CX3CR1 Deficiency Impairs Dendritic Cell Accumulation in Arterial Intima and Reduces Atherosclerotic Burden

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Objective—Dendritic cells (DCs) have recently been found in atherosclerosis-predisposed regions of arteries and have been proposed to be causal in atherosclerosis. The chemokine receptor CX3CR1 is associated with arterial injury and atherosclerosis. We sought to determine whether a link exists between arterial DC accumulation, CX3CR1, and atherosclerosis.

Methods and Results—Mouse aortas were isolated and subjected to en face immunofluorescence analysis. We found that DCs were located predominantly in the intimal regions of arterial branch points and curvatures. Consistent with the increased accumulation of intimal DCs in aged and ApoE−/− aortas compared with young WT aortas (P=0.004 and 0.05, respectively), the incidence of atherosclerosis was 88.9% for aged WT and 100% for ApoE−/− mice compared with 0% for young WT mice. CX3CR1 was expressed on intimal DCs and DC numbers were decreased in CX3CR1-deficient aortas of young, aged, and ApoE−/− mice (P=0.0008, 0.013, and 0.0099). The reduced DC accumulation in CX3CR1-deficiency was also correlated with decreased atherosclerosis in these animals.

Conclusions—The accumulation of intimal DC increases in aged and ApoE−/− aortas and correlates with the generation of atherosclerosis. CX3CR1-deficiency impairs the accumulation of DC in the aortic wall and markedly reduces the atherosclerotic burden. (Arterioscler Thromb Vasc Biol. 2008;28:243-250)

Key Words: atherosclerosis ■ chemokine and chemokine receptor ■ dendritic cells ■ macrophages ■ animal models of human disease

Atherosclerosis is an inflammatory disease featured with intense immunologic activities. Macrophages and T cells are immune cells found in atherosclerotic plaques. While macrophages uptake oxidized low-density lipoprotein (oxLDL) through scavenger receptors to perform the first line of host defense, antigen-specific T cells influx in atherosclerotic lesions to elicit an adaptive immune response. Although macrophages are capable of presenting antigens to T cells, dendritic cells (DCs) are the only antigen-presenting cell capable of activating the naïve T cell, thereby playing a crucial role in triggering adaptive immunity.

Recently, DCs have been identified in atherosclerotic plaques in patients with atherosclerosis and in animal models of atherosclerosis. DCs have been suggested to participate in the immune response in advanced atheroma by colocalizing with T cells. Interestingly, DCs have also been detected in the arterial intima of healthy young children and in normal wild-type mice, giving rise to the intriguing possibility that preexisting DCs in the arterial wall contribute to the generation of atherosclerosis.

Monocytes are major precursors of vascular macrophages and DCs, and the chemokine receptor CX3CR1 with its ligand CX3CL1 (fractalkine) is a key regulator of monocyte adhesion and migration. CX3CR1 is expressed on monocytes, whereas CX3CL1 is a transmembrane chemokine on activated endothelium. Membrane-tethered CX3CL1 mediates the rapid capture and firm adhesion of monocytes under physiological conditions. CX3CL1 can also be shed by proteolysis to act as a potent chemoattractant. Both human genetic studies and animal models have implicated an important role for CX3CR1 and CX3CL1 in atherosclerosis. In humans, a polymorphism of CX3CR1 coding for a dysfunctional receptor is associated with reduced prevalence of atherosclerosis and coronary artery disease. When fed with a high-fat diet, mice that lack both CX3CR1 and ApoE exhibit a reduction in atherosclerotic lesion formation compared with ApoE-deficient mice. CX3CL1-deficient mice also have reduced atherosclerotic burden in the innominate artery. As demonstrated by defective monocyte recruitment to the vessel wall in response to vascular injury in CX3CR1-deficient mice,
CX3CR1 is a critical factor for monocyte trafficking to the vasculature, thereby a strong candidate for mediating the infiltration and accumulation of vascular DCs.

In this study, we investigated DC arterial accumulation and atherosclerosis during aging in WT and CX3CR1−/− mice. ApoE−/− mice were also used to characterize DCs in atherogenesis and define the mechanism by which CX3CR1 may play a role. Our data demonstrate that CX3CR1 deficiency impairs the accumulation of vascular DCs and markedly reduces the atherosclerotic burden. DCs in atherosclerosis-prone areas were involved in aging and ApoE deficiency.

Materials and Methods

Animal Care and the Generation of CX3CR1 Knockin Mice

CX3CR1−/− mice were backcrossed onto the C57BL/6J background for 12 generations, and age-matched CX3CR1−/−/WT mice generated by crossing CX3CR1−/− mice were generated by crossing CX3CR1−/− with a gene encoding human CX3CR1 fused at 3′ end to green fluorescence protein. SlowFade Antifade Kit were obtained from Molecular Probes (Invitrogen); Tyramide signal amplification kits (TSA, Fluorescein Isothiocyanate-Cy3) were purchased from PerkinElmer. Propidium Iodide, TOTO-3, and a SlowFade Antifade Kit were obtained from Molecular Probes, Propidium Iodide, TOTO-3, and a

Antibodies and Reagents

Biotin-conjugated anti-mouse antibodies targeting CD11c, hamster IgG (BD Biosciences, San Jose, Calif), CD83 (eBioscience, San Diego, Calif) and CD68 (Serotec Inc., Raleigh, NC); anti-mouse CD32/CD16, allophycocyanin (APC)-conjugated anti-mouse B220, Tri-color conjugated anti-mouse CD11b, PE-conjugated anti-mouse DX-5 (BD Biosciences), anti-CX3CL1 (R&D Systems, Minneapolis, Minn), anti-mouse IgG (Sigma-Aldrich, St. Louis, Mo) antibodies and secondary antibody; biotin–anti-goat IgG (Vector Laboratories, Burlingame, Calif) were used. Ribonuclease A and Oil Red O were purchased from Sigma-Aldrich, Propidium Iodide, TOTO-3, and a SlowFade Antifade Kit were obtained from Molecular Probes (Invitrogen); Tyramide signal amplification kits (TSA, Fluorescein Isothiocyanate and Cyanine3) were purchased from PerkinElmer.

Tissue Preparation, Histology, and Morphometry

Anesthetized mice were perfused with ice-cold PBS for 5 minutes, followed by 4% paraformaldehyde in PBS for 10 minutes via cannulation of the left ventricle. Aortic roots and innominate arteries were isolated and embedded in OCT and 10-μm frozen sections were used for analysis. The aortic root was opened longitudinally and pinned to a wax surface. Atherosclerotic plaques were visualized by Oil Red O staining and measured by computerized morphometry (Image J, NIH) using an Olympus BX-60 microscope and an attached Olympus DP-70 microscope digital camera.

En Face Immunofluorescent Microscopy

Surface microscopy was performed as described previously with modifications. Briefly, fresh isolated aortas were further fixed in 4% paraformaldehyde in PBS for 30 minutes at 4°C, and permeabilized with 0.2% Triton X-100 and 0.1 mol/L glycine in PBS for 7 minutes at RT. Single or double immunostaining was performed using Tyramide amplification. After quenching endogenous peroxidase activity with 3% H2O2, tissue segments were incubated with anti-CD32/CD16 FC-block (1:100) and 10 μg/mL mouse IgG. Primary antibody and isotype control IgG were incubated respectively overnight at 4°C. Samples were then incubated with streptavidin-HRP, followed by fluorescein isothiocyanate (FITC)-conjugated Tyramide. For double staining, Cy3-Tyramide was used. After quenching the remaining HRP activity with 3% H2O2, samples were then incubated again with streptavidin-HRP, followed by FITC-conjugated Tyramide. Nuclei were stained with 2 μg/mL propidium iodide or 0.2 μmol/L TOTO-3.

Aortas including ascending, arch, and thoracic regions were collected. The ascending aorta and aortic arch before the bifurcation of carotid artery (designated as aorta AA) as one piece was opened in a highly reproducible manner using the method described by Liyama et al. The remaining aortic arch and thoracic aorta (designated as aorta AT) were opened longitudinally from the upper wall away from the spine. Flattened segments of the aorta were mounted on glass slides with mounting media. En face immunofluorescence images were obtained with a Zeiss 510 Meta Inverted Laser Scanning Confocal microscope equipped with Argon, HeNe, and Infra-red lasers.

Immunohistochemistry

Immunohistochemical analysis was used to identify the expression of CX3C1/L in frozen sections of aortas following a method described previously.

Statistical Analysis

Numerical data presented in text and figure were expressed as mean±SEM. All sections and images subjected to measurement were analyzed by two investigators, one blinded and one unblinded. Student unpaired t test was used to compare average numbers of cells or percentages between experimental groups. In all cases, P≤0.05 was considered significant.

For online supplemental Methods and Figures, please see http://atvb.ahajournals.org.

Results

CX3CR1 Deficiency Reduced Atherosclerotic Burden in ApoE−/− Mice With Normal Chow Diet

CX3CR1 deficiency has been shown to protect mice on a high-fat diet from generating atherosclerosis. However, a high-fat diet is suggested to switch the immune process of atherogenesis in ApoE−/− mice from a Th1 to a Th2-dependent response, which may alter the immune profile, thereby affecting vascular immune cell function. Therefore, to simulate atherogenesis analogous to clinical disease in humans, we analyzed CX3CR1 impact on atherosclerosis in mice with a normal chow diet.

Atherosclerotic plaques were found on the luminal surface of aortas, aortic roots, and the opening of innominate, carotid, and left subclavian arteries. In CX3CR1−/−/ApoE−/− mice, the amount of the atherosclerotic plaques in the aortic root (0.08±0.01, n=9) and luminal surface of aorta (0.41±0.06, n=10) decreased by 33% and 71%, respectively, compared with ApoE−/− mice (0.12±0.01 for aortic root, n=8, P=0.02; 1.39±0.02% for aortic surface, n=12, P=0.00006; Figure 1A and 1B). Lesions in the innominate arteries of CX3CR1−/−/ApoE−/− mice were 61% smaller than lesions in ApoE−/− mice (0.07±0.02, n=9 versus 0.17±0.03, n=10, P=0.007; Figure 1C). These results demonstrate that normal chow diet did not change the overall effect of CX3CR1 deficiency on reducing atherosclerotic burden in ApoE−/− mice.
CX3CR1 Is Involved in the Recruitment of DCs to the Aortic Wall

CX3CR1-expressing monocytes are major precursors for vascular DCs and macrophages. To study the effect of CX3CR1 on DC arterial recruitment, focal accumulation of DCs was characterized by CD11c staining of fresh isolated arteries and analyzed by en face immunoconfocal microscopy. Consistent with previous findings, in wild-type mice with morphologically healthy aortas, the majority of CD11c cells were located immediately beneath the endothelium in the intima. Instead of covering the entire luminal surface, intimal DCs localized preferentially in the inner curvature of the ascending aorta and aortic arch (Figure 2A). CD11c cells also appeared at the ostia of the innominate, carotid, and left subclavian arteries but were rarely found in the descending aorta and the adventitia. These CD11c cells phenotypically resembled immature DCs as they were negative for CD83, a marker for mature DCs (Figure 2B). CD68 was expressed by macrophages and most of DCs (Figure 2C). However, the number of macrophages was small compared with the number of DCs in the wild-type intima.

CX3CR1 is expressed on DCs in small intestine, kidney, and skin. Using a CX3CR1-GFP KI mouse generated in our laboratory, we found that CX3CR1 was coexpressed with CD11c by intimal DCs (Figure 3A and supplemental Figure II) with the same morphology and distribution as those CD11c+ cells in wild-type and ApoE−/− arteries. To test whether CX3CR1 plays a role in recruiting vascular DCs, we first examined the expression of CX3CR1 ligand CX3CL1 in the aorta. CX3CL1 mRNA was expressed low in young wild-type aortas (20 weeks) and upregulated in aged (∼60 weeks) and ApoE−/− aortas (20 weeks) (supplemental Figure III). Immunohistochemical staining of CX3CL1 revealed that CX3CL1 was expressed by endothelial cells lining the luminal surface and atherosclerotic plaques (Figure 3B). Then we compared the abundance of intimal CD11c+ cells in young (20 weeks) and aged (∼60 weeks) CX3CR1−/− mice with their age-matched wild-type controls. The number of intimal CD11c+ cells in CX3CR1−/− mice was decreased by approximately 40% for young mice (541.1 ± 93.6 versus 904 ± 79.5 for WT, *P* = 0.0008) and 41% for aged mice (1295.31 ± 99.22 versus 2196.2 ± 253.98 for WT, *P* = 0.013; Figure 4). Finally, we assessed the number of intimal CD11c+ cells in ApoE−/−/CX3CR1−/− aortas at 10 weeks of age. Again, significantly fewer CD11c+ cells were detected in ApoE−/−/CX3CR1−/− aortas than that in ApoE−/− aortas (1321 ± 202.73 versus 544.42 ± 69.73 for ApoE−/−/CX3CR1−/− *P* = 0.0099) (Figure 4 and supplemental Figure IV).

Taken together, these data suggest that DCs are recruited to the intima of atherosclerosis-predisposed areas in normal and

![Figure 1. CX3CR1 deficiency reduces atherosclerotic burden of ApoE−/− mice on normal chow diet. Atherosclerotic plaques in sections of the aortic root (A), the aortic luminal surface (B), and the innominate artery (C) of ApoE−/− and ApoE−/−/CX3CR1−/− mice were stained with Oil Red O (arrows). The amount of plaque is plotted in the corresponding bar graphs. **P<0.05.](image-url)
atherosclerosis-prone arteries. CX3CR1-deficiency impairs the intimal accumulation of vascular DCs.

**Aging Increases the Accumulation of Intimal DCs and the Incidence of Atherosclerosis**

Aged individuals are more susceptible to atherosclerosis, and this is largely attributed to chronic arterial inflammation. To study the biological consequences resulting from vascular DC accumulation, we analyzed DCs in aged mice. Because DCs are found in the intima of young healthy aortas and their location is restricted to atherosclerosis-predisposed areas, we investigated the accumulation and distribution of DCs in normal aortas during the process of aging. In contrast to the healthy 20-week-old aorta, CD11c+ cells in the aorta from

![Figure 2. Immunoconfocal images of intimal DCs and macrophages in WT mice. Images demonstrate the morphology of CD11c+ cells (green) and nuclei (red) in A. Coexpression of CD11c (green) with CD83 (red) and CD68 (red) is shown in Band C, respectively. Images are representative of at least 3 animals per group.](image)

![Figure 3. Expression of CX3CR1 and CX3CL1 in the aortas. A. The expression of CX3CR1 (green) is shown in the CX3CR1-GFP KI aorta that was stained with CD11c (red) antibodies. Coexpression of CX3CR1 and CD11c is shown (orange) in the overlapping image. B. Immunohistochemical staining of CX3CL1 and endothelial cells (von Willebrand factor) is shown in an atherosclerotic lesion within the aorta.](image)
mice over 60 weeks old expanded to a larger area and invaded into the thoracic aorta. CD11c+ cells were also found in aged adventitia. Quantitatively, the number of CD11c+ cells in the intima increased gradually and substantially in a time course of aging (Figure 5). Wild-type aged mice at 60 weeks old recruited 58% more CD11c+ cells to their aortas than younger mice at 20 weeks old (2196.2±154 versus 904±79.5, P<0.004; Figure 4).

Although wild-type mice are exceptionally resistant to the induction of atherosclerosis, we speculated that the increased accumulation of intimal DCs in aged aortas might influence atherogenesis in aged mice. Therefore, we examined the incidence of atherosclerosis in mice over 60 weeks old. Oil Red O staining showed that 7 of 8 (88.9%) aged mice developed atherosclerotic lesions in the aortic root in locations where DCs were found to be most abundant (supplemental Figure V).

Together, these data suggest that the recruitment and retention of intimal DCs correlates with the development of atherosclerosis in aged mice.

Intimal DCs Are Involved in Atherosclerotic Formation

The relative frequency of vascular DCs has been shown to increase in atherosclerotic lesions compared with surrounding areas. To further address the involvement of vascular DCs in the development of atherosclerosis, we analyzed their intimal presence in localization, morphology, and quantity in the aortas of atherosclerotic ApoE−/− mice. In addition to their appearance in the adventitia, in which very few DCs resided in young healthy arteries, CD11c+ cells in ApoE−/− arteries were located in the intimal areas of arterial branch points and curvatures of atherosclerosis-prone areas in a pattern similar to normal aortas. However, the morphology of these DCs was dramatically different from normal vascular DCs. They were enlarged and often fused together in ApoE−/− aortas especially in and around atherosclerotic plaques (Figure 6A and supplemental Figure VI). The number of CD11c+ cells in ApoE−/− aortic intima increased significantly compared with control wild-type aortas at 20 weeks of age (2697.5±214.3 versus 904±79.5, P=0.05; Figure 6B). These data suggest that intimal DCs are involved in the pathogenesis of atherosclerosis.

Discussion

Atherosclerosis is a chronic inflammatory disease mediated by immune dysfunctions. The recent findings of DCs in
atherosclerotic plaques as well as in normal arteries arouse enormous interest in their roles in atherogenesis.

Immune cell recruitment plays a critical role in the development of atherosclerosis. Little is known about the mechanisms that regulate the homing of DCs to the vessel wall. Recent evidence shows that the accumulation of intimal DCs and macrophages is a result of continued recruitment of bone marrow–derived blood monocytes. All subsets of monocytes express CX3CR1 and like CCR2, one of the major functions of CX3CR1 is to mediate monocyte adhesion and migration. We have previously shown that CX3CR1 deficiency leads to a decrease in monocyte infiltration into injured arteries in the model of intimal hyperplasia. Therefore, we hypothesized that CX3CR1 is involved in vascular DC recruitment. Indeed, intimal DCs strongly expressed CX3CR1 in wild-type healthy vessels, and the accumulation of intimal DCs was significantly reduced in the absence of CX3CR1. In ApoE−/− mice, we observed robust expression of CX3CR1 on DCs, and the CX3CR1 ligand CX3CL1 was greatly upregulated in endothelial cells that cover both plaque-predisposed arterial surface and plaque itself. CX3CR1 deficiency in ApoE−/− mice significantly reduced DC accumulation in the intima of atherosclerotic arteries. A surprising observation is that DCs outnumbered macrophages in the intima of young healthy aortas. The early appearance of DCs in atherosclerotic-prone regions of healthy arteries may suggest that DCs regulate a balance between host defense and inflammation in the arterial wall.

Clustering of DCs is considered an indicator of autoimmune processes. Vascular DCs are thought to initiate and maintain T cell responses in autoimmune disease giant cell arteritis. In our study, DCs were not only clustered in the atherosclerotic-prone intima and in atherosclerotic plaques, they also accumulated in the adventitia in aging and ApoE−/− arteries. In echoing DC clusters, T cells were shown residing in the adventitia in normal arteries with DCs and macrophages and their numbers doubled in ApoE−/− arteries. The same study also demonstrated that antigen-activated DCs induced T lymphocyte proliferation within the aorta 72 hours after adoptive transfer. Substantial evidence has indicated that autoantigens, such as oxidized low-density lipoprotein (oxLDL), oxidized phosphatidylcholine, heat shock protein, and beta2 glycoprotein 1, play an important role in atherosclerosis. Our data support the hypothesis that vascular...
DC activation by autoantigens are critical for initiating immune responses in the arterial wall that lead to atherosclerotic alteration.

According to Wick et al., the arterial wall is a vascular-associated lymphoid tissue (VALT) analogous to the mucosa-associated lymphoid tissue to protect arteries from potentially danger signals. Indeed, we found DCs were present in the VALT of normal arteries in areas that are prone to atherosclerotic plaques, and their abundance changed correspondingly in the process of atherogenesis. In a time course of aging, the number of intimal DCs increased significantly in aged mice, and at the same time, approximately 89% of the 60-week-old mice developed atherosclerotic lesions, suggesting that the increased accumulation of vascular DCs may be one of the factors involving in plaque generation. This finding is supported by our data as well as other studies that ApoE−/− arteries harbor more DCs. Interestingly, in ApoE−/− vessels, DCs not only significantly enlarged but also fused. Considering the substantially larger plaques in ApoE−/− than in aged arteries, the morphological changes of vascular DCs in ApoE−/− mice may indicate further DC activation, thereby leading to more severe lesions.

In summary, DCs in the intima of arteries were not static but dynamic. While the abundance of DCs in normal arteries increased in the process of aging, they varied in morphology and quantity in ApoE−/− arteries and atherosclerotic plaques. CX3CR1-deficiency impaired the recruitment of DCs to the arterial wall. These findings not only substantiate our understanding of the vascular mechanisms in response to atherogenic dangers, but they may also provide biological basis for immunologic intervention toward prevention and control of atherosclerosis.

Acknowledgments

The authors sincerely thank the Michael Hooker Microscopy Facility at UNC-Chapel Hill for technique support on using its confocal microscope; Drs Mildred Kwan, Nobuyo Maeda, Shinja Kim, and Rishi Rampersad and James Ellinger for their technical assistance; and Dr Teresa Tarrant for constructive discussions and suggestions.

Sources of Funding

These studies were supported by NIH grants HL077406, CA098110, and T32-AR07416 and by an Arthritis Foundation Postdoctoral Fellowship.

Disclosures

None.

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Arterioscler Thromb Vasc Biol. 2008;28:243-250; originally published online December 13, 2007;
doi: 10.1161/ATVBAHA.107.158675
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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Supplementary Methods

*Generation of CX3CR1-GFP KI Mice*

Briefly, four individual DNA segments, including murine 3’-flanking region, neomycin resistance gene flanked by loxp sites, hCX3CR1-GFP, and murine 5’ flanking region were cloned into pPNT vector in the sequence listed. First, the 3’-flanking region was generated by PCR using Ecolig2, a genomic clone containing murine CX3CR1 (This genomic clone for mouse CX3CR1 derived from 129/sv genomic library that was kindly provided by Dr. Christophe Combadiere) as template with mV28 K-E-5’ (5’-tcgaccggtaccttgccatcgtcc-3’) and mV28 K-E-3’ (5’-tccttggaattcgacatgacatgg-3’) as primer sets. The segment was ligated into pPNT via KpnI and EcoR1. Second, the neomycin resistance gene flanked by loxp sites (ploxp2, kindly provided by Dr. Yuan Zhuang) was cloned into into pPNT by blunt-ended ligation into KpnI site. Third, to ensure proper transition from murine non-coding to human coding gene sequence and preservation of splicing motif, a DNA segment containing murine genomic DNA immediate upstream of murine CX3CR1 start codon was fused in frame to human CX3CR1-GFP. This was generated by PCR amplification using hCX3CR1-GFP in pEGFP-N1 vector (Clontech, Palo Alto, CA) as template and mV28 X-C-5’ (5’-tgcttactcggaggcagccagtgaactcataaataaagggtcagctgtttcttgtctctcccctgagaccttaaaacttctgattgaatg-3’) and mV28 X-C-3’ (5’-ggttggatcattaactaagttgatgtg-3’) as primers. The amplified product was cloned into Xhol and Clal sites of pBlueScript SK vector (Stratagene, La Jolla, CA). The hCX3CR1-GFP segment was cloned into pPNT via Xho1 and Xba1/SacII blunt-end ligation. Last the DNA segment containing the majority of murine 5’-flanking region (~6Kb) was constructed from 2 segments. To ensure proper transition from murine 5’-flanking region that contains motif required for proper splicing to human CX3CR1-GFP coding region, an endogenous Mun1 restriction located ~120bp upstream of murine CX3CR1 start codon was utilized. A segment containing sequence from
MunI site to an engineered Xhol site was generated by PCR using Ecolig2 as template and mV28 M-X-5' (5'-tagttccaatgttcaccctttcagtg-3') and mV28 M-X-3' (5'-ggtgcctcgagtaagcaggrcca-3') as primer set. The segment was initially cloned into pBluescript SK vector via BamH1 and XhoI sites. This segment was combined with majority of 5'-flanking region from EcoR1-Mun1 to form a ~6Kb DNA segment. This was cloned into pPNT via Not1 and Xho restriction sites. The fidelity of all DNA segments generated by PCR was confirmed by sequencing.

To generate ES cell clones and mice, the final pPNT construct containing all fragments was linearized by Not1 digestion and electroporated into R1 ES cells, derived from 129X1/5vj x 129S1/sv+pt+cmgr+s1-j/x. The clones surviving G418 selection were screened by PCR using a primer located within the 3'-flanking sequence of CX3CR1 (mV28-mock-3'-KI: 5'-ttttggactacagtctccttgtct-3') and second primer within the neomycin resistance gene (ploxNeo-mock-5'-start: 5'-agagcagccgattgtctgtgtgc-3'). Only those containing the construct inserted in the proper location within the genome yielded a product. The positive clone was then injected into blastocysts to generate the knockin mice. The neomycin resistance gene was removed by breeding to CRE transgenic mice (from Jackson).

CX3CR1-GFP KI mice allow for the ability to track CX3CR1 expressing cells. Leukocyte subsets in spleen and blood of KI mice are similar in number and percentage compared to wild type mice (Figure SIA). Furthermore, CX3CR1 positive leukocytes from KI animals adhere to CX3CL1 (Figure SIB). ApoE−/−/CX3CR1-GFP KI mice were generated by crossing ApoE−/− and CX3CR1-GFP KI mice. The incidence of atherosclerosis of ApoE−/−/CX3CR1-GFP KI mice is 100% at age of 40 weeks, which was the same with ApoE−/− mice.
**Real-time PCR**

Total RNA was isolated from mouse kidneys and aortas using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was generated using the First-Strand cDNA Synthesis Superscript II RT (Invitrogen, Carlsbad, CA) for RT-PCR. Quantitative real-time PCR cDNA was performed using SYBR Green Master Mix and iCycler (BioRad Laboratories Hercules, CA). The $2^{-\Delta\Delta Ct}$ method\(^1\) was used for analyzing real-time PCR data.

**Flow Cytometry**

PBMC and splenocytes were isolated from mice using Histopaque 1083 (Sigma, St. Louis, MO) and a manual tissue homogenizer, respectively. Red blood cells (RBC) were lysed with RBC lysis buffer (0.14M NH\(_4\)Cl, 0.017M Tris-HCl adjust to pH7.2). Cells were washed with PBS containing 1% bovine serum albumin and passed through a 70 µm nylon filter. 5x10\(^5\) cells were incubated with rat anti-mouse B220, CD11b, DX-5 (NK) and parallel IgG controls for 30 minutes on ice. After washing, flow cytometry analysis was performed using a FACScan flow cytometer (BD Biosciences) and CellQuest Software.

**Adhesion Assay**

A static adhesion assay was performed as described with minor modification\(^2\). Briefly, FKN-SEAP fusion proteins were isolated and concentrated from 293-EBNA cultured supernatants. 10 µg/ml anti-SEAP antibody (Sigma, St. Louis, MO) in 50mM Tris pH9.5 is coated on the bottom of a 6 well plate for overnight at 4°C. Next, the plates were blocked using 1% BSA. After washing in PBS, 10nM FKN-SEAP fusion protein was added and incubated for 2 hours at room temperature. SEAP only was used as a negative control. Following washes with PBS, 1 X 10\(^5\) PBMC were added to each well and incubated for 30 minutes at RT.
Nonadherent cells are carefully removed by PBS washes and cells remaining firmly bound were counted in four random fields under a microscope.

Supplementary Figures

Figure SI. Evaluation of CX³CR1-GFP knockin mice

A. Dot plots of GFP expression on leukocyte subsets in WT and knockin animals as determined by flow cytometry. GFP expression is shown on the X-axis. The Y-axis shows expression of B220 (B cells), CD11b (monocytes) and DX-5 (NK cells).  B. Leukocyte adhesion of WT and knockin mice to SEAP (open bar) and CX³CL1-SEAP (filled bar).  **, P<0.05. Error bars represent Means ± SEM.
Figure SII. Coexpression of CX3CR1 and CD11c in aged WT and ApoE−/− mice

Aged CX3CR1-GFP KI (A) and ApoE−/−/CX3CR1-GFP KI (B) aortas were stained with anti-CD11c (red) antibodies. Co-expression of GFP-CX3CR1 (green) and CD11c is shown in orange in the overlapping image. The scale bar is 50 μm.
Figure SIII. CX₃CL1 mRNA expression in the aortas

Aortas from young WT (20 weeks), aged WT (60 weeks), and ApoE⁻⁻ (20 weeks) mice were isolated for RNA preparation. Real-time PCR was performed in duplicates and CX₃CL1 mRNA expression levels shown are averages of two individual experiments. Error bars represent Means ± SEM.
Figure SIV. CD11c+ cells in ApoE−/− and ApoE−/−/CX3CR1−/− mice

Aortas of 10-week-old mice of ApoE−/− (A) and ApoE−/−/CX3CR1−/− (B) were isolated and stained with anti-CD11c antibodies using en face immunoconfocal microscopy described in Materials and Methods. Shown is morphology of CD11c+ cells (green) in the aortic intima in representative images. Nuclei are showing in red and the scale bar is 50 μm.
Figure SV. Atherosclerotic lesions in aged WT mice

Oil Red O staining of aortic root sections of WT mice at 20 and > 60 weeks of age are shown. Arrows point at the atherosclerotic lesions positive for Oil Red O in aged mice.
Figure SVI. CD11c + cells in an atherosclerotic plaque

The distribution of the intimal CD11c+ cells (green) in and around an atherosclerotic plaque in the aorta of an Apo E−/− mouse. Nuclei are shown in red and the scale bar is 50 μm.

References