The Dissociation of Pentameric to Monomeric C- Reactive Protein Localizes and Aggravates Inflammation: In vivo Proof of a Powerful Pro-Inflammatory Mechanism and a New Anti-Inflammatory Strategy

Running title: Thiele et al.; CRP-dissociation localizes/aggravates inflammation

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Abstract

**Background**—The relevance of the dissociation of circulating pentameric C-reactive protein (pCRP) to its monomeric subunits (mCRP) is poorly understood. We investigated the role of conformational CRP changes *in vivo*.

**Methods and Results**—We identified mCRP in inflamed human striated muscle, human atherosclerotic plaque and in infarcted myocardium (rat and human) and its co-localization with inflammatory cells, suggesting a general causal role of mCRP in inflammation. This was confirmed in rat intravital microscopy of lipopolysaccharide-induced cremasteric muscle inflammation. Intravenous pCRP administration significantly enhanced leukocyte rolling, adhesion and transmigration via localized dissociation to mCRP in inflamed but not in non-inflamed cremaster muscle. This was confirmed in a rat model of myocardial infarction. Mechanistically, this process was dependent on exposure of lysophosphatidylcholine on activated cell membranes, which is generated following phospholipase A2 (PLA2) activation. These membrane changes could be visualized intravitaly on endothelial cells, as well as the colocalized mCRP generation. Blocking PLA2 abrogated CRP dissociation and thereby blunted CRP’s pro-inflammatory effects. Identifying the dissociation process as a therapeutic target, we stabilized pCRP using 1,6-bis-phosphocholin-hexane, which prevented dissociation *in vitro* and *in vivo*, and consequently inhibited generation and pro-inflammatory activity of mCRP and notably, inhibited mCRP deposition and inflammation in rat myocardial infarction.

**Conclusions**—These results provide *in vivo* evidence for a novel mechanism localizing and aggravating inflammation via PLA2-dependent dissociation of circulating pCRP to mCRP. mCRP is proposed as a pathogenic factor in atherosclerosis and myocardial infarction. Most importantly, the inhibition of pCRP dissociation represents a promising novel anti-inflammatory therapeutic strategy.

**Key words**: C-reactive protein, inflammation, microcirculation, atherosclerosis, myocardial infarction, Phospholipase-A2
Introduction

C-reactive protein belongs to the pentraxin family of protein and circulates as a pentamer of five identical non-covalently linked subunits in plasma. Recently a dissociation mechanism on activated platelets that leads to a conformational change from the circulating native, pentameric CRP (pCRP) to its monomeric subunits (mCRP) has been identified. This dissociation is mediated by bioactive lipids found on activated or damaged cells or platelets and leads to an alteration of the pro-inflammatory profile of the protein.

With respect to the inflammatory properties of the two isoforms it has been shown that dissociation of p- to mCRP leads to increased activation, adhesion and transmigration of monocytes, as well as formation of reactive oxygen species in vitro, which represent major pathophysiological factors contributing to tissue injury in inflammation.

Recent animal work has suggested that injection of human pCRP can increase myocardial infarct size in a rat myocardial infarction model, which can be reduced by a CRP-blocking reagent. In a vascular injury model in transgenic mice, CRP led to increased neointima formation. Animal models of myocardial infarct, stroke and intestinal ischemia/reperfusion have found that infusion of CRP increases tissue damage. To date it remains unclear whether the recently described pCRP dissociation to mCRP is of relevance in vivo and how it potentially contributes to the aforementioned findings. Furthermore, our understanding of the pathophysiology of CRP dissociation is mainly based on in vitro findings and overall the mechanisms and mediators involved are poorly understood.

Phospholipase A2 (PLA2) enzymes modify the composition of cellular membranes by releasing fatty acids from the sn2 position of phospholipids, which leads to the formation of arachidonic acid and lysophospholipids. Arachidonic acid is further processed into bioactive

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eicosanoids including prostaglandins and leukotrienes. Lysophospholipids can be further modified to platelet-activating factor (PAF). All these molecules are potent regulators of inflammation \(^{10,11}\). Since binding of CRP is enhanced on lysophosphatidylcholine enriched membranes, we hypothesized that there is a link of PLA2 activation and CRP dissociation.

Here we investigated the role of conformational changes in CRP in vivo and aimed to elucidate the underlying mechanisms including the role of PLA2-mediated membrane changes. Furthermore, we aimed to identify the CRP dissociation process as a therapeutic target.

**Methods**

**Reagents**

pCRP purified from human ascites (Merck, Darmstadt, Germany) was dialyzed against phosphate-buffered physiological saline solution with Ca\(^{2+}\) and Mg\(^{2+}\) (PBS-Ca-Mg, Biochrom, Berlin, Germany) prior to use, utilizing a Slide-A-Lyzer Dialysis Cassette (Pierce Biotechnology, Rockford, USA). For subsequent application, the final concentration of pCRP was determined (BCA, Sigma-Aldrich, St. Louis, USA) \(^{12,13}\). mCRP (1 mg/ml in Na-PBS) was used in the soluble, citraconylated form, and prepared as described previously \(^{14}\). In awareness of potential problems with bacterial contamination in CRP preparations \(^{15}\) all reagents were tested for LPS contamination with a Limulus assay (Sigma-Aldrich) and found to be below detection limit (0.125 U/ml or 0.01 ng/ml LPS). CRP purity and spontaneous mCRP formation were ruled out by Western blotting with 1/20\(^{th}\) of normal levels of SDS of pCRP and mCRP preparations to avoid confounding by contamination of other CRP isoforms.

All animal studies were approved by the institutional review board of the University of Freiburg Medical Centre and procedures were performed in accordance with institutional
guidelines. Studies involving human tissue samples were approved by the institutional review committee and the subjects gave informed consent to the use of the tissue samples for this study.

**Statistical analysis**

Analysis of data was performed using GraphPad Prism v5.0 software (GraphPad Software, San Diego, USA). For comparison of two groups, a two-tailed t-test was employed. A p value of less than 0.05 was considered statistically significant. All experiments were performed at least three times. The data are expressed as mean ± standard error of the mean (SEM). A one-way ANOVA to compare effects of different treatments was used, if more than two groups were compared. In case of significance, Tukey’s test was used for pair wise comparison. Only significant results for both ANOVA and Tukey’s test are presented. For analysis of the aptamer specific binding to mCRP but not pCRP we used a one-way ANOVA with repeated measures to compare the groups with different concentrations. 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. Additionally, the random factor “animal” was included in the model. In case of a non-significant interaction and significant treatment effect, pair-wise 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.

An expanded Methods section is available in the Online Data Supplement.

**Results**

**CRP is deposited in inflamed human tissue as mCRP and co-localizes with monocytes/macrophages**

In the human muscle biopsies of ischemia/reperfusion injury we detect mCRP, but not pCRP in
the post-ischemic tissue samples, which co-localizes with CD68-positive monocytes (Figure 1A). The postischemic increases in mCRP expression and CD68-positive cells show a similar tendency (Figure 1B).

To analyze the potential relevance of mCRP deposition in the pathogenesis of tissue damage in humans, infarcted myocardial tissue samples were obtained from autopsy specimens originating from patients, who had suffered a myocardial infarction 2-4 days prior to death. Sections taken from infarcted and non-infarcted tissue were stained with antibodies specific for mCRP and pCRP. There is extensive deposition of mCRP in infarcted regions with very limited deposition in non-infarcted areas (Figure 1C). We identified a peri-vascular distribution of mCRP staining.

Inflamed atherosclerotic plaques obtained from human carotid endarterectomy samples were stained with pCRP, mCRP and CD68-specific antibodies (Figure 1D). mCRP can be detected in the necrotic core of atherosclerotic lesions and co-localizes with macrophages. CRP aggravates the inflammatory response via interaction with the complement system, but CRP does not show intrinsic pro-inflammatory properties.

In an intravital microscopy model of microcirculation changes in the rat cremaster muscle low-dose LPS superfusion causes a mild inflammatory response resulting in increased leukocyte rolling and adhesion (Figure 2A+B), as well as endothelial membrane changes, detected by in vivo Annexin V staining of endothelial cells (Figure 2E). pCRP infusion does not induce leukocyte rolling or adhesion in the resting muscle tissue. In inflamed muscle tissue after LPS superfusion CRP aggravates the pre-existing inflammatory response as measured by leukocyte rolling, adhesion (Figure 2A+B) and monocyte transmigration (Figure 2C+D). Similar effects could be observed in isolated rat monocytes ex vivo (Figure 2F).
The pro-inflammatory, tissue damaging effects of human CRP in vivo are dependent on complement. In order to show that the observed effects are dependent on this CRP-complement interaction and to exclude confounding by endogenous rat CRP we analyzed the role of the complement system in CRP induced leukocyte activation via depletion of complement. Intraperitoneal injection of CVF leads to a significant reduction of complement activity compared to the control (Online Supplemental Figure 1). Complement depletion induces a significant decrease in leukocyte rolling after 60 min and adhesion at 80 min in the CRP driven inflammation (Online Supplemental Figure 1). These results indicate a dominant in vivo role of the complement system in the mCRP-triggered pro-inflammatory cascade.

CRP is transported into areas of inflammation on transmigrating leukocytes and is deposited in inflamed tissue

Western blot analysis shows a deposition of CRP in LPS challenged cremasteric muscle tissue, but not in resting tissue as evaluated for the triceps muscle, and heart and lung tissue (Figure 3A-C). CRP in vivo tracking reveals that CRP can be detected on transmigrating leukocytes and is deposited in inflamed tissue (Figure 3D). Intravital staining of CRP and endothelial membrane changes (Annexin V) shows that CRP/leukocyte transmigration is found in areas with endothelial membrane changes. In resting tissue neither Annexin V nor CRP can be detected.

CRP is deposited as mCRP in inflamed tissue

Rat cremaster muscle samples were examined for CRP deposition by native Western blotting and immunohistochemistry by conformation specific antibodies. Native Western blot analysis reveals that a majority of CRP deposited in the inflamed cremasteric tissue is in monomeric form. In non-inflamed tissue (without LPS superfusion) CRP is found to a much reduced extent and only as pCRP (Figure 3E+F). This is verified by immunohistochemistry of rat cremasteric muscle
after microcirculation experiments. In the control group and LPS group no CRP is detected. After pCRP injection, without inflammatory stimulus to the cremasteric tissue, trace amounts of pCRP were detected by immunohistology. LPS induced localized cremasteric inflammation leads to deposition and detection of mCRP (Figure 3G+H).

mCRP induces adhesion of human and rat monocytes and generation of ROS
In an in vitro monocyte adhesion assay mCRP induces a significant increase in leukocyte adhesion to a fibrinogen matrix compared to the control that is comparable to high dose stimulation with LPS in both rat (Figure 4A) and human monocytes (Figure 4B). This finding underlines the physiological relevance of data obtained in the rat model to human disease processes. mCRP induces ROS generation as a major component of inflammatory tissue damage (Figure 4F). Furthermore, these results indicate that mCRP is the pro-inflammatory isoform of CRP responsible for producing the inflammatory effects.

mCRP is responsible for the pro-inflammatory activity of CRP
Pre-dissociated mCRP induces leukocyte rolling (after 30 min) and adhesion (at 60 min) in cremasteric muscle venules (Figure 4C+D). Without existing inflammatory tissue damage, pCRP injection does not show effects on leukocyte rolling and adhesion.

Pro-inflammatory mCRP effects on monocytes are mediated by Fcγ-RI and III signaling
Fcγ receptors have previously been implicated in the signaling of mCRP effects 1. To establish the specific role of these receptors, siRNA knock-downs of the three major Fcγ-RI (CD64), IIa (CD32) and III (CD16) were performed on harvested donor monocytes. RT-PCR revealed successful knock-down of the three receptors in the target groups (t) compared to the control groups (c) that were transfected with scrambled siRNA sequences (Online Supplemental Figure 2A). The reduction in surface expression of the respective receptors was confirmed by
flow cytometry (Online Supplemental Figure 2B). Knock-down of Fcγ-RI and III significantly reduced mCRP binding to monocytes compared to the corresponding scrambled control as determined by confocal microscopy, exemplarily shown for Fcγ-III in Online Supplemental Figure 2F. Addition of mCRP leads to receptor clustering and co-localization with mCRP in native monocytes. In static monocyte adhesion assays, pro-inflammatory mCRP effects were significantly reduced in the Fcγ-RI knock-down (Online Supplemental Figure 2C) and Fcγ-RIIIa knock-down group when compared to the corresponding mCRP control group (Online Supplemental Figure 2E). Knock-down of Fcγ-RIIa did not have a significant effect on mCRP-mediated monocyte adhesion (Online Supplemental Figure 2D).

**Inhibition of PLA2 prevents CRP dissociation on activated cells**

Several inhibitors of enzymes of the PLA2 family were used to analyze the effects on CRP dissociation. By native gel Western blot analysis we are able to detect CRP dissociation on activated monocytes as described previously \(^1\). Inhibition of PLA2 by ONO-RS, MAFP and AA reduces mCRP formation, suggesting that CRP dissociation is mediated by LPC expression on cells through activation of PLA2 (Figure 5A+B).

*In vitro* experiments demonstrated that LPS-induced PLA2 activity in mononuclear leukocytes can be decreased by pre-incubation of cells with ONO-RS (Figure 5E) thereby inhibiting the mCRP formation in a concentration-dependent manner as evaluated by native gel electrophoresis. Concentrations of 10 μM ONO-RS were able to produce a near complete inhibition of CRP dissociation (Figure 5C+D).

**Inhibition of PLA2 in vivo inhibits localized mCRP formation and leukocyte activation**

Using a concentration of 10 μM ONO-RS superfusion of the cremasteric muscle tissue, we are able to demonstrate a complete inhibition of CRP effects on leukocyte rolling at 120 min and
adhesion after 60 min. In contrast, LPS-mediated pro-inflammatory effects were not significantly inhibited compared to the LPS control (Figure 6A+B). This demonstrates that blocking of PLA2 in vivo inhibits mCRP generation and thereby prevents amplification of the inflammatory response. Localized CRP dissociation was observed intravitally by tracking of fluorescently labeled pCRP and detection of mCRP by a mCRP specific fluorescently labeled aptamer. Control experiments demonstrated that the aptamer was specific for mCRP and did not possess potential pro-inflammatory confounding effects (Figure 6D+E). In vivo mCRP generation and transmigration into inflamed tissue was observed. This is abolished by blocking PLA2 with ONO-RS (Figure 6C). Due to the inhibition of mCRP generation, the fluorescently labeled pCRP remains detectable. This is associated with a lack of CRP detection in the inflamed tissue.

Stabilization of CRP and prevention of CRP dissociation inhibits pro-inflammatory CRP properties in vivo

1,6-bis PC is able to stabilize pCRP in a decameric conformation and is able to inhibit its interactions with complement. We demonstrate here that 1,6-bis PC also inhibits CRP dissociation to mCRP (Figure 7A+B) in vitro. Using this stabilization of pCRP prior to injection we can demonstrate by native Western blotting of inflamed cremasteric tissue that 1,6-bis PC inhibits CRP deposition in inflamed tissue (Figure 7C). This data is confirmed by immunohistochemistry, which demonstrates that mCRP deposition in the inflamed muscle tissue is markedly decreased after stabilization of CRP with 1,6-bis PC (Figure 7D). This blocking effect results in inhibition of the CRP-mediated increase in leukocyte rolling after 60 min and adhesion at 120 min (Figure 7E+F). This demonstrates the feasibility of this potential therapeutic approach and the central importance of pCRP dissociation in the unmasking of pro-inflammatory CRP effects.
mCRP and pCRP deposition is localized to infarcted myocardial tissue in a rat model of ischemia/reperfusion injury

In order to confirm the relevance of CRP dissociation in a disease model, we examined the proposed mechanism and its inhibition by 1,6-bis PC in a rat LAD-ligation model of myocardial infarction. Histological examination of rat myocardium shows extensive deposition of mCRP in infarcted tissue as shown in Figure 7H. In contrast, in non-infarcted right ventricular segments (Figure 7H, row 2) no mCRP could be detected. Furthermore, control samples, which were not infused with human pCRP (Figure 7H, row 3), did not show any staining for mCRP. Small amounts of pCRP could be detected in ischemic/reperfused tissue but in significantly lower amounts compared to mCRP. These findings were quantified using imaging analysis software and the results are shown in Figure 7I. There is significantly more mCRP than pCRP identified in ischemic tissue segments. The non-ischemic and vehicle controls do not show specific staining.

1,6-bis-PC inhibits the formation and deposition of mCRP in infarcted tissue and reduces the localized inflammatory response

In the myocardial ischemia/reperfusion model a third experimental group was infused with human pCRP together with 1,6-bis-PC. This agent was able to completely abolish the formation and deposition of mCRP (Figure 7H). The amount of both mCRP and pCRP detected was not significantly different to the vehicle and non-ischemic controls as shown in Figure 7I.

The reduced mCRP formation and deposition by 1,6-bis-PC leads to a significant reduction of leukocyte infiltration, expression of Caspase 3, as marker of apoptosis and expression of the pro-inflammatory cytokines IL-6 and TNFα (which served as surrogate parameters for the degree of tissue injury) compared to the CRP-control (Online Supplemental Figure 3).
Discussion

Here we have identified and characterized the role of pCRP dissociation to mCRP for the first time *in vivo* in an animal model of acute inflammation. This points to the CRP dissociation process as a potential therapeutic target. This is supported by the following findings: (1.) mCRP can be detected in human tissue samples of inflamed striated muscle tissue, human atherosclerotic plaques and areas of infarcted rat and human heart tissue, and is typically co-localized with inflammatory cells. These findings are consistent with a ubiquitous causal role in inflammation. (2.) CRP is deposited in inflamed or ischaemic tissue, but not in healthy tissue beds. It aggravates the pre-existing inflammatory response by inducing pathological leukocyte-endothelial interaction and generation of reactive oxygen species. pCRP does not show any intrinsic inflammatory properties without pre-existing inflammatory tissue damage. (3.) CRP is deposited in the area of inflammation as mCRP. (4.) Localized pCRP dissociation is dependent on PLA2-mediated endothelial membrane changes and exposure of LPC, localizing mCRP induced inflammation to areas of PLA2 activation. Blocking PLA2 by pharmacological inhibitors abrogates these membrane changes and results in inhibition of CRP dissociation and prevention of the pro-inflammatory effects of CRP *in vivo*. (5.) mCRP has marked pro-inflammatory properties *in vitro* and *in vivo*. mCRP deposits in tissue promote monocyte chemotaxis, recruiting circulating leukocytes to areas of inflammation via Fcγ-RI and IIIa signaling. (6.) mCRP is transported into inflamed tissue bound to transmigrating leukocytes. (7.) 1,6-bis-PC inhibits pCRP dissociation and inhibits CRP deposition in inflamed tissue. By preventing mCRP formation 1,6-bis-PC inhibits pro-inflammatory CRP effects in inflamed tissue and consequently inhibits pro-inflammatory CRP effects. This hypothesis is confirmed in a rat model of myocardial infarction where the therapeutic use of 1,6-bis-PC prevents localized mCRP
formation and deposition within the infarcted tissue and in turn reduces parameters of tissue injury and inflammation. (8.) mCRP induces leukocyte rolling and adhesion via a complement dependent mechanism. These results are summarized in the schematic drawing in Figure 8.

To verify the pathophysiological relevance of mCRP in vivo we utilized a rat model, as even though rats have abundant CRP (300–600 µg/ml in normal healthy pathogen-free rats) it does not activate rat complement 17. This is in contrast to human CRP that activates both rat and human complement, but not mouse complement 18, which is a major limitation for using mice in CRP-research. Depletion of complement abrogates CRP effects, which confirms the crucial role that complement plays in CRP pathophysiology 6, 7.

One limitation of the use of rats is the limited availability of knock-out animals. Therefore we utilized a pharmacological approach to suppress PLA2 activity with a range of specific inhibitors to characterize its fundamental mechanistic role in CRP dissociation. However this approach does not completely rule out contribution of other unknown factors to the observed effects. As PLA2 inhibition shows no effect on LPS induced inflammation without the addition of pCRP our results show that PLA2 inhibition influences the effects of CRP mediated exacerbation of inflammation. Further interpretations of the role of PLA2 in this context have to be done on the basis of the natural limitations of the experimental strategy, as the mechanism of the PLA2 inhibitors may be indirect or confounded by unidentified off-target effects.

To prove the ubiquitous character of the pro-inflammatory mechanism of localized pCRP dissociation, we analyzed mCRP in human infarcted myocardial tissue and in atherosclerotic plaques. In human myocardial tissue pCRP could not be detected, indicating that all bound pCRP underwent dissociation within hours of infarction. These findings were confirmed in rat model of LAD-ligation. The local deposition of mCRP limited to areas of ischemic/necrotic tissue. In
human atherosclerotic plaques we can show that mCRP deposition is localized to the necrotic core of the lesion. Overall, these findings support the notion that CRP dissociation is modulating inflammation in acute (cardiac ischemia/reperfusion) and chronic (atherosclerosis) inflammatory processes.

PLA2 enzymes are regulators of inflammation\(^{11}\), which is supported by the finding of reduced post-ischemic brain injury in a knock-out mouse model deficient in cytosolic PLA2\(^{19}\). Conversely iPLA2 beta over-expression led to increased vascular inflammation in a vascular ligation model in mice\(^{20}\). In addition to the release of arachidonic acid activation of PLA2 results in the production of LPC. Generation of LPC via activation of the Ca\(^{2+}\) independent phospholipase A2 (iPLA2) and exposure of LPC on the cell surface is a mechanism generally identified in activated or apoptotic cells\(^{21}\). Lauber et al. identified iPLA2 activation in apoptotic cells and subsequent LPC generation as a “find-me” signal for macrophages\(^{22}\). We visualized these membrane changes \textit{in vivo} by expression of phosphatidylserine (PS) on endothelial cells detected by annexin V binding. PS surface expression and LPC generation both are events in the early phase of cell damage\(^{23}\) and present an “eat-me” signal for macrophages\(^{24}\). Both phospholipids (PS and LPC) are central to the signaling pathways of apoptosis and are both expressed on activated cell membranes and microparticles\(^{25,26}\). Thus annexin V binding is a suitable parameter to monitor membrane changes and a suitable surrogate marker for the surface expression of LPC as the expression of LPC and PS are concurrent events. For the first time we have been able to observe localized CRP dissociation \textit{in vivo} by intravital imaging of the microcirculation of inflamed tissue by means of a specific fluorescently labeled anti-mCRP aptamer\(^{16}\). This localized mCRP generation is completely inhibited after blocking PLA2 \textit{in vivo}.

In our experiments LPS was used to activate PLA2\(^{27}\) and facilitate LPC formation. This
enables the binding of circulating pCRP\textsuperscript{28} and subsequent conformational changes of the protein\textsuperscript{1,2}. Our findings complete the understanding of this inflammatory pathway. In addition to direct chemoattraction by LPC\textsuperscript{22}, the conformational change of CRP is an activation signal to circulating leukocytes that marks the area of inflammation by localized deposition. These findings identify a novel mechanism by which PLA2 regulates inflammation.

Recently a role for lipoprotein associated PLA2-generated LPC has been postulated for human atherosclerotic plaque formation\textsuperscript{29}. We previously described the localized dissociation and deposition of mCRP in atherosclerotic plaques\textsuperscript{1}. PLA2-mediated membrane changes and subsequent CRP dissociation may therefore be a ubiquitous mechanism in chronic inflammatory conditions such as atherosclerosis. This event is dependent on the expression of bioactive lipids, namely LPC. In our \textit{in vivo} experiments we show that endothelial cells in inflamed areas undergo these membrane changes and are likely to be the mediators of CRP dissociation, whereas for our \textit{in vitro} experiments, we use membrane changes of mononuclear cells to demonstrate the activity of PLA2 inhibitory agents and 1,6-bis PC on CRP dissociation, again demonstrating the ubiquity of this process. In our previous work we demonstrated that activated platelet membranes could mediate this dissociation process in chronic inflammation\textsuperscript{1}. Therefore, this mechanism is independent of the cell type and represent a general mechanism in various pathophysiological settings by which membrane changes due to cell activation or apoptosis can activate an immune response via binding and dissociation of CRP, thereby resulting in leukocyte activation and infiltration of the area of cellular damage. Our findings potentially link the novel mechanistic role of pCRP to mCRP dissociation with emerging data regarding phospholipase activity and LPC generation being associated with increased cardiovascular risk\textsuperscript{30}.

In accordance with our previous data, our current findings do not indicate that pCRP
possesses intrinsic pro-inflammatory potential and CRP is not deposited outside areas of focal inflammation, which confirms the findings of a recent study in which pCRP infusions in healthy volunteers showed no pro-inflammatory effect \(^{31}\). The ability of CRP to mediate any inflammatory effects was dependent on a pre-existing localized inflammatory microenvironment with PLA2-mediated membrane changes. In the clinical setting this is reflected by the fact that levels of circulating pCRP rise 6-12 hours after the inflammatory insult \(^{32}\). This indicates that CRP dissociation is a modulator, in particular amplifier, of an established inflammatory response rather than a first line defense mechanism. Conversely, patients with a chronic mild elevation of circulating CRP levels have been found in numerous studies to be at increased cardiovascular risk \(^{33}\). In this setting, local dissociation of circulating pCRP in the inflamed intima of pre-atherosclerotic lesions might drive atherogenesis.

As a further proof for the relevance of the CRP dissociation process in aggravating the inflammatory response, the recently described compound 1,6-bis-PC was used. This agent acts by stabilizing CRP in a decameric form \(^{5}\). This stabilization prevents CRP dissociation \textit{in vitro} \(^{25}\). We are able to show that the inhibition of pCRP dissociation by 1,6-bis-PC abolishes pro-inflammatory CRP effects, a powerful inductive proof demonstrating the relative importance of CRP in promoting innate immunity. The identification of this novel therapeutic approach may support the development of further compounds, which are suitable for clinical use. However, it has to be taken into consideration that the therapeutic inhibition of mCRP generation might reduce the pro-angiogenic effect of mCRP that has been recently described \(^{34}\). Inflammation as such is not necessarily harmful, but uncontrolled inflammation may be detrimental in conditions such as ischemia/reperfusion injury. Similarly, the pro-angiogenic effects of mCRP may be beneficial. However, in the context of an exaggerated inflammatory response they may have less
relevance. Therefore, targeting mCRP effects may be a valuable therapeutic strategy in these conditions.

Whereas the cremasteric muscle inflammation model served as a proof of concept model to identify the mechanism of CRP aggravated inflammation, we performed a rat LAD-ligation model to confirm the relevance of this mechanism in a myocardial infarction. Previous animal models of myocardial infarction have found that CRP directly contributes to myocardial cell death and necrosis. This finding is consistent with observational studies following MI in humans, which have found that the peak serum (p)CRP level correlates with adverse clinical outcomes. The mechanistic link establishing how CRP contributes to inflammation has been missing and is now elucidated by our study. Histological studies have found that CRP and complement components (most often component C1q) can be found to be co-localized in myocardial tissue during acute myocardial infarction. We show that the depletion of serum complement abrogates any adverse effect of CRP, strongly implicating complement involvement in CRP pathogenesis. However, both pCRP and C1q are found independently in the plasma with no recognized interaction. In contrast to pCRP, mCRP is a potent ligand of the complement component C1q. The identification of an intermediate conversion step in the injured myocardial tissue is an elegant solution to these conflicting observations. The data obtained in our animal model supplements those previously published by Pepys et al as we provide data to suggest that the mechanism of action of 1,6-bis-PC is to prevent mCRP formation by inhibiting pCRP binding and dissociation, which is in turn reducing localized inflammation and tissue injury.

We conclude that in inflamed tissue, pCRP is dissociated and the resulting mCRP is deposited and it aggravates as well as localizes inflammatory responses such as leukocyte
activation/infiltration. In inflammation, local membrane changes occur and our results indicate that these are induced by activation of PLA2 and subsequent exposure of bioactive lipids that mediate the conformational changes of pCRP. Therefore, blocking pCRP dissociation to a monomeric form or inhibiting pro-inflammatory mCRP activity is a promising therapeutic strategy with the potential to offer a novel treatment approach in a wide range of inflammatory disorders.

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**Conflict of Interest Disclosures:** None.

**References:**


4. Eisenhardt SU, Thiele JR, Bannasch H, Stark GB, Peter K. C-reactive protein: How


11. Teslenko V, Rogers M, Lefkowith JB. Macrophage arachidonate release via both the cytosolic ca(2+)-dependent and independent phospholipases is necessary for cell spreading. *Biochim Biophys Acta.* 1997;1344:189-199.


Figure Legends:

Figure 1. Accumulation of mCRP in inflamed human muscle tissue. (A) Conformation specific detection of C-reactive protein and CD68+ cells in striated human muscle tissue before (pre-ischemia) and after free tissue transfer (post-reperfusion) by immunohistochemistry. Left panel staining for pCRP and middle panel staining for mCRP. Goat anti-mouse horseradish peroxidase-coupled antibody was used as secondary antibody. Typical results are given. (B) Quantification of immunohistochemical results. Given are relative values of immunoreactivity for mCRP and CD68. At least three non-overlapping visual fields were evaluated and averaged from each sample. The average counted as value for one sample. Values are mean ± SEM; n=11; *p<0.05. The inflammatory response in ischemia-reperfusion leads to a significant increase of mCRP deposition, that co-localizes (arrow) with CD68+ leukocytes. (C) Human post-mortem samples following myocardial infarction demonstrate extensive deposition of mCRP in ischemic tissue. Whereas there is strong staining for mCRP in ischemic tissue, in non-infarcted areas there is only a small amount of mainly peri-vascular staining for mCRP. There is no significant staining of pCRP identified in any of the tissue segments or control samples. One example out of 4 is shown. (D) Detection of mCRP, but not pCRP, in human atherosclerotic plaques obtained by carotid endarterectomy. mCRP is localized to the necrotic core and co-localizes with CD68-positive cells. One example out of 3 is shown. Positive staining is shown in green.

Figure 2. pCRP significantly increases leukocyte rolling after 60 min (A) and adhesion at 120 min (B) in LPS-induced inflammation in rat cremasteric postcapillary venules. Leukocyte-endothelial interaction were analyzed by intravital microscopy under superfusion with LPS (25 ng/ml) ± i.v. injection of pCRP (25 μg/ml). Leukocytes were labeled with Rhodamine 6G.
Counts at 0 min was set to 100%. Dot plots and mean of 6 rats are shown; # p<0.05. (C) Immunofluorescence detection of monocyte/macrophage transmigration in the inflamed cremasteric tissue using FITC-anti rat monocyte antibody. The vessel wall was stained by a Cy3-anti α-SMA antibody. Representative results are shown. n=6 rats per group. (D) Quantification of the immunofluorescence results. 3 slides of each animal were analysed and averaged. The average counted as value for one rat (n=6 rats per group) Mean cell count of the control was set to 100%; # p<0.05. Values are mean ± SEM. pCRP application significantly increases transmigration in the LPS-stimulated tissue. (E) In vivo Phosphatidylsernie (PS) expression in LPS-stimulated cremasteric tissue. PS expression was detected by i.v. application of Alexa488-AnnexinV (500 μl/kg BW) 20 min before addition of LPS. Representative results are shown (n=3 rats). Endothel-associated PS expression is strongly induced by LPS. (F) Static rat monocyte adhesion assay after stimulation with LPS (25 ng/ml) ± pCRP (25 μg/ml). Adherent cells were quantified in a phosphatase-substrate reaction. Values are mean ± SEM. Experiments were performed in triplicates (n=3 rats). Representative results are shown. Addition of pCRP significantly increases monocyte adhesion under LPS. pCRP alone has no effect. (G) Flow chart of the intravital experimental protocol.

**Figure 3.** pCRP accumulates in inflammation and binds to transmigrating leukocytes. (A-C) Western blot analysis of rat tissue after cremasteric superfusion with LPS (25 ng/ml) ± i.v. application of pCRP (25 μg/ml) by 20 min. Anti-CRP antibody clone 8 was applied. GAPDH served as loading control. A typical result of cremasteric tissue from 3 different animals is shown (A) and quantification is illustrated (B). Relative values of immunoreactivity for clone 8 related to the signal of GAPDH are given. Values are mean ± SEM; # p<0.05. Deposition of CRP in the
cremasteric tissue is significantly increased through LPS-induced inflammation. Triceps muscle, heart and liver (C) do not accumulate CRP. (D) CRP in vivo tracking by intravital microscopy. Prior to i.v. injection, pCRP (25 μg/ml) was fluorescently labeled with Alexa594 (L-pCRP). Local inflammation was achieved by cremasteric superfusion with LPS (25 ng/ml; left lane). Endothelial activation/impairment was confirmed via Alexa488-Annexin V (500 μl/kg body weight) positive phosphatidylserine expression. In the inflamed (left lane) but not in the resting (right lane) cremasteric tissue, pCRP binds to activated leukocytes (arrow) and, with them extravasates into the perivascular tissue. Representative images are shown, n=3 per group. (E) Dissociation of pCRP to mCRP in acute inflammation. Western blot of native PAGE (1/20 SDS) of rat cremasteric tissue after treatment with LPS (25 ng/ml) for 120 min ± i.v. application of pCRP after 20 min. Both pCRP and mCRP were detected with the anti-CRP antibody clone 8. pCRP solution and pre-dissociated mCRP solution served as controls. A typical result out of 3 experiments is shown. (F) Quantification of the Western blot results. Relative values of immunoreactivity for mCRP normalized to the signal intensity of anti-GAPDH staining are shown. Values are mean ± SEM; # p<0.05. In inflamed cremasteric tissue accumulated pCRP is in large part dissociated to mCRP. mCRP is not detectable in resting tissue. (G) Immunohistochemical staining of the cremaster muscle with HistoGreen after superfusion with LPS (25 ng/ml) ± i.v. application of pCRP (25 μg/ml). Clone 8D8 was used to detect pCRP and clone 9C9 was used to detect mCRP. Representative results are shown (n=6 per group). (H) Quantification of the immunohistochemical results. Relative values of immunoreactivity for pCRP and mCRP are shown. Three non-overlapping visual fields were evaluated and averaged for each rat. The average counted as value for one rat (n=6); # p<0.05. pCRP can be detected in the cremasteric tissue 120 min after i.v. application ± treatment with LPS, though positive
staining is more pronounced in the resting muscle tissue. LPS-triggered inflammation leads to a significant increase of interstitial mCRP deposition.

**Figure 4.** Pro-inflammatory effects of ex vivo pre-dissociated mCRP in vitro and in vivo. Static adhesion assay of rat (A) and human (B) monocytes that were incubated with either pCRP or mCRP at 25 μg/ml for 60 min. Adherent cells were quantified. PBS control was set to 100%. 50 μg/ml LPS served as positive control. Mean ± SEM values are given. Experiments were performed in triplicates; n=3; # p<0.05. Representative results are shown. mCRP induces a significant increase in monocyte adhesion. (C-E) In vivo leukocyte-endothelial interaction under i.v. injection of either mCRP or pCRP (25 μg/ml) analyzed by intravital microscopy. Superfusion of the cremaster muscle with LPS (1 μg/ml) served as positive control. Counts at 0 min were set to 100%. Dot plots and mean of 6 rats are shown; # p<0.05. mCRP significantly increases leukocyte rolling after 30 min (C) and leukocyte adhesion at 60 min (D) whereas pCRP shows no significant effect when compared to the control. Images shown in (E) are typical examples for venules of the four groups after 30 min. (F) Reactive oxygen species (ROS) formation in rat monocytes after *in vivo* exposure to p- and mCRP. Following intravital microscopy, electron spin resonance spectroscopy (ESR) was performed in isolated monocytes using CMH (25 μg/ml) as a spin label. Values are mean ± SEM of 4 different rats; # p<0.05. ROS formation in rat monocytes is significantly induced by mCRP in vivo.

**Figure 5.** Prevention of mCRP formation by inhibition of PLA2. (A) Western blot of native PAGE with 1/20 SDS of human monocytes stained for mCRP with clone 9C9 after incubation with the PLA2 inhibitors ONO-RS-082 (ONO; 20 μM), methyl arachidonyl fluorophosphonate.
(MAFP; 20 μM) and aristolochic acid (AA; 10 μM) for 20 min followed by incubation with pCRP (25 μg/ml) ± LPS (25 ng/ml) for another 60 min. β-Actin served as loading control. A typical result out of 3 experiments is shown. (B) Quantification of the Western blot results. Relative values of immunoreactivity for mCRP normalized to β-Actin signal are shown. Values are mean ± SEM; # p<0.05. Inhibition of PLA2 by ONO and MAFP of activated human monocytes significantly reduces mCRP formation. (C) Western blot of native PAGE with 1/20 SDS of human monocytes stained for mCRP with clone 9C9 after incubation with ONO at concentrations from 10 to 0.01 μM for 20 min followed by incubation with pCRP (25 μg/ml) + LPS (25 ng/ml) for another 60 min. A typical result out of 3 experiments is shown. (D) Quantification of the Western blot results. Given are relative values of immunoreactivity for mCRP normalized to β-Actin signal. Values are mean ± SEM; # p<0.05. ONO inhibits mCRP formation in a concentration dependent manner. (E) Quantification of PLA2 activity in mononuclear leukocytes after treatment with 25 ng/ml LPS ± 20 μM ONO. Induction of PLA2 activity by exposition to LPS is significantly decreased by addition of ONO as measured by cleavage of a PLA2 specific fluorescent substrate. Mean ± SEM are given. Experiments were performed in triplicates; n=3; # p<0.05. Representative results are shown.

**Figure 6.** Prevention of CRP-mediated inflammation by inhibition of PLA2. Evaluation of leukocyte rolling (A) and adhesion (B) by intravital microscopy. After cremasteric superfusion with LPS (25 ng/ml) and ONO (10 μM) ± i.v. application of pCRP (25 μg/ml). Dot plots and mean of 6 rats are shown; # p<0.05. ONO prevents the CRP-induced pro-inflammatory potential on circulating leukocytes in LPS-triggered inflammation with a significant decrease of leukocyte rolling (A) at 120 min and a significant reduction of leukocyte adhesion (B) after 60 min. (C)
Conformation specific immunofluorescence detection of p- and mCRP in vivo. pCRP was fluorescently labeled with Alexa594 (L-pCRP). In vivo detection of mCRP was conducted by i.v. injection of mCRP binding Alexa488 labeled aptamer (mCRP-Apt.; 2,5 μg/ml) after 100 min and subsequent intravital imaging after 120 min. mCRP can be detected in vivo in inflamed tissue by mCRP specific aptamer binding. In LPS-triggered inflammation, mCRP is detected intravascularly and on transmigrating leukocytes (arrows). Cremasteric superfusion with ONO abrogates the formation of mCRP in acute inflammation. Representative results are shown; n=3. (D) In vitro binding of mCRP-Apt. to p- and mCRP quantified by fluorescence spectroscopy. Mean ± SEM are given. # p<0.05 compared to the corresponding value under pCRP. mCRP could be specifically detected by the aptamer with a significant increase in fluorescence signal at concentrations of 0,5 μg/ml compared to the pCRP group. (E) Pro-inflammatory testing of mCRP-Apt. in a static monocyte adhesion assay in the presence or absence of CRP-isoforms after 60 min treatment. Experiments were performed in triplicates, PBS control was set to 100%. Mean ± SEM are given; # p<0.05. mCRP-Apt. has no intrinsic pro-inflammatory potential and shows no significant impact on the pro-inflammatory potential of mCRP. (F) Flow chart of the intravital experimental protocol.

**Figure 7.** Prevention of pCRP dissociation by decameric stabilization. (A) Western blot of native PAGE (1/20 SDS) of human monocytes stained for mCRP after stabilization of pCRP with 1,6-bis PC (PC) that was administered for 60 min after pre-incubation with LPS (25 ng/ml; 20 min). A typical result out of 3 experiments is shown. 1,6-bis-PC prevented dissociation of pCRP to mCRP on activated monocytes. (B) Chemical structure of 1,6-bis-PC. (C) Detection of mCRP in the LPS-inflamed cremasteric tissue after i.v. application of pCRP vs. PC-pCRP. Western blot
of native PAGE with 1/20 SDS stained for mCRP. pCRP solution and pre-dissociated mCRP solution served as controls. A typical result out of 3 experiments is shown. (D) Immunohistochemical staining of the cremaster muscle with HistoGreen after treatment with LPS + pCRP ± PC. Clone 8D8 was used to detect pCRP and clone 9C9 was used to detect mCRP. Representative results are shown (n= 6 for each sample). pCRP dissociation and interstitial deposition in LPS-triggered inflammation is abrogated by stabilization of pCRP with PC. (E) Quantification of leukocyte-endothelial interaction in postcapillary venules of the inflamed cremasteric tissue by intravital microscopy under i.v. application of pCRP (25 μg/ml) ± PC by 20 min. Dot plots and mean of 6 rats are shown; # p<0.05. By preventing CRP dissociation PC masks the pro-inflammatory potential of CRP in acute inflammation and induces a significant decrease in leukocyte rolling after 60 min (E) and a significant decrease in leukocyte adhesion (F) after 120 min compared to the LPS+pCRP group. 1,6-bis-PC alone has neither pro- nor anti-inflammatory potential. (G) Flow chart of the intravital experimental protocol. (H) Human mCRP but not pCRP can be detected in infarcted rat myocardium following administration of human pCRP into the circulation. Non-infarcted right ventricular tissue from the same animal was used as a control and showed no evidence of mCRP or pCRP deposition. When pCRP was pre-incubated with 1,6-bis-PC (PC) (50:1 molar ratio) there was no significant deposition of mCRP or pCRP in either the infarcted or non-infarcted tissue. This result did not differ significantly from the vehicle control displayed in the third column. n=6 rats per group, typical examples are given. (I) Quantification of results shown in 7.H using image analysis software as described in Methods. 3 slides of each animal were analysed and averaged. The average counted as value for one rat (n=6).
**Figure 8.** Proposed mechanism of PLA2-mediated LPC generation and consecutive pCRP dissociation in LPS-induced inflammation. LPC mediates the dissociation of pCRP and is expressed on activated membranes via induction of PLA2. mCRP triggers leukocyte-endothelial interaction and is deposited in areas of inflammation. The pro-inflammatory effects of the resulting mCRP can be abrogated by inhibition of PLA2 with ONO-RS and thus inhibition of LPS generation or by inhibition of pCRP dissociation by decameric stabilization of pCRP with 1,6-bis-PC.
Figure 1
Figure 1, cont’d
Figure 2
Figure 3
Figure 3, cont’d
Figure 4
Figure 5
Figure 7
Figure 7, cont'd
Figure 8
The Dissociation of Pentameric to Monomeric C-Reactive Protein Localizes and Aggravates Inflammation: In vivo Proof of a Powerful Pro-Inflammatory Mechanism and a New Anti-Inflammatory Strategy

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Methods

Reagents

The anti-pCRP antibody clone 8D8 and anti-mCRP antibody clone 9C9 were generated and characterized for conformation-specificity as described \(^1\) and used as hybridoma supernatant. The aforementioned reagents were kindly provided by Dr. Larry Potempa (College of Pharmacy, Roosevelt University, Schaumburg, USA). Anti-CRP antibody clone 8, Lipopolysaccharide from Escherichia coli serotype O127:B8 (LPS), phosphatase substrate, MAFP (methyl Arachidonyl fluorophosphonate) and ONO-RS-082 were all obtained from Sigma-Aldrich. Alexa488 labeled Annexin V solution was obtained from Life Technologies (Carlsbad, USA), mouse anti-human CD68 from Dako. mCRP binding RNA aptamer was purchased from Integrated DNA Technologies (Leuven, Belgium), and labeled with Alexa488. The aptamer sequence was: 5'-Alexa488-GCC UGU AAG GUG GUC GGU GUG GCG AGU GUG UUA GGA GAG AUU GC-3'. Stocks were reconstituted with DEPC-treated water and stored at -20°C until used. Cobra venom factor (CVF) was obtained from Quidel (Santa Clara, USA) and stored at -70°C until use. 1,6-bis(phosphocholine)-hexane (PC) was synthesized by Syngene International, Bangalore, India.

Immunohistology of human tissue samples

Tissue samples were obtained from 11 patients receiving reconstruction of soft tissue defects after traumatic injuries of the lower extremity by means of free muscle transfer as described previously \(^2\). The first sample was taken intra-operatively
immediately prior to interruption of the blood supply and the second one after ischemia and reperfusion on post-operative day 5. The study was approved by the ethic committee of the University of Freiburg Medical Centre and informed consent was obtained from each patient. Immunohistological staining and analysis by planimetry were performed according to previously published protocols. Antibody clone 8D8 was used for the detection of pCRP and clone 9C9 for the detection of mCRP.

Human atherosclerotic plaques derived from carotid endarterectomy were stained as described above. After incubation with HRP-labeled anti-mouse antibody (Dako, Glostrup, Denmark) reaction products were stained with HistoGreen substrate kit for peroxidase (Linaris, Dossenheim, Germany) resulting in a green reaction product.

For staining of human myocardial tissue samples were obtained from the CVPath Institute (Gaithersburg, Maryland, USA). All samples had been referred for post-mortem examination and cases where myocardial infarction had been determined to occur between 2 and 5 days prior to death were selected. Myocardial tissue was fixed in formalin prior to examination and histology was performed as described previously.

Samples were prepared as described previously with slight modification. Slides were de-parafinized in xylene and rehydrated in graded alcohol solutions followed by distilled water. Antigen retrieval was performed by 30 min of heating at 37°C in 2M hydrochloric acid solution. Samples were rinsed and the staining protocol continued as published previously.

Animal model for intravital microscopy studies
Animal studies were approved by the institutional animal care committee of the University of Freiburg, Germany. Male Wistar rats (Charles River, Sulzfeld, Germany) weighing 120 - 180 g were used. A detailed protocol has been previously described\(^6\).

Leukocyte-endothelial interactions in the cremaster muscle were observed by intravital microscopy. Leukocytes were labeled by intravenous injection of rhodamine 6G (0.4 mg/kg body weight, Sigma-Aldrich)\(^7\). Leukocytes were considered adherent to the endothelium if they remained stationary for 20 s or more. Rolling leukocytes were defined as those moving at a velocity less than that of erythrocytes within a given vessel. One to three postcapillary venules with a diameter of 20 - 50 \(\mu\)m were chosen for observation; to minimize variability, the same section of cremasteric venule was observed throughout the experiment.

Following stabilization of the tissue, a recording of 30 seconds was made to establish baseline values for leukocyte rolling and adherence. To minimize the influence of pre-activation of the tissue, only vessels in which leukocyte rolling was <20 cells/30 seconds and the number of adherent cells <10 cells/200 \(\mu\)m of endothelium were utilized for study. Animals observed over a 120 min protocol were randomly assigned to 8 different groups as follows (n=6 for each group). LPS: The cremaster muscle was constantly superfused with low dose (25 ng/ml) LPS for 120 min. Control: vehicle control (PBS-Ca-Mg). pCRP: A pCRP-solution bolus (25 \(\mu\)g/ml serum concentration) was injected at 20 min. Weight-dependent rat serum volume was calculated\(^8\) and serum levels were verified by a particle-enhanced immunoturbidimetric assay (Modular Analytics, Roche Diagnostics, Basel, Switzerland). LPS+pCRP: pCRP was injected after 20 min (at 25 \(\mu\)g/ml serum concentration) in addition to the superfusion with LPS (25 ng/ml). In some groups, we added ONO-RS-082 (ONO) at 10 \(\mu\)M to the superfused solution for reversible PLA 2 blockade. Superfusion was initiated at 0 min. For decameric stabilization 1 mg/ml...
pCRP solution was incubated with 1,6-bis(Phosphocholine)-hexane (PC) in 100 to 1 molar ratio for 30 min at 37°C. Animals observed over a 60 min protocol and were randomly assigned to 4 different groups (n=6 for each group). pCRP and mCRP were both injected at 25 µg/ml serum concentration after establishing baseline values of leukocyte rolling and adhesion. Constant superfusion with LPS (1 µg/ml) served as a positive control and PBS-CA-Mg as vehicle control.

To investigate the role of the complement system in CRP mediated inflammation *in vivo*, rats were treated with CVF (250 U/kg bodyweight) 24 hours before intravital imaging via i.p. injection. Deactivation of the hemolytic complement activity in rat serum was measured by calculation from the dilution producing 50% lysis of optimally sensitized sheep erythrocytes using the CH 50 test from Diamedix (Miami, USA).

*Rat myocardial infarction/reperfusion model, histology and quantitative PCR*

Adult male Wistar rats were weighed and anaesthetized. Following a sub-costal incision the left anterior descending coronary artery was ligated with a quick release suture. Myocardial ischemia was confirmed via electrocardiograph changes and myocardial pallor. Lignocaine 1mg/kg was administered intravenously to minimize electrical instability. After 40 min of ischemia the coronary suture was released and human CRP (200 µg), human CRP incubated with PC (200 µg CRP, 50:1 molar ratio PC:CRP) or vehicle control were infused intravenously. Animals remained anaesthetized and were sacrificed after a further hour by exsanguination. The hearts were excised, suspended in OCT compound and fresh frozen in liquid nitrogen prior to storage at -80°C. All experiments were approved by and performed in accordance with the institutional animal ethics committee.
6µm fresh frozen sections were analyzed by immunohistochemistry with conformation-specific anti-CRP antibodies as described above. Histological analysis was performed on infarcted and non-infarcted myocardium. Infarcted areas were identified anatomically from the left anterior descending artery territory. Right ventricular free wall tissue supplied by the non-ligated right coronary artery was used as a matched non-ischemic control in all samples reported. For determination of myocardial damage, sections were stained with Alexa 488 anti-rat CD45 antibody (1:50 dilution, Biolegend). Vascular margins were displayed via Cy3-conjugated monoclonal anti α-smooth muscle actin (SMA) antibody clone 1A4 (1:200 dilution, Sigma-Aldrich). The number of leukocytes in tissue was quantified in at least three non-overlapping visual fields of each sample to determine the corresponding value (n=3). Nuclei were stained blue with DAPI mounting medium (Vector Laboratories). For determination of CRP deposition, Alexa 488 labeled clone 8 antibody (Sigma-Aldrich) was used in a 1:100 dilution. Analysis was carried out by planimetry. For the quantification of mRNA-expression, 30mg frozen infarcted and non-infarcted myocardium was disintegrated and homogenized mechanically by using the Ultra-Turrax (IKA-Werke, Staufen, Germany). The RNA isolation was done by RNeasy Fibrous Tissue Mini Kit (Quiagen). RNA was dissolved in 30µl RNase-free water and concentration and purity of RNA were determined spectrophotometrically. cDNA synthesis and TaqMan PCR assays were performed as described under “Fcγ-Receptor studies”. Rat-specific primers and probes were obtained from Applied Biosystems (Primerset Caspase 3, Primerset IL-6, Primerset TNF; TaqMan Gene Expression Assays). Data were analyzed using the relative standard curve method, with each sample being normalized to GAPDH.
**In vivo Phosphatidylserine detection**

Alexa488 labeled Annexin V solution (Life Technologies) was intravenously injected in male Wistar rats at 1 ml/kg BW. Cremasteric superfusion with LPS (25 ng/ml) was initiated 20 min later. Endothelial associated phosphatidylserine expression in the cremaster muscle was detected via Annexin V specific binding as previously described \(^{10}\). The fluorescence signal was measured by digital intravital epifluorescence microscopy in 20 min intervals (n=3).

**In vivo pCRP tracking and mCRP detection**

For some intravital experiments, pCRP was fluorescently labeled with Alexa594 according to the manufacturer’s protocol (Protein Labeling Kit, Life Technologies) to form stable dye-protein conjugates (L-pCRP).

For the detection of mCRP Alexa488 labeled mCRP binding aptamer (mCRP-Apt.) was intravenously injected (2.5 µg/ml serum concentration) 100 min after LPS superfusion (25 ng/ml) and application of L-pCRP (25 µg/ml) as previously described in the presence or absence of ONO (10 µM). The fluorescence signal was assessed after 120 min (n=3). For *in vitro* testing a collagen matrix was coated with pCRP and mCRP (25 µg/ml) followed by incubation at 4°C overnight. Blockade with 1% BSA was followed by addition mCRP-Apt. for 1 hour at RT at various concentrations. After extensive washing against PBS-Ca-Mg aptamer binding was quantified using fluorescence spectroscopy.

Pro-inflammatory testing of mCRP-Apt. was conducted in a static monocyte adhesion assay described below in the presence or absence of mCRP-Apt. at 1 and 5 µg/ml.
**Measurement of ROS generation**

After intravital microscopy rats were killed by exsanguination. Mononuclear cells were isolated from whole blood by Ficoll density gradient centrifugation. ROS formation was determined by electron spin resonance spectroscopy (ESR) using CMH (1 mM, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine, Noxygen, Elzach, Germany) as the spin label and was prepared as described by Kuzkaya et al. Cell count was determined and 500 µl of cell suspension were incubated at 37°C for 30 min with 25 µg/ml CMH (1 mM). The oxidation of spin probe CMH by ROS generated stable 3-methoxycarbonyl-proxyl radicals (CM) was quantified by ESR referring to standard CM solutions. Measurements were performed using a MiniScope MS 200 ESR Spectrometer (Magnettech, Berlin, Germany) with following instrument settings: center field, 3340 G; sweep wide, 60 G; sweep time, 5 milliseconds over 10 scans; modulation amplitude, 2.4 G; microwave power, 10 mW. Results were charged against the cell concentration and expressed as percentage of values of the control group.

**Experimental protocol of the Fcγ-receptor studies**

Monocytes were isolated from peripheral venous blood of healthy human volunteers and cultured for 24 hours (37°C / 5% CO₂). After transfection with the respective siRNA cells were harvested, centrifuged and TaqMan RT-PCR was performed. Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Total RNA (0.5 µg) was treated with 3 units of desoxyribonuclease I (DNase I, Invitrogen, Karlsruhe, Germany) to digest genomic
DNA contamination. Random-primed cDNA synthesis was performed using 0.5 µg of DNase I-treated total RNA and 50 units of AffinityScript reverse transcriptase according to the manufacturer's instructions (Stratagene, La Jolla, USA). TaqMan PCR assays were performed in 384-well optical plates on a LightCycler (Roche, Mannheim, Germany) using Absolute qPCR ROX Mix (Abgene, Hamburg, Germany) according to the manufacturer's instructions. Oligonucleotide primers and probes for human GAPDH (GAPDH Forward Primer, GAPDH-875F; GAPDH Reverse Primer, GAPDH-946R; GAPDH-probe-899; Eurofins MWG Operon, Ebersberg, Germany), human Fcγ-Receptor I (Primerset Fcγ-RI, TaqMan Gene Expression Assays, Applied Biosystems, Foster City, USA), IIA (Primerset Fcγ-RIIA, TaqMan Gene Expression Assays), IIIA (Primerset Fcγ-RIIIA, TaqMan Gene Expression Assays) were used.

Data were analyzed using the relative standard curve method, with each sample being normalized to GAPDH to correct for differences in RNA quality and quantity.

Flow cytometry

Transfected cells were harvested, cleaned and the pellet was resuspended in PBS + 2% FCS. For each sample tubes (BD Falcon 5ml Polystyrene Round-Bottom Tube, BD Biosciences) were prepared containing 100 µl cell suspension and 5 µl FITC-labeled antibody (Monoclonal anti-human CD16, Clone 3G8, Sigma-Aldrich; Anti-Human CD32-FITC, Clone IV.3, Stemcell Technologies, Grenoble, France; Human CD64 FITC Conjugate Mouse IgG1, Invitrogen) or 5 µl FITC-labeled isotype control antibody (IgG1-FITC Isotype Control, Clone MOPC 21, Sigma-Aldrich; FITC Mouse IgG2b, Clone MPC-11, BioLegend, San Diego, USA; Mouse IgG1 FITC Conjugate Negative Control, Invitrogen), respectively. After an incubation period of 30 min, 1 ml
of PBS +2 % FCS was added and flow cytometry was performed using BD LSRFortessa Cell Analyzer (BD Biosciences).

**Immunohistology of rat cremasteric tissue**

After intravital microscopy the cremaster muscle was excised, placed in tissue freezing medium (Leica Microsystems, Nussloch, Germany), and stored at -20°C. Specimens were cut vertically into 6 µm thick serial sections and immobilized on pre-cleaned microscope slides (SuperFrost, R. Langenbrinck, Emmendingen, Germany). Anti-macrophage/monocyte antibody clone ED-1 (Millipore, Billerica, USA) was used in a 1:100 dilution to bind transmigrating rat monocytes/macrophages and fluorescently detected with FITC-anti-mouse secondary antibody (1:1000 dilution, Sigma-Aldrich). Venule margins were displayed via Cy3 conjugated monoclonal anti-α-smooth muscle actin (SMA) antibody clone 1A4 (1:200 dilution, Sigma-Aldrich). Macrophages/monocytes level with the α-SMA⁺ layer or beyond within a 50 µm distance were regarded as transmigrated. At least 9 venules of 50-150 µm in diameter were quantified per rat at non-overlapping levels (n=3). Nuclei were stained blue with DAPI mounting medium (Vector Laboratories, Burlingame, USA). For detection of CRP specimens were cut horizontally. Staining was performed as described previously. Antibody clone 8D8 was used for the detection of pCRP and 9C9 for the detection of mCRP. Analysis was carried out by planimetry.

**Static monocyte adhesion assay**

Static monocyte adhesion assays were conducted as previously described. Briefly, monocytes from healthy human volunteers or from male Wistar rats were purified by
Ficoll gradient centrifugation (1×10^6/ml) and incubated with either forms of CRP at 25 μg/ml for 60 min at 37°C. mCRP-Apt was added at 1 or 5 μg/ml to the CRP isoforms. 100 μl/well of this suspension was added to fibrinogen coated 96-well plates (Nunc MaxiSorp ®, Sigma-Aldrich) in triplicates and allowed to adhere for 60 min. After extensive washing with PBS-Ca-Mg phosphatase substrate stain (50 mM sodium acetate pH 5.0, 1% Triton X-100 and 6 mg/ml of phosphatase substrate, all from Sigma) was added and incubated for 1 h at 37°C. Staining was terminated with 1 M NaOH and absorbance measured at 405 nm by spectrophotometry. Monocytes incubated with 25 μg/ml LPS served as positive control.

**Determination of PLA2 activity**

Phospholipase A2 activity in lysates of mononuclear leukocytes was assayed by EnzChek Phospholipase A2 Assay Kit (Invitrogen). Briefly, freshly isolated human mononuclear leukocytes (2×10^6/ml in RPMI 1640 medium) were incubated with PLA2 inhibitor ONO-RS (10 mM) for 30 min followed by LPS (25 ng/ml) for 2 h at 37°C. Cells were washed in PBS and resuspended in 200μl PLA2 assay buffer. Cell lysis was accomplished by repeated freezing and thawing in liquid nitrogen and pressing the lysate through a 27G needle. Lysates were cleared of cell debris by centrifugation and the protein concentration was adjusted to 4mg/ml. PLA2 activity was measured by the intensity increase of a single wavelength at 515nm according to the manufacturer’s protocol.

**Western blot analysis**
For CRP detection in rats, tissue was homogenized on ice using an ultra turrax (Ika, Staufen, Germany). Cells from rat tissue or rat monocytes were lysed by addition of lysis buffer (1 % NP-40, 0.5% sodiumdeoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.2 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 mM sodium orthovanadate). Particulate material was removed by centrifugation and protein concentration of the supernatants was determined using a BCA protein assay kit (Sigma-Aldrich). About 20 µg of total protein were separated by SDS gel electrophoresis, transferred to Hybond ECL nitrocellulose membranes (GE Healthcare, Munich, Germany), and probed with anti-CRP antibodies for 1 h at RT. To confirm equal loading of the gel, the membrane was stripped and re-probed with monoclonal antibodies against β-actin (Sigma-Aldrich) or GAPDH (abcam, Cambridge, UK). Detection was performed after incubation of the membrane with an anti-mouse horseradish peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany) for 1 h at RT using enhanced chemiluminescence (ECL, GE Healthcare) as described by the manufacturer and captured on Hyperfilm ECL (GE Healthcare). The signal intensity per millimeter squared was quantified by ImageJ version 1.46 (background values were subtracted). For Western blotting with SDS reduced to 1/20th of normal levels cell lysis buffer containing 20mM Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane, 500 mM ε-aminocaproic acid, 20 mM NaCl, 10% glycerol, 0,5% Triton, and protease inhibitors were used. SDS levels in all solutions were reduced to 1/20th of normal levels (0,4% SDS in sample buffer). Buffers were produced without Dithiothreitol (DTT) and β-Mercaptoethanol.
A. Di.p. injection 24 hours before imaging

B. Rhodamine 6G

C. CVF [250 U/kg BW]

D. 120 min LPS-superfusion [25ng/ml]

Online Supplemental Figure 1
Online Supplemental Figure 2

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FITC-FcγR IIa
Alexa594-mCRP
FITC-FcγR IIa
control
Online Supplemental Figure Legends

Supplemental Figure 1

(A) Prevention of CRP-mediated inflammation by depletion of the complement system. Leukocyte-endothelial interaction under superfusion with LPS (25 ng/ml) ± i.v. application of pCRP (25 μg/ml) in complement-depleted rats quantified by intravital microscopy. Dot plots and mean of 6 rats are shown; # p<0.05. Depletion of the complement system via i.p. injection of CVF (250 U/kg BW) 24 hours prior to intravital imaging prevents the CRP-induced pro-inflammatory potential on circulating leukocytes in LPS-triggered inflammation with a significant decrease of leukocyte rolling after 60 min (A) and adhesion (B) at 120 min. (C) Quantification of the complement system activity by measuring the 50% hemolytic complement (CH50) activity of rat serum. Experiments were performed in triplicates. Mean ± SEM are given. # p<0.05. Application of CVF at 250 U/kg BW induces a significant decrease in complement system activity 24 hours after i.p. injection (n=3 rats). (D) Flow chart of the intravital experimental protocol.

Supplemental Figure 2

(A,B) Fcγ receptor knockdown in human monocytes evaluated on mRNA and protein expression level. (A) Fcγ-R mRNA-expression is significantly lower in all knockdown groups. (B) Quantification of Fcγ-R surface-expression via flow cytometry shows an equivalent reduction after Fcγ-R-knockdown. # p<0.05; values are mean ± SEM (n=3). (C-E) Results of static monocyte adhesion assays under Fcγ-RI (C), Fcγ-RIIA (D) and Fcγ-RIIIA (E) knockdown. Receptor knockdown results in a significant reduction of the pro-inflammatory potential of mCRP for Fcγ-RI and Fcγ-RIII, whereas Fcγ-RIIA knockdown has no significant effect. # p<0.05; * indicates a
p<0.05 compared to the corresponding LPS control (n=3). (F) Fcγ-RIIIa knockdown reduces mCRP binding to monocytes. Addition of mCRP leads to receptor clustering and Fcγ-RIIIa co-localization with mCRP in native monocytes. Fcγ-RIIIa expression on monocytes was visualized by FITC labeled anti-Fcγ-RIIIa antibody. mCRP binding was visualized with Alexa594 labeled anti-CRP antibody (clone 8). FITC and Alexa Fluor594 isotype control show no binding. Representative results of confocal microscopy are shown.

Supplemental Figure 3

Stabilization of pCRP with 1,6-bis PC prior to i.v. application in rats prevents the CRP-induced tissue damage in infarcted myocardium. (A) Immunofluorescence detection of tissue leukocytes in rat myocardium using Alexa488-anti CD45 antibody (upper panel) and detection of CRP (lower panel) using Alexa488-clone 8 antibody. Vascular margins are displayed via Cy3-anti α-SMA stain, nuclei are stained with DAPI. Representative results of the infarcted myocardium are shown. (B,C) Quantification of the immunofluorescence results. (B) Mean leukocyte count of the CRP group in infarcted tissue was set to 100%; # p<0.05. (C) Relative values of immunofluorescence for CRP are shown. At least three non-overlapping visual fields were evaluated of each sample to determine the corresponding value. n=3 rats / group; # p<0.05. Values are mean ± SEM. Addition of 1,6-bis PC prevents deposition of CRP and significantly decreases the number of leukocytes in the infarct, which were increased in number under i.v. application of CRP alone. (D-F) Relative mRNA expression of Caspase 3, IL-6 and TNF-α in infarcted rat myocardium and non-infarcted myocardium. Values were normalized to GAPDH to correct for differences in RNA quality and quantity. n=3 rats / group; # p<0.05. Values are mean ± SEM.
CRP significantly increases the expression of Caspase 3 and TNF-α in infarcted tissue. IL-6 induction under CRP does not reach statistical significance compared to the vehicle control. 1,6-bis PC prevents the CRP-induced tissue damage in myocardial infarction and significantly decreases Caspase 3, TNF-α and IL-6 expression.

References

1. Wang MS, Black JC, Knowles MK, Reed SM. C-reactive protein (crp) aptamer binds to monomeric but not pentameric form of crp. Anal Bioanal Chem. 2011;401:1309-1318


5. Strang F, Scheichl A, Chen YC, Wang X, Htun NM, Bassler N, Eisenhardt SU, Habersberger J, Peter K. Amyloid plaques dissociate pentameric to monomeric c-


