Structural biochemistry of the pentraxins

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5 A pipeline for screening CRP based therapies: a nanobody and TRAP based approach

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Abstract

C-reactive protein (CRP) is an acute phase protein with important functions in immunity and inflammation, as well as being linked to disorders such as atherosclerosis. However, therapies targeting CRP have focused either on inhibition of the association with phosphocholine or depletion of total serum levels of CRP, thereby ablating all CRP effector functions. As such, more specific inhibitors of particular problematic aspects of CRP function such as complement activation, could maintain other potential beneficial processes such as phagocytosis. Structural and biochemical analysis in the previous chapters and in the literature suggest there are certain structural hotspots on CRP for particular functions, and that targeting these hotspots could inhibit specific CRP functions. Here we present a biochemical screen for testing potential CRP inhibitors, where we test a range of recently published αCRP nanobodies and establish that they selectively inhibit different effector functions of CRP, suggesting that they bind to distinct structural hotspots. As such, these nanobodies could form the basis of more selective and efficacious treatments for CRP related disorders, such as systemic lupus erythematosus and atherosclerosis. In turn, this could be complemented by structural studies to clarify structure to function relationships for various subdomains of CRP and CRP effector functions. Additionally, we also present the results of a peptide screen of interactors for CRP, which could then also be analysed using the same biochemical pipeline. As a result, the presented study could form the foundation for the screening a range of molecules targeting CRP, from small molecules to monoclonal antibodies, whilst deconvoluting which specific CRP functions are inhibited.

Introduction

C-reactive protein (CRP) is an acute phase soluble pattern recognition receptor (PRR) of the pentraxin family, that consists of five non-covalently associated pentraxin domains forming a disc like structure(34). CRP can activate the classical complement cascade representing its function in the immune system(27, 30, 136) and it is associated with the development of both atherosclerosis(171) and systemic lupus erythematosus (SLE) (64, 66, 128, 182). The canonical ligand of CRP is the phosphocholine head group (PC) of phosphatidylcholine lipids, which CRP can recognise in response to environmental cues associated with danger, such as lipid

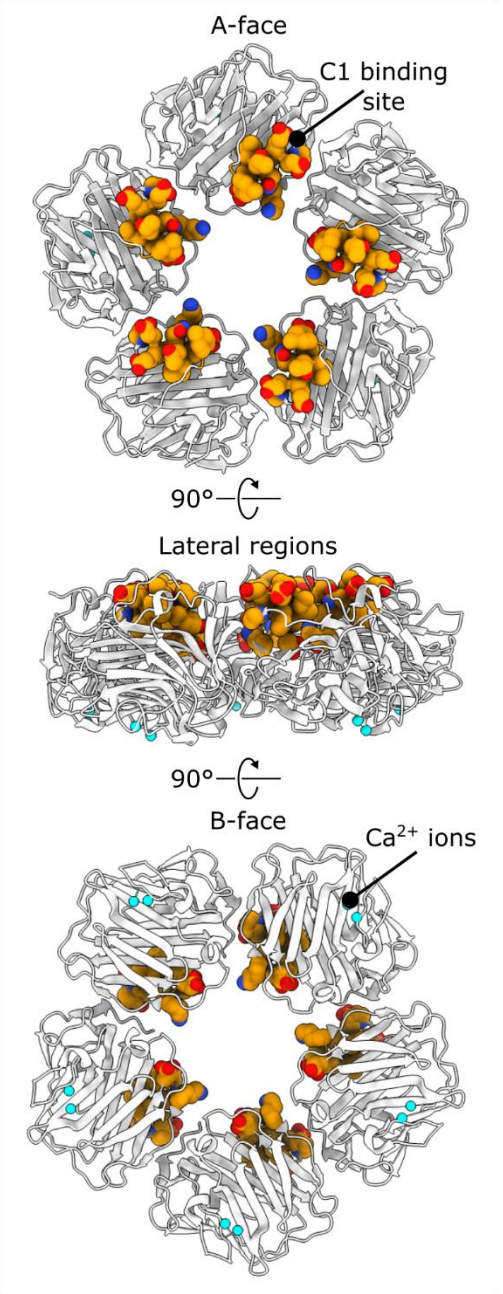

Figure 1: CRP structure showing the A-face, lateral regions and B-face. *C1 binding residues on the A-face are highlighted as orange spheres and the B-face Ca^{2+} ions as cyan spheres. (PDB:7PKE).*

oxidation (130). CRP-bound lipid membranes are directed for removal by the immune system via CRP effector functions; namely self-association with other CRP pentamers to agglutinate lipid membranes together to facilitate phagocytosis (***chapter 3***)(145), as well as binding to the C1 complex to activate the classical complement cascade (***chapter 3***)(136). These two aspects of CRP function occur on different faces of the pentameric disc, with immune effector ligands such as C1(136) and Fcγ receptors(22, 24) binding to the activating face (A-face) and PC binding being mediated by the binding face (B-face) via coordinated Ca^{2+} ions (**Fig. 1**). Additionally, lateral interactions between CRP pentamers on a 2D plane, such as on a lipid membrane, have been suggested to be important in higher order multimer formation that may precede complement activation (***chapter 3***). As such, steric hinderance of either the A-face, B-face or lateral regions by molecules such as peptides or nanobodies could selectively inhibit different functions of CRP, biasing it towards different physiological outcomes such as phagocytosis or complement activation. Several drugs based on PC mimics have been published (**Table 1**), however these have focused on the B-face, thereby resulting in non-specific inhibition of all CRP functions (111, 123). In addition, several methods of reducing total CRP in the serum have been trialled (**Table 1**), although this also prevents CRP performing potential beneficial functions. For example, the silent clearance of apoptotic cells via phagocytosis of cellular debris has been highlighted as protective against SLE, contrasting to the negative effects of complement activation, suggesting selective inhibition of complement activation could provide better results(58, 64, 67). In addition, biochemical data, in conjunction with structural studies of inhibitory molecules bound to CRP, could reveal insights into structure function relationships between the various subdomains of CRP and CRP effector functions.

Nanobodies are monomeric variable antibody domains derived from camelids such as llamas, with a range of advantages as potential therapeutics due to their small size, thermal stability, high affinity and solubility(183). Recently, a range of αCRP nanobodies were published showing tight and specific binding to CRP(184), and as such we sought to biochemically

characterise these nanobodies bound to CRP, allowing preliminary epitope mapping of these molecules in order to explore the possibilities of their therapeutic potential. The result was the development of a biochemical pipeline to measure the effect of biomolecules on the various functions of CRP, which could be applied to a range of therapeutics such as monoclonal antibodies or small molecules. Accordingly, given that peptides also show great therapeutic potential due to their high specificity and ability to disrupt protein-protein interactions(185), a peptide screen using the transcription–translation coupled with association of puromycin linker (TRAP) system was performed to obtain preliminary peptide that showed specific binding to CRP. These could also be submitted to the same pipeline presented herein to screen them for which structural hotspots of CRP they associate with and ultimately their ability to modulate CRP function. Taken together, the study below presents nanobodies that affect the biological function of CRP in different ways, with some initial peptide sequences that could be analysed using the same series of biochemical assays. In turn this takes the field closer to the ultimate goal of better understanding how CRP can be biased towards different effector functions, in order to produce better clinical outcomes in diseases such as SLE.

Drug type	Binding site	Mode of action
Bivalent PC(111)	B-face	Prevents lipid binding Occludes A-face
PC mimic(123)	B-face	Prevents lipid binding
Peptide-PC conjugate(186)	B-face	Prevents lipid binding
ASO(187, 188)	N/A	Prevents CRP translation
PC decorated agarose(189, 190)	B-face	Depletes CRP from serum

Table 1: Current CRP targeting biomolecules, their binding sites on CRP and modes of action. *Abbreviations: PC, phosphocholine; ASO, antisense oligonucleotides.*

Result

CRP binds to its canonical ligand PC in lipid membranes via B-face coordinated Ca^{2+} ions. As a result, chelating agents such as EDTA or competitive inhibitors like free PC can be used to block B-face binding to PC-containing liposomes. Similarly, prospective therapeutics that aim to neutralise this face of CRP would also prevent association with lipid membranes(111, 123). To measure the association between CRP and liposomes in the presence or absence of nanobodies, a spin filter with a 300 kDa molecular weight cut-off was used to separate free CRP and nanobodies from liposomes. After washing, the liquid remaining at the top of the spin filters was analysed by SDS-PAGE to detect CRP that co-precipitated with liposomes, indicating binding (**Fig. 2A**). Preincubation of CRP with nanobodies at a fivefold excess led to bands of equal intensity to positive controls for A6, E12 and H7 (**Fig. 2B**). Interestingly, H7 appeared to cause extra bands at approximately 60 and 150 kDa (**Fig. 2B**). This was unexpected given that the samples were denatured and reduced before application to the gel, so any conventional non-covalent or disulfide bonds should be broken. This could relate to aggregation induced by the nanobody between CRP and the lipids, as membrane associated proteins can become SDS resistant when aggregated(191). However, the nanobody A12 seemed to substantially reduce co-sedimentation of CRP with liposomes,

suggesting that this nanobody binds to or at least partially occludes the PC binding site on the B-face, in turn preventing association with the lipid membrane.

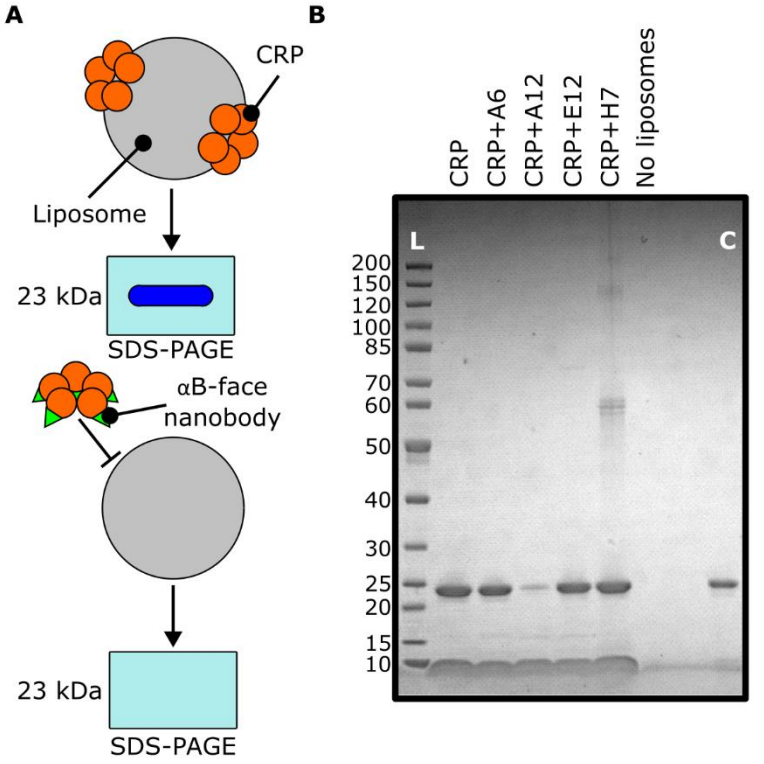

Figure 2: Inhibition of the association of CRP with LPC containing liposomes. *(**A**) Schematic of the assay showing that CRP binding to liposomes results in a band as detected via SDS-PAGE, whereas if nanobodies inhibit liposome binding no band is visualised. (**B**) SDS-PAGE analysis of the effect of preincubation of CRP (500 nM) with nanobodies (2.5 µM) on liposome binding (DMPC:LPC 80:20 mol%). Gels were stained with Coomassie. L and C signify ladder and CRP control respectively.*

Furthermore, C1 is known to bind to residues on the A-face of CRP(90-92) and as such nanobodies binding to this region should sterically hinder C1 binding (**Figs. 1&3A**). This can be measured via enzyme linked immunosorbent assays (ELISA), where CRP is immobilized to a plate and normal human serum (NHS) can be added on top as a source of C1. CRP was immobilized to the plate after preincubation with nanobodies or was added to the plate directly, with nanobodies added at a later step (**Fig. 3B&C**). When CRP was preincubated with A12, C1q bound at levels simsilar to positive controls (**Fig. 3B**). There was a slight reduction if CRP was immobilized prior to addition of the nanobody (**Fig. 3C**), although this was marginal. Conversely, when preincubated with CRP both A6 and H7 resulted in decreases of C1q binding to levels similar to negative controls (**Fig. 3B**). However, this was much less stark if the nanobody was added to CRP after immobilization on the plate (**Fig. 3C**), possibly relating to mild denaturation that can occur when proteins bind to the plastic surface of ELISA plates(192). Interestingly, E12 appeared to partially reduce C1q binding regardless of whether it was preincubated with nanobodies or not, showing the smallest change in effect between the two conditions (**Fig. 3B&C**). This indicates the binding site of E12 contains only partial overlap with the C1 binding site on CRP and may bind to a distinct location compared to the other nanobodies (**Fig. 3B**).

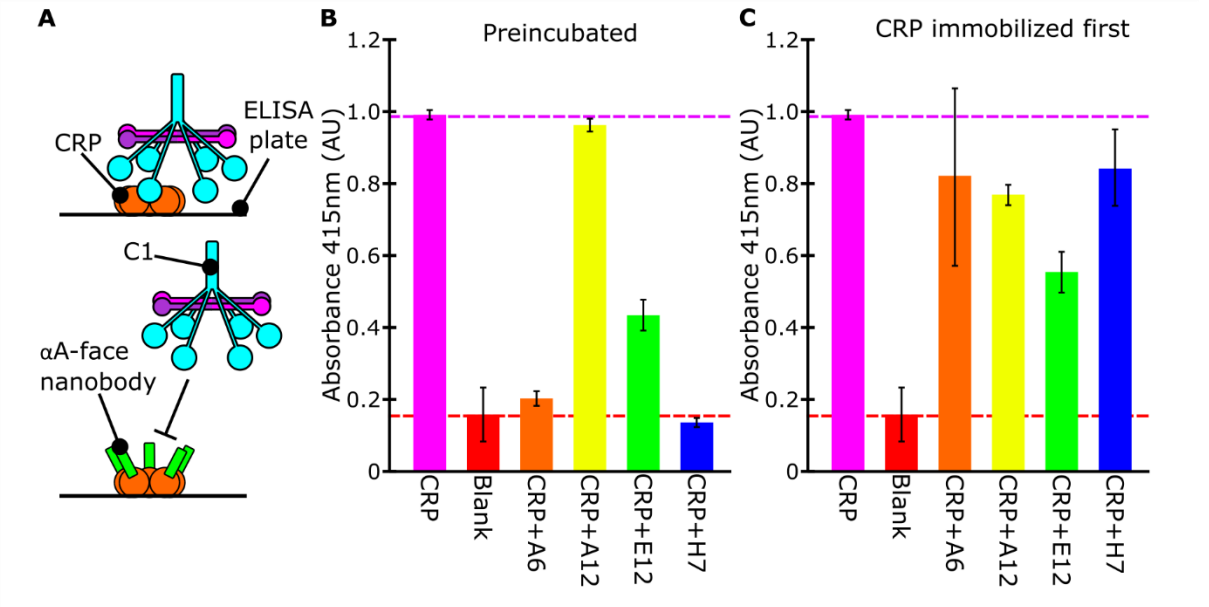

Figure 3: ELISA measuring the inhibition of C1q-CRP interactions by nanobodies. *(**A**) Schematic of the ELISA showing that CRP bound to the ELISA plate can normally bind to C1 from normal human serum (NHS). However, A-face binders that overlap with the C1q binding site would prevent this interaction. (**B**) ELISA showing C1q binding to CRP (10 µg/µl) immobilised on ELISA plates. Nanobodies were preincubated with CRP at a 10 fold molar excess (870 and 87 nM respectively). (**C**) ELISA showing C1q binding to CRP (10 µg/µl) immobilised on ELISA plates, with nanobodies added after immobilization at a 10 fold molar excess. NHS was used as a source of C1q (1%). Error bars represent the standard deviation of triplicate wells.*

CRP is known to bind to C1 and activate the classical complement cascade(***chapter 3***) (73, 136). Upon binding to complement activators such as CRP, the protease domains of C1 - C1r and C1s - are activated, causing proteolysis of complement factors C4 and C2(7). The rate of proteolysis of the C1 complex, stimulated via liposome bound CRP, can be monitored to see how nanobodies effect this activity. The rate of the proteolytic activity of C1 could be reduced in three ways; inhibition of CRP binding to the liposome (B-face binder), inhibition of C1 binding to CRP (A-face binder) or disruption of the formation of higher order oligomers via lateral interactions required for complement activation as seen in ***chapter 3*** (**Fig. 4A**). CRP was incubated with each nanobody at a five-fold molar excess, bound to liposomes and C1 was added. Subsequently, the proteolytic activity of C1 was monitored over a four-hour period (**Fig. 4B**). A12 appeared to cause large reductions in C1 activity close to levels of fluid phase activation of CRP with C1 (**Fig. 4B**), consistent with the idea that A12 blocks CRP association with the lipid membrane (**Fig. 2B**). Similarly, H7 caused a large decrease in C1 activation, supporting previous data from ELISA that it blocks association with C1 directly. However, E12 and A6 only showed minor reductions in C1 proteolytic activity (**Fig. 4B**), which is in contrast to the ELISA data for A6, as this nanobody appeared to inhibit C1 binding to a similar extent to H7 (**Fig. 3B**). As E12 also caused a small decrease in the proteolytic activity of C1 (**Fig. 4B**), this was consistent with ELISA data showing E12 did not completely ablate C1q binding (**Fig. 3B**).

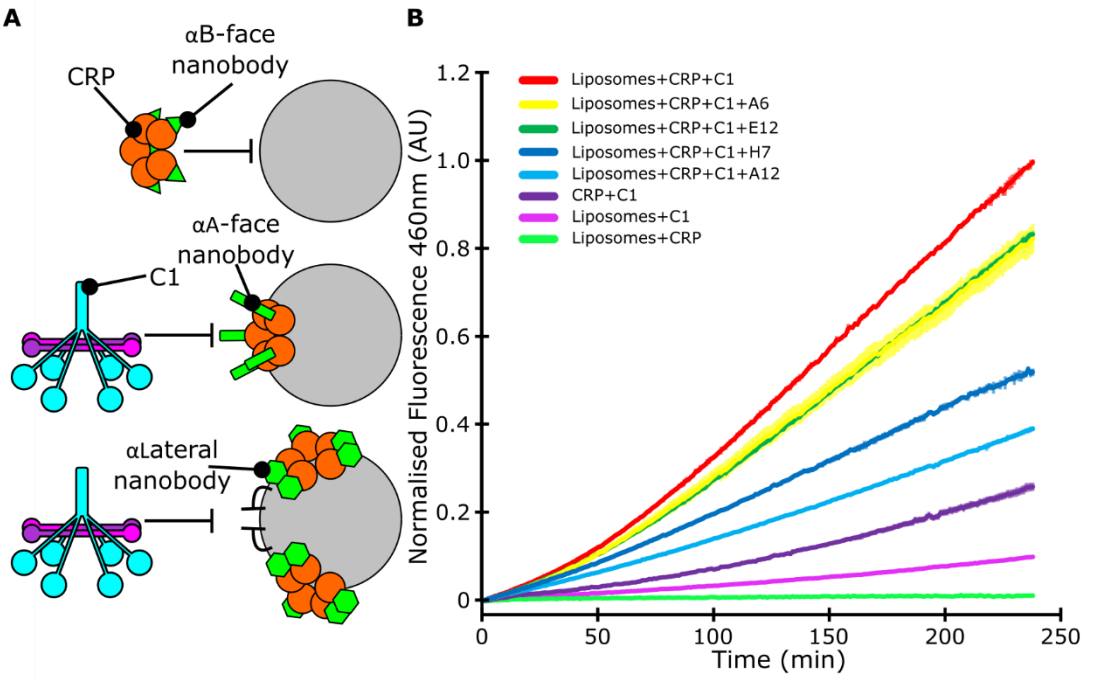

Figure 4: Effect of preincubation of the nanobodies with CRP on CRP mediated stimulation of the protease domains of C1 on a lipid membrane. *(**A**) Schematic of the possible reasons for inhibition of CRP mediated stimulation of C1; inhibition of liposome binding, inhibition of C1q association with CRP and inhibition of lateral interactions of CRP to create higher order oligomers for C1 binding and activation. (**B**) CRP (2.5 µM) activation of purified C1 (40 nM) on liposomal surfaces (DMPC:LPC 80:20 mol%) in the presence and absence of nanobodies (12.5 µM). Error bars represent the standard deviation of duplicate wells.*

In order to facilitate the phagocytosis of PC containing membranes, CRP has been shown to agglutinate liposomes together via formation of an offset decameric species (***chapter 3,*** **Fig. 5A**). Decamers are formed via A-face interactions(35, 87) and it has been shown that mutation of ionic bonds regulating this interface disrupts the ability of CRP to agglutinate liposomes (***chapter 3***). In a similar manner, nanobodies binding to these regions would inhibit decamerisation and therefore agglutination (**Fig. 5B**). Nanobodies were incubated with CRP at a two-fold excess and added to PC containing liposomes. A12 and H7 appeared to reduce agglutination, consistent with their respective hypothesised binding sites at the B and A-face respectively (**Fig. 5C**). However, A6 and E12 did not cause large decreases in agglutination (**Fig. 5B**), suggesting these nanobodies may bind to a region outside of the decamer interface. In conclusion, we have presented four nanobodies which appear to produce different outcomes on the functions of CRP, suggesting they bind to different structural hotspots on CRP (**Table 2**).

Here we have presented a pipeline for monitoring the biochemical effects of molecules that can bind to CRP. This same pipeline could be used for other molecules, such as monoclonal antibodies or peptides. Peptides show advantages over small molecules as drugs due to their high specificities and increased size, which allows greater inhibition of protein-protein interactions(185). This has resulted in peptides representing a steadily increasing proportion of the total number of therapeutics sold worldwide (185). The TRAP system(193) represents a fast, cell free, in vitro method to screen a library of peptides for binding to a target of interest (**Fig. 6A**). In this system, peptides are translated and transcribed from a DNA library and

concurrently linked to a cDNA barcode via a puromycin linker. Additionally, the incorporation of a non-canonical amino acid, N-chloroacetyl-tyrosine, allows macrocyclization of the peptides, which can in turn result in greater affinities and specificities to targets, resistance to hydrolysis and greater membrane permeability(194). The peptides are then added to beads conjugated to a protein target of interest or to unconjugated beads in order to positively and negatively select candidates, respectively. DNA barcodes can then be detected via qPCR and used to retrieve sequences that bound to the target in order to iteratively enrich a pool of peptide hits. To achieve this, streptavidin coated beads were bound with CRP biotinylated (biotin-CRP) via the non-specific linkage of biotin N-hydroxysuccinimide ester conjugates to surface lysines on CRP or left blank. Next, libraries containing D- or L- N-chloroacetyl-tyrosine were applied to the beads over four rounds, showing enrichment for both libraries, in particular for the D-peptide library (**Fig. 6B**). This resulted in the sequencing of 16 potential hits of peptide CRP binders. These hits could be subjected to the presented pipeline above to screen for their effects on the different functions of CRP, thereby increasing the arsenal of tools that could be used in the lab or clinic to modulate CRP, or indeed for diagnostic use in disorders ranging from COVID-19 infection to type 2 diabetes(184).

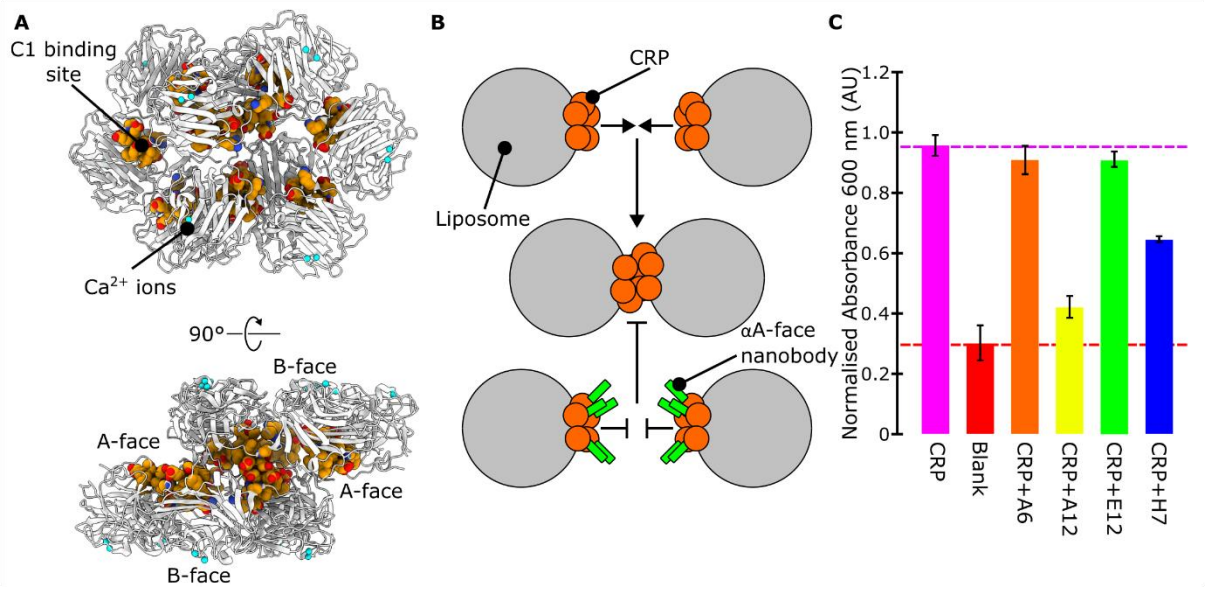

Figure 5: Effect of preincubation of the nanobodies with CRP on CRP mediated agglutination of LPC containing liposomes. *(**A**) Structure of the offset decamer of CRP formed by A-face interactions (PDB:7PK9). (**B**) Schematic showing how decamers of liposome bound CRP pentameric rings can cause the aggregation of lipid membranes together. Nanobodies occupying the region of the A-face mediating this would inhibit this interaction. (**C**) CRP (1.7 µM) based aggregation of liposomes (DMPC:LPC 80:20 mol%) together in the presence or absence of nanobodies (3.4 µM).*

	A6	A12	E12	H7
Liposome binding	NE	--	NE	NE
C1q binding (preincubated/immobilized CRP)	--/NE	NE/-	--/--	--/NE
Complement activation	-	--	-	--
Agglutination	NE	--	NE	-

Table 2: Summary of the effects of the nanobodies A6, A12, E12 and H7 on the biological functions of CRP. *Abbreviations: --, strong inhibition; -, weak inhibition; NE, No effect.*

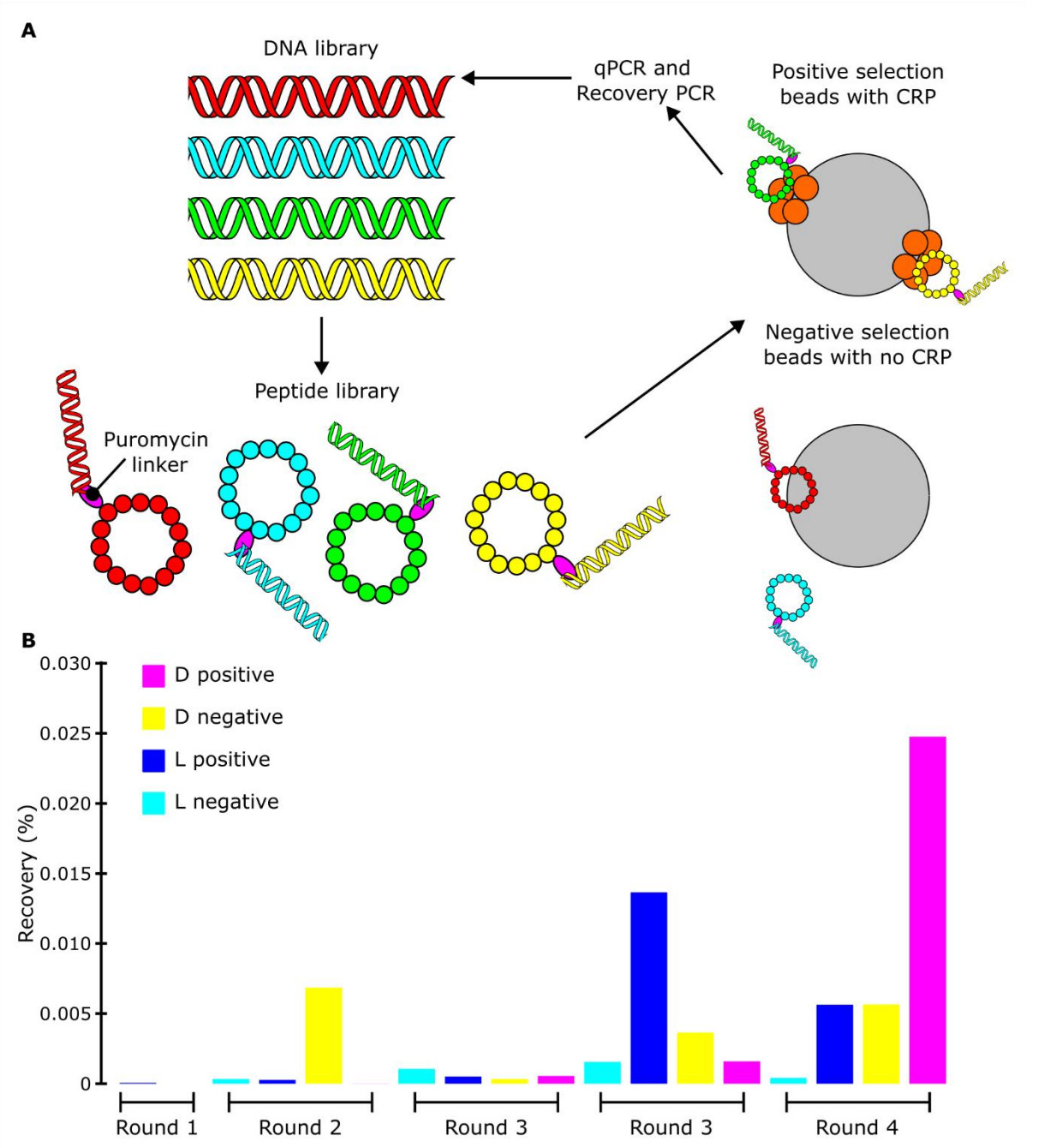

Figure 6: Enrichment of peptides binding to CRP by the TRAP system (transcription–translation coupled with association of puromycin linker). *(**A**) Schematic of the TRAP method for detecting small peptide binders to protein ligands. Briefly, a large DNA library of peptides is translated and transcribed whilst being linked to cDNA barcodes via puromycin linkers after reverse transcription. Peptides are then added to streptavidin coated beads with or without biotinylated CRP (biotin-CRP), which are taken as positive or negative hits, respectively. The sequences are identified by qPCR and recovered for the next round. This process is iterated several times before sequencing. (**B**) Enrichment of L- and D-peptide libraries binding to biotin-CRP coated streptavidin beads.*

Discussions

This study presents a biochemical pipeline that can screen different molecules for their effects on the biological functions of CRP. In this instance, nanobodies(184) were used, but this could

be adapted to other potentially therapeutic agents such as monoclonal antibodies, peptides and small molecules. Four published αCRP nanobodies were screened to see how they affected the function of CRP(184). First, A12 appeared to inhibit association of CRP with liposomes, which indicates that it likely binds to or occludes the B-face of CRP as this region is known to be crucial in interacting with PC in lipid membranes(104) (**Fig. 2C**). Secondly, whilst H7 did not prevent CRP-lipid interactions, H7 did prevent binding to C1q when preincubated with CRP as measured by ELISA, as well as substantially reducing stimulation of the protease domains of C1 by CRP bound to a lipid membrane (**Figs. 3B & 4B**). Taken together this indicates that H7 binds to or at least obscures the inner regions of the A-face known to be important in binding to C1 (**Fig. 1**)(90-92). These results suggest each nanobody has differing effects on the biochemical activity of CRP (**Table 2**).

On the other hand, A6 seemed to lead to conflicting results with ELISA showing that preincubation with CRP inhibited C1q binding in a similar manner to H7 (**Fig. 3B**), but that this did not translate to equivalent inhibition of the proteolytic activity of C1 bound to CRP on liposomes (**Fig. 4B**). Additionally, C1q binding was less affected if A6 was added directly to CRP immobilized on the ELISA plate (**Fig. 3B**). This could be due to the fact that binding to a plastic or lipid surface has been reported to change the epitope structure of CRP. For instance, binding to plastic plates has been demonstrated to induce a structural change in CRP, converting it to a hypothesised form called monomeric CRP (mCRP)(99). Moreover, mCRP contains specific epitopes differing from native pentameric CRP (pCRP), allowing differentiation between mCRP and pCRP with antibodies to epitopes particular to each CRP subtype(99, 133). Additionally, pCRP binding to lipid membranes has been postulated to induce a intermediate form of CRP, termed modified CRP (pCRP*), which displays dual antigenicity with elements of both mCRP and pCRP, whilst maintaining pentameric symmetry(107, 195). In contrast, mCRP or pCRP* is not formed when binding to free PC, suggesting only certain surfaces cause structural changes in CRP that affect what epitopes it may contain(99, 133). Whilst direct evidence for monomeric or modified forms of CRP via structural methods are sparse (***chapter 2 and 3***), this may still suggest there are subtle changes in CRP which could result in the modification of epitopes between the fluid phase, lipid bound and immobilized forms of CRP. Taken together this indicates that A6 binds to an epitope that is present on fluid phase CRP involved in C1q binding, but that this epitope is partially and completely disrupted in both membrane and immobilized CRP, respectively.

Similarly, E12 showed no effect on liposome binding and it partially inhibited C1q binding to CRP as measured via ELISA, with preincubation resulting in marginally greater inhibition than adding to immobilized CRP (**Fig. 3B&C**). However, this translated to only slight reductions in the activation of the C1 complex bound to CRP (**Fig. 4B**). This may indicate that E12 binds to regions outside of the B- or A-face such as the lateral regions, though partial overlap with the A-face cannot be ruled out given the intermediate inhibition of C1q binding seen via ELISA (**Fig. 3B&C**). This could mean E12 can disrupt the lateral contact seen in ***chapter 3***, that may precede and lead to C1 activation. However, given the modest effect on proteolytic activity of C1, this may suggest that a greater concentration or a larger molecule such as a monoclonal antibody or nanobody-conjugated to a larger construct, may be required to further disrupt higher order oligomer formation of CRP on lipid membranes. Ultimately, structural techniques such as X-ray crystallography or cryoelectron microscopy (cryoEM) could be used

to exactly determine the epitopes that these nanobodies bind to, and would in turn reveal structure-function relationships between structural motifs on CRP and effector functions. Therefore, future studies should focus on obtaining high resolution structures of these nanobodies bound to CRP, much as has been achieved previously with fluid phase CRP at different pHs (***chapter 2***)(35). This study also obtained several peptide hits for CRP binding as detected by TRAP(193) (**Fig. 6**). As peptides can be used as highly effective and specific therapies(185), these could be taken through the biochemical pipeline presented above to extend the arsenal of CRP modulatory molecules. Given that CRP is also linked to a range of disorders from COVID-19 to type 2 diabetes(184), these molecules could also be used as diagnostics for these pathologies. Additionally, as suggested for the nanobodies, cryoEM of peptides bound to CRP would reveal greater insight into their modes of action.

Exploring CRP as a possible target for therapeutic agents could help resolve disorders ranging from cardiovascular disease to SLE. For example, CRP has been linked to SLE and patients are often characterised by reduced levels of CRP in their serum(64). As SLE has been postulated to result from the defective silent clearance of apoptotic cellular debris(58), reduced CRP levels could lead to dysfunctional cellular clearance that results in the manifestation of SLE due to the pro-phagocytic properties of CRP (129, 144-146). Reinforcing this are the reported clinical benefits of CRP in mouse models of SLE(66). Additionally, SLE patients often have aberrant complement activation, suggesting dampening of the complement cascade may also be beneficial(67, 68). As such, creation of either recombinant CRP or therapeutic molecules that bias CRP towards phagocytosis opposed to complement activation, could build on the already-established benefits of CRP for this disorder(66, 126). In addition, as discussed in ***chapter 4*** in more detail, CRP is linked to many heart conditions such as cardiovascular disease(196), thrombosis(171) and atherosclerosis (170). In fact, CRP has already been used as a target to treat these disease states with the development of small molecules(111) and targeting it as a general anti-inflammatory(123) (**Table 1**). However, all the small molecules to date target the B-face exclusively, thereby knocking out all CRP function. As suggested above, by selecting more precise epitopes that disrupt only particular functions of CRP using this pipeline and cryoEM, more precise treatments could be developed. Additionally, some of the small molecules targeting CRP suffer from a short half-life (90 minutes) and high IC_{50} (2-20μM)(111), suggesting that other constructs such as nanobodies may provide greater efficacy in a clinical setting.

To conclude, we present a biochemical pipeline, with the potential to be augmented with structural studies, to interrogate biomolecules for their ability to modulate CRP with potential therapeutic benefits for pathologies such as cardiovascular disease and SLE, as well as gaining greater insight into the structural and biochemical mechanisms of CRP.

Methods

Liposome production

Liposomes were prepared using dimyristoylphosphatidylcholine (DMPC) and 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC) purchased from Avanti Polar Lipids (USA). Lipid films were composed of DMPC–LPC (80 : 20 mol%). Components were dissolved in

chloroform–methanol (9 : 1 v/v) before drying under nitrogen gas and desiccation overnight. Films were rehydrated at 60°C for 1 hour in Tris 50 mM NaCl 150 mM $CaCl_2$ 5 mM pH 7.4 (TBS) to a final lipid concentration of 0·8 mg/ml. Lipid films were then pipetted off the sides of the glass vials and were vortexed for 10 seconds. Liposomes were then extruded through a 400 and then 100 nm membrane respectively with 11 passes each using an extruder from Avanti Polar Lipids (USA).

Liposome co-precipitation assay

First, CRP at 1000 nM, preincubated with or without nanobodies, was incubated with 150 µl of liposomes for 15 mins at 37°C and 400 RPM. These mixtures were then added to Vivaspin™ spin filters (Sartorius, Germany) with a 300 kDa cut off and made up to 500 µl with TBS, before spinning at 14,000 g at RT for 5 minutes. Flow through was discarded and the spin filter was topped up with 500 µl TBS. This was repeated two more times before the dead volumes were used for further analysis via SDS-PAGE.

SDS-PAGE

Sample 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 (Bolt™, Invitrogen™, 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).

Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed using 96 well microtiter plates coated with 50 µl of CRP at 10 µg/ml in 0.1 M sodium carbonate (pH 9.3). For coating controls, only the sodium carbonate solution was added. For preincubated assays, CRP was preincubated with nanobodies (87 and 8700 nM respectively) at 37°C and 1000 RPM for 15 minutes prior to this step. For non-preincubated assays, only CRP was added. These were incubated for one hour at 37°C, then washed three 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. For non-preincubated assays, nanobodies were then added at 8700 nM and incubated as previously before washing. Next, NHS (10%, Complement Technology, USA) diluted in RPMI (Thermofisher, USA) was incubated for one hour at 37°C before washing. To detect C1q, 50 µl of a rabbit polyclonal anti-human C1q (Dako, Denmark) diluted 1:1000 was added and incubated for one hour before being washed as described above. Thereafter, 50 µl of a goat anti-rabbit conjugated to HRP diluted 1:5000 (Dako, Denmark) was added, then incubated for one hour and washed. Absorbance at 415 nm was measured 60 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) H_2O_2 using a Clariostar plus plate reader (BMG Labtech, Germany). Error bars represent standard deviation of triplicate wells.

C1 protease activity assay

All steps were done at 4° C until measurements were started with liposomes added at half the total reaction volume. CRP at 2500 nM was added to liposomes composed of DMPC-LPC (DMPC-LPC, 80 : 20 mol%) in TBS (reaction buffer). If nanobodies were present, CRP was preincubated with nanobodies (12500 nM) at 37°C and 1000 RPM for 15 minutes prior to this step. Next, a fluorescent peptide substrate, Boc-Leu-Gly-Arg-Amino Methyl Cumarin (LGR-AMC, PeptaNova GmbH, Germany), dissolved in DMSO (5% DMSO final concentration) was added at 500 µM, before buffer exchanged purified C1 complex (Complement Technology, USA) was added to the reaction at 40 nM. Subsequently, proteolytic activity of purified C1 complex was measured in real time via cleavage of LGR-AMC on a Clariostar plus plate reader (BMG Labtech, Germany). Measurements were taken every minute for a period of four hours at room temperature, with excitation and emission set at 360 and 460 nm respectfully. The first measurement of each condition was subtracted as background, before normalizing the entire plate to the highest value. Duplicate wells were then averaged and the standard deviation between them was calculated and represented as error bars.

Agglutination assay

The OD_{600} of liposomes (DMPC:LPC, 80 : 20 mol%) were measured for two minutes to establish a baseline for each well on a Clariostar plus plate reader (BMG Labtech, Germany). Next, CRP (1000 nM) was added to the liposomes and if nanobodies were present, CRP was preincubated with nanobodies (10,000 nM) at 37°C and 1000 RPM for 15 minutes prior to this step. The mixtures were left at room temperature for >10 minutes and then the OD_{600} was measured again for two minutes. The initial baseline was subtracted as background and the values across triplicate wells were averaged and the standard deviation was calculated.

CRP biotinylation

For the biotinylating of CRP the EZ-Link™ Sulfo-NHS-LC-Biotinylation kit (Thermofisher, USA) was used according to the manufacturer's instructions. First, CRP was buffer exchanged to PBS and then NHS-PEG_4-Biotin was added at a 40-fold molar excess, before the sample was incubated at room temperature for 45 minutes. Excess NHS-PEG_4-Biotin was then removed from the CRP using a desalting column.

Flexizyme and initiator tRNA production

The assembly of the DNA templates for flexizyme (eFx) and initiator tRNA ($tRNA^{fMet}_{CAU}$) production were performed as previously described(197, 198).

Amino acylate tRNA

First, EFX (250 µM) was mixed with initiator tRNA (250 µM) and incubated 95° for 2 minutes then at room temperature for 5 minutes. Subsequently, 3 M of $MgCl_2$ was added and the sample was left for a further 5 minutes at room temperature, before the mixture was put on ice. When ice cold, N-chloroacetyl-L-tyrosine or N-chloroacetyl-D-tyrosine (25 mM) was added and the sample was incubated for 1 hour on ice. The reaction was then quenched with

acetic acid (0.3 M) and the reaction products were purified via ethanol precipitation. Samples were stored at -80°C

***In vitro* selection of macrocyclic peptides on immobilized CRP using TRAP display**

The selection rounds of the L and D library in parallel was carried out as described previously(193). Recovered DNA was sequenced according using Nanopore flongle sequencing protocol described previously (199) with the Flongle flow cell (Oxford nanopore technologies, UK).

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