T Cell CX3CR1 Mediates Excess Atherosclerotic Inflammation in Renal Impairment

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ABSTRACT
Reduced kidney function increases the risk for atherosclerosis and cardiovascular death. Leukocytes in the arterial wall contribute to atherosclerotic plaque formation. We investigated the role of fractalkine receptor CX3CR1 in atherosclerotic inflammation in renal impairment. Apoe<sup>−/−</sup> (apolipoprotein E) CX3CR1<sup>−/−</sup> mice with renal impairment were protected from increased aortic atherosclerotic lesion size and macrophage accumulation. Deficiency of CX3CR1 in bone marrow, only, attenuated atherosclerosis in renal impairment in an independent atherosclerosis model of LDL receptor–deficient (LDLr<sup>−/−</sup>) mice as well. Analysis of inflammatory leukocytes in atherosclerotic mixed bone-marrow chimeric mice (50% wild-type/50% CX3CR1<sup>−/−</sup> bone marrow into LDLr<sup>−/−</sup> mice) showed that CX3CR1 cell intrinsically promoted aortic T cell accumulation more than CD11b<sup>+</sup>CD11c<sup>+</sup> myeloid cell accumulation and increased IL-17-producing T cell counts. In vitro, fewer Th<sub>17</sub> cells were obtained from CX3CR1<sup>−/−</sup> splenocytes than from wild-type splenocytes after polarization with IL-6, IL-23, and TGFβ. Polarization of Th<sub>17</sub> or T<sub>REG</sub> cells, or stimulation of splenocytes with TGFβ alone, increased T cell CX3CR1 reporter gene expression. Furthermore, TGFβ induced CX3CR1 mRNA expression in wild-type cells in a dose- and time-dependent manner. In atherosclerotic LDLr<sup>−/−</sup> mice, CX3CR1<sup>+/−</sup> T cells upregulated CX3CR1 and IL-17A production in renal impairment, whereas CX3CR1<sup>−/−</sup> T cells did not. Transfer of CX3CR1<sup>+/−</sup> but not Il17a<sup>−/−</sup> T cells into LDLr<sup>−/−</sup>CX3CR1<sup>−/−</sup> mice increased aortic lesion size and aortic CD11b<sup>+</sup>CD11c<sup>+</sup> myeloid cell accumulation in renal impairment. In summary, T cell CX3CR1 expression can be induced by TGFβ and is instrumental in enhanced atherosclerosis in renal impairment.

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cells can attenuate lesion formation. The role of T_{H17} cells appears to be highly context-dependent. We have shown that the T_{H17} cell cytokine IL-17A aggravates atherosclerosis in renal impairment and is required for enhanced aortic CD11b^+CD11c^+ myeloid cell accumulation in this condition.

Myeloid cells that phagocytose lipids and form foam cells accumulate in the atherosclerotic lesion due to both monocyte immigration and local proliferation. During atherogenesis, CD11c expression on aortic CD11b^+ myeloid cells increases. This cell type is capable of both lipid phagocytosis and antigen presentation and can productively interact with T cells in the murine aorta. In moderate renal impairment, aortic CD11b^+CD11c^+ myeloid cell numbers expand significantly. Aortic myeloid cells mostly derive from peripheral blood monocytes at early stages of atherosclerosis development. Multiple chemokines have been implicated in this, including fractalkine receptor CX3CR1, which promotes lesion progression. CX3CR1 is expressed on leukocytes, including monocytes, dendritic cells, T cells, and natural killer cells, but also nonmyeloid cells such as aortic smooth muscle cells. CX3CR1 deficiency (CX3CR1^{−/−}, mostly studied in CX3CR1^{fl/fl} mice) decreases atherosclerotic lesions in ApoE^{−/−} mice. This protection is transferred by deficient bone marrow, indicating a major role of myeloid CX3CR1 in lesion development. In ApoE^{−/−} and LDLr^{−/−} mice, pharmacologic CX3CR1 inhibition moderately decreases aortic root lesion size.

Two main mechanisms for the proatherogenic function of CX3CR1 have been proposed. First, CX3CR1 is highly expressed on monocytes and mediates their adhesion to endothelial cells and smooth muscle cells. The absence of systemic CX3CR1 impedes monocyte accumulation in the plaque in an aorta transplantation model. Its role in aortic accumulation of other leukocytes has not been reported. Second, CX3CR1 inhibits apoptosis of smooth muscle cells and monocytes ex vivo, and under proatherogenic conditions in vivo. During atherosclerosis development, CX3CR1^{low}Gr1^{high} inflammatory monocyte blood counts increase in ApoE^{−/−}, but not ApoE^{−/−}/CX3CR1^{−/−} mice. In resting mice, however, mostly CX3CR1^{high}Gr1^{low} monocytes are affected but only under certain conditions. CX3CR1^{−/−} macrophages undergo excess cell death in hepatic fibrosis and renal candidiasis and fibrosis. CX3CR1 is upregulated on T cells in inflammation, CX3CR1 protects T_{H2} and T_{H1} cells from apoptosis, and cytokine production is higher in CX3CR1^{+} than CX3CR1^{−} T_{H1} cells. However, whether CX3CR1 modifies other T cell subtypes and its mechanism of regulation on T cells has not been reported.

Our initial experiments showed that ApoE^{−/−}/CX3CR1^{−/−} mice were completely protected from increase in atherosclerotic lesion size in renal impairment. We therefore investigated role and regulation of leukocyte CX3CR1 in atherosclerosis and renal impairment.

**RESULTS**

**Absence of Fractalkine Receptor CX3CR1 Abrogates Excess Atherosclerotic Lesion Formation in Renal Impairment**

Atherosclerotic lesions in renal impairment were studied in ApoE^{−/−} mice after unilateral nephrectomy, which significantly decreases their renal function. Renal impairment increased atherosclerotic aortic lesions as previously described. Absence of CX3CR1 (CX3CR1^{fl/fl}) term CX3CR1^{−/−} throughout this manuscript) impeded atherosclerosis development as previously reported. In addition, it completely prevented an increase of lesion size in renal impairment in all parts of the aortic vessel, namely aortic arch, thoracic, and abdominal aorta (Figure 1B). Changes in male and female mice were very similar (Supplemental Figure 1, A and B). Histologic aortic root lesions were moderately but significantly increased in renal impairment as previously described (Figure 1C). CX3CR1 deficiency prevented additional lesion growth in renal impairment. At this stage of atherosclerosis, CX3CR1 deficiency did not alter histologic aortic root lesion size in mice with normal renal function. This differs from an earlier time point, suggesting that the role of CX3CR1 for total lesion size may be more obvious at early stages in the respective aortic region. Lesion composition in regards to collagen contents was unaltered (Supplemental Figure 1, C and D).

To investigate the inflammatory vascular infiltrate, leukocytes were assessed in blood and the atherosclerotic aortic root. Monocytes express CX3CR1 and increase in blood during high-fat-diet feeding and atherosclerosis development in ApoE^{−/−} mice. We made a similar observation (Supplemental Figure 2). Renal impairment, however, did not significantly alter total blood monocyte levels in either ApoE^{−/−} or ApoE^{−/−}/CX3CR1^{−/−} mice if all four groups were compared (Supplemental Figure 2, Supplemental Table 1).

In contrast to blood, in the atherosclerotic aortic root, renal impairment significantly increased F4/80^+ macrophage content (Figure 1D), a cell type that also expresses CD11c in the atherosclerotic aorta (data not shown). It also increased aortic root cell proliferation (Figure 1E). This was completely prevented in the absence of CX3CR1. Both increased lesion size and macrophage infiltration propose CX3CR1 as a central mediator of aggravation of atherosclerosis and vascular inflammation in renal impairment.

**Absence of CX3CR1 on Bone Marrow–Derived Cells Prevents Aggravated Atherosclerosis in Renal Impairment**

CX3CR1 is highly expressed on myeloid cells, most prominently on monocytes in the blood, but also on vascular cells. Monocytes are the main precursors of lesional macrophages that are increased in renal impairment. We wanted to examine if bone marrow CX3CR1 plays a role in atherosclerosis aggravation in renal impairment. An independent atherosclerosis
model of \( \text{LDLr}^{-/-} \) mice were reconstituted with either wild-type (WT) or \( \text{CX3CR1}^{-/-} \) bone marrow (characteristics in Supplemental Table 2). Renal function was significantly reduced after unilateral nephrectomy (Supplemental Figure 3).\(^5\) Atherosclerotic root lesions quantified after 10 weeks on a high-fat diet were significantly smaller in the absence of bone marrow \( \text{CX3CR1}^{-/-} \) mice than WT mice with renal impairment (Figure 2C).

These results suggest a central role for leukocyte CX3CR1 in lesion development in renal impairment. We next generated mixed bone-marrow chimeric mice to better understand the role of CX3CR1 in individual leukocyte fate in atherosclerosis. A mix of 50% WT (CD45.1) and 50% \( \text{CX3CR1}^{-/-} \) (CD45.2) bone marrow was transferred into lethally irradiated \( \text{LDLr}^{-/-} \) mice (characteristics in Supplemental Table 3). Flow cytometric analysis of atherosclerotic aortas after 10 weeks on a high-fat diet showed no significant difference in numbers of WT and \( \text{CX3CR1}^{-/-} \) CD11b\(^+\)CD11c\(^+\) myeloid cells (Figure 2D). The tendency towards an increase in renal impairment was very similar for both genotypes. Monocytes are precursors of most early plaque macrophages, however, during later stages of atherosclerosis, most myeloid cells are derived locally.\(^13\) To test for a role of CX3CR1 in monocyte recruitment, aorta analysis was conducted earlier, after 6 weeks of high-fat diet. Atherosclerotic aortic root lesion size increased with renal impairment (Figure 2E). The tendency towards an increase in renal impairment was very similar for CD11b\(^+\)CD11c\(^+\) myeloid cells of both genotypes in the identical environment of mixed bone-marrow chimeric mice. \( \text{CX3CR1}^{-/-} \) cells were even more common than WT cells among aortic CD11b\(^+\)CD11c\(^+\) myeloid cells (Figure 2F). This argues against an individual monocyte recruitment defect in the absence of CX3CR1.

The results in Figure 2F constitute an overrepresentation of CD45.2\(^+\) cells among aortic myeloid cells compared with peripheral blood, where counts did not significantly differ from the expected 50% (Supplemental Figure 4). Replacement of recipient cells was very good in both blood and bone marrow (see Concise Methods). Still, we separately addressed locally derived CD45.2\(^+\)CD11b\(^+\)CD11c\(^+\) myeloid cells of donor origin in the aorta by a single injection of clodronate liposomes to deplete phagocytes from \( \text{LDLr}^{-/-} \) mice transplanted with mixed bone marrow (50% WT [CD45.1] and 50% \( \text{CX3CR1}^{-/-} \) [CD45.2]) before induction of atherosclerosis.\(^41\) When aortic CD11b\(^+\)CD11c\(^+\) myeloid cells were assessed after 6 weeks on a high-fat diet, very similar proportions of WT and \( \text{CX3CR1}^{-/-} \) cells were recovered from the aortas (Figure 2G). Compared with controls with normal kidney function but otherwise the same treatment, aortic CD11b\(^+\)CD11c\(^+\) myeloid cells of both genotypes again
similarly tended towards an increase in renal impairment, although this was statistically significant only in WT cells (Figure 2G). These experiments show a role of bone marrow CX3CR1 in aggravation of atherosclerosis in renal impairment, however, they argue against a major individual cell–specific monocyte or CD11b+CD11c+ myeloid cell progenitor effect of CX3CR1 in aortic homing.

Impaired Aortic Accumulation and TH17 Polarization of CX3CR1<sup>−/−</sup> T cells

Among bone marrow–derived cells, CX3CR1 is also expressed on T cells. Given the impact of T helper cells in atherogenesis, we analyzed aortic lymphocytes in mixed bone-marrow chimeric LDLr<sup>−/−</sup> mice. CX3CR1<sup>−/−</sup> T cells were underrepresented in the atherosclerotic aortas compared with WT cells from the same animal (Figure 3A). Also, aortic CX3CR1<sup>−/−</sup> T cells were relatively less frequent than in the spleen (Figure 3B). Third, aortic CX3CR1<sup>−/−</sup> T cells were decreased compared with aortic and splenic CX3CR1<sup>−/−</sup> B cells (a CX3CR1-negative cell type) (Figure 3, A–D). The CX3CR1<sup>−/−</sup> T cells are CD45.2<sup>+</sup> therefore, their deficiency in accumulating in the aorta may even be underestimated as any residual recipient leukocytes are CD45.2<sup>+</sup> and would obscure the observed diminution (Figure 3A).

T cell polarization toward phenotypes with known roles in atherogenesis, namely TH1, TH17, and T<sub>REG</sub> cells, was investigated in the spleens of the same mice (Figure 3, E–G). While proportions of WT and CX3CR1<sup>−/−</sup> cells among TH1 cells (Figure 3E) closely resembled their proportion among all T cells (Figure 3B), CX3CR1<sup>−/−</sup> cells were significantly less abundant among both TH17 (Figure 3F) and T<sub>REG</sub> cells (Figure 3G) in mice with renal impairment. Similarly, in LDLr<sup>−/−</sup> mice with

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**Figure 2.** Bone marrow CX3CR1 promotes atherosclerosis but not individual monocyte aortic homing in LDLr<sup>−/−</sup> mice with renal impairment. (A–C) LDLr<sup>−/−</sup> mice reconstituted with either WT or CX3CR1<sup>−/−</sup> bone marrow were kept on a high-fat diet for 10 weeks after unilateral nephrectomy. (A, B) Aortic root lesion size was assessed by histology (n=5–6, scale bar: 500 μm). (C) Aortic CD11b<sup>+</sup>CD11c<sup>+</sup> myeloid cells were investigated by flow cytometry (n=4–5). (D–G) LDLr<sup>−/−</sup> mice reconstituted with 50% WT (CD45.1) and 50% CX3CR1<sup>−/−</sup> (CD45.2) underwent surgery (unilateral nephrectomy [RI] and control [ctrl]). (D) After 10 weeks on a high-fat diet, aortic CD11b<sup>+</sup>CD11c<sup>+</sup> myeloid cells were assessed by flow cytometry for WT (CD45.1) and CX3CR1<sup>−/−</sup> (CD45.2) genotype and numbers compared in RI and ctrl mice (n=4–7, two independent experiments). (E, F) After 6 weeks on a high-fat diet, aortic root lesion size was assessed by histology (E, n=4) and aortic CD11b<sup>+</sup>CD11c<sup>+</sup> myeloid cells quantified by flow cytometry (F, n=5–6, two independent experiments). (G) A single injection of clodronate liposomes was applied on day 0 of a 6-week high-fat diet. Aortic CD11b<sup>+</sup>CD11c<sup>+</sup> myeloid cells were measured by flow cytometry (n=5). *P<0.05, **P<0.01, ***P<0.001.
renal impairment that had been reconstituted with either WT or CX3CR1<sup>2/2</sup> bone marrow, the proportion of splenic TH17 cells was significantly lower in mice without bone marrow CX3CR1 (Figure 3, H–J). A nonsignificant trend toward reduction was observed in TREG cells, however, TH1 cells were not significantly altered. These in vivo data indicate a defect in aortic CX3CR1<sup>2/2</sup> T cell accumulation and TH17 polarization in atherosclerosis and renal impairment.

**Impaired IL-17A Production and TH17 Polarization of CX3CR1<sup>2/2</sup> Cells In Vitro**

*In vitro* TH<sub>H17</sub>, T<sub>H17</sub>, and T<sub>REG</sub> cell polarization was conducted for WT and CX3CR1<sup>1/−/−</sup> splenocytes in parallel. T<sub>H17</sub> and T<sub>REG</sub> polarization increased WT, but not CX3CR1<sup>1/−/−</sup> T cell survival (Figure 4, A–C). No significant difference was seen in cell proliferation assessed by intracellular dye dilution (n=2, data not shown). Marker cytokines of T cell lineages were measured in the supernatants (Figure 4, D–F). Compared with WT cells cultured in parallel, supernatants of CX3CR1<sup>1/−/−</sup> splenocytes contained markedly less IL-17A, but not IL-10 or IFNγ. After restimulation and intracellular staining, the proportion of T<sub>H17</sub> cells was significantly lower in polarized CX3CR1<sup>1/−/−</sup> than WT splenocytes (Figure 4, G–I). A similar trend was observed in T<sub>REG</sub> cells. There was no significant impact of CX3CR1 genotype on TH1 cell polarization. Taken together, TH17 cell polarization was impaired in the absence of the CX3CR1 gene.

**TGFβ Induces T Cell CX3CR1 Expression**

After T<sub>H17</sub> polarization, we noted an increase of CX3CR1 promoter-driven green fluorescent protein (GFP) in TH17 cells compared with T<sub>H17</sub> cells obtained from cultures without polarizing cytokines (Figure 5A). Therefore the cytokines that were part of the T<sub>H17</sub> polarization protocol were tested separately. CX3CR1 promoter-driven GFP was measured in IL-17A<sup>+</sup> T cells. Expectedly, cultures with single cytokines resulted in markedly fewer IL-17A<sup>+</sup> T cells, but CX3CR1 promoter-driven GFP expression among them was induced even more strongly by TGFβ alone than by the T<sub>H17</sub> cocktail. Neither IL-6 nor IL-23 exerted a significant effect (Figure 5, A and B).
Also, after TREG polarization with TGFβ, CX3CR1 promotor-driven GFP significantly increased among TREG cells defined either by FoxP3+ (Figure 5C) or CD4+CD25+ with better discrimination on the latter, non-permeabilized cells (Figure 5, D and E).

To further define the TGFβ effect, CX3CR1 mRNA was assessed in WT splenocyte culture. TGFβ induced CX3CR1 mRNA both dose- and time-dependently (Figure 5F). TGFβ receptor–mediated SMAD activation was inhibited pharmacologically.42,43 This reduced CX3CR1 mRNA expression in WT cells to baseline (Figure 5G). Also in isolated CD4+ T cells, TGFβ significantly induced CX3CR1 mRNA (Figure 5H). These data suggest TGFβ is a mediator of CX3CR1 expression in T cells.

**T Cell CX3CR1 and IL-17 Expression is Upregulated in Renal Impairment**

In atherosclerotic mice transplanted with CX3CR1<sup>−/−</sup> bone marrow, no consistent change of leukocyte CX3CR1 levels was measured with renal impairment as mean fluorescence intensity of the GFP reporter gene (data not shown). However, given the role of CX3CR1 in cell survival (Figure 4, A–C),32 we reasoned cells that increased their GFP expression as an indicator of CX3CR1 promoter activity might have vanished. To test for CX3CR1 expression in atherosclerosis in the presence of a functional CX3CR1, we generated strains of LDLr<sup>−/−</sup>CX3CR1<sup>+/−</sup> and LDLr<sup>−/−</sup>CX3CR1<sup>−/−</sup> mice.

In LDLr<sup>−/−</sup>CX3CR1<sup>−/−</sup> mice, aortic CD11b<sup>+</sup>CD11c<sup>+</sup> myeloid cells but not T cell numbers increased significantly in renal impairment (Figure 6A), similar to our earlier studies with bone marrow–transplanted LDLr<sup>−/−</sup> mice (Figures 2 and 3), while in direct comparison, aortic CD11b<sup>+</sup>CD11c<sup>+</sup> cells in LDLr<sup>−/−</sup>CX3CR1<sup>−/−</sup> mice were unchanged (Figure 6F). CX3CR1 expression was significantly enhanced on aortic T cells and CD11b<sup>+</sup>CD11c<sup>+</sup> myeloid cells (Figure 6, B and C), while in the absence of CX3CR1, neither aortic T cell nor CD11b<sup>+</sup>CD11c<sup>+</sup> CX3CR1 promotor-driven GFP cell number increased in renal impairment (Figure 6, G and H). Also the proportion of T<sub>H17</sub> cells increased significantly in renal impairment (Figure 6, D and E). Among CX3CR1 promotor-driven GFP-positive T cells, the proportion of IL-17 producers was significantly higher than in all T cells (P=0.02 for control and P<0.001 for mice with renal impairment) and further

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**Figure 4.** Impaired T<sub>H17</sub> and T<sub>REG</sub> differentiation of CX3CR1<sup>−/−</sup> cells in vitro. In vitro T<sub>H</sub> cell polarization was conducted in WT and CX3CR1<sup>−/−</sup> splenocytes in parallel as described in the Concise Methods section. (A–C) Cell viability is depicted as proportion of live T cells among all events after 4 days of polarization and restimulation in T<sub>H17</sub> (A), T<sub>REG</sub> (B), and T<sub>H1</sub> polarized cells (n=6–8, from three to four experiments). (D–F) Cell culture supernatants were assessed for T cell markers IL-17A (D), IL-10 (E), and IFNγ (n=3 polarizations). (G–I) Intracellular staining after polarization was used to assess proportion of polarized T cells. IL-17A was stained after restimulation in T<sub>H17</sub> polarized and control T<sub>H0</sub> cells (G, n=12, from six independent parallel experiments). The proportion of FoxP3<sup>+</sup> T cells was studied after T<sub>REG</sub> polarization and in control T<sub>H0</sub> cells (H, n=5–8, from three independent experiments). Intracellular IFNγ production assessed in T<sub>H17</sub> polarized WT and CX3CR1<sup>−/−</sup> T cells (I, n=6, from three independent experiments).

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increased in renal impairment (Figure 6E). In contrast, in *LDLr<sup>−/−</sup>CX3CR1<sup>−/−</sup>* mice, splenic IL-17A producers were unaltered in renal impairment (Figure 6, I and J). The proportion of splenic IL-17A producers was significantly lower in *LDLr<sup>−/−</sup>CX3CR1<sup>−/−</sup>* than *CX3CR1<sup>−/−</sup>* mice with renal impairment (Figure 6, D and I, *P*=0.02). This demonstrates that CX3CR1

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**Figure 5.** TGFβ induces T cell CX3CR1 expression. (A, B) In vitro T cell culture in CX3CR1<sup>−/−</sup> splenocytes was performed in the absence of exogenous cytokines (*T<sub>H0</sub>*), with IL-6, IL-23, and TGFβ (*T<sub>H17</sub>*), or in the presence of individual cytokines (IL-6, IL-23, or TGFβ). CX3CR1 promotor-driven GFP expression was assessed by flow cytometry in IL-17A<sup>+</sup>GFP<sup>+</sup> live T cells (A, examples and % of GFP<sup>+</sup> among IL-17A<sup>+</sup> live T cells, B, Mean fluorescence intensity of IL-17A<sup>+</sup>GFP<sup>+</sup> live T cells from n=4–16, from two to seven independent experiments, Dunnett test after ANOVA). (C–E) CX3CR1<sup>−/−</sup> splenocytes were polarized toward T<sub>REG</sub> (IL-2 and TGFβ) and the proportion of CX3CR1<sup>GFP</sup> among FoxP3<sup>+</sup> T cells (C, n=6–8, from three independent experiments) and CD4<sup>+</sup>CD25<sup>+</sup> cells (D), and the mean fluorescence intensity of CD4<sup>+</sup>CD25<sup>+</sup>GFP<sup>+</sup> cells assessed (E, n=6, from three independent experiments). (F–H) WT splenocytes were stimulated with TGFβ. (F) Dose and time response of CX3CR1 mRNA was assessed by quantitative PCR (one of two experiments, with parallel results). (G) Splenocytes were stimulated with TGFβ (1 ng/ml) for 2 hours in the presence of inhibitor SB and CX3CR1 mRNA expression assessed relative to DMSO ctrl (n=4, two independent experiments). (H) CD4<sup>+</sup> sorted splenic T cells were stimulated with TGFβ (1 ng/ml) and CX3CR1 mRNA expression was assessed (n=6, three independent experiments).
and IL-17A are induced in T cells during atherosclerosis in renal impairment and that a functional CX3CR1 is required for this.

**CX3CR1⁺ T Cells Promote Atherosclerotic Inflammation in Renal Impairment**

To test whether T cell CX3CR1 was mechanistically relevant for the increased atherosclerotic lesion in renal impairment, LDLr⁻/⁻ CX3CR1⁻⁻ mice with renal impairment and controls received a single adoptive transfer of CX3CR1⁺/⁺ CD4⁺ T cells. CX3CR1⁻⁻/⁻ CD4⁺ T cells restored the increase in atherosclerotic lesion size in renal impairment (Figure 7, A and B). Also, the number of aortic myeloid cells was significantly higher (Figure 7C). To test whether IL-17A production in CX3CR1-competent T cells was required for this process, we adoptedly transferred II17a⁻⁻ T cells into LDLr⁻/⁻ CX3CR1⁻⁻ mice after renal impairment or control surgery. II17a⁻⁻ T cells failed to increase lesion size (Figure 7, A and B) or CD11b⁺CD11c⁺ myeloid cell numbers (Figure 7C) in renal impairment. These results support the hypothesis that both T cell CX3CR1 and T cell IL-17A are required for enhanced atherosclerosis in renal impairment.

**DISCUSSION**

Our data demonstrate that T cell CX3CR1 mediates excess atherosclerotic inflammation in renal impairment. They also define TGFβ as a novel inducer of CX3CR1 in T cells. Absence of CX3CR1 prevented increase in atherosclerotic lesion size in renal impairment in both Apoe⁻⁻/⁻ and LDLr⁻/⁻ mice. The absence of bone marrow CX3CR1 significantly decreased aortic myeloid cell numbers and lesion size in renal impairment. Contrary to our primary hypothesis, monocyte myeloid cells with high CX3CR1 expression did not have a major defect in homing to the atherosclerotic aorta. Instead, CX3CR1⁻⁻ T cells were significantly underrepresented. This is reminiscent of impaired intradural T cell accumulation in a model of atopic dermatitis. Increased T cell CX3CR1 in CKD has previously been associated with carotid intima-media thickness. Also in our experiments, aortic T cells upregulated CX3CR1. Beyond this, adoptive transfer of CX3CR1-competent T cells into LDLr⁻/⁻ CX3CR1⁻⁻ mice sufficed to restore excess atherosclerotic inflammation in renal impairment, demonstrating their mechanistic importance.

T cells express CX3CR1 in mice and humans. Our data demonstrate TGFβ as inducer of CX3CR1 during T cell polarization both by reporter gene and mRNA expression. TGFβ increased CX3CR1 T cell intrinsically. However, its action was not limited to T cells. A similar response has been described during glial differentiation and we found that TGFβ also increased CX3CR1 expression in murine blood monocytes and bone marrow– derived macrophages (unpublished data). CX3CR1⁻⁻ cells undergo excess apoptosis in TGFβ-rich environments such as renal and hepatic fibrosis, while its role has been debated at rest. Upregulation by TGFβ may contribute to increased CX3CR1 sensitivity in these conditions.

In vitro T_{H17} polarization induced less IL-17A secretion and a smaller proportion of T_{H17} cells among CX3CR1⁻⁻ splenocytes. T_{H17}, T_{H17}, and T_{REG} cells modulate atherosclerosis progression. In our experiments, increase in atherosclerotic lesion size in renal impairment paralleled upregulation of IL-17A in WT T cells. T_{H17} cells were decreased in the absence of CX3CR1. Transfer of CX3CR1-competent T cells alone sufficed to restore atherosclerotic lesion size and increase myeloid cells in renal impairment in LDLr⁻/⁻ CX3CR1⁻⁻ mice. Consistent with the role of IL-17A in aggravation of atherosclerosis in renal impairment demonstrated previously, this was absent if CX3CR1-competent II17a⁻⁻ T cells were transferred.

![Figure 6](https://www.jasn.org)
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TREG and TH17 cells and their intermediates. Apoe-/- mice contribute to the in...se outcome. In summary, our data show promotion of atherosclerotic lesion size has been previously described. For histologic root lesions were assessed by histology (A shows example [bar indicates 500 µm thick sections from the aortic...Figure 7. CX3CR1-/- but not Il17a-/- T cells increase atherosclerosis in LDLr-/- CX3CR1-/- mice with renal impairment. LDLr-/- CX3CR1-/- mice after renal impairment (RI) or control surgery (ctrl) were adoptively transferred with either CX3CR1+/+ or Il17a-/-/CD4+ T cells. Lesions and aortic leukocytes were analyzed after 6 weeks of a high-fat diet starting on the day of adoptive transfer. (A, B) Atherosclerotic aortic root lesions were assessed by histology (A shows example [bar indicates 500 µm]). B shows statistical analysis of n=5 per group for CX3CR1+/+ and n=6-7 for Il17a-/-/CD4+ T cells. Two-way ANOVA showed significant differences between RI mice receiving CX3CR1+/+ T cells and all other groups, asterisks indicate significant differences between RI and ctrl mice that received CX3CR1+/+ T cells. (C) Aortic C11b+/CD11c+ myeloid cells were analyzed by flow cytometry (n=5, three independent transfers for CX3CR1+/+, n=6-7, three independent transfers for IL17a-/-, Bonferroni test after two-way ANOVA).

Published data in other disease models show significantly reduced disease severity and Th17 infiltration in collagen-induced arthritis in the absence of CX3CR1, and trends toward less IL-17A in mucosal candidiasis and nephrotic nephritis. In contrast, in experimental autoimmune encephalitis, both IL-17 and IFNγ production and disease severity were enhanced in TGFβ-/- mice compared with WT controls, however, this effect was not T cell intrinsic. In colitis, IL-17A production was not affected or even increased in CX3CR1-/- mice, which was attributed to defective intramural macrophage populations. Regarding the other main TGFβ-promoted T cell subgroup, these tended to or were significantly reduced in CX3CR1-/- mice with colitis and T cell-mediated skin immunity. These published results are consistent with the hypothesis that TGFβ-mediated CX3CR1 upregulation in T cells is functionally relevant beyond the atherosclerosis phenotype described here. Given the role of TGFβ during T cell polarization, this would affect mostly TREG and Th17 cells and their intermediates. It is conceivable that an abundance of TGFβ, the relevance of CX3CR1 on homing to the affected organ, and pathophysiologic importance of TREG versus Th17 cell subsets (versus other leukocytes such as monocytes) will contribute to the influence of CX3CR1 on disease outcome. In summary, our data show promotion of atherosclerosis by Th cell CX3CR1 in renal impairment.

CONCISE METHODS

Animals
WT C57Bl/6, CX3CR1-/- (termed CX3CR1eff/effpsa), LDLr-/-, Apoe-/- (all CD45.2 on a C57Bl/6 background) and congenic B6.SJL-Ptprc<sup>pep</sup>b/Boy (CD45.1) mice (Jackson Labs, Bar Harbor, ME), Il17a-/- mice (kindly provided by Dr. Y. Iwakura, University of Tokyo), and CX3CR1+/+ crossed with Apoe-/- and LDLr-/- to obtain double-deficient mice were genotyped by PCR and used in age- and sex-matched groups. Mice were kept in specific-pathogen-free conditions. Animal experiments were approved by the Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Lower Saxony, Germany. Mice were maintained on a high-fat diet (Harlan Teklad 88137, 40% of kcal from fat, 1.5% cholesterol, 0.68% calcium, 0.56% phosphorous) or normal chow diet for the indicated time periods. Serum urea, creatinine, electrolytes, and lipids were measured by an Olympus AU400 Chemistry Immuno Analyzer (Olympus, Hamburg, Germany), and blood counts by an automatic analyzer (VetABC animal blood counter, ScilVet, Viernheim, Germany). FITC-sinistrin clearance was determined using the NIC-Kidney Device according to the manufacturer’s instructions (Mannheim Pharma and Diagnostics GmbH, Mannheim, Germany).

Bone Marrow Transplantation and Kidney Surgeries
Procedures were performed essentially as described. Lethal irradiation was performed in a <sup>137</sup>Cs irradiator (10 Gray) at 6 weeks of age, mice were reconstituted with unfractioned bone marrow. Recipient chimerism was assessed in mixed bone-marrow chimeric (50% WT [CD45.1] and 50% CX3CR1+/+ [CD45.2] bone marrow) LDLr-/- mice. Recipients were CD45.2+.. As among blood monocytes, all CX3CR1+/+ (CD45.2) monocytes are GFP<sup>+</sup>, any GFP<sup>+</sup> CD45.1<sup>+</sup> blood monocyte would be a recipient cell; 98±0.2% of GFP<sup>+</sup> blood monocytes (i.e., non-CX3CR1+/+ cells) was CD45.1<sup>+</sup> (i.e., of donor origin) in these mice. Similarly, among GFP<sup>+</sup> bone marrow monocytes, 94±1% were of donor (CD45.1) origin (n=9 for both measurements), indicating a very good replacement efficacy. For nephrectomy, mice were anesthetized by intraperitoneal injection of ketamine (125 mg/kg), xylazine (12.5 mg/kg), and atropine (0.025 mg/kg) and procedures were performed as described. Postoperative analgesia was with intraperitoneal buprenorphine as needed. Mice were rested for 1 week after surgery before a high-fat diet was started. If indicated, mice were injected intravenously with 200 µl of liposomal cladribine (5 mg/ml) once on day 0 of the high-fat diet. For adoptive transfer experiments, 1.5×10<sup>6</sup> CD4<sup>+</sup> splenocytes per recipient were isolated using a BD FACSAria and injected intravenously into recipients irradiated with 3 Gray on day 0 of the high-fat diet.

Quantification of Atherosclerosis and Aortic and Histologic Analysis
The procedure for en face and histologic assessment of murine atherosclerotic lesion size has been previously described. For histologic aortic root analysis, frozen 5-µm thick sections from the aortic...
valve plane in 50-µm intervals covering a total of 400 µm were stained with oil-red-O with hematoxylin and light-green counterstain or Picrosirius red. For immunofluorescence, F4/80 (BM8) (BD Bioscience), rabbit anti-mouse Ki67 (SP6, Thermo Fisher Scientific, Fremont, CA), and the following secondary antibodies were used: anti-FITC Alexa Fluor 488 (Molecular Probes), goat anti-rabbit Cy3 (Jackson Immunoresearch, Newmarket, UK). Images were obtained with a Leica DMI3000B microscope with 5×, and 20× original magnification using Leica Application Suite version 3.5.0 (Leica, Wetzlar, Germany). Quantification was conducted with National Institutes of Health ImageJ software and the GNU Image Manipulation Program (version 2.8). For aortic roots, a data point represents a mean of all sections’ lesion sizes from one mouse.

**Cell Culture, Stimulation, and T Cell Polarization**

Total and CD4+–enriched (CD4+ T cell isolation kit, Miltenyi, Bergisch Gladbach, Germany) mouse splenic lymphocytes were cultured on plate-bound purified anti-CD28 and anti-CD3 (Biologend, San Diego, CA) without exogenous cytokines (TREG), in the presence of 10 ng/ml IL-12 and anti-IL-4 (3 µg/ml) (T eff), or in the presence of IL-6 (50 ng/ml), TGFβ (1 ng/ml), IL-23 (20 ng/ml), anti-IFNγ (3 µg/ml), and anti-IL-4 (3 µg/ml) for T H17 polarization complete Iscove’s Modified Dulbecco’s Medium. For T eff polarization, culture was in RPMI with TGFβ (10 ng/ml) and IL-2 (10 ng/ml; all from Peprotech, Biologend, or ebioscience, San Diego, CA). FoxP3 was detected in 53±1% of CD4+CD25+ T cells after 3 days of TREG polarization (n=4). CellTrace Far Red Cell Proliferation Kit (Life Technologies, Carlsbad, CA) without exogenous cytokines (TH0), in the presence of 1 ng/ml, IL-23 (20 ng/ml), anti-IFNγ, and for live cells after cell culture. Data were analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

**RNA Isolation, Real-Time PCR, and ELISA**

RNA was isolated using Nucleospin RNAII Kit (Macherey-Nagel, Düren, Germany) and reverse-transcribed with M-MLV Reverse Transcriptase (Promega, Mannheim, Germany) according to the manufacturer’s instructions. Real-Time PCR was performed on a LightCycler480 using SYBR Green (Roche Diagnostics, Grenzach-Wyhlen, Germany). Primers were selected using PrimerBank as follows: CX3CR1: fw: GGACTCCTACCTCATCACGCC, rev: TCCGGTTGTTCATGAGTTGG, HPRT: fw: CAGTCC-CACGGTCGATTCA, rev: AGCAAGTCTTCAGTCTGCT. Products were confirmed by melting curve analysis and gel electrophoresis. Transcript levels were normalized to HPRT using the ΔΔct method. Cytokine concentrations were determined using BD Enhanced Flex Cytometric bead assay according to the manufacturer’s instructions.

**Enzymatic Digestion of Tissues and Flow Cytometry**

Preparation and enzymatic digestion of tissues was performed as described.5 The following antibodies were used: anti-CD4 (GK1.5), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD25 (PC61), anti-CD45 (30-F11), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD115 (AFS98), anti-Gr1 (RB6-8C5), anti-T cell receptor β (H57–597), anti-CD19 (6D5), anti-MHCII (M5/114.15.2), anti-FoxP3 (150D), anti-IL-17A (TC11–18H10.1), and anti-IFNγ (XMGl.2) (obtained from Biolegend, ebioscience, or R&D systems). Yellow and near-infrared LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen, Carlsbad, CA) was used according to the manufacturer’s instructions. Flow cytometry analysis was performed on a Becton-Dickinson FACS Canto or LSRII. Gating was performed for live CD45+ cells in all organ digests, and for live cells after cell culture. Data were analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

**Statistical Analyses**

Two-tailed t test was used to compare two conditions. If more than two conditions were compared, Bonferroni test of selected conditions was applied after ANOVA. P <0.05 was considered statistically significant. Data are expressed as mean ±SEM. P values are indicated as follows: *P<0.05, **P<0.01, ***P<0.001.

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**DISCLOSURES**

None.

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