C-REACTIVE PROTEIN IN AGE-RELATED DISORDERS

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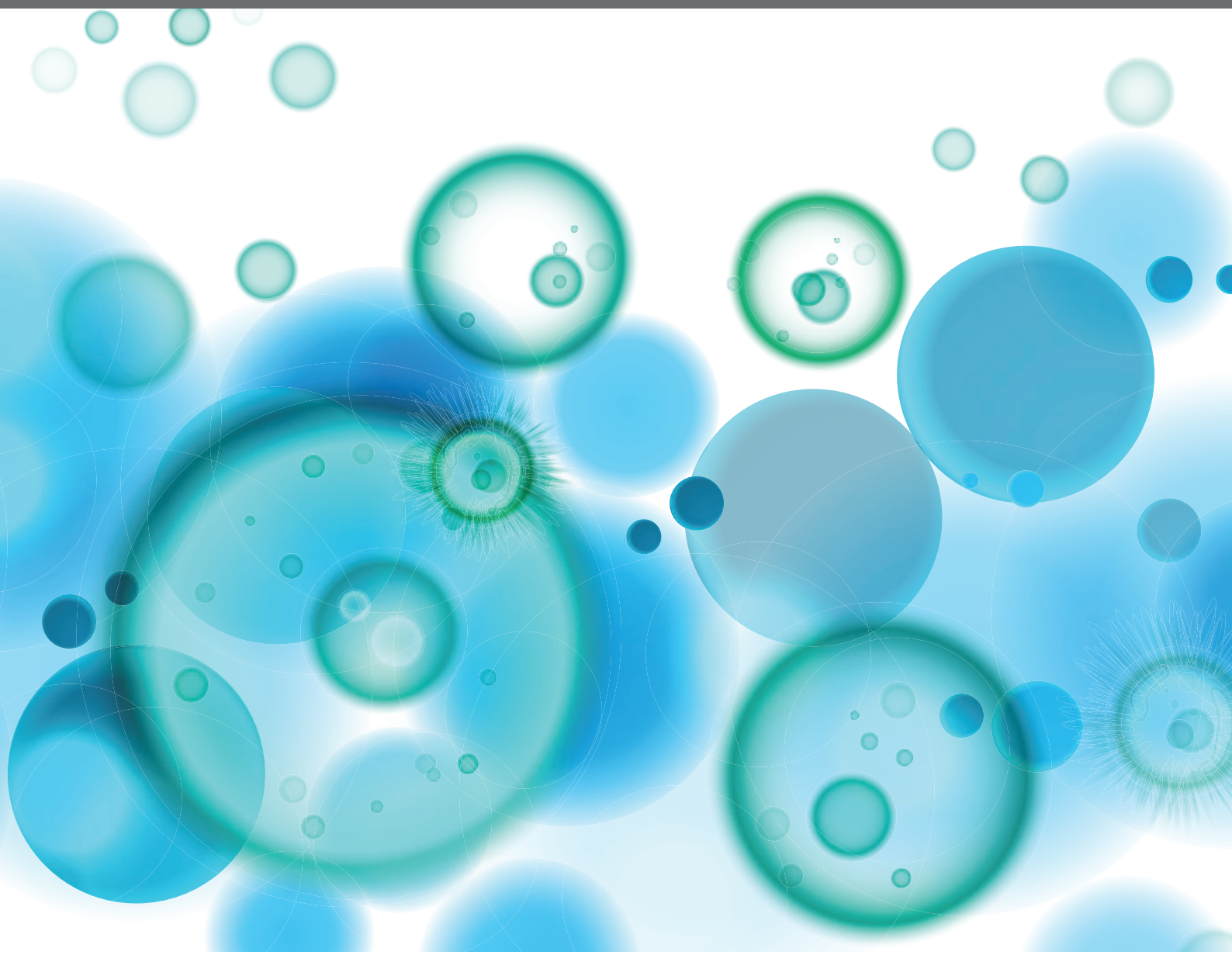

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C-REACTIVE PROTEIN IN AGE-RELATED DISORDERS

Topic Editors:
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Over recent years, native pentameric C-reactive protein (pCRP) and its biologically active dissociated form, monomeric CRP (mCRP) have assumed an important role in disease development and pathophysiology.

In this series, we have highlighted the thoughts and research of the most eminent scientists in the field of CRP research. This eBook is a collection of original articles and reviews on the subject, creating an archive of current knowledge and understanding.

This Research Topic provides new findings of the role of CRP in the fields of neuroscience, cardiovascular disease, inflammation, and macular degeneration as well as defined links to stages in pathological disease progression. These articles explain the mechanisms and pathways through which the dissociated mCRP interacts with a variety of cells, and provide possible prognostic implications and new methods for analysis.

Over the coming years, the importance and fascination of the active role of CRP in health and disease is set to rise, and we hope this collection will serve as a valuable reference for these future investigations.

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EDITORIAL
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Editorial: C-Reactive Protein in Age-Related Disorders

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Keywords: dissociation, c-reactive protein, cell signaling, inflammation, biomarker

Editorial on the research topic

C-Reactive Protein in Age-Related Disorders

Over the last decade, native pentameric C-reactive protein (pCRP), and its biologically active dissociated monomer monomeric CRP (mCRP), have hit the headlines as it has become realized that they may not play such a passive role in disease development and pathophysiology as was once thought. In this series we have composed the thoughts and research of the most eminent scientists in the field of CRP-research in a mixture of original articles and reviews on the subject with a view to creation of an archive of current knowledge and understanding.

Both acute and chronic inflammation are hallmarks of potential injury and illness and are critical pathophysiological fingerprints of major disease development such as in cardiovascular disease, diabetes, obesity, and cancer (Gupta et al.). Circulating levels of pCRP have been used in conjunction with other biomarkers to measure accurately systemic levels of inflammation but too often only poorly, the prediction of future pathobiological events or consequences of the acute immune response. As our understanding of the biological importance of mCRP has increased, so has the suggestion that measurement of its circulating levels associated with microparticles, macrophages, and platelets of plasma, in patients with both acute and chronic inflammatory conditions could represent a much more accurate event predictor. Here, for the first time, Zhang et al. have produced a quantifiable ELISA assay accurately measuring mCRP levels in plasma. Furthermore, they went on to test this on a small cohort of patients with skin-related immune disorders showing that mCRP indeed predicted the course of illness far better than pCRP measurements. In acute coronary syndromes, autoimmune diseases (e.g., Lupus, giant cell arteritis) and possibly even some solid tumors, measurements of plasma mCRP may prove to be a significantly better prognostic indicator (1, 2).

Recent publications have highlighted important novel biological mechanisms associated with both native pCRP and mCRP signaling and pathophysiological action. For example, the work of Wu et al. (3) and Thiele et al. on understanding CRP conformational changes associated with induction of inflammation, and lipid binding details to apoptotic cell membranes by Alnaas et al. (4). In this series, we have included a group of focussed reviews relating the current state of knowledge of the role of CRP in major disease progression. Articles by Molins et al. Chirco and Potempa and Fernandez-Robredo et al. have discussed local inflammation and immune activation in age-related macular degeneration (AMD), a major cause of blindness, highlighting its pathogenic role via activation of complement; and identifying an association of A69S SNIP with high levels of CRP and degeneration in wet AMD.

Other reviews have discussed the impact of cellular and tissue deposition of mCRP on atherosclerotic plaque development, dementia and AMD (McFadyen et al.), and others the

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inflammatory links to diabetes and Parkinson's (Luan and Yao). Previously cited work by Strang et al. (5) and Slevin et al. (6) have already provided strong evidence of a possible causative involvement of mCRP in development of beta-amyloid plaques and neurodegeneration-associated cognitive impairment linked to Alzheimer's and dementia, suggesting that CRP manipulation could represent a future therapeutic target for helping to protect against development of these conditions.

Sproston and Ashworth discussed details of a novel mechanism for a role of CRP in response to infection, recruitment of leukocytes (also shown by Thiele et al. (7) in an ischemia-reperfusion injury model) and induction of nitric oxide (NO) (mCRP), following this up with a detailed original article measuring CRP associated changes in NO and iNOS using a monocyte-macrophage model of aging and inflammation. Additional reviews in our series have focussed on the ability of mCRP to activate endothelial cells, stimulating angiogenesis, increase plaque instability in coronary artery disease and potentially catalyze thrombosis leading to myocardial infarction (Badimon et al.). Di Napoli et al.. also discussed the utility of CRP as a biomarker for, and predictor of both long and short-term outcomes in intracerebral hemorrhage. Release of CRP and possible accumulation of mCRP in the brain after stroke, could contribute significantly to inflammation during the acute phase, and neurodegeneration thereafter (8) and hence further investigation of its role in these processes and the others described above is warranted.

Finally, we include in this series a group of articles focussed on detailing distinct novel biological properties of CRP. Sudhakar et al. showed increased CRP in non-morbidly obese individuals and preferred mCRP binding to the leptin receptor in human plasma and this could link to low-grade systemic infection on over-weight individuals. Jia et al. demonstrated binding of mCRP to receptor activator of NF-κB associated with blocking osteoclast differentiation with potential protective action in joint inflammation associated with rheumatoid arthritis. On the other hand Jimenez et al. showed that CRP may also play a role in the maintenance of peripheral T cell tolerance as they showed the ability of CRP to suppress development, maturation, and function of dendritic cells. Interest has arisen with regard to the potential interaction between CRP and nicotinic/acetylcholine receptors. CRP is known to affect innate immunity operating through the nicotinic acetylcholine receptors and here, Richter et al. (9) showed that pCRP associated with phosphocholine, could block macrophage-induced cytokine release potentially protecting trauma-associated injuries *in vivo*. In contrast, mCRP potently stimulates macrophage-associated inflammation, and further work detailed in this series showed that acetylcholine inclusion, within an *in vitro* model of inflammation, could effectively block mCRP-induced production of tumor necrosis factor-alpha (10).

A detailed analysis of crystal binding structure of mCRP by Caprio et al. concluded that various phospholipase A2 inhibitors might have therapeutic potential to block the early interaction of the molecule at the cell membrane thus negating activation of harmful biological effects. Future work could focus on effective prevention of mCRP binding to activated cell surfaces; or even more excitingly, either systemically or in a targeted form, blocking the primary pCRP-mCRP dissociation especially during acute inflammatory conditions.

In conclusion, the series provides an encyclopedia of current knowledge within the field of CRP research, it highlights new findings and major links to critical disease processes. It defines clear and opposing actions of the pentameric protein when compared with the dissociated monomer, and it expresses the complexity of cell and tissue specific interactions of this highly conserved molecule. Publications within this topic will undoubtedly increase over the coming years and we hope this collection will serve as a valuable reference for this future investigation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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A Conformational Change in C-Reactive Protein Enhances Leukocyte Recruitment and Reactive Oxygen Species Generation in Ischemia/Reperfusion Injury

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Introduction: C-reactive protein circulates as a pentameric protein (pCRP). pCRP is a well-established diagnostic marker as plasma levels rise in response to tissue injury and inflammation. We recently described pro-inflammatory properties of CRP, which are mediated by conformational changes from pCRP to bioactive isoforms expressing pro-inflammatory neo-epitopes [pCRP* and monomeric C-reactive protein (mCRP)]. Here, we investigate the role of CRP isoforms in renal ischemia/reperfusion injury (IRI).

Methods: Rat kidneys in animals with and without intraperitoneally injected pCRP were subjected to IRI by the time of pCRP exposure and were subsequently analyzed for monocyte infiltration, caspase-3 expression, and tubular damage. Blood urea nitrogen (BUN) was analyzed pre-ischemia and post-reperfusion. CRP effects on leukocyte recruitment were investigated *via* intravital imaging of rat-striated muscle IRI. Localized conformational CRP changes were analyzed by immunohistochemistry using conformation specific antibodies. 1,6-bis(phosphocholine)-hexane (1,6-bisPC), which stabilizes CRP in its native pentameric form was used to validate CRP effects. Leukocyte activation was assessed by quantification of reactive oxygen species (ROS) induction by CRP isoforms *ex vivo* and *in vitro* through electron spin resonance spectroscopy. Signaling pathways were analyzed by disrupting lipid rafts with nystatin and subsequent ROS detection. In order to confirm the translational relevance of our findings, biopsies of microsurgical human free tissue transfers before and after IRI were examined by immunofluorescence for CRP deposition and co-localization of $CD68^+$ leukocytes.

Results: The application of pCRP aggravates tissue damage in renal IRI. 1,6-bisPC reverses these effects *via* inhibition of the conformational change that leads to exposure of pro-inflammatory epitopes in CRP (pCRP* and mCRP). Structurally altered CRP induces

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leukocyte–endothelial interaction and induces ROS formation in leukocytes, the latter can be abrogated by blocking lipid raft-dependent signaling pathways with Nystatin. Stabilizing pCRP in its native pentameric state abrogates these pro-inflammatory effects. Importantly, these findings are confirmed in human IRI challenged muscle tissue.

Conclusion: These results suggest that CRP is a potent modulator of IRI. Stabilizing the native pCRP conformation represents a promising anti-inflammatory therapeutic strategy by attenuation of leukocyte recruitment and ROS formation, the primary pathomechanisms of IRI.

Keywords: C-reactive protein, ischemia/reperfusion injury, reactive oxygen species, therapeutic targets, conformational change, translational medical research, leukocyte recruitment, rat models

INTRODUCTION

Ischemia/reperfusion injury (IRI) is an inflammatory response that occurs when tissue is reperfused following a prolonged period of ischemia (1). The main responsible pathomechanisms of this inflammation are often overshooting leukocyte activation (2, 3), complement activation (4), and generation of reactive oxygen species (ROS) (5–7) that lead to the release of pro-inflammatory cytokines and increased vascular permeability and consequently result in tissue damage. Renal IRI is inevitable in many clinical situations such as renal transplantation, vascular surgery (8), acute ischemic renal injury, and delayed graft function (9). Due to the relatively limited understanding of the pathophysiology, there is to date no specific treatment of this devastating clinical condition. The current research, therefore, addresses the major medical need to identify new therapeutic approaches to IRI.

Recently, we have been able to show that C-reactive protein (CRP), an acute phase reactant that is elevated after tissue injury, undergoes conformational changes from its circulating native pentameric isoform (pCRP) to a bioactive conformation (pCRP*). pCRP* binds complement C1q and activates the classical complement pathway. It then further dissociates into monomeric CRP subunits [monomeric C-reactive protein (mCRP)], which exert further pro-inflammatory actions before they are cleared by phagocytes (10–14). These conformational changes are mediated by bioactive lipids (15) on activated or damaged cells or platelets (10, 16). Conformation-specific antibodies can detect a neo-epitope (that is, residues 199–206 of CRP become accessible), which is present on both pCRP* and mCRP, but not on pCRP (10, 17). This neo-epitope mediates most of the pro-inflammatory CRP effects (12, 18). pCRP* is the major pro-inflammatory isoform *in vivo*, but exists only on biological surfaces and thus cannot be purified for *in vitro* use. Therefore, mCRP is commonly used as surrogate to study pro-inflammatory pCRP* effects *in vitro* as it presents the same bioactive epitopes. mCRP leads to increased monocyte activation, adhesion, and transmigration, as well as formation of ROS (10) and activation of the complement system (12), which represent major pathophysiological factors contributing to tissue injury in IRI. Thus, we hypothesized that the conformational change of pCRP and the consecutive aggravation of inflammation might be a pathophysiological mechanism by which inflammation is regulated and localized in IRI and thus represents a therapeutic target to reduce tissue damage in IRI.

Abbreviations: CRP, C-reactive protein; mCRP, monomeric C-reactive protein; pCRP, pentameric C-reactive protein; pCRP*, neo-epitope expressing CRP; IRI, ischemia/reperfusion injury; 1,6-bisPC, 1,6-bis(phosphocholine)-hexane; PBMC, peripheral blood mononuclear cells; PMN, polymorphonuclear cells; D-PBS, Dulbecco's physiological phosphate-buffered saline; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

MATERIALS AND METHODS

Reagents

Human pCRP was purchased from Calbiochem (Nottingham, UK; purified from human ascites) and was dialyzed against Dulbecco's phosphate buffered saline with Ca^{2+}/Mg^{2+} (D-PBS) (ThermoFisher Scientific) to prevent potential contaminations and tested as described before (11, 12). 1,6-bisPC was synthesized by Syngene International, Bangalore, India. Lipopolysaccharide (LPS) from *E. coli* serotype O127:B8 for intravital microscopy was obtained from Sigma-Aldrich. As described previously, we utilized and prepared mCRP (1 mg/ml) in soluble, citraconylated form (19). Conformation-specific CRP antibodies clone 8D8 and 9C9 were kindly provided by Dr. Larry Potempa (College of Pharmacy, Roosevelt University, Schaumburg, IL, USA) (20).

Animals

Male Wistar rats were purchased from Charles River Research Models and Services (Sulzfeld, Germany). For the renal IRI-model, all rats were 6 weeks old and body weight was between 180 and 220 g. Male Wistar rats for intravital microscopy were selected and handled as previously described (11). Animals were housed in light controlled rooms (12 h light/dark cycle) and allowed access to food and water *ad libitum*. This study was carried out in accordance with the recommendations of the animal ethic committee of the University of Freiburg Medical Center, Germany. The protocol was approved by the animal ethic committee of the University of Freiburg Medical Center, Germany.

Human Studies

For immunohistology of human ischemia/reperfusion-injured tissue, biopsies of 15 patients receiving free muscle flap reconstruction of posttraumatic soft tissue defects of the lower extremity were taken between September 2008 and March 2010. Informed consent was obtained from each patient. The study was approved by the ethic committee of the University of Freiburg Medical Center (Application number: 67/08) and conducted in accordance with the declaration of Helsinki.

Renal Ischemia/Reperfusion-Injury Model

Prior to surgery, 30 Wistar rats were randomly allocated to one of five designated groups; (1) sham-operated controls receiving flank incisions without renal clamping. Animals received i.p. vehicle D-PBS solution treatment; (2) IRI-treated rats were subjected to the surgical procedure described hereafter. IRI rats received i.p. 500 µl D-PBS application; (3) IRI + pCRP-treated rats: the same surgical procedure as in group (2) was performed. Animals received i.p. pCRP application in a 25 µg/ml serum concentration instead of D-PBS; (4) IRI + pCRP + 1,6-bisPC-treated group: as in group (3) rats received i.p. pCRP application in a 25 µg/ml serum concentration. pCRP was incubated with 1,6-bisPC (1:100 molar ratio) before administration; (5) IRI + 1,6-bisPC-treated group: the same surgical procedure as in group (2) was performed. Animals received i.p. 1,6-bisPC application only ($n = 6$ per group).

Experimental Protocol of Renal IRI

The surgical procedure was a modification of the renal IRI-model described by Delbridge et al. (21). A bilateral ischemic AKI modification was considered more relevant to human pathological conditions (22). In brief, Wistar rats were anesthetized with 1.5–2 vol% isoflurane (Abbott, Wiesbaden, Germany) *via* silicone mask and received subcutaneous buprenorphine (0.05 mg/kg body weight) (23) for pain relief. Buprenorphine is a convenient option for analgesics in IRI-models since it is long-acting with a high therapeutic index and metabolized in the liver (24). Adequate depth of anesthesia to commence following surgery was achieved by loss of reflexes to toe pinch test and distinct slowing of respiratory rate. An eye lubricating ointment (Bepanthen, Bayer Vital GmbH, Leverkusen, Germany) was used to avoid postoperative blinding of the rat. Animals were placed in lateral recumbency on a heated surgical table to maintain core body temperature at 37°C (anal probe-controlled) to avoid effects of the body temperature on the severity of IRI (21, 22). Both renal pedicles were exposed *via* two paravertebral flank incisions and clamped with nontraumatic micro vessel clips for 45 min followed by 24 h reperfusion. A gradual change in color from light red to dark purple served as a surrogate parameter for a successfully induced ischemia of the kidney. The kidneys were embedded in saline solution soaked gazes during the period of exposure. Simultaneously, weight-adapted volume of group-corresponding solution was administered intraperitoneally. Serum volume was estimated as described before (11) as a function of the body weight (25). A second bolus was injected i.p. after 12 h of reperfusion and constant serum levels of pCRP were verified by immunologic turbidity measurements. Immediately after surgery, subcutaneous saline supplementation was given to avoid dehydration of the rats. All microsurgical procedures were conducted using a stereo microscope (Stemi 2000, Carl Zeiss).

Measurement of Blood Urea Nitrogen (BUN)

Renal function was assessed by BUN concentration (26, 27). Blood samples were obtained *via* lateral tail vein sampling in micro tubes with clotting activator (Micro tube 1.3 ml Z, Clotting Activator/Serum, Sarstedt, Nümbrecht, Germany). Preparation of clotted blood samples was conducted using a precooled tabletop centrifuge (Eppendorf centrifuge 5427R, Eppendorf AG, Hamburg, Germany). To receive designated serum, probes were centrifugated at 2,000 × *g* for 10 min at 5°C. Measurement of BUN concentration was performed using cobas 8000 modular analyzer (cobas 8000 modular analyzer series, Roche, Basel) by the central laboratories of the University Medical Center, Freiburg. To avoid invalidation, samples that showed macroscopic levels of hemolysis were excluded.

Immunostaining and Histomorphological Evaluation

Immunohistochemistry and histomorphological evaluation of the renal tissue was performed on formalin-fixed paraffin-embedded renal tissue sections (5 µm thick serial sections). Previously, both kidneys were flushed till bloodlessness with D-PBS followed by 4% formalin for fixation. Kidneys were then excised and examined in blinded fashion by two researchers using a Zeiss microscope (Carl Zeiss Microscopy Axio Imager. M2, Germany). Staining was performed as described previously (17, 28, 29) with minor modifications. Paraffin-embedded sections were de-paraffinized in xylol, rehydrated, and boiled for 20 min in concentrated citric acid (pH 6.0). Antigen unmasking for anti-monocyte detection was done by application of pepsin solution (Digest-All™ 3, life technologies) at room temperature for 20 min (30). Histomorphological changes were evaluated on Periodic acid–Schiff stained sections by quantitative measurement of tubulointerstitial injury, which was assessed by loss of tubular brush border and cast formation following an established protocol (31, 32). In brief, the morphological assessment was scaled in five steps: with not present (0), mild (1), moderate (2), severe (3) to very severe (4). Transmigrated leukocytes were detected by anti-monocyte/macrophage antibody clone ED-1 (Millipore, Billerica, MA, USA) in a 1:100 dilution and renal inflammation was evaluated by counting ED-1$^+$ cells in 20 randomized areas of interest of the renal cortex at ×200 magnification. The number of apoptotic cells was evaluated using anti-caspase-3 antibody (Novus Biologicals, Abingdon, UK) in a 1:1,000 dilution (33). Sections were counterstained with Mayer's hematoxylin. Negative immunocontrols were issued by sections to which primary antibodies had not been added. Each parameter was determined on at least five different animals per group. As a proof of concept, detection of human CRP on the renal tissue sections was performed using anti-pCRP*/mCRP

antibody 9C9 (1:100 dilution). The immunostaining for CRP conformations in cremaster muscle was conducted following the surgical procedure described below. The cremaster muscle was excised, snap-frozen, and conserved in tissue freezing medium (Leica Microsystems, Nussloch, Germany). Tissue samples were cut serially in horizontal direction into 6 µm sections. For conformation specific detection of pCRP, we utilized antibody clone 8D8 and antibody clone 9C9 was used for the detection of conformationally altered CRP (pCRP*/mCRP) (1:100 dilution) (34). Immunostaining proceeded as described in earlier work (10).

Intravital Microscopy Studies of Rat Cremaster Muscle

As previously described (11, 12) and published in a detailed protocol (35), leukocyte–endothelial interaction was observed in the microcirculation of the cremaster muscle in male Wistar rats (weighing 120–180 g) using intravital microscopy. Briefly, the rats were anesthetized with 1.5–2 vol% isoflurane and volume controlled ventilated *via* tracheotomy (Servo Ventilator 900C, Maquet, Rastatt, Germany; settings: frequency 35–45 breaths/min, tidal volume 4.5–5 ml, FiO_2 0.35–0.5). Vital parameters (heart rate, mean arterial pressure, blood gases) were monitored through a cannulated carotid artery. Intravenous injection of rhodamine 6G (0.4 mg/kg body weight, Sigma-Aldrich) (36) *via* an established jugular vein port stained the circulating leukocytes and enabled for intravital tracking. After externalization of the cremaster muscle and visualization of the cremasteric microcirculation, leukocyte rolling and adherence was assessed. Leukocyte rolling was defined as significantly slower moving leukocytes compared to erythrocytes within the same vessel. Adherent leukocytes remained stationary for 20 s or more.

Western Blot Analysis

Native Western blot analysis was conducted for CRP detection in the cremaster muscle as described previously (11). Briefly, muscle tissue was excised and homogenized on ice using a high-power disperser (Ultra-Turrax® IKA, Staufen, Germany). The purification of the cell lysates was determined by a BCA protein assay kit (Sigma-Aldrich) and portioned. After the separation by SDS gel electrophoresis and the transfer to Hybond ECL nitrocellulose membranes (GE Healthcare, Munich, Germany), samples were probed with anti-pCRP*/mCRP antibody for 1 h at RT. To ensure equilibration, we used monoclonal antibodies against GAPDH (abcam, Cambridge, UK). An anti-mouse horseradish peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany) was utilized for detection using enhanced chemiluminescence (ECL, GE Healthcare) and conserved on Hyperfilm ECL (GE Healthcare). Cell lysis and Western blotting buffer were used as described earlier (11).

Electron Spin Resonance (ESR) Spectroscopy for the Study of Leukocyte-Derived ROS

For *ex vivo* experimental procedures, we utilized ESR spectroscopy to identify and quantify ROS formation in rat leukocytes. CMH (1 mM, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine, Noxygen, Elzach, Germany) was adopted as a spin label suitable for biological utilization as described before (37). After the intravital experimental procedure, whole blood samples were collected in EDTA tubes by cannulation of the abdominal aorta and mononuclear cells [peripheral blood mononuclear cells (PBMC) and polymorphonuclear cells (PMN)] were isolated by Ficoll density gradient (Bicoll Separating Solution, Biochrom, Berlin, Germany; density 1.077 kg/m^3) centrifugation as described before (11). MiniScope MS 200 ESR Spectrometer (Magnettech, Berlin, Germany) was used for measurements with following instrument settings: center field, 3,340G; sweep wide, 60G; sweep time, 5 ms over 10 scans; modulation amplitude, 2.4G; microwave power, 10 mW. Positive controls were conducted by cremasteric superfusion with LPS in concentration 1 µg/ml. For the pCRP/mCRP groups, 25 µg/ml pCRP or mCRP was administrated intravenously. Blood samples were taken 60 min after the treatment.

For *in vitro* experiments, whole blood samples were collected from rats and healthy human donors and PBMC and PMN were isolated as described above. The cell suspensions were then incubated at 37°C for 30 min with PBS only (control), LPS (50 µg/ml), pCRP (10 µg/ml), and mCRP (10 µg/ml), respectively.

Statistical Analysis

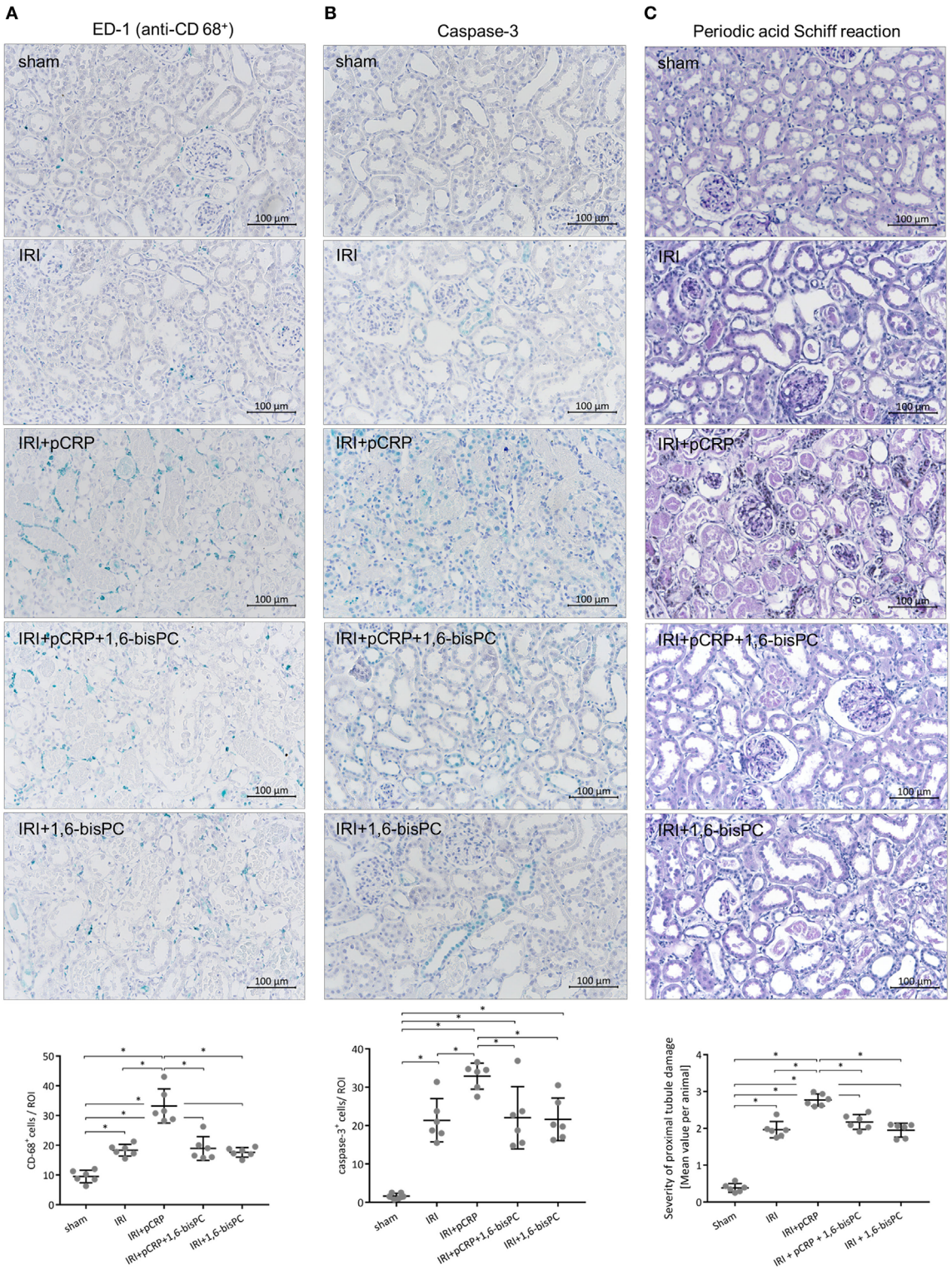
Statistical analysis was performed using GraphPad Prism v7.0 software (GraphPad Software, San Diego, CA, USA). For comparison of two groups, a two-tailed *t*-test was employed. Experimental data were compared using one-way ANOVA to compare effects of different treatments, if more than two groups were compared. In case of significance, Turkey's test was performed for pairwise comparison. To analyze treatment effects over time, we performed a two-way repeated measures (mixed model) ANOVA with the fixed factors "time," "treatment," and the corresponding interaction term. In case of a non-significant interaction and significant treatment effect, pairwise Bonferroni adjusted comparisons were performed at each time point. Significant results for both two-way repeated measures (mixed model) ANOVA and Bonferroni *post hoc* tests are presented. A p-value <0.05 statistical significance level was accepted. All data are expressed as scatter plot with mean ± SEM.

RESULTS

CRP Increases Tissue Damage in Renal IRI

Rat renal IRI was quantified by determination of BUN, immunohistological staining for caspase-3 and monocytes as well as evaluation of morphological changes after reperfusion. All experimental readouts consistently demonstrate that intraperitoneal (i.p.) injection of pCRP potentiates renal IRI leading to elevated BUN, increased caspase-3 expression, and aggravated IRI-specific alterations in tissue morphology. A significant

increase in the number of infiltrated monocytes could be detected (**Figures 1A–E**). Immunohistological staining with anti-pCRP*/mCRP-antibody 9C9 demonstrates that the injected pCRP is not deposited in healthy renal tissue, however, accumulates in IRI-damaged tubules in its conformationally altered isoforms (**Figure 1D**).



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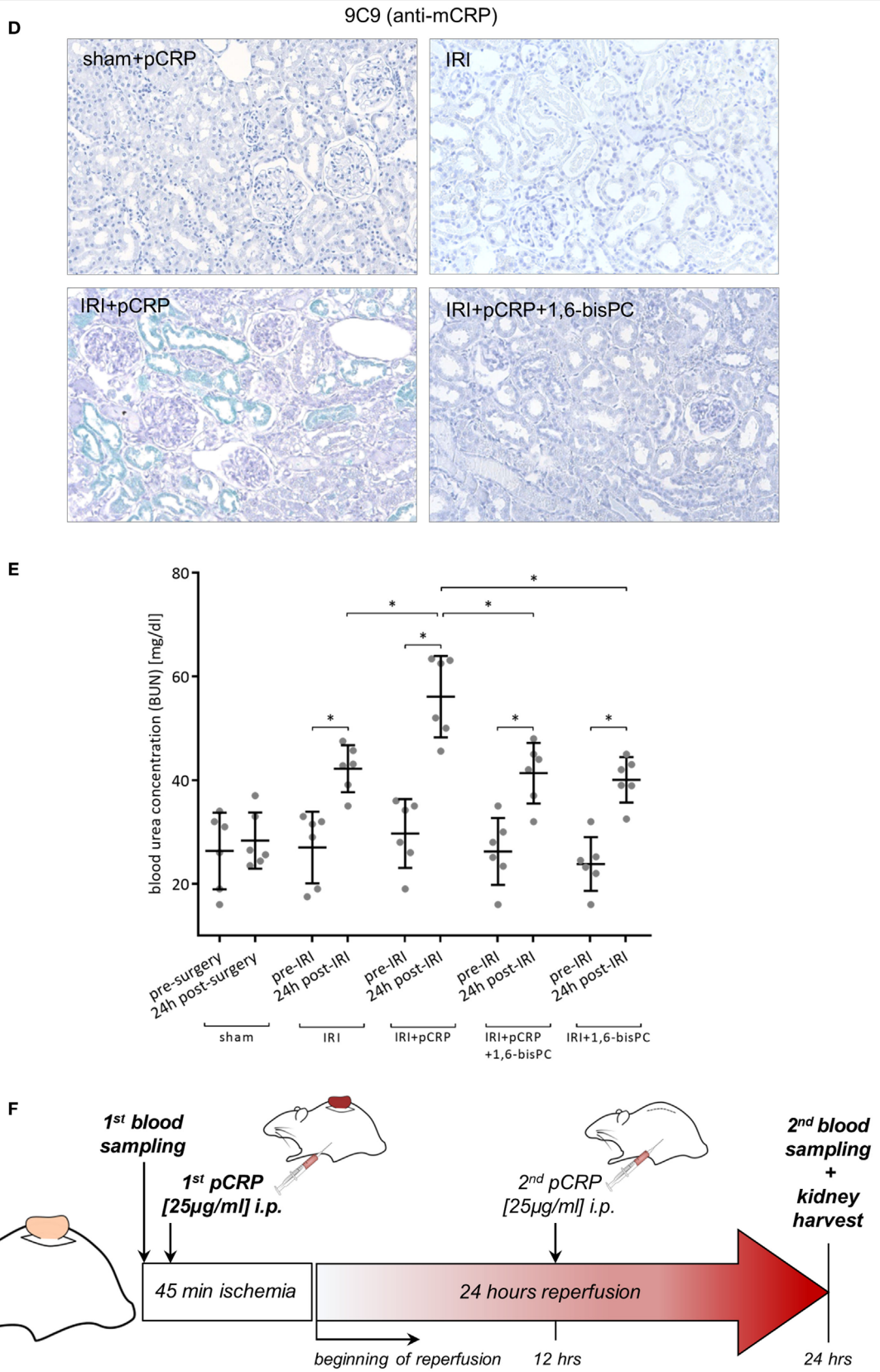

FIGURE 1 | Continued

FIGURE 1 | C-reactive protein (CRP) aggravates renal ischemia/reperfusion injury (IRI) in rats. **(A)** Immunohistochemical detection of $CD68^+$ cells in the renal cortex and quantification of the results. Antibody binding was detected with the HistoGreen substrate kit. $CD68^+$ cells were quantified in randomly chosen areas of the renal cortex at ×200 magnification. Given are mean cell counts/area per animal. $^*p < 0.05$ for IRI + pentameric C-reactive protein (pCRP) vs. IRI, IRI + pCRP + 1,6-bisPC, and IRI + 1,6-bisPC. Values are mean ± SEM; $n = 6$. The number of $CD68^+$ cells transmigrating in the I/R-injured tissue is significantly increased under pCRP application; stabilization of pCRP with 1,6-bisPC abrogates this effect. **(B)** Localization and quantification of caspase-3 protein in the tubular epithelium. Anti-caspase-3 antibody and HistoGreen staining of apoptotic cells in the renal tubules. Given are counts of positive cells. Randomly selected areas of the renal cortex were evaluated from each sample. $^*p < 0.05$ for IRI + pCRP vs. IRI/IRI + pCRP + 1,6-bisPC/IRI + 1,6-bisPC. Values are mean ± SEM; $n = 6$. CRP significantly increases the number of apoptotic cells in IRI. This is inhibited by addition of 1,6-bisPC. **(C)** Histomorphological evaluation of renal tissue after IRI using Periodic acid–Schiff (PAS) staining. A minimum of ten fields per slide was examined at ×200 magnification. Shown are mean values per animal for the severity of proximal tubule damage. $^*p < 0.05$ for IRI + pentameric C-reactive protein (pCRP) vs. IRI/IRI + pCRP + 1,6-bisPC/IRI + 1,6-bisPC. Not significant for IRI vs. IRI + pCRP + 1,6-bisPC and IRI vs. IRI + 1,6-bisPC. **(D)** Immunohistochemical detection of CRP neo-epitopes with anti-pCRP*/monomeric C-reactive protein (mCRP) antibody 9C9 and HistoGreen after IRI and i.p. application of pCRP ± 1,6-bisPC compared with IRI without pCRP application and sham-operated kidneys with pCRP application. After i.p. application of pCRP, conformationally altered CRP is deposited in renal tubules. This is not seen when pCRP is pre-stabilized with 1,6-bisPC. **(E)** Analysis of blood urea nitrogen. Blood samples were taken before ischemia/surgery and after the 24 h reperfusion period or 24 h after surgery, respectively. Renal IRI induces a significant increase in BUN, a functional hallmark of renal function in acute kidney injury. CRP-dissociation aggravates the functional tissue damage, 1,6-bisPC prevents pro-inflammatory alterations of CRP and partially preserves kidney function. $^*p < 0.05$ for IRI + pCRP vs. IRI/IRI + pCRP + 1,6-bisPC/IRI + 1,6-bisPC; $^*p < 0.05$ for all groups pre-ischemia vs. postischemia. **(F)** Flow chart representing the experimental protocol of the renal IRI model.

CRP Aggravates Leukocyte Recruitment in IRI, but Does Not Show Intrinsic Pro-Inflammatory Potential in Sham-Operated Animals

In a model of IRI, leukocyte–endothelial interaction in the microcirculation of the rat cremaster muscle was observed *via* intravital microscopy.

Moderate inflammatory response was induced by ischemic occlusion of the cremaster muscle for 30 min, resulting in an increase in leukocyte rolling that reaches statistical significance after 60 min and in leukocyte adhesion by 120 min. pCRP infusion has no impact on leukocyte recruitment in the resting muscle tissue. However, tissue alteration through IRI results in a marked aggravation of the IRI-induced inflammatory response through application of pCRP. Thus, the number of rolling leukocytes in IRI significantly increases after 60 min and more than doubles to the end of the imaging period (**Figure 2A**). Likewise, leukocyte adhesion increases in IRI in the presence of pCRP; however, effects are timely delayed compared to rolling leukocytes. Values do not reach statistical significance compared to the control until 120 min after infusion of pCRP (**Figures 2B**,**G**).

In IRI of Striated Muscle Tissue, Conformationally Altered CRP Is Deposited in the Interstitial Space

Rat cremaster muscle samples were examined for deposition of CRP by immunohistochemistry after intravital imaging using conformation specific antibodies. Staining reveals that the infused native pCRP can only weakly be detected in the interstitial space of healthy tissue by anti-pCRP 8D8 antibodies (**Figure 2C**). This is in contrast to the IRI-mediated inflamed muscle tissue, where total amounts of CRP deposition increases (**Figure 2D**). Here, CRP is in large parts detectable by anti-pCRP*/mCRP 9C9 antibodies and thus deposited in its conformationally altered isoforms.

Blocking the CRP Conformational Change Abrogates Pro-Inflammatory Effects

The small molecule inhibitor 1,6-bisPC is able to stabilize pCRP in a decameric confirmation, thereby inhibiting the pro-inflammatory conformational change (pCRP*/mCRP) (11). Here, we show that the decameric stabilization prevents deposition of CRP in IRI-altered tubules of the kidney (**Figure 1D**). Conformation-specific staining for CRP as well as native Western blotting of the cremasteric tissue (**Figures 2C–F**) reveals that 1,6-bisPC prevents the dissociation of pCRP in IRI, thereby impending the deposition of pCRP*/mCRP in the interstitial space. This in turn masks the pro-inflammatory contribution of CRP to the inflammatory reaction in renal IRI as well as in IRI of striated muscle tissue. Significant decreases in caspase-3 expression, monocyte infiltration, and tubular damage clearly demonstrate the protective effect of 1,6-bisPC in the CRP-induced tissue damage in renal IRI (**Figures 1A–C**). This is also reflected in the significant decrease in BUN under 1,6-bisPC compared to the elevated concentrations in IRI under CRP alone (**Figure 1E**). 1,6-bisPC does not only soften the CRP driven inflammatory response in IRI detected after a 24 h reperfusion period but also modulates the immediate inflammatory reaction after ischemia. This is reflected in a mitigated leukocyte–endothelial interaction in the microcirculation of the IRI-challenged cremasteric tissue after pretreatment of pCRP with 1,6-bisPC, showing significant decreases in leukocyte rolling and adhesion after 120 min. 1,6-bisPC alone, however, shows neither pro- nor anti-inflammatory potential (**Figures 2A,B**).

Exposition of Pro-Inflammatory Neo-Epitopes in Inflamed Tissue Can Be Mimicked by Pre-Dissociated CRP

In order to analyze the pro-inflammatory potential of CRP when neo-epitopes are exposed, we used pre-dissociated mCRP in rat intravital microscopy. Superfusion of the cremaster muscle with (LPS) (1 μg/ml) served as positive control. mCRP but not pCRP infusion leads to a rapid increase of leukocyte rolling in the cremasteric microcirculation with significant differences after 20 min compared to the control and to the pCRP group (**Figures 3A,C**). This is in line with the increase in the number of adherent leukocytes under mCRP, though values do not reach statistical significance before 50 min (**Figures 3B,D**). Subsequent conformation-specific

immunohistochemical staining of the cremaster muscle reveals that the infused mCRP is extensively deposited in and around cremasteric vessels. pCRP can only be detected in trace amounts in the cremasteric tissue after pCRP infusion (**Figures 3E,F**).

mCRP Induces Respiratory Burst in Rat and Human Leukocytes Which Is Mediated by Lipid Rafts in PBMC

Generation of ROS was assessed *ex vivo* in rat PBMC (**Figure 4A**) and PMN (**Figure 4B**) *via* ESR spectroscopy.

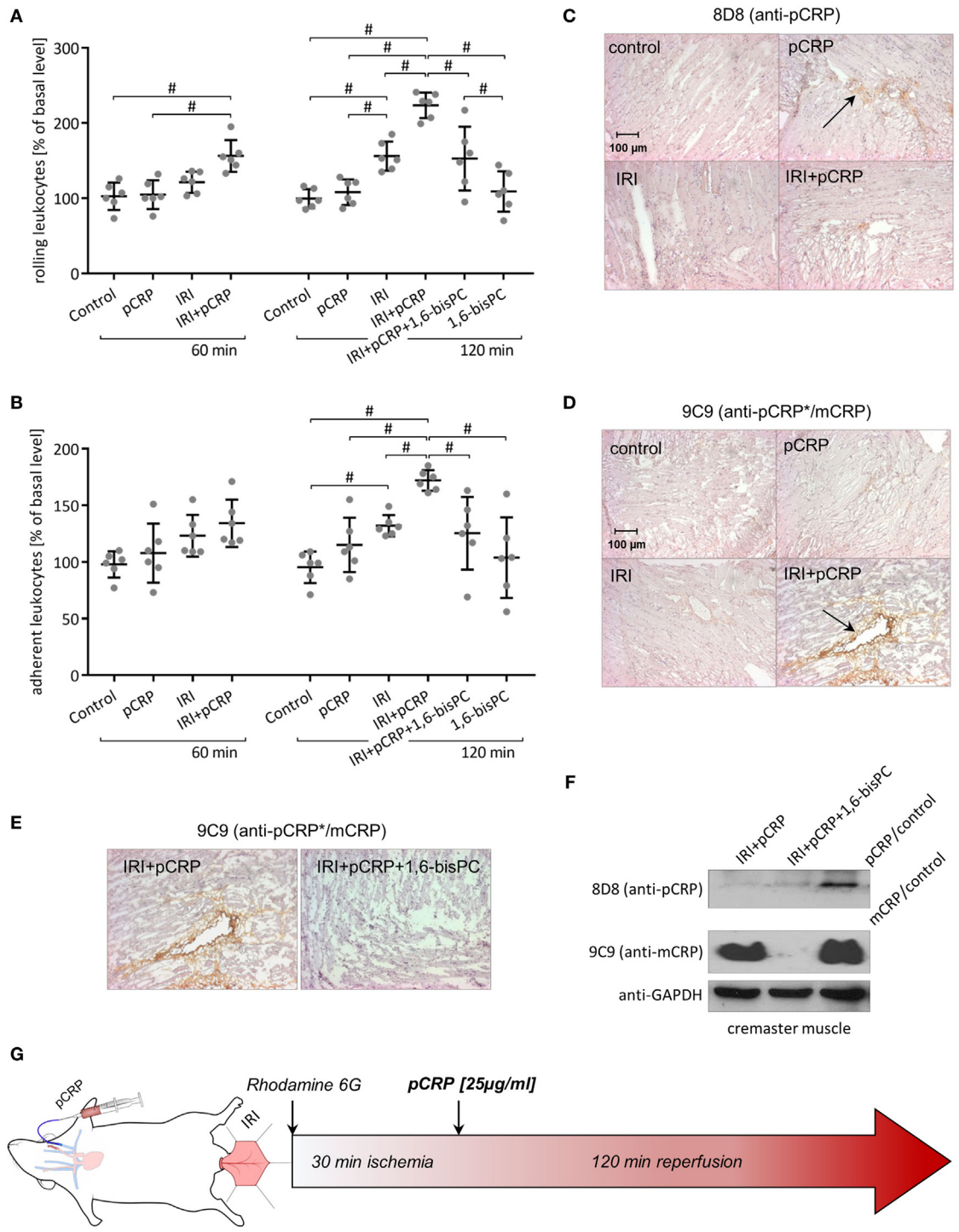

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FIGURE 2 | C-reactive protein (CRP)-induced aggravation of ischemia/reperfusion injury (IRI) is controlled by a localized conformational change regulating leukocyte recruitment. **(A,B)** Intravital microscopy of rat cremasteric postcapillary venules to determine leukocyte–endothelial interaction in IRI ± i.v. injection of pentameric C-reactive protein (pCRP) (25 µg/ml). Leukocytes were labeled with Rhodamine 6G. Counts at 0 min was set to 100%. Values are mean ± SEM of 10–14 observed venules in 6 rats. $^{\#}p < 0.05$. i.v. application of pCRP aggravates IRI and significantly increases leukocyte rolling **(A)** and adhesion **(B)**. 1,6-bisPC masks the pro-inflammatory potential of CRP in IRI and induces a significant decrease in leukocyte rolling **(A)** and adhesion **(B)** compared to the IRI + pCRP group. 1,6-bisPC alone has neither pro- nor anti-inflammatory potential. **(C,D)** Immunohistochemical staining of the cremaster muscle with DAB after IRI ± i.v. application of pCRP. Clone 8D8 **(C)** was used to detect pCRP and clone 9C9 **(D)** was used to detect conformationally altered CRP. Representative results are shown ($n = 6$ for each sample). After i.v. application, pCRP can be detected in the cremasteric tissue (arrow). Staining gets less pronounced in IRI. Neo-epitope expressing CRP is strongly detectable after pCRP injection in IRI (arrow), though trace amounts are detected in healthy tissue. IRI induces an alteration of the CRP conformation and a deposition in the inflamed tissue. **(E)** Immunohistochemical staining of the cremaster muscle with DAB after IRI and i.v. application of pCRP ± 1,6-bisPC. Stabilization of pCRP by 1,6-bisPC abrogates the formation and deposition of conformationally altered CRP. **(F)** Western blot analysis of native PAGE (1/20 SDS) of I/R-injured rat cremaster muscle tissue stained for mCRP with antibody clone 9C9 and 8D8. In the I/R-injured muscle tissue, altered CRP accumulates after the dissociation of pCRP. 1,6-bisPC stabilizes pCRP in its native conformation and, therefore, altered CRP is not detectable in 1,6-bisPC-treated rats. **(G)** Flow chart of the intravital experimental protocol.

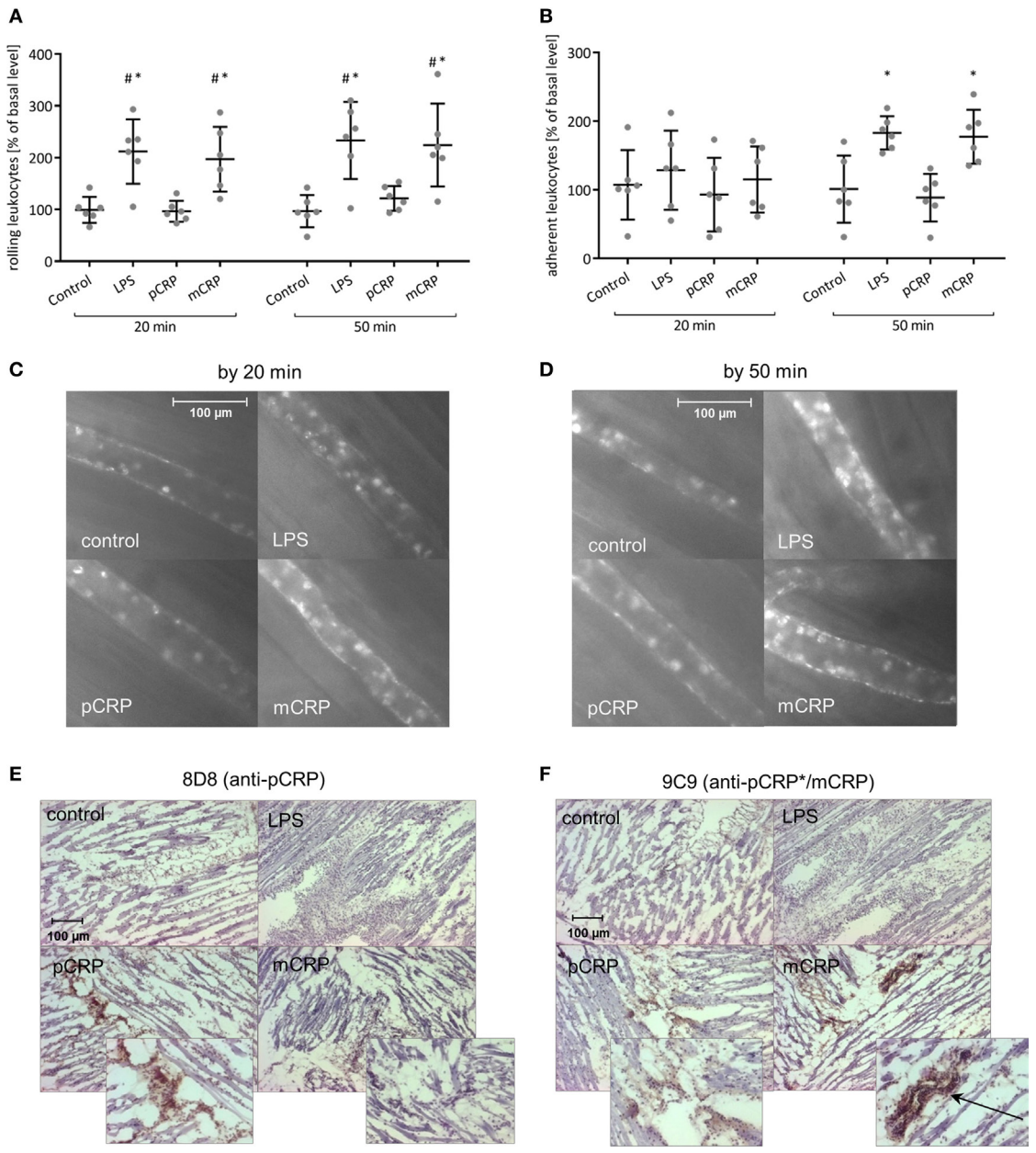

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FIGURE 3 | Monomeric C-reactive protein (mCRP) is the pro-inflammatory tissue-appealing isoform of C-reactive protein (CRP). **(A,B)** Leukocyte–endothelial interaction under i.v. injection of either mCRP or pentameric C-reactive protein (pCRP) (25 μg/ml) quantified by intravital microscopy. Superfusion of the cremaster muscle with lipopolysaccharide (LPS) (1 μg/ml) served as positive control. Counts at 0 min was set to 100%. Values are mean ± SEM of 10–14 observed postcapillary venules in six rats. $^{\#}p < 0.05$ compared to the control; $^{*}p < 0.05$ compared to the pCRP group. mCRP significantly increases leukocyte rolling **(A)** and leads to a rapid increase of leukocyte adhesion **(B)** during the course of the experimental protocol whereas pCRP shows no significant effect. Images shown under **(C,D)** are typical venules of the four groups after 20 and 50 min. **(E,F)** Immunohistochemical staining of the cremaster muscle with DAB after i.v. application of pCRP (25 μg/ml) and mCRP (25 μg/ml). Clone 8D8 was used to detect pCRP **(E)** and clone 9C9 was used to detect neo-epitope expressing CRP (pCRP*/mCRP) **(F)**. Representative results are shown ($n = 6$ for each sample). Trace amounts of pCRP can be detected in the cremasteric tissue in the pCRP group. mCRP application results in a pronounced positive staining for mCRP particularly around cremasteric vessels (arrow).

In both leukocyte subsets, intravenous treatment with mCRP significantly increases ROS formation. In contrast, the infusion of pCRP showed no such effect. This is in line with the *in vitro* stimulation of previously isolated PBMC (**Figure 4C**) and PMN (**Figure 4D**) where mCRP also significantly induces ROS formation. These results can be reproduced for human isolated leukocytes (**Figures 4E,F**). **Figure 4G** shows representative ESR spectra. To investigate the significance of lipid rafts in the CRP-mediated respiratory burst, isolated PBMC and PMN were treated with Nystatin prior to incubation with CRP. ESR spectroscopy reveals that disruption of lipid rafts on PBMC masks the pro-inflammatory potential of mCRP resulting in a significant decrease in ROS formation (**Figure 4I**). On PMN, however, treatment with Nystatin failed to eliminate the pro-inflammatory potential of mCRP (**Figure 4J**) indicating alternative signaling pathways.

CRP Is Deposited in the IRI of Human Striated Muscle Tissue and Co-Localizes With CD68^{+} Leukocytes

Immunofluorescence with conformation specific detection of CRP and CD68^{+} cells in striated human muscle tissue shortly before (pre-ischemia) and after free tissue transfer (post-reperfusion) revealed extensive deposition of neo-epitope expressing CRP in the IRI-challenged tissue (**Figure 5A**). pCRP is detected in the reperfused muscle; however, deposition shows no significant increase when compared to pre-ischemic values. Elevated numbers of CD68^{+} leukocytes co-localize with neo-epitope expressing CRP in the inflamed tissue (**Figure 5B**).

DISCUSSION

Here, we identify and characterize the role of CRP in the pathological cascade of IRI by two distinct *in vivo* models of IRI, renal IRI, and IRI of striated muscle tissue. We further analyze the underlying mechanisms of CRP mediated tissue damage. Based on our findings, inflammatory tissue impairment in IRI can potentially be targeted by the prevention of molecular changes in CRP structure. This is supported by the following findings: (1) CRP aggravates renal IRI in a rat model and aggravates ischemia induced renal damage. (2) CRP undergoes a conformational change in renal IRI leading to exposure of pro-inflammatory epitopes (pCRP*/mCRP). (3) mCRP induces significant leukocyte activation in the microcirculation of the rat cremaster muscle. (4) Accumulated CRP in IRI consists mostly of conformationally altered isoforms. (5) The pro-inflammatory potential of CRP in renal IRI and IRI of striated muscle tissue can be blocked by preventing the conformational change of pCRP. (6) mCRP, but not pCRP, induces ROS generation *in vivo* and *ex vivo*. (7) mCRP-mediated ROS formation in PBMC is mediated by lipid rafts. (8) In human IRI of striated muscle tissue, neo-epitope expressing CRP accumulates and co-localizes with inflammatory cells, suggesting a transferability of our results into the *in vivo* situation in humans.

There is a growing body of evidence suggesting a causal role for CRP in IRI. Padilla and coworkers were able to show that CRP is an activator of complement in a rat model of intestinal IRI (38). Another report revealed that CRP exacerbates renal IRI in transgenic human CRP mice compared to a wild-type control (39). Only recently, the same group showed in their mouse model that myeloid-derived suppressor cells might participate in the CRP-driven inflammation in renal IRI (40). Our work provides the underlying mechanism by which CRP contributes to IRI. We are able to show that pCRP does not exert any pro-inflammatory effects, which is in line with previous findings (41–43). Moreover, we can show that a paramount requirement for the aggravation of inflammation by CRP is the expression of the pro-inflammatory neo-epitope, thus defining the molecular basis for the effects observed in the aforementioned publications. A rat model was used, as even though rats have abundant CRP (300–600 μg/ml in normal healthy pathogen-free rats), it does not activate rat complement. This is in contrast to human CRP that, similar to the *in vivo* situation in humans, activates rat complement and thus makes the rat the ideal animal model for CRP research (44).

We have previously shown that the conformational change of circulating pCRP is a localized process limited to the area of inflammation (11) and is mediated by activated cell membranes carrying bioactive lipids, such as lysophosphatidylcholine (10, 15). In our recent work, we identify an initial structural change in the pentameric protein (pCRP*) after binding to activated monocytes that leads to the expression of pro-inflammatory neo-epitopes resembling those of mCRP (12). The pathophysiological cascade from pCRP binding to activated membranes, consecutive generation of pCRP* and finally mCRP formation has proven its pro-inflammatory potential *in vitro* (12) and in an *in vivo* model of acute inflammation (11). Here, we demonstrate the significance of structural alterations in CRP for the first time in clinically highly relevant renal IRI. Inhibiting the conformational change of pCRP with a compound that stabilizes pCRP in a decameric conformation, first described by Pepys et al. (45) abrogates all CRP effects. We have recently shown that in this decameric conformation, CRP is not able to undergo its conformational change with exposure of pro-inflammatory epitopes (11, 46).

In the IRI models that we investigated herein, 1,6-bisPC blunts the pro-inflammatory effects of CRP that promoted an aggravated inflammatory reaction beforehand. 1,6-bisPC itself, however, shows no intrinsic anti-inflammatory potential. We thereby confirm the conformational change as "*conditio sine qua non*" for the pro-inflammatory properties and provide prove of the feasibility of therapeutically locking pCRP in its native isoform in order to attenuate IRI-induced tissue damage.

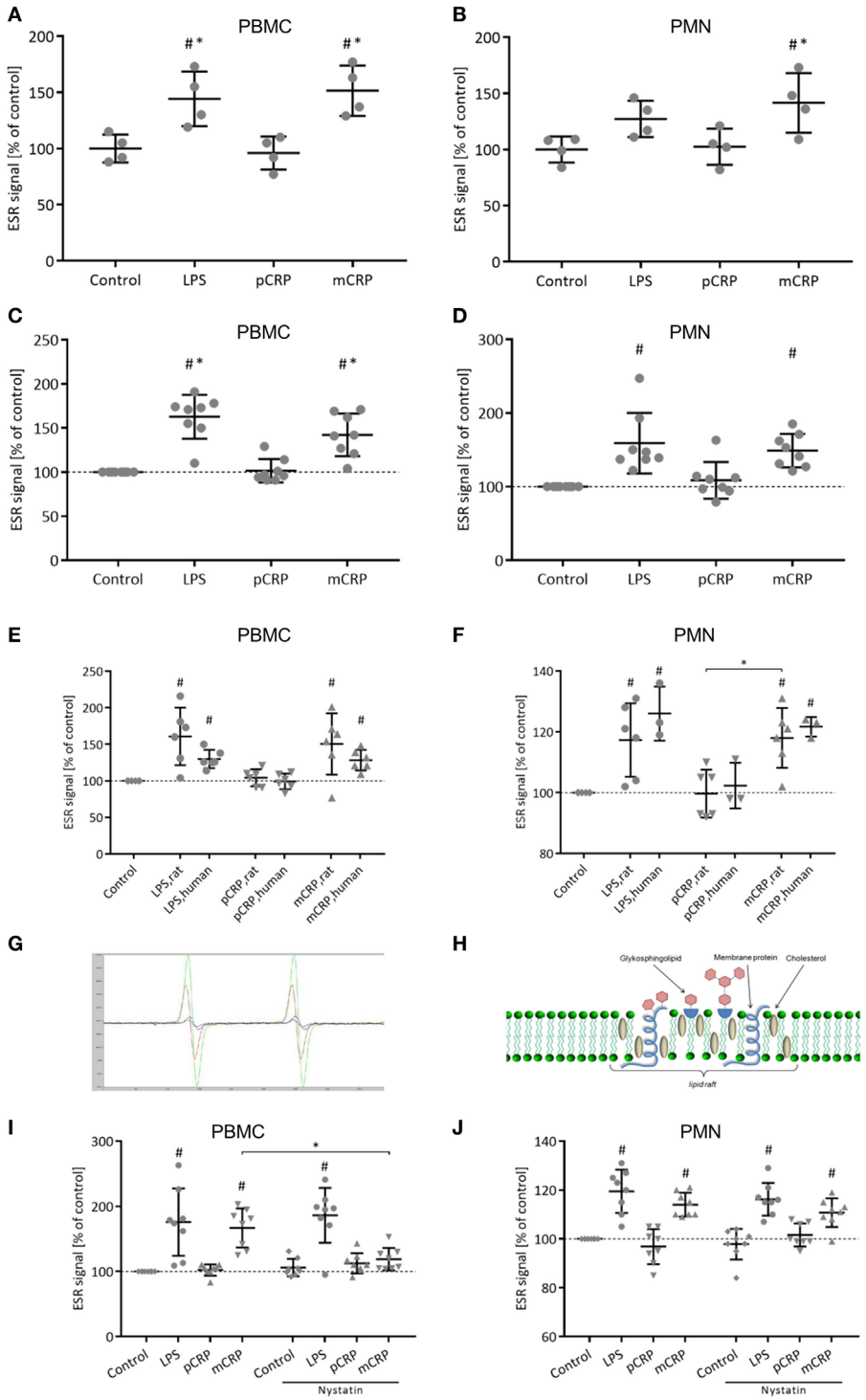

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FIGURE 4 | Monomeric C-reactive protein (mCRP) increases reactive oxygen species (ROS) formation in rat leukocytes. This is reproducible for human leukocytes. Nystatin disrupts the pro-inflammatory signaling of mCRP in rat peripheral blood mononuclear cells (PBMC). **(A,B)** *Ex vivo* electron spin resonance (ESR) detection using CMH (25 µg/ml) as spin label in rat PBMC and polymorphonuclear cells (PMN) after *in vivo* treatment with p-/mCRP (25 µg/ml). Values are mean ± SEM of four different rats. $^{\#}p < 0.05$ compared to the control group. $^{*}p < 0.05$ compared to the pentameric C-reactive protein (pCRP) group. mCRP induces a significant stimulation of ROS in PBMC **(A)** as well as in PMN **(B)**. Cremasteric superfusion with lipopolysaccharide (LPS) (1 µg/ml) served as control. **(C,D)** *In vitro* ESR detection in isolated rat PBMC and PMN after incubation with p-/mCRP (10 µg/ml). LPS (50 µg/ml) served as positive control. Values are mean ± SEM of eight different rats. $^{\#}p < 0.05$ compared to the control group. $^{*}p < 0.05$ compared to the pCRP group. mCRP induces a significant stimulation of ROS in PBMC as well as in PMN. **(E,F)** *In vitro* ESR detection in isolated human and rat PBMC/PMN after incubation with p-/mCRP (10 µg/ml). LPS (50 µg/ml) served as positive control. Values are mean ± SEM of at least three different rats/human donors. $^{\#}p < 0.05$ compared to the control group. $^{*}p < 0.05$ for pCRP vs. mCRP. ROS stimulation by mCRP in rat leukocytes can be reproduced in human leukocytes. **(G)** Representative spectra of electron spin resonance (ESR) spectroscopy. **(H)** Schematic drawing of a lipid raft. **(I,J)** *In vitro* ESR detection using CMH (25 µg/ml) as spin label in isolated rat PBMC **(I)** and PMN **(J)** after incubation with p-/mCRP (10 µg/ml) ± prior treatment with nystatin (25 µg/ml) intending to disrupt lipid rafts. LPS (50 µg/ml) served as positive control. Values are mean ± SEM of eight different rats. $^{\#}p < 0.05$ compared to the control group. $^{*}p < 0.05$ for mCRP vs. mCRP + nystatin. Disruption of lipid rafts by nystatin prior to incubation with mCRP significantly decreases ROS generation in PBMC. Signal drop did not reach statistical significance in PMN.

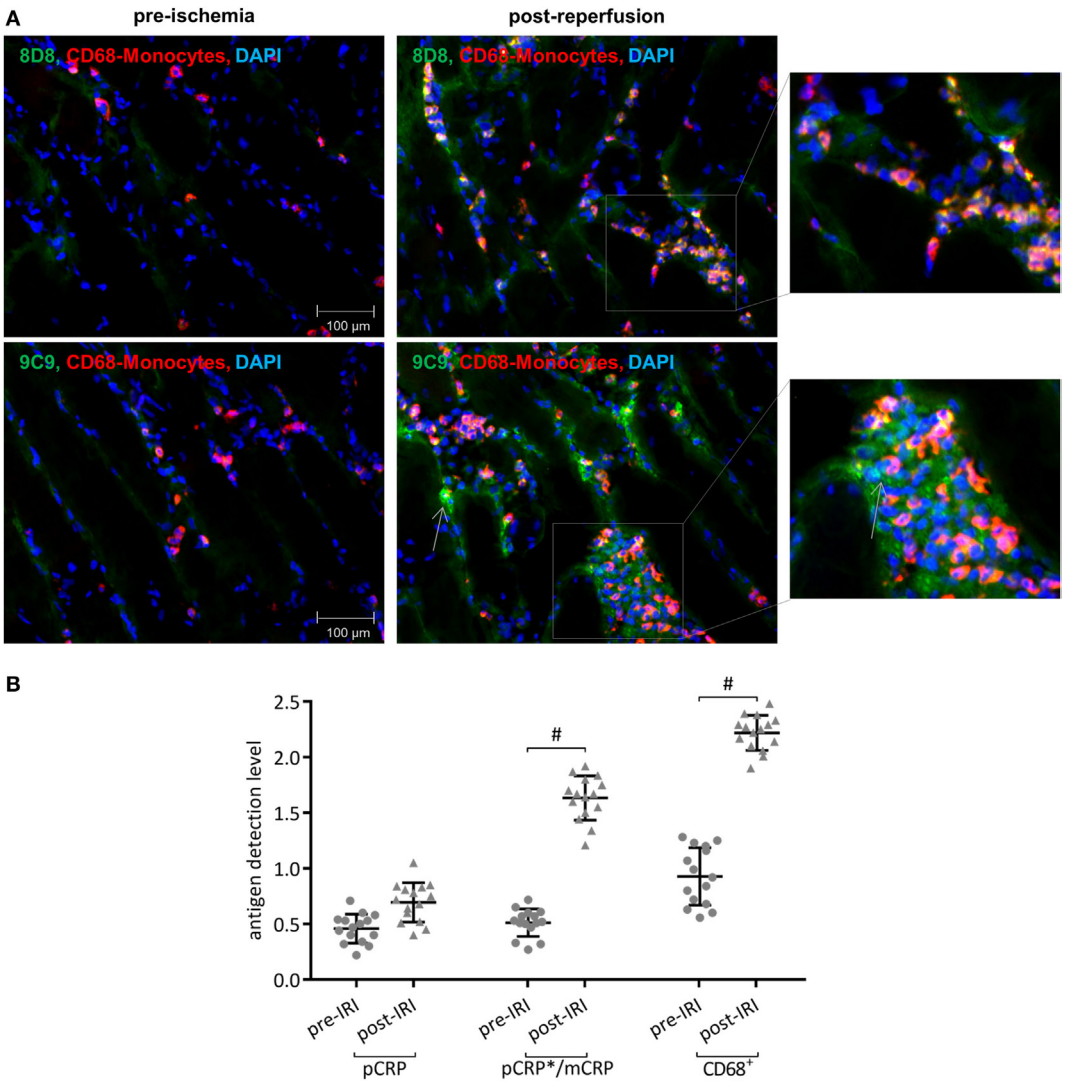

FIGURE 5 | Conformationally altered C-reactive protein (CRP) accumulates in inflamed human muscle tissue. **(A)** Immunofluorescence conformation-specific detection of C-reactive protein and CD68^{+} cells in striated human muscle tissue shortly before (pre-ischemia) and after free tissue transfer (post-reperfusion). Conformation-specific antibody clone 8D8, which recognizes exclusively pentameric C-reactive protein (pCRP) and clone 9C9, recognizing neo-epitopes expressed by pCRP*/monomeric C-reactive protein (mCRP), were used. Typical results are given. **(B)** Quantification shows relative values of immunoreactivity for CD68^{+} cells and pCRP and pCRP*/mCRP, respectively. At least three non-overlapping images were evaluated from each sample to determine the corresponding value. $^{\#}p < 0.05$ for pre-ischemia vs. ischemia/reperfusion injury (IRI). Values are mean ± SEM; $n = 15$. IRI leads to a significant increase of p- and especially conformationally altered CRP deposition, which co-localizes with CD68^{+} leukocytes.

The mCRP-induced ROS generation *in vitro* is a significant finding of our work, which is also confirmed *ex vivo*. The formation of oxygen radicals is of causal relevance in various diseases, such as atherosclerosis (47), myocardial infarction (48, 49), and other inflammatory diseases (50). We investigated the generation of ROS *ex vivo* in leukocytes by ESR spectroscopy following *in vivo* exposition to p-/mCRP, thereby indicating leukocyte activation and oxidative stress. This was further supported by the *in vitro* analysis of CRP-induced radical formation in leukocytes. Our findings demonstrate that mCRP induces oxidative stress in different leukocyte subsets, which potentially aggravates tissue damage in the course of IRI associated inflammation.

Recent literature proposes mCRP–lipid raft interaction as an important mechanism in mediating cellular responses to mCRP in human cells (**Figure 4H**) (10, 51). Lipid rafts represent dynamic, detergent-resistant plasma membrane microdomains that are highly enriched in cholesterol and sphingolipids and play critical roles in cellular signaling (52, 53). For the interaction of mCRP with lipid rafts, a direct membrane integration of mCRP has been proposed (51). At the same time, the FcγR-I can be found in lipid raft microdomains (54) and has previously been identified as a potent pro-inflammatory mediator of mCRP on human monocytes (11) and potent inductor for ROS formation (55) *via* induction of NADPH oxidases (56). Disruption of lipid rafts with nystatin abrogates the potential binding sites for mCRP in lipid rafts microdomains. This inhibits the mCRP-induced respiratory burst in PBMC, however, fails to abrogate the pro-inflammatory CRP effects in PMN. This might be explained through distinct receptor patches on different leukocyte subsets such as a higher significance of FcγR-IIIa in PMN that has shown to mediate mCRP signaling (11). The inhibition of mCRP signal transduction by nystatin in PBMC, which does not inhibit LPS-induced generation of ROS, furthermore confirms the specificity of the mCRP effects, as it rules out potential influences of contaminating bacterial products as described by other authors (57).

In conclusion, we demonstrate that CRP aggravates IRI *via* various pro-inflammatory mechanisms. Formation of neo-epitope expressing CRP leads to significant renal damage and induces leukocyte–endothelial interaction and generation of

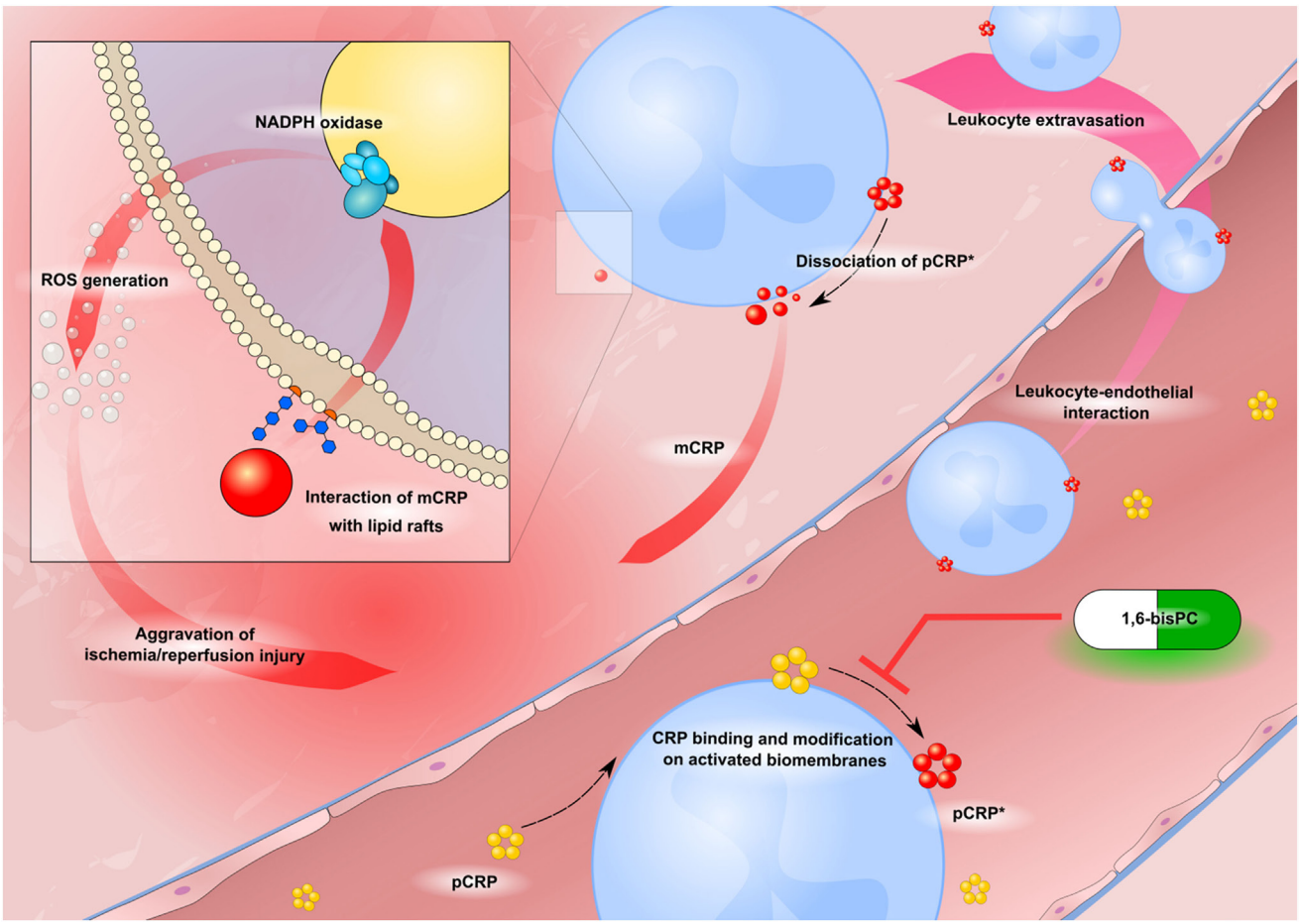

FIGURE 6 | Schematic drawing of CRP-driven leukocyte response in ischemia/reperfusion injury (IRI). Circulating pentameric C-reactive protein (pCRP) (yellow) binds to activated biomembranes in the microcirculation of inflamed tissue. It is subsequently conformationally altered to bioactive pCRP*, dissociates and forms mCRP (red circles). Neo-epitope expressing CRP induces leukocyte–endothelial interaction and activation of the ROS producing NADPH oxidase enzyme complex. 1,6-bisPC (green–white pill) prevents the CRP-mediated leukocyte activation by stabilizing the native pentameric isoform of CRP.

ROS. These effects are in part mediated by lipid raft signaling and can be therapeutically targeted by blocking pCRP dissociation with 1,6-bisPC (**Figure 6**).

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the animal ethic committee of the University of Freiburg Medical Center, Germany. The protocol was approved by the animal ethic committee of the University of Freiburg Medical Center, Germany. For immunohistology of human ischemia/reperfusion-injured tissue, biopsies of 15 patients receiving free muscle flap reconstruction of posttraumatic soft tissue defects of the lower extremity were taken between September 2008 and March 2010. Informed consent was obtained from each patient. The study was approved by the ethic committee of the University of Freiburg Medical Centre (Application number: 67/08) and conducted in accordance with the declaration of Helsinki.

AUTHOR CONTRIBUTIONS

JT: conducted main part of experiments and authored the manuscript with JZ. JZ: conducted main part of experiments and authored the manuscript with JT. JK, DB, and YL, HB, and KP: contributed to the experiments and the authoring of the manuscript. SK: provided support in lab work and contributed to the experiments. LP: provided conformation-specific CRP antibodies. FG: supported in establishment of renal IRI model and contributed to the authoring of the manuscript. TH: supported in establishment of renal IRI model and contributed to the authoring of the manuscript. MH-L: contributed to the interpretation of data, authoring and final approval of the manuscript. GS: contributed to the authoring of the manuscript. SE: planned the experimental procedures, contributed in main parts to the experiments, and the authoring of the manuscript.

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An ELISA Assay for Quantifying Monomeric C-Reactive Protein in Plasma

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Native C-reactive protein (nCRP) is a non-specific marker of inflammation being claimed as a bystander in several chronic disorders. Accumulating evidence indicates that nCRP dissociates to and acts primarily as the monomeric conformation (mCRP) at inflammatory loci. This suggests that mCRP may be a superior disease marker with improved specificity and clear causality to the underlying pathogenesis. However, the lack of a feasible assay to quantify mCRP in clinical samples precludes the assessment of that suggestion. Here we report the development of a sandwich ELISA assay for quantification of plasma mCRP using commercially available reagents. Our assay is reproducible and highly conformation-specific showing a reliable detection limit of 1 ng/mL. We further show that mCRP appears to be a better marker than nCRP in several skin-related autoimmune disorders. This assay thus provides a useful tool to examine the clinical significance and utility of mCRP.

Keywords: inflammation, autoimmune diseases, urticaria, psoriasis, C-reactive protein, monomeric C-reactive protein

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INTRODUCTION

Native C-reactive protein (nCRP) is a major human acute phase reactant that responds to tissue damage or infection by rapidly increasing its blood concentrations (1, 2). It thus has been widely used as a non-specific marker of inflammation in clinical practice. Moreover, a minor increase in the circulating level of nCRP, originally considered as non-pathological, is found to be associated with the risk and prognosis of several chronic inflammatory disorders, including cancer (3) and cardiovascular disease (4). However, those associations are usually moderate and are shown to be non-causal by large-scale genetic studies (5, 6). Therefore, it appears plausible that nCRP may simply be a bystander in these diseases (3, 4).

Native C-reactive protein is composed of five identical subunits that are induced to dissociate at inflammatory loci (7–11) upon encountering damaged membranes (12–21), amyloid aggregates (22), neutrophil extracellular traps (23), or acidic pH (24). The dissociated conformation is termed as monomeric CRP (mCRP) and could be the major actor in local inflammation (7–11). This argues that mCRP may be a marker more specific to the underlying pathological processes. Indeed, circulating or microparticle-bound mCRP has been shown to be a better diagnostic index than nCRP in myocardial infarction (20, 25) and peripheral artery disease (26). However, the homemade assays used in these studies prohibit broad evaluation of the above argument. To clear that hurdle, here we

develop a highly specific ELISA assay to measure plasma levels of mCRP based on commercially available reagents.

MATERIALS AND METHODS

Reagents

Native C-reactive protein (purity > 97%) purified from human ascites was purchased from the BindingSite (Birmingham, UK; catalog number: BP300.X; lot number: 361639 and 404353) and repurified with p-Aminophenyl Phosphoryl Choline Agarose (Thermo Fisher Scientific, Rockford, IL, USA; catalog number: 20307). mCRP was prepared by treatment of nCRP with 8-M urea-EDTA (27) or by recombinant expression and purification (28, 29). Our assay worked well for both forms of mCRP. Proteins were dialyzed to remove NaN_3, and passed through Detoxi-Gel Columns (Thermo Fisher Scientific, Rockford, IL, USA; catalog number: 20344) to remove endotoxin when necessary. Mouse antihuman CRP Abs 1D6 and 3H12 were generated as described (30, 31).

ELISA Assay Quantifying nCRP

The sheep antihuman CRP polyclonal antibody (BindingSite; catalog number: PC044; lot number: 352325, 076682) was immobilized onto microtiter wells (Corning, NY, USA; catalog number: 42592; lot number: 10917007) at 2.5 μg/mL in coating buffer (10-mM sodium carbonate/bicarbonate, pH 9.6) overnight at 4°C. All the following steps were conducted at 37°C. Wells were washed with TBS (10-mM Tris, 140-mM NaCl, 2-mM Ca, pH 7.4) containing 0.02% NP-40, and then blocked with 1% BSA in TBS (blocking buffer). Samples diluted in blocking buffer were added into wells for 1 h. Captured CRP was detected with 1D6 mAb (1:300 in blocking buffer) that specifically recognizes the native conformation and an HRP-labeled goat anti-mouse IgG (H + L) (1:20,000 in blocking buffer) (Jackson ImmunoResearch, West Grove, PA; catalog number: 115-035-003; lot number: 125229). Wells were developed with TMB (Sigma-Aldrich; catalog number: T2885; lot number: WXBC2414V) and stopped with 1-M H_2SO_4. OD570 and OD450 nm were measured with a microplate reader. The OD value of each sample was calculated as OD450–OD570 nm. 100-μL volume was used at all incubation steps, while 300-μL volume was used for washing after each incubation step.

ELISA Assay Quantifying mCRP

The mouse antihuman CRP mAb CRP-8 (Sigma-Aldrich, St. Louis, MO, USA; catalog number: C1688; lot number: 025M4863V) was immobilized onto microtiter wells (Thermo Fisher Scientific; catalog number: 468667, 442404; lot number: 148860, 148034; Corning, NY, USA; catalog number: 42592; lot number: 10917007) at 1:1,000 in coating buffer (10-mM sodium carbonate/bicarbonate, pH 9.6) overnight at 4°C. The performance of the assay was comparable regardless of the type of microtiter well used. 3H12 (1:200) was also immobilized as the capture antibody to compare its performance with that of CRP-8 as shown in **Figure 1B**. All the following steps were conducted at 37°C. Wells were washed with TBS containing 0.02% NP-40, and then blocked with 1% BSA in TBS (blocking buffer). TBS was made of 10-mM Trizma base (Sigma; catalog number: V900483), 140-mM NaCl (Amresco, Solon, OH, USA; catalog number: X190), and 2-mM $CaCl_2$ (Sinopharm, Shanghai; catalog number: 10005861) in ultrapure water (>18.2 MΩ.cm) with pH adjusted to 7.4. Samples diluted in blocking buffer were added into wells for 1 h. Captured mCRP was detected with a sheep antihuman CRP polyclonal antibody (1:2,000 in blocking buffer) (BindingSite; catalog number: PC044; lot number: 352325, 076682) and an HRP-labeled donkey anti-sheep IgG (H + L) from Abcam (1:10,000 in blocking buffer) (Cambridge, UK; catalog number: ab6900; lot number: GR272029-6) or from Abbkine (1:20,000 in blocking buffer) (Wuhan, China; catalog number: A21060-1; lot number: ATQMA0601, ATQJN0701). Wells were developed with TMB and stopped with 1-M H_2SO_4. OD570 and OD450 nm were measured with a microplate reader. The OD value of each sample was calculated as OD450–OD570 nm. 100-μL

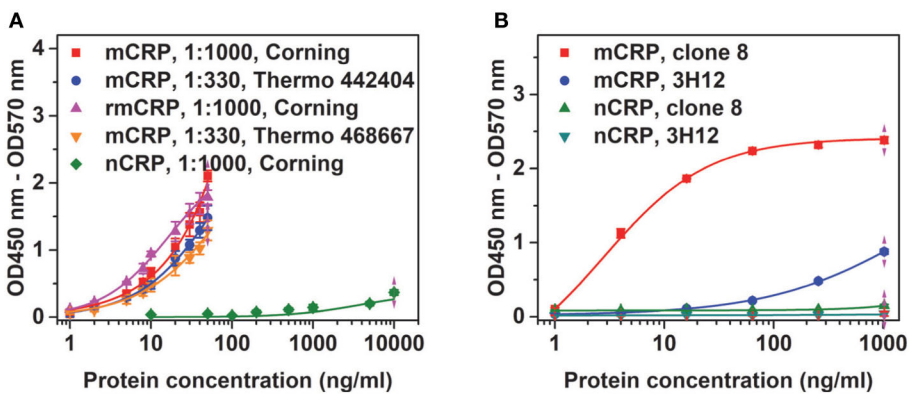


FIGURE 1 | Conformation specificity of the assay. **(A)** Native C-reactive protein (nCRP), monomeric CRP (mCRP), or recombinant mCRP (rmCRP) at the indicated concentrations were added to the indicated wells coated with 1:1,000 or 1:330 CRP-8 mAb, and the captured proteins were detected with a polyclonal CRP antibody from the BindingSite. **(B)** nCRP or mCRP at the indicated concentrations were added to wells (Thermo Fisher; catalog number: 442404) coated with 1:330 CRP-8 (clone 8) or 1:200 3H12 mAbs, and the captured proteins were detected with a polyclonal CRP antibody from the BindingSite.

volume was used at all incubation steps, while 300-μL volume was used for washing after each incubation step. Plasma samples were obtained from the First (urticaria) and Second Affiliated Hospitals (eczema and psoriasis) of Xi'an Jiaotong University. Informed consents for blood sampling were signed by all participants, and the research was in compliance with the Declaration of Helsinki and approved by the local ethical committee.

Statistical Analysis

Data were presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA, Kruskal–Wallis ANOVA or

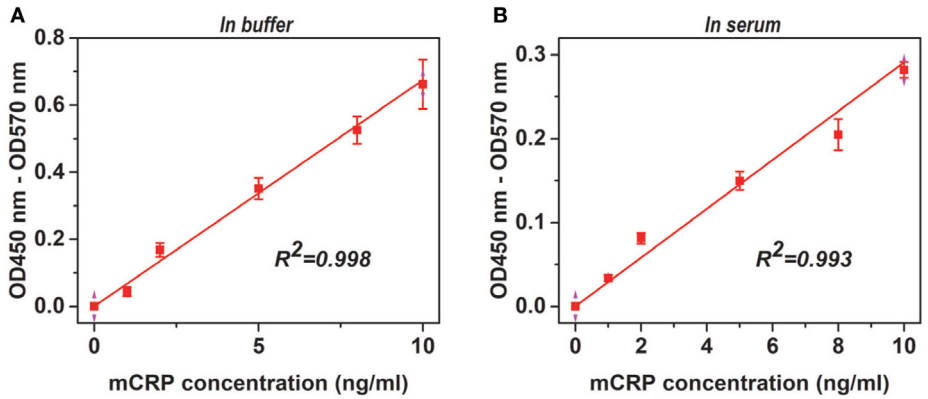
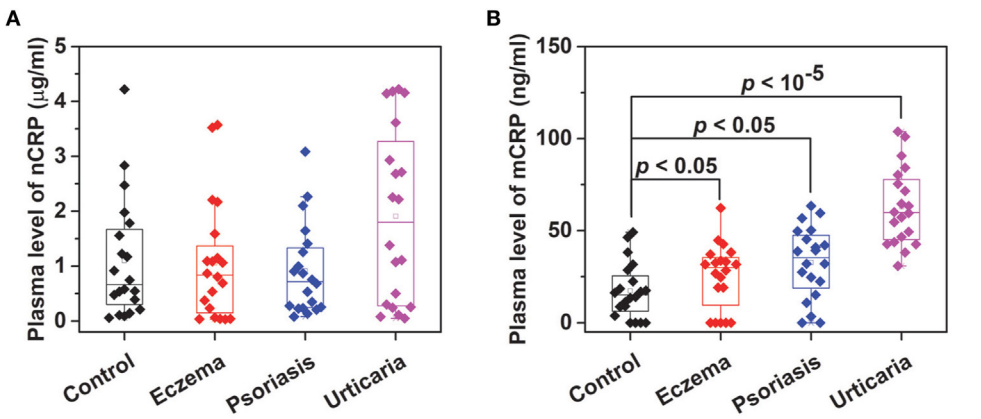


FIGURE 2 | Adaptability of the assay. Monomeric CRP (mCRP) at the indicated concentrations in TBS buffer **(A)** or reference human sera (1:100) **(B)** were added to wells (Corning) coated with 1:1,000 CRP-8 mAb (clone 8), and the captured proteins were detected with a polyclonal CRP antibody from the BindingSite. The endogenous mCRP concentrations in the reference human sera were <10 ng/mL and therefore was undetectable following 1:100 dilution. The Pearson's correlation coefficients (*R*) were also indicated.



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	Control (n=20)	Eczema (n=20)	Psoriasis (n=20)	Urticaria (n=20)	*p* value
Age (year)	32.3±7.8	33.9±9.1	33.2±8.0	32.0±8.8	0.879
Male (%)	50	50	50	50	/
nCRP (μg/ml)	0.66 (0.26, 1.73)	0.84 (0.10, 1.48)	0.72 (0.24, 1.37)	1.80 (0.27, 3.44)	0.235
mCRP (ng/ml)	15.2 (5.05, 27.1)	30.0 (4.77, 36.3)	35.4 (17.0, 48.6)	59.8 (44.5, 79.1)	6.58×10^{-8}

FIGURE 3 | Determination of monomeric CRP (mCRP) levels in clinical samples. The plasma levels of native C-reactive protein (nCRP) **(A)** and mCRP **(B)** were determined in healthy controls ($n = 20$) and patients with eczema ($n = 20$), psoriasis ($n = 20$), and urticaria ($n = 20$) using Corning microtiter wells coated with 1:1,000 CRP-8. **(C)** The summarized results.

Kolmogorov–Smironv tests as appropriate. Differences were considered significant at values of $p < 0.05$.

RESULTS AND DISCUSSION

Sandwich ELISA is a convenient yet reliable assay for laboratory quantification of antigens in clinical samples without demanding equipment requirements. In case of measuring mCRP, the key to the success is to identify an appropriate pair of capture and detection antibodies. Such an antibody pair should be highly specific and sensitive to accurately quantify low level of mCRP [probably in ng/mL (25)] in the background of high level nCRP (usually in μg/mL). Polyclonal antibodies can bind both nCRP and mCRP, and therefore may not represent suitable candidates for being the capture antibody. Indeed, most screened pairs with polyclonal capture antibodies worked well for purified mCRP, but all failed in reconstituted mixtures or clinical samples containing both nCRP and mCRP (not shown).

For screened pairs with monoclonal capture antibodies, those using polyclonal detection antibodies performed better. A monoclonal capture antibody from Sigma (CRP-8) and a polyclonal detection antibody from BindingSite emerged as the best choice. This pair was highly selective, reaching half maximal signals for mCRP at ~20 ng/mL but generating only background signals for nCRP at 1 μg/mL (**Figure 1A**). The performance of CRP-8 as the capture antibody was even superior than that of 3H12, an established mAb of mCRP (30) (**Figure 1B**). This assay could reliably report mCRP as low as 1 ng/mL in both purified (**Figure 2A**) and complex samples (**Figure 2B**). Its robustness was further validated by using different batches of reagents and microtiter wells, by performing in laboratories at different cities by distinct colleagues over months.

We next determined plasma levels of nCRP and mCRP in healthy controls and patients with skin-related autoimmune disorders, including eczema, psoriasis and urticaria (**Figure 3**). The levels of nCRP in patients did not differ significantly from that in controls (**Figure 3A**), whereas the levels of mCRP were significantly higher in patients than in controls (**Figure 3B**). Moreover, though mCRP was increased in all three disorders, the extents of increase differed significantly with the strongest increase observed in patients with active urticaria. These results suggest that mCRP is not only more sensitive to local status of inflammation but may also be specific to the underlying pathogenesis. Therefore, large-scale investigations and thorough analysis are warranted to establish the clinical significance of mCRP in diagnosis and prognosis, and the assay developed herein provides a means for that purpose.

ETHICS STATEMENT

The study was approved by the Ethics Committee of Xi'an Jiaotong University. Informed consents for blood sampling were signed by all of the participants.

AUTHOR CONTRIBUTIONS

YW and S-RJ designed the research. LZ, H-YL, WL, Z-YS, and Y-DW performed the research. YW, S-RJ, LZ, and H-YL analyzed the data and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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C-Reactive Protein (CRP) and Leptin Receptor in Obesity: Binding of Monomeric CRP to Leptin Receptor

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While leptin deficiency or dysfunction leads to morbid obesity, obese subjects are characterized paradoxically by hyperleptinemia indicating lack of response to leptin. C-reactive protein (CRP) has been suggested to be a key plasma protein that could bind to leptin. To examine whether CRP interferes with leptin action, mediated through its cell surface receptor, docking studies of CRP with the extracellular domain of the leptin receptor were done employing bioinformatics tools. Monomeric CRP docked with better *Z*-rank score and more non-bond interactions than pentameric CRP at the CRH2–FNIII domain proximal to the cell membrane, distinct from the leptin-docking site. Interaction of CRP with leptin receptor was validated by solid phase binding assay and co-immunoprecipitation of CRP and soluble leptin receptor (sOb R) from human plasma. Analysis of the serum levels of leptin, CRP, and sOb R by ELISA showed that CRP levels were significantly elevated ($p < 0.0001$) in non-morbid obese subjects ($n = 42$) compared to lean subjects ($n = 32$) and correlated positively with body mass index (BMI) ($r = 0.74$, $p < 0.0001$) and leptin ($r = 0.8$, $p < 0.0001$); levels of sOb R were significantly low in obese subjects ($p < 0.001$) and showed a negative correlation with BMI ($r = -0.26$, $p < 0.05$) and leptin ($r = -0.23$, $p < 0.05$) indicating a minimal role for sOb R in sequestering leptin.

Keywords: obesity, monomeric C-reactive protein, leptin, leptin receptor, protein–protein docking

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INTRODUCTION

Chronic low grade inflammation underlies the development and progression of a number of pathological conditions associated with obesity and insulin resistance in human subjects. An increase in plasma levels of inflammatory markers and acute phase proteins such as C-reactive protein (CRP) is observed in subjects with obesity and associated diseases (1). It has been suggested that these proteins are not merely markers or mediators of the inflammatory process but they also affect the action of adipokines, thus having a direct role in the regulation of adiposity. In this context, the potential role of CRP in modulating the action of leptin in obesity is relevant. Human CRP is a 115 kDa pentameric calcium dependent ligand binding plasma protein comprising of five identical polypeptide subunits. The protomers, each containing 206 amino acid residues, associate non-covalently. It binds to a variety of autologous and extrinsic ligands (2). The pentameric form has been reported to dissociate into a more physiologically active and pro-inflammatory monomeric form (mCRP) which can bind to cell surface receptors (3) and has been implicated in the pathogenesis of inflammatory diseases (4).

Leptin is an important adipocyte-derived lipostatic polypeptide hormone which regulates food intake and energy consumption by modulating anorexigenic pathways in the hypothalamus and

regulating peripheral tissue metabolism. It is a 16 kDa non-glycosylated protein made up of 146 amino acids arranged in four α-helix and two loop bundle structures (5–7). It acts through a membrane receptor belonging to the interleukin six receptor families which activates the intracellular JAK/STAT signaling pathway (6, 8, 9). The extracellular part of the leptin receptor (Ob R) comprises of an N-terminal domain, a cytokine receptor homology domain 1 (CRH1), an immunoglobulin domain, a second CRH domain (CRH2), and two FN type III domains proximal to the plasma membrane (10, 11). Leptin binds with high affinity to the CRH2 domain while the proximal FN type III domains seem to be critical in Ob R activation (8, 12). Of the different isoforms of Ob R, the soluble form present in the plasma is reported to be formed by constitutive shedding of the extracellular domain of the membrane-anchored Ob R (13).

Plasma levels of leptin are altered in several pathological conditions, including cardiovascular diseases (14–17), obesity (7, 18), and reproductive disorders (19). Congenital leptin deficiency leads to uncontrolled appetite and morbid obesity in humans (6). However, a majority of obese subjects are characterized, paradoxically, by elevated levels of leptin in plasma suggesting that leptin fails to exert its effect in spite of being present in abundance (20, 21). This state of hyperleptinemia with a reduced response to circulating hormone is independently associated with insulin resistance and coronary heart disease in human subjects (22). Investigations into the molecular mechanism of leptin resistance, carried out mainly in experimental animals and cell-based systems (20, 23, 24), suggested that binding of plasma leptin to circulating molecules such as soluble leptin receptor (sOb R) and acute phase proteins could be an important mechanism that limits the availability and action of leptin at target tissue.

Several clinical studies have shown a correlation between increased plasma levels of both CRP and leptin in subjects with obesity, CVD, and diabetes (25–30). Although a concomitant increase in CRP and plasma leptin has been observed in obesity, it is not clear whether elevation in CRP is due to acute inflammation or due to adipose tissue expansion or both. While leptin is produced by adipocytes (31), CRP is produced primarily by liver and vascular cells (32, 33). Changes in leptin levels were also found to be independently associated with CRP (after adjustment for age, gender, smoking, alcohol consumption) (34) indicating a relationship between leptin and CRP production. This has been further confirmed by demonstrating a direct effect of leptin on CRP production by hepatocytes and suggests that circulating leptin and CRP levels are linked by a regulatory loop (35). This seems to indicate the existence of an adipo–hepato axis whereby leptin produced by adipocytes enhances CRP expression which in turn may antagonize leptin action by limiting its tissue availability (24).

As CRP is a circulating factor which can bind to leptin, its interaction with leptin has been suggested to be critical in regulating the availability of leptin in the hypothalamus and a factor that might contribute to leptin resistance (36). However, no direct proof for a role of CRP in regulating leptin action inside the CNS is available. CRP has also been shown to attenuate leptin signaling in cells overexpressing Ob R (36), however it was not clear whether this was a result of binding with leptin or a direct effect of CRP on Ob R. Therefore, in the present study we have examined the possible interaction of CRP with the leptin receptor and its implication in obesity. Results of the study showed that CRP, particularly the monomeric CRP, binds to sOb R, whose level in the plasma is decreased in obese subjects.

MATERIALS AND METHODS

Materials

All the chemicals used were high quality analytical grade reagents procured from Merck, Mumbai, India, Spectrochem, Mumbai and SRL Mumbai, India. Plastic wares used were products of Becton & Dickinson, Tarson India and NUNC. ELISA kits for assay of leptin and leptin receptor were procured from Invitrogen USA. Human CRP and recombinant leptin receptor (synthesized in *Escherichia coli*) were procured from Thermo Fischer, USA. Purified antibody against leptin receptor (rabbit IgG, HPA030899), human CRP (mouse IgG, C1688), HRP conjugated anti-rabbit IgG, HRP conjugated anti-mouse IgG, protein A-sepharose and o-phenylamine diamine (OPD), and DAB were the products of Sigma Aldrich, USA. NC membrane and Clarity Western ECL substrate were products of Bio Rad, USA.

Methods

Protein–Protein Docking Studies

Interaction of leptin, CRP, and Ob R was studied by protein–protein docking tools using Discovery Studio 4.0. The crystal structures of CRP (PDB ID. 1GNH), human leptin (PDB ID. 1AX8), and Ob R (PDB ID.3v6O) were taken from PDB database. The Z dock protocol (37) in Discovery studio 4.00 (DS) was used to perform docking of proteins. The protein structures downloaded from the PDB database were prepared for docking using "Prepare protein protocol." The monomer structure was taken from the prepared pentameric structure of CRP. The missing amino acid residues 24–39 in the leptin structure were inserted using optimize side chain conformation for residues with inserted atoms. Leptin receptor was modeled as described below and optimized for docking. Z Dock algorithm in DS used the Fast Fourier Transform correlation technique to search all possible binding positions of the docking proteins; it performed an exhaustive multi-dimensional search in the translational and rotational space between two docking molecules. The scoring function of Z Dock is a geometrical measure according to the degree of shape complementarity between the docking proteins. The Z Dock prediction was re-ranked to yield *Z*-rank score which is a linear weighted sum of van der Waal's energy, electrostatic attractive and repulsive energies, and degree of solvation. The Z-Dock score is expressed as positive values and the *Z*-rank score is expressed as negative values. An evenly distributed rotational search gave 2,000 poses of which the best ranked pose represented by the lowest *Z*-rank score (38, 39) in the largest cluster was taken for comparison and the analysis of the docking interactions.

Homology Modeling of Leptin Receptor

Homology modeling of leptin receptor was done using the crystal structure of CRH2 domain of Ob R (PDB ID 3V6O) and the FnIII domain of gp130 (PDB ID.3L5I_A) as templates.

Using psi BLAST program, sequence similarity search was done against protein sequences in PDB depository and the templates for residues 431–841 in the CRH2 and Fn III domains of the extracellular domain of leptin receptor were identified. 3D model was built using MODELER program in DS and compared the model structure with the template to evaluate the model score. The verified score value of the modeled receptor (92.2499) was higher than the expected low score value (84.3719).

Binding Assay

For binding studies, monomeric CRP (mCRP) was prepared from human serum CRP or recombinant CRP by treatment with 8 M urea/10 mM EDTA at 37°C for 2 h as described in Ref. (40). Binding of leptin receptor to CRP was assayed following an ELISA method similar to that described before (3). 96-well microtiter plate was coated with different concentrations of mCRP by incubating at 4°C overnight in Tris/HCl buffer (0.05 M, pH 7.4). Free binding sites were blocked by incubating with 0.5% gelatin in PBS (0.15 M NaCl, 0.005 M phosphate buffer, pH 7.2, 0.05% Tween20) at room temperature for 2 h. Soluble recombinant human leptin receptor (100 ng/ml) in 50 µl PBS was added to the wells and incubated at RT for 1 h. Wells without CRP coating served as control. Non bound Ob R was removed, washed with PBS as above, and each well was treated with 50 µl anti-Ob R antibody (1:1,000) for 1 h; the antibody was removed at the end of the incubation, the wells washed thrice with PBS and treated with HRP conjugated anti-rabbit IgG (1:1,000) for 1 h. At the end of the incubation, HRP was removed; the wells washed thrice with PBS, and developed using OPD/H_2O_2 as substrate. The bound Ob R was quantified by measuring the absorbance at 480 nm.

Co-Immunoprecipitation of sOb R and CRP From Serum

To 50 µl freshly isolated human serum pre-treated with protein A sepharose, 5 µl of 10× PBST (1.5 M NaCl, 0.05 M phosphate buffer, pH 7.2, 0.5% Tween 20) and 0.5 µl of anti-Ob R or anti-CRP were added and incubated overnight at 4°C followed by incubation at room temperature with protein A sepharose for 2 h. The protein A beads were collected by centrifugation at 500 *g* for 10 min and washed three times with PBST and finally with PBS. The beads were then treated with 50 µl of 0.1% SDS in PBS, centrifuged and the co-precipitated CRP or sOb R determined in an aliquot by ELISA with anti-CRP or anti-Ob R antibody (1:1,000) using HRP-conjugated anti-mouse IgG or anti-rabbit IgG as described above. Serum samples treated with protein A sepharose without adding antibody were taken as control. The specificity of the anti-Ob R antibody tested by Western blotting using human serum showed the presence of major and a minor band in molecular size 120–150 kDa similar to that reported earlier (41).

For Western blot analysis, serum samples pre-treated with protein A beads were immunoprecipitated with anti-Ob R antibody as above, the immuno-precipitate captured with protein A beads, the beads washed with PBST, extracted with electrophoresis sample buffer, subjected to SDS-PAGE (42), and electro-blotted onto nitrocellulose membrane (43) using a Biometra Transblot apparatus. Blotted protein was probed with anti-CRP antibody (1:1,000 in PBST) followed by HRP conjugated second antibody (1:1,000 in PBST/0.5% BSA) and developed using ECL reagent. A negative control without addition of the primary antibody while probing was also carried out.

Analysis of CRP, Leptin, and sOb R in Serum—Study Design and Subjects

A total of 79 subjects, 50 males and 29 females, attending the master health check-up at Sri Ramachandra medical college hospital, Chennai were part of this sub-group study. Subjects were aged between 22 and 45 years. There were 32 lean subjects (21 males and 11 females), 5 overweight subjects, and 42 obese subjects (28 males and 14 females). Subjects were considered lean if their body mass index (BMI) was less than 23 kg/m^2, overweight if it was between 23 and 25 kg/m^2, and obese if it was greater than 25 kg/m^2 (according to World Health Organization, Western Pacific region guidelines). Those with a known history of diabetes mellitus, hypertension/systolic blood pressure of >140 mm Hg or diastolic blood pressure >90 mm Hg at the time of examination, coronary artery disease, cerebrovascular accident, bronchial asthma, cancer as well as those on lipid lowering therapy, anti-thrombotic therapy, steroids, those who had surgery within the previous 3 months, pregnant women, and lactating mothers were excluded. The study was approved by the Institutional Ethics Committee of the Sri Ramachandra University (Ref. IEC-NI/12/DEC/31/62) and all subjects provided informed written consent. The study was carried out in accordance with the ethical standards of the Institutional Ethics Committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Anthropometric Measurements

The height, weight, waist circumference, and the hip circumference were measured and BMI calculated as weight (kg)/square of height (m). The blood pressure was measured in the left brachial artery in the sitting position by auscultatory method using a sphygmomanometer.

Biochemical Analyses

Blood samples were collected from subjects, after an overnight fast, in yellow topped BD vacutainers with a separator gel, allowed to stand for 30 min, centrifuged at 5,000 rpm for 10 min, and the serum separated into two aliquots. The first was immediately analyzed for lipids and CRP, while the other was stored at −40°C and after completion of sample collection, analyzed for leptin and sOb R. Another blood sample was collected in a sodium fluoride containing gray topped BD vacutainers and centrifuged immediately to obtain plasma for analysis of fasting plasma glucose. Plasma glucose, total cholesterol, HDLc, LDLc, and triglycerides were measured by the enzymatic photometric methods on the Siemens Advia 1800 auto analyzer using commercial kits following manufacturer's recommendations. Serum CRP was measured by particle enhanced turbidimetric immunoassay using the Dade Behring RXL Max autoanalyzer. Concentration of leptin in serum was determined using the Invitrogen Hu Leptin ELISA kit (the sensitivity of the assay was 3.5 pg/ml; intra- and inter-assay coefficient of variations for lowest and highest values were 3.0 and 3.8% and 3.9 and 4.6%, respectively) while the levels of sOb R were assayed by Quantikine sOb R ELISA kit of R&D systems

(the sensitivity of the assay was 0.057 ng/ml; intra- and inter-assay coefficient of variations for lowest and highest values were 6.1 and 4.9% and 8.6 and 6.8%, respectively).

Statistical Analysis

All serum values are expressed as mean ± SEM or median (IQR). After data were tested for normality by the Shapiro–Wilk test, comparison was done either by the unpaired "*t*" test with Welch's correction or Mann–Whitney *U* test. Correlation analysis was done using the Spearman coefficient of correlation. A "*p*" value of less than 0.05 was considered significant. Statistical analysis was done using Graph Pad Prism 5.01.

RESULTS

Interaction of CRP With Leptin Receptor; Docking of CRP to Leptin Receptor

The cellular effect of leptin involves its binding to the extracellular domain of the cell surface receptor followed by downstream signaling. To examine whether CRP interacts with the leptin receptor, docking studies of CRP to the extracellular domain of the receptor was done. For this, initially, the CRH2 domain of the receptor that has been shown to be sufficient to induce leptin response (8) was employed in the docking with mCRP and the results are shown in **Figure 1**. The best ranked pose in the largest cluster (*Z*-rank score −115.571) was found to involve 22H-bond interactions between the two docking partners (Table S1A in Supplementary Material); mCRP interacted with the amino acid residues 619(L), 620(G), 621(Y), 622(W), and 625(W), mostly present in the C-terminal region of CRH2 domain of the Ob R. To examine whether the mCRP docking site is same or different from that of leptin-binding site on CRH2, docking of leptin to the CRH2 domain was done (**Figure 2**). The best pose in the largest cluster, (*Z*-rank score of −113.578), showed leptin interacting through 12H-bond interactions predominantly with residues 431N, 433N, 434I, 435S, 567N, 589Y, and 614K of the CRH2 domain (Table S1B in Supplementary Material) indicating that leptin and CRP dock at different sites on the leptin receptor. However, similar docking studies using pentameric CRP with leptin receptor showed that the best pose gave a poor *Z*-rank score and less number of non-bond interactions than those shown by mCRP (Table S1C in Supplementary Material). These results suggest that leptin mostly docks to the residues toward the N-terminal region of the CRH2 domain, while mCRP docks to residues toward its C-terminal region closer to the plasma membrane.

Since mCRP was found to dock to C-terminal region of the CRH2 domain which is linked to the FN type III domain of the Ob

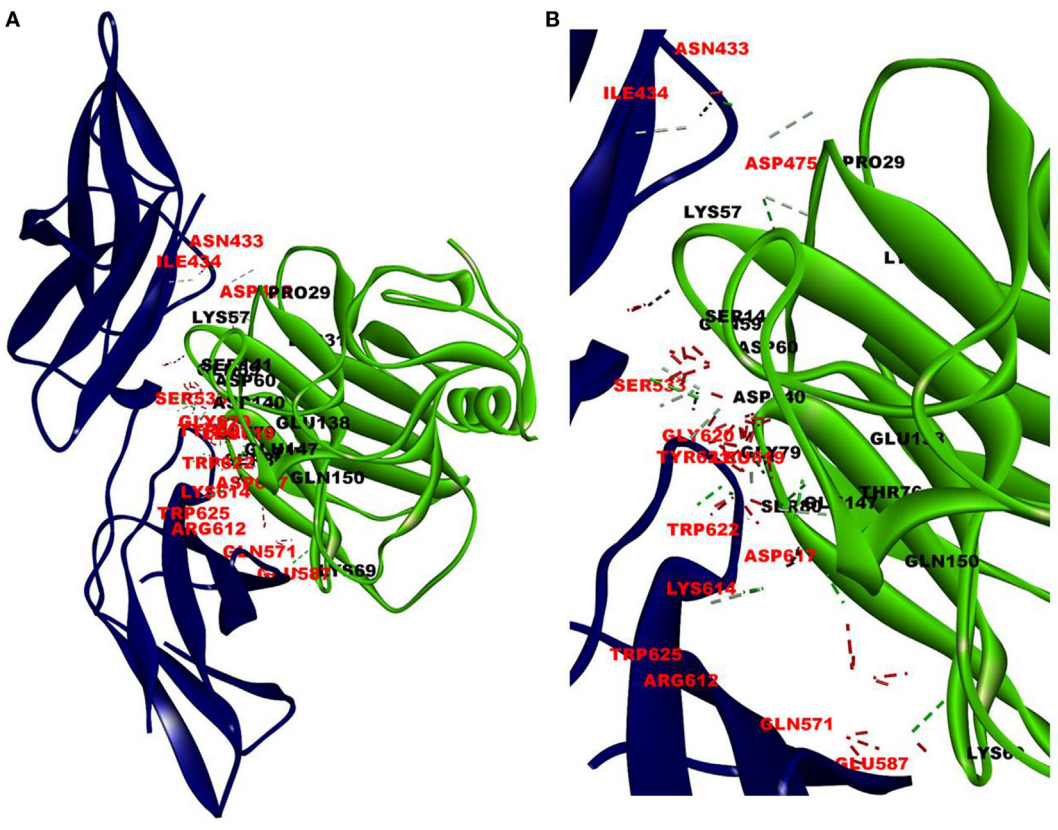

FIGURE 1 | Docking of C-reactive protein (CRP) to leptin receptor. Protein–protein docking was done by employing Z Dock Protocol in Discovery Studio as described in the text. The image of the pose with the best *Z*-rank score in the largest cluster is shown in **(A)** leptin receptor, PDB ID 3V6O, (CRH2 domain), (deep blue) docked (blind) with CRP monomer, PDB Id 1GNH, (single chain) (green) and **(B)** enlarged image of docked amino acid skeleton.

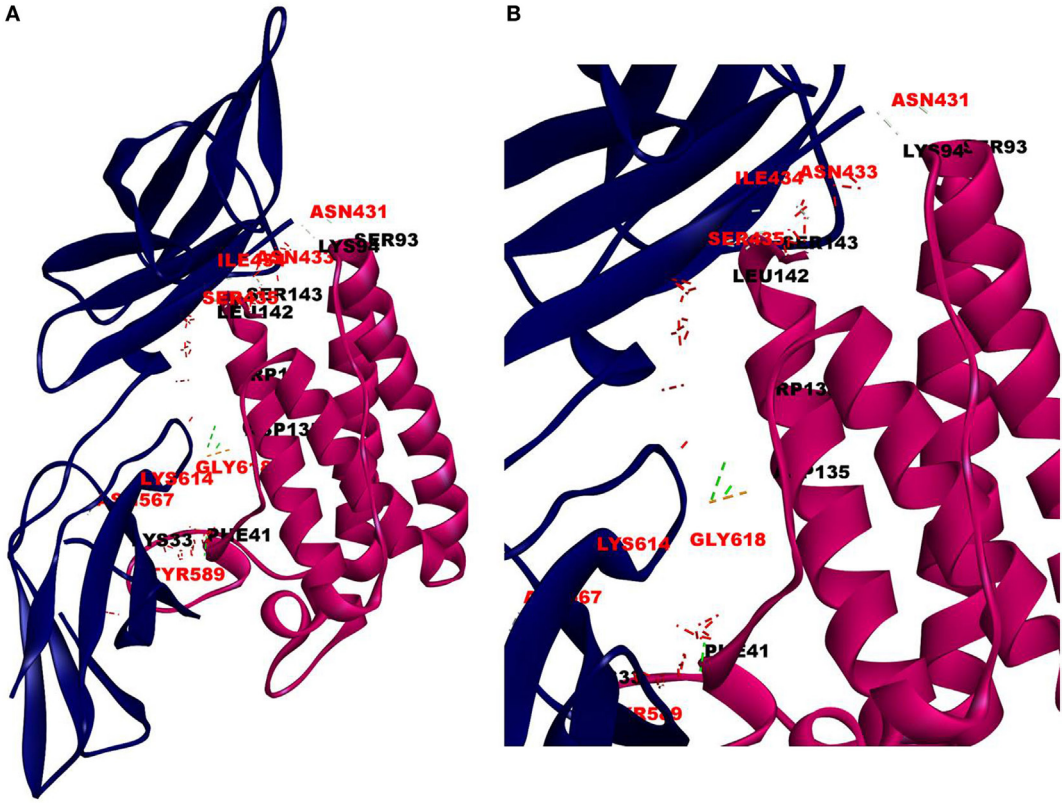

FIGURE 2 | Docking of leptin to leptin receptor. Protein–protein docking was done between leptin and leptin receptor (CRH2, PDB ID 3V6O) as described in legends to **Figure 1**. The image of the pose with the best *Z*-rank score in the largest cluster is shown in **(A)** leptin, PDBID-1AX8, (pink) docked (blind) with leptin receptor, PDB ID 3V6O, (CRH2 domain) (deep blue) and **(B)** enlarged image of docked amino acid skeleton.

R and is reported to be essential for Ob R activation and signaling (8), the possible role of this domain if any in the interaction of mCRP was examined. The Ob R (CRH2–FNIII) was modeled and prepared for docking as described above. mCRP docked to the FNIII domain (*Z*-rank score of −121.633) with 13H-bond interactions with the amino acid residues (764)NYKL(767), 794K, (803)PIE(805), 807Y in FN type III domain and 474D, (477) PSIH(480), 517H, 518S, 522l, 523D, 525P, and 526P in the CRH2 domain (**Figure 3**) (Table S1D in Supplementary Material). These results indicate that mCRP can interact with the membrane proximal FN type III domain and CRH2 domain, unlike leptin which docked to the membrane distal region of the CRH2 domain of Ob R. In the light of the earlier report on binding of CRP to leptin (36), it was proposed to examine whether CRP docks to leptin or its receptor in the leptin–receptor complex. For this, a leptin-Ob R structure was created *via* Z dock, using the Ob R containing the CRH2–FN type III domain model described above. The best ranked pose was energy minimized using standard dynamics cascade and the prepared proteins were further processed for docking with mCRP. The best ranked pose had a *Z*-rank score of −129.234 and involved 17H-bond interactions. This showed that CRP docked predominantly to the receptor component (762P, 763S, 765Y, 766K, 768M, 769Y, 790S, 829T, 830Q, and 832D) (Table S1E in Supplementary Material) on the FN III domain. However, certain residues on leptin (35K, 117S, 118G, and 119Y), which are not involved in docking to the Ob R, dock with CRP in the leptin–receptor complex (**Figure 4**).

A comparison of the *Z*-rank scores which is indicative of the energy, and thus of the stability of the structures, showed that mCRP docked best with the Ob R having CRH2–FN III domain (**Table 1**) and the amino acid residues predominantly in the membrane proximal domain of the leptin receptor were involved in docking. The Z-dock score, which is a geometrical measure of the degree of shape complementarity and thus a reflection of the binding ability was better for docking of CRP to Ob R than that of leptin to Ob R.

Binding of mCRP to Leptin Receptor

Results of docking studies were tested using an ELISA type solid phase binding assay and the results are shown in **Figure 5**. Leptin receptor bound to immobilized mCRP in a concentration-dependent manner (**Figure 5A**). mCRP prepared from human serum CRP showed better binding than recombinant mCRP. The K_d for binding to human mCRP was 1.02×10^{-8} M. Heat-treated mCRP did not show any binding to the leptin receptor indicating that the native structure of CRP is critical in binding to the leptin receptor. Native pentameric CRP was also found to bind to

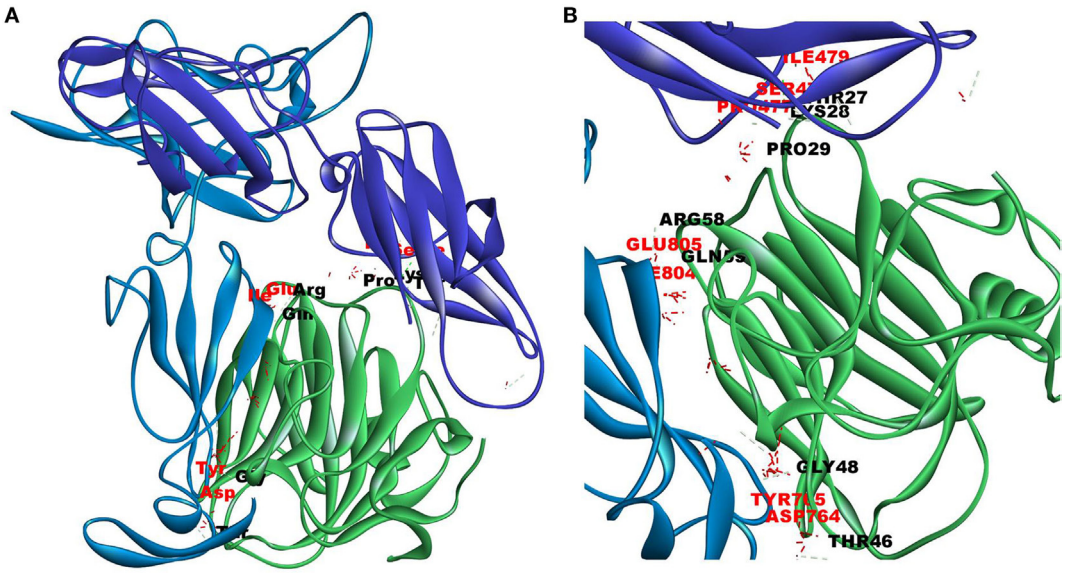

FIGURE 3 | Docking of C-reactive protein (CRP) to modeled leptin receptor. Protein–protein docking was done between modeled leptin receptor (CRH2–FNIII) and CRP (monomer) (PDB Id 1GNH) as described in **Figure 1**. The image of the pose with the best *Z*-rank score in the largest cluster is shown in **(A)** leptin receptor modeled [CRH2 (deep blue) –Fn III domains (light blue)] docked with CRP monomer (green) and **(B)** enlarged image of docked amino acid skeleton.

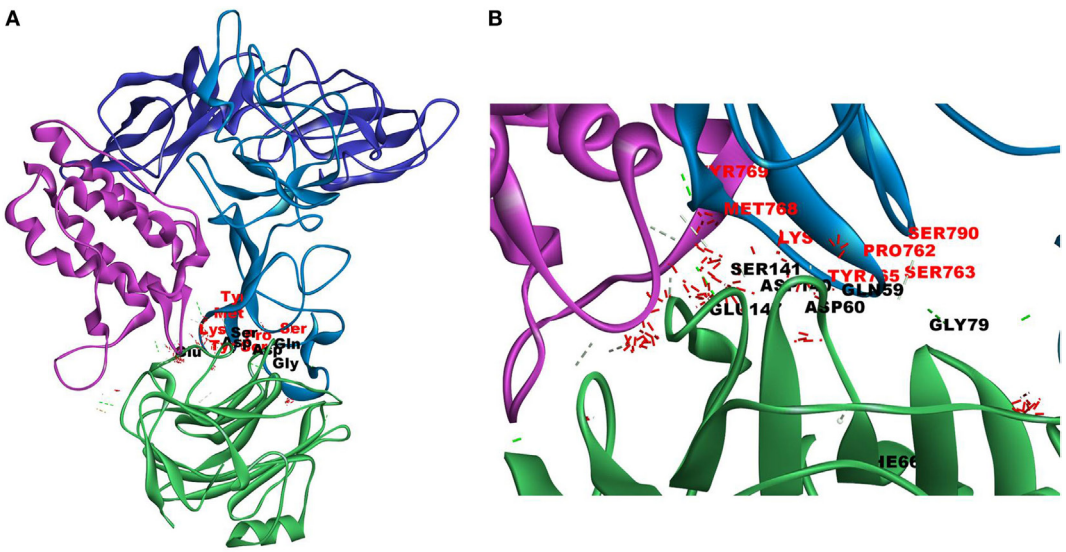

FIGURE 4 | Docking of C-reactive protein (CRP) to leptin–leptin receptor complex. Leptin (PDB Id 1AX8) –leptin receptor (CRH2–FNIII) complex was prepared, the complex was energy minimized, prepared for further docking as described in Section "Materials and Methods" and docked with CRP (PDB Id 1GNH). Protein–protein docking was done as described in **Figure 1**. The image of the pose with the best *Z*-rank score in the largest cluster is shown. **(A)** Leptin (pink) was docked (site specific) to modeled leptin receptor [CRH2 (deep blue)–Fn III (light blue)], CRP monomer (green). **(B)** Enlarged image of docked amino acid skeleton.

the leptin receptor, though to a lesser extent than mCRP (80%). Presence of Ca^{2+}, Mg^{2+}, or Mn^{2+} ions did not affect the binding. The binding appeared to be specific as non-relevant proteins such as serum albumin or lysozyme did not show significant binding (**Figure 5B**). However, amino acids, such as His, Tyr, Asp, and Gln, which were predicted to be involved from docking studies, did not cause any significant effect at the concentrations tested. However, a decrease in pH 5 in the binding assay medium caused a significant reduction in the binding, indicating that protonation of certain residues might have affected the binding.

TABLE 1 | Docking scores and H-bonding interactions of CRP and LR.

Sl No	Molecules docked	*Z*-rank score	Non bond interactions (<3Å)
1	L–LR (CRH2)	−113.578	12H
2	LR (CRH2)–CRP monomer	−115.571	22H
3	LR (CRH2)–CRP pentamer	−109.101	8H
4	LR (CRH2, Fn)–CRP monomer	−121.633	8H
5	L LR(CRH2, Fn)–CRP	−129.234	16H

L, leptin; LR, leptin receptor; CRP, C-reactive protein; LR(CRH2), cytokine receptor homology domain 2 of LR, LR(CRH2-Fn), CRH2-fibronectin type III domains of LR. Residues within a distance of 3Å involved in H-bond interactions were considered.

Co-Immunoprecipitation of sOb R and CRP of Serum

To further examine binding of CRP to Ob R, sOb R was immunoprecipitated from human serum and the amount of co-precipitated CRP estimated; the results are shown in **Figure 6**. About 5% of serum CRP was co-preciptated with sOb R. Spiking with different concentrations of sOb R followed by immunoprecipitation showed an increase in the amount of CRP co-precipitated from serum with increase in the amount of added sOb R. Similar analysis of immunoprecipitated CRP showed that about 1.25% of the total sOb R was co-precipitated with CRP. Co-immunopreciptation of CRP with sOb R was confirmed by SDS -PAGE and Western blot analysis which showed the presence of a 24 kDa band corresponding to the monomeric subunit of plasma CRP (**Figure 6D** lane 1) in the immunoprecipitated sOb R. To further confirm that CRP was co-precipitated, the immuno-precipitate captured on protein-A beads was extracted with 0.1% SDS in PBST, the CRP immunoprecipitated and subjected to Western blotting. Presence of an identical band (**Figure 6D** lane 2) corresponding to molecular size 24 kDa confirmed the presence of CRP. An identical band was also obtained when whole serum was subjected to similar analysis using anti-CRP antibody (**Figure 6D** lane 3). Binding of CRP to sOb R was further confirmed by co-immunoprecipitation of sOb R with CRP. CRP was immunoprecipitated from serum by anti-CRP and the immunoprecipitate subjected to electrophoresis and immunoblotting using antibody against sOb R. Two bands in the molecular weight region 121 and 136 kDA, corresponding to sOb R, were found suggesting co-precipitation of sOb R with CRP (Figure S1 in Supplementary Material).

In both instances, another band corresponding to an approximate molecular weight 53 kDa was observed. This appeared to be the immunoglobulin detected by the secondary antibody as suggested by its persistence in negative controls which did not use a primary antibody for probing. The antigens of interest, CRP and sOb R were not detected in negative controls confirming specificity of the primary antibody (Figure S1 in Supplementary Material).

Serum Levels of leptin, CRP, and sOb R in Lean and Obese Human Subjects

As an important protein in plasma that binds to leptin, the level of sOb R in plasma is critical in determining the availability of leptin and its response. Earlier studies in human subjects with obesity associated metabolic diseases obtained contrasting results with either a increase, no change, or a decrease in plasma levels of sOb R (44). It is not clear whether these variations are due to associated metabolic complications of obesity. To assess the relation, if any, between the concentration of sOb R and its binding proteins, particularly CRP and leptin, the concentrations of these proteins in serum of lean and obese human subjects without any history of co-morbidities, as described in Section "Materials and Methods," was determined. The anthropometric and biochemical profile of the subjects are given in the Table S2 in Supplementary Material. None of the subjects had any history of acute or chronic inflammatory disorders or infection. There was no significant difference between the age of obese and lean subjects. While there was no significant difference between levels of total cholesterol of the two groups, there was a significant decrease in HDLc and an increase in LDLc levels and triglyceride levels in obese subjects. Although the FBS levels were higher in obese subjects, none of the subjects were diabetic.

The levels of leptin, sOb R, and CRP in the serum of these subjects are given in **Table 2**. A significant increase in the levels of leptin in serum of obese subjects, compared to lean subjects, was observed, consistent with the earlier reports (45). There was a positive correlation between levels of leptin and BMI (**Figure 7A**). In agreement with earlier reports, serum levels of CRP showed a significant increase in obese subjects when compared to that of lean subjects. The levels of CRP correlated positively with both BMI and serum leptin levels (**Figures 7B,C**). The levels of sOb R in the serum of obese subjects was significantly lower than that in lean subjects; it showed a negative correlation with BMI (**Figure 7D**) and serum levels of leptin ($r = -0.23$, $p < 0.05$). Though there was decrease in sOb R, no significant negative correlation between CRP and sOb R levels was observed. Increase in levels of serum CRP and leptin were observed in both male and female obese subjects (Table S3 in Supplementary Material). However, no significant difference in sOb R was found between lean and obese female subjects.

To examine whether the binding of sOb R to CRP in plasma was affected in obese condition, the levels of sOb R and CRP co-immunoprecipitated were analyzed as done above. The amount of CRP co-precipitated with sOb R from serum was not significantly different in obese subjects compared to lean subjects (**Figure 8**). The amount of sOb R co-precipitated with CRP was also not different.

DISCUSSION

Investigations into the molecular mechanisms of leptin resistance have led to the suggestion that circulating leptin-binding proteins could limit its availability at target sites. Among the different proteins in the plasma, CRP, a major acute phase protein has been considered as the principal leptin-binding protein that could limit the action of leptin. Results presented above suggest that CRP does interact with the leptin receptor. The evidence in support of binding of CRP with the extracellular domain of the Ob R includes (a) molecular docking of CRP to extracellular CRH2–FN type III domain of Ob R (b) binding of the recombinant leptin receptor to CRP, particularly to mCRP, in a solid phase

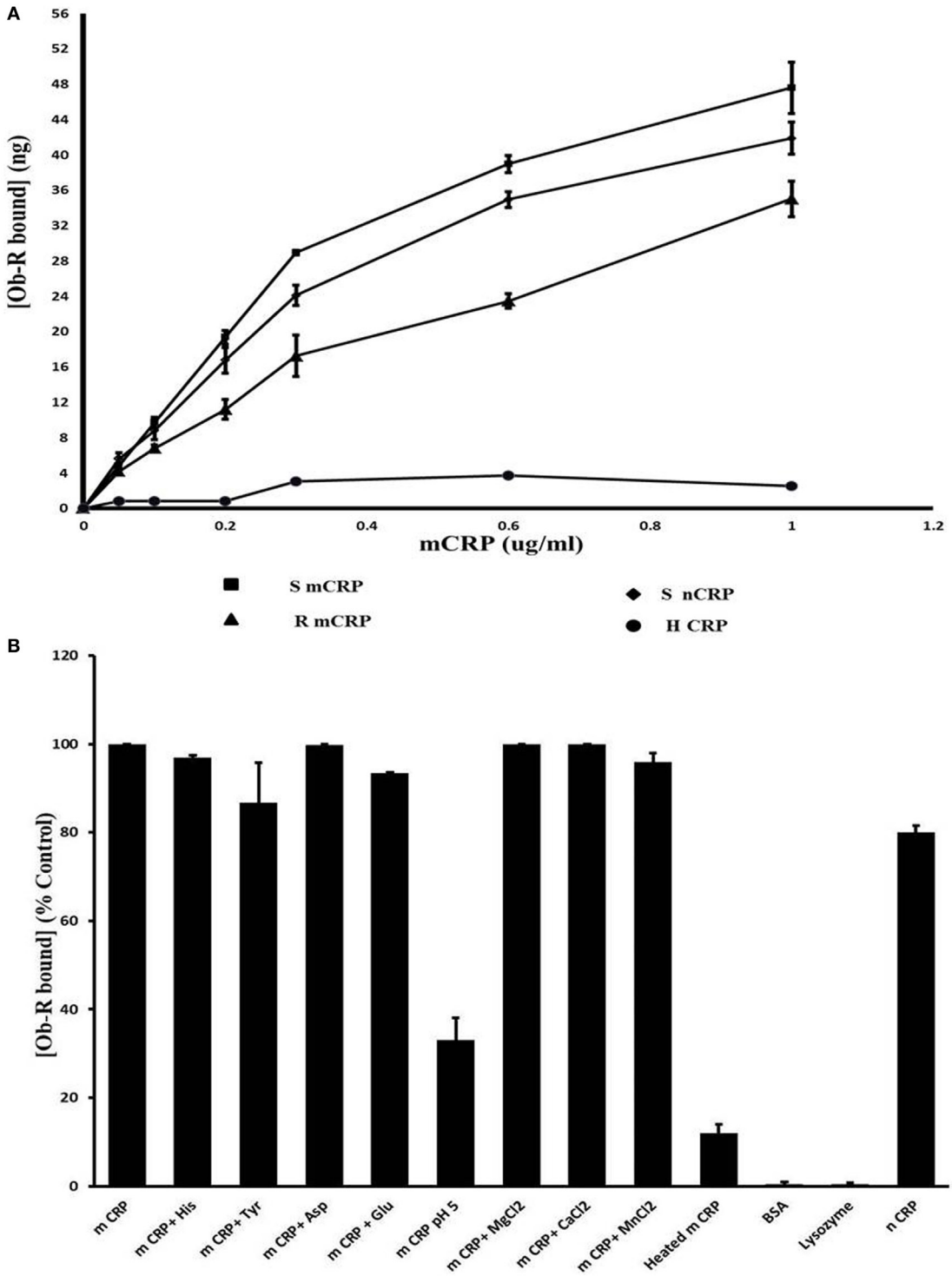

FIGURE 5 | Solid phase binding assay of leptin receptor to C-reactive protein (CRP). **(A)** Concentration dependence: different concentrations of monomeric CRP (mCRP) from serum (s-mCRP), recombinant CRP (R-mCRP), native serum CRP (n-CRP), and heat treated (h-mCRP) were coated on to 96-well micro titer plate incubated with leptin receptor (100 ng of Ob R) and the amount of Ob R bound was quantitated by ELISA using anti-Ob R antibody and HRP-conjugated secondary antibody as described in text. Values given are the average of 3–6 experiments done in duplicate ± SD. **(B)** Effect of different substances: mCRP (0.6 μg/ml) was coated on to 96-well micro titer plate and binding of leptin receptor in the presence of different cations (1 μM each of $MnCl_2$, $MgCl_2$, and $CaCl_2$), or different amino acids (1 μM each of His, Tyr, Asp, and Glu) or in buffer pH 5.0 was assayed. Binding of leptin receptor to wells coated with BSA (100 μg/ml), lysozyme (100 μg/ml) native CRP (nCRP), or heat-treated CRP (0.6 μg/ml) was also analyzed. Results are expressed as percent of untreated mCRP control. Each value represents mean ± SD of three experiments.

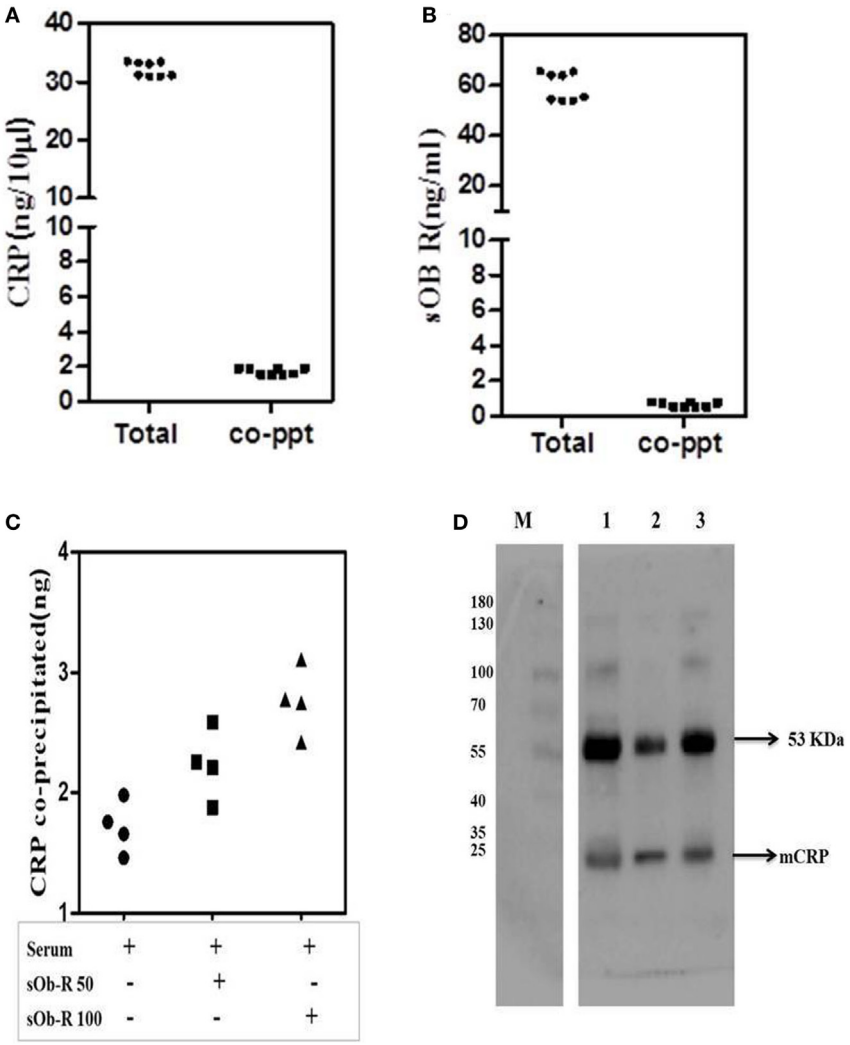

FIGURE 6 | Co-precipitation of soluble leptin receptor (sOb R) and C-reactive protein (CRP) from serum. **(A)** Co-precipitation of CRP with sOb R: sOb R was immunoprecipitated from fresh human serum and the amount of CRP present in the immunoprecipitate was assayed by ELISA. Total CRP in serum was also assayed. Scatter plot represents results of multiple experiments in replicates. (mean ± SD of total CRP was 32.18 ± 1.24 and that of co-precipitated CRP was 1.74 ± 0.17). **(B)** Co-precipitation of soluble leptin receptor with CRP: CRP was immunoprecipitated from freshly isolated human serum and the amount of soluble leptin receptor present in the immunoprecipitate was determined by ELISA. Total soluble leptin receptor in serum was also determined. [mean ± SD of total soluble leptin receptor (sOb R) was 59.6 ± 5.6 and that of co-precipitated sOb R was 0.67 ± 0.14]. **(C)** Co-precipitation of CRP from serum spiked with different concentrations of soluble leptin receptor. To 50 µl of freshly isolated serum, different concentrations (50, 100 ng) of leptin receptor were added, incubated for 30 min, immunoprecipitated leptin receptor, and estimated the amount of CRP co-precipitated by ELISA as above. Scatter plot represents results of multiple assays and shows an increase in co-precipitated CRP with increase in concentrations of added leptin receptor. **(D)** Immunoblot analysis of co-precipitated CRP. sOb R was immunoprecipitated from human serum, subjected to 7.5% SDS-PAGE followed by Western blotting, probed with anti-CRP, and located by ECL as described in the text. Lane 1: sOb R co-precipitate. Lane 2: immune-precipitated CRP from sOb R co-precipitate. Lane 3: immunoprecipitated CRP from whole serum. M: molecular weight markers.

binding assay, and (c) co-immunoprecipitation of CRP with sOb R from human serum.

Molecular docking studies revealed that mCRP docks to the extracellular domain of the leptin receptor. Detailed analysis of the docking interactions revealed the following: (a) mCRP, but not the larger pentameric CRP, docks to the C-terminal region of the CRH2–FNIII domain of Ob R proximal to the plasma membrane. (b) mCRP docking site is distinct from the leptin-docking site which is located more toward the N-terminal

TABLE 2 | Serum levels of leptin, soluble leptin receptor (sOb R), and C-reactive protein (CRP) in lean and obese subjects.

	Body mass index (BMI) <23	BMI >25	*p*
N	32	42	
Leptin (ng/ml)	18.0 (7.6–21.5)	54.6 (40.95–62.2)	<0.0001
sOb R (ng/ml)	26.38 (19.8–35.36)	21.37 (15.65–28.04)	<0.05
CRP (mg/dl)	0.16 (0.11–0.23)	0.60 (0.465–0.95)	<0.0001

Values expressed as median (interquartile range). Comparison was done by the Mann–Whitney U-test.

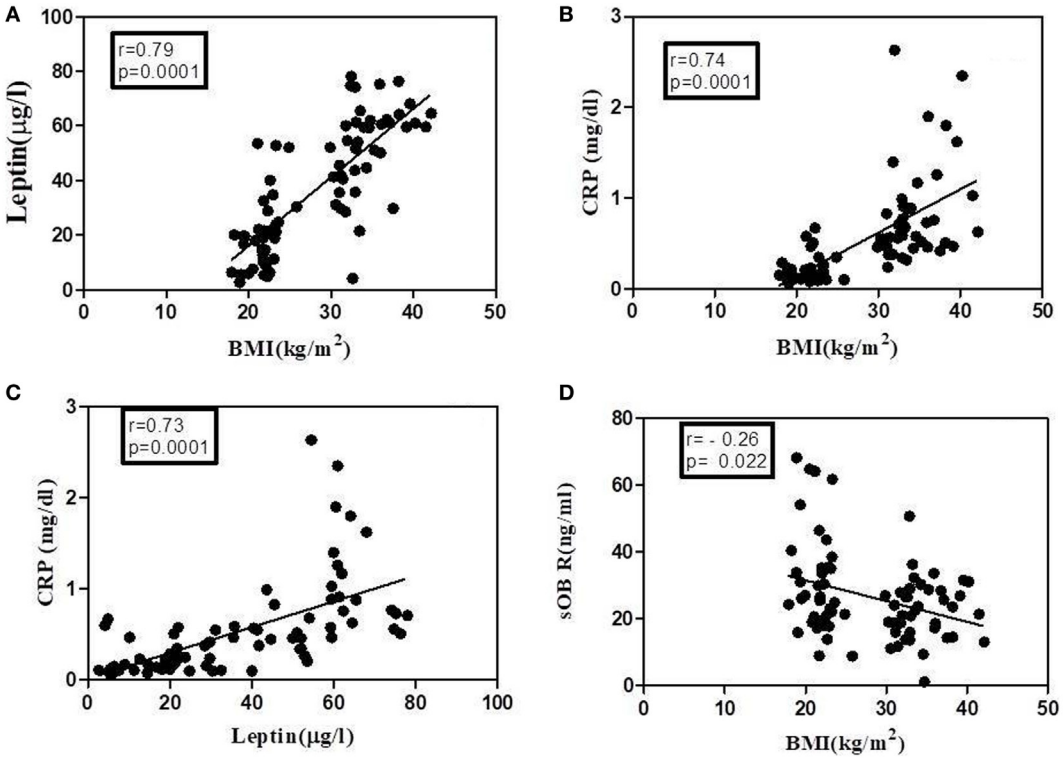

FIGURE 7 | Correlation of leptin, C-reactive protein (CRP), and soluble leptin receptor (sOb R) with body mass index (BMI) analyses of correlation of BMI with serum levels of leptin, CRP, and sOb R as well as that between leptin and CRP was done using Spearman coefficient of correlation. A value of $p < 0.05$ was considered significant. Dark circles represent individual values. **(A)** BMI and leptin ($n = 78$, $r = 0.79$), **(B)** BMI and CRP ($n = 78$, $r = 0.74$), **(C)** leptin and CRP ($n = 77$, $r = 0.73$), and **(D)** BMI and sOb R ($n = 79$, $r = -0.26$).

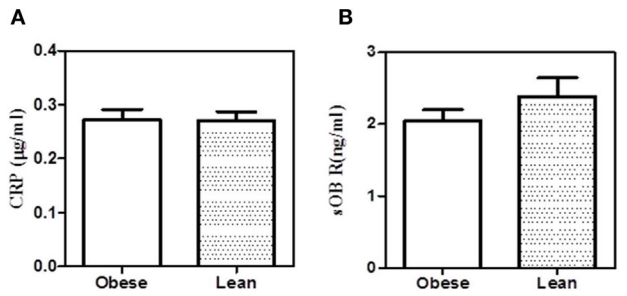

FIGURE 8 | Co-precipitation of leptin receptor and C-reactive protein (CRP) from serum of obese and lean subjects. **(A)** Soluble leptin receptor (sOb R) was immunoprecipitated from the serum of lean ($n = 6$) (body mass index, BMI <23) and obese ($n = 5$) (BMI >25) male subjects and CRP estimated by ELISA as shown in **Figure 3**. **(B)** CRP was immunoprecipitated from the serum of lean and obese male subjects and estimated sOb R by ELISA. Values given are the mean ± SD. $p > 0.05$ not significant.

region of the CRH2 domain of Ob R. (c) Docking studies with leptin–leptin receptor complex predict that mCRP docks directly to CRH2FNIII domain of the receptor and not through leptin; it docks predominantly to the receptor component with amino acids predominantly in the FNIII domain with minimal interaction with the leptin molecule. (d) A better *Z*-rank score (**Table 1**), which is a linear weighted sum of the van der Waal's energy, electrostatic energy, and degree of solvation reflecting the binding energy, predicted that the mCRP–Ob R interaction is ranked comparably with that of leptin, the natural ligand. Comparison of the *Z*-rank scores for docking of mCRP with Ob R (CRH2–FNIII) and leptin–Ob R complex (−121.633 and −129.234) further suggests that leptin may not affect interaction of mCRP with the Ob R. However, rigid docking studies such as these offer limited insights into ligand-induced changes in the binding characteristics of the receptor. (e) Analysis of the leptin-docking residues on CRH2 domain, which has been shown to be critical in leptin binding and signal generation (8) suggest that our docking results agree to a certain extent with the experimental data on the effect of leptin on cells. Leptin docked predominantly to the residues in the N-terminal part of the CRH2 domain which has been reported to contain the leptin-binding site (8, 9, 46); the AA residue 567 (Asn), found to be involved in docking with leptin, has been reported in earlier mutation studies to be critical in signal transduction (47). Furthermore, earlier mutation studies have reported that the amino acid residues 35(Lys in human), 41 (Phe), 138 (Trp in human), 142/143 (leu/Ser in human) in leptin that were involved in docking with the receptor in our study, were critical in leptin-receptor binding and signaling (47, 48). Further, the amino acids identified to interact with CRP are

present in the internal CRH2–FN III domain 2 which is reported to be critical in receptor dimerization and activation (8). Earlier cell-based studies had suggested that CRP attenuated leptin signaling (36). Since CRP is reported to bind to leptin, this effect could either be due to sequestration of leptin by CRP thereby reducing its availability, or due to a direct effect of CRP on the leptin receptor, a distinction which could not be resolved in an experimental system. The latter possibility is supported by our docking studies, which showed that mCRP docks to membrane proximal FN III domain, which is critical in receptor activation and signaling.

There is increasing evidence indicating dissociation of the pentameric CRP to the mCRP in tissues (49–51) including brain (52) and the role of mCRP in inflammation (4, 51, 53) and angiogenesis (54, 55). mCRP has been reported to bind to cells through cell surface molecules, such as CD16 (56) and $\alpha_V\beta_3$ integrin (3). Presence of modified CRPs in plasma has been reported in obese human subjects (57). It, therefore, appears that the CRP, particularly the smaller mCRP, could attenuate leptin receptor activation independent of its ability to bind to leptin which might contribute to leptin resistance. Further experiments would be required to demonstrate its effect on leptin signaling.

Soluble leptin receptor to which CRP is found to bind is generated by shedding of the extracellular domain of membrane-anchored Ob R. Immunoblotting studies showed two bands of 121 and 136 kDa, corresponding to sOb R. These were also found in the CRP co-precipitate. Human sOb R has been reported to have a molecular weight of around 140 kDa. However, molecular weights ranging from 110 kDa to 290–300 kDa have also been reported (58–61). This difference has been attributed to differences in glycosylation of the core protein (61, 62). The data in this study are comparable to that reported earlier using a different antibody against sOb R (41). sOb R in plasma is considered as an important protein that binds to leptin and regulates its availability at target sites. But it is not clear how CRP-sOb R binding can influence the availability of sOb R for leptin sequestration.

The relevance of these binding studies was further examined by analyzing the levels of leptin, CRP, and sOb R in serum of non-obese and obese non-morbid human subjects. While a positive correlation between the levels of leptin and CRP was observed, there was a negative correlation between the levels of leptin and its soluble receptor. As reported in earlier studies (45), this appears to be related to obesity as indicated by a positive correlation between BMI and leptin and CRP. Although previous studies have demonstrated elevated CRP levels in obesity, the possibility of obesity associated co-morbidities contributing to this elevation could not be excluded. In the present study, the subjects did not have any co-morbidities, hence the increase in CRP levels is more likely to be linked to the expansion of adipose tissue. Unlike CRP, serum level of sOb R, is decreased in obese subjects compared to that of lean subjects. The levels of sOb R in plasma have been reported to be differentially regulated in various metabolic diseases (44). While our results on decrease in sOb R in obese subjects agree with those reported earlier (63), there are reports showing no change (64) and increased (65) levels of sOb R. These variations may be due to associated morbidity as evidenced by increase in plasma levels of sOb R in obese subjects with non-alcoholic steatosis (65). In the present study, obese subjects did not have any co-morbidity and any confounding effects were minimal. It has been shown that ADAM10/17 dependent cleavage of extracellular domain of the cell surface Ob R is the source of plasma sOb R (13). Change in ADAM 10 activity as in lipotoxicity or apoptotic conditions can contribute to changes in levels of sOb R (44). Changes in levels of cell surface Ob R is another factor determining the extent of its shedding and the serum level of sOb R is a reflection of its level of membrane expression (44). Thus the decrease in level of sOb R in obese subjects without any co-morbidity, observed in the present study, may reflect a lower level of membrane expression of Ob R. It is also possible that binding of CRP to membrane Ob R may protect against proteolytic cleavage resulting in lower levels of sOb R.

The negative correlation between BMI and sOb R suggests that the contribution of sOb R in the plasma in impeding the flow of leptin is relatively less compared to the effect of CRP which is elevated in obese subjects. In the light of these results on binding of CRP, particularly mCRP, to the cell surface domain of Ob R, it is possible that increased levels of CRP in obese subjects might contribute to diminished response to leptin in obese subjects.

The interaction of CRP with Ob R assumes particular significance in the context of the role of leptin in the interface of immune function, inflammation, and metabolism. Apart from its well-known effect as key regulator of metabolism, leptin, a pro-inflammatory cytokine and an activator of the immune system, is implicated in a number of inflammatory and immune disorders (66). Its levels are elevated in obese conditions and it mediates the pro-inflammatory state of obesity and associated pathophysiology. As leptin can stimulate CRP production by hepatocytes and the vasculature (24), these results suggest a possible regulatory system where binding of CRP to the leptin receptor may modulate the pleiotropic effects of leptin at multiple target sites. This may have implications in both adipose tissue physiology as well as pathogenesis of obesity related diseases.

ETHICS STATEMENT

The study was approved by the Institutional Ethics Committee of the Sri Ramachandra University (Ref. IEC-NI/12/DEC/31/62) and all subjects provided informed written consent. The study was carried out in accordance with the ethical standards of the Institutional Ethics Committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

AUTHOR CONTRIBUTIONS

MS, SS, and RR conceived and designed the study. Docking and binding studies and analysis by AS, AP, and MS. Sample collection and data analysis by MS, SS and RR. Manuscript written by MS, SS, and RR.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01167/full#supplementary-material.

FIGURE S1 | Immunoblot analysis of co-precipitated soluble leptin receptor (sOb) R C-reactive protein (CRP) was immunoprecipitated from human serum, subjected to 7.5% SDS-PAGE, followed by Western blotting, probed with anti-sOb R, and located by ECL **(B)** Negative controls: anti-CRP immunoprecipitate probed without anti-sOb R (only HRP conjugated secondary antibody) served as negative control **(C)** for identification of co-precipitated sOb R **(B)**. Anti-sOb R immunoprecipitate, probed without anti-CRP (only HRP conjugated secondary antibody) served as negative control **(A)** for identification of co-precipitated CRP (Figure 6D).

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C-Reactive Protein As a Mediator of Complement Activation and Inflammatory Signaling in Age-Related Macular Degeneration

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Age-related macular degeneration (AMD) is a devastating neurodegenerative disease affecting millions worldwide. Complement activation, inflammation, and the loss of choroidal endothelial cells have been established as key factors in both normal aging and AMD; however, the exact mechanisms for these events have yet to be fully uncovered. Herein, we provide evidence that the prototypic acute phase reactant, C-reactive protein (CRP), contributes to AMD pathogenesis. We discuss serum CRP levels as a risk factor for disease, immunolocalization of distinct forms of CRP in the at-risk and diseased retina, and direct effects of CRP on ocular tissue. Furthermore, we discuss the complement system as it relates to AMD pathophysiology, provide a model for the role of CRP in this disease, and outline current therapies being developed and tested to treat AMD patients.

Keywords: C-reactive protein, age-related macular degeneration, inflammation, complement, complement factor H, membrane attack complex

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INTRODUCTION

Age-related macular degeneration (AMD) is a progressive neurodegenerative disease affecting more than 8% of the global population (1), with roughly 11 million cases in the United States alone (2). Inflammation and complement activation are recognized as prominent events in the manifestation and progression of AMD, with C-reactive protein (CRP) as a potential mediator in these processes. Here, we review AMD pathophysiology, the complement system, and the current evidence supporting CRP as a contributor to complement activation and inflammation in the context of AMD. Finally, we will present a model for the role of CRP in this disease and provide insight into future therapies for AMD patients.

AMD PATHOPHYSIOLOGY

Early AMD is clinically identified by the presence of extracellular deposits, called drusen, and/or pigmentary changes within the macula, the central 6 mm of the retina. If the disease progresses, vision loss can occur either from atrophy of the retina (termed advanced dry AMD or atrophic AMD) or aberrant growth of vessels beneath the retina (termed wet AMD or neovascular AMD). The primary pathology in AMD includes dysfunction and/or loss of (1) the photoreceptor cells, which are the light-sensing cells of the retina, (2) the retinal pigment epithelium (RPE), which forms the outer blood–retinal barrier and consists of a single layer of epithelial cells that support photoreceptor cell function, and (3) the choriocapillaris, the capillary bed that lies just outside the RPE (and forms

the innermost layer of the choroid) and provides oxygen and nutrients to both the RPE and photoreceptor cells.

While the pathologic changes that occur within the macula during AMD are incompletely understood, evidence supports complement-mediated changes and the loss of choroidal endothelial cells (CECs) as early events in both normal aging and disease (3, 4). CECs are believed to be lost early in disease progression, which may accompany or even precede dysfunction and loss of the RPE (4–9). Loss of these supporting cells eventually leads to loss of photoreceptor cells within the macula, causing significant and irreversible visual decline (10).

THE COMPLEMENT SYSTEM IN AMD

Although AMD is a complex, multifactorial disease, many genetic factors have been associated with disease risk. To date, 19 genetic loci have been identified, including genes involved in regulating complement activity (11). The discovery of a genetic link between AMD and the complement system supports previous histological and proteomic data identifying complement proteins as constituents of drusen in postmortem eyes (12–14).

The complement system, which is important for eliminating pathogens, cellular debris, and dying host cells, consists of three distinct arms: the classical pathway, the alternative pathway, and the lectin pathway. After pathway-specific initiation, followed by a series of cleavage events, the pathways continue, similarly, through to cleavage of C5 and entrance into a common terminal pathway. The terminal pathway culminates in formation of the membrane attack complex (MAC), which promotes cell lysis. Similar to other arms of the immune system, the complement system must be carefully regulated, and various serum- and tissue-derived complement proteins aid in the regulation of one or more pathways. One of the major genetic risk loci for AMD includes the complement factor H (CFH) gene, which encodes an alternative pathway regulator and harbors multiple disease-associated variants (15). Importantly, the Y402H single nucleotide polymorphism (SNP) in *CFH* is a common variant that significantly increases AMD risk (15–18). The Y402H substitution occurs in the SCR7 region of the complement factor H (FH) protein, resulting in impaired protein binding to various substrates, including proteoglycans (19–22) and CRP (23).

Another component of the complement system, the MAC, has been linked to AMD through various histological studies. MAC deposition becomes increasingly elevated within the choriocapillaris with advancing age in human postmortem eyes, and this phenomenon is specific to the eye (24). MAC accumulation is further elevated in *CFH* Y402H high-risk and AMD maculae beyond what is observed in normal aging (3). Compromised binding of the FH Y402H protein to its extracellular matrix substrates, such as heparan sulfate proteoglycans, may increase complement activation in the human retina (21), and may be one cause for elevated MAC deposition in the macula in AMD (3, 4). The potential consequences of MAC accumulation in the choriocapillaris are twofold. First, MAC formation on CECs *in vitro* results in significant cell lysis. Second, the cells that survive complement attack adopt an angiogenic phenotype (25). While complement activation and MAC accumulation in the choriocapillaris in AMD are well-established events (26), the precise details for how and why these events occur remain to be fully elucidated.

CRP IN COMPLEMENT ACTIVATION AND INFLAMMATION

C-reactive protein is an established component of drusen in human postmortem eyes (14, 27–29) and it plays a role in both complement activation and regulation (23, 30, 31), implicating CRP as an intriguing candidate for disease involvement.

Studies examining the role of CRP as a regulator of complement activation and other inflammatory response pathways have recently evolved to evaluate and distinguish the bioactivities of two distinct conformational forms of the protein. The widely appreciated serum-associated form is a hepatically synthesized pentameric discoid protein (pCRP) with high aqueous solubility and calcium-dependent phosphocholine (PC) binding affinity. pCRP can bind to PC groups exposed on disrupted plasma membranes, as occurs when lysophospatidyl choline is formed (32). Upon binding to disrupted cell membranes, as shown with activated platelets and apoptotic monocytes, pCRP undergoes rapid dissociation into monomeric form (mCRP) (33, 34) with distinctive solubility, antigenicity, tissue localization, binding ligands, and functions compared to pCRP (30, 35, 36). It is now known that mCRP rather than pCRP is an efficient activator of the classical complement pathway involving C1 (*via* binding to C1q), C4, C2, and C3 (37, 38). mCRP also acts as a regulator of the alternative complement pathway, *via* recruitment of FH to injured tissues. This binding to FH was shown to occur in a dose-dependent manner and at a site that does not interfere with mCRP binding to C1q (38). Importantly, the high-risk FH Y402H protein has reduced mCRP binding by up to 45% when compared to the FH Y402 protein (28, 39, 40).

In the context of atherosclerosis and coronary artery disease, mCRP has been shown to be strongly pro-inflammatory, differentiating monocytes toward a pro-inflammatory M1 phenotype (41), delaying neutrophil apoptosis, and stimulating leukocytes pro-inflammatory effector responses (42). In addition, Zouki and colleagues revealed a mCRP-mediated upregulation of CD11b/CD18 expression on the surface of human neutrophils, which results in enhanced adhesion of these cells to activated endothelium (43). Monomeric CRP can also activate human coronary artery endothelial cells *in vitro*, resulting in production of IL-8 and MCP-1 (44). Moreover, mCRP stimulates angiogenesis and promotes tube formation in brain microvasculature (45). While CRP has been widely studied outside the eye, the bioactivities of both forms of CRP are now beginning to be elucidated within ocular tissue in the context of AMD.

SERUM-ASSOCIATED CRP AS A RISK FACTOR IN AMD

Seddon and colleagues began examining a potential link between CRP and AMD, by assessing CRP levels in the serum of AMD and control patients. They found CRP levels to be higher in

individuals with intermediate and advanced AMD compared to controls (46), and they discovered a positive correlation between serum CRP levels and AMD progression (47). Furthermore, they found the lowest risk of AMD progression associated with CRP levels <0.5 mg/L, little change to AMD risk for CRP levels within the range of 0.5–10.0 mg/L, and the highest risk for AMD progression when CRP levels reached at least 10 mg/L (47). Finally, Seddon's group examined the relationship between serum CRP levels, *CFH* genotype, and AMD risk (48), and they found CRP and *CFH* genotype to be independently associated with AMD risk. In fact, both the *CFH* variant and high CRP levels have an additive effect on AMD risk.

Interestingly, others have gone on to assess the relationship between serum CRP levels and AMD status in individuals harboring variants in the promoter region of the *CRP* gene, all of which either increase or decrease CRP levels in the serum. However, regardless of whether the SNP increased or decreased CRP levels, the authors found no direct association between any of the variants and AMD status (49–51). While none of these variants result in an amino acid change to the CRP protein and, therefore, do not affect biological function of the protein, these studies suggest that serum CRP levels alone may not be enough to alter AMD risk.

CRP IN THE AMD RETINA

The association between serum CRP levels and AMD risk may be important for defining disease biomarkers; however, the primary pathology of AMD occurs in the retina. Prior to the realization that CRP exists in more than one form (e.g., mCRP and pCRP), work done by Johnson and colleagues examined the presence of total CRP in the retina of postmortem eyes genotyped at the *CFH* locus (27). They showed that high-risk eyes (homozygous for the Y402H SNP) had more CRP immunoreactivity in the choroid compared to low-risk eyes (homozygous for Y402), especially in regions containing drusen-like deposits (27). A similar study aimed to determine differences in total CRP levels in the retina based on AMD status (52). CRP immunoreactivity differed based on disease status, with early and wet AMD eyes having more intense CRP immunolabeling compared to controls. However, in advanced dry AMD eyes with geographic atrophy (GA), CRP immunoreactivity in the non-atrophic area was similar to that of age-matched controls, with CRP levels significantly reduced within the atrophic lesions. CRP was primarily immunolabeling extending into Bruch's membrane in early and wet AMD eyes (52). Interestingly, FH immunoreactivity was significantly reduced in the choroid in AMD eyes compared to controls, regardless of disease stage, suggesting an imbalance in CRP and FH levels, especially in early and wet AMD.

A recent study of human postmortem eyes examined CRP in the retina to determine which form of CRP is present in the tissue and to begin teasing out its precise role in AMD pathogenesis (29). Using monoclonal antibodies that clearly differentiate pCRP and mCRP antigens (53), the study found that mCRP is the primary form of CRP in the choroid, and it is predominantly localized to the choriocapillaris and Bruch's membrane. Similar to previous work looking at total CRP (27), mCRP is more abundant in donor eyes with the high-risk *CFH* polymorphism compared to age-matched controls. Monomeric CRP also exhibits a direct effect on CECs *in vitro*, including increasing CEC migration and increasing monolayer permeability. Furthermore, mCRP treatment of human RPE-choroid tissue *ex vivo* results in a significant upregulation of pro-inflammatory gene expression, including an increase in *ICAM1*, which has been associated with AMD previously (54). These data suggest a role for mCRP in promoting inflammation in the choroid, which may be especially true in individuals at risk for AMD development. This study revealed co-localization between mCRP and the MAC in the choriocapillaris of human postmortem eyes, providing evidence to support the hypothesis that mCRP increases complement activation in the choriocapillaris (29).

The work outlined above shows that mCRP primarily localizes to the choroid; however, other retinal cell types may be affected by the presence of this potent pro-inflammatory molecule within ocular tissue. For example, Molins et al. propose a role for mCRP in disruption of the outer blood–retinal barrier. They found that treatment with mCRP *in vitro* led to a disruption in tight junction integrity in ARPE-19 monolayers (55). Exposing ARPE-19 cells to mCRP also stimulates *IL8* and *CCL2* expression, two molecules that are important for leukocyte recruitment and blood–retinal barrier integrity (40, 56). Monomeric CRP binds necrotic RPE cells *in vitro* and enhances recruitment of FH to those cells by over 100% compared to FH recruitment without mCRP. In contrast, mCRP increases the recruitment of the Y402H variant of FH by just 50%. The mCRP-mediated recruitment of FH results in a 35% decrease in TNFα secretion from necrotic RPE cells, which is reduced to a 1% decrease for the high-risk FH molecule (28). These data indicate that without efficient recruitment of FH to RPE cells, the pro-inflammatory effects of mCRP may override the protective effects, leading to exacerbation of AMD pathology.

MODEL FOR THE ROLE OF CRP IN THE PATHOGENESIS OF AMD

The amount of CRP that gets made during an acute phase response is up to 1,000-fold beyond baseline levels. While it is unlikely that all of the pCRP in serum gets into the tissue, it is possible that much of it does reach the target tissue and dissociate into monomers. Furthermore, local production of mCRP cannot be ruled out; however, more work is required to determine the precise source(s) of mCRP in the choriocapillaris. Regardless of the source, once mCRP reaches the choriocapillaris, it may presumably outnumber the regulatory FH molecules present, which could create an imbalance in complement regulation in favor of more complement activation. Furthermore, in the choriocapillaris, where complement injury is highest in AMD, FH is the primary regulator of the complement system. This is in contrast to the RPE, which has multiple complement regulators present and, therefore, may be better armed against complement-mediated injury (57, 58). In an individual who harbors the high-risk *CFH* polymorphism, their ability to control complement, at least the alternative pathway, is further limited due to

altered binding capabilities of mutant FH to its tissue-associated ligands (e.g., glycosaminoglycans, such as heparan sulfate, within Bruch's membrane). Since mCRP is shown to be present in these tissues, it is possible that mCRP may affect complement regulation resulting in the increased MAC deposition observed in the choriocapillaris with advanced age and disease. Together, these data propose a mechanism for CEC loss in AMD pathogenesis, *via* complement-mediated attack (**Figure 1**).

Evidence suggests that mCRP also plays a key role in promoting an inflammatory environment in AMD eyes. For example, mCRP increases *ICAM1* mRNA and protein levels in human postmortem RPE-choroid tissue *ex vivo* (29). ICAM-1, which is constitutively expressed in the choriocapillaris with highest levels in the macula (59), acts to promote leukocyte recruitment in the choroid (60) and elevated levels of ICAM-1 have been associated with AMD (54). Ultimately, combining the evidence for (1) its localization to the choriocapillaris in high-risk and AMD patients, (2) its role in complement system activation and its interaction with AMD-associated complement proteins, (3) its ability to directly promote CEC activation *in vitro* and *ex vivo*, and (4) its pro-inflammatory effects on RPE-choroid tissue, mCRP is a promising target for the treatment of AMD.

THERAPEUTIC DEVELOPMENT TO TARGET INFLAMMATION AND COMPLEMENT ACTIVATION IN AMD

Treatment options for AMD are currently limited, with the most effective therapies consisting of AREDS supplements (antioxidants plus zinc), which have been shown to reduce the risk of progression beyond early or intermediate AMD by about 25–30% over 5 years (61), and intravitreal anti-vascular endothelial growth factor injections, which can help ameliorate the symptoms of wet AMD (62). The absence of a more effective and universal therapy has been a driver for the continued pursuit of novel therapeutic targets. Based on the evidence for mCRP and complement activation as key players in AMD pathogenesis, future therapies may need to target both mCRP-mediated effects in addition to complement proteins to effectively treat the disease. Many clinical trials are already completed or underway to assess treatments that target inflammation or complement activation in individuals with AMD [**Table 1**; reviewed in Ref. (63, 64)]. For example, a handful of trials have aimed to target inflammation in participants with AMD using corticosteroids such as dexamethasone [NCT01162746] and fluocinolone

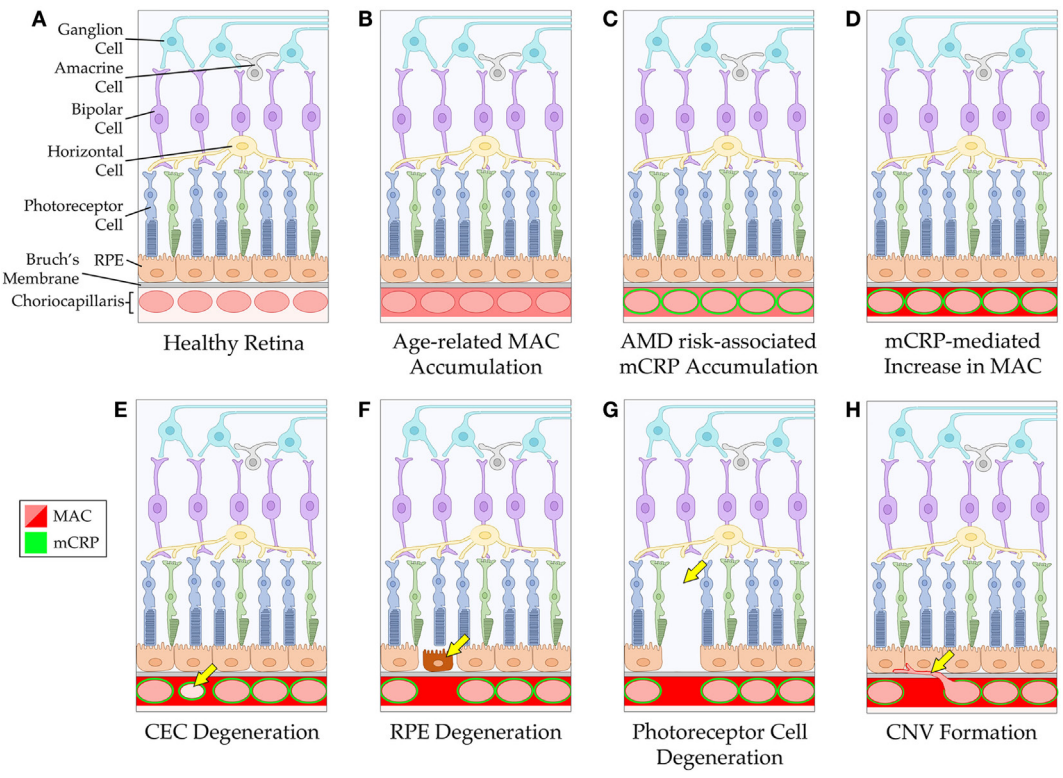

FIGURE 1 | Schematic of mCRP-associated age-related macular degeneration (AMD) pathogenesis. The healthy retina and choriocapillaris is depicted in **(A)**. With advancing age, the membrane attack complex (MAC) accumulates around the vessels of the choriocapillaris **(B)**. In individuals with an increased genetic risk for AMD (*via* the *CFH* Y402H polymorphism), mCRP accumulates around the vessels of the choriocapillaris **(C)**, and this may lead to increased complement activation and subsequent elevation in MAC levels in the tissue **(D)**. The mCRP- and/or MAC-mediated changes to the tissue environment may result in CEC death and degeneration of the choriocapillaris **(E)**. Loss of the vessels of the choriocapillaris can cause dysfunction and degeneration of the RPE **(F)**, and eventually the photoreceptor cells **(G)**. Alternatively, loss of choriocapillaris vessels can lead to choroidal neovascularization formation **(H)**. RPE, retinal pigment epithelium; CEC, choroidal endothelial cell; CNV, choroidal neovascularization.

TABLE 1 | Completed and ongoing clinical trials to reduce inflammation and complement activation in age-related macular degeneration (AMD).

Therapy	Mechanism	Route	Target	Trial identifier
Ranibizumab + dexamethasone	Anti-VEGF + corticosteroid	Intravitreal injection	CNV	NCT00793923 NCT01162746
Dexamethasone	Corticosteroid	Intravitreal implant	CNV	NCT00511706
Fluocinolone acetonide (iluvien)	Corticosteroid	Intravitreal implant	AMD	NCT00605423
Eculizumab	Humanized monoclonal antibody targeting C5	IV infusion	GA	NCT00935883
ARC1905	Anti-C5 RNA aptamer	Intravitreal injection	GA CNV	NCT00950638 NCT00709527
Zumira®	Anti-C5 aptamer	Intravitreal injection	GA	NCT02686658
LFG316	Humanized monocloncal antibody targeting C5	Intravitreal injection	GA CNV	NCT01527500 NCT01535950
LFG316 + CLG561	Humanized monocloncal antibody targeting C5 + anti-properdin antibody	Intravitreal injection	GA	NCT02515942
POT-4/Compostatin	Inhibitor of C3 cleavage	Intravitreal injection	CNV	NCT00473928
Lampalizumab	Humanized monocloncal antibody targeting CFD	Intravitreal injection	GA	NCT02247479 NCT02247531
AAVCAGsCD59	sCD59 overexpression	Intravitreal injection	GA	NCT03144999

GA, geographic atrophy; CNV, choroidal neovascularization.

acetonide (iluvien) [NCT00605423]. In addition to steroid therapies to reduce inflammation, various studies have taken aim at regulating complement system activation in AMD patients, including drugs targeting complement components C5, C3, and CFD.

Despite the promise these ongoing trials provide, many hurdles still exist in the therapeutic regulation of inflammation and complement in AMD patients, including delivery method, dose, and disease stage at time of treatment. The use of gene therapy to treat AMD may help resolve these current issues and provide a promising option for future treatments. Early studies to examine gene therapy-mediated treatments are already underway in mice [e.g., Cr2-fH fusion protein (65) and FH overexpression (66)] and in human clinical trials (AAVCAGsCD59; NCT03144999). Additional gene therapy options, such as CRISPR/Cas9-mediated gene editing, may be useful to correct high-risk variants in AMD patients, including the *CFH* Y402H polymorphism. Since advanced AMD pathology includes loss of multiple cell types within the macula, cell replacement therapy, possibly in combination with other gene editing or drug therapies, could provide the greatest promise for improved visual acuity in AMD patients. As we continue to advance our understanding of the key mediators in AMD pathogenesis, we continue to move closer to the development of life-changing treatments for millions of individuals.

AUTHOR CONTRIBUTIONS

KC and LP wrote and edited the manuscript.

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C-Reactive Protein as a Therapeutic Target in Age-Related Macular Degeneration

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Age-related macular degeneration (AMD), a retinal degenerative disease, is the leading cause of central vision loss among the elderly population in developed countries and an increasing global burden. The major risk is aging, compounded by other environmental factors and association with genetic variants for risk of progression. Although the etiology of AMD is not yet clearly understood, several pathogenic pathways have been proposed, including dysfunction of the retinal pigment epithelium, inflammation, and oxidative stress. The identification of AMD susceptibility genes encoding complement factors and the presence of complement and other inflammatory mediators in drusen, the hallmark deposits of AMD, support the concept that local inflammation and immune-mediated processes play a key role in AMD pathogenesis that may be accelerated through systemic immune activation. In this regard, increased levels of circulating C-reactive protein (CRP) have been associated with higher risk of AMD. Besides being a risk marker for AMD, CRP may also play a role in the progression of the disease as it has been identified in drusen, and we have recently found that its monomeric form (mCRP) induces blood retinal barrier disruption *in vitro*. In this review, we will address recent evidence that links CRP and AMD pathogenesis, which may open new therapeutic opportunities to prevent the progression of AMD.

Keywords: C-reactive protein, macular degeneration, aging, inflammation, retina

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INTRODUCTION

Age-related macular degeneration (AMD) is the primary cause of irreversible vision loss among the aging population worldwide. The disease affects up to 1.75 million individuals alone in the United States, and this number could increase up to 3 million by 2020 (1–3). Worldwide, the projected number of people with AMD in 2020 is 196 million (95% CrI 140–261), which increases to 288 million in 2040 (205–399) (4). AMD is a complex, degenerative, and progressive disease involving multiple genetic and environmental factors, which can ultimately result in severe visual loss. The disease-causing molecular mechanisms remain unknown, although inflammatory processes have been implicated by the identification of AMD susceptibility genes encoding complement factors (5, 6) and by the presence of complement proteins in drusen, the hallmark deposits associated with

AMD and other features of immune activation, including inflammasome activation (7–10).

The pathology of AMD is characterized by vision loss due to alterations in the macula, the central zone of the retina. Visual dysfunction in AMD is associated with the degeneration of the outer portion of the retina, the outer blood retinal barrier (oBRB), which includes the retinal pigment epithelium (RPE), the Bruch's membrane, and the choriocapillaris. This is followed (or in some cases preceded) by degeneration of the light-sensing photoreceptor cells supported by the oBRB. Degeneration of the RPE seems to begin with impaired clearance of cellular waste. The initial clinical manifestations of AMD are characterized by the presence of drusen, deposits of extracellular matrix, and pigment that form most commonly within the macula at the choroid–RPE interface. Based on the size and number of drusen, the presence of atrophy, and/or neovascularization, AMD is classified into five stages of increasing severity (11). Early and intermediate AMD are characterized by the presence of small or large drusen and RPE irregularities. Forms of late AMD include geographic atrophy and neovascularization, both of which can lead to severe central visual impairment and legal blindness due to degenerative and neovascular alterations in the macula, respectively (11, 12). Although, currently, neovascular AMD can be controlled with antiangiogenic agents that block vascular endothelial growth factor, most treated patients still suffer from visual impairment as they develop fibrosis and atrophy, and more than one-third of them show long-term loss of effect (13). Most concerning is that there is still no approved treatment for geographic atrophy.

Age is the primary risk factor for AMD. Physiological changes that occur with aging may impair cellular function in those at risk of the disease (14). In addition, other genetic and environmental risk factors are associated with AMD, most significantly smoking (15). A variety of complement pathway-associated gene variants, such as complement factor H (CFH) (16), factor B, and the complement components C2 and C3 have associations with AMD pathogenesis (17). Smoking increases the risk of the exudative type of AMD both in females and men (18, 19), and there is a direct association between AMD and raised concentration of cholesterol (20). In addition, small increases in the plasma levels of C-reactive protein (CRP) are an additional associated risk factor for AMD (21). Dietary interventions with carotenoids, oral supplementation with high levels of antioxidants and minerals, or high intake of omega-3 fatty acids and fish arguably slow the course of the disease and are implemented clinically to various degrees worldwide (22, 23). Light and photosensitization reactions may also play a role in the development of AMD *via* synthesis of reactive oxygen species, with consequent damage to the RPE and Bruch's membrane (24). Finally, chronic systemic disorders such as atherosclerosis (25), diabetes (26), and cardiovascular diseases (27) contribute to the risk for AMD development.

Although the etiology of AMD in terms of multifactorial risk factors are increasingly well documented, the patho-etiology of how oxidative stress, atherosclerotic-like changes, RPE cell dysfunction, genetic variants, and inflammation/altered tissue immune responses interlink is less well defined (28–30). One notion to enquire further is the influence of systemic immunity or alarming, acute phase responses in the progression of AMD, not dissimilar to dementia (31). In this context, elevated CRP levels are found both in the blood of AMD patients and in the eyes of carriers of a *CFH* polymorphism associated to the risk for developing the disease, providing a molecular clue to AMD pathogenesis and to how genetic risk factors may influence its course (21, 32). In this review, we summarize the main findings that support the implication of CRP in the pathogenesis of AMD and its connection with aging.

INFLAMMATION AND AMD

Chronic inflammation is a prolonged condition in which tissue injury and attempts at repair coexist, leading to tissue remodeling and dysfunction. It is the common pathological basis for age-associated diseases such as cardiovascular disease, diabetes, cancer, Alzheimer's disease, but also AMD. A multitude of bodily changes occur with aging that contribute to the initiation and development of inflammation. In particular, the immune system of elderly individuals is characterized by a basal systemic inflammatory state, as increased levels of proinflammatory cytokines and acute phase reactants are observed with aging (33). Local inflammation and immune-mediated processes play a central role in AMD pathogenesis (34–36).

A competent immune system in the eye is necessary to maintain intraocular health. The network of macrophages and microglia along with the RPE and choroidal endothelial cells maintain tissue homeostasis allowing cellular debris removal and pathogen surveillance. Besides the presence of tissue-resident immune cells, inflammatory molecules are constitutively expressed in the subretinal space, meaning that there is a persistent inflammatory state, known as para-inflammation, which deals with danger signals and protects the tissue against over-inflammation and destruction. Proteomic and histochemical analysis of ocular drusen have shown that these deposits contain inflammatory proteins and complement components that mediate local inflammation, such as C5, C9, CRP, amyloid A, fibrinogen, and vitronectin (7, 37, 38). The complement system is one of the main effectors of the innate immune response. The activation of the complement system culminates in the formation of the membrane attack complex and, potentially, cell lysis. Accumulation of membrane attack complex in the macula increases with aging and in AMD patients compared to age-matched controls (39–42). On the other hand, some diseases that are associated with complement activation have been independently linked to AMD (43), and a number of complement pathway-associated genes have been recognized as important driving factors of AMD pathogenesis. Some of these genetic variants might cause the complement system to be overactive, resulting in a chronic inflammatory condition (42, 44). This abnormal inflammatory stimulus adversely affects RPE cells and promotes drusen formation (45). The strongest genetic risk factor for AMD known to date is a common polymorphism in the CFH gene (c.1277T > C, p.Tyr402His); the CFH p.Tyr402His variant (in following termed CFH_{H402}) increases the risk for AMD approximately twofold to fourfold for heterozygous and fivefold to sevenfold for homozygous individuals (5, 16, 46).

FH is a major inhibitor of the alternate complement pathway that regulates complement activation in plasma, host cells, and

tissue, in particular, at sites of tissue inflammation, following injury or during degeneration (47). The protein is essentially comprised of 20 tandem Sushi domains, also known as short consensus repeat (SCR) or complement control protein modules. The exchanged residue in the FH_{H402} variant is located in domain SCR7, which mediates the binding to CRP, malondialdehyde (MDA), and to cell surfaces through interactions with heparan sulfate (HS) chains (48, 49). The "at risk" variant of FH shows an impaired binding to these ligands, which could result in increased complement activation and chronic local inflammation. MDA is a toxic by-product of lipid peroxidation and Weismann et al. showed that FH binds MDA through SCR7 and protects from oxidative stress. Notably, the "at risk" variant resulted in severely reduced factor-I-mediated C3 cleavage when bound to MDA (50). Regarding HS, the "non-risk" variant of FH can bind to multiple sites on HS chains in BM due to its wide specificity. Instead, the 402H variant only binds to highly sulphated motifs within HS (51, 52). Thus, if insufficient FH is present in BM, as is the case for the 402H variant, there will be increased activation of the complement cascade and the release of pro-inflammatory mediators. However, increased inflammation could be also due to the impaired binding of CRP to the FH from the "at risk" variant.

STRUCTURE AND FUNCTION OF CRP

CRP is the prototypical acute-phase reactant and an active regulator of the innate immune system; CRP levels increase rapidly in response to infection, inflammation, and tissue injury (53). It is a highly conserved protein of the pentraxin family, mainly produced in the liver. Among the multiple functions ascribed to CRP are activation of the classical complement pathway and inactivation of the alternative pathway (53). In plasma, CRP exists as a cyclic, noncovalent pentamer of 125 kDa composed of five identical subunits (pCRP), and which is stabilized by numerous electrostatic and Van-der-Waals interactions (54, 55). Native pCRP binds in a Ca^{2+}-dependent manner to phosphocholine (PCh)-containing ligands such as pneumococcal cell wall C-polysaccharide, but also to the surface of necrotic cells and parasites (55–58). Oxidative stress, low pH, and bioactive lipids from activated or damaged cells can dissociate the CRP pentamer into its 23-kDa subunits (59–62). This poorly soluble, tissue-based monomeric form (mCRP) possesses distinct biological functions compared to pCRP (60, 63–67). The dissociation mechanism of CRP requires, first, a reversible structural transition within pCRP subunits, but without disrupting the pentameric symmetry (60, 68, 69). This rapid conversion to the modified form (pCRP*) may contribute to acute phase amplification of the inflammatory response. Then, the pCRP* → mCRP irreversible transition is likely to occur at sites of persistent chronic inflammation, where the inflammatory microenvironment—characterized by acidic conditions, oxidative stress, and presence of bioactive phospholipids—continuously favors dissociation of the pentameric arrangement. mCRP would then effectively trigger proinflammatory responses and regulate complement (68). Indeed, the dissociation of circulating pCRP to mCRP in areas of inflammation has been observed *in vivo* in a rat model of acute inflammation. Mechanistically, this process is dependent on exposure of lysophosphatidylcholine (LysoPCh), a bioactive lipid that is generated after phospholipase A2 activation on activated cell membranes (62, 70).

The crystal structure of native pCRP in complex with PCh shows how large PCh-containing ligands may be specifically bound by CRP and offers clues to the mechanism of mCRP formation (54). The PCh ligand-binding site is located in a groove of a β-sheet on the so-called "B face" of the pentamer (**Figure 1**). Multipoint attachment of this planar face of the CRP molecule to a PCh-bearing surface would leave available, on the opposite A face, the recognition sites for complement C1q. In the absence of Ca^{2+}, residues 140–150 form a loop that projects away from the body of each CRP subunit exposing a normally hidden proteolysis site. Cleavage at this site facilitates that individual CRP subunits move apart, thus exposing a neoepitope (residues 199–206, colored yellow in **Figure 1C**) that is recognized by anti-mCRP-specific antibodies (9C9 or 3H12) (68, 71). The globular head of C1q is then able to insert itself into the inner annular void of pCRP* (the relaxed conformation) forcing the subunits further apart (noteworthy, C1q is unable to bind to the "strained" pCRP conformation) (68). Finally, the CRP subunits might dissociate, likely accompanied by partial unfolding to generate the mCRP form (72, 73). This process would enable CRP to target physiologically and/or pathologically significant complement activation.

MOLECULAR CHANGES IN THE OBRB IN AMD: INTERACTION BETWEEN CRP AND FH

The fact that patients with AMD and individuals with the CFH_{H402} variant show increased systemic and local levels of CRP, respectively, provides a molecular hint on the pathogenesis of AMD. AMD lesion formation has been proposed to share mechanisms with atherosclerotic plaque formation, which is initiated with low-density lipoprotein retention within the arterial wall (74). Although a thorough discussion of the cross-connections between CRP and cardiovascular diseases is beyond the scope of this review, it is noteworthy that patients with clinical evidence of atherosclerosis (stroke, coronary, and peripheral artery disease) show modestly but significantly increased CRP levels (25, 75, 76).

Seddon and coworkers were the first to address the relationship between elevated CRP concentrations and AMD progression. They found a significant increase in circulating CRP levels as the disease progressed, and showed that low-, medium-, and high-risk AMD groups are associated with serum CRP concentrations below 0.5, between 0.5 and 10.0, and over 10.0 mg/L, respectively (21, 77, 78). However, this association has not been universally confirmed (79). A more recent study by the Seddon group shows that high levels of circulating CRP are associated with a higher risk of AMD, regardless of the *CFH* genotype (80).

Other authors have recently attempted to triangulate the association between plasma concentrations of CRP, four *CRP* genetic variants reported to influence CRP circulating levels, and the risk of advanced AMD (81). They found that two of the genetic variants do share some association with plasma CRP concentrations.

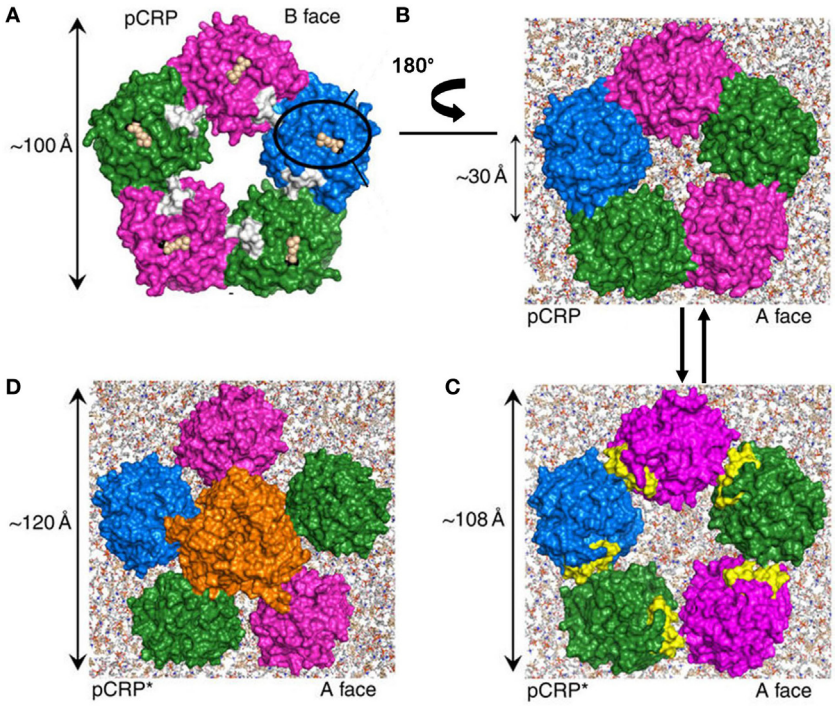

FIGURE 1 | Proposed model of the conversion from the strained to the relaxed conformations of pentameric C-reactive protein (CRP). **(A)** Solid surface representation of the crystal structure of human CRP in its "strained" conformation (pCRP) bound to phosphocholine (PCh) (PDB entry 1B09) (54). The view shown is from the membrane binding "B face" of pCRP. The individual subunits are represented color-coded, with PCh (cream spheres) and Ca^{2+} ions (black spheres) occupying the ligand binding site on each subunit. **(B)** Modeled interaction of pCRP with a phospholipid bilayer. View is from "above," looking down onto the pCRP "A face." Each pCRP subunit can independently bind to a PCh head group of the bilayer. Exposure to lysoPCh triggers reversible conversion of pCRP to pCRP*. **(C)** Pentameric pCRP*, same view as in **(B)**. As the individual CRP subunits move apart, a neoepitope (colored yellow) is exposed. **(D)** The globular head of C1q inserts itself into the inner annular void of pCRP* forcing the subunits further apart [adapted from Braig et al. (68)].

However, none of the four variants was significantly associated with the risk of AMD. Their findings have important implications for our understanding of the pathophysiology of AMD, in particular, for the distinct roles played by local and systemic inflammation in this regard. However, it must be considered that other factors such as *C3* genotype and smoking strongly affect circulating CRP levels. Thus, these results do not preclude a direct link between AMD pathophysiology through complement activation and chronic inflammation, and plasma CRP concentrations might still be useful as an AMD biomarker. Important differences exist between systemic inflammation and the local inflammatory macular tissue microenvironment.

Since CFH risk haplotypes are associated with increased complement activation in human macular tissue (82) but not in the circulation (83, 84), it is important to determine the localization and abundance of both CRP and FH in the extra-macular choroid of individuals homozygous for the high-risk CFH_{H402} genotype, as compared to those homozygous for the low-risk CFH_{Y402} variant. This investigation, reported by Johnson and colleagues, showed that the localization and abundance of FH do not differ between *CFH* genotypes (32). However, choroidal immuno-staining of CRP was significantly higher in the CFH_{H402} eyes compared to the CFH_{Y402} eyes. Interestingly, these differences between the *CFH* homozygotes were independent of AMD status. Because the high-risk allele affects binding of FH to CRP (85), deficient FH binding could potentially increase the pro-inflammatory activity of CRP in choroidal tissue, contributing to AMD pathogenesis. Also along these lines, Bhutto and colleagues have reported distinct patterns of localization for FH and CRP in the aging eye. Most notably, these authors found an inverse relationship between CRP and FH levels in macular tissue from patients with advanced AMD as compared to age-matched control individuals (86). In AMD patients, Bruch's membrane, drusen, and choroidal vessel walls all showed increased labeling of CRP and decreased labeling of FH compared to controls. These findings support the idea that the macula of AMD patients has an increased inflammatory microenvironment with decreased capacity for complement inhibition.

Although FH is known to bind CRP, there was certain controversy regarding the relevance of the monomeric and pentameric forms in this regard. For instance, two separate binding sites for pCRP were located on domains SCR4-6 and SCR16-20, respectively (49). On the other hand, FH showed strong binding to denatured, monomeric CRP, rather than to the native multimeric form (87, 88). We have recently shown that mCRP, but not the pentameric form, contributes to oBRB disruption *in vitro* (89). The functional integrity of the RPE, critical for the maintenance of the specialized environment of the neural retina, is dependent

on the structures of tight junctions. Exposure to mCRP, but not pCRP, significantly increased the paracellular permeability of the RPE compared with that of untreated cells, suggesting that mCRP could compromise the barrier function of the RPE monolayer. Notably, mCRP was also able to disturb the expression and distribution of the TJ proteins, ZO-1, and occludin. In another study, we also showed that mCRP confers a proinflammatory phenotype to RPE cells as it increases production of the proinflammatory cytokines IL-8 and CCL2 (**Figure 2**) (90). The mCRP-induced pro-inflammatory phenotype was further demonstrated by the significantly increased rates of peripheral blood mononuclear cells migration treated with conditioned medium from RPE cells after being exposed to mCRP, but not with conditioned media from either untreated cells or from cells exposed to pCRP. The oBRB disruption induced by mCRP could conceivably permit passage of inflammatory cells into the retina, further contributing to chronic inflammation and accelerating tissue damage.

Moreover, we also showed that the "non-risk" FH variant (CFH_{Y402}) can effectively bind to mCRP to dampen mCRP pro-inflammatory activity. Notably, FH from AMD patients carrying the risk polymorphism for AMD shows an impaired binding to mCRP and, therefore, its proinflammatory effects remain unrestrained (**Figure 2**) (90). In line with and highlighting our findings, Chirco et al. have recently shown that mCRP is the more abundant form of CRP in human RPE-choroid, and that mCRP levels are elevated in individuals with the high-risk CFH genotype (91), which could thus sustain chronic inflammation contributing to the progression of AMD in CFH_{H402} individuals. Moreover, pro-inflammatory mCRP significantly affects endothelial cell phenotypes, suggesting a role for mCRP in choroidal vascular dysfunction in AMD as well.

It is also interesting to note that, in our cohort of AMD patients, those carrying the risk variant of *CFH* had significantly higher levels of systemic IL-8 and CRP than healthy subjects carrying the non-risk allele (90). Further, the levels of these proteins were positively correlated in AMD patients homozygous for the risk CFH_{H402} variant. These results are in conflict with previous studies showing that CRP levels and the CFH_{H402} polymorphism were independent risk factors for AMD (80). We observed differences in circulating CRP concentrations between subjects carrying the different *CFH* variants, albeit in a smaller population. Our results

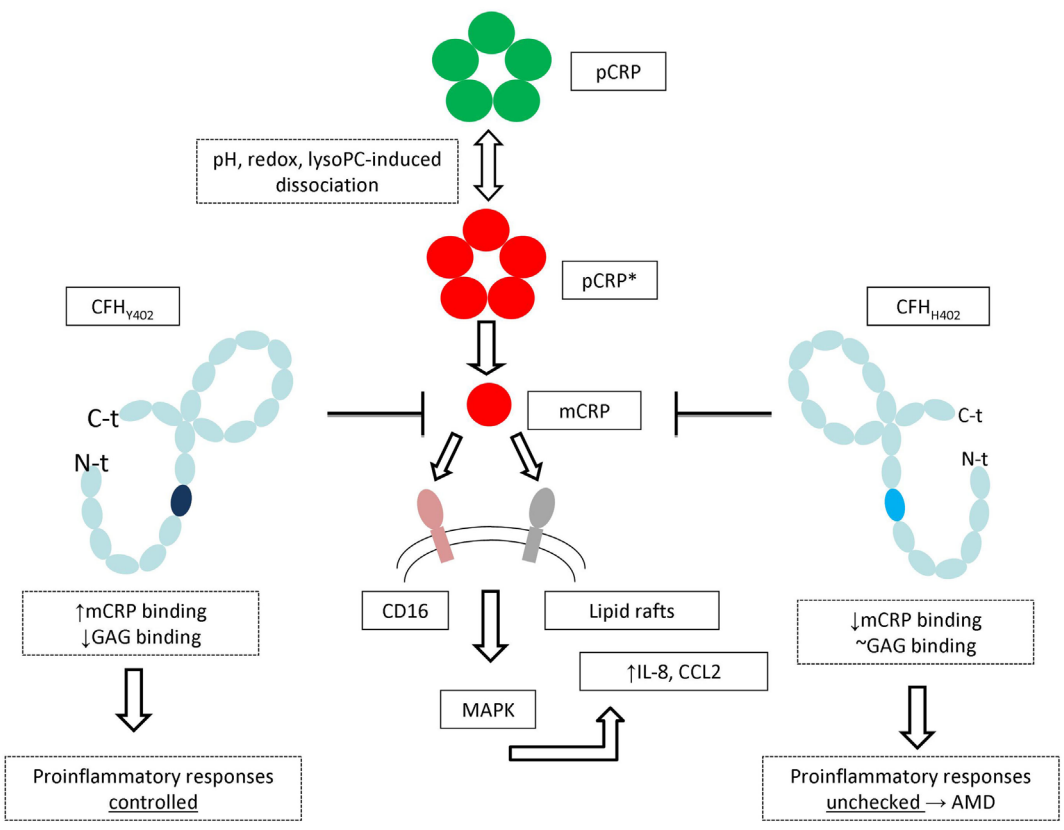

FIGURE 2 | A unified mechanism of mCRP-induced proinflammatory responses and the role of the *CFH* p.Tyr402His polymorphism in age-related macular degeneration (AMD). Generation of mCRP is accelerated *in vivo* under inflammatory conditions by bioactive lipids such as lysophosphatidylcholine (lysoPC) exposed on the surface of microparticles, activated or damaged cells. mCRP is recognized on the cell surface, leading to activation of MAPK pathways and ultimately enhances expression of proinflammatory cytokines and disrupts the outer blood retinal barrier. Binding of FH to mCRP attenuates this inflammatory response, but the FH_{H402} variant is less effective in this regard, both because of its altered binding to glycosaminoglycans (52), but, in particular, due to its markedly lower affinity for mCRP. The unchecked inflammatory response leads eventually to progression of AMD and vision loss [figure adapted from Molins et al. (90)].

might explain the previously reported higher risk of AMD within genetically susceptible individuals when CRP concentrations are high (80). We hypothesize that higher levels of circulating CRP could derive in higher mCRP concentrations in microenvironments that favor dissociation, such as inflammatory or apoptotic conditions, which in the case of patients carrying the CFH_{H402} risk variant would further cause unchecked inflammation. However, it is unclear where, when, and how mCRP dissociates within the oBRB. mCRP could either dissociate distantly in activated endothelium or locally within the RPE. Unchecked mCRP activity may sustain chronic inflammation thus favoring AMD progression. Whether this provokes disease or not requires validation, but this process alone may not be sufficient to explain all the immune-related changes we observe in AMD and, therefore, further research is warranted.

TARGETING MONOMERIC CRP IN AMD

The recent findings from us and others discussed above reinforce the importance of mCRP in chronic inflammation and point to the pCRP dissociation process and/or mCRP itself as novel therapeutic targets for AMD. Indeed, therapies associated with a reduction in systemic CRP levels are successfully used in other chronic inflammatory diseases such as atherosclerosis, where CRP is an important player (76). However, given that CRP may have a more important role in the macular tissue, it might be more appropriate to target local CRP for AMD treatment. Blocking the dissociation of pCRP with 1,6-bis-PCh, a compound that stabilizes CRP in a decameric form, abolished the proinflammatory effects of mCRP *in vivo* (62). Restrictively, 1,6-bis PCh is not suitable for clinical purposes due to its pharmacokinetics and its low affinity for pCRP (150 nM) (62). Thus, a more potent drug with higher oral bioavailability, higher affinity for pCRP, and prolonged half-time needs to be designed to efficiently target the pCRP dissociation process as an innovative therapeutic strategy. Blocking LysoPCh formation with PLA2 inhibitors may be another interesting approach to inhibit pCRP dissociation. Alternatively, therapeutic approaches aimed to enhance FH-mCRP binding could be developed to block mCRP proinflammatory activities, thus preventing the progression of AMD.

CONCLUSION

The reduced ability to control the balance between pro- and anti-inflammatory signals associated with aging might promote a switch to chronic inflammation in the macular tissue. This scenario could then favor CRP dissociation and mCRP accumulation further fueling chronic inflammation and tissue damage, especially in those patients with the "risk" FH variant, CFH_{H402}, where FH is unable to dampen mCRP proinflammatory activity and to localize to HS in Bruch's membrane. A combination of poor binding of the FH H402 variant to Bruch's membrane and mCRP, combined with aging associated processes such HS loss and an increased proinflammatory environment, may eventually result in complement activation, persistent mCRP-induced inflammation, and thereby contribute to AMD progression. Future research is warranted to confirm the contribution of mCRP to disease etiology and progression, and eventually to test the therapeutic potential of compounds that either prevent CRP dissociation or stimulate FH binding to mCRP.

AUTHOR CONTRIBUTIONS

BM, PF-P, SR-V, AA, and AD contributed to the design of the project and manuscript preparation, and all authors reviewed the manuscript.

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C-Reactive Protein Impairs Dendritic Cell Development, Maturation, and Function: Implications for Peripheral Tolerance

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C-reactive protein (CRP) is the prototypical acute phase reactant, increasing in blood concentration rapidly and several-fold in response to inflammation. Recent evidence indicates that CRP has an important physiological role even at low, baseline levels, or in the absence of overt inflammation. For example, we have shown that human CRP inhibits the progression of experimental autoimmune encephalomyelitis (EAE) in CRP transgenic mice by shifting $CD4^+$ T cells away from the T_H1 and toward the T_H2 subset. Notably, this action required the inhibitory Fcγ receptor IIB (FcγRIIB), but did not require high levels of human CRP. Herein, we sought to determine if CRP's influence in EAE might be explained by CRP acting on dendritic cells (DC; antigen presenting cells known to express FcγRIIB). We found that CRP (50 μg/ml) reduced the yield of $CD11c^+$ bone marrow-derived DCs (BMDCs) and CRP (≥5 μg/ml) prevented their full expression of major histocompatibility complex class II and the co-stimulatory molecules CD86 and CD40. CRP also decreased the ability of BMDCs to stimulate antigen-driven proliferation of T cells *in vitro*. Importantly, if the BMDCs were genetically deficient in mouse FcγRIIB then (i) the ability of CRP to alter BMDC surface phenotype and impair T cell proliferation was ablated and (ii) CD11c-driven expression of a human *FCGR2B* transgene rescued the CRP effect. Lastly, the protective influence of CRP in EAE was fully restored in mice with CD11c-driven human FcγRIIB expression. These findings add to the growing evidence that CRP has important biological effects even in the absence of an acute phase response, i.e., CRP acts as a tonic suppressor of the adaptive immune system. The ability of CRP to suppress development, maturation, and function of DCs implicates CRP in the maintenance of peripheral T cell tolerance.

Keywords: acute phase response, aging, autoimmunity, inflammaging, inflammation, transgenic

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INTRODUCTION

Inflammation is a normal local response to tissue injury and infection. If the insult is sufficiently strong there will follow a systemic response, termed the acute phase response (APR), during which leukocytes release inflammatory mediators (primarily IL-6, IL-1, and/or TNFα) into the circulation that sequentially propel a diversity of effects. During the APR, the liver increases the synthesis of a

number of pattern recognition proteins. Among these C-reactive protein (CRP) is the prototype; it is maintained at low levels in normal sera (1–5 μg/ml) (1), but can reach upwards of ~500 μg/ml during inflammation (2). CRP's ability to activate complement, opsonize microbes, bind to phosphatidylserine on apoptotic cells, and bind Fc receptors is well known (2–4) and these biological actions have been studied extensively in the context of CRP's upregulation during inflammation. Increasing evidence indicates that CRP also exerts important biological influences even when its levels remain low as in healthy individuals and when it is only slightly raised as in aging individuals (4).

Previously, we have shown that human CRP transgenic mice (CRPtg) are resistant to experimental autoimmune encephalomyelitis (EAE), a disease comparable to human multiple sclerosis (MS) i.e., they have delayed onset of disease and milder clinical symptoms compared to wild type (WT) mice. Notably, despite the ability of CRPtg to mount a robust human CRP acute phase response, this protection does not require high levels of human CRP. We initially attributed CRP's protective action in EAE to inhibition of encephalitogenic T cells, since *in vitro* CRP reduced T cell proliferation and shifted their cytokine production toward a less noxious T_H2 profile (5). Our subsequent studies demonstrated that FcγRIIB$^{-/-}$ mice, which lack expression of this inhibitory receptor, were refractory to CRP's protective action (6), but we did not identify the FcγRIIB-expressing cell(s) that CRP relied upon. Herein, we demonstrate that CRP impairs the development of bone marrow (BM) cells into CD11c$^+$ dendritic cells (DCs), professional antigen presenting cells that express ample FcγRIIB (7), are paramount for robust T cell responses (8), and are known to contribute to EAE/MS (9, 10, 11).

At doses as low as 5 μg/ml, CRP significantly prohibited bone marrow-derived DCs (BMDC) activation/maturation in response to stimulation with lipopolysaccharide (LPS), and impaired the ability of BMDCs to promote antigen-specific T cell proliferation. These suppressive actions of CRP were not evident using FcγRIIB$^{-/-}$ BMDCs, but BMDCs from FcγRIIB$^{-/-}$ mice genetically reconstituted to express a CD11c-driven human FcγRIIB transgene ($_{cd11c}$FcγRIIB^{hu}) were responsive to CRP, i.e., CRP prohibited their activation/maturation in response to LPS and suppressed their ability to promote T cell proliferation. As we previously reported, CRPtg were more resistant to EAE compared to WT, whereas CRPtg lacking expression of endogenous FcγRIIB (FcγRIIB$^{-/-}$/CRPtg), were not. For the latter, however, expression of the CD11c-specific human FcγRIIB transgene fully reconstituted human CRP-mediated protection from EAE.

Based on these new findings, we propose that CRP acts as an endogenous down-regulator of DC development and activation/maturation, thereby acting as a brake on T cell mediated immunity and shifting the balance toward tolerance. Given that many of the effects of CRP on DCs were observed using ≤10 μg/ml, it is likely that even modest elevation of blood CRP—like that associated with aging (12)—is sufficient to significantly affect T cell tolerance.

MATERIALS AND METHODS

Mice

Our human CRPtg have been fully described elsewhere (13, 14). In brief, CRPtg (C57BL/6 background) carry a 31-kb human DNA fragment encoding the *CRP* gene and all the *cis*-acting elements required for tissue specificity and acute phase inducibility, while the *trans*-acting factors required for its human-like pattern of regulation are conserved from mouse to man (13, 14). Consequently, unlike WT, CRPtg exhibit a robust human CRP acute phase response during inflammation. FcγRIIB deficient mice (FcγRIIB$^{-/-}$; B6.129S4-*Fcgr2b*^{tm1TtK} N12) (15) were purchased from Taconic Farms (Germantown, NY). 2D2 mice [C57BL/6-Tg(Tcra 2D2, Tcrb 2D2) 1Kuch/J] (16) are transgenic for a T cell receptor (TCR) that recognizes residues 35–55 of myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) and were purchased from Jackson Laboratories (Bar Harbor, ME, USA; JAX 006912). OT-II mice [B6.Cg-Tg(Tcra Tcrb)425Cbn/J] (17) are transgenic for a TCR that recognizes residues 323–339 of ovalbumin ($OVA_{323–339}$) and were purchased from Jackson Laboratories (Bar Harbor, ME, USA; JAX 004194). FcγRIIB$^{-/-}$ mice expressing a human *FCGR2B* transgene driven by a mouse CD11c minimal promoter ($_{cd11c}$FcγRIIB^{hu}) were generated herein and are fully described in the Section "Results." To date, no embryonic lethality or unusual phenotype has been observed for $_{cd11c}$FcγRIIB^{hu}. C57BL/6 mice (WT) were obtained from the Jackson Laboratories (Bar Harbor, ME, USA; JAX 000664). All mice were housed in the same vivarium at constant humidity (60 ± 5%) and temperature (24 ± 1°C) with a 12-h light cycle (6:00 a.m. to 6:00 p.m.), and maintained *ad libitum* on sterile water and regular chow (Harlan Teklad). Mice were 8–12 weeks old when used and both sexes were combined unless specifically noted. All animal use protocols were approved by the Institutional Animal Care and Use Committees at the University of Alabama at Birmingham and were consistent with the *Guide for the Care and Use of Laboratory Animals; Eighth Edition* (NIH Academies Press, 2011).

BMDC Cultures

Bone marrow progenitors were grown under conditions known to drive DC generation and expansion (18, 19). Briefly, BM was harvested from femurs, the red blood cells lysed (Hybri-Max Red Blood Cell Lysing Buffer; Sigma, Salem, MA, USA), and the marrow passed through a 70 μM cell strainer and brought to single-cell suspension in RPMI 1640 (Gibco, Grand Island, NY) containing 5% fetal bovine serum (Gibco), 1% Penicillin/Streptomycin (Gibco), 2 mM GlutaMAX™ (Invitrogen), non-essential amino acids (Gibco), 55 μM β-mercaptoethanol (Gibco), and 20 ng/ml granulocyte macrophage-colony stimulating factor (Shenandoah Biotechnology, Warwick, PA, USA). BM progenitors were then added to 12-well tissue culture plates (1×10^6 cells in 1 ml per well) that were incubated at 37°C, 5% CO_2 for 7 days. The culture medium was replaced on days 3 and 5. On day 5, cells were exposed to 50 μg/ml of highly purified human CRP (endotoxin and azide-free CRP from US Biological; Salem, MA, USA), purified chicken $OVA_{323–339}$ peptide (MISC-011; CPC Scientific, San Jose, CA, USA), or purified $MOG_{35–55}$ peptide (12668-01; Biosynthesis Inc., Lewisville, TX, USA). $OVA_{323–339}$ and $MOG_{35–55}$ loaded BMDCs were subsequently used in BMDC:T cell co-cultures with OT-II and 2D2 T cells, respectively, as described below. To trigger BMDC maturation in some experiments LPS from *Escherichia coli,* serotype 055:B5 (Sigma Aldrich) was added (1 μg/ml) on day 6. Alternatively,

culture medium was supplemented with 100 ng/ml interleukin-4 (IL-4; Shenandoah Biotechnology, Warwick, PA, USA). IL-10 and IL-12p70 production was assessed by ELISA (88-7105-22 and 88-7121-22; Invitrogen, Eugene, OR, USA) according to the manufacturer protocol. Flow cytometry was performed on a BD LSR-II cytometer (described below) and, after excluding dead cells and aggregated cells, BMDCs were identified as $CD11b^+$ $CD11c^+$ cells. For detailed analysis of cell death, cells were stained with Annexin V and 7-AAD and were defined as early apoptotic (Annexin V^+ $7\text{-}AAD^-$), late apoptotic (Annexin V^+ $7\text{-}AAD^+$), necrotic (Annexin V^- $7\text{-}AAD^+$), or live (Annexin V^- $7\text{-}AAD^-$).

T Cells and BMDC:T Cell Cocultures

From OT-II and 2D2 mice, the spleens and lymph nodes (axillary, brachial, inguinal) were harvested and mechanically homogenized, the red blood cells lysed, and the homogenate passed through a 70 μM cell strainer, and brought to single-cell suspension in media at 1×10^8 cells/ml. $CD4^+$ T cells were enriched by negative selection according to the manufacturer's guidelines using a kit from StemCell Technologies (Vancouver, BC, Canada). Enriched $CD4^+$ T cells were then stained for 20 min with 1 μM CellTrace™ carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Eugene, OR, USA). BMDCs, cultured as described above, were treated with $MOG_{35–55}$ or $OVA_{323–339}$ peptide on day 6. On day 7, the peptide-loaded BMDCs were mixed with the freshly isolated and CFSE-stained $CD4^+$ T cells (1:5 ratio in triplicate), placed into 96-well round bottom plates, and incubated for 3 days before analysis of $CD4^+$ T cell proliferation (dilution of CFSE). BMDC:T cell co-cultures exposed to plate-bound anti-CD3ε and soluble anti-CD28 antibodies (both from Biolegend, San Diego, CA, USA) served as positive controls.

Antibodies and Flow Cytometry

Cells were washed with PBS, spun down at 300 × *g* for 5 min at 4°C, stained with the viability dye eFluor 780 (eBioscience, San Diego, CA, USA) for 30 min at room temperature, fixed in Fixation Buffer (Biolegend, San Diego, CA, USA) for 10 min at room temperature, blocked with anti-mouse CD16/32 (clone 93; eBioscience) at 4°C for 15 min, and stained with specific antibodies at 4°C for 30 min. For BMDCs, we used anti-mouse CD11c (clone N418), MHC class II IA/IE (clone M5/114.15.2), CD40 (clone HM40-3), CD80 (clone 16–10 A1), CD86 (clone GL-1) (all from Biolegend), and FcγRIIB (clone AT 130-5, Bio Rad, Hercules, CA, USA), and anti-human FcγRIIB (clone AT 10, AbD Serotec, Raleigh, NC, USA). For T cells we used anti-mouse CD4 (clone RM4-5) (Biolegend). Stained and labeled cells were run on a BD LSR-II cytometer and the acquired data analyzed using BD FACSDiva version 6.1.3 and FlowJo version 10.3. For all gating analyses, debris was gated out using a FSC by SSC dot plot, followed by selection of single cells using a SSC-A by SSC-H dot plot, and live cells were selected based on the viability dye eFluor 780 dot plot. For assessment of T cell proliferation (CFSE dilution), the bounds for the $CD4^+$ $CFSE^+$ "parents" gate was determined using unstimulated T cells and the bounds for the "progeny peaks" were based on anti-CD3ε/anti-CD28 stimulated T cells (see **Figure 3A**). As T cells divide, the progeny:parent ratio increases.

Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis was induced as we described previously (5, 6, 20). Briefly, 10–12-week-old mice were immunized subcutaneously with 150 μg $MOG_{35–55}$ emulsified in Freund's complete adjuvant plus 400 μg heat-killed *Mycobacterium tuberculosis* (Difco, Detroit, MI, USA). On days 0 and 2, mice received an intraperitoneal injection of 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA). For 30 days thereafter the development of EAE was monitored daily. EAE symptoms were scored on a clinical scale ranging from 0 to 6 as follows: 0, asymptomatic; 1, loss of tail tone; 2, flaccid tail; 3, incomplete paralysis of one or two hind limbs; 4, complete hind limb paralysis; 5, moribund (at which case the mouse was humanely euthanized); 6, dead. For mice that developed EAE, the day of onset was defined as the first of two consecutive days, wherein the clinical score was ≥2.

Statistical Analysis

Raw data were pooled and are expressed graphically as the mean ± SEM or SD, as noted. Group comparisons were done using one-way analysis of variance (*ANOVA*) followed by *post hoc* Bonferroni's and Tukey's multiple comparison tests, or using linear trends tests. Differences were considered significant when p was <0.05. For EAE, the maximum clinical score achieved by each animal during the 30-day observation period was used to calculate the average maximum clinical score (a measure of severity). To study the time-course of disease, average clinical scores were calculated and plotted daily for each group of mice, and cumulative disease index (CDI) was calculated by area under the curve analysis. Statistical analyses were done using GraphPad Prism version 7.00.

RESULTS

CRP Suppresses Generation and Maturation of BMDCs

We first examined the influence of human CRP on the generation of DCs from BM progenitors. On day 7 of culture, nearly 90% of all cells were viable (dashed horizontal lines in **Figure 1A**) and the cultures routinely achieved a yield of nearly 75% BMDCs (dashed horizontal line in **Figure 1B**). Whether CRP at 10 or 100 μg/ml was added on day 0 or 6 of culture it had no significant effect on cell viability (**Figure 1A**). CRP treatment also had no effect on the proportion of early apoptotic, late apoptotic, and necrotic BM cells (data not shown). However, CRP treatment did significantly decrease (by 10–15%) the proportion of $CD11b^+$ $CD11c^+$ BMDCs that developed (**Figure 1B**). Notably, when CRP was added at the initiation of culture, the inhibitory effect on the final yield of BMDCs was strongest (**Figure 1B**) and was dose-dependent (**Figure 1C**). These results show that while CRP has no significant influence on the viability of cultured BM progenitors, it does significantly impede the generation of $CD11b^+$ $CD11c^+$ BMDCs in both a temporal and dose-dependent manner.

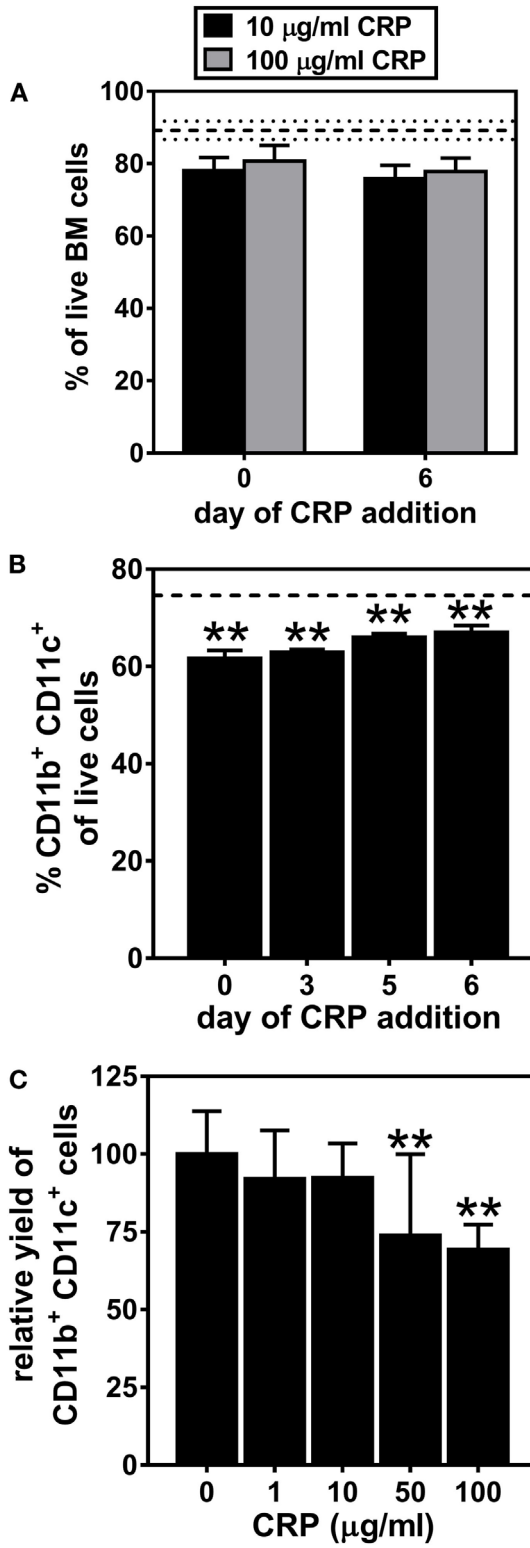

FIGURE 1 | Continued

FIGURE 1 | C-reactive protein (CRP) impedes the generation of CD11b$^+$ CD11c$^+$ bone marrow-derived dendritic cells (BMDCs) in a temporal and dose-dependent fashion. **(A)** CRP addition to bone marrow cultures on day 0 or on day 6 had no significant effect on cell viability. The horizontal dashed lines indicate cell viability of 89.2% ± 2.52 (mean ± SD) without CRP. **(B)** The proportion of live cells that were CD11b$^+$ CD11c$^+$ BMDCs was significantly reduced by addition of CRP (50 μg/ml) on the indicated day of culture. The horizontal dashed line indicates the average proportion of BMDCs generated in the absence of CRP (74.6% ± 0.57 SD). **(C)** The relative yield of CD11c$^+$ BMDCs was reduced in a dose-dependent fashion by CRP (1–100 μg/ml) added on day 0 of culture. The symbols indicate the results of one-way analyses of variance with Tukey's multiple comparisons tests compared to cultures not treated with CRP, $p < 0.005$ (**) ($n = 3$–9 per group).

Next, we assessed the influence of CRP on activation/maturation of BMDCs. Treating immature BMDCs with CRP (50 μg/ml) had no effect on their surface expression of MHC class II, CD86, CD40, and CD80 (**Figure 2A**), whereas treatment of immature BMDCs with LPS (1 μg/ml) significantly upregulated these markers (**Figure 2A**), indicative of BMDC maturation. While CRP did not trigger BMDC maturation, CRP did significantly inhibit the LPS-triggered increase in surface expression of MHC class II and the co-stimulatory markers, CD86 and CD40 (**Figure 2A**). Also, the suppressive effect of CRP on LPS-triggered BMDC maturation was dose-dependent, as evidenced by a stepwise reduction of MHC class II, CD86, and CD40 (**Figure 2B**). This suppressive effect was specific as CRP had no effect on the expression of CD80, CD11b, or CD172a (**Figure 2B**). Finally, BMDCs treated with LPS (1 μg/ml) robustly produced both the T cell suppressive cytokine IL-10 and the T cell stimulatory cytokine IL-12p70 (225.7 ± 8.8 and 1245.8 ± 191.0 ng/ml, respectively), but the production of both cytokines was significantly suppressed by CRP (no detectable IL-10 and 773.2 ± 13.2 ng/ml IL-12p70; $p < 0.05$, *t*-tests). These data demonstrate that CRP dose-dependently prohibits LPS-triggered activation/maturation of BMDCs and limits their production of IL-10 and IL-12p70, cytokines with pleiotropic effects in immunoregulation.

CRP Inhibits BMDC-Mediated Stimulation of Antigen-Specific T Cell Proliferation

We next sought to determine if the observed effects of CRP on BMDC activation/maturation phenotype and cytokine production affects their T cell stimulatory function. We found that CRP (1–100 μg/ml) had no significant effect on the proliferation of OT-II T cells co-cultured with BMDCs in the absence of any stimulus (**Figure 3B**; nil) or in the presence of T cell activating antibodies (**Figure 3B**; CD3/CD28). Importantly, however, when BMDCs loaded with $OVA_{323–339}$ peptide were used as APCs, the addition of CRP caused a dose-dependent inhibition of OT-II T cell proliferation (**Figure 3B**; OVA). Using the MOG TCR-transgenic model (2D2) we obtained similar results, i.e., CRP (50 μg/ml) significantly inhibited the proliferation of 2D2 T cells co-cultured with BMDCs loaded with $MOG_{35–55}$ peptide (**Figure 3C**). These data confirm that CRP's prohibition of BMDC activation/maturation and cytokine production reduces their ability to stimulate antigen-specific T cell proliferation. The fact that in both model systems, CRP had no effect on T cells directly stimulated with anti-CD3ε/anti-CD28 antibodies shows that CRP's influence on T cell proliferation must be *via* its actions on BMDCs.

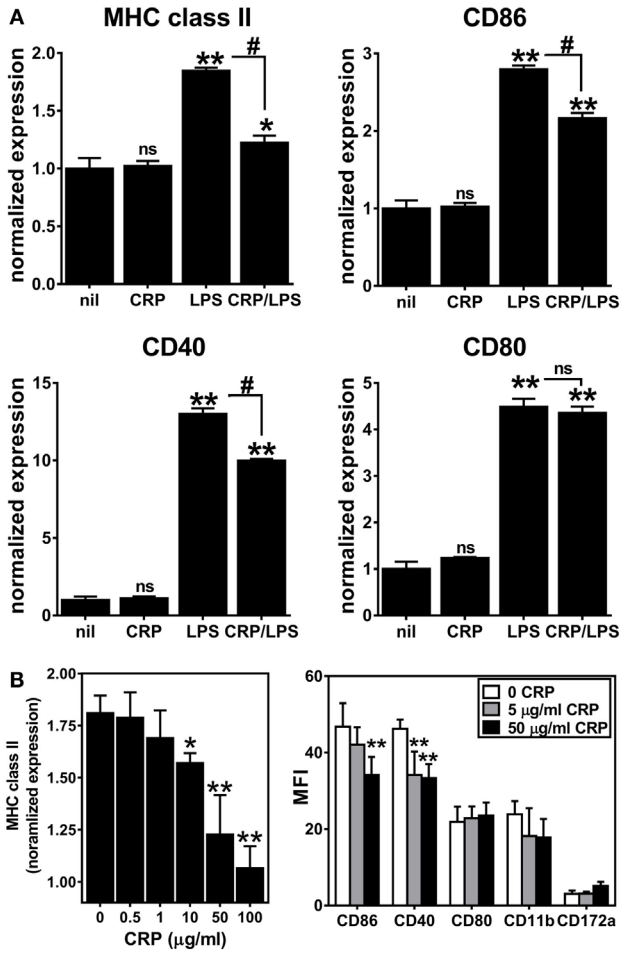

FIGURE 2 | C-reactive protein (CRP) suppresses expression of MHC class II, CD86, and CD40 on lipopolysaccharide (LPS)-matured bone marrow-derived dendritic cells (BMDCs) in a dose-dependent manner. **(A)** Surface expression of MHC class II, CD86, CD40, and CD80 on immature CD11b$^+$ CD11c$^+$ BMDCs left untreated (nil) or treated with CRP (50 μg/ml on day 5), and on BMDCs matured with LPS (1 μg/ml on day 6) or treated with CRP (50 μg/ml on day 5) and LPS (1 μg/ml on day 6) (CRP/LPS). Expression of each marker (MFI of flow cytometry) is normalized to expression on untreated immature BMDCs (nil). The symbols above each bar indicate not significant (ns), $p < 0.05$ (*), or $p < 0.005$ (**) compared to "nil." The symbols above each bracket indicate ns or $p < 0.005$ (#) for the LPS versus CRP/LPS groups. One-way analyses of variance (ANOVAs) with Tukey's multiple comparisons tests. **(B)** CRP dose-dependent suppression of expression of MHC class II (left) and CD86 and CD40 (right) by LPS-treated BMDCs. MHC class II expression is normalized as in **(A)**. The symbols indicate the results of one-way ANOVAs with Tukey's multiple comparisons tests, $p < 0.05$ (*) and $p < 0.005$ (**) compared to no CRP ($n = 2$–6 per group).

CRP Does Not Prohibit the Activation/Maturation of FcγRIIB$^{-/-}$ BMDCs

C-reactive protein binds to both activating and inhibitory Fc receptors, thereby triggering a diversity of cellular responses *in vitro* (2, 21) and many of the *in vivo* biological actions of human CRP in CRPtg are fully supported by FcγRIIB (6, 22). Since FcγRs *per se*, and FcγRIIB in particular, are widely expressed by both human and mouse DCs (7), we generated

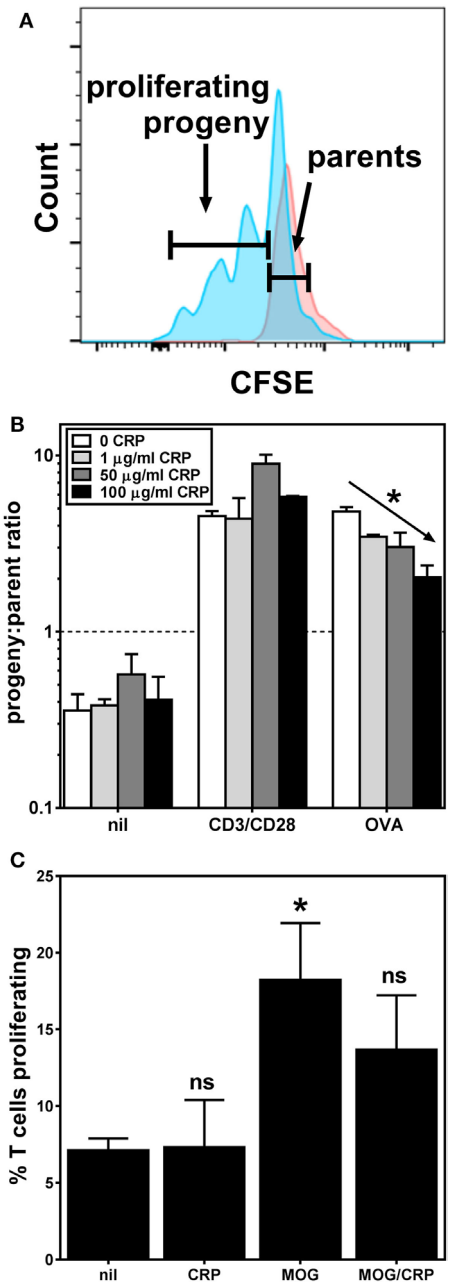

FIGURE 3 | C-reactive protein (CRP) inhibits bone marrow-derived dendritic cell (BMDC)-mediated/antigen-driven T cell proliferation. **(A)** Typical flow cytometry histograms for carboxyfluorescein succinimidyl ester-labeled OT-II T cells harvested 3 days after co-culture with BMDCs without antigen (parental generation, red) and with BMDCs loaded with $OVA_{323–339}$ peptide (progeny generations, blue). **(B)** Proliferation of OT-II T cells co-cultured with antigen-naïve BMDCs and no other stimulant (nil), or with anti-CD3ε/anti-CD28 antibodies (CD3/CD28), and co-cultured with $OVA_{323–339}$ peptide-loaded BMDCs (OVA), without or with addition of CRP. The diagonal arrow indicates $p < 0.0001$ (*) for a linear trend test of column means in left-to-right column order. **(C)** 2D2 T cell proliferation in the presence of antigen-naïve BMDCs without (nil) or with 50 μg/ml CRP, or in the presence of myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide-loaded BMDCs without (MOG) or with 50 μg/ml CRP (MOG/CRP). The symbols indicate not significant or $p < 0.05$ (*) for one-way analyses of variance with Tukey's multiple comparisons tests compared to nil ($n = 3$–6 per group).

DCs using FcγRIIB$^{-/-}$ BM to test if CRP's influence on BMDC phenotype and function required FcγRIIB. Like the expression on immature WT BMDCs (**Figure 2A**), expression of MHC class II, CD80, CD40, and CD86 on immature FcγRIIB$^{-/-}$ BMDCs was unaffected by CRP alone (50 μg/ml), and LPS triggered their increase (**Figure 4A**). However, in stark contrast to its effect on LPS-matured WT BMDCs (**Figure 2A**), CRP did not impair the LPS-triggered upregulation of MHC class II, CD86, and CD40 by FcγRIIB$^{-/-}$ BMDCs (**Figure 4A**). Like for WT BMDCs, expression of IL-10 by LPS-treated FcγRIIB$^{-/-}$ BMDCs (308.5 ± 12.5 ng/ml) was lowered by CRP (69.6 ± 8.7 ng/ml). However, unlike for WT BMDCs, for FcγRIIB$^{-/-}$ BMDCs treated with LPS the amount of IL-12p70 produced (948.9 ± 25.3 ng/ml) was not reduced by CRP (1017.1 ± 51.6 ng/ml). These findings strongly suggest that FcγRIIB expression is required for CRP to prohibit LPS-induced activation/maturation of BMDCs and to suppress production of the T cell stimulatory cytokine IL-12p70. As expected, when $MOG_{33–55}$ peptide-loaded FcγRIIB$^{-/-}$ BMDCs were used as APCs, CRP (50 μg/ml) did not impair their proliferation (**Figure 4B**). In our hands, FcγRIIB$^{-/-}$ BMDCs did not stimulate OT-II T cell proliferation even when loaded with $OVA_{323–339}$ (data not shown), precluding us from assessing if CRP requires FcγRIIB in the OT-II model system. Nevertheless, the results from the 2D2 model confirmed that CRP's ability to prohibit BMDC stimulation of an antigen-specific T cell response is facilitated by FcγRIIB expressed on BMDCs.

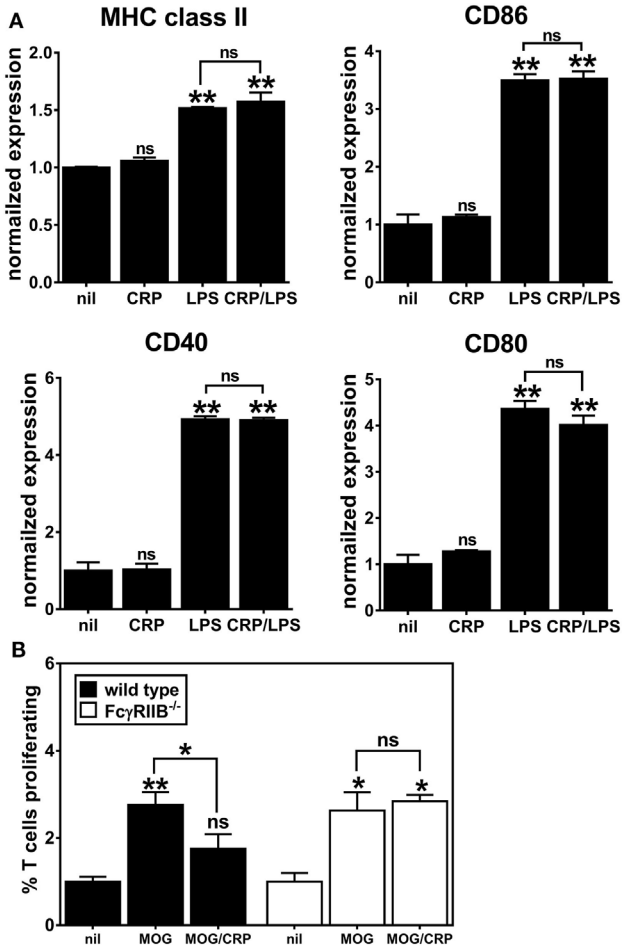

FIGURE 4 | C-reactive protein (CRP)-mediated prohibition of lipopolysaccharide (LPS)-induced maturation of bone marrow-derived dendritic cells (BMDCs) and inhibition of BMDC-mediated/antigen-driven proliferation of 2D2 T cells is FcγRIIB-dependent. **(A)** Surface expression of MHC class II, CD86, CD40, and CD80 on FcγRIIB$^{-/-}$ BMDCs left untreated (nil), or treated with CRP (50 μg/ml), LPS (1 μg/ml), or CRP and LPS. Each marker's expression is normalized to the nil group and the symbols directly above each bar indicate not significant (ns), $p < 0.05$ (*), or $p < 0.005$ (**) compared to nil. There was no significant difference between the LPS versus CRP/LPS groups. One-way analyses of variance (ANOVA) with Tukey's multiple comparisons tests ($n = 3$ experiments). **(B)** 2D2 T cell proliferation in the presence of wild type versus FcγRIIB$^{-/-}$ BMDCs. BMDCs were antigen-naïve (nil) or myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide-loaded and CRP was at 50 μg/ml. The symbols above the bars indicate ns, $p < 0.05$ (*) and $p < 0.005$ (**). The symbols above the brackets compare the MOG/CRP versus MOG groups. One-way ANOVA with Tukey's multiple comparisons tests ($n = 3$ per group).

Transgenic Expression of Human FcγRIIB Supports Human CRPs Actions on Mouse FcγRIIB$^{-/-}$ BMDCs

The apparent requirement of mouse FcγRIIB for human CRP-mediated prohibition of BMDC activation/maturation and 2D2 T cell proliferation prompted us to investigate this biology further. Accordingly, we generated FcγRIIB$^{-/-}$ mice that express a human *FCGR2B* transgene. Expression of the human FcγRIIB receptor was restricted to DCs by using a vector that contains the CD11c minimal promoter (kindly provided by Dr. Thomas Brocker, Institute for Immunology, LMU Munich Goethestr. 31, D-80336 Munich, Germany) (8). Briefly, a full-length cDNA clone encoding human *FCGR2B* (23) was inserted into the vector (**Figure 5A**) to drive *FCGR2B* expression on CD11c^{+} DCs in all mouse tissues. Transgenic mice ($_{cd11c}$FcγRIIB^{hu}) were then established by injecting the construct directly into fertilized FcγRIIB$^{-/-}$ eggs in the UAB Transgenic & Genetically Engineered Models Core. Offspring were screened for presence of the human transgene by PCR and flow cytometry was used to detect surface expression of human FcγRIIB on peripheral blood mononuclear cells (**Figure 5B**, left). Of the three potential founders identified (M27-1, F6-5, and F6-4; **Figure 5B**, left), only one (F6-5) showed germline transmission of the transgene. Transgenic descendants of F6-5 showed uniform expression of human FcγRIIB (**Figure 5B**, right) and were used for all further experiments.

We generated $_{cd11c}$FcγRIIB^{hu} BMDCs and confirmed that they upregulated expression of MHC class II, CD86, CD40, and CD80 after LPS-triggered activation/maturation (**Figure 6A**) and that CRP alone had no effect on expression of these markers (**Figure 6A**). Expression of human FcγRIIB partly reconstituted the CRP prohibitory effect on BMDC maturation, i.e., upon LPS-stimulation, CRP prohibited the expression of MHC class II and CD40 (**Figure 6A**). CRP inhibited IL-10 production by LPS-stimulated $_{cd11c}$FcγRIIB^{hu} BMDCs (459.4 ± 3.1 ng/ml without CRP and no detectable amounts with CRP), but not IL-12p70 production (485.9 ± 94.8 ng/ml and 689.9 ± 235.9 ng/ml without or with CRP, respectively). Although the effect was not significant (ns), when $MOG_{35–55}$ peptide-loaded $_{cd11c}$FcγRIIB^{hu} BMDCs were used

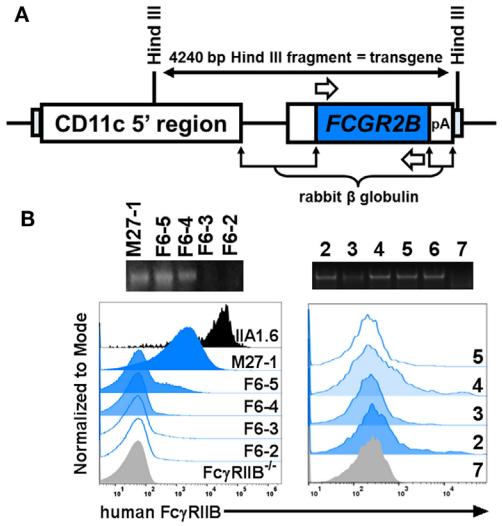

FIGURE 5 | Generation of $_{cd11c}$FcγRIIB^{hu} mice. **(A)** The targeting vector (fully described in (8)) encodes the mouse CD11c minimal promoter driving the human *FCGR2B* open reading frame. **(B)** Left panels: agarose gel electrophoresis of PCR amplified gDNA from a male (M27-1) and four female (F6-2, -3, -4, and -5) potential founders; three of which carried the *FCGR2B* transgene as indicated by presence of a 658 base pair amplicon generated using human *FCGR2B*-specific primers [white arrows in panel **(A)**]. Expression of human FcγRIIB was detected on mouse peripheral blood cells on M27-1, F6-5, and F6-5, using flow cytometry with an anti-human FcγRIIB antibody (clone AT 10). Murine B cell lymphoma (IIA1.6) cells transfected with a plasmid containing cDNA encoding human FcγRIIB (fully described in (24)) and peripheral blood cells from an FcγRIIB$^{-/-}$ mouse served as controls. Right panels: agarose gel electrophoresis of PCR amplified gDNA from six littermates descended from F6-5 (which transmitted the transgene). Their respective expression of human FcγRIIB on CD11c^{+} splenocytes is uniform.

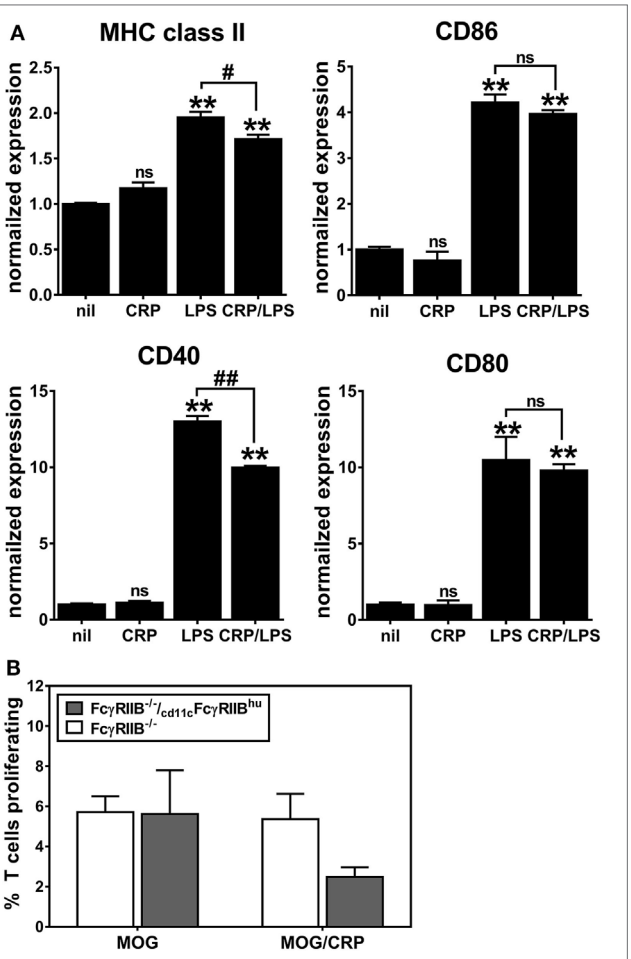

FIGURE 6 | CD11c-specific expression of human FcγRIIB reconstitutes C-reactive protein (CRP)-mediated suppression of **(A)** expression of MHC class II and CD40 on lipopolysaccharide (LPS)-matured bone marrow-derived dendritic cells (BMDCs) and **(B)** BMDC-mediated/myelin oligodendrocyte glycoprotein-driven 2D2 T cell proliferation. The symbols directly above each bar indicate not significant or $p < 0.005$ (**) compared to nil. The symbols above each bracket indicate $p < 0.05$ (#), or $p < 0.005$ (##) comparing LPS versus CRP/LPS groups. One-way analyses of variance with Tukey's multiple comparisons tests ($n = 3$–6 per group).

as APCs, their ability to stimulate the proliferation of 2D2 T cells was reduced by CRP (**Figure 6B**). These data generally support the premise that CRP's influence on DCs requires their expression of FcγRIIB, since some of the effects of CRP on FcγRIIB$^{-/-}$ BMDCs are recovered by expression of human FcγRIIB. Interestingly, although reconstitution of FcγRIIB$^{-/-}$ BMDCs with human FcγRIIB restored their ability to promote $OVA_{323-339}$-driven OT-II T cell proliferation, CRP (50 μg/ml) did not have a significant effect (data not shown).

Human FcγRIIB Supports Human CRP's Protective Actions in EAE

We had previously shown that CRPtg undergoing EAE have delayed onset and reduced severity of disease compared to WT and that this beneficial effect of CRP is FcγRIIB-dependent (5, 6, 20, 25), and herein we provide new evidence that this FcγRIIB-dependency extends to BMDCs *in vitro*. Moreover, although not all the observed effects of human CRP on BMDCs were supported by human FcγRIIB, CD11c-specific expression of human FcγRIIB was sufficient to fully reconstitute human CRP's beneficial actions in EAE (**Figure 7**; **Table 1**). Given that human CRP can utilize human FcγRIIB expressed by CD11c^{+} cells in transgenic mice, it is possible that the same or a similar CRP→FcγRIIB pathway operates in humans to regulate tolerance and prevent autoimmunity.

DISCUSSION

Previously, we showed that human CRP protects CRPtg mice from EAE triggered either directly by immunization with MOG or indirectly by the transfer of MOG-specific T cells (5, 20, 25) and that this protection was FcγRIIB-dependent manner (6). Although human CRP can have direct effects on T cells (25), the initial evidence of CRP inhibiting EAE suggested that CRP most likely conferred protection by acting on an intermediary APC. This study provides strong evidence to this effect, i.e., *in vivo* human CRP protects mice from EAE by acting on CD11c^{+} FcγRIIB-expressing DCs. We propose that the beneficial effect of

transgenically expressed human CRP in EAE, and perhaps other T cell-mediated diseases like lupus and collagen-induced arthritis (26–37), is achieved *via* its capacity to inhibit DC development and function, thereby diminishing the stimulation of pathogenic T cells.

Our *in vitro* data reveal several separate, but likely additive, mechanisms by which CRP impacts the T cell stimulating actions of DCs. First, CRP dose-dependently decreased the proportion of BM progenitors that developed into BMDCs, suggesting that CRP influences the fate of hematopoietic stem cells. Native pentameric CRP is likely required for this effect as heat denatured CRP did not have any effect (data not shown). Furthermore, CRP did not significantly affect early or late apoptosis or necrosis during the course of BM culture, demonstrating that CRP binding to phosphatidylserine on dying cells does not play a significant role and that CRP's influence is likely not due to selective killing of certain BM progenitors. Indeed, in separate studies we have also observed that CRP dose-dependently promotes the development of myeloid-derived suppressor cells (MDSC) at the expense of DCs (Figure S1 in Supplementary Material), and that the spleens of healthy CRP knockout mice (27) have an increased number of plasmacytoid ($CD11c^+$ $CD11b^{+/-}$ Siglec H^+) and conventional ($CD11c^+$ $CD11b^+$ Siglec H^-) DCs compared to WT and CRPtg (Figure S2 in Supplementary Material). The mechanism by which CRP alters myeloid progenitor cell developmental fate is still under investigation, but the fact that CRP shifts the myeloid balance away from DCs (which can promote T cell proliferation) and toward MDSCs (which can suppress it) directly implicates CRP in the regulation of the balance between adaptive immunity and tolerance. Second, CRP dose-dependently prohibits the LPS-triggered (TLR4-triggered) activation/maturation of BMDCs as evidenced by its ability to limit expression of MHC class II and co-stimulatory markers. Notably, CRP had no effect in the absence of a maturation signal (i.e., immature BMDCs) or in the presence of the TLR9 agonist CpG oligodeoxynucleotides (data not shown). This implies that *in vivo* CRP attenuates the responses of mature DCs in the periphery (i.e., those not participating in central tolerance) and does not impact immature DCs. Third, CRP impairs the production of IL-10 and IL-12p70 by BMDCs, two pleiotropic cytokines that can suppress (29) or promote (30) T cell functions, respectively. Fourth, CRP inhibited the ability of peptide-loaded mature BMDCs to stimulate antigen-driven T cell proliferation. Unexplored was whether CRP impairs the ability of BMDCs to uptake, process, and present antigen, but others have shown that CRP can also impact these processes (31–33).

We previously showed that in the absence of FcγRIIB, human CRP cannot protect mice against EAE (6). That observation led us here to test whether the CRP-responsive, FcγRIIB-expressing cell that might promote CRP's beneficial effects in EAE are DCs (7). In preliminary studies, we showed that CRP dose-dependently decreased the yield of both WT and $FcR\gamma^{-/-}$ $CD11c^+$ BMDCs,

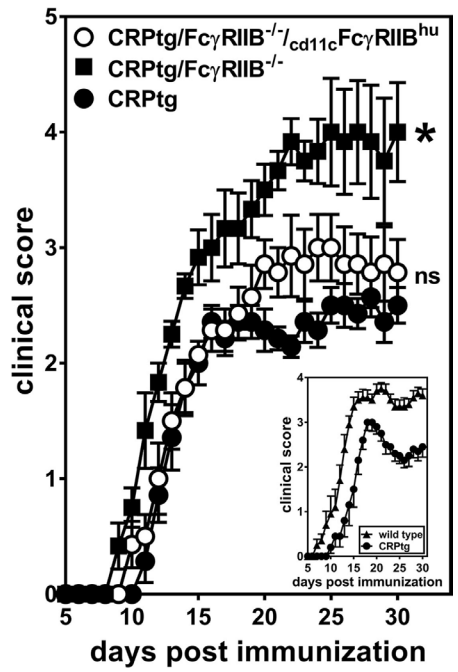

FIGURE 7 | CD11c-specific expression of human FcγRIIB restores resistance to experimental autoimmune encephalomyelitis (EAE) in CRP transgenic mice (CRP)tg/$FcγRIIB^{-/-}$ mice. Mice were immunized with myelin oligodendrocyte $glycoprotein_{35–55}$ and their ensuing EAE symptoms were monitored for 30 days for CRPtg (●), CRPtg/$FcγRIIB^{-/-}$ (■), and CRPtg/$FcγRIIB^{-/-}/_{cd11c}FcγRIIB^{hu}$ (○). The asterisk indicates the course of disease in CRPtg/$FcγRIIB^{-/-}$ is significantly worse (see **Table 1** for details). The inset shows the course of EAE in CRPtg compared to wild type from a separate experiment (n = 6–10 mice per group).

TABLE 1 | Experimental autoimmune encephalomyelitis (EAE) outcomes in C-reactive protein transgenic mice (CRPtg) lacking mouse FcγRIIB and/or expressing human FcγRIIB.

Genotype (n)	Day of onset[a] (mean ± SEM)	CDI[b] (mean ± SEM)	Maximum score[c] (mean ± SEM)
CRPtg (7)	14.0 ± 0.43	41.71 ± 0.94	2.86 ± 0.09
CRPtg/$FcγRIIB^{-/-}$ (6)	11.67 ± 0.33[d]	67.17 ± 5.15[d]	4.17 ± 0.40[d]
CRPtg/$FcγRIIB^{-/-}/_{cd11c}FcγRIIB^{hu}$ (7)	13.71 ± 0.52 [ns]	48.43 ± 3.79 [ns]	3.43 ± 0.28[ns]
Analysis of variance	p = 0.004	p = 0.0005	p = 0.0135

[a]*The day the clinical score attained a value ≥2 and remained ≥2 for at least 2 days.*

[b]*Cumulative disease index: the sum of clinical scores from day 0–31.*

[c]*The maximum clinical score attained by each mouse. Mice that succumbed to EAE were assigned a score of 6.*

[d]*Tukey's multiple comparisons test, $p < 0.05$.*

[ns]*Tukey's multiple comparisons test, not significant ($p > 0.05$).*

but not FcγRIIB$^{-/-}$ ones (Figure S3 in Supplementary Material). In alignment with those initial data, we showed herein that FcγRIIB$^{-/-}$ BMDCs maintain their ability to mature in response to LPS and to subsequently stimulate 2D2 T cell proliferation when loaded with MOG_{35-55}, but are refractory to inhibition by CRP. Importantly, in the absence of FcγRIIB expression, CRP was unable to downregulate BMDC production of the T cell stimulating cytokine IL-12p70. These findings highlight the importance of FcγRIIB for the inhibitory action of CRP on the development, maturation, cytokine production, and antigen-specific T cell stimulatory function of BMDCs. Since, human CRP can bind both mouse and human FcγRs *in vitro* and *in vivo* (2, 21), we generated a mouse completely deficient in endogenous mouse FcγRIIB, but expressing human *FCGR2B* on CD11c$^+$ cells. Using bone marrow from these $_{cd11c}$FcγRIIB^{hu} mice we showed that human CRP utilized human FcγRIIB to evoke impairment of BMDC activation and T cell stimulating function, but not to regulate IL-12p70 production. Nevertheless, CRP protection from EAE was fully reconstituted in CRPtg/$_{cd11c}$FcγRIIB^{hu} mice. We recognize that mouse CD11c, and, therefore, human FcγRIIB in the $_{cd11c}$FcγRIIB^{hu} mice, might be expressed at low levels on cell types other than DCs and that other DC subtypes may not express CD11c at all [e.g., plasmacytoid DCs and DCs with tolerogenic phenotypes (34)]. Nevertheless, this study is the first to show that human CRP interaction with human FcγRIIB expressed *in vivo* on CD11c$^+$ cells can modulate EAE.

We suspect that CRP regulates the generation and actions of DCs in the periphery (i.e., those not directly involved in central tolerance), thereby limiting the activation of auto-reactive T cells especially in the setting of tolerance breakdown. Withal, CRP promotes the number and generation of myeloid-derived suppressor cells (MDSCs) [Figure S1 in Supplementary Material (35)], a cell type known to potently suppress T cell proliferation (36). Simply by modulating the myeloid lineage development away from DCs and toward MDSCs, CRP could thus profoundly impact T cell immunity and the maintenance of peripheral tolerance. This role is unlikely to be restricted to EAE/MS and should also be manifest in the setting of immunosenescence and aging, for example [reviewed in Ref. (37)]. Indeed, some of the prominent features of immunosenescence are inflammation, decreased T cell numbers, and decreased naïve and memory T cell responsiveness (37, 38, 39), and in the aged, inflammaging can contribute to dysregulated DC responses and a consequent breakdown of tolerance that can predispose them to autoimmunity (40, 41). We propose that in this context, modest elevation of CRP due to biological aging (12) might act as a tonic suppressor of DC activation and thus limit autoreactivity.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals; Eighth Edition* (NIH Academies Press, 2011) and the Institutional Animal Care and Use Committees at the University of Alabama at Birmingham.

AUTHOR CONTRIBUTIONS

AS, RJ, TW, and NJ designed the experiments and RJ, TW, and NJ performed them. JW and AG aided in the generation of the $_{cd11c}$FcgRIIB^{hu} mouse. RJ and AS wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00372/full#supplementary-material.

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C-Reactive Protein in Atherothrombosis and Angiogenesis

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C-reactive protein (CRP) is a short pentraxin mainly found as a pentamer in the circulation, or as non-soluble monomers CRP (mCRP) in tissues, exerting different functions. This review is focused on discussing the role of CRP in cardiovascular disease, including recent advances on the implication of CRP and its forms specifically on the pathogenesis of atherothrombosis and angiogenesis. Besides its role in the humoral innate immune response, CRP contributes to cardiovascular disease progression by recognizing and binding multiple intrinsic ligands. mCRP is not present in the healthy vessel wall but it becomes detectable in the early stages of atherogenesis and accumulates during the progression of atherosclerosis. CRP inhibits endothelial nitric oxide production and contributes to plaque instability by increasing endothelial cell adhesion molecules expression, by promoting monocyte recruitment into the atheromatous plaque and by enzymatically binding to modified low-density lipoprotein. CRP also contributes to thrombosis, but depending on its form it elicits different actions. Pentameric CRP has no involvement in thrombogenesis, whereas mCRP induces platelet activation and thrombus growth. In addition, mCRP has apparently contradictory pro-angiogenic and anti-angiogenic effects determining tissue remodeling in the atherosclerotic plaque and in infarcted tissues. Overall, CRP contributes to cardiovascular disease by several mechanisms that deserve an in-depth analysis.

Keywords: c-reactive protein, pentameric C-reactive protein, monomeric C-reactive protein, atherosclerosis, thrombosis, angiogenesis, ischemic heart disease, cardiovascular disease

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INTRODUCTION

C-reactive protein (CRP) is a short pentraxin belonging to the highly conserved family of calcium-dependent ligand-binding plasma proteins of the superfamily of soluble pattern-recognition molecules, and it is mainly found as a pentamer in the circulation. It is synthesized in the liver induced by interleukin (IL)-6 (1), IL-1β, and tumor necrosis factor (TNF) (2), although other tissues such as adipose tissue may be able to synthesize CRP under pro-inflammatory stimuli (3). The native circulating form of CRP is pentameric (pCRP), that is a disc of five identical subunits non-covalently bounded around a central pore (4). When pCRP bounds to one of its ligands [for instance lysophosphatidylcholine *via* activation of phospholipase A2 (5) or in denaturizing or oxidative environment (6)] it dissociates in a non-reversible manner into its non-soluble monomers, leading to a potential functional activation (7). pCRP and monomeric CRP (mCRP) are shown to exhibit different functions, although the specific physiopathological functions of CRP are still unknown and are a focus of intense research. It is believed that mCRP is involved in the innate immune system by

activating the complement cascade (8), in angiogenesis (9) and in thrombosis (10), whereas pCRP is mostly released to the circulation after an inflammatory stimuli (1).

CVD is mainly caused by atherosclerosis, which starts from lipid infiltration in the vessel wall, endothelial dysfunction, and chronic low-grade inflammation causing plaque development that ends with clinical ischemic complications. Levels of pCRP in serum ≥3 μg/mL are used in the clinical setting as unspecific marker for inflammation, infection, and tissue injury, associated with an acute-phase response (11). Indeed, CRP is considered a predictor of future cardiovascular events (12), and in current guidelines is classified as Class III B level of evidence (13), although there are some discrepancies (14). CRP is a downstream biomarker of elevated IL-1, IL-6, and TNF-α. It can increase 10,000-fold within 6 h and has a half-life of 19 h, and its catabolic rate is independent of its plasma concentration (15). Besides its role in humoral innate immune response, CRP recognizes and binds multiple intrinsic ligands, such as the complement system, resulting in a significant increase in infarct size, cell receptors, apoptotic cells, growth factors, and extracellular matrix components, and thus contributing to cardiovascular disease progression. On those grounds, we aimed to highlight the implication of CRP and its forms on the pathogenesis of atherothrombosis and angiogenesis.

CRP IN ATHEROTHROMBOSIS

Atherothrombosis is a complex inflammatory pathological process initiated by lipid deposition in the arterial wall with a subsequent recruitment of circulating leukocytes. The growing atheromatous plaque may become unstable and rupture, triggering the formation of a thrombus by accumulation of platelets and coagulation proteins. Occlusive thrombi may eventually induce an ischemic event (16). In this process, inflammation has a pivotal role in all phases, and CRP actively participates by activating the complement system, and inducing apoptosis, vascular cell activation, leukocyte recruitment, lipid accumulation, platelet aggregation, and finally thrombosis (17). mCRP is detectable in the vessel wall in early stages of atherogenesis but not in healthy vessels, and accumulates during the progression of atherosclerosis, whereas pCRP is not detectable in healthy or atherosclerotic vessels (18). In this context, complement activation by enzymatically modified low-density lipoprotein (LDL) plays an important role in atherogenesis (19). Enzymatic modification of LDL confers the capacity to bind pCRP, and CRP-binding enhances complement activation through C3 cleavage (20). Both pCRP and mCRP are able to activate and amplify the classical pathway of the complement system by interacting with the complement factor C1q, significantly activating C1. Only mCRP is able to interact with complement factor H and C4b-binding protein (21), thus provoking local inflammatory responses and contributing to the establishment and progression of atherosclerosis or to the tissue damage following myocardial infarction. In addition, pCRP can promote inflammation by binding to modified or oxidized LDL and (non) oxidized phosphatidylcholine from apoptotic cells (22), promoting the transformation from macrophages to foam cells.

C-reactive protein contributes to endothelial dysfunction and hypertension by inhibiting nitric oxide (23), increasing endothelin-1 production, and thus impairing endothelial-dependent vascular relaxation (24). In the setting of chronic local inflammation in atherosclerosis, the addition of mCRP to apical but not basolateral surfaces of intact human coronary artery endothelial cell monolayers, upregulated monocyte chemotactic protein (MCP)-1, IL-8, and IL-6 expression and activated endothelial cells through the polarized induction of phospholipase C, p38 mitogen-activated protein kinase, and nuclear factor (NF)-κB signaling pathways (25). Therefore, tissue-associated mCRP induces endothelial cell activation and dysfunction, and spatial localization is determinant for the highly context-dependent actions of CRP isoforms within vessels.

In addition, pCRP contributes to plaque instability by activating NF-κB and, therefore, increasing endothelial cell adhesion molecules expression such as vascular cellular adhesion molecule-1, vascular E-selectin, and MCP-1 (26, 27). pCRP also induces monocyte polarization to M1 and conversion from M2 to M1 phenotype (2), thus promoting monocyte recruitment into the plaque. Indeed, circulating pCRP binds to the cell membrane of activated, but not resting monocytes (28), and activated but not resting platelets and apoptotic leukocytes are able to dissociate pCRP to mCRP *via* lysophosphatidylcholine inducing reactive oxygen species (ROS) production and monocyte chemotaxis, activation and adhesion (18), being mCRP and not pCRP the responsible of these effects, even at low concentrations. In neutrophils, mCRP but not pCRP increases IL-8, CD11b/CD18, and superoxide production, and induces endothelial nitric oxide synthase-mediated nitric oxide formation. This leads to enhanced peroxynitrite formation, and to the activation of NF-κB and activator protein (AP)-1 (29), as well as enhanced neutrophil adhesion to activated endothelial cells (30), thus aggravating the inflammatory response at injured vascular sites and contributing to plaque destabilization. Overall, mCRP is able to aggravate the preexisting inflammatory response by inducing leukocyte rolling, adhesion, and transmigration to the endothelium and generation of ROS (5), which in turn, modifies the structure and ligand recognition function of CRP (31).

In addition, CRP also contributes to plaque instability by inducing the expression of metalloproteinases (MMP) 1, 2, and 9 (32, 33). On those grounds, CRP mRNA was detected in potentially vulnerable ulcerated carotid artery plaques but not in hemorrhagic ulcerated plaques independently of the circulating levels of CRP. In non-complicated ulcerated carotid artery plaques, CRP was mainly localized in infiltrated and endothelial cells around areas of newly formed microvessels (34), potentially contributing to plaque neovascularization and rupture resulting in thrombosis.

C-reactive protein also contributes to thrombosis, but depending on its form it elicits different actions. CRP at 10–100 mg/L is able to increase 75-fold tissue factor (TF) procoagulant activity of monocytes, with a parallel increase in TF antigen levels (35). CRP at 2–24 mg/L activates both inflammation and coagulation through increasing circulating levels of E-selectin, von Willebrand factor, IL-6, IL-8, serum amyloid A protein, type II secretory phospholipase A_2, prothrombin F1 +2, D-dimer, and

plasminogen activator inhibitor type-1 (36). It has been shown that circulating microvesicles can bind pCRP and dissociate it to mCRP, and patients with myocardial infarction have circulating microvesicles carrying mCRP (37). In addition, pCRP binding to activated cell-derived microvesicles also undergoes a structural change leading to the expression of neoepitopes without disrupting the pentameric symmetry activating the classical complement pathway through C1q binding and enhancing leukocyte recruitment to inflamed tissues (28). pCRP has no involvement in thrombogenesis, whereas mCRP is able to promote thrombosis by inducing platelet activation (38), platelet adhesion by upregulating P-selectin (10), and thrombus growth (39). Additionally, mCRP has been found in platelet aggregates and stimulates further platelet deposition (38). Blocking glycoprotein IIb-IIIa on activated platelets prevented the dissociation of pCRP to mCRP and reduced platelet deposition at the arterial wall (38).

As depicted in **Figure 1**, the dissociation of pCRP into mCRP could be interpreted as a master switch for the inflammatory processes involved in atherogenesis. Both mCRP bound to phosphorylcholine of activated cell membranes and mCRP present within the advanced atherosclerotic plaque may play a critical role in the further development of the plaque and thrombus formation and propagation upon mechanical or spontaneous atherosclerotic plaque rupture. Nevertheless, it is worth mention that some *ex vivo* experiments suggest anti-atherosclerotic functions of CRP. As previously stated, CRP binds to enzymatically modified LDL at the same binding site as phosphocholine; therefore, it could prevent the formation of foam cells and limit complement activation (40, 41). Indeed, when CRP binds to lysophosphatidylcholine, this complex triggers a less potent generation of ROS and less activation of the transcription factors AP-1 and NF-κB by macrophages in comparison to free CRP or lysophosphatidylcholine, reducing the pro-atherogenic effects of macrophages (42). Finally, it has also been shown that CRP also affects the physicochemical properties of LDL and inhibits further oxidation of ox-LDL (43, 44), although the mechanism remains unknown. In addition, mCRP has been found to decrease the uptake of acetylated LDL by endothelial cells independently of CD16, CD32, or the receptor for oxidized LDL (45).

CRP IN ISCHEMIA AND ANGIOGENESIS

Monomeric CRP has been involved in ischemic heart disease (46), therefore pCRP dissociation to mCRP modulates inflammation in both acute (cardiac ischemia/reperfusion) and chronic (atherosclerosis) inflammatory processes. Local inflammatory response during myocardial ischemia contributes to myocardial damage and infarct size, and plays a major role in angiogenesis and tissue remodeling. Infiltrated macrophages at the border site of the cardiac ischemic lesion express mCRP (47, 48), and CRP in monocytes upregulates vascular endothelial growth factor (VEGF)-A expression *in vitro via* binding to its Fc-gamma receptors (49). In fact, myocardial ischemia activates mCRP expression in myocardial infiltrated macrophages but not in peripheral blood mononuclear cells (47), and cardiac mCRP expression remains elevated after 1 week of acute myocardial infarction (48), potentially contributing to cardiac remodeling and in perpetuating and/or amplifying the inflammatory process. Along this line, circulating CRP has been shown to correlate with infarct size and left ventricle remodeling 2 months after percutaneous coronary intervention, and patients with persistent microvascular obstruction presented increased circulating CRP levels 2 days after percutaneous coronary intervention (50).

Angiogenesis also has a role in plaque instability and disruption, favoring leukocyte and macrophage infiltration in growing atherosclerotic lesions. Indeed, the adventitial *vasa vasorum* facilitates neovascularization related to progression of atherosclerosis (51). Although some authors have observed that CRP inhibits VEGF production and angiogenesis (23, 52), several studies suggest that mCRP may be a mediator of neovessel formation in the intima of vulnerable plaques, as it has been localized in the adventitia and intimal neovessels from complicated regions of unstable carotid plaques (53). In the setting of atherosclerosis, CRP upregulates VEGF expression *via* activating hypoxia

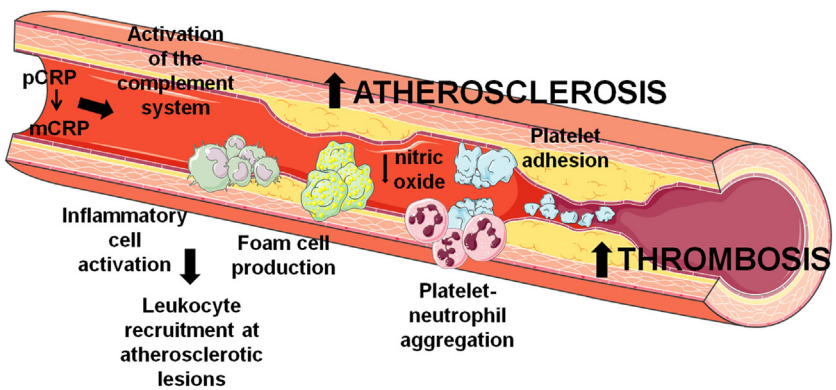

FIGURE 1 | Involvement of C-reactive protein (CRP) in atherothrombosis. CRP contributes to the development and progression of atherosclerosis and thrombosis by several mechanisms that induce endothelial dysfunction, leukocyte recruitment at atherosclerotic lesions, and thrombus formation through platelet activation and aggregation.

inducible factor-1α, and MMP-2 expression and in adipose-derived stem cells, significantly increasing endothelial cell tube formation and *vasa vasorum* proliferation (54). As previously explained, mCRP has been localized around newly formed microvessels in carotid artery plaques and in peri-infarct regions after an acute ischemic stroke (34, 55), promoting angiogenesis and inducing inflammation and increased permeability of abnormally developing microvessels after tissue injury (56), potentially leading to an increased risk of dementia.

In stroke patients, mCRP colocalized with endoglin (CD105), a marker of angiogenesis in regions of revascularization, and stimulated phosphorylation of extracellular signal-regulated kinase (ERK)1/2, inducing cell migration and formation of tube-like structures independently of the CD16 axis (55). mCRP exerts potent angiogenic effects on microvascular endothelial cells. CRP dissociates into mCRP on the endothelial cell membrane and mCRP induces angiogenic effects by increasing TF expression and activation of the axis F3-TF-ETS1-CCL2 (9), and by increasing endothelial expression of CD32 and CD64 (57), thus promoting migration, wound repair, and tube-like formation. In parallel, it has been demonstrated that mCRP has the ability to promote angiogenesis by increasing proliferation, migration, and tube-like structure formation *in vitro* and by stimulating blood vessel formation *in vivo* with the chorioallantoic membrane assay. mCRP induced vascular VEGFR2/KDR, platelet-derived growth factor (PDGF-BB), inhibitor of DNA binding/differentiation-1 (ID1) gene expression, notch family transcription factors (Notch1 and Notch3), and also induced stabilization and maturation of cysteine-rich angiogenic inducer 61 (CYR61/CCN1), overall playing a central role in the main stages of blood vessel formation and remodeling (58). Along this line, mCRP induces Notch-3 and N-cadherin expression and down-regulates VE-cadherin expression. mCRP and Notch-3 act in a co-operative manner in vascular endothelial cells, exerting a role in the remodeling and maturation of the vascular development by increasing endothelial cell proliferation, migration, and tube formation and also stabilizing vascular structures through modulating VE-cadherin and N-cadherin expression (59). On the other hand, CRP has several deleterious effects on endothelial progenitor cells, which account for about 26% of endothelial cells in newly formed blood vessels (60), by decreasing their survival and inducing apoptosis, by impairing their differentiation through the inhibition of the expression of tyrosine-protein kinase receptor for angiopoietin (Tie)-2, endothelial cell-specific lectin, and VE-cadherin, and by impairing nitric oxide-dependant angiogenesis *via* decreasing endothelial nitric oxide synthase (61, 62). Therefore, as depicted in **Figure 2**, angiogenesis plays a dual role in CVD progression, and further research should focus on the mechanisms by which CRP may contribute to the atherosclerotic process and/or tissue repair.

CRP AND CVD PROGNOSIS

Although CRP response is unspecific and is triggered by many disorders unrelated to cardiovascular disease, mathematical models that incorporate high-sensitivity CRP (hsCRP) improve CV risk prediction. Increased levels of CRP strongly predict the thrombotic complications of atherosclerosis, principally myocardial infarction (1) and its adverse outcomes such as left ventricular failure, cardiac death, and ventricle rupture (7). In fact, CRP may have a role in risk stratification of patients with established CVD. hsCRP levels > 3 mg/L are predictive of major adverse cardiac events at 1 year, and are also associated with higher coronary plaque burden and volume (63). In addition, low, average and high CV risk categories can be stratified by hsCRP levels (<1.0, 1.0 to 3.0, and >3.0 mg/L, respectively) (64), and in the general population CRP levels are able to independently predict the risk of all-cause and cardiovascular mortality (65).

In subjects at intermediate risk of CVD, incorporation of CRP to a model of assessment of CV risk improves the prognostic power for myocardial infarction presentation (66), and could help prevent 1 additional CV event in 10 years from 400–500 screened subjects (12). In patients with previous CVD and in asymptomatic subjects, hsCRP was a moderated predictor of coronary heart disease at the long term (67). In fact, the combination of troponin I, N-terminal pro-brain natriuretic peptide (NT pro-BNP), cystatin C and CRP improved significantly the risk stratification for cardiovascular death (68). In patients with

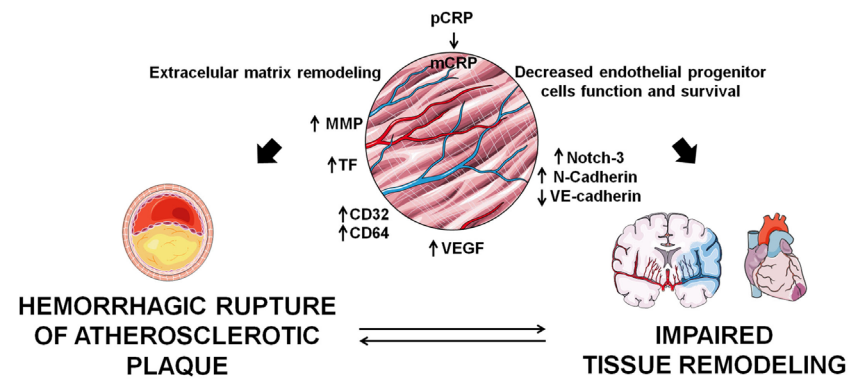

FIGURE 2 | Implications of C-reactive protein (CRP) in angiogenesis. monomeric CRP (mCRP) has apparently contradictory pro-angiogenic and anti-angiogenic effects which determine tissue remodeling in the atherosclerotic plaque and in infarcted tissues.

stable and unstable angina, elevated CRP levels are predictive of future coronary events (69). Indeed, in ST-elevation myocardial infarction patients CRP levels predicted heart failure and cardiovascular mortality the year after the CV event (70), and in patients with non-ST-elevation myocardial infarction, in-hospital mortality was four times higher in patients with a CRP > 10 mg/L compared to patients with <3 mg/L CRP levels, and this association persisted at the long term (71).

High-sensitivity CRP can be quantified by immunonephelometry sensitized techniques routinely used to measure circulating pCRP with a lower detection limit than former procedures. However, as stated in this review it is unlikely that circulating pCRP elicit a direct role in CHD progression (7), because no major prothrombotic or pro-inflammatory effects have been found for circulating pCRP, and no association between genetically elevated CRP and risk of CHD has been found (72). Therefore, it seems plausible that mCRP would be the responsible for the observed associations between CRP and CVD.

CONCLUSION

As reviewed, mCRP is a potential regulator of signaling pathways associated with thrombosis, angiogenesis, and inflammation. The ability of CRP to bind and interact with multiple ligands underscores its implication in different steps of atherosclerosis and CVD. CRP contributes to atherosclerosis progression by exerting pro-inflammatory effects, modulating the innate immune response and activating the complement system, promoting platelet activation, thrombus formation, vascular remodeling, and angiogenesis. However, whether CRP acts as regulator or amplifier of the innate immune response remains to be fully elucidated. Determining whether increased pCRP production merely reflects atherosclerosis or does indeed participate in its pathogenesis and complications is of utmost importance in order to definitively consider hsCRP as a clinical biomarker of CVD.

The study of the molecular mechanisms by which CRP contributes to atherothrombosis, angiogenesis, and CVD has a major pitfall. Human CRP does not interact with C1q in mice, and mice do not produce large amounts of CRP after an inflammatory stimuli (46). The study of CRP function has largely been performed with administration of exogenous, heterologous CRP or with mice transgenic for rabbit or human CRP. Therefore, caution should be taken when extrapolating from animal models to humans, and more research toward a more appropriate animal model is still warranted. Taking this into consideration, further research is required in order to differentially characterize the roles of CRP isoforms (pCRP, facilitator, versus mCRP, effector) in CVD onset and progression, and the binding ligands to circulating pCRP which can lead to CRP dissociation and induction of local inflammation in order to develop more potent and orally bioavailable "CRP inhibitors" for the treatment of inflammation and atherosclerosis.

AUTHOR CONTRIBUTIONS

GC-B prepared the main body of the manuscript and figures. EP, GA, TP, MS, and GV wrote different sections of the manuscript. LB revised and prepared the manuscript. All authors listed critically revised the paper for intellectual content.

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REVIEW
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Dissociation of C-Reactive Protein Localizes and Amplifies Inflammation: Evidence for a Direct Biological Role of C-Reactive Protein and Its Conformational Changes

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C-reactive protein (CRP) is a member of the pentraxin superfamily that is widely recognized as a marker of inflammatory reactions and cardiovascular risk in humans. Recently, a growing body of data is emerging, which demonstrates that CRP is not only a marker of inflammation but also acts as a direct mediator of inflammatory reactions and the innate immune response. Here, we critically review the various lines of evidence supporting the concept of a pro-inflammatory "CRP system." The CRP system consists of a functionally inert circulating pentameric form (pCRP), which is transformed to its highly pro-inflammatory structural isoforms, pCRP* and ultimately to monomeric CRP (mCRP). While retaining an overall pentameric structure, pCRP* is structurally more relaxed than pCRP, thus exposing neoepitopes important for immune activation and complement fixation. Thereby, pCRP* shares its pro-inflammatory properties with the fully dissociated structural isoform mCRP. The dissociation of pCRP into its pro-inflammatory structural isoforms and thus activation of the CRP system occur on necrotic, apoptotic, and ischemic cells, regular β-sheet structures such as β-amyloid, the membranes of activated cells (e.g., platelets, monocytes, and endothelial cells), and/or the surface of microparticles, the latter by binding to phosphocholine. Both pCRP* and mCRP can cause activation of platelets, leukocytes, endothelial cells, and complement. The localization and deposition of these pro-inflammatory structural isoforms of CRP in inflamed tissue appear to be important mediators for a range of clinical conditions, including ischemia/reperfusion (I/R) injury of various organs, cardiovascular disease, transplant rejection, Alzheimer's disease, and age-related macular degeneration. These findings provide the impetus to tackle the vexing problem of innate immunity response by targeting CRP. Understanding the "activation process" of CRP will also likely allow the development of novel anti-inflammatory drugs, thereby providing potential new immunomodulatory therapeutics in a broad range of inflammatory diseases.

Keywords: inflammation, C-reactive protein, cardiovascular diseases, ischemia/reperfusion, Alzheimer disease

INTRODUCTION

C-reactive protein (CRP) is a member of the pentraxin superfamily and was first discovered in 1930 by Tillett and Francis (1). Indeed, the first characterization of this protein was based on the initial observation that a distinct third fraction identified from the sera of patients with pneumococcus infection could precipitate the "C" polysaccharide derived from the pneumococcus cell wall. Subsequently, Avery and McCarty described CRP as an acute phase reactant after demonstrating that CRP levels were elevated in patients with a range of inflammatory conditions (2–4). Some 40 years after the original description of CRP, phosphocholine (PC) was shown to be the specific ligand for CRP binding within the pneumococcal cell wall (5). Today, CRP is widely used in the clinic as a marker of inflammation (6).

However, importantly, there is now a large body of evidence from prospective clinical trials that CRP levels may serve as a predictor of cardiovascular events, thus bringing the biological role of CRP into focus (7–9). This review discusses how insights gained into the different structural isoforms of CRP have led to a greater appreciation of its pro-inflammatory and prothrombotic role, which is relevant to a broad range of disease states.

CRP STRUCTURE AND FUNCTION

C-reactive protein is predominantly synthesized by the liver as a pentamer composed of five identical, non-covalently linked 23 kD protomers—each one folded into two antiparallel β-sheets with a "jelly roll" topology (10, 11). Each protomer has a binding face with a PC-binding site, which binds apoptotic cell membranes and bacterial cell walls (12). The two key residues of the hydrophobic binding pocket essential for mediating PC binding are Phe-66 and Glu-81. Phe-66 regulates hydrophobic interactions with the methyl group of PC, while Glu-81 interacts with the positively charged nitrogen (12). The opposite face of the binding face, known as the effector face, binds the globular domain of the complement factor 1q (C1q) and Fc gamma receptors, thus providing a mechanism to activate the innate immune system (13). However, the location of these binding sites on the pentameric form of CRP (pCRP) appears to be cryptic, thus supporting the concept that pCRP does not possess intrinsic pro-inflammatory properties.

CONFORMATIONAL CHANGE OF CRP CREATES HIGHLY PRO-INFLAMMATORY MOLECULES

pCRP dissociates into monomeric CRP (mCRP) after exposure to heat, urea, or an acidic microenvironment (14, 15). The dissociation of pCRP into its subunits exposes a range of neoepitopes that are likely to account for the distinct pro-inflammatory function of mCRP (16, 17) (see below). More recently, the *in vivo* generation and consequences of mCRP production have begun to be elucidated. Indeed, we and others have demonstrated that pCRP can be dissociated by calcium-dependent binding to liposomes and cell membranes (16–18). Activated platelets (19), endothelial cells (20), and monocytes (17) may provide the requisite PC-binding sites to facilitate the dissociation of pCRP to mCRP. This process is phospholipase A2 dependent since PC exposure and hence pCRP dissociation are dependent upon PLA2 generation (20).

The dissociation of pCRP to mCRP also produces marked changes in the solubility of the respective structural isoforms. While pCRP is soluble, the dissociation produces a shift from a predominantly β-sheet tertiary protein conformation to protomers with an α-helical tertiary structure and the exposure of previously cryptic interprotomer contacts (17). As such, mCRP has little solubility, and this led to the concept that this isoform was predominantly a tissue-bound form of CRP.

IDENTIFICATION OF THE PRO-INFLAMMATORY pCRP* STRUCTURAL ISOFORM

Very recent work from our group has provided additional new insights into the mechanism of pCRP dissociation. The conformational change of pCRP that occurs on the PC-rich and highly curved MP membranes (derived from activated monocytes, platelets, or endothelial cells) initiates a spatial separation of the five CRP monomers in relation to each other (17). Therefore, MPs with bound, dissociated pCRP* act as transport vehicles of circulating pCRP* to distant sites. In this regard, microparticles with bound dissociated CRP can be detected in patients with acute coronary syndromes (21). In contrast, mCRP appears to be rapidly cleared from the circulation due to its unfolded and more disordered state. Moreover, while most studies have investigated the presence of mCRP as the dissociated structural isoform in inflamed tissue, we have shown that pCRP* is the dominant isoform in injured tissue, including inflamed human muscle, burn wounds, and human atherosclerotic plaques.

Crucially, both pCRP* and mCRP have pro-inflammatory functions; however, the structural isoform-specific antibodies commonly used to distinguish pCRP from dissociated isoforms cannot differentiate between mCRP and pCRP*. Although still existent as a pentamer, this intermediate form—termed pCRP*—exposes functionally active neoepitopes, which are recognized by isoform-specific antibodies and allow the binding of C1q (1, 17). These studies have led to the concept of conformation-dependent regulation of the innate response by CRP. While pCRP does not display any pro-inflammatory properties, as discussed below both mCRP and pCRP* are potent pro-inflammatory structural isoforms of CRP, which can mediate immune, inflammatory, and prothrombotic responses in a range of diseases (**Figure 1**).

LOCATION AND REGULATION OF CRP PRODUCTION

The vast majority of CRP is produced in the liver (22). While the production of CRP has also been reported in an array of other cell types including leukocytes (23), adipocytes (24), neuronal cells (25), renal cells (26), and respiratory epithelial cells (27), the extrahepatic production of CRP is not thought to influence plasma CRP concentrations significantly. In health, plasma CRP levels may be undetectable; however, in the context of inflammation,

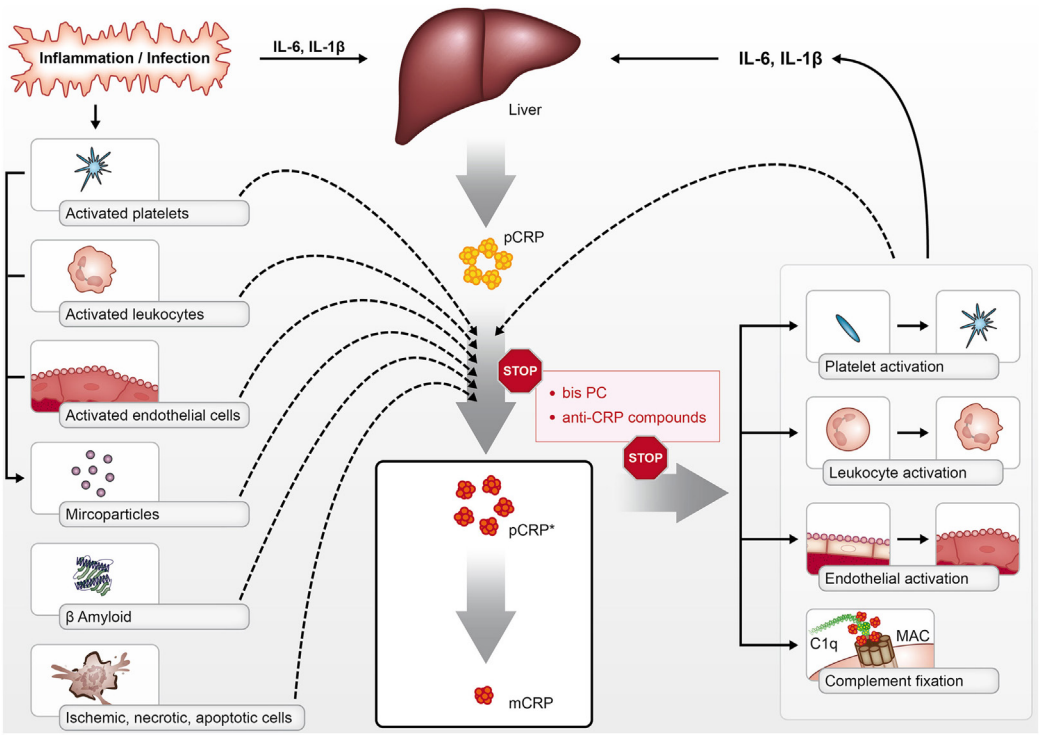

FIGURE 1 | pCRP is produced by the liver in the context of inflammation and infection in response to cytokines such as interleukin 6 (IL-6) and IL-1β. pCRP can be dissociated into its pro-inflammatory structural isoforms [pCRP* and monomeric CRP (mCRP)] on the surface of activated platelets, leukocytes, endothelial cells, phosphocholine-rich MPs, dying cells, and beta-amyloid. pCRP* and mCRP can activate platelets, leukocytes, and endothelial cells, in addition to activating complement *via* C1q binding. The pro-inflammatory effects of pCRP*/mCRP can be inhibited with compounds such as bisPC that inhibit the dissociation of pCRP and block mCRP effector binding.

they can increase by 1,000-fold within 24–72 h (28). Thus, the CRP plasma level is widely used as a marker in reporting on the general inflammatory status of patients (29–32).

The induction of CRP expression and secretion by hepatocytes is principally regulated by interleukin 6 (IL-6) and to a lesser extent interleukin 1β (IL-1β) (33). In contrast, interferon-α, statins, and nitric oxide suppress the induction of pCRP expression by pro-inflammatory cytokines, resulting in a weak correlation of serum CRP levels with viral infections or systemic lupus (34–36). Indeed, the pro-inflammatory cytokines induce hepatic production of several acute phase proteins. This upregulation occurs *via* the transcriptional activation of the STAT3, C/EBP, and NF-κB pathways (33, 37, 38). In the context of CRP, the recruitment and activation of the C/EBP family members C/EBPβ and C/EBPα appear critical to the induction of CRP. Moreover, the STAT3 and Rel proteins bind to the proximal CRP promoter, with the ensuing interactions resulting in enhanced binding of C/EBP and thereby facilitating maximal CRP induction.

THE PRO-INFLAMMATORY AND PROTHROMBOTIC PROPERTIES OF CRP

As discussed, dissociated mCRP and pCRP* are considered the pro-inflammatory components of the "CRP system." While most work has focused on the biological effects of mCRP, pCRP* exhibits similar effects. Consistent with this, mCRP has been demonstrated to activate monocytes, thus leading to Mac-1 activation and resulting in enhanced monocyte adhesion *in vitro* (19). Moreover, mCRP-stimulated monocytes induce reactive oxygen species generation (19). While the binding of mCRP to monocytes is at least partially dependent upon the Fc gamma receptors (CD64, CD32, CD16), it also appears that lipid rafts are essential for mCRP binding since disruption of these with agents such as nystatin completely inhibits mCRP binding (39–41). In accordance with the pro-inflammatory properties of mCRP, neutrophils in response to mCRP stimulation upregulate Mac-1 binding, which correlates with an increase in neutrophil adhesion on activated endothelial cells *in vitro* (42). In addition, mCRP stimulates the production of interleukin 8 (IL-8) from neutrophils *via* intracellular peroxynitrite production (43).

Monomeric CRP can also activate endothelial cells (41, 44). Under normal conditions, the endothelium maintains a quiescent state to prevent the unwanted adhesion of platelets and leukocytes. However, upon activation, inflamed endothelium upregulates the expression of many adhesion receptors, which facilitate platelet and leukocyte interaction. Two of the main endothelial adhesion receptors, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), are upregulated by endothelial cells in response to mCRP stimulation

(18, 41, 44). ICAM-1 serves as an important counter-receptor for the leukocyte integrin lymphocyte function-associated antigen 1 and thereby enhances leukocyte recruitment to sites of endothelial inflammation (45). Moreover, ICAM-1 can bind fibrinogen and thus serves as an important counter-receptor for the platelet integrin GPIIb/IIIa and thus mediates stable platelet adhesion to the inflamed endothelium (46). Likewise, VCAM-1 binds to the leukocyte integrin very late antigen 4 and serves to promote leukocyte adhesion (47). In addition, mCRP has been demonstrated to stimulate endothelial chemokine production, with both IL-8 and monocyte chemoattractant protein-1 release being increased in the context of mCRP activation (41, 44, 48).

Platelets, which mediate thrombosis and hemostasis, also have important innate immune functions (49). In this regard, mCRP has been shown to activate platelets as demonstrated by its ability to induce GPIIb/IIIa activation and alpha granule exocytosis (as measured by P-selectin expression) in a process dependent upon p38 MAPK and JNK signaling (50, 51). The receptor responsible for mediating the prothrombotic effects of mCRP on platelets has not been clearly elucidated. However, the platelet scavenger receptor CD36 plays a vital role given that CD36 inhibition blocks some of the effects of mCRP (50). Interestingly, the membrane of activated platelets appears to be an essential substrate mediating pCRP dissociation and, in this context, growing thrombi *in vitro* can dissociate CRP, thus promoting thrombus growth (50, 51).

The complement cascade is an essential arm of the immune system. In keeping with the concept that mCRP modulates the innate immune response, mCRP can bind and activate the complement system (52). mCRP and pCRP* readily bind to C1q and thus lead to robust activation of the classical complement cascade, which ultimately culminates in the formation of the membrane attack complex (17). Recent experimental work has demonstrated that the globular head of C1q can only bind to dissociated mCRP or spatially altered pCRP*, but not to pCRP, since this appears to represent the crucial structural determinant regulating complement activation (17). Given the highly conserved nature and universal presence of PC in eukaryotic cells, the ability of PC to bind CRP may serve to direct the opsonization of apoptotic and necrotic cells by the complement system, thus facilitating their clearance (28, 53, 54).

Interestingly, mCRP appears to demonstrate a dual role in regulating the complement system, since it not only serves to activate the complement cascade but also functions as a regulator of the degree of complement activation. In this regard, mCRP can also bind complement regulatory proteins such as Factor H and direct these to sites of cellular damage (55, 56). This process acts to enhance C3b inactivation, thus limiting the further generation of inflammatory products and aiding in the opsonization and subsequent clearance of damaged cells (55, 57). However, under pathological conditions, these responses may be maladaptive and promote the pathogenesis of inflammatory and immune conditions.

As will be discussed, these observations that pCRP* and mCRP can enhance innate immune responses have relevance to a range of clinical conditions and, as such, they may represent potentially novel therapeutic targets for a diverse spectrum of diseases.

CRP IN ISCHEMIA/REPERFUSION

Ischemia/reperfusion injury (IRI), referring to the restoration of blood flow to a previously ischemic organ, significantly contributes to morbidity and mortality in a range of clinical scenarios, including myocardial infarction and ischemic stroke (58). Indeed, experimental evidence suggests that up to 50% of the final infarct volume from myocardial infarction is due to reperfusion injury (59). One of the classic features of IRI is the initiation of a maladaptive immune response, which leads to widespread microvascular dysfunction and exacerbation of organ injury (58). A large body of data now exists demonstrating that CRP plays an active role in exacerbating IRI (**Figure 2**). The administration of pCRP to rats after the onset of myocardial ischemia has been shown to increase infarct volume by 40% in a complement-dependent manner (60). In accordance, pCRP administered to rats prior to the onset of cardiac reperfusion results in the marked deposition of mCRP in the infarcted myocardium, which correlates to the degree of leukocyte infiltration, apoptosis (as measured by caspase-3), and expression of IL-6 and TNF-alpha (20).

Critically, mCRP, but not pCRP, is abundant within infarcted human myocardium and also co-localizes with complement staining (20, 61). Our group has recently confirmed these observations in a rat model of IRI where rats administered pCRP displayed more severe renal tubular damage, which correlated with increased mCRP deposition, leukocyte infiltration, and caspase-3 activation after IRI (manuscript under review). Interestingly, the effects of CRP here appear primarily dependent upon facilitating endothelial–leukocyte interactions and enhancing leukocyte ROS generation in a process linked to mCRP binding to leukocyte lipid raft domains. These findings are consistent with previous work utilizing transgenic mice that express human CRP where, after renal IRI, these mice displayed more severe renal injury associated with a diversion of the monocyte/macrophage profile away from a protective M2 profile toward a "pro-inflammatory," deleterious M1 profile in the context of IRI (62).

Studies using intravital microscopy have been used to delineate the spatiotemporal aspects of CRP conformational changes in the context of tissue inflammation. mCRP becomes deposited on apoptotic endothelial cells after LPS challenge, and this leads to enhanced leukocyte rolling, adhesion, and transmigration (20). CRP is also transported into the inflamed tissue by transmigrating leukocytes, with leukocyte infiltrates in inflamed tissue co-localizing with mCRP deposition (17, 20). These pro-inflammatory effects of CRP are principally complement-dependent, since depletion of complement *in vivo* is associated with a marked inhibition of leukocyte adhesion in the context of exogenous pCRP administration. Moreover, monocytes in rats administered exogenous pCRP in the context of LPS-induced endothelial inflammation also demonstrated increased ROS generation *in vivo*, thus demonstrating another mechanism, in addition to complement activation, by which mCRP can mediate tissue damage (20). In accordance with these *in vivo* findings, sections of human striated muscle, which have undergone IRI also demonstrated strong mCRP staining, which co-localized with monocytes/macrophages (20). Interestingly, recent findings from our group demonstrate that circulating pCRP binds to the

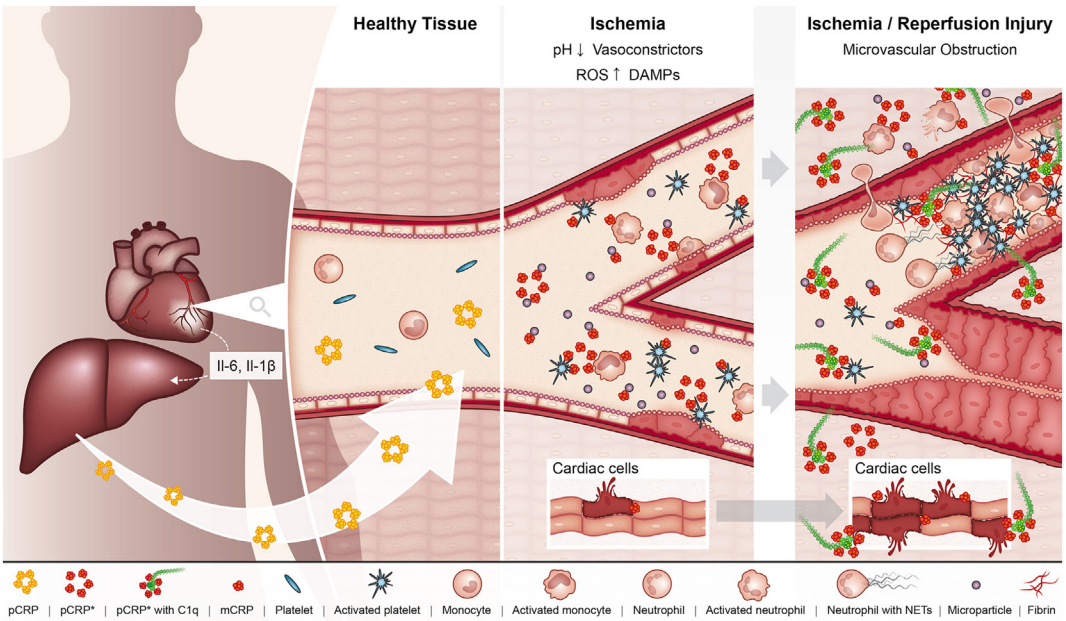

FIGURE 2 | In the setting of myocardial ischemia, the hepatic production of pCRP is upregulated under the regulation of interleukin 6 (IL-6). pCRP circulates in the blood, where it can interact with MPs, activated platelets, leukocytes, and endothelial cells in the ischemic myocardium, which triggers the dissociation of pCRP to its pro-inflammatory forms pCRP* and monomeric CRP (mCRP). In turn, these pro-inflammatory structural isoforms of CRP aggravate tissue injury by promoting the adhesion of leukocytes and platelet deposition to the ischemic endothelium, ultimately resulting in the formation of microvascular thrombi and thus microvascular obstruction. In addition, pCRP*/mCRP enhances leukocyte transmigration where infiltrating leukocytes degranulate and exacerbate tissue injury. pCRP*/mCRP also activate complement, which can directly mediate tissue injury *via* formation of the membrane attack complex. These pro-inflammatory effects of CRP ultimately result in enhanced myocardial injury and impaired organ function.

cell membrane of LPS-activated, but not resting, monocytes *in vitro* and *in vivo* (17). Here, CRP binds to stimulated monocytes, which release pro-inflammatory microvesicles loaded with pCRP*, thus allowing binding of the complement factor C1q and ultimately causing complement activation.

The acidic pH at sites of inflammation or ischemia can also lead to exposure of CRP neoepitopes (14–16). Emphasizing the broad biological relevance of CRP deposition and dissociation in damaged tissue, CRP has also been demonstrated to co-localize with complement factor C3d and leukocytes in tissue samples from burned wounds (63). Moreover, mCRP deposition has not only been found exclusively in areas of damaged tissue co-localized with CD68-positive monocytes and complement but also correlates with the predicted burn depth (64). Complementary *in vitro* data suggest that mCRP opsonizes necrotic cells and accelerates phagocytosis by macrophages (64). These observations, combined with previous data supporting a role of complement in mediating wound healing, suggest that the role of mCRP in this context likely extends beyond its direct pro-inflammatory effects and it may promote wound scarring and contracture development.

CRP IN ALLOGENEIC TRANSPLANTATION

Allogeneic solid organ transplantation is a curative therapy for patients with end-stage organ failure. Despite improvements in immunosuppressive protocols and supportive care, close to half of all patients lose their allografts within 10 years after transplantation due to chronic graft rejection and graft dysfunction (65). Based on multiple retrospective studies of human kidney transplantation, an acute rejection episode has been shown to be associated with a significantly decreased 1-year graft survival (66, 67), in addition to being linked to adverse long-term survival and graft outcomes (68).

Allograft rejection triggered by major histocompatibility complex incompatibility is dependent on T-cell activation. However, it has recently been demonstrated that innate recognition of allogeneic non-self by the recipient's monocytes can also initiate graft rejection (69). Importantly, it is now apparent that other factors such as IRI play a critical role in mediating the immune response to allogeneic transplantation. In solid organ transplantation, the impacts of IRI on both acute rejection and long-term allograft survival have been extensively investigated (70–72). For instance, increased rates of acute rejection episodes have been reported in experimental renal transplant models when ischemic times were prolonged (73) and large clinical trials have shown that the duration of cold ischemia time adversely correlates with allograft survival (72, 74). However, the relevance of the innate response to acute and chronic rejection in allogeneic transplantation is poorly understood.

Donor endothelial cells are the predominant target of the alloimmune response because these cells represent the first barrier to the recipient's immune system. Thus, allograft rejection typically manifests in the microvasculature of the transplanted tissue (75). IRI generates damage-associated molecular patterns (DAMPs) such

as heat shock proteins and ROS, heparin sulfate, or fibrinogen (76). DAMPs, in turn, can bind and activate toll-like receptors, which results in a strong inflammatory response and release of IL-1β, IL-6, and tumor necrosis factor, as well as CRP production (77).

With respect to the pro-inflammatory effects of CRP and its contribution to postischemic tissue injury, we have investigated the impact of CRP on innate allogeneic recognition and graft rejection in a hind limb transplantation model in rats (unpublished data). Based on our previous studies, we hypothesized that CRP-aggravated tissue damage and inflammation are associated with higher rates of acute and chronic rejection. Furthermore, we investigated the potential of inhibiting the conformational change of pCRP with bisPC as a therapeutic target for allogeneic transplantation *in vivo*. In our study, the administration of pCRP significantly accelerated clinical allograft rejection. We identified that the tissue damage-induced conformational change of pCRP led to increased systemic activation and localized transmigration of monocyte subpopulations. Most importantly, the stabilization of pCRP with bisPC abrogated its immunomodulatory effects and consequently inhibited aggravation of the acute transplant rejection (unpublished data). Thus, the inhibition of pCRP dissociation represents a promising, novel immunomodulatory therapeutic strategy in allogeneic solid organ transplantation.

CRP IN ATHEROSCLEROSIS

Inflammation incites and promotes the progression of atherosclerosis (78). In this regard, the role of CRP as a biomarker reflecting cardiovascular risk has received much attention (79, 80). Supporting the concept that CRP may reflect cardiovascular risk, data from the Women's Health Study and Physicians' Health Study demonstrated that CRP is a strong predictor of future cardiovascular events (81, 82). Moreover, the large-scale JUPITER trial has shown that treating patients with asymptomatic elevations in CRP provides benefit in reducing the cardiovascular event rate and death (7). Most interestingly, in the recent highly publicized CANTOS trial, the extent of the reduction of CRP achieved with the anti-Il-1β antibody canakinumab correlated directly with the extent of reduction in primary endpoint events, mainly myocardial infarction (8).

However, some controversy persists regarding whether CRP is a useful predictive biomarker in cardiovascular disease and this may reflect the fact that standard assays measure total CRP, rather than explicitly measuring the pro-inflammatory monomeric structural isoform (83). Aside from acting as a potential biomarker for cardiovascular disease, there is now a wealth of experimental data suggesting a direct causative role for CRP in promoting cardiovascular disease. Indeed, vulnerable plaques have been demonstrated to release CRP, which correlated with neutrophil Mac-1 activation (84). Additionally, CRP is present within atherosclerotic plaques and here is co-localized with macrophages, complement, and oxidized LDL (85). Significantly, we have demonstrated that mCRP, but not pCRP, is present within atherosclerotic plaques and that mCRP-bearing microparticles are significantly increased in patients with acute coronary syndromes (19, 21). Importantly, mCRP, but not pCRP, is detected within infarcted human myocardium, where it co-localizes with macrophages (20). More recently, *in vivo* work has demonstrated that mCRP accumulates at sites of endothelial inflammation and this results in enhanced leukocyte adhesion and transmigration (17). Demonstrating the importance of mCRP in exacerbating tissue injury, inhibition of pCRP dissociation with bisPC has been shown to reduce infarct size in a rat model of myocardial infarction (86).

These findings support the concept that mCRP may not only be a valuable biomarker in cardiovascular disease but also directly acts to promote a deleterious inflammatory response in the context of atherogenesis and myocardial infarction.

CRP IN ALZHEIMER'S DISEASE (AD)

Alzheimer's disease represents the leading cause of dementia (87). The underlying pathogenesis of AD appears to involve the accumulation of amyloid beta (Aβ) plaques in extracellular spaces and within the walls of the vasculature (88). The later stages of AD typically involve the accumulation of neurofibrillary tangles—comprised of Tau protein aggregates—in neurons (89). Previous studies have demonstrated that neuronal tissue from patients with AD expresses higher amounts of CRP compared to non-AD controls and that CRP staining co-localizes with senile plaques (90, 91). Recently, evidence is beginning to emerge that CRP may actively contribute to the pathogenesis of AD. Aβ plaques can dissociate pCRP *in vitro* and, in accordance with this, mCRP and C1q are observed to co-localize with Aβ plaques in human AD sections (92).

Supporting the concept that mCRP may directly contribute to AD pathogenesis, recent data demonstrate that the hippocampal injection of mCRP in a mouse model of AD induces cognitive decline and behavioral changes (93). These features correlate with structural changes, with mCRP-treated mice displaying enhanced p-Tau and p-β amyloid plaque production (93). mCRP has also been demonstrated to induce Tau filament polymerization and directly trigger potentially neurodegenerative signaling pathways in rat cortical neurons *in vitro*. Interestingly, mCRP is also abundant within the microvasculature of patients with AD and co-localizes with β amyloid and CD105 (93). This observation appears most prominent in patients with prior ischemic stroke, which raises the interesting possibility that mCRP deposition in these regions promotes dysregulated angiogenesis and, therefore, promulgates the small vessel vasculopathy characteristic of AD.

CRP IN AGE-RELATED MACULAR DEGENERATION (AMD)

Age-related macular degeneration is a leading cause of blindness in developed countries (94). It has previously been demonstrated that elevated CRP levels serve as an independent risk factor for the development and progression of AMD (95). Recently, it was shown that mCRP plays a potentially crucial pathogenic role in patients with the high-risk CFH SNP (Y402H) (96). This SNP involving the CFH gene is considered one of the most significant genetic risk factors for AMD development (97). Indeed, levels of mCRP, but not pCRP, are increased in the choroid of patients with the high-risk CFH mutation (96). Moreover, the application of mCRP to choroidal endothelial cells *in vitro* results in enhanced endothelial cell permeability and migration, both of which are

important for AMD development (96). Further supporting the notion that mCRP may promote AMD development, human choroidal tissue exhibits an altered transcriptome in response to mCRP stimulation. The expression of both ICAM-1 and carbonic anhydrase 4 (CA4) is altered at the mRNA and protein level, with mCRP resulting in increased ICAM-1 expression and decreased CA4 (96). These are important observations, given that these changes are observed in patients with AMD and are linked to the development of AMD (98, 99).

THERAPEUTIC TARGETING OF CRP

The powerful pro-inflammatory properties of pCRP* and mCRP in mediating a broad range of disease states make inhibition of CRP an attractive therapeutic strategy. In this regard, 1,6 bis-phosphocholine (bisPC) has previously been shown to ameliorate the deleterious effects of CRP in mediating myocardial IRI (20, 86). Indeed, bisPC prevents mCRP formation and deposition in infarcted myocardium in addition to preserving myocardial function in the context of IRI. bisPC abrogates the pro-inflammatory effects of CRP by binding to and preventing CRP dissociation, in addition to blocking the binding of pCRP to MPs, thus inhibiting the interactions of CRP with complement (17, 86). However, the pharmacokinetic profile of bisPC is unfavorable, given its relatively low affinity for CRP (Kd = 150 nM) and relatively short half-life of 90 min in mice (86). Therefore, there remains much interest in developing novel, more potent inhibitors of CRP with improved bioavailability.

PERSPECTIVE

Deepening our understanding of the role of CRP, as an essential arm of the innate immune response and a central player in the pathogenesis of a range of inflammatory conditions, will be vitally important in translating potential anti-inflammatory, anti-CRP approaches to the clinic. However, to date, an important limitation in exploring the direct effects of CRP inhibition in these chronic diseases is the lack of suitable mouse models. Currently, the most widely used approach to study the role of CRP *in vivo* is the exogenous administration of pCRP to mice or rats prior to an acute inflammatory challenge. This is due to basic phylogenic differences in CRP biology between rodents and humans. For example, mice express very low levels of CRP, while rat CRP cannot activate complement (100, 101).

Therefore, while the administration of exogenous human CRP to rodents has afforded essential insights regarding CRP biology, it does not allow for *in vivo* mechanistic studies of CRP in chronic inflammatory conditions such as IRI, transplant rejection, atherosclerosis, or AMD. Thus, the development of novel animal models that express human CRP will allow more detailed studies regarding the precise mechanism of CRP dissociation in inflammation and its potential role as a novel therapeutic target.

AUTHOR CONTRIBUTIONS

JM, JK, SE, and KP conceived and co-wrote the manuscript. JL-S, DB, and LP co-wrote the manuscript.

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Effect of Roux-en-Y Bariatric Surgery on Lipoproteins, Insulin Resistance, and Systemic and Vascular Inflammation in Obesity and Diabetes

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Purpose: Obesity is a major modifiable risk factor for cardiovascular disease. Bariatric surgery is considered to be the most effective treatment option for weight reduction in obese patients with and without type 2 diabetes (T2DM).

Objective: To evaluate changes in lipoproteins, insulin resistance, mediators of systemic and vascular inflammation, and endothelial dysfunction following Roux-en-Y bariatric surgery in obese patients with and without diabetes.

Materials and methods: Lipoproteins, insulin resistance, mediators of systemic and vascular inflammation, and endothelial dysfunction were measured in 37 obese patients with (n = 17) and without (n = 20) T2DM, before and 6 and 12 months after Roux-en-Y bariatric surgery. Two way between subject ANOVA was carried out to study the interaction between independent variables (*time since surgery* and *presence of diabetes*) and all dependent variables.

Results: There was a significant effect of *time since surgery* on (large effect size) weight, body mass index (BMI), waist circumference, triglycerides (TG), small-dense LDL apolipoprotein B (sdLDL ApoB), HOMA-IR, CRP, MCP-1, ICAM-1, E-selectin, P-selectin, leptin, and adiponectin. BMI and waist circumference had the largest impact of *time since surgery.* The effect of *time since surgery* was noticed mostly in the first 6 months. Absence of diabetes led to a significantly greater reduction in total cholesterol, low-density lipoprotein cholesterol, and non-high-density lipoprotein cholesterol although the effect size was small to medium. There was a greater reduction in TG and HOMA-IR in patients with diabetes with a small effect size. No patients were lost to follow up.

Conclusion: Lipoproteins, insulin resistance, mediators of systemic and vascular inflammation, and endothelial dysfunction improve mostly 6 months after bariatric surgery in obese patients with and without diabetes.

Clinical Trial Registration: www.ClinicalTrials.gov, identifier: NCT02169518. https://clinicaltrials.gov/ct2/show/NCT02169518?term=paraoxonase&cntry1=EU%3AGB&rank=1.

Keywords: Roux-en-Y, lipoproteins, insulin resistance, vascular inflammation, diabetes

INTRODUCTION

Globally, mean body mass index (BMI) has increased progressively since 1980 (1). Moreover, a 33% increase in obesity prevalence and a 130% increase in severe obesity prevalence have been projected over the next 2 decades (2). Excess mortality above the BMI range of 22.5–25 kg/m^2 is mainly attributed to vascular disease (3). The outcome of the Swedish obese subjects (SOS) trial indicated that bariatric surgery, when compared with usual care, was associated with a long-term reduction in overall mortality and reduced incidence of type 2 diabetes (T2DM), myocardial infarction, stroke, and cancer (4). Indeed 72% of patients with T2DM at baseline were in remission 2 years after surgery and high baseline insulin and/or high glucose predicted favorable treatment effects, whereas high BMI did not (4).

There is evidence that high-sensitivity C-reactive protein (hsCRP), tumor necrosis factor-alpha (TNFα), monocyte chemoattractant protein 1 (MCP-1), intercellular adhesion molecule 1 (ICAM-1), E-selectin, P-selectin, resistin, and leptin may be mediators of insulin resistance, vascular inflammation, and endothelial dysfunction in those with obesity (5–9). It has been suggested that adiponectin increases insulin sensitivity and reduces the risk of atherosclerosis (10). Leptin and adiponectin are primarily released from the adipocytes whereas hsCRP, TNFα, MCP-1, and ICAM-1 are chiefly released from non-fat cells in adipose tissue (11).

While some studies have shown a significant reduction in mediators of vascular inflammation after bariatric surgery at 6 months (12) and 12 months (13–16), but not at 1 month (17). Other studies have reported no significant change in mediators of vascular inflammation 6 months after bariatric surgery (18).

In this study, we hypothesized a significant improvement in lipids, lipoproteins, insulin resistance, mediators of systemic and vascular inflammation, and endothelial dysfunction at 6 and 12 months after Roux-en-Y bariatric surgery in obese patients with and without diabetes. Outcomes:

1. Effect size of time since surgery on dependent variables associated with lipoproteins, insulin resistance, mediators of systemic and vascular inflammation, and endothelial dysfunction at 6 and 12 months after Roux-en-Y bariatric surgery (as small, medium, and large).
2. Effect size of presence/absence of diabetes on dependent variables associated with lipoproteins, insulin resistance, mediators of systemic and vascular inflammation, and endothelial dysfunction at 6 and 12 months after Roux-en-Y bariatric surgery (as small, medium, and large).

MATERIALS AND METHODS

Participants

The study was approved by the local research ethics committee. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. We recruited 37 obese patients (17 with T2DM) awaiting bariatric surgery at Salford Royal Hospital (Salford, UK). Informed consent was obtained from all individual participants included in the study. Patients with anemia, acute coronary syndrome within 6 months, any malignancy, active infections, autoimmune diseases, HIV, and other chronic diseases were excluded. All smokers were required to stop smoking at least 2 weeks prior to the date of surgery. Each participant attended The Wellcome Trust Clinical Research Facility (Manchester, UK) where blood samples were taken at baseline, 6, and 12 months. Hypertension was defined as clinic blood pressure 140/90 mmHg or higher and subsequent ambulatory blood pressure monitoring daytime average or home blood pressure monitoring average blood pressure of 135/85 mmHg or higher (NICE Clinical Guideline127, https://www.nice.org.uk/guidance/CG127/chapter/1-Guidance#diagnosing-hypertension-2).

Separation of Serum and Plasma

Blood samples were collected between 09:00 and 11:00 hr after participants had fasted from 22:00 hr the previous day. Serum and EDTA-plasma were isolated by centrifugation at 2,000 × *g* for 15 min at 4°C within 2 h of collection and were maintained at that temperature until further use. Aliquots for biochemical analysis were frozen at −80°C.

Laboratory Analyses

Total cholesterol (TC) was measured using the cholesterol oxidase phenol 4-aminoantipyrine peroxidase method, triglycerides (TG) by the glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase method, and apolipoprotein A1 (apo-A1) and apolipoprotein B (apo B) were assayed using immunoturbidimetric assays (ABX Diagnostics, Shefford, UK). High-density lipoprotein cholesterol (HDL-C) was assayed using a second-generation homogenous direct method (Roche Diagnostics, Burgess Hill, UK). All these tests were performed on a Cobas Mira analyser (Horiba ABX Diagnostics, Nottingham, UK). The laboratory participated in the RIQAS (Randox International Quality Assessment Scheme; Randox Laboratories, Dublin, Ireland) scheme which is

CRC calibrated. Low-density lipoprotein cholesterol (LDL-C) was estimated using the Friedewald formula. Non-HDL-C was estimated using the formula: non-HDL-C = (TC) − (HDL-C).

Small-dense LDL apolipoprotein B (sdLDL apoB) (density range 1.044–1.063 g/mL) was isolated from plasma adjusted to density of 1.044 g/mL and ultracentrifuged at 100,000 rpm (435,680 × *g*) for 5 h at 4°C using a Beckman Optima TLX bench top ultracentrifuge fitted with TLA 120.2 fixed angle rotor (Beckman Coulter UK) (19). ApoB in SdLDL was determined using the method described above.

Biomarkers

An in-house, antibody sandwich ELISA technique using anti-human CRP antibodies, calibrators, and controls from Abcam (Cambridge, UK) was used to measure hsCRP. TNFα, adiponectin, leptin, resistin, MCP-1, E-selectin, P-selectin, resistin, and ICAM-1 were all measured using DuoSet ELISA development kits from R&D Systems (Abingdon, UK).

Glycated hemoglobin (HbA1c) and fasting blood glucose were measured using the standard laboratory methods in the Department of Clinical Biochemistry, Central Manchester University Hospitals National Health Service Foundation Trust. Insulin was determined in plasma using Mercodia ELISA kits from Diagenics Ltd. (Milton Keynes, UK). Homeostatic model assessment was used to assess insulin resistance (HOMA-IR) using the following formula (20):

$$\text{HOMA} - \text{IR} = \left[\text{insulin}(\text{mU/l}) \times \text{glucose}(\text{mmol/l})\right]/22.5.$$

Statistical Analyses

Statistical analysis was performed with SPSS for Windows, Version 16.0 (Chicago, SPSS Inc.). Two way between subject ANOVA was carried out to study the interaction between independent variables (*time since surgery* and *diabetes)* and all dependent variables (e.g., weight, LDL-C, HOMA-IR, and TNFα). Univariate analysis was done to calculate effect size. R-E-G-W-Q was selected as the *post hoc* test for *time since surgery* but not *diabetes* as the latter independent variable had only two levels (i.e., presence or absence of diabetes). As sample sizes were similar, this test offered good control of type 1 error and a superior ability to detect the difference if present. Partial *eta* squared (η^2) was calculated to establish the variance in dependent variables attributed to independent variables. η^2 effect was graded as 0.01 = small, 0.06 = medium, and 0.14 = large. If effect of independent variables on dependent variables was significant, pairwise comparisons were reviewed to

TABLE 1 | Characteristics of all patients, non-diabetic group and diabetes group at baseline, 6 months, and 12 months after surgery.

	All patients (*n* = 37)			Non-diabetic patients (*n* = 20)			Diabetes patients (*n* = 17)		
	Baseline	**6 months**	**12 months**	**Baseline**	**6 months**	**12 months**	**Baseline**	**6 months**	**12 months**
Weight (kg)	140 (21)	105 (25)	93 (22)	138 (29)	105 (25)	91 (22)	143 (32)	105 (26)	95 (23)
Body mass index (kg/m^2)	52 (9)	39 (7)	35 (7)	51 (9)	38 (7)	34 (7)	53 (9)	40 (8)	36 (7)
% EWL		46 (40–60)	63 (51–77)		46 (42–59)	69 (51–77)		49 (33–62)	60 (48–74)
Waist circumference (cm)	141 (17)	116 (15)	106 (15)	141 (16)	114 (13)	104 (15)	141 (18)	119 (18)	109 (15)
SBP (mmHg)	135 (22)	127 (20)	124 (20)	140 (25)	124 (21)	128 (23)	129 (19)	131 (19)	119 (14)
DBP (mmHg)	74 (13)	75 (13)	69 (10)	76 (15)	73 (13)	71 (11)	71 (90)	77 (12)	68 (10)
Total cholesterol (mmol/l)	4.84 (1.30)	4.94 (1.31)	4.57 (0.80)	5.26 (1.51)	5.00 (91.22)	4.76 (0.71)	4.36 (0.80)	4.87 (1.46)	4.36 (0.88)
Triglycerides (TG) (mmol/l)	1.75 (0.84)	1.45 (0.45)	1.16 (0.39)	1.55 (0.63)	1.37 (0.47)	1.12 (0.32)	1.98 (1.00)	1.54 (0.43)	1.22 (0.46)
Low density lipoprotein cholesterol (mmol/l)	2.81 (1.19)	3.04 (1.15)	2.56 (0.77)	3.28 (1.37)	3.17 (1.10)	2.75 (0.75)	2.26 (0.61)	2.89 (1.22)	2.34 (0.75)
Non-high density lipoprotein cholesterol (HDL-C, mmol/l)	3.61 (1.21)	3.66 (1.19)	3.09 (0.75)	3.99 (1.44)	3.71 (1.11)	3.25 (0.71)	3.17 (0.68)	3.59 (1.31)	2.90 (0.77)
Small-dense LDL apolipoprotein B (mg/dl)	23.19 (12.01)	13.86 (7.51)	11.57 (5.39)	24.23 (14.75)	13.46 (5.57)	11.10 (5.07)	22.04 (8.24)	14.34 (9.54)	12.10 (5.84)
ApoB (g/l)	0.96 (0.25)	1.01 (0.31)	0.88 (0.20)	1.01 (0.30)	1.01 (0.30)	0.91 (0.20)	0.90 (0.16)	1.02 (0.34)	0.84 (0.20)
HDL-C (mmol/l)	1.22 (0.29)	1.28 (0.34)	1.4 (0.34)	1.26 (0.34)	1.28 (0.37)	1.51 (0.35)	1.18 (0.22)	1.27 (0.31)	1.46 (0.34)
ApoA (g/l)	1.33 (0.23)	1.31 (0.24)	1.36 (0.22)	1.28 (0.27)	1.27 (0.25)	1.35 (0.24)	1.39 (0.17)	1.35 (0.23)	1.38 (0.21)
Adiponectin (mg/l)	1.59 (0.70)	2.15 (0.88)	2.74 (1.16)	1.64 (0.75)	2.22 (0.93)	2.90 (1.22)	1.54 (0.66)	2.07 (0.83)	2.56 (1.09)
Leptin (ng/l)	85.87 (49.50)	32.63 (26.86)	23.72 (24.06)	91.86 (51.04)	33.37 (27.45)	22.64 (15.28)	78.82 (48.19)	31.71 (26.98)	25.00 (31.98)
HOMA-IR	8.12 (6.69)	3.80 (4.27)	2.68 (2.79)	6.02 (3.48)	3.57 (5.34)	1.95 (1.63)	10.60 (8.63)	4.10 (2.50)	3.53 (3.61)
CRP (mg/l)	10.25 (10.66)	5.42 (7.69)	2.20 (2.51)	9.23 (9.52)	4.83 (5.83)	2.00 (2.08)	11.46 (12.05)	6.16 (9.70)	2.43 (2.99)
Tumor necrosis factor-alpha (pg/ml)	33.50 (39.50)	30.50 (51.09)	11.33 (29.90)	33.04 (38.36)	31.49 (40.50)	15.89 (39.17)	34.04 (41.94)	29.26 (63.32)	5.96 (11.53)
Monocyte chemoattractant protein 1 (pg/ml)	288 (147)	222 (129)	154 (84)	273 (148)	207 (116)	162 (80)	307 (149)	240 (146)	145 (91)
Intercellular adhesion molecule 1 (ng/ml)	226 (104)	184 (75)	134 (38)	224 (73)	176 (54)	135 (39)	228 (134)	193 (96)	134 (38)
E-selectin (ng/ml)	12.05 (5.80)	9.50 (3.87)	3.10 (3.67)	11.89 (5.32)	7.33 (3.11)	7.52 (3.41)	12.30 (6.48)	7.71 (4.75)	6.61 (4.00)
P-selectin (ng/ml)	36.22 (13.95)	30.37 (10.75)	23.16 (7.52)	37.13 (14.47)	29.30 (9.90)	22.87 (6.03)	35.15 (13.68)	31.70 (11.91)	23.48 (9.13)
Resistin (ng/ml)	14.78 (6.20)	14.60 (6.54)	11.30 (5.32)	14.39 (3.78)	14.66 (5.33)	12.02 (4.91)	15.23 (8.32)	14.53 (7.98)	10.44 (5.80)

Values in mean ± SD. No patients were lost to follow-up.

check in which period (0–6 months post surgery or 6–12 months post surgery) the main effect lay. Effect has been reported as F (dF, error) = F value, P-value, η^2 (effect size) [where F is effect and dF is degrees of freedom]. Differences were considered as statistically significant at $P < 0.05$.

RESULTS

Of the 37 obese patients in the study, the mean age was 49 (range, 26–63) years, mean BMI 52 (9), 17 (45%) had T2DM, 23 (60%) had hypertension, 8 (20%) were smokers, 5 (15%) were known to have ischemic heart disease, and 22 (60%) patients took a statin. In the non-diabetes patients ($n = 20$), eight (40%) were on statins (Atorvastatin equivalent dose 10–40 mg/day), whereas in the diabetes patients ($n = 17$), 15 (90%) patients were on a statin (Atorvastatin equivalent dose 10–40 mg/day). Characteristics of all patients, non-diabetes patients and diabetes patients at baseline, 6, and 12 months have been presented in **Table 1** and **Figure 1**.

Time since Surgery

There was a significant effect of *time since surgery* on lipoproteins, insulin resistance, mediators of systemic and vascular inflammation, and endothelial dysfunction (**Table 2**). A large effect size was seen on weight, BMI, waist circumference, TG, sdLDL ApoB, HOMA-IR, CRP, MCP-1, ICAM-1, E-selectin, P-selectin, leptin, and adiponectin. Medium effect size was seen on HDL-C, TNFα, and resistin and small effect size was seen on non-HDL-C. BMI and waist circumference had the largest impact of *time since surgery.* The effect of *time since surgery* was noticed mostly in the first 6 months.

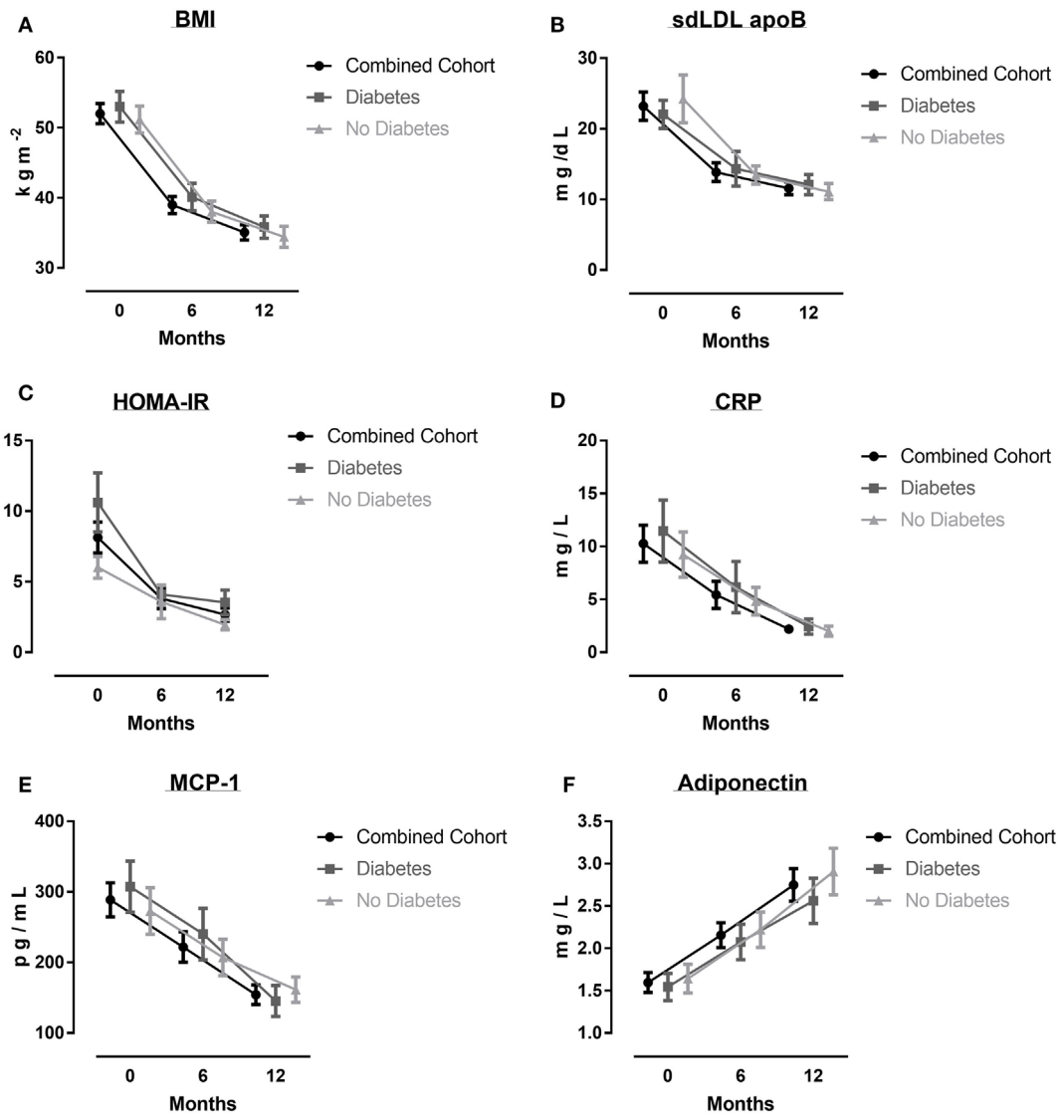

FIGURE 1 | Various parameters before, and 6 and 12 months after bariatric surgery: body mass index (BMI) **(A)**, small-dense LDL apolipoprotein B (sdLDL apoB) **(B)**, HOMA-IR **(C)**, CRP **(D)**, MCP-1 **(E)**, and adiponectin **(F)**. Results are given as mean (±SEM).

TABLE 2 | Shows effect size of time since surgery on dependent variables as small, medium, and large (only data with significant *P* values shown).

	F (dF; error)	*P*-value	Partial eta square (effect size)	*P*-value for effect at 6 months since surgery	*P*-value for effect between 6 and 12 months after surgery
Weight	31.93 (2; 70,843)	<0.0001	0.38 (large)	<0.0001	0.167
Body mass index	49.65 (2; 5,992)	<0.0001	0.49 (large)	<0.0001	0.095
Waist circumference	47.98 (2; 2,5091)	<0.0001	0.48 (large)	<0.0001	<0.05
Triglycerides (TG)	9.39 (2; 36)	<0.0001	0.15 (large)	<0.05	0.129
Non-high density lipoprotein cholesterol (HDL-C)	3.27 (2; 116)	<0.05	0.005 (small)	1.0	<0.05
Small-dense LDL apolipoprotein B	16.82 (2; 7,820)	<0.0001	0.25 (large)	<0.0001	0.86
HDL-C	6.19 (2; 11)	<0.005	0.10 (medium)	1.0	<0.05
HOMA-IR	14.32 (2; 2,319)	<0.0001	0.21 (large)	<0.0001	0.983
CRP	10.09 (2; 6,336)	<0.0001	0.16 (large)	<0.05	0.23
Tumor necrosis factor-alpha	3.19 (2; 178,782)	<0.05	0.06 (medium)	1.0	<0.05
Monocyte chemoattractant protein 1	11.14 (2; 1,599,516)	<0.0001	0.17 (large)	<0.05	<0.05
Intercellular adhesion molecule 1	12.74 (2; 632,746)	<0.0001	0.20 (large)	<0.05	<0.05
E-selectin	13.17 (2; 2,214)	<0.0001	0.20 (large)	<0.0001	1.0
P-selectin	9.34 (2; 12,327)	<0.0001	0.17 (large)	0.123	<0.05
Leptin	31.81 (2; 132,717)	<0.0001	0.38 (large)	<0.0001	0.90
Resistin	3.9 (2; 3,875)	<0.05	0.071 (medium)	1.0	<0.05
Adiponectin	13.38 (2; 92)	<0.0001	0.20 (large)	<0/05	<0.05

Also shows P values for effect at 6 months since surgery and from 6 to 12 months after surgery.
F, effect that is being reported; dF, degrees of freedom.

Diabetes

Absence of diabetes led to a significantly greater reduction in TC, LDL-C, and non-HDL-C although the effect size was small to medium (**Table 3**). There was a greater reduction in TG and HOMA-IR in patients with diabetes with a small effect size (**Table 3**). No patients were lost to follow-up.

TABLE 3 | Shows effect size of diabetes on dependent variables as small, medium, and large (only data with significant *P* values shown).

	F (dF, error)	*P*-value	Partial eta square (effect size)	Comments
Total cholesterol	4.71 (1, 137)	<0.05	0.043 (small)	Absence of diabetes led to greater reduction
Triglycerides	4.18 (1, 36)	<0.05	0.039 (small)	Presence of diabetes led to greater reduction
Low-density lipoprotein cholesterol	8.51 (1, 107)	<0.005	0.076 (medium)	Absence of diabetes led to greater reduction
Non–high-density lipoprotein cholesterol	4.5 (1, 116)	<0.05	0.042 (small)	Absence of diabetes led to greater reduction
HOMA-IR	6.07 (1, 2319)	<0.05	0.05 (small)	Presence of diabetes led to greater reduction

F, effect that is being reported, dF, degrees of freedom.

DISCUSSION

In this study of morbidly obese patients with or without diabetes followed over 6 and 12 months after Roux-en-Y gastric bypass surgery, we found a significant reduction in pro-atherosclerotic lipoproteins, insulin resistance, mediators of systemic and vascular inflammation, and endothelial dysfunction.

Bariatric surgery has been shown to consistently achieve significant and sustained weight loss; however, the effect on various markers of inflammation is quite variable. A recent meta-analysis evaluated the changes in blood levels of CRP and TNFα after bariatric surgery (21). There was a 54 and 81% reduction in CRP at 6 and 12 months, respectively, which is comparable to our study where we show a 47 and 78% reduction in CRP. While TNFα was only reduced by 1.3 and 1.2% at 6 and 12 months, respectively, in our study we report a 9 and 66% reduction. The much greater reduction in TNFα in our study compared with the meta-analysis could be due to a higher baseline BMI which was 52 kg/m^2 compared to other studies included in the meta-analysis where the average BMI was <50 kg/m^2. It could also reflect the use of Roux-en-Y surgery in our study compared to a variety of procedures, such as laparoscopic-adjustable gastric banding (LAGB), biliopancreatic diversion (BPD), or sleeve gastrectomy (SG) in the meta-analysis. There could also be significant differences in the number of patients with non-alcoholic steatohepatitis or ectopic adipose tissue, where the effect of weight loss may differ. Indeed Bachmayer et al. have reported no significant change in TNFα, MCP-1, or adiponectin, 12 months after Roux-en-Y, SG, and BPD (22). Auguet et al. have demonstrated a significant reduction in weight, insulin resistance, and CRP and circulating TNF receptors at both 6 and 12 months, with an increase in adiponectin at 6 but not 12 months after bariatric surgery (23). Sdralis et al. have demonstrated a significant fall in insulin with an increase in adiponectin with no change in TNFα even at 12 months (24). Thus, this area of research needs larger trials with a more uniform baseline weight and postoperative weight loss as well as surgical intervention such as Roux-en-Y surgery as it may lead to an exaggerated glucagon-like peptide-1 response, which may not be seen with LAGB, SG, and BPD (25).

In human studies, the role of resistin in insulin resistance and glucose metabolism is inconclusive (26, 27). Bariatric surgery results in a significant reduction in resistin levels at 12 months which correlates with insulin resistance (28). Our study confirms the significant reduction in resistin levels 12 months after bariatric surgery.

Tumor necrosis factor-alpha may play a direct role in the development of atherosclerosis through induction of ICAM-1, MCP-1, P-selectin, and E-selectin in endothelial and vascular smooth muscle cells resulting in endothelial cell apoptosis (29). Moreover, resistin augments the expression of endothelin-1, MCP-1, and ICAM-1 in endothelial cells (30, 31). The continued reduction in resistin and TNFα in the obese diabetic group up to 12 months after surgery may explain the reduction in HOMA-IR, MCP-1, and ICAM-1 in this group over the same period.

SdLDL apoB is a LDL subtype closely associated with diabetes and atherosclerosis (32), because of its greater susceptibility to undergo oxidative modification and glycation compared with more buoyant LDL (33, 34). While LAGB has been shown to modestly reduce sdLDL apoB at 12 months (35), our study shows a much more robust reduction in sdLDL apoB at 6 months with Roux-en-Y bariatric surgery.

We also demonstrate no change in apo-A1 but a significant increase in HDL-C after surgery which may indicate increased cholesterol cargo of HDL lipoproteins returning from the peripheral vasculature back to the liver, i.e., reverse cholesterol transport.

In the DM group, TC and LDL-C did not change significantly probably reflecting the fact that 90% of the DM group were already on long-term statins.

Limitations

The 12-month follow-up of patients was relatively short. All patients recruited for this study underwent Roux-en-Y bariatric surgery; therefore, these results may not be generalized to all categories of bariatric surgery. The number of patients included in this study was limited by availability.

CONCLUSION

Lipoproteins, insulin resistance, mediators of systemic and vascular inflammation, and endothelial dysfunction improve most 6 months after bariatric surgery in obese patients with and without diabetes. The greater reduction in inflammatory markers in our study compared to published meta-analyses may reflect a greater impact of Roux-en-Y surgery on weight loss achieved and may in the long-term translate to more pronounced cardiovascular benefit.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of NRES Committee North West—Greater Manchester Central with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the NRES Committee North West—Greater Manchester Central.

AUTHOR CONTRIBUTIONS

HS, PD, and BA designed the study. RY, AS, SH, RA, JN, SA, and JH recruited patients and organized follow-ups. HS, RY, SH, YL, JS, MF, SA, TS, JH, PP, MJ, and RD did laboratory work and data analysis. RY, SH, RM, RD, PD, BA, JS, TS, RA, SD, and HS prepared the first draft. All authors contributed in revising the manuscript for important intellectual content. All authors approved of the version to be published. All authors agreed to be accountable for all aspects of the work.

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Inflammation, a Double-Edge Sword for Cancer and Other Age-Related Diseases

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Increasing evidence from diverse sources during the past several years has indicated that long-term, low level, chronic inflammation mediates several chronic diseases including cancer, arthritis, obesity, diabetes, cardiovascular diseases, and neurological diseases. The inflammatory molecules and transcription factors, adhesion molecules, AP-1, chemokines, C-reactive protein (CRP), cyclooxygenase (COX)-2, interleukins (ILs), 5-lipooxygenase (5-LOX), matrix metalloproteinases (MMPs), nuclear factor (NF)-kB, signal transducer and activator of transcription 3 (STAT3), tumor necrosis factor (TNF), and vascular endothelial growth factor (VEGF) are molecular links between inflammation and chronic diseases. Thus, suppression of inflammatory molecules could be potential strategy for the prevention and therapy of chronic diseases. The currently available drugs against chronic diseases are highly expensive, minimally effective and produce several side effects when taken for long period of time. The focus of this review is to discuss the potential of nutraceuticals derived from "Mother Nature" such as apigenin, catechins, curcumin, ellagic acid, emodin, epigallocatechin gallate, escin, fisetin, flavopiridol, genistein, isoliquiritigenin, kaempferol, mangostin, morin, myricetin, naringenin, resveratrol, silymarin, vitexin, and xanthohumol in suppression of these inflammatory pathways. Thus, these nutraceuticals offer potential in preventing or delaying the onset of chronic diseases. We provide evidence for the potential of these nutraceuticals from pre-clinical and clinical studies.

Keywords: cancer, chronic disease, cytokine, inflammation, nutraceutical

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The term "inflammation" that means "to set on fire" can be both acute and chronic. Although acute inflammation is beneficial, chronic inflammation is a source for several chronic diseases including cancer, diabetes, and obesity (1). The modern science has delineated the molecular basis of inflammation. The inflammatory molecules and transcription factors such as 5-LOX, adhesion molecules, chemokines, COX-2, C-reactive protein, cytokines, MMPs, NF-κB, prostate-specific antigen (PSA), STAT3, TWIST, and vascular endothelial growth factor (VEGF) are known molecular links between inflammation and chronic diseases (**Figure 1**) (1). The pro-inflammatory transcription factors (NF-κB and STAT3) are the crucial regulators of inflammation (1, 2). For example, more than 500 cancer related genes are known to be regulated by NF-kB (3, 4).

The epidemiological, genetic and pharmacological studies support the association of inflammation with chronic diseases (5). For example, accumulating evidence suggest that chronic

inflammation is precursor to most tumors. The gastritis (inflammation of lining of stomach) can lead to gastric cancer (6). It is estimated that almost 20% of smokers with bronchitis (inflammation of the mucous membrane in the bronchial tubes) can develop lung cancer in their lifetime (7). Similarly, colitis (inflammation of colon) is a precursor to colon cancer (8). Chronic inflammation plays a crucial role in various aspects of tumor development including cellular transformation, survival, proliferation, invasion, metastasis, and angiogenesis (5, 9). The healthy lifestyle can significantly reduce the risk of developing cancer, cardiovascular diseases, type 2 diabetes, and stroke (10).

The lifestyle factors such as alcohol, infectious agents, obesity, radiation, stress, tobacco, and toxicants are known activators of inflammatory pathways. The dietary intake of low-density lipoproteins can induce inflammation of the arteries. Omega-6 essential fatty acids commonly present in dietary vegetable oils, is known to induce inflammation. However, omega-3 fatty acids can lower inflammation. The dietary dairy protein (casein) and wheat protein (gluten) can also induce inflammation. The environmental sources of inflammation are toxicants such as adhesives, air fresheners, cleaning products, glues, latex, plastics, and synthetic fibers. The inflammation can also be induced by hormonal changes such as estrogen, progesterone, and testosterone. The lifestyle factors are known to modulate the production of inflammatory molecules (11). Lifestyle factors can also induce production of reactive oxygen species (ROS), which in turn lead to inflammation (12–15). ROS can regulate production of several inflammatory molecules such as chemokines, cyclooxygenase-2, cytokines, and pro-inflammatory transcription factors (16).

It is now well known that chronic inflammation is a cause for most chronic diseases. Thus, chronic treatment is required for most chronic diseases. In addition, dysregulation in multiple inflammatory molecules contribute to the development of chronic diseases. Yet, drugs for most of the chronic diseases are based on the modulation of more specifically a single target. Thus, these drugs are less likely to be effective. In addition, these drugs are highly expensive and are associated with numerous side effects when taken for long period of time (17–20). The implication of these facts necessitates the development of agents that are cost-effective, multi-targeted, and readily available. Because of their affordability, safety, and long-term use, agents derived from natural sources (nutraceuticals) possess enormous potential (21, 22). The sources of nutraceuticals include cereals, fruits, nuts, pulses, spices and vegetables. A recent study suggests that more than 70% of the drugs introduced over the past 25 years have been originated from nature (23).

The evidence from pre-clinical and clinical studies support the role of nutraceuticals in suppressing inflammatory pathways. Curcumin, which is derived from the golden spice turmeric, is known to modulate the production as well as activity of a number of inflammatory molecules (24). Curcumin can also directly bind to a number of inflammatory molecules. For example, the molecular docking studies have revealed that curcumin can bind at the receptor-binding sites of TNF-α by forming both noncovalent and covalent interactions (25). Curcumin can also directly bind and inhibit the activities of COX-1, COX-2, and MMP (26, 27). The potential anticancer activities of nutraceuticals by modulating NF-kB activation pathway has been documented by numerous lines of evidence. The nutraceuticals are known to suppress NF-κB activity by modulating several steps such as IKK activation, phosphorylation and degradation of IκBa, p65 nuclear translocation, phosphorylation and acetylation of p65, and p65 DNA binding. The most common nutraceuticals known to inhibit NF-κB activation include caffeic acid phenethyl ester (CAPE) (28), capsaicin (29), curcumin (30), emodin (31), epigallocatechin gallate (EGCG) (32, 33), guggulsterone (34), resveratrol (35, 36), and sanguinarine (29). Some nutraceuticals such as guggulsterone (34) and EGCG (33) act by inhibiting IKK activation. Curcumin (34, 37, 38), guggulsterone (34), capsaicin (29, 39), sanguinarine (29), emodin (31), and EGCG (33) are known to prevent phosphorylation and degradation of IκBα, which is a central point in NF-κB activation. Capsaicin (1, 29, 39) and EGCG (33) are known to inhibit nuclear translocation of NF-κB p65. Nutraceuticals can also inhibit the binding of p65 with DNA. For example, in human myeloid leukemia cells, curcumin was found to inhibit p65-DNA binding (30). Caffeic acid phenethyl ester can suppress the direct binding of the p50-p65 complex with DNA (28). In HeLa cells, emodin can oxidize the redox-sensitive site on NF-κB and thereby can prevent NF-κB-DNA binding (40). Plumbagin can inhibit NF-κB-DNA binding in breast cancer cells (41, 42). Nutraceuticals are also known to sensitize cancer cells to the chemotherapeutic agents and to induce apoptosis through modulation of NF-κB activation pathway. The most common nutraceuticals among this category are anacardic acid (43), 1'-acetoxychavicol acetate (44), noscapine (45), evodiamine (46), indirubin (47), thymoquinone (48), isodeoxyelephantopin, and withanolides (49).

Nutraceuticals are also known to inhibit STAT3 activation pathway and to suppress survival of tumor cells. For example, emodin was found to suppress STAT3 activation and to induce apoptosis in human myeloid cells (50). Similarly, suppression of STAT3 activation by capsaicin was found to induce apoptosis in multiple myeloid cells (51). Curcumin can suppress STAT3 activation pathway and tumor growth in an orthotopic murine model of ovarian cancer (52). Similarly, deguelin induced apoptosis in HTLV transformed T cells by inhibiting STAT3 phosphorylation (53). Quercetin can suppress STAT3 tyrosine phosphorylation and angiogenesis (54).

The clinical studies also support the potential of nutraceuticals in suppressing inflammatory pathways and chronic diseases. The safety, pharmacokinetics, and efficacy of nutraceuticals against numerous chronic diseases has been addressed in a number of human clinical trials. For example, EGCG, which is derived from green tea is reported to have potential against several chronic diseases (55). In prostate cancer patients, tea polyphenols are known to suppress serum levels of PSA, VEGF, and hepatocyte growth factor (HGF) (56, 57). The consumption of green tea is reported to reduce the risk of prostate adenocarcinoma (58). Similarly, black tea is known to decrease the levels of inflammatory biomarkers in colon cancer patients (59). The consumption of tea can also reduce the risk of breast cancer (60), gastric cancer (61), and lung cancer (62). Pomegranate, which is rich in isoflavonoid, such as

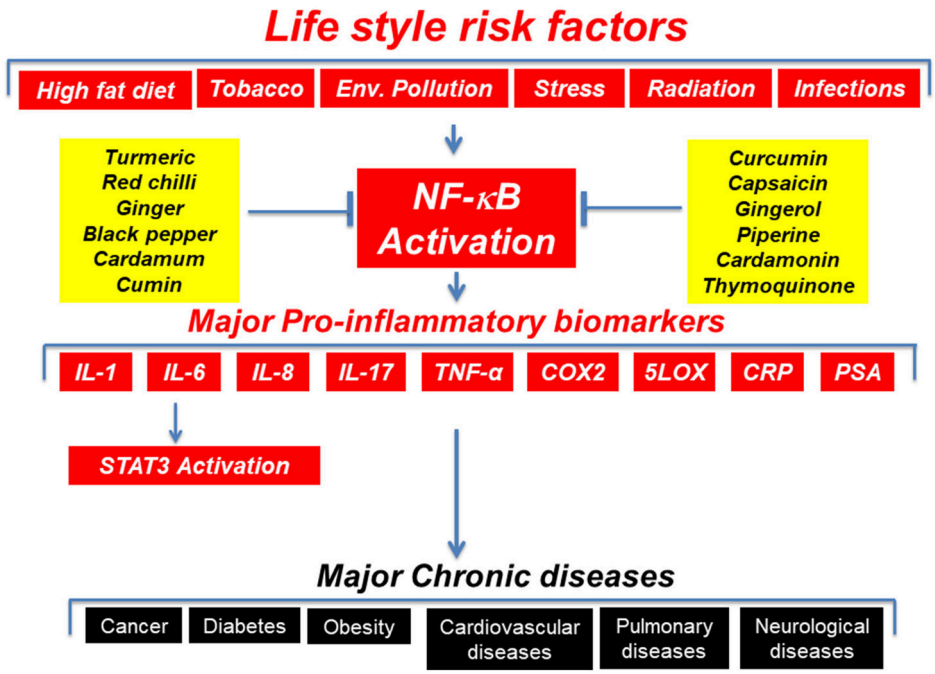

FIGURE 1 | Major risk factors and inflammatory molecules associated with chronic diseases.

quercetin, kaempferol, and luteolin, has been used for centuries for medicinal purposes (63). The consumption of pomegranate juice is known to significantly increase PSA doubling time in a phase II clinical trial of prostate cancer patients (64). Furthermore, pomegranate juice can decrease cell proliferation and induce apoptosis (64). The incidence of colorectal, prostate, and lung cancer can be reduced by selenium supplementation (65). The nutraceuticals have shown promise for several other chronic diseases such as acquired immunodeficiency syndrome, acute coronary syndrome, arthritis, atherosclerosis, biliary dyskinesia, cardiovascular disease, cholecystitis, chronic bacterial prostatitis, Crohn's disease, Dejerine-Sottas disease, diabetes, diabetic microangiopathy, diabetic nephropathy, gastric inflammation, gastric ulcer, idiopathic orbital inflammatory pseudotumor, irritable bowel disease, lupus nephritis, oral lichen planus, peptic ulcer, renal conditions, tropical pancreatitis, ulcerative colitis, ulcerative proctitis, uveitis, vitiligo, psoriasis, and β-thalassemia (66). In clinical trials, nutraceuticals have been used as an individual agent and also in combination with other agents. The formulations of nutraceuticals such as capsules, emulsions, liposomes, nanoparticles, powder, and tablets have been used for clinical trials.

In addition to cancer, nutraceuticals are also known to produce beneficial effects in other disease models. For example, an oral administration of curcumin at 375 mg (three times a day for 2 weeks) produced beneficial effects in patients with uveitis (67). Curcumin is also effective in patients with rheumatoid arthritis as demonstrated in clinical trials (68, 69). A short-term, double-blind, crossover study examined the efficacy of this polyphenol in 18 rheumatoid arthritis patients (68). The efficacy of curcumin was also compared with that of phenylbutazone, which is a prescription drug. The patients were administered with phenylbutazone (0.3 g/d) or curcumin (1.2 g/d) for 2 weeks. The anti-rheumatic activities of curcumin were identical with that of phenylbutazone. Furthermore, the polyphenol was very well tolerated and produced no adverse effects in patients. The polyphenol also produced anti-rheumatic activities when combined with diclofenac sodium (69). Additionally, curcumin is known to produce symptomatic relief in patients with peptic ulcers (70). One study examined the potential of curcumin against vitiligo, which is characterized by white patches over the skin on the different body parts (71). A statistically significant repigmentation was observed after 8–12 weeks of curcumin treatment. The polyphenol is known to exhibit anti-psoriatic activity possibly through modulation of phosphorylase kinase (PhK) activity (72). The efficacy of curcumin in Alzheimer's disease patients was examined in a randomized, double-blind, placebo-controlled study (73). The patients were administered with the polyphenol at 1 or 4 g doses. Although curcumin was unable to improve mental status and the serum Aβ40 levels, vitamin E level was increased in patients without any adverse effects (73). The polyphenol also reduces total cholesterol and LDL cholesterol, and increases HDL cholesterol in patients with acute coronary syndrome (74). Overall, these results suggest the beneficial effects of curcumin in patients with acute coronary syndrome. When the polyphenol was administered to 10 healthy volunteers for 7 days, reduction in serum lipid peroxides and total serum cholesterol levels, and an increase in HDL cholesterol was observed (75). In one study, the potential of curcuminoids (NCB-02) in 72 patients

with type 2 diabetes (T2DM) was examined (76). The patients were randomized to receive atorvastatin (10 mg, once a day), NCB-02 (300 mg of curcumin, twice a day), or placebo for 8 weeks. The administration of curcumin was associated with an improvement in endothelial function and reduction in oxidative stress (MDA) and inflammatory markers (endothelin-1, IL-6, TNFα) suggesting the potential of curcuminoids against T2DM. However, larger, randomized clinical trials are required to confirm these observations. Like curcumin, resveratrol is also beneficial in T2DM patients (77). More specifically, administration of resveratrol at 1 g/day for 45 days suppressed fasting blood glucose, haemoglobinA1c (HbA1c), insulin and insulin resistance. Furthermore, a significant rise in high density lipoprotein cholesterol was observed after resveratrol treatment (77). In patients with non-alcoholic fatty liver disease (NAFLD), resveratrol significantly reduces the levels of glucose, cholesterol, and liver enzymes ALT and aspartate aminotransferase (78). Resveratrol also decreases the levels of ALT and hepatic steatosis in NAFLD patients (79). Conversely, resveratrol was unable to produce beneficial effects in another clinical trial of NAFLD patients (80). The post-menopausal women are at increased risk of breast cancer owing to reduced expression of sex steroid hormone binding globulin (SHBG). Furthermore, a lower ratio of 2-hydroxyestrone (2-OHE1) and 16α-hydroxyestrone (16α-OHE1) in postmenopausal cohort correlate with the higher breast cancer risk (81). An administration of resveratrol at 1 g/day for 12 weeks is known to increase SHBG levels in obese postmenopausal women (82). Resveratrol also elevates 2-OHE1/16α OHE1 ratio. Thus, it can be concluded that resveratrol has beneficial effects in postmenopausal women (82).

In conclusion, chronic inflammation is a cause for several chronic diseases. Thus, treatment of chronic diseases requires chronic treatment. Modern science has delineated the molecular links of chronic inflammation and chronic diseases. The drugs developed by pharmaceutical companies are highly expensive, produce side effects and cannot be afforded by more than 80% of world population. Nutraceuticals have also been successfully used in combination with other agents. Nutraceuticals are readily available and can modulate multiple cell signaling pathways. In addition, nutraceuticals and their sources have been consumed since ancient time. Thus, their safety is well tested. Conversely, nutraceuticals have been reported to produce undesired adverse effects by some studies. For example, oral intake of curcumin is associated with diarrhea, headache, rash, and yellow stool in some healthy volunteers. When curcumin was administered in combination with gemcitabine, abdominal pain was reported by some pancreatic cancer patients. Furthermore, nutraceuticals such as curcumin and resveratrol are associated with poor bioavailability. Overall, nutraceuticals offer promise to prevent or delay the onset of chronic diseases. However, none of the nutraceuticals have been approved for human use by regulatory entities. Moreover, nutraceuticals have been reported to produce adverse effects by some studies. More studies are required before these agents can be prescribed by clinicians for therapeutic purpose.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Monomeric C-Reactive Protein and Cerebral Hemorrhage: From Bench to Bedside

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C-reactive protein (CRP) is an important mediator and a hallmark of the acute-phase response to inflammation. High-sensitivity assays that accurately measure levels of CRP have been recommended for use in risk assessment in ischemic stroke patients. Elevation of CRP during the acute-phase response in intracerebral hemorrhage (ICH) is also associated with the outcomes such as death and vascular complications. However, no association has been found with the increased risk of ICH. The aim of this review is to synthesize the published literature on the associations of CRP with acute ICH both as a risk biomarker and predictor of short- and long-term outcomes as well as its role as a pathogenic determinant. We believe before any clinical utility, a critical appraisal of the strengths and deficiencies of the accumulated evidence is required both to evaluate the current state of knowledge and to improve the design of future clinical studies.

Keywords: CRP, inflammation, stroke, intracerebral hemorrhage, SAP, outcomes, risk assessment, biomarkers

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INTRODUCTION

Inflammation is recognized to play a major role in the pathogenesis of cerebrovascular disease (1, 2). C-reactive protein (CRP), which is produced in the liver, is a hallmark of the acute inflammatory response, and represents an extensively studied systemic marker of inflammation (3). The significance of elevated CRP as a marker of inflammation in the clinical setting has been suggested in the literature (4–6). Notably, over recent decades CRP has been the focus of an intense investigation to explore its role in the setting of intracerebral hemorrhage (ICH) and currently is proposed as a risk assessment tool and prognostic marker (7).

The aim of this review is to synthesize the available literature examining the association of monomeric CRP with acute ICH as a risk assessment biomarker and predictor of short- and long-term outcomes as well as its role as a pathogenic determinant.

STRUCTURE AND FUNCTION OF CRP

CRP and serum amyloid P (SAP) component are the main pentraxins (PTX) in humans. PTX represent an evolutionarily conserved family of proteins mostly involved in immunological responses. They have a unique pentameric structure and bind to their ligands in a calcium-dependent manner (8, 9). CRP and SAP are soluble pattern-recognition proteins of pathogenic

bacteria or damaged cells and, therefore, interact with the complement pathway and Fcγ receptors to activate the innate immune system (10, 11). CRP, also known as PTX1, primarily exists in two structurally and functionally independent forms: (1) net anti-inflammatory, serum-associated native pentameric CRP (npCRP), and (2) pro-inflammatory tissue-associated, monomeric CRP (mCRP) (10, 12).

DEFINITION OF npCRP/mCRP

npCRP is an acute phase PTX produced mainly by the liver in response to inflammatory infection in humans. It is composed of five none-covalently linked identical subunits. It is plasma-soluble, and its concentrations are linked to level of systemic inflammatory response. Serum npCRP concentrations have been shown to increase from <3 to $>100\,\mu g/ml$ within 8–72 h from the onset of an inflammatory response (13, 14). Its physiological role is to bind to phosphocholine, expressed on the surface of dead or dying cells, stimulating the activation of the complement system via the C1Q complex.

mCRP is a monomer of the parent molecule npCRP. mCRP is mainly tissue-associated, as its formation requires a conformational change that renders the protein largely insoluble (15, 16). The circulating 115 kDa npCRP homopentamer irreversibly dissociates into 23 kDa monomers upon binding to pathogenic membranes, damaged or apoptotic cell membranes, activated platelets, or blood-derived microparticles (MPs). Due to contact with cells and tissue, it converts to the monomeric-biological form where it can remain chronically. Specifically, on contact with cell membranes, npCRP undergoes an intermediate conformational change through hydrophobic insertion, maintaining pentameric status but acquiring the properties of mCRP, this is followed by loss of the pentameric form by breakage of the intrasubunit disulphide bond. The reduced form of mCRP (rmCRP), where the intra-subunit disulphide bridge is broken (e.g., on contact of reducing agents such as thioredoxin), binds strongly to the cholesterol binding motif of membrane lipid rafts and actively stimulates cell signaling, following unlocking of the lipid raft interaction motif through multiple mechanisms (17). Amongst others, rmCRP/mCRP has been shown to stimulate inflammation directly, induce aberrant angiogenesis and promote platelet activation and aggregation. Importantly, standard clinical high sensitive (hs)-CRP assays cannot detect mCRP or urea-solubilized mCRP on MPs. hsCRP assays, therefore, can only measure the portion of total CRP in plasma that is in the soluble pentameric form (soluble npCRP). Currently, only by using flow cytometry and antibodies it is possible to measure both forms of CRP (npCRP and mCRP) on MPs to revealing a compartment of CRP not currently measured with hs-CRP technique (18).

ROLE OF CRP IN INFLAMMATION

CRP shows unique properties amongst the PTX family. Thiele et al. (19) published information showing that dissociation of pentameric to monomeric CRP localizes and aggravates inflammation. This work provides *in vivo* evidence of a powerful pro-inflammatory effect of mCRP in striated muscle, atherosclerotic plaque and infarcted myocardium (human and rat tissues). Agrawal et al. (20, 21) reported that in an *in vitro* acidic solution, npCRP was able to bind to various types of proteins with altered conformations in a calcium-independent manner. Taking into account acidic pH at inflammatory sites, npCRP protects against toxic conditions caused by protein misfolding and aggregation maintaining extracellular proteostasis of the inflammatory site by inhibiting the aggregation of unfolded/misfolded proteins (22). In addition, a dramatic increase in expression of mCRP has been observed in blood vessels of damaged brain regions. Furthermore, mCRP is highly angiogenic, unlike the native molecule, and may affect tissue survival and development by influencing vascularization and remodeling (23). Krupinski et al. (24) demonstrated a strong expression of mCRP within microvessels with unstable plaques whilst normal looking arteries and stable fibrous lesions contained a significantly lower expression. The mCRP was mainly associated with inflammatory cells and newly formed angiogenic microvessels within unstable plaque regions (24). This suggested that mCRP may have a pathological role in the development of unstable atherosclerosis and/or increased risk of plaque thrombosis. mCRP specifically increases the activation of the inflammation both *in vitro* and *in vivo*, getting deposited chronically within the brain after ICH (25), and may play a role in perpetuating neuroinflammation after brain injury.

The liver is the primary site of CRP synthesis in humans. However, it is also expressed, albeit at low levels, in the CNS. In the brain, mCRP is produced in large quantities after an ischemic insult and in response to inflammation, becoming indefinitely attached to cellular components and within the extracellular matrix (ECM) (26). After ICH onset, CRP could be clearly localized inside blood vessels and in the cytoplasm of activated astrocytes and neurons within the perihematoma regions (27). CRP has also been detected in a number of neurodegenerative diseases like Alzheimer's disease (AD) (28–31), amyotrophic lateral sclerosis (32), and multiple sclerosis (33). Using unspecific and specific CRP antibodies against mCRP, immunoreactivity has been detected in the neurofibrillary tangles of AD patients (31) and elevated CRP concentrations have been associated with increased risk of developing dementia in older people. Increased concentrations of serum CRP have recently been associated with cerebral microstructural disintegration, suggesting an involvement of CRP in silent stroke-associated vascular dementia and, possibly, hemorrhagic stroke (27, 34). However, these findings do not exclude the systemic origin of CRP. It has been shown that CRP may reach the brain tissue from the circulation by activating the endothelial contractile machinery in both an *in vitro* blood-brain barrier (BBB) model and in an isolated whole brain preparation (35, 36). In summary, these data are consistent with the hypothesis that npCRP may be a member of extracellular chaperones protecting against toxic conditions caused by protein misfolding and aggregation in acidic inflammatory environments. npCRP dissociates to mCRP at sites of inflammation, which is then deposited in brain

parenchima at the level of ECM and via its proinflammatory effects acts to amplify and localize inflammation.

npCRP-mCRP CELLULAR BINDING AND MECHANISM OF ACTION

mCRP binds to macrophages via integrin α5β3 and subsequent AKT signaling inducing a pro-inflammatory status and chemotaxis (37). In addition, the immuno-modulatory effect of CRP is also mediated through its ability to interact with FcγRs (similar to the G class of IgGs), and to stimulate phagocytic cells (e.g., macrophages). CRP acts as an opsonin for *N. meningitides* and other bacteria. Therefore, as CRP-opsonized bacteria showed increased uptake by human macrophages and neutrophils, this could be useful regarding potential clearing mechanisms via enhanced macrophage engulfment and phagocytosis after antibody binding. Reduction of the intra-subunit disulfide bond which occurs on cellular contact (after calcium-dependent npCRP binding), allows unlocking of the lipid raft interaction motif enhancing significantly the cellular uptake and signal activation in macrophages/endothelium (38, 39). In endothelial cells, mCRP and specifically rmCRP is known to bind to membrane-associated enriched lipid rafts and stimulate cellular activation through phospholipase C, MAP kinase, and NF-κB (39). In addition, as previously shown in a monocyte cell line (37), integrins may also mediate the binding of monocytic cells and other cell types via α5β3 integrin binding to mCRP, and mediate pro-inflammatory signaling upon binding to mCRP.

ANATOMO-PATHOLOGICAL STUDY IN ICH

Published data has shown that mCRP is strongly expressed in the brain parenchyma (neuronal nucleus and cytoplasm and angiogenic microvessels co-localized with CD105) of patients following ischemic stroke in the damaged core and penumbral regions (26). The mCRP remained visible and in significant quantities several months after the event. mCRP levels were also increased in brain tissue parenchyma after ICH (27), and was present in large amounts in perihematomal regions and within neurons and glia of patients who died within 12 h of spontaneous ICH (7, 27). Circulating levels of npCRP also predicted hematoma growth and outcome. mCRP was abundant in the walls of microvessels from the perihematoma region of the metabolic penumbra of patients who died soon (2 days) after stroke (**Figure 1**). mCRP may be responsible for increases in vascular permeability and aberrant angiogenesis leading to vessel structural instability and hemorrhagic conversion after stroke. *In vivo*/*ex-vivo* effects on vascular development include the findings that NCAM becomes over-activated, linked to pathological, aberrant angiogenesis, the FITC-dextran permeability assay showed that mCRP increased monolayer permeability, sprouting angiogenesis assays and gap junction spacing between cells, whilst dorsal matrigel implants containing mCRP produced hemorrhagic lesions (while nCRP and VEGF did not show a similar effect; see **Figure 2**).

EPIDEMIOLOGICAL STUDIES OF CRP AND ICH RISK

Spontaneous ICH accounts for approximately 20% of all strokes, and it is characterized by high rates of mortality and residual disability among survivors (43, 44). Furthermore, unlike ischemic stroke, ICH typically has no identifiable premonitory signs or symptoms. Accordingly, the identification of individuals at risk of ICH, prior to the occurrence of the event, represents a striking clinical issue. On these grounds, older age, male sex, hypertension, diabetes mellitus, alcohol intake abuse and smoking have been associated with higher risk of ICH (45). Nonetheless, predictive models based upon conventional risk factors are still inadequate and, as such, there is a need for developing early preventive strategies. Low-grade inflammation is increasingly recognized as a key player in the pathophysiology underpinning many different medical conditions. Serum biomarkers related to increases in systemic inflammatory activity are significant predictors of cardiovascular diseases (CVD) and mortality (46). In this respect, the serum CRP concentration has close associations with the risk of coronary heart disease, ischemic stroke, and vascular mortality (47). On the contrary, there is no evidence providing a clear-cut relationship with the risk of ICH. In the last decade, a number of epidemiological studies across multiple ethnicities have been conducted, but none demonstrated a meaningful link between circulating CRP levels and ICH risk.

Based on 6,430 participants as part of the elderly population-based Rotterdam Study, Bos et al. (48). evaluated whether the risk of stroke varied with baseline CRP serum levels and whether that can helpful in the prediction of risk in individual stroke. During an average follow-up of 8.2 years, 498 first-ever strokes occurred, of which 51 (10%) were sub-classified as hemorrhagic. CRP levels were significantly associated with incident stroke and ischemic stroke, but not with ICH. The sex-specific hazard ratios for the associations between one standard deviation (SD) increase in logarithmically transformed CRP and ICH were 1.36 (0.87–2.14) and 0.81 (0.57–1.17) in men and women, respectively.

In a 12-years follow-up examination of a Japanese population in the town of Hisayama, from December 1988 through to November 2000, Wakugawa et al. (49) demonstrated that elevation of serum CRP levels was an independent risk factor for future ischemic stroke in men, whereas no association between serum CRP levels and the risk of future hemorrhagic stroke in either sex was observed. The age-adjusted incidence rates of first-ever hemorrhagic stroke according to quintiles of baseline serum CRP were 2.4, 1.1, 2.2, 1.9, and 2.7 per 1,000 person-years for men, and 1.1, 2.6, 1.0, 1.3, and 1.6 per 1,000 person-years for women with no significant trends in either sex.

Associations of CRP levels with risks of total stroke and its subtypes were examined in the Circulatory Risk in Communities Study (49), prospective nested case-control study of 13,521 Japanese men and women aged 40–85. A total of 261 incident strokes were identified, of which 67 were ICH and 29 were subarachnoid hemorrhage (SAH). After adjustment for known cardiovascular risk factors, CRP predicted the incidence of total

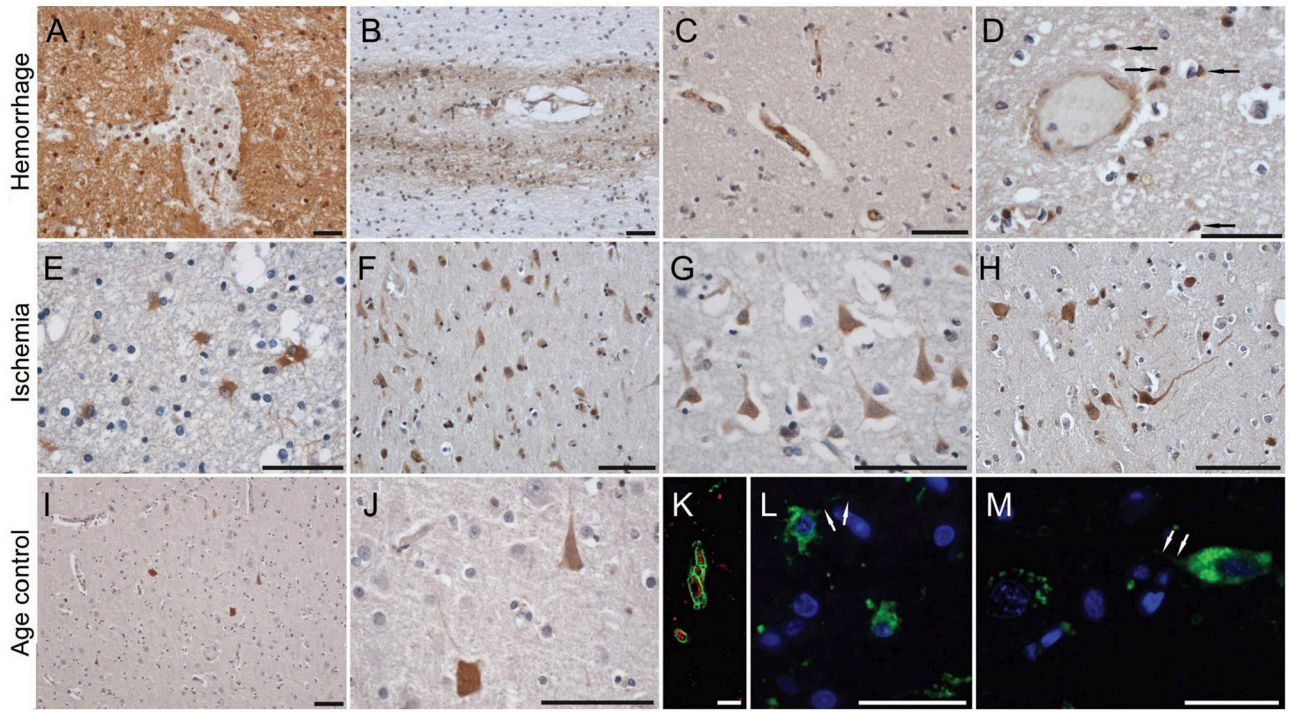

FIGURE 1 | C-reactive protein staining in human brain after hemorrhagic and ischemic stroke and in normal aged control subjects. In the perihematoma areas, a diffuse neuropil staining was present, together with almost all cells' silhouettes taking up the stain **(A)**. Further away from the hemorrhagic core, the diffuse staining pattern diminished, but with some neurons clearly retaining an affinity for the antibody. Although distant from the hemorrhage, white matter fiber tracts showed relatively intense staining for CRP, especially those surrounding intracallosal vessels **(B)**. Focal intravascular staining sometimes was observed **(C)**, and only on occasion were microglia-like cells immunoreactive for CRP **(D)**. By immunofluorescence, CRP could be clearly localized inside blood vessels **(K)**, and in the cytoplasm of activated astrocytes **(L)** and neurons **(M)**. On the contrary, in the region immediately surrounding an ischemic area, a high number of gemistocytic astrocytes was noted **(E)**, while further away from the lesion core numerous neuronal silhouettes were observed **(F–H)**. On occasion, diffuse staining could be noted along the white matter tracts and blood vessels. In the control brain, and in the hemispheres contralateral to the lesion, only occasional neuronal silhouettes were stained, with their respective densities being clearly lower compared to the lesioned (ipsilateral) hemispheres **(I,J)**. Scale bars, 20 μm. Reproduced with permission from (27).

and ischemic strokes, while no associations were associated with the risk of hemorrhagic stroke.

The role of hemostatic and inflammatory indices as predictors of ICH was addressed by analyzing data from the Cardiovascular Health Study (50), a randomly selected population-based cohort study from four US communities, which recruited 5,201 participants aged 65 years or older. Among the considered biomarkers, fibrinogen and von Willebrand factor but not CRP, factor VII, and white blood cell count were significantly associated with incidence of ICH.

In a prospective, population-based study nested within the Northern Sweden Cohorts, Andersson et al. (40) explored the role of CRP as a determinant of first-ever stroke and the relationships between the 1444C > T polymorphism, CRP levels, and stroke. CRP concentrations were categorized as <1, 1–3, and >3 mg/L for low, average, and high risk, respectively. Three hundreds and eight cases of ischemic stroke, 61 cases of ICH with time from ischemic and hemorrhagic strokes were 52 and 50 months, respectively. A total of 749 matched referents were defined as control. In a multivariate model including traditional risk factors, CRP was significantly associated with the risk of having a first ischemic but not ICH [OR for the highest CRP group: 2.58 (1.74–3.84) for ischemic stroke and 0.97 (0.30–3.11) for ICH]. Additionally, the CRP 1444 (CC/CT vs. TT) polymorphism was associated with plasma levels of CRP but not with ischemic stroke or ICH.

CRP levels and risk of mortality from CVD were investigated as part of the Japan Collaborative Cohort Study for evaluation of cancer risk (51). A total of 39,242 subjects of 40–79 years of age provided serum samples at baseline between 1988 and 1990; controls were matched for sex, age, residence area and year of serum storage. During the 13-years follow-up, the total strokes cases comprised of 214 hemorrhagic (119 ICH and 95 SAH) and 294 ischemic strokes. Higher serum CRP levels were associated with higher mortality from CVD, but not ICH. Multivariable odd ratios for one SD increment of CRP were 1.36 (0.75–2.48) in men and 1.50 (0.99–2.28) in women. The impact of CRP on risk of stroke, stroke subtypes, and ischemic heart disease in middle-aged Japanese was further explored in a prospective, population-based Study (52). A total of 1,341 CVD events occurred between 1990 and 2007 among subjects included in the study, including 494 hemorrhagic strokes (344 ICH and 150 SAH). The main finding was the lack of any association between CRP and risk of hemorrhagic stroke.

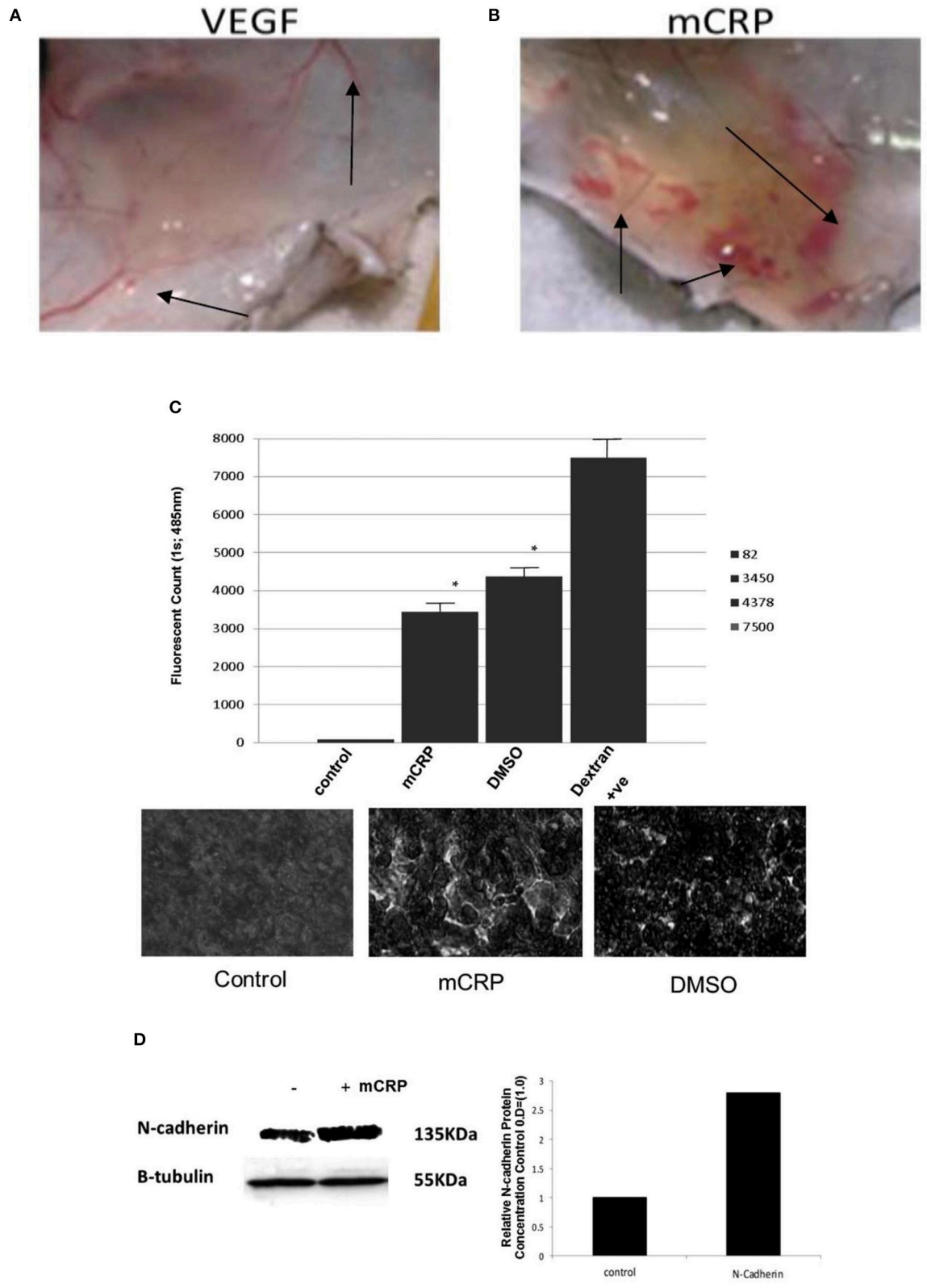

FIGURE 2 | mCRP increases vascular permeability and aberrant angiogenesis. Top shows hemorrhagic blood vessels produced within a matrigel murine skin implant-compared with normal vessels after VEGF incorporation. Dorsal matrigel implants containing mCRP (10 μg/ml; 72 h) produced strong and visible hemorrhagic angiogenesis (**B**; arrows) compared with a typical, normal looking vascular response seen in the presence of VEGF (**A**; 25 ng/ml). In **(C)** the graph shows a significant

(Continued)

FIGURE 2 | increase in monolayer permeability in the presence of mCRP (10 μg/ml; 8 h) using a Millipore-based filter assay, similar to that produced by 10% DMSO ($p < 0.01$ increase in FITC dextran penetrating the monolayer in the presence of either mCRP or the positive control DMSO), and lighter regions in the images shown indicate areas of increased permeability. Lower images show intact control monolayer (left); mCRP-treated monolayer and (right) DMSO-treated monolayer showing enhanced permeability and surface damage. Lighter regions in the images shown indicate areas of increased permeability. **(D)** Expression of adhesion molecules was examined in BAEC treated with mCRP (10 μg/ml; 24 h). NCAM expression was increased by approximately 2.8-fold whilst VCAM, ICAM and integrins were not affected (data not shown). β-tubulin was used as the house keeping control (gel and bar chart shown). Reproduced with permission from (34).

A longitudinal study from June 2006 to October 2007 based on the Kailuan community, in Tangshan city, included 90, 517 Chinese adults, with no history of stroke or myocardial infarction (MI) at baseline (53). During a total follow-up of 362,163 person-years and a median of 49 months per participants, 1,472 incident strokes were documented, of which 383 (26.0%) were ICH and 40 (2.7%) were SAH. The age- and sex-standardized incidence per 1,000 person-years of ICH increased with CRP concentrations, being 3.04, 4.36 and 4.61 (p-value for trend = 0.04) for <1, 1–3 and >3 mg/L groups, respectively. After adjusting for covariates, CRP concentrations ≥1 mg/L were associated with increased risks of all stroke but were not associated with ICH or SAH, regardless of hypertensive status.

A recent aggregate data meta-analysis considered prospective observational studies that enrolled participants from the general population, measured hs-CRP levels and reported adjusted estimates of stroke risk in at least three CRP categories (54). Four studies were included in the meta-analysis with a total of 40,002 subjects that 655 were ICH cases. Elevated CRP levels were associated with excessive risk of ischemic but not hemorrhagic stroke. The pooled adjusted risk ratio of hemorrhagic stroke comparing the highest with the lowest CRP category group was 0.82 (0.59–1.13; $p = 0.217$) in a fixed-effect model, with no evidence of heterogeneity. Although serum levels of CRP do not seem to predict the risk of future ICH, it is noteworthy that none of the aforementioned studies provided data on ICH etiology. The main causes of ICH (i.e., hypertension, lipohyalinosis of small vessels, and amyloid angiopathy) have different risk factor profiles and underlying pathophysiological pathways (55). Accordingly, the involvement of the inflammatory response and, in turn, the predictive role of serum CRP may differ according to the ICH subtype. Indeed, associations between serum CRP levels, cerebral small vessel disease, and lobar or deep micro-bleeds have been inconstantly reported highlighting the need for further investigations before drawing concrete conclusions (56–58). Finally, although hypertension and smoking may accelerate the development and growth of intracranial aneurysms (59), which are a main cause of SAH, the relationship between an atherosclerosis and the intracranial aneurysm is weak (60). This can explain the lack of a clear association between CRP levels and SAH. Finally, the lack of a significant association between ICH and CRP can also be due to clinical hs-CRP assays that only measure the portion of total CRP in plasma in the soluble pentameric form (18). The insoluble component of CRP, npCRP and mCRP, on MP is not currently measured in clinical settings that may have a relevant pathogenetic role that is different from soluble pentameric form in ischemic vascular disease (18).

CEREBRAL HEMORRHAGE AND GENETICS OF CRP

Several studies of CRP genetics have focused on genetic polymorphisms and development of CVD. The impact of genetic variants of CRP single-nucleotide polymorphisms (SNPs) such as rs1800947, rs1417938, rs1130864, and rs3093077 on circulating protein level and outcome has been recently assessed in a cohort of in-patients with CVD. It was found that both CRP level ≥5 mg/L and SNP rs1800947 of the CRP gene were independent risk factors for further adverse vascular events among patients with CVD within a 3 years follow-up (61).

The 717A > G polymorphism, which is located in the promoter region of the CRP gene, has been associated with ischemic stroke and it was an independent predictor of cerebral ischemia among Chinese population. Although in hemorrhagic stroke patients the frequency of haplotype H5 (A-T-C) was lower compared to controls, it was not an independent protective factor against cerebral hemorrhage. Accordingly, H3 haplotype (G-C-C) could be an independent risk marker for ischemic stroke, and the H5 haplotype (A-T-C) could act as a prognostic marker of hemorrhagic stroke (38, 62).

CRP levels differ in individuals and this inter-individual variation is genetically controlled with a substantial heritability score (63, 64). The CRP gene that controls its blood levels is localized at chromosome position 1q21–1q23, spans 1.9 kb and exhibits two exons. Twenty-nine different polymorphisms have been assessed within the CRP gene, however, so far there is a paucity of robust studies clarifying the genetic consequences of CRP gene vis-à-vis ICH. Four genetic studies have been conducted hitherto, one of which has shown that haplotype ATC of CRP SNPs (rs2794521, rs3091244, and rs1205) is an independent prognostic marker for hemorrhagic stroke (38).

Another prospective population-based study on a Swedish cohort has examined the contribution of 1444C/T polymorphism of the CRP gene for the risk of ischemic stroke and ICH. Using a multivariate model including traditional risk factors, CRP levels were found to be significantly associated with ischemic stroke (OR 2.06, 95%CI 1.29–3.29), whilst the 1444C/T polymorphism failed to correlate with ICH (40). Das et al. (41) investigated the 1059G/C polymorphism of the CRP gene in relation to its contribution for the risk of ischemic and hemorrhagic stroke in an Indian population. Results of this study showed that CRP levels were increased significantly in hemorrhagic stroke patients ($p < 0.001$) when compared with controls, but the CRP gene polymorphism was not found to be associated with hemorrhagic stroke. A case-control study involving 236 hemorrhagic stroke patients in a community of Han Chinese population revealed that SNP rs3091244 (−286C/T/A) of the

TABLE 1 | Genetic studies of CRP polymorphisms in intra-cerebral hemorrhage stroke patients.

Population	CRP-SNPs	Sample size	Diagnostic criteria	Results	References
Swedish	1444C/T (rs1130864)	Ischemic stroke: 308 ICH: 61	TOAST criteria	CRP levels were associated with ischemic stroke in multivariable model; however, 1444C/T polymorphism was not associated with any type of stroke.	(40)
Chinese	−757A/G (rs3093059), −717A/G (rs2794521), −286C/T/A (rs3091244), 2147C/T (rs1205)	Ischemic stroke:431 ICH: 67	CT and/or MRI	−717A allele showed protective effect for ischemic stroke. Haplotype H3 (GCC) influenced the risk for IS (OR 1.05; 95%CI 1.00–1.10, $P = 0.04$), whereas, haplotype H5 (ATC) influenced the risk for ICH ($P = 0.0001$).	(38)
Indian	1059G/C (rs1800947)	Ischemic stroke:200 ICH:200	CT and/or MRI	hsCRP levels were significantly increased ($P < 0.001$) in ICH patients, however, CRP gene polymorphism (rs1800947) did not correlate with CRP levels.	(41)
Chinese	−757A/G (rs3093059), −717A/G (rs2794521), −286C/T/A (rs3091244), 3′UTR-T/C (rs876537)	ICH: 236 Controls:993	CT and/or MRI	−286C/T/A (rs3091244) was significantly associated with higher hs-CRP levels whereas, rs2794521 showed negative correlation with its levels in ICH patients.	(42)

IS, ischemic stroke; ICH, intracerebral hemorrhage.

CRP gene was significantly associated with elevated CRP levels in male patients (42). Summarizing the above data (see **Table 1**), the studies investigating CRP gene polymorphism as the genetic determinant of ICH are inadequate. The inferences drawn from these studies can only be considered as suggestive rather than conclusive due to the unchecked false positive cases and lack of proper statistical power. More genetic studies in different populations after adjusting risk concomitants will enhance our knowledge regarding the contribution of the CRP gene and its underlying contribution to the prognosis and prediction of ICH.

CRP STRATIFICATION IN ACUTE ICH: SHORT AND LONG-TERM PROGNOSIS

Early prediction of ICH outcome is a clinical priority to stratify patient risks, objectively design individual management, assess patient prognosis with reasonable accuracy, and standardize communication among healthcare providers. The evidence that ICH prognosis cannot be fully explained by traditional baseline hematoma features alone has prompted to look for adjunctive biochemical (65, 66), radiological (67, 68), clinical (67–70), and therapeutic (71, 72) variables that could characterize and inform the secondary-induced brain injury and improve outcome prediction.

The local and systemic inflammatory response that takes place shortly after the ICH onset can enhance the brain injury and post-stroke complications (73). Immune biomarkers, including fever, white blood cell count, neutrophil-to-lymphocyte ratio, interleukin-6 and fibrinogen have been shown to improve outcome prognostication (74–77). On these grounds, the specific relationship between CRP and short- and long-term ICH outcome has been also extensively explored over the past few years.

In a cohort of 399 patients presenting with primary or vitamin K antagonist (VKA)-associated ICH plasma CRP levels, measured within 6 h from onset were significantly associated with the occurrence of hematoma growth and neurological worsening (7). In the final model accounting for age, sex, VKA use, GCS score, ICH volume, midline shift, intra-ventricular hemorrhage, time of sampling and white blood cells count, CRP > 10 mg/L independently predicted hematoma growth [OR 4.71 (2.75–8.06); $p < 0.0001$] and neurological worsening [OR 2.70 (1.50–4.84); $p = 0.0009$), both of which increased the risk of mortality. Alexandrova et al. (78) examined 46 patients with ICH within 48 h after onset of symptoms and found admission serum CRP level to be a strong predictor of short-term fatal outcome. A 5.2% increase in the odds of first-week mortality was associated with an increase of CRP concentration by 1 mg/L. A prospective, multicenter, international, longitudinal study analyzed CRP kinetics and its association with short-term prognosis after ICH (27). A total of 223 patients were recruited, and CRP was evaluated at admission, 24, 48, and 72 h after symptom onset. Plasma CRP increased markedly from 48 to 72 h from admission, and the magnitude of the response was related to hematoma volume at later time points. Higher levels of CRP were independently associated with higher mortality and poor functional outcome at 30 days. From a clinical perspective, it is noteworthy to

mention that serial CRP measurements during the acute ICH stages could provide different predictive utility: admission CRP values were only weakly related to mortality and did not predict functional status, CRP at 24 h was a better predictor of mortality and unfavorable outcome, and predictability improved further with the obtained CRP levels at 48 or 72 h that was stronger for mortality than for functional recovery (27, 79, 80).

CRP has also been demonstrated within neurons and glial cells around the hemorrhagic lesion in an immunostaining analysis of brain tissue of patients who died within 12 h of ICH (27). The early presence of CRP—which could reflect either the local synthesis triggered by the hematoma or the conversion of the circulating soluble pentameric form to its insoluble and monomeric form—further supports the active role of CRP in defining the extent of damage. The addition of CRP concentration to the ICH-score significantly increased the ability to predict 30-days mortality by about 8% (80). The net benefit in prognostic accuracy was greater in patients classified as being at low to moderate risk using the ICH-score alone compared with the highest risk patients, in whom the severity of ICH could reasonably be the major determinant of death, and the lowest risk category, in which comorbidities could represent the major determinants of prognosis. Acute phase reaction biochemical markers including CRP were significantly associated with a 3-months good prognosis [modified Rankin scale (mRS) score ≤ 2] in the bivariate analysis performed by Castellanos et al. (81) to identify outcome predicting variables in patients presenting with primary medium to large spontaneous hemispheric ICH within the first 12 h of symptom onset.

Löppönen et al. (82) identified 807 subjects who suffered primary ICH between 1993 and 2008 among the population of Northern Ostrobothnia, Finland, and extracted the CRP values within 24 h after symptom onset from the records. The analysis confirmed high serum CRP levels as an independent predictor of unfavorable 3-months outcome. The interconnections between infections, CRP levels, and ICH outcome were addressed by Diedler and colleagues (83). In a cohort of 103 patients with supratentorial hematoma, infections occurred during a hospital stay in 52 cases. Patients classified as having poor status (mRS > 2) at discharge had a higher incidence of infections and higher baseline (median 6.2 vs. <0.2 mg/dl; $p = 0.001$) and maximal CRP levels (median 79.6 vs. 7.6 mg/dl; $p < 0.001$) compared to patients with good prognosis. A similar pattern was also observed for the 1-year assessment [median baseline CRP 7.0 vs. 1.0 mg/dL ($p = 0.006$) and median maximal CRP 91.3 vs. 9.5 mg/dL ($p < 0.001$) for poor vs. good outcome]. The multivariate logistic regression model showed that maximal CRP levels were independent predictors of scanty functional outcome and mortality at discharge and long-term follow-up.

In a prospective study conducted at Sarawak General Hospital, Malaysia, from April 2013 to April 2015, 60 patients with a diagnosis of supratentorial ICH within 24 h after symptom onset were recruited to determine mortality and morbidity at 6 months (84). Admission and 72-h CRP levels were found to be the main determinants of 6-months functional outcome and overall survival, alongside baseline GCS, hematoma volume, and total leukocytes (84). The relationship between CRP and long-term ICH outcome was further explored in a prospective, multi-center, case-cohort study for the assessment of stroke risk factors, which enrolled 291 patients with first-ever stroke (196 ischemic and 95 hemorrhagic) between November 2000 and July 2001 from five medical centers in Hubei province in China (85). Patients were followed for 5 years, and the primary outcome included the occurrence of acute MI, ischemic or hemorrhagic stroke, and death. The mean plasma CRP levels in patients with and without endpoint events were 4.4 and 2.7 mg/L ($p < 0.01$), respectively, and the relative risk of death or vascular events during the 5-years follow-up was 3.01 (1.60–5.64; $p = 0.001$) in patients with CRP > 3 mg/L compared to patients with CRP < 1 mg/L.

CONCLUSIONS

CRP seems to be an independent predictor of ICH outcome that is a reliable, easily accessible prognostic tool. The values of CRP concentrations for the prediction of ICH prognosis have been recognized in a variety of ethnic groups with conflicting results, so there is a possibility that it predicts different ICH phenotypes in different populations. The concentrations of CRP and its predication values on variety of stroke phenotypes are influenced by the ethnic genome background in the different racial groups, sex, and the environment. Based on the current report, CRP should be used while taking in mind these limitations. Furthermore, it is important to understand whether CRP polymorphisms affect the expression level or function of the CRP and whether single plays a key role or multiple polymorphisms act together. Finally, before routine use of CRP measurements in ICH prognostication, the clinical bioassays of circulating CRP should be refined. Actual hs-CRP assays do not reveal all the components of CRP subtypes. As discussed here, this aspect appears more relevant in ICH than in ischemic stroke. However, the inclusion of CRP subtype assays to aid in diagnosis or treatment of ICH relies on further studies. CRP does not simply reflect the strength of the acute-phase reaction but also correlates with the inflammatory response in the brain tissue. CRP can directly be involved with neuroinflammation and secondary-induced damage by activating the complement cascade and microglia, promoting leukocyte chemotaxis and adhesion molecule expression, stimulating cytokines release, inducing caspase-dependent apoptosis, BBB disruption, and brain edema formation. The downstream effects of mCRP within the damaged brain tissue can be blocked by preventing its cellular binding via lipid rafts and associated cell signaling soon after ICH onset. In this way, we can nullify the neurodegenerative cascade via a dual action of protecting neurons and preventing further re-bleeding thereby reducing the risk of hematoma expansion. This should be achievable through production of a specific blocking antibody, which could be delivered intra-arterially to bind to mCRP released into the extracellular matrix to phagocytic clearance of apoptotic cells from the

brain parenchyma. The further understanding of the underlying signaling pathways may be useful to identify new targets for neuroprotection and develop future strategies for treatment of ICH.

AUTHOR CONTRIBUTIONS

MD, MS, AP-W, PS, SL, and AD have designed the study, managed the work, and drafted the script.

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Monomeric C-Reactive Protein Binds and Neutralizes Receptor Activator of NF-κB Ligand-Induced Osteoclast Differentiation

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C-reactive protein (CRP) is an established marker of rheumatoid arthritis (RA) but with ill-defined actions in the pathogenesis. Here, we show that CRP regulates the differentiation of osteoclasts, a central mediator of joint inflammation and bone erosion in RA, in a conformation- and receptor activator of NF-κB ligand (RANKL)-dependent manner. CRP in the native conformation is ineffective, whereas the monomeric conformation (mCRP) actively modulates osteoclast differentiation through NF-κB and phospholipase C signaling. Moreover, mCRP can bind RANKL, the major driver of osteoclast differentiation, and abrogate its activities. The binding and inhibition of RANKL are mediated by the cholesterol binding sequence (CBS) of mCRP. Corroborating the *in vitro* results, CRP knockout exacerbates LPS-induced bone resorption in mice. These results suggest that mCRP may be protective in joint inflammation by inhibiting pathological osteoclast differentiation and that the CBS peptide could be exploited as a potential RANKL inhibitor.

Keywords: inflammation, rheumatoid arthritis, osteoclast, receptor activator of NF-κB ligand, C-reactive protein

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic joint inflammation and bone erosion (1). Autoimmunity in RA is likely triggered first at mucosal sites in response to environmental insults but needs to specifically target joints to initiate the disease. Osteoclasts play a major part in homing systemic autoimmunity to joints (2). These cells locate in the bone compartments, and are differentiated from myeloid precursors driven primarily by receptor activator of NF-κB ligand (RANKL) (3). They respond to autoantibodies frequently found in RA by secreting cytokines that evoke and amplify joint inflammation. Moreover, osteoclasts mediate bone resorption directly causing joint damage (4). Therefore, modulating the differentiation and actions of osteoclasts represents a promising strategy for treatment of RA (2, 5, 6).

C-reactive protein (CRP) is an established biomarker of RA (7, 8) with its blood levels closely associated with disease severity and progression (9). However, it remains elusive how CRP acts in the underlying pathological process. CRP appears to be protective in mice with collagen-induced arthritis (10, 11), but fails to influence autoantibody responses (11). This suggests that the protection by CRP might be exerted at joint level. Therefore, recent studies have examined the effects of CRP on the differentiation of osteoclasts, but yielding opposite conclusions (12, 13). Here, we address this

controversy by demonstrating the conformation- and RANKL-dependent actions of CRP. Only with the monomeric conformation (mCRP) that is converted specifically at inflammatory loci (14–18), can this protein regulate osteoclast differentiation and neutralize the activities of RANKL. Therefore, exploiting mCRP-RANKL interactions might provide a novel osteoclast-targeting strategy in RA.

MATERIALS AND METHODS

Reagents

Native CRP (nCRP; purity > 97%) purified from human ascites was purchased from the BindingSite (Birmingham, UK; catalog number: BP300.X; lot number: 361639 and 404353) and repurified with p-aminophenyl phosphoryl choline agarose (Thermo Fisher Scientific, Rockford, IL, USA; catalog number: 20307). Wide-type and mutant mCRP were prepared as described (19, 20). Proteins were dialyzed to remove NaN_3, and passed through Detoxi-Gel Columns (Thermo Fisher Scientific, Rockford, IL, USA; catalog number: 20344) to remove endotoxin when necessary. CRP peptides (purity > 98%) were synthesized by Science Peptide Biological Technology (Shanghai, China). Lyophilized peptides were reconstituted aseptically with DMSO at 40 mg/ml and stored at −20°C in aliquots or kept at 4°C for a maximum of 1 week. Mouse antihuman mCRP mAbs were generated as described (21). 2.5 μg/ml polymyxin B (PMB, Inalco Pharmaceuticals, San Luis Obispo, CA, USA; catalog number: 1758-9325; lot number: R1/51/121) was included in all cell response experiments to neutralize residual endotoxin.

Osteoclast Differentiation

RAW 264.7 murine macrophage cells (13) and bone marrow-derived macrophages (BMDMs) (22) were prepared and cultured as described. Briefly, bone marrow cells were isolated from femurs and tibias of 6-week-old male C57BL/6 mice. Following removal of red blood cells, the remaining cells were cultured in α-MEM (Hyclone, Logan, UT, USA; catalog number: SH30265.01; lot number: AC10207087, AB10164750) containing 10% FBS and 5 ng/ml M-CSF (R&D Systems, Minneapolis, MN, USA; catalog number: 416-ML; lot number: ME2915062 and ME3919011) for 12 h. Nonadherent cells were cultured for another 3 days with 10 ng/ml M-CSF. After vigorous washing, adherent cells were harvested and seeded in 24-well plates for 1–2 days and used as BMDMs. 10 ng/ml M-CSF was copresent in all treatments of BMDMs.

Raw cells or BMDMs were treated with nCRP, mCRP, RANKL (R&D Systems, Minneapolis, MN, USA; catalog number: 462-TEC; lot number: CWA1815111), or their combinations to induce osteoclast differentiation. Culture media were changed every two days. In some experiments, BMDM were pretreated with signaling inhibitors and then stimulated with mCRP or RANKL for 24 h. The used inhibitors were: Bay11-7082 (10 μM, 1 h pretreatment; NF-κB inh), SB20358 (20 μM, 15 min pretreatment; p38 MAPK inh), SP600125 (10 μM, 1 h pretreatment; JNK inh), U0126 (10 μM, 1 h pretreatment; ERK inh), U73122 [10 μM, 0.5 h pretreatment; phospholipase C (PLC) inh], LY294002 (50 μM, 1 h pretreatment; PI3K inh), MK-2206 2HCl (5 μM, 1 h pretreatment; Akt inh), Piceatannol (10 μM, 1 h pretreatment; Syk inh), and GW5074 (20 μM, 0.5 h pretreatment; Raf inh).

Total RNA was extracted with RNAiso Plus reagent (Takara, Shiga, Japan; catalog number: 9109; lot number: AKA3402, AKA5802). cDNA was synthesized from 2 μg total RNA using PrimeScript RT Master Mix system (Takara; catalog number: RR036A; lot number: AK4102, AK4403). The expression of osteoclast marker genes was determined with quantitative PCR using RealStar Green Power Mixture (Genestar, Beijing, China; catalog number: A311; lot number: 7AB01) in a StepOne Plus real-time PCR system (Thermo Fisher Scientific). The gene expression levels were normalized to that of GAPDH. The primer sequences used were: TRAP (forward: 5′-GCAACATCCCCTGGTATGTG-3′; reverse: 5′-GCAAACGGTAGTAAGGGCTG-3′); Cathepsin K (forward: 5′-GCATTACCAACATGGCCAGC-3′; reverse: 5′-CTCCCTTCCAAAGCCACCAA-3′); RANKL (forward: 5′-CAGCATCGCTCTGTTCCTGTA-3′; reverse: 5′-CTGCGTTTTCATGGAGTCTCA-3′); NFKB2 (forward: 5′-GGCCGGAAGACCTATCCTACT-3′; reverse: 5′-CTACAGACACAGCGCACACT-3′); SOCS1 (forward: 5′-CTGCGGCTTCTATTGGGGAC-3′; reverse: 5′-AAAAGGCAGTCGAAGGTCTCG-3′); SOCS3 (forward: 5′-ATGGTCACCCACAGCAAGTTT-3′; reverse: 5′-TCCAGTAGAATCCGCTCTCCT-3′); PPARG (forward: 5′-GGAAGACCACTCGCATTCCTT-3′; reverse: 5′-GTAATCAGCAACCATTGGGTCA-3′); GAPDH (forward: 5′-GGGCTACACTGAGGACCAGGTT-3′; reverse: 5′-TGCTGTAGCCGTATTCATTGTCA-3′).

Gene expression profiles were determined with SurePrint G3 Mouse Gene Expression 8*60K Microarray (Agilent Technologies, Santa Clara, CA, USA) by CapitalBio Technology (Beijing, China). Expression ratios were calculated, first normalized to the 75th percentile per chip, and finally normalized to medians per gene.

Following differentiation for 6 days, cells were fixed with formaldehyde and the number of osteoclasts was determined with a TRAP staining kit (Sigma-Aldrich, St. Louis, MO, USA; catalog number: 387A-1KT; lot number: SLBP7795V) according to the manufacture's instruction. TRAP-positive multinucleated cells (>3 nuclei/cell) were counted by a light microcopy as osteoclasts.

Bone Resorption

RAW cells or BMDMs were seeded on fresh bovine femur slices of 20-μm thick in 24-well plates. Osteoclast differentiation was induced by treatment of nCRP, mCRP, RANKL or their combinations with culture media changed every 2 days. Six days later, the slices were washed with 1 M ammonia for 3 min to break the osteoclasts, then fixed with 50% glutaric acid for 3 min, and finally stained with 1% toluidine blue for 5–10 min. Bone resorption was measured by pit area.

RANKL Binding

The interaction of mCRP and RANKL was determined with ELISA. Briefly, microtiter wells were coated with 1 μg/ml RANKL overnight at 4°C. All the following steps were performed at 37°C, and after each incubation step wells were washed 3 times with TBS (10 mM Tris, 140 mM NaCl, pH 7.4) containing 0.02% NP-40. Wells were washed and blocked with 1% BSA in TBS. nCRP, mCRP, or mCRP mutant was added to immobilized RANKL

for 1 h followed by washing. Binding were then detected with a sheep antihuman CRP polyclonal antibody (BindingSite; catalog number: PC044; lot number: 352325) and a donkey antisheep IgG (H + L) secondary antibody (Abbkine, Wuhan, China; catalog number: A21060; lot number: ATQMA0601).

Inflammatory Osteolysis

CRP knockout (KO) mice of C57BL/6 background were generated by insertion of a floxed STOP cassette at the translation start site of CRP gene *via* homologous recombination using CRISPR/Cas9 technique (Shanghai Biomodel Organism Science & Technology Development, Shanghai, China). CRP KO mice are fertile and grossly healthy. Inflammatory osteolysis was induced in wild-type or CRP KO mice (25 ± 2 g) of 7–8 weeks age as described (23). Briefly, 5 mg/kg LPS (Sigma-Aldrich; catalog number: L2880, lot number: 25M4040V), 2.5 mg/kg mCRP or vehicle was injected into the subcutaneous tissues overlying calvaria. The injections were performed every other day for 7 days. The calvaria were harvested and fixed in 4% paraformaldehyde for 2 days, followed by decalcification with 10% neutral buffered EDTA and embedding in paraffin. Samples were sectioned and TRAP and hematoxylin and eosin (HE) staining were performed to evaluate osteoclastogenesis and bone damage. The experiments conformed to the Guide for the Care and Use of Laboratory Animals published by NIH and were conducted according to the protocols approved by the Ethics Committee of Animal Experiments of Xi'an Jiaotong University.

Fluorescence Imaging

Raw264.7 and BMDM cells cultured on coverslips were rinsed twice with sterile PBS and incubated with FITC-labeled nCRP or mCRP for 30 min at 4°C. After gently rising, cell membrane was marked with FM 4-64 (Invitrogen, Carlsbad, CA, USA; catalog number: F34653; lot number: 1814727) at 4°C for 1 min. Nuclei were counterstained with DAPI (SouthernBiotech, Birmingham, AL, USA; catalog number: 0100-20; lot number: F0617-S327). Samples were examined by a LSM 710 confocal microscopy (Zeiss, Jena, Germany).

Statistical Analysis

Data were presented as mean ± SEM. Statistical analysis was performed by two-tailed Student's *t*-test, one-way ANOVA with Tukey *post hoc* or Kolmogorov–Smironv tests as appropriate. Values of $p < 0.05$ were considered significant.

RESULTS

mCRP Induces Osteoclast Differentiation

Circulating CRP is composed of five identical subunits, but dissociates into the monomeric conformation, i.e., mCRP, upon entering local lesions (14–18). Indeed, mCRP has been identified as the major conformation present in synovium tissues of RA patients (24). CRP in different conformations exhibit distinct or even contrasting activities (14–18), which may account for the controversies on its role in osteoclast differentiation (12, 13). We thus first examined this issue using Raw 264.7 macrophage cell line. Treating Raw cells with nCRP for 2 days did not alter the expression of osteoclast maker genes TRAP (also called ACP5) and Cathepsin K (**Figure 1A**). By contrast, these genes were markedly upregulated by mCRP treatment.

The effects of mCRP were not due to endotoxin contaminant because experiments were performed in the presence of PMB. Moreover, boiling or deleting the key recognition motif, i.e., cholesterol-binding sequence (CBS; a.a. 35–47) (20) that interacts with the lipid raft receptor (25, 26), impaired the actions of mCRP (**Figure 1B**). mCRP also showed much stronger binding to Raw cells than nCRP (**Figure 1C**), and evoked substantial responses at a concentration of 2 μg/ml (**Figure 1D**). The capability of mCRP to drive osteoclast differentiation was further confirmed by functional assays of TRAP staining (**Figures 1E,F**) and bone resorption (**Figures 1G,H**), in which nCRP was ineffective.

We next validated the conformation-dependent actions of CRP using mouse BMDMs. nCRP showed only weak binding to BMDMs and was unable to drive osteoclast differentiation; while mCRP bound BMDMs intensely (**Figure 2A**) and induced strong expression of TRAP (**Figure 2B**), leading to the formation of multinucleated osteoclasts (**Figures 2C,D**) with bone resorption activities (**Figures 2E,F**). Consistent with the *in vitro* results, subcutaneous injection of mCRP on calvaria of healthy mice resulted in increased number of osteoclasts (**Figures 2G,H**) and obvious trabecular damage (**Figures 2I,J**). We thus conclude that the induction of osteoclast differentiation by CRP depends on the monomeric conformation.

mCRP Does Not Act *via* Induction of RANKL

RANKL is considered as the major inducer of osteoclast differentiation (3) and has been reported to be upregulated by CRP (12). To clarify whether the effects of mCRP are mediated *via* downstream RANKL, we performed expression profiling on mCRP-treated BMDMs by DNA microarray. Osteoclast differentiation emerged as one of the top-ranked pathways activated by mCRP. Of the 35 relevant genes, 11 were differentially expressed in response to mCRP following a 4-h treatment (**Figures 3A,B**). Importantly, mCRP did not induce the expression of RANKL, its activating receptor RANK, or the inhibitory receptor LGR4 (22), but downregulated the expression of the decoy receptor OPG, suggesting little involvement of the canonical RANKL pathway in mediating the downstream effects of mCRP.

Additional analysis revealed remarkable differences between the gene expression profiles induced by mCRP and RANKL (**Figure 3C**). Moreover, screening with signaling inhibitors demonstrated that they activated distinct pathways (**Figure 3D**). mCRP acted primarily through NF-κB and phospholipase C, while a more extensive network was involved in RANKL signaling (**Figure 3E**). Of particular interest, ERK inhibition with U0126 markedly enhanced the effects of mCRP but suppressed that of RANKL. These results together demonstrate that the induction of osteoclast differentiation by mCRP does not depend on RANKL.

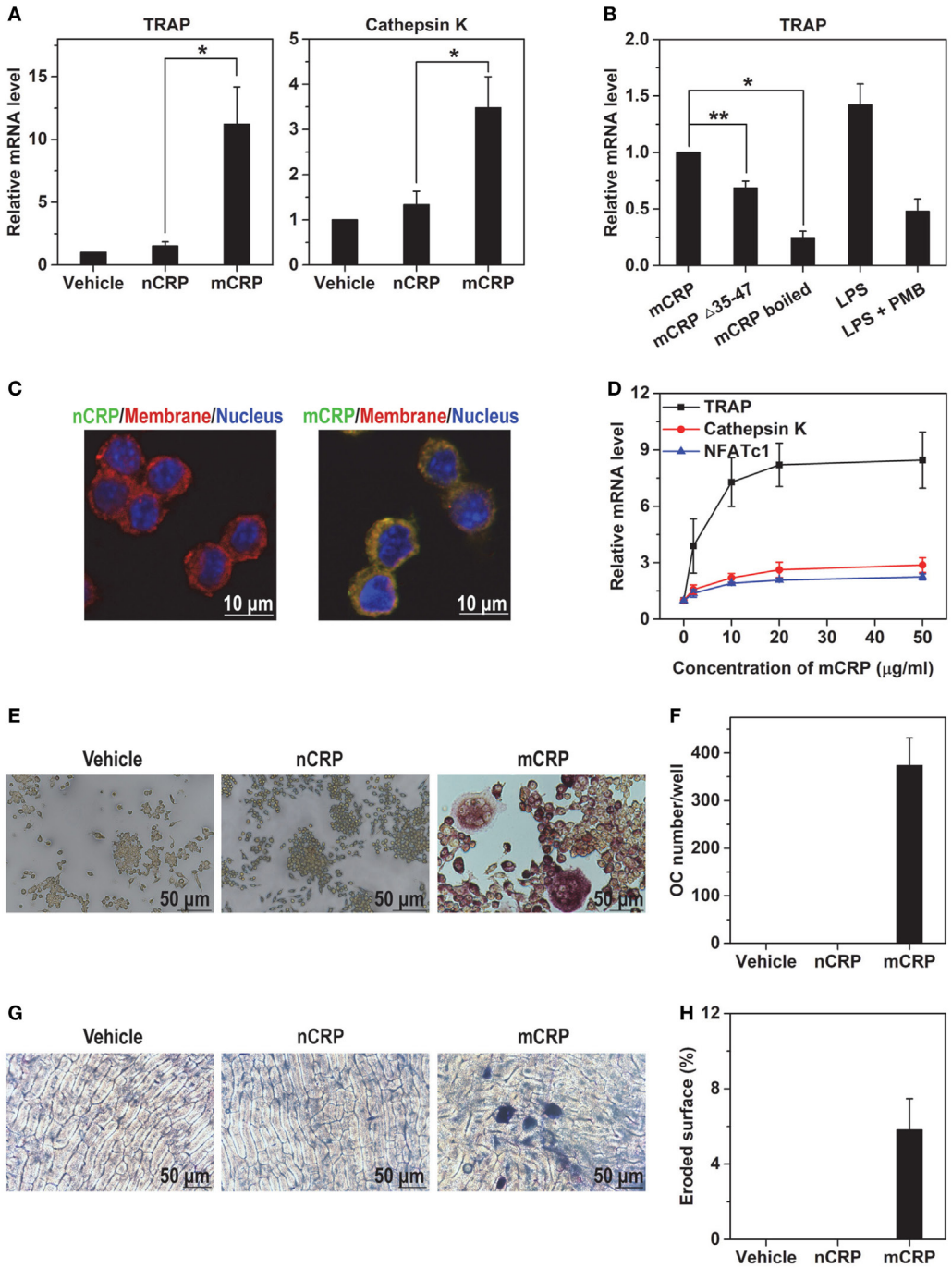

FIGURE 1 | mCRP but not native C-reactive protein (nCRP) induces osteoclast differentiation of Raw 264.7 cell line. Raw cells were treated with the indicated reagents for 2 days and the expression of osteoclast marker genes was determined with q-PCR. **(A)** 100 μg/ml mCRP upregulated the expression of TRAP and Cathepsin K, while nCRP at the same concentration was ineffective. **(B)** mCRP mutant lacking cholesterol-binding sequence motif (Δ35–47) or boiled wild-type mCRP showed impaired capacity to upregulate the expression of TRAP. The effects of LPS at 100 ng/ml, which is 50-fold higher than the residual level of endotoxin in our mCRP preparation, could be abrogated by the copresence of 2.5 μg/ml polymyxin B (PMB) that was included in all differentiation experiments. **(C)** FITC-labeled nCRP and mCRP were incubated with Raw cells at 4°C and visualized by confocal microscopy. Cell membranes and nuclei were counterstained with FM-4-64 and DAPI, respectively. mCRP showed intense binding to Raw cells, but nCRP did not. **(D)** Dose-dependent induction of TRAP expression by mCRP. Following treatment with 100 μg/ml nCRP or mCRP for 6 days, Raw cells were stained for TRAP **(E)** and osteoclast number was counted as TRAP-positive multinucleated cells **(F)**. Raw cells were plated on bone slices and treated with nCRP or mCRP for 6 days. The slices were then stained by toluidine blue **(G)** to measure eroded surface **(H)**. mCRP but not nCRP induced the differentiation of Raw cells to mature osteoclasts with bone resorption activities.

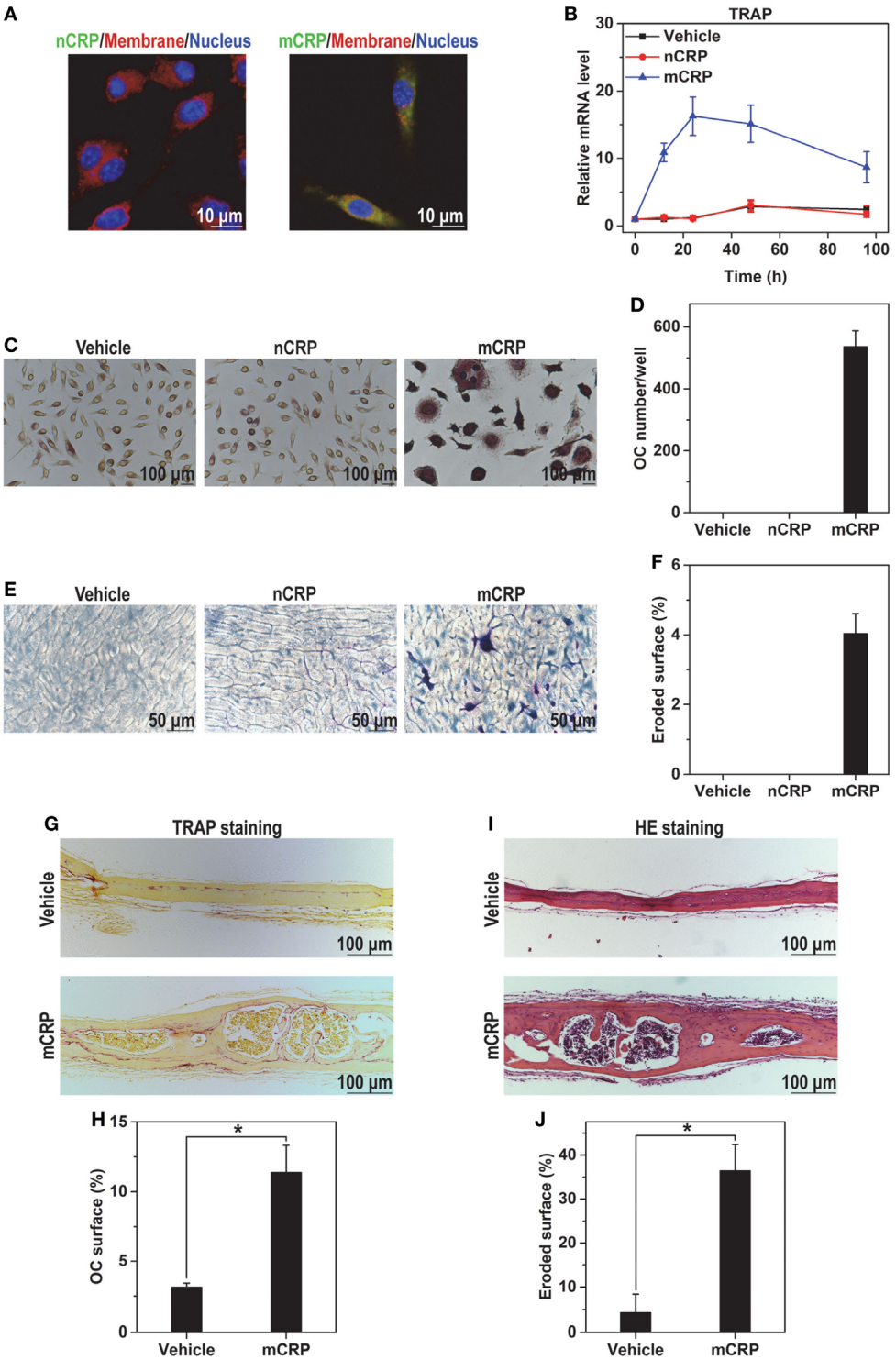

FIGURE 2 | mCRP but not native C-reactive protein (nCRP) induces osteoclast differentiation of bone marrow-derived macrophages (BMDMs) and in mice. **(A)** FITC-labeled nCRP or mCRP was incubated with BMDMs at 4°C and visualized by confocal microscopy. Cell membranes and nuclei were counterstained with FM-4-64 and DAPI, respectively. mCRP bound BMDMs strongly, while nCRP did not. **(B)** BMDMs were treated with 100 μg/ml nCRP or mCRP for the indicated times. TRAP expression was induced by mCRP but not nCRP. BMDMs were treated with 100 μg/ml nCRP or mCRP for 6 days, and then stained for TRAP **(C)** to count the number of TRAP-positive multinucleated cells as osteoclasts **(D)**. BMDMs were plated on bone slices and treated with nCRP or mCRP for 6 days. The slices were then stained by toluidine blue **(E)** to measure eroded surface **(F)**. mCRP but not nCRP induced the differentiation of BMDMs to mature osteoclasts with bone resorption activities. mCRP (2.5 mg/kg) or saline buffer (Vehicle) was s.c. injected on calvaria of healthy wild-type mice every 2 days for 1 week. TRAP **(G,H)** and hematoxylin and eosin staining **(I,J)** were conducted to evaluate osteoclastogenesis and bone damage, respectively. Osteolysis was actively induced by mCRP injection.

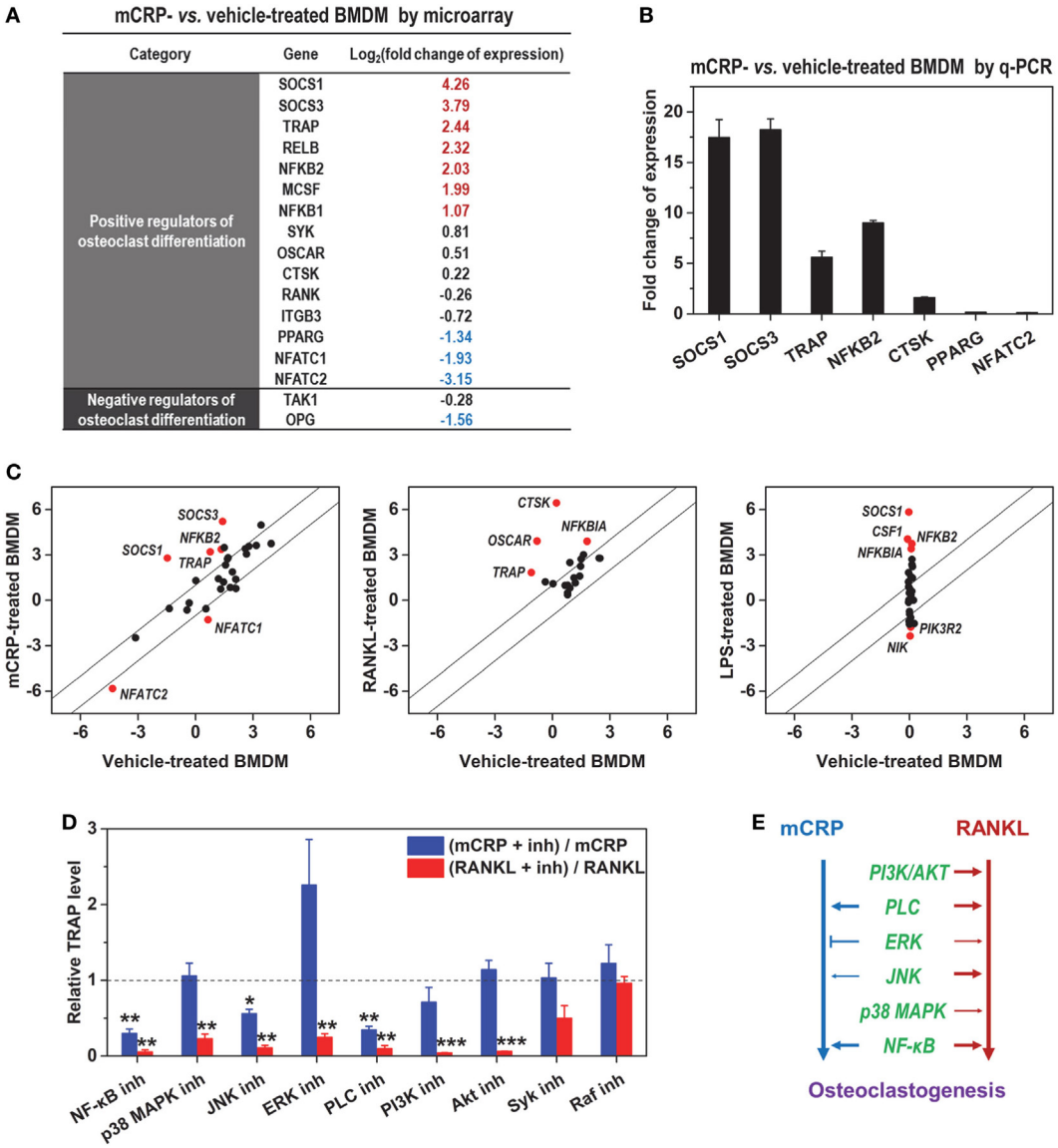

FIGURE 3 | mCRP acts *via* receptor activator of NF-κB ligand (RANKL)-independent pathway. **(A)** The expression profiles of mCRP- and vehicle-treated bone marrow-derived macrophages (BMDMs) were determined with DNA microarray. The expression changes of confidently determined genes relevant to osteoclast differentiation were listed. mCRP did not induce the expression of RANKL or its activating receptor RANK. **(B)** q-PCR validation of gene expression induced by mCRP. **(C)** Comparison of the effects of mCRP, RANKL (GSE57468), and LPS (GSE21895) on the expression of osteoclastogenesis-relevant genes. Remarkable differences in their effects were evident. **(D)** BMDMs were treated with mCRP or RANKL in the presence or absence of the indicated signaling inhibitors: Bay11-7082 (10 μM, NF-κB inh), SB20358 (20 μM, p38 MAPK inh), SP600125 (10 μM, JNK inh), U0126 (10 μM, ERK inh), U73122 (10 μM, Phospholipase C (PLC) inh), LY294002 (50 μM, PI3K inh), MK-2206 2HCl (5 μM, Akt inh), Piceatannol (10 μM, Syk inh), and GW5074 (20 μM, Raf inh). The mRNA levels of TRAP were determined by q-PCR and normalized to that of controls treated only with inhibitor. Results are shown as (TRAP level of cells treated with mCRP or RANKL plus the indicated inhibitor)/(TRAP level of cells treated with mCRP or RANKL). **(E)** The signaling pathways evoked by the two inducers differed significantly.

mCRP Inhibits RANKL-Induced Osteoclast Differentiation

As mCRP appeared to act differently from RANKL, we asked whether these two inducers have any synergy in osteoclast differentiation. When applied alone, RANKL was 3–10-fold more potent than mCRP in inducing the expression of osteoclast maker genes in BMDMs (**Figure 4A**). When applied together with mCRP, however, the effects of RANKL was completely absent with the net responses comparable to that of mCRP acting alone. Similar findings were also obtained in TRAP staining (**Figures 4B,C**) and bone resorption assays (**Figures 4D,E**). These results suggest that mCRP might instead inhibit the activities of RANKL on osteoclast differentiation.

mCRP is specifically converted in inflamed tissues, but does not exist in normal tissues (14–18). Therefore, it is plausible that the actions of mCRP are confined to pathological osteoclast

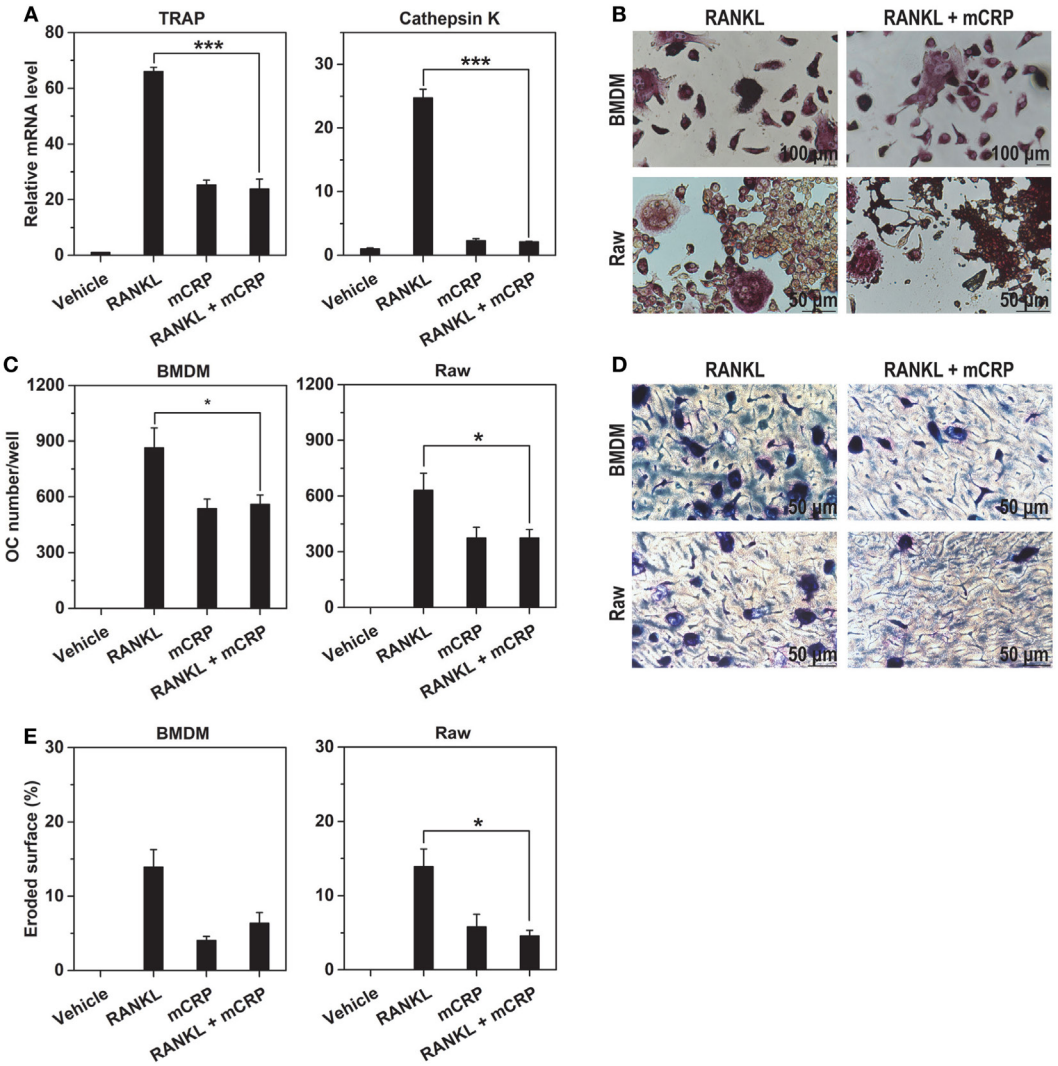

FIGURE 4 | mCRP neutralizes the effects of receptor activator of NF-κB ligand (RANKL). Bone marrow-derived macrophages (BMDMs) were treated with 10 ng/mL of M-CSF and 50 ng/ml RANKL in the presence or absence of 100 μg/mL of mCRP. After treatment for 2 days, the expression of TRAP and Cathepsin K were determined by q-PCR **(A)**. After treatment for 6 days, cells were stained for TRAP **(B)** and counted for the number of osteoclasts **(C)**. Their bone resorption activities were evaluated by toluidine blue staining **(D)** and the quantification of eroded surface **(E)**. The potent effects of RANKL on BMDMs were absent when treated together with mCRP. Comparable results were also obtained with Raw cells **(B–E)**.

differentiation where overproduced RANKL also plays a predominant role (4–6). The overall effects of mCRP in such a scenario, however, are likely suppressive due to inhibition of RANKL. In line with this speculation, the bone resorption phenotype of CRP KO mice (**Figures 5A,B**) is indistinguishable from that of wild-type mice in normal calvaria, but manifested increased number of osteoclasts (**Figures 5C,D**) and enhanced damage of trabeculae (**Figures 5E,F**) following subcutaneous injection of LPS.

CBS Mediates the Binding and Inhibition of RANKL

The inhibition of RANKL by mCRP could be due to interference between signaling pathways evoked by them. However, the complete and persistent inhibition observed in mouse BMDMs (**Figure 6A**) and peripheral blood mononuclear cells (PBMCs) (**Figure 6B**) made this possibility unlikely. Rather, inhibition due to their direct physical interactions appeared more likely. Indeed, mCRP bound RANKL with high affinity (**Figure 6C**). Deleting CBS (a.a. 35–47), the major recognition motif of mCRP (20), or competing with the synthetic CBS peptide markedly impaired the binding (**Figure 6D**). Moreover, the CBS peptide efficiently inhibited RANKL-induced osteoclastogenesis (**Figures 6E–H**). Therefore, the physical interactions between mCRP and RANKL are mediated by CBS.

DISCUSSION

The effects of CRP on osteoclast differentiation have been controversial. One study reports that CRP inhibits RANKL-induced

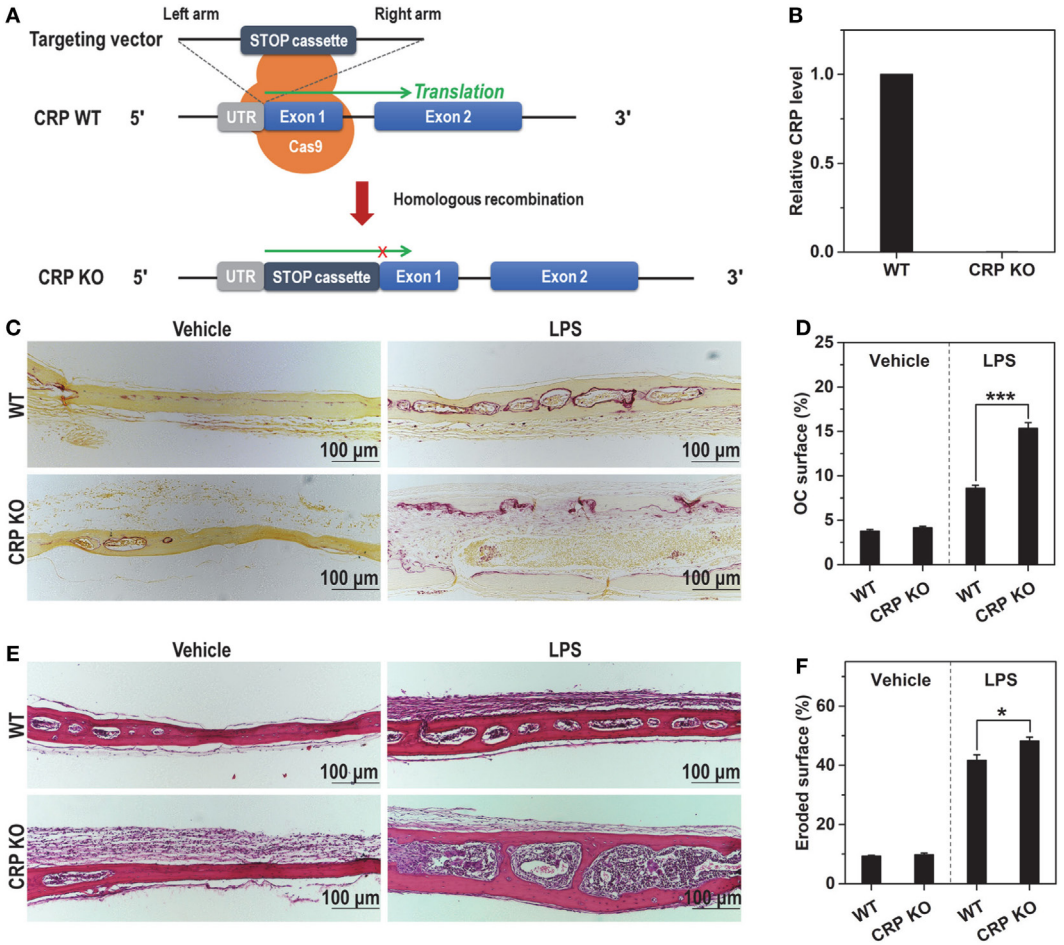

FIGURE 5 | C-reactive protein (CRP) knockout (KO) aggravates proinflammatory bone damage in mice**. (A)** The design of CRP KO mice. **(B)** The mRNA level of CRP in liver tissues of wild-type (WT) and CRP KO mice. No CRP expression could be detected in CRP KO mice. LPS (5 mg/kg) or saline buffer (Vehicle) was s.c. injected on calvaria of healthy wild-type and CRP KO mice every 2 days for 1 week. TRAP **(C,D)** and hematoxylin and eosin staining **(E,F)** were conducted to evaluate osteoclastogenesis and bone damage, respectively. These pathological indices were aggravated in CRP KO versus wide type mice.

osteoclast differentiation of Raw 264.7 cells *via* TLR signaling (13). However, residual endotoxin is a notorious contaminant that confuses the interpretation of CRP's activity (27, 28). Another study reports that CRP promotes osteoclast differentiation of human PBMCs *via* induction of RANKL (12). However, the prolonged incubation time (3–21 days) and the prominent death of primary cells cultured *ex vivo* would favor the conversion of nCRP to mCRP (29–34). Interestingly, the authors claim that the actions of CRP are mediated at least partly by CD16 (12), an established receptor for mCRP (35).

In the present study, with tight control of endotoxin contaminant and protein conformation, we show that CRP in its native conformation, i.e., nCRP, has no effect on osteoclast differentiation of Raw 264.7 cells, mouse BMDMs or PBMCs; while mCRP actively regulates their differentiation. The remarkable difference between gene expression profiles induced by mCRP and LPS further exclude possible confounding of endotoxin. Our findings thus establish that the effects of CRP on osteoclast differentiation are conformation-dependent. This may account for the aforementioned controversy and argues that conformation control is critical for interpreting the actions of CRP.

The actions of mCRP in osteoclastogenesis appear to depend on crosstalk among NF-κB, phospholipase C and ERK signaling pathways. Indeed, NF-κB, phospholipase C, and p38 MAPK have been shown to be responsible for mCRP-induced cytokine induction in endothelial cells (36, 37), while ERK and PI3K/Akt are more important in mediating the effects of mCRP on angiogenesis (38–41) and survival of neutrophils (42). These findings reveal a cell type- and biological process-dependent signaling evoked by mCRP. Such a versatility in activating various signaling pathways is likely duo to the capacity of mCRP to interact with lipid rafts (25, 26), signaling platforms on cell surfaces. Therefore, adaptor(s) in lipid rafts that mediates the context-specific actions of mCRP warrants further investigation.

It has been increasingly recognized that the actions of CRP also depend on localization (14–18). nCRP circulates in the

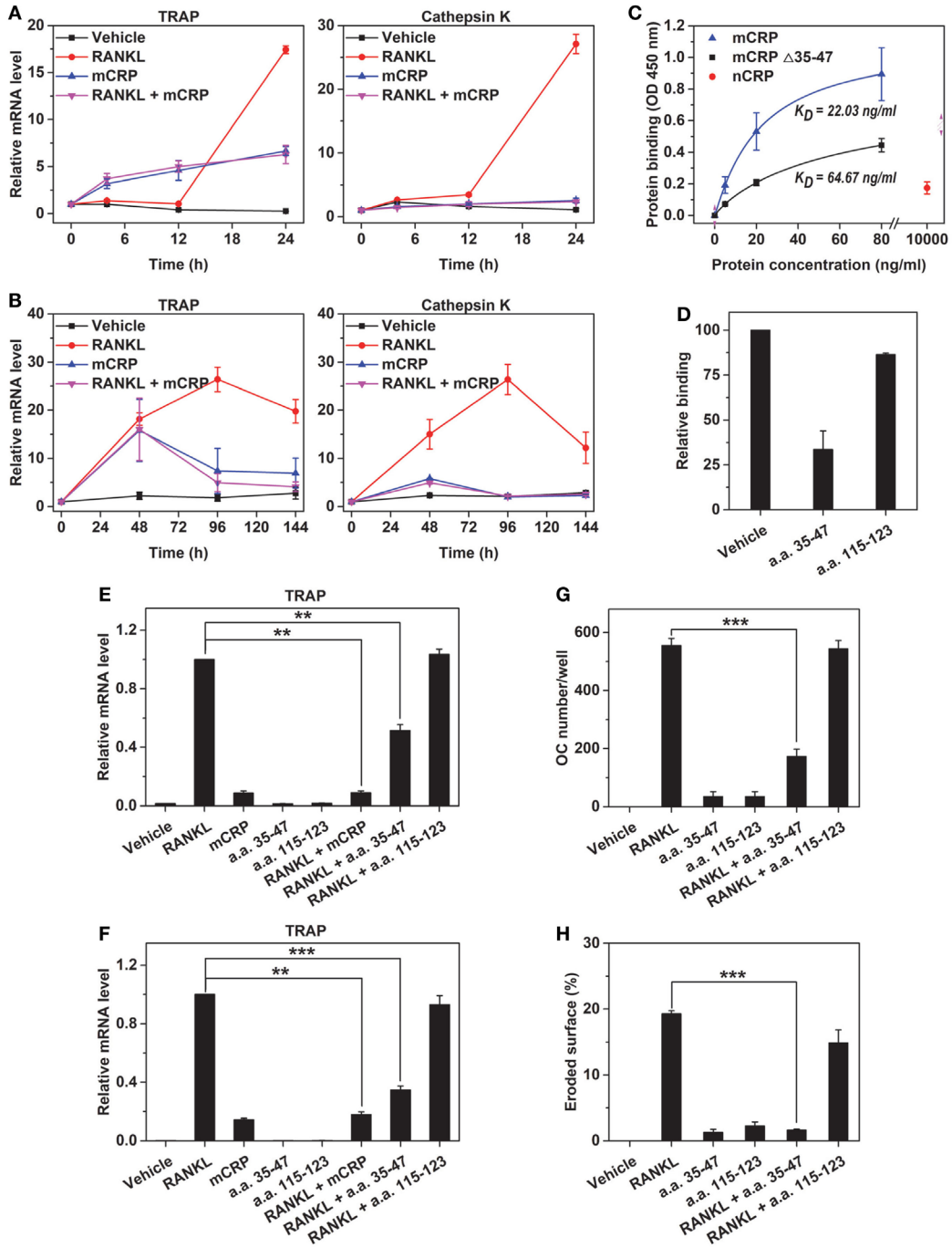

FIGURE 6 | mCRP binds receptor activator of NF-κB ligand (RANKL) *via* cholesterol-binding sequence. Bone marrow-derived macrophages (BMDMs) **(A)** or peripheral blood mononuclear cells **(B)** were treated with RANKL, mCRP, or their combination for the indicated times. In both cell types, the effects of RANKL were abrogated by cotreatment of mCRP at all time points tested. **(C)** mCRP, mCRP Δ35–47, or native C-reactive protein (nCRP) at the indicated concentrations were added to RANKL immobilized on microtiter wells. mCRP avidly bound immobilized RANKL, but this binding was impaired by deleting a.a. 35–47. Weak binding of nCRP was only detected at very high concentration. a.a. 35–47 peptide (20 μg/ml) nearly abrogated the binding of mCRP to RANKL **(D)**, halved RANKL-induced TRAP expression in Raw **(E)** and BMDM **(F)**, and greatly suppressed RANKL-induced differentiation of osteoclasts **(G)** and their bone resorption activities **(H)**. By contrast, another CRP peptide, i.e., a.a. 115–123 (20 μg/ml), showed no effect in these assays.

blood as a pentamer, but undergoes irreversible conformation changes, forming mCRP in inflamed tissues due to interaction with damaged membranes (29–34, 43–46), amyloid aggregates (47), neutrophil extracellular traps (48), or acidic pH (49). mCRP exhibits markedly enhanced activities, and is consequently considered to be the major conformation acting in

local lesions (14–18), such as synovium tissues of RA patients (24). Therefore, the interaction of mCRP with osteoclast precursors would be expected to occur in inflamed but not normal joints.

In inflamed joints, dysregulated RANKL signaling likely plays a major role in driving pathological osteoclast differentiation (4–6), and its neutralizing mAb, Denosumab, has become an approved therapeutic (50) that prevents bone loss in RA (51). As such, the overall effects of mCRP in pathological osteoclast differentiation may be protective through antagonizing RANKL. This speculation is supported by exacerbated inflammatory bone resorption in CRP KO versus wild-type mice, and is also consistent with the beneficial role of CRP in collagen-induced arthritis (10, 11). In this regard, CBS that mediates the interaction of mCRP with RANKL and exhibits anti-inflammatory activities as a synthetic peptide (20, 52), might emerge as a potential RANKL inhibitor to target pathological osteoclast differentiation.

Though mCRP is a potent proinflammatory molecule, it also possesses anti-inflammatory actions (53, 54). Therefore, the net contribution of mCRP in diseases might be context-dependent. Indeed, a protective role of mCRP in early atherogenesis has been reported (55). Recently, we have demonstrated that mCRP may be protective in lupus nephritis by recruiting CFH, and autoantibodies against CBS abrogating this effect predicts worse prognosis (52). Of note, dysregulated complement activation is critically involved in both atherosclerosis and lupus nephritis, which may explain the beneficial role of mCRP. In RA, however, dysregulated osteoclast differentiation is a central pathogenic mechanism (2) and mCRP may thus exert protective effects by neutralizing RANKL-induced osteoclastogenesis.

ETHICS STATEMENT

The experiments conformed to the Guide for the Care and Use of Laboratory Animals published by NIH and were conducted according to the protocols approved by the Ethics Committee of Animal Experiments of Xi'an Jiaotong University.

AUTHOR CONTRIBUTIONS

YW and S-RJ designed the research. Z-KJ, H-YL, and Y-LL performed the research. YW, S-RJ, Z-KJ, H-YL, and LP analyzed the data and wrote the article. All authors reviewed the results and approved the final version of the manuscript.

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Novel Association of High C-Reactive Protein Levels and A69S at Risk Alleles in Wet Age-Related Macular Degeneration Women

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Purpose: To explore the relationship between plasma C-reactive protein (CRP) levels, the main *ARMS2* gene single nucleotide polymorphism (SNP), and gender in patients with neovascular age-related macular degeneration (wet AMD).

Methods: Our study included 131 patients with wetAMD [age-related eye disease study (AREDS) category 4] and 153 control participants (AREDS category 1) from two Spanish retinal units. CRP levels were determined on blood samples by high-sensitivity ELISA assay. According to their CRP level, subjects were categorized into three well-established CRP categories: low (<1.00 mg/L, L-CRP), moderate (1–2.99 mg/L, M-CRP), and high (>3.00 mg/L, H-CRP). Genomic DNA was extracted from oral swabs using QIAcube (Qiagen, Hilden, Germany) and the A69S; rs10490924 of *ARMS2* gene was genotyped by allelic discrimination with validated TaqMan assays (Applied Biosystems, Foster City, CA, USA). Univariate and multivariate logistic regression adjusted for age was used to analyze the genomic frequencies and to calculate odds ratio (OR) using SNPStats software.

Results: Considering CRP risk categories, H-CRP group showed a significant [OR 4.0 (1.9–8.3)] association with wetAMD compared to L-CRP group. The risk genotypes of A69S (TT) SNPs showed an association with wetAMD risk [OR 14.0 (4.8–40.8)]. Interestingly, the gender stratification of the CRP categories showed a significant increase in CRP levels in wetAMD women compared with control women [OR 6.9 (2.2–22.3)] and with wetAMD men [OR 4.6 (1.3–16.9)]. In addition, the subgroup analysis of CRP within A69S genotype and gender showed a link in women between the A69S and CRP levels in the AMD group compared to controls [OR 4.2 (1.4–12.6)].

Conclusion: Our study shows, for the first time, that a different genetic association related with gender could contribute to AMD risk. As a consequence, the risk of female gender in the different CRP levels and A69S SNP frequencies could be taken into consideration to the established risk relationship of high levels of CRP and its association with risk A69S genotype.

Keywords: C-reactive protein, wet macular degeneration, polymorphism, single nucleotide, gender differences, case–control studies

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss among people more than 55 years of age in developed countries and can be divided into early and late stages. Late AMD is characterized by geographic atrophy or choroidal neovascularization (CNV) (wet AMD) both of which may lead to central visual impairment or irreversible blindness (1, 2). Inflammation and components of innate immunity, like complement activation, play a major role in the pathophysiology of AMD (3–5). Normal, adaptive parainflammation (defined as low-grade chronic inflammation) exists in the aging retina under physiologic conditions, but unbalanced and uncontrolled parainflammation leads to a detrimental chronic inflammatory response and contributes to the development of early and advanced AMD forms. This chronic process is mediated by many genetic and environmental factors (6).

Inflammatory factors, such as C-reactive protein (CRP), interleukin (IL)-6, and amyloid beta levels are increasingly being associated with AMD in the scientific literature (7–13). Among them, elevated CRP serum levels have been widely associated with both AMD development (9, 12, 14, 15) and progression (16). Similarly, several single nucleotide polymorphisms (SNP) in the complement pathway, particularly in the complement factor H (CFH), as well as in the *ARMS2* gene region, have been strongly associated with prevalence and incidence of AMD (17–19). More specifically, the A69S polymorphism has been related to the wet form of AMD rather than the atrophic form of the disease (20). Systemic CRP, along with genetic variants in the inflammatory pathway (CFH) and other pathway (ARMS2) have been associated with advanced AMD in age-related eye disease study (AREDS) sub-population (15). Although the relationship between CFH and CRP has been thoroughly studied by several authors, there is a paucity of high quality data directed to study the association between *ARMS2* and CRP levels.

Moreover, recent studies have shown controversial data about the influence of gender in the development and onset of early and late AMD. Whereas some studies have not found any relationship (1), a recent meta-analysis revealed that some individual studies suggested higher rates of neovascular AMD in women, mainly based on potentially higher rates of cerebrovascular events (2, 21). Interestingly, cerebrovascular and cardiovascular incidents have been related to increased systemic CRP levels (22).

To elucidate potential relationships between these factors, we aimed to investigate the influence of systemic CRP, A69S genotype, and gender in a large cohort of wetAMD patients and age-matched controls that attended in the Retinal units of two tertiary referral centers.

MATERIALS AND METHODS

Study Population

Our study included 131 patients with wetAMD (AREDS category 4) and 153 control participants (AREDS category 1) from 2 tertiary referral hospitals: Clinica Universidad de Navarra and Hospital Clinic de Barcelona (from the Spanish Multicenter Group on AMD and the "Red Temática de Investigación Cooperativa en Salud," RD07/0062, OFTARED RD12/0034 and RD16/0008). All participants were of Caucasian origin, completed a demographic questionnaire and gave permission for their inclusion in a database (data protection consent). All procedures were performed in accordance with the ethical standards of the Institutional Ethics Review Board of the Clínica Universidad de Navarra and with the 1964 Helsinki Declaration and its later amendments, or comparable ethical standards. All subjects gave written informed consent. Inclusion criteria for patients with wetAMD included the following: diagnosis of AMD with active subfoveal or juxtafoveolar CNV confirmed by fluorescein angiography (FA) and/or optical coherence tomography (OCT) (AREDS category 4). For control participants, inclusion criteria were the following: absence of drusen or no more than 5 small drusen (≤65 μm), absence of retinal pigment abnormalities in the macular area, and absence of chorioretinal macular atrophy or any other form of CNV (AREDS category 1). Exclusion criteria for this study (for both patients with wetAMD and control participants) included: age younger than 55 years, the presence of other CNV-related retinal diseases (i.e., angioid streaks, nevus in the macular area, toxoplasmosis scars, photocoagulation scars in the posterior pole, or polypoidal choroidal vasculopathy), history of retinal surgery, retinal disease in the studied eye (i.e., diabetic retinopathy or hereditary retinal dystrophies), and more than 6 diopters of myopia. DNA analysis was performed in 131 controls and 115 wetAMD subjects, the remaining samples were not analyzed because of technical problems with DNA (e.g., poor quality of DNA, DNA degradation, and sample collection issues). All cases underwent detailed ophthalmologic examination, including visual acuity assessment, dilated slit-lamp biomicroscopy, automatic objective refraction, color fundus photography, FA, and/or OCT. Controls underwent visual acuity assessment, mydriatic fundus examination, and measurement of refractive error and axial length.

CRP Analysis

Samples were obtained from peripheral blood in EDTA containing tubes and processed accordingly (1,500 g, 15 min). Plasma high sensitive hsCRP levels were determined using a high-sensitivity ELISA assay (ICN Pharmaceuticals, Costa Mesa, CA, USA) as previously reported (23, 24) and following manufacturer instructions. All assays were performed in duplicates by investigators blinded to the clinical or genetic characteristics of the study subjects. Intra- and inter-assay coefficients of variations were <10%.

As previously reported by other authors (24) the distribution of CRP values was not normal (skewness = 1.130, kurtosis = 0.73), but the values transformed to the natural logarithm approximated a normal distribution (skewness = −0.846, kurtosis = 1.38). Logarithmic transformations were used for CRP for analyses with continuous variables in order to decrease the effect of extreme observations (25).

Genotyping

The DNA samples from 131 controls and 115 wetAMD subjects were used in this study. Genomic DNA was extracted from oral swabs using QIAcube (Qiagen, Hilden, Germany). The control and wetAMD cohorts were genotyped for the rs10490924 of

ARMS2 gene by allelic discrimination with a validated assay (TaqMan; Applied Biosystems, Foster City, CA, USA) using real-time PCR (PE7300; Applied Biosystems), according to the manufacturer's instructions.

Statistical Analysis

Age analysis was performed by student's *t*-test and chi-square test was used for gender distribution analysis. Levels of hsCRP and LogCRP were analyzed by lineal regression adjusted by age. The frequencies of alleles, genotypes, and haplotypes were calculated in all groups and were compared using a chi-square test and Fisher's exact test, and corresponding ORs were calculated. A69S was in Hardy–Weinberg equilibrium.

Univariate logistic regression, adjusted by age and gender, and only by age for gender-based analysis, was used to estimate the ORs and 95% confidence intervals (95% CI) using SNPStats software (26). Analyses for each genetic variant were performed independently of other variants using codominant, dominant, recessive, and/or overdominant genetic models in base Akaike information, which chooses the inheritance model that best fits the data. A p value <0.05 was considered statistically significant.

RESULTS

CRP Analysis

Demographics for gender distribution and mean age in control and wetAMD groups for the total population and the individuals that underwent A69S genotyping analysis are shown in **Table 1**. The mean age (years ± SEM) was 73.60 ± 0.57 for controls and 76.56 ± 0.61 for wetAMD group ($p = 0.0005$). The percentage of women was balanced in both controls and wetAMD patients (53.6 vs. 59.5%, respectively). No differences in age were observed between men and women both in control (73.30 ± 0.86 and 73.87 ± 0.77, respectively; $p = 0.622$) and wetAMD groups (76.83 ± 0.93 and 76.38 ± 0.80, respectively; $p = 0.721$). In men, the mean age was 73.30 ± 0.86 in controls and 76.83 ± 0.93 in wetAMD group ($p = 0.007$) and 73.87 ± 0.77 in control women vs. 76.38 ± 0.80 in wetAMD women ($p = 0.025$). Mean LogCRP levels, CRP distribution, and A69S genotypes according to wetAMD cases and controls are shown in **Table 2**. Regarding CRP systemic levels, significantly greater mean hsCRP (mg/L) levels normalized by Log function (24) were observed in wetAMD vs. controls (1.43 vs. 1.26, $p < 0.001$). Without normalization, these greater mean hsCRP levels were still statistically significant (3.47 vs. 2.71 mg/L, $p < 0.001$) and wetAMD individuals showed a 28% greater hsCRP level compared to controls. Usually, CRP levels are stratified into low (L-CRP, <1.0 mg/L), moderate (M-CRP, 1.0–3.0 mg/L), and high (H-CRP, 3.0–10.0 mg/L) (23, 24). In our study, we found that there was a statistical difference ($p = 0.001$) in CRP distribution between control and wetAMD participants. H-CRP levels were observed in 46.6% of wetAMD individuals compared to a 31.4% in controls, whereas L-CRP levels were observed in 9.9% of wetAMD group compared to 26.8% of controls. Compared to the L-CRP group, a greater wetAMD risk was observed for the M-CRP group (OR 2.8 95% CI, 1.3–5.8, $p = 0.007$) and H-CRP group (OR 4.0 95% CI, 1.9–8.3, $p = 0.0001$).

TABLE 1 | Demographics for the total population and the subgroup which underwent A69S genotyping analysis.

		Control	WetAMD	*P* value (control vs. wetAMD)
Total population				
Men	*n* (%)	71 (46.4)	53 (40.5)	
	Age (years)	73.30 ± 0.86	76.83 ± 0.93	**0.007**
Women	*n* (%)	82 (53.6)	78 (59.5)	0.314
	Age (years)	73.87 ± 0.77	76.38 ± 0.80	**0.025**
Total	*n*	153	131	
	Age (years)	73.60 ± 0.57	76.56 ± 0.61	**0.0005**
Subgroup DNA analysis				
Men	*n* (%)	63 (48.1)	48 (41.7)	
	Age (years)	72.79 ± 0.89	77.10 ± 0.93	**0.001**
Women	*n* (%)	68 (51.9)	67 (58.3)	0.369
	Age (years)	73.87 ± 0.89	76.36 ± 0.91	0.052
Total	*n*	131	115	
	Age (years)	73.35 ± 0.63	76.67 ± 0.66	**0.0003**

Age is shown in years (mean ± SEM). No significant differences were observed in gender distribution for the total population (p = 0.314) and the genotyped participants (p = 0.369). Comparisons in age within the total population between control men and women (p = 0.622), wetAMD men and women (p = 0.721) were not statistically significant. Comparisons within the genotyped population between control men and women (p = 0.395), wetAMD men and women (p = 0.577) were not statistically significant. Age analysis was performed by student's t-test and chi-square was used for gender distribution. Significant p-values were highlighted in bold.

TABLE 2 | C-reactive protein (CRP) levels, number of subjects per CRP stratification, and A69S genotype for control and wetAMD groups.

	Control	WetAMD	*P* value	OR (95%CI)
Mean LogCRP ± SEM	1.26 ± 0.04	1.43 ± 0.03	**0.001**	
CRP—*n* (%)			**0.001**	
L-CRP (<1 mg/L)	41 (26.8)	13 (9.9)		1.00
M-CRP (1.0–3.0 mg/L)	64 (41.8)	57 (43.5)	**0.007**	**2.8 (1.3–5.8)**
H-CRP (3.0–10.0 mg/L)	48 (31.4)	61 (46.6)	**0.0001**	**4.0 (1.9–8.3)**
ARMS2 A69S—*n* (%)	*n* = 131	*n* = 115	**<0.0001**	
GG	85 (64.9)	36 (31.3)		1.00
GT	41 (31.3)	54 (47.0)	$\mathbf{9.0 \times 10^{-5}}$	**3.7 (2.0–6.8)**
TT	5 (3.8)	25 (21.7)	$\mathbf{1.0 \times 10^{-7}}$	**14.0 (4.8–40.8)**
MAF	T: 0.19	T: 0.45	$\mathbf{7.6 \times 10^{-10}}$	**3.4 (2.3–5.1)**

*Mean LogCRP between controls and wetAMD participants was statistically significant (p < 0.001) based on lineal regression test and data on single nucleotide polymorphisms and CRP levels were analyzed by univariate logistic regression adjusted by age and gender. *L-CRP (low C-reactive protein, <1 mg/L), M-CRP (moderate C-reactive protein, 1.0–3.0 mg/L), and H-CRP (high C-reactive protein, 3.0–10.0 mg/L). SEM; OR, odds ratio; CI, confidence interval; MAF, minor allele frequency. Letters T and G: nucleotides for the ARMS2 A69S. ORs >1 imply that was associated with increased risk of wetAMD when compared with the control (reference) group. Significant p-values were highlighted in bold.*

ARMS2 A69S Genotyping

The distribution of genotypes for the A69S polymorphism was in Hardy–Weinberg equilibrium in the study population. The distribution of *ARMS2* A69S genotypes was different between controls and wetAMD cases ($p < 0.0001$, **Table 2**), as we have described previously (20). The study of allelic frequencies showed

significant differences between wetAMD and control groups (OR 3.4 CI: 2.3–5.1; $p = 7.6 \times 10^{-10}$, **Table 2**). The percentage of risk genotypes (A69S GT and TT) was greater in the wetAMD group compared to the control group (68.7 vs. 35.1%). GT genotype showed a strong association with risk of wetAMD (OR 3.7 CI: 2.0–6.8; $p = 9.0 \times 10^{-5}$) which was even greater for the TT-risk genotype, with a 14-fold increase in wetAMD group vs. controls (OR 14.0 CI: 4.8–40.8; $p = 1.0 \times 10^{-7}$, **Table 2**).

CRP and *ARMS2* A69S Genotypes and Gender Influence

CRP Analysis by Gender

Descriptive statistics divided by gender, control and wetAMD groups, CRP levels, genotype frequencies, and demographic factors are disclosed in **Tables 3–6**. Significantly higher LogCRP and hsCRP levels were observed in women between control and wetAMD groups ($p = 0.002$ and $p = 0.009$, respectively, **Table 3**), while men did not show differences between these groups ($p = 0.140$ and $p = 0.275$, respectively, **Table 3**). Women showed greater wetAMD risk in M-CRP [OR 5.7 (1.8–18.7); $p = 0.002$] and H-CRP groups [OR 6.9 (2.2–22.3); $p = 0.0004$] compared to the L-CRP group (**Table 3**). The percentage of patients with H-CRP levels was greater in wetAMD vs. controls in women [52.6 vs. 37.8%, OR = 6.9 (2.2–22.3), $p = 0.0004$], but no differences were observed in men [37.7 vs. 23.9%, OR = 2.6 (0.9–7.2), $p = 0.08$] (**Table 3**).

ARMS2 A69S Genotypes Analysis by Gender

Subgroup analysis by A69S genotypes and gender revealed a risk association for TT genotype and wetAMD in men [OR 9.7 (1.9–50.1); $p = 0.003$, **Table 3**]. However, this association was observed in women for both the GT [OR 8.8 (3.7–21.4); $p = 1.2 \times 10^{-5}$] and TT [OR 22.8 (5.4–95.2); $p = 8.3 \times 10^{-6}$, **Table 3**] genotypes when comparing control and wetAMD groups. The allelic frequency studies also corroborated the genotyping results, and differences between the control group vs. wetAMD were observed in men [OR 2.5 (1.4–4.6), $p = 0.002$] and women, who showed a greater association [OR 4.2 (2.4–7.3), $p = 1.1 \times 10^{-7}$; **Table 3**].

The results obtained from the division of control and wetAMD individuals into men and women are shown in **Table 4**. The analysis of the LogCRP levels showed that wetAMD women had significantly higher CRP levels compared with wetAMD men (1.49 vs. 1.36, $p = 0.036$, **Table 4**). Further, no differences were reported in the control group with regards to CRP stratification levels between women and men (**Table 4**; **Figure 1**). However, women with H-CRP levels showed higher risk of wetAMD compared to men [52.6 vs. 37.7%, OR 4.6 (1.3–16.9); $p = 0.02$]. No differences were observed for M-CRP or L-CRP groups, and interestingly, L-CRP levels were observed only in 5.1% of wetAMD women compared to 17% of wetAMD men (**Table 4**; **Figure 1**).

With regards to A69S genotypes and gender of the same group, differences between wetAMD men and women were only observed in GT genotype frequencies [OR 3.1 (1.3–7.6); $p = 0.016$, **Table 4**; **Figure 2**]. The allelic study did not show significant differences for the T risk allele between wetAMD women and men, although the allelic frequency in women was higher than in men [*T*; 0.39 vs. 0.50, $p = 0.10$; OR 1.6 (0.98–2.7), **Table 4**; **Figure 2**].

ARMS2 A69S Genotypes and CRP Combined Analysis by Gender

The distribution of A69S genotypes and stratified CRP levels divided by gender in both control and wetAMD patients is shown in **Tables 5** and **6**, respectively. These tables also show *p*-values of the interaction between CRP levels and A69S, based on a model controlling for dominant genotypes (GT/TT vs. GG) and CRP (L-CRP and M/H-CRP) adjusted by age. Normal population usually shows L-CRP levels and taking into account that a CRP level

TABLE 3 | Subgroup analysis by gender.

	Men				Women			
	Controls	**WetAMD**	***P* value**	**OR (95% CI)**	**Controls**	**wetAMD**	***P* value**	**OR (95% CI)**
Number per group—*n*	71	53			82	78		
Mean hsCRP (mg/L) ± SEM	2.63 ± 0.31	3.13 ± 0.32	0.275		2.79 ± 0.24	3.71 ± 0.25	**0.009**	
Mean LogCRP ± SEM	1.21 ± 0.06	1.36 ± 0.05	0.140		1.30 ± 0.04	1.49 ± 0.03	**0.002**	
C-reactive protein (CRP)—*n* (%)								
L-CRP	20 (28.2)	9 (17.0)		1.00	21 (25.6)	4 (5.1)		1.00
M-CRP	34 (47.9)	24 (45.3)	0.5	1.6 (0.6–5.0)	30 (36.6)	33 (42.3)	**0.002**	**5.7 (1.8–18.7)**
H-CRP	17 (23.9)	20 (37.7)	0.08	2.6 (0.9–7.2)	31 (37.8)	41 (52.6)	**0.0004**	**6.9 (2.2–22.3)**
ARMS2 A69S—*n* (%)	*n* = 63	*n* = 48			*n* = 68	*n* = 67		
GG	40 (63.5)	21 (43.8)		1.00	45 (66.2)	15 (22.4)		1.00
GT	21 (33.3)	17 (35.4)	0.39	1.5 (0.6–3.51)	20 (29.4)	37 (55.2)	**1.2×10^{-5}**	**8.8 (3.7–21.4)**
TT	2 (3.2)	10 (20.8)	**0.003**	**9.7 (1.9–50.1)**	3 (4.4)	15 (22.4)	**8.3×10^{-6}**	**22.8 (5.4–95.2)**
MAF	*T*; 0.20	*T*; 0.39	**0.002**	**2.5 (1.4–4.6)**	*T*; 0.19	*T*; 0.50	**1.1×10^{-7}**	**4.2 (2.4–7.3)**

Mean C-reactive protein (CRP) level and number of men and women divided into control and wetAMD groups according to CRP groups and genotype frequencies. L-CRP (low C-reactive protein, <1 mg/L), M-CRP (moderate C-reactive protein, 1.0–3.0 mg/L), and H-CRP (high C-reactive protein, 3.0–10.0 mg/L). LogCRP levels were analyzed by lineal regression and adjusted by age. CRP groups and A69S genotyping were analyzed by univariate logistic regression adjusted by age. SEM; OR, odds ratio; CI, confidence interval; MAF, minor allele frequency. Letters T and G: nucleotides for the ARMS2 A69S. ORs >1 imply that was associated with increased risk of wetAMD when compared with the control (reference) group. Significant p-values were highlighted in bold.

TABLE 4 | Subgroup analysis for control and wetAMD individuals divided into gender, according to CRP groups and genotype frequencies.

	Control				WetAMD			
	Men	**Women**	***P* value**	**OR (95% CI)**	**Men**	**Women**	***P* value**	**OR (95% CI)**
Number per group—*n*	71	82			53	78		
Mean hsCRP (mg/L) ± SEM	2.63 ± 0.31	2.79 ± 0.24	0.696		3.13 ± 0.32	3.71 ± 0.25	0.154	
Mean LogCRP ± SEM	1.21 ± 0.06	1.30 ± 0.04	0.427		1.36 ± 0.05	1.49 ± 0.03	**0.036**	
C-reactive protein (CRP)—*n* (%)								
L-CRP	20 (28.2)	21 (25.6)		1.00	9 (17.0)	4 (5.1)		1.00
M-CRP	34 (47.9)	30 (36.6)	0.86	1.2 (0.5–2.6)	24 (45.3)	33 (42.3)	0.12	3.1 (0.8–11.2)
H-CRP	17 (23.9)	31 (37.8)	0.28	1.7 (0.7–4.0)	20 (37.7)	41 (52.6)	**0.02**	**4.6 (1.3–16.9)**
ARMS2 A69S—*n* (%)	*n* = 63	*n* = 68			*n* = 48	*n* = 67		
GG	40 (63.5)	45 (66.2)		1.00	21 (43.8)	15 (22.4)		1.00
GT	21 (33.3)	20 (29.4)	0.70	0.9 (0.4–1.8)	17 (35.4)	37 (55.2)	**0.016**	**3.1 (1.3–7.6)**
TT	2 (3.2)	3 (4.4)	1.00	1.3 (0.2–8.3)	10 (20.8)	15 (22.4)	0.19	2.2 (0.8–6.2)
MAF	*T*; 0.20	*T*; 0.19	1.00	0.9 (0.5–1.7)	*T*; 0.39	*T*; 0.50	0.10	1.6 (0.9–2.7)

L-CRP (low C-reactive protein, <1 mg/L), M-CRP (moderate C-reactive protein, 1.0–3.0 mg/L), and H-CRP (high C-reactive protein, 3.0–10.0 mg/L). LogCRP levels were analyzed by lineal regression and adjusted by age. CRP groups and A69S genotyping were analyzed by univariate logistic regression adjusted by age. SEM; OR, odds ratio; CI, confidence interval; MAF, minor allele frequency. Letters T and G: nucleotides for the ARMS2 A69S. ORs >1 imply that was associated with increased risk of wetAMD when compared with the control (reference) group. Significant p-values were highlighted in bold.

TABLE 5 | Dominant model of the frequencies observed for the *ARMS2* A69S genotype within CRP groups dividing total population, men and women into control and wetAMD groups.

ARMS2-A69S	**Total population**				**Men**				**Women**			
	Control	**WetAMD**	***P* value**	**OR (95% CI)**	**Control**	**WetAMD**	***P* value**	**OR (95% CI)**	**Control**	**WetAMD**	***P* value**	**OR (95% CI)**
L-CRP												
GG	22	5		1.00	8	4		1.00	14	1		1.00
GT/TT	12	7	0.19	2.6 (0.7–9.9)	9	5	0.95	1.1 (0.2–5.6)	3	2	0.14	9.3 (0.6–139.6)
MAF	*T*; 0.18	*T*; 0.42	**0.03**	**3.2 (1.1–8.9)**	*T*; 0.25	*T*; 0.39	0.35	1.9 (0.6–6.4)	*T*; 0.09	*T*; 0.50	**0.03**	**10.3 (1.4–75.7)**
M/H-CRP												
GG	63	31		1.00	32	17		1.00	31	14		1.00
GT/TT	34	72	$\mathbf{6.5 \times 10^{-7}}$	**4.3 (2.4–7.8)**	14	22	**0.014**	**3.0 (1.2–7.2)**	20	50	$\mathbf{2.4 \times 10^{-5}}$	**5.5 (2.4–12.5)**
MAF	*T*; 0.20	*T*; 0.46	$\mathbf{5.3 \times 10^{-8}}$	**3.3 (2.1–5.2)**	*T*; 0.17	*T*; 0.38	**0.001**	**3.0 (1.5–6.0)**	*T*; 0.22	*T*; 0.50	$\mathbf{2.0 \times 10^{-5}}$	**3.4 (1.9–6.1)**

L-CRP (low C-reactive protein, <1 mg/L) and M/H-CRP (moderate-high C-reactive protein, 1.0–10.0 mg/L). Data were analyzed by univariate logistic regression adjusted by age and gender for the total population and by age for men and women subgroups. OR, odds ratio; CI, confidence interval; MAF, minor allele frequency. Letters T and G: nucleotides for the ARMS2 A69S. ORs >1 imply that was associated with increased risk of wetAMD when compared with the control (reference) group. Significant p-values were highlighted in bold.

higher than 1 mg/L is considered a cardiovascular risk factor. Therefore, we combined both categories (M-CRP and H-CRP) into a single one (M/H-CRP) to assess the influence of CRP levels on the risk of wetAMD, depending on genotypes. A very strong relationship was observed between A69S risk genotypes, CRP levels, and female gender. In the total study cohort, the allele frequency study showed a significant association of the T risk allele in both CRP levels analyzed [L-CRP; OR = 3.2 (1.1–8.9), $p = 0.03$, and M/H-CRP; OR = 3.3 (2.1–5.2), $p = 5.3 \times 10^{-8}$, **Table 5**]. In M/H-CRP level, women showed a greater association of T risk allele compared with men [OR 3.4 (1.9–6.1), $p = 2.0 \times 10^{-5}$ and OR 3.0 (1.5–6.0), $p = 0.001$, **Table 5**; **Figure 3**]. Considering the GT/TT risk genotypes this difference is even greater. The total population showed a 4.3-fold increase likelihood of wetAMD in GT/TT genotypes compared to GG [OR 4.3 (2.4–7.8), $p = 6.5 \times 10^{-7}$, **Table 5**], however, was women carrying GT/TT distribution where the risk of wetAMD vs. control was the greatest [women; OR 5.5 (2.4–12.5), $p = 2.4 \times 10^{-5}$ and men; OR 3.0 (1.2–7.2), $p = 0.014$, **Table 5**]. We further explored these differences and found that, women with M/H-CRP and GT/TT genotypes have a threefold greater risk of wetAMD than men with such conditions [OR 2.8 (1.2–6.6), $p = 0.03$, **Table 6**; **Figure 3**]. Finally, a trend for a protective association was in the control group for women carrying GG and L-CRP compared to men [OR 0.2 (0.04–1.0), $p = 0.07$, **Table 6**].

DISCUSSION

The present study describes, for the first time, a novel association between gender, CRP (a systemic marker of inflammation), the A69S SNP in the *ARMS2* gene, and wet AMD. We found that CRP is a multiplying wet AMD risk factor to the *ARMS2* SNP in women, and we observed a threefold higher wetAMD risk for women carrying the GT or TT at-risk allele and with intermediate or high CRP levels compared to men.

Age-related macular degeneration is a chronic and complex disease affecting people older than 55 years (1) in which several genetic and environmental factors have an influence on its

TABLE 6 | Dominant model of the frequencies observed for the *ARMS2* A69S genotype within CRP groups between control and wetAMD in men and women separately.

ARMS2-A69S	Control				WetAMD			
	Men	Women	*P* value	OR (95% CI)	Men	Women	*P* value	OR (95% CI)
L-CRP								
GG	8	14		1.00	4	1		1.00
GT/TT	9	3	0.07	0.2 (0.04–1.0)	5	2	1.0	1.6 (0.1–24.6)
MAF	*T*; 0.25	*T*; 0.09	0.10	0.3 (0.06–1.1)	*T*; 0.39	*T*; 0.50	0.9	1.5 (0.2–10.3)
M/H-CRP								
GG	32	31		1.00	17	14		1.00
GT/TT	14	20	0.4	0.7 (0.3–1.6)	22	50	**0.03**	**2.8 (1.2–6.6)**
MAF	*T*; 0.17	*T*; 0.22	0.5	0.7 (0.3–1.5)	*T*; 0.38	*T*; 0.50	0.11	1.6 (0.9–2.8)

L-CRP (low C-reactive protein, <1 mg/L) and M/H-CRP (moderate-high C-reactive protein, 1.0–10.0 mg/L). Data were analyzed by univariate logistic regression adjusted by age. OR, odds ratio; CI, confidence interval; MAF, minor allele frequency. Letters T and G: nucleotides for the ARMS2 A69S. ORs >1 imply that was associated with increased risk of wetAMD when compared with the control (reference) group. Significant p-values were highlighted in bold.

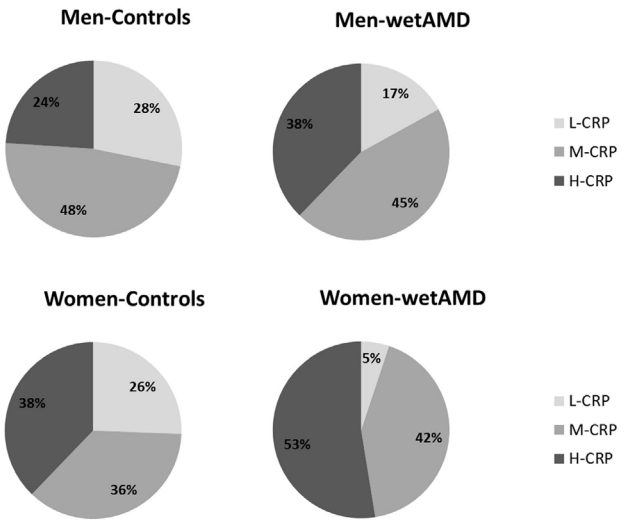

FIGURE 1 | Percentage of individuals according to C-reactive protein (CRP) level stratification divided by gender (men: upper row; women: lower row) and study groups (controls: left column and wet age-related macular degeneration: right column). L-CRP (low C-reactive protein, <1 mg/L; light gray), M-CRP (moderate C-reactive protein, 1.0–3.0 mg/L; medium gray), and H-CRP (high C-reactive protein, 3.0–10.0 mg/L; dark gray). Letters T and G: nucleotides for the *ARMS2* A69S.

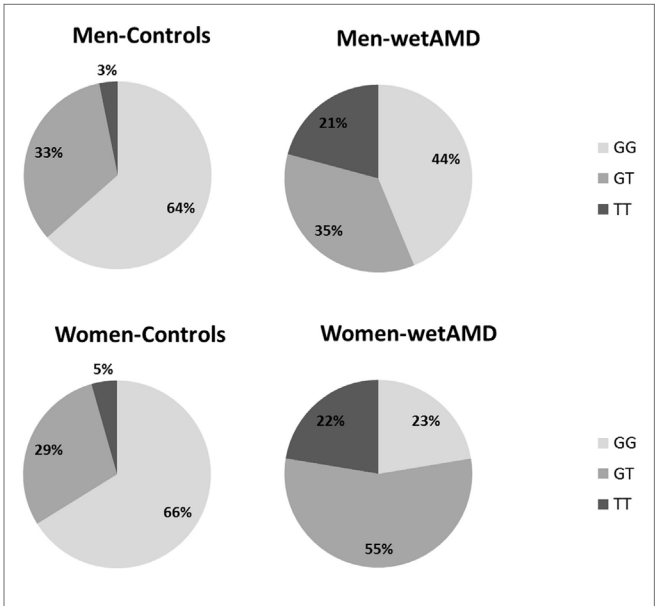

FIGURE 2 | Percentage of individuals according to A69S genotypes divided by gender (men: upper row and women: lower row) and study groups (controls: left column and wetAMD: right column). L-CRP (low C-reactive protein, <1 mg/L; light gray), M-CRP (moderate C-reactive protein, 1.0–3.0 mg/L; medium gray), and H-CRP (high C-reactive protein, 3.0–10.0 mg/L; dark gray). Letters T and G: nucleotides for the *ARMS2* A69S.

progression (27, 28). Although serum CRP levels have gained greater importance as a risk marker in cardiovascular disease (CVD), the magnitude of its risk potential has been debated (29). Consistent evidence of a significant association between elevated serum CRP and late AMD supports the hypothesis that higher levels of CRP (>3 mg/L) are associated with a twofold higher likelihood of late AMD. In contrast, the evidence of an association between CRP and early AMD is weaker (23, 30). This difference has been previously investigated for other diseases in which CRP is considered an inflammatory marker, such as CVD (31), obstructive sleep apnea (32), and obesity (33), with controversial results. In the present study, we confirmed our previous results where a relationship between A69S and the risk of wetAMD was observed (20). We have found a fourfold increase of wetAMD likelihood in GT and a 14-fold increase for the at-risk TT genotype. Since elevated CRP confers a higher risk of AMD (30), the combination of these findings suggests that the risk allele might contribute to the development of AMD. In our study, we observed that *ARMS2* A69S heterozygous and homozygous risk alleles along with moderate CRP levels seem to be associated with wet AMD in men and women.

ARMS2 protein is a component of the extracellular matrix of the choroid that some authors locate in the mitochondria (34). Recent studies in rats, using electron microscopy, have demonstrated disorganization and swelling of mitochondria's after CRP injection in cardiomyocytes from ischemic carotids (35). Other authors have found that ARMS2 is located in the cytosol colocalized with cellular skeleton (microtubules and actin filaments),

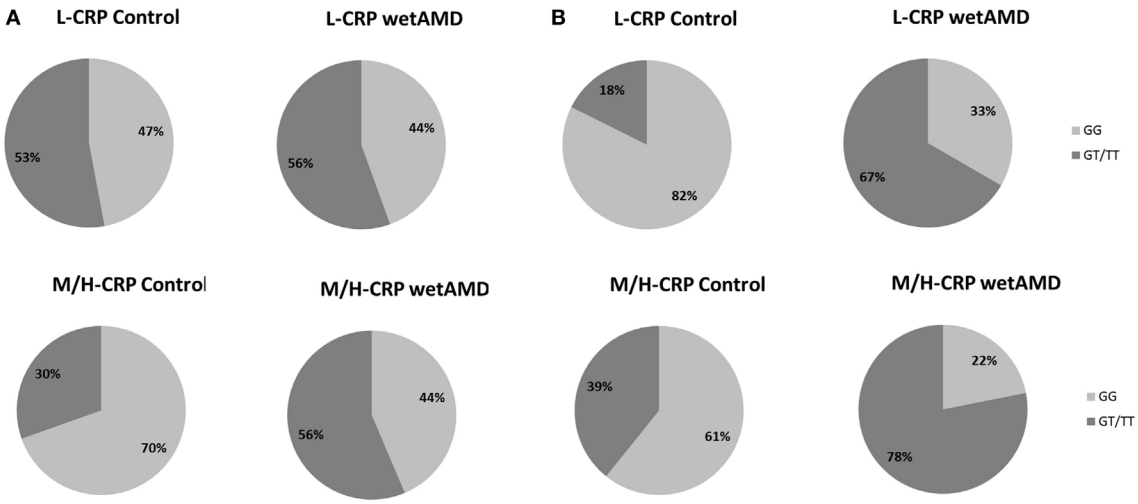

FIGURE 3 | Percentage of individuals according to men **(A)** and women **(B)** and to C-reactive protein (CRP) stratified groups (L-CRP: upper row and M/H-CRP: lower row) and A69S genotype (light gray: GG and dark gray: GT/TT). L-CRP (low C-reactive protein, <1 mg/L) and M/H-CRP (moderate-high C-reactive protein, 1.0–10.0 mg/L). Letters T and G: nucleotides for the *ARMS2* A69S.

suggesting that A69S may gain function to interact with the cytoskeleton (36). Furthermore, CRP is involved in microtubules stabilization by inhibiting neutrophil movement through the kinases pathway (37). Finally, some authors have found significantly higher CRP deposits in the Bruch's membrane and retinal and choroid vessels of wet AMD eyes compared to aged controls and AMD human donors (38).

Currently, there is great concern about the role of gender as potential bias that may influence results of clinical trials and affect diagnostic or treatment decisions. As a consequence, gender perspective has recently become a major factor to be considered in clinical research and especially in genetics (39), since some differences related to gender may commonly be missed and/or remain unrevealed. To ascertain these potential differences, we considered the parameters separately for each gender. After dividing the study cohort into men and women, we observed a strong association between high levels of CRP and wetAMD in women, which was not seen in men. Moreover, in the subanalysis performed by gender in control and wetAMD groups, higher levels of CRP were more frequently observed in women compared with men within the wetAMD group. These results could suggest an association of female gender and high CRP levels and allegedly, inflammation, in the development of wet AMD. These findings highlight the potential usefulness of systemic CRP as a marker of wet AMD in women, as a novel risk factor that may also be considered along with the well-established factors for AMD. Interestingly, wetAMD men only showed a 56.2% of heterozygous and at-risk homozygous genotypes compared to the 77.6% observed in wetAMD women. Furthermore, women with GT genotype had a threefold increase risk of wetAMD compared to men.

Understanding the combined effects and interactions among specific genotypes and risk factors in wet AMD may provide new insights into the complex etiology of such chronic condition. In fact, the study of the association between AMD genetics, inflammatory factors, and gender is a rapidly evolving research field. A recent study, for example, described that *DAPL1* is an AMD-associated gene and that this disease association is female-specific (40).

Despite the growing consensus that chronic inflammation is an important factor in the pathogenesis of AMD, few studies have studied the association of *ARMS2* and systemic inflammatory markers such as CRP. Hence, we also analyzed the correlation between serum CRP categories and *ARMS2* A69S genotypes in the total population and stratified by gender. In our study, in agreement with the results observed by Seddon et al. (15), we observe that the *ARMS2* at-risk allele for AMD is strongly associated with moderate and high CRP systemic levels with a fourfold increased risk, suggesting that probably this association is related to low-grade chronic systemic inflammation (22).

The most novel finding in our study is related to gender. We found that women with intermediate-high CRP and carrying the A69S at-risk alleles had almost a sixfold greater risk of wetAMD compared to the threefold risk observed in men. This was confirmed when genders were compared within groups, and an almost threefold higher risk of wetAMD was observed in women with the at-risk GT/TT alleles and moderate-high CRP levels compared to men.

We hypothesized that a plausible biological explanation to these gender-related differences reported could rely on the different hormone levels observed in men and women. Oral hormone therapy and CRP levels in postmenopausal women have shown a direct relationship (41). Further, a biological pathway for AMD that features hormone replacement therapy includes estrogen receptors 1 and 2. Both proteins have been observed in the human retina, suggesting that estrogens play a role in the pathogenesis of AMD (42, 43). Estrogen controls the expression of chitinase 3-like-1 protein (YKL-40), a molecule found in choroidal neovascular membranes. Lower levels of estrogen may trigger the upregulation of YKL-40 and play a role in the development of

neovascular AMD (44). Moreover, estrogens have an antioxidant effect by inhibiting lipid peroxidation, which provides protection against oxidative damage in the retina caused by the aging process (45). Women are mostly exposed to hormonal changes with aging and especially after their mid-life, which may contribute to a greater risk compared to men who usually show more stable hormonal levels. However, the exact mechanisms underlying these differences are still unclear and these preliminary results come from preclinical investigations using cell culture and animal models.

This is the first study directed to evaluate the relationship between the *ARMS2* A69S distribution, serum CRP levels, and gender in AMD patients and controls. Interestingly, we have found a very strong association in women between the *ARMS2* A69S and CRP levels in the AMD group compared to controls. The mechanisms and associations related to the *ARMS2* locus and CRP remain to be elucidated. In our study, it seems that the *ARMS2* and intermediate-high CRP levels provide greater risk of wetAMD, especially in women.

However, it should be noted that these findings may be considered within the context of a selected population and some limitations should be acknowledged such as sample size. Therefore, more studies to replicate this association in an independent sample set in a stronger powered analysis are needed. Moreover, as other authors have also reported for CVD, CRP levels can be confounded by obesity, ethnicity, gender, and other comorbidities (31). Future studies must consider the inclusion of factors, such as smoking, body mass index, serum cholesterol, hypertension, and the use of hormone replacement therapy in the variance component model.

In spite of the limitations described above, our study shows several strengths including that this is the first time that a gender-based analysis on A69S genotype frequencies is performed. Despite the relatively low number of samples in the gender subgroups, the magnitude of the associations and the significance values obtained are strong enough to consider these aspects for future prospective and retrospective studies. These data can serve as a starting point for prospective trials evaluating such associations. Taking into account these results, a potential therapeutic approach could be directed to modify this systemic inflammation status, especially among those genetically high-risk individuals carrying the at-risk alleles in order to reduce the likelihood of wet AMD and, according to other studies, also other forms of late AMD (46).

In conclusion, we have observed that women with high CRP levels showed higher risk of wetAMD than men, suggesting that systemic CRP levels could be useful as an important multiplying factor to the already established genetic factors involved in the development of wet AMD. These individuals should be prioritized for interventional studies directed to prevent the progression of the disease (47). One proposal for those at-risk populations has been the inhibition of monomeric-CRP for local treatment of vascular disease (48) and reduction of myocardial infarct size (49) in at-risk areas. In line with this, monomeric-CRP has shown to induce an inflammatory phenotype (50) and blood–retinal barrier disruption in RPE cells (51). Given the similarities of risk factors between CVD and AMD, this approach could be considered as a local adjuvant therapy in future new strategies for patients at greater risk, such as women with high CRP levels and at-risk A69S genotype (52). This could contribute to a better quality of life and reduce personal burden for AMD patients.

ETHICS STATEMENT

All procedures were performed in accordance with the ethical standards of the Institutional Ethics Review Board of the Clínica Universidad de Navarra and with the 1964 Helsinki Declaration and its later amendments, or comparable ethical standards. All subjects gave written informed consent.

AUTHOR CONTRIBUTIONS

Conceptualization: PF-R, AG-L, SR, and JZ-V. Data curation: PF-R, SR, and JZ-V. Funding acquisition: AG-L and PF-R. Investigation: JZ-V, RC-M, AA, SR, and AG-L. Methodology: BM, SR, MV, JZ-V, and PF-R. Resources: JZ-V, RC-M, AA, and AG-L. Supervision: AG-L, PF-R, and AA. Writing—original draft: PF-R, SR, and MH. Writing—review and editing: PF-R, MV, BM, MH, AG-L, SR, AA, and JZ-V.

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MINI REVIEW
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pCRP-mCRP Dissociation Mechanisms as Potential Targets for the Development of Small-Molecule Anti-Inflammatory Chemotherapeutics

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Circulating C-reactive protein (CRP) is a key acute-phase protein and one of the main clinical biomarkers for inflammation and infection. CRP is an important upstream mediator of inflammation and is associated with the onset of a number of important disease states including cardiovascular disease and neurodegenerative disorders such as Alzheimer's disease. This pentraxin exerts pro-inflammatory properties *via* dissociation of the pentamer (pCRP) to a monomeric form (mCRP). This dissociation is induced by binding of pCRP to cell surface phosphocholine residues exposed by the action of phospholipase A_2 (PLA_2). Given the association of CRP with the onset of a range of serious disease states this CRP dissociation process is a tempting drug target for the development of novel small-molecule therapeutics. This review will discuss potential targets for chemotherapeutic intervention elucidated during studies of CRP-mediated inflammation and provide an up-to-date summary of the development of small molecules, not only targeted directly at inhibiting conversion of pCRP to mCRP, but also those developed for activity against PLA_2, given the key role of this enzyme in the activation of CRP.

Keywords: CRP, inflammation, chemotherapy, phospholipid, phospholipase

INTRODUCTION

Pentameric C-reactive protein (p-CRP) is a pentraxin, composed of five identical subunits, linked by van der Waals and H-bonding, each weighing around 23 kDa with, what is described as, a jelly role shape with the subunits arranged around a central, hydrophobic pore. The pentamer presents two faces, each distinguished by their binding capabilities. Thus, the A face (effector face) binds to globular head groups of compliment c1q and Fcγ cell surface receptors on leukocytes while the B (binding) face exhibits one binding site per subunit which undergoes Ca^{2+}-mediated binding with phosphocholine moieties exposed on lipid membranes (1). pCRP is synthesized in the liver and is freely circulating. While normally present at negligible levels, plasma concentrations rise 6–12 h after acute inflammatory insult to 1,000-fold levels after 24–48 h, focused at sites of inflammation (2, 3).

As a result, CRP is used as a biomarker for inflammation and infection. It was long thought that pCRP was a direct mediator of inflammation leading to upregulation of endothelial cellular adhesion molecules, activation of the compliment system, phagocytosis, and release of a range of inflammatory signaling proteins (4, 5). However, it has recently been shown that the dissociation into the monomeric form, mCRP, is the key pro-inflammatory event (6). Further work has shown that this event is localized to sites of inflammation and mCRP plays an important role in the pathogenesis of inflammation interacting with endothelial cells, neutrophils, macrophages, and platelets (7). mCRP, rather than pCRP, induces upregulation of IL-8, MCP-1, E-selectin, ICAM-1, and VCAM-1 in endothelial cells resulting in increased adhesion of neutrophils (8). These studies reveal that this process is mediated *via* p38 MAPK signaling. Interestingly, recent work indicates that the interaction with endothelial cells is initiated *via* binding to lipid rafts rather than receptors, such as FcγRs on the cell surface (9, 10). CRP is a ligand for LOX-1 which mediates the entry of oxidized low-density lipoprotein (ox-LDL) across the endothelium (11). Furthermore, mCRP is implicated in the uptake of ox-LDL by macrophages leading to foam cell formation (12). mCRP can also activate monocytes to adhere to endothelia and transmigrate—a process mediated *via* binding with integrin receptors (13, 14). High local levels of mCRP have been detected in the myocardium of patients suffering from acute coronary syndrome (15) and the choroids obtained from donors at high risk of developing age-related macular degeneration (16). Furthermore, it has been shown there is an accumulation of mCRP in pertinent brain regions, arising from poststroke inflammation (17) and evidence that this observation explains the known link between ischemic stroke and onset of AD (18). In addition, Aβ plaques have been demonstrated to cause dissociation of pCRP to mCRP leading to a buildup of the latter in cortical tissue of AD patients (19).

The dissociation of pCRP to mCRP has now been delineated in some detail. The dissociation is mediated by binding of pCRP subunits to phosphocholine residues of lysophosphotidylcholines (LPC) exposed on cell membranes (**Figure 1**). LPC is generated by the action of pro-inflammatory phospholipase (PLA_2) enzymes acting on cell surface lysophospholipids. This link between PLA_2 and CRP-mediated inflammation is backed up by the 6–12 h delay observed between inflammatory insult and onset of high levels of CRP. Furthermore, CRP formation is prevented by pre-incubation of monocytes with ONO-RS-82, a well-known inhibitor of PLA_2 enzymes (20). Dissociation is also mediated *via* interaction with phosphocholine present on the surface of activated platelets, which acts to localize mCRP generation to areas of inflammation such as atherosclerotic plaques (13). Localized dissociation may also arise from binding of pCRP to lysophosphocholine residues exposed on the surface of ox-LDL, by lipoprotein-associated PLA_2 (Lp-PLA_2) (11). The most recent studies have provided a more detailed mechanism of dissociation (21). Binding of pCRP on activated monocytes, in addition to docking with phosphocholine, also involves interactions between hydrophobic regions of the pentamer and lipid rafts on the cell surface. The protein is then released onto microvesicles and undergoes a conformational change to an activated pentamer designated pCRP*. This moiety, while still pentameric, exists in a more open form and undergoes binding with a globular head group of complement C1q, which inserts into the central cavity forcing the subunits of the pentamer further apart to ultimately cause dissociation to mCRP.

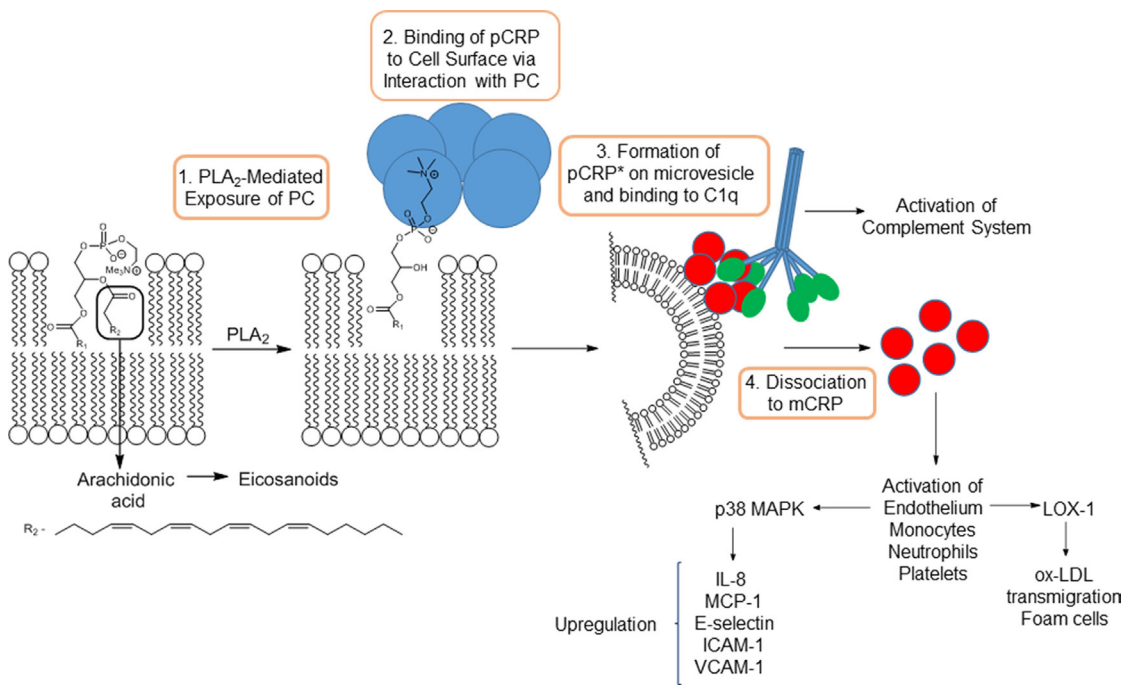

FIGURE 1 | Action of PLA_2 on arachidonic acid-containing phospholipids and subsequent mechanism of dissociation of pCRP to mCRP.

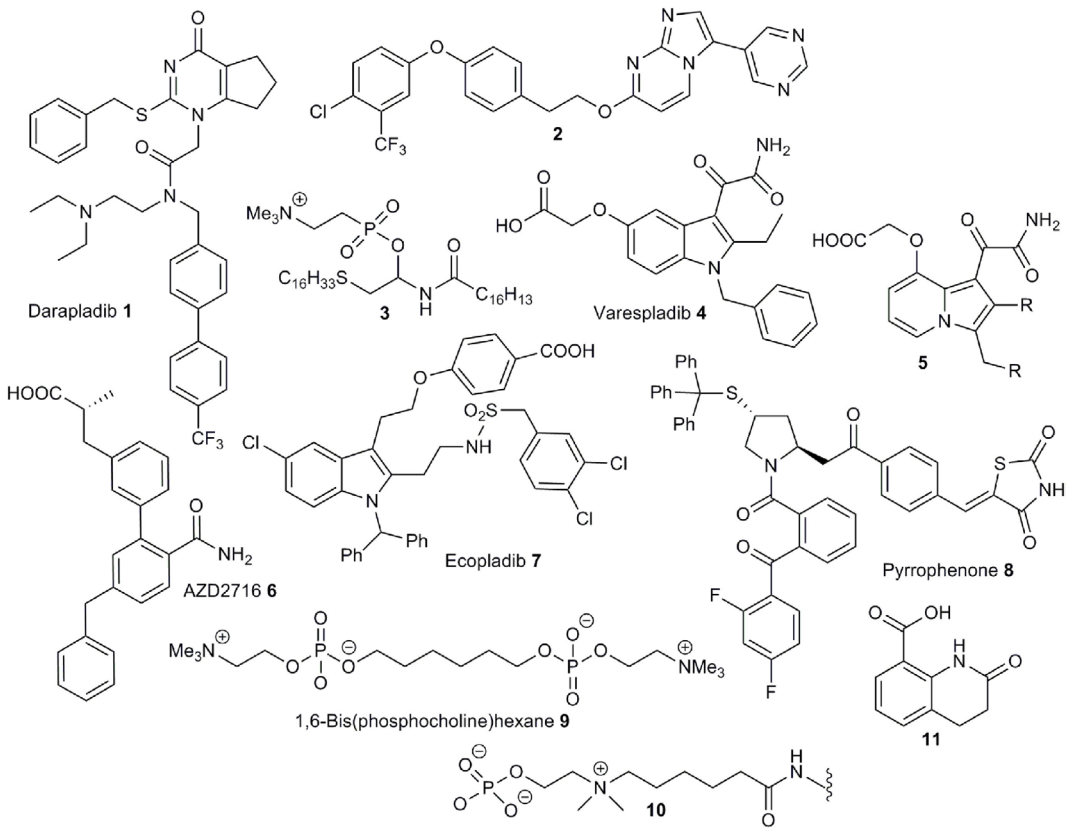

FIGURE 2 | Example structures of anti-PLA_2 drugs and small-molecule binders to CRP.

CHEMOTHERAPEUTIC TARGETS IN CRP DISSOCIATION

The clear link between pCRP-mCRP dissociation and the onset/mediation of inflammation indicates that inhibition of this process is, potentially, a valuable chemotherapeutic strategy for the treatment of a range of conditions associated with the inflammatory response. A number of key stages, from initial exposure of cell surface phosphocholine residues to mCRP-mediated activation of monocytes/platelets/endothelia potentially provide an opportunity for chemotherapeutic inhibition. However, an understanding of these various processes at the molecular level is an important prerequisite for the development of small molecules abrogation. Fortunately, investigations have provided information on amino acid–ligand interactions by *in silico* modeling, site-directed mutagenesis studies, and X-ray crystallographic information. For instance, an X-ray crystal structure of pCRP bound to phosphocholine reveals key amino acids involved in ligand binding (1). Significantly, a hydrophobic cavity is shown to exist, adjacent to the binding region, providing a potential blueprint for the design of inhibitors of pCRP–phosphocholine binding. Furthermore, an X-ray crystal structure of a CRP dissociation inhibitor, 1,6-bis(phosphocholine)-hexane, a drug discussed further below, bound to the active of two CRP pentamers has also been determined (22). A crystal structure of the globular head group of C1q has been solved, and the information used to provide a model for the interaction of this domain with p-CRP and to postulate amino acid residue interactions involved in complement-pentamer binding (23). Site-directed mutagenesis studies have also been directed toward identifying the key CRP–C1q interactions (24). mCRP-mediated activation of monocytes via binding to integrins αvβ3 and α4β1 has also been simulated by *in silico* modeling yielding identification of potential binding sites (14). Significantly, this study, while predicting favorable mCRP-integrin binding, indicates significant steric interactions in pCRP-integrin models of binding. The identification of lipid raft interactions as key to mCRP binding to a range of targets, including endothelia, *via* cholesterol binding sequence (9, 10) offers an additional target for small-molecule intervention-although this interaction has not been studied at similar levels of details to some of those discussed above.

These studies provide information that can be used to develop small-molecule agents to inhibit the interaction between pCRP and phosphocholine, complement C1q-induced dissociation to mCRP and subsequent activation of monocytes. However, to date, the only stage which has which has been perturbed by small-molecule agents is the initial binding of pCRP to phosphocholine, to be discussed herein. Nevertheless, an important stage of CRP activation is exposure of phosphocholine residues on cell surfaces by PLA_2 and the action of this enzyme has been

linked to CRP-mediated inflammation (20). A large number of small molecules have been developed to inhibit phospholipase activity although only a small number have been shown to lower levels of mCRP (20). However, the use of PLA_2 inhibitors to treat neuroinflammation, *via* suppression of pro-inflammatory lysophospholipid formation, has been postulated (25) and, given the clear links between mCRP formation and lysophospholipid exposure, further implicates the use of PLA_2 inhibitors to prevent CRP dissociation. Thus, this review will focus on summarizing work in this area.

SMALL-MOLECULE INHIBITORS OF PHOSPHOLIPASE A_2

Among the various subgroups within the phospholipase A_2 superfamily, secreted phospholipase A_2 ($sPLA_2$), cytosolic phospholipase A_2 ($cPLA_2$), and lipoprotein-associated phospholipase A_2 ($LpPLA_2$) have been the most popular targets for the development of inhibitors. The development of small molecules against the PLA_2 family has been extensively reviewed and this mini review will seek to provide a brief, up-to-date overview of only the most successful drug candidates against s-, c-, and $LpPLA_2$ (26).

All PLA_2 enzymes catalyze the hydrolysis of phospholipids at cell membranes or the surface of lipoproteins, to produce free fatty acids and exposing lysophospholipids, including LPC, on the cell surface (**Figure 1**). The former may include arachidonic acid, which is converted to inflammatory-mediating eicosanoids, indicating a dual pro-inflammatory role for PLA_2 enzymes.

Lipoprotein-associated phospholipase ($LpPLA_2$) hydrolyzes oxidized phospholipids present on the surface of ox-LDL producing pro-inflammatory oxidized fatty acids and lysophospholipids (27). A plausible link between $LpPLA_2$ activity and CRP activation is supported by the detection of CRP/ox-LDL complexes in the plasma of atherosclerosis patients (28). The central role of this enzyme in the development of inflammation has led to its use as a predictive biomarker for the onset of atherosclerosis (29). A diversity of structures have been discovered to exhibit $LpPLA_2$ inhibition (30–35). The most successful drugs against $LpPLA_2$ are pyrimidin-4-ones of the darapladib class **1** (**Figure 2**) (36) discovered by modification of lead compounds unearthed by high throughput screening programs at GSK (37–39). A range of analogs, based on the darapladib motif have been studied but do not display improved activity (40, 41), although some imidazopyrimidine derivatives, such as **2**, do exhibit improved bioavailability (42). Unfortunately, darapladib failed Phase III clinical trials due to a failure to alleviate the risk of cardiovascular death or stroke in coronary heart disease patients (43, 44).

Secretory phospholipase A_2 ($sPLA_2$) is an extracellular phospholipase catalyzing the hydrolysis of phospholipids at cell surfaces. The association of this enzyme with the development of inflammatory conditions, and even some cancers, has driven the development of a number of small-molecule inhibitors (45). Unsurprisingly, phospholipid derivatives do serve as inhibitors given the natural substrates for this enzyme class (46–48). For instance, the thioether analog **3** is a potent inhibitor (49). The phosphocholine group has been successfully substituted with a carboxylic acid moiety, which appears to function as a bioisostere for this group, to provide compounds with excellent anti-$sPLA_2$ activity (50) and substitution of the trimethylammonium group with an amide provides more permeable compounds with some inhibitory properties (51). The most successful molecules against $sPLA_2$ are those based on an indole-3-acetamide structure. Structure-activity studies based around this central motif (52, 53), aided by an X-ray crystal structure of recombinant enzyme co-complexed with a lead compound (54, 55) led to the development of the 3-glyoxamide derivative varespladib **4** (56). Unfortunately, as with darapladib, varespladib failed to negotiate Phase III trials due to lack of efficacy (57). Significantly, indole-based compound, closely related to **4**, are also potent inhibitors of group X $sPLA_2$, mammalian phospholipases, which are particularly active pro-inflammatory members of this enzyme family (58). Furthermore, X-ray structures of these inhibitors bound to the active site have been obtained (59). Related indolizines such as **5** also exhibit potent anti-$sPLA_2$ activity (60) and the importance of a central heterocyclic aromatic core to this activity is reflected by the use of this information to develop potent inhibitors based around pyrazole fragments (61). This concept was later expanded to the study of amide-functionalized aromatic fragments leading to the development of the preclinical candidate AZD2716 **6** (62). Compound **6** exhibits better oral bioavailability than varapladib, which requires deployment as a methyl ester prodrug.

In contrast to $sPLA_2$, $cPLA_2$ functions is an intracellular enzyme and specifically interacts with arachidonyl phospholipids and is thus especially responsible for the formation of pro-inflammatory arachidonic acid in addition to lysophospholipids. This enzyme has been identified as a key mediator of inflammation leading to a range of disease states (63). A range of relatively simple compounds have been found to act as potent inhibitors of activity. The design of these is largely based on mimicking the arachidonoyl phosphonate structure and a knowledge of the serine-based mechanism of phospholipid hydrolysis. While a hydrophobic chain or aromatic group acts as a replacement for the arachidonate moiety, an activated ketone serves to disrupt serine hydrolysis and, as is the case with $sPLA_2$ inhibitors, a carboxylate is an effective surrogate for the phosphonate group (64). The early, anthranilic acid-based broad spectrum, PLA_2 inhibitors such as *N*-(p-amylcinnamoyl)anthranilic acid (ACA) and ONO-RS-82 (65), widely used as tools to probe PLA_2 activity, partially fit this model for inhibitor design as does the selective $cPLA_2$ inhibitor arachidonyltrifluoromethylketone ($AA\text{-}COCF_3$) (66). A design strategy based on phospholipid binding has also led to the development of linear 2-oxoamides (67) and 2-oxoesters (68) linked *via* nitrogen or oxygen, respectively, to aliphatic carboxylic acid group, and bis-aryloxypropanones, where both aliphatic groups around a central carbonyl group have been replaced with aromatic moieties (64). The disubstituted propanones serve as useful motifs for inhibitor design and replacement of one aromatic group with a thiazole (69), or suitably substituted indoles (70), have yielded $cPLA_2$ inhibitors with good activity. The indole moiety has been identified as a suitable substitute for the arachidonate section of the phospholipid substrate, and this strategy has led to the development of the ecopladib **7** class of $cPLA_2$ inhibitors (71). Structural modification of **7** led to the development of the closely

related efipladib (72) and giripladib (73). The latter compound was advanced to Phase II trials but terminated at this stage. High-throughput screening approaches have also led to the discovery of potent $cPLA_2$ inhibitors. Compound library screening yielded two fragments—a pyrrolidine and a thiazolidinylidene, combination of which provided a series of compounds, such as pyrrophenone **8**, with very high inhibitory activity (74, 75).

SMALL-MOLECULE INHIBITORS OF PCRP DISSOCIATION

The only small molecule demonstrated to inhibit dissociation of pCRP to mCRP is the bis-phosphocholine dimer 1,6-bis (phosphocholine)-hexane (bis(PC)-H) **9** (22). The design of this compound utilized a similar strategy used in the development of drugs targeted toward serum amyloid P component (SAP) which act to crosslink two SAP molecules and is based on the utilization of moieties chemically similar to phosphocholine head groups that bind to the same active site to disrupt LPC-mediated CRP activation. Crucially, a X-ray crystal structure of the pCRP-bis(PC)-H drug complex was obtained revealing binding of five drug molecules to phosphocholine binding sites to link two pentamers. This interaction abrogates binding of pCRP to known ligands such as LDL and blocks CRP-mediated complement C1q activation. Additionally, bis(PC)-H was demonstrated to reduce CRP-mediated effects in rat models. Despite demonstration of some clinical efficacy in animal models bis(PC)-H suffers from a low half-life, low CRP affinity and other suboptimal pharmacokinetic parameters.

While bis(PC)-H is the only small molecule that has been demonstrated to effectively disrupt CRP dissociation, *via* direct binding, other compounds have been shown to undergo chemical interactions with this pentamer and thus provide potential blueprints for the future design of inhibitors. For instance, a polypeptide conjugated with the phosphocholine linker **10** is a high-affinity binder to CRP demonstrating that phosphocholine mimics, free from the cell surface, can effectively interact with the active sites of the pentamer (76, 77). Furthermore, effective binding of **10** indicates that the CRP active sites may tolerate phosphocholine analogs with larger, extended alkyl chains as has been indicated previously by the X-ray crystal structure of the CRP-phosphocholine complex (1). Further work in this area has revealed that conjugates bearing heterocycles such as **11** also function as high-affinity binders (78). The dissimilarity between **11** and phosphocholine, and the competition experiments, indicates that there are alternate regions on the surface of CRP that may provide targets for future inhibitor design. Finally, rosuvastatin inhibits CRP-mediated inflammation in rat models expressing human CRP (79). As this treatment does not reduce circulating levels of CRP, effects are not solely down to inhibition of gene expression but rather to inhibition of CRP-mediated pathways. Direct binding to CRP has not been established however.

CONCLUSION

The dissociation of pCRP to mCRP is clearly an important event in the onset of inflammatory processes implicated in major disease states and inhibition is thus an important chemotherapeutic goal. It is surprising that only one compound has been developed that successfully inhibits dissociation *via* direct binding to CRP and the lack of follow-up studies Thus, the use of PLA_2 inhibitors to indirectly affect dissociation is potentially the most promising current strategy given the range of structures available and proven efficacy. However, few have been demonstrated to exert effects on mCRP formation, and the failure of all anti-PLA_2 drugs evaluated in advanced trials is a cause for concern. Nevertheless, studies have revealed a range of well-characterized potential chemotherapeutic targets for inhibition of CRP dissociation and, given the recent discoveries of non-natural small-molecule binders to CRP, it is anticipated that the search for drugs that abrogate CRP-mediated inflammation will be a rich area of research in the future.

AUTHOR CONTRIBUTIONS

VC prepared a large part of the body of text and figures. LB, MN, W-HF, GF, BG, RI, DL, YZ, and MS assisted in manuscript preparation and provided critical evaluation of the work.

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REVIEW
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Role of C-Reactive Protein at Sites of Inflammation and Infection

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C-reactive protein (CRP) is an acute inflammatory protein that increases up to 1,000-fold at sites of infection or inflammation. CRP is produced as a homopentameric protein, termed native CRP (nCRP), which can irreversibly dissociate at sites of inflammation and infection into five separate monomers, termed monomeric CRP (mCRP). CRP is synthesized primarily in liver hepatocytes but also by smooth muscle cells, macrophages, endothelial cells, lymphocytes, and adipocytes. Evidence suggests that estrogen in the form of hormone replacement therapy influences CRP levels in the elderly. Having been traditionally utilized as a marker of infection and cardiovascular events, there is now growing evidence that CRP plays important roles in inflammatory processes and host responses to infection including the complement pathway, apoptosis, phagocytosis, nitric oxide (NO) release, and the production of cytokines, particularly interleukin-6 and tumor necrosis factor-α. Unlike more recent publications, the findings of early work on CRP can seem somewhat unclear and at times conflicting since it was often not specified which particular CRP isoform was measured or utilized in experiments and whether responses attributed to nCRP were in fact possibly due to dissociation into mCRP or lipopolysaccharide contamination. In addition, since antibodies for mCRP are not commercially available, few laboratories are able to conduct studies investigating the mCRP isoform. Despite these issues and the fact that most CRP research to date has focused on vascular disorders, there is mounting evidence that CRP isoforms have distinct biological properties, with nCRP often exhibiting more anti-inflammatory activities compared to mCRP. The nCRP isoform activates the classical complement pathway, induces phagocytosis, and promotes apoptosis. On the other hand, mCRP promotes the chemotaxis and recruitment of circulating leukocytes to areas of inflammation and can delay apoptosis. The nCRP and mCRP isoforms work in opposing directions to inhibit and induce NO production, respectively. In terms of pro-inflammatory cytokine production, mCRP increases interleukin-8 and monocyte chemoattractant protein-1 production, whereas nCRP has no detectable effect on their levels. Further studies are needed to expand on these emerging findings and to fully characterize the differential roles that each CRP isoform plays at sites of local inflammation and infection.

Keywords: C-reactive protein, native C-reactive protein, monomeric C-reactive protein, inflammation, infection

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C-REACTIVE PROTEIN (CRP)

C-reactive protein is a homopentameric acute-phase inflammatory protein, a highly conserved plasma protein that was initially discovered in 1930 by Tillet and Francis while investigating the sera of patients suffering from the acute stage of *Pneumococcus* infection and was named for its

reaction with the capsular (C)-polysaccharide of *Pneumococcus* (1). In the presence of calcium, CRP binds to polysaccharides such as phosphocholine (PCh) on microorganisms and triggers the classical complement pathway of innate immunity by activating C1q (2). CRP has many homologs in vertebrates and some invertebrates (3) and is a member of the pentraxin family, which includes other structurally related molecules such as serum amyloid A (4). Transcriptional induction of the *CRP* gene mainly occurs in hepatocytes in the liver in response to increased levels of inflammatory cytokines, especially interleukin-6 (IL-6) (5).

C-reactive protein exhibits elevated expression during inflammatory conditions such as rheumatoid arthritis, some cardiovascular diseases, and infection (6). As an acute-phase protein, the plasma concentration of CRP deviates by at least 25% during inflammatory disorders (7). The highest concentrations of CRP are found in serum, with some bacterial infections increasing levels up to 1,000-fold (8). However, when the stimuli ends, CRP values decrease exponentially over 18–20 h, close to the half-life of CRP (9). CRP plasma levels increase from around 1 μg/mL to over 500 μg/mL within 24–72 h of severe tissue damage such as trauma and progressive cancer (10). IL-6 is reported to be the main inducer of *CRP* gene expression, with IL-1 enhancing the effect (11). However, although IL-6 is necessary for *CRP* gene induction, it is not sufficient to achieve this alone (12).

There are many factors that can alter baseline CRP levels including age, gender, smoking status, weight, lipid levels, and blood pressure (13). The average levels of CRP in serum in a healthy Caucasian is around 0.8 mg/L, but this baseline can vary greatly in individuals due to other factors, including polymorphisms in the *CRP* gene (14). The human *CRP* gene can be found at 1q23.2 on the long arm of chromosome 1, and to date, there have been no allelic variations or genetic deficiencies discovered for this gene although some polymorphisms have been identified (13). For example, up to 50% of baseline variance in CRP is associated with the number of dinucleotide repeats found in an intronic region of the gene (15).

There is no significant seasonal variation in baseline CRP concentration; however, twin studies show a significant heritable component in baseline CRP values that is independent of age and body mass index (16). Pankow et al. (17) found evidence that interindividual variation in blood CRP levels is 35–40% heritable. Increased CRP levels are typically associated with disease, but liver failure is one condition observed to impair CRP production. Very few drugs reduce elevated CRP levels unless they treat the underlying pathology that is causing the acute-phase stimulus (16).

There is emerging research that oral hormone replacement therapy (HRT) causes background levels of circulating CRP to increase in postmenopausal women, increasing the risk of thrombotic events such as clots (18). Corcoran et al. (19) found that a combination of estrogen and oxidized low-density lipoproteins (oxLDLs) increased CRP expression in a model of coronary heart disease in both older men and postmenopausal women, but no effect on CRP expression was seen when estrogen supplementation was replaced with testosterone. Ridker et al. (20) found that healthy postmenopausal women had nearly twofold increased levels of circulating CRP when they were taking oral HRT and that CRP was the most affected inflammatory marker. Numerous studies have confirmed that CRP is a predictive marker for cardiovascular disease and that HRT use in postmenopausal women increases the risk of stroke and blood clots (20–23).

Interestingly the mode of HRT delivery appears to influence the effect on circulating CRP levels. Vongpatanasin et al. (23) found that estrogen administered orally increases circulating CRP levels twofold, whereas estrogen administered transdermally had no effect on circulating CRP levels. Similarly, patients taking oral HRT containing estrogens combined with progestogens had an increase in circulating CRP levels in the first 12 months of therapy compared to those using transdermal therapy who demonstrated no change in circulating CRP levels (22). In contrast, several other studies have instead shown that circulating CRP levels are reduced in humans treated with transdermal estrogen (24, 25). A reduction in CRP levels following peripheral estrogen administration supports the findings of Ashcroft et al. (26) demonstrating that estrogen reduces the inflammatory response during wound healing. The effect of transdermal administration of estrogen on local CRP levels in peripheral tissues such as skin has not yet been elucidated, with previous studies measuring only circulating levels of CRP.

ISOFORMS OF CRP

The pentameric protein, termed native CRP (nCRP), is characterized by a discoid configuration of five identical non-covalently bound subunits, each 206 amino acids long with a molecular mass of about 23 kDa. These five subunits lie in the same orientation around a central pore and arranged in a characteristic "lectin fold" with a two-layered beta sheet (15). Each subunit lies with the PCh binding site facing the "recognition" face of the nCRP molecule (27). The molecule has a ligand-binding face that has a characteristic feature of having two calcium ions per protomer. The calcium ions are important for the stability and binding of ligands. The "opposite" face interacts with the C1q aspect of the complement pathway as well as interacting with Fc receptors (6).

The pentameric protein is synthesized primarily in liver hepatocytes but has also been reported to be synthesized in other cell types such as smooth muscle cells (28), macrophages (29), endothelial cells (30), lymphocytes, and adipocytes (31). CRP is first synthesized as monomers and then assembled into the pentamer in the endoplasmic reticulum of the source cell. In hepatocytes, the pentameric protein is retained in the endoplasmic reticulum by binding to two carboxylesterases, gp60a and gp50b (32). While in a resting (non-inflammatory) state, CRP is released slowly from the endoplasmic reticulum, but following an increase in inflammatory cytokine levels, the binding CRP to the carboxylesterases decreases and CRP is secreted rapidly (6). The stimulation of CRP synthesis mainly occurs in response to pro-inflammatory cytokines, most notably IL-6 and to a lesser degree IL-1 and tumor necrosis alpha (TNF-α) (33).

Pentameric CRP can be irreversibly dissociated, with the resultant free subunits termed monomeric (or modified) CRP (mCRP). The dissociation of nCRP into free subunits has been observed at either high concentrations of urea (34) or high temperatures in the absence of calcium (35). The mCRP molecules are distinguished from nCRP by their different antigenic, biological,

and electrophoretic activities (36) and by the fact that they express different neoepitopes (37). The two isoforms of CRP have been shown to have distinct biological functions in the inflammatory process. For example, Khreiss et al. (37) provided evidence that nCRP suppresses the adherence of platelets to neutrophils, whereas mCRP enhances these interactions. This difference in function can be explained by the two isoforms binding to differing types of Fcgamma (Fcγ)-receptor involved in the signaling process. The mCRP isoform utilizes the low-affinity immune complex binding immunoglobulin G (IgG) receptor called FcγRIIIb (CD16b) on neutrophils and FcγRIIIa (CD16a) on monocytes, while nCRP binds to the low-affinity IgG receptor FcγRIIa (CD32) (38).

Evidence is emerging of new structural intermediates of CRP with biological function. Ji et al. (39) found that the native protein first dissociates into subunits while retaining some of the native conformation before fully dissociating into mCRP. This intermediate, termed $mCRP_m$, is formed when the nCRP is bound to cell membranes and then dissociates, allowing the subunits to retain some of the conformation before fully dissociating into mCRP subunits on detachment from the membrane. It is suggested that this transitional process allows for more effective regulation of CRP function, with $mCRP_m$ allowing for the enhanced activation of the classical complement pathway (39). Further work needs to be conducted to determine the biological functions of the $mCRP_m$ intermediate, but initial findings suggest that it behaves in a similar manner to mCRP, typically promoting pro-inflammatory activity.

CRP IN DISEASE PATHOLOGY

The majority of CRP research has focused on the role of CRP and its isoforms on cardiovascular disease and stroke. CRP is used as a clinical marker of inflammation, with elevated serum levels being a strong independent predictor of cardiovascular disease in asymptomatic individuals (40). CRP levels have been linked to prognosis in patients with atherosclerotic disease, congestive heart failure, atrial fibrillation, myocarditis, aortic valve disease, and heart transplantation, suggesting that it has an active role in the pathophysiology of cardiovascular disease (41). High-sensitivity assays, such as nephelometric assays, are used to detect baseline levels of CRP and patients who are at risk of cardiovascular disease. An individual with a CRP level higher than 3 mg/L has an increased risk of coronary heart disease (42), and this risk increases in those with type 2 diabetes (43).

Increased levels of CRP have been found in patients with appendicitis, cholecystitis, pancreatitis, and meningitis (44). In patients suffering possible symptoms of appendicitis, acute appendicitis can be excluded in those with CRP levels lower than 25 mg/L in blood taken 12 h after the onset of symptoms (45). When clinical symptoms of cholecystitis occur concurrently with CRP levels of over 30 mg/L, an accurate diagnosis of cholecystitis can be obtained with 78% sensitivity, suggesting that CRP is a more sensitive marker than erythrocyte sedimentation rate and white cell count in supporting cholecystitis diagnosis (46). In terms of acute pancreatitis, CRP levels of more than 210 mg/L were able to discriminate between mild and severe cases, with 83% sensitivity and 85% specificity (47). Serum CRP is elevated in bacterial meningitis, and resolution of symptoms following treatment with antibiotics is slow in those with the highest CRP levels (48). Measurement of CRP in cerebrospinal fluid has a sensitivity of 100% and a specificity of 94% for differentiating between patients with bacterial meningitis, viral meningitis, and no infection (49).

Although studies have shown that CRP levels increase during infections and inflammatory diseases, the precise role of CRP isoforms in their development and progression remains largely unknown. Thus, urgent investigations are required to determine the effects of each CRP isoform on specific cellular processes during disease development. Evidence shows that in general nCRP tends to exhibit more anti-inflammatory activities relative to the mCRP isoform, possibly because nCRP limits the generation of the membrane attack complex (MAC) and C5a, thus inhibiting the alternative complement activation (50). In contrast, mCRP can have marked pro-inflammatory properties both *in vitro* and *in vivo* by promoting monocyte chemotaxis and the recruitment of circulating leukocytes to areas of inflammation *via* Fcy-RI and Fcy-RIIa signaling (50). Thus, in addition to therapeutic strategies to inhibit CRP activity (51), more targeted therapies have been proposed for the treatment of CRP-mediated pathologies, including inhibiting mCRP activity (52) or preventing the dissociation of nCRP into mCRP (53).

CRP AND INFLAMMATION

C-reactive protein levels are known to increase dramatically in response to injury, infection, and inflammation (**Figure 1**). CRP is mainly classed as an acute marker of inflammation, but research is starting to indicate important roles that CRP plays in inflammation. CRP is the principal downstream mediator of the acute-phase response following an inflammatory event and is primarily synthesized by IL-6-dependent hepatic biosynthesis (54, 55). The main role of CRP in inflammation tends to focus around the activation of the C1q molecule in the complement pathway leading to the opsonization of pathogens. Although CRP can initiate the fluid phase pathways of the host defense by activating the complement pathway, it can also initiate cell-mediated pathways by activating complement as well as to binding to Fc receptors of IgG (54). CRP binds to Fc receptors with the resulting interaction leading to the release of pro-inflammatory cytokines (56). CRP also has the ability to recognize self and foreign molecules based on the pattern recognition, something that other activators of complement such as IgG cannot achieve because these molecules only recognize distinct antigenic epitopes (56).

Evidence suggests that CRP is not only just a marker of inflammation but also plays an active role in the inflammatory process. However, most early research in the literature only refers to CRP and does not distinguish between the two isoforms. Thus, unlike more recent publications, the findings of early work on CRP can seem somewhat unclear and at times conflicting since it was often not specified which CRP isoform was measured or utilized in experiments, whether responses attributed to nCRP were in fact possibly due to partial/full dissociation into mCRP or if lipopolysaccharide (LPS) contamination could be present. More recent studies generally distinguish between the differential

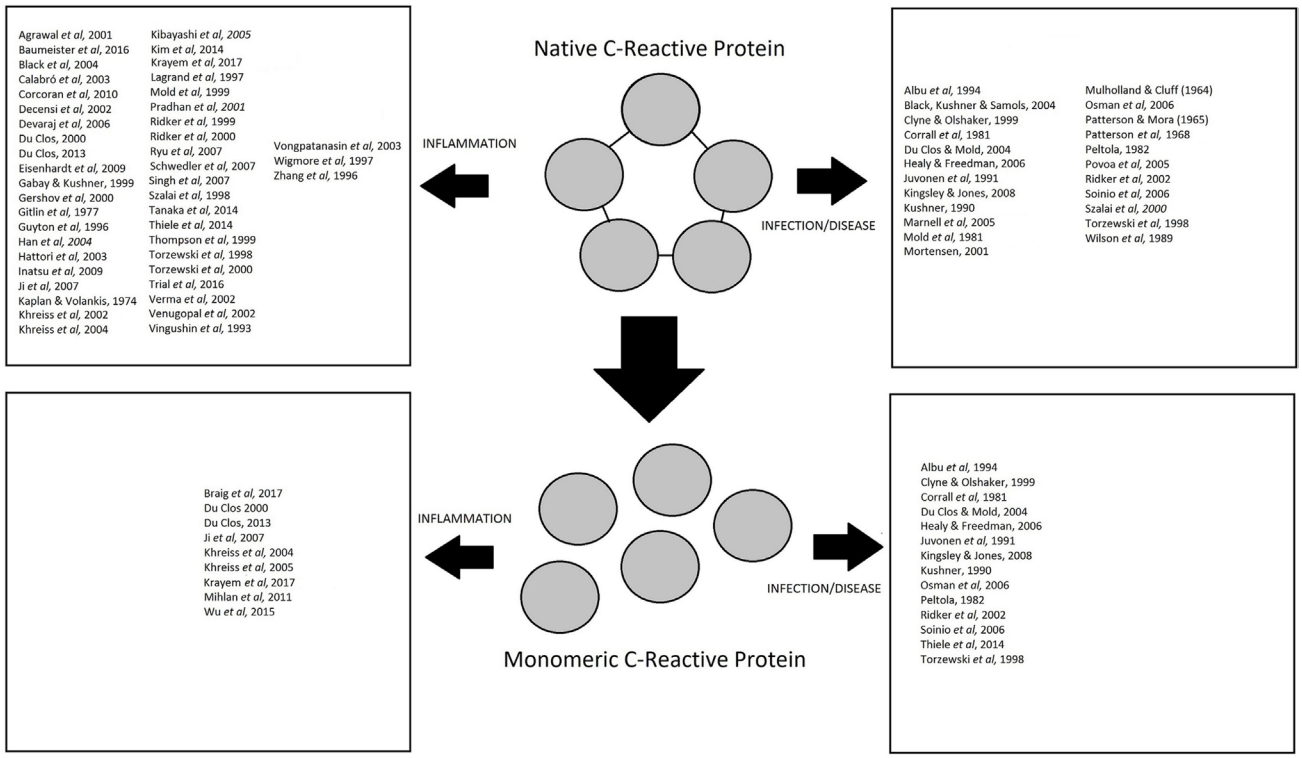

FIGURE 1 | Summary of studies investigating the role of native C-reactive protein (CRP) and monomeric CRP in inflammation, infection, and disease.

effects of each CRP isoform on inflammatory processes, but since antibodies for mCRP are not commercially available to date, few laboratories are able to conduct studies investigating the mCRP isoform.

There is increasing evidence that CRP has a functional role in the inflammatory process. It is well established that CRP is an acute marker of inflammation and that its concentration increases in circulation during inflammatory events. CRP is deposited at sites of inflammation and tissue damage in both naturally occurring and experimental conditions (57). However, there is a raft of published data investigating CRP that does not consider its two different isoforms. Understandably, when some of these studies were conducted, the existence of two CRP isoforms was not well established and available antibodies would have been raised against the pentameric nCRP alone. Another issue with published data is that CRP localization is often investigated in only a narrow range of inflammatory conditions and tissue types. Although the mCRP isoform has been shown to be insoluble in plasma, it becomes localized in inflamed tissues and amplifies a pro-inflammatory response by a positive feedback loop (58).

The literature suggests that CRP binds to damaged cell membranes and contributes to the inflammatory response (59), with CRP molecules becoming associated with terminal complement complexes, especially in atherosclerotic lesions (60). Lagrand et al. (61) provided evidence that CRP localizes to infarcted heart tissue and promotes local complement activation, triggering further damage to the heart tissue. Gitlin et al. (62) concluded that CRP was localized to the nuclei of cells within the synovium of rheumatoid arthritis patients, but the cell type was not identified at the time. However, other studies indicate no significant localization of CRP in a number of pathologies, suggesting that CRP is found predominantly in the fluid phase rather than becoming deposited in tissues at sites of inflammation or injury (63). There has been little research conducted on the localization of CRP in inflammatory cells to date. There is a correlation between the localization of CRP in neutrophil infiltrates, especially in lesions of vasculitis and allergic encephalomyelitis (64, 65).

CRP AND INFECTION

C-reactive protein is a marker for inflammation, and its levels increase during bacterial infection (66). Kingsley and Jones (67) stated that CRP increases during infection in response to monocytic mediators such as IL-1 and IL-6 and that it has a stable decay rate. It is thought that most of the interaction between CRP and the immune response to pathogens involves the binding of CRP to PCh and the activation of the classical complement pathway (68). Mold et al. (69) showed that CRP provides mice with protection against infection by the gram-positive pathogen *Streptococcus pneumoniae* by binding to a PCh determinant of the pathogen cell wall and activating the complement pathway. Mice pretreated with 200 μg CRP before being infected showed an increase in percentage survival across all pathogen doses tested. The study concluded that the ability of CRP to protect against infection lies

in its ability to bind to pneumococcal polysaccharide C in the bacterial cell wall (69).

Szalai et al. (70) showed that CRP can confer protective benefits against *Salmonella enterica* serovar Typhimurium, a gram-negative pathogen that provides a model of typhoid fever in mice. By using transgenic mice expressing human CRP, the study found that CRP offered protection against a low dose of Typhimurium and increased resistance to a fatal infection with a low dose of Typhimurium. Szalai et al. (70) concluded that CRP increases the early clearance of intravenously injected bacteria from the blood and reduces dissemination of bacteria to the liver and spleen during the initial stages of infection, thus allowing the mice to survive infection.

Marnell et al. (71) reviewed the protective role CRP against *Haemophilus influenza* infection in both transgenic and wild-type mice treated by passive inoculation. CRP was shown to bind the pneumococcal C-polysaccharide of bacteria and opsonize them for phagocytosis. This process did not require the use of the Fcγ receptors, suggesting that CRP is not primarily protective by direct opsonization but more likely through activation of complement and subsequent opsonophagocytosis.

Kingsley and Jones (67) tested whether CRP could be used to distinguish different types of infections. They discovered that mean CRP levels in a spreading infection were higher than those in other colonized, critically colonized, and locally infected groups. All cases of infection showed an increase in CRP levels compared to non-infected controls, but CRP levels could not distinguish between the infection types, showing that it is infection in general that causes CRP levels to increase, rather than the type of infection. This was also noted by Healy and Freedman (66) who showed that CRP levels can be used only as a method of detecting infection, rather than distinguishing it.

C-reactive protein can mediate host responses to *Staphylococcus aureus* including some protective function against infection and an increase in phagocytosis of this pathogen. Povoa et al. (72) stated that the normal CRP level for the healthy population is about 0.08 mg/dL, and this increases to more than 8.7 mg/dL during chronic *S. aureus* infection. Thus, CRP can be used as an indicator of infection, alongside a body temperature of more than 38.2°C. Patterson and Mora (73) observed that enhanced resistance to intraarticular infection with *S. aureus* in chickens was associated with an increase in serum CRP and that isolated preparations of the protein produced antibacterial activity. Mulholland and Cluff (74) discovered that endotoxin-induced changes in resistance to local infection with *S. aureus* in rabbits were correlated with the circulating levels of leukocytes in the blood. The study showed that induced resistance was paralleled by an increase in CRP and leukocytes. This was collaborated by Patterson et al. (75) who found an association between CRP and non-specific resistance to infection, including *S. aureus* and showed that CRP was acting upon the polysaccharide bacterial cell wall. Black et al. (3) stated that CRP enhances the *in vitro* phagocytosis of many microorganisms (including *S. aureus*) by leukocytes. Their work confirmed this finding even in the absence of complement, suggesting that the enhancement of phagocytosis by CRP is due to the interactions with Fcγ receptors.

In summary, evidence shows that CRP is not only a marker of infection and inflammation but that CRP also has a protective role against bacterial infections (**Figure 1**), principally through the activation of complement and subsequent opsonization of pathogens.

CRP AND COMPLEMENT

Complement is one of the major defenses of the human immune system that is involved in the clearance of foreign particles and organisms after recognition by antibody. The complement pathway is made up of 35 plasma or membrane proteins that is an important system in immunity and the defense of the host against microbial infection. The components of the complement pathway can be activated in three different pathways to trigger a cascade of proteins, which are used to help bind microbial surfaces for the immune system to recognize and activate phagocytosis (76, 77). The classical pathway is triggered by a target bound antibody, whereas the lectin pathway is triggered by microbial repetitive polysaccharide structures and the alternative pathway is triggered by recognition of other foreign surface structures. Even though the triggers are different, the three pathways merge at a pivotal activation of the C3 and C5 convertases. A majority of the components are synthesized in the liver, C1 in the intestinal epithelium, and factor D in the adipose tissue (76).

The role of CRP in activating the complement pathway has been extensively investigated. In 1974, Kaplan and Volanakis first described the ability of CRP to activate the classical complement pathway using C-polysaccharide and phospholipid ligands (59). The activation of complement by CRP is considered a crucial step since when complement was depleted, and the effects of CRP were abrogated (50).

The opposite face of the CRP molecule, which is typically complexed with polyvalent ligand or chemically cross-linked, binds to C1q and activates the classical complement pathway (56). C1q is a large 460-kDa molecule made up of six identical subunits, each made up of three structurally similar but distinct polypeptide chains (78). This process requires the use of calcium ions for the stable formation of the C1 complex (79). CRP is most effective during the early classical pathway activation of C1, C4, and C2 (80). This is because the ligand-bound interaction with C1q leads to the formation of C3 convertase, triggering the complement activation of C1–C4 but with little activation of the late complement proteins C5–C9 (15).

Activation of complement by CRP varies from activation by antibody in that CRP has selective activation of early components without the need to form the MAC. In addition to activating the classical complement pathway, CRP can inhibit the alternative complement pathway by decreasing C3 and C5 convertase activities and inhibiting the complement amplification loop. This is achieved by the recruitment of factor H to the cell surface and by preventing C5 convertase cleaving C5 to recruit neutrophils and prevent the formation of the MAC (71). As the levels of CRP increase, this causes decreased binding of C3b and C5b-9 to liposomes, possibly also explaining the lack of C5–C9 consumption by CRP during classical pathway activation (80).

Both the initiator (C1q) and the inhibitor (C4bp) of the classic complement pathway compete for mCRP binding, with the competition controlling the local balance of activation and

inhibition of the pathway in tissues (58). Interestingly, mCRP but not nCRP binds the C4bp inhibitor, suggesting that mCRP rather than nCRP is able to provide a high degree of control over the classic complement pathway (58).

CRP AND APOPTOSIS

There has been little research conducted into the effect of CRP on the proliferation process. However, there is evidence that CRP has a major role in the apoptosis process. Devaraj et al. (81) showed that CRP stimulates the production of pro-apoptotic cytokines and inflammatory mediators *via* the activation of Fc-γ receptors. The pro-apoptotic cytokines and inflammatory mediators induced by CRP include interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα), and reactive oxygen species (82, 83).

C-reactive protein induces the upregulation of p53 in monocytes and affects cell cycle kinetics of monocytes through CD32 (FcγRII), inducing apoptosis by G_2/M arrest in the cell cycle (84). CD32 receptors have been shown to trigger apoptotic signals and are expressed in a subset of monocytes that polarize to pro-inflammatory macrophages, suggesting that CRP may dampen macrophage-driven pro-inflammatory responses by inducing apoptosis (85).

C-reactive protein is elevated in cardiovascular disorders and is a mediator of atherosclerosis. CRP localizes directly in the atherosclerotic plaques where it induces the expression of genes that are directly involved in the adhesion of monocytes and the recruitment of intracellular molecules such as E-selectin and monocyte chemoattractant protein-1 (MCP-1). CRP has also been shown to play a role in mediating low-density lipoprotein uptake in macrophages and activating the complement system, which is implicated in atherogenesis (86). Apoptosis occurs in atherosclerotic plaques and the number of apoptotic cells increase as lesions become more advanced. As cells become apoptotic, they start to cause plaque disruption, leading to the expression of growth arrest- and DNA damage-inducible gene 153 (*GADD153*). *GADD153* upregulation has been shown to induce G_1 arrest or apoptosis in some cancer cell lines (87). Blaschke et al. (88) found that CRP can induce the apoptosis of human coronary vascular smooth muscle cells through a caspase-mediated mechanism, especially through increased caspase-3 activity. CRP was co-localized to the *GADD153* gene product in atherosclerotic lesions suggesting that CRP is triggering the caspase cascade and apoptosis by inducing the expression of the *GADD153* gene.

There is little research on how the two isoforms of CRP interact with the apoptosis process. It is suggested that CRP can exert anti-apoptotic activity but only when the cyclic pentameric structure is lost. This would suggest that the apoptotic activity of CRP is induced through the native isoform. Native CRP (nCRP) can bind to low-affinity IgG FcγRIIa (CD32) and IgG FcγRI (CD64), leading to depressed functional activities, degranulation, and the generation of superoxide by inducible respiratory burst. On the other hand, mCRP binds to low-affinity IgG FcγRIIIb (CD16) that can delay apoptosis by triggering the cell survival pathway in neutrophils, even at low concentrations (89).

The nCRP isoform has the ability to opsonize apoptotic cells and induce the phagocytosis of damaged cells. Removal of nCRP-bound apoptotic monocytes and macrophages may be *via* FcγR-mediated phagocytosis (84). CRP binds to apoptotic cells, inhibits the assembly of terminal complement components, and promotes the opsonization of apoptotic cells (89, 90).

CRP AND NITRIC OXIDE (NO)

C-reactive protein has the ability to attenuate NO production with a marked reduction in *in vitro* angiogenesis, cell migration, and capillary-like tube formation by CRP at concentrations known to cause cardiovascular risk (91). Eisenhardt et al. (15) showed that CRP upregulated the expression of adhesion molecules and inhibited endothelial nitric oxide synthase (eNOS) expression, indicating a role for CRP in the production of NO. Several studies have revealed that CRP inhibits NO production *via* downregulation of eNOS in cardiovascular endothelial cells, thereby inhibiting angiogenesis *in vitro* and promoting the pathogenesis of atherosclerotic vascular disease through vasoconstriction, leukocyte adherence, and inflammation (14, 91–93). Another study found that it was in fact the nCRP isoform that downregulated eNOS and thus impaired endothelial function in ApoE knockout mice, *via* a mechanism thought to involve iNOS (94). Eisenhardt et al. (15) provided evidence that nCRP suppresses endothelium-dependent NO-mediated dilation by activating the p38 mitogen-activated protein kinase (MAP kinase) pathway and NADPH oxidase, suggesting that multiple pathways could be interacting with this process.

In contrast, mCRP has the opposite effect, enhancing NO production in neutrophils *via* upregulation of eNOS (95) with reverse transcription polymerase chain reaction showing an amplification of eNOS mRNA, but not iNOS or nNOS mRNA. This study highlighted that mCRP initiates calcium (Ca^{2+}) mobilization and activation of calmodulin and PI3 kinase to induce NO formation in neutrophils (95). The effect of CRP isoforms on other inflammatory cells, such as monocytes or macrophages, has not been investigated to date.

CRP ISOFORMS AND INFLAMMATORY CYTOKINES

There has been increasing evidence of a relationship between CRP and several pro-inflammatory cytokines.

IL-6 and CRP

Interleukin-6 is a pro-inflammatory cytokine secreted by various cells including inflammatory cells, keratinocytes, fibroblasts, and endothelial cells. It regulates the acute-phase response, and its main role involves the host response to infection (96). Even though it is predominantly a pro-inflammatory cytokine, in some cells, IL-6 can have regenerative and anti-inflammatory effects through the activation of membrane-bound IL-6 receptor signaling (97).

Interleukin-6 is synthesized in the initial stages of inflammation and induces a number of acute-phase proteins, including CRP (98). IL-6 can also reduce the production of fibronectin, albumin, and transferrin as well as the promotion of $CD4^+$ T

helper cells, which initiates the linking of innate and acquired immunity (98). There is a correlation between increasing levels of IL-6 during inflammation and increasing levels of CRP (11), with IL-6 inducing the *CRP* gene (12). However, most investigations of CRP production by IL-6 generally fail to indicate which isoforms of CRP are generated. In some cases, the antibodies used suggest that nCRP is present, but given IL-6 occurs at the sites of inflammation, the pentameric CRP may be dissociating into mCRP.

When CRP levels become elevated in atheroma, this leads to the induction of IL-6 by macrophages indicating that CRP may have a direct effect on IL-6 release (99). Krayem et al. (100) found that a combination of mCRP, nCRP, and oxLDL decreases IL-6 production in a model of atherosclerosis. This triple combination suggests that nCRP might downregulate the IL-6 release by macrophages that have been stimulated by both mCRP and oxLDL.

Interleukin-8 (IL-8) and CRP

Interleukin-8 is a cytokine produced by numerous cell types including inflammatory cells, keratinocytes, fibroblasts, and endothelial cells. IL-8 acts as a potent chemoattractant of neutrophils (101) and is overexpressed in chronic inflammatory diseases and during septic shock (102). IL-8 stimulates the release of granules from neutrophils by a process called degranulation. These granules contain a range of antimicrobial effectors that can help combat infection (103). Neutrophils are the first inflammatory cells to arrive at the site of inflammation, and they carry out the phagocytosis of bacteria and release chemotactic mediators that recruit other leukocytes to the affected tissue (103).

Kibayashi et al. (104) indicated that CRP plays a role in atherosclerosis *via* enhanced IL-8 production and increased expression of IL-8 mRNA in a CRP dose-dependent manner. They showed that CRP promotes IL-8 production *via* the activation of the ERK, p38 MAPK, and JNK pathways. Conversely, Wigmore et al. (105) indicated that IL-8 induces CRP production in hepatocytes, providing a potential feedback loop. The effect of the different CRP isoforms on IL-8 production has been investigated. Khreiss et al. (37) showed that nCRP had no detectable effect on the production of IL-8, whereas mCRP increased IL-8 production and IL-8 gene expression, promoting pro-inflammatory activity through a p38 MAPK-dependent mechanism. When treated with anti-CD16, there was inhibition of mCRP-stimulated NO formation and IL-8 release.

MCP-1 and CRP

Monocyte chemoattractant protein-1 is a cytokine that plays a role in the regulation of migration and infiltration of monocytes and macrophages (106). It is released by a number of cell types in response to events such as oxidative stress, cytokine release, and growth factor release (107). Human MCP-1 is known to bind to at least two receptors, and its production can be induced by interleukin-4 (IL-4), IL-1, TNF-α, bacterial LPS, and IFN-γ (107). There is increasing evidence that MCP-1 influences T-cell immunity by enhancing the secretion of IL-4 by T cells, as well as having a role in the migration of leukocytes (106). This in turn has a regulatory function on monocytes and macrophages, which are the major source of MCP-1 (107). MCP-1 is known to recruit monocytes to the vessel wall (99) and cause the arrest of rolling monocytes on endothelial monolayers that express E-selectin (108).

Evidence suggests that CRP stimulates endothelial cells to express MCP-1 (99) in addition to being a direct chemoattractant of monocytes itself (109). CRP can promote monocyte chemotactic activity in response to MCP-1 *via* upregulation of the monocyte chemotaxis receptor CCR2, with elevated CRP levels promoting the accumulation of monocytes in the atherogenic arterial wall (99). When vascular smooth muscle cells are exposed to increasing levels of CRP, MCP-1 mRNA substantially increased within 2 h and remained elevated for at least 24 h (110). Incubation with mCRP increases the secretion of MCP-1, leading to pro-inflammatory activity through a p38 MAPK-dependent mechanism, whereas nCRP had no detectable effect (37).

TNF-α and CRP

Tumor necrosis factor-α is a component of the acute-phase response and is mainly produced by monocytes and macrophages but can be produced by numerous other immune cells such as neutrophils, natural killer cells, and eosinophils. TNF-α is not usually detectable in a healthy host, but levels become elevated in a number of inflammatory and infectious conditions (111). The main stimulant of TNF-α production is LPS, but many other pathological conditions such as trauma infection, impaired wound healing, and heart failure also induce its production (111, 112). TNF-α mediates various processes such as cell proliferation, differentiation, and apoptosis.

Studies have shown a correlation between TNF-α production and the concentration of CRP. TNF-α induces a dose-dependent secretion of CRP in hepatocytes, which corresponds to an increase in CRP mRNA (28). Conversely, elevated CRP levels in atheroma leads to the induction of IL-1β, IL-6, and TNF-α production by macrophages (99). Research shows a close relationship between TNF-α and IL-6 levels in inflammation (113), with both TNF-α and IL-6 inducing the transcription of CRP (33). However, there is some contradictory evidence showing a potential inhibitory effect of CRP on TNF-α production, suggesting that there could be a negative feedback mechanism whereby elevated levels of CRP inhibit further stimulation of CRP by reducing the TNF-α production (114). A combination of mCRP, nCRP, and oxLDL also causes a decrease in both TNF-α and IL-6 production in a macrophage model of atherosclerosis (100). This triple combination suggests that nCRP might downregulate TNF-α and IL-6 production by macrophages stimulated by both mCRP and oxLDL.

CONCLUSION

C-reactive protein is a homopentameric acute-phase inflammatory protein that exhibits elevated expression during inflammatory conditions such as rheumatoid arthritis, some cardiovascular diseases, and infection. Evidence suggests that CRP is an important regulator of inflammatory processes and not just a marker of inflammation or infection. Key areas of inflammation and host responses to infection mediated by CRP include the complement pathway, apoptosis, phagocytosis, NO release, and cytokine

production. However, most research to date has investigated the role of CRP in the vascular tissues, highlighting the need to conduct further work to determine the precise role of CRP in peripheral tissues.

C-reactive protein is synthesized primarily in liver hepatocytes but also other cell types such as smooth muscle cells, macrophages, endothelial cells, lymphocytes, and adipocytes. Evidence also suggests that the sex steroid hormone estrogen can influence CRP levels, with HRT having a profound influence on CRP levels in the elderly. Administration of oral HRT increases background levels of CRP in circulation, whereas evidence suggests that transdermal estrogen supplementation either reduces or has little effect on circulating CRP levels. A reduction in CRP levels following local administration of estrogen supports findings showing that estrogen reduces the inflammatory response in peripheral tissues such as skin.

There are two distinct isoforms of CRP, nCRP and mCRP, and the nCRP isoform can irreversibly dissociate at sites of inflammation, tissue damage, and infection into five mCRP subunits. Evidence indicates that nCRP often tends to exhibit more anti-inflammatory activities compared to mCRP. The nCRP isoform activates the classical complement pathway, induces phagocytosis, and promotes apoptosis. On the other hand, mCRP promotes the chemotaxis and recruitment of circulating leukocytes to areas of inflammation and can delay apoptosis. The nCRP and mCRP isoforms inhibit and induce NO production *via* downregulation and upregulation of eNOS, respectively. In terms of pro-inflammatory cytokine production, mCRP increases IL-8 and MCP-1 production, whereas nCRP has no detectable effect on their levels. CRP can also induce IL-6 and TNF-α production at sites of inflammation, again suggesting probable involvement of mCRP from the dissociation of nCRP. Further studies are needed to expand on these emerging findings and to fully characterize the differential roles that each CRP isoform play at sites of local inflammation and infection.

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The Clinical Significance and Potential Role of C-Reactive Protein in Chronic Inflammatory and Neurodegenerative Diseases

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C-reactive protein (CRP) is an acute-phase protein synthesized by hepatocytes in response to pro-inflammatory cytokines during inflammatory/infectious processes. CRP exists in conformationally distinct forms such as the native pentameric CRP and monomeric CRP (mCRP) and may bind to distinct receptors and lipid rafts and exhibit different functional properties. It is known as a biomarker of acute inflammation, but many large-scale prospective studies demonstrate that CRP is also known to be associated with chronic inflammation. This review is focused on discussing the clinical significance of CRP in chronic inflammatory and neurodegenerative diseases, such as cardiovascular disease, type 2 diabetes mellitus, age-related macular degeneration, hemorrhagic stroke, Alzheimer's disease, and Parkinson's disease, including recent advances on the implication of CRP and its forms specifically on the pathogenesis of these diseases. Overall, we highlight the advances in these areas that may be translated into promising measures for the diagnosis and treatment of inflammatory diseases.

Keywords: C-reactive protein, chronic inflammation, biomarker, cardiovascular disease, diabetes

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INTRODUCTION

C-reactive protein (CRP) belongs to the family of pentraxins and exists in at least two conformationally distinct forms—such as the native pentameric CRP (pCRP) and monomeric CRP (mCRP). Studies suggest that pCRP possesses both pro-inflammatory and anti-inflammatory properties in a context-dependent manner (1). By contrast, mCRP exerts potent pro-inflammatory actions on endothelial cells, endothelial progenitor cells, leukocytes, and platelets and may amplify the inflammatory response (1). The dissociation of pCRP into pro-inflammatory mCRP might directly link CRP to inflammation. CRP is considered as a serum biomarker in patients undergoing acute inflammatory response (2–4). The elevation in baseline CRP level was shown to be useful to gauge chronic inflammation and tissue damage resulting from excessive inflammation or failure of the initial inflammatory response (5). Higher CRP concentrations may be indicative of an acute infection or inflammation and therefore are often excluded in studies of chronic inflammation. Higher CRP concentration over time, rather than spikes in CRP, may result in cardiovascular diseases (CVDs) and problems leading to atherosclerosis (6). Furthermore, some chronic inflammatory diseases such as hemorrhagic stroke, Alzheimer's disease (AD), and Parkinson's disease (PD) are also associated with CRP formation (7–10) (**Table 1**). In this review, we emphasize recent advances that may explain how conformational changes in CRP are linked to chronic inflammation and neurodegeneration.

TABLE 1 | Chronic inflammatory diseases associated with CRP levels.

Disease category	Pathology/disease type	CRP (mCRP/nCRP) levels	Role and clinical significance of CRP
CVD	Atherosclerosis, chronic heart failure	Elevated mCRP levels (11)	Inflammatory biomarker, risk predictor, participant
T2DM	Insulin resistance	Elevated CRP levels (12)	Inflammatory biomarker, risk predictor, mediator
AMD	Progressive visual impairment, senile macular degeneration, blinding disease	Elevated CRP levels (13)	Inflammatory biomarker, risk predictor
Hemorrhagic Stroke	Intracerebral hemorrhage, subarachnoid hemorrhage, brain injury	Elevated CRP levels (14)	Inflammatory biomarker, risk predictor
AD	Neurodegenerative disorder, dementia	Reduced/elevated CRP levels (15)	Inflammatory biomarker, no causal role
PD	Neurodegenerative disorder, motor symptoms	Elevated CRP levels (16)	Inflammatory biomarker, risk predictor

CRP plays an important role in the progression of various chronic inflammatory diseases. This table lists diseases associated with nCRP and monomeric CRP (mCRP) levels. Although any potential mechanism underlying the effect of CRP on these processes is incompletely elucidated, its clinical significance appears to be positive.
CVD, cardiovascular disease; T2DM, type 2 diabetes mellitus; AD, Alzheimer's disease; PD, Parkinson's disease; AMD, age-related macular degeneration; CRP, C-reactive protein; nCRP, native CRP.

CRP AND CVDs

Elevated levels of numerous inflammatory biomarkers have been implicated to predict adverse cardiovascular events. Several studies indicate the predictive CRP values in patients with acute coronary syndrome (ACS). The routine biomarkers of CVD include troponin I (cTnI) and creatine kinase isoenzyme (CK-MB) that are mainly synthesized in cardiac muscle cells. Both cTnI and CK-MB may be detected in the blood during severe ischemia, degeneration, and necrosis of cardiomyocytes; but lack the sensitivity to detect minute damage to cardiomyocytes (17, 18). CRP is the biomarker that most strongly correlates with future cardiovascular events and may be slightly elevated during the early stage of myocardial vascular inflammation (7, 19). Unlike other markers of inflammation, CRP levels are stable over long periods and display no diurnal variation. In addition, these may be inexpensively measured with available high-sensitivity assays and have shown specificity in terms of the prediction of CVD risk (20). At present, high-sensitivity CRP (hs-CRP) is gaining popularity under clinical settings, as it allows the detection of lower levels of CRP. A moderate increase in hs-CRP is thought to predict an increased risk of coronary events in unstable angina pectoris patients and its detection is a reliable predictor of the risk of CVDs (21–23). During the course of atherosclerotic plaque formation, the stimulation of the local inflammatory response may lead to elevated levels of hs-CRP, which has been identified as a risk factor for atherosclerosis (24). These findings have highlighted the significant importance of hs-CRP for the evaluation of atherosclerotic inflammation. However, a recent meta-analysis called into question the clinical value of CRP as a predictor of CVD risk. In their prospective study, Danesh et al. (25) revealed CRP as a relatively modest predictor of CVD. Many studies have identified elevated serum CRP levels in response to cardiovascular events and that CRP levels may strongly and independently predict adverse cardiovascular events, including myocardial infarction (MI), ischemic stroke, and sudden cardiac death (26).

Although CRP is an inflammatory biomarker, its involvement in the pathogenesis of CVD is questionable. CRP has been demonstrated to exhibit prothrombotic property, and high concentrations of CRP may activate the coagulation system, resulting in an increase in the level of prothrombin and D-dimer (27, 28). In a clinical study with a long follow-up, the investigators reported high predictive values of CRP in atherosclerotic thrombotic events after the implantation of drug-eluting stents (29). Studies investigating the relationship between stent thrombosis, hs-CRP levels, and statin therapy revealed that statins may only reduce the formation of early stent thrombosis in patients with high levels of hs-CRP. Rosuvastatin was similarly shown to markedly reduce the incidence of CVD and CVD-associated mortality and decrease levels of CRP and low-density lipoprotein (LDL) (30). Furthermore, Eisenhardt et al. (31) found a localized dissociation mechanism of pCRP into mCRP mediated by activated platelets, thereby resulting in the deposition of mCRP in atherosclerotic plaques. A clinical trial has demonstrated the effectiveness of statins in reducing the incidence of future cardiovascular events is associated with decreased pCRP level (32). Ruptured plaques and inflamed tissues in patients with ACS were shown to be more prone to opsonization by mCRP, leading to the consumption of autoantibodies against mCRP (33). In addition, multiple epidemiological studies have revealed the participation of CRP in the pathogenesis of CVDs based on genetic polymorphisms that affect CRP levels (34). However, some epidemiological studies failed to support the notion that the common variation in CRP gene had an alternative effect on the occurrence of coronary heart diseases. A Mendelian randomization study of over 28,000 cases and 100,000 controls found that a lack of concordance between the effect of the CRP genotypes on the risk of coronary heart diseases and CRP levels argued against a causal association of CRP with coronary heart disease (28). Using genetic variants as the unconfounded proxies of CRP concentration, Mendelian randomization meta-analysis of individual participant data from 47 epidemiological studies showed that CRP concentration itself was unlikely to be the modest causal factor in coronary heart disease (35). Steady state CRP levels in serum are influenced by CRP gene haplotypes. Although elevated CRP level has lately been found to be a consistent and relatively strong risk factor for CVD, no association was observed between CRP gene haplotypes and coronary heart disease (36). A prospective, nested case-control study design from the Physicians' Health Study cohort demonstrated that none of the single nucleotide polymorphisms related to higher CRP levels showed any association with the risk of incident MI or ischemic stroke (37). Therefore, these epidemiological data

indicate a significant interaction between both genetic and environmental factors and increased CRP levels that predict a greater risk of CVD events.

Some basic research shows that the inflammatory response plays a central role in various phases of atherosclerosis, i.e., from the initial recruitment of circulating leukocytes to the arterial wall to the rupture of unstable plaques, thereby resulting in the clinical manifestations of the disease. CRP may be critically involved in each of these stages by directly influencing processes, including complement activation, apoptosis, endothelial nitric oxide (NO) synthase inhibition, vascular cell activation, monocyte recruitment, lipid accumulation and thrombosis, and pro-inflammatory cytokine formation (38). Taken together, CRP appears to play a pivotal role in many aspects of CVD, as described below (**Figure 1**):

- mCRP induces endothelial cells to produce monocyte chemoattractant protein 1, interleukin-8 (IL-8), intercellular adhesion molecule 1, and vascular cell adhesion molecule 1 (39).
- mCRP inhibits the apoptosis of neutrophils that is partly meditated by the activation of the low-affinity immunoglobulin G immune complex receptor FcγRIII (CD16) *via* stimulation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B and extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK)-ERK (MEK) signaling pathways, leading to the inhibition of caspase-3. This process is partly

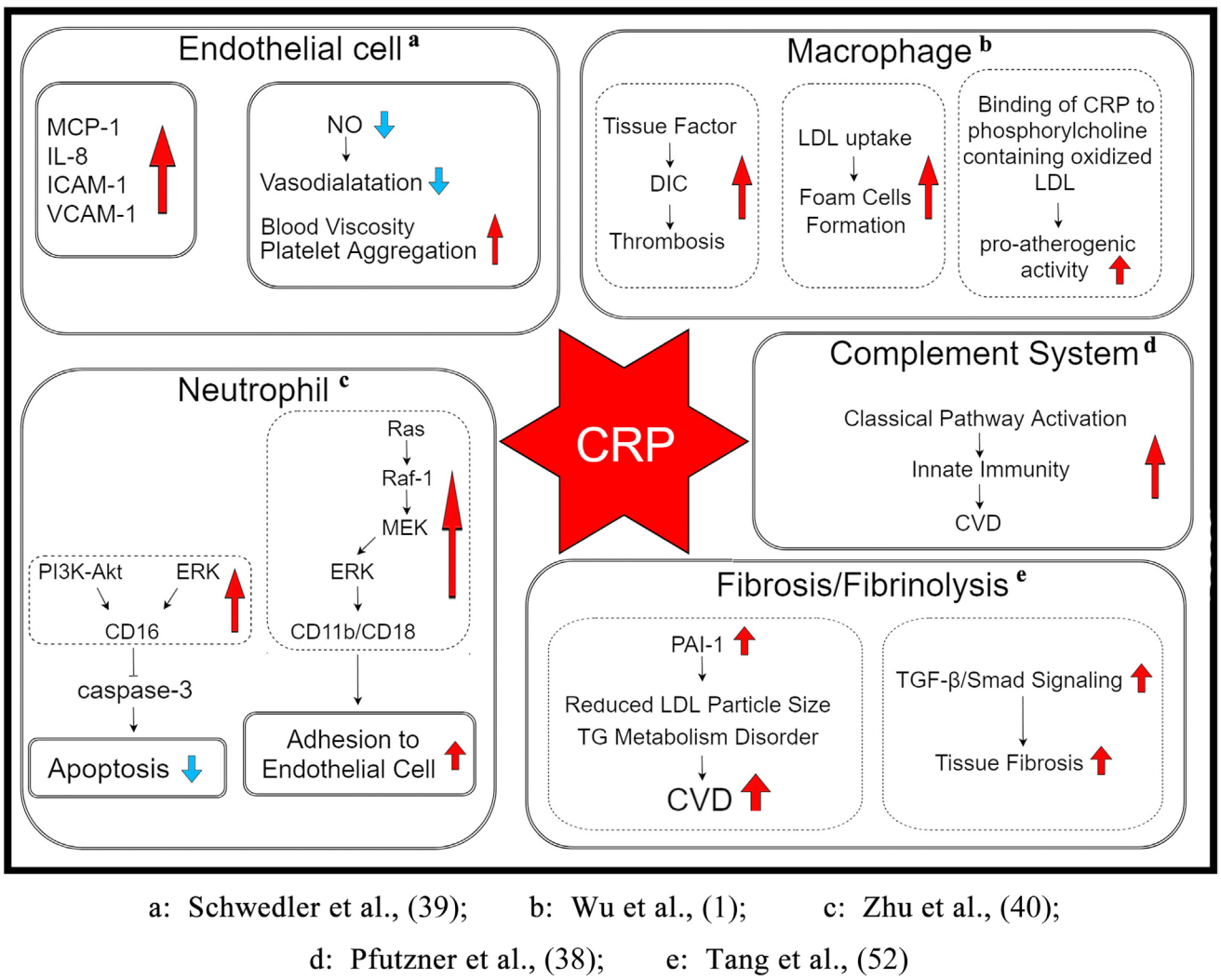

FIGURE 1 | The potential mechanism underlying the role of CRP in the pathogenesis of CVDs. CRP may be involved in various stages through its direct influence on pathophysiological processes such as the activation of endothelial cells and macrophages, inhibition of apoptosis of neutrophils and expression of endothelial NO synthase, stimulation of the complement cascade, enhancement of PAI-1 activity and LDL uptake, accumulation of lipid and thrombosis, and upregulation of pro-inflammatory cytokine expression. Abbreviations: DIC, disseminated intravascular coagulation; LDL, low-density lipoprotein; CVD, cardiovascular diseases; TG, triglyceride; MCP-1, monocyte chemoattractant protein 1; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; NO, nitric oxide; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PAI-1, plasminogen activator inhibitor-1; TGF-β, transforming growth factor-β; CRP, C-reactive protein.

mediated by the activation of neutrophil ERK *via* the Ras/Raf-1/MEK cascade that increases CD11b/CD18 expression, thereby promoting adhesion to endothelial cells (40).
- C-reactive protein activates macrophages to secrete tissue factor, a powerful procoagulant, which may lead to disseminated intravascular coagulation and thrombosis during inflammatory states.
- C-reactive protein increases the uptake of LDL into macrophages and enhances the ability of macrophages to form foam cells. It binds to phosphocholine of oxidized LDL.
- Through the above action, CRP induces the classical complement pathway and directly activates and amplifies the innate immunity, a process that has already been associated with the initiation and progression of CVD.
- C-reactive protein inhibits endothelial NO synthase expression. NO, an important signaling molecule, is closely associated with the regulation of vasodilatation, blood rheology, platelet aggregation, and other physiological as well as pathological processes.
- C-reactive protein upregulates plasminogen activator inhibitor-1 (PAI-1) expression and activity. PAI-1 reduces LDL particle size in conjunction with triglyceride metabolism disorder, leading to an increased risk of CVD (41).
- C-reactive protein mediates tissue fibrosis in CVD by activating transforming growth factor-β (TGF-β)/Smad signaling *via* TGF-β1-dependent and -independent mechanisms.

CRP AND TYPE 2 DIABETES MELLITUS (T2DM)

Type 2 diabetes mellitus is a serious disease associated with high morbidity and mortality. CRP, tumor necrosis factor-alpha, and IL-6 may be triggered by the excessive adipose tissue to activate insulin signaling pathways, resulting in insulin resistance that eventually progresses into T2DM (42). Cross-sectional and prospective studies have demonstrated a relationship between elevated CRP levels and increased risk for T2DM (43). Higher levels of hemoglobin A1c (HbA1c, %), the indicator of overall glycemic control in diabetics, were positively correlated with elevated CRP levels even after adjustment, as seen in elderly patients with T2DM (44, 45). In comparison to subjects with normal fasting glucose, patients with diabetes and impaired fasting glucose level (defined by the reduction in glucose from 110 to 100 mg/dL and/or elevated plasma glucose level at 2 h in the oral glucose tolerance test) showed a strong increase in hs-CRP (46, 47). Furthermore, clinical studies showed elevated serum concentrations of fetuin-A, vascular endothelial growth factor, and CRP in T2DM patients with diabetic retinopathy (DR), suggestive of the increased risk of DR with high CRP level (48).

Based on the multitude of clinical observations, CRP appears to be not only a marker of chronic inflammation but also a media tor of kidney diseases in basic research. CRP is known to bind to its receptor FcγRII (CD32/CD64) and directly activate or interact with a number of signaling pathways in the process of inflammation, fibrosis, and aging. CRP may activate the mechanistic target of rapamycin (mTOR) signaling under diabetic conditions (49). To date, the most consistent animal data in CRP research were obtained with the diabetic mouse model of CRP; CRP was shown to clearly enhance renal fibrosis in diabetic nephropathy (T2DN) *via* CD32b-Smad3-mTOR signaling (50). In addition, CRP could activate Smad3 *via* both TGF-β-dependent and ERK/MAPK crosstalk mechanisms, leading to the direct binding of Smad3 to p27. The suppression of Smad3 or FcγRII CD32 may result in the inhibition of CRP-induced p27-dependent G1 cell cycle arrest and promotion of CDK2/cyclin E-dependent G1/S transition of tubular epithelial cells (51). Moreover, CRP could markedly mediate tissue fibrosis in several cardiovascular and kidney diseases by activating the TGF-β/Smad3 pathway (52).

CRP AND AGE-RELATED MACULAR DEGENERATION (AMD)

Age-related macular degeneration, an acquired disease of the macula, is characterized by progressive visual impairment, owing to the late-onset neurodegeneration of the photoreceptor-retinal pigment epithelial complex (53–57). Chronic inflammation is thought to be critically involved in the pathophysiology of AMD. A cross-sectional study documented an obviously higher CRP level in the exudative form of AMD (eAMD) as compared to that observed in the early form (56). Higher CRP levels were closely associated with the higher risk of exudative AMD (55, 58–60). In comparison with pCRP, pro-inflammatory mCRP strongly influenced endothelial cell phenotypes, indicative of its potential role in choroidal vascular dysfunction in AMD (61). A recent observational study of elderly European patients by Cipriani et al. (62) suggested no causal association between CRP concentrations and AMD. However, complement activation may be involved in the development of AMD. It was found that mCRP played a role in choroidal vascular dysfunction in AMD by influencing endothelial cell phenotypes *in vitro* and *ex vivo* (63). In addition, high levels of CRP could activate the complement system at the retina/choroid interface and contribute to chronic inflammation and subsequent tissue damage (64). These clinical results indicate that CRP plays an important role in the pathogenesis of AMD and may be used to assess the severity of AMD. Plasma levels of CRP are independently associated with the risk of AMD, but whether CRP is causally associated with AMD or acts as a mere marker of AMD is uncertain.

CRP AND HEMORRHAGIC STROKE

During the initial stages of hemorrhagic stroke, including intracerebral hemorrhage and subarachnoid hemorrhage, the reflex mechanisms are activated to protect cerebral perfusion. The inflammatory process and hyperglycemia are involved in the spontaneous intracerebral hemorrhage (sICH) as well as the progression of sICH-induced brain injury (65, 66). Several prospective studies have reported the association between higher CRP levels and increased disability risk of ischemic stroke (67). CRP elevation displays negative prognostic implications for many conditions, while elevations in CRP as a consequence of the major acute-phase response following ischemic or hemorrhagic stroke are associated with death and vascular complications

(68). In a cross-sectional study, an increase in hs-CRP level was observed in patients with ischemic infarction but not in those with hemorrhagic stroke, suggestive of the role of hs-CRP in the initial diagnosis of the stroke type (69). Moreover, CRP was an independent predictor of mortality and its expression was significantly correlated with poor clinical outcomes in sICH (70–72). Although several studies have shown the higher level of CRP in patients with ischemic stroke, its potential role in various stroke types, particularly in ischemic and hemorrhagic stroke, needs to be investigated.

CRP AND AD

Alzheimer's disease is a neurodegenerative disorder characterized by gradually progressive cognitive decline and functional impairment. Neuroinflammation may play a potential role in AD pathogenesis (73, 74). However, the precise mechanism related to AD phenotype remains unclear. CRP was clearly recognized in the senile plaques (SP) from patients with AD using immunostaining, implying that the process of SP formation may include an acute-phase inflammatory state and/or the formation of CRP (75). A meta-analysis included 10 cross-sectional studies and showed no significant difference in the serum CRP level between AD patients and normal controls, whereas patients with mild and moderate AD had lower serum CRP levels as compared with the healthy controls by Mini-Mental State Examination scores (15), indicating that the diagnostic value of CRP for mild and moderate AD may be useful in clinical practice. Most studies support the reduced plasma CRP levels in mild and moderate AD patients and indicate its potential role as a representative systemic inflammatory marker for the diagnosis of AD. In addition, lower CRP levels are associated with more rapid cognitive and functional decline (76). The elevated CRP level was associated with an increased risk of AD (77), while such elevation appeared to diminish and fall below the level observed in nondemented controls after the clinical manifestation of the disease. In addition, the influence of CRP and homocysteine (Hcy) on patients suffering from AD was assessed and both CRP and Hcy were found to play no role in the development of AD (78). Strang et al. (79) recently demonstrated the association between mCRP in AD patients and beta-amyloid (A-β) plaques, which may induce the dissociation of pCRP into individual monomers. Moreover, basic research of human AD/stroke patients revealed that high mCRP levels from infarcted core regions were associated with the reduced expression of A-β/Tau, suggestive of the role of mCRP in promoting dementia after ischemia (80). Taken together, a direct functional effect elicited by CRP may, at least in part, explain the pathogenesis of AD.

CRP AND PD

Parkinson's disease is a neurodegenerative disorder pathologically characterized by dopaminergic neuronal death and the presence of Lewy bodies (81). Previous studies have highlighted the key role of neuroinflammatory reactions in the pathogenesis of PD and patients with PD were shown to exhibit higher levels of serum hs-CRP. Patients with more severe PD, classified according to the Hoehn–Yahr staging system, had significantly higher levels of hs-CRP than those at an earlier stage and non-Parkinson's control subjects (82). A cross-sectional study suggested that CRP may play an important role in the development of PD and elevations in the plasma CRP level correlated with an increased risk of PD. Baseline CRP concentrations were recently shown to be associated with the risk of death and predicted life prognosis of patients with PD (81). A retrospective analysis further supported the association between baseline plasma CRP levels and motor deterioration and predicted motor prognosis in patients with PD; these associations were independent of sex, age, PD severity, dementia, and use of antiparkinsonian agents (83). Although formal demonstration of the mechanism of action of CRP in the pathogenesis of PD is currently lacking, there is a continuous increase in the experimental data, which is in line with the aforementioned concept.

CONCLUSION

As a nonspecific marker of inflammation, CRP plays a vital role in the monitoring of bacterial infection, inflammation, neurodegeneration, tissue injury, and recovery. Chronic inflammation may be a continuation of an acute or a prolonged low-grade form, which is increasingly recognized as an important issue with social and economic implications. CRP levels are observed to be increased during acute-phase inflammation as well as chronic inflammatory diseases. From both experimental and clinical data, increasing evidence suggest that elevated CRP concentrations are associated with an increased risk of CVD, T2DM, AD, hemorrhagic stroke, PD, and AMD. Moreover, CRP is not only an excellent biomarker of chronic inflammation but also acts as a direct participant in the pathological process (84). The differentiation between the physiological and pathophysiological CRP levels may allow better management of inflammation-related diseases. Although the clinical significance and underlying mechanisms of CRP in chronic inflammatory and neurodegenerative diseases are incompletely elucidated, further research is required in order to differentially characterize the roles of CRP isoforms (pCRP, facilitator, versus mCRP, effector) in chronic inflammation onset and progression. A better understanding of CRP activation and dissociation is essential to develop therapeutic strategies to minimize tissue injury, which may further improve the outcome of chronic inflammatory diseases.

AUTHOR CONTRIBUTIONS

We affirm that all authors have contributed to, seen, and approved the final, submitted version of the manuscript and are willing to convey copy right to Frontiers in Immunology.

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The Effect of C-Reactive Protein Isoforms on Nitric Oxide Production by U937 Monocytes/Macrophages

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Inflammation is regulated by many endogenous factors including estrogen, a steroid hormone that declines with increasing age, leading to excessive inflammation in the elderly. C-reactive protein (CRP) is an acute phase inflammatory protein that exists in two forms, native CRP (nCRP) and monomeric CRP (mCRP), which mediate distinct biological activities. It is unclear how each CRP isoform mediates nitric oxide (NO), a signaling molecule generated by NO synthase (NOS). This study investigated whether CRP isoforms have distinct effects on NO production by unstimulated and lipopolysaccharide (LPS)-activated monocytes/macrophages and whether estrogen mediates CRP-induced NO production in an *in vitro* model of aging. NO and inducible NOS (iNOS) were measured ($n = 12$) by the Griess assay and an enzyme-linked immunosorbent assay, respectively following incubation (24 h) of human-derived U937 monocytes/macrophages with CRP isoforms [(nCRP) = 500 and 1,000 μg/ml; (mCRP) = 100 and 250 μg/ml] in the absence or presence of 17 beta-estradiol (1×10^{-7}, 1×10^{-8}, and 1×10^{-9} M). The response to each CRP isoform and estrogen was dependent on the differentiation and activation status of cells. Monocytes with or without prior LPS-activation significantly increased ($P < 0.01$) NO/iNOS production when treated with mCRP. The mCRP isoform had no effect ($P > 0.05$) on NO/iNOS production by unactivated or LPS-activated macrophages, whereas nCRP significantly ($P < 0.05$) reduced NO/iNOS production by macrophages, with or without prior LPS-activation. The nCRP isoform had opposing actions on monocytes, significantly ($P < 0.01$) increasing and reducing NO/iNOS by unactivated and LPS-activated monocytes, respectively. Estrogen significantly ($P < 0.01$) reversed nCRP-mediated NO inhibition by unactivated macrophages but decreased CRP-induced NO by unactivated monocytes treated with nCRP or mCRP and LPS-activated monocytes treated with mCRP. NO was differentially mediated by CRP isoforms in a cell-type/state-specific manner, with production corresponding to concomitant changes in iNOS levels. Collectively, the findings indicate nCRP and estrogen predominantly reduce NO production, whereas mCRP increases NO production. This supports growing evidence that mCRP exacerbates inflammation while nCRP and estrogen dampen the overall inflammatory response. Therapeutic strategies that restore estrogen levels to those found in youth and promote the stability of nCRP or/and prevent the formation of mCRP may reduce NO production in age-related inflammatory conditions.

Keywords: C-reactive protein, nitric oxide, inflammation, native C-reactive protein, monomeric C-reactive protein, estrogen

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INTRODUCTION

There is increasing evidence that C-reactive protein (CRP) may play a role in inflammatory processes. CRP is a homopentameric acute-phase inflammatory protein that exhibits elevated expression during inflammatory conditions, such as rheumatoid arthritis, some cardiovascular diseases, and infection (1). The pentameric protein, termed native CRP (nCRP) is characterized by a discoid configuration of five identical non-covalently bound subunits, each 206 amino acids long with a molecular mass of about 23 kDa (1).

The nCRP isoform is synthesized primarily in liver hepatocytes but has also been reported to be synthesized in other cell types, such as smooth muscle cells (2), macrophages (3), endothelial cells (4), lymphocytes (5), and adipocytes (6). CRP is first synthesized as monomers and then assembled into the pentamer in the endoplasmic reticulum of the cell. The stimulation of CRP synthesis mainly occurs in response to pro-inflammatory cytokines, most notably interleukin-6 (IL-6), IL-1, and tumor necrosis factor-alpha (TNF-α) (7,8).

Pentameric CRP can irreversibly dissociate, with the resultant-free subunits termed monomeric (or modified) CRP (mCRP). The mCRP isoform is distinguished from nCRP by its distinct antigenic, biological, and electrophoretic activities (9) and the fact it expresses different neoepitopes (10). Evidence suggests nCRP which often exhibits a more anti-inflammatory function in tissues relative to the mCRP isoform. It is suggested that this occurs by nCRP binding at sites of tissue injury to limit the generation of the membrane attack complex (MAC) and C5a, thus inhibiting complement activation (11). On the other hand, mCRP generally has marked pro-inflammatory properties *in vitro* and *in vivo* by promoting the recruitment of circulating leukocytes to areas of inflammation through interaction with receptors of the Fcγ family (12) and lipid raft microdomains in cell membranes (13).

There has been some evidence that CRP interacts with nitric oxide (NO), a short-lived, pleiotropic-free radical regulator of various biological functions, including vasodilation, neurotransmission, inflammation, and macrophage-mediated immunity (14). NO has properties that allow it to be easily soluble and diffuse across biological membranes to conduct its intracellular processes (15). This intercellular signaling molecule is important for the immune system, as well as generating free oxygen radicals called peroxynitrites ($ONOO^-$) which act in a cytotoxic manner causing tissue damage and apoptosis (16). NO also plays a functional role in processes, such as leukocyte adhesion and transmigration, proliferation, apoptosis, and cytokine expression (17–20). NO is generated by NO synthase (NOS) enzymes (21); neuronal NOS, constitutive eNOS, and inducible NOS (iNOS). NO production in inflammatory conditions and following infection is predominantly through iNOS (22).

The versatility of NO at inducing variable responses suggests it has both pro- and anti-inflammatory effects. However, the inhibition of NO pathways is often beneficial in the treatment of inflammatory disease (23). Several factors modify the effect of NO including its concentration, the rate of reactive nitrogen species formation and the physiological environment. During the inflammatory response, pro-inflammatory cytokines stimulate NO production in monocytes, macrophages, and neutrophils and *in vivo* levels can increase by up to 1,000-fold in severe cases of bacterial sepsis (22). NO acts as a positive feedback molecule when released by tissue macrophages during phagocytosis, leading to the recruitment of further phagocytes. However, excessive NO can lead to tissue destruction as seen in autoimmune diseases (24). The level of NO produced is regulated at the transcriptional level, depending on the cell type and the nature of the stimulation involved. The mitogen-activated protein kinase and PI3 kinase pathways mediate NO production (25) but there is conflicting evidence if the p38 pathway is involved, with studies showing upregulation, downregulation, and no effect on NO production (26, 27). NO can also regulate its own production *via* positive and negative feedback loops. The positive feedback utilizes the increase in cAMP levels to activate the production of iNOS and subsequent increase in NO production. The negative feedback loop uses the inhibition of NFκβ to lower NO production (25).

Several studies that have highlighted a relationship between NO production and CRP. Hattori et al. (28) showed CRP mediates both NO and iNOS gene expression. Schwedler et al. (21) found that nCRP treatment *in vivo* caused impairment in endothelial function in the aortic rings that was associated with increased iNOS activity. Several cardiovascular studies have revealed that nCRP inhibits NO production by downregulating endothelial NOS (eNOS) in endothelial cells, thereby inhibiting angiogenesis (29). Inhibition of endothelial-derived NO promotes the pathogenesis of atherosclerotic vascular disease through vasoconstriction, leukocyte adherence, and inflammatory cell activation (29–32), highlighting the pro-inflammatory nature of nCRP in the circulatory system. Little work has been published on the effect of CRP on NO production by cells and tissues outside the cardiovascular system. Borderie et al. (33) showed that CRP concentration correlates with the number of iNOS-positive synovial fluid leukocytes in patients with rheumatoid arthritis but specific CRP isoforms were not distinguished. The mCRP isoform has subsequently been shown to enhance NO production in isolated human neutrophils *via* upregulated NOS activity (34). Consequently, this study assessed whether CRP isoforms have distinct effects on iNOS and NO production by human-derived U937 monocytes/macrophages.

Nitric oxide is also regulated by many endogenous factors including the steroid hormone estrogen (35). Estrogen is known to have beneficial effects during wound repair following tissue injury, accelerating healing, and reducing inflammation in both male and female humans (36, 37). Moreover, the profound decline in estrogen levels in the elderly is implicated in age-related impaired healing, leading to delayed repair with excessive inflammation (38). Studies have shown that estrogen has the ability to stimulate NO production and vasodilation in the cardiovascular system by inducing eNOS (39). In contrast, Hassouna et al. (40) showed that the loss of estrogen following ovariectomy in a rat model of inflammation significantly increased levels of both CRP and iNOS, while subsequent estrogen supplementation reversed this effect. This study investigated whether exogenous estrogen can mediate CRP-induced NO production by human-derived U937 monocytes/macrophages in an *in vitro* model of aging.

MATERIALS AND METHODS

Purification of CRP Isoforms

The CRP isoforms were purified from a high purity commercial source of human nCRP (MBS536586; MyBioSource, San Diego, CA, USA). Purified mCRP was generated using the Potempa method (41). In this method, 1 ml commercial CRP protein was chelated in a 1:1 ratio with EDTA/urea buffer (10 mM EDTA, 8 M ultrapure urea) and incubated at 37°C for 2 h prior to dialysis (20 kDa MWCO) in buffer (25 mM Tris–HCl, 50 mM NaCl; pH 8.3) for 24 h.

Commercial nCRP (1 ml) was purified further by hydrophobic interaction chromatography using a Sepharose HiTrap column (GE Healthcare, Buckinghamshire) according to the manufacturer's instructions. The eluted protein was immediately placed inside a 50 kDa MWCO float-a-lyzer (Medicell, London) and dialyzed overnight into a 2 mM calcium chloride buffer (2 mM $CaCl_2$, 25 mM Tris–HCl, 150 mM NaCl; pH 7.4).

Both the nCRP and mCRP samples were tested for purity by immunoblotting using published methods (42). The immunoblotting procedure consisted of placing 5 µl of purified CRP samples onto two separate nitrocellulose membranes. A similar volume blot (5 µl) of 1 mg/ml bovine serum albumin (BSA) was used as a negative control protein and 5 µl of the original commercial CRP at 5 mg/ml was used as a positive control on each membrane. The samples were allowed to dry before placing the membranes in blocking buffer (1% BSA in TBS-Tween, pH 7.4) for 1 h at room temperature with 60 rpm rotation. Monoclonal mouse anti-human nCRP 1D6 [clone I-15-1D6 (isotype: IgG2a, κ)] and monoclonal mouse anti-human mCRP 8C10 [clone III-26-8C10 (IgG1, κ)] were primary antibodies kindly provided by L.A. Potempa (Roosevelt University, IL, USA; ImmTech Inc.) to detect specific CRP isoforms (43). Membranes were incubated overnight at 5°C with 50 rpm rotation in primary antibody diluted 1:10 with blocking buffer before washing five times in TBS-Tween buffer for 5 min. The membranes were then incubated in a 1:1,000 dilution of horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-mouse immunoglobulins (secondary antibody) (P0260; Dako, Cambridge) in 5% milk/TBS-tween solution for 1 h with 60 rpm rotation. The membranes were washed again five times in TBS-Tween buffer for 5 min. The membranes were then treated with the EZ-ECL chemiluminescence detection kit for HRP (K1-0172; Geneflow, Lichfield) according to the manufacturer's instructions. The chemiluminescent image was then captured in a Syngene GBox with a 1 min exposure time using Genesnap software (Version 7.07, Syngene).

Purified CRP samples were quantified using bicinchronic acid (BCA) analysis (BCA1-1KT; Sigma-Aldrich, Poole) according to the manufacturer's instructions to determine the yield. A standard curve was generated for the BCA analysis using a BSA standard. CRP samples were incubated with BCA working buffer for 30 min at 37°C prior to reading absorbance measurements at 595 nm on a Biotech Synergy HT plate reader. CRP concentrations were quantified by interpolation from the standard curve and then normalized at 2 mg/ml. Normalized CRP samples (2 mg/ml) were then sterile filtered and confirmed endotoxin-free using a commercial endotoxin detection E-TOXATE (*Limulus* amebocyte lysate) kit (ET0100; Sigma Aldrich, Poole) according to the manufacturer's instructions. All CRP isoforms were stored at 4°C until use in cell assays.

Cell Culture

The monocyte-like cell line U937 was cultured in RPMI-1640 media supplemented with 10% FBS and 2% penicillin–streptomycin. In some experiments, U937 cells were differentiated into a macrophage-like phenotype using 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 72 h and/or pre-activated for 24 h with 0.5 mg/ml lipopolysaccharide (LPS). All cell culture experiments were performed in a class II safety cabinet according to local biosecurity and safety procedures. **Figure 1** outlines the different treatment paths used to activate and differentiate the U937 cells. Cell viability was assessed using 0.4% trypan blue exclusion in which cellular uptake of the dye indicates cell death.

Flow Cytometry

Differentiation of U937 monocytes into a macrophage-like phenotype was confirmed by flow cytometry through detection of the CD11c macrophage-specific surface antigen. Following differentiation with PMA, cells were treated with 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS) for 10 min at room temperature. Cells were washed twice with DPBS and stained for 30 min at room temperature with FITC-conjugated anti-human CD11c antibody (Clone Bu15; BioLegend, UK) diluted 1:40 with 10% fetal bovine serum (FBS) in DPBS. Cells were then washed twice in DPBS before suspension in 500 µL DPBS. CD11c expression was assessed on 10,000 events (live, individual cells) with a BD Accuri C6F1 cytometer using BD Accuri C6 software (Biosciences, USA) after gating in the forward scatter/side scatter and fluorescence parameter 1 (FL1-A) windows. Data were presented as average percentage $CD11c^+$ cells (%) and median fluorescence intensity (MFI) relative to unstained monocytes from three independent experiments.

NO and iNOS Assays

Cells at a concentration of 1×10^6 cells/ml were incubated with physiological concentrations of mCRP (100 µg/ml or 250 µg/ml) or nCRP (500 µg/ml or 1,000 µg/ml) for 24 h. Negative controls were incubated with equivalent concentrations of BSA for 24 h. In inhibitor treatments, cells were co-incubated with CRP isoforms and the inhibitor nystatin at a concentration of 25 µg/ml. In the aging model, cells were co-incubated with CRP isoforms and estrogen at a concentration of 1×10^{-7}, 1×10^{-8}, and 1×10^{-9} M. Estrogen at 1×10^{-8} M is typical of physiological levels found in circulation during youth and a concentration range from 1×10^{-9} to 1×10^{-7} M is frequently utilized under experimental conditions (44, 45). Furthermore, cultured human inflammatory cells, including U937 cells, have previously been utilized as *in vitro* models of estrogen-mediated aging (46, 47).

Nitric oxide production was measured in each treatment group ($n = 12$) by the Griess method (48) with the absorbance of a 1:1 ratio of sample and Griess reagent read using a Biotech Synergy HT plate reader at a wavelength of 540 nm. Concentrations were calculated against a standard curve of sodium nitrite standards (0, 1, 10, 25, 50, 75, and 100 µM).

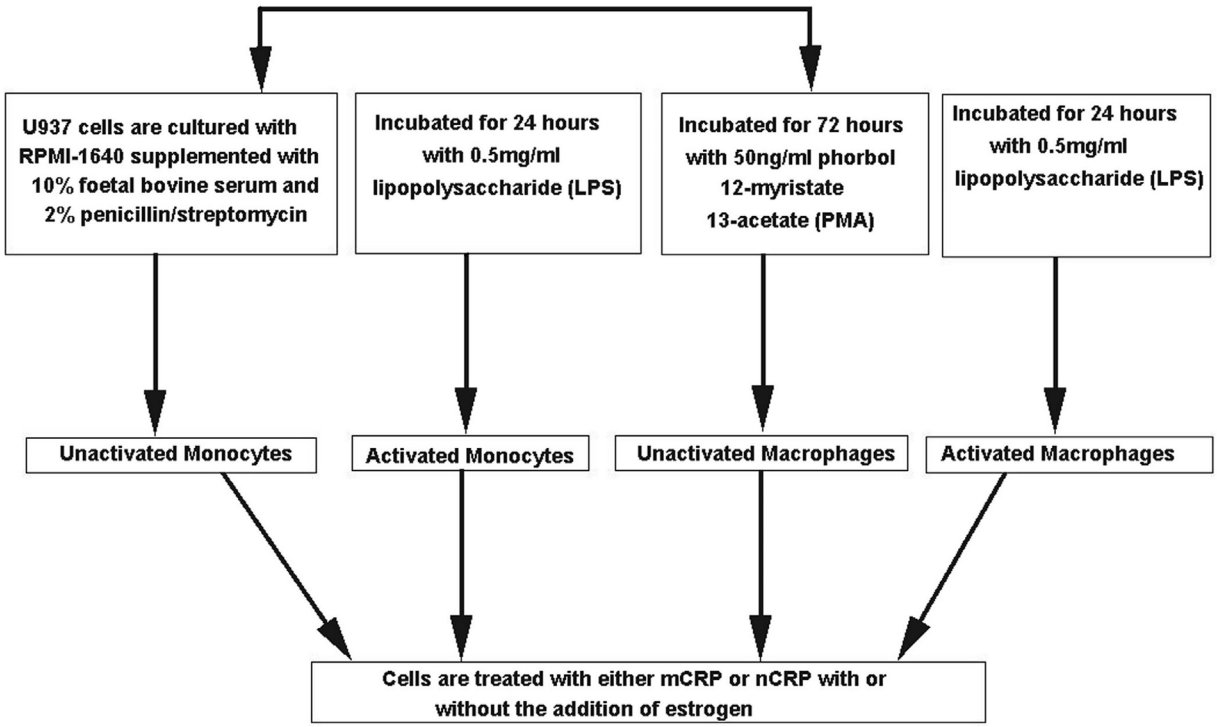

FIGURE 1 | Flow chart of different treatment paths used to activate and differentiate the U937 cells. U937 cells were differentiated into a macrophage-like phenotype using 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 72 h and/or pre-activated for 24 h with 0.5 mg/ml lipopolysaccharide (LPS).

The human iNOS sandwich-enzyme-linked immunosorbent assay (Elabscience, USA) was used to measure iNOS levels in each treatment group ($n = 12$) according to the manufacturer's instructions and published work (49). Optical density readings were performed using a Biotech Synergy HT plate reader at a wavelength of 450 nm and concentrations were calculated against the standard curve generated within the assay.

Statistical Analysis

Readings for NO and iNOS were measured in four replicate sample wells and average concentrations were determined from three independent experiments ($n = 12$ in total). Treatment groups were analyzed by ANOVA and *t*-tests (parametric data) or Mann–Whitney *U*-tests (non-parametric data) using SPSS (Version 22). A probability (*P*) value of $P < 0.05$ was considered statistically significant in all cases.

RESULTS

Purification of CRP Isoforms

Immunoblotting confirmed the purity of CRP isoforms (**Figure 2**) used in the experiments. There was no detection of mCRP in the purified nCRP sample and no detection of nCRP in the purified mCRP sample. Purification yields for nCRP and mCRP were 4.5 and 2.3 mg/ml, respectively prior to normalization at 2 mg/ml.

Moreover, both the nCRP and mCRP samples at 2 mg/ml were confirmed effectively endotoxin-free (<20 ρg/ml), thus excluding LPS contamination in the experimental treatments. In essence, the deliberate pre-activation of some U937 cells with 0.5 mg/ml LPS exposed those cells to at least 25 million times more endotoxin than was present in normalized CRP samples, and at least 50 and 200 million times more endotoxin than was present in experimental concentrations of nCRP (<10 ρg/ml LPS) and mCRP (<2.5 ρg/ml LPS), respectively. Therefore, the negligible endotoxin levels in CRP treatments cannot account for observed experimental changes in NO.

Flow Cytometry

The conversion of U937 monocytic cells into a distinct population of macrophage was confirmed *via* detection of the CD11c surface marker by flow cytometry (**Figure 3**). PMA-differentiated cells almost exclusively (99.6% $CD11c^+$) expressed the CD11c macrophage marker, whereas monocytes predominantly lacked the CD11c surface marker (0.2% $CD11c^+$). The MFI was significantly ($P < 0.001$) higher in PMA-treated cells compared to undifferentiated U937 monocytes, confirming PMA transformed U937 monocytes into $CD11c^+$ macrophages.

The Effect of Exogenous CRP Isoforms on NO and iNOS Production

The NO values obtained in the study were in line with published findings utilizing unstimulated and LPS-activated U937 cells

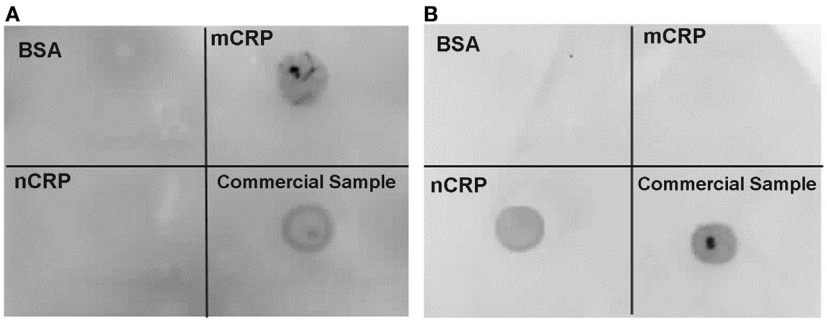

FIGURE 2 | Immunoblot confirming purity of C-reactive protein (CRP) isoforms. Immunoblotting confirmed the presence of monomeric CRP (mCRP) alone **(A)** and native CRP (nCRP) alone **(B)** in purified samples. Both membranes included blots for a non-specific bovine serum albumin negative control protein, the purified mCRP sample, the purified nCRP, and the original commercial CRP sample. The mCRP and nCRP isoforms were detected with monoclonal anti-mCRP 8C10 [membrane **(A)**] and monoclonal anti-nCRP 1D6 [membrane **(B)**] respectively.

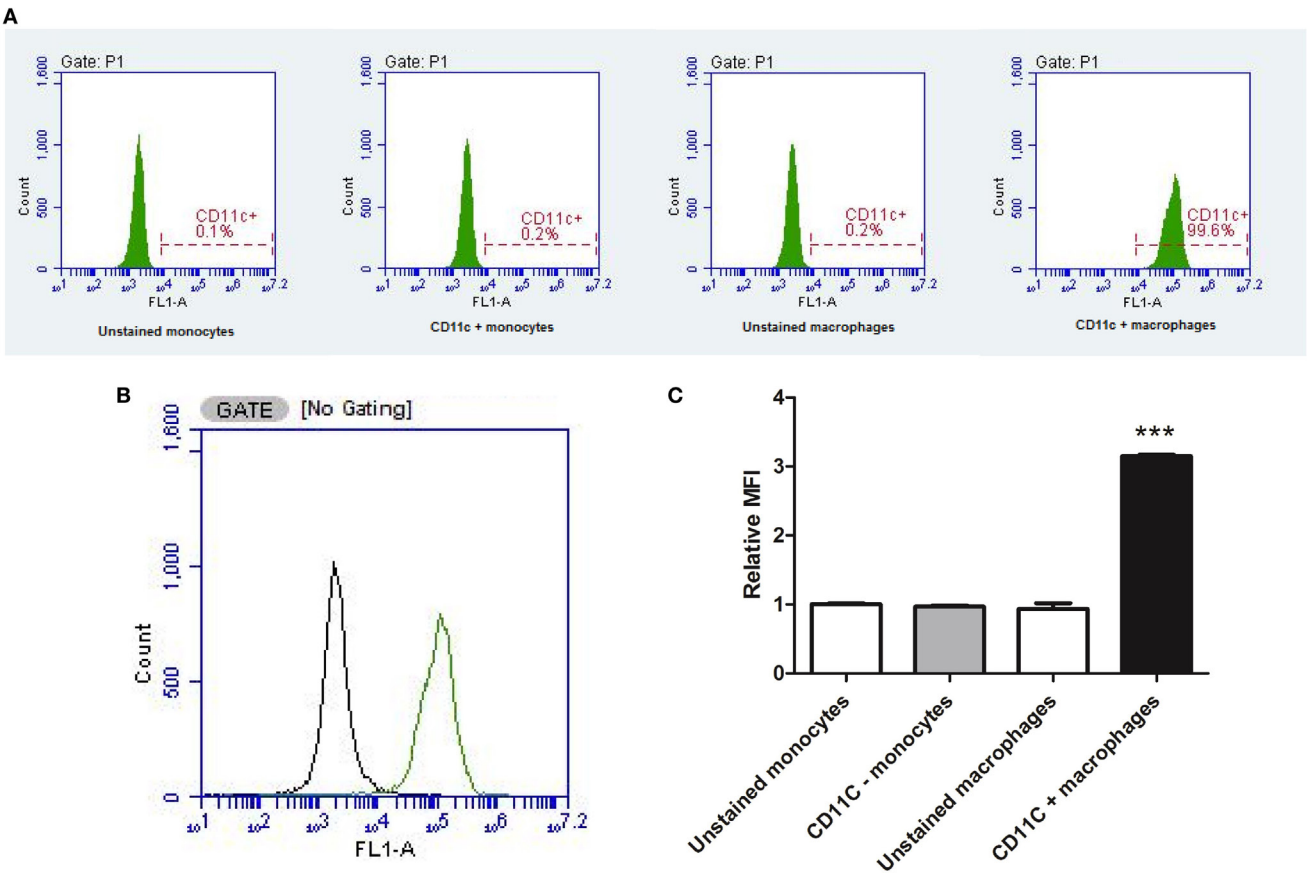

FIGURE 3 | Flow cytometry confirming macrophage differentiation. The conversion of U937 monocytic cells into a distinct population of macrophage was confirmed *via* detection of the CD11c surface marker by flow cytometry (data represent an average of three independent experiments). Phorbol 12-myristate 13-acetate-differentiated cells almost exclusively expressed the CD11c macrophage marker, whereas monocytes predominantly lacked the CD11c surface marker **(A)**. Two distinct populations of cells were detected **(B)** with significantly higher median fluorescence intensity (MFI) in PMA-treated cells compared to undifferentiated U937 monocytes **(C)**. ***Indicates significant difference in MFI ($P < 0.001$; $n = 3$). Errors bars represent the SEM.

(50–52). Monocytic cells exhibited a substantial (more than 1.5-fold) concentration-dependent increase ($P < 0.05$) in NO and iNOS production when treated with mCRP (**Figures 4A** and **5A**), regardless of their activation state. The mCRP isoform did not have any effect ($P > 0.05$) on NO or iNOS production by unactivated or LPS-activated macrophages, whereas nCRP significantly

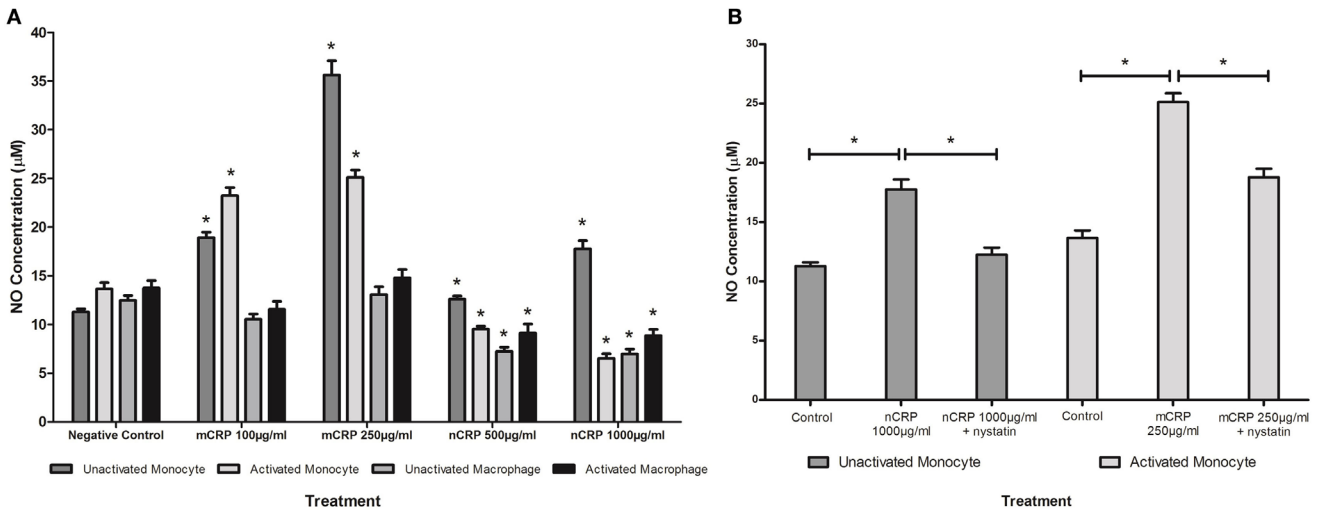

FIGURE 4 | Effect of C-reactive protein (CRP) isoforms on nitric oxide (NO) production. NO production by several cell states (monocyte, lipopolysaccharide (LPS)-activated monocyte, macrophage, and LPS-activated macrophage) was differentially mediated by CRP isoforms in a concentration-dependent manner **(A)**. Nystatin significantly reversed CRP-mediated NO production in unactivated monocytes treated with native CRP (Ncrp) and activated monocytes treated with mCRP **(B)**. *Indicates significant differences ($P < 0.05$; $n = 12$) in NO production compared with corresponding negative controls **(A)** or CRP/nystatin treatments **(B)**. Errors bars represent the SEM.

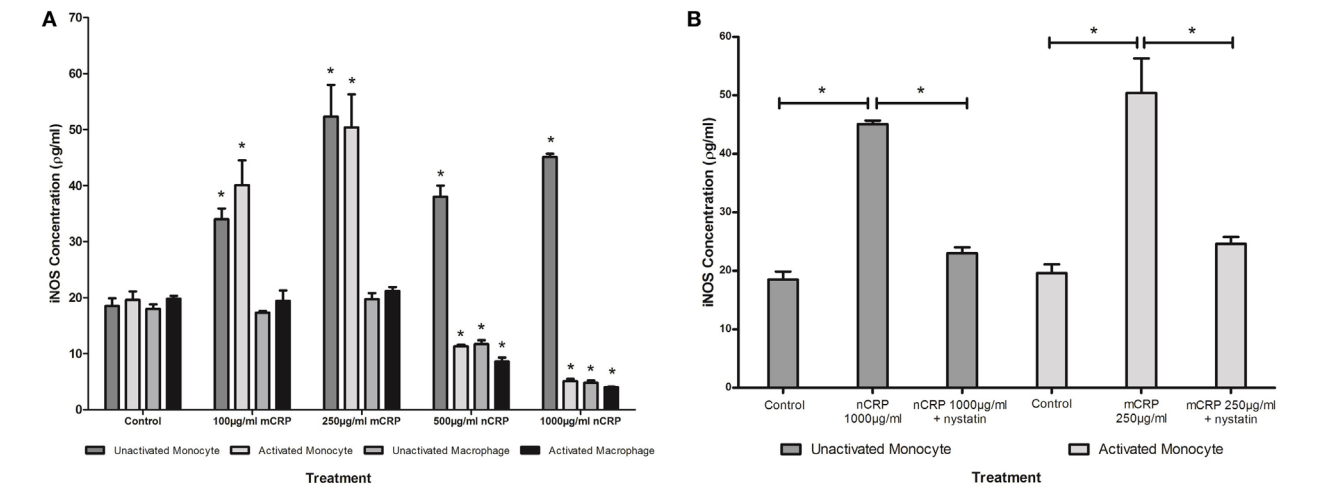

FIGURE 5 | Effect of C-reactive protein (CRP) isoforms on inducible nitric oxide synthase (iNOS). Levels of iNOS production by several cell states (monocyte, lipopolysaccharide (LPS)-activated monocyte, macrophage, and LPS-activated macrophage) were differentially mediated by CRP isoforms in a concentration-dependent manner **(A)**. Nystatin significantly reversed CRP-mediated iNOS production in unactivated monocytes treated with nCRP and activated monocytes treated with mCRP **(B)**. *Indicates significant differences ($P < 0.05$; $n = 12$) in iNOS production compared with corresponding negative controls **(A)** or CRP/nystatin treatments **(B)**. Errors bars represent the SEM.

($P < 0.05$) reduced NO and iNOS production by both unactivated and LPS-activated macrophages. However, the greatest reduction in NO (from 13.7 to 6.5 μM) and iNOS (from 19.6 to 5.1ρg/ml) was observed by LPS-activated monocytes following treatment with (1,000 μg/ml) nCRP. Interestingly, the nCRP isoform had the opposite effect in unactivated monocytes, significantly ($P < 0.05$) increasing NO and iNOS following treatment with nCRP, but more modestly than the mCRP isoform.

The Effect of Nystatin on CRP-Induced NO and iNOS Production

Sample treatments that exhibited significant changes in NO production were repeated with the nystatin inhibitor to determine whether the CRP-mediated responses involved interaction with lipid rafts in the cellular membrane of monocytes/macrophages. **Figures 4B** and **5B** show significant reversal of NO and iNOS production following inhibition with nystatin. Non-significant

results ($P > 0.05$) were excluded from **Figures 4B** and **5B** for clarity, including treatment with nystatin alone (NO = 12.3 ± 0.4 and 12.9 ± 0.4 μM; iNOS = 18.3 ± 1.5 and 20.0 ± 1.0 ρg/ml in unactivated and activated monocytes respectively).

When unactivated monocytes were treated with nCRP there was a significant increase ($P < 0.05$) in the production of NO and iNOS. When co-treated with nystatin there was a significant reversal in both NO ($P = 0.001$) and iNOS ($P < 0.001$) production by unactivated monocytes treated with 1,000 μg/ml nCRP.

Treatment of LPS-activated monocytes with mCRP induced a significant ($P < 0.001$) increase in NO and iNOS production. Again, nystatin significantly reversed the mCRP-induced NO ($P < 0.05$) and iNOS ($P < 0.001$) production by LPS-activated monocytes back to levels observed in the corresponding control.

The Effect of Estrogen on CRP-Induced Nitric Oxide Production

Treatment with estrogen controls in the absence of CRP isoforms (**Figure 6**) showed that physiological concentrations of estrogen reduced NO production by monocytes and macrophages exposed to endotoxin. Treatment of unactivated monocytes with either CRP isoform alone significantly ($P < 0.001$) increased NO production. However, all concentrations of estrogen co-supplementation significantly decreased ($P < 0.01$) CRP-induced NO production by unactivated monocytes (**Figure 7**) to levels similar or below those of the untreated control. Moreover, the reduction in NO by LPS-activated monocytes following treatment with estrogen alone (in the absence of CRP isoforms) was relatively modest compared to the estrogen-mediated reversal of NO production following co-incubation with mCRP.

Similar to the response by unactivated monocytes, mCRP significantly ($P < 0.001$) increased NO production by LPS-activated monocytes. In direct contrast, there was a significant ($P < 0.01$) dose-dependent decrease in NO production by LPS-activated monocytes following treatment with nCRP. Estrogen significantly ($P < 0.05$) reversed mCRP-induced NO production to levels below that of the control while having no effect on the nCRP-mediated inhibition of NO production by LPS-activated monocytes (**Figure 8**).

Estrogen had no significant ($P > 0.05$) effect on NO production when co-supplemented with mCRP in unactivated (**Figures 9A,B**) and LPS-activated (**Figures 10A,B**) macrophages, mirroring the fact treatment with mCRP alone had no significant effect on NO production by macrophages.

The nCRP isoform significantly reduced NO production by unactivated and LPS-activated macrophages. Estrogen significantly reversed ($P < 0.01$) the nCRP-mediated reduction in NO production by unactivated macrophages treated with low (**Figure 9C**) but not high (**Figure 9D**) levels of nCRP. Co-incubation with estrogen maintained the nCRP-mediated reduction in NO production by LPS-activated macrophages (**Figures 10C,D**), in line with modest reductions in NO following treatment with estrogen alone (in the absence of CRP isoforms).

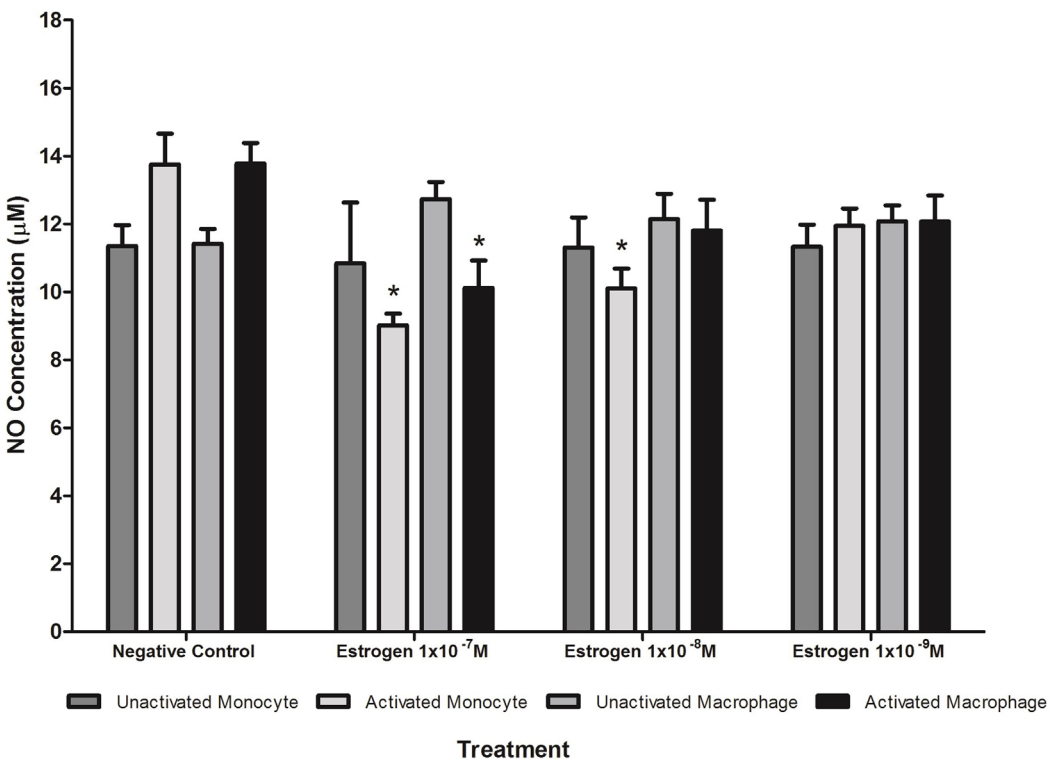

FIGURE 6 | Effect of estrogen on nitric oxide (NO) production. Estrogen reduced NO production by lipopolysaccharide (LPS)-activated U937 monocytes and macrophages. * denotes a significant ($P < 0.05$; $n = 12$) difference in NO production followed by treatment with estrogen (1×10^{-7} 1×10^{-8}, or 1×10^{-9} M) compared to the negative control. Errors bars represent the SEM.

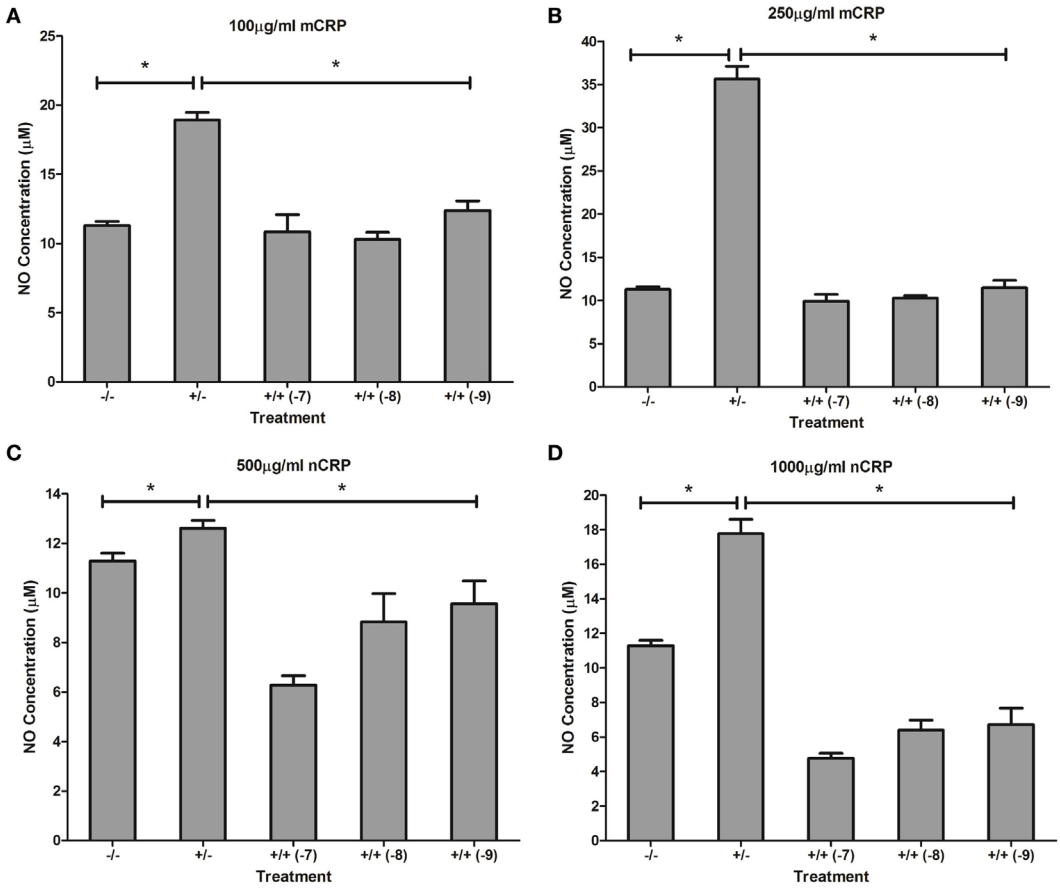

FIGURE 7 | Effect of estrogen on C-reactive protein (CRP)-mediated nitric oxide (NO) production by unactivated U937 monocytes. Estrogen reversed CRP-induced NO production by unactivated U937 monocytes **(A–D)**. $^{-/-}$ represents the untreated negative control with no CRP or estrogen supplementation. $^{+/-}$ represents treatment with CRP alone. $^{+/+}$ represents treatment with both CRP and estrogen. The number in parentheses indicates the concentration (1×10^{-7}, 1×10^{-8}, or 1×10^{-9} M) of estrogen. *Denotes a significant ($P < 0.05$; $n = 12$) difference in NO production following treatment with CRP or co-supplementation with estrogen. Errors bars represent the SEM.

DISCUSSION

The Effect of Exogenous CRP Isoforms on NO Production

The response to each CRP isoform appeared to be dependent on the differentiation and activation status of cells. The mCRP isoform stimulated NO and iNOS production by monocyte-like U937 cells, regardless of the activation state. This agrees with studies showing CRP can induce NO production in both the presence and absence of infection (28, 53). The mCRP isoform has previously been shown to stimulate NO production in neutrophils (34). In stark contrast, the nCRP isoform reduced NO and iNOS production by macrophage-like cells, both in the presence and absence of endotoxin. Furthermore, in LPS-activated monocytes, nCRP reduced NO and iNOS levels in a concentration-dependent manner. These findings support growing evidence that nCRP often has opposing actions to mCRP and dampens the overall inflammatory response relative to that induced by mCRP following tissue injury or infection (54, 55).

Collectively, these findings suggest that CRP isoforms mediate NO levels in U937 monocytes/macrophage *via* changes in the levels of iNOS. Previous work has also showed that CRP can mediate NO production in cardiac myocytes *via* iNOS (53) but responses to each specific CRP isoform were not determined. The data indicate that nCRP may have an inhibitory effect on NO and iNOS production by circulating monocytes following activation by bacterial endotoxin, and by tissue macrophages with or without prior LPS-activation. In direct contrast, the data suggest mCRP may have a stimulatory effect on NO and iNOS production by circulating monocytes with or without prior activation by bacterial endotoxin but this effect is lost once monocytes differentiate into tissue macrophages. In the absence of infection, the findings indicate both nCRP and mCRP increase NO and iNOS production by monocytes but the stimulation by nCRP is relatively modest compared to that produced by mCRP at CRP concentrations typical of an acute inflammatory response. The relative proportion of each CRP isoform *in vivo* could possibly influence the inflammatory response generated, with the formation of mCRP from the dissociation of nCRP promoting

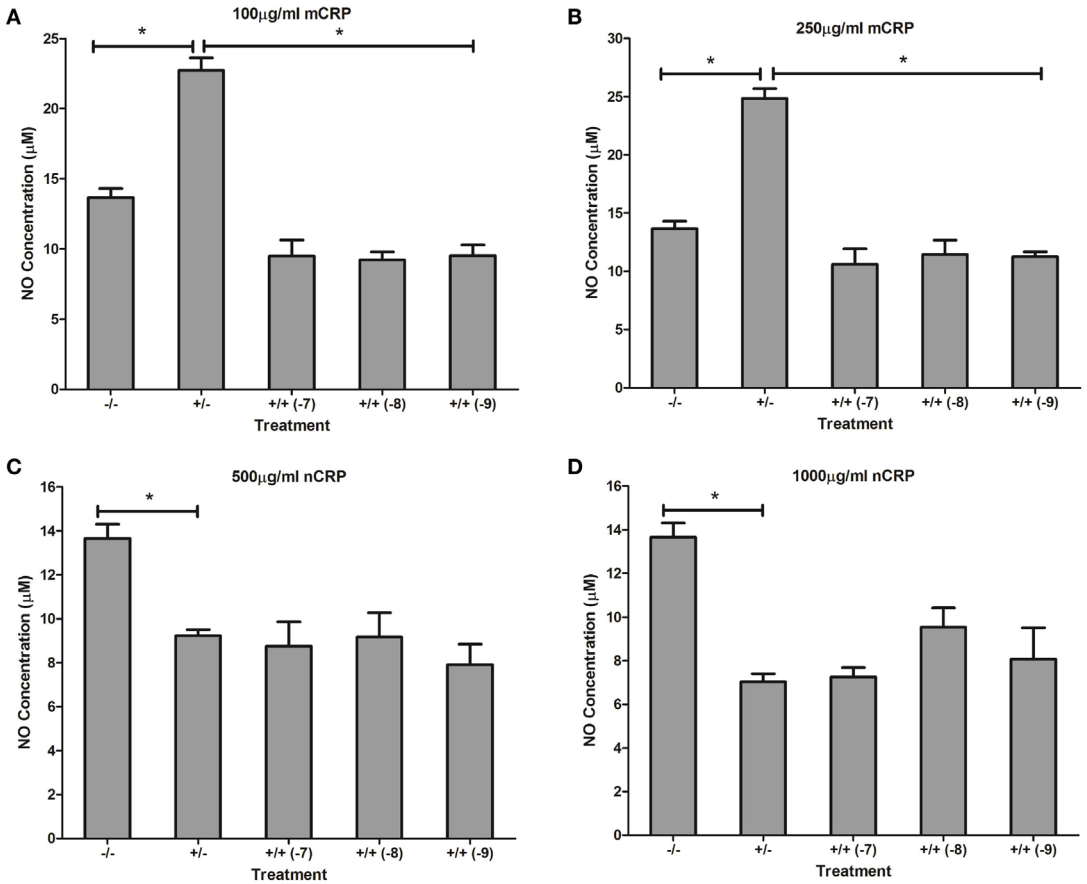

FIGURE 8 | Effect of estrogen on C-reactive protein (CRP)-mediated nitric oxide (NO) production by lipopolysaccharide (LPS)-activated U937 monocytes. Estrogen reversed monomeric CRP (mCRP)-induced NO production **(A,B)** but had no effect on native CRP (Ncrp)-mediated inhibition of NO production **(C,D)** by activated U937 monocytes. $^{-/-}$ represents the untreated negative control with no CRP or estrogen supplementation. $^{+/-}$ represents treatment with CRP alone. $^{+/+}$ represents treatment with both CRP and estrogen. The number in parentheses indicates the concentration (1×10^{-7}, 1×10^{-8}, or 1×10^{-9}M) of estrogen. *Denotes a significant ($P < 0.05$; $n = 12$) difference in NO production following treatment with CRP or co-supplementation with estrogen. Errors bars represent the SEM.

inflammation. Elevated levels of NO are known to induce the production of pro-inflammatory cytokines (56) including IL-6 and tumor necrosis factor alpha (TNF-α) by both monocytes/macrophages (57, 58) and neutrophils (59). The release of such cytokines can stimulate iNOS, leading to further NO production (60) and increased phagocytosis (61).

Effect of Nystatin on CRP-Induced NO Production

Nystatin was the only inhibitor applied in this study. Several other inhibitors had been considered but could not be utilized, since they directly affect NO production (62–64). Nystatin is a relatively mild inhibitor that binds to cholesterol and disassembles lipid rafts in cellular membranes (65). The mCRP isoform is known to associate with lipid rafts in the cell membranes (13, 66).

In this study, nystatin reversed nCRP-induced NO and iNOS production by unactivated monocytes and mCRP-induced NO and iNOS production by LPS-activated monocytes. Thus, given nystatin disrupts lipid rafts and these findings suggest nCRP may interact with lipid rafts in monocytes in the absence of infection, whereas mCRP may associate with lipid rafts in monocytes following infection. Given the binding of mCRP to integrins αvβ3 and α4β1 influences its pro-inflammatory actions (67), antagonizing these integrins may help to elucidate mechanisms by which mCRP mediates NO production.

In both unactivated and activated macrophages, nystatin had no significant ($P > 0.05$) effect on the nCRP-mediated reduction in NO and iNOS. This suggests that once monocytes become mature macrophages, nCRP might no longer associate with lipid rafts. The mCRP isoform had no effect on NO or iNOS production by macrophages in the presence or absence of LPS, so nystatin inhibition was irrelevant.

Treatment of U937 monocytes and macrophages with nystatin alone had no significant effect on NO levels, suggesting this inhibitor did not directly disrupt NO pathways through its effect on cell membranes. Indeed, nystatin has been routinely utilized in various investigations of CRP activity and studies have confirmed

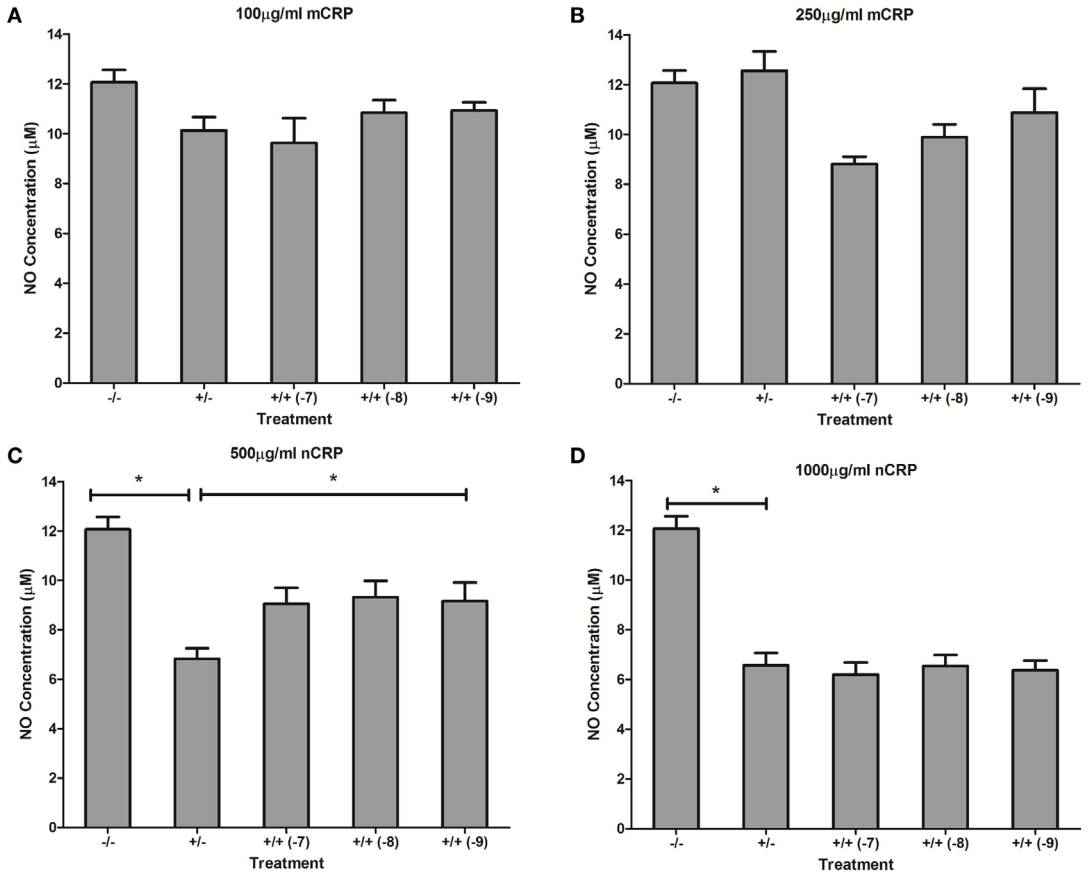

FIGURE 9 | Effect of estrogen on CRP-mediated NO production by unactivated U937 macrophages. Estrogen had no effect on NO production when co-supplemented with mCRP in unactivated **(A,B)** macrophages. However, estrogen reversed the nCRP-mediated reduction in NO production by unactivated macrophages treated with low **(C)** but not high **(D)** levels of nCRP. $^{-/-}$ represents the untreated negative control with no CRP or estrogen supplementation. $^{+/-}$ represents treatment with CRP alone. $^{+/+}$ represents cells treatment with both CRP and estrogen. The number in parentheses indicates the concentration (1×10^{-7}, 1×10^{-8}, or 1×10^{-9}M) of estrogen. * denotes a significant ($P < 0.05$; $n = 12$) difference in NO production following treatment with nCRP or co-supplementation with estrogen. Errors bars represent the SEM.

it does not cause a functional disturbance of cellular cholesterol homeostasis (13, 68).

Effect of Estrogen on CRP-Induced NO Production

Physiological concentrations of estrogen had distinct effects on CRP-mediated NO production, dependent on cell differentiation and activation state, agreeing with studies showing that estrogen can have both suppressive and stimulatory effects on iNOS and NO production (35). The decrease in CRP-induced NO levels by estrogen in unactivated monocytes treated with nCRP or mCRP supports evidence showing estrogen reduces the inflammatory response, including the production of NO/iNOS (37, 40). Estrogen reversed mCRP-induced NO production by LPS-activated monocytes, while the nCRP-mediated reduction in NO production was maintained by physiological concentrations of estrogen in LPS-activated monocytes. This supports previous work showing estrogen suppresses pro-inflammatory effects, including NO production (69, 70) and inflammatory cytokines such as IL-6 in activated monocytes (71). Indeed, treatment of LPS-activated monocytes with estrogen in the absence of CRP also reduced NO levels but the reduction was modest compared to the estrogen-mediated reversal of NO production following treatment with mCRP.

In both unactivated and LPS-activated macrophages, nCRP significantly decreased NO production, indicative of the relative anti-inflammatory properties of nCRP in tissues (11). Reversal of the nCRP-mediated reduction in NO production by estrogen in unactivated macrophages concurs with findings showing increased iNOS expression and NO production by peritoneal macrophages and splenocytes exposed to estrogen (35, 72). Furthermore, some studies have shown that estrogen can induce interferon-gamma (IFN-γ) secretion and subsequent NO production by macrophages (35, 73, 74). The nCRP-induced reduction in NO by activated macrophages was sustained by physiological concentrations of estrogen, concurring with the reduction in NO production following treatment of LPS-activated macrophages with estrogen in the absence of CRP isoforms. These findings agree with recent evidence showing estrogen reduces the inflammatory response, including NO production, in LPS-activated macrophages (75).

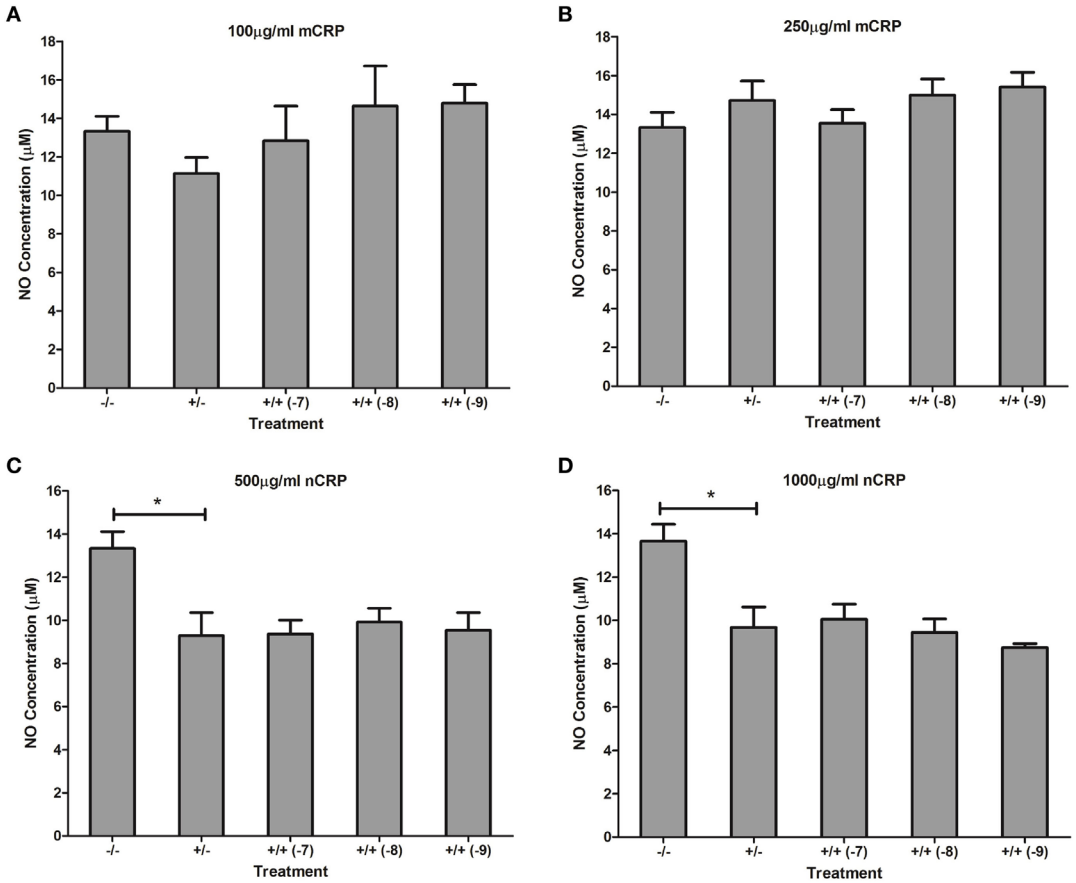

FIGURE 10 | Effect of estrogen on CRP-mediated NO production by LPS-activated U937 macrophages. Estrogen had no effect on NO production when co-supplemented with mCRP **(A,B)** or nCRP **(C,D)** in activated U937 macrophages. $^{-/-}$ represents the negative control with no CRP or estrogen supplementation. $^{+/-}$ represents treatment with CRP alone. $^{+/+}$ represents treatment with both CRP and estrogen. The number in parentheses indicates the concentration (1 x 10^{-7}, 1 x 10^{-8}, or 1 x 10^{-9}M) of estrogen. * denotes a significant ($P < 0.05$) reduction in NO production following treatment with nCRP compared to the control ($n = 12$). Errors bars represent the SEM.

CONCLUSION

In conclusion, the findings suggest that CRP isoforms are potential targets for mediating NO production by human monocytes and macrophages. Each CRP isoform induced a distinct profile of NO responses that was dependent on cell differentiation and activation status. In the presence or absence of endotoxin, nCRP had an inhibitory effect on NO/iNOS production in macrophages whereas mCRP had no effect. The mCRP had a stimulatory effect on NO/iNOS synthesis in monocytes, with or without LPS-activation. The nCRP isoform had opposing effects in monocytes, reducing NO/iNOS production following LPS-activation but stimulating NO by unactivated monocytes. These findings warrant further investigation to confirm the results in additional cell lines and *ex vivo* primary human leukocytes, together with the use of selective inhibitors to elucidate the pathways involved. Animal models of age-related impaired healing could assess the effect of CRP isoforms on *in vivo* NO production during aging. The predominant inhibition of NO/iNOS production by nCRP in all cell states except unactivated monocytes and the stimulation of NO/iNOS by mCRP in monocytes supports growing evidence that mCRP initiates a greater inflammatory response relative to nCRP. Furthermore, the findings highlight the importance of the age-related decline in hormones (notably estrogen) on CRP-mediated responses and have implications for therapeutic strategies aimed at the elderly. With the exception of NO stimulation by estrogen in unactivated macrophages treated with nCRP, the predominant reduction of CRP-induced NO levels by estrogen supports evidence that estrogen reduces the inflammatory response following injury and infection. Thus, therapeutic strategies that restore estrogen levels to those found in youth and promote the stability of nCRP and/or prevent the formation of mCRP from the dissociation of nCRP may reduce overall NO production in age-related inflammatory conditions.

AUTHOR CONTRIBUTIONS

NS and ME conducted the experimental work, analysed and interpreted the data, and prepared the paper. WG and MS contributed to the conception and design of the study and revising the paper. JA was involved with all aspects of the work including

conception/design of the study, supervising experimental work, analysing and interpreting the data, and writing and revising the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01500/full#supplementary-material.

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Acetylcholine Inhibits Monomeric C-Reactive Protein Induced Inflammation, Endothelial Cell Adhesion, and Platelet Aggregation; A Potential Therapeutic?

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Objectives: In this study, we examined the possibility of using targeted antibodies and the potential of small molecular therapeutics (acetylcholine, nicotine and tacrine) to block the pro-inflammatory and adhesion-related properties of monomeric C-reactive protein (mCRP).

Methods: We used three established models (platelet aggregation assay, endothelial leucocyte binding assay and monocyte inflammation via ELISA and Western blotting) to assess the potential of these therapeutics.

Results: The results of this study showed that monocyte induced inflammation (raised tumor necrosis factor-alpha-TNF-α) induced by mCRP was significantly blocked in the presence of acetylcholine and nicotine, whilst tacrine and targeted antibodies (clones 8C10 and 3H12) had less of or no significant effects. Western blotting confirmed the ability of acetylcholine to inhibit mCRP-induced cell signaling phosphorylation of extracellular signal regulated kinase 1/2 (ERK1/2), p38 and nuclear factor-kappa B (NF-κB). There was no evidence of direct binding between small molecules and mCRP. mCRP also induced endothelial cell-monocyte adhesion in a dose dependent fashion, however, both acetylcholine and nicotine as well as targeting antibodies notably inhibited adhesion. Finally, we investigated their effects on mCRP-induced platelet aggregation. All three small molecules significantly attenuated platelet aggregation as did the antibody 8C10, although 3H12 had a weaker effect.

Discussion: Acetylcholine and to a lesser extent nicotine show potential for therapeutic inhibition of mCRP-induced inflammation and cell and platelet adhesion. These results highlight the potential of targeted antibodies and small molecule therapeutics to inhibit the binding of mCRP by prevention of membrane interaction and subsequent activation of cellular cascade systems, which produce the pro-inflammatory effects associated with mCRP.

Keywords: CRP, inflammation, cell adhesion, acetylcholine, nicotine

INTRODUCTION

Activated platelets and endothelial leucocyte interactions represent an important link between pro-thrombotic and pro-inflammatory association of monomeric C-reactive protein (mCRP). These interactions may play a significant role in atherogenesis of cardiovascular diseases leading to acute ischemic stroke and a more chronic role in the development of brain lesions in vascular dementia and associated diseases (1).

It has been shown that cell membranes, liposomes, and static activated platelets can dissociate pentameric or native C-reactive protein (nCRP) into the monomeric and highly pro-inflammatory form via binding to phosphocholine groups on lysophosphatidylcholine. The main proposed mechanism of mCRP-associated interaction with cellular membranes and receptors is via its cholesterol binding domain (cystathionine-β-synthase; CBS; a.a 35–47). In this mechanism, the hydrophobic region of the mCRP inserts into lipid rafts of the plasma membrane binding to cholesterol molecules (2). Similarly, deposition of mCRP on to endothelial cell (EC)-circulating micro particles seems to be associated with chronic inflammation and linked to macrophage activation and T cell polarization (3).

Recently, Thiele et al. (4) described a phospholipase-A2 blocking mechanism using 2-(p-amylcinnamoyl)-amino-4-chlorobenzoic acid, (ONO-RS) that effectively prevented nCRP association with lysophosphatidylcholine on the cell surface and subsequent dissociation to mCRP concomitantly, significantly attenuating the pro-inflammatory effects of the protein both *in vitro* and *in vivo*. This work suggested that effective blocking of the binding of mCRP to the cell membrane could inhibit dissociation and abrogate the detrimental effects known to be associated with neurological inflammation and subsequent stroke worsening and/or dementia, as well as cardiovascular instability and complication (5). However currently considered anti-inflammatory molecules such as interleukin-1 receptor (IL-1R) antagonist may not be effective therapeutic agents being difficult to pass through the blood-brain-barrier (6) and/or having possible toxic and off pathway side-effects when given systemically at doses that could be useful therapeutically (7). Given the structural similarity between phosphocholine and acetylcholine we became interested in examining the potential of this neurotransmitter and two representative cholinergic small molecules, nicotine and tacrine, to perturb the actions of mCRP.

Here, we investigated the potential of a number of compounds anticipated to interact with mCRP/phosphatidylcholine in an effort to understand their capability and, subsequently, mechanism of action in blocking mCRP-mediated inflammation, EC-monocyte activation and platelet aggregation.

MATERIALS AND METHODS

Cell Culture and Differentiation

U937 cells were maintained in RPMI 1640 medium supplemented with 10% de-complemented Fetal Bovine Serum (FBS) under humidified 5% CO_2 air at 37°C in a T-75 flask. The media was changed every 3 days. Cell viability was estimated using a Biorad TC1 automatic cell counter. Cell viability was maintained above 90% for the experiment. To induce monocyte differentiation into adherent macrophages, the U937 cells were seeded at an initial density of 2×10^6 in 2 ml of differential media/well [growth media with phorbol-12-myristate 13-acetate (PMA) at 50 ng/ml for 72 h in a 6-well plate]. Following differentiation, the cells were washed twice with warm Dulbecco's Phosphate Buffered Saline (DPBS). Next, the macrophages were starved in RPMI 1640 medium supplemented with 2% FBS under humidified 5% CO_2 air at 37°C for at least 24 h. Next, the macrophages were stimulated for 8 min with mCRP (for Western blotting based on our previously published observations) and 24 h for inflammation assays following 2 h pre-incubation with acetylcholine (10–100 μM), nicotine (0.93 μM) or tacrine (1 μM). Concentrations of small molecules were chosen based on published literature showing their use as inhibitors in macrophages/glia, [acetylcholine, (8)]; [nicotine, (9)]; [tacrine, (10)] and our own pilot observations and optimization (toxicity assay assessment of viability using a range of concentrations for the three molecules showed that the concentrations above were non-toxic to the U937 cells). For the monocyte-EC adhesion assay, immortalized human brain microvascular EC cells (HbMEC), were kindly donated by Prof. Babette Weksler (Division of Hematology and Medical Oncology, Weill Medical College, Cornell University, New York). Cells were cultured routinely before use in microvascular EC medium-2 (EBM-2) from Clonetics (Lonza, Germany), supplemented with growth factors as recommended by the manufacturer.

ELISA Assay

Human promonocytic leukemia U937 cells were grown in RPMI 1640 medium supplemented with 10% FBS, (Sigma-Aldrich) in a humidified incubator with 5% CO_2 at 37°C. U937 monocytes (2×10^6 cells/well) were fully differentiated into macrophages after 72 h incubation with 50 ng/ml phorbol-12-myristate 13-acetate (PMA) in 6-well culture plates. After washing twice with DPBS, macrophages were pre-treated with acetylcholine (10 μM), nicotine (0.93 μM), tacrine (5 μM), methyllycaconitine (10 μM), anti-CD16/32/64 (1:100), anti-mCRP antibody 3H12 clone (1:10), or anti-mCRP antibody 8C10 clone (1:10) for 2 h, followed by stimulation with mCRP (100 μg/mL) for an additional 24 h. Mouse monoclonal antibodies to human mCRP sub-unit (8C10/3H12) were obtained from Dr L.A. Potempa and fully characterized as described previously (11). We have previously shown that 8C10 (N-terminal aa-22-45) pre-incubation of EC was sufficient to block angiogenesis and associated cell signaling (12). Here, we employed the use of this antibody and a second similar one (3H12; C-terminal aa-198-206) as "potential" blocking antibodies in U937 inflammatory response.

The production of tumor necrosis factor alpha (TNF-α), IL-6, and IL-10 in the supernatant was quantified using ELISA kits (R&D Systems) according to the manufacturer's instruction. Stimulation with lipopolysaccharide (LPS) (10 ng/mL) for 24 h was used as the positive control for macrophage cytokine

production. Samples were tested in triplicate and results are presented as the mean ± SD from a representative example of three independent experiments, unless specified otherwise in the text. $^{*}P \leq 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ using ANOVA.

Western-Blot Protocol

A general RIPA buffer containing a protease and phosphatase inhibitor cocktail was used to make the cell lysates. Following this, the cell lysates were sonicated for 20 s and centrifuged for 10 min at 10000 RFC at 4°C. The supernatant protein samples were collected and the protein concentrations were estimated using the BCA protein assay. Then, the samples were frozen at −80°C for later use.

Equal quantities of proteins (30 μg) were mixed with 2× Laemmli sample buffer, boiled in a water bath for 15 min and then centrifuged. Samples were separated along with pre-stained molecular weight markers (32,000–200,000 kDa) by 12% SDS-PAGE. Proteins were electro-transferred (Hoefer, Bucks, UK) onto nitrocellulose filters (1 h) (Whatman, Protran BA85, Germany) and the filters were blocked for 1 h at room temperature in TBS-Tween (pH 7.4) containing 1% bovine serum albumin (BSA). Filters were then stained with the primary antibodies diluted in the blocking buffer, overnight at 4°C on a rotating shaker. The following primary antibodies were applied at 1:1,000 dilution: phospho/total-extracellular signal-regulated kinase 1/2 (ERK1/2) (thr202-tyr204; mab/4370 and mab/4695, respectively; from Cell Signaling Antibodies, Bio-rad, Hertfordshire, UK); phospho/total-jun N-terminal kinase 1/2 (JNK1/2) (t183, y185, mab/1205 and mab ab179461, respectively; from Bio-Techne Ltd., Minneapolis, USA); phospho/total-p38 (t180, y182 ab4822, and ab27986, respectively; from Abcam, West Sussex, UK); and phospho/total-nuclear factor kappa B (NFκb) (p65, S529 and p65, ab16502; from Abcam, West Sussex, UK).

After washing (5 × 10 min in TBS-Tween at room temperature), filters were stained with either goat anti-rabbit or rabbit anti-mouse HRP-conjugated secondary antibodies diluted in TBS-Tween containing 5% de-fatted milk (1:2,000, 1 h, room temperature) with continuous mixing. After a further five washes in TBS-tween, proteins were visualized using enhanced chemiluminescence detection (ECL, Thermo Scientific, UK), and semi-quantitatively identified fold differences compared with house-keeping controls (α-tubulin, ab7291, Abcam, West Sussex, UK) were determined using Image-Lab software (Bio-rad, UK). All experiments were repeated three times and a representative example is shown.

NF-κB Translocation Assay

Macrophages were cultured alone or in the presence of LPS (10 μg/ml) as a positive control or mCRP (100 μg/ml) with and without small molecules (2 h pre-incubation as described above) on glass coverslips for 1 h prior to a 5 min wash in PBS. Samples were fixed in 100% methanol at−20°C for 5 min and following evaporation, stored at −80°C.

Prior to staining, cells were rehydrated with 0.05% PBST. Non-specific binding of the secondary antibody was blocked using 4% goat serum (Vector laboratories, Peterborough, UK) for 30 min. Cells were washed with PBST (2 × 5 min) then incubated overnight with NF-κB p65 rabbit mAb 16502 (Cell Signaling, MA, USA) at 1:400 dilution. Cells were then rinsed twice with PBST for 5 min, and incubated with goat anti-rabbit IgG secondary antibody (Alexa Fluor 488; Thermo-Fisher scientific, Runcorn, UK) at 1:250 dilution. Slides were washed with PBS for 5 min, mounted with vector shield (H1200 with DAPI) and left to dry for 20 min before microscopy. Fluorescence images were captured on a Zeiss Z1 AxioObserver fluorescence microscope. Three coverslips/wells were used for each test and 500 cells were counted from each coverslip; and the experiment performed twice. Differences in relative translocation were analyzed using one-way ANOVA with Bonferroni post-test analysis.

Cytoselect Monocyte-Endothelium Adhesion Assay

This was applied according to the manufacturer instruction and referring to the work of Kapitsinou et al. (13). Briefly, human brain microvessel EC (HbMEC) (1×10^5/per well) were added applied to a 96-well plate. After 48 h, when the EC monolayer was formed, they were treated with mCRP (1–100 μg/ml) for 6 h. After removing the medium, they were washed once with serum free medium, and 200 μl of the monocyte suspension already labeled with Leuko-Tracker added to each well and incubated for 90 min. After removing the medium and a further three washes, 150 μl of 1X lysis buffer was added to each well containing cells. Fluorescence was measured using a fluorescence plate reader at 480 nm/520 nm. In these experiments we added nCRP since possible effects on EC-monocyte interactions have not previously been examined. However since published data clearly shows a lack of inflammatory activity on macrophages (3, 14), we did not include it within the other experimental protocols. Each test was conducted in triplicate, repeated three times, and a representative example is given. $^{*}p \leq 0.05$ using Wilcoxon matched pair test.

Platelet Aggregation Assay

Venous blood was taken from non-smoking (since smoking is known to affect/increase platelet aggregation) (15), healthy volunteers with informed consent (carried out with internal ethical approval obtained through our local University ethical committee). Monomeric C-reactive protein-induced platelet activation was evaluated using the platelet aggregation assay, and its coagulation was measured by light-transmission aggregometry (LTA) using platelet rich plasma (PRP). Blood was centrifuged (20 min, 150 g, 20°C) to obtain PRP.

A total of 250 μL of PRP adjusted to 250×10^6 platelets/mL was incubated with 250 μL solution containing mCRP (100 μg/ml) or control buffer and small molecules. Adenosine diphosphate (ADP) at 10 μM was used as a positive control. Antibodies were used at 1:100 dilution. The following concentrations were used for the small molecules, mCRP 100 μg/mL + nicotine 0.93 μM, mCRP 100 μg/mL + acetylcholine 10 μM and mCRP 100 μg/mL + tacrine at 10 μM. Each experiment was performed in triplicate. Results are presented as the mean ± SD of maximum platelet aggregation (%), from a representative example of three independent experiments. $^{*}P \leq 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ using ANOVA.

CRP Purity Testing

In all of the experiments CRP treated with detoxi-gel columns (CRPdt) containing immobilized polymyxin B was used, to ensure the absence of pyrogens/endotoxin (AffinityPak™ detoxi-Gel™ column; Pierce, Rockford, IL) and removal was confirmed using the Limulus assay. Sources were purchased free from sodium azide.

Dot-Blot Assay

Incubation of mCRP With Small Molecules

mCRP was incubated for 2 h with tacrine (10 μM), nicotine (0.93 μM) or acetylcholine (100 μM) prior to binding assessment on nitrocellulose described below.

Dissociation of nCRP to mCRP

The nCRP commercial sample was purchased from Yo protein laboratories (Aachen, Germany). 250 μL of mCRP was added 250 μL of 10 mM EDTA and 8 M urea for chelation and the mixture incubated at 37°C for 2 h, with or without tacrine (10 μM), nicotine (0.93 μM), or acetylcholine (100 μM).

A grid was drawn on a piece of nitrocellulose membrane using a pencil and indicating the region for the blots. Next, using a narrow-mouth pipette tip, 2 μl of samples was placed onto the nitrocellulose membrane at the center of the grid. The penetration area was kept to a minimum by applying it slowly. After drying, non-specific sites were blocked with 5% BSA in TBS-T (0.5–1 h, RT). A 10 cm petri dish was used as a reaction chamber, and samples were incubated with primary anti-mCRP/nCRP antibodies [1:10] dissolved in BSA/TBS-T for 30 min at RT. After washing three times with TBS-T (3 × 5 min), samples were incubated with secondary anti-mouse antibody conjugated with HRP (1:500 for 30 min at RT).

After washing, (TBS-T; 15 min × 1 and 5 min × 2), then once with TBS (5 min), membranes were incubated with ECL reagent for 1 min, covered with Saran-wrap (after removal of excessive solution from the surface), and exposed using the G-box/Image Lab software. All blots were performed in triplicate and experiments were repeated three times with a representative example being shown.

Surface Plasmon Resonance Testing

Surface plasmon resonance (SPR) was used to assess the binding of mCRP to nicotine, acetylcholine, tacrine and 3H12 polyclonal antibody. Monomeric CRP was first buffer exchanged using a 0.5 ml Zeba™ spin column (Thermo) which was pre-equilibrated in PBS. mCRP was then incubated with a 1:2 ratio of NHS-Peg4-Biotin (Thermo) for 30 min at room temperature before purifying the free biotin with another Zeba™ spin column in PBS. SPR was performed on a Biacore T200 (GE life sciences) and a streptavidin coated SA chip. The instrument was equilibrated in Biacore buffer, 10 mM HEPES buffer pH 7.4 with 0.05% tween 20 and the SA chip was washed with 10 mM EDTA and 50 mM NaOH prior to loading with biotinylated mCRP. For the antibody binding test the chip was loaded with ∼100 response units of mCRP. For the small molecule tests the chip was loaded to a maximum loading of ∼3,500 response units of biotinylated mCRP. Analytes, either antibody at 1:100 dilution, 1 mM nicotine, 1 mM acetylcholine, or 10 μM tacrine diluted in Biacore buffer were injected at 30 μl/min over the mCRP surface and the response monitored in real time. The results are shown as a subtracted response where flow cell 1 was used as a reference with no mCRP added which was subsequently subtracted from the data.

Statistical Analyses

Data are presented as the mean ± SD of individual representative experiments carried out in triplicate. Statistical analysis was performed using GraphPad Prism software version 7.0 for Windows (GraphPad Software). The values were compared using paired Student's *t*-test, non-parametric Wilcoxon test, or one-way ANOVA with Bonferroni post-test analysis. $^{*}P \leq 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

RESULTS

mCRP Modulated TNF-α, IL-6, and IL-10 Expression in U937-Derived Macrophages

To determine the effect of mCRP on the expression of inflammatory mediators, U937-derived macrophages were exposed for 24 h to 100 μg/ml mCRP and secreted protein levels of TNF-α, IL-6, and IL-10 in the supernatant were quantified using ELISA. As shown in **Figure 1**, mCRP significantly increased the secretion of pro-inflammatory cytokines including TNF-α ($P < 0.05$, **Figure 1A**) and IL-6 ($P < 0.01$, **Figure 1B**), exerting a similar response to LPS. Surprisingly, mCRP significantly decreased the levels of anti-inflammatory cytokine IL-10 by 25% (derived from our included representative experiment and similarly decreased in repeated tests; $P < 0.05$, **Figure 1C**), opposite to LPS which augmented the production of IL-10 by 2.2-fold ($P < 0.001$, **Figure 1C**).

We then investigated whether the small molecules (acetylcholine, nicotine, tacrine) and anti-mCRP antibodies (3H12 and 8C10) would affect macrophage cytokine profiles induced by mCRP. Pre-treatment with acetylcholine, nicotine, tacrine and anti-mCRP antibody 3H12 significantly inhibited TNF-α production induced by mCRP, with the strongest inhibition by acetylcholine (63.8% reduction), followed by nicotine (52.6% reduction), 3H12 (31.8% reduction), and tacrine (25.9% reduction) (derived from our included representative experiment and similarly decreased in repeated tests; ANOVA, $P < 0.05$, **Figure 1A**). Both acetylcholine and nicotine also significantly decreased IL-6 levels induced by mCRP ($P < 0.05$, **Figure 1B**). In addition, acetylcholine and 3H12 tended to restore the mCRP repressed IL-10 levels back to normal ($P < 0.05$, **Figure 1C**). Small molecules alone did not significantly increase macrophage cytokine expression (values for TNF-α shown in **Figure 1D**). A small increase in TNF-α was seen in the presence of 3H12/8C10 alone (in the absence of mCRP) and this was probably due to a residual low concentration of endotoxin found in the supernatant. To confirm receptor interaction of mCRP on the cell surface of macrophages we pre-incubated U937 with a pharmacological

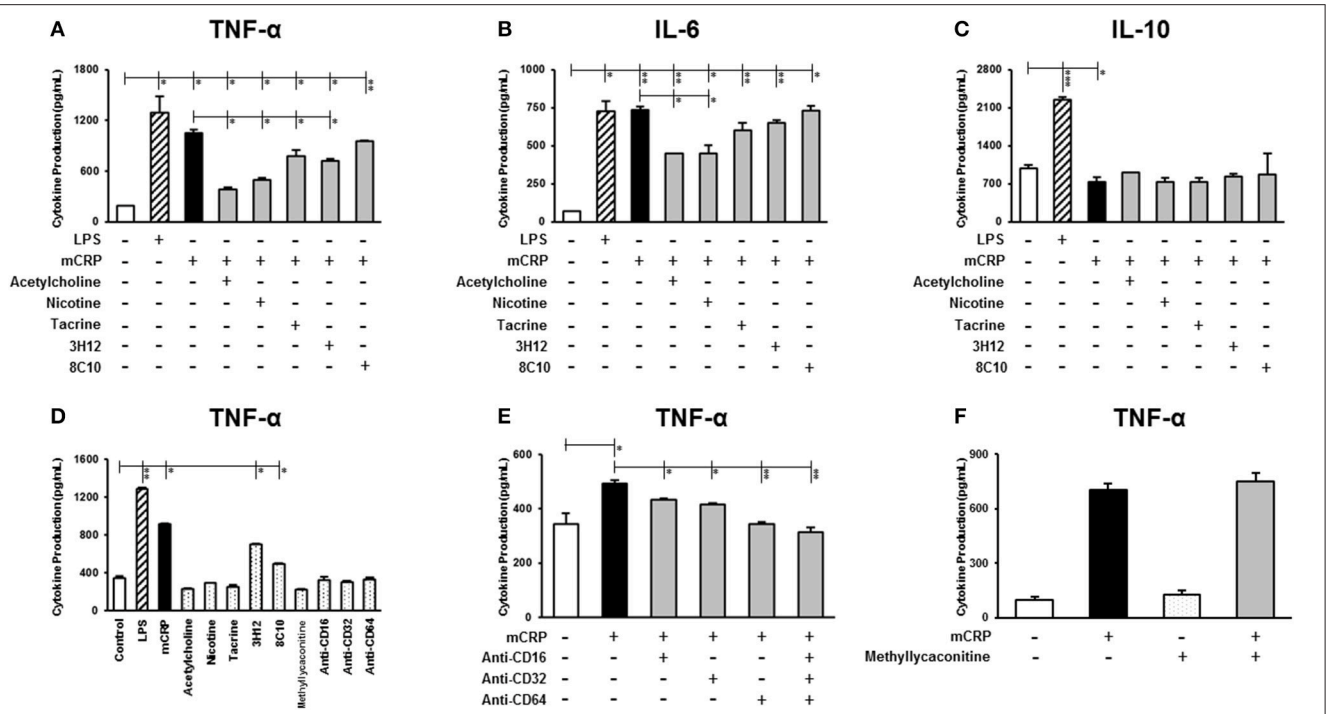

FIGURE 1 | The effect of mCRP on macrophage cytokine production. The differentiated U937 macrophages were pre-treated with acetylcholine (10 μM), nicotine (0.93 μM), tacrine (5 μM), anti-mCRP antibodies 3H12 (1:10) or 8C10 (1:10) for 2 h, followed by stimulated with mCRP (100 μg/mL) for an additional 24 h. The production of TNF-α **(A)**, IL-6 **(B)**, and IL-10 **(C)** in the supernatant was quantified using ELISA kits (R&D System). The stimulation with LPS (10 ng/mL) for 24 h was used as the positive control for macrophage cytokine production. **(D)** Shows that treatment with small molecules alone, nicotinic acid receptor antagonist methyllycaconitine (10 μM) or CD16/32/64 to FC-ϫ receptors (1:100) did not affect cytokine (TNF-α) production in U937 cells, although there was a marginal increase in TNF-α in the presence of the mCRP antibodies probably due to residual contamination of low levels of LPS. When cells were pre-treated with CD16/32/64 to FC-ϫ receptors (1:100) for 2 h, complete abrogation of mCRP-induced TNF-α production was shown **(E)**, whilst pre-incubation with the nicotinic acid receptor antagonist methyllycaconitine (10 μM) for 2 h in the presence of either acetylcholine or nicotine did not reverse the anti-inflammatory capacity **(F)**. Results are presented as the mean ± SD from a representative example of three independent experiments. $^{*}P \leq 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ using one-way ANOVA with Bonferroni post-test analysis.

antagonist of nicotinic α7 receptor (methyllycaconitine; 10 μM) or blocking antibodies CD16/32/64 to FC-ϫ receptors (1:100) for 2 h. A combination of antibodies CD16/32/64 was able to completely abrogate mCRP-induced TNF-α production ($P < 0.01$, **Figure 1E**), whilst pre-incubation with the nicotinic receptor antagonist methyllycaconitine (10 μM shown and 1–100 μM tested) for 2 h in the presence of nicotine did not reverse the anti-inflammatory effects suggesting a pathway not working through the nicotinic receptor signaling (**Figure 1F**). Acetylcholine, nicotine and methyllycaconitine cytotoxicity studies are included as **Supplementary Figure 1**, and no cytotoxicity was observed at any of the concentrations used.

mCRP Induced the Phosphorylation of MAPK and Activation of NF-κB in Cultured Macrophages

Differentiated U937 macrophages were pre-treated with acetylcholine (100 μM, lane 4; 10 μM, lane 5) or nicotine (0.93 μM, lane 6) for 2 h, followed by stimulation with mCRP (100 μg/mL, lanes 3–6) for an additional 8 min (**Figure 2A**). Since tacrine was not effective in blocking macrophage-induced inflammation (**Figure 1**) we did not include it in these signaling experiments. The first lane was loaded with the extract from untreated macrophages as control. The cultured macrophages treated with LPS (10 ng/mL) for 8 min were used as a positive control (lane 2). Results show that acetylcholine was effective in blocking phosphorylation of ERK1/2, NF-κB, and JNK1/2 but not p-38 MAP kinase. Nicotine similarly inhibited phosphorylation of ERK1/2 JNK1/2 and NF-κB but not p38 signaling molecules. **Figures 2B–E** shows densitometric analysis of immunoblot quantification. Interestingly, acetylcholine alone increased NF-κB phosphorylation as well as that of p-38 MAP kinase, whilst increased p-38 phosphorylation was also seen after addition of nicotine. No change in the expression of "total" proteins was seen.

To confirm activation of NF-κB, we performed immunofluorescent staining on cultured U937 macrophages exposed to mCRP (100 μg/ml; 1 h) or LPS as a positive control (10 ng/ml). Nuclear translocation of NF-κB was clearly seen in LPS-treated cells (∼63% of cells; iii) and also mCRP treated U937 (∼30%; ii) but not in control untreated cells (<1%; i) as shown in **Figures 2F,G** ($P < 0.05$ from a representative experiment, which was repeated giving similar results). None

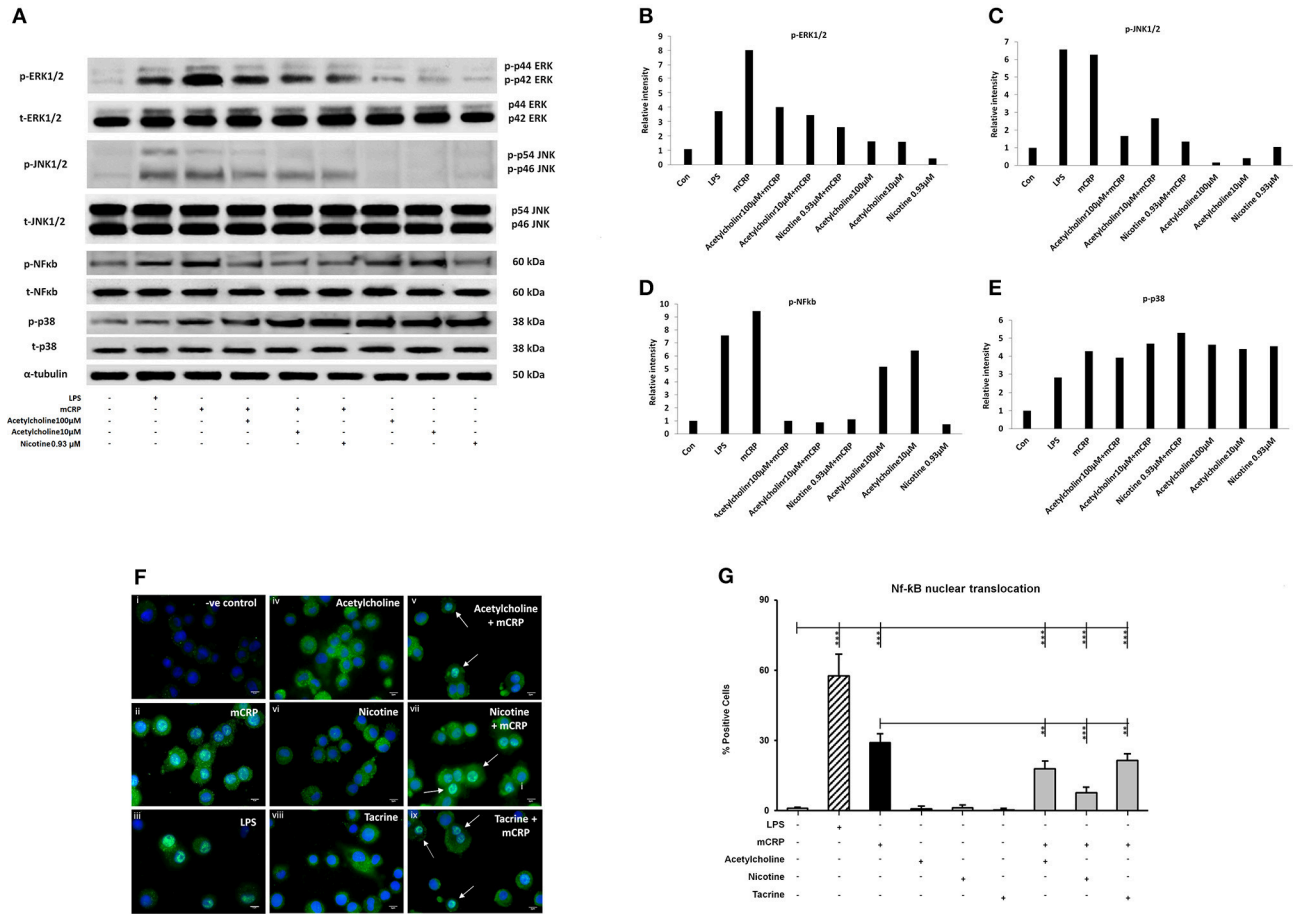

FIGURE 2 | The expression of selected phospho-proteins by macrophages. 2×10^6 (U937 cells) were differentiated in one well of a 6-well plate. After 72 h, when the macrophage monolayer had formed, they were treated with various concentrations of acetylcholine (10, 100 μM) or nicotine (0.93 μM) with or without mCRP (100 μg/ml) for 8 min. After removing the medium, they were washed twice with ice-cold PBS, followed by addition of 200 μl of 1X lysis Buffer to each well. Lane 1, control; Lane 2, LPS; Lane 3, mCRP; Lane 4, acetylcholine (10 μM) + mCRP; Lane 5, acetylcholine (100 μM) + mCRP; Lane 6, nicotine + mCRP; Lane 7, acetylcholine alone (100 μM); Lane 8, acetylcholine alone (10 μM); Lane 9, nicotine alone. **(A)** Expressions of selected proteins. **(B)** Bar chart shows phospho-ERK1/2 expression. **(C)** Bar chart shows phospho-JNK expression. **(D)** Bar chart shows phospho-p38 expression. **(E)** Bar chart shows phospho-NFκb expression. **(F)** Shows immunofluorescent demonstration of nuclear translocation of NFκb FITC green with DAB-blue positively stained nuclei (I, negative control; ii, mCRP treated for 1 h; iii, LPS positive control; iv, acetylcholine; v, acetylcholine + mCRP; vi, nicotine; vii, nicotine + mCRP; viii, tacrine; ix, tacrine + mCRP), and **(G)** is a representative bar chart showing the percentage of cells showing nuclear translocation from 500 counted cells/coverslip. Pre-incubation with nicotine (0.93 μM; 1 h) significantly reduced NFκb translocation. Each test was carried out in triplicate and the experiments conducted twice. A representative example is given here. $^{**}P < 0.01$; $^{***}P < 0.001$ using one-way ANOVA with Bonferroni post-test analysis.

of the small molecules alone had any effect on translocation (iv, vi, and viii), however pre-incubation of cells with nicotine (0.93 μM; 1 h) significantly reduced the translocation of NF-κB ($p < 0.05$; from 30 to 18% in our presented experiment which was performed twice giving similar results). This data confirmed mCRP-induced activation of the NF-κB signaling pathway with gene transcriptional involvement.

mCRP-Induced EC-Monocyte Adhesion Tends to Be Inhibited by 8C10 Antibody

Monocyte adherence to the endothelium is a strong indicator of abnormal activity/activation and potential for inflammatory signaling associated with vascular damage that may ultimately lead to atherosclerosis. mCRP significantly increased EC adhesion to monocytes in a dose dependent manner whilst native CRP (nCRP) had no significant effect (**Figure 3A**). Acetylcholine and nicotine alone significantly promoted EC adhesion to monocytes ($P < 0.05$), however, in the presence of mCRP, they tended to antagonize mCRP-mediated adhesion of EC to monocytes (non-significant; **Figure 3B**). mCRP-induced EC-monocyte adhesion tended to be inhibited by 8C10 particularly at 1:10 dilution (**Figure 3C**). **Figure 3D** shows that mCRP specific antibody 3H12 had a weak but non-significant inhibitory effect at 1:10 dilution only. Each test was conducted in triplicate and statistical analysis performed using the Wilcoxon matched pair test. Experiments were repeated three times and a representative example is given.

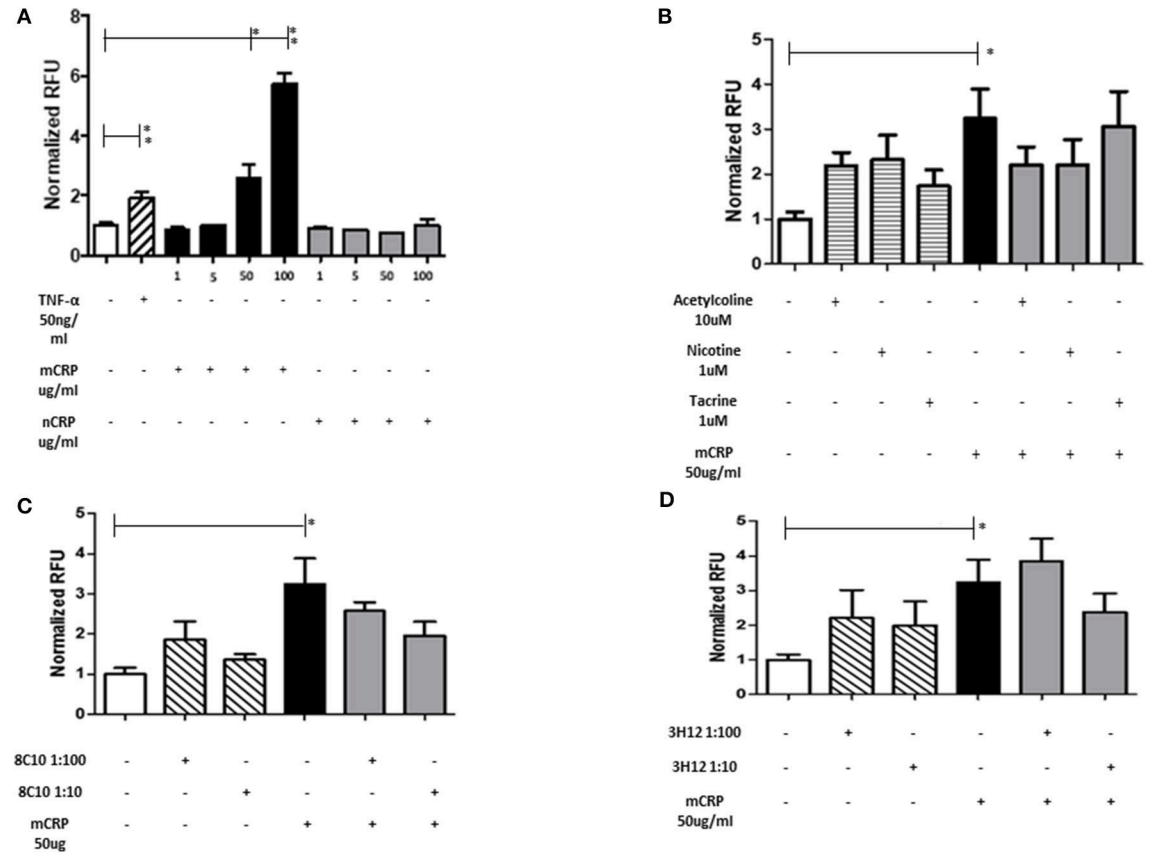

FIGURE 3 | The effect of mCRP on endothelial cell adhesion to U937. After treating with mCRP for 6 h (10–100 μg/ml), monolayer HBmEC were incubated with U937 cells labeled with Leuko-Tracker for 60 min. Fluorescence of the control wells containing EC and U937 without any treatment were arbitrarily set as 1. **(A)** mCRP significantly increased EC adhesion to U937 in a dose dependent manner, however nCRP had no significant effect. **(B)** Acetylcholine (10 μM), and nicotine (0.93 μM), alone significantly promoted EC adhesion to U937, however, in the presence of mCRP, they antagonized mCRP-mediated adhesion of EC to U937 (non-significant). **(C)** Monomeric C-reactive protein specific antibody 8C10 also inhibited mCRP-mediated EC adhesion to U937. **(D)** mCRP specific antibody 3H12 reduced the aggregation but to a lesser extent than 8C10 (non-significant). Each test was conducted in triplicate, repeated three times, and a representative example is given. $^{*}p \leq 0.05$, $^{**}p < 0.01$ using Wilcoxon matched pair test. Using ANOVA, no statistical differences were found in the inhibition of mCRP-driven EC adhesion to U937 cells.

Small Molecules and Antibodies Effectively Blocked Platelet Aggregation

The measurement of platelet aggregation is a strong indicator of the potential for thrombus or clot formation in acute coronary syndromes. Platelet aggregation analysis revealed that mCRP (100 μg/ml; 5 min), induced platelet aggregation (40–50%), as seen in **Figure 4A**. Monomeric C-reactive protein-induced platelet aggregation was blocked in the presence of the anti-mCRP antibody (8C10), and partially prevented by anti-mCRP antibody (3H12). All three small molecules almost completely inhibited mCRP-induced platelet aggregation ($P < 0.05$), but did not block aggregation induced by ADP (10 μM; **Figure 4B**). Monomeric C-reactive protein-induced aggregation of platelets was independently tested using a second healthy donor and similar levels of aggregation were produced (**Supplementary Figure 2**).

Antibody and Protein Binding Studies Indicated No Direct Interaction Between Small Molecules and mCRP

Here, we used dot blotting with our specific mCRP antibody 3H12 in order to examine possible interactions that would affect surface structure change/binding. No significant change in specific antibody binding was found between the mCRP control sample and mCRP samples pre-treated for either 2 or 4 h with any of the three small molecules (**Figure 5A**). After incubation of nCRP with urea at 37°C, dot blots obtained following incubation with mCRP antibody 3H12 indicated that urea mediated dissociation of nCRP to mCRP was not affected by acetylcholine, tacrine or nicotine (**Figure 5B**).

SPR binding analysis was used to identify any adsorption of material on to the mCRP-coated electrodes. Results indicated that under these conditions there was no observable direct interaction of any of the small molecules to biotinylated mCRP. The 3H12

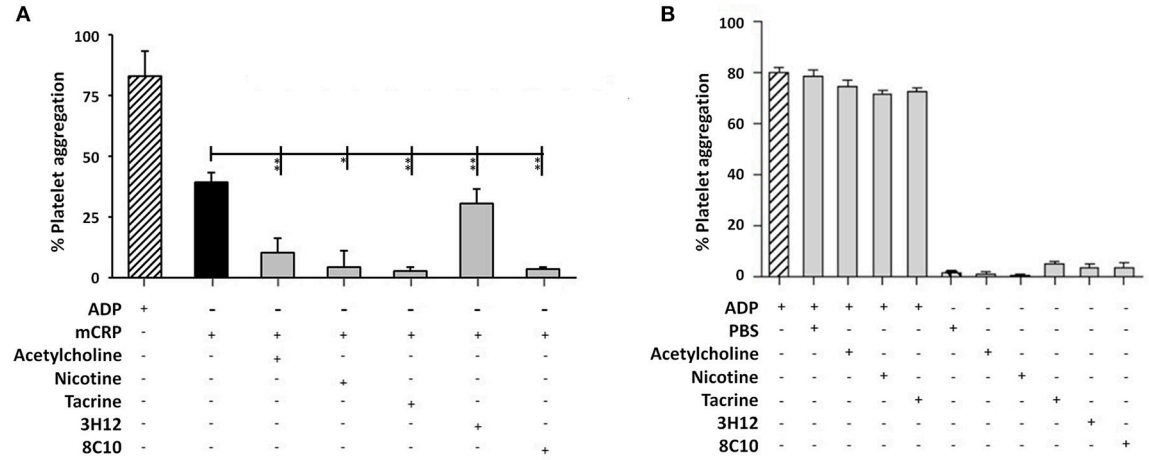

FIGURE 4 | Platelet aggregation assay: **(A)** Platelet aggregation/ coagulation was measured by light-transmission aggregometry (LTA) using PRP derived from volunteers fresh whole blood. Monomeric C-reactive protein (100 μg/ml)-induced platelet aggregation was blocked by anti-mCRP antibody (8C10) and partially inhibited by anti-mCRP (3H12). Acetylcholine (10 μM), nicotine (0.93 μM), and tacrine (5 μM), almost completely inhibited mCRP-induced platelet aggregation ($P < 0.05$), whilst having no effect either alone or on ADP-induced platelet aggregation **(B).** The experiments were each performed in triplicate. Results are presented as the mean ± SD from a representative example of three independent experiments. $^{*}P \leq 0.05$; $^{**}P < 0.01$; using one-way ANOVA with Bonferroni post-test analysis.

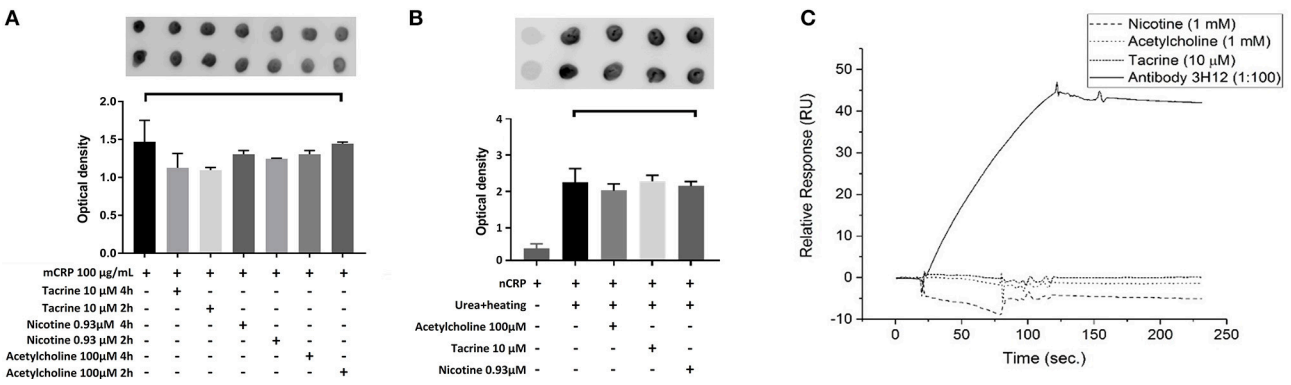

FIGURE 5 | Dot-blot and cell binding assays: **(A)** After Incubation of mCRP with and without tacrine, nicotine, and acetylcholine for 4 h, dot blotting with 3H12 showed no significant specific antibody binding. **(B)** Following incubation of nCRP with urea at 37°C dot blotting using 3H12 to bind to the mCRP indicated that dissociation of nCRP to mCRP was not affected by the presence of any of the three small molecules. **(C)** The results of SPR showed that there was no observable interaction of the small molecules to biotinylated mCRP. The 3H12 antibody exhibited a strong response with a slow off-rate (kd) of $\sim 7.8 \times 10^{-4}$ s^{-1}. Experiments were performed in duplicate and repeated three times. Results are presented as the mean ± SD from a representative example. No significant differences were found using one-way ANOVA with Bonferroni post-test analysis.

antibody, however, exhibited a strong response with a slow off-rate (kd) of $\sim 7.8 \times 10^{-4}$ s^{-1} (**Figure 5C**). For each experiment, duplicates of each sample were made, and three repeats of each experiment were carried out with a representative example being shown here.

DISCUSSION

mCRP is involved in significant perpetuation of tissue associated inflammation, potentially associating it to thrombosis in atherosclerosis (14), neurological degradation and dementia (16), macular degeneration (17), and sepsis (18). Therefore, antagonists of either, native CRP breakdown and dissociation into mCRP, or small molecules that would inhibit mCRP binding and activation via the cell membrane phosphocholine docking site, could prove useful as future therapeutic agents.

Apart from our characterized antibodies (3H12 and 8C10; 8C10 having been previously shown by ourselves to block mCRP-induced cell signaling through p-ERK1/2 and angiogenesis in bovine aortic EC); (16), we investigated the potential of acetylcholine, nicotine and tacrine to modulate mCRP-induced inflammation. Acetylcholine is very similar in structure to phosphocholine. Work by Nazarov et al. (19) indicates that CRP could bind to acetylcholine, as evidenced, by CRP-based inhibition of breakdown of this molecule and subsequent influence on cardiovascular systemic inflammation. In order to gain some insight into potential CRP/acetylcholine interactions and how this compares to binding at known cholinergic

receptors/enzymes; we also chose to examine the effects of nicotine – the putative agonist at nicotinic receptors and tacrine, a therapeutically useful inhibitor of acetylcholinesterase.

The induction of inflammation in U937-derived macrophages *in vitro* is a reliable indication of the activity of mCRP, and increases in IL-6 and TNF-α are attributed to detrimental tissue-related complications (20). Here we showed that both acetylcholine and nicotine were able to attenuate, significantly, both TNF-α and IL-6 activity, whilst neither tacrine nor the targeting antibodies were effective. In addition, a small decrease in production of the anti-inflammatory cytokine IL-10 was noticed, in the presence of mCRP, but this tended to return to basal levels in the presence of acetylcholine or 3H12 antibodies. A reduction in anti-inflammatory cytokines elicited by mCRP could potentially alter the vascular micro-environment leading to enhanced inflammation and hence molecules that could block this effect systemically could have some therapeutic interest (21). Il-1β was also tested and substantially increased in the presence of mCRP, however, neither the antibodies nor the small molecules used showed any significant inhibition of cytokine expression (data not shown).

Previous work has demonstrated mCRP induced production of IL-6 and TNF-α in U937 macrophages via Fc-gamma receptor-associated signaling and that co-incubation with oxidized LDL antagonized this inflammatory response (3). This pair of cytokines are linked in systemic inflammation/acute and chronic infection and are associated with increased risk of atherosclerosis and thrombosis and therefore blocking their production with a novel inhibitor such as acetylcholine could protect highly at risk individuals (22).

To confirm that macrophage cell signaling was perturbed, we carried out Western blotting experiments. Previously, Li et al. (23) showed that EC stimulation with mCRP induced MAP kinase signaling and p-38, NF-κB, associated with increase in IL-6 cytokine expression. Only one published study investigating the effects of mCRP on macrophage signaling was carried out previously by Eisenhardt et al. (24) who performed proteomic analysis on THP-1 macrophages, but did not identify any critical signaling intermediates associated with pro-inflammatory gene expression. We pre-incubated macrophages with acetylcholine or nicotine (since they produced a strong inhibitory inflammatory response in the presence of mCRP) and observed a reduction in p-ERK1/2 by both, and NF-κB phosphorylation in the presence of both acetylcholine and nicotine (p-JNK was weakly inhibited whilst AKT/p-p38 were not affected—AKT not shown). mCRP also caused nuclear translocation of NF-κB (by immunofluorescent analysis, for which the data are derived from only duplicate experiments)-a process associated with phosphorylation and degradation of IκBα normally allowing translocation of NFkB into the nucleus where it regulates gene transcription).

The phosphorylation of NF-κB by acetylcholine when applied alone is difficult to explain but this was previously reported following incubation with a bronchial epithelial cell line, and linked to increased IL-8 production, although the mechanism responsible for this is not clearly understood (25). Oenema et al. showed stimulation of IκB in smooth muscle cells through the muscarinic receptors indicating a possible signaling mechanism for this surprising finding (26).

Previous work has shown that both p-38 and NF-κB are required for IL-6/TNF-α processing in multiple cell types (27), and hence our work provides an indication that, particularly, acetylcholine, may block mCRP binding and signaling pathways associated with its powerful pro-inflammatory action.

We assessed whether there was a direct interaction between mCRP/nCRP and small molecules to indicate if direct binding possibly leading to structural modification, may be responsible for imparting biological inhibition. Dot blots performed on nitrocellulose bound with specific mCRP antibodies showed no ability of the small molecules to block binding directly to the antibody, nor to inhibit native CRP dissociation in the presence of urea. Similarly SPR could not show any direct interaction apart from the antibody (which we used as a positive control), thus indicating the interaction of these substances with mCRP may be at the membrane-phosphotidylcholine binding site rather than specific binding to the CRP which should be the subject of further investigation.

To further investigate the effects on macrophage activation and the relationship with EC adhesion, we conducted the Cytoselect monocyte-EC adhesion assay. Previous work has shown an important role for mCRP in stimulation of neutrophil attachment to human coronary artery EC [HCAEC; (28)], whilst Khreiss et al. (29), showed mCRP-induced HCAEC through enhanced MCP-1 and IL-8 secretion with concomitant phospho-p-38 expression, although there is no specific literature describing the link between EC and macrophages. Recently, mCRP was shown to activate angiogenesis and trigger F3 gene transcription, upregulating tissue factor signaling (30). Here we show that mCRP induced EC-monocyte adhesion and this was notably inhibited in the presence of both nicotine and acetylcholine (although not tacrine), and also, similarly using our two targeting antibodies, although these trends were non-significant. Inflammation and cell "stickiness" linked to mCRP are known to encourage monocyte attachment to the vascular cell wall for example at the early stages of atherosclerosis (31), and later as a precipitant of thrombosis with platelet aggregation involvement (32).

Regarding platelet aggregation, mCRP at 100 μg/ml was previously shown to cause CD62-platelet aggregation and adhesion to fibrinogen (33). Using our standardized thrombotic assay, we showed that mCRP (100 μg/ml) significantly induced thrombosis within 2 min of application (∼39%). In the presence of small molecules/antibodies, whilst the 3H12 was ineffective, the 8C10 antibody and all three small molecules significantly attenuated platelet aggregation, whilst the positive control ADP was not blocked. Mollins et al. (32), partially explained the mechanism of mCRP action through surface P-selectin activation, CD63 exposure, and glycoprotein IIb-IIIa activation. Although we are not sure of the characteristics of our small molecules, the thrombotic pathway appears to be driven at least partially through p-38 activation and at least acetylcholine was able to block this pathway hence this could help to explain our findings. Regarding the anti-mCRP antibodies, the 8C10 binds to the N-terminal part of mCRP through aa 22–45 thereby covering

the cholesterol binding site and probably explaining a mechanism for prevention of mCRP from entering lipid rafts (2). This may explain its greater effectiveness when compared with the 3H12 antibody which binds the C-terminal octapeptide aa 198–206. Since this epitope becomes hidden after mCRP enters a lipid zone e.g., on the surface of a cell membrane or lipid rafts of platelets, mCRP may have bound to the platelets prior to an effective influence of the antibodies or small molecules since there was no pre-incubation phase in this experiment.

We confirm here that small molecules like acetylcholine and nicotine could potentially be developed or optimized as protectors in cardiovascular and other inflammatory debilitating conditions. Nonetheless acetylcholine administration may have considerable severe side effects when administered systemically, (e.g., inhibition of other normal CNS functions by blocking serotonin function) leading to enhanced anxiety and depression (34). In addition, since the normal half-life of acetylcholine in the blood is 1–2 min, treatment requiring prolonged action systemically would require additional anticholinesterase therapy, and in fact, tacrine, is an example of a drug previously tested for management of Alzheimer's within the USA (35).

It is worthy of note that whilst several years ago, there was some controversy over the existence of mCRP *in vivo*, and identification of the active rmCRP; an intermediate form produced on contact of the native protein with cell membranes and liposomes (36, 37). More recent work from Thiele et al. (4) and others, demonstrated manipulation of CRP using a specific phosphocholine inhibitor (1-6-bis(phosphocholine)-hexane), *in vivo*. They showed the existence of mCRP in tissue and pharmacologically successfully blocked this dissociation directly at the cell surface, thereby validating our studies here and indicating a possible novel therapeutic strategy to abrogate inflammatory disease.

In conclusion, orphan, off target molecules such as acetylcholine or more specific small molecules, of similar structure may have potential for blocking the pro-inflammatory effects of CRP.

AUTHOR CONTRIBUTIONS

MS, MD, W-HF, RA, XZ, and GM designed the project, experiments, managed the work, and drafted the script. RI and BG conducted the ELISA and inflammation assays. DL and RI conducted the Western blotting. GF did the adhesion assay. YZ and NB performed and managed the platelet aggregation assay. VC organized all the small molecule studies and NP carried out the SPR.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02124/full#supplementary-material

Supplementary Figure 1 | Shows trypan blue cytotoxicity screening and no significant cell death or loss of viability after 24 h in the presence of the following concentrations of the small molecules /nicotinic acid receptor inhibitors used within this study (acetylcholine-0–500 μM; nicotine-0–150 ng/ml; methyllycaconitine citrate-0–200 μM). Each experiment was performed three times in triplicate and a representative example is shown here.

Supplementary Figure 2 | Results for a second donor for the platelet aggregation assay showing a similar pattern of results for mCRP and the effects of the small molecules on aggregation. Anti-mCRP antibody 8C10 blocked effectively the aggregation of platelets by mCRP whilst 3H12 only partially abrogated the effects ($P < 0.05$). Acetylcholine (10 μM), nicotine (0.93 μM), and tacrine (5 μM), all significantly inhibited mCRP-induced platelet aggregation. Results are presented as the mean ± SD from a representative example (different donor) of three independent experiments. $^{*}P \leq 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ using one-way ANOVA with Bonferroni post-test analysis.

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