Increased Atherosclerotic Lesion Formation and Vascular Leukocyte Accumulation in Renal Impairment Are Mediated by Interleukin-17A

Shuwan Ge, Barbara Hertel, Ekaterina K. Koltsova, Inga Sörensen-Zender, Jan T. Kielstein, Klaus Ley, Hermann Haller, Sibylle von Vietinghoff

Rationale: Atherosclerosis is a major cause of death in patients with chronic kidney disease. Chronic inflammation of the arterial wall including invasion, proliferation, and differentiation of leukocytes is important in atherosclerotic lesion development. How atherosclerotic inflammation is altered in renal impairment is incompletely understood.

Objective: This study analyzed leukocytes of the atherosclerotic aorta in mice with impaired and normal renal function and studied a mechanism for the alteration in aortic myeloid leukocytes.

Methods and Results: Unilateral nephrectomy significantly decreased glomerular filtration rate and increased atherosclerotic lesion size and aortic leukocyte numbers in 2 murine atherosclerosis models, apolipoprotein E (ApoE−/−) and low-density lipoprotein (LDL) receptor–deficient (LDLr−/−) mice. The number of aortic myeloid cells increased significantly. They took up less oxidized LDL, whereas CD11c expression, interaction with T cells, and aortic T cell proliferation were significantly enhanced in renal impairment. In human peripheral blood mononuclear cell cultures, chronic kidney disease serum decreased lipid uptake and increased human leukocyte antigen II (HLA II) expression. Supplementation with interleukin-17A similarly increased HLA II and CD11c expression and impaired oxidized LDL uptake. Interleukin-17A expression was increased in atherosclerotic mice with renal impairment. Ablation of interleukin-17A in LDLr−/− mice by lethal irradiation and reconstitution with HLA II−/− bone marrow abolished the effect of renal impairment on aortic CD11b+ myeloid cell accumulation, CD11c expression, and cell proliferation. Atherosclerotic lesion size was decreased to levels observed in normal kidney function.

Conclusions: Kidney function modifies arterial myeloid cell accumulation and phenotype in atherosclerosis. Our results suggest a central role for interleukin-17A in aggravation of vascular inflammation and atherosclerosis in renal impairment. (Circ Res. 2013;113:965-974.)

Key Words: atherosclerosis ■ interleukin-17 ■ leukocytes ■ renal insufficiency ■ vascular inflammation

In patients with impaired renal function, risk of death, mostly from cardiovascular events, is significantly elevated.1-5 Acute hospital admissions for cardiac causes are common, with patients with kidney disease presenting with less specific symptoms than the general population.6 At most stages of chronic kidney disease (CKD), patients are more likely to die from cardiovascular disease than to proceed to terminal renal failure and require renal replacement therapy.4 At a 50% decrease in renal function (glomerular filtration rate [GFR] of ≤60 mL/min), a significant association with cardiovascular mortality was consistently observed after correction for other risk factors.1,3 This degree of CKD (CKD stages III and IV) affects ≈8% of the adult United States population.7

Unilateral nephrectomy significantly increased atherosclerotic lesion size in apolipoprotein E (ApoE−/−) mice,8,11 in which (different from wild-type [WT] mice) serum creatinine was significantly elevated after unilateral nephrectomy.8,11 Renal glomerular number was decreased in ApoE−/− compared with WT mice, but there was no evidence for accelerated progression of kidney disease.11 Blood pressure was unaffected by unilateral9,10,12 and even 5/6 nephrectomy in ApoE−/− mice.9,12,13 Blood pressure treatment with the angiotensin II blocker losartan and the vasodilator hydralazine was compared in ApoE−/− mice after unilateral nephrectomy.10 Losartan was more antiatherogenic despite similar blood pressure levels and despite higher cholesterol.

Original received June 2, 2013; revision received July 31, 2013; accepted August 1, 2013. In July 2013, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.24 days.

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The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.113.301934/-/DC1.

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.113.301934

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Vascular calcification is prominent in end-stage renal disease.\textsuperscript{14,15} In contrast, histological findings of the atherosclerotic lesions at CKD stages III and IV or \textit{Apoe}^{-/-} mice after unilateral nephrectomy to a large extent resemble that of the lesions in normal kidney function.\textsuperscript{8–10} Specific histopathologic features such as medial calcification that are observed in dialysis-dependent patients’ arteries\textsuperscript{14,15} and severely uremic murine atherosclerosis models\textsuperscript{8,9,13,16} are not found. The mechanism of increased atherosclerosis in patients with mild-to-moderate renal impairment (RI) is incompletely understood.

Vascular wall infiltration by both innate and adaptive immune cells contributes to atherosclerotic lesion progression.\textsuperscript{17} Alterations of the immune system by, for example, splenectomy, ablation of B cells, or specific T helper cell subsets can change the size and also the composition of atherosclerotic lesions, and alter collagen, lipid, or smooth muscle cell contents.\textsuperscript{17,18} Monocyte-derived myeloid cells that give rise to foam cell–forming macrophages and also fully functional antigen-presenting cells are central in plaque development.\textsuperscript{17,18} Their number within the arterial wall increases markedly during atherosclerosis development because of both immigration and local proliferation. Oxidized low-density lipoprotein (oxLDL) upregulates the expression of the \( \beta_2 \) integrin subunit CD11c, a marker of antigen-presenting cells in mice, on monocytes.\textsuperscript{19} CD11c-deficiency decreases atherosclerotic lesion size.\textsuperscript{19} In atherogenesis, CD11b\textsuperscript{+}CD11c\textsuperscript{−} cells are capable of both lipid phagocytosis and antigen presentation to CD4\textsuperscript{+} T cells.\textsuperscript{20–24} This occurs in lymphatic organs;\textsuperscript{24} however, T cells are also located in close proximity to antigen-presenting cells in the atherosclerotic arteries of mice and humans\textsuperscript{25,26} and interact with each other in the vascular wall.\textsuperscript{27} Close interaction with CD11b\textsuperscript{+}CD11c\textsuperscript{−} cells in the vascular wall was limited to \textit{Apoe}^{-/-}CD4\textsuperscript{+} T cells and did not occur with WT CD4\textsuperscript{+} T cells, suggesting an antigen-specific process.\textsuperscript{27} The number of T lymphocytes increases during the atherosclerosis progression in the vessel.\textsuperscript{17,18} T cells are differentially regulated by myeloid and plasmacytoid dendritic cells\textsuperscript{24} and can modulate atherosclerotic inflammation by cytotoxicity and cytokine secretion. While some T-cell cytokines such as interferon-\( \gamma \) have a proven proatherogenic role in diverse settings,\textsuperscript{17,18} the effect of others such as interleukin (IL)-17A is controversial.\textsuperscript{28,29} Enhancement of atherosclerotic lesion formation by IL-17A has been reported in some,\textsuperscript{30–34} but not all, models.\textsuperscript{35–38} Although some studies were limited by available reagents, the role of IL-17A may also differ depending on the environment, for example, lesion localization\textsuperscript{14,15} or regulation of other immune mediators.\textsuperscript{39} We found an induction of atherosclerosis by IL-17A in pharmacological immunosuppression\textsuperscript{39} and also in IL-17A overproduction in the absence of the IL-27 receptor.\textsuperscript{40}

In patients with RI, some serum inflammatory markers are increased and correlate with mortality; among them is IL-6, which can enhance IL-17 production and can be induced by

![Figure 1. Renal impairment (RI) increases atherosclerotic lesion size and leukocyte infiltration. A and B, En face aortic atherosclerotic lesion size was assessed in \textit{Apoe}^{-/-} mice (12 weeks of high-fat diet; A) and quantified as percentage of aortic surface area (B; \( n=6–10; 3 \) independent experiments). C and D, Aortic root lesion size in the 300 \( \mu m \) following the aortic valve (C; \( n=8; 4 \) independent experiments and D; examples, \( \times 4 \) and \( \times 20 \) original magnification, arrows mark the same areas as in Figure 2). E–G, Aortic leukocyte numbers were analyzed by flow cytometry (gated for live, CD45\textsuperscript{+} cells; \( E; n=9–11; 4 \) independent experiments). Staining for CD11b (myeloid cells), CD19 (B cells), and \( \alpha \beta \) TCR (T cells; example in F) was used for quantification of these subgroups (G; \( n=5–11 \) per group; 4 independent experiments). Ctrl indicates sham-operated control. *\( P<0.05 \), **\( P<0.01 \).]
IL-17 itself. Increased IL-17A in serum in a cohort of patients receiving hemodialysis for renal replacement was recently reported. Cellular immune alterations in renal failure include, among others, peripheral blood lymphopenia and neutrophilia. Circulating dendritic cells are depleted. However, data from the atherosclerotic vascular wall are required for a better understanding of the inflammatory disease process.

Here, we examined the aortic leukocyte infiltrate in atherosclerotic Apoe−/− and LDL receptor–deficient (LDLr−/−) mice with surgically induced impaired renal function (unilateral nephrectomy). Our initial screening revealed a significant increase in aortic leukocytes. We investigated myeloid cell phenotype, function, and mechanism of accumulation in RI.

Methods
Detailed methods are available in the Online Data Supplement.

Animals
Wild-type (wt) C57Bl/6, LDLr−/−, Apoe−/− mice (both on C57Bl/6 background) (Jackson Labs, Bar Harbor, ME), CD11cYFP, kindly provided by Dr M. Nussenzweig, Rockefeller University, NY and crossed with Apoe−/−, and mice lacking IL-17A (Il17a−/−), 96% C57Bl/6 background, kindly provided by Dr Y. Iwakura, University of Tokyo, 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 Animal Care Committee at La Jolla Institute for Allergy and Immunology and Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Lower Saxony, Germany. High-fat diet contained 40% kcal from fat and 1.5% cholesterol.

Bone Marrow Transplantation and Unilateral Nephrectomy
Experiments were commenced at 6 weeks of age. Bone marrow transplantations were performed as described. Unilateral nephrectomy or decapsulation of the kidney were performed 1 week before high-fat diet was started.

Quantification of Atherosclerosis, Histological, and Aortic Leukocyte Analysis
Aortic en face staining and histological assessment of aortic roots, plasma lipid fast protein liquid chromatography, and measurement of GFR by FITC-Inulin are described in the Online Data Supplement. Preparation and flow cytometry of aortic leukocytes39 and 2-photon imaging of aortic CD11c+T-cell interaction were performed as described.

Cell Culture Experiments
Cell culture procedures are described in the Online Data Supplement, and serum for stimulation experiments was obtained after written informed consent from patients with nondiabetic CKD described in the Online Data Supplement.

Statistical Analysis
Data are expressed as mean±SEM. Two-tailed Student t test and Mann–Whitney test were used to compare 2 conditions. One-way ANOVA and post hoc tests as indicated were used if >2 conditions were compared. P<0.05 was considered significant.

Results
Impaired Renal Function Increases Atherosclerotic Lesion Size and Aortic Leukocyte Infiltration
Unilateral nephrectomy significantly increased serum creatinine (Online Table I) and decreased GFR measured by inulin clearance in Apoe−/− mice (Online Figure I). This degree of RI significantly increased aortic atherosclerotic lesion size in Apoe−/− mice (Figure 1A and 1B), with similar affection of both sexes (Online Figure IIA). Aortic root lesion size also increased (Figure 1C and 1D). Relative collagen contents
and composition were essentially unaltered (Online Figure IIB and IIC). No significant differences in body or spleen weight occurred, and serum calcium, phosphorus, total leukocytes as a marker of systemic inflammation, thrombocyte, and erythrocyte counts were unaltered. Total serum cholesterol was increased, because of LDL, but not HDL levels (Online Table I). No alteration was observed in renal leukocytes (data not shown).

In Apoe<sup>−/−</sup> mice with RI, significantly more leukocytes were recovered from the aorta by flow cytometry analysis as described<sup>39</sup> (Figure 1E). All investigated leukocyte types, myeloid cells (CD11b<sup>+</sup>), B cells (CD19<sup>+</sup>), and αβTCR<sup>+</sup> T cells were significantly more abundant (Figure 1F and 1G).

These data show that the increase in atherosclerotic lesion size in Apoe<sup>−/−</sup> mice with RI is accompanied by an extended inflammatory vascular infiltrate.

### Enhanced Accumulation of Aortic Myeloid Cells and CD11c Expression in RI

We further investigated aortic accumulation of myeloid leukocytes in RI. Numbers of CD11b<sup>+</sup> myeloid cells in the aorta were significantly elevated already early in atherosclerosis development in Apoe<sup>−/−</sup> mice after 3 weeks of high-fat diet (Figure 2A). Aortic cell proliferation is highly elevated at this stage in mice with normal renal function.<sup>27,39</sup> We labeled proliferating cells with BrdUrd and measured incorporation into CD11b<sup>+</sup> leukocytes. RI significantly increased proliferation of aortic myeloid leukocytes (Figure 2B). Expression of the antigen-presenting cell marker CD11c on aortic CD11b<sup>+</sup> myeloid cells<sup>19,20,39</sup> was enhanced in RI (Figure 2A).

Both myeloid cell numbers and the proportion that expressed CD11c had further increased in lesions after 12 weeks of high-fat diet (Figure 2C). Similar results were observed in male and female mice (Online Figure III). Cell localization in the vascular wall by confocal imaging showed large CD11c<sup>+</sup> and CD11b<sup>+</sup> neointimal areas (Figure 2D) in both lipid-rich and cellular plaque areas in the animals with RI.

### RI Alters Myeloid Cell Function in the Atherosclerotic Aortic Wall

Next, we investigated lipid uptake and antigen-presenting function of myeloid aortic cells. Uptake of labeled exogenous oxLDL in aortic leukocytes was measured by flow cytometry (Figure 3A). Uptake into total CD11b<sup>+</sup> and the CD11b<sup>+</sup>CD11c<sup>+</sup> subgroup of aortic leukocytes was significantly lower in Apoe<sup>−/−</sup> mice with impaired renal function (Figure 3B).

We studied CD11c<sup>+</sup> cell/CD4<sup>+</sup> T-cell movement and interactions in the aortic wall of mice with normal and impaired renal function. First, antigen presentation involves prolonged interactions with T cells and slowing of cell speed. We used a recently developed method<sup>37</sup> (Online Figure IV) to investigate this in the atherosclerotic aorta of mice with normal and impaired renal function. CD4<sup>+</sup> T cells interacted with CD11c<sup>+</sup> cells in aortas of mice with RI for an increased proportion of the observation time (11±1% in control, 19±1% in RI; n=2; P<0.02). Second, productive T-cell interactions with antigen-presenting cells decreased the speed of both cell types.<sup>47</sup> The average speed of both CD11c<sup>+</sup> cells and CD4<sup>+</sup> T cells was significantly lower in aortas from animals with RI than in controls (CD11c<sup>+</sup> in Figure 3C, similar to spleen cells (Figure 3D). T-cell stimulation by antigen-presenting cells decreases proliferation. Aortic CD11b<sup>+</sup>CD11c<sup>+</sup> enhanced CD4<sup>+</sup> T-cell proliferation during mixed lymphocyte reaction in vitro similar to spleen cells (Figure 3E). RI significantly increased

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**Figure 3.** Altered lipid uptake and interaction with T cells of aortic myeloid leukocytes in renal impairment (RI).

A and B, Uptake of Dil-oxLDL by aortic leukocytes was assessed 24 hours after injection into Apoe<sup>−/−</sup> mice with and without RI by flow cytometry (A, shown in all live leukocytes; B, quantified as proportion of CD11b<sup>+</sup> and CD11b<sup>+</sup>CD11c<sup>+</sup> cells; n=4–7; 2 independent experiments). C and D, Aortic antigen presentation was assessed by live-cell 2-photon microscopy after coincubation of explanted aortas from CD11c<sup>+</sup>Apoe<sup>−/−</sup> mice (control [ctrl] and RI) with SNARF-labeled Apoe<sup>−/−</sup>CD4<sup>+</sup> T cells (red). CD11c<sup>+</sup> antigen–presenting cell (APC; C) and T-cell (D) velocities were assessed in control and in RI aortas in 3 dimensions (z axis shown in ctrl; stills from Online Movies; 1–4 average cell velocities given; 1 of 2 independent experiments; unpaired t tests). E, Proliferation of BALB/c lymphocytes in mixed lymphocyte reaction with splenic and aortic CD11b<sup>+</sup>CD11c<sup>+</sup> cells from atherosclerotic Apoe<sup>−/−</sup> mice was assessed by CFSE dilution (1 of 2 independent experiments with similar results; 4–5 aorta donors per group). F, Proliferation of Apoe<sup>−/−</sup> aortic lymphocytes after 3 weeks of high-fat diet (BrdUrd<sup>+</sup>cells/aorta; n=8–10 from 3 independent experiments). *P<0.05, **P<0.01.

Online Movies 1 and 2; CD4<sup>+</sup> in Figure 3D, Online Movies 3 and 4. Third, T-cell stimulation by antigen-presenting cells induces proliferation. Aortic CD11b<sup>+</sup>CD11c<sup>+</sup> enhanced CD4<sup>+</sup> T-cell proliferation during mixed lymphocyte reaction in vitro similar to spleen cells (Figure 3E). RI significantly increased
proliferation of aortic αβ TCR+ lymphocytes but not CD19+ B lymphocytes (Figure 3F).

**RI and IL-17A Alter Myeloid Cell Differentiation In Vitro**

To test whether the altered aortic myeloid cell phenotype in mice with RI could be replicated in human cells, human adherent peripheral blood mononuclear cell cultures were supplemented with 10% serum from patients with stable chronic kidney disease (CKD; glomerular filtration rate [GFR] >60 mL/min=CKD stages I and II, n=5; GFR 30–60 mL/min=CKD stage III, n=11; and healthy controls [HC], n=9; 3 independent experiments; Bonferroni after 1-way ANOVA). B, human leukocyte antigen II (HLA II) surface expression on human PBMC-derived macrophages assessed by flow cytometry (CKD stages I and II, n=11; CKD stage III, n=19; healthy controls, n=8; 6 independent experiments; Bonferroni after 1-way ANOVA). C and D, HLA II expression on PBMC-derived macrophages cultured with and without recombinant IL-17A (C; n=5, linear trend after 1-way ANOVA) and dendritic cells (D; promoted by culture with GM-CSF and IL-4, n=4; linear trend after 1-way ANOVA). E and F, Murine macrophages were generated by culture of adherent bone marrow cells. The effect of recombinant mouse IL-17A on CD11c expression was investigated in both wild-type (WT; 4 independent experiments) and Il17a−/− compared with Il17ra−/− mice (F; t test after 1-way ANOVA; 3 independent experiments). G, Il17a−/− macrophage mRNA expression was assessed on day 3 of culture in the presence of recombinant IL-17A (1 ng/mL) and compared with control cells (t tests; 3 independent experiments). H, WT macrophage oxLDL uptake on day 7 after culture with different concentrations of recombinant IL-17A (Dunnett’s after 1-way ANOVA; n=12; 3 independent experiments). *P<0.05, **P<0.01, ***P<0.001.

**IL-17A Expression in RI**

Next, we assessed T-cell polarization in vivo. Among T-cell lineage markers, the T_{h}17 transcription factor RORγt was
expressed significantly higher in aortas of atherosclerotic APOE-/- mice with RI (Figure 5A), and the proportion of splenic IL-17A producers was significantly increased (Figure 5B and 5D). No significant change was seen in T-bet and interferon-γ, markers of TH1 cells, and well-described proatherogenic mediators (Figure 5A–5C) or Foxp3 regulatory T cells with known antiatherogenic function (Figure 5A and 5E). The increase in IL-17 production was not limited to CD4+ T cells; it also was observed in the CD4-CD3+ compartment that includes multiple lineages (data not shown). In serum from patients with impaired renal function (CKD stage III), many IL-17A measurements were below detection limits, similar to cohorts of patients with hypertension and the acute coronary syndrome. However, in 16 of 32 patients, but in only 3 of 9 of healthy volunteers, serum IL-17A was detectable (>6 pg/mL), and IL-17A effects on macrophage differentiation started at low concentrations (Figure 4C and 4D). Similarly, most other cytokine concentrations were below detection limits; however, there was a trend toward elevated tumor necrosis factor TH17-related IL-6 levels (data not shown).

Recent data have shown that angiotensin induced increase in blood pressure and tissue damage was mediated by IL-17A. Blockade of angiotensin signaling is among the most successful pharmacological interventions in RI. IL-17A producers were increased in APOE-/- mice with RI (135±47 pg/mL [RI] and 48±14 pg/mL [control]; n=6–7; P=0.02). We investigated the effect of angiotensin II on T-cell cytokines implicated in atherosclerosis development. No significant effect on interferon-γ production was observed, but angiotensin II significantly increased the number of IL-17A–producing T cells under TH17 polarizing conditions (Figure 5F and 5G). No effect on the proportion of IL-17A was observed if angiotensin II was applied either without TH17 favoring conditions (TH10; Figure 5F and 5G) or during restimulation of T cells (data not shown). The effect on TH17 polarization was reverted by the angiotensin receptor blocker Losartan (Figure 5H).

**Increased Aortic Lesion Size and CD11b+CD11c+ Leukocyte Accumulation in Impaired Renal Function Is IL-17A–Dependent**

To directly investigate the role of IL-17A in atherosclerosis in RI, we reconstituted LDLR-/- mice with either WT or II17a-/- bone marrow. Unilateral nephrectomy significantly decreased renal function as measured GFR in this mouse model of atherosclerosis (Online Figure I). Online Table II shows that no significant differences occurred in body weight, circulating leukocytes and serum triglyceride, and total cholesterol levels. In RI, there was a tendency toward an increase in LDL and VLDL levels as determined by fast protein liquid chromatography (Online Figure VI). Aortic lesion size was significantly increased in impaired renal function similar to the APOE-/- atherosclerosis model (Figure 7A–7C).

Reconstitution with II17a-/- bone marrow abolished IL-17A-producing cells in the spleen (Online Figure VIIA). Comparing control LDLR-/- mice that underwent transplantation with WT and II17a-/- bone marrow, aortic root lesion size was similar. However, in the absence of IL-17A, RI no longer increased atherosclerotic lesion size (Figure 6A and 6B; Online Figure VIIB and VIIC). Lesional collagen content
was similar in all 4 experimental groups (Figure 6C and 6D), similar to what was observed in Apo e−/− mice (Online Figure III). However, lesional macrophage accumulation was significantly enhanced in RI in WT mice (Figure 6E). This was abolished in the absence of IL-17A.

RI increased CD11b and, more markedly, CD11c immunofluorescence staining in the aortic root of LDLr−/− mice with WT bone marrow (Figure 7A). This was abrogated in the absence of IL-17A. Also, by flow cytometry, significantly enhanced CD11b+CD11c+ leukocytes were detected in the aortas of WT LDLr−/− mice with RI compared with controls with normal renal function (Figure 7B). This increase was completely abolished in Il17a−/−-reconstituted LDLr−/− mice. Similar to the Apoe−/− model (Figure 2), RI significantly increased aortic cell proliferation in control LDLr−/− mice (Figure 7C and 7D). The proliferation increase was also abolished in the absence of IL-17A. Collectively, these results suggest a central role for IL-17A in aggravation of atherosclerosis and vascular leukocyte accumulation in RI.

Discussion

Kidney disease is frequent and an independent risk factor for the development of atherosclerosis and its complications. The inflammatory response is increased but dysfunctional in patients receiving renal replacement therapy.51,56 However, inflammatory leukocytes within the vascular wall have not been systematically explored in patients receiving renal replacement therapy or with lesser degrees of RI. Our study shows a significant increase of total aortic leukocytes and CD11b+CD11c+ myeloid cells in 2 independent murine atherosclerosis models, Apoe−/− and LDLr−/− mice, with a 50% decrease in renal glomerular number and significantly decreased GFR. Our study also identifies IL-17A as a critical mediator of atherosclerosis enhancement in RI.

The role of IL-17A in atherosclerosis in general is controversial.28,29 A proatherogenic role is suggested by antiatherogenic effects of IL-17–receptor blockade and IL-17A blockade in some, albeit not all, models28,29 and proatherogenic IL-17A effects in immunosuppressed Apoe−/− mice in which IL-17A participated in myeloid cell accumulation in the arterial wall.39 The expressions of IL-17A and CD11b+ myeloid cell accumulation in the aorta of mice with impaired kidney function were enhanced. Published data suggest that IL-17A can enhance antigen-presenting cell functions30,31 and macrophage cytokine secretion32 in fully differentiated cells. In our in vitro model, IL-17A supplementation during differentiation significantly increased dendritic cell marker expression on mouse and human myeloid cells. It was demonstrated that CD11b+CD11c+ cells can function as fully functional antigen-presenting cells within the aortic wall.24,27 We confirmed their ability to induce CD4+ T-cell proliferation.24 We also demonstrate that RI not only enhanced accumulation of CD11b+CD11c+ cells but also increased interactions with T cells. Productive interactions could locally promote T-cell cytotoxicity and cytokine production, further increasing atherosclerotic inflammation, including activation of other cell types such as smooth muscle cells and endothelium. In addition, activated lymphocytes and antigen-presenting cells migrating from the vascular wall to secondary and tertiary lymphatic organs19,22 could amplify systemic immune response.

Not only the recruitment but also the lipid scavenging function of aortic CD11b+CD11c+ cells were significantly affected by RI. A previous report has described impaired efflux of cholesterol from peritoneal macrophages from mice with impaired renal function attributable to a decrease in ABCA1 gene expression.58 The authors concluded that decreased cholesterol efflux would promote foam cell formation and thereby the enhancement of atherosclerosis in RI. We are able to expand this finding to the aorta in vivo. We also observed a decrease in lipid uptake in human macrophages exposed to serum from patients with CKD in vitro. Although multiple cytokines and other plasma components lipids may be differentially present in patients with RI, IL-17A by itself was sufficient for similar effects on human macrophages. IL-17A also decreased oxLDL uptake in murine bone marrow–derived macrophages. Both CD36 and ABCA1 cholesterol trafficking molecules gene expression was diminished by IL-17A. This change in lipid uptake may contribute to the altered lipid profile in Apoe−/− mice.
with RI as observed here and by others.8,9,13,16,58 The changes in circulating lipid levels were significant in Apoe−/−, but not in LDLr−/− mice in our study. It is possible that the extreme lipid overload in Apoe−/− mice may make the changes in transport molecules more visible in systemic lipid levels. However, circulating lipid levels do not always correlate with disease severity. For example, in double ABCA1−/−/ABCG1−/− mice, a decrease in cholesterol efflux decreased macrophage lipid clearance abilities and increased atherosclerosis levels despite lower circulating lipid levels.59 Also in impaired renal function, angiotensin II blockade was more antiatherogenic than a vasodilator despite higher cholesterol levels.10 IL-17A influenced molecules responsible for both cholesterol influx and efflux and therefore may have local proatherogenic actions by increasing lipid deposition in the vascular wall, which are not necessarily reflected in plasma levels.

We found more IL-17A–producing T cells in Apoe−/− mice with decreased kidney function. IL-17A serum levels were also increased in a cohort of patients requiring hemodialysis.42 Most importantly, RI had no effect on atherosclerotic lesion size and aortic CD11b+CD11c+ leukocytes of LDLr−/− mice reconstituted with Il17a−/− bone marrow, suggesting a mechanistic role rather than an association of IL-17A in enhanced atherogenesis in RI. Several factors may confer atherosclerosis-promoting function to IL-17A in RI not observed under baseline conditions in LDLr−/− mice here or by others.7 First, our results show enhancement of IL-17A production by angiotensin II under TH17 polarizing conditions. Angiotensin II blockade is a major disease-modifying factor in patients with kidney disease54,55 and more effective than blood pressure treatment with other agents in 5/651 and unilaterally nephrectomized mice.10 Angiotensin II levels were elevated in mice with RI. The TH17-regulating cytokine transforming growth factor-β was induced by angiotensin II in several forms of vascular inflammation.60 Possibly, an enhancement by angiotensin II increases IL-17A levels enough to become a determinant of atherosclerosis severity. However, an amplification or alteration of the IL-17A signal in RI may also occur by other yet to be determined factors, for example, cytokines on IL-17A–responsive cells such as leukocytes.

In conclusion, our data show that RI increases atherosclerotic inflammation, alters aortic myeloid cell phenotype and their interaction with T lymphocytes, and suggests IL-17A as a key mediator for this increase in disease severity.

Acknowledgments
We thank Dr Witztum and Jennifer Pattison for performance of fast protein liquid chromatography.
Disclosures
This work was supported by National Science Foundation of China (#81200531 to S. Ge), American Heart Association (#10POST1461042-01 to E.K. Koltsova), National Institutes of Health (HL58108 and HL55798 to K.F. Ley), and Deutsche Forschungsgemeinschaft (V1508/4-1) and TUI-Stiftung (to S. von Vietinghoff).

References
Unilateral nephrectomy in Apoe−/− mice significantly decreases kidney function and aggravates atherosclerosis. During renal impairment, more myeloid cells, T lymphocytes, and B lymphocytes accumulate, and antigen-presenting cells interact more with T cells in the arterial wall. Absence of interleukin-17 abrogates enhancement of myeloid cell accumulation and atherosclerosis that accompany renal impairment.

What Is Known?
- Chronic kidney disease increases the risk of atherosclerosis and its complications.
- During arterogenesis, leukocytes accumulate in the arterial wall and contribute to plaque growth.
- Apor−/− mice after unilateral nephrectomy can serve as a mouse model of atherosclerosis with moderate renal impairment.

What New Information Does This Article Contribute?
- Unilateral nephrectomy in Ldlr−/− mice significantly decreases kidney function and aggravates atherosclerosis.
- During renal impairment, more myeloid cells, T lymphocytes, and B lymphocytes accumulate, and antigen-presenting cells interact more with T cells in the arterial wall.
- Absence of interleukin-17 abrogates enhancement of myeloid cell accumulation and atherosclerosis that accompany renal impairment.
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*Circ Res.* 2013;113:965-974; originally published online August 1, 2013;
doi: 10.1161/CIRCRESAHA.113.301934

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/113/8/965

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Increased atherosclerotic lesion formation and vascular leukocyte accumulation in renal impairment are mediated by Interleukin 17A

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Detailed Methods:

Animals

Wild-type (wt) C57Bl/6, BALB/c, LDLr<sup>−/−</sup>, Apoe<sup>−/−</sup> mice (both on C57Bl/6 background) (Jackson Labs, Bar Harbor, ME), CD11c<sup>YFP</sup> mice, kindly provided by Dr. M. Nussenzweig, Rockefeller University, NY and crossed with Apoe<sup>−/−</sup> to obtain double-deficient mice, and mice lacking IL-17A (Il17a<sup>−/−</sup>),<sup>2</sup> 96% C57Bl/6 background, kindly provided by Dr. Y. Iwakura, University of Tokio, 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 Animal Care Committee at LIAI and Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Lower Saxony, Germany. Mice were maintained on high fat diet (Harlan Teklad 88137)(40% of kcal from fat, 1.5% Cholesterol, 0.68% calcium, 0.56% phosphorous) or normal “chow” diet (Pico lab Rodent diet 20 with 0.81% calcium, 0.63% phosphorous). Plasma lipids were measured at UCSD murine core laboratory or Olympus AU400 ChemistryImmunoAnalyzer (Olympus, Hamburg, Germany) and blood counts by automatic analyzers (Hemavet 950FS, DREW Scientific, Oxford, CT and VetABC animal blood counter, ScilVet, Viernheim, Germany). Plasma FPLC was conducted as described.<sup>3</sup>

Bone marrow transplantation, nephrectomy and measurement of renal function

Lethal irradiations were performed in a <sup>137</sup>Cs irradiator (10 Gray), mice were reconstituted with unfractioned bone marrow and treated with trimethoprim-sulfomethoxazole in drinking water for 2 weeks after transplantation. For nephrectomy, mice were anesthetized by intraperitoneal injection of ketamine (125 mg/kg), xylazine (12.5 mg/kg), and atropine (0.025 mg/kg). The left kidney was approached and removed after ligation of vessels and ureter. Care was taken to avoid damage of adrenal gland and ovary. Sham surgery consisted of de-capsulation of the kidney. Post-operative analgesia was with buprenorphine i.p. as needed. Surgery was well tolerated and the abdominal site was without signs of inflammation. Unilateral nephrectomy after bone marrow transplantation in LDLr<sup>−/−</sup> mice was conducted after two weeks when peripheral blood neutrophils have started to recover,<sup>4</sup> high fat diet was started on week later. Injection with Dil-oxLDL i.p. (10 µg/mouse) was 24 h before aorta harvest. Glomerular filtration rate (GFR) was determined after injection of fluorescent inulin as described.<sup>5</sup> Angiotensin II was determined by ELISA (RayBio, Norcross, GA, detection limit: 20pg/ml) according to the manufacturer’s description in serum from Apoe<sup>−/−</sup> mice after 12 weeks high fat diet.

Quantification of atherosclerosis and histologic analysis

For en face staining, mice were sacrificed by CO<sub>2</sub> suffocation and aortas were excised, fixed and stained with SudanIV.<sup>6</sup> Digital images were obtained using a moticam 1000 (Motic, Richmond, Canada) on an Olympus S267 dissection scope (Olympus, Center Valley, PA) and analyzed using NIH Image J. Aortic lesion size is expressed as percentage of total surface area. For histologic aortic root analysis, frozen sections were prepared. For quantification, 5 µm sections were taken from the aortic valve plane in 50 µm intervals covering a total of 300 µm. Photomicrographs were taken with a 4x objective on a Nikon eclipse 80i microscope after oil-red-O staining with hematoxylin and light-green counterstain.
and Picrosirius red stain. Lesion size in each section was determined using NIHImageJ. Each data point represents a mean of all sections’ lesion sizes from one mouse. For immunofluorescence, CD11b-FITC (M1/70) and hamster-anti-CD11c (BD Bioscience), rabbit-anti-mouse-Ki67 (Sp6, Thermo Scientific, Fremont, CA, USA) and the following secondary antibodies were used: anti-FITC-AF488 (Molecular Probes), donkey-anti-rat IgG-AF488 (H+L) (Invitrogen), goat-anti-rabbit-Cy3, goat-anti-hamster-Cy3 and anti-hamster-IgG-DyLight649 (Jackson immunoresearch, Newmarket, UK) were used. Images were acquired on a Leica DM6000 upright microscope with DIC optics using a HCX PLAPO 2x and 40x oil-immersion objectives at 488 and 633 nm excitation wavelength or a Zeiss Axioplan-2 imaging microscope using AxioVision 4.6 (Zeiss, Jena, Germany). NIH Image J was employed to adjust brightness and one-step smoothing on all images in parallel.

**Enzymatic digestion of tissues**

Mice were sacrificed and perfused with PBS containing heparin (20 U/ml). Complete thoracic and abdominal aortas were prepared with very close removal of adventitial fat and digested as described. Briefly, organ dissects were incubated for 50 min at 37°C and 150 rpm with a mixture of 450 U/ml collagenase type I, 250 U/ml collagenase type XI, 120 U/ml hyaluronidase type I-s and 120 U/ml DNAse1 (all Sigma-Aldrich, Saint Louis, MO). Single cell suspensions were obtained by the use of a 70µM cell strainer.

**Mixed lymphocyte reaction, bone marrow macrophage differentiation, splenocyte culture and in vitro T cell polarization**

For mixed lymphocyte reaction, live CD45+CD11b+CD11c+ cells were sorted (FACS-Aria) from spleens and aortas (without adventitial tissues) of atherosclerotic Apoe^-/- mice (high fat diet for at least 12 weeks, 4-5 donors per experiment) and co-incubated with magnetically enriched (Miltenyi Biotec, Bergisch Gladbach, Germany) BALB/c CD4+T cells at a ratio of 1:2 for 96 hrs. Cell proliferation was assessed by CFSE dilution. Un-stimulated CD4+ T cells served as controls.

For macrophage differentiation, adherent mouse bone marrow cells were cultured in full RPMI in the presence or absence of recombinant murine IL-17A (Peprotech, Rocky Hill, NY) for seven days. Oxidized LDL (oxLDL) labeled with 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI) was added at a concentration of 10 μg/ml (Biomedical Technologies) for 4 h. Mouse splenic lymphocytes were cultured in complete RPMI on plate-bound purified anti-CD28 and anti-CD3 (Biolegend, San Diego, CA, USA) without exogenous cytokines (T_{10}), in the presence of 16 ng/ml IL-12 (T_{11}) or in the presence of IL-6 (50 ng/ml), TGF-β (1 ng/ml; Peprotech, Hamburg, Germany), and IL-23 (20 ng/ml; eBioscience, San Diego, CA, USA) for T_{17} polarization as described. Angiotensin II and Losartan (Sigma-Aldrich) were dissolved in PBS and added in the indicated concentrations on day 0 and day 3 of culture. Re-stimulation was with PMA/ionomycin as described.

**Differentiation of macrophages from human peripheral blood mononuclear cells**

Human peripheral blood cells and serum was obtained after informed consent according to the declaration of Helsinki, and local ethics board approval (MHH 2010/807). Human peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Biocoll 1.077, Biochrom, Berlin, Germany) and adherent cells cultivated for
seven days in full RPMI supplemented with 10%FCS or human serum and recombinant human IL-17A (Peprotech, Rocky Hill, NY) as indicated. Dendritic cell polarization was with 1000 U/ml IL-4 (R&D systems, Wiesbaden, Germany) and 500 U/ml GM-CSF (Novartis Pharma, Nürnberg, Germany) in 2% autologous serum with partial media exchange on day 2, 4 and 6 of differentiation for a total of seven days. Serum for stimulation experiments was obtained from patients with non-diabetic chronic kidney disease and stable renal function from the outpatient clinic at Hannover Medical School (CKDI-II 4/11 male, age 46±17 years, CKDIII 11/19 male, mean age 54±15 years, difference not significant). The underlying renal disease was 8 ANCA associated vasculitis, 5 systemic lupus erythematoses, 3 IgA nephropathy, 3 FSGS, 2 membranous GN, 2 minimal change GN, 6 other and unknown.

**RNA isolation and Real Time PCR**


**Flow cytometry**

The following antibodies were used: Anti- mouse: anti-CD45 (30-F11) (Becton-Dickinson), anti-CD11c (N418), anti-CD11b (M1/70), anti-CD19 (1D3), anti-IL-17RA (5G4) (eBioscience, San Diego, CA), anti-Foxp3 (150D), anti-IL17A (TC11-18H10.1), anti-IFNγ (XMG1.2), anti-TCRβ (H57-597)(Biolegend, San Diego, CA), anti-human: anti-HLADR (G46-6)(BD). BD-Fix-Perm (BD Pharmingen, San Jose, CA, USA), LIVE/DEAD® Fixable Dead Cell Stain Kit (Invitrogen, Carlsbad, CA) and BrdU flow kit (BD Pharmeding, San Jose, CA) were used according to the manufacturer’s instructions. BrdU was given 24 h before tissue harvest. The gate for BrdU+ cells was set by the identical cell type from non-BrdU–injected animals after identical preparation and antibody treatment. Flow cytometry analysis was performed on a Becton-Dickinson FACS Calibur, Canto or LSRII. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR). Gating was performed for live, CD45+ events.

**T cell sorting and labeling, two-photon microscopy and cell tracking of the explanted aorta**
Procedures were essentially as described.\textsuperscript{10} Splenic CD4\textsuperscript{+} T cells were purified by Robosep negative selection (StemCell Technology, Vancouver, CA), labeled with SNARF (Molecular Probes, 2.5mM) and resuspended at 1.5x10\textsuperscript{6}/ml. T cells were incubated with the explanted aortic arch and thoracic aorta of CD11c\textsuperscript{YFP} Apoe\textsuperscript{-/-} mice with normal and impaired renal function after 6 weeks on high fat diet for 12 hrs in complete RPMI1640 media. For image acquisition, aortas were placed in a Petri dish, maintained at 37°C and superfused with RPMI1640 equalized with 95%O\textsubscript{2}/5% CO\textsubscript{2}.

Two-photon imaging was performed using a DM6000 upright microscope with 4 non-descanned detectors (Leica Microsystems, Wetzlar, Germany) and a Chameleon Ultra Ti:Sapphire laser (Coherent) tuned at 900 to 1000 nm for acquisition using a water-dipping objective Olympus XLUMPLFL 20XW, NA0.95. Emitted fluorescence was split with 2 dichroic mirrors (560 nm and 593 nm) and passed through filters (Semrock, Rochester, NY) 535/22 nm, 585/40 nm and 624/40 nm. Typically, 10 to 20 z-planes spaced 10 to 15 μm apart were acquired at 512x512 pixels/1 min. Movies were registered in x,y, and z directions using a vector field convolution on the 2D projection.\textsuperscript{11} Velocities were observed over the whole time of acquisition to avoid potential bias from manual classification of interacting versus non-interacting cells.
Supplementary figure I: Renal function after unilateral nephrectomy

Glomerular filtration rate (GFR) was assessed by FITC-Inulin clearance in sham operated (ctrl) and mice after unilateral nephrectomy (RI) in Apoe<sup>-/-</sup> and LDLr<sup>-/-</sup> (C,D) mice. Results are given as total GFR (A,C) and per 100g of body weight (B,D) (n= 4-6 Apoe<sup>-/-</sup>, n=6 LDLr<sup>-/-</sup> mice, unpaired t-tests).
**Supplementary figure II: ApoE⁻/⁻ male and female aortic en face lesion size and lesional collagen contents after 12 weeks high fat diet**

(A) Aortic en face lesion size increased similarly in male and female ApoE⁻/⁻ mice with impaired renal function (12 weeks high fat diet, subgroup analysis of figure 1B).

(B-D) Aortic roots were stained for collagen with Picrosirius red after twelve weeks on high fat diet (B). Lesion collagen contents was quantified as described for Sudan IV (C, n=4 mice per group). Polarized light was used to assess collagen structure (D).
Supplementary figure III: Aortic leukocytes in male and female ApoE⁻/⁻ mice

Results of aortic leukocyte flow cytometry analysis from male (A) and female mice (B) (12 weeks high fat diet) as depicted in figure 2C are plotted separately.
Supplementary figure IV: Experimental design of 2-photon imaging

CD4+ splenocytes were isolated from an Apoe−/− mouse after 6 weeks on high fat diet by negative selection with magnetic beads and labeled with SNARF dye. Aortic arches with macroscopically visible atherosclerotic lesions were explanted from CD11cYFPApoe−/− mice (controls and renal impairment) and co-incubated with labeled CD4+ T cells. The aorta was subjected to imaging in full RPMI at 37°C and 5% CO2.
Supplementary figure V: Lipid levels in patients with renal impairment

Results of lipid analysis from n=7 healthy controls, n=10 pts with a GFR > 60 l/min and n=19 pts with a GFR of 30-60 ml/min (Dunnett’s after One way ANOVA).
Supplementary figure VI: Plasma lipid levels in LDLr⁻/⁻ mice

FPLC results from n= 2 ctrl and n=2 mice with renal impairment (RI) after 6 weeks high fat diet.
Supplementary figure VII: Analysis of IL-17A expression after bone marrow transplantation and subgroup analysis of aortic lesion size in male and female LDLr^{-/-} mice transplanted with wild type and Il17a^{-/-} bone marrow.

(A) IL-17A expression in αβ TCR^{+} splenocytes was studied by intracellular staining after stimulation by PMA/ionomycin (PMA/iono) six weeks after lethal irradiation and reconstitution with wildtype and Il17a^{-/-} bone marrow (control: unstimulated cells).

(B,C) The experimental groups depicted in figure 6B are plotted separately for male (A) and female mice (B) (total numbers: wild type (wt) n=8 ctrl (6 male, 2 female), renal impairment (RI) n=11 (8 male, 3 female) and Il17a^{-/-}, control n=7 (5 male, 2 female), RI n=11 (7 male, 4 female) from 4 independent experiments each. P values are given from Bonferroni after One-way-ANOVA).
### Tables:

**Suppl. table I: Characteristics of Apoe<sup>−/−</sup> mice after 12 weeks high fat diet**

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>Renal impairment</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>29.4±1.7 (13)</td>
<td>26.2±1.1 (20)</td>
<td>0.11</td>
</tr>
<tr>
<td>Spleen weight (g)</td>
<td>0.21±0.01 (10)</td>
<td>0.20±0.02 (12)</td>
<td>0.7</td>
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<tr>
<td>Kidney weight (g)</td>
<td>0.19±0.005 (10)</td>
<td>0.22±0.009 (12)</td>
<td>0.007**</td>
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<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.13±0.01 (6)</td>
<td>0.18±0.01 (8)</td>
<td>0.01**</td>
</tr>
<tr>
<td>Serum calcium (mg/dl)</td>
<td>9.2±0.7 (7)</td>
<td>9.8±0.4 (9)</td>
<td>0.48</td>
</tr>
<tr>
<td>Serum phosphorus (mg/dl)</td>
<td>8.2±0.5 (7)</td>
<td>8.8±0.5 (9)</td>
<td>0.46</td>
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<tr>
<td>Blood leukocytes (10&lt;sup&gt;7&lt;/sup&gt;/µl)</td>
<td>8.5±0.48 (15)</td>
<td>9.7±0.28 (21)</td>
<td>0.2</td>
</tr>
<tr>
<td>Blood monocytes (/µl)</td>
<td>503±56 (15)</td>
<td>550±33 (21)</td>
<td>0.5</td>
</tr>
<tr>
<td>Blood thrombocytes (10&lt;sup&gt;6&lt;/sup&gt;/µl)</td>
<td>931±71 (15)</td>
<td>1111±126 (21)</td>
<td>0.06</td>
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<tr>
<td>Blood erythrocytes (10&lt;sup&gt;6&lt;/sup&gt;/µl)</td>
<td>9.1±0.25 (15)</td>
<td>9.3±0.24 (21)</td>
<td>0.27</td>
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<tr>
<td>Total cholesterol (mg/dl)</td>
<td>1251±91 (7)</td>
<td>1642±107 (7)</td>
<td>0.02*</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>131±7 (7)</td>
<td>200±21 (7)</td>
<td>0.01**</td>
</tr>
</tbody>
</table>

Values given as ± SEM (n)
**Suppl. table II: Characteristics of LDLr−/− mice after 6 weeks high fat diet**

<table>
<thead>
<tr>
<th>BM genotype</th>
<th>control</th>
<th>Renal impairment</th>
<th>p-value (ctrl vs RI)</th>
<th>p-value (wt vs Il17a−/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt</td>
<td>Il17α−/−</td>
<td>wt</td>
<td>Il17α−/−</td>
</tr>
<tr>
<td>Body (g)</td>
<td>18±1(8)</td>
<td>19±1(7)</td>
<td>17±1(10)</td>
<td>20±1 (11)</td>
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<tr>
<td>Spleen (g)</td>
<td>0.09±0(8)</td>
<td>0.07±0(4)</td>
<td>0.09±0(10)</td>
<td>0.08±0(7)</td>
</tr>
<tr>
<td>Kidney (g)</td>
<td>0.12±0(8)</td>
<td>0.12±0(4)</td>
<td>0.13±0(10)</td>
<td>0.16±0(7)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.28±0(7)</td>
<td>0.33±0(4)</td>
<td>0.32±0 (5)</td>
<td>0.34±0(7)</td>
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<tr>
<td>Leukocytes</td>
<td>16±2.2(8)</td>
<td>12±1.6(5)</td>
<td>17±2.1(10)</td>
<td>15±1.7(8)</td>
</tr>
<tr>
<td>Monocytes (10^3/µl)</td>
<td>1.4±0.2(8)</td>
<td>0.9±0.2(5)</td>
<td>1.5±0.2(10)</td>
<td>1.2±0.1 (8)</td>
</tr>
<tr>
<td>Thrombocytes (10^9/µl)</td>
<td>293±64 (8)</td>
<td>364±83 (5)</td>
<td>283±67 (10)</td>
<td>409±71 (8)</td>
</tr>
<tr>
<td>Erythrocytes (10^9/µl)</td>
<td>7.8±1 (8)</td>
<td>8.0±1 (5)</td>
<td>8.2±0 (10)</td>
<td>8.8±1 (8)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1436±143 (8)</td>
<td>1477±69 (4)</td>
<td>1549±181 (6)</td>
<td>1622±170 (7)</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>500±65(8)</td>
<td>551±62(4)</td>
<td>570±54(6)</td>
<td>647±81 (7)</td>
</tr>
</tbody>
</table>

Values are means ± SEM (n). Serum creatinine, cholesterol and triglycerides are given in mg/dl. P values are given for individual student’s T tests, ANOVA of all four groups were not significant.
Supplemental References:


List of supplemental movies:

Movie 1: Tracking of CD11c$^+$ in the atherosclerotic aorta of an Apoe$^{-/-}$ mouse
Movie 2: Tracking of CD11c$^+$ in the atherosclerotic aorta of an Apoe$^{-/-}$ mouse with impaired renal function

Movie 3: Tracking of CD4$^+$ in the atherosclerotic aorta of an Apoe$^{-/-}$ mouse
Movie 4: Tracking of CD4$^+$ in the atherosclerotic aorta of an Apoe$^{-/-}$ mouse with impaired renal function