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Monomeric CRP contributes to complement control in fluid phase and on cellular surfaces and increases phagocytosis by recruiting factor H

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Complement forms the first defense line of innate immunity and has an important role in the non-inflammatory clearance of apoptotic and necrotic cells. Factor H is one essential complement inhibitor that binds to the acute phase reactant C-reactive protein (CRP). By using recombinant proteins, calcium-independent binding of Factor H to monomeric CRP (mCRP), but not to pentameric CRP (pCRP), was shown. In addition to the two known CRP-binding sites, a novel third site was localized within the C-terminus. This region is frequently mutated in the hemolytic uremic syndrome and the mutant proteins show reduced mCRP binding. In this study, we show that mCRP directs Factor H to the surface of apoptotic and necrotic endothelial cells and identify phosphocholine as one binding moiety for this complex. Factor H–mCRP complexes enhance C3b inactivation both in the fluid phase and on the surface of damaged cells and inhibit the production of pro-inflammatory cytokines. By recruiting the soluble complement inhibitor Factor H to the surface of damaged cells, mCRP blocks the progression of the complement cascade beyond the step of the C3 convertase, prevents the formation of inflammatory activation products, and thus contributes to the safe removal of opsonized damaged cells and particles.
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Damaged cells, in the form of apoptotic or necrotic cells, are cleared from the circulation in a safe, non-inflammatory manner. The complement system, as a central part of innate immunity, marks modified cells by opsonization and enhances uptake by phagocytic cells.

The central complement inhibitor Factor H controls complement activation at the level of the C3 convertase by acting as a cofactor for the serine protease Factor I for cleavage of C3b and by accelerating the decay of the C3 convertase C3bBb.[1,2] Factor H controls complement activation in plasma and attaches to host cells as well as to biological surfaces, such as the glomerular basement and the retinal Bruchs' membrane. Factor H is composed of 20 domains, termed short consensus repeats (SCR), and has two important functional regions. The N-terminal four domains (SCRs 1–4) mediate complement regulation and the C-terminal domains, that is, SCRs 19–20, are relevant for surface attachment.[3,4]

Factor H binds to the potent inflammatory marker C-reactive protein (CRP).[5] CRP plasma levels increase during inflammation, tissue damage and infection from undetectable levels to more than 500 mg/l.[6] CRP is a member of the pentraxin protein family and is composed of five identical subunits that are stabilized by calcium ions.[7,8] This 125-kDa pentameric form (pCRP) is modified on calcium depletion, heat or urea treatment, surface attachment, by inflammatory conditions, oxidative stress, low pH or by proteases, and dissociates into monomeric units of 23 kDa, termed monomeric CRP (mCRP).[9–11] The exact biological functions of pCRP and mCRP are currently unclear. Both mCRP and pCRP bind to damaged cells. mCRP, but apparently not pCRP, increases the respiratory burst of neutrophils and displays prothrombotic activity on platelet activation.[12,13]

CRP also binds complement components such as the classical pathway effector C1q.[14,15] Bound to apoptotic and necrotic cell surfaces, CRP binds C1q and activates the classical complement pathway to the level of the C3 convertase.[16,17] This results in C3b deposition and opsonization. However, further amplification, C5 convertase formation and terminal pathway activation is inhibited. This blockade is likely because of the attachment of the soluble regulators, Factor H and C4BP, which inhibit complement at the level of C3 convertase and block further progression.[18,19]

In this study, we characterize the Factor H–CRP interaction and compare the binding of the two CRP isoforms with immobilized Factor H. mCRP, but not pCRP, binds to Factor H in a calcium-independent manner. A novel third mCRP-binding site was localized to the C-terminus of Factor H. We show for the first time that mCRP enhances the regulatory activity of Factor H both in the fluid phase and on surfaces. Thus, by recruiting Factor H to the surface of damaged endothelial cells, mCRP blocks further complement amplification. We show further that Factor H bound to apoptotic

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Abbreviations: PC, phosphocholine; aHUS, atypical hemolytic uremic syndrome; mCRP, monomeric C-reactive protein; pCRP, pentameric CRP
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particles increases phagocytosis and uptake by human macrophages, and inhibits production of the pro-inflammatory cytokine TNF-α and the chemokine IL-8.

Results

Binding of Factor H to mCRP and pCRP. Binding of the two CRP isoforms to immobilized Factor H was analyzed by ELISA. A characterization of newly generated mCRP is shown in Supplementary Figure 1. Under physiological conditions, pCRP did not, but mCRP did, bind to immobilized Factor H (compare Figure 1a and b column 1). Binding of mCRP is independent of Ca^{2+}, as similar intensities were observed in the presence and absence of Ca^{2+}. Thus, mCRP, but not pCRP, binds to immobilized Factor H in a Ca^{2+}-independent manner.

Next, the binding of CRP to immobilized Factor H deletion constructs was analyzed. pCRP showed a relatively weak Ca^{2+}-dependent binding to constructs SCRs 1–7, SCRs 8–20, SCRs 8–11, SCRs 11–15 and SCRs 15–20 and no binding to SCRs 15–18 (Figure 1a, columns 2–6). mCRP binding was more pronounced and was Ca^{2+} independent (Figure 1b, columns 2–6). Two CRP-binding sites within Factor H were previously localized to SCR 7 and SCRs 8–11.[5] In this study, by analyzing mCRP binding to Factor H, a novel C-terminal site was identified.

To further localize this third binding site, additional C-terminal deletion fragments were used. pCRP showed rather weak interaction, but mCRP bound Ca^{2+} independently to SCRs 15–20, SCRs 15–19 and SCRs 19–20 but not to SCRs 15–18 (Figure 1c and d). Thus, mCRP contacts Factor H at three sites, which are located in SCR 7, SCRs 8–11 and SCRs 19–20 (Figure 1e).

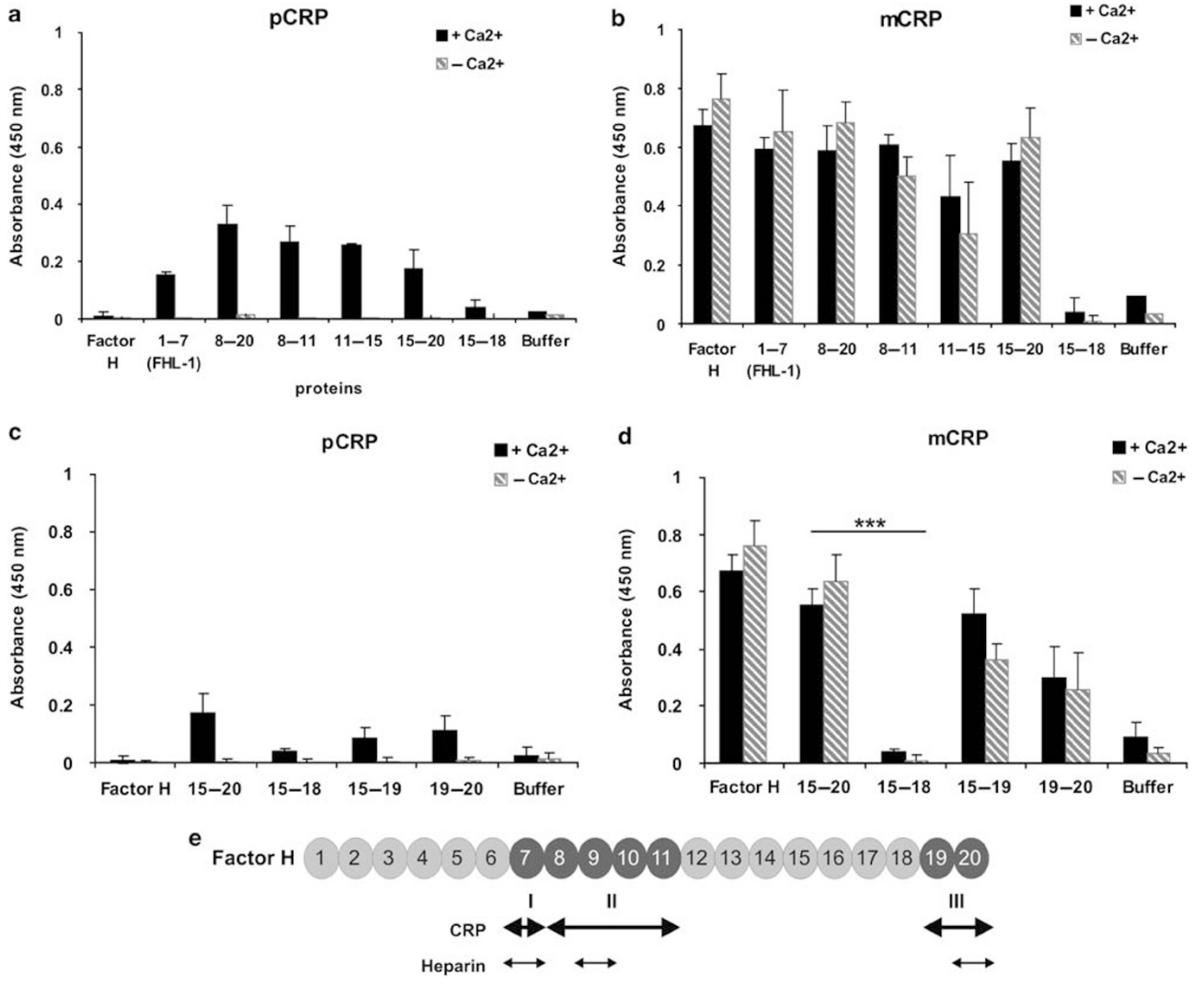


Figure 1 mCRP binds to Factor H through three separate binding sites. Factor H and Factor H deletion fragments were immobilized to a microtiter plate and binding of pCRP as well as mCRP was assayed. After extensive washing, bound CRP was detected with CRP antiserum. (**a**, **c**) In the presence of Ca^{2+}, pCRP bound weakly to Factor H deletion fragments, but not to intact Factor H (black bars) and did not bind in the absence of Ca^{2+} (striped bars). (**b**, **d**) mCRP bound to immobilized intact Factor H and to various Factor H deletion constructs in the presence (black bars) and absence of Ca^{2+} (striped bars) (***$P<0.001$). Buffer without protein revealed background levels. (**e**) Domain composition of Factor H. The binding regions for CRP and the heparin binding sites are indicated

Role of the C-terminus and of the atypical hemolytic uremic syndrome (aHUS)-associated Factor H mutations for mCRP binding. Binding through the C-terminus was confirmed by blocking the interaction with mAb C18, which binds to SCRs 19–20 of Factor H. mAb C18 reduced mCRP binding to full-length Factor H by about 70% and to SCRs 15–20 by ~95% (Figure 2a).

The novel C-terminal mCRP-binding site in Factor H was further characterized using surface plasmon resonance. mCRP was immobilized and equimolar amounts of purified Factor H and C-terminal Factor H deletion mutants were added as analytes. Factor H bound to immobilized mCRP, as indicated by the prominent association and slower dissociation (Figure 2b). Similarly, SCRs 15–20 and SCRs 15–19 showed association. The stronger binding of full-length Factor H is explained by way of an additive effect of the three binding regions. The stronger binding of SCRs 15–20 when compared with that of SCRs 15–19 indicates an important role of SCR 20 for mCRP binding. SCRs 15–18 and the HepG mutant, which has five residues exchanged in SCR 20, did not bind to mCRP (Figure 2b).

Lack of Factor H–HepG mutant binding to immobilized mCRP was confirmed by ELISA (Figure 2c, column 3). SCR 20 is a hot spot of aHUS-related mutations; therefore, four aHUS-associated Factor H mutants were assayed that showed reduced mCRP interaction. Binding of mutant Factor H_{W1183L} was reduced by 86%, Factor H_{V1197A} by 65%, Factor H_{R1210C} by 80% and Factor H_{R1215G} by 72% (Figure 2c). Thus, aHUS-associated C-terminal single amino acid exchanges strongly reduced Factor H–mCRP interaction.

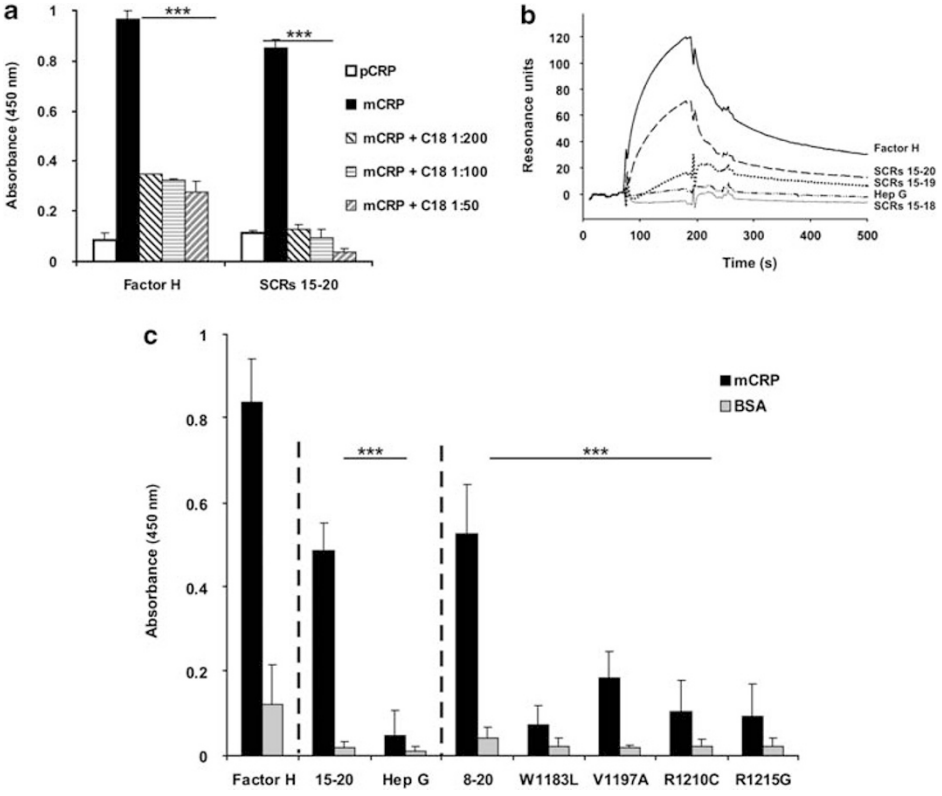
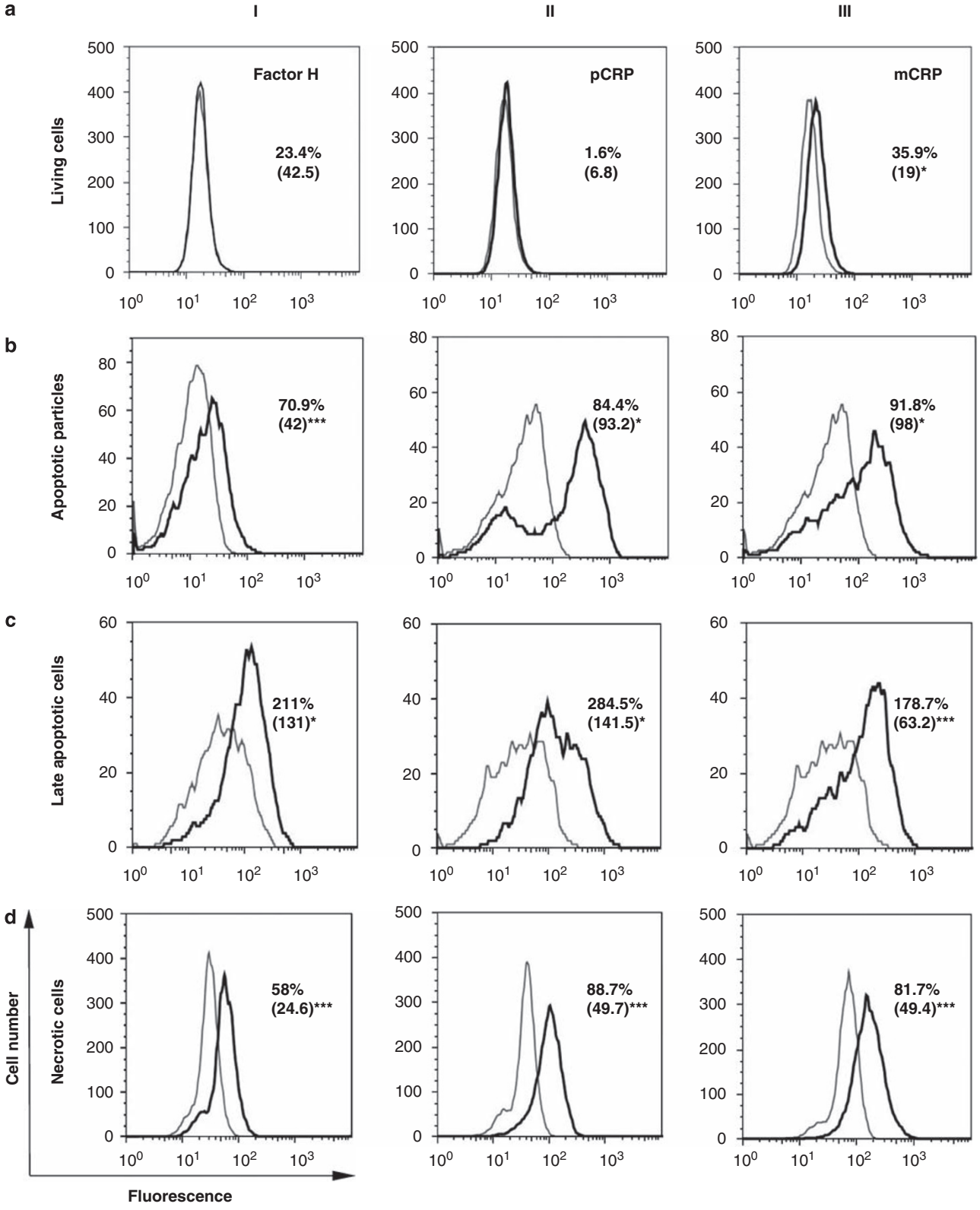


Figure 2 The aHUS-associated mutations of Factor H inhibit binding to mCRP. (**a**) Dose-dependent blockade of Factor H binding to mCRP by monoclonal Factor H antibody C18, which binds to SCRs 19–20. Mean values ± S.D. derived from three independent experiments are shown. The difference is significant (***$P<0.001$). (**b**) Surface plasmon resonance analyses of Factor H binding to immobilized mCRP. Factor H and Factor H deletion fragments SCRs 15–20, SCRs 15–19, 15–18 and HepG (400 nM) were analyzed in the presence of Ca^{2+}. The signal obtained with Factor H on a nontreated surface is subtracted. (**c**) Binding of Factor H, Factor H deletion constructs SCRs 15–20, the HepG mutant (Factor $H_{SCRs15-20}$), SCRs Factor $H_{SCRs8-20}$ and recombinant aHUS mutants (W1183L; V1197A; R1210C; R1215G) to immobilized mCRP. The HepG mutant showed lower binding as the intact Factor $H_{SCRs15-20}$ (compare columns 2 and 3). Similarly, the recombinant aHUS-derived single mutants showed reduced binding compared with Factor $H_{SCRs8-20}$ (***$P<0.001$) to mCRP (column 4). The gray bars show the background binding of Factor H and aHUS mutants to immobilized BSA. The mean values ± S.D. of three independent experiments are shown

Figure 3 Factor H and both CRP isoforms bind to apoptotic and necrotic cells. Binding of Factor H (columns I), pCRP (columns II) and mCRP (columns III) (each 30 μg/ml) to HUVEC cells was analyzed by flow cytometry using the appropriate antisera (thick black lines). Antibody binding in the absence of specific proteins are shown by thin gray lines. (**a**) pCRP did not and Factor H and mCRP bound weakly to intact HUVEC cells. (**b**, **c**) Apoptosis was induced by staurosporine. Factor H, pCRP and mCRP bound to apoptotic particles (Annexin V-positive and DAPI-negative) and to late apoptotic cells (Annexin V-positive, DAPI-positive and caspase 3/7-positive). (**d**) Necrosis was induced by heat treatment. All three proteins bound to necrotic cells (DAPI-positive and caspase 3/7-negative). The numbers represent the increase of MFI values of six independent experiments and the standard deviations are shown in parentheses. To compare the results from six separate independent experiments, the mean values were normalized and the antibody control was set to 100%. Statistical analyses *$P<0.05$, **$P<0.01$, ***$P<0.001$ were determined by unpaired *t*-test

Factor H and both CRP variants bind to the surface of damaged cells. To address the physiological relevance of Factor H–mCRP interaction, we assayed the binding of Factor H and of the two CRP variants to intact and to damaged endothelial cells. The three proteins bound relatively weakly to intact Human umbilical vein endothelial cells (HUVEC) (Figure 3a). However, Factor H, pCRP or mCRP bound to apoptotic particles (Annexin V-positive and



DAPI-negative) (Figure 3b), to late apoptotic cells (Annexin V-positive, DAPI-positive and caspase 3/7-positive) (Figure 3c) and also to necrotic cells (DAPI-positive and caspase 3/7-negative) (Figure 3d and Supplementary Figure 2).

mCRP enhances Factor H binding to the surface of apoptotic particles and necrotic cells. We next studied whether mCRP recruits Factor H to the surface of damaged cells. mCRP increased the surface attachment of Factor H to apoptotic particles, as shown by the increase in mean fluorescence (from 100 to 169%) (Figure 4a and b). This effect is specific, as pCRP had no effect. Rather similar results were observed for late apoptotic and necrotic cells. mCRP increased Factor H binding to late apoptotic cells by approximately 80%. The effect of pCRP was not significant (Figure 4c and d). Similarly, mCRP, but not pCRP, increased Factor H binding to necrotic cells (approximately 40%) (Figure 4e and f). When binding was analyzed by western blotting using cell lysate, mCRP increased Factor H binding to necrotic cells (Figure 4g, lane 3).

Factor H–mCRP complexes bind to phosphocholine (PC). To identify a cellular ligand, the binding of preformed Factor H–mCRP complexes to immobilized PC was assayed. Bound complexes were eluted, separated by SDS-PAGE, transferred to a membrane and analyzed by western blotting. Factor H was identified in the adsorbed sample (Figure 5, lane 3) and showed identical mobility as purified Factor H. No interaction was observed when pCRP was used (Figure 5, lane 2). The faint signal observed when Factor H was incubated with buffer (Figure 5, lane 1) suggests that Factor H in the absence of mCRP does also bind to PC. These results show that Factor H–mCRP complexes are formed in solution and bind to PC. Attachment of Factor H and enhanced binding in the presence of mCRP was also observed in synthetic phosphatidylethanolamine, phosphatidic acid and phosphatidylserine (data not shown).

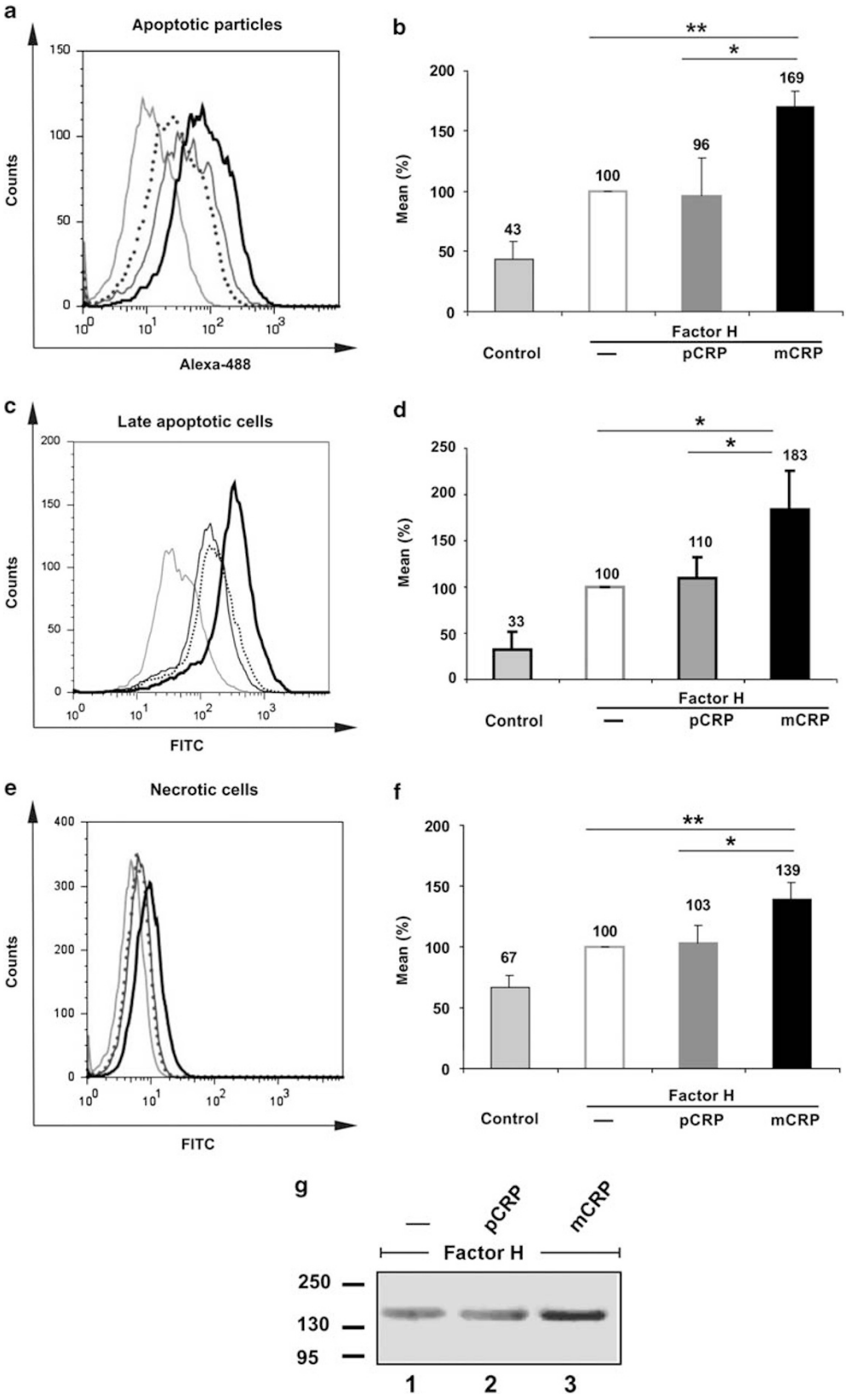
mCRP enhances Factor H cofactor activity in fluid phase and on cell surfaces. Next, we asked whether mCRP affects the complement regulatory function of Factor H. First, the fluid phase cofactor activity of Factor H in the presence of either pCRP or mCRP was assayed. Cleavage of C3b is indicated by a decrease in the intact α' chain and an increase in the α' 46 and α' 43 degradation fragments (Figure 6a, lane 3 and Figure 6c, lane 3). pCRP did not affect cleavage based on the constant intensity of the α' 46 and α' 43 bands (Figure 6a and b, lanes 5 to 7). However, mCRP enhanced cofactor activity, as evidenced by the dose-dependent decrease in density of the uncleaved α' chain and the increased density of the α' 43 band (Figures 6c and d, lanes 5 to 7). At the highest concentration, the density of the α' 43 band was increased almost threefold (Figure 6d, compare column 7 with column 3). Alone, both CRP variants lacked cofactor activity (Figure 6a and c, lanes 4) and did not influence C3b binding to Factor H (Supplementary Figure 3).

The effect of mCRP on Factor H cofactor activity was further evaluated on the surface of necrotic endothelial cells. Cell surface-attached Factor H showed cofactor activity (Figure 6e, lane 3) and, in the presence of mCRP C3b cleavage, was enhanced by $\sim$45% (Figure 6e and f, lanes 6). Again, mCRP alone had no effect (Figure 6e, compare lane 4 with lane 1). The additional band of $\sim$70 kDa, which was detected in samples that included cells treated in bovine serum albumin (BSA) buffer, is considered unspecific and likely represents BSA (Figure 6e, lanes 3–6). Thus, mCRP recruits Factor H and increases Factor H-mediated complement inactivation on the surface of damaged cells.

Factor H attached to apoptotic particles enhances phagocytosis and blocks cytokine release of macrophages, such as TNF-α and IL-8. The biological effect of Factor H was assayed by measuring phagocytosis and uptake of apoptotic particles, as well as cytokine response from activated human macrophages on incubation with Factor H-coated or Factor H–mCRP-coated apoptotic particles. DiD-stained apoptotic particles were coated with mCRP, Factor H or a combination of both, then added to human macrophages and after 1 h the amount of ingested and adsorbed particles was determined by flow cytometry. Phagocytes recognized and ingested apoptotic particles (Figure 7a, compare columns 1 and 2). This effect is not influenced by mCRP (Figure 7a, column 3). In contrast, Factor H on the surface of the particles increases the uptake by about 16% (Figure 7a, column 4) and the Factor H–mCRP complex increases phagocytosis even further by about 40% (Figure 7a, column 5).

To determine whether the increased adsorption and phagocytosis of particles affects the cytokine response of macrophages, TNF-α and IL-8 were determined in the culture supernatant after coincubation. Apoptotic particles induced the release of both cytokines (Figure 7b and c, columns 1). mCRP coating did not affect cytokine induction (Figure 7b and c, columns 2). However, Factor H-coated particles inhibited cytokine release, and this inhibitory effect was more pronounced when Factor H and mCRP were combined (Figure 7b and c, compare columns 3 and 4). Factor H inhibited TNF-α release by 66% and the Factor H–mCRP combination

Figure 4 mCRP enhances Factor H binding to apoptotic and necrotic cells. (**a**) Factor H bound to apoptotic particles (dotted line). mCRP, but not pCRP, increased Factor H binding (thick vs thin black line). Control sample with antibody alone showed weak signal (thin gray line). (**b**) The mean fluorescence values ± S.D. of three independent experiments are shown. The difference between Factor H binding in the absence or presence of mCRP is statistically significant (**$P<0.01$). (**c**, **d**) Similarly, Factor H bound to late apoptotic HUVEC cells. (**d**) The mean fluorescence values ± S.D. of three independent experiments are shown. The difference between Factor H binding in the absence or presence of mCRP is statistically significant (*$P<0.05$). (**e**) Factor H binding to necrotic HUVEC cells. (**f**) The differences of Factor H binding to necrotic cells in the absence or presence of mCRP are statistically significant. (**$P<0.01$) (**g**) The role of mCRP on Factor H surface binding was assayed by western blotting. Necrotic HUVEC cells were first incubated with pCRP or mCRP. After the addition of Factor H, cells were lysed and the lysate was separated by SDS-PAGE and transferred to a membrane. Factor H bound the cells (lane 1). pCRP did not affect binding (lane 2). However, mCRP increased Factor H binding (lane 3). The data are representative of three independent experiments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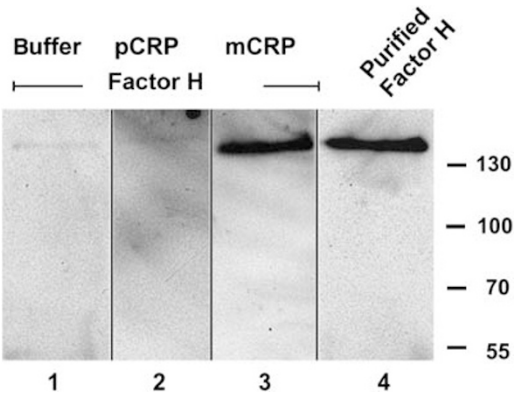


Figure 5 Factor H–mCRP complexes bind phosphocholine. pCRP and mCRP were added to Factor H in solution and the mixture was bound to phosphocholine. Bound complexes were eluted, separated by SDS-PAGE, transferred to a membrane and Factor H was identified by specific antiserum. Factor H was coabsorbed when mCRP, but not pCRP, was present (compare lane 3 and lane 2). The faint band detected in samples with pCRP (lane 2) and with buffer (lane 1) indicates weak binding of Factor H to phosphocholine. Purified Factor H was separated in lane 4. Representative data of three independent experiments are shown

by 80% (Figure 7b). Cytokine release was not detectable for culture supernatant derived from THP-1 cells, and neither was it detectable from apoptotic particles alone (Figure 7b and c, columns 5–6). A similar but not as pronounced an effect was observed for the chemokine IL-8 (Figure 7c).

Discussion

Factor H binding to immobilized CRP was initially described in 1993. In contrast, CRP did not bind to immobilized Factor H.[20,21] This discrepancy is explained by the structural modification of pCRP on immobilization (data not shown).[14,22] In this study, by comparing the binding of mCRP and pCRP to immobilized Factor H, we show that mCRP but not pCRP binds to immobilized Factor H (Figure 1).[23] The Factor H–mCRP interaction is detected in both settings when Factor H or mCRP is immobilized (Figure 1b and d, Figure 2b and data not shown).[22,24] mCRP binding to Factor H is calcium independent (Figure 1b) and is weakly affected by ionic strength or by heparin (Supplementary Figure 4). However, CFHR4A, an additional Factor H family protein, binds pCRP but not mCRP.[25] CRP contacts Factor H at three sites. In addition to the two previously identified sites in SCR 7 and SCRs 8–11, a novel third C-terminal-binding region was identified (Figure 1a–e and 2b).[5]

On calcium-dependent recruitment of the inflammatory marker pCRP to the surface of damaged cells, bound pCRP is modified and structurally altered.[9] This can occur during inflammation, at the surface of apoptotic and necrotic cells, at low pH, in the presence of oxygen radicals or in a proteolytic environment.[11,13,26] Structurally modified CRP, ultimately representing monomeric units, exposes novel binding sites.

Both mCRP and pCRP bind to the surface of necrotic and late apoptotic cells and to apoptotic particles, but not to early apoptotic cells (Figure 3). Surface-bound mCRP, but not pCRP, recruits Factor H and increases the Factor H concentration by approximately 40% (necrotic cells) or 70% (late apoptotic cells and particles) (Figure 4). In this study, we identify PC, which is exposed on the surface of damaged cells, as a ligand for the Factor H–mCRP complex (Figure 5).[27]

CRP binds several complement components and regulators such as C1q.[22] CRP-bound C1q initiates complement activation to the level of the C3 convertase, which results in C3b surface deposition and opsonization.[18,28] However, further progression of the complement cascade, for example, C5 convertase formation, C5a release, progression of the terminal pathway, membrane attack complex (MAC) assembly and membrane insertion does not occur. The initiation of the initial steps and blockade of further steps is explained by recruitment of the regulators Factor H and C4BP. Consequently, Factor H–mCRP and likely also C4BP–CRP complexes inhibit complement activation at the C3 convertase level. Thus, the inflammatory mediator CRP acts as a local complement modulator, which enhances C3b deposition at damaged surfaces, but blocks C5a generation and MAC formation. This results in a non-inflammatory clearance of damaged cells.

Complexed to mCRP and bound to the cell surface, Factor H maintains complement regulatory activity (Figure 6c–f). A similar functional effect was observed for Factor H bound to immobilized mCRP (Supplementary Figure 5). In this study, we show that mCRP affects Factor H-mediated cofactor activity, as mCRP enhances the complement inhibitory function of Factor H both in the fluid phase and at the cell surface. Densitometric analyses show that in the presence of mCRP, cleavage of the C3 α' chain is enhanced almost threefold (Figure 6d). Similarly, mCRP inhibits C1q-mediated complement activation through the classical pathway in fluid phase.[14]

In addition, binding of Factor H increases the uptake of apoptotic particles by human phagocytic cells. mCRP recruits Factor H to the surface of apoptotic particles (Figure 4a), and the combination of mCRP and Factor H enhances the uptake of these particles (Figure 7a). However, Factor H–mCRP complexes attached to the surface of apoptotic particles inhibit cytokine release by human macrophages. Factor H-coated particles inhibited TNF-α release by 66%, and the inhibitory effect was more pronounced (80%) for the combination of mCRP and Factor H (Figure 7b). This is the first direct anti-inflammatory effect reported for Factor H attached to apoptotic particles.

Factor H mutations are associated with the disease and the C-terminus of Factor H represents a hot spot for aHUS-associated mutations.[29] aHUS is also associated with enhanced apoptosis of erythrocytes and human endothelial cells.[30,31] The reduced mCRP interaction of aHUS-associated Factor H mutants (Figure 2c) suggests that under inflammatory conditions this reduced surface binding causes increased cellular damage and enhances inflammation.

CRP is associated with atherosclerosis and opposing effects in plaque development were reported for the two isoforms.[32] In ApoE knockout mice, pCRP increases and mCRP reduces the plaque development.[33] In addition, in atherosclerotic lesions, mCRP colocalizes with complement proteins C3b, iC3b and the terminal complement complex, as well as with oxidized and modified LDL.[27,34,35] This colocalization suggests a cooperative effect of mCRP and

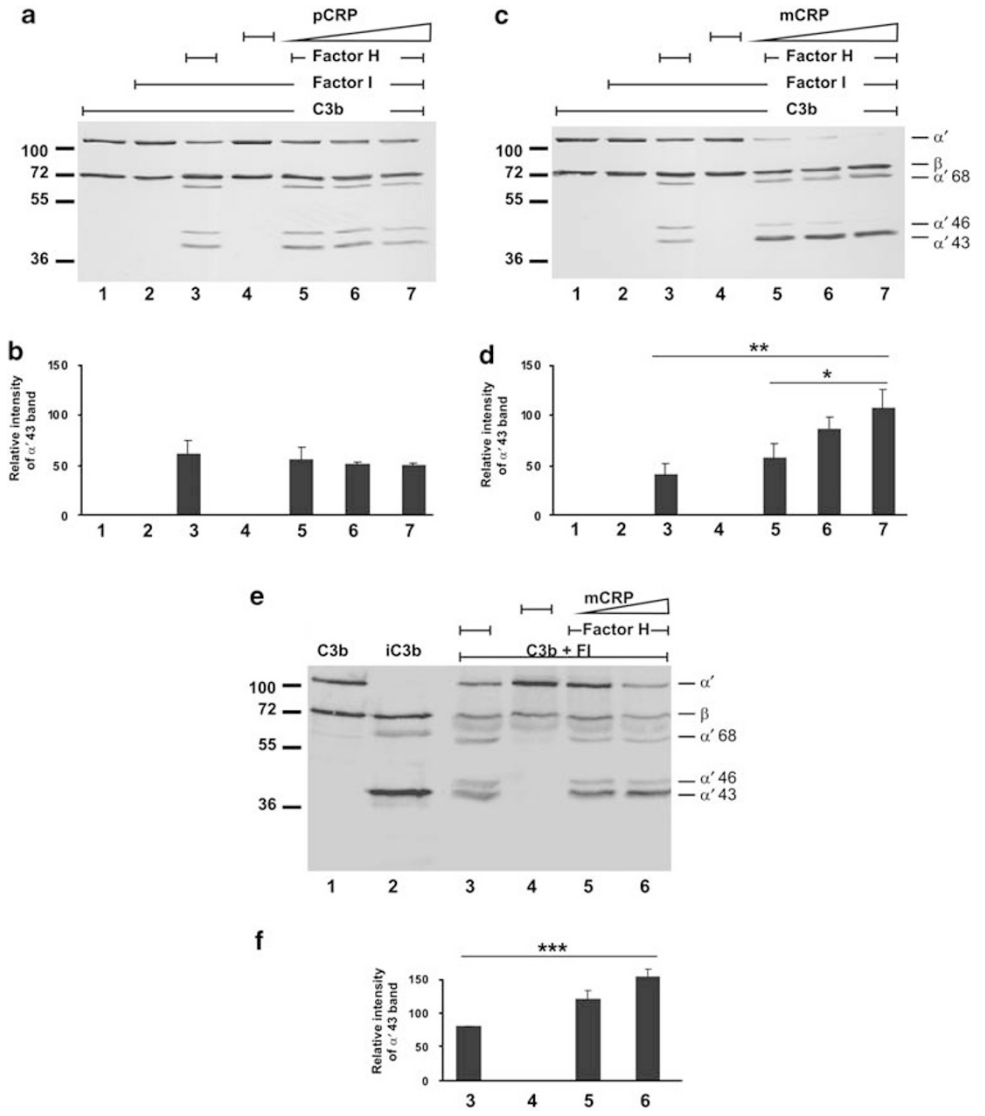


Figure 6 mCRP enhances Factor H-mediated complement control. mCRP enhances cofactor activity of Factor H in the fluid phase. (**a**) The mobility of the C3b α'- and β-chain are shown in lanes 1 and 2 in the absence of Factor I and Factor H. Factor H-mediated cofactor activity for protease Factor I is shown by the decreased intensity of α'-chain and the appearance of cleavage fragments α' 68, α' 46 and α' 43 (lane 3). pCRP used at different concentrations did not influence C3b cleavage (lanes 5–7). (**b**) Densitometry of the α' 43 band showed constant amounts. The results of three independent experiments are shown. The bars show the normalized relative light units of the fragment. (**c**) mCRP enhances Factor H-mediated cofactor activity for C3b cleavage (lane 5–7). This effect is dose dependent over a range of 0.5–1.5 μg mCRP ($^*P<0.05$) (compare lanes 5–7). (**d**) Densitometric analyses of the α' 43 band. Results are derived from three independent experiments. The increase in intensity of the α' 43 band is statistically significant ($^{**}P<0.01$). pCRP and mCRP without Factor H lack cofactor activity, and no cleaving products were detected (panels **a** to **d**, lanes 4). (**e**) mCRP affects Factor H cofactor activity on the surface of necrotic cells. Factor H–mCRP complex was bound, C3b and Factor I were added, and the cells were incubated. The reaction was stopped, cells were lysed, the cell lysate was separated by SDS-PAGE and C3b cleavage products were identified. Factor H-mediated cofactor activity on the cell surface is revealed by the appearance of the α' 68, α' 46 and α' 43 bands and the disappearance of the 110-kDa α' band (lane 3). In the presence of mCRP, the intensity of the α' 43 band is increased, and similarly the decrease in the intensity of the α' chain is dose dependent (lanes 5–6). The cleavage pattern is identical to that of iC3b (lane 2). mCRP alone did not affect cofactor activity (lane 4). (**f**) Densitometric analyses of the α' 43 band. The results of three independent experiments are shown ($^{***}P<0.001$)

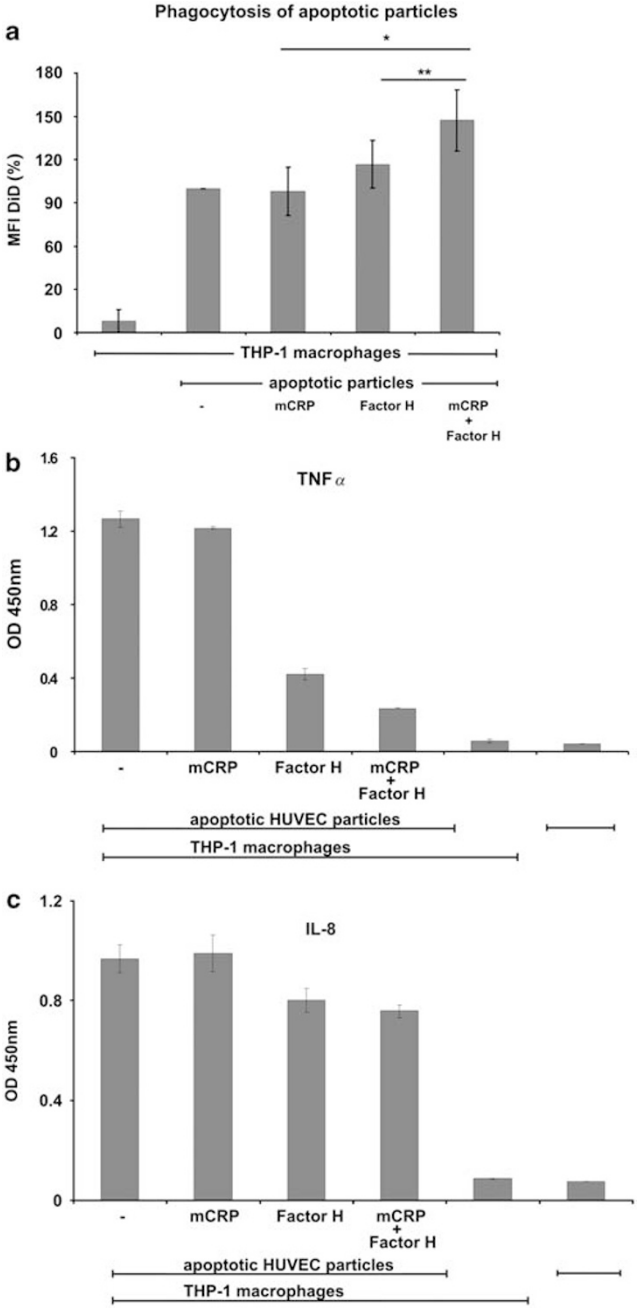
complement in pathology, and also a protective role of mCRP in early atherosclerotic lesions. Later phases of atherosclerosis and thus pronounced inflammation and complement action may override an initial regulatory effect of Factor H (Supplementary Figures 6 and 7).[14,36]

In this study, we identify for the first time that mCRP, but not pCRP, has a complement-modulating effect. mCRP recruits the complement inhibitor Factor H to the surface of damaged cells or particles. mCRP enhances local complement inhibition both in the fluid phase and on the cellular surfaces. Thus, by recruiting C1q to the surface of damaged cells, mCRP triggers complement activation resulting in the formation of C3 convertases and C3b surface deposition. However, by binding the inhibitor Factor H and enhancing the inhibitory activity, further complement activation, amplification, cytokine release, C9 deposition and terminal membrane attack assembly are inhibited (Supplementary Figure 7). Furthermore, the phagocytosis of apoptotic particles is increased.

This shows how CRP can contribute to an anti-inflammatory scenario and explains how mCRP contributes to the safe removal of damaged apoptotic particles and necrotic cells. Apparently, these functions are relevant for diseases, including the aHUS hemolytic uremic syndrome and atherosclerosis.[37]

Materials and Methods

CRP isoforms and complement components. Recombinant pCRP expressed in *E. coli* or pCRP derived from ascites fluid was used (Merck Biosciences, Schwalbach, Germany; Sigma-Aldrich, Taufkirchen, Germany). pCRP was stored in a $CaCl_2$-containing buffer. mCRP was generated by treating pCRP with 8 M urea in the presence of 10 mM EDTA for 1 h at 37 °C. mCRP was thoroughly dialysed in phosphate buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 15 mM NaCl, pH 7.4).[10]



Factor H deletion constructs SCRs 1–7 (FHL1), SCRs 8–11, SCRs 8–20, SCRs 11–15, SCRs 15–20, SCRs 15–18, SCRs 15–19 and SCRs 19–20 were expressed as described.[38] Purified full-length Factor H, Factor I and C3b were purchased from Merck Biosciences. Recombinant mutant Factor H proteins, with single amino acid residues exchanges in SCR 20, representing aHUS-associated mutations, that is, W1183L, V1197A, R1210C and R1215G, were expressed on an SCRs 8–20 backbone.[39] The HepG Factor H mutant has five amino acid residues exchanged (that is, R1203E, R1206E, R1210S, K1230S and R1231A) and was expressed on an SCRs 15–20 backbone.[40]

mCRP and pCRP binding to immobilized Factor H. Binding of mCRP and pCRP to immobilized Factor H was analyzed by ELISA using a TC buffer (Tris-calcium-buffer 140 mM NaCl, 10 mM Tris, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4)[18] or phosphate-buffered saline.[39] In brief, Factor H and the various Factor H constructs (10 μg/ml) were immobilized, and pCRP or mCRP (10 μg/ml) was added in the presence or absence of $CaCl_2$ (2 mM) for 1 h at 37 °C. Bound CRP was detected with goat CRP antiserum (Sigma-Aldrich).

Binding of Factor H mutants to mCRP by ELISA. mCRP (10 μg/ml) was coated in DPBS to Maxisorp ELISA plates (Nunc GmbH & Co. KG, Langenselbold, Germany). After the addition of blocking buffer (AppliChem, Darmstadt, Germany), Factor H mutants were added in the fluid phase (10 μg/ml). Bound Factor H and Factor H mutants were detected using goat Factor H antiserum (Merck, Schwalbach, Germany) followed by peroxidase-conjugated rabbit anti-goat IgG.

Surface plasmon resonance assays. Surface plasmon resonance was used to analyze the binding of Factor H, Factor H constructs SCRs 15–18, SCRs 15–19, SCRs 15–20 and the HepG mutant to immobilized mCRP using a Biacore 3000 instrument (GE Healthcare Biacore, Freiburg, Germany) as described.[41]

Formation of Factor H–mCRP complexes. Factor H was incubated for 15 min with either mCRP or pCRP in solution and then added to PC-coated Maxisorp microtiter wells. Bound complexes were eluted, separated by SDS-PAGE, transferred to a membrane and Factor H was detected by western blotting using goat antiserum (Merck).

Cell culture and induction of apoptosis and necrosis. Human umbilical vein endothelial cells were cultivated in DMEM, as described.[41] Apoptosis

Figure 7 Factor H attached to apoptotic particles increases their uptake by phagocytes, and reduces TNF-α and IL-8 release. Human macrophage-like THP-1 cells were treated for 24 h with PMA. (**a**) Human THP-1 macrophage cells were stained with DiO and coincubated with DiD-stained apoptotic HUVEC particles for 1 h. Unbound particles were removed and macrophages were assayed for DiO and DiD double-positive cells by flow cytometry. Adhesion and phagocytosis of the labeled apoptotic particles by the macrophages are indicated by the increase in DiD signal (column 2). Treatment of the apoptotic particles with mCRP did not (column 3), but incubation with Factor H increased the uptake of apoptotic particles by 16% (column 4). In addition, when the particles were treated with the combination of mCRP and Factor H, phagocytosis was significantly enhanced by ~40% (column 5). The mean values and standard deviation of four independent experiments are shown. DiD MFI values were normalized to values measured with untreated particles. Statistical analyses $^*P<0.05$, $^{**}P<0.01$ were determined by unpaired *t*-test. (**b**) Apoptotic HUVEC particles were coincubated with macrophages for 20 h, and the culture supernatant was harvested and assayed for the cytokine TNF-α and the chemokine IL-8. Apoptotic particles induced TNF-α production (column 1). Attachment of mCRP to the particles did not influence the level of cytokines released (column 3). However, when macrophages were treated with Factor H-coated particles, the TNF-α content was reduced by 66% (column 3). This decrease was more pronounced (~80%) when the particles were treated with a combination of mCRP and Factor H (column 4). Neither THP-1 cells alone in the absence of apoptotic particles (column 5) nor apoptotic particles alone (column 6) showed TNF-α production. (**c**) Similar effects were observed for IL-8. Apoptotic particles induced IL-8 production (column 1), which was reduced when the particles were treated with Factor H (18%) and with a combination of Factor H and mCRP (23%) (columns 3 and 4). mCRP alone had no influence on IL-8 production (column 2). The data are derived from one experiment where triplicate assays were performed. This experiment is representative and two additional independent experiments showed the same results

was induced with staurosporine (Sigma-Aldrich) (0.6 μg/ml) overnight at 37°C in DMEM lacking FCS. Apoptotic particles and cells were distinguished by flow cytometry by forward and side scatter profile (LSR II Becton Dickinson, San José, CA, USA). Cells were stained with Annexin V (BD Biosciences, Heidelberg, Germany) and DAPI (Sigma-Aldrich GmbH, Taufkirchen, Germany) to distinguish living, necrotic, early apoptotic and late apoptotic cells, as well as apoptotic particles. Necrosis was induced by heating at 65°C for 35 min.[18] Measurement of caspase 3/7 activity was performed with Caspase-Glo 3/7 assay (Promega, Madison, WI, USA).

Flow cytometry. Binding of Factor H and of the two CRP isoforms to intact, apoptotic and necrotic cells was analyzed by flow cytometry. HUVEC cells ($\sim 1 \times 10^6$) were incubated with the indicated concentrations of either Factor H, or pCRP, or mCRP in TC buffer supplemented with 1% BSA for 20 min at 37 °C. Binding of Factor H and the CRP isoforms was studied by incubating the cells first with either pCRP or mCRP, and then Factor H (30 μg/ml) was added. The washing steps and all subsequent reactions were performed in TC buffer in the presence of Ca^{2+}. After addition, CRP or Factor H antiserum (Merck), the corresponding FITC-conjugated secondary antibody (Dako, Hamburg, Germany) or Alexa Fluor 488-labeled antibodies (Invitrogen, Karlsruhe, Germany) were applied.

Cofactor assay for C3b inactivation. The effects of mCRP and pCRP on Factor H-mediated cofactor activity in the fluid phase and on the surface of necrotic HUVEC cells was assayed.[41] Necrotic HUVEC cells were incubated with Factor H or mCRP–Factor H complex, and after washing C3b and factor I were added. After incubation, the cells were washed, lysed and the presence of C3b fragments in the lysate was detected by western blotting using C3 antiserum.

Phagocytosis analysis. THP-1 monocytes (0.6×10^6 cells/well) were stimulated with phorbol 12-myristate 13-acetate (PMA) overnight to induce a macrophage phenotype. These cells were cultivated for 2 days in RPMI medium supplemented with fetal calf serum. Adherent macrophages were stained with Vybrant DiO cell-labeling solution (Invitrogen). HUVEC cells were treated with staurosporine (0.6 μg/ml) for 12 h to induce apoptosis. Afterward, apoptotic particles were isolated by a two-step centrifugation. First, the particles were centrifuged at $1000 \times g$ to obtain a particle-containing supernatant. This supernant was further centrifuged at $25000 \times g$. Apoptotic particles were incubated with mCRP, Factor H or a combination of both as described. After extensive washing, the particles were stained with Vybrant DiD cell-labeling solution (Invitrogen) and added to DiO-stained THP-1 macrophages (10^6 cells/well) in plain RPMI. After coincubation for 1 h, free particles were removed by washing and macrophages were detached from the surface with accutase (PAA, Marburg, Germany). The extent of phagocytosis was analyzed by determination of DiO/DiD double-positive macrophages using flow cytometry.

Cytokine release. THP-1 monocytes (10^6 cells/well) were stimulated with PMA. Apoptotic HUVEC particles treated with mCRP, Factor H or a combination of the two proteins were then added in FCS-free RPMI (3×10^6 cells/well). After coincubation for 20 h, the culture supernatant was collected, and the TNF-α and IL-8 content was determined using the high-sensitivity human ELISA Set (Immuno Tools, Friesoythe, Germany).

Statistical analysis. Data are represented as mean ± S.D. Significant differences between the two groups were analyzed by the unpaired *t*-test. Values of $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ were considered statistically significant.

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Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)