Leukocyte-Specific CCL3 Deficiency Inhibits Atherosclerotic Lesion Development by Affecting Neutrophil Accumulation


Objective—Despite common disbelief that neutrophils are involved in atherosclerosis, evidence is accumulating for a causal role of neutrophils in atherosclerosis. CC chemokine ligand (CCL)3 is an inflammatory chemokine and its expression is significantly increased during atherosclerotic lesion formation in mice. It has recently been shown that under conditions of inflammation neutrophils can migrate along a CCL3 gradient. In this study, we aimed to elucidate the role of leukocyte-derived CCL3 in atherogenesis.

Methods and Results—Irradiated low density lipoprotein receptor –/– mice, reconstituted with CCL3 –/– or littermate bone marrow showed markedly reduced CCL3 response to lipopolysaccharide treatment, establishing the critical relevance of leukocytes as source of CCL3. Hematopoietic deficiency of CCL3 significantly reduced aortic sinus lesion formation by 31% after 12 weeks of western-type diet. Interestingly, whereas plaque macrophages, collagen, and vascular smooth muscle cell content were unchanged, neutrophil adhesion to and presence in plaques was significantly attenuated in CCL3 –/– chimeras. These mice had reduced circulating neutrophil numbers, which could be ascribed to an increased neutrophil turnover and CCL3 –/– neutrophils were shown to be less responsive toward the neutrophil chemoattractant CXC chemokine ligand 1.

Conclusion—Our data indicate that under conditions of acute inflammation leukocyte-derived CCL3 can induce neutrophil chemotaxis toward the atherosclerotic plaque, thereby accelerating lesion formation. (Arterioscler Thromb Vasc Biol. 2013;33:e75-e83.)

Key Words: atherosclerosis ■ chemokines ■ inflammation ■ neutrophils
CCL3 appears to be the macrophage, although evidence also points to the release of this chemokine by activated platelets, neutrophils, and mast cells.\textsuperscript{14-16} CCL3 can induce chemotaxis of different leukocyte subsets, including monocytes/macrophages and T-lymphocytes via CC chemokine receptor (CCR)1, CCR4, or CCR5. Although initial observations showed that neutrophil migration is not under influence of CCL3, it is becoming increasingly clear that tumor necrosis factor-α (TNFα)-stimulated neutrophils are responsive to CCL3 and become migratory by upregulation of the integrins CD11b and CD18.\textsuperscript{17} Furthermore, it was shown that CCL3 is a mediator of firm adherence and (subsequent) transmigration of neutrophils, as a result of lipid mediator production, which, in turn, directly activate neutrophils.\textsuperscript{16}

Several clinical studies have proposed CCL3 as a marker of clinical atherosclerosis,\textsuperscript{18} and we have shown it to be an independent predictor of future ischemia.\textsuperscript{20,21} Although these findings suggest an important role for CCL3 in atherosclerosis, no experimental data are available as yet to substantiate such a role. In this study, we therefore aimed to establish the effect of leukocyte-specific CCL3 deficiency on atherosclerotic lesion development.

Materials and Methods

Animals
Low density lipoprotein receptor−/− mice were obtained from the local animal breeding facility. C57Bl6 mice were from Charles River, Maastricht, the Netherlands, and CCL3-deficient mice were obtained from the Jackson Laboratory, Bar Harbor, United States. Mice were maintained on sterilized regular chow (RM3; Special Diet Services, Essex, United Kingdom), and drinking water was supplied ad libitum. Animal experiments were performed at the animal facilities of the Gorlaeus laboratories of Leiden University. All experimental protocols were approved by the ethics committee for animal experiments of Leiden University.

Temporal Expression Profile
Male LDLr−/− mice were fed WTD containing 0.25% cholesterol and 15% cacaobutter (Special Diet Services, Sussex, UK) 2 weeks prior to surgery and throughout the experiment. To determine plaque gene-expression patterns, atherosclerotic carotid artery lesions (n=20) were induced by perivascular collar placement, as described previously.\textsuperscript{22} Subsets of mice (n=4) were euthanized at 0, 2, 4, 6, and 8 weeks after collar placement. Both common carotid arteries were removed and snap-frozen in liquid nitrogen for optimal RNA preservation. The specimens were stored at −80°C until further use.

RNA Isolation
Two carotids were pooled and pools homogenized by grounding in liquid nitrogen with a pestle. Total RNA was extracted from the tissue using Trizol reagent according to manufacturer’s instructions (Invitrogen, Breda, the Netherlands). RNA was reverse transcribed using moloney murine leukemia virus reverse transcriptase (RevertAid, MBI Fermentas, Leon-Roth). Genetic profiling of carotid artery lesions was performed using Illumina Bead-Chip Whole Genome Microarrays (ServiceX, Leiden, the Netherlands).

Bone Marrow Transplantation
To induce bone marrow aplasia, male LDLr−/− recipient mice were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation using an Andros Smart 225 Röntgen source (YXLON International) with a 6-mm aluminum filter 1 day before transplantation. Bone marrow was isolated from male CCL3−/− or wild-type (WT) littermates by flushing the femurs and tibias. Irradiated recipients received 0.5×10\textsuperscript{7} bone marrow cells by tail vein injection and were allowed to recover for 6 weeks. Drinking water was supplied ad libitum and supplemented with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymyxin B sulfate) and 6.5 g/L sucrose. Animals were placed on a WTD containing 0.25% cholesterol and 15% cacao butter (SDS) diet for 12 weeks and subsequently euthanized. Twenty-four hours before euthanization, a subset of animals were injected intraperitoneally with lipopolysaccharide (100μg/kg LPS; Salmonella minnesota R595 (Re); List Biological Laboratories Inc, Campbell, CA).

Histological Analysis
Cryostat sections of the aortic root (10 μm) were collected and stained with Oil-red-O. Lesion size was determined in 7 to 10 sections of the aortic valve leaflet area. Corresponding sections on separate slides were stained immunohistochemically with an antibody directed against a macrophage-specific antigen (anti-monoocyte + macrophage antibody-2, monoclonal rat IgG2b, dilution 1:50; Serotec, Oxford, U.K.). Goat anti rat anti IgG-alkaline phosphatase (dilution 1:100; Sigma, St. Louis, MO) was used as secondary antibody and nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (Dako, Glostrup, Denmark) as enzyme substrate. T-lymphocytes were stained using CD3 as a marker (CD3 clone SP7, dilution 1:150, Immunologic, Duiven, the Netherlands). Antibody IgG-poly-horseradish peroxidase (Powervision, Leica, Rijswijk, the Netherlands) was used as secondary antibody and Nova-Red (Vector labs, Peterborough, United Kingdom) as enzyme substrate. Cells were counted manually. Masson trichrome staining (Sigma, St. Louis, MO) was used to visualize collagen (blue staining). Neutrophils were visualized by Naphtol AS-D Chloroacetate Esterase stain, according to the manufacturer’s protocol (Sigma).

Cell Isolation and Stimulation
Peritoneal leukocytes were harvested by peritoneal lavage. Platelets were isolated as described earlier.\textsuperscript{23} Neutrophils were isolated by negative selection from bone marrow as described earlier.\textsuperscript{24} In short, bone marrow was isolated from CCL3−/− and WT animals by flushing the femurs and tibias. Cells were incubated at 4°C with erythrocyte lysis buffer (155 mmol/L NH4CL in 10 mmol/L Tris/HCl, pH 7.2) for 5 minutes to remove residual erythrocytes. After washing, cell suspensions were incubated with an antibody cocktail containing α-CD5, CD45R, CD49b, CD117, Fc/E80, and TER119 (4°C, 10 minutes under constant shaking). After washing, cells were incubated with α-biotin microbeads (Miltenyi, Leiden, the Netherlands, 4°C, 10 minutes under constant shaking). Subsequently, neutrophils were isolated by magnetic bead isolation (magnetic-activated cell sorting LS column, Miltenyi). We obtained neutrophils at ≥90% purity, as validated by flow cytometry and histology (Figure IIIA in the online-only Data Supplement). Subsequently, cells were stimulated with LPS (1μg/mL Salmonella minnesota R595 (Re) for 16 hours) and supernatant was collected for CCL3 measurements.

ELISA
Levels of CCL3 were determined by sandwich ELISA (Biosource, Carlsbad, CA), according to the manufacturer’s protocol. Levels of monocyte chemotactic protein-1, TNFα, and CXC chemokine ligand 1 (CXCL1) in peritoneal lavage fluid, 24 hours post-LPS injection, were determined by sandwich ELISA (Ebioscience, San Diego, CA; BD Biosciences, San Diego, CA; and Biosource, respectively), according to the manufacturer’s protocol.

Cyclophosphamide-Induced Neutropenia
Female CCL3−/− mice or WT control received an intraperitoneal injection of cyclophosphamide (6 mg/mouse) to deplete blood neutrophils, as described previously.\textsuperscript{25,26} Blood samples were taken via the tail vein
regularly and blood cell differentiation was determined on a Sysmex differential cell counter (Goffin Meyvis, Etten-Leur, the Netherlands).

**Apoptosis Assay**

Neutrophils were isolated from bone marrow and stained with Ly6G/Gr-1 (clone 1A8, BD biosciences) for neutrophil gating. Apoptotic cells were detected by Annexin-V/propidium iodide (PI) staining kit, and according to manufacturers protocol (Ebioscience). Cells were analyzed by flow cytometry (FACSCanto, BD biosciences). Annexin-V−propidium iodide-deficient cells were considered apoptotic.

**Static Adhesion**

Neutrophils were isolated from bone marrow as described above and used for static adhesion assays essentially as described previously. In short, 5x10⁴ WT or CCL3−/− neutrophils were plated in either uncoated wells or wells coated with 5 µg/mL fibronectin or 5 µg/mL collagen (both Roche Diagnostics, n=3). After overnight incubation at 37°C and 5% CO₂, neutrophil adhesion was monitored by manual scoring of the number of adhered cells (average of 3 high-power microscopic fields per sample).

**Flow Cytometry**

Peritoneal leukocytes were harvested by peritoneal cavity lavage with PBS. Crude peripheral blood mononuclear cells and peritoneal leukocytes were incubated at 4°C with phycoerythrin (PE) or allophycocyanin (APC) (Biolegend, Leiden, the Netherlands) and according to manufacturers protocol (BD Biosciences). Cells were analyzed by FACSCanto (BD Biosciences). Fluorescence-activated cell sorting data were analyzed with CELLQuest software (BD Biosciences).

**Statistical Analysis**

Data are expressed as means±SEM. A 2-tailed Student t test was used to compare individual groups, whereas multiple groups were compared by 1-way ANOVA and subsequent Newman–Keuls multiple comparisons test. Nonparametric data were analyzed using a Mann–Whitney U test. A level of P<0.05 was considered significant.

**Results**

Temporal expression analysis of atherosclerotic lesions in LDLr−/− mice showed a persistent upregulation of CCL3 (from 2 weeks after collar placement). This expression pattern shows similarities with the expression profile of macrophage marker CD68 and the neutrophil-related genes CXCR2, CXCL1 (Figure 1A–1C), colony stimulating factor 3 receptor, and neutrophil gelatinase-associated lipocalin/lipocalin-2 (Figure I in the online-only Data Supplement). This expression profile suggests that CCL3 may be involved in the critical recruitment of inflammatory cells, such as monocytes and neutrophils, to atherosclerotic lesion sites.

To assess effects of hematopoietic CCL3 deficiency on leukocyte migration and activation, as well as on atherogenesis, we reconstituted LDLr−/− mice with CCL3−/− bone marrow, or WT littermate bone marrow as a control. CCL3 deficiency did not influence body or total cholesterol levels during the course of the experiment (data not shown). Hematopoietic CCL3 deficiency led to lowered plasma CCL3 under basal conditions, albeit that this effect did not reach significance (2.4±0.8 pg/mL in WT versus 0.9±0.6 pg/mL in CCL3−/− chimeras; P=0.1; Figure 1D). However, CCL3 deficiency sharply affected plasma CCL3 levels under inflammatory conditions. Circulating CCL3 levels were robustly increased, 24 hours after LPS treatment, in WT but not in CCL3−/− chimeras (14.7±0.4 pg/mL in control compared with 2.1±1.0 pg/mL in CCL3−/− chimeras; P=0.00005; Figure 1D), establishing that hematopoietic cells are primarily responsible for LPS-induced CCL3 release. To further clarify the specific cellular source of CCL3, we isolated macrophages, neutrophils, and platelets. On stimulation with LPS, both macrophages and neutrophils release CCL3 (Figure 1E and 1F). Macrophages appear the major source of CCL3 as they release approximately 10 times more CCL3 compared with neutrophils. The neutrophil chemoattractant cytokine CXCL1 slightly induced CCL3 release from neutrophils (P=0.13; Figure 1G). We were unable to detect any platelet-derived CCL3 (data not shown).

Next, we assessed the effect of CCL3 deficiency on atherosclerotic lesion formation. Lesion development in the aortic root of CCL3−/− chimeras was reduced by a significant 31% (135±77×10³ μm² in CCL3−/− chimeras compared with 198±51×10³ μm² in controls; P=0.04; Figure 2A, with representative pictures). The intimal anti-macrophage + macrophage antibody-2a area was slightly reduced in CCL3−/− chimeras (29.6±6.4×10³ μm² in CCL3−/− compared with 39.9±6.6×10³ μm² in controls, n.s.; Figure 2B), whereas relative macrophage content was not different between groups (19.3±2.6% in controls versus 22.9±3.0% in CCL3−/− chimeras; Figure 2C), suggesting that CCL3 is not critically involved in monocyte/macrophage accumulation and proliferation in the atherosclerotic plaque. Interestingly, plaque neutrophil numbers (2.9±0.8 in WT compared with 0.9±0.3/mm² intimal tissue in CCL3−/− chimeras plagues; P=0.04; Figure 2D), as well as the number of adherent neutrophils (5.9±0.8 in WT compared with 2.5±0.9/mm² intimal tissue in CCL3−/− chimeras plagues; P=0.01; Figure 2E) were significantly reduced in CCL3−/− chimeras. CD3+ T-cell numbers were not influenced by CCL3 deficiency (2.9±1.2 T-cells/mm² in controls and 2.6±1.5 T-cells/mm² in CCL3−/− chimeras; Figure 2F). As measure of lesion-progression stage, intimal collagen content and necrotic core size was determined. The relative collagen content in CCL3−/− plaques was not influenced by CCL3 deficiency (7.5±1.4 in WT compared with 5.7±1.0% in CCL3−/− chimeras; Figure 2G), nor was the area of necrosis (5.9±1.5 in WT compared with 6.7±1.1% in CCL3−/− chimeras; Figure 2H). Furthermore, TUNEL staining did not reveal any differences in the number of apoptotic cells in the plaque (Figure 2I). These latter observations suggest that CCL3 deficiency interferes with...
It has been shown that neutrophils require priming by inflammatory stimuli, such as TNFα, to become responsive to CCL3. To establish if the effects we observed on neutrophils are not reflected by differences in inflammatory status of the CCL3+/− chimeras, we measured TNFα, monocyte chemotactic protein-1, and CXCL1 release in the peritoneum after LPS challenge. Monocyte chemotactic protein-1 response to LPS was similar in WT and CCL3−/− chimeras (Table). Likewise, TNFα content of peritoneal fluid, which was already quite high at baseline, was induced to a similar extent in both groups on LPS challenge (Table). Both basal and LPS stimulated levels of CXCL1, a major neutrophil chemoattractant, did not differ between WT and CCL3−/− chimeras either (Table). Apparently, the observed neutrophil effects are not attributable to a difference in inflammatory status, but are directly related to CCL3 deficiency.

The decreased neutrophil numbers may result from accelerated turnover, or from impaired differentiation and stromal egress of neutrophils. To investigate this, CCL3+/− or WT animals on chow diet were treated with a single injection of cyclophosphamide and neutrophil elimination/repopulation kinetic was monitored for 10 days. Basal white blood cell number and cellular composition was not different between WT controls and CCL3−/− mice (data not shown). Neutrophil kinetics on cyclophosphamide are represented in Figure 4A. Neutrophil repopulation, initiated 5 days postinjection, was similar between CCL3+/− and WT controls (Figure 4B), suggesting that neutrophil release from the bone marrow was not altered by CCL3 deficiency. In contrast, CCL3-deficient neutrophils were slightly more sensitive to cyclophosphamide treatment, as their decay was considerably faster in CCL3−/− mice (1.9±0.4 days in WT compared with 1.0±0.1 days in CCL3−/−; P=0.11; Figure 4C). Interestingly, the accelerated decay was associated with higher activation status, as indicated by enhanced surface expression of CD1828 and decreased expression of CD62L29 compared with WT neutrophils (18.0±1.7 in CCL3−/− compared with 8.5±2.3% CD18+ CD62L− Gr1hi CD71− neutrophils in WT; P=0.02; Figure 4D), which fits with the notion that nonactivated have a decreased neutrophil half life of 2 to 4 days, whereas activated neutrophils survive for only 1 to 2 days. Next, we assessed if the decreased half life of CCL3−/− neutrophils was because of enhanced apoptosis of these cells. Neutrophils were isolated from bone marrow of
WT and CCL3−/− animals by negative selection.24 Before isolation animals received WTD for 2 weeks to assess the effects of WTD on neutrophil apoptosis. Neutrophils were labeled with Annexin-V and propidium iodide, and assessed by flow cytometry. Annexin-V–positive, propidium iodide-negative neutrophils were regarded apoptotic neutrophils. The CCL3−/− neutrophil population was significantly enriched in apoptotic cells (31.6±3.7% in CCL3−/− compared with 13.4±2.2% in WT neutrophils; \( P = 0.002 \); Figure 4E). Interestingly, WTD feeding significantly induced neutrophil apoptosis in WT neutrophils (13.4±2.2% on chow versus 30.5±3.1 on WTD; \( P = 0.001 \); Figure 4E) and even further enhanced apoptosis of CCL3−/− neutrophils (31.6±3.7% on chow versus 47.7±1.2% on WTD; \( P = 0.004 \); Figure 4E).

Yet, we wondered if this increased turnover is the sole reason for the decreased accumulation of neutrophils in the atherosclerotic lesion. The fact that the amount of apoptosis and necrotic cores size were not altered in CCL3−/− chimeras suggests that other mechanisms were involved as well. These mechanisms may likely involve neutrophil adhesion and mobility. First, we assessed adhesion properties of CCL3−/− neutrophils to different matrix proteins. CCL3−/− neutrophils are clearly impaired in their adhesion to collagen (34.8±0.6 adherent CCL3−/− neutrophils versus 62.7±2.3 adherent WT neutrophils; \( P = 0.0003 \); Figure 5A) and to fibronectin (44.1±3.5 adherent CCL3−/− neutrophils versus 60.9±2.0 adherent WT neutrophils; \( P = 0.02 \); Figure 5A). A next logical step was to investigate if the mobility of CCL3−/− neutrophils was altered as well. First, we measured the surface expression of CCR1 and CCR5, the receptors that neutrophils employ to enter the atherosclerotic lesion.7 Again, we also

Figure 3. Circulating neutrophil numbers (A) and neutrophil percentages (B) were decreased. White bars represent wild-type (WT) chimeras and black bars CCL3−/− chimeras. * \( P < 0.01 \) compared with WT chimeras (n=10).
assessed the effects of WTD on chemokine receptor expression. White blood cells were obtained by lysis of red blood cells, and neutrophils were identified as CD11b+ and Ly6G/Gr-1 high-expressing cells (Figure 5B). CCL3−/− neutrophils express more CCR1 (mean fluorescence intensity: 642±46 on CCL3−/− neutrophils compared with 432±29 on WT neutrophils; \( P = 0.003 \); Figure 5C and 2.3±0.2% on CCL3−/− neutrophils compared with 1.5±0.2% on WT neutrophils; \( P = 0.03 \); Figure 5E) and CCR5 (MFI: 1812±172 on CCL3−/− neutrophils compared with 1169±60 on WT neutrophils; \( P = 0.004 \); Figure 5D and 22.2±3.1% on CCL3−/− neutrophils compared with 8.7±0.6% on WT neutrophils; \( P = 0.001 \); Figure 5F) on their cell surface. This observation may be directly related to the absence of CCL3 binding and signaling in these cells. Interestingly, CCR5 expression (MFI: 1169±60 on chow versus 1538±100 on WTD; \( P = 0.01 \); Figure 5D and 8.7±0.6% on chow compared with 14.7±1.4% on WTD; \( P = 0.003 \); Figure 5F) is upregulated on WTD feeding in WT neutrophils, but not in CCL3−/− neutrophils. CCR1 expression is unaltered by WTD feeding (Figure 5C). This suggests that CCR5 may play a more pronounced role in atherosclerosis compared with CCR1. To exclude a more general effect on chemokine receptor expression in CCL3−/− neutrophils, we also measured the expression of CXCR2, which was unaltered on these cells and unaffected by WTD feeding (Figure IIIB in the online-only Data Supplement). CXCR4 expression was unaltered on CCL3−/− neutrophils, but did slightly decrease on WTD feeding (Figure IIIC in the online-only Data Supplement) To establish if the CCL3−/− neutrophil have altered chemotactic responses, we measured in vivo neutrophil influx toward a gradient of CXCL1 in WT and CCL3−/− mice. Two hours after intraperitoneal injection of CXCL1, white blood cells and peritoneal leukocytes were isolated and analyzed for neutrophil content by flow cytometry. Circulating neutrophil numbers were similar between WT and CCL3−/− animals (Figure 5H). CXCL1 injections robustly induced neutrophil migration toward the peritoneum of control animals (Figure IIID in the online-only Data Supplement). Despite our observation that CCL3−/− neutrophils express normal levels of CXCR2, peritoneal neutrophil influx was strongly impaired in CCL3−/− animals (20× induction in WT compared with 7.5× induction in CCL3−/−; \( P = 0.003 \); Figure 5I). More detailed analysis showed that chemotactic response to CXCL1 was impaired in CXCR2+ CCR5− neutrophils (Figure 5J) and was even more pronounced in CCR5+ CXCR2− (Figure 5K) neutrophils, whereas the influx of CXCR2− CCR5+ neutrophils was not affected (data not shown).

**Table. Inflammatory Status in WT and CCL3−/− Mice**

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<th>WT</th>
<th>CCL3−/−</th>
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<th></th>
<th>WT</th>
<th>CCL3−/−</th>
<th>( P )-Value</th>
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<td>TNFα (ng/mL)</td>
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<td>0.17±0.06</td>
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LPS indicates lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; and WT, wild-type.

**Discussion**

Chemokine-mediated migration of leukocytes into the vessel wall is an essential step in atherosclerotic lesion formation and progression.\(^{30}\) The CC chemokine CCL3 can interact with chemokine receptors CCR4, CCR1, and CCR5, of which the latter two have been implicated in atherogenesis.\(^{31–35}\) Combined with the upregulated aortic expression during atherogenesis,\(^{13}\) and its potent chemotactic effect on T-cells, macrophages, and neutrophils,\(^{17}\) a role of this chemokine in atherogenesis is conceivable.

**Figure 4.** Kinetics of cyclophosphamide-induced transient neutropenia (A). Repopulation of neutrophils is similar as in CCL3−/− chimeras (B), whereas their elimination is accelerated (C; \( n = 8 \)). CCL3−/− neutrophils are more activated (D, \( n = 4 \)) and have increased apoptosis (E, \( n = 6 \)). Neutrophil apoptosis is further enhanced by western-type diet feeding in both wild-type (WT) and CCL3−/− neutrophils (E). White bars represent WT mice and black bars represent CCL3−/− mice. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) compared with WT neutrophils.
Here, we demonstrate that CCL3 is almost exclusively produced by leukocytes (macrophages and neutrophils) during LPS-elicited inflammatory responses. Moreover, temporal profiling of gene expression during atherosclerotic lesion development revealed a marked upregulation of CCL3 with disease progression, in a pattern similar to macrophages (CD68) and neutrophils (CXCR2/CXCL1). These data suggest a prominent role of leukocyte CCL3 in the inflammatory responses relevant to atherosclerosis. Indeed, atherogenesis in CCL3–/– mice was significantly attenuated, but we did not observe any effects on macrophage or T-cell content. Interestingly, it was demonstrated that deficiency of the CCL3 receptor CCR1 led to accelerated atherosclerosis. Plaques of CCR1-deficient mice contained more macrophages and T-cells, and CCR1+ T-cells produced more interferon-γ. However, functional deficiency of CCR5 was shown to reduce atherosclerotic lesion development, and plaques contained less macrophages and T-cells. In our study, CCL3 deficiency had major impact on neutrophils.

Until recently, the involvement of neutrophils in the pathogenesis of atherosclerosis was, at best, controversial. However, evidence is mounting for an active role of this leukocyte subset in this disease. Neutrophils are potent inflammatory cells acting in a narrow time span, and plaque neutrophils are associated with increased intimal apoptosis and a proinflammatory phenotype. Conceivably, neutrophil accumulation in atherosclerotic lesions can induce plaque destabilization as a result of enhanced inflammation, intimal apoptosis, necrotic core formation, and matrix degradation.

CCL3 has been reported to be able to augment neutrophil chemotaxis induced by the proinflammatory cytokine TNFα in a CCR5-dependent manner. In concurrence with these findings, we show attenuated neutrophil migration to and accumulation into the plaque in hematopoietic CCL3 deficiency. This impaired migration was not because of differences in TNFα production in these mice. Moreover, in vivo neutrophil migration toward CXCL1 was reduced in CCL3–/– mice and involved both CXCR2- and CCR5-expressing neutrophils. Exactly how CCL3 deficiency influences CXCR2-mediated...
neutrophil migration cannot be fully explained by our data. The complexity of chemokine system (pleiotropic ligand–receptor interactions, scavenging function, mediators of chemokine release) makes it difficult to fully elucidate the interaction between CCL3 and CXCR2 in neutrophils. The influx of neutrophils on CXCL1 in WT mice was more robust for CXCR5- than CXCR2-expressing neutrophils. It must be noted that this difference may be because of abundant presence of CXCR2 neutrophils under basal conditions already. Still, the fact that the observed reduction of influx was most pronounced in CXCR5-expressing neutrophils suggests that it is directly related to the CCL3 deficiency. These observations indicate that CXCL1 can induce the release of CCL3, from resident macrophages or neutrophils, leading to a progressive neutrophil migration. We did not observe differences in influx of CXCR2/CXCR5 double-positive neutrophils. This is likely a reflection of their relatively small (≈2%) contribution to the total peritoneal neutrophil pool. Next to impaired mobility, we also show that CCL3-/- neutrophils displayed reduced adhesion to matrix proteins as compared with WT neutrophils, and these 2 pathways may be the major contributors to impaired neutrophil migration and influx into the atherosclerotic lesion.

A different, more indirect mechanism by which CCL3 deficiency may have led to augmented neutrophil recruitment relates to its surprising effect on neutrophil turnover. Apoptosis of neutrophils is regarded as a protective measure to dampen acute inflammatory responses and prevent unwanted tissue damage. Surprisingly, although CCL3 has not yet been implicated in neutrophil turnover, we observed a clear effect of CCL3 deficiency on neutrophil elimination kinetics. The half life of CCL3-deficient neutrophils was almost 2-fold decreased, and apoptosis rate and activation status were increased. With age, neutrophils upregulate CXCR4 on their cell surface to enable re-entry into bone marrow stroma. Blockade of CXCR4 augments neutrophil homing to the bone marrow, whereas homing of CXCR4-deficient neutrophils to the bone marrow is reduced. These observations clearly suggest that CXCR4-mediated neutrophil kinetics may be affected in our CCL3-/- chimeras. However, despite the accelerated decay of CCL3-/- neutrophils, we did not observe differences on cell surface expression of CXCR4 on CCL3-/- neutrophils. Suggestive that the elimination of neutrophils in our model is not confined to the bone marrow, which concurs with the observation that clearance of CXCR4-/- neutrophils is comparable with WT neutrophils. Repopulation of neutrophils was not influenced by CCL3 deficiency, implying that neutrophil maturation and stromal release per se are not influenced.

Taken together, our data clearly establish that macrophages, and to a lesser extent neutrophils, are the primary source of CCL3 under conditions of inflammation and identifies CCL3 as a new actor in neutrophil turnover and function. Furthermore, leukocyte CCL3 deficiency results in attenuated plaque development by altering neutrophil half life and reducing neutrophil adhesion to, and accumulation in, the plaque.

Acknowledgments
The authors gratefully acknowledge M.M. Westra, and J. Krom for excellent technical assistance.

References


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Arterioscler Thromb Vasc Biol. 2013;33:e75-e83; originally published online January 3, 2013; doi: 10.1161/ATVBAHA.112.300857

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Temporal expression of neutrophil related genes. Temporal profiling in collar induced carotid artery plaques showed induction of colony stimulating factor 3 receptor (A) and neutrophil gelatinase-associated lipocalin /Lipocalin-2 (B) in a pattern similar to CCL3. *p<0.05, **p<0.01, ***p<0.001 compared to time point 0 (n=3)
Supplemental figure II

**White blood cell and monocyte numbers.** Total number of white blood cells (A), monocytes numbers (B) and monocyte percentages (C) were not different in CCL3−/− mice. White bars represent WT chimeras and black bars CCL3−/− chimeras (n=10).
Supplemental figure III

Neutrophil characterization. Neutrophil isolation by negative selection from bone marrow. Purity assessment by flow cytometry and microscopy (A). Average expression of CXCR2 (B) and CXCR4 (C) per cell is similar on WT and CCL3−/− neutrophils. The expression of CXCR4 is reduced by western type diet feeding (C). White bars represent WT mice and black bars represent CCL3−/− mice. **p<0.01 compared to chow diet (n=5). CXCL1 induced neutrophil influx assessed by flow cytometry. Neutrophils were gated as CD11b+ GR1 high expressing cells (P2) (D).