CXCL4 Downregulates the Atheroprotective Hemoglobin Receptor CD163 in Human Macrophages

Christian A. Gleissner, Iftach Shaked, Christian Erbel, Dittmar Böckler, Hugo A. Katus, Klaus Ley

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<sup>−/−</sup> 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)<sup>−</sup>– 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<sup>−</sup>CD163<sup>+</sup> and CD68<sup>+</sup>CD163<sup>+</sup> 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;106:00-00.)

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. 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. All these functions make the plaque macrophage an interesting target for prevention and therapy of atherosclerotic disease.

Over the past several years, it has become evident that macrophages do not represent a homogenous cell population. 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. 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. This basic M1/M2 scheme has been enriched by definition of various additional differentiation types including activation through Fc receptors or Toll-like receptors.

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. 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. The finding that macrophages show different polarization patterns induced by specific stimuli in vitro is supported by in
vivo data demonstrating the presence of phenotypically and potentially functionally different macrophage subsets in human atherosclerotic lesions (recently reviewed elsewhere). The first evidence for the presence of M2 polarized macrophages within human atherosclerotic lesions was presented by Bouchel 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. 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). 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. 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). The platelet-derived chemokine CXCL4 has also been demonstrated to promote macrophage differentiation from human blood–derived monocytes. Interestingly, presence of CXCL4 in atherosclerotic lesions correlates with clinical parameters in patients with atherosclerosis. 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.

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. CD163 engagement has been described to induce up-regulation of heme oxygenase-1, an enzyme that protects from atherosclerosis. 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.

**Real-Time RT-PCR**

Real-time PCR was performed as described previously.

**Flow Cytometry**

Flow cytometry was performed as described previously.

**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.

**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.

**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.
CXCL4 Downregulates CD163 in Macrophages

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.\(^1^,\)\(^2\) 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.\(^3\)\(^,\)\(^4\) 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 LPII,\(^5\) 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 down-regulate CD163 surface expression and that maximal down-regulation was achieved at a concentration of 1 μmol/L (Figure 2A). Studies of the time course of CXCL4-induced CD163 downregulation revealed 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 down-regulated 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).
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; however, much higher concentrations are necessary to see this effect in the absence of serum as used in our experimental setting. 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. 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±heparin (2

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±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±SEM (n=3).
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 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. 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; 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
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. 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, monocyes were cultured with M-CSF 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.

A

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.

B

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. 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. 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).

A

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).

B

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, 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-
induced modulation of the macrophage phenotype was relevant in vivo, we measured CD163 and PF4 gene expression in 18 atherosclerotic plaques obtained by carotid endarterectomy. 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 observations 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.8 In 2000, Scheuerer et al demonstrated that CXCL4 prevents monocyte apoptosis and promotes differentiation toward macrophages.4 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.5 Sachais et al recently demonstrated that lack of CXCL4 in mice results in reduced lesion formation in the ApoE−/− model,16 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).34 Blocking the formation of CXCL4-CCL5 heterodimers significantly reduces lesion size in the ApoE−/− mouse model of atherosclerosis.35 CXCL4 also inhibits binding of native LDL to its receptor and subsequent internalization, thereby potentially promoting LDL oxidation, which makes LDL more atherogenic.36 In addition, CXCL4 binds to oxidized LDL and mediates its binding to endothelial cells of the vascular wall.37 Finally, macrophages differentiated under the influence of CXCL4 express high levels of surface HLA-DR and completely lack CD163. Therefore, the potential of CXCL4 to lose surface expression of CD163 was demonstrated by (1) the inability of CD163+ macrophages to express HLA-DR under the influence of CXCL4; (2) the inability of CD163+ macrophages to express HLA-DR under the influence of CXCL4; and (3) the inability of CD163+ macrophages to express HLA-DR under the influence of CXCL4.

CXCL4-induced modulation of CD163 expression was demonstrated by in vivo observations that CXCL4 downregulates CD163 expression in a dose-dependent manner. This supports our in vitro observations that CXCL4 downregulates CD163 expression in a dose-dependent manner.
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\textsuperscript{45} and CXCL4\textsuperscript{47} 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.\textsuperscript{27} However, it is unclear how CXCL4 effects are of CXCR3, and CXCL4\textsuperscript{47} 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 differenciate 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 summary, we present evidence for a novel CD163\textsuperscript{+} 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
None.

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References

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Disclosures
None.

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We thank Dr. Maria-Beatriz Lopes, University of Virginia, Charlottesville, for providing post mortem coronary artery specimens, and Keely Arbenz-Smith, and Nadine Wambganss for excellent technical assistance.


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

**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% paraformaldehyde 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).
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.
## Tables

**Online Table I:** Primers use for real-time PCR

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

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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
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

Forward scatter

Lymphocytes

Monocytes

% of Max

$10^0$ $10^1$ $10^2$ $10^3$

$10^0$ $10^1$

$10^0$ $10^1$

$10^0$ $10^1$ $10^2$ $10^3$

CXCR3 Cy5

B

Relative CD163 mRNA expression

NCSE

CXCL4 + IgG1

CXCL4 + anti-CXCR3

Online Figure V