C-Reactive Protein Polarizes Human Macrophages to an M1 Phenotype and Inhibits Transformation to the M2 Phenotype

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Objective—Inflammation is pivotal in atherosclerosis. Monocyte-macrophages are crucial in atherosclerosis. Monocytes can develop into subsets: classically (M1) or alternatively (M2) activated cells. Several studies point to a proinflammatory role for C-reactive protein (CRP). Because there is a paucity of data on the effects of CRP on macrophage phenotype, we tested effects of CRP on macrophage polarization.

Methods and Results—Monocytes were incubated with CRP (0 to 50 μg/mL) and differentiated into macrophages for 7 days. Phenotypic characterization of M1 and M2 macrophages was performed using flow cytometry. CRP treatment resulted in increased population of M1 macrophages (tumor necrosis factor [TNF]/interleukin [IL]-12/C-C chemokine receptor 2, TNF/IL-12/monocyte chemotactic protein-1, or TNF/IL-1/IL-12). These effects were not abrogated by polymyxin B or small interfering RNA to Toll-like receptor-4, but they were abrogated by boiled CRP. Administration of human CRP to rats in vivo increased polarization of macrophages to M1 phenotype compared with human serum albumin. When macrophages were primed to the M2 phenotype with IL-4, addition of CRP resulted in significantly increased secretion of TNF-α, MCP-1, and IL-1 and conversion of macrophages from the M2 to the M1 phenotype. CRP failed to prime macrophages to the M1 phenotype in presence of CD32/CD64 small interfering RNA or dominant-negative nuclear factor kappa B.

Conclusion—Collectively, these results further support a role for CRP in promoting differentiation of human monocytes toward a proinflammatory M1 phenotype. (Arterioscler Thromb Vasc Biol. 2011;31:1397-1402.)

Key Words: cytokines • macrophages • vascular biology

Inflammation plays a pivotal role in atherosclerosis.1 Monocyte-macrophages are present in all stages of atherosclerosis, from the nascent fatty streak lesion to the culmination in acute coronary syndromes.1-3 A key early step in this inflammatory process is the infiltration of monocytes into the subendothelial space of large arteries and their differentiation into tissue macrophages. Also, macrophage infiltration is observed in adipose tissue of obese subjects and appears to contribute to adipokine-induced inflammation.3,5

Monocytes often function as control switches of the immune system and maintain the balance between pro- and antiinflammatory activities. There is a novel concept emerging from numerous lines of evidence that these cells can develop into different subsets: classically (M1) or alternatively (M2) activated cells.6-8 M1 monocytes produce high levels of proinflammatory cytokines, including interleukin (IL)-1, IL-6, IL-12/IL-23, and tumor necrosis factor (TNF), and play a role in tissue destruction. Their M1 polarization can be mediated by lipopolysaccharide (LPS) and interferon-γ. M2 monocytes are more prominent producers of antiinflammatory cytokines, IL-1 receptor antagonist, IL-10, and transforming growth factor-β. They also express increased levels of scavenger and the mannose receptor (CD206) and appear to be involved in tissue remodeling. M2 polarization is mediated via IL-4 and IL-13. The number of M1 cells increases with obesity in rodents, and analysis of macrophages that infiltrate adipose tissue of obese mice suggests that these cells are classically M1 phenotype and correlate inversely with insulin sensitivity of these mice.6-11 Monocytes of the M1 phenotype infiltrate atherosclerotic lesions. In hypercholesterolemic apolipoprotein E−/− mice, monocyteosis is mainly of the M1 subtype.9,12,13

Among the numerous inflammatory biomarkers, the largest amount of published data supports a role for C-reactive protein (CRP) as a robust and independent risk marker in predicting adverse cardiovascular events in both primary and secondary prevention.14 In addition to being a risk marker, there is much evidence indicating that CRP may indeed participate in atherogenesis.15 CRP induces endothelial dysfunction.16 CRP also induces proinflammatory cytokine release from human monocytes and upregulates CCR-2 and NADPH oxidase activity.17-19 Ballou and Lozanski20 incubated human monocytes with CRP at different doses for 16 hours and were able to demonstrate significantly increased
levels of IL-1, TNF, and IL-6. We have shown that incubation of human monocyte-derived macrophages (HMDM) with CRP significantly decreased LPS-induced second-wave IL-10 mRNA and intracellular and secreted IL-10 protein and destabilized IL-10 mRNA.20–22 Also, CRP alone increased secretion of IL-8, IL-6, and TNF from HMDM and in vivo from peritoneal macrophages of Wistar rats.21,22 However, there are no data examining the direct effect of CRP on macrophage phenotypes. In the present study, we examined the hypothesis that CRP may exert its proinflammatory effects on human monocytes by priming to a proinflammatory M1 phenotype and prevent agonist-induced polarization to an M2 phenotype.

**Materials and Methods**

CRP purified from human ascitic or pleural fluids was passed through a Detoxigel column to remove LPS and dialyzed extensively to remove azide as described previously.23 Our CRP preparations have been shown to have proinflammatory effects in Toll-like receptor-4 small interfering RNA (siRNA) knocked-down cells, whereas LPS loses its effect in these cells.23 Boiled CRP was also used as a control.23

**Cell Culture**

After informed consent was obtained, human peripheral blood mononuclear cells were isolated from the blood of healthy volunteers by Ficoll density gradient centrifugation. Monocytes were isolated by negative magnetic separation as described previously and were seeded onto 6-well plates following suspension in RPMI 1640. Using this method, 92% of cells isolated are CD14+ 21,22 After 2 hours of incubation (37°C, 5% CO2) for adherence, the medium was replaced with RPMI 1640 supplemented with 20% autologous human serum. Adhered monocytes were incubated at 37°C in 5% CO2 for 7 days to induce differentiation into macrophages, with a change of medium every 3 days. CRP (0 to 50 μg/mL) or human serum albumin (HuSA) was added during the 7 days of monocyte differentiation into macrophages.

**CRP-Specific Effects**

To ensure that the effects were in fact due to CRP per se and not some contaminant, we used boiled CRP as negative control. To examine whether these effects were attributable to LPS/endotoxin contamination, we added polymixin B along with CRP or HuSA as described previously.21,23

**Phenotypic Characterization of Classical (M1) and Alternately Activated Monocytes (M2)**

Phenotypic characterization of M1 and M2 cells from subjects was performed using 4-color flow cytometry. For fluorescence-activated cell sorting analysis, cells (5x10⁶ cells) were washed twice and resuspended in 100 μL of 1% bovine serum albumin in PBS and incubated with their respective fluorochrome-labeled antibodies and isotype controls. Cells were then assessed by flow cytometry using the Becton Dickinson FACSArray. M1 cells were characterized by positivity for TNF and IL-12/23, and M2 cells were characterized by positivity for CD206 (mannose receptor) and IL-10. The percentage of classically versus alternatively monocytes was estimated. Furthermore, we also characterized M1 as TNF+/IL-1β+/ or TNF+/IL-12+ cells and M2 as IL-10+/IL-1 receptor antagonist+/ or IL-10+/CD163+ cells.

Fluorescently labeled macrophages were analyzed under the same instrument settings (to eliminate measurement bias), and all data were corrected for background fluorescence intensity obtained with isotype control antibodies (all positive cells were over the first quadrant in the Becton Dickinson FACSArray) and were usually less than 10%. Although high positive expression of TNF, IL-12/23, and IL-1β was used for the M1 cells, these cells were also very low expressors of IL-10 and CD 206 (less than 10%) and hence were classified as the M1 phenotype. For the M2 phenotype, characterization by flow cytometry used the same criteria.

**In Vivo Effects of CRP in Rat Macrophages**

Wistar rats were injected with either human CRP (20 mg/kg of body weight) for 3 days or vehicle control (HuSA) as described previously.18–21 The number of peritoneal macrophages that were of M1 phenotype (IL-23, TNF, and IL-1 positive) versus M2 phenotype (CD163/IL-10/CD206 positive) was assessed by flow cytometry, and greater than 10% over isotype control was considered positive.

**Flow Cytometric Analysis**

The intracellular cytokine expression in HMDM was determined by flow cytometry, and cytokine levels in secreted supernatants were quantitated using a multiplex high-sensitivity cytokine array from Milliplex as described previously.21–23

**Role of Fc-γ Receptors and NFKb**

Cells were incubated with siRNA to CD32, CD64, CD16, or nuclear factor kappa B (NFκB) dominant-negative or scrambled oligodeoxynucleotide for 48 hours, and the M1/M2 phenotype in presence of CRP was examined.21–23

**Statistical Analysis**

The Student paired t test was used for parametric data, and the Wilcoxon signed rank test using Graph Pad Prizm software was used if data were nonparametric. ANOVA followed by the appropriate multiple-comparisons post test was carried out for experiments having more than 2 experimental groups. Data are presented as mean±SD. P<0.05 was considered statistically significant.

**Results**

To evaluate the effect of CRP on macrophage phenotype, we characterized the percentage of M1/M2 macrophages with increasing doses of CRP, boiled CRP, or polymixin B (25 μg/mL) as control. CRP treatment resulted in an increased population of M1 macrophages (Figure 1A to 1C). We used different combinations of biomarkers to define the M1 phenotype. In Figure 1A, M1 phenotype was defined by positivity for TNF/IL-12 and CCR-2. In Figure 1B, we defined M1 phenotype as positivity for TNF/IL-12 and MCP-1, and in Figure 1C, the M1 phenotype was defined by positivity for TNF/IL-1/IL-12. Using any of these combinations, CRP treatment resulted in a significant polarization of macrophages to the M1 phenotype. Addition of polymixin B did not abrogate CRP’s effects, and boiled CRP had no effect (Figure 1). Furthermore, to prove that these effects of CRP were not due to LPS contamination, because LPS signals through Toll-like receptor-4, we transfected cells with siRNA to Toll-like receptor-4 or control siRNA before addition of LPS or CRP. We demonstrated that siRNA to Toll-like receptor-4 failed to abrogate the effects of CRP but did abrogate the effects of LPS (Supplemental Figure I, available online at http://atvb.ahajournals.org). Next, we evaluated levels of intracellular cytokines from these macrophages and also showed that CRP treatment resulted in increased proinflammatory cytokines/chemokines from HMDM, by flow
We have previously shown in the rat model that administration of human CRP versus HuSA results in proatherogenic effects in macrophages, including activation of NADPH oxidase, NFKb, matrix metalloproteinase-9, tissue factor activity, and the proinflammatory cytokines IL-1 and IL-6.18–21 Furthermore, the Peps group24 has shown that human CRP in the rat increases infarct size, and this is prevented with a small molecule inhibitor to CRP, bisphosphocholine. Human CRP administration in the rat model results several proinflammatory, proatherogenic effects in vivo.24–27 Thus, we tested the effect of human CRP on macrophage phenotype in the rat model. As shown in Figure 3, intraperitoneal administration of human CRP to rats resulted in increased polarization of macrophages to the M1 phenotype compared with HuSA administration.

To determine whether CRP could convert an M2 macrophage into an M1 phenotype, we primed macrophages to an M2 phenotype in presence of IL–4 and then tested the effect of CRP coinubcation on these cells. Incubation of M2 macrophages with CRP resulted in significantly increased secretion of the proinflammatory molecules TNF-α, MCP-1, and IL-1 and conversion of macrophages from the M2 to the M1 phenotype (Figure 4).

CRP has been shown to exert its effects via binding to the Fc-γ receptors CD32 and CD64 on human monocyte-macrophages. Also, CRP has been shown to augment NFKb, the master switch of inflammation, which regulates expression of several proinflammatory cytokines in monocytes. Thus, we tested whether downregulation of Fc-γ receptors or NFKb in HMDM results in abrogation of CRP’s effects. As shown in Figure 5, CRP failed to prime monocytes to an M1 phenotype in presence of CD32 or CD64 siRNA but not CD16 siRNA. Furthermore, dominant-negative NFKb also abrogated CRP’s effects on priming toward an M1 phenotype. Collectively, our results suggest that CRP-induced M1 phenotype polarization is mediated largely through CRP binding to Fc-γ receptors on HMDM and also is dependent on NFKb signaling. Taken together, these results provide further support for a role for CRP in promoting differentiation of monocytes toward a proinflammatory M1 phenotype.

**Discussion**

Monocytes function as control switches of the immune system, maintaining the balance between pro- and antiinflammatory activities. Emerging evidence points to at least 2 different subsets of macrophages: classically (M1) or alternatively (M2) activated cells.8–11 The prototypic downstream marker of inflammation in humans, CRP, is an accepted cardiovascular risk marker. Furthermore, both in vitro and in vivo data support a proatherogenic role for CRP via its effects on both endothelial and monocyte-macrophage biology.15 In this report, we provide novel in vitro and in vivo data further underscoring its proinflammatory effects by showing that it promotes the differentiation of monocytes into the proinflammatory M1 macrophage phenotype. In addition, we provide evidence that CRP treatment inhibits agonist-induced polarization to M2 macrophages and instead promotes differentiation into the proinflammatory M1 phenotype. It should be noted that

cytometry, including TNF-α, IL-6, IL-1β, and MCP-1 (Figure 2A to 2E). Although IL-6 levels were significantly increased with CRP concentrations ≥12.5 μg/mL, IL-1β, TNF, MCP-1, and CCR-2 levels were significantly increased with CRP concentrations ≥25 μg/mL. Also, levels of secreted cytokines in supernatants of macrophages treated with CRP were significantly increased with CRP treatment, and these effects were not abrogated by addition of polymixin B (Table). All secreted biomediators were significantly increased at a CRP concentration of ≥12.5 μg/mL. The observed changes in protein levels of M1 markers suggests that CRP primes macrophages into an M1 phenotype.

**Figure 1.** Effect of CRP treatment on the HMDM phenotype. Monocytes were isolated from healthy human volunteers and differentiated into macrophages for 7 days in the absence and presence of different doses of CRP (0, 12.5, 25, and 50 μg/mL) (C, C12.5, C25, C50, respectively), boiled CRP (25 μg/mL) (BCRP25), or CRP and polymixin B (C25+PMB) (25 μg/mL), and macrophage phenotype was assessed by flow cytometry as described in Methods. n=9, *P<0.05 compared with control (C). A, M1 is expressed as positivity for TNF/IL-12 and CCR-2; B, M1 is expressed as positivity for TNF/IL-12 and MCP-1; C, M1 is expressed as positivity for TNF/IL-12 and IL-1. Data are presented as mean±SD. mfi indicates mean fluorescence intensity.
CRP levels of ≈50 to 100 μg/mL are attained in patients with acute coronary syndromes, and high levels of CRP augur a poor prognosis in these patients.28–30 These effects in the vessel wall could result in an accumulation of proinflammatory M1 macrophages. Furthermore, monocytes of the M1 phenotype have been shown to be predominant in human atheroma and in apolipoprotein E−/− mice and thus could be implicated in atherogenesis.9,12,13

We have previously shown in the rat model that administration of human CRP versus HuSA results in proatherogenic effects on macrophages, including activation of NADPH oxidase, NFKb, matrix metalloproteinase-9, tissue factor activity, and the proinflammatory cytokines IL-1 and IL-6.18–21 Furthermore, the Pepys group24 has shown that human CRP in the rat increases infarct size following coronary artery ligation, and this is prevented with a small molecule inhibitor to CRP, bisphosphocholine. Also, human CRP administration in the rat model increased stroke volume following middle cerebral artery occlusion and adenoviral vector–induced human CRP synthesis in rats resulted in hypertension and was accompanied by decreased NO and impairment of endothelium-dependent vasorelaxation.24–27 Thus, the rat is the preferred model with which to test the proatherogenic effects of CRP. We demonstrate in vivo, in this rat model, that human CRP administration results in polarization of macrophages to the M1 phenotype. This is in support of all the in vitro and in vivo data described previously.18–21,24–27

CRP's proatherogenic activity in monocytes has been shown to be via the Fc-receptors CD32 and CD6431; therefore, we used the siRNA strategy to examine mechanisms by which CRP promoted an M1 phenotype.21–23

Figure 2. Effect of CRP treatment on cytokine/chemokine release from HMDM. Monocytes were isolated from healthy human volunteers and differentiated into macrophages for 7 days in the absence and presence of different doses of CRP (0, 12.5, 25, and 50 μg/mL) (C, C12.5, C25, C50, respectively) or boiled CRP (25 μg/mL) (BCRP25), and macrophage cytokine release was assessed by multiplex analysis of supernatants and expressed per mg of cell protein as described in Methods. n=9, *P<0.05 compared with control (C). A, IL-6 release; B, TNF-α release; C, MCP-1 release; D, IL-1β release; E, CCR-2 expression. Data are presented as mean±SD. mfi indicates mean fluorescence intensity.
We showed that both CD32 and CD64 blockade but not CD16 blockade prevented the CRP-induced transition of monocytes to the M1 phenotype.

At the molecular level, previous studies have shown that CRP promotes NFKb activation, resulting in increased proinflammatory cytokines; therefore, we tested whether inhibition of NFKb would abrogate CRP’s effects. Here, we show that NFKb downregulation using dominant-negative NFKb abrogated CRP’s effects of promoting the polarization of HMDM to an M1 phenotype. Human atherosclerotic lesions appear to contain both M1 and M2 macrophages. Native monocytes, in the presence of cytokines such as IL-4, can be primed toward an M2 phenotype.9,12,13 Thus, in presence of IL-4, which resulted in an increased M2 phenotype, we tested whether CRP could prevent polarization of such antiinflammatory/reparative phenotype. We demonstrated that CRP indeed polarizes IL-4 M2 macrophages to an M1 phenotype, providing another molecular pathway by which it exerts proinflammatory effects and potentially promotes atherosclerosis by creating an abundant repository of M1 macrophages in the intima.

Previously, 2 groups have shown that adiponectin, an antiinflammatory adipokine, promotes the M2 macrophage phenotype but fails to induce an M2 phenotype when differentiated macrophages have already differentiated into an M1 phenotype.32–35 In this report, we show that CRP, in addition to polarizing monocytes to an M1 phenotype, also prevents macrophages, in the presence of an agonist, IL-4, from differentiating to an M2 phenotype macrophage. Because CRP is increased and adiponectin is decreased in the metabolic syndrome, in diabetes and coronary artery disease this unfavorable ratio could promote atherosclerosis via accrual of M1 macrophages in the intima.34,35

In conclusion, the present study demonstrates that CRP polarizes human monocyte differentiation toward proinflammatory M1 macrophages via NFKb and by binding to the Fc-γ receptors CD32 and CD64. Furthermore, it reverses agonist-induced M2 macrophages by promoting their polarization to a proinflammatory M1 phenotype. These data, in addition to previous studies, further provide evidence for the role of CRP in atherothrombosis. Future studies will examine a complete chemokine array of the 2

Table. Cytokine Release From Human Macrophages With CRP

<table>
<thead>
<tr>
<th></th>
<th>IL-1β (pg/mg protein)</th>
<th>IL-6 (pg/mg protein)</th>
<th>TNF (pg/mg protein)</th>
<th>MCP-1 (pg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48±11</td>
<td>330±101</td>
<td>451±101</td>
<td>89±33</td>
</tr>
<tr>
<td>12.5 µg/mL CRP</td>
<td>98±37*</td>
<td>756±126*</td>
<td>1245±345†</td>
<td>124±44‡</td>
</tr>
<tr>
<td>25 µg/mL CRP</td>
<td>142±71†</td>
<td>890±319†</td>
<td>1879±541‡</td>
<td>198±83§</td>
</tr>
<tr>
<td>50 µg/mL CRP</td>
<td>156±77‡</td>
<td>1124±345†</td>
<td>2141±801†</td>
<td>225±45†</td>
</tr>
<tr>
<td>25 µg/mL CRP+25 µg/mL polymixin B</td>
<td>138±43†</td>
<td>829±411†</td>
<td>1779±468†</td>
<td>186±76†</td>
</tr>
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*P<0.0001 compared with control.
†P<0.001 compared with control.
‡P<0.05 compared with control.

Figure 3. Effect of CRP administration in vivo on the HMDM phenotype. Peritoneal macrophages were obtained from Wistar rats (n=5/group) administered human CRP (20 mg/kg of body weight) or HuSA for 3 days, and macrophage phenotype was assessed by flow cytometry as described in Methods. *P<0.01 compared with HuSA. Data are presented as mean±SD. mfi indicates mean fluorescence intensity.

Figure 4. Effect of CRP on IL-4 primed macrophages. HMDM were primed with IL-4 to polarize to an M2 phenotype as described in Methods, followed by incubation with CRP (0, 12.5, 25, and 50 µg/mL) (C, C12.5, C25, C50, respectively). *P<0.01 compared with control; †P<0.01 compared with C+IL-4; ‡P<0.05 compared with C+IL-4. n=7 experiments. Data are presented as mean±SD. mfi indicates mean fluorescence intensity.

Figure 5. Mechanisms for CRP polarization of HMDM to M1 phenotype. HMDM were transfected with siRNA to CD32, CD64, or CD16 (CD32si, CD64si, and CD16si, respectively) or dominant-negative (DN) to NFKb or scrambled dominant-negative (Scr Dn) as described in Methods, followed by incubation with 25 µg/mL CRP (C25). *P<0.05 compared with C; †P<0.05 compared with 25 µg/mL CRP. Data are presented as mean±SD. mfi indicates mean fluorescence intensity.
subsets of monocytes to determine which additional biomarkers best predict whether monocytes are in a proinflammatory M1 state or in an antiinflammatory M2 state in our quest to identify cellular biomarkers of cardiovascular disease risk.

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**Disclosures**
None.

**References**
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Supplement I:

Cells were treated with CRP 25 μg/mL or Boiled CRP 25 μg/mL or with scrambled siRNA or TLR4 siRNA. Another set of cells was treated with LPS (1 μg/ml) in absence and presence of TLR4siRNA. M1/M2 phenotype was assessed by flow cytometry.

* * * *
* p<0.05 compared to C

![Bar chart showing M1/M2 phenotype (mfi) for different treatments](chart)

- M2 (CD206/163/IL-10)
- M1 (TNF/IL-12/CCR2)