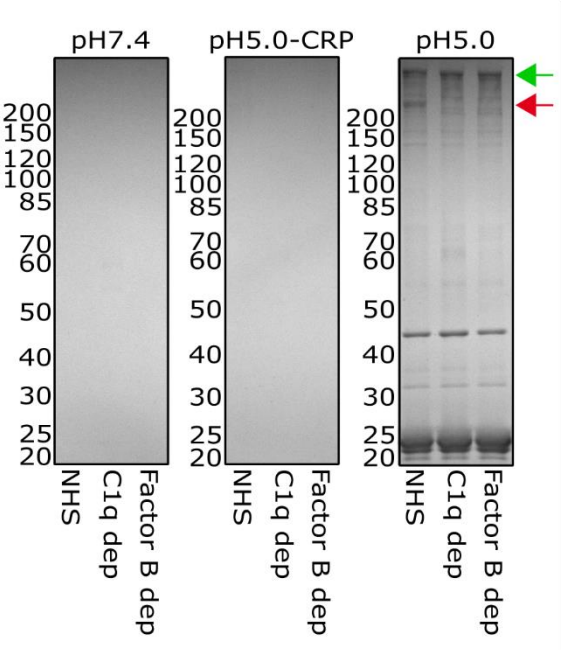

Figure 2. The classical and alternative pathways are required for proteolysis resulting from CRP incubation with normal human serum (NHS)*. SDS-PAGE analysis of the effect of incubation of CRP (2000 nM) with NHS, C1q depleted serum (C1q dep) or factor B depleted serum (factor B dep, 10%) separated from the fluid phase at pH 7.4 and pH 5.0. The different serums alone incubated with pH 5.0 are also shown. Gels were stained with Coomassie. Green and red arrows show the uncleaved and cleaved complement substrate respectively.*

CRP binds to lipid components of NHS via the B-face interactions with apolipoproteins(174), which would leave the A-face free to interact with effector ligands such as other CRP pentamers or C1q. As such, it was hypothesised that A-face interactions could be driving the sedimentation and possible complement activation seen above. To investigate this, a cocktail of polyclonal αApoA and αApoB antibodies were preincubated with NHS, before incubation with CRP at pH 5.0. This led to a dramatic reduction in the amount of protein detected in the pelleted fraction after centrifugation (**Fig. 3**) Moreover, αApoA/B antibodies also appeared to prevent complement mediated proteolysis of the >200 kDa bands, suggesting complement is activated via interactions between CRP and the lipid components of NHS such as LDL (**Fig. 3**).

We previoulsy postulated that the decameric species was able to agglutinate lipids together (***chapter 3***)(35), and this may also play a role in aggregating the protein and lipids, producing a large pelletable mass of material. A mutant previously shown to be deficient in the ability to agglutinate lipids together (R188A; ***chapter 3***) was used to explore this and indeed incubation of CRP R188A (2000 nM) with NHS (10%) at pH 5.0 resulted in substantially lower amounts of total protein detected by SDS-PAGE (**Fig. 3**). In contrast, the >200 kDa band was present only in its lower molecular weight form, indicating that complement may still activated in the absence of agglutination (**Fig. 3**) and supports the idea that pH dependent interactions between apolipoprotein and CRP drive complement mediated deposition or proteolysis of an unidentified protein factor (**Fig. 3**). Together this data supports a model whereby CRP binds to lipid vesicles in NHS via interactions with apolipoproteins under conditions of acidosis, which in turn results in aggregation of the lipid nanoparticles via decamer formation and complement activation.

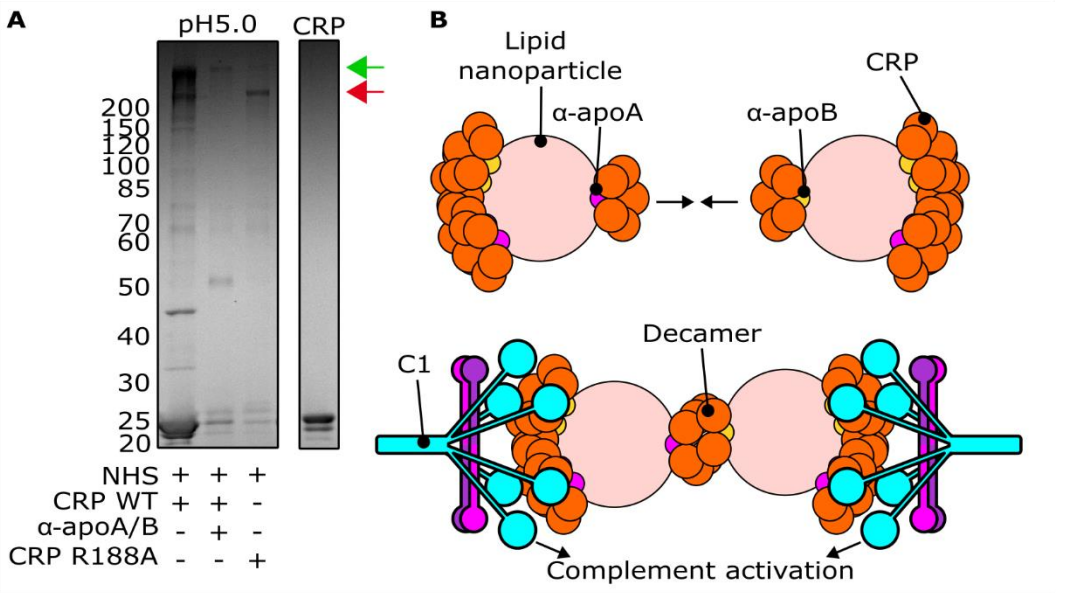

Figure 3. CRP binds to Apolipoprotein B (ApoB) or Apolipoprotein A (ApoA) and requires agglutination to form large aggregates where complement is activated*. (**A**) SDS-PAGE analysis of the effect of incubation of CRP (2000 nM) with NHS (10%), CRP with NHS and αapoA/B antibodies (0.09 mg/ml), CRP R188A with NHS separated from the fluid phase at pH 7.4 and pH 5.0. Gels were stained with Coomassie. Green and red arrows show the uncleaved and cleaved or undeposited and deposited complement substrate respectively. (**B**) Schematic of the mechanism of action. Briefly, CRP binds top apolipoproteins on lipid nanoparticles in the serum leading to decamerisation and agglutination as well as complement activation via binding C1.*

Discussions

Here we present data that indicates that CRP binds to apolipoproteins in a pH dependent manner (**Fig. 1**), which may result in the proteolysis or deposition of unidentified protein factors by the classical and alternative pathways (**Figs. 2**). Moreover, it was shown that complement dependent hydrolysis or membrane deposition of the unidentified protein factor is dependent on interactions between apolipoproteins and CRP (**Fig. 3**). Two higher molecular

weight bands (>200 kDa) were present in the presence of NHS, but only one with the depleted sera (**Fig. 2**), which could indicate either proteolysis or membrane deposition of the protein. As proteolysis would result in the depletion of one of the protein species, techniques such as densitometry could be used to measure the band intensities to see if the higher molecular weight band reduces in intensity when the lower band appears in the presence of NHS. Additionally, mass spectrometry could be used to identify this protein and show if it is membrane bound or not. Furthermore, it was shown that formation of decameric CRP resulted in agglutination of apolipoprotein containing material (**Fig. 3**). Together, this implies that at an acidic pH CRP will aggregate lipid nanoparticles present in NHS, such as LDL, and activate the proinflammatory complement system, representing a bona fide link between inflammation, CRP, LDL aggregation and immune activation. *In vivo* this could be triggered by lifestyle factors documented to increase both the risk of cardiovascular disease and chronic inflammation(167), such as a western diet(175) and obesity(168) (**Fig. 4**). As the inflammatory microenvironment is characterised by an acidic pH(75, 80-82), this would lead to the alteration of the biochemical properties of CRP, including extending the ligand binding repertoire and increasing complement activation(30, 85) (**Fig. 4**). Under normal homeostatic conditions, this may represent a form of regulation, ensuring CRP is only activated at the sites of inflammation to prevent aberrant complement activation given the near ubiquitous presence of its canonical ligand PC(127, 176). However, during states of prolonged inflammation due to inflammatory lifestyle risk factors for cardiovascular disease, CRP may bind excessively to apolipoproteins in NHS and form aggregates (**Figs. 1,3&4**) resulting in cardiovascular disease. Indeed, Rosuvastatin, a drug shown to lower cholesterol and LDL levels in humans(177), has also been reported to reduce proinflammatory effects of CRP independently of CRP serum concentration in spontaneously hypertensive rats(178), suggesting a link between a reduction in serum levels of LDL and inflammation, via a CRP based mechanism. Additionally, Rosuvastatin has been shown to reduce cardiovascular risk in patients with high circulating CRP levels(179), suggesting CRP may play an active role in the pathogenesis of cardiovascular disease via its interactions with LDL (**Fig. 4**).

Use of CRP-R188A, a mutant unable to agglutinate lipids together (***chapter 3***), resulted in less sedimented material after centrifugation, indicating that the aggregates contained less mass than aggregates from WT CRP (**Fig. 3**). This suggests that the ability of CRP to decamerise is the driving factor of the agglutination of apolipoproteins seen above (**Fig. 3**). The decameric species has been previously identified via structural methods(35, 87), which has been reported to form to a greater extent when bound to a B-face ligand and at an acidic pH (***chapter 3***)(35). This is may be due to the ionic nature of the inter-A-face interactions between CRP pentamers. Coupled with reports showing CRP interacts with Apo B via the B-face(174), the current study provides data that the decameric species may form at an acidic pH to aggregate apolipoproteins (**Figs. 2&3**).

Moreover, hydrolysis or deposition of an unidentified protein factor by the classical and alternative pathways was dependent on CRP binding to apolipoproteins present in NHS (**Fig. 2&3**), suggesting that these pathways were activated. Whilst formation of the decameric

species discussed above would occlude the C1 binding site (***chapter 3***), large aggregates would still contain bound pentameric CRP at the peripheries (**Fig. 4**). This would in turn allow C1q binding and complement activation, leading to the deposition of opsonins such as C3b. Complement activation also results in the production of the anaphylatoxins, C3a and C5a, which act as chemoattractants for leukocytes(29) (**Fig. 4**). This could represent a mechanism for the initial infiltration of the inflamed area by leukocytes, mediated via CRP binding to lipid vesicles such as LDL. Additionally, CRP could cause the phagocytosis of LDL containing aggregates via direct interactions with phagocytic receptors(23, 24, 91) or indirectly via deposited complement proteins such as C3(20). *In vivo* macrophages phagocytose aggregates of LDL(180), with defective phagocytosis leading to foam cell formation(164) and ultimately formation of an atheroma (**Fig. 4**). This indicates that CRP could play a causative role in foam cell formation and cardiovascular pathologies.

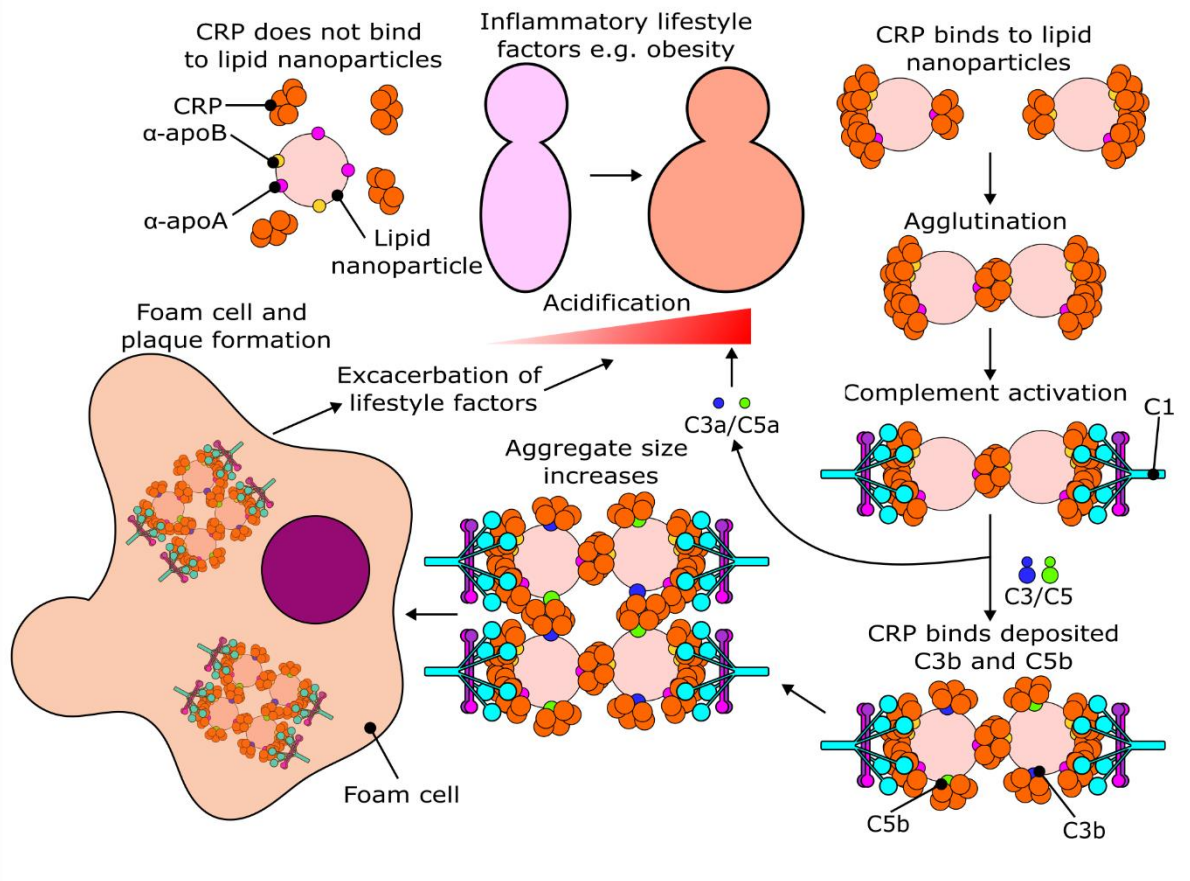

Figure 4: Schematic representation of the CRP based mechanism of atherosclerotic progression. *Under normal homeostatic conditions CRP does not associated with lipid nanoparticles in the serum. However, inflammatory lifestyle conditions like obesity acidify parts of the serum leading to biochemical changes in CRP which allow binding to the apolipoprotein content of the serum. This leads to complement activation and more CRP deposition on deposited complement factors C3b and C5b. Additionally, the anaphylatoxins cause further decreases in local pH and recruit leukocytes to the region. The aggregate increases in size and is phagocytosed by white blood cells such as macrophages leading to foam cell and plaque formation. This is in turn can worsen lifestyle factors by causing disabilities and reduced movement in the patient.*

The sequence of events presented above could lead to a positive feedback loop characterised by iterative localised acidification, as both complement activation(181) and leukocyte metabolism(80) have been shown to cause reductions in extracellular pH. Additionally, the increased association of CRP with complement proteins such as C3 at an acidic pH (**Fig. 1**), could reinforce this positive feedback loop as complement activation would itself result in more CRP binding and subsequent complement activation.

This model opens up the possibility of CRP as a therapeutic target, and previous reports have targeted the B-face of CRP with some success in rat models of myocardial infarction(111). Rather than non-specifically inhibiting all CRP functions in this way, perhaps more selective inhibition of CRP mediated complement activation or agglutination could produce efficacious results with less side effects. Utilising knowledge gained in ***chapter 3*** that lateral interactions between CRP pentamers may mediated complement activation, mutants that can agglutinate ligands without activating complement could be rationally designed as will be discussed in the general discussions of this thesis (***chapter 8***). This would enable comparisons with non-agglutinating mutants such as R188A in mouse models of cardiovascular disorders(111) to see if biasing CRP function towards phagocytosis or complement activation could produce better clinical outcomes. As such, future studies should focus on the rational design of CRP mutants with monofunctionality, or alternatively focussing on molecules capable of selectively inhibiting different CRP functions such as nanobodies or small molecules. To conclude, this study presents a link between an inflammatory environment and CRP mediated aggregation of LDL with subsequent complement activation, with wide ramifications for potentially targeting CRP as a therapeutic.

Methods

Protein sequence for a single-chain gC1q protein.

A single-chain gC1q protein with the following sequence was synthesised by U-Protein Express BV (Netherlands) in HEK293E-253 cells. The signal peptide is in bold and cleaved off during secretion. The triple strep-tag is underlined.

MARPLCTLLLLMATLAGALAGSDQPRPAFSAIRRNPPMGGNVVIFDTVITNQEEPYQNHSGRFVC
TVPGYYYFTFQVLSQWEICLSIVSSSRGQVRRSLGFCDTTNKGLFQVVSGGMVLQLQQGDQVWVE
KDPKKGHIYQGSEADSVFSGFLIFPSAGSGKQKFQSVFTVTRQTHQPPAPNSLIRFNAVLTNPQG
DYDTSTGKFTCKVPGLYYFVYHASHTANLCVLLYRSGVKVVTFCGHTSKTNQVNSGGVLLRLQVG
EEVWLAVNDYYDMVGIQGSDSVFSGFLLFPDGSAKATQKIAFSATRTINVPLRRDQTIRFDHVIT
NMNNNYEPRSGKFTCKVPGLYYFTYHASSRGNLCVNLMRGRERAQKVVTFCDYAYNTFQVTTGGM
VLKLEQGENVFLQATDKNSLLGMEGANSIFSGFLLFPDMEAAAWSHPQFEKGAAWSHPQFEKGAA
WSHPQFEKGAA

ELISA

ELISAs were performed using 96 well Maxisorp microtiter plates (Thermofisher, USA) coated with 100 µl of either gC1q (see above), C3 (Complement technology, USA), sMAC

(Complement technology, USA), HSA (Thermofisher, USA) or BSA (Thermofisher, USA) at 10 µg/ml in 0.1 M sodium carbonate (pH 9.3). For coating controls, only the sodium carbonate solution was added. These were incubated for 1 hour at 37°C, then washed 3 times in phosphate buffered saline (PBS) with 0.05% Tween-20. Subsequently, wells were blocked with 100 µl of 0.1 M spermidine, before being incubated and washed as previously described. Next, 50 µl CRP (Merck , USA) was added at the concentration ranges and buffer conditions described in the figures. Briefly, CRP (1 mg/ml in 20 mM Tris, 140 mM NaCl, 2 mM $CaCl_2$, 0.05% NaN3, pH 7.5) was diluted in either HEPES buffer at pH 7.5 (20 mM HEPES, 140 mM NaCl, 2 mM $CaCl_2$) or acetate buffer at pH 5 (140 mM NaAcetate, 40 mM Acetic Acid, 140 mM NaCl, 2 mM $CaCl_2$) and was incubated for 1 hous at 37°C before washing. To detect CRP binding, 50 µl of a mouse polyclonal anti-human CRP antibody (Thermofisher, USA) was added at 2 µg/µl, incubated for 1 hour, and washed as described above. Thereafter, 50 µl of a goat anti-mouse antibody conjugated to horseradish peroxidase was added and then incubated for 1 hour and washed. Absorbance at 415 nm was measured 15 minutes after addition of 50 µl of 2.5 mg/ml 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in citric acid buffer (0.15 M, pH 4.2) with 0.015% (v/v) H2O2 using a Clariostar plus plate reader (BMG Labtech, Germany). The data set consisted of three independent triplicate plates for each ligand. All values were normalized to 1500 nM of CRP pH 5 binding to gC1q, with the values of the 0 nM CRP in each condition subtracted as background.

CRP NHS interactions

Liposomes (50 µl per reaction) were incubated with CRP (2000 nM) for 30 minutes at 4°C before being mixed with ice-cold NHS, factor B depleted serum or C1q depleted serum (Complement Technology, USA, 10%) and made up to 70 µl with buffer. Antibodies for Apo A and B (R&D systems, USA) were incubated with serum on ice for 15 minutes before application to the CRP solution. Negative controls only contained CRP or serum. This was incubated on ice for 30 minutes before being spun down at 21,460 g and 4°C for 15 minutes. Next, the supernatant was discarded and the pellet resuspended in 70 µl of buffer. Subsequently, the sample was spun as before and the pellet resuspended in 20 µl of buffer, which was then used for further analysis. This was done at pH 7.4 or pH 5.0 with the following respective buffers; tris-HCl 50 mM, NaCl 150 mM, $CaCl_2$ 5 mM at pH 7.4 or Na-4cetate 140 mM, acetic acid 40 mM, NaCl 150 mM, $CaCl_2$ 5 mM at pH 5.0.

For SDS-PAGE analysis, samples were diluted in 2× Laemmli buffer (65.8 mM Tris-HCl, 26.3% (w/v) glycerol, 2.1% SDS, 0.01% bromophenol blue, pH 6.8) with DTT (50 mM) and heated to 99°C for 10 mins, briefly centrifuged at 700 g, and then loaded onto onto a 4-12% pre-cast Bis-Tris Protein Gels (BoltTM, InvitrogenTM, Thermo Fischer scientific, USA) and run at room temperature for 35 minutes at 200 V. Gels were stained with SimplyBlue™ to visualise protein content (Thermofisher, USA).

Western Blot

For Western Blotting, samples were separated using PAGE as described above. Samples were transferred to nitrocellulose membranes in Tris-Glycine buffer (Tris 12 mM, Glycine 96 mM)

with 10% methanol. The membranes were then blocked using phosphate buffered saline (PBS) with 0.1% Tween 20 and 5% milk (blocking buffer). After washing three times with blocking buffer, proteins were detected with a polyclonal mouse anti-CRP primary antibody (Thermofisher, USA) diluted 1:1000 at 4°C overnight. The membranes were then washed as before, and subsequently a HRP linked goat anti-mouse antibody (Dako, Denmark) diluted 1:1000 was added and incubated with the membranes at room temperature for 30 minutes. The membranes were washed twice in blocking buffer and four times in PBS with 0.1% Tween 20. Detection was performed with ECL™ reagent and imaged on a ChemiDoc™ gel imaging system (Thermofisher, USA).

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