Identification of therapeutic targets in coronary artery disease: from patient to mice and back

Kraaijeveld, A.O.

CHAPTER 6

Leukocyte specific CCL3 deficiency inhibits atherosclerotic lesion development by attenuation of intimal neutrophil accumulation

Saskia C.A. de Jager
Adriaan O. Kraaijeveld
Ilze Bot
Shalini O'Donnell
Martine Bot
Marijke M. Westra
Josan Krom
Peter J. van Santbrink
Theo J.C. van Berkel
Erik A.L. Biessen

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ABSTRACT

Introduction: Migration of leukocytes into the vessel wall is an essential step in atherosclerotic lesion formation and progression, and chemokines are regarded as key regulators of this process. Macrophage Inflammatory Protein 1 alpha (MIP1α or CCL3) can bind and signal via chemokine receptors such as CCR1, and CCR5, which were previously shown to be implicated in atherogenesis. In this study we aimed to elucidate the role of leukocyte derived CCL3 in atherogenesis.

Methods and results: Irradiated LDLr$^{-/-}$ mice, reconstituted with CCL3$^{-/-}$ or littermate bone marrow showed markedly reduced CCL3 response to LPS treatment ($P<0.001$), establishing the critical relevance of leukocytes as source of CCL3. Lesion formation in the aortic sinus in CCL3$^{-/-}$ chimeras after 12 weeks of western type diet feeding was greatly impaired ($P<0.05$). While collagen, macrophage and T cell content of plaques of CCL3$^{-/-}$ chimeras were essentially similar to that of littermate controls, neutrophil adhesion to and presence in plaques was significantly attenuated. Under non inflammatory conditions circulating neutrophil numbers did not differ between WT and CCL3$^{-/-}$ mice, whereas they were markedly decreased in CCL3$^{-/-}$ mice upon LPS treatment. Kinetic analysis of neutrophils after cyclosphosphamide treatment showed accelerated depletion in CCL3$^{-/-}$ mice pointing to a reduced neutrophil half life. CCL3$^{-/-}$ neutrophils were less responsive towards the neutrophil chemo-attractant KC.

Conclusion: Our data indicate that under conditions of acute inflammation leukocyte derived CCL3 can induce neutrophil chemotaxis towards the atherosclerotic plaque, thereby accelerating lesion formation.

INTRODUCTION

Atherosclerosis is a progressive multi-factorial disease of middle and large sized arteries. In recent years it has become increasingly clear that atherosclerosis is a lipid storage disorder, with features of chronic inflammation[1, 2]. Migration of leukocytes into the vessel wall is an essential step in atherosclerotic lesion initiation and progression, and chemokines are considered key regulators orchestrating this process[3]. Chemokines are members of the cytokine family of small chemotactic proteins that orchestrate cellular migration responses. Chemokines and their receptors have conventionally been categorized into four families on the basis of the structural arrangement of the N-terminal conserved cysteine residues (CXC, CC, C and CX3C) [4, 5]. Next to their structural classification, chemokines can also be functionally classified as homeostatic or inflammatory.

Macrophage Inflammatory Protein 1 alpha (MIP1α or CCL3) is an inflammatory chemokine of the CC subfamily. It binds and signals via chemokine receptors CCR1, CCR4 and CCR5. MIP1α and its co-ordinately regulated partner Macrophage Inflammatory Protein 1 beta (MIP1β) are known to form heterodimers which interact with the cognate receptors [6]. The major source of CCL3 appears to be the macrophage, although recent evidence also points to the release of this chemokines, in analogy to CCL2 [7], by activated platelets [8]**.** Moreover, platelet derived interleukin-7 (IL-7) was seen to induce CCL3 production by monocytes, which in turn can stimulate IL-7 release from platelets, amplifying the inflammatory response [9]**.** CCL3 release can induce chemotaxis of different leukocyte subsets including monocytes/macrophages and T lymphocytes. It has been recently shown that CCL3 can also be a product of neutrophils [11] and mast cells [10-12]. It is triggered by the Toll Like Receptor 4 ligand LPS and shown to be elevated in patients with high circulating HDL levels [13]. Furthermore exposure of macrophages to VLDL up-regulates CCL3 expression in a dose dependent manner [14], and a similar effect is noticed by angiotensin AT1 receptor activation [15]. In support of this finding, CCL3 expression was significantly increased during atherogenesis in aortas of ApoE$^{-/-}$ mice [16].

Recent clinical studies have proposed CCL3 as a marker of atherosclerosis [17], while we have shown its up-regulation during episodes of acute myocardial ischemia next to its predictive value of future cardiac events [18]. Although collectively these findings suggest an important role in atherosclerosis, no experimental data are available as yet to substantiate such a role. In this study we therefore aimed to establish the role of leukocyte CCL3 in atherogenesis. We show that leukocyte specific CCL3 deficiency attenuates atherosclerotic lesion formation, mainly by inhibition of neutrophil migration to the plaque.

MATERIALS AND METHODS

Animals

LDLr$^{-/-}$ mice were obtained from the local animal breeding facility. Mice were maintained on sterilized regular chow diet (RM3; Special Diet Services, Essex, U.K.). Drinking water was supplied with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymyxin B sulfate) and 6.5 g/L sucrose and was provided ad libitum. Animal experiments were performed at the animal facilities of the Gorlaeus laboratories of the 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 a Western type diet containing 0.25% cholesterol and 15% cacaobutter (Special Diet Services, Sussex, UK) two weeks prior to surgery and throughout the experiment. To determine gene expression levels in (n=20) mouse plaques, atherosclerotic carotid artery lesions were induced by perivascular collar placement as described previously [19]. Mice were anaesthetized by subcutaneous injection of ketamine (60 mg/kg, Eurovet Animal Health, Bladel, the Netherlands), fentanyl citrate and fluanisone (1.26 mg/kg and 2 mg/kg respectively, Janssen Animal Health, Sauderton, UK). From 0 to 8 weeks after collar placement every two weeks a subset of 4 mice was sacrificed. The animals were anaesthetized as described above and perfused through the left cardiac ventricle with PBS and exsanguinated by femoral artery transsection. Subsequently, 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 or three carotids were pooled per sample and 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 M-MuLV reverse transcriptase (RevertAid, MBI Fermentas, Leon-Roth) and used for quantitative analysis of gene expression with an ABI PRISM 7700 Taqman apparatus (Applied Biosystems, Foster City, CA) as described previously [23], using murine hypoxanthine phosphoribosyltransferase (HPRT) and cyclophilin A (CypA) as standard housekeeping genes (Table 1).

Table 1. RT-PCR primer sequences and sources.

Gene	forward primer (5'-3')	reverse primer (5'-3')
CCL3	GCCACATCGAGGGACTCTTCA	GATGGGGGTTGAGGAACGTG
CD36	GTTCTTCCAGCCAATGCCTTT	ATGTCTAGCACACCATAAGATGTACAGTT
CD68	CCTCCACCCTCGCCTAGTC	TTGGGTATAGGATTCGGATTTGA
HPRT	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
CypA	CCATTTCAAGAAGCAGCGTTT	ATTTTGTCTTAACTGGTGGGTCTGT

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 Andrex Smart 225 Röntgen source (YXLON International) with a 6-mm aluminum filter 1 day before transplantation. Bone marrow was isolated from male CCL3$^{-/-}$ or littermates by flushing the femurs and tibias. Irradiated recipients received 0.5x10^{7} bone marrow cells by tail vein injection and were allowed to recover for 6 weeks. Animals were placed on a western type diet containing 0.25% cholesterol and 15% cacao butter (SDS) diet for 12 weeks and subsequently sacrificed. Twenty four hours prior to sacrifice a subset of animals were injected intraperitoneally with lipopolysaccharide (LPS) (*Salmonella minnesota* R595 (Re) (List Biological Laboratories Inc., Campbell, CA)). Plasma levels of CCL3 were determined by sandwich Elisa (Biosource, Carlsbad, CA) according to the manufacturer's protocol.

Histological analysis

Cryostat sections of the aortic root (10 μm) were collected and stained with Oil-red-O. Lesion size was determined in 5 sections of the aortic valve leaflet area. Corresponding sections on separate slides were stained immunohistochemically with an antibody directed against a macrophage specific antigen (MOMA-2, monoclonal rat IgG2b, dilution 1:50; Serotec, Oxford, UK). Goat anti-rat IgG-AP (dilution 1:100; Sigma, St. Louis, MO) was used as secondary antibody and NBT-BCIP (Dako, Glostrup, Denmark) as enzyme substrates. Masson's 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).

Macrophage stimulation

Serum deprived RAW264.7 macrophages were stimulated with 10μg/ml ox-LDL or 1ng/ml LPS for 24 hours. Total RNA was isolated for real time PCR to assess CCL3 expression. Serum deprived RAW 264.7 macrophages were stimulated with recombinant CCL3 (10 or 100ng/ml) for 24 hours. Subsequently [^{3}H]-thymidine (1 μCi/well, specific activity 24 Ci/mmol; Amersham Biosciences, the Netherlands) was added to each well and cells were allowed to proliferate for another 24 hours. Cells were rinsed twice with cold PBS and subsequently lysed with 0.1M NaOH. The amount of [^{3}H]-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R).

Cyclophosphamide induced neutropenia

Female CCL3$^{-/-}$ mice or WT control received an intraperitoneal (i.p) injection of cyclophosphamide (6 mg/mouse) to deplete blood neutrophils as described previously [20, 21]. Blood samples were taken via the tail vein regularly and blood cell differentiation was determined on a Sysmex cell differentiation apparatus (Goffin Meyvis, Etten-Leur, the Netherlands).

In Vivo Chemotaxis

Female CCL3-/- mice or WT control received an i.p. injection of 500ng recombinant KC (Peprotech, Rocky Hill, NJ) or PBS. Two hours later blood and peritoneal cells were isolated and analyzed for neutrophil composition by flow cytometry.

Flow Cytometry

Peritoneal leukocytes were harvested by peritoneal cavity lavage with PBS. Crude peripheral blood mononuclear cells (PBMC) and peritoneal leukocytes were incubated at 4°C with erythrocyte lysis buffer (155mM NH_4CL in 10mM Tris/HCL, pH 7.2) for 5 minutes. Cells were centrifuged for 5 minutes at 1500 rpm, resuspended in lysis buffer to remove residual erythrocytes. Cells were washed twice with PBS. Cell suspensions were incubated with 1% normal mouse serum in PBS and stained for the surface markers CD11b, GR1 and CD71 (eBioscience, San Diego, CA) at a concentration of 0.25 µg Ab/200,000 cells. Subsequently cells were subjected to flow cytrometric analysis (FACSCalibur, BD Biosciences, San Diego, CA). FACS data were analyzed with CELLQuest software (BD Biosciences).

Statistical analysis

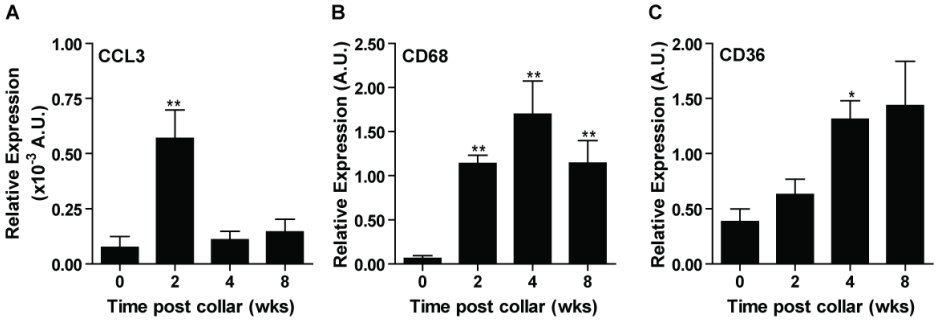
Data are expressed as mean ± SEM. A 2-tailed Student's t-test was used to compare individual groups, while multiple groups were compared with a one-way ANOVA and a subsequent Student-Newman-Keuls multiple comparisons test. Non-parametric data were analyzed using a Mann-Whitney U test. A level of $P<0.05$ was considered significant.

RESULTS

Temporal expression analysis of atherosclerotic lesions in LDLr-/- mice showed a clear-cut, transient up-regulation of CCL3 in initial plaques (2 weeks after collar placement). At more advanced stages of lesion progression CCL3 is returning to its original level. This expression is initially accompanied by increased expression of macrophage marker CD68 of which its levels remain high at later time points. The expression of CD36 is somewhat delayed as compared to CD68 and CCL3 (Figure 1). The expression profiles suggest that CCL3 may be involved in the initial recruitment of inflammatory cells to atherosclerotic lesion sites. *In vitro* exposure of RAW 264.7 macrophages to ox-LDL leads to a moderate induction of CCL3 expression, while the TLR4 ligand LPS strongly induces CCL3 at mRNA level (Figure 2).

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. CCL3 deficiency did not influence body weight or total cholesterol levels during the course of the experiment (data not shown). Plasma CCL3 levels were not significantly different between CCL3-/- chimeras

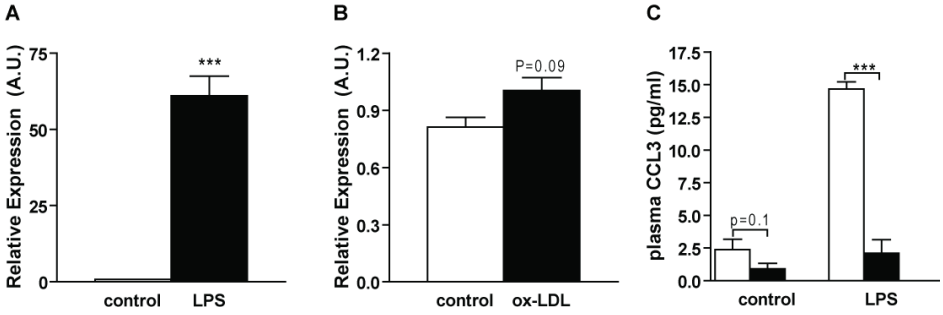
Figure 1.



Temporal profiling of CCL3 expression in collar induced carotid artery plaques shows increased CCL3 production 2 weeks after collar placement (A). Rapid and steady CD68 induction is observed (B), while induction of CD36 is somewhat delayed (C). **P<0.01 compared to baseline (t=0).

and littermate controls (2.4 ± 0.8 pg/ml in WT vs. 0.9 ± 0.6 pg/ml in CCL3$^{-/-}$ chimeras; p = 0.1, Figure 2C). The CCL3 deficient phenotype was much more pronounced after *in vivo* treatment with LPS. Circulating CCL3 levels 24h after LPS treatment were robustly increased in WT but not in CCL3-/- chimeras (14.7 ± 0.4 pg/ml in control compared to 2.1 ± 1.0 pg/ml in CCL3$^{-/-}$ chimeras; p=0.00005, Figure 3A).

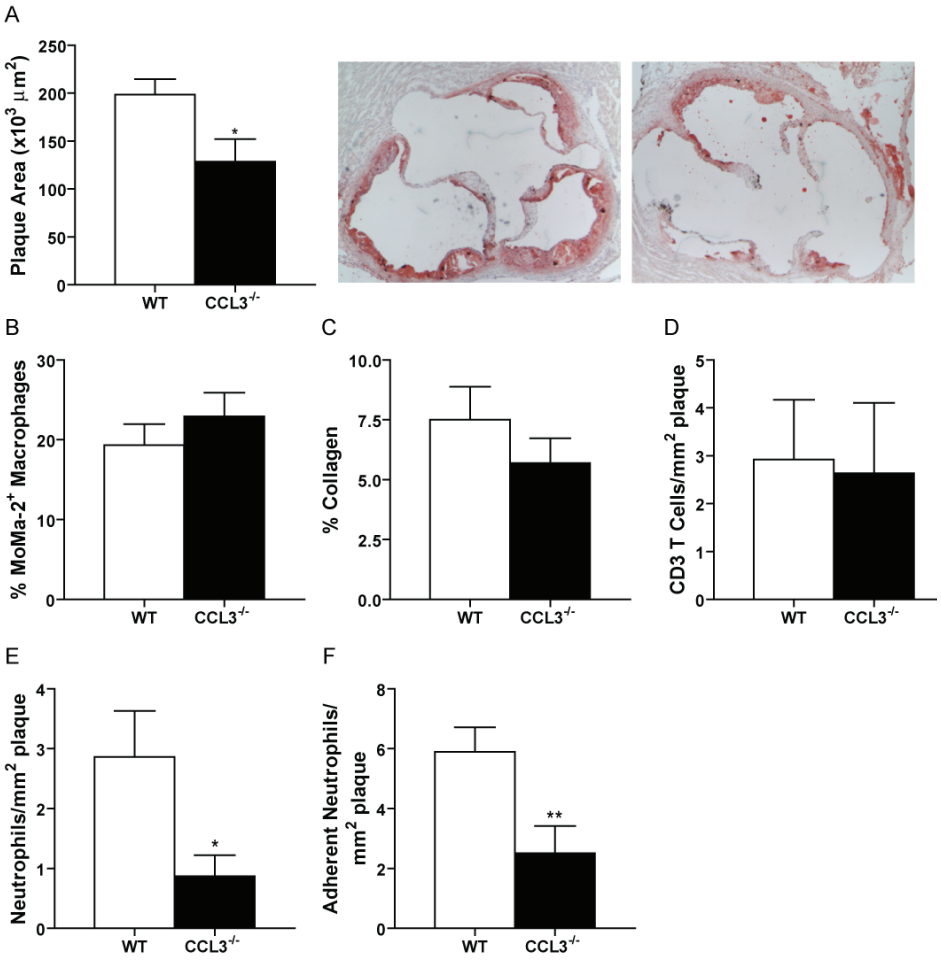
Figure 2.



CCL3 expression in macrophages is strongly up-regulated upon LPS (50 ng/ml) but not ox-LDL (10 ug/ml) stimulation (A,B). LPS induced CCL3 response in vivo is ablated in CCL3-/- chimeras (C, black bars). ***P<0.001

Lesion development in the aortic root of CCL3$^{-/-}$ chimeras was reduced by a significant 31% (135.1 ± 76.5x10^3 μm^2 in CCL3$^{-/-}$ compared to 198.4 ± 51.4x10^3 μm^2 in controls; P = 0.04, Figure 4A). The percentage of intimal MoMa-2$^+$ macrophages was not different between groups (19.3 ± 2.6% in controls vs. 22.9 ± 3.0% in CCL3$^{-/-}$, Figure 3B), suggesting that CCL3 alone may not be very critical in macrophage accumulation and proliferation in the atherosclerotic plaque. CD3$^+$ T-cell numbers were not influenced by CCL3 deficiency (2.9 ± 1.2 T-cells/mm^2 plaque in controls and 2.6 ± 1.5 T-cells/mm^2 plaque in CCL3-/-, Figure 3D). In contrast, the amount of plaque neutrophils (7.0 ± 0.7 in WT compared to 2.9 ± 0.8/mm^2 intimal tissue in CCL3$^{-/-}$ plaques; p=0.001, Figure 3E), as well as neutrophil adherence were significantly reduced in CCL3$^{-/-}$ plaques (Figure

Figure 3.

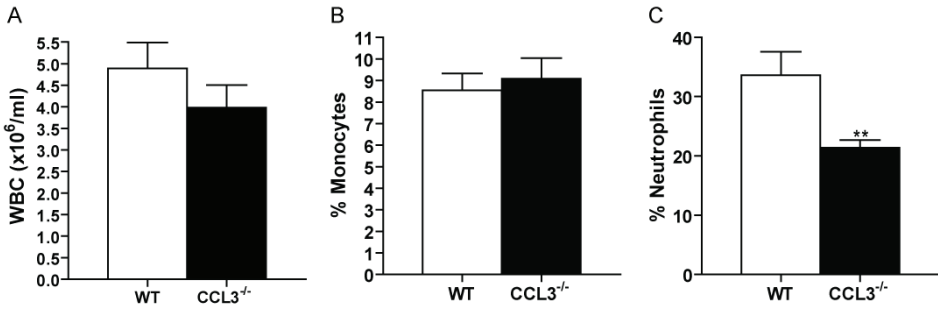


Atherosclerotic lesions are significantly smaller in CCL3$^{-/-}$ chimeras compared to WT controls (A with representative pictures). Macrophages (B), collagen (C) and T-cell content (D) is similar between WT and CCL3$^{-/-}$ chimeras. Neutrophil influx (E) and adhesion (F) is significantly attenuated in CCL3$^{-/-}$ chimeras. *P<0.05, **P<0.01. For color figure see page: 223

3F). As a measure of lesion progression stage, intimal collagen deposition was determined. The percentage of collagen in CCL3$^{-/-}$ plaques was not influenced by CCL3 deficiency (7.5 ± 1.4 in WT compared to 5.7 ± 1.0% in CCL3$^{-/-}$ chimeras, Figure 3C).

CCL3 deficiency did not influence the total number of circulating white blood cells in WT and CCL3$^{-/-}$ transplanted animals (4.4 ± 0.7 in WT vs. 3.9 ± 0.6x10^6 cells/ml in CCL3$^{-/-}$, Figure 4A) and the number of circulating monocytes was not affected by CCL3 deficiency as well (7.7 ± 1.1 in WT vs. 8.9 ± 1.0% in CCL3$^{-/-}$ chimeras, Figure 4B). Interestingly, the percentage of circulating

Figure 4.



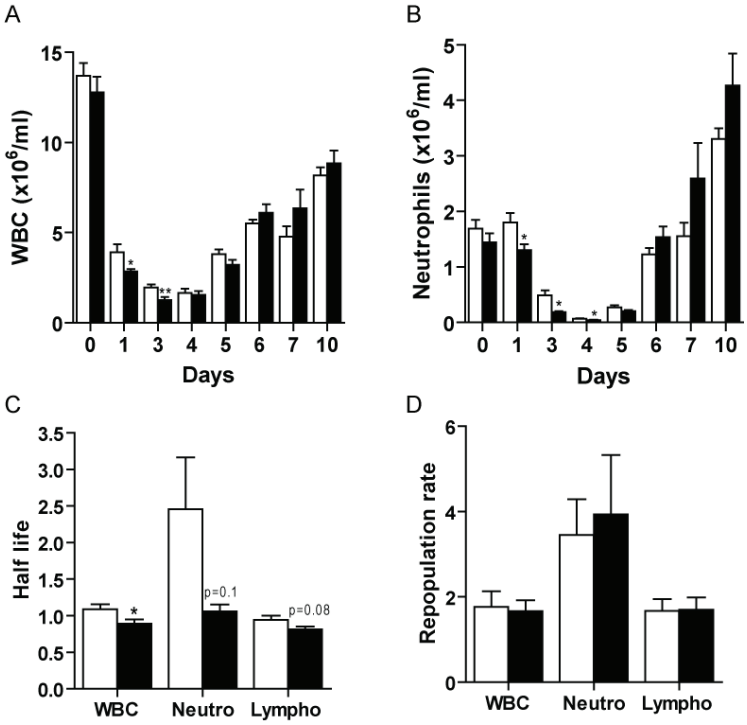
Total number of white blood cells (A) and monocytes (B) is not different in CCL3-/- mice, whereas neutrophil numbers (C) are significantly decreased. **P<0.01, ***P<0.001

neutrophils was significantly decreased in CCL3$^{-/-}$ chimeras (35.3 ± 3.9 in WT vs. 23.6 ± 2.5% in CCL3$^{-/-}$ chimeras; p=0.02, Figure 4C).

The decreased neutrophil numbers may result from a reduced half life or an impaired differentiation and stromal egress of neutrophils. To investigate this, animals were treated with a single injection of cyclophosphamide and the neutrophil elimination/repopulation kinetics was monitored for 10 days. Basal white blood cell number and cellular composition was not different between WT controls and CCL3$^{-/-}$ mice. CCL3 deficient cells were slightly more sensitive to cyclophosphamide treatment (Figure 5A,B) as white blood cell half life was significantly enhanced in CCL3$^{-/-}$ mice compared to WT (1.09 ± 0.07 days in WT compared to 0.89 ± 0.06 days in CCL3$^{-/-}$; p=0.04, Figure 5C) and appeared equally distributed over the neutrophil and lymphocyte subset (Figure 5C). Thus CCL3 deficient mice show a decreased neutrophil half life which concurs with the reduced numbers of circulating and plaque neutrophils in this strain. Repopulation of cells initiated 5 days post injection was similar between CCL3$^{-/-}$ and WT controls (Figure 5D).

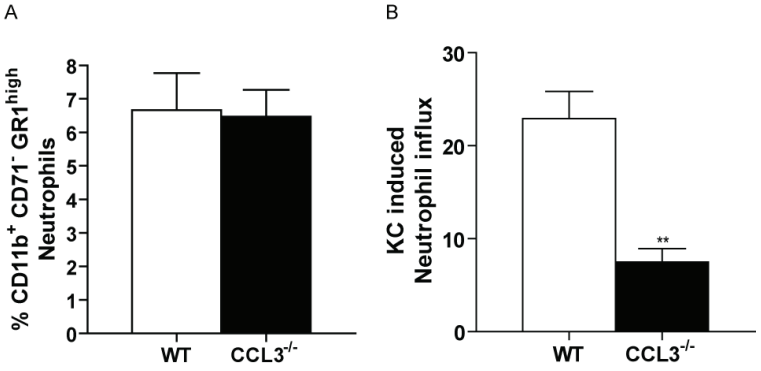
Next, we assessed the chemotactic response of WT and CCL3$^{-/-}$ neutrophils towards a gradient of the major chemokine in neutrophil recruitment, KC (murine IL8 analogue). Two hours after i.p. injection of KC, WBCs and peritoneal leukocytes were isolated and analyzed for neutrophil content. Circulating neutrophil numbers were similar between WT and CCL3$^{-/-}$ animals (6.1 ± 1.0 in WT compared to 5.3 ± 1.0 in CCL3$^{-/-}$, Figure 6A). Surprisingly, given the similar circulating neutrophil numbers, CCL3$^{-/-}$ animals had slightly enhanced neutrophil numbers in the peritoneum under normal conditions (0.6 ± 0.5% in WT compared to 1.4 ± 0.07, p=0.2). KC injections robustly induced neutrophil migration towards the peritoneum of control animals. Peritoneal neutrophil counts after KC injections in CCL3$^{-/-}$ animals were only marginally lower compared to WT animals (12.3 ± 0.4 in controls compared to 10.2 ± 1.9 in CCL3$^{-/-}$ animals, data not shown). However, the induction of neutrophil influx was decreased in CCL3$^{-/-}$ animals (20x induction

Figure 5.



Kinetics of cyclophosphamide induced transient leukopenia (A) and neutropenia (B) in control (white bars) and CCL3$^{-/-}$ mice (black bars). Elimination of neutrophils is accelerated in CCL3$^{-/-}$ chimeras (C), while repopulation is similar (D). *P<0.05, **P<0.01

Figure 6.



Intra-peritoneal injection of KC does not affect circulating CD11b^{+} CD71^{-} Gr1^{high} neutrophil numbers in WT and CCL3$^{-/-}$ mice (A). KC elicited induction of neutrophil influx to the peritoneal cavity is »2.5 times lower in CCL3$^{-/-}$ mice compared to WT mice (B). **P=0.003

in WT compared to 7.5x induction in CCL3$^{-/-}$, p=0.003; Figure 6B), suggestive of impaired chemotaxis of CCL3$^{-/-}$ neutrophils under conditions of inflammation.

DISCUSSION

Chemokine mediated migration of leukocytes into the vessel wall is an essential step in atherosclerotic lesion formation and progression [3]. The CC chemokine CCL3 can interact with chemokine receptors CCR4, CCR1 and CCR5, of which the latter two have been implicated in atherogenesis. Combined with the up-regulated aortic expression during atherogenesis [16], and its potent chemotactic effect on T-cells, macrophages and neutrophil-released TNF-α [11], a role of CCL3 in atherogenesis is conceivable. Here we show that leukocytes are the primary source of CCL3 under conditions of inflammation and that leukocyte CCL3 deficiency attenuates plaque development by altering neutrophil half life and reducing neutrophil accumulation.

In vitro experiments clearly showed that activated macrophages are a rich source of CCL3, which is in concurrence with earlier data [22]. Moreover, circulating baseline levels of CCL3 in the circulation were seen to be only partly of leukocyte origin but almost exclusively produced by leukocytes during LPS elicited inflammatory responses [23, 24]. Expression profiles of atherosclerotic lesion development revealed that CCL3 is mainly up-regulated during early lesion progression, suggesting that CCL3 is involved in plaque initiation [16]. Atherogenesis in $CCL3^{-/-}$ mice was significantly attenuated, but no effects on macrophage or T-cell content were apparent. Interestingly, hematopoietic and systemic deficiency of one of the CCL3 receptors, CCR1, has been shown to lead to accelerated atherosclerosis [25, 26]. CCR1 deficient plaques contained more macrophages and T-cells and $CCR1^{-/-}$ T-cells produced more IFNγ [25]. Conversely, functional deficiency of CCR5, either in the hematopoietic lineage or systemically, was shown to reduce atherosclerotic lesion development and plaques contained less macrophages and T-cells [26, 27]. Antagonism of CCR5 by use of Met-RANTES similarly attenuated atherosclerosis development, macrophage and T-cell content. Furthermore, Met-Rantes treatment resulted in lower expression levels of CCR5, but not of its ligand CCL3 [28]. CCL3 was shown to have a higher binding affinity for CCR5 [29, 30], suggestive that CCR5 mediated effects are primary during a chronic low rate inflammation, while acute substantial inflammation might exert effects via CCR1 signalling. The phenotypic change seen in hematopoietic CCL3 deficiency seems to be more consistent with that of impaired CCR5 function, albeit that we did not see any noticeable effects on plaque macrophage content. This indicates that, although CCL3 might influence inflammatory cell migration, it is not crucial in monocyte or T-cell migration towards the plaque.

Neutrophils were, until recently, not implicated in the pathogenesis of atherosclerosis. However, more and more data support an active role of this subset of white blood cells. Naruka et al. showed plaque neutrophil infiltrates to be associated with acute coronary events [31]. Experimental support came from van Leeuwen et al., showing the abundant presence of neutrophils in advanced mouse plaques [32], and from a collaborative expansion after blockage of CXCR4 [33]. Plaque neutrophils are potent inflammatory cells acting in a narrow time span. Neutrophils are associated with increased intimal apoptosis and a pro-inflammatory phenotype [33]. Conceivably, neutrophil accumulation in atherosclerotic lesions can induce plaque destabilization as a result of enhanced inflammation, necrotic core formation as a consequence of oxidative injury

and matrix degradation by release of neutrophil elastases. CCL3 has been reported to be able to augment neutrophil chemotaxis, induced by the pro-inflammatory cytokine TNFα in a CCR5 dependent manner [11]. In concurrence with these findings, we show attenuated neutrophil migration to and diapedesis into the plaque in hematopoietic CCL3 deficiency. Moreover, *in vivo* neutrophil migration towards KC was reduced in CCL3$^{-/-}$ mice. This indicates that IL-8, similar to TNFα, can induce CCL3 mediated neutrophil migratioAnother intriguing option is that CCL3 affects neutrophil homeostasis. During inflammation, circulating neutrophil numbers were significantly lower in CCL3$^{-/-}$ mice, which fits well with the notion that apoptosis of neutrophils is regarded as a protective measure to dampen acute inflammatory responses and prevent unwanted tissue damage [34]. Terminally matured neutrophils therefore show a sharply reduced half life. Moreover, they have impaired migration and degranulation[35, 36]. We observed a clear effect of CCL3-/- on neutrophil elimination kinetics, as the half life of CCL3 deficient neutrophils was decreased. However, repopulation of neutrophils was not influenced by CCL3 deficiency, showing that neutrophil maturation and stromal release per se are not influenced. These data suggest that CCL3$^{-/-}$ neutrophils are more sensitive to cyclophosphamide, and perhaps other pro-apoptotic signals leading to a reduced half life.

Taken together, our data clearly establish a causal role for neutrophils in the development of atherosclerosis. Furthermore, we hypothesize that under inflammatory conditions, leukocyte derived CCL3 can alter neutrophil homeostasis and enhance neutrophil chemotaxis towards the atherosclerotic plaque to accelerate lesion formation.

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