CXCL4 Downregulates the Atheroprotective Hemoglobin Receptor CD163 in Human Macrophages

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Rationale: CXCL4 is a platelet-derived chemokine that promotes macrophage differentiation from monocytes. Deletion of the *PF4* gene that encodes CXCL4 reduces atherosclerotic lesions in *ApoE*$^{-/-}$ mice.

Objective: We sought to study effects of CXCL4 on macrophage differentiation with possible relevance for atherogenesis.

Methods and Results: Flow cytometry for expression of surface markers in macrophage colony–stimulating factor (M-CSF)– and CXCL4-induced macrophages demonstrated virtually complete absence of the hemoglobin scavenger receptor CD163 in CXCL4-induced macrophages. mRNA for CD163 was downregulated as early as 2 hours after CXCL4. CD163 protein reached a minimum after 3 days, which was not reversed by treatment of cells with M-CSF. The CXCL4 effect was entirely neutralized by heparin, which bound CXCL4 and prevented CXCL4 surface binding to monocytes. Pretreatment of cells with chlorate, which inhibits glycosaminoglycan synthesis, strongly inhibited CXCL4-dependent downregulation of CD163. Similar to recombinant CXCL4, releasate from human platelets also reduced CD163 expression. CXCL4-differentiated macrophages were unable to upregulate the atheroprotective enzyme heme oxygenase-1 at the RNA and protein level in response to hemoglobin–haptoglobin complexes. Immunofluorescence of human atherosclerotic plaques demonstrated presence of both CD68$^+$CD163$^+$ and CD68$^+$CD163$^-$ macrophages. *PF4* and *CD163* gene expression within human atherosclerotic lesions were inversely correlated, supporting the in vivo relevance of CXCL4-induced downregulation of CD163.

Conclusions: CXCL4 may promote atherogenesis by suppressing CD163 in macrophages, which are then unable to upregulate the atheroprotective enzyme heme oxygenase-1 in response to hemoglobin. (*Circ Res*. 2010;106:203-211.)

Key Words: atherosclerosis ■ macrophage ■ CXCL4 ■ CD163

Atherosclerosis is an inflammatory disease of the arterial wall. Atherogenesis is characterized by monocytes entering the subendothelial space where they differentiate into macrophages.[1,2] These macrophages represent a major cellular component of the atherosclerotic lesion and promote plaque development by secreting numerous inflammatory mediators like proteases, cytokines and chemokines. The resulting inflammatory milieu leads to recruitment of smooth muscle cells and additional immune cells.[3] All these functions make the plaque macrophage an interesting target for prevention and therapy of atherosclerotic disease.[4]

Over the past several years, it has become evident that macrophages do not represent a homogenous cell population.[5] The first evidence suggesting the existence of different macrophage subtypes dates back to 1992, when Gordon et al described an alternative macrophage phenotype induced by interleukin-4, which was characterized by high mannose receptor expression.[6] Since then, the paradigm of "classic" M1 macrophages (obtained through activation by interferon-γ and lipopolysaccharide [LPS]) and the "alternative" M2 macrophages (obtained through alternative activation by interleukin [IL]-4 or IL-13) has developed.[7] This basic M1/M2 scheme has been enriched by definition of various additional differentiation types including activation through Fc receptors or Toll-like receptors.[8]

In vitro studies using gene arrays have thoroughly investigated the differentiation of human blood–derived monocytes toward M1 or M2 macrophages and found distinct sets of genes that are specifically upregulated in either M1 or M2 macrophages.[9] We previously studied the effects of specific chemokines as well as oxidized or minimally modified low density lipoprotein on macrophage differentiation and showed a gene expression pattern in foam cells that resembled that of inflammatory dendritic cells.[10]

The finding that macrophages show different polarization patterns induced by specific stimuli in vitro is supported by in

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Non-standard Abbreviations and Acronyms

ApoE	apolipoprotein E
Hb-Hp	hemoglobin–haptoglobin
HMOX-1	heme oxygenase-1
IL	interleukin
LDL	low-density lipoprotein
LPS	lipopolysaccharide
M-CSF	macrophage colony–stimulating factor

vivo data demonstrating the presence of phenotypically and potentially functionally different macrophage subsets in human atherosclerotic lesions (recently reviewed elsewhere[11]). The first evidence for the presence of M2 polarized macrophages within human atherosclerotic lesions was presented by Bouhlel et al who showed that expression of M2 markers like IL-10 or mannose receptor in atherosclerotic plaques correlated with peroxisome proliferator-activated receptor γ expression.[12] Similarly, our own group recently showed that a large number, but not all plaque macrophages, express aldose reductase, an enzyme that is associated with oxidative stress and can be induced by high glucose levels or oxidized low-density lipoprotein (LDL).[13] Another description of differentially polarized macrophages within atherosclerotic plaques was published by Waldo et al who describe the presence of different macrophage subsets defined by the levels of CD14 expression as well as differential ability to take up oxidized low density lipoprotein.[14] Although all these data strongly suggest the presence of 2 or possibly even more differentially polarized macrophage subsets in atherosclerotic lesions, the significance of these subsets for atherogenesis and plaque progression is not entirely clear. Furthermore, the significance of specific factors like cytokines or growth factors for macrophage polarization during atherogenesis has not been studied in detail.

Macrophage differentiation from monocytes is induced by macrophage colony–stimulating factor (M-CSF).[15] The platelet-derived chemokine CXCL4 has also been demonstrated to promote macrophage differentiation from human blood–derived monocytes.[16] Interestingly, presence of CXCL4 in atherosclerotic lesions correlates with clinical parameters in patients with atherosclerosis.[17] Furthermore, deletion of the *PF4* gene encoding CXCL4 results in reduced lesion size in atherosclerotic apolipoprotein E–null ($ApoE^{-/-}$) mice suggesting a significant role for this chemokine during atherogenesis.[18]

Based on the important role of macrophages in atherosclerosis as well as the accumulating evidence that CXCL4 may be an important player in atherogenesis, we hypothesized that macrophages differentiated under the influence of this chemokine may display specific characteristics relevant for atherogenesis. In a gene expression and surface marker screening we found that CXCL4-induced macrophages completely lack expression of CD163, a scavenger receptor for hemoglobin and hemoglobin–haptoglobin (Hb-Hp) complexes.[19] CD163 engagement has been described to induce upregulation of heme oxygenase-1,[20] an enzyme that protects from atherosclerosis.[21] Here, we tested the hypothesis that CXCL4 suppresses CD163 expression, thus generating a proinflammatory macrophage type in atherosclerotic lesions.

Methods

Monocyte-Derived Macrophages

Human peripheral blood was obtained with approval from the institutional review board from healthy volunteer donors as described previously.[10]

Real-Time RT-PCR

Real-time PCR was performed as described previously.[10]

Flow Cytometry

Flow cytometry was performed as described previously.[10]

Soluble CD163 ELISA

Soluble CD163 was measured in cell culture supernatants of macrophages induced by M-CSF (100 ng/mL) or CXCL4 (1 μmol/L) by ELISA (BMA Biomedicals, Augst, Switzerland) as indicated by the manufacturer.

Platelets

Platelets were isolated and activated as described previously.[23]

Hemoglobin

For assessment of the macrophage response to hemoglobin response, macrophages were cultured for 6 days with either M-CSF or CXCL4 as described. At the time of monocyte isolation, red blood cell lysates and plasma of individual donors were kept and stored at −20°C. On day 6 after monocyte isolation, macrophages were incubated with macrophage serum-free medium (Gibco, Carlsbad, Calif) containing a final concentration of 500 μg/mL autologous hemoglobin and 20% autologous serum to ensure sufficient amounts of haptoglobin. After 4 hours, cells were harvested and *HMOX1* gene expression was measured by real-time PCR as described above. For assessment of heme oxygenase-1 protein expression by flow cytometry, cells were harvested after 18 hours.

Plaque Tissue Processing

Carotid arteries were obtained with approval by the institutional review board from 18 consecutive patients undergoing endarterectomy at the Department of Vascular Surgery (University of Heidelberg). Intraoperatively, carotid plaques were removed en bloc to preserve plaque structure. Tissue samples were shock frozen in liquid nitrogen and stored at −80°C until use. RNA extraction and real-time PCR were performed as described above. Demographic and clinical patient data are shown in Online Table II, which is available in the Online Data Supplement at http://circres.ahajournals.org.

Immunofluorescence

Human coronary arteries from patients with cardiovascular disease were obtained with approval by the institutional review board post mortem from the University of Virginia Department of Pathology/ Tissue bank (Charlottesville, Va) as described previously.[13]

Statistical Analysis

Statistical analysis was performed using Prism software (GraphPad, La Jolla, Calif). Paired *t* tests were used for single comparisons, 1-way ANOVA with post hoc Tukey test or Dunnett's test for multiple comparisons where appropriate. For variables lacking normal distribution, Kruskal–Wallis test with post hoc Dunn's test for multiple comparisons was used. Correlation was determined by nonparametric Spearman's testing. All analyses were done 2-sided, $P<0.05$ was considered significant.

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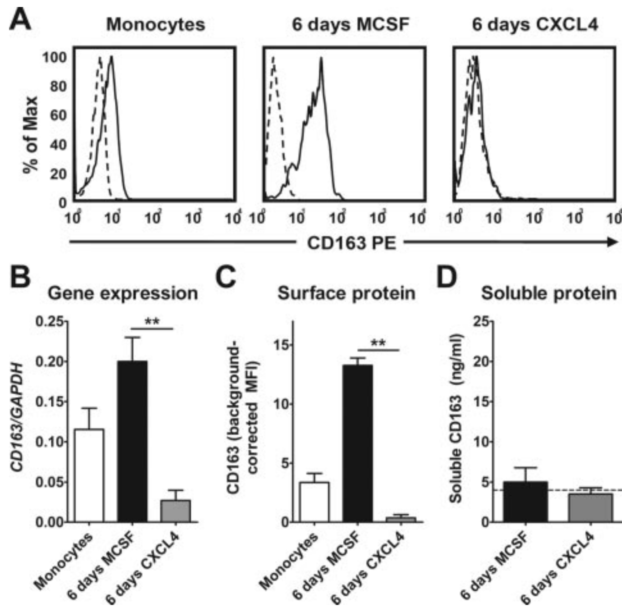

Figure 1. CXCL4 downregulates CD163 during macrophage differentiation. A, Histograms of CD163 surface expression as measured by flow cytometry in freshly isolated monocytes and macrophages differentiated for 6 days with M-CSF (100 ng/mL) or CXCL4 (1 μmol/L), respectively. All cells are derived from the same donor. Dotted line indicates isotype control. B, *CD163* gene expression relative to *GAPDH* as determined by real-time PCR in freshly isolated monocytes or macrophages differentiated with M-CSF or CXCL4 as described above. Means+SEM (n=3 to 8). $^{*}P<0.05$, $^{**}P<0.01$. C, Summary of CD163 background-corrected mean fluorescence intensity in monocytes and M-CSF– or CXCL4-induced macrophages. Means+SEM (n=3 to 5). $^{**}P<0.01$, $^{***}P<0.001$. D, Soluble CD163 was measured by ELISA in macrophage cell culture supernatants after 6 days exposure to M-CSF or CXCL4. The dotted line indicates the detection limit of the assay.

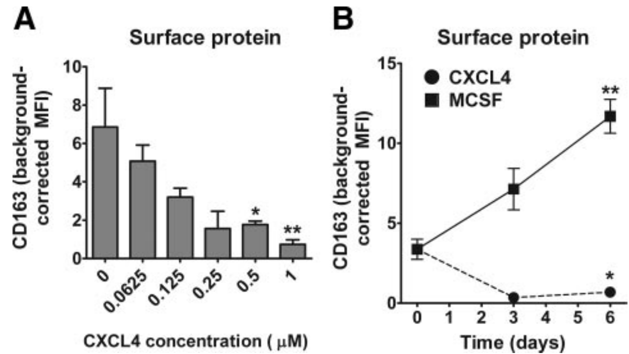

Figure 2. Dose response and time course of CD163 expression during macrophage differentiation with CXCL4. A, Dose response of CD163 background-subtracted mean fluorescence intensity in monocytes treated with CXCL4 for 3 days. Means±SEM (n=3). $^{*}P<0.05$, $^{**}P<0.01$ vs concentration 0. B, Time course of CD163 background-subtracted mean fluorescence intensity in monocytes and M-CSF– or CXCL4-induced macrophages. Means±SEM (n=3 to 6). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs time 0.

Results

CXCL4 Downregulates CD163 Expression During Macrophage Differentiation

To study the differential effects of M-CSF and CXCL4 on monocyte–macrophage differentiation, human peripheral blood monocytes were cultured with 100 ng/mL M-CSF or 1 μmol/L CXCL4 for 6 days. These dosages had previously been demonstrated to induce macrophage differentiation.[13,14] When studying gene and protein expression of various macrophage surface receptors, we found CD163 to be differentially expressed in macrophages differentiated with M-CSF or CXCL4. As described previously, M-CSF significantly increased CD163 at the mRNA and surface expression level during monocyte-macrophage differentiation.[23] By contrast, CXCL4 significantly downregulated CD163 at the mRNA and protein level. Accordingly, CD163 was virtually absent in CXCL4-induced macrophages (Figure 1A through 1C). As CD163 surface expression is known to be in part regulated by shedding, eg, after exposure to LPS,[25] we assessed whether treatment of macrophages with CXCL4 resulted in significant levels of soluble CD163 in cell culture supernatants. In M-CSF–treated macrophages, soluble CD163 was barely detectable and did not increase in CXCL4-treated macrophages, suggesting that CXCL4 does not induce shedding of surface CD163 (Figure 1D). Notably, the expression of other markers like CD45 (leukocytes), CD14 (monocytes), or CD11b (macrophages) was not differentially regulated by the 2 growth factors (Online Figure II).

CXCL4-Induced CD163 Downregulation Is Not Reversible and Is Not Mediated by Reduced Secretion of IL-10

Dose response experiments revealed that a CXCL4 concentration of 0.5 μmol/L was sufficient to significantly downregulate CD163 surface expression and that maximal downregulation was achieved at a concentration of 1 μmol/L (Figure 2A). Studies of the time course of CXCL4-induced CD163 downregulation showed that *CD163* gene expression was significantly reduced as early as 2 hours after adding CXCL4 and reached its minimum after 24 hours (data not shown). CD163 surface expression was significantly downregulated after 3 days and stayed low thereafter (Figure 2B).

To assess whether CXCL4-induced downregulation of CD163 is reversible, monocytes were treated with either M-CSF or CXCL4 for 3 days and then switched to CXCL4 or M-CSF, respectively (Figure 3A). These experiments showed that M-CSF induced CD163 upregulation as seen on day 3. This was reversed when cells were switched to CXCL4 (Figure 3B). By contrast, M-CSF was unable to significantly induce CD163 expression once monocytes had been exposed to CXCL4 for 3 days (Figure 3C).

IL-10 is known to strongly upregulate CD163 expression in macrophages.[2] To test whether CXCL4 downregulates CD163 indirectly via reduction of IL-10 secretion, we tested whether CXCL4 has any effect on IL-10 mRNA or protein expression. Although there was no difference in mRNA expression, CXCL4-treated macrophages secreted significantly lower levels of IL-10 protein (Online Figure III, A). However, addition of exogenous IL-10 was not able to rescue CD163 surface expression on macrophages differentiated with CXCL4. This excludes CXCL4-induced downregulation of IL-10 secretion as the underlying mechanism of CD163 downregulation (Online Figure III, B).

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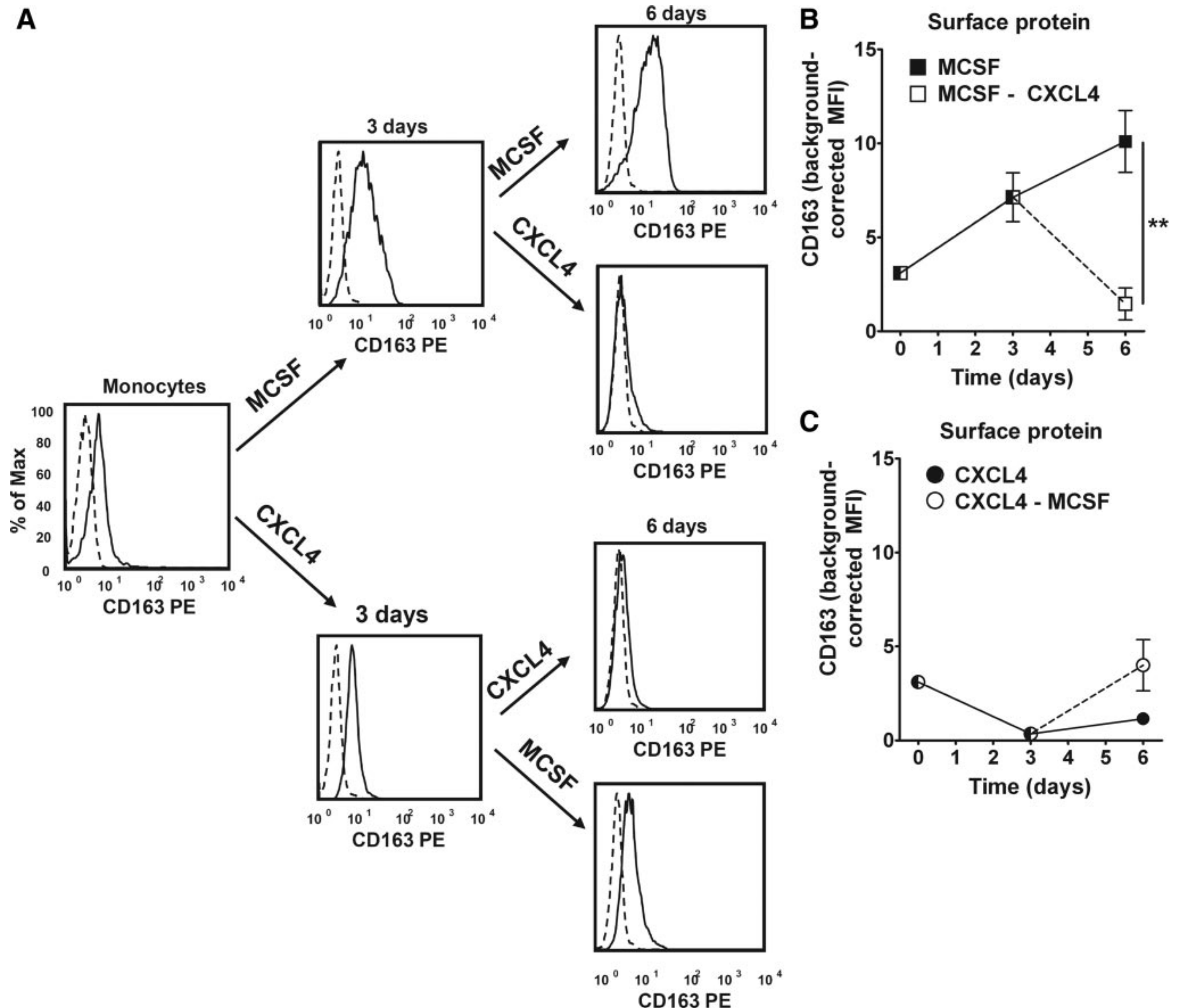

Figure 3. CXCL4 actively downregulates CD163. A, Histograms of CD163 surface expression in freshly isolated monocytes and macrophages treated for 6 days with M-CSF (100 ng/mL) or CXCL4 (1 μmol/L), switched from M-CSF to CXCL4 or from CXCL4 to M-CSF on day 3 as indicated. B, Line graph indicating CD163 surface expression as determined by flow cytometry in monocytes/macrophages treated with M-CSF for 6 days (solid squares) or switched from M-CSF to CXCL4 on day 3 (open squares). Means$\pm$SEM (n=3). **$P<0.01$. C, Line graph indicating CD163 surface expression as determined by flow cytometry in monocytes/macrophages treated with CXCL4 for 6 days (solid circles) or switched from CXCL4 to M-CSF on day 3 (open circles). Means$\pm$SEM (n=3).

CXCL4 Effects Are Not Attributable to LPS Contamination

Similar to our findings with CXCL4, LPS (1 ng/mL) has been demonstrated to downregulate CD163 expression in the presence of serum[24]; however, much higher concentrations are necessary to see this effect in the absence of serum as used in our experimental setting.[24] To test whether the effects seen in the present study were attributable to LPS contamination of the recombinant CXCL4, two approaches were chosen. (1) The maximum possible LPS content of the recombinant CXCL4 preparation used in the experiments was 0.1 ng/μg, which translates to 780 pg/mL for 1 μmol/L CXCL4. This LPS concentration was unable to suppress CD163 gene expression under our experimental conditions (Online Figure IV). (2) We repeated the experiments with CXCL4 isolated from human platelets to exclude LPS that may be present in recombinant CXCL4. These experiments gave the same results as those with recombinant CXCL4 and showed complete absence of CD163 on the cell surface after 3 days.

CXCL4-Induced Downregulation of CD163 Is Mediated by Surface Glycosaminoglycans

Heparin is known to specifically bind and inactivate CXCL4.[26] We therefore reasoned that adding heparin to the culture medium may prevent CXCL4 effects on CD163 expression. We first studied whether CXCL4 surface binding to monocytes was affected by heparin. In fact, heparin completely abrogated surface binding to monocytes at 4°C, as assessed by flow cytometry (Figure 4A).

We then treated monocytes with M-CSF for 3 days to induce robust CD163 expression. After this period, cells were kept in medium alone or switched to CXCL4$\pm$heparin (2

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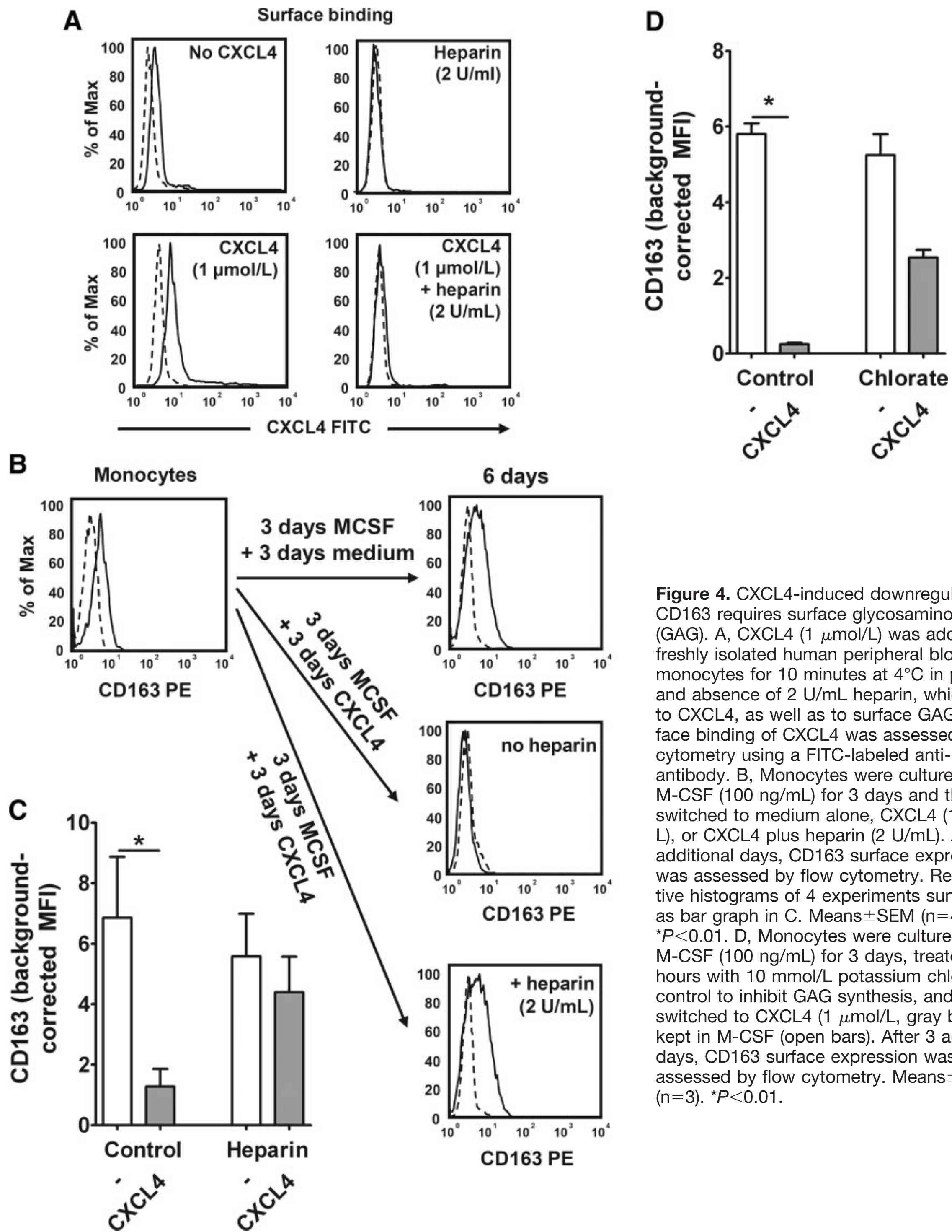

Figure 4. CXCL4-induced downregulation of CD163 requires surface glycosaminoglycans (GAG). A, CXCL4 (1 μmol/L) was added to freshly isolated human peripheral blood monocytes for 10 minutes at 4°C in presence and absence of 2 U/mL heparin, which binds to CXCL4, as well as to surface GAGs. Surface binding of CXCL4 was assessed by flow cytometry using a FITC-labeled anti-CXCL4 antibody. B, Monocytes were cultured with M-CSF (100 ng/mL) for 3 days and then switched to medium alone, CXCL4 (1 μmol/L), or CXCL4 plus heparin (2 U/mL). After 3 additional days, CD163 surface expression was assessed by flow cytometry. Representative histograms of 4 experiments summarized as bar graph in C. Means±SEM (n=4). *$P<0.01$. D, Monocytes were cultured with M-CSF (100 ng/mL) for 3 days, treated for 4 hours with 10 mmol/L potassium chlorate or control to inhibit GAG synthesis, and then switched to CXCL4 (1 μmol/L, gray bars) or kept in M-CSF (open bars). After 3 additional days, CD163 surface expression was assessed by flow cytometry. Means±SEM (n=3). *$P<0.01$.

U/mL). Presence of heparin in the culture medium completely abrogated CXCL4-dependent downregulation of CD163 (Figure 4B and 4C). These findings suggest that CXCL4 binding to heparan-sulfate expressing surface receptors[26] is required for CD163 downregulation. This conclusion was also supported by experiments in which macrophages cultured in M-CSF for 3 days were pretreated with 10 mmol/L chlorate for 4 hours and then either switched to CXCL4 or kept in M-CSF–containing medium, both in the presence of chlorate. Chlorate is known to significantly reduce glycosaminoglycan synthesis.[28] After chlorate pretreatment, the effect of CXCL4 on CD163 expression was reduced and did not reach statistical significance (Figure 4D).

In endothelial cells, CXCL4 has been reported to bind to the chemokine receptor CXCR3B[28]; however, CXCR3 was undetectable by real-time RT-PCR (data not shown) or flow cytometry in freshly isolated monocytes (Online Figure IV). Even after 6 days in culture with either M-CSF or CXCL4, no CXCR3 expression could be detected (data not shown). However, as CXCR3 expression may be too low to be

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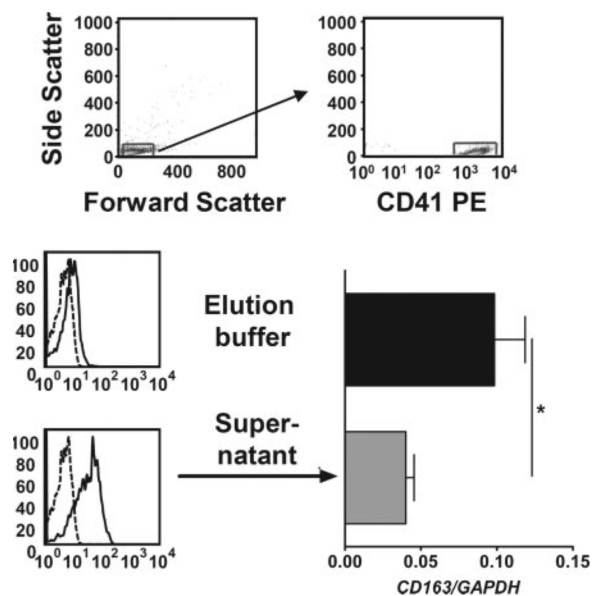

Figure 5. Releasate from activated platelets induces CD163 downregulation in macrophages. Platelets were gated by forward and side scatter and CD41 (top row). Monocytes were cultured with M-CSF (100 ng/mL) for 3 days to induce robust CD163 expression and then treated with releasate from platelets activated with a combination of TRAP-7 and ADP resulting in robust P-selectin expression (bottom row). Controls were treated with elution buffer only (middle row). After 2 hours, CD163 gene expression was assessed by real-time PCR and normalized for *GAPDH*. Means+SEM (n=3 to 6). $^{**}P<0.01$.

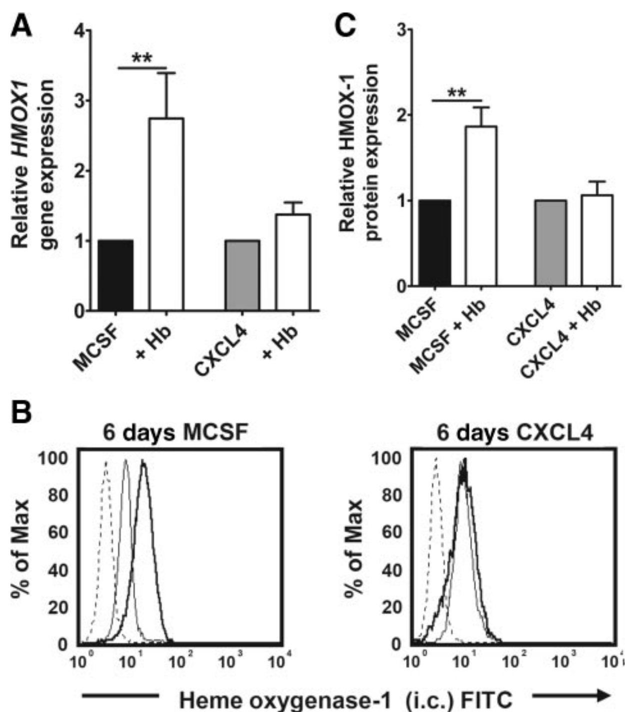

Figure 6. CD163^{-} macrophages do not upregulate heme oxygenase-1 in response to Hb-Hp complexes. Macrophages were differentiated from monocytes for 6 days with M-CSF (100 ng/mL) or CXCL4 (1 μmol/L) and exposed to 500 μg/mL autologous hemoglobin in culture medium supplemented with 20% autologous serum providing sufficient amounts of haptoglobin (Hb-Hp). A, After 4 hours, *HMOX1* gene expression was measured by real-time RT-PCR. Means±SEM (n=5 to 6). $^{**}P<0.01$. B, Intracellular heme oxygenase-1 protein expression was assessed by flow cytometry after 18 hours of exposure to Hb-Hp. Dotted line indicates isotype control; fine line, no Hb-Hp; bold line, Hb-Hp. The results of 4 independent experiments are summarized as a bar graph in C. $^{*}P<0.01$ (n=4).

detected by flow cytometry, we also performed blocking experiments, in which cells were treated with a blocking antibody against CXCR3 or control antibody before exposure to CXCL4. These experiments showed no effect of anti-CXCR3 antibody on CXCL4-induced downregulation of CD163 (Online Figure V).

Releasate From Activated Platelets Can Downregulate CD163 Expression in Macrophages

Platelet releasate contains high amounts of CXCL4 but also a variety of other chemokines.[30] To test whether the CXCL4 concentrations resulting from platelet degranulation in the context of other factors contained in the platelet releasate were able to downregulate CD163, monocytes were cultured with M-CSF for 3 days to induce robust CD163 expression. On day 3, cells were treated with cell-free releasate of ADP- and TRAP-7-activated platelets. Platelet activation was verified by flow cytometric assessment of CD62P (P-selectin) surface expression (Figure 5). Platelet releasate, but not control buffer significantly reduced *CD163* gene expression as early as 2 hours after addition supporting the physiological relevance of our findings with recombinant CXCL4 (Figure 5).

CXCL4-Induced Suppression of CD163 Results in Inability to Upregulate Heme Oxygenase-1

Engagement of CD163 by Hb-Hp complexes has been described to induce heme oxygenase-1, an enzyme linked to atheroprotection.[20] Accordingly, CD163 expressing macrophages have been demonstrated to exert antiinflammatory effects in response to CD163 engagement. We therefore assessed whether CXCL4-induced loss of CD163 on macrophages suppressed expression of heme-oxygenase-1. M-CSF– and CXCL4-induced macrophages were treated with Hb-Hp complexes for 4 hours. After this period, *HMOX1* gene expression was assessed by real-time RT-PCR. As expected, M-CSF–induced CD163^{+} macrophages robustly upregulated *HMOX1* after exposure to Hb-Hp, whereas CXCL4-induced CD163^{-} macrophages were not able to upregulate *HMOX1* (Figure 6A). This was also true on the protein level, where M-CSF–induced macrophages showed a 2-fold upregulation of heme oxygenase-1 protein in response to Hb-Hp complexes, whereas CXCL4-induced macrophages were unable to respond (Figure 6B and 6C). This supports the notion that CXCL4-induced downregulation of CD163 is functionally relevant and prevents an important antiinflammatory mechanism relevant in macrophages.

Expression of CXCL4 and CD163 Within Human Atherosclerotic Lesions Is Inversely Correlated

Presence of CD163^{+} and CD163^{-} macrophages within atherosclerotic plaques has been described previously,[33] which we confirmed by showing CD163^{+} and CD163^{-} macrophages in human coronary atherosclerotic lesions (Figure 7A and 7B). To assess whether our in vitro finding of CXCL4-

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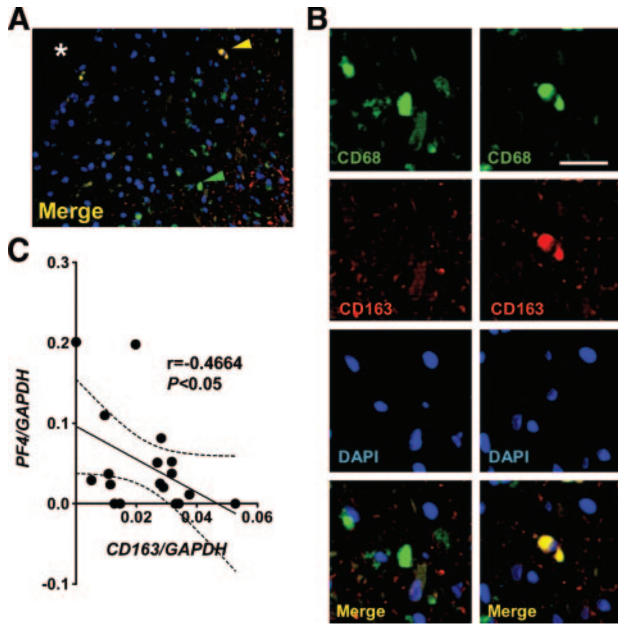

Figure 7. Negative correlation between *PF4* and *CD163* in human atherosclerotic plaques. A, Coronary artery sections from patients with cardiovascular disease were obtained post mortem. Paraffin sections were stained for the macrophage marker CD68 (FITC) and CD163 (phycoerythrin). DAPI was added as nuclear stain. Vascular lumen is indicated by *. B, Magnification of a $CD68^{+}CD163^{+}$ (yellow arrow in A, top row) and $CD68^{+}CD163^{-}$ macrophage (green arrow in A, bottom row). Scale bars: 20 μm. C, RNA was isolated from carotid atherosclerotic plaques immediately after carotid endatherectomy. Expression of *CD163* and *PF4* message was measured by real-time PCR and normalized to *GAPDH*.

induced modulation of the macrophage phenotype was relevant in vivo, we measured *CD163* and *PF4* gene expression in 18 atherosclerotic plaques obtained by carotid endatherectomy. Whereas *CD163* gene expression was detectable in all but 1 plaque, *PF4* mRNA was detected in 13 lesions. *CD163* and *PF4* expression showed a significant negative correlation ($r=-0.4664$, $P<0.05$), supporting the notion that high *PF4* expression levels are correlated with low *CD163* mRNA expression (Figure 7C). This supports our in vitro findings that CXCL4 downregulates CD163 expression in a dose-dependent manner.

Discussion

Here, we show that macrophages differentiated under the influence of the platelet chemokine CXCL4 lose surface expression of CD163. The physiological relevance of CXCL4-induced loss of CD163 was demonstrated by (1) the ability of releasate from activated platelets to downregulate CD163; (2) the inability of $CD163^{-}$ macrophages to respond to exposure to Hb-Hp complexes by upregulating heme oxygenase-1 (HMOX-1); and (3) an inverse correlation between CXCL4 and CD163 expression within human atherosclerotic lesions supporting an in vivo relevance for our in vitro observations.

CXCL4 is released from platelets on activation at micromolar concentrations.[30] In 2000, Scheuerer et al[16] demonstrated that CXCL4 prevents monocyte apoptosis and promotes differentiation toward macrophages.[16] Presence of CXCL4 within atherosclerotic lesions has been associated with clinical parameters including lesion grade and presence of symptoms, suggesting an important role of this chemokine in atherogenesis.[17] Sachais et al[18] recently demonstrated that lack of CXCL4 in mice results in reduced lesion formation in the $ApoE^{-/-}$ model,[18] thus showing that CXCL4 has a net proatherogenic effect.

Several biological effects of CXCL4 may be important for plaque development. CXCL4 promotes recruitment of monocytes toward the arterial wall through formation of heterodimers with CCL5 (RANTES).[35] Blocking the formation of CXCL4-CCL5 heterodimers significantly reduces lesion size in the $ApoE^{-/-}$ mouse model of atherosclerosis.[36] CXCL4 also inhibits binding of native LDL to its receptor and subsequent internalization, thereby potentially promoting LDL oxidation, which makes LDL more atherogenic.[37] In addition, CXCL4 binds to oxidized LDL and mediates its binding to endothelial cells of the vascular wall.[38] Finally, macrophages differentiated under the influence of CXCL4 express high levels of surface HLA-DR and completely lack CD86.[16] Here, we show that CXCL4-induced macrophages completely lose CD163 expression and that this macrophage phenotype is found in atherosclerotic lesions. The downregulation of CD163 expression in monocyte-derived macrophages may explain a mechanism by which CXCL4 has proinflammatory effects in atherosclerosis. CD163 is an important scavenger receptor for Hb-Hp complexes and, with lower affinity, for uncomplexed hemoglobin.[20] Decreased expression of CD163 on peripheral blood mononuclear cells has been demonstrated in patients with a specific haptoglobin genotype (Hp 2 to 2), which is know be associated with a significantly increased risk of cardiovascular disease in diabetic patients.[34]

Engagement of CD163 results in upregulation of HMOX-1,[20] an enzyme with antioxidative and antiinflammatory effects. HMOX-1 is atheroprotective, which was demonstrated by knocking out the *Hmox1* gene in mice, resulting in increased lesion size in both $ApoE^{-/-}$ and $Ldlr^{-/-}$ mice[32,33,40] Bone marrow transplant experiments have clearly shown that heme oxygenase-1 in hematopoietic cells, which include monocytes and macrophages, is responsible for this effect.[33] Furthermore, macrophages from $Hmox1^{-/-}$ mice were more likely than those from $Hmox1^{+/+}$ mice to take up oxidized LDL, generate reactive oxygen species, and secrete inflammatory cytokines.[33] HMOX-1 is also present in human atherosclerotic lesions. *HMOX1* gene expression was found to be higher in carotid arteries from asymptomatic patients,[41] but high levels of CD163 and HMOX-1 were also found in symptomatic plaques.[42] Our findings suggest that macrophages differentiated in presence of CXCL4 have an impaired ability to process hemoglobin that may accumulate from small intravascular hemorrhages. Boyle et al have recently suggested that intracoronary hemorrhage may evoke a novel atheroprotective macrophage phenotype characterized by high expression of CD163 and low expression of HLA-DR, whereas foam cells express low levels of CD163.[43] They induced the $CD163^{+}$ macrophage phenotype by Hb-Hp complexes, IL-10, or dexamethasone.[43] Here, we show that CXCL4 has the

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opposite effect on macrophage polarization, characterized by absence of CD163 and inability to upregulate the atheroprotective enzyme HMOX-1 in response to intraplaque hemorrhage. The fact that releasate from activated platelets is able to mimic this proatherogenic phenotype as well as the fact that presence of platelets[44] and CXCL4[17] have been demonstrated in atherosclerotic lesions both support the physiological relevance of this finding. Taken together, it is likely that macrophages differentiated under the influence of CXCL4 differentiate toward a more proatherogenic macrophage phenotype and thereby promote lesion formation. The fact that CD163 and CXCL4 message expression within human atherosclerotic lesions are inversely correlated strongly suggests that the in vitro findings described in this report are relevant in the in vivo setting of an atherosclerotic lesion.

In microvascular endothelial cells and T lymphocytes, CXCL4 has been demonstrated to act via engagement of CXCR3B, a splice variant of the chemokine receptor CXCR3.[29] However, it is unclear how CXCL4 effects are mediated in monocytes. We found no evidence for CXCR3 expression in monocytes at the mRNA or protein levels, and even after fully differentiation toward macrophages no CXCR3 expression could be detected. Furthermore experiments using blocking antibody against CXCR3 did not result in abrogation of the CXCL4 effect on CD163 expression. These findings make involvement of CXCR3 or CXCR3B in CXCL4-induced downregulation of CD163 unlikely. In an in vitro study, Yu et al have demonstrated CXCL4-induced upregulation of E-selectin in human umbilical vein endothelial cells mediated by the LRP (LDL receptor–related protein) through activation of nuclear factor κB.[45] Similar mechanisms may be involved in CXCL4-dependent downregulation of CD163, but our data suggest that CD163 downregulation by CXCL4 requires binding to a glycosaminoglycan present on the monocyte/macrophage cell surface.[27] This conclusion is based on the finding that heparin and chlorate treatment both inhibit CXCL4 surface binding and CD163 downregulation.

In summary, we present evidence for a novel $CD163^-$ proinflammatory macrophage phenotype that is induced by the platelet-chemokine CXCL4 and is present in atherosclerotic lesions in vivo. This work adds to the growing body of evidence that atherosclerotic plaque macrophages do not represent a homogeneous entity and are composed of phenotypically and functionally distinct subsets and suggests CXCL4 as favorable target for atheroprotective therapeutic or prophylactic interventions.

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Disclosures

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Supplement Material

Methods

Monocyte-derived macrophages

Briefly, monocytes were isolated from human peripheral blood by gradient centrifugation (Histopaque, Sigma, St. Louis, MO) and subsequent negative bead isolation (Miltenyi, Auburn, CA) yielding >97% purity as determined by flow cytometry for CD14. A red blood cell lysis step was added to ensure that cells were free from hemoglobin contamination. After several wash steps with 1 mmol/L EDTA, monocytes showed little platelet contamination as demonstrated by low CD41 expression by flow cytometry (Online Figure I). Monocytes were cultured in macrophage serum-free medium (Gibco, Carlsbad, CA) supplemented with Nutridoma SP (Roche, Indianapolis, IN) and penicillin/streptomycin (Sigma, St. Louis, MO) for six days in the presence of 100 ng/mL M-CSF (recombinant, Peprotech, Rocky Hill, NJ) or 1 µmol/L CXCL4 (either recombinant (Peprotech, Rocky Hill, NJ) or isolated from human platelets (Athens Research and Technology, Athens, GA)). Some experiments were done in the presence of 2 U/ml heparin (ratiopharm, Ulm, Germany) or included pre-incubation of cells with 10 mM potassium chlorate (Sigma, Steinheim, Germany) or 2 ng/ml interleukin-10 (R&D Systems, Minneapolis, MN).

Real-time RT-PCR

RNA was isolated using columns including a DNAse-step followed by reverse transcription (all Qiagen, Valencia, CA). Real-time PCR on a Light Cycler 480 (Roche, Indianapolis, IN) was performed in duplicates using *GAPDH* as housekeeping gene.

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Product specificity was assessed by melting curve analysis or agarose gel. Primer sequences (Online Table I) were obtained from primer bank[i].

Platelets

Platelets were isolated from platelet-rich human plasma by sepharose column and activated for 10 minutes with TRAP-7 (10 µmol/L (Bachem, Torrance, CA)) and ADP (1 mmol/L (Sigma, St. Louis, MO)). Activation was confirmed by flow cytometric assessment of CD62P (P-selectin) positivity by FACS (clone AK-4, BD Biosciences, San Jose, CA). Platelet-free supernatants were added to freshly isolated monocytes so that each macrophage was treated with the releasate of 1000 platelets. Controls were treated with the same amount of elution buffer.

Flow cytometry

For flow cytometry, cells were treated with Fc receptor block (Miltenyi, Auburn, CA), washed and subsequently stained with antibodies against CD11b (clone ICRF44), CD14 (clone M5E2), CD45 (clone 2D1, all BD Biosciences, San Jose, CA), CD163 (clone GHI/61c, eBioscience, San Diego, CA), CXCR3 (clone 2Ar1, Abcam, Cambridge, MA), heme oxygenase-1 (clone HO-1-1, Abcam, Cambridge, MA) or appropriate isotype controls. Surface binding of CXCL4 to monocytes was assessed by exposing freshly isolated monocytes to CXCL4 (1 µmol/L) for 10 min at 4˚C in the presence or absence of 2 U/mL heparin. Subsequently, cells were washed and stained with a FITC-labeled antibody against CXCL4 (clone 170138, R&D Systems, Minneapolis, MN). For intracellular staining, cells were fixed with 2% paraformaledhyde and subsequently stained in buffer containing saponin and the appropriate antibodies. Fluorescence intensity was analyzed on a Fascalibur (Becton Dickinson, Sparks, MD), data analysis was done using FloJo software (Treestar, Ashland, OR).

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Immunofluorescence

Immunofluorescence was performed to determine the colocalization of CD163 and CD68. Briefly, coronary arteries were embedded in paraffin and 5 µmol/L sections were prepared. After heat-induced antigen retrieval using antigen unmasking solution (Vector Laboratories), sections were incubated with antibodies against CD68 (clone KP-1) and CD163 (clone C-16, both Santa Cruz Biotechnology, Santa Cruz, CA). KP-1 was FITC-labeled, C-16 was visualized with a Texas red-labeled anti-goat secondary. DAPI (Millipore, Billerica, MA) was used as nuclear stain.

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Tables

Online Table I: Primers use for real-time PCR

Gene	Forward sequence	Reverse sequence
GAPDH	GGCTCATGACCACAGTCCAT	GCCTGCTTCACCACCTTCT
CD163	ACTTGAAGACTCTGGATCTGCT	CTGGTGACAAAACAGGCACTG
HMOX1	CTGACCCATGACACCAAGGAC	AAAGCCCTACAGCAACTGTCG
CXCR3	GTACGGCCCTGGAAGACTG	CATTTAGCACTTGGTGGTCACT

Online Table II: Demographic and clinical data of patients undergoing carotid endatherectomy (mean ± SEM or percentage)

Age (years)	68.8 ± 7.7
Gender (male)	82.3 %
Risk factors	
- Hypertension	82.4 %
- Diabetes mellitus	62.5 %
- Hyperlipidemia	53.9 %
- Smoking history	36.4 %

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Figures

Online Figure I: Isolated monocytes display low platelet contamination. (A) Purity of monocytes isolated from peripheral blood as assessed by positivity for CD14 in flow cytometry. Platelet contamination of cell culture was assessed in $CD14^+$ cells by flow cytometry for CD41. (B) CD41 Expression on platelets as positive control.

Online Figure II: Leukocyte surface markers in monocytes and macrophages differentiates with M-CSF or CXCL4. Surface expression of (A) CD45, (B) CD14, and (C) CD11b in freshly isolated monocytes, macrophages after six days differentiation with M-CSF (100 ng/mL) or CXCL4 (1 µmol/L) as determined by flow cytometry. Isotype controls as dotted lines. All cells derived from the same donor.

Online Figure III: CXCL4-dependent downregulation of CD163 is not mediated through downregulation of IL-10 expression. (A) IL-10 concentration in supernatants from monocyte-derived macrophages differentiated for six days with M-CSF (100 ng/mL) or CXCL4 (1 µmol/L) as determined by ELISA. The dotted line indicates the detection limit of the assay. *P<0.05 by paired t test. (B) Histogram of CD163 surface expression as determined by flow cytometry in monocyte-derived macrophages differentiated for six days with M-CSF (100 ng/mL, solid line), CXCL4 (1 µmol/L, thin dotted line) or CXCL4 (1 µmol/L) with addition of recombinant human IL-10 (2 ng/mL, thick dotted line).

Online Figure IV: CD163 downregulation during macrophage differentiation is not caused by LPS contamination of recombinant CXCL4. Monocytes were cultured with M-CSF (100 ng/mL) for 6 days (black bars) and or switched to medium with 780 pg/mL LPS (corresponding to the maximum possible LPS contamination of recombinant

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CXCL4, white bars) or 1 µmol/L recombinant human CXCL4 (grey bars) on day 3. Gene expression of *CD163* was measured by real-time PCR. *IL8* expression was measured as positive control for the LPS effect. *** P<0.001, **P<0.01, *P<0.05 by ANOVA with post-hoc Tukey test.

Online Figure V: The chemokine receptor CXCR3 is not involved in CXCL4 effects on macrophage CD163 expression. (A) Lymphocytes, but not monocytes express CXCR3 on the cell surface. Freshly isolated human peripheral blood mononuclear cells were stained with antibody against CXCR3 and expression was analyzed by flow cytometry. Lymphocytes and monocytes were identified by forward and side scatter. (B) Blocking antibody against does not prevent CXCL4-induced downregulation of CD163. Monocytes were treated with M-CSF for 3 days to induce CD163 expression; after this period they were switched to CXCL4 in the presence of irrelevant IgG control or blocking antibody against CD163. *** P<0.001, **P<0.01 by ANOVA with post-hoc Tukey test.

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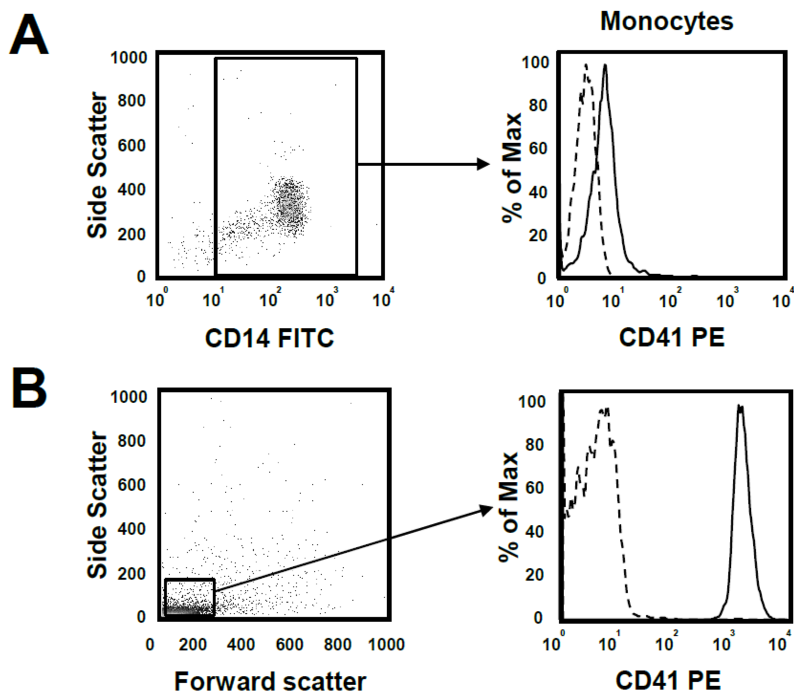

Online Figure I

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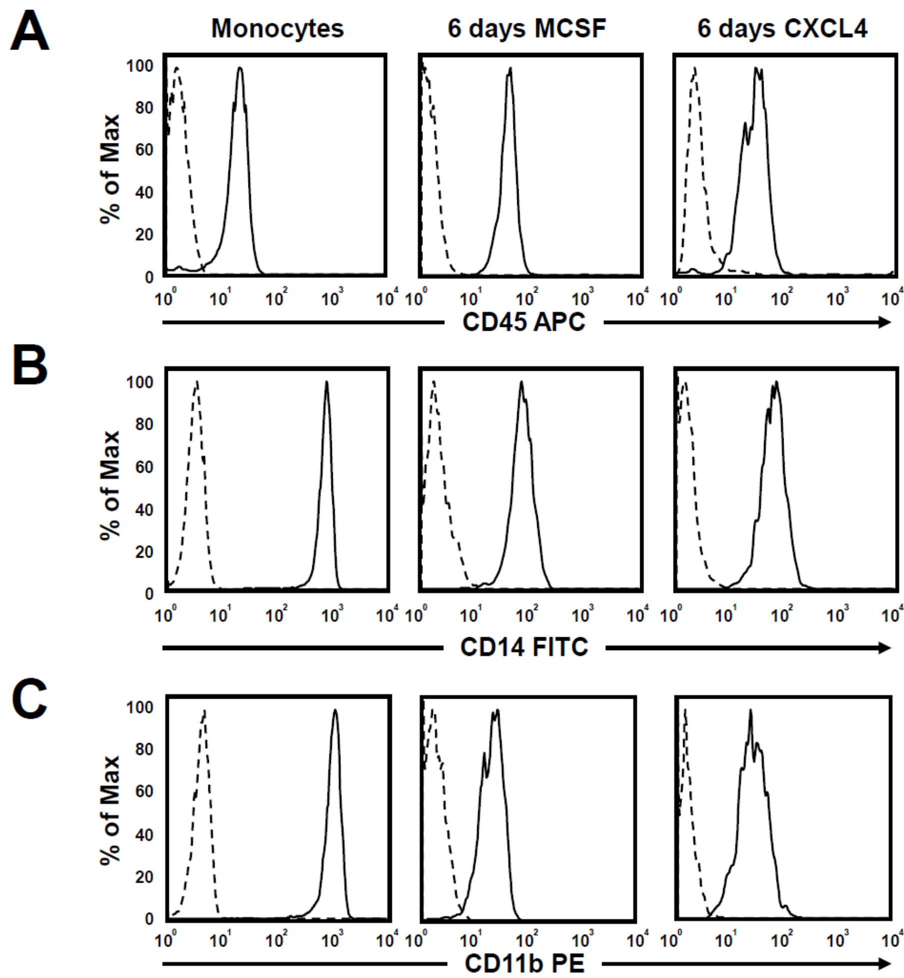

Online Figure II

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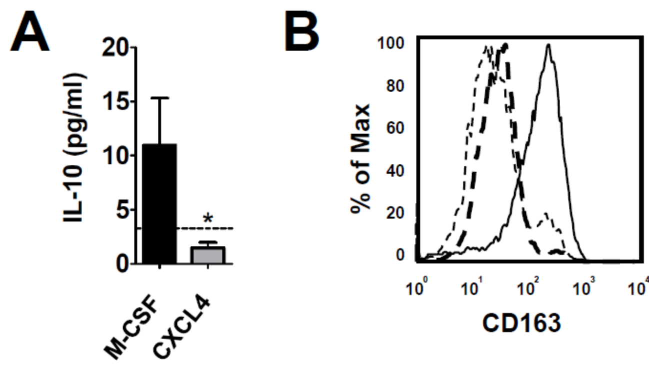

Online Figure III

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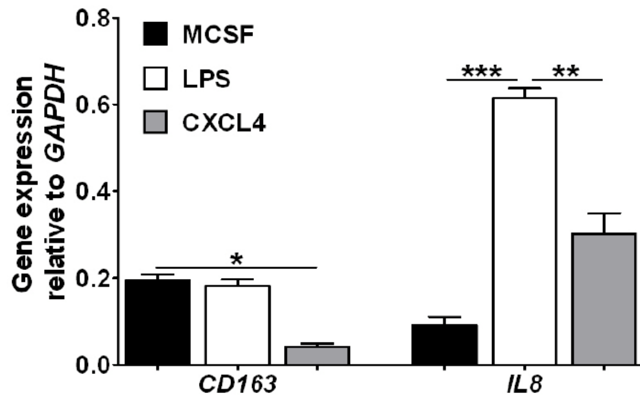

Online Figure IV

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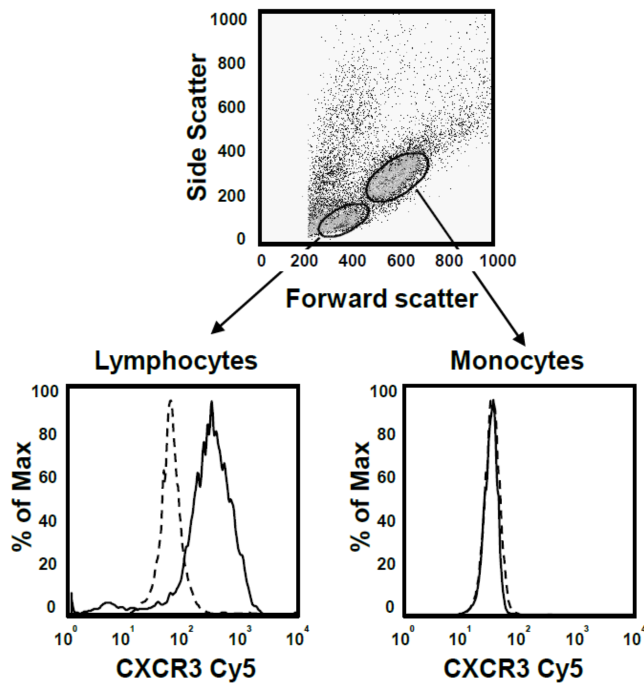
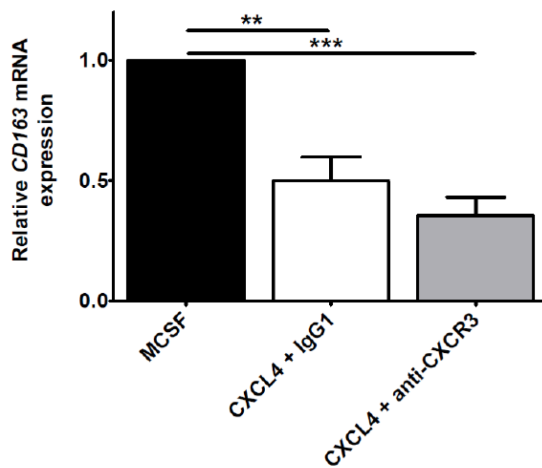
A



B



Online Figure V

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