C-reactive protein (CRP) induces chemokine secretion via CD11b/ICAM-1 interaction in human adherent monocytes

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Abstract: Several studies support C-reactive protein (CRP) as a systemic cardiovascular risk factor. The recent detection of CRP in arterial intima suggests a dual activity in atherosclerosis as a circulating and tissue mediator on vascular and immune cells. In the present paper, we focused on the inflammatory effects of CRP on human monocytes, which were isolated by Ficoll-Percoll gradients and cultured in adherence to polystyrene, endothelial cell monolayer, or in suspension. Chemokine levels, adhesion molecule, and chemokine receptor expression were detected by ELISA, flow cytometry, and real-time RT-PCR. Migration assays were performed in a Boyden chamber. Stimulation with CRP induced release of CCL2, CCL3, and CCL4 in adherent monocytes through the binding to CD32a, CD32b, and CD64, whereas no effect was observed in suspension culture. This was associated with CRP-induced up-regulation of adhesion molecules membrane-activated complex 1 (Mac-1) and ICAM-1 on adherent monocytes. Blockade of Mac-1/ICAM-1 interaction inhibited the CRP-induced chemokine secretion. In addition, CRP reduced mRNA and surface expression of corresponding chemokine receptors CCR1, CCR2, and CCR5 in adherent monocytes. This effect was a result of chemokine secretion, as coincubation with neutralizing anti-CCL2, anti-CCL3, and anti-CCL4 antibodies reversed the effect of CRP. Accordingly, a reduced migration of CRP-treated monocytes to CCL2 and CCL3 was observed. In conclusion, our data suggest an in vitro model to study CRP activities in adherent and suspension human monocytes. CRP-mediated induction of adhesion molecules and a decrease of chemokine receptors on adherent monocytes might contribute to the retention of monocytes within atherosclerotic lesions and recruitment of other circulating cells. *J. Leukoc. Biol.* 84: 1109–1119; 2008.

Key Words: *atherosclerosis · inflammation · adhesion molecules · leukocytes*

INTRODUCTION

Increasing evidence suggests a pivotal role for inflammatory processes in all phases of atherosclerosis from the fatty streak lesion to plaque rupture [1]. Local and systemic soluble inflammatory mediators are pivotal players in regulating atherosclerotic plaque development [1]. Cytokines, chemokines, growth factors, and hormones orchestrate recruitment and activities of inflammatory cells within the plaque, with subsequent induction of a systemic proinflammatory state involving adipose tissue and liver [2]. T lymphocytes and monocytes are influenced by several factors, with local and systemic proatherosclerotic activities [2]. Among these, recent work supports C-reactive protein (CRP) as a proatherosclerotic factor with this dual action [3]. CRP is a nonglycosylated protein and a member of the pentraxin family, known to be classically synthesized by the liver as an acute-phase reactant [4, 5]. CRP is capable of activating cells in immunoinflammatory responses, through the binding of FcγRI (CD64) and FcγRII (CD32) on cell membranes [6]. The strong association between CRP serum levels and the risk of future atherosclerotic events supports a role for CRP as a factor contributing to atherogenesis [7]. Among several in vitro and in vivo studies, the most important finding was the demonstration of CRP production, not only by liver cells but also within atherosclerotic lesions, rheumatoid synovium, kidney, neurons, and lung, suggesting a new role for CRP as a local inflammatory factor [8–12]. Different cell populations, localized in the atherosclerotic plaque and in other tissues, have been found to produce and release CRP, as determined on the mRNA level or in its secreted protein form [8, 11, 13–15]. To better clarify the activity of CRP as a paracrine (local) and endocrine (systemic) atherosclerotic factor on monocytes, we stimulated these cells in the presence of CRP in different culture dishes, mimicking circulating condition or adherence to the vessel wall. In particular, we investigated chemokine secretion, chemokine receptor, and adhesion molecule expression as well as chemotaxis, which are crucial processes during atherosclerotic plaque development.

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MATERIALS AND METHODS

Isolation of human monocytes and cell culture

Human monocytes were isolated from buffy coats of healthy volunteers without clinical signs of inflammatory disorders after informed consent. The Local Ethics Committee approved the investigation protocol, and it was conformed with the principles outlined in the Declaration of Helsinki. After centrifugation on Ficoll-Hypaque density gradient, mononuclear cells were collected from the interface and washed with 0.9% (w/v) sodium chloride. Then, monocytes were purified from the upper interface of a hypotonic Percoll density gradient, as described previously [16]. Viability and purity of monocytes were determined by flow cytometric analysis (CD14 staining), confirming that at least 85% purity was achieved in all experiments. Cells were cultured in polystyrene (to culture cells in adherent conditions) or Teflon dishes (to culture cells in suspension conditions) at a concentration of 5×10^6 cells/ml in serum-free RPMI-1640 medium containing 25 mmol/L Hepes and 500 ng/mL polymixin B (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified atmosphere with 5% CO_2, unless otherwise specified.

Recombinant and human (rh)CRP immunodepletion

rhCRP was from R&D Systems Europe Ltd. (Abingdon, UK). hCRP, obtained from human pleural fluid, was from Lee Biosolutions, Inc. (St. Louis, MO, USA). As shown by the manufacturers (R&D Systems Europe Ltd. and Lee Biosolutions, Inc.), the purity of the compound was >97% (for rCRP) and >98% (for hCRP; determined by SDS-PAGE and visualized by silver stain), and endotoxin level was <1.0 EU per 1 μg rCRP (determined by the Limulus amoebocyte lysate method). However, to exclude a possible effect of contaminants, the reconstituted rCRP (at 10 μg/mL and 100 μg/mL) and hCRP (at 10 μg/mL) were immunodepleted (ID) and used as a vehicle control in all experiments. rCRP and hCRP were incubated with 1 μg (for CRP reconstituted at 10 μg/mL) or 10 μg (for rCRP reconstituted at 100 μg/mL) anti-hCRP mAb in the presence (ID) or the absence [ID without Protein A-agarose (IDWA)] of Protein A-agarose (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) on a rotating wheel (overnight at 4°C), as described previously [17, 18]. Prior to immunodepletion, the anti-hCRP mAb and hCRP were dialyzed against the buffer for 1 h at 4°C using Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, IL, USA) to avoid a contamination of the CRP compound with sodium azide. Immunoprecipitated CRP was removed, and the supernatant was collected and stored at –20°C. The CRP levels in the ID compound were measured by ELISA (R&D Systems Europe Ltd.) and found undetectable (<0.78 ng/mL).

Chemokine secretion assay

Monocytes (5×10^6/mL) were cultured in the presence or absence of rCRP or hCRP (0.1, 0.3, 1, 3, 10 μg/mL) for 12 h. In selective experiments, cells were preincubated for 30 min with 20 μg/mL-blocking anti-hCD11b (BD PharMingen, Franklin Lakes, NJ, USA) and 10 μg/mL anti-hICAM-1, 50 μg/mL anti-hCD32a, 50 μg/mL anti-hCD32b, or 50 μg/mL anti-hCD64 antibodies (all from R&D Systems Europe Ltd.), followed by 12 h of incubation in the presence or absence of 10 μg/mL rCRP. CCL2, CCL3, CCL4, and CXCL8 levels were measured in supernatants of monocyte cultures in polystyrene, Teflon, and polystyrene coated with a monolayer of HUVEC (Cambrex Bio-Science, Walkersville, MD, USA) dishes by using ELISA kits (R&D Systems Europe Ltd.). HUVEC were maintained in RPMI-1640 medium in the presence of 10% heat-inactivated FCS (Invitrogen, Basel, Switzerland), 10% heat-inactivated newborn calf serum (Invitrogen), 1% penicillin/streptomycin (Invitrogen), 15 μg/mL endothelial cell growth supplement (BD Biosciences, Allschwil, Switzerland), and 50 IU/ml heparin (Drossapharm AG/SA, Basel, Switzerland) in flasks precoated with 1% gelatin (Sigma, Poole, UK). For experiments, HUVEC were used at the fourth or fifth passage in culture. Then, HUVEC culture medium was removed, and HUVEC-monocyte coculture experiments were conducted by applying 5×10^6 monocytes to gelatine-coated polystyrene dishes containing a monolayer of confluent HUVEC, and HUVEC and monocytes were coincubated for 12 h in the presence of different stimuli in RPMI-1640 medium containing 25 mmol/L Hepes and 500 ng/mL polymixin B. In selective experiments, monocytes were preincubated for 60 min with 20 μg/mL-blocking anti-CD11b, 10 μg/mL anti-ICAM-1 antibody, 50 μg/mL anti-CD32a, 50 μg/mL anti-CD32b, or 50 μg/mL anti-CD64 antibodies, followed by 12 h of incubation in the presence or absence of 10 μg/mL rCRP.

Flow cytometry

Monocytes were cultured in polystyrene dishes in the presence or absence of 10 μg/mL rCRP or 10 μg/mL hCRP for 30 min (CD11b and CD18 analysis) or 24 h (ICAM-1 analysis), respectively, or different doses of rCRP for 12 h to study CCR1, CCR2, and CCR5 expression. fMLP (100 nmol/L; Sigma-Aldrich) or 100 U/mL IFN-γ (R&D Systems Europe Ltd.) were used as positive controls [19, 20]. In selective experiments, monocytes were preincubated for 30 min with 50 μg/mL anti-CD32a, 50 μg/mL anti-CD32b, or 50 μg/mL anti-CD64 antibodies and then stimulated with 10 μg/mL rCRP. In parallel experiments, monocytes were incubated with rCCL2 (1 ng/mL), CCL3 (1 ng/mL), or CCL4 (2 ng/mL; all from R&D Systems Europe Ltd.) for 8 h or in the presence of 10 μg/mL CRP plus neutralizing anti-hCCL2 (2 μg/mL), anti-hCCL3 (2 μg/mL), or anti-hCCL4 (10 μg/mL) antibodies (R&D Systems Europe Ltd.) for 12 h. After incubation time, culture supernatants were removed, and cells were washed with PBS to remove nonadherent cells. Adherent monocytes were collected by scraping with a plastic policeman (Costar Cambridge, MA, USA) and energetically pipetting to stain FITC- or PE-labeled antibodies to anti-hCCR1, -hCCR2, -hCCR5, -hCD11b, and -hCD18 (R&D Systems Europe Ltd.) and anti-hCD54 and -hCD14 (BD PharMingen), as well as corresponding isotype controls. CellQuest software was used for acquisition and analysis on a FACSCalibur (BD Biosciences, Heidelberg, Germany). Data were expressed as mean fluorescence intensities (MFI), compared with baseline expression (defined as 100%).

CRP-binding assay

For estimation of specific CRP binding to human monocytes, we adapted the previously published method for THP-1 monocytes [6]. Briefly, after isolation, 10^5 monocytes were incubated with different concentrations of rCRP in PBS containing 1% BSA (Sigma-Aldrich) at 4°C for 30 min. In selective experiments, to displace CRP binding to its receptors, monocytes were incubated with 50 μg/mL rCRP and blocking anti-hCD32a, anti-hCD32b, anti-hCD64, anti-hCD11b, or anti-hICAM-1 antibodies (1, 50, and 100 μg/mL). Then, cells were washed once, and surface-bound CRP was detected by rabbit polyclonal anti-hCRP IgG (Sigma) or rabbit serum (as isotype control), followed by PE-conjugated goat IgG anti-rabbit IgG (Invitrogen). Nonspecific CRP binding was determined by incubating labeled cells with an excess of CRP (100 μg/tube). Specific binding of CRP to monocytes was calculated by subtracting nonspecific binding from total binding of CRP. Data were expressed as MFI values determined by flow cytometry. K_d of CRP binding was calculated by using Prism 4 GraphPad Software (San Diego, CA, USA).

Cytotoxicity assay

Cell death was determined by quantification of lactate dehydrogenase (LDH) release in cell culture supernatants of adherent and suspension cultures after 12 and 24 h (BioVision, Mountain View, CA, USA).

Real-time RT-PCR

Monocytes were cultured in the presence or absence of 10 μg/mL CRP ± neutralizing anti-hCCL2 (2 μg/mL), anti-hCCL3 (2 μg/mL), or anti-hCCL4 (10 μg/mL) antibodies for 12 h. Total RNA was extracted using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and reverse-transcribed using the Quantitect kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Real-time PCR was performed with the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). hCCR2 primers and probe were designed with Primer Express software (Applied Biosystems): 5′ GCGTTTAATCACATTCGAGTGTTT (forward), 5′ CCACTGGCAAATTAGGGAACAA (reverse), 5′ FAM AGTGCTTCGCAGATGTCCTTGATGCTC TAMRA (probe). hCCR1, hCCR5, and hypoxanthine guanine phosphoribosyl transferase primers and probes were described previously [21, 22].

Modified Boyden chamber migration assay

Monocytes were collected after 12 h of incubation in the presence or absence of 10 μg/mL CRP. Culture supernatants were removed, and cells were washed with PBS to remove nonadherent cells. Then, adherent mono-

cytes were collected by scraping with a plastic policeman (Costar Cambridge) and energetically pipetting. After washing three times in chemotaxis medium (RPMI containing 25 mmol/L Hepes and 1% BSA, Sigma-Aldrich), cells were tested for migration to 10 nmol/L CCL2 or 10 nmol/L CCL3. Monocyte chemotaxis was assessed in a 48-well microchemotaxis-modified Boyden chamber (NeuroProbe, Gaithersburg, MD, USA) using a 5-μm pore size, 5-μm-thick polyvinylpyrrolidone-free polycarbonate filter (NeuroProbe). Cells were seeded in upper wells, and medium or chemoattractant solutions were added to the lower wells. The chamber was incubated for 60 min at 37°C in a humidified atmosphere with 5% CO_2. Then, filters were removed from the chambers and stained with Diff-Quick (Baxter, Rome, Italy). Cells in five random oil-immersion fields were counted at 1000× magnification (blinded observer), and the chemotaxis index calculated from the number of cells migrated to the chemokine divided by the number of cells migrated to the medium.

Statistical analysis

All data were expressed as mean ± SEM. One-way ANOVA with Bonferroni's post-test was performed using GraphPad InStat, Version 3.05 (GraphPad Software). Differences between P values below 0.05 were considered significant.

RESULTS

CRP induces secretion of the chemokines CCL2, CCL3, and CCL4 only in human adherent monocytes through the binding with its cognate receptors CD32 and CD64

To study a possible CRP-mediated induction of chemokine secretion under different culture conditions, adherent and suspension monocyte cultures were treated with increasing concentrations of CRP. The incubation of monocytes in adherent conditions with 10 μg/mL rCRP resulted in a significant (25-fold) induction of CCL2 secretion as compared with untreated cells (**Fig. 1A**). This concentration corresponds to high-sensitivity CRP serum levels that may be found under pathophysiological conditions [23]. Conversely, no rCRP-mediated increase of CCL2 secretion was observed in suspension cultures, which mimicked circulating cell condition (Fig. 1A). Similar to the effect on CCL2, 10 μg/mL rCRP also induced a significant increase of CCL3 (30-fold) and CCL4 (17-fold) secretion in adherent cells (Fig. 1, B and C). The basal levels of chemokine release from untreated cells were comparable between adherent and suspension cultures. The ID CRP compounds had no effect (Fig. 1, A–C), indicating that the induction of chemokine secretion in human monocytes was indeed mediated by CRP and not a result of a contamination of the vehicle. Comparable results were observed in the presence of hCRP for CCL2, CCL3, and CCL4 secretion (**Fig. 2, A–C**). Preincubation with anti-CD32a, -CD32b, and -CD64 antibodies significantly inhibited rCRP-induced chemokine secretion in adherent monocytes (**Fig. 3, A–C**). Furthermore, increasing concentrations of anti-CD32a, CD32b, and CD64 antibodies displaced rCRP binding to monocytes (Fig. 3, D–F). These data indicate that CRP-induced CCL2, CCL3, and CCL4 secretion in human adherent monocytes is dependent on the binding with its cognate receptors CD32 and CD64, which is supported by studies published previously [6]. In none of the experimental conditions (adherent and suspension cultures) did we observe an increased cytotoxicity or cell viability, as determined by release of LDH and Trypan blue staining (data not shown). To assess functional responsiveness of monocytes cultured in ad-

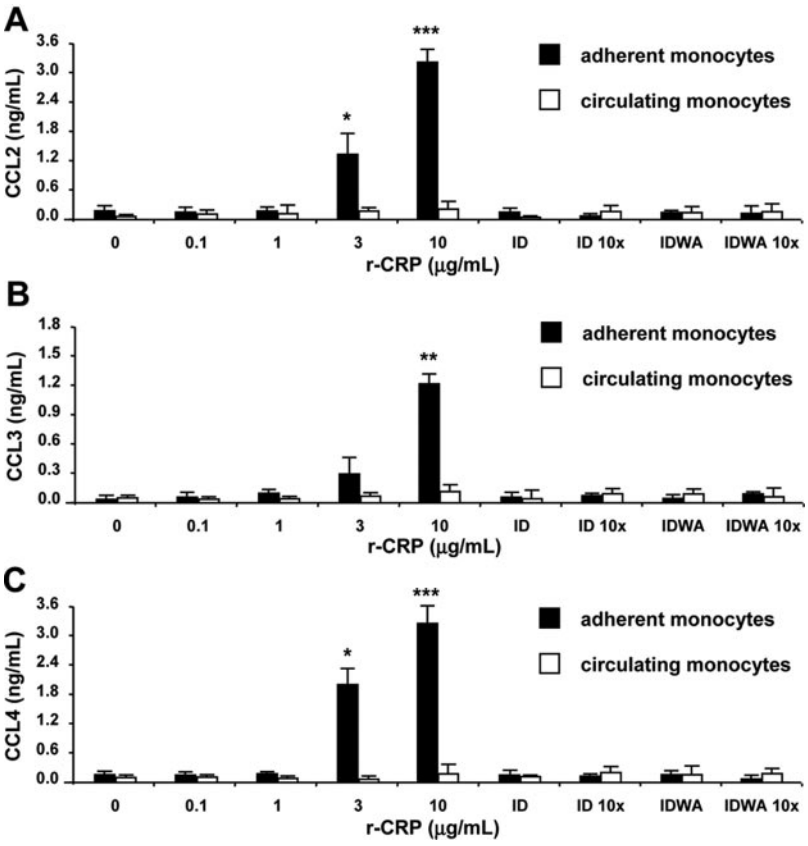

Fig. 1. rCRP induces CCL2, CCL3, and CCL4 secretion in adherent human monocytes. Chemokine secretion in adherent versus circulating monocytes (suspension culture) treated with increasing concentrations of rCRP [$n=12$ for rCRP from 0 to 10 μg/mL and rCRP (10 μg/mL) ID, and $n=6$ for rCRP (100 μg/mL) ID compound (ID 10×), rCRP (10 μg/mL) IDWA, and rCRP (100 μg/mL) IDWA (10×)]. (A) CCL2: ***, $P < 0.001$; *, $P < 0.05$, versus medium alone or rCRP ID compounds. (B) CCL3: **, $P < 0.01$, versus medium alone or rCRP ID compounds. (C) CCL4: ***, $P < 0.001$; *, $P < 0.05$, versus medium alone or rCRP ID compounds.

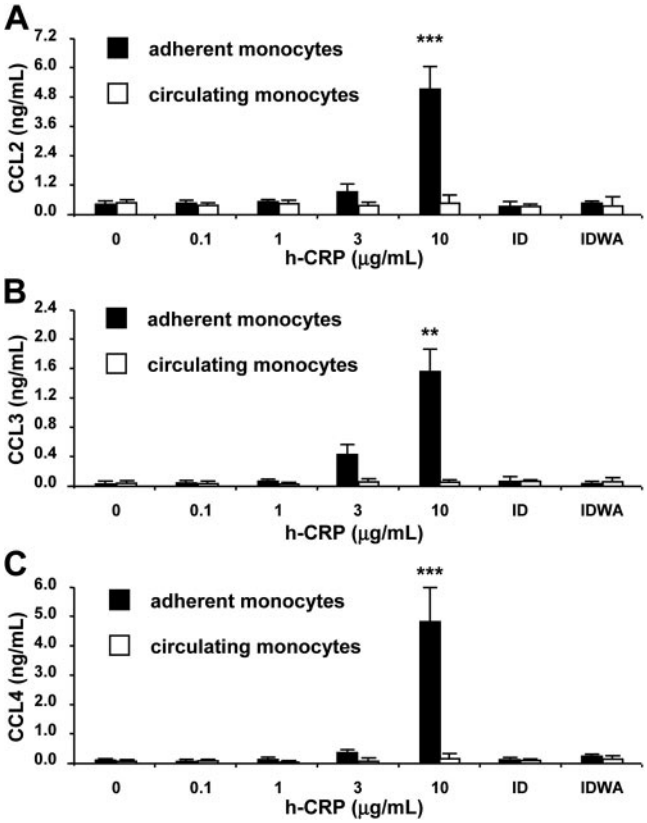

Fig. 2. hCRP induces CCL2, CCL3, and CCL4 secretion in adherent human monocytes. Chemokine secretion in adherent versus circulating monocytes (suspension culture) treated with increasing concentrations of hCRP ($n=7$). (A) CCL2: ***, $P < 0.001$, versus medium alone, hCRP (10 μg/mL) ID, or hCRP (10 μg/mL) IDWA. (B) CCL3: **, $P < 0.01$, versus medium alone or hCRP ID compounds. (C) CCL4: ***, $P < 0.001$, versus medium alone or hCRP ID compounds.

herence (polystyrene dishes) or in suspension (Teflon dishes), CXCL8 secretion assay (as a functional positive control) was performed [24]. Twelve hours of incubation with 10 μg/mL rCRP or hCRP increased CXCL8 secretion in comparison with control medium in both culture conditions (mean±SEM; $n=2$; adherence: rCRP vs. CTL: 88.75±29.10 vs. 17.80±9.5 ng/mL, hCRP vs. CTL: 69.90±11.50 vs. 17.80±9.50 ng/mL; suspension: rCRP vs. CTL: 41.48±14.37 vs. 11.63±2.58 ng/mL, hCRP vs. CTL: 31.05±13.45 vs. 11.63±2.58 ng/mL).

CRP induces CD11b, CD18, and ICAM-1 up-regulation only on the surface of adherent human monocytes via CD32 and CD64

We next investigated the effect of CRP on CD11b, CD18, and ICAM-1 adhesion molecule expression in the two different culture conditions. In adherence, treatment with 10 μg/mL rCRP or 10 μg/mL hCRP for 30 min increased the surface expression of integrins CD11b and CD18 with a magnitude comparable with the positive control fMLP (**Fig. 4, A, B**, and **D**). This effect was not observed in suspension culture (Fig. 4, A and B). Similarly, 24 h of incubation with 10 μg/ml rCRP or 10 μg/mL hCRP induced a significant increase of ICAM-1 expression versus medium alone in adherence but not in suspension culture (Fig. 4, C and D). On the other hand, treatment of monocytes with IFN-γ (positive control) induced a strong (~2.5-fold) up-regulation of ICAM-1 in adherence and a weak (~1.5-fold), but still significant, increase in suspension culture (Fig. 4C). In all of these experiments, ID CRP did not have any effect (Fig. 4, A–C). rCRP-induced CD11b, CD18, and ICAM-1 up-regulation was reversed by pretreatment with anti-hCD32a, anti-hCD32b, and anti-hCD64 antibodies (**Fig. 5, A–C**). These data indicate that CRP induced up-regulation of adhesion molecules on the adherent monocyte surface via CD32a, CD32b, and CD64.

CRP-induced CCL2, CCL3, and CCL4 secretion in adherent human monocytes also depends on the costimulatory CD11b/ICAM-1 interaction

To further clarify the underlying mechanisms of CRP on chemokine secretion, we performed experiments with adhesion-blocking anti-CD11b and anti-ICAM-1 antibodies. We found that anti-CD11b and anti-ICAM-1 antibodies significantly inhibited the CRP-induced secretion of CCL2 (**Fig. 6A**). A similar effect was observed for CCL3 and CCL4 (Fig. 6, B and C). Coincubation with anti-CD11b or anti-ICAM-1 with anti-CD32a, anti-CD32b, and anti-CD64 further inhibited chemokine secretion (Fig. 6, A–C). Figure 6, D and E, shows that the binding of CRP to the monocyte surface was not displaced by an increasing concentration on blocking anti-CD11b or anti-ICAM-1 antibodies, suggesting that CRP did not bind CD11b or ICAM-1. Furthermore, coincubating human monocytes and the HUVEC monolayer in the presence of 10 μg/mL rCRP or 10 μg/mL hCRP induced chemokine secretion by human monocytes (**Fig. 7, A–C**). The blockade of CD32a, CD32b, and CD64 (CRP receptors) or CD11b/ICAM-1 interaction significantly inhibited CRP-induced chemokine secretion (Fig. 7, A–C). No significant increase of chemokine secretion was detected in cultures with HUVEC alone (without adding monocytes) in the presence or the absence of rCRP (Fig. 7, A–C). These findings strongly support that a costimulatory signal between CRP receptors (CD32a, CD32b, and CD64) and CD11b/ICAM-1 is necessary for the secretion of CCL2, CCL3, and CCL4 by adherent monocytes.

rCRP reduces CCR1, CCR2, and CCR5 expression and chemokine-induced migration

As chemokine receptors are crucial for monocyte recruitment to inflammatory sites, we studied a possible rCRP-mediated effect on the expression of CCL2, CCL3, and CCL4 chemokine receptors CCR1, CCR2, and CCR5. We found that treatment with 10 μg/mL rCRP induced a significant decrease of CCR1, CCR2, and to a lesser extent, CCR5 surface expression on adherent, but not circulating, monocytes (**Fig. 8, A–D**). The rCRP-induced decrease of CCR1, CCR2, and CCR5 was associated with a modulation of monocyte migration in response to the classic chemoattractants CCL2 and CCL3. Pretreatment with CRP in adherent, but not suspension, conditions resulted in a significant reduction of monocyte chemotaxis to CCL2 (**Fig. 9A**). Similar results were observed for the migration of adherent monocytes to CCL3 (Fig. 9B).

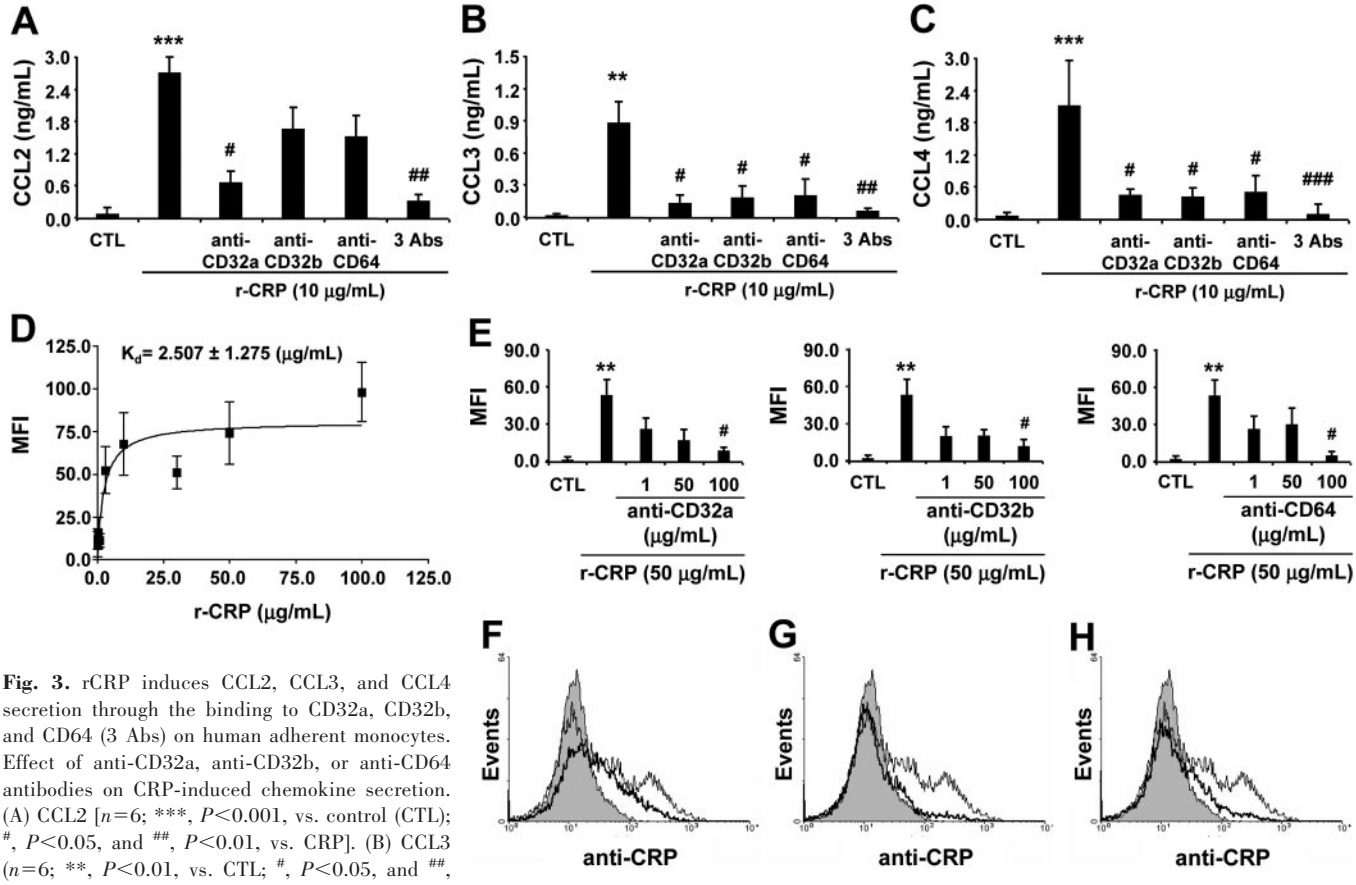

Fig. 3. rCRP induces CCL2, CCL3, and CCL4 secretion through the binding to CD32a, CD32b, and CD64 (3 Abs) on human adherent monocytes. Effect of anti-CD32a, anti-CD32b, or anti-CD64 antibodies on CRP-induced chemokine secretion. (A) CCL2 [n=6; ***, P<0.001, vs. control (CTL); #, P<0.05, and ##, P<0.01, vs. CRP]. (B) CCL3 (n=6; **, P<0.01, vs. CTL; #, P<0.05, and ##, P<0.01, vs. CRP). (C) CCL4 (n=8; ***, P<0.001, vs. CTL; #, P<0.05, and ###, P<0.001, vs. CRP). (D) Binding of rCRP to human monocytes, which were incubated with increasing concentrations (0.01–100 μg/mL) of rCRP. Cell-bound CRP was labeled with rabbit polyclonal anti-hCRP and PE-conjugated goat anti-rabbit IgG. MFI was determined by flow cytometry (n=7; mean±SEM). (E) Displacing of the binding of rCRP to human monocytes. Increasing concentrations (1–100 μg/mL) of blocking anti-hCD32a, anti-hCD32b, or anti-hCD64 were incubated in the presence of 50 μg/mL rCRP (n=6; mean±SEM; **, P<0.01, vs. CTL; #, P<0.05, vs. CRP). (F–H) Representative flow cytometric analyses of CRP binding to human monocytes. The respective histograms show isotype control (solid filled-in gray) and staining of 50 μg/mL CRP-treated (thin black line, unfilled) or 50 μg/mL CRP-treated in the presence of (F) 100 μg/mL anti-CD32a antibody, (G) 100 μg/mL anti-CD32b antibody, or (H) 100 μg/mL anti-CD64 antibody (bold black line each) for the anti-CRP MFI analysis.

The lower expression of CCR1, CCR2, and CCR5 in rCRP-treated adherent monocytes is a consequence of CCL2, CCL3, and CCL4 secretion

It is well known that the interaction of a transmembrane receptor with its cognate ligand can induce the internalization of its receptor with the consequent reduction of its expression on the cell surface [25, 26]. The chemokines CCL2, CCL3, and CCL4, which are secreted by adherent monocytes in response to rCRP, may bind directly to CCR1, CCR2, and CCR5 on the same cells and consequently, modulate their own receptor expression. To verify this hypothesis, we tested the effect of neutralizing antichemokine antibodies in the presence of rCRP or recombinant chemokines alone on chemokine receptor expression in adherent conditions. Indeed, the effect of rCRP on CCR1 expression was reversed partially by anti-CCL2, anti-CCL3, and to a lesser extent, anti-CCL4-neutralizing antibodies (**Fig. 10A**). Accordingly, treatment with recombinant chemokine CCL2, CCL3, or CCL4 partially mimicked the effect of CRP, and treatment with the three chemokines together had an additional effect (Fig. 10A). Similarly, anti-CCL2 and less-pronounced anti-CCL3 and anti-CCL4 reversed the effect of rCRP on CCR2 expression, and treatment with the three chemokines together synergistically inhibited CCR2 (Fig. 10B). Finally, anti-CCL2, anti-CCL3, and less-powerful anti-CCL4 reversed the rCRP effect on CCR5, and parallel treatment with the three chemokines synergistically inhibited CCR5 expression (Fig. 10C). These data suggest that the reduction of CCR1, CCR2, and CCR5 on adherent monocytes incubated in the presence of CRP is rather induced by the chemokine release and not a direct effect of rCRP.

We further investigated if the chemokine-induced receptor down-regulation was a result of a reduction of mRNA levels. Therefore, adherent and suspension monocyte cultures were treated with increasing concentrations of CRP. We found a marked, but not significant, reduction of CCR2 and to a lesser extent, CCR1 and CCR5 mRNA levels in adherent monocytes treated with 10 μg/mL rCRP (**Fig. 11A**). The effect of rCRP on CCR2 was reversed by using neutralizing anti-CCL2, anti-CCL3, and anti-CCL4 antibodies (Fig. 11B). On the contrary, only neutralizing anti-CCL2 and anti-CCL3 antibodies, but not anti-CCL4 antibody, reversed the rCRP-mediated effect on CCR1 and CCR5 (Fig. 11B).

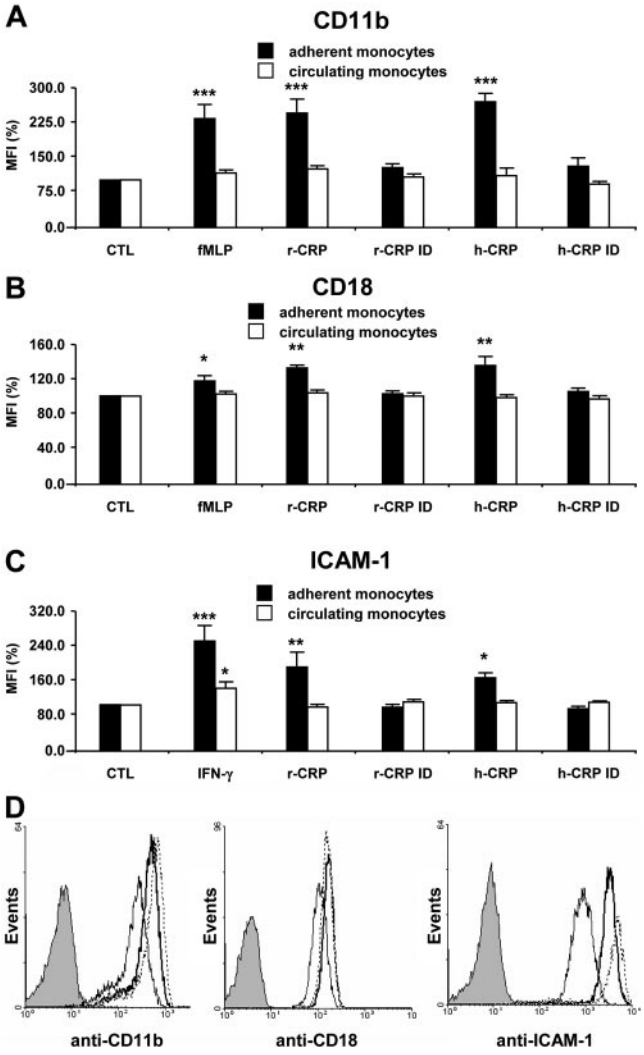

Fig. 4. CRP induces CD11b, CD18, and ICAM-1 surface expression on adherent monocytes. Adhesion molecule expression in adherent versus circulating monocytes (suspension culture) in response to 10 μg/ml rCRP, 10 μg/ml hCRP, or positive controls (fMLP or IFN-γ, respectively). (A) CD11b [$n=10$ for CTL, fMLP, rCRP, and rCRP ID, and $n=6$ for hCRP and hCRP ID: ***, $P<0.001$, vs. medium alone (CTL), rCRP ID, or hCRP ID]. (B) CD18 ($n=10$ for CTL, fMLP, rCRP, and rCRP ID, and $n=6$ for hCRP and hCRP ID: *, $P<0.05$, and **, $P<0.01$, vs. CTL, rCRP ID, or hCRP ID). (C) ICAM-1 (adherent: $n=11$ for CTL, IFN-γ, rCRP, and rCRP ID, and $n=5$ for hCRP and hCRP ID; circulating: $n=7$ for CTL, IFN-γ, rCRP, and rCRP ID, and $n=3$ for hCRP and hCRP ID); *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, vs. CTL, rCRP ID, or hCRP ID. (D) Representative flow cytometric analyses of CD11b, CD18, and ICAM-1 expression on adherent monocytes. The respective histograms show isotype control (solid filled-in gray) and staining of untreated (black line, unfilled), CRP-treated (bold line), or fMLP-treated monocytes (dotted line) for the CD11b and CD18 expression analysis. ICAM-1 expression was measured in untreated (black line, unfilled), CRP-treated (bold line), or IFN-γ-treated monocytes (dotted line).

DISCUSSION

Recruitment of monocytes to inflammatory sites is critical for establishing host defense as well as for the development of chronic inflammatory disorders, such as atherosclerosis and rheumatoid arthrits. Monocyte migration is characterized by firm adhesion to endothelium and diapedesis into the subendothelial space. The first step is regulated by various adhesion molecules, i.e., selectins (E-selectin, P-selectin), ICAM-1, VCAM-1, and integrins such as membrane-activated complex 1 (Mac-1; CD11b/CD18), LFA-1 (CD11a/CD18), and P150,95 (CD11c/CD18) [27]. The second step is dependent on chemokine gradients and chemokine binding to their cognate receptors [27]. Therefore, the modulation of adhesion molecules and chemokine receptors on the monocyte surface membrane is crucial in inflammatory processes underlying atherosclerosis. Before discussing our results, we have to explain some methodological aspects necessary for the validation of the present experimental model. We investigated the potential proinflammatory role of CRP (present in bloodstream and atherosclerotic plaques) on human monocytes in two different in vitro conditions: in adherence (adhesion to polystyrene and endothelial monolayer) and in suspension (mimicking circulation in the bloodstream) [28–32]. The different conditions of cell cultures are crucial for our study, as they may influence monocyte functions in terms of gene expression, cytokine production, chemotaxis, cytoplasmic calcium mobilization, and differentiation to macrophages [33–38]. However, monocyte chemokinesis and differentiation to dendritic cells were not affected by adherence in vitro [36, 39]. To avoid adherence during monocyte isolation, which may cause well-documented alterations of cell morphology and function, we used Ficoll and Percoll gradients [40–42]. A possible major criticism focused on the specificity of the effects mediated by CRP in vitro has been raised in various in vitro studies [6], including the possibility of contaminations of the vehicle with IgG fragments or bacterial products (endotoxin) [6, 43]. To address this question, we performed control experiments in which the rCRP-ID preparations served as a negative control. CRP was immunoprecipitated in the presence or absence of protein A-agarose to ensure specificity of the method [17, 18]. The compound tenfold more concentrated (CRP at 100 μg/mL) than the dose effective in our experiments (CRP at 10 μg/mL) was also ID to prove the absence of a possible dilution effect in the ID supernatant. The ID compounds did not exert any effects, confirming that CRP itself directly mediated all of the observed effects on monocyte functions. However, Pepys and coworkers [43] reported previously that transgenic mice expressing hCRP did not present a proinflammatory phenotype. Moreover, they showed that in vitro stimulation with natural hCRP did not induce proinflammatory responses in monocytic cell lines (THP-1 and human embryo kidney 293 cells) [43]. The authors concluded that the absence of any proinflammatory activity in natural hCRP for macrophage cell lines and mouse cells indicated the nonspecific role of rCRP contaminants in CRP-mediated, proinflammatory activities observed in the literature. To further clarify the specificity of CRP-mediated effects in our study, we additionally tested natural hCRP, and we observed similar results of the ones obtained using rCRP, while no effect was observed after immunodepletion. The lack of proinflammatory effects after immunodepletion of rCRP or hCRP supports the direct proinflammatory activity of CRP in human primary monocytes. The discrepancies in the available data in the literature could reflect differences in the experimental cell models, in particular, the use of human primary cells as compared with transgenic animals and cell lines [43–45]. This is also supported by two other independent findings: CRP serum levels have been

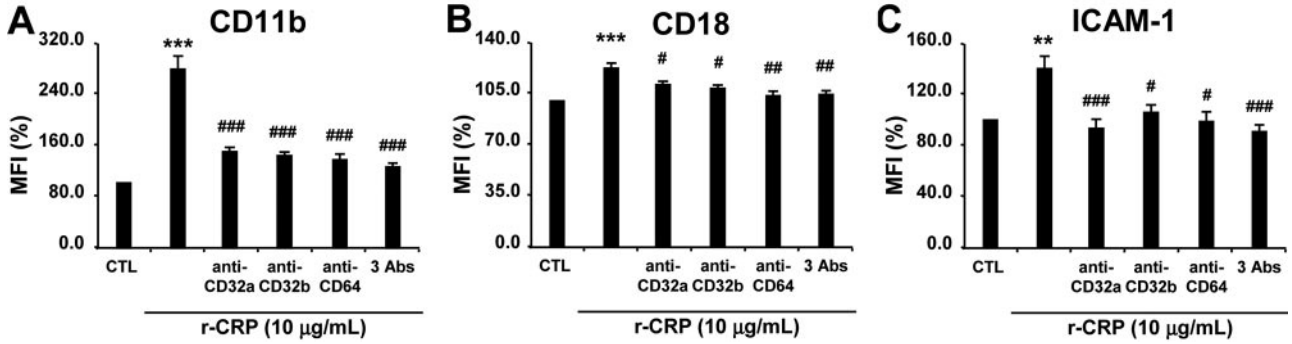

Fig. 5. CRP induces CD11b, CD18, and ICAM-1 surface expression on adherent monocytes via CD32a, CD32b, and CD64. Effect of anti-CD32a, anti-CD32b, and anti-CD64 antibodies on rCRP-induced (A) CD11b, (B) CD18, and (C) ICAM-1 expression on human adherent monocytes ($n=5$: ***, $P<0.001$; **, $P<0.01$, vs. medium alone; #, $P<0.05$; ##, $P<0.01$; ###, $P<0.001$, vs. CRP).

shown to increase atherosclerosis in humans at concentrations tenfold lower than in hCRP-transgenic mice [23, 46, 47]; and CRP induces controversial effects in human primary monocytes and monocytic cell lines, indicating that they indeed represent different cell models [48, 49]. Therefore, the present paper shows that CRP specifically activates primary human monocytes at concentrations within the range of human serum levels found in clinical conditions [23].

The major findings of the present study were that CRP induced CCL2, CCL3, and CCL4 secretion in human monocytes only in adherent conditions. These data are in agreement with a previous study showing that human monocytes secreted CCL2 in the presence of a synthetic peptide derived from CRP [50]. Similar data have been shown by Zhang and Wahl [51] in human monocytes activated by cytokines. Our data about CRP-induced CCL3 and CCL4 secretion from human primary monocytes are novel. The poor effect of CRP in suspension cultures suggests that adhesion is a crucial step in monocyte activation. Additional experiments support the hypothesis that induction and interaction of adhesion molecules are the main mechanisms implicated in CRP-induced chemokine secretion in adherent monocytes: CRP induced Mac-1 and ICAM-1 up-regulation, and antibody-mediated adhesion blockade inhibited chemokine secretion. Furthermore, the increase of Mac-1/ICAM-1 interactions coincubating human monocytes and HUVEC [52] results in a consequent CRP-mediated up-regulation of chemokine secretion by human monocytes. No significant CRP-induced increase in chemokine secretion by HUVEC alone has been observed. These data are in accordance with a recent paper [53] showing that contaminants

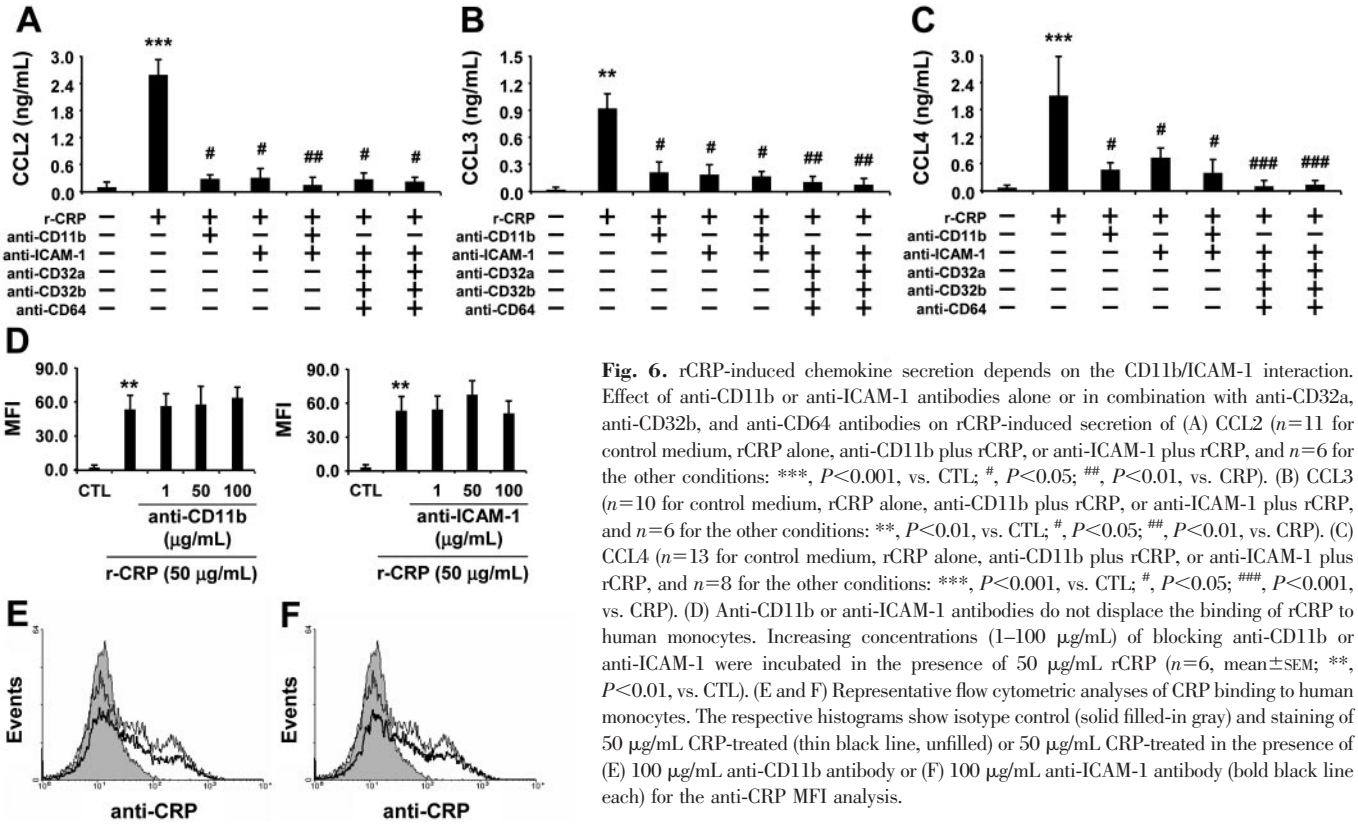

Fig. 6. rCRP-induced chemokine secretion depends on the CD11b/ICAM-1 interaction. Effect of anti-CD11b or anti-ICAM-1 antibodies alone or in combination with anti-CD32a, anti-CD32b, and anti-CD64 antibodies on rCRP-induced secretion of (A) CCL2 ($n=11$ for control medium, rCRP alone, anti-CD11b plus rCRP, or anti-ICAM-1 plus rCRP, and $n=6$ for the other conditions: ***, $P<0.001$, vs. CTL; #, $P<0.05$; ##, $P<0.01$, vs. CRP). (B) CCL3 ($n=10$ for control medium, rCRP alone, anti-CD11b plus rCRP, or anti-ICAM-1 plus rCRP, and $n=6$ for the other conditions: **, $P<0.01$, vs. CTL; #, $P<0.05$; ##, $P<0.01$, vs. CRP). (C) CCL4 ($n=13$ for control medium, rCRP alone, anti-CD11b plus rCRP, or anti-ICAM-1 plus rCRP, and $n=8$ for the other conditions: ***, $P<0.001$, vs. CTL; #, $P<0.05$; ###, $P<0.001$, vs. CRP). (D) Anti-CD11b or anti-ICAM-1 antibodies do not displace the binding of rCRP to human monocytes. Increasing concentrations (1–100 μg/mL) of blocking anti-CD11b or anti-ICAM-1 were incubated in the presence of 50 μg/mL rCRP ($n=6$, mean±SEM; **, $P<0.01$, vs. CTL). (E and F) Representative flow cytometric analyses of CRP binding to human monocytes. The respective histograms show isotype control (solid filled-in gray) and staining of 50 μg/mL CRP-treated (thin black line, unfilled) or 50 μg/mL CRP-treated in the presence of (E) 100 μg/mL anti-CD11b antibody or (F) 100 μg/mL anti-ICAM-1 antibody (bold black line each) for the anti-CRP MFI analysis.

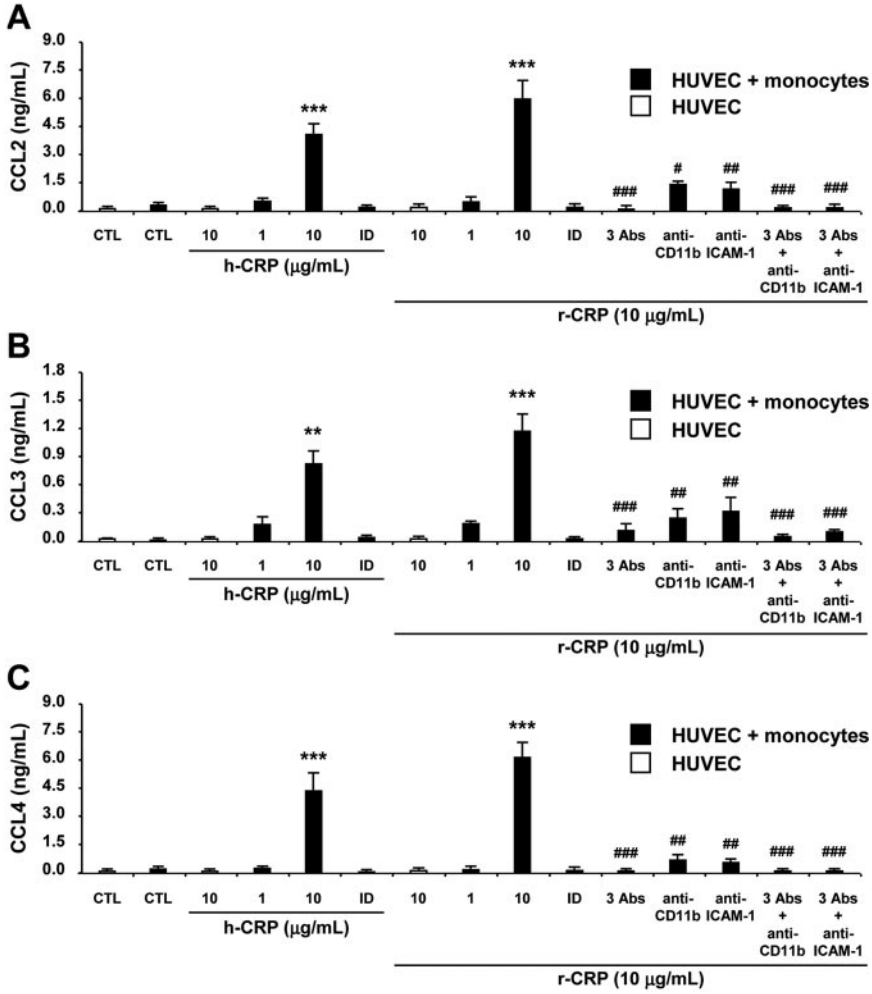

Fig. 7. CRP induces CCL2, CCL3, and CCL4 secretion in human monocytes adherent to the HUVEC monolayer. One hour before coincubation with HUVEC, human monocytes were incubated with anti-CD32a, anti-CD32b, and anti-CD64 and anti-CD11b or anti-ICAM-1 alone or in combination. Then, the control medium alone, rCRP (1 and 10 μg/mL), hCRP (1 and 10 μg/mL), rCRP (10 μg/mL) ID, or hCRP (10 μg/mL) ID were added ($n=6$). (A) CCL2: ***, $P < 0.001$, versus medium alone or CRP ID compounds; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$, versus 10 μg/mL hCRP or 10 μg/mL rCRP. (B) CCL3: ***, $P < 0.001$; **, $P < 0.01$, versus medium alone or CRP ID compounds; ##, $P < 0.01$; ###, $P < 0.001$, versus 10 μg/mL hCRP or 10 μg/mL rCRP. (C) CCL4: ***, $P < 0.001$, versus medium alone or CRP ID compounds; ##, $P < 0.01$; ###, $P < 0.001$, versus 10 μg/mL hCRP or 10 μg/mL rCRP.

rather than CRP induced chemokine secretion in endothelial cells and further support the purity of the rCRP used in our study. Thus, combined signaling of the two CRP receptors (CD32 and CD64) together with Mac-1/ICAM-1 is necessary for inducing CCL2, CCL3, and CCL4 secretion. Adhesion molecules are well known to influence leukocyte functions [54]. CD11b is expressed on human monocytes and binds different ligands [19, 55, 56]. Among the molecules capable of binding CD11b, ICAM-1 is probably the most important one for adhesion to endothelium [20]. The effect of CRP on CD11b

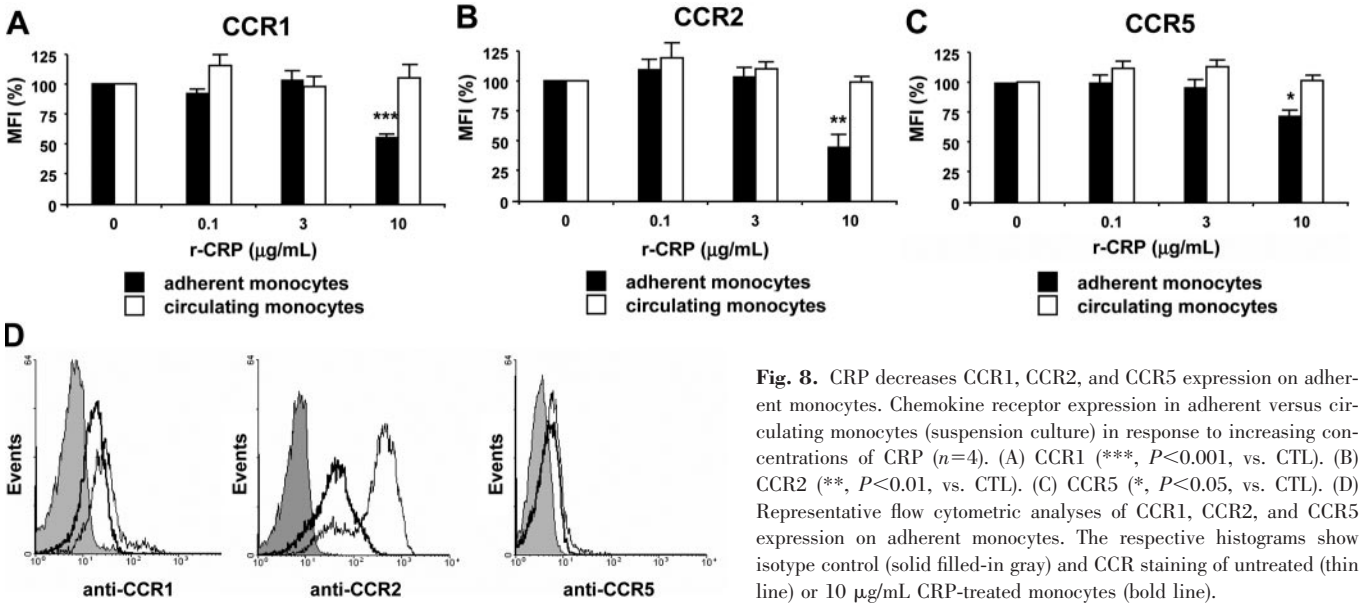

Fig. 8. CRP decreases CCR1, CCR2, and CCR5 expression on adherent monocytes. Chemokine receptor expression in adherent versus circulating monocytes (suspension culture) in response to increasing concentrations of CRP ($n=4$). (A) CCR1 (***, $P<0.001$, vs. CTL). (B) CCR2 (**, $P<0.01$, vs. CTL). (C) CCR5 (*, $P<0.05$, vs. CTL). (D) Representative flow cytometric analyses of CCR1, CCR2, and CCR5 expression on adherent monocytes. The respective histograms show isotype control (solid filled-in gray) and CCR staining of untreated (thin line) or 10 μg/mL CRP-treated monocytes (bold line).

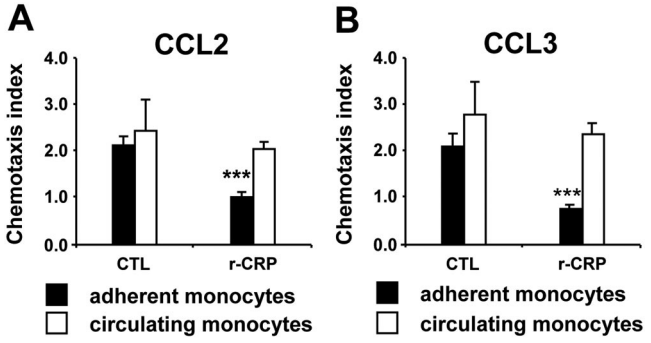

Fig. 9. Effect of CRP on monocyte migration to CCL2 or CCL3. Migration of monocytes to chemokines after pretreatment with 10 μg/mL CRP in adherence (n=10) or suspension culture (circulating monocytes, n=6). (A) Migration to CCL2 (***, P<0.001, vs. CTL). (B) Migration to CCL3 (***, P<0.001, vs. CTL).

and ICAM-1 has not been well investigated in the past. Recent studies reported a modulation of CD11b integrin expression induced by CRP [49, 57], which has been described previously to promote monocyte migration to the classical chemoattractant CCL2 [6]. On the other hand, CRP has been described as a monocyte chemoattractant itself [31]. This activity might lead to a cross-desensitization of cells for migration in response to other chemoattractants, which may explain different observations on monocyte migration [58]. Han and co-workers [6] have shown that CRP promotes monocyte migration to CCL2 through CCR2 up-regulation. This is in conflict with our finding that CRP inhibited monocyte migration to CCL2 and CCL3 through the down-regulation of their cognate receptors CCR1 (CCL3 receptor), CCR2 (CCL2 receptor), and CCR5 (CCL3 and CCL4 receptors). These opposite results might be explained by the different methodological approaches used for monocyte isolation and culture. This supports the notion that the methods and materials may have a crucial effect on cell function and should be considered carefully when interpreting in vitro data. In our model, we also provide evidence for the mechanism underlying CCR1, CCR2, and CCR5 down-regulation. The recombinant chemokines CCL2, CCL3, and CCL4 synergistically mimicked the CRP-induced down-regulation of CCR1, CCR2, and CCR5. In line with these findings, the effect of CRP was reversed by neutralizing antichemokine antibodies, and anti-CCL2 and anti-CCL3 are more efficient than anti-CCL4. These data suggest that CCL2, CCL3, and CCL4 are synergistically involved, at least in part, in chemokine receptor down-regulation. The finding that chemokines, upon binding with their cognate receptors, trigger receptor down-regulation, not only through receptor internalization, has already been reported for CCL3 [59, 60]. In agreement with this, our data indicate that chemokine receptor down-regulation is not only a result of receptor internalization but also the reduction of their mRNA level.

To summarize, we provide evidence that adherence and adhesion molecules are crucial for CRP-mediated effects on human monocytes. Although highly speculative, CRP-mediated effects on adherent cells might suggest a proinflammatory role, not only for circulating CRP but also for CRP deposits in the atherosclerotic plaque on adherent monocytes.

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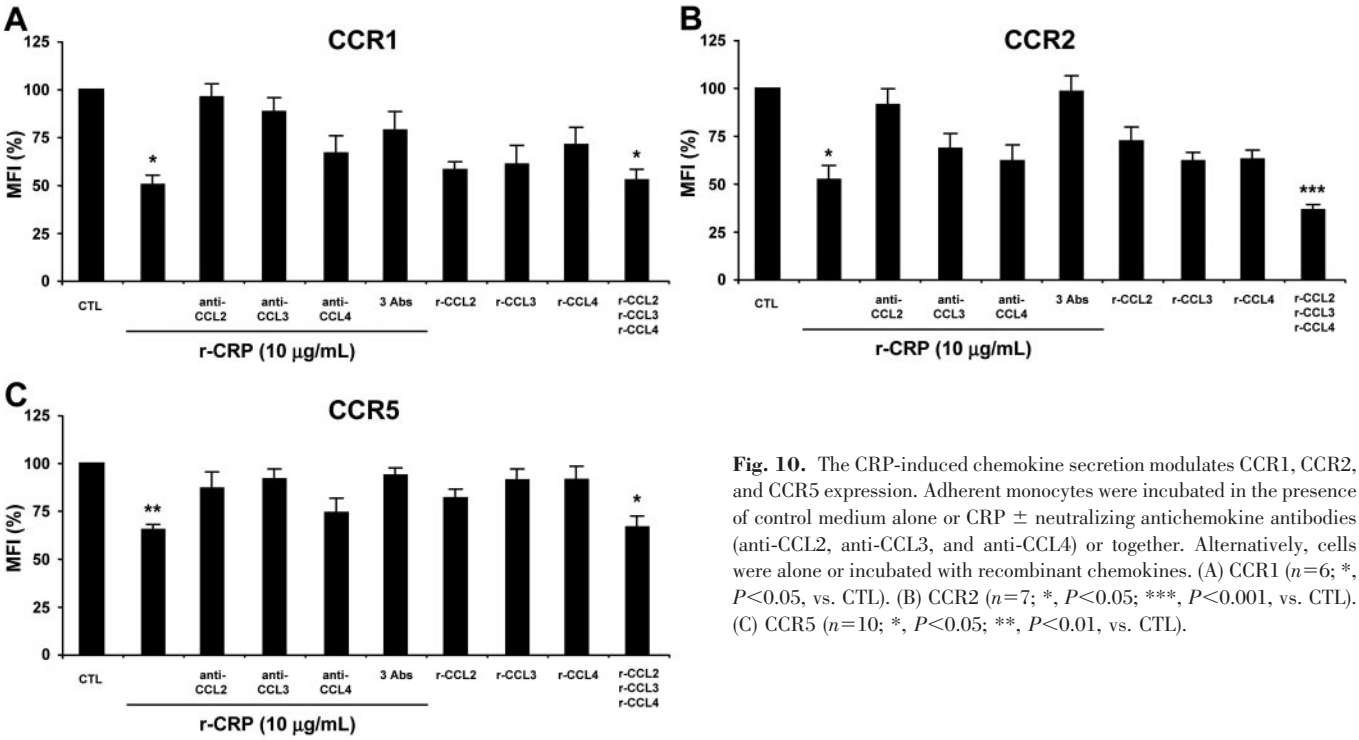

Fig. 10. The CRP-induced chemokine secretion modulates CCR1, CCR2, and CCR5 expression. Adherent monocytes were incubated in the presence of control medium alone or CRP ± neutralizing antichemokine antibodies (anti-CCL2, anti-CCL3, and anti-CCL4) or together. Alternatively, cells were alone or incubated with recombinant chemokines. (A) CCR1 (n=6; *, P<0.05, vs. CTL). (B) CCR2 (n=7; *, P<0.05; ***, P<0.001, vs. CTL). (C) CCR5 (n=10; *, P<0.05; **, P<0.01, vs. CTL).

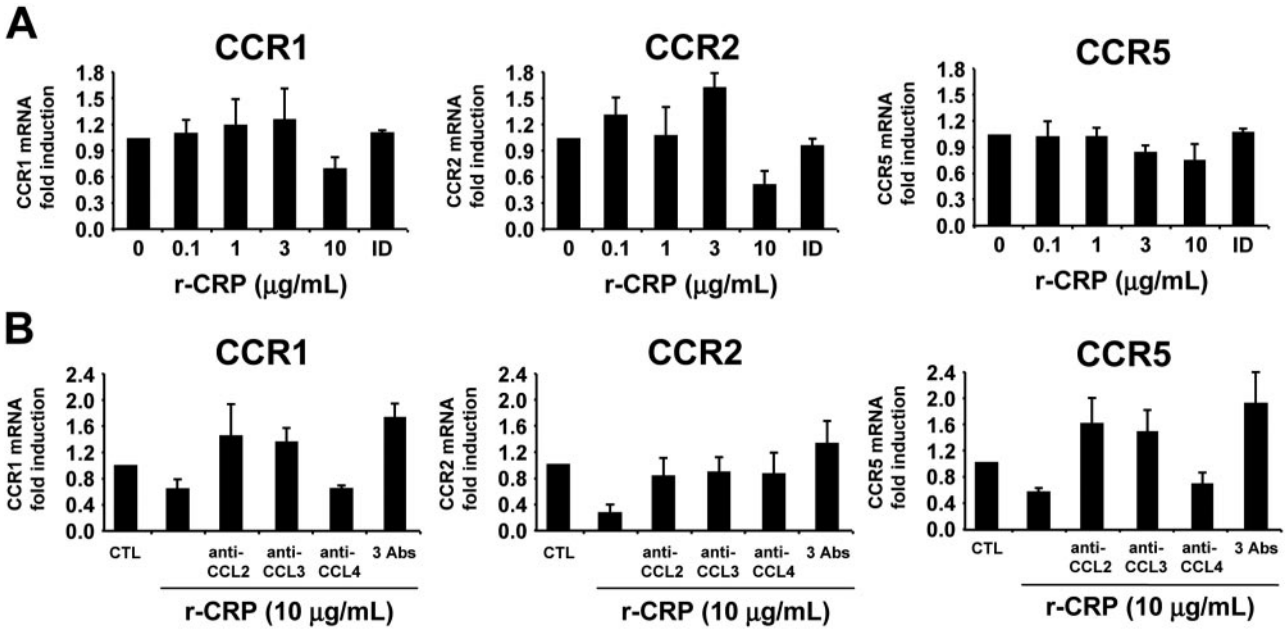

Fig. 11. Chemokines induced by CRP modulate CCR1, CCR2, and CCR5 expression of the mRNA level. (A) CCR1, CCR2, and CCR5 mRNA expression in adherent monocytes after treatment with increasing concentrations of CRP ($n=4$ for CCR1; $n=3$ for CCR2 and CCR5). (B) CCR1, CCR2, and CCR5 mRNA expression in adherent monocytes incubated in medium alone or with CRP ± neutralizing antichemokine antibodies alone (anti-CCL2, anti-CCL3, and anti-CCL4) or together ($n=3$ for CCR1 and CCR2; $n=4$ for CCR5).

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